Supplemental Methods

Cell lines

HEK293T (human embryonic kidney) , Raji (Burkitt's lymphoma) and MV-4-11 (myelomonocytic leukemia) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher Scientific Waltham, MA; 293T), Roswell Park Memorial Institute (RPMI; ThermoFisher Scientific; Raji) or Iscove's Modified Dulbecco's Medium (IMDM; ThermoFisher Scientific; MV-4-11) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). MOLM-13 cell line was purchased from the Leibniz Institute (DSMZ, German Collection of Microoganisms and Cell Cultures) and cultured in RPMI supplemented with 10% FBS. CD123 expressing Raji cells (Raji.CD123) were created by first subcloning the full length human CD123 coding sequence into a pCDH lentiviral backbone. Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped lentiviral particles were produced using the pPACKH1 HIV Lentivector Packaging Kit (System Biosciences, Palo Alto, CA) according to the manufacturer's instructions and used for Raji cell modification. CD123-positive cells were isolated using fluorescence-activated cell sorting (FACS) and antigen surface expression verified prior to use. All cells used for BLI-based cytotoxicity assays and/or our xenograft models were transduced with a retroviral vector carrying an enhanced green fluorescent protein (GFP) firefly luciferase fusion gene (GFP.ffLuc).[1] GFP-positive cells were sorted and maintained in the appropriate culture medium. Luciferase expression was confirmed using D-luciferin and quantification of bioluminescence. All cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C.

Determination of Vector Copy Number (VCN)

Primer/probe-FAM was designed to the MMLV-derived psi present in pSFG and purchased from ThermoFisher Scientific. RNAseP primer/probe-VIC/TAMRA mix (Applied Biosystems #4403326) was used as comparison. Genomic DNA was isolated from CAR-NK cells and 25 ng used for amplification with TaqMan Universal PCR Mastermix (ThermoFisher) and the above primer/probe mixes on a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). The following amplification conditions were used: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. No-template, unmodified NK cells and a condition containing only plasmid were used as controls. Vector copy number calculation was performed using the $2^{-\Delta Ct}$ method.[2]

Cytotoxicity assay

Bioluminescence (BL) based: NK cells were co-cultured with target cells expressing ffLuc at the indicated E:T ratios. D-luciferin was added to plate and BL measured per well. Mean percentage of specific lysis of triplicate samples was calculated as 100*(spontaneous death–experimental death)/(spontaneous death-background). Spontaneous death was measured with control wells containing only target cells. Flow cytometric: NK cells were cultured with target cells. NK and target cell numbers were measured using flow cytometric analysis and NK or target-cell specific markers as above, with dead cell exclusion.

Cytokine secretion assay

100,000 NK cells were plated with an equivalent number of target cells in 0.2 mL media and cultured for 24 hours. Supernatant was collected and IFNγ quantification performed via ELISA (R&D Systems, Minneapolis, MT). For measurement of IL15 secretion, 1 million NK cells were

plated in 2 mL media. After 24 hours, supernatant was collected and cytokine quantification was performed with ELISA (R&D Systems) according to the manufacturer's instruction.

Library preparation and RNA sequencing

For RNA sequencing experiments, RNA samples were converted to double stranded cDNA using the Ovation RNA-Seq System v2.0 kit (Tecan, Männedorf, Switzerland), which utilizes a proprietary strand displacement technology for linear amplification of mRNA without rRNA/tRNA depletion as per the manufacturer's recommendations. This approach does not retain strand specific information. Quality and quantity of the resulting cDNA was monitored using the Bioanalyzer High Sensitivity kit (Agilent) which yielded a characteristic smear of cDNA molecules ranging in size from 500 to 2000 nucleotides in length. After shearing 500 nanograms of cDNA to an average size of 250 nucleotides with the Covaris S4 (Covaris Inc., Woburn, MA) library construction was completed with the Truseq Nano kit (Illumina; San Diego, CA) according to the manufacturer's instructions. mRNA libraries were sequenced on an Illumina Novaseq 6000 instrument using 150bp paired-end dual indexed reads and 1% of PhiX control. Reads were aligned to GRCh38 using rsem version 1.3.0 with the following options – star-calc-ci-star-output-genome-bam-forward-prob 0.5.

1. Vera, J., *et al.* T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. *Blood* **108**, 3890-3897 (2006).
2. Kunz, A., *et al.* Optimized Assessment of qPCR-Based Vector Copy Numbers as a Safety Parameter for GMP-Grade CAR T Cells and Monitoring of Frequency in Patients. *Mol Ther Methods Clin Dev* **17**, 448-454 (2020).