Therapeutic targeting of tumor-associated myeloid cells synergizes with radiation therapy for glioblastoma

Peng Zhang$, Jason Miska$, Catalina Lee-Chang#, Aida Rashidi#, Wojciech K. Panek#, Shejuan An#, Markella Zannikou#, Aurora Lopez-Rosasa, Yu Han$, Ting Xiao#, Katarzyna C. Pituch#, Deepak Kanojia#, Irina V. Balyasnikova#, and Maciej S. Lesniak#$

$Department of Neurological Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

Edited by W. Mark Saltzman, Yale University, New Haven, CT, and accepted by Editorial Board Member Ruslan Medzhitov October 6, 2019 (received for review April 15, 2019)

Tumor-associated myeloid cells (TAMCs) are key drivers of immunosuppression in the tumor microenvironment, which profoundly impedes the clinical response to immune-dependent and conventional therapeutic modalities. As a hallmark of glioblastoma (GBM), TAMCs are massively recruited to reach up to 50% of the brain tumor mass. Therefore, they have recently been recognized as an appealing therapeutic target to blunt immunosuppression in GBM with the hope of maximizing the clinical outcome of antitumor therapies. Here we report a nano-immunotherapy approach capable of actively targeting TAMCs in vivo. As we found that programmed death-ligand 1 (PD-L1) is highly expressed on glioma-associated TAMCs, we rationally designed a lipid nanoparticle (LNP) formulation surface-functionalized with an anti–PD-L1 therapeutic antibody (αPD-L1). We demonstrated that this system (αPD-L1-LNP) enabled effective and specific delivery of therapeutic payload to TAMCs. Specifically, encapsulation of dinaciclib, a cyclin-dependent kinase inhibitor, into PD-L1-targeted LNPs led to a robust depletion of TAMCs and an attenuation of their immunosuppressive functions. Importantly, the delivery efficiency of PD-L1–targeted LNPs was robustly enhanced in the context of radiation therapy (RT) owing to the RT-induced up-regulation of PD-L1 on glioma-infiltrating TAMCs. Accordingly, RT combined with our nano-immunotherapy led to dramatically extended survival of mice in 2 syngeneic glioma models, GL261 and CT2A. The high targeting efficiency of αPD-L1-LNP to human TAMCs from GBM patients further validated the clinical relevance. Thus, this study establishes a therapeutic approach with immense potential to improve the clinical response in the treatment of GBM and warrants a rapid translation into clinical practice.


Significance

Tumor-associated myeloid cells (TAMCs) are a key driver of immunosuppression and therapy resistance in glioblastoma (GBM). The fact that TAMCs compose up to 50% of the brain tumor mass further highlights the urgent need to develop therapeutic strategy for effective targeting of TAMCs in GBM. Here we report a lipid nanoparticle (LNP) platform capable of actively targeting and delivering therapeutics to mouse and human TAMCs by recognizing highly expressed PD-L1 in TAMCs. We show that LNP encapsulated with dinaciclib robustly eliminated TAMCs from glioma and significantly extended survival of mice in glioma models in combination with radiation therapy. This nanomedicine platform holds great potential for improved treatment of GBM and rapid translation into clinical practice.


Competing interest statement: There is a pending patent pertaining to the work presented in this manuscript.

This article is a PNAS Direct Submission. W.M.S. is a guest editor invited by the Editorial Board.

Published under the PNAS license.

To whom correspondence may be addressed. Email: maciej.lesniak@northwestern.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1906346116/-/DCSupplemental.
PD-L1 over other subsets of immune cells, and even tumors themselves (25, 28). Interestingly, although PD-L1 has drawn considerable attention as a therapeutic target in immune checkpoint blockade therapy, it rarely has been employed as a targeted moiety for therapeutic delivery to PD-L1 expressing cells as tumor cells, which might be due to the lack of evidence to show that binding of ligand to PD-L1 could promptly activate transmembrane transport mechanisms. However, the inherent phagocytic functions and scavenging capabilities of the CD11b⁺ myeloid lineage cells (e.g., TAMCs) (29) raise the possibility that active binding of nanoparticles to highly expressed PD-L1 on such cells could be a feasible approach to trigger active uptake of nanoparticles for targeted therapeutic delivery to TAMCs.

Here we describe a nano-immunotherapy approach capable of actively targeting TAMCs in vivo to blunt the immunosuppression in GBM (Fig. 1). We hypothesized that surface functionalization of a lipid nanoparticle (LNP) formulation with anti–PD-L1 antibody (αPD-L1) might enable effective delivery of LNP-encapsulated therapeutics to TAMCs. Current immunotherapy using αPD-L1 as an immune checkpoint inhibitor only blocks the functions of the inhibitory ligand without degrading the ligands or eradicating PD-L1⁺ cells, likely limiting the overall therapeutic benefits of the treatment. The knowledge of continuous recycling of PD-L1 (30) suggests that targeting of PD-L1⁺ cells with a payload that could inhibit de novo synthesis of PD-L1 and/or eliminate these immunosuppressive cells would be beneficial in context of antiglioma treatment. A recent study highlighted the critical role of cyclin-dependent kinase 5 (CDK5) in interferon gamma (IFNγ)-stimulated PD-L1 production in tumor cells (31). In this work, we demonstrated that treatment of TAMCs with dinaciclib (Dina), a small-molecule CDK5 inhibitor, effectively attenuated PD-L1 expression on TAMCs at a dose as low as 25 nM and induced an apoptosis of TAMCs at a higher dose. Encapsulation of Dina into PD-L1-targeting LNPs created a dual-action system allowing a specific delivery of therapeutic antibodies and drug payloads to PD-L1-expressing TAMCs. Local intracranial treatment with this system resulted in robust TAMC depletion and attenuation of their immunosuppressive functions. Interestingly, radiation therapy (RT), a standard of care in the treatment of GBM, induced up-regulation of PD-L1 in glioma-infiltrating TAMCs, which is considered an important mechanism of tumor radio-resistance. Here we demonstrate that the RT-elicited increase in PD-L1 expression further enhanced the targeting efficiency of PD-L1-targeting nanoparticles. Accordingly, RT combined with our nano-immunotherapy led to dramatically extended survival of tumor-bearing mice in 2 different syngeneic glioma models, GL261 and CT2A. The high targeting efficiency of αPD-L1-LNP to human TAMCs isolated from tumor and blood samples of GBM patients further validated the clinical relevance of the proposed system. Thus, this study establishes a therapeutic approach with great potential to improve the clinical response in the treatment of GBM and warrants a rapid translation into clinical practice.

Results

Engineering of LNPs Targeted to Glioma-Associated TAMCs. Our first step was to identify a receptor that is highly expressed on TAMCs over other subsets of immune cells, which could be...
readily recognized by the complementary ligand functionalized on nanoparticles. Emerging evidence indicates that PD-L1 is over-expressed on myeloid lineage in several tumor models (25, 32). Herein we comprehensively analyzed an array of tumor-infiltrating immune cells in the GL261 syngeneic mouse glioma model. Flow cytometric analysis revealed a PD-L1 expression by several immune cell subsets with a profound overexpression on TAMCs. Particularly, monocyte MDSCs (M-MDSCs) showed the highest expression of PD-L1, followed by TAMS and polymorphonuclear MDSCs (PMN-MDSCs), as judged by the percentage of PD-L1–positive cells (Fig. 1B) and mean fluorescence intensity (MFI) (Fig. 1C). These data suggest that PD-L1 might be a viable targeted molecule for specific therapeutic delivery to glioma-associated TAMCs.

Next, we designed a system allowing for simultaneous and specific drug and antibody delivery to TAMCs. A lipid-based nanoparticle formulation was prepared (Fig. L1). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cardiolipin, and cholesterol are 3 components which constitute the hydrophobic membrane with a phospholipid bilayer structure, in which the small hydrophobic molecule Dina can be well accommodated. The surface of formed LNPs was engineered with DSPE-PEG2000 to provide high phobic molecule Dina can be well accommodated. The surface of glycero-3-phosphocholine (DOPC), cardiolipin, and cholesterol (mAbs) (Fig. 1A) demonstrates a particle size distribution of the αPD-L1–functionalized lipid nanoparticles (αPD-L1-LNP) that are around 90 nm in diameter, only slightly larger than nonmodified LNPs (Fig. 1D, Lower, and SI Appendix, Fig. S1). Zeta-potential analysis indicates a slightly negatively charged surface of nanoparticles.

αPD-L1–Functionalized LNPs Demonstrate a High Avidity and Specificity to Glioma-Associated TAMCs and Impair Recycling of PD-L1 in TAMCs. As an initial step to evaluate if surface conjugation of αPD-L1 could be an efficacious approach to target the therapeutic delivery to TAMCs, we generated glioma-associated TAMCs in vitro as our test system, as depicted in Fig. 2A. The in-vitro–generated TAMCs showed a high purity and highly expressed PD-L1 (SI Appendix, Fig. S2), among which M-MDSCs were more prevalent over PMN-MDSCs, which is consistent with the in vivo phenotyping of GL261 glioma model (SI Appendix, Fig. S3). αPD-L1-LNPs demonstrated high binding to TAMCs, traced by Rhod-PE–labeled phospholipids, as compared to IgG isotype control conjugated LNP (Iso-LNP) as well as LNP without mAb decoration (Fig. 2B). In our experiments, all cells were pre-incubated with Fc receptor binding inhibitors to block nonspecific binding of αPD-L1 to myeloid cells. Importantly, the enhancement in the cellular binding of αPD-L1-LNPs was significantly impeded by preblocking of TAMCs with an excess amount of free αPD-L1 mAbs (Fig. 2B), further validating that the targeting of LNPs was largely mediated by surface interaction of αPD-L1 and PD-L1 on TAMCs.

To confirm if PD-L1–mediated surface binding could effectively trigger internalization of nanoparticles into TAMCs, we tracked the cellular uptake and intracellular distribution of nanoparticles in TAMCs. Fig. 2C shows a more robust accumulation of αPD-L1-LNPs in TAMCs after only 1 h of incubation at 37 °C, and the intracellular distribution of αPD-L1-LNPs was further indicated by wheat germ agglutinin (WGA) cell membrane staining and NucBlue cell nucleus staining (Fig. 2 D, Left). Moreover, LysoTracker staining demonstrated a high colocalization of αPD-L1-LNPs with lysosomes, suggesting that binding of αPD-L1-LNPs to PD-L1 on TAMCs efficiently and promptly triggered nanoparticle internalization through the endocytosis/phagocytosis pathway in myeloid cells (Fig. 2 D, Right).

To further demonstrate the preferential uptake of αPD-L1-LNPs by TAMCs over T cells expressing PD-L1 at lower levels, we analyzed the interactions of αPD-L1-LNPs in a coculture of TAMCs and T lymphocytes. Not surprisingly, owing to a higher expression of PD-L1 and phagocytic activity, TAMCs exerted dramatically stronger capability to engulf αPD-L1-LNPs as indicated by flow cytometry analysis, whereas T cells showed a minimal ability (SI Appendix, Fig. S4). More importantly, we also evaluated the interactions of αPD-L1-LNPs in a coculture of TAMCs and GL261 glioma cells since PD-L1 is also well known to be expressed on tumor cells (SI Appendix, Fig. S5). Interestingly, decoration by αPD-L1 did not elicit robust enhancement in targeting efficiency of LNP to GL261 glioma cells, which showed much lower cellular uptake of αPD-L1-LNPs than TAMCs (Fig. 2E). Altogether, these data strongly indicate the high avidity and specificity of αPD-L1–functionalized LNPs toward glioma-associated TAMCs.

The interaction between αPD-L1-LNP and PD-L1 on the plasma membrane of TAMCs was further assessed, using free unconjugated αPD-L1 as a control (Fig. 2F). An efficient blockade of cell-surface PD-L1 on TAMCs was achieved after incubation with αPD-L1-LNP or free αPD-L1 at 4 °C. Interestingly, binding of αPD-L1-LNP induced substantial loss of PD-L1 from the surface of TAMCs after subsequent incubation at 37 °C to allow internalization, whereas free αPD-L1–treated TAMCs regained a high level of PD-L1 on the cell membrane, which could be largely reduced by a treatment with primaquine (PM), an inhibitor of endocytic recycling (33) (Fig. 2G). These data demonstrate a continuous internalization and recycling of PD-L1 on plasma membrane, and binding with αPD-L1-LNP may substantially impair the recycling pathway of PD-L1. Furthermore, our data suggest that αPD-L1-LNP may direct PD-L1 to lysosomal degradation, as evidenced by high lysosomal accumulation of αPD-L1-LNP after only 1 h of incubation (Fig. 2D). In support of this hypothesis, incubation at 37 °C induced a dramatic internalization and thus reduction of cell-surface–bound αPD-L1 in αPD-L1-LNP–treated TAMCs; however, such reduction was not observed in cells treated with free αPD-L1 (Fig. 2H), which could be recycled back to the cell surface with PD-L1 through endocytic recycling (30). This was further evidenced by the substantial loss of cell-surface–bound free αPD-L1 in the presence of recycling inhibitor (Fig. 2H). The capability of αPD-L1-LNP to reroute the endocytic/recycling pathway of PD-L1 is yet to be fully understood, but may be due to the clustering of PD-L1 on plasma membrane (SI Appendix, Fig. S6) caused by multivalent interactions (34) with LNP conjugation of antibodies.

Therapeutic LNPs Induce Cytotoxicity and Attenuate Immunosuppressive Functions of TAMCs. The effects of PD-L1–targeting LNPs carrying therapeutic payload were first evaluated in vitro. To construct the therapeutic LNPs, Dina, a small-molecule CDK5 inhibitor, was readily encapsulated into the phospholipid bilayers (Fig. 3A). The resulting Dina-loaded LNPs (αPD-L1-LNP/Dina) demonstrated high effectiveness in inhibiting TAMCs in terms of viability as well as immunosuppressive activities. As shown in Fig. 3B, αPD-L1-LNP/Dina induced cytotoxicity in TAMCs in a dose-dependent manner. The treatment with αPD-L1-LNP/Dina at a Dina concentration of 12.5 and 25 nM for 24 h induced apoptosis in less than 20% of TAMCs; however, when the dose was increased to 50 nM, a vast majority of the TAMCs were effectively eliminated. Treatment of cells with free Dina demonstrated comparable cytotoxicity, confirming that the cytotoxic effect is caused by the payload drug (Fig. 3B). In contrast, drug-free nanoparticles did not affect the viability of TAMCs. Compared to TAMCs, GL261 glioma cells demonstrated lower sensitivity to Dina treatment (SI Appendix, Fig. S7).

As an important mechanism to dampen T cell activity and induce immunosuppression, PD-L1 is highly up-regulated on
TAMCs, which is known to be inducible by IFN\(\gamma\). Treatment with a low dose of Dina at 25 nM, a sublethal dose, was sufficient to remarkably inhibit the IFN\(\gamma\)-stimulated production of PD-L1 in TAMCs, as measured by both mRNA (Fig. 3C) and protein levels (Fig. 3D). Interestingly, compared to free drug, \(\alpha\)PD-L1-LNP/Dina demonstrated a remarkably enhanced capacity of PD-L1 inhibition in TAMCs (Fig. 3D), which may be due to the synergistic effect of Dina + \(\alpha\)PD-L1-LNP to simultaneously impair the de novo synthesis of PD-L1 and induce its lysosomal degradation. In addition to PD-L1, a variety of key factors associated with immunosuppressive...
Therapeutic LNPs Actively Target and Eliminate TAMCs In an Ex Vivo Model. The targetability of αPD-L1-LNPs was further assessed using an ex vivo model, in which immune infiltrates were isolated by Percoll gradient from the intracranial GL261 glioma tumors in mice (Fig. 4A). Consistent with the in vitro results, surface-modified αPD-L1 actively targeted LNPs to TAMCs and dramatically increased the cellular uptake in comparison to control LNPs (Fig. 4B). Among all of the examined immune cell subsets, M-MDSCs and TAMs were the major targets and presented the highest efficiency in taking up αPD-L1–decorated LNPs. In contrast, LNPs were not highly distributed in PMN-MDSCs, the phagocytic activity of which is known to be much lower (35).

Building on the capability of highly efficient and selective delivery to TAMCs, ex vivo treatment with αPD-L1-LNP/Dina effectively eliminated TAMCs without showing significant off-target toxicity to tumor-infiltrating lymphocytes (TIL) (Fig. 4C), as further quantified by the change in cell abundance (Fig. 4D), whereas free Dina caused severe off-target cytotoxicity and non-specific elimination of all immune cell populations (SI Appendix, Fig. S10). Among subsets of MDSCs, αPD-L1-LNP/Dina presented a higher potency in eliminating M-MDSCs (SI Appendix, Fig. S11), which is correlated with the highest targetability toward M-MDSCs as demonstrated by such nanoparticles (Fig. 4B). It is also worth noting that the frequency of CD4+ Foxp3+ Tregs was decreased by the treatment (SI Appendix, Fig. S11), which might be a downstream effect of the elimination/inactivation of TAMCs.

Therapeutic LNPs Effectively Target TAMCs In Vivo and Extend Survival of Glioma-Bearing Mice. We next determined the in vivo ability of αPD-L1-LNP/Dina to target TAMCs and to control tumor progression in GL261 glioma-bearing mice. A cannula implantation system was established for multiple intracranial injections of nanoparticles into mice. Biodistribution of TAMC-targeting LNPs in brain tumor was tracked by Rhod-PE–tagged phospholipids 24 h post intracranial administration. As shown in Fig. 4E, αPD-L1-LNPs were highly retained at the brain tumor site and, importantly, substantially colocalized with TAMCs. In contrast, much lower retention of control LNPs was observed at the tumor site (SI Appendix, Fig. S12).

The therapeutic efficacy of the proposed nano-immunotherapy strategy was evaluated in mice bearing the GL261 glioma. GL261 is known as an aggressive murine glioma model, which led to a short median survival of 20 d in glioma-bearing mice (Fig. 4F). Intracranial injection of drug-free LNPs (αPD-L1-LNP) and nontargeting nanoparticles (Iso-LNP/Dina) showed no significant benefit on overall survival. However, administration of αPD-L1-LNP/Dina led to a dramatically enhanced therapeutic effect (P < 0.05). Only 2 injections of αPD-L1-LNP/Dina at a dose of 2.5 mg Dina/kg substantially extended the median survival of glioma-bearing mice to 28.5 d. In comparison, administration of free Dina at 2 different doses (2.5 and 5 mg/kg) did not lead to notable improvement in animal survival (SI Appendix, Fig. S13), likely due to the lack of specificity and off-target toxicity.

Irradiation Up-Regulates PD-L1 Expression on TAMCs and Enhances Delivery Efficiency to TAMCs. RT, which induces apoptosis of tumor cells through damage of DNA, has been widely used as a mainstay treatment of GBM in clinic (36). Indeed, irradiation caused cytotoxicity in GL261 glioma cells in a dose-dependent manner (SI Appendix, Fig. S14). Importantly, the ability of irradiation to shape TME and host immunity has also been recognized, indicating an impact of RT on both tumor cells and immune cells (37–39). As shown in Fig. 5A, irradiation profoundly up-regulated the expression of PD-L1 on in-vitro–generated TAMCs, as determined by both flow cytometry and RT-qPCR analysis. Notably, irradiation reduced the abundance of PMN-MDSCs, a PD-L1low subpopulation of TAMCs, while further elevating PD-L1 expression in the PD-L1high subpopulation.

activities of TAMCs, including arginase 1, inducible nitric oxide synthase, and transforming growth factor beta, were also dramatically suppressed by the treatment of αPD-L1-LNP/Dina (SI Appendix, Fig. S8).

Since one of the major immunosuppressive mechanisms of TAMCs is to inhibit the proliferation of cytotoxic T lymphocytes (CTLs) (21), we next evaluated if our LNP treatment affects CTL proliferation. TAMCs were treated with 25 nM of αPD-L1-LNP/Dina, αPD-L1-LNP, or Dina. (C) Expression of PD-L1 on TAMCs 24 h post stimulation with IFNγ, as determined by RT-qPCR. mRNA levels were normalized to beta-actin and reported relative to control TAMC expression. (D) Flow cytometric analysis of PD-L1 expression on TAMCs 24 h post stimulation with IFNγ, as presented by MFI. (E) Representative histograms of proliferating CD8+ T cells 72 h after being cocultured with nontreated TAMCs (blue) or 25 nM of αPD-L1-LNP/Dina-treated TAMCs (red), as traced by Cell Trace Violet and compared to CD8+ T cells alone (gray-shaded region). Data are represented as mean ± SEM; n = 3; *P < 0.05; **P < 0.001; determined by 1-way ANOVA with Tukey’s multiple comparisons test.

![Diagram of TAMC targetability and activity](image-url)
M-MDSCs (SI Appendix, Fig. S15). This suggests that the remaining radio-resistant subset of TAMCs (M-MDSCs) are more targetable by PD-L1–targeting LNPs. Indeed, the percentage of targetable TAMCs by αPD-L1-LNPs was dramatically increased from 62 to 96% post irradiation (Fig. 5B), which was highly correlated with the capability of RT to reshape the composition and

Fig. 4. Therapeutic LNPs actively target TAMCs in an ex vivo and an in vivo glioma model and extend survival of glioma-bearing mice. (A) Schematic of isolating immune infiltrates in GL261 glioma model. (B) Flow cytometric analysis of distribution of Rhod-PE–labeled LNPs among immune cell subsets, as represented by MFI (n = 3). (C and D) Flow cytometric analysis of glioma-associated immune cells after treatment with αPD-L1-LNP/Dina at a Dina concentration of 0, 25, and 50 nM for 72 h (n = 4). (C) Representative gating of CD45<sup>high</sup> CD11b<sup>−</sup> TIL, CD45<sup>high</sup> CD11b<sup>+</sup> TAMC, and CD45<sup>int</sup> CD11b<sup>+</sup> microglia. (D) The cell abundance was determined by cell counts and flow cytometry analysis, as normalized to nontreated control. (E) Distribution of Rhod-PE–labeled αPD-L1-LNPs at a brain tumor site 24 h post injection. (Scale bar, 100 μm.) (F) Survival curves of GL261-bearing mice after 2 administrations of saline, drug-free αPD-L1-LNP, Iso-LNP/Dina, or αPD-L1-LNP/Dina at 2.5 mg/kg Dina on days 7 and 14 after intracranial implantation of 5 × 10<sup>5</sup> GL261 glioma cells; n = 7–8 mice per group. Data are represented as mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001; determined by 1-way ANOVA in D or 2-way ANOVA in B with Tukey’s multiple comparisons test or log-rank method with P values adjusted by Bonferroni correction in F.

Fig. 5. Irradiation up-regulates PD-L1 expression on TAMCs and enhances targeted delivery to TAMCs. (A) RT-qPCR and flow cytometric quantification of PD-L1 expression on TAMCs as normalized to control TAMC expression. (B) Flow cytometric quantification of cellular uptake of Rhod-PE–labeled LNPs in TAMCs after 1 h of incubation, as presented by the percentage of NP<sup>+</sup> cells (blue, nontreated TAMCs; red, NP-treated TAMCs). (C) Flow cytometric quantification of percentage of PD-L1–positive TAMCs (blue, Iso control; red, PE anti-PD-L1). (D) Cell circle analysis of TAMCs treated with phosphate-buffered saline, RT (8 Gy), αPD-L1-LNP, αPD-L1-LNP/Dina (25 nM Dina), or RT+αPD-L1-LNP/Dina (25 nM Dina). Data are represented as mean ± SEM; n = 3; *P < 0.05; ***P < 0.001; determined by Student’s t test in A or 1-way ANOVA with Tukey’s multiple comparisons test in D.
PD-1 expression of TAMCs (Fig. 5C). Overall, irradiation induced a 3.5-fold increase in accumulative cellular uptake of αPD-L1-LNPs by TAMCs (SI Appendix, Fig. S16), leading to an increased cytotoxicity in TAMCs after combination therapy over monotherapy (Fig. 5D).

**Therapeutic LNPs Synergize with Radiation Therapy to Eliminate TAMCs and Promote Antitumor Responses in Glioma-Bearing Mice.** To test the potential of using TAMC-targeting therapeutic LNPs as a combination therapeutic strategy with RT, different treatment regimens were assessed in C57BL/6 mice bearing the GL261 glioma model (Fig. 6A). Compared to RT monotherapy, combination with drug-free αPD-L1-LNPs provided limited improvement in animal survival; however, encapsulation of Dina into αPD-L1-LNPs significantly enhanced the therapeutic efficacy of RT (SI Appendix, Fig. S17). We then generated a more aggressive glioma model in C57BL/6 mice by increasing the number of inoculated GL261 glioma cells by 4-fold. As shown by Fig. 6B, a short life span was observed in a control group of mice with a median survival of only 17 d. Monotherapy, either 4 daily 2-Gy fractions of irradiation or 2 injections of αPD-L1-LNP/Dina, moderately improved animal survival to 22.5 d. However, combination therapy extended the median survival of GL261-bearing mice to 32 d, a 2-fold increase in comparison to the control group. The treatment-induced apoptosis/necrosis of glioma cells and decreased tumor size were also indicated by histopathological analysis of glioma tissue (SI Appendix, Fig. S18).

The ability of treatments to shape the immunosuppressive TME was also assessed in GL261 glioma-bearing mice. RT largely caused infiltration of TAMCs into glioma, which were dramatically eliminated by treatment with αPD-L1-LNP/Dina (Fig. 6C), particularly M-MDSCs and TAMs (Fig. 6 D and E), whereas the treatment had no significant effect on PMN-MDSCs (SI Appendix, Fig. S19). Such elimination was heavily relied on PD-L1–targeted therapeutic delivery. PD-L1–expressing TAMCs were almost depleted after 2 injections of αPD-L1-LNP/Dina (Fig. 6F), and the remaining TAMC subset demonstrated a low level of PD-L1 expression (Fig. 6G). As a result of the elimination of TAMCs, the abundance of Tregs was also profoundly reduced, which is consistent with in vitro and ex vivo data, without dramatically affecting CD4+ T effectors (SI Appendix, Fig. S19).

To foster a rapid clinical translation of these nanoparticle formulations, we also administrated the therapeutic LNPs through a...
noninvasive intranasal delivery approach (Fig. 6I). Similar to the results of intracranial delivery, a regimen combining intranasally administrated αPD-L1-LNP/Dina daily for 8 d with 4 doses of irradiation led to an improved animal survival in a GL261 glioma model over monotherapies (Fig. 6J). In addition, the described nano-immunoradiation combination therapy regimen (Fig. 6A) was also evaluated in mice bearing a different syngeneic glioma model termed CT2A (Fig. 6I). CT2A is well known as an aggressive murine glioma model with brain tumor stem cell-like properties (40). Indeed, monotherapies did not seem to work well and showed only marginal therapeutic effects. However, excitingly, the combination therapy contributed to a largely extended animal survival compared to the control group (34 vs. 20 d), and 30% of glioma-bearing animals were observed to have long-term survival.

αPD-L1-LNPs Show High Targeting Efficiency toward Human TAMCs from GBM Patients. To evaluate the potential of our TAMC-targeting strategy for clinical translation, targeting efficiency of αPD-L1-LNPs was tested in human TAMCs, which were harvested from tumor samples of GBM patients (Fig. 7A). The clinical tumor/blood samples were collected from GBM patients with diverse demographic, treatment, and molecular characteristics (SI Appendix, Table S1). Consistent with the results from murine glioma models, a more prevalent population of CD11b+CD33+CD14+ M-MDSCs over CD11b+CD33+CD15+ PMN-MDSCs was observed in human GBM samples (Fig. 7B), which is a unique characteristic of GBM in comparison to most types of cancers.

Owing to the highly expressed PD-L1 (Fig. 7C), a predominate population (~90%) of M-MDSCs, from GBM case NU02056, was effectively targeted by LNPs surface-functionalized with antihuman PD-L1 mAb, which was dramatically higher than control LNPs (Fig. 7D). Quantification by MFI further identified M-MDSCs as the primary target of αPD-L1-LNP (Fig. 7E). Comparable target specificity was observed in tumor-infiltrating myeloid cells in GBM case NU02033 (Fig. 7F). In addition, αPD-L1 also highly efficiently targeted LNPs to circulating M-MDSCs in peripheral blood of the same patient, which expressed highest the PD-L1 over other subsets of peripheral blood mononuclear cells (PBMCs) (Fig. 7G). Similar characteristics with regards to PD-L1 expression and target specificity were observed in glioma-infiltrating myeloid cells as well as in PBMCs in GBM cases NU01794 and NU01761 (SI Appendix, Fig. S20). Collectively, these data confirm that our nanoparticles are effective in targeting human TAMCs from GBM patients, in which M-MDSCs highly expressing PD-L1 are likely the major target.

Discussion
TAMCs have been recently highlighted as a pivotal contributor to the generation of immunosuppression in the TME, tolerance to antitumor therapies, and tumor relapse and metastasis (29, 41). Therefore, they have become an attractive therapeutic target with a great potential to ameliorate the tumor-associated immunosuppressive microenvironment and to unleash the full potential of antitumor therapeutic modalities. The fact that TAMCs are largely recruited into GBM to reach up to 50% of the tumor mass further emphasizes the importance and necessity of developing new
approaches to therapeutically target and eliminate TAMCs for the treatment of glioma.

Progress in nanomedicine-based therapy has clearly indicated the essential role of ligand-receptor interaction-mediated therapeutic delivery to the desired subset of cells. Cell-specific targeting could be readily achieved through surface functionalization of nanoparticles with targeting ligands, including small molecules, peptides, and monoclonal antibodies, that could recognize and bind with high affinity to receptors highly expressed in cells of interest (42, 43). The research finding that PD-L1 is highly expressed on glioma-associated TAMCs lends support to the possibility that PD-L1 could be a potential novel target for therapeutic delivery of TAMCs, which can be used to therapeutically modulate the immunosuppression in glioma TME as a nano-immunotherapy approach.

One of the major concerns about the use of PD-L1 as a target molecule for therapeutic delivery might be that so far there is no solid evidence to demonstrate that binding of ligands to PD-L1 could actively trigger transmembrane transport pathways such as endocytosis in PD-L1-expressing cells, especially tumor cells. However, interaction of αPD-L1-decorated LNPs and PD-L1 on the surface of TAMCs may greatly enhance and accelerate the engulfment of nanoparticles by such cells with strong phagocytic and scavenging capabilities, as suggested by initial clues from pilot studies (44). Indeed, our data indicate that LNPs demonstrated high effectiveness and specificity in targeting TAMCs throughout comprehensive in vitro, ex vivo, and in vivo assessments. Among TAMCs, the most efficient targeting was achieved in M-MDSCs, which is consistent with the highest expression level of PD-L1 on these cells.

As a major component of TAMCs, MDSCs are characterized into 2 subsets: M-MDSCs and PMN-MDSCs. In most tumor models, PMN-MDSCs represent a predominant population of MDSCs; however, as a unique characteristic of GBM, the M-MDSC subset is more prevalent at the tumor site (22). More importantly, recent evidence has indicated that M-MDSCs are more potent in promoting immunosuppression over PMN-MDSCs (35). Therefore, M-MDSCs have great potential as a therapeutic target with the aim to correct immune defects in glioma, and the capability of our αPD-L1-LNPs to actively target M-MDSCs indeed imparted therapeutic benefit to gliomas (26, 27). In addition, αPD-L1-LNPs were also highly effective in targeting TAMs, another major component of TAMCs with highly expressed PD-L1.

A unique advantage of our therapeutic approach is the ability to recognize and deliver therapeutics to PD-L1+ TAMCs. Upregulated expression of PD-L1 has been regarded as one of the major negative regulatory mechanisms deployed by TAMCs to blunt antitumor activity of T cells and NK cells (26, 27). Although great advances have been achieved in current anti–PD-L1 immunotherapy using checkpoint-blocking antibodies, the fact that it blocks only the expressed inhibitory ligand on target cells may largely limit the overall therapeutic outcome of the treatment. To address this challenge, we created a dual-action nano-delivery system allowing a simultaneous delivery of the therapeutic antibodies and drug payloads to PD-L1–expressing TAMCs. The surface-engineered anti-PD-L1 antibody not only efficiently targeted LNPs to PD-L1+ TAMCs, but also functionally neutralized PD-L1 on TAMCs as a therapeutic antibody. More importantly, binding of αPD-L1-LNPs, rather than unconjugated free antibodies, may largely impair endocytic recycling of PD-L1 on TAMCs by rerouting the ligand to lysosomal degradation, which is a unique mechanism of our nanoparticle system. Also, we demonstrated that the payload drug, Dina, potently inhibited IFNγ-stimulated de novo synthesis of PD-L1 in TAMCs, which further enabled a synergistic and profound inhibition of PD-L1 in TAMCs through 2 distinct mechanisms. The treatment of αPD-L1-LNP/Dina significantly induced cytotoxicity in TAMCs when the dose was further increased and led to a robust depletion of TAMCs and survival benefit of glioma-bearing mice.

Another advantage of our TAMC targeted nano-immunotherapy strategy is the capability to synergize with radiation therapy. RT has been widely used in the treatment of various cancers and, particularly, as a standard of care for GBM. However, RT could dramatically induce infiltration of TAMCs into the tumor site, which strongly suppresses RT-elicited immune response and is believed a crucial resistance mechanism to RT (9, 10). Interestingly, while RT induced dramatic cytotoxicity to PMN-MDSCs, radio-resistant M-MDSCs up-regulated PD-L1, which actually allows for enhanced delivery of our nanoparticle treatment. Supporting this notion, administration of therapeutic nanoparticles to glioma-bearing mice post RT caused dramatic elimination of TAMCs, particularly M-MDSCs and TAMs, leading to an extended survival of animals in 2 different glioma models compared to RT monotherapy. These data strongly suggest that our therapeutic nanoparticle therapy could be used as a combination therapy strategy to synergize with radiotherapy for GBM therapy.

Finally, our therapeutic nanoparticles demonstrate a great potential with respect to rapid translation into clinical practice, relying on the high efficiency in performance and simplicity in manufacturing. LNPs, to date, are the most clinically successful nano-formulation with well-demonstrated safety and efficiency (45, 46). Thus, a rapid transition from the benchtop research findings to bedside application could be expected. Excitingly, the high targeting efficiency of αPD-L1-LNPs to human TAMCs isolated from tumor and blood samples of GBM patients further validates the clinical relevance of the proposed system. Moreover, our therapeutic nanoparticle is a versatile platform that could be readily tailored by switching the payload therapeutics or surface-functionalized targeting ligands with the aim of targeting variable subsets of cells of interest in different disease models. A successful attempt to administer our therapeutic nanoparticles through an intranasal delivery method further extended the potential application of our therapeutic approach in different routes of drug administration.

In summary, we have developed a viable nano-immunotherapy approach that could actively target both murine and human glioma-associated TAMCs and lead to robust TAMC depletion and attenuation of their immunosuppressive functions. This nanomedicine platform establishes a therapeutic strategy with great potential to improve the clinical response in the treatment of GBM and holds great promise for a rapid translation into clinical application.

Materials and Methods
LNPs were synthesized through a thin-film hydration method followed by surface functionalization with αPD-L1. In vitro TAMCs were generated from bone marrow progenitor cells of C57BL/6 mice. Ex vivo studies were performed using immune infiltrates isolated by Percoll gradient from the intracranial GL261 glioma tumors in mice. Experimental animals were of both sexes and randomly assigned. All animal-related experiments were performed in full compliance with animal protocols approved by the Northwestern University Institutional Animal Care and Use Committee. All human tumor and peripheral blood samples were collected by the Nervous System Tumor Bank of Northwestern University under institutional review board protocol no. STU00202003 with obtained informed consent. A detailed description of materials and methods is provided in SI Appendix, Materials and Methods.

Data Availability. Necessary materials are available from the corresponding author on reasonable request.

ACKNOWLEDGMENTS. We thank the Northwestern Nervous System Tumor Bank, supported by Specialized Program of Research Excellence (SPORE) Grant P50CA221747 for Translational Approaches to Brain Cancer, for
17. A. L. Chang et al., CCL2 produced by the glioma microenvironment is essential for the recruitment of regulatory T cells and myeloid-derived suppressor cells. Cancer Res. 76, 5671–5682 (2016).