Engineered tumor-specific T cells using immunostimulatory photothermal nanoparticles

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ABSTRACT

Background: Adoptive T cell therapy (ATCT) has been successful in treating hematological malignancies and is currently under investigation for solid-tumor therapy. In contrast to existing chimeric antigen receptor (CAR) T cell and/or antigen-specific T cell approaches, which require known targets, and responsive to the need for targeting a broad repertoire of antigens in solid tumors, we describe the first use of immunostimulatory photothermal nanoparticles to generate tumor-specific T cells.

Methods: Specifically, we subject whole tumor cells to Prussian blue nanoparticle-based photothermal therapy (PBNP-PTT) before culturing with dendritic cells (DCs), and subsequent stimulation of T cells. This strategy differs from previous approaches using tumor cell lysates because we use nanoparticles to mediate thermal and immunogenic cell death in tumor cells, rendering them enhanced antigen sources.

Results: In proof-of-concept studies using two glioblastoma (GBM) tumor cell lines, we first demonstrated that when PBNP-PTT was administered at a “thermal dose” targeted to induce the immunogenicity of U87 GBM cells, we effectively expanded U87-specific T cells. Further, we found that DCs cultured ex vivo with PBNP-PTT–treated U87 cells enabled 9- to 30-fold expansion of CD4+ and CD8+ T cells. Upon co-culture with target U87 cells, these T cells secreted interferon-γ in a tumor-specific and dose-dependent manner (up to 647-fold over controls). Furthermore, T cells manufactured using PBNP-PTT ex vivo expansion elicited specific cytolytic activity against target U87 cells (donor-dependent 32–93% killing at an effector to target cell (E:T) ratio of 20:1) while sparing normal human astrocytes and peripheral blood mononuclear cells from the same donors. In contrast, T cells generated using U87 cell lysates expanded only 6- to 24-fold and killed 2- to 3-fold less U87 target cells at matched E:T ratios compared with T cell products expanded using the PBNP-PTT approach. These results were reproducible even when a different GBM cell line (SNB19) was used, wherein the PBNP-PTT–mediated approach resulted in a 7- to 39-fold expansion of T cells, which elicited 25–66% killing of the SNB19 cells at an E:T ratio of 20:1, depending on the donor.

Conclusions: These findings provide proof-of-concept data supporting the use of PBNP-PTT to stimulate and expand tumor-specific T cells ex vivo for potential use as an adoptive T cell therapy approach for the treatment of patients with solid tumors.

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Introduction

Adaptive T cell therapy (ATCT) has emerged as a promising strategy for the treatment of cancers otherwise resistant to standard therapies. Chimeric antigen receptor T cells and antigen-specific T cells (e.g., against virus-associated antigens) are designed based on a priori known targets expressed by tumor cells and have been successfully used for the treatment of patients with hematologic malignancies [1–4]. However, solid tumors, and specifically brain tumors, have proven more difficult to treat with chimeric antigen receptor T cell approaches [5,6].

We have previously demonstrated that Prussian blue nanoparticle-based photothermal therapy (PBNP-PTT) decreases tumor burden, increases long-term survival and generates immunological protection against tumor rechallenge in syngeneic mouse models of solid tumors (neuroblastoma and melanoma) [7–12]. These responses were driven by increased endogenous T cell infiltration into PBNP-PTT–treated tumors and subsequent T cell activation [7–11]. When tumor-infiltrating T cells were isolated from long-term surviving mice previously treated with PBNP-PTT, these T cells were specifically cytotoxic to the tumor cell targets [12]. Importantly, these studies used PBNP-PTT administered at thermal doses validated to elicit immunogenic cell death (ICD) [13]. ICD is characterized by the recruitment and engagement of dendritic cells (DCs) and other antigen-presenting cells for subsequent T cell activation and is characterized by the release of ATP, the increase of cell-surface calreticulin and the release of HMGB1 from tumor cells [14–16].

Motivated by these observations that PBNP-PTT can trigger potent endogenous T cell immunity in vivo, we sought to replicate this approach in an ex vivo manufacturing protocol to generate multiantigen-specific T cells in an antigen-agnostic manner. We hypothesized that DCs co-cultured with PBNP-PTT–treated tumor cells would be primed for enhanced antigen uptake and presentation, to facilitate the generation of a potent, multiantigenic, tumor-specific T cell population in an antigen-agnostic manner for future use in ATCT.

For proof-of-concept feasibility of the PBNP-PTT–based T cell expansion platform, we used glioblastoma (GBM) as a tumor cell model. GBM is the most commonly diagnosed brain or central nervous system tumor in the United States, impacting ~12 000 people per year [17]. Despite intense standards-of-care that include surgery, radiation and chemotherapy (e.g., temozolomide), the prognosis for GBM remains dismal, with a 5-year survival rate of 5% [17]. Therefore, there is an urgent need to develop novel therapies for this vulnerable patient population. The heterogeneity of GBM cells is an important limiting factor to immune therapies [18–20] and is a major source of treatment failure [21–23]. We anticipate that our PBNP-PTT–based T cells will overcome this treatment limitation, as each T cell product will be designed to recognize the patient’s tumor, thereby potentially targeting diverse, patient-tumor-specific antigens. We envision that autologous GBM-specific T cells generated using this approach may be clinically administered to patients with poor-prognosis GBM in combination with standards-of-care and/or in the setting of minimal residual disease post-surgery.

The overall strategy to generate GBM-specific T cells using PBNP-PTT is illustrated in Figure 1. First, we investigated the effect of PBNP-PTT to generate thermal effects and immunoactivation in the well-characterized GBM cell line U87. We then expanded U87-specific T cells using healthy donor peripheral blood mononuclear cells (PBMCs) that were matched with the tumor cells at the HLA-A*02 antigen using our novel PBNP-PTT–mediated expansion platform. Next, we evaluated the expansion and phenotype of the resultant T cell products. Finally, we demonstrated the specificity and cytotoxic functionality of the U87-specific T cells toward the target U87 cells, evaluated for off-target effects by culturing the T cell products with normal human astrocytes (NHAs) and PBMCs, and verified the broad applicability of the manufacturing approach by replicating the results in another GBM cell line (SNB19). Together, these data provide us with a compelling rationale to proceed to small animal studies to characterize the efficacy of these novel GBM-specific T cell products in vivo.

**Figure 1.** PBNP-PTT–mediated tumor-specific T cell expansion scheme. Timeline for expanding tumor-specific T cells using PBNP-PTT. Day 0: Monocytes from individual donors are isolated using a CD14 isolation MACS beads kit. The isolated CD14-positive (CD14+) cells are cultured in DC media in the presence of IL-4 and GM-CSF. The CD14-negative (CD14−) fraction is frozen at −80°C. Day 2: PBNP-PTT is administered to target GBM tumor cells (i.e., U87, SNB19) in vitro and added to DCs. DCs are then matured with GM-CSF, TNF-α, IL-1β, IL-4, IL-6, IFN-γ, and LPS overnight. Day 3: CD14+ PBMCs are thawed and stimulated with harvested DCs at a 1:5 (DC:T cell) ratio in T cell media supplemented with IL-6, IL-7, IL-12 and IL-15 and incubated at 37°C. Day 9 or 13: T cells are fed with IL-6, IL-7 and IL-15. Day 13: Fresh or frozen CD14+ cells are thawed and cultured with IL-4 and GM-CSF. Day 15: Day 2 protocol is repeated. Day 16: Day 3 protocol is repeated and treated DCs are added to the ongoing T cell culture with IL-7 and IL-2. Day 20 (or as needed based on T cell proliferation): T cells are fed with IL-15. Day 23: T cells are harvested for phenotyping and functional assays. GM-CSF, granulocyte–macrophage colony-stimulating factor; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α. (Color version of figure is available online.)
Materials and Methods

Cell lines and cell culture

U87 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SNB19 cells were obtained from the National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository through a Materials Transfer Agreement. NHAs were purchased from Thermo Fisher Scientific (Waltham, MA, USA). U87 cells were grown in Minimum Essential Medium Eagle (MEM media; Millipore Sigma, Burlington, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Cyiva, Marlborough, MA, USA), 1% Glutamax (Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco). SNB19 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Cyiva), 1% Glutamax (Gibco) and 1% penicillin–streptomycin (Gibco). NHAs were cultured on Geltrex matrix–coated plates in Astrocyte Medium (Thermo Fisher Scientific). Cells were expanded as per the specifications provided by the supplier. Healthy donor peripheral blood leukopaks were purchased from AllCells (Alameda, CA, USA) with the corresponding human leukocyte antigen (HLA) reports. PBMCs were matched at the HLA-A*02 antigen expressed by both U87 and SNB19 cells. Supplementary Table 1 lists the available HLA typing of healthy donors and cell lines [24–26]. As blood was sourced from a commercial vendor, donors were de-identified before receipt to ensure privacy. PBMCs were isolated by density gradient centrifugation using lymphocyte-separation media (LSM; Corning, Corning, NY, USA). PBMCs were cryopreserved for future use. DCs were cultured in DC media (CellGenix, Freiburg im Breisgau, Germany) supplemented with 1% Glutamax (Gibco). T cells were cultured in 46.5% Click’s media (Millipore Sigma), 46.5% RPMI-1640 (Gibco), 5% human AB serum (Gemini Bio-products, Sacramento, CA, USA), 1% Glutamax (Gibco) and 1% penicillin–streptomycin (Gibco).

PBNP-PTT treatment of tumor cell lines

PBNPs used for PTT were synthesized as described previously [27, 28]. Potassium hexacyanoferrate (II) trihydrate (K₃[Fe(CN)₆]·3H₂O) and iron (III) chloride hexahydrate (Fe(Cl)₃·6H₂O) were purchased from Millipore Sigma. To summarize in brief, an aqueous solution of 1.0 mmol/L FeCl₃·6H₂O in 20 mL of deionized (DI) water was added under vigorous stirring to an aqueous solution containing 1.0 mmol/L of K₃[Fe(CN)₆]·3H₂O in 20 mL of DI water. After stirring for 15 min, the precipitate was isolated by centrifugation in equal parts DI water and acetone (10 000g for 10 min) at room temperature, and rinsed by sonication (5 s, 40% amplitude) in DI water using a Q500 sonicator (QSonica LLC, Newtown, CT, USA). The isolation and rinsing steps were repeated three times before the nanoparticles were resuspended by sonication in DI water. This process yielded stable and monodisperse PBNPs as measured by dynamic light scattering on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) (Supplementary Figure 1). To administer PBNP-PTT to the GBM tumor cells for calreticulin and cytokine analysis, five million U87 or SNB19 cells were suspended in 500 μL of their respective media with 0.15 mg/mL PBNPs. The samples were then illuminated using a near-infrared laser (808 nm; Laserglow Technologies, Toronto, Canada) for 10 min at varied powers (0.75, 1.0, 1.5, 2.0 W) to administer a range of thermal doses. The laser power used was confirmed using a power meter (Thorlabs, Newton, NJ, USA). The maximum temperature of the cell suspension was measured using a thermal camera (FLIR, Arlington, VA, USA), and recorded every minute for 10 min. The PBNP-PTT scheme is illustrated in Supplementary Figure 2. The PBNP-PTT–treated GBM cells were then mixed using a pipette and added to a 6-well plate containing either U87 or SNB19 media and incubated at 37°C for 24 h before further analysis was performed. When PBNP-PTT was performed during the T cell culture protocol, 5 million U87 or SNB19 cells were treated with PBNP-PTT (0.15 mg/mL PBNPs and 1.5 W laser power) in 500 μL of DC media. PBNP-PTT–treated cells were then mixed using a pipette and added directly to DCs in DC media in a 1:1 ratio of DC/tumor cells (500 000 DCs and 500 000 tumor cells) in a 24-well plate, immediately after PBNP-PTT was completed. The DC and tumor cell co-culture was then incubated at 37°C overnight.

Heat treatment of tumor cell lines

In order to compare the heat effects of PBNP-PTT versus heating by other means, five million U87 cells were suspended in 500 μL of media. The samples were then placed in a heat block set at 60°C, 70°C or 80°C for 10 min. The maximum temperature of the cell suspension was measured using a thermal camera (FLIR, Arlington, VA, USA), and recorded every minute for 10 min. Following incubation in the heat block, the samples were split into two aliquots. In one aliquot, 0.15 mg/mL PBNPs were added, to evaluate the effect of PBNPs on resulting tumor cell immunogenicity. The second aliquot received an equivalent volume of water. Then, the cells were then mixed using a pipette and added to a 6-well plate containing U87 cell media and incubated at 37°C for 24 h before further analysis was performed.

Calreticulin expression analysis

To assess calreticulin expression levels on treated tumor cells as a measure of immunogenicity, tumor cells were harvested after 24 h incubation at 37°C post-PBNP-PTT or post-heating by heat block, stained with Zombie Violet Fixable viability dye (BioLegend, San Diego, CA, USA), blocked with human TruStain FC block (BioLegend) and stained with a fluorescent antibody against calreticulin (clone FMC75; Enzo Life Sciences, Farmingdale, NY, USA) [29]. Flow cytometry was performed on a BD FACS Cealetta (Franklin Lakes, NJ, USA) flow cytometer, and cytometric analysis was done using FlowJo software (Ashland, OR, USA).

Cytokine release analysis

To evaluate the release of cytokines on treated tumor cells as a measure of immunogenicity, supernatants from tumor cells were harvested after 24 h incubation at 37°C post-PBNP-PTT or cell lysis. The Legendplex Human Immune Response Panel (BioLegend) assay was performed as per manufacturer’s instructions, and visualized on the Cytoflex flow cytometer (Beckman Coulter, Brea, CA, USA). Median fluorescence intensity (MFI) values that exceeded the limit of detection are listed as upper limit of detection; MFI values that fell below the limit of detection are listed as lower limit of detection.

T cell expansion protocol

T cell lines were generated from PBMCs matched at HLA-A*02 to U87 or SNB19 cells using monocyte-derived DCs based on a previously established protocol [30] with minor modifications, as illustrated in Figure 1. Three or four individual donors were used to generate both U87- and SNB19-specific T cells. To summarize, monocytes were isolated using a CD14 isolation MACS MicroBeads kit (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany) per the manufacturer’s instructions. The isolated CD14 positive (CD14+) cells were cultured in DC media in the presence of interleukin (IL)-4 (1000 U/mL) and granulocyte–macrophage colony-stimulating factor (800 U/mL) (R&D Systems, Minneapolis, MN, USA). The CD14-negative (CD14−) fraction was cryopreserved for later use. Two days later (day 2), the CD14+ cells (now termed DCs) from each donor were pulsed with GBM cells subjected to PBNP-PTT as described herein and previously [13, 31] at 1.5 W laser power, or GBM cell
lysates generated using multiple freeze-thaw cycles using a dry-ice-ethanol mixture and a 37°C water bath [32,33]. The PBNP-PTT-treated GBM cells or GBM cell lysates were added to DCs at a ratio of 1:1. GBM cells surviving post-PBNP-PTT or post-lysis were not viable in DC media, and cell death was confirmed using Trypan Blue solution, 0.4% (Thermo Fisher Scientific) visualized on the LUNA-FL automated cell counter (Logos Biosystems; Dongan-gu Anyang-si, South Korea). On the same day that the treated GBM cells were added to DCs (day 2), DCs were matured with granulocyte-macrophage colony-stimulating factor (800 U/mL), tumor necrosis factor-α (10 ng/mL), IL-1β (10 ng/mL), IL-4 (1000 U/mL), IL-6 (100 ng/mL), interferon gamma (IFN-γ; 100 U/mL) (R&D Systems) and lipopolysaccharide (30 ng/mL; Millipore Sigma) overnight. The following day (day 3), the cryopreserved CD14+ PBMCs were thawed and stimulated with the primed and harvested DCs at a 1:5 (DC/T cell) ratio in T cell media supplemented with IL-6 (100 ng/mL), IL-7 (10 ng/mL), IL-12 (10 ng/mL) and IL-15 (5 ng/mL) (R&D Systems). These cells were incubated at 37°C and were expanded and fed with IL-6 (100 ng/mL), IL-7 (10 ng/mL) and IL-15 (5 ng/mL) (R&D Systems) as needed. On day 13, frozen PBMCs from the same donor were thawed, and CD14 isolation was performed as described previously. On day 15, DCs were again pulsed with GBM cells subjected to PBNP-PTT or cell lysis at a ratio of 1:1 (tumor cell/DC), and matured as described previously. The following day (day 16) these matured DCs were added to the ongoing T cell culture at a ratio of 1:5 (DC/T cell) in the presence of IL-7 (10 ng/mL) (R&D Systems) and IL-2 (50 U/mL) (Proluclin; Clingen Group, London, UK). T cells were fed and split with fresh media and IL-15 (5 ng/mL) (R&D Systems) when necessary. On day 23, cells were harvested for phenotyping and functional assays. Expansion was quantified by harvesting and counting with acridine orange/propidium iodide cell viability kit (Logos Biosystems) on a LUNA-FL automated cell counter (Logos Biosystems) periodically throughout the development scheme. Control PHA-stimulated lymphocytes (PHA-L) were generated using CD14+ cells from the same donor PBMCs. CD14+ cells were stimulated with lectin, PHA-L, Phaseolus vulgaris (PHA; 5 μg/mL; Millipore Sigma) on day 1, and cultured for 11 days in RPMI-1640 (Gibco) supplemented with 5–10% human AB serum (Gemini Biologicals), 1% Glutamax (Gibco) and 1% penicillin streptomycin (Gibco) and IL-2 (100 U/mL; Clingen Group). T cell phenotype, specificity and cytotoxic functionality was measured by flow cytometry, ELISPOT and calcein AM cytotoxicity assays, respectively, as described to follow.

**T cell phenotyping**

T cells were harvested on day 23 of the expansion protocol (or day 11 for PHA-L) and assessed for viability and phenotypic markers. The cells were stained with Zombie green fluorescent viability dye (BioLegend), blocked with human TruStain Fc block (BioLegend) and stained with fluorescent antibodies from BioLegend against human CD3 (clone HIT3a), CD4 (clone RPA-T4) and CD8 (clone SK1). Flow cytometry was performed on either the Beckman Couter Cytoflex or BD FACSCelesta flow cytometer and cytometric analysis was performed using Flowjo software.

**T cell specificity analysis**

T cell specificity was determined by IFN-γ ELISPot. Multiscreen HTS IP filter plates (Millipore Sigma) were coated with an IFN-γ capture antibody (clone 1-D1K; Mabtech, Stockholm, Sweden). On the following day, the target cells (U87 or SNB19) were collected from 37°C incubation and used directly. T cells were either plated alone or in presence of target cells at varied effector-to-target cell (E:T) ratios (i.e., 1:1, 2:1, 5:1, 10:1, 20:1, 50:1), keeping the number of T cells constant at 100 000 cells per well and varying the number of target cells. Then, 1 μg/mL actin was administered to the T cells as a negative control (JPT peptide technologies, Berlin, Germany); 1 μg/mL PHA (Millipore Sigma) was administered to the T cells as a positive control for T cell activation. Cells were incubated at 37°C and the ELISPOT plate was developed 24 h later using the manufacturer’s protocol. The number of IFN-γ spot-forming units were quantified by Zellnet Consulting (Fort Lee, NJ, USA). The frequency of tumor-specific T cells was expressed as spot-forming cells per 100 000 cells.

**T cell cytotoxicity analysis**

To assess the cytotoxicity of T cells against target cells, we conducted a calcein release assay. Target cells (U87, SNB19, NHA or PBMCs from donor 4) were labeled with calcein AM (Thermo Fisher Scientific) for 30 min at 37°C. Spontaneous and total release of calcein dye from the target cells was measured using a SpectraMax 13X microplate reader ( Molecular Devices, San Jose, CA, USA) by treating the cells with media or 2% Triton X-100 (Millipore Sigma), respectively [34,35]. Expanded T cells were resuspended at different concentrations and were mixed with the aforementioned target cells to generate E:T ratios of 2.5:1, 5:1, 10:1 or 20:1. Target cell concentration remained constant in all E:T ratios (10 000 target cells per well), and T cells were varied (10 000–500 000 T cells per well depending on the E:T ratio). To quantify T cell cytotoxicity-based calcein release, the co-cultures of T cells and tumor cells were incubated for 4 h at 37°C whereupon the supernatants were harvested and analyzed for calcein dye release using the SpectraMax 13X microplate reader. Specific lysis was calculated using the formula: (sample measurement-spontaneous release) / (total release-spontaneous release) × 100.

**Statistics**

Statistically significant differences between groups were determined using analysis of variance and pairwise t-tests in Microsoft Excel (Redmond, WA, USA). Values were considered statistically significantly different when P values were less than 0.05. To ensure rigor and reproducibility, n = 3–4 individual donors were used for biological replicates for all analyses, and n = 2 technical replicates were used for each donor.

**Results**

PBNP-PTT heats tumor cells, generating increased tumor cell death and immunogenicity in a thermal dose-dependent manner

PBNP-PTT was administered to U87 cells to evaluate the impact of varied thermal dose on the ex vivo expansion of tumor-specific T cells. Upon administration of PBNP-PTT, the temperature of U87 cells increased in a laser power-dependent manner over time (Figure 2A), which is a measure of the thermal dose. At the same time, U87 cell viability decreased in a laser power- and thermal dose-dependent manner (Figure 2B), with 23.2% U87 cells remaining viable at the highest laser power of 2.0 W PBNP-PTT. Previous literature, including several of our own published studies, has demonstrated that the expression of calreticulin on the surface of dying cells is critical to engage immunogenic antitumor signaling [29,36,37]. Consequently, as a measure of the immunogenic response generated by PBNP-PTT, we assessed the levels of calreticulin expressed on the surface of U87 cells. In response to PBNP-PTT, calreticulin expression levels significantly (P < 0.05) increased on the surface of live U87 cells in a laser power- and thermal dose-dependent manner (Figure 2C, Supplementary Figure 3). Calreticulin expression was observed to be maximal after PBNP-PTT using a laser power of 1.5 W based on MFI (Figure 2C). Administering a laser power of 1.5 W during PBNP-PTT also resulted in significant GBM cytotoxicity (71% U87 cell death; P = 5.98E−5 compared with vehicle), and, as such, represents the
optimal PBNP-PTT condition to enable both immunogenicity and target cell death.

To determine whether PBNP-PTT elicits differential immunogenicity in U87 cells compared with U87 cell lysis, U87 cell supernatants were harvested after treatment with either PBNP-PTT or lysis and evaluated for the presence of secreted or released cytokines. PBNP-PTT facilitated the increased release of several pro-inflammatory cytokines (i.e., IL-4, tumor necrosis factor-α, MCP1, IL-17A, IL-6, IFN-γ, IL-12p70 and IL-8) from U87 cells, compared with U87 cell lysis (Supplementary Figure 4).

To evaluate whether heating by other means elicits similar cytotoxic and immunogenic responses as PBNP-PTT, U87 cells were heated in a heat block to 60, 70 or 80°C and then subsequently incubated with media or media containing PBNPs. Heating by the heat block at all temperatures facilitated >70% U87 cell death, and no associated increases in calreticulin expression on the remaining live cells (Supplementary Figure 5). Compared with U87 cells heated to the same temperatures in the absence of PBNPs, the presence of PBNPs elicited significantly higher release of ATP (measured by a decrease in intracellular ATP), and a decreased viability, but there were no associated changes in immunogenicity as measured by calreticulin expression. These data suggest that unlike conventional cell heating, PBNP-PTT can heat tumor cells and elicit cytotoxicity in a thermal dose-dependent manner, while simultaneously upregulating calreticulin, a signal involved in antigen engulfment by DCs [14].

After confirming the thermal dose-dependent heating by PBNP-PTT and its effects on tumor cell viability and immunogenicity in vitro, we conducted studies to test whether PBNP-PTT could effectively expand tumor-specific T cells. Accordingly, PBNP-PTT was administered to U87 cells using 0.15 mg/ml PBNPs and 1.5 W laser power, as this condition was measured to be optimal in terms of generating significant cytotoxicity and maximal expression of calreticulin on the surface of U87 cells (Figure 2). The PBNP-PTT-treated U87 cells were then co-cultured with DCs from four donors (matched at HLA-A*02 to target U87 cells) and subsequently, primed DCs were added to CD14+ cells derived from the corresponding donors to expand tumor-specific T cells, as detailed in the Materials and Methods section. Three to four biological replicates (i.e., from three or four individual donors), as well as two technical replicates, were used for all T cell-based assays to ensure robust and reproducible results and to evaluate statistical significance. T cells developed using our PBNP-PTT-based approach to target U87 cells expanded 14.7-, 25.2-, 29.8- and 8.9-fold (donors 1-4, respectively) by day 23 (Figure 3A). By contrast, T cells developed using U87 cell lysates expanded 8.5-, 24.3- and 6.3-fold in 23 days (donor 1, 3, 4, respectively; Donor 2 was excluded due to lack of available PBMCs) (Figure 3B). The expanded T cell products generated using PBNP-PTT or cell lysis were analyzed for T cell phenotype by flow cytometry. PBNP-PTT-mediated ex vivo

Figure 2. PBNP-PTT heats, kills and increases immunogenicity of GBM cells in a thermal dose-dependent manner. U87 cells were subjected to 0.15 mg/ml PBNPs and illuminated with a NIR laser for 10 minutes at the listed laser powers, varying from 0.75-2.0 W. (A) Temperatures, measured at 1-minute intervals, attained by U87 cells as a function of time in response to varying PBNP-PTT laser powers. (B) Viability of U87 cells in response to varying laser powers measured 24 h post-PBNP-PTT by flow cytometry. (C) Calreticulin expression on live U87 cells in response to varying laser powers, measured by flow cytometry 24 h post-PBNP-PTT, as described by MFI. Values represent mean ± standard deviation (n = 3/group). *P < 0.05 compared with vehicle. (Color version of figure is available online.)

PBPN-PTT mediates efficient expansion of potent tumor-specific T cells

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expansion resulted in a mean of 90.1% CD3+ T cells. Of the CD3+ cells, we identified 33.5% CD4+ and 57.3% CD8+ T cells (Figure 3C, Supplementary Figure 6). T cell products expanded using U87 cell lysates comprised a mean of 80.1% T cells, 65.9% CD4+ T cells, and 28.5% CD8+ T cells (Supplementary Figure 7).

Having established the efficient expansion of PBNP-PTT–mediated T cells using multiple donors, we analyzed the specificity and functionality of the resulting T cell products in co-culture assays with the target tumor cells. All donors’ T cell products targeted U87 cells significantly (P < 0.05 compared with actin) secreted IFN-γ in response to exposure to target U87 cells in an E:T ratio-dependent manner (Figure 3D, Supplementary Figure 8, Supplementary Table 2). For each E:T ratio, the number of T cells was held constant, whereas the number of target U87 cells varied. At an E:T ratio of 1:1, 870 ± 427 T cells secreted IFN-γ in response to target U87 cells, depending on the donor. At E:T ratios of 2:1, 5:1, 10:1, 20:1 or 50:1, 614 ± 45, 406 ± 160, 324 ± 169, 283 ± 181 or 196 ± 129 T cells secreted IFN-γ, respectively, in response to target U87 cells, depending on the donor. To compare these effects to the intrinsic capability of T cells to respond to GBM cells, we expanded T cells from three donors non-specifically using PHA stimulation (termed “PHA lymphocytes” or “PHA-L”). At E:T ratios of 1:1, 2:1, 5:1, 10:1, 20:1 or 50:1, only 20 ± 12, 23 ± 8, 10 ± 6, 8 ± 4, 4 ± 3 or 2 ± 2 PHA-L secreted significantly (P < 0.05) lower levels of IFN-γ, respectively, in response to U87 cells, depending on the donor, compared to T cell products manufactured using the PBNP-PTT approach (Supplementary Figure S9). Similarly, 118 ± 59 T cells expanded using U87 cell lysates (donors 1, 3, 4) secreted IFN-γ in response to U87 cells at an E:T ratio of 1:1, significantly lower than T cell products expanded using the PBNP-PTT strategy from the same donors (Figure 3E). At E:T ratios of 2:1, 5:1, 10:1, 20:1 or 50:1, 118 ± 50, 79 ± 49, 61 ± 38, 41 ± 42 or 32 ± 27 T cells developed using U87 cell lysates secreted IFN-γ, respectively, in response to target U87 cells. Additional description of the ELISpot results can be found in Supplementary Tables S2-S3. Together, these results illustrate the ability of PBNP-PTT–mediated T cell development to enable efficient expansion over 23 days. The expanded T cells were observed to potently activate in response to U87 cell exposure by secreting IFN-γ in an E:T ratio-dependent manner and exhibited greater specificity than T cell products expanded using U87 cell lysates, or via non-specific (PHA) expansion.

T cells developed via PBNP-PTT–mediated expansion exhibit specific and dose-dependent cytotoxicity toward target cells

Critical to their effector function, we evaluated whether IFN-γ secretion from U87-specific T cells developed via PBNP-PTT corresponded with an increased cytotoxicity toward target cells. To test the specificity of the resulting PBNP-PTT–derived T cells to tumor cells and not healthy central nervous system–derived or off-target cells, we also assessed their cytotoxicity toward NHAs and PBMCs. T cell products expanded using PBNP-PTT were harvested on day 23 and co-cultured with target U87 cells. At E:T ratios of 2.5:1, 5:1, 10:1, and 20:1, these T cell products killed 15.5–49.5%, 20.2–69.6%, 24.5–86.0% and 32.3–92.7% of target U87 cells in the co-culture after...
4 hours, respectively, depending on the donor (Figure 4A). In contrast, non-specific T cells (PHA-L) derived from the same donors did not elicit cytotoxicity against U87 cells (Supplementary Figure 9). T cells derived through U87 cell lysis killed U87 cells 8.4–36.3%, 7.7–48.7% and 11.2–58.2% at E:T ratios of 2.5:1, 5:1, 10:1 and 20:1, respectively (Figure 4B). At an E:T ratio of 20:1, T cells developed via PBNP-PTT killed 1.6-fold (donor 1), 2.6-fold (donor 3) and 2.9-fold (donor 4) more target U87 cells than did T cells developed via U87 cell lysis.

Importantly, the same T cell products (developed to target U87 cells via PBNP-PTT) killed only 2.4%, 5.2%, 3.6% and 2.7% of NHA (donor 1) and 2.1%, 1.0%, 3.1% and 3.8% of autologous PBMCs (donor 4), when co-cultured at E:T ratios of 2.5:1, 5:1, 10:1 and 20:1 (Figure 4C). These values reflect a mean of 13.0-, 6.8-, 11.3- and 16.2-fold increased cytotoxicity of T cells developed via PBNP-PTT on U87 cells versus NHA, and 7.4-, 20.5-, 7.9- and 8.5-fold increased cytotoxicity on U87 cells versus PBMCs, supporting the specificity of T cells developed via PBNP-PTT to specifically kill tumor cells without resulting in cytotoxicity against non-tumor cells, nor autoreactive cytotoxicity.

**PBNP-PTT–mediated expansion generates potent, tumor-specific T cells against a second GBM tumor cell line**

To evaluate the robustness of the PBNP-PTT–mediated T cell generation strategy, we implemented the same platform to generate T cells targeting another GBM tumor cell line, SNB19. Accordingly, PBNP-PTT was administered to SNB19 cells, which resulted in increased heating, cytotoxicity and immunogenicity in a thermal dose-dependent manner (Supplementary Figures 10 and S11) similar to U87 cells. The PBNP-PTT–treated SNB19 cells were then co-cultured with DCs derived from three donors (all matched at HLA-A*02 to target SNB19 cells) and subsequently, primed DCs were added to CD14− cells from the corresponding donors to enable the development of tumor-specific T cells, as detailed in the Materials and Methods section. T cells expanded using our PBNP-PTT–based approach to target SNB19 via the PBNP-PTT–mediated scheme expanded 39.1-, 812, 336, 399 and 257, 311, 328 and 268 ± 219 T cells secreted IFN-γ, respectively, in response to target SNB19 cells, depending on the donor. At E:T ratios of 2:1, 5:1, 10:1, 20:1 or 50:1, 887 ± 812, 483 ± 336, 399 ± 257, 311 ± 312 or 268 ± 219 T cells secreted IFN-γ, respectively, in response to target SNB19 cells, depending on the donor. In contrast, at all E:T ratios tested, a mean of two or fewer PHA-L products secreted IFN-γ in response to SNB19 cells, suggesting that these non-specifically expanded PHA-L products derived from the same donors did not elicit an alloreactive response to the target cells (Supplemental Figure 9). We next evaluated whether IFN-γ secretion from PBNP-PTT expanded SNB19-specific T cells corresponded with an increased cytotoxicity toward target cells. Consequently, T cells manufactured using PBNP-PTT were harvested on day 23 and co-cultured with target SNB19 tumor cells. At E:T ratios of 2.5:1, 5:1, 10:1 and 20:1, T cells killed 9.2−23.3%, 15.0−33.4%, 20.0−43.2% and 25.3−66.4% of target SNB19 cells in a co-culture after 4 hours, respectively, depending on the donor (Figure 5D), unlike non-specifically activated T cell products (PHA-L) derived from the same donors which were unable to elicit a cytolytic effect against SNB19 cells (Supplemental Figure 9). These data suggest that the PBNP-PTT–mediated T cell expansion strategy is eliciting a tumor-specific T cell response and may be broadly applicable to different tumor types.

**Discussion**

In this paper, we describe a novel platform to manufacture tumor-specific T cells for potential use in immunotherapy through the ex vivo–modulated ablation of tumor cells via PBNP-PTT (Figure 1). Antigen-specific T cells used in ATCT for cancer have traditionally been developed by pulsing autologous DCs with either tumor-associated antigen peptides or tumor cell lysates [38–42]. Here, we applied PBNP-PTT to GBM cells ex vivo to generate tumor cell death and immunogenicity (Figure 2, Supplementary Figure 4B). Having determined the expansion of PBNP-PTT–mediated T cells using multiple donors, the specificity and functionality of the resulting T cells were analyzed by co-culture of T cells with the target tumor cells. T cells developed from all donors significantly (P < 0.05 compared with actin) secreted IFN-γ in response to exposure to target SNB19 cells in an E:T ratio–dependent manner (Figure 5C, Supplementary Table 2). At an E:T ratio of 1:1, 1581 ± 1815 T cells secreted IFN-γ in response to target SNB19 cells, depending on the donor. At E:T ratios of 2:1, 5:1, 10:1, 20:1 or 50:1, 887 ± 812, 483 ± 336, 399 ± 257, 311 ± 312 or 268 ± 219 T cells secreted IFN-γ, respectively, in response to target SNB19 cells, depending on the donor. In contrast, at all E:T ratios tested, a mean of two or fewer PHA-L products secreted IFN-γ in response to SNB19 cells, suggesting that these non-specifically expanded PHA-L products derived from the same donors did not elicit an alloreactive response to the target cells (Supplemental Figure 9). We next evaluated whether IFN-γ secretion from PBNP-PTT expanded SNB19-specific T cells corresponded with an increased cytotoxicity toward target cells. Consequently, T cells manufactured using PBNP-PTT were harvested on day 23 and co-cultured with target SNB19 tumor cells. At E:T ratios of 2.5:1, 5:1, 10:1 and 20:1, T cells killed 9.2−23.3%, 15.0−33.4%, 20.0−43.2% and 25.3−66.4% of target SNB19 cells in a co-culture after 4 hours, respectively, depending on the donor (Figure 5D), unlike non-specifically activated T cell products (PHA-L) derived from the same donors which were unable to elicit a cytolytic effect against SNB19 cells (Supplemental Figure 9). These data suggest that the PBNP-PTT–mediated T cell expansion strategy is eliciting a tumor-specific T cell response and may be broadly applicable to different tumor types.

**Figure 4.** T cells developed via PBNP-PTT–mediated expansion exhibit specific and dose-dependent cytotoxicity toward target cells. (A) U87-specific T cells developed via PBNP-PTT were co-cultured with U87 cells at the listed E:T ratios for 4 h. Cytotoxicity was measured by calcein release (n = 2 replicates/donor). (B) U87-specific T cells developed via U87 cell lysis were co-cultured with U87 cells at the listed E:T ratios for 4 h. Cytotoxicity was measured by calcein release (n = 2 replicates/donor). Each data point in (B) is signiﬁcantly different (P < 0.05) compared with U87-specific lysis of corresponding donor T cells developed via PBNP-PTT at the equivalent E:T ratio (with the exception of donor 1 at the E:T ratio of 2.5:1). (C) U87-specific T cells developed via PBNP-PTT (donor 1 or donor 4) were co-cultured with NHA (donor 1) or PBMCs from the corresponding healthy donors (donor 4) at the listed E:T ratios. Cytotoxicity was measured by calcein release. Values represent mean ± standard deviation (n = 2/group). (Color version of figure is available online.)
S10), building on our published work in different solid tumor models in vivo [8,13]. We observed that DCs co-cultured with PBNP-PTT-treated GBM cells facilitated the expansion of GBM-specific T cells ex vivo in multiple donors using two distinct tumor cell lines (Figures 3-5).

One important feature of an effective ATCT for cancer is the discriminatory ability of the T cell product to preferentially kill cancer cells while sparing normal cells. We demonstrated that T cells developed to target GBM cells via PBNP-PTT were not cytotoxic toward NHA, despite likely sharing multiple antigens [43], nor were they cytotoxic toward PBMCs from the same healthy donor, suggesting the absence of autoreactivity (Figure 4). Additionally, non-specifically expanded (PHA-stimulated) T cell products (PHA-L) derived from the same donors were neither specific nor cytotoxic toward the GBM cells evaluated (Supplementary Figure 9). These findings suggest that T cells developed using the PBNP-PTT manufacture scheme are highly specific to the tumor cells they were designed to target.

We also demonstrated that T cells developed using PBNP-PTT had enhanced expansion (Figure 3A,B), specificity (Figure 3D,E) and cytotoxicity (Figure 4A,B) compared with T cell products generated using tumor cell lysates. Preclinical and clinical investigation has demonstrated that tumor lysates are a source of tumor-specific antigens for downstream DC and T cell activation [44-48], but this strategy has limitations. Tumor lysates may secrete immunoregulatory cytokines (e.g., transforming growth factor-β) that create tolerogenic DCs in the absence of other immunostimulatory cytokines and/or signals [49], and tumor antigens may require a vehicle for adequate delivery to DCs [50-52]. Like tumor lysates, PTT via other photothermal agents has been shown to induce multi-antigen release from tumor cells [53]. Importantly, other research suggests that the mechanism through which the cells die, as well as the presence of immunological adjuvants (e.g. calreticulin, HMGB1, ATP, DAMPs), will play a critical role in the response of anti-tumor immune responses elicited via tumor lysate-based vaccination approaches [36,54-56]. Here, we exploit the multi-antigen release from tumor cells observed in tumor lysis studies, but improve the downstream effects by providing pro-immunogenic signaling via ICD from tumor cells in the context of the tumor-specific antigens.

Although our proof-of-concept studies used allogeneic healthy donor immune cells matched to the tumor cell lines at one HLA, we envision that ATCT using this PBNP-PTT strategy may be most optimally clinical translated in an autologous manner. Patients with malignancies receiving a poor prognosis (e.g., GBM) will undergo
routine surgery or biopsy as determined by their oncologist and/or surgeon. PBNP-PTT will be administered to the biopsied or surgically resected tumor tissue, using previously determined thermal doses to induce ICD and immunogenicity of the tumor cells. PBNP-PTT–treated tumor cells will then be co-cultured with autologous immature monocyte-derived DCs from the patient, in the presence of cytokines, for tumor-specific T cell expansion.

The studies presented in this paper illustrate a proof-of-concept strategy for the manufacture of multitargeted tumor-specific T cells for ATCT, but further investigations are required to verify that the manufacturing platform is broadly applicable in other tumor types, and to characterize the antigens targeted by the resulting T cell products. There is evidence in the literature that nanoparticles have the ability to both capture antigens on their surfaces to enable immune cell engagement and stimulation [57] and are intrinsically susceptible to cellular uptake based on their physical characteristics [58–60]. These properties of nanoparticles could therefore confer a specific advantage to prime DCs with tumor-specific antigens, since antigens released from the tumor cells via PBNP-PTT would be immobilized on the surface of PBNPs to be taken up by DCs for processing and presentation [57,61]. Ongoing studies are therefore investigating the role of PBNPs on DC antigen uptake in the novel T cell manufacture. Future studies also aim to validate the efficacy of this novel PBNP-PTT–mediated expansion platform using PBMCs from patients with poor prognosis malignancies.

In summary, this study illustrates a novel platform for manufacturing a mixed population of personalized tumor-specific T cells for potential clinical use as an ATCT for patients with solid tumors, such as GBM. By using the immunogenicity elicited by ex vivo PBNP-PTT, we could robustly manufacture multitargeted tumor-specific T cell products in an antigen-agnostic manner. Here, GBM-specific T cells engineered using PBNP-PTT demonstrate robust cytolytic activity in vitro.

Declaration of Competing Interest

CMB and CRYC are co-founders and scientific advisory board members of Mana Therapeutics, a biotechnology company that uses ex vivo–expanded T cells as a therapy for cancer. CMB is a past scientific advisory board member for NextImmune and Repertoire Immune Medicines, both antigen-specific T cell companies. CRYC has equity interest in Mana Therapeutics. CMB has stock or ownership in Cabaletta Bio, Catamaran Bio, NextImmune, and Mana Therapeutics. EES and RF are co-founders of ImmunoBlue, a biotechnology company focused on developing PBNP-based nonimmunotherapies.

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Author Contributions

Conception and design of the study: EES, CRYC, RF. Acquisition of data: EES, PS, JC, DT, SJC. Analysis and interpretation of data: EES, PS, JC, DT, SJC, KBC, CES, CMB, CRYC, RF. Drafting or revising the manuscript: EES, PS, JC, DT, SJC, KBC, CES, CMB, CRYC, RF. All authors have approved the final article.

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Supplementary materials

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