# 2B4 (CD244) Signaling by Recombinant Antigen-specific Chimeric Receptors Costimulates Natural Killer Cell Activation to Leukemia and Neuroblastoma Cells

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**Abstract**

**Purpose:** Novel natural killer (NK) cell–directed strategies in cancer immunotherapy aim at specifically modulating the balance between NK cell receptor signals toward tumor-specific activation. The signaling lymphocyte activation molecule–related receptor 2B4 (CD244) is an important regulator of NK cell activation. We investigated whether 2B4-enhanced activation signals can redirect the cytolytic function of human NK cells to NK cell–resistant and autologous leukemia and tumor targets.

**Experimental Design:** *In vitro*–stimulated NK cells from healthy donors and pediatric leukemia patients were gene modified with CD19 or $G_{D2}$-specific chimeric receptors containing either the T-cell receptor ζ or 2B4 endodomain alone or combined.

**Results:** Chimeric 2B4 signaling alone failed to induce interleukin-2 receptor up-regulation and cytokine secretion but triggered a specific degranulation response. Integration of the 2B4 endodomain into T-cell receptor ζ chimeric receptors significantly enhanced all aspects of the NK cell activation response to antigen-expressing leukemia or neuroblastoma cells, including CD25 up-regulation, secretion of IFN-γ and tumor necrosis factor-α, release of cytolytic granules, and growth inhibition, and overcame NK cell resistance of autologous leukemia cells while maintaining antigen specificity.

**Conclusion:** These data indicate that the 2B4 receptor has a potent costimulatory effect in NK cells. Antigen-specific 2B4ζ-expressing NK cells may be a powerful new tool for adoptive immunotherapy of leukemia and other malignancies.

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Besides antigen-specific CTLs, cellular components of the innate immune system can contribute to immune surveillance of malignant cell growth. In particular, natural killer (NK) cells can eliminate abnormal cells without priming or sensitization (1). Their activity is determined by the balance of signals from inhibitory and activating NK cell receptors. Inhibitory receptors [e.g., killer immunoglobulin receptors (KIR)] interact with self-MHC class I antigens and protect normal cells from NK cell attack. Many malignant cells express MHC class I antigens and are thus naturally resistant to lysis by autologous NK cells. Accordingly, the first clinical trials using adoptive transfer of autologous NK cells have failed to produce significant therapeutic effects (2, 3). For these reasons, NK cell–based immunotherapies have mostly focused on the haploidentical hematopoietic stem cell transplantation setting, where KIR:MHC class I mismatches between the donor and recipient were found by many investigators to contribute to the control of residual myeloid leukemia cells (4, 5). Increasing awareness of the role of activating receptors in the recruitment of NK effector functions has motivated new efforts to target autologous malignancies. Indeed, engagement of activating NK cell receptors by ligands expressed on tumor cells can overcome inhibitory signals and stimulate NK cell responses even in the presence of autologous MHC class I (6, 7). This mechanism is efficiently counteracted in many human tumors, where cells evade NK cell–mediated killing by shedding or intracellular retention of ligands for activating receptors (8). Furthermore, inhibitory cytokines secreted within the malignant microenvironment result in systemic downmodulation of either NK cell receptors or their ligands in cancer patients, further contributing to NK cell resistance (9). Attempts at increasing the susceptibility of malignant cells to NK cell–mediated lysis have focused on modulating the balance between inhibitory and activating NK cell receptor signals using agonistic cytokines or drugs (6, 10).

An alternative approach is the genetic modification of NK cells with chimeric receptors (CARs) that retarget cellular activation

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### Translational Relevance

In recent years, natural killer (NK) cell strategies for cancer immunotherapy have stimulated increasing interest but their clinical application has largely been restricted to the haploidentical hematopoietic stem cell transplantation setting, where natural killer immunoglobulin receptor mismatches between the donor and recipient are exploited to enhance the graft-antileukemia effect. In this article, we describe a gene engineering strategy that efficiently modulates the balance of human NK cell signals toward tumor-specific activation. This strategy promises to extend the use of NK cells in cancer therapy beyond allogeneic mismatched hematopoietic stem cell transplantation. Due to the high selectivity of the gene-modified NK cells for their tumor targets, NK cells from fully matched donors as well as autologous NK cells can be used. Our data thus pave the way toward the clinical application of NK cells for the efficient and specific elimination of minimal residual tumor cells in various malignancies.

pathways to tumor surface antigens. Tumor-specific CARs were first established in T cells (11). They consist of an antibody-derived single-chain Fv domain linked to a cytoplasmic signaling domain, generally derived from the T-cell receptor ζ (TCRζ) chain, thereby redirecting T-cell effector function to a defined target antigen. Expression of ζ-based CARs in NK cell lines (12, 13) and primary human NK cells (14, 15) can indeed trigger powerful stimulatory signals, inducing cytolysis of otherwise NK cell–resistant tumor cell lines. In T cells, the CAR-mediated antitumor functionality was significantly enhanced when integrated costimulatory signaling components were provided (16), emphasizing the importance of considering the full activation requirements of the effector cell for optimal CAR design. The stimulatory needs for overcoming the inhibitory effects of autologous MHC recognition in NK cells have not yet been established in detail. However, evidence exists that the NK cell activation response can be positively modulated by the combination of multiple (co) stimulating signals (17).

The signaling lymphocyte activation molecule–related receptor 2B4 (CD244) is an important immune regulator, mediating potent stimulatory and costimulatory signals in both T cells and NK cells (18–20). Thus, 2B4 could potentially enhance signaling in NK cells retargeted to malignant cells. Using $CD19^+$ B-cell precursor acute lymphoblastic leukemia (ALL) and neuroblastoma as models, we investigated the capacity of antigen-specific CARs with integrated 2B4 signaling components to overcome resistance of malignant cells against NK cell lysis.

## Materials and Methods

*Cell lines.* The packaging cell line Phoenix-ampho (21) was provided by Gary P. Nolan (Stanford University, Stanford, CA). FLYRD18 (provided by E. Vanin, Northwestern University, Chicago, IL) is an amphotropic retrovirus packaging cell line that provides viral recombinants with the RD114 envelope. REH and SUP-B15 (both from the American Type Culture Collection) are ALL cell lines, and ML-2 (American Type Culture Collection) is a human acute myelomonocytic leukemia cell line. JF (kindly provided by Malcolm K. Brenner, Baylor College of Medicine, Houston, TX) is a human neuroblastoma cell line. K562 (American Type Culture Collection) is a human erythroleukemia line that is sensitive to lysis by NK cells. The generation of the K562-mb15-41BBL stimulator cells was described previously (14).

*Constructs.* All CD19-specific CARs contain the single-chain Fv antibody domain of the monoclonal antibody FMC-63 (22). The gene fragment encoding the transmembrane and cytoplasmic domains of 2B4 was cloned by PCR from cDNA obtained from peripheral blood mononuclear cells (PBMC) of a healthy donor. To generate 19-2B4ζ, the 2B4 gene fragment was subcloned upstream of the cytoplasmic domain of the *TCRζ* gene while replacing the transmembrane domains of ζ. For 19-2B4, the entire ζ fragment was replaced by the transmembrane and cytoplasmic domains of 2B4. The same strategy was applied to generate 14.G2a-2B4ζ from the previously published $G_{D2}$-specific CAR 14.G2a-ζ (23). The truncated fragment t2B4 was generated by site-directed mutagenesis as previously described by Eissmann et al. (24) by inserting a stop codon at base pair number 892 of wild-type human 2B4 where the A of the start codon is base pair number one. To generate 19-t2B4ζ, the truncated 2B4 gene fragment up to base pair number 891 was subcloned upstream of the cytoplasmic domain of the ζ gene. All CAR genes were subcloned into the *Age*I and *Not*I sites of the retroviral vector SFG-IRES-GFP, which was generated by M. Pule by inserting the IRES-GFP expression cassette into the retroviral vector SFG (provided by R.C. Mulligan, Harvard Medical School, Cambridge, MA; ref. 25). A previously published construct with FMC-63 monoclonal antibody–derived CD19 specificity, the transmembrane domain of CD8α and the intracellular domains of 41BB and CD3ζ (26), was used for comparative experiments.

*Production of recombinant retrovirus.* To generate stable retroviral producer cell lines, fresh retroviral supernatants collected from transiently transfected Phoenix-ampho cells were used to infect the packaging cell line FLYRD18 by overnight incubation at 37°C in the presence of polybrene (4 μg/mL). Viral supernatants were generated on the resulting bulk producer cell line by adding Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 20% FCS. After 24 h of incubation at 32°C, the supernatants were filtered through a 0.45-μm filter and used to transduce the NK cells.

*Expansion and transduction of human NK cells.* Approval for using peripheral blood samples of both healthy donors and pediatric leukemia patients was obtained from the University of Muenster Ethical Board. PBMCs were incubated at $1 \times 10^6$ per well in a 24-well tissue culture plate in the presence of 40 IU/mL recombinant human interleukin-2 (rhIL-2) in RPMI 1640 and 10% FCS and stimulated once with $0.75 \times 10^6$ irradiated (120 Gy) K562-mb15-41BBL stimulator cells, as previously described (14). For retroviral transductions, the cells were transferred to 24-well nontissue culture–treated plates coated with recombinant fibronectin fragment FN CH-296 (Retronectin, Takara Shuzo) at 4 μg/$cm^2$ and coincubated with viral supernatant for 48 h followed by expansion in the presence of rhIL-2 (40 IU/mL).

*Flow cytometry.* Transduction efficiencies with CD19 constructs were determined by flow cytometric identification of GFP-expressing cells. Surface expression of 14.G2a-ζ and 14.G2a-2B4ζ was analyzed by staining with a biotinylated goat anti-mouse antibody specific for IgG $F(ab')_2$ fragment (Jackson ImmunoResearch) and secondary phycoerythrin (PE)-labeled streptavidin antibody (Becton Dickinson). For immunophenotyping, cells were stained with fluorescein-conjugated monoclonal antibodies directed against CD3, CD56, CD16 (Becton Dickinson), CD19, CD48 (Immunotools), MHC I (Dako), and CD244 (eBioscience) surface proteins. For each sample, 20,000 cells were analyzed with FACSCalibur and CellQuest software (Becton Dickinson). Up-regulation of CD25 in response to interaction with tumor targets was determined by staining of stimulated NK cells with

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PE-marked CD25 antibody (Becton Dickinson) and subsequent analysis of PE-positive cells within the $GFP^{+}CD56^{+}CD3^{-}$ gate.

***Intracellular cytokine assay.*** NK cells were seeded at $1 \times 10^6$ per well in a 24-well plate and stimulated with $1 \times 10^6$ irradiated tumor target cells for 4 h. Cytokine secretion was blocked with 10 μg brefeldin A (Sigma) per $2 \times 10^6$ cells for 2 h. Permeabilization of the cells was done using a proprietary solution (Becton Dickinson). Cells were stained with IFN-γ–specific and tumor necrosis factor-α (TNF-α)–specific antibodies according to the manufacturer's recommendations.

***CD107a assay.*** The cytolytic ability of transduced NK cells was assessed by flow cytometric analysis of CD107a expression after 4 h of coincubation with various tumor cell targets at a 1:1 stimulator to responder ratio in the presence of PE-labeled CD107a antibody (Becton Dickinson). After 1 h, 5 μL of 2 mmol/L monensin (Sigma) were added. The NK cells were washed and stained with PE-Cy7–labeled anti-CD56 antibody followed by analysis of PE-positive cells within the $GFP^{+}CD56^{+}$ gate.

***Long-term cytotoxicity assays.*** To allow for quantification of cytotoxicity by the low numbers of transduced effector cells obtained after fluorescence-activated cell sorting (FACS) purification from patient samples, sensitive 16-h coincubation assays were used. Target cells ($2 \times 10^5$) were seeded into 96-well U-bottomed tissue culture plates and incubated with NK cells at various E:T ratios. JF neuroblastoma cells were labeled with carboxyfluorescein diacetate succinimidyl ester before coincubation. Transduced NK cells were purified by cell sorting based on either GFP expression or F(ab) positivity using a FACSVantage (Becton Dickinson) before incubation. Cells were harvested after 16 h. The percentages of viable leukemia cells were determined by staining with PE-labeled CD19 antibody (Immunotools), and viable JF cells were assessed by carboxyfluorescein diacetate succinimidyl ester staining. To adequately consider the number of leukemia cells undergoing spontaneous apoptosis, the numbers of target cells recovered from cultures without NK cells were used as a reference.

***Statistical analysis.*** The Student's $t$ test was used to test for significance in each set of values, assuming equal variance. Mean values ± SD are given unless otherwise stated.

## Results

***Expression of chimeric receptor genes in human NK cells.*** NK cells were selectively expanded from PBMCs using a previously reported method (14). Briefly, PBMCs were stimulated with irradiated K562 cells gene modified to express membrane-bound IL-15 and 41BB ligand in the presence of low-dose (40 IU/mL) rhIL-2. Under these conditions, NK cells were selectively expanded from the peripheral blood of five healthy donors to 14- to 61-fold (mean, 30 ± 18–fold) after 10 days of culture, resulting in a relative increase of $CD3^{-}CD56^{+}$ NK cells to 76% to 95% (89.5 ± 7.0%) within the stimulated bulk populations

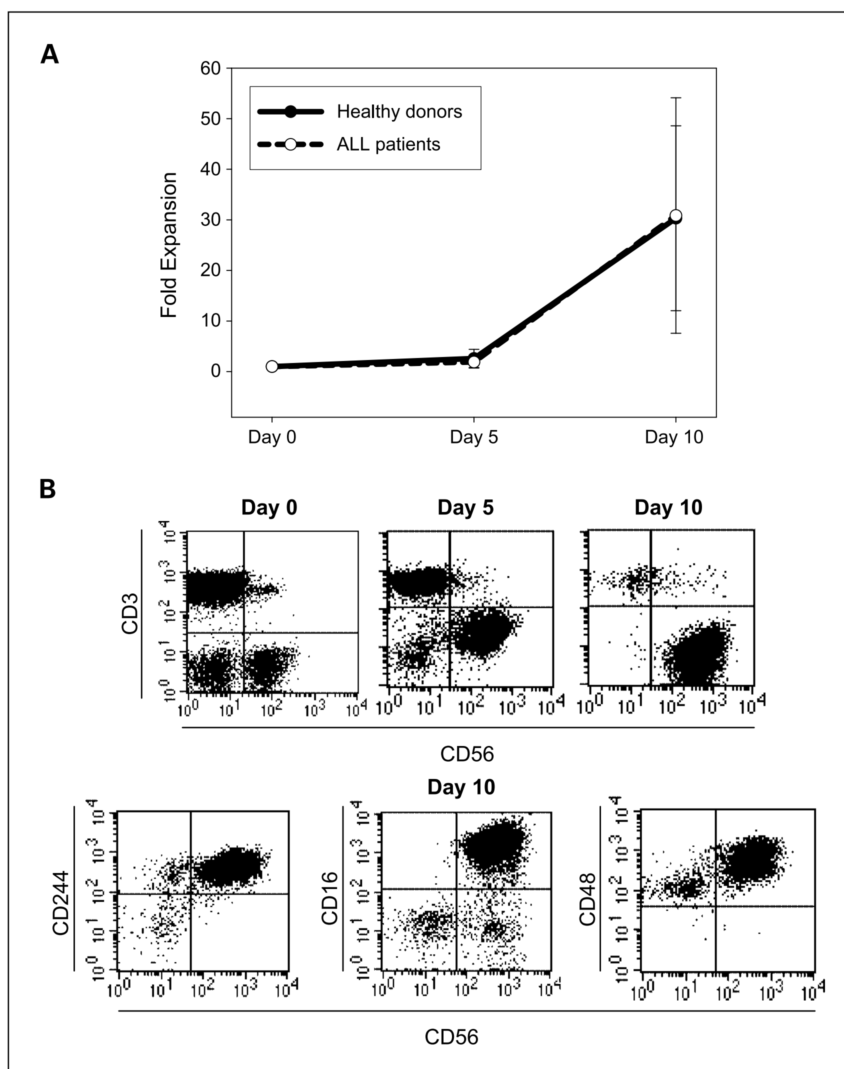


**Fig. 1.** Expansion and immunophenotypes of NK cells cocultured with K562-mb15-41BBL cells. *A,* PBMCs from five healthy donors and from four pediatric patients in first hematologic remission of B-cell precursor ALL were cocultured with irradiated K562-mb15-41BBL at a 0.75:1 ratio in the presence of rhIL-2 (40 IU/mL), and absolute numbers of NK cells were calculated every 5 d after staining of viable cells with CD3- and CD56-specific antibodies. *B,* cell surface expression of CD3 and CD56 before and on days 5 and 10 after stimulation by flow cytometry, and expression of CD56, CD16, CD244 (2B4), and CD48 on day 10 of coculture with K562-mb15-41BBL cells. Shown are representative histograms for one of five cell cultures obtained from four donors.

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on day 10 (Fig. 1A), whereas the proportions of $CD3^+$ T cells on day 10 ranged between 3.3% and 26.2% (10.2 ± 7.0%).

These data were reproduced using peripheral blood samples of four pediatric patients with $CD19^+$ B-cell precursor leukemia who were in complete hematologic remission of their disease during maintenance treatment or within the first year after therapy. Although the starting numbers of NK cells in the patients were substantially lower than in the healthy donors (1-13%, mean 6 ± 5%, versus 15-19%, mean 17 ± 1%), comparable expansion rates were obtained in five independent cell cultures (14- to 72-fold; mean, 31 ± 23–fold; Fig. 1A). The expanded cells coexpressed CD56 and $CD16^{high}$, characteristic for a highly cytolytic, activated NK cell subpopulation. Both the 2B4 receptor (CD244) and its ligand (CD48) were detected at high densities on the cell surface (Fig. 1B). Reduced 2B4 expression in NK cells has been suggested to play a role in tumor immune escape (27). In our patients, the surface expression densities of 2B4 were comparable with healthy donors at the initiation of the cultures, with mean fluorescence intensities of 113 ± 28 in patients versus 117 ± 50 in healthy donors. Stimulation and expansion resulted in 2B4 up-regulation in NK cells from both donor populations, resulting in mean fluorescence intensities of 201 ± 140 in patients and 315 ± 149 in healthy donors on day 10 (difference not statistically significant).

To compare the capacity of ζ and 2B4 for inducing functional antileukemia responses in NK cells, we generated CARs with identical extracellular specificity for the B-cell lineage antigen CD19 combined with various intracellular signaling domains (Fig. 2). In CD19-ζ, which was previously described by our group and by others (14, 22, 28, 29), the cytoplasmic signaling domain is derived from the ζ chain and thus contains three immunoreceptor tyrosine-based activation motifs. CD19-2B4 provides signaling by the full 2B4 receptor cytoplasmic domain, containing four immunoreceptor tyrosine-based switch motifs (ITSM). In CD19-2B4ζ, both endodomains are combined. Based on data showing the inhibitory effect of the third ITSM of 2B4 and the lack of significant stimulatory capacity of the fourth (24), further CARs were generated containing 2B4 endodomains truncated after the second ITSM (CD19-t2B4

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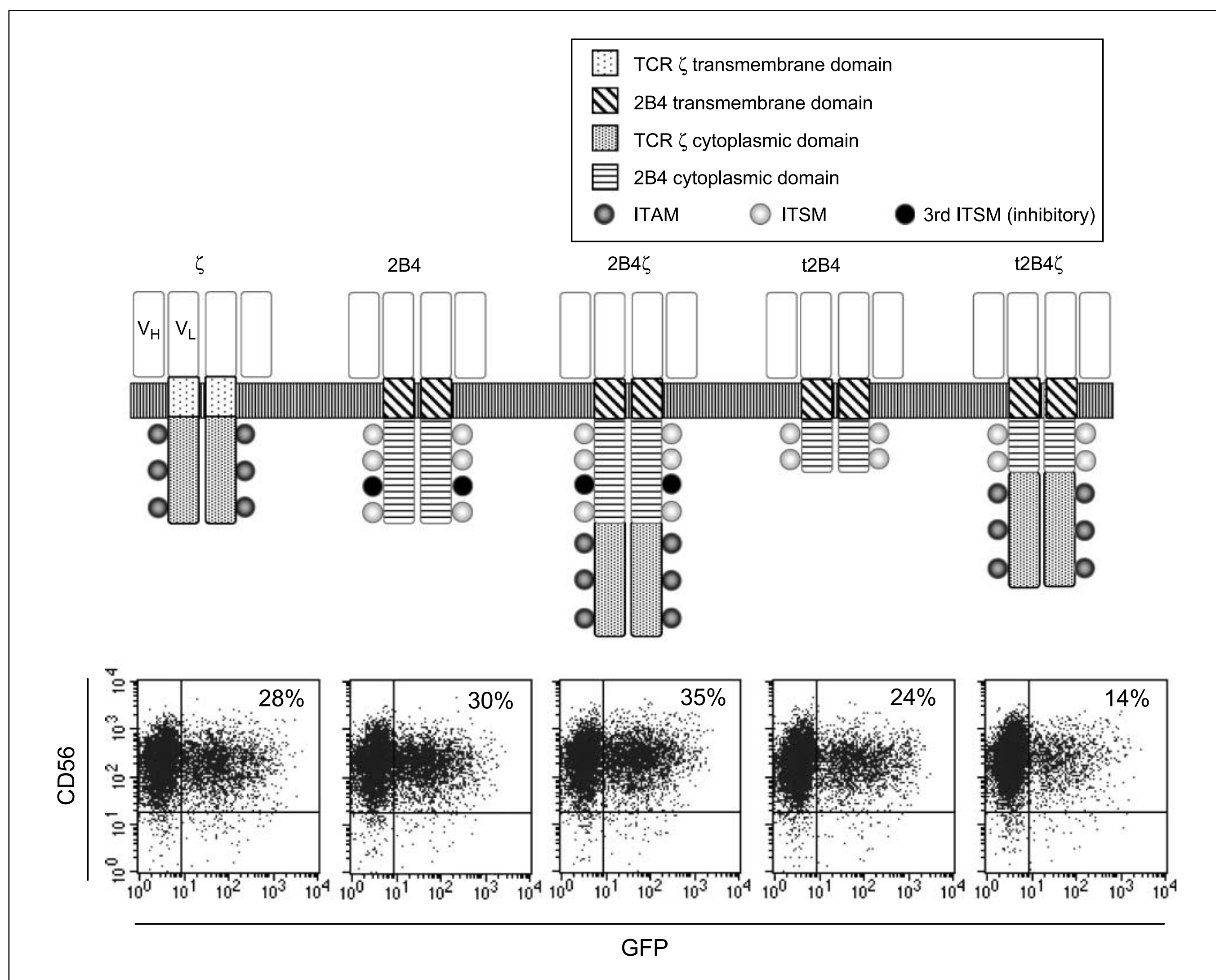


**Fig. 2.** NK cells expanded from PBMCs are efficiently transduced with the CAR genes. The various CAR constructs are depicted schematically. Following retroviral transduction of NK cells, gene expression was determined by flow cytometric quantification of GFP-expressing cells. Shown is one representative example of four.

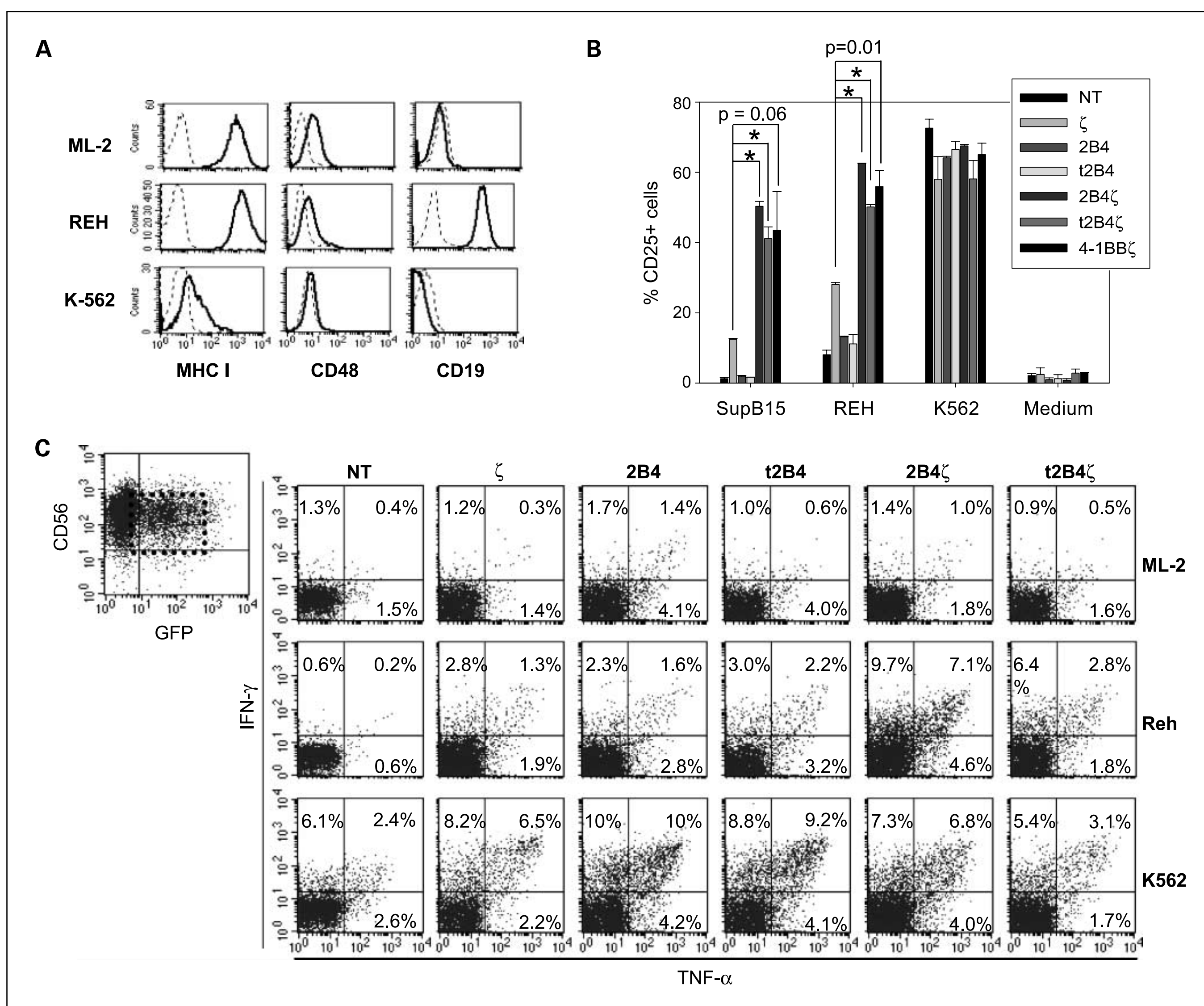


**Fig. 3.** CAR-transduced NK cells functionally interact with antigen-expressing tumor targets. *A,* expression of the 2B4 ligand CD48 on the leukemia target cell lines. *Dashed line,* isotype control; *solid line,* antibody. CD25 up-regulation (*B*) and intracellular IFN-γ and TNF-α expression (*C*) by nontransduced (*NT*), CD19-ζ–transduced, CD19-2B4–transduced, CD19-t2B4–transduced, CD19-2B4ζ–transduced, CD19-t2B4ζ–transduced, and CD19-41BBζ–transduced NK cells were quantified by flow cytometry after 24-h (*B*) or 6-h (*C*) coincubation with $CD19^+$ REH or SupB15 leukemia cells, CD19-negative ML-2 leukemia cells, K562, or medium alone, as indicated. To exclude non-NK lymphocytes and nontransduced NK cells within the cultures from analysis, the gate was set on GFP/CD56-coexpressing cells. Shown is one representative experiment of three. *, $P < 0.01$.

and CD19-t2B4ζ). The IRES-linked GFP within the transgenes permits quantification of transduced cells based on GFP fluorescence. Retroviral transduction was done on day 5 after initial stimulation with K562-mb15-41BBL. Flow cytometric analysis revealed comparable transduction efficiencies in NK cells transduced with the individual receptor genes (Fig. 2). In detail, the percentages of $GFP^+$ NK cells were 16.7 ± 7.6% for CD19-ζ, 16.2 ± 8.8% for CD19-2B4, 12.9 ± 7.1% for CD19-t2B4, 24.0 ± 7.4% for CD19-2B4ζ, and 18.0 ± 8.4% for CD19-t2B4ζ. The phenotype of the NK cells, as described above, was not affected by modification with CAR genes. Subsequent to transduction, NK cells could be kept in culture for at least 21 to 28 days without further stimulation. These results confirm that human NK cells from both healthy donors and pediatric leukemia patients can be efficiently expanded and gene modified to express leukemia antigen-specific chimeric receptors.

*CD19-induced 2B4 signaling costimulates specific NK cell activation.* Functional NK cell activation responses are characterized by up-regulation of the IL-2 receptor (CD25) as well as production of cytokines and secretion of cytolytic granules, resulting in the specific cytolysis of target cells. To compare the capacity of the various CARs for inducing antileukemic NK cell responses, we analyzed these parameters in response to interaction of gene-modified NK cells with antigen-expressing leukemia targets. In all flow cytometry–based assays, non-NK lymphocytes and nontransduced NK cells within the cultures were excluded from analysis by gating on GFP/CD56-coexpressing cells, and in direct cytolysis assays, the cells were purified by FACS for GFP expression. To adequately consider potential KIR-mediated interactions or natural engagements of the 2B4 receptor naturally expressed at high densities on the expanded NK cells (Fig. 1B), MHC class I and CD48 expression was

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determined on all target cell lines. K562 cells expressed low levels of MHC class I, in agreement with the previous observation that interactions between HLA-C molecules on K562 cells and the CD160 NK cell activating receptor contribute to their susceptibility to NK cell lysis (30). REH and ML-2 leukemia cell lines were MHC class I positive. Although CD48 was reported to be down-regulated on most leukemias (7), we detected CD48 surface expression on both REH and ML-2 leukemia cell lines but not on K562 (Fig. 3A) and SupB15 cells (data not shown).

Although exposure of CD19-ζ–transduced NK cells to CD19$^+$ B-cell precursor ALL cell lines resulted in significant up-regulation of CD25 (Fig. 3B) as well as intracellular secretion of both IFN-γ and TNF-α (Fig. 3C) compared with nontransduced NK cells, the signal mediated by the CD19-2B4 receptor failed to induce either CD25 up-regulation or cytokine secretion (Fig. 3B and C). Elimination of the potentially inhibitory components of the 2B4 receptor endodomain in the truncated variant did not increase the activation response to the leukemia targets (Fig. 3B and C). By contrast, a significant stimulatory effect of 2B4 became apparent in the receptor combining both signaling domains: Compared with ζ chain alone, 2B4ζ potently increased the specific NK cell response to CD19$^+$ leukemia cells in both assay systems. The CD25 up-regulation response to 2B4ζ was comparable with that observed with a previously described 41BB signal-enhanced CAR (Fig. 3B). Importantly, the activation responses of transduced NK cell cultures toward K562 were not affected by CAR expression (Fig. 3B and C). Cells within the GFP-negative subpopulations of transduced NK cells failed to functionally interact with target cells, confirming that GFP is a prerequisite for antigen-specific functionality and thus provides an adequate surrogate parameter for CAR expression of transduced NK cells (data not shown). The specificity of the transduced NK cell populations for the CAR-determined target antigen was confirmed by the lack of response to the CD19-negative cell line ML-2. Despite their high 2B4 receptor expression (Fig. 1B), nontransduced NK cells failed to functionally interact with the CD48-expressing leukemic targets REH and ML-2 (Fig. 3), although the relatively low expression of CD48 on these two targets does not allow any conclusions regarding potential NK cell activation in response to high levels of surface CD48. In summary, although the capacity of 2B4 signaling alone in the absence of the ζ chain to mediate NK cell activation is clearly limited, 2B4 significantly costimulates the functional response to ζ-mediated signals.

*2B4 signaling augments cytolytic NK cell responses to leukemia cells.* NK cells expressing a CD19-ζ CAR exert potent cytolytic responses against CD19-expressing leukemia target cells (Fig. 4A). Engagement of the receptor CD19-2B4 also triggered a significant CD19-specific degranulation response, which was further enhanced with the truncated 2B4 receptor chain (CD19-t2B4). Thus, whereas 2B4 triggering in the absence of additional signals fails to induce full NK cell activation (Fig. 3), the signal mediated by the 2B4 cytoplasmic domain is sufficient for inducing an antigen-specific NK cell degranulation response. Consistent with the synergistic capacity of 2B4 and ζ to induce cytokine secretion and CD25 up-regulation, both combinations of the ζ chain with the full or truncated 2B4 endodomain resulted in a significantly enhanced potency of the degranulation response against CD19$^+$ leukemia targets. When used in combination with TCRζ, truncated 2B4 was not consistently superior to the nontruncated domain, and the truncation even reduced

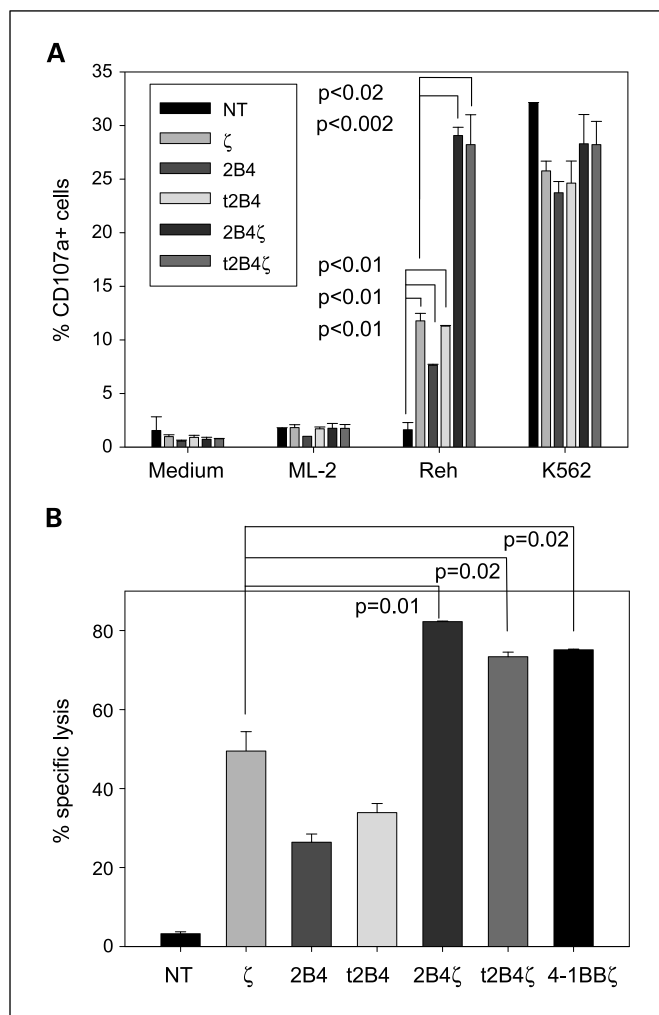


**Fig. 4.** CAR-transduced NK cells show powerful cytolytic responses against antigen-expressing tumor targets. *A,* the percentages of CD107a-expressing, degranulating NK cells were determined by flow cytometry after 4-h coincubation of nontransduced (*NT*), CD19-ζ–transduced, CD19-2B4–transduced, CD19-t2B4–transduced, CD19-2B4ζ–transduced, and CD19-t2B4ζ–transduced NK cells with CD19-negative ML-2 leukemia cells, CD19$^+$ REH leukemia cells, or K562 cells at an E:T ratio of 1:1 or in the presence of medium alone, as indicated. To exclude non-NK lymphocytes and nontransduced NK cells within the cultures from analysis, the gate was set on GFP/CD56-coexpressing cells. Shown is one representative experiment of two. *B,* to directly assess the cytotoxic effects of transduced NK cells on CD19$^+$ leukemia cells, FACS-purified (GFP$^+$) NK cells expressing the various CARs were coincubated for 16 h with the CD19$^+$ leukemia cell line REH at a 1:3 E:T ratio. Each data point represents the mean percentage of target cytolysis compared with parallel cultures of REH cells in the absence of NK cells. Nontransduced NK cells were used as negative controls. Shown is one representative experiment of two, each done in duplicate wells.

the capacity of the combined receptor to induce CD25 up-regulation. Again, the antigen specificity of the response was preserved in the presence of the 2B4 signal, as shown by the lack of any significant background degranulation of the transduced NK cells in response to antigen-negative target cells (ML-2). Furthermore, all transduced NK cell populations maintained vigorous degranulation responses toward K562 cells (Fig. 4A). The enhancement of CD19-specific target cytolysis by 2B4 was further reflected by an increased potency of NK cells transduced with CD19-2B4ζ or CD19-t2B4ζ versus CD19-ζ alone to significantly and specifically lyse cocultured

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REH cells (Fig. 4B). Direct comparison with the 41BB-containing CAR showed equal performance of the signal-enhanced CARs (Fig. 4B). Altogether, these data show that 2B4 has both stimulatory as well as potent costimulatory effects on NK cytolytic responses against leukemia targets.

***CAR gene-modified NK cells efficiently lyse autologous leukemia cells.*** An important question with regard to the potential clinical application of this strategy regards the susceptibility of autologous leukemia cells to the cytolytic effects of natural or gene-modified NK cells. To address this issue, bone marrow leukemia cells obtained at diagnosis from two pediatric patients with $CD19^+$ B-cell precursor cell ALL were cocultured with *in vitro*–activated autologous NK cells in three independent experiments. The functionality of patient-derived NK cells was confirmed by their efficient degranulation response to K562 target cells. Nontransduced NK cells failed to exert degranulation (Fig. 5A) and cytolytic (Fig. 5B) responses against autologous leukemia cells and lysis of leukemia cells. Thus, the stimulatory signal induced by natural ligation of the 2B4 receptor in activated NK cells is insufficient for overcoming resistance in our model. Although signaling via the ζ chain mediated significant lysis of autologous leukemic cells in the presence of transduced NK cells, it failed to induce a degranulation response to autologous targets, revealing an important lack of potency of the CD19-ζ receptor. By contrast, potent enhancement of both NK cell degranulation as well as cytolysis was obtained by combined 2B4 and ζ signaling even in the autologous setting. Thus, retargeted 2B4 signaling can overcome resistance of autologous leukemia cells to NK cell–mediated cytolysis and potentiate NK cell responses induced by a primary activation signal.

***2B4 signaling enhances NK cell activation responses to neuroblastoma cells.*** To extend our findings to a solid tumor model, we investigated whether 2B4 signal-enhanced CARs may retarget NK cells to NK cell–resistant neuroblastoma cells. Neuroblastoma is the most common extracranial solid tumor of childhood. Although many neuroblastoma cell lines are susceptible to NK cell–mediated lysis, patient-derived primary neuroblastoma cells were considerably more resistant (31). We genetically modified four NK cell lines generated from three healthy donors with CARs containing the extracellular single-chain Fv domain of the $G_{D2}$-specific monoclonal antibody 14.G2a and endodomains of either the TCRζ alone or in combination with 2B4. In contrast to the CD19-specific CARs, 14.G2a CARs are detectable on the cell surface by staining with F(ab)-specific antibody, allowing for the direct assessment of transgene expression. Transduction with 14.G2a-ζ and 14.G2a-2B4ζ resulted in surface expression on 51.9 ± 12.6% and 11.2 ± 9.3% of NK cells, respectively, confirming the proper assembly and transport of both ζ and 2B4ζ CARs to the cell surface (Fig. 6A). Genetic modification with 14.G2a-2B4ζ, but not 14.G2a-ζ, significantly enhanced the activation response of NK cells to the neuroblastoma cell line JF (Fig. 6B). Furthermore, whereas JF cells were susceptible to lysis by two of three unmodified NK cell cultures, resistance to the third NK cell line was successfully overcome by the signal-enhanced receptor (Fig. 6C).

## Discussion

The first promising NK cell–based therapeutic strategies have been based on NK alloreactivity between leukemia patients and their donors (4, 5). Targeted activation of NK cells may establish immune control of autologous malignancies and thus extend the use of NK cell therapies beyond the mismatched allotransplantation context. We present a strategy that allows to specifically and efficiently redirect NK cell effector functions

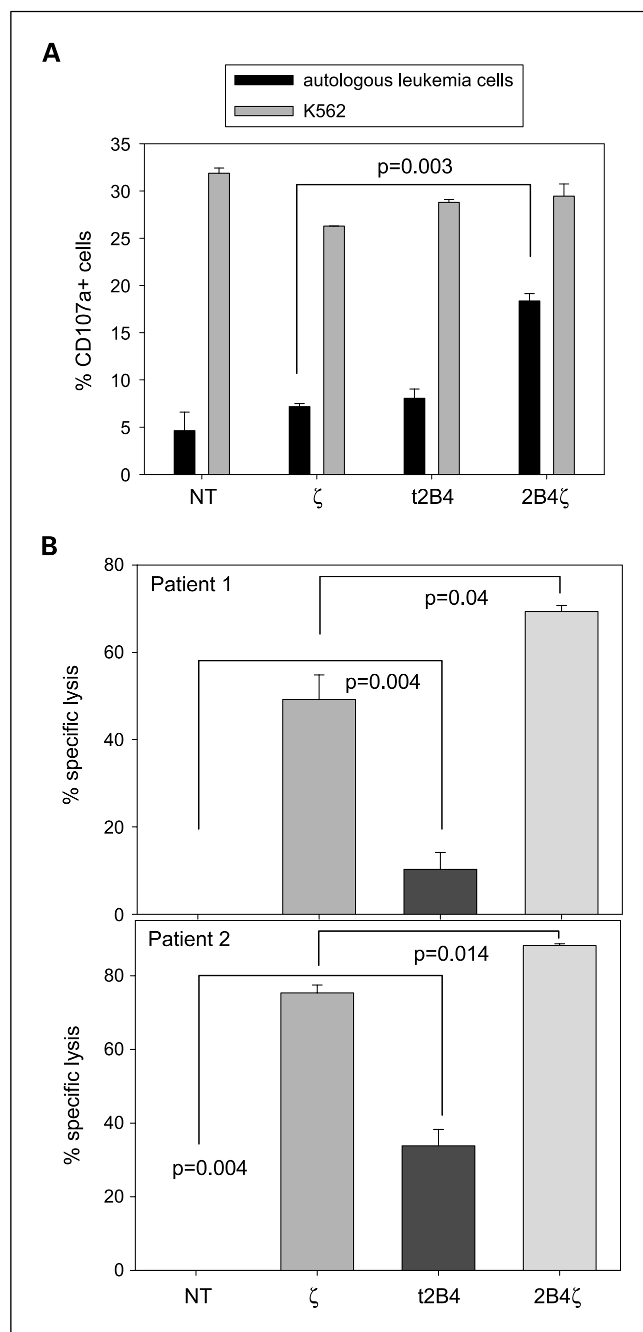


**Fig. 5.** NK cells obtained from pediatric leukemia patients and gene modified with CARs are highly cytolytic against autologous leukemia cell lines. *A,* the percentages of CD107a-expressing NK cells were determined by flow cytometry after 4-h coincubation of nontransduced (*NT*), CD19-ζ–transduced, CD19-2B4ζ–transduced, and CD19-t2B4–transduced NK cells at a 1:1 E:T ratio. To restrict the analysis to transduced NK cells, the gate was set on GFP/CD56-coexpressing cells. Shown is one representative experiment of three, done with three individual donors. *B,* FACS-purified ($GFP^+$) NK cells expressing the various CARs were incubated for 16 h with autologous leukemic cells at an E:T ratio of 1:8. Each data point represents the mean percentage of lysis of autologous leukemia cells compared with parallel cultures in the absence of NK cells. Nontransduced NK cells were used as negative controls.

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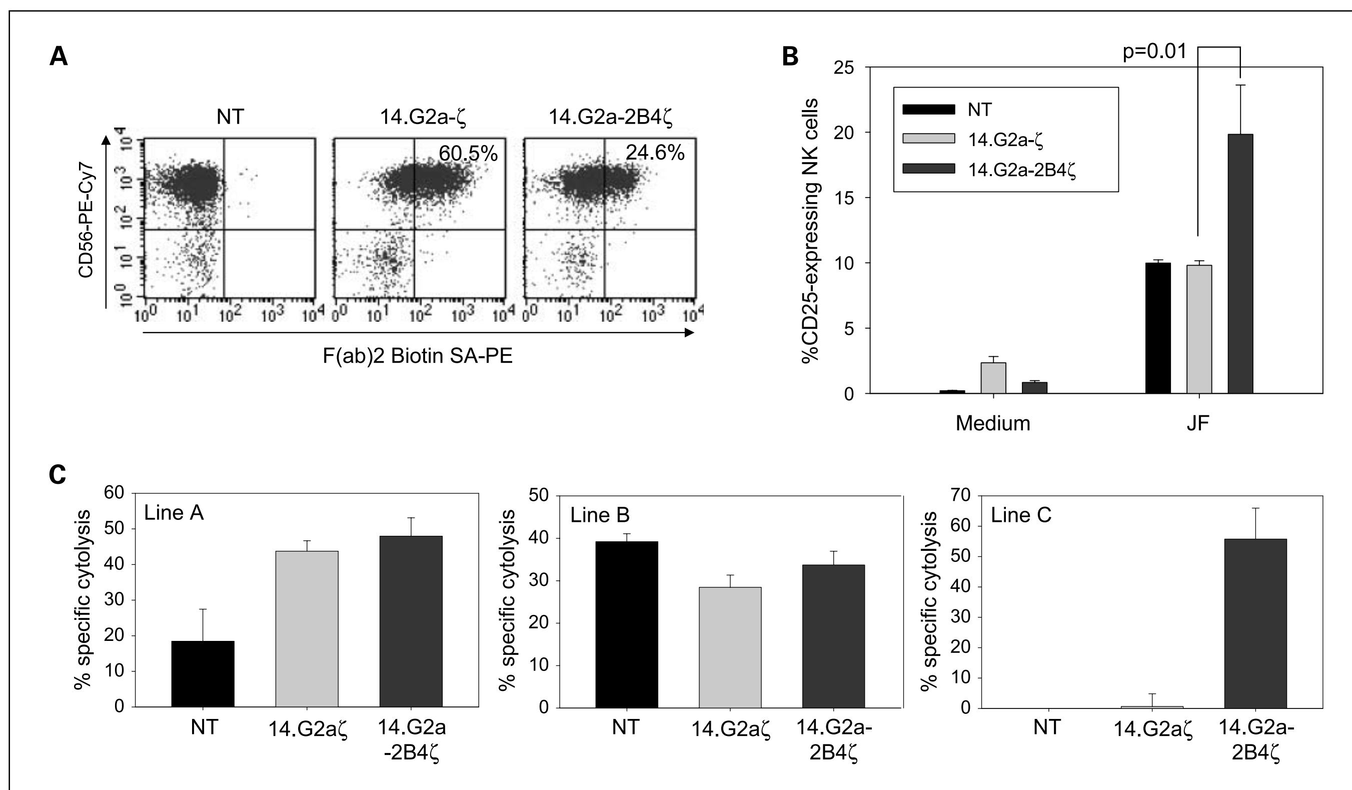


**Fig. 6.** 14.G2a-2B4ζ expression in NK cells enhances the activation response to $G_{D2}^{+}$ neuroblastoma cells and can overcome their resistance to NK cell lysis. *A,* 14.G2a-ζ and 14.G2a-2B4ζ CAR surface expression was determined by staining with F(ab)-specific antibody. One of four representative experiments. *B,* CD25 up-regulation by nontransduced (*NT*), 14.G2a-ζ–transduced, and 14.G2a-2B4ζ–transduced NK cells was quantified by flow cytometry after 24-h coincubation with $G_{D2}^{+}$ JF neuroblastoma cells at an E:T ratio of 1:1. To exclude nontransduced NK cells within the cultures from analysis, the cells were FACS purified based on F(ab) staining before analysis. Shown is one representative experiment of two. *C,* FACS-purified transduced NK cells from three individual healthy donors were incubated for 16 h with autologous leukemic cells at an E:T ratio of 2:1. Each data point represents the mean percentage of lysis of JF cells compared with parallel cultures in the absence of NK cells. Nontransduced NK cells were used as negative controls.

to autologous leukemia cells via activating signaling pathways, overcoming their natural NK cell resistance.

The concept of redirecting non-MHC–restricted immune effector function toward tumor cells by genetic engineering with antigen-specific CARs has been widely studied in T cells (11) and has now entered the first clinical trials (32–35). More recently, the potential of CARs was explored in cells of the innate immune system, including NK T cells (36) and γδ T cells (22). Although NK cells lack a TCR and thus fail to express a TCR-associated ζ chain, ζ is expressed in association with CD16 (37), and engineered ζ signaling was shown to induce functional responses in the NK cell line NK-92 (13) as well as in primary human NK cells (14, 15). Our data confirm that triggering of ζ in NK cells induces a potent and antigen-specific activation response to allogeneic targets that overcomes inhibitory signals that otherwise prevent lysis of tumor target cells by unmodified allogeneic NK cells. However, in response to autologous leukemia targets, the CD19-ζ–mediated NK cell response was limited, and more potent receptors will likely be needed for eradication of residual disease *in vivo*.

"Second-generation" receptors with integrated costimulatory signaling have substantially enhanced the redirected functionality of T cells against tumor targets (16, 29). Activation responses of NK cells can also be amplified by coengagement of receptors with stimulatory properties (17). Indeed, the signaling domain of 41BB, a molecule with known costimulatory function in T cells, was shown to contribute to ζ-mediated NK cell activation responses to leukemia cells (14). Adjusting the cytoplasmic components of CARs to the specific activation requirements of NK cells is likely to maximize the outcome of their interaction with the tumor target cell. We chose the NK cell receptor 2B4 (CD244) as a signal amplifier of leukemia-specific activation responses based on the following considerations. First, 2B4-induced signaling pathways are important in both innate and adaptive immune control as shown by the fatal immune dysfunction in patients with X-linked lymphoproliferative disease, who have genetic deficiencies in a molecule involved in 2B4 signaling (38). Second, engagement of 2B4 on NK cells by its ligand CD48 is critical for optimal NK cell function (39) and potently enhances activation induced by other NK receptors (40). Finally, a role for 2B4 in tumor immunity was suggested by its contribution to eliminating tumor cells in mice (19) and by the association of reduced 2B4 expression in NK cells from multiple myeloma patients with tumor immune escape (41). We found that integrated signals from 2B4 combined with ζ significantly enhance all aspects of the NK cell activation response, including CD25 up-regulation, secretion of IFN-γ and TNF-α, release of cytolytic granules, and target-specific growth inhibition. Importantly, integration of 2B4 efficiently overcame the limited activation response to autologous leukemia target cells induced by CD19-ζ. The antigen specificity of the interaction was fully preserved, ruling out autoproliferation and promiscuity of

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killing as a potential consequence of signal leakage by the multiple signaling components of the receptor. Furthermore, the capacity of gene-modified NK cells to functionally respond to K562 cells was fully maintained. Compared with thepreviously described 41BB signal-enhanced CAR (14), the functional assays did not reveal superiority of either construct. However, beyond *in vitro* functionality, the costimulatory signal provided by the CAR is highly likely to affect the *in vivo* performance of gene-modified NK cells, regarding antitumor activity, robustness of cytokine and cytolytic responses, and persistence *in vivo*. Only clinical trials can show which of the available second-generation CARs has the highest potential of inducing sustained antitumor NK cell responses. In summary, CD19-2B4ζ is a novel CAR with improved signaling characteristics for mediating powerful and specific antileukemic NK cell effector functions and efficiently breaking resistance to autologous NK cell lysis.

Besides their potential in anticancer immunotherapy, CARs are an excellent tool for identifying the functional consequences of receptor triggering in individual cell populations. To this end, our findings might help elucidating open questions regarding the role of 2B4 in NK cells. It is still controversial whether activation by 2B4 is strong enough to break NK cell tolerance in the presence of KIR signals, and some investigators have even reported 2B4 to provide inhibitory signals (42, 43). Indirect evidence against a dominant stimulatory role of 2B4 in NK cells is the broad expression of its ligand CD48 on all nucleated hematopoietic cells. We found that natural interactions between CD48 and 2B4 on activated NK cells failed to overcome resistance of leukemia cells to NK cell–mediated cytolysis. Furthermore, assessment of the direct consequences of enforced 2B4 signaling via CARs revealed incomplete activation responses to 2B4 with an inferior functional outcome compared with ζ triggering. Thus, 2B4 emerges from these experiments as a receptor with weak stimulatory and potent costimulatory function, exerting its full potential in human NK cells in combination with additional activating signals.

As a member of the signaling lymphocyte activation molecule family, the cytoplasmic tail of 2B4 contains four ITSMs (18). Whereas phosphorylation of the first and second ITSMs results in activation of stimulatory downstream pathways, the third motif was suggested to have a negative influence on human NK cell activation (44). However, in our system, the truncation did not consistently improve the functionality of the gene-modified T cells and even had a negative effect on antigen-specific CD25 up-regulation. Although we can only speculate about the potential reasons, it is conceivable that NK cell activation before expansion and transduction with 2B4-containing constructs may have overcome the inhibitory effect of the third ITSM by up-regulating cellular SAP expression, which was shown to positively modulate 2B4 function (45, 46).

One obvious potential clinical application of CAR gene-modified NK cells is the prevention of leukemia relapse following hematopoietic stem cell transplantation in high-risk patients. In contrast to existing NK cell–based strategies, fully matched related and unrelated donors can be used regardless of KIR typing. We further propose that autologous 2B4ζ gene-modified NK cells may efficiently target minimal residual leukemia cells after conventional chemotherapy or high-dose therapy. Besides hematologic diseases requiring allogeneic transplantation, genetic manipulation of NK cells allows to extend their use to various other cancers because CARs with various specificities have been generated in previous years. Using neuroblastoma as an example, we showed that 2B4ζ CAR gene-modified NK cells receive potent activation stimuli by antigen-expressing tumor cells and are capable of overcoming resistance of an individual neuroblastoma cell line to NK cell cytolysis. Critical aspects for the clinical translation of CD19-2B4ζ–redirected NK cells regard the feasibility and safety of the approach as well as factors ensuring maximum efficacy. We confirmed that despite the immunosuppressed state of pediatric patients during cytostatic maintenance therapy for leukemia, NK cells with a highly cytolytic, activated phenotype can be expanded and gene modified with comparable efficiencies to healthy donors. Good Manufacturing Practice–compliant protocols for the large-scale *in vitro* expansion of human NK cells have been developed (47, 48). Although alloreactive residual T cells may have to be removed from bulk cultures of expanded NK cells by clinical-grade sorting strategies in the mismatched allogeneic setting, enriched cultures of autologous, retargeted NK cells can be used adoptive immunotherapy without further selection.

A key issue regarding long-term control of leukemia is the *in vivo* persistence of the transferred effector cell populations. Although haploidentical donor NK cell infusions have indeed resulted in persistence and expansion of NK cells *in vivo* (4), concomitant administration of IL-2 will likely be required to explore the full potential of 2B4ζ gene-modified NK cells to survive *in vivo*. Comparable with T-cell–based strategies, embedding of 2B4ζ-transduced NK cell transfusions in a clinical context of preceding depletion of regulatory T cells and maximum cytoreduction may further enhance their therapeutic potential.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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