Click Treg Cell Engineering for Carrying DNPS Increasing the Effect of Tumor-Targeting

Zheng Liu

Lanzhou University

Hangcheng Zhao

Lanzhou University

Qian'ge Zhao

Lanzhou University

Jun Zhang (✉ zhangjun17@lzu.edu.cn)

Chongqing High-tech Zone People's Hospital

Research Article

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Abstract

The targeting properties of nanomaterials generally lead to poor efficacy. To address this issue, using the characteristic of Treg enriching in tumor, we achieved high enrichment of DOX in tumor tissue by using Treg as a nanodrug delivery carrier. The results showed that, compared with nanomedicine, Treg-DNPS can transport eight times DOX to tumor tissues than DNPS and reduce the accumulation of DOX in other organs, thereby enhancing tumor-killing by DNPs as well as improving biosafety. By using this platform, more drugs can achieve targeted delivery and tumor-killing effect.

Introduction

Solid tumors are one of the main causes of morbidity and mortality worldwide, with hundreds of thousands of people dying each year from cancer(1–3). Therefore, the development of effective therapeutic drugs against cancer is highly significant and of broad interest. Presently, many drugs have very high inhibitory effects on tumors in vitro. However, due to the complexity of the body and metabolism by the liver and kidney, many drugs cannot effectively enter tumor tissues (4-6). In order to improve the efficacy of antitumor drugs, a range of nanomaterials are used as carriers for drug delivery. However, a shortcoming of conventional drug delivery nanosystems is the lack of targeting to specific sites (7-10).

Although the role of regulatory T cells (Tregs) in cancer development remains unclear, studies have shown that CD4(+)FOXP3(+)CD25(+) Tregs accumulate in cancer tissues and promote tumor growth by inhibiting immune responses. On the contrary, it has also been observed that Tregs suppress inflammation caused by bacteria, thereby inhibiting cancer growth (11-15). These results suggest that Tregs can play a dual role in cancer progression. This may explain why Tregs are associated with a poor prognosis in some cancers and favorable outcomes in others. The clinical and prognostic relevance of Tregs in cancer patients depends on environmental factors, such as infectious agents, tumor-derived products, and locally produced cytokines, which determine the nature of the immune responses, including Treg production, recruitment, and survival. Interestingly, Treg cells can, however, be recruited into the tumor microenvironment, implying that Treg cells may be targeted to tumors.

In this study, we developed a well-defined drug-carrier approach to achieve targeted drug transport for cancer therapy. Treg carrying DOX-loaded polymer nanoparticles (DNPs), whereby the DOX was encapsulated in polylactic-co-glycolic acid (PLGA) to form a core covered with lecithin and DSPE-PEG-NH₂, were generated(Fig1 A-B). These homologous targeting nanoparticles (abbreviated as DNPs, for DOX-loaded PLGA nanoparticles) were connected to Treg cells by Ac4Ga1Naz, and the Treg-DNPs exhibited better targeting and tumor-suppressive effects in all experiments.

Results

DNPs Synthesis and Characterization.

The particle size measurement showed that the DNPs were 200 nm in size (SFig1 A). The fluorescence (FL) spectra of the DNPs showed that the emission peak of the DNPs was at 591 nm (SFig1B), which was close to the emission peak of free DOX. The UV–Visible absorption peaks of DOX in the DNPs were consistent with those of free DOX (490 nm) (SFig1 C). Therefore, the fluorescent properties of DOX were retained after encapsulation of the DNPs. Zeta potential analysis showed that the DNPs were stable in water (SFig 1D).

To investigate the DOX release behavior of the DNPs, we examined the DOX release levels at different times. As shown in SFig.1E, the DNPs released DOX continuously and efficiently in an acidic environment.

Validating Treg cells carrying DNPs

To enable Treg to carry DNPs, Tregs were processed by Ac4Ga1NAz. we first investigated the proper concentration of Ac4Ga1NAz to the Tregs. After two days of in vitro culture, we detected the proportion of Treg cells attached with Ac4Ga1NAz, and the results showed that 25 μ M of Ac4Ga1NAz was sufficient for most cells to have Ac4Ga1NAz attached to the membrane surface (Fig2 A-B). To determine the cellular damage caused by Ac4Ga1NAz binding to membrane, we used CCK8 to detect cell viability. The results showed that Ac4Ga1NAz did not cause damage to cells (Fig2 C).

Using DBCO-NH₂ as a cross-linker, we linked Treg cells to the DNPs. The results of CCK8 showed that DNPs did not damage the cells (Fig2 D). Further, we used electron microscopy to determine cell-particle attachment, and results revealed many DNPs attached to the cell surface, whereas Tregs without DBCO-NH₂ had no DNPs attached (Fig2 E). Immunofluorescence experiments yielded similar results (Fig2 F and SFig2).

Treg-DNPs can inhibit the proliferation, migration, and invasion of tumor cells in vitro, and promote tumor apoptosis

In order to determine whether Tregs target tumors, we selected CCL22, a protein highly expressed in tumors and capable of enriching Tregs. We verified the migration and invasion of Tregs by transwell assay, and we determined the number of cells using CCK8 assay. The results showed that the Tregs targeted tumors (Fig3 A). In addition, EDU experiments showed that the Treg-DNPs inhibited more tumor cell proliferation than Treg + DNPs and DOX (Fig3 B-C). Transwell experiments showed that the Treg-DNPs inhibited tumor cell migration and invasion more than Treg + DNPs and DOX (SFig3 A-C). Using AKT, bcl2, and capase3 as protein markers for cell proliferation and apoptosis, we determined the expression levels of related proteins in tumor cells treated under different conditions. The results showed that the levels of Bcl2 and p-AKT decreased, whereas the expression level of capsase3 increased (Fig3 D-E). This is consistent with the cell test results.

Enrichment level of DOX

Treg-DNPs accumulation in tumors and major organs was investigated using fluorescence imaging of DOX 48 h after intravenous injection (Fig4 A) in mice. In the DOX and Treg + DNP groups, DOX signal

mainly accumulated in the kidneys and tumors. However, reduced DOX signal was observed in the kidneys of the group treated with Treg-DNPs. In addition, DOX accumulation was significantly enhanced in the tumors of the Treg-DNPs group, as the DOX signal in the Treg-DNPs group was higher than that in the Treg + DNPs and DOX groups. These results confirmed that Treg-DNPs have an excellent tumor-targeting effect.

***In vivo* antitumor effect**

To verify the anti-tumor effect of Treg-DNPs *in vivo*, we used K1654 and B16-F10 mouse tumor models. *In vivo* imaging showed that the anti-tumor effect of Treg-DNPs *in vivo* was higher than that of the Treg + DNPs and DOX groups (Fig4 B-D). The survival curve of the mice showed that all the mice in the PBS and the DOX groups died at 30 days, while the survival rates of the groups treated with Treg + DNPs were approximately 35% (B16-F10) and 20% (K1375), respectively, and the survival rate of the Treg-DNPs group was 100% (B16-F10) and 70% (K1375) (SFig4 A). This indicates that Treg-DNPs can exhibit a better therapeutic effect by selective tumor targeting. There was no obvious variation in the body weights of the mice in any of the treated groups, suggesting that all of the experimental treatments were well tolerated (Fig4 B).

After two weeks of treatment, the tumor tissues were sectioned and EDU staining was performed to detect the level of tumor cell proliferation in the tissues. Compared with the Treg+DNPs and DOX group, significantly inhibition was noted in the tumors of the Treg + DNPs group, which was attributed to higher DOX accumulation via Treg targeting (SFig5 A-B). This result was also verified by Western blotting (SFig5 C-D).

As expected, Treg + DNPs showed the highest level of cancer cell damage as a result of Treg targeting.

To determine whether the therapeutic effect of Treg + DNPs was related to the enrichment of Tregs in the tumors, we analyzed the Treg content of the tumor tissues. Compared to the PBS and DOX groups, the Treg content in the Treg-DNPs and Treg + DNP tissues was higher (SFig6 A-E).

Biocompatibility assay

The biosafety was assessed by histology of the organs (heart, liver, spleen, lung, and kidney) at the end of the experiment (SFig7 A-B). Visual evidence of myocardial tissue damage was observed in the hearts of the free-DOX group, suggesting cardiotoxicity of free DOX, whereas the DNPs had no discernible toxic effects on the heart. Further quantification of serum cTnI (cardiac troponin I, a marker of cardiac function) (SFig7 A), alanine aminotransferase/aspartate aminotransferase (markers of liver function) (SFig7 B-C), creatinine, and blood urea nitrogen (markers of renal function) (SFig7 D-E) concentrations revealed that the levels of cTnI in the DNPs group were similar to those in the control group, whereas elevated cTnI concentrations in the free-DOX group indicated cardiac damage. These results suggest that cancer cell membrane-biomimetic DHCNPs can deliver highly effective chemotherapy with minimal toxicity to normal tissues.

Discussion

In this study, we produced nanoparticle attached regulatory T cells which targets tumor tissue. As a result, the Treg enrichment in tumor tissues enhances the targeting of nanoparticles. The experimental results show that Treg-DNPs can effectively transport DOX to tumor cells or tissues and reduce the accumulation of DOX in other organs, thereby enhancing tumor cell killing by DNPs as well as improving biosafety.

Drug delivery and target remains a problem, because tumor accumulation is dependent on the presence of a leaky tumor vasculature, and particles often become trapped perivascularly in tumors (16–21). Nanoparticle has been reported to be a promising strategy for this, however, how to deliver nanoparticles to target tumor tissues is still an obstacle. To overcome this obstacle, we generated a nanoparticle carrying system by Tregs. The nanoparticles were able to be delivered and accumulated around the tumor cells. Although whether promotive or inhibitory role on tumor tissue played by Tregs was under debate, the interact between Tregs and tumor cells was sure. This inspired us to develop this delivery system.

The results suggest that Tregs carrying DNPs were successfully generated, and DNPs were delivered to the target tumor tissues. We also demonstrate that inhibitory effects were observed after the delivery of DNPs by Tregs.

Conclusion

This study reported development of a well-defined drug-carrying approach for targeted drug transport for cancer therapy. Treg-DNPs were generated that consist of Treg cells carrying DOX-loaded polymer nanoparticles. These Treg-DNPs utilize the characteristic that Treg cells are enriched in vivo, thereby achieving precise delivery of antitumor drugs. Both the in vivo and in vitro experiments showed inhibitory effects on tumor cells. As a new type of living drug, Treg-DNPs not only exhibit high targeting and biological safety but also good tumor killing. Overall, this work provides a new approach to cancer therapy that uses cells to selectively carry anticancer drugs to tumors.

Materials And Methods

Materials

DOX (CAS [23214-92-8](#)), lecithin (CAS [8002-43-5](#)), DSPE-PEG-NH₂ (CAS [474922-26-4](#)), and PLGA (CAS [26780-50-7](#)) were purchased from Sigma-Aldrich (USA). Transwell was purchased from Corning (USA).

DNPs synthesis and characterization

Lecithin (10mg/mL, 12μL, dissolved in alcohol), DSPE-PEG-NH₂ (10mg/mL, 18μL, dissolved in alcohol), and DOX (1mg/mL–12μL dissolved in alcohol) were added to 2mL of 4% aqueous EtOH, and 1 mL of PLGA solution (dissolved in acetonitrile, 2mg/mL) was then added dropwise under sonication using an

ultrasonic processor (VCX130, USA) at a frequency of 20 kHz and 32 W power for 5 min to form the DNPs. Ultrafiltration was then performed using a dialysis membrane.

The size distribution of the nanoparticles was determined by dynamic light scattering (DLS) using a Malvern Zetasizer (Nano ZS, Malvern, USA) at 25 °C. The morphology and structure were determined using transmission electron microscopy (TEM; F20, TECHNI, USA). Absorption spectra were obtained by UV-Vis spectroscopy (Lambda25, Perkin-Elmer, USA), and fluorescence spectra were obtained by fluorescence spectroscopy (F900, Edinburgh Instruments Ltd., UK) at an emission wavelength of 480 nm. 6 Protein detection of DHCNPs. The zeta potential was analyzed using a Delsa Nano C Zeta Potential Analyzer (Beckman Coulter Inc., USA).

Western blot assay

Proteins were subjected to 10% SDS-PAGE gels and then transferred to PVDF membranes. For immunoblotting, the membranes were incubated overnight with anti-AKT, anti-P-AKT, anti-ERK, anti-P-ERK, anti-caspase3, and anti-Bcl2 antibodies (Abcam, USA) followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h. The antigens were then detected by enhanced chemiluminescence (ECL, Pierce, USA).

Cells culture

Treg cells were isolated from the spleens of treated WT mice and expanded according to a Treg expansion kit protocol (Miltenyi Biotec). Tumor cells were cultured in DMEM medium containing 10% heat-inactivated FBS, 1% penicillin, and 1% streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Treg cell content analysis

For flow cytometry, tumors were harvested and dissected into fragments by cutting, digested in tumor digestion buffer for 2 h at 37 °C. The cells were collected by centrifugation for 3 min at 500 × g and then stained following the protocol of the mouse Treg flow kit (FOXP3 Alexa Fluor 488/CD4 APC/CD25 (BioLegend) and identified using a FACSCalibur™ flow cytometer (BD).

For confocal laser microscopy, frozen sections (10 μm) were prepared from tumor-bearing mice. Double immunofluorescence staining was performed on frozen sections of anti-Foxp3 antibody (Abcam) and DAPI using a Zeiss LSM510 confocal laser-scanning microscope with a 63× objective

Treg-DNPs preparation

DBCO-NHS and DNPs (1:1) were incubated for 1 h at 37 °C. Free DBCO-NHS was removed by ultrafiltration in centrifuge tubes (Millipore) for 10 min. After Ac4Ga1NAz treatment for 2 days, a total of 2 × 10⁶ Treg cells were incubated with DBCO-NHS-DNPs for 1 h and then washed three times with PBS.

EDU assay

For cells, Treg-DNPs, PBS, DOX, or Treg + DNPS were added in the upper chambers of a transwell with 100 μ L per chamber, and the cancer cells were seeded in the lower compartment. After 48 h, EDU staining was performed using a EDU assay kit (Beyotime, C1088).

For tissues, between two weeks of treatment, the mice were injected with EdU (50mg/kg EdU in PBS) for five consecutive days. Then, frozen tissue sections (10 μ M) were prepared from mice bearing tumors. EDU staining was performed using a EDU assay kit.

Migration and invasion assays

Treg-DNPs, PBS, DOX, or Treg + DNPS were added into the upper chambers of the transwell as 100 μ L per chamber, and the cancer cells were seeded in the lower compartment. After 48 h, the cancer cells (5×10^4) were added to the upper chambers of a new transwell with low-serum medium (2%), and the cells were incubated for another 12–24 h. Cells that migrated through the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet for 15 min at room temperature, and imaged by microscopy

Statistical analysis. All the results are reported as mean $\pm$ SD. The differences among groups were determined using one-way ANOVA analysis and Student's t-test; (*) $P < 0.05$, (**) $P < 0.01$.

Declarations

Ethics approval and consent to participate. All animals received standard care, and animal procedures in this study were approved by lanzhou University.

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Not applicable.

Authors' contributions

JZ designed the research; ZL performed the research; HZ and QZ analyzed the data and wrote the paper. The author(s) read and approved the final manuscript.

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Availability of data and materials

The materials can be applied to the corresponding author for use.

Consent for publication

All authors agree to publish.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

The design and function of Treg-DNPS. (A) Synthesis of Treg-DNPS. DNPS were prepared by DOX-PLGA, lecithin, DSPE-PEG-NH₂, and then were linked to Treg cells (N₃) by DBCO-NHS. (B) cellular functions of Treg-DNPS, including tumor-targeted, inhibiting cancer cell proliferation, migration and invasion, and effecting tumor-related protein expression.

Figure 2

Preparation and characterizations of Treg-DNPS. (A) Flow cytometry analysis

of Ac4Ga1NAz-Treg. (B) Statistical results of *positive* cells proportion. (C) the result of Ac4Ga1NAz-Treg cells activity tested by CCK8. (D) the result of Treg-DNPS cells activity tested by CCK8. (E) SEM images of Treg, Treg+DNPS and Treg-DNPS. Scale bar = 1 μ m. After incubation with DNPS, N3-labeled Treg cells

were successfully conjugated with DBCO-NHS by the click reaction. For interpretation of the references to colour in this figure legend, the reader is referred to the Webversion of this article. (F) Confocal laser scanning microscopy (CLSM) images of Treg-DNPS. DNPS was conjugated to the Treg cell surface via the DBCO/N3 click reaction. Scale bar = 5 μ m. Data are presented as the means and standard errors. Scale bar = 20 mm. Significance was calculated using one-way ANOVA with Tukey's posttest, $n = 5$. * $p < 0.05$, ** $p < 0.01$.

Figure 3

The tumor suppression of Treg-DNPS in vitro. (A) The Treg recruitment of CCL22 tested by transwell and CCK8. (B) The inhibition of cell proliferation by Treg-DNPS (NC, DOX, Treg+DNPS). (C) Statistical results of cell proliferation proportion. (D) The expression of BCL2, Caspsae3, P-AKT, AKT, and GAPDH. (E) Statistical results of protein expression. Data are presented as the means and standard errors. Scale bar = 20 μ m. Significance was calculated using one-way ANOVA with Tukey's posttest, n = 5. *p < 0.05, **p < 0.01.

Figure 4

The anti-tumor effect of Treg-DNPS in vivo. (A) Treg-DNPS accumulation in tumors and major organs was investigated using fluorescence imaging of DOX. (B) Tumor-bearing mice were monitored by bioluminescence imaging. (C) Individual tumor growth curves were constructed by measuring tumor volume using calipers at the indicated time points. (D) weights of excised tumors at the end of

experiments. Significance was calculated using one-way ANOVA with Tukey's posttest, $n = 5$. * $p < 0.05$, ** $p < 0.01$.

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