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Combined treatment of tumor-tropic human neural stem cells containing the CD suicide gene effectively targets brain tumors provoking a mild immune response

SE JEONG LEE[1-3*], YONGHYUN KIM[1,2*], MI-YOUNG JO[1,2], HYEONG-SEOK KIM[1,2], YOUNGGEON JIN[1,2], SEUNG U. KIM[4,5], JUYOUN JIN[1,2], KYEUNG MIN JOO[2,3] and DO-HYUN NAM[1,2]

[1]Department of Neurosurgery, Samsung Medical Center, Sungkyunkwan University School of Medicine; [2]Cancer Stem Cell Research Center, Samsung Biomedical Research Institute; [3]Department of Anatomy, Seoul National University College of Medicine, Seoul, South Korea; [4]Division of Neurology, UBC Hospital, University of British Columbia, Vancouver, Canada; [5]Medical Research Institute, Chung-Ang University College of Medicine, Seoul, South Korea

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Abstract. Previous studies showed promise of coupling genetically engineered neural stem cells (NSCs) with blood-brain barrier permeable prodrugs as an effective anti-brain tumor therapy. Here, we further advance those findings by testing the suicide gene therapeutic system in syngenic glioblastoma immunocompetent mice. After intracranial injection of HB1.F3.CD, an immortalized human NSC cell line engineered to constitutively produce cytosine deaminase (CD), the prodrug 5-fluorocytosine (5-FC) was administered for five days, q.d., via intraperitoneal injection. The HB1.F3.CD hNSCs migrated specifically to the brain tumor site via the corpus callosum and significantly reduced the tumor volume (67%) by converting 5-FC into the cytotoxic 5-fluorouracil. A corresponding increase in F4/80-positive population was observed in the treatment group, although CD3-positive population remained unchanged compared to control. No toxic effects or morphological changes were observed in the spleen and the lymph nodes. The data suggest that the NSC-enzyme/prodrug treatment is an effective anti-tumor therapeutic strategy that specifically targets only the tumor site with little or no systemic side effects. In addition, the treatment modeled here successfully elicited a macrophagic immune response which seemed to have a synergistic role in reducing tumor volume, thus showing promise for treatment-mediated enhancement of inherent immune responses against brain tumors.

Correspondence to: Dr Kyeung Min Joo, Department of Anatomy, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-799, South Korea
E-mail: kmjoo@snu.ac.kr

Professor Do-Hyun Nam, Department of Neurosurgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea
E-mail: nsnam@skku.edu

*Contributed equally

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Introduction

Glioblastoma, the most common and aggressive form of brain tumor arising from glial cells, is often treated with surgical resection, radiotherapy, and/or chemotherapeutic agents as first-line treatment. However, surgical resection and radiation is sometimes not a viable option when the tumor is diffused and infiltrative (1,2). In these cases, systemic chemotherapy is widely used, but the intact blood-brain barrier is largely impermeable, making many chemotherapeutic drugs ineffective (2).

To overcome such problems, more specific targeted delivery of therapeutic agent is desirable, and the recent discovery that neural stem cells (NSCs) possess tumor-tropic properties made it viable to use them as delivery agent (3-6). In particular, previous studies showed that NSCs genetically engineered to possess suicide genes can be an effective treatment against animal models with glioblastoma, medulloblastoma, metastatic neuroblastoma, and breast cancer brain metastasis (6-13). We and others previously showed that the NSCs engineered with therapeutic cytosine deaminase (CD) gene can specifically target brain tumors and convert the systemically administrated prodrug 5-fluorocytosine (5-FC) into the toxic anticancer agent 5-fluorouracil (5-FU) (9,10).

These previous studies, however, were performed with immunodeficient animal models. To bring this promising therapeutic closer to the clinic, the subsequent question then was to see NSC interaction in systems with intact immune system. In particular, we were curious if the delivery of CD+5-FC would invoke the immune system to synergistically target brain tumors. Therefore, in this study, we address the use of NSCs engineered with the CD gene in syngenic glioblastoma-bearing immunocompetent mice. We show that

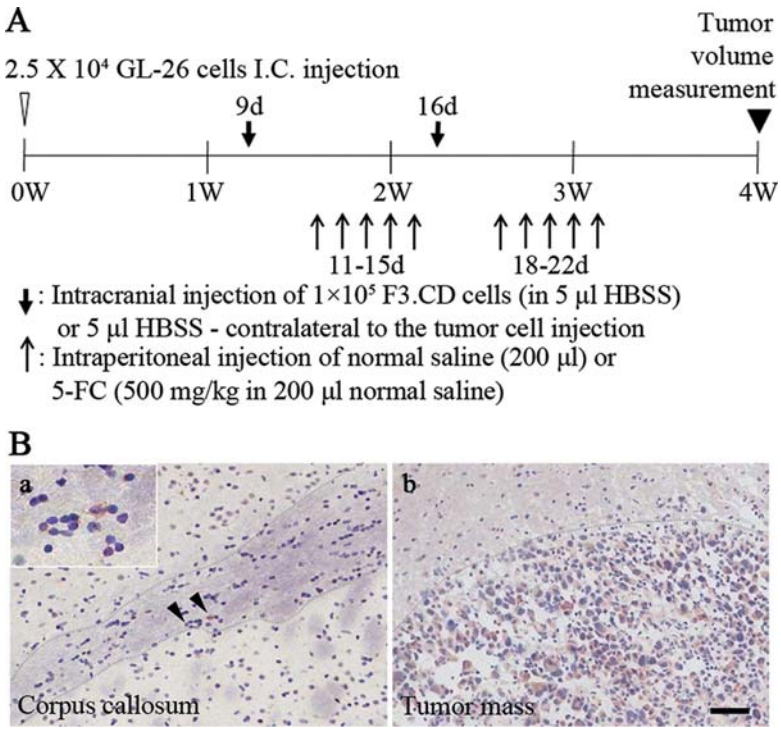

Figure 1. Combined treatment of HB1.F3.CD hNSCs and 5-fluorocytosine (5-FC) specifically target brain tumor mass in immunocompetent C57BL/6 mice. (A) Timeline for the syngenic brain tumor animal model and schematic of treatment using HB1.F3.CD hNSCs and prodrug 5-FC. (B) HB1.F3.CD hNSCs were localized specifically in corpus callosum (panel a) and tumor mass (panel b). Closed arrowheads indicate cytosine deaminase (CD) immunoreactive HB1.F3.CD hNSCs and the inset shows a magnified field. (Scale bar, 50 μm; HBSS, Hanks' balanced salt solution; i.p., intraperitoneal).

the NSC-enzyme/prodrug therapy is similarly effective in reducing tumor volume as it was in immunodeficient glioma mouse model, and that the treatment triggers only a mild macrophagic response.

Materials and methods

Cell culture. The clonal HB1.F3.CD human NSC (hNSC) line was derived from the parental HB1.F3, an immortalized hNSC line derived from human fetal brain (the ventricular zone) at 15 weeks of gestation and immortalized using an amphotropic, replication-incompetent retroviral vector containing v-*myc* (7,9). An expression plasmid encoding *Escherichia coli* CD was constructed using the retroviral pBABEpuro backbone and the 1.5 kb CD cDNA. Vectors were packaged by cotransfection of pA317 cells with the CD puro plasmid and the MV12 envelope-coding plasmid. CD puro retroviral supernatant was used for multiple infections of F3 cells. Transduced HB1.F3.CD cells were selected with 3 μg/ml puromycin (Invitrogen, Grand Island, NY) over four weeks (7,9). HB1.F3.CD cells were maintained as adherent cultures in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen). The murine glioma cell line GL-26, derived from C57BL/6 (14), were maintained RPMI-1640 containing 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen).

Syngenic glioblastoma animal model. All animal experiments were approved by the Institutional Review Boards of the Samsung Medical Center (Seoul, Korea) and conducted in accord with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised in 1996). For syngenic GBM model, anesthetized 6-week-old male C57BL/6 (H-2^b) mice were secured in a rodent stereotactic frame. A hollow guide screw was implanted into a small drill hole made at 2 mm left and 1 mm anterior to the bregma, and 2.5×10^4 GL-26 cells in 5 μl Hanks' balanced salt solution (HBSS) were injected through this guide screw into the white matter at a depth of 2 mm [anterior/posterior (AP) +0.5 mm, medial/lateral (ML) +1.7 mm, dorsal/ventral (DV) -3.2 mm].

In vivo therapeutic efficacy of HB1.F3.CD human NSCs. Thirteen and 20 days after GL-26 tumor cell injection, animals were subjected to contralateral injection (AP +0.5 mm, ML -1.7 mm, DV -3.2 mm) of 5 μl HBSS (groups 1 and 3; n=6 each) or 1×10^5 HB1.F3.CD hNSCs in 5 μl HBSS (groups 2 and 4; n=6 each). Forty-eight hours after the injection, the mice were further given intraperitoneal injections of control normal saline (NS; 200 μl in groups 1 and 2) or 5-FC (500 mg/kg in 200 μl NS in groups 3 and 4), q.d., for 5 days. Two days after the final intraperitoneal injection, brain, spleen, and lymph nodes were removed and cut into 4-mm thick slices. The portion of the brain with the largest tumor size was set aside for tumor volume calculation, while the remaining portion was cryoembedded into frozen blocks. For paraffin embedding, the brain slices were fixed in 10% formalin solution, embedded in paraffin, sectioned into 4-μm coronal sections using a microtome and stained with hematoxylin and eosin (H&E).

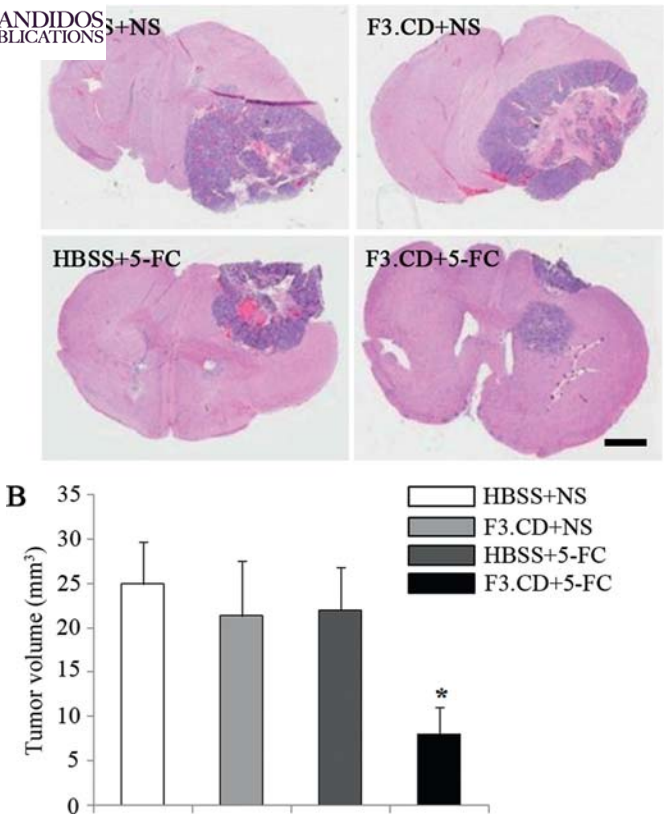

Figure 2. Combined treatment of HB1.F3.CD hNSCs and 5-FC has therapeutic effects in mouse glioma model. (A) Representative histology of mouse brains with the four treatment groups. The mice treated with HB1.F3.CD hNSCs and 5-FC showed significantly reduced glioma tumor volumes. (Scale bar, 2 mm) (B) Mean (columns) ± SE (bars) tumor volume for each group (n=6 per group). *p<0.05 compared to HBSS+NS control group.

Tumor volume was approximated as largest width^2 x largest length x 0.5. The spleen and lymph node were similarly fixed, embedded in paraffin, sectioned, and stained with H&E.

Immunohistochemistry of brain, spleen, and lymph node CD, CD3, and macrophage. Frozen blocks of brain, spleen, and lymph nodes were prepared by embedding in optimal cutting temperature compound (Miles, Elkhart, IN), then frozen rapidly in liquid nitrogen, and were cut into 8-μm coronal sections using a cryostat (Leica, CM3050S, USA). Paraffin blocks were prepared by embedding in paraffin and were sectioned using a microtome into 4-μm coronal sections. Immunohistochemistry staing was performed as previously described (9). Briefly, a rabbit anti-CD polyclonal antibody (1:100; kind gift from Dr Karen Aboody, City of Hope Medical Center, Duarte, CA), anti-CD3ε (Abcam, USA), or anti-F4/80 (MCA497, AbD Serotec, UK) was attached, followed by the avidin-biotin complex (Vector Laboratories, Burlingame, CA). The attached antibodies were visualized using 3,3'-diaminobenzidine (Sigma).

Statistical analyses. Statistical comparisons between groups were performed using one-way ANOVA or multiple comparison tests. $p<0.05$ was considered statistically significant.

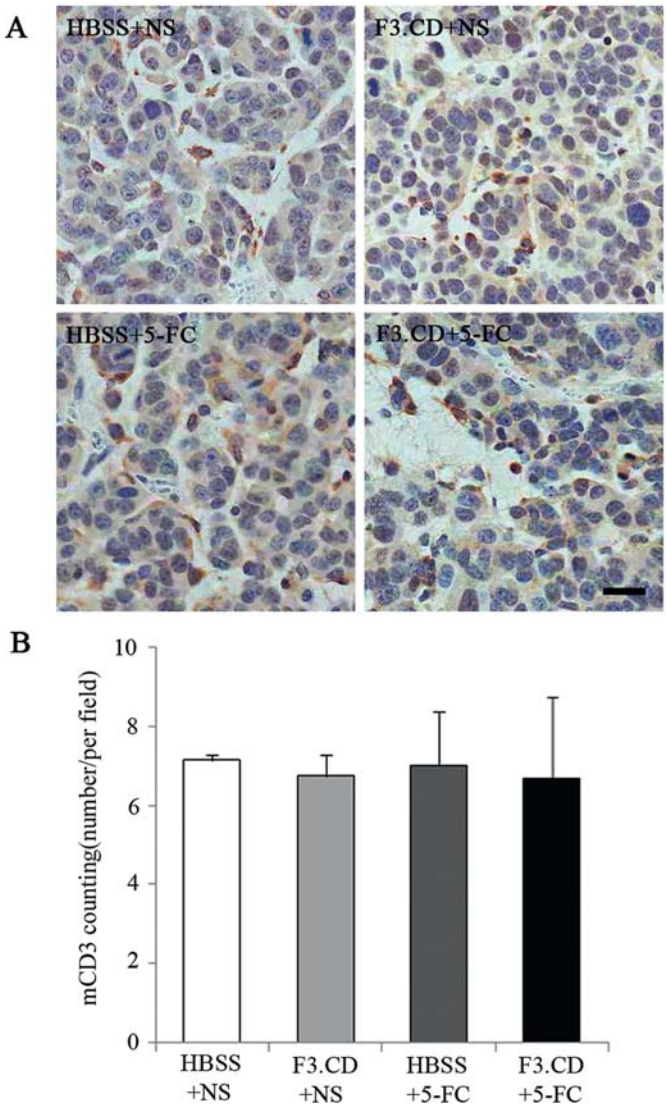

Figure 3. Combined treatment of HB1.F3.CD hNSCs and 5-FC does not effect T cell expression changes in mouse glioma model (A). Representative paraffin sections of tumors stained for mouse T cells using anti-mouse CD3 antibody (B). There was no qualitative difference in the number of T cell-positive cells between groups. (Scale bar, 50 μm, n=10; HBSS+NS; HB1.F3.CD+NS; HBSS+5-FC; HB1.F3.CD+5-FC).

Results

Therapeutic efficacy of HB1.F3.CD in syngenic mouse glioma model. To propose migrating potential and to reduce the tumor volume in mouse glioma syngenic model, C57BL/6 animals were given stereotactic intracranial injection of mouse glioma cells (2.5x10^4 GL-26 cell line). Thirteen days after the injection, HB1.F3.CD hNSCs were injected into the brain in the contralateral hemisphere to the tumor cell injection site (Fig. 1A). The mice were given five-day 5-FC q.d. treatment, and the distribution of HB1.F3.CD hNSCs and alterations in tumor volumes were determined after the final 5-FC treatment (Fig. 2). There were no physical abnormalities visible in any of the mice, including those with HB1.F3.CD injection (data not shown). CD-immunoreactive HB1.F3.CD cells were found specifically in the corpus callosum and tumor bed, confirming their tumor-tropic characteristics (Fig. 1B). Through histological analysis, the mean ± SE tumor volumes

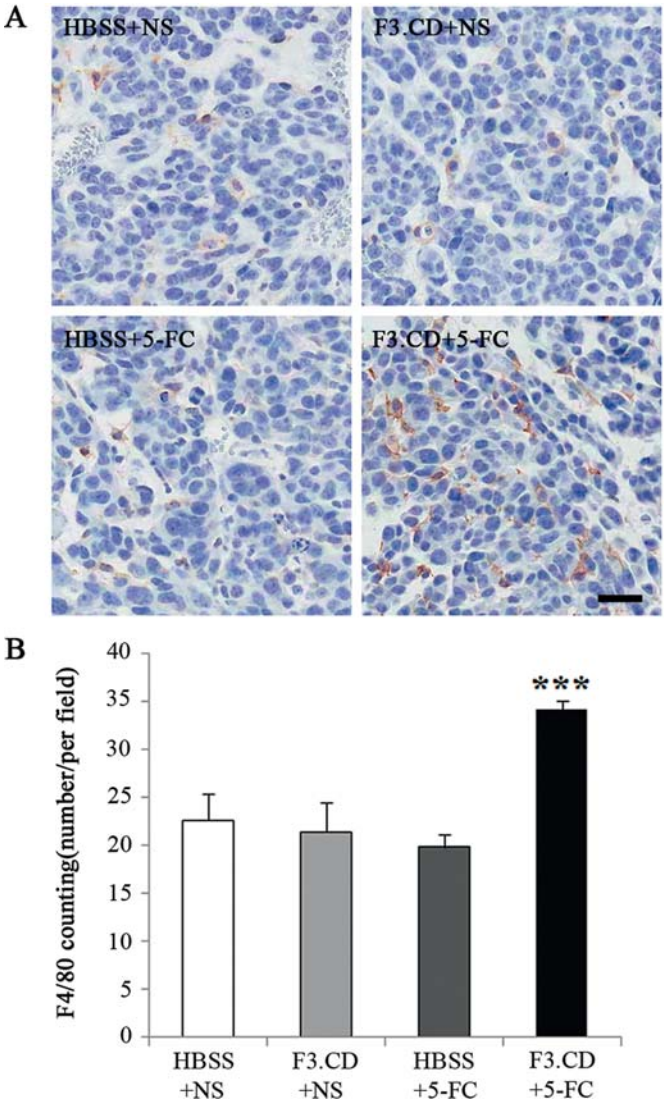

Figure 4. Combined treatment of HB1.F3.CD hNSCs and 5-FC increases macrophage population in mouse glioma model (A). Paraffin sections of tumors stained for mouse macrophage specific marker, F4/80 antibody (B). Immunostaining analysis of tumor-infiltrating macrophage showed an increased number of macrophage-positive cells in HB1.F3.CD+5-FC treatment groups. (Scale bar,50 μm, n=10, HBSS+NS; HB1.F3.CD+NS; HBSS+5-FC; HB1.F3.CD+5-FC; $^{***}p<0.001$).

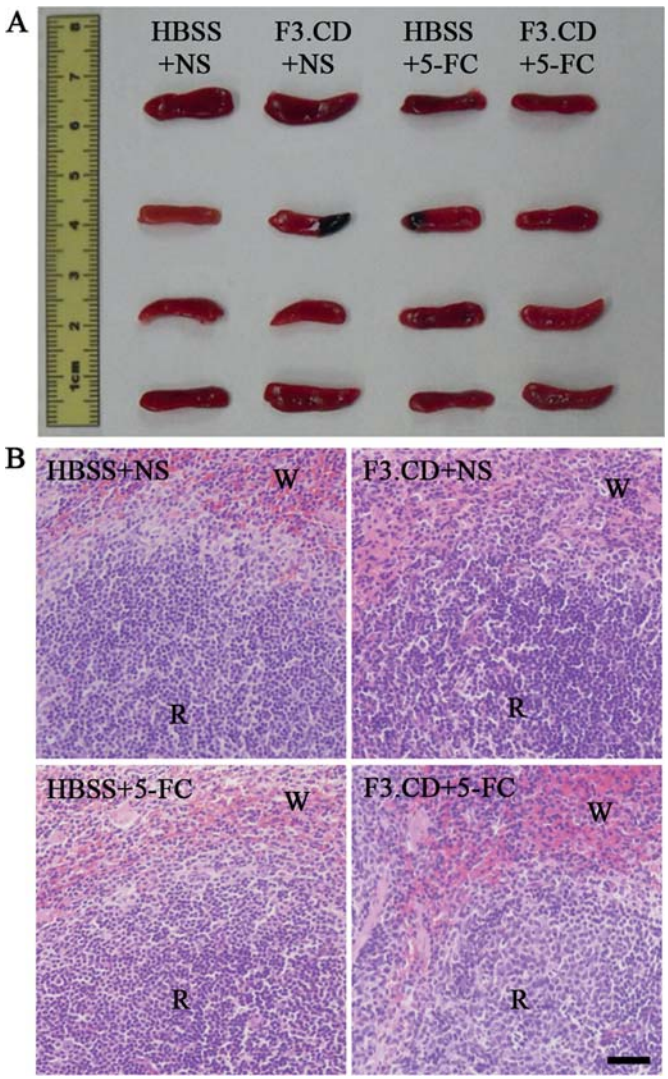

Figure 5. Combined treatment of HB1.F3.CD hNSCs and 5-FC does not effect morphological changes or toxicity in mouse glioma model. (A) Spleen from each mouse in the following groups is represented. There were no observable changes in the HB1.F3.CD cell treatment group. (B) Hematoxylin and eosin staining of mouse spleen. There were no toxicity and morphological changes in any group. (Scale bar, 50 μm, HBSS+NS; HB1.F3.CD+NS; HBSS+5-FC; HB1.F3.CD+5-FC; R, red pulp; W, white pulp).

were calculated as 24.9±4.7, 21.3±6.1, 22.0±4.7, and 8.2±3.00 mm^3 for the HBSS+normal saline (NS) (group 1), HB1.F3.CD+NS (group 2), HBSS+5-FC (group 3), and HB1.F3.CD+5-FC (group 4) groups. In other words, the tumor volume was significantly reduced (67%) only in the brains of 5-FC-treated, HB1.F3.CD injected mice (Fig. 2).

Immunological response in HB1.F3.CD cell treatment in syngenic mouse glioma model. To analyze host immune response in HB1.F3.CD treated group, we examined the expression of mouse T cell marker (CD3) and macrophage specific membrane marker (F4/80) in the brain tumor by immunohistochemical staining. We observed a similar level of mouse CD3-positive T cells in the brains of all four groups (Fig. 3). However, there was a significantly increased number of F4/80-positive macrophages (per field of view) in HB1.F3.CD+5-FC treated brain tumors compared to the other groups (31% increase; Fig. 4).

Effects of HB1.F3,CD treatment on the systemic immune system. To further investigate the effects of the HB1.F3.CD treatment on the systemic immune system, we analyzed the morphological changes of spleen and cervical, axillary lymph nodes. There was no detectable splenic size difference and morphological changes in the four groups (Fig. 5). Also, the cervical and axillary lymph nodes in HB1.F3.CD cell treatment group showed no detectable level of histological changes compared to the other groups (data not shown). There was no discernable difference in the mouse T cell- and macrophage-positive cells in the spleen or lymph nodes in the four groups (Figs. 6 and 7).

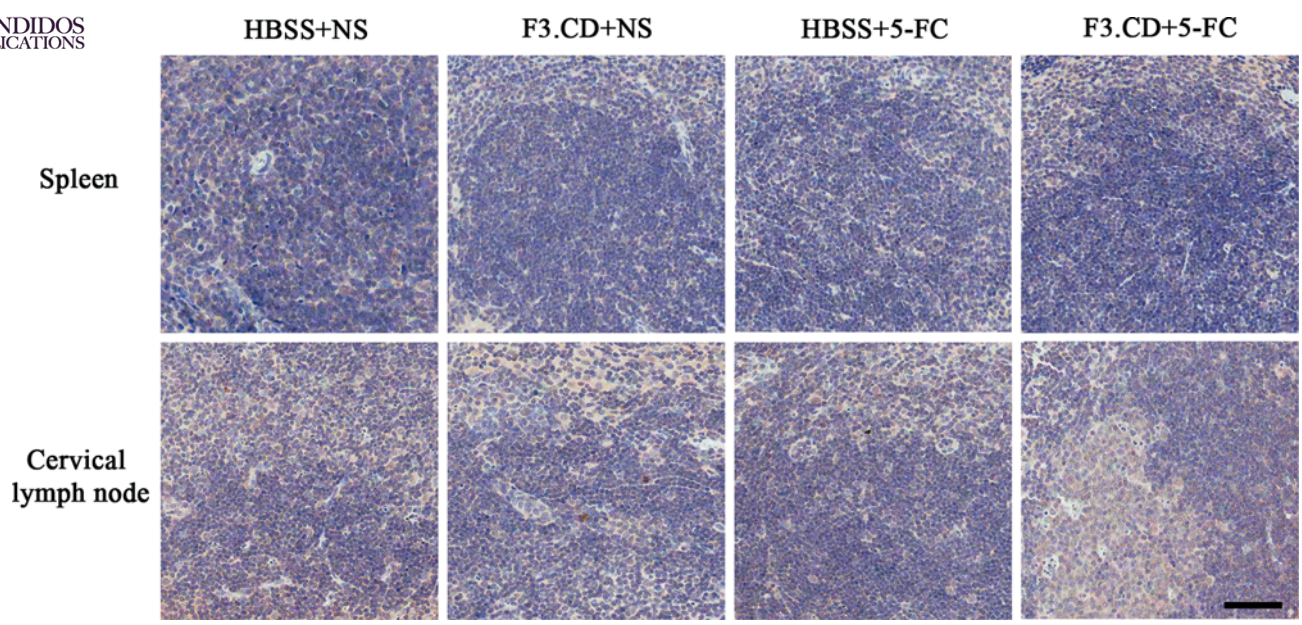

Figure 6. Immunostaining of mouse T cells in spleen and lymph node. Immunostaining of spleen and lymph node using mouse T cell marker-specific CD3 antibody. There was no difference in the number of positive mouse CD3 cells in the spleen and the cervical lymph node. (Scale bar, 50 μm, n=10).

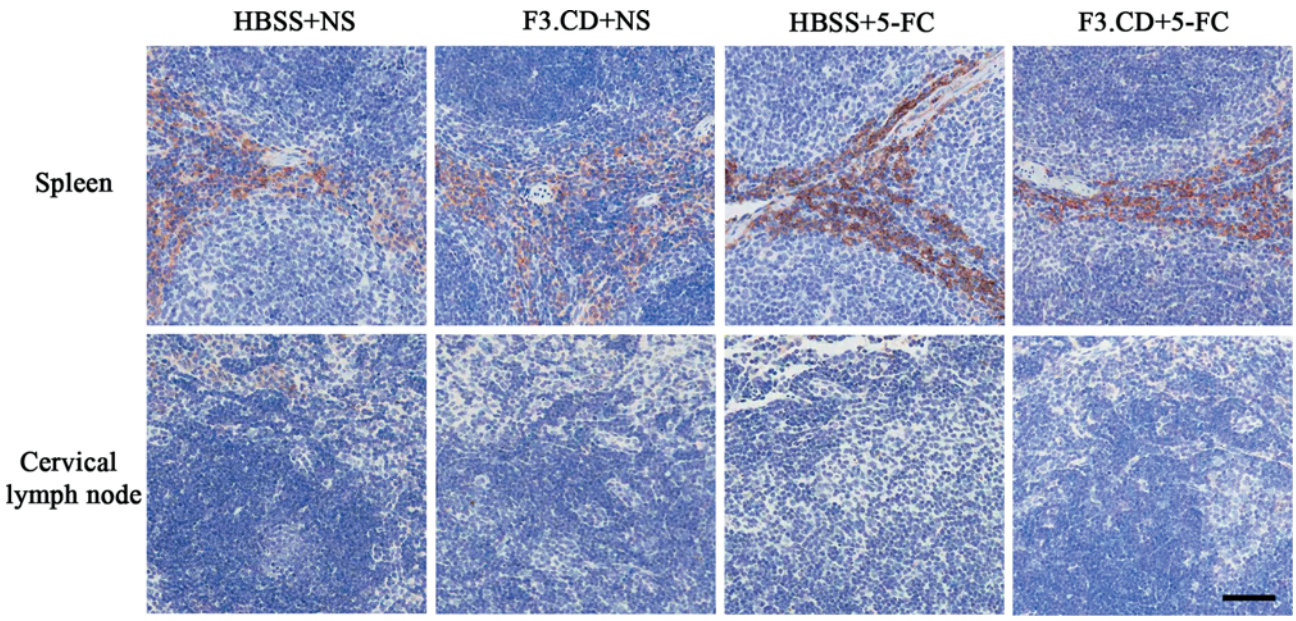

Figure 7. Immunostaining of macrophage in spleen and lymph node. Immunostaining of spleen and lymph node using the mouse macrophage-specific marker F4/80 antibody. There was no difference in the number of mouse macrophage-positive cells in the spleen and the cervical lymph node. (Scale bar, 50 μm, n=10).

Discussion

In this study, we show the possibility of using a combined treatment of the prodrug 5-fluorocytosine (5-FC) with tumor-tropic hNSCs carrying the CD suicide gene to effectively target a brain tumor in immunocompetent systems. Our data here and our previous study (9) both show that the hNSCs indeed migrate along the corpus callosum towards the tumor mass and effectively convert the prodrug to result in a dramatic decrease in brain tumor volume (Figs. 1B and 2). What has not been addressed previously was whether this treatment strategy would also be effective when the immune system is intact. Here, we showed that the engineered hNSCs and 5-FC caused a macrophagic response in the C57BL/6 mouse brain tumor (Fig. 4). There was a greater percent of tumor volume reduction in the C57BL/6 (this study) than in the BALB/c-nu (9), suggesting that the macrophages might have a synergistic therapeutic role. The macrophage increase was only seen in the tumor-bearing brain and not in the spleen and the lymph node (Fig. 7), suggesting that the hNSC-enzyme/prodrug

treatment elicited local, tissue-resident, and antigen-non-specific macrophage activation against the tumor cells, akin to a general inflammation response (15-19). On the other hand, we did not observe a detectable increase of CD3-positive T cells in the tumor location (Fig. 3), thus suggesting only a mild immune response. This, we speculate, may be due to tumor cells expressing and secreting immune suppressing molecules, such as TGF-ß which suppress T cell activation (20). In the future, if we could overcome such immune suppressing microenvironment, we may acquire even greater therapeutic effects with our system.

Prior to bringing this therapeutic strategy to the bedside, however, we must first address potential risks of using our v-*myc* immortalized hNSC (21). Our data here and previous experience with HB1.F3.CD did not thus far result in any detectable abnormalities or *de novo* tumor formation despite the use of the myc gene. Since the treatment results in suicide of the NSCs, we speculate that the risk posed by the myc gene will be minimal. Nevertheless, caution is warranted and further toxicity tests and long-term follow-up studies using syngenic NSCs will be required as further validation. Alternatively, using induced pluripotent stem cells (iPS) from adult fibroblasts (22,23) and NSC-like cells from adult bone marrow (24,25) may help bypass the use of the myc gene.

In summary, we report a promising therapeutic treatment against difficult-to-treat brain tumors by using a combined treatment of genetically engineered hNSCs with the prodrug 5-FC. The treatment was effective in our syngenic mouse model and successfully elicited a macrophagic immune response. It is therefore speculated that this mode of therapy can cooperate with the patients' immune system to effectively reduce the tumor mass.

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