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Continuously expanding CAR NK-92 cells display selective cytotoxicity against B-cell leukemia and lymphoma

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Running title: CAR NK-92 cells targeting B-cell malignancies

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Abstract

Background. Natural killer (NK) cells can rapidly respond to transformed and stressed cells, and represent an important effector cell type for adoptive immunotherapy. In addition to donor-derived primary NK cells, continuously expanding cytotoxic cell lines such as NK-92 are also being developed for clinical applications. *Methods*. To enhance their therapeutic utility for the treatment of B-cell malignancies, we engineered NK-92 cells by lentiviral gene transfer to express chimeric antigen receptors (CARs) that target CD19 and contain human CD3ζ (CAR 63.z) or composite CD28-CD3ζ or CD137-CD3ζ signaling domains (CARs 63.28.z and 63.137.z). *Results*. Exposure of CD19-positive targets to CAR NK-92 cells resulted in formation of conjugates between NK and cancer cells, NK-cell degranulation and selective cytotoxicity towards established B-cell leukemia and lymphoma cells. Likewise, the CAR NK cells displayed targeted cell killing of primary pre-B-ALL blasts that were resistant to parental NK-92. While all three CAR NK-92 cell variants were functionally active, NK-92/63.137.z cells were less effective than NK-92/63.z and NK-92/63.28.z in cell killing and cytokine production, pointing towards differential effects of the costimulatory CD28 and CD137 domains. In a Raji B-cell lymphoma model in NOD-SCID IL2R γ^{null} (NSG) mice, treatment with NK-92/63.z cells but not parental NK-92 cells inhibited disease progression, indicating that selective cytotoxicity was retained *in vivo*. *Conclusions*. Our data demonstrate that it is feasible to generate CAR-engineered NK-92 cells with potent and selective antitumor activity. These cells may become clinically useful as a continuously expandable off-the-shelf cell therapeutic agent.

Key words: adoptive immunotherapy; B-cell malignancies; CD19; chimeric antigen receptor; natural killer cells

Abbreviations:

B-ALL, B-cell acute lymphoblastic leukemia

CAR, chimeric antigen receptor

CV, calcein violet

EGFP, enhanced green fluorescent protein

E/T, effector to target

FBS, fetal bovine serum

IHC, immunohistochemistry

NK, natural killer

NSG, NOD-SCID IL2R γ^{null}

PI, propidium iodide

SFFV, Spleen Focus Forming Virus

Introduction

Chimeric antigen receptor (CAR)-engineered T cells targeting the B-cell differentiation antigen CD19 have demonstrated remarkable clinical efficacy in patients with lymphomas and leukemias of B-cell origin [1-7]. Natural killer (NK) cells represent another important cell type for adoptive immunotherapy [8, 9], but CAR-mediated retargeting of NK cells has been attempted less frequently and so far no clinical data for such an approach are available [10]. NK cells are part of the innate immune system and play an important role in cancer immunosurveillance [11, 12]. They do not require prior sensitization and recognition of peptide antigens presented in complex with MHC molecules, but are controlled in their activity by distinct germline-encoded cell surface receptors that allow discrimination between healthy and diseased tissues [13, 14]. In cancer patients, NK-cell responses are often impaired due to the immunosuppressive tumor microenvironment. Hence, current NK-cell therapies are typically based on adoptive transfer of *ex vivo* expanded allogeneic NK cells derived from a suitable donor [9, 15]. While displaying graft-versus-leukemia or graft-versus-tumor activity, such donor-derived NK cells do not carry the risk of graft-versus-host-disease frequently associated with donor lymphocyte infusion of allogeneic T cells, which may also be a limitation of allogeneic CAR-engineered T lymphocytes [16].

Unlike primary NK cells which have a limited life span and expansion potential, the human NK cell line NK-92 can be continuously expanded in the presence of IL-2 in a GMP-compliant process [17]. Safety and clinical activity of NK-92 cells as an allogeneic cell therapeutic has been demonstrated in early phase clinical trials in patients with advanced malignancies [18-20]. The feasibility of generating CD19-specific CAR NK cells has been investigated in preclinical studies employing both primary human NK cells as well as NK-92 [21-23]. Thereby the use of NK-92 allows isolation and expansion of CAR-engineered cells from a bulk of non-transduced cells [24-26], which bypasses the problem of limited efficiency of gene transfer into NK cells when compared to T cells [22, 27]. For primary CAR NK cells targeting CD19 it was previously demonstrated that by inclusion of a costimulatory protein

domain derived from CD137 (4-1BB) or CD244 (2B4) in the CAR in addition to CD3ζ, activation and cytotoxicity were markedly enhanced in comparison to cells carrying a CD3ζ-only CAR [21, 23]. In contrast, for the establishment of CD19-specific CAR NK-92 cells so far only first-generation CARs were employed, which utilized a murine CD3ζ domain for signaling [22, 28, 29].

To investigate the impact of different CAR designs on activation and cytotoxicity of NK-92 cells, here we engineered the cells by lentiviral gene transfer to express CD19-specific CARs that harbor human CD3ζ (CAR 63.z) or composite CD28-CD3ζ or CD137-CD3ζ domains (CARs 63.28.z and 63.137.z) for signaling. We analyzed *in vitro* cytotoxicity of the CAR NK-92 cells against established cancer cell lines and primary pre-B-ALL blasts, and dependence of cell killing on expression of the CD19 target antigen and CAR activation. Due to their more pronounced cytotoxicity *in vitro*, NK-92/63.z cells expressing a humanized first-generation CAR were chosen for analysis of *in vivo* antitumor activity in comparison to unmodified parental NK-92 cells in a Raji B-cell lymphoma model in NOD-SCID IL2R γ^{null} (NSG) mice.

Materials and Methods

Cells and culture conditions

Human MDA-MB453 breast carcinoma and HEK 293T cells (both ATCC, Manassas, VA) were propagated in DMEM (Lonza, Cologne, Germany). Human Raji and WSU-NHL lymphoma, BV173 leukemia and C1R-neo B-cell lymphoblastoid cells (all ATCC) were cultured in RPMI 1640 medium (Lonza). Media were supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Darmstadt, Germany). MDA-MB453/CD19 breast carcinoma cells were generated by transduction with GALV pseudotyped retroviral vector encoding truncated human CD19 (kindly provided by Malcolm Brenner and Gianpietro Dotti, Baylor College of Medicine, Houston, TX) following standard protocols [30]. Primary human pre-B-ALL blasts were grown in Iscove's modified Dulbecco's medium (IMDM; Lonza) supplemented with 1 µg/mL bovine insulin (Sigma-Aldrich, Taufkirchen, Germany), 50 µM β-mercaptoethanol, 200 µg/mL Fe^{3+}-saturated human apo-transferrin (Invitrogen, Karlsruhe, Germany), 0.6% human serum albumin (Sanquin, Amsterdam, The Netherlands), 2.0 mM L-glutamine, and 20 µg/mL cholesterol [31]. Human NK-92 cells (kindly provided by NantKwest, Inc., Culver City, CA) were cultured in X-VIVO 10 (Lonza) containing 5% heat-inactivated human plasma (German Red Cross Blood Donation Service Baden-Württemberg - Hessen) and 100 IU/mL IL-2 (Proleukin; Novartis Pharma).

Generation of CAR-expressing NK-92 cells

CARs 63.z, 63.28.z and 63.137.z consist of an immunoglobulin heavy chain signal peptide, a scFv fragment of CD19-specific antibody FMC63 [32], and a modified CD8α hinge region, either followed by CD3ζ transmembrane and intracellular domains (CAR 63.z), CD28 transmembrane and intracellular domains and CD3ζ intracellular domain (CAR 63.28.z), or CD137 (4-1BB) transmembrane and intracellular domains and CD3ζ intracellular domain (CAR 63.137.z). Codon-optimized CAR sequences were synthesized (GeneArt, Thermo

Fisher Scientific, Braunschweig, Germany) and inserted into lentiviral transfer plasmid pHR'SIN-cPPT-SIEW (pSIEW) upstream of IRES and EGFP sequences [33], yielding the vectors pS-63.z-IEW, pS-63.28.z-IEW, and pS-63.137.z-IEW. As a control, the CAR signaling domains were removed from the pS-63.28.z-IEW construct by digestion with *Nae*I and religation, with the resulting pS-63.TM-IEW vector containing a premature stop codon following the transmembrane domain of CD28. VSV-G pseudotyped vector particles were produced using HEK 293T cells and NK-92 cells were transduced as described [25, 34]. EGFP-expressing NK-92 cells were enriched by flow cytometric cell sorting with a FACSAria fluorescence-activated cell sorter (BD Biosciences, Heidelberg, Germany). CAR surface expression in the obtained cell pools was confirmed by flow cytometry with Myc-tag-specific antibody (9E10; Santa Cruz, Heidelberg, Germany) using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with CellQuest Pro software (BD Biosciences).

CD19 expression and cytotoxicity assays

CD19 expression on the surface of target cells was analyzed by flow cytometry using FITC-coupled CD19-specific antibody (SJ25-C1; Invitrogen). Cytotoxicity of NK-92 cells towards established cancer cell lines and primary human pre-B-ALL blasts was analyzed in FACS-based assays as described [34]. Target cells were labeled with calcein violet AM (CV) (Molecular Probes, Invitrogen) and incubated with effector cells at various effector to target (E/T) ratios for 2 h at 37°C. Then 150 µL of a 1 µg/mL propidium iodide (PI) solution were added to each sample before flow cytometric analysis in a FACSCanto II flow cytometer (BD Biosciences). Dead target cells were identified as CV and PI double positive. Spontaneous target cell lysis in the absence of NK cells was subtracted to calculate specific cytotoxicity. Data were analyzed using FACSDiva software (BD Biosciences).

Degranulation assay and conjugate analysis

Degranulation of NK cells upon exposure to target cells at an E/T ratio of 1:1 for 1 h at 37°C was analyzed as described [35], detecting surface expression of lysosomal-associated

membrane protein LAMP-1 (CD107a) using BD FastImmune CD107a APC antibody (BD Biosciences) according to the manufacturer's instructions. As controls, NK cells were stimulated with 1 µg/mL phorbol 12-myristate 13-acetate (PMA) and 1 µg/mL ionomycin (Sigma-Aldrich), or incubated without target cells. Conjugate formation and redistribution of cytotoxic granules were assessed by confocal laser scanning microscopy. NK cells and target cells were mixed at a 1:1 ratio and kept on poly-L-lysine-coated cover slips (Life Technologies) for 1 h at 37°C. Then the cells were fixed for 10 min with phosphate-buffered 4% formaldehyde solution and permeabilized for 5 min with 0.1% Triton X-100 in PBS. After washing and blocking of unspecific binding sites for 30 min with 10% FBS in PBS, the samples were incubated for 1 h with perforin-specific antibody (δG9; Santa Cruz Biotechnology) and Alexa Fluor 594-coupled anti-mouse secondary antibody (Invitrogen) in blocking buffer at room temperature. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). After washing with PBS, cells were embedded with Mowiol 4-88 (Roth, Karlsruhe, Germany) and analyzed using a Leica SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Cytometric bead array

Cytokine release by NK-92 cells was measured using a BD Cytometric Bead Array (CBA) and a BD FACSArray bioanalyzer (BD Biosciences) according to the manufacturer's recommendations. Briefly, 5 x 10^5 NK-92 cells were co-cultured for 6 h at 37°C with target cells at an E/T ratio of 1:1 in a total volume of 1 mL. Cytokine concentrations in supernatants were measured using BD CBA Flex Sets for IFN-γ, IL-10, GM-CSF and MIP-1α/CCL3 (all BD Biosciences). Data were analyzed with BD FCAP Array software (BD Biosciences).

***In vivo* lymphoma model**

Raji Burkitt's lymphoma cells were transduced with VSV-G pseudotyped SEW-luc2 lentiviral vector encoding firefly luciferase and EGFP linked via a 2A peptide [36], and EGFP-positive cells were enriched by flow cytometric cell sorting. Six to 8 week old male NOD-SCID IL2R

γ^{null} (NSG) mice were intravenously (i.v.) injected with 1 x 10^3 Raji/Luc cells. At days 3, 4, 8, 11, 13 and 22 after tumor cell inoculation animals were treated by i.v. injection of 1 x 10^7 NK-92/63.z or parental NK-92 cells. Control mice received PBS. Disease development was monitored by imaging with an IVIS Lumina II *in vivo* imaging system (Perkin Elmer, Rodgau, Germany) 10 min after intraperitoneal injection of 75 mg/kg of D-luciferin (Promega, Mannheim, Germany). For *in vivo* experiments all applicable guidelines for the care and use of animals were followed. All animal experiments were approved by the responsible government committee (Regierungspräsidium Darmstadt, Darmstadt, Germany).

Immunohistochemistry

Liver tissue of sacrificed mice was fixed with phosphate-buffered 4% formaldehyde solution (Roth) and paraffin-embedded. Sections of 3 µm thickness were deparaffinized and hydrated, and then stained using a standardized staining protocol (Bond Polymer Refine IHC protocol, IHC-F; Leica Microsystems) with CD19-specific antibody (HIB19, 1:100; eBioscience, Frankfurt, Germany) and polymeric HRP-conjugated anti-mouse secondary antibody (DAB Polymer Refine Detection Kit; Leica Microsystems). Slides were then stained with hematoxylin and embedded in mounting medium.

Statistical Analysis

Data were analyzed by two-tailed unpaired Student's *t*-test. *P* values < 0.05 were considered statistically significant. Prism 5 software (GraphPad Software, La Jolla, CA) was used for statistical calculations.

Results

Lentiviral transduction of NK-92 cells results in surface expression of CD19-specific chimeric antigen receptors

For comparative analysis of different protein designs, we generated codon-optimized CAR sequences which all harbor a CD19-specific scFv fragment derived from antibody FMC63 [32], linked to human CD3ζ (CAR 63.z), or composite CD28-CD3ζ (CAR 63.28.z) or CD137-CD3ζ (CAR 63.137.z) signaling domains via a CD8α hinge region [25, 34]. A corresponding truncated CAR (CAR 63.TM) containing the transmembrane domain of CD28 but lacking intracellular signaling domains served as a control. The CAR sequences were inserted into the self-inactivating lentiviral vector pSIEW, where they are encoded under the control of the Spleen Focus Forming Virus (SFFV) promoter and coexpressed with an EGFP marker gene (Figure 1A). VSV-G pseudotyped lentiviral vector particles were produced and used for transduction of human NK-92 cells. CAR NK-92 cells were enriched by two rounds of flow cytometric cell sorting resulting in homogeneous EGFP-positive NK cell populations (Figure 1B, upper panels), with highest CAR surface expression found for NK-92/63.28.z cells, more moderate expression for NK-92/63.z cells and less pronounced expression for NK-92/137.z cells (Figure 1B, lower panels).

CAR composition influences cytotoxic activity against established cancer cells

Cytotoxic potential and specificity of CAR NK-92 cells were first assessed using established human Raji and WSU-NHL lymphoma, BV173 leukemia and C1R-neo B-cell lymphoblastoid cells as targets that express varying levels of CD19 (Figure 2A). CD19 highly positive Raji and BV173 cells showed no or only moderate sensitivity to parental NK-92 cells after 2 hours of co-incubation, but were readily killed by NK-92/63.z, NK-92/63.28.z and NK-92/137.z cells (Figure 2B, left panels). Interestingly, especially at low E/T ratios of 5:1 and 1:1, NK-92/63.z cells displayed more potent cell killing activity than NK-92/63.28.z cells, with NK-92/137.z cells being the least effective of the three CAR NK cell populations. This was even more

apparent for intrinsically NK-sensitive WSU-NHL and C1R-neo target cells, which express considerably lower levels of CD19 but were also most effectively killed by NK-92/63.z cells (Figure 2B, right panels). Cytotoxicity of NK-92 cells expressing the second-generation CARs 63.28.z or 63.137.z was less pronounced, in the case of C1R-neo target cells not differing significantly from that of parental NK-92 cells.

Targeted cytotoxicity depends on CD19 recognition and CAR signaling

To verify that increased cytotoxicity of CAR NK-92 cells was due to CAR-mediated recognition of CD19, similar experiments were performed with MDA-MB453 breast carcinoma cells transduced with a CD19-encoding retroviral vector (MDA-MB453/CD19) (Figure 3A). Thereby NK-92/63.z cells again displayed most potent killing of otherwise NK-resistant MDA-MB453/CD19 cells, with marked but less pronounced cytotoxicity observed for NK-92/63.28.z and NK-92/137.z cells. In contrast, CD19-negative MDA-MB453 cells were not lysed by any of the NK-92 derivatives, thus confirming CAR specificity. Furthermore, NK-92/63.TM cells which express a truncated CD19-specific CAR without signaling capabilities failed to kill MDA-MB453/CD19 cells, demonstrating that CAR-mediated signaling is required to trigger lytic activity (Figure 3B). Likewise, engagement of the functional CARs 63.z, 63.28.z and to a lesser extent 63.137.z but not of signaling-deficient CAR 63.TM induced CD19-dependent degranulation of NK-92 cells (Figure 3C). When analyzed by confocal microscopy after 1 hour of co-incubation, NK-92 cells expressing functional CARs as well as parental NK-92 and NK-92/63.TM cells had all formed contacts with MDA-MB453/CD19 cells. However, only conjugate formation between NK-92/63.z and NK-92/63.28.z with MDA-MB453/CD19 cells rapidly triggered concentration of perforin-containing cytotoxic granules at the immunological synapse (Figure 3D), confirming the reduced responsiveness of NK-92/63.137.z and the lack of activation of NK-92/63.TM and parental NK-92 cells observed in the degranulation experiments.

CAR 63.28.z induces high-level cytokine secretion

In addition to the release of cytotoxic granules, CAR activation in NK cells can trigger secretion of cytokines and chemokines [25, 26, 37]. To test cytokine production of CD19-specific CAR NK-92 cells, we co-incubated NK-92/63.z, NK92/63.28.z and NK-92/63.137.z cells with CD19-positive Raji lymphoma cells for 6 hours at an E/T ratio of 1:1 and analyzed culture supernatants using a cytometric bead array (Figure 4). Parental NK-92 and NK-92/63.TM cells were included as controls. While all NK-92 variants secreted measurable levels of the chemokine MIP-1α/CCL3 after prolonged contact with Raji cells, only NK-92/63.z and NK92/63.28.z cells produced high levels of the pro-inflammatory cytokines IFN-γ and GM-CSF. These cells also generated more pronounced levels of immunoregulatory IL-10 than parental NK-92, NK-92/63.137.z and NK-92/63.TM cells. Of note, levels of IFN-γ, GM-CSF and IL-10 produced by NK92/63.28.z cells were thereby considerably higher than those secreted by NK-92/63.z cells, despite the superior activity of the latter against Raji cells observed in cytotoxicity assays (see Figure 2B).

CAR NK-92 cells lyse NK-resistant primary B-ALL blasts

Next we analyzed a panel of primary pre-B-ALL blasts previously shown to retain the characteristics of the original tumors during long-term culture [31]. All primary target cells investigated expressed elevated surface levels of CD19 (Figure 5A). KW, DW and BV pre-B-ALL cells displayed pronounced resistance to parental NK-92 cells after 2 hours of co-incubation. In contrast, even at low E/T ratios of 5:1 and 1:1, the primary cells were readily killed by NK-92/63.z and NK-92/63.28.z, and to a lesser extent by NK-92/63.137.z cells (Figure 5B), confirming the observations made with established lymphoma and leukemia cell lines as targets.

NK-92/63.z cells inhibit *in vivo* lymphoma growth

Due to their enhanced antitumoral activity *in vitro*, NK-92/63.z cells were chosen for evaluation of the *in vivo* activity of CD19-specific CAR NK-92 cells in an aggressive

lymphoma xenograft model in NOD-SCID IL2R γ^{null} (NSG) mice. Animals were intravenously injected with 1 x 10^3 luciferase-expressing Raji cells. At days 3, 4, 8, 11, 13 and 22 after tumor cell inoculation, the mice were treated by intravenous injection of 1 x 10^7 parental NK-92 or CAR-expressing NK-92/63.z cells. Control animals received PBS. Lymphoma development was monitored at frequent intervals by *in vivo* bioluminescence imaging (BLI) (Figure 6A, B). While parental NK-92 cells had little effect on tumor development in comparison to PBS treatment resulting until day 32 in marked lymphoma growth in liver, joints and lymph nodes (Figure 6A), therapy with NK-92/63.z cells was effective and significantly delayed disease development, with remaining lymphoma mainly restricted to small areas in the liver. This was confirmed by immunohistochemical analysis demonstrating heavy infiltration of the livers of mice treated with parental NK-92 cells or PBS with CD19-positive lymphoma cells, while liver tissue from NK-92/63.z-treated animals showed more confined lymphoma growth (Figure 6C).

Discussion

In this study we generated a panel of CAR-engineered NK-92 natural killer cell variants targeting the B-cell differentiation antigen CD19 and investigated their activity against lymphoma and leukemia cells in preclinical *in vitro* and *in vivo* models. Immune cells in cancer patients are often functionally compromised due to the immunosuppressive activity of the malignant cells. Hence, for adoptive cancer immunotherapy with NK cells, donor-derived allogeneic cells are being preferred which do not recognize tumor cells as 'self', thereby bypassing inhibitory signals [9, 15]. This advantage may be extended to NK-92 cells that do not express most of the inhibitory KIRs and phenotypically resemble activated NK cells [17, 38]. We and others have previously described CAR-engineered NK-92 cells which target antigens expressed by cancer cells of solid tumor origin or hematological malignancies, indicating the potential utility of this approach [22, 24, 25, 34, 39-42]. In most of these cases, however, prototypic CD3ζ-based CARs were employed without additional costimulatory protein domains such as CD28 and CD137, which are utilized in the CD19-specific constructs of CAR-T cells currently in advanced stages of clinical development and crucial for the T cells' long-term engraftment and functionality [43].

To investigate the possible contribution of CD28 and CD137 signaling to CAR-mediated activity of NK-92 cells, we generated CD19-specific CARs harboring the same codon-optimized scFv antibody fragment and CD8α hinge region, either linked to human CD3ζ, or composite CD28-CD3ζ or CD137-CD3ζ signaling domains. Gene-modified NK-92 cell lines obtained after lentiviral gene transfer and flow cytometric cell sorting displayed homogeneous and stable CAR surface expression, and showed high and specific cytotoxicity against CD19 overexpressing established lymphoma and leukemia cells that were resistant or only moderately sensitive to parental NK-92 cells. This confirms earlier reports with NK-92 cells expressing a CD19-specific first-generation CAR based on murine CD3ζ [22, 28, 29], and demonstrates that the human signaling domains, as expected, are functionally active. Importantly, NK-92/63.z, NK-92/63.28.z and NK-92/63.137.z cells even at low E/T ratios also

displayed marked cell killing activity against primary human pre-B-ALL blasts, which were completely resistant to parental NK-92 cells. Thereby recognition of CD19 and CAR activation were required for specific cytotoxicity as evidenced by the observed lysis of otherwise NK-resistant MDA-MB453 breast carcinoma cells upon ectopic expression of CD19, and absence of cell killing if a CD19-specific CAR without signaling domains was employed. Likewise, contact of NK-92/63.z and NK-92/63.28.z cells, and to a lesser extent of NK-92/63.137.z cells with MDA-MB453/CD19 cells triggered NK-cell degranulation, which was not observed with parental NK-92 and NK-92/63.TM cells.

For CD19-targeted primary NK cells expanded on K562 feeder cells expressing membrane-anchored IL-15 and 4-1BB ligand, inclusion of costimulatory CD137 (4-1BB) or CD244 (2B4) domains in the CAR in addition to CD3ζ was shown to enhance both, specific cytotoxicity and production of IFN-γ and GM-CSF [21, 23]. In our study, NK-92/63.137.z cells were less effective than NK-92/63.z and NK-92/63.28.z in cell killing and cytokine production, pointing towards a differential requirement for costimulatory signals between primary NK cells and established NK-92. While we observed significantly increased cytokine production by NK-92/63.28.z in comparison to NK-92/63.z cells, there was no enhancement in cell killing if the CD28 domain was present. In contrast, depending on the type of target cells cytotoxicity of NK-92/63.28.z cells was in most cases less pronounced than that of NK-92/63.z. This may in part be explained by differences in the threshold for CAR signaling. CAR 63.z utilizes the endogenous transmembrane domain of CD3ζ, which allows formation of disulfide-linked CAR homodimers and heterodimers of CAR and endogenous CD3ζ [24, 25, 39]. Such preformed receptor complexes may get activated more rapidly and by lower target antigen densities than CAR 63.28.z that utilizes the transmembrane domain of CD28 and cannot form covalent dimers. Also sterical effects can play a role in determining the activation threshold of individual CARs [44]. While CARs 63.z and 63.28.z contain the same scFv antibody fragment and CD8α hinge domain, their different membrane anchors may influence flexibility and access to the target antigen.

Due to their enhanced cytotoxic activity *in vitro*, NK-92/63.z cells were chosen for further evaluation in a Raji lymphoma xenograft model in NSG mice. While repeated systemic injections of parental NK-92 cells had little effect resulting in extensive lymphoma growth in liver, joints and lymph nodes, disease development was controlled to a large extent by NK-92/63.z cells demonstrating that they retained specific antitumor activity *in vivo*. In this immunodeficient mouse model analysis is restricted to direct antitumor effects of the CAR NK cells. Nevertheless, in a clinical setting also interaction with endogenous immune cells may contribute to their therapeutic activity. Thereby production of cytokines such as IFN-γ, GM-CSF and the chemokine MIP-1α (CCL3) as particularly observed after exposure of NK-92/63.28.z cells to CD19-positive targets will likely be important for NK-mediated enhancement of adaptive immunity [45]. Indeed, in a recent study treatment with NK-92 cells expressing an ErbB2/HER2-specific CAR with CD28 and CD3ζ domains in addition to direct antitumor effects induced long-lasting endogenous antitumor immunity in an immunocompetent glioblastoma model [26].

As a truly off-the-shelf therapeutic, continuously expanding CAR NK-92 cells may become clinically useful for the treatment of cancer patients especially in cases where the infrastructure or suitable donors for a CAR T cell approach are not available. Our data demonstrate high and consistent cytotoxic activity of NK-92 cells expressing CD19-specific CARs that employ CD3ζ or CD28-CD3ζ signaling domains against established and primary B-cell lymphoma and leukemia cells, while a similar CD137-CD3ζ CAR was functional but less effective. At low E/T ratios NK-92/63.z cells displayed more pronounced cell killing than NK-92/63.28.z cells, while the latter secreted higher cytokine levels upon CAR triggering. This may contribute to direct and indirect antitumor effects and should be considered during further development of this approach towards clinical applications.

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Disclosure of interest: H.K. is a co-founder and employee of NantKwest, Inc., an entity commercially developing NK-92 cells. C.Z., T.T. and W.S.W. are named as inventors on patents in the field of cancer immunotherapy owned by their respective institutions. The other authors declare that they have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Figure legends

Figure 1. Generation of CAR-engineered NK-92 cells. (A) Lentiviral transfer plasmids encoding different chimeric antigen receptors under the control of the Spleen Focus Forming Virus promoter (SFFV). The CAR sequences are followed by an internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) cDNA. In pS-63.z-IEW the CAR consists of an immunoglobulin heavy chain signal peptide (SP), a CD19-specific scFv antibody fragment, a Myc-tag (M), a CD8α hinge region (CD8α), and transmembrane and intracellular domains of CD3ζ (CAR 63.z). pS-63.28.z-IEW and pS-63.137.z-IEW vectors encode similar CARs with transmembrane and intracellular domains of CD28 and the intracellular domain of CD3ζ (CAR 63.28.z), or transmembrane and intracellular domains of CD137 (4-1BB) and the intracellular domain of CD3ζ (CAR 63.137.z), respectively. (B) Enhanced green fluorescent protein expression of sorted NK-92/63.z, NK-92/63.28.z and NK-92/63.137.z cells was determined by direct flow cytometry (upper panel, filled areas). CAR surface expression was analyzed by flow cytometry with Myc-tag-specific antibody (lower panel, filled areas). Parental NK-92 cells (open areas) and cells stained with an irrelevant isotype-matched antibody (dashed lines) served as controls.

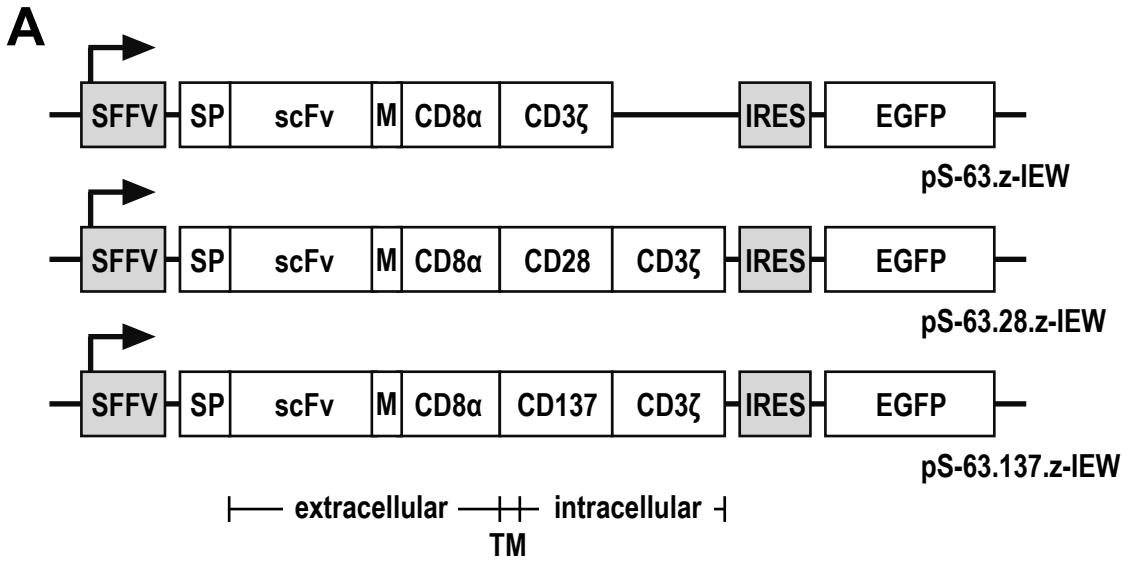
Figure 2. Specific cytotoxicity of CAR NK cells against established lymphoma and leukemia cells. (A) Expression of CD19 on the surface of Raji and WSU-NHL lymphoma, BV173 leukemia and C1R-neo B-cell lymphoblastoid cells was determined by flow cytometry with CD19-specific antibody (filled areas). Unstained cells served as controls (open areas). (B) Cell killing by parental NK-92 (open circles) and CAR-expressing NK-92/63.z (filled circles), NK-92/63.28.z (open triangles) and NK-92/63.137.z cells (filled triangles) was investigated in FACS-based cytotoxicity assays after co-incubation with target cells for 2 h at the indicated E/T ratios. Mean values ± SEM are shown; n=4. *, $p < 0.05$; ns, $p \geq 0.05$.

Figure 3. Dependence of target cell killing on CAR-mediated NK-cell activation. (A) To confirm specificity, cytotoxicity of parental NK-92 (open circles) and CAR-expressing NK-92/63.z (filled circles), NK-92/63.28.z (open triangles) and NK-92/63.137.z cells (filled triangles) was investigated after co-incubation for 2 h at the indicated E/T ratios with CD19-negative MDA-MB453 human breast carcinoma cells and MDA-MB453/CD19 cells transduced with a CD19-encoding retroviral vector as targets. (B) Cytotoxicity of CAR NK cells against MDA-MB453/CD19 cells at an E/T ratio of 10:1 was compared to that of NK-92/63.TM cells which express a truncated CD19-specific CAR without signaling domains. (C) Degranulation of NK-92/63.z, NK92/63.28.z and NK-92/63.137.z upon activation by CD19-expressing cancer cells was analyzed by flow cytometry determining CD107a surface expression after 5 h of co-culture with MDA-MB453 or MDA-MB453/CD19 target cells at an E/T ratio of 1:1. Parental NK-92 and NK-92/63.TM cells were included for comparison. NK cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Iono) served as controls. Mean values ± SEM are shown in panels a, b, c; n=5. **, $p < 0.01$; ns, $p \geq 0.05$. (D) Conjugate formation between CAR NK cells and MDA-MB453/CD19 target cells was investigated by confocal microscopy. Tumor (T) and CAR NK-92 (N) cells were co-incubated for 1 h. Cells were stained for perforin (red) to identify cytotoxic granules. Nuclei were labeled with DAPI (blue). Parental NK-92 and NK-92/63.TM cells served as controls. Scale bar: 10 µm.

Figure 4. Secretion of cytokines by CAR NK cells upon activation by CD19-expressing lymphoma cells. 5 x 10^5 NK-92, NK-92/63.z, NK92/63.28.z, NK-92/63.137.z or NK-92/63.TM cells were incubated for 6 h with Raji lymphoma cells at an E/T ratio of 1:1. Supernatants were collected and the levels of (A) IFN-γ, (B) GM-CSF, (C) MIP-1α/CCL3, and (D) IL-10 were measured using a cytometric bead array. NK cells kept without target cells were included as controls. Mean values ± SEM are shown; n=3. **, $p < 0.01$; *, $p < 0.05$; ns, $p \geq 0.05$.

Figure 5. Killing of pre-B-ALL blasts by CAR NK cells. (A) Expression of CD19 on the surface of primary pre-B-ALL blasts from three different patients (KW, DW, BV) was determined by flow cytometry with CD19-specific antibody (filled areas). Unstained cells served as controls (open areas). (B) Specific lysis of pre-B-ALL blasts by parental NK-92 (open circles) and CAR-expressing NK-92/63.z (filled circles), NK-92/63.28.z (open triangles) and NK-92/63.137.z cells (filled triangles) was investigated in FACS-based cytotoxicity assays after co-incubation with target cells for 2 h at the indicated E/T ratios. Mean values ± SEM are shown; n=3. ns, $p \geq 0.05$.

Figure 6. Inhibition of *in vivo* lymphoma growth by CD19-specific CAR NK-92 cells. NSG mice were intravenously injected with 1 x 10^3 Raji/Luc lymphoma cells. At days 3, 4, 8, 11, 13 and 22 after tumor cell inoculation, the animals were treated by intravenous injection of 1 x 10^7 parental NK-92 or CAR-expressing NK-92/63.z cells. Control mice received PBS. (A) Lymphoma development was monitored by *in vivo* bioluminescence imaging. Images taken at day 32 of the experiment are shown (exposure time of 1 sec). (B) Average radiance values measured over time. Mean values ± SEM are shown; n=4; **, $p < 0.01$. (C) Sections of liver tissues from mice treated with NK-92/63.z, parental NK-92 cells or PBS were stained with CD19-specific antibody for detection of lymphoma cells. Scale bar: 500 µm.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SFFV
SP
scFv
M
CD8α
CD3ζ
IRES
EGFP
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CD28
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CD137
pS-63.137.z-IEW
extracellular
intracellular
TM

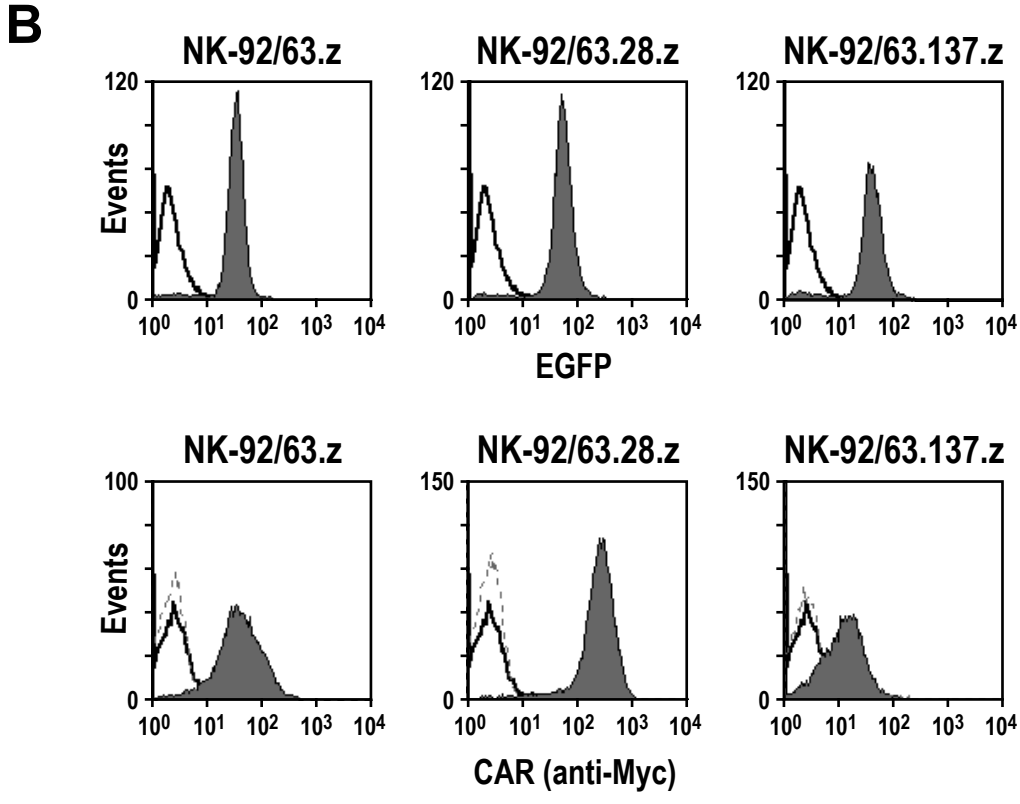
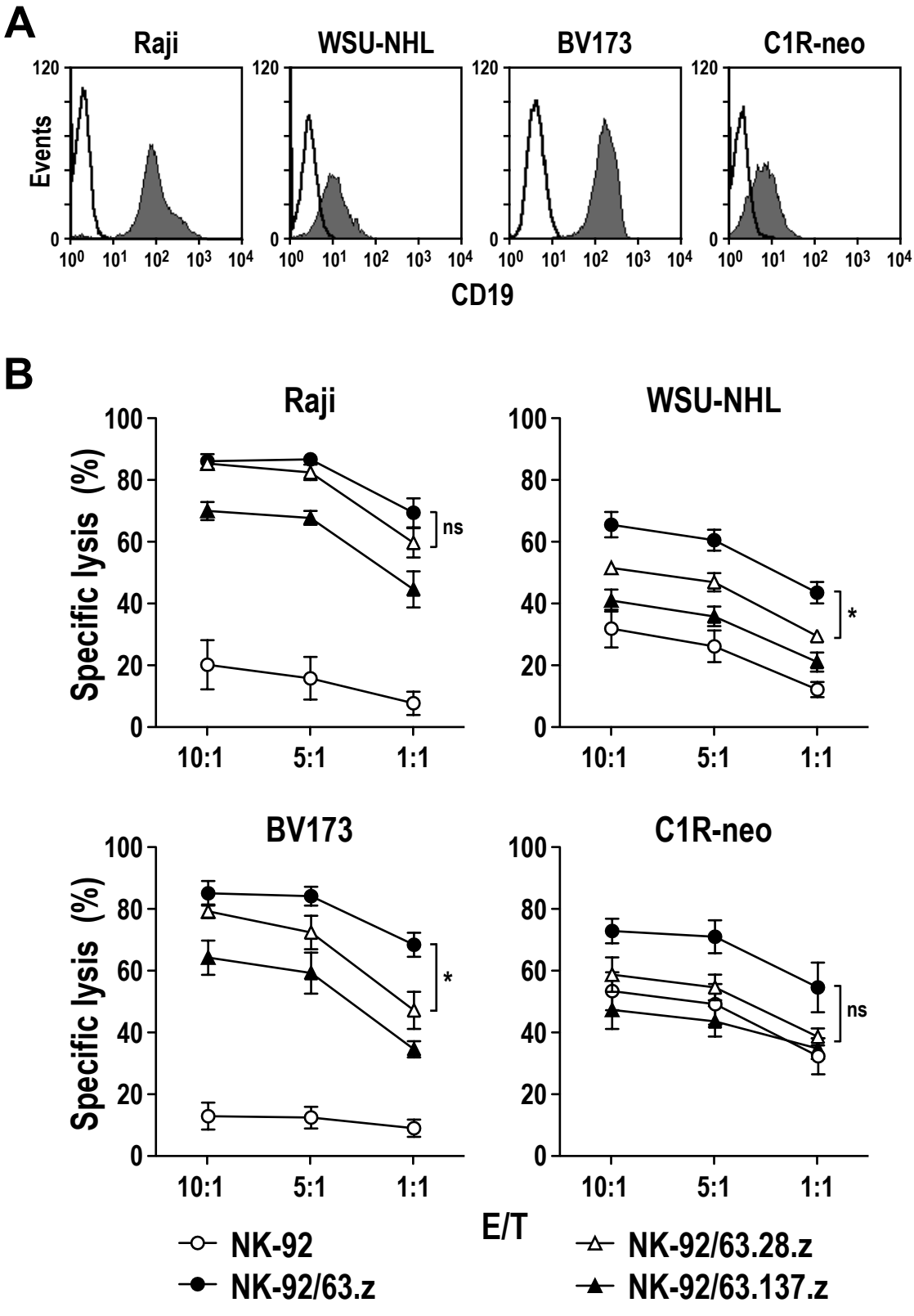

B
NK-92/63.z
NK-92/63.28.z
NK-92/63.137.z
Events
EGFP
CAR (anti-Myc)

Figure 1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Raji
WSU-NHL
BV173
C1R-neo
Events
120
0
10^0 10^1 10^2 10^3 10^4
CD19
B
Raji
WSU-NHL
BV173
C1R-neo
Specific lysis (%)
100
80
60
40
20
0
10:1
5:1
1:1
ns
*
*
ns
E/T
NK-92
NK-92/63.z
NK-92/63.28.z
NK-92/63.137.z

Figure 2

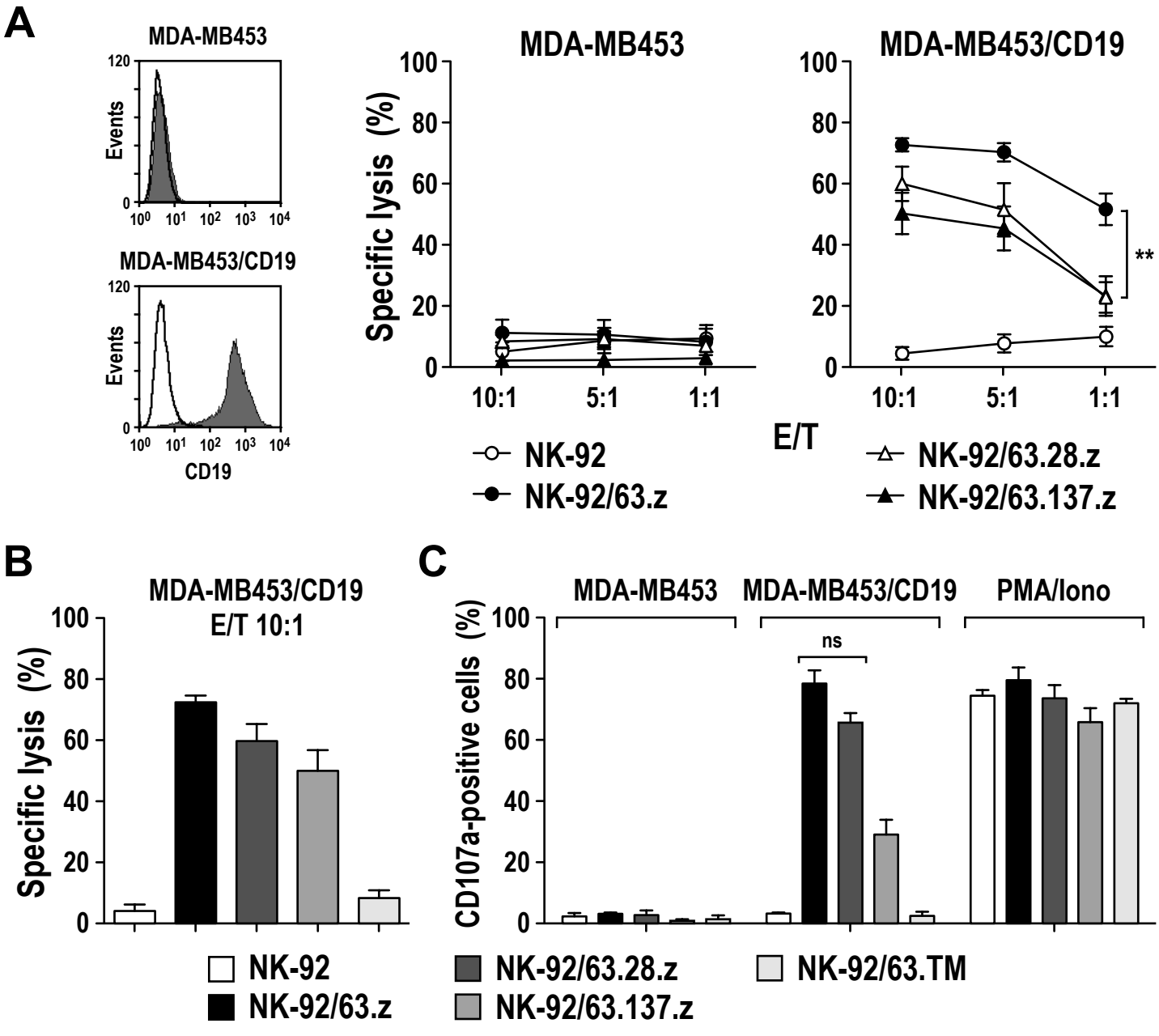
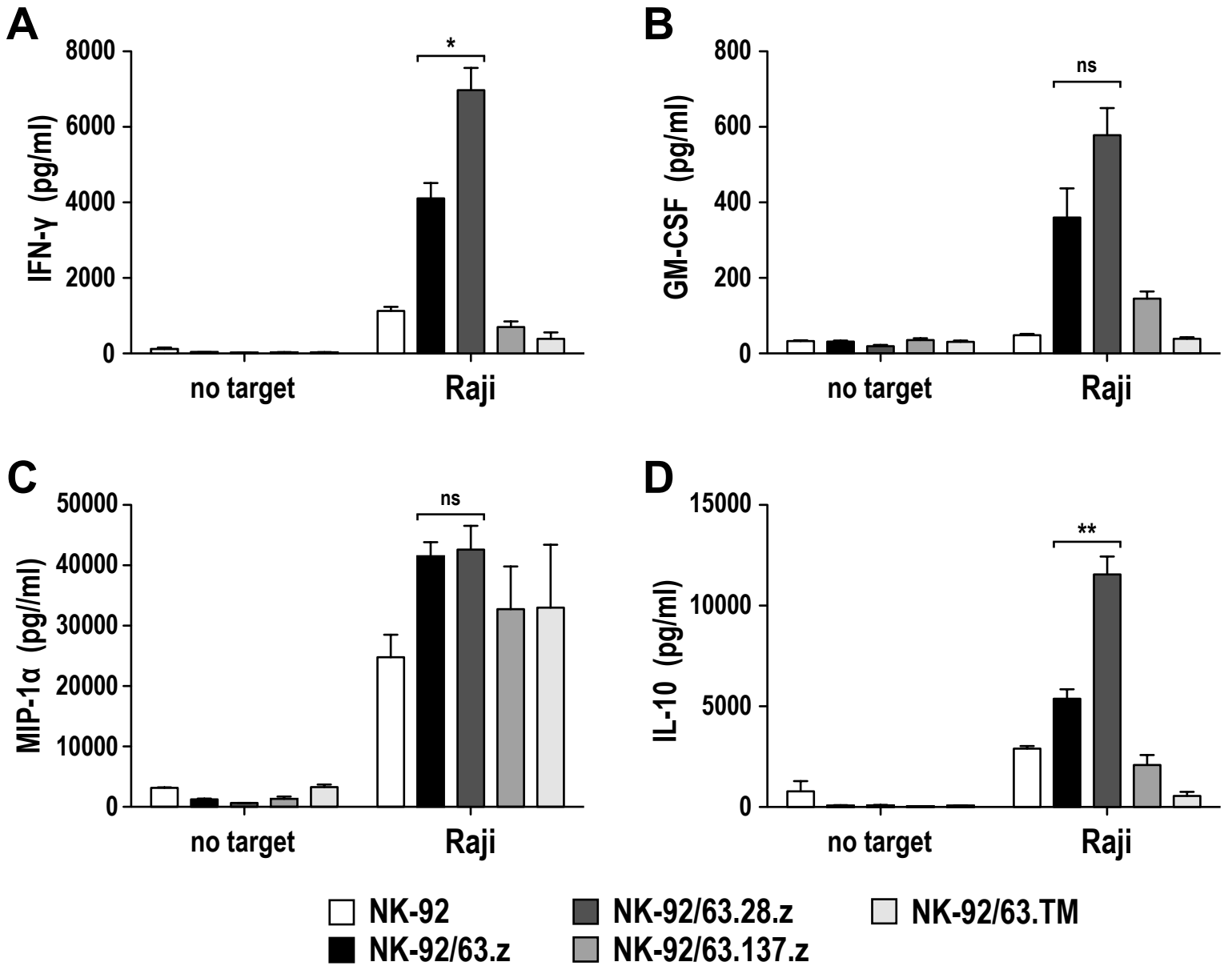

A
MDA-MB453
Events
MDA-MB453/CD19
CD19
MDA-MB453
Specific lysis (%)
MDA-MB453/CD19
**
10:1
5:1
1:1
E/T
NK-92
NK-92/63.z
NK-92/63.28.z
NK-92/63.137.z
B
MDA-MB453/CD19
E/T 10:1
Specific lysis (%)
NK-92
NK-92/63.z
C
MDA-MB453
MDA-MB453/CD19
PMA/Iono
ns
CD107a-positive cells (%)
NK-92/63.28.z
NK-92/63.137.z
NK-92/63.TM
D
NK-92
NK-92/63.z
NK-92/63.28.z
NK-92/63.137.z
NK-92/63.TM
N
T

Figure 3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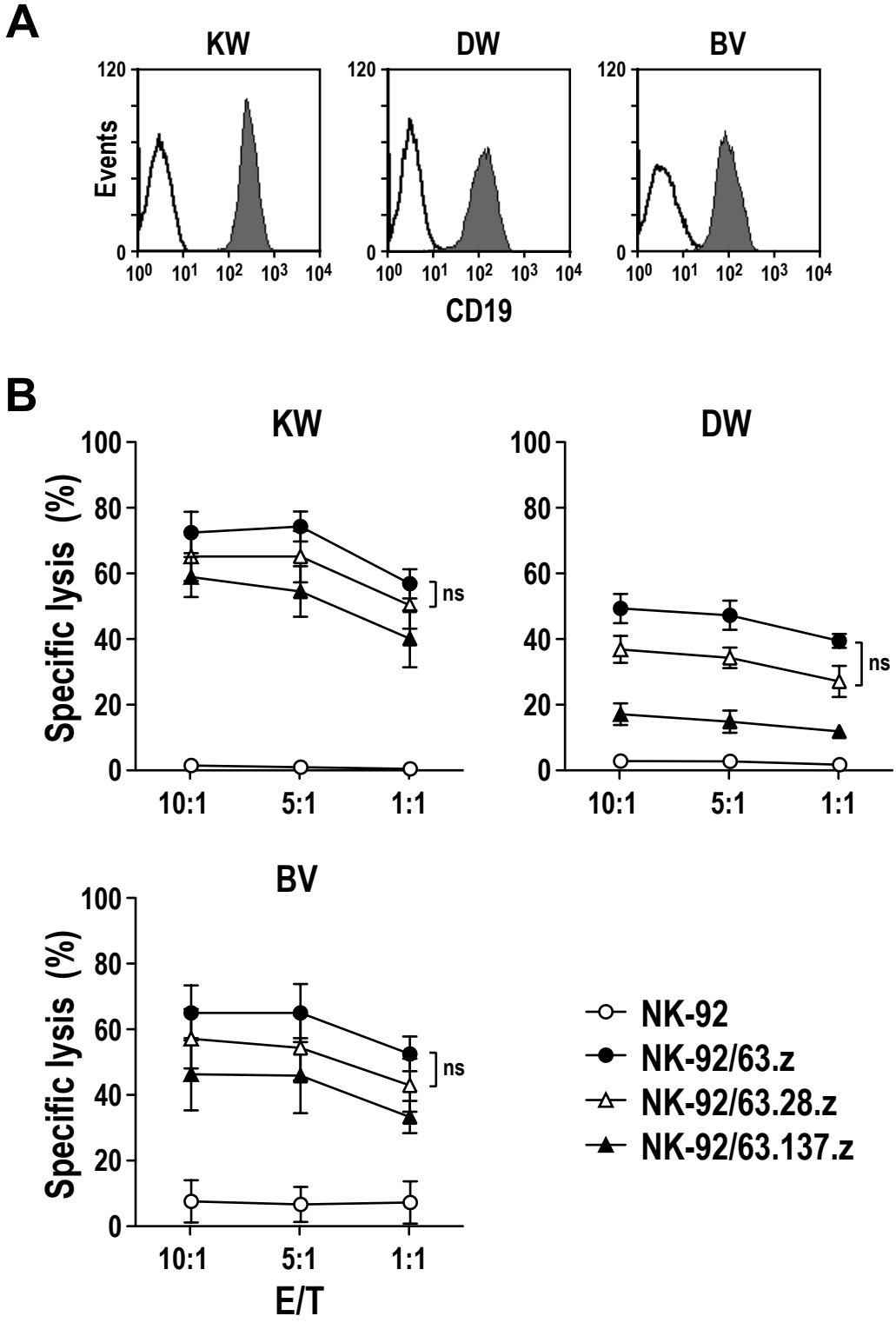
6000
4000
2000
0
IFN-γ (pg/ml)
*
no target
Raji
B
800
600
400
200
0
GM-CSF (pg/ml)
ns
no target
Raji
C
50000
40000
30000
20000
10000
0
MIP-1α (pg//ml)
ns
no target
Raji
D
15000
10000
5000
0
IL-10 (pg/ml)
**
no target
Raji
NK-92
NK-92/63.z
NK-92/63.28.z
NK-92/63.137.z
NK-92/63.TM

Figure 4


A
KW
DW
BV
120
Events
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10^0 10^1 10^2 10^3 10^4
CD19
B
KW
DW
BV
Specific lysis (%)
100
80
60
40
20
0
10:1
5:1
1:1
ns
E/T
NK-92
NK-92/63.z
NK-92/63.28.z
NK-92/63.137.z

Figure 5

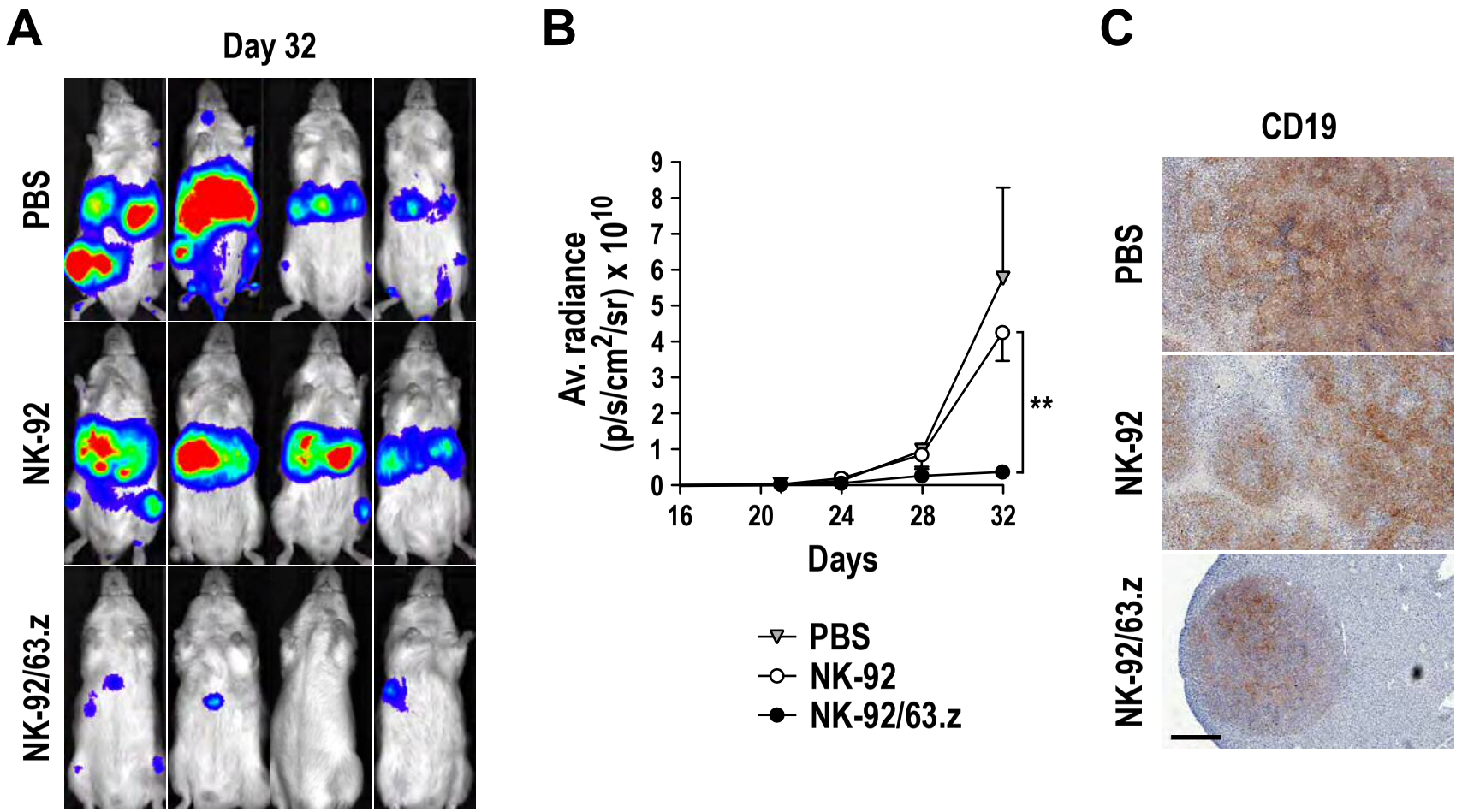
A
Day 32
PBS
NK-92
NK-92/63.z
B
Av. radiance (p/s/cm²/sr) x 10¹⁰
9
8
7
6
5
4
3
2
1
0
16
20
24
28
32
Days
**
PBS
NK-92
NK-92/63.z
C
CD19
PBS
NK-92
NK-92/63.z

Figure 6