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Aptamer-Engineered Natural Killer Cells for Cell-Specific Adaptive Immunotherapy

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Abstract

Natural Killer (NK) cells are a key component of the innate immune system as they can attack cancer cells without prior sensitization. However, due to lack of cell-specific receptors, NK cells are not innately able to perform targeted cancer immunotherapy. Aptamers are short single-stranded oligonucleotides that specifically recognize their targets with high affinity in a similar manner to antibodies. To render NK cells target-specificity, synthetic CD30-specific aptamers were anchored on cell surfaces to produce aptamer-engineered NK cells (ApEn-NK) without genetic alteration or cell damage. Under surface-anchored aptamer guidance, ApEn-NK specifically bound to CD30-expressing lymphoma cells, but did not react to off-target cells. The resulting specific cell binding of ApEn-NK triggered higher apoptosis/death rates of lymphoma cells compared to parental NK cells. Additionally, experiments with primary human NK cells demonstrated the potential of ApEn-NK to specifically target and kill lymphoma cells, thus presenting a potential new approach for targeted immunotherapy by NK cells.

Keywords

adaptive immunotherapy; aptamer-engineering; lymphoma; Natural Killer (NK) cells; oligonucleotide aptamers

Natural Killer (NK) cells are a unique subset of cytotoxic lymphocytes and a key component of the innate immune system[1, 2]. In addition, the transfer safety of autologous, allogeneic,

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and cultured NK cells in adaptive cancer immunotherapy has been demonstrated[3–5]. In contrast to T-lymphocytes, NK cells do not express antigen-specific T cell receptors[6], but instead recognize target cells via an array of germ-line encoded surface ligands[7, 8]. Therefore, NK cells are able to attack cancer cells without prior sensitization or clonal expansion[6, 9]. NK cells can induce the death of targeted cancer cells through exocytosis of cytotoxic granules and can trigger apoptosis by activating cellular signaling pathways[10, 11]. These unique, inherent properties confer high value to NK cells in adaptive cancer immunotherapy. However, due to their lack of cell-specific receptors, NK cells are not able to selectively target tumor cells, thus diminishing their immunotherapeutic potential. Chimeric antigen receptor (CAR)-T cell technology has emerged as a growing field and was recently FDA-approved for immunotherapy in certain cancer types[12, 13]. Similarly, CAR-NK cells have also shown promising results for cancer immunotherapy[14–16]. However, the genetic manipulation used to generate CAR-T/NK cells may carry risks for patients, including transgene insertional mutagenesis[17, 18]. Also, the production of CAR-NK cells is laborious and costly[19, 20]. The development of a simpler and safer technology is necessary to overcome these technical and clinical challenges.

Aptamers are a group of short, single-stranded oligonucleotides with unique three-dimensional structures[21, 22]. Similar to protein antibodies, aptamers specifically recognize their targets, which can include nucleic acids, proteins, cells, and even tissue, with high affinity[23–28]. Aptamers are considered “chemical antibodies,” given that they are chemically synthesized and have the ability to specifically bind their targets through three-dimensional recognition[29–31]. To target lymphoma in particular, CD30-specific aptamers have been previously developed and clinically validated [32, 33].

In this study, we developed aptamer-engineered NK cells (ApEn-NK) for targeted immunotherapy. To create cell-specificity, synthetic aptamers were anchored on the surface of NK cells via a simple biophysical reaction without genetic alteration. Under surface-anchored aptamer guidance, ApEn-NK specifically bound to lymphoma cells and subsequently triggered cell apoptosis and death. This exciting result opens a new and powerful avenue for cancer immunotherapy.

RESULTS

Generation of aptamer-engineered NK cells

To render NK cells target-specificity, the ApEn-NK were generated by simply engineering synthetic aptamer-anchor structures on the cell surfaces via biophysical intercalation into the cell membrane (Fig 1A). Lipophilic linkers have been widely used to anchor functional molecules on the membranes of living cells for different purposes, with no adverse impact on cell viability[34–37]. In this study, a CD30-specific ssDNA aptamer sequence[33] was synthesized to different lipophilic anchors, including single- or double-C18 hydrocarbon chains, cholesterol, or vitamin E, to produce the aptamer-anchor structures Apt-C18, Apt-2xC18, Apt-Chol, and Apt-VitE, respectively (Fig 1B). For tracking purposes, fluorochrome Cy3 was attached to the 5' end of the aptamer sequence. Because of their amphiphilic properties, the formed aptamer-anchor structures had the ability to precisely

present hydrophilic aptamer sequences on the cell surface through intercalation of their hydrophobic anchors into the cell membrane.

To generate ApEn-NK, cultured NK92 cells were incubated with individual aptamer-anchoring structures under a physiological condition. Efficacy of cell surface-anchoring by synthetic Apt-2xC18, which contained two lipophilic chain anchor structures, was first studied. After a short incubation, confocal microscopy revealed fluorescent signals from the surface-anchored Apt-2xC18 on ApEn-NK with intact cell morphology (Fig 1C). Quantitative analysis by flow cytometry demonstrated that the anchoring reaction rapidly achieved maximal levels in 30 min, and reached a plateau at a final concentration of 2 μ M. For validation studies, the ApEn-NK were treated with DNase to digest the aptamers anchored on the cell surface. Fig 1D shows that the DNase treatment resulted in a greater than 10-fold decrease in cell fluorescent signals derived from Apt-2x18C, thus confirming surface-anchoring of Apt-2x18C on ApEn-NK.

In addition, Apt-Chol, which contains a non-linear anchoring structure, was also used to produce ApEn-NK under the same reaction conditions. Confocal microscopy confirmed cell surface-anchoring of Apt-Chol as well as cytoplasmic penetration, likely due to cholesterol receptor-mediated endocytosis (Fig 1E). Quantitative analysis demonstrated that the cell-anchoring reaction of Apt-Chol occurred rapidly and was dose-dependent. Similarly, exposure of the ApEn-NK to DNase treatment significantly reduced surface-anchored aptamer signals derived from Apt-Chol (Fig 1F). In contrast, synthetic Apt-C18, which had single lipophilic chain anchor, failed to produce ApEn-NK (Fig 1G), although it was able to be surface-anchored on cultured carcinoma cells under the same conditions (unpublished data). Moreover, Apt-VitE showed a minimal capacity to produce ApEn-NK, and an anchoring reaction plateau could not be reached even at a final concentration of up to 3 μ M (Fig 1H). Taken together, these findings indicate the potential of Apt-2xC18 and Apt-Chol structures to produce ApEn-NK.

For biostability studies, the produced ApEn-NK were incubated in cell culture media, and changes in fluorescent signals of surface-anchored aptamers were monitored. Flow cytometry analysis revealed that ApEn-NK made with Apt-2x18C were stable, and a greater than 90% cellular signal of the anchored aptamers remained 60 min post-production (Fig 2A). The remaining intact ApEn-NK carrying surface-anchored aptamers were also confirmed by confocal microscopy. In contrast, ApEn-NK made of Apt-Chol were not stable, as a greater than 80% reduction of the anchored aptamer signals was detected as early as 15 min post-production (Fig 2B). Confocal microscopy also revealed nearly complete loss of the cell surface-anchored Apt-Chol signals in 60 min, despite the presence of scattered cytoplasmic signals. The observed instability limited Apt-Chol utility, and thus only ApEn-NK made of Apt-2xC18 were investigated in subsequent studies.

For extended biostability validation, the ApEn-NK were incubated in cell culture media over a time course. Quantitative flow cytometry revealed that more than 40% of cell signals of anchored aptamer remained on ApEn-NK 10 h post-production (Fig 2C). To evaluate the biocompatibility of synthetic aptamer-anchor structures, cultured NK92 cells were continuously exposed to Apt-2xC18 for three days while changes in cell growth rates were

monitored. Figure 2D shows that the presence of Apt-2xC18 in cultures had no adverse effects on NK92 cell growth, as compared to non-treatment or aptamer sequences with control cells.

Finally, to confirm that NK cell-anchoring of Apt-2×18C was through anchor structures, NK92 cells were incubated with only Cy3-labeled aptamer sequences (Fig 2E). Flow cytometry analysis showed that sole aptamer sequences could neither be anchored on NK92 cells nor bind to NK92 cells that lack CD30 expression. To rule out non-specific aptamer-cell interaction, additional CD30-negative cells were also tested, and no cell binding of aptamer sequences was detected by flow cytometry analysis (Fig 2F). To confirm targeting specificity, CD30-positive lymphoma cells were treated with aptamer sequences[33] and the resulting specific cell-binding by aptamers was determined by flow cytometry (Fig 2G). These findings indicate that cell surface-anchoring of Apt-2×18C was mediated by its anchor structures.

Specific binding of ApEn-NK to lymphoma cells

It is expected that under the guidance of surface-anchored aptamers, ApEn-NK would be able to specifically target lymphoma cells as illustrated in Fig 3A. To test this hypothesis using cell binding assays, CD30-expressing K299 lymphoma cells were pre-stained with Calcein-AM (green fluorescence), and ApEn-NK cells were tracked by the red fluorescence of surface-anchored Apt-2xC18. Equal amounts of ApEn-NK (Effector cells) and lymphoma cells (Target cells) were mixed. In control experiments, parental NK92 cells were pre-stained with Red-Orange AM and used to replace ApEn-NK in the cell mixture. Resultant cell binding was analyzed by flow cytometry 30 min post-incubation. As showed in Fig 3B, different cell populations were separated and gated, specifically, including Effector cells (E) in red, Target cells (T) in green, and E/T clusters containing both red and green signals[38]. Quantitative analysis revealed that ApEn-NK specifically targeted lymphoma cells and formed E/T clusters significantly more than that observed in control experiments containing NK92 cells (12.38% vs. 8.22% mean cluster formation rates, $p < 0.05$). Notably, in mixtures of CD30-negative U937 cells, similar E/T cluster formation baselines were detected with ApEn-NK or NK92 cells (7.11% vs. 6.2% mean cluster formation rates).

For further characterization, cell mixtures were examined under a fluorescence microscope, confirming E/T cluster formation (Fig 3C). In addition, time course studies with CD30-positive lymphoma cells (K299, SUDHL-1, and HDLM2 cell lines) also revealed that ApEn-NK induced significantly higher numbers of E/T clusters per well of culture plates than that by parental NK92 cells (Fig 3D). However, ApEn-NK and NK92 cells had very similar E/T cluster formation rates in mixtures containing CD30-negative cells (U937, Maver-1, and Jeko-1 cell lines), indicating that ApEn-NK were target cell-specific. Moreover, cell numbers of each E/T cluster were manually counted under a fluorescence microscope. In comparison to parental NK92 cells, ApEn-NK induced larger E/T clusters that contained more total cells (Fig 3E) and higher numbers of both ApEn-NK and target lymphoma cells (Fig 3F, 3G). Taken together, these findings demonstrated the specific binding capacity of ApEn-NK to target lymphoma cells.

Specific killing of lymphoma cells by ApEn-NK

Given that NK cells are able to attack cancer cells without prior sensitization, acquired specific cell-binding capacity should result in higher killing efficacy of ApEn-NK (Fig 4A). For cell functional assays, ApEn-NK were incubated with lymphoma cells at E/T ratios of 3:1, 1:1, or 1:3, and the resultant changes in apoptosis and death rates of target cells were evaluated. For identification purposes, lymphoma cells were pre-stained with green fluorescence and ApEn-NK cells were tracked by red fluorescence of anchored aptamers, as described above. After incubation, cells were harvested and stained with Cy5 Annexin V and eFluor 450 dyes to mark apoptotic and dead cells, respectively. To evaluate killing efficacy, individual cell populations were first separated and gated by flow cytometry, including lymphoma cells, ApEn-NK, and formed cell clusters (Fig 4B, left). Subsequently, the gated lymphoma cells were further analyzed to determine changes in cell apoptosis/death (Fig 4B, right).

In comparison to parental NK92 cells, ApEn-NK induced significant increases in apoptosis/death of target lymphoma cells (K299, SUDHL-1, and HDLM-2 cells lines that express CD30) at all tested E/T ratios (Fig 4C). In contrast, no enhanced killing effects on off-target control cells (U937, Maver-1, and Jeko-1 cell lines) were observed, demonstrating that the ApEn-NK killing effect was target cell-specific. Interestingly, statistical analysis of all tested lymphoma cells revealed the highest enhancement of killing effects at the lowest E/T ratio. In comparison to non-specific background rates determined by parental NK92 cells, 44%, 62%, and 168% increases in lymphoma cell killing at E/T ratios 3:1, 1:1, and 1:3, respectively, were detected (Fig 4D).

Characterization of ApEn-NK from primary human NK cells

To determine if these results would replicate in human models, primary NK cells were isolated from three healthy human donors and cultured for *in vitro* expansion. After culturing for three weeks, primary NK cells from individual donors were expanded up to 377, 436, and 552 folds, respective to each donor (Fig 5A). Subsequently, ApEn-NK were produced in these cells with Apt-2xC18 aptamers, and successful surface-anchoring of aptamers on ApEn-NK was confirmed by confocal microscopy, as previously described (Fig 5B). In addition, changes in fluorescent signals of ApEn-NK derived from surface-anchored aptamers were dynamically monitored by flow cytometry analysis. Time course assays demonstrated that the aptamer-anchoring reaction on primary NK cells occurred rapidly, and achieved a maximal level within 90 min (Fig 5C). Moreover, dose course studies showed that the aptamer-anchoring reaction was dose-dependent, and reached a plateau at 2 μ M final concentration of Apt-2xC18 structures (Fig 5D).

For cell binding assays, target cells (K299 and U937) were pre-stained with Calcein-AM, and ApEn-NK cells were tracked by fluorescent signal of surface-anchored aptamers. In control experiments, primary human NK cells were pre-stained with Red-Orange AM and used instead of ApEn-NK. ApEn-NK or primary NK cells were incubated with target cells and resultant E/T clusters were then quantified by flow cytometry, as previously described. In comparison to the non-specific baseline of cell binding established by primary NK cells, ApEn-NK induced significantly more E/T clusters with CD30-expressing K299 cells

(14.32% vs. 39.81%, $p < 0.5$) (Fig 5E). However, similar E/T cluster formation with CD30-negative U937 cells were observed in cell mixtures containing primary NK cells or ApEn-NK under the same conditions (Fig 5F). These findings indicated that ApEn-NK derived from primary NK cells were able to specifically bind lymphoma cells and did not react to off-target cells.

Finally, to evaluate immunotherapeutic potential, target cells (K299 or U937) were treated with ApEn-NK derived from the three healthy donors at the previously defined E/T ratios. In control experiments, parental primary NK cells derived from the same donor were used to replace ApEn-NK. The resultant apoptosis/death rates of target cells were then quantified by flow cytometry analysis, as previously described. Fig 5G shows that in comparison to paired parental NK cells, ApEn-NK treatment induced a significant increase in apoptosis/death of CD30-expressing K299 lymphoma cells. Notably, although enhanced killing effects were observed in all tested E/T ratios, the highest increased killing effect was observed at the lowest E/T ratio. Quantitative analysis of the killing effects of three ApEn-NK lines revealed a 28% increase (mean $\pm$ 6%) at E/T ratio 3:1, a 76% increase at E/T ratio 1:1 (mean $\pm$ 26%), and a 201% increase at E/T ratio 1:3 (mean $\pm$ 39%) from the baseline effects. In contrast, no enhanced killing effect of ApEn-NK on CD30-negative U937 cells was observed under the same conditions (Fig 5H). Taken together, these findings demonstrate the immunotherapeutic potential of ApEn-NK to specifically attack lymphoma cells.

DISCUSSION

This study validates a unique approach to render NK cells target-specificity by simply anchoring oligonucleotide aptamers on the cell surface without genetic alteration or cell damage. Under the guidance of surface-anchored aptamers, ApEn-NK cells specifically targeted and subsequently induced apoptosis/death of targeted lymphoma cells. This ApEn-NK platform has several unique features as compared to CAR-T/NK technology[19, 20, 39]. First, generation of ApEn-NK cells is a simple, time- and labor-efficient process as it involves a single-step engineering reaction and can be completed in 30 min. Notably, the engineering reaction was highly effective, and all NK cells were surface-anchored with amphiphilic aptamer-anchor structures without alteration of cellular properties. The simplicity and efficiency of the engineering process render ApEn-NK cells clinically valuable for rapid personalized adaptive immunotherapy. Secondly, because they are composed of natural nucleic acids, aptamer-anchor structures are biocompatible and biodegradable, and will be risk-free for clinical use. Importantly, manufacturing of ApEn-NK cells does not involve genetic manipulation and/or viral gene transfection, which are required for CAR-T/NK generation[2, 18, 40] and can carry undefined risks for patients[41–43]. Finally, *in vitro* studies demonstrated that ApEn-NK were able to efficiently kill target lymphoma cells, with the highest enhancement in the lowest tested E/T ratio 1:3 (Fig 4D). These findings strongly suggest the suitability of ApEn-NK for immunotherapy as the *in vivo* E/T ratios at tumor sites are expected to be significantly lower than those in *in vitro* experiments.

Phospholipids are major components of cell membranes, constituting the membrane's bilayer structure. Cholesterol is an essential component of the membrane and is dispersed

between phospholipids to maintain membrane integrity and flexibility[44]. Notably, phospholipid and cholesterol derivatives have the capacity to intercalate between cell membranes via hydrophobic interactions, and thus have been used as linkers for fluorescence labeling of membranes[36, 37, 45]. In this study, different phospholipid and cholesterol derivatives were tested in the production of amphiphilic aptamer-anchor structures (Fig 1B). The single hydrocarbon chain anchor failed to engineer aptamers on NK cells, although this strategy is widely used for linkers carrying single fluorochrome molecules. Interestingly, Apt-2xC18 achieved rapid and stable surface-anchoring of aptamers on NK cells. These findings suggest that an optimal balance between the hydrophobic and hydrophilic properties of amphiphilic aptamer-anchor structures is critical for successful membrane intercalation. Cholesterols instead of phospholipids were also studied in the generation of aptamer-anchor structures. Apt-Chol had high efficacy in the surface-anchoring of aptamers on NK cells (Fig 1E), but poor stability on cell membranes, which is likely due to receptor-mediated internalization (Fig 2B). In addition, vitamin E, an essential component of membrane lipids[46], was tested as an anchor; however, Apt-VitE showed minimal capacity for surface-anchoring on NK cells. Moreover, our unpublished results showed that the surface-anchoring capacity of different linkers varied significantly among engineered cells. These findings demonstrated that precision optimization of aptamer-anchor structures is essential to achieve maximum efficacy of cell surface-anchoring. Thus, it is necessary to carefully validate the aptamer-anchor structures under each set of clinical conditions.

Under surface-anchored aptamer guidance, the ApEn-NK specifically targeted lymphoma cells, leading to the formation of stable E/T clusters. Notably, the surface-anchored aptamers enhanced cell-specific binding, but had no effect on NK cell activity because of the lack of intracellular signaling domains. Therefore, it is reasonable to conclude that the observed immunotherapeutic effects on targeted lymphoma cells resulted from aptamer-mediated specific cell binding and the innate killing capacity of ApEn-NK. To improve immunotherapeutic potential, ApEn-NK cells could be stimulated and activated *in vitro* prior to adoptive transfer. Theoretically, the aptamer-anchoring system can be combined with a cell activation approach, creating ApEn-NK specific cell-binding and enhancing targeted cell killing for improved immunotherapy. It would be of interest to compare the immunotherapeutic efficacy of ApEn-NK derived from autologous- and allogeneic-donors because autologous NK cells will have no risk for the patients.

Biostability studies revealed that the surface-anchored aptamers were stable up to 10 h post-ApEn-NK production (Fig 2C), with only a moderate decrease likely due to nuclease degradation and/or dilution as NK cells divided. To enhance *in vivo* biostability, nuclease-resistant aptamer sequences can be synthesized through chemical modifications. Although the minimal number of surface-anchored aptamers required for cell targeting is unknown, the use of high-affinity aptamer sequences can improve the cell-binding capacity and functional lifetime of ApEn-NK upon dividing *in vivo*. Finally, our preclinical studies demonstrated the possibility of producing ApEn-NK using primary human NK cells derived from healthy donors for specific killing of target lymphoma cells.

METHODS

Reagents and cells:

Cell lines Karpas 299 (K299, T-cell lymphoma), SUDHL-1 (diffuse histiocytic lymphoma), HDLM2, (Hodgkin lymphoma), U937 (histiocytic lymphoma), Jeko-1 (B-cell lymphoma), and Maver-1 (mantle cell lymphoma) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 medium (Thermo Fisher Scientific, Rockford, IL, USA) containing 10% FBS and 100 IU/mL penicillin-streptomycin. NK92 cells (NK cell lymphoma) from ATCC were cultured in alpha minimum essential medium (α MEM) (Gibco, Grand Island, NY, USA) supplemented with 12.5% Fetal Bovine Serum (FBS) (Atlanta Premium, Atlanta, GA, USA), 12.5% horse serum from ATCC, 0.2 mmol/L inositol (Sigma Aldrich, St. Louis, MO, USA), 0.1 mmol/L β -mercaptoethanol (Sigma Aldrich), 0.02 mmol/L folic acid (Sigma Aldrich), 200 U/mL recombinant IL-2 (PeproTech, Rocky Hill, NJ, USA), and 100 IU/mL penicillin-streptomycin (Corning, Corning, NY, USA).

To isolate primary human NK cells, peripheral blood from anonymized healthy donors was used with an IRB-approved protocol. Mononuclear cells in blood buffy coats were isolated by a density-gradient technique (Ficoll-Histopaque; Sigma, St Louis, MO, USA) and CD56+ NK cells were then purified using an NK isolate kit (Miltenyi Biotec, San Diego, CA, USA). For cell expansion, primary NK cells were cultured in RPMI 1640 medium (Corning, NY, USA) in the presence of irradiated (100 Gy) feeder cells of K562-mb15-41 BBL obtained from St. Jude's Children's Research Hospital at 2:1 ratio of feeder:NK cells, and supplemented with 5% human AB serum (Sigma), recombinant human IL2 (200 IU/mL, PeproTech, Rocky Hill, NJ, USA), recombinant human IL15 (10 ng/mL, PeproTech), and recombinant human IL21 (1 ng/mL, PeproTech). Cultures were expanded every other day with fresh media and irradiated feeder cells were replaced every week. NK cell expansion was monitored by cell counting on day 7, 14, and 21.

Cell-binding assay of aptamer sequences:

The CD30-specific ssDNA aptamer sequence, 5'-ACTGGGCGAAACAAGTCTATTGACTATGAGC-3', was labeled with fluorochrome Cy3 at the 5' end for tracking purposes, as previously reported[33]. Cultured cells (5×10^5), including the human NK cell line (NK92), CD30-expressing lymphoma cell lines (K299, SUDHL-1, and HDLM2), and CD30-negative lymphoma/leukemia cell lines (U937, Jeko-1, and Maver-1) were incubated with 200 nM aptamer probes at room temperature (RT) for 30 min in Dulbecco's phosphate-buffered saline (DPBS) (GE Healthcare, Chicago, IL, USA). Cells were washed once with DPBS and resulting cell binding of aptamer probes was quantified by flow cytometry (LSRII, BD Biosciences, San Jose, CA, USA). The same set of cells treated with random ssDNA sequences of the same length were set as negative/baseline controls for the cell-binding assay.

Design of aptamer-anchor structures:

To identify optimal aptamer-anchor structures for engineering of ApEn-NK, the aptamer sequences were conjugated at the 3' end to different lipophilic anchor molecules, including:

single and double C18 hydrocarbon chains, cholesterol (via a 15-atom triethylene glycol spacer), or vitamin E (via a 12-carbon spacer), as shown in Fig 1B. For tracking purposes, fluorochrome Cy3 was conjugated at the 5' end as a reporter. All aptamer-anchor structures were synthesized and purified by Integrated DNA Technologies (IDT, Coralville, IA, USA), and stored in nuclease-free water at -20°C until used.

Production and characterization of ApEn-NK:

Cultured NK92 cells or fresh primary human NK cells (5×10^5) were incubated with individual aptamer-anchor structures in DPBS at RT with intermittent mixing to produce ApEn-NK cells. The concentrations of aptamer-anchor structures and reaction times are described in the individual experiments. After the anchoring reaction, the produced ApEn-NK were washed once and resuspended in DPBS. Both dose and time course studies were conducted to determine the optimal reaction conditions and optimal concentration of aptamer-anchor structures for ApEn-NK production.

Surface-anchoring of aptamer sequences on ApEn-NK was confirmed with a confocal microscope (FluoView™ FV1000, Olympus America, Melville, NY, USA) at x60 magnification on bright field and the Cy3 channel. In addition, cellular fluorescence signals derived from surface-anchored aptamers on ApEn-NK were quantified by flow cytometry analysis. Intact parental NK92 or primary human NK cells were used for assay baseline controls. To evaluate biostability, ApEn-NK cells were maintained in culture media, and residual cellular signals of surface-anchored aptamers were quantified at different time points, as described in the experiments.

To validate the presence of aptamer sequences on the exterior of the cell membranes, ApEn-NK were treated with 250 U/mL DNase (Sigma Aldrich) in DPBS at 37°C for 30 min. ApEn-NK cells were washed with DPBS once, and residual cellular signals of surface-anchored aptamers were quantified by flow cytometry.

Cell proliferation assay:

To rule out cytotoxicity, NK92 cells (5×10^4) were incubated with aptamer-anchor structures Apt-2xC18 under the same conditions to produce ApEn-NK or equimolar amounts of CD30 aptamer sequences alone in control experiments. Cells were then seeded into complete culture media, and cultured in 96-well plates. Changes in cell proliferation were monitored via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at 24, 48, and 72 h. The absorbance in each well at 570 nm was measured with a microplate reader (GE Healthcare).

Quantification of cell cluster formation:

For identification purpose, lymphoma cells (Target cells, T) were pre-stained with 100 nmol/L Calcein-AM (green fluorescence), and ApEn-NK (Effector cells, E) were tracked by red fluorescence signals from the surface-anchored aptamers. In control experiments, parental NK cells were pre-stained with 100 nmol/L Calcein Red-Orange AM (Life Technologies, Carlsbad, CA, USA) to replace AnEn-NK. For the cell binding reaction, equal amounts of effector and target cells (1×10^5) were mixed at different ratios in 500 μL RPMI

1640 containing 10% FBS. Subsequently, the mixture was incubated in a 5 mL polystyrene round-bottom tube with constant gentle shaking on a shaking-bed (GE Healthcare) at RT for 30 min. Constant shaking mimics *in vivo* dynamic blood flow and also prevents non-specific cell clotting due to gravity-induced precipitation. Cell mixtures were analyzed by flow cytometry, and the formed E/T clusters were quantified. Given that E/T clusters emitted fluorescence signals with both effector and target cells, they were selectively gated, and calculated as the percentage of total detected cell events[38]. Each set of samples was analyzed in triplicate, and the analysis was repeated 3 times.

For the time course study, equal amounts of effector and target cells (2×10^4) were mixed in 2 mL RPMI 1640 containing 10% FBS. Cell mixtures were incubated in a 12-well plate with constant gentle shaking for 15, 30, or 45 min. Individual wells were then examined under a fluorescence microscope to count the number of formed E/T clusters that were aggregates of more than 4 cells and contained both effector (red signal) and target (green signal) cells. In addition, the number of total, effector, and target cells per formed E/T cluster were manually counted, and a mean value with $\pm$ SD was calculated from 8 or more randomly observed clusters.

Cell killing assays:

ApEn-NK were produced by incubating 5×10^5 cultured NK92 or cells with 1 μ mol/L Apt-2xC18 in DPBS at RT for 30 min. For tracking purposes, lymphoma (K299, HDLM2, and SU-DHL-1 cell lines) or control cells (U937, Jeko-1, and Maver-1 cell lines) were labeled with 1 μ mol/L Calcein-AM. ApEn-NK cells were mixed with target cells in 2 mL RPMI 1640 containing 10% FBS in 12-well plates at E:T ratios of 3:1, 1:1, and 1:3. Notably, all cell mixtures contained equal amounts of target cells (3×10^4) with variable numbers of ApEn-NK cells to achieve different E:T ratios as noted in the figure. Cell mixtures were gently shaken on a shaking bed at RT for 30 min to avoid non-specific cell binding, then incubated in a humidified atmosphere with 5% CO₂ at 37°C for 4 h. Finally, cell mixtures were labeled with Cy5 Annexin V (BD Bioscience, Waltham, MA, USA) and Dye eFluor 450 (eBioscience, Waltham, MA, USA) for 30 min, and analyzed by flow cytometry. First, different cell populations were gated, including ApEn-NK bearing the red fluorescent signals from the surface-anchored aptamers, lymphoma cells pre-stained with green fluorescence Calcein-AM, and E/T clusters that emitted both red and green signals. Subsequently, the gated lymphoma cell population was further analyzed for cell apoptotic and death rates based on cellular signals of Cy5 Annexin V and eFluor 450, respectively. The resulting apoptosis/death rates over the total gated lymphoma cells (%) with $\pm$ SD were calculated. Each set of samples was analyzed in triplicate and the same experiments were repeated 3 times with similar findings.

For primary NK cell killing assay, expanded primary NK cells were incubated with 1 μ mol/L Cy3-Apt-2xC18 in DPBS at RT for 30 min. Target cells K299 and U937 were labeled with 100 nmol/L Calcein-AM in DPBS at RT for 30 min. After washing twice, NK cells and target cells were mixed in RPMI1640 supplemented with 10% FBS and 500 IU/ml recombinant human IL2 in V-bottom 96-well plate. Plate was centrifuged for 1 min at 100 g to initiate cell contact and incubated at 37 °C for 4 h. Cells were mixed by pipetting

with a 100 μ L pipetter in order to uniformly suspend the released calcein followed with spinning down plate at 100g for 5 minutes to pellet the cells, and then 100 μ L of the supernatant was transferred to a new plate. Plate was read using a fluorescent plate reader (excitation filter 485 nm, emission filter 530 nm) and the percent specific lysis was calculated according to the formula $[(\text{test release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$. Each set of samples was analyzed in triplicate and the same experiments were repeated 3 times with similar findings.

Statistical analysis:

The data were presented as the mean $\pm$ standard deviation (SD). Sample size (n) for each statistical analysis was a minimum of three. Statistical analysis and graph plotting were performed with OriginPro 8.5. Differences between groups were determined by one-way Analysis of variance (ANOVA). *p* values of $* < 0.05$ and $** < 0.01$ were considered statistically significant. All the experiments have been repeated for at least three times with similar findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of cell-specific ApEn-NK cells.

a) ApEn-NK formation scheme. b) Aptamer-anchor structures. The CD30-specific ssDNA aptamer sequence was conjugated to different lipophilic anchors, specifically single C18 hydrocarbon chains (Apt-C18), dual C18 hydrocarbon chains (Apt-2xC18), cholesterol (Apt-Chol), or vitamin E (Apt-VitE). For tracking purposes, the aptamer sequence was labeled with fluorochrome Cy3. c) ApEn-NK with surface-anchored Apt-2xC18. Cultured NK92 cells were used for ApEn-NK production. Left: confocal microscopy image of the formed ApEn-NK under light and fluorescent views; Middle and right: time and dose courses of the surface-anchoring of aptamers on NK92 cells detected by flow cytometry. d) Degradation of surface-anchored aptamers by DNase treatment. Left: confocal microscopy image of ApEn-NK post-DNase treatment; Right: changes in surface-anchored aptamers post-DNase treatment by flow cytometry. Parental NK92 cells were used in control experiments. e) ApEn-NK with surface-anchored Apt-Chol. Left: confocal microscopy;

Middle and right: time and dose courses of the surface-anchoring of aptamers. f) Degradation of surface-anchored aptamers by DNase. Left: confocal microscopy post-DNase treatment; Right: changes in surface-anchored aptamers post-DNase treatment. Parental NK92 cells were used in control experiments. g) ApEn-NK with Apt-C18. Left: confocal microscopy; Middle and right: time and dose courses of the cell surface-anchoring of aptamers. h) ApEn-NK with Apt-VitE. Left: confocal microscopy; Middle and right: time and dose courses of the surface-anchoring of aptamers.

Figure 2. Characterization of ApEn-NK.

a) Stable ApEn-NK with Apt-2xC18. No changes in surface-anchored aptamers of ApEn-NK cells were detected by flow cytometry analysis (left) or confocal microscopy (right) in 60 min post-ApEn-NK production. b) Unstable ApEn-NK with Apt-Chol. Rapid decrease of surface-anchored aptamers (> 80% reduction) was detected by flow cytometry in 15 min post-ApEn-NK production (left), and near complete loss was observed by confocal microscopy in 60 min (right). c) Stability time course study of ApEn-NK with Apt-2xC18. Quantitative flow cytometry revealed that the ApEn-NK cells were stable and > 40% of surface-anchored aptamer signals remained 10 h post-ApEn-NK production. d) Cell proliferation assays. No toxicity of Apt-2xC18 structures in NK92 cells was observed in cultures up to 72 h. e) f) and g) Cell-binding specificity of the aptamer sequences. Cell binding assays revealed that synthetic aptamers did not react with NK92 cells (e) or CD30-negative U937, Maver-1, or Jeko-1 cells (f); synthetic aptamers only specifically bound to

K299, SUDHL1, and HDM2 cells, which express CD30 (g). Random ssDNA sequences of the same length were used in negative (–) controls. Data shown are Mean $\pm$ SD, n=3; Statistical analysis and graph plotting were performed with OriginPro 8.5. Differences between groups were determined by one-way Analysis of variance (ANOVA). *p* values of *<0.05 and **<0.01 were considered statistically significant; NS, no statistically significant difference.

Figure 3. Specific binding of ApEn-NK to lymphoma cells.

a) Schematic of specific interactions between ApEn-NK and target cells. b) Specific E/T cluster formation of ApEn-NK. Equal amounts of Effector cells (ApEn-NK or parental NK92 cells) and Target cells (CD30-expressing K299 or CD30-negative U937 cells) were mixed and resulting E/T cell clusters were detected. Left: individual cell populations in the cell mixtures were gated by flow cytometry, including single Effector cells (E), single Target cells (T), and the E/T cell clusters via fluorescence emission of red, green, and both signals, respectively. Right: Percentages (%) of E/T clusters in all cell events were calculated. Parental NK92 cells were used as a baseline for background controls. c) Fluorescence microscopy images of E/T cell clusters. ApEn-NK showed the red fluorescent signal of surface-anchored aptamers, and K299 or U937 cells were pre-stained in green fluorescence. d) Time course analysis of E/T cluster formation. K299, SUDHL-1, and HDLM2 cells are CD30-expressing lymphoma cells; U937, Maver-1, and Jeko-1 are CD30-negative control

cells. e) Number of total cells per formed E/T cluster. f) Effector cells (E) per formed E/T cluster. g) Target cells (T) per formed E/T cluster. Data shown are Mean $\pm$ SD, n=3; Statistical analysis and graph plotting were performed with OriginPro 8.5. Differences between groups were determined by one-way Analysis of variance (ANOVA). *p* values of * <0.05 and ** <0.01 were considered statistically significant; NS, no statistically significant difference.

Figure 4. Specific killing of lymphoma cells by ApEn-NK.

a) Schematic of ApEn-NK effects on targeted cells. b) Schematic of cell killing assays. The pre-stained lymphoma or control cells were treated with ApEn-NK or parental NK92 cells. Post incubation, individual cell populations of the mixtures were initially separated and gated by flow cytometry (left). Subsequently, the gated single lymphoma cells were further analyzed for their apoptosis/death rates by cellular staining of Cy5 Annexin V and eFluor 450, respectively (right). c) Specific killing of lymphoma cells by ApEn-NK. Apoptotic/dead rates (%) of individual CD30-expressing lymphoma cells (K299, SUDHL1, and HDLM2 cells) at different E/T ratios were detected, and shown as mean $\pm$ SD (upper row). In control experiments, individual CD30-negative cells (U937, Maver, and Jeko cells) were tested under the same treatment conditions (lower row). Parental NK92 cells were used as baseline background controls. d) Highest killing effect occurred at the lowest E/T ratio. The average mean $\pm$ SD of ApEn-NK cellular effects on all three lymphoma cell lines was

calculated and normalized against baseline resulting from parental NK92 cells. Results from three off-target control cell lines were also shown. Data shown are Mean $\pm$ SD, n=3; Statistical analysis and graph plotting were performed with OriginPro 8.5. Differences between groups were determined by one-way Analysis of variance (ANOVA). *p* values of * <0.05 and ** <0.01 were considered statistically significant; NS, no statistically significant difference.

Figure 5. Functional study of ApEn-NK derived from primary human NK cells.

a) *In vitro* expansion of primary NK cells. Primary NK cells were isolated from fresh peripheral blood samples from healthy human donors and cultured *in vitro*. Viable primary NK cells were counted at day 7, 14, and 21, and cell expansion results from three donors are shown. Primary NK cells cultured for 14–21 days were used for ApEn-NK production. b) Confocal microscopy of ApEn-NK. Light view of ApEn-NK derived from primary human NK cells and fluorescent view of cell surface-anchored aptamer signals. c) Time course study of aptamer anchoring reaction. Expanded primary NK cells were incubated with 1 μ mol/L Apt-2xC18 at room temperature and cellular signals derived from surface-anchored aptamers were quantified by flow cytometry at different time points as indicated. d) Dose course study of aptamer anchoring reaction. Expanded primary NK cells were treated for 30 minutes with Apt-2xC18 at different concentrations as indicated, and cellular signals derived from surface-anchored aptamers were quantified by flow cytometry. e) and f) Specific

binding of ApEn-NK to lymphoma cells. E/T cell cluster formation was analyzed by flow cytometry. The single target cells (K299 or U937), single effector cells (primary NK or ApEn-NK), and resulting E/T clusters containing both target and effector cells were quantified. In comparison to primary NK cells, ApEn-NK induced a significant high binding rate to K299 cells, but caused little change in binding to off-target U937 cells. g) and h) Specific killing of lymphoma cells by ApEn-NK. Target cells (K299 and U937) were treated with ApEn-NK or primary NK cells derived from three healthy donors at different E/T ratios as indicated. Cell killing effects were determined by quantifying apoptosis/death rates of target cells using flow cytometry. In comparison to primary NK cells derived from the same donors, ApEn-NK had significantly higher killing efficacy of lymphoma cells (K299), but little effect on off-target U937 cells. Notably, the highest killing efficacy was observed at the lowest E/T ratio. Differences between groups were determined by one-way Analysis of variance (ANOVA). *p* values of * <0.05 and ** <0.01 were considered statistically significant; NS, no statistically significant difference.