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Novel vaccine adjuvant LPS-Hydrogel for truncated basic fibroblast growth factor to induce antitumor immunity

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ABSTRACT

The need to enhance the immunogenicity of tumor-associated antigens and modulate the resulting immune responses has prompted the development of new adjuvants. We prepared a novel adjuvant, lipopolysaccharides (LPS) loaded thermosensitive hydrogel (LPS-Hydrogel), for truncated basic fibroblast growth factor (tbFGF) peptide to enhance immunological responses and improve therapeutic effects in cancer. When co-formulated with tbFGF, LPS-Hydrogel formed antigen-adjuvant complexes, which enhanced antibody and cell-mediated responses in mice, thus promoting a more balanced antibody-mediated and cytotoxic T lymphocyte (CTL)-mediated immune response to inhibit tumor growth and metastases *in vivo*. Furthermore, the secretion of IFN-γ and IL-4 was detected, confirming activation of the two immune responses *in vivo*. There were no significant systemic toxicities observed with tbFGF-LPS-Hydrogel treatment. These results suggested that the thermosensitive and biodegradable LPS-Hydrogel was a novel adjuvant and carrier for peptide vaccines in cancer immunotherapy.

1. Introduction

Vaccine development has focused on the use of pathogen-derived protein/subunit or peptide antigens. These antigens, however, are often weakly immunogenic and rely heavily on the stimulation of innate immune mechanisms through the use of adjuvants to ensure a vigorous adaptive response and consequent vaccine efficacy (Kovacs-Nolan et al., 2009). Thus, there is a continuing need for powerful adjuvants that are safe to use. Technologies that incorporate adjuvants to increase the immunogenicity of antigens have been recently cited as one of the top 10 technologies that will significantly impact global health (Mutwiri, Nichani, Babiuk, & Babiuk, 2004). On the basis of their principal mechanisms of action, adjuvants can be broadly divided into 2 classes: vaccine delivery systems and immunostimulatory adjuvants (Singh & O'Hagan, 2003). Ideal adjuvants should have both actions.

Lipopolysaccharides (LPS), derived from the cell wall of Gram-negative bacteria, has been used as an adjuvant for years, because it activates innate immunity through the recognition of antigen-presenting cells such as macrophages, dendritic cells, and murine B lymphocytes (Seppala & Makela, 1984). The proliferation of spleen cells has been observed after LPS was injected into mice (Franzl & McMaster, 1968). LPS elicits an immune response through its interaction with Toll-like receptor 4 (TLR4) (Thompson, Chilton, Ward, Evans, & Mitchell, 2005). After LPS injection, the titer of serum immunoglobulins IgM and IgG were elevated approximately 6- and 4-fold above the pre-injection levels, respectively (Izui, Eisenberg, & Dixon, 1981; Makela & Peterfy, 1983). However, its extreme toxicity precludes its use in clinical settings (Thompson et al., 2005). Therefore, alternative compounds with similar immunostimulatory properties, which could increase tolerance to LPS in humans, are required.

Abbreviations: ATCC, American Type Culture Collection; bFGF, basic fibroblast growth factor; CTL, cytotoxic T lymphocyte; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FGFR, fibroblast growth factor receptor; FTIR, Fourier transforms infrared spectroscopy; GPC, gel permeation chromatography; ^1H-NMR, Nuclear magnetic resonance analysis; LDH, lacto-dehydrogenase; LL/2, Lewis lung carcinoma; LPS, lipopolysaccharides; LPS-Hydrogel, lipopolysaccharides loaded poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) hydrogel; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PECE, poly(ethylene glycol)-poly(ε-caprolactone)-poly (ethylene glycol); tbFGF, truncated basic fibroblast growth factor; tbFGF-FA, tbFGF loaded Freund's adjuvant; tbFGF-LPS-Hydrogel, truncated basic fibroblast growth factor and lipopolysaccharides loaded poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) hydrogel; TLR4, Toll-like receptor 4.

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Tumor growth is partly determined by tumor-associated angiogenesis, which is mediated by an angiogenic switch (Folkman, 1985; Liotta, Steeg, & Stetler-Stevenson, 1991). A balance between inhibitors and stimulators of angiogenesis is maintained in the homeostatic state *in vivo*. In contrast, tumor angiogenesis is controlled by positive and negative modulators; there is a shift in this balance towards the angiogenic phenotype during neoplasia (Ribatti, Vacca, & Dammacco, 1999). Among them, basic fibroblast growth factor (bFGF) is one of the first angiogenic growth factors identified (Moscatelli, Presta, & Rifkin, 1986; Shing et al., 1984), which is not only an essential stimulator of angiogenesis (Bremnes, Camps, & Sirera, 2006; Danielsen & Rofstad, 1998; Kos & Dabrowski, 2002; Przybylski, 2009; Rogala et al., 2001), but also highly expressed in many tumor cells, and could form an autocrine loop to promote tumor cell proliferation (Bisping et al., 2003; Tsunoda et al., 2001; Uria et al., 1998). The critical roles of bFGF in angiogenesis and tumor cell proliferation have been reported in a number of contributions (Gaudric, N'Guyen, Moenner, Glacet-Bernard, & Barritault, 1992; Kibbey, Grant, & Kleinman, 1992; Watanabe et al., 1997; Wilting & Christ, 1992). Thus, a rationale for targeting specific immune responses to appropriate epitopes on the bFGF molecule was developed for endogenous control of tumor growth (Plum et al., 2000). To screen the ability of bFGF-stimulated proliferation of endothelial and tumor cells, we derived peptides from the functional domains of bFGF (truncated basic fibroblast growth factor, tbFGF) that could block the proliferation of endothelial cells, but not tumor cells, *in vitro*.

In our previous contributions, we have prepared a biodegradable thermosensitive hydrogel based on a poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) (PEG-PCL-PEG, PECE) copolymer (Gong, Shi, Wu, et al., 2009; Gong, Shi, Dong, et al., 2009; Gong, Wu, Dong, et al., 2009). PECE hydrogel exhibits reverse thermal gelation, where aqueous solutions are liquid sol at or below ambient temperature and form nonflowing gels at physiological temperature. We also found that antigen loaded PECE hydrogel could significantly improve the immuno-response (Luo et al., 2011). In the present study, we used a LPS loaded PECE hydrogel (LPS-Hydrogel) system to increase tolerance of LPS *in vivo*. We investigated the combined immunostimulatory effect of LPS-Hydrogel as a combination adjuvant and used with tbFGF as anti-tumor antigen, as well as the effect of substituted indolicidin derivatives to further enhance both cellular and humoral immune responses. A combination of truncated basic fibroblast growth factor (tbFGF) peptide and LPS-Hydrogel vehicle exhibited an anti-tumor effect in primary and metastatic tumors when administered to mice.

2. Experimental

2.1. Animals and cell lines

C57BL/6J mice, 6–8 weeks old, were purchased from the West China Experimental Animal Center and kept in pathogen-free cages. The ambient temperature was 20–22 °C and the relative humidity was 55–60%. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number: 20100612). All mice were treated humanely throughout the experimental period. Murine Lewis lung carcinoma (LL/2), murine embryonic fibroblast (NIH-3T3), and murine pancreatic islet endothelial (MS1) cell lines were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum and 100 μg/ml Amikacin. Cells were maintained in a humidified chamber at 37 °C in 5% CO_2 atmosphere.

2.2. Preparation of truncated bFGF peptide

The tbFGF peptide (a.a. 24–115), containing a bFGF receptor binding site and part of the heparin-binding site, can effectively bind FGF receptor (FGFR) on the cell surface, but cannot stimulate cell proliferation. To obtain the tbFGF peptide, the tbFGF cDNA was inserted into the prokaryotic expression plasmid pQE30 (Qiagen, Santa Clara, USA) to get a 6 × His and tbFGF fusion protein. Recombinant tbFGF was expressed in *E. coli* TG-1 as an inclusion body. Inclusion bodies were solubilized using buffer A (50 mM Tris–HCl [pH 7.0], 8 M urea). The supernatant was then applied to a Ni-chelating sepharose column (GE Healthcare Science), and the purified and unfolded tbFGF was dialyzed against refolding buffer (150 mM NaCl, 20 mM sodium phosphate buffer [pH 6.0], 10% glycerol). After one-step chromatography, up to 99% the purity of the recombinant bFGF was achieved. The characterization of tbFGF was confirmed by Western blot with tbFGF immunized mice serum. The molecular weight of the obtained tbFGF was approximately 10.9 kD, and the tbFGF was stored at −80 °C.

2.3. Detection of tbFGF bioactivity

NIH-3T3 cells were used to measure the bioactivity of tbFGF. NIH-3T3 cells were suspended in DMEM, supplemented with 2% heat-inactivated fetal bovine serum (Hyclone, UT, USA) at a concentration of 2.5×10^4 cells/ml. Cells were then plated in 96-well (100 μl/well) culture plates (Costar, MA, USA) and incubated for 24 h at 37 °C in 5% CO_2. Cells were incubated with either the bFGF or tbFGF at 3.05×10^{-5}, 1.22×10^{-4}, 4.88×10^{-4}, 1.95×10^{-3}, 7.81×10^{-3}, 3.125×10^{-2}, 0.125, 0.5, 2, and 8 μg/ml for 72 h. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the manufacturer's instructions (Mosmann, 1983).

2.4. Preparation of tbFGF loaded LPS-Hydrogel (tbFGF-LPS-Hydrogel)

PECE copolymers were synthesized and purified as reported previously (Gong, Shi, Wu, et al., 2009; Gong, Shi, Dong, et al., 2009). Briefly, ring-opening polymerization of ε-CL initiated by MPEG was used to prepare MPEG-PCL diblock copolymer, and $Sn(Oct)_2$ was used as a catalyst. PECE triblock copolymers were then synthesized by coupling MPEG-PCL diblock copolymer using HMDI as coupling agent (shown in Fig. 1). Nuclear magnetic resonance analysis (^{1}H-NMR, Varian 400 spectrometer, Varian, USA), Fourier transforms infrared spectroscopy (FTIR, 200SXV Infrared Spectrophotometer, Nicolet, USA), and gel permeation chromatography (GPC, Agilent 110 HPLC, USA) were used to characterize the prepared PECE copolymer.

The PECE copolymer was dissolved in sterile water at a specific temperature and then cooled to 4 °C to form a sol state. Then, LPS and tbFGF were added to the PECE solution to form a homogeneous solution, with final LPS and tbFGF concentrations of 50 and 100 μg/ml, respectively, and 25 wt% of PECE.

Sol-gel-sol phase transition diagram of tbFGF-LPS-Hydrogel was recorded using test tube-inverting method and rheometry (AR Rheometer 2000ex, TA Instruments, USA). The aqueous PCEC copolymer solution was placed between parallel plates of 40 mm diameter and a gap of 31 μm. The data were collected under a controlled stress (4.0 dyn/cm^2) and a frequency of 1.0 rad/s. The heating rate was 1 °C/min.

2.5. Release of LPS and tbFGF from thermosensitive PECE hydrogel

FITC-labeled LPS (Sigma, USA) or FITC-labeled tbFGF (Protein Fluorescence Label Kit; Roche Inc., Basel, Switzerland) was loaded

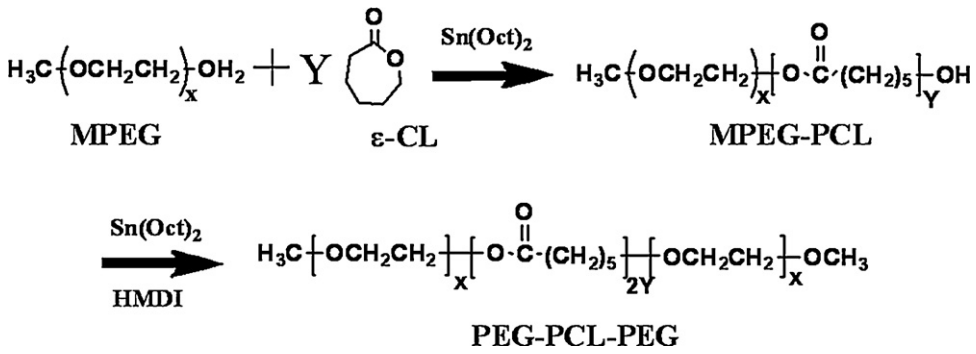

Fig. 1. Structure and synthesis scheme of PECE copolymers.

into thermosensitive PECE hydrogel to detect the release of LPS or tbFGF, respectively. Two hundred microliters of FITC-LPS-Hydrogel or FITC-tbFGF-Hydrogel was injected into the flank of the C57 mice. Then, mice were sacrificed on days 1, 3, 5, 7, and 14 to observe the absorption of PECE hydrogel, After mice were sacrificed, unabsorbed PECE hydrogel were made into frozen sections, and the release of LPS or tbFGF in the unabsorbed PECE hydrogel frozen sections was identified with a inverted fluorescence microscope.

2.6. Immunization and tumor models

Mice were immunized with tbFGF-LPS-Hydrogel (containing 20 μg of tbFGF). Subcutaneous (s.c.) injections were administered 3 times once every 2 weeks with tbFGF-LPS-Hydrogel, tbFGF loaded Freund's adjuvant (tbFGF-FA), or phosphate-buffered saline (PBS; nonimmunized/control mice). Mice in all groups were weighed once every 5 days. Fourteen days after the last immunization, 5 mice from each group were challenged with 3×10^5 LL/2 tumor cells s.c. in the right flank. Tumor dimensions were measured every 3 days with callipers, and tumor volumes were calculated according to the following formula: width^2 × length × 0.52. Another 5 mice from each group were challenged with 3×10^5 LL/2 tumor cells intravenously (i.v.) through the tail vein. Eighteen days after injection of tumor cells, mice were sacrificed to determine the number of tumor nodules and weight of the lung species, respectively.

2.7. Detection of anti-bFGF antibodies in serum

The serum samples were obtained from mice every week during the immunization period and stored at −80 °C. Serum concentrations of anti-bFGF antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, the anti-bFGF antibody ELISA was prepared by coating Immunolon-3 plates (Dynatech Laboratories, VA, USA) with a solution of 0.5 μg/ml bFGF in 50 mM carbonate/bicarbonate buffer at a pH of 9.6 and incubating overnight at 4 °C. Wells were blocked with a 5% solution of non-fat dried milk in PBS. The plasma samples were diluted 1:10, 1:100, 1:1,000, and 1:10,000 in buffer (PBS with 5% milk) and incubated at 37 °C for 60 min. After washing, the plates were incubated with protein A-HRP (Jackson ImmunoResearch, PA, USA) for 60 min at 37 °C. After washing, the substrate was developed with 3, 3, 5, 5-tetramethylbenzadine (Sigma). The colorimetric reaction was stopped with 1 M hydrochloric acid and the optical density was determined by using an ELISA plate reader (Thermo LabSystems, MA, USA).

2.8. CTL activity and specificity assessment

T lymphocytes derived from the different mice groups were tested for their cytotoxicity against MS1 cells by using the lacto-dehydrogenase (LDH) cytotoxicity assay (LDH Cytotoxicity Detection Kit; Roche, Germany) in several effector-to-target cell ratios. Briefly, 5×10^4 MS1 cells were incubated in 96-well flat bottom plates with the different effector ratios for 4 h at 37 °C in 5% CO_2. Then, the cells were processed as instructed by the manufacturer. Controls included medium, cytotoxic T lymphocytes (CTLs), and target cells for spontaneous and maximal LDH release.

2.9. Detection of cellular response against tbFGF

The IFN-γ and IL-4 enzyme-linked immunospot (ELISpot) assay was performed as previously described (Davies et al., 2009). Briefly, each well received 10^5 spleen T cells of immunized mice, followed by 2 μg/well of tbFGF for T cell stimulation. No antigen was added to the negative control well. Concanavalin A, (Sigma) at a final concentration of 100 ng/ml, was included as a positive control. Spots were counted under the microscope (Carl Zeiss Microimaging Inc.).

2.10. Detection of microvessel density

Primary tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 3–5 μm sections. The methods and procedures were according to the method described by Weidner, Semple, Welch, and Folkman (1991), the procedure of immunostaining for CD31 was previously described in detail (Yang et al., 2009). The following antibodies were used: goat anti-mouse CD31 monoclonal antibody (mAb) (1:200; Santa Cruz Biotechnology, CA, USA) and biotinylated polyclonal rabbit anti-goat antibody (1:100; Santa Cruz Biotechnology).

2.11. Statistical analysis

SPSS 13.0 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by Student's *t*-test and ANOVA. Findings were regarded as statistically significant if $P < 0.05$.

3. Results

3.1. Bioactivity of the tbFGF peptide

Bioactivity of tbFGF was measured by an NIH-3T3 cell proliferation assay. After the cells were incubated with either bFGF or tbFGF at 3.05×10^{-5}, 1.22×10^{-4}, 4.88×10^{-4}, 1.95×10^{-3}, 7.81×10^{-3}, 3.125×10^{-2}, 0.125, 0.5, 2 and 8 μg/ml for 72 h, cell proliferation was detected by MTT assay. The results showed that the ED50 of native bFGF was 30 ng/ml, whereas that of the tbFGF was not

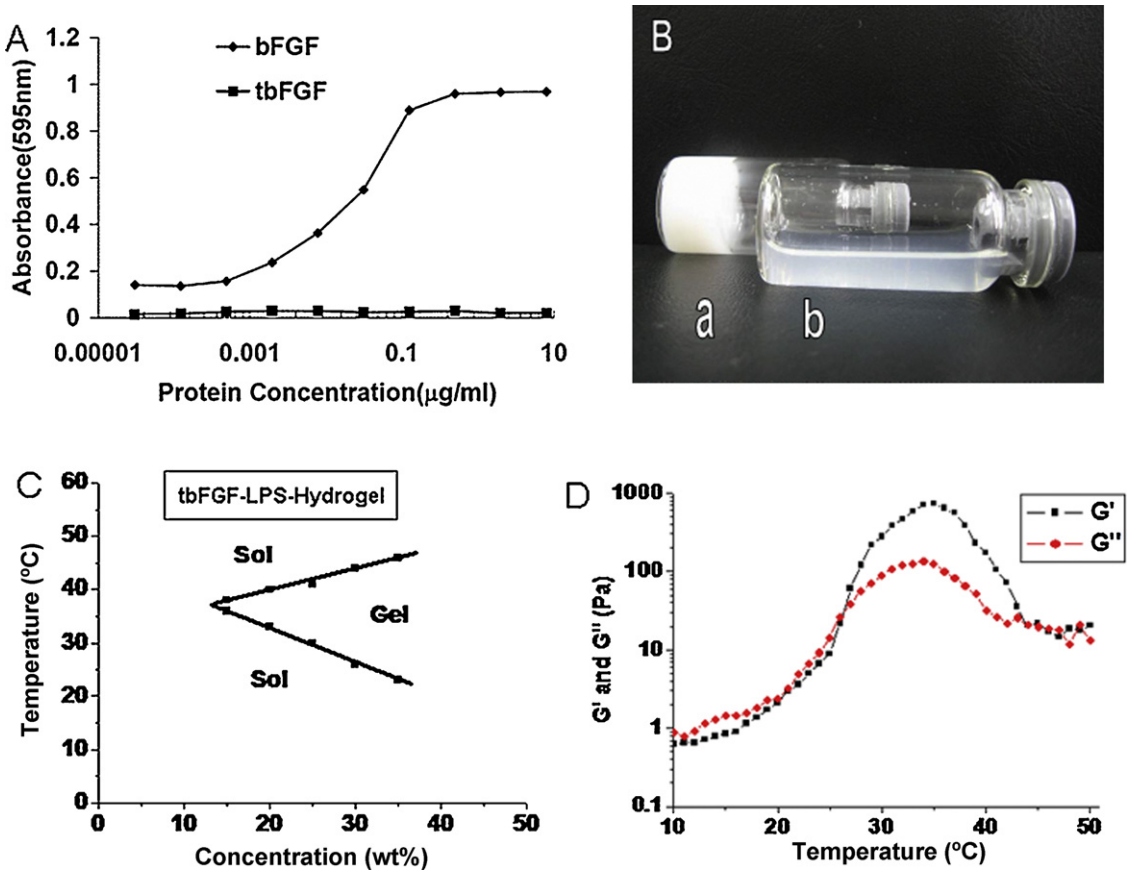

Fig. 2. The properties of tbFGF and characters of LPS and tbFGF encapsulated PECE hydrogel. (A) Bioactivity of tbFGF measured by NIH-3T3 cell proliferation assy. The definition of ED50 is 30 ng/ml. Recombinant tbFGF could not stimulate NIH-3T3 fibroblast proliferation. The bioactivity of tbFGF was much lower than that of bFGF ($P < 0.01$). (B) Morphology of prepared LPS (50 μg/ml) and tbFGF (100 μg/ml) loaded thermosensitive hydrogels (25 wt%) at 37 °C (a) and 10 °C (b), respectively. (C) Phase transition behavior of the tbFGF-LPS-Hydrogel. With an increased of PECE copolymer concentration (>15 wt%), the lower critical gelation temperature gradually decreased and the upper critical gelation temperature gradually increased. (D) Rheological analysis of tbFGF-LPS-Hydrogel system.

detected, indicating that the recombinant tbFGF could not stimulate NIH-3T3 fibroblast proliferation ($P < 0.001$) (Fig. 2A).

3.2. Characterization of tbFGF-LPS-Hydrogel

The biodegradable PECE copolymer was successfully synthesized by ring-opening polymerization and coupling reaction according to Fig. 1. The molecular weight of prepared PECE copolymer calculated from ^{1}H-NMR spectra were 3408, and repeating units of PEG and PCL were 12.5 and 29 respectively. When LPS and tbFGF were incorporated into the PECE hydrogel, the sol-gel-sol phase transition behavior of tbFGF-LPS-Hydrogel was investigated. According to Fig. 2B, tbFGF-LPS-Hydrogel is an injectable sol at or below ambient temperature (Fig. 2B-b) and converts into non-flowing gel at body temperature (Fig. 2B-a). With an increase in the copolymer concentration (>15 wt%) the lower critical gelation temperature gradually decreased and the upper critical gelation temperature gradually increased. We applied a PECE hydrogel at a concentration of 25 wt%, which had a sol-gel transition temperature of 30 °C and a gel–sol transition temperature of 41 °C (Fig. 2C). Sol–gel–sol transition of tbFGF-LPS-Hydrogel accompanies with a significant change in modulus. Fig. 2D showed the change in storage modulus (G') and loss modulus (G'') of tbFGF-LPS-Hydrogel (25 wt %) as a function of temperature. The G' in sol state was less than 1 Pa and increased abruptly to greater than 600 Pa by due to the sol-gel transition as the temperature increased. The dramatic decrease in the G' at about 41–45 °C demonstrated the gel–sol transition of the aqueous copolymer solution.

3.3. Release of tbFGF and LPS from thermosensitive PECE hydrogel

Mice were sacrificed on days of 1, 3, 5, 7, and 14 after injected with tbFGF-LPS-Hydrogel, FITC-LPS-Hydrogel, or FITC-tbFGF-Hydrogel in the right flank. The bodies of the mice gradually absorbed the tbFGF-LPS-Hydrogel, FITC-LPS-Hydrogel, and FITC-tbFGF-Hydrogel (Fig. 3A, tbFGF-LPS-Hydrogel *in vivo*). In order to investigate the release of LPS and tbFGF, unabsorbed Hydro-FITC-LPS and Hydro-FITC-tbFGF was identified with frozen sections. The density of PECE hydrogel in both FITC-LPS-Hydrogel and FITC-tbFGF-Hydrogel gradually decreased over the 14 days (Fig. 3A, microscopic inspection); the FITC-LPS or FITC-tbFGF was released gradually from the PECE hydrogel (Fig. 3A, FITC-LPS, FITC-tbFGF). The release process is illustrated in Fig. 3B.

These results showed that the prepared hydrogel is a free-flowing sol at low temperature, which could be mixed with drugs and injected by syringe easily. Then, the hydrogel forms a gel by a syringe injection at a target location, serving as a sustained delivery depot of the drugs.

3.4. The effect of anti-tumor immunization

We summarized the tumor growth rates in mice s.c. injected with control vehicle (PBS), tbFGF-FA, and tbFGF-LPS-Hydrogel in the right flank. tbFGF-LPS-Hydrogel and tbFGF-FA treatment effectively reduced tumor volume compared to PBS treatment ($P < 0.05$) (Fig. 4A). To assess the ability of vaccinated mice to inhibit tumor development in a metastatic lung model, we challenged treated

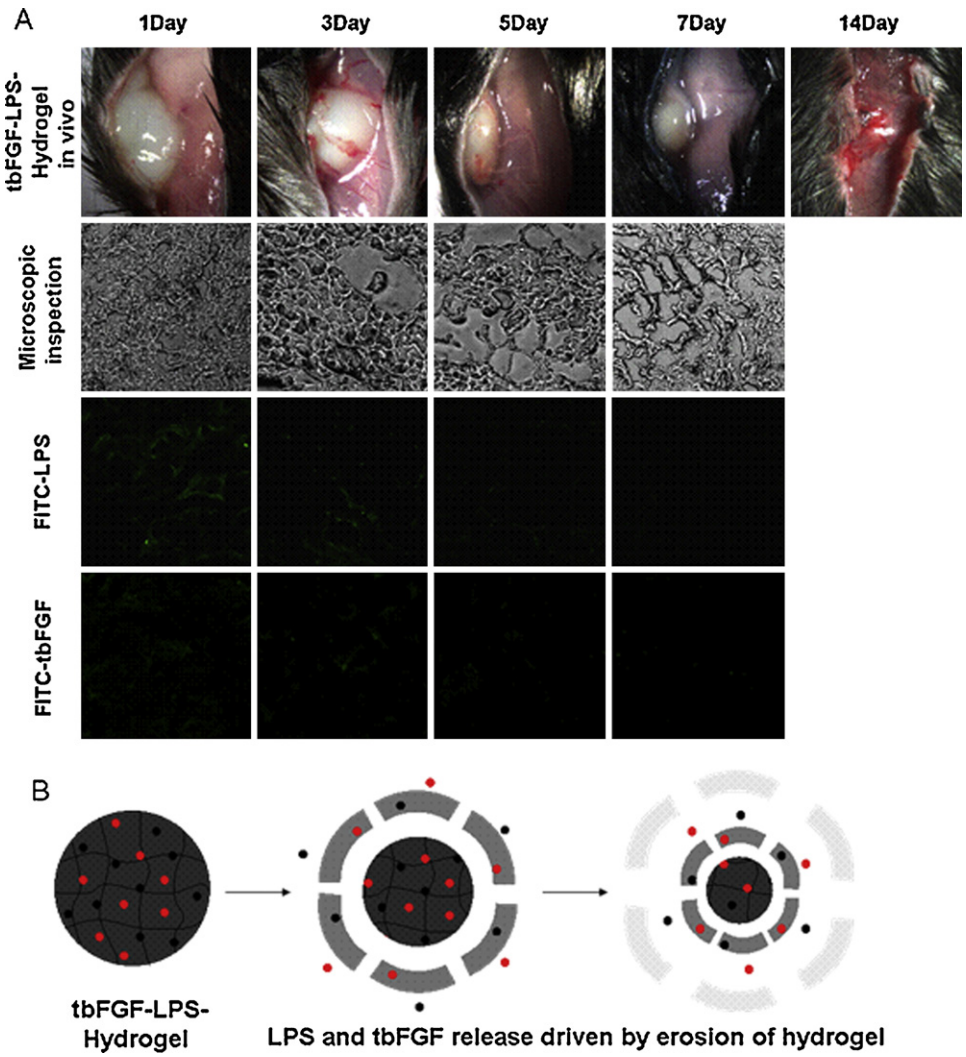

Fig. 3. The release behavior of LPS and tbFGF from the biodegradable and thermosensitive hydrogels. Mice were sacrificed on days 1, 3, 5, 7, and 14 after FITC-LPS-Hydrogel injection in the right flank. (A) FITC-LPS-Hydrogel or FITC-tbFGF-Hydrogel was gradually absorbed by the body and disappears *in vivo*. Unabsorbed FITC-LPS-Hydrogel or FITC-tbFGF-Hydrogel was identified in frozen sections. The density of hydrogel gradually decreased over the 14 days; and FITC-LPS and FITC-tbFGF were gradually released from the hydrogel. (B) Modeling and simulation in the FITC-LPS-Hydrogel absorption processes. Diffusion of LPS and tbFGF out of the hydrogel matrix was due to the interior porous structure of the hydrogel, and because of the erosion of the hydrogel (absorption by the body), LPS and tbFGF incorporated in the eroded region was absorbed by the body.

mice with LL/2 through the tail vein. Fourteen days after intravenous challenge with LL/2, mice were sacrificed, lungs were removed and weighed, and the number of surface metastases was counted. Inhibition of macroscopic LL/2 metastases in the lungs was significantly higher in the tbFGF-LPS-Hydrogel and tbFGF-FA groups than the control group ($P < 0.01$), and nodules with diameters over 5 mm were only found in the control group ($P < 0.01$) (Fig. 4B).

3.5. Detection of serum anti-bFGF antibodies

tbFGF-LPS-Hydrogel, tbFGF-FA, and PBS were separately used to treat mice 3 times, once every 2 weeks. Following the treatments, mice were bled and sera were pooled to analyze serum immunoreactivity to native bFGF in an ELISA format. The antibody reaction against the native bFGF molecule increased gradually from the first week to the sixth week in the tbFGF-LPS-Hydrogel and tbFGF-FA groups. In contrast, sera collected from control group mice did not show immunoreactivity to the native bFGF molecule (Fig. 5A). Next, we determined the titer of anti-native FGF antibody in the sixth week in all 3 groups. The tbFGF-FA and tbFGF-LPS-Hydrogel groups achieved a titer of 1:10,000 and 1:4,000, respectively (Fig. 5B).

3.6. CTL-mediated cytotoxicity assay

Specific CTL activity was tested by LDH cytotoxicity assay; the results revealed that T lymphocytes from mice immunized with tbFGF-LPS-Hydrogel and tbFGF-FA were more cytotoxic to MS-1 cells than those from mice injected with PBS ($P < 0.01$) (Fig. 5C). These findings indicate the activation of anti-MS-1 cellular immunity.

3.7. Detection of a cellular response against tbFGF

The ELISpot assay was explored to measure Th1 and Th2 responses to bFGF by detecting IL-4 and IFN-γ. The experiments revealed that more IL-4 and IFN-γ were secreted in the tbFGF-LPS-Hydrogel and tbFGF-FA groups than in the control group ($P < 0.01$) (Fig. 5D). However, there was no difference in secreted IL-4 and IFN-γ levels between the tbFGF-LPS-Hydrogel and tbFGF-FA groups,

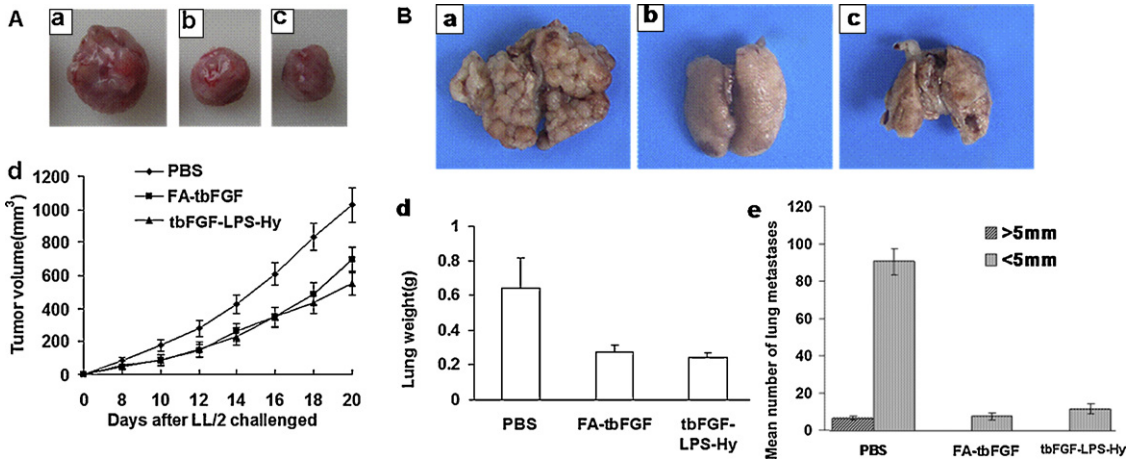

Fig. 4. Induction of protective anti-tumor effects in immunized mice. (A) S.c. induction of protective anti-tumor immunity. (a–c) Tumors of mice in each group. C57BL/6J mice were injected with PBS (♦), FA-tbFGF (■), and Hy-LPS-tbFGF (▲) once a week for 6 weeks and were then challenged with LL/2 cells (3×10^5) on day 7 after the last immunization. (d) There was a difference in tumor volume ($P < 0.05$) between the control group and other treatment groups, while there was no difference between the FA-tbFGF and Hy-LPS-tbFGF groups ($P > 0.05$). Points, mean ($n = 5$); bars, SD. The experiment was repeated twice. (B) Induction of protective anti-tumor metastatic immunity. C57BL/6J mice were injected with PBS (♦), FA-tbFGF (■), and Hy-LPS-tbFGF (▲) once a week for 6 weeks and were then challenged with LL/2 cells (3×10^5) on day 7 after the last immunization. (a–c) Lungs of mice in each group. (d) Lung weight of each group. (e) Metastatic lung nodules from each group. There was a significant difference in lung weight ($P < 0.01$) and the number of lung metastases ($P < 0.01$) between the control group and other treatment groups, and there was no difference between the FA-tbFGF and Hy-LPS-tbFGF groups ($P > 0.05$). Columns, mean ($n = 5$); bars, SD. The experiment was repeated twice.

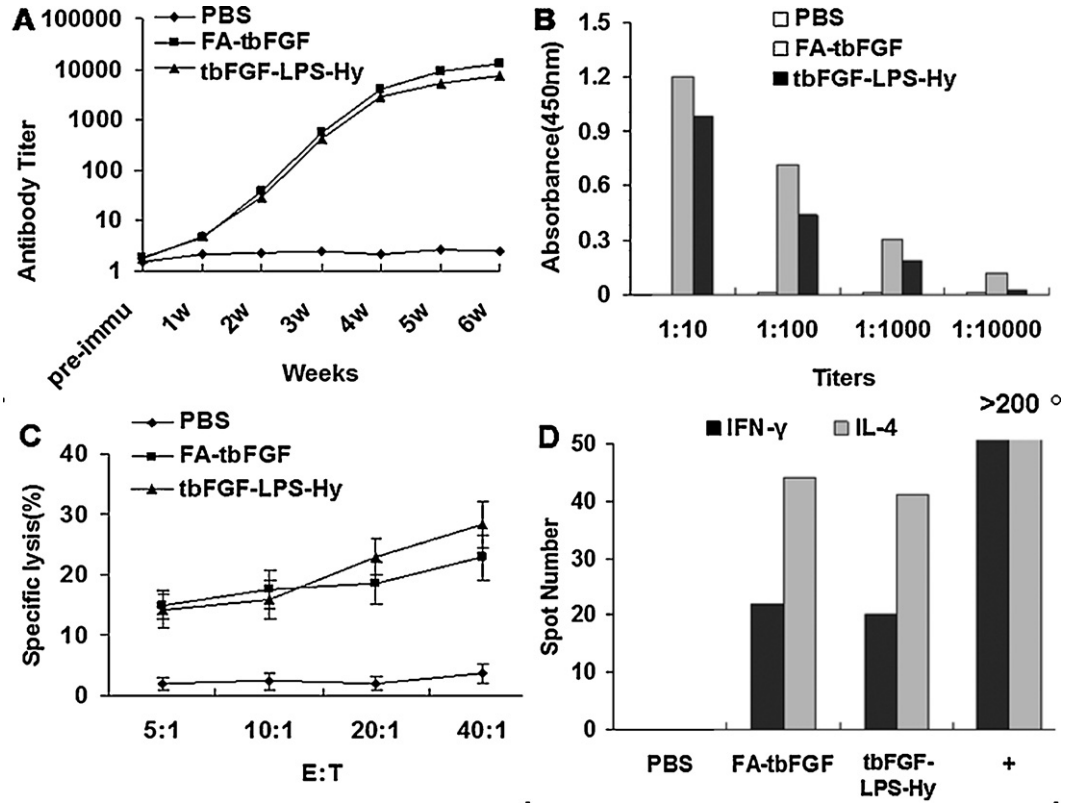

Fig. 5. Cellular and humoral immune responses induced by Hy-LPS-tbFGF. (A) Anti-bFGF antibody titer of the 3 groups from pre-immunization to the sixth week (titer, 1:100). The samples at pre-immunization, first week, and second week were negative for anti-bFGF antibodies in all 3 groups; anti-bFGF antibodies at the third, fourth, fifth, and sixth weeks were positive in the FA-tbFGF and Hy-LPS-tbFGF groups. The titer of anti-bFGF antibodies increased over the 6 weeks in the FA-tbFGF and Hy-LPS-tbFGF groups. (B) Anti-bFGF antibodies titer of the 3 groups ranged from 1:10 to 1:10000 (the sixth week). The titers of the FA-tbFGF and Hy-LPS-tbFGF groups were more than 1:1000. (C) Specific CTL activity was tested by LDH cytotoxicity assay. T lymphocytes from mice immunized with Hydro-LPS-tbFGF and FA-tbFGF were more cytotoxic to MS-1 cells than mice injected with PBS ($P < 0.05$). With the increase of E:T (effector cells: target cells), effector cells enhanced the ability to kill target cells. (D) Potent T cell response against bFGF as determined by ELISpot assays. Secretion of IFN-γ and IL-4 by CD4+ and CD8+ T cells was examined. Secretion of IFN-γ and IL-4 from the Hydro-LPS-tbFGF and FA-tbFGF groups was significantly higher than that from the control group ($P < 0.05$). +, positive control (Concanavalin A stimulated of T cells).

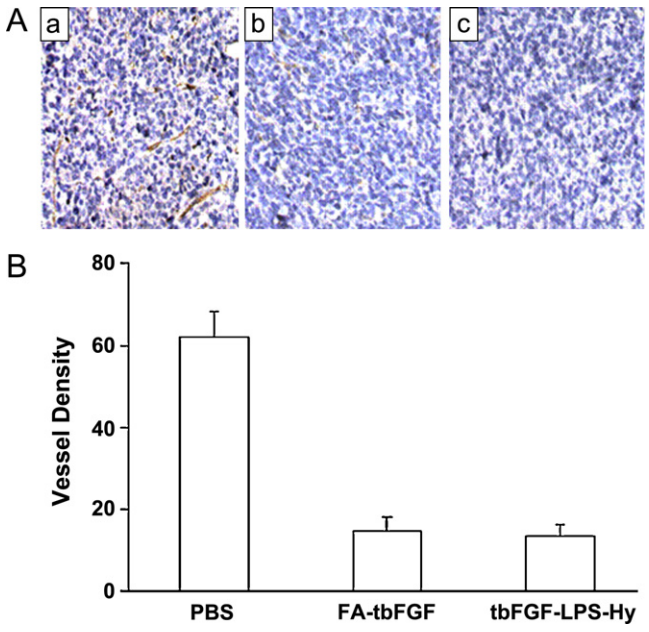

Fig. 6. Effect of inhibition of angiogenesis in tumors. After mice treatment was stopped, tumors samples were frozen and sectioned. (A) Frozen tumor tissue sections were tested by immunohistochemical analysis using anti-CD31 antibody and vascular density was quantified by counting the number of microvessels per high-power field (400×). (a) Control group, (b) FA-tbFGF group, and (c) Hy-LPS-tbFGF group. (B) CD31-positive microvessels in all 3 groups. The vessel density of tumor tissues from the FA-tbFGF and Hy-LPS-tbFGF groups indicated a decrease in tumors compared with the control group ($P < 0.05$). Columns, mean (5 high-power fields/slide); bars, SD.

suggesting that both humoral and cellular immunity against bFGF was induced by tbFGF-LPS-Hydrogel and tbFGF-FA.

3.8. Histologic analysis of tumor microvessel density

Tumor sections of each group were stained with anti-CD31 antibody to evaluate the tumor microvessel density (MVD). Control group tumors demonstrated high MVD, while those of tbFGF-LPS-Hydrogel and tbFGF-FA groups were significantly lower (Fig. 6A). The number of microvessels was also significantly different between the control group and the tbFGF-LPS-Hydrogel and tbFGF-FA groups ($P < 0.01$) (Fig. 6B).

3.9. Identification of tbFGF-LPS-Hydrogel side effects

Mice were weighed once every 5 days prior to immunization to evaluate the toxicity of the LPS-Hydrogel adjuvant system (Fig. 7A). There was no significant difference in mouse weight between the control and tbFGF-LPS-Hydrogel groups ($P > 0.05$). After immunization, in addition to body mass, heart and respiratory rates were also monitored (Fig. 7B and C). No significant differences in mouse weight, heart rates and respiratory rates were observed between the control and tbFGF-LPS-Hydrogel groups after immunization ($P > 0.05$). Examination of the histopathologies of vital organs (heart, liver, spleen, lung, and kidney) failed to detect any differences between the control and tbFGF-LPS-Hydrogel groups (data not shown).

4. Discussion

Adjuvants can be broadly divided into 2 classes according to their principal mechanisms of actions: vaccine delivery systems and immunostimulatory adjuvants (O'Hagan & Singh, 2003; Singh & O'Hagan, 2003). Vaccine delivery systems are generally particulate, for example, emulsions, liposomes, micro particles, and ISCOMS; the main function of vaccine delivery systems is to target the associated antigens on antigen presenting cells (Singh & O'Hagan, 2003). Here, we employed a new vaccine delivery system, biodegradable thermosensitive PECE hydrogel. The safety of the PECE hydrogel was confirmed safe in our previous study (Gong, Wu, Dong, et al., 2009). The PECE hydrogel was investigated as controlled drug delivery system previously, and the drug release behavior of the PECE hydrogel was driven by two forces: diffusion effect and erosion effect (Gong, Shi, Peng, et al., 2009; Weich, Iberg, Klagsbrun, & Folkman, 1990). On the basis of this characteristic, PECE hydrogel was applied as a depot for sustained antigen release. Moreover, proteins encapsulated in the PECE hydrogel matrix have been shown to retain their native configuration (Gong, Shi, Wu, et al., 2009; Gong, Shi, Dong, et al., 2009; Gong, Wu, Dong, et al., 2009). The strong intermolecular interactions between proteins and the PECE hydrogel, along with the low diffusion rate *in vivo*, decreased the release rate of proteins form the PECE hydrogel system. During the release process *in vivo*, erosion of the PECE hydrogel dominated the drug release behavior. The delayed protein release indicates the potential application of the PECE hydrogel as a protein vaccine carrier to improve immune responses (Gong, Shi, Peng, et al., 2009).

In contrast to vaccine delivery systems, immunostimulatory adjuvants are predominantly derived from pathogens and often

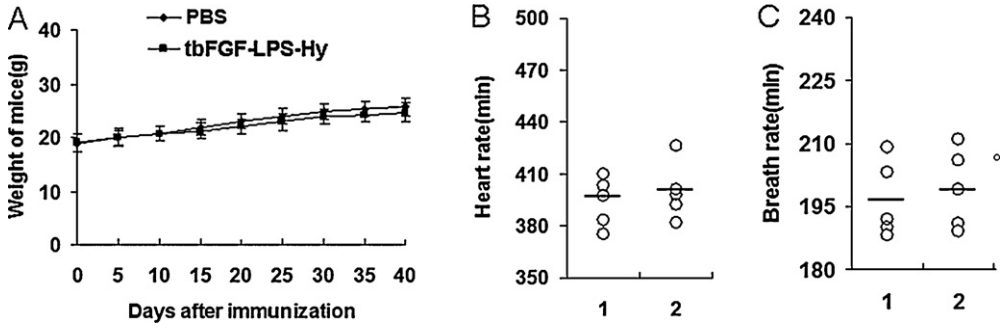

Fig. 7. Physiological monitoring of mice immunized with Hy-LPS-tbFGF. (A) Mice mean weight of the 2 groups. Mice from all groups were weighed once every 4 days. There was no difference in weight between the control and Hy-LPS-tbFGF groups from the beginning to the end of the immunization. On the fortieth day of immunization, the mean weight in the control and Hy-LPS-tbFGF groups was 23.7 (SD 1.9) g and 21.6 (SD 2.2) g, respectively ($P > 0.05$). Points, mean ($n = 5$); bars, SD. (B) Heart rate of mice immunized with control and Hy-LPS-tbFGF. There was no difference in the heart rate between the control and Hy-LPS-tbFGF groups. The mean heart rate of control and Hy-LPS-tbFGF groups was 196.4 and 199.2 beats per minute, respectively ($P > 0.05$). Bars, mean ($n = 5$). (C) Respiratory rate of mice immunized with control and Hy-LPS-tbFGF. There was no difference in respiratory rate between the control (mean, 393.6 breaths per minute) and Hy-LPS-tbFGF (399.8 breaths per minute) groups ($P > 0.05$). Bars, mean ($n = 5$).

represent pathogen-associated molecular patterns, such as LPS, MPL, and CpG DNA, which activate cells of the innate immune system (Singh & O'Hagan, 2003). LPS has been used as an adjuvant for years (Inoue & Yokochi, 1990; van Berkel et al., 2007), because it activates innate immunity through the recognition of antigen-presenting cells such as macrophages, dendritic cells, and murine B lymphocytes (Seppala & Makela, 1984). In recent years, it has been used to stimulate DC cells *in vitro* in some clinical trials (http://clinicaltrials.gov; ClinicalTrials.gov Identifier: NCT01189383, ClinicalTrials.gov Identifier: NCT00796770, and ClinicalTrials.gov Identifier: NCT00637117) for cancer and AIDS therapy. However, due to its toxicity, the application of LPS has been constrained *in vivo*. In this study, we loaded LPS into the PECE hydrogel to make a vaccine adjuvant complex; the LPS-Hydrogel was able to persist for approximately 14 days *in vivo* by s.c. administration. The delayed release of LPS resulted in a distinct decrease in LPS concentration *in vivo*, and its duration of effectiveness was prolonged. Furthermore, this prolonged release and uptake may result in a low concentration of LPS *in vivo*, and is most likely responsible for the observed decrease in toxicity. LPS increased the titer of serum immunoglobulins in our study, and the LPS-Hydrogel adjuvant had an effect similar to that of Freund's adjuvant (FA).

Angiogenesis plays an important role in tumor growth and metastasis, and bFGF is one of the most important cytokines for angiogenesis. Additionally, bFGF has been reported to play an important role in pathologic processes, including cancer (Brattstrom et al., 2004; Bremnes et al., 2006; Gravdal, Halvorsen, Haukaas, & Akslen, 2006; Guddo et al., 1999; Kuhn et al., 2006; Peyrat et al., 1992). Thus, bFGF is an ideal target for cancer therapy; however, native bFGF might remain in the body if used as a protein vaccine. Native bFGF has a 2-component receptor system: a transmembrane protein tyrosine kinase FGF receptor and heparin sulfate proteoglycan at the cell surface (Sheehan & Allen, 1999). Alterations in either the receptor binding domain or heparin binding domain of FGF-2 may abrogate FGF-2 activity (Plum et al., 2000). In our study, a truncated bFGF peptide (a.a. 24–115) corresponding to the heparin binding domain and part of the receptor binding domain of murine FGF-2 was generated and shown to block FGF-2-induced endothelial cell proliferation. This suggested that the truncated bFGF peptide possessed antigenic characteristics *in vivo*.

It is widely accepted that subunit vaccines elicit more potent and durable antigen-specific immunity if combined with an adjuvant (Ko et al., 2005; Moss et al., 1998; Schlick, Hewetson, & Ruffmann, 1986). A vaccine would require optimal adjuvants and delivery systems. In this study, using the truncated bFGF peptide as an antigen, we have shown that the novel LPS-Hydrogel combination adjuvant can enhance antigen-specific antibody and cell-mediated immune responses. Furthermore, mice vaccinated with tbFGF-LPS-Hydrogel inhibited tumor growth and development of LL/2 in both subcutaneous and lung metastases model.

Our research had yielded insight into the initiation of immune responses and the ways in which immunostimulatory adjuvants might enhance this process in mice. Our results showed that mice immunized with the inhibitory tbFGF peptide induced an immune response against bFGF and blocked the induction of neovascularization by bFGF, as observed in tumor histological slices. This was because the generation of antibody in Hydro-LPS-tbFGF-treated mice could target the key mediator of the pathological angiogenesis, bFGF, and subsequently control tumor growth through restoration of the homeostatic balance (Plum et al., 2000). Moreover, T cells isolated from mice immunized with tbFGF-LPS-Hydrogel showed increased cytotoxicity against the target MS1 cells, indicating that tbFGF-LPS-Hydrogel elicited the cytotoxic T lymphocyte response. The ELISpot revealed that tbFGF-LPS-Hydrogel elicited not only type 2 responses but also type 1 responses. Freund's adjuvant is considered the gold standard for adjuvants by many immunologists, and the effect of tbFGF-LPS-Hydrogel was almost as powerful as that of Freund's adjuvant.

5. Conclusions

The identification of novel adjuvants that can effectively enhance and modulate the antigen-specific immune response is of prime importance for successful vaccine development for both human and veterinary applications (Kovacs-Nolan et al., 2009). This study demonstrates that our newly designed LPS-Hydrogel system elicited antigen-specific antibody and cell-mediated immune responses. The effects of anti-tumor growth and metastases might be associated with humoral and cell-mediated immune responses. The thermosensitive and biodegradable PECE hydrogel is a promising in situ gel-forming controlled drug delivery system. tbFGF-LPS-Hydrogel could induce a very potent and robust anti-bFGF-specific immune response. LPS-Hydrogel could be a potent adjuvant and carrier for future peptide vaccines in cancer immunotherapy.

Conflict of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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