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Research paper

In situ administration of cytokine combinations induces tumor regression in mice

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ABSTRACT

Background: Recent advances in cancer immunotherapy suggest a possibility of harnessing the immune system to defeat malignant tumors, but the complex immunosuppressive microenvironment confines the therapeutic benefits to a minority of patients with solid tumors.
Methods: A lentivector-based inducible system was established to evaluate the therapeutic effect of cytokines in established tumors. Intratumoral injection of certain cytokine combination in syngeneic tumor models was conducted to assess the therapeutic potentials.
Findings: Doxycycline (Dox)-induced local expression of cytokine combinations exhibites a strong synergistic effect, leading to complete regression of tumors. Notably, IL12 + GMCSF+IL2 expression induces eradication of tumors in all mice tolerated with this treatment, including those bearing large tumors of ~15 mm in diameter, and generates intensive systemic antitumor immunity. Other combinations with similar immune regulatory roles also induce tumor elimination in most of mice. Moreover, intratumoral injection of chitosan/IL12 + GMCSF+IL2 solution induces a complete response in all the tested syngeneic tumor models, regardless of various tumor immunograms.
Interpretation: Administration of certain cytokine combinations in tumor microenvironment induces a strong synergistic antitumor response, including the recruitment of large amount of immune cells and the generation of systemic antitumor immunity. It provides a versatile method for the immunotherapy of intractable malignant neoplasms.
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1. Introduction

In recent years, cancer immunotherapy, including immune checkpoint inhibition and chimeric antigen receptor T cell (CAR-T), has become a powerful weapon against malignant neoplasms [1–8]. The results from experimental mouse tumor models and human clinical trials indicated that the immune system can eradicate malignant tumors, even those at the advanced stage. Although cancer immunotherapy has come a long way, there is still room for further improvement due to the percentage of non-responders, e.g., the low response rate of immune checkpoint therapeutics and ineffectiveness of CAR-T in solid tumors [9–11]. There remains a need for new modalities that are available to harness the immune system to defeat solid tumors.

Cytokines are important biomolecules in the crosstalk among immune cells [12]. The destination of an immune response can be greatly affected by the cytokine network. Based on the powerful immune regulation capacity, immunotherapy of cancers with cytokines has been attempted for decades [13]. The therapeutic effects of interferons, which are considered important in cancer immunoediting, have been evaluated in many clinic trials [14]. Type I interferon has been approved by the US Food and Drug Administration (FDA) for the application in the treatment of certain malignant tumors. In preclinical studies, some interleukins have exhibited good antitumor activities, providing attractive candidates to be translated into the clinic. Several phase II trials have indicated the effectiveness of interleukin 12 (IL12) in cancer immunotherapy, and interleukin 2 (IL2) has been approved by the FDA for some tumor indications. Because of its the ability to stimulate the proliferation and differentiation of immune cells, granulocyte macrophage colony stimulating factor (GMCSF) has been approved for the adjuvant treatment of malignant tumors. Moreover, GMCSF can promote the antigen presentation of dendritic cells, making it widely used in tumor vaccines [15].

Although the antitumor activities of cytokines have been observed in many studies, it is rare that the administration of a cytokine alone induces complete tumor regression. In fact, combination usage of cytokines often generates synergistic effects [16]. Here, we construct a

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Research in context

Evidence before this study

Cytokine therapy has been attempted in cancer treatment for decades, but improved therapeutic effects are still desperately desired. We searched publications in Pubmed using the keywords: "cytokine" and "cancer", especially focusing on those studies using cytokine combinations in preclinical tumor models. There are a few of papers describing the synergistic effects of cytokines used in a combinational manner decades ago. The enthusiasm in this area gradually reduces, due in part to the lack of a convenient method to precisely evaluate the therapeutic effects of cytokine combinations. The selection of cytokines and duration of administration may greatly affect the therapeutic outcome.

Added value of this study

Our study provides a platform to rapidly evaluate the therapeutic effects of cytokine combinations and demonstrates that the sustained administration of certain cytokine combinations efficiently induces tumor regression and antitumor immunity. Enrichment of Cytokines at tumor site by induced expression can unmask the maximal efficacy of the cytokine combinations.

Implications of all the available evidence

Cytokine combinations display surprising synergistic therapeutic potentials in malignant tumors. Many cytokines have been used in the clinic. The modality can be translated into the clinic based on the previous clinical investigation of these cytokines.

system to rapidly estimate the antitumor effects of cytokine combinations. Because cytotoxic T cells are pivotal effector cells to kill tumors, we tried to identify cytokine combinations that can induce tumor cell death, promote the presentation of tumor antigens, expand tumor-specific cytotoxic T cells and disrupt the immune suppression in the tumor microenvironment. The results described herein demonstrate that the in situ administration of certain cytokine combinations can efficiently evoke antitumor immunity and eliminate primary tumors.

2. Materials and methods

2.1. Cell culture

The mouse B16F10 melanoma cell line (RRID:CVCL_0159), Lewis lung carcinoma (LLC) cell line (RRID:CVCL_4358), EL4 lymphoma cell line (RRID:CVCL_0255) and CT26.WT colon carcinoma cell line (RRID:CVCL_7256) were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS (Life Technologies), and penicillin/streptomycin (Life Technologies) at 37 °C in a 5% CO_2 incubator. The mouse 4T1 mammary carcinoma cell line (RRID:CVCL_0125) was maintained in RPMI1640 medium supplemented with 10% FBS (Life Technologies), and penicillin/streptomycin (Life Technologies) at 37 °C with 5% CO_2. The human embryonic kidney cell line 293 (RRID:CVCL_0045) and 293FT (RRID:CVCL_6911) were maintained in DMEM with 10% FBS (Life Technologies), and penicillin/streptomycin (Life Technologies) at 37 °C in a 5% CO_2 incubator.

2.2. Plasmid construction and cell transduction

The DNA sequence encoding a reverse tet transactivator protein (rtTA) was synthesized (Genscript) and subcloned into the lentiviral vector pLentis-CMV-MCS-IRES-Bsd between the *Bam*HI and *Xho*I sites, generating the lentiviral vector pLentis-CMV-rtTA-IRES-Bsd. GFP, iRFP, IFNα, IFNγ, lymphotoxin, IL12, GMCSF, FLT3L, IL2, IL15 and IL21 coding sequences were synthesized and subcloned into the lentiviral vector pLentis-TRE-MCS-PGK-PURO between the *Bam*HI and *Xho*I sites, generating the inducible vector pLentis-TRE-Genes-PGK-PURO. The transcription of inserted genes was controlled by the tet-responsive TRE promoter. The two subunits of lymphotoxin, Lτα and Lτβ, were jointed using a P2A sequence. The two subunits of IL12, IL12α and IL12β, were jointed using a T2A sequence. The cytokine expression vectors were constructed by ligated IL12, GMCSF, FLT3L or IL2 coding sequences into the *Bam*HI and *Xho*I sites of pLentis-CMV-MCS-IRES-PURO, resulting in pLentis-CMV-IL12-IRES-PURO, pLentis-CMV-GMCSF-IRES-PURO, pLentis-CMV-FLT3L-IRES-PURO and pLentis-CMV-IL2-IRES-PURO.

The lentiviral particles were produced by the cotransfection of pMD2.G, psPAX2 and lentiviral vectors into 293FT cells. B16F10 cells were first tranduced with pLentis-CMV-rtTA-IRES-Bsd virus and selected with 8 μg/ml blasticidin, generating B16F10-rtTA cells. Subsequently, B16F10-rtTA cells were transduced with pLentis-TRE-Genes-PGK-PURO virus and selected with 3 μg/ml puromycin, generating the doxycycline (DOX) inducible cell line, B16F10-rtTA (TRE-Genes). The transduced cells were selected with blasticidin and puromycin every two passages. To construct cytokine-producing cells, 293 cells were transduced with pLentis-CMV-IL12-IRES-PURO, pLentis-CMV-GMCSF-IRES-PURO, pLentis-CMV-FLT3L-IRES-PURO or pLentis-CMV-IL2-IRES-PURO virus and selected using 3 μg/ml puromycin, the resulting cell lines were termed 293(IL12), 293(GMCSF), 293(FLT3L) and 293(IL2), respectively.

2.3. Fluorescence induction

B16F10-rtTA (TRE-GFP) cells were plated into a 24 well plate at 2×10^4 cells/well. GFP expression was induced by the addition of 100 ng/ml of dox immediately after plating. The cells were photographed using a fluorescence microscope (Olympus IX71).

Next, 10^5 B16F10-rtTA (TRE-iRFP) cells were subcutaneously inoculated into the right flank of C57BL/6 mice. When the tumor grew to a long diameter of 10 mm, the mice were fed with water containing 2 g/l of dox. The fluorescence was detected using the IVIS spectrum in vivo imaging system (Caliper Quantum FX).

2.4. Mice

C57BL/6 and BALB/c mice were purchased from the animal center of the Third Military Medical University. Mice aged 6–10 weeks were used for tumor inoculation. All the animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals. At the signs of illness (lethargy, hunched posture, scruffy coat, social isolation, inactivity or weight loss), the mice were removed from the experiments. The investigators were not blinded in the experimental conduction and data analysis. Animal experiments were conducted in at least 2 separate environments to avoid the result bias caused by the microbiome.

2.5. Mouse experimental design

There were five mice in one group and the trials were replicated two or three times, generating a 10 or 15 mice for each experiment. Particularly, in the experiments to assess the effects of induced IL12 + FLT3L + IL2, IL12 + FLT3L + IL15 and IL12 + FLT3L + IL21 expression, 8–12 mice were inoculated with tumor cells at the beginning, to ensure that five mice with an established tumor can be randomly allocated.

The purpose of this study was to identify a method to completely eradicate tumors, instead of suppressing tumor growth. The major

endpoint was the overall survival of mice after treatment. If a mouse died, even the tumor grew slower than the control, treatment failure was indicated. Mice keeping tumor free for over two months after treatment were considered successful tumor clearance. The survival of each mouse was recorded and indicated as fractions number in figures. However, only a portion of the tumor growth data was recorded and presented in the figures and elucidated in the legends.

2.6. Assessment of the effects of induced cytokine expression on tumors

In experiments evaluating the effects of single cytokine expression on tumor growth, 10^5 inducible tumor cells were subcutaneously injected into the right flank of mice. In experiments assessing the effects of cytokine combinations, tumor cell mixtures containing 5×10^4 of each inducible tumor cell line were subcutaneously injected into the right flank of mice. When a noticeable tumor nodule was formed, dox was continuously administered by adding 2 g/l dox into drinking water. The perpendicular diameters of the tumors were measured using a caliper every three days, and tumor area was calculated by $a \times b$, where a is the long diameter and b is the short diameter. In experiments investigating the therapeutic effects of induced IL12 + GMCSF +IL2 expression on large tumors, dox was administered after the long diameter reached 10-15 mm or over 15 mm.

To detect the existence of immune memory, 2×10^5 parental B16F10 cells were intravenously injected or subcutaneously inoculated into the contralateral flank of cured mice two months after primary tumor clearance. Subcutaneous tumor formation and mouse survival were recorded daily.

In experiments exploring the effects of induced IL12 + GMCSF+IL2 expression on tumors at the contralateral flank, the mice were allocated into three groups based on the tumor size. 1, Inducible tumor cells were s.c. injected at the right flank 10 days prior to inoculation of 10^5 parental tumor cells at the contralateral flank. At the time of induction, the inducible tumors were larger (~10 mm in diameter) and the parental tumors were smaller (~3 mm in diameter). 2, Inducible tumor cells were s.c. injected at the right flank one day prior to the inoculaton of 10^5 parental tumor cells at the contralateral flank. At the time of induction, the inducible tumors were smaller (~5 mm in diameter) and the parental tumors were larger (~15 mm in diameter). 3, Inducible tumor cells were s.c. inoculated at the right flank of mice. When the tumors were palpable, 10^5 parental tumor cells were s.c. injected at contralateral flank. At the time of induction, the sizes of the tumors at both flanks were comparable (~10 mm in diameter).

In experiments exploring the effects of induced IL12 + GMCSF+IL2 expression on i.v. infused tumor cells, the mice were allocated into two groups based on the time of induction. Inducible tumor cells were s.c. inoculated at the right flank of mice. Next, 2×10^5 parental tumor cells were i.v. injected after tumor nodules were formed. Dox was administered immediately or two days later. Mouse survival was monitored daily.

2.7. Detection of cytokine secretion

Inducible tumor cells were plated into a 24 well plate at 5×10^4 cells/well in 700 μl of medium. Next, 100 ng/ml of dox was separately administered at 24 h, 48 h or 72 h, and all supernatants were collected at 96 h after cell plating.

293 cells expressing IL12, GMCSF or IL2 were plated into a 24 well plate at 5×10^4 cells/well in 700 μl of medium. The supernatants were collected 96 h later.

The concentration of IL12p70, GMCSF, FLT3L, IL2, IL15 or IL21 in the supernatants was measured using ELISA Kits (Neobioscience), according to the manufacturer's instructions.

2.8. Tumor infiltrating immune cell analysis

Mice bearing tumors of ~6 mm in diameter were used in this experiment. At 0 days, the first day, the second day and the third day after dox administration, mice bearing inducible tumors were sacrificed. In the control group, mice bearing parental tumors were sacrificed at 0 days and the third day after dox addition. Tumor tissues were isolated with a scalpel and fixed in 1% paraformaldehyde overnight. After rinsing in PBS, the samples were hydrated in 30% sucrose/PBS overnight. The tissues were mounted in OCT embedding compound and cut in 10 μm tissue sections using a cryostat. The sections were fixed in PBS /1% PFA, permeabilized in PBS /0.2% Tween-20 /0.3 M glycine and blocked with PBS /0.2% Tween-20/5% heat-inactivated FBS/0.05% NaN3. Next, the sections were incubated with 1:500–1:1000 dilution for each primary antibody in PBS/0.2% Tween-20/5% heat-inactivated FBS/0.05% NaN3. After washing, the sections were incubated with 1:500 dilution for the corresponding secondary antibodies in PBS/0.2% Tween-20/5% heat-inactivated FBS/0.05% NaN3, together with a trace amount of DAPI. The photographs were captured using an inverse research microscope (Nikon Ti-E).

The primary antibodies used in this study were rat anti-CD3(R&D, MAB4841-SP, RRID:AB_358426), rat anti-CD19(abcam, ab25232, RRID: AB_470414), hamster anti-CD11c(AbD Serotec, MCA1369, RRID:AB_324490), and rat anti-F4/80(AbD Serotec, MCA497G, RRID:AB_872005). The secondary antibodies used in this study were goat anti-hamster IgG (H + L) Secondary Antibody, Alexa Fluor 647 (Invitrogen, A-21451, RRID:AB_2535868), donkey anti-rat IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Invitrogen, A-21208, RRID:AB_2535794).

2.9. Cytokine solution production

293 cells expressing IL12, GMCSF, FLT3L or IL2 were plated into 15 cm dishes in complete DMEM medium. After the cells grew to over 90% confluence, the culture medium was replaced with 25 ml of CDM4HEK293 medium (Hyclone) supplemented with 4 mM L-glutamine. Ninety-six hours later, the supernatants were collected and filtered through a 0.45 μm filter (Millipore). Next, each cytokine supernatant was concentrated to 1 ml using an Amicon Ultra-15 centrifugal filter unit (Millipore), according to the manufacturer's instructions. The cytokine solutions were aliquoted and stored at −20 °C.

2.10. Murine tumor treatment

B16F10 (10 [5]), LLC (2×10 [5]) or EL4 (10 [6]) cells were s.c. injected into the right flank of C57BL/6 mice. 4 T1 (5×10 [5]) or CT26 (5×10 [5]) cells were s.c. injected into the right flank of BALB/c mice. Treatment was initiated when the tumor reached ~6 mm in diameter.

In experiments using cells for treatment, 293 cells expressing IL12, GMCSF or IL2 were digested and resuspended in complete DMEM medium at a density of 1.5×10^5 293-IL12 + 1.5×10^5 293-GMCSF +1.5×10^5 293-IL2/45 μl. After the addition of 1.5 μl 2 M $CaCl_2$, 45 μl of 1.5% alginate (Sigma Aldrich, A0682) solution was added and mixed immediately to form hydrogel, which was slowly injected into tumors at 1.5 μl/mm^2 using a 29G insulin syringe (Becton).

In experiments using cytokine solution for treatment, 45 μl of solution containing 15 μl of each cytokine were mixed with 45 ul of 3% chitosan (Chitosan glutamate, Protosan G 213, NovaMatrix) solution. Next, the chitosan/cytokine solution was slowly injected into tumors at 1.5 μl/mm^2 using a 29G insulin syringe.

At the signs of disease progression, e.g., tumor size increase or new nodule appearance, additional injections were conducted until the tumor reached 12 mm in diameter.

A fifty-week-old BALB/c mouse bearing a 10 mm × 10 mm spontaneous tumor at ear root was intratumorally injected with 100 μl of chitosan/IL12 + GMCSF+IL2.

2.11. Dog tumor treatment

Two dogs, diagnosed with advanced-stage cancer, were treated with chitosan/IL12 + GMCSF+IL2 at Animal Hospital of Beijing Chaoyang District Animal Disease Control Center. The protocol was approved by the ethics committee of the hospital. Signed informed consent signature from the dog owners was required before treatment. Canine IL12, GMCSF and IL2 (Novas Biologicals) were reconstituted to 150 ng/μl. The cytokine solution for injection was prepared by mixing IL12, GMCSF, IL2 and 3% chitosan at a volume ratio of 1:1:1:3. The dose of injection was 1 μl of cytokine solution per mm^2 tumor area. The solutions were slowly injected into the nodules using a 29G insulin syringe and the status of the dogs was closely monitored. Treatment response was defined as follows: complete response (CR), disappearance of all tumors; partial response (PR), at least 30% reduction in the sum of the long tumor diameter; progressive disease (PD), at least 20% increase in the sum of the long tumor diameter.

2.12. Statistics

Statistical analysis was carried out using GraphPad Prism 5 software. Survival curves were analysed using the log-rank (Mantel-Cox) test. The comparison of growth curves was conducted with two-way ANOVA. $p < .05$ was considered to indicate statistical significance, $^{*}p < .05$,$^{**}p < .01$, $^{***}p < .001$.

3. Results

3.1. Evaluation of the antitumor activities of cytokine combinations using a lentivector-based inducible system

Inducible expression systems have been used to evaluate the influences of some genes or siRNAs on tumor development [17–20]. We constructed a lentivector-based doxycycline (dox)-inducible system comprising two components: 1, a lentiviral vector for the constitutive expression of reverse tet transactivator (rtTA); 2, a lentiviral vector harboring a tet-responsive TRE promoter and an MCS site, into which cytokine genes are easy to be cloned (Fig. 1a). After transduction and antibiotics selection, B16F10 cells carrying various inducible genes were generated. The addition of dox induced significant expression of the cloned gene in vitro (fig. S1). Using iRFP as a marker [21], we tested the availability of this system in vivo. Fluorescence could be detected 24 h post induction, and gradually increased on the subsequent days (Fig. 1b). Notably, survival analysis suggested that the tumorigenicity of B16F10 cells was not altered by lentiviral transduction and dox administration (fig. S2). Thus, the therapeutic effects of some cytokines, which can elicit immune activation, were assessed with this system. Although it has been approved in cancer therapy in the clinic [22], monotherapy of some cytokines could only suppress tumor growth, not eliminate established tumors in this system. IL12, IFNα and IFNγ induced tumor regression in some of the tested mice (Fig. 1c, fig. S3). Subsequently, the synergistic antitumor effects of the cytokine combinations were investigated. In antitumor immunity, macrophages and T cells play pivotal roles, and dendritic cells are important for tumor antigen presentation. Three cytokines, IL12, IL2 and GMCSF, which can activate these cells, were selected to evaluate the combination effects. All the mice in the IL12 + IL2 group, 86.7% in the IL12 + GMCSF group and 40% in the GMCSF+IL2 group were cured, indicating a strong synergistic effect (Fig. 1d). Furthermore, the combination of IL12, GMCSF and IL2 exhibited superior antitumor activity, such that all the tumors were eliminated within 10 days after induction (Fig. 1e, fig. S4). Ulceration of the tumor area suggested the existence of acute immune attack (Fig. 1f). Considering the importance of IFNγ in antitumor immunity, we additionally tested the curative effects of the IL12 + GMCSF+IL2 + IFNγ combination. Interestingly, the addition of IFNγ attenuated the antitumor activity of the IL12 + GMCSF+IL2 combination,

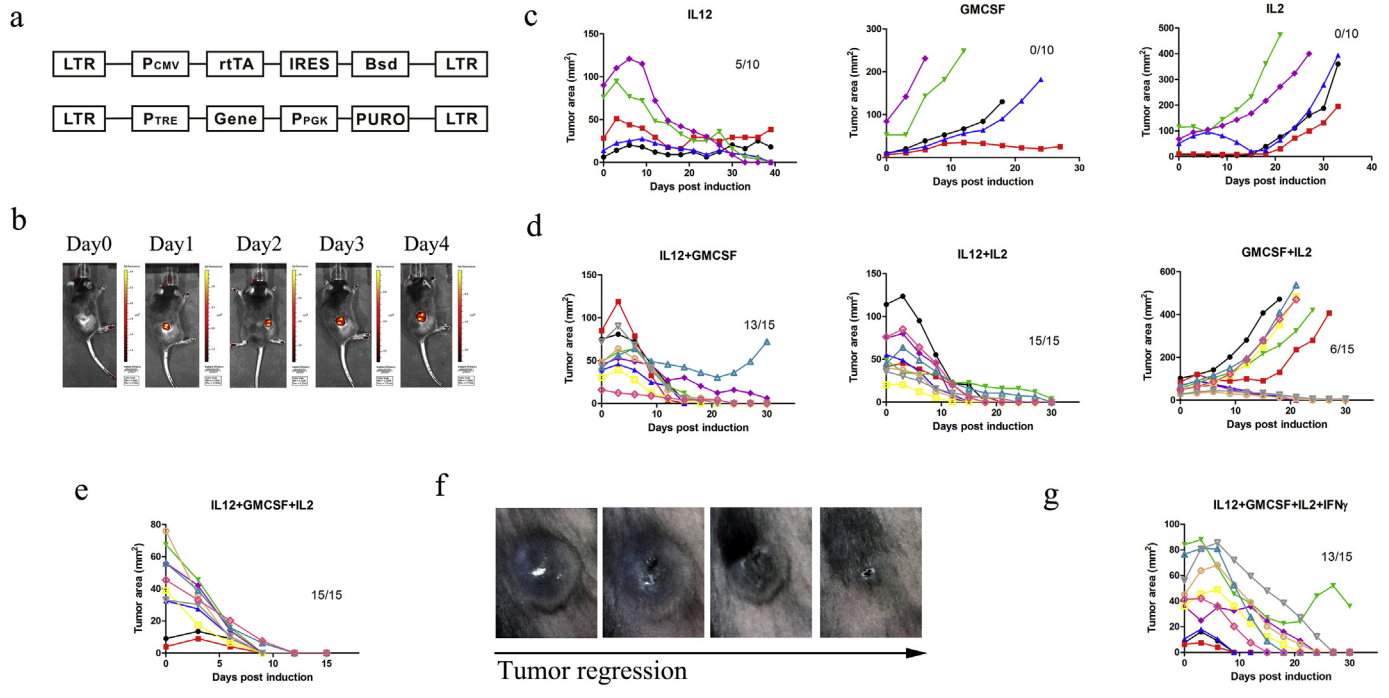

Fig. 1. A lentivector-based inducible system to evaluate the antitumor effects of cytokines. (a) Schematic representation of the two lentivectors of the inducible system. (b) Dox-induced iRFP expression in B16F10-rtTA(TRE-iRFP) tumors. (c) Effects of dox-induced single cytokine expression, including IL12, GMCSF or IL2, on the growth of established tumors. The tumor growth curves are data from one of two replicate experiments (five mice in each replicate). (d) Effects of dox-induced two-cytokine combinations expression, including IL12 + GMCSF, IL12 + IL2 or GMCSF+IL2, on the growth of established tumors. The tumor growth curves are data from two of three replicate experiments (five mice in each replicate). (e) Effects of dox-induced expression of the IL12 + GMCSF+IL2 combination on the growth of established tumors. The tumor growth curves are data from two of three replicate experiments (five mice in each replicate). (f) Representative photographs of tumor regression after induced IL12 + GMCSF+IL2 expression. (g) Effects of dox-induced expression of the IL12 + GMCSF+IL2 + IFNγ combination on the growth of established tumors. The tumor growth curves are data from two of three replicate experiments (five mice in each replicate). Each curve represented the tumor growth of an individual mouse after dox administration. The numbers in the graphs indicate tumor-cleared mice/total dox-treated mice.

leading to delayed tumor regression (Fig. 1g) and suggesting that an elaborate design of cytokine combination was required for the maximal stimulation of antitumor immunity.

3.2. Local expression of IL12 + GMCSF+IL2 induces systemic antitumor immunity

Next, we assessed the therapeutic potential of the IL12 + GMCSF +IL2 combination in late stage melanoma. The addition of dox-induced tumor cells to secrete high level cytokines (fig. S5), provided a sustained cytokine supply in inducible tumors. For advanced tumors (longest diameter range from 10 mm to 15 mm), induction of cytokine expression led to complete clearance in all tumor bearing mice (Fig. 2a). In the group of mice with a larger tumor burden (longest diameter over 15 mm), dox addition induced the elimination of tumors in all tolerated mice (Fig. 2a, fig. S6). Of note, except for the curative effects, the other mice died within four days after induction, likely due, in part, to overwhelming inflammation from an immune attack on large tumors. Next, we rechallenged the IL12 + GMCSF+IL2 cured mice, subcutaneously or intravenously, with parental B16F10 cells over two months later. All the mice rejected the inoculated tumor cells, suggesting immune memory targeting endogenous tumor antigens (Fig. 2b). To investigate whether the immune response could erase established tumors, parental tumor cells were inoculated contralaterally to inducible tumors. Induction of cytokine expression significantly prolonged the survival time of mice bearing a small parental tumor and a large inducible tumor (Fig. 2c, Fig. S7a). By contrast, antitumor immunity from cytokine expression in small inducible tumors had little effect on the growth of large parental tumors on the contralateral flank (Fig. 2c, Fig. S7b). However, if the parental and inducible tumors were both larger in size, all the mice died within six days after induction (Fig. 2c, Fig. S7c). Thereafter, we evaluated the effects of the antitumor immune response on metastatic tumor cells. Parental B16F10 cells were intravenously injected into mice bearing inducible tumors. Long-term survival was achieved in all the injected mice if dox was administered just after injection (Fig. 2d, Fig. S8a). If dox was administered two days post injection, 40% of mice cleared intravenous tumor cells and the remaining died within four days after induction (Fig. 2d, Fig. S8b). The cause was likely due to systemic inflammation elicited from the immune response targeting visceral metastatic lesions. Unlike other immunotherapy modalities [23,24], the side effects after cytokine treatment were much weaker, such as vitiligo that was well restricted at the tumor site (Fig. 2e).

To identify cell types participating in the tumor clearance mediated by IL12 + GMCSF+IL2 expression, the tumor masses were collected and subjected to immunohistochemistry (IHC) analysis. Dox induction did not alter the immune cell infiltration in parental tumors. Before induction, there were slightly more F4/80+, CD11c + and CD3+ cells in the tissues of inducible tumors than those in parental tumors, likely due to the background expression of cytokines. After induction, extensive macrophage and T cells infiltration was observed in the tumors, accompanied with an increased number of dendritic cells (fig. S9). This founding was consistent with a previous conclusion that T cells are the predominant effector cells in cancer immunotherapy [25,26].

3.3. Identification of other cytokine combinations with strong antitumor activity

Cytokines are multifunctional biomolecules in the immune system, indicating that some cytokines have overlapped activities. Like GMCSF, FLT3L promotes the activation and antigen presentation of dendritic cells. In addition to IL2, other cytokines, including IL15 and IL21, can promote T cell activation and expansion [13]. Therefore, after substituting GMCSF with FLT3L, and IL2 with IL15 or IL21, we tested the antitumor activities of the other five cytokine combinations: IL12 + GMCSF +IL15 (Fig. 3a), IL12 + GMCSF+IL21 (Fig. 3b), IL12 + FLT3L + IL15 (Fig. 3c), IL12 + FLT3L + IL21 (Fig. 3d) and IL12 + FLT3L + IL2 (Fig. 3e). All the combinations exhibited strong antitumor activities (fig. S10). Specifically, background secretion of IL12 + FLT3L + IL2 displayed marked suppressive effect in the process of tumor engraftment, as only 50% mice inoculated with these tumor cells generated a palpable tumor lesion. Although tumor growth was markedly inhibited in these groups, only IL12 + GMCSF+IL21 induced complete regression in all the mice. Generally, compared with FLT3L, GMCSF exhibited a

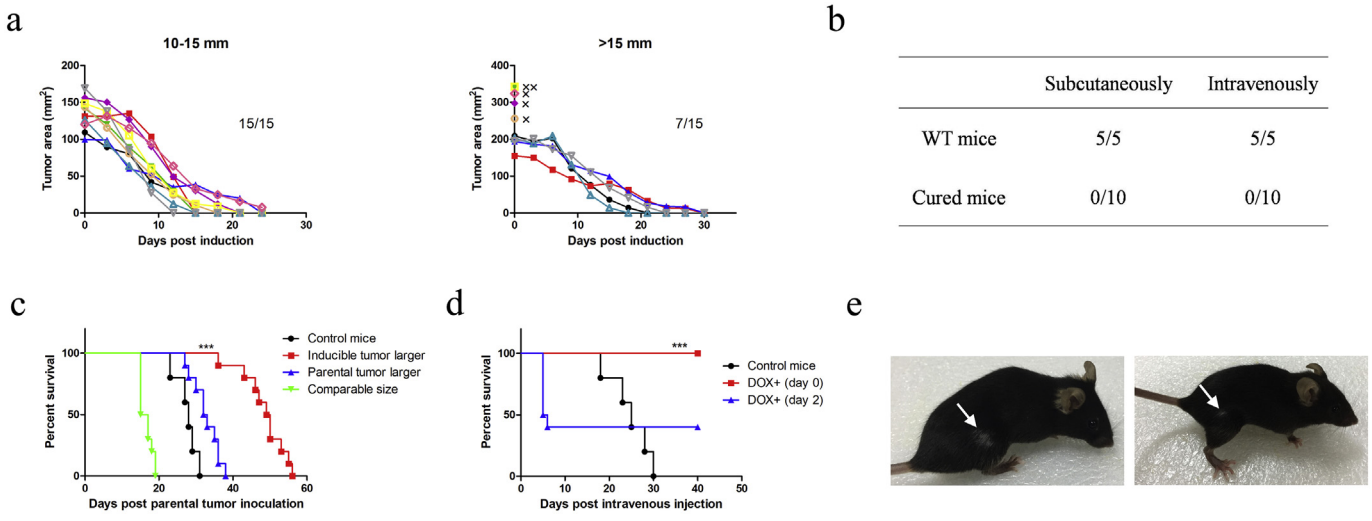

Fig. 2. Induced expression of the IL12 + GMCSF + IL2 combination elicites systemic antitumor immunity. (a) Effects of dox-induced expression of the IL12 + GMCSF+IL2 combination on the growth of large established tumors (10 mm < diameter < 15 mm or diameter > 15 mm). Each curve represented the tumor growth of an individual mouse after dox administration. The numbers in the graphs indicate tumor-cleared mice/total dox-treated mice. X indicates a death case within four days after induction. The tumor growth curves are data from two of three replicate experiments (five mice in each replicate). (b) Cured mice rejected rechallenged B16F10 tumor cells, either subcutaneously or intravenously injected. The numbers indicated death case/injected mice. (c) At different times post subcutaneous inoculation of inducible tumor cells into the flank of C57BL/6 mice, parental tumor cells were subcutaneously injected into the contralateral side. Dox induction was initiated when the size of bilateral tumors was at different ratios (inducible tumor larger, parental tumor larger, or size of both tumors were comparable). Mice bearing only parental tumors served as the control. The survival of tumor bearing mice was recorded. $n = 5$ for the control group and $n = 10$ for the other groups. ***, $p < .001$. (d) Parental tumor cells were intravenously injected into mice bearing subcutaneously established inducible tumors. Dox induction was initiated just after injection or two days later. Mice without inducible tumors served as the control. The survival of tumor-bearing mice was recorded. n = 5 for the control group and n = 10 for the other groups. ***, $p < .001$. (e) Vitiligo at the tumor site after tumor regression caused by the induced expression of IL12 + GMCSF+IL2.

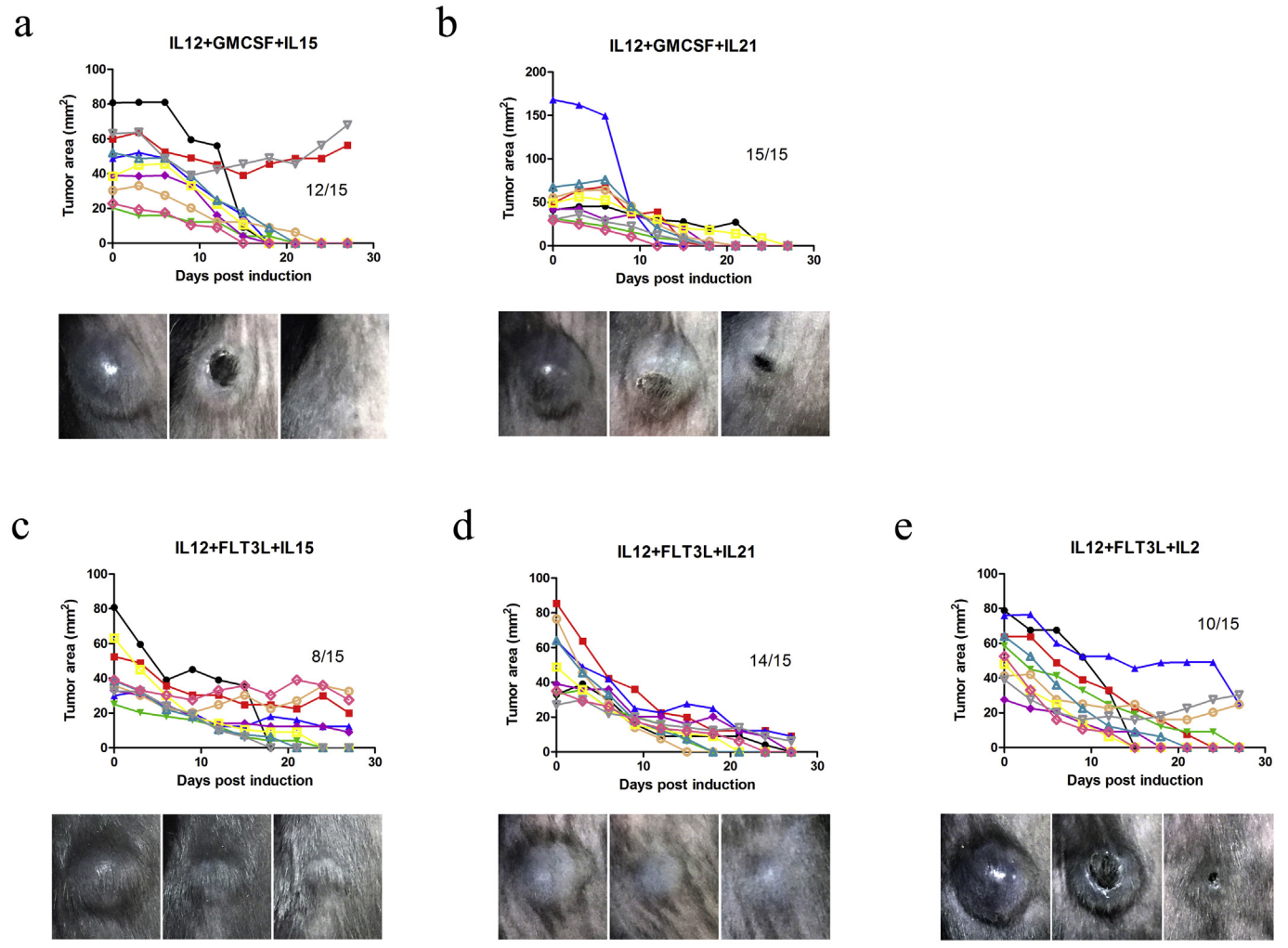

Fig. 3. Induced expression of cytokine combinations eliminates established tumors. Mixtures of different cytokine inducible B16F10 cells, including IL12 + GMCSF+IL15 (a), IL12 + GMCSF +IL21 (b), IL12 + FLT3L + IL15 (c), IL12 + FLT3L + IL21 (d), IL12 + FLT3L + IL2 (e), were subcutaneously inoculated at the flank of C57BL/6 mice. Dox induction was initiated after tumor nodule formation. Tumor growth was recorded after induction. Each curve represented the tumor growth of an individual mouse after dox administration. The numbers in the graphs indicate tumor-cleared mice/total dox treated mice. The tumor growth curves are data from two of three replicate experiments (five mice in each replicate). The photographs indicate a representative tumor regression progress.

higher efficiency of stimulating the immune clearance of established tumors (Overall survival: IL12 + GMCSF+IL2 vs IL12 + FLT3L + IL2, 100% vs 66.7%, respectively; IL12 + GMCSF+IL15 vs IL12 + FLT3L + IL15, 80% vs 53.3%, respectively; IL12 + GMCSF+IL21 vs IL12 + FLT3L + IL21, 100% vs 93.3%, respectively;). Unlike IL12 + GMCSF+IL2, vitiligo was rarely observed in the cured mice of these groups (fig. S11). The IL12 + GMCSF+IL2 induced tumor regression process was also faster than that in these groups (Fig. 1e). Taken together, the data showed that the local administration of the cytokine combination by induced expression can eradicate large established tumors with advanced malignancy, and a better therapeutic effect is usually accompanied by a higher side effect.

3.4. Intratumoral injection of IL12 + GMCSF+IL2 induces complete tumor regression

The induction of cytokine expression from tumor cells provides a sustained and concentrated cytokine supplement in the tumor microenvironment, a function that is important for persistent immune stimulation and reduced peripheral toxicity. Some slow release materials, e.g., alginate microsphere and chitosan, have been used in cancer therapy to recapitulate this effect [27,28]. First, we constructed 293 cells expressing cytokines by lentiviral transduction (fig. S12a). Using alginate gel as a carrier, cells expressing IL12, GMCSF and IL2 were injected into B16F10 melanoma lesions. After 3–4 rounds of injection, 70% of mice successfully cleared established tumors (fig. S13). To further improve the versatility of cytokine treatment, IL12and GMCSF, as well as IL2 expressed from 293 cells, were concentrated by ultrafiltration (fig. S12b). After mixing with chitosan, the concentrated cytokines were injected into tumors for long-term retention. Compared with cytokine-expressing cells, this treatment was more efficient as one or two injections induced B16F10 melanoma regression in all the mice (Fig. 4a). Although there were relapses in some mice, secondary injection caused regression. Thus, there was no resistance to this therapeutics in recurrent tumors. Despite the high antitumor activity in the process of tumor engraftment, delivery of IL12 + FLT3L + IL2 with chitosan could not induce the elimination of established B16F10 tumors (fig. S14).

Next the therapeutic potential of chitosan/IL12 + GMCSF+IL2 was evaluated in other murine syngeneic tumor models, including lymphoma cells (EL4), Lewis lung carcinoma cells (LLC), colon carcinoma cells (CT26) and breast cancer cells (4 T1). Injection of chitosan alone could not suppress the tumor growth (fig. S15). A single injection of chitosan/IL12 + GMCSF+IL2 led to complete regression of EL4 and CT26 tumor lesions (Fig. 4b, Fig. 4d). All LLC tumors regressed after two rounds of intratumoral injections (Fig. 4c). Some 4 T1 tumors relapsed

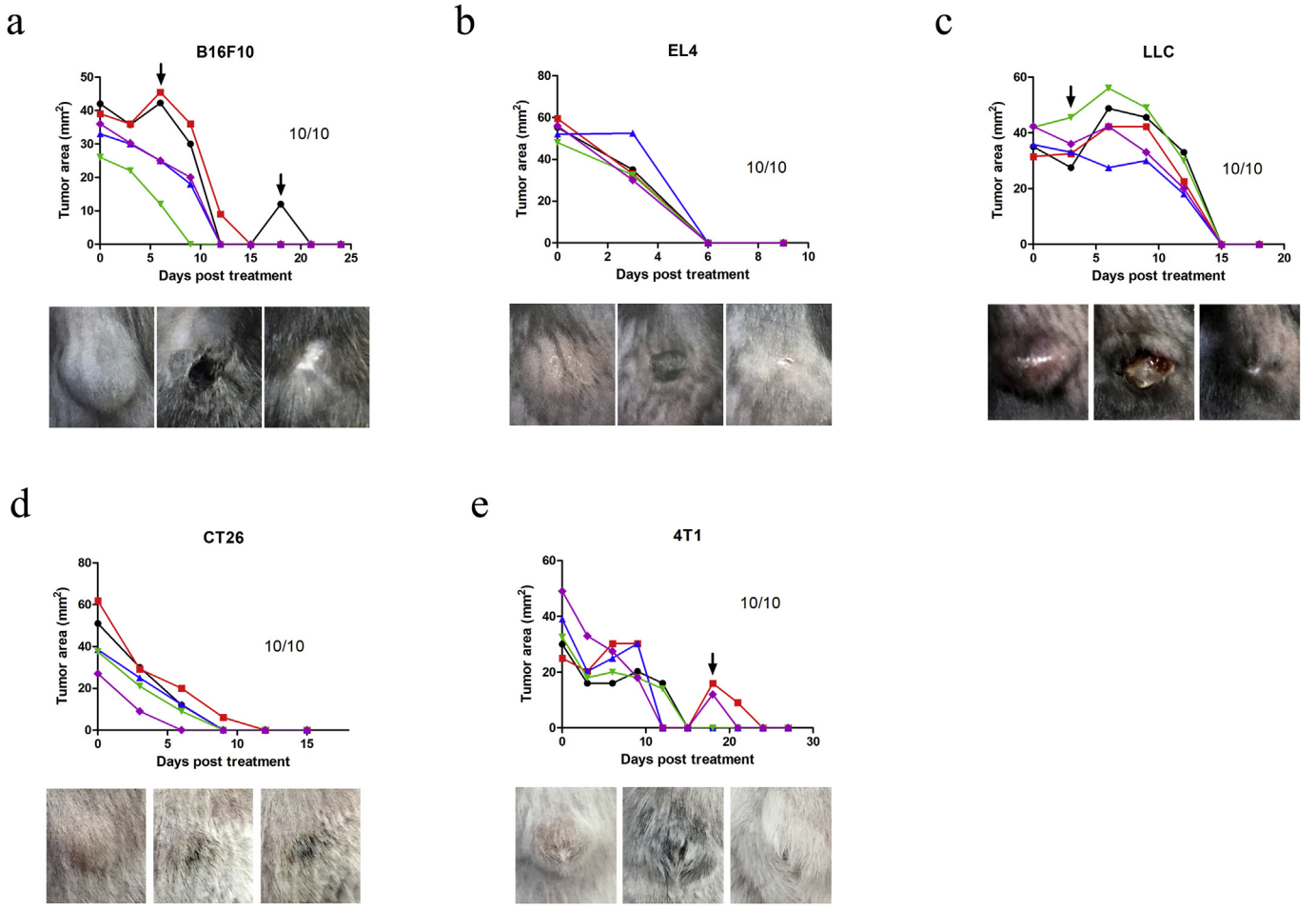

Fig. 4. Treatment of syngeneic tumors by intratumoral injection of chitosan/IL12 + GMCSF + IL2. B16F10 (a), EL4 (b), LLC (c), CT26 (d) and 4 T1 (e) tumors were established by subcutaneous injection of tumor cells into the flank of syngeneic mice. Intratumoral injection of chitosan/IL12 + GMCSF+IL2 was performed when the tumor diameter reached ~6 mm. Tumor growth was monitored and additional injections were carried out at the signs of tumor size increase or relapse after regression, indicated by arrows in the graphs. X indicates a death case. The numbers in the graphs indicated tumor-cleared mice/total treated mice. The tumor growth curves are data from one of two replicate experiments (five mice in each replicate). The photographs indicate a representative tumor regression progress.

1–2 weeks after regression. Apart from two mice that died within four days post secondary injection, the remaining showed clearance of the relapsed 4 T1 tumors, generating a tumor-free long-term survival (Fig. 4e). Regardless of various cancer immunograms, all tumor bearing mice were curable by intratumoral injection once the therapeutics were tolerated. In addition, an old mouse harboring a spontaneous tumor at the ear root was treated with chitosan/IL12 + GMCSF+IL2. After injection, the tumor gradually regressed within ten days (fig. S16) demonstrating that this treatment, targeting the immune system, could be applied on various malignant tumors. To test the therapeutic potential in large animals, two dogs were treated by intratumoral injection of chitosan/IL12 + GMCSF+IL2. CR in the dog with oral malignant melanoma and PR in the dog with mammary carcinoma were induced by a single injection, without serious adverse effects (fig. S17).

4. Discussion

Based on the complementary immune-stimulating capabilities of different cytokines, the combinational usage usually generates strong synergistic effects [16]. Simultaneous administration of other cytokines, including IL2, IL15, IL21, and GMCSF, can greatly enhance the antitumor activities of IL12. The mechanism of the synergistic effects is very complex due to the existence of reciprocal target cells for these cytokines. For example, IL18 + IL12 induces Th1 polarization, but IL18 + IL2 induces Th2 cytokines [29,30]. The application of certain cytokines and inhibition of others with opposing functions is another choice of generating synergistic effects [31,32]. In fact, the consequences of cytokine combinations are affected by factors such as the dose, administration route, schedule, and strategies. The IL12/pulse IL2 treatment regimen, not simultaneous infusion, has exhibited great antitumor effects and has been translated into the clinic [33]. It is difficult to predict the therapeutic outcome of cytokine combinations by previous understanding of the molecular mechanisms of a single cytokine. In our study, it was unexpected that the addition of IFNγ impairs the competency of IL12 + GMCSF+IL2. Consistent with previous research, two cytokine combinations, IL12 + IL2 and IL12 + GMCSF, efficiently suppress tumor growth [34]. The rationale of the IL12 + GMCSF+IL2 combination is to provide all components to induce tumor cell death, promoting antigen presentation and expanding tumor-reactive CTLs. Fortunately, this combination exihibits superior antitumor activities in practice.

There are great variations in genomic mutations and immune cell infiltration among different tumor types [35]. The availability of IL12 + GMCSF+IL2 in various tumor models indicates it is a versatile and active therapeutics, which can recruit tumor reactive immune cells even in a tumor lacking immune cell infiltration. These characteristics well address the concern of tumor heterogeneity in human patients [36]. Unlike immune checkpoint therapy, which relies on pre-existing tumor antigen-specific T cells, the cytokine combination regimen theoretically can be applied on all solid tumors without pre-determination of immune profiles.

0.01w?>Cytokines have been used in cancer treatment clinics for decades, mostly in systemic administration. Poor therapeutic outcomes and increased side effects have greatly restricted their extensive application [37,38]. Local delivery of cytokines using biomaterials has been attempted in some murine tumor models, and a synergistic effect was observed in combination usage [39–41]. Because of the infinite cytokine combinations, it is unaffordable to screen cytokines with high antitumor activities using those methods. However, the lentivector based inducible system presented here resolved these problems. Induction of cytokine expression in tumor cells provides sustained local cytokine enrichment, greatly improving the lesion/serum ratio of the drug concentration, which ultimately unmasks the therapeutic potential of cytokines. Albeit inferior to inducible expression, chitosan/IL12 + GMCSF +IL2 treatment erased all the tested murine tumors, suggesting the practicability of this screen system. It is noteworthy that in situ administration is crucial because systemic IL2 delivery attenuates the antitumor effect of IL12 + GMCSF [34]. The lethal toxicity observed in mice with a high tumor burden might be tolerated in human patients because of the much lower tumor/body weight ratio compared with small animals.

In this study, chitosan is used to recapitulate the feature of a sustained release of cytokines in the induced expression system, providing a microenvironment favorable for antitumor immune responses. Biodegradable materials have been widely used in tumor treatment. Alginate scaffold delivery greatly improves the efficacy of adoptive T cell therapy [42]. Vaccination with polylactide-*co*-glycolide (PLG) matrices incorporating GMCSF, CpG and tumor lysates induces strong antitumor immunity [39]. Delivery of alginate microspheres encapsulated endostatin-secreting cells are effective for the treatment of implanted human glioblastoma [27]. Although convenient to use, chitosan is not the optimal choice as a cytokine delivery vehicle. It can be envisaged that the therapeutic effects of cytokine combinations can be further improved by the utilization of advanced biomaterials [43].

Recently, the T cell response to immune checkpoint therapy in human patients was found to be similar to that in the murine tumor model [44], paving the way for translating cancer immunotherapeutics from murine models to human carcinoma. IL12 is in late-stage clinical trials [45], and new drug application (NDA) of GMCSF and IL2 have been approved, providing a possibility to initiate an exploratory clinical investigation of a regimen using the IL12 + GMCSF+IL2 combination. Certainly, some more optimizations can be attempted to ameliorate the drawbacks of the method in the current status. Compared with the cytokine mixture, the construction of fusion protein is more suitable for drug development. On the other hand, the addition of a tumor targeting subunit, e.g., antibody conjugation, is likely to generate a formulation suitable for intravenous infusion, which increases the operation convenience. Nevertheless, our study is valuable to rapidly improve immune therapeutic strategies targeting human malignant neoplasms.

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Declarations of interests

Jinyu Zhang has a patent PCTCN2018080479 pending, and a patent 2018100138344 pending. The other authors declare no conflicts of interests.

Author contributions

Jinyu Zhang designed the study and carried out most of the experiments. Haochen Jiang performed IHC staining of tumor tissues. Haiyun Zhang organized the treatment in dog patients.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.09.050.

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