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Nanodrug enhances post-ablation immunotherapy of hepatocellular carcinoma via promoting dendritic cell maturation and antigen presentation

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ABSTRACT

Thermal ablation (TA) as an effective method treating hepatocellular carcinoma (HCC) in clinics is facing great challenges of high recurrence and metastasis. Although immune-checkpoint blockade (ICB)-based immuno-therapy has shown potential to inhibit recurrence and metastasis, the combination strategy of ICB and thermal ablation has shown little progress in HCC treatments. The tremendous hurdle for combining ICB with thermal ablation lies with the insufficient antigen internalization and immaturity of tumor-infiltrating dendritic cells (TIDCs) which leads to an inferior immune response to distant tumor growth and metastasis. Herein, an antigen-capturing nanoplatform, whose surface was modified with mannose as a targeting ligand, was constructed for co-delivering tumor-associated antigens (TAAs) and m6A demethylases inhibitor (*i.e.*, fat mass and obesity associated gene (FTO) inhibitor) into TIDCs. *In vivo* results demonstrate that the intratumoral injection of nanodrug followed by HCC thermal ablation promotes dendritic cells (DCs) maturation, improves tumor infiltration of effector T cells and generates immune memory, which synergize with ICB treatment to inhibit the distant tumor growth and lung metastasis. Therefore, the antigen-capturing and FTO-inhibiting nanodrug holds potential to boost the ICB-based immunotherapy against HCC after thermal ablation.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed malignancy ranking the fourth leading cause of cancerrelated deaths [1,2]. Although thermal ablation (TA) with a great advantage of minimal invasiveness has been widely used for HCC treatment nowadays, most HCC patients eventually die of tumor metastasis and recurrence after ablation [3–5]. It is well known that thermal ablation can lead to the release of tumor-associated antigens (TAAs) activating antitumor immunological responses, with which the immune checkpoint blockade (ICB) starts to unleash the brake of T cell response for tumor cell killing [6–10]. Hence, the combination of thermal ablation and ICB therapy may be a promising strategy for HCC treatment [11,12]. Unfortunately, as reported in a previous preclinical study, non-effective tumor inhibition can be expected when applying ICB after ablation because the immune responses arising from thermal ablations are too weak to induce an efficient inhibition of tumor metastasis and recurrence [13,14].

The activation and maturation of dendritic cells (DCs) are pivotal for activating antitumor effector T cells to induce a strong immune response

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in ICB-based immunotherapy [15–18]. Typically, after thermal ablation, TAAs are released from apoptotic tumor tissue and then captured by DCs to induce the activation and maturation of DCs [7,19]. However, only a small amount of TAAs can be recognized and processed by DCs for presentation to T cells, which eventually leads to inferior immune response [13,20–22]. Studies have found that tumor antigen-captured nanoparticles could improve the efficiency of antigen uptake and the capability of DCs to generate antitumor response [23,24]. Nevertheless, the tumor-infiltrating dendritic cells (TIDCs) usually exhibit an immunosuppressive phenotype, which contributes to the failure of antitumor activity even when tumor antigens were engulfed [25–27]. Effectively activating TIDCs is critical for improving the antitumor immune responses of ICB after tumor thermal ablation.

On the other hand, the regulation of N6-methyladenosine (m6A) has become an emerging focus of cancer research [28,29]. The mechanism involves that the "writer" or methyltransferases (*i.e.*, METTL3, METTL14 and WTAP) catalyzes m6A modification of RNA, while the "erasers" or demethylases, including FTO and ALKBH5, are responsible for removing the methyl group [30–32]. The latest research showed that the abnormality of m6A mRNA was found in tumor-infiltrating immune cells such as TIDCs, which powerfully aggravated tumor immunosuppression and immune escape [33]. For instance, it was reported that m6A mRNA levels in mature DCs were higher than those in immature DCs, and down-regulation of m6A methylation levels *via* knocking-out "writer" METTL3 could inhibit the activation and maturation of DCs [34,35]. Although some studies have investigated the interaction between m6A methylation and DCs function, few have examined the role of the "eraser" FTO enzyme in innate immunity. Since the inhibition of the FTO enzyme can up-regulate m6A methylation, we assume that the FTO inhibitor would promote TIDCs activation and maturation following antigen uptake.

In the present study, we prepared an antigen-capturing nanodrug for co-delivering the TAAs and a FTO inhibitor into TIDCs, in a hope that the nanodrug could synergize with the ICB therapy to better inhibit the HCC metastasis and recurrence after thermal ablation (Fig. 1). FTO inhibitor (FB23-2) was encapsulated in the pores of mesoporous polydopamine (MPDA) nanoparticle, and maleimide (mal) as an antigen-capturing agent and mannose as active DCs-targeting ligand were anchored on the surface of MPDA through the poly(ethylene glycol) (PEG) linker. Hopefully, nanodrug intratumorally injected into tumor could capture TAAs released upon tumor thermal ablation through reacting with the mal groups and then was endocytosed by TIDCs through mannosemediated targeting effect. TAAs and FTO inhibitor co-delivered to TIDCs was expected to trigger a significant antitumor immune response by increasing m6A methylation, promoting DC maturation and activating antitumor immunity after HCC thermal ablation, thereby assisting ICB to inhibit distant tumor growth and metastasis.



Fig. 1. Schematic illustration of the preparation of tumor antigen-capture nanoplatforms (Man/mal-MPDA@FB23-2 (M/m-MP@F)) and their highly efficient activation of tumor-infiltrating dendritic cells (TIDCs) via increasing m6A abundance in mRNA of TIDCs.



Fig. 2. Characterization and antigen capture efficiency of M/m-MP@F. a) The schematic and transmission electron microscopy (TEM) images of nanodrugs, scale bar, 200 nm. The particle sizes (b) and zata potentials (c) of nanodrugs. d) *In vitro* FB23-2 release from M/OVA/FITC-m-MP@F (n = 3, means \pm SD). e) Hepa1-6 cells were heated using a microwave antenna, and the temperature was monitored real-time and was maintained at 60 °C for 2 min. Then, M/m-MP@F and M-MP@F were added and incubated for 10 min, after the cells were removed, the nanodrug was collected. The contents of HMGB1 and HSP60 captured by nanoparticle were determined by ELISA kits (n = 4; means \pm SD). Cellular uptake of M/OVA/FITC-m-MP and OVA/FITC-m-MP in DC2.4 cells determined by CLSM (f) and flow cytometry (g). Green fluorescence indicates OVA/FITC, and cell nuclei are labeled with Hoechst 33342 (blue fluorescence). h) Lysosomal escape of OVA-FITC after incubation with M/OVA/FITC-m-MP for 1 h and 2 h in DC2.4 cells. Red fluorescence indicates lysosome, green fluorescence indicates OVA/FITC, and cell nuclei are labeled with Hoechst 33342 (blue fluorescence).

2. Results and discussions

2.1. Characteristics and antigen capture efficiency of nanodrugs

Mesoporous particulate MPDA (MP) was prepared according to our previous study [36], and loaded the FTO inhibitor (FB23-2) in the pores, which was named MP@F (Fig. S1). Then, amidation reaction between the catechol groups of MP and mal-PEG-NH₂/Mannose-PEG-NH₂ was carried out, which introduced the antigen-capturing maleimide (mal) and DCs-targeting mannose to the surface of nanoparticles (M/m-MP@F). As shown in Fig. S2, nanoparticle without PEG modification (MP@F) precipitated after storing for 24 h. In contrast, while nanodrug modified with Mannose-PEG-NH2 and mal-PEG-NH2 (M/m-MP@F) was fairly stable over storing time, which indicated that the PEG coating significantly increased the stability of the sample in aqueous solution. In its UV-visible spectrum, M/m-MP@F showed an absorbance peak of FB23-2 at 301 nm, indicating that the FTO inhibitor was successfully encapsulated into nanoparticle (Fig. S3). Transmission electron microscopy (TEM) observation revealed the spherical morphology and mesoporous structure of MP@F (Fig. 2a). After loading FB23-2 into the pores and decorating the surface with Mannose-PEG-NH₂ & mal-PEG-NH₂, the mesoporous structure of MP (M/m-MP@F) turned somewhat imperceptible, which might be attributed to the existence of polymer layer on the surface of MP after PEG modification [37]. To test whether M/m-MP@F could capture antigen, M/m-MP@F was incubated for 30 min with ovalbumin (OVA) as a replacement for tumor antigen. As shown in Fig. 2a, the mesoporous structure disappeared completely, indicating OVA binding to the surface of M/OVA-m-MP@F. The particle sizes and zeta potentials of nanoparticles were detected by dynamic light scattering (DLS) measurements. As shown in Fig. 2b, both the MP@F and M/m-MP@F showed particle sizes around 247 nm. After OVA binding, the particle size increased to 288 nm. Moreover, the OVA binding decreased the zeta potential of nanodrug to -29 mV from -11 mV (Fig. 2c).

Then, the drug release profiles of the M/OVA-m-MP@F nanoplatform were investigated *in vitro* at pH 7.4 and pH 5.0 for normal physiological environment and lysosomal environment, respectively. As shown in Fig. 2d, at pH 7.4, the FB23-2 release was very slow, showing a cumulative release of only 3.4% over 12 h or 5.3% over 48 h. However, at pH 5.0, the FB23-2 release was obviously much faster, showing 31.4% release over 12 h or 45.1% release over 48 h.

The ability of M/m-MP@F to capture the protein from tumor cell lysate was evaluated in vitro. After M/m-MP@F was incubated for 30 min in aqueous solution added with Hepa1-6 cell lysates obtained from thermal ablation, the nanodrug was collected and subjected to the ELISA analysis. As the main immunostimulatory signals for activation of antitumor immune response, heat shock protein 60 (HSP60) and high mobility group box 1 (HMGB1) in the cell culture supernatant were produced after thermal ablation of tumor cells [38,39]. As shown in Fig. 2e, high levels of HSP60 and HMGB1 were captured by M/m-MP@F due to the existence of large amount of maleimide groups on the nanodrug surface. The capability of M/m-MP@F to capture the HSP60 and HMGB1 from the ablated tumor tissues was further investigated in vivo. Similar to the in vitro results, the M/m-MP@F + TA group exhibited higher HSP60 and HMGB1 levels than M-MP@F + TA group (Fig. S4). These results demonstrated that the maleimide groups on the nanodrug surface could enhance the capture efficiency of thermal ablation generated-immunostimulatory proteins, which might increase the retention time of these proteins in ablation site to improve their internalization opportunity into DCs.

2.2. Cytotoxicity and DCs-targeting effect of nanodrug in vitro

The cytotoxicity of blank MP on DC2.4 cells was evaluated by MTT assay. As shown in Fig. S5, MPDA showed almost no cytotoxicity even at the high concentration of 200 μ g/mL, indicating the good bio-

compatibility of the vector. Previous studies have shown that mannose modification could mediate DCs-targeted endocytosis of nanodrugs [23, 40]. The targeting efficiency of antigen-captured M/m-MP in DC2.4 cells was confirmed via CLSM and flow cytometry analyses using FITC-labeled OVA as an indicator replacing antigens. Cells incubated with targeted M/OVA/FITC-m-MP displayed much more intense FITC green fluorescence than cells treated with OVA/FITC-m-MP at 2 h (Fig. 2f), and consistent results were obtained in quantitative analysis by flow cytometry (Fig. 2g). The DC2.4 cells incubated with OVA/FITC-m-MP for 1 h and 2 h only exhibited 23.74% and 35.9% cell uptake, respectively. However, the DC2.4 cells incubated with M/OVA/FITC-m-MP displayed much higher levels of cell uptake reaching 67.02% and 100% at 1 h and 2 h, respectively. These results indicated that mannose on the nanoparticle surface played a key role in improving the DCs-targeting efficiency.

Internalized antigens escaped from endosomes into cytosol for crosspresentation in DCs had been demonstrated to be critical players in the process of anti-tumor immune response [41]. To investigate whether the antigen captured by nanodrug could escape from lysosomes after endocytosis by DCs, the intracellular distribution of FITC-labeled OVA was evaluated with CLSM. As shown in Fig. 2h, the OVA green fluorescence overlapped with the red fluorescence from lysosomes after incubating DCs with M/OVA/FITC-m-MP for 1 h, and the green fluorescence separated from the red fluorescence at 2 h, which indicated that the M/m-MP-captured OVA could escape from lysosomes inside DCs.

2.3. M/Ag-m-MP@F increases m6A modification level to promote DC maturation

The m6A modification plays an important role in the maturation of DCs [33]. A knockdown (KD) of METTL3 can result in down-regulation of m6A modification in DCs to suppress their maturation [34]. Moreover, the FTO enzyme causes m6A demethylation to impede the m6A modification in DCs. Therefore, inhibiting the activity of the FTO enzyme could enhance the m6A modification to induce DC maturation. In the present study, a FTO inhibitor (FB23-2) was loaded into the nanocarrier (M/m-MP@F), then M/m-MP@F captured antigen from tumor cell lysate to obtain the final nanodrugs were referred to as M/Ag-m-MP@F. After incubating the bone marrow-derived dendritic cells (BMDCs) with nanodrugs, the m6A modification level was investigated. As shown in Fig. 3a, cells transfected with METTL3 siRNA showed a decrease in the level of m6A modification, whereas cells receiving FTO siRNA or M/Ag-m-MP@F showed an obviously increase in the level of m6A modification at the mRNA level. Then, the effect of silencing FTO on BMDCs activation was investigated. As shown in Fig. 3b–d, the levels of key cytokines for innate immunity (e.g., IL-6 and IL-12) and the key pro-inflammatory cytokines for the activation of cellular immunity (e.g., $TNF-\alpha$) were significantly decreased when BMDCs received the METTL3 siRNA treatment, which was in line with the previous report [34]. However, high expression levels were detected for all these cytokines in the supernatant when BMDCs were treated with M/Ag-m-MP@F. Meanwhile, the RT-qPCR (Fig. 3e) and Western blot assays showed obviously up-regulated expression levels of co-stimulatory molecules CD80 and CD86 in BMDCs receiving the M/Ag-m-MP@F treatment (Fig. 3f).

The antigen-presenting abilities of DCs were positively correlated with the density of major histocompatibility complex class II (MHC-II) molecules on cell surface [42,43]. To investigate whether the M/Ag-m-MP@F treatment could promote the antigen-presenting performance of DCs, the expression level of MHC-II on BMDCs was analyzed with RT-qPCR, Western blot and CLSM. As shown in Fig. 3e, f and g, BMDCs increased the expression of MHC-II upon the treatment with M/Ag-m-MP@F. Notably, CLSM observation showed that BMDCs receiving the M/Ag-m-MP@F treatment not only increased MHC-II expression but also resulted in typical dendriform morphologic characteristics by formating of numerous pseudopods, which could improve



Fig. 3. *In vitro* antigen presentation effect of M/Ag-m-MP@F (dose of FB23-2, 5 μ M). a) Relative m6A levels of mRNA were evaluated using m6A RNA methylation quantification kit upon different treatment (n = 3; means \pm SD; ***P* < 0.01, ****P* < 0.001). b-d) Increased expressions of the IL-6, IL-12 and TNF- α in BMDCs by M/ Ag-m-MP@F determined by ELISA kits (n = 4; means \pm SD; **P* < 0.05, ***P* < 0.01). Increased expressions of the CD80, CD86 and MHC-II in BMDCs by M/Ag-m-MP@F determined by RT-qPCR (e) and Western blot assay (f) (n = 4; means \pm SD; **P* < 0.05, ***P* < 0.05, ***P* < 0.01). g) CLSM images show that M/Ag-m-MP@F significantly increases the expression of MHC-II and modifies the morphological aspects of BMDCs. CD11c: Alexa Fluor 488 labeled (green), MHC-II: Alexa Fluor 555 labeled (red), nuclei: Hoechst 33342 labeled (blue). h) Quantification of CD80⁺CD86⁺ expression *via* flow cytometry for various samples (gate on CD11c⁺ cells).

the contact area with other immune cells to promote the antigen-presenting ability of DCs [44]. The efficiency of M/Ag-m-MP@F to induce DCs maturation was further investigated *via* analyzing the expressions of the co-stimulatory molecules CD80 and CD86 with flow cytometry. As shown in Fig. 3h, the M/Ag-m-MP treatment showed a higher level of DCs maturation (34.0%) than the M/m-MP treatment (13.5%) and the M/m-MP@F (17.3%) treatment. Moreover, the M/Ag-m-MP@F treatment led to the highest level of DCs maturation (68.6%), whereas the Ag-m-MP@F treatment resulted in a much lower

level of DCs maturation (39.3%) due to the inefficient nanodrug endocytosis. These results suggested an efficient internalization of the TAAs adsorbed on the nanodrug into DCs *via* mannose targeting, and the FTO inhibitor delivered by nanocarrier could promote antigen presentation and DCs maturation *via* up-regulating the m6A modification level.

2.4. Migration of nanodrug to lymph nodes in vivo

In vitro experiments had demonstrated that M/Ag-m-MP@F

efficiently promoted DCs maturation, which is required for DCs migration to draining lymph nodes to activate antigen-specific T cells in the process of antigen-specific immune response. To further verify the DCs migration to lymph nodes *in vivo*, DiR-loaded nanodrug was intratumorally injected into the subcutaneous tumor, and then the fluorescence intensity in inguinal lymph nodes was evaluated on small animal imaging system. As shown in Fig. 4a and b, mice injected with the mannose-targeted nanodrug (M/m-MP@DiR) showed stronger DiR fluorescence at 6 h, 12 h, and 24 h in the right inguinal lymph nodes than mice injected with the non-targeted one (m-MP@DiR). Meanwhile, similar result was obtained for the left inguinal lymph nodes (Fig. 4a and c). Likely, the intratumorally injected nanodrug M/Ag-m-MP@F capable of transfecting DCs efficiently through mannose targeting as demonstrated *in vitro* could promote the DCs maturation and their migration to draining lymph node.

To support this notion, the Nile red (NR) labeled nanodrugs were intratumorally injected into the subcutaneous tumor in the right leg, and the lymph node sections were then observed under CLSM at different post-injection time points. As shown in Fig. 4d and e, a small amount of red fluorescence was found (red arrow) to separate from the green fluorescence-labeled DCs at 6 h and 12 h in the inguinal lymph in the animal injected with m-MP@NR, indicating little nanodrug could directly migrate to lymph nodes. In contrast, as shown in Fig. 4d and f, much more red fluorescence showing high level of overlapping with green fluorescence was observed in the animal injected with M/m-

MP@NR group (yellow arrow), implying that the mannose-targeted nanodrug was delivered into the lymph nodes by DCs. In addition, the animals were sacrificed to collect major organs for *ex vivo* fluorescence imaging at 24 h after nanodrug injection. As shown in Fig. S6, injecting the mannose-targeted nanodrug or the non-targeted one made no significant difference in the distribution of nanodrug in major organs.

2.5. Nanodrug inhibited tumor recurrence and distal tumor growth

The effects of nanodrug treatments on tumor recurrence and distal tumor growth after thermal ablation were explored on both the subcutaneous distal tumor model (Fig. 5a) and the orthotopic distal tumor model (Fig. 5d). First, in the subcutaneous distal tumor model (Fig. 5a), the mal-MPDA@FB23-2 (m-MP@F) treatment and the Man-MPDA@FB23-2 (M-MP@F) treatment after the ablation of primary tumor resulted in lower recurrence rates than the PBS or Man/mal-MPDA (M/m-MP) treatment, and no tumor recurrence was observed in mice receiving the M/m-MP@F treatment (Fig. S7). In comparison with PBS, M/m-MP only showed a tiny inhibitory effect on the growth of subcutaneous distal tumor (secondary tumor). Moreover, m-MP@F, M-MP@F and M/m-MP@F showed much more obvious inhibitory effect on the growth of subcutaneous distal tumor, with M/m-MP@F showing the most prominent effect (Fig. 5b). Consistently, the M/m-MP@F treatment also showed the best effect to prolong the animal survival (Fig. 5c). Similar results were obtained in the distal orthotopic liver tumor model.



Fig. 4. Nanoparticle internalized-TIDCs emigrated from primary tumor sites to draining lymph nodes. a) The fluorescence images of lymph nodes isolated at various time points (pre, 6, 12 and 24 h) after M/m-MP@DiR and m-MP@DiR intratumoral injection into the right tumor (dose of M/m-MP@DiR or m-MP@DiR, 200 μ g/kg body weight). Quantitative analysis of fluorescence intensities of right lymph nodes (b) and left lymph nodes (c) at various time points (pre, 6, 12 and 24 h) after M/m-MP@DiR and m-MP@DiR and m-MP@DiR injection into the right tumor (n = 3; means ± SD; **P* < 0.05, ***P* < 0.01). d) CLSM images of lymph node sections exhibiting the colocalization of CD11c and nanodrug on DCs after M/m-MP@NR and m-MP@NR intratumoral injection into the right tumor (dose of M/m-MP@NR or m-MP@NR, 300 μ g/kg body weight). Fluorescence intensity profiles along the selected line (indicated by a red line (f) and green line (e) in d).



Fig. 5. Anti-distal tumor effect of nanodrugs. a) Illustration of anti-distal tumor study schedule. b) Tumor volume and survival curve (c) of mice treated with different treatments (PBS, M/m-MP, m-MP@F, M-MP@F and M/m-MP@F) (n = 6; means \pm SD; ***P < 0.001). d) Illustration of anti-distal orthotopic tumor study schedule. e) Distal orthotopic tumor growth is monitored by MRI at different time points (7 d, 14 d and 28 d) after receiving different treatments (tumors are marked with dash lines). Representative images of excised orthotopic tumors (f) and quantified tumor weights of excised orthotopic tumors (g) (n = 3; means \pm SD; *P < 0.05). h) Cumulative survival of mice bearing orthotopic tumors after receiving different treatments (n = 6). i) *Ex vivo* pathological H&E staining of distal orthotopic tumor tissue in mice receiving different treatment.

In comparison with the PBS treatment and the M/m-MP treatment, the m-MP@F treatment and the M-MP@F treatment exhibited better inhibitory effects on the growth of distal orthotopic liver tumor (Fig. 5e–g). Moreover, the M/m-MP@F treatment most effectively inhibited the growth of distal orthotopic liver tumor, which led to the longest survival time of mice exceeding 50 d (Fig. 5h). According to the H&E staining, mice receiving the M/m-MP@F treatment showed the lowest cell density in the orthotopic tumor tissue (Fig. 5i). These results strongly demonstrated that the M/m-MP@F treatment effectively suppressed the recurrence of ablated primary tumor and the growth of distal secondary tumor.

To further understand the different efficacies of these nanodrugs against distal tumors, we evaluated the development of mature TIDCs in the tumor-draining lymph nodes using flow cytometric analysis. In comparison with the PBS treatment and the M/m-MP treatment,

treatments using the FB23-2-loaded nanodrugs m-MP@F, M-MP@F and M/m-MP@F effectively up-regulated the expression levels of the costimulators CD80 and CD86 for mature TIDCs, and the M/m-MP@F treatments led to the highest expression levels of CD80 and CD86 (Fig. 6a and b). Obviously, *in vivo* results demonstrated that M/m-MP@F-induced inhibition of the FTO activity increased the m6A RNA methylation, eventually resulting in the high efficiency of DCs maturation. In line with the TIDCs maturation results, a significant enhancement in the secretion levels of IL-6 and IL-12 was also found in the distal tumor of mice receiving the M/m-MP@F treatment (Fig. 6c and d). Based on the above results, the M/m-MP@F nanodrug captured the tumor-associate antigens released after tumor ablation, and then efficiently delivered them into TIDCs *via* mannose receptor-mediated endocytosis to eventually result in an effective FTO inhibition to promote TIDCs maturation.



Fig. 6. Nanodrugs promote DC maturation to enhance the infiltration of CD8⁺ T cells into tumors. a) Quantification of CD80⁺CD86⁺ expression in tumor-draining lymph nodes *via* flow cytometry for various samples (gated on CD11c⁺ cells). b) Quantitative analysis of the percentage of CD11c⁺CD80⁺CD86⁺ cells in tumor-draining lymph nodes (n = 3, mean \pm SD; ***P* < 0.01, ****P* < 0.001). The expression levels of IL-6 (c) and IL-12 (d) in Hepa1-6 tumors of mice were measured by ELISA kits (n = 4, mean \pm SD; ***P* < 0.001). e) Quantification of CD8⁺ T cells in Hepa1-6 tumors analyzed by flow cytometry (gated on CD3⁺ T cells). f) Quantitative analysis of the percentage of the CD8⁺ T cells gating on CD3⁺ T cells in tumor tissue obtained during treatment study (n = 3, mean \pm SD; ***P* < 0.01, ****P* < 0.001). The expression levels of TNF- α (g) and IFN- γ (h) in Hepa1-6 tumors of mice were measured by ELISA kits (n = 4, mean \pm SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001). i) Immunofluorescence of Hepa1-6 tumor tissues shows tumor infiltration of CD8⁺ T cells. CD8⁺ T cells: Alexa Fluor® 488 labeled (green), nuclei: DAPI labeled (blue).

Since the tumor infiltration of CD8⁺ T lymphocytes plays critical role in antitumor immunotherapy, the infiltration of $CD8^+$ T cells into the distal orthotopic tumor was analyzed with flow cytometry and immunofluorescence. As shown in Fig. 6e, f and i, the treatments using FB23-2 loaded nanodrugs (m-MP@F and M-MP@F) promoted the tumor infiltration of CD3⁺CD8⁺ T cells in comparison with the PBS treatment and the M/m-MP treatment. Moreover, the M/m-MP@F treatment, which caused the highest level of TIDCs maturation, also resulted in the highest proportions of CD3⁺CD8⁺ T cells in tumor. Most likely, promoting the maturation of TIDCs increased their antigen presentation for T cell activation, which then improved the infiltration of CD8⁺ T cells into the tumor. It is well-known that anti-programmed cell death ligand 1 (aPD-L1) antibody strengthens antitumor immunity by releasing the "brakes" for CD8⁺ T cells to secrete tumor cell-killing IFN- γ and TNF- α [45,46]. Therefore, the secretion of those cytokines in distal tumor was determined using ELISA kits. As shown in Fig. 6g and h, both cytokines were obviously increased in the distal tumor upon the M/m-MP@F treatment. Consequently, the combination treatment of M/m-MP@F and aPD-L1 antibody most significantly inhibited the growth of both distal subcutaneous tumor and distal orthotopic tumor (Fig. 5).

2.6. Immune memory against tumor metastasis elicited by nanodrug

With the experiment design shown in Fig. 7a, the effector memory T cells (Tems, CD44⁺CD62⁻ cells) and central memory T cells (Tcms, CD44⁺CD62⁺ cells) in bloodstream were analyzed to assess immunological memory critical for distal tumor inhibition and tumor metastasis prevention. In comparison with the PBS or M/m-MP treatment, both the m-MP@F treatment and the M-MP@F treatment elevated the proportion of Tcms. obviously, and the M/m-MP@F treatment increased the proportion of Tcms most effectively (Fig. 7b and c). Meanwhile, the M/m-MP@F treatment remarkably increased the proportions of Tems as well (Fig. 7b and d). These results indicated that the M/m-MP@F treatment resulted in a strong immune memory effect.

Then, we compared the inhibitory effects of different nanodrugs on tumor pulmonary metastasis. As shown in Fig. 7e, multiple large metastases could be observed in the PBS treatment group and the M/m-MP treatment group. In contrast, much fewer metastases were seen in the m-MP@F treatment group and the M-MP@F treatment group. Notably, no visible metastasis was detected in the M/m-MP@F treatment group showing the longest survival (Fig. 7f).

The above results indicated that the M/m-MP@F treatment after incomplete ablation could induce a strong immune memory response to prevent tumor metastasis, which accounted for its prominent efficacy in prolonging the survival time of mice. In addition, the H&E staining assay on major organs including heart, spleen, lung, liver and kidney revealed no pathological change for all treatment groups, demonstrating the low side effect of nanodrugs (Fig. S8).

3. Conclusion

A mannose-targeted and antigen-capturing nanodrug was prepared for co-delivering the TAAs and a FTO inhibitor into TIDCs after thermal ablation of HCC. The nanodrug featured the MPDA mesopores for FTO inhibitor (FB23-2) encapsulation and the PEG shell decorated with mannose and maleimide for TIDCs targeting and antigen capturing,



Fig. 7. Anti-tumor immune memory effect and anti-metastasis effect of nanodrugs. a) Illustration of anti-metastasis tumor study schedule. b) Representative flow cytometric analysis of memory T cells (gated on $CD3^+CD8^+$ T cells) in blood. Proportion of central memory T cells ($CD62L^+CD44^+$) (c) and effect memory T cells ($CD62L^-CD44^+$) (d) over $CD3^+CD8^+$ T cells in blood (n = 3, mean \pm SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001). e) H&E-stained lung tissues were collected from the mice received different treatments. Black circles indicate the foci of tumor metastases. f) Survival curves of mice bearing metastasis tumors treated with different treatments (n = 6).

respectively. After primary tumor ablation, the maleimide groups of nanodrug (M/m-MP@F) captured the released TAAs and then effectively co-delivered the TAAs and FTO inhibitor into TIDCs with the mannose-mediated targeting effect. The FTO inhibitor intracellularly released from nanodrug promoted antigen presentation and TIDC maturation *via* up-regulating the m6A modification level. Then, the matured TIDCs migrated to local draining lymph nodes to present antigens to naive T cells, which eventually activated antitumor immunity. *In vivo* studies demonstrated that this nanodrug synergized with ICB therapy to prevent tumor recurrence and metastasis after the primary tumor ablation therapy.

4. Materials and methods

4.1. Preparation of Man/mal-MPDA@FB23-2

The synthesized MP@F nanoparticles were modified with amino polyethylene glycol mannose and amino polyethylene glycol maleimide (HN_2 -PEG₂₀₀₀-Man, NH₂-PEG₂₀₀₀-Mal) through covalently linking reaction between phenolic hydroxyl and amino. Briefly, 1 mL of MP@F (1 mg/mL) solution was placed in a 5 mL centrifuge tube and the pH value was adjusted to 10 using 1 M of NaOH solution. Then, 10 mg of HN₂-PEG₂₀₀₀-Man and 10 mg NH₂-PEG₂₀₀₀-Mal dissolved in 1 mL deionized ice water were added into the MP@F solution followed by stirring overnight. The resulting product was ultrafiltered by a 10 kDa ultrafiltration tube and centrifuged at 9000 rpm for three times to discard the excess PEG molecules. Finally, the obtained Man/mal-MPDA@FB23-2 (M/m-MP@F) nanodrug were stored in a 4 °C refrigerator for future use.

4.2. The release profile of FB23-2 in vitro

To determine the release of FB23-2 from M/m-MP@F, the obtained M/m-MP@F nanodrug (100 μ g/mL) were incubated at pH 5.0 and pH 7.4 for different times, and the supernatant FB23-2 were obtained by centrifugation at 9000 rpm for 10 min. The amount of released FB23-2 was evaluated at an ultraviolet wavelength of 301 nm, and the cumulative drug release was calculated according to the following formulas: Cumulative drug release = (mass of drug in supernate)/(mass of drug in nanoparticle) × 100%.

4.3. Protein from tumor cell lysate capture by M/m-MP@F

Hepa1-6 cells (1 \times 10⁸) were heated using a microwave antenna, and the temperature was real-time monitored and maintained at 60 °C for 2 min. Then, nanodrug of M/m-MP@F or M-MP@F was added followed by an incubation for 10 min. Subsequently, after the cells were removed, the nanodrug was collected by centrifuged. The contents of protein captured by nanoparticles from tumor cell lysate (HMGB1 and HSP60) were determined by ELISA kits following the manufacturer's instructions.

4.4. Cell uptake of nanodrugs

DC2.4 cells were seeded on confocal dishes at a density of 1×10^4 cells/mL and cultured overnight. Then, cells were incubated with M/ OVA/FITC-m-MP and OVA/FITC-m-MP for 1 h and 2 h, respectively. After three washes in PBS, nanodrug internalized by DC2.4 cells were observed using CLSM. For flow cytometric analysis, DC2.4 cells were seeded at a density of 1×10^5 cells per well (12-well plate) and cultured overnight. After nanodrug treatment for 1 h or 2 h, DC2.4 cells were washed with PBS, collected for flow cytometry detection (NovoCyte 2060).

4.5. Intracellular distribution of nanoparticles

DC2.4 cells were plated at 1×10^4 cells/well overnight in confocal

dishes. The M/OVA/FITC-m-MP was added and incubated with cells for 1 h or 2 h. After three washes in PBS, intracellular distribution of nanoparticles was imaged using CLSM (Nikon C2). Before observation, Hoechst 33342 was added to stain nuclei for 15 min, and Lyso-Tracker Red DND-99 was added to stain lysosomes for 3 min, respectively.

4.6. Determination of m6A level in BMDCs

BMDCs were plated at 1×10^4 cells/well overnight in six-well plates. Then, cells were divided into four groups and treated with PBS + M/Ag-m-MP, METTL3 siRNA (METTL3 KD) + M/Ag-m-MP, FTO siRNA (FTO KD) + M/Ag-m-MP and M/Ag-m-MP@F, respectively. After 24 h incubation, total RNA was extracted from BMDCs, and using the EpiQuik m6A RNA Methylation Quantification Kit (Epi-gentek, Farmingdale, NY, USA) to measure the level of m6A in RNA according to the manufacturer's instructions.

4.7. Cytokine secretion of BMDCs

BMDCs were plated at 5 \times 10⁴ cells/well overnight in 12-well plates. Then, cells were divided into five groups and treated with PBS, PBS + M/Ag-m-MP, METTL3 siRNA (METTL3 KD) + M/Ag-m-MP, FTO siRNA (FTO KD) + M/Ag-m-MP and M/Ag-m-MP@F, respectively. After 24 h incubation, the supernatant was collected and the level of IL-6, IL-12 and TNF- α were assayed by ELISA kit (Westang, Shanghai, China) following the manufacturer's instructions.

4.8. Western blot assay

BMDCs were plated at 1×10^6 cells/well overnight in 12-well plates. Then, cells were divided into five groups and treated with PBS, PBS + M/Ag-m-MP, METTL3 siRNA (METTL3 KD) + M/Ag-m-MP, FTO siRNA (FTO KD) + M/Ag-m-MP and M/Ag-m-MP@F, respectively. After 24 h incubation, total proteins were isolated from BMDCs with a total extraction kit. The isolated protein was analyzed by SDS gel electrophoresis. After transferred onto PVDF membrane, incubation with 5% BSA for 1 h, and incubated with primary antibodies, *e.g.*, CD80, CD86, MHC-II and GAPDH overnight at 4 °C. The membranes were incubated with secondary antibody for 1 h at room temperature. Finally, the immunoreactive protein bands were visualized on a chemiluminescence system (ImageQuant LAS 500).

4.9. Cell immunofluorescence assay

BMDCs were plated at 1×10^4 cells/well overnight in confocal dishes. Then, cells were divided into six groups and treated with PBS, M/m-MP, M/Ag-m-MP, M/m-MP@F, M/Ag-m-MP@F and Ag-m-MP@F, respectively. 24 h later, BMDCs were fixed with 4% paraformaldehyde first. After three washes in cold PBS, cells were incubated with BSA for 30 min at 37 °C and incubated with primary antibodies, *e.g.*, CD11c, and MHC-II overnight at 4 °C. Then, cells were incubated with corresponding secondary antibody for 1 h at 37 °C following PBS washing, the nuclei were counterstained with DAPI and the immunofluorescence imaging was taken using CLSM (Nikon C2).

4.10. Activation of BMDCs

BMDCs were plated at 1×10^5 cells/well overnight in six-well plates. Then, cells were divided into six groups and treated with PBS, M/m-MP, M/Ag-m-MP, M/m-MP@F, M/Ag-m-MP@F and Ag-m-MP@F, respectively. 24 h later, BMDCs were washed with PBS and stained with flow antibodies (*e.g.*, CD11c, CD80 and CD86) for 30 min at 4 °C. After washing with PBS, the expression of surface molecules was analyzed by flow cytometry (NovoCyte 2060).

4.11. Lymph node homing assay

DiR was loaded into nanoparticles to get the M/m-MP@DiR and m-MP@DiR. After intratumoral injection, the inguinal lymph nodes and major organs, including the heart, liver, spleen, lung and kidney were collected for *ex vivo* fluorescence imaging using small animal imaging system at 6 h, 12 h and 24 h. Furthermore, Nile red (NR) were loaded into nanoparticles to get the M/m-MP@NR and m-MP@NR. After intratumoral injection, the inguinal lymph nodes were collected for immunofluorescence staining. In short, lymph nodes were embedded in optimal cutting temperature compound (OCT) to make cryosectioned tissue (8 µm). After being stained with CD11c antibody, corresponding secondary antibody and DAPI, observation was conducted under CLSM (Nikon C2).

4.12. Animal model and antitumor response

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Second Affiliated Hospital of Guangzhou Medical University and approved by the Animal Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University. All animals were handled in accordance with ARRIVE guidelines. For the bilateral subcutaneous tumor model, 1×10^6 Heap1-6 cells were subcutaneously injected into the right thigh of the C57BL/6j mouse, 6 days later, an equal number of Heap1-6 cells was subcutaneously inoculated in the left thigh of mice as the distant tumor. After another 6 days, the Hepa1-6 tumor-bearing mice were intratumorally injected with nanodrugs (PBS, M/m-MP, m-MP@F, M-MP@F and M/m-MP@F) into the right tumor and treated with thermal ablation (dose of FB23-2, 1 mg/kg body weight). After different treatments, all mice were supplied with 5 mg/kg aPD-L1 antibody every three days and the distant tumors were monitored every three days during the process of treatment. For distant orthotopic liver tumor model, $1\times 10^{6}\,\text{Heap1-}6$ cells were subcutaneously injected into the right thigh of the C57BL/6j mouse, 6 days later, an equal number of Heap1-6 cells were orthotopically injected in the liver of mouse, after intratumoral injection with nanodrug and receiving incomplete ablation, all mice were supplied with 5 mg/kg aPD-L1 antibody every three days and the change of distal orthotopic tumor volume was monitored by MRI every seven days.

4.13. Flow cytometry in vivo

Tumors were collected and dispersed into single-cell suspensions. Then, the cells were filtered using 70-mesh screens and washed with PBS. After being stained with different antibodies, *e.g.*, CD11c, CD80, CD86, CD3, CD4 and CD8, cells were harvested by centrifugation and resuspended in PBS for flow cytometry analysis. For the *in vivo* memory analysis, mice were euthanized and the blood was collected for the next experiments. Live T cells were purified from blood by Ficoll gradient centrifugation. Then, cells were stained with different antibodies, *e.g.*, CD3, CD8, CD44 and CD62L. After that, cells were harvested by centrifugation and resuspended for flow cytometry analysis.

4.14. Enzyme-linked immunosorbent assay in vivo

Mice were sacrificed and tumors were collected for further experiments. Tumors were dispersed in PBS, after centrifugation, the supernatant was collected. The level of IL-6, IL-12, TNF- α and IFN- γ in the supernatant were assayed by ELISA kits following the manufacturer's instructions.

4.15. Immunofluorescence staining

Tumor tissues and lungs were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin and sliced into 2 μ m sections. After dewaxed and rehydrated, the sections were treated with citrate buffer to retrieve

antigen and treated with 3% hydrogen peroxide (5%) for 15 min. Next, the sections were incubated with 5% BSA for 30 min at 37 °C and incubated with CD8 antibody overnight at 4 °C. Afterwards, the sections were washed with PBST three times and treated with goat anti-rabbit Alexa Fluor® 488 secondary antibody for 1 h at 37 °C. After washing with PBST again, nuclei were stained with DAPI for 5 min and the sections were sealed with anti-fluorescence. Finally, the stained sections were observed using CLSM (Nikon C2).

4.16. Lung metastasis models

 1×10^6 Heap1-6 cells were subcutaneously injected into the right thigh of the C57BL/6j mouse, 12 days later, mice were intratumorally injected with nanodrugs into the tumor and treated with incomplete ablation, aPD-L1 antibody was injected *via* the tail vein at the same time, and once again after 7 days. After treatment, mice were *i.v.* injected with 1×10^6 Hepa1-6 cells at day 14, the immune memory assessment and lung metastasis tumor analysis were determined at days 14 and 28, respectively.

4.17. Histological analysis

Tumor tissues and organs were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin and sliced into 2 μ m sections. The sections were stained with H&E staining kit following the manufacturer's instructions. Then, the stained sections were observed on a Pathology Imaging System (Perkin-Elmer Vectra, USA).

4.18. Statistical analysis

Data were presented as means \pm SD and analyzed using one-way analysis of variance (ANOVA). ANOVA was followed by Tukey's test with GraphPad Prism 8.0 software (GraphPad Software Inc., USA). Survival curves were analyzed using the Kaplan–Meier method (Statistically significant differences (relative to PBS group) were marked with asterisks. *P* < 0.05 was considered statistically significant.

Data availability

Correspondence and requests for materials should be addressed to corresponding author. All data needed to evaluate the conclusions in the paper are present in the paper and the Supporting Informations. Additional data related to this paper may be requested from the authors.

Ethics approval and consent to participate

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Second Affiliated Hospital of Guangzhou Medical University and approved by the Animal Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University. All animals were handled in accordance with ARRIVE guidelines.

CRediT authorship contribution statement

Zecong Xiao: Methodology, Writing – original draft, Investigation, Funding acquisition. Tan Li: Methodology, Formal analysis. Xinyao Zheng: Methodology, Investigation. Liteng Lin: Investigation. Xiaobin Wang: Investigation, Validation. Bo Li: Validation, Funding acquisition. Jingjun Huang: Funding acquisition. Yong Wang: Project administration, Validation, Funding acquisition. Xintao Shuai: Supervision, Project administration, Conceptualization, Funding acquisition. Kangshun Zhu: Visualization, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.07.027.

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