

In situ PEGylation of CAR T cells alleviates cytokine release syndrome and neurotoxicity

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Chimeric antigen receptor T (CAR T) cell immunotherapy is successful at treating many cancers. However, it often induces life-threatening cytokine release syndrome (CRS) and neurotoxicity. Here, we show that in situ conjugation of polyethylene glycol (PEG) to the surface of CAR T cells ('PEGylation') creates a polymeric spacer that blocks cell-to-cell interactions between CAR T cells, tumour cells and monocytes. Such blockage hinders intensive tumour lysing and monocyte activation by CAR T cells and, consequently, decreases the secretion of toxic cytokines and alleviates CRS-related symptoms. Over time, the slow expansion of CAR T cells decreases PEG surface density and restores CAR T cell–tumour-cell interactions to induce potent tumour killing. This occurs before the restoration of CAR T cell–monocyte interactions, opening a therapeutic window for tumour killing by CAR T cells before monocyte overactivation. Lethal neurotoxicity is also lower when compared with treatment with the therapeutic antibody tocilizumab, demonstrating that in situ PEGylation of CAR T cells provides a materials-based strategy for safer cellular immunotherapy.

Chimeric antigen receptor T (CAR T) cell immunotherapy is a revolutionary cancer treatment technique^{1,2}. There are six CAR T cell therapies that have been approved by the US Food and Drug Administration (FDA) and a variety of candidates currently being assessed in clinical or pre-clinical studies^{3,4}. CAR T cells targeting CD19 have shown remarkable anti-tumour efficacy against B cell malignancies^{5–7}. However, treatment often induces cytokine release syndrome (CRS)^{8,9} and neurotoxicity^{10,11}. Studies have shown that 20–70% of patients receiving CD19 CAR T cell therapy develop CRS^{12,13}. Severe CRS usually develops within a day after CAR T cell injection, with symptoms such as high fever, vomiting, and

cardiovascular and respiratory insufficiency^{14–16}. If left untreated, this can result in multiple organ dysfunction or patient death^{17,18}. CRS is also accompanied by neurotoxicity¹³. However, neurotoxicity is usually delayed for weeks after the disappearance of CRS-related symptoms and may induce patient death^{19,20}. Tocilizumab, an anti-IL-6 receptor (IL-6R) antibody, is effective at CRS management in the clinical setting^{21,22} but has failed at preventing neurotoxicity^{19,23}.

Recently, several studies have demonstrated that monocytes/macrophages play a vital role in the development of these adverse conditions^{24–26} (Supplementary Fig. 1) with increased numbers of monocytes

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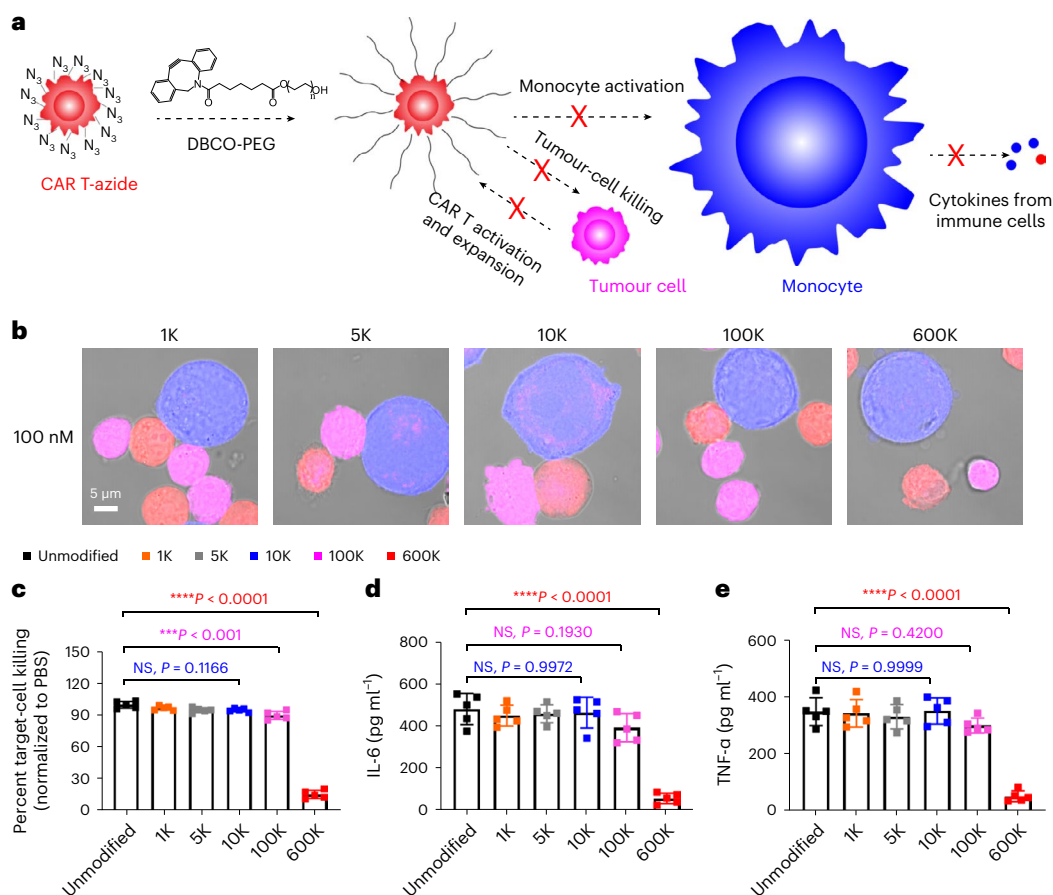


Fig. 1 | PEGylation of CAR T cells alters cell-to-cell interactions and cytokine release. a–e, Schematic showing how conjugation of DBCO-PEG to azido-glycan modified CAR T cells affects cell-to-cell interactions. **a**, PEGylated CAR T cells (red) were co-incubated with both Raji tumour cells (pink) and monocytes (blue) for 4 h and cells were examined under confocal microscopy (**b**). **c**, Raji target-cell

viability. **d**, Monocyte cytokine IL-6 concentration and **e**, T cell cytokine TNF- α concentration in cell-culture media were measured. Data in **c**, **d** and **e** are shown as mean \pm s.d. ($n = 5$ biologically independent experiments, one-way ANOVA with Tukey's post hoc test). P values are indicated (blue: 10K versus unmodified; pink: 100K versus unmodified; red: 600K versus unmodified).

in humanized mice associated with severe CRS²⁵. Moreover, overactivation of monocytes/macrophages is a source of toxic cytokines such as IL-6 and IL-1 that induce CRS and neurotoxicity^{25,27}. While CAR T cells failed to cause CRS in monocyte-depleted mice, anti-tumour efficacy was also attenuated²⁵, indicating monocytes may be necessary for effective CAR T cell therapy. Collectively, these findings highlight the complex role of monocytes in CAR T cell therapy as they are simultaneously crucial for CAR T cell-mediated toxicity and tumour-cell killing. Studies have shown that the overactivation of monocytes during CRS is a result of unwanted CAR T cell–monocyte interactions²⁸. For example, T cell surface proteins CD40 ligand^{24,29,30}, CD69 (ref. 31), lymphocyte activation gene³², and membrane-expressed TNF- α (refs. 33,34) can activate monocytes and macrophages via cell-to-cell interaction mechanisms^{28,35,36}. Therefore, decreasing monocyte overactivation via control of CAR T cell–monocyte interactions could provide a potential solution for the treatment of CRS and neurotoxicity.

Here, we validated in situ PEGylation on the surface of CAR T cells as a potential way to abolish the overactivation of monocytes by CAR T cells during CRS and prevent subsequent inflammatory effects by blocking cell-to-cell interactions between CAR T cells, monocytes and tumour cells.

PEGylation blocks cell-to-cell interactions in vitro

We first evaluated whether modification of CAR T cells with polyethylene glycol (PEG) can block cell-to-cell interactions in vitro. PEG was chosen as the polymeric spacer because it is a biomaterial that has

been FDA-approved for various biomedical applications³⁷. Human CD19-targeted CAR T cells were prepared, and CAR expression was evaluated (Supplementary Fig. 2)³⁸. Metabolic labelling was utilized to label the surface of CAR T cells with azide groups³⁹ by culturing CAR T cells with azido glycans (*N*-azidoacetylmannosamine-tetraacylated, Ac4ManNAz) for 48 h (Supplementary Fig. 3). The metabolic labelling strategy was found to be non-toxic⁴⁰ and did not affect the tumour-cell killing capacity of CAR T cells (Supplementary Fig. 4). To conjugate PEG to azide-labelled CAR T cells, dibenzocyclooctyne (DBCO)-modified PEG was synthesized⁴¹ (Supplementary Fig. 5). The DBCO group reacts with the azide group present on the CAR T cell surface under culture conditions⁴² (Fig. 1a) or in living animals⁴³. DBCO-PEGs with molecular weights (MWs) of 1,000 (1K), 5,000 (5K), 10,000 (10K), 100,000 (100K) and 600,000 (600K) were used to modify CAR T cells (Supplementary Fig. 6). We found that PEGylated CAR T cells with PEGs of MW 1K, 5K, 10K and 100K were able to bind to anti-CD3 antibody-coated 96-well plates, but PEGylated CAR T cells with a PEG of MW 600K failed to bind to the well plate (Supplementary Fig. 6a,b), demonstrating that PEG600K can potentially be used to block cell-to-cell interactions (Supplementary Fig. 6a,b). Confocal images (Fig. 1b) showed that PEGylated CAR T cells with PEGs of MW 1K, 5K, 10K and 100K failed to block interactions between CAR T cells, Raji tumour cells and monocytes, whereas PEGylated CAR T cells with a PEG MW of 600K substantially blocked all cell-to-cell interactions (Fig. 1b). Moreover, PEG600K modification also decreased target tumour-cell killing (Fig. 1c). The levels of IL-6, a cytokine produced by activated monocytes/macrophages, and TNF- α ,

a cytokine produced by activated CAR T cells, were also substantially decreased (Fig. 1d,e). Further, we found that PEG600K modification did not induce CAR T cell toxicity (Supplementary Fig. 6c–e). These results suggest that modification of CAR T cells with PEG600K block cell-to-cell interactions and decrease both monocyte activation and cytokine release *in vitro*. Encouraged by these data, we sought to test whether PEGylation of CAR T cells *in vivo* may reduce CRS.

Conjugation of PEG to the surface of CAR T cells *in vivo*

We first constructed a humanized mouse model following a previous report (Supplementary Fig. 7a–i)²⁵. We then constructed a CRS model in tumour-bearing mice and demonstrated that CAR T cell infusion induced severe CRS *in vivo* (Supplementary Fig. 8a–m). Before testing whether DBCO-PEG600K can conjugate to CAR T-azide cells in mice, we first evaluated the expansion levels of CAR T cells *in vivo*. Because azide-labelled CAR T cells may divide after infusion¹⁹, it may affect DBCO-PEG600K conjugation. We found that in the low, medium and high tumour burden groups, CAR T cells had expanded by approximately one-and-a-half, two and six times at day 7 (peak expansion), respectively (Supplementary Fig. 9a–c). We further demonstrated that after one, six or even 20 times expansion, PEGylation was unaffected and prevented binding of CAR T cells to an antibody-coated plate (Supplementary Fig. 9d). These data illustrate that the azide group modification on the surface of CAR T cells is sufficient for DBCO-PEG600K conjugation even after CAR T cell expansion.

We then sought to investigate whether DBCO-PEG600K can conjugate to CAR T-azide cells *in vivo*. We first tested the biocompatibility of DBCO-PEG600K using a haemolysis assay (Supplementary Fig. 10). We then investigated the blood circulation of a Cy7-labelled DBCO-PEG600K (DBCO-PEG600K-Cy7) and found that DBCO-PEG600K-Cy7 has a 18 h half-life in the bloodstream in normal mice (Fig. 2a,b), and it spreads throughout the whole body after injection (Fig. 2c), which is similar to previous reports⁴⁴. We also investigated whether DBCO-PEG600K-Cy7 can conjugate to fluorophore (CFSE)-modified CAR T-azide cells *in vivo* (Fig. 2d–i). CAR T-CFSE cells were mainly detected in the liver, spleen and lymph nodes (Fig. 2d,e), which are the tissues where tumour cells localize. Cy7 signal was also detected in these tissues, indicating potential conjugation of DBCO-PEG600K-Cy7 to CAR T-azide cells (Fig. 2d,e). A flow cytometry experiment (Fig. 2f–m) showed that DBCO-PEG600K-Cy7 conjugated to CAR T cells in the blood circulation, liver, spleen and lymph node within 60 min (Fig. 2f–m). These results demonstrate the rapid conjugation of DBCO-PEG600K to CAR T-azide cells *in vivo*.

PEGylation of CAR T cells alleviates cytokine release syndrome

Next, we constructed a Raji tumour-bearing mouse model and intravenously (*i.v.*) infused CAR T-azide cells at day 0 (Supplementary Fig. 11a). After 24 h, all mice developed high fever ($\Delta T > 2^\circ\text{C}$, T: temperature). Immediately following high fever, DBCO-PEG600K at different doses (0, 1, 5, 10, 50 mg kg⁻¹) was *i.v.* injected into mice. We found that a low dose of DBCO-PEG600K (1 and 5 mg kg⁻¹) did not affect weight loss, body temperature and cytokine release compared with a phosphate-buffered saline (PBS) infusion (Supplementary Fig. 11b–g). Excitingly, higher doses of DBCO-PEG600K (10 and 50 mg kg⁻¹) greatly reversed weight loss, high fever and cytokine release (Supplementary Fig. 11b–g). To investigate the mechanism, we sorted CAR T cells from mouse blood at day 7 and cocultured them with luciferase and green fluorescence protein (GFP)-expressing Raji (Raji–Luc–GFP) cells and human monocytes for 24 h. CAR T cells sorted from mice that received 0, 1 and 5 mg kg⁻¹ DBCO-PEG600K induced substantial Raji–Luc–GFP cell lysis and the production of both IL-6 and TNF- α (Supplementary Fig. 11h–j). However, CAR T cells collected from mice receiving 10 and 50 mg kg⁻¹ DBCO-PEG600K exhibited very

low levels of target-cell killing and decreased cytokine production (Supplementary Fig. 11h–j). These results suggest the potential of a 10 and 50 mg kg⁻¹ injection of DBCO-PEG600K for controlling CRS *in vivo*. Since injections of 10 mg kg⁻¹ and 50 mg kg⁻¹ of DBCO-PEG600K displayed similar effects in decreasing CRS symptoms, we dosed mice with 10 mg kg⁻¹ of DBCO-PEG600K for all remaining studies.

Next, we investigated whether PEG600K must be conjugated to CAR T cells and whether a short DBCO-PEG could have a similar effect. We generated the CRS mouse model (Fig. 3a), and upon observation of high fever (Fig. 3b), 10 mg kg⁻¹ DBCO-PEG1K, unmodified PEG600K and DBCO-PEG600K were injected *i.v.* into mice. As expected, treatment with DBCO-PEG1K and unmodified PEG600K did not affect weight loss, temperature or cytokine release compared with a PBS infusion (Fig. 3c–i). However, DBCO-PEG600K treatment greatly reversed weight loss, high fever and cytokine release (Fig. 3c–i). The number of CAR T cells, tumour cells and monocytes in mouse blood was also measured (Fig. 3j–l). In both DBCO-PEG1K- and unmodified PEG600K-treated mice, almost all tumour cells were cleared within 2–3 days post-CAR T cell administration. However, in animals treated with DBCO-PEG600K there was a significant delay in tumour-cell clearance, but all tumour cells were eventually cleared at day 35 post-CAR T cell injection (Fig. 3j,p,q). Along with the clearance of tumour cells, we found a rapid increase in CAR T cell and CD14⁺ monocyte numbers in the PBS, DBCO-PEG1K, and PEG600K groups (Fig. 3k,l). However, in mice treated with DBCO-PEG600K, CAR T cell and CD14⁺ monocyte expansion was greatly reduced (Fig. 3k,l). Moreover, the amount of IL-6⁺, IL-1⁺ and TNF- α monocytes at day 7 was greatly decreased in mice treated with DBCO-PEG600K compared with those in the PBS-, PEG600K- or DBCO-PEG1K-treated mice (Supplementary Fig. 12a–j). Since monocyte activation is a well-known sign of CRS, these results provide further support for the use of this PEGylation strategy in alleviating CRS-related symptoms.

To further verify whether the polymeric spacer is present on azide-labelled CAR T cell surfaces after DBCO-PEG600K injection, we sorted CAR T cells from treated mice and performed the three-cell coculture experiment mentioned previously. We found that CAR T cells isolated from mice that received DBCO-PEG1K and unmodified PEG600K induced substantial Raji–Luc–GFP cell lysis and cytokine release, whereas CAR T cells collected from DBCO-PEG600K-treated mice did not lead to high target-cell killing and cytokine release (Fig. 3m,n,o). Confocal images of CAR T cells from mice treated with DBCO-PEG600K that were cocultured with tumour cells and monocytes suggested that cell-to-cell interactions were blocked (Supplementary Fig. 13a). These results support DBCO-PEG600K-induced CRS alleviation due to the conjugation of DBCO-PEG600K to CAR T cells.

We further explored why tumour cells were eventually cleared even after the PEG spacer blocked cell-to-cell interactions. We found that CAR T cell expansion *in vivo* induced dilution of the PEG spacer (Supplementary Fig. 13b–g). We also cocultured these sorted cells with both Raji–Luc–GFP cells and monocytes and found that in the DBCO-PEG600K-treated group, CAR T cells collected at day 10 did not induce tumour-cell killing and cytokine (IL-6, IL-1, TNF- α) release compared with other groups (Supplementary Fig. 14a–h), indicating that cell-to-cell interactions were still blocked at day 10. However, the CAR T cells collected on days 15 and 20 induced substantial target-cell killing and cytokine release (Supplementary Fig. 14a–h). We believe that, as DBCO-PEG600K became gradually diluted on the surface of CAR T cells, cell-to-cell interactions were gradually restored. Interestingly, at day 15, CAR T cells induced substantial tumour-cell killing and release of CAR T cell-associated cytokine TNF- α , but not monocyte-related cytokines (IL-6 and IL-1), suggesting the restoration of CAR T cell–tumour-cell interactions (day 15) before the recovery of CAR T cell–monocyte interactions (day 20). This is likely due to the smaller size of the Raji tumour cells (7–10 μm) and T cells (5–8 μm) compared with monocytes (30–50 μm). The size-dependent sequential cell-to-cell interaction

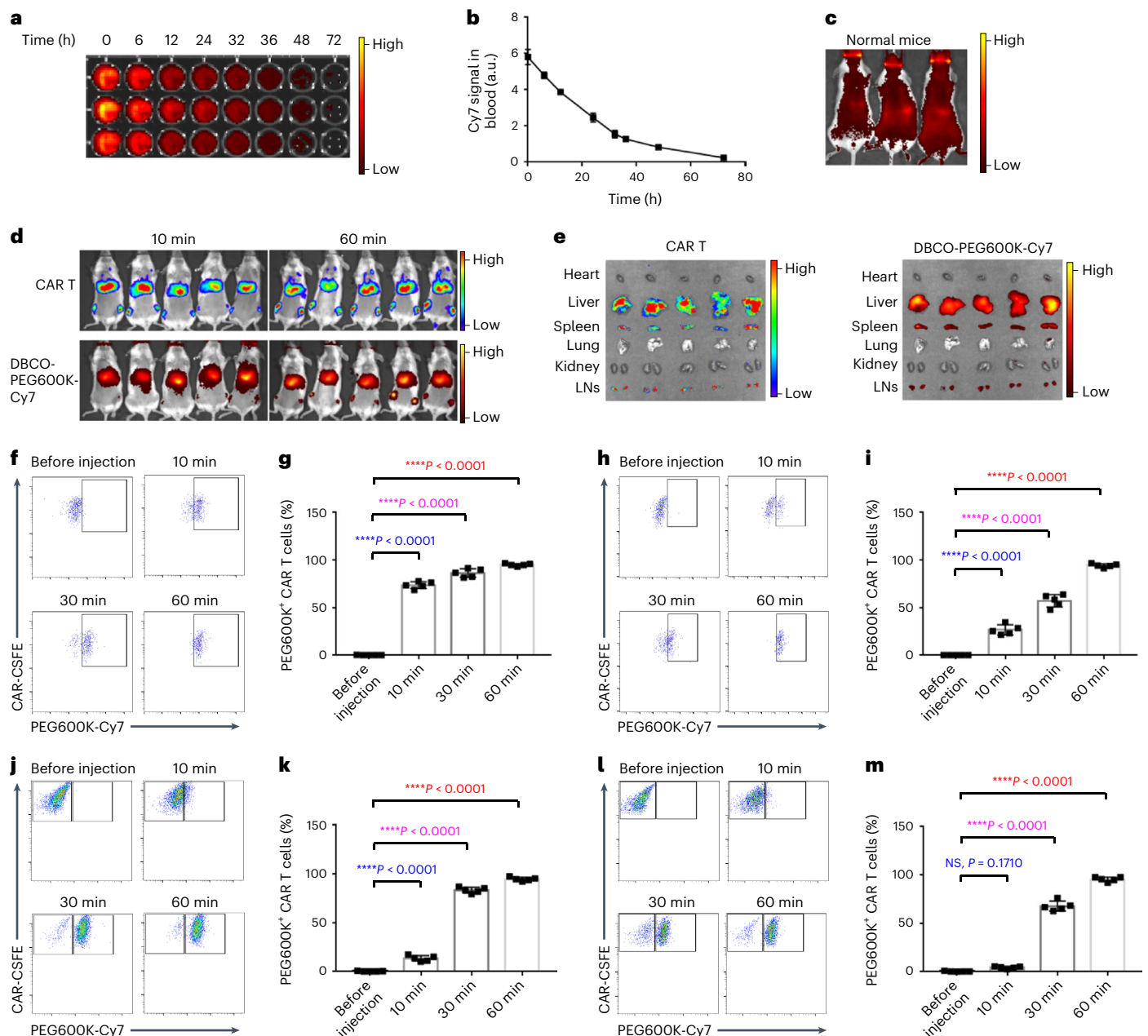


Fig. 2 | Conjugation of DBCO-PEG600K to CAR T cells in vivo. **a**, Cy7-labelled DBCO-PEG600K (DBCO-PEG600K-Cy7) was i.v. injected into normal mice and 10 μ L of peripheral blood was collected at various time points (0 h, 6 h, 12 h, 24 h, 32 h, 36 h, 48 h and 72 h) and the Cy7 signal in the blood was detected using IVIS. **b**, quantification of **a**. Data in **b** are shown as mean \pm s.d. ($n = 3$ biologically independent animals). **c**, Whole-body IVIS images of mice were taken 6 h following injection. **d**, Fluorophore (CSFE)-labelled CAR T cells (CAR T-CSFE) were used to treat tumour-bearing mice and the biodistribution of CAR T cells and DBCO-PEG600K-Cy7 in vivo were examined. **e**, Mouse hearts, livers, spleens, lungs, kidneys and lymph nodes were isolated and imaged using

IVIS. Mouse blood, lymph nodes (LNs), liver and spleen were collected before injection, 10 min, 30 min and 60 min after injection. **f–m**, The conjugation of DBCO-PEG600K-Cy7 to CAR T cells in the blood (**f,g**), liver (**h,i**), spleen (**j,k**) and LNs (**l,m**) was investigated using flow cytometry. Representative dot plots show the conjugation of DBCO-PEG600K-Cy7 to CAR T cells in the blood (**f**), liver (**h**), spleen (**j**) and LNs (**l**) at indicated time points. Quantification of Cy7⁺ CAR T cells in the blood (**g**), liver (**i**), spleen (**k**), and LNs (**m**). The data in **g,i,k,m** are shown as mean \pm s.d. ($n = 5$ biologically independent animals, one-way ANOVA with Tukey's post hoc test). P values are indicated (blue: 10 min versus before injection; pink: 30 min versus before injection; red: 60 min versus before injection).

restoration was also confirmed with an in vitro DBCO-PEG600K density experiment (Supplementary Fig. 15).

CAR T cell expansion in vivo is highly dependent upon the recognition of tumour cells. However, in our study, the DBCO-PEG600K modification blocked CAR T cell–tumour-cell interactions, and thus tumour cells could not induce rapid PEGylated CAR T cell expansion. Nevertheless, we found that DBCO-PEG600K-modified CAR T cells slowly expand in response to tumour-cell lysate (Supplementary Fig. 16),

allowing for the slow dilution of PEG on the CAR T cell surface to enable tumour-cell killing before the initiation of CAR T cell-induced monocyte activation.

PEGylation of CAR T cells alleviates neurotoxicity

Even though tocilizumab is effective at alleviating some symptoms related to severe CRS⁴⁵, it has failed to treat neurotoxicity⁴⁶. We sought to investigate whether a DBCO-PEG600K injection has advantages

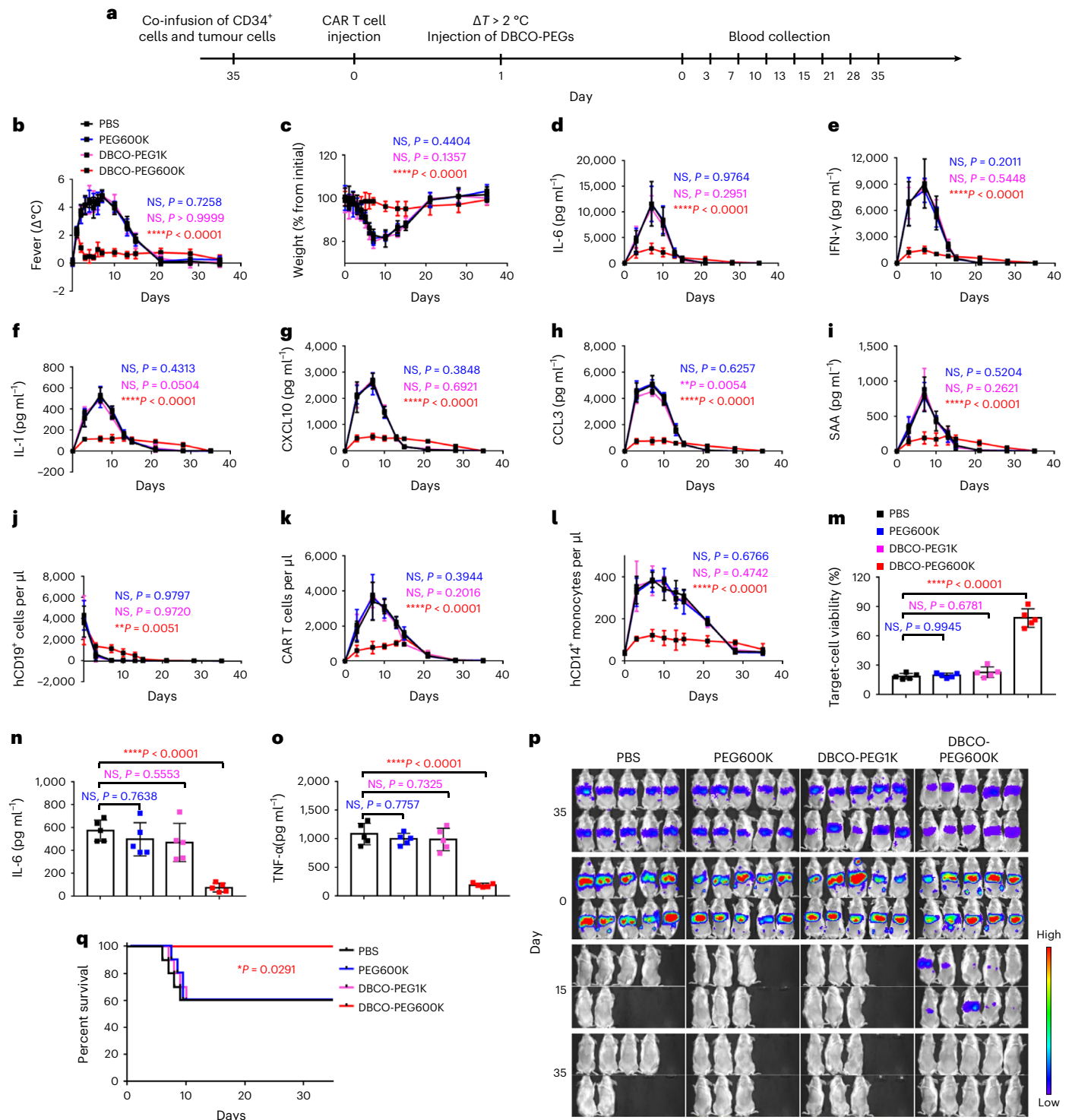


Fig. 3 | In situ PEGylation of CAR T cells alleviates cytokine release syndrome. **a**, CRS mouse model was constructed and mice were treated with PBS, PEG600K, DBCO-PEG1K or DBCO-PEG600K. **b,c**, Mouse temperature (**b**) and body weight (**c**) were monitored. **d–l**, The levels of IL-6 (**d**), IFN- γ (**e**), IL-1 (**f**), CXCL10 (**g**), CCL3 (**h**), SAA (**i**) and the numbers of human CD19⁺ cells (**j**), human CAR T cells (**k**) and human CD14⁺ monocytes (**l**) were monitored. **m**, CAR T cells isolated from mice were cocultured with Raji-Luc-GFP cells and monocytes, and after 24 h, Raji-Luc-GFP cell viability was determined. **n,o**, The levels of IL-6 (**n**) and TNF- α

(**o**) in cell-culture medium were detected. Data in **b–o** are shown as mean \pm s.d. ($n = 5$ biologically independent experiments, two-way ANOVA with Tukey's post hoc test). P values are indicated (blue: PEG600K versus PBS; pink: DBCO-PEG1K versus PBS; red: DBCO-PEG600K versus PBS). **p**, In a separate animal survival experiment, the tumour-cell burden level was monitored using IVIS imaging. **q**, Mouse survival data are shown. Exact P value indicated in the figure is from a Mantel-Cox two-sided log-rank test ($n = 10$ biologically independent animals). DBCO-PEG600K versus PBS, $*P = 0.0291$.

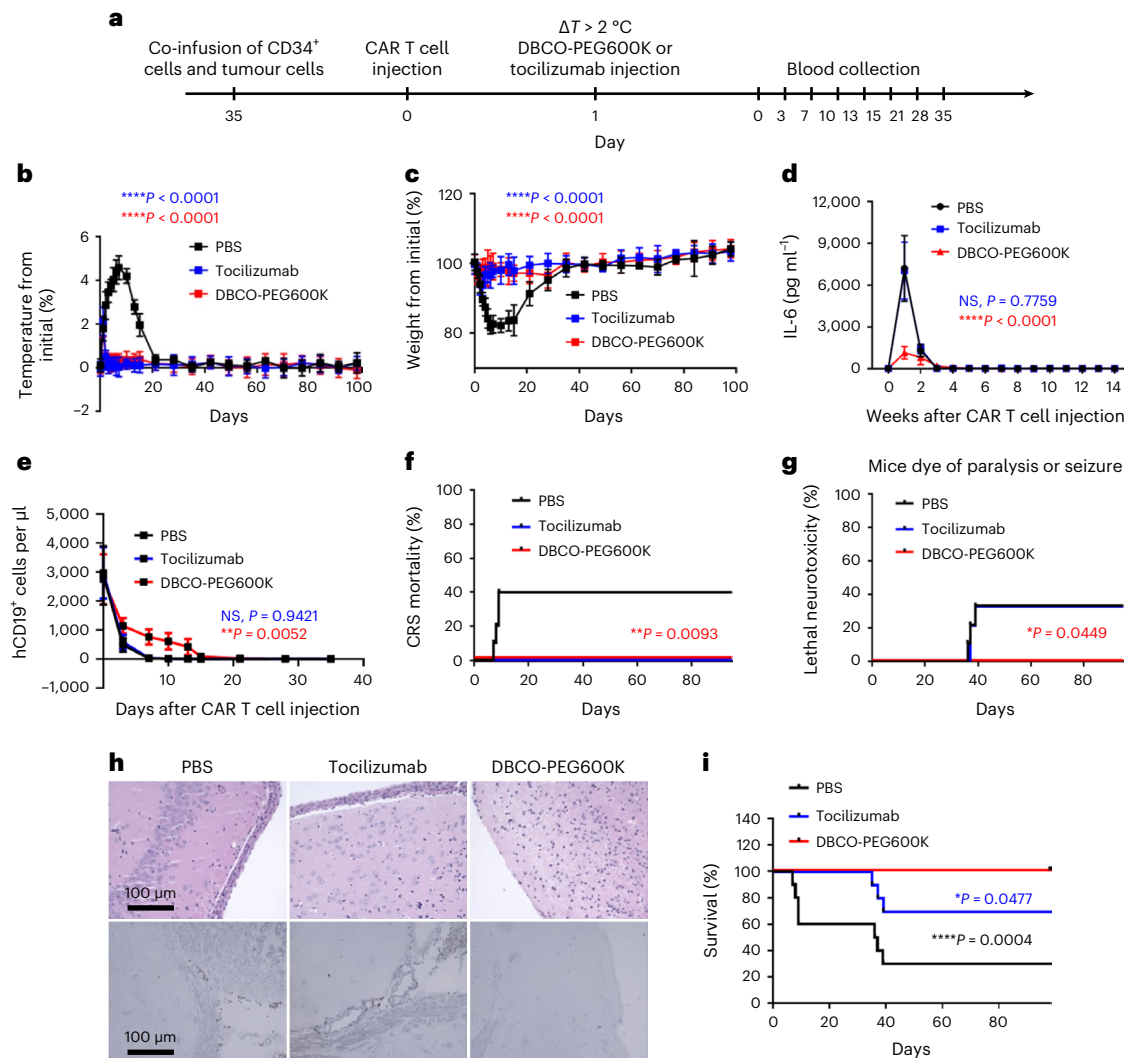


Fig. 4 | In situ PEGylation of CAR T cells abolishes neurotoxicity.

a, Experimental timeline of construction of the CRS model and the following treatments: **a**, 2×10^6 CAR T cells were infused into tumour-bearing mice at day 0. After the onset of high fever (day 1), mice were infused with DBCO-PEG600K (10 mg kg^{-1}) or treated with a tocilizumab *in vivo* bio-similar antibody (10 mg kg^{-1}). Control mice were treated with PBS. **b–e**, Mouse temperature (**b**), weight (**c**), IL-6 (**d**) and human CD19⁺ cell levels (**e**) in mouse blood were monitored. Data in **b–e** are shown as mean \pm s.d. ($n = 10$ biologically independent animals, two-way ANOVA with Tukey's post hoc test). *P* values indicated in the

figure are from the comparisons on day 7 (blue: tocilizumab versus PBS; red: DBCO-PEG600K versus PBS). **f**, CRS mortality curves. **g**, Lethal neurotoxicity curves. *P* values shown in the figure are from a Mantel–Cox two-sided log-rank test. **h**, Brain haematoxylin and eosin (H&E) staining and CD68 (human) immunohistochemistry (IHC) images of mice that received different treatments at day 37. Images are representative of three independent experiments. **i**, Kaplan–Meyer survival plots. *P* values are calculated from a Mantel–Cox two-sided log-rank test: for DBCO-PEG600K versus PBS, **** $P = 0.0004$; for DBCO-PEG600K versus tocilizumab, * $P = 0.0449$.

over a tocilizumab injection to treat CRS symptoms and neurotoxicity (Fig. 4a). We found that both tocilizumab- and DBCO-PEG600K treatment can greatly control CRS-related symptoms such as high fever and weight loss (Fig. 4b–d). However, while tumour cells were completely cleared in mice that received either tocilizumab or DBCO-PEG600K by day 35 (Fig. 4e), only DBCO-PEG600K treatment substantially decreased CAR T cell expansion levels and cytokine (interferon gamma (IFN- γ), IL-6 and IL-1) production (Supplementary Fig. 17a–c). Since IL-1 is a cytokine that mainly contributes to neurotoxicity, these results demonstrate that DBCO-PEG600K could be used for neurotoxicity management.

After approximately 35 days postinjection of CAR T cells, tumour-bearing mice that received either PBS or tocilizumab developed sudden paralysis (Supplementary Fig. 17d) or seizure (Supplementary Fig. 17e), which are signs of lethal neurological syndrome²⁵. However, mice injected with DBCO-PEG600K did not show any signs of

paralysis or seizure. No signs of xenogeneic graft-versus-host disease (X-GVHD) in the skin and liver were detected according to postmortem analysis (Supplementary Fig. 18). Both tocilizumab and DBCO-PEG600K protected mice from CRS mortality (Fig. 4f) without inducing severe toxicity (Supplementary Fig. 19), but only DBCO-PEG600K prevented mice from developing lethal neurotoxicity (Fig. 4g). PBS-treated and tocilizumab-treated mice did show brain meningeal thickening (Fig. 4h) and human monocyte infiltration in the subarachnoid space (Fig. 4h). However, these were not detected in mice treated with DBCO-PEG600K. These data demonstrate that DBCO-PEG600K protected mice from severe neurotoxicity that tocilizumab was not able to protect against. As a result, only DBCO-PEG600K—and not tocilizumab—significantly prolonged mice survival (Fig. 4i).

We also asked if conjugation of PEG600K to CAR T cells *ex vivo* and infusion of the cells back into mice could treat CRS and neurotoxicity (Extended Data Fig. 1a). We constructed the Raji tumour model and

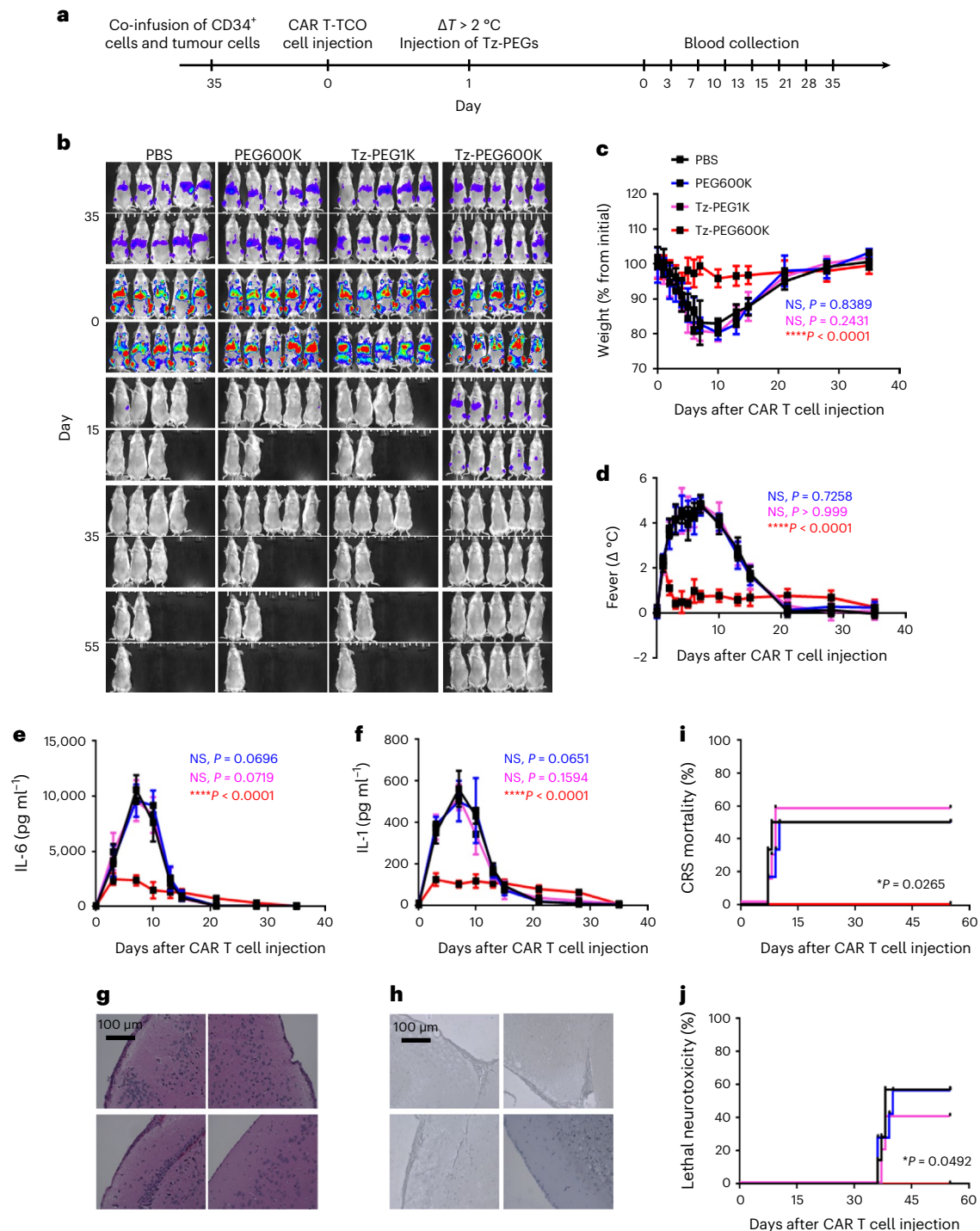


Fig. 5 | In situ PEGylation-induced CRS and neurotoxicity alleviation can also be achieved using the tetrazine (Tz)-TCO reaction. **a**, Raji tumour mouse model was constructed and TCO-modified CAR T (CAR T-TCO) cells were i.v. infused into mice on day 0. After the onset of high fever ($\Delta T > 2^\circ\text{C}$, day 1), PEG600K, Tz-PEG1K, Tz-PEG600K or PBS were i.v. infused into mice. **b–f**, Tumour burden (**b**), mouse body weight (**c**), temperature (**d**) and blood IL-6 (**e**) as well as IL-1 (**f**) were monitored. **g**, Brain H&E staining and **h**, human CD68 IHC images of mice that received different treatments (tissues were collected on day 37).

Images are representative of three independent experiments. **i**, CRS mortality curves. **j**, lethal neurotoxicity curves. Data in **c–f** are shown as mean \pm s.d. ($n = 10$ biologically independent animals, two-way ANOVA with Tukey's post hoc test). P values indicated in the figure are from comparisons at day 7 (blue: PEG600K versus PBS; pink: Tz-PEG1K versus PBS; red: Tz-PEG600K versus PBS). Comparison in **i** and **j** was conducted using a Mantel–Cox two-sided log-rank test ($n = 10$). P values are indicated.

treated mice with either ex vivo PEGylated CAR T cells or regular CAR T-azide cells at day 0. On day 1, we found that mice treated with CAR T-azide cells developed high fever, so we injected DBCO-PEG600K into mice (Extended Data Fig. 1b). However, mice that received ex vivo PEGylated CAR T cells failed to develop high fever. Tumour growth was

monitored using IVIS (Extended Data Fig. 1c) and blood IL-6 (Extended Data Fig. 1d), and CAR T cell levels in blood (Extended Data Fig. 1e) were documented. We found that ex vivo PEGylated CAR T cells failed to induce IL-6 storm in mice. However, ex vivo PEGylated CAR T cells did not inhibit tumour growth compared with PBS treatment (Extended

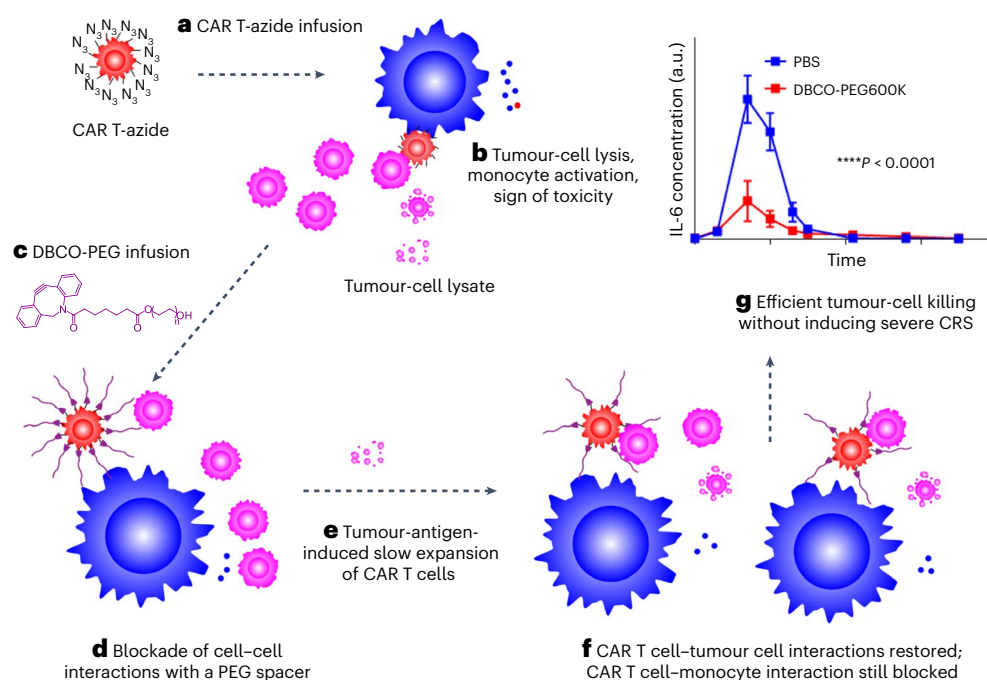


Fig. 6 | Mechanistic illustration of the in situ PEGylation strategy for managing cytokine release syndrome and neurotoxicity. **a**, CAR T-azide cells were infused into mice. Similar to conventional CAR T cells, they can recognize tumour cells, induce tumour-cell lysis and activate monocytes through direct cell-to-cell interactions. **b–d**, Here, upon the first signs of CRS (high fever, **b**), DBCO-PEG600K is infused into mice, which can conjugate to the CAR T cell surface (**c**), forming a polymeric spacer on CAR T cell surfaces to block cell-to-cell interactions (**d**). This prevents CAR T cells and monocytes from expanding too quickly and becoming overactivated. **e**, Over time, small tumour antigens can

reach CAR T cells to slowly activate and expand them. The PEG600K spacer on the CAR T cell surface then becomes diluted and cell-to-cell interactions are gradually restored. **f**, CAR T cell–tumour cell interactions are restored earlier than CAR T cell–monocyte interactions because of the relatively smaller size of B cell lymphoma cells compared with monocytes. **g**, This creates a therapeutic window for tumour-cell killing without inducing monocyte overactivation. Data in **g** are shown as mean \pm s.d. ($n = 10$ biologically independent animals, two-way ANOVA with Tukey's post hoc test).

Data Fig. 1f). In contrast, DBCO-PEG600K treatment greatly decreased the peak IL-6 concentration and completely cleared tumour cells in vivo (Extended Data Fig. 1c). Even though the ex vivo approach did not induce CRS, the fast growth of tumour cells led to rapid mouse death (Extended Data Fig. 1g). This is because the surfaces of ex vivo PEGylated CAR T cells were fully coated with PEG, preventing the cells from reaching any tumour cells, thus prohibiting CAR T cell expansion (Extended Data Fig. 1d). However, with the in situ PEGylation strategy, the initial CAR T cell-induced cancer-cell killing led to the release of tumour antigens, which can penetrate between the PEG spacers, while slow expansion of CAR T cells restored the CAR T–tumour-cell interactions (Supplementary Fig. 16). These results demonstrate that the in situ PEGylation strategy can alleviate CAR T cell-induced CRS and neurotoxicity while not hampering tumour-killing ability.

Tetrazine-trans-cyclooctene reaction for in situ PEGylation

To explore whether this in situ PEGylation strategy could be generalized to chemistries beyond DBCO-azide, we investigated the use of the tetrazine (Tz)-trans-cyclooctene (TCO) reaction for its ability to control CAR T cell-mediated CRS and neurotoxicity (Fig. 5a–j). PEG600K, Tz-PEG1K, Tz-PEG600K, or PBS was i.v. infused into the mice. Similar to what was seen in the DBCO-azide click-chemistry-mediated in situ PEG modification (Fig. 3), treatment with Tz-PEG1K and unmodified PEG600K did not affect tumour growth, weight loss, high fever and cytokine release compared with PBS infusion (Fig. 5b–f). However, Tz-PEG600K treatment greatly reversed weight loss, high fever and cytokine release (Fig. 5c–f). Moreover, tumour cells in the Tz-PEG600K-treated mice were cleared at around day 35 post-CAR T cell injection (Fig. 5b) Further extending the duration of the experiment, we found that a

high proportion of mice in PBS-, PEG600K- and Tz-PEG1K-treated groups showed signs of neurological toxicity. However, mice in the Tz-PEG600K treated group did not show such neurological toxicity (Fig. 5g,h). Moreover, Tz-PEG600K greatly decreased CRS mortality and lethal neurotoxicity (Fig. 5i,j). Based on these results using DBCO-based click chemistry and Tz-TCO chemistry, in situ PEGylation of CAR T cells can act to alleviate CRS and neurotoxicity in vivo.

CAR T cell therapy is a revolutionary cancer immunotherapy used in the clinical setting. However, CRS and neurotoxicity restrict its broader application. In this study, we show that in situ PEGylation of CAR T cells (Fig. 6a–c) can greatly decrease interactions between CAR T cells, tumour cells and monocytes, which overall decreased intensive tumour-cell lysis and monocyte overactivation by CAR T cells (Fig. 6d). Over time, CAR T cells slowly expand and the PEG600K spacer becomes diluted, gradually restoring cell-to-cell interactions (Fig. 6e,f). CAR T cell–tumour-cell interactions were restored earlier than CAR T cell–monocyte interactions. In this way, CAR T cells completely clear tumour cells but do not induce severe IL-6 production (Fig. 6g). In the modern drug discovery field, researchers focus mostly on developing therapeutics for gene-, protein-, metabolic- or epigenetic-level-modulating cell behaviours. This work provides an example that disease treatment can be achieved by modulating cell-to-cell interactions with materials alone. A benefit of this system is that the polymeric spacer can be added on an as-needed basis after CAR T-azide cells have been administered—so that the tumour-killing ability is not dampened unless the patient presents symptoms of severe CRS. Our results demonstrate that in situ PEGylation of CAR T cells holds great promise for safer CAR T cell therapy. As the use of PEG may induce the generation of anti-PEG antibodies in vivo, future work will evaluate in situ conjugation of different polymers⁴⁷ or nano-/microparticles⁴⁸ as alternative ‘spacers’ to

control CRS and neurotoxicity. Moreover, as cell-to-cell interactions are involved in many important physiological and pathological processes⁴⁹, future studies will explore the use of in situ biomaterial-conjugation strategies to treat various diseases.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41563-023-01646-6>.

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Methods

Chemicals

Polyethylene glycol (PEG) with molecular weight of 1K, 5K, 10K, 100K and 600K were purchased from Sigma. DBCO-acid was purchased from Sigma. *N*-azidoacetylmannosamine-tetraacylated (catalogue no. 88904) was obtained from Fisher. CD19 CAR plasmid was a gift from Scott McComb (Addgene no. 135991). The packaging plasmids pMD2.G (Addgene no. 12259) and psPAX2 (Addgene no. 12260) were from Didier Trono. CSFE dye and e450 dye were purchased from Fisher. FirePlex-96 Key Cytokines (Human) Immunoassay Panel (catalogue no. ab243549) was purchased from Abcam. Enzyme-linked immunosorbent assay kit for mouse serum amyloid A (catalogue no. KMA0021) was obtained from Thermo Fisher. Human CD68 antibody (catalogue no. 76437 S) was purchased from Cell Signalling Technology and was used as 1:1,000 dilutions. Anti-CD3 antibody (catalogue no. 85061) for T cell binding experiment was obtained from CST. Anti-human CD3-PE (catalogue no. 980008), anti-human CD4-BV711 (catalogue no. 317439), anti-human CD8-APC (catalogue no. 344721), anti-human CD19-BV711 (catalogue no. 302245) and anti-human CD14-APC (catalogue no. 325607) antibodies were purchased from Biolegend. Anti-human F(ab')₂-biotin antibody (catalogue no. 109-066-006) was purchased from Jackson ImmunoResearch. All flow antibodies were used at 1:100 dilutions. Anti-PEG antibody (ab53449) was purchased from Abcam and was used at 1:100 dilutions. StemCell EasySep human T cell isolation kit (catalogue no. 17951), Dynabead Human T-Activator CD3/CD28 (catalogue no. 11132D), retronectin (TAKARA Bio, catalogue no. T100B) and Lipofectamine 2000 (catalogue no. 11668019) were obtained from Thermo Fisher.

Cell lines and animals

Raji cells were originally ordered from ATCC (CCL-86). Raji-Luc-GFP cells were originally purchased from Creative Bio Labs (CAR-STC-ZP43). 293T cells were purchased from ATCC (CRL-3216). The cells were tested mycoplasma negative before use. Human fetal liver CD34⁺ cells were purchased from the Tissue Bank of University of Pennsylvania. Around 200 female triple transgenic NSG (SGM3) mice (6–8 weeks) expressing human stem-cell factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)IEav/MloySzJ) were obtained from the Jackson lab and housed in a specific pathogen-free-grade animal facility with air humidity 40–70%, ambient temperature (22 ± 2 °C) and 12-h dark/12-h light cycle. All protocols performed on animals in this study were approved by the institutional animal care and use committee of the University of Pennsylvania (protocol no. 806540).

Lentivirus production

293T cells were cultured in a 10 cm dish, and after 80% confluence was reached, the cells were treated with Opti-MEM medium containing 80 µl lipofectamine 2000 mixed with CAR plasmid (10 µg) and two packaging plasmids (pCMV-VSV-G, 7.5 µg; psPAX2, 5 µg). After 6 h, old medium was removed and 8 ml of prewarmed medium was added gently without disturbing the cells. After 24 h, virus-containing supernatant was collected and was passed through a 0.45 µm filter. Virus-containing supernatant was then aliquoted and stored at –80 °C for further use.

Preparation of CAR T cells

Some 24-well untreated plates were treated with 20 µg ml⁻¹ of Retronectin in PBS at 4 °C overnight. Then lentiviral supernatants were added, and the plate was centrifuged at 1,000g for 1 h for lentiviral adhesion. The spleens of humanized mice were collected, and CD8⁺ T cells were isolated using a human CD8⁺ T cell isolation kit. T cells were activated with Dynabeads Human T-Activator CD3/CD28 at a bead-to-cell ratio of 1:1, and 50 IU ml⁻¹ recombinant IL-2 was added to the cell-culture medium. After 48 h of activation, Dynabeads were removed, and the T cells were added to lentiviral-coated 24-well plates (1 million cells

per well). At 2 days after T cells were added, transduction efficiencies were determined by flow.

Synthesis of DBCO-PEG

Dibenzocyclooctyne (DBCO)-acid was mixed with PEG 1K, 5K, 10K, 100K or 600K in dichloromethane (DCM) under N₂ protection, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 4-(dimethylamino)pyridine (DMAP) were used as catalysts. After 24 h, DCM was removed under reduced pressure and the free DBCO-acid and catalysts were removed by dialysis.

Azido glycans modification on CAR T cell surfaces

The surfaces of CAR T cells were labelled with azido glycans by culturing CAR T cells with Ac4ManNAz for 48 h. Confocal images were obtained using a Zeiss LSM 710 confocal with ZEN2010 software.

Construction of humanized immune system NSG-SGM3 mouse model

Recently, a study developed a murine model of CRS, where they found that CAR T cells injected into humanized mice bearing leukaemia can induce severe CRS and neurotoxicity²⁵. Here, we also constructed a humanized mouse model following their method, with modification. NSG-SGM3 mice were treated with busulfan (40 mg kg⁻¹) to remove the bone marrow, and then 10⁵ human fetal liver CD34⁺ cells were i.v. injected into mice. The development of human immune cells including T cells, B cells and monocytes in blood was determined by flow cytometry and histological analysis. X-GVHD was monitored for daily by assessing mouse activity, weight loss, fur texture and skin integrity. To construct the CRS model in tumour-bearing mice, 1 × 10⁴ Raji cells and 1 × 10⁵ human fetal liver CD34⁺ cells were co-infused into NSG-SGM3 mice to develop human immune cells and to allow tumour growth. After 7 weeks, 2 × 10⁶ human T cells isolated from a non-tumour-bearing humanized mouse were transduced with CD19 CAR (1928z). These cells were then injected i.v. into tumour-bearing mice. Body weight and temperature were recorded every 3 days. Blood was also collected to determine the levels of various cytokines and the numbers of tumour cells, CAR T cells and monocytes. The concentrations of several human cytokines, such as interleukin 6 (IL-6), IL-1, tumour necrosis factor alpha (TNF-α), IL-8 and mouse serum amyloid A (SAA, a murine homologue to the human CRS biomarker C-reactive protein) in the blood were also monitored. Mouse IL-6 and TNF-α levels were also measured. CRS mortality was defined as death preceded by the following criteria: more than 20% body weight loss, ΔT > 2 °C and serum IL-6 > 2,500 pg ml⁻¹. Neurotoxicity-induced lethality was defined as death in the absence of CRS criteria and preceded by either seizures or paralysis. The maximal tumour size/burden permitted by the ethics committee is 2 cm; we confirmed that during this study the size limit was not reached.

Flow cytometry and cell sorting experiments

A total of 50–100 µl mouse blood was collected in ethylenediaminetetraacetic acid (EDTA)-pretreated tubes using the orbital bleeding method. Cells were spun down, the red blood cells were lysed with ACK lysing buffer, and the cell suspensions were passed through a 70 µm cell strainer before being stained with antibodies for flow cytometry (BD LSR II). CAR T cell sorting from mouse blood was performed using a BD FACSAria.

IVIS imaging

Raji-Luc-GFP tumour growth in mice was monitored using a Perkin Elmer in vivo imaging system (IVIS, Lumina 3). Mice were intraperitoneally injected with *D*-luciferin (150 mg kg⁻¹) for 15 min, and then were imaged in the IVIS system under inhaled isoflurane anaesthesia.

Statistics

Statistical analysis was performed using Graphpad prism v.7.0 software. Error bars represent mean ± standard deviation (s.d.). One- or two-way analyses of variance (ANOVAs) were used as appropriate to compare

means, with Tukey's post hoc test. The differences in animal survival experiments were calculated using the Kaplan–Meyer method and the *P* values were determined using the log-rank test.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All relevant data of this study are available within the paper and its Supplementary Information files. Source data are provided with this paper.

Acknowledgements

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Author contributions

N.G. and M.J.M. conceived and designed the experiments. N.G., X.H., L.X. and A.G.H. performed the experiments. N.G., L.X., X.H. and R.E.M.

analysed the data. N.G. and M.J.M. wrote the manuscript. M.M.B. and A.E.M. edited the manuscript. All authors discussed the results and commented on the manuscript.

Competing interests

N.G. and M.J.M. have filed a patent application related to this study. The remaining authors declare no competing interests.

Additional information

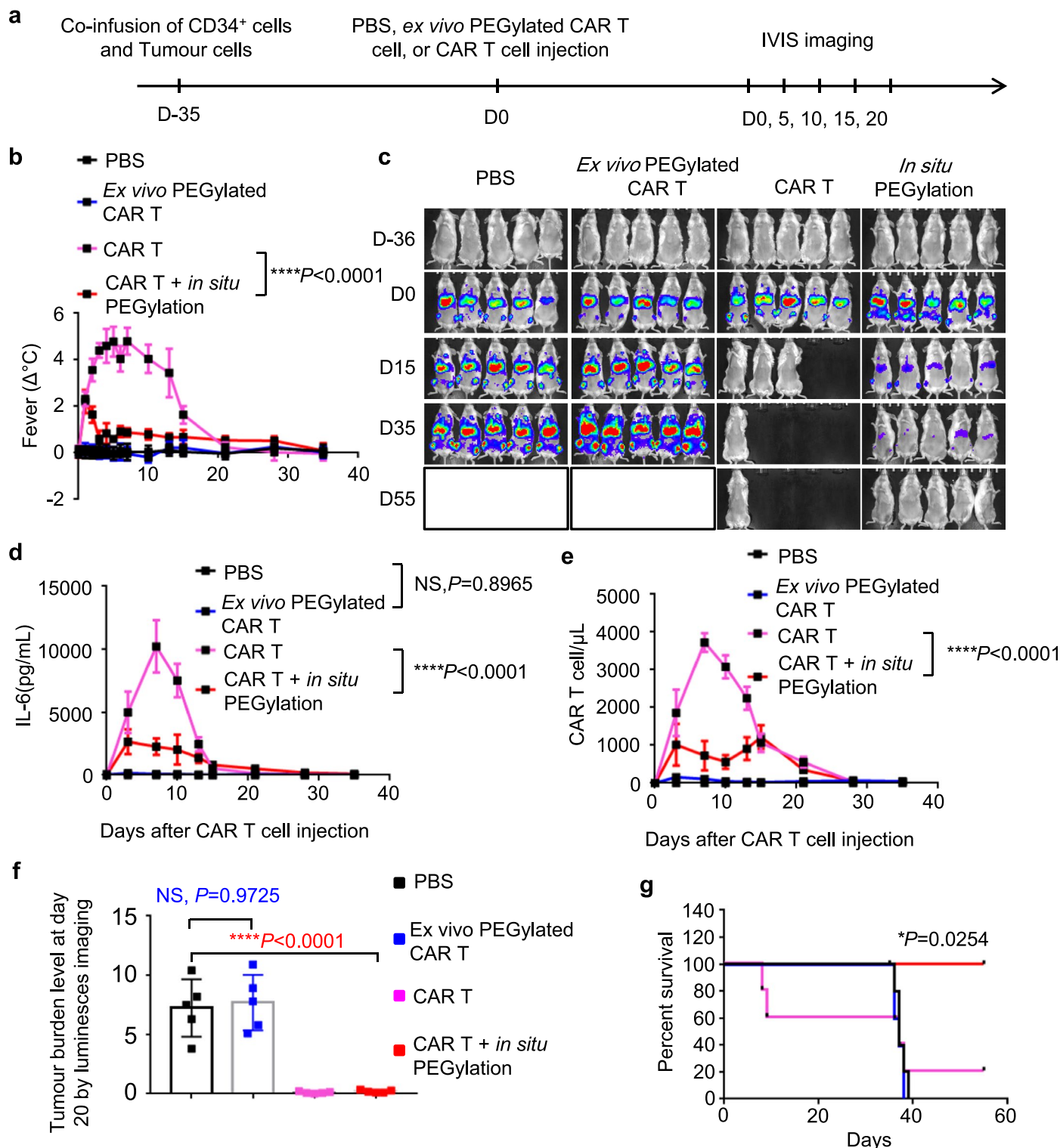
Extended data is available for this paper at <https://doi.org/10.1038/s41563-023-01646-6>.

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Extended Data Fig. 1 | Comparison of the CAR T cell ex vivo PEGylation strategy with the in situ PEGylation strategy. **a**, Raji tumour-bearing mouse model was constructed, and mice were treated with either ex vivo PEGylated CAR T cells or regular CAR T-azide cells at day 0. On day 1, the mice receiving regular CAR T-azide cells developed high fever ($\Delta T > 2^{\circ}\text{C}$), after which DBCO-PEG600K (*in situ* PEGylation) was injected. Mouse body temperature (**b**), tumour burden (c), blood IL-6 levels (**d**) and CAR T cell levels (**e**) were monitored. **f**, quantification

of tumour burden in different groups at day 35. **g**, Kaplan–Meyer survival plots. Data in (**b**), (**d**), (**e**), and (**f**) are shown as mean \pm s.d. ($n = 5$). Statistical differences in (**b**), (**d**), and (**e**) were calculated using two-way ANOVA with Tukey’s post hoc test. Statistical differences in (**f**) were calculated using one-way ANOVA with Tukey’s post hoc test. P values indicated in the figure are from the comparisons on day 7. Statistical differences in (**g**) were conducted using a Mantel–Cox two-sided log-rank test ($n = 5$). P values are indicated.

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Data exclusions	No animals and/or data were excluded.
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Randomization	For animal experiments, dosing groups were filled by randomly selecting from the same pool of animals. Groups in all the in vitro and in vivo experiments were selected randomly.
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<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used	Anti-human CD68 antibody (Catalog# 76437S, clone# D4B9C, 1:1000 dilution) and anti-human CD3 antibody (Catalog# 85061, clone# D7A6E, 1:1000 dilution) were obtained from Cell Signaling Technology. Anti-human CD3-PE (Catalog: 980008, clone# UCHT1, 1:100 dilution), anti-human CD4-BV711 (Catalog: 317439, clone# OKT4, 1:100 dilution), anti-human CD8-APC (Catalog: 344721, clone# SK1, 1:100 dilution), anti-human CD19-BV711 (Catalog: 302245, clone# HIB19, 1:100 dilution), and anti-human CD14-APC (Catalog:
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325607, clone# HCD14, 1:100 dilution) antibodies were purchased from Biolegend. Anti-human F(ab')₂ fragment- DyLight™ 405 antibody (Catalog: 109-475-006, clone# Polyclonal, 1:100 dilution) was purchased from Jackson ImmunoResearch. Anti-PEG antibody (Catalog: ab53449, 1:100 dilution) was purchased from Abcam.

Validation

All the antibodies used are from commercial sources and have been validated by the vendors. Validation data are available on the manufacturer's website.

1. Anti-human CD68 antibody has been validated to be used for Immunohistochemistry analysis and mentioned species reactivity with human. (ref. Fan C S, et al. *Oncotarget*, 2018, 9(4): 4998. DOI: 10.18632/oncotarget.23578)
2. Anti-human CD3 antibody has been validated to be used for Immunohistochemistry analysis and mentioned species reactivity with human (ref. Gong T J, *Frontiers in Oncology*, 2021, 11: 728437. DOI: 10.3389/fonc.2021.728437)
3. Anti-human CD3-PE antibody has been validated to be used for flow cytometric analysis and mentioned species reactivity with human. (Data is provided on their website: <https://www.biolegend.com/en-us/products/pe-anti-human-cd3-antibody-17523>)
4. Anti-human CD4-BV711 antibody has been validated to be used for flow cytometric analysis and mentioned species reactivity with human. (Data is provided on their website: <https://www.biolegend.com/en-us/products/brilliant-violet-711-anti-human-cd4-antibody-7942>)
5. Anti-human CD8-APC antibody has been validated to be used for flow cytometric analysis and mentioned species reactivity with human. (Data is provided on their website: <https://www.biolegend.com/en-us/products/apc-anti-human-cd8-antibody-6531>)
6. Anti-human CD19-BV711 antibody has been validated to be used for flow cytometric analysis and mentioned species reactivity with human. (Data is provided on their website: <https://www.biolegend.com/en-us/products/brilliant-violet-711-anti-human-cd19-antibody-8519>)
7. Anti-human CD14-APC antibody has been validated to be used for flow cytometric analysis and mentioned species reactivity with human. (Data is provided on their website: <https://www.biolegend.com/en-us/products/apc-anti-human-cd14-antibody-3953>)
8. Anti-human F(ab')₂ fragment-Biotin antibody has been validated to be used for flow cytometric analysis and mentioned species reactivity with human. (Data is provided on their website: <https://www.jacksonimmuno.com/catalog/products/109-066-006>. Also in this paper: Guo X, et al. *Frontiers in pharmacology*, 2018, 9: 1118. DOI: 10.3389/fphar.2018.01118)
9. Anti-PEG antibody has been validated to be used for flow cytometric analysis. (Data is provided on their website: <https://www.jacksonimmuno.com/catalog/products/109-066-006> <https://www.abcam.com/products/primary-antibodies/biotin-polyethylene-glycol-antibody-peg-b-47b-ab53449.html>)

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)

Raji cells were originally ordered from ATCC (CCL-86). Raji-Luc-GFP cells were originally from Creative Bio Labs (CAR-STC-ZP43). 293T cells were purchased from ATCC (CRL-3216). and all of the cell lines were tested negative for mycoplasma in University of Pennsylvania cell center.

Authentication

Raji cells were authenticated by ATCC (<https://www.atcc.org/products/ccl-86>)
Raji-Luc-GFP cells were authenticated by Creative Bio Labs (https://www.creative-biolabs.com/car-t/human-burkitt-lymphoma-cell-line-raji-luciferase-gfp-puro-overexpressed-93090.htm?gclid=Cj0KCQjw4s-kBhDqARIsAN-ipH0WVVOey3g_YN0rmwPP8VBZ7iqW92Bnh5L00KkUwe4g2xY_MvDZDAKkaApaEALw_wcB)
293T cells were authenticated by ATCC (<https://www.atcc.org/products/crl-3216?nt=wobj-20-q>)

Mycoplasma contamination

All cell lines were tested for mycoplasma contamination. No mycoplasma contamination was found.

Commonly misidentified lines
(See [ICLAC](#) register)

Raji, Raji-luc-gfp cell lines cell lines are not listed in the database.

Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

Around 200 Female Ttriple transgenic NSG (SGM3) mice (6-8 week) expressing human stem cell factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ) were obtained from the Jackson lab and were housed in a specific pathogen free-grade animal facility with air humidity 40%–70%, ambient temperature (22 ± 2 °C), and 12-h dark/12-h light cycle.

Wild animals

No wild animal was used in this study.

Reporting on sex

Female mice were used in this study

Field-collected samples

The study did not involve samples collected from field.

Ethics oversight

All animal experiment protocols were reviewed and approved by the institutional animal care and use committee of the University of Pennsylvania.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Mice blood was incubated with ammonium chloride buffer for erythrocyte lysis and washed with PBS. Single-cell suspensions were obtained by filtering through a 70 μ M cell strainer. Then the suspensions and stained with antibodies according to the manufacturer's protocols, and then analyzed by flow cytometry.

Instrument

BD LSR II

Software

FlowJo software package (Flowjo V10)

Cell population abundance

The absolute cells around 8000-10000 were analyzed for fluorescent intensity in the defined gate.

Gating strategy

In general, cells were first gated on FSC/SSC. Singlet cells were gated using FSC-H and FSC-A. Dead cells were then excluded and further surface and intracellular antigen gating was performed on the live cell population (Supplementary Figure 23).

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.