Endowing universal CAR T-cell with immune-evasive properties using TALEN-gene editing

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Universal CAR T-cell therapies are poised to revolutionize cancer treatment and to improve patient outcomes. However, realizing these advantages in an allogeneic setting requires universal CAR T-cells that can kill target tumor cells, avoid depletion by the host immune system, and proliferate without attacking host tissues. Here, we describe the development of a novel immune-evasive universal CAR T-cells scaffold using precise TALEN-mediated gene editing and DNA matrices vectorized by recombinant adeno-associated virus 6. We simultaneously disrupt and repurpose the endogenous TRAC and B2M loci to generate TCRαβ- and HLA-ABC-deficient T-cells expressing the CAR construct and the NK-inhibitor named HLA-E. This highly efficient gene editing process enables the engineered T-cells to evade NK cell and alloresponsive T-cell attacks and extend their persistence and antitumor activity in the presence of cytotoxic levels of NK cell in vivo and in vitro, respectively. This scaffold could enable the broad use of universal CAR T-cells in allogeneic settings and holds great promise for clinical applications.
Universal chimeric antigen receptor (CAR) expressing T cells have great potential to democratize and improve the treatment of cancer patients worldwide. Reasons for such potential are multiple but all stem from the source of the biological material used to produce them. Because universal CAR T cells are engineered out of third-party healthy donor T cells, donors can be carefully chosen for potency, and cells can be manufactured, formulated, and controlled thoroughly before being adoptively transferred to multiple patients in an allogeneic setting.

To realize their full potential in an allogeneic setting, universal CAR T cells must not induce two detrimental and potentially toxic phenomena: the graft-versus-host (GvH) reaction and the Host-versus-Graft (HvG) reaction. The GvH reaction can be readily addressed by the transient or constitutive inactivation of T-cell receptor αβ (TCRαβ) expression in CAR T cells1,2. In contrast, preventing the depletion of CAR T cells due to the HvG reaction is less straightforward. The preconditioning regimen that is typically used to lymphodeplete patients prior CAR T-cell transfer (cyclophosphamide and fludarabine)3,4 can delay the HvG reaction and create a first window of opportunity for CAR T-cell engraftment. However, because this lymphodepletion is transient, it may not fully prevent the HvG reaction. In addition to human leukocyte antigen (HLA) matching between CAR T-cell donors and recipients5,6, two main engineering strategies have been thoroughly assessed for their ability to further inhibit or delay the HvG reaction. The first strategy relies on developing drug-resistant CAR T cells wherein TCRαβ and genes that modulate sensitivity to lymphodepleting drugs (CD52 and dCK which are responsible for alemtuzumab binding and fludarabine metabolism7,8, respectively) are inactivated. This strategy, which is designed to allow for CAR T-cell engraftment and proliferation under prolonged lymphodepletion of the allogenic host, showed encouraging antitumor potency in clinical trials in the presence of alemtuzumab9,10. The second strategy relies on the genetic inactivation of beta-2 microglobulin (B2M) in CAR T cells. The inactivation of B2M prevents the expression of the HLA Class-I surface marker that is responsible, in part, for the host T-cell-mediated HvG reaction11,12. TCRαβ(−) HLA-ABC(−) CAR T cells can be efficiently generated via several gene-editing methods, are hypoimmunogenic with respect to allosreactive T cells13,14, and are currently being evaluated in a phase I clinical trial.

This second approach, often presented as the next generation of universal CAR T-cell treatment, offers the potential advantage of extending the engraftment of CAR T cells without relying on prolonged lymphodepletion which heightens the risk of opportunistic infections and reduces any potential benefits provided by endogenous immune effectors. However, while highly attractive, this strategy is likely to be impaired by the presence of host NK cells, which recognize and readily deplete HLA-ABC(−) T cell through the missing self-response15. Clinical studies investigating the antitumor potential of HLA-ABC(−) CAR T cells will be informative but it is difficult to predict whether NK cells will recover in number and fitness to mediate a missing self-response following lymphodepletion. Therefore, realizing the full potential of universal HLA-ABC(−) CAR T cells will require new engineering strategies that can enable them to evade host NK-cell attacks.

Here, we report the development of an immune-evasive universal CAR T-cell scaffold named ΔTRAC-ΔB2MHLAE, which incorporates disruptive insertions of a CAR and HLA-E, a non-polymorphic NK inhibitor16 into the TRAC and B2M loci, respectively. Using a combination of multiplex TAL Effector Nucleases (TALEN) and recombinant adeno-associated virus 6 (AAV6) treatments, we show that ΔTRAC-ΔB2MHLAE can be efficiently produced, displays antitumor activity, and resists primary allosreactive T cells and primary NK cells sourced from healthy donors and from acute myeloid leukemia (AML) patients. These findings demonstrate the immune-evasive properties of ΔTRAC-ΔB2MHLAE and support its utilization as an off-the-shelf universal CAR T-cell product that is compatible with adoptive cell transfer in allogeneic settings. Further process development of ΔTRAC-ΔB2MHLAE production needs to be performed with relevant CAR constructs, GMP media, and materials before moving forward to clinical evaluation.

Results

Efficient production of ΔTRAC-ΔB2MHLAE by disruptive insertions of CAR and HLA-E at the TRAC and B2M loci, respectively. Multiple approaches could be used to inhibit the cytolytic function of NK cells toward HLA-ABC(−) T cells17,18. To develop a robust and straightforward approach that is compatible with clinical applications, we chose to use the B2M gene to re-express the engineered NK inhibitor HLA-E, via a disruptive gene insertion approach (i.e., a knockout-by-push-in). Based on the approach of ref. 16, we derived an AAV6 promoter-less DNA repair matrix that is specific for the B2M locus; we named this matrix HLA-Em. The HLA-Em matrix includes a nonameric peptide derived from HLA-G (VMAPRTLIL 16,20,21), codon-optimized B2M, and HLA-E domains covalently linked together by GS linkers (HLA-E16,22, Fig. 1a), and 300-bp left and right homology arms that are specific for exon 1 of the B2M locus. HLA-Em was designed to be used in combination with a B2M-specific TALEN that would inactivate the endogenous B2M gene and repurpose its regulatory elements and reading frame to express the engineered HLA-E. In the following, the B2M-specific TALEN and HLA-Em will be used in combination with the TRAC TALEN and the AAV6 promoter-less CAR matrix (CARm, Fig. 1a), described earlier to mediate the disruptive insertion of a CAR construct at the TRAC locus23.

To assess the efficiency of TALEN-mediated CARm and HLA-Em insertion at their respective loci, we simultaneously transfected T cells with mRNA encoding the TRAC and B2M TALEN and transduced them with CARm and HLA-Em, using the protocol described in Fig. 1b23. For this proof of concept, two different CARm-encoding CAR tool constructs specific for antigens that are expressed in hematological malignancies (tool CAR123 and tool CAR22, which are specific for CD123 and CD22, respectively) were used to assess the robustness of our engineering strategy. Control samples transduced with each single repair matrix after TALEN transfection were included to accurately identify gene-edited cell subpopulations using flow cytometry. TALEN efficiently co-inactivated the TRAC and B2M genes with up to 96% of TCRαβ(−) HLA-ABC(−) T cells obtained in the presence of both repair matrices (Fig. 1c, top panel, black box, Fig. 1d black box plots, median ~90%). CAR and HLA-E expression were observed in TCRαβ(−) HLA-ABC(−) T cells (Fig. 1c, red or green boxes, respectively, Fig. 1d, red or green box plot, respectively) indicating successful disruptive insertion of both transgenes at TRAC and B2M loci. The efficiency of double targeted insertion reached as much as 68% with the two different CAR constructs (Fig. 1c blue box and 1d, blue box plots, median ~50%). Therefore, this TALEN/AAV6-mediated editing strategy enables the efficient expression of CAR and HLA-E by TCRαβ(−) HLA-ABC(−) cells after a single-transfection/transduction step. For the sake of clarity, we refer to this engineered T-cell scaffold as ΔTRAC-ΔB2MHLAE in the following sections. Of note, similar levels of CAR expression and TRAC and B2M inactivation were reached using a CAR construct vectorized by recombinant lentivirus particle (rlv, Supplementary Fig. 1, TLA sample 8).
Specifity of TALEN- and AAV6-mediated engineering of ΔTRACCARΔB2MHLAE. To investigate the specifity of TALEN cleavage and AAV6 matrix insertion, we performed an oligo capture assay (OCA) and targeted locus amplification (TLA) analysis of engineered T cells. We also quantified translocations between B2M and TRAC loci by qPCR. OCA analysis of TRAC and B2M TALEN co-treated T cells identified candidate off-target sites that were then validated or invalidated using high-throughput DNA sequencing of amplicon-specific PCRs (Supplementary Fig. 1a and Supplementary Table 1). High-throughput DNA sequencing of the 22 top-scoring candidate off-target sites showed insertions/deletions (indels) frequencies falling under the threshold of relevant detection (threshold = 0.16, see “Methods”), indicating that the TRAC and B2M TALEN co-treatment does not promote significant off-site targeting. As expected, simultaneous transfection of T cell by both TALEN, promoted translocation between the TRAC and B2M loci in up to 4% of cells (2T-TCR centromeric, Supplementary Fig. 1b and Supplementary Tables 3 and 4). When the CARm and HLA-Em AAV6 matrices were added to the TALEN treatment, translocations were detected at a level similar to that observed in mock-treated T cells. Because our qPCR method could not amplify translocation events containing the HLA-Em or CARm matrices, we cannot draw conclusions about the presence of translocation events integrating all or part of the AAV6 payload.

TLA analysis of engineered T cells with the double insertion of CARm and HLA-Em at the TRAC and B2M loci showed that the transgenes were precisely inserted at their proper locations (CARm at chr14 and HLA-Em at chr15, Supplementary Fig. 1d, f, Sample 7, red boxes), as reported earlier with similar editing strategies.
integrations at chr15 and ch14, respectively (CARm at chr15 and HLA-Em at chr14, Supplementary Fig. 1d, f, Sample 7, blue box). This suggests that a small number of integrations arose from the homology-independent insertion of AAV6 matrices, as documented in former reports. Notably, such homology-independent insertion events were found to occur at markedly lower rates than those observed in control experiments performed with a single AAV6 matrix and an unpaired TALEN (CARm with B2M TALEN or HLA-Em and TRAC TALEN, compare the blue boxes in Sample 2 or 4 from Supplementary Fig. 1c, f to Sample 7 from Supplementary Fig. 1d). Of note, homology-independent insertion events obtained with AAV6 were found to occur at markedly lower rates than those obtained using CAR construct control vectorized by lentivirus particles along with B2M and TRAC TALEN co-transfection (Compare blue boxes in sample 7 to sample 8 Supplementary Fig. 1d, e).

\[ \Delta TRACm\Delta B2M_{HLAE} \text{ cells display antitumor activity in vivo and in vitro.} \]

To investigate the impact of B2M depletion and HLA-E expression on CAR T-cell antitumor activity, we first determined whether CAR T cells engineered with these changes showed cytotoxic activity against leukemia cell lines in vitro. Engineered CAR T cells specific for CD22 or CD123 showed similar antitumor activity toward their respective leukemia cell line targets (RAJI and MOLM13 tumor cells, respectively, Fig. 2a, b) regardless of the number of edited features (\( \Delta TRACm\Delta B2M \), and \( \Delta TRACm\Delta B2M_{HLAE} \)). This activity was found significantly higher than MOCK control cells lacking CAR expression. By design, the background antitumor activity of TRAC and TRACB2M toward RAJI cells was significantly lower than Mock and \( \Delta B2M \) control groups, in agreement with the low, nonspecific and TCR-dependent-cytolytic activity of T cells (Fig. 2a). We then evaluated the antitumor activity of \( \Delta TRACm\Delta B2M_{HLAE} \) T cells in vivo using MOLM13 tumors xenografted in immunodeficient NSG mice (Fig. 2c). A single administration of \( \Delta TRACm\Delta B2M_{HLAE} \) T cells led to significantly extended survival in leukemia-bearing mice (Fig. 2d). Consistent with our in vitro results in the absence of host T cells and NK cells, we observed no significant difference in overall survival between mice treated with \( \Delta TRACm\Delta B2M \) T cells and mice treated with \( \Delta TRACm\Delta B2M_{HLAE} \) T cells or those treated with \( \Delta TRACm\Delta B2M \) T cells and \( \Delta TRACm\Delta B2M_{HLAE} \) T cells. Altogether, these in vitro and in vivo results indicate that neither the inactivation of B2M nor the disruptive targeted insertion of HLA-E at the B2M locus affect the antitumor activity of engineered CAR T cells. This conclusion was reached with two different tool CAR constructs indicating that our engineering process could be used with other CAR constructs including those used in the clinic.

**Targeted knock-out of the B2M locus enables engineered T cells to resist allogeneic T-cell attacks.** We next verified that the depletion of HLA-ABC from the surface of engineered T cells prevented their elimination by allospecific T cells. Allospecific T cells primed for 3 weeks to recognize and attack T cells from three different donors were used to perform mixed lymphocyte reactions (MLR) with TRAC and B2M TALEN-engineered T cells (Supplementary Fig. 2a). T cells were intentionally engineered to obtain a mixture of engineered T-cell subpopulations (∼50% HLA-ABC(−) and 50% HLA-ABC(+) ) and to assess their relative sensitivity to allospecific T-cell attack within each sample. The HLA-ABC(−) subpopulation of engineered T cells (Supplementary Fig. 2b) was significantly enriched by up to 23-fold over the HLA-ABC(+) subpopulation in the presence of allospecific T cells in MLR assays (Supplementary Fig. 2c). Consistent with former reports, these results indicate that the HLA-ABC(−) subpopulation is resistant to allogeneic T-cell-mediated cytolytic attack and confirms the hypoinmunogenic properties of HLA-ABC(−) cellular scaffold.

**Targeted insertion of HLA-E at the B2M locus enables engineered \( \Delta TRAC\Delta B2M_{HLAE} \) to resist NK-cell attacks in vitro.** To assess whether HLA-E expression can prevent the depletion of HLA-ABC-deficient T cells by NK cells, we compared the sensitivity of \( \Delta TRAC\Delta B2M \) and \( \Delta TRAC\Delta B2M_{HLAE} \) cells to the cytotoxic activity of healthy donor NK cells in vitro (Fig. 3a). First, as a proof of principle, we intentionally engineered T cells in suboptimal conditions to obtain balanced engineered CAR T-cell subpopulations (HLA-ABC(−)/(+) and HLA-E(−)/(+) ). This allowed us to assess the relative sensitivity of these subpopulations to NK-cell attack. After 3 days in culture, HLA-ABC(−) HLA-E(−) T cells were selectively and markedly depleted by NK cells in both \( \Delta TRAC\Delta B2M \) T cells and \( \Delta TRAC\Delta B2M_{HLAE} \) T cells, consistent with the missing self-mediated activation of NK cells (Fig. 3b). Such depletion was correlated with significant enrichment of the HLA-ABC(−) HLA-E(+) T-cell subpopulation of \( \Delta TRAC\Delta B2M_{HLAE} \) T cells, as demonstrated by a fourfold increase in the HLA-E(+) to HLA-E(−) ratio among HLA-ABC(−) T cells compared to untreated control (Fig. 3c, left panel). This result indicates that the expression of HLA-E at the surface of T cell successfully inhibited the cytolytic activity of NK cells. HLA-E(+) T cells were also enriched among HLA-ABC(−) T cells when the CD123 tool CAR was substituted for a CD22 tool CAR (about a threefold increase, \( \Delta TRAC\Delta B2M \) and \( \Delta TRAC\Delta B2M_{HLAE} \), Fig. 3c, right panel), confirming the protective role of HLA-E against NK-cell activity and demonstrating the transposable nature of this feature. It is to be noted that the values of the HLA-E(+) /HLA-E(−) ratio is more dispersed in the dataset for CD123 CAR, possibly due to a larger dataset (8 points vs. 4 points) where the variability in the cytolytic activity of NK cells is more likely to be observed from donor to donor.

To further confirm that HLA-E expression efficiently protects HLA-ABC-deficient T cells from NK-cell attack, we next engineered T cells to obtain optimal HLA-ABC inactivation and HLA-E expression (Fig. 3d). The resulting \( \Delta TRAC\Delta B2M_{HLAE} \) harboring a majority of HLA-ABC(−)/HLA-E(+) subpopulation (∼90%) were then co-cultured with NK cells for 3 days and the remaining HLA-ABC(−) subpopulation was quantified. Consistent with the data described in Fig. 3b, c, the HLA-ABC(−) subpopulation of \( \Delta TRAC\Delta B2M_{HLAE} \) control T cells was significantly depleted (80% depletion compared to untreated control). The extent of this depletion was variable, suggesting that some NK-cell donors may be tolerant toward HLA-ABC(−) HLA-E(−) T cells. In stark contrast, the HLA-ABC(−) subpopulation of \( \Delta TRAC\Delta B2M_{HLAE} \) T cells remained resistant to NK-cell attack, confirming the protective role of HLA-E (Fig. 3e, f and supplementary Fig. 3). As expected, \( \Delta TRAC\Delta B2M_{HLAE} \) T cells were not killed by NK cells harvested from donors with more NKG2A(C) NK cells than NKG2C(C) NK cells (NKG2A(C>−) donors, see Supplementary Fig. 3, donor 1, 3, 4, and 5), but from those harboring more NKG2C(C) NK cells than NKG2A(C) NK cells (NKG2C(C>−) donors, Supplementary Fig. 3, donor 2). This trend was confirmed by investigating the level of CD107a/b degranulation of total NK cells, NKG2A(C) NK cells or NKG2C(C) NK cells subpopulations after being co-cultivated with \( \Delta TRAC\Delta B2M_{HLAE} \) (Supplementary Fig. 4). This
phenomenon is consistent with the dual specificity of HLA-E for NKG2C and NKG2A, two orthogonal surface-exposed NK receptor that are known to activate and inhibit NK cytotoxic activity, respectively, upon HLA-E engagement.31,32. Noteworthy, NKG2A>C donors (n = 15 out 17, 88%, Fig. 3g, h) were significantly more prevalent than NKG2C>A donors (n = 2 out 17, 12%, Fig. 3g, h), suggesting that HLA-E activity was correlated to a decrease CAR T-cell activity in the presence of activated NK cell in vitro. We demonstrated in two independent sets of experiments that targeted insertion of HLA-E at the B2M locus did not affect the antitumor activity of TRACCARΔB2MHLAE. T cells were widely hypoinmunogenic toward healthy donor NK cells.

Targeted insertion of HLA-E at the B2M locus prolongs the antitumor activity engineered TRACCARΔB2MHLAE in the presence of activated NK cell in vitro. We demonstrated in two independent sets of experiments that targeted insertion of HLA-E at the B2M locus did not affect the antitumor activity of TRACCARΔB2MHLAE and allowed them to resist to NK-cell attack. We then sought to test if these two properties held true when CAR T cell were repeatedly challenged by tumor and NK cells (Fig. 4). To do so, we set up a serial killing assay where the antitumor activity of the three different engineered versions of CAR T cell (TRACCARΔC22, TRACCARΔC22ΔB2M, TRACCARΔC22ΔB2MHLAE) were challenged over 4 days, by daily addition of RAJI and NK cells (NKG2A>C donor, Fig. 4a). Two scenarios of NK-cell addition were investigated (NK day 1 to mimic the different physiological conditions that may be encountered by CAR T cell following patients’ preconditioning. Our results showed that while TRACCARΔB2M and TRACCARΔB2MHLAE showed similar antitumor activity in the absence of NK cells (Fig. 4b, left panel), daily addition of NK led to complete or partial abolition of TRACCARΔB2M activity (Fig. 4b, middle and right panels, respectively). In stark contrast, TRACCARΔB2MHLAE remained highly active and behaved similarly to TRACCARΔC22. By design, the NK-dependent drop of TRACCARΔB2M activity was correlated to a marked decrease CAR T-cell counts and to an increase of RAJI cell counts (Fig. 4c, d). Further analysis of the cell populations remaining at the end of the serial killing assay showed that the B2M(−) sub-population of TRACCARΔB2M was selectively depleted by NK cells while remaining constant in the case of TRACCARΔB2MHLAE. Similar observations were made with different CAR to RAJI and CAR to NK-cell ratio (Supplementary Fig. 5). Taken together, these
results indicate that the targeted insertion of HLA-E at the B2M locus prolongs the antitumor activity of engineered ΔTRACCARΔB2MHLAE in the presence of cytotoxic levels of activated NK cell.

NK cells from AML/ALL patients and healthy donors display similar phenotypical characteristics. Cancer patient NK cells may display different fitness and cytotoxic activity compared to healthy donor NK cells. Thus, their ability to attack and deplete the ΔTRACCARΔB2MHLAE scaffold should be evaluated to assess the robustness and potential clinical translatableity of the B2M/HLA-E engineering embedded in this scaffold. To gauge the magnitude of this attack, we further investigated the phenotype, fitness, and cytotoxicity of clinically relevant primary NK cells obtained from several ALL and AML patients. Because NK-cell numbers and fitness can be affected by previous treatment and by the nature and stage of each disease, we selected patient samples obtained before and after conventional frontline therapy. We first selected a cohort 23 ALL patients and 27 AML patients treated with induction therapy (7 + 3 anthracycline, cytarabine), and thoroughly investigated the phenotype of their NK-cell subpopulations using mass cytometry (Fig. 5a).

The frequencies (Fig. 5b, left panel) and absolute counts (Supplementary Fig. 6) of NK cells were comparable between the different cohorts. One exception could be however noticed for...
AML patients in complete remission (AML CR), who displayed a significantly lower frequency of total NK cells (Fig. 5b, left panel) and median absolute counts that fell under the normal range observed in healthy donors (Supplementary Fig. 6b). Frequencies of NKG2A(+) and NKG2C(+) NK-cell subpopulations were also comparable between the different cohorts (except for newly diagnosed ALL patients, P value <0.00001) with a majority of patient being NKG2A>C (92% of patients observed across diagnostic, complete remission and relapse/refractory status were NKG2A>C, Fig. 5b, middle and right panels). Therefore, these results suggest that HLA-E expression at the surface of TRACAR22MHLAE T cells is likely to inhibit NK cells from most ALL and AML patients, regardless of the stage of their disease.

As described earlier for healthy donors (Fig. 3g and Supplementary Fig. 3b), some NKG2C>A outlier patients could be identified in the different cohorts studied. Indeed, these patients were identified in 4 out of 27 (15%) and 2 out of 12 (17%) of newly diagnosed and relapsed refractory AML patients, respectively (Fig. 5b, right panel). Because we observed that NK cells from NKG2C>A healthy donors depleted TRACAR22MHLAE T cells (Fig. 3 and Supplementary Fig. 3), we hypothesized that NKG2C>A patient NK cells would exhibit similar behavior.

To predict the cytolytic activity of NKG2C>A patient NK cells toward TRACAR22MHLAE T cells, we further dissected the phenotypic characteristics of their NKG2C(+) NK cells. To do so, we investigated the fitness of NKG2C(+) NK-cell subpopulations by quantitatively analyzing their maturation, activation/migration, and proliferation markers (Fig. 5c, d). Deep phenotyping analysis performed on several extracellular and intracellular relevant markers, indicated that the NKG2C(+) subpopulation was similar in overall fitness and maturation in AML and ALL patients compared to healthy donors. Indeed, we observed conventional expression of CD56, NKG2A, KIRs, CD57, and of the triggering receptors NKP30, NKP46, and NKG2D (Fig. 5d). These triggering receptors were markedly more prevalent in NKG2C(+) NK cells than in NKG2C(-) NK cells, used here as a control subpopulation. The NKG2C(-) control subpopulation was altered in newly diagnosed AML patients, as evidenced by the lower expression of NKP30, NKP46, and NKG2D activating receptors compared to healthy donors (Fig. 4c, left panel; compare the density plots of a healthy donor and AML patients). This trend was consistent with a former report33, validating the robustness and reproducibility of our dataset. Finally, we observed no difference in the intrinsic fitness and maturation profile of NKG2C(+) cells in both AML and ALL cohorts after chemotherapy, suggesting that NKG2C(+) NK cells lack the classical changes described in individuals undergoing chemotherapy (Fig. 5c)33. Altogether, our deep phenotyping results indicate that NKG2C>A outlier patients may be equipped to deplete TRACAR22MHLAE T cells, although they represent a minority among the studied cohorts.

To validate our phenotypical investigation, we assessed the cytolytic activity of NK cells from AML patients against TRACAR123ΔB2MHLAE. To do so, we co-cultivated TRACAR123ΔB2MHLAE or TRACAR123ΔB2M with or without human peripheral blood mononuclear cells (PBMCs) from healthy donors and AML patients for 24 h and determined their absolute number at the end of the culture (Fig. 5e). Our results showed that TRACAR123ΔB2MHLAE was significantly more enriched than TRACAR123ΔB2M at the end of the co-culture (Fig. 5f). This HLA-E-driven enrichment was correlated with a decrease of NK-cell activation probed by IFNγ release, although the number of PBMCs specimens tested was too low to reach statistical significance. These results confirmed that HLA-E expression at the surface TRACAR123ΔB2MHLAE could inhibit the missing self-mediated cytolytic activity of NK cells from AML patients. Taken together, these findings confirm that TRACAR22MHLAE T cells are widely hypoimmunogenic toward primary NK cells isolated from multiple donors, including cancer patients.

To confirm the HLA-E inhibits NK cells in a more complex model, we evaluated the persistence and enrichment of TRACAR123ΔB2MHLAE in hIL-15 NOG mouse model. To confirm the HLA-E inhibits NK cells in a more complex model, we evaluated the persistence and enrichment of TRACAR123ΔB2MHLAE in hIL-15 NOG mice engrafted with PBMCs from a healthy allogeneic donor. We first verified that this in vivo model supported efficient engraftment and expansion of human NK cells as described earlier34. To do so, we intravenously injected hIL-15 NOG mice with either PBMCs or PBMCs depleted of NK cells as negative control (Fig. 6a and Supplementary Fig. 7a). Flow cytometric analysis confirmed the successful engraftment of human CD45(+) immune cells and CD56(+) NK cells (Fig. 6b and Supplementary Fig. 7b) in agreement with data reported in ref. 34. We then confirmed that the mouse cohort injected with PBMCs partially depleted of NK cells displayed significantly lower NK-cell engraftment than did the cohort injected with PBMCs (Fig. 6b). As expected, this difference in NK levels did not impact the composition and engraftment of other immune cell compartments (Supplementary Fig. 7b).

In parallel, we engineered T cells from a different donor using suboptimal HLA-E knock-in conditions to generate TRACAR123ΔB2MHLAE cells with both HLA-ABC(−) HLA-E(−) and HLA-ABC(−) HLA-E(−) subpopulations (in an approximate ratio 1:1; Fig. 6c, i). This allowed us to assess the
relative enrichment of HLA-E(+) over HLA-E(−) subpopulations in mice engrafted with allogeneic PBMCs. Four days after CAR T-cell engraftment, we observed an enrichment of HLA-ABC(−) HLA-E(+) ΔTRACCARΔB2MHLAE cells in the spleen relative to their HLA-ABC(−) HLA-E(−) counterparts (Fig. 6c and Supplementary Fig. 7d). Quantification of this phenomenon translated into a fourfold increase of HLA(+)HLA(−) CAR T-cell frequency ratio compared to the one observed pre-injection (Fig. 6d, compare black and blue circles). This enrichment was significantly lower in the mice engrafted with NK cells-depleted
PBMCs (1.5-fold, Fig. 6d, compare black and red circles), indicating (i) that the depletion of HLA-ABC(−)-HLA-E(−) subpopulation of CAR T cells and (ii) that HLA-E expression protects HLA-ABC(−) CAR T cells from NK cells attack, in agreement with the earlier results obtained in vitro.

Together, our results show the ΔTRACCARΔB2MHLAE can be generated by precise TALEN-mediated gene insertion of a CAR and a HLA-E construct at the TRAC and B2M loci in primary T cells. This process generates TCRαβ- and HLA-ABC-deficient engineered T cells expressing the CAR construct and the NK inhibitor HLA-E. Engineered CAR T cell displays immune-evasive properties toward alloreactive T cells and NK cells and shows efficient antitumor activity even in the presence of cytolytic levels of NK cells.

**Discussion**

The goal of this work was to develop an immune-evasive universal CAR T-cell scaffold that is able to resist both NK-cell and alloreactive T-cell attacks that would be compatible with adoptive cell transfer in an allogenic setting. Using a combination of TALEN-mediated gene editing and AAV6-dependent gene insertion, we developed a hypoimmunogenic universal CAR T-cell scaffold that we mediated gene editing and AAV6-dependent gene insertion, we transferred in an allogeneic setting. Using a combination of TALEN-active T-cell attacks and that would be compatible with adoptive cell CAR T-cell scaffold that is able to resist to both NK-cell and alloreactive T cells toward RAJI-Luc cells in the presence or absence of activated NK cells. The three different serial killing assay scenarios investigated are illustrated (no NK, NK day 0, and NK day 1). Black, blue, and red arrows indicate addition of RAJI cells, CAR T cells (ΔTRACCAR22, ΔTRACCARΔB2M and ΔTRACCARΔB2MHLAE, n = 1 donor) and NK cells (n = 1 donor), respectively. Luminescence and flow-cytometry analysis of cell populations are indicated at the different measurement time points. b Frequency of RAJI-Luc cells killing observed by luminescence with the three different serial killing scenarios performed with a CAR T cell to RAJI ratio of 2.5:1 and NK to CAR T-cell ratio of 1:1. Each point represents one experiment performed with a given T-cell donor and a given donor NK. c Representative flow-cytometry plots showing the different cell populations remaining at the end of the serial killing assay in absence of NK cell (left panel) and in the presence of NK cells added at day 0 and at day 1 (middle and right panel, respectively). The gating strategy is indicated by blue boxes. d Absolute CAR T cells and RAJI cells counts obtained by flow-cytometry analysis and cell counts in the same condition as in (b). Source data are provided as a Source Data file.
initial level, 1 to 2 years after treatment onset. Thus, host CD8(+) T cells and NK cells are expected to play a key role in controlling the length of the allogeneic CAR T-cell therapeutic window by being the first and primary contributors to rejection.

Although CD8(+) T cells need to go through a clonal expansion to mount an efficient alloresponse, they may be the first subset to reject allogeneic CAR T cells. In this way, the inactivation of HLA class I at the surface of CAR T cells could efficiently blunt such rejection and offer an initial therapeutic window to eradicate cancer cells. The subsequent reconstitution of host NK cells may complicate this scenario by recognizing and depleting HLA class-I-deficient CAR T cells without the need for clonal expansion. Embedding an NK inhibitor within CAR T cells could further extend their persistence. We therefore focused on engineering a CAR T cell that could...
Fig. 5 Deep phenotyping and functional characterization of NK cells from healthy donors and AML or ALL patients undergoing standard induction chemotherapy. **a** Total lymphocytes obtained from healthy donors (HD, n = 8), newly diagnosed ALL patients (ALL D, n = 23) and AML patients selected at the time of diagnosis (AML D, n = 27), complete remission (AML CR, n = 8), and in relapse/refractory status (AML R/R, n = 11) were characterized using mass cytometry. **b** Frequencies of NK-cell subsets among total lymphocytes (left panel), frequencies of NKG2A(+) and NKG2C(+) subpopulations among total NK-cell subset (middle panel), and ratio of NKG2A(+) over NKG2C(+) subpopulations (right panel) characterized by mass cytometry. For quantitative comparisons, data were analyzed using an ordinary one-way ANOVA test, a tukey’s multiple comparison test with single pooled variance and a confidence interval of 95%. **c, d** For each group described in **a**, NKG2C(−) and NKG2C(+) NK cells from each individual were exported and concatenated in order to generate consensus files. **c** The optimized parameters for T-distributed stochastic neighbor embedding (opt-SNE) algorithm were used to cluster NK-cell populations based on the expression of markers of interest in healthy donors and in AML patients at the time of diagnosis (left panel). Expression of markers of interest defining the different clusters are projected on opt-SNE maps (right panel). **d** The heatmap displays the mean frequencies of NK-cell markers in NKG2C(−) and NKG2C(+) NK cells, relative to pooled NKG2C(−) and NKG2C(+) NK cells. **e** Schematic showing the experimental design to investigate the susceptibility of ΔTRACCARΔB2M and ΔTRACCARΔB2MHLAE cells (n = 1 T-cell donor), engineered using optimal conditions, to NK-cell-dependent depletion in vitro using PBMC from healthy donors (n = 7) AML patient (n = 7) at diagnostic. **f** Box plots represent the ratio of ΔTRACCARΔB2M and ΔTRACCARΔB2MHLAE counts obtained in the presence of PBMCs over the counts obtained in the absence of PBMCs. Dotted lines indicate data obtained from the same PBMC donor. In each box plot, the central mark indicates the median, the bottom and top edges of the box indicate the interquartile range (IQR), and the whiskers represent the maximum and minimum data point. For quantitative comparisons, data were analyzed using a two-sided non-parametric Wilcoxon matched-pairs signed-rank test with a confidence interval of 95%. **g** Values are indicated as “ns”. Source data are provided as a Source Data file.

![Image](https://example.com/image.png)

**Fig. 6** The targeted insertion of HLA-E at the B2M locus allows for efficient engraftment of ΔTRACCARΔB2MHLAE in hIL-15 NOG mice adoptively transferred with human NK cells. **a** Strategy for assessing the resistance of ΔTRACCARΔB2MHLAE T cells to NK cells in a xenotransplantation model using hIL-15 NOG mice intravenously injected with human PBMCs. **b** Quantitation of human CD45(+) immune cell and CD56(+) NK-cell engraftment in the spleen of hIL-15 NOG mice injected with human PBMCs (n = 1 PBMC donor, n = 5 mice) or NK-depleted human PBMCs (n = 1 PBMC donor, n = 5 mice). **c** Representative flow-cytometry plots showing the expression of HLA-E by ΔTRACCARΔB2MHLAE T cells at the time of injection and 4 days post intravenous injection in PBMC-engrafted hIL-15 NOG mice. **d** Box plots representing the ratio of HLA-E(+/−)/HLA-E(−) computed from the CD3(−)/HLA-ABC(−)/HLA-E(+) subpopulations observed in **c** (n = 5). For quantitative comparisons, data from **b, d**, were analyzed using a two-sided parametric unpaired t test with a confidence interval of 95%. On each box plot, the central mark indicates the median, the bottom and top edges of the box indicate the interquartile range (IQR), and the whiskers represent the maximum and minimum data point. Each point represents the HLA-E(+/−)/HLA-E(−) among CD3(−)/HLA-ABC(−) subpopulations obtained in one mouse. P values are indicated on the figures. Nonsignificant P values are indicated as “ns”. Source data are provided as a Source Data file.

evade the cytolytic activities of NK cells and alloresponsive CD8(+) T cells. To achieve this goal, we aimed to prevent HLA Class-I expression in CAR T cells by inactivating B2M and to endow these cells with a surface-exposed NK inhibitor (HLA-E). We also considered the inactivation of TCRαβ to prevent GvHD, a major risk of allograft transplants. We reasoned that TRAC and B2M genes could be concomitantly inactivated and used as landing pads for transgene insertion (CAR and HLA-E). We forecasted that this strategy would simplify and speed up the CAR T-cell production process and mitigate potential genetic adverse events. The resulting cellular scaffold, ΔTRACCARΔB2MHLAE, can be efficiently generated via a simultaneous double knockout by knock-in strategy. The combination of TRAC and B2M TALEN treatment along with an optimized design of AAV6 repair matrices enabled us to obtain a high...
frequency of TCRαβ(−) HLA-ABC(−) T cells expressing CAR and HLA-E. This engineering strategy is compatible with standard universal CAR T-cell production processes that already includes TCRαβ(+) T-cell depletion, does not require any HLA-E (+) CAR T-cell enrichment or antibiotic-dependent selection step and thus, appears suitable for clinical product manufacturing. Co-treatment of T cells with TRAC and B2M TALEN did not result in significant off-site cleavage activity. This was demonstrated by an unbiased OCA assay and high-throughput DNA sequencing. As expected, this co-treatment induced translocations between Chr14 and Chr15, to an extent similar to the one reported previously with different gene-editing tools.24,35,51,52 A search of gene fusion databases, including the Atlas of Genetics and Cyto genetics in Oncology and Hematology, did not show any record of pathogenic translocation between the TRAC and B2M loci (14q11 and 15q21.1, respectively), suggesting that serious adverse events associated with such gene fusion are unlikely. Although this limitation could be mitigated by further process development, consisting in uncoupling TRAC and B2M editing by two to three days, further preclinical studies should be carried out to assess the potential toxicity of such translocations before moving to clinical studies. In addition, the homology-dependent insertion of AAV6 repair matrices was specific to the TRAC and B2M loci and correlated with robust transgene expressions. Nevertheless, we found rare homology-independent insertions of HLA-Em and CARm at the TRAC and B2M loci, respectively. Because both repair matrices are devoid of promoter, these improper insertions would not be expected to lead to transgene expression unless they inserted in frame with the edited gene. We did not observe CAR expression at the B2M locus (Supplementary Fig. 1c, TLA sample 4), but we did find low but detectable expression of HLA-E at the TRAC locus (Supplementary Fig. 1c, TLA sample 2). Although we do not envision critical adverse events related to HLA-E expression by the TRAC locus, this dataset points out one of the limitations of this multiplex gene-editing strategy that must be considered in the development of other cell therapy products. It is noteworthy that this AAV6-mediated insertion approach was markedly more specific than random transgene insertion mediated by lentivirus particles (Supplementary Fig. 1c, TLA sample 8).

We further report that efficiently targeted insertion of HLA-Em at the B2M locus leads to a robust HLA-E surface expression that inhibits the cytolytic activity of NK cells. Consistent with former reports,16,22, this outcome results from the structure and identity of the different domains of the engineered HLA-E. The inhibitory potency of HLA-E toward NK cells is intimately linked to the nature of the nonamer peptide it exposes. Its sequence was shown to significantly influence the binding affinity of HLA-E complex to the inhibitory receptor NKG2A and the activating receptor NKG2C, and thus to strongly alter the fine balance controlling NK activation and inhibition.30,21 We chose to expose at the surface of the HLA-E engineered construct, the nonamer sequence VMAPRTLFL, because it promotes stronger engagement of NKG2A than NKG2C, as opposed to other conventional peptides including VMAPRFLFL (HLA-G-peptide20,22. By design, our in vitro results showed that our construct efficiently inhibits NKG2A(+) NK cells from multiple healthy donors and from AML patients. However, despite our choice of peptide, HLA-E can still activate NKG2C(+) NK cells, leading to the swift depletion of TRACCARΔB2MHLAE T cells in NKG2C(−) donors. For the reasons described earlier, we believe that one way to mitigate this activation would be to identify a noncanonical nonamer peptide displaying orthogonal specificity for NKG2A and substitute it for VMAPRTLFL within the engineered HLA-E construct.

Nevertheless, in a clinical perspective, our results suggest that the HLA-E-mediated protection of ΔTRACCARΔB2MHLAE is likely to occur in the vast majority of AML and ALL patients. Indeed, most of these patients are NKG2A(+)/(+)- (like healthy donors) and thus harbor a greater frequency of NKG2A (+) NK cells than NKG2C(+) NK cells. This imbalance, observed at different stages of disease (92% of NKG2A(+)/NK cells observed across diagnostic, complete remission and relapse refractory status, Fig. 5b), is expected to promote evasion of ΔTRACCARΔB2MHLAE from NK cells and thus, potentially extend their overall persistence, although this needs to be demonstrated in clinical settings. Determining the NKG2A(+)NKG2C(+) ratio prior to patient injections would be beneficial for fully exploiting the therapeutic potential of ΔTRACCARΔB2MHLAE.

Expressing HLA-E on the surface of HLA-ABC(−) CAR T cells is not the only strategy to evade the cytolytic activity of NK cells. Indeed, one alternate approach would be to prevent the expression of key T-cell surface receptors involved in NK-cell activation. These receptors include MIC-A/B, SLAM family receptors CD48 and CD229, NKP46 ligand, B7H3 and CD155, different receptors known to activate NK cells through the engagement of their cognate receptors CD244, CD229, NKP46, IL20ra and KIR2DL5A, respectively.36,53–56 Genetic abrogation or downregulation of these receptors may dampen the activation of NK cells triggered by the absence of MHC Class I. Although further work is needed to evaluate the robustness and clinical translatableity of this approach, a recent report demonstrated that overexpression of the Human Herpes Virus-8 ubiquitin ligase E5 in K562 cell lines or T cells, could mitigate their depletion by NK cell through an hypothetical downregulation of MIC-A/B.57 Another alternate strategy would be to endow engineered T cells with cytolic activity toward NK cells. This strategy was elegantly explored by Mo et al.58 who recently showed that engineering CAR T cells with an alloseimmune defense receptor (ADR) specific for 4-1BB, enabled them to efficiently blunt the HvG reaction by actively targeting activated NK cells and alloresponsive T cells.

The ultimate goal of a universal CAR T-cell product is to allow for efficient and specific depletion of cancer cells in allogeneic settings with minimal biological and toxicological footprints. Our strategy was designed to mitigate such footprints by allowing CAR T cells to evade the host immune system passively and locally without relying on active, systemic, and prolonged lymphodepletion. One potential advantage over the previously described strategies2,9,58 is to spare endogenous immune effectors and allow them to work in concert with CAR T cells in the fight against cancer cells. Such collaboration could be especially useful in the context of solid tumor treatments, where endogenous immune effectors, including tissue-resident memory cells,59,60 tumor-infiltrating lymphocytes,61 and other cellular subsets are already equipped and poised to deplete tumor antigen- and neoantigen-expressing cells. The maintenance of functional endogenous immune effectors could be also a key advantage to improve the potency of CAR T-cell therapies by allowing combination therapies with oncolytic viruses,62, vaccine boosting agents,63, bispecific effectors,64 or other antibody-based immunotherapeutics.65 We believe ΔTRACCARΔB2MHLAE could allow for multiple relevant combination therapies that will leverage the full potential of the human immune system and improve the therapeutic outcome of adoptive cell therapies in allogeneic settings.

In summary, we report here the development of an immune-evasive universal CAR T-cell scaffold that is deficient for TCRαβ and HLA Class I and endowed with a surface-exposed HLA-E NK inhibitor. These features render it compatible with adoptive cell transfer in allogeneic settings by preventing GvHD and allowing it to evade the cytolytic activities of NK cells and alloresponsive
CD8(+) T cells, the two major actors of HVG rejection. These hypoinnmunogenic properties could potentially extend the persistence of universal CAR T cells and therefore, increase their antitumor potency in an immune-competent host, although it must be demonstrated in clinical settings. Our engineering strategy is efficient and specific, transportable to different CAR constructs and adaptable to conventional CAR T-cell manufacturing processes. We believe this next generation of universal CAR T cells has the potential to improve the therapeutic outcome of off-the-shelf therapeutic T-cell products and to allow their large-scale utilization against multiple malignancies for the benefit of a broader range of patients.

Methods

Materials. Cryopreserved human PBMCs were acquired from ALLCELLS (cat# PB006F). PBMCs were cultured in CTS OpTmizer media (obtained from Gibco, cat# 15440-039) or CTS OpTmizer IL-2 (obtained from Miltenyi Biotec, cat# 130-748) or IL-7 and IL-15 (obtained from Miltenyi Biotec, cat#130-995-361 and -130-995-764), human serum AB (obtained from Seralab, cat# GEM-100-318), and CTS Immune Cell SR (obtained from Gibco, cat# A2596101). Human T Cell TransAct from Miltenyi Biotec (cat# 130-111-160) was used to activate T cells. Antibody screening was carried out with antibodies summarized in Supplemen- tary Table 7. Luminescence of tumor cell lines was assessed in vitro using NANO-Glo and oneGlo reagents (Promega, cat# N1110 and cat# E6110, respectively) and in vivo using Xenolight D-luciferin (obtained from Perki- nellier, cat#770504). PBMCs from ALL and AML patients were obtained from the HEMATOMBIO cohort (NCT02320656). PBMCs were cryopreserved in 90% albumin/10% DMSO. Samples of human origin and associated data were obtained from the IPC/CRCM/UMR 1068 Tumour Bank, that operates under the authorization # AC-2007-33 granted by the French Ministry of Research (Ministère de la Recherche et de l’Enseignement Supérieur). Prior to the scientific exploration of this data, patients were adequately informed and asked to consent in writing, in compliance with French and European regulations. The project was approved by the IPC Institutional Review Board (Comité d’Ori- entation Stratégique, COS) as well as the Committee for the Protection of Persons South Mediterranean I (#2013-A14137-38).

Cell lines. MOLM13-nanoLuc-GFP and RAJI-Luc-GFP were engineered out of MOLM13 and RAJI cells (DSMZ, cat# ACC 554 and ATCC, cat# CCL-86, respectively) using an in house rLV encoding NanoLuc_T2A_EGFP construct and AMSho cat# LV19253-PSB, respectively, using the manufacturer’s protocols.

Targeted integration of CAR and HLA-E constructs. Targeted insertion of CAR and HLA-E at the TRAC and B2M loci were performed as described previously23 with minor variations. Briefly, PBMCs were thawed, washed, resuspended, and cultured in CTS OpTmizer complete media (reconstituted CTS OpTmizer media, 5% human AB serum, 20 ng/mL IL-2). One day later, the cells were activated with Human T Cell TransAct (25 μL of beads) and IL-2 (1000 U/mL) and resuspended at a final concentration of 28 × 106/mL (1 × 106 cells/mL) in complete media at 37 °C in the presence of 5% CO2. The cells were then split into fresh complete media and transduced/transfected the next day according to the following procedure. On the day of transduction-transfection, the cells were washed twice in Cytoporation buffer T (BTX Harvard Apparatus, Holliston, Massachusetts) and resuspended in final Cytoporation solution according to the manufacturer’s guidelines.

Identification and detection of candidate off-site targeting by oligo capture assay and high-throughput DNA sequencing. Oligo capture assays (OCA)24 were performed to assess the specificity of the CARm and/or HLA-Em matrices and seeded in 48-well plates. After 2 h of culture, primary T cells were co-transfected with mRNA encoding TRAC and B2M TALEN and transduced by AAV6 encoding CARm and/or HLA-Em matrices. Targeted integration events of TRAC and B2M TALEN treatment in the presence or absence of CARm and HLA-Eα AAV6 matrices. Translocation events between the TRAC and B2M loci were detected via targeted integration-specific primers described in Supplementary Table 4. Reactions were performed using PowerUp SYBR Green Master Mix (ThermoScienCe Cat # A25742) and the Bio-rad CFX qPCR instrument (Bio-Rad) on genomic DNA extracted from each experimental group. Amplification efficiencies and copy numbers were determined using reference control matrices designed to min the expected translocation events (Supplementary Table 3). Genomic DNA and control matrices were quantified using PicoGreen dsDNA quantitation assay (Thermo Fisher). Within these matrices, an XhoI restriction site was introduced at breakpoints between TRAC and B2M TALEN target sites to control for potential contaminations of experimental samples from control matrices. The four potential translocation quadruplets (T-TA) were engineered into technical quadruplets, using sets of genomic DNA obtained from two independent T-cell donors. The two donors were either mock-treated (negative control), co-treated with mRNA encoding the TRAC and B2M TALEN (positive control), or co-treated with mRNA encoding the TRAC and B2M TALEN and transduced by AAV6 encoding CARm and HLA-Eα matrices.

Targeted locus amplification assay. TLA analysis of engineered T cell was performed by Cergentis (Utrecht, Netherlands) as described23,24 using the engineered T-cell groups described in Supplementary Table 5 and Supplementary Fig. 1f and a primers described in Supplementary Table 6. Two or three specific primer sets were used for each engineered T-cell groups. PCR products were sequenced and analyzed by Illumina Nوج-Seq and library preparation. The Illumina-sequenced reads were mapped on an Illumina sequencer. Reads were mapped using BWA-SW, version 0.7.15-r1140, settings bowtie -b 7. The NGS reads were aligned to the matrix sequences and host genome. Human genome build hg19 was used as a host reference genome sequence. Integration sites were detected based on a coverage peaks in the genome and on the identification of fusion-reads between the matrices sequence and the host genome as described in ref. 23.

In vitro T-cell antitumor activity assay. The antitumor activity of the engineered T cells was assessed by cytotoxicity assays. Engineered T cells were mixed with a suspension of 5 × 105 MOLM13-Nano-Luc or RAJi-Nano-Luc tumor cells at an effector to target ratio (E:T) ranging from 10:1 to 1:0.1 in a final volume of 0.1 mL of CTS OpTmizer media supplemented with 5% human AB serum. The mixture was incubated for 24 h, and cells were lysed using Zwittergent® solution according to the manufacturer protocol. The luminescence of the remaining viable RAJi- Nano-Luc or MOLM13-Nano-Luc cells was determined after incubating the cell lysate with NANO-Glo reagent at a 1:1 volume ratio for 3 min. The nano-lucerase signal obtained after co-culture was normalized to the one obtained for tumor cells fully depleted by Zwittergent® to determine the frequency of tumor cells lysed reported in “Results”.

In vitro NK-cell cytotoxicity assay with NK cells purified from healthy donors. After thawing, cryopreserved purified human CD56+ NK cells (ALLCells) (1 × 106 cells/mL) in a complete medium (NK MACS medium supplemented with 1% NK MACS supplement (Miltenyi Biotec) and 5% human AB serum) were cultured in a 24-well plate (100 μL/well) at 37 °C, 5% CO2, and incubated overnight. On day 1, IL-2 (60 ng/mL) was added to NK cells. After an additional 5 h of incubation at 37 °C, 5% CO2, the NK cells were washed and placed in NK expansion medium.
In vitro expansion and activation of NK cells used in the serial killing assay.

To perform an in vitro serial killing assay in the presence of NK cells (see below), a large-scale activation and expansion of NK cells were set up according to the following method. After thawing, cryopreserved purified human CD56+ NK cells, negatively selected (AlloCells) (5 × 10⁶ cells) were resuspended at 1 × 10⁶ cells/mL in expansion NK MACS medium (NK MACS medium supplemented with 1% NK MACS supplement (Miltenyi Biotec), 5% human AB serum, IL-2 (500 IU/mL) and IL-15 (14-15 IU/mL)). Cells were then cultured in a 24-well plate (700 µL/well) and incubated at 37 °C, 5% CO₂ undisturbed for the first 5–6 days. At day 5 or 6, 300 µL of fresh complete Xvivo-15 media containing 40 ng/mL IL-2 was added without disturbing the cells. On day 7, a fresh expansion NK MACS medium was added to cells to dilute to a final concentration of 5 × 10⁵ cells/mL and cells were cultured in a six-well plate (2.5 mL/well). Starting on day 10, a fresh expansion NK MACS medium was added every 2 days to dilute cells to a final concentration of 5 × 10⁵ cells/mL and cells were cultured in T75 flasks (75 mL/flask) or T175 flasks (200 mL/flask). Expanded cells were utilized in serial killing assays starting at day 13 or 14.

In vitro serial killing assay in the presence of activated NK cells.

To assess the persistence of ΔTRACRABΔHLAE T cells in vitro, a serial killing assay was performed. ΔTRACRARB T cells, ΔTRACRABΔRBM T cells, and ΔTRACRABΔHLAE T cells were co-cultured with RAJI-luc tumor cells (1 × 10⁵) at CAR T to RAJI ratio = 5:1 and 2.5:1 in the presence or absence of in vitro-expanded and activated NK cells at a CAR T cell to NK-cell ratio of 1:1 and 0.5:1 in a total volume of 500 µL of Xvivo-15 media supplemented with 5% AB serum in a 48-well plate. The cell mixture was incubated for 24 h before determining the luminescence of 25 µL of cell suspension using 25 µL of one-Go reagent (Promega). The cell mixture was then spun down, the supernatant was discarded and replaced by 250 µL of fresh complete Xvivo-15 media containing 1 × 10⁵ RAJI-luc cells, additional 250 µL of media was added to each well with or without NK cells depending on the experimental group. The resulting cell mixture was incubated for 24 h. This protocol was repeated for 4 days. An additional 20 µL from the cell culture was used for flow-cytometry analysis at days 2, 3, and 4.

Flow-cytometry analysis. Cells in U-bottom 96-well plate were spun down and washed with PBS (150 µL/well) at 300 × g for 2 min. Prior to surface staining, cells were stained with Fixable Viability Dye eFluor 450 or eFluor 780 (ebiosciences) according to the manufacturer’s instructions. The cells were then stained with antibodies diluted in FACS buffer (4% FBS + 5 mM EDTA + 0.05% azide in PBS, 20 mL/well) for at least 15 min in the dark at 4 °C. Cells were washed with PBS (150 µL/well), spun at 300 × g for 2 min, and resuspended in fix buffer (4% paraformaldehyde in PBS, 100 µL/well). Sample collection was performed on a MacsQuant (Miltenyi), FACScanto II cytometer (BD) or NovoCyte Penteon flow cytometer (Agilent), which enables direct volumetric absolute count without the need for reference counting beads.

Mouse models. All procedures involving animals were approved by The Mispro Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of the PHS (Public Health Service) Policy on Humane Care and Use of Laboratory Animals, OLAB (Office of Laboratory Animal Welfare), and the USDA (United States Department of Agriculture) AWIA (Animal Welfare Act). Experimental/control animals were co-housed. The method for euthanasia was CO₂ asphyxiation followed by cervical dislocation to assure death. Humane end-point criteria for tumor models were (i), weight loss greater than or equal to 20% from baseline, (ii), abnormal gait, paralysis, or inability to ambulate properly, (iii), respiratory distress/laboring breathing, (iv), lethargy or persistent recumbency, (v), loss of righting reflex or other abnormal neurological behaviors.

Mass cytometry analysis of NK cells from AML, ALL, and healthy donors.

PBMCs were processed as previously described with slight modifications29. PBMCs were isolated and washed with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and incubated in RPMI 1640 with 2% FCS and 1/100000 Pierce Universal Nuclease 5kU (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO₂ for 30 min. Cells were incubated with cisplatin 0.1 M to stain dead cells. Non-specific epitopes were blocked using 0.5 mg/mL Human Fc Block (BD Biosciences, San Jose, CA, USA). Cells were stained for 20 min at 4 °C with a mix of extracellular antibodies (see Supplemental Table 7) and barcoded with the Cell-ID™ 20-Plex Paired Protocol (see Supplemental Table 6). The mix was collected into 15 mL tubes labeled with 125 nM iodosid (Fluidigm, Cambridge, MA, USA) according to the manufacturer’s recommendations. Cells were washed with Maxpa® cell staining buffer (CSM) (Fluidigm) and samples were combined and stained with metal-based flow-cytometry conditions used for direct volumetric absolute count without the need for reference counting beads.
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Author contributions

A.W., L.F., P.D., and J.V. conceived the study. A.W., S.D., S.J., B.J., J.V., J.P.F., D.L., I.S.C., A.L., T.P., S.R., E.D., and A.C. designed and performed experiments. A.G., M.D., and R.G. supervised D.L. and I.C.S. A.C. designed and performed the mass cytometry analysis of Healthy donor and AML/ALL patient samples. A.L. and T.P. perform functional assays with healthy donors and AML/ALL patient PBMCs. S.J., J.P.F., S.R., and E.D. performed cytotoxicity and serial killing assays. E.M. provided critical biological reagents. D.O. and R.D. coordinated selection and access to AML/ALL patient specimens. D.O. supervised A.C., A.L., T.P., and R.D. J.V., S.D., S.J., A.C., A.L., T.P., D.L., L.P., and A.D. analyzed experiments. J.V. led the project, coordinated the collaborative work, and wrote the manuscript with support from all authors.

Competing interests

S.D., I.S.C., B.J., I.C.S., A.G., M.D., R.G., J.P.F., A.D., P.D., and J.V. areCellectis employees and hold patents related to the engineering process of CAR T cells andTALEN technology. TALEN is a Cellectis patented technology. D.O. declares competing interests as being the co-founder and shareholder of Imcheck Therapeutics, Alderzian Biotechnology and Emergence Therapeutics and has research funds from Imcheck Therapeutics, Alderzian Biotechnology, Cellex Ia, and Emergence Therapeutics. A.W., B.J., and M.D. are former Cellectis employees. The remaining authors declare no competing interests.

Additional information

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