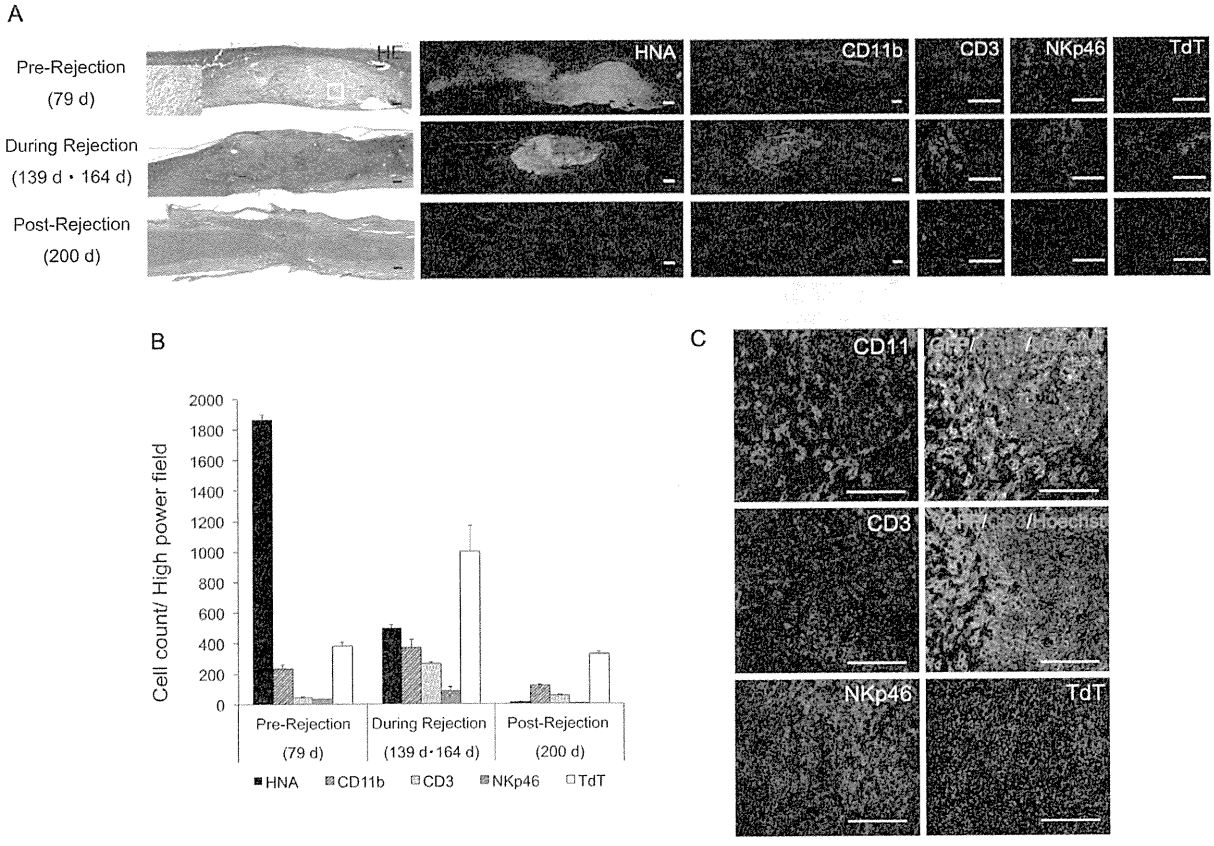

Fig 5. Analysis of inflammatory cell infiltration and apoptotic cells during the immune rejection of hiPSC-NS/PC-derived tumors. Immunohistochemical (A) and quantitative (B) analyses of human nuclear antigen (HNA)-, GFP-, cluster of differentiation (CD) 11b-, CD3-, NKp46-, and terminal deoxynucleotidyl transferase (TdT)-positive cells after discontinuing immunosuppressant treatment on day 100. After discontinuing the immunosuppressants, HNA-positive cells were gradually rejected. Inflammatory cells, including CD11b-, CD3-, and NKp46-positive cells, increased up to day 122 and then gradually decreased thereafter. TdT-positive cells also became prominent by day 122 and then gradually disappeared (C). Scale bars in A, 200 μm. hiPSC-NS/PC, human induced pluripotent stem cell-derived neural stem/progenitor cell.

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Notably, undifferentiated iPSCs can become immunogenic upon autografting [37]; however, the immunogenicity of iPSC-derived somatic cells can be different from that of undifferentiated iPSCs [4][38]. This is partly because the autografting study mentioned here utilized undifferentiated iPSCs and focused on immune responses against iPSC-generated teratomas rather than gliomas. Nevertheless, the *in vitro* differentiation of iPSCs into neural lineages prior to their transplantation is likely to prevent or at least attenuate their rejection by the host immune system [4].

Recent reports demonstrated that the immunogenicity of iPSCs and iPSC-derived products is as low as that of ESCs in syngeneic settings, whereas iPSCs become more immunogenic in allogeneic settings [38–42]. These reports were again directed toward immune responses against teratomas, as well as against skin grafts, and iPSC immunogenicity may differ in the context of the immune-privileged spinal cord. Furthermore, iPSC-NS/PC immunogenicity increases during the *in vivo* differentiation of NS/PCs in the CNS [43,44]. On the other hand, the NS/PCs utilized in the present study were differentiated *in vitro* and then xenografted into an immunosuppressed host, conditions under which the transplanted cells are less likely to be rejected [16]. Nonetheless, xenografted human NS/PCs were not rejected following their

transplantation into a cerebral infarction animal model, even in the absence of immunosuppressants [45]. However, a critical weakness in the methodology of this previous study (i.e., the implanted cells were labeled with Hoechst 33342 prior to transplantation [45]) renders it difficult to draw clear conclusions about the survival of xenografts.

Prevention of immune rejection of xenografted iPSC-NS/PC-derived tumors in immunosuppressed animals

According to previous reports [10,11], there is an optimal time window for the transplantation of cells in regenerative medicine strategies for SCI therapy. Considering the constraints of this time window (i.e., cell transplantation must be performed within several weeks after SCI), autologous transplantation upon the generation of host iPSCs and subsequent neural differentiation into NS/PCs is not a particularly realistic goal at this time. The situation, as it stands, necessitates the performance of allogeneic transplantation of iPSC stock-derived stem cells in clinical applications for SCI patients, along with combinatorial immunosuppressive treatment [4,46],[47].

Swijnenburg and colleagues used FK506 (tacrolimus), sirolimus, and mycophenolate mofetil (MMF) as immunosuppressant agents during the subcutaneous transplantation of human ESCs into mice [48]. FK506 monotherapy failed to support the successful engraftment of the xenografted cells, and combination therapy with MMF did not improve ESC survival. Similarly, the survival of xenografted hiPSC-NS/PCs was low when cyclosporin A was used as monotherapy [16]. Furthermore, our unpublished data revealed a low survival rate for xenografted hiPSC-NS/PCs in the mouse spinal cord when FK506, a clinically employed IL-2 blocker, was used as monotherapy (data not shown). For this reason, the current study used FK506 and anti-CD4 mAb as combination immunosuppressant therapy along with stem cell transplantation for SCI with reference to recent studies [49,50].

Antigen recognition is the first immunological barrier facing transplanted cells. Antigen recognition can be divided into two distinct pathways, direct and indirect recognition [51]. The indirect pathway is generally the most relevant in regenerative medicine applications. Hematopoietic stem cell transplantation, which occurs almost without the contamination of donor antigen-presenting cells, is the exception. The affinity of murine TCRs for human MHC molecules is low; therefore, it is reasonable to assume that transplanted human cells will be phagocytosed in murine antigen-presenting cells, followed by the presentation of human antigens to CD4-positive murine T-cells, and finally, the induction of the host immune response. In support of this idea, a study recently compared transgenic T-cell models of allogenic transplantation by grafting human ESCs into CD8-positive T-cell-knockout mice versus CD4-positive T-cell-knockout mice. The ESCs were immune rejected in both models, but the survival period was significantly longer in the CD4-positive T-cell-knockout mice than in the CD8-positive T-cell-knockout mice [52]. These observations provided the basis for our use of the anti-CD4 mAb together with FK506 in the present study. Indeed, the combination of these two immunosuppressants permitted the survival of unsafe hiPSC-NS/PCs in the mouse spinal cord for at least 200 days, with evidence of significant tumor growth in randomly selected spinal cord sections.

Auchincloss and Sachs suggested that the actions of NK cells are involved in immune rejection after organ transplantation [27]. However, transplantation of *in vitro*-differentiated ESCs is associated with increased expression of stem cell MHCs and decreased expression of ligands for NKG2D, an activating receptor found on NK cells [53–55]. Other investigations regarding allogeneic transplantation environments indicated that CD4-/CD8-positive T-cells play an important role in the immunity around the transplanted cells [20,52,56,57]. In agreement with

these earlier reports, the present study showed that CD3-positive T-cells occupied a large proportion of the invasive hiPSC-NS/PC-derived tumors in the grafted mouse spinal cord, whereas there were fewer NKp46-positive cells.

The hiPSC-NS/PC-derived tumors observed in this investigation exhibited the histological features of low-grade, glioma-like tumor, with no endodermal or mesodermal components characteristic of teratomas. Histological analyses also revealed the differentiation of the transplanted cells into neurons, astrocytes, and oligodendrocyte precursors in the mouse spinal cord. However, the hiPSC-NS/PC-derived tumors consisted mainly of undifferentiated, Nestin-positive cells.

A previous report exploring the somatic cell origin of mouse iPSCs and their associated tumorigenicity showed that contamination of cell grafts with *Nanog*-GFP-positive cells increased the risk of teratoma formation [22]. However, the present study utilized hiPSCs derived from skin fibroblasts and failed to detect teratomas. Therefore, we considered that Oct4-positive cells rather than Nanog-positive cells might have been the cause of 253G1-NS/PC tumorigenicity. Oct4-positive cells were observed within the 253G1-NS/PC-derived tumors, suggesting that the transcription factor was reactivated within the invasive growths.

In addition, gene insertion by retroviruses, incomplete reprogramming of the somatic cells used to generate iPSCs, genomic instability owing to high iPSC passage numbers, and cell differentiation/induction methods must all be taken into account when considering the potential capability of iPSCs/iPSC-NS/PCs to form tumors. For example, mutations in the tumor suppressor gene in transplanted NS/PCs increased the risk of astrocytoma in a mouse model [58]. In addition, intracranial transplantation of neural precursors derived from transformed but not normal human ESCs yielded glioma in immunodeficient NOD/SCID mice [59]. Thus, further research is urgently required to shed light on the mechanisms of both teratoma and glioma generation following stem cell transplantation into the CNS.

Tumor rejection following the discontinuation of immunosuppressants

Immunosuppressant treatment was discontinued in the present study at 100 days after iPSC-NS/PC transplantation. CD4-positive T-cells began to recover 12 days later, as assessed by FACS analysis, and ffLuc-positive hiPSC-NS/PCs gradually fell below the limit of BLI detection by 64 days after the cessation of immunosuppressants. The BLI technique cannot detect fewer than 1,000 cells; therefore, we also evaluated the presence of the transplanted stem cells by immunohistochemistry. At 64 days after FK506 and anti-CD4 mAb discontinuation, transplanted HNA-positive cells were still observed, but they were few in number and completely disappeared by 97 days. Two hundred days later, there was still no tumor recurrence.

Over the course of iPSC-NS/PC-derived tumor rejection, inflammatory cells (e.g., CD11b-positive cells, CD3-positive cells, and NKp46-positive cells) showed marked infiltration into the tumor. However, immune rejection with a focus on T-cells rather than NK cells was expected, based on the data described above. During the next phase of immune rejection, TdT-positive cells became prevalent, and then declined along with the numbers of transplanted cells and infiltrating immune cells.

As noted above, immune rejection following immunosuppressant discontinuation was hypothetically triggered by indirect antigen presentation (rather than MHC molecules) by hiPSC-NS/PCs and hiPSC-NS/PC-derived tumor cells to host T-cells. Nonetheless, regardless of the presence or absence of MHC molecules on the surface of the transplanted cells or their degree of differentiation, immune rejection occurred in all the transplanted cells. We postulate that other factors in addition to or instead of MHC molecules may be associated with the mechanism of immune rejection.

The BBB largely isolates the CNS from the rest of the body; therefore, inflammatory reactions rarely occur in the brain or the spinal cord without its collapse. However, even at 100 days after iPSC-NS/PC transplantation, immune rejection of the iPSC-NS/PC-derived tumor could be induced by the discontinuation of immunosuppressant treatment. It is possible that the tumor itself or our cell transplantation process stimulated breakdown of the BBB. For instance, earlier work showed that the use of a glass needle similar to the Hamilton syringe used herein for NS/PC transplantation disrupted the BBB. However, the BBB repaired itself in this earlier study within 12 days after cell transplantation, as evidenced by Evans blue dye transfer in rats[60]. Therefore, it is unlikely that transplantation of iPS-NS/PCs caused the continued collapse of the BBB at 100 days. Interestingly, another report indicated that activated lymphocytes can pass through the BBB[61], and that immune rejection can occur at 3 months after transplantation under xenograft settings.

The limitations of this study are as follows. The ablation of the tumor after iPS-NS/PC transplantation was observed in xenogeneic settings rather than allogeneic settings. The other limitation is that iPSC-NS/PCs were transplanted into the intact spinal cord rather than the injured spinal cord. In comparison, the immune response to allogeneic transplantation into the injured spinal cord greatly differs. Therefore, it must be determined whether the findings of the current study are replicated following allogeneic transplantation into the injured spinal cord. Furthermore, we must also examine the immunogenicity of iPSC-NS/PCs and the regulation of immune rejection. Additionally, the ultimate goal in cell replacement therapy is to minimize immune rejection and the use of immunosuppressants by matching HLA types between the donor cells and the host; however, it is practically difficult for iPSC banks to prepare iPSCs of all HLA types. Therefore, Yamanaka's group is obtaining cells from a donor with homozygous alleles of the HLA-A, -B, and -DR loci to establish iPSC stocks in Japan. In terms of renal and bone marrow transplantation, survival rates would not be 100% even if grafts from a HLA 6 allele-matched donor were transplanted into an immunosuppressed recipient. Furthermore, it is unclear whether immune rejection of iPSC derivatives occurs in these settings. In addition, immune rejection might occur due to a mismatch between the donor and recipient in a locus other than HLA-A, -B, and -DR or in the minor histocompatibility antigen. Therefore, immunosuppressant treatment could still be necessary in current clinical trials using iPSC derivatives in Japan; however, because of their toxicity, we must consider minimizing the use of immunosuppressants. Recent clinical trials of fetal NSCs for SCI used transient immunosuppression. Assuming that transient immunosuppression is sufficient to graft iPSC derivatives, the discontinuation of immunosuppressant treatment will be time-limited. Moreover, tumorigenesis can occur even after the achievement of graft tolerance. In this case, other treatment strategies will be needed to ablate iPSC-derived tumors. Although other treatments, aside from surgery, have been proposed for brain and spinal cord tumors, including graft-derived tumors, none are curative. Immunotherapies have also been considered, but their effects are currently limited. In the present study, we induced the immune rejection of tumors derived from transplanted allogenic iPS-NSCs by discontinuing immunosuppressant treatment, which resulted in complete tumor rejection and no recurrence. This is the first report regarding the immune rejection of iPSC derivatives. Although there are various limitations, as mentioned above, this is a simple strategy with no side effects and should therefore be further tested as a means to ablate tumors following the transplantation of iPSC derivatives. We believe that these observations can lead to the control of the immune rejection of grafts and assist the realization of regenerative medicine.

Conclusions

In this study, hiPSC-NS/PCs were xenografted into the spinal cords of immunosuppressed mice, resulting in the formation of tumors with the histological characteristics of low-grade gliomas rather than teratomas. The hiPSC-NS/PC-derived tumors were successfully rejected by discontinuation of immunosuppressant treatment. The issue of hiPSC-NS/PCs tumorigenicity remains a major concern in regenerative medicine applications; therefore, we propose that the discontinuation of immunosuppressants can provide a "safety lock" to ablate the grafted cells in the case of tumor development after stem cell transplantation therapy for SCI.

Supporting Information

S1 ARRIVE Checklist.
(PDF)

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Author Contributions

Conceived and designed the experiments: GI YK HO MN. Performed the experiments: GI YK SN HI MT. Analyzed the data: GI YK HO MN. Contributed reagents/materials/analysis tools: GI YK. Wrote the paper: GI AI YT HO MN.

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Control of the survival and growth of human glioblastoma grafted into the spinal cord of mice by taking advantage of immunorejection.

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Abstract

Recent studies have demonstrated that transplantation of induced pluripotent stem cell-derived neurospheres can promote functional recovery after spinal cord injury in rodents, as well as in non-human primates. However, the potential tumorigenicity of the transplanted cells remains a matter of apprehension prior to clinical applications. As a first step to overcome this concern, this study established a glioblastoma multiforme xenograft model mouse. The feasibility of controlling immune suppression to ablate the grafted cells was then investigated. The human glioblastoma multiforme cell line U251 MG was transplanted into the intact spinal cords of immunodeficient NOD/SCID mice, or into those of immunocompetent C57BL/6J H-2kb mice treated with or without immunosuppressants (FK506 plus anti-cluster of differentiation (CD) 4 antibody (Ab), or FK506 alone). *In vivo* bioluminescent imaging was used to evaluate the chronological survival of the transplanted cells. The graft survival rate was 100% (n=9/9) in NOD/SCID mice, 0% (n=6/6) in C57BL/6J mice without immunosuppressant treatment, and 100% (n=37/37) in C57BL6/J mice with immunosuppressant treatment. After confirming the growth of the grafted cells in the C57/BL6J mice treated with immunosuppressants, immune suppression was discontinued. The grafted cells were subsequently rejected within 3 days in C57BL/6J mice treated with FK506 alone, as opposed to 26 days in C57BL/6J mice treated with FK506 plus anti-CD4 Ab. Histological evaluation confirmed the ablation of the grafted

cells. Although this work describes a xenograft setting, the results suggest that this immunomodulatory strategy could provide a safety lock against tumor formation stemming from transplanted cells.

Key words

Spinal cord injury, cell transplantation, spinal glioblastoma, xenograft model

INTRODUCTION

The central nervous system (CNS), including the brain and the spinal cord, has traditionally been considered a representative example of an organ system in which regeneration is difficult. However, this view is being overturned by recent progress in stem cell biology. Several studies recently demonstrated the effectiveness of neural stem/progenitor cell (NS/PC) transplantation for the repair of spinal cord injury (SCI) (14,20,28). However, clinical application of NS/PCs has not been realized in Japan due to ethical issues related to the use of cells derived from aborted fetal tissues. Under such circumstances, Yamanaka *et al.* (37) introduced several genes into somatic cells to create induced pluripotent stem cells (iPSCs) with embryonic stem cell (ESC)-like pluripotency and proliferative capacity. Recently, we showed the reparative efficacy of iPSC-derived NS/PCs in mouse and non-human primate SCI models (18,27,42).

Nonetheless, before clinical application of such cells, several concerns must be addressed. Perhaps the most important is the potential tumorigenicity of iPSC-derived NS/PCs. Genetic and epigenetic abnormalities acquired from their origin, in addition to reprogramming stress and the culture environment, all increase the tumorigenicity of human iPSC-derived cells. Moreover, a number of investigations reported the increasing incidence of teratomas and gliomas due to transplantation of "dangerous" ESC- and iPSC-derived NS/PC clones (4,9,17,21,44). To overcome the risk of tumor formation, safety screening of iPSC-derived NS/PCs prior to transplantation is essential. However, despite such screenings, the risk of tumorigenicity after transplantation is not eliminated. A recently developed Ganciclovir (GCV)-mediated cell ablation system employing

Herpes Simplex Virus type 1 thymidine kinase (TK) (the delta TK/GCV system) shows the potential to reduce the cancer risk (6). On the other hand, this system could still augment the risk of tumorigenesis resulting from gene transfer and/or insertional mutagenesis, and it has not yet used in clinical practice. Furthermore, a spinal cord tumor model to evaluate tumorigenesis has not been established.

From the viewpoints of the timing of the transplantation after the injury, safety concerns and cost performance, we assume allogeneic iPS-NSC transplantation into the injured spinal cord in first-in man study rather than autologous transplantation. Therefore, we hypothesized that allo-grafted iPS-NSCs derived tumors could be eliminated by inducing immunorejection without any adverse events. As a first step, to confirm the feasibility of this concept, we used human glioblastoma multiforme (GBM) U251 MG cells in this study because GBM is the most aggressive and toughest tumor against the immunorejection. The purpose of this study is to determine whether xeno-grafted GBM could be eliminated by discontinuing immunosuppressant without any adverse events such as deterioration of motor function. Here, we developed a novel spinal cord tumor model mouse by implanting U251 cells, followed by bioluminescence imaging (BLI). BLI accurately monitored grafted tumor growth, in addition to immunorejection. We transplanted the cells into the intact spinal cords of immunodeficient NOD/SCID mice, as well as those of immunocompetent C57BL/6J H-2kb mice with or without immunosuppressant treatment (FK506 plus anti-cluster of differentiation (CD4) antibody (Ab), or FK506 alone). We successfully induced the immunorejection of the grafted U251 cells in the xenograft setting of the C57BL/6J

mouse solely by discontinuing immunosuppressant treatment without any adverse events, suggesting that tumors arising from currently employed cell transplantation practices for the management of SCI could be ablated by withdrawing immunosuppressants.

MATERIALS AND METHODS

Cell culture and lentivirus transduction

Human male GBM U251 MG cells (kindly gifted from professor Ikeda, Yamaguchi University Graduate School of Medicine, Japan) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum and 5% penicillin/streptomycin, passaged using trypsin-EDTA, and then seeded into fresh culture medium. For transplantation data about human iPS-NS/PC (hiPS-NS/PC), cell culture and neural differentiation of hiPSCs (clone 253G1, Caucasian, 36 y/o, Female, human dermal fibroblast) were performed as previously described (18,21,27). The lentivirus was prepared and transduced into U251 cells as described previously (22,46). Briefly, a fusion HIV-1-based lentiviral vector (kindly gifted from Dr. Hara, Brain Science Institute, RIKEN, Japan) expressing ffLuc (Venus fused to firefly luciferase) under the control of the elongation factor promoter was used (10). This vector enabled grafted cells to be detected as strong bioluminescent ffLuc signals in live mice and also as green fluorescent protein (GFP)-positive cells by using an anti-GFP Ab in fixed spinal cord sections, because the Venus protein was originally modified from GFP (25). The concentrated virus was added to the culture

medium to infect U251 cells and hiPS-NS/PC.

Cell transplantation

Adult female NOD/SCID (N=9), C57BL/6J H-2kb (N=54) mice and BALB/cA H-2kd (N=5) mice (8–9-weeks old, 18–22 g; Clea Japan Inc., Tokyo, Japan) were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg body weight; Sankyo Life Tech Co., Ltd., Tokyo, Japan) and xylazine (10 mg/kg body weight; Bayer, Leverkusen, Germany). After laminectomy at the tenth thoracic spinal vertebra, the dorsal surface of the dura mater was exposed. U251 cells and hiPS-NS/PC with phosphate buffered saline (PBS) for vehicle (5×10^5 cells/2 μl) were injected with a glass micropipette at a rate of 1 μl/min with a Hamilton syringe (25 μl) and a stereotaxic microinjector KDS 310; Muromachikikai Co., Ltd., Tokyo, Japan). All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Keio University (Assurance No. 13020) and the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA).

BLI

The Xenogen-IVIS spectrum-cooled charge-coupled device optical macroscopic imaging system (Summit Pharmaceuticals International Corporation, Tokyo, Japan) was used for *in vitro* BLI, as well as for *in vivo* BLI to confirm the survival of transplanted cells. Monitoring was performed 1–2 times/week beginning 1 week after transplantation.

Briefly, D-luciferin (Promega KK Corporation, Tokyo, Japan) was administered by i.p. injection at a dose of 300 mg/kg body weight. Animals were placed in a light-tight chamber, and photons emitted from luciferase-expressing cells were collected. The integration time was fixed at 1 min for each image. All images were analyzed with Living Image software (Caliper Life-Sciences, Hopkinton, MA, USA). The BLI signal was quantified in maximum Radiance units (photons/sec/cm^2/steradian (p/s/cm^2/sr)) and presented as log10 (photons/sec).

Immunosuppressive therapy

For the immunosuppressive therapy protocol, female C57BL/6J H-2kb mice were randomized to receive FK506 (Prograf®; Astellas Pharma Inc., Tokyo, Japan) plus anti-CD4 Ab (BioXcell, New Hampshire, USA) (N=37), or FK506 alone (N=11). FK506 was administered at a dose of 5 mg/kg once daily by subcutaneous injection, and anti-CD4 Ab was administered at a dose of 10 mg/kg by intraperitoneal injection on day −2 before transplantation and day 7 after transplantation. For hiPS-NS/PC transplantation study, female BALB/cA H-2kd mice (N=5) were randomized to receive FK506 and anti-CD4 Ab by same protocol and continuing once per week until 100 d after transplantation.

Motor function analysis

Hind limb motor function was evaluated using the locomotor rating test of the Basso Mouse Scale (3). Well-trained investigators, blinded to the treatments, performed

the behavioral analysis, and the Basso Mouse Scale scores were determined at the same time each day.

Histological analysis

Animals were anesthetized and transcardially perfused with 0.1 M phosphate buffered saline (Nacalai tesque Inc.) containing 4% paraformaldehyde (Nacalai tesque Inc., Kyoto, Japan). The spinal cords were removed, embedded in Optimal Cutting Temperature compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan), and sectioned in the sagittal plane on a cryostat (CM3050; Leica Microsystems, Wetzlar, Germany). Sections were stained with hematoxylin-eosin (HE) and the following primary Abs: anti-GPF Ab (rabbit IgG, 1:200; Frontier Institute Co., Ltd., Hokkaido, Japan), anti-human nuclear antigen (HNA) Ab (mouse IgG, 1:200; Chemicon), anti-Ki-67 Ab (rabbit IgG, 1:200; Novocastra, Newcastle upon Tyne, UK), anti-CD11b Ab (rat IgG2b 1:200; BD Pharmingen, Franklin Lakes, NJ, USA), anti-NKp46 Ab (rat IgG2a, κ, 1:500, BioLegend, San Diego, CA, USA), anti-terminal deoxynucleotidyl transferase (TdT) Ab (Apop Tag plus Fluorescein *in situ* apoptosis detection kit; Chemicon, Temecula, CA, USA), and anti-CD3 Ab (rat IgG1, 1:100; AbD Serotec). The samples were incubated with primary Abs at 4°C overnight. They were then incubated with secondary Abs conjugated to Alexa488, Alexa555, or Alexa633 (Invitrogen, Carlsbad, CA, USA) as appropriate for the primary Ab host species at a 1:500 dilution for 1 h at room temperature. Nuclei were stained with Hoechst 33258 (10 μg/ml; Sigma-Aldrich). Samples were examined on an inverted fluorescence microscope (BZ 9000; Keyence

Co., Osaka, Japan) or a confocal laser-scanning microscope (LSM 700, Carl Zeiss, Oberkochen, Germany).

Flow cytometry

Isolated peripheral blood leukocytes were analyzed by triple immunofluorescence staining followed by flow cytometry. The following Abs were all purchased from eBiosciences (San Diego, CA, USA): anti-CD3-APC Ab (clone 145-2C11), anti-CD4-FITC Ab (clone GK 1.5), and anti-CD8-PE Ab (clone 53-6.7), Armenian Hamster IgG Isotype Control APC (clone eBio299Arm), Rat IgG2b K Isotype Control FITC (eB149/10H5), Rat IgG2a K Isotype Control PE (eBR2a). The cells were stained with a mixture of these Abs at 4°C for 30 min. Flow cytometric analysis was performed on a fluorescence-activated cell sorting (FACS) Calibur (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

All data are presented as the mean ± the standard error of the mean (SEM). Repeated measures ANOVA followed by Bonferroni post-hoc test was used to determine significant differences in the BLI analysis. For all statistical analyses, the significance level was set at $p < 0.05$.

RESULTS

***In vitro* BLI of lentivirally transduced human GBM U251 cells**

U251 cells were cultured and labeled with ffLuc genes (Venus fused to luciferase) via lentiviral transduction, as reported previously (10) (Fig. 1A). Signals detected from these cells were sufficient for qualitative analysis by fluorescence microscopy (Fig. 1B, C). To examine the sensitivity of the BLI technique, the Xenogen-IVIS system was used to detect the luminescence intensity of various numbers of cells (5×10^5–3.5×10^6 cells per well) in the presence of D-luciferin, as reported previously (29). Quantitative analysis clearly revealed that the luminescence intensity *in vitro* was directly proportional to the cell number (Fig. 1D, E). The minimum number of cells that could be measured by their photon counts with *in vitro* BLI was approximately 1000. FACS caliber analysis revealed that >90% of these cells were GFP-positive (data not shown).

Survival of human GBM cells grafted into the spinal cords of immunodeficient and immunocompetent mice

Transduced U251 cells (5×10^5) were transplanted into the intact spinal cords of immunodeficient NOD/SCID (n=9) and immunocompetent C57BL/6J H-2kb (n=6) mice. The photon counts of the U251 cells grafted into C57BL/6J mice without immunosuppressant treatment decreased to background levels at 15 days after transplantation, suggesting that immunorejection of the cells occurred within 2 weeks in all six C57BL/6J mice. By contrast, the photon counts of the U251 cells grafted into the NOD/SCID mice sharply increased at 2 weeks after transplantation, suggestive of the survival and growth of the grafted cells in each of the immunodeficient mice (Fig. 2). These findings indicate that even in the immune-privileged CNS of C57BL/6J mice,

xenografted glioma cells were rejected at about 2 weeks without immunosuppressant treatment. Consistently, histological analysis revealed that U251 cells grafted into NOD/SCID mice survived well and migrated into the host spinal cord at 28 days after transplantation. The resulting tumor showed nuclear atypia, microvascular proliferation, necrosis, and Ki-67 index was 18% (Fig. 3A, B).

Immunorejection of human GBM U251 cells grafted into C57BL/6J mice after discontinuation of immunosuppressant treatment, resulting in functional recovery

To avoid the immunorejection of the xenografts, human GBM U251 cells were transplanted into C57BL/6J mice treated with immunosuppressants. Long-term survival of the grafted cells was achieved, similar to the situation in NOD/SCID mice. The graft survival rate was 82% (N=9/11) in the FK506 group, 100% (N=37/37) in the FK506/anti-CD4 Ab group, and 100% in NOD/SCI mice (Table 1). With the increase in the size of the tumor, almost all mice showed complete paralysis of the hind limbs at 21–28 days after transplantation.

To induce the immunorejection of the xenografted tumor cells, the administration of immunosuppressants was discontinued at different time points after transplantation. In the FK506 group, the immunosuppressive therapy was discontinued at 28 days after cell transplantation, which induced the immunorejection of grafted U251 cells within 1 week (Fig. 4A). By contrast, in the FK506/anti-CD4 Ab group, immunosuppressive therapy was discontinued at 7 or 14 days after transplantation. In five of 17 mice in the FK506/anti-CD4 Ab group, immunorejection of grafted U251 cells was achieved by