Engineering genetic devices for in vivo control of therapeutic T cell activity triggered by the dietary molecule resveratrol

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Chimeric antigen receptor (CAR)–engineered T cell therapies have been recognized as powerful strategies in cancer immunotherapy; however, the clinical application of CAR-T is currently constrained by severe adverse effects in patients, caused by excessive cytotoxic activity and poor T cell control. Herein, we harnessed a dietary molecule resveratrol (RES)–responsive transactivator and a transrepressor to develop a repressible transgene expression (RES\textsubscript{rep}) device and an inducible transgene expression (RES\textsubscript{ind}) device, respectively. After optimization, these tools enabled the control of CAR expression and CAR-mediated antitumor function in vitro and in vivo. Our study demonstrates the utility of RESrep and RESind devices as effective tools for transgene expression and illustrates the potential of RES\textsubscript{rep}-CAR and RES\textsubscript{ind}-CAR devices to enhance patient safety in precision cancer immunotherapies.

T cell therapy with tumor-targeted chimeric antigen receptor (CAR)–engineered T cells has proven to be a transformative cancer treatment for a range of target indications (1, 2). CARs are constructed via a surface-displayed single-chain antibody variable domain (scFv) coupled to intracellular signaling components, to endow patient-derived T cells with the ability to recognize antigens and subsequently eliminate tumor cells (3). Ever since the approval of CD19-specific CAR T cells for the treatment of B cell leukemias and lymphomas, dramatic clinical successes have been achieved and numerous CAR T cell products are emerging (4–7).

Despite initial encouraging results, several challenges remain for CD19 CAR T cells, one main challenge being a lack of control over these engineered cells (8, 9). Rapid responses after large cell infusion doses can induce several immune-mediated side effects, such as cytokine release syndrome (CRS), tumor lysis syndrome, and neurotoxicity (10, 11). However, achieving potent therapeutic effects requires the robust expansion and sequential activation of a sufficient number of CAR T cells. Accordingly, it is a significant challenge to administrate appropriate doses of this “living drug” in order to balance in vivo efficacy and toxicity, further complicated by the highly divergent responses of patients (12–14).

Synthetic devices have been shown to improve the safety and feasibility of CAR T cell therapy by regulating CAR expression and controlling T cell activation (15, 16). To mitigate immunotherapy toxicity, several safety devices have been developed to control engineered T cells in a “function-off” or “function-on” manner. Previous studies have reported several user-controlled function-off devices to eliminate infused T cells from patients exhibiting severe toxicity, including suicide devices (17, 18), antibody-based elimination devices (9, 19, 20), small molecule-controlled STOP-CAR devices (21), and photoswitchable CAR devices (22). However, function-off devices may be insufficient to ensure safety against toxicities induced immediately upon cell infusion.

Complementary strategies using a function-on approach include several inducible devices with tunable CAR T cell function control: small molecule-inducible CAR devices (23–27), intermediary antibody or protein-based CAR devices (28–30), as well as light/ultrasound-responsive noninvasive devices (31–33). Synthetic Notch (synNotch) receptor-based circuits were also designed to enhance the precision of tumor recognition (34–38). Although these devices theoretically enable safer CAR T cell therapies, synthetic modules controlled by even safer natural molecules capable of diffusing throughout the entire body would offer a more versatile mechanism of control. Further development of orthogonally regulated devices that enable facile control of CAR T cell

Significance

Chimeric antigen receptor (CAR)–engineered T cell therapies have shown tremendous success in the clinic, but excessive cytotoxic activity and poor control over engineered T cells limit the application of CAR-T therapies. Here we have developed resveratrol (RES)–triggered regulation devices (on/off) that could be installed into CAR-T cells, which allow precise control over T cell activity through adjustment of RES dosage. We further demonstrated RES-inducible/repressible CAR expression and reversible control over T cell activation via a RES-titratable mechanism. Our results reveal that RES\textsubscript{ind}-CAR T cells can be dose-dependently activated by RES with strong anticancer cytotoxicity. Our RES-controlled systems establish proof of concept for strategies to control cancer immunotherapies based on the RES-regulated repression/induction of therapeutic immune cells.

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Competing interest statement: H.Y., L.Y., and J.Y. are inventors of patent applications (Chinese patent application no. 202110671259.9) submitted by East China Normal University that covers the RES\textsubscript{rep} and RES\textsubscript{ind} devices. This article is a PNAS Direct Submission.

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functions to ensure safer device control in immunotherapy is needed.

The stilbenoid natural product resveratrol (RES, 3,4′,5-trihydroxystilbene), notably present in red wine, grapes, peanuts, and berries, has been shown to be a safe inducer compound for genetic devices designed for therapeutic applications (39). Beyond its demonstrated utility in genetic engineering applications, this molecule possesses wide-ranging beneficial effects, including clinical evidence for activities against aging, cancer, cardiovascular diseases, and inflammation (40–43).

To enable safer strategies for cancer immunotherapy with both a function-off and function-on modality, we designed a resveratrol-repressible transgene expression (RESrep) device and a resveratrol-inducible transgene expression (RESind) device on resveratrol-responsive regulatory elements. We demonstrate that our RESrep and RESind devices can reversibly and flexibly control transgene expression in vitro and in vivo in a highly tunable manner. We further apply these devices to control CAR expression and CAR-mediated cell activities by developing a resveratrol-repressible CAR expression (RESrep-CAR) device and a resveratrol-inducible transgene expression (RESind-CAR) device. We then demonstrate that the RESrep-CAR device can repress CAR expression and CAR-mediated cell activation in primary human T cells and a xenograft mouse model of leukemia, all with triggerable T cell inactivity in case of severe toxicity. We further confirm the titratable and reversible capabilities of the RESrep-CAR device for both CAR expression and T cell activity in vitro and in vivo; this is a feature that could allow physicians to precisely control T cell activity by adjusting the dosage of resveratrol according to the clinical responses of individual patients. These resveratrol-controlled transgene expression devices extend synthetic biology-inspired tools to clinically relevant applications and provide potential strategies for improving the safety of T cell immunotherapies by preventing fatal toxicity in clinical settings.

Results
Design, Construction, and Characterization of an RESrep Device in Mammalian Cells and Mice. Capitalizing on resveratrol-responsive TtgR, a specific transcriptional repressor of efflux pump (TtgABC) in Pseudomonas putida (44), we engineered a RESrep device in mammalian cells (Fig. 1A). Our designed RESrep device consists of a resveratrol-dependent transactivator ResA3 (TtgR-VPR) that we fused to a synthetic activator VPR (comprising three transcriptional activators: VP16, VP64, and Rta) (45) via the C terminus of TtgR. The chimeric transactivator can bind to the resveratrol-dependent promoter PResA1, which contains a ResA3 binding site (OTrc1; reverse complement and site-directional mutation of OTrg) positioned in front of a minimal human cytomegalovirus immediate-early promoter (PcmvMin); Transactivator binding results in recruitment of the transcription initiation complex to trigger transgene expression; subsequently, resveratrol releases ResA3 from PResA1, thereby silencing transgene expression (Fig. 1A). We initially optimized the synthetic transactivator ResA by fusing TtgR to different transcriptional domains (VP16, VP64, and VPR) driven by Psv40 (simian virus 40 promoter) or Pcmv (human cytomegalovirus promoter; SI Appendix, Fig. S1 A–H) as well as optimizing the promoter PResA with various iterations of OTrc1 tandem repeats (SI Appendix, Fig. S1 I–N). Ultimately, we found that a combination of Pcmv-driven ResA3 and Pcmv-driven secreted human placental alkaline phosphatase (SEAP) demonstrated the best induction performance; this version of the RESrep device also achieved significant induction performance in five human cell lines (Fig. 1B). Importantly, assessment of resveratrol-mediated toxicity in mammalian cells showed that resveratrol had no negative effect on cell viability (SI Appendix, Fig. S2), using resveratrol concentrations of 0 to 50 μM.

We next explored the potential of the RESrep device for cell/gene therapy, by stably integrating the RESrep device driving SEAP into HEK293 cells. Although the device achieved success across a series of clonal cell lines, the best inhibition (~15.5-fold) was in a monoclonal HEKRESrep-SEAP cell line (Fig. 1C). The HEKRESrep-SEAP cell line showed excellent resveratrol dose- and time-dependent transgene expression (Fig. 1D). Moreover, this cell line exhibited sustained output over a 14-d culture (Fig. 1E) and displayed fully reversible transgene expression kinetics (Fig. 1F).

To enable safer strategies for cancer immunotherapy with both a function-off and function-on modality, we designed a resveratrol-repressible transgene expression (RESrep) device and a resveratrol-inducible transgene expression (RESind) device on resveratrol-responsive regulatory elements. We demonstrate that our RESrep and RESind devices can reversibly and flexibly control transgene expression in vitro and in vivo in a highly tunable manner. We further apply these devices to control CAR expression and CAR-mediated cell activities by developing a resveratrol-repressible CAR expression (RESrep-CAR) device and a resveratrol-inducible transgene expression (RESind-CAR) device. We then demonstrate that the RESrep-CAR device can repress CAR expression and CAR-mediated cell activation in primary human T cells and a xenograft mouse model of leukemia, all with triggerable T cell inactivity in case of severe toxicity. We further confirm the titratable and reversible capabilities of the RESrep-CAR device for both CAR expression and T cell activity in vitro and in vivo; this is a feature that could allow physicians to precisely control T cell activity by adjusting the dosage of resveratrol according to the clinical responses of the patients. These resveratrol-controlled transgene expression devices extend synthetic biology-inspired tools to clinically relevant applications and provide potential strategies for improving the safety of T cell immunotherapies by preventing fatal toxicity in clinical settings.

Characterization of Resveratrol-Repressible CAR Expression (RESrep-CAR) and CAR-Mediated T Cell Activity. Given previous studies reporting that resveratrol exerts potent antitumor effects against some cancers (46, 47) and may enhance human T cell activity (48, 49), we profiled the effects of resveratrol on the Jurkat T cells that we planned to use as the recipient cells for our devices to exclude any unanticipated impacts from the induction/repression agent (resveratrol). A total of 30 μM resveratrol had no significant effect on CAR expression or CD19 antigen-induced activation of unmodified Jurkat T cells (negative control) or conventional CAR-engineered Jurkat T cells (positive control; SI Appendix, Fig. S3).

We next developed a RESrep-CAR device to inhibit CAR expression in engineered Jurkat T cells and primary T cells; this device was capable of selectively inactivating its therapeutic effects in the presence of resveratrol (Fig. 2A). First, we optimized the RESrep-CAR device in Jurkat T cells (JurkatRESrep-CAR) and found that a combination of PmPGK (mouse phosphoglycerate kinase gene promoter)-driven ResA3 and PResA1-driven CAR-P2A-EGFP achieved the best resveratrol-mediated performance in Jurkat T cells (SI Appendix, Fig. S4). These JurkatRESrep-CAR cells, which were transduced with the optimal RESrep-CAR device, exhibited dose-dependent resveratrol-repressible CAR expression (Fig. 2B). These responses include CD69 expression (a general readout for effector T cell activation; Fig. 2C) and hIL-2 production (a general readout for effector T cell activation; Fig. 2D). Moreover, the RESrep-CAR device enables fully reversible CAR expression in Jurkat T cells (Fig. 2E). We also found that the presence of resveratrol reduced the extent that the CD19 antigen stimulated the activation of the JurkatRESrep-CAR. These results demonstrate that the RESrep device can fine tune the repression of both CAR expression levels and the CAR-mediated cell activation of Jurkat T cells.

We subsequently optimized the RESrep-CAR device in primary human T cells, which required the development of a particularly resveratrol-sensitive TtgR mutant (H67A) (50). The combination of PmPGK-driven ResA3 (TtgRH67A-VPR) and PResA1-driven CAR-P2A-EGFP achieved excellent transgene inhibition upon incubation with 15 μM resveratrol in primary human T cells (SI Appendix, Fig. S5). These RESrep-CAR T cells derived from primary human T cells exhibited titrable resveratrol-repressed CAR expression (Fig. 2F) as well as excellent CAR-regulated cell deactivation upon CD19 antigen stimulation in the presence of increased concentration of resveratrol, assessed via hIL-2 (Fig. 2G), and hIFN-γ (Fig. 2H) expression.

To test the cytotoxic performance of the engineered primary human T cells, we labeled CD19+ K562 cells with mCherry (CD19+ K562mCherry) and CD19− K562 cells with EGFP (CD19− K562EGFP) and then mixed them as a population of two target cell types that could be identified and measured by flow cytometry (SI Appendix, Fig. S6). The cytotoxic activity of the...
CARs were quantified as the selective reduction of CD19+ T cells equipped with RESrep-CAR into the mice. Subsequently, human primary T cells (negative control) or engineered human and dendritic cell function. After 10 h, we injected i.p. unmodified B, and natural killer [NK] cells, along with reduced macrophage viability.

We investigated whether the cytotoxic activity of RESrep-CAR T cells could be regulated by resveratrol in mice, we evaluated target cell lethality and cytokine release in a rapid xenograft mouse tumor model of B cell leukemia (23) (Fig. 3). In this model, we implanted C57BL/6J mice implanted with 2 × 10⁶ K562EGFP cells into NCG mice (NOD−Prkdcem26Cd52Il2rgem26Cd22−/−) (SI Appendix, Fig. S7). Importantly, we confirmed that 15 μM resveratrol alone did not have any significant effects on either target cell lethality or cytokine production of non-RESrep regulated primary T cells modified with conventional CAR (SI Appendix, Fig. S8). In summary, we successfully constructed a RESrep-CAR device for fine tuning CAR expression and applied it in Jurkat T cells and primary T cells.

Anticancer Performance of RESrep-CAR T Cells in NCG Mice. To investigate whether the cytotoxic activity of RESrep-CAR T cells could be regulated by resveratrol in mice, we evaluated target cell lethality and cytokine release in a rapid xenograft mouse tumor model of B cell leukemia (23) (Fig. 3). In this model, we implanted a mixture of CD19+ K562mCherry and CD19+ K562EGFP cells into the peritoneal cavity of NCG mice (NOD-Prkdcem26Cd52Il2rgem26Cd22−/− NjuCrl: triple immunodeficiency and lacking functional/mature T, B, and natural killer [NK] cells, along with reduced macrophage and dendritic cell function). After 10 h, we injected i.p. unmodified human primary T cells (negative control) or engineered human T cells equipped with RESrep-CAR into the mice. Subsequently, we administered various doses of resveratrol (50, 100, or 200 mg/kg/d) or vehicle control by i.p. injection at the indicated times. Mice were killed after 48 h for peritoneal lavage collection, from which recovered cells were analyzed by flow cytometry; serum cytokine release analysis occurred at 24 h and 48 h (Fig. 3A).

Mice injected with RESrep-CAR T cells showed resveratrol-repressible depletion of the CD19+ K562mCherry cell population upon treatment with different resveratrol dosages; no depletion was observed for mice injected with control T cells (Fig. 3B). Further, comparison of the two dosages showed that serum cytokine levels (hIL-2 and hTNF) and extent of target cytotoxicity were tunable based on in vivo resveratrol concentration (Fig. 3 C–G). We confirmed that the high dosage of resveratrol (200 mg/kg/d) had no significant effect on the cytotoxicity capability in the positive control CAR T cells transplanted into mice (SI Appendix, Fig. S9). In summation, RESrep-CAR T cells were able to achieve tunable cancer cell lethality in a mouse leukemia model.

Design, Construction, and Characterization of a RESind Transgene Expression Device in Mammalian Cells and Mice. Our RESrep-CAR T cells enable a strategy for negatively regulating T cell function, an attractive approach when the clinical response from a constitutively activated cell type requires inactivation; nevertheless, a RESind-CAR expression device should enable fine tuning therapeutic activity in a function-on manner. Pursuing this, we engineered a resveratrol-inducible on device (RESind) for controlling transgene expression in mammalian cells. Our designed RESind device comprises a resveratrol-dependent transrepressor ResR1 (Tgr-R-KRAB), based on fusing a Krüppel-associated box protein (KRAB) to the C terminus of TgrR; this can bind to the synthetic resveratrol-inducible promoter (PResind), harboring a constitutive expression promoter embedded with transrepressor ResR1 binding sites (OTRCl, a variant of OTtgR), thereby repressing transgene expression. Resveratrol releases transrepressor ResR1 from PResind and induces transgene expression (Fig. 4A).
To obtain the envisioned RESind device with minimal leakage in the absence of resveratrol and strong resveratrol-induced transgene expression, we optimized the RESind device by testing different OTtgR variants, different variants of resveratrol-responsive promoters, and different strengths of constitutive promoters for driving TgR expression (SI Appendix, Figs. S10 and S11). Due to its superior induction performance, the variant OTtgR (reverse complement and site-directional mutation of OTtgR) was initially chosen as the optimal binding sequence for the subsequent optimization of resveratrol-responsive promoters. Ultimately, we determined that a combination of PhCMV-driven TgR with the resveratrol-responsive promoter PResR4 (OTtgR1745/OTtgR1745) exhibited optimal induction performance in HEK293 cells (SI Appendix, Fig. S11O), and this version of the RESind device also achieved significant induction performance across five human cell lines (Fig. 4B) and strong time-course–dependent performance (SI Appendix, Fig. S12).

As in our characterization of the RESrep device in assessing suitability for cell/gene therapy, we evaluated the most monocular HEKRESind-SEAP Cell line (378-fold induction; Fig. 4C), from the examined clones and found that it showed excellent resveratrol dose- and time-dependent transgene expression (Fig. 4D). Moreover, this cell line exhibited sustained resveratrol-induced transgene output throughout a 14-d culture (Fig. 4E) and fully resveratrol-reversible transgene expression kinetics (Fig. 4F).

We then microencapsulated HEKRESind-SEAP cells and implanted them into mice to assess resveratrol-induced in vivo transgene expression from the RESind device. After mice were intraperitoneally administrated with resveratrol at doses ranging from 0 to 250 mg/kg daily, we observed that resveratrol could control SEAP production in a dose-dependent manner (Fig. 4G), over an induction window of 15 d (Fig. 4H). These results confirm that the RESind device can fine tune transgene induction in mammalian cells and in mice.

Seeking to decrease the amount of RES needed to trigger the RESind device, and ultimately aiming to achieve controlled transgene expression based on the oral administration of resveratrol, we attempted to further improve the sensitivity of our RESind device by testing various mutants of the TtgR protein (SI Appendix, Fig. S13). The RESind-mut device proved to be substantially more sensitive to resveratrol than our initial device. The combination of PhCMV-driven ResR4 (TtgR1671A–KRB) and PResR4–driven SEAP exhibited the best induction performance in HEK293 cells with dose- and time-course–dependent induction kinetics, especially at lower resveratrol concentrations (0 to 5 μM; SI Appendix, Fig. S13 A and B). We next constructed and selected a highly sensitive and inducible isogenic cell clone that allowed for the RES-inducible transgene expression of SEAP (HEKRESind-mut–SEAP). The RESind-mut device continued to show superior transgene induction at low resveratrol concentrations (0 to 6 μM; SI Appendix, Fig. S13C). When the microencapsulated HEKRESind-mut–SEAP cells were implanted into mice, regardless of delivery method (intraperitoneal injection or oral intake of resveratrol), resveratrol controlled transgene expression in a dose-dependent manner (SI Appendix, Fig. S13 D–G). These results demonstrate that the RESind-mut device is successful in being more sensitive to resveratrol.
We next developed a RESind-CAR expression device to positively regulate CAR expression in engineered Jurkat T cells and primary T cells; this device was capable of selectively activating therapeutic effects in the presence of resveratrol and upon CD19 antigen stimulation (Fig. 5A).

To characterize the performance of the RESind-CAR device in T cells and obtain an optimal RESind-CAR device, with low transgene expression leakage in the absence of resveratrol and high induction rates in the presence of resveratrol, we used the RESind-CAR device to control the transcriptional activation of a transgene cassette, comprising both enhanced green fluorescent protein (EGFP) and CAR, and optimized the resveratrol-inducible CAR-P2A-EGFP expression device in Jurkat T cells (JurkatRES-ind-CAR-EGFP) by measuring the EGFP expression level. Guided by a previous study (51), we optimized the resveratrol-inducible promoter and the constitutive promoters for driving ResR1 expression with P<sub>mPGK</sub> and P<sub>hEF1α</sub> (human extension factor).

**Tunability and Kinetics of RES<sub>ind</sub>-CAR Expression in Jurkat T Cells.** We next developed a RES<sub>ind</sub>-CAR expression device to positively regulate CAR expression in engineered Jurkat T cells and primary T cells; this device was capable of selectively activating therapeutic effects in the presence of resveratrol and upon CD19 antigen stimulation (Fig. 5A).

To characterize the performance of the RES<sub>ind</sub>-CAR device in T cells and obtain an optimal RES<sub>ind</sub>-CAR device, with low transgene expression leakage in the absence of resveratrol and high induction rates in the presence of resveratrol, we used the RES<sub>ind</sub>-CAR device to control the transcriptional activation of a transgene cassette, comprising both enhanced green fluorescent protein (EGFP) and CAR, and optimized the resveratrol-inducible CAR-P2A-EGFP expression device in Jurkat T cells (JurkatRES-ind-CAR-EGFP) by measuring the EGFP expression level. Guided by a previous study (51), we optimized the resveratrol-inducible promoter and the constitutive promoters for driving ResR1 expression with P<sub>mPGK</sub> and P<sub>hEF1α</sub> (human extension factor).

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**Fig. 3.** Resveratrol-repressible activation of primary human T cells engineered with RES<sub>rep</sub>-CAR in NCG mice. (A) Experimental design for in vivo assessment of resveratrol-controlled RES<sub>rep</sub>-CAR T cell-mediated cytotoxicity against K562 cells in a rapid xenograft mouse tumor model. NCG mice inoculated with 3 × 10<sup>7</sup> mixed K562 cells (CD19<sup>−</sup> K562<sub>EGFP</sub> Cells:CD19<sup>+</sup> K562<sub>mCherry</sub> Cells = 1:1) were injected intraperitoneally with 2 × 10<sup>7</sup> unmodified primary human T cells (control) or RES<sub>rep</sub>-CAR T cells, followed by the administration of various resveratrol doses (50, 100, or 200 mg/kg/d) or vehicle control at the indicated times. After 48 h, blood was drawn for cytokine examination and peritoneal cells were quantified by flow cytometry to measure in vivo cytotoxicity. (B) Representative flow cytometry data showing the resveratrol-repressible depletion of the CD19<sup>+</sup> K562<sub>mCherry</sub> cell population. (C) Quantified target cytotoxicity results for all experimental groups. Cytotoxicity values were calculated from the ratios of CD19<sup>+</sup> K562<sub>mCherry</sub> cells to CD19<sup>−</sup> K562<sub>EGFP</sub> cells. n = 4 mice. (D–G) In vivo hIL-2 (D and F) and hTNF (E and G) release at 24 h and 48 h. The data in C–G are mean ± SEM. P values were calculated with Student’s t test (n = 4 mice). *P < 0.05; **P < 0.01; ***P < 0.001 versus control.
JurkatRES-ind-CAR-EGFP cells, which express CAR in a resveratrol-EGFP showed the best induction performance in Jurkat T cells (Appendix and CAR-mediated cell activation in Jurkat T cells. An optimal RESind-CAR-EGFP device, exhibited excellent resveratrol with pLF50 (ITR-PResR12-SEAP-pA::PhCMV-TtgR-KRAB-P2A-PuroR-pA-ITR) and the transposase expression vector pCMV-T7-SB100 (PhCMV-SB100X-pA). The selected cell clones were profiled for their resveratrol-inducible SEAP regulation performance. The blue frame marks the best-in-class clone chosen for the following experiments. (D) Resveratrol dose-dependent SEAP production in HEKRES-ind-SEAP cells. Different color bars represent different time periods for profiling SEAP production. (E) Long-term SEAP production kinetics in HEKRES-ind-SEAP stable cells. (F) Reversibility of HEKRES-ind-SEAP-mediated SEAP production. HEKRES-ind-SEAP cells were cultivated for 6 d while altering the resveratrol concentrations from 0 to 20 μM. Cell density was adjusted to 5 × 10^4 every 48 h and SEAP production was profiled every 12 h. (G) Dose-dependent resveratrol-inducible SEAP production in mice. C57BL/6J mice implanted with 2 × 10^6 microencapsulated HEKRES-ind-SEAP cells were intraperitoneally injected with resveratrol once a day (0 to 250 mg/kg/d). SEAP production in blood was profiled after 48 h. (H) Long-term resveratrol-inducible SEAP production kinetics in mice. SEAP production was profiled on days 2, 4, 6, and 15. The data in B–F are mean ± SD, n ≥ 3 independent experiments. The animal data G and H are mean ± SEM. P values were calculated with Student’s t test (n = 6 mice). **p < 0.01; ***p < 0.001 versus control.

α gene promoter). We found that a combination of P_mPGK-driven ResR1 and P_resR1 (O_TCR1-P_EF1a-O_TCR1)-driven CAR-P2A-EGFP showed the best induction performance in Jurkat T cells (SI Appendix, Fig. S14).

JurkatRES-ind-CAR-EGFP cells, which were transduced with the optimal RESind-CAR-EGFP device, exhibited excellent resveratrol-dose-dependent control of CAR and EGFP expression, with extremely strong transgene induction at a low (15 μM) resveratrol concentration (Fig. S5B). Moreover, by observing changes in EGFP expression via flow cytometry, we found that JurkatRES-ind-CAR-EGFP cells possessed excellent long-time induction performance with negligible leakage and nonsaturating increases in EGFP expression over 14 d (Fig. 5C and SI Appendix, Fig. S15). Moreover, experiments in which we periodically added then removed resveratrol demonstrated that the device enables fully reversible transgene expression (Fig. 5 D and E and SI Appendix, Fig. S16). We also verified that the induced JurkatRES-ind-CAR-EGFP cells, which express CAR in a resveratrol-dose-dependent manner, could be activated by CD19-positive K562 cells (CD19^+ K562mCherry), as assessed by CD69 induction (Fig. 5F) and hIL-2 production (Fig. 5G). We also observed that the RESind-CAR device showed CD19 antibody density-dependent response in Jurkat T cells (SI Appendix, Fig. S17). These results demonstrate that the RESind device can fine tune CAR expression and CAR-mediated cell activation in Jurkat T cells.

Resveratrol-Inducible CAR Expression in Primary Human T Cells. Next, we further optimized the RESind-CAR device in primary human T cells with different configurations of resveratrol-inducible promoters and various mutants of the TtgR protein (SI Appendix, Figs. S18 and S19) guided by data from previously reported studies (44, 50, 52). A combination of P_EF1a-driven ResR4 (TtgRH67A-KRAB) and P_resR15 (O_TCR1-P_EF1a-O_TCR1)-driven CAR showed the best induction performance and was used for further studies in primary T cells.

Primary T cells engineered with the optimized RESind-CAR device exhibited titratable CAR expression controlled by resveratrol (Fig. 5H) and resveratrol-controlled CAR-related cell activation upon antigen stimulation, as assessed by hIL-2 production (Fig. 5I) and hIFN-γ production (Fig. 5J). Cytotoxicity assays demonstrated that the cytotoxic efficacy of RESind-CAR T cells against CD19^+ K562mCherry is excellent and is tunable on resveratrol dosage (Fig. 5K). Moreover, CAR-induced T cell activation can be mediated by the RESind-CAR device in a time-dependent manner as well (SI Appendix, Fig. S20). In summary, we generated a RESind-CAR device to control transgene induction, CAR T cell activation, and cancer cell lethality in T cells.

Resveratrol-Controlled Cancer Cell Lethality Mediated by RESind-CAR T Cells in NCG Mice. To evaluate antitumor efficacy mediated by the RESind-CAR device in vivo, we established two xenograft tumor mouse models. We first investigated whether the activity of RESind-CAR T cells could be regulated by resveratrol in a dose-dependent manner in a rapid xenograft mouse model of B cell leukemia (Fig. 6). In this model, unmodified T cells (Ctrl) or RESind-CAR T cells were injected i.p. after NCG mice were implanted with a mixture of CD19^+ K562mCherry and CD19^-K562EGFP cells. These mice were then administrated with various...
RESind-CAR T cells

A

RESind-CAR T cells

Inactive

-RES

Active

CD19*

K562

CD19*

K562mCherry

CAR

Expression

- Target cell killing

CD69 expression

Cytokine production

Activation

CD19* K562 cells

CD19* K562mCherry cells

Fig. 5. Resveratrol-titratable activation of Jurkat T and primary T cells engineered with RESind-CAR. (A) Schematic of the RESind-CAR device. Jurkat T cells or human primary T cells were engineered with lentivirus to express RESind-CAR. In the absence of resveratrol, CAR expression was repressed and T cells were insensitive to antigen-mediated activation. The presence of resveratrol induced CAR expression, and these CAR+ T cells were activated by antigen-positive (CD19+) K562 target cells (assessed based on cytokine production, CD69 expression, and cell killing assays). (B) Dose-dependent resveratrol-inducible CAR and EGFP expression in Jurkat T cells (JurkatRESind-CAR-EGFP). JurkatRESind-CAR-EGFP engineered with lentiviral pLF355 (LTR-PResR15-CAR-LTR) and pLF64 (LTR-PmPGK-ResR4-LTR) were cultivated with different concentrations of resveratrol for 48 h. Expression levels of CAR and EGFP were quantified by flow cytometry. (C) Long-term JurkatRESind-CAR-EGFP-mediated EGFP expression. (D and E) Reversibility of JurkatRESind-CAR-EGFP-mediated EGFP expression. Cells were cultivated without (gray rectangles) or with 20 μM resveratrol (blue rectangles) periodically; EGFP expression was profiled every 12 h. Cell density was adjusted to 1 × 10^6 cells/mL every 48 h. (F and G) Resveratrol and antigen-triggered JurkatRESind-CAR-EGFP cell activation. Cells were cultivated with various concentrations of resveratrol for 48 h and then incubated with CD19+ K562EGFP cells (white squares) or CD19+ K562mCherry cells (red squares) at an E:T ratio of 2:1. After overnight incubation, the surface expression of the T cell activation markers CD69 (F) and hIL-2 secretion (G) were quantified. (H) Dose-dependent CAR surface expression of the optimized RESind-CAR device in human primary T cells (RESind-CAR T cells). RESind-CAR T cells engineered with lentiviral pLF355 (LTR-PResR15-CAR-LTR) and pLF361 (LTR-PmPGK-ResR4-LTR) were cultivated in medium containing 0 to 15 μM resveratrol, and CAR expression was quantified by flow cytometry after 48 h. (I and J) Resveratrol-controlled cytokine release from RESind-CAR T cells. RESind-CAR T cells were cultivated with various concentrations of resveratrol for 48 h and then incubated with CD19+ K562EGFP cells (white squares) or CD19+ K562mCherry cells (red squares) at an E:T ratio of 5:1. hIL-2 (I) and hIFN-γ (J) production were quantified after overnight incubation. (K) Resveratrol-inducible target cytotoxicity of RESind-CAR T cells. Cells were cultivated with various concentrations of resveratrol for 48 h and then incubated with a mixture of target cells containing CD19+ K562EGFP cells and CD19+ K562mCherry cells at an E:T ratio of 10:1. After 18 h, cells were collected for flow cytometry and cytotoxicity values were calculated based on ratios of CD19+ K562mCherry cells to CD19+ K562EGFP cells. The data in B–K are mean ± SD, n = 3. N.D., not detectable.

doses of resveratrol (5 to 100 mg/kg/d) or vehicle control. Serum cytokine release was analyzed every 24 h and the cytotoxicity of RESind-CAR T cells was quantified by flow cytometry at 48 h (Fig. 6A). Mice injected with RESind-CAR T cells showed resveratrol-dependent depletion of the CD19+ K562mCherry cell population (Fig. 6B). RESind-CAR T cells killed target cells in a resveratrol-inducible manner, with the strongest cytotoxicity observed with a resveratrol dosage of 10 mg/kg/d (Fig. 6C). Consistently, monitoring in vivo hIL-2 and hTNF cytokine release demonstrated that the activity of RESind-CAR T cells was tunable to the resveratrol concentration (Fig. 6D–G).

We further assessed antitumor efficacy mediated by RESind-CAR T cells in an aggressive leukemia xenograft model in NSG mice (Fig. 7). We engrafted mice with Renilla luciferase (Rluc)–expressing CD19+ K562 cells intravenously. After 5 d, mice received control unmodified T cells (Ctrl), conventional CAR T cells (Conv. CAR), or RESind-CAR T cells intravenously, followed by daily intraperitoneal (50 mg/kg) or oral (250 mg/kg) resveratrol (Fig. 7A). Serum cytokines were quantified after T cell infusion on day 3 and significant secretion of hIL-2 and hTNF was found in mice infused with RESind-CAR T cells and resveratrol (Fig. 7B and C). Additionally, tumor burden was demonstrated by bioluminescence imaging, and the results demonstrated that tumor growth was significantly suppressed in mice that received RESind-CAR T cells with resveratrol treatment for 28 d (Fig. 7D–F). A similar suppression was seen in mice that received conventional CAR T cells. The observed tumor flux in mice that received the RESind-CAR T cells in the presence of resveratrol was significantly lower than those that received the RESind-CAR T cells in the absence of resveratrol (Fig. 7F). In contrast, tumor flux in mice that received RESind-CAR T cells in the absence of resveratrol increased similarly to mice that received unmodified T cells. Furthermore, we observed prolonged survival in mice infused with RESind-CAR T cells and resveratrol (Fig. 7G).

Collectively, these results demonstrate that the cytotoxic activity of RESind-CAR T cells against tumor cells can be fine
tuned by resveratrol in mice, suggesting potential applications for controlling CAR T cell activity in patients.

**Discussion**

Here, we report the development of resveratrol-controlled transgene expression devices (RES<sub>reg</sub> and RES<sub>ind</sub>) based on the TtgR regulatory device to precisely control transgene expression in mammalian cells and mice. We further demonstrate that the RES<sub>reg</sub> and RES<sub>ind</sub> devices are effective in controlling CAR expression in T cells to guide the recognition and eradication of tumor cells, both in vitro and in vivo. These resveratrol-controlled devices establish a proof of concept for cancer immunotherapy strategies based on the resveratrol-regulated repression or induction of immune cells.

In recent years, synthetic biology-inspired therapeutic programs have achieved favorable outcomes in the treatment of various diseases, including diabetes (53–55), insulin resistance (56), liver disorders (57), bacterial infections (58), and cancer (59–62); several of these treatments require effective, long-term therapeutic outputs, achieved by the sustained delivery of trigger molecules.
molecules. The natural dietary molecule resveratrol, an antioxidant in red wine, has been a part of the human diet for thousands of years and is generally recognized as safe; resveratrol has already seen use in cosmetics and some healthcare products. This study demonstrates that RESind and RESrep devices can achieve titratable, reversible, and long-term control of low concentration resveratrol-regulated transgene expression in mammalian cells and mice.

CAR T cell therapy is a paradigm-shifting therapeutic approach for cancer treatment that is achieving tremendous success in the clinic; however, its increasing utilization is revealing many unpredictable and/or uncontrollable side effects from on-target/off-tumor toxicity, including severe CRS (sCRS) and neurotoxicity (13), tumor lysis syndrome, and the eradication of normal cell types expressing low levels of an antigen (63). Our RESrep-CAR expression device can be used to block the activity of infused CAR T cells, potentially being deployed to prevent toxicity-associated morbidity and mortality when patients are considered at a high risk for clinical complications in early-stage CAR T therapy. We verified that T cells engineered with RESrep-CAR provide effective and titratable inhibition over T cell activation, while retaining their inducible therapeutic efficacy upon resveratrol removal. The pattern and extent of functional inhibition achieved by resveratrol in our study highlights the clinical potential of RESrep-CAR.

We envision the RESind-CAR device to be implemented with patients who are in danger of developing severe responses to cell...
infusion. Our results demonstrate that T cells engineered with RESrep-CAR can fine tune CAR expression levels and associated CAR-mediated cell activation, in vitro and in vivo, by adjusting administered resveratrol concentrations. In practice, the use of RESrep-CAR in clinical trials could be coincident with monitoring cell infiltration toxicity responses and real-time CAR T cells efficacy, enabling ongoing resveratrol-mediated CAR T cell activity adjustment to achieve highly precise, personalized, therapeutic outcomes.

Despite the excellent regulation performance of resveratrol-controlled transgene expression devices in vitro and in vivo, there is still room for improvement from a translational perspective. For example, given the low bioavailability of resveratrol by oral administration, the response sensitivity of resveratrol-controlled devices will require further engineering and optimization. We anticipate that more sensitive devices could be obtained by optimizing the operator sequences and directed evolution approaches, or via rational protein design to alter the transactivator and/or transrepressor properties. Furthermore, combination with our previously engineered and orthogonal systems (53) could enable multilayered control and even safer therapies.

With its broad potential uses, promising therapeutic impact for numerous diseases, and nutraceutical properties, resveratrol is a particularly suitable trigger molecule for clinical applications. Our work shows that resveratrol-controlled genetic devices can expand the toolbox of cybergenetics and highlights them as a safe, robust, and convenient strategy for the dynamic control of therapeutic outputs for future gene- and cell-based precision medicine. Considering that the resveratrol-responsive TtgR, a transcriptional repressor from

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might present potential immunomodulatory, the potential immunogenicity of RESrep-CAR or RESrep-CAR needs to be further evaluated before clinical trials. In conclusion, our proof of concept and initial in vivo application of two resveratrol-sensitive devices for fine tuning the activity of transgene expression and CAR T cell therapeutic activity illustrates how safe dietary molecules can usher in a new era of precision oncoimmunotherapies.

**Materials and Methods**

**Generation of Stable Cell Lines.** The HEKRESrep-SEAP cell line, transgenic for resveratrol-repressed SEAP expression, was constructed by cotransfecting 5 \( \times 10^5 \) HEK293 cells with 190 ng of pLF51 (LTR-Tet-O1×α-EGFP-LTR) and 10 ng of Sleeping Beauty transposase expression vector pCMV-T7-SB100 (P\text{CMV}\_SB100X-pA) and 100 μg/mL polybrene. The HEKRESrep-SEAP cell line, transgenic for resveratrol-triggered SEAP expression, was constructed by cotransfecting 5 \( \times 10^5 \) HEK293 cells with 190 ng of pLF50 (LTR-P\text{resR12×α}-SEAP-pA/P\text{CMV}\_SB100X-pA) and 100 μg/mL polybrene. The HEKRESind-SEAP cell line, transgenic for resveratrol-triggered SEAP expression, was constructed by cotransfecting 5 \( \times 10^5 \) HEK293 cells with 190 ng of pLF365 (P\text{resR12×α}-SEAP-pA/P\text{CMV}\_SB100X-pA). The HEKRESind-SEAP cell line, transgenic for resveratrol-triggered SEAP expression, was cotransduced 10\(^{4}\) Jurkat T cells with two lentiviruses: plF63 (LTR-P\text{resR12×α}-Res-LTR) and plF64 (LTR-P\text{resR12×α}-P2A-GFP-LTR). Infected Jurkat T cells were cultured for 5 d and then incubated with 10 μM resveratrol for 24 h. EGFP-positive cells were selected based on EGFP fluorescence intensity by FACs-mediated cell sorting using a Becton Dickinson FACSAria Cell Sorter (BD Biosciences). After the EGFP selection, the transduced Jurkat T cells were expanded for further experiments. CD19-negative K562 target cells, labeled with the EGFP (CD19\(^{-}\) K562egfp) cell line or CD19-positive K562 target cells labeled with the mCherry (CD19\(^{+}\) K562mCherry) cell line, were transduced by cotransducing 1 \( \times 10^5 \) K562 cells with lentiviral plF367 (LTR-P\text{resR12×α}-Rluc-P2A-Puro-LTR) and the Rluc-expressing cell sorting using a Becton Dickinson FACSAria Cell Sorter (Becton Dickinson). K562 cells were expanded and selected on EGFP or mCherry fluorescence intensities by FACs-mediated cell sorting using a Becton Dickinson FACSAria Cell Sorter (Becton Dickinson).

**Cytotoxicity Assay of RESrep-CAR or RESrep-CAR Devices in Engineered Primary Human T Cells.** CD19\(^{+}\) K562mCherry and CD19\(^{-}\) K562egfp target cells were mixed at a 1:1 ratio and then incubated with primary human T cells expressing RESrep-CAR or RESrep-CAR at an E:T ratio of 10:1 (1 \( \times 10^5 \) cells:1 \( \times 10^5 \) cells) in 48-well flat-bottom plates. After overnight incubation, cells were washed in cell staining buffer and stained with a Fixable Viability Dye eFluor 660. Stained cells were washed three times and analyzed by BD LSRFortessa flow cytometry. Singlets were gated using forward versus side scatter and followed by gating based on forward scatter area (FSC-H) versus forward scatter height (FSC-A) characteristics. Live cells were gated via the allophycocyanin (APC) channel, and K562 target cells were gated by excluding T cells via the TexasRed channel (mCherry) versus the fluorescein isothiocyanate (FITC) channel (EGFP). Cytotoxicity was calculated by normalized cytotoxicity as calculated by the following formula:

\[
\text{Cytotoxicity (\%)} = \left(1 - \% \text{CD19}^{-}\right) \times \% \text{K562mCherry cells} / \% \text{K562egfp cells} \times 100
\]

**Isolation, Culture, and Transduction of Primary Human CD4\(^{+}\) and CD8\(^{+}\) T Cells.** Human peripheral blood mononuclear cells (PBMCs) were isolated from the leukocyte concentrate of the blood from deidentified healthy donors (a byproduct from blood banks in the manufacturing of red blood cell and thrombocyte concentrates from antigenauglated whole blood; Shanghai Blood Center) using Ficol gradients (Cedarlane Laboratories), as approved by the Medical Ethics Committee of Yangpu Hospital affiliated with Tongji University (approved ID: LL-2018-SCI-003). Primary human CD4\(^{+}\) and CD8\(^{+}\) T cells were then isolated from PBMCs using a CD4\(^{+}\) and CD8\(^{+}\) T cell isolation kit (Milteny, 130-045-201 and 130-045-101). T cells were cryopreserved in 90% Roswell Park Memorial Institute (RPMI) 1640 medium and 10% dimethyl sulfoxide (DMSO). Before lentiviral transduction, isolated T cells were thawed, cultured in serum-free hematopoietic cell culture medium X-Vivo 15 (Lonza, 04-4180), and subsequently activated with anti-CD3/CD28 antibodies (Stem Cell Technologies, 10971) and 50 units/mL recombinant human IL-2 (Peprotech, AF-200-02) for 2 d. Activated primary T cells were resuspended with cell culture medium supplemented with 8 μg/mL polybrene and then transduced with concentrated lentivirus mixture with a multiplicity of infection (MOI) of ~10 for each virus and spun for 90 min at 1,800 × g.

Infected primary T cells were maintained at a density of ~1 \( \times 10^6 \) cells/mL in X-Vivo 15 medium supplemented with 50 units/mL hIL-2 for 9 d, after which, they were rested and could be used in assays.

**Quantification of CAR Expression on T Cells.** Jurkat T or primary human T cells were washed, stained with biotin conjugated anti-mouse CD19-specific monoclonal scFv antibodies (FM63, Bioswan, R1998-100), followed by streptavidin-phycocerythrin (PE) staining (Bioswan, R1998-100), and finally stained with a live/dead viability stain. Stained cells were washed three times and then analyzed by flow cytometry. PE fluorescence was quantified by excitation at 561 nm and emission at 586 nm. The percentage of gated cells and their median fluorescence intensity (MFI) were analyzed by the FlowJo software. Weighted CAR expression was evaluated as the percentage of gated cells multiplied by their median fluorescence. Unmodified cells were used as controls.

**Quantification of hIL-2 or hIFN-γ Production.** Cytokine release assays were performed using hIL-2 or hIFN-γ enzyme-linked immunosorbent assay (ELISA) kits. Engineered Jurkat T cells or human primary T cells were incubated with CD19\(^{+}\) K562mCherry or CD19\(^{-}\) K562egfp target cells at an effector-to-target (E:T) ratio of 5:1 (5 \( \times 10^5 \) cells:1 \( \times 10^5 \) cells) in duplicate wells in 96-well flat-bottom plates. After incubation for 18 h, the supernatants were collected and analyzed for human hIL-2 or hIFN-γ by ELISA (BioLegend, 431804 or 430101).

**Cytotoxicity Assay of RESrep-CAR or RESrep-CAR Devices in Engineered Primary Human T Cells.** CD19\(^{+}\) K562mCherry Cells and CD19\(^{-}\) K562egfp target cells were mixed at a 1:1 ratio and then incubated with primary human T cells expressing RESrep-CAR or RESrep-CAR at an E:T ratio of 10:1 (1 \( \times 10^5 \) cells:1 \( \times 10^5 \) cells) in 48-well plates. After overnight incubation, cells were washed in cell staining buffer and stained with a Fixable Viability Dye eFluor 660. Stained cells were washed three times and analyzed by BD LSRFortessa flow cytometry. Singlets were gated using forward versus side scatter and followed by gating based on forward scatter area (FSC-H) versus forward scatter height (FSC-A) characteristics. Live cells were gated via the allophycocyanin (APC) channel, and K562 target cells were gated by excluding T cells via the TexasRed channel (mCherry) versus the fluorescein isothiocyanate (FITC) channel (EGFP). Cytotoxicity was calculated by normalized cytotoxicity as calculated by the following formula:

\[
\text{Cytotoxicity (\%)} = \left(1 - \% \text{CD19}^{-}\right) \times \% \text{K562mCherry cells} / \% \text{K562egfp cells} \times 100
\]

**Animal Experiments.** Production of mice. The HEKRESrep-SEAP and HEKRESind-SEAP stable cell lines engineered for RES-controlled SEAP repression and expression were
Resveratrol was administered intraperitoneally at a dose of 50 mg/kg or by CAR, RESrep-CAR, or RESind-CAR) or unmodified T cells were intraperitoneally. Ten hours after cell implantation, engineered primary T cells (conventional CAR T cells (Conv. CAR), or RESind-CAR T cells, intravenously. 400 procedure described previously (64). Collected cells were centrifuged at cytokine release level using a Cytometric Bead Array (CBA) kit (BD Biosciences). Data analysis and target cell quantification were performed using Prism software (GraphPad). CD19+ K562 xenograft tumor model in NSG mice. Were performed using the vehicle alone. Two days after doses ranging from 0 to 200 mg/kg/d by intraperitoneal injection, twice each day. Conv. CAR T cells were injected with the vehicle alone. Two days after resveratrol induction, blood samples were collected to determine in vivo cytokine level release using a Cytometric Bead Array (CBA) kit (BD Biosciences 558270 and 560112). Mice were killed by carbon dioxide asphyxiation followed by cervical dislocation. Peritoneal cells were collected based on a procedure described previously (64). Collected cells were centrifuged at 400 g for 10 minutes, washed with the vehicle alone. Two days after resveratrol induction, blood samples were collected to determine in vivo cytokine level release using a Cytometric Bead Array (CBA) kit (BD Biosciences). 5 min at room temperature, centrifuged again at 400 g for 8 min, stained with eFluor 660 conjugated live/dead viability stain, fixed in 4% paraformaldehyde, and finally washed twice and resuspended with cell staining buffer. Samples were analyzed with a BD LSRFortessa cytometer. Data analysis and target cell quantification were performed using FlowJo software (TreeStar), as described for in vitro cell killing experiments. Data plotting and statistical analysis (Student’s t test) were performed using Prism software (GraphPad).

CD19+ K562 xenograft tumor model in NSG mice. Briefly, female immunodeficient NSG mice (6 wk old) were inoculated with 1 × 106 RmL-expressing CD19+ K562 cells intravenously. After 5 d, mice were randomly divided into five groups and each mouse was injected with 1 × 106 unmodified T cells (Ctrl), conventional CAR T cells (Conv. CAR), or RESind-CAR T cells, intravenously. Resveratrol was administered intraperitoneally at a dose of 50 mg/kg or by oral administration at a dose of 250 mg/kg. Murine blood was drawn 3 d after T cell infusion, and the secretion of human cytokines in serum was measured using a CBA kit (BD Biosciences, 558270 and 560112). Tumor progression was regularly monitored by bioluminescent imaging (BLI) using an IVIS Lumina imager (PerkinElmer) and quantified as the photon flux over the entire mouse body. For in vivo imaging, each mouse was intraperitoneally injected with 6 mg/kg coelenterazine substrate solution (Yeasen Biotech, Chemical Abstracts Service [CAS] no. 55779-48-1) immediately before imaging.

Ethics. All experiments involving animals were performed in accordance with the protocol approved by the ECNU Animal Care and Use Committee and in direct accord with the Ministry of Science and Technology of the People’s Republic of China on Animal Care Guidelines. The protocol used in this study was approved by the ECNU Animal Care and Use Committee (protocol ID: m20160305).

Statistical Analysis. All in vitro data are represented as the mean ± SD of three independent experiments (n = 3). For the animal experiments, each treatment group consisted of randomly selected mice (n = 4 to 6). Blood sample analysis was blinded. Comparisons between groups were performed using Student’s t test, and the results are expressed as means ± SEM. Differences were considered statistically significant at P < 0.05. Prism 6 software (GraphPad Software, Inc.) was used for statistical analysis. n and P values are indicated in the figure legends.

Data Availability. All data needed to evaluate the conclusions in the paper are present in the paper and/or SI Appendix, Supplementary Methods. All genetic components related to this paper are available with a material transfer agreement and can be requested from H.Y. (inf@bio.ecnu.edu.cn).

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