

T-cell receptor engineering of primary NK-cells to therapeutically target tumours and tumour immune evasion

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Abstract

TCR-engineered cells can be powerful tools in the treatment of malignancies. However tumor-resistance by HLA-class I downregulation can negatively-impact the success of any TCR-mediated cell therapy. Allogeneic NK-cells have demonstrated efficacy and safety against malignancies without inducing GvHD highlighting the feasibility for an “off the shelf” cellular therapeutic. Furthermore, primary NK-cells, sourced from the peripheral blood or umbilical-cord blood, can target tumours using a broad array of intrinsic activation mechanisms. Here, we developed a unique NK:TCR cell therapy from peripheral blood derived NK-cells, which enhances NK-efficacy against tumours through additional TCR-mediated lysis. Upon loss of HLA-class I, NK:TCR will lyse the resistant tumour cells in an NK-mediated manner. Our NK:TCR technology is the first cellular therapy which incorporates TCR-based targeting of tumours and the associated TCR immune-evasion.

Introduction

In unmodified T-cells, the T-cell receptor (TCR) confers specificity and engages antigen which has been processed and presented as peptide by HLA molecules. The effectiveness of TCR-mediated adoptive cell therapy (ACT) has been demonstrated by donor-lymphocyte infusion (DLI)¹⁻³, infusions of tumour infiltrating lymphocytes (TILs)⁴ or virus-specific T-cells⁵ and TCR-engineered T-cells⁶⁻⁸. With the careful selection of tumour-specific targets, TCR-engineered cells have the potential to be extremely effective against malignancies⁹⁻¹². Despite this, overcoming tumour immune-evasion strategies remains a challenge for all T-cell based immune therapies. One such strategy is HLA-class I loss, observed in patients after TIL therapy whereby unresponsive patients had dysfunctional β -2-microglobulin (B2M), required for stable HLA-class I expression¹³. Furthermore, analysis of relapsed patients following immune checkpoint blockade(ICB) revealed acquired defects in antigen-presentation, including B2M mutations, are contributing factors to immune resistance^{14,15}. Increasing TCR-mediated immune pressure on tumours, through ICB and T-cell therapies, can therefore promote HLA-class I loss as a tumour immune-evasion mechanism through immunoediting¹⁶.

Recently, NK-cells have gained interest in ACT and thanks to advances in *ex-vivo* NK expansion protocols sufficient numbers can be achieved for infusion^{17,18}. Once activated, NK-cells share similar effector functions with T-cells, including production of cytotoxic granules and inflammatory cytokines, however activation is independent of antigen. Instead, activation relies on a balance between activating and inhibitory signals from germ-line encoded receptors¹⁹. To prevent non-specific activation, NK-cells express killer-cell immunoglobulin-like receptors (KIRs) as well as the NKG2A/CD94 heterodimer which provide inhibitory signals upon engagement with HLA-class I molecules on healthy cells. This control mechanism allows NK-cells to become activated by malignant cells lacking HLA-class I expression due to absence of inhibitory signals²⁰. This anti-tumour effector mechanism has been exploited in NK-cell based ACT through KIR-mismatching. KIR-mismatched NK-cells have been beneficial therapeutically and has correlated with survival advantages in AML patients receiving T-cell depleted allogeneic stem cell

transplant (alloSCT) ²¹. Multiple studies have since demonstrated the efficacy of allogeneic NK-ACT as a standalone therapy for the treatment of haematological and solid malignancies ²²⁻²⁷. Unlike T-cells, allogeneic NK-cells do not cause graft-versus-host-disease (GvHD) highlighting the potential for an “off the shelf” cell product allowing broader patient applicability. Enhancement of NK effector function is now a focus and genetically engineering NK-cells to improve efficacy, persistence and homing is part of ongoing research ²⁸⁻³¹.

We hypothesized that the combination of intrinsic, anti-tumour effector functions of NK-cells with TCR-engineering (NK-TCR) would create a novel therapeutic strategy to avoid TCR-associated immune resistance. Recently, TCR expression in an FDA-approved NK-cell line, NK-92, demonstrated TCR-mediated efficacy can be achieved in non T-cells ^{32,33}. Despite the advantages of a cell line as an “off the shelf” cell product, NK-92 must be irradiated prior to infusion to prevent tumorigenesis which affects the *in vivo* efficacy ³⁴. Furthermore, NK-92 cells lack expression of KIRS and have low expression of NKG2A which, without further modification, would limit efficacy against malignant cells with HLA-class I loss.³⁵ Described in this study is a two-step retroviral (RV) transduction protocol to allow functional TCR expression in primary NK-cells derived from the peripheral blood of healthy donors. As a model of efficacy, we focus on the expression of a promising TCR targeting BOB1, a transcription factor highly expressed in all healthy and malignant B-cell lineages, including multiple myeloma ¹¹. We aimed to demonstrate NK-cells expressing the BOB1-specific TCR enhances NK effector functions through additional antigen-specific activation whilst retaining NK-mediated effector functions which can be engaged upon HLA-class I loss as an immune-evasion strategy.

Materials And Methods

Genetic modification of NK-cells.

NK-stimulator cell line K562-mbIL21-41BBL clone 41 was generated by transducing K562 with genes encoding CD137L and membrane-bound IL21 as described in ³⁶ and single cell sorted by FACS. NK-cells were isolated from frozen PBMC using NK-cell isolation Kit (Miltenyi Biotec, Germany). In a 24 well, 1×10^6 isolated NK-cells were immediately co-cultured with 0.5×10^6 irradiated (100GY) K562-mbIL21-41bbL clone 41 in NK medium (NK-M). NK-M consisted of IMDM (Lonza, Switzerland) supplemented with 5% heat-inactivated FBS (Gibco, Thermo Fisher Scientific, U.S), 5% human serum (Sanquin, The Netherlands), 1.5% 200 mM l-glutamine (Lonza, Switzerland), 1% 10,000 U/ml penicillin/streptomycin (Lonza, Switzerland), 5 ng/ml IL15 (Miltenyi Biotec, Germany) and 100 IU/ml IL-2 (Novartis, Switzerland). NK-cells were then stimulated weekly with irradiated (100GY) K562-mbIL21-41bbL at a 2:1 effector:stimulator (E:S) ratio and kept between $0.5-1 \times 10^6$ cells/ml in culture and medium was refreshed every 2–3 days. Expansion of NK-cells was calculated by live cell counts at regular timepoints following stimulation. Retroviral transduction of NK-cells occurred on day 2/3 post stimulation. Transduced NK-cells were MACS™ (Miltenyi Biotec, Germany) enriched on Day 6/7 post stimulation and immediately re-stimulated as described above. NK-cell were used in effector assays between Day 7–14 post stimulation.

Transgenic CD8 T-cells

CD8 T-cells (CD8T) were isolated from frozen PBMCs using CD8-microbeads (Miltenyi Biotec, Germany) and subsequently stimulated with TransAct™ (Miltenyi Biotec, Germany) at 10 µl/1x10⁶ cells in NK-M without IL15. Where specified, CD8T were CRISPR/Cas9 edited on day 2 with *TRAC* and *TRBC* targeting RNP as described previously³⁷. On Day 3, CD8 T-cells were retrovirally transduced with murinised tgTCR and MACS™ (Miltenyi Biotec, Germany) and enriched on Day 7 for mTCRβ expression. Enriched CD8T were cultured and used in effector assays between Day 10–12 post stimulation.

CRISPR/Cas9 genome editing.

Ribonucleoprotein (RNP) were generated by complexing crRNA:trRNA (Integrated DNA technologies (IDT) Coralville, Iowa) with *Streptococcus pyogenes* (sp)Cas9 (Integrated DNA technologies (IDT) Coralville, Iowa) as described previously³⁷. B2M-RNP were electroporated into cell lines using the NEON transfection system (Thermo Fisher Scientific, Waltham, Massachusetts) using transfection settings: UM9 (1600v 10 ms 3x pulses), BV-ALL (1550v 10 ms 3x pulses) and EBV-LCL (1500v 20 ms 2x pulses). B2M-KO cells were FACS sorted according to loss of HLA-class I.

B2M crRNA sequence³⁸ :5'-GGCCACGGAGCGAGACAUCUGUUUUAGAGCUAUGCU-3'.

In vitro Effector assays

Cytotoxicity :1250 Target cells were labelled with 100 µCi Na₂⁵¹CrO₄ for 1 hour at 37 °C, and co-cultured with NK or T-cells at multiple effector-to-target (E:T) ratios in 100 µl IMDM supplemented with 10% FBS. After 6 hours incubation at 37 °C, 25 µl supernatants were harvested). ⁵¹Cr release was measured on a 2450 Microbeta² plate counter (PerkinElmer, Waltham, Massachusetts). % cytotoxicity was calculated as follows: (Test sample ⁵¹Cr release minus the spontaneous ⁵¹Cr release)/ (maximum ⁵¹Cr release minus the spontaneous ⁵¹Cr release) x100. TCR-dependent killing was calculated as the difference in killing between NK/CD8T:BOB1 and NK:TCRneg/CD8T:MOCK at the highest E:T ratio. **Degranulation and cytokine production**: Effector cells were stimulated with PMA (5 ng/ml) and Ionomycin (400 ng/ml) or co-cultured with stimulator cells at an effector-to-stimulator (E:S) ratio of 1:4 in 100 µl NK-M(IL15) in the presence of anti-CD107a. After 1 hour incubation at 37°C, Brefeldin-A (5 µg/ml) was added and subsequently incubated for 12-14hours. Cells were then stained with zombie-aqua (Biolegend) followed by cell surface antibodies (anti-CD56(NK)/anti-CD8(CD8)) and subsequently fixed for 12 min in 1% paraformaldehyde and permeabilised with 0.1% saponin for 20 minutes at 4°C. Intracellular antibody staining was then performed in 0.1% saponin for 20 min at 4°C and analysed by FACS.

In vivo anti-myeloma efficacy

On Day 0, non-irradiated female NOD.Cg-*Prkdc*(scid)*Il2rg*(tm1Wjl)/SzJ (NOD *scid* gamma, NSG) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were infused with Luciferase positive UM9 multiple myeloma cells alone or simultaneously with NK cells in 200 µl of PBS supplemented with 0.5ug human

GMP-grade IL15 (Miltenyi Biotec Bergisch Gladbach, Germany). Tumour cells and NK-cells were prepared separately and mixed directly before infusion. 0.5ug human GMP-grade IL15 (Miltenyi Biotec, Bergisch Gladbach, Germany) was then infused via i.p. injection (200 µl) every 2–3 days for 3 weeks. To monitor tumor growth, mice were anesthetized with 3–4% isoflurane after they received an i.p. injection of 200µL 7.5 mM D-luciferine (Cayman Chemical company, Ann Harbor, MI, USA). Whole body bioluminescence images were obtained using a CCD camera (IVIS spectrum, PerkinElmer, Waltham, Massachusetts).

Flow cytometry.

FACS analysis was performed on either the LSRII (BD Biosciences, New Jersey, U.S) or Fortessa X20 (BD Biosciences, New Jersey, U.S) and data was analysed using flowjo V10 software (TreeStar, Oregon, U.S). For each stain, 0.05×10^6 NK-cells were aliquoted into 96-well v-bottom plates, washed and incubated with 10 µl antibody-mixes for 20 min at 4 °C. For tetramer binding experiments, NK-cells were first incubated with 10 µl PE-labelled pMHC-tetramers for 20 min at 4 °C, followed by antibody staining.

Statistical analysis

Statistical analysis was performed using GraphPad Prism7 software. When comparing 2 groups, statistical significance was calculated using unpaired T-test. *In vivo* efficacy was calculated using one-way ANOVA with Tukey's multiple comparison test on log transformed data. Kaplan-Meier curve was used to depict survival with Gehan-Breslow-Wilcoxon test.

Results

Generation of TCR expressing NK-cells following a two-step retroviral production protocol.

Here, we genetically engineered NK-cells using retrovirus (RV) to generate a final cell product containing TCR expressing NK-cells (NK-TCR) (Fig. 1A). NK-cells were sourced from the peripheral blood of healthy donors and stimulated weekly using a modified NK-sensitive K562 cell line, expressing a membrane-bound form of IL21 and co-stimulatory receptor 41BBL (K562-mbIL21-41BBL) (Fig. 1A) ¹⁷. Functional TCR expression is challenging in NK cells because all missing components must be introduced alongside the TCRαβ chains, including CD8αβ co-receptor and the CD3ζγεδ signalling chains. To reduce the number of transductions, we first introduced TCRαβ and CD8αβ in one RV-construct and transduction efficiency was measured by cell surface expression of CD8β, as without CD3 the TCR cannot reach the cell surface (Fig. 1B and Supplemental Fig. 1A). Introducing the BOB1-specific TCR (NK:BOB1) resulted in efficient and reproducible CD8β expression frequencies (BOB1-TCR $34 \pm 15.7\%$, Mean \pm SD) which could be enriched for CD8β expression (Supplemental Fig. 1A). CD8β^{pos} NK cells were subsequently transduced with the 4 invariant chains of the CD3 signalling complex in a separate RV-construct to permit cell surface expression of the introduced TCRαβ (Fig. 1C and supplemental Fig. 1B). In these experiments, each transgenic TCR (tgTCR) was murinised (mTCR) to allow distinction between any contaminating T-cells present in the culture which may influence functional data. Transduction efficiencies were consistent resulting in expression of mTCRβ (BOB1-TCR $34 \pm 13\%$)(supplemental Fig. 1B), which were enriched

before a final stimulation and further expansion. To test reproducibility, NK-BOB1 cell products were generated from 4 donors and repeated twice for each donor. High expression of CD8 β , mTCR β and CD3 ϵ was repeatedly observed in NK-TCR cell products and NK-TCR specificity was confirmed using peptide-MHC tetramers (Fig. 1D-G). All NK:BOB1 remained negative for human TCR $\alpha\beta$ indicating any residual T-cells present at isolation did not expand *in vitro* (Fig. 1E and G). Following this stepwise method, pure NK-TCR was generated from multiple healthy donors, within 21 days and total fold expansion of 4385 ± 2026 SEM was observed (supplemental Fig. 2). Furthermore, this protocol could be easily adapted to express different clinically relevant TCRs such as CMV-specific or PRAME-specific TCRs (Supplemental figure S3 and S4A-E)

NK-TCR express a diverse array of receptors required for NK-cell activation.

The phenotype of final NK-TCR cell products revealed high frequency expression of activation receptors CD2, DNAM1, CD16, NKG2D (Supplemental figure S5). Natural cytotoxicity receptors (NCRs) NKp30 and NKp46 were also highly expressed whereas NKp44 was expressed on only $29 \pm 3.8\%$ (Mean \pm SD) of NK:BOB1 (Supplemental figure S5). Additionally, high frequency expression of inhibitory receptor NKG2A was repeatedly observed in NK:BOB1 ($93.5 \pm 3.3\%$ Mean \pm SD) and the activating receptor NKG2C was found on a small fraction of cells ($12.8 \pm 4.8\%$ Mean \pm SD). KIR expression was similarly expressed on a small percentage of cells indicating the expansion protocol did not select for a particular KIR expressing population (Supplemental figure S5). As a negative control, TCR negative NK-cells (NK:TCRneg) were expanded in parallel to NK:BOB1 from the same healthy donors. The phenotype of NK:TCRneg did not significantly differ except for an increased frequency of NKp44 expressing cells ($54.3 \pm 2.2\%$ Mean \pm SD) suggesting they were more activated. This expression profile suggested NK-TCR can be activated via many pathways which would permit anti-tumour effector functions.

NK-TCR elicit potent HLA-dependent, antigen-specific cytotoxicity against tumour targets

Functional capabilities of our generated NK-TCR cell products were investigated on day 21–24 without further stimulation. We validated the NK-mediated cytotoxic potential of NK:BOB1 and NK:TCRneg against HLA-class I negative K562 cell line which was always equally potent and therefore no advantage was observed when BOB1-TCR was expressed (Fig. 2). To explore antigen-specific killing we designed a panel of EBV-LCLs, 2 endogenously expressing the BOB1 T-cell epitope in HLA-B*07:02 and 2 negative for HLA-B*07:02. NK:TCRneg demonstrated low levels of killing against all 4 EBV-LCLs representing the background NK-mediated cytotoxicity (Fig. 2A). In contrast, NK:BOB1 demonstrated increased killing of HLA-B*07:02 + but not HLA-B*07:02- EBV-LCLs indicative of TCR-mediated lysis (Fig. 2A). We confirmed antigen-dependence using HLA-B*07:02 + fibroblasts which were negative for BOB1 antigen and did not induce TCR-mediated cytotoxicity (Fig. 2B). The expression of TCR on NK:BOB1 also increased cytotoxicity against HLA-B*07:02 + cell lines representing B-cell acute lymphoblastic leukemia (B-ALL) and multiple myeloma (MM) which were previously insensitive to NK-mediated cytotoxicity (Fig. 2C). To expand on this further NK:BOB1 was investigated for its ability to lyse HLA-B*07:02 + leukapheresis samples from patients with B-cell chronic lymphocytic leukaemia (B-CLL) or B-ALL. All samples

contained > 70% malignant cells and variable levels of NK-mediated cytotoxicity was demonstrated by NK:TCRneg against each of the malignancies (Fig. 2D and E). As before, increased cytotoxicity was demonstrated by NK:BOB1 and the benefit of NK-TCR was more pronounced when the primary malignancy did not induce an NK-mediated response (Fig. 2D and E). This TCR-mediated cytotoxicity was consistently demonstrated by NK-BOB1 cell products from multiple donors (Fig. 2F). Furthermore, antigen-specific killing was not restricted to the BOB1-TCR, and NK-TCR expressing PRAME-specific TCR (NK:PRAME) or CMV-specific TCR (NK:CMV) were also able to specifically lyse antigen-expressing target cells (Supplemental figures S4F and S6).

NK-TCR enabled improved efficacy in a pre-clinical *in vivo* model of multiple myeloma.

Next, we investigated if TCR-mediated activation of NK:BOB1 would improve efficacy of NK-cell therapeutics *in vivo*. NSG mice were injected with HLA-B*07:02 + multiple myeloma cell line UM9, previously shown to have low NK-mediated activity (Fig. 2). NK:BOB1 and NK:TCRneg were generated from a KIR-matched healthy donor and co-injected with UM9 in the presence of IL15. Efficacy was measured by tumour outgrowth and NK:BOB1 demonstrated delayed tumour outgrowth and increased overall survival compared to NK:TCRneg and untreated mice (Fig. 3). These data validated the *in vitro* results and demonstrated TCR expression in NK-TCR permits an additional activation pathway that ultimately improves NK-cell therapy efficacy *in vivo*.

The cytotoxicity of NK-TCR is comparable to CD8 T-cells expressing the same TCR.

To further understand the potency of NK-TCR we compared cytotoxicity relative to CD8 T-cells (CD8T) expressing the same TCR. To remove the influence of the endogenous TCR on tgTCR expression, the endogenous TCR $\alpha\beta$ chains of CD8T were deleted using CRISPR/Cas9, as described previously³⁷. Despite the presence of a residual human TCR $\alpha\beta$ + cell population co-expressing mTCR β in CD8T:BOB1 (Fig. 4A), the overall frequency and expression of mTCR β + cells was comparable between NK:BOB1 and CD8T:BOB1 (Fig. 4B). This was not observed in comparison to CD8T without endogenous TCR $\alpha\beta$ knock-out (Supplemental figure S7A and B). Upon investigation of antigen-specific cytotoxicity, NK:BOB1 consistently demonstrated a ratio-dependent, higher overall cytotoxicity against HLA-B*07:02 + B-cell targets endogenously expressing BOB1 than did CD8T:BOB1 (Fig. 4C). However, TCR-dependent killing of NK:BOB1 and T:BOB1 was calculated to be comparable and suggested the enhanced NK:BOB1 killing was the accumulative effect of both TCR and NK mediated activation (Fig. 4E). This effect was also demonstrated when compared to CD8T without endogenous TCR knock-out (Supplemental figure S7C-E). Interestingly, peptide-titration revealed CD8T:BOB1 were cytolytic towards targets cells presenting low concentrations of exogenously loaded antigen, whereas NK:BOB1 was more effective at higher concentrations (Fig. 4D). This was more apparent for NK:CMV as the antigenic-peptide is more readily exogenously loaded onto target cells (supplemental figure S8). Unique dual-activation of NK-TCR may therefore offer an advantage over other TCR-engineered cell therapeutics by enhancing cytotoxic potency when antigen is highly expressed.

Multifunctional, antigen-specific effector functions are elicited by NK-TCR.

To understand the functional capabilities of NK:BOB1 further, we analysed degranulation and cytokine production of NK:BOB1 and CD8T:BOB1 cell products. All cell products were capable of degranulation, as measured by CD107a, and production of TNF α and IFN- γ inflammatory cytokines except for one CD8T donor that produced low levels of cytokine (Supplemental figure S9). NK-mediated responses were demonstrated by NK:BOB1 and NK:TCRneg which, unlike CD8T, degranulated and produced TNF α and IFN- γ in response to K562 (Fig. 5). Antigen-specific degranulation and cytokine production was also demonstrated by NK:BOB1 and CD8T:BOB1 following stimulation with HLA-B*07:02 + B-cell targets (Fig. 5). Furthermore, these findings were not limited to the BOB1-TCR and NK:CMV and CD8T:CMV also showed antigen-specific responses (supplemental figure S10). Interestingly, CD8T:BOB1 demonstrated significantly increased degranulation against multiple myeloma cell line UM9 (Fig. 5A). This trend was also observed for cytokine production, and suggests the effector functions of NK-TCR may be influenced by the target cell itself (Fig. 5B and C). Nonetheless, these data demonstrate NK-TCR can evoke multifunctional, antigen-specific effector functions.

NK-mediated effector functions of NK-TCR can be engaged upon HLA-class I loss on tumour targets.

Finally, we modelled HLA-class I loss in tumours by generating HLA-B*07:02 + EBV-LCLs, B-ALL and MM cell lines with β -2-microglobulin knock-out (B2M KO). B2M KO prevented cell surface expression of HLA-class I molecules in all cell lines (Fig. 6A). B2M KO or wild-type cells were then co-cultured with NK:BOB1, NK:TCRneg, CD8T:BOB1 and CD8T:MOCK. In this model of HLA-class I loss, CD8T:BOB1 killed the wild-type cells but was unable to kill the B2M KO cells due to absence of TCR-specific epitope at the cell surface (Fig. 6B and C). In contrast, NK:BOB1 and NK:TCRneg demonstrated equally potent killing of B2M KO cells due to activation of NK-mediated cytotoxicity (Fig. 6B and C). As demonstrated previously, only NK:BOB1 was able to kill wild-type cells representing TCR-mediated killing. Next, we compared BOB1-TCR efficacy in CD8T and NK-cells *in vivo* using the same UM9 multiple myeloma model previously described (Fig. 3A). KIR-matched NK-cells and CD8T were isolated from the same healthy donor and as a negative control, the CMV-TCR was also expressed in both NK-cell and CD8T in parallel to the BOB1-TCR (Fig. 7A). CD8T:BOB1 cells are capable of controlling high tumour burdens in xenograft models¹¹, therefore we doubled the number of tumour cells infused to further understand the capabilities of NK:BOB1 in comparison to CD8T:BOB1. NK:BOB1 and CD8T:BOB1 treated mice demonstrated reduced tumour outgrowth of wildtype UM9 compared to untreated mice, whilst NK:CMV and CD8T:CMV treated mice did not (Fig. 7B). Furthermore, no significant difference was found between NK:BOB1 and CD8T:BOB1 treated mice until day 26 post infusion, demonstrating comparable TCR-mediated control of the tumour. However, this effect did not persist in NK:BOB1 treated mice as demonstrated by increased tumour outgrowth whilst CD8T:BOB1 maintained control of the tumour (Fig. 7B). In parallel to this, mice were similarly infused with a 50/50 mix of UM9 wildtype and UM9 B2M KO cells alongside NK-TCR and CD8T (Fig. 7A). In contrast to UM9 wildtype mice, NK:CMV significantly reduced tumour outgrowth compared to untreated mice, demonstrative of NK-mediated targeting of HLA-class I negative tumour cells (Fig. 7C). Furthermore, NK:BOB1 demonstrated an enhanced effect as both HLA-class I negative and positive cells

can be targeted (Fig. 7C). CD8T:BOB1, and not CD8T:CMV, also significantly controlled tumour outgrowth, however CD8T:BOB1 was no longer able to maintain control of tumour outgrowth as only half the tumour expressed HLA-class I (Fig. 7C). These data demonstrate the NK-mediated effector functions of NK-TCR can also be engaged in the absence of TCR-specific epitope following loss of HLA-class I and has potential to prevent outgrowth of TCR-resistant malignancies.

Discussion

The data presented in this study demonstrates the enhancement of NK effector function by genetically engineering primary NK-cells to express a TCR. High expression of TCR was repeatedly observed in modified NK-cells and the protocol can be used to express different TCRs targeting many tumour types. NK-TCR permits an alternative NK-activation mechanism against malignancies which are insensitive to NK-mediated attack. To date, autologous NK-ACT has not shown clinical-efficacy and inhibition by self-HLA is likely to contribute to its ineffectiveness^{39,40}. Equipping autologous NK-cells with tumour-specific TCR may therefore override inhibition signals and boost therapeutic efficacy. Furthermore, the cumulative effect of NK-mediated and TCR-mediated activation increased overall cytotoxicity against tumour targets when compared with tgTCR expression in T-cells (Fig. 4C). This increased potency could be beneficial in clinical contexts in which NK-cells are already associated with anti-tumour effects, such as alloSCT. Graft versus leukaemia (GvL) effects are described in patients transplanted with alloreactive KIR mis-matched NK-cells^{21-23,41}. Our data infers that tgTCR expression in infused NK-cells could enhance this effect. Another application could be early infusion of allogeneic NK-cells expressing virus-specific and tumour-reactive TCR following T-cell depleted alloSCT. This would potentially allow preservation of GvL effects and protection against harmful viral re-activities in the absence of T-cells, without the risk of GvHD⁵. Our data does indicate NK-TCR may not be as potent against low antigen expression when compared to CD8T expressing the same TCR (Fig. 4D and Supplemental figure S8). For this reason, BOB1 is an ideal target for NK-TCR for it is highly expressed in most B-cell malignancies¹¹, nevertheless antigen expression must be carefully considered for alternative TCR-specificities.

To our knowledge this is the first study demonstrating TCR expression in primary NK-cells derived from the peripheral blood (PB) of healthy donors. To date, functional TCR expression in non-T-cells has been restricted to the NK-cell line, NK-92^{32,33}, largely owing to low transduction efficiencies observed in primary NK-cells. As demonstrated in this study, improvements to NK-expansion protocols have now enabled efficient retroviral transduction of primary NK-cells (supplemental figure S1). PB offers a readily available source of mature NK-cells with a potent cytotoxic profile, leading to their use in multiple clinical studies⁴². In allogeneic settings, alternative NK sources such as cord blood (CB) or haematopoietic stem cells have also been used due to the reduced risk of T-cells being present in the graft^{28,43}. In this study T-cells present after NK-isolation did not expand upon stimulation and were absent from final NK-TCR cell products (Fig. 1). Although not extensively studied, we have demonstrated functional TCR could also be expressed in CB-NK suggesting our protocol is not limited to PB-NK (data not shown). Primary NK-cells can offer certain advantages for cellular therapy when compared to NK-92 cell lines. Firstly, it is

necessary to irradiate NK-92 cells prior to patient infusion to circumvent tumorigenesis, which can negatively impact on therapeutic persistence and efficacy and often requires multiple doses³⁴. In contrast, it was recently demonstrated a single dose of CB-NK expressing CD19-IL15 CAR achieved complete responses in patients with CLL and NHL and the NK-cells persisted at least 12 months after infusion⁴⁴. Secondly, although NK-92 express a wide array of activation receptors they lack expression of CD16, KIRs and have low expression of NKG2A which can limit the tumour targeting potential compared to a primary NK population. Here, we demonstrate NK-TCR derived from PB have high expression of CD16 which can therefore be activated by monoclonal antibody and induce antibody-dependent-cellular-cytotoxicity (ADCC) in combinational therapies (Supplemental figure S5). Furthermore, NK-TCR has a diverse array of KIRs, and high expression of NKG2A, required for NK-cell activation in the absence of HLA-class I on tumour cells (Supplemental figure S5). Therefore, our NK-TCR approach using primary NK-cells, is unique in its ability to target tumours specifically as well as the associated immune resistance mechanisms following increased immune pressure.

The implications of immune pressure is particularly evident in CD19 CAR-T therapy through the appearance of antigen-negative relapses which is a common feature of antibody-based approaches targeting non-essential proteins^{45,46}. For TCR-mediated approaches, specific targeting of essential proteins from intracellular processes can be achieved. However, loss of antigenicity can occur due to HLA-class I loss/downregulation as a result of immunoediting and has been described in patients treated with ICB and TILs^{13,14,47}. The duality of NK-TCR means that acquired loss of HLA-class I, as a result of TCR-mediated immune pressure activates innate NK responses. Our data supported this as NK-cells with or without BOB1-TCR expression were cytotoxic against target cells with a β -2-microglobulin knock-out, whilst the wild-type counterpart was insensitive to NK-mediated attack and could only be targeted via the BOB1-TCR (Figs. 6 and 7). In theory, the TCR-mediated and NK-mediated pathways of NK-TCR could provide feedback for each other and prevent a selective growth advantage for tumours with defects in antigen presentation¹⁴. However, tumours can also acquire specific HLA-allele loss following immune pressure, as seen in patients who relapsed following HLA-haploidentical SCT⁴⁸. HLA-dependence of NK-TCR may therefore be considered a limitation and as with most TCR-mediated approaches a multi-HLA targeting cell product will be paramount to its broader application.

For any ACT, it is important to reduce toxicity to allow infusion of clinically-effective doses. NK-cells have a reduced cytokine profile which limits their capacity to cause cytokine release syndrome even when expressing CD19-CAR⁴⁴. We demonstrate NK-TCR have reduced cytokine production in response to antigen compared to NK-mediated activation, suggesting TCR stimulation would not be harmful (Fig. 5), and tgTCR expression in T-cells has not yet shown such toxicities^{6,7}. Furthermore, evidence that allogeneic NK-cells do not cause graft-versus-host-disease (GvHD), makes them a safer alternative when T-cells are considered too high risk^{26,27}. Together, these properties make NK-TCR an interesting candidate for an "off the shelf" TCR-based ACT. Although generating an NK-TCR cell product from primary NK-cells is feasible, this protocol is laborious and translating to the clinic may be challenging, although not impossible. A single-transduction step would simplify the approach, however this method needs to be

optimised and so far has resulted in lower tgTCR expression and efficacy (data not shown). Moreover, there are several limitations to overcome to become a standardised clinical approach. For instance, an obstacle for “off the shelf” allogeneic ACT is avoiding elimination by the host immune system and currently allogeneic NK-cells are given to patients who have undergone lymphodepletion regimens^{22, 44}. NK-cells themselves are short lived and, although an advantage in terms of toxicity, this may reduce clinical efficacy. It is known that in the absence of IL15 primary NK-cells do not persist⁴⁹ and in this study, tumour outgrowth occurred in NK:BOB1 treated mice following IL15 withdrawal (Fig. 7B). This was suggestive of decreased NK-cell persistence and also makes direct comparison with CD8T *in vivo* difficult. Increasing persistence by inclusion of autonomous IL15 production in NK-TCR would therefore be important to its development. There is also limited clinical success of NK-cell targeting solid malignancies, despite many solid malignancies demonstrating down-regulated HLA-class I⁵⁰. This is often associated with insufficient migration and trafficking of NK-cells into these sites. Modifying NK-cells to express molecules that enhance persistence and homing is possible but further NK-TCR manipulation maybe technically challenging. Finally, other than HLA-class I loss, tumours can exhibit many immune evasion strategies⁵¹ including immune-checkpoint inhibition which can directly impact on NK function^{52, 53}. Therefore, combination therapy with ICB should also be considered in future NK therapeutics⁵⁴. The associated resistance to immune-checkpoint blockade may in turn be overcome by NK-TCR by preventing a selective growth advantage for tumours with defects in antigen presentation.

In conclusion, following our described two-step retroviral transduction protocol, TCR can be feasibly expressed in primary NK-cells and used for ACT. The data presented in this study demonstrate TCR-mediated NK responses can enhance NK-cell efficacy against malignancies, particularly when resistant to NK-mediated attack. In addition, NK-TCR can also target HLA-class I loss, an associated immune-evasion strategy of TCR-mediated approaches which makes NK-TCR a unique cellular therapeutic.

Declarations

Competing-interest-statement

The authors declare no competing financial interests.

Ethical statements:

For this study, materials were used from the Leiden University Medical Center Biobank for Hematological Diseases. This study was approved by the Institutional Review Board of the Leiden University Medical Center (IRB LUMC approval number B16.039). Materials from patients and healthy individuals were collected after written informed consent according to the Declaration of Helsinki. All animal studies were conducted in accordance with institutional guidelines after obtaining permission from the national Ethical Committee for Animal Research (AVD116002017891) and in accordance with Dutch laws on animal experiments.

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Figures

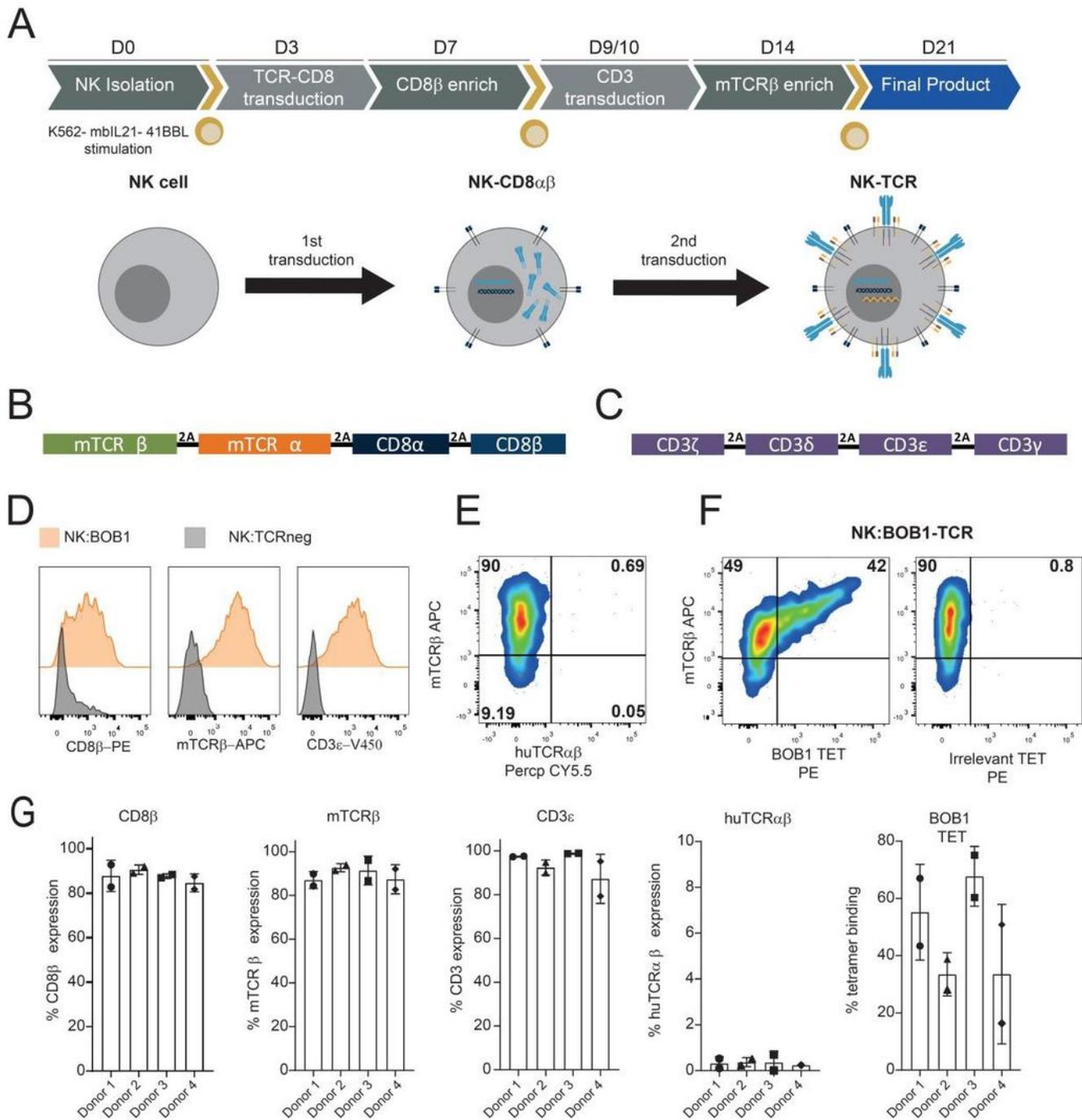


Figure 1

Figure 1

Generation of TCR expressing NK cells following a two-step retroviral production protocol. A) Schematic of 21-day production protocol to generate TCR expressing NK cells (NK-TCR). Retroviral construct design for B) TCR linked via 2A sites to CD8ab (TCR-CD8) and C) CD3 ζ δ ϵ γ invariant chains linked via 2A sites. D) Representative histograms of CD8 β , mTCR β , CD3 ϵ and human TCR $\alpha\beta$ expression in a final NK-TCR cell product expressing BOB1-specific TCR (NK:BOB1) or NK-cells without TCR expression (NK:TCRneg)

on Day 21. Representative FACS plot of NK:BOB1 stained for E) mTCR β and human(hu) TCR $\alpha\beta$ expression and with F) BOB1 or an irrelevant pMHC tetramer on day 21 post isolation . G) Summary of expression frequencies of CD8 β , mTCR β , CD3 ϵ , huTCR $\alpha\beta$ expression and BOB1-specific tetramer binding frequencies on Day 21 of NK:BOB1 cell products generated from 4 different donors. NK:BOB1 was generated twice for each donor. Error bars represent mean and standard deviation.

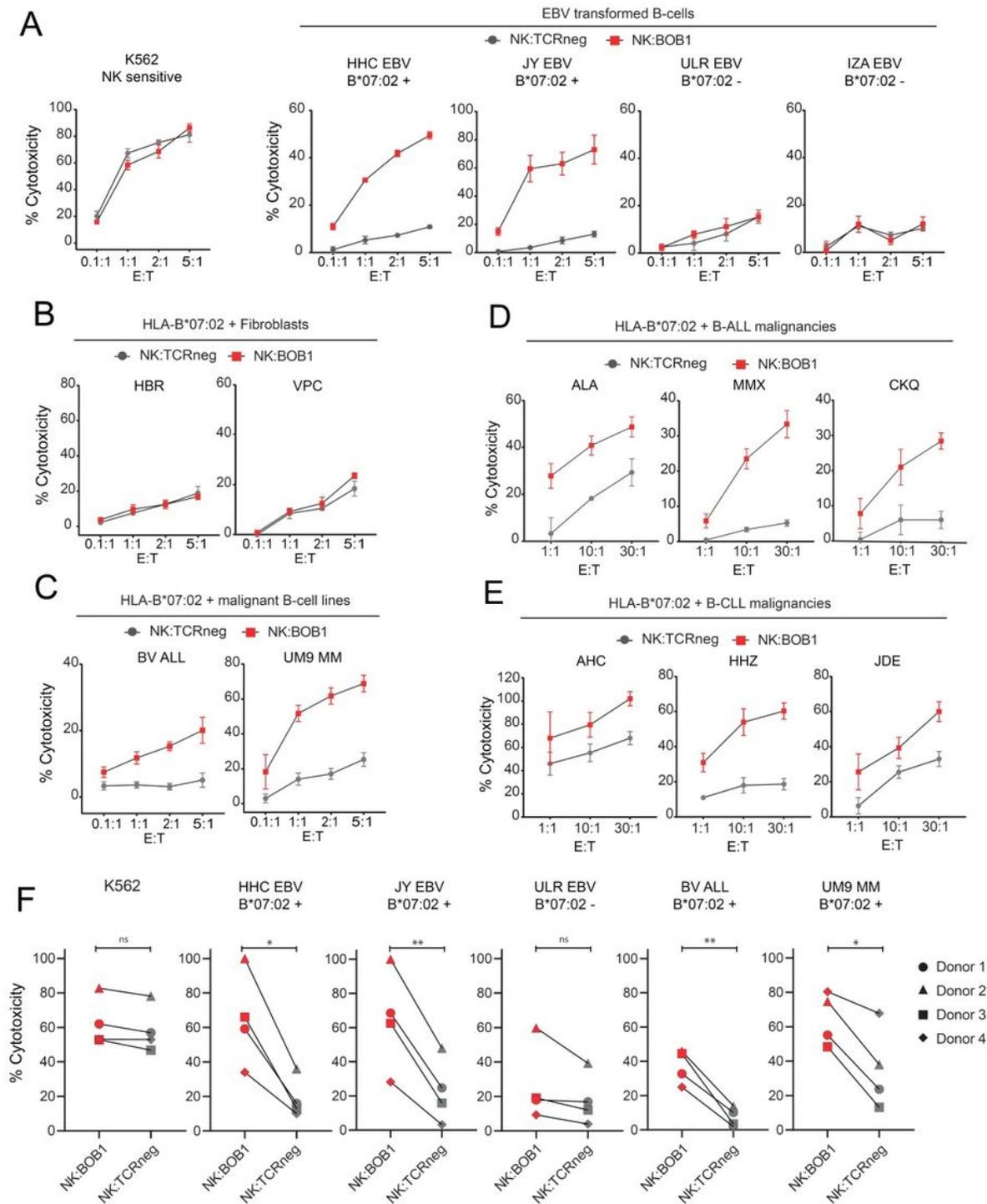


Figure 2

Figure 2

NK-TCR elicit antigen-specific cytotoxicity against tumour targets. NK cells expressing the HLA-B*07:02 restricted, BOB1-specific TCR (NK:BOB1) or negative for TCR expression (NK:TCRneg) were co-cultured, at multiple effector:target (E:T) ratios, with ⁵¹Cr labelled A) K562-mbIL21-41BBL or HLA-B*07:02 +/- EBV-LCLs, B) HLA-B*07:02+ fibroblasts negative for BOB1 expression, C) HLA-B*07:02+ B-ALL cell line (BV-ALL) or HLA-B*07:02+ Multiple myeloma cell line UM9 and leukapheresis samples from HLA-B*07:02+ patients with D) B-cell acute lymphocytic leukemia (B-ALL) or E) B-cell chronic lymphocytic leukemia (B-CLL). Each patient material had >70% malignant cells present and was untreated. Figure A-E error bars represent SEM of technical replicates. F) Summary of % Cytotoxicity at the highest E:T ratio (5:1) from 4 NK cell products generated from different donors. Error bars represent mean and SEM of biological replicates. Statistical test used was unpaired T-test.

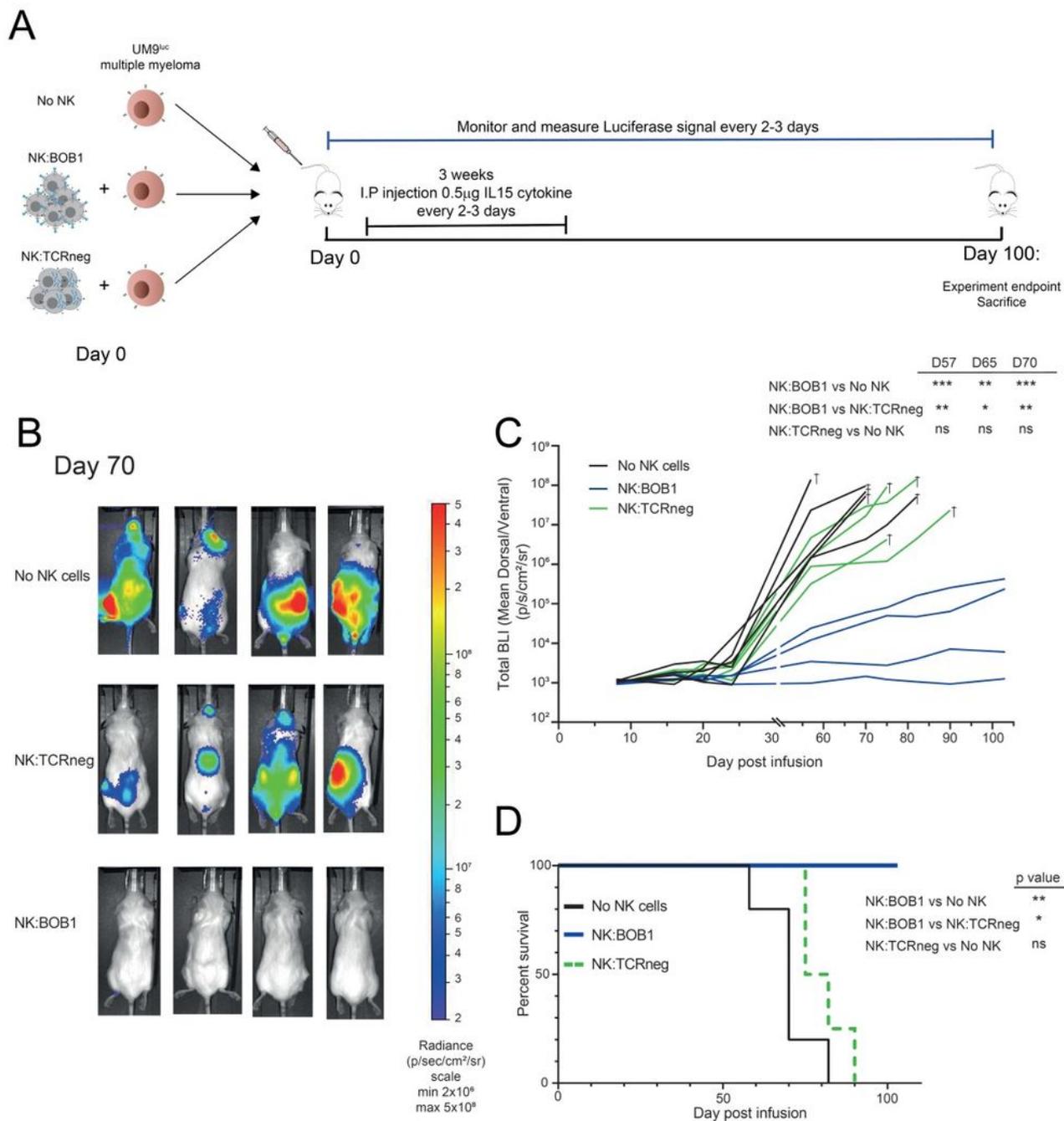


Figure 3

Figure 3

NK-TCR increases survival of treated mice in a pre-clinical model of multiple myeloma. A) Schematic of experiment set-up, NSG mice were infused intravenously with 0.4×10^6 HLA-B*07:02+, luciferase-expressing UM9 multiple myeloma cell line alone (No NK, $n = 5$) or simultaneously with 6×10^6 NK cells expressing BOB1-TCR (NK:BOB1, $n = 4$) or TCR negative NK cells (NK:TCRneg, $n = 4$) in the presence of human IL15 cytokine. Mice were injected every 2-3 days with $0.5 \mu\text{g}$ IL15 for 3 weeks. For measurement

mice were injected I.P with luciferin and measured for Bioluminescence. B) Bioluminescent images of mice at Day 70. C) Tumour burden as measured by bioluminescence of UM9 over time. Each line represents an individual mouse and crosses indicate sacrifice of the mouse. Statistical analysis was calculated using one-way ANOVA with Tukey's multiple comparison on Day 57,65 and 70. C) Kaplan-Meier survival curve of mice receiving No NK cells, NK:BOB1 and NK:TCRneg. Remaining mice were sacrificed on day 100 at experiment end. This experiment has been repeated twice.

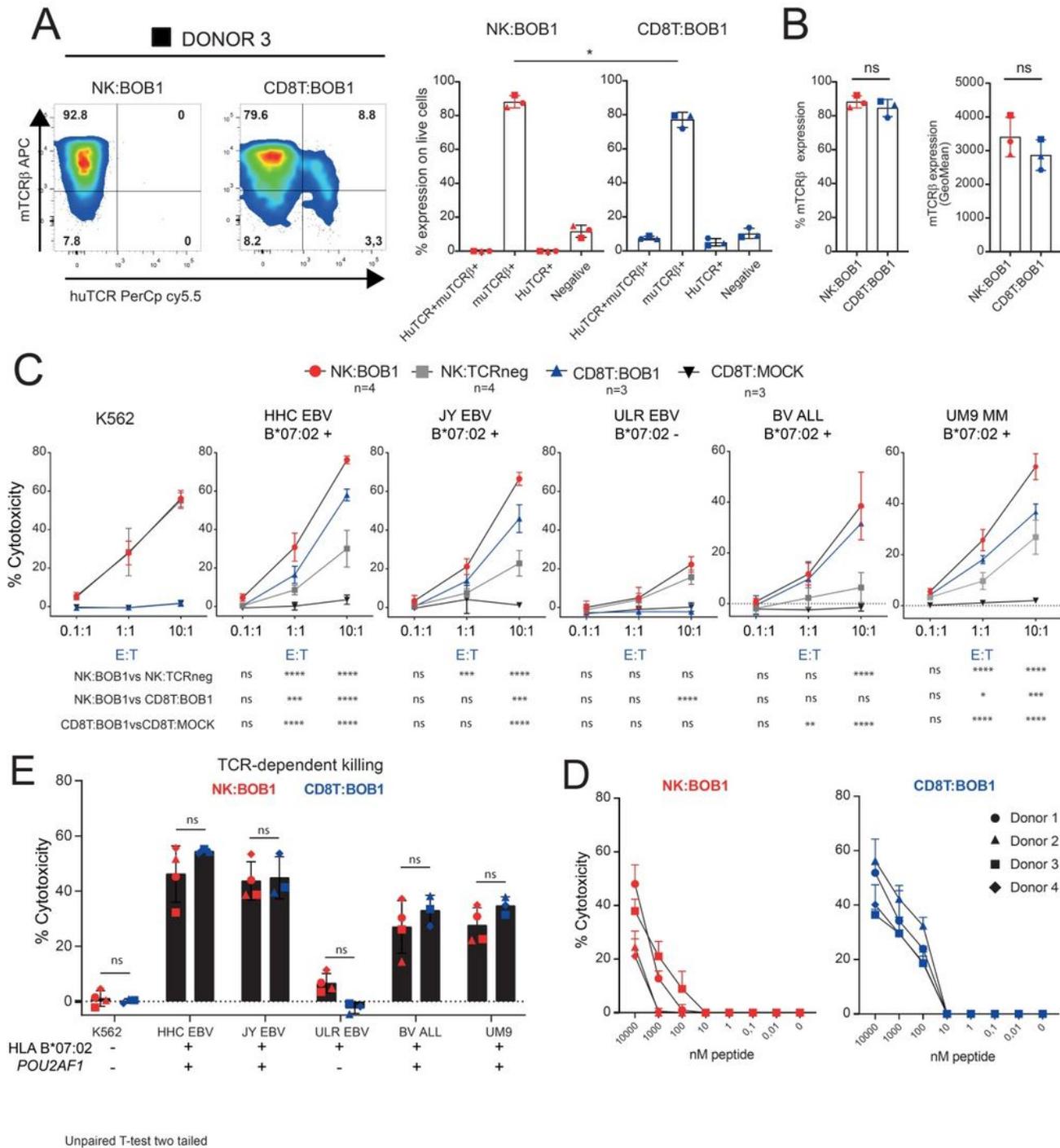


Figure 4

Figure 4

NK-TCR demonstrate increased overall cytotoxicity against tumor targets compared to tgTCR expressing CD8 T-cells. Alongside NK:BOB1, CD8 T-cells (CD8T), with CRISPR-mediated knock out of endogenous TCR $\alpha\beta$ chains, were transduced to express murinised BOB1-specific TCR (CD8T:BOB1) or mock transduced with murinised CMV-specific TCR (CD8T:MOCK). A) Representative FACS plot and summary data of mTCR β and huTCR $\alpha\beta$ expression in NK:BOB1 (D7 post stimulation) and CD8T:BOB1 (D10 post stimulation) generated from the same 3 healthy donors. B) Overall expression frequencies and geometric mean of mTCR β expression in CD8T:BOB1 and NK:BOB1. Error bars represent mean and SD from biological replicates. C) Combined data depicting the % cytotoxicity at multiple effector:target ratios of NK:BOB1 (n=4), NK:TCRneg (n=4), CD8T:BOB1 (n=3) and CD8T:MOCK (n=3) co-cultured with HLA-class I negative K562, HLA-B*07:02 +/- EBV-LCLs, HLA-B*07:02+ B-ALL cell line (BV-ALL) and HLA-B*07:02+ Multiple myeloma cell line UM9. E) TCR-dependent killing of NK:BOB1 and CD8T:BOB1 calculated as the difference between NK:BOB1\NK:TCRneg and CD8T:BOB1\CD8T:MOCK cytotoxicity at the highest E:T ratio. POU2AF1 encodes the BOB1 protein. D) % cytotoxicity of NK:BOB1 and CD8T:BOB1 against HLA-B*07:02 CD8T target cells, exogenously loaded with BOB1(B7)-peptide (APAPTAVVL) at a 10:1 E:T ratio. Each symbol represents a different donor and error bars represent mean and SD of biological replicates. Statistical test used was unpaired T-test.

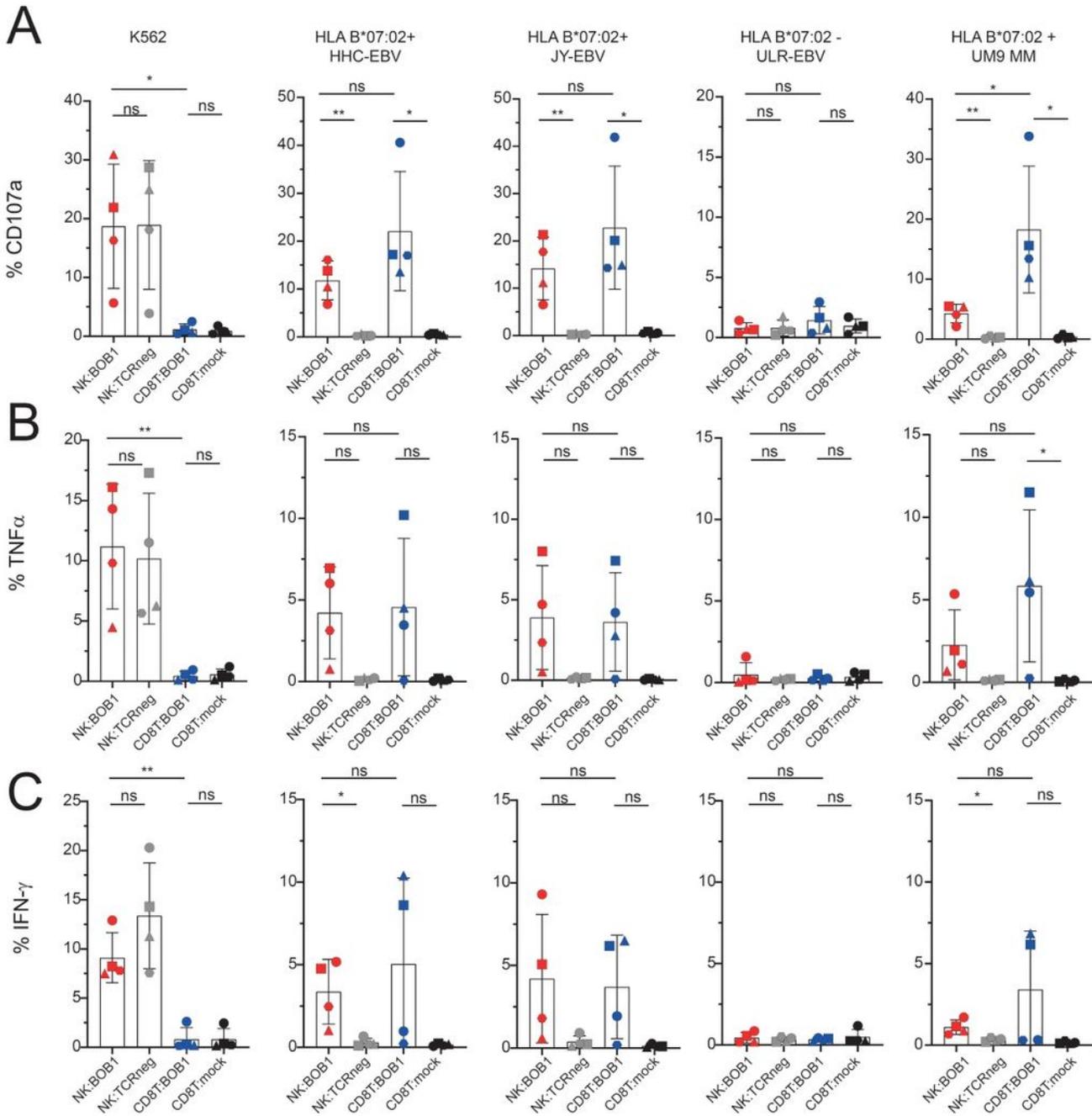


Figure 5
unpaired t-test mean and sd

Figure 5

NK-TCR demonstrate comparable antigen-specific degranulation and cytokine production compared to tgTCR expressing CD8T. CD8T expressing BOB1-TCR or CMV-TCR (D10 post stimulation) and NK-cells expressing BOB1-TCR or TCR negative NK-cells (Day 7 post stimulation) were co-cultured overnight with HLA-class I negative K562, HLA-B*07:02 +/- EBV-LCLs, HLA-B*07:02+ B-ALL cell line (BV-ALL) and HLA-B*07:02+ Multiple myeloma cell line UM9 in the presence of Brefeldin-A and anti-CD107a. After 12-14

hours incubation, cells were stained for inflammatory cytokines TNF α and IFN- γ and assessed by FACS. Depicted is the combined data of cell products derived from different donors for A) CD107a, B) TNF α and C) IFN- γ expression. Live NK-cells were gated on CD56 expression and Live CD8T were gated on CD8 expression. Positive cells were gated according to unstimulated. Target cells were td-tomato positive and were removed from analysis. Each symbol represents a different donor and error bars represent mean and SD of biological replicates. Statistical test used was unpaired T-test.

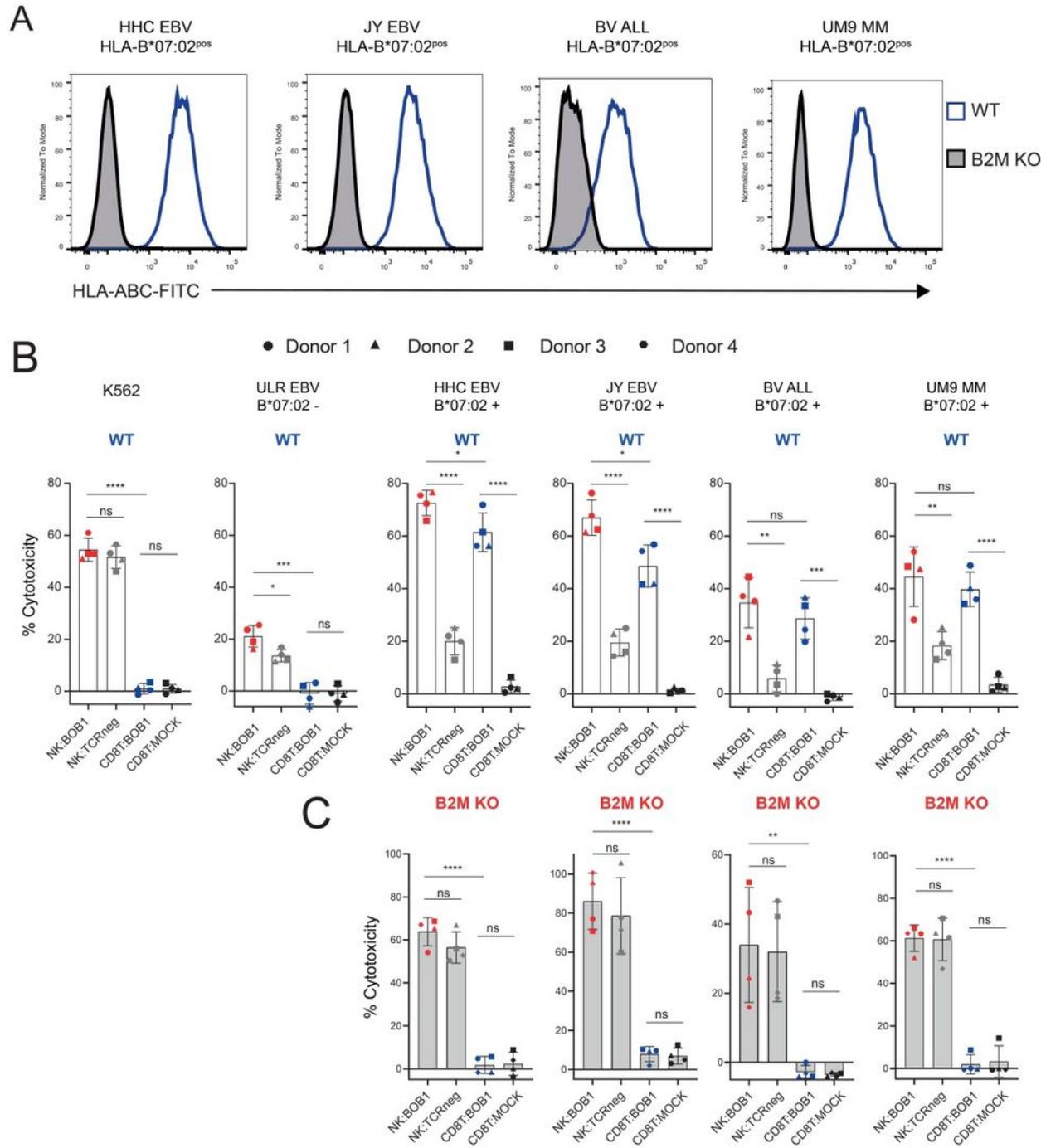


Figure 6

Figure 6

NK-TCR demonstrate NK-mediated killing of tumour targets after HLA-class I loss. HLA-B*07:02+ tumour targets including EBV-LCLs (HHC EBV and JY EBV), B-cell ALL cell line (BV ALL) and multiple myeloma cell line (UM9) were generated with a β -2-microglobulin knock-out (B2M KO) using CRISPR/Cas9 genome editing. B2M KO cells were sorted based on absence of HLA-class I. A) wild-type (WT) and B2M KO cell lines were analyzed for HLA-class I expression by FACS. B) Cytotoxicity was determined using a 6 hour Cr-release assay. NK cells expressing the BOB1-specific TCR (NK:BOB1), TCR negative NK-cells (NK:TCRneg), CD8T expressing BOB1-TCR (CD8T:BOB1) or CMV-TCR (CD8T:MOCK) were co-cultured with HLA-B*07:02+ tumour targets which were either B) wildtype or C) B2M KO. Depicted is the combined cytotoxicity data at 10:1 E:T ratio by cell products derived from 4 different healthy donors. Each symbol represents a different donor and error bars represent mean and SD of biological replicates. Statistical test used was unpaired T-test.

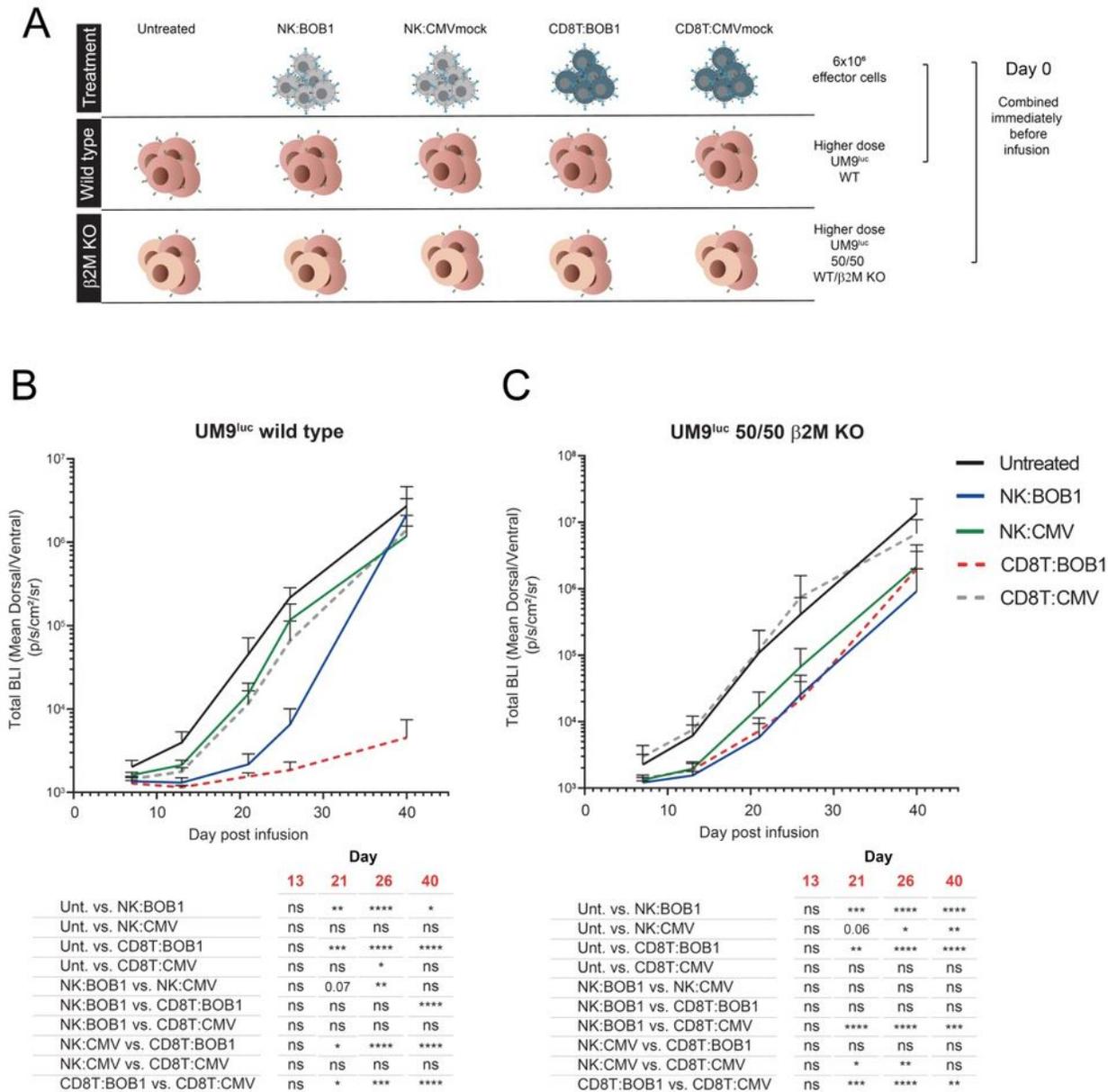


Figure 7

Figure 7

NK-TCR demonstrate in vivo targeting of multiple myeloma cells with HLA-class I loss. A) Schematic representation of experiment design. B) non-irradiated NSG mice were infused intravenously with a total $0,8 \times 10^6$ (higher dose) HLA-B*07:02+, Luciferase-expressing(luc) UM9 multiple myeloma cell line alone (untreated, n =4) or simultaneously with 6×10^6 KIR-matched NK cells or CD8 T-cells expressing BOB1-TCR (NK:BOB1, n=5, CD8T:BOB1, n=5) or CMV-TCR (NK:CMV, n=4, CD8T:CMV, n=5). C) NSG mice were infused

intravenously with a 50/50 mix of $0,4 \times 10^6$ wildtype UM9luc and $0,4 \times 10^6$ UM9luc B2M KO cells alone (untreated, $n = 4$) or simultaneously with 6×10^6 KIR-matched NK cells or CD8 T-cells expressing BOB1-TCR (NK:BOB1, $n=7$, CD8T:BOB1, $n=7$) or CMV-TCR (NK:CMV, $n=6$, CD8T:CMV, $n=4$). All cells were injected in the presence of human IL15 cytokine. Mice were then injected every 2-3 days with $0.5 \mu\text{g}$ IL15 for 3 weeks. For measurement mice were injected I.P with luciferin and measured for Bioluminescence. Tumour burden as measured by bioluminescence of UM9luc over time. Error bars represent Mean and SD and statistical analysis was calculated using one-way ANOVA with Tukey's multiple comparison.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalMethods.docx](#)
- [supplementalfiguresnatcomm.pdf](#)