Magnetic Nano-Clusters Armed with Responsive PD-1 Antibody Synergistically Improved Adoptive T Cell Therapy for Solid Tumors

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Magnetic Nano-Clusters Armed with Responsive PD-1 Antibody Synergistically Improved Adoptive T Cell Therapy for Solid Tumors

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ABSTRACT: Although adoptive T-cell therapy has been successful in hematological malignancy treatment, its application in solid tumors remains a great challenge. Here, using a pH sensitive benzoic-imine bond and inverse electron-demand Diels-Alder cycloaddition, we prepared magnetic nano-clusters (NCs) armed with responsive PD-1 antibody (aP), which could then bind onto effector T cells due to their PD-1 expression. After adoptive transfer, the magnetization and superparamagnetism of NCs enabled us to magnetically recruit effector T cells and aP simultaneously to tumor sites with MRI guiding. Owing to the acidic intratumoral microenvironment, the benzoic-imine bond then hydrolyzed, leading to the release of aP. The therapeutic effects of adoptive T cells and free aP could thus be spatiotemporally coupled. As a result, we achieved inhibition of tumor growth with few side effects, demonstrating the great promise of such a chemical approach for safe and high-performance adoptive T-cell therapy against solid tumors.
As an immune-based approach that can harness the body’s natural defenses, adoptive T-cell therapy is a potential alternative for cancer treatment. In a typical process, autologous tumor-specific T cells are first isolated from patients. After \textit{ex vivo} activation and expansion, these tumor-reactive T cells, such as cytotoxic T lymphocytes (CTLs), tumor infiltrating lymphocytes (TILs), and chimeric antigen receptor T Cells (CAR-T), are then infused back into the individual to eliminate tumors.\textsuperscript{1,2} Although this treatment modality has demonstrated its great success in treating hematological malignancies,\textsuperscript{3,4} its application in solid tumors still is a challenge.\textsuperscript{5}

To attack the solid tumors, a prerequisite for the adoptively transferred T cells is their tumor tropism. Unfortunately, the complicated circulation process and harsh physical barriers impede the migration of these adoptive T cells to tumor sites. In this case, almost no T cells can be detected in the solid tumors after transfer.\textsuperscript{6-8} To solve this problem, T cells are usually genetically modified to express tumor chemokines to redirect them toward solid tumors.\textsuperscript{9,10} However, such single genetic engineering-based strategy is still unsatisfactory. Moreover, after infiltration, the T cells have to further navigate the
complicated tumor microenvironment (TME), which consists of numerous immunosuppressive cells and signals.\textsuperscript{11,12} For example, the local accumulation and expansion of regulatory T cells (Tregs) at tumor tissues can oppose the activity of infiltrated effector T cells.\textsuperscript{13,14} In another aspect, the immune checkpoint PD-1 (programmed cell death protein 1) can also be activated by its ligand PD-L1 in TME, which causes the anergy or exhaustion of infiltrated effector T cells.\textsuperscript{15-19} One popular way to eradicate the TME obstacles for adoptive T-cell therapy is the combinational use of cytokines and/or antibodies.\textsuperscript{20} However, the performance of these biomolecules is always compromised due to their poor intratumoral accumulation after intravenous injection.\textsuperscript{21,22} The very limited availability thus requires repeated injections,\textsuperscript{23,24} which raises the concern of side effects and high cost. Moreover, the distinct distribution and metabolism of T cells and cytokines/antibodies fails to retain them at tumor sites in a spatiotemporal coupling manner, which is also unfavorable for synergistic immune response. Therefore, an ideal regimen should be capable of synergistically guiding both adoptive T cells and assisting therapeutic agents to the tumor sites. Meanwhile, noninvasive tracking of their
dynamic deliver process \textit{in vivo} would be highly useful for providing valuable information for clinicians to tailor the most suitable regimen for personalized treatment \textsuperscript{25}.

Herein, we report a chemical strategy that can improve the performance of adoptive T cell therapy for solid tumors. Typically, magnetic nano-clusters (NCs) were firstly armed with PD-1 antibody (aP) through pH-sensitive benzoic-imine bond and inverse electron-demand Diels-Alder cycloaddition. The formed NC-aP could then bind to effector T cells due to their PD-1 expression. After adoptive transfer, both the T cells and aP could be magnetically enriched to solid tumors with magnetic resonance imaging (MRI) guiding. During intratumoral infiltration, the slightly acidic extracellular microenvironment triggered hydrolysis of the benzoic-imine bond, leading to the release of aP for PD-1 blocking. As a result, the adoptive T cells and aP worked in a synergistic manner, and the growth of solid tumors could be delayed with minor side effects (Scheme 1).

\textbf{RESULTS AND DISCUSSION}

We previously developed a hydrothermal approach to synthesize uniform-sized NCs, which consisted of stacked \sim 10 \text{ nm} particle building units.\textsuperscript{26} Such an architecture thus
endowed them with both superior magnetization and superparamagnetism for magnetic control and MRI guiding, respectively. The amino group (-NH₂) sourced from doped polyethylenimine (PEI) also facilitated subsequent modification. Specifically, benzaldehyde-PEG2000-tetrazine (BD-PEG-Tz) was synthesized as a linker (Figure S1), wherein BD could react with NH₂-NC to form pH-sensitive benzoic-imine bond (Figure S2-3),²⁷,²⁸ and trans-cyclooctene (TCO)-modified aP (aP-TCO) (Figure S4-5) could be anchored at the other terminal via the inverse electron-demand Diels-Alder cycloaddition.²⁹-³¹ These modifications could be verified by the gradually increased particle size, reversed zeta potential, and fluorescent aP corona around NC in confocal laser scanning microscope (CLSM) and stochastic optical reconstruction microscopy (STORM) image (Figure 1b-c, S6-7). After optimization, the binding capacity could be tuned to ~ 3.2 µg aP for ~50 µg NC (Figure 1d, S8). Such an abundant modification had little effect on the particle morphology and magnetism properties (Figure 1e, S9). As shown in Figure S9, the high saturation magnetization (~70 emu·g⁻¹) enabled the rapid enrichment of NC-aP by a commercial magnetic scaffold within 1 min, and its T₂ contrast signal intensity was directly proportional to the concentration, which enabled the magnetic
enrichment to solid tumors with MRI guiding. Meanwhile, the release rate of aP was much faster at pH 6.5 than pH 7.4, promising the cleavage of benzoic-imine bond in the slightly acid tumor microenvironment, while maintaining stability at normal tissues (Figure 1f, S10).

Having successfully prepared the NC-aP, we next incubated them with CTLs (OVA-specific) to form CTL: NC-aP through the recognition of aP decorated on NC and PD-1 expressed on CTLs (Figure S11). As indicated by TEM, CLSM and SEM, which were shown in Figure 1 g, h and S12, respectively, many NCs-aP could be observed on the CTLs surface, and the amount could be well controlled in a dose dependent manner (Figure S13). Taking 50 \( \mu \text{g/mL} \) NCs-aP (iron concentration) exposed to \( 10^6 \) CTLs as an example, up to \(~3713\) NCs-aP could bind to an individual CTL (Figures S14 and S18). In this case, the activity of the CTLs remained more than 90% (Figure S15), which could be verified by the dominant green CTLs after Live/Dead assay (Figure 1i). Moreover, the IFN-\( \gamma \) secretion and granzyme-B expression levels of these CTLs decreased by less than 10% (Figure 1j; S16), further indicating that NC-aP binding led to almost no damage under
the iron concentration of 50 μg/mL and a 30-min incubation. Owing to the benzoic-imine bond, a pH-dependent release of NCs from CTLs could also be observed (Figure S17-18), again demonstrating the satisfactory release selectivity at the acidic tumor sites.

To reveal the important role of the pH-sensitive benzoic-imine bond in CTL:NC-aP, we also constructed CTL:NC-aP(-) as a control for comparative study, wherein a pH-insensitive amide bond was utilized. After in vitro incubation with target E.G7 (OVA+) cells, different CTL formulations exhibited distinct cytotoxicities as evaluated by the lytic activity of cells (Figure 2a). In pH=7.4 cell culture medium, the decoration of either NC-aP(-) or NC-aP showed little effect compared with pristine CTLs. In pH=6.5 medium, the released aP in CTL:NC-aP group could block the resident PD-1 on the CTLs (Figure S19), thus inhibit the PD-1:PD-L1 pathway. In this aspect, the cytotoxicity could be improved, which was much superior to that of pristine CTLs and CTL:NC-aP(-). For further verification, these CTL formulations were administered to E.G7-tumor-bearing mice via a single intratumoral injection with a dose of 2×10^6. As shown in Figure 2b and S20, the CTL:NC-aP group showed significantly improved tumor inhibit ability than that of CTL and
CTL:NC-aP(-) groups, and the tumor growth was delayed over 14 days. The CTL:NC-aP(-) group also showed better antitumor efficacy than that of CTL group, possibly because that aP could block part of PD-1 on the CTLs surface and reduce the PD-1:PD-L1 interaction. Deeper insights could also be obtained at the histology level (Figure 2c).

In the CTL:NC-aP(-) group, most NCs and aPs were well overlapped with the colocalization rate (CR) value up to 82.7%. In contrast, the majority of these two signals separated in the CTL:NC-aP group due to the hydrolysis of benzoic-imine bond in the acidic tumor tissue, and the CR value dropped to 18.8%. In this case, the released free aP could be utilized to block the PD-1:PD-L1 pathway throughout the tumor tissue. Consequently, the conversion of naive T cells to Foxp3+ Tregs may be inhibited and the effector T cell development may be promoted.\textsuperscript{32-35} As a result, the percentage of CD8+ CTLs in the tumors of mice was obviously increased, and the percentage of Tregs (CD4+CD25+Foxp3+) in the tumor was significantly decreased after CTL:NC-aP treatment compared to other groups. As a result, the ratio of CD8+ T cells to Tregs in the tumors from CTL:NC-aP group was also greatly increased (Figure 2d, S21-S22).
Encouraged by the above results, we continued to evaluate the \textit{in vivo} fate of these adoptive CTLs. We were glad to see the decoration of NC-aP endowed CTLs with the talents of magnetic control (Figure 3a) as well as MR imaging (Figure S23). Subsequently, the CTLs labeled with DIR were intravenously injected into E.G7 tumor-bearing mice and monitored by \textit{in vivo} fluorescence imaging. As shown in Figure 3b, both pristine CTL and CTL:NC-aP shared the feature of poor tumor accumulation. Once a magnetic field was added in the CTL:NC-aP(m) group, much more CTLs could be enriched at tumor sites within 12 h and retained for a longer period. And the area under the curve of fluorescence intensity thus improved by 11.4 fold compared with that of pristine CTL group (Figure 3c; S24). Therefore, a large amount of infiltrated CTLs could be observed in the tumor tissue after CTL:NC-aP(m) treatment (Figure 3d). To gain a deeper insight of systemic distribution, we also examined excised organs (Figure 3e, S25). As expected, the CTL:NC-aP(m) group exhibited the highest accumulation in tumors while the lowest distribution in other organs. ICP-MS quantitative analysis results showed that much more (~3.0 fold) CTL:NC-aP could be enriched at the tumor sites by using an external magnetic field. Owing to the superparamagnetism of NCs, similar results could also obtain through
in vivo MRI (Figure 3f-g). The CTL:NC-aP(m) group still outperformed the CTL and CTL:NC-aP groups in $T_2$ imaging at the tumor sites, again demonstrating the success of utilizing magnetic control to improve target delivery of adoptive CTLs.

Finally, we were in the position to investigate the in vivo therapeutic performance. The mice were challenged with E.G7 tumor cells and then infused with various CTL formations. Compared with PBS control, CTLs alone yielded only a slight inhibition of the tumor growth due to poor tumor accumulation (Figure 4a). The combination with free aP showed little improvement since the effects of immune cytotoxicity and checkpoint blockade failed to work in a synergistic way. This dilemma could be ameliorated when these two components were responsively integrated in the CTL:NC-aP group. Upon a further magnetic field, much more CTLs, together with aP could be recruited to tumor sites, leading to the complete suppression of tumor development in the CTL:NC-aP(m) group. Additionally, no death occurred during 35 days, whereas all mice died within 25 days in the other groups (Figure 4b). If the magnetic field was applied only to the CTLs (CTL:NC(m) + free aP group), the tumor inhibit capability was also clearly inferior to the
CTL:NC-aP(m) group (Figure S31), again confirmed that the nano-clusters facilitated the synergetic therapy of CTL and aP through a low pH responsive manner. Similar results could also be found at the histology level where the CTL:NC-aP(m) group again achieved the most potent apoptosis effect and the lowest proliferation of tumor cells (Figure 4c). In addition to the improved therapeutic effect, the target accumulation of CTLs and aP in a spatiotemporal coupling manner could also reduce the administration dose and frequency compared with previous regimens.\textsuperscript{36,37} Therefore, treatment with CTL:NC-aP(m) resulted in few abnormalities in the weight loss, body temperature, immune storm indicators, organ histology, and blood biochemical markers, further confirming its safe use (Figure S26-29).

To confirm the universality of our strategy, we also investigated the feasibility for adoptive TIL therapy. To this end, TILs separated from malignant 4T1 tumor tissues were bound to NC-aP, and then infused back to the same tumor model. Similarly, TILs alone showed little inhibition of the tumor growth (Figure 5a). Owing to the assistance of responsively bound aP, a further delay could be observed in the TIL:NC-aP group. With further magnetic recruitment (TIL:NC-aP(m)), we observed notable necrosis in the tumor
tissues. Correspondingly, tumor development could be almost totally inhibited, and all mice remained alive during the observation period (Figures 5b-c, S31). As metastasis to distant lung and bone are always observed for breast cancer in clinic, the anti-metastasis performance was also evaluated in this model. As shown in Figures 5d-f and S30, metastatic foci in lung and metastasis-induced erosion in tibia were found to different extents in other groups. In contrast, no metastasis signs were observed in the mice treated with TIL:NC-aP(m), again confirming its superior performance over traditional adoptive T cell therapy.

CONCLUSIONS

In summary, we developed a chemical strategy for improving the treatment performance of adoptive T cells for solid tumors, wherein the magnetic NCs were armed with aP through an acidic pH-sensitive benzoic-imine bond and then bound to the T cells NC via the interaction between aP and PD-1. Owing to the superior magnetization and superparamagnetism, the T cells, along with aP could be magnetically recruited to tumor sites with MRI guiding. During the intratumoral infiltration in the acidic extracellular...
microenvironment, the benzoic-imine bond hydrolyzed, leading to the release of aP. In this case, the functions of immune cytotoxicity and checkpoint blockade could work in a synergistic manner. As a result, potent therapeutic effects with few side effects were demonstrated on different models, which could fit various requirements for adaptive T cell therapy against solid tumors.

EXPERIMENTAL SECTION

**Key chemicals:** Ferrous sulfate heptahydrate (FeSO$_4$·7H$_2$O) was purchased from Aladdin (China). Polyethyleneimine (PEI), ethylene glycol (EG) and diethylene glycol (DEG) were purchased from Alfa Aesar (UK). Sodium hydroxide (NaOH) was purchased from Acros (Belgium). Benzaldehyde-PEG2000-NHS ester (BD-PEG-NHS) was obtained from Ponsure (China). Tetrazine-amine hydrochloride (Tz-NH$_2$·HCl) was obtained from Bioconebio (China). Trans-cyclooctene-PEG4-NHS ester (TCO-PEG$_4$-NHS) was purchased from Click Chemistry Tools (USA). PD-1 antibody (aP) was purchased from Biolegend (USA). All chemicals were used as received without purification.
Preparation of magnetic nanoclusters: Magnetic nanoclusters (NCs) were prepared through a solvothermal treatment method, as we previously reported. Briefly, FeSO₄·7H₂O (0.128 g) and PEI (0.06 g) were dissolved in a mixture solution of EG/DEG (1 mL/14 mL) and heated to 160 °C with vigorous stirring in an argon atmosphere, and part of Fe²⁺ was oxidized to Fe³⁺ during this process. Next, a NaOH/DEG stock solution was rapidly added, with primary Fe₃O₄ nanocrystal nucleation occurred in the PEI due to the coprecipitation of formed Fe²⁺ and Fe³⁺ in alkaline conditions. The reaction continued for 1 h at 220 °C to allow nanocrystals to aggregate and spontaneously form primary clusters. The resulting NCs with primary amine groups on the surface (NC-NH₂) were collected through magnetic separation and were washed with ethanol and deionized water.

Synthesis of benzaldehyde-PEG2000-tetrazine: BD-PEG-NHS (10 μmol) was dissolved in methanol/triethylamine solution (2 mL, pH 8.0) containing Tz-NH₂·HCl (11 μmol). The solution was stirred at room temperature for 5 hours, after which it was added dropwise to ethyl ether and centrifuged at 5,000 × g for 30 min to collect the crude product.
The product was re-precipitated by cold ethyl ether and then dialyzed with a dialysis bag
(Mw: 500) for 3 days, dried with a vacuum freeze dryer, and analyzed by \(^1\)H NMR (Bruker,
Advance III 500).

**Modifying NC-NH\(_2\) with benzaldehyde-PEG2000-tetrazine:** NC-NH\(_2\) was dispersed in
0.1 M citric acid buffer (pH 5.0) by sonication. An excess of benzaldehyde-PEG2000-
tetrazine (BD-PEG2000-Tz) was then added and shaken at room temperature for 7 hours.

The modified product, NC-Tz, was collected by magnetic separation and washed with
PBS. The zeta potential and hydrodynamic diameter were tested by dynamic light
scattering (Malvern, ZS90). FT-IR spectra were analyzed with a Thermo Scientific Nicolet
iS50 spectrometer.

**Modification of PD-1 antibody with TCO:** An aliquot of 20 \(\mu\)g of aP was diluted in 200
\(\mu\)L of PBS, followed by the addition of a 50-fold molar excess of TCO-PEG\(_4\)-NHS. The
mixture was allowed to react for 1 hour at room temperature with mild stirring, and
unreacted TCO-PEG\(_4\)-NHS was then separated from TCO-derivatized PD-1 antibody
(aP-TCO) by centrifugation (Millipore Co, Amicon Ultra-0.5) at 7000 rpm at 4 °C for 30
min. The purified aP-TCO suspended in PBS was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Axima Performance MA). To verify the activity of aP-TCO, activated cytotoxic lymphocytes (CTLs) were incubated with aP-TCO or aP on ice for 30 minutes and then washed with PBS. Subsequently, the Alexa Fluor 488 secondary antibody was added, incubated and washed. The labeled CTLs were analyzed by flow cytometry (BD, FACSCelesta) and confocal laser scanning microscope (CLSM) (Leica, TCS SP5).

**Preparation of PD-1 antibody-decorated NCs:** Decoration of NC-Tz with aP-TCO was realized through an inverse electron-demand Diels–Alder cycloaddition reaction. Briefly, NC-Tz (Fe: 50 μg) and aP-TCO (3 to 12 μg) were mixed in PBS and reacted overnight with gentle rotation at 4 °C. The final NC-aP product was purified through magnetic separation to remove unbound antibody and then redissolved in PBS and stored at 4 °C. The saturation binding of anti-PD-1 was determined by a micro-BCA Protein Assay Kit (Thermo Scientific). The NC-aP was characterized by transmission electron microscopy (TEM) (HITACHI, HT7700) and dynamic light scattering measurement. Magnetic-
hysteresis curves (M-H curves) were measured by a VSM (Lake Shore, 730T). Nuclear magnetic resonance images (MRIs) of the NC-aP solutions (iron concentrations: 0, 31.25, 62.5, 125, 250, and 500 μM) were recorded on a Micro-MRI Scanner (BRUKER, Biospec70/20USR). The stochastic optical reconstruction microscopy (STORM, Nikon) images were acquired by labeling the aP on the NCs with goat anti-rat IgG (Alexa Fluor 647) (Abcam).

**Tumor cell culture.** The E.G7-OVA (C57BL/6 mouse lymphoma cell line) and 4T1 (mouse breast cancer cell line) were kindly obtained from Institute of Process Engineering (China). All cell lines were maintained in a 37°C / 5% CO₂ humidified chamber. The 4T1 cells were maintained in RPMI-1640 media (Gibco) with containing 10% fetal bovine serum (FBS) (Gibco) and 2% penicillin–streptomycin, and E.G7 cells were maintained in RPMI-1640 media (Gibco) containing 10% FBS, 2 mM L-glutamine, 4.5 g/L glucose, 0.05 mM 2-mercaptoethanol and 0.4 mg/ml G418 (Amresco).

**Activation and expansion of CTLs and tumor-infiltrating lymphocytes:** Splenocytes were isolated from the spleens of OT-1 mice (aged 6–8 weeks), and CD8+ T cells were isolated
using a CD8+ no-touch isolation kit (Miltenyi) according to the manufacturer’s guidelines.

The CD8+ T cells were cultured in complete media composed of BIOTARGET-1 SFM supplemented with L-glutamine (4 mM) and penicillin-streptomycin (2%). For stimulation and expansion, the artificial antigen presenting cells (aAPC) were first constructed, as we previously reported \(^{38}\), and the aAPCs were then incubated with \(1 \times 10^6\) CD8+ T cells at a concentration of 100 \(\mu\)g per well in 24-well plates for 5-6 days. The obtained CTLs were resuspended by pipetting up and down and were finally separated from the aAPCs through magnetic separation.

To separate tumor infiltrating lymphocytes (TILs), the tumor tissues from 4T1 tumor-bearing BALB/c mice were cut into 1 mm\(^3\) fragments and enzymatically digested for 1 h in HBSS (Gibco) containing collagenase type 1A (1 mg/mL), hyaluronidase (1 mg/mL) and DNase (20 U/mL) (Sigma). The digestion mixtures were filtered through a 40 \(\mu\)m cell strainer to obtain single-cell suspensions and overlaid over mouse lympholyte-M media (Solarbio) for lymphocytes isolation according to the manufacturer’s protocols. The TILs were cultured in complete medium plus IL2 (6000 IU/ml) (Biolegend) for two weeks.
Thereafter CD8+ TILs were isolated using a FlowComp Mouse CD8 kit (Dynabeads) according to the manufacturer’s guidelines and then released from the beads using the provided release buffer. The resulting CD8+ TILs were cultured for another week in complete medium plus IL2 (6000 IU/ml).

**Preparation of NC-aP armed T cells:** NC-aP (Fe: 50 μg) was incubated with 1 × 10^6 activated CD8+ T cells (CTLs or TILs) in 100 μL of PBS for 30 min at 37 °C with gentle shaking every 10 min, after which 200 μL of PBS was added and the mixture transferred to a Transwell chamber to remove excess NC-aP. The NC-aP-armed T cells (CTL:NC-aP or TIL:NC-aP) were characterized by TEM, scanning electron microscope (SEM) (HATICHI, SU8020), energy dispersive spectrometer (EDS)-elemental mappings (HORIBA, EX-350), CLSM and MRI. The quantity of iron on the cell surface was determined by ICP-AES (PerkinElmer, Optima 5300DV).

Acidic-stable CTL:NC-aP (-) and TIL:NC-aP (-) were also prepared as a control. Briefly, NC-Tz linked by an amide bond [NC-Tz (-)] was synthesized from NC-NH₂·HCl and Tz-PEG2000-NHS. Next, NC-Tz (-) (Fe: 50 μg) was reacted with 9 μg of aP-TCO in PBS by
gentle rotation overnight at 4 °C. The product, NC-aP (-), was collected and incubated with $1 \times 10^6$ CTLs or TILs in 100 μL of PBS for 30 min at 37 °C. The excess NC-aP (-) was removed through a Transwell chamber.

To prepare CTL:NC or TIL:NC, $1 \times 10^6$ CTLs or TILs were incubated with maleimide-PEG2000-TCO (50 μM) in 200 μL of PBS for 30 min at 37 °C. The resulting TCO modified CTLs or TILs were collected and incubated with NC-Tz (Fe: 50 μg) in 100 μL of PBS for 30 min at 37 °C with gentle shaking every 10 min, after which 200 μL of PBS was added and the mixture transferred to a Transwell chamber to remove excess NC-aP.

**Granzyme-B and IFN-γ analysis:** $1 \times 10^6$ CTL cells were incubated with varying amounts of NC-aP (0, 10, 25, 50 or 100 μg Fe), and the resultant CTL:NC-aP was individually cultured in complete media at 37 °C for 24 h.

For granzyme-B analysis, each group of CTL:NC-aP was collected, washed with ice-cold PBS, and fixed in fixation buffer in the dark for 20 minutes at room temperature. The fixed cells were washed and stained with PE/Cy7 anti-mouse Granzyme B Antibody (Biolegend) according to their intracellular flow cytometry staining protocol. Thereafter,
the cells were washed with perm/wash buffer and analyzed by a flow cytometer (BD, FACSCelesta).

For IFN-γ analysis, the cell supernatant of each group was collected and analyzed by an ELISA Kit (Invitrogen) according to the manufacturer’s guidelines.

**pH-dependent release of PD-1 antibody from NC-aP or NC from CTL:NC-aP:** To determine the release of aP from NC-aP, NC-aP was stained by goat anti-rat IgG (Alexa Fluor 488) and then dissolved in pH 7.4 or 6.5 PBS. At different time intervals, the NC-aP was magnetically separated and analyzed by flow cytometry. Meanwhile, the fluorescence intensity of the supernatants was measured by a fluorescence spectrometer (HORIBA Jobin Yvon, FluoroMax-4) and the antibody concentration was calculated from a standard curve.

To determine the release of NC from CTL:NC-aP, CTL:NC-aP was cultured in complete media with pH 7.4 or 6.5 in Transwell chambers. At desired time points, the lower media was collected and analyzed by ICP to determine the released iron content.
**In vitro cytotoxicity evaluation:** CTLs, CTL:NC-aP(-) or CTL:NC-aP at a density of $1.5 \times 10^5$ cells/well were individually co-cultured with 5,000 E.G7 cells in complete media with pH 7.4 or 6.5 at 37 °C for 24 h. The lytic activity of different CTL-based formations against E.G7 target cells was evaluated by a lactate dehydrogenase (LDH) release assay (Dojindo) according to the manufacturer’s guidelines.

**In vitro Tregs quantitative analysis:** Freshly separated splenocytes were cocultured with E.G7 cells at pH 7.4 or 6.5; meanwhile, different CTL-based formations were individually added and incubated for 48 h, after which the mixed cells were stained with anti-CD8-PE (eBioscience), anti-CD4-eFluor450 (eBioscience), and anti-CD25-FITC (eBioscience) on ice for 20 minutes in the dark. Subsequently, the cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and then stained with anti-Foxp3-Alexa Fluor 700 (eBioscience) according to their intracellular staining protocol. Finally, the number of CD4+ CD25+ Foxp3+ Treg cells as a percentage of the total CD8+ T cell population was analyzed by flow cytometry.
Intratumoral performance evaluation of CTL:NC-aP: C57Bl/6 mice were subcutaneously inoculated with E.G7 cells (1 × 10^6 cells) and the tumors were allowed to grow for 6 days. After this time, the mice were randomly divided into four groups and intratumorally injected with (1) PBS, (2) CTLs, (3) CTL:NC-aP(-) or (4) CTL:NC-aP (5 × 10^6 cells per mouse). The tumor volume was measured every 2 days thereafter. To verify the release of aP from CTL:NC-aP in vivo, the tumors were dissected at 72 h post injection and snap frozen in optimal cutting temperature (OCT) medium. Subsequently, released aP was labeled using goat anti-rat IgG (Alexa Fluor 488). The colocalization rates between aP and NC were calculated using the following formula: Colocalization Rate = Colocalization Area / Area Foreground, where Area Foreground = Area Image - Area background. For immunofluorescence assay of Treg and CD8+ T cells, the sections were stained with Foxp3 and CD8 primary antibodies (Abcam) (1:200 in 1% BSA/TBS) overnight and then washed with 0.025% Triton-X-100/TBS. The sections were then incubated with Alexa Fluor 488 and Alexa Fluor 594 secondary antibody, diluted in 1% BSA/TBS, at room temperature in the dark for 1 h, and the nuclei were stained with DAPI. The fluorescence images of each tumor section were obtained with an automated
quantitative pathology imaging system (Vectra 3, PerkinElmer). To quantify the Treg and
CD8+ T cells, the tumors were steriley dissected to prepare single-cell suspensions
and overlaid over mouse lympholyte-M media (Solarbio) for lymphocyte isolation
according to the manufacturer’s protocols, followed by centrifugation at 2500 rpm. The
resulting buffy coat layer was removed, washed, and subsequently stained with anti-CD8-
Alexa Fluor 450 (Biolegend), anti-CD4-PE (Biolegend), and anti-CD25-FITC (Biolegend)
on ice for 20 minutes in the dark. Subsequently, the cells were fixed and permeabilized
using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and then stained
with anti-Foxp3-APC (eBioscience) according to their intracellular staining protocol.
Finally, the number of CD8+ CTLs and CD4+ CD25+ Foxp3+ Treg cells as a percentage
was analyzed by flow cytometry.

*In vivo* fluorescence and MR imaging: CTLs or CTL:NC-aP were first labeled with DiR.
E.G7 tumor-bearing mice were randomly divided into three groups. The first group was
injected (i. v.) with CTLs, and the other two groups were injected (i. v.) with CTL:NC-aP
(2×10^6 cells per mouse). At the beginning of the treatment, a commercial neodymium
magnet (Grade: N35) was fixed to the tumor site in the CTL:NC-aP(m) group by using medical adhesive tapes, and kept for 24 hours after the i.v. injection. At different time intervals, the mice were anesthetized using isoflurane/O$_2$ (2% v v$^{-1}$) and scanned by an in vivo fluorescence imaging system (Kodak, FX Pro). To further investigate the intratumoral biodistribution of adoptive transferred CTLs, the mice were sacrificed at 12 h post injection, and the tumors were dissected from the mice and snap frozen in OCT medium for fluorescence staining. To quantify the biodistribution of CTL:NC-aP in tumors and different organs, the mice were sacrificed at 12 h post injection, and then the dissected tumors and organs were used for ICP-MS analysis.

For MR imaging, the CTLs or CTL:NC-aP without DiR labeling were used at a dose of 1×10$^7$ cells per mouse and then imaged at 24 h by an MR imaging system (Bruker, BioSpec 70/20 USR).

**In vivo therapeutic efficacy and safety evaluation of CTL:NC-aP in E.G7 tumor models:**

A total of 5 × 10$^5$ E.G7 cells were inoculated into the right flanks of female C57BL/6 mice (6 weeks) on day 0. The mice were then randomly divided into five groups (9 mice per
group) and intravenously injected with (1) PBS, (2) CTL, (3) CTL+ free aP (4) CTL:NC-aP and (5) CTL:NC-aP(m) (CTL dose: $1 \times 10^7$ cells per mouse) on day 6 and 9. The body weight, temperature and tumor volume of each mouse was monitored every 2 days. Animals were euthanized when the tumor volume was greater than 1000 mm$^3$, and the tumor tissues and major organs were collected for immunohistochemical and TUNEL assay. For safety evaluation, the serum cytokine levels (IL-6 and TNF-α) were analyzed using enzyme-linked immunosorbent assay kits (Invitrogen).

**In vivo therapeutic efficacy of TIL:NC-aP TIL for 4T1 tumor metastatic models:** A total of $5 \times 10^5$ 4T1 cells were subcutaneously injected into BALB/c mice (6 weeks) on day 0. The mice were randomly divided into four groups (9 mice per group), and then intravenously injected with (1) PBS, (2) TILs, (3) TIL+ free aP, (4) TIL:NC-aP, or (5) TIL:NC-aP(m) (TIL dose: $5 \times 10^6$ cells per mouse) on day 4 and 13. The tumor volumes were measured every 2 days. To evaluate distant metastases, the mice were euthanized on day 35, and the lungs and hind legs of each mouse were collected. The lungs were
characterized by optical photographs and H&E staining, and the tibia bones were imaged by computed tomography (CT) (Quanturum FX, PerkinElmer).

**Statistical analysis:** Statistical evaluations of the data were performed using Student’s t test. All of the results were expressed as the mean ± standard error; *P < 0.05, **P < 0.01, ***P < 0.001.
Scheme 1. Schematic illustration of magnetic nano-clusters armed with PD-1 antibody improved adoptive T cell therapy for solid tumors. Magnetic NCs were firstly armed with aP through a pH-sensitive benzoic-imine bond, then the resultant NC-aP could bind to T cells. After adoptive transfer, both T cells and aP could be magnetically enriched to solid tumors with MRI guiding. During intratumoral infiltration, the slightly acidic
microenvironment triggered the release of aP for PD-1 blocking. As a result, the adoptive T cells and aP worked in a synergistic manner.

**Figure 1.** Fabrication and characterization of pH-responsive NC-aP and CTL:NC-aP. a, Schematic illustration of NC-aP. b, Hydrodynamic size aP change following NC-aP. c, Confocal microscopy image of NC-aP. d, aP bound (µg) with total aP added (µg). e, Transmission electron microscopy image of NC-aP. f, Time course of released aP at pH 6.5 and pH 7.4. g, Immunofluorescence image of Granzyme-B expression. h, Immunofluorescence image of IFN-γ expression. i, Semi-quantitative analysis of Granzyme-B and IFN-γ expression.
fabrication. c, STORM image of NC-aP labeled with fluorescent secondary antibody (red) (Scale bar: 50 nm). d, The micro BCA protein assay of aP on NC. The binding capacity could be tuned to ~ 3.2 μg aP for ~50 μg NC. e, TEM image of NC-aP (Scale bar: 500 nm). f, The release profiles of aP from NC-aP. g, TEM images of CTL:NC-aP. NCs-aP could be observed on the CTLs surface (Scale bars: 2 μm). h, The representative confocal and corresponding 3D reconstruction images of CTL:NC-aP (Green: CTLs; Red: NC-aP. Scale bars: 3 μm). i, Live (green)/Dead (red) staining image of CTL:NC-aP (Scale bar: 100 μm). j, IFN-γ release and granzyme-B expression levels of CTL:NC-aP under the iron concentration of 50 μg/mL and a 30-min incubation. All error bars indicate SD (n=3).

Figure 2. Effects of pH-responsive linker on the synergistic performance of CTLs and aP. a, Cytotoxicity of different CTL formations on E.G7 cells at pH = 7.4 or 6.5. Error bars
indicate SD (n=3). b, Tumor volume of E.G7 tumor-bearing C57BL/6 mice with different treatments. Error bars indicate SD (n=6). c, CLSM images of tumor tissues infiltrated with CTL:NC-aP (-) or CTL:NC-aP (Red: NC; Green: fluorescent secondary antibody-labeled aP; Blue: nuclei. Scale bars: 30 μm). d, Immunofluorescence staining images of the intratumoral CD8+ T cells and Tregs (Scale bar: 50 μm).
Figure 3. In vivo visual and magnetic targeting transfer of CTL:NC/AgP. a, Magnetic manipulation of CTL:NC-aP in vitro monitored by CLSM (Scale bars: 50 μm). The DiO-labeled CTL:NC/AgP could be enriched after attaching a commercial magnet onto the top
side of the chamber. b, Visualization of the tumor-targeting ability of DiR-labeled CTL formations after injection (i. v.). Images were representative of three independent experiments. c, Quantitative statistics of the median fluorescence intensity in tumor region in b. d, Typical CLSM imaging of tumor tissue sections after 12 h injection (i.v.) (Red: CTLs; Blue: nuclei. Scale bar: 100 μm). e, Ex vivo fluorescence images of tumor and major organs excised from tumor-bearing mice treated with different formulations after 48 h injection (i.v.). f, T2-MR imaging of mice before and after injection (i.v.) with different CTL formations. g, Quantification of signal intensity changes in f. All error bars indicate SD (n=3).

**Figure 4.** In vivo therapeutic performance of CTL:NC-aP in E.G7 tumor model. a-b, Average tumor growth curves and survival percentages of mice after different treatment. All error bars indicate SD (n=9). c, Representative immunohistochemical analysis of the tumor sections using TUNEL assay and Ki67 staining (Scale bars: 300 μm).
Figure 5. *In vivo* therapeutic performance of TIL:NC-aP in 4T1 tumor metastasis model. a, b, Average tumor growth curves and survival rates of mice after different treatments. Error bars indicate SD (n=9). c, Representative H&E staining analysis of tumor sections (Scale bars: 300 μm). d, Representative photographs of lung tissues at the end of experiment. The black arrows indicate the metastatic nodules. NMN means the average number of metastatic nodules (n=6). e, Representative H&E staining images of collected lung tissues at the end of the experiment (Scale bar: 200 μm). f, Typical CT images of
collected tibia bones at the end of the experiment. Regions bounded by red imaginary lines indicate the metastasis-induced erosion in tibia, which confirmed the efficient \textit{in vivo} anti-metastasis performance of the TIL:NC-aP.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website.

Supplemental figures showing other characterizations, quantitative analysis, biodistribution of CTL:NC-aP, flow cytometry analysis, histology analysis, statistical analyses of typical blood biochemical markers, \textit{et al}.

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