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Drug-loaded bispecific T cell nanoengager overcomes T cell exhaustion for potent cancer immunotherapy

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Bispecific T cell engager (BiTE) therapeutics that link T cells and tumor cells to induce tumor cell lysis have demonstrated great success in the clinic for the treatment of many cancers. However, T cell exhaustion in the tumor microenvironment leads to tumor cell escape and BiTE therapy resistance. Herein, we developed a drug-loaded bispecific T cell nanoengager (NanoBiTE) to overcome this obstacle. NanoBiTE is composed of a mesoporous silica nanoparticle encapsulating the adenosine A2A receptor antagonist PBF-509 as a core, with a lipid layer surface coating as a shell and modification with anti-CD19 and anti-CD3 antibodies for tumor and T cell binding, respectively. Like the traditional BiTE blinatumomab, NanoBiTE can engage T cells with $CD19^+$ tumor cells to promote tumor cell lysis. However, unlike blinatumomab, which tends to induce T cell exhaustion, we showed that the release of PBF-509 from NanoBiTE suppressed the A2AR pathway and substantially improved tumor cell killing induced by NanoBiTE. Moreover, NanoBiTE treatment led to substantially reduced tumor burden in vivo in a humanized mouse model. Our results demonstrate that NanoBiTE is a safe and potent bispecific therapy that can also reduce T cell exhaustion for cancer immunotherapy.

nanomedicine | immunotherapy | cancer | T cell

Significance

Persistent tumor antigen exposure leads to T cell exhaustion during bispecific T cell engager (BiTE) therapy. T cell exhaustion is a condition in which T cells lose the ability to effectively kill tumors. To address this, we have developed a drug-loaded bispecific T cell nanoengager (NanoBiTE) that can directly link T cells to tumor cells while releasing the A2A receptor antagonist PBF-509 to overcome T cell exhaustion. This approach offers a more precise and effective strategy to enhance the efficacy of BiTE-based immunotherapy.

Bispecific T cell engager (BiTE) therapeutics that leverage cancer cell and T cell surface markers to enhance interaction and recognition between these cells have demonstrated promise for the treatment of many cancers (1). Examples of previously investigated BiTEs include blinatumomab for B cell acute lymphoblastic leukemia (2), catumaxomab for epithelial cancers (3), Imdelltra for small-cell lung cancer (4), lunsumio for late follicular lymphoma (5), and elrexfio for multiple myeloma (6). However, studies have shown that BiTE therapy readily induces T cell exhaustion (1, 7), a condition in which T cells lose their ability to kill cancer cells, contributing to BiTE therapy resistance and tumor recurrence (8, 9).

Many strategies have been developed to overcome T cell exhaustion during BiTE therapy. Combining BiTEs with anti-PD-1 or anti-CTLA-4 antibodies can enhance T cell activation and improve tumor control in preclinical models and clinical trials (10–12). Another strategy to avoid T cell exhaustion involves providing cytokine support. Cytokines including interleukin-2 (IL-2) or IL-15 can promote T cell survival, proliferation, and effector functions. Incorporating these cytokines into the treatment regimen can mitigate T cell exhaustion and enhance the persistence of BiTE-activated T cells within the tumor microenvironment (13, 14). A different strategy involves the combination of regulatory T cell (Treg) depletion with BiTE therapy (15). For example, removal of Tregs in tumor tissue was demonstrated to convert blinatumomab nonresponders to responders (15). Others have shown that engaging costimulatory receptors on T cells can improve the efficacy of BiTE therapy by enhancing T cell function and persistence (16, 17). Although the above strategies have been encouraging, reductions in T cell exhaustion remain marginal, limiting the potential of BiTEs and their clinical efficacy.

Recent studies have shown that BiTE therapy induces extensive tumor cell lysis within the tumor microenvironment and leads to the release of substantial amounts of adenosine triphosphate (ATP) (18). ATP can be subsequently converted into adenosine through enzymatic degradation by CD39 and CD73 expressed on cancer cells (19). Adenosine can be recognized by the A2A receptor (A2AR) and lead to A2AR-cAMP signaling pathway activation and the shift of T cell metabolism from oxidative phosphorylation toward glycolysis (20, 21), a process that is associated with T cell exhaustion (22, 23). Specifically, adenosine-A2AR pathway activation triggers a signaling cascade that increases intracellular cAMP levels and leads to reduced production of proinflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (24, 25). Moreover, adenosine-A2AR signaling activation contributes to the

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upregulation of immune inhibitory receptors such as PD-1, CTLA-4, and LAG-3 (26). Therefore, targeting the adenosine-A2AR pathway to reduce T cell exhaustion could be a promising strategy to enhance the efficacy of BiTE therapy. However, designing therapeutic strategies that can integrate T cell and tumor cell engagement and adenosine-A2AR pathway inhibition is challenging.

Herein, we developed a bispecific T cell nanoengager (NanoBiTE) to overcome the obstacle of BiTE-associated T cell exhaustion (Fig. 1*A*). In vitro assays were first employed to assess functional tumor cell lysis induced by NanoBiTE as well as effects on T cell exhaustion. A humanized immune system mouse model (27) was constructed, and the antitumor efficacy of NanoBiTE was tested in a human B cell lymphoma model along with the

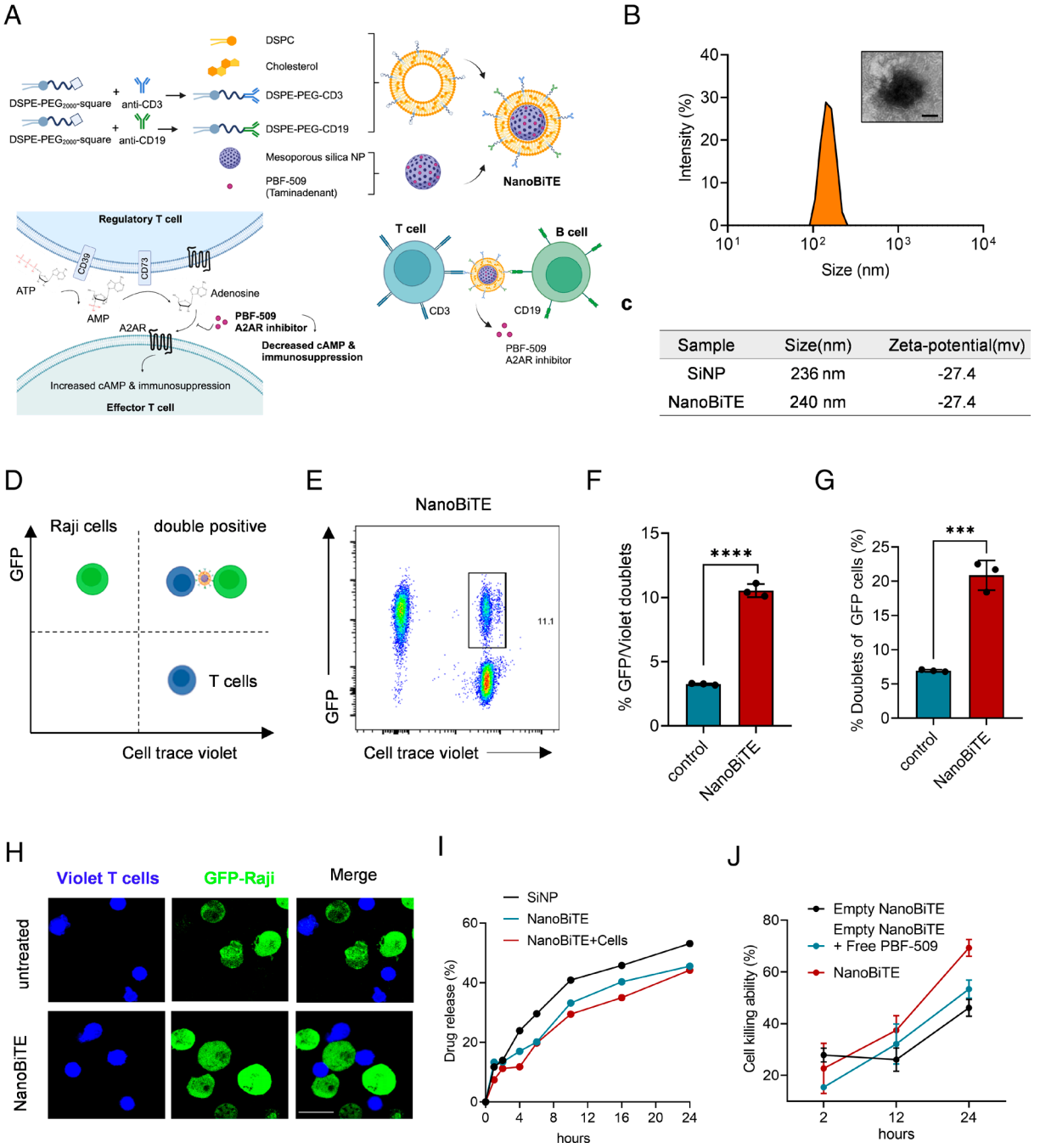

Fig. 1. Formulation and characterization of drug-loaded bispecific T cell nanoengager (NanoBiTE). (*A*) Schematic depicting the composition and formulation of NanoBiTE and NanoBiTE engagement of $CD3^+$ T cells and $CD19^+$ B cells inducing the release of PBF-509, an adenosine A2A receptor (A2AR) antagonist. PBF-509 can reduce cAMP accumulation and immunosuppression in the tumor microenvironment. (*B*) Representative transmission electron microscopy (TEM) image (Scale bar, 100 nm.) and dynamic light scattering (DLS) histogram of NanoBiTE. (*C*) Summary characterization data for NanoBiTE and silica nanoparticle (SiNP). (*D*) Schematic describing interpretation of flow cytometry data for assay of NanoBiTE cell bridging. (*E*) Representative flow cytometry plots demonstrating NanoBiTE-induced cell bridging. (*F* and *G*) Cell bridging assay of NanoBiTE-treated cells. Raji-GFP-Luc cells and Violet cell tracer-labeled primary human T cells were mixed at a 1:1 ratio and were incubated at 4 °C for 2 h. The fraction of $Violet^+$ GFP^+ doublets was quantified by flow cytometry. (*H*) Visualization of Raji-GFP-Luc tumor cells and Violet-labeled primary human T cells cocultured in the presence or absence of NanoBiTE (1 μg/mL) via confocal microscopy (Scale bar, 100 μm). (*I*) In vitro drug release profiles of SiNP, NanoBiTE, and NanoBiTE mixed with cells. (*J*) Tumor cell killing ability of primary human T cells following treatment with Empty NanoBiTE, Empty NanoBiTE + free PBF-509, or NanoBiTE, measured after 2, 12, and 24 h of incubation. The data in *F*, *G*, and *J* are shown as the mean ± SD (n = 3). Statistical differences were analyzed by two-tailed unpaired Student's *t* test.

immune profile of the tumor microenvironment. Overall, the results demonstrate the potential of NanoBiTE as a safe and potent agent for cancer immunotherapy.

Results

Synthesis and Characterization of NanoBiTE. The adenosine-A2AR pathway is a critical mechanism by which tumors escape immune cell attack (28). This study aimed to develop a bispecific nanoengager that can overcome adenosine-induced T cell exhaustion to improve BiTE therapy. We first prepared mesoporous silica nanoparticles (MSNs) following a previously published method (29). The A2AR antagonist PBF-509 (30) was loaded into silica nanoparticles (SiNPs) via ultrasonication for 10 min. PBF-509-loaded MSNs were rinsed with PBS to remove free drug. The surface of the nanoparticle was then coated with DSPC, cholesterol, and DSPE-PEG2000, to form a lipid layer using chloroform evaporation. Anti-CD3 conjugated DSPE-PEG2000-Square and anti-CD19 conjugated DSPE-PEG2000-Square were then added. The antibody-coated nanocomplex was again rinsed with PBS to remove free antibody, yielding NanoBiTE. NanoBiTE demonstrated a spherical structure with an average diameter of 240 nm under both TEM and DLS (Fig. 1 *B* and *C*) and a polydispersity index (PDI) of 0.384 according to DLS measurement. Quantification of PBF-509 loading efficiency was performed using UV–Vis spectroscopy, demonstrating a loading efficiency of approximately 8.84% and encapsulation efficiency of approximately 97% (*SI Appendix*, Fig. S1).

To investigate whether NanoBiTE can lead to T cell and tumor cell engagement, T cells were labeled with a Violet cell tracer dye and subsequently incubated with Raji-GFP-Luc cells in the presence or absence of 1 μg/mL NanoBiTE at 4 °C for 2 h. We employed flow cytometry and confocal imaging to assess cellular interactions mediated by NanoBiTE. We observed that the percentage of Raji-T cell doublets was increased, demonstrating that NanoBiTE can bind Raji-GFP-Luc cells and human T cells (Fig. 1 *D*–*G*). We further used confocal imaging to visualize cellular engagement. Contact between T cells and Raji-Luc-GFP cells was observed in treatment groups that received NanoBiTE treatment but not those receiving PBS treatment, demonstrating successful NanoBiTE-mediated engagement between T cells and cancer cells (Fig. 1*H*). To explore whether cell bridging was specifically induced by NanoBiTE, unmodified SiNPs, single-antibody-modified SiNPs (anti-CD3 or anti-CD19 SiNPs), and dual-antibody-functionalized SiNPs (anti-CD3/anti-CD19 SiNPs) were used to treat cells. Only the dual-antibody-functionalized SiNPs induced significant cell conjugation (*SI Appendix*, Fig. S2). These findings confirm that the cell bridging is not due to nonspecific interactions with the nanoparticle platform or individual antibodies alone.

We then assessed the drug release capability of NanoBiTE. We evaluated the drug release profiles of SiNP, NanoBiTE, and NanoBiTE mixed with Raji-T cells. Samples from release medium were collected at different time points throughout incubation for UV absorption analysis. Our results showed that antibody-conjugation or cell interaction does not alter the release rate of PBF-509 (Fig. 1*I*). We then explored whether PBF-509 in NanoBiTE can affect NanoBiTE-induced tumor cell lysis. Primary human T cells were used as effector cells and Raji-GFP-Luc cells were used as target cells. Effector cells and target cells were mixed at a 5:1 ratio and treated with empty NanoBiTE, NanoBiTE, or empty NanoBiTE + free PBF-509. To mimic the in vivo environment, cells were cocultured in the upper chamber of a transwell system, and the medium in the bottom chamber was replaced with fresh medium after 2 h treatment. T cell killing ability at 2, 12, and 24 h was determined by using a luciferase-based assay. NanoBiTE resulted in increased tumor cell lysis compared to empty NanoBiTE + free PBF-509 treatment and empty NanoBiTE alone after 24 h (Fig. 1*J*). To assess the role of drug treatment on cAMP production, we further assessed the cAMP levels in human T cells following the treatment with empty NanoBiTE, empty NanoBiTE + free PBF-509, or NanoBiTE. Our results demonstrated that NanoBiTE led to a decreased cAMP concentration compared to cells treated with empty NanoBiTE + free PBF-509 and empty NanoBiTE (*SI Appendix*, Fig. S3). These results demonstrate that sustained release of PBF-509 inhibited A2AR-mediated cAMP accumulation and enhanced T cell killing of cancer cells in vitro via NanoBiTE treatment.

PBF-509 Loading Reduces BiTE-Associated T Cell Exhaustion. To evaluate whether PBF-509 released from NanoBiTE reduces T cell exhaustion, activated primary human T cells and Raji-GFP-luc cells were cocultured with empty NanoBiTE, NanoBiTE, or empty NanoBiTE + free PBF-509 for 24 h in a transwell system (Fig. 2*A*). After that, cells were collected for flow cytometry analysis. Our results demonstrated that NanoBiTE decreased the expression of PD-1 and LAG-3 on T cells compared to other treatments, suggesting that NanoBiTE has potential for mitigating T cell exhaustion (Fig. 2 *B*–*I*). Moreover, we investigated T cell proliferation through flow cytometry and found that NanoBiTE induced an increase in the proportion of $Ki67^+$ T cells (*SI Appendix*, Fig. S4). These results suggest that PBF-509 encapsulated in NanoBiTE mitigated T cell exhaustion resulting from tumor cell and T cell engagement and promotes T cell proliferation.

NanoBiTE Reduces Tumor Burden In Vivo. Encouraged by the results from in vitro experiments, we next investigated whether NanoBiTE could overcome T cell exhaustion and improve cancer immunotherapy in vivo. First, we established a humanized NOD scid gamma (NSG) mouse model following a reported protocol (*SI Appendix*, Fig. S5) (27). After confirming the engraftment of human immune cells (*SI Appendix*, Fig. S5), Raji tumor cells were subcutaneously injected to construct a tumor model (Fig. 3*A*). After allowing tumors to grow to approximately 200 mm^3, PBS, free PBF-509, empty NanoBiTE + free PBF-509 (1 mg/kg), or NanoBiTE was injected (1 mg/kg) intratumorally (Fig. 3*A*) and mice were imaged to monitor tumor size at different times (Fig. 3*B*). While free PBF-509 and empty NanoBiTE + free PBF-509 induced moderate tumor growth inhibition, NanoBiTE led to substantial tumor growth inhibition (Fig. 3*C*). Furthermore, extended survival was observed in mice treated with NanoBiTE (Fig. 3*D*), suggesting reduced tumor burden following NanoBiTE treatment. We found that mouse body weight was maintained in normal ranges during the period of treatment, indicating limited toxicity (*SI Appendix*, Fig. S6).

To assess the effect of NanoBiTE on T cells at the tumor sites, we analyzed tumor-infiltrating T cell profiles by flow cytometry (Fig. 4). We found that both free PBF-509 and empty NanoBiTE + free PBF-509 induced slightly increased T cell infiltration in tumor tissue compared to the PBS group. However, NanoBiTE treatment led to substantially improved T cell infiltration in tumors compared to the PBS group (Fig. 4 *A* and *B*). NanoBiTE also decreased the level of PD-1 expression on $CD3^+$ T cells (Fig. 4 *C* and *D*) at the tumor site compared to other groups. In addition, we further evaluated effector memory T cells and found that NanoBiTE led to an expansion of memory T cell populations in vivo (Fig. 4 *E* and *F*). The percentage of regulatory T cells was decreased following NanoBiTE treatment (Fig. 4 *G* and *H*). These results indicate that drug-loaded NanoBiTE holds promise in

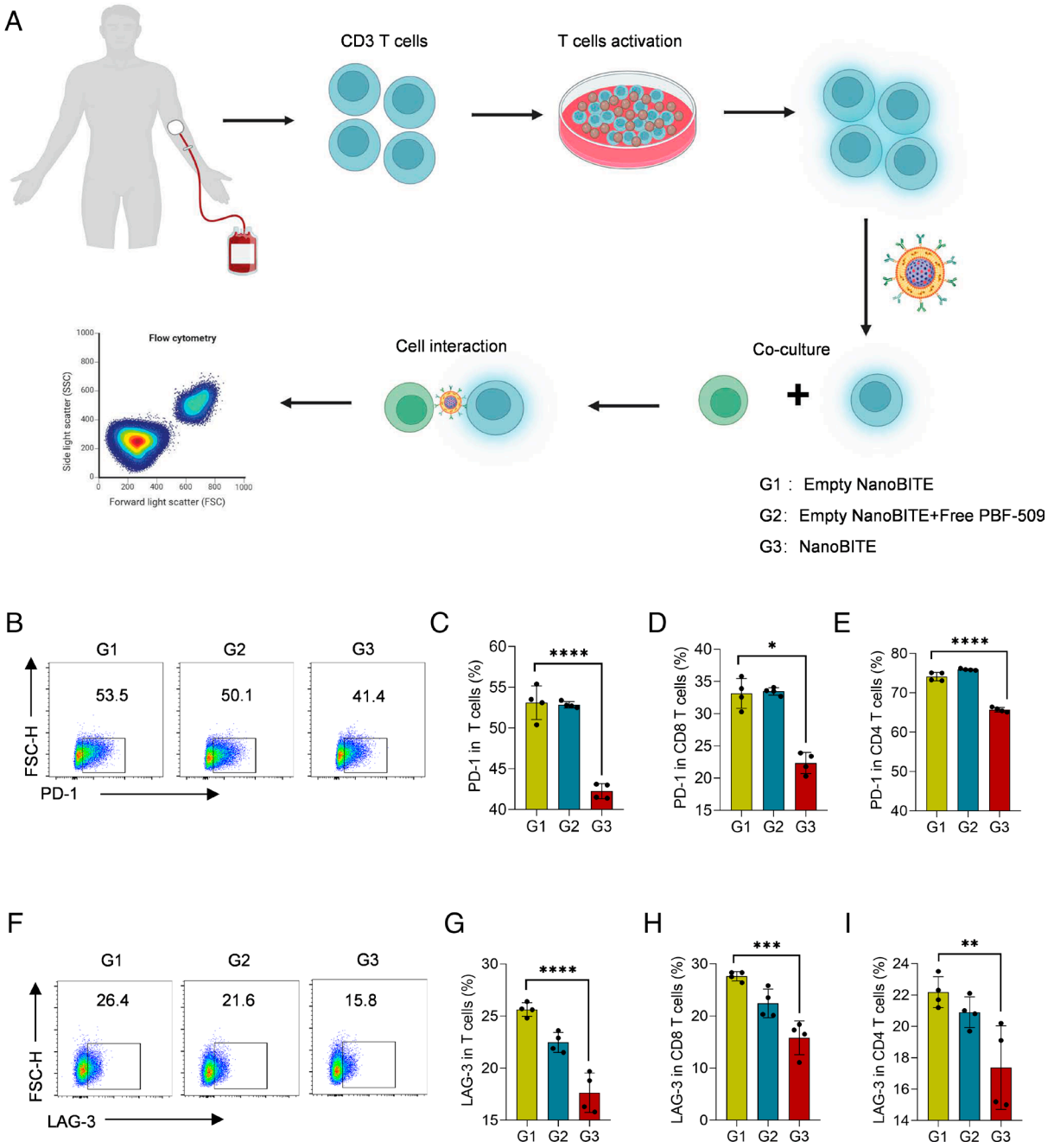

Fig. 2. NanoBiTE reduces T cell exhaustion in vitro. Raji-Luc-GFP cells were cocultured with primary human T cells and treated with Empty NanoBiTE, Empty NanoBiTE + free PBF-509 or NanoBiTE. The expression of PD-1 and LAG-3 by T cells was evaluated using flow cytometry. (*A*) Schematic depicting T exhaustion assay. Human T cells were isolated, activated, cocultured with Raji-Luc-GFP cells using a transwell model, and analyzed by flow cytometry. (*B–I*) After 24 h of incubation, PD-1 expression (*B–E*) and LAG-3 expression (*F–I*) were analyzed on total $CD3^+$ T cells, $CD8^+$ T cells, and $CD4^+$ T cells by flow cytometry. Representative flow plots showing PD-1 (*B*) and LAG-3 (*G*) expression on T cells. The data in *B–I* are shown as the mean ± SD (n = 4). Statistical differences were analyzed by using one-way ANOVA, followed by post hoc Student's *t* tests.

cancer immunotherapy by reversing T cell exhaustion and enhancing T cell effector function.

Considering BiTEs may lead to cytokine release syndrome in vivo, which hinders cancer immunotherapy, we analyzed serum IL-6 and TNF-α levels using ELISAs (Fig. 5*A*), observing that NanoBiTE did not induce obvious cytokine storm in humanized immune system mice (Fig. 5 *B* and *C*). Moreover, hematoxylin and eosin (H&E) staining showed that NanoBiTE treatment did not cause damage in major organs including the heart, liver, spleen, lungs, and kidneys (Fig. 5*D*), demonstrating the biocompatibility of NanoBiTE. Altogether, our results suggest that NanoBiTE holds potential for safe and potent cancer immunotherapy.

Discussion

BiTEs are an innovative form of immunotherapy designed to direct the immune system, specifically T cells, to target and kill cancer cells. BiTEs have shown promising results in hematological malignancies, such as acute lymphoblastic leukemia, with blinatumomab being the first BiTE approved for clinical use (31). However, the effectiveness of BiTE is restricted by T cell exhaustion. Exhausted T cells are less effective at killing tumor cells (32), reducing the therapeutic impact of BiTEs. Furthermore, exhausted T cells have reduced longevity (33), which is critical for sustained antitumor responses. Therefore, therapeutic strategies that can

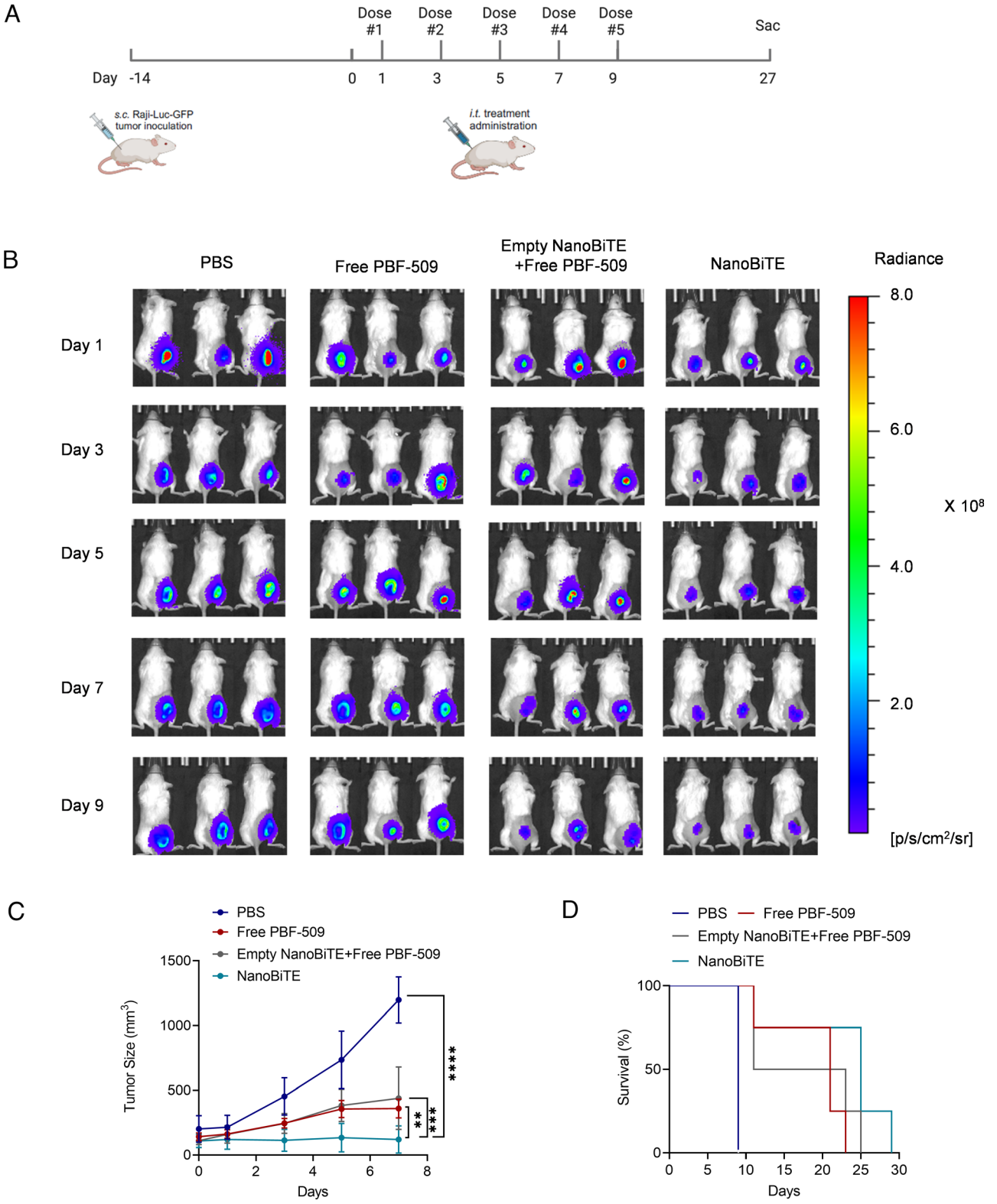

Fig. 3. NanoBiTE inhibits tumor growth and extends survival in mice. (*A*) 10^6 Raji-Luc-GFP cells were administered via subcutaneous (*s.c.*) injection to humanized NSG mice to establish the tumor model. 14 d later, when tumor sizes reached 200 mm^3, mice were treated intratumorally (*i.t.*) with PBS, PBF-509 (equivalent to the amount of PBF-509 loaded in NanoBiTE), Empty NanoBiTE + free PBF-509 (1 mg/kg), or NanoBiTE (1 mg/kg) on days 1, 3, 5, 7, and 9. (*B*) Representative IVIS images of tumor-bearing mice on days 1, 3, 5, 7, and 9. (*C* and *D*) Mouse tumor growth curves (*C*) and Kaplan–Meier survival curves (*D*) from the humanized NSG mouse model. Data in *C* are shown as the mean ± SD (n = 4). Statistical differences were analyzed by using two-way ANOVA followed by Tukey's multiple comparisons test.

overcome T cell exhaustion during BiTE therapy are an unmet need. Herein, we show that the release of PBF-509 from NanoBiTE suppressed T cell exhaustion and substantially improved BiTE-induced tumor cell killing. Moreover, NanoBiTE treatment led to substantially improved tumor growth inhibition in vivo in a humanized mouse model. Our results demonstrate that the drug-loaded bispecific T cell nanoengager is a safe and potent strategy for cancer immunotherapy.

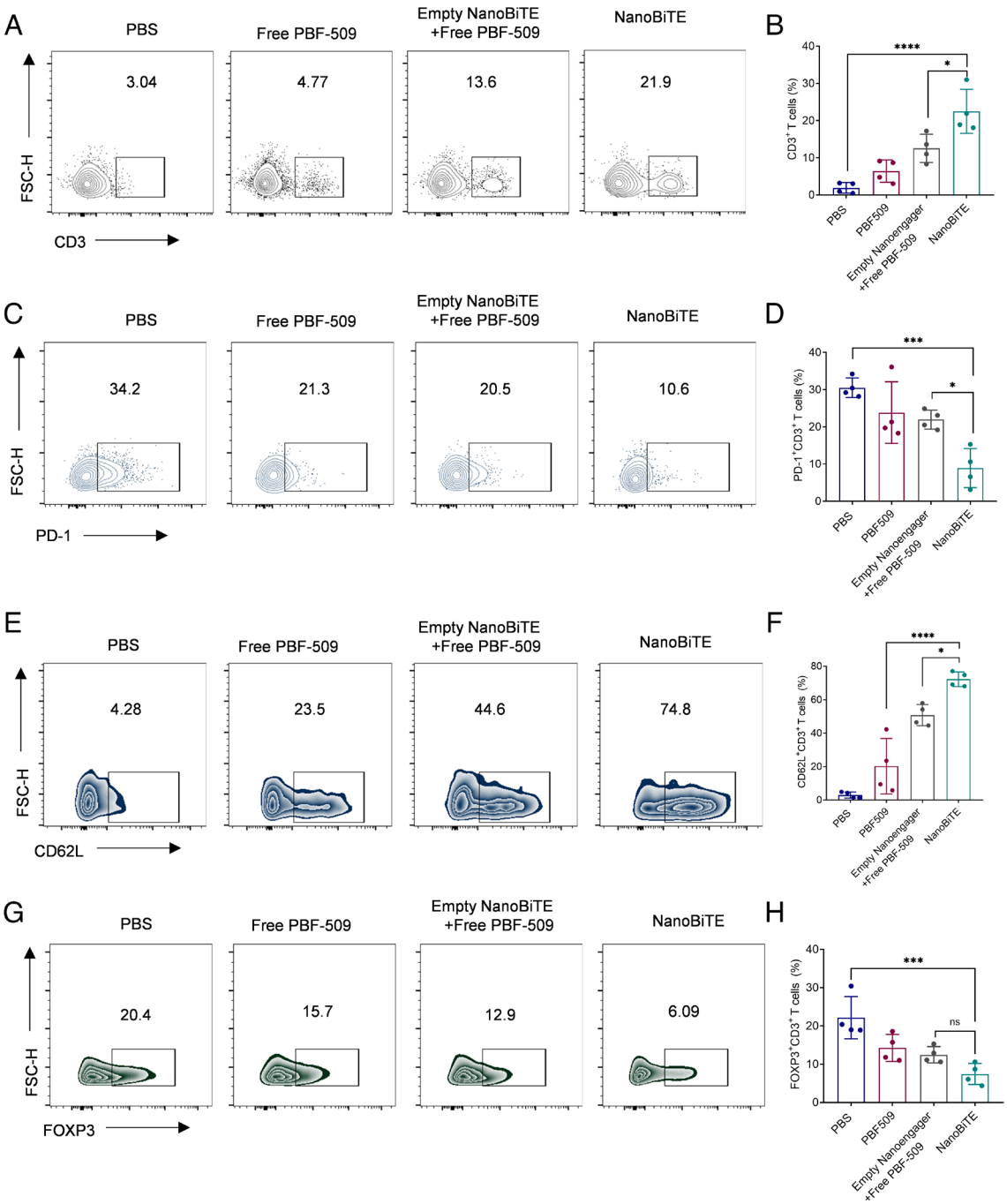

Fig. 4. NanoBiTE reduces T cell exhaustion and enhances T cell effector function. Mice were inoculated subcutaneously with Raji-Luc-GFP cells. Mice were treated intratumorally (*i.t.*) with various treatments. On day 9, tumor tissue was collected for flow cytometric analysis of tumor-infiltrating T cells. (*A*) Representative flow plots of $CD3^+$ T cell populations in tumor tissue. (*B*) Quantification of T cell infiltration in the tumor across treatment groups. (*C*) Representative flow plots of PD-1^+ T cell populations in tumor tissue. (*D*) Quantification of PD-1^+ T cell infiltration in the tumor. (*E*) Representative flow plots of memory $CD62L^+$ T cell populations in tumor tissue. (*F*) Quantification of memory T cell infiltration in the tumor. (*G*) Representative flow plots of $Foxp3^+$ regulatory T cell populations in tumor tissue. (*H*) Quantification of regulatory T cell infiltration in the tumor in each group. Data in *B*, *D*, *F*, and *H* are shown as the mean ± SD. In *B*, *D*, *F*, and *H*, n = 4. Statistical differences were analyzed by using one-way ANOVA with post hoc Student's *t* tests.

Despite success in treating hematological cancers, the application of BiTEs in treating solid tumors has been challenging due to several factors (34). First, solid tumors create a hostile microenvironment that suppresses immune responses (35). Components such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and immunosuppressive cytokines (e.g., TGF-β, IL-10) inhibit T cell activity (36). In addition, the dense extracellular matrix and abnormal vasculature in solid tumors impede the infiltration and distribution of BiTEs. Moreover, solid tumors often exhibit heterogeneous expression of TAAs, leading to incomplete targeting and resistance (37). Given the limitations of current BiTEs in treating solid tumors, future studies should explore the use of NanoBiTE in solid tumor applications. Overcoming T cell exhaustion is crucial for improving the therapeutic outcomes of BiTEs for solid tumor treatment. First, reversing exhaustion can restore the effector functions of T cells, enhancing their cytotoxic activity against tumor cells. Second, strategies that prevent or reverse exhaustion can prolong the persistence and survival of T cells, ensuring sustained antitumor activity. This approach demonstrates an enhanced tumor infiltration by T cells and reduced

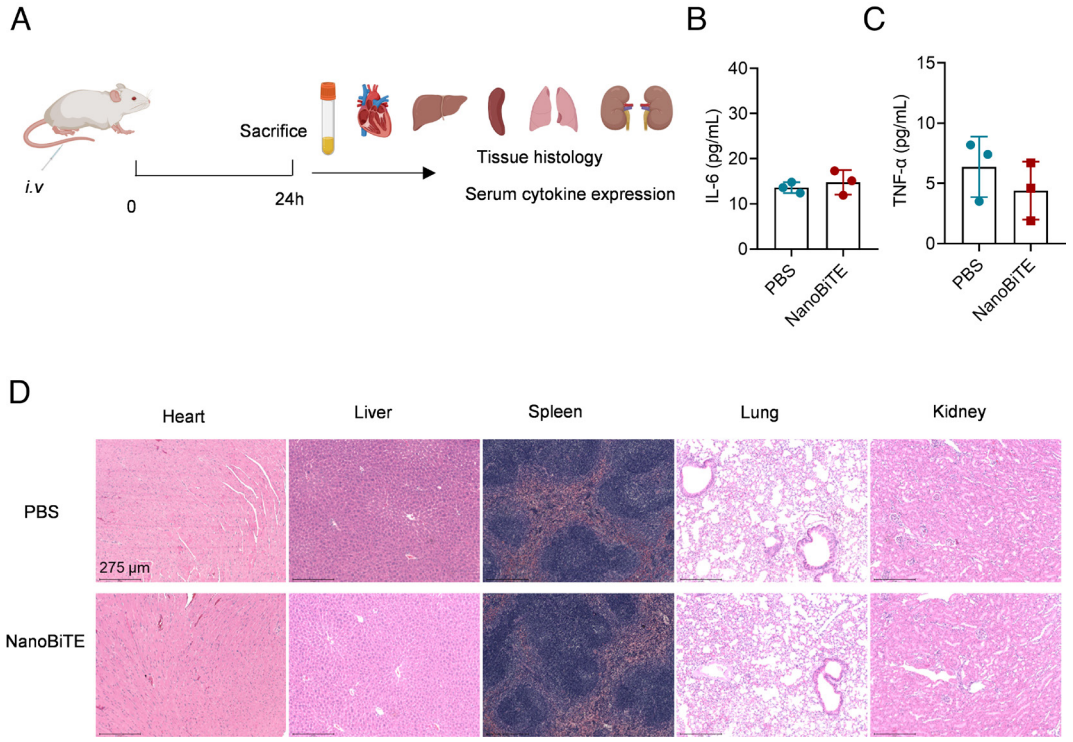

Fig. 5. Evaluation of in vivo NanoBiTE biocompatibility. (*A*) Humanized NSG mice were treated with PBS or NanoBiTE for 24 h, following which serum was collected. The concentration of cytokines interleukin-6 (IL-6) (*B*) and tumor necrosis factor-α (TNF-α) (*C*) in serum was determined via ELISA. (*D*) Major organs (heart, liver, spleen, lung, kidney) from mice treated with PBS or NanoBiTE were collected, sectioned, stained with H&E, and imaged via brightfield microscopy. (Scale bar, 275 μm.) Data in *B* and *C* are shown as the mean ± SD (n = 3).

exhaustion compared to previously reported platforms. This will require a multifaceted approach, combining BiTEs with other modalities that enhance T cell function and longevity within the challenging microenvironment of solid tumors.

Materials and Methods

All the detailed materials and methods are included in *SI Appendix*.

Cells. Raji–GFP-Luc cells were obtained from Dr. Alan Epstein (University of Southern California). The cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured in incubators maintained at 37 °C with 5% CO_2. Human primary T cells were purchased from the Human Immunology Core at the University of Pennsylvania.

Animals. Female NSG mice, aged 6 to 8 wk, were procured from the Jackson Laboratory and housed in a specific-pathogen-free animal facility under controlled conditions: ambient temperature (22 ± 2 °C), air humidity of 40 to 70%, and a 12-h dark/12-h light cycle.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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