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Tumor-Associated Macrophage-Mediated Targeted Therapy of Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is the most aggressive form of breast cancer. TNBC is often infiltrated with a large number of macrophages, which in turn promote tumor growth and metastasis. In this study, tumor-associated macrophages (TAMs) were exploited as a target to deliver doxorubicin (DOX), a chemotherapeutic agent, to TNBC using nanoparticles surface-functionalized by i) acid-sensitive sheddable PEGylation and ii) modifying with mannose (i.e. DOX-AS-M-PLGA-NPs). In mice with orthotopic M-Wnt triple-negative mammary tumors, a single intravenous injection of DOX-AS-M-PLGA-NPs significantly reduced macrophage population in tumors within 2 days, and the density of the macrophages recovered slowly. Repeated injections of DOX-AS-M-PLGA-NPs can help maintain the population of the macrophages at a lower level. In M-Wnt tumor-bearing mice that were pre-treated with zoledronic acid to non-selectively deplete macrophages, the TAM-targeting DOX-AS-M-PLGA-NPs were not more effective than the DOX-AS-PLGA-NPs that were not surface-modified with mannose, and thus do not target TAMs, in controlling tumor growth. However, in M-Wnt tumor-bearing mice that were not pre-treated with zoledronic acid, the TAM-targeting DOX-AS-M-PLGA-NPs were significantly more effective than the non-targeting DOX-AS-PLGA-NPs in controlling the tumor growth. The AS-M-PLGA-NPs or other nanoparticles surface-functionalized similarly, when loaded with chemotherapeutic agents commonly used in adjuvant therapy of TNBC, may be developed into targeted therapy of TNBC.

Keywords

Nanoparticles; macrophage depletion; tumor growth inhibition

Introduction

Breast cancer is among the most common cancers affecting women in the U.S [1]. It is a group of heterogeneous diseases characterized by different molecular subtypes, risk factors,

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clinical behaviors, and responses to treatments. In research settings, breast cancers are classified into luminal A, luminal B, HER2-enriched, and basal-like, based on their molecular subtypes [2, 3]. Based on treatment options, breast cancers are roughly divided into three groups, however: (i) hormone receptor-positive (ER^+ and/or PR^+) (i.e. luminal A and B); (ii) HER2-positive; and (iii) triple-negative (ER^-, PR^-, $HER2^-$) [4]. Triple-negative breast cancer (TNBC) accounts for about 15% of all breast cancers, but is responsible for a disproportionally large share of morbidity and mortality [5]. TNBC is significantly more aggressive than breast tumors of other molecular subtypes and have a poor prognosis [3, 6]. The majority of TNBC are either basal-like (39 to 54%) or claudin-low (25 to 39%) [7]. The triple-negative nature renders TNBC patients non-respondent to hormonal therapy (e.g. tamoxifen or aromatase inhibitors) [8], or to therapies that target HER2 receptors (e.g. Herceptin) [9]. The only systemic therapy currently available for patients with TNBC is adjuvant chemotherapy with various combinations of anthracyclines (e.g. doxorubicin), taxanes (e.g. paclitaxel or docetaxel), or cyclophosphamide [10, 11]. However, further study revealed that claudin-low TNBC shows a lower pathological complete response (pCR) rate after anthracycline/taxane-based chemotherapy than basal-like tumors [7], suggesting that claudin-low TNBC shows some chemotherapy sensitivity, but has an overall poor prognosis and may not be managed effectively with existing chemotherapy regimens.

Herein we report a potential targeted therapy for TNBC by targeting tumor-associated-macrophages (TAMs). TAMs are innate immune effector cells recruited to tumor tissues, and contribute to tumor growth and metastasis by promoting angiogenesis, producing stromal breakdown factors, and suppressing adaptive immunity [12, 13]. Moreover, TAMs also reduce tumor response to chemotherapy [14-18]. There is strong clinical evidence that high density of TAMs in tumor tissues correlates with poor prognosis of TNBC and high risk of metastasis [19-22]. In fact, TAMs are increasingly considered as a viable target for cancer therapy [23-25]. By taking advantage of the slightly lower pH in solid tumor microenvironment and the fact that TAMs overexpress mannose receptors, we previously developed poly (lactic-*co*-glycolic) acid (PLGA) nanoparticles that are PEGylated with acid-sensitive sheddable polyethylene glycol (PEG) and surface-modified with mannose (i.e. AS-M-PLGA-NPs) to actively target the nanoparticles to TAMs via mannose-mannose receptor recognition after acid-sensitive “shedding” of the PEG in the relatively lower pH tumor microenvironment. We incorporated doxorubicin (DOX), a chemotherapeutic agent commonly used in TNBC adjuvant chemotherapy, into the nanoparticles to prepare DOX-AS-M-PLGA-NPs, and showed that the DOX-AS-M-PLGA-NPs significantly increase the distribution of DOX in tumor tissues in a mouse model [26]. Importantly, in mice with subcutaneous B16-F10 melanoma, DOX-AS-M-PLGA-NPs increase the uptake of DOX by TAMs, decrease TAM population in tumors, but do not significantly affect macrophage population in the mononuclear phagocyte system (MPS, e.g. liver and spleen) [26]. In the present study, using a mouse model with orthotopic triple-negative mammary tumors that have high density/population of TAMs, we studied the effect of the DOX-AS-M-PLGA-NPs on TAMs and evaluated the nanoparticle's ability to inhibit triple-negative mammary tumor growth as compared to DOX alone.

Materials and methods

Materials

PLGA (Resomer 752H) and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (St. Louis, MO). Zoledronic acid monohydrate was from the US Pharmacopoeia (Rockville, MD). O-stearoyl mannose (M-C18) and polyethylene glycol 2000-hydrazone-C18 (PHC) were synthesized following our previously published methods [27, 28]. Doxorubicin hydrochloride was from LC Labs (Woburn, MA). RM0029-11H3 was from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-F4/80 and APC-labeled anti-CD206 were from BD Biosciences (San Jose, CA). Solvents used in chemical synthesis were of analytical grade.

Cell lines and animals

J774A.1 macrophage cells were from the American Type Culture Collection (ATCC, Manassas, VA). MMTV-M-Wnt-1 (M-Wnt) mammary tumor cells were cloned from spontaneous mammary tumors in MMTV-Wnt-1 transgenic mice in a congenic C57BL/6 background [29]. J774A.1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), while M-Wnt cells were cultured in RPMI 1640 medium, both at 37°C and 5% CO_2. Both media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. All cell culture media and reagents were from Invitrogen (Carlsbad, CA).

Female C57BL/6 mice (6-8 weeks) were from Charles River Laboratories (Wilmington, MA). Animal studies were performed in accordance with the U.S. National Research Council Guidelines for the Care and Use of Laboratory Animals. Animal protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. M-Wnt tumors were established by injecting M-Wnt (basal-like, triple-negative, claudin-low) tumor cells (5×10^5 cells/mouse) in the ninth mammary fat pad of C57BL/6 mice; and Met-1fvb2 tumors (luminal) were established similarly by orthotopically implanting Met-1 tumor cells as reported previously [30]. Mammary tumor tissues were also harvested from genetically-modified mice with two other mammary tumors subtypes (i.e. MMTV-Neu ($HER2^+$) [31] and MMTV-*Wnt*-1 (basal-like, triple-negative) [32, 33]).

Preparation and characterization of doxorubicin (DOX)-loaded nanoparticles

Nanoparticles were prepared following our previously reported method with slight modifications [27]. Briefly, 0.9 ml of tetrahydrofuran (THF) containing PLGA 752H (3 mg) and DOX (0.3 mg) was added drop-wise into 4.5 ml of water under stirring. The nanoparticles were collected by centrifugation (13 000 × *g*, 10 min, 4° C) after the evaporation of THF. For the purpose of surface modification, M-C18 (1.2 mg) and/or PHC (3.6 mg) were dissolved together with PLGA and DOX in THF and added into water [27]. The particle size and zeta potential of the nanoparticles were determined using a Malvern Zeta Sizer Nano ZS (Westborough, MA). The entrapment efficiency (EE) of DOX was determined by measuring the concentration of free drug (unentrapped) in the supernatant after centrifugation at 14 000 rpm for 10 min. The particle size, zeta potential, and entrapment efficiency of the TAM-targeting DOX-AS-M-PLGA-NPs were 152 ± 17 nm, -27

± 2 mV, and 63.0 ± 0.3%, respectively; and they were 138 ± 15 nm, -24 ± 5 mV, and 70.0 ± 2.3%, respectively for the non-targeting DOX-AS-PLGA-NPs [26].

The effect of macrophages that are pre-incubated with DOX-AS-M-PLGA-NPs on the proliferation of M-Wnt cells

J774A.1 macrophages were seeded in a flask at a density of 5×10^5 cells/plate. M-Wnt cells were seeded into a 24-well plate at a density of 5×10^3 cells/well. After 24 h, J774A.1 cells were incubated in the presence of DOX-AS-M-PLGA-NPs (DOX concentration, 50 mM) for 2 h. The high concentration of DOX-AS-M-PLGA-NPs allows the J774A.1 macrophages to internalize and/or bind a sufficient amount of the DOX-AS-M-PLGA-NPs within 2 h, without causing cell death. The DOX-AS-M-PLGA-NPs were pre-incubated in pH 6.8 phosphate-buffered saline (PBS, 10 mM) overnight to facilitate PEG shedding [26]. Cells were collected, washed, and seeded in cell culture inserts (Thinsert, Greiner Bio-one, Monroe, NC) at 5×10^5 cells/well, which were then attached to the 24-well plate with M-Wnt cells. Twenty-four hours later, the inserts were removed, and the number of M-Wnt cells alive was determined using MTT assay [34]. Controls include DOX-AS-M-PLGA-NPs alone, J774A.1 cells that were not pre-incubated with DOX-AS-M-PLGA-NPs, or just the medium alone, all added directly into the Thinsert cell culture inserts.

The effect of DOX-AS-M-PLGA-NPs on macrophages in M-Wnt tumors in mice

Three separate experiments were carried out to study the effect of the DOX-AS-M-PLGA-NPs on macrophage population in M-Wnt tumors. In the first experiment, M-Wnt tumor-bearing mice were intravenously (i.v.) injected with DOX-AS-M-PLGA-NPs (DOX dose, 15 mg/kg) or sterile PBS (i.e. Control) 21 days after tumor cells were implanted in mice. Mice were euthanized 0 (i.e. immediately), 2, 5, or 12 days after the injection. Tumors were harvested, fixed, sectioned, and stained using an anti-F4/80 antibody at the University of Texas MD Anderson Cancer Center Science Park Research Division (Smithville, TX). Slides were examined under a light microscope, and positive staining was analyzed using an Aperio ImageScope Software (Leica Biosystems, Inc., Buffalo Grove, IL).

In the second experiment, M-Wnt tumor-bearing mice (tumor diameter, ∼7 mm) were i.v. injected with a single dose of DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs, at a DOX dose of 20 mg/kg. Control mice were injected with sterile PBS (10 mM, pH 7.4). Mice were euthanized 24 h later to collect tumors. Single tumor cell suspensions were stained with APC-labeled anti-CD206 antibody (1:200 dilution) for 20 min on ice, washed 3 times with PBS, and then analyzed using a BD FACS Aria Flow Cytometer (San Jose, CA). The percent of CD206-positive cells was analyzed using the Flow Jo software (Tree Star Inc., Ashland, OR). Cells were gated based on the single cell suspension from tumor tissues harvested from mice that were injected with sterile PBS and were not further stained with anti-CD206 antibody. As controls, M-Wnt tumor-bearing mice were pretreated with zoledronic acid (200 μg/kg, 3 times a week for 2 weeks, intraperitoneal injection (i.p.)), starting on the day when tumor cells were implanted. The next day after the last injection of zoledronic acid, mice were randomized and i.v. injected with the DOX formulations as mentioned above (i.e. DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs), and the

percent of CD206-positive cells in tumors was also determined using flow cytometry 24 h after the injection.

In the third experiment, M-Wnt tumor-bearing mice were i.v. injected with DOX-AS-M-PLGA-NPs, DOX, or sterile PBS, once in every 2-3 days for a total of four times, starting 18 days after tumor cell implantation. The dose of DOX was 10 mg/kg per injection. As a control, mice were i.v. injected with zoledronic acid (5 mg/kg) every other day for a total of 5 times. Twenty-four hours after the last injection, mice were euthanized, tumor tissues collected, and single tumor cell suspensions were prepared and stained with APC-labeled anti-CD206 antibody and analyzed using a flow cytometer as mentioned above.

The effect of DOX-AS-M-PLGA-NPs on the growth of M-Wnt tumors in mice

When orthotopic M-Wnt tumors in C57BL/6 mice reached 3∼4 mm in diameter, mice were randomized and i.v. injected with DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs, all in sterile PBS, 3 times a week for a total of 6 doses. The dose of DOX was 5 mg/kg. Tumor growth and mouse health were monitored. Twenty-four hours after the last dose, mice were euthanized to collect tumor tissues, liver, and spleen, which were fixed in formalin, embedded, sectioned, and stained with anti-F4/80 antibody (tumors, often used to stain against mouse TAMs) or RM0029-11H3 (a pan-macrophage antibody, liver and spleen). In another study, mice were i.p. injected with zoledronic acid (3 times a week for 4 weeks, 11 doses total; 200 μg/kg for the first 5 doses, and 300 μg/kg for the remaining 6 doses), starting on the day when the tumor cells were implanted. Starting 2 weeks after tumor implantation (i.e. after 5 doses of zoledronic acid), mice were randomized (n = 7) and i.v. injected with the same DOX formulations as mentioned above, 3 times a week for 2 weeks. Tumor tissues were collected 24 h after the last injection and stained using anti-F4/80 antibody as well.

Statistics

Statistical analyses were completed by performing analysis of variance followed by Fisher's protected least significant difference procedure. A p value of ≤ 0.05 (two-tail) was considered significant.

Results and Discussion

Triple-negative mouse M-Wnt mammary tumors contain a high density/population of macrophages

The M-Wnt cell line was derived from mammary tumors spontaneously developed in MMTV-*Wnt*-1 genetically-modified mice [29]. It possesses many features of claudin-low, TNBC cells, such as inconsistent expression of basal keratins (e.g. keratins 5, 14 and 17) and low expression of claudin 3, claudin 7, HER2, and luminal markers such as ER and PR [29]. When implanted into syngeneic mice, M-Wnt cells poorly differentiated to tumors with metaplastic morphology, intratumoral adipocytes, and are significantly invasive [29]. M-Wnt cell line is among the few reported murine mammary cancer cell lines that closely mimic the pathology and molecular profile of human claudin-low TNBC cells [29]. First, we estimated the density of TAMs in orthotopic M-Wnt tumors in mice by staining tumor tissues with

RM0029-11H3, a rat anti-mouse macrophage antibody. As controls, the densities of TAMs in mammary tumors from MMTV-*Wnt*-1 (i.e. ER^-, PR^-, $HER2^-$) genetically-modified mice, MMTV-neu (i.e. $HER2^+$) genetically-modified mice, and orthotopically implanted Met-1 tumors were also estimated. As shown in Fig. 1, the extent of positive staining was high in the MMTV-*Wnt*-1 and M-Wnt tumors, but relatively lower in MMTV-neu tumors; and positive staining was rarely detectable in the Met-1 tumors, showing that the triple-negative mouse mammary tumors MMTV-Wnt-1 and M-Wnt have a relative higher density/population of TAMs. Therefore, the orthotopic M-Wnt mammary tumor model was used for further studies.

TAM-targeting DOX-AS-M-PLGA-NPs decrease the density/population of TAMs in orthotopic M-Wnt tumors

Previously, using mice with subcutaneous B16-F10 tumors, we showed that the DOX-AS-M-PLGA-NPs can target tumors by interacting with TAMs, because depletion of mouse macrophages using zoledronic acid, a potent bisphosphonate known to induce macrophages to undergo apoptosis and is commonly used for the non-selective depletion of macrophages in mouse models [35, 36], decreases the distribution of DOX-AS-M-PLGA-NPs in tumors [26]. In addition, the DOX-AS-M-PLGA-NPs increase the uptake of the DOX by TAMs, as compared to DOX alone or DOX-AS-PLGA-NPs, which do not actively target TAMs due to the lack of surface-modification with mannose [26]. In the present study, using the orthotopic M-Wnt triple-negative mouse mammary tumor model, we studied the effect of the DOX-AS-M-PLGA-NPs on TAMs. Initially, we examined the density of macrophages in M-Wnt tumors at different time after tumor-bearing mice were i.v. injected with DOX-AS-M-PLGA-NPs. Mice with orthotopic M-Wnt tumors of ~7 mm were i.v. injected with DOX-AS-M-PLGA-NPs and then euthanized immediately or 2, 5, or 12 days after the injection. Tumors were stained with an anti-F4/80 antibody, as F4/80 is often used as marker of mouse TAMs. As shown in Fig. 2, the density of $F4/80^+$ cells in M-Wnt tumor tissues decreased significantly 2 days after the injection of DOX-AS-M-PLGA-NPs, and then slowly increased and recovered on day 12 to a level similar to the original level (i.e. the level on day 0).

We then compared the effect of DOX-AS-M-PLGA-NPs on TAMs in orthotopic M-Wnt tumors to that of DOX-AS-PLGA-NPs or DOX using flow cytometry. Again, mice with orthotopic M-Wnt tumors of ~7 mm were i.v. injected with DOX-AS-M-PLGA-NPs, DOX-AS-PLGA-NPs, or DOX, but tumor tissues were harvested 24 h later to prepare single cell suspensions, which were then stained with APC-labeled anti-CD206 antibody and analyzed using a flow cytometer (CD206 is a marker of M2 macrophages). As shown in Fig. 3, more than 50% of the cells in M-Wnt tumors were CD206-positive. A single dose of DOX alone did not significantly reduce the percent of CD206-positive cells, but a single dose of DOX-AS-M-PLGA-NPs reduced the percent of CD206-positive cells by about 50% (Fig. 3). DOX-AS-PLGA-NPs also reduced the percent of CD206-positive cells, but to a less extent than the DOX-AS-M-PLGA-NPs (Fig. 3). Moreover, after the M-Wnt tumor-bearing mice were treated with zoledronic acid to deplete macrophages (Fig. 3), treatment with DOX-AS-M-PLGA-NPs no longer significantly further reduced the percent of CD206-positive cells (Fig. 3).

Finally, to understand the effect of multiple doses of DOX-AS-M-PLGA-NPs on macrophage population in tumors, orthotopic M-Wnt tumor-bearing mice were i.v. injected with DOX-AS-M-PLGA-NPs once in every 2-3 days for 4 times, and the percent of CD206-positive cells in tumors was measured using flow cytometry 24 h after the last injection. As shown in Fig. 4, repeated injections of DOX-AS-M-PLGA-NPs can maintain the population of CD206-positive cells in M-Wnt tumors at a significantly lower level (i.e. for 9 days after the first injection).

Taken together, using both immunohistostaining and flow cytometry, we showed that i.v. injection of our TAM-targeting DOX-AS-M-PLGA-NPs significantly reduced the density/population of TAMs in orthotopic M-Wnt tumors within 1-2 days, and the density/population of TAMs then recovered slowly; however, repeated injections of the DOX-AS-M-PLGA-NPs can help maintain the density/population of TAMs at a lower level.

TAM-targeting DOX-AS-M-PLGA-NPs are more effective than DOX alone or the DOX-AS-PLGA-NPs that do not target TAMs in controlling orthotopic M-Wnt tumor growth

Previously, using mice with subcutaneously implanted B16-F10 tumor, we showed that our TAM-targeting DOX-AS-M-PLGA-NPs are more effective than DOX alone in controlling tumor growth [26]. The triple-negative M-Wnt mouse mammary tumors contain a high population of TAMs (Fig. 1). In this study, we tested whether the DOX-AS-M-PLGA-NPs can be used to more effectively control M-Wnt tumor growth than DOX alone. C57BL/6 mice with orthotopic M-Wnt tumors were treated with DOX-AS-M-PLGA-NPs, DOX alone, or DOX-AS-PLGA-NPs (as a control). As shown in Fig. 5A-B, DOX-AS-M-PLGA-NPs significantly inhibited M-Wnt tumor growth. The equivalent dose of DOX alone or DOX-AS-PLGA-NPs also slightly inhibited the tumor growth, but not significant, when compared to PBS (as a vehicle control) (Fig. 5A-B). Data in Fig. 5C showed that repeated injections of DOX-AS-M-PLGA-NPs did not significantly affect mouse body weight.

At the end of the study, mice were euthanized; and tumors, liver, and spleen were collected to examine the effect of the DOX-AS-M-PLGA-NPs on macrophages in them using immunohistostaining. As shown in Fig. 6, DOX-AS-M-PLGA-NPs significantly reduced the density of macrophages in tumors (Fig. 6A), but did not show significant effect on the density of macrophages in mouse liver and spleen (Fig. 6B). The DOX-AS-PLGA-NPs, which do not target TAMs, appeared to be less effective than DOX-AS-M-PLGA-NPs, but more effective than DOX alone, in reducing the density of macrophages in tumors (Fig. 6A). This finding is in agreement with our previous finding using the B16-F10 melanoma tumor-bearing mouse model [26].

The TAM-targeting DOX-AS-M-PLGA-NPs are not more effective than the non-targeting DOX-AS-PLGA-NPs in controlling M-Wnt tumor growth in mice wherein macrophages were depleted by treatment with zoledronic acid

To further understand the role of TAMs on the antitumor activity of DOX-AS-M-PLGA-NPs, the antitumor activity of DOX-AS-M-PLGA-NPs was compared to that of DOX or DOX-AS-PLGA-NPs in orthotopic M-Wnt tumor-bearing mice that were pretreated with zoledronic acid to non-selectively deplete macrophages, including TAMs.

Immunohistostaining and flow cytometry confirmed the reduction of the density of macrophages in M-Wnt tumors using zoledronic acid (Fig. 6C and Fig. 4). As shown in Fig. 7, in mice pre-treated with zoledronic acid to deplete macrophages, DOX-AS-M-PLGA-NPs remained more effective than DOX in controlling tumor growth, but they were no longer more effective than DOX-AS-PLGA-NPs. After the TAM population in M-Wnt tumors was significantly reduced using zoledronic acid, the ability of the DOX-AS-M-PLGA-NPs and DOX-AS-PLGA-NPs to deliver DOX into tumor tissues and TAMs may not be different enough to cause a significant difference in their ability to control M-Wnt tumor growth. Nonetheless, data in Fig. 5 and Fig. 7 clearly showed that it is advantageous to use the DOX-AS-M-PLGA-NPs to inhibit the triple-negative M-Wnt mouse mammary tumor growth, regardless whether the TAMs in the tumors were reduced or depleted by treating the tumor-bearing mice with zoledronate. This is significant as there may be heterogeneity in the relative population of TAMs in different TNBC patients, although there is strong clinical evidence that high density of TAMs in tumor tissues correlates with poor prognosis of TNBC and high risk of metastasis [19–22].

Unlike previous efforts in targeting cytotoxic agents directly into tumor cells *per se* to more effectively kill them; our DOX-AS-M-PLGA-NPs are designed to target DOX into TAMs instead [26, 27]. Since M-Wnt tumors contain a large number of macrophages, the stronger antitumor activity of the DOX-AS-M-PLGA-NPs may be attributed to their ability to more effectively deliver DOX into TAMs and then reduce macrophage population in tumor tissues, as compared to the non-targeting DOX-AS-PLGA-NPs or DOX alone (Fig. 3B). However, it is worth noting that targeting of TAMs does not preclude the cytotoxicity of DOX in the DOX-AS-M-PLGA-NPs to tumor cells. For example, DOX that is released from the DOX-AS-M-PLGA-NPs before or after the nanoparticles reach tumors can be cytotoxic to tumor cells. In addition, DOX-AS-M-PLGA-NPs that are bound to TAMs, or even those that are internalized by TAMs, may be cytotoxic to tumor cells as well. In fact, in an *in vitro* study, we showed that murine J771A.1 macrophages that were pre-incubated with DOX-AS-M-PLGA-NPs for 2 h (and then washed) significantly inhibited the growth of M-Wnt tumor cells (Fig. 8), even as expected [12, 37], the J774A.1 macrophage cells alone promoted M-Wnt tumor cell growth (Fig. 8). More experiments will have to be carried out to fully elucidate the mechanism underlying the stronger antitumor activity of the TAM-targeting DOX-AS-M-PLGA-NPs. Nonetheless, it is clear that delivering DOX, which is commonly used in combination adjuvant chemotherapy of TNBC, using the TAM-targeting AS-M-PLGA-NPs significantly increased the antitumor activity of DOX. It is expected that delivering other chemotherapeutic agents that are commonly used in TNBC chemotherapy, e.g. taxanes and cyclophosphamide, using the TAM-targeting AS-M-PLGA-NPs, will also enhance their antitumor activity. Of course, due to differences in the physicochemical properties of different chemotherapeutic agents, the nanoparticles may need to be tailored accordingly to efficiently entrap the agents in the nanoparticles, but we expected that similar surface-functionalization will enable the nanoparticles to target TAMs. Therefore, our TAM-targeting nanoparticles can potentially make targeted therapy of TNBC possible.

Finally, the TAM-targeting nanoparticles may also be adopted to improve the chemotherapy of other solid tumors enriched with TAMs. In addition, since it is known that TAMs reduce tumor cell response to chemotherapy, and our DOX-AS-M-PLGA-NPs can selectively

reduce the density of TAMs, it is expected that the DOX-AS-M-PLGA-NPs may be used to improve tumor cell response to chemotherapy with DOX or other chemotherapeutic agents (e.g. taxanes or cyclophosphamide).

Conclusion

Previously, we developed TAM-targeting PLGA nanoparticles by acid-sensitive sheddable PEGylation and surface-modification with mannose (i.e. AS-M-PLGA-NPs). In the present study, we show that the AS-M-PLGA-NPs incorporated with DOX, a chemotherapeutic agent, can be used to reduce TAM population or density in orthotopic, triple-negative, M-Wnt mammary tumors in a mouse model. More importantly, the DOX-AS-M-PLGA-NPs were more effective than DOX alone in inhibiting the M-Wnt tumor growth in the mouse model, and their effectiveness against the M-Wnt tumors, relative to the DOX-AS-PLGA-NPs that do not target TAMs, was dependent on the presence of a high population of TAMs. We conclude that the high density of TAMs in triple-negative breast tumors can be potentially exploited to design a targeted therapy to more effectively control the tumor growth.

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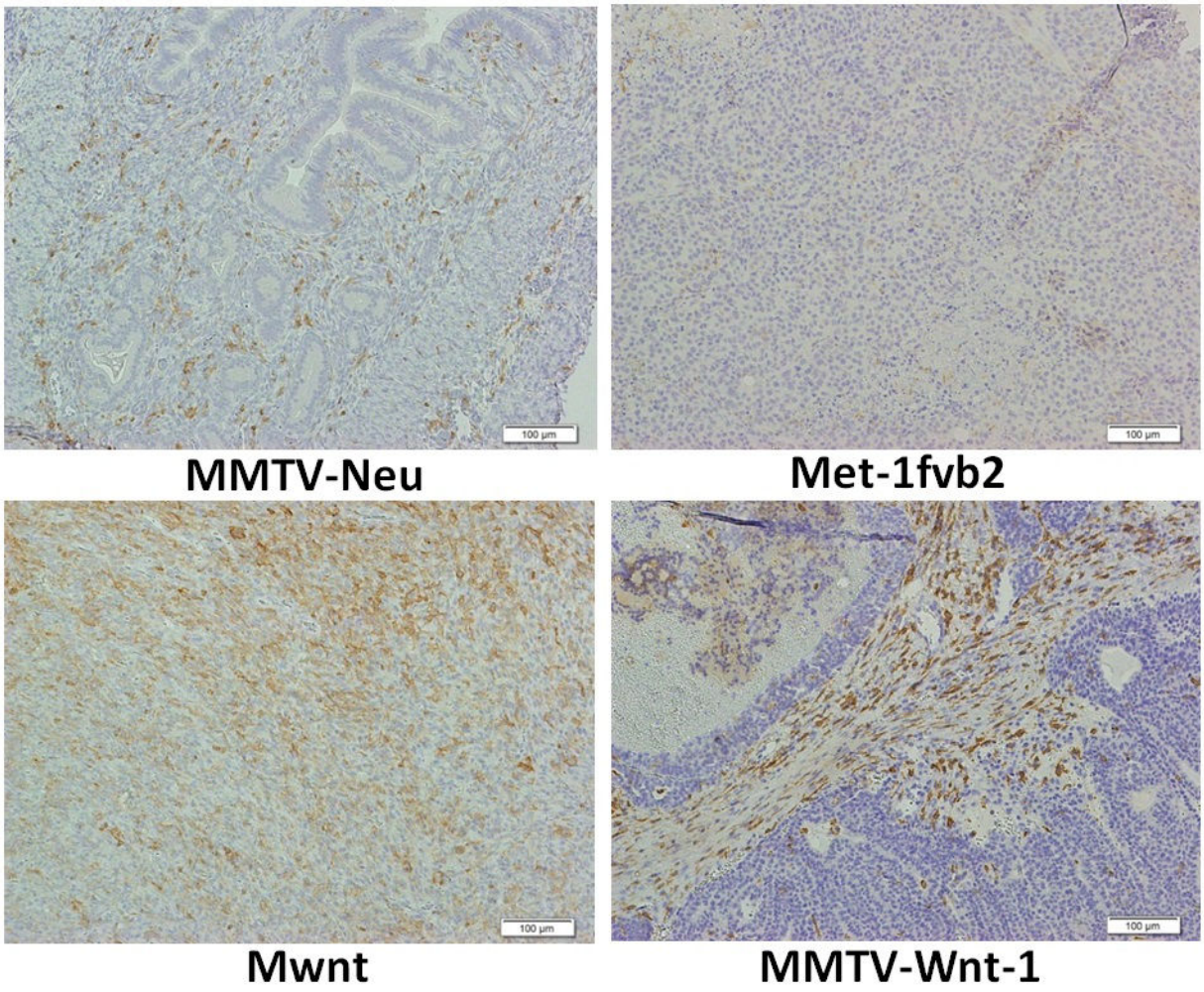


Fig. 1.

Representative images of mouse mammary tumors of different molecular subtypes after stained with an anti-F4/80 antibody. F4/80 is a macrophage marker. Bar = 100 μm.

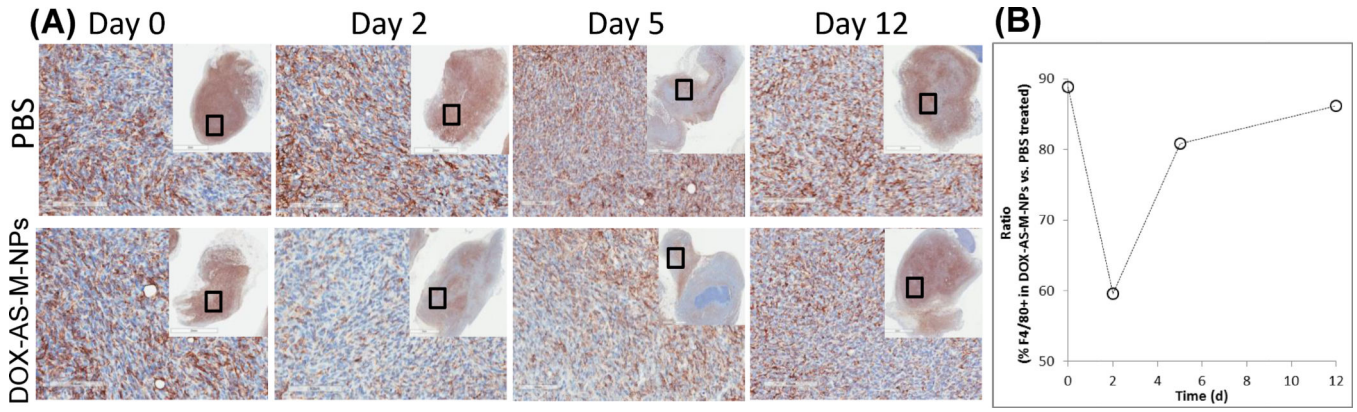
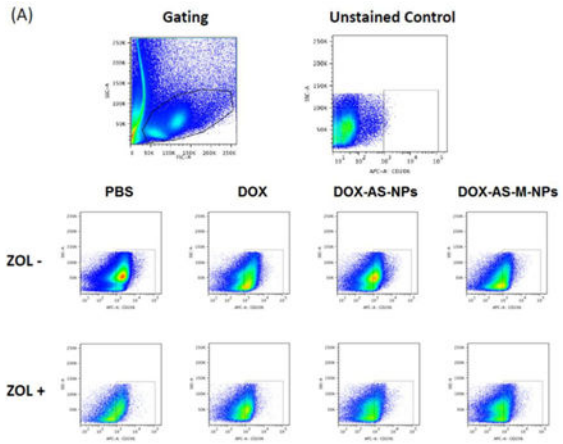


Fig. 2.

(A) Representative images of M-Wnt tumors stained with an antiF4/80 antibody when tumors were harvested 0, 2, 5, or 12 days after tumor-bearing mice were i.v. injected with a single dose of DOX-AS-M-PLGA-NPs or PBS (as a control). (B) The percent of $F4/80^{+}$ cells in M-Wnt tumors in mice treated with DOX-AS-M-PLGA-NPs, relative to in mice injected with PBS. Data are mean ± S.D. (n = 3).

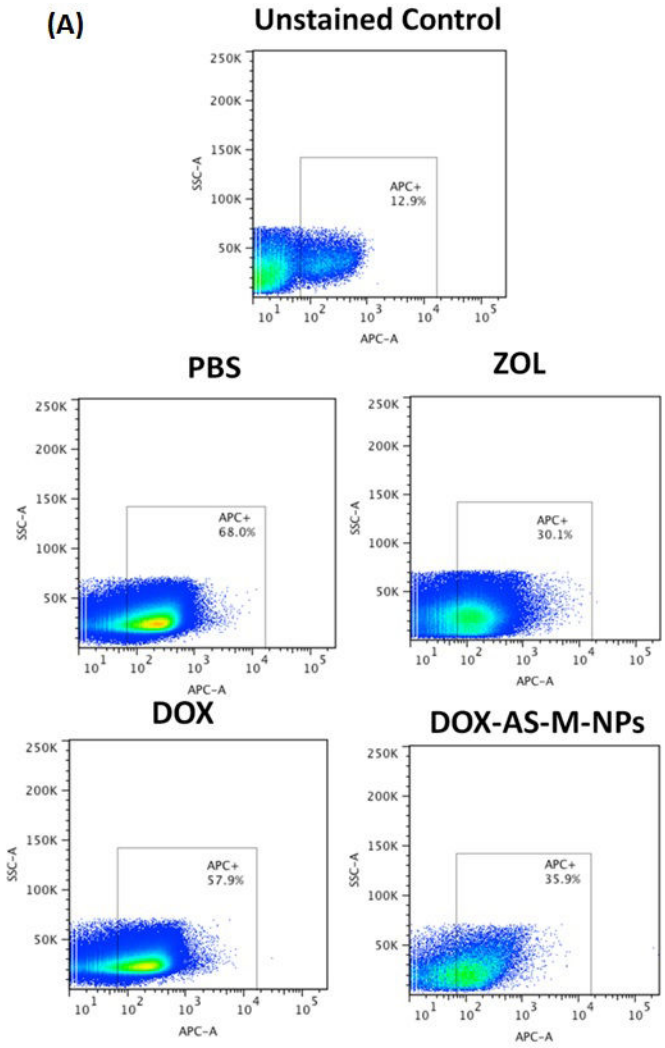


(B)

Treatment	Formulation	Macrophage %
ZOL -	PBS	57.2 ±9.1 [a]
	DOX	49.6 ± 2.5 [a]
	DOX-AS-NPs	39.2 ± 5.1 [b]
	DOX-AS-M-NPs	31.1 ± 2.1 [c]
ZOL +	PBS	31.5 ± 8.4
	DOX	29.3 ± 6.7
	DOX-AS-NPs	23.0 ± 13.2
	DOX-AS-M-NPs	21.4 ± 8.0

Fig. 3.

Percent of macrophages in M-Wnt tumors in mice treated with PBS, DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs at a single dose of 20 mg/kg. Mice that were pre-treated with zoledronic acid (i.e. ZOL+) were used as controls. Tumor cell suspensions were stained with APC-labeled anti-CD206 and analyzed using a flow cytometer. (A) Representative flow cytometry graphs. (B) The percent of macrophages in tumors ($^{a\text{-}c}$ $p \leq 0.05$). Data ae mean ± S.D. (n = 4).



(B)

Formulation	Macrophage %
PBS	46.4±11.0
DOX	36.8 ± 14.1
DOX-AS-M-NPs	27.2 ± 6.4 *
ZOL	21.8 ± 5.2 *

Fig. 4.

Percent of macrophages in M-Wnt tumors in mice that were treated with multiple doses of DOX-AS-M-PLGA-NPs, DOX, or zoledronic acid. Single tumor cell suspensions were stained with APC-labeled anti-CD206 and analyzed using a flow cytometer. (A) Representative flow cytometry graphs. (B) The percent of macrophages in tumors (* $p \leq 0.05$ vs. PBS). Data are mean ± S.D. ($n \geq 3$). Numbers shown are after subtraction of the mean of the Unstained Control.

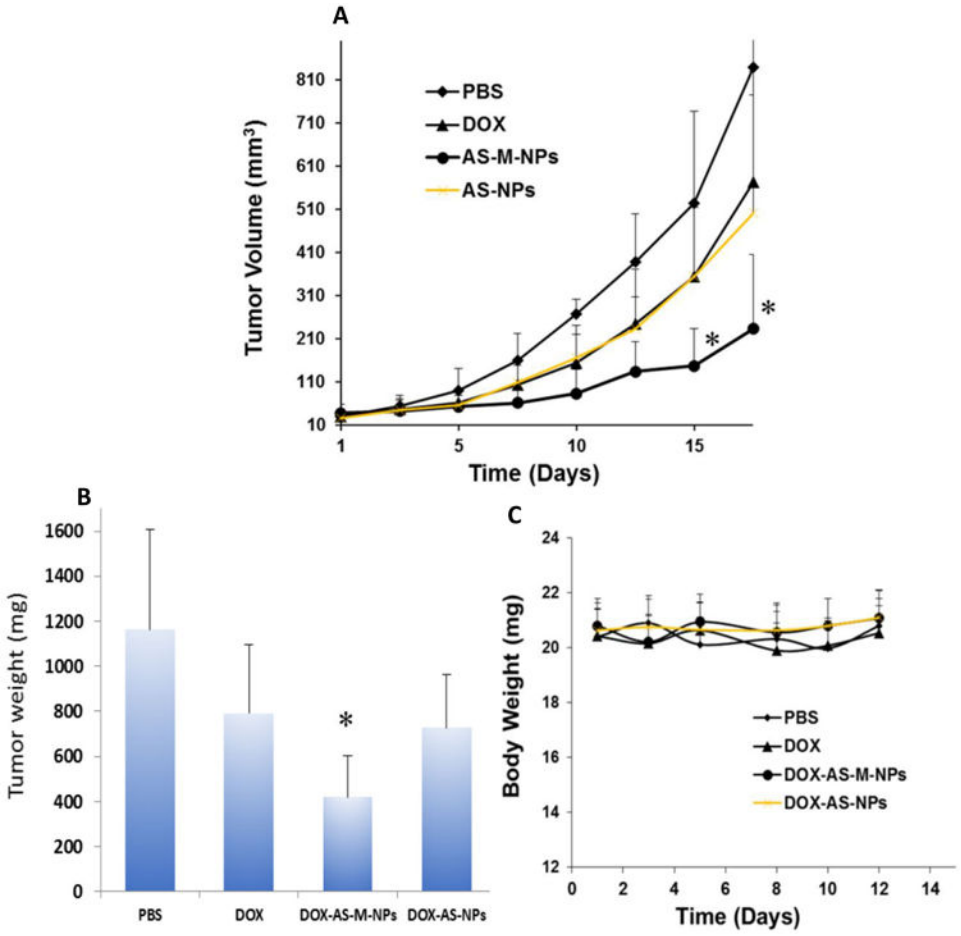


Fig. 5.

DOX-AS-M-PLGA-NPs are more effective than DOX alone or DOX-AS-PLGA-NPs in inhibiting M-Wnt tumor growth. M-Wnt tumor bearing mice were i.v. injected with PBS, DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs 3 times per week, at DOX dose of 5 mg/kg, for 2 weeks. Shown are tumor volumes (A), tumor weights at the end of the study (B), and mouse body weights (C) (* $p < 0.05$ vs. others). Data are mean ± S.D. ($n = 5$).

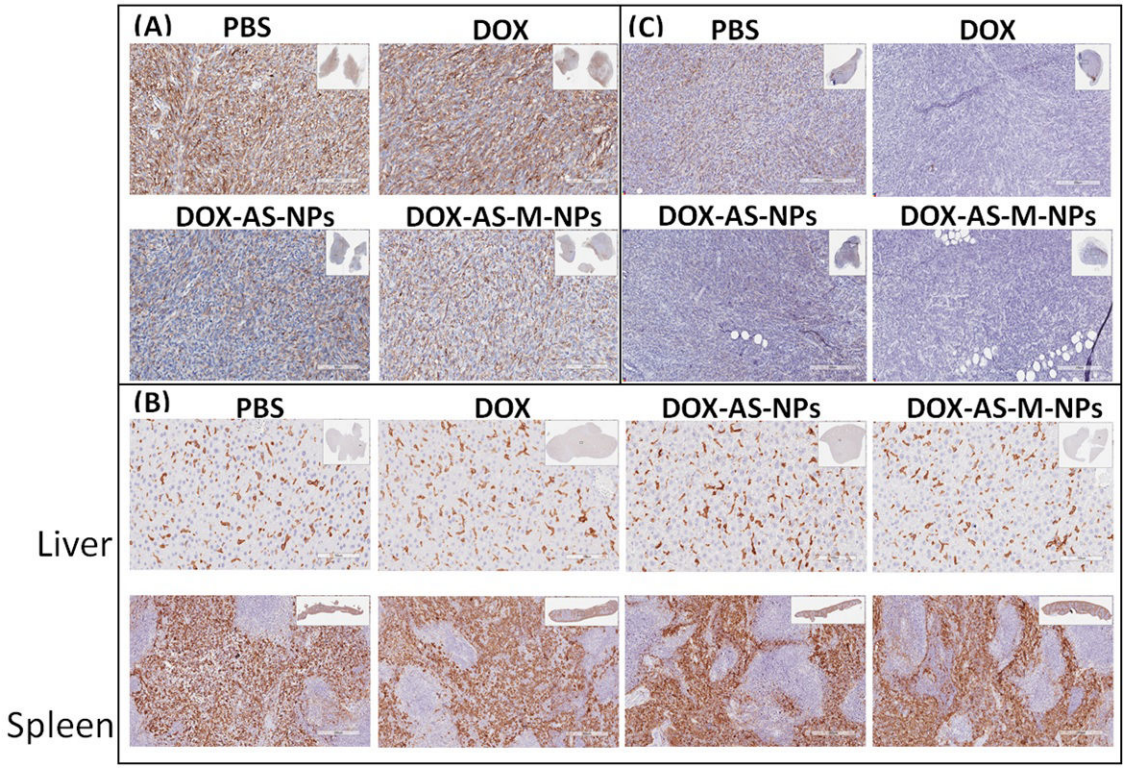


Fig. 6.

Representative images of M-Wnt tumor tissues (A) and major organs (B) after stained with an anti-F4/80 antibody (A) or RM0029-11H3 (B). Major organs and tumor tissues were collected from M-Wnt tumor-bearing mice treated with PBS, DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs. (C) As controls, tumor from mice that were treated with zoledronic acid before treating with PBS, DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs were also stained with an anti-F4/80antibody. Bar = 200μm.

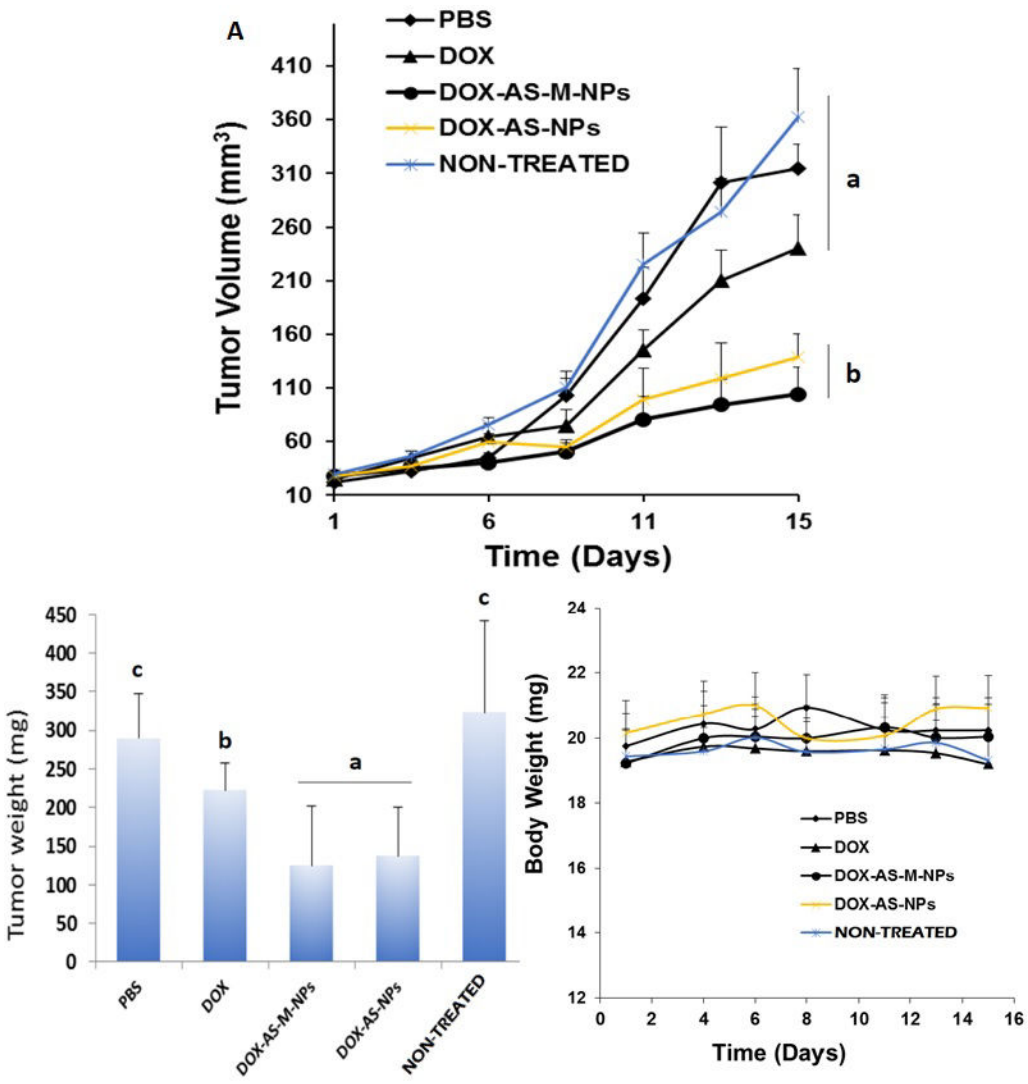


Fig. 7.

In M-Wnt tumor-bearing mice that were pre-treated with zoledronic acid to deplete macrophages, DOX-AS-M-PLGA-NPs are no longer more effective than DOX-AS-PLGA-NPs in inhibiting tumor growth. Mice were treated with zoledronic acid for 4 weeks starting right after tumor cell implantation and i.v. injected (2 weeks after tumor implantation) with PBS, DOX, DOX-AS-PLGA-NPs, or DOX-AS-M-PLGA-NPs 3 times a week, at DOX dose of 5 mg/kg, for 15 days. Shown are tumor volumes (A), tumor weights at the end of the study (B), and mouse body weights (C) ($^{a\text{-}c}$ $p < 0.05$). Data are mean ± S.D. (n = 7). NON-TREATED indicates mice in the group were implanted with tumors but did not receive zoledronic acid or any other treatments.

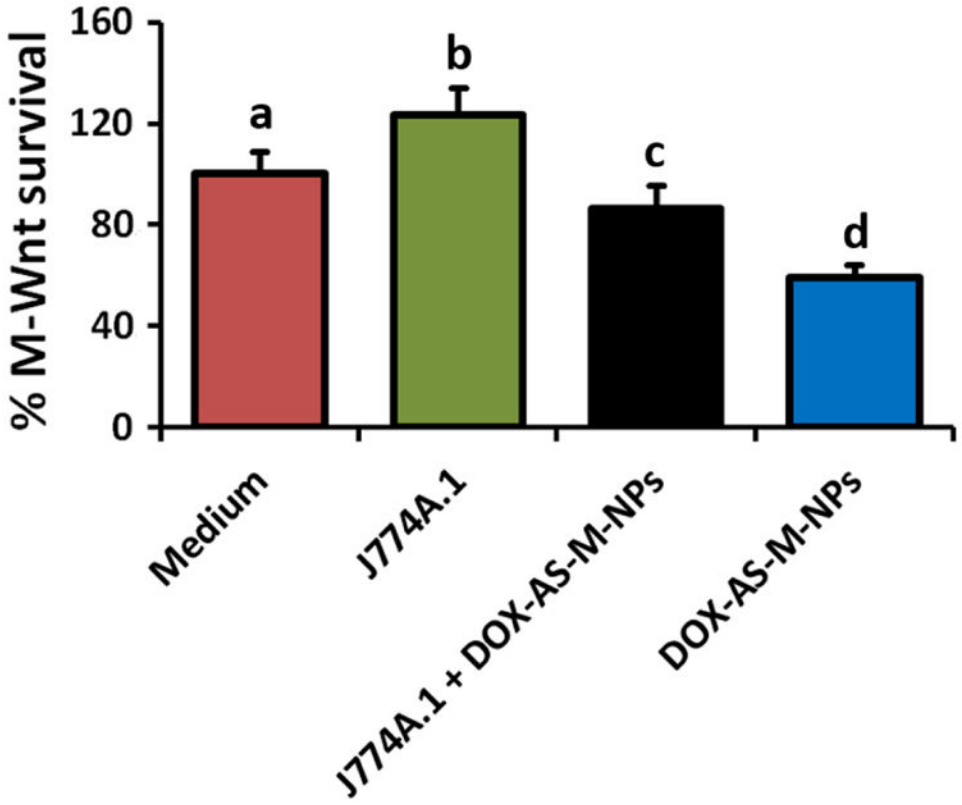


Fig. 8.

The effect of J774A.1 macrophages pre-incubated with DOX-AS-M-PLGA-NPs on M-Wnt tumor cell proliferation. As controls, J774A.1 cells alone, DOX-AS-M-PLGA-NPs alone, or cell culture medium alone were used ($^{a\text{-}d}$ $p < 0.05$). Data are mean ± S.D. ($n = 5$).