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Surface modification of polymeric nanoparticles with M2pep peptide for drug delivery to tumor-associated macrophages

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Abstract

Purpose: Tumor-associated macrophages (TAMs) with immune-suppressive M2-like phenotype constitute a significant part of tumor and support its growth, thus making an attractive therapeutic target for cancer therapy. To improve the delivery of drugs that control the survival and/or functions of TAMs, we developed nanoparticulate drug carriers with high affinity for TAMs.

Methods: Poly(lactic-co-glycolic acid) nanoparticles were coated with M2pep, a peptide ligand selectively binding to M2-polarized macrophages, via a simple surface modification method based on tannic acid-iron complex. The interactions of M2pep-coated nanoparticles with macrophages of different phenotypes were tested *in vitro* and *in vivo*. PLX3397, an inhibitor of the colony stimulating factor-1 (CSF-1)/CSF-1 receptor (CSF-1R) pathway and macrophage survival, was delivered to B16F10 tumors via M2pep-modified PLGA nanoparticles.

Results: In bone marrow-derived macrophages polarized to M2 phenotype, M2pep-coated nanoparticles showed greater cellular uptake than those without M2pep. Consistently, M2pep-coated nanoparticles showed relatively high localization of CD206⁺ macrophages in B16F10 tumors. PLX3397 encapsulated in M2pep-coated nanoparticles attenuated tumor growth better than the free drug counterpart.

Conclusion: These results support that M2pep-coating can help nanoparticles to interact with M2-like TAMs and facilitate the delivery of drugs that control the tumor-supportive functions of TAMs.

Keywords

Tumor-associated macrophages; PLGA nanoparticles; drug delivery; M2pep; PLX3397

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INTRODUCTION

Traditional cancer chemotherapy mainly targets malignant tumor cells. However, increasing evidence indicates that non-cancerous cells and extracellular matrix in the tumor microenvironment also play critical roles in tumor progression (1). For example, endothelial cells support self-renewing cancer cells and accelerate tumor growth (2), cancer-associated fibroblasts promote tumor progression and metastasis by secreting growth factors and cytokines (1, 3), and tumor extracellular matrix is remodeled and stiffened to provide a tumor-supportive microenvironment (4). In particular, tumor-associated macrophages (TAMs), which constitute a large portion of the tumor cell population, serve as major tumor-promoting immune cells, along with regulatory T cells (5). Several clinical studies have demonstrated that the density of TAMs is linked to poor prognosis for cancer patients (6). TAMs can activate and protect tumor stem cells, stimulating their proliferation, and promote angiogenesis and metastasis (7, 8). Furthermore, TAM-derived cytokines and other proteins, such as transforming growth factor- β , interleukin-10, arginase-1, and galectin-3, contribute to suppressing T cell-dependent immunity (9, 10). Therefore, TAMs make an attractive target for cancer therapy.

Efforts have been made to deliver a drug to TAMs to deplete them (11) or reprogram the tumor-supportive M2-like phenotype into anti-tumorigenic M1-like phenotype (12–14). Nevertheless, it is challenging to deliver drugs to TAMs because they overexpress P-glycoprotein (P-gp), a drug efflux transporter (15), and have limited phagocytic activities (16). To overcome these challenges, several ligands targeting M2 macrophages such as mannose or folate have been explored as part of drug carriers. Mannose and folate are ligands for mannose receptor and folate receptor β , respectively, and shown to have high affinity for TAMs and increase the uptake of a drug when conjugated to the drug (17, 18). To increase the selectivity of TAM targeting, Pun et al have screened a peptide phage library and identified M2pep (YEQDPWGVKWWY) as a promising alternative to the traditional ligands (19). M2pep was conjugated to a proapoptotic peptide as monovalent (19) or multivalent ligands (20) to concentrate the toxic effect of the peptide to M2 macrophages. These fusion peptides have shown toxicity to M2 macrophages, reduced the M2/M1 ratio in tumors, and extended the survival time of tumor-bearing mice in a M2pep-specific manner (19, 20). However, the drug loading capacity and the avidity of M2 macrophage-binding remain to be improved (21).

We hypothesize that polymeric nanoparticles may be an effective partner of M2pep for targeted drug delivery to M2-like TAMs (Fig.1), because their surface can provide a room for multivalent binding of M2pep and the matrix can accommodate various types of payloads ranging from small molecules to macromolecules. The surface modification of polymeric nanoparticles with peptide ligands can be challenging if the particle surface does not expose reactive functional groups. We have previously shown that polyphenols such as polydopamine (pD) or tannic acid (TA) aggregates can deposit on nanoparticle surface and serve as an intermediate layer to help conjugate peptide ligands (22–24). This process does not require the nanoparticle platform to possess pre-existing functional groups such as maleimide, thiol, or azide and enables the modification of nanoparticle surface irrespective of its chemical nature. For example, TA instantaneously interact with Fe^{3+} in neutral

aqueous medium to form an adhesive TA-Fe³⁺ complex (pTA) layer on the surface of nanoparticles, including non-reactive ones, and accommodate thiol- or amine-terminated ligands via Michael addition and/or Schiff base reactions to provide additional functionality (22, 25, 26). Therefore, we expect that M2pep can be conjugated to the surface of polymeric nanoparticles by the TA-Fe³⁺ complexation method via free amine groups in N-terminus and/or Lys residue to facilitate the delivery of anti-TAM drugs to M2-like TAMs and suppress their tumorigenic functions.

In this study, we modify poly(lactic-co-glycolic acid) (PLGA) nanoparticles, a widely explored drug carrier due to the biocompatibility and biodegradability, with M2pep by the simple TA-Fe³⁺-based surface modification method. We investigate how the M2pep-conjugated PLGA nanoparticles interact with macrophages of different phenotypes *in vitro* and *in vivo* and test if an inhibitor of the colony stimulating factor-1 (CSF-1)/CSF-1 receptor (CSF-1R) pathway, PLX3397, delivered to tumors via M2pep-modified PLGA nanoparticles help attenuate tumor growth. The results support that M2pep conjugation increases the uptake of PLGA nanoparticles by M2-polarized macrophages and improves the anti-tumor efficacy of PLX3397 in a mouse B16F10 melanoma model.

MATERIALS AND METHODS

Materials

Poly(lactic-co-glycolic) acid (PLGA, LA:GA = 85:15, 30 kDa, ester end-capped) and Rhodamine-labeled PLGA (LA:GA = 50:50, 30 kDa, Rhodamine B end-capped) were purchased from Akina, Inc. (West Lafayette, IN). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33342 were purchased from Invitrogen (Eugene, OR). Human serum albumin was purchased from Sigma-Aldrich (St. Louis, MO). M2 peptide (M2pep, YEQDPWGVKWWY; N to C terminus) and WR12 (RWWRWRRWRR; N to C terminus) were custom-synthesized by GenScript USA (Piscataway, NJ). APC-labeled anti-mouse CD11c antibody and FITC-labeled anti-mouse CD86 antibody were obtained from Biolegend (San Diego, CA). PE-conjugated anti-human/mouse arginase-1 antibody was purchased from R&D Systems (Minneapolis, MN). APC-conjugated anti-human/mouse iNOS antibody was purchased from ThermoFisher (Waltham, MA). Rabbit anti-CD206 antibody and Alexa Fluor 488TM-conjugated goat polyclonal anti-rabbit secondary antibody were purchased from Abcam (Cambridge, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Nanoparticle preparation

Core PLGA nanoparticles (NP) were produced by the single emulsion evaporation method. Briefly, 100 mg of PLGA or rhodamine-labeled PLGA was dissolved in 4 mL dichloromethane (DCM) and emulsified in 16 mL of 5% PVA solution by probe sonication (Sonics Vibracell, Newton, CT) for 2 min at 40% amplitude with 4s-on and 2s-off cycle. The emulsion was dispersed in 40 mL of deionized (DI) water, stirred for 3 h, and evaporated by a rotary evaporator to remove DCM. PLGA nanoparticles were collected by centrifugation at 34,000 rcf and washed twice with DI water. The rhodamine labeling was indicated by *. For the preparation of PLX3397-loaded PLGA nanoparticles (PLX3397@NP), 1 mg of

PLX3397 dissolved in 100 μ L DMSO was added to the polymer phase and processed in the same way.

For the preparation of pTA-coated NP (NP-pTA), PLGA nanoparticles were dispersed in bicine buffer (10 mM, pH 7.4) containing 0.2 mg/mL TA and vortex-mixed for 30 s. FeCl_3 was then added to 0.05 mg/mL and vortex-mixed for 30 s. Nanoparticles were collected by centrifugation and washed twice with DI water. NP-pTA was modified with albumin or M2pep. For albumin modification, NP-pTA was resuspended in bicine buffer (10 mM, pH 7.4) at 1 mg/mL. Albumin was added to the suspension at 5 mg/mL. After 3 h incubation at room temperature with rotation, the albumin-coated NP-pTA (NP-pTA-Al) was collected by centrifugation and washed with DI water. The M2pep-coated NP-pTA (NP-pTA-M2pep) was prepared in a similar manner except that NP-pTA and M2pep were added to 1.5 and 1 mg/mL, respectively, and incubated for 1 h. PLX3397@NP-pTA-Al and PLX3397@NP-pTA-M2pep were prepared with PLX3397@NP by the same surface modification process.

For the preparation of pD-coated NP (NP-pD), PLGA nanoparticles were incubated in dopamine solution in Tris buffer (10 mM, pH 8.5) for 3 h at room temperature with rotation. The concentrations of PLGA nanoparticles and dopamine were 0.5 mg/mL. NP-pD was collected by centrifugation and washed twice with DI water. For M2pep modification, NP-pD was resuspended in Tris buffer containing M2pep, with the concentrations of NP-pD and M2pep fixed at 1.5 and 1.0 mg/mL, respectively. After 1 h incubation at room temperature with rotation, the M2pep-coated NP-pD (NP-pD-M2pep) was collected by centrifugation and washed with DI water.

Nanoparticle characterization

The hydrodynamic diameter and zeta potential of nanoparticles were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS90 (Worcestershire, UK). Particles were dispersed in 10 mM NaCl for the size measurement and in phosphate buffer (5 mM, pH 7.4) for the zeta potential. The morphology of particles was imaged by transmission electron microscopy (TEM) using a Tecnai F20 (FEI, Hillsboro, OR). Samples were negatively stained by 1% uranyl acetate prior to imaging.

The albumin content in NP-pTA-Al was determined by SDS-PAGE electrophoresis. NP-pTA-Al (5 mg/mL) or albumin solutions in PBS of known concentrations were mixed with 4 $\times$ Laemmli sample buffer in 3:1 ratio and heated at 95 $^{\circ}\text{C}$ for 5 min. The samples were resolved in a 12% polyacrylamide gel. The gel was stained with QC Colloidal Coomassie Stain (BioRad, CA) and imaged by Azure C300 (Dublin, CA). The albumin bands were quantified by the AzureSpot Analysis Software. The albumin content was determined by comparing the albumin band intensities of samples and standard albumin solutions.

The amount of M2pep conjugated to NP-pTA was indirectly determined. The supernatant was collected by centrifugation after incubating NP-pTA with M2pep, and the M2pep remaining in the supernatant was quantified by an Agilent 1100 HPLC system (Palo Alto, CA), equipped with Phenomenex Luna C18 column (25 cm $\times$ 4.6 mm, particle size: 5 μm). The mobile phase was a mixture of water and acetonitrile containing 0.05% trifluoroacetic acid and flowed at 0.7 mL/min. M2pep was detected by a UV detector at a wavelength of

280 nm. The total amount of M2pep conjugated to NP-pTA was calculated as the fed M2pep - M2pep remaining in the supernatant.

The PLX3397 content in nanoparticles was determined by HPLC. Nanoparticles with a premeasured mass were dissolved in DMSO and 0.1 M HCl sequentially, vortexed and filtered through 0.45 μ m syringe filter. HPLC analysis of PLX3397 was performed in the same condition as M2pep.

Macrophage model screening

RAW264.7, J774.1, and peritoneal macrophage cell lines were obtained from ATCC (Manassas, VA). All the cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with recommended concentrations of FBS (10% for RAW264.7 and J774.1; 5% for peritoneal macrophages), 100 units/mL penicillin, and 100 μ g/mL streptomycin. The cells were seeded in a 12 well plate at a density of 2×10^5 per well. After overnight culture, the cells were incubated with 40 ng/mL of IL-4 for 24 h and collected by scraping. A million cells were incubated with a recommended dose of APC-anti-mouse CD11c antibody or PE-anti-human/mouse arginase-1 antibody in 100 μ L incubation buffer and analyzed by an Accuri C6 flow cytometer (BD bioscience, San Jose, CA).

Primary macrophages were derived from the bone marrow of male C57BL/6 mice. All animal procedures were approved by Purdue Animal Care and Use Committee, in conformity with the NIH guidelines for the care and use of laboratory animals. C57BL/6 male mice at the age of 6–8 weeks were purchased from Envigo (Indianapolis, IN) and acclimatized for 1 week prior to the procedure. Animals were sacrificed by CO₂ asphyxiation followed by cervical dislocation. The femur and tibia were separated and flushed through the cavity with warm Dulbecco's PBS. The obtained bone marrow was pipetted and passed through the cell strainer to form a single cell suspension. Cell pellets were collected by centrifugation at 500 g for 10 min at room temperature and treated with the red blood cell lysis buffer. The cells were differentiated into macrophages in DMEM containing 30 ng/mL macrophage colony-stimulating factor (M-CSF; supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin), counted and cultured for 5 days. Floating cells were removed, and adherent cells ("M0 macrophages") were cultured for another 24 h in new media (containing 100 ng/mL LPS and 20 ng/mL IFN- γ for M1 macrophages; 40 ng/mL IL-4 for M2 macrophages). The IL-4-treated cells were probed with APC-anti-mouse CD11c antibody and PE-anti-human/mouse arginase-1 antibody. The LPS/IFN- γ -treated cells were incubated with FITC-anti-mouse CD86 antibody and APC-anti-human/mouse iNOS antibody. To confirm the differential polarization status, the M0, M1, and M2 macrophages were incubated with FITC-anti-mouse CD86 antibody or PE-anti-human/mouse arginase-1 antibody. The cells were analyzed by the Accuri C6 flow cytometer.

In vitro macrophage uptake of M2pep-modified nanoparticles

To examine NP uptake efficiency *in vitro*, the bone-marrowed derived macrophages (BMDMs; M0, M1, and M2) were seeded in a 12 well plate at a density of 2×10^5 per well, incubated overnight and treated with 100 μ g/mL of rhodamine-labeled nanoparticles (*NP-

pTA, *NP-pTA-Al, or *NP-pTA-M2pep). After 30 min incubation, free or loosely bound nanoparticles were removed, and the cells were rinsed twice with PBS. Finally, the cells were collected, and their fluorescence intensity was analyzed by flow cytometry.

For microscopic observation of nanoparticle uptake, M2-polarized BMDMs were seeded at a density of 5×10^5 per 35 mm petri dish. The cells were incubated with *NP-pTA, *NP-pTA-Al, and *NP-pTA-M2pep for 30 min and rinsed with PBS twice. The cells were stained with Calcein AM (10 μ M) for 30 min, then with Hoechst 33342 (2 μ g/mL) for 15 min prior to imaging. The cells were imaged with a Nikon-A1R confocal microscope (Nikon America Inc., Melville, NY). The nanoparticles (rhodamine-labeled), cell nuclei (Hoechst 33342), and cytoplasm (Calcein AM) were excited and detected at $\lambda_{ex}/\lambda_{em}$ of 561/570–620 nm, 408/425–475 nm, and 488/500–550 nm, respectively.

Uptake of M2pep-modified nanoparticles by tumor-associated macrophages

For analysis of the nanoparticle uptake by TAMs *in vivo*, the particles were injected to mice bearing B16F10 melanoma via tail vein. The mouse model of melanoma was prepared with B16F10 cells and 6–8 week old C57BL/6 male mice. A million B16F10 cells were inoculated subcutaneously in the upper flank of the right leg. When the tumor volume reached 1000 mm³, 6 mg of *NP-pTA, *NP-pTA-Al, and *NP-pTA-M2pep suspended in 100 μ L of PBS were injected into the tumor. Twenty four hours after injection, animals were humanely sacrificed. Tumors were collected, fixed in 10% formalin for 24 h and kept in 30% sucrose solution until the tissues completely sank. The tumors were transferred to a cryomold with OCT matrix and snap frozen in 2-methyl butane chilled by liquid nitrogen. The frozen tissues were cryosectioned into 5 μ m thick sections and stored at -80°C . Immunohistochemistry was performed with a 5 min PBS wash at 4°C . Following heat-induced antigen retrieval with Diva Decloaker (DV2004; Biocare Medical), sections were blocked with 5% normal goat serum in PBS for 45 min at room temperature. Tissues were incubated with a rabbit polyclonal anti-mannose receptor (CD206) antibody (1:1000; Abcam ab64693) for 18 h at 4°C . Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, Abcam, ab150077) was used as the secondary antibody and incubated for 45 min at room temperature. Slides were mounted with ProLong Gold Antifade mounting medium with DAPI (Molecular Probes, P36935). Slides were scanned by using Leica Versa 8 40 $\times$ objective. Ten 40 $\times$ fields per slide were acquired using Leica Aperio Image Analysis software. CD206⁺ macrophages and CD206⁺ macrophages containing rhodamine-labeled nanoparticles per field were counted by Image J software.

Drug screening

M2-polarized BMDMs were treated with three candidate drugs (paclitaxel, WR12, and PLX3397) in different concentrations. After 24 h incubation with each drug, cells were rinsed with PBS twice. Cell viability was determined by the MTT assay. After treating the cells with 15 μ L of MTT solution (5 mg/mL) for 3 h, the formazan crystals formed in the cells were dissolved in stop/solubilization solution (50% dimethylformamide, 20% sodium dodecyl sulfate, pH 5) and quantified by a SpectraMax M3 microplate reader (Molecular Devices, CA) at the wavelength of 562 nm.

***In vivo* efficacy of PLX3397-loaded M2pep-modified nanoparticles**

C57BL/6 mice (male, 6–8 weeks old) with subcutaneous B16F10 melanoma were prepared in the same manner as above. When the average tumor volume reached 170 mm³, the animals were randomly assigned to 3 groups (n = 5 per group) and treated with intratumoral injection of PLX3397, PLX3397@NP-pTA-AI and PLX3397@NP-pTA-M2pep (all equivalent to 30 ng of PLX3397 per mm³) suspended in PBS every other day for 5 times. The tumor growth was monitored by measuring length (L) and width (W) with a digital caliper every other day, and the volume (V) was calculated according to the modified ellipsoid formula: $V = (L \times W^2)/2$. When tumor volume reached 2000 mm³, mice were humanely sacrificed for tumor tissue analysis. The collected tumors were fixed in 4% formalin solution overnight, paraffin imbedded and sliced into 5-μm thick sections. The sections were stained with hematoxylin and eosin (H&E) for histological evaluation.

Statistical analysis

All statistical analysis was performed with GraphPad Prism 7 (La Jolla, CA). All data were analyzed by t-test or one-way ANOVA followed by the recommended *post-hoc* multiple comparisons test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and characterization of nanoparticles

PLGA nanoparticles were modified with M2pep via TA-Fe³⁺ coordination complex (NP-pTA-M2pep) as a potential drug carrier to M2-like TAMs. Unmodified nanoparticles (NP), pTA-coated intermediate nanoparticles (NP-pTA), and albumin-coated nanoparticles (NP-pTA-AI) were also prepared for comparison. Albumin was chosen based on its traditional use as a blocking agent to reduce non-specific binding (27), thereby making an NP control with low affinity for TAMs. We have previously verified that NPs coated with albumin in a similar manner show negligible interactions with macrophages (28). The particle properties including size, zeta potential, polydispersity index, and morphology are summarized in Table 1 and Fig. 2.

After incubation with Fe³⁺ and TA, the nanoparticles showed dark blue color, indicating the formation of pTA. TEM images showed wrinkled layers of pTA on the particle surfaces (Fig. 2), similar to other polyphenol coatings demonstrated previously (22, 24). The relatively high negative charge of NP-pTA resulting from abundant galloyl (trihydroxyphenyl) groups of TA also indicates the presence of pTA. Additional incubation with albumin or M2pep reduced the negative charge, suggesting the shielding of pTA layer by the surface modifiers. The sizes of the surface-modified nanoparticles (NP-pTA, NP-pTA-AI, and NP-pTA-M2pep) measured by DLS were slightly larger than the unmodified particles (NP); however, TEM images showed little difference in particle size. We speculate that the 20–50 nm difference between NP and the surface-modified nanoparticles reflects a mild degree of particle aggregation in the medium the size was measured (pH 7.4, 10 mM NaCl solution) rather than the coating layer.

NP-pTA-A1 had 0.5 ± 0.03 of albumin per 100 nm^2 surface area, comparable to those coated with albumin via polydopamine, another polyphenol frequently used for NP surface modification (NP-pD-A1, 0.4 ± 0.2 per 100 nm^2) (22, 23). M2pep was coated on NP-pTA more efficiently than albumin, yielding 272.7 ± 58.3 per 100 nm^2 , likely due to the small size: M2pep is a peptide consisting of 12 amino acids with a molecular weight of 1,657 Da, which occupies smaller space than albumin (66,458 Da). Moreover, the peptide's reactive functional groups (primary amines) are more accessible than those of albumin, which may be partly hidden in a globular structure of the protein. The density of M2pep on NP-pTA-M2pep was twice as high as NP-pD-M2pep (NP coated with M2pep via polydopamine: 122.4 ± 12.1 per 100 nm^2), suggesting that additional mechanisms such as hydrogen bonding and hydrophobic interaction may have contributed to the interactions between pTA and M2pep (29). This result demonstrates that pTA allows for efficient surface modification of PLGA nanoparticles.

Cell model screening

Macrophages may be classified along a linear scale according to their functions (30). M1-macrophages secrete proinflammatory cytokines such as IL-12 and TNF- α and help destroy foreign organisms and tumor cells (31). Conversely, M2-macrophages secrete anti-inflammatory cytokine IL-10 and promote angiogenesis and tissue remodeling (31). TAMs are considered to have an M2-like phenotype (32). Accordingly, M2 macrophages were pursued as a cell model for *in vitro* evaluation of NP-pTA-M2pep as a drug carrier. We initially screened commercially available macrophage cell lines. RAW264.7, J774.1 and peritoneal macrophage cell lines were incubated with IL-4 for 24 h for polarization to M2 phenotype, and examined with respect to the expression of M2-macrophage biomarkers, such as CD11c and arginase-1. None of the immortalized cell lines responded to IL-4 treatment to express the two biomarkers (Supporting Fig. 1), which may be due to their oncogenic transformation and relatively more monocytic phenotype (33). We then resorted to primary macrophages derived from the mouse bone marrow. Bone marrow-derived cells (BMDMs) were first differentiated into macrophages and polarized into M1 or M2 phenotypes by different stimuli. The polarization status was confirmed by the expression of respective biomarkers: BMDMs treated with LPS and IFN- γ expressed CD86 and iNOS (M1 phenotype biomarkers) (30), whereas those with IL-4 expressed CD11c and arginase-1 (M2 biomarkers) (19, 34) (Fig. 3). Simultaneous staining of three macrophages confirmed relatively high expression of CD86 in M1 BMDMs compared to M0 and M2 BMDMs and higher arginase-1 in M2 BMDMs relative to M0 and M1 BMDMs (Supporting Fig. 2). These BMDMs were used in the *in vitro* evaluation of NP uptake and the screening of anti-macrophage drugs.

In vitro interaction between macrophages and nanoparticles

We first observed the uptake of *NP-pTA-M2pep by M2-polarized BMDMs by confocal microscopy. *NP-pTA and *NP-pTA-A1 were also tested as control nanoparticles without M2pep or with albumin as a non-specific protein, respectively. As shown in Fig. 4 (and Supporting Fig. 3), many *NP-pTA were seen in a field of view, but the majority was located outside the cells. This is likely due to the adhesive pTA surface, which makes *NP-pTA adhere to the plate surface. Albumin rather reduced the binding of particles to the plate

surface, as indicated by the minimum NP signals observed with *NP-pTA-AI. The number of *NP-pTA-M2pep seen in a field of view was comparable to that of *NP-pTA, but most signals were detected in the cytoplasm, indicating that M2pep facilitated the uptake of nanoparticles by M2 BMDMs. The percent of nanoparticle signal nested in the cells (% area of nanoparticle signals / area of cytoplasm) acquired by image analysis reflects this trend, with *NP-pTA-M2pep-treated cells showing the highest value followed by *NP-pTA and *NP-pTA-AI-treated ones ranking last (Fig. 4).

To test whether the nanoparticle uptake depends on the macrophage polarization state, we incubated *NP-pTA-M2pep and *NP-pTA-AI (as a non-specific control) with M0-, M1- and M2-polarized BMDMs and measured their fluorescence increase by flow cytometry as an estimation of particle uptake. In general, M1 BMDMs showed the greatest phagocytosis activity among the three types of macrophages. Although M2 BMDMs were not as active as M1 BMDMs in particle uptake, they consistently preferred *NP-pTA-M2pep to *NP-pTA-AI, evident from the difference in the ratios of M2 uptake relative to M1 or M0 uptake (Fig. 5, Supporting Fig. 4). This result is consistent with confocal microscopy and supports that M2pep was superior to albumin in improving the interaction between the nanoparticles and M2 macrophages.

***In vivo* interaction between TAMs and NP-pTA-M2pep**

To test if the uptake of *NP-pTA-M2pep by M2-like TAMs occurs in tumor microenvironment with other cell populations, such as tumor cells, lymphocytes, fibroblasts, and endothelial cells (35), we injected *NP-pTA, *NP-pTA-AI and *NP-pTA-M2pep to mice bearing B16F10 melanoma intravenously and observed their uptake by TAMs. Tumors were collected 24 h after the injection, TAMs were identified by anti-CD206 antibody (36), and nanoparticles were located relative to the CD206⁺ cells. All three nanoparticles were seen in tumors. However, the extents of particle uptake by TAMs, quantified as % CD206⁺ cells containing the particles, were slightly different (Fig. 6), showing the highest percentage of particle-containing CD206⁺ cells with *NP-pTA-M2pep. Due to the large variance, the difference between groups did not reach statistical significance, but the trend was consistent with the *in vitro* result (Fig. 4).

Anti-macrophage agent screening

Based on the efficient uptake of *NP-pTA-M2pep by M2-like TAMs, we sought to deliver a TAM-suppressing drug to tumors via NP-pTA-M2pep. Paclitaxel (anti-cancer drug blocking cell mitosis), WR12 (cytotoxic cationic peptide) and PLX3397 (CSF-1R inhibitor) were tested with respect to the cytotoxicity to M2-polarized BMDMs. Paclitaxel had no apparent toxicity to M2-BMDMs at a concentration as high as 8.5 µg/mL (10 µM) (Supporting Fig. 5a), consistent with an earlier report, which attributes the resistance to the increased expression of P-gp and the terminally differentiated status of macrophages (37). WR12 reduced the metabolic activity of M2 BMDMs at 200 µg/mL (Supporting Fig. 5b). The rather modest toxicity of WR12 is likely due to its relatively large size (2,072 Da) and polarity, which make it difficult to enter the cells. On the other hand, PLX3397 inhibited the proliferation of M2 macrophages with an IC₅₀ of 30.4 µg/mL (Supporting Fig. 5c). PLX3397 is a tyrosine kinase inhibitor targeting CSF-1R, a receptor of CSF-1 and IL-34

(38). The activation of CSF-1/CSF-1R pathway is critical to the differentiation of cells of monocyte/macrophage lineage and the migration, proliferation, survival and function of macrophages (39, 40), and its inhibition reduces the macrophage population (39) or reprograms TAMs (41). We thus chose PLX3397 as a model drug for nanoparticle encapsulation. The drug loading efficiency in NP-pTA-M2pep was 4%. PLX3397 loading did not significantly change the particle properties (Table 2) as compared with blank particles (Table 1).

***In vivo* anti-tumor efficacy**

Free PLX3397, PLX3397@NP-pTA-AI, and PLX3397@NP-pTA-M2pep equivalent to 30 ng PLX3397 per mm³ tumor were administrated to B16F10 tumors by intratumoral injection at q2d × 5 schedule when the average tumor volume reached 170 mm³. We used 30 ng/mm³ of PLX3397 to match the IC₅₀ value against M2-polarized BMDMs (30.4 µg/mL). It was quite close to IC₅₀ value against B16F10 cells (39.7 µg/mL, Supporting Fig. 6) that we could not exclude its direct effect on tumors; nevertheless, any differential effects, if observed, would be interpreted as the contribution of NP-mediated delivery of PLX3397 to M2-like TAMs. The treatments were administered by intratumoral injection to avoid confounding difference in biodistribution.

The tumors of free PLX3397-treated animals grew quickly and reached the end point in 8 days (Fig. 7a), similar to non-treated animals observed in our previous study (42). PLX3397@NP-pTA-AI seemed to have enhanced the effect of PLX3397 compared to free PLX3397, likely due to improved intratumoral retention of the drug; however, the difference did not reach statistical significance. PLX3397@NP-pTA-M2pep treatment showed significant delay in tumor growth as compared to free PLX3397 at the equivalent dose (Fig. 7b). According to the histological evaluation of H&E stained tumor tissues (Fig. 7c), the most distinct change was identified in the samples treated with PLX3397@NP-pTA-M2pep. These tissues demonstrated multifocal to coalescing bands of necrosis composed of eosinophilic fibrillar material and erythrocytes. The necrotic tissue was infiltrated by a mixed inflammatory population composed of macrophages, lymphocytes, and plasma cells. Necrosis was also evident in the PLX3397@NP-pTA-AI group, but the distribution was less severe than the PLX3397@NP-pTA-M2pep-treated group. The PLX3397@NP-pTA-AI group demonstrated multiple necrotic foci composed of eosinophilic materials and infiltrated by macrophages, lymphocytes, and plasma cells. Free PLX3397 exhibited very little effect on the tumor tissue. Major histologic findings included sheets of polygonal to roughly ovoid neoplastic epithelial cells with anisocytosis and anisokaryosis. The cytoplasm of the neoplastic cells was distinctly eosinophilic or vacuolated. Necrotic foci were interspersed between neoplastic cells. Of note, tumor-infiltrating macrophages were more noticeable with the nanoparticle-treated groups than free PLX3397-treated group. Considering the adverse effect of PLX3397 on existing TAMs, the tumor-infiltrating macrophages may represent the cells recruited to tumors as a secondary effect of tissue necrosis.

Remaining challenges

The *in vivo* anti-tumor efficacy study supports that M2pep-modified PLGA nanoparticles can enhance the effect of PLX3397. However, PLX3397@NP-pTA-M2pep did not

completely suppress the tumor growth. The modest efficacy of PLX3397@NP-pTA-M2pep treatment may be attributable to multiple reasons. First, the dosing regimen has not been optimized. We used a conservative dose to focus on the effect of PLX3397 on TAMs and minimize its direct effect on tumor cells. In preclinical studies of PLX3397, it was administered by daily oral gavage at 50 – 100 mg/kg to achieve significant attenuation of tumor growth compared to the vehicle control (43–45). Our intratumoral dose (30 ng per mm³ tumor volume) is not directly comparable to the oral dose (50 – 100 mg/kg), but the frequency and the number of dosing (q2d × 5) are far less than the reference regimen (daily for >10 d) (43). Second, the efficacy of PLX3397 as a monotherapy may be inherently limited (46). Most preclinical studies of CSF-1R inhibitors have seen significant anti-tumor effects when additional drugs or treatments are combined to address tumor cells (45, 46) or supplement tumor-infiltrating lymphocytes (43, 44). PLGA nanoparticles offer a sufficient room for the delivery of drug combinations; thus, it may be worthwhile to combine second drugs such as anticancer drugs or immune checkpoint inhibitors in addition to PLX3397 in future studies. Third, the PLX3397 effect on TAMs may have been transient and reversed with time. Discher et al reported that highly phagocytic, engineered macrophages delivered to tumors repolarized toward non-phagocytic TAMs in the stiffness of tumors, indicating the plasticity of macrophage phenotype (47). PLX3397@NP-pTA-M2pep showed initial burst release in the first few hours (data not shown). It is possible that the PLX3397@NP-pTA-M2pep may not have provided a long-lasting effect of PLX3397, permitting TAMs to recover or infiltrating macrophages to reprogram during the dosing interval. If this is the case, it will be beneficial to reformulate the core nanoparticles to provide sustained drug release. Finally, potential effects of pTA on TAMs may not be overlooked. A recent study indicates that tannic acid inhibits NF- κ B signaling in BMDMs to suppress IL-1 β secretion (48) and a derivative of tannic acid inhibits protein tyrosine phosphatase 1B to induce M2 polarization of macrophages (49). Although the pTA included in the final NP product is relatively minor, a systematic study remains to be performed to determine whether it offsets the effect of PLX3397@NP-pTA-M2pep on TAMs and it is necessary to replace pTA with alternative polyphenols.

CONCLUSION

M2pep-coated nanoparticles were produced as a drug carrier targeted to tumor-supportive TAMs. M2pep was conjugated to PLGA nanoparticles via tannic acid/Fe³⁺ complex as an adhesive layer. The M2pep-coated nanoparticles better entered M2-polarized BMDMs *in vitro* and CD206⁺ macrophages in B16F10 tumors *in vivo* than those without M2pep. As a carrier of PLX3397, a CSF-1/CSF-1R inhibitor, M2pep-coated nanoparticles helped retain the drug in B16F10 tumors and attenuated tumor growth to a greater extent than free drug. These results support that M2pep-coated nanoparticles provide a useful means to deliver drugs to tumor-supportive TAMs and control their functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Schematic illustration of M2pep-coated nanoparticles and their interaction with tumor-associated macrophages in tumor.

Fig. 2. Transmission electron microscopic images of nanoparticles. See Table 1 for nanoparticle description. Negatively stained with 1% uranyl acetate. Scale bars: 100 nm.

Fig. 3.
Phenotypes of bone marrow derived macrophages polarized to M1 or M2-macrophages (CD86, iNOS: M1 biomarkers; CD11c, arginase-1: M2 biomarkers).

Fig. 4.

Confocal microscopic images of bone marrow derived M2 macrophage after 30 min incubation with Rhodamine-labeled nanoparticles. Red: nanoparticles; blue: Hoechst 33342-stained cell nuclei; green: Calcein AM stained cytoplasm. Scale bar: 50 μm . For all captured images, see Supporting Fig. 2. The graph shows the % area of nanoparticle signal / area of cytoplasm. 4–5 images per treatment (Supporting Fig. 2) were analyzed by ImageJ. p-values by Tukey's multiple comparisons test.

Fig. 5.

Cellular interactions between Rhodamine-labeled nanoparticles and bone marrow derived macrophages after 30 min incubation. Measured by flow cytometry and shown as ratios of interaction with M2 macrophages indicated by cellular fluorescence intensity relative to the interactions with M1 (a) or M0 (b). $n = 3$ independent experiments with nanoparticles from different batches. Means $\pm$ s.d. Each data point is an average of three replicates per experiment. p -values by two-tailed paired t test. The result of each experiment, expressed as raw fluorescence intensity of macrophages interacting with the labeled nanoparticles, is shown in Supporting Fig. 4.

Fig. 6. Colocalization of nanoparticles and tumor associated macrophages in B16F10 tumor tissues at 24 h after intravenous injection of the nanoparticles. Red: Rhodamine labeled nanoparticles; blue: DAPI-stained nuclei; green: anti-CD206 antibody labeled tumor associated macrophages.

Fig. 7.

In vivo activity of free PLX3397, PLX3397@NP-pTA-AI and PLX3397@NP-pTA-M2pep in C57BL/6 mice bearing subcutaneous B16F10 tumors. n=5/group. (a) Tumor volumes recorded every other day; (b) Specific growth rate of B16F10 tumor. $\log V/t$ (V : tumor volumes; t : time in days). p-values by Uncorrected Fisher's LSD. (c) Histopathologic features of the tumor parenchyma treated with free PLX3397, PLX3397@NP-pTA-AI, or PLX3397@NP-pTA-M2pep. Scale bar: 300 μ m: (left) free PLX3397-treated tumor composed of sheets of neoplastic epithelial cells with scattered foci of necrosis and hemorrhage; (center) PLX3397@NP-pTA-AI-treated tumor composed of neoplastic epithelial cells with a central core of necrosis expanded by fibroblasts, fibrin, and hemorrhage; (right) PLX3397@NP-pTA-M2pep-treated tumor composed of coalescing bands of necrosis composed of eosinophilic fibrillar material, erythrocytes, and a mixed inflammatory population.

Table 1.

Size and zeta potential of nanoparticles

Nanoparticles	Description	Size (nm)	PDI	Zeta potential(mV)
NP	Bare PLGA nanoparticles	171.6 ± 6.2	0.05 ± 0.02	-1.6 ± 0.1
NP-pTA	pTA-coated NP	202.7 ± 3.0	0.21 ± 0.02	-22.0 ± 0.3
NP-pTA-Al	NP coated with albumin via pTA	199.4 ± 0.9	0.15 ± 0.02	-16.6 ± 0.7
NP-pTA-M2pep	NP coated with M2pep via pTA	217.4 ± 2.9	0.17 ± 0.04	-14.0 ± 0.5

n = 3 identically and independently produced batches. Means ± s.d.

Table 2.

Size, zeta potential, and drug loading of PLX3397-loaded nanoparticles

	Size (nm)	PDI	Zeta Potential (mV)	Drug loading (%)
PLX3397@NP	177.5 ± 3.1	0.04 ± 0.01	-1.4 ± 0.2	4.2±0.1
PLX3397@NP-pTA	191.2 ± 1.3	0.13 ± 0.07	-19.8 ± 1.0	4.1±0.1
PLX3397@NP-pTA-Al	201.3 ± 0.6	0.21 ± 0.03	-20.2 ± 0.7	3.2±0.3
PLX3397@NP-pTA-M2pep	210.3 ± 2.0	0.18 ± 0.02	-16.1 ± 0.6	4.0±0.2

n = 3 identically and independently produced batches. Means ± s.d.

Drug loading: % Drug loaded / nanoparticle mass