Natural blood plasma-based hydrogels as tumor vaccines delivery systems to enhance biomimetic recruitment of antigen presenting cells for tumor immunotherapy

Linghong Huang a,1, Sufen Peng a,1, Zonghua Liu a, Juncheng Zhang b, Ning Liu b,*, Jiansheng Lin c,*

a Department of Biomedical Engineering, Jinan University, Guangzhou, 510632, China
b Department of Bone and Joint Surgery, The First Affiliated Hospital of Jinan University, Guangzhou, 510632, China
c Department of Anatomy, Hunan University of Chinese Medicine, Changsha, 410208, China

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ABSTRACT

Tumor vaccines can inhibit or eliminate tumors by vaccinating hosts with tumor antigens to activate antigen-specific immune responses and have gained wild attention. However, their clinical application efficacy is often comprised due to the low safety and poor efficiency of vaccine adjuvants/carriers. Specifically, the adjuvants/carriers usually could not efficiently recruit antigen presenting cells (APCs) to capture the vaccines or directly damage these cells. Therefore, ideal tumor vaccine adjuvants/carriers should effectively recruit APCs and be friendly to the cells for well keeping their bio-functions. In this work, injectable natural blood plasma hydrogel was used for the first time to encapsulate tumor antigens and adjuvant (Mn2+) for the construction of a personalized tumor vaccine. This kind of natural hydrogel with extremely high bio-safety has great potential to friendly recruit APCs in a biomimetic manner by simulating the natural degradation process of subcutaneous blood stasis. The obtained results show that the natural blood plasma hydrogel-based tumor vaccines could significantly promote the recruitment of APCs, well maintain the immuno-functions of the cells, and finally induce efficient anti-tumor immune responses. Compared with traditional tumor vaccines, this natural blood plasma-based hydrogel provides a new strategy for the development of safe and effective tumor vaccines.

1. Introduction

Tumor vaccines exert anti-tumor function by immunizing tumor antigens to trigger antigen-specific immune responses [1]. In recent years, tumor vaccines have attracted considerable attention, and preventive tumor vaccines (Gardasil, Cervarix) and therapeutic tumor vaccines (Provenge) have been approved for marketing. The latest US clinical trial database (Clinical Trials) shows that more than 1000 tumor vaccines have entered phase I and II clinical studies and that more than 100 tumor vaccines have entered phase III and IV clinical studies. The clinical results show that these tumor vaccines can activate antitumor immune responses to different degrees and prolong the survival time of vaccinated patients. Although so many tumor vaccines have entered clinical research, very few have entered the market in the past ten years, mainly because the safety and effectiveness of their adjuvants/carriers are not qualified [2]. Unsafe adjuvants/carriers often cause local reactions such as necrosis, granulomas, ulcers, and sterile abscesses, as well as systemic reactions such as fever, arthritis, allergies, and tissue toxicity [3–5]. In particular, the unsafe adjuvants/carriers can damage antigen presenting cells (APCs) and hence hinder their bio-functions. Therefore, it is urgent to develop extremely safe and efficient tumor vaccine adjuvants/carriers.

To trigger an effective immune response in tumor vaccine therapy, the first and probably the most important step is to friendly recruit APCs to capture the vaccine components at injection sites. In the natural physiological process, the invading pathogens recruit APCs through the pathogen associated molecular patterns (PAMP) on microbes [6]. In the vaccination process, the administered vaccines recruit APCs through their PAMP-like immune stimulation effect, which mainly relies on the

* Corresponding author.
** Corresponding author.
E-mail addresses: liuning@163.com (N. Liu), linjiansheng1020@163.com (J. Lin).
1 These authors contributed equally to this work and should be considered as co-first authors.

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non-antigen components added to the vaccines, i.e., adjuvants/carriers [7]. In this way, it is particularly important to develop ideal vaccine adjuvants/carriers to friendly and efficiently recruit APCs. However, many vaccine adjuvants/carriers under research cannot meet both two criteria due to their inefficient recruitment ability or intrinsic toxicity to the APCs.

Among various vaccine adjuvants/carriers candidates, Mn\(^{2+}\)-containing blood plasma hydrogel seems ideal to meet both two criteria. In the normal physiological process, the formed subcutaneous blood stasis recruit macrophages and other immune cells for its uptake and degradation [8–10]. This phenomenon is due to the presence of sphingosine-1-phosphate molecules on the surface of platelet membranes and red blood cell membranes, which can act as “find me” signaling molecules to recruit macrophages [10,11]. Moreover, Fei [12] and Fan et al. [13] used blood clot-based gels for antigen and adjuvant delivery and induce a robust anticaner immune response. Inspired by this natural phenomenon, we propose that blood plasma hydrogel could also be used as vaccine adjuvants/carriers to effectively recruit APCs in this biomimetic manner. Platelet-containing plasma is absolutely safe and easily obtained by centrifuging blood to remove blood cells. In addition, it was recently found that Mn\(^{2+}\) plays an adjuvant effect in the process of immunization. After the pathogen invades, Mn\(^{2+}\) will be released into the cytoplasm to trigger the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway to induce anti-DNA virus and anti-tumor responses [14,15]. But Mn\(^{2+}\) alone may be rapidly metabolized in the body, requiring multiple administrations. Moreover, direct administration of Mn\(^{2+}\) would lead to potential toxicity if Mn\(^{2+}\) diffuses into other tissues [16]. Therefore, in this research, the injectable natural blood plasma hydrogels were used to encapsulate tumor antigen (Ovalbumin, OVA) and adjuvant (Mn\(^{2+}\)) to construct a novel personalized tumor vaccine to further enhance the immune response. The encapsulation of blood plasma hydrogels can easily achieve the storage of antigens and Mn\(^{2+}\), reduce the number of administrations, and is expected to achieve safe recruitment of immune cells. This provides a new idea for the development of novel tumor vaccines with both good bio-safety and high efficacy.

2. Methods

2.1. Materials

Manganese chloride monohydrate (MnCl\(_2\cdot\)H\(_2\)O) and calcium chloride (CaCl\(_2\)) was bought from Macklin (Shanghai, China). OVA protein and Collagenase type IV were bought from Sigma-Aldrich (Saint Louis, MO, USA). The mouse monoclonal fibrin antibody was obtained from Novus (St. Louis, USA). The Limulus amebocyte lysate (LAL) assay was obtained from Biofroxx (Germany). DNase was obtained from TIANGEN (Beijing, China). IFN-γ antibody was obtained from Biox (Beijing, China). Cell culture medium 1640 RPMI, fetal bovine serum, and penicillin-streptomycin were obtained from Gibco (CA, USA). DAPI staining solution and red blood cell lysis buffer were obtained from Beyotime (Shanghai, China). The cell counting kit-8 (CCK8) was obtained from Dojindo (Kyushu, Japan). FITC TUNEL cell apoptosis detection kit and CD8-GB13429 were obtained from Servicebio (Wuhan, China). CFSE staining solution, all flow cytometry antibody dyes, and enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioLegend (CA, USA). Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and murine IL-4 were purchased from PeproTech (NJ, USA).

All female C57BL/6 mice (4–6 weeks) used in the research were purchased from Beijing HFK Laboratory Animal Technology Co. (Beijing, China). In addition, all animal experiments were determined eligible for the study and consented to by the ethical committees of Jinan University.

2.2. Preparation and characterization of plasma hydrogel

Firstly, the blood was extracted from the female C57BL/6 mice and mixed with sodium citrate for anticoagulation, and then centrifuged (3000 rpm, 10 min) to obtain plasma. Subsequently, 30 μL of the CaCl\(_2\) (0.2 M) solution was added to 330 μL of plasma and incubated for 20 min at 37 °C to obtain plasma hydrogel. Similarly, 5 μL MnCl\(_2\cdot\)H\(_2\)O (2 M) was added to the above plasma solution to prepare plasma/Mn hydrogel. The morphology of obtained plasma hydrogel was observed with a scanning electron microscope (SEM, Zeiss, Germany), and the clotting time of plasma and plasma/Mn were measured with a thromboelastography (TEG, Haemscope, USA). The rheological properties were characterized by a rotary rheometer (Malvern, KINEXUS Pro, the UK).

The preparation of plasma/OVA hydrogel: The plasma was obtained as described above. Then, 15 μL OVA (6 mg/mL) was added to the 330 μL of plasma and mixed. Finally, 30 μL of the CaCl\(_2\) (0.2 M) solution was added to the plasma solution and incubated for 20 min at 37 °C to obtain plasma/OVA hydrogel. In addition, the endotoxin content in OVA was determined by Limulus amebocyte lysate (LAL) assay.

2.3. In vitro recruitment of DCs and macrophages by plasma hydrogel vaccine formulations

Referring to the experimental method for in vitro recruitment of DCs by polypeptide hydrogel implemented by Song et al. [17], the ability of plasma hydrogel vaccine formulations to recruit DCs and macrophages in vitro was determined. Firstly, the OVA, plasma/OVA hydrogel, or plasma/Mn/OVA hydrogel (OVA: 5 μg/well, plasma: 220 μL) were placed in the bottom well, while 200 μL DC2.4 and RAW 264.7 (without FBS) were seeded into the upper transwell at 5 × 10\(^4\). Then, 600 μL of media (with 20% FBS) was placed inside the bottom well. Cells were incubated at 37 °C with 5% CO\(_2\) for 24 h and then fixed with formaldehyde. After removing the non-migrated cells in the transwell, the DC2.4 that migrated on the underside of the transwell was stained with crystal violet, and the RAW 264.7 that migrated in the bottom plate was stained with DAPI staining. Finally, all cells were observed with an inverted optical microscope (Leica DMi6000, Germany). All images were taken at 200 × magnification. The cells were counted from five random microscope fields.

2.4. Generation and stimulation of BMDCs in vitro

BMDCs were isolated from the hind limb bones of female C57BL/6 mice according to previously described procedures [18]. Briefly, both distal bone ends were excised and the marrow cells were flushed using RPMI 1640. Red blood cells were lysed and the remaining cells were centrifuged at 1500 rpm for 10 min. Subsequently, the cells were seeded into 6-well plates and cultured for 6 d with RPMI1640 complete medium (containing 20 ng/mL GM-CSF and 10 ng/mL IL-4). The medium was replaced every 2 days. On the 6th day, the immature BMDCs were seeded into 24-well low attachment surface plates (2 × 10\(^5\) cells/well) and treated for 24 h with OVA, plasma/OVA hydrogel, plasma/Mn/OVA hydrogel or Alum/OVA formulations (OVA: 5 μg/well, plasma: 220 μL). Then, the fluorescent dye-labeled antibodies solutions (anti-CD11c APC, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE, anti-H\(_2\)K\(_b\)/SIINFEKL (MHC I) PE) were used to detect the expression of OVA-specific MHC I, MHC II, CD86 and CD40 molecules on CD11c \(^{+}\) DCs with a flow cytometer (Beckman Coulter, USA).

2.5. Detection of the retention of the antigen at the injection site

C57BL/6 female mice (4–6 weeks old) were randomly divided into three groups (n = 4), and immunized in their left legs with 50 μL of
Cy5.5-OVA, plasma/Cy5.5-OVA hydrogel, or plasma/Mn/Cy5.5-OVA hydrogel formulations (15 μg OVA/mouse). At 1, 24, 48 h, and 7 d, the fluorescence intensity of the antigen at the vaccine injection site was observed with a small animal bioluminescence imaging system (IVIS Lumina III, PerkinElmer, USA). On the 7th day after immunization, the lymph nodes of the mice were collected, and the sizes of the lymph nodes were observed.

2.6. In vivo recruitment of DCs and macrophages by plasma hydrogel vaccine formulations

The C57BL/6 female mice (4–6 weeks old) were randomly divided into five groups (n = 3) and subcutaneously immunized with 50 μl of OVA, plasma/OVA hydrogel, plasma/Mn/OVA hydrogel or Alum/OVA formulations (15 μg OVA/mouse). On 24 h, 48 h, and 7 d after vaccination, the skins of the mouse injection site were separated, sliced, and stained with DNase and stained with CD11c and CD68 antibody dyes. Finally, the corresponding quantitative analysis of cells expressing CD11c and CD68 molecules in the cells was detected with the flow cytometer.

2.7. Immunization evaluations of the vaccine formulations in vivo

The C57BL/6 female mice (4–6 weeks old) were randomly divided into five groups (n = 5), and subcutaneously immunized with 100 μl of OVA, plasma/OVA hydrogel, plasma/Mn/OVA hydrogel or Alum/OVA formulations (30 μg OVA/mouse). The mice were vaccinated twice at an interval of 7 days. On the 7th day after the second vaccination, the sera were obtained from the mice, and the splenocytes were separated from the spleens of the mice.

The titers of OVA-specific antibodies in the sera were detected by the enzyme-linked immunosorbent assay (ELISA), and the optical density was detected with a microplate reader (Multiskan MK3, Thermo, USA). In addition, the obtained splenocytes were seeded in 96-well plates (5 × 10⁴ cells/well) of anti-CD11c-PE, anti-CD44-PE, anti-CD8a-PerCP-Cy5.5, and anti-CD4-FITC) staining solution. Finally, the slices were stained with CD11c and CD68 antibody dyes. Finally, the obtained data were statistically analyzed using GraphPad Prism 5 software. The obtained data were statistically analyzed using one-way ANOVA test. The data were expressed as the mean ± standard deviation (Mean ± SD). *p < 0.05, **p < 0.01, and ***p < 0.001 were used to indicate the significant differences.

3. Results and discussion

3.1. Formation and characterization of the plasma hydrogel

The sodium citrate-anticoagulant plasma used in this work contained citrate that chelated with endogenous Ca²⁺ in plasma to block blood coagulation. However, along with the addition of exogenous Ca²⁺, the coagulation process would be restarted to trigger the agglutination reaction, promote the production of insoluble fibrin, and finally form a hydrogel [19]. As shown in Fig. 1A, we first confirmed in vitro that Ca ions can successfully induce the formation of the blood plasma hydrogel. In addition, the SEM images of the blood plasma hydrogel (Fig. 1B) showed that fibrin formed a well-structured hydrogel-like scaffold. Moreover, the plasma hydrogel was cut into slices and stained with fibrin-specific antibody dye. As shown in Supporting Information Fig. S1, the plasma gel showed a green fluorescent signal, which proves the formation of the insoluble fibrin. In addition, the SEM images of plasma/Mn hydrogel (Fig. S1A of Supporting Information) showed that the plasma/Mn hydrogel also has a scaffold structure. In addition, the elemental analysis (Fig. S1B of Supporting Information) showed that the plasma/Mn hydrogel contained the Mn element, which indicates that Mn²⁺ was successfully encapsulated in the plasma hydrogel. It is well known that the plasma proteins are negatively charged, which maintains their repulsion to each other and to the vascular endothelial cells. In this way, the positively charged Mn²⁺ ions in the plasma hydrogel can be quickly transformed into hydrogel in the body. Then, TEG was used to detect the exact time when the plasma formed a hydrogel. The TEG traces of plasma coagulation were shown in Fig. 1D.
and the main coagulation parameters are listed in Fig. 1E. Ca^{2+} could cause plasma and plasma/Mn coagulation, and their initial coagulation times were 9.7 and 9.4 min, respectively, and were within the normal range. Those results indicate that the plasma hydrogel is injectable and that the presence of Mn^{2+} doesn’t affect Ca^{2+}-induced the formation of plasma hydrogel.

The rheological characteristics of plasma hydrogel were measured through a rotary rheometer. The results were shown in Fig. 1F and G, about 10min after calcium ion was added to the plasma, the elastic modulus (G') of the plasma hydrogel was much higher than the viscosity modulus (G''), indicating that the plasma gel was formed stably and remained in a stable gel state in the subsequent detection time (Fig. 1F). The Frequency-scan curves of the hydrogels was shown in Fig. 1G. When the frequency >20 Hz, the gel state remains stable, while when the frequency is >20 Hz, the hydrogel state is destroyed.
3.2. Blood plasma hydrogel-based vaccine formulations can recruit DCs and macrophages in vitro

Sphingosine 1-phosphate (S1P), a metabolite produced by many cell types, such as platelets, is present in high concentrations in serum and can act as a “find me” signaling molecule to recruit immune cells [10,11, 21–24]. In this paper, the transwell chambers were used to test whether platelets in plasma gels can also actively recruit macrophages and DC cells. Blood plasma hydrogel was placed in the bottom well, and macrophages or DCs were seeded in the upper transwell chambers. The downward migration of macrophages or DCs were shown in Fig. 2, compared with the OVA alone group, the plasma hydrogel-containing group significantly enhanced the downward migration of DCs and macrophages. This result indicates that the antigen-encapsulated plasma hydrogel can recruit DCs and macrophages in large quantities. This effect will help enhance the level of antigen uptake and antigen presentation.

Several literature have reported that S1P has an inherent potential to recruit DCs and macrophages, while the migration-inducing effect of S1P could be severely hampered by the application of the Fingolimod (FTY720) [21–24]. Therefore, FTY720 was used as an S1P inhibitor to study their effects on DC migration in this work. As shown in Fig. S3 of Supporting Information, compared with the OVA alone group, the plasma hydrogel groups significantly enhanced the downward migration of DCs. By contrast, the addition of FTY720 significantly inhibited the DC migration. This indicates that the S1P in plasma could help recruit DCs.

3.3. Plasma hydrogel-based vaccine formulations can activate DCs

Firstly, considering that endotoxin in OVA may affect DC activation, the endotoxin content in the OVA was detected to be 0.043 EU/mg (equivalent to 5.8 ng/mg) with the Limulus amebocyte lysate (LAL) assay. As reported, LPS concentrations higher than 0.05 ng/mL could up-regulate the expression of CD80, CD40, and MHC II on DC surface [25]. The OVA concentration used in the BMDCs experiments was 5 μg/mL, and correspondingly LPS concentration was 0.029 ng/mL. Therefore, the LPS concentration had little effect on the bioactivity of the BMDCs. After confirming that the plasma hydrogel-based vaccine formulations can recruit APCs, we next detected whether the antigen-encapsulated plasma hydrogels can activate and promote the maturation of DCs. As shown in Fig. 3, compared with the OVA alone and plasma hydrogel groups, the plasma/Mn hydrogel group could significantly increase the levels of MHC II, CD86, and CD80. The plasma and plasma/Mn hydrogel groups could significantly increase the level of MHC I. These results indicated that after the antigen was encapsulated by the plasma hydrogel, the presentation of the antigen can be increased, and the introduction of Mn²⁺ can further increase antigen presentation and promote DC maturation.

Subsequently, we investigated whether Mn²⁺ could activate the cGAS/STING pathway in DCs by detecting IFN-β as an important indicator of the STING pathway activation. As shown in Fig. S3C of Supporting Information, the concentration of IFN-β in the plasma/Mn/OVA group was significantly higher than those in other groups. The results indicate that Mn²⁺ could activate the cGAS/STING pathway.

3.4. Plasma hydrogel-based vaccine formulations can recruit DCs and macrophages in vivo

The recruitment of APCs at the injection site will help promote
antigen presentation in the body and strengthen the subsequent immune response. As shown in Fig. 4, the OVA alone group recruited only a small number of DCs and macrophages at 24 h, and its capability to recruit DCs and macrophages gradually decreased from 24 h to 7 days. However, the two plasma hydrogel groups recruited a large number of DCs and macrophages within 24 h and maintained a high DCs and macrophages recruitment number within 48 h. On the 7th day, its ability to recruit DCs and macrophages only declined slightly. Subsequently, the corresponding quantitative analysis of cells expressing CD11c and CD68 molecules among the cells collected from the injected skin tissue was performed by using flow cytometry. As shown in Fig. S2 of Supporting Information, the expression levels of CD11c and CD68 on the cells in the OVA alone group were lower than those of the plasma/OVA and plasma/Mn/OVA hydrogel groups at 24 h, 48 h, and 7 d. In addition, the expressed CD11c and CD68 molecules in the OVA alone group gradually decreased from 24 h to 7 d. By contrast, the plasma/OVA and plasma/Mn hydrogel groups maintained high expression of CD11c and CD68 until 48 h and only slightly declined on day 7. These results are consistent with Fig. 4. These results confirm that the formation of plasma hydrogel would recruit APCs to take up antigens.

3.5. Plasma hydrogel-based vaccine formulations increase antigen retention

Generally, free antigens are easily diffused and metabolized in body fluids, and only a small part of antigens are taken up by APCs for immune response. Therefore, the prolonged retention of antigens in injection sites could greatly increase the uptake of antigens by APCs and enhance the immune response effect. The retention of the antigen OVA at the injection site and its migration to peripheral immune organs were studied. As shown in Fig. 5A&B, the fluorescence of OVA alone at the injection site decreased rapidly, was weak at 24 h, and was almost invisible on day 7. However, the plasma and plasma/Mn hydrogel groups still displayed strong fluorescence signals at 48 h. This result implies that the plasma hydrogel prolonged antigen retention, which is beneficial for improving the immune response. In addition, on the seventh day after immunization, the lymph nodes of the mice were separated. As shown in Fig. 5C, the lymph nodes in the plasma-containing groups were bigger than those in the OVA alone group. These results indicated that the plasma hydrogel assisted antigen retention, and promoted lymphocyte proliferation.
3.6. OVA-specific IgG titers

Antibody titer is a key indicator of the level of the immune response. The OVA-specific antibody titers in the sera of the immunized mice were measured by using ELISA. As shown in Fig. 6A–C, compared with OVA alone and plasma hydrogel groups, the IgG and IgG1 antibody titers of the plasma/Mn hydrogel group had significantly increased. Moreover, the plasma/Mn hydrogel group has the highest IgG2a antibody titer level. This result indicates that the plasma/Mn hydrogel displayed strong vaccine adjuvant efficacy, and significantly enhanced humoral immunity and cellular immune responses. This may be attributed to that the plasma gel prolongs the residence time of antigens in inoculation and recruits APCs to uptake antigens.

3.7. Splenocyte proliferation

The splenocyte proliferation assay is another important immunological indicator. When exposed to the same antigen again, splenocytes proliferate rapidly, and T cells produce rapid and effective immune responses [26]. Therefore, the splenocyte proliferation assay can indirectly evaluate the degree of immune responses induced by vaccination. As shown in Fig. 6D, compared with OVA alone and plasma hydrogel groups, the splenocyte proliferation indexed of the plasma/Mn hydrogel group significantly increased. This result demonstrates that plasma-encapsulated Mn and OVA can cause strengthened immune responses to antigen re-exposures.

3.8. Levels of the secreted cytokines

After re-stimulation by the same antigen, immune cells secrete cytokines to regulate and maintain immune responses [27]. IFN-γ secreted by T lymphocytes promotes the production of the antibody IgG2a and the differentiation of CD8+ T cells into CTLs [28–30]. Similarly, tumor necrosis factor-α (TNF-α), which is secreted by macrophages, is highly related to anti-tumor immunity [31–33] and can activate innate immunity [32,34]. In addition, IL-4 can regulate the production of the antibody IgG1, and promote the growth and differentiation of B cells and monocytes, as well as promote humoral immunity [35,36]. Other factors, such as IL-6, can participate in the regulation of cellular and humoral immune responses [35,37–41]. Here, ELISA was used to determine the levels of IFN-γ, TNF-α, IL-4, and IL-6 secreted by splenocytes after antigen restimulation. These cytokines represent important indexes for the successful
induction of antitumor immune responses. As shown in Fig. 6E–H, compared with those in the OVA alone and plasma hydrogel groups, the levels of IFN-γ, TNF-α, and IL-4 in the plasma/Mn hydrogel group were the highest. In addition, the levels of IL-6 in the plasma and plasma/Mn hydrogel groups were all significantly higher than those in the OVA alone group. These results indicate that plasma hydrogel can effectively enhance the immune response of OVA, while plasma hydrogel encapsulating Mn²⁺ and OVA triggers strong cellular and humoral immunity.

3.9. Immune memory T cells

Immune memory T cells play a key role in immune surveillance by rapidly and potently responding to antigen re-exposures [42]. Memory T cells can be classified as effector memory T cells (TEM cells, which express CD44highCD62Llow) and central memory T cells (TCM cells, which express CD44highCD62Lhigh) [43,44]. Here, the percentage of TEM cells in the splenocytes was detected. As shown in Fig. 6I–K, compared with the saline and OVA alone groups, the plasma/Mn group induced slightly higher percentages of CD8⁺ TEM and CD4⁺ TEM cells, while the plasma/Mn group induced significantly higher percentages of CD8⁺ TEM and CD4⁺ TEM cells. These results indicate that the addition of Mn²⁺ to plasma greatly increased the proportion of immune memory T cells and prolonged the immune response time.

3.10. Histocompatibility of the vaccine formulations

The bio-safety of the plasma hydrogel in vivo is critical for its clinical applications. On the seventh day after the last immunization, the heart, liver, spleen, lung, and kidney of the immunized mice were collected to evaluate the histopathological toxicity of the vaccine formulations. As shown in Fig. 7, all of the organs did not show obvious pathological changes, indicating the bio-safety of the plasma and plasma/Mn hydrogels for further clinical translation.

3.11. Therapeutic vaccination for anti-tumor evaluation

Evaluating antitumor effects based on the potent induction of antigen-specific immune responses is necessary. The schematic diagram of anti-tumor therapy immunity was shown in Fig. 8A. The body weight curve of the mice was shown in Fig. 8B, no significant weight fluctuations in all of the mice were observed. The tumor growths (Fig. 8C) in the plasma and plasma/Mn hydrogel groups were delayed compared with that in the saline group. Tumor growth in the plasma/Mn hydrogel group was slower than that in the plasma hydrogel group. Fig. 8D shows, the tumor weights of the plasma and plasma/Mn hydrogel groups were significantly lighter than those of the saline group, and the tumor weight of the plasma/Mn hydrogel group was the lightest. These results all corresponded to Fig. 8E&F, indicating that the addition of plasma and Mn²⁺ played an important role in tumor immunotherapy.

Some blood biochemical indexes (AST, ALT, LDH, and BUN) can be used to reflect the effectiveness and biosafety of vaccine formulations. The growth of melanomas causes an increase in ALT, AST, and LDH levels [45–48]. An increase in BUN level is an indicator of kidney damage [49]. As shown in Fig. 8G–I, the levels of AST and LDH in the plasma hydrogel group were significantly lower than those in the saline group. Moreover, the levels of AST, ALT and LDH in the plasma/Mn hydrogel group were significantly lower than those in the saline group and the levels of AST and ALT in the plasma/Mn hydrogel group were significantly lower than those in the OVA alone group. This result reflects that the OVA-encapsulated plasma/Mn hydrogel can improve antitumor capability. In addition, melanoma growth caused a certain degree of kidney damage, whereas the OVA alone and plasma hydrogel vaccine formulations significantly reduced kidney damage. Among the formulations, the plasma/Mn hydrogel vaccine formulation could reduce the level of BUN to normal levels (Fig. 8J), proving that it can also maintain normal renal function. The tumors were collected and stained with TUNEL dye as shown in Fig. 9A. Compared with saline and OVA alone groups, the
Fig. 6. The serum OVA-specific IgG (A), IgG1 (B), and IgG2a (C) titers were detected by ELISA. (D) Splenocyte proliferation detected by CCK-8 kit. (E-H) The levels of IFN-γ, TNF-α, IL-4, and IL-6 cytokines in the splenocytes supernatant were detected by ELISA. (I) The percentages of CD44hi CD62low cells in CD4⁺ T and CD8⁺ T cells of collected splenocytes, and (J-K) the corresponding statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001.
plasma hydrogel vaccine formulations obviously inhibited the growth of melanoma cells.

Next, we investigated whether the vaccine formulations inhibited tumor growth by promoting CTLs proliferation. First, the tumors were collected and stained with CD8$^+$-specific dye (CD8-GB13429 dye). As shown in Fig. 9B, compared with those in the other groups, the tumors in the plasma/Mn hydrogel group had the highest number of CD8$^+$ T cells, which accounted for the strongest anti-tumor effect of the plasma/Mn hydrogel formulation. The CD8$^+$/CD4$^+$ T cell ratio in the CD3$^+$ T lymphocyte subset is a significant indicator of the outcome of adaptive T cell immunotherapy [50]. As shown in Fig. 9C&D, the CD8$^+$/CD4$^+$ T cell ratios in the plasma and plasma/Mn hydrogel groups were significantly
higher than those in the OVA alone group. The proliferation of CD8\(^+\) T cells among the antigen-restimulated splenocytes was detected by using CFSE dye. As shown in Fig. 9E&F, compared with saline groups, the fluorescence intensities in the OVA alone, plasma hydrogel, and plasma/Mn hydrogel groups were significantly decreased, indicating obvious CD8\(^+\) T cell proliferation. The proliferation of CD8\(^+\) T cells in the plasma/Mn hydrogel group was significantly higher than that in the OVA alone and plasma/Mn hydrogel groups. These results indicated that the plasma/Mn hydrogel greatly increased the number of CTLs. This effect is beneficial for the direct killing of tumor cells.

Finally, The staining of IFN-\(\gamma\) and CD8\(^+\) T cells in the tissues of the tumors, spleens, and draining lymph nodes were conducted. As shown in Figs. S4–6 of Supporting Information, compared with the Saline and OVA groups, the IFN-\(\gamma\) expression in the three tissues of the Plasma/OVA and Plasma/Mn/OVA groups were higher and the IFN-\(\gamma\) expression in CD8\(^+\) T cells of the Plasma/OVA and Plasma/Mn/OVA groups (white arrows) were also higher. Among them, the IFN-\(\gamma\) expression in CD8\(^+\) T cells was highest in the Plasma/Mn/OVA group. However, some IFN-\(\gamma\) was outside of CD8\(^+\) T cells in the three tissues. On one hand, IFN-\(\gamma\) could be secreted by other immune cells, such as NK cells. On the other hand, IFN-\(\gamma\) could
also be secreted by CD8+ T cells outside of the cells.

4. Conclusion

In this research, a pure natural blood plasma hydrogel was firstly used to encapsulate the antigen and adjuvant (Mn2+) for constructing a highly biosafe and injectable personalized tumor vaccine. The hydrogel released the antigen slowly at the injection site, simulated the natural degradation process of subcutaneous blood stasis, recruited immune cells for antigen uptake, and promoted the presentation of antigen. At the same time, the Mn2+ produced by the degradation of the hydrogel exerted a vaccine adjuvant effect, significantly enhanced the immune response, and prolonged the duration of the immune response. Compared with traditional tumor vaccines, this natural blood plasma-based hydrogel provides new ideas for the development of safe and effective vaccines.

Credit author statement
Linghong Huang: Investigation, data curation, writing-original draft, formal analysis. Sufen Peng: Investigation, conceptualization, methodology, formal analysis. Zonghua Liu: Methodology, funding acquisition. Ning Liu: Investigation, conceptualization, writing-review & editing. Jiansheng Lin: Conceptualization, investigation, project administration, writing-review & editing.

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.

Data availability
Data will be made available on request.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100497.

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