Journal for ImmunoTherapy of Cancer

Generation of non-genetically modified, CAR-like, NK cells

Loïs Coënon ,[1] Emilie Rigal,[2] Hortense Courot ,[2] Caroline Multrier ,[1] Sara Zemiti,[1] Jennifer Lambour,[1] Martine Pugnière ,[3] Marion de Toledo ,[4] Guillaume Bossis ,[4] Guillaume Cartron ,[5] Bruno Robert ,[3] Pierre Martineau ,[3] Bénédicte Fauvel ,[2] Jessy Presumey ,[2] Martin Villalba [1]

To cite: Coënon L, Rigal E, Courot H, *et al*. Generation of non-genetically modified, CAR-like, NK cells. *Journal for ImmunoTherapy of Cancer* 2024;**12**:e009070. doi:10.1136/jitc-2024-009070

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-009070).

ER and HC contributed equally.

Accepted 27 June 2024

[1]IRMB, INSERM U1183, University of Montpellier, CHU Montpellier, Montpellier, France
[2]CYTEA BIO, Montpellier, France
[3]IRCM, INSERM U1194, University of Montpellier, ICM, Montpellier, France
[4]IGMM, CNRS, University of Montpellier, Montpellier, France
[5]Department of Clinical Hematology, CHU Montpellier, Montpellier, France

Correspondence to
Dr Martin Villalba;
martin.villalba@inserm.fr

Dr Jessy Presumey;
jessy.presumey@cytea.bio

ABSTRACT

Background Natural killer (NK) cell therapy is considered an attractive and safe strategy for anticancer therapy. Nevertheless, when autologous or allogenic NK cells are used alone, the clinical benefit has been disappointing. This is partially due to the lack of target specificity. Recently, CD19-specific chimeric antigen receptor (CAR)-NK cells have proven to be safe and potent in patients with B-cell tumors. However, the generation of CAR-NK cells is a complicated manufacturing process. We aim at developing a targeted NK cell therapy without the need for cellular genetic modifications. We took advantage of the natural expression of the IgG Fc receptor CD16a (FcγRIIIa) to induce strong antigen-specific effector functions through antibody-dependent cell-mediated cytotoxicity (ADCC). We have generated the new technology "Pin", which enables the arming of modified monoclonal antibodies (mAbs) onto the CD16a of ex vivo expanded NK (eNK) cells.

Methods Ex vivo eNK were prepared from umbilical cord blood cells and expanded using interleukin (IL)-2/IL-15 and Epstein-Barr virus (EBV)-transformed B-lymphoblastoid feeder cells. mAbs were engineered with four substitutions called Pin mutations to increase their affinity to CD16a. eNK were incubated with anti-CD20 or anti-CD19 Pin-mAbs to generate "armed" eNK and were used to assess effector functions in vitro on cancer cell lines, lymphoma patient cells and in vivo.

Results CD16a/Pin-mAb interaction is stable for several days and Pin-mAb eNK inherit the mAb specificity and exclusively induce ADCC against targets expressing the cognate antigen. Hence, Pin-mAbs confer long-term selectivity to eNK, which allows specific elimination of the target cells in several in vivo mouse models. Finally, we showed that it is possible to arm eNK with at least two Pin-mAbs simultaneously, to increase efficacy against heterogenous cancer cell populations.

Conclusions The Pin technology provides an off-the-shelf NK cell therapy platform to generate CAR-like NK cells, without genetic modifications, that easily target multiple tumor antigens.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Allogeneic natural killer (NK) cells can be universally and safely used for cell therapy. Chimeric antigen receptor (CAR)-NKs, with improved target-specificity, have also shown clinical benefits in patients with cancer, but require complex manufacturing process, limiting their use. NK cells naturally express the Fc gamma receptor CD16a that mediates antibody-dependent cellular cytotoxicity (ADCC), an innate functionality that can be exploited to generate CAR-like NK cells without genetic modifications.

WHAT THIS STUDY ADDS

⇒ For the first time, our study demonstrates that it is possible to specifically harness the physiological mechanism of ADCC by arming ex vivo expanded NK cells (eNK) with clinically relevant engineered monoclonal antibodies (Pin-mAbs) that bind persistently to CD16a. Pin-mAb armed eNK are highly potent to kill tumor cells expressing the Pin-mAb cognate antigen in vitro and in vivo. Moreover, multispecificity can be achieved by arming eNK with at least two Pin-Mabs.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our new Pin platform allows the generation of CAR-like NK cells with the major advantages of not being genetically modified, of drastically lightening manufacturing process and, of opening possibilities for targeting multiple antigens to address tumor heterogeneity.

INTRODUCTION

Natural killer (NK) cell therapy using ex vivo expanded allogeneic NK cells is an established anticancer strategy that has already proven to be safe, and in contrast to therapy with other immune cells, having very limited undesirable side effects.[1–3] However, clinical results show that the adoptive transfer of NK cells alone has a limited outcome.[4 5] NK cells' role in cancer immunosurveillance is governed by a balance between inhibitory and activating receptors that recognize aberrant protein expression in cancer cells. These activating receptors include CD16a (FcγRIIIa), NKG2D and the natural cytotoxicity receptors NKp30,

NKp44, NKp46.[6] Moreover, NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) on target recognition through CD16a/FcγRIIIa interaction with its ligand, the Fc moiety of IgGs. This cytotoxic mechanism is harnessed therapeutically through the action of several therapeutic monoclonal antibodies (mAbs), such as rituximab (a chimeric monoclonal antibody targeted against the pan-B-cell marker CD20 and approved for the treatment of B-cell malignancies[7]). However, therapeutic mAbs require high doses to be clinically efficient, which often lead to side effects.[8] Additionally, deficiency in endogenous NK cell number and function in patients with cancer has been observed,[4 9] dampening the potency of ADCC-dependent mAbs. One alleviation strategy is to administer patients with ex vivo expanded donor-derived NK cells in association with mAb-based therapies.[4 10] Recently, several clinical trials using this strategy have given promising results, highlighting the need for refining NK cell therapy combined with the targeting activity of mAbs.[11–13] Targeting specificity is achieved by chimeric antigen receptor (CAR)-NK cells, and results from anti-CD19 CAR-NK clinical trials in patients with B-cell tumor showed no toxicity and clinical benefits for patients.[14 15] CAR-NK cells have the ability to target diverse antigens but their complex manufacturing reduces their flexibility as new products have to be made for each target. To circumvent this issue, universal strategies involving CAR or other receptors could be developed.[16]

We aim at improving current NK cell therapy options without the need for cell genetic manipulations, using phenotypically "normal" cells. In order to do so, we developed the Pin platform that allows the assembly of a cell therapy product composed of an Fc-engineered mAb (Pin-mAb) spontaneously bound to CD16a-expressing ex vivo expanded donor-derived NK cells. We previously reported the development of expanded NK cells (eNK) that induce an ADCC effect with multiple clinical mAbs,[17–19] including in in vivo mice models.[20 21] Here, to arm eNK cells, we took advantage of four amino acid mutations situated in the mAb Fc region, known to increase the affinity to CD16a,[22 23] to generate Pin-mAbs.

We report the preclinical validation of eNK armed with anti-CD20 Pin-mAb (Pin-CD20 eNK) targeting B-cell lymphoma. We show that Pin-CD20 is stably armed on eNK for several days, and that Pin-CD20 eNK exhibit superior cytotoxic potency in vitro against a panel of cancer cell lines and patient samples and in vivo in engrafted mice models compared with unarmed eNK. Finally, we report that the Pin technology allows the arming of two Pin-mAbs at the same time, enabling the prospect of increased efficiency against heterogenous cancer cell populations.

RESULTS

eNK are armed with Pin-CD20 and the resulting assembly is stable in vitro and in vivo

The amino acid substitutions S239D/H268F/S324T/I332E in an IgG1 CH2 domain dramatically increase the affinity towards CD16a (>30-fold) and enhance ADCC mechanism compared with unmodified IgG1.[23] We therefore produced a modified anti-CD20, or Pin-CD20, by mutating the sequence of rituximab with these four mutations. First, we confirmed that this Fc-engineering allows increasing affinity for CD16a and other FcγR (CD16b, CD32a) (online supplemental figure S1A). Moreover, CD16a has a single nucleotide polymorphism resulting in two subtypes of high affinity (V158) and low affinity (F158) receptors, which influence the in vitro and clinical activity of antibodies.[7 24–28] In addition, whereas unmodified rituximab affinity for F158 variant is 1.43 lower than for V158 variant, Pin-CD20 displayed a comparable binding to both variants with a 1.18 ratio. Hence, Pin mutation reduces affinity variation between the two variants.

Having established that Pin-CD20 affinity to CD16a is strongly increased, we then investigated whether Pin-CD20 could remain bound to eNK and thus generate "armed" eNK (Pin-mAb eNK). First, NK were expanded from umbilical cord blood using feeder cells and interleukin (IL)-2/IL-15 as previously described.[17] At the end of the expansion protocol, eNK contained a pure population of cytotoxic $CD56^+$ NK cells (>95% $CD56^+CD3^-$ cells) (figure 1A,B). eNK expressed the inhibitory receptor NKG2A and activating receptors and coreceptors phenotypically associated with cytotoxic cells (NKG2D, NKp30, NKp46, CD2, CD69, and CD16; figure 1C). Interestingly, eNK exhibited high levels of the chemokine receptors CCR5 and CXCR3, which are important for cell migration to tumor sites, and low levels of the immune checkpoint receptors T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT) and programmed cell death protein-1 (PD-1), known to inhibit NK cell activity when expressed at high levels.

To generate eNK armed with Pin-mAb (Pin-mAb eNK), eNK were incubated with Pin-mAbs, allowing for the spontaneous anchoring of Pin-mAbs on eNK through interaction with CD16a (figure 1D). The optimal Pin construct arming concentration was determined by using increasing doses of a fluorescent Pin-Fc. Using a 10 µg/mL Pin-Fc concentration, more than 80% of eNK were armed and saturated with Pin-Fc compared with 30% with unmodified Fc (online supplemental figure S1B). We next co-incubated eNK with Pin-CD20 to generate Pin-CD20 eNK and demonstrated by flow cytometry that more than 80% of eNK were armed directly after incubation. Unmodified anti-CD20 (rituximab) was not able to persistently bind to eNK (figure 1E,F). The frequency and geometric mean of fluorescence intensity (gMFI) of Pin-CD20 eNK decreased between 1 hour and 8 hours after arming and then remained very stable, as more than 50% of the cells were still positive for Pin-CD20 after 72 hours of culture. Of note, non-armed eNK corresponded to CD16 negative eNK within the population, confirming that the Pin-mAb binding occurs only on CD16 positive cells (online supplemental figure S2). To assess for stability in vivo,

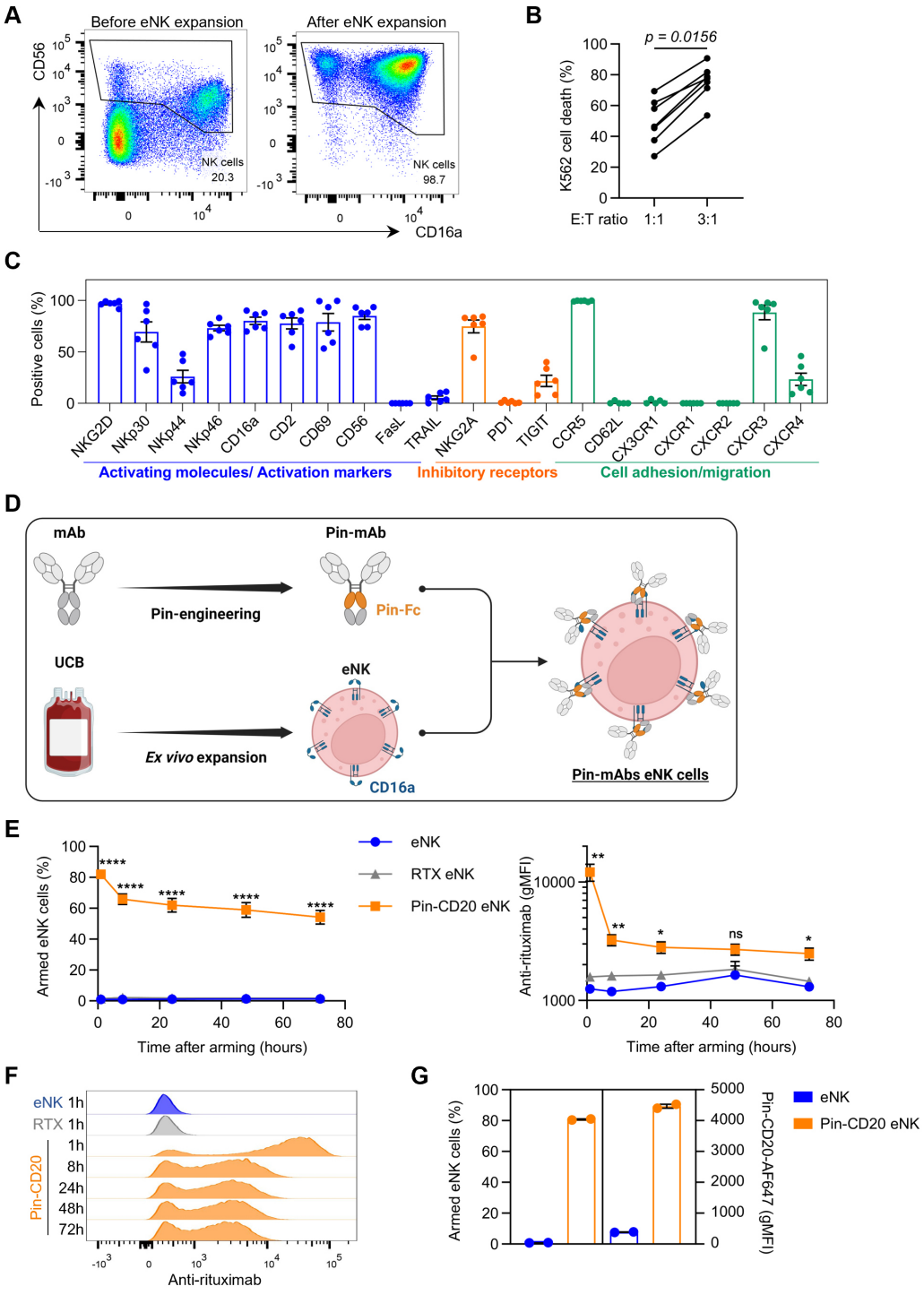


Figure 1 eNK cells armed with Pin-CD20 exhibit long-term binding in vitro and in vivo. (A) Representative dot plot showing UCB-derived NK cells purity before (left) and after 14 days of expansion (right). NK cell frequency is indicated within the dot plot. (B) eNK cytotoxicity against K562 cell lines at effector to target ratio 1:1 and 3:1 at 4 hours. n=7 individuals eNK donors represented, Wilcoxon matched-pairs test. (C) eNK phenotypic analysis of indicated markers. The bar graph shows individual eNK donors (n=6) and mean±SEM is shown. (D) Pin-mAbs eNK cells manufacturing scheme. Mutated Fc region (Pin-Fc): red; FcγR CD16a: blue. UCB-derived eNK cells are incubated with Pin-mAbs, allowing its arming onto CD16a expressing eNK cells. Created with BioRender.com. (E and F) Comparison of arming efficiency in rituximab-armed eNK (RTX eNK) and Pin-CD20-armed eNK (Pin-CD20 eNK) over a 72 hours kinetic, eNK cells represent the baseline. Representative histogram plot for one eNK donor (F) and bar graphs representing the frequency of armed cells (left) and the arming geometric mean of fluorescence intensity (gMFI, right) detected by an anti-rituximab idiotype antibody (E) are shown. n=9 eNK donors; two-way analysis of variance with Tukey's test, difference between RTX eNK and Pin-CD20 eNK groups is shown; mean±SEM is shown. (G) Arming stability in vivo. Pin-CD20 eNK cells arming efficiency was assessed 24 hours after intraperitoneal injection in SCID mice. eNK, n=2; Pin-CD20 eNK, n=2. mean±SEM is shown. eNK, expanded NK; mAb, monoclonal antibodies; NK, natural killer; UCB, umbilical cord blood.

Pin-CD20 eNK were intraperitoneally injected into SCID mice and analysis showed that 80% of eNK remain armed after 24 hours, suggesting that in vivo environment is not likely to lead to an extensive loss of Pin-CD20 binding (figure 1G; online supplemental figure S3).

In addition, we evaluated whether human IgGs present in plasma (estimated at 10 mg/mL) or the presence of other cell types expressing FcγR could affect the Pin-mAb binding to eNK through competition or displacement if Pin-mAb eNK were intravenously injected into patients. Arming stability was assessed either by incubating Pin-CD20 eNK with 10 mg/mL of human IgG or by incubating Pin-Fc with peripheral blood mononuclear cells (PBMCs) from healthy donors for 4 hours (online supplemental figure S4A,B). In both cases, arming was stable although a slight but significant decrease of arming was observed when co-incubated with human IgG. Moreover, Pin-Fc binding on other PBMC cell types was observed, especially on monocytes and to a lesser extent on NK cells (online supplemental figure S4B). Nevertheless, the majority of the Pin-CD20 mAb remained bound to eNK suggesting that Pin-mAb displacement is unlikely to adversely affect the potency of Pin-CD20 eNK in vivo. Interestingly, monocytes and monocyte-derived macrophages could efficiently be armed with Pin-CD20 (online supplemental figure S5A), inducing antibody-dependent cell phagocytosis of CD20-positive target cells. Once armed with Pin-CD20, both cell types engulfed more efficiently target material compared with no antibody condition (online supplemental figure S5B), showing that the Pin technology could be used on other FcγR expressing cell types than NK cells.

Pin-CD20 eNK induce Pin-mAb dependent cell cytotoxicity (Pin-ADCC) in vitro on B-lymphoma cell lines

Classical ADCC occurs when CD16a-expressing NK cells encounter IgG-opsonized target cells. An immune synapse forms, inducing NK cell cytotoxicity and, subsequently, target cell death. In our setting, Pin-mAb eNK are already bound to antibodies, and whether the formation of an immune synapse in a different sequence would still induce mAb-specific cell targeting and cytotoxicity remained to be elucidated. We therefore investigated Pin-CD20 eNK cytotoxicity and activation compared with eNK in co-culture experiments (figure 2A). Cytotoxicity assays were performed with 12 cancer cell lines, expressing various levels of CD20, at an effector to target (E:T) ratio of 1:1 (online supplemental figure S6A). Notably, specific cell killing was induced by Pin-CD20 eNK on all CD20-positive cell lines and did not seem to correlate with the number of CD20 molecules at the target cell surface, suggesting that even a limited amount of antigen effectively induces cytotoxicity. Daudi and Toledo cell lines were selected as high and low CD20 expressing, respectively, for further characterization and tested in cytotoxicity assays using increasing E:T ratios. Pin-CD20 eNK showed greatly enhanced cytotoxicity against both cell lines at almost all E:T ratios compared with eNK (figure 2B). Indeed, E:T ratio where 50% of the target cells is killed (E:T50) is reached at ratio 1.4:1 for Pin-CD20 eNK versus 7:1 for eNK on Daudi cells, and at ratio 1:1 for Pin-CD20 eNK versus 9.1:1 for eNK on Toledo cells, meaning that five times more (for Daudi cells) or nine times more (for Toledo cells) eNK than Pin-CD20 eNK were needed to obtain the same cytotoxic activity (figure 2C).

Next, we evaluated the relationship between arming concentration and cytotoxic effect by arming eNK with a range of Pin-CD20 concentrations from 0.01 µg/mL to 40 µg/mL (figure 2D) and co-culturing them with Daudi and Toledo cell lines using the ratio corresponding to the E:T80 calculated in figure 2C. Sigmoidal dose-response curves show that arming concentration where 50% of the target cells are killed (AC50) is 0.49 µg/mL for Daudi cells and 0.71 µg/mL for Toledo cells. In addition, arming eNK with higher concentrations than 10 µg/mL does not lead to higher cell killing, implying that 10 µg/mL is an optimal concentration under these conditions as previously suggested (online supplemental figure S1B). Nevertheless, AC50 values suggest that using lower mAb concentrations would still produce potent cytotoxic Pin-CD20 eNK.

Pin-CD20 eNK selectivity was assessed first on CD20 negative cell lines (MOLM-13 and NALM6) and Pin-ADCC was not observed when the target expression was absent (online supplemental figure S6A). Second, the specificity of Pin-mAb eNK was tested using Pin-CD20 and two irrelevant Pin-mAbs, Pin-HER2 and Pin-EGFR, based on trastuzumab (anti-HER2) and cetuximab (anti-EGFR) Fab sequences, respectively. Cytotoxicity assay was performed on Daudi cells that do not express neither HER2 nor EGFR (figure 2E). Hence, we demonstrated that using eNK armed with a Pin-mAb against a target cell that does not express the cognate antigen neither improves eNK cytotoxicity nor inhibits the natural cytotoxicity of eNK.

Next, we assessed eNK or Pin-CD20 eNK activation in the absence or presence of target cells. In the absence of target cells, Pin-CD20 eNK did not show elevated CD107a surface expression compared with eNK (figure 2F). Similarly, no cytokine or granule toxins secretion was observed (figure 2G). However, when co-cultured with Daudi cells, Pin-CD20 eNK showed a significant increase in CD107a expression compared with eNK (36% vs 19%, respectively) and secreted significantly more tumor necrosis factor (TNF)-α and interferon-γ than eNK (figure 2F,G). However, no difference regarding granule toxins released was found. Of note, this enhanced activation was restricted to CD20-expressing cells since CD20-negative K562 cells equally activated eNK and Pin-CD20 eNK (online supplemental figure S6B). Moreover, the binding of Pin-CD20 on the eNK cell surface did not trigger classical complement pathway activation, as complement C3b binding was not observed on Pin-CD20 eNK, whereas C3b was found to bind to target cells already opsonized by Pin-CD20 (online supplemental figure S6C). Finally, Pin-CD20 eNK did not show any decrease in viability compared with eNK

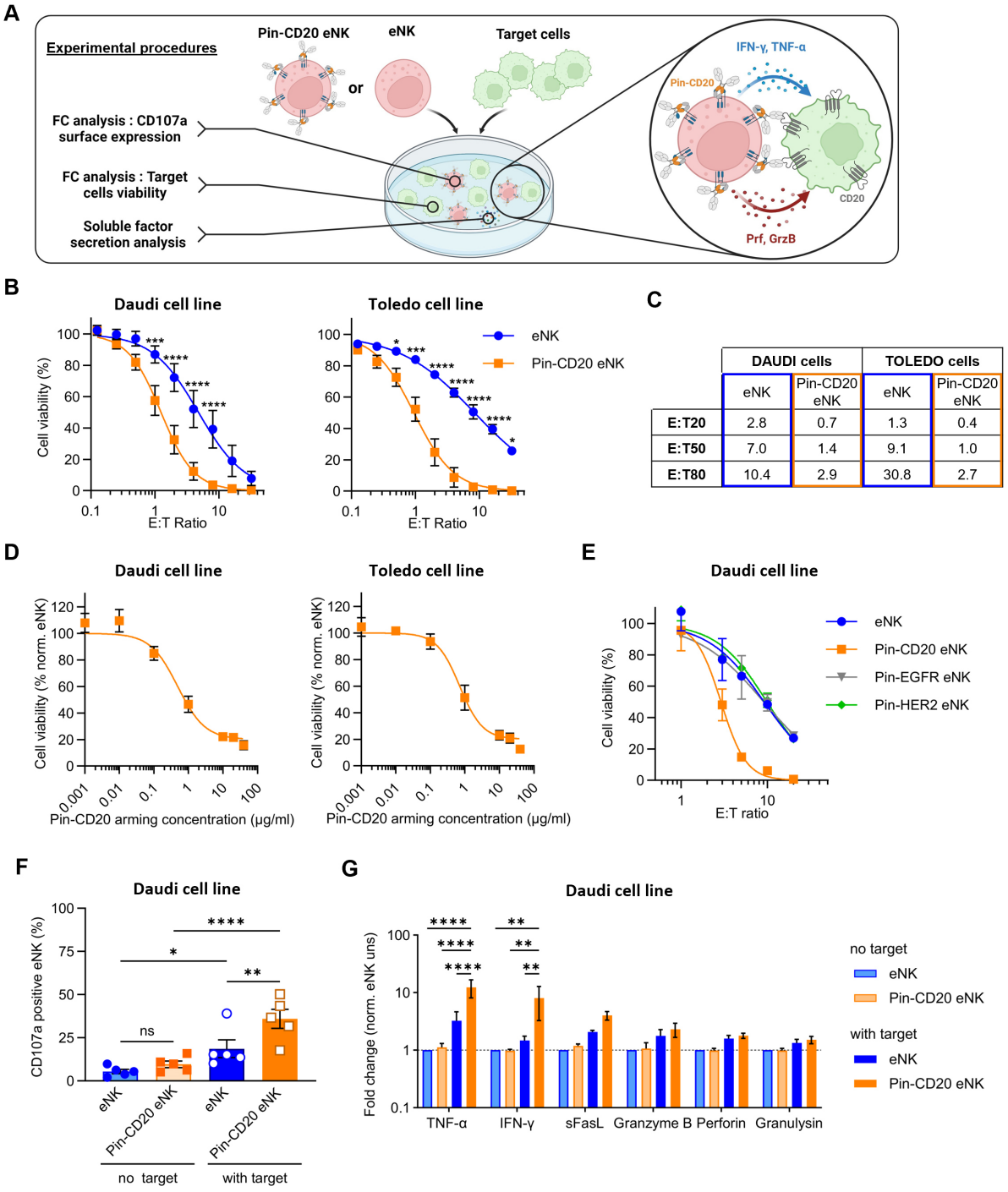


Figure 2 Pin-CD20 eNK cells display enhanced and specific antibody-dependent cellular cytotoxicity and activation on CD20 positive B-lymphoma cell lines recognition. (A) Schematic overview of in vitro functional assays performed to evaluate Pin-CD20 eNK cells. Flow cytometry: FC; perforin: Prf; granzyme B: GrzB. Created with BioRender.com. (B and C) Cytotoxicity assays comparing eNK versus Pin-CD20 eNK were performed on Daudi (n=6 eNK donors) and Toledo (n=3 eNK donors) B-lymphoma cell lines at different E:T ratios by flow cytometry. Two-way ANOVA with Sidak's test and mean±SEM are shown (B). Four parameter logistic model was used to measure E:T ratios that lyse 20% (E:T20), 50% (E:T50) or 80% (E:T80) of the target cells (C). (D) eNK cells were armed with increasing concentration of Pin-CD20 mAbs and cytotoxicity was measured using E:T ratio ET80 measured for two eNK donors (from b, c). Mean±SEM is shown. (E) Cytotoxicity assay against Daudi Luciferase cell line using one eNK donor armed with Pin-CD20, Pin-EGFR, Pin-HER2 or unarmed. Mean±SEM is shown. (F) CD107a expression on eNK or Pin-CD20 eNK in the presence or absence of Daudi cells assessed by flow cytometry after 4 hours incubation at E:T ratio 3:1. n=5 eNK donors; Two-way ANOVA with Tukey's test; mean±SEM is shown. (G) Cytotoxic mediators secretion from eNK or Pin-CD20 eNK in presence or absence of Daudi cells at E:T ratio 1:1. Data are shown as fold change normalized with eNK unstimulated (norm. eNK uns). n=8 eNK donors; Two-way ANOVA with Tukey's test mean±SEM is shown. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. ANOVA, analysis of variance; eNK, expanded natural killer; E:T, effector to target; IFN, interferon; TNF, tumor necrosis factor.

up to 72 hours (online supplemental figure S6D). Taken together, these data demonstrate that Pin-CD20 eNK are able to potently engender Pin-mAb ADCC (Pin-ADCC) only in the presence of Pin-mAb target expressing tumor cells.

Pin-CD20 eNK display long-term enhanced cytotoxicity

Given that Pin-CD20 binding on eNK is remarkably persistent for several days following arming (figure 1F), we sought to evaluate Pin-CD20 eNK functional activity at different time points after cell arming by measuring Pin-ADCC. We performed cytotoxicity assays against Daudi cells, at E:T ratios 10:1, 3:1 and 1:1, at day 0, day 1 and day 2 after arming using the same batch of armed eNK every day. At E:T ratio 10:1, we found that Pin-CD20 eNK are statistically more potent than eNK at every time point after the arming procedure (D0: 97% vs 69%; D1: 89% vs 59%; D2: 78% vs 44% of cytotoxicity) (figure 3A). In addition, we observed the same patterns for the other E:T ratios tested (online supplemental figure S7A,C). After 2 days in culture without cytokine stimulation, Pin-CD20 eNK are still highly potent, even though a time-dependent decrease in efficacy is observed (figure 3B; online supplemental figure S7B,D).

As mentioned earlier, CD16a F158V polymorphism influence mAbs activity.[7 24–28] We, therefore, explored the potential impact of this polymorphism on eNK arming stability and activity. eNK donors were genotyped by quantitative PCR for the presence of the alleles CD16a V158 or F158. Donors homozygous for CD16a V158 (V/V) or F158 (F/F), or heterozygous (F/V) were selected and tested in a cell arming kinetic experiment. Although no statistical differences are observed, there was a consistent tendency of the V/V polymorphism to remain more stably armed than F/F (figure 3C). Indeed, 2 days after the arming procedure, 74% of V/V eNK were still armed whereas 52% of F/F eNK remained armed (vs 78% for both groups at day 0), while F/V eNK were similar to V/V eNK. Whether this arming efficiency difference has an impact on target cell killing efficiency was tested in cytotoxicity assay and specific Pin-ADCC of Pin-CD20 eNK was used to compare eNK donors with F/F, F/V and V/V polymorphism. We did not find any statistical difference between the three groups for each time point (figure 3D, online supplemental figure S7E), highlighting that Pin-ADCC is not impacted by CD16a polymorphism in vitro. Taken together, these data indicate that Pin-CD20 eNK has potential as a universal long-term active product and supports the contention that donor selection with respect to CD16a polymorphism will be unnecessary.

We further investigated ADCC durability after the target encounter. For this assay, eNK and Pin-CD20 eNK were co-cultured with carboxyfluorescein succinimidyl ester (CFSE) labeled Daudi targets for 24 hours at E:T ratio 3:1, then CellTrace Far Red labeled Daudi targets were added at ratio 3:1 for another 24 hours in order to distinguish the newly introduced target cells. Pin-CD20 eNK cell arming was determined at 24 hours and target cell killing was determined at 24 hours and 48 hours. Even though cell arming dropped in the presence of target cells (figure 3F), independently of CD16 shedding (figure 3E), an average of three Pin-CD20 eNK was needed to kill one Daudi cell during initial co-culture compared with six eNK cells. After 24 hours, when the cells were challenged with new target cells, Pin-CD20 eNK were still able to kill with the same activity. Although these results are not significantly different, they indicate that, after killing all the tumor cells, Pin-CD20 eNK remain active in eradicating newly emerging tumor cells. However, the loss of arming after target encounter encourages a multiple doses strategy for therapeutic use.

Pin-CD20 eNK efficiently eliminate CD20-positive targets in vivo

The first proof of concept of Pin-CD20 eNK efficiency in vivo was designed to eliminate CD20-positive B cells in a humanized NCG mouse model. Preliminary in vitro cytotoxicity assay on human PBMC from healthy donors showed that only B cells but no other immune cell population were killed by Pin-CD20 eNK, whereas eNK had no cytotoxic effect (figure 4A). In humanized mice, 7 days after a single eNK or Pin-CD20 eNK intravenous injection, close to 50% of B cells were depleted by Pin-CD20 eNK compared with eNK while no effect was observed on the T-cell population (figure 4B). To further investigate the specificity of Pin-CD20 eNK in B-lymphoma models, we next designed an in vivo cytotoxicity experiment using two cell lines harboring different phenotypes: Raji cells, expressing high levels of CD20, and Nalm6 cells which are CD20 negative (online supplemental figure S5A). eNK and Pin-CD20 eNK were intraperitoneally co-injected with both cell lines and cell content of peritoneal lavage was analyzed after 4 hours. Flow cytometry analysis showed that eNK killed both cell lines indiscriminately while Pin-CD20 eNK selectively targeted and destroyed CD20 positive Raji cells compared with vehicle treated group (figure 4C). These data demonstrated in vivo that Pin-CD20 eNK target preferentially CD20 expressing cells compared with eNK.

Finally, we compared antitumor efficacy of eNK and Pin-CD20 eNK in a xenograft B-lymphoma model. Raji cells were implanted intravenous in SCID mice that received two doses of intravenous eNK or Pin-CD20 eNK treatment after 4 and 8 days (figure 4D). After 28 days, Raji cells were detected in the lungs, bone marrow and liver of vehicle-treated mice. In contrast, both eNK and Pin-CD20 eNK successfully cleared Raji cells in the lungs and only Pin-CD20 eNK completely eliminated tumor cells in the bone marrow and to a lesser extent in the liver, as shown by the frequency of Raji cells detected in each organ (figure 4E) and the frequency of lymphoma incidence (figure 4F). Altogether, these results show that Pin-mAb arming enables eNK selectivity toward target cells expressing the cognate antigen in vivo and generates potent antitumor activity.

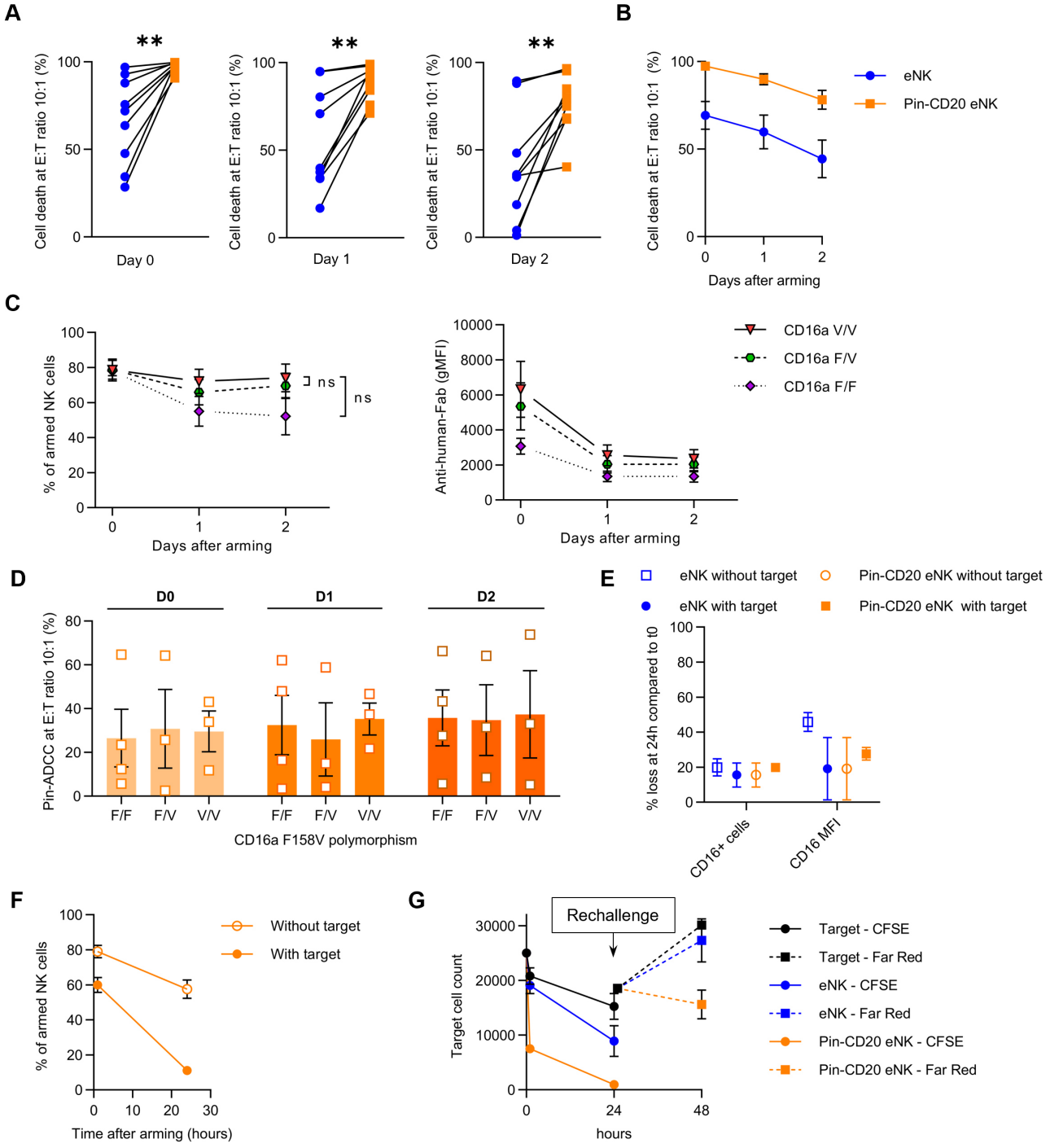
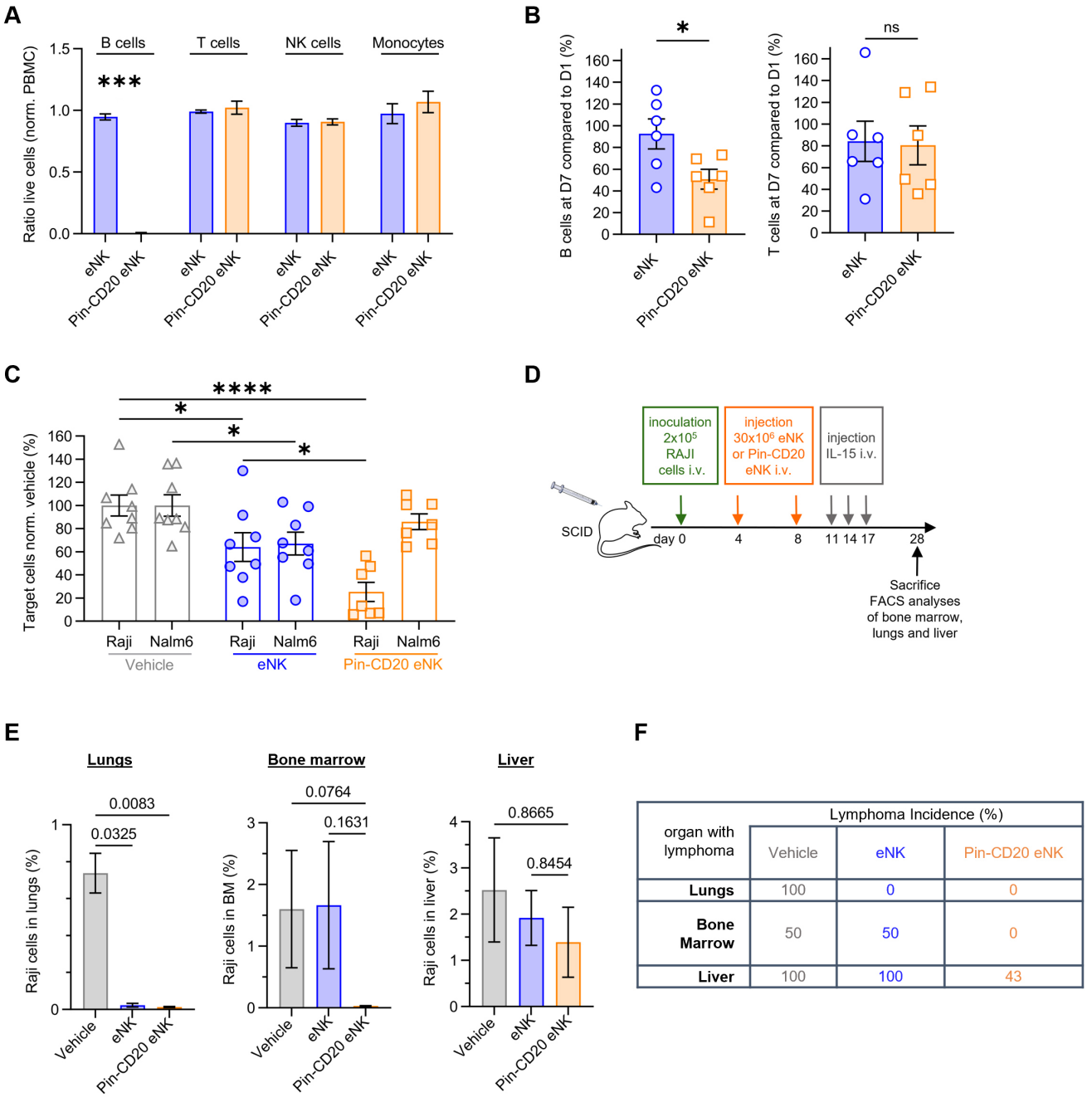


Figure 3 Pin-mAbs eNK are highly functional for several days, independently of the CD16a F158V polymorphism. (A and B) eNK cells were armed with Pin-CD20 or not and used in cytotoxicity assays against Daudi cells after 1 day (Day 1), 2 days (Day 2) incubation or no incubation time (Day 0) at E:T ratio 10:1. (A) Target cell death at E:T ratio 10:1 was measured by flow cytometry. N=10 eNK donors; paired t-test mean±SEM is shown; **p≤0.01. (B) Mean cytotoxicity of eNK and Pin-CD20 eNK over time. Mean±SEM is shown. (C) Comparison of Pin-CD20 arming efficiency in CD16a V/V, F/V or F/F donors over a 48 hours kinetic. Dot graphs represent the frequency of armed cells (left) and the arming geometric mean of fluorescence intensity (gMFI, right) detected by an anti-human Fab antibody. n=6 F/F, 6 F/V, 5 V/V eNK donors. Two-way analysis of variance with Tukey's test mean±SEM is shown; ns=not significant. (D) Pin-ADCC effect of Pin-CD20 eNK at D0, D1 and D2 in CD16 V/V, F/V or F/F eNK donors at E:T ratio 10:1. Pin-ADCC was calculated by subtracting eNK natural cytotoxicity to Pin-CD20 eNK total cytotoxicity. n=4 F/F, 3 F/V and 3 V/V eNK donors. Mean±SEM is shown. (E, F and G) eNK and Pin-CD20 eNK were cultured or not with CFSE Daudi at E:T ratio 3:1 for 24 hours. CD16 expression (frequency and gMFI) was compared between t0h and t24h. (E) Frequency of armed eNK cells was measured at t1h and t24h (F). At 25 hours, eNK and Pin-CD20 eNK were challenged a second time with CellTrace Far Red Daudi at E:T ratio 3:1 for another 24 hours. Target cell count (CFSE, line; Far Red, dotted line) was measured at 24 hours and 48 hours (G). ADCC, antibody-dependent cellular cytotoxicity; CFSE, carboxyfluorescein succinimidyl ester; eNK, expanded NK; E:T, effector to target; NK, natural killer.



organ with lymphoma	Lymphoma Incidence (%)		
	Vehicle	eNK	Pin-CD20 eNK
Lungs	100	0	0
Bone Marrow	50	50	0
Liver	100	100	43

Figure 4 Pin-CD20 shows target specificity and enhanced cytotoxicity in vivo compared with unarmed eNK. (A) in vitro validation of CD20⁺ B cells targeting compared with other PBMC cell types (T cells, NK cells, monocytes) when eNK or Pin-CD20 eNK were co-incubated with PBMCs from healthy donors during 16 hours at effector to target ratio 1:1. PBMCs populations were then assessed by flow cytometry. n=6 samples (two experiments: one eNK donor tested on one PBMC donor and two eNK donors tested on two PBMC donors). Multiple paired t-tests and mean±SEM are shown. (B) 3×10^6 eNK or Pin-CD20 eNK were injected intravenously into human CD34+reconstituted humanized NCG mice (Hu-NCG, 28 days after reconstitution). Relative percentage difference of peripheral blood CD20+B cells (left) and CD3+T cells (right) number at D7 compared with D1 was assessed by FC. eNK, n=6 mice; Pin-CD20 eNK, n=6 mice. Unpaired t-tests and mean±SEM are shown. (C) 1×10^6 eNK or Pin-CD20 eNK were co-injected intraperitoneally in NSG mice with 1×10^6 Raji and 1×10^6 Nalm6 cells. Cells were harvested after 4 hours, and target cell number was measured by flow cytometry. Vehicle, n=8 mice; eNK, n=8 mice; Pin-CD20 eNK, n=7 mice. Two-way analysis of variance with Tukey's test and mean±SEM are shown. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. (D, E and F) In vivo leukemia mouse model. (D) In vivo model timeline. (E and F) At day 0, 2×10^5 Raji cells were inoculated intravenous into SCID mice. At day 4 and 8, mice were treated intravenous with saline (vehicle), 30×10^6 eNK or Pin-CD20 eNK, followed by intravenous injection of IL-15 at day 11, 14 and 17. At day 28, bone marrow, lungs and liver were harvested for detection of CD20⁺ Raji cells by FC. (E) Bar graphs represent the frequency of Raji cells in each organ. (F) Table reporting the frequency of mice that present Raji cells in each organ (lymphoma incidence). Vehicle, n=4 mice; eNK, n=6 mice; Pin-CD20 eNK, n=7 mice. Kruskal-Wallis tests, p values and mean±SEM are shown. eNK, expanded NK; IL, interleukin; NK, natural killer; PBMC, peripheral blood mononuclear cells.

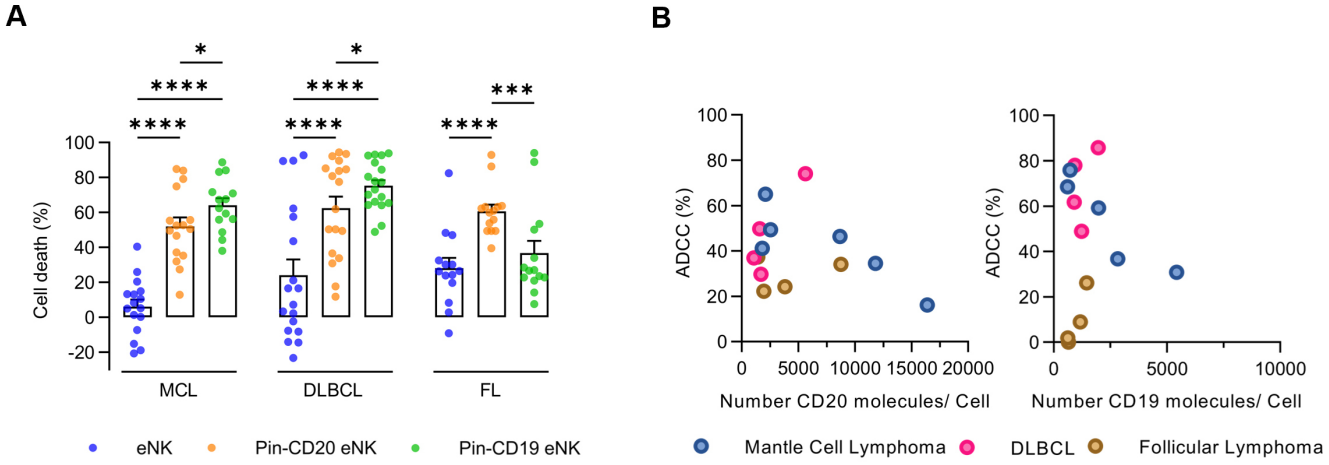


Figure 5 Pin-CD20 eNK and Pin-CD19 eNK efficiently kill patient with B-lymphoma cells. Patient with lymphoma cells from three B-lymphoma subtypes were used: mantle cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL). Patient cells were co-cultured with eNK, Pin-CD20 eNK or Pin-CD19 eNK for 16 hours at effector to target ratio 3:1 and target cell death was assessed by FC. (A) Frequency of target cell death in the three lymphoma subtypes. MCL, n=6 patients; DLBCL, n=6 patients; FL, n=5 patients; for each patient sample, two to four eNK donors were tested. Two-way analysis of variance with Tukey's test and mean±SEM are shown. *p≤0.05; ***p≤0.001; ****p≤0.0001. (B) Correlation between the average number of CD20 and CD19 molecules per cell and ADCC efficiency of Pin-CD20 eNK (left) and Pin-CD19 eNK (right). ADCC is the cytotoxicity measured above natural cytotoxicity from eNK cells. MCL, n=6 patients; DLBCL, n=4 patients; FL, n=4 patients. ADCC, antibody-dependent cellular cytotoxicity; eNK, expanded natural killer.

Pin-mAbs enhance eNK cell toxicity in patient with primary B-lymphoma cells

CD19 is another potential therapeutic target for patients with B-lymphoma, that has been previously selected in CAR-T and CAR-NK cells.[14] It has a broader expression profile than that of CD20 and has been shown to be expressed in cases of patient resistance to rituximab due to CD20 downregulation.[29] Therefore, we generated an anti-CD19 Pin-mAb (Pin-CD19) based on the anti-CD19 single-chain fragment variable (scFv) sequence of blinatumomab (a CD3×CD19 bispecific T-cell engager[30]). We next challenged Pin-CD20 eNK and Pin-CD19 eNK with primary patient cells from mantle cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL), and follicular lymphoma (FL). Compared with eNK that displayed modest cytotoxicity (MCL: 6%; DLBCL: 23%; FL: 28%), Pin-CD20 eNK showed enhanced cytotoxicity against all lymphoma subtypes (MCL: 52%; DLBCL: 62%; FL: 60%), whereas Pin-CD19 eNK efficiently eliminated primary cells, except from FL (MCL: 64%; DLBCL: 75%; FL: 37%) (figure 5A). Of note, in these samples from MCL and DLBCL patients, Pin-CD19 eNK were significantly more cytotoxic than Pin-CD20 eNK, suggesting that depending on the lymphoma subtypes, CD19 or CD20 could be a more adequate therapeutic target, at least in this patient cohort. Interestingly, we again observed that antigen density on target cells does not correlate with Pin-mAbs eNK activity (figure 5B). However, this observation fails to explain why Pin-CD19 eNK exerts lower efficiency against FL samples compared with DLBCL samples.

Although the superiority of Pin-mAbs eNK was shown on a large panel of patient with B-lymphoma samples, Pin-CD19 eNK can fail to efficiently destroy FL samples while they are more efficient against MCL and DLBCL than Pin-CD20 eNK. Hence, we hypothesized that eNK armed against multiple targets could be beneficial in the context of heterogeneous expression of tumor target proteins.

Dual arming of eNK increases Pin platform potential

To further characterize and demonstrate the full potential of our arming strategy, we simultaneously armed eNK with two Pin-mAbs directed against two different antigens to generate double-armed eNK. We achieved successful arming of eNK concomitantly with Pin-CD20 and Pin-CD19 as shown by flow cytometry (figure 6A; online supplemental figure S8A). Next, a binding kinetic experiment was conducted. First, we found that Pin-CD19 displays a similar binding kinetic as Pin-CD20 (figure 6B; online supplemental figure S8B). Second, similar frequencies of Pin-mAb positive cells and gMFI were observed for individual Pin-mAbs whether eNK were mono-armed or double-armed. Remarkably, when eNK were incubated simultaneously with Pin-CD20 and Pin-CD19, all the armed cells were positive for both Pin-mAbs (figure 6A,C). As described for mono-armed cells, about 50% of eNK were still double-armed 72 hours after the arming procedure (figure 6C). This shows that double-arming does not impact the overall binding efficiency.

Functionally, on patient with primary lymphoma samples, double-armed eNK exerted similar potency compared with Pin-CD20 or Pin-CD19 eNK suggesting that arming eNK with two different Pin-mAbs does not reduce their ability to recognize their target (figure 6D). However, Pin-ADCC efficiency was not enhanced, probably because all samples expressed both proteins CD20

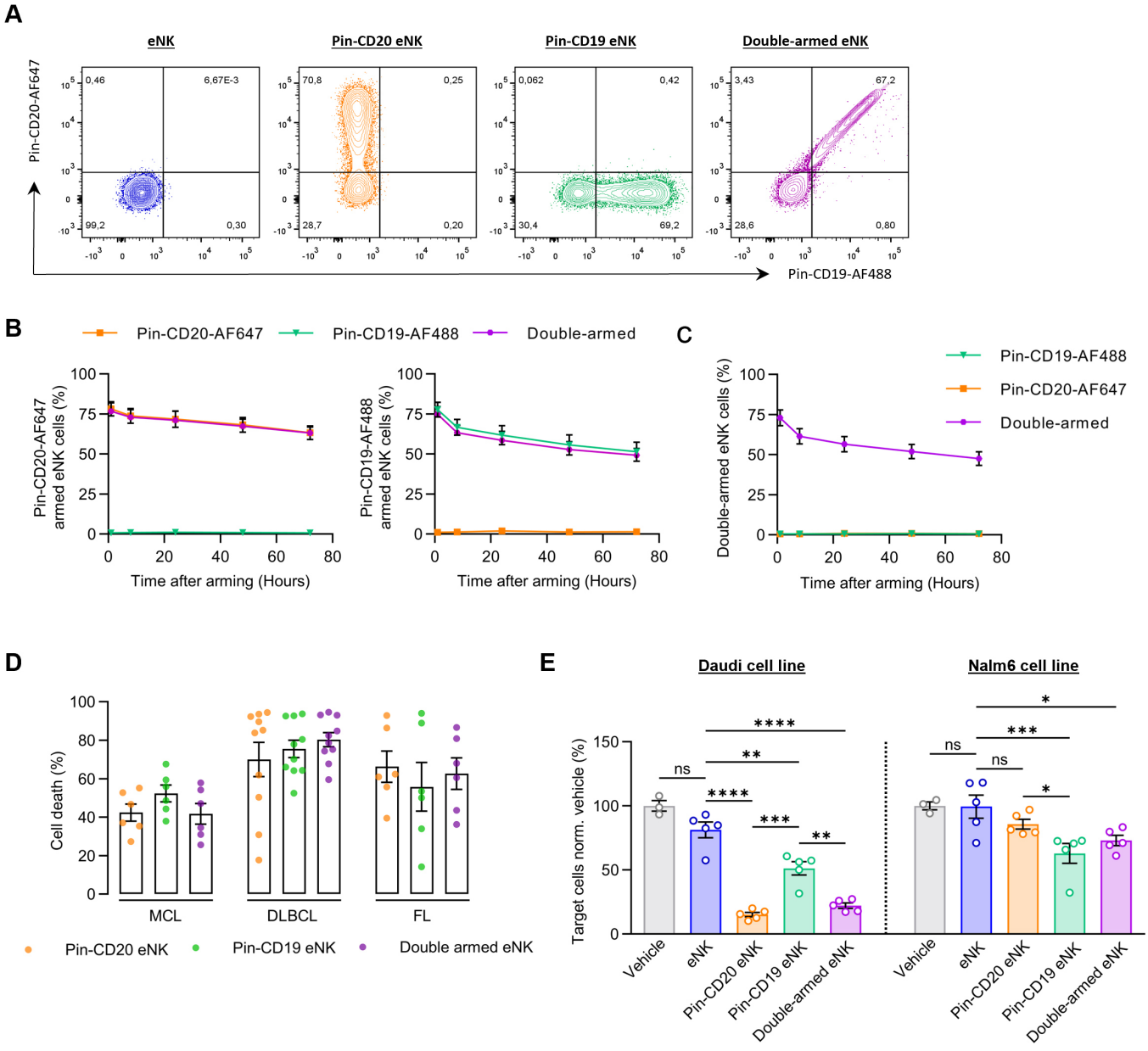


Figure 6 eNK cells can efficiently be armed with multiple Pin-monoclonal antibodies and remain actively cytotoxic. (A, B and C) eNK cells were armed with Pin-CD20, Pin-CD19 or both (double-armed eNK) using fluorescently labeled pin-CD20 (Pin-CD20-AF647) and pin-CD19 (Pin-CD19-AF488) and analyzed 1 hour, 8 hours, 24 hours, 48 hours and 72 hours after. (A) Representative dot plots of Pin-CD20-AF647 and Pin-CD19-AF488 arming for each condition. (B) Frequency of eNK cells armed with Pin-CD20 (left) or Pin-CD19 (right). (C) Frequency of eNK cells armed simultaneously with both Pin-CD20 and Pin-CD19 (double-armed). n=4 eNK donors. Data are shown as mean±SEM. (D) Patient with lymphoma cells were co-cultured with eNK or Pin-CD20 and/or Pin-CD19 eNK for 16 hours. Tumor cell death was assessed by flow cytometry. Mean±SEM is shown, MCL, n=3; DLBCL, n=4; FL, n=3. For each patient sample, two to three eNK donors were tested. (E) 1×10^6 eNK, Pin-CD20 eNK, Pin-CD19 eNK or double-armed eNK were intraperitoneally co-injected with 1×10^6 Daudi and 1×10^6 Nalm6 cells into NSG mice. Peritoneal lavage were performed after 4 hours and the number of target cell was analyzed by flow cytometry. Vehicle, n=3 mice; eNK, n=5 mice; Pin-CD20 eNK, n=5 mice; Pin-CD19 eNK, n=5 mice; double-armed eNK, n=5 mice. Two-way analysis of variance with Sidak's test and mean±SEM are shown, *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001; ns, not significant. eNK, expanded natural killer; DLBCL, diffuse large B cell lymphoma; FC, follicular lymphoma; MCL, mantle cell lymphoma.

and CD19. We hypothesized that dual arming could be beneficial in heterogenous cell populations expressing only one protein target or the other, or both. To test this hypothesis, we selected cell lines that are CD20 and CD19 positive (Daudi cells) or CD20 negative and CD19 positive (Nalm6 cells). Both cell lines were labeled and mixed before being intraperitoneally injected with eNK, Pin-CD20 eNK, Pin-CD19 eNK or double-armed eNK. After 4 hours, cell count analysis of each target cell line revealed that double-armed eNK killed both targets, whereas Pin-CD20 eNK failed to target the CD20-negative Nalm6 cells (figure 6E). Therefore, double-armed eNK

would particularly benefit in treating tumors which exhibit significant phenotype heterogeneity.

Finally, we performed a double-arming using Pin-HER2 and Pin-EGFR used in figure 2. We observed that both mAbs equally armed eNK (online supplemental figure S9A) as observed for Pin-CD20 and Pin-CD19 double-arming (figure 6A). Moreover, both Pin-HER2 and Pin-EGFR were able to induce Pin-ADCC against targets expressing the cognate antigen, that is, HCT116 cells express both HER2 and EGFR (online supplemental figure S9B). Remarkably, double-armed eNK did not lose cytotoxicity against targets expressing only one antigen, that is, MCF7 express only HER2 and MDA-MB-468 express only EGFR (online supplemental figure S9C). Therefore, we are able to give any target specificity to an NK cell provided that there is an mAb against a specific antigen.

DISCUSSION

NK cells are becoming attractive therapeutic tools for cancer treatment, either by the recruitment of patient's own NK cells through NK cell engagers via CD16a, NKG2D or NKp46,[31 32] or by adoptively transferring autologous or donor-sourced genetically modified CAR-NK cells.[33 34] NK cell engagers display efficiency but entirely rely on patients' NK cells and their level of expression for these receptors. Like CAR-NK cells, our Pin platform ensures specific targeting capacity for allogeneic NK cells, but does not rely on artificial receptors induced by genetic modifications using complex manufacturing.[35] Moreover, the Pin platform is more flexible or versatile than CAR-NK cells as modified antibodies can be easily interchanged depending on the indication or patient. Therefore, our strategy represents a promising therapeutic and industrial alternative to CAR-NK cells.

Recently, others have developed technologies allowing cancer cell specific targeting, via chemical antibody conjugation or tetravalent molecules containing anti-CD16 domains, bound to NK cell surfaces as ligands and not requiring cell genetic modifications.[36–39] Although efficient, those technological platforms still necessitate NK cell surface functionalization and complex molecule development and manufacturing. Here, to our knowledge, we present for the first time a technology relying only on natural interactions between CD16a and modified mAbs, which preserves effector cell function. Moreover, whereas other technologies show limited binding of their targeting molecules after 48 hours (less than 40%[36]), Pin-mAb remain bound, on average, on more than 60% of the cells, and most of the cells that do not bind it lack CD16a expression. Hence, using an anti-CD16 seems less efficient than directly harnessing a naturally occurring event such as CD16a/Fc IgG interaction. Moreover, our approach does not require the generation of new and complex bispecific or multispecific constructs.

Pin-mAbs interaction with eNK cells seems to be persistent over a period of 3 days and only decreases from 80% to 50–60% in that period, therefore more than half the cells remains armed after 72 hours in culture. The processes leading to the loss of arming are not entirely resolved and can involve various mechanisms. Low CD16a cleavage as well as internalization could be happening. Additionally, since the arming is non-covalent, it is possible that certain CD16a/Fc bonds are stronger than others and, hence, certain molecules stay longer on the CD16a. However, this loss appears to not impede Pin-mAbs eNK potency. We still postulate that the Pin technology could be further improved in the future since CD16a/IgG Fc interaction is not fully understood, as highlighted by the recent observations of the involvement of the Fab region in the binding of mAbs to CD16a.[40] On the other hand, arming loss over time could also act as a natural "off-switch" for Pin-mAbs eNK, without the need to use inducible suicide genes or drug administration.[41 42] In this sense, NK cells lifespan is short and allogeneic NK cell survival after patient engraftment ranges between 7 and 10 days in several clinical contexts.[4 43 44] For these reasons, our approach and many others involving NK cells would require multiple doses for clinical application to maximize patients' chances of remission.

The Pin technology displays a promising safety profile, as Pin-mAb eNK are harmless to host immune cells and the lack of eNK cell proliferation and their short life span would probably decrease the formation of immune reactions towards them. Moreover, the process of cell arming with Pin-mAbs does not negatively impact eNK cells, and only the presence of Pin-mAb epitope-expressing target cells induce Pin-ADCC.

Double-arming is an appealing concept since it could prevent tumor resistance through selective antigen loss. In our study, double-armed cells were as efficient as mono-armed eNK, although we did not find any improvement in cytotoxicity potency. This suggests that, for the treatment of heterogenous tumors, eNK arming with multiple Pin-mAbs could offer an advantage. Some authors reported dual-targeted CAR-NK with superior activity than their single-target counterparts,[45 46] while others did not find any differences.[47] Further studies comparing multiple mAbs and various levels of expression of their targets will determine the full potential of the dual arming approach. It will be interesting, for example, to compare dual-armed cells with mixed populations of single-armed cells.

We show, in this study, the preclinical development of a novel antitumor cell therapy product, Pin-mAb eNK, that uses the powerful ADCC capacity of NK cells to kill B-lymphoma cells by bringing together effector cells and specific targeting of mAbs. We report that multiple Pin-mAbs are efficient in vitro and in vivo to induce Pin-ADCC, providing a superior effect than unarmed eNK. In addition to the mAbs described here, the Pin technology could combine eNK with virtually any mAb for a relevant tumor target. The Pin technology could also be applied to other effector cells that were modified or naturally express CD16a. Finally, there is a broad range of Fc-based constructs carrying the Pin mutations that could

be designed. These could include proteins or peptides recognizing target cell receptors.

The advantages of our approach lie in its simplicity, with an easily executable protocol for generating armed NK cells applicable to a broad range of clinically relevant mAbs. However, limitations include its dependency on FcγR-expressing cells and the relatively short lifespan of the cells and the mAb's stability after target cell encounter, which will imply the injection of repeated doses in patients. Nevertheless, our findings collectively indicate that the Pin platform is poised to produce effective antitumor cell therapy products.

METHODS

Pin-mAbs production and purification

Pin-mAbs are human IgG1 containing four amino acid substitutions in the upper CH2 of the Fc: S239D/H268F/S324T/I332E.[23] All Pin-mAbs possess the same Pin-modified Fc. All Pin-mAbs were produced by RD-Biotech (Besançon, France). Pin-mAbs were produced in CHO cells and purified by protein A. All batches contained less than <1 EU/mg of endotoxins. All Pin-mAbs Fab come from clinical mAbs: Pin-CD20 Fab sequence is from rituximab; Pin-HER2 Fab sequence is from trastuzumab; Pin-EGFR Fab sequence is from cetuximab; Pin-CD19 scFv sequence is from blinatumomab.

Cells

K562, Daudi, Toledo, Raji, MOLM-13, Namalwa, Ramos, Karpas, TDM8, SUDHL-10, SUDHL-6 and SUDHL-4 were purchased at ATCC or ECACC. Nalm6 were kindly gifted by Pascale Plence, IRMB Montpellier, France. All suspension cell lines were cultured in RPMI-1640 GlutaMAX supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO_2. HCT116, MDA-MB-468 and MCF7 were obtained from the "Site de Recherche Intégré sur le Cancer (SIRIC)" of Montpellier and grown in Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 10% FBS and incubated at 37°C with 5% CO2. *Mycoplasma* contamination was routinely verified every month using MycoAlert Kit (Lonza). The patients with lymphoma's cells were obtained from the platform CRB-CHUM in CHU of Montpellier (Biobank—BB-0033–00031).

Antigen quantification

CD20 and CD19 absolute numbers of molecules on cell lines and patient samples were quantified using BD Quantibrite PE Phycoerythrin Fluorescence Quantitation Kit (BD Biosciences) according to manufacturer's instructions. Briefly, 2.10^5 cells were stained with 10 µL of PE-conjugated anti-CD20 or anti-CD19 FACS antibodies. After 20 min incubation in the dark at 4°C, cells were washed, resuspended in phosphate-buffered saline (PBS) containing 2% FBS and acquired on a BD FACSCanto II (BD Biosciences) flow cytometer. Antigen absolute numbers were calculated according to the manufacturer's instructions.

eNK expansion

eNK expansion protocol was adapted from a previously described protocol.[17] Briefly, CD3 positive cells were removed from umbilical cord blood mononuclear cells (UCBMCs) using EasySep Human CD3 Positive Selection Kit II (STEMCELL), according to manufacturer's instructions. CD3 negative cells were cultured at 1×10^6 cells/mL in eNK medium (RPMI1640 GlutaMAX or NK MACS (Miltenyi) supplemented with 10% FBS or 5% human serum, 100 IU/mL rhIL-2 (PeproTech) and 5 ng/mL hrIL-15 (Miltenyi). From day 5–7 and every 2–3 days until day 14–21, the medium was removed and freshly prepared eNK medium was added to reach 0.6×10^6 cells/mL. 70 Gy-irradiated B-lymphoblastoid Epstein Barr virus (EBV)-transformed feeder cells at appropriate ratios were added.

At the end of the expansion, the eNK cells phenotype was analyzed by flow cytometry. 2.10^5 cells were stained in PBS containing 2% FBS using the following FACS antibodies (BioLegend): CD56-PE-Cy7 or BV421; CD45-BV510; CD57-FITC; CD16-PerCP-Cy5.5; Nkp30-PE-Cy7; NKG2A-AF488; NKG2C-PE; NKG2D-APC; CD2-BV421; Nkp46-FITC; Nkp44-PE; CD69-APC; CD62L-AF488; FasL-PE; TIGIT-PerCP-Cy5.5; TRAIL-APC; PD-1-BV421; CXCR1-FITC; CXCR2-PE; CXCR4-PerCP-Cy5.5; CXCR3-APC; CCR5-BV421; CX3CR1-BV510. Cells were incubated for 20 min at 4°C in the dark, followed by two washes using PBS containing 2% FBS. Cells were then resuspended in PBS containing 2% FBS and acquired on a BD FACSCanto II (BD Biosciences) flow cytometer.

eNK arming

eNK were counted using a Muse Cell Analyzer (CYTEK) and then incubated at 2.10^6 cells/mL in RPMI 1640 GlutaMAX (Gibco) with 10 µg/mL of Pin-CD20, Pin-CD19, Pin-HER2 or Pin-EGFR mAbs for 1 hour at 37°C and 5% CO_2. For double-arming, both Pin-CD20 and Pin-CD19 or Pin-HER2 and Pin-EGFR were added at 10 µg/mL simultaneously. In some cases, Pin-CD20 or Pin-HER2 and Pin-CD19 or Pin-EGFR were labeled with Alexa Fluor 647 and Alexa Fluor 488, respectively using Lightning-Link Antibody Conjugation Kits (Abcam, ab236553 and ab269823) following the manufacturer's instructions. Briefly, 100 µg of Pin-mAbs at 1 mg/mL were labeled by adding Modifier Reagent and transferred to the lyophilized fluorochrome. After 15 min incubation, Quencher was added and incubated for 15 more min. Additionally, eNK cells were armed with Pin-Fc-AF647 construct or with Pin-HER2 or Pin-EGFR mAbs. In all conditions, eNK were next washed twice with RPMI containing 10% FBS to remove the excess of soluble Pin-mAbs. Armed eNK were then ready to use for further experiments.

Arming detection

For kinetic experiments, Pin-mAbs eNK were incubated in RPMI 1640 GlutaMAX containing 100 UI/mL

rhIl-2 (PeproTech) and 5 ng/mL rhIL-15 (Miltenyi) for 24 hours, 48 hours or 72 hours. In some cases, eNK and Pin-CD20 eNK were incubated in the presence of 10 mg/mL of polyclonal human IgG (Privigen). For cell analysis, cells were stained for extracellular markers using anti-CD56-PE-Vio770 (Miltenyi), anti-CD16-PerCP-Cy5.5 (clone B73.1, BioLegend, 360712), anti-CD3-VioBlue (Miltenyi), eFluor780. For Pin-mAbs detection staining, either anti-idiotype anti-rituximab-AF488 or AF647 (RnD systems, FAB9630G and FAB9630R) or anti-human-Fab-AF647 (CliniScience, CSA3835) were used.

Cytotoxicity assay

After arming, eNK cells or Pin-mAb eNK were co-cultured in 96-well plates with CFSE or CellTrace Far Red-labeled target cells for 24 hours at the indicated E:T ratios. For kinetic studies, eNK and Pin-mAb eNK were added to the target cells culture at indicated days after arming procedure. After co-culture, cells were stained for viability and CD56 expression and resuspended in 200 µL of PBS containing 2% FBS. 20 µL of Precision Count Beads (BioLegend, 424902) were added in each sample and samples were analyzed on a BD FACSCanto II (BD Biosciences) flow cytometer. The absolute number of target cells was calculated using the formula provided by the supplier:

$$\text{Absolute cell count} = \frac{\textit{Cell count} \times \textit{Precision Count}}{\textit{Neads Volume}} \times \frac{\text{Precision Count}}{\text{Beads Concentration}}$$
$$\textit{Precision Count Beads Counts} \times \textit{Cell volume}$$

The percentage of cytotoxicity was calculated by comparing treated target cells to untreated target cells as a 100% viable cells control. E:T50, E:T20 and E:T80 were determined by fitting the data with non-linear regression curve model (log(agonist) vs response–variable slope (four parameters)) with GraphPad Prism Software V.10.0.1.

For the CD107a degranulation assay, anti-CD107a-APC antibody (BioLegend) and monensin (Abcam, ab193381) were directly added in the co-culture, in the presence or absence of Daudi cells at 3:1 E:T ratio. To measure soluble cytotoxic mediators, supernatants were collected 4 hours or 24 hours after co-culture and analyzed with LEGENDplex (BioLegend).

Concerning co-cultures of PBMCs from healthy donor with eNK or Pin-CD20 eNK, cells were incubated for 16 hours at an E:T ratio of 1:1.

In vivo binding

10 million AF647-labeled Pin-CD20 eNK cells were injected intraperitoneally into SCID mice. After 24 hours, mice were sacrificed and eNK cells were harvested through peritoneal lavage and stained with the following antibodies: anti-human CD45-AF488; anti-human CD16-PerCP-Cy5.5; anti-human CD56-PE-Vio770; viability dye eFluor780; anti-murine CD45-VioGreen and acquired on a BD FACSCanto II (BD Biosciences) flow cytometer.

In vivo cytotoxic experiments

NOD/LtSz-SCID/IL-2Rγchain null (NSG) mice were produced at the Institute of Molecular Genentic of Montpellier (IGMM) Animal Facility (Montpellier-France) using breeders coming from Charles River Laboratories. Mice were housed in sterile conditions using HEPA-filtered microisolators under a 12 hours light/dark cycle and given ad libitum irradiated regular chow diet (A04; SAFE) and sterile water.

10-week-old female NSG mice received intraperitoneal injection of eNK, Pin-CD20 eNK, Pin-CD19 eNK or double-armed eNK along with Nalm6 cells (pre-stained with 5 µM of CFSE) and Raji or Daudi cells (pre-stained with 0,5 µM CFSE) diluted in PBS. The different CFSE dilutions allow for the identification of each cell line by flow cytometry. Four hours later, mice were euthanized, and cells were harvested by peritoneal lavage using 3 mL of cold PBS containing 3% FBS. Red blood cells were removed using RBC Lysis Buffer (BioLegend) according to the manufacturer's instructions. Murine FcγR were blocked with murine Fc block (eBiosciences, clone93, 14-0161-85). Cells were then stained with anti-human CD56-PE-Vio770 (Miltenyi), anti-human CD45-VioGreen (Miltenyi), anti-murine CD45-BV421 (BioLegend, 103133) and eFluor 780 (eBioscience). Cells were acquired on a BD FACSCanto II (BD) flow cytometer using Precision Counting Beads (BioLegend, 424902). Gating strategy analysis is described in online supplemental figure S10. Absolute number of living target cells was calculated using Precision Counting Beads as previously explained in the "Cytotoxicity assay" methods.

In vivo humanized mice

All procedures and housing were performed at TransCure bioServices (Archamps, France) and have been reviewed and approved by their local ethics committee. eNK cells or Pin-CD20 eNK were intravenously injected into NCG humanized mice. At day 1 and day 7, blood sampling was performed and PBMC were analyzed by flow cytometry using the following antibodies: hCD3-BV421; hCD45-BV510; hCD8-BV605; mCD45-BV711; HLADR-BV785; anti-rituximab-AF488; hCD16-PerCP-Cy5.5; hCD14-PE; hCD20-PE-Dazzle594; CD56-PE-Cy7; CD19-APC; FVS780. The relative percentage of B cells and T cells was measured at day 7 compared with the number of cells present at day 1.

In vivo leukemia model

All procedures and housing were performed at C-RIS Pharma (Saint Malo, France) and have been reviewed and approved by their local ethics committee. Raji cells ($0{,}2.10^5$) were intravenously inoculated in CB17 SCID mice. At day 4 and 8, 30.10^6 eNK or Pin-CD20 eNK cells resuspended in PBS were injected by the same route. Injection with PBS was used as a control. At day 11, 14 and 17, rhIL-15 was administrated intravenously. At day 27, all mice were sacrificed and lung, bone marrow and blood were harvested. Lungs were dissociated in 5 mL Hank's

Balanced Salt Solution (HBSS) containing 2 mg/mL of dispase II using gentleMACS. Next, lungs were incubated for 20 min at 37°C on a shaker and then passed through a 40 µM cell strainer to obtain a single cell suspension.

Bone marrow was aspirated from femoral bones and centrifuged. Pellets were resuspended in RBC Lysis Buffer for 5 min and then washed with PBS.

Blood was collected in EDTA tubes, then mixed with RBC Lysis Buffer and incubated for 5 min before being washed.

All samples were stained with anti-CD20-PE and anti-CD20-HLA-A/B/C-PerCP-Cy5.5 antibodies for 1 hour at 4°C protected from light. Cells were then washed and resuspended in FACS buffer before analysis on a Guava easyCyte flow cytometer. The frequency of Raji cells in each organ was determined.

Statistics and analysis

All flow cytometry data were analyzed using FlowJo software. All statistical analyzes were performed using GraphPad Prism software V.10.0.1 and are described in each figure legend. To determine adequate statistical tests, samples populations normality was assessed with the d'Agostino-Pearson omnibus normality test. The two-sided Wilcoxon matched pair signed-rank test was used if the data were not normally distributed. n is the number of samples used in the experiments. The means are shown, with error bars indicating the SEM. Significance is indicated as follows: *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. P value≤0.05 was considered statistically significant. Four-parameter non-linear regression analysis was used to calculate the E:T50, E:T20, E:T80 and AC50.

Acknowledgements We acknowledge the imaging facility MRI, a member of the National Infrastructure France-BioImaging supported by the French National Research Agency (ANR-10-INBS-04, Investments for the future). The "Région Languedoc Roussillon" supports the clinical data and samples (HEMODIAG_2020). We acknowledge the "Site de Recherche integrated sur le Cancer" SIRIC of Montpellier.

Contributors Conceptualization: LC, BF, JP, MV. Methodology: LC, JL, JP. Investigation: LC, ER, HC, SZ, CM, MP, MT. Visualization: LC, JP. Formal analysis: LC, ER, HC, BF, JP. Funding acquisition: GC, BR, PM, MV. Project administration: JP, MV. Supervision: JP, MV. Writing—original draft: LC, JP. Writing—review and editing: LC, GB, GC, BR, PM, BF, JP, MV. MV, JP acted as guarantor.

Funding This work was supported by "Institut National Du Cancer" (INCA) PRT-K program 2021 (MV, PM and GC; 2021-014). A "prematuration" support of the « sociétés d'accélération du transfert de technologies » (SATT) AxLR (MV). The "Investissements d'avenir" Grant LabEx MAbImprove: ANR-10-LABX-53 (GC/MV/PM/BR). The 2021 AAP Companies On Campus by the Montpellier Université d'Excellence (MUSE; MV). LC is a recipient of a fellowship from MRT, France, and from "Fondation pour la Recherche Médicale" (FDT202304016765), France.

Competing interests The patent of PIN™ technology has been licensed to CYTEA BIO (WO2022023581A1 "Armed NK cells for universal cell therapy"). ER, HC, BF and JP are currently employees of CYTEA BIO. BR, PM and MV were initial creators of CYTEA BIO.

Patient consent for publication Not applicable.

Ethics approval Before initiating the experiments, we received ethical clearance from the French National Ethics Committee (Approval No. 21PLER2018-0069 for peripheral blood (PB); French national blood center "Etablissement Français du Sang"; and BB-0033-00031 for umbilical cord blood (UCB) CHU Montpellier). Samples from patients with B-cell Non-Hodgkin Lymphoma were provided by the CRB-CHUM platform from the CHU of Montpellier (HEMODIAG_2020 cohort, Biobank BB-0033-00031). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iDs
Loïs Coënon http://orcid.org/0000-0002-9533-3320
Hortense Courot http://orcid.org/0000-0001-8661-9557
Caroline Multrier http://orcid.org/0009-0003-4207-477X
Martine Pugnière http://orcid.org/0000-0002-2049-2909
Marion de Toledo http://orcid.org/0000-0002-7638-0041
Guillaume Bossis http://orcid.org/0000-0002-3349-8250
Guillaume Cartron http://orcid.org/0000-0003-0659-9635
Bruno Robert http://orcid.org/0000-0001-5573-6945
Pierre Martineau http://orcid.org/0000-0002-7993-7183
Bénédicte Fauvel http://orcid.org/000-0003-1166-8391
Jessy Presumey http://orcid.org/0009-0001-1770-7112
Martin Villalba http://orcid.org/0000-0002-4385-4888

REFERENCES

1 Olson JA, Leveson-Gower DB, Gill S, *et al*. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* 2010;115:4293–301.
2 Bachanova V, Burns LJ, McKenna DH, *et al*. Allogeneic natural killer cells for refractory lymphoma. *Cancer Immunol Immunother* 2010;59:1739–44.
3 Koehl U, Kalberer C, Spanholtz J, *et al*. Advances in clinical NK cell studies: donor selection, manufacturing and quality control. *Oncoimmunology* 2016;5:e1115178.
4 Villalba M, Alexia C, Bellin-Robert A, *et al*. Non-genetically improving the natural cytotoxicity of natural killer (NK) cells. *Front Immunol* 2019;10:3026.
5 Allison M, Mathews J, Gilliland T, *et al*. Natural killer cell-mediated immunotherapy for leukemia. *Cancers (Basel)* 2022;14:843.
6 Cózar B, Greppi M, Carpentier S, *et al*. Tumor-infiltrating natural killer cells. *Cancer Discov* 2021;11:34–44.
7 Cartron G, Dacheux L, Salles G, *et al*. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG FC receptor FcgammaRIIIa gene. *Blood* 2002;99:754–8.
8 Paul F, Cartron G. Infusion-related reactions to Rituximab: frequency, mechanisms and predictors. *Expert Rev Clin Immunol* 2019;15:383–9.
9 Vo DN, Constantinides M, Allende-Vega N, *et al*. Dissecting the NK cell population in hematological cancers confirms the presence of tumor cells and their impact on NK population function. *Vaccines (Basel)* 2020;8:727.
10 Coënon L, Villalba M. From CD16A biology to antibody-dependent cell-mediated cytotoxicity improvement. *Front Immunol* 2022;13:913215.
11 Lim CM, Liou A, Poon M, *et al*. Phase I study of expanded natural killer cells in combination with Cetuximab for recurrent/metastatic Nasopharyngeal carcinoma. *Cancer Immunol Immunother* 2022;71:2277–86.
12 Ishikawa T, Okayama T, Sakamoto N, *et al*. Phase I clinical trial of adoptive transfer of expanded natural killer cells in combination with

IgG 1 antibody in patients with gastric or colorectal cancer. *Int J Cancer* 2018;142:2599–609.
13 Lee S-C, Shimasaki N, Lim JSJ, *et al*. Phase I trial of expanded, activated Autologous NK-cell infusions with Trastuzumab in patients with Her2-positive cancers. *Clin Cancer Res* 2020;26:4494–502.
14 Liu E, Marin D, Banerjee P, *et al*. Use of CAR-transduced natural killer cells in CD19-positive Lymphoid tumors. *N Engl J Med* 2020;382:545–53.
15 Marin D, Li Y, Basar R, *et al*. Safety, efficacy and determinants of response of allogeneic CD19-specific CAR-NK cells in CD19+ B cell tumors: a phase 1/2 trial. *Nat Med* 2024;30:772–84.
16 Mitwasi N, Feldmann A, Arndt C, *et al*. 'Unicar'-Modified off-the-shelf NK-92 cells for targeting of GD2-expressing tumour cells. *Sci Rep* 2020;10:2141.
17 Sanchez-Martinez D, Allende-Vega N, Orecchioni S, *et al*. Expansion of allogeneic NK cells with efficient antibody-dependent cell cytotoxicity against multiple tumor cells. *Theranostics* 2018;8:3856–69.
18 Reina-Ortiz C, Constantinides M, Fayd-Herbe-de-Maudave A, *et al*. Expanded NK cells from umbilical cord blood and adult peripheral blood combined with Daratumumab are effective against tumor cells from multiple myeloma patients. *Oncoimmunology* 2020;10:1853314.
19 Calvo T, Reina-Ortiz C, Giraldos D, *et al*. Expanded and activated allogeneic NK cells are cytotoxic against B-chronic lymphocytic leukemia (B-CLL) cells with sporadic cases of resistance. *Sci Rep* 2020;10:19398.
20 Allende-Vega N, Marco Brualla J, Falvo P, *et al*. Metformin sensitizes Leukemic cells to cytotoxic lymphocytes by increasing expression of Intercellular adhesion Molecule-1 (ICAM-1). *Sci Rep* 2022;12:1341.
21 Belkahla S, Brualla JM, Fayd'herbe de Maudave A, *et al*. The metabolism of cells regulates their sensitivity to NK cells depending on P53 status. *Sci Rep* 2022;12:3234.
22 Lazar GA, Dang W, Karki S, *et al*. Engineered antibody FC variants with enhanced effector function. *Proc Natl Acad Sci U S A* 2006;103:4005–10.
23 Moore GL, Chen H, Karki S, *et al*. Engineered FC variant antibodies with enhanced ability to recruit complement and mediate effector functions. *MAbs* 2010;2:181–9.
24 Dall'Ozzo S, Tartas S, Paintaud G, *et al*. Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. *Cancer Res* 2004;64:4664–9.
25 Tout M, Gagez A-L, Leprêtre S, *et al*. Influence of FCGR3A-158V/F genotype and baseline CD20 antigen count on target-mediated elimination of Rituximab in patients with chronic lymphocytic leukemia: a study of FILO group. *Clin Pharmacokinet* 2017;56:635–47.
26 Weng WK, Levy R. Two immunoglobulin G fragment C receptor Polymorphisms independently predict response to Rituximab in patients with follicular lymphoma. *JCO* 2003;21:3940–7.
27 Zhang W, Gordon M, Schultheis AM, *et al*. FCGR2A and FCGR3A Polymorphisms associated with clinical outcome of Epidermal growth factor receptor–expressing metastatic colorectal cancer patients treated with single-agent Cetuximab. *JCO* 2007;25:3712–8.
28 Musolino A, Naldi N, Bortesi B, *et al*. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/ neu –positive metastatic breast cancer. *JCO* 2008;26:1789–96.
29 Hiraga J, Tomita A, Sugimoto T, *et al*. Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with Rituximab-containing combination Chemotherapies: its prevalence and clinical significance. *Blood* 2009;113:4885–93.
30 Bargou R, Leo E, Zugmaier G, *et al*. Tumor regression in cancer patients by very low doses of a T cell–engaging antibody. *Science* 2008;321:974–7.
31 Gauthier L, Virone-Oddos A, Beninga J, *et al*. Control of acute myeloid leukemia by a Trifunctional NKp46-CD16A-NK cell engager targeting CD123. *Nat Biotechnol* 2023;41:1296–306.
32 Gauthier L, Morel A, Anceriz N, *et al*. Multifunctional natural killer cell engagers targeting NKp46 trigger protective tumor immunity. *Cell* 2019;177:1701–13.
33 Chockley PJ, Ibanez-Vega J, Krenciute G, *et al*. Synapse-tuned cars enhance immune cell anti-tumor activity. *Nat Biotechnol* 2023;41:1434–45.
34 Cichocki F, Bjordahl R, Goodridge JP, *et al*. Quadruple gene-engineered natural killer cells enable multi-antigen targeting for durable antitumor activity against multiple myeloma. *Nat Commun* 2022;13:7341.
35 Page A, Chuvin N, Valladeau-Guilemond J, *et al*. Development of NK cell-based cancer immunotherapies through receptor engineering. *Cell Mol Immunol* 2024;21:315–31.
36 Reusch U, Ellwanger K, Fucek I, *et al*. Cryopreservation of natural killer cells pre-complexed with innate cell engagers. *Antibodies (Basel)* 2022;11:12.
37 Wang X, Luo X, Tian Y, *et al*. Equipping natural killer cells with Cetuximab through metabolic Glycoengineering and Bioorthogonal reaction for targeted treatment of KRAS mutant colorectal cancer. *ACS Chem Biol* 2021;16:724–30.
38 Frank MJ, Olsson N, Huang A, *et al*. A novel antibody-cell conjugation method to enhance and characterize cytokine-induced killer cells. *Cytotherapy* 2020;22:135–43.
39 Li H-K, Hsiao C-W, Yang S-H, *et al*. A novel off-the-shelf Trastuzumab-armed NK cell therapy (Ace1702) using antibody-cell-conjugation technology. *Cancers (Basel)* 2021;13:2724.
40 Yamaguchi Y, Wakaizumi N, Irisa M, *et al*. The Fab portion of immunoglobulin G has sites in the CL domain that interact with FC gamma receptor Iiia. *MAbs* 2022;14:2038531.
41 Jan M, Scarfò I, Larson RC, *et al*. Reversible ON- and OFF-switch Chimeric antigen receptors controlled by Lenalidomide. *Sci Transl Med* 2021;13:eabb6295.
42 Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* 2021;11:69.
43 Strauss-Albee DM, Blish CA. Human NK cell diversity in viral infection: ramifications of ramification. *Front Immunol* 2016;7:66.
44 Shevtsov M, Multhoff G. Immunological and translational aspects of NK cell-based antitumor immunotherapies. *Front Immunol* 2016;7:492.
45 Hegde M, Mukherjee M, Grada Z, *et al*. Tandem CAR T cells targeting HER2 and IL13Rα2 mitigate tumor antigen escape. *J Clin Invest* 2016;126:3036–52.
46 Hegde M, Corder A, Chow KKH, *et al*. Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma. *Mol Ther* 2013;21:2087–101.
47 Zanetti SR, Velasco-Hernandez T, Gutierrez-Agüera F, *et al*. A novel and efficient Tandem CD19- and CD22-directed CAR for B cell ALL. *Mol Ther* 2022;30:550–63.