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Attenuating Metabolic Competition of Tumor Cells for Favoring the Nutritional Demand of Immune Cells by a Branched Polymeric Drug Delivery System

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Abstract: Tumor cells are dominant in the nutritional competition in the tumor microenvironment (TME), and their metabolic abnormalities often lead to microenvironmental acidosis and nutrient deprivation, thereby impairing the function of immune cells and diminishing the anti-tumor therapeutic effect. Herein, we report a branched polymeric conjugate and its efficacy in attenuating the metabolic competition of tumor cells. Compared with the control nanoparticles prepared from its linear counterpart, the branched conjugate-based nanoparticles (branched NPs) could more efficiently accumulate in the tumor tissue and interfere with the metabolic processes of tumor cells to increase the concentration of essential nutrients and reduce the level of immunosuppressive metabolites in the TME, thus creating a favorable environment for infiltrated immune cells. Its combined treatment with an immune checkpoint inhibitor (ICI) achieved an enhanced anti-tumor effect. Our work presents a promising approach for targeting the metabolic competition in the TME to enhance the chemo-immunotherapeutic effect against cancers.

1. Introduction

Cancer cells rewire their metabolic pathways, such as the metabolism of glucose and amino acids, to maximize the utilization of nitrogen/carbon sources for macromolecular metabolic synthesis to meet the demand of nutrients for their rapid growth ^[1]. Their aggregative nutrition consumption results in the deprivation of nutrients for tumor-infiltrating immune cells in the tumor microenvironment (TME) ^[2]. Besides, the metabolism including glycolysis by tumor cells at a high rate is accompanied with the production of lactic acid, kynurenine, and other metabolites, and these metabolites could inhibit the anti-tumor immune response after they are exported into the extracellular milieu ^[3]. Therefore, the anti-tumor immune system is reprogrammed to be ineffective and the tumor-promoting phenotypes are acquired. Strategies for targeting the metabolic syndrome in the TME, such as targeted regulation of competitive depletion of glucose and generation of immunosuppressive metabolites, have been demonstrated to enhance the anticancer immunotherapeutic effect ^[4].

Although success has been witnessed in targeting some of the metabolic pathways by small molecule metabolic regulators, the inhibition of one single metabolic pathway has a low broad-spectrum efficacy in inhibiting the production of a myriad of immunosuppressive metabolites ^[3c, 5]. Furthermore, these small-molecule metabolic modulators often have inevitable systemic toxicity, and they may have the issue of drug resistance due to the network of the tumor metabolism ^[6]. Hence, realization of targeted modulation for broad metabolic to extensively inhibit the tumor cell metabolism would improve the immunotherapeutic effect by supplying adequate interstitial

nutrients for tumor immune cells, while reducing the generation of immunosuppressive metabolites [7].

Apart from assistance in reducing systemic toxicity and side effects of small molecular drugs, drug delivery systems could play a role in the disruption of tumor cell metabolic processes. Our previous study demonstrated that a degradable dendronized polymeric prodrug with a branched structure effectively interfered with the metabolic processes in tumor cells, including the tricarboxylic acid cycle (TCA) and the amino acids metabolism [8]. This altered metabolic stress in tumor cells could weaken the dominant role of tumor cells in metabolic competition, thus lessening the nutritional competition between tumor cells and immune cells in the TME. The resulting favorable environment for immune cells could help induce potent anti-tumor immune responses [9].

Herein, we synthesized a conjugate derived from degradable and branched poly[hydroxypropyl methacrylate] (polyHPMA) containing disulfide bonds, and a chemotherapeutic agent, epirubicin (Epi), was covalently bound to the polymer chain via a pH-sensitive bond. A conjugate with a linear structure was also prepared as a control. We simulated the assembly process of the branched polymeric conjugate and drug release from the branched conjugate in silico. The potent efficacy of the resulting branched conjugate-based nanoparticles (branched NPs) against tumor cells was demonstrated in comparison with the nanoparticles prepared from the conjugate with a linear structure. It was found that the branched NPs self-assembled from the polymeric conjugate with a large molecular weight could achieve a high accumulation level in tumor, and the incorporated drug could be released to induce immunogenic cell death (ICD), which enhanced the anti-tumor immune response. The immune response was shown to be strengthened after the branched NPs were combined with an ICI [10]. Through metabolomic analysis and flow cytometry, the branched NPs were shown to disrupt the metabolism of tumor cells, and the resulting rich nutrients in the extracellular tumor environment could be utilized by tumor-infiltrating immune cells for potent anti-tumor immune responses (**Figure 1**). This work on using a polymer-based drug delivery system for regulating the metabolic competition between tumor cells and immune cells presents a new promising chemo-immunotherapeutic platform for effective treatment of cancers.

2. Results

2.1. Synthesis and characterization

Two polymeric prodrug conjugates based on the HPMA polymers with branched (**Figure 2A**) and linear (**Figure 2B**) structures were synthesized by RAFT polymerization ^[11]. The detailed synthesis routes, characterizations and computer simulation methods of the HPMA-based polymeric prodrug conjugates are supplemented in the Supplementary Materials (Schemes S1-S5, Figure S1-S6, and Tables S1, S2). Both linear (an Epi content of 8.29 wt%) and branched (an Epi content of 8.74 wt%) polymeric prodrug conjugates self-assembled in water to form NPs, with a negative zeta potential of -5.04 mV and -2.34 mV, respectively (**Figure 2C**). **Figure 2D** shows that the hydrodynamic size of the branched NPs (~ 59 nm) was larger than that of the linear one (~ 24 nm), which was consistent with the TEM results as shown in **Figure 2E** and **2F**. A larger size of NPs resulting from the branched polymeric prodrug conjugate was further consolidated by computer simulation (**Figure 2G, 2H**, and **S7-S9**). The branched three-dimensional structure with a high molecular weight could contribute to stronger van der Waals interactions with each other in an aqueous solution, leading to the formation of a stable spatial structure ^[12]. The stable spatial structure acted as a well-defined nucleation site, which drew the neighboring molecules to form a larger compact three-dimensional nanostructure. However, the linear conjugates agglomerated to form a less compact nanostructure as compared with that formed by the branched conjugates. A larger size has been reported to effectively reduce the glomerular metabolism ^[13].

The UV spectra of both NPs are shown in **Figure S10A**. The maximum absorption of Epi was seen at a wavelength of ~486 nm. After Epi was conjugated to the polymer with a linear or branched structure via an acid-labile hydrazone bond, there was a marginal change in the maximum absorption wavelength for the resulting NPs, indicated successful loading of free Epi in the NPs. **Figure 2I** and **S10B, S10C** show no significant changes in the hydrodynamic size, zeta potential or polydispersity (PDI) for both NPs formed from the linear and branched polymeric conjugates after 48 h in a PBS buffer, indicative of their high colloidal stability. As shown in **Figure 2J** and **Figure S10D**, the release profile of Epi from both NPs in an acidic condition was considerably enhanced compared with that in a neutral pH condition. Under the simulated acidic condition containing glutathione (GSH), the linear or branched polymeric conjugates were able to release more than 70% of the conjugated Epi within 48 h, indicating both drug conjugates were responsive to release Epi at a low

pH value. The release rate of Epi was slightly rapid and the accumulative release amount of the drug from the branched NPs was higher in comparison with those from the linear one, which may attribute to the disulfide bonds cleavage in the branched conjugate in the presence of GSH ^[14]. According to the computer simulation results shown in Figure 2K, 2L and S8, S9, when breakage of disulfide bonds and protonation were introduced, the branched NPs could be completely dissociated. The radial distribution function (RDF) results for the interaction between Epi and the branched polymer chain suggested that there were only a small number of drugs on the polymeric chain, indicative of almost complete drug release. This is because the cleavage of disulfide bonds in the branched polymeric structure facilitated the NP disintegration that increased the accessibility of acid-labile hydrazone bonds for easier cleavage, leading to efficient drug release. DLS (Figure S10E) and TEM (Figure 2M) results confirmed evident changes in the polymer structure of the branched conjugates under an acidic condition in the presence of GSH as well as hydrodynamic size changes of nanoaggregates. The degradation product from the branched polymeric prodrug conjugate had a molecular weight of about 20 KDa with a PDI of 1.24, and its size was less than the degradation product of linear one (Figure S10E and S10F). The molecular weight of the degradation product was lower than the reported threshold of 45-50 Kda ^[15], suggesting the HPMA-based branched polymeric conjugate could be effectively cleared in the body.

2.2. Intracellular delivery and in vitro cytotoxicity

First, we examined the cellular uptake and internalization pathways of both NPs and free Epi by 4T1 cells. Quantitative analysis of the fluorescence intensity by flow cytometry in **Figure 3A** and S11A showed that the internalization efficiency of the branched NPs was higher than that of the linear ones. Confocal results shown in Figure S11B suggested that the intracellular fluorescence intensity of free Epi was rapidly enhanced over time with efficient nuclear localization. As comparison, Figure S11C and S11D showed that both NPs gradually accumulated in the cytoplasm and the Epi fluorescent signal in the nucleus increased at a much slower rate, whilst the branched NPs were more efficient in Epi delivery than the linear ones, which is consistent with the flow cytometry result in Figure 3A and S11A. After staining the acidic compartments including endosomes and lysosomes with LysoTracker, it was seen in Figure 3B and S11E that the red Epi fluorescent signal was colocalized with green LysoTracker signal, indicating that Epi could be effectively released from the

polymeric prodrug conjugates after intracellular internalization and cleavage of acid-labile hydrazone bonds in these acidic compartments after intracellular internalization. Three dimensional (3D) multicellular tumor spheroids (MTS) models by 4T1 cells were also established to examine the penetration of both NPs and free Epi. As shown in Figure S12A-S12C, the branched NPs showed stronger and broader signals than linear NPs, indicating it could penetrate into the 3D MTSs more deeply.

Subsequently, we investigated the level of apoptosis and subsequent ICD of 4T1 cells after treatment with the linear and branched NPs. To reveal the toxic effects of these NPs, 4T1 cells were co-incubated with free Epi and the NPs. The half-maximal inhibitory concentration (IC₅₀) value of free Epi, the linear and branched NPs against 4T1 cells was 0.14 $\mu\text{g/mL}$, 0.62 $\mu\text{g/mL}$, and 0.33 $\mu\text{g/mL}$, respectively (Figure 3C). As comparison, both drug-free polymers did not show obvious toxicity toward HUVECs (Figure S13A). The flow cytometry analysis in Figure 3D and S13B indicated that the linear NPs, the branched NPs, and free Epi induced a distinct G0/G1 phase cell cycle arrest. Apoptosis of tumor cells is a prerequisite for effectively triggering ICD^[16], another key indicator of drug cytotoxicity. We evaluated the effectiveness of NPs in inducing ICD through tumor cell apoptosis. As shown in Figure S13C and S13D, the percentage for the late apoptotic cells after treatment with the branched NPs, the linear NPs, and free Epi was $28.4 \pm 0.58\%$, $21.27 \pm 1.19\%$ and $29.67 \pm 1.72\%$, respectively. To probe the mechanism of branched NPs-induced apoptosis, the expression level of PARP, caspase-3, Bcl-2 and cleaved caspase-3, was detected by the western blotting technique after different treatments. As shown in Figure 3E and S14, a significantly reduced expression level of PARP, caspase 3 and Bcl-2, but an increase in the expression level of cleaved caspase-3 was observed in 4T1 cells treated with the branched NPs. A very similar trend was also found for the linear NPs and free Epi. These results indicated that branched NPs could effectively induce a similar level of apoptosis of tumor cells as Epi. Overall, these results supported that both NPs entered cells through the endocytic pathway, released Epi after internalization to result in apoptosis and arrest the cell cycle. The apoptosis mechanism may be due to DNA damage in the nucleus by the released Epi from the linear or branched NPs^[17].

ICD, a mode of cell death that plays a critical role in anti-tumor immunity activation, is characterized with calreticulin (CRT) exposure, HMGB1 release, and adenosine triphosphate (ATP)

secretion^[18]. Thus, apart from cytotoxicity induced by the apoptotic effect of the branched NPs, we examined whether the branched NPs could ultimately trigger ICD through apoptosis. As shown in Figure 3F and S15, the percentage of CRT exposure on 4T1 cells induced by the branched NPs reached $24.53 \pm 1.80\%$, distinctively higher than that induced by the linear NPs ($10.65 \pm 2.12\%$) and that of the blank control ($3.87 \pm 0.84\%$), while it was comparable to that induced by free Epi ($22.63 \pm 2.32\%$). The intracellular and extracellular ATP concentrations were measured to confirm the induced ICD by the branched NPs. As shown in Figure 3G, the intracellular ATP in the control group was higher than that of free Epi, the branched or linear NPs treated groups. However, ATP secreted into the extracellular medium in the group treated with the branched NPs was 3.69-fold higher than that in the control group. The extracellular HMGB1 was quantified via ELISA. As shown in Figure S16A, the HMGB1 in the control group was lower than that of free Epi, the branched or linear NPs-treated groups. The concentration of released HMGB1 in branched NPs-treated group reached 1.26 ng/mL, which was significantly higher than control group (0.33 ng/mL) and linear NPs-treated group (0.65 ng/mL), and slightly higher than Epi group (1.15 ng/mL). Meanwhile, the intracellular HMGB1 expression level was detected in Figure S16B. The results showed that the HMGB1 expression level in control group was higher than that of free Epi, the branched or linear NPs-treated groups, indicating that HMGB1 was released extracellular after treated with Epi, branched and linear NPs. These results could be explained by that the branched NPs could induce apoptosis of tumor cells, resulting in effective ICD (Figure 3H).

2.3. Anti-tumor activity in a murine tumor model

PD-L1 or cytotoxic T-lymphocyte-associated protein 4 (CTLA4), predominantly targeting at PD-1, has been the mainstream therapy in recent years^[19]. However, it often fails to achieve the expected therapeutic effect because ICI has no strong immune activation effect. In this study, we combined an anti-PD-1 antibody (α -PD-1) for immune checkpoint inhibition and the polymeric conjugate for induction of ICD to synergistically boost the tumor immune response and achieve effective, potent anti-tumor therapeutic effect. α -PD-1 was combined with the branched NPs for the treatment of 4T1 cells-bearing mice and the treatment outcome was compared with the groups treated with saline, free Epi, α -PD-1, the linear NPs, the branched NPs, and the linear NPs + α -PD-1, respectively. The schematic illustration of the treatment plan was shown in Figure 3I. To assess the safety profiles of

the branched NPs, we monitored the changes in the body weight of the treated mice and excised their major organs and tumors for histopathological examination (H&E). As shown in Figure 3J, the body weight of the mice injected via tail vein with free Epi decreased significantly, while a slight change or even increased in the body weight was found in the other groups, suggesting that the incorporation of Epi in responsive polymeric carriers could reduce its side effects. The synergistic treatment with α -PD-1 and the linear or branched NPs did not have evident side effects in the treated mice. The H&E staining results shown in Figure S17 further confirmed that no significant toxicity toward normal tissues or organs was seen in each treatment group. The branched NPs combined with α -PD-1 had the best inhibition effect on subcutaneous 4T1 tumor cells compared with the other six groups. As shown in Figure 3K and 3L, compared with the control group, an inhibition rate of $23.46 \pm 0.11\%$ and $37.58 \pm 0.19\%$ in the tumor volume was seen in the groups after treatment with Epi and α -PD-1, respectively. Thus, a single therapeutic agent, Epi or α -PD-1, was not sufficiently potent to eradicate tumor cells. Treatment with the linear NPs resulted in a reduction of the tumor volume ($57.23 \pm 0.06\%$), which was lower than that after the branched NPs treatment ($75.56 \pm 0.32\%$). Treatment with the linear NPs in combination with α -PD-1 resulted in a further reduction by 10.98% in the tumor volume compared with the treatment with the linear NPs only, which could be due to the combination of ICI via α -PD-1 and the ICD effect by the linear NPs. The synergistic treatment effect was also observed after the combinational treatment by the branched NPs and α -PD-1, with an additional 17.30% reduction in the tumor volume compared with the treatment with the branched NPs only. Thus, this combinational treatment had a superior antitumor efficacy with an about 92.88% reduction in the tumor volume. The tumor tissue in each mouse of the above experimental groups was harvested and weighed, and the tumor growth inhibition (TGI) was calculated. As shown in Figure 3M and 3N, the results of the tumor weights and the corresponding TGI values further confirmed the excellent anti-tumor effect of the combinational treatment by the branched NPs and α -PD-1. Analysis of the biodistribution of the branched NPs at 24 h post-injection via in vitro fluorescence imaging suggested that the branched NPs could retain in the tumor tissue for exerting their anti-tumor function. In the group treated with the branched NPs, the fluorescence intensity of Epi in the tumor was remarkably stronger than that in the groups treated with the linear NPs or free Epi (Figure S18A). This could be ascribed to a slower clearance

rate *in vivo* of the branched NPs by glomeruli, as confirmed in Figure S18B and Table S3, because of a branched structure with a higher molecular weight.

To further study the *in vivo* anticancer mechanism of the branched NPs, TUNEL staining and HMGB1 immunohistochemical staining were performed on the tumors harvested from the above treatment groups. Figure S19 revealed a bigger apoptotic positive area (positive TUNEL staining, brown) in the group treated with the branched NPs compared with the groups treated with the linear NPs and free Epi. This may be due to a greater accumulation level of the branched NPs in tumors, which led to a higher level of tumor apoptosis and ICD. Free Epi did not effectively induce tumor apoptosis or ICD because it was easily metabolized, leading to its poor tumor accumulation. The experimental results indicated that treatment with α -PD-1 did not efficiently induce ICD. However, treatment with α -PD-1 in combination with the branched NPs enhanced the ICD effect because α -PD-1 could block the immune checkpoint on the T cell surface to improve the secretion of IFN- γ by CD8⁺ T cells [20]. The HMGB1 staining results shown in Figure S19 also confirmed that the branched NPs in combination with α -PD-1 significantly increased the expression of HMGB1. The expression level after the combinational treatment was higher than that treated with the branched NPs only, consolidating that α -PD-1 could improve ICD by enhancing the IFN- γ expression of CD8⁺ T cells. We utilized immunofluorescence staining to detect the CD3 and PD-1 expression level in tumor tissues. As shown in Figure 3O and S20, PD-1 was expressed on CD3⁺ tumor-infiltrating lymphocytes, which suggested the treatment with both the branched NPs and the ICI be a promising approach to enhancing anti-tumor immune responses. In addition, those results also showed that the branched NPs could effectively improve the immune infiltration of CD3⁺ cells. Overall, the treatment with the branched NPs combined with α -PD-1 was potent in inhibiting tumor growth in the subcutaneous animal model.

2.4. *In vivo* immunomodulatory effect

We conducted comprehensive evaluation of immune cells in the cancer tissue from the treated 4T1 tumor-bearing mice to explore the immunomodulatory effect of the synergistic chemo-immunotherapeutic strategy based on the branched NPs and α -PD-1. First, maturation and activation of dendritic cells (DCs) is a prerequisite to induce the anti-tumor immunity [21]. **Figure 4A-**

4D and S21A, S21B showed that treatment with free Epi, α -PD-1, and the linear NPs led to an increase in the expression of CD86 from $32.10 \pm 1.82\%$ in the control group to $40.00 \pm 4.15\%$, $39.14 \pm 2.70\%$ and $35.24 \pm 3.11\%$, respectively, while an increase in the MHC II expression from $32.94 \pm 1.68\%$ in the control group to $47.40 \pm 2.66\%$, $46.12 \pm 3.42\%$ and $45.44 \pm 3.11\%$, respectively. It was interesting to note that the expression of CD86 and MHC II further increased to $47.72 \pm 1.94\%$ and $52.34 \pm 2.33\%$, respectively, after treatment with the branched NPs. Encouragingly, the combinational treatment with the branched NPs and α -PD-1 resulted in an additional increase in the expression of CD86 ($49.95 \pm 3.51\%$) and MHC II ($63.38 \pm 4.23\%$). A similar trend was observed for the expression of CD86 ($40.92 \pm 4.63\%$) and MHC II ($49.98 \pm 5.10\%$) after combinational treatment with the linear NPs and α -PD-1. t-SNE analysis of DCs infiltration in tumor tissues from the mice after different treatments (Figure 4E) revealed an increase in the infiltration of CD86⁺ DCs and MHC II⁺ DCs into the tumor tissue during the treatment with the branched NPs, suggesting that the branched NPs could not only induce potent ICD of tumor cells, but also promote the infiltration and maturation of DCs.

T cells are the main cellular components that contribute to the anti-tumor immune response, and the ratio of tumor-infiltrating CD4⁺ to CD8⁺ T cells is an important indicator of the anti-tumor immunity^[22]. As shown in Figure 4F, 4G and S21C, there was no significant change in the ratio of CD8⁺ to CD4⁺ T cells in the groups treated with free Epi, α -PD-1 and the linear NPs, since these treatments could not induce sufficient ICD *in vivo* for stimulating the anti-tumor immune response. However, the anti-tumor immunity was distinctively activated due to effective ICD induction after the treatment with the branched NPs, as evidenced by an increase in the ratio of CD8⁺ to CD4⁺ T cells from 1.02 ± 0.10 folds (control) to 1.90 ± 0.15 folds. The combinational treatment with the branched NPs and α -PD-1 led to a more increase in the ratio to 3.01 ± 0.29 folds, which could be ascribed to the inhibition of the PD-1/PD-L1 axis by α -PD-1^[23]. These results suggested that the combinational treatment of the branched NPs with α -PD-1 could improve the activation effect of T cells, which could be attributed to the synergistic action of a potent ICD effect induced by the branched NPs, and an immuno-suppression weakening effect by α -PD-1.

Regulatory T (T_{reg}) cells play an essential role in inhibiting the anti-tumor immune response^[24]. Figure 4H, 4I and S21D exhibited that the percentage of Foxp 3⁺ cells relative to CD4⁺ T cells

decreased from $40.50 \pm 1.49\%$ for the control group down to $26.53 \pm 2.68\%$ for the branched NP-treated group, in comparison with $34.30 \pm 1.89\%$ for the free Epi-treated group, $32.90 \pm 0.95\%$ for the linear NP-treated group, and $33.06 \pm 2.18\%$ for the α -PD-1-treated group. This suggested that the branched NPs were most efficient in reducing the percentage of T_{reg} cells relative to the $CD4^+$ T immune cells, thus improving the immune suppression. When α -PD-1 was combined with the branched NPs, the percentage of the $Foxp3^+$ cells relative to $CD8^+$ T cells further decreased to $19.40 \pm 1.80\%$, indicating that α -PD-1 could further reduce the percentage of T_{reg} cells.

Effector T (T_{eff}) cells are the main effector cells in anti-tumor immune response. IFN- γ , the product of T_{eff} cells, is a key factor for the anti-tumor mechanism of $CD8^+$ T cells, and is often used for assessing the anti-tumor potency of $CD8^+$ cells^[25]. We assayed the percentage of IFN- γ^+ / $CD8^+$ T cells in the tumors after different treatments to reflect the vitality of T_{eff} cells. As shown in Figure 4J, 4K and S21E, the treatment with the branched NPs could boost the percentage of IFN- γ^+ cells in $CD8^+$ T cells to $8.14 \pm 0.77\%$, in comparison with those groups treated with the control ($3.54 \pm 1.09\%$), free Epi ($4.02 \pm 0.68\%$), the linear NPs ($5.13 \pm 0.70\%$) and α -PD-1 ($3.01 \pm 0.69\%$), respectively. The effective activation of the anti-tumor immune response by the branched NPs could be attributed to their inhibition of tumor metabolic competitiveness, thus enriching the nutrients in the tumor stroma and promoting the activity of $CD8^+$ T cells. Combined treatment with the branched NPs and α -PD-1 further increased the percentage of IFN- γ^+ cells in $CD8^+$ T cells to $11.47 \pm 0.74\%$, indicating that α -PD-1 could further activate T_{eff} cells.

UMAP analysis of T cells infiltration in the tumor tissues (Figure 4L) indicated that treatment with the branched NPs increased the number of $CD8^+$ T cells and $CD8^+$ /IFN- γ^+ T cells, and decreased the number of $CD4^+$ T_{reg} cells infiltrating into the tumor tissues. These results were summarized in Figure 4M, suggesting that the branched NPs in combination with α -PD-1 could improve the anti-tumor immune response, leading to a potent therapeutic effect. Compared with the other treatment groups, the branched NPs has a more significant effect on regulating immune cell subtypes. We have reported that a degradable dendronized polymeric prodrug with a branched structure effectively interfered with the metabolic processes^[8]. The change in immune cell subtypes reported herein could be induced by the interfering effect of the branched NPs on cell metabolism, which could result in a change in the constitutional compositions of the immune microenvironment. This was

consolidated through quantitative determination of the extracellular and intracellular metabolite compositions by metabolomic analysis. The effect of the nutrients in the intra-tumoral environment on the immune cell types was detailed below.

2.5. Nutrient competition between tumor cells and immune cells

The metabolic characteristics of tumor cells are essential for the growth, survival and function of immune cells. A growing number of studies have shown that abnormal tumor metabolism can affect the function of immune cells in the TME. It has been demonstrated that the level of tumor metabolism indirectly determines immune cell activity through nutrient competition^[26]. Before entering the tumor site, the immune cells in the lymphatic system have sufficient nutrition. After arriving at the tumor site, the immune cells start to compete with the tumor cells in gaining adequate nutrients for their survival and function. Given the intense metabolism of the tumor cells, the tumor-infiltrating immune cells experience nutritional deficiency, which may affect their physiological function. We hypothesized that functional differences caused by material structure could significantly reduce the nutritional competitiveness of tumor cells, leading to an increase in the concentration of metabolites in the tumor tissue, which could reshape the profile of the tumor-associated immune cells. To test the hypothesis, the intracellular and extracellular metabolites after treatment with the branched NPs were analyzed via metabolomics sequencing (**Figure 5A**). As shown in **Figure 5B**, principal component analysis (PCA) indicated significant differences in the concentrations of intracellular metabolites in the cells treated with the branched NPs compared with the blank control. These metabolites with significant differences were identified via the Wilcoxon rank sum test ($p < 0.1$) and orthogonal partial least squares discriminant analysis (OPLS-DA, $VIP \geq 1$). The intracellular metabolites with the above two indexes (VIP and p -value) in the form of Volcano plot were shown in **Figure 5C**. Compared with the control, the concentration of the majority of the metabolites decreased in the branched NPs-treated group. These intracellular metabolites were listed in **Figure 5D**, such as a large number of amino acids, energy-related metabolism intermediates, and nucleotides. Notably, the metabolism rate of essential metabolites for tumor cells was reduced, including glutamic acid, aspartic acid, histidine, tyrosine, serine, proline, arginine, alanine, tryptophan, isoleucine, leucine, threonine, and lactic acid, indicating that the branched NPs significantly inhibited the metabolism level of tumor cells. As comparison, **Figure S22** displayed that

treatment with the linear NPs and free Epi did not show a significant impact on the metabolism rate of tumor cells, suggesting that only the branched NPs could contribute to the interfering effect on the intracellular metabolism of tumor cells, which was evidenced by distinct changes in the metabolites in the metabolic processes by the branched NPs (Figure 5E), including alanine, aspartate and glutamate metabolism, butanoate metabolism, glycine, serine and threonine metabolism, arginine and proline metabolism, and other metabolic pathways obtained from the enrichment analysis.

The same metabolomics sequencing method (Figure 5A) was applied for analysis of the extracellular metabolites and the results were shown in Figure 5F-5H and S23-S28. It was noted that main nutrients for tumor cells including sugars, amino acids and nucleotides, including cystine, lysine, glutamine, serine, threonine, glucose, glycine, leucine, methionine, phenylalanine, isoleucine, asparagine, and tyrosine were rich in the extracellular environment. However, extracellular immunosuppressive metabolites of tumor cells, such as kynurenine and methyl adenosine, decreased significantly, indicating that the branched NPs could inhibit the metabolic consumption of essential nutrients by the tumor cells, thus reducing the production of the immunosuppressive metabolites in the extracellular environment. Subsequently, the contents of glucose and lactic acid in the supernatant were determined, and the results shown in Figure S29A and S29B were in agreement with the aforementioned metabolomics sequencing results. The concentration of lactic acid in the supernatant of tumor cells treated with the branched NPs was lower than that in the other groups, while the glucose concentration was higher. Among these extracellular metabolites, glucose and glutamine, main sources of carbon and nitrogen for cells, were rich, while the concentration of lactic acid, a critical immunosuppressive metabolite, decreased, suggesting that the branched NPs could inhibit the intracellular metabolism, thereby restraining the generation of extracellular immunosuppressive by-products.

From the intracellular and extracellular metabolite analysis, the branched NPs could inhibit the metabolic competitiveness of tumor cells, reduce the consumption of nutrients and production of lactic acid in the extracellular microenvironment, and improve their immuno-supportive pathway. The changes in the metabolism of tumor cells after treatment with the branched NPs could be due to impairment of amino acid and lactic acid transporters by the branched NPs. To confirm this, those

transporters, which are highly expressed by breast cancer cells, including SLC7A11 for cystine and glutamate ^[27], SLC6A14 for neutral and basic amino acids ^[28], and MCT for lactic acid ^[29], were detected by western blotting. As shown in Figure 5I and S30, a significant reduction in the expression level of SLC7A11, SLC6A14 and MCT was observed in the 4T1 cells after co-incubation with the branched NPs, the linear NPs and free Epi, respectively, while treatment with the branched NPs resulted in the most significant inhibitory effect on SLC7A11 and MCT expression, indicating that the branched NPs could effectively restrain the transportation of amino acids from an extracellular environment into tumor cells for their growth, maintenance and migration, and reduce the transportation of lactic acid from inside the tumor cells to the extracellular environment. Abnormal activation of signaling networks such as the phosphatidylinositol 3-kinase (PI3K)-AKT signaling network regulates the expression of key components in metabolic pathways through direct regulation of nutrient transporters and metabolic enzymes or control of transcription factors ^[30]. We detected the expression levels of two key proteins, Akt and pAkt. As shown in Figure 5I and S31, a similar expression level of Akt and pAkt was detected after PBS treatment (control) and the linear NPs, respectively, whilst a significantly lower expression level of Akt and pAkt in the cells treated with the branched NPs and free Epi. These results supported that the branched NPs could effectively inhibit the tumor energy metabolism and uptake of essential nutrients, resulting in an increase in the concentration of extracellular nutrients and a decrease in the level of immunosuppressive metabolites in the extracellular environment.

2.6. Effect of immune competition on immune cell activity and subtypes

Extracellular metabolites have been reported to have a significant effect on immune cell function ^[31], suggesting that the metabolic competitiveness of tumor cells inhibited by the branched NPs could have an impact on the activity and populations of tumor immune cells. The maturation and activation of DCs are essential for inducing the anti-tumor immunity ^[32]. The effect of ICD on DCs after treatment with the branched NPs was examined. As shown in **Figure 6A**, after co-incubation of DCs with tumor cells treated with the branched NPs, the linear NPs and free Epi, the CD80 and CD86 expression levels on the DCs surface were detected by flow cytometry. Figure 6B and 6C demonstrated that the treatment of tumor cells with the branched NPs could considerably up-regulated the expression level of CD80 and CD86, two co-stimulatory molecules, on the DCs surface,

while there was a slight increase in the expression level of CD80 and CD86 after the treatment with free Epi or the linear NPs. These results suggested that treatment of 4T1 tumor cells with the branched NPs could induce DCs maturation by promoting ICD. The role of glucose, a major nutrient, in the maturation process of DCs was also explored. Lipopolysaccharide (LPS), an immune-stimulatory ligand, was used to stimulate DCs. The LPS-stimulated DCs were cultured with glucose at different concentrations. The results in Figure 6D and 6E revealed that the expression level of CD80 and CD86 was positively correlated with the glucose concentration in the extracellular environment of DCs, indicating that the glucose concentration in the TME may play a role in the maturation of DCs. These results suggested that the intervention with the branched NPs could decrease the metabolic competitiveness of tumor cells, leading to an increase in the concentration of amino acids, sugars and other nutrients in the tumor microenvironment and the consequent facilitation of the anti-tumor immune response.

Although DCs maturation is typically used as an indicator of immune response, a single type of the immune cell may not be representative of the complex immune environment in the body^[33]. Splenocytes, consisting of myeloid as well as lymphoid cells, were used to mimic the complex immune microenvironment inside the tumor. As shown in Figure 6F, we co-incubated splenocytes with tumor cells treated with the branched NPs, and detected the CD80 and CD86 expression levels on the DCs surface, and CD3, CD8, CD4, IFN- γ , Foxp 3 in the T cells by flow cytometry. As shown in Figure 6G-6J, similarly to the effect of the branched NPs on the isolated DCs, the CD80 and CD86 expression levels on the surface of DCs in a mixture of immune cells were up-regulated, while the expression of IFN- γ in the CD8⁺ T cells was up-regulated and the expression of Foxp 3⁺ in the CD4⁺ T cells down-regulated. These results indicated that the branched NPs could enhance the anti-tumor immune response in the presence of a variety of immune cells. The effect of nutrients, including glucose, glutamine, amino acids, and lactic acid, at different concentration gradients on the immune cells activity and their populations was investigated by flow cytometry after incubation of splenocytes with these nutrients (Figure 6K). As shown in Figure 6L and S32-S34, the concentrations of glucose, glutamine, amino acid and lactic acid were positively correlated with the degree of DC maturation, and glucose had the most significant effect on DC maturation. The Foxp 3⁺ expression in CD4⁺ T cells were up-regulated in the presence of lactic acid, but its expression level was very similar

after exposure to glucose, glutamine and amino acids. The expression of IFN- γ in the CD8⁺ T cells was positively influenced in the presence of glucose, glutamine, amino acids, and lactic acid. Amino acids played a predominant role in inducing IFN- γ expression. These results suggested that the branched NPs could regulate the immune cell populations due to rich extracellular nutrients in the tumor microenvironment after they inhibited the metabolic competitiveness of tumor cells.

Overall, we elucidated the mechanisms of attenuating the metabolic competition of tumor cells by the branched NPs and creating a favorable nutritional microenvironment for infiltrating immune cells. First, the branched NPs accumulated in the tumor tissue. Epi was released from the NPs to induce apoptosis, which triggered intense ICD and promoted the maturation of DCs. Second, the branched NPs attenuated the metabolic competitiveness of tumor cells, evidenced by the impaired metabolic activity and a reduced level of transporters expression in tumor cells. Consequentially, amino acids, glutamine, glucose and other nutrients in the tumor microenvironment were enriched, which could be essential for favoring the immune cell populations to enhancing the immune response. Third, treatment of the branched NPs could lead to a decrease in the immunosuppressive metabolite, lactic acid, which helped reducing the population of T_{reg} cells. Finally, the combination of an ICI with the branched NPs could enhance ICD of tumor cells, further reducing the metabolic consumption of nutrients by tumor cells in the tumor immune microenvironment and improving the chemotherapy-immunotherapy outcomes.

3. Conclusion

In summary, we established a novel treatment regimen of tumor metabolism inhibition combined with ICI based on a new perspective of nutrient competition between tumor cells and immune cells. We have successfully designed and synthesized a stimuli-responsive polymeric conjugate and the nanoparticles formed from the polymeric conjugate could effectively inhibit the metabolic activity of tumor cells and reduce their nutritional competitiveness, resulting in an increase in the nutrients in the TME. This metabolic inhibitory function may be attributed to the attenuated expression of Akt and pAkt, as well as amino acid transporters by branched NPs. Meanwhile, the drug released from the NPs could induce ICD of tumor cells, which in turn triggered an antitumor immune response and recruited various immune cells into the TME. These infiltrating immune cells could be proliferative

with sufficient supply of nutrients in the TME and their populations could also be regulated to create a favorable anti-tumor immunity microenvironment. Treatment with an ICI in combination with the branched NPs could diminish the inhibitory effect of tumor cells on the anti-tumor immune response and significantly inhibited tumor growth. In summary, our results supported successful application of a branched polymeric conjugate could attenuate the nutritional competition between tumor cells and immune cells to form a potent anti-tumor immune microenvironment. Our work presents a promising platform for chemo-immunotherapeutic treatment of cancers.

4. Experimental Section

Materials, methods, preparation and characterization of the prodrug conjugates, imaging studies, *in vitro* and *in vivo* anticancer studies and their relative anticancer mechanism were provided in Supporting Information. The 4T1 cell line and HUVEC cell line were cultured in RPMI 1640 medium (Gibico) containing 10% (volume percentage) fetal bovine serum (Gibico), 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin (Hyclone), and they were kept in a humidified chamber supplemented with 5% CO₂ under 37 °C. 6-8 weeks female BALB/c mice weighing 18.0-22.0 g used in this study were purchased from Chengdu DaShuo Biological Technology Co., Ltd. All animal experiments were conducted in accordance with Chinese national regulations, and approved by the Animal Experiment Ethics Committee of the West China Hospital of Sichuan University (Approval No. 2018148A and 2018150A).

Data were shown as mean $\pm$ SD. Statistical significance was analyzed by calculating variances for multivariate groups or performing two-tailed Student's t-tests for paired groups. Significant difference was defined as a *p*-value less than 0.05.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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Captions of Figures

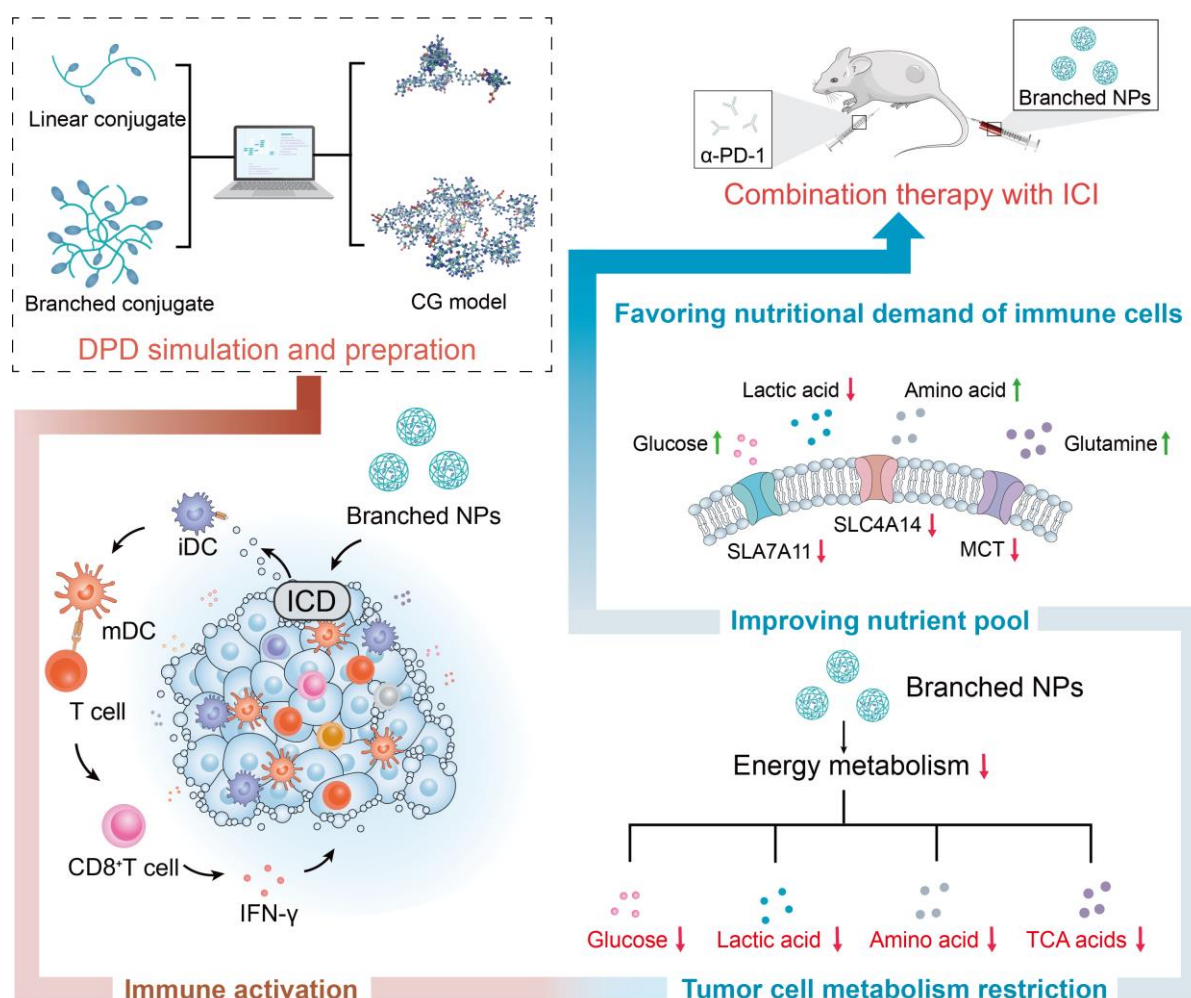
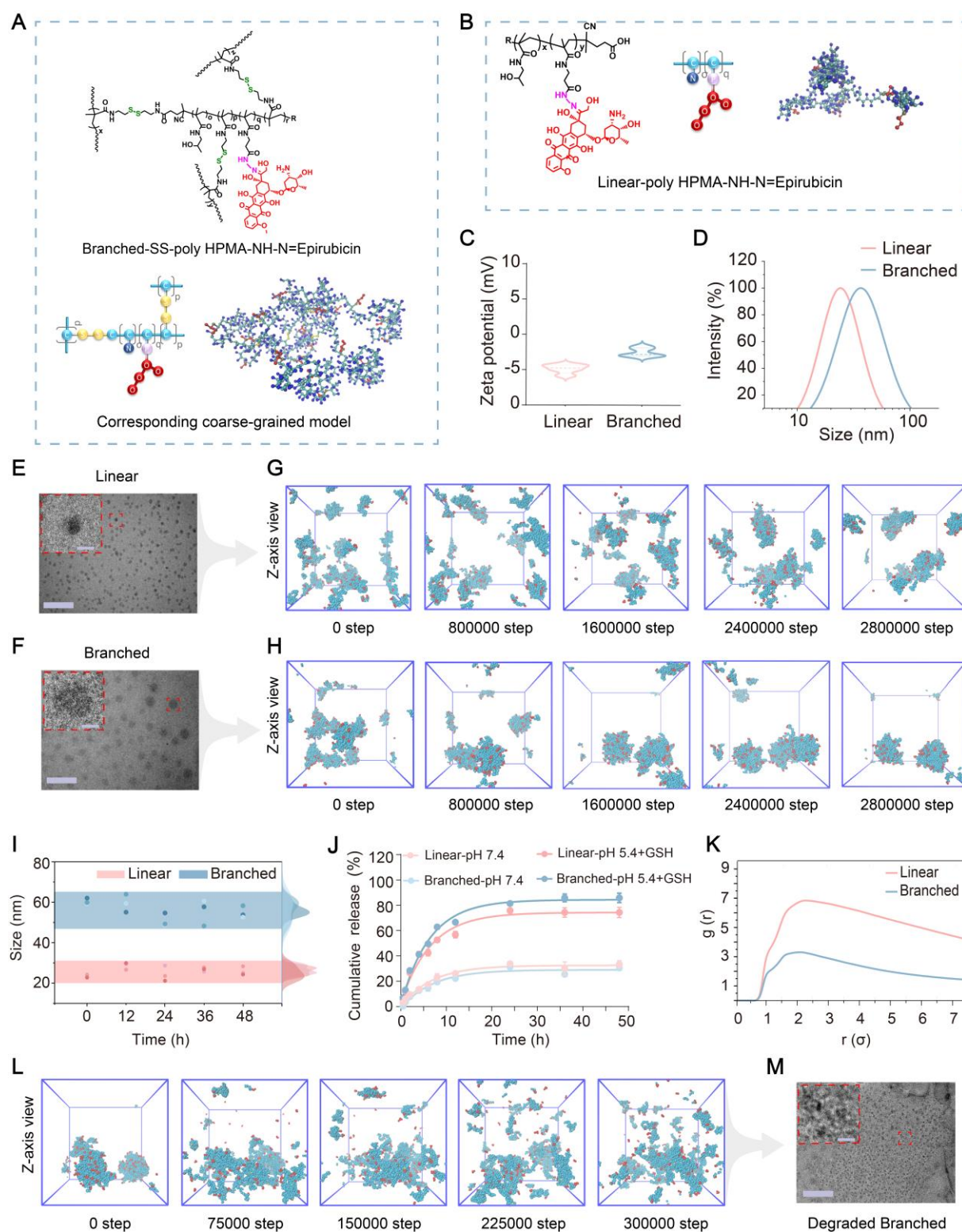


Figure 1. Schematic illustration of a proposed mechanism of the branched polymer-based NPs to attenuate metabolic competition of tumor cells for favoring immune system nutritional demand, resulting in an enhanced chemo-immunotherapeutic effect. Chemical structural formula and the CG models of the branched polymeric prodrug conjugate were used for DPD simulations. The branched polymer-based NPs could induce ICD of tumor cells and subsequent activating antitumor immune response, including DC maturation, an increase in CD8⁺ cells and enhanced IFN- γ secretion. Next, the branched NPs could simultaneously impair the metabolic competitiveness of tumor cells, thus increasing the concentration of extracellular nutrients for favoring metabolic demand of immune cells. Eventually, the metabolically remodeled tumor microenvironment could enhance the immune

response and weaken the immunosuppression to achieve an enhanced therapeutic effect for combinational chemotherapy and immune checkpoint therapy.



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Figure 2. Chemical structures, in silico computational results and physicochemical characterizations of polymeric prodrug conjugates and their NPs. (A and B) Chemical structural formula and the CG models of branched (A) and linear (B) polymeric prodrug conjugates. (C and D) The zeta-potential (C) and hydrodynamic size (D) of the linear and branched NPs in an aqueous solution. (E and F) TEM images of the nanostructure formed from the linear (E) and branched (F) polymeric prodrug conjugates (scale bar: 200 nm, and 20 nm for the inset image). (G and H) Temporal self-aggregation behavior of the linear (G) and branched (H) polymeric prodrug conjugates in the Z-axis view. (I) Changes in the size of the linear and branched NPs incubated in pH = 7.4 PBS for two days. (J) *In vitro* drug release curves from the linear and branched polymeric prodrug conjugates under different mimicking release conditions, including a pH 7.4 neutral environment and a pH 5.4 acidic environment containing GSH. (K) Radial distribution functions (RDFs) of the interaction between the HPMA polymer backbone and the drug Epi in an equilibrium state during disassembly and drug release from DPD simulations. Differences in the RDF values between the branched and linear polymeric prodrugs indicated more drugs could be released from the branched structure. (L) Temporal disassembly and drug release behavior of the nanostructure formed from the branched polymeric prodrug conjugates in the Z-axis view. (M) TEM image of the degraded products from the branched NPs (scale bar: 200 nm and 20 nm for the inset image).

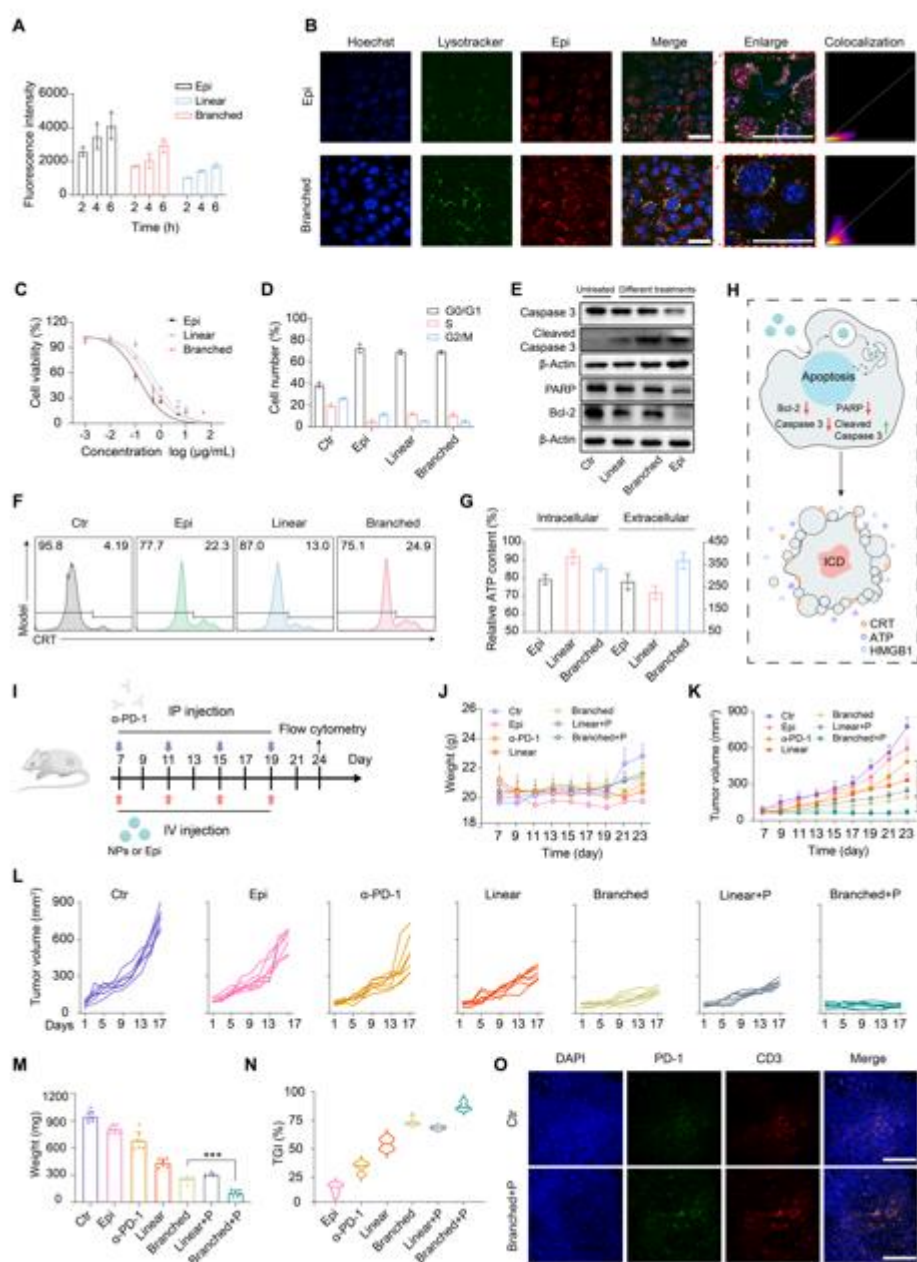


Figure 3. Anti-tumor effect of the branched NPs and their combinational therapeutic effect with α -PD-1 *in vivo*. (A) The quantitative level of cellular uptake of Epi, the linear and branched NPs at different time-points. (B) Representative CLSM images of cellular internalization and intracellular distribution of the branched NPs and free Epi in 4T1 cells (scale bar = 100 μ m). (C) The cytotoxicity of

Epi, the linear and branched NPs against 4T1 cells. **(D)** Quantitative analysis of the 4T1 cell cycle at different phases after treatment with Epi, the linear and branched NPs. **(E)** The expression of key proteins for apoptosis in 4T1 cells, including Bcl-2, PARP, caspase 3 and cleaved caspase 3, after treatment with Epi, the linear and branched NPs. **(F)** Representative flow cytometric images of CRT exposure on the 4T1 cell surface after treatment with Epi, the linear and branched NPs. **(G)** Intracellular and extracellular ATP levels in 4T1 cells after treatment with Epi, the linear and branched NPs. **(H)** Schematic diagram of ICD of tumor cells induced by the polymeric prodrug conjugates. **(I)** Schematic diagram of experimental plans for *in vivo* study. **(J)** The body weight changes of treated mice during the treatment period. **(K)** Changes in the tumor volume in each group, including PBS (Ctr), Epi (Epi), anti-PD-1 antibody (α -PD-1), the linear NPs (linear), the branched NPs (branched), the linear NPs with α -PD-1 (linear + P), and the branched NPs with α -PD-1 (branched + P). **(L)** Changes in the tumor size of every mouse in each group after different treatments. **(M)** The average tumor weight of the harvested tumors in each group. **(N)** The TGI value of each mice group. **(O)** Representative immunofluorescence images of CD3 and PD-1 in the tumor from mice treated with the branched NPs and the control (scale bar = 200 μ m). Data were presented as mean $\pm$ SD ($n = 7$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

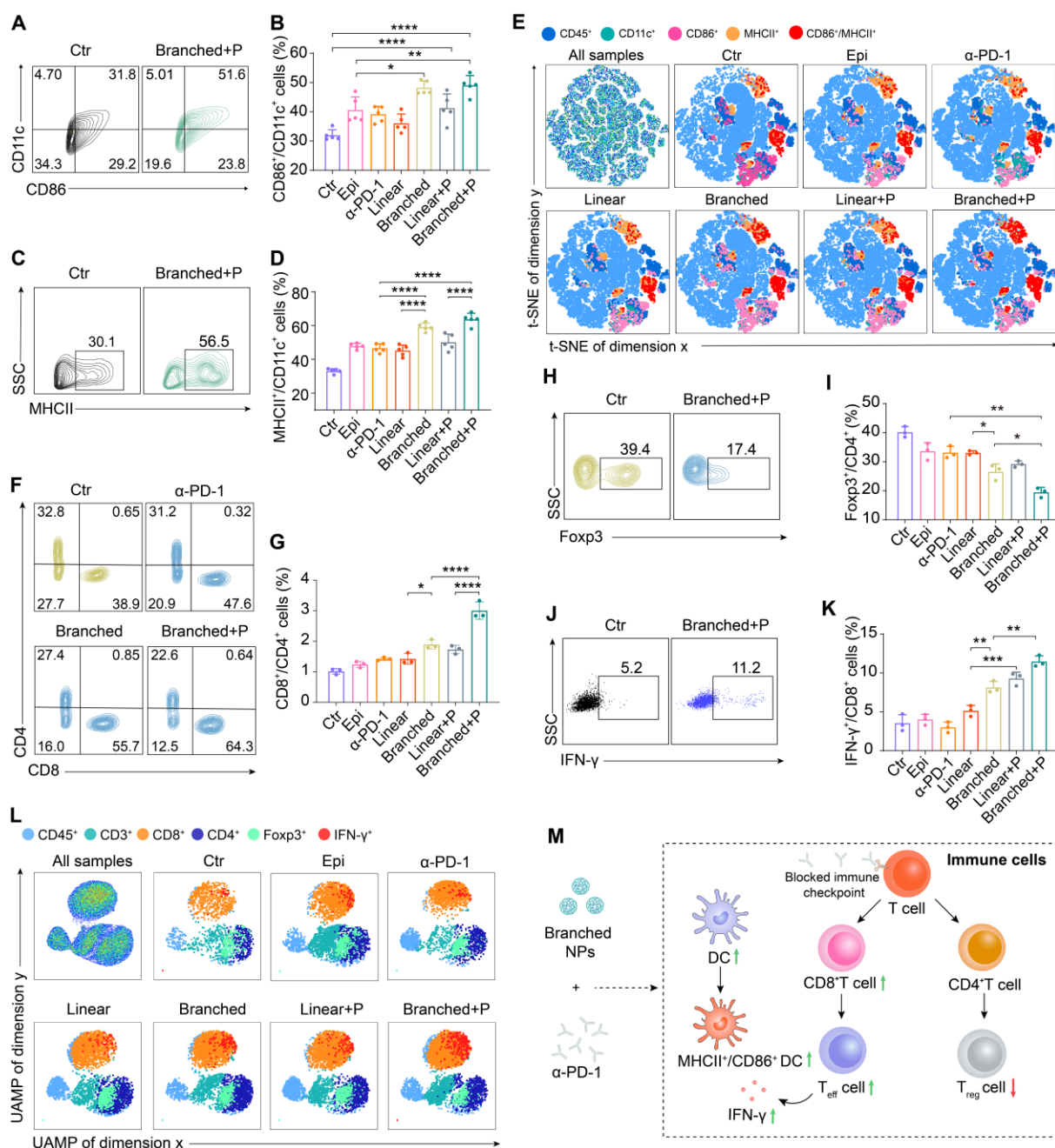


Figure 4. Combinational therapeutic effect of the branched NPs with α-PD-1 for an improved tumor immune microenvironment in the mice. (A and B) Representative flow cytometric images (A) and quantitative data (B) of CD86⁺ DCs in CD11c⁺ cells. **(C and D)** Representative flow cytometric images (C) and quantitative data (D) of MHC II DCs in CD11c⁺ cells. **(E)** tSNE visualization of the

expression of CD45, CD11c, CD86 and MHC II of lymphocytes. **(F)** Representative flow cytometric images of CD4⁺ and CD8⁺ T cells in CD3⁺ cells. **(G)** The corresponding quantitative ratios of CD4⁺/CD8⁺. **(H and I)** Representative flow cytometric images (H) and quantitative data (I) of IFN- γ ⁺ cells in CD4⁺ T cells. **(J and K)** Representative flow cytometric images (J) and quantitative data (K) of Foxp 3⁺ cells in CD8⁺ T cells. **(L)** UMAP visualization of the expression of CD45, CD3, CD4, CD8, IFN- γ and Foxp 3 of lymphocytes. **(M)** Schematic illustration of the combinational therapeutic effect of the branched NPs with α -PD-1 for improving the tumor immune microenvironment in the mice. All the detected immune cells were from tumors in mice after treatment with PBS (Ctr), Epi (Epi), anti-PD-1 antibody (α -PD-1), the linear NPs (linear), the branched NPs (branched), the linear NPs with α -PD-1 (linear + P), and the branched NPs with α -PD-1 (branched + P). Data were presented as mean $\pm$ SD (n = 3, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

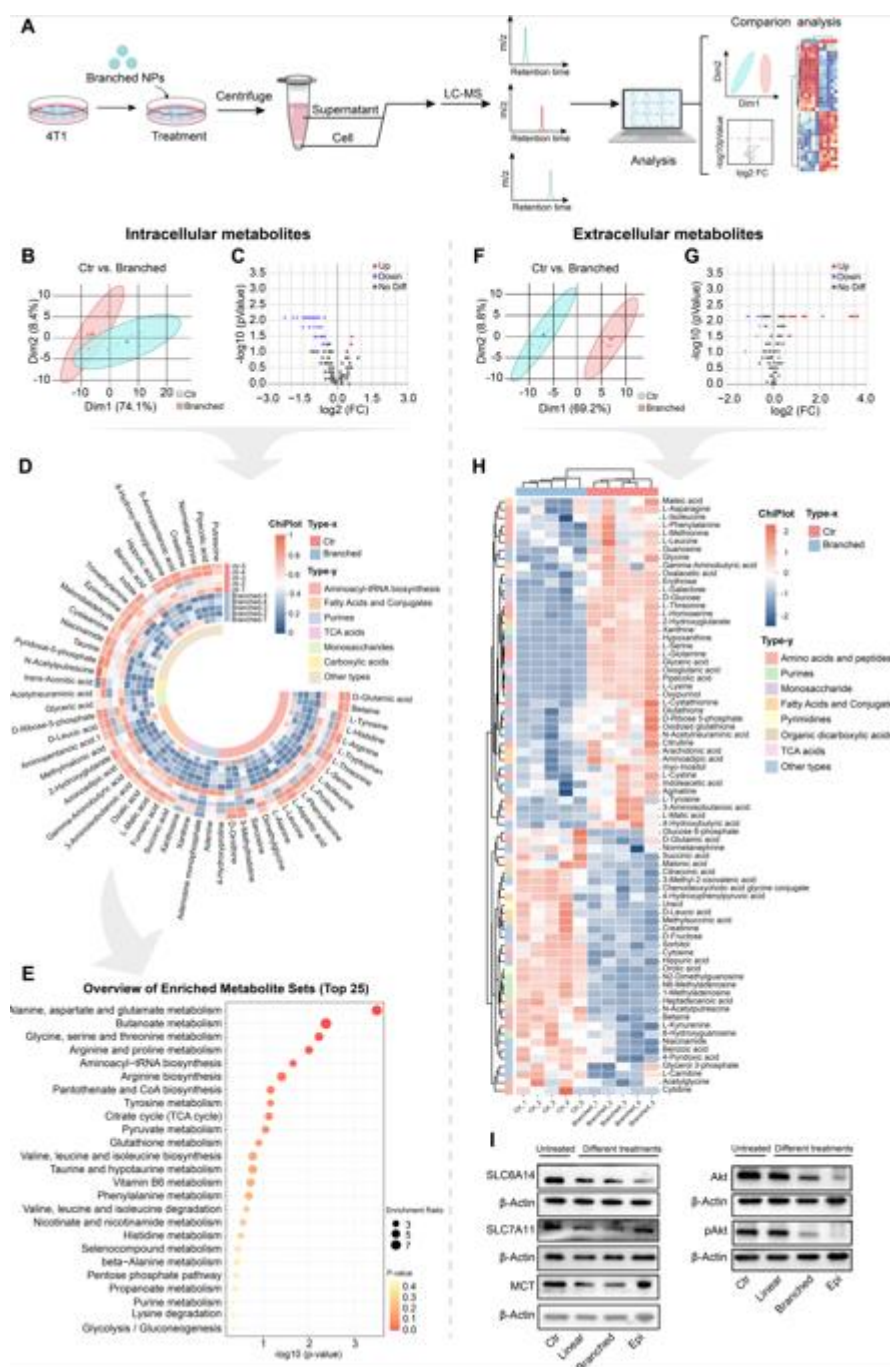


Figure 5. Weakening the metabolic competitiveness of tumor cells via the branched NPs. (A) Schematic diagram of experimental procedures for metabolomic analysis. **(B)** Principal component

analysis (PCA) of the normalized metabolomic data within 4T1 cells treated with the branched NPs versus the control (PBS). **(C)** Volcano plot of differentially abundant metabolites in 4T1 cells treated with the branched NPs versus the control. **(D)** Heatmaps for z-scores of differential metabolites in the 4T1 cells. **(E)** Enriched pathways in the samples treated with the branched NPs via the KEGG pathway enrichment analysis. **(F)** PCA of the normalized data for the metabolites in the extracellular medium after treatment with the branched NPs versus the control (PBS). **(G)** Volcano plot of differentially abundant metabolites in the cell supernatant after treatment with the branched NPs versus the control. **(H)** Heatmaps for z-scores of differential metabolites within extracellular medium after treatment with the branched NPs versus the control (PBS). **(I)** Expression of AKT, pAKT, SLC7A11, SLC6A14, and MCT detected by Western blots after treatment with Epi, the branched and linear NPs.

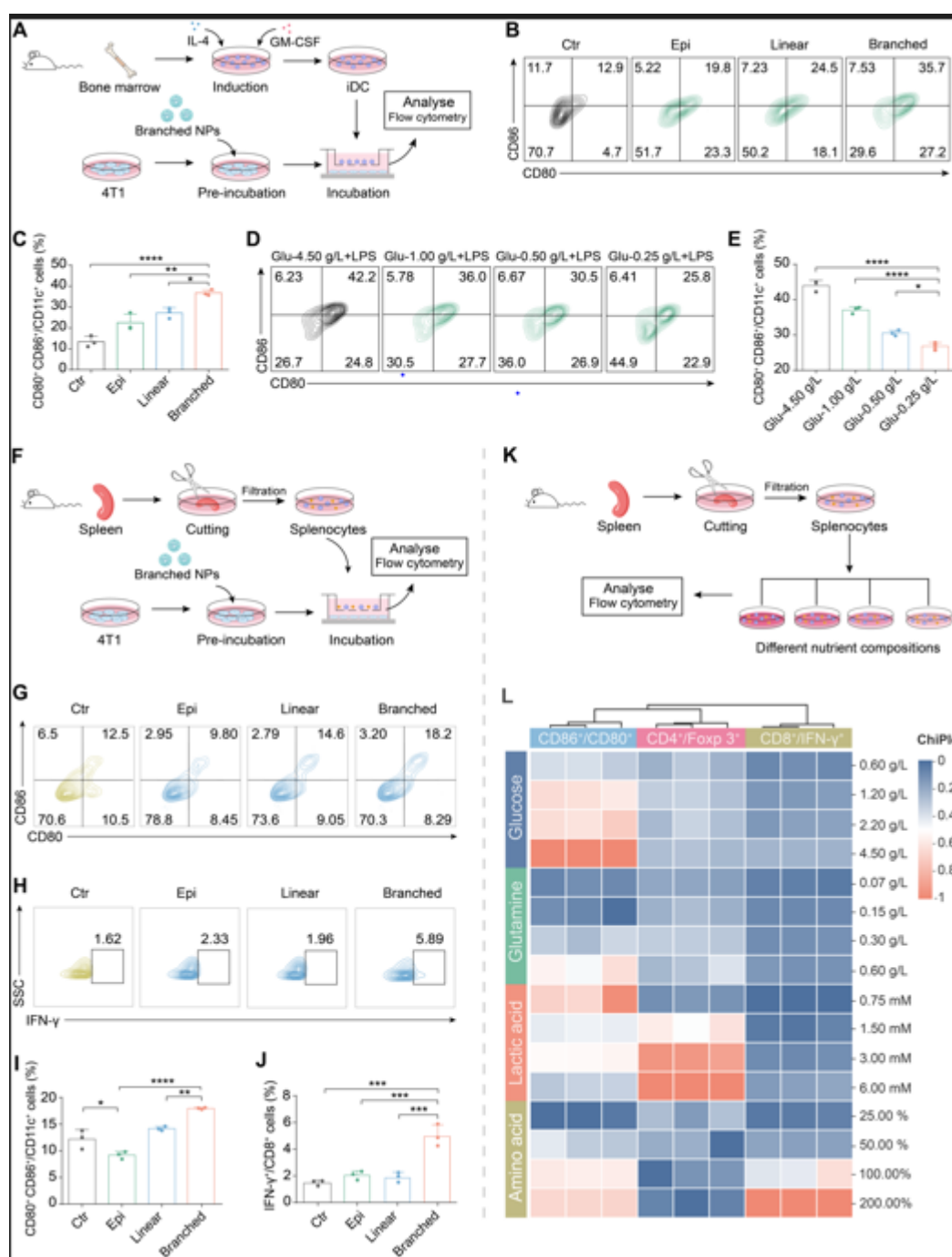


Figure 6. Effects of the branched NPs on immune cells by remodeling the nutrient environment.

(A) Schematic diagram for co-culture of DCs and pretreated 4T1 cells with different formulations. (B) Representative flow cytometry results of mature DCs after co-incubation with 4T1 cells pretreated with the control (PBS), Epi, the linear and branched NPs. (C) Quantitative data for the proportion of CD86⁺ CD80⁺ cells to CD11c⁺ cells in DCs after co-culture with pretreated 4T1 cells with different formulations. (D) Representative flow cytometry analysis of CD86⁺ CD80⁺ DCs stimulated by LPS after culture with glucose at different concentrations. (E) Quantitative data for the proportion of CD86⁺ CD80⁺ cells to CD11c⁺ cells in DCs after co-culture with 4T1 cells at different glucose concentrations. (F) Schematic diagram for co-culture of splenocytes and pretreated 4T1 cells with different formulations. (G) Representative flow cytometry results of CD86⁺ CD80⁺ DCs after co-incubating with 4T1 cells pretreated with the control (PBS), Epi, the linear and branched NPs. (H) Representative flow cytometry results of IFN- γ ⁺ cells in CD8⁺ T cells after co-culture with 4T1 cells with the control (PBS), Epi, the linear and branched NPs. (I) Quantitative data of the proportion of CD86⁺ CD80⁺ cells to CD11c⁺ cells in splenocytes after co-culture with pretreated 4T1 cells with different formulations. (J) Quantitative data of the proportion of IFN- γ ⁺ cells in CD8⁺ T cells to CD11c⁺ cells in splenocytes after co-culture with pretreated 4T1 cells with different formulations. (K) Schematic diagram of the experimental design of incubating splenocytes with nutrients at different concentrations. (L) Heat map of CD86⁺/CD80⁺, Foxp 3⁺/CD4⁺, and IFN- γ ⁺/CD8⁺ in splenocytes incubated with glucose, glutamine, lactic acid, and amino acids at different concentrations. Data were presented as mean $\pm$ SD (n = 3, * p < 0.05, ** p < 0.01, **** p < 0.0001).

The table of content

A branched polymeric drug delivery system can be used as a previously unknown metabolic modulator to improve the tumor immune microenvironment via interfere with the metabolic processes of tumor cells to increase the concentration of essential nutrients and reduce the level of immunosuppressive metabolites in the TME, thus creating a favorable environment for infiltrated immune cells.

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Attenuating Metabolic Competition of Tumor Cells for Favoring the Nutritional Demand of Immune Cells by a Branched Polymeric Drug Delivery System

ToC figure

