

# Synthetic High-density Lipoprotein Nanodiscs for Personalized Immunotherapy Against Gliomas

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## ABSTRACT

**Purpose:** Gliomas are brain tumors with dismal prognoses. The standard-of-care treatments for gliomas include surgical resection, radiation, and temozolomide administration; however, they have been ineffective in providing significant increases in median survival. Antigen-specific cancer vaccines and immune checkpoint blockade may provide promising immunotherapeutic approaches for gliomas.

**Experimental Design:** We have developed immunotherapy delivery vehicles based on synthetic high-density lipoprotein (sHDL) loaded with CpG, a Toll-like receptor-9 agonist, and tumor-specific neoantigens to target gliomas and elicit immune-mediated tumor regression.

**Results:** We demonstrate that vaccination with neoantigen peptide-sHDL/CpG cocktail in combination with anti-PD-L1

immune checkpoint blocker elicits robust neoantigen-specific T-cell responses against GL261 cells and eliminated established orthotopic GL261 glioma in 33% of mice. Mice remained tumor free upon tumor cell rechallenge in the contralateral hemisphere, indicating the development of immunologic memory. Moreover, in a genetically engineered murine model of orthotopic mutant IDH1 (mIDH1) glioma, sHDL vaccination with mIDH1 neoantigen eliminated glioma in 30% of animals and significantly extended the animal survival, demonstrating the versatility of our approach in multiple glioma models.

**Conclusions:** Overall, our strategy provides a general roadmap for combination immunotherapy against gliomas and other cancer types.

## Introduction

Gliomas are devastating brain cancers with a median survival rate of approximately 15 months (1). Currently, the standard of care for patients diagnosed with glioma includes surgery, radiation, and chemotherapy, but they remain ineffective at significantly increasing median survival. Treatment effectiveness for glioma has been limited because of tumor heterogeneity, an immunosuppressive tumor micro-environment (TME), and the presence of the blood-brain barrier, which hampers the transport of therapeutics to the central nervous system (CNS; refs. 2, 3). Despite surgical resection, patients invariably develop disease progression and tumor recurrence due to residual tumor cells (4, 5).

Immunotherapy has recently emerged as a novel and attractive therapeutic platform for glioma (6–8). Immune checkpoint blockade

designed to reinvigorate immune responses against tumor has shown promising results across multiple types of solid cancer (9, 10). There are various ongoing clinical trials assessing therapeutic benefits of anti-PD-1 and anti-CTLA-4 therapies in patients with primary and recurring gliomas (11); however, clinical trial results have been unimpressive thus far. Phase III trial NCT02667587 reported in 2019 that nivolumab (anti-PD-1) combined with radiotherapy did not prolong overall survival of patients with glioma, compared with temozolomide and radiation (12). Initial results from a phase III trial (NCT02017717) examining the efficacy of nivolumab with or without ipilimumab (anti-CTLA-4) in patients with recurrent glioma have shown that nivolumab alone did not prolong overall survival, but the combination of nivolumab and ipilimumab is still under investigation (13). Hence, there is an urgent need for developing novel immune-mediated combination approaches for treating glioma.

A complementary approach to immune checkpoint blockade is to vaccinate patients against their own tumor cells using endogenous tumor-specific antigens or neoantigens (NeoAg; ref. 14). Initial clinical trials examining neoantigen-based vaccines against advanced melanoma and glioma have shown promising results (15–17). However, current neoantigen delivery methods, such as direct injection of neoantigen peptides admixed with adjuvants such as oil emulsions, often result in precipitation, accumulation, and sustained inflammation at the injection site with minimal lymphatic drainage, which can lead to immune tolerance and deletion of antigen-specific T cells at the injection site (18–20). Thus, new strategies are needed to improve the delivery of neoantigens and adjuvant molecules to antigen-presenting cells (APC) in lymphoid tissues to achieve potent antitumor immunity (21). An ideal neoantigen vaccine system should promote stable and efficient transport of neoantigen peptides to APCs in lymph nodes (LN) while allowing for colocalized delivery of both antigens and adjuvant molecules to the same APCs without causing an unwanted inflammatory response (21, 22).

To address these challenges, various “nano-vaccines” are under development for lymphatic trafficking and targeted delivery to

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### Translational Relevance

Only a subset of patients currently benefits from immune checkpoint inhibitors, thus highlighting an urgent need to improve cancer immunotherapy. Combination immunotherapies, including cancer vaccines, could boost T-cell immunity, but the efficacy of cancer vaccines has been limited, especially for gliomas. Here, we present a new strategy for personalized cancer vaccination against gliomas. Briefly, we have developed synthetic high-density lipoprotein (sHDL) loaded with CpG, a Toll-like receptor-9 agonist, and tumor-specific neoantigens to target gliomas and elicit immune-mediated tumor regression. We report that sHDL vaccination in combination with anti-PD-L1 immunotherapy elicits potent neoantigen-specific T-cell responses and leads to tumor regression, long-term survival, and immunologic memory. Our strategy provides a general roadmap for personalized vaccination for immunotherapy against gliomas and other cancer types.

APCs (22–25). In particular, we have previously demonstrated that synthetic high-density lipoprotein (sHDL) nanodiscs can effectively deliver neoantigens and a Toll-like receptor-9 (TLR9) agonist CpG to APCs in draining LNs and generate potent cytotoxic CD8 $\alpha^+$  T-cell lymphocyte (CTL) responses with promising antitumor efficacy (26). Here, we have employed the sHDL vaccine platform to generate neoantigen-specific CTLs against glioma and examined their antitumor efficacy in syngeneic models of GL261, which is a well-established murine model of glioma (27). We also examined whether neoantigen-based nanodisc vaccination can synergize with anti-PD-L1 immunotherapy. This is motivated by prior reports showing that some gliomas overexpress PD-L1 (28, 29), as well as recent encouraging results from an ongoing phase II clinical trial (NCT02336165) studying the combination of durvalumab and radiotherapy in patients with newly diagnosed glioma (30). Here, we report that the nanodisc platform in combination with anti-PD-L1 blockade induced robust infiltration of neoantigen-specific CD8 $\alpha^+$  T cells into the GL261 TME and achieved potent antitumor efficacy with long-term antitumor immunity in syngeneic mouse GL261 glioma model. Furthermore, we have also shown the therapeutic efficacy of nanodisc vaccination in a genetically engineered orthotopic model of mutant IDH1 (mIDH1) glioma expressing IDH1-R132H, shATRX, and shTP53 (31).

## Materials and Methods

### Materials

Neoantigen peptides were synthesized by RS Synthesis. Female C57BL/6 mice were purchased from Jackson Laboratory. Antibodies against mouse CD40 and PD-L1 were purchased from BioXCell. Polyinosine-polycytidylic acid (polyIC) was purchased from InvivoGen. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from NOF America. 22A Apolipoprotein-A1 mimetic peptide was synthesized by GenScript. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridylidithio)propionate] (DOPE-PDP) was purchased from Avanti Polar Lipids. Both cholesterol-modified CpG1826 (cho-CpG) and unmodified CpG1826 were synthesized by Integrated DNA Technologies. IFN $\gamma$  ELISPOT Kits were purchased from Thermo Fisher Scientific. Cell media were purchased from Invitrogen. The following antibodies for flow cytometry were

purchased from BD Biosciences: rat anti-mouse CD107a-APC clone 1D4B; hamster anti-mouse CD69-PE clone H1.2F3; rat anti-mouse CD8 $\alpha$ -Brilliant Violet 605, clone 53–6.7; rat anti-mouse Foxp3-PE, clone MF23; and rat anti-mouse CD25-PE-Cy7, clone PC61. The following antibodies for flow cytometry were purchased from BioLegend: anti-mouse CD8 $\alpha$ -APC, clone 53–6.7; anti-mouse CD279 (PD-1)-PE/Cy7; rat anti-mouse CD4-Brilliant Violet 605, clone: GK1.5; anti-mouse CD3-FITC, clone 17A2; rat anti-mouse CD86-PE/Cy7; anti-mouse CD11c-FITC, clone N418; and anti-mouse CD103-APC, clone 2E7. The following was purchased from eBioscience: anti-mouse MHC Class II (I-A/I-E)-PE, clone M5/114.15.2.

### Screening of GL261 neoantigen peptides

Neoantigen peptide sequences chosen from an immunogenomics study on murine glioma models published in 2016 (32) were computationally screened for predicted MHC reactivity using the artificial neural network method tool. Six of 10 neoantigen peptides identified in the study were synthesized and screened for *in vivo* immunogenicity according to the results from predicted MHC-binding affinities produced by the Immune Epitope Database Analysis Resource (Supplementary Table S1). Female C57BL/6 mice aged 6–7 weeks were shaved and inoculated with  $1 \times 10^6$  GL261 cells subcutaneously in the flank. On days 4 and 11 after tumor inoculation, 50  $\mu$ g of each neoantigen peptide was coadministered intraperitoneally with anti-CD40 (50  $\mu$ g) and Toll-like receptor-3 (TLR3) agonist polyIC (100  $\mu$ g). On day 26 after inoculation, all mice were euthanized for spleen extraction and IFN $\gamma$  ELISPOT analysis to evaluate and compare the immunogenicity of the neoantigen peptide candidates.

### Screening of mIDH1 neoantigen peptides

Peptide epitopes encompassing the mutant IDH1 region were reported previously (33), and from these, we chose two neoantigen epitopes (mIDH1<sub>123–132</sub> and mIDH1<sub>126–141</sub>) according to the results from predicted MHC-binding affinities produced by the Immune Epitope Database Analysis Resource.

### Formulation and characterization of sHDL nanodiscs carrying neoantigen peptides

sHDL nanodiscs were prepared by dissolving DMPC and 22A in acetic acid, lyophilizing the mixture, and rehydrating the mixture in 10 mmol/L sodium phosphate buffer, followed by thermocycling (34). Size of nanodisc was measured by dynamic light scattering (DLS). For incorporating neoantigen peptides into nanodiscs, neoantigen peptides were modified with a cysteine-serine-serine (CSS) sequence at the N-terminus for conjugation to thiol-modified lipids. Modified neoantigen peptides were mixed with pyridyl disulfide-modified phospholipid (DOPE-PDP) for 2–3 hours on an orbital shaker to form a lipid-peptide conjugate. To incorporate lipid-peptide conjugates into sHDL, their mixture was incubated on an orbital shaker at 200 rpm for 1 hour. cho-CpG was added by simple mixing of antigen-loaded nanodiscs and cho-CpG at a DMPC:cho-CpG weight ratio of 50:1. All nanodisc formulations were analyzed by Zetasizer to measure hydrodynamic size and zeta potential; by reverse-phase ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) and high-performance liquid chromatography (HPLC) to measure the extent of lipid-peptide conjugation and incorporation; and by gel permeation chromatography (GPC) to assess incorporation of cho-CpG.

### Combination immunotherapy in a subcutaneous model of GL261 tumor

All animal experiments were conducted in accordance with the approval by the Institutional Animal Care and Use Committee at the University of Michigan (Ann Arbor, MI). Immunocompetent female C57BL/6 mice (6–8 weeks old, Jackson Laboratory) were inoculated subcutaneously with  $1.2 \times 10^6$  GL261 cells in the flank. When tumors were palpable, mice were administered subcutaneously at the tail base with neoantigen peptide-sHDL/CpG cocktail, soluble neoantigen peptide cocktail + CpG, or PBS. Vaccines were given with a 7-day interval. A subset of animals received anti-PD-L1 intraperitoneally on days 1 and 4 after each vaccination. Injection dose was 15  $\mu$ g for each peptide, 15  $\mu$ g for CpG, and 100  $\mu$ g for anti-PD-L1 IgG. Mice were euthanized when tumors reached 1.5 cm in diameter. Long-term survivors that exhibited complete tumor regression were rechallenged 72 days after the boost vaccination with  $1.2 \times 10^6$  GL261 cells at the contralateral flank. For analysis of the TME, mice were treated as indicated and on day 8 after vaccination, tumors were harvested for flow cytometric analysis on a Bio-Rad Zeti Flow Cytometer. Tumors were digested into single-cell suspensions using a cocktail of DNase I and collagenase, followed by antibody staining and flow cytometry analyses.

### IFN $\gamma$ ELISPOT analysis

Six days after either prime or boost vaccination, blood samples were taken from the submandibular vein, or spleens were excised. Red blood cells were lysed and removed from the samples. For analysis of peripheral blood mononuclear cells (PBMC; ref. 35),  $0.1 \times 10^6$  PBMCs were plated in each well of an anti-IFN $\gamma$ -coated 96-well immunospot plate in RPMI media + 10% FBS + 1% penicillin/streptomycin. For analysis of splenocytes,  $0.5 \times 10^6$  splenocytes were plated in each well of a 96-well immunospot plate coated with anti-IFN $\gamma$  IgG in RPMI media + 10% FBS + 1% penicillin/streptomycin. Neoantigen peptides were dissolved in water, diluted in RPMI media, and incubated with PBMCs or splenocytes for 18 hours at 37°C. Plates were then processed according to the manufacturer's instructions and later read at Cancer Center Immunology Core at the University of Michigan (Ann Arbor, MI). The maximum detectable signal was 3,000 spots in each well.

### Combination immunotherapy in an orthotopic model of GL261 tumor

Immunocompetent female C57BL/6 or immunocompromised CD4<sup>-/-</sup> and CD8<sup>-/-</sup>-knockout (KO) mice (Jackson Laboratory) were stereotactically injected with 20,000 GL261 cells into the right striatum using a 22-gauge Hamilton syringe (1  $\mu$ L over 1 minute) with the following coordinates: +1.00 mm anterior, 2.5 mm lateral, and 3.00 mm deep to establish brain tumors (36–39). Mice were vaccinated subcutaneously at the tail base with the nanodisc vaccine or free neoantigen peptides and administered with anti-PD-L1 IgG intraperitoneally at indicated time points. Long-term survivors in the nanodisc treatment group were rechallenged by inoculating mice with GL261 cells in the contralateral (left) hemisphere. To assess the immune cell population within the GL261 TME in the brain, mice were euthanized 2 days after the third vaccination, and brains were extracted. Tumor mass was dissected and homogenized using Tenbroeck (Corning) homogenizer in DMEM containing 10% FBS. Immune cell populations in the TME were enriched with 30%–70% Percoll (GE Lifesciences) density gradient. Live/dead staining was carried out using fixable viability dye (eBioscience). Nonspecific antibody binding was blocked with CD16/CD32, followed by staining with the following antibodies. Macrophages were labeled with CD45, F4/80, and CD206

antibodies. T cells were labeled with CD45, CD3, CD8 $\alpha$ , and CD4 antibodies. All antibodies were purchased from BioLegend. M1 macrophages were identified as CD45<sup>+</sup>/F4/80<sup>+</sup>/CD206<sup>low</sup>, and M2 macrophages were identified as CD45<sup>+</sup>/F4/80<sup>+</sup>/CD206<sup>high</sup>. Effector CD8 $\alpha$ <sup>+</sup> T cells were identified as CD45<sup>+</sup>/CD3<sup>+</sup>/CD8 $\alpha$ <sup>+</sup> and CD4<sup>+</sup> Th cells were identified as CD45<sup>+</sup>/CD3<sup>+</sup>/CD4<sup>+</sup>. T-cell exhaustion was assessed by staining for PD-1. Regulatory T cells (Treg) were identified as CD45<sup>+</sup>/CD3<sup>+</sup>/CD4<sup>+</sup>/CD25<sup>+</sup>/Foxp3. Antibody staining was carried out for 30 minutes at 4°C. Flow cytometry was performed using FACSaria Flow Cytometer (BD Biosciences) and analyzed using FlowJo version 10 (TreeStar; ref. 40).

### Nanodisc vaccination in an orthotopic model of mIDH1 tumor

Immunocompetent female C57BL/6 mice were stereotactically injected with 25,000 mIDH1 neurospheres (termed NPAI) into the right striatum using a 22-gauge Hamilton syringe (1  $\mu$ L over 1 minute) with the following coordinates: +1.00 mm anterior, 2.5 mm lateral, and 3.00 mm deep to establish brain tumors (36–39). Mice were vaccinated subcutaneously at the tail base with the nanodisc vaccine or free neoantigen peptides at indicated time points. Long-term survivors in the nanodisc treatment group were rechallenged by inoculating mice with mIDH1 cells in the contralateral (left) hemisphere.

### Statistical analysis

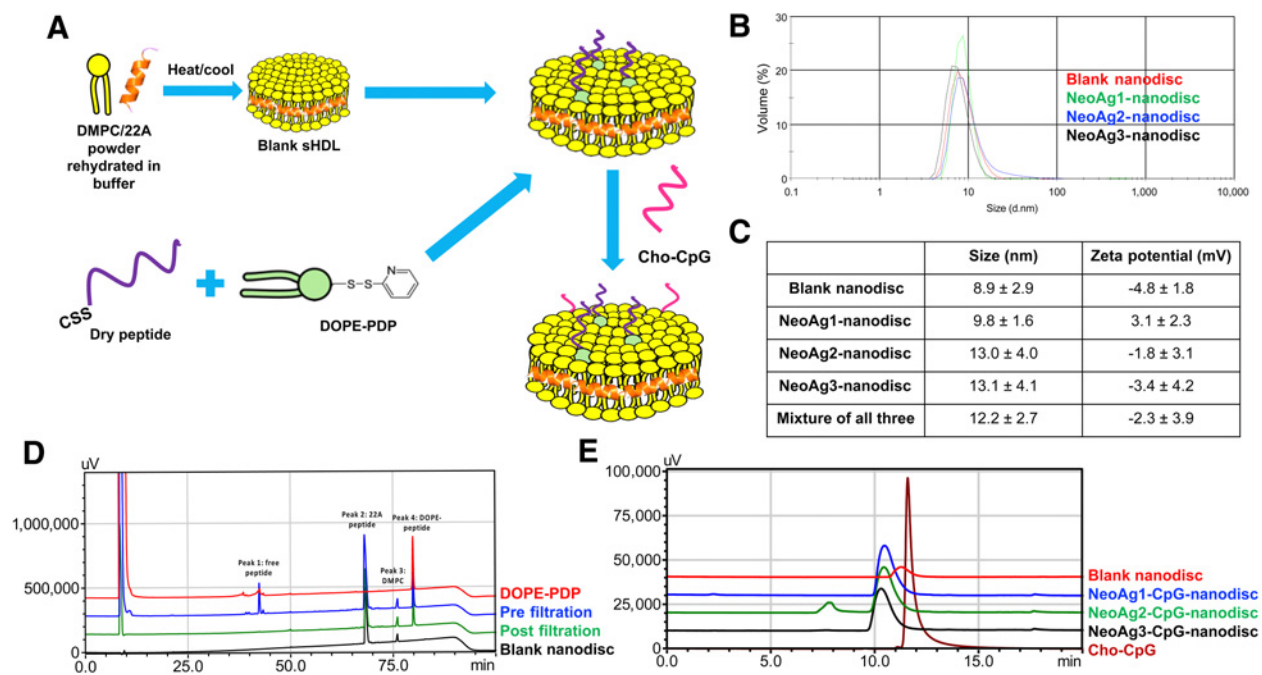
Sample sizes were chosen based on preliminary data from pilot experiments. For animal studies, the mice were randomized to match similar primary tumor volume, and all procedures were repeated at least twice in a nonblinded fashion. The results are expressed as mean  $\pm$  SEM. Statistical analysis was performed with two-tailed *t* tests for individual group comparisons or one-way ANOVA, followed by Tukey *post hoc* analyses for multiple comparison tests with Prism 8.0 Software (GraphPad Software). Analyses of survival differences were performed using Kaplan–Meier survival analyses with log-rank Mantel–Cox. Statistical significance is indicated as \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; and \*\*\*\* *P* < 0.0001.

## Results

### Selection and validation of GL261 neoantigens

Employing recently published 12 neoantigen sequences from GL261 murine tumors by Johanns and colleagues (32), we first subjected these neoantigen peptide sequences for predicted binding to MHC-I using an MHC-binding prediction tool (IEBD) and selected six neoantigens with low predicted IC<sub>50</sub> values and mutated residues residing between the third and seventh peptides in the epitope sequence (refs. 41, 42; Supplementary Table S1). To further narrow down neoantigen candidates, we examined immunogenicity of the top six neoantigens in GL261 tumor-bearing mice. C57BL/6 mice were inoculated subcutaneously at flank with  $10^6$  GL261 cells. On days 4 and 11 after tumor inoculation, 50  $\mu$ g of each neoantigen peptide was administered intraperitoneally with 50  $\mu$ g anti-CD40 IgG and 100  $\mu$ g polyinosine-polyIC. Anti-CD40 IgG and polyIC are a potent adjuvant combination known to amplify antigen-specific T-cell responses (43, 44). Anti-CD40 IgG enhances dendritic cell (DC) survival, cytokine release, and upregulation of costimulatory receptors (45), while a TLR3 agonist polyIC promotes cytokine release from DCs. Three of six peptides coadministered with anti-CD40 IgG and polyIC elicited strong T-cell responses, as evidenced by high IFN $\gamma$  ELISPOT counts, comparable with the positive control (Supplementary Table S1). In particular, we have identified three neoantigen epitopes with robust *in vivo* immunogenicity, namely AALLNKYLA

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**Figure 1.** Development and characterization of neoantigen-loaded sHDL nanodiscs. **A**, Schematic of the formulation process for neoantigen-loaded sHDL nanodiscs. **B**, Representative DLS profiles for each glioma neoantigen-sHDL formulation. **C**, The hydrodynamic size and zeta potential of each glioma neoantigen-sHDL formulation. **D**, Representative HPLC chromatograms for one glioma neoantigen-sHDL formulation. **E**, GPC chromatogram set for all glioma neoantigen-sHDL formulations after loading cholesterol-modified CpG.

(NeoAg1, H2-D<sup>b</sup>-restricted), MSLQFMTL (NeoAg2, H2-K<sup>b</sup>-restricted), and GAIFNGFTL (NeoAg3, H2-D<sup>b</sup>-restricted).

### Synthesis of nanodiscs carrying GL261 neoantigens

Having identified three top candidate neoantigens, we next synthesized sHDL nanodiscs incorporated with each neoantigen. **Figure 1A** shows the overall schematic for the synthesis of sHDL nanodiscs coloaded with neoantigens and CpG. Blank sHDL nanodiscs were first prepared using DMPC and 22A apolipoprotein-A1 mimetic peptide. Next, neoantigen peptides premodified with a CSS linker were conjugated with DOPE-PDP, and the peptide-lipid conjugate was added to blank nanodiscs (**Fig. 1A**). Each nanodisc formulation was analyzed with DLS to assess the particle size. Blank nanodiscs as well as nanodiscs carrying each neoantigen peptide all had similar particle sizes ranging 9–13 nm, indicating that the addition of neoantigens did not significantly change the size of sHDL. NeoAg1-nanodisc had a positive charge of  $3.1 \pm 2.3$  mV, while NeoAg2-nanodisc and NeoAg3-nanodisc had negative charges of  $-1.8 \pm 3.1$  mV and  $-3.4 \pm 4.3$  mV, respectively (**Fig. 1C**). We also examined whether nanodiscs carrying each neoantigen were compatible when mixed all together. Three nanodisc formulations combined into one cocktail yielded a stable mixture of nanodiscs with an average diameter of  $12.2 \pm 2.7$  nm and a negative charge of  $-2.3 \pm 3.9$  mV (**Fig. 1C**).

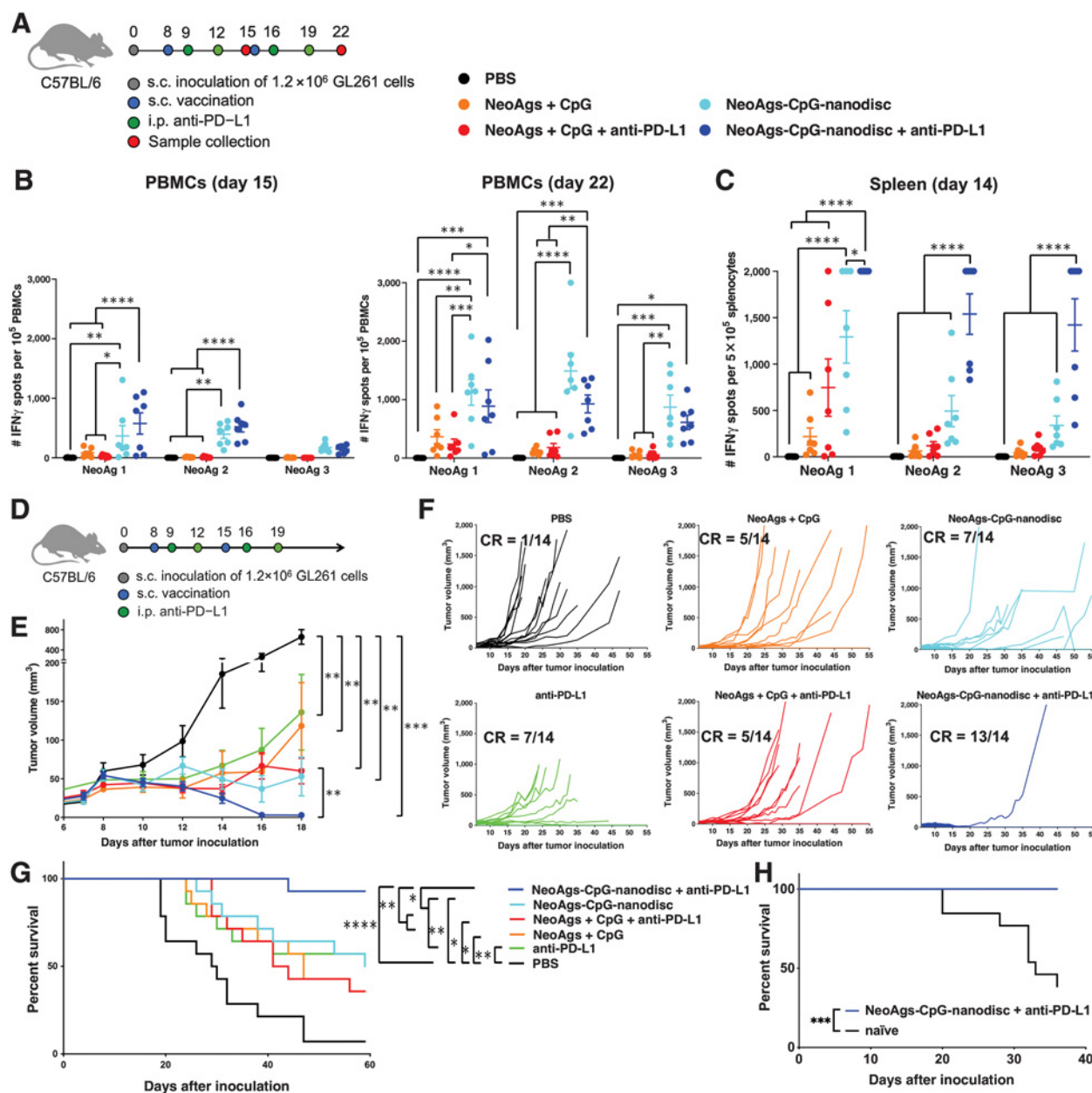
We quantified the amount of neoantigen peptides loaded into nanodiscs using UPLC/MS and HPLC. We observed successful conjugation of all three neoantigen peptides to DOPE lipid with >99% efficiency and >90% incorporation efficiency of neoantigen-lipid conjugates into nanodiscs (**Fig. 1D**), as quantified by the amount of neoantigen-lipid conjugates remaining before and after filtration of

nanodiscs. HPLC chromatograms also showed disappearance of the free peptide peaks after filtration, which indicated efficient removal of free peptide from neoantigen-loaded nanodiscs. Nanodiscs were subsequently incubated with cho-CpG, a TLR9 agonist, by simple mixing at a DMPC:cho-CpG weight ratio of 50:1. GPC analysis confirmed >99% incorporation of cho-CpG, resulting in nanodiscs coloaded with neoantigens and CpG (NeoAgs-CpG-nanodisc; **Fig. 1E**).

### Therapeutic efficacy of nanodisc vaccination combined with immune checkpoint therapy

To identify the optimum neoantigen dose and combination, we next evaluated the immunogenicity and antitumor effects of sHDL nanodisc vaccination combined with anti-PD-L1 IgG immune checkpoint therapy. C57BL/6 mice were inoculated with  $1.2 \times 10^6$  GL261 tumor cells at subcutaneous flank and vaccinated on days 8 and 15 with NeoAg1, NeoAg2, and NeoAg3 in either soluble or nanodisc forms. A subset of animals also received intraperitoneal administration of 100  $\mu$ g anti-PD-L1 IgG on days 1 and 4 after each vaccination (**Fig. 2A**). Soluble peptide vaccination with neoantigens + CpG induced detectable levels of T-cell responses after the boost immunization as shown by IFN $\gamma$ <sup>+</sup> ELISPOT assay performed with PBMCs (**Fig. 2B**). Notably, compared with soluble vaccination with neoantigens + CpG, NeoAgs-CpG-nanodisc vaccination significantly improved T-cell responses against all three neoantigens, generating approximately 3-fold ( $P < 0.01$ ), 6-fold ( $P < 0.0001$ ), and 4-fold ( $P < 0.01$ ) higher IFN $\gamma$ <sup>+</sup> T-cell responses to NeoAg1, NeoAg2, and NeoAg3, respectively (**Fig. 2B**). ELISPOT assay performed with splenocytes also indicated potent IFN $\gamma$ <sup>+</sup> T-cell responses against all three neoantigens (**Fig. 2C**). The addition of anti-PD-L1 IgG therapy to nanodisc vaccination further augmented



**Figure 2.**

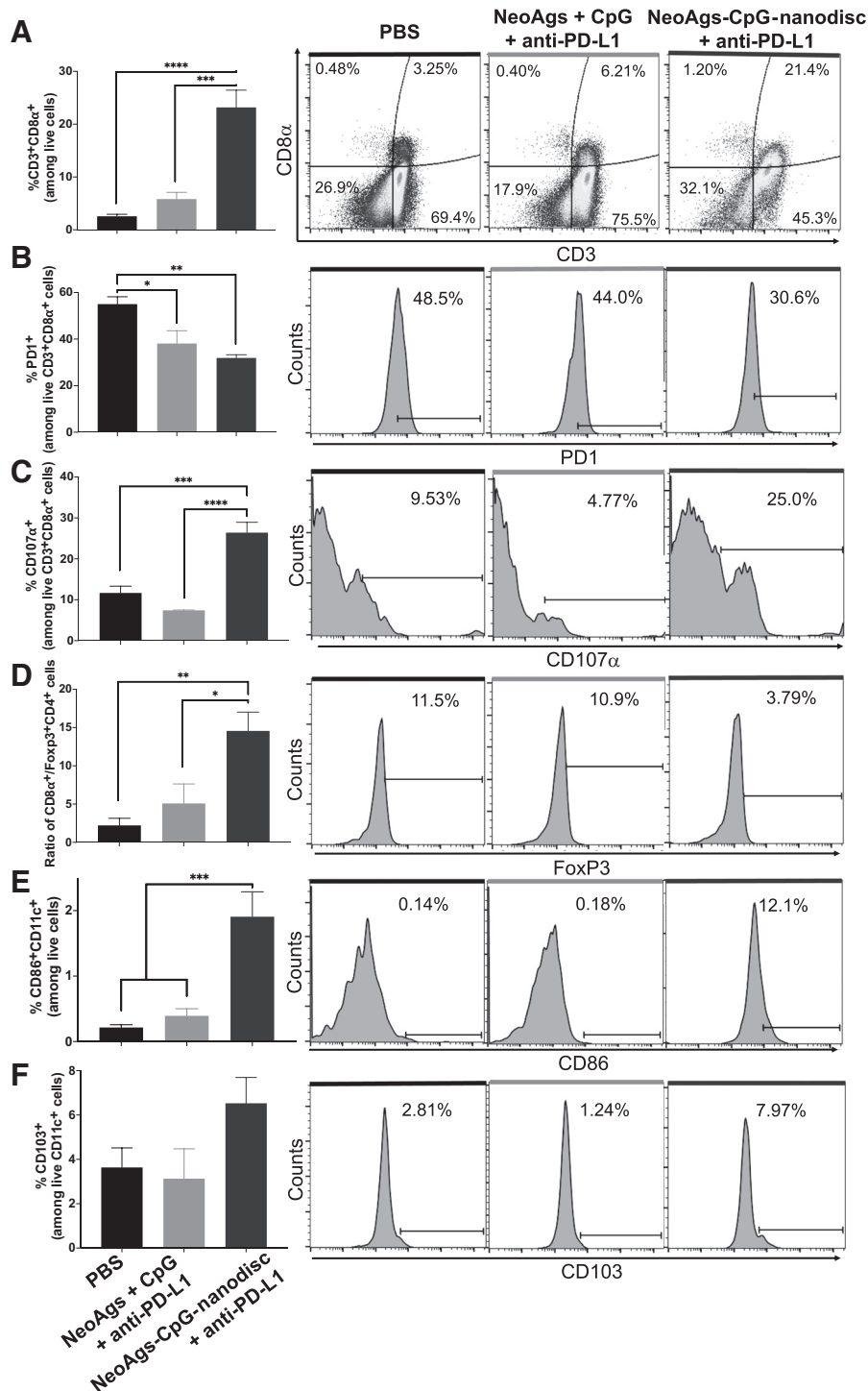
Neoantigen-specific T-cell responses and antitumor efficacy exerted by NeoAgs-CpG-nanodisc and anti-PD-L1 therapy in subcutaneous flank GL261 tumor model. **A**, Treatment regimen and study timeline ( $n = 7$  per group). **B**, IFN $\gamma$  ELISPOT assays were performed using PBMCs on 7th day after prime (left) and boost (right) vaccinations. **C**, IFN $\gamma$  ELISPOT assays were performed using splenocytes on 6th day after prime vaccination. **D**, Treatment regimen and study timeline for combination therapy. **E**, Tumor growth summary for all treatment groups ( $n = 14$  per group). **F**, Individual tumor growth curves for all animals in the study. **G**, Kaplan-Meier overall survival curves. **H**, On day 90, surviving mice from the NeoAgs-CpG-nanodisc + anti-PD-L1 group were rechallenged with GL261 cells in the contralateral flank. CR, complete response; i.p., intraperitoneal; s.c., subcutaneous. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

neoantigen-specific T-cell responses, as shown by the splenocyte ELISPOT assay (Fig. 2C).

We next examined the therapeutic efficacy of nanodisc vaccination combined with anti-PD-L1 IgG therapy. C57BL/6 mice bearing GL261 at subcutaneous flank were treated as above (Fig. 2D). Nanodisc vaccination alone efficiently slowed tumor growth (Fig. 2E) and eliminated tumors in 7 of 14 animals (Fig. 2F). As T-cell exhaustion is widely reported in GL261 tumors (46), we combined nanodisc vac-

ination with anti-PD-L1 IgG therapy, which led to stronger antitumor efficacy ( $P < 0.01$ ; Fig. 2E) and elimination of established tumors in 13 of 14 animals (Fig. 2F). On the other hand, soluble neoantigen vaccination with or without anti-PD-L1 IgG therapy had only 5 of 14 mice with complete response (Fig. 2F). Overall, nanodisc vaccination plus anti-PD-L1 IgG therapy resulted in approximately 90% animal survival rate (Fig. 2G), representing a significant improvement over all other treatment conditions. When

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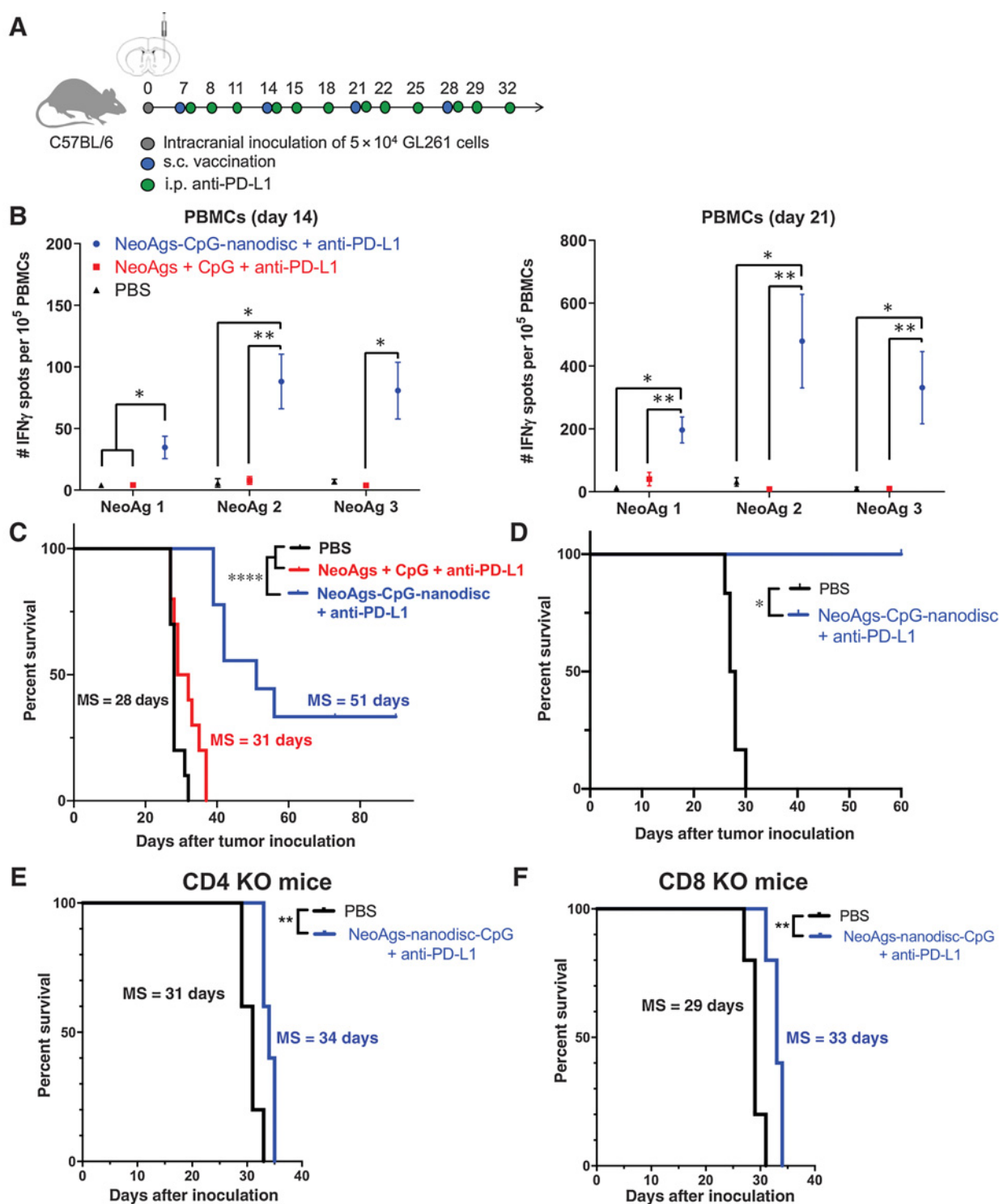
**Figure 3.**

Immune activation within the TME after NeoAgs-CpG-nanodisc plus anti-PD-L1 therapy. **A-F**, C57BL/6 mice inoculated subcutaneously with  $1.2 \times 10^6$  GL261 tumor cells were vaccinated on day 20 and administered anti-PD-L1 on days 21 and 24. On day 26, tumor tissues were stained with antibodies and analyzed by flow cytometry for CD8 $\alpha$ <sup>+</sup> T cells, Tregs, and DCs ( $n = 4$  per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

rechallenged with GL261 tumor cells on day 72 after the boost vaccination, all surviving animals from the nanodisc plus anti-PD-L1 IgG group were protected against tumor growth (Fig. 2H), indicating long-term antitumor memory response. Taken together, these results demonstrated that nanodisc vaccination combined with immune checkpoint blockade therapy exerted potent and durable T-cell responses with robust antitumor efficacy against subcutaneous GL261 tumors.

#### Immune activation within the TME

We next performed flow cytometry analyses on subcutaneous GL261 tumor-bearing mice and examined the impact of combination immunotherapy on the TME. Nanodisc vaccination combined with anti-PD-L1 IgG therapy promoted robust intratumoral infiltration of CD8 $\alpha$ <sup>+</sup> T cells into subcutaneous GL261 tumors (Fig. 3A). Intratumoral CD8 $\alpha$ <sup>+</sup> T cells in the nanodisc + anti-PD-L1 IgG group exhibited 1.5-fold decrease in PD-1 expression ( $P < 0.01$ , compared

**Figure 4.**

Antitumor efficacy exerted by NeoAgs-CpG-nanodisc and anti-PD-L1 therapy in an orthotopic GL261 tumor model. **A**, Treatment regimen and study timeline. **B**, IFN $\gamma$  ELISPOT assays were performed using PBMCs on day 7 after prime (left) and boost (right) vaccinations ( $n = 3$  per group). **C**, Kaplan-Meier overall survival curves for all treatment groups ( $n = 10$  per group). **D**, Kaplan-Meier survival analysis of long-term survivors from **C** rechallenged with GL261 cells in the contralateral hemisphere. **E**,  $CD4^{-/-}$ -KO mice (**E**) or  $CD8^{-/-}$ -KO mice (**F**) carrying orthotopic GL261 tumors were treated with nanodisc vaccine + anti-PD-L1 therapy as in **A** and monitored for survival ( $n = 5$  per group). i.p., intraperitoneal; s.c., subcutaneous. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ .

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with PBS; **Fig. 3B**) and 2.5-fold increase expression of degranulation marker CD107 $\alpha$ , compared with soluble neoantigen vaccination ( $P < 0.0001$ ) or PBS control ( $P < 0.001$ ; **Fig. 3C**). Nanodisc + anti-PD-L1 IgG also increased the absolute number of CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells, PD-1<sup>+</sup>CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells, and CD107 $\alpha$ <sup>+</sup>CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells within the tumor tissues, compared with the PBS control ( $P < 0.05$ ; Supplementary Fig. S1). We also observed a 6-fold increase in the ratio of CD8 $\alpha$ <sup>+</sup> T cells to CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in animals treated with nanodisc + anti-PD-L1 IgG, compared with soluble vaccine + anti-PD-L1 IgG ( $P < 0.05$ ) or PBS control ( $P < 0.01$ ; **Fig. 3D**). Moreover, intratumoral DCs in animals treated with nanodisc + anti-PD-L1 IgG treatment exhibited an activated phenotype with increased expression of CD86 and CD103 (**Fig. 3E and F**).

### Therapeutic efficacy of nanodisc vaccination in an orthotopic GL261 glioma model

Having shown immunogenicity and potency of nanodisc vaccination in the subcutaneous flank model, we proceeded to assess the therapeutic efficacy of nanodisc vaccination in an orthotopic glioma model. C57BL/6 mice were inoculated with 20,000 GL261 cells via stereotactic injection into the right striatum on day 0. Animals received 4 weekly immunizations of soluble or nanodisc vaccines at subcutaneous tail base, starting day 7 post-tumor implantation. Animals also received intraperitoneal administration of 100  $\mu$ g anti-PD-L1 on days 0, 1, and 4 after each vaccination (**Fig. 4A**).

ELISPOT assay performed on PBMCs indicated that nanodisc vaccine + anti-PD-L1 therapy elicited potent IFN $\gamma$ <sup>+</sup> T-cell responses against all three neoantigens (**Fig. 4B**). A single cycle of nanodisc vaccination and anti-PD-L1 therapy improved IFN $\gamma$ <sup>+</sup> T-cell responses against NeoAg1, NeoAg2, and NeoAg3 by 7-fold ( $P < 0.05$ ), 8-fold ( $P < 0.01$ ), and 15-fold ( $P < 0.05$ ), compared with soluble vaccine + anti-PD-L1 (**Fig. 4B**). Neoantigen-specific T-cell responses were further augmented after the second cycle of nanodisc vaccine + anti-PD-L1 therapy, as shown by 5-fold, 100-fold, and 30-fold higher IFN $\gamma$ <sup>+</sup> T-cell responses (NeoAg1, NeoAg2, and NeoAg3, respectively;  $P < 0.01$ ; **Fig. 4B**), compared with the soluble vaccine + anti-PD-L1 group.

All animals treated with soluble vaccine + anti-PD-L1 succumbed to tumor growth within 40 days without any statistical difference from the PBS control group (**Fig. 4C**). In stark contrast, nanodisc vaccine + anti-PD-L1 therapy exerted significantly enhanced antitumor efficacy, resulting in complete response in 3 of 9 mice (33% complete response) without any signs of recurrence until day 90 ( $P < 0.0001$ ; **Fig. 4C**). To assess for long-term immunity, survivors in the nanodisc vaccine + anti-PD-L1 group were rechallenged on day 90 by stereotactic injection of GL261 cells into the contralateral hemisphere; the animals did not show any signs of neurologic deficits during 60 days of observation (**Fig. 4D**). Moreover, we also tested an abbreviated treatment regimen (three cycles of vaccination plus three administrations of anti-PD-L1 therapy). Nanodisc vaccine + anti-PD-L1 group had a slightly reduced complete response rate of approximately 15%, which still represented a significant improvement over the soluble vaccine + anti-PD-L1 therapy group ( $P < 0.001$ ; Supplementary Fig. S2). Overall, nanodisc vaccine combined with anti-PD-L1 therapy exerted strong antitumor efficacy in a murine model of orthotopic glioma. To evaluate whether the efficacy of this treatment was dependent on the host's T cells, we inoculated GL261 cells in the brains of CD4<sup>-/-</sup> or CD8<sup>-/-</sup>-KO mice and then treated the animals with nanodisc vaccine + anti-PD-L1 as above. Nanodisc vaccine + anti-PD-L1 therapy had minimal impact on animal survival (**Fig. 4E and F**). These results indicate the critical role played by the CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> T cells

in mediating a therapeutic response for NeoAgs-CpG-nanodisc + anti-PD-L1 therapy.

### Intratumoral infiltration of CD8 $\alpha$ <sup>+</sup> T cells within CNS

We performed flow cytometric analyses on GL261 glioma tumors isolated from CNS on day 23 from the above experiment. Nanodisc vaccine plus anti-PD-L1 therapy promoted a significant (~3.4-fold) increase in the frequency of intratumoral CD8 $\alpha$ <sup>+</sup> T cells ( $P < 0.0001$ ) with approximately 2-fold lower expression level of PD-1 ( $P < 0.0001$ ), compared with the PBS control group (**Fig. 5A and B**; Supplementary Fig. S3A). Nanodisc + anti-PD-L1 therapy also significantly decreased the frequency of CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs ( $P < 0.001$ ; **Fig. 5C**; Supplementary Fig. S3B), resulting in 6.7-fold increase in the ratio of CD8 $\alpha$ <sup>+</sup> T cells to Tregs ( $P < 0.05$ ; **Fig. 5D**). We also observed 4-fold higher ratio of M1-like macrophages (CD45<sup>+</sup>F4/80<sup>+</sup>CD206<sup>-</sup>) to M2-like macrophages (CD45<sup>+</sup>F4/80<sup>+</sup>CD206<sup>+</sup>) in the TME of GL261 tumor-bearing mice treated with nanodisc + anti-PD-L1 combination immunotherapy ( $P < 0.001$ ; **Fig. 5E**). On the other hand, we did not observe difference in the levels of activation markers (CD80, CD86, and MHC-II) on intratumoral DCs in nanodisc vaccine plus anti-PD-L1 versus PBS treatment groups (Supplementary Fig. S3C).

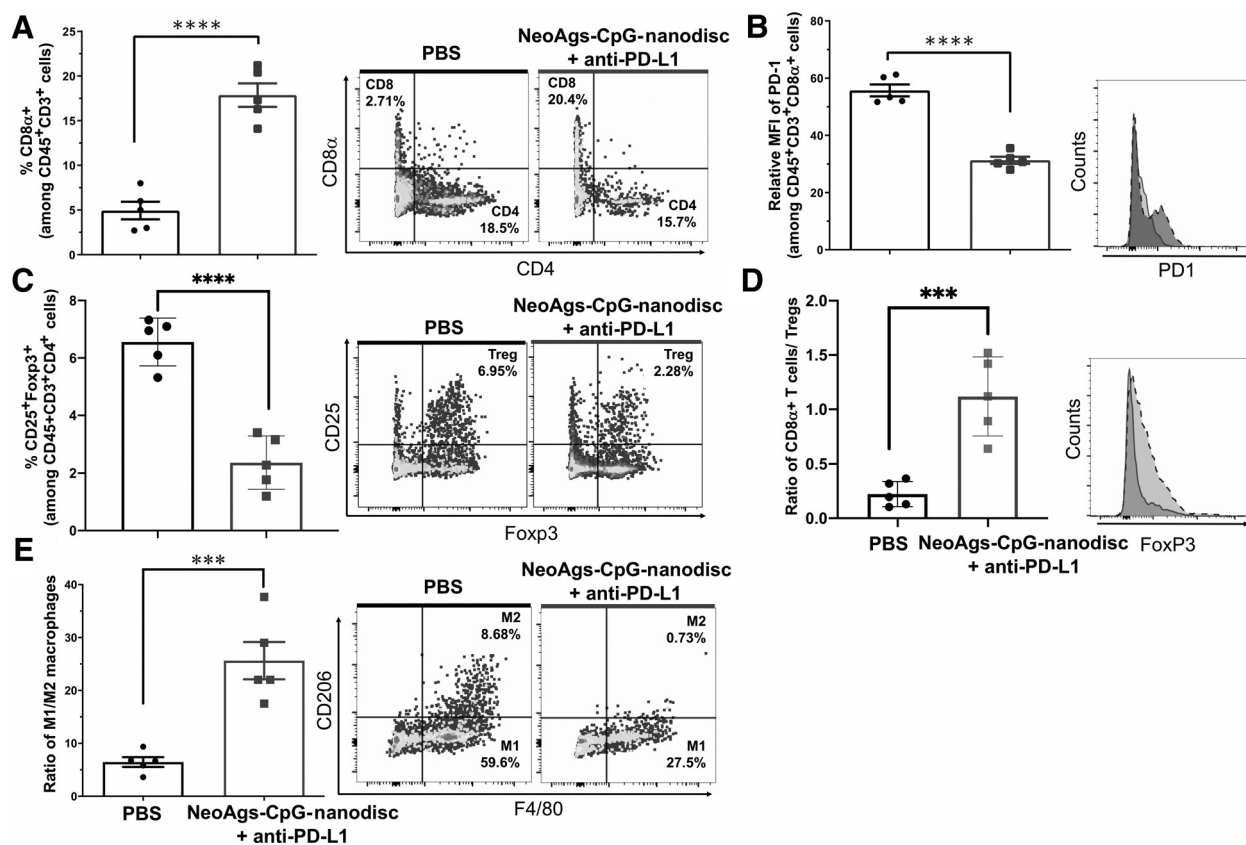
### Therapeutic efficacy of nanodisc vaccination in an orthotopic mIDH1 glioma model

Having shown immunogenicity and potency of nanodisc vaccination in an orthotopic GL261 glioma model, we proceeded to assess the therapeutic efficacy of nanodisc vaccination in a genetically engineered murine model of mIDH1 glioma (31). C57BL/6 mice were inoculated with 25,000 mIDH1 neurospheres via stereotactic injection into the right striatum on day 0. Animals received three weekly immunizations of soluble or nanodisc vaccines at subcutaneous tail base, starting day 7 post-tumor implantation (**Fig. 6A**). We first tested nanodiscs carrying CpG with either mIDH1<sub>123-132</sub> or mIDH1<sub>126-141</sub> neoantigens, which are predicted to be good binders for MHC-I in C57BL/6. Nanodisc vaccination with either mIDH1<sub>123-132</sub> or mIDH1<sub>126-141</sub> significantly extended the survival with the median survival (MS) of 65 days and 59 days ( $P < 0.01$ ; **Fig. 6B**), compared with the MS of 35 days for PBS and 45 days for nanodisc-CpG. Moreover, when the survivors from both nanodisc groups were rechallenged with mIDH1 neurospheres in the contralateral hemisphere, 100% of the animals resisted tumor recurrence ( $P < 0.05$ ; **Fig. 6C**), suggesting nanodisc-mediated immune memory against mIDH1 glioma. However, the combination of nanodiscs delivering both mIDH1<sub>123-132</sub> and mIDH1<sub>126-141</sub> did not further extend the animal survival (MS = 63 days; Supplementary Fig. S4), potentially due to the overlapping T-cell responses against mIDH1<sub>123-132</sub> and mIDH1<sub>126-141</sub> epitopes.

## Discussion

In this work, we have successfully developed a nanodisc platform for neoantigen-based personalized vaccination against gliomas. We have demonstrated that (i) neoantigens with different physicochemical properties can be loaded onto sHDL nanodiscs with CpG (26); (ii) neoantigen-loaded sHDL nanodiscs in combination with anti-PD-L1 immune checkpoint blockade can elicit significantly greater systemic neoantigen-specific CD8 $\alpha$ <sup>+</sup> T-cell expansion when compared with soluble neoantigen peptides in both flank and orthotopic GL261 tumor models; and (iii) neoantigen-loaded sHDL nanodiscs when used in combination with anti-PD-L1 immune checkpoint blockade drive intratumoral infiltration of neoantigen-specific CD8 $\alpha$ <sup>+</sup> T cells and exert potent antitumor effects in an orthotopic model of glioma.

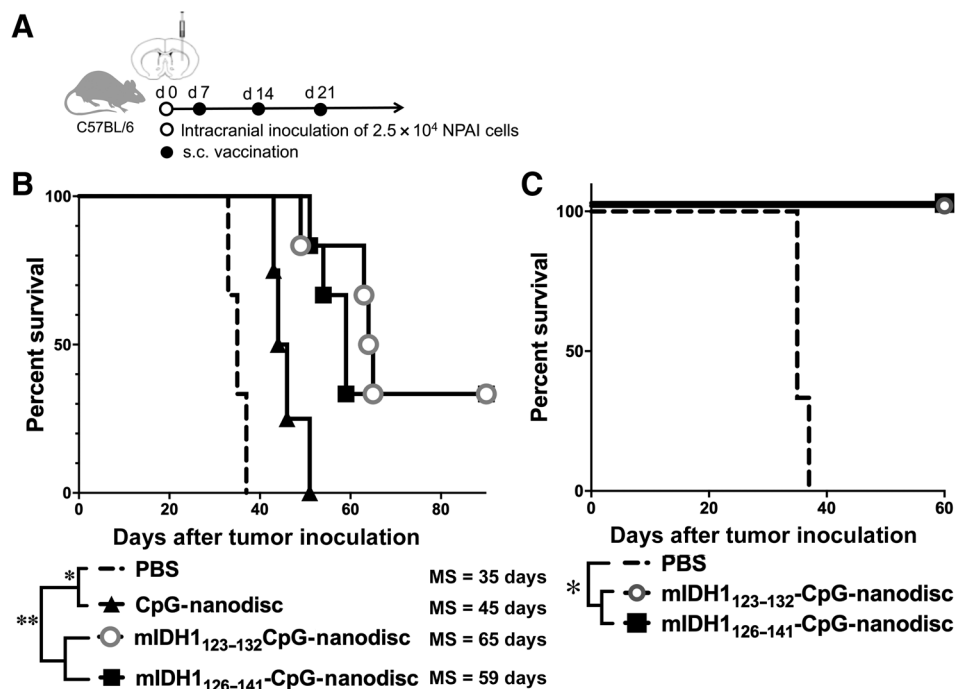


**Figure 5.**

TME analysis of GL261 tumors in CNS. **A-E**, C57BL/6 mice were inoculated with GL261 cells and treated as in Fig. 4, and tumors were isolated on day 23 and analyzed by flow cytometry ( $n = 5$  per group). CD8 $\alpha^+$  T cells among all T cells (**A**), PD-1 receptor expression on CD8 $\alpha^+$  T cells (**B**), CD25 $^+$ Foxp3 $^+$  Tregs among CD4 $^+$  T cells (**C**), ratio of CD8 $\alpha^+$  T cells to Tregs (**D**), and the ratio of M1 (CD206 $^-$ F4/80 $^+$ ) to M2 (CD206 $^+$ F4/80 $^+$ ) macrophages (**E**). MFI, mean fluorescence intensity. \*\*\*\*,  $P < 0.0001$ .

**Figure 6.**

Survival analysis of mIDH1 tumor (termed NPAI)-bearing mice vaccinated with mIDH1 neoantigens. **A**, Treatment regimen and study timeline. **B**, Kaplan-Meier overall survival curves for mice treated with the indicated vaccine formulations. **C**, On day 90, surviving mice were rechallenged with mIDH1 neurospheres in the contralateral hemisphere and monitored for survival ( $n = 3$  for PBS;  $n = 4$  for CpG-nanodisc; and  $n = 6$  for mIDH1-CpG-nanodisc). s.c. subcutaneous. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



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In particular, our initial screening of C57BL/6-derived GL261 neoantigen candidates narrowed our choices of neoantigens from the previously identified pool (32) to three unique peptides based on their significant immunogenicity in GL261 tumor-bearing mice. During optimization of the vaccine formulations, we found that all three neoantigen-loaded nanodisc formulations could be combined into one cocktail without precipitation (Fig. 1), which allowed us to test the antitumor potential of these neoantigens using a single formulation. In both flank and orthotopic glioma tumor models, we found that administration of a prime boost NeoAgs-CpG-nanodisc vaccination induced robust systemic expansion of IFN $\gamma$ -producing, neoantigen-specific CD8 $\alpha^+$  T cells (Figs. 2B and 4B). We have previously shown that the nanodisc platform allowed for efficient codelivery of neoantigens and CpG to DCs in draining LNs in various models of subcutaneous flank tumors (26, 47, 48); thus, based on these, we hypothesized that this general phenomenon would also induce CTL responses in orthotopic tumors located in the brain. Herein, we report that sHDL nanodisc platform provides a powerful and convenient strategy to generate neoantigen-specific T-cell responses against glioma. While nanodisc vaccine alone was not effective at extending the survival of GL261 tumor-bearing mice, potentially due to T-cell exhaustion (46), nanodisc vaccination in combination with anti-PD-L1 immune checkpoint blockade produced a complete response rate of 93% in mice bearing subcutaneous flank GL261 glioma tumor (Fig. 2D). Nanodisc combined with anti-PD-L1 induced the maturation of intratumoral DCs, followed by intratumoral infiltration of CD8 $\alpha^+$  T cells with CD107 $\alpha$  effector phenotype into the TME (Fig. 4). This led to tumor regression by promoting neoantigen presentation by DCs to CD8 $\alpha^+$  T cells and subsequent CD8 $\alpha^+$  T-cell-dependent tumor cell killing. NeoAgs-CpG-nanodiscs also promoted a significant increase in the ratio of cytotoxic (CD8 $\alpha^+$ ) T cells to Tregs (Foxp3 $^+$ CD4 $^+$ ; Fig. 4). These results indicated vaccine-induced shifts in the balance of effector T cells in the TME, leading to improved survival outcomes and protective immunity against tumor relapse.

Extending our findings from the subcutaneous flank model, we have also demonstrated the therapeutic efficacy of NeoAgs-CpG-nanodisc + anti-PD-L1 treatment in a murine model of GL261 orthotopic glioma. IFN $\gamma$  ELISPOT analysis of PBMCs revealed significantly higher frequencies of neoantigen-specific CD8 $\alpha^+$  T cells across all three neoantigens in mice treated with nanodisc vaccination, compared with soluble neoantigen peptides (Fig. 4B). This increased frequency of circulating neoantigen-specific CD8 $\alpha^+$  T cells in turn led to their robust infiltration into CNS with glioma tumors (Fig. 5A). Nanodisc vaccine combined with anti-PD-L1 therapy also significantly decreased the frequencies of immunosuppressive Tregs, tumor-associated macrophages (TAM), and PD-1 $^+$ -exhausted T cells in the TME (Fig. 5). In addition, when the long-term survivors from the nanodisc + anti-PD-L1 group were rechallenged with GL261 tumors in the contralateral hemisphere, they remained tumor free without further treatment (Fig. 4D), suggesting establishment of immunologic memory. Moreover, we have also demonstrated the therapeutic efficacy of nanodisc vaccination in a second genetically engineered mouse glioma model. Using a genetically engineered murine mIDH1 glioma model (31), we have shown that nanodisc vaccination against mIDH1<sub>123-132</sub> or mIDH1<sub>126-141</sub> significantly extended animal survival and established long-term immunity against mIDH1 tumors (Fig. 6). To the best of our knowledge, our work is the first to show a personalized neoantigen vaccine platform that can elicit effective antitumor immunity and promote lasting immunologic memory to prevent tumor recurrence in murine orthotopic models of glioma.

Despite these exciting results, there are still challenges to overcome before clinical translation of personalized nanodisc vaccination against gliomas. First, more in-depth cytokine and chemokine analyses should be conducted to elucidate the mechanisms by which immunosuppression was reversed and immune memory was generated to improve the overall survival after treatment with NeoAgs-CpG-nanodisc + anti-PD-L1. Second, we have tested only one immune checkpoint blockade antibody in our study. Combinations of different or multiple immune checkpoint blockade antibodies with the nano-vaccine should be tested to determine how a multi-pronged approach could be optimized to treat tumors as aggressive as glioma. Clinical trials studying anti-PD-1 and anti-CTLA-4 therapies in patients with both primary and recurring glioma are ongoing but have no conclusive results yet. Because of the possibility that certain tumors might be unresponsive to immune checkpoint blockade, future work on our combinatorial approach to immunotherapy should include investigation of the mechanisms of resistance to immune checkpoint blockade and elucidation of immune cell activation, which will facilitate identification of ideal therapeutics and their treatment regimens. Third, as genomic profiling of primary and recurrent gliomas has shown that recurrent tumors possess significantly more mutations (49), it would be interesting to evaluate our neoantigen-based approach for combination immunotherapy in the setting of recurrent gliomas. Finally, the current rate-limiting step of the nano-vaccine formulation is neoantigen peptide identification and synthesis. It is estimated that 6–8 weeks would be required for neoantigen identification and production of GMP-grade peptides. We are currently streamlining the sHDL formulation process so that antigen loading and characterization can be performed in 1 week after completion of peptide synthesis. Overall, the sHDL nanodisc provides a promising and versatile platform for minimally invasive (subcutaneous) delivery of neoantigens and adjuvant molecules. As the sHDL formulation process has been proven scalable and safe in prior phase I trials (34), we anticipate that our strategy outlined here will provide a general roadmap for personalized vaccination for immunotherapy against glioma and other cancer types.

### Disclosure of Potential Conflicts of Interest

A. Schwendeman is listed as an inventor on a patent describing nanodisc technology or delivery of peptide antigen licensed to EVOQ Therapeutics. J.J. Moon reports receiving commercial research grants from and holds ownership interest (including patents) in EVOQ Therapeutics. No potential conflicts of interest were disclosed by the other authors.

### Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L. Scheetz, X. Sun, S. Son, A. Hassani Najafabadi, M. Aikins, M.G. Castro

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. Scheetz, P. Kadiyala, X. Sun, S. Son, M. Aikins, P.R. Lowenstein, M.G. Castro, J.J. Moon

**Writing, review, and/or revision of the manuscript:** L. Scheetz, P. Kadiyala, X. Sun, M. Aikins, P.R. Lowenstein, A. Schwendeman, M.G. Castro, J.J. Moon

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** L. Scheetz, P. Kadiyala

**Study supervision:** L. Scheetz, P.R. Lowenstein, A. Schwendeman, M.G. Castro, J.J. Moon

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# Clinical Cancer Research

## Synthetic High-density Lipoprotein Nanodiscs for Personalized Immunotherapy Against Gliomas

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