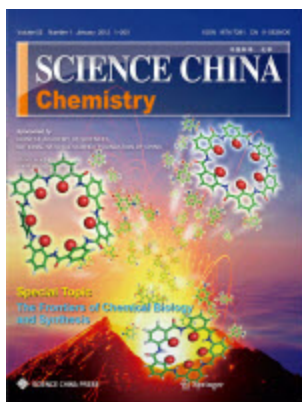




Repolarization-gated potentiation and imaging of macrophages with engineered oncolytic bacteria for on-demand immunotherapy

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Repolarization-gated potentiation and imaging of macrophages with engineered oncolytic bacteria for on-demand immunotherapy

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Abstract Immune checkpoint blockade (ICB) on macrophages can improve their anti-tumor immune activity, yet it is hampered by on-target off-tumor toxicity and the lack of direct visualization of macrophages response. This work develops a kind of engineered oncolytic bacteria (pEDp@MM) for repolarization-gated ICB and real-time monitoring of macrophage. Enzyme-activated DNA prodrug (Dp) containing ICB drug is anchored on macrophage membranes-encapsulated catalase-expressing *Escherichia coli* (pE.coli). After targeting tumor-associated macrophages (TAMs), pEDp@MM induce oxygen production for photoacoustic imaging-guided self-localization and repolarize TAMs toward M1 phenotype with pE.coli, triggering nuclear-to-cytoplasmic translocation of apurinic/apyrimidinic endonuclease 1 (APE1) and elevation of *IL-6* mRNA within macrophages. The translocated APE1 triggers the releases of ICB drug from Dp for on-demand immunotherapy, while the *IL-6* mRNA initiates the fluorescence recovery of cleaved Dp for visualization of macrophage phenotype change and drug release. In vivo studies demonstrate the phenotype-dependent ICB induces effective immune activation with real-time macrophage imaging, offering a promising approach for on-demand immunotherapy and precision diagnostics.

Keywords repolarization, immunotherapy, engineered bacteria, SIRP α -CD47 axis, self-reporting

1 Introduction

Tumor-associated macrophages (TAMs), a major immune constituent predominantly exhibiting M2 phenotype in tumor microenvironment (TME), can secrete immunosuppressive factors to drive tumor progression by promoting angiogenesis, suppressing T-cell activation, and recruiting additional macrophages [1,2]. Repolarization of TAMs to M1 phenotype restores their phagocytic and antigen-presentation capacity [3]. However, their phagocytic activity is generally limited by the “do not eat me” signal mediated between CD47 on tumor cells and signal regulatory protein α (SIRP α) on macrophages. Immune checkpoint blockade (ICB) of the CD47-SIRP α axis to potentiate the phagocytosis of cancer cells by macrophages can activate cytotoxic T-cell and initiate an antitumor immune response [4,5]. Nevertheless, clinical application has revealed considerable interpatient heterogeneity in response to ICB therapy, whereby even patients with high expression of tumor-derived biomarkers often fail to achieve favorable responses due to poor immune cell reactivity [6]. Furthermore, non-selective inhibition, resulting from the extensive expression of both SIRP α and CD47 on normal cells (e.g., hematopoietic and pulmonary macrophages), is associated with severe systemic hematological disorders and pneumonitis [7,8].

Activatable ICB by using tumor markers (pH [9], matrix metalloproteinases 2 [10]) highly expressed in TME, or external stimuli (light [11], magnetism [12]) as a switch has attracted widespread attention for targeted immune modulation. Despite advances, these strategies still face several challenges. Firstly, exogenous physical interventions are difficult to adjust dynamically based on interindividual differences. Secondly, a single tumor-intrinsic indicator provides only relatively static spatial information within the TME, which results in inconsistent efficacy of drug activation due to the heterogeneous distribution of target molecules [13], and on-target off-tumor (OTOT)

toxicity to adjacent normal tissues [14,15]. In addition, insufficient tools for direct and real-time visualization of immune cell responses during treatment also aggravates the risk of off-target toxicity [16,17], underscoring the necessity to develop spatially resolved in vivo imaging technologies for dynamically activated immune cell-associated biomolecules [18,19].

As highly plastic immune cells, macrophages typically reside in the bone marrow in an inactivated M0 phenotype. They can be polarized into the pro-inflammatory M1 phenotype under inflammatory conditions and the anti-inflammatory (yet pro-tumorigenic) M2 phenotype in the TME [20]. Notably, this polarization process involves dynamic changes in key molecules, with apurinic/apyrimidinic endonuclease 1 (APE1) and interleukin-6 (*IL-6*) mRNA serving as prominent markers of M1-like polarization [18]. Normally, APE1 resides in the nucleus and initiates the base excision repair pathway by cleaving apurinic/apyrimidinic (AP) sites [21]. However, M1-like polarization triggers the translocation of APE1 from nucleus to cytoplasm, along with a marked increase in interleukin-6 (*IL-6*) mRNA expression, which enhances their phagocytic capacity toward tumor cells. Consequently, real-time monitoring of APE1 and *IL-6* mRNA dynamics offers a direct and accurate readout of macrophage responses during immunotherapy, enabling immune-responsive ICB regulation and precise evaluation of immune outcomes.

Bacteria have emerged as a new kind of biovectors in tumor therapy with the advantages such as intrinsic immunostimulatory properties for repolarization of macrophages, bacterial motility for improving intratumoral distribution and genetic tractability for functional protein expression [22–25]. Nevertheless, the clinical translation of live bacterial therapeutics remains great challenges, including dose-dependent virulence, rapid clearance by the reticuloendothelial system, and limited controllability after systemic administration [26–28]. Cell-membrane camouflage strategy has shown promise in improving biosafety

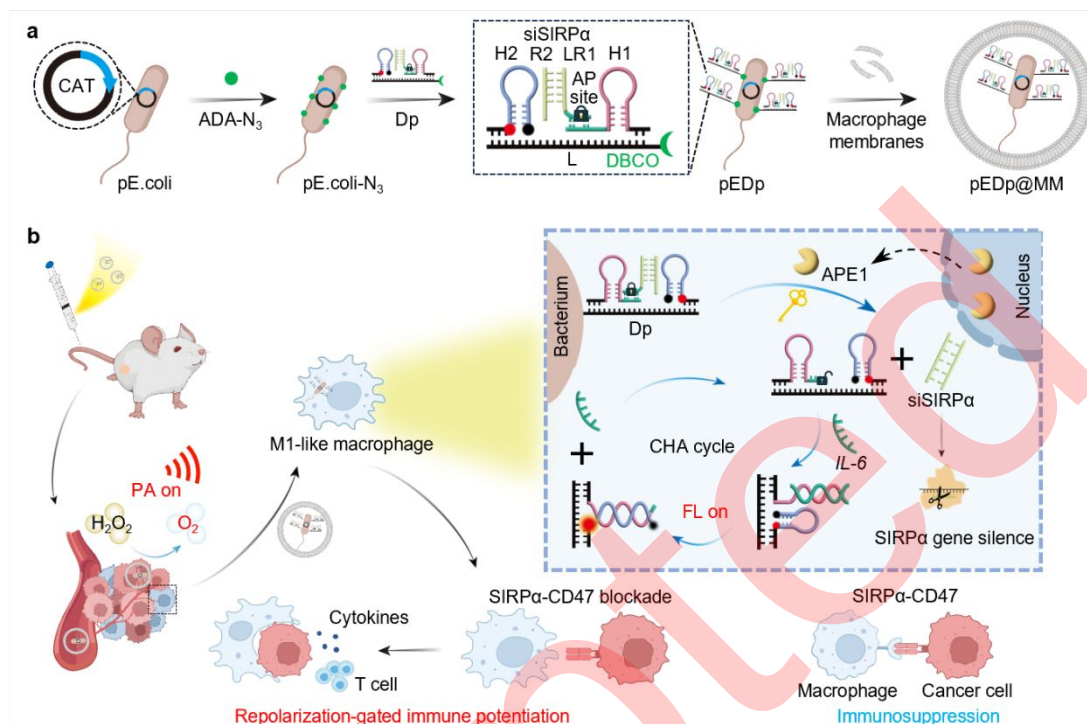


Figure 1 Schematic illustration of oncolytic bacteria for on-demand immunotherapy. (a) Preparation of pEDp@MM. (b) Repolarization-gated macrophage potentiation and imaging in tumor immunotherapy. Under the tumor-targeting effect of macrophage membranes, pEDp@MM selectively migrate to the TME to catalyze hydrogen peroxide into oxygen and induce M1-like repolarization of macrophages, which triggers the nuclear-to-cytoplasmic translocation of APE1 and elevates *IL-6* mRNA expression. APE1 cleaves the AP sites in Dp anchored on bacteria to release siSIRPα for ICB, while *IL-6* mRNA initiates CHA cycle of cleaved Dp for fluorescence recovery and amplification.

and prolonging circulation, and macrophage membranes in particular have been reported to enhance tumor targeting [29,30]. Aim to the challenges in on-demand immune modulation with real-time monitoring while improving the translational potential of live bacterial therapeutics, here we designed a repolarization-gated cascade strategy to potentiate macrophage-mediated tumor immunotherapy and proposed a fluorescence/photoacoustic (FL/PA) bimodal self-reporting technology to in situ visualize the cell phenotype change and related intracellular signatures by engineering catalase (CAT)-expressing *Escherichia coli* (pE.coli) with an APE1-responsive DNA prodrug (Dp) and then tumor-targeting macrophage membranes (MM) (Figure 1a). After intravenous injection of the constructed pEDp@MM, the engineered bacteria could actively migrate to the TME according to homing effect of MM [31,32] to induce M1 phenotypic transformation of macrophages and trigger both the nuclear-to-cytoplasmic translocation of APE1 and the elevation of *IL-6* mRNA expression (Figure 1b), which minimized OTOT toxicity. The translocated APE1 cleaved the AP sites of LR1 in Dp to release siSIRPα (a small-interfering RNA targeting SIRPα that was assembled by LR1 and R2), which silenced the immunosuppressive SIRPα gene to potentiate the phagocytic ability of M1-like macrophages via ICB. Meanwhile, the *IL-6* mRNA recognized the cleaved Dp to recover the Cy5 fluorescence of hairpin 2 (H2) for visualizing the cell phenotype change and siSIRPα release. In addition, the pE.coli could convert hydrogen peroxide into oxygen in TME due to the presence of expressed CAT, which alleviated hypoxia to promote the infiltration of immune cells and bacteria, and provided PA signal [33,34] for in situ monitoring of bacterial self-localization and immunotherapy. Furthermore, the exposed hybridization site of hairpin 1 (H1) could initiate *IL-6* mRNA triggered-catalytic hairpin assembly [35] (CHA) to amplify the fluorescence signal.

Therefore, this work enables phenotype-selective and dual-modal image-guided cancer immunotherapy.

2 Experimental

Mice used in this work were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine. The ethical review number was 202601A054. All experimental methods were in accordance with the Helsinki Declaration. BALB/c mice were purchased from Jiangsu Cavens Laboratory Animal Co., Ltd. (China). The animal model of 6-week-old BALB/c female mice was established by subcutaneous injection of 4T1 cell suspensions (2×10^6 cells) into the flank region of mice. The in vivo fluorescence and PA imaging were conducted when tumors grew to a capacity of ~ 120 mm³.

3 Results and discussion

3.1 Preparation and characterization of pEDp@MM

Firstly, bacteria were engineered to stably express CAT by introducing a plasmid encoding CAT gene into *Escherichia coli* BL21 (DE3) (*E. coli*) according to a previous report [36]. SDS-PAGE analysis of the obtained pE.coli revealed a distinct band at the expected molecular weight (~ 60 kDa), which increased upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction (Figure S1a), confirming successful CAT expression. To further verify the enzymatic activity of CAT expressed by pE.coli, 2×10^6 colony-forming units (CFU) of bacteria were incubated in 1 mL of medium containing 1% H₂O₂, which produced visible bubbles compared to the wild-type strain, indicating effective

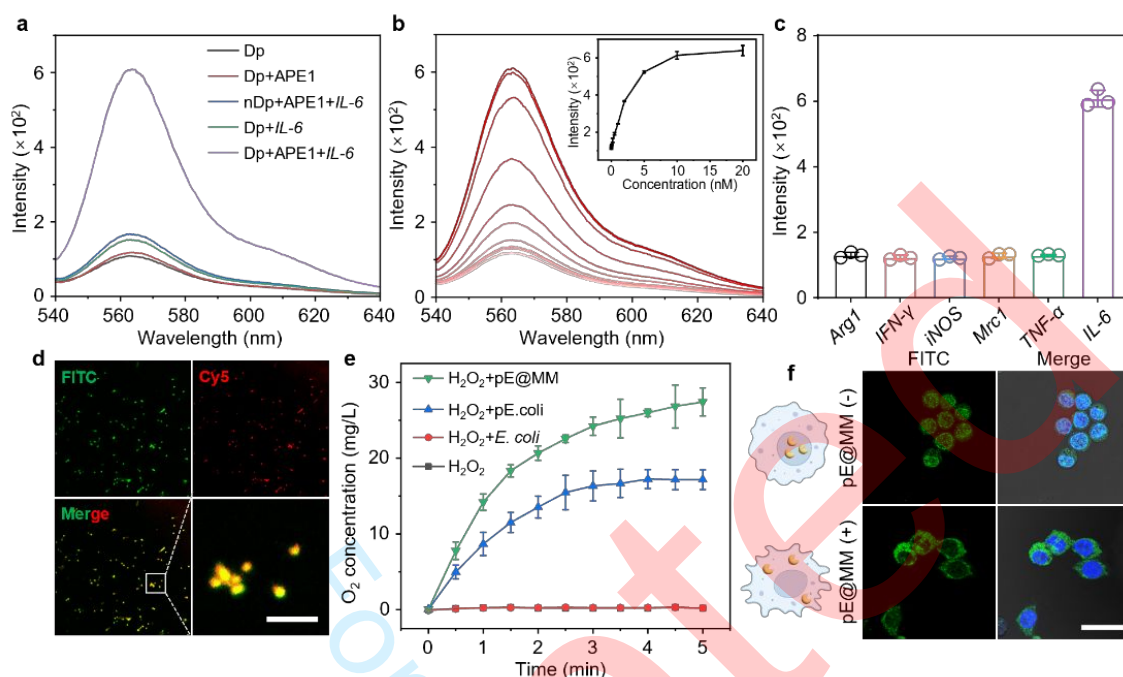


Figure 2 Characterization of sensing and repolarization ability of the engineered bacteria in vitro. (a) Fluorescence spectra of Dp or non-cleavable Dp (nDp, 50 nM) in response to addition of *IL-6* mRNA (20 nM) and/or APE1 (1 U/mL). (b) Fluorescence spectra of Dp in the presence of APE1 (1 U/mL) and *IL-6* mRNA with various concentrations. Inset: plot of fluorescence intensity at 563 nm vs *IL-6* mRNA concentration. $n = 3$, $\lambda_{\text{ex}} = 520$ nm. (c) Fluorescence intensity of Dp (50 nM) and APE1 (1 U/mL) at 563 nm in response to different mRNAs (20 nM). (d) CLSM images of FITC-bound pEDp without presence of BHQ3. Red, Cy5; green, FITC. Scale bar, 5 μm . (e) O_2 generation in 0.06% H_2O_2 containing *E. coli*, pE.coli or pE@MM (2×10^6 CFU/mL). $n = 3$. (f) Fluorescence imaging of APE1 in RAW 264.7 cells after incubated with pE@MM (10^8 CFU/mL) or PBS for 12 h. Green, FITC-labelled APE1; blue, Hoechst 33342. Scale bar, 25 μm .

catalysis of CAT to the reaction of H_2O_2 into O_2 (Figure S1b). Dp was constructed by assembling a linker (L), a pair of hairpins (labelled H1 and H2), and siSIRP α , where the toehold of H1 was sealed by LR1 containing an apurinic/apyrimidinic (AP) site. 12% polyacrylamide gel electrophoresis (PAGE) analysis (Figure S2) showed a clear mobility shift of Dp (Lane 3), verifying successful synthesis. Upon addition of APE1, a faster-migrating band and a new band at low molecular weight corresponding to free siRNA occurred (Lane 4), demonstrating effective cleavage of AP site to release siSIRP α .

IL-6 mRNA-activated CHA of cleaved Dp was evaluated in test tubes containing APE1 and Dp constructed with Cy3 and BHQ2 labelled H2 (Figure 2a). Both the Dp and its mixture with APE1 showed very low fluorescence signal due to the quenching of BHQ2 on Cy3. The mixture of *IL-6* mRNA with Dp also showed low Cy3 fluorescence, indicating that *IL-6* mRNA could not directly open H2 assembled in Dp due to the steric hindrance of bound siSIRP α , which was similar to the mixture of *IL-6* mRNA, APE1 and nDp without the presence of AP site. Therefore, the cleavage of AP sites by APE1 to expose the toehold of H1 for *IL-6* mRNA hybridization played a key role in fluorescence recovery, which resulted in greatly increased fluorescence signal as observed from the mixture of *IL-6* mRNA, APE1 and Dp. Interestingly, the recovered fluorescence became stronger with the increasing time and trended toward a stable value at about 60 min (Figure S3), implying the presence of CHA cycle, which also resulted in *IL-6* mRNA concentration-dependent fluorescence (Figure 2b) and a sensitive method for detection of *IL-6* mRNA concentration with a limit of detection of 97 nM (Figure S4). Moreover, Dp displayed high specificity toward *IL-6* mRNA over other macrophage mRNA markers, including arginase 1 (*Arg1*) mRNA, interferon-gamma (*IFN- γ*)

mRNA, inducible nitric oxide synthase (*iNOS*) mRNA, mannose receptor C-type 1 (*Mrc1*) mRNA, and tumor necrosis factor-alpha (*TNF- α*) mRNA (Figure 2c).

The anchoring of Dp onto pE.coli was achieved via a click reaction [37] between $-\text{N}_3$ and dibenzocyclooctyne (DBCO) (Figure 1a), which was verified by labelling H2 with only Cy5 and pE.coli with FITC. The confocal laser scanning microscopic (CLSM) images showed strong overlapped fluorescence of Cy5 and FITC (Figure 2d), confirming the successful anchoring. From the calibration curve (Figure S5), the average number of Dp on per attained pEDp was estimated to be about 3.3×10^5 (Figure S5b). To further examine the possibility of endogenous biomolecules in healthy tissues to nonspecifically activate the CHA cycle of pEDp, pEDp was incubated with normal mouse liver lysates, which did not result in obvious fluorescence enhancement compared with 1% BSA/PBS control (Figure S6), indicating that the basal levels of endogenous components in healthy tissues were insufficient to activate the prodrug. In contrast, simultaneous addition of *IL-6* mRNA and APE1 restored a strong fluorescence signal, demonstrating that the Dp-anchored bacteria remained responsive of *IL-6* mRNA and APE1 in a complex biological environment. After capsulated with MM, the average size of prepared pEDp@MM increased from 1.0 μm to ~ 1.5 μm (Figure S7a), and a surface zeta potential shift from -15.4 mV to -17.8 mV (Figure S7b). The hydrodynamic diameter of pEDp@MM did not significantly change after incubated in 0.01 M PBS (pH 7.4) for different times (Figure S8), indicating its good stability under physiological ionic conditions. Notably, the bacterial anchoring and MM capsulation protected the Dp from enzymatic degradation, which led to 80.7% of Dp remaining intact state after 48 h incubation in 10% fetal bovine serum (FBS) (Figure S9), far exceeding 40% of intact state for

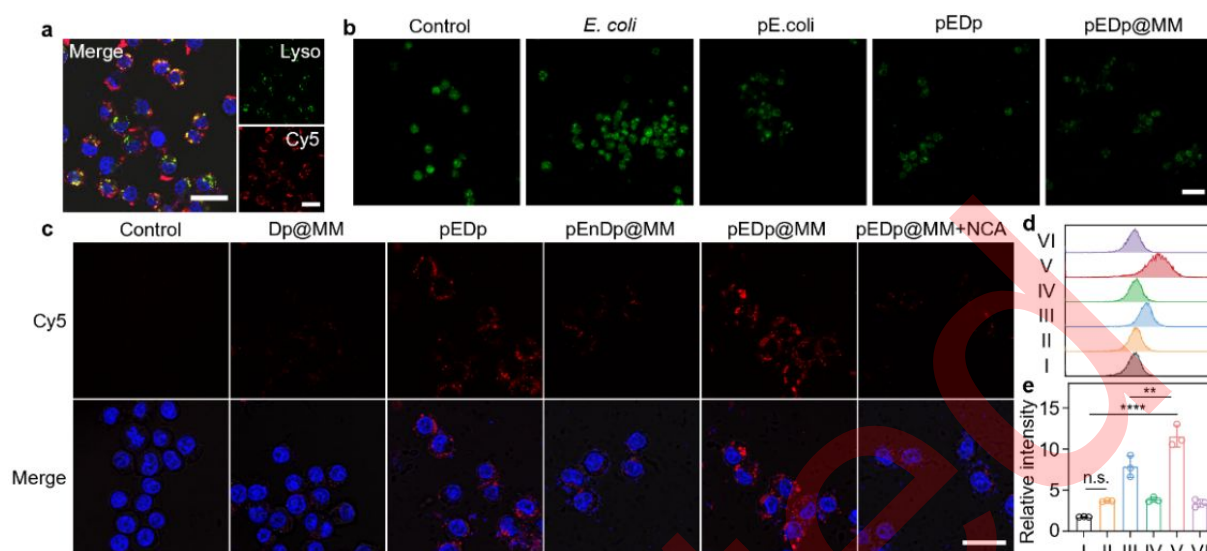


Figure 3 (Color online) Cellular uptake of pEDp@MM and APE1 triggered FL imaging of *IL-6* mRNA. (a) CLSM images of RAW 264.7 cells incubated with only Cy5 labelled pEDp@MM (10⁸ CFU/mL) at 37 °C for 12 h. Green, LysoTracker Green; red, Cy5; blue, Hoechst 33342. Scale bar, 25 μm. (b) CLSM images of oxygen levels in RAW 264.7 cells stained with [Ru(dpp)₃]Cl₂ as O₂ probe for 6 h and then treated with the marked formulations (10⁸ CFU/mL) for 4 h. Scale bar, 25 μm. (c) CLSM images of RAW 264.7 cells treated with marked formulations (I to VI, 10⁸ CFU/mL) for 12 h. Red, Cy5; blue, Hoechst 33342. Scale bar, 25 μm. (d) Flow cytometric analysis of the cells in (c). (e) Statistical Cy5 fluorescence intensities from (c). *n* = 3, ***p* < 0.01, *****p* < 0.0001.

naked DNA strands [38].

Furthermore, the engineering of p*E. coli* did not impair bacterial proliferation (Figure S10), and enhanced CAT activity possibly due to the presence of MM to facilitate substrate accessibility (Figure 2e). To assess the ability of pEDp@MM to repolarize macrophages toward an M1-like phenotype, MM and tumor cell membranes (CM) capsulated p*E. coli* (pE@MM and pE@CM) were prepared to incubate M0 and M2-like RAW 264.7 cells for 12 h and then extract the total RNA for qPCR analysis of *IL-6* mRNA, which revealed a significant upregulation of *IL-6* mRNA in pE@MM treated cells compared to p*E. coli* or pE@CM treated group, though the latter could also upregulate *IL-6* mRNA due to the intrinsic immunostimulatory properties of the bacteria (Figure S11). Notably, the stronger upregulation could be attributed to homologous targeting ability of MM [39]. Furthermore, pE@MM stimulation of RAW 264.7 cells triggered the translocation of APE1 from nucleus to cytoplasm (Figure 2f), demonstrating the promoting effect of pE@MM on repolarization of both M0 and M2-like macrophages toward an M1-like phenotype.

3.2 Cellular uptake of pEDp@MM by macrophages and APE1 triggered FL imaging of *IL-6* mRNA

To validate the feasibility of cellular uptake of pEDp@MM by macrophages, the cytotoxicity of pEDp@MM to RAW 264.7 cells was firstly evaluated. After incubated with pEDp@MM for 24 h, RAW 264.7 cells did not show obvious death even at a high concentration of 10⁹ CFU/mL (Figure S12), indicating negligible cytotoxicity of pEDp@MM. The RAW 264.7 cells exhibited time- and concentration-dependent behaviors after they were treated with only Cy5 labelled-pEDp@MM (Figures S13, S14), while Cy5 fluorescence primarily localized in the cytoplasm instead of lysosomes (Figure 3a), indicating the cellular uptake of pEDp@MM likely due to cell membrane fusion, which enabled direct cytosolic delivery and evaded lysosomal capture [40]. Interestingly, pEDp@MM could ameliorate hypoxia, which was

demonstrated with CLSM images of RAW 264.7 cells after culturing under hypoxic condition for 12 h, incubating with [Ru(dpp)₃]Cl₂ as O₂ probe [41] for 6 h, and then treating with different engineered bacteria (10⁸ CFU/mL) for 4 h. The decreased green fluorescence of [Ru(dpp)₃]Cl₂ indicated CAT mediated oxygen production (Figure 3b), which provided the possibility to perform PA imaging. After M0-like, M1-like, or M2-like RAW 264.7 cells were incubated with Dp or nDp for 4 h, only Dp treated M1-like RAW 264.7 cells showed strong Cy5 fluorescence (Figure S15), indicating high *IL-6* mRNA expression, which activated CHA of APE1 cleaved Dp similar to that in solution (Figure 2a). Notably, the M1-like RAW 264.7 cells treated with both nDp and the mixture of Dp and NCA (an APE1 inhibitor [42]) showed very low fluorescence signal, confirming the function of both translocated APE1 and *IL-6* mRNA to specifically trigger the fluorescence recovery of Cy5 in M1-like macrophages. This function was further demonstrated by incubating M0-like RAW 264.7 cells with pEDp@MM, which also showed the fluorescence recovery of Cy5 (Figure 3c, d). No obvious fluorescence was observed in Dp@MM treated group, indicating that the translocation of APE1 and upregulation of *IL-6* mRNA resulted from bacteria-induced M1 phenotype repolarization. Moreover, the presence of MM enhanced the fluorescence by approximately 1.47 folds due to its targeting effect (Figure 3e). Contrarily, pEnDp@MM and pEDp@MM+NCA treated groups showed weak fluorescence, further confirming the key role of APE1 in fluorescence recovery. Similar fluorescence changes were also observed during treatments of M2-like macrophages, indicating the effective repolarization capacity of the engineered bacteria toward both M0- and M2-like macrophages (Figure S16). Therefore, pEDp@MM provided an effective FL imaging protocol for in vitro monitoring *IL-6* mRNA in repolarization of macrophages.

3.3 Repolarization-gated potentiation of macrophages by pEDp@MM

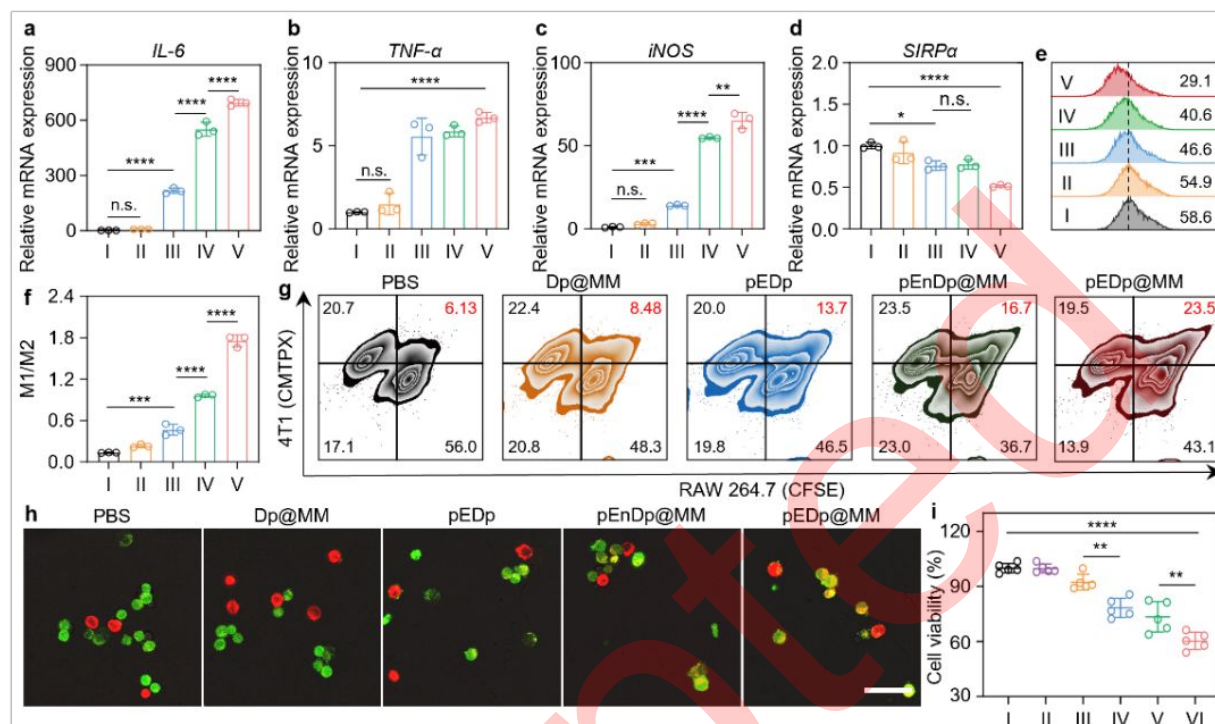


Figure 4 (Color online) Repolarization-gated phagocytosis potentiation of M0-like macrophages. (a-d) qRT-PCR analysis of *IL-6* (a), *TNF-α* (b), *iNOS* (c) and *SIRPα* (d) mRNA expression in RAW 264.7 cells treated with (I) PBS, (II) Dp@MM, (III) pEDp, (IV) pEnDp@MM and (V) pEDp@MM (10^8 CFU/mL) for 12 h. $n = 3$. (e) Flow cytometric analysis of *SIRPα* on RAW 264.7 cells after different treatments for 24 h. (f) Quantification of M1- ($CD86^+CD206^-$ cells) and M2-like ($CD86^+CD206^+$ cells) phenotypic proportions by flow cytometric analysis from Figure S16. (g) Flow cytometric analysis and (h) CLSM images of macrophage phagocytosis after different treatments. Green, CFSE-labelled RAW264.7 cells; red, CMTPX-labelled 4T1 cells. Scale bar, 50 μ m. (i) Cell viability of 4T1 cells after incubation with (I) DMEM or (II-VI) the supernatants of PBS (II), Dp@MM (III), pEDp (IV), pEnDp@MM (V) and pEDp@MM (VI) treated RAW 264.7 cells for 24 h. $n = 5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The repolarization potentiation effects of pEDp@MM were examined with both M0- and M2-like RAW 264.7 cells. After incubating them with PBS, Dp@MM, pEDp, pEnDp@MM or pEDp@MM for 12 h, qPCR analysis revealed gradual increase in mRNA markers of M1-like macrophages, including *IL-6*, *TNF-α* and *iNOS* mRNA (Figure 4a-c and Figure S17a-c), while *Mrc1* and *Arg1* mRNAs, the markers of M2-like macrophages, gradually declined (Figure S17d, e), indicating the repolarization of macrophages toward M1-like phenotype by these engineered bacteria. Except for pEDp@MM treated group, other cells retained high *SIRPα* levels (Figure 4d, e and Figure S17f, g), which suggested the existence of the immunosuppressive CD47–*SIRPα* axis that may promote immune tolerance, indicating the pEDp@MM-mediated ICB of macrophages for tumor immunotherapy. Upon pEDp@MM-mediated *SIRPα* silencing, macrophages exhibited significantly enhanced phagocytosis of the engineered bacteria (Figure S18) and resulted in the highest proportion of M1-like repolarization compared to the non-responsive groups (Figure 4f, Figure S17h, i and Figure S19). The phagocytosis of cancer cells by macrophages was evaluated by incubating M0-like RAW 264.7 cells with different engineered bacteria, and then labelling the cells with CFSE (green) to co-culture them with CMTPX-stained (red) 4T1 cancer cells. Compared to 6.13% for PBS-treated group, pEDp@MM treated group showed 23.5% CFSE⁺/CMTPX⁺ cells (Figure 4g), similar to that observed from CLSM images (Figure 4h). The phagocytosis was also observed in M2-like RAW 264.7 cells (Figure S17j) or bone marrow-derived macrophages (BMDMs) isolated from mice, even if BMDMs showed less increase (Figure S20) due to the fact that MM derived from RAW

264.7 cells possessed higher targeting efficiency toward homologous cell lines [43]. These results indicated the pEDp@MM potentiated phagocytosis ability of macrophages to cancer cells. Besides the phagocytosis, pEDp@MM mediated secretion of proinflammatory factors from macrophages could also enhance tumor immunotherapy [44]. After incubated with the supernatants of RAW 264.7 cells treated with different concentrations of pEDp@MM, 4T1, Hepa 1-6 and B16-F10 cancer cells, representing tumor models with varying macrophage infiltration levels [45], all exhibited a concentration-dependent reduction in viability, while untreated RAW 264.7 cells caused negligible viability change. These results suggested that the engineered bacteria effectively enhanced macrophage-mediated tumor cell killing across tumor models with different macrophage infiltration characteristics (Figures 4i, S21-S23).

3.4 FL/PA bimodal imaging of pEDp@MM-mediated tumor immunotherapy

After intravenous administration of pEDp@MM (2×10^8 CFU/mL, 100 μ L) for 36 h, 4T1 tumor-bearing mice showed robust FL signal at the tumor site, which reached the maximum at 72 h (Figure 5a, b), indicating the efficient nuclear-to-cytoplasmic translocation of APE1 and the elevated *IL-6* mRNA expression in TAMs to trigger the cleavage of AP site in anchored Dp and then CHA cycle of cleaved Dp. The mice treated with either pEnDp@MM or Dp@MM exhibited significantly lower FL signal at the tumor site after 72 h, which was attributed to the APE1 insensitivity of nDp and the lack of

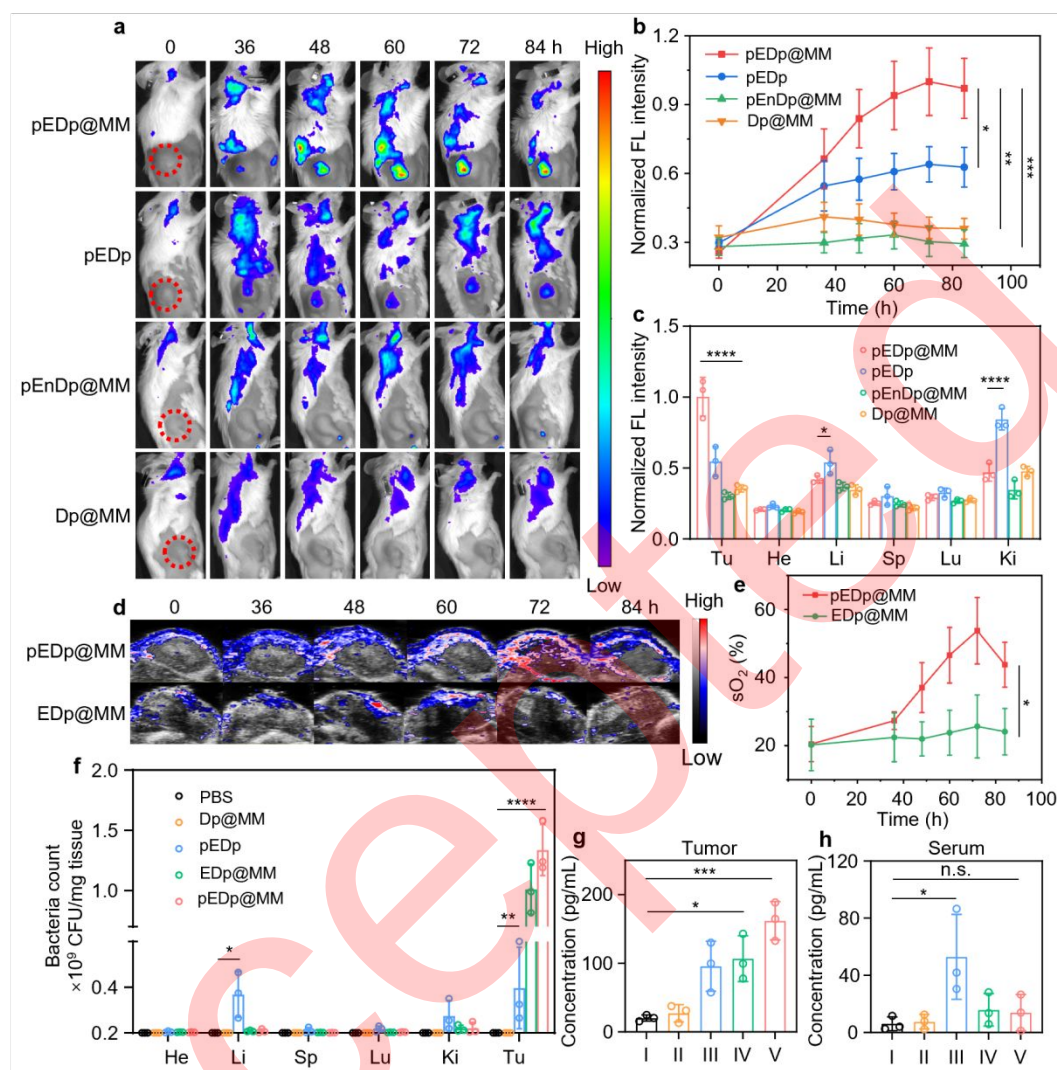


Figure 5 (Color online) FL and PA bimodal imaging of pEDp@MM-mediated tumor immunotherapy. (a) FL imaging of 4T1 tumor-bearing mice and (b) normalized FL intensity at tumor site after intravenous injection of pEDp@MM, pEDp, pEnDp@MM or Dp@MM ($100 \mu\text{L}$, 2×10^8 CFU/mL bacteria or 500 nM Dp@MM) for different times. (c) Normalized FL intensities in tumor (Tu) and heart (He), liver (Li), spleen (Sp), lung (Lu), and kidney (Ki) tissues at 84 h injection. $\lambda_{\text{ex/em}} = 640/680$ nm. (d) PA imaging and (e) corresponding quantification of tumor sO₂ recorded in oxy-hemo mode after intravenous injection of pEDp@MM or EDp@MM ($100 \mu\text{L}$, 2×10^8 CFU/mL) for different times. (f) Biodistribution of bacteria in major organs and tumor tissue from mice after injected with indicated formulations for 72 h. (g, h) ELISA quantification of IL-6 in tumor (g) and sera (h) of mice after intravenous injection of (I) PBS, (II) Dp@MM, (III) pEDp, (IV) pEnDp@MM and (V) pEDp@MM for 72 h. $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

bacterial stimulation, respectively. The FL intensity at the tumor site of pEDp treated group was 36% lower than that of pEDp@MM treated group demonstrated the tumor targeting of MM, which resulted in considerably higher FL intensity in tumor tissue and lower FL intensity in major organs, particularly the liver and kidney, dissected from pEDp@MM-treated mice than those from pEDp-treated mice (Figure 5c, Figure S24). Therefore, the targeting and repolarization ability of pEDp@MM to TAMs for tumor immunotherapy could be in situ visualized by FL imaging.

Given that excess H₂O₂ could be catalyzed by CAT to generate O₂, as demonstrated in cellular testing with pEDp@MM treatment (Figure 3b), the in situ PA monitoring of blood oxygen saturation (sO₂) alteration in the TME could be achieved upon the binding of O₂ to hemoglobin [46]. After intravenous injection of pEDp@MM, 4T1 tumor-bearing mice showed obvious increase of tumor sO₂ from an initial 20.5% to a peak value of 53.8% at 72 h (Figure 5d, e), consistent with the time showing the maximum FL imaging signal, which demonstrated the in situ

FL/PA bimodal imaging ability of pEDp@MM. The minor sO₂ fluctuations of EDp@MM treated group, ranging from 20.2% to 25.7%, could be attributed to inflammation-associated vascular and metabolic changes induced by bacterial stimuli. To further verify bacterial colonization at the tumor site, tumor tissue and major organs dissected from different groups after 72 h injection were homogenized for bacterial plate count analysis, which showed bacterial growth in pED, EDp@MM, and pEDp@MM treated groups, and 7 folds greater bacterial count of pEDp@MM treated group than pED treated group (Figure 5f and Figure S25). Moreover, pED treated group exhibited greater accumulation of pED in the liver and kidney, which correlated with the elevated systemic levels of proinflammatory factor IL-6 rather than localized tumor (Figure 5g, h).

3.5 Therapeutic efficacy of pEDp@MM on 4T1 tumors

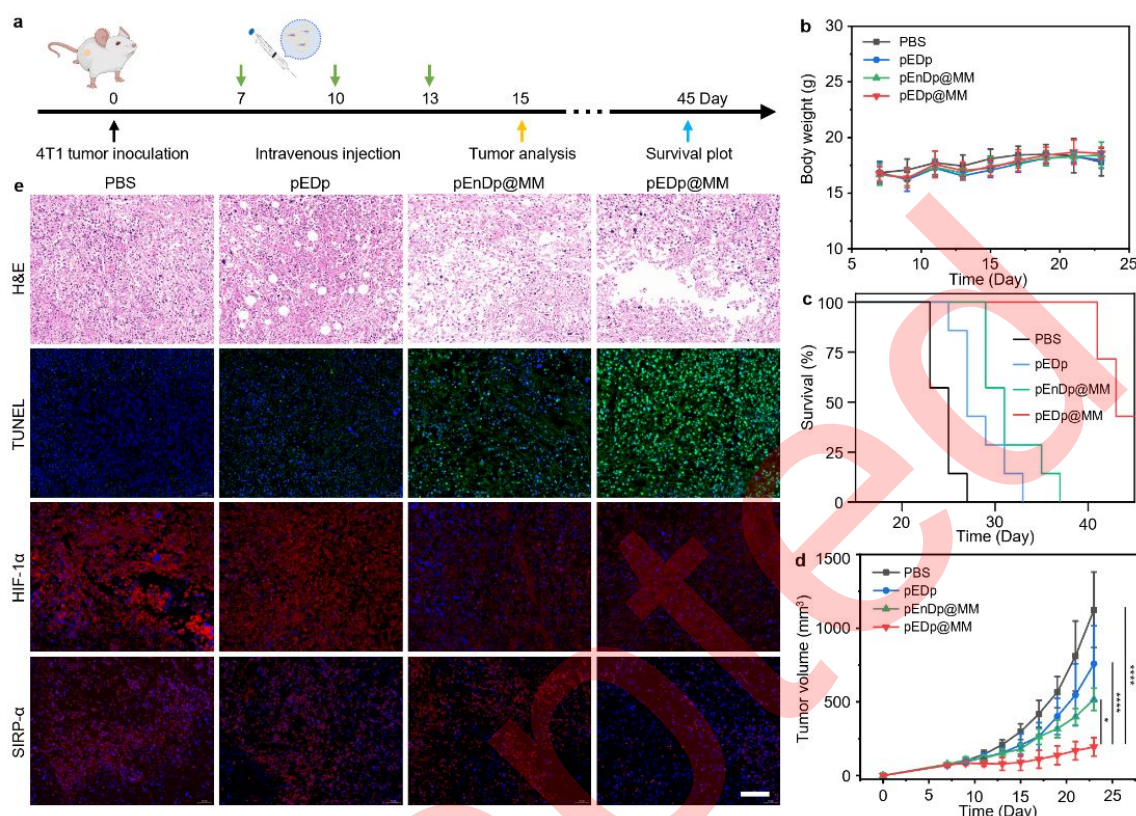


Figure 6 (Color online) In vivo antitumor efficiency of pEDp@MM on subcutaneous 4T1 tumor-bearing mice. (a) Schematic illustration of treatment process. (b-d) Body weight (b) and survival curves (c) of mice, and tumor growth (d) after i.v. injection with (I) PBS, (II) pEDp, (III) pEnDp@MM, or (IV) pEDp@MM (100 μ L, 2×10^8 CFU/mL), $n = 7$. * $p < 0.05$, *** $p < 0.0001$. (e) Tumor tissues from treated mice after H&E, TUNEL, HIF-1 α , and SIRP α staining. Scale bar: 100 μ m.

Based on the dual-modal imaging results, which showed the maximal bacterial accumulation and macrophage activation at 72 h after administration, a 3-day dosing interval was selected for therapeutic evaluation. 4T1 tumor-bearing BALB/c mice were randomized into 4 groups and intravenously injected with PBS, pEDp, pEnDp@MM, and pEDp@MM every 3 days for three times (Figure 6a). 48 h after the last injection (day 15) of diverse formulations, a subset of mice from each group were euthanized and their tumors and adjacent lymph nodes were harvested for different analyses.

Histological and biochemical analyses were firstly performed to evaluate the biosafety of pEDp@MM. Hematoxylin & eosin (H&E) staining of liver and kidney exhibited partial cell necrosis (indicated by yellow arrows) in mice treated with pEDp (Figure S26). In contrast, pEnDp@MM and pEDp@MM did not show obvious tissue damage or morphological alterations in major organs, indicating that the presence of MM effectively shielded bacterial pathogen-associated molecular patterns (PAMPs) during systemic circulation, thereby significantly reducing off-target tissue injury. Liver and renal functions were further evaluated by measuring the levels of alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CREA) in serum samples. All of them remained within normal ranges (Figure S27). Furthermore, the mice in pEDp@MM group exhibited minor body weight fluctuations in the treatment period (Figure 6b), indicating that the encapsulation process significantly enhanced biocompatibility, which also resulted in the longest median survival of over 40 days compared to the other groups (Figure 6c). The mice treated with pEDp@MM also exhibited the most prominent antitumor efficacy with maximum inhibition of tumor growth, while the

treatment with pEDp or pEnDp@MM induced a modest delay in tumor growth compared to saline control (Figure 6d and Figure S28), likely due to the immunostimulatory nature of bacteria. The limited antitumor efficacy was attributed to the lack of ICB compared to pEDp@MM group, suggesting that this immune activation was counteracted by the immunosuppressive TME. H&E and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining of resected tumor tissue slices confirmed that pEDp@MM treatment caused more significant tumor cell death than the other treatments, and profound remodeling of the immunosuppressive TME due to the reduced expression of SIRP α , accompanied by a marked alleviation of tumor hypoxia, as evidenced by the downregulation of the hypoxia marker hypoxia-inducible factor 1- α (HIF-1 α) (Figure 6e). These coordinated changes synergistically restored immune surveillance and enabled immune cells to exert more potent antitumor activity within TME.

3.6 Immunological evaluation of anti-tumor efficacy

The ability of pEDp@MM to repolarize TAMs and activate T cells was further investigated with 4T1 tumor-bearing BALB/c mice to elucidate the mechanisms underlying its anti-tumor efficacy by identifying different phenotypes of macrophages and T cells, such as M1-like macrophages (CD11b⁺F4/80⁺CD80⁺), M2-like macrophages (CD11b⁺F4/80⁺CD206⁺), cytotoxic T cells (CD3⁺CD8⁺), and helper T cells (CD3⁺CD4⁺), and quantifying the levels of immune cytokines (Figure 7).

Total macrophages were defined by co-expression of CD11b and F4/80, while T cells were defined by CD3 expression. Flow cytometric results revealed that pEDp treated mice (~5.9%) and

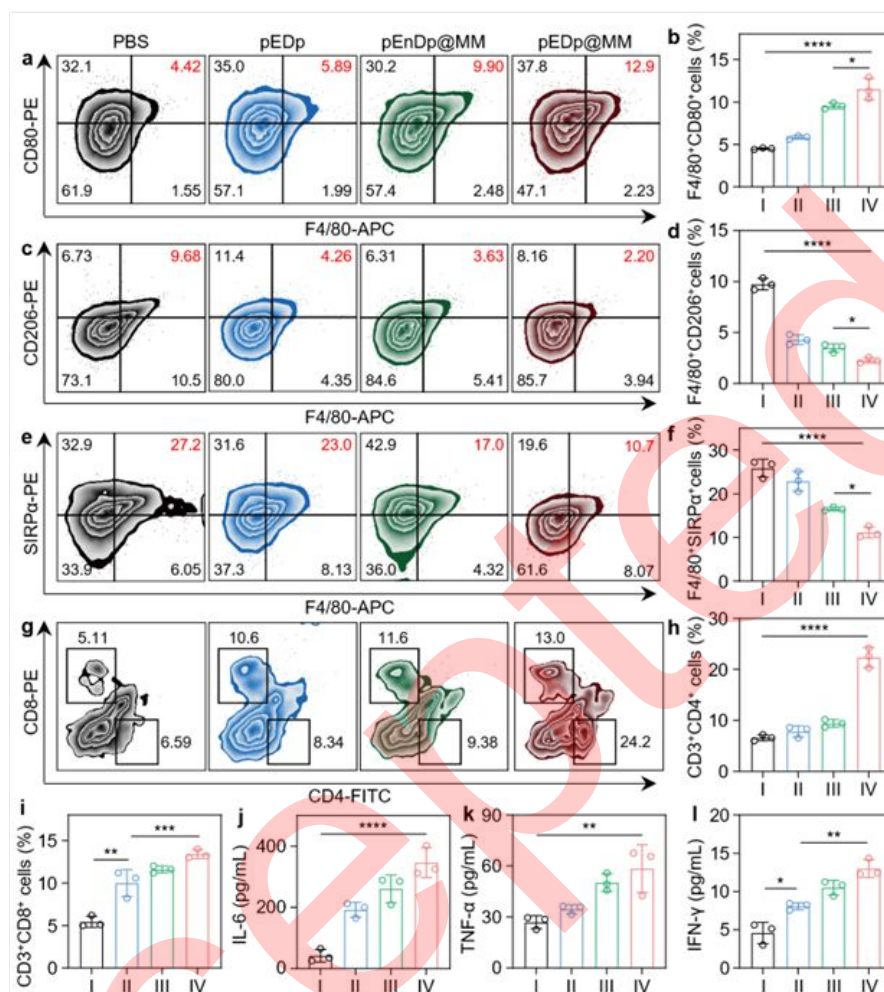


Figure 7 (Color online) Immune stimulation effect of pEDp@MM on subcutaneous 4T1 tumor-bearing mice. (a-f) Representative flow cytometric images and corresponding statistical analysis of M1-like (CD80⁺) (a, b), M2-like (CD206⁺) (c, d) and SIRPα⁺ (e, f) macrophages in the tumors of mice i.v. injected with (I) PBS, (II) pEDp, (III) pEnDp@MM, or (IV) pEDp@MM (100 μL, 2 × 10⁸ CFU/mL). (g-i) Representative flow cytometric images (g) and corresponding statistical analysis of the CD4⁺ (h) and CD8⁺ (i) T cells in the tumor tissues. (j-l) ELISA quantification of IL-6 (j), TNF-α (k) and IFN-γ (l) levels in tumor tissues. *n* = 3, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

pEnDp@MM treated mice (~9.9%) had a slight change in M1-like macrophages, whereas pEDp@MM treated group exhibited a marked increase of M1-like macrophages to 12.9%, compared to 4.4% in PBS treated tumors (Figure 7a, b). The proportion of M2-like macrophages decreased from 9.7% to 2.2% after treatment with pEDp@MM (Figure 7c, d), indicating an effective repolarization of TAMs toward M1 phenotype in TME. This finding was further corroborated by immunofluorescence staining of tumor sections, which showed increased CD86 expression and decreased CD206 expression (Figure S29). Notably, the expression of SIRPα on macrophages was concurrently reduced to 10.7% following treatment with pEDp@MM, compared to 17.0% of pEnDp@MM treated group (Figure 7e, f), which confirmed the repolarization-gated release of siSIRPα. In addition to phagocytosis, macrophages also acted as antigen-presenting cells to secrete immunomodulatory cytokines, thereby potentiating T cell-mediated anti-tumor immunity, which was demonstrated by the most significant increase in the proportions of both CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in the tumor tissues and the draining lymph nodes occurring in mice after treated with pEDp@MM (Figure 7g-i and Figure S30). In addition, the maximum increasing levels of immune cytokines, such as IL-6, TNF-α, and IFN-γ, in tumor tissues upon

pEDp@MM treatment (Figure 7j-l) further confirmed the robust antitumor immune response upon macrophage repolarization.

4 Conclusions

In summary, we present a repolarization-gated cascade strategy with engineered oncolytic bacteria (pEDp@MM) to potentiate macrophage-mediated immunotherapy and achieve FL/PA bimodal imaging for in situ visualization of cell phenotype change and related intracellular signatures. The pEDp@MM are ingeniously constructed through anchoring APE1-responsive Dp onto CAT-expressed *E. coli*, followed by encapsulation with tumor-targeting MM. After targeting tumor, pEDp@MM repolarize TAMs to M1 phenotype, which leads to APE1 translocation and IL-6 mRNA increase to trigger the release of siSIRPα from Dp and IL-6 mRNA-mediated fluorescence signal amplification. The released siSIRPα promotes ICB of immunosuppressive SIRPα-CD47 axis to potentiate the phagocytosis of cancer cells by macrophages. Meanwhile, PA imaging can be achieved by CAT-catalyzed oxygen generation for bacterial self-monitoring, which also alleviates tumor hypoxia for the infiltration of immune cells and bacteria. The repolarization-gated strategy that uses dynamically activated molecules within immune cells as switches to control ICB drug

release with real-time immune monitoring has been successfully validated in a 4T1 tumor-bearing mouse model. Future studies involving larger experimental cohorts and additional tumor models may further validate the versatility of this strategy. Moreover, the incorporation of patient-derived autologous macrophage membranes for personalized immunotherapy and the feedback capability of the theranostic system for image-guided optimization of the combination of bacterial therapy with other therapeutic modalities may further improve therapeutic efficacy. Overall, the engineered oncolytic bacteria provide an avenue for on-demand immunotherapy with imaging-feedback and minimization of OTOT toxicity.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

The supporting information is available online at <http://chem.scichina.com> and <http://link.springer.com/journal/11426>. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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