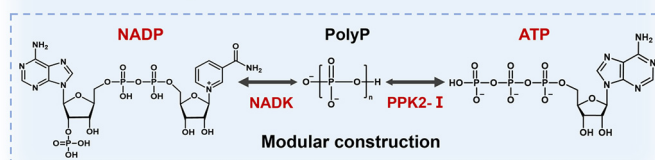
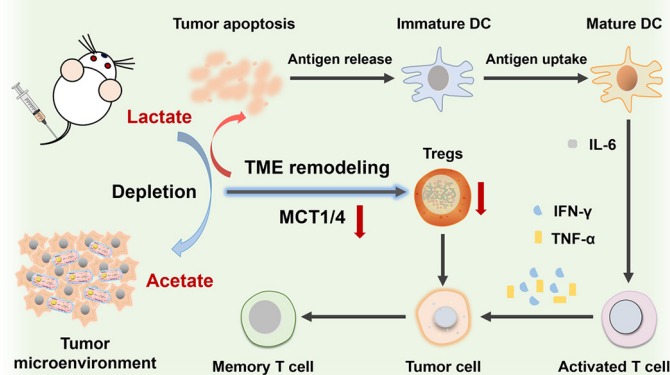


Research Article

Reprogramming bacterial energetics reverses lactate-mediated immunosuppression for synergistic cancer immunotherapy

(A) Construction of engineered *S. oneidensis*

(B) Immune activation therapy



Lactate accumulation drives tumor immunosuppression. We engineered *Shewanella oneidensis* MR-1 with polyphosphate-driven energy rewiring to enhance lactate clearance, boost antigen presentation and T cell infiltration, suppress regulatory T cells, and synergize with α PD-1—providing a bioenergetic strategy for metabolic cancer immunotherapy.

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Highlights

Engineered *Shewanella oneidensis* MR-1 enhances ATP and NADH/NAD⁺ production to improve tumor lactate clearance.

Reprogrammed bacteria exhibit prolonged intratumoral persistence, accelerate lactate depletion, and suppress lactate transport via downregulation of monocarboxylate transporters MCT1 and MCT4.

Lactate depletion reverses immunosuppression, enhances antigen presentation and CD8⁺ T cell infiltration, and reduces regulatory T cells.

Combination with anti-programmed cell death protein 1 (α PD-1) immunotherapy promotes tumor eradication and establishes durable immune memory.

This bioenergetic engineering strategy bridges bacterial therapy and metabolic immunotherapy.

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Research Article

Reprogramming bacterial energetics reverses lactate-mediated immunosuppression for synergistic cancer immunotherapy

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The immunosuppressive tumor microenvironment (TME), driven by lactate accumulation, critically limits cancer immunotherapy efficacy. In this research article, we engineered *Shewanella oneidensis* (*S. oneidensis*) MR-1 by reprogramming energy metabolism to reverse lactate-driven immunosuppression in the TME. Integration of polyphosphate kinase 2 and NAD kinase markedly augmented bacterial bioenergetics, boosting ATP production by 287.5% and elevating the NADH/NAD⁺ ratio by 299.9% compared with the 'wild-type' (WT) strain. This bioenergetic enhancement accelerated targeted lactate depletion and increased intratumoral bacterial persistence, while impairing tumor lactate transport via downregulation of monocarboxylate transporters MCT1 and MCT4. Crucially, metabolic remodeling reversed immunosuppression by enhancing antigen presentation and CD8⁺ T cell infiltration, and reducing regulatory T cells, thereby converting immunologically 'cold' tumors into immunoreactive phenotypes. When combined with anti-programmed cell death protein 1 (αPD-1) immunotherapy, this strategy synergistically amplified antitumor efficacy and established durable immunological memory against recurrence. Collectively, our work establishes a paradigm for bacterial bioenergetic engineering to advance cancer immunotherapy.

Introduction

Cancer immunotherapy has transformed oncology, yet its clinical efficacy remains constrained by the immunosuppressive **tumor microenvironment (TME)** (see [Glossary](#) [1,2]. Excessive lactate accumulation within the TME drives immunosuppression through multifaceted mechanisms, including suppressing cytotoxic T cell function, promoting regulatory T cell (Treg) expansion, and impairing antigen presentation [3–5]. Beyond its role as a metabolic byproduct, lactate functions as a potent immunosuppressant through multiple mechanisms. It drives epigenetic alterations, disrupts mitochondrial energetics, subverts immune cell function to facilitate tumor evasion, and promotes stromal fibrosis [6–9].

Current strategies targeting lactate dynamics, such as small-molecule monocarboxylate transporter (MCT1/4) inhibitors and siRNA-mediated suppression, have demonstrated promising antitumor effects in preclinical models by disrupting lactate shuttling [10–12]. However, their clinical translation faces persistent challenges: limited target specificity can lead to off-target toxicity; rapid clearance restricts sustained activity within the TME; and heterogeneous biodistribution hinders spatially precise modulation. These constraints highlight an unmet need for precisely

Technology readiness

We engineered *Shewanella oneidensis* (*S. oneidensis*) MR-1 to coexpress polyphosphate kinase 2 and NAD kinase, generating a bioenergetically enhanced strain that depletes lactate, downregulates MCT1/4, and remodels the immunosuppressive tumor microenvironment. The engineered strain showed antitumor activity as monotherapy and in combination with anti-programmed cell death protein 1 (αPD-1), with durable immunity. The current technology readiness level is 3–4: proof of concept has been established in a single syngeneic murine model under controlled laboratory conditions. Clinical translation requires fail-safe biocontainment, spatiotemporal control of bacterial activity, optimized dosing/combination regimens, scalable Good Manufacturing Practice (GMP) manufacturing, and long-term safety data to meet regulatory requirements for live biotherapeutic products.

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targeted, self-renewing lactate depletion systems capable of synergizing with established immunotherapies.

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Bacterial therapy shows unique advantages in cancer treatment, including intrinsic tumor tropism and favorable biosafety profiles [13–15]. By enabling localized lactate catabolism directly within tumors, they offer a paradigm-shifting alternative [5,16–18]. *Shewanella oneidensis* (*S. oneidensis*) MR-1 is a uniquely suited chassis for this purpose, leveraging its bidirectional **extracellular electron transfer (EET)** system to oxidize lactate into immunostimulatory metabolites independent of synthetic nanocomponents or oxygen constraints [19–21]. Nevertheless, prior engineering efforts failed to address a fundamental bottleneck: insufficient ATP flux and redox imbalances that severely limit heterologous pathway efficiency and TME-remodeling capacity [22,23].

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To overcome this bioenergetic limitation, we harnessed **polyphosphate (polyP)**, an evolutionarily conserved and ubiquitous energy currency in bacteria, as a metabolic driver [24–26]. Through synthetic coexpression of polyphosphate kinase 2 (PPK2-I) and NAD kinase (NADK), we rationally rewired the core metabolism of *S. oneidensis* to establish a self-sustaining energy regeneration system. This engineered strain drives a dual lactate-targeted therapeutic strategy: (i) accelerated lactate depletion coupled with enhanced intratumoral persistence and (ii) suppression of tumor lactate transport via downregulation of MCT1/4. When combined with **anti-programmed cell death protein 1 (αPD-1)**, our approach reverses immunosuppressive networks and triggers durable antitumor immunity.

Results

Abductive learning-guided prediction of polyP-mediated energy metabolism in *S. oneidensis*

We first characterized endogenous polyP biosynthesis in *S. oneidensis* MR-1 by identifying its polyphosphate kinase 1 (PPK1) system through comparative genomics. Sequence analysis revealed a chimeric *ppkA* locus (3120 bp) comprising three functional domains: ISSod11 transposase *tnpA* (957 bp), N-terminal *ppkA-N* (492 bp), and C-terminal *ppkA-C* (1671 bp) (Figure S1A in the Supplemental information online). Phylogenetic analysis clustered *ppkA* within the *ppk1* clade, showing high similarity to orthologs from diverse bacteria (Figure S1B). To explore the function of these domains, we generated two engineered strains: SPPKA1 (overexpressing *ppkA-N*) and STNPA (overexpressing *tnpA*) (the *ppkA-C* fragment lacks a complete open reading frame in the native genome). Compared with ‘WT’ strain, SPPKA1 showed a 33.7% increase in intracellular polyP content with a broader distribution, coupled with a 70.4% decrease in ATP levels, whereas STNPA displayed a 47.0% decrease in polyP content and a 173.5% increase in ATP levels (Figure S1C,D). These results collectively establish *ppkA-N* as the catalytic core responsible for polyP synthesis.

To predict polyP-mediated metabolic regulation, we developed a neuro-symbolic framework based on **abductive learning (ABL)** tailored for *S. oneidensis* MR-1 (Figure 1A) [27,28]. This model simulated bioenergetic alterations induced by the expression of polyP metabolic enzymes and predicted significant upregulation of genes involved in NAD⁺ synthesis (*fdhD*, *fdhE*), lactate metabolism (*crp*, *cyaC*), and cytochrome biogenesis (*mtrB/C/F*, *omcA*) (Figure 1B–D and Figure S2 in the Supplemental information online). These predictions provided a critical theoretical foundation for our subsequent polyP-driven metabolic engineering in *S. oneidensis* MR-1.

Engineering *S. oneidensis* with polyP-utilizing enzymes to enhance bioenergetics

We next engineered *S. oneidensis* to augment energy metabolism through heterologous expression of polyP metabolic enzymes, all driven by the ribosome binding site (RBS)-B0034 (Figure 1E

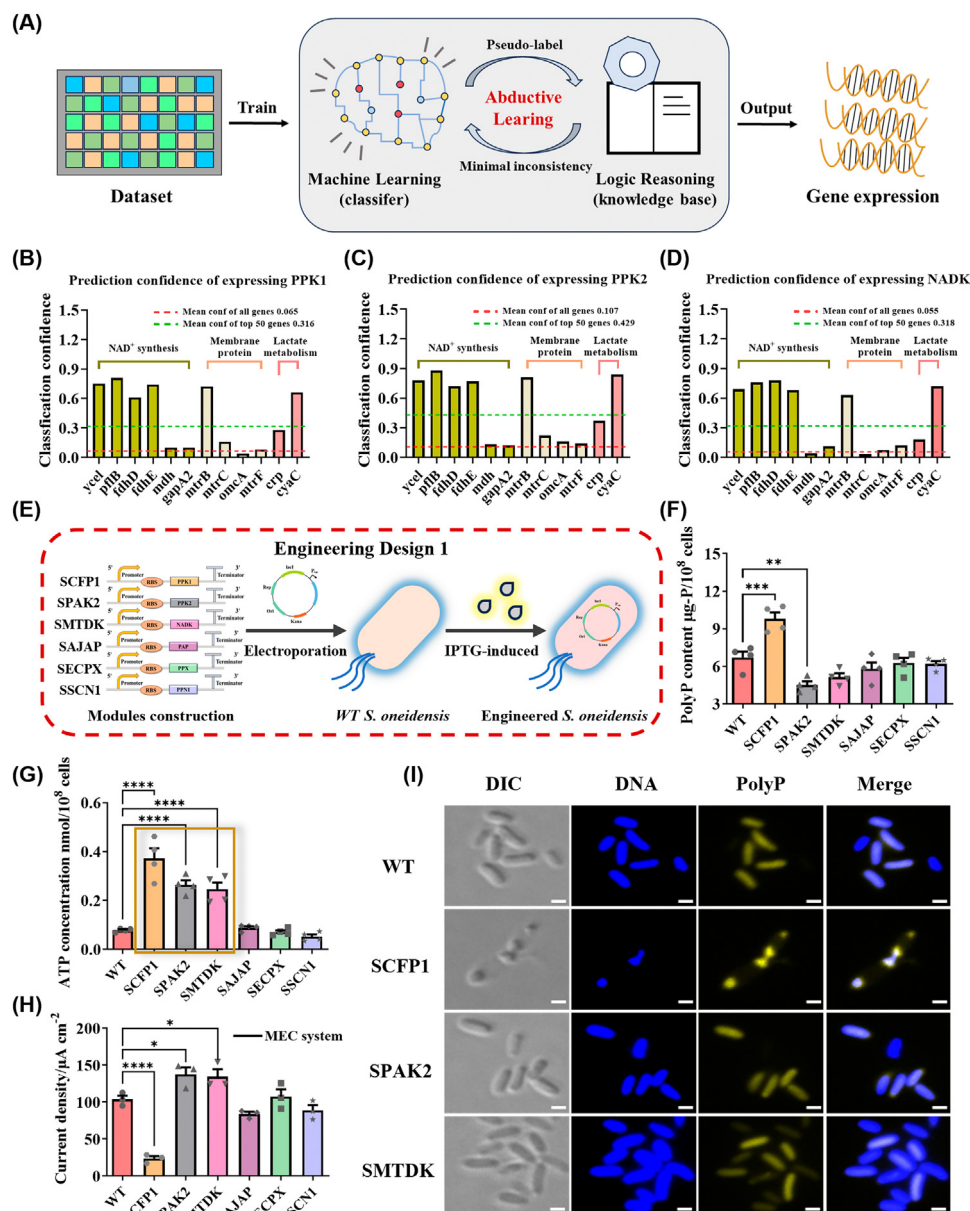


Figure 1. Prediction and construction of polyP metabolic enzymes in *S. oneidensis* MR-1. To investigate whether polyP can regulate the bioenergetic metabolism of *S. oneidensis*, we developed an ABL model to predict its physiological functions (A). Based on the predictions (B–D), we constructed engineered strains expressing polyP metabolic enzymes for experimental validation (E). The resulting data confirmed that ABL predictions were consistent with the observed changes in polyP/ATP levels and electrochemical performance (F–I), demonstrating that modulating polyP levels can effectively enhance the bioenergetic metabolism of *S. oneidensis*. (A) Schematic representation of the gene expression prediction model integrating Gene Ontology and ABL. (B–D) ABL model-predicted confidence scores for the expression of PPK1, PPK2-I, and NADK in *S. oneidensis* MR-1. (E) Schematic diagram of the modular design of polyP metabolic enzymes in *S. oneidensis* MR-1. (F and G) Intracellular polyP and ATP levels in WT and engineered *S. oneidensis* strains ($n = 4$). (H) Maximum output current densities generated by WT and engineered *S. oneidensis* strains in the microbial electrochemical cell (MEC) system ($n = 3$). (I) Fluorescence microscopy images of strains captured under different fluorescence channels. Scale bar: 1 μm . All data are presented as mean $\pm$ standard error of the mean (SEM).

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Glossary

Abductive Learning (ABL): a neuro-symbolic learning paradigm that combines machine learning with logical reasoning; it leverages both data-driven model predictions and background knowledge (e.g., Gene Ontology) to infer plausible explanations for observed phenomena and outputs confidence scores.

Anti-programmed cell death protein 1 ($\alpha\text{PD-1}$): a monoclonal antibody that blocks the programmed cell death protein 1 (PD-1) immune checkpoint receptor, thereby restoring the function of tumor-infiltrating T cells and enhancing antitumor immune responses.

Extracellular electron transfer

(EET): a process by which certain bacteria (e.g., *S. oneidensis*) transfer metabolically generated electrons to external solid acceptors, such as electrodes or minerals, enabling bio-electrochemical energy conversion.

Pathogen-associated molecular patterns (PAMPs):

a conserved molecular structure on pathogens, such as lipopolysaccharide or flagellin, that are recognized by host pattern recognition receptors to trigger innate immune responses.

Polyphosphate (polyP): a biopolymer formed by multiple orthophosphate units linked through high-energy phosphoanhydride bonds; it occurs naturally in living organisms and can also be synthesized artificially for industrial and medical applications.

Tumor microenvironment (TME): the local environment in which a tumor resides, including cancer cells, surrounding supporting cells, extracellular matrix, blood vessels, immune cells, and signaling molecules; it is dynamic and complex, influencing tumor initiation, progression, and metastasis.

and Table S1 in the Supplemental information online). PolyP quantification confirmed successful enzyme activity in three engineered strains: SCFP1 (expressing *Citrobacter freundii* PPK1) [29], SPAK2 (expressing *Pseudomonas aeruginosa* PPK2-I) [30], and SMTDK (expressing *Mycobacterium tuberculosis* NADK) [31] (Figure 1F). Consistent with their distinct catalytic functions, PPK1 primarily synthesizes polyP, whereas PPK2-I and NADK promote polyP utilization for energy cofactor regeneration. These engineered strains exhibited markedly elevated ATP levels (376.6, 238.2, and 215.3% higher than WT, respectively), which aligned with computational predictions that PPK1/PPK2-I/NADK-mediated regulation enhances energy cofactor synthesis (Figure 1G).

However, enhanced bioenergetic output did not uniformly improve EET efficiency. Electrochemical half-cell analysis revealed divergent EET phenotypes: SPAK2 and SMTDK showed 32.6 and 29.8% increases in maximum currents, whereas SCFP1 exhibited a 77.2% reduction (Figure 1H). We attribute this discrepancy to polyP overaccumulation burden, as evidenced by growth impairment and yellowish discoloration in SCFP1 (Figure S3 in the Supplemental information online). Fluorescence microscopy revealed polyP and DNA colocalization within phase-separated aggregates, and material migration was further corroborated through high-angle annular dark-field scanning transmission electron microscopy-energy dispersive spectroscopy (HAADF-STEM-EDS) and Bio-TEM mapping (Figure 1I and Figure S4 in the Supplemental information online). Transcriptomics further demonstrated downregulation of genes involved in electron transport (formate dehydrogenase activity and heme/quinone binding), amino acid metabolism, the tricarboxylic acid (TCA) cycle, and pyruvate metabolism (Figure S5 in the Supplemental information online). These results establish PPK1-mediated polyP overaccumulation as a multifaceted metabolic disruptor causing redox imbalance, consistent with growth-metabolism decoupling in *Escherichia coli* (*E. coli*) [32,33].

To balance bioenergetic flux and prevent polyP overaccumulation, we engineered three dual-enzyme coexpression systems: (i) PPK1 (RBS-B0031 tuned) with PPK2-I (RBS-B0034 tuned), (ii) PPK1 (RBS-B0031 tuned) with NADK (RBS-B0034 tuned), and (iii) PPK2-I (RBS-B0034 tuned) with NADK (RBS-B0034 tuned) (Figure 2A). Quantitative analysis uncovered distinct polyP profiles among these strains: polyP levels rose by 67.8% in SPK1-2 (PPK1 + PPK2-I) and 38.2% in SPK1DK (PPK1 + NADK), while decreasing by 32.2% in SPK2DK⁺ (PPK2-I + NADK) (Figure 2B). Concurrently, ATP content was significantly elevated by 277.7% (SPK1-2), 279.8% (SPK1DK), and 287.5% (SPK2DK⁺) (Figure 2C). Notably, only SPK2DK⁺ exhibited 54.4% higher current output than WT, contrasting with SPK1-2's 40.3% reduction and SPK1DK's 35.0% reduction—findings that underscore metabolic imbalance induced by polyP accumulation (Figure 2D). Importantly, PPK2-I and NADK coexpression maintained full biocompatibility, with no signs of DNA condensation or protein aggregation (Figure S6 in the Supplemental information online), validating its suitability for synthetic biology applications.

Mechanism underlying enhanced energy metabolism in engineered *S. oneidensis*

To elucidate the molecular mechanism driving bioenergetic enhancement in the SPK2DK⁺ strain, we performed an integrated analysis of the SPK2DK⁺ strain alongside the single-enzyme controls, SCFP1 and SPAK2. Given the pivotal role of the NAD⁺/NADH pool in cellular redox balance and the ability of NADK to convert NAD⁺ to NADP⁺ using polyP-derived phosphate [19,34], we hypothesized that coordinated action of PPK2-I and NADK would expand intracellular

One-way analysis of variance (ANOVA) was used, followed by Dunnett's multiple comparisons test for comparisons against the WT group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. DIC: differential interference contrast; IPTG: isopropyl β-D-1-thiogalactopyranoside.

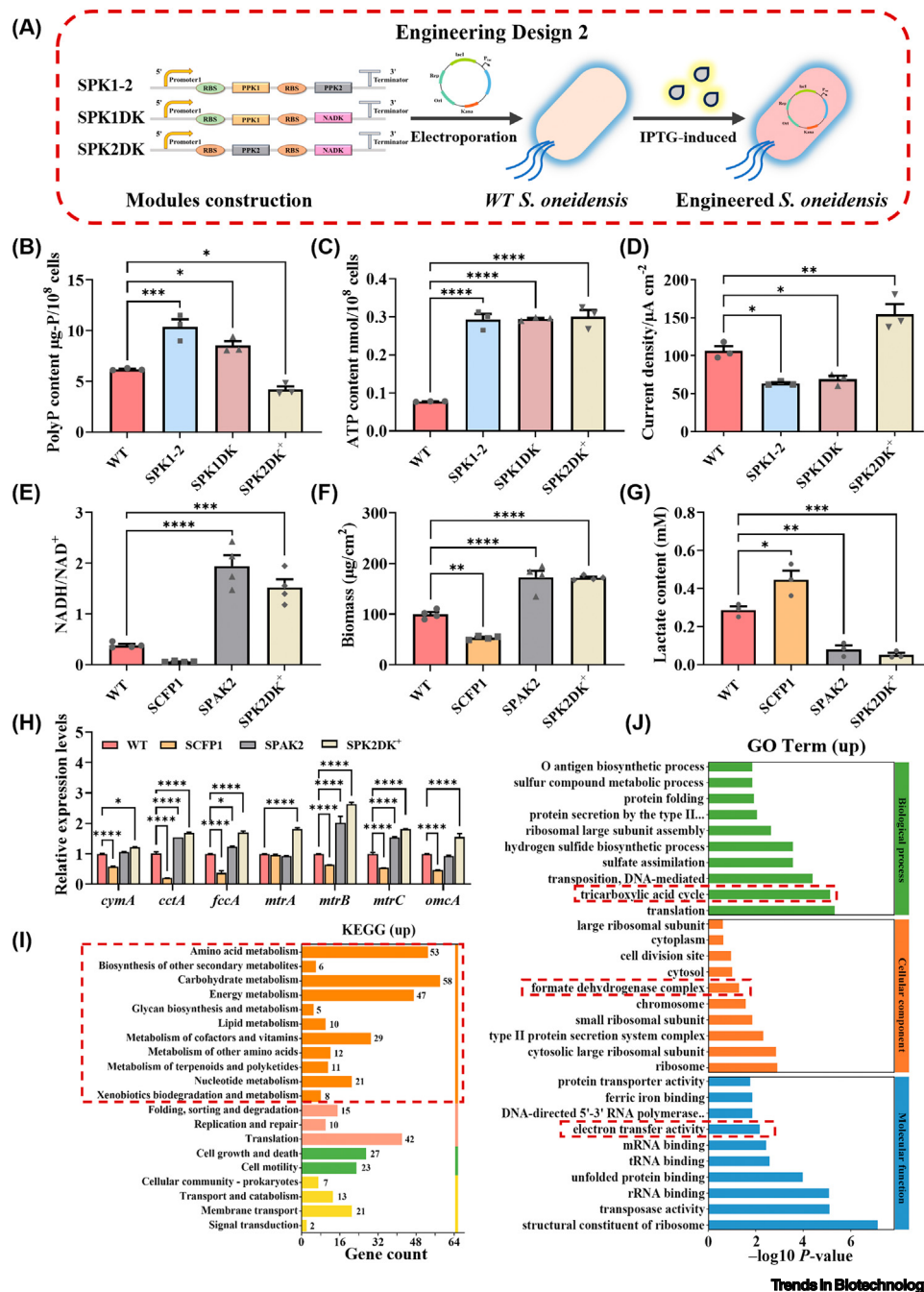


Figure 2. Optimization and functional characterization of engineered *S. oneidensis* for enhanced bioenergetics. To further enhance the bioenergetic metabolism of *S. oneidensis*, we constructed dual-gene coexpression systems for polyP metabolic enzymes (A). The engineered strain with significantly improved bioenergetics (SPK2DK⁺) exhibited increased ATP levels, higher NADH/NAD⁺ ratios, enhanced maximum output current densities, biofilm formation and lactate consumption (B–G), and upregulation of c-type cytochrome genes (c-Cyts) (H). Moreover, transcriptomic analyses revealed global metabolic reprogramming, with upregulation of key pathways involved in energy metabolism (I and J). (A) Schematic illustration of dual-enzyme coexpression strategies for polyP metabolic enzymes. (B and C) Intracellular polyP and ATP levels in WT and engineered *S. oneidensis* strains (*n* = 3). (D) Maximum output current

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reducing equivalents. Biochemical assays confirmed this: relative to the WT strain, SPAK2 and SPK2DK⁺ strains exhibited elevated NADH/NAD⁺ ratios (with a 411.8% and a 299.9% increase, respectively), increased NADH abundance and NADP⁺ biosynthetic capacity, and reduced NAD⁺ levels, indicating a shift toward a more reduced intracellular redox state (Figure 2E and Figure S7 in the Supplemental information online). Functionally, this redox remodeling was associated with the upregulation of genes involved in central carbon metabolism and lactate transport (Figure S8 in the Supplemental information online), which aligned with the observed phenotypic improvements, including enhanced lactate utilization, biofilm formation, and respiratory chain maturation (Figure 2F–H and Figure S9 in the Supplemental information online).

Integrated transcriptomics and metabolomic analyses revealed extensive metabolic reprogramming in SPK2DK⁺ strain. Principal component analysis clearly separated WT and SPK2DK⁺ groups (Figure S10A in the Supplemental information online), with 728 genes upregulated and 491 downregulated in response to polyP hydrolysis (Figure S10B). Differentially expressed genes spanned key pathways, including electron transport (e.g., formate dehydrogenase complex), the TCA cycle, ribosomal translation, and mRNA binding (Figure 2I,J). Metabolomics profiling further demonstrated enhanced flux through oxidative phosphorylation, two-component signaling, and pyruvate metabolism in the SPK2DK⁺ strain (Figure S10C), alongside the accumulation of amino acids, phosphate metabolites, and reducing power mediators such as nicotinic acid (Figure S10D). Collectively, these findings establish SPK2DK⁺ as a metabolically optimized chassis for bioenergy applications.

In vitro antitumor activity by engineered *S. oneidensis*

We next evaluated the therapeutic potential of the engineered *S. oneidensis* strain SPK2DK⁺. Cytotoxicity assays confirmed its selective tumor-killing activity *in vitro*, which was most pronounced against CT26 cells, with no significant cytotoxicity observed in other cell types (Figure 3A and Figure S11 in the Supplemental information online). Fluorescence microscopy revealed more severe membrane disruption in CT26 cells after treatment with SPK2DK⁺ compared with the WT strain (Figure 3B). This differential cytotoxicity likely stems from tumor-type-specific reliance on lactate metabolism: SPK2DK⁺ induced a pronounced reduction in extracellular lactate specifically in CT26 cocultures, but not in 4T1 cocultures (Figure 3C), consistent with known metabolic heterogeneity between tumor types [35,36]. Supporting this notion, supplementation with 10 mM exogenous lactate upregulated the lactate transporters MCT1 and MCT4 in CT26 cells [11,37] (Figure S12 in the Supplemental information online). Taken together, these findings suggest that CT26 cells are more sensitive to lactate depletion and that SPK2DK⁺ preferentially targets these cells by leveraging this metabolic vulnerability.

Given the immunosuppressive role of lactate [6,12,38], we then investigated whether SPK2DK⁺ could promote macrophage polarization toward an antitumor phenotype. To distinguish lactate-specific effects from general **pathogen-associated molecular pattern (PAMP)**-driven activation, we included an *E. coli* TOP10 carrying an empty vector in a transwell coculture

densities generated by WT and engineered *S. oneidensis* strains in a MEC system ($n = 3$). (E) Quantification of intracellular NADH/NAD⁺ ratios of the WT and the engineered strains ($n = 4$). (F) Biofilm biomass on carbon cloth anode ($n = 4$). (G) Quantitative measurements of lactate content in the supernatant after completion of the MEC reaction ($n = 3$). (H) The relative expression levels of *c*-type cytochromes (*c*-Cyts)-related genes of the WT and the engineered strains ($n = 3$). (I) KEGG pathway enrichment analysis of upregulated genes in SPK2DK⁺ compared with WT. (J) GO enrichment analysis of the top 30 genes with significant enrichment in SPK2DK⁺ compared with WT. All data are presented as mean $\pm$ SEM. One-way or two-way ANOVA was used as appropriate, followed by Dunnett's multiple comparisons test for comparisons against the WT group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. GO: Gene Ontology; IPTG: isopropyl β -D-1-thiogalactopyranoside; KEGG: Kyoto Encyclopedia of Genes and Genomes.

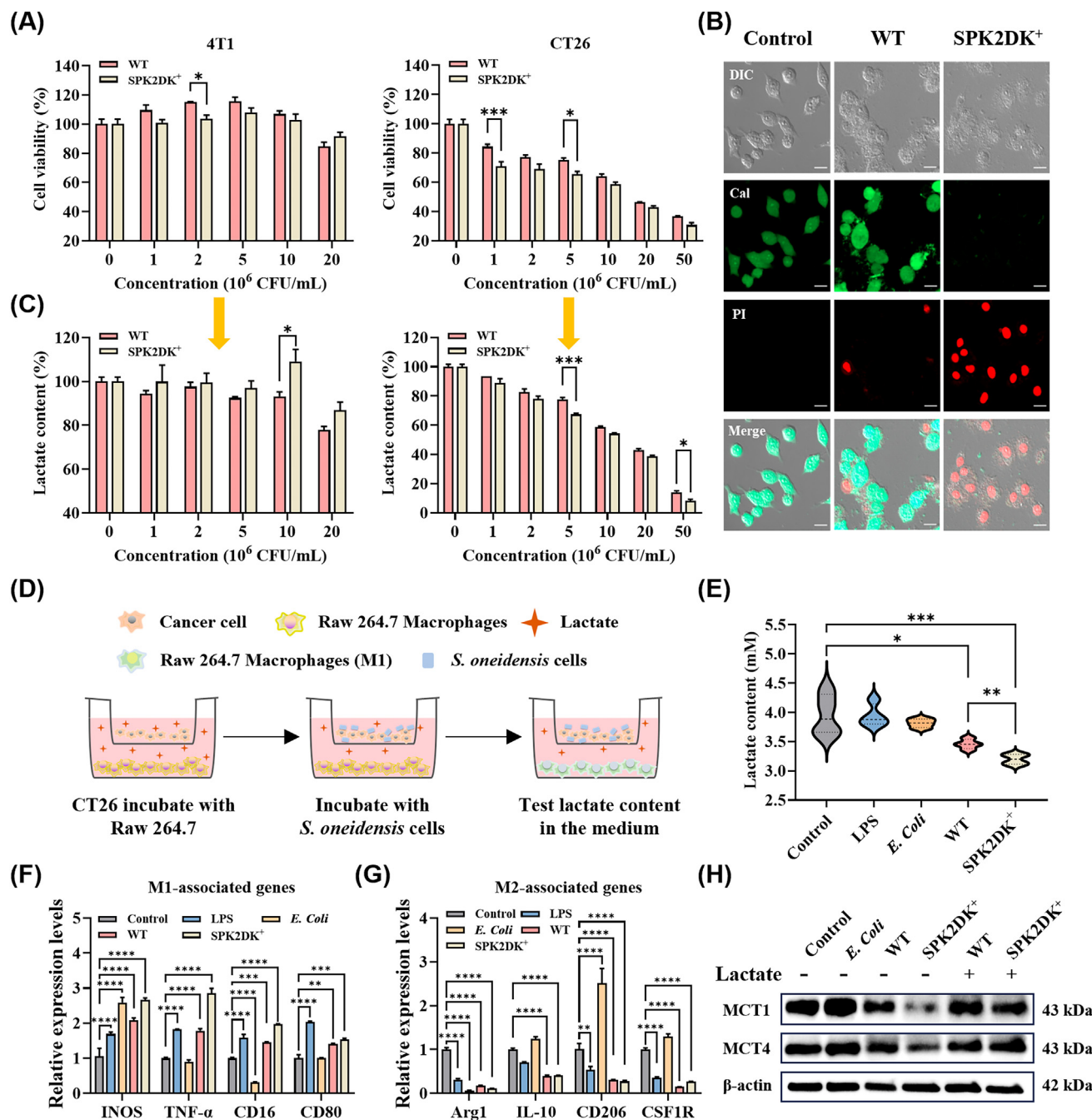


Figure 3. Antitumor efficacy of the engineered *S. oneidensis* SPK2DK⁺ *in vitro*. To evaluate the *in vitro* antitumor activity of SPK2DK⁺, we performed cytotoxicity assays and live/dead staining. SPK2DK⁺ selectively killed CT26 cells and induced membrane disruption (A–C). To assess its immunomodulatory potential, we cocultured SPK2DK⁺ with macrophages in a transwell system. SPK2DK⁺ significantly reduced extracellular lactate (D), and promoted M1-like polarization and suppressed M2 markers (D–G). Moreover, western blot analysis showed that SPK2DK⁺ downregulated MCT1 and MCT4 in a lactate-dependent manner, inhibiting lactate transport (H), demonstrating that SPK2DK⁺ exerts its antitumor effect through direct lactate depletion and blockade of lactate transport, leading to enhanced immune cell activation. (A) Tumor cell viability after 24 h coculture with indicated *S. oneidensis* strains ($n = 3$). (B) Fluorescence microscopic assessment of cell viability: Calcein AM (green, live cells); propidium iodide (red, dead cells). Scale bars: 20 μ m. (C) Extracellular lactate concentration in culture supernatants after 24 h of coculture ($n = 3$).

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system of CT26 cells and RAW 264.7 macrophages (Figure 3D) [39]. SPK2DK⁺ reduced extracellular lactate by 19.1% (relative to untreated controls), outperforming both WT *S. oneidensis* (12.5%) and *E. coli* (3.5%) (Figure 3E). This enhanced lactate consumption was associated with a robust shift toward M1-like polarization, as evidenced by significant upregulation of M1 markers and downregulation of M2 markers compared with both control groups (Figure 3F,G). ELISA further confirmed markedly elevated secretion of the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) (Figure S13 in the Supplemental information online), indicating that the enhanced M1 polarization observed with SPK2DK⁺ is closely linked to its superior lactate-scavenging capacity.

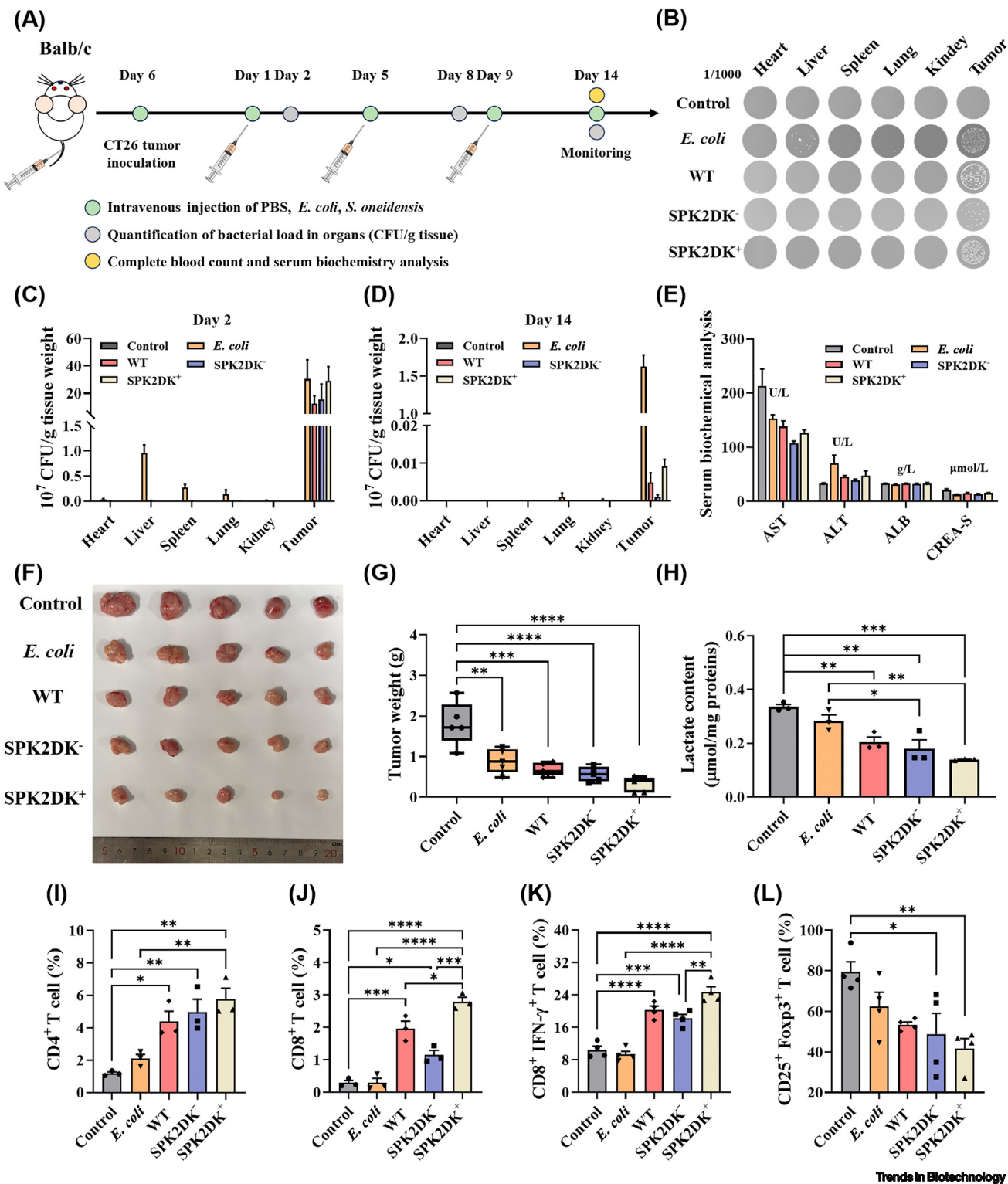
To elucidate how SPK2DK⁺ modulates lactate dynamics, we analyzed the expression of the lactate transporters MCT1 and MCT4 after 24 h of bacterial treatments. Western blot analysis showed that *E. coli* treatment had no significant effect, whereas both WT and SPK2DK⁺ *S. oneidensis* reduced MCT1 and MCT4 expression, with the greatest downregulation observed in SPK2DK⁺-treated cells (Figure 3H and Figure S14 in the Supplemental information online). Moreover, a lactate rescue assay demonstrated that supplementing the medium with 10 mM lactate prior to bacterial treatment effectively restored MCT1 and MCT4 protein levels, confirming that lactate depletion is a critical regulator of MCT1/MCT4 downregulation. These results indicate that SPK2DK⁺ exerts its effect through two complementary mechanisms: direct scavenging of extracellular lactate and lactate-dependent downregulation of MCT1/MCT4. Together, these dual actions on lactate flux likely contribute to the enhanced antitumor efficacy of SPK2DK⁺ against CT26 cells.

In vivo biosafety and antitumor efficacy of engineered *S. oneidensis*

We then assessed the biosafety and *in vivo* therapeutic efficacy of engineered *S. oneidensis* in a CT26 tumor-bearing BALB/c mouse model. As *S. oneidensis* is largely cleared from the host within 72 h after administration, we chose a dosing interval of every 4 days to maintain sustained bacterial presence at the tumor site [40]. Mice were treated with phosphate-buffered saline (PBS), empty-vector *E. coli*, WT *S. oneidensis*, the bioenergetically enhanced SPK2DK⁺ strain, and its uninduced *S. oneidensis* SPK2DK⁻ (bioenergetically comparable to WT) (Figure 4A and Figure S15 in the Supplemental information online). Biodistribution (colony-forming unit [CFU]) analysis showed that by Day 2, all bacterial strains preferentially colonized tumors, with *S. oneidensis* exhibiting minimal dissemination to healthy organs (Figure 4B,C). Clearance was evident by Day 8 (Figure S16 in the Supplemental information online), and by Day 14, *S. oneidensis* was nearly undetectable in host tissues, whereas *E. coli* persisted at higher systemic levels (Figure 4D and Figure S17 in the Supplemental information online). Comprehensive hematological and serum biochemical analyses confirmed that liver/kidney function markers (aspartate aminotransferase [AST]; alanine aminotransferase [ALT]; albumin [ALB]; and serum creatinine [CREA-S]) and systemic inflammation indicators (procalcitonin [PCT] and C-reactive protein [CRP]) remained within normal ranges across all *S. oneidensis*-treated groups, indicating no overt systemic toxicity (Figure 4E and Figures S18 and S19 in the Supplemental information online).

Critically, the SPK2DK⁺ strain exhibited superior intratumoral persistence compared with WT and SPK2DK⁻ strains, suggesting that enhanced bioenergetics prolongs tumor retention. This

(D) Schematic diagram of the transwell coculture system consisting of CT26 cells, RAW 264.7 macrophages, and bacteria (1×10^7 CFU). (E) Lactate concentration in culture supernatants from the transwell system after 24 h coculture with different strains ($n = 4$). (F and G) qPCR analysis of macrophage polarization markers: (F) M1-associated genes (*iNOS*, *TNF- α* , *CD16*, and *CD80*) and (G) M2-associated genes (*Arg1*, *IL10*, *CD206*, and *CSF1R*) ($n = 3$). (H) Western blot analysis of lactate transporter proteins (MCT1 and MCT4) in CT26 cells after 24 h of bacterial treatment. All data are presented as mean $\pm$ SEM. One-way or two-way ANOVA was used as appropriate, followed by Dunnett's multiple comparisons test for comparisons against the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Cal: calcein acetoxyethyl ester; LPS: lipopolysaccharide; PI: propidium iodide.



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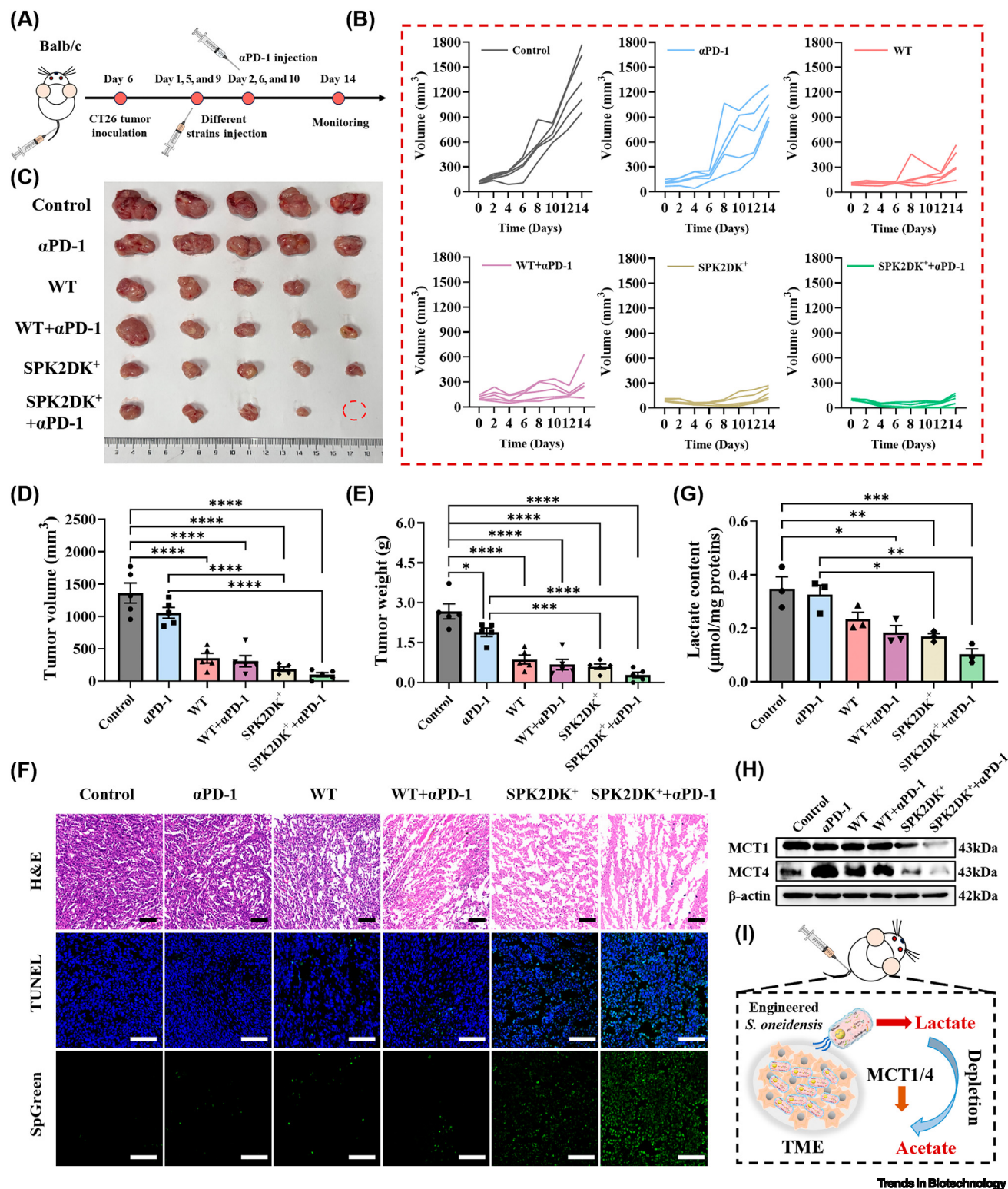
sustained colonization correlated with the strongest therapeutic outcome: SPK2DK⁺ treatment reduced final tumor weight by 82.6%, significantly outperforming *E. coli* (50.6%), WT (62.2%), and SPK2DK⁻ (68.7%) (Figure 4F,G and Figure S20 in the Supplemental information online). The superior antitumor activity was paralleled by a profound depletion of intratumoral lactate, with levels in the SPK2DK⁺ group reduced by 58.6% of the PBS group (Figure 4H). Furthermore, analysis of the immune cell populations in the tumor immune microenvironment revealed strain-specific remodeling, with SPK2DK⁺ inducing the most robust changes, including increased infiltration of CD4⁺ and CD8⁺ T cells, a higher frequency of CD8⁺ interferon- γ (IFN- γ)⁺ T cells, and a corresponding reduction in Tregs (Figure 4I–L and Figures S21 and S22 in the Supplemental information online).

Building on the differential cytotoxicity observed *in vitro*, we further evaluated antitumor efficacy in the 4T1 tumor model *in vivo*. *S. oneidensis* maintained favorable tumor targeting, with intratumoral burdens comparable to those in the CT26 model (Figure S23 in the Supplemental information online). However, clearance kinetics differed: by day 14, bacterial loads remained detectable in 4T1 tumors but were nearly undetectable in CT26 tumors, suggesting slower clearance in the 4T1 setting—likely due to its highly immunosuppressive and metastatic microenvironment [41,42]. As anticipated, SPK2DK⁺ strain showed greater intratumoral persistence than the WT and SPK2DK⁻ strains, correlating with enhanced tumor growth inhibition and lactate depletion (Figures S24 and S25 in the Supplemental information online). Nevertheless, its therapeutic advantage over other *S. oneidensis* strains was less pronounced in 4T1 than in the CT26 model. This observation aligns with our *in vitro* findings and supports the conclusion that therapeutic efficacy is dictated by tumor-intrinsic metabolic sensitivity to lactate depletion. Together, these results suggest that the antitumor efficacy of engineered bacteria is influenced by the interplay between strain-specific pharmacokinetics and tumor-intrinsic microenvironmental features.

Synergistic antitumor efficacy of engineered *S. oneidensis* and α PD-1 immunotherapy

Building on the established safety profile of *S. oneidensis* and the rationale that combining bacterial therapy with immune checkpoint blockade can enhance antitumor efficacy, we next investigated whether its therapeutic effect could be enhanced by combining it with α PD-1 in CT26 tumor-bearing mice (Figure 5A) [5,14,40]. Hematological analysis and hematoxylin and eosin (H&E) staining of major organs after 14 days confirmed that all hematological and metabolic parameters remained within normal physiological ranges (Figures S26 and S27 in the Supplemental information online), consistent with the favorable biosafety profile observed in prior monotherapy studies. SPK2DK⁺ monotherapy elicited a substantial antitumor response, reducing tumor growth by 77.8% relative to the PBS control and by 30.8% compared with WT monotherapy. The observed efficacy was moderately lower than that recorded in a prior experimental batch

Figure 4. *In vivo* biosafety and antitumor efficacy of engineered *S. oneidensis* in CT26 tumor-bearing mice. To evaluate the biosafety and therapeutic efficacy of the engineered SPK2DK⁺ strain in a CT26 tumor-bearing mouse model, we assessed its tumor-targeting, biodistribution, and antitumor activity. The strain exhibited favorable tumor-targeting properties with minimal dissemination to healthy organs (B–D), and no overt systemic toxicity was observed (E). SPK2DK⁺ monotherapy significantly reduced tumor weight (F and G) and intratumoral lactate levels (H), accompanied by increased infiltration of CD4⁺ and CD8⁺ T cells and reduced Treg proportions (I–L). (A) Experimental timeline for therapeutic and safety assessment in CT26 tumor-bearing mice ($n = 5$). (B) Representative images of bacterial colonies from homogenates of indicated organs (liver, spleen, lung, and kidney) plated on LB-kanamycin agar at Day 2 in CT26 tumor-bearing mice (top left: dilution factor). (C and D) Quantification of bacterial loads (CFU) in tumors and major organs at Day 2 (C) and Day 14 (D) in CT26 tumor-bearing mice ($n = 3$). (E) Serum biochemistry analysis at Day 14 after treatment in CT26 tumor-bearing mice ($n = 3$). (F) Subcutaneous tumor images following different treatments in CT26 tumor-bearing mice ($n = 5$). (G) Final tumor weights of CT26 tumor-bearing mice after different treatments ($n = 5$). (H) Intratumoral lactate concentrations following different treatments from CT26 tumor-bearing mice ($n = 3$). (I and J) Proportions of CD4⁺ T cells and CD8⁺ T cells in tumor tissues from CT26 tumor-bearing mice ($n = 3$). (K and L) Proportions of CD8⁺ IFN- γ ⁺ T cells and CD25⁺ Foxp3⁺ Tregs in tumor tissues from CT26 tumor-bearing mice ($n = 4$). All data are presented as mean $\pm$ SEM. One-way ANOVA was used, followed by Tukey's multiple comparisons test for pairwise comparisons among all groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.



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(82.6% vs. PBS control and 53.8% vs. WT monotherapy), a variation likely attributable to increased interindividual variability and differences in tumor cell viability between batches (Figure 5B–E and Figure S28 in the Supplemental information online). Moreover, the addition of α PD-1 to SPK2DK⁺ treatment led to a marked improvement in outcome, achieving 89.5% inhibition versus the control and 67.4% versus WT monotherapy. Histopathological evaluation correlated with these findings: both the SPK2DK⁺ monotherapy and combination groups exhibited extensive tumor necrosis and suppressed proliferation, with the most severe damage evident in the combination cohort (Figure 5F).

Mechanistically, SPK2DK⁺ monotherapy decreased intratumoral lactate levels by 51.4% relative to the control and by 27.4% relative to WT treatment, while the combination therapy achieved even greater reductions of 70.7 and 56.2%, respectively (Figure 5G). Consistent with this metabolic shift, the expression of the lactate transporters MCT1 and MCT4 was significantly downregulated in tumors from both the SPK2DK⁺ monotherapy and the SPK2DK⁺ + α PD-1 combination groups, with a more pronounced reduction observed in the latter (Figure 5H and Figure S29 in the Supplemental information online). Collectively, these findings suggest that lactate-targeting bacterial therapy not only depletes lactate within the TME but also suppresses lactate transport and uptake by tumor cells via downregulation of MCT1 and MCT4. This dual action synergizes with immune checkpoint blockade to potentiate antitumor efficacy (Figure 5I).

Metabolic reprogramming-mediated antitumor immunity of engineered *S. oneidensis*

To delineate how metabolic remodeling reshapes antitumor immunity, we performed comprehensive immunophenotyping. In tumor-draining lymph nodes, SPK2DK⁺ monotherapy elevated the frequency of mature dendritic cells (DCs) (CD80⁺ CD86⁺) by 96.1% over the control level and by 20.4% over the WT level, an effect that was further enhanced by α PD-1 co-administration (224.5% increase over control and 99.3% increase over WT) (Figure 6A,B), indicating improved antigen-presenting capacity. Within tumors, SPK2DK⁺ monotherapy significantly promoted infiltration of CD4⁺ T cells (127.3% increase over control; 22.5% increase over WT), CD8⁺ T cells (126.8% increase over control; 45.9% increase over WT), and CD8⁺ IFN- γ ⁺ T cells (112.5% increase over control; 30.4% increase over WT) (Figure 6C–E and Figures S30 and S31 in the Supplemental information online). The combination therapy SPK2DK⁺ + α PD-1 induced even more robust T cell expansion, with CD4⁺ T cells showing a 145.5% increase relative to control and a 32.2% increase relative to WT, CD8⁺ T cells showing a 219.2% increase relative to control and a 105.4% increase relative to WT, and CD8⁺ IFN- γ ⁺ T cells showing a 158.6% increase relative to control and a 58.7% increase relative to WT.

A key feature of the remodeled tumor immune microenvironment was the marked reduction in immunosuppressive Tregs [8,43,44]. SPK2DK⁺ monotherapy decreased Treg proportions by

Figure 5. Evaluation of antitumor efficacy of *S. oneidensis* and α PD-1 antibody combination therapy in CT26 tumor-bearing mice. To evaluate whether combining the engineered SPK2DK⁺ strain with α PD-1 immune checkpoint blockade could further enhance antitumor efficacy, we performed a combination therapy experiment (A). The combination therapy produced stronger tumor growth inhibition than either monotherapy alone (B–E), leading to increased tumor necrosis and apoptosis (F). Mechanistically, the combination synergistically reduced intratumoral lactate levels (G) and downregulated MCT1 and MCT4 expression (H), with a proposed mechanistic model illustrated in (I). (A) Schematic diagram of the treatment regimen for CT26 tumor-bearing mice. (B and C) Individual tumor volume growth curves and subcutaneous tumor images of CT26 tumor-bearing mice following different treatments ($n = 5$). (D and E) Final tumor volumes and tumor weights of CT26 tumor-bearing mice following different treatments ($n = 5$). (F) H&E and TUNEL staining of tumors harvested from mice 14 days following intravenous injection. Scale bar: 100 μ m. (G) Intratumoral lactate content in CT26 tumor-bearing mice following different treatments ($n = 3$). (H) Western blot analysis of MCT1 and MCT4 expression in tumor tissues following different treatments. (I) Mechanistic illustration of TME remodeling by engineered *S. oneidensis* SPK2DK⁺. All data are presented as mean $\pm$ SEM. One-way ANOVA was used, followed by Tukey's multiple comparisons test for pairwise comparisons among all groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

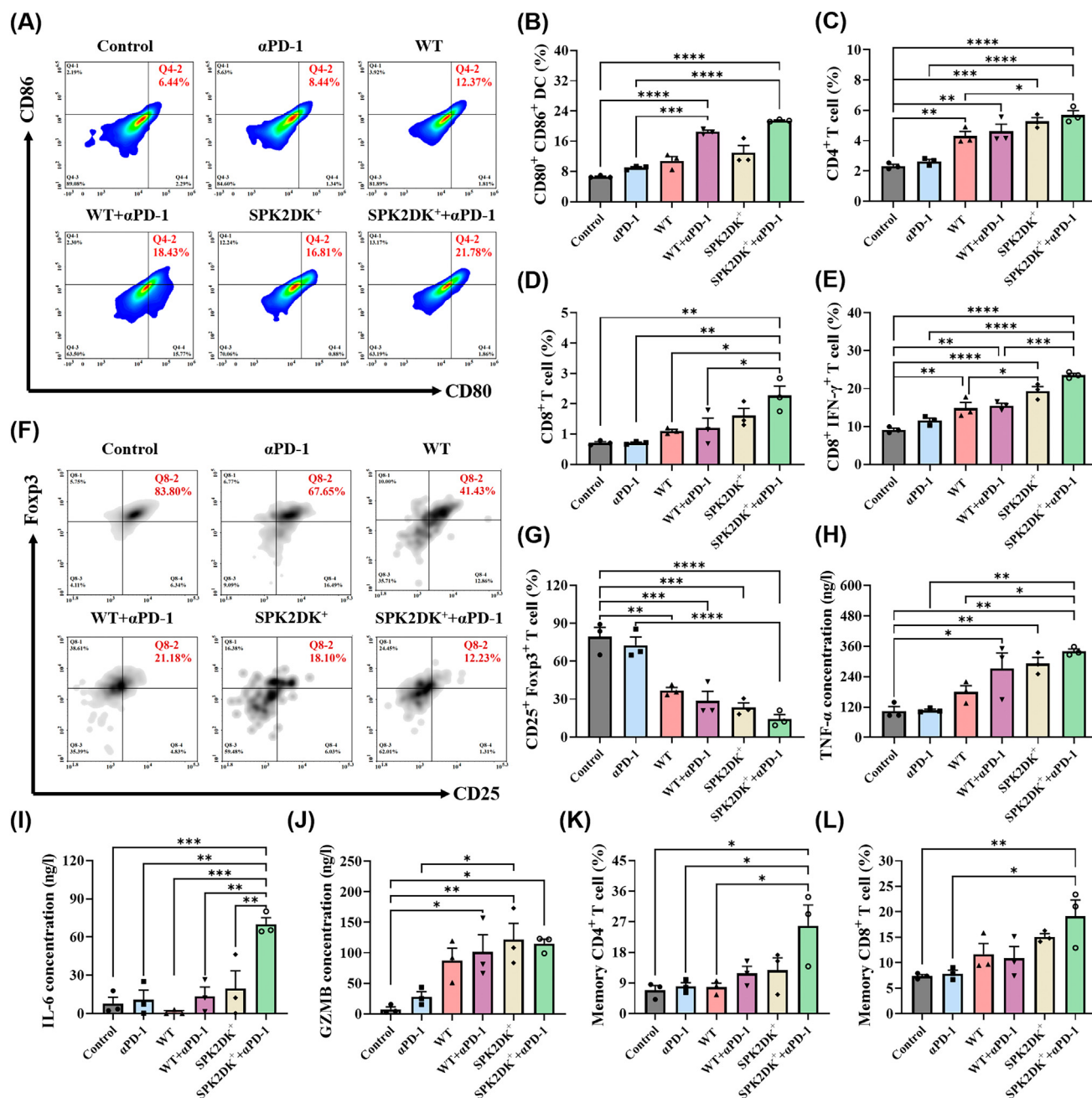


Figure 6. Evaluation of immune activation induced by *S. oneidensis* and combined with αPD-1 therapy in CT26 tumor-bearing mice. To evaluate the *in vivo* immune activation status following monotherapy or combination therapy with αPD-1, we analyzed immune cell populations and cytokine levels. The combination treatment significantly enhanced DCs maturation in lymph nodes (A and B), increased intratumoral CD4⁺ and CD8⁺ T cell infiltration (C and D), and elevated the frequency of CD3⁺ CD8⁺ IFN-γ⁺ T cells (E). It also reduced Treg proportions (F and G) and raised serum levels of effector cytokines and granzyme B (H–J). Moreover, effector memory T cells (CD44⁺ CD62L⁺) were increased in the spleen (K and L), indicating durable immunological memory and collectively demonstrating that the combination of SPK2DK⁺ with αPD-1 induces potent and long-lasting antitumor immunity. (A and B) Statistical percentages of matured DCs (CD80⁺ CD86⁺) in lymph nodes from CT26 tumor-bearing mice (*n* = 3). (C and D) Statistical percentages of CD4⁺ and CD8⁺ T cells in tumor tissues from CT26 tumor-bearing mice (*n* = 3). (E) Statistical percentages of CD3⁺ CD8⁺ IFN-γ⁺ T cells in tumor tissues from CT26 tumor-bearing mice (*n* = 3). (F and G) Statistical analysis and percentages of CD25⁺ Foxp3⁺ Tregs in tumor tissues from CT26 tumor-bearing mice (*n* = 3). (H–J) Serum levels of TNF-α, IL-6, and GZMB following different treatments from CT26 tumor-

(Figure legend continued at the bottom of the next page.)

70.6% relative to the control and 36.5% relative to the WT, while the combination therapy reduced them by 82.1% and 61.3%, respectively (Figure 6F,G). This shift was associated with lactate depletion in the TME, consistent with the hypothesis that metabolic reprogramming contributes to treatment efficacy. In addition, systemic T cell analysis in the spleen validated these intratumoral trends (Figures S32–S34 in the Supplemental information online). ELISA quantification confirmed that both SPK2DK⁺ monotherapy and combination with αPD-1 significantly elevated proinflammatory cytokines (IFN-γ, TNF-α, and IL-6) and granzyme B (GZMB), consistent with an activated antitumor immune state (Figure 6H–J and Figure S35 in the Supplemental information online) [45,46]. Moreover, SPK2DK⁺ treatment induced long-term immunological memory, with flow cytometry revealing significantly lower frequencies of splenic central memory T cells (CD3⁺ CD4⁺ CD44⁺ CD62L⁺ and CD3⁺ CD8⁺ CD44⁺ CD62L⁺) and higher frequencies of effector memory T cells (CD3⁺ CD4⁺ CD44⁺ CD62L[−] and CD3⁺ CD8⁺ CD44⁺ CD62L[−]) than other groups (Figure 6K,L and Figure S36 in the Supplemental information online), a phenotype associated with protection against secondary tumor challenge [47,48]. In summary, lactate scavenging by the PPK2-I and NADK-engineered strain SPK2DK⁺ contributes to reprogramming of the immunosuppressive TME. This metabolic intervention, in synergy with αPD-1 blockade, promotes a potent and durable tumor-specific immune response.

Discussion

Despite transformative advances in cancer immunotherapy, its clinical efficacy remains limited by the prevalence of immunologically ‘cold’ tumors characterized by lactate-driven immunosuppression [49,50]. To address this challenge, we engineered *S. oneidensis* through rational enzymatic rewiring of its core bioenergetics, creating an energetically enhanced bacterial platform that overcomes key metabolic limitations of live therapeutics while concurrently remodeling the immunosuppressive TME. By harnessing polyP, an evolutionarily conserved high-energy metabolite, we synthetically integrated PPK2-I and NADK to reconfigure metabolic flux. This design generated a coupled energy-generating circuit, driving a 287.5% increase in ATP production and a 299.9% elevation in the NADH/NAD⁺ ratio. Crucially, this reprogrammed metabolism mediated a dual-pronged action on lactate: (i) accelerated intratumoral lactate clearance via enhanced bacterial catabolism and (ii) suppressed tumor lactate transport through downregulation of monocarboxylate transporters MCT1 and MCT4.

Bioenergetic remodeling not only accelerated targeted lactate depletion in the TME but also prolonged the intratumoral persistence of the engineered strain. By impairing tumor lactate flux, the bacteria further potentiated antitumor immunity. Combination with αPD-1 blockade synergistically amplified antitumor efficacy and induced durable immunological memory against rechallenge, illustrating that engineering microbial bioenergetics can integrate metabolic intervention with checkpoint immunotherapy.

Additionally, to assess the advantage of *S. oneidensis* as a lactate-depleting chassis, we also compared it with the widely used bacterial vector *E. coli*. Although *E. coli* retained some tumor-targeting ability, a notable portion disseminated to healthy organs, whereas *S. oneidensis* displayed limited systemic distribution and more rapid clearance, highlighting its favorable biosafety profile. Consistently, *E. coli* only marginally reduced intratumoral lactate, while *S. oneidensis*, especially SPK2DK⁺ achieved potent lactate depletion and tumor inhibition. Thus, *S. oneidensis* offers distinct advantages as a tumor-targeting, lactate-depleting platform.

bearing mice ($n = 3$). (K and L) Statistical percentages of effector memory T cells (CD44⁺ CD62L[−]) among CD4⁺ and CD8⁺ T cells in the spleen after different treatments from CT26 tumor-bearing mice ($n = 3$). All data are presented as mean ± SEM. One-way ANOVA was used, followed by Tukey's multiple comparisons test for pairwise comparisons among all groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

In particular, bioenergetically reprogrammed *S. oneidensis* can serve as a self-sustaining, tumor-targeting lactate scavenger, a capability that conventional small-molecule inhibitors (which only block transport without consuming existing lactate) cannot achieve. This opens therapeutic possibilities not only for colorectal cancer but also for other solid tumors where lactate-driven immunosuppression is a major barrier. In addition, we believe that the polyP-based energy-rewiring strategy is potentially transferable to other probiotic or electroactive bacterial chassis, enabling the design of live biotherapeutics for metabolic and immune-related diseases.

Collectively, this study establishes engineered bacterial energetics as a strategic platform for synergistic cancer therapy. By reprogramming microbial energy metabolism to drive sustained lactate scavenging and disrupt tumor metabolic adaptation, we provide a proof of concept that targeting bioenergetic pathways in live therapeutics offers a promising avenue for overcoming immunosuppression in solid tumors.

Concluding remarks

Lactate accumulation in the TME suppresses immune cell function and is a key reason why many patients fail to respond to immunotherapy. Here, we used synthetic biology to reprogram energy metabolism in *S. oneidensis* MR-1 by harnessing polyP—an evolutionarily conserved high-energy metabolite—enabling the bacterium to function as an enhanced lactate-scavenging platform within tumors. By introducing PPK2-I and NADK, we established a bioenergetically augmented strain that increased ATP production by 287.5% and raised the NADH/NAD⁺ ratio by 299.9% compared with the WT strain. This enhancement increased the capacity for lactate depletion and was associated with prolonged intratumoral persistence.

The engineered strain reduced intratumoral lactate, downregulated tumor MCT1 and MCT4 expression, and shifted the immune landscape—enhancing both antigen presentation and CD8⁺ T cell infiltration, while reducing Tregs. When combined with αPD-1, it further enhanced antitumor efficacy and established durable immunity against rechallenge. This self-sustaining, tumor-homing lactate scavenger offers a live therapeutic alternative that can potentially reduce off-target toxicity and be extended to other solid tumors where lactate-driven immunosuppression is a major barrier. Our work demonstrates a synthetic biology strategy to actively remodel lactate-rich immunosuppressive microenvironments, offering a promising approach to address metabolic barriers in cancer immunotherapy (see [Outstanding questions](#)).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Bacterial culture
 - Plasmid construction
 - Cell culture
 - Animals
- METHOD DETAILS
 - Comparative genomic analysis
 - ABL model
 - Biofilm formation assay and biomass quantification
 - Quantification of intracellular polyP
 - Fluorescence imaging of intracellular polyP

Outstanding questions

Can the metabolism of live biotherapeutics be synthetically programmed to dynamically sense and respond to multiple immunosuppressive signals in the tumor microenvironment—not just lactate, but also factors such as adenosine or hypoxia—while maintaining metabolic homeostasis?

Will the polyphosphate-driven bioenergetic circuit (polyphosphate kinase 2 and NAD kinase) function robustly in human tumors, which exhibit greater heterogeneity in oxygenation, pH, and nutrient availability than murine models?

How can engineered bacteria be rationally combined with emerging therapies (e.g., adoptive T cell transfer, nanocarriers, or radiotherapy) to achieve coordinated metabolic and immune activation with precise spatio-temporal control?

- Electrochemical analysis
- Morphological characterization
- Qualification of ATP and NADH/NAD⁺
- Qualification of lactate
- qPCR quantification
- Cell viability assay
- Western blot analysis
- Quantification of bacterial loads
- *In vivo* immune activation and memory detection
- ELISA
- Biosafety assessment
- Transcriptomic analysis
- Metabolomic analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to, and will be fulfilled by, the lead contact, Professor Wei Wei, weiwei@nju.edu.cn.

Materials availability

This study did not generate new or unique reagents.

Data and code availability

Transcriptomic data have been deposited in Gene Expression Omnibus (GEO), and metabolomic data have been deposited in National Genomics Data Center (NGDC). Both datasets are publicly available as of the date of publication, and accession numbers are listed in the Key Resources Table. The original code is available at <https://github.com/yfxiang0112/EGOAL>. Any additional information required to reanalyze the data reported in this study is available from the lead contact upon request.

Author contributions

J.Z. designed and performed experiments, analyzed data, and wrote the manuscript. N.Z. and L.Q. performed experiments and analyzed data. J.L. and J.W. performed fluorescence microscopy and confocal fluorescence microscopy. Y.X. wrote the AI model and ran the program. Y.H., C.L., J.Z., and X.W. conceived and supervised the study, designed experiments, and analyzed data. W.W. conceived and supervised the study, designed experiments, and revised the manuscript.

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Declaration of interests

The authors declare no competing interests.

Supplemental information

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STAR★METHODS

KEY RESOURCES TABLE

Reagent or resource	Source	Identifier
Antibodies		
Beta Actin Recombinant antibody	Proteintech	CAT#81115-1-RR; RRID: AB_2923704
MCT1 Polyclonal antibody	Proteintech	CAT#20139-1-AP; RRID: AB_2878645
MCT4 Polyclonal antibody	Proteintech	CAT#22787-1-AP; RRID: AB_11182479
HRP-labeled Goat Anti-Rabbit IgG (H+L)	Beyotime	CAT#A0208
FITC anti-mouse CD3	BioLegend	CAT#100204; RRID: AB_312661
PE anti-mouse CD8a	BioLegend	CAT#100708; RRID: AB_312747
APC anti-mouse CD8a	BioLegend	CAT#100712; RRID: AB_312751
APC anti-mouse IFN- γ	BioLegend	CAT#505810; RRID: AB_315404
PE anti-mouse CD4	BioLegend	CAT#100512; RRID: AB_312715
Pacific Blue™ anti-mouse/human CD44	BioLegend	CAT#103020; RRID: AB_493683
APC anti-mouse CD25	BioLegend	CAT#102012; RRID: AB_312861
Alexa Fluor® 488 anti-mouse/rat/human FOXP3	BioLegend	CAT#320012; RRID: AB_439748
APC anti-mouse CD11c	BioLegend	CAT#117310; RRID: AB_313779
FITC anti-mouse CD80	BioLegend	CAT#104706; RRID: AB_313127
PE anti-mouse CD86	BioLegend	CAT#105008; RRID: AB_313151
APC anti-mouse CD62L	BioLegend	CAT#104412; RRID: AB_313099
InVivoMAb anti-mouse PD-1 (CD279)	Bio X Cell	CAT#RMP1-14; RRID: AB_10949053
Bacterial and virus strains		
<i>S. oneidensis</i> MR-1	CGMCC	ATCC 700550
WT (harbouring pYYDT)	This study	N/A
SPPKA1 (harbouring pYYDT- <i>ppkA-N</i>)	This study	N/A
STNPA (harbouring pYYDT- <i>TnpA</i>)	This study	N/A
SCFP1 (harbouring pYYDT- <i>Bba_B0034-ppk1</i>)	This study	N/A
SECPX (harbouring pYYDT- <i>ppx</i> (<i>Saccharomyces cerevisiae</i> S288C- <i>ppx</i>))	This study	N/A
SPAK2 (harbouring pYYDT- <i>ppk2</i> (<i>Pseudomonas aeruginosa</i> - <i>ppk2</i>))	This study	N/A
SMTDK (harbouring pYYDT- <i>nadk</i> (<i>Mycobacterium tuberculosis</i> - <i>ppnk</i>))	This study	N/A
SSCN1 (harbouring pYYDT- <i>ppn1</i> (<i>Saccharomyces cerevisiae</i> S288C- <i>ppn1</i>))	This study	N/A
SAJAP (harbouring pYYDT- <i>pap</i> (<i>Acinetobacter johnsonii</i> - <i>pap</i>))	This study	N/A
SPK1-2 (harbouring pYYDT- <i>Bba_B0031-ppk1-Bba_B0034-ppk2</i>)	This study	N/A
SPK1DK (harbouring pYYDT- <i>Bba_B0031-ppk1-Bba_B0034-nadk</i>)	This study	N/A
SPK2DK (harbouring pYYDT- <i>Bba_B0034-ppk2-Bba_B0034-nadk</i>)	This study	N/A
Chemicals, peptides, and recombinant proteins		
LB Broth Powder	Sangon	CAT#A507002-0250
DAPI	Sigma	CAT#28718-90-3
Kanamycin sulfate	Sangon	CAT#A506636-0100
IPTG	Sangon	CAT#A600168-0025
Bovine serum albumin	Sangon	CAT#A600332-0100

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Reagent or resource	Source	Identifier
Sodium DL-lactate solution	Aladdin	CAT#C2208115
1 × PBS buffer	Fdbio	CAT#FD7032
20 × TBST buffer (pH 7.4)	Fdbio	CAT#FD9061
RIPA Lysis Buffer	Fdbio	CAT#FD008
Stripping Buffer	Fdbio	CAT#FD0050
Dulbecco's Modified Eagle Medium, High Glucose (P/S)	KeyGEN	CAT#KGL1206-500
PAN FBS	PAN	CAT#ST30-2602
Critical commercial assays		
2 × Phanta® Flash Master Mix (Dye Plus)	Vazyme	Cat#P520
ClonExpress Ultra One Step Cloning Kit V2	Vazyme	CAT#C116
HiScript II Reverse Transcriptase	Vazyme	CAT#R222
HisyGo RT Red SuperMix for qPCR (+gDNA Wiper)	Vazyme	CAT#RT101
SDS-PAGE gel	Sangon	CAT#C651404-0001
FastPure® Cell/Tissue Total RNA Isolation Kit V2	Vazyme	CAT#RC112
Calcein/PI Cell Viability/Cytotoxicity Assay Kit	Beyotime	CAT#C2015S
Enhanced ATP Assay Kit	Beyotime	CAT#S0027
NAD ⁺ /NADH Assay Kit with WST-8	Beyotime	CAT#S0175
Lactate Acid (L-LA) Content Assay Kit	Solarbio	CAT#BC5340
Mouse TNF α Elisa Kit	GenXspan	CAT#XL-EM0359
Mouse IL-6 Elisa Kit	GenXspan	CAT#XL-EM0196
Mouse IFN-γ Elisa Kit	GenXspan	CAT#XL-EM0173
Mouse GZB Elisa Kit	GenXspan	CAT#XL-EM1374
Mouse PCT Elisa Kit	GenXspan	CAT#XL-EM1003
Mouse CRP Elisa Kit	GenXspan	CAT#XL-EM0076
Tanon TM High-sig ECL Western Botting Substrate	Tanon	CAT#180-501
Deposited data		
Transcriptomic data	OE Biotech	GEO Accession: GSE330449
Metabolomic data	OE Biotech	NGDC Accession: OMIX016772
Code for Abductive Learning (ABL) model	This study	https://github.com/yfxiang0112/EGOAL
Experimental models: Cell lines		
Cell: Cell lines	KeyGEN	N/A
Mouse: BALB/c mice	Ziyuan	N/A
Oligonucleotides		
Primers for PCR/qRT-PCR	Sangon	Table S1
Recombinant DNA		
Plasmid: pYYDT	Northeastern University	Donated by Professor Song Hao
Plasmid: pYYDT- <i>ppkA-N</i>	This study	N/A
Plasmid: pYYDT- <i>TnpA</i>	This study	N/A
Plasmid: pYYDT- <i>Bba_B0034-ppk1</i>	This study	N/A
Plasmid: pYYDT- <i>ppx</i> (<i>Saccharomyces cerevisiae</i> S288C- <i>ppx</i>)	This study	N/A
Plasmid: pYYDT- <i>ppk2</i> (<i>Pseudomonas aeruginosa</i> - <i>ppk2</i>)	This study	N/A
Plasmid: pYYDT- <i>nadk</i> (<i>Mycobacterium tuberculosis</i> - <i>ppnk</i>)	This study	N/A

(continued)

Reagent or resource	Source	Identifier
Plasmid: pYYDT- <i>ppn1</i> (<i>Saccharomyces cerevisiae</i> S288C- <i>ppn1</i>)	This study	N/A
Plasmid: pYYDT- <i>pap</i> (<i>Acinetobacter johnsonii</i> - <i>pap</i>)	This study	N/A
Plasmid: pYYDT- <i>Bba_B0031-ppk1-Bba_B0034-ppk2</i>	This study	N/A
Plasmid: pYYDT- <i>Bba_B0031-ppk1-Bba_B0034-nadk</i>	This study	N/A
Plasmid: pYYDT- <i>Bba_B0034-ppk2-Bba_B0034-nadk</i>	This study	N/A
Software and algorithms		
GraphPad Prism 10	GraphPad software	N/A
SnapGene 7.0.2	SnapGene software	N/A
MEGA5	MEGA software	N/A
Chi1040c	CHI Instrument	N/A
Image lab 6.1	Image lab software	N/A
ZEISS Zen	ZEN Microscopy software	N/A
NovoExpress	NovoExpress software	N/A
Other		
Electrochemical workstation	Chenhua (Shanghai, China)	CHI1040C
Fluorescence microscope	ZEISS (German)	Axio Observer Z1
Quantitative real-time PCR instrument	Thermo Fisher Scientific (USA)	Applied Biosystems Cyclar
PCR amplification instrument	BIORAD (USA)	T100 Thermal Cycler
UV-visible spectrophotometer	Shimadzu (Japan)	UV-1800
Refrigerated benchtop centrifuge	Eppendorf (Germany)	5430R

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial culture

All *S. oneidensis* strains were cultured overnight in LB medium at 30 °C. For induction of engineered strains, fresh overnight cultures were inoculated into fresh LB medium containing kanamycin (50 µg/mL). When the optical density at 600 nm (OD₆₀₀) reached approximately 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. The cultures were then incubated overnight at 30 °C.

Plasmid construction

Coding sequences of all target genes were retrieved from the National Center for Biotechnology Information (NCBI) database, codon-optimized, and synthesized by Beijing Tsingke Biotech Co., Ltd. The amplified fragments were then inserted into the pYYDT vector (primers were listed in Table S1), which had been digested by *NdeI* and *XhoI* restriction enzymes. Plasmids with verified correct sequence alignment were transformed into *S. oneidensis* MR-1 competent cells via electroporation at 1.5 kV, and transformants were selected on medium supplemented with 50 µg/mL kanamycin [51].

Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), authenticated by short tandem repeat (STR) typing, and confirmed to be mycoplasma-free by KeyGEN Biotech Co., Ltd. (Nanjing, China). Cells were maintained in Dulbecco's Modified Eagle Medium, High Glucose (P/S) (DMEM) supplemented with 10% heat-inactivated PAN-FBS, under standard culture conditions (37°C, 5% CO₂ humidified atmosphere).

Animals

5- to 6-week-old Balb/c mice were purchased from Hangzhou Ziyuan Laboratory Animal Science and Technology Co., Ltd. (Hangzhou, China), subcutaneously inoculated in the flank with approximately 1×10^6 CT26 or 4T1 tumor cells. When the tumor volume reached approximately 80 mm³ (calculated as (length/2) × (width²)), typically around day 6 post-inoculation, mice were

randomly assigned to treatment groups. This time point was designated as Day 0. Bacterial therapy was administered via tail vein injection on Days 1, 5, and 9 over a 14-day treatment period. Each injection consisted of 100 μ L of an overnight-induced bacterial suspension at a concentration of 6.7×10^8 CFU/mL. Tumor dimensions and body weight were measured and recorded every two days from Day 0 to Day 14. For the combination therapy with α PD-1 antibody, mice additionally received 100 μ g of anti-PD-1 antibody (Bio X Cell, USA) via tail vein injection on Days 2, 6, and 10. Tumor dimensions and body weight in this group were monitored on the same schedule (every two days from Day 0 to Day 14) as described for the monotherapy groups.

METHOD DETAILS

Comparative genomic analysis

Given the high conservation of the polyphosphate kinase 1 (PPK1) sequence, the amino acid and nucleotide sequences of a previously characterized PPK1 from *Citrobacter freundii* were used as query sequences to perform BLASTP and BLASTN searches against genomes of the *Shewanella* genus in the National Center for Biotechnology Information (NCBI) database. To elucidate the evolutionary relationships of this homologous gene, ppk1 nucleotide sequences were retrieved from multiple representative bacterial species. A phylogenetic tree was constructed via the neighbor-joining method in MEGA 5 software; the numbers at branch nodes represent bootstrap values, which indicate the reliability of the inferred evolutionary relationships.

ABL model

The Abductive Learning (ABL) model is comprised of a machine learning component and a knowledge component. We used a random forest classifier for the learning component, and a Gene Ontology derived knowledge graph in the knowledge component. The predictions are made by the learning component, and the confidence score is a vector with the size of genome, representing the class probability of involved in or expression changed in the experiment for each gene, given the input of experiment conditions and observations (The input is formalized as GO terms). Training procedure of the ABL model involves supervision signal from both transcriptomic data and the knowledge graph, therefore, high confidence score for a gene represents that it either exhibits a significant effect in the dataset, or is related with the experiment conditions and observations in the knowledge graph. Concerning reproducibility of the ABL model, its code, complete workflow of training and inferencing are available at <https://github.com/yfxiang0112/EGOAL>.

Biofilm formation assay and biomass quantification

IPTG-induced bacterial cells ($OD_{600} = 1.0$) were collected and mixed with 200 μ L M9 buffer; the mixture was incubated in sterile black 96-well plates (Corning, USA) at 30°C for 24 h to induce biofilm formation. Floating planktonic cells were discarded, and adherent biofilms were stained with 150 μ L of 0.1% crystal violet solution. After staining, the wells were washed repeatedly with sterile water, and excess liquid was aspirated completely. Plates were air-dried, and 95% ethanol was added to solubilize the stained biofilm; the optical density (OD) of each sample was measured at 590 nm (OD_{590}) [52,53]. Anode-associated biofilm biomass was quantified as previously described [54,55].

Quantification of intracellular polyP

Intracellular polyP levels was quantified according to previous protocol described by Kulakova *et al.* and type 45 polyP (Sigma-Aldrich, USA) was used as the standard reference [56]. Briefly, IPTG-induced bacterial cells ($OD_{600} = 1.0$) were collected by centrifugation in 1 mL HEPES buffer and heat-treated in 95°C for 10 min to release intracellular polyP. A 10 μ L cell cultures were added to 900 μ L of 20 mM HEPES buffer and 100 μ L of 100 μ M DAPI buffer. The resulting mixture was vortexed thoroughly every 5 min for 2 successive intervals, and the fluorescence intensity was measured with an excitation wavelength of 415 nm and an emission wavelength of 550 nm using a fluorescence microplate reader (Zeiss, Germany).

Fluorescence imaging of intracellular polyP

Briefly, bacterial cells were collected at an OD_{600} of 1.0 by centrifugation at 12,000 $\times$ g for 5 min and washed once with 20 mM HEPES buffer. The resulting cell pellet was resuspended in a staining solution consisting of 900 μ L HEPES buffer and 100 μ L 100 μ M DAPI solution. After 30 min of incubation at room temperature for staining, fluorescence images were acquired using a fluorescence microscope with the AF430-polyP filter set (excitation wavelength: 277 nm; emission wavelength: 540 nm).

Electrochemical analysis

Electrochemical analyses were performed using a single-chamber microbial electrochemical cell (MEC) system with a 28 mL working volume, configured with a standard three-electrode system. The working electrode was carbon cloth, the reference electrode was a 3 M KCl Ag/AgCl electrode, and the counter electrode was a graphite rod (Ci Yue, Shanghai, China). The electrolyte comprised M9 buffer supplemented with 20 mM sodium lactate and 5% LB broth. The maximum output current was recorded via an electrochemical workstation (CHI1040C, Chen Hua, China) by current-time (I-t) measurements (200 mV vs. reference electrode).

Morphological characterization

IPTG-induced bacterial cells were harvested by centrifugation at 12000 rpm for 5 min, then fixed with 5% glutaraldehyde at 4°C for 12h. Following fixation, the samples were subjected to gradient dehydration in an ethanol series of increasing concentrations (25%, 50%, 75%, 95%, and 100% v/v). Dehydrated samples were then prepared for scanning electron microscopy (SEM) imaging using a SU8020 SEM system (Hitachi, Japan). Transmission electron microscopy (TEM) images were acquired using a FEI Tecnai G2 F30 transmission electron microscope (USA), while energy-dispersive X-ray spectroscopy (EDS) mapping was performed on a high-resolution transmission electron microscope (XPLORE, UK).

Qualification of ATP and NADH/NAD⁺

IPTG-induced bacterial cells ($OD_{600} = 1.0$) from LB cultures were harvested and centrifuged at 8000 rpm for 10 min. The supernatant was discarded, and the precipitate was resuspended in ddH₂O. The levels of ATP and NADH/NAD⁺ were quantified by the Enhanced ATP Assay Kit and NADH/NAD⁺ Assay Kit with WST-8 (Beyotime Biotechnology, China).

Qualification of lactate

In Vitro Lactate Assay: Subsequent to electrochemical analysis, bacterial cells ($OD_{600} = 1.0$) were collected by centrifugation at 8000 rpm for 10 min and washed twice with ddH₂O. Lactate levels were determined using the Lactate Acid (L-LA) Content Assay Kit (Solarbio, China). For cellular co-culture experiments, lactate concentrations were measured in the cell culture supernatant collected after medium centrifugation.

In Vivo Lactate Assay: For the quantification of intratumoral lactate in animal experiments, lactate levels were assayed using homogenates prepared from excised tumor tissues, with the same Lactate Acid (L-LA) Content Assay Kit (Solarbio, China).

qPCR quantification

The total RNA was extracted by FastPure® Cell/Tissue Total RNA Isolation Kit according to the manufacturer's protocol, and then reverse transcription to cDNA for next step (Vazyme, China). The $2^{-\Delta\Delta CT}$ method was used for data analysis and the *gyrB* was chosen as the reference gene. The cell experiments involved qPCR assays that were collected from co-cultured cells and the *gapdh* was chosen as the reference gene.

Cell viability assay

For the cell viability assay using the CCK-8 method: Cells were seeded in culture plates at a density of 5×10^4 cells/mL, allowed to adhere overnight, and then co-cultured with bacteria at a different concentration of strains for 24 hours. After co-incubation, cellular viability was quantified using a CCK-8 cell proliferation assay kit following the manufacturer's instructions (KeyGEN BioTECH, China).

For live/dead cell staining: CT26 cells (5×10^4 cells/mL) were seeded in confocal dishes and cultured overnight at 37°C. Bacteria were then added at a concentration of 1×10^7 CFU/mL for 24-hour co-culture. After incubation, cell viability was evaluated using a live/dead cell staining kit (Calcein AM/PI method; KeyGEN BioTECH, China) according to the manufacturer's protocol, and images were captured using a confocal laser scanning microscope (CLSM).

Western blot analysis

Tumor cells with a density of 5×10^4 cells/mL were seeded into 6-well plates (2 mL per well) and cultured overnight at 37°C for adherent growth. On the following day, bacteria at a dose of 1×10^7 CFU/mL were added to each well for 24 h of co-culture with tumor cells. After co-culture, the culture supernatant was discarded, and the cells were rinsed with sterile PBS. Then 300 μ L of RIPA lysis buffer (Fdbio Science, China) was added to each well, and the cells were lysed on ice; the cell lysate was collected subsequently, and

the protein concentration was determined using a BCA Protein Quantification Kit (Vazyme, China). Protein samples (6 μ g per sample, adjusted to a uniform loading volume of 15 μ L) were prepared for electrophoresis. For protein expression analysis, β -Actin was used as the internal reference. The primary antibodies against β -Actin, MCT1 and MCT4 (Proteintech, China) were used for incubation, respectively. For tumor tissue samples, total proteins were extracted using a Tissue Protein Extraction Kit (Fdbio Science, China).

Quantification of bacterial loads

Bacterial loads in tissues were quantified on days 2, 8, and 14 post-treatments. Major organs (heart, liver, spleen, lung, and kidney) and tumors were harvested and weighted. Approximately 0.1 g of each tissue was weighed, homogenized in 1 mL of sterile PBS, and subsequently filtered through a 60- μ m cell strainer. The homogenates were then subjected to serial 10-fold dilutions. A 10- μ L aliquot of each dilution was spotted onto LB-Kana agar plates. After overnight incubation at 30°C, plates were photographed, and bacterial colonies were counted to determine colony-forming units (CFU).

In vivo immune activation and memory detection

After the 14-day treatment period, lymph nodes, spleen and tumor tissues were collected from mice, ground, and analyzed using flow cytometry. Lymph nodes were stained with anti-CD11c APC, anti-CD86 PE, and anti-CD80 FITC antibodies. Lymphocytes infiltrating the tumor were stained with anti-CD3 FITC, anti-CD4 PE, and anti-CD8 α APC antibodies. Effector CD8⁺ T cells were stained with anti-CD3 FITC, anti-CD8 α PE, and anti-IFN- γ APC antibodies. Regulatory T cells (Tregs) were stained with anti-CD4 PE, anti-CD25 APC, and anti-Foxp3 FITC antibodies. Spleen memory T cell analysis staining antibodies used: anti-CD3 FITC, anti-CD8 α PE/anti-CD4 PE, anti-CD44 Pacific Blue, and anti-CD62L APC. All antibodies were sourced from BioLegend and all flow cytometry gating methods refer to previous articles [57].

ELISA

The culture supernatant from Transwell co-culture experiments was collected, and the ELISA was performed strictly following the manufacturer's instructions. For cytokine quantification in serum samples, mouse blood was collected and centrifuged at 4,000 rpm at 4°C to separate the serum. The levels of cytokines (IFN- γ , Granzyme B, TNF- α , and IL-6) in the serum supernatant were detected using commercial ELISA kits (GenXspan, China).

Biosafety assessment

Blood samples were collected from all experimental groups for comprehensive hematological and serum biochemical analyses (Nanjing Boshuo Biotechnology Co., Ltd, China). In detail, hematological profiling was conducted using an automated five-part differential hematology analyzer (MEK-8222K, Nihon Kohden). Serum biochemistry parameters were quantified with commercial assay kits (Jiancheng Bioengineering, Nanjing, China). Major organs (liver, spleen, kidneys, heart, lungs, tumor) were harvested, fixed in 10% neutral buffered formalin (24 h, 4°C), and processed through standard paraffin embedding. Sections were stained with hematoxylin and eosin (H&E) and examined by bright-field microscopy (Nikon Eclipse E100) (Nanjing Boshuo Biotechnology Co., Ltd, China).

Transcriptomic analysis

To analyze the global transcriptional regulation in engineered bacteria, total RNA was extracted from *S. oneidensis* samples (n = 3) induced with IPTG. Bacterial cells were collected by centrifugation at 4°C and 8000 $\times$ g, followed by immediate flash-freezing in liquid nitrogen. After total RNA extraction, ribosomal RNA was removed using a ribodepletion kit, and the remaining RNA was chemically fragmented. First-strand cDNA was synthesized from the fragmented RNA using random hexamer primers, and second-strand cDNA was synthesized with dUTP substituted for dTTP. Sequencing adapters were ligated, and the dUTP-containing second strand was selectively digested with uracil-DNA glycosylase to retain strand-specific information. The purified first-strand cDNA was subjected to end repair, poly(A) tailing, and adapter ligation, followed by size selection and PCR amplification to construct the sequencing library. Library quality was assessed using an Agilent 2100 Bioanalyzer, and paired-end sequencing was performed on an Illumina platform. The RNA libraries were sequenced by OE Biotech, Inc., (Shanghai, China) for assisting in sequencing and/or bioinformatics analysis.

Metabolomic analysis

To systematically characterize the metabolic phenotype of engineered bacteria, a widely-targeted metabolomic analysis was performed on IPTG-induced bacterial samples (n = 6) using ultra-high-performance liquid chromatography coupled with high-

resolution mass spectrometry (UPLC-HRMS). Bacterial cells were flash-frozen in liquid nitrogen, and metabolites were extracted with pre-cooled methanol: water (4:1, v/v). Analysis was conducted using a Waters ACQUITY UPLC I-Class plus/Thermo QE plus mass spectrometer. Differential metabolites were identified with a significance threshold of $P < 0.05$ (Student's *t*-test) and a variable importance in projection (VIP) value greater than 1. Metabolomic and data analyses were implemented by OE Biotech, Inc. (Shanghai, China).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 10 software was used for data analysis and visualization. The sample sizes to derive statistics and numbers of biologically independent experiments ($n \geq 3$) are provided in the figure legends. All data are presented as mean $\pm$ standard error of the mean (SEM). One-way or two-way analysis of variance (ANOVA) was used as appropriate, followed by Dunnett's multiple comparisons test for comparisons against the control group, or by Tukey's multiple comparisons test for pairwise comparisons among all groups. A P value < 0.05 was considered statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$).