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Nanoparticle Targeting of Neutrophils for Improved Cancer Immunotherapy

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Abstract

Cancer immunotherapy using tumor specific monoclonal antibodies (mAbs) presents a novel approach for cancer treatment. A monoclonal antibody TA99 specific for gp75 antigen of melanoma, initiates neutrophil recruitment in tumor responsible for cancer therapy. Here we report a strategy for hijacking neutrophils *in vivo* using nanoparticles (NPs) to deliver therapeutics into tumor. In a mouse model of melanoma, we showed that systemically delivered albumin NPs increased in tumor when TA99 antibody was injected; and the nanoparticle tumor accumulation was mediated by neutrophils. After the administration of pyropheophorbide-a (Ppa) loaded albumin NPs and TA99, photodynamic therapy significantly suppressed the tumor growth and increased mouse survival compared with treatment with the NPs or TA99. The study reveals a new avenue to treat cancer by nanoparticle hitchhiking of immune systems to enhance delivery of therapeutics into tumor sites.

Keywords

Albumin nanoparticles; neutrophils; antibodies; tumor; immunotherapy

Over the past two decades, remarkable progress has been made in the development of nanoparticle drug delivery systems to treat cancer.[1] The nanoparticle-based cancer therapy was proposed based on a concept of enhanced permeability and retention (EPR) effect because tumor vasculatures are more permeable than normal tissues, which facilitates drug-loaded nanoparticles (NPs) to efficiently accumulate into tumor vasculature, therefore

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the Publications website:

Experimental Section; SDS-page and immunoprecipitation analysis; particle size and its distribution of the NPs; flow cytometry of neutrophils in tumor and blood; Number of neutrophils in blood and percentage of neutrophils in tumor after depletion. Percentage of neutrophils containing NPs and MFI of neutrophils in blood after the injection of Alexa Fluor-488-BSA NPs and Ppa loaded Alexa Fluor-488-BSA NP.

The authors declare no competing financial interest.

increasing therapeutic efficacy compared with free drugs.[2] This passive drug delivery is strongly dependent on the size of nanoparticle carriers, and their circulation time in the bloodstream.[3] Active tumor targeting using NPs could enhance cancer therapy. Here we propose a strategy in which NPs are able to hijack leukocytes to actively deliver drugs to tumor sites.

The use of monoclonal antibodies (mAbs) as therapeutics for cancer treatment has increased dramatically in the past decade.[4] They can be designed to specifically target tumor-associated antigens and initiate a response of several effector cells that eliminate tumor cells.[5] For instance, anti-HER-2 mAb trastuzumab used to treat breast cancer has demonstrated tremendous benefits to cancer patients.[6] In a mouse model of B16 melanoma, it is found that monoclonal antibody TA99 specific for gp75 antigen can induce neutrophil recruitment in tumor sites as a mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC) for cancer therapy, and neutrophils are a major component of ADCC effect rather than other immune effector cells.[7, 8] The interaction of neutrophils with tumor cells is mediated by the binding of antibody Fc portion to Fc receptors expressed on immune cells.[9] Targeting neutrophils in vivo using NPs could enhance the delivery of drugs in the tumor microenvironment.

Using intravital microscopy of TNF-α-induced inflammation of cremaster venules, we have shown that intravenously (i.v.) injected denatured albumin NPs can selectively target activated neutrophils. Using these NPs we are able to deliver anti-inflammation drugs to neutrophils, thereby preventing the acute lung inflammation/injury.[10, 11]

Here, we combine cancer immunotherapy and nanotechnology to develop a method for hitchhiking activated neutrophils to increase therapeutic nanoparticle deposition in tumor sites. Using a mouse melanoma model, we demonstrate the feasibility of this approach to improve cancer therapy.

Preparation of the antibody and albumin NPs

Antibody TA99 was produced using TA99 hybridoma in serum free medium. After purification, the antibody was characterized by reduced SDS-PAGE and immuno-precipitation. The result (Figure S1) indicates that the antibody was successfully made with a high quality as the commercial standard. Furthermore, the immuno-precipitation studies showed a comparative binding ability of our antibody with the standard to the antigen of TYRP1/gp75, which has a molecular weight of 75 kDa (Figure S2).[12]

Several techniques have been successfully employed to prepare albumin NPs.[13] However, the biological properties of albumin NPs strongly depend on the preparation techniques. The desolvation approach using ethanol causes the denaturation of albumin proteins which consequently aggregate and form into NPs.[14] This makes a unique feature of albumin NPs that are specifically internalized by activated neutrophils when we administered the NPs in a mouse.[10] We also found that albumin nanoparticle uptake is independent of fluorescent labeling on albumin NPs, but only determined by the denaturation of albumin.[10, 11] Using the same nanoparticle preparation approach we made Cy5-conjugated bovine serum albumin

(Cy5-BSA) NPs for imaging. To demonstrate cancer therapy, we loaded Ppa, a photosensitizer in BSA NPs and the loading efficiency was 5 ± 0.4 wt%. The particle sizes were characterized using dynamic light scattering, indicating that the sizes were 134, 153 and 224 nm for BSA, Cy5-BSA and Ppa-loaded BSA NPs, respectively (Figure S3A and S3B).

Antibody increases the accumulation of NPs in tumor mediated by neutrphils

We address whether TA99 could increase neutrophil accumulation in a mouse tumor site. First, we studied a time course of neutrophil infiltration after administration of TA99. 24 and 48 h after the administration of TA99 at 40 mg/kg,[8] we collected mouse tumor tissues and performed the flow cytometry after staining with Alexa-Fluor-488-labeled anti-mouse Gr-1 to mark neutrophils. It is noted that the infiltration of neutrophils in tumor tissues was strongly dependent on TA99, and the percentage of neutrophils dramatically increased 48 h after the administration of TA99 (Figure 1A and S4A–S4D), suggesting that TA99 can facilitate the infiltration of neutrophils into tumor, which is consistent with the previous study.[7]

This neutrophil migration in tumor could be a means to deliver therapeutics for cancer therapy if nanoparticles could specifically target neutrophils *in vivo*. In a mouse model of acute lung inflammation, we have showed that albumin NPs are precisely internalized by activated neutrophils.[10] Here we address whether albumin NPs could hijack neutrophils to arrive at tumor tissues. Based on the study in Figure 1A, we designed our experiments (in the inset of Figure 1) in which neutrophils efficiently take up albumin nanoparticles before they infiltrate into tumors. The fluorescently-labeled albumin NPs were injected 24 h after TA99 administration. 24 h later we collected mouse tumor tissues and isolated neutrophils. We performed the fluorescence confocal microscopy and flow cytometry to investigate whether neutrophils in a tumor site contained albumin NPs. We found in the confocal images that neutrophils internalized albumin NPs after the administration of TA99 (Figure 1B). However, we observed few neutrophils internalizing albumin NPs in the absence of TA99 (Figure 1B). The flow cytometry further showed that the mean fluorescence index (MFI) of albumin NPs after the co-administration of TA99 and NPs dramatically increased compared with NPs alone (Figure 1C). The results of confocal imaging and flow cytometry clearly indicate that TA99 can promote the accumulation of albumin NPs in tumor sites mediated by neutrophil uptake of NPs.

The question is whether albumin nanoparticle tumor deposition is specifically mediated by neutrophils, so we performed an experiment of neutrophil deleption in a mouse.[11] In the control, we observed that neutrophils were completely depleted in the circulation after i.p. injection of anti-Gr-1 antibody (Figure S5A). Futhermore, we observed that the neutrophil recruitment was also robustly prevented in tumor (S5B). The result confirmed that administration of anti-Gr-1 antibody can abolish neutrophils in a mouse. Then, we investigated whether neutrophil depletion affected the nanoparticle tumor accumulation. The concentrations of NPs in tumor tissues were analyzed by measuring the fluorescence of NPs.

It is noted that administration of TA99 markedly increased the accumulation of albumin NPs in tumor tissues compared with those in the absence of TA99 (Figure 1D). When neutrophils were depleted using anti-Gr-1 antibody, the amount of albumin NPs in tumor decreased to the level at injection of NPs alone. This study indeed indicates that the increase of albumin NPs in tumor tissue is specifically mediated by the neutrophil recruitment when TA99 was administered.

Antibody increases the internalization of NPs by neutrphils in blood

To define how albumin NPs hitchhiked neutrophils leading to the increase of albumin NPs in tumor, we investigated neutrophil uptake of albumin NPs in blood. Cy5-BSA NPs were intravenously injected in mice bearing melanoma 24 h after the administration of TA99. 24 h later we collected peripheral blood neutrophils and performed the flow cytometry. It was found that the percentage of nanoparticle-laden neutrophils markedly increased from 0.7% in the NPs only group to 6.2% for co-administration of TA99 and albumin NPs (Figure 2A and S6A–6C). The MFI of neutrophils also showed the increase of nanoparticle uptake induced by TA99 (Figure 2B). The confocal images of neutrophils in blood further confirmed that TA99 antibody increased the neutrophil uptake of albumin nanoparticles (Figure 2C). Combining with the studies of Figure 1, the results suggest that nanoparticle-laden neutrophils could be recruited in tumor tissues via a mediator of TA99, and therefore we observed the accumulation of albumin NPs in tumor tissues.

Neutrophil uptake of BSA NPs does not affect the function of neutrophils

To address whether neutrophil uptake of NPs will affect neutrophil recruitment in tumor sites, we quantitatively measured the percentage of neutrophils in tumor after nanoparticle injection. We found that neutrophil uptake of albumin NPs or Ppa-loaded albumin NPs did not alter the neutrophil recruitment in tumor sites compared with the absence of NPs (Figure 3 and S7). We also investigated the effect of drug loading on neutrophil uptake of BSA NPs *in vivo*. It was found that in terms of percentage of neutrophils internalizing NPs and MFI of neutrophils in blood, there were no significant differences between blank NPs and drug-loaded NPs ($P > 0.1$) (Figure S8A and S8B). The result combined with Figure 3 suggests that neutrophils might be a carrier to actively deliver therapeutic NPs in the tumor microenvironment.

Antibody increases the efficacy of drug loaded albumin NPs for tumor treatment

To demonstrate the usefulness of albumin nanoparticle hijacking of neutrophils in cancer therapy, we loaded Ppa, a photosensitizer[15] in albumin NPs. The photodynamic therapy was established in the mouse melanoma model. [16] In TA99 group, the tumor size was slightly decreased compared with the control group (PBS/5% glucose). The reason would be that the multiple doses of TA99 were required for the marked suppression of tumor growth during the treatment.[8, 17] When we combined TA99 and Ppa-loaded albumin NPs (Figure 4A), the tumor size was significantly smaller than TA99 alone or NPs alone. This would be associated with the increased accumulation of albumin NPs in tumor mediated by TA99-

induced neutrophil recruitment (Figure 1 and 2). For survival studies, we also did not observe obvious therapeutic benefit when mice were treated with TA99 compared with the control. Although the photodynamic therapy of Ppa-loaded albumin NPs extended the mouse lifespan (Figure 4B), the combination of TA99 and Ppa-loaded albumin NPs significantly increased the survival rate (Figure 4B). During the survival studies, we also monitored the body weight changes. The mice in all groups gained their weight with time and the weight increase was independent of administration of antibody or NPs (Figure 4C). The result implies that mice tolerated TA99 and Ppa-loaded BSA NPs at the doses used. In contrast to the treatment of TA99 or NPs alone, the combination of cancer immunotherapy and active delivery of NPs dramatically improves the cancer treatment.

In summary, we have demonstrated a novel approach for cancer therapy by combining immunotherapy and nanotechnology. Our studies indicate that albumin NPs are capable of in situ hitchhiking neutrophils and delivering therapeutics into tumor sites. The approach has showed the marked improvement of photodynamic therapy when we loaded a photosensitizer in albumin NPs. Human neutrophils account for 50–70% of all circulating leukocytes.[18] Therefore, the finding would have a great impact on treating a wide range of cancer with the combination of specific antibodies in cancer immunotherapy and engineering of nanoparticles. Furthermore, our studies reveal a new strategy for hijacking of immune systems as a route to deliver therapeutic NPs into the tumor microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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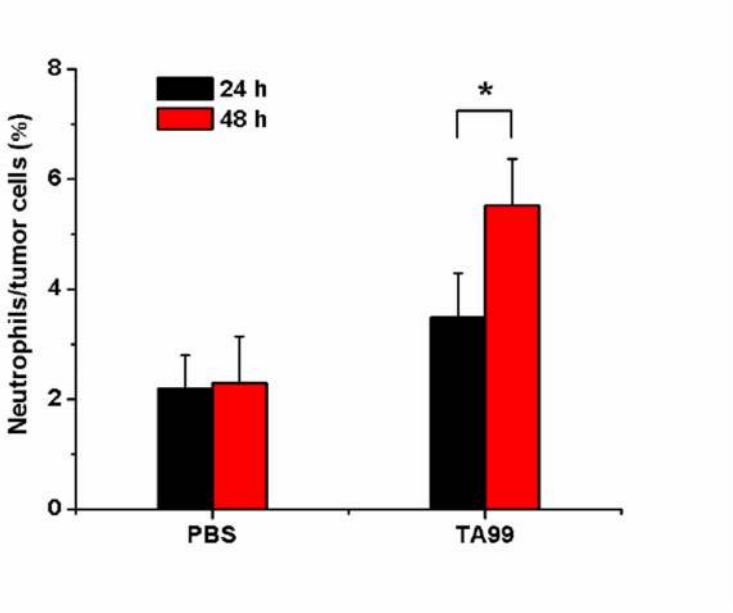

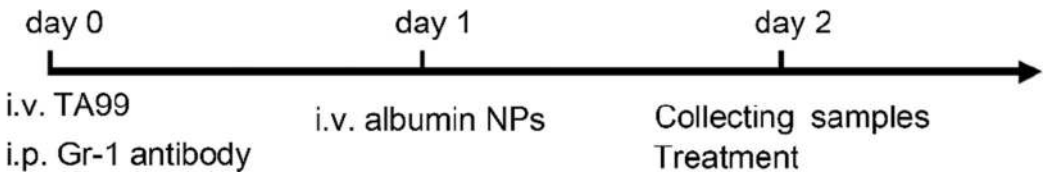

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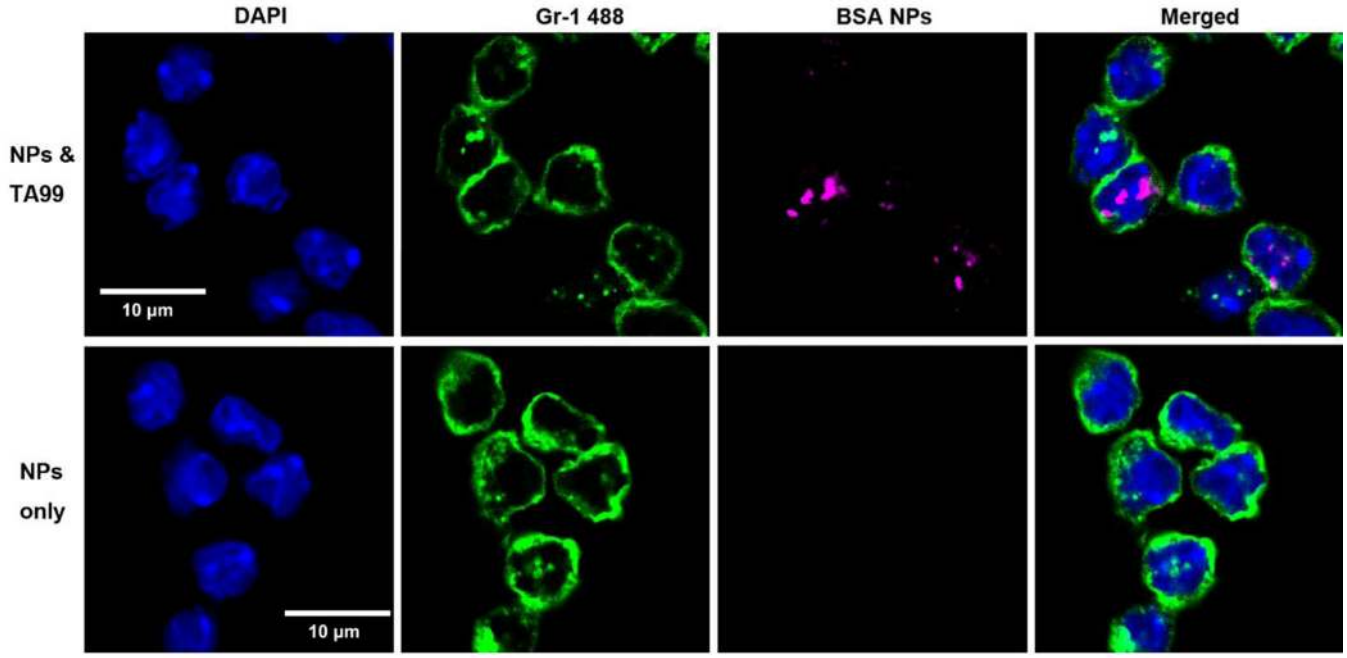

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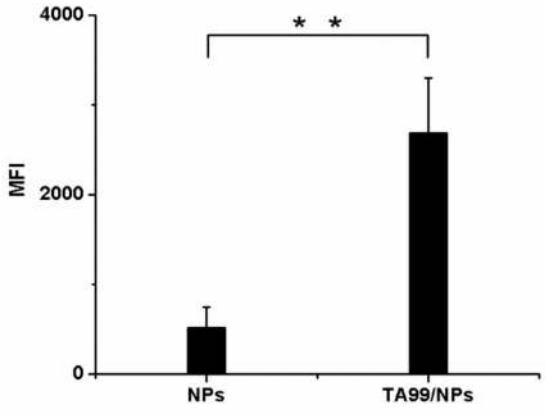

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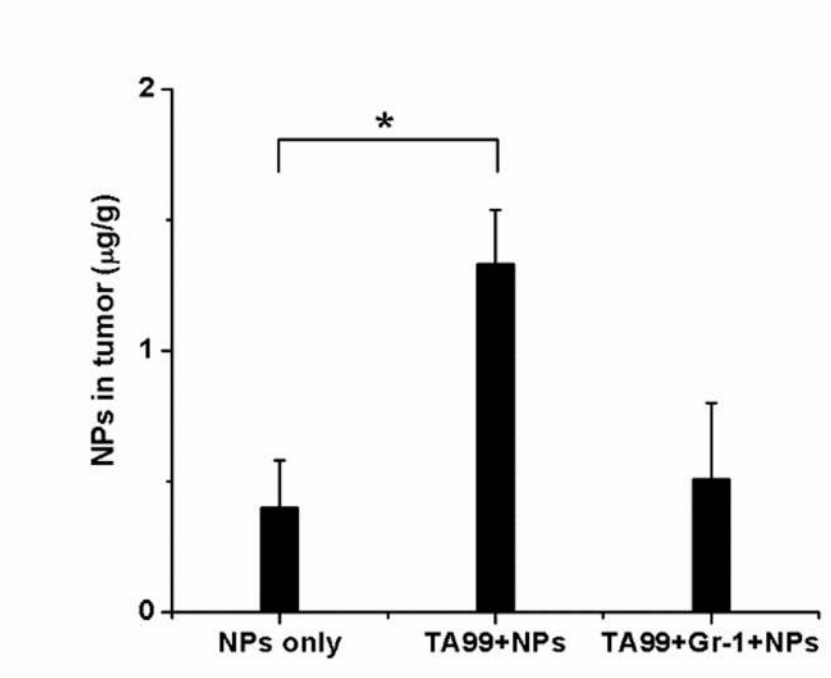

Figure 1. TA99 increases the accumulation of NPs in tumor mediated by neutrphils

(A) Percentage of neutrophils in tumor tissue measured by flow cytometry after i.v. injection of PBS and TA99, respectively (n=3). The samples were single cell suspension prepared from mouse tumor 24 or 48 h after mice were treated with PBS and TA99. Neutrophils were labelled by Alexa Fluor-488 anti-mouse Gr-1 antibody. (B) Fluorescence confocal microscopy of neutrophils (green by Alexa Fluor-488 anti-mouse Gr-1 antibody) from tumor 48 h after administration of both TA99 and Cy5-BSA NPs (red) or NPs alone. Nucleus was stained by DAPI (blue). Experiment scheme is shown above the figure. (C) MFI of NPs in neutrophils in tumor by flow cytometry 48 h after the injection of Cy5-BSA NPs or both TA99 and Cy5-BSA NPs, respectively (n=3). Neutrophils in tumor were isolated by Pluriselect anti-mouse-Ly6G S-pluribeads. (D) Concentrations of Cy5-BSA NPs in tumor tissue with PBS, TA99 or TA99/Gr-1 (n=3). The tumor was removed from the mice one day after the administration of Cy5-BSA NPs in 5% glucose. In all expriments the doses of TA99, Cy5-BSA NPs and Gr-1 antibody were 40, 8 and 20 mg/kg, respectively. Statistics were performed by a two-sample student's t-test (*, $P < 0.05$; **, $P < 0.01$).

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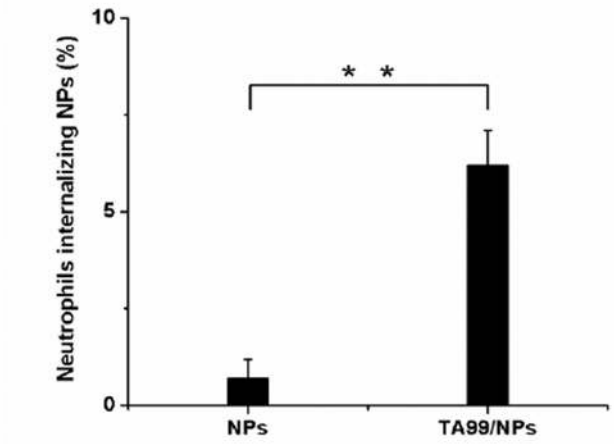

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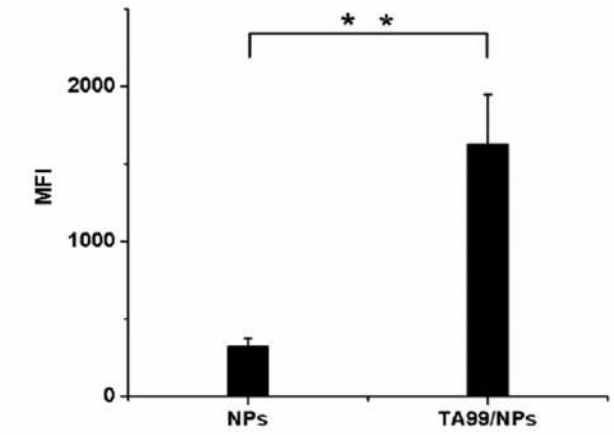

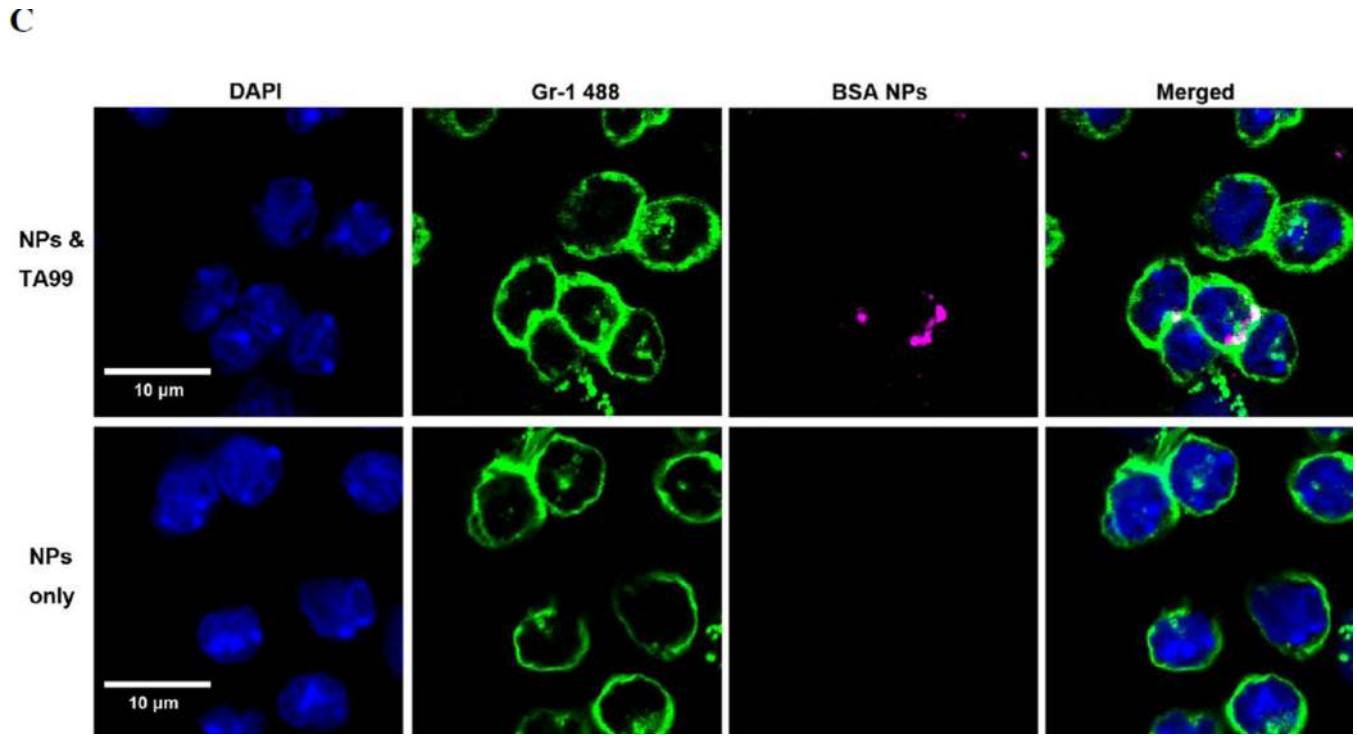

Figure 2. TA99 increases the internalization of NPs by neutrphils in blood

(A) Percentage of neutrophils containing Cy5-BSA NPs, (B) MFI of NPs in neutrophils measured by flow cytometry, (C) Fluorescence confocal microscopy of neutrophils (green by Alexa Fluor-488 anti-mouse Gr-1 antibody) after the injection of NPs or both of NPs and TA99, respectively (n=3). The experimental procedure is the same as Figure 1. Neutrophils were stained with Alexa Fluor-488 anti-mouse Gr-1 antibody, and nucleus was stained by DAPI (blue). Neutrophils in blood were isolated by Pluriselect anti-mouse-Ly6G S-pluribeads. The dose of TA99 and Cy5-BSA NPs were 40 and 8 mg/kg, respectively. Statistics were performed by a two-sample student's t-test (**, $P < 0.01$).

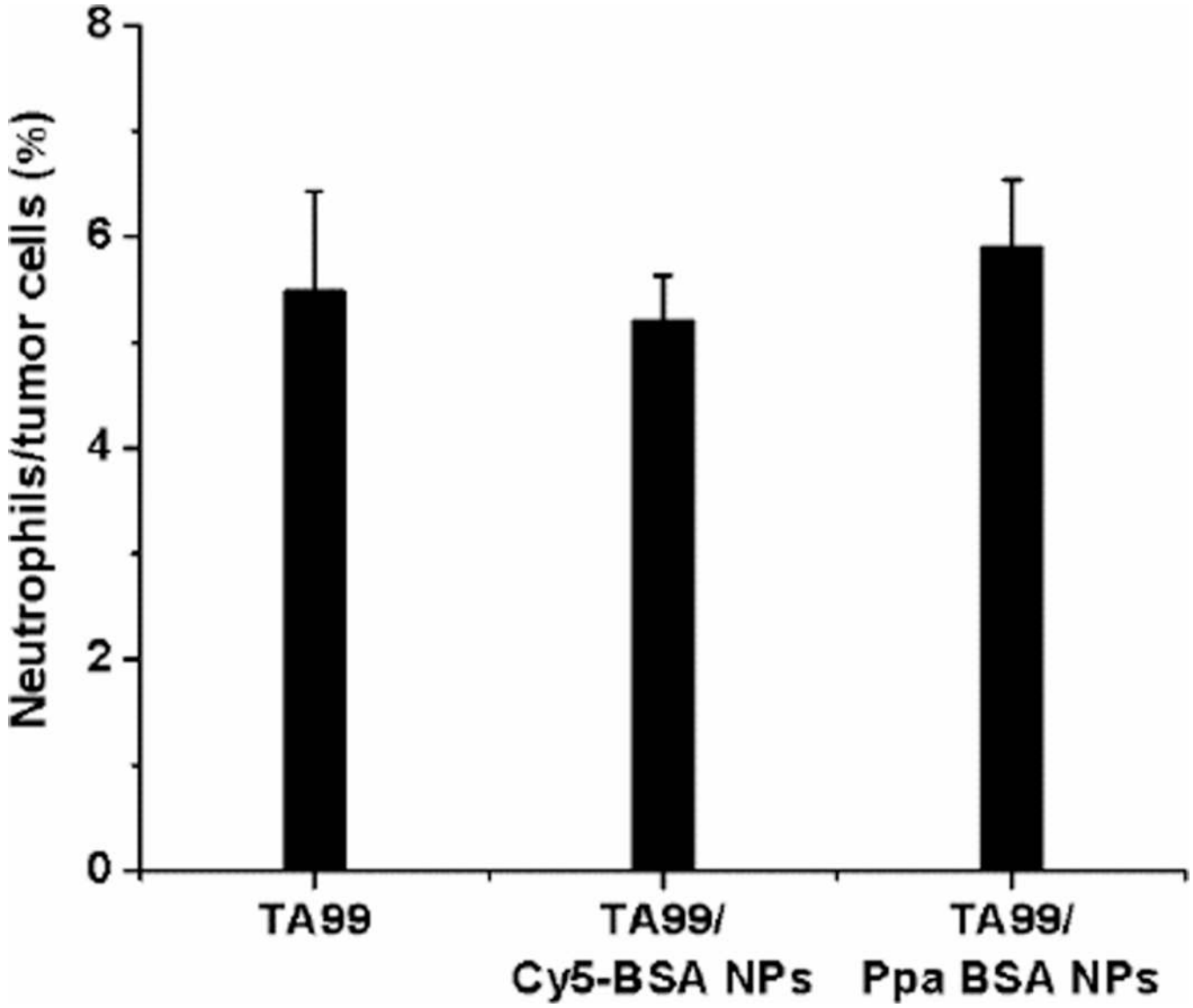

Figure 3. Neutrophil uptake of BSA NPs does not affect neutrophil recruitment in tumor

Percentage of neutrophils in tumor tissue measured by flow cytometry after the injection of TA99, both of TA99 and Cy5-BSA NPs or Ppa BSA NPs, respectively (n=3). The experimental procedure is the same as Figure 1. Neutrophils were labelled by Alexa Fluor-488 anti-mouse Gr-1 antibody.

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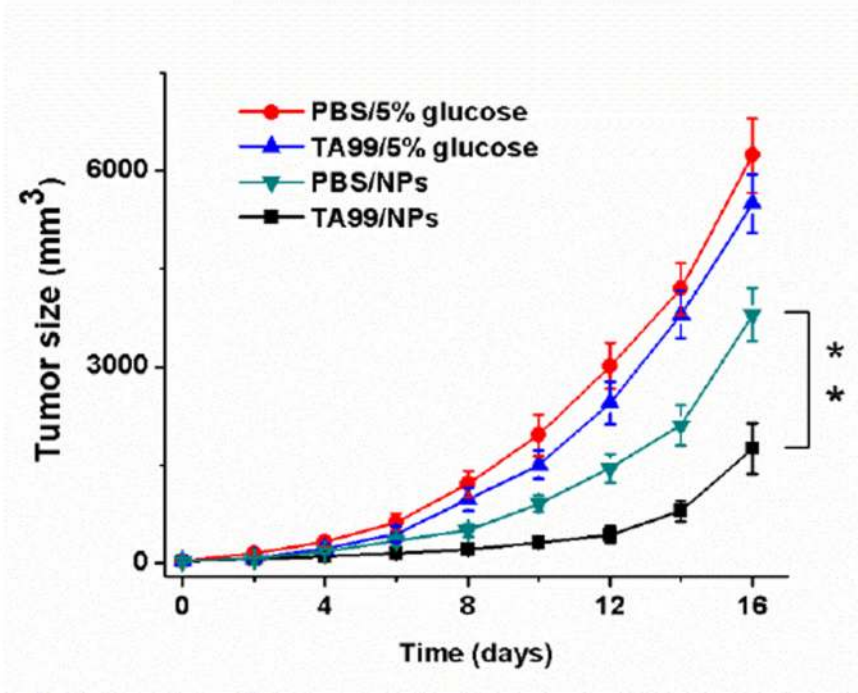

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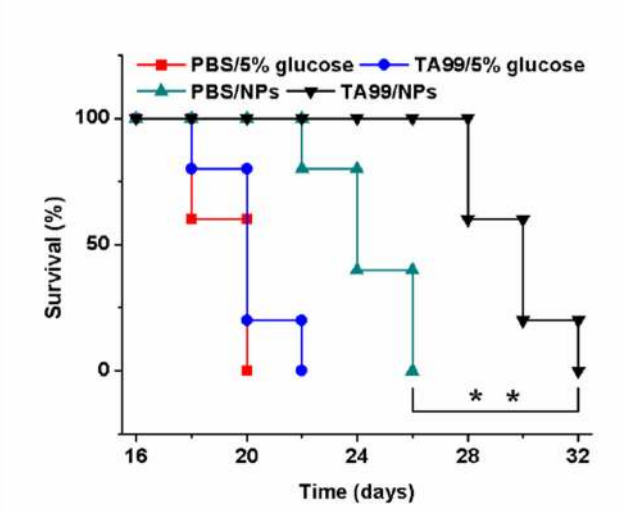

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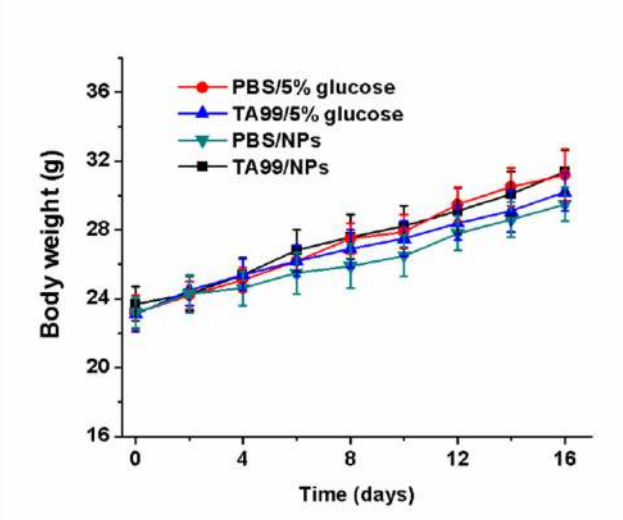

Figure 4. TA99 increases the efficacy of drug-loaded albumin NPs in tumor treatment

(A) Tumor size, (B) survival rates and (C) body weight of the mice bearing melanoma illuminated with 660 nm laser at day 2 after the injection of vehicles, TA99, Ppa-loaded NPs or both of TA99 and Ppa-loaded NPs. The doses of TA99 and Ppa were 40 and 2 mg/kg, respectively. Statistics for tumor sizes were performed by a two-sample student's t-test (**, $P < 0.01$). Significance of survival rates between treatment groups (**, $P < 0.01$) was

assessed by a log-rank (Mantel–Cox) survival analysis test with 95% CIs using GraphPad Prism® software (GraphPad Software, Inc., CA).