Thermoreversible Poly(ethylene glycol)-*g*-Chitosan Hydrogel as a Therapeutic T Lymphocyte Depot for Localized Glioblastoma Immunotherapy
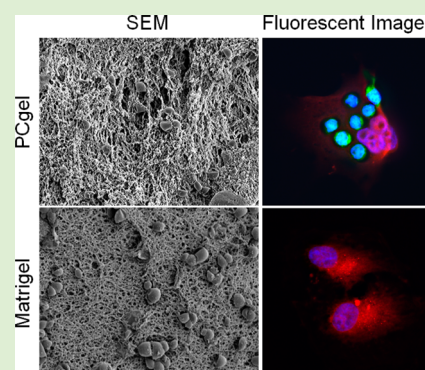
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ABSTRACT: The outcome for glioblastoma patients remains dismal for its invariably recrudesces within 2 cm of the resection cavity. Local immunotherapy has the potential to eradicate the residual infiltrative component of these tumors. Here, we report the development of a biodegradable hydrogel containing therapeutic T lymphocytes for localized delivery to glioblastoma cells for brain tumor immunotherapy. Thermoreversible poly(ethylene glycol)-*g*-chitosan hydrogels (PCgels) were optimized for steady T lymphocyte release. Nuclear magnetic resonance spectroscopy confirmed the chemical structure of poly(ethylene glycol)-*g*-chitosan, and rheological studies revealed that the sol-to-gel transition of the PCgel occurred around ≥ 32 °C. T lymphocyte invasion through the PCgel and subsequent cytotoxicity to glioblastoma were assessed *in vitro*. The PCgel was shown to be cellular compatible with T lymphocytes, and the T lymphocytes retain their anti-glioblastoma activity after being encapsulated in the PCgel. T lymphocytes in the PCgel were shown to be more effective in killing glioblastoma than those in the Matrigel control. This may be attributed to the optimal pore size of the PCgel allowing better invasion of T lymphocytes. Our study suggests that this unique PCgel depot may offer a viable approach for localized immunotherapy for glioblastoma.

INTRODUCTION

Glioblastoma (WHO grade IV) is the most aggressive and fatal form of brain tumor.[1,2] Despite the intensive treatment of current therapies such as surgical resection, followed by radio- and chemotherapies, glioblastoma remains incurable.[3,4] Glioblastomas rarely metastasize; instead, almost all types of these tumors recur locally within centimeters of the original resection cavity. Even after gross total resection (as confirmed by pathology), glioblastomas invariably recur within 2 cm of the original lesion because of their infiltrative nature.[5] This has inspired the development of various loco-regional treatment approaches such as Gliadel wafer (biodegradable polyanhydride wafers loaded with the chemotherapeutic carmustine) that are placed in the resection cavity and have shown some success as a drug delivery implant for glioblastoma.[6] Unfortunately, the two-month survival benefit gained with Gliadel wafers provides a very short survival advantage to glioblastoma patients, and thus, new therapies including better delivery approaches are urgently needed.

Many other approaches to local tumor treatment have been evaluated,[5−7] including direct introduction of chemotherapeutic agents by controlled release polymers placed in the tumor resection cavity,[8,9] direct infusion of toxin conjugates into the tumor,[10] and application of virus-producing cells for suicide gene therapy.[11,12] Nevertheless, chemotherapies and gene therapies are limited by tumor resistance and a lack of drug delivery specificity causing systemic toxicity and other off-target side effects. Immunotherapy that attempts to stimulate the immune system to specifically reject and destroy tumors with minimal harm to health tissues can be an alternative approach to chemotherapies and gene therapies in brain cancer treatment. Immunotherapy is a rapidly emerging treatment strategy for multiple malignancies[13] and is especially attractive for treating glioblastoma, where tumor cells residing beyond the resection cavity must be selectively targeted without damaging the normal brain tissue.[14] Adoptive T lymphocyte transfer involves the activation of patient-derived T lymphocytes toward a selective tumor cell-surface antigen, which has shown tremendous promise in preclinical studies.[13,15,16] Anti-glioblastoma adoptive T lymphocyte transfer has been shown to be safe in numerous clinical trials.[17] The key to the successful immunotherapy of glioblastoma will be dependent on the persistent existence of T lymphocytes to fully eradicate individual glioblastoma cells that have invaded regions throughout the brain. Therefore, developing approaches for locally sustained delivery of T lymphocytes is highly desirable.

Here we report the development of a biodegradable, thermal reversible hydrogel containing therapeutic T lymphocytes for localized delivery to glioblastoma cells for brain tumor immunotherapy. The hydrogel is made of poly(ethylene glycol)-g-chitosan (PCgel). Chitosan, a biodegradable, natural polysaccharide derived by the partial deacetylation of chitin, shares structural similarities with the glycosaminoglycans (GAGs) present in the native extracellular matrix (ECM). Poly(ethylene glycol) (PEG) is a neutral, water-soluble, and nontoxic polymer approved by the Food and Drug Administration for internal consumption and injection in a variety of foods, cosmetics, personal care products, pharmaceuticals, and biomedical applications.[18] The properties of the PCgel such as viscosity, pore size, and gelation time and temperature can be tailored by varying the amount of the PEG on chitosan or the concentration of the hydrogel. The PCgel introduced in our previous studies is a liquid at low temperatures and forms a gel readily at body temperature.[19,20] This unique property allows the gel to incorporate T lymphocytes and introduce them to the site of action (e.g., the brain tumor site) without the need for surgical intervention, for localized and sustained release. The PCgel made in our previous studies was stable in PBS but not in cell culture media. In this study, we aimed to produce a PCgel that is stable in cell culture media, has a pore diameter of a few micrometers, and can provide steady release of viable T lymphocytes while maintaining the same thermally reversible property. The chemical bonding between chitosan and PEG and the viscosity of the PCgel were characterized via nuclear magnetic resonance (NMR) analysis and rheological measurements, respectively. The biocompatibility of the PCgel was assessed by a culture with T lymphocytes. The ability of the PCgel to act as a depot for sustained release of cytotoxic T lymphocytes was tested using a transwell invasion assay. The cytotoxic activity of the invasive T lymphocytes on glioblastoma cells was examined using a live/dead assay.

■ EXPERIMENTAL SECTION

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Chitosan (85% deacetylated, medium molecular weight) and methoxy-poly(ethylene glycol) (PEG, 2000 Da) were used as received.

RPMI Media 1640 (RPMI), antibiotic-antimycotic, Dulbecco's phosphate-buffered saline (PBS), Lipofectamine 2000 Reagent, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

Preparation of PEG-g-chitosan. On the basis of our previous studies,[19,20] the poly(ethylene glycol)-g-chitosan (PEG-g-chitosan) was prepared with a slight modification to decrease the ratio of PEG to chitosan and thus to increase the hydrophobicity of chitosan and produce a firmer PEG-g-chitosan gel that would be stable in cell culture media. Specifically, PEG-aldehyde was prepared by oxidation of PEG with dimethyl sulfoxide (DMSO) and acetic anhydride.[21] After 20 g of PEG completely dissolved in an anhydrous chloroform/DMSO mixture [0.125 (v/v)], 10 mL of acetic anhydride was added to the solution. The mixture was steadily stirred for 16 h at room temperature under a nitrogen atmosphere. Then the solution was precipitated with excess diethyl ether. The precipitate was dissolved with chloroform and then reprecipitated with diethyl ether. After the sample had been dried under vacuum, white PEG-aldehyde powder was obtained.

PEG-g-chitosan was prepared by alkylation of chitosan followed by Schiff base formation.[22] The mixture of PEG-aldehyde and chitosan [0.32 (w/w)] were added to a mixture of methanol and 2% acetic acid [0.25 (v/v)]. A 5% cyanoborohydride (NaCNBH$_3$) aqueous solution was then added dropwise to the mixture of chitosan and PEG-aldehyde at pH 5.5 [NaCNBH$_3$/PEG-aldehyde, 0.2 (w/w)]. The resultant

mixture was dialyzed with a dialysis membrane (molecular weight cutoff of 12000−14000) against deionized (DI) water and 0.05 M NaOH, and then DI water was again added until a neutral pH was reached. The solution was subsequently freeze-dried. PEG-g-chitosan was obtained by removal of PEG-aldehyde residue with excess acetone. EtO gas was used to sterilize the PEG-g-chitosan powder.

Characterization of PEG-g-chitosan. The chemical bonding between chitosan and PEG in PEG-g-chitosan was confirmed via ^{1}H NMR spectroscopy, and ^{1}H NMR spectra were acquired with a Bruker AV-301 spectrometer with 500 MHz at 50 °C. PEG-g-chitosan samples of 10−20 mg each were dissolved in D$_2$O (0.7 mL) with the addition of one drop of 0.5 M DCl in D$_2$O.

The thermoreversible gelation behavior of PCgel was further studied by rheological measurements. Water-soluble PEG-g-chitosan was reconstituted with RPMI to make a PEG-g-chitosan hydrogel solution [PCgel, 1.5% (w/v)]. The solution was put on ice for 4 h with periodic vortexing to make sure PEG-g-chitosan was fully dissolved. The continuous viscosity change of PCgel was measured as a function of time and temperature using an Anton Paar MCR 301 stress-controlled rheometer with a 25 mm cone and plate configuration and a 1° cone angle. The shear viscosity of the PCgel was measured over a temperature range of 0−45 °C at a fixed shear rate of 30 s^{-1}. In a typical experiment, a constant oscillation frequency of 1 Hz and a strain of 10% (will not affect the formation of the gel) were used. The temperature was ramped at a rate of 1 °C/min to obtain values for the storage and loss modules (G' and G'', respectively) and to allow sufficient time for the sample temperature to equilibrate.[23] The gelation time of PCgel was 8−12 min as tested in a water bath maintained at 37 °C.

Preparation of Genetically Engineered Cytotoxic T Lymphocytes. T lymphocytes were isolated from primary peripheral blood mononuclear cells (PBMCs). Platelet apheresis products were collected from healthy donors at the Puget Sound Blood Center. Cells were diluted in a 1/1.5 ratio with PBS containing 0.562 mM EDTA (Thermo Scientific, Waltham, MA). PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.), washed twice in PBS-EDTA, washed once in PBS, and resuspended in Automacs Running Buffer (Miltenyi) at a density of 10^6 cells/mL. The PBMC population after depleting CD8− cells and CD45 RA+ cells using the MicroBeads (Miltenyi Biotech) was enriched for CD62L+ to isolate CD8+ central memory T lymphocytes (CD8+CD45RO+CD62L+). T lymphocytes were expanded in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 50 IU/mL recombinant human interleukin 2 (IL-2), and 1 ng/mL recombinant human interleukin 15 (IL-15). At the completion of T lymphocyte isolation, the cells were stimulated with anti-CD3/CD28 Dynabeads (Life Technologies). Lentiviral spinoculation of T lymphocytes was conducted on day 3 of the culture by adding a viral volume necessary to obtain a multiplicity of infection (MOI) of 3 and centrifuging at 800g for 30 min at 32 °C. An anti-EGFR chimeric antigen receptor molecule, which recognizes cell-surface EGFR on target glioblastoma cells, was introduced into isolated CD8+ central memory T lymphocytes using lentivirus generated in HEK 293 cells. Transduced T lymphocytes were purified using a surrogate marker of transduction (truncated nonsignaling EGFR encoded by the lentivirus construct). For the sake of simplicity, these genetically modified T lymphocytes were termed to T lymphocytes, and the T lymphocyte without the genetic modification was termed Mock.

Transfection and Cell Culture of U-87 MG. U-87 MG cells were transiently transfected with pRFP-N2 using Lipofectamine 2000 reagent, according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed with PBS and supplied with fresh medium and then selected with G418-rich medium (500 μg/mL). Two weeks after being selected, the cells were sorted by fluorescence-activated cell sorting (FACS) (Vantage SE). For the sake of simplicity, U-87 MG+RFP is abbreviated as U-87 MG hereafter. U-87 MG were maintained in DMEM supplemented with 10% FBS and 1% AA. The medium was refreshed every 2 days.

Assessment of Cell Proliferation. Proliferation of T lymphocytes was assessed using the oxidation−reduction indicator AlamarBlue

(Alamar BioSciences, Sacramento, CA), according to the manufacturer's protocol. Briefly, prior to the assay, cells on a tissue culture plate (TCP) or in the PCgel or Matrigel are replaced with 1 mL of 9-fold diluted AlamarBlue (diluted with PBS, 110 μg/mL) for immersion for 8 h. The diluted AB solution was then collected, and the centrifuged supernatant was transferred to a 96-well black-walled plate. The absorbance of the solution was measured spectrophotometrically with a microplate reader at 540 nm. The cell number was calculated on the basis of standard curves of known numbers of T lymphocytes. The results are presented as means with the standard deviation ($n = 6$).

T Lymphocyte Invasion Assay. T lymphocyte invasion assessment was adopted from a previous study.[24] T lymphocyte invasion was evaluated using a 24-well, 3.0 μm pore size transwell plate (Costar, Cambridge, MA) precoated with 200 μL of PCgel. The density of T lymphocytes were adjusted to 2×10^7 cells/mL in regular medium without FBS. An aliquot (50 μL) of the cell suspension containing 1×10^6 T lymphocytes was placed in the top of the PCgel-precoated transwell; 500 μL of regular medium with 10% FBS was added to the bottom chamber of the transwell. After incubation for specific time intervals of 0.5, 1 3, 5, 19, 24, 48, and 96 h at 37 °C in a 5% CO$_2$ atmosphere, the top chamber was removed, and the number of T lymphocytes that had invaded the bottom chamber was counted under a microscope.

Scanning Electron Microscopy (SEM) Images of Cells. T cells on the PCgel were fixed before SEM analysis. Briefly, samples were fixed with 2.5% glutaraldehyde in complete medium for 30 min at 37 °C. After being fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C overnight and dehydration in serial ethanol washes (0, 30, 50, 70, 85, 95, and 100%), the samples were dried by critical point, mounted, and sputter coated with platinum. Samples were imaged with a JSM-7000F SEM instrument (JEOL, Tokyo, Japan) at 10 kV and 5 nA. The average diameter of the pore size of the gel was determined by measuring the diameters of the pores at 100 different points in three 2000× SEM images using ImageJ (National Institutes of Health, Bethesda, MD) as reported previously.[25]

Fluorescence Images and Flow Cytometry. Fluorescent imaging and flow cytometry were applied to confirm the invasive T lymphocytes retained the anti-glioblastoma activity. For fluorescent imaging, the interaction between the invasive T lymphocytes (Green Cell Tracker-labeled) and U-87 MG was imaged using a fluorescence microscope (Nikon TE 300) after the T lymphocytes invaded from the PCgel or Matrigel precoated transwell plates. For flow cytometry, the cells from all conditions were stained with LIVE/DEAD Fixable Dead Cell Stain Kit (Violet Dead Cell Stain, Invitrogen) and analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed and plotted using FlowJo (Tree Star Inc., Ashland, OR).

Statistical Analysis. The results are presented as means of triplicate samples ± the standard deviation. The statistical difference was determined by an unpaired, two-tailed Student's t test. Values were considered to be statistically significant at $P < 0.05$ (asterisks).

■ RESULTS AND DISCUSSION

Physicochemical Properties of the PCgel. Panels a and b of Figure 1 show the chemical structure of PEG-g-chitosan and the ^{1}H NMR spectra of PEG-g-chitosan and pure chitosan, respectively. The characteristic signals of PEG-g-chitosan were as follows: δ 4.9−5.0 (H-1), 3.6−4.3 (H-3, H-4, H-5, H-6, H-8, H-9, and H-10), 3.4 (H-11), 3.1−3.3 (H-2), and 2.1 ppm (H-7). The characteristic signals of pure chitosan were as follows: δ 5.0−5.2 (H-1), 3.7−4.2 (H-3, H-4, H-5, and H-6), 3.3−3.5 (H-2), and 2.25 ppm (H-7).[20,21] Compared to those of pure chitosan, the peaks of PEG-g-chitosan in the range of 3.6−3.9 ppm were not well separated, because of the overlap of the more intense peak of the PEG methylene groups with those of the saccharide backbone of chitosan. Furthermore, the methyl group of PEG in PEG-g-chitosan was seen clearly at 3.4 ppm.[26] The observed changes in NMR analysis confirmed the
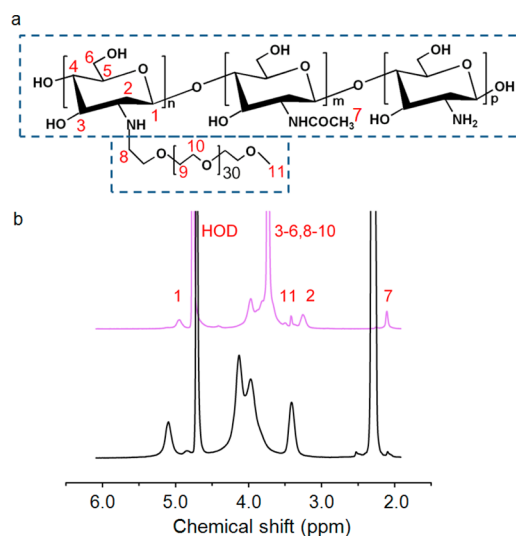

Figure 1. Chemistry of PEG-g-chitosan. (a) Chemical structure of PEG-g-chitosan. PEG is framed by the small dashed rectangle and chitosan by the large dashed rectangle. (b) NMR analysis of PEG-g-chitosan (purple) and pure chitosan (black). HOD is the abbreviation for deuterium oxide.

successful grafting of PEG onto chitosan, and the grafted PEG in PEG-g-chitosan was determined to be 60 wt %.

The sol-to-gel transition behavior of PCgel was tested by rheological analyses. Figure 2a shows the viscosity of the PCgel
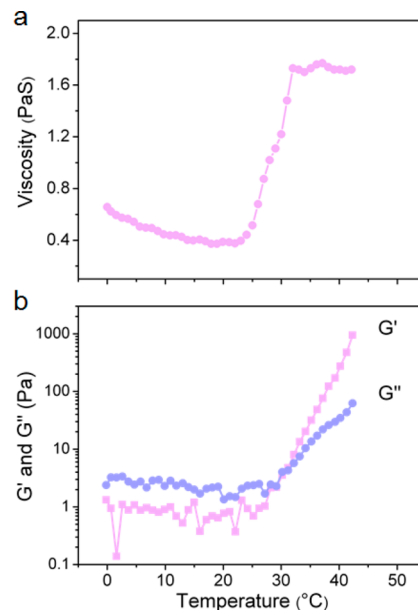

Figure 2. Rheological properties of PCgel. (a) Shear viscosity and (b) storage modulus (G') and loss modulus (G'') as a function of temperature.

as a function of temperature. The viscosity slightly decreased as the temperature increased from 4 to ~25 °C. When the temperature approached 25−30 °C, an abrupt and significant increase in viscosity was observed. The viscosity remained at the elevated constant level after the temperature reached 32 °C until the temperature reached 45 °C. Figure 2b represents the changes in the storage modulus (G') and the loss modulus (G'') for the PCgel as a function of temperature.

When the sample temperatures were below 32 °C, the G' and G'' values remained around 1 Pa, indicating that the samples remained in a liquid state. When the temperature increased above 32 °C, both modulus values increased drastically to 100 Pa (G'') and 1000 Pa (G'), respectively. The transition from liquidlike behavior to elastic gel-like behavior occurred at the crossover point of G' and G'', which was observed at approximately 32 °C.

T Lymphocyte Viability in the PCgel. Cell viability is the most fundamental feature of cellular compatibility for biomedical materials. The viability of genetically modified T lymphocytes in the PCgel was assessed with the AlamarBlue assay. Specifically, 3×10^5 T lymphocytes were loaded evenly in 400 μL of the liquid PCgel onto 24-well cell culture plates. The T lymphocyte-loaded PCgel was then placed in an incubator for solidification. An additional 400 μL of culture medium was added on top of the solidified PCgel 2 h after T lymphocyte cell seeding. A blank standard TCP and Matrigel were used as two-dimensional and three-dimensional controls, respectively. Twenty-four hours after T lymphocyte seeding, the culture medium was replaced with AlamarBlue for 8 h. The AlamarBlue solution was then collected, and the centrifuged supernatant was transferred to a 96-well black-walled plate for quantifying the number of live cells. Figure 3 shows the number of T cells
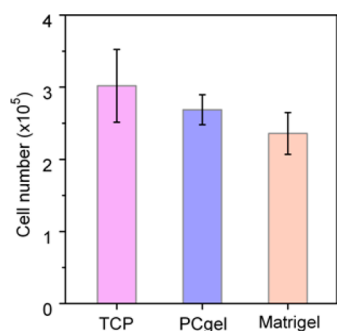

Figure 3. Biocompatibility of the PCgel. The numbers of T lymphocytes cultured on a TCP, PCgel, and Matrigel quantified using AlamarBlue after a 24 h culture.

on a TCP, in the PCgel, and in Matrigel. Though the number of T lymphocytes in the PCgel was observed to be slightly lower than that of a TCP (89%; $p = 0.353$) and slightly higher than that of Matrigel (113%; $p = 0.184$), there was no statistical difference among the three tested materials ($p = 0.156$). This confirmed that the PCgel had good cellular compatibility with T lymphocytes.

T Lymphocyte Invasion through the PCgel. To act as a depot for sustained T lymphocyte release, the PCgel must have appropriate pore sizes to allow for T lymphocyte invasion out of the matrix. Previous studies have demonstrated lymphocyte invasion through three-dimensional gels of native collagen fibers.[27] The kinetics of penetration of lymphocytes into the gel matrix indicated that lymphocytes migrate in a "random-walk" fashion through this collagen matrix that had a pore size of 2 μm, comparable to that of T lymphocytes (approximately 1−2 μm in diameter). This large pore size likely explains the random-walk movement of T lymphocytes because they were able to freely move throughout the collagen gel with pore sizes slightly larger than the cells. To act as a controlled release depot for T lymphocytes, the pore sizes must be small enough to prevent the random walk so the cells must actively invade out of the gel but large enough for the cells to invade through. SEM images of the PCgel (Figure 4a,b) and Matrigel (Figure 4d,e) revealed larger pores in the PCgel and in Matrigel. The 0.5−1 μm pore size distribution of the PCgel (Figure 4c) was more suitable for T lymphocyte invasion than that of Matrigel that had more small pore sizes [0.1−0.5 μm (Figure 4f)].

T lymphocytes at the surface of both matrices 20 h after seeding were observed to retain a similar characteristic morphology of a uniformly round, small shape. Moreover, there was an apparent reduction in the density of T lymphocytes remaining on the surface of the PCgel as compared that on the surface of Matrigel (Figure 4a,d), suggesting that T lymphocytes were more effective in infiltrating the PCgel than Matrigels. This might be due to the fact that the PC gel has pore sizes larger than those of Matrigel.
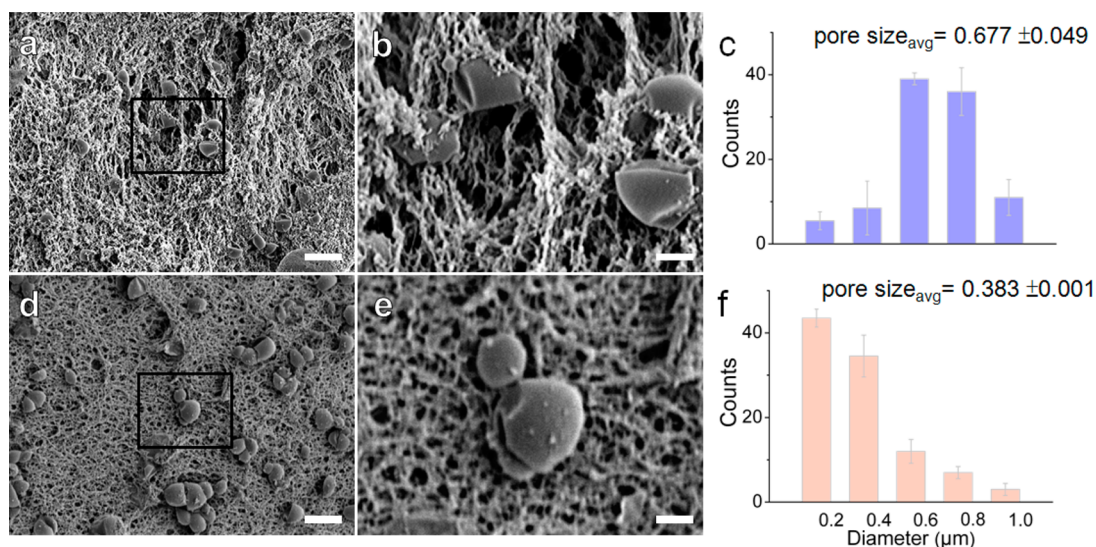

Figure 4. Pore size evaluation of gels. SEM images of T lymphocytes in the process of invading through (a and b) the PCgel and (d and e) Matrigel after 20 h. The scale bars in panels a and d are 4 μm and in panels b and e 1 μm. (c and f) Distributions of the pore sizes of the (c) PCgel and (f) Matrigel.

The increased capacity of the PCgel for T lymphocytes to invade was further confirmed through quantification of cells in a transwell invasion assay (Figure 5). It was found that 4 times
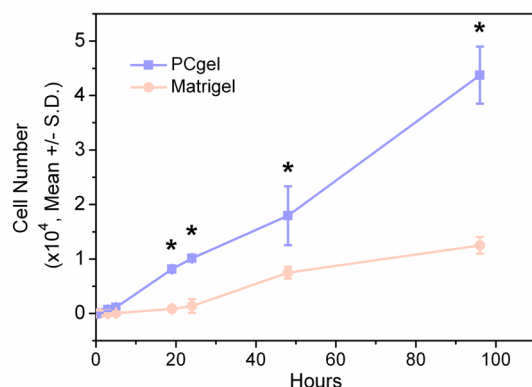

Figure 5. Time course of therapeutic T lymphocyte invasion through the PCgel and Matrigel. The statistical differences in therapeutic T lymphocyte invasion through the PCgel vs through Matrigel are labeled with asterisks ($p < 0.05$).

as many T lymphocytes invaded through the PCgel as compared to Matrigel after 100 h. In addition, sustained invasion of T lymphocytes through the PCgel was observed with an approximately linear increase in the number of invaded T lymphocytes over time. This was likely a result of the appropriate pore size and biocompatibility of the PCgel for T lymphocytes.

Specific Binding to and Effective Killing of U-87 MG by Invading T Lymphocytes. To decide the ratio of T cells to U-87 MG cells for optimized tumor cell death, different amounts of T lymphocytes were added 2 h after 1×10^4 U-87 MG cells had been seeded. AlamarBlue was used to quantify the number of viable U-87 MG cells, which was quantified by AlamarBlue after T lymphocytes were added for 24 h and each well was washed thrice with PBS to ensure no residual T lymphocytes. The tumor killing ability measurement revealed that the highest tumor cell kill was observed at the highest T lymphocyte/U-87 MG ratio (100/1) and not the smaller ratios (50/1, 25/1, 10/1, and 3/1), following coculture of therapeutic T lymphocytes and U-87 MG on a TCP (Figure 6). The same
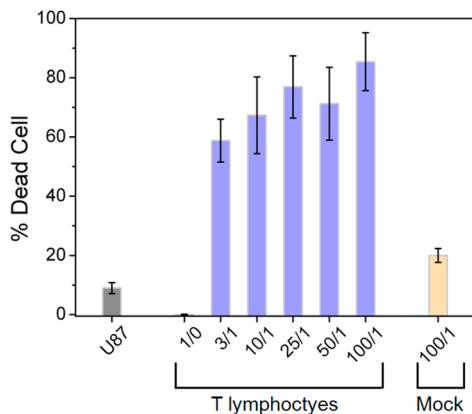

Figure 6. Percentage of U-87 MG tumor cell death after T lymphocyte treatment at various effector/target (T lymphocyte/U87) ratios (1/0, 3/1 to 100/1). Mock was applied as a negative control.

ratio of T cells to U-87 MG cells of 100/1 was also applied to Mock as a positive control. Therefore, the ratio of T cells to U-87 MG cells of 100/1 was selected for the following study.

Once the T lymphocytes invade through the PCgel, they must remain functionally active to specifically target glioblastoma cells. To investigate if T lymphocytes invading through gels specifically bind to tumor cells, 10^4 U-87 MG cells were seeded on a TCP as the lower chamber of the transwell device and T lymphocytes were seeded onto transwell plates precoated with the PCgel or Matrigel (as control) the following day. The upper chamber was dissembled from the lower chamber 20 h after the addition of T lymphocytes. The interaction between the invasive T lymphocytes and U-87 MG cells was imaged using a fluorescence microscope. The number of T lymphocytes bound to U-87 MG cells was quantified by counting on 10 random fields and was reported in number per field of view. Panels a and b of Figure 7 show the interaction of invasive T lymphocytes with the U-87 MG cells after invading through the transwell PCgel and Matrigel, respectively. Green T lymphocytes were observed binding to red U-87 MG cells in the PCgel condition, but very few T lymphocytes were observed in the Matrigel condition. Indeed, quantification of bound T lymphocytes revealed there were significantly more T lymphocytes bound to U-87 MG cells in the PCgel transwell invasion condition than in the Matrigel transwell invasion condition [$p = 0.0258$ (Figure 7c)].

To confirm T lymphocytes that bound to target U-87 MG cells could induce cell death, a live/dead flow cytometry assay was performed to determine the number of U-87 MG cells killed by T lymphocytes that invaded through the PCgel or Matrigel (control). For this study, 10^4 U-87 MG cells were first seeded on a TCP as the lower chamber of the transwell device and T lymphocytes were seeded onto transwell plates precoated with 200 μL of the PCgel or Matrigel for 1 day. The upper chamber was then dissembled from the lower chamber 20 h after the addition of T lymphocytes. U-87 MG cells without any treatment were used as negative control cells, and U-87 MG wells were treated with heat (60 °C, 4 h) and 2 mM H_2O_2 for 4 h as positive control wells. Cells from all conditions were harvested after treatments, stained with the LIVE/DEAD kit, and analyzed by flow cytometry. U-87 MG +RFP cells were gated and analyzed for the percentage of dead cells (i.e., BV 450-A+).

Figure 8a shows that there were more dead U-87 MG cells (BV 450-A+) under the PCgel transwell invasion condition than in the Matrigel. Figure 8b shows that there were a large number of dead U-87 MG cells killed by heat or H_2O_2. Moreover, quantitative analysis of the flow cytometry data (Figure 8c) shows that the frequency of U-87 MG death was significantly higher under the PCgel transwell invasion condition than for untreated cells ($p = 0.0003$) and under the Matrigel transwell invasion condition ($p = 0.0057$). This confirms the T lymphocytes that invaded through the PCgel and bound to U-87 MG cells were active and could induce a therapeutic response. Therefore, our study shows the utility of the PCgel as a depot for release of T lymphocytes and a convenient and valuable *in vitro* model for studying clinically relevant improvements to glioblastoma immunotherapy.

■ **CONCLUSIONS**

We have demonstrated the utilization of the thermoreversible PCgel as a suitable depot for delivery of T lymphocytes for brain tumor immunotherapy. The PCgel had appropriate pore
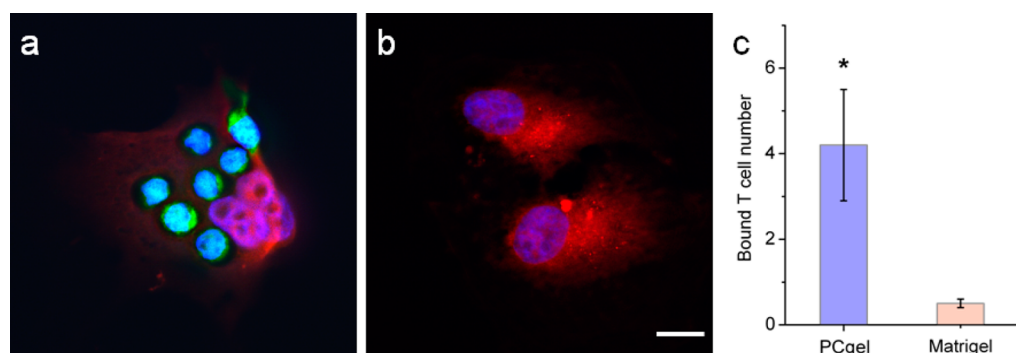
Figure 7. Recognition of U-87 MG cells and invasive T lymphocytes. Fluorescence images of T lymphocytes (green) attached to U-87 MG cells (red) after invading through the transwell under (a) PCgel and (b) Matrigel conditions. Nuclei were counterstained with DAPI (blue). The scale bar is 10 μm. (c) Quantification of bound T lymphocytes through either the PCgel or Matrigel to U-87 MG cells (at 60× magnification from 10 different fields of view on fluorescent images). Data are representative of two experiments conducted in duplicate. The statistical difference with Matrigel was labeled with an asterisk ($p < 0.05$).
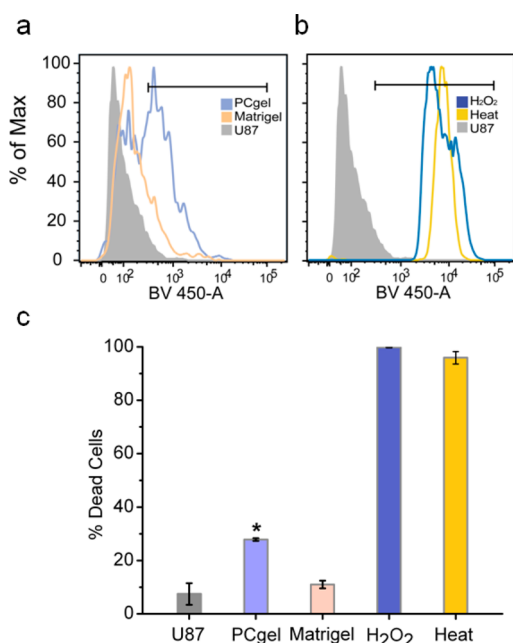

Figure 8. Evaluation of U-87 MG cell death by the live/dead flow cytometry assay. (a and b) Flow cytometry histograms of the gated U-87 MG cell population (RFP+) assessed for the percentage of dead cells (BV 450-A+) after (a) invasive T lymphocyte through PCgel or Matrigel and (b) H_2O_2 and heat as positive controls. Data for the untreated U-87 MG cells are labeled as U87. (c) Quantification of the percentage of dead cells based on BV 450-A positivity. Data are representative of triplicate samples. The statistical difference with untreated tumor cells (U87) is labeled with an asterisk ($p < 0.05$).

sizes that could release viable T lymphocytes. The PCgel is cellular compatible with T lymphocytes and retains the anti-glioblastoma activity of T lymphocytes. When the PCgel containing T lymphocytes contacts glioblastoma cells, T cells released from the gel could effectively kill GBM cells. In addition, the T cells in the PCgel were more effective in killing glioblastoma than those in Matrigel. The PCgel is clinically preferable over Matrigel and any other animal-sourced gels because of its excellent biocompatibility, biodegradability, low immunogenicity, low potential for pathogen transfer, low cost, and consistent properties from batch to batch. Our study suggests that this unique PCgel depot may offer a viable approach for localized immunotherapy for glioblastoma and have the potential to improve glioblastoma immunotherapy.

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Notes

The authors declare no competing financial interest.

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