




SHORT REPORT

Full chimaeric CAR.CIK from patients engrafted after allogeneic haematopoietic cell transplant: Feasibility, anti-leukaemic potential and alloreactivity across major human leukocyte antigen barriers

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Summary

Cytokine-induced killer lymphocytes (CIK) are a promising alternative to conventional donor lymphocyte infusion (DLI), following allogeneic haematopoietic cell transplantation (HCT), due to their intrinsic anti-tumour activity and reduced risk of graft-versus-host disease (GVHD). We explored the feasibility, anti-leukaemic activity and alloreactive risk of CIK generated from full-donor chimaeric (fc) patients and genetically redirected by a chimeric antigen receptor (CAR) (fcCAR.CIK) against the leukaemic target CD44v6. fcCAR.CIK were successfully *ex-vivo* expanded from leukaemic patients in complete remission after HCT confirming their intense pre-clinical anti-leukaemic activity without enhancing the alloreactivity across human leukocyte antigen (HLA) barriers. Our study provides translational bases to support clinical studies with fcCAR.CIK, a sort of biological bridge between the autologous and allogeneic sources, as alternative DLI following HCT.

KEYWORDS

AML, CAR, cell therapy, cytokine-induced killer lymphocytes, HSC transplantation

Paola Circosta and Chiara Donini contributed equally to this work.

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INTRODUCTION

Allogeneic haematopoietic stem cell transplantation (allo-HCT) represents for many haematological malignancies the only curative treatment; most of its efficacy relies on the action of donor immune effector cells against tumour (graft-versus-tumour effect — GVT). Unfortunately, disease relapse is the major cause of transplant failure. Donor lymphocyte infusion (DLI) can be used in this setting to treat early-stage relapse, with the aim of restoring allogeneic immune responses against residual tumoural cells. Its overall success rate, however, is relatively low due to several causes; among the major are: limited GVT effect, unwanted adverse events, including severe graft-versus-host disease (GVHD), and lack of timely 'off the shelf' availability of donor lymphocytes.¹ To further increase the curative potential of allo-HCT, addressing all these issues will be fundamental.

Chimaeric antigen receptor (CAR) T-cell therapy has brought a paradigm shift in the management of haematological malignancies and in this perspective approaches combining allo-HCT and CAR-T cell therapies are an attractive area of research.² Their successful use has been already described³; however, relapses after CAR-T cell treatment have been reported.⁴ The most common and potentially severe toxic effects with CAR-T cell therapy are cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome, along with the known risk of GVHD when considering the allogeneic setting after HCT.

Effective and safe approaches, exploiting alternative immune effectors, are needed to promote the application of CAR-based strategies in HCT settings. The infusion of cytokine-induced killer lymphocytes (CIK) has been proposed as an appealing alternative to conventional DLI.⁵ CIK are mixed T-NK killer lymphocytes, endowed with intrinsic human leukocyte antigen (HLA)-independent tumour killing ability, and reduced alloreactivity across HLA barriers.⁶ CIK represent a promising platform for CAR-based strategies, alternative to conventional CAR-T, with supporting preclinical data^{7–10} and also initial clinical reports in adult and paediatric patients showing that they can offer target specificity but at the same time retain their broader cell-killing properties.¹¹ CIK are generated from donor; therefore, despite their favourable profile, they do not resolve the issue of prompt availability of donor immune effector cells.

We hypothesized that CIK, and consequentially CAR.CIK, may be successfully generated from full-donor chimaeric patients who are in complete response in the early post-HCT setting, and cryopreserved for possible future applications as clinically needed.

The underlying idea is that full chimaeric CAR.CIK (fcCAR.CIK) might represent a sort of biological bridge between the autologous and allogeneic sources. fcCAR.CIK may potentially have favourable implications in terms of availability and alloreactive safety, while conjugating their

HLA-independent intrinsic anti-tumour activity with the engineered CAR-anti-tumour specificity.

Here we explore and provide the first translational proof of concept on the generation of fcCAR.CIK, assessing their pre-clinical anti-leukaemic activity without enhancing their alloreactivity across major HLA barriers. As experimental platform we targeted the isoform variant 6 of the hyaluronic acid receptor CD44 (CD44v6), already reported as a valuable and safe CAR target in acute myeloid leukaemia (AML) and myeloma.¹²

MATERIALS AND METHODS

Research hypothesis

We aim at exploring the preclinical feasibility, anti-leukaemic activity and alloreactive risk of CIK generated from full-donor chimaeric (fc) patients and genetically redirected by a CAR (fcCAR.CIK) against the leukaemic target CD44v6. We hypothesize to conjugate the cell-intrinsic anti-tumour activity of CIK with the potent CAR specificity, enhancing the overall anti-tumour potential and minimizing the chances of tumour immune evasion by leukaemic clones not expressing the CAR target.

From the safety perspective, we explored if fcCAR.CIK do retain the favourable alloreactive profile known for CIK lymphocytes as compared to conventional T lymphocytes.

Full chimaeric CAR.CIK and target leukaemia cells

We ex-vivo generated full chimaera (fc) CIK from peripheral blood mononuclear cells (PBMC) of full-donor chimaeric patients (lymphocyte count $\geq 1000 \text{ mm}^{-3}$) who were in complete response following HCT ($n = 3$ HLA-identical, $n = 6$ HLA-haploidentical, $n = 6$ matched unrelated donor HCT). Baseline PBMC and mature CIK were phenotyped by flow cytometry and donor chimaerism on mature CIK assessed by short tandem repeat (STR)-fragment length analysis (AmpFlSTR®Identifiler®PCR Amplification Kit, Applied Biosystems), with full chimaerism defined as more than 97% donor cells.

Full chimaera (fc) CAR.CIK (CD44v6-CAR.CIK) have been generated from four patients of our cohort by engineering the baseline PBMC with a lentiviral vector encoding for the second-generation CD44v6 CAR construct with CD28 signalling domain, along with the inducible suicide gene HSV-TK, as previously described.^{7,12}

All procedures in this study were performed in accordance with the ethical standards of the institutional research committees and with the Declaration of Helsinki and its later amendments.

The leukaemia and lymphoma cell lines ($n = 11$) used are listed in the Supporting information data. Primary patient-derived leukaemic blasts were obtained from peripheral or marrow blood samples.

Leukaemic cell-killing assay

Anti-leukaemic activity in vitro of CD44v6–fcCAR.CIK and fc non-transduced (NTD) CIK was assessed at different effector:target ratios (from 5:1 to 1:4) for 48 h. Target cells were stained with vital dye PKH26 (Sigma-Aldrich), according to the manufacturer's protocols. Immune-mediated killing was analysed after 48 h by flow cytometry (Cyan ADP, Dako) and measured by the DAPI (4',6-diamidino-2-phenylindole) permeability of target cells (PKH26⁺ gate). Untreated target cells were used to evaluate spontaneous mortality. The cytotoxic activity was calculated as follows: [(experimental – spontaneous mortality)/(100 – spontaneous mortality) × 100].

CD44v6–fcCAR.CIK alloreactivity within allogeneic mixed lymphocyte reaction

CD44v6–fcCAR.CIK and unmodified fcNTD.CIK were used as effectors and labelled with vital dye PKH26 (Sigma-Aldrich).

Mixed lymphocyte reactions (MLRs) were set by seven-day cocultures with irradiated PBMCs as stimulators (30 Gy, ratio = 2:1), collected either from full HLA-mismatched unrelated donors or from autologous pre-HCT peripheral blood samples (generating HLA-haploidentical mismatch with fcCAR.CIK).

Data were acquired by flow cytometry gating on PKH-26⁺CD3⁺ viable effectors by FACS Cyan (Cyan ADP, Beckman Coulter s.r.l.) and analysed with Summit Software.

Representative MLR images were acquired by an automated live-cell imaging system (LIPSI, Nikon).

Statistical analysis

Data were analysed using GraphPad Prism 8.0 (GraphPad Software). Descriptive data are presented as mean values ± standard errors. Data were analysed by either two-tailed Student's *t*-tests or by two-way analyses of variance (ANOVA).

Additional information about materials and methods is present in the Supporting information (Data S1).

RESULTS

We first demonstrated the feasibility to successfully ex-vivo generate recipient-derived fcCIK from 15 full-donor chimeric patients (median age 49, range 21–76) in complete response following allo-HCT and confirmed with full donor chimaerism (*n* = 3 HLA-identical, *n* = 6 HLA-haploidentical, *n* = 6 matched unrelated donor HCT). At the time of PBMC collection 3/15 patients were off immunosuppression while all the others were tapering FK506 (median days off

immunosuppression +76). The main T-cell subsets within the baseline PBMC collection (median day +65, range: 51–180 days) were CD3⁺CD4⁺ lymphocyte (18.10% ± 2.34%) and CD3⁺CD8⁺ (30.78% ± 6.18%).

Phenotype analysis in mature CIK showed that the expanded CD3⁺CD56⁺ fraction was 40.29% ± 2.59% (range 22.87%–54.66%) and CD3⁺CD8⁺ cells were 82.41% ± 3.63% (range 46.05%–96.01%). We confirmed that mature CIK retained the full donor chimaerism.

From four patients we could successfully generate fcCIK expressing the CD44v6–CAR with the CD28 costimulatory endodomain.

CAR expression in fcCIK (CD44v6–fcCAR.CIK) was 35% ± 9% (mean ± SEM) (Figure S1), their ex-vivo expansion (median 21-fold, range 16–33) and phenotypic characterization were not affected by the genetic modification (Table S1), with the effector memory-like subpopulation being the main subset (Figure S1).

We evaluated the rate of CD44v6 surface expression, already validated as a relevant clinical target in AML, on a panel of eight human leukaemia and three lymphoma cell lines along with nine primary circulating patient-derived AML blasts (Table S1; Figure S2). CD44v6 was expressed at variable levels in all leukaemia cell lines (100%) (CD44v6⁺ cell range: 6%–78%), in two of the three lymphoma cell lines (67%, range 7%–70%) and six of the nine primary AML blasts (67%, range 4%–70%).

Recipient-derived CD44v6–fcCAR.CIK in vitro revealed significantly superior killing capability against myeloid (*n* = 5) and lymphoid (*n* = 2) cell lines compared with unmodified fcNTD.CIK (*n* = 14; *p* < 0.0001) (Figure 1A).

Activity and CAR antigen specificity were confirmed by cytotoxic assays with recipient-derived CD44v6–fcCAR.CIK against target with no detectable expression of CD44v6 (Daudi cell line: Burkitt's lymphoma; *n* = 2, *p* = 0.9) (Figure 1B) and with fcCIK expressing an irrelevant anti-CSPG4 CAR against target expressing CD44v6 but not CSPG4 (MV4-11 cell line: biphenotypic B myelomonocytic leukaemia; *n* = 1; *p* = 0.03). We confirmed the absence of unspecific CAR activity (Figure 1C).

In a dedicated experiment, we analysed in vitro the anti-leukaemic response of CD44v6–fcCAR.CIK and CD44v6–fcCAR-T cells, obtained from the same patient, against the THP-1 cell line (human monocytic leukaemia; *p* = 0.0001). CD44v6–fcCAR.CIK and CD44v6–fcCAR-T cells equally and successfully killed leukaemia cells even at the lowest E:T ratios (*p* = 0.8, 95% confidence interval –14,85 to 16,96). (Figure 1D). We investigated CD44v6–CAR-specific IFN γ , TNF α , IL-1 β , IL6 and IL4 production after coculture with CD44v6⁺ target cells. CD44v6-expressing leukaemia cells evoked a comparable production of IFN γ , IL-1 β and IL6 by CD44v6–fcCAR.CIK and CD44v6–fcCAR-T cells, while a trend towards reduced release of IL-4 (1.6-fold) and TNF α (1.8-fold) was evoked by CD44v6–fcCAR.CIK compared to CD44v6–fcCAR-T cells (Figure S3). No relevant killing activity and cytokine production were detected by control T cells.

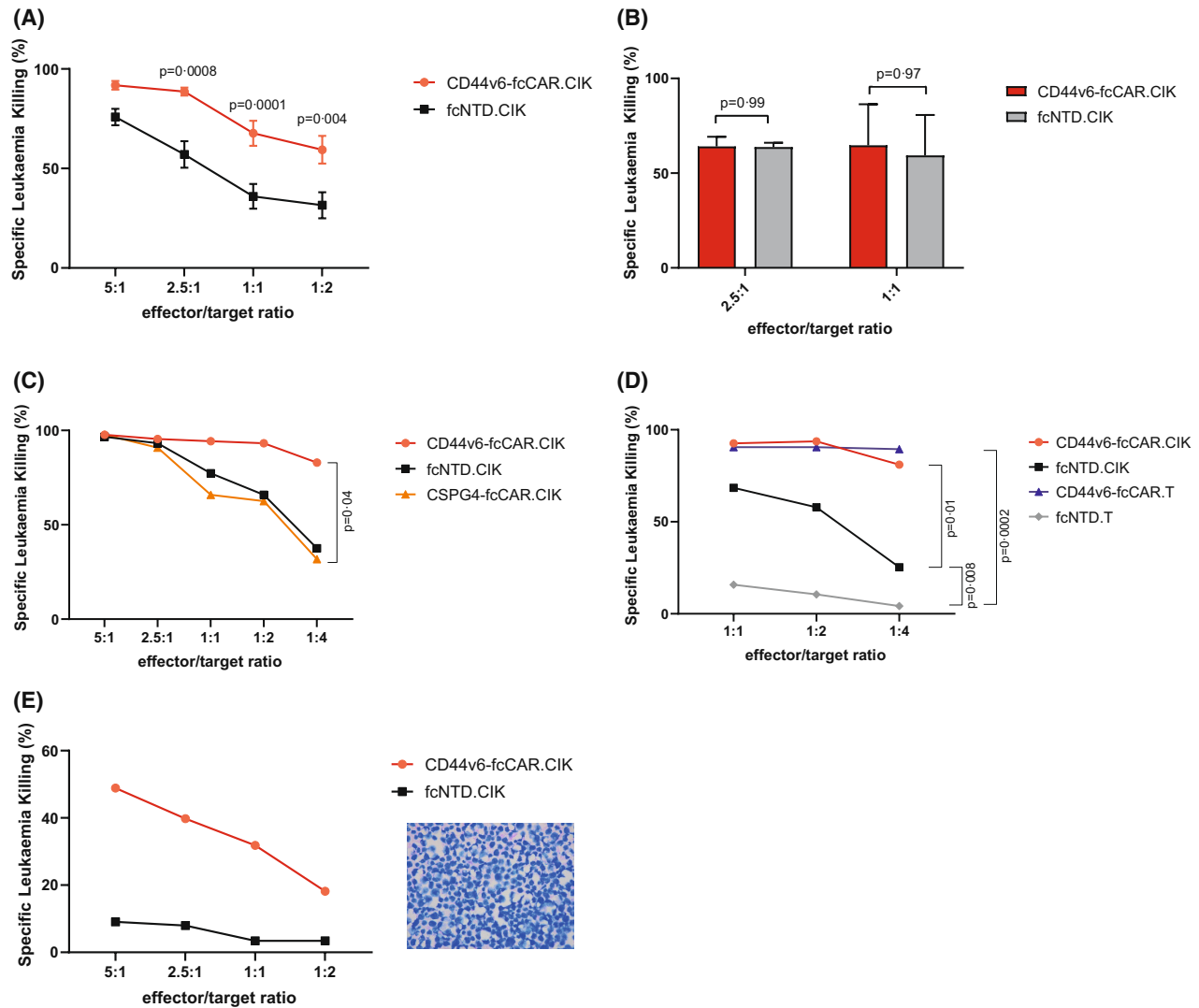


FIGURE 1 CD44v6-CAR.fcCIK effectively and specifically targets CD44v6⁺ acute myeloid leukaemia (AML) cells in vitro. CD44v6-fcCAR.CIK revealed significantly superior in vitro cytotoxicity compared with unmodified fcNTD.CIK against leukaemia cell lines at different effector to target ratios ($n = 14$; $p < 0.0001$) (A). Activity and antigen-specificity were confirmed with CD44v6-fcCAR.CIK against target with no detectable expression of CD44v6 (Daudi cell line) ($n = 2$, $p = 0.9$) (B) and with fcCIK expressing an irrelevant anti-CSPG4 CAR against target expressing CD44v6 but no CSPG4 (MV4-11 cell line) ($p = 0.03$) (C). CD44v6-fcCAR.CIK and CD44v6-fcCAR.T cells equally and successfully killed leukaemia cells ($p = 0.0001$) (D). CD44v6-fcCAR.CIK showed significantly superior killing activity compared with fcNTD.CIK against autologous AML blasts collected from peripheral blood of relapsed patient ($p = 0.01$). Haematoxylin-eosin staining of AML blasts is shown. (E) All cytotoxicity assays were analysed by two-way ANOVA and Tukey post-hoc test analysis; statistical significance is reported as $p \leq 0.05$.

Notably, in a selected experiment we confirmed the intense killing of CD44v6-fcCAR.CIK, significantly superior ($p = 0.01$) as compared with fcNTD.CIK, against autologous AML blasts (#Pt4, Table S2) collected at the time of disease relapse (Figure 1E).

Several studies have already demonstrated that CIK present a reduced alloreactivity across major HLA barriers^{13,14}; in this context we wondered whether this stands also for CD44v6-fcCAR.CIK as compared with fcNTD.CIK. Importantly, we did not detect any modification in alloreactivity between CD44v6-fcCAR.CIK and fcNTD.CIK in allogeneic mixed leukocyte reaction (alloMLR), when full HLA-mismatched unrelated donors were used as stimulators ($n = 6$, $p = 0.4$, 95% confidence interval -16,71 to 7031)

(Figure 2A). In selected experiments, CD44v6-fcCAR.CIK showed reduced alloreactivity against pre-HCT autologous stimulators as compared with full HLA-mismatched stimulators, in agreement with data already reported for allogeneic CIK¹⁵ ($n = 3$, $p = 0.02$, 95% confidence interval 3030 to 29,07) (Figure 2B; Figure S4A).

DISCUSSION

We report that fcCAR.CIK can be effectively generated ex vivo from patients engrafted after allo-HCT, and may exert an intense anti-leukaemic activity without enhancing the alloreactivity across HLA barriers. Donor CIK lymphocytes

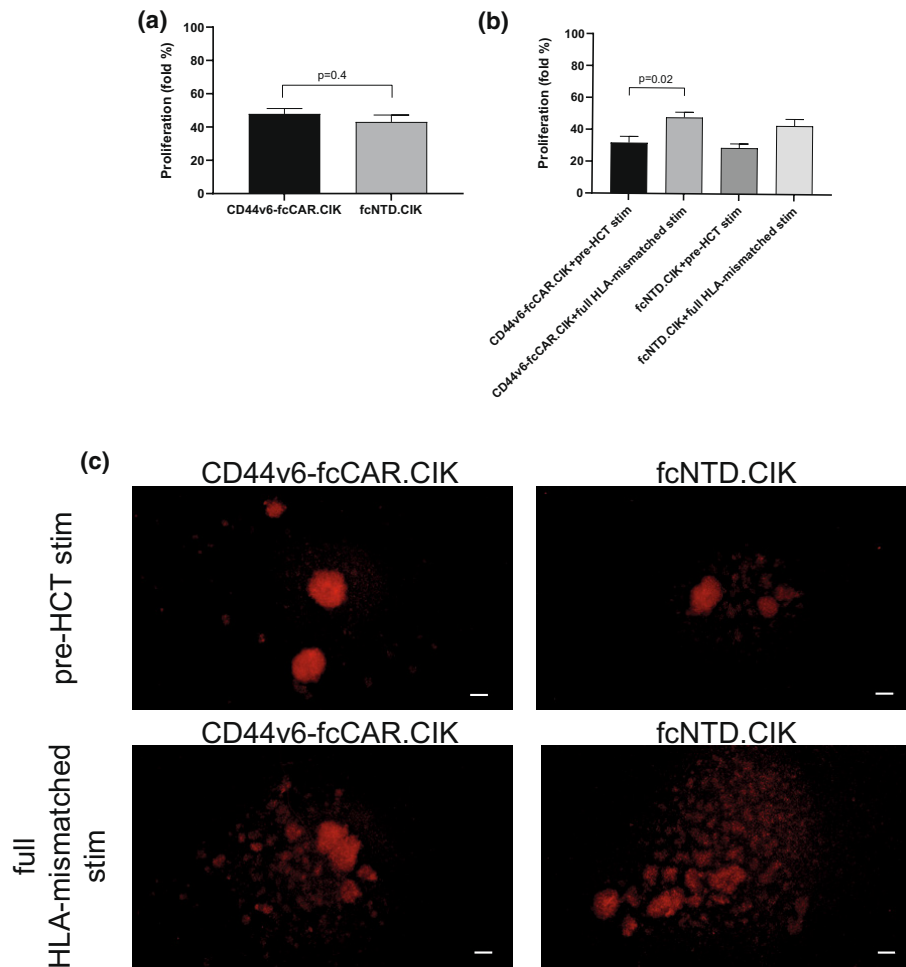


FIGURE 2 CD44v6-CAR.fcCIK showed a reduced alloreactivity across major HLA-barrier. CD44v6-fcCAR.CIK alloreactivity was comparable with fcNTD.CIK ($n = 6$, $p = 0.4$, 95% confidence interval -1671 to 7031) within an allogeneic Mixed Lymphocyte Reaction (MLR). MLR were set by seven days cocultures with irradiated full HLA mismatched stimulators (30 Gy, ratio = 2:1) (A). CD44v6-fcCAR.CIK showed reduced alloreactivity against pre-HCT stimulators compared with full HLA- mismatched stimulators ($n = 3$, $p = 0.02$, 95% confidence interval 3030 to 2907) (B). Representative images of live imaging microscopy of CD44v6-fcCAR.CIK and fcNTD.CIK in allo-MLR. CD44v6-fcCAR.CIK and fcNTD.CIK were stained with PKH26 vital dye (red). Magnification 4 \times , scale bar 500 μ m (C). Statistical significance is reported as $p \leq 0.05$.

are considered a valuable alternative to conventional DLI, with reduced risks of GVHD. We built on this concept enhancing the anti-leukaemic potential of CIK by CAR redirection and proposing fully chimaeric patients as a valuable alternative and highly accessible source for CIK and CAR. CIK in order to overcome the delay always present in the clinical scenario between the need of such a therapy and the time necessary to collect the lymphocytes from the donor. Our findings support that the ex-vivo expansion rates and phenotype of fcCAR.CIK are comparable with conventional CIK, while the anti-CD44v6 CAR confers a superior anti-leukaemic potential confirmed against autologous blasts from AML relapse as compared to unmodified standard CIK. Data from HLA-mismatched MLR support that CAR engineering does not enhance the alloreactivity of chimaeric CIK, neither across fully mismatched or haploidentical HLA barriers, with important positive implications in terms of GVHD risk. In principle, chimaeric PBMC may represent a favourable 'alloreactive compromise' between pure

autologous and allogeneic donor sources for adoptive cell therapy. With respect to CAR engineering, CIK are emerging as an intriguing platform integrative or alternative to conventional T lymphocytes, favoured by their cost-effective ex-vivo expansibility and innate (CAR-independent) tumour killing activity. CAR-engineered CIK would exploit a dual tumour cell-killing capability, namely through both the NKG2D receptor and the CD44v6-CAR, that could help counteracting the immune evasion by leukaemia clones with low or negative CD44v6 expression.

Clinically relevant rates of fcCAR.CIK might be easily generated from fully chimaeric patients in complete response after HCT, and cryopreserved for subsequent clinical infusions. As CAR target, CD44v6 is confirmed to be extremely effective against leukaemia, and the choice is also supported by its association with cancer stemness features, which could provide further relevance in perspective of clinical applications. Previous preclinical data demonstrated that CD44v6 is expressed on keratinocytes and monocytes at significantly

lower levels compared with AML cells, while it is absent from progenitor haematopoietic stem cells (HSC). CD44v6–CAR-T cells mediated potent antileukemia and antimyeloma effects sparing HSC¹² with only transitory monocytopenia.

Concerns may be raised for potential skin toxicity as previously reported in a study with bivatuzumab mertansine (monoclonal antibody against CD44v6 conjugated with a potent chemotherapy agent).¹⁶ However, preclinical data^{12,17} support that keratinocytes may be relatively resistant to CAR-T cell recognition even if the clear mechanistic explanation is still partially unclear. As additional consideration, in clinical perspective, it is important to underscore that CD44v6–fcCAR.CIK are endowed with an inducible suicide gene (HSV-TK) that represents an important safety tool in case of undesired reactions, allowing the inactivation of CAR lymphocytes by treatment with ganciclovir.^{7,12} In conclusion, our study provides translational bases to support clinical studies of CD44v6–fcCAR.CIK in patients with early AML relapse following allogeneic HCT.

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CONFLICT OF INTERESTS

The authors do not have any conflict of interest to disclose with regard to publication of this manuscript.

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SUPPORTING INFORMATION

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

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