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ORIGINAL ARTICLE

Mutated GM-CSF-based CAR-T cells targeting CD116/CD131 complexes exhibit enhanced anti-tumor effects against acute myeloid leukaemia

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Abstract

Objectives. As the prognosis of relapsed/refractory (R/R) acute myeloid leukaemia (AML) remains poor, novel treatment strategies are urgently needed. Clinical trials have shown that chimeric antigen receptor (CAR)-T cells for AML are more challenging than those targeting CD19 in B-cell malignancies. We recently developed *piggyBac*-modified ligand-based CAR-T cells that target CD116/CD131 complexes, also known as the GM-CSF receptor (GMR), for the treatment of juvenile myelomonocytic leukaemia. This study therefore aimed to develop a novel therapeutic method for R/R AML using GMR CAR-T cells. **Methods.** To further improve the efficacy of the original GMR CAR-T cells, we have developed novel GMR CAR vectors incorporating a mutated GM-CSF for the antigen-binding domain and G4S spacer. All GMR CAR-T cells were generated using a *piggyBac*-based gene transfer system. The anti-tumor effect of GMR CAR-T cells was tested in mouse AML xenograft models. **Results.** Nearly 80% of the AML cells predominant in myelomonocytic leukaemia were found to express CD116. GMR CAR-T cells exhibited potent cytotoxic activities against $CD116^{+}$ AML cells *in vitro*. Furthermore, GMR CAR-T cells incorporating a G4S spacer significantly improved long-term *in vitro* and *in vivo* anti-tumor effects. By employing a mutated GM-CSF at residue 21 (E21K), the anti-tumor effects of GMR CAR-T cells were also improved especially in long-term *in vitro* settings. Although GMR CAR-T cells exerted cytotoxic effects on normal monocytes, their lethality on normal neutrophils, T cells, B cells

and NK cells was minimal. **Conclusions.** GMR CAR-T cell therapy represents a promising strategy for $CD116^{+}$ R/R AML.

Keywords: AML, CD116, GM-CSF, GM-CSF receptor, GMR, low affinity

INTRODUCTION

In spite of the modern advances in intensive chemotherapy and haematopoietic stem cell transplantation (HSCT), the prognosis of acute myeloid leukaemia (AML) remains poor.[1–3] Approximately 10-20% of AML patients experience induction failure,[4] and 5-year survival is only 60% in paediatric AML patients[5,6] and 40% in adults.[1,2] AML outcomes further deteriorate in patients with such poor prognostic factors as FMS-like tyrosine kinase 3 (*FLT3*) mutations.[7,8] Thus, effective and safe therapies are urgently needed for AML patients.

Recent clinical trials have shown impressive results for CD19 antigen-specific chimeric antigen receptor-T (CAR-T) cell therapies in B-cell malignancies.[9–13] However, AML-directed CAR-T cell treatments have been challenging to date in clinical studies.[14–17] Specifically, the 'on target off tumor effect' is almost inevitable when CAR-T cells are targeted to AML blasts since the AML-associated target antigens are generally expressed on normal myeloid cells as well.[18–21] New strategies are therefore needed to improve the safety and efficacy of CAR-T cell therapy for AML.

The granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor (GMR) consists of two subunits: an α subunit (CD116) and a common β subunit (CD131) that is shared with IL-3 and IL-5 receptors. As the α subunit of the GMR, CD116 is expressed in juvenile myelomonocytic leukaemia (JMML),[22] AML and normal myeloid cells.[23] Specifically, hypersensitivity to GM-CSF is a hallmark of JMML, with mutually exclusive genetic abnormalities in the GMR signalling pathway.[24,25] A recent study revealed that hypersensitivity to GM-CSF was also found in chronic myelomonocytic leukaemia (CMML).[26] Moreover, approximately 30% of CMML patients obtained clinical benefit from the GM-CSF inhibitor lenzilumab in a phase I/Ib clinical trial.[27] We recently demonstrated the antiproliferative effects of ligand-based GMR-specific CAR-T cells against JMML with minimal toxicity to normal myeloid progenitor cells.[22] Since CD116 is overexpressed in 63-78% of AML cases,[23,28] and especially in the *FLT3*-mutated AML associated with poor prognosis,[29] we hypothesise that CAR-T cells targeting CD116/CD131 complexes may be a promising strategy for the treatment of high-risk and relapsed/refractory (R/R) AML.

The residue 21 of GM-CSF plays a critical role in its biological activity.[30] Mutated analogs of GM-CSF at this residue, such as E21K and E21R, exhibit distinct biological functions as compared with the wild type.[31] Thus, it is possible that mutated ligand-based GMR CAR-T cells can alter the biological interactions between CAR-T cells and their targets, which may enhance anti-tumor activity.

In the present study, we aimed to demonstrate that GMR CAR-T cells could elicit anti-tumor effects against AML both *in vitro* and *in vivo*. By employing both an optimised spacer and a specifically mutated GM-CSF at residue 21, we also sought to improve the *in vivo* anti-tumor effects of GMR CAR-T while maintaining minimal toxicity on normal cells.

RESULTS

The GM-CSF receptor α chain, CD116, is highly expressed in AML cells

To determine whether the GM-CSF receptor α chain (CD116) could be used as a target of CAR-T therapy for AML, we examined CD116 expression in 5 AML cell lines by flow cytometry. CD116 was highly expressed in the myelomonocytic leukaemia lines of THP-1 (French–American–British [FAB] classification M5), MV4-11 (M5) and ShinAML-1 (M4) and was partially expressed in Kasumi-1 (M2) and HL-60 (M3) cell lines (Figure 1a). In contrast, CD116 was minimally expressed in the B-acute lymphoblastic leukaemia (ALL) cell line (KOPN57bi) (Figure 1a). We next evaluated CD116 expression in primary leukaemic cells by analysing peripheral blood or bone marrow samples obtained from 29 AML patients with various FAB subtypes (M1-M7) (Figure 1b, c) (Supplementary figure 1 and Supplementary table

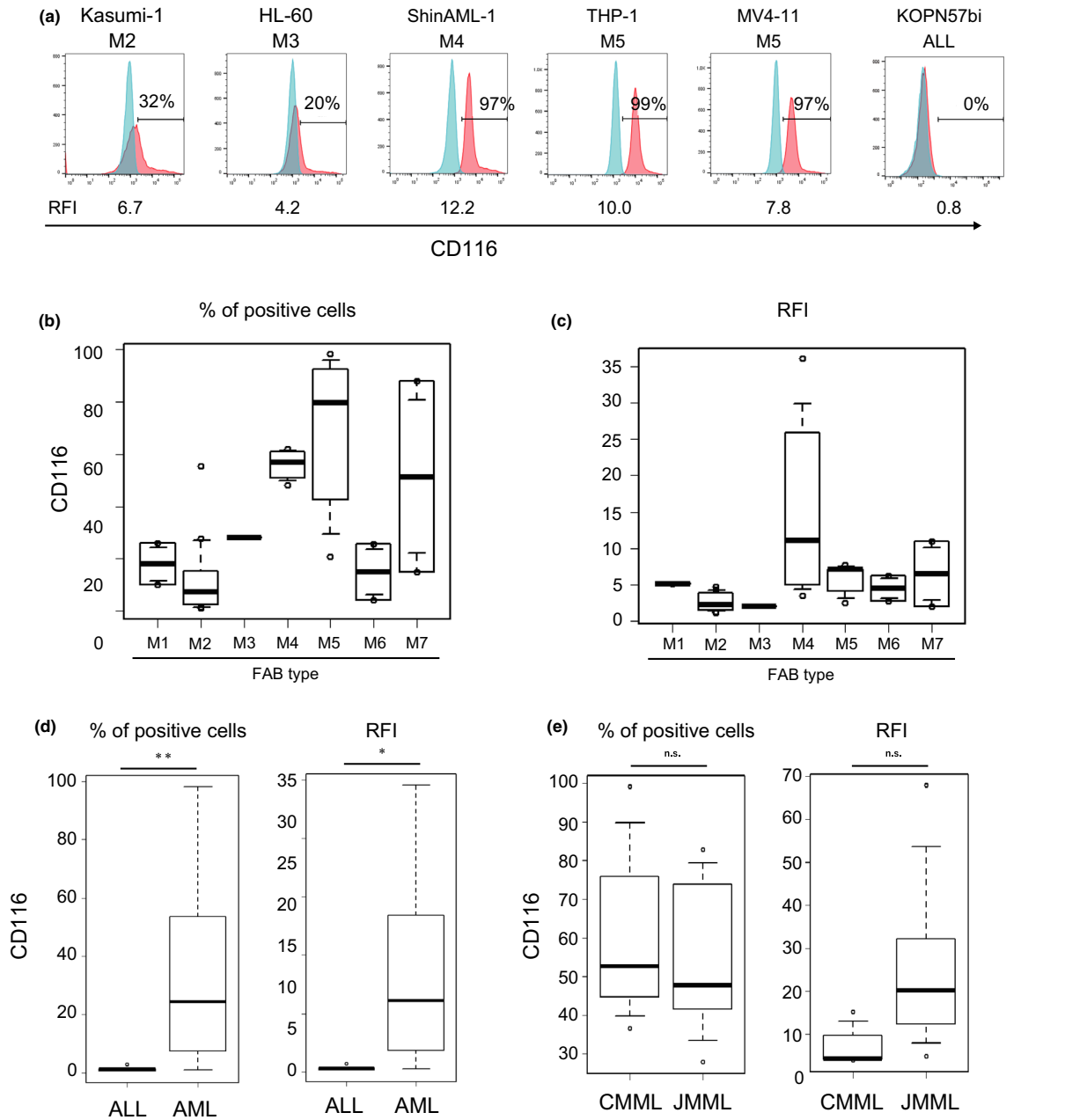

Figure 1. CD116 (GMRα) is expressed in AML. CD116 expression was examined in both AML cell lines and primary leukaemic samples from AML patients. **(a)** Surface CD116 (GMRα) expression relative to the isotype control of 5 AML cell lines: Kasumi-1, HL-60, shinAML-1, THP-1, MV4-11 and 1 ALL cell line (KOPN57bi). RFI was calculated by dividing the MFI of samples with that of the isotype control **(b, c)**. Summary results of CD116 expression on primary leukaemia cells from 29 AML patients according to FAB classification. The percentage of CD116-positive cells **(b)** and RFI **(c)** is shown. **(d)** Comparison of CD116 expression between 29 AML patients and 5 ALL patients. The percentage of CD116-positive cells and RFI is shown. **(e)** Comparison of CD116 expression between 3 CMML patients and 5 JMML patients. The percentage of CD116-positive cells and RFI is shown. Mann–Whitney *U*-tests were used to identify significant differences. $*P < 0.05$, $**P < 0.01$, n.s., not significant.

1). Overall, the median (range) percentage of $CD116^+$ cells was 24.4 (1.0–98.1) % in AML patients (Figure 1d), and 24 of 29 (83%) AML patients showed a CD116 relative fluorescence intensity (RFI) of ≥ 2 (Figure 1d). Specifically, myelomonocytic and monocytic leukaemia (FAB M4 and M5) displayed significantly higher percentages (median 60.1%, range 20.5–98.1%,

$P < 0.001$) of $CD116^+$ cells than those (median 11.5%, range 1.0–87.8% $P < 0.01$) of other subtypes (M1-3 and M6-7) (Figure 1b). CD116 expression levels were consistently and significantly higher (RFI: median 7.1 range 2.3–36.0, $P < 0.01$) in FAB M4 and M5 subtypes as compared with those (RFI: median 2.6, range 1.1–11.0) of other subtypes (Figure 1c). These results indicated that AML, especially of the myelomonocytic lineage, highly expressed CD116. CD116 was overexpressed in AML, whereas negligible percentages (median 1.4%, range 0.6–2.8%) and intensities (RFI: median 1.5, range 1.2–1.6) of CD116 were observed in ALL cells (Figure 1d). Additionally, both CMML and JMML expressed high levels of CD116 (Figure 1e). Based on the above findings, we proceeded to investigate the anti-tumor effects of GMR CAR-T therapy for AML, particularly of myelomonocytic lineage.

GMR CAR-T cells exert anti-tumor effects on AML cells *in vitro*

We previously described the antiproliferative effects of ligand-based GMR CAR-T cells against JMML cells.[22] However, our original GMR CAR construct contained GM-CSF as an antigen-binding site and an IgG1 CH2CH3 hinge lesion as a spacer (GMR^{WT} CAR [CH2CH3]) (Figure 2a), which might abrogate the *in vivo* efficacy of CAR-T cells.[32–34] As expected, GMR^{WT} CAR[CH2CH3]-T cells showed no anti-tumor effects *in vivo* (Supplementary figure 2). Therefore, we constructed 2 new plasmid vectors of GMR^{WT} CAR (Figure 2a) by either removing the CH2CH3 region (ΔCH2CH3) or replacing it with a G4S linker (G4S) (Figure 2a). As shown in Figure 2b, GMR^{WT} CAR-T cells with either the ΔCH2CH3 spacer (GMR^{WT} CAR [ΔCH2CH3]-T cells) or G4S spacer (GMR^{WT} CAR [G4S]-T cells) displayed similar CAR expression levels (37–42%), which were comparable to that of the original GMR CAR-T cells (46%) (data not shown).

To examine the anti-tumor effects of GMR^{WT} CAR-T cells on AML cells *in vitro*, GMR^{WT} CAR-T cells were co-cultured with 4 different subtypes of AML cell lines: FAB M2, M3, M4 and M5. On day 5 of co-culture, GMR^{WT} CAR-T cells with either the ΔCH2CH3 or G4S spacer had eradicated the $CD116^+$ ShinAML-1 and THP-1 cells (Figure 2c). The GMR^{WT} CAR-T cells also exhibited specific anti-tumor effects on $CD116^+$ in the MV4-11 (M5) cell line and could eradicate Kasumi-1 (M2) cells having only partial CD116 expression (Figure 2c). These anti-tumor effects were comparable for the ΔCH2CH3 and G4S spacers. In contrast, neither of the GMR^{WT} CAR-T cell types exerted anti-tumor effects on HL-60 (M3) cells weakly expressing CD116 (Figure 2c).

We next explored the long-term killing ability of the GMR^{WT} CAR-T cells with different spacers. Against multiple challenges of leukaemia cells, the GMR^{WT} CAR[ΔCH2CH3]-T cells initially reduced, but ultimately failed, to control tumor growth, whereas the GMR^{WT} CAR[G4S]-T cells could sustain their anti-tumor activity against both MV4-11 and THP-1 even after a second or a third challenge (Figure 2d) (Supplementary figure 3). Thus, the G4S spacer appeared to provide superior long-term anti-tumor effects to GMR^{WT} CAR-T cells *in vitro*.

To compare the *in vivo* anti-tumor activities of GMR^{WT} CAR-T cells with different spacers, THP-1 firefly Luciferase (ffLuc)-bearing NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/*SzJ* (NSG) mice were treated with intravenous injection of 1.2×10^6 of CAR-T cells (Figure 2e). In mice receiving PBS, tumor progression was evident (Figure 2f–g), and all mice had succumbed to leukaemia by day 60 (Figure 2h). Similarly, CD19.CAR-T cells were unable to control leukaemia progression, and all mice had died by day 80. Conversely, GMR^{WT} [G4S] CAR-T cells significantly suppressed leukaemia progression as compared with CD19.CAR-T cells as well as with GMR^{WT} CAR [ΔCH2CH3]-T cells (both, $P < 0.01$) (Figure 2g). These substantial anti-tumor effects translated to a significant prolongation of survival in GMR^{WT} CAR[G4S]-T cells versus PBS and CD19.CAR-T cells (both, $P < 0.01$), with all the mice surviving more than 145 days (Figure 2h). Together with the long-term *in vitro* co-culture results, GMR^{WT} CAR [G4S]-T cells were considered to have robust and durable anti-tumor activities against $CD116^+$ AML.

CAR-T cells with a mutated GM-CSF ligand exhibit superior anti-tumor effects

We simultaneously sought to further improve the anti-tumor activity of GMR CAR by modulating the binding affinity of the antigen recognition site. Based on studies performed by Lopez *et al*.,[30,31] GM-CSF mutations at residue 21 in GMR CAR vectors were used to fine-tune the antigen-binding ability of GMR CAR-T cells.

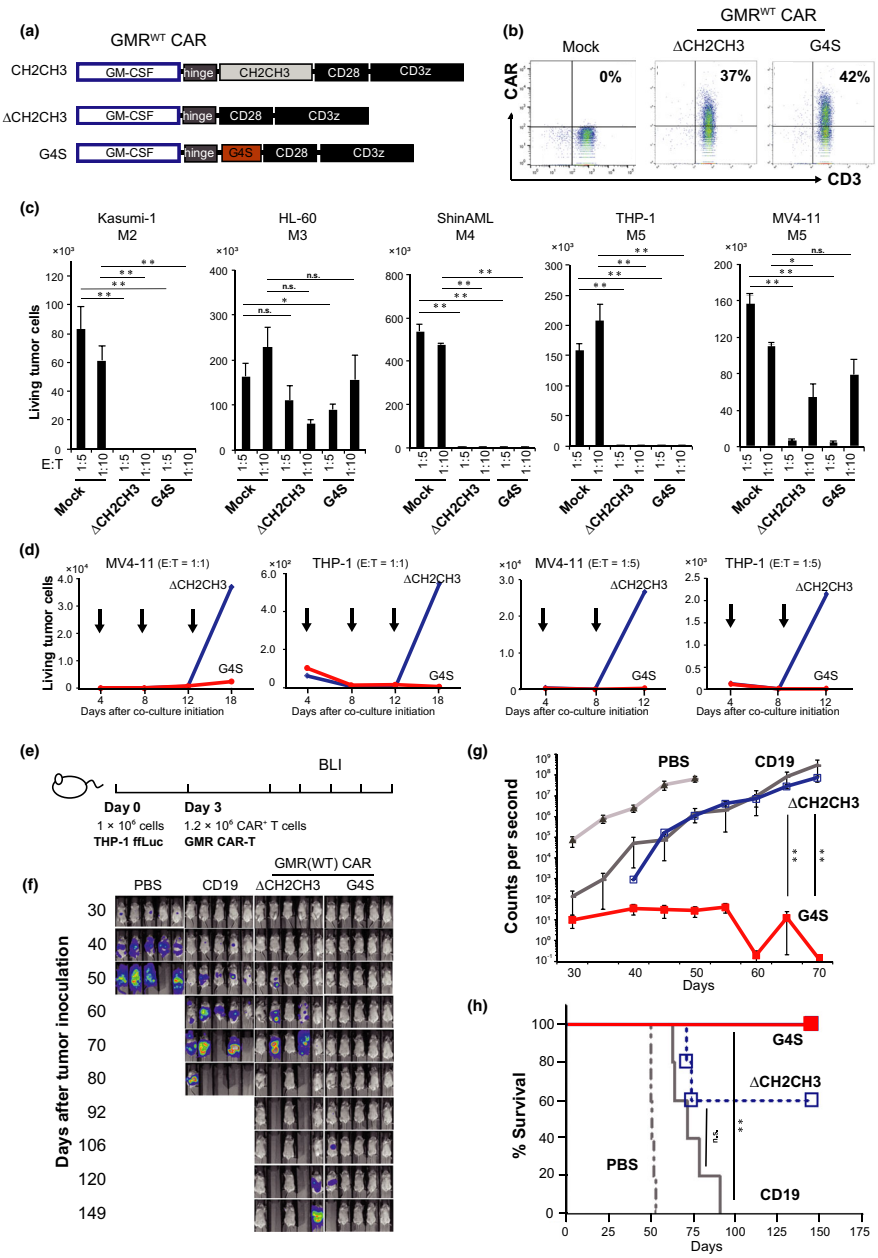

Figure 2. GMR CAR-T with modified G4S spacer exhibited superior anti-leukaemic effects against AML cells. **(a)** Schematic diagram of the GMR CAR constructs with spacer modifications. The CH2CH3 region was removed from the original GMR CAR (CH2CH3) construct and replaced with either an IgG1 hinge region (ΔCH2CH3) or 3 repeated sequences of GGGGS (G4S). **(b)** Representative surface CAR expression of 2 GMR CAR-T cells with different spacers (ΔCH2CH3 and G4S). Mock-T cells were used as a negative control. **(c)** *In vitro* anti-tumor effects of the spacer-modified GMR CAR-T cells. GMR CAR-T cells were co-cultured with 5 different AML lines at an E:T ratio of 1:5 or 1:10. The numbers of residual live leukaemia cells at 5 days after co-culture are shown. Data represent the mean ± SEM (n = 3). Student's t-tests were employed to identify significant differences. *P < 0.05, **P < 0.01, n.s., not significant. **(d)** Long-term *in vitro* killing ability of GMR CAR-T by serial co-culture assays. GMR CAR-T cells were co-cultured with THP-1 or MV4-11. On day 3 or 4 of serial co-culture, the cells were harvested and analysed by flow cytometry to quantify the residual leukaemic cells and T cells. Fresh leukaemia cells were repeatedly added at the defined E:T ratio (n = 1). **(e)** Experimental plan of the THP-1 ffLuc xenograft model. On day 3 after leukaemia inoculation, the mice were treated with 1.2 × 10^6 GMR CAR-T cells (dose was adjusted by CAR^+ cells). **(f)** Sequential BLI study in the THP-1 ffLuc xenograft model. BLI was performed approximately every 10 days until day 120. **(g)** Summary of the BLI results in each treatment group. Data represent the mean ± SEM of each group. *P < 0.05, **P < 0.01. **(h)** Kaplan–Meier analysis of survival for each treatment group. Log-rank tests were used for statistical analysis of survival between groups. *P < 0.05, **P < 0.01. Data are representative of one experiment (n = 5 mice per group) **(g, h)**.

Screening analysis of 7 mutated GMR CAR constructs (Figure 3a) with the CH2CH3 spacer revealed that GMR CAR-T cells with GM-CSF E21R or E21K mutations (GMR^{E21R} or GMR^{E21K}) exhibited superior anti-tumor activity *in vitro* (Figure 3b) against THP-1 cells. Accordingly, we constructed GMR CAR vectors with either E21K or E21R mutations and the ΔCH2CH3 spacer (Figure 3c). Both mutated GMR^{E21K} and GMR^{E21R} CAR [ΔCH2CH3]-T cells showed equivalent CAR expression (Figure 3d). To test the anti-tumor activity of the mutated GMR CAR-T cells, THP-1-bearing NSG mice were intravenously treated with 5×10^6 total T cells (Figure 3e). All control group mice had died from leukaemia by day 49 (PBS) or day 53 (CD19.CAR-T cells) (Figure 3f) because of the leukaemia progression, as confirmed by pathological examination (Supplementary figure 4). The mice treated with GMR^{WT} CAR[ΔCH2CH3]-T cells had succumbed to leukaemia by day 63 (Figure 3f). In contrast, the animals treated with both GMR^{E21R} and GMR^{E21K} CAR[ΔCH2CH3]-T cells showed significantly prolonged survival over controls (PBS or CD19.CAR-T cells) (all, $P < 0.01$) as well as over GMR^{WT} CAR[ΔCH2CH3]-T cells (E21R, $P < 0.01$; E21K, $P = 0.02$), with some surviving more than 150 days. These results indicated that modifying the antigen-binding site could further improve the anti-tumor effects of GMR CAR-T cells.

Fine-tuned GMR CAR-T cells display enhanced anti-tumor effects

To further optimise GMR CAR-T cells, the mutated GMR CAR-T cells were incorporated with the G4S spacer (Figure 4a). Both the mutated GMR^{E21K} and GMR^{E21R} GMR CAR[G4S]-T cells showed similar CAR expression levels (Figure 4b, c) and comparable *ex vivo* expansion of transduced T cells (Figure 4d), as compared to GMR^{WT} CAR [G4S]-T cells as well as GMR CAR[ΔCH2CH3]-T cells (Figure 4c, d). To examine whether the mutated GM-CSF ligands could ameliorate long-term *in vitro* anti-tumor effects, GMR CAR-T cells were sequentially co-cultured with THP-1 cells or MV4-11 cells. After multiple leukaemia cell challenges, GMR^{E21K}CAR[G4S]-T cells maintained superior anti-tumor activity to GMR^{WT}CAR[G4S]-T cells against MV4-11 cells, but not against THP-1 cells (Figure 4e) (Supplementary figure 5). Furthermore, GMR^{E21K}CAR[G4S]-T cells showed significantly better proliferation in response to both THP-1 cells and MV4-11 cells than GMR^{WT}CAR[G4S]-T cells after 2–5 stimulations. Next, MV4-11-bearing NSG mice were intravenously treated with 1.2×10^6 GMR CAR [G4S]-T cells to evaluate *in vivo* anti-tumor effects (Figure 4f). On day 45, peripheral blood samples were collected from the mice, and leukaemia cell burden was quantified by detecting the *MLL-AF4* fusion gene using qRT-PCR. In control group mice (PBS and CD19.CAR-T cells), substantial amounts of leukaemic *MLL-AF4* fusion transcripts were observed on day 45 (Figure 4g), and most animals had died by day 60 (Figure 4g). *MLL-AF4* fusion transcripts were also detected in the mice treated with GMR^{E21R} CAR[G4S]-T cells regardless of the spacer, and approximately half of them had died by day 75. No leukaemic *MLL-AF4* transcripts were detected in mice treated with GMR^{WT}CAR[G4S]-T cells or GMR^{E21K}CAR[G4S]-T cells (Figure 4h), which resulted in significantly longer survival than in control groups (both $P < 0.05$) (Figure 4h) (Supplementary table 2).

GMR CAR-T responses correlated with the CD116 expression levels of target cells

To investigate whether the expression levels of CD116 correlated with the cytotoxic effects of GMR CAR-T cells, we co-cultured GMR^{E21K} CAR [G4S]-T cells with 3 cell lines (MV4-11, Kasumi-1 and K562) possessing different CD116 levels. As expected, cytotoxicity against the 3 cell lines was correlated to the CD116 expression levels of the target cells (Supplementary figure 6). Furthermore, IFN-γ, IL-2 and TNF-α production by GMR^{E21K} CAR[G4S]-T cells correlated with CD116 expression levels (Supplementary figure 6). To confirm these observations, we co-cultured GMR^{E21K} CAR[G4S]-T cells with 7 leukaemic primary cells exhibiting different CD116 expression levels (Supplementary figure 7). The cytotoxicity of the GMR^{E21K} CAR[G4S]-T cells were closely correlated to the % of CD116-positive cells in the target cells ($R^2 = 0.7444$, $P = 0.07$) (Supplementary figure 8). In agreement, IFN-γ production levels against leukaemia cells were also related to target cell CD116 expression levels ($R^2 = 0.7404$, $P = 0.013$) (Supplementary figure 8). These results confirmed that the cytotoxic and cytokine responses of GMR^{E21K} CAR[G4S]-T cells correlated strongly with the CD116 levels of the target cells.

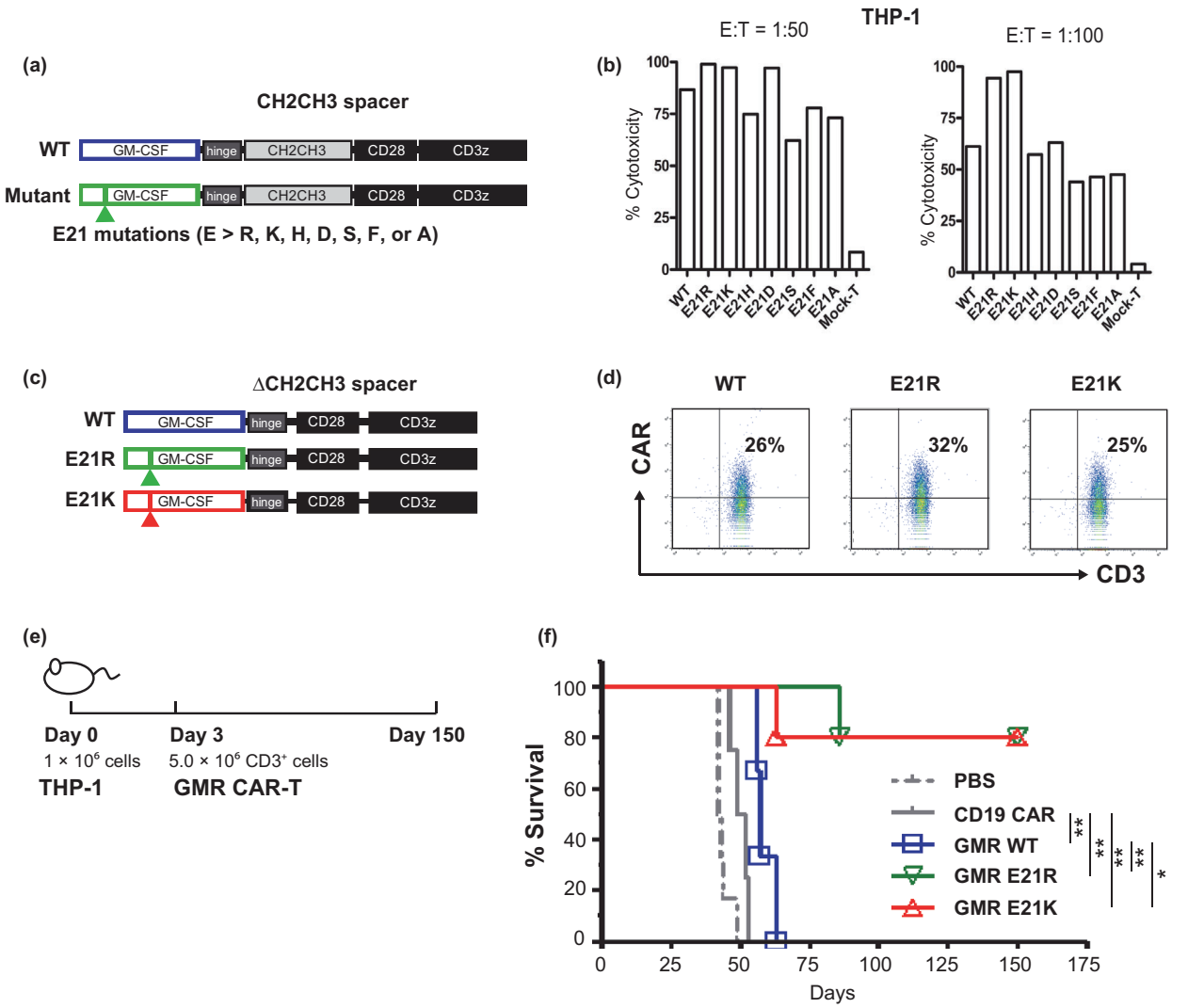

Figure 3. GMR CAR-T with mutated ligand-binding site exhibited superior anti-leukaemia activity against AML. **(a)** Schematic diagram of the WT or mutated GMR CAR constructs with the CH2CH3 hinge region. **(b)** Screening co-culture experiment of WT or mutated GMR CAR-T and THP-1. GMR CAR-T was co-cultured with THP-1 cells at E:T ratios of 1:50 or 1:100. At 5 days of co-culture, the living leukaemia cells were quantified by flow cytometry and counting beads (n = 1). The percentage of cytotoxicity was calculated by the following formula: (100 – [count of $CD33^+$ cells with CAR-T cells/count of $CD33^+$ cells without CAR-T cells]) × 100%. **(c)** Schematic diagram of the WT and E21R- and E21K-mutated GMR CAR constructs with the ΔCH2CH3 spacer. **(d)** Representative surface expression of CAR in GMR CAR-T cells with the WT or mutated GM-CSF ligand and incorporating the ΔCH2CH3 spacer. **(e)** Experimental plan of the THP-1 xenograft model. NSG mice were intravenously injected with 1 × 10^6 THP-1 cells on day 0. The mice received 5 × 10^6 of GMR CAR-T cells (dose was adjusted by $CD3^+$ cells) on day 3. **(f)** Kaplan–Meier analysis for each treatment group). Log-rank tests were used for statistical analysis of survival between groups. *P < 0.05, **P < 0.01. Data are representative of one experiment (PBS, n = 6; CD19, n = 4; GMR WT, n = 3; GMR E21R and E21K, n = 5 mice per group).

Safety of GMR CAR-T cells

To characterise the safety profile of GMR CAR-T cells, CD116 expression was examined in normal haematopoietic cells. As expected, nearly 100% of monocytes and neutrophils expressed CD116 (Figure 5a, b), while T, B and NK cells expressed only negligible levels. We observed a significantly higher percentage and RFI for CD116 in monocytes than in neutrophils (Figure 5b, c). We subsequently co-cultured GMR CAR-T cells with PBMCs or polymorphonuclear leukocytes (PMNs) to determine whether GMR CAR-T cells affected normal haematopoietic cells. All of the tested

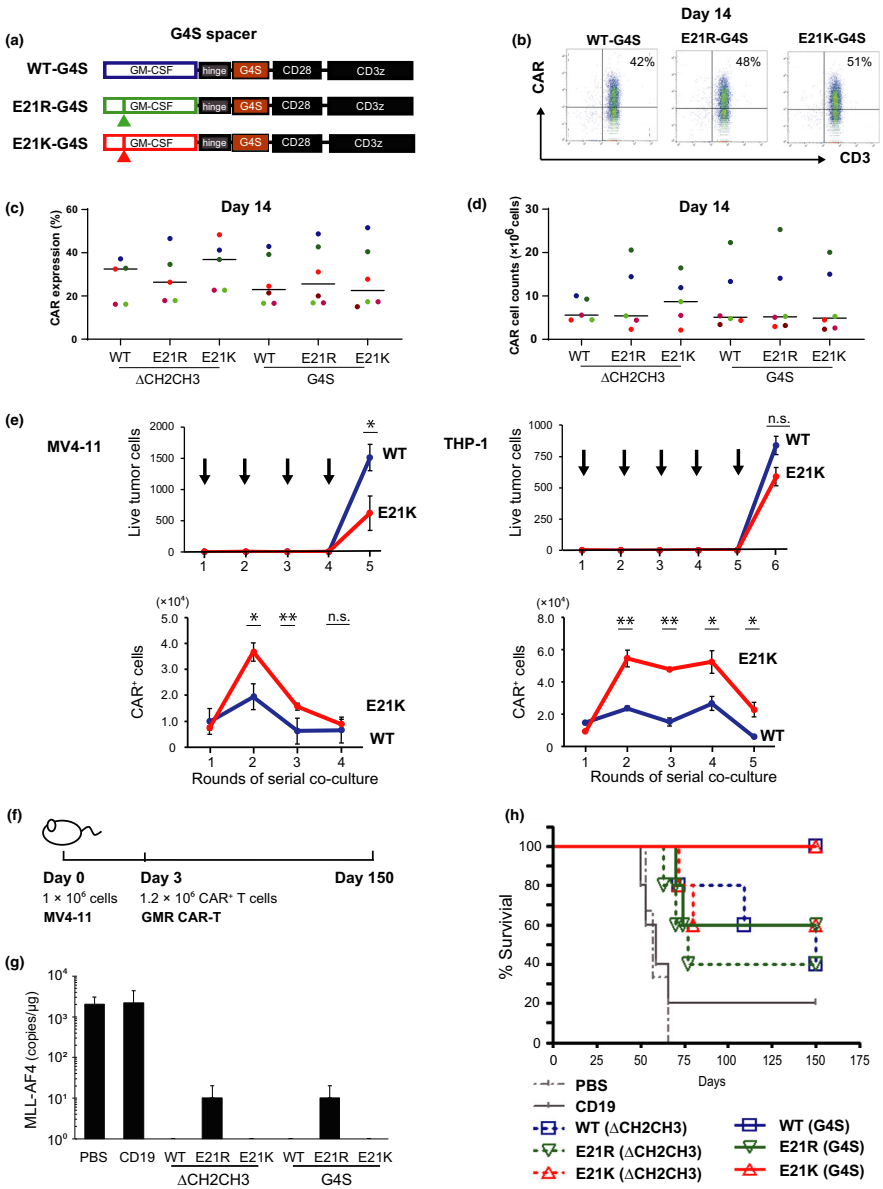

Figure 4. GMR^{E21K} CAR-T cells incorporating a G4S spacer exhibited potent anti-leukaemic effects. **(a)** Schematic diagram of the CAR constructs of GMR CAR with a mutated GMR ligand-binding site and G4S spacer. **(b)** Representative surface expression of GMR CAR-T cells generated from a healthy donor with wild-type GMR or with mutated GMR and the G4S spacer. **(c)** Comparison of CAR expressions among GMR CAR-T cells with 6 different constructs (ΔCH2CH3 or G4S × WT, E21K or E21R). Data represent CAR expression in GMR CAR-T cells generated from 5 (ΔCH2CH3) or 6 (G4S) healthy donors 14 days after the culture initiation. Each colour represents a different donor. **(d)** Total CAR$^+$ T cell numbers generated by the *piggyBac*-modified CAR-T generation system from 10 × 10^6 PBMCs after 14 days of culture. Data represent the numbers of CD3$^+$CAR$^+$ cells obtained from 5 (ΔCH2CH3) or 6 (G4S) healthy donors. Each colour represents a different donor. **(e)** Long-term *in vitro* killing ability of the mutated GMR CAR-T cells with the G4S spacer by serial co-culture assays. GMR CAR-T cells were co-cultured with 1 × 10^5 THP-1 or MV4-11 cells in each well of 48-well plates. On day 3 or 4 of serial co-culture, the cells were harvested and analysed by flow cytometry to quantify the residual leukaemic cells and CAR$^+$ T cells. CAR-T cells were repeatedly re-challenged with fresh leukaemia cells at the defined E:T ratio. The mean ± SEM values from 3 independent experiments are shown. Student's *t*-tests were used to identify significant differences. *$P < 0.05$, **$P < 0.01$. **(f)** Experimental plan of the MV4-11 xenograft model. NSG mice were treated with 1.2 × 10^6 GMR CAR-T cells (dose was adjusted by CAR$^+$ cells) on day 3 after tumor inoculation. **(g)** Leukaemic *MLL-AF4* transcripts in peripheral blood samples were examined by qRT-PCR. Data represent the median (range) of each group. **(h)** Kaplan–Meier analysis of each treatment group. Log-rank tests were used for statistical analysis of survival among the groups. *$P < 0.05$, **$P < 0.01$. Data are representative of one experiment (PBS, $n = 3$; CD19, $n = 5$; GMR, $n = 5$ mice per group) (**g**, **h**).

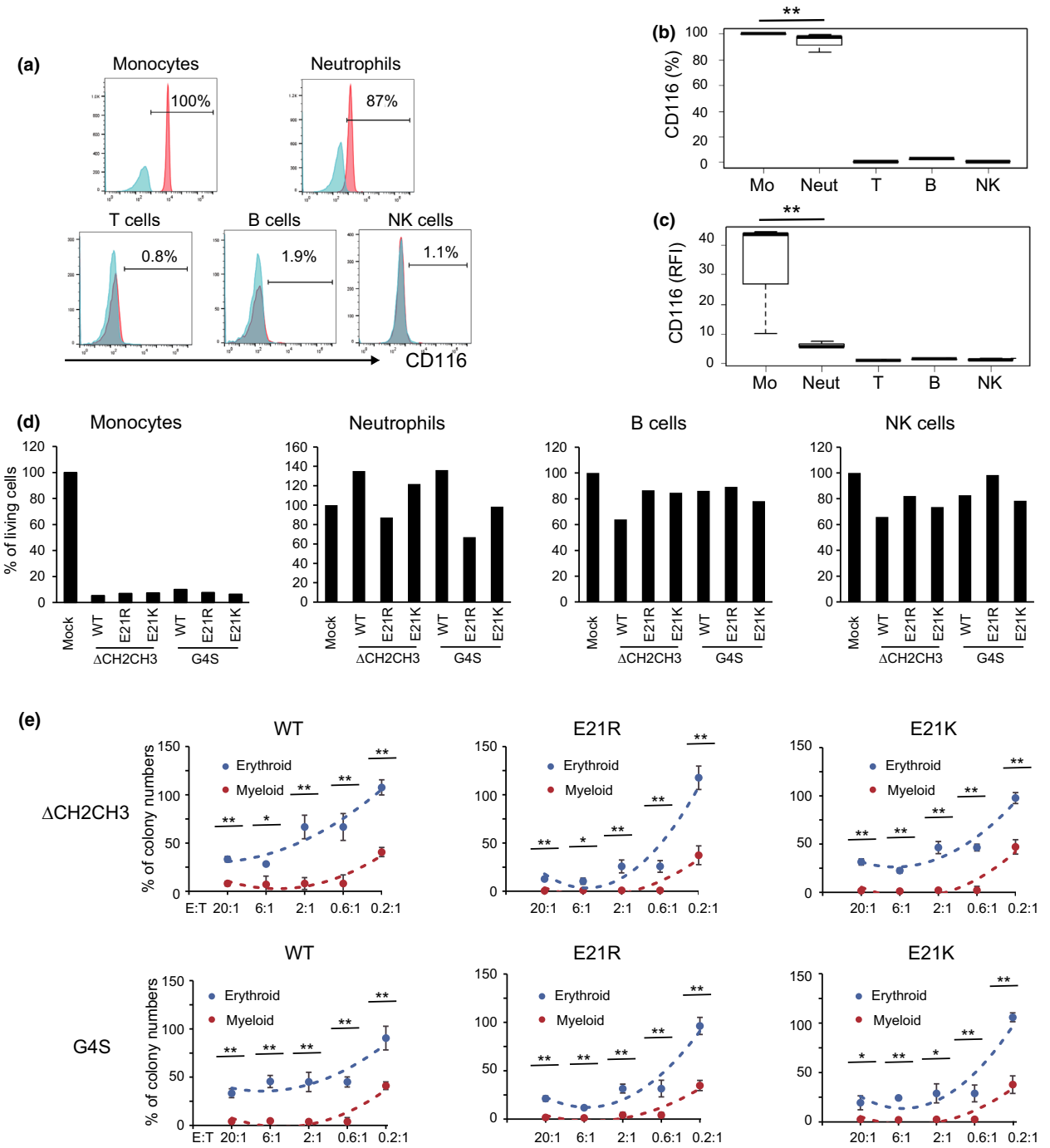

Figure 5. Safety profile of GMR CAR-T cells. **(a–c)** CD116 expression in normal haematopoietic cells of healthy donors. Representative histogram plots **(a)** and summary results of CD116 (%) **(b)** and CD116 (RFI) **(c)** are shown. Data represent the median (range) values from 3 donors. Mann–Whitney *U*-tests and Kruskal–Wallis tests were used to identify significant differences between treatment groups. **P* < 0.05, ***P* < 0.01, n.s., not significant. **(d)** PBMCs (monocytes and T, B and NK cells) or PMNs (neutrophils) were co-cultured with GMR CAR-T cells at an E:T = 1:1 for 3 days, and then B cells ($CD19^+CD3^-$), NK cells ($CD16^+CD3^-$), neutrophils ($CD11b^+CD3^-$) and monocytes ($CD11b^+CD3^-$) were quantified by flow cytometry using counting beads. **(e)**. Samples of 500 human cord blood $CD34^+$ cells and effector cells (Mock-T cells or GMR CAR-T cells) were co-cultured at the indicated E:T ratios. On day 7, colony numbers of erythroid and myeloid colonies from the co-cultures were quantified using STEMvision™. The mean ± SD values are shown (n = 3). Student's *t*-tests were employed to identify significant differences. **P* < 0.05, ***P* < 0.01.

GMR CAR-T cells exerted substantial cytotoxic effects on monocytes, but only negligible cytotoxicity on T, B and NK cells (Figure 5d). Surprisingly, GMR CAR-T cells minimally affected neutrophils despite high neutrophil CD116 expression. Although GMR CAR-T cells induced higher cytotoxic effects on CD116 highly expressing dendritic cells (DCs) than on control CD19 CAR-T cells, they did not eradicate DCs, even at high E:T ratios (Supplementary figure 9).

To examine the toxic effects of GMR CAR-T cells on haematopoietic stem cells (HSCs)/progenitor cells (HPCs), CD116 expression in those cells was analysed by flow cytometry. Although HSCs and HPCs partially expressed CD116, their expression levels were remarkably lower than that in bone marrow myeloid cells (Supplementary figure 10). We next assessed the impact of GMR CAR-T cells on HSCs by colony-forming assays. All of the tested GMR CAR-T cells were comparably toxic to myeloid progenitor cells but showed limited cytotoxicity on erythroid progenitor cells (Figure 5e). Statistical analysis showed that myeloid progenitor cells were significantly more susceptible to GMR CAR-T cells than were erythroid progenitor cells.

Optimised GMR CAR-T cells exhibit a favorable phenotype

To characterise the phenotype of the optimised GMR^{E21K} CAR[G4S]-T cells, immunophenotypes were examined by flow cytometry. Consistently with our previous reports,[35,36] *piggyBac*-modified GMR CAR-T cell products contained 68.7 $\pm$ 11.6% of $CD3^+$ cells with $CD8^+$ dominance and a low percentage (10.4 $\pm$ 4.3%) of $CD3^-CD56^+$ cells (Supplementary figure 11a, b). Moreover, GMR CAR-T cells contained approximately 20.2 $\pm$ 2.4% of the $CCR7^+CD45RA^+$ naïve/stem cell memory population (Supplementary figure 11c, d). Although 48.5 $\pm$ 11.2% of GMR CAR-T cells expressed the exhaustion marker TIM3, they minimally expressed other markers, such as PD-1 and LAG3 (Supplementary figure 11e, f). These phenotypical characteristics were comparable among GMR CAR-T cells with 6 different CAR configurations (Supplementary figure 12). These findings suggested that the *piggyBac*-modified GMR CAR-T cells possessed a relatively favorable phenotype that might be beneficial in clinical trials.

DISCUSSION

The present study comprehensively demonstrated that GMR CAR-T cells redirected to a GMR complex exhibited specific and potent anti-tumor activity against $CD116^+$ AML cells both *in vitro* and *in vivo*. Although various kinds of CAR-T cells have been developed for AML, there remain challenges in AML targeting, mostly because of the heterogeneous and weak expression of target antigens and myelotoxicity. We herein described novel GMR CAR-T cells specifically targeted $CD116^+$ AML cells for eradication at very low E:T ratios *in vitro*. Not only did the GMR CAR-T cells demonstrate durable cytotoxic activity against multiple challenges of AML cells *in vitro*, they also completely suppressed the progression of $CD116^+$ AML cells in multiple AML xenograft models. Furthermore, fine-tuning of GMR CAR-T cells with the E21K mutation of GM-CSF and a G4S spacer produced a CAR configuration and phenotype suitable for clinical testing.

This study showed that 82% of AML primary cells overexpressed CD116, which was consistent with recent studies[23,28] showing CD116 expression in 63%–78% of AML cells. Specifically, higher CD116 expression was observed in AML with the M4-5 FAB subtypes in accordance with the remarkably high CD116 expression in JMML and CMML. These AML subtypes may be of clinical relevance because of the potential suboptimal response to venetoclax with hypomethylating agents.[37] The CD116 expression pattern in AML subtypes differs from those of CD33 and CD123,[38] both of which have been promising targets in CAR-T therapy for AML.[39] The unique therapeutic range provided by GMR CAR-T cells represents an attractive alternative CAR-T treatment for R/R AML.

The design of spacers connecting the antigen recognition site and transmembrane region is known to affect the persistency and efficacy of CAR-T cells.[32,33,40,41] Specifically, GMR CAR-T cells incorporating the G4S spacer exhibited superior long-term *in vitro* anti-tumor activity over the ΔCH2CH3 spacer consistently with a greater long-term *in vitro* killing ability. Although the precise mechanism of the difference between CH2CH3 and G4S was not addressed in this study, one possible explanation was that a different spacer length could provide the optimal distance between target antigens and CAR-T cells.[42]

The present study also demonstrated that the use of a mutated GM-CSF (E21K) significantly improved long-term *in vitro* anti-tumor activities. Recent structure–function studies have revealed that GMRs may be binary complexes of GMRα alone (low-affinity receptors) or hexamer/dodecamer complexes consisting of both GMR α and β chains (high-affinity receptors).[43] The E21K-mutated GM-CSF analog has been reported as harbouring a significantly decreased binding ability to high-affinity receptors but maintaining an equivalent binding ability to low-affinity ones.[30] Given that most AML cells express both high- and low-affinity receptors, the GMR^{E21K} CAR[G4S]-T cells are considered to have a lower binding ability to AML cells than do GMR^{WT} CAR-T cells. Recent studies of low-affinity CARs targeting CD19,[44] ErbB2[45] and ICAM-1[46] showed comparable or superior anti-tumor effects to high-affinity CARs in preclinical and clinical trials in spite of conflicting data in preliminary research stages.[47,48] Although the mechanism of how low-affinity CARs improve the anti-tumor activity of CAR-T cells remains undetermined, it has been postulated that a shorter duration of receptor–ligand interactions may cause repeated stimulations of CARs, thus leading to enhanced intracellular proliferative signalling.[44] A lower binding ability to leukaemia cells could therefore have contributed to the improved long-term *in vitro* anti-tumor effects of the GMR^{E21K} CAR[G4S]-T cells. Additionally, Lopez *et al*. have described that GM-CSF analogs with mutations at residue 21 (E21R and E21K) function only as antagonists, without inducing agonistic effects.[30,31] Therefore, the avoidance of unwanted signals through the GMR in leukaemia cells may also support the enhanced anti-tumor effects of GMR^{E21K} CAR[G4S]-T cells.

Safety tests revealed that the toxicity of GMR CAR-T cells was restricted to normal monocytes. Surprisingly, GMR CAR-T cells minimally affected normal neutrophils despite their CD116 expression being similar to that on monocytes. The clinical characteristics of MonoMAC syndrome patients who congenitally lack monocytes have been well described.[49,50] Based on those features, transient monocytopenia caused by GMR CAR-T cells should be manageable if the patient is bridged to HSCT. Meanwhile, GMR CAR-T cells showed significantly higher cytotoxic effects on myeloid progenitor cells than on erythroid progenitor cells. Since myelotoxicity could not completely be ruled out, bridging HSCT will be required for the clinical application of GMR CAR-T cells.

A variety of safety switch systems have been developed for eliminating CAR-T cells in the case of excessive reactivation, some of which have already been translated into clinical trial settings, such as truncated epidermal growth factor receptor + cetuximab and iCasp9 + AP1903.[51] Although no safety switch systems were applied in the present study, the incorporation of such a system would further enhance the safety of CAR-T therapy and should therefore be studied specifically in patients who are ineligible for HSCT.

Lastly, we employed a *piggyBac* system to transfect GMR CAR genes into T cells. Consistently with our previous studies,[35,36] *piggyBac*-modified GMR CAR-T cells showed a naïve/stem cell memory T cell-dominant phenotype as indicated by the $CD45RA^{+}CCR7^{+}$ population, which has been related to the increased persistency and anti-tumor effects of CAR-T cells in both preclinical[52] and clinical studies.[53] Furthermore, the present investigation revealed that the *piggyBac*-modified GMR CAR-T cells minimally expressed such exhaustion markers as PD-1 and LAG3. This favorable phenotype of *piggyBac*-modified GMR CAR-T cells may have contributed to the long-term anti-tumor effects observed in our preclinical trials.

The limitations of this study include the restricted expression of CD116 in myelomonocytic AML. Approximately 20-30% of AML cases cannot be targeted by GMR CAR-T cells and require alternative therapeutic approaches. Another limitation is the potential safety issues of GMR CAR-T cells. Although we extensively examined their toxicity on haematopoietic cells, the adverse effects on other tissues were not investigated in this study. Since the GMR has not yet been tested as a target of immunotherapy, its safety must carefully be evaluated in a phase I clinical trial.

CONCLUSIONS

Optimised GMR CAR-T cells containing an E21K GM-CSF mutant and G4S spacer exhibited potent anti-tumor effects against $CD116^{+}$ AML both *in vitro* and *in vivo*. GMR CAR-T cells minimally affected normal haematopoietic cells apart from monocytes. Further testing is warranted to determine the safety and efficacy of *piggyBac*-modified GMR CAR-T therapy on R/R AML.

METHODS

This study was conducted in accordance with the Helsinki Declaration and was approved by the institutional review board of Shinshu University School of Medicine.

Plasmids

The *PiggyBac* transposase plasmid (pCMV-*piggyBac*)[54] and original GMR CAR with a CH2CH3 hinge construct (CH2CH3) were as described previously.[22] In order to improve *in vivo* persistence, we modified the original GMR CAR vector by replacing the CH2CH3 hinge region with either an IgG1 hinge region (ΔCH2CH3) or 3 repeated sequences of GGGGS (G4S). To modulate the antigen-binding ability of GMR CAR, 7 new types were generated by replacing E with each of the following amino acids: R, K, H, D, S, F and A. The mutated GMR CAR constructs with E21R or E21K were further modified by incorporating either a ΔCH2CH3 or G4S spacer.

PiggyBac transposon-based gene transfer and culture method

GMR CAR-T cells were generated from healthy donor peripheral blood mononuclear cells (PBMCs) using *piggyBac* transposon-mediated gene transfer, as described previously.[35] Briefly, 15×10^6 PBMCs were electroporated with 5 μg of a pIRII-GMR CAR transposon plasmid and a pCMV-*piggyBac* transposase plasmid using a 4D-Nucleofector device (program FL-115) and P3 primary cell 4D-Nucleofector X kit (Lonza, Basel, Switzerland). Electroporated cells were then cultured with 2×10^6 irradiated PBMCs pulsed by viral peptide pools. Cells were maintained in serum-free TexMACS™ medium (Miltenyi Biotec, Inc., Auburn, CA) supplemented twice a week with IL-7 and IL-15. On day 7, the cells were transferred to G-REX10 culture flasks (Wilson Wolf, New Brighton, MN) and stimulated with 3.0×10^6 OKT3 blasts pulsed by viral peptide pools, as previously reported.[35] On days 14–16, the cells were harvested for further experiments.

Flow cytometry

Anti-human GM-CSF-PE, CD3-APC, CD33-PE (Miltenyi Biotec, Auburn, CA); CD45RA-Pacific blue, CCR7-APC/cy7, CD4-Pacific blue, CD8-APC/cy7, CD19-APC-Cy7, CD3-FITC, CD11b-APC, CD38-BV421, CD33-BV605, CD34-APC, CD45RA-BV786 and CD16-Pacific Blue (Biolegend, San Diego, CA); and CD116-PE (BD Biosciences, Franklin Lakes, NJ) antibodies were used for analysis. Flow cytometric data were acquired by BD FACSCanto™II, BD FACSCelesta™ or BD Accuri™ C6 Plus (BD Biosciences San Jose, CA) and analysed by FlowJo (TOMY Digital Biology, Tokyo, Japan).

Leukaemia cells

The AML cell lines (THP-1, MV4-11, Kasumi-1 and HL-60) were all purchased from ATCC (Manassas, VA). ShinAML-1 was established in our laboratory from a patient with AML FAB M4. Primary leukaemia samples were analysed from AML patients after obtaining informed written consent. The THP-1-ffLuc cell line was established in our laboratory using lentiviral transduction.

Co-culture experiments

Samples of $1.0–2.5 \times 10^5$ target cells (tumor cells or PBMCs) were co-cultured with effector cells at the indicated E:T ratios in RPMI 1640 medium supplemented with 10% foetal bovine serum. On day 1 or 5, the cells were harvested and analysed by flow cytometry to quantify the numbers of leukaemia cells and T cells using Count Bright Absolute Counting Beads® (Invitrogen, Carlsbad, CA).

For serial co-culture experiments, leukaemia cells and 1.0×10^5 T cells were co-cultured at E:T ratios of 1:1 and 1:5 in each well of 48-well plates. On day 2–4, half of the cells were analysed by flow cytometry. Aliquots of 1.0×10^5 tumor cells were then added to the remaining cells.

Cytokine production assay

Co-culture supernatants were collected 24 h after culture initiation and analysed in cytokine production assays. IFN-γ concentrations were measured using a Human IFN gamma High Sensitivity ELISA Kit (Abcam, Cambridge, UK).

To determine the multiple cytokine responses of GMR CAR-T cells to target cells, the production levels of IL-2, TNF-α and IFN-γ were measured using a Cytometric Bead Array Kit (BD Biosciences, San Jose, CA). After 24 h of co-culture, cell culture supernatants were collected, and cytokine concentrations were measured and analysed. Data were acquired on BD Accuri™ C6 Plus (BD Biosciences) and analysed by FCAP Array™ Ver.3.0 (BD Biosciences).

Animal experiments

All animal studies were approved by the Institutional Animal Care and Usage Committee of Shinshu University School of Medicine. NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice were purchased from Charles River Laboratories (Wilmington, MA).

In the THP-1-ffLuc xenograft model, 1.0×10^6 THP-1-ffLuc cells were intravenously injected into NSG mice. Three days after tumor inoculation, the mice were treated with intravenous injection of 1.2×10^6 GMR CAR-T cells or control CAR-T cells (dose was adjusted by CAR$^+$ cells). Bioluminescence imaging (BLI) was performed twice per week to track leukaemia burden after intraperitoneal injection of D-Luciferin (OZ Bioscience, San Diego, CA) using the Night OWL II LB983 system (Berthold Oak, Ridge, TN).

In the THP-1 xenograft model, 1.0×10^6 THP-1 cells were intravenously injected into NSG mice. Three days after tumor inoculation, the mice were treated with intravenous injection of 5.0×10^6 CAR-T cells (dose was adjusted by CD3$^+$ cells).

In the MV4-11 xenograft model, 1.0×10^6 MV4-11 cells were intravenously injected into NSG mice on day 0. On day

3, the mice were treated with intravenous injection of 1.2×10^6 GMR CAR-T cells (dose was adjusted by CAR^+ cells). To quantify the leukaemic cell load *in vivo*, blood samples were obtained from the mice, and *MLL-AF4* chimeric transcripts were examined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) by SRL Inc. (Tokyo, Japan) on day 52 using the primers presented in the Supplementary Data.

All mice were sacrificed according to predefined ethical criteria.

Dendritic cell generation

$CD14^+$ cells were isolated from healthy donor peripheral blood mononuclear cells using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA). Mature and immature dendritic cells (DCs) were generated as previously described [55]. Briefly, $CD14^+$ cells were supplemented with IL-4 (1000 IU mL^{-1}) and GM-CSF (800 IU mL^{-1}) on days 0, 3 and 5. For generating mature DCs, cells were further supplemented with IL-1β (10 ng mL^{-1}), TNF-α (10 ng mL^{-1}), IL-6 (10 ng mL^{-1}) and PGE2 (1 μg mL^{-1}) on day 5.

MTT assay for evaluating toxicity of GMR CAR-T cells on normal human dendritic cells

Normal human dendritic cells (NHDC) were purchased from Lonza (Basel, Switzerland). Aliquots of 2×10^6 GMR CAR-T cells were co-cultured with NHDCs at the indicated effector:target ratios. Three days after co-culture, the cells were used for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using an MTT cell quantification kit (Nacalai Tesque, Kyoto, Japan).

Colony-formation assays

Samples of 500 human cord blood $CD34^+$ cells (STEMCELL™ Technologies, Vancouver, Canada) were co-cultured with effector cells (Mock-T cells or GMR CAR-T cells) at indicated E:T ratios. Samples of 300 MV4-11 cells were also co-cultured with effector cells as a positive control. Co-cultures were maintained in 100 μL of RPMI 1640 medium supplemented with IL-3 (10 ng mL^{-1}), stem cell factor (10 ng mL^{-1}) and thrombopoietin (10 ng mL^{-1}) in each well of 96-well round-bottom plates.

On day 2 of co-culture, the cells were transferred to SmartDish 6-well plates with an additional 1 mL of MethoCult H4434 classic (STEMCELL Technologies). On day 7, the erythroid and myeloid colonies were quantified using STEMvision™ (STEMCELL Technologies).

Primer sets for detecting MLL-AF4 fusion transcripts in peripheral blood of MV4-11-bearing mice

Forward primer: GGTCCAGAGCAGAGCAAACAGAAA, reverse primer: GGGTTACAGAACTGACATGC and Probe 5′FAM: TGGCTCCCCGCCCAAGTATCCCTG TAMRA 3′.

Statistical analysis

Statistical analysis was performed using EZR Ver. 1.37 (Saitama Medical Center, Jichi Medical University, Saitama, Japan).[56] Statistical significance was determined as $P < 0.05$. Mann–Whitney *U*-tests and Kruskal–Wallis tests were used to identify significant differences between treatment groups. Student's *t*-tests were employed to identify significant differences for comparing the anti-tumor effects and proliferation ability of GMR CAR-T cells in *in vitro* experiments and for comparing the toxicity of GMR CAR-T cells on myeloid and erythroid progenitor cells. For the mouse experiments, survival rates under each condition were analysed using Kaplan–Meier curves and log-rank testing.

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CONFLICT OF INTEREST

SNar and SNak are employees of Kissei Pharmaceuticals, Co., Ltd SNar, SNak, HO and YI are members of the Department of Drug Discovery Science, a collaborative research laboratory between Shinshu University and Kissei Pharmaceuticals, Co., Ltd, Shinshu University and Kissei Pharmaceuticals, Co., Ltd have filed joint patent applications related to the GMR CAR-T for $CD116^+$ myeloid malignancies. AH, HM, KM, MT and YN are named as the inventors of the applications. The other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Aiko Hasegawa involved in data curation; formal analysis; investigation; writing—original draft; writing—review and editing. **Shoji Saito** contributed to conceptualisation; formal analysis; investigation; methodology; supervision; writing—original draft; and writing—review and editing. **Shogo Narimatsu** contributed to data curation; formal analysis; investigation; methodology; and writing—review and editing. **Shigeru Nakano** contributed to conceptualisation; data curation; formal analysis; investigation; funding acquisition; writing—review and editing. **Mika Nagai** contributed to data curation; formal analysis; investigation; methodology; and writing—review and editing. **Hideki Ohnota** contributed to conceptualisation; formal analysis; investigation; methodology; supervision; and writing—

review and editing. **Yoichi Inada** contributed to conceptualisation; formal analysis; investigation; methodology; supervision; and writing—review and editing. **Hirokazu Morokawa** contributed to data curation; formal analysis; investigation; methodology; and writing—review and editing. **Ikumi Nakashima** contributed to data curation; formal analysis; and writing—review and editing. **Daisuke Morita** contributed to investigation; formal analysis; methodology; and writing—review and editing. **Yuichiro Ide** contributed to data curation; formal analysis; and writing—review and editing. **Kazuyuki Matsuda** contributed to investigation; formal analysis; methodology; and writing—review and editing. **Haruko Tashiro** contributed to data curation; formal analysis; investigation; methodology; writing—original draft; and writing—review and editing. **Shigeki Yagyu** contributed to data curation; formal analysis; investigation; methodology; writing—original draft; and writing—review and editing. **Miyuki Tanaka** contributed to data curation; conceptualisation; formal analysis; formal analysis; investigation; methodology; and writing—review and editing. **Yozo Nakazawa** contributed to conceptualisation; formal analysis; funding acquisition; investigation; methodology; and project administration.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.