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A non-viral gene therapy for melanoma by staphylococcal enterotoxin A

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ABSTRACT

Staphylococcal enterotoxin A (SEA) derived from *Staphylococcus aureus*, as a superantigen, shows potential for cancer immunotherapy, but systemic immunotoxicity restricts its clinical application. Targeted delivery of SEA to tumor site provides a promising option for reducing the systemic toxicity. Here, we constructed an iRGD peptide (H-[Cys-Arg-Gly-Asp-Lys-Gly-Pro-Asp-Cys]-NH_2) modified nanoparticle (iDPP) to deliver plasmids encoding SEA for melanoma treatment. The iDPP/SEA nanocomplexes efficiently mediated SEA expression in B16-F10 cells *in vivo* and *in vitro* and induced the activation of lymphocytes and maturation of murine bone marrow-derived dendritic cells (BMDCs) *in vitro*. In the subcutaneous B16-F10 melanoma model, the iDPP/SEA nanocomplexes could effectively enhance immune response and T lymphocytes infiltration in tumor site after intravenous administration, thereby considerably decreased melanoma growth. Meanwhile, no obvious adverse effect was observed after intravenous administration of the iDPP/SEA nanocomplexes *in vivo*. Our findings demonstrated that gene therapy of SEA is a potential candidate for melanoma treatment.

Melanoma is one of the most common fetal skin tumors that exhibits a high degree of malignancy, attributed to its severe invasiveness, and resistance to systemic radiation and chemotherapy [1,2]. In 2020, approximately 325,000 new cases of melanoma and 57,000 deaths were reported globally [3]. Immunotherapy provides alternative treatment options for tumors, typically involving T lymphocytes that are reactive to tumor-specific neoantigens or tumor-associated antigens (TAAs) [4–8]. The application of immune checkpoint treatment has produced impressive outcomes, such as long-term therapeutic effects on advanced metastatic malignancies including melanoma [9–12]. Nevertheless, only a few cancer patients who are sensitive to immune checkpoint therapy can benefit from these treatments, as most common types of cancers do not show abundant mutations and lack infiltrating immune cells [13–15]. Thus, new strategies for the treatment of melanoma still need to be developed.

Superantigens, a type of polyclonal activator for T cells, have the potential to serve in cancer immunotherapy given the fact that they could bypass the requirement for conventional intracellular antigen presentation and lack histocompatibility complex class (MHC) restriction [16–18]. *Staphylococcus aureus*, as one of the clinically common pathogens, secretes enterotoxins which act as superantigens [19,20]. Staphylococcal enterotoxin A (SEA) is the most common type of enterotoxins that has been widely reported, which could cause massive T cell activation with extremely low concentrations (1–10 ng/L) [20]. SEA binds major histocompatibility complex II (MHC-II) on antigen presenting cells (APCs) and interferes with T-cell receptor (TCR) Vβ chain on T cells without presenting process in APCs, which shows a difference from normal antigens. Consequently, T lymphocytes release a large amounts of cytokines like tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which could stimulate immune system and have a powerful killing effect on tumor cells [21]. Therefore, SEA is considered as a good candidate for cancer immunotherapy. Thamm *et al.* created a conjugation of lipid-SEA-canine-interleukin 2 (Lip-SEA-canine-IL2) for soft tissue sarcoma treatment [22]. However, the activation of staphylococcal enterotoxin to lymphocytes is non-

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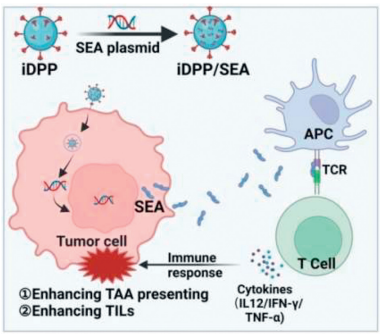
specific, and the excessive activation may cause uncontrolled inflammation, multiple organ failure syndrome (MODS), and toxic shock syndrome (TSS) [23–25]. Thus, it is necessary to develop a tumor-targeted superantigen strategy.

Gene therapy, as a promising alternative strategy, provides a potential option to address the challenges of SEA-derived immunotherapy [26,27]. The key point of gene therapy is that the vectors can efficiently deliver gene to the targeted tumor tissue for expression. Compared to viral vectors, non-viral vectors possess lower immunogenicity and higher safety [28]. In our previous research, we designed a non-viral gene delivery system-iRGD/DOTAP/MPEG-PDLLA (iDPP), which is a neutral targeted gene vector by complexing cationic nanoparticles with the therapeutic plasmids through electrostatic interaction with high transfection efficiency on melanoma cell lines [29]. In current study, we developed the iDPP/SEA nanocomplexes to deliver SEA to B16-F10 melanoma cells. Subsequently, we investigated the effect of iDPP/SEA nanocomplexes on the activation of the immune system both *in vitro* and *in vivo*. The iDPP/SEA nanocomplexes, as a potential immune agonist, can effectively inhibit tumor growth by activating immune response, which provides a novel option for melanoma treatment (Scheme 1).

Initially, the nucleic acid sequence encoding SEA was synthesized and constructed into the empty vector pVAX1. Previous research had confirmed that iDPP nanoparticles can efficiently deliver genes to melanoma cells for expression (Fig. 1a) [29]. Following that, we prepared the iDPP/SEA nanocomplexes by previous method. The image of transmission electron microscope (TEM) showed that iDPP/SEA nanocomplexes possessed a sphere-like morphology with a mean particle size of 50 nm (Fig. 1b). And the mean hydrodynamic particle size and zeta potential of the iDPP/SEA nanocomplexes were 147 ± 1 nm and -0.4 ± 0.4 mV, respectively (Figs. 1c and d). Then, green fluorescent protein plasmid (pGFP) was used as a reporter plasmid to evaluate the transfection efficiency, and the result showed that iDPP nanoparticles could mediated the expression of GFP in B16-F10 cells with high efficiency (Fig. 1e). To investigate the expression of SEA in tumor cells, we detected mRNA and protein levels of SEA in melanoma cell B16-F10 of normal saline (NS) group, iDPP/pVAX group (empty vector control), and iDPP/SEA group, respectively. After treatment for 48 h, both mRNA and protein expression of SEA were detected in iDPP/SEA group, indicating that the iDPP nanoparticles successfully delivered SEA plasmid into melanoma cells for expression (Figs. 1f and g). In order to assess the cytotoxicity of iDPP/SEA nanocomplexes, we treated B16-F10 cells with different dosages of iDPP/SEA nanocomplexes and iDPP/pVAX nanocomplexes, respectively, and measured cell viability through cell counting kit-8 (CCK-8) assay. The results illustrated that the cell viability of iDPP/SEA groups was comparable to that of the iDPP/PVAX group, which showed that the SEA overexpression did not cause additional cytotoxicity (Fig. 1h).



Scheme 1. iDPP/SEA enhances immunity for melanoma treatment. The picture was generated with Biorender.

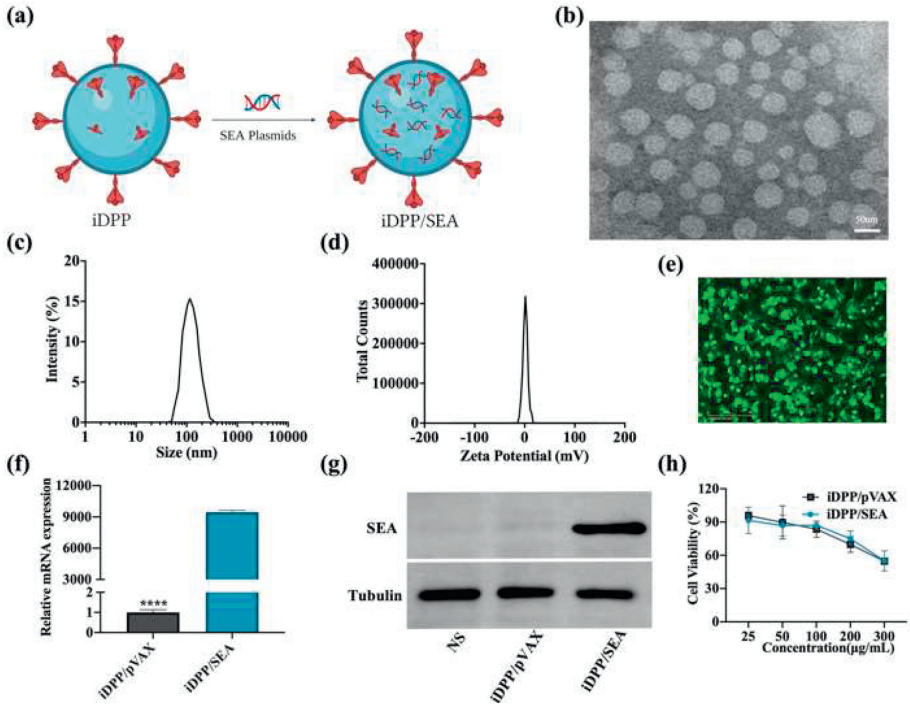


Fig. 1. iDPP/SEA nanocomplexes mediate SEA expression in B16-F10 cells. (a) Scheme of the assembly of iDPP/SEA nanocomplexes including iRGD, mPEG2000-PLA2000, DOTAP and pVAX-SEA plasmids. (b) Transmission electron microscopic image of iDPP/SEA nanocomplexes. Scale bar: 50 nm. (c) Size distribution of iDPP/SEA nanocomplexes. (d) Zeta potential of iDPP/SEA nanocomplexes. (e) Fluorescent image of B16-F10 cells transfected with iDPP/GFP nanocomplexes. Scale bar: 200 μm. (f) The mRNA expression level of SEA in B16-F10 cells transfected with iDPP/pVAX nanocomplexes, iDPP/SEA nanocomplexes. $^{****}P < 0.0001$. (g) The protein expression of SEA in NS group, iDPP/pVAX group, iDPP/SEA group analyzed by Western blot. (h) Cell viability of B16-F10 cells treated with iDPP/pVAX nanocomplexes, iDPP/SEA nanocomplexes (MTT assay, $n = 5$). Data are mean ± standard deviation (SD).

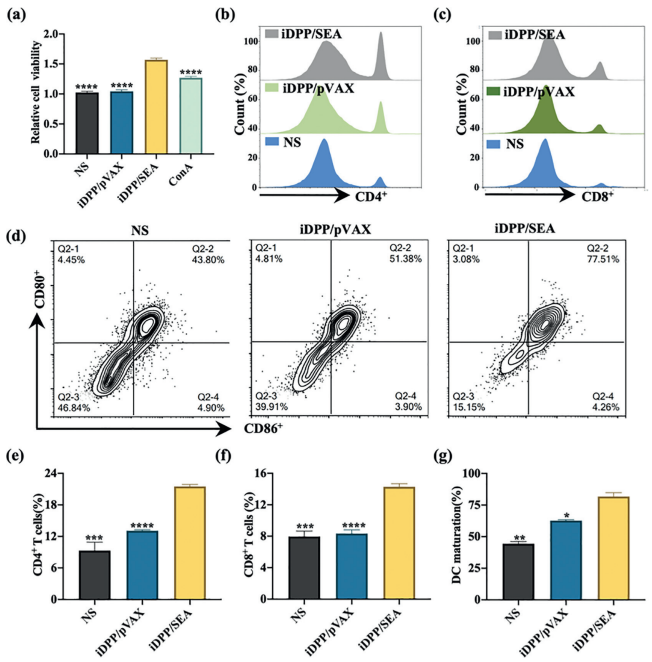


Fig. 2. SEA expression in B16-F10 induces lymphocytes activation and DCs maturation *in vitro*. (a) Cell viability of splenic lymphocytes cells that co-cultured with B16-F10 cells of NS, iDPP/pVAX, iDPP/SEA or concanavalin A group. (b, c, e, f) Detection of $CD4^+/CD8^+$ splenic lymphocytes cells that co-cultured with B16-F10 cells of NS, iDPP/pVAX, iDPP/SEA ($n = 3$). (d, g) DCs maturation when incubation with B16-F10 cells of NS, iDPP/pVAX, iDPP/SEA group ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data are mean ± SD.

SEA mainly serves as a superantigen to activate immune response and attenuate tumorigenesis. To assess the ability of iDPP/SEA nanocomplexes to activate the immune system, we explored how iDPP/SEA nanocomplexes affected splenic lymphocyte proliferation *in vitro*. When splenic lymphocytes were incubated with the supernatant of iDPP/SEA group, its cell viability was increased, which was higher than the positive control concanavalin A (Con A) group (Fig. 2a). Con A (50 μg/mL) is a type of carbohydrate-binding protein with strong mitogenic effect that can promote the lymphocyte transformation [30]. Furthermore, we detected the subsets of splenic lymphocytes. Flow cytometry analysis revealed that co-culture of splenic lymphocytes and B16-F10 cells treated by iDPP/SEA nanocomplexes resulted in an increase in the number of $CD4^+$ and $CD8^+$ T cells in splenic lymphocytes (Figs. 2b and c). The ratio (21.5%) of $CD4^+$ T cells in splenic lymphocytes that incubated with B16-F10 cells of iDPP/SEA group was 1.65-fold to that of the iDPP/pVAX group (13%) and 2.31-fold to that of the NS group (9.3%), respectively (Fig. 2e). The ratio (14.2%) of $CD8^+$ T cells in splenic lymphocytes that incubated with B16-F10 cells of iDPP/SEA group treatment was 1.71-fold to that of the iDPP/pVAX group (8.3%) and 1.79-fold to that of the NS group (7.9%), respectively (Fig. 2f). Both ratios of $CD4^+$ and $CD8^+$ T cells were significantly higher than the control group. Our results above demonstrated that B16-F10 cells transfected with SEA can efficiently enhance the proliferation and differentiation of splenic lymphocytes.

Following that, the activation of dendritic cells (DCs) by B16-F10 cells treated by iDPP/SEA was examined. Murine bone-marrow dendritic cells (BMDCs) were co-cultured with B16-F10 cells of NS, pVAX, and iDPP/SEA group for 24 h, respectively. Flow cytometry was used to investigate the maturation of DCs. When incubated with B16-F10 cells of the iDPP/SEA group, the ratio of mature DCs were considerably higher than those of the iDPP/pVAX and NS groups (Fig. 2d). The proportion of $CD11c^+CD80^+CD86^+$ DCs in the iDPP/SEA group (84.4%) was 1.57-fold higher than that in the pVAX group (53.7%) and 1.85-fold higher than that in the NS group (45.6%) (Fig. 2g). Our findings implied that cancer cells treated with iDPP/SEA nanocomplexes could effectively promote the maturation of DCs.

The anti-tumor efficiency of iDPP/SEA nanocomplexes *in vivo* was evaluated using the B16-F10 subcutaneous melanoma model. All the animal experiments were approved by the Animal Care Committee of Sichuan University. The plasmid pGL6 was utilized as a reporter to assess the tumor targeting capabilities of the iDPP delivery system. After tail vein injection, the iDPP/pGL6 nanocomplexes could concentrate to the melanoma tissue and express firefly luciferase, demonstrating that iDPP delivery system has good targeting ability *in vivo* (Fig. 3a). In the B16-F10 subcutaneous melanoma model, the growth rate of tumor volume decreased significantly in iDPP/SEA group compared to the NS and iDPP/pVAX (Figs. 3b and c). After measuring the tumor weight of each group, the tumor inhibition rate of each group was calculated. The tumor weights were 0.99 ± 0.19 g in NS group, 0.61 ± 0.11 g in iDPP/pVAX group, and 0.21 ± 0.18 g in iDPP/SEA group (Fig. 3d). The tumor inhibition rate of iDPP/SEA gene preparation was about 68%, which was significantly higher than that of iDPP/pVAX group. The mice treated with iDPP/SEA nanocomplexes also survived longer than other groups (Fig. 3e). Furthermore, hematoxylin-eosin (H&E) staining showed that the density of tumor cells in melanoma tissues of iDPP/SEA group was significantly lower than that in other groups (Fig. 3f). In addition, a high number of inflammatory cells were observed in melanoma tissue in the iDPP/SEA group.

Furthermore, we analyzed T cells infiltration in tumor tissues. The results of flow cytometry analysis demonstrated that the proportion of $CD4^+$ T cells in the tumor tissues of the NS group, iDPP/pVAX group, and iDPP/SEA were 0.07% ± 0.02%, 0.2% ± 0.06%, and 6.52% ± 0.7%, respectively. The $CD8^+$ T cells in the tumor tissues of the NS group, iDPP/pVAX group, and iDPP/SEA were 0.45% ± 0.03%, 0.42% ± 0.03%, and 5.69% ± 0.65%, respectively (Figs. 3g–i). Meanwhile, we observed more $CD8^+$ T cells and $CD4^+$ T cells in the tumor tissues of the iDPP/SEA group through immunofluorescence staining (Fig. 3j). Therefore, the T cells infiltration in the tumors of the iDPP/SEA group was significantly higher than that of the iDPP/pVAX group. All the results demonstrated that SEA could effectively recruit and activate cytotoxic T cells for melanoma treatment.

The safety of the iDPP/SEA formulation was examined from several perspectives *in vivo*. We did not observe obvious pathological changes, such as edema, inflammation, degeneration, or necrosis, in heart, liver, spleen, lung, and kidney of each treatment group through H&E staining (Fig. 4a). Furthermore, the body weight of mice treated with iDPP/SEA nanocomplexes was not substantially different from that of NS (Fig. 4b) after treatment. The biochemical markers, such as alanine aminotransferase (ALT), creatinine (CREA), and aspartate aminotransferase (AST) of iDPP/SEA group were not significantly different from those in NS group (Figs. 4c–e). As all mentioned above, the intravenous injection of iDPP/SEA nanocomplexes did not exhibit obvious side effect *in vivo*.

Superantigens have potential in tumor therapy as an immunoadjuvant [23]. In recent research, superantigen was used to enhance chimeric antigen receptor T cell (CAR-T) potency and boost CAR-T efficacy against solid tumors [31]. A lot of work has been carried out to enhance the tumor targeting of superantigens to reduce the side effects. Yousefi *et al.* developed a method of fusing the third loop of transforming growth factor α (TGFαL3) with staphylococcal enterotoxin type B (SEB) to effectively inhibit the growth of breast tumors [32]. Golob-Urbanc *et al.* designed a superantigen fusion proteins which split a superantigen into two fragments, individually inactive, until both fragments came into close proximity and reassembled into a biologically active

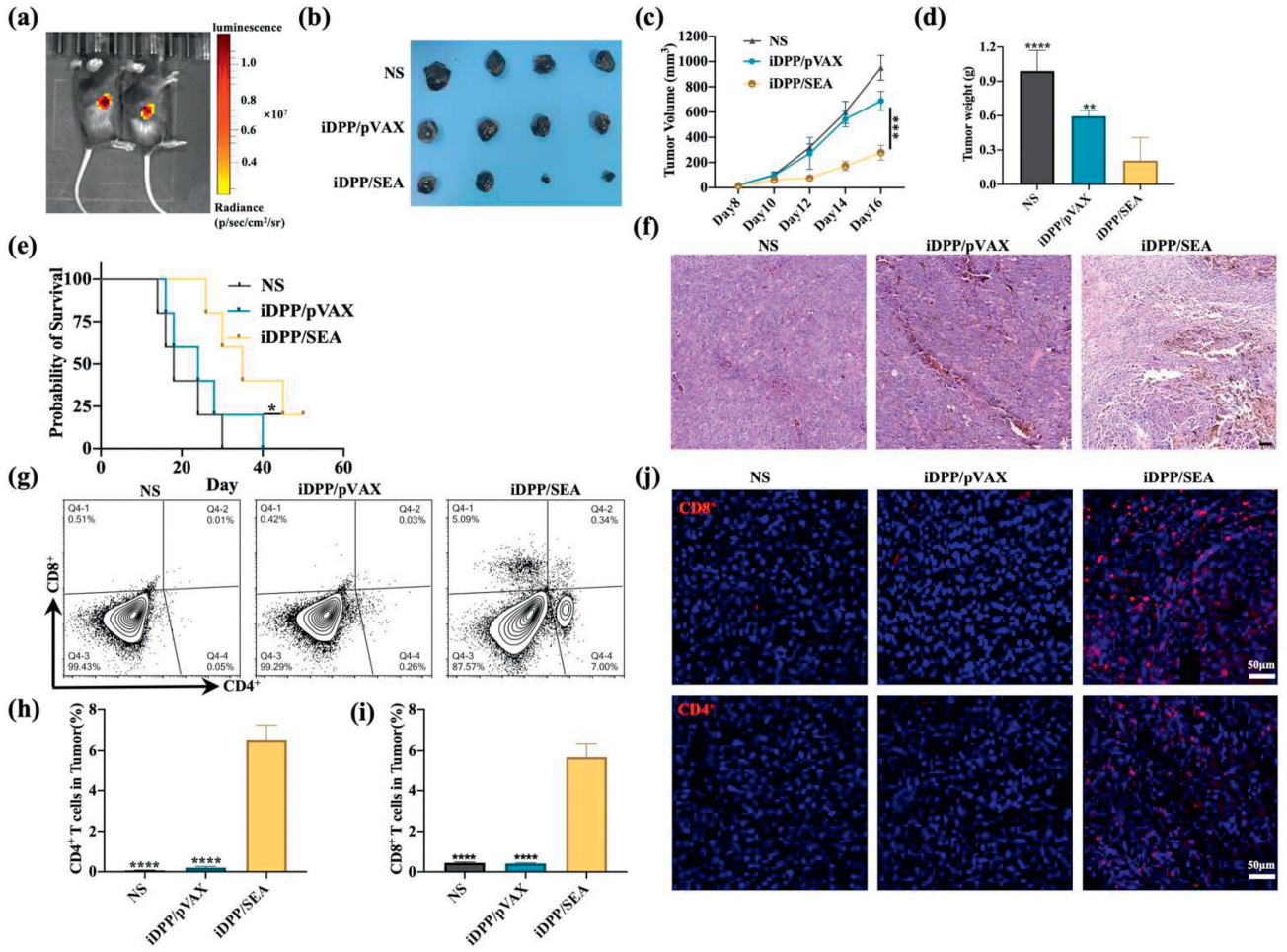


Fig. 3. Antitumor activity of iDPP/SEA by intravenous injection *in vivo*. (a) *In vivo* imaging of luciferase expression in B16-F10 cells-bearing mice treated with iDPP/pGL6 at 72 h post-injection. (b) Tumor masses. (c) Tumor volume ($n = 7$). (d) Tumor weights in each treatment group ($n = 7$). (e) Survival analysis of mice after 50 d of initial tumor cell inoculation ($n = 5$). (f) Representative histological sections of tumors stained with H&E. Scale bar: 50 μm. (g) The numbers of $CD8^+$ T cells and $CD4^+$ T cells in tumor tissues were examined by flow cytometry. (h, i) Statistics of $CD8^+$ T cells and $CD4^+$ T cells ratio of different groups ($n = 3$). (j) Fluorescence microscopic images of $CD8^+$ T cells and $CD4^+$ T cells in tumor tissues. Scale bar: 50 μm ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data are mean ± SD.

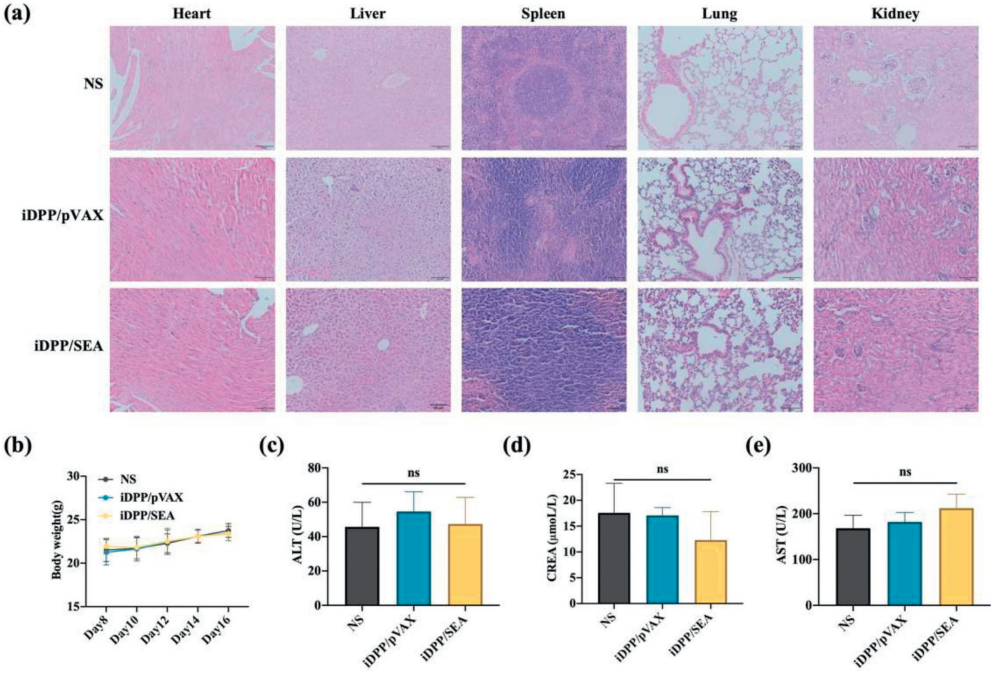


Fig. 4. Safety assessment. (a) H&E staining of vital organs of each group. Scale bar: 50 μm. (b) Body weight of each group after treatment ($n = 7$). (c–e) The analyses of important blood biochemistry indexes of each group ($n = 3$). ns, $P > 0.05$. Data are mean ± SD.

form capable of activating T cell response [33]. Distinguished from strategies like tumor-specific antibody conjugates, tumor-targeting nanoparticle iDPP was used to deliver SEA in this work. It could express SEA at tumor sites, trigger T cells infiltration which shows great potential to enhance lymphocytes infiltration at tumor site, which might change “cold tumor” to “hot tumor”.

In summary, we have developed a new gene formulation based on iDPP nanoparticles to deliver SEA plasmids at tumor site for melanoma treatment. These nanoparticles could actively accumulate in melanoma tumor sites due to their specific targeting capability and stimulate anti-tumor immune response after expressing superantigen SEA in both *in vivo* and *in vitro*. Overall, this study devised a promising approach derived from superantigen SEA for melanoma treatment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108822.

References

[1] L.E. Davis, S.C. Shalin, A.J. Tackett, Cancer Biol. Ther. 20 (2019) 1366–1379.
[2] C. Garbe, T. Amaral, K. Peris, et al., Eur. J. Cancer 170 (2022) 236–255.
[3] M. Arnold, D. Singh, M. Laversanne, et al., JAMA Dermatol. 158 (2022) 495–503.
[4] Z. Duan, Y. Luo, Transduct. Target Ther. 6 (2021) 127.
[5] K. DePeaux, G.M. Delgoffe, Nat. Rev. Immunol. 21 (2021) 785–797.
[6] N. Gong, N.C. Sheppard, M.M. Billingsley, et al., Nat. Nanotechnol. 16 (2021) 25–36.
[7] Z. Hong, X. Zan, T. Yu, et al., Chin. Chem. Lett. 34 (2023) 107603.
[8] L. Wen, X. Zan, Q. Pang, et al., Chin. Chem. Lett. 34 (2023) 108224.
[9] A.C. Huang, R. Zappasodi, Nat. Immunol. 23 (2022) 660–670.
[10] K.A. Lee, A.M. Thomas, L.A. Bolte, et al., Nat. Med. 28 (2022) 535–544.
[11] M.S. Carlino, J. Larkin, G.V. Long, Lancet 398 (2021) 1002–1014.
[12] Y. Hu, L. Lin, Z. Guo, et al., Chin. Chem. Lett. 32 (2021) 1770–1774.
[13] P. Sharma, B.A. Siddiqui, S. Anandhan, et al., Cancer Discov. 11 (2021) 838–857.
[14] S. Vukadin, F. Khaznadar, T. Kizivat, et al., Biomedicines 9 (2021) 835.
[15] M.T.Q. Duong, Y. Qin, S.H. You, J.J. Min, Exp. Mol. Med. 51 (2019) 1–15.
[16] M. Brown, N. Bhardwaj, Nat. Rev. Immunol. 21 (2021) 72.
[17] Y. Sagi, M. Shahar, M. Pinsker, J. Immunother. Cancer 9 (2021) A605.
[18] R. Kaempfer, Toxins 10 (2018) 459.
[19] D.L. Hu, S. Li, R. Fang, H.K. Ono, Animal Diseases 1 (2021) 7.
[20] A. Shivaee, M. Sedighi, A. Imani Fooladi, Ann. Ig. 32 (2020) 648–663.
[21] N. Benkerroum, Crit. Rev. Food Sci. Nutr. 58 (2018) 1943–1970.
[22] D.H. Thamm, I.D. Kurzman, E.G. Macewen, et al., Cancer Immunol. Immunother. 52 (2003) 473–480.
[23] S. Berger, A. Kunerl, S. Wasmuth, et al., Lancet Infect. Dis. 19 (2019) e313–e321.
[24] R.A. Porritt, L. Paschold, M.N. Rivas, et al., bioRxiv (2020), doi:10.1101/2020.11.09.372169.
[25] O. Yamasaki, S. Sugihara, A. Kajita, et al., J. Dermatol. 48 (2021) 547–550.
[26] T.M. Belete, Biologics 15 (2021) 67–77.
[27] C.E. Dunbar, K.A. High, J.K. Joung, et al., Science 359 (2018) eaan4672.
[28] H. Zu, D. Gao, AAPS J. 23 (2021) 78.
[29] L. Luo, Y.P. Yang, T. Du, et al., Adv. Funct. Mater. 28 (2018) 1800173.
[30] R. Palacios, J. Immunol. 128 (1982) 337–342.
[31] B. Scheidt, M. Wang, A.J. Oliver, et al., Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 25229–25235.
[32] F. Yousefi, S. Siadat, A. Saraji, et al., Tumour Biol. 37 (2016) 5305–5316.
[33] A. Golob-Urbanc, U. Rajčević, Ž. Strmšek, R. Jerala, J. Biol. Chem. 294 (2019) 6294–6305.