Localized cocktail chemoimmunotherapy after in situ gelation to trigger robust systemic antitumor immune responses

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Currently, there is a huge demand to develop chemoimmunotherapy with reduced systemic toxicity and potent efficacy to combat late-stage cancers with spreading metastases. Here, we report several “cocktail” therapeutic formulations by mixing immunogenic cell death (ICD)-inducing chemotherapeutics and immune adjuvants together with alginate (ALG) for localized chemoimmunotherapy. Immune checkpoint blockade (ICB) antibody may be either included into this cocktail for local injection or used via conventional intravenous injection. After injection of such cocktail into a solid tumor, in-situ gelation of ALG would lead to local retention and sustained release of therapeutics to reduce systemic toxicity. The chemotherapy-induced ICD with the help of immune adjuvant would trigger tumor-specific immune responses, which are further amplified by ICB to elicit potent systemic antitumor immune responses in destructing local tumors, eliminating metastases and inhibiting cancer recurrence. Our strategy of combining clinically used agents for tumor-localized cocktail chemoimmunotherapy possesses great potential for clinical translation.

INTRODUCTION

Cancer immunotherapy by using, activating, and training the body’s own immune system to attack cancer cells is a breakthrough technology, which has attracted tremendous attention in oncology over the past several years (1, 2). Among several mainstream cancer immunotherapy strategies, immune checkpoint blockade (ICB) is an important type that has demonstrated encouraging clinical results to even cure some patients with late-stage metastatic cancer (3). Currently, antibodies against cytotoxic T lymphocyte–associated antigen 4 (anti-CTLA4) and programmed death protein 1 (anti-PD1) and its ligand (anti-PDL1) have been approved for clinical use (4, 5). Although ICB therapy has the potential to produce durable clinical responses, its clinical response rate (~20%) is far from satisfactory in that a large proportion of patients are not responsive (6, 7). Meanwhile, ICB, by triggering a nonspecific immune response, would often lead to side effects such as cytokine storm that could even be lethal (8, 9). Therefore, further improvement of ICB therapy, as well as its combination with other therapeutic protocols, is being intensively explored in both fundamental research and clinical trials (10, 11).

Chemotherapy that uses cytotoxic drugs to kill tumor cells is a conventional treatment method for cancer (12). However, many chemotherapeutic drugs that target fast dividing cells including cancer cells and immune cells may result in immunosuppression of the body (13). Fortunately, it has been found that certain types of chemotherapeutic drugs could cause immunogenic cell death (ICD) of cancer cells to elicit certain levels of antitumor immune responses, which resulted from tumor-associated antigens exposed in cancer cell debris after chemotherapy (14, 15). These drugs, including doxorubicin (DOX), oxaliplatin (OXA), bleomycin, bortezomb, and cyclophosphamide, may be used in combination with cancer immunotherapy, especially ICB therapy (16, 17). Encouraged by the abundant preclinical literature on ICD-inducing chemotherapeutics, a great number of clinical trials on combinatorial regimens relying on ICD inducers plus checkpoint inhibitors are ongoing (18). However, the safety profile of the combination therapy is still the focus of clinical trials, and some combinatorial regimens may result in further increased side effects (19, 20). Moreover, the antitumor immune response after chemotherapy-induced ICD may not be potent enough (21). Thus, we reason that the ICD-triggered antitumor immune responses may be further amplified if immune adjuvants are introduced so as to achieve in situ generation of tumor vaccines.

Systemic administration as a commonly used administration mode in the clinic can cause notable toxicity and side effects on normal tissues (22). Although systemic administration is necessary for conventional chemotherapy to attack cancer cells throughout the body, systemic antitumor immune responses may be triggered in immunotherapy by topical administration, which may be safer and requires a lower dose than systematic administration (23, 24). It has been reported that systemic chemotherapy could lead to systemic and intratumoral lymphodepletion, while localized chemotherapy could potentiate antitumor immune responses and further improve therapeutic efficacy of anti-PD1 therapy (25, 26). Immunostimulating adjuvants, which are more suitable for topical administration (to avoid cytokine storm that may occur after systemic administration), can be introduced to the combination therapy strategies (27, 28). In recent studies by us and other groups, it was found that immune adjuvants such as Toll-like receptor 7 (TLR7) agonist imiquimod (R837) and TLR9 agonist CpG oligodeoxynucleotides (CpG-ODNs) could be introduced topically into tumors to boost antitumor immune responses to inhibit distant tumors after radiotherapy (29, 30), photothermal therapy (31, 32), or photodynamic therapy (33, 34) to destruct local primary tumors. Inspired by those findings, we thus hypothesize that localized chemotherapy with ICD drugs, together with immune adjuvants and ICB therapy, may perform function synergistically to trigger a robust systemic antitumor therapeutic outcome with reduced systemic toxicity.
In this work, a “cocktail” chemoimmunotherapeutic composite containing an ICD-inducing chemotherapeutic drug (DOX or OXA), an immune adjuvant (R837), and a pharmaceutical excipient alginate (ALG) is designed for the localized chemoimmunotherapy. These cocktail therapeutic composites (DOX/R837/ALG or OXA/R837/ALG) could be stored in lyophilized powder and redissolved in water before use. The ICB antibody (anti-PDL1) could also be added into the injection solution for local coadministration into tumors or intravenously injected into treated mice following the clinical protocol. The injectable drug composite with the help of ALG could rapidly form a hydrogel in the presence of calcium ions (Ca$^{2+}$) within the tumor. After this in situ gelation, the therapeutic agents would show sustained release, with chemo-drugs, immune adjuvant R837, and anti-PDL1 released subsequently to trigger ICD, amplify immune responses, and further modulate the immune balance to enhance antitumor immunity, respectively (Fig. 1A). We found that localized chemotherapeutics with the help of ALG had better therapeutic efficacy to destroy local tumors and lower side effects than systemic chemotherapeutics through intravenous injection or local chemotherapy in the absence of ALG. Moreover, efficient abscopal effect and immune memory effect of localized chemoimmunotherapy through local release of OXA, R837, and anti-PDL1 with the help of ALG were observed on mice bearing CT26 colon tumors. The therapeutic advantage of this localized chemoimmunotherapy was also demonstrated on an orthotopic murine breast cancer 4T1 model with spontaneous metastasis using DOX to replace OXA as the chemo-drug in the cocktail. Excitingly, even for an orthotopic murine breast tumor model, known as king of cancer, the localized chemoimmunotherapy could induce rather effective antitumor immune responses and cured most of the mice. Last but not least, all components involved in the drug composition are U.S. Food and Drug Administration–approved, and standardization and mass production of products have been achieved, presenting tremendous potential for industrialization and clinical translation of this technology.

**RESULTS**

**Preparation and characterization of the drug composition**

The drug composites containing several kinds of therapeutic agents and ALG, a pharmaceutical excipient, were prepared upon simple mixing, which should be suitable for scale-up production and industrialization. Every step of the preparation process was optimized, especially the order by which the drug was added and the way the drug was dissolved. We first prepared an aqueous solution of ALG, into which immune adjuvant imiquimod (R837) was added under stirring for 3 hours to form an emulsion. Afterward, ICD-inducing chemotherapeutics such as DOX or OXA was added and stirred for another 3 hours. Thereafter, the whole solution was frozen dried into a powder for storage. Various drug combinations with different ICD-inducing drugs, including OXA/R837/ALG, DOX/R837/ALG, and bortezomib/R837/ALG, were successfully prepared. Meanwhile, we also demonstrated large-scale preparation of OXA/R837/ALG in the form of lyophilized powder (fig. S1).

The obtained lyophilized drug composites could be easily dispersed in aqueous solutions. Notably, in the presence of Ca$^{2+}$ at the physiological concentration (0.5%), this ALG-containing drug composite solution would be rapidly transformed into a hydrogel (Fig. 1B and fig. S2). Porous network structure of the hydrogel was confirmed by scanning electron microscopy (SEM) analysis (Fig. 1C and fig. S3). Furthermore, rheological properties during the gel formation were determined (Fig. 1, E to H). It was found that when the ALG concentration was higher than 5 mg/ml, the elastic modulus (G’) would rapidly increase and eventually exceed the viscous modulus (G”), demonstrating the formation of hydrogel. Notably, proteins such as anti-PDL1 added into the drug composite solution could be efficiently encapsulated inside the formed hydrogel in the presence of Ca$^{2+}$, with a nearly 100% encapsulation efficiency (fig. S4).

Next, we examined the release profiles of various ICD-inducing chemotherapeutics, immune adjuvant, and ICB antibody from the formed ALG/Ca$^{2+}$ hydrogels (Fig. 1, I to L). As expected, the release rates of these agents decreased as the ALG concentration increased from 1 to 20 mg/ml. When the ALG concentration was more than 20 mg/ml, ~80% of OXA and ~60% of DOX or R837 were released within 2 days, whereas ~30% of anti-PDL1 was released within 7 days. Notably, the concentrations of drugs in the drug compositions within our tested range would not significantly affect their release rate (fig. S5).

**Localized chemoimmunotherapy for eliciting immunogenic tumor phenotype**

Next, we measured the responses of immune tumor microenvironment induced by localized chemoimmunotherapy. As expected, both OXA/ALG and OXA/R837/ALG could effectively trigger immunogenic death of CT26 cancer cells (figs. S6 and S7). To further assess the in vivo immune stimulation effects of the OXA/R837/ALG cocktail, which was locally injected into tumors, we examined the subsequent infiltration of various immune cells within the tumors including leukocytes, dendritic cells (DCs), tumor-associated macrophages (TAMs), and tumor-infiltrating lymphocytes (TILs) (Fig. 2A and figs. S8 and S9). The percentages of overall immune cells (CD45$^{+}$) were significantly increased after the OXA/R837/ALG treatment (Fig. 2B). Furthermore, we observed a significant promotion of DCs (CD45$^{+}$CD11b$^{+}$MHCI$^{+}$) and tumor-associated macrophage (CD45$^{+}$CD11b$^{+}$F4/80$^{+}$) infiltration within the tumor after local injection of the cocktail (Fig. 2, C and D). Notably, a remarkable promotion of TILs, especially cytotoxic T lymphocyte (CD45$^{+}$CD3$^{+}$CD8$^{+}$), was also observed in mice treated with OXA/R837/ALG (Fig. 2, E and F).

In addition to the infiltration of various immune cells within the tumors, DC maturation (CD80$^{+}$CD86$^{+}$) in lymphonodus was also greatly improved after the OXA/R837/ALG treatment (Fig. 2G and fig. S10). Cytokines including tumor necrosis factor–α (TNF–α) and interferon-γ (IFN–γ) were also measured before and after the OXA/R837/ALG treatment (Fig. 2H), which resulted in a significant increase in the serum levels of these two types of cytokines.

To prove the rationale of combining our cocktail chemoimmunotherapy with checkpoint blockade, PDL1 expression on cancer cells or PD1 expression on immune cells was assessed by flow cytometry after intratumoral injection of OXA/R837/ALG (Fig. 2I). It was found that the OXA/R837/ALG treatment could result in increased PDL1 expression on cancer cells, DCs, and macrophages compared to the untreated tumors and tumors treated with gel. Meanwhile, the PD1 expression on both CD4$^{+}$ and CD8$^{+}$ TILs within the tumor was also found to be increased after local injection of OXA/R837/ALG (Fig. 2J and figs. S11 and S12). These results together substantiate that the OXA/R837/ALG treatment can elicit an inflamed and immunogenic tumor microenvironment, and it would be reasonable to combine this localized cocktail chemoimmunotherapy with anti-PDL1 ICB therapy.
Localized chemoimmunotherapy to achieve abscopal effect for the CT26 colon tumor model

For in vivo experiments, we first proved that in situ gelation of ALG would lead to long-term retention of therapeutic agents in tumors. The fluorescent dye Cy5.5 with or without mixing with ALG was intratumorally injected into CT26 mouse colon tumor–bearing mice (n = 3) for in vivo fluorescence imaging. With the help of ALG, the fluorescence signals of Cy5.5 showed obviously prolonged retention in the tumor for up to 24 hours, much longer than that of free Cy5.5 molecules without ALG (fig. S13). Next, we measured the blood levels of therapeutics at different time points after intratumoral injection of free OXA or OXA/ALG into CT26 tumor–bearing mice. It was found that the blood concentrations of OXA in the drug/ALG group were obviously lower at earlier time points but were maintained at higher levels at later time points compared to that in the free drug group, demonstrating the sustained drug release after in situ gelation of OXA/ALG (fig. S14).

Thereafter, we tested the efficacy of localized chemotherapy in comparison to systemic chemotherapy using OXA as the model chemotherapy drug to treat subcutaneous CT26 mouse colon tumors.
Fig. 2. Local administration of chemoimmunotherapeutic drug composite to elicit immunogenic tumor phenotypes. (A) CT26 tumors harvested from mice with intravenous injection of OXA/R837/ALG were analyzed by flow cytometry 7 days after the treatment. (B to E) Representative flow cytometric plots of immune cell infiltrations within the tumor and (F) corresponding quantification results. (G) Representative flow cytometric plots of DC maturation in lymphonodus. (H) TNF-α and IFN-γ concentrations in mouse sera before and 7 days after the OXA/R837/ALG treatment. (I) PDL1 expression on tumor cells and PD1 expression on TILs after the OXA/R837/ALG treatment and (J) the corresponding quantification of PDL1- and PD1-specific fluorescence signal intensities on different types of cells after various treatments are indicated. Data are means ± SEM. Statistical significance was calculated via two-tailed Student’s t test (F and J). *** P < 0.001. MFI, mean fluorescence intensity; a.u., arbitrary units.
When the tumor volume reached ~70 mm³, Balb/C mice bearing CT26 tumors were randomly divided into six groups (n = 6): (i) saline, (ii) OXA [intravenous (iv) injection dose = 3 mg/kg], (iii) OXA [intratumoral (it) injection dose = 1.5 mg/kg], (iv) OXA/ALG [OXA (0.375 mg/kg, it)], (v) OXA/ALG [OXA (0.75 mg/kg, it)], and (vi) OXA/ALG [OXA (1.5 mg/kg, it)] (Fig. 3A). Compared to OXA treatment alone through either intravenous (group 2) or intratumoral (group 3) injection, which resulted in only partly delayed tumor growth, OXA/ALG treatment at the same dose of OXA (1.5 mg/kg, in group 6) offered a stronger inhibitory effect to the tumor growth (Fig. 3B). Even with halved dose of OXA (0.75 mg/kg, group 5), the antitumor efficacy OXA/ALG treatment was still found to be better than that with free OXA at the dose of 1.5 mg/kg. In addition, obvious body weight loss was observed for mice that received OXA treatment through intravenous injection, indicating apparent side effects of these systemically adminitratred chemotherapeutics (Fig. 3C).

In contrast, mice treated through intratumoral injection in groups 3 to 6 showed no appreciable body weight drop after treatment. Therefore, localized chemotherapy using OXA/ALG with in situ formed ALG gel to allow localized and sustained drug release would be able to provide enhanced therapeutic efficacy in treating local tumors while reducing systemic toxicity.

While gel-based localized chemotherapy could be a powerful method to destruct local tumors, it may not be a viable approach to manage spreading metastatic tumors. We next introduce gel-based chemoimmunotherapy to achieve systemic antitumor responses after localized treatment. A bilateral CT26 tumor model was introduced in our experiments, with tumors on the right side designated as “primary tumors” for localized therapy with injection of the drug composite and those on the left side designated as “distant tumors” without direct drug administration (Fig. 3D). When the primary tumors reached 70 mm³, mice were randomly divided into seven groups (n = 6) with their primary tumors treated with: (i) saline, (ii) OXA/R837/anti-PDL1 [OXA (1.5 mg/kg), R837 (5 mg/kg), and anti-PDL1 (1 mg/kg)], (iii) OXA/anti-PDL1/ALG [OXA (1.5 mg/kg) and anti-PDL1 (1 mg/kg)], (iv) OXA/ALG [OXA (1.5 mg/kg) + anti-PDL1 (intravenous)], (v) OXA/R837/ALG [OXA (1.5 mg/kg) and R837 (5 mg/kg)]), (vi) OXA/R837/anti-PDL1/ALG [OXA (1.5 mg/kg), R837 (5 mg/kg), and anti-PDL1 (1 mg/kg)], and (vii) OXA/R837/ALG [OXA (1.5 mg/kg) and R837 (5 mg/kg)] + anti-PDL1 (intravenous). For mice in groups 4 and 7, mice were intratumorally injected with anti-PDL1 (1 ng/kg) for three times on days 1, 5, and 9. The sizes of tumors on both sides were closely monitored after treatment.

As expected, with the help of ALG to enable local retention and sustained release of therapeutics, tumor growth of primary tumors for mice in groups 3 to 5 showed stronger inhibition than that observed for mice treated with OXA/R837/anti-PDL1 without ALG in group 2 (Fig. 3E). Notably, the OXA/R837/ALG treatment in combination with anti-PDL1, which was either coadministred within the “coil-tail” drug composite solution (group 6) or separately administrated by intravenous injection (group 7), could completely suppress the growth of primary groups. Distant tumors in these two groups (6 and 7) were also completely eliminated after their primary tumors were treated by gel-based localized chemoimmunotherapy (OXA/R837/ALG) in combination with anti-PDL1 (groups 6 and 7). In contrast, for mice treated with OXA/R837/anti-PDL1 without ALG-induced in situ gelation, OXA/ALG in combination with anti-PDL1 but no R837 (groups 3 and 4), as well as OXA/R837/ALG in the absence of anti-PDL1 (group 5), the observed abscopal effects, although strong enough in delaying the growth of distant tumors, were far from comparable to that achieved in groups 6 and 7. In addition, for mice in group 6 or 7, which were intratumorally injected with OXA/R837/anti-PDL1/ALG or OXA/R837/ALG plus further intravenous injection with anti-PDL1, respectively, the two treatment strategies had similar inhibitory effects to the primary tumors and distant tumors, although the total dose of anti-PDL1 used in group 7 was three times as much as that used in group 6 (Fig. 3G).

To find out the mechanism of abscopal effects induced by localized chemoimmunotherapy, we first examined infiltrating T cells in distant tumors, which were collected after various treatments to measure tumor-infiltrating CD8⁺ (CD3⁺CD4⁻CD8⁺) and CD4⁺ (CD3⁺CD4⁺CD8⁻) T cells by flow cytometry (Fig. 3H). It was found that the localized ICD-inducing chemotherapy in combination with immune adjuvant R837 and ICB antibody anti-PDL1 (groups 2, 6, and 7) could effectively increase the frequency of CD8⁺ and CD4⁺ T cells within the distant tumors, especially in combination with immune adjuvant R837 (Fig. 3I to K, and fig. S15). Notably, the ratios of tumor-infiltrating CD8⁺ killer T cells to immunosuppressive regulatory T cells (Treg) (CD3⁺CD4⁺Foxp3⁺), which could be an indicator of antitumor immune balance, were found to be the highest in groups 6 and 7 (Fig. 2L), consistent to the observed strongest abscopal anti-tumor effects in these two groups.

In addition to responses of immune cells, PDL1 expression in distant tumors of mice after various treatments was further assessed (fig. S16). It was found that for mice in group 5 with OXA/R837/ALG-treated primary tumors, increased PDL1 expression in distant tumors showed up compared to untreated tumors in group 1. In contrast, for groups 6 and 7 with the combination of anti-PDL1 treatment, the PDL1 expression on tumor cells in distant tumors could be largely blocked, favorable for T cell–mediated immune attack of tumor cells. Furthermore, cytokines including TNF-α, IFN-γ, interleukin-6 (IL-6), and IL-12p70 in sera of mice after various treatments were also measured by enzyme-linked immunosorbent assay (ELISA) (fig. S17). Both TNF-α and IFN-γ levels in groups 6 and 7 were the highest among all groups, suggesting the favorable antitumor immune responses.

To demonstrate the importance of tumor-infiltrating T cells in achieving the observed abscopal effects, we used anti-CD4 (αCD4) and anti-CD8 (αCD8) antibodies to deplete the respective immune cells. Bilateral CT26 tumor–bearing mice were treated with OXA/R837/αPDL1/ALG as described before and intravenously injected with αCD4, αCD8, or mouse immunoglobulin G (IgG) (as the control) at a dose of 20 μg per mouse on days 0 and 5. For the local tumors, blocking of either CD4⁺ T cells or CD8⁺ T cells, especially the latter, would largely diminish the tumor growth inhibition effect of the cocktail chemoimmunotherapy for both local and abscopal tumors (fig. S18). Therefore, our results demonstrate that the tumor infiltration of T lymphocytes after the localized gel-based chemoimmunotherapy would be critical to promote the antitumor therapeutic effect against both local and distant abscopal tumors (fig. S19).

**Long-term immune memory effect after localized chemoimmunotherapy**

To further investigate the potency of the localized chemoimmunotherapy, we evaluated long-term immune memory effect on the CT26 tumor model. Memory T cells, transformed from effector T cells, could
Fig. 3. Localized chemoimmunotherapy to achieve abscopal effect for CT26 colon tumors. (A) Schematic to show the use of drug composition containing OXA/R837/anti-PDL1/ALG for treatment of CT26 colon tumor model. (B and C) Tumor growth curves (B) and body weight (C) of CT26 tumor–bearing mice after different treatments indicated. (D) Schematic illustration of localized chemoimmunotherapy to inhibit the tumor growth at distant sites. (E and F) Tumor growth curves of primary tumors (E) and distant tumors (F) of mice after various treatments indicated. (G) Body weight of mice after various treatments indicated. (H) Representative flow cytometric plots showing different groups of T cells in secondary tumors. (I to K) Proportions of tumor-infiltrating CD3+ T cells, CD8+ killer T cells, and CD4+ T cells according to data in (H). (L) CD8+ CTL:Treg ratios in the distant tumors. Statistical significance was calculated via two-tailed Student’s t test (I to L). ***P < 0.001. Photo credit (A): Yu Chao, Soochow University.
offer long-term protection against rechallenged pathogens. In our experiments, mice bearing CT26 tumors were randomly divided into three groups \((n = 5)\), and the first inoculated tumors were removed by (i) surgery, (ii) OXA/R837/ALG \([\text{OXA} (1.5 \text{ mg/kg, it}) \text{ and R837} (5 \text{ mg/kg, it})] + \text{anti-PDL1} (1 \text{ mg/kg, iv})\) for three times on days 1, 5, and 9, or (iii) OXA/R837/anti-PDL1/ALG \([\text{OXA} (1.5 \text{ mg/kg, it}), \text{R837} (5 \text{ mg/kg, it}), \text{and anti-PDL1} (1 \text{ mg/kg, it})]\). Two months later, mice were rechallenged with the secondary CT26 tumors (Fig. 4A). Compared to the rapid growth of the secondary tumors in group 1, greater inhibitory effects were observed for secondary tumors in groups 2 and 3 (Fig. 4B). Notably, 80% of mice in group 3 and 60% of mice in group 2 survived for 120 days, in marked contrast to mice in group 1, which all died within 80 days (Fig. 4C).

To assess T cell memory response induced by localized chemoimmunotherapy, we harvested spleens of mice before inoculation of the secondary tumors. It was found that the percentages of effector T memory \((T_{EM})\) cells \(\left(\text{CD3}^+\text{CD8}^+\text{CD44}^+\text{CD62L}^-\right)\) in groups 2 and 3 were 56.9 and 54.7%, respectively, which were much higher than that in group 1 (Fig. 4E). In addition, the serum levels of cytokines such as TNF-α and IFN-γ in groups 2 and 3 were found to be much higher than that in group 1 (Fig. 4, F and G). These data indicated that effective T cell memory response could be achieved after localized chemoimmunotherapy.

**Localized chemoimmunotherapy to treat orthotopic breast tumor models**

To further demonstrate that our proposed localized chemoimmunotherapy could be broadly applicable, we performed experiments with the 4T1 murine breast tumor model, a triple-negative breast cancer model. DOX, an ICD-inducing chemotherapeutic commonly used to treat breast cancer, was adopted for these experiments. We first studied the therapeutic efficacy and side effects of localized chemotherapy using DOX/ALG for the subcutaneous 4T1 tumor model. When the tumor volume reached 200 mm\(^3\), mice were randomly divided into six groups \((n = 6)\): (i) saline, (ii) DOX (3 mg/kg, iv), (iii) DOX (3 mg/kg, it), (iv) DOX/ALG [DOX (0.75 mg/kg, it)], (v) DOX/ALG [DOX (1.5 mg/kg, it)], and (vi) DOX/ALG [DOX (3 mg/kg, it)] (Fig. 5A). Note that the sizes of 4T1 tumors at the beginning of treatment were much bigger than that of CT26 tumors. Because of the relative large size of tumors, DOX treatment alone through intravenous or intratumoral injection showed no obvious tumor growth inhibitory effect. In contrast, DOX/ALG treatment showed a remarkable DOX dose-dependent inhibitory effect to 4T1 tumors.

\[\text{Fig. 4. Long-term immune memory effect after localized chemoimmunotherapy.} \quad \text{(A)} \quad \text{Schematic illustration to evaluate the immune memory effect after localized chemoimmunotherapy to treat CT26 tumors with DOX/R837/anti-PDL1/ALG or OXA/R837/ALG together with intravenously injected anti-PDL1.} \quad \text{(B to D)} \quad \text{Tumor growth curves (B), percent survival (C), and average body weights (D) of different groups of mice after various treatments, as indicated (n = 5).} \quad \text{(E) Proportions of T}_{EM} \text{ in the spleen analyzed by flow cytometry (gated on CD3}^+\text{CD8}^+\text{ T cells) at day 60, right before rechallenging mice with secondary tumors.} \quad \text{(F and G) Cytokine levels including IFN-γ (F) and TNF-α (G) in sera from mice isolated 5 days after mice were rechallenged with secondary tumors. Statistical significance was calculated via two-tailed Student's t test (E to G).} \quad ** P < 0.01; *** P < 0.001.\]
(Fig. 5B). In addition, compared to the obvious side effect of DOX treatment as indicated by the instant body weight drop after intravenous injection, intratumoral injection of DOX/ALG showed no appreciable effect to the mouse body weight, demonstrating the improved safety of this gel-based localized chemotherapy (Fig. 5C).

Next, we further evaluated the therapeutic efficacy of our gel-based chemoimmunotherapy on an orthotopic 4T1 murine breast cancer model with spontaneous metastasis. Firefly luciferase (fLuc)–expressing 4T1 murine breast cancer cells were inoculated into the mouse breast pad of each female Balb/C mouse. Two weeks later, when spontaneous metastases would have occurred, mice were randomly divided into six groups (n = 10) and the orthotopic 4T1 tumors were treated by (i) surgery, (ii) DOX/R837/anti-PDL1 (intratumorally), (iii) DOX/R837/ALG (intratumorally), (iv) DOX/anti-PDL1/ALG (intratumorally), (v) DOX/R837/anti-PDL1/ALG (intratumorally), and (vi) DOX/R837/ALG (intratumorally) plus anti-PDL1 (intravenously) (Fig. 5D). The doses of DOX, R837, and anti-PDL1 were 6, 10, and 1 mg/kg (intratumorally), respectively. For group 6, however, anti-PDL1 was intravenously injected at the dose of 1 mg/kg for three times on days 16, 20, and 24. In the following days, metastatic tumors were monitored by the bioluminescence signals from fLuc-expressing 4T1 cells (Fig. 5E). For mice with primary breast tumors removed by surgery, metastatic tumors were not affected; therefore, whole-body metastases showed up at later time points, resulting in rapid death of mice (Fig. 5F). Meanwhile, DOX/R837/anti-PDL1 treatment without ALG (group 2), DOX/R837/ALG plus R837 without anti-PDL1 (group 3), or DOX/anti-PDL1/ALG without R837 (group 4) all failed in eliminating metastatic tumors and extending mouse survival. Excitingly, for mice in groups 5 and 6 with DOX/R837/anti-PDL1/ALG (intratumorally) or DOX/R837/ALG (intratumorally) plus anti-PDL1 (intravenously) treatment, no detachable metastatic fLuc-4 T1 cells were found at later time points (day 25 and beyond). Impressively, while mice in other control groups died in 25 to 35 days, all the mice in groups 5 and 6 survived for more than 120 days (Fig. 5F). Those results strongly evidenced that our proposed localized chemoimmunotherapy would trigger robust systemic antitumor
Localized chemoimmunotherapy to treat orthotopic brain tumor model

In addition to the orthotopic murine breast cancer model with spontaneous metastasis, we further applied our localized gel-based chemoimmunotherapy in treating a more challenging orthotopic brain tumor model. To establish an isogenic mouse glioma model, P5 C57 neural stem cells were isolated and expanded in N2 and B27 Supplement liquid (N2/B27) containing serum-free medium with 20 ng of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The cells were then transformed by transducing with lentCRISPR v2 (Addgene plasmid no. 52961) lentivirus containing P53 and NF1 guide sequences to obtain isogenic glioma cells. To establish orthotopic brain tumor model, these engineered glioma cells (2 × 10^5) were injected into the brain of each female C57 black mice using a stereotactic apparatus.

Four days after tumor cell inoculation within the mouse brains, those mice were randomly divided into nine groups (n = 8): (i) saline, (ii) temozolomide (TMZ) (6 mg/kg, iv), (iii) R837/anti-PDL1/ALG [R837 (1 mg/kg, it) and anti-PDL1 (0.2 mg/kg, it)], (iv) DOX/ALG [DOX (0.6 mg/kg, it)], (v) DOX/R837/anti-PDL1 [DOX (0.6 mg/kg, it), R837 (1 mg/kg, it), and anti-PDL1 (0.2 mg/kg, it)], (vi) DOX/R837/ALG [DOX (0.6 mg/kg, it) and R837 (1 mg/kg, it)], (vii) DOX/anti-PDL1/ALG [DOX (0.6 mg/kg, it) and anti-PDL1 (0.2 mg/kg, it)], (viii) DOX/R837/anti-PDL1/ALG [DOX (0.6 mg/kg, it), R837 (1 mg/kg, it), and anti-PDL1 (0.2 mg/kg, it) and (ix) DOX/R837/ALG [DOX (0.6 mg/kg, it) and R837 (1 mg/kg, it)] plus anti-PDL1 (anti-PDL1 (0.2 mg/kg, iv)). Localized chemoimmunotherapy for the orthotopic brain tumor was realized by injecting therapeutics through the same way used to inject tumor cells (Fig. 6A). It was found that localized immunotherapy using R837/anti-PDL1/ALG without any chemotherapeutics through intratumoral injection had no effect on survival of mice bearing brain tumors (Fig. 6B). Furthermore, mice in group 4 with localized chemotherapy using DOX/ALG through intratumoral injection had a longer overall survival than TMZ treatment (intravenous injection), the first-line chemo-drug for brain cancer. Excitingly, all the mice in groups 8 and 9 with our proposed localized chemoimmunotherapy survived for 90 days, indicating powerful systemic antitumor immune response induced by ICD inducer in combination with immune adjuvant and ICB inhibitor. Notably, the TNF-α and IFN-γ levels in mouse sera were highest in groups 8 and 9 compared to the other control groups (Fig. 6, C and D), suggesting that the strongest antitumor immune responses occurred in these two groups.

To further evaluate therapeutic efficacy of localized chemoimmunotherapy, the whole brain of each mouse was harvested for immunohistochemical analysis. It was found that CD4^+ and CD8^+ cells were significantly increased in groups 8 and 9 compared to those in the other seven groups (fig. S20 and Fig. 6, F and G). Notably, the CD8^+ to CD4^+ ratio was also found to be the highest in groups 8 and 9 compared to the other groups. To further validate immune memory effect of our proposed localized chemoimmunotherapy, mice in groups 8 and 9 were rechallenged with the second tumor by subcutaneous injection of isogenic glioma cells on day 60 (Fig. 6F). The tumor growth on mice in groups 8 and 9 was significantly inhibited compared to the control group (Fig. 6G). All these results demonstrated that our proposed localized chemoimmunotherapy can be used to treat brain glioma tumors and could also offer a strong immune memory effect, similar to that observed with other types of tumor models.

DISCUSSION

Here, a cocktail therapeutic formulation for chemoimmunotherapy is designed by mixing ICD-inducing chemotherapeutics (OXA, DOX, and other possible candidates), immune adjuvant R837, and a pharmaceutical excipient ALG to formulate lyophilized powder. ICB antibody such as anti-PDL1 may be added together into this cocktail for local injection or applied by conventional intravenous administration. This cocktail therapeutic solution after local injection into tumors would form ALG-Ca^{2+} hydrogels in the presence of endogenous calcium ions, enabling retention and sustained release of therapeutic components within the tumors. It was demonstrated that localized chemoimmunotherapy using OXA/R837/anti-PDL1/ALG could eliminate primary tumors, inhibit distant tumors, and further prevent tumor recurrence in the CT26 tumor model. Using the other cocktail formulation with OXA replaced by DOX, this localized chemoimmunotherapy showed exceptional therapeutic effects in treating an orthotopic murine breast cancer model with spontaneous metastasis, as well as an orthotopic brain tumor model by a single injection.

This unique advantage of this localized chemoimmunotherapy strategy is to achieve systemic antitumor immune responses after local administration of the cocktail therapeutic solution. The in situ gelation by ALG is important in this strategy to allow local retention and sustained release of therapeutic agents. Thus, by local injection of therapeutic agents and subsequent in situ gelation, the systemic toxicity of chemo-drugs could be largely avoided. Moreover, unlike ICB therapy alone that induces nontumor-specific immune responses, this cocktail chemoimmunotherapy would trigger tumor-specific immune effects, similar to that achieved with whole tumor cell–based cancer vaccines (35–37), but with far simpler procedures. Different from the conventional combination of ICD-based chemotherapy with ICB therapy (38, 39), the immune adjuvant induced in this system is critical and could greatly enhance the tumor-specific immune responses.

In our work, ICB therapy with anti-PDL1 could be given by either intravenous injection after localized chemoimmunotherapy or intratumoral injection together with our drug composites. Compared to the separated use of intravenously injected anti-PDL1 plus intratumorally injected drug composites, local ICB therapy by including anti-PDL1 in the cocktail would require a lower antibody dose by only one injection to achieve the comparable therapeutic outcome. However, for clinical transformation, including antibodies into these cocktail drug composites would largely complicate the drug formulation process. Therefore, it would be more realistic to focus on the clinical transformation of the cocktail therapeutics containing the ICD inducer R837 and ALG at the current stage. Moreover, in addition to anti-PDL1 used in this work, it is expected that anti-PD1 (by intravenous injection) may also be combined with the cocktail drug composites developed in our work for enhanced chemoimmunotherapy.

In this cocktail chemoimmunotherapeutic formulation, various components are mixed together to prepare the lyophilized powder. The formulation process is rather simple and could be easily scaled up, while the lyophilized powder could allow a long shelf-life. Regarding the production under the good manufacturing practices standard, the large-scale sterilization and endotoxin control of ALG have both been realized. Because all components in these systems
Fig. 6. Localized chemoimmunotherapy to treat orthotopic brain tumors. (A) Schematic illustration of localized chemoimmunotherapy to treat the orthotopic brain tumor model. (B) Survival of mice bearing orthotopic brain tumors after various treatments indicated (n = 10 per group). (C) and (D) Cytokine levels including IFN-γ (C) and TNF-α (D) in sera from mice isolated 5 days after the treatment. (E) Tumor slices of brains from different groups of mice after various treatments indicated. (F) and (G) Number of CD4+ and CD8+ cells per tumor slice according to data in (E) and (F). (H) CD8+ to CD4+ ratio in tumor slice. (I) Schematic illustration of evaluating the immune memory effect after localized chemoimmunotherapy. (J) and (K) Tumor growth curves (J) and survival data (K) of different groups of mice after various treatments applied on day 0 to eliminate their orthotopic brain tumors (n = 5). The same type of tumor cells was subcutaneously inoculated on those mice on day 60. Statistical significance was calculated via two-tailed Student’s t test (C, D, and F to H). **P < 0.01; ***P < 0.001.
have been already approved for clinical use, our chemoimmunotherapeutic cocktail formulations are quite promising for future clinical translation. On the basis of the current technique, a start-up company has been established, aiming at pushing this cocktail chemoimmunotherapeutic formulation into clinical trials in 2 to 3 years.

MATERIALS AND METHODS

Materials

All the chemicals, except additionally mentioned, were purchased from Sigma-Aldrich. Pharmaceutical-grade ALG was obtained from Qingdao Bright Moon Seaweed Group Co. Ltd. Clinically used chemotherapeutics, including DOX hydrochloride and OXA, were purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. All antibodies were purchased from BioLegend (San Diego, CA) and used as received.

Preparation and characterization of the drug composition

The drug composites containing several kinds of therapeutic agents and ALG were prepared upon simple mixing. For the immune adjuvant imiquimod (R837) added into these drug composites, 800 mg of water-insoluble R837 was pretreated by hydrochlorination with 10 ml of 1 M HCl for 5 min to obtain R837 HCl with better water dispersibility, and the solution was lyophilized for later use. Four grams of ALG powder was then dissolved in 200 ml of water under stirring. Afterward, ICD-inducing chemotherapeutics such as 480 mg of DOX or 240 mg of OXA was added together with 800 mg of R837 lyophilized powder into the ALG solution and stirred for 3 hours. Thereafter, the whole solution was freeze-dried into a powder for final use. Various drug combinations with different ICD-inducing drugs including OXA/R837/ALG, DOX/R837/ALG, and bortezomib/R837/ALG were successfully prepared. The rheological properties of drug composites or gels were determined by a rotational rheometer (Haake RheoStress 6000, Germany). The morphologies of OXA/R837/ALG-Ca$^{2+}$ hydrogels and DOX/R837/ALG-Ca$^{2+}$ hydrogels were characterized by SEM (Gemini 500).

Drug release from ALG-based hydrogels

Drug release tests were carried out at 37°C in phosphate-buffered saline (PBS) under stirring. The released DOX was analyzed with an ultraviolet-visible spectrophotometer (PerkinElmer). The released OXA was measured by inductively coupled plasma atomic emission spectroscopy (Thermo Fisher Scientific). The released R837 was detected by high-performance liquid chromatography (Agilent 1200 Infinity Series), and the released anti-PDL1 was determined with the Rat IgG Total ELISA Kit (eBioscience).

Mice

Female BALB/c mice (6 to 8 weeks) and C57 black mice (6 to 8 weeks) were purchased from JOINN Laboratories Co. Ltd. (China). All mouse studies were performed according to the protocols approved by the Soochow University Laboratory Animal Center. Institutional Review Board and/or Institutional Animal Care and Use Committee guidelines were followed with human or animal subjects. All the mice used in our experiments were randomly divided. Tumor size was calculated as follows: volume = (tumor length) × (tumor width)$^2$/2.

In vivo experiments

For in vivo fluorescence imaging, Cy5.5 was intratumorally injected into CT26 mouse colon tumor–bearing mice ($n = 3$) with or without ALG. In vivo fluorescence imaging was carried out at different time points after injection (0.5, 1, 3, 6, 12, and 24 hours).

To determine the blood levels of OXA, OXA or OXA/ALG was intratumorally injected into CT26 tumor–bearing mice ($n = 3$), and blood was taken from the orbit of mice at different time points after injection (0.5, 1, 3, 6, 12, 24, and 48 hours). The blood was weighed and solubilized in a lysis buffer. OXA concentration was measured by inductively coupled plasma–mass spectrometry.

For in vivo local chemotherapy, CT26 tumor–bearing mice were randomly divided into six groups ($n = 6$): (i) saline, (ii) OXA (3 mg/kg, iv), (iii) OXA (3 mg/kg, it), (iv) OXA/ALG (0.75 mg/kg, it), (v) OXA/ALG (1.5 mg/kg, it), and (vi) OXA/ALG (3 mg/kg, it).

For local chemoimmunotherapy of OXA/R837/anti-PDL1/ALG in CT26 mice tumor model, CT26 tumor–bearing mice were randomly divided into seven groups ($n = 6$): (i) saline, (ii) OXA/R837/anti-PDL1 (intratumoral), (iii) OXA/ALG and anti-PDL1 (intratumoral), (iv) OXA/ALG and anti-PDL1 (intravenous), (v) OXA/R837/ALG (intratumoral), (vi) OXA/R837/ALG and anti-PDL1 (intratumoral), and (vii) OXA/R837/ALG (intratumoral) and anti-PDL1 (intravenous). The intraperitoneal injection doses were 60, 100, 500, and 20 μg for OXA, R837, ALG, and anti-PDL1, respectively. For group 7, anti-PDL1 antibody (20 μg per mice) was systemically administered on days 1, 5, and 9.

Bilateral CT26 tumor–bearing mice were treated with OXA/R837/aPD1/ALG as described before and intravenously injected with αCD4, αCD8, or mouse IgG (as the control) at a dose of 20 μg per mouse on days 0 and 5.

For long-term immune memory effect, CT26 tumor–bearing mice were randomly divided into three groups: (i) surgery, (ii) OXA/R837/ALG and anti-PDL1 (intratumoral), and (iii) OXA/R837/ALG (intratumoral) and anti-PDL1 (intravenous). Sixty days after the primary tumors were removed, mice were rechallenged with the same 2 × 10$^5$ CT26 cells.

For local chemotherapy of DOX/ALG in 4T1 mice tumor model in vivo, 4T1 tumor–bearing mice were randomly divided into six groups ($n = 6$): (i) saline, (ii) DOX (3 mg/kg, iv), (iii) DOX (3 mg/kg, it), (iv) DOX/ALG (0.75 mg/kg, it), (v) DOX/ALG (1.5 mg/kg, it), and (vi) DOX/ALG (3 mg/kg, it).

For local chemoimmunotherapy of DOX/R837/anti-PDL1/ALG, the 4T1 orthotopic mouse breast cancer model had a spontaneous metastasis, according to our previous reports (29) (Fig. 5, D to F). Fifteen days after tumor inoculation, mice were randomly divided into six groups ($n = 10$ per group): (i) surgery, (ii) DOX/R837/anti-PDL1, (iii) DOX/R837/ALG, (iv) DOX/anti-PDL1/ALG, (v) DOX/R837/anti-PDL1/ALG, and (vi) DOX/R837/ALG and anti-PDL1 (intravenous). Lyophilized powder of DOX/R837/ALG drug composite and anti-PDL1 were mixed together for intraperitoneal injection at doses of 60, 100, 500, and 20 μg, respectively. For group 6, anti-PDL1 antibody (20 μg per mice) was systemically administered on days 1, 5, and 9. After various treatments, spontaneous metastases were imaged by an IVIS Spectrum system.

To establish an isogenic glioma mouse glioma model, P5 C57 neural stem cells were isolated and expanded in N2/B27 containing serum-free medium with 20 ng of EGF and bFGF. The cells were then transfected by transducing lentivirus containing P53 and NFI guide sequences subsequently with lentiCRISPR v2 (Addgene plasmid no. 52961): sgTrp53, CCTCGAGTCCCTCTGAGCC (40); sgNf1, GTTGTGCTCGGTGACTT.
To establish orthotopic brain tumor model, 2 × 10^5 isogenic glioma cells were injected into the brain of each female C57 black mice using a stereotactic apparatus. Four days later, mice were randomly divided into nine groups (n = 8): (i) blank, (ii) TMZ (60 mg/kg intraperitoneally), (iii) R837/anti-PDL1/ALG (intratumoral), (iv) DOX/ALG (intratumoral), (v) DOX/R837/anti-PDL1 (intratumoral), (vi) DOX/R837/ALG (intratumoral), (vii) DOX/anti-PDL1/ALG (intratumoral), (viii) DOX/R837/anti-PDL1/ALG (intratumoral), and (ix) DOX/R837/ALG (intratumoral) and anti-PDL1 (intravenous). The intraperitoneal injection doses of DOX, R837, ALG, and anti-PDL1 were 6, 10, 50, and 2 μg, respectively, for each mouse. For group 9, anti-PDL1 antibody (2 μg per mouse) was intravenously administered on days 1, 5, and 9. Survival of all mice was monitored. All the brains of mice were collected for immunohistochemical staining. For long-term immune memory effect, mice in groups 8 and 9 were rechallenged with the same isogenic glioma cells in the right flank.

Cytokine detection

TNF-α (Dakewe Biotech), IFN-γ (Dakewe Biotech), IL-12p70 (IL12p70, Dakewe Biotech), and IL-6 (Dakewe Biotech) in mouse serum samples were analyzed with ELISA kits according to the vendors’ protocols.

Ex vivo analysis of different groups of T cells

To analyze immune cells by flow cytometry, tumors or spleens of mice after various treatments were collected and stained according to the manufacturer’s protocols. In brief, cells from tumors were blocked with CD16/32 antibody (BioLegend, catalog no. 103102) and then stained with antibodies against CD45 (BioLegend, catalog no. 103108, clone 30-F11), CD11b (BioLegend, catalog no.101208, clone M1/70), CD206 (BioLegend, catalog no. 141716, clone C068C2), F4/80 (BioLegend, catalog no. 123116, clone BM8), CD80 (BioLegend, catalog no. 104722, clone 16-10A1), CD3 (clone 17A2, catalog no. 100204), CD8a (clone 53-6.7, catalog no. 100712), and CD4 (clone GK1.5, catalog no. 100432). To analyze memory T cells, spleens of mice were stained with anti-CD3–FITC (fluorescein isothiocyanate), anti-CD8–APC (allophycocyanin), anti-CD62L–PerCP (peridinin chlorophyll protein)–Cy5.5 (eBioscience, clone MEL-14, catalog no. 17-0621), and anti-CD44–PE (phycoerythrin) (eBioscience, clone IM7, catalog no. 12-0441) antibodies to identify T_CM (central memory) T cells (CD3+CD8+CD62L−CD44+) and T_EM cells (CD3+CD8+CD62L+CD44+).

Statistical analysis

All results are expressed as means ± SEM or SD as indicated. Student’s t test was used when more than two groups were compared.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/10/eaaz4204/DC1

Fig. S1. Photographs showing the obtained lyophilized drug composites. Fig. S2. Photographs showing the obtained lyophilized drug composites. Fig. S3. A SEM image of the gel scaffold loaded with DOX and R837. Fig. S4. Photographs showing the obtained lyophilized drug composites. Fig. S5. Confocal immunofluorescence images of CT26 cells with OXA/ALG in the form of chemotherapy.

Fig. S5. Cumulative drug release profiles of R837.

Fig. S6. Representative flow cytometric analysis of immunogenic death of CT26 cells and corresponding quantification results.

Fig. S7. Confocal immunofluorescence images of CT26 cells with OXA/ALG or OXA/R837/ALG treatment.

Fig. S8. Representative flow cytometric analysis of immune cell infiltration within the tumor.

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Acknowledgments
Funding: This work was partially supported by the National Basic Research Programs of China (973 Program) (2016YFA0201200), the National Natural Science Foundation of China (51525203 and 51761145041), a Collaborative Innovation Center of Suzhou Nano Science and Technology, and a “111” program from the Ministry of Education of China.

Author contributions: Y.C. and Z.L. designed the project. Y.C., C.L., and H.T. performed the experiments and collected the data. Y.C., Y.D., D.W., and Z.D. performed cellular and in vitro experiments. Y.C., Q.J., G.C., J.X., Z.K., Q.C., and W.J. discussed the data. Y.C. and Z.L. wrote the paper. Z.L. directed the research and provided financial support.

Competing interests: Z.L. and Y.C. are inventors on several pending patents related to the technology described here, filed by the China National Intellectual Property Administration (201811634727.X, 201811632248.4, and 201811632451.1). The authors declare that they have no other competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. Correspondence and requests for materials should be addressed to Z.L. (zliu@suda.edu.cn).

Submitted 7 September 2019
Accepted 11 December 2019
Published 4 March 2020
10.1126/sciadv.aaz4204

Citation: Y. Chao, C. Liang, H. Tao, Y. Du, D. Wu, Z. Dong, Q. Jin, G. Chen, J. Xu, Z. Xiao, Q. Chen, C. Wang, J. Chen, Z. Liu, Localized cocktail chemoimmunotherapy after in situ gelation to trigger robust systemic antitumor immune responses. Sci. Adv. 6, eaaz4204 (2020).