**Targeting CD20+ B cell non-Hodgkin Lymphoma by anti-CD20 CAR mRNA Modified Expanded Natural Killer (NK) Cells in vitro and in NSG Mice**

**Supplementary Methods, Figures, and Table**

**Supplementary Methods**

**Retrovirus production**

The pMSCV-IRES-anti-CD20-BB-, pEQPAM3(–E), and pRDF constructs were kindly provided by Dr. Dario Campana (St Jude Children’s Research Hospital, University of Tennessee College of Medicine, Memphis). The retrovirus was generated as previously described.18 We used calcium phosphate DNA precipitation to transfect 293T cells with pMSCV-IRES-anti-CD20-BB- or pMSCV-IRES-GFP with pEQ-PAM3(–E); and pRDF. Conditioned medium containing retrovirus was harvested at 48 hours and 72 hours after transfection. Concentrated viral medium was used to infect NK92 cells.

**NK cell expansion and isolation**

To induce the expansion of NK cells, PBMNCs were stimulated with irradiated genetically modified K562-mb15 - 41BBL (geK562) cells as previously described (generously supplied by D. Campana, MD, PhD, St. Jude’s Children’s Hospital, Memphis, TN, USA) [(17)](#_ENREF_18). Briefly,geK562 or wildtype K562 (WTK562) were lethally irradiated with 100 Gray and then co-cultured with washed PBMNCs at a 1:1.5 in RPMI-1640 (Invitrogen) + 10% FBS in 40 IU/ml of recombinant IL-2 (Invitrogen).

Expanded PBNK cells were isolated by negative selection using Miltenyi NK cell isolation kit (Miltenyi Biotec, Cambridge, MA) following the manufacturer instructions. NK cell purity was confirmed by flow cytometry using anti-CD56 and anti-CD3 antibodies (Becton Dickinson). Purified NK cells were maintained in culture medium with IL-2 (40 IU/ml) (Invitrogen) until use.

**Intracelluar CD107a and IFN- assays**

CD107a expression was measured by flow cytometry, as we have previously described [(20)](#_ENREF_22" \o "Ayello, 2009 #227). ExPBNK cells were mixed with medium or target cells at a ratio of 10:1 in the absence of exogenous cytokines in RPMI1640 medium. Anti-CD107a-FITC (BD Biosciences) was added to each well and incubated for 1 hour at 37°C. After 1 hour, brefeldin A (Golgi Plug; BD Biosciences) was added to each well, and the cells were incubated for an additional 3 hours. Cells were then washed, fixed, permeabilized using Cytofix/Cytoperm reagent kit (BD Biosciences), and resuspended in staining buffer containing anti-CD56-PE (BD Biosciences).

Intracellular interferon-gamma was analyzed after exPBNK cells were mixed with medium or tumor cells, at a ratio of 10:1 in the absence of exogenous cytokines in RPMI1640 medium with brefeldin A for 4 hours (Golgi Plug; BD Biosciences). Cells were then washed, fixed, permeabilized using Cytofix/Cytoperm reagent kit (BD Biosciences), and re-suspended in staining buffer containing anti-CD56-PE-Cy5 and anti-INF-PE.

All experiments included appropriate isotype controls. Medium alone served as a control and a stained control sample with target cells was included to detect spontaneous degranulation. Samples were analyzed on a FACScan flow cytometer (BD Biosciences) and a minimum of 10,000 events collected.

**Tumor cell recovery**

MOCK or CAR exPBNK cells were incubated for overnight with Ramos at E:T=3:1 in culture medium RPMI-1640 supplemented with 10% fetal bovine serum. Ramos cells incubated with culture medium only were used as control. After incubation, cells were washed in PBS with 10% heat-inactivated FBS and 1% sodium azide and stained with CD19-PE (BD Biosciences) and 7-AAD (BD Biosciences). The cells were analyzed by flow cytometry on a BD FACScan (BD Biosciences). The total living cells were gated by 7-AAD negative. The living Ramos cells were gated by CD19+ 7-AAD- by flow cytometry analysis.

***In vitro* cytotoxicity**

Tumor targets were labeled with BATDA (acetyoxymethyl ester-enhancing ligand) according to the manufacturer's instructions. Labeled target cells (1x 105) were placed in 96-well U-bottom tissue culture plates and incubated with expanded NK cells at various effector:target (E:T) ratios for 2-4 hrs at 37oC. Cytolytic activity was evaluated using a time-resolved fluorometer (Perkin Elmer). Percentage of specific release was calculated as follows:

% Specific release= 100x (Experimental release – spontaneous release)I / I(Maximum release – spontaneous release)

All tests were run in triplicate or quadruplicate.

**Flow cytometry–based phenotyping of NK activating and inhibitory receptors**

The expanded PBNK and anti-CD20 CAR modified exPBNK populations were analyzed for phenotypic expression of inhibitory NK receptors (CD94, NKG2A), inhibitory NK KIR receptors (CD158a, CD158b, CD158e), activating C-lectin NK receptors (CD69, NKG2D), and activating natural cytotoxicity receptors (Nkp46, NKp30, NKp44) by flow cytometry. In brief, 5x105 cells were washed in PBS with 10% heat-inactivated FBS and 1% sodium azide (Sigma Aldrich). Fluorescent-conjugated monoclonal antibodies (CD3, CD56, CD16, CD69, NKp30, NKp44, Nkp46, CD158a, CD158b, CD158e, CD94, NKG2A and NKG2D: BD Biosciences, San Jose, CA, USA, Biolegend and R and D systems, respectively) were added. Cells were incubated in the dark at 40C for 30-45 minutes, and washed in azide buffer. Samples were analyzed on a BD FACScan (BD Biosciences) and MACSQuant Analyzer (Miltenyi Biotec). No stain, isotype controls and single color controls were used for compensation and gating. A minimum of 10,000 events was collected and analyzed using FlowJo (Treestar Inc) or MACSQuantify™ Software.

**Histology (H&E) staining and Immunofluorescence staining**

Tumors were collected in O.C.T. Compound (Fisher Scientific, Inc) and frozen at -80oC.

10m sections were cut in a cryostat microtome (Leica CM 1850) and were stained by H&E after they were fixed with cold acetone for 10 minutes.

10m cryosections were fixed with cold acetone. Tissue sections were blocked with blocking buffer (10% normal goat serum in PBS) and incubated with rabbit anti-human CD56 (Abcam) overnight at 4°C. The next day, tissue sections were blocked and incubated with goat anti-rabbit Alexa fluor 488 secondary antibody (Life Technologies) for 1 hour at room temperature. Rat IgG was used as the negative control. Tumor sections were mounted in mounting medium with DAPI (Vector Labs). Slides were visualized under the Nikon eclipse 90i fluorescence microscope (Nikon, Melville, NY).

**Supplementary Figure Legends**

**Supplementary Fig.S1:** **Anti-CD20 CAR enhances NK92 cells in-vitro cytolytic activity against CD20+ B-NHL cells by retroviral transduction.** (A)Schematic representation of the anti-CD20 chimeric receptor in an RD114-pseudotyped retroviral vector. LTR indicates long terminal repeat; AMP, ampicillin resistance. (B)-(C) Expression of anti-CD20 CAR was confirmed in infected Jurkat cellsby flow cytometry after staining with a goat anti–mouse (Fab)2 polyclonal antibody conjugated with biotin followed by streptavidin PE (y-axes); expression of GFP is also shown (x-axes) (B) and it was also confirmed in infected PBMC by western blot analysis with an anti-human CD3 antibody (C). (D) NK92 cells expressing anti-CD20 chimeric receptors or expression GFP only were incubated with BATDA labeled targets: CD20+ NK sensitive Ramos, CD20+ NK resistant Daudi and the CD20- pre-B-ALL cell line RS4;11 at the indicated E/ T ratios for 4 hours. Europium release assays were performed according to the manufacturer's instructions. Each data point represents the mean (SEM; n=3). Average values are reported as the mean ± SEM. p values using unpaired student t test were noted in (D).

**Supplementary Fig.S2:** **nucleofection of anti-CD20 CAR mRNA does not affect the expression of endogenous receptors on exPBNK cells**

PBNK cells were expanded ex vivo by co-culturing PBMC with irradiated geK562 feeder cell lines in the presence of 40 IU/mL IL-2 for 14 days and isolated by depleting CD3+ T cells. The expanded NK cells were nucleofected with anti-CD20 CAR mRNA (CAR) or without anti-CD20 CAR mRNA (mock). CAR exPBNK or MOCK exPBNK cells were stained with indicated monoclonal antibodies. The expression of receptors on viable exPBNK Cells were compared by flow cytometry analysis.

**Supplementary Fig.S3:** **U-698-M stimulates the expression of activating receptors on mock exPBNK and CAR exPBNK similarly**

The expanded NK cells were nucleofected with anti-CD20 CAR mRNA (CAR) or without anti-CD20 CAR mRNA (mock). MOCK exPBNK or CAR exPBNK cells were cultured with medium only, or CD20+ U-698-M at a 3:1 ratio overnight. The activating receptors (NKp30, NKp44, CD69, and NKG2D) and inhibitor receptors (CD94 and KIR2DL2/3) as indicated on MOCK exPBNK and CAR exPBNK were examined and compared by flow cytometry analysis.

**Supplementary Fig.S4:** ***In vitro* bioluminescence to detect luciferase expression in Raji and Raji-2R cells**

Raji and Raji-2R cells were electroporated with ffLUCZeo-pcDNA and selected for stable clones expressing luciferase (Luc) with Zeocin. (A) The bioluminescence intensity increased proportionally with increasing Raji-Luc cell numbers. A strong correlation between number of cells and light emission was obtained (R2= 0.99). (B) The bioluminescence intensity increased proportionally with increasing Raji-2R-Luc cell numbers. A strong correlation between number of cells and light emission was obtained (R2= 0.989). (C)-(D) Dynamic change of the bioluminescent signal in Raji-Luc and Raji-2R-Luc after over 2 months selection. Signal intensities are shown as a function of time after addition of D-luciferin.

**Supplementary Fig.S5: exPBNK cells tracking and persistence in vivo**

(A) exPBNK cells were labeled with DID and iv injected into NSG mice. 8 days later after NK injection, mice were sacrificed and organs (lung, liver, spleen, kidney, heart) and bones were collected and washed with D-PBS. Fluorescent imagings were acquired and analyzed by using LIVING IMAGE (Xenogen). (B) Single cell suspensions were obtained from lung, liver and spleen and analyzed by flow cytometry to detect DID labeled exPBNK cells. (C)1x10\*7mock exPBNK or CAR exPBNK cells were IV injected to Raji-Luc xenografted mice through the tail veins. Peripheral blood was collected at the indicated time point after NK NK injection. Circulated NK cells from mock exPBNK group and CAR exPBNK group were quantified using the Trucount method by flow cytometry analysis with anti-CD56-PE antibodies.

**Supplementary Fig.S6:** **The purified expanded PBNK cells have higher activating receptors expression**

PBNK cells were expanded ex vivo by co-culturing PBMC with irradiated geK562 feeder cell lines in the presence of 40 U/mL IL-2 for 14 days and isolated by depleting CD3+ T cells. NK cells were stained with indicated monoclonal antibodies immediately after isolation. Cell surface expression on viable cells is shown by flow cytometry analysis.

**Supplementary Fig.S7:** **Proposed scientific model**

We propose that the interaction between anti-CD20 CAR on exPBNK and CD20 on the surface of B-L/L transmits activating signal back to exPBNK and significantly stimulates exPBNK degranulation and release of cytokine such as IFN- to lyse resistant tumor targets.