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CXCR1 Expression to Improve Anti-Cancer Efficacy of Intravenously Injected CAR-NK Cells in Mice with Peritoneal Xenografts

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One reason underlying the failure of current chimeric antigen receptor (CAR) immune therapy to treat solid tumors adequately is insufficient tumor infiltration of CAR immune cells. To address the issue, we electroporated natural killer (NK) cells with two mRNA constructs encoding the chemokine receptor CXCR1 and a CAR targeting tumor-associated NKG2D ligands. The CXCR1-modified NK cells displayed increased migration toward tumor supernatants *in vitro* and augmented infiltration into human tumors *in vivo* in subcutaneous and intraperitoneal xenograft models. Most importantly, the cytotoxicity of the CAR-NK cells was not affected by CXCR1 transgene expression, and the enhanced tumor trafficking following intravenous injection resulted in significantly increased antitumor responses in mice carrying established peritoneal ovarian cancer xenografts. Collectively, our findings suggest that the coexpression of CXCR1 and a CAR may provide a novel strategy to enhance therapeutic efficacy of NK cells against solid cancers.

INTRODUCTION

Cancer immunotherapy employing genetically engineered T lymphocytes that express chimeric antigen receptors (CARs) is an effective approach in treating several hematological cancers. The translation of CAR immune cell therapy to nonhematological malignancies is, however, challenging, due to the special pathophysiological characteristics of solid tumors, including target antigen heterogeneity, obstacles to CAR immune cell trafficking, and intrinsic negative regulatory mechanisms of the tumor microenvironment (TME).

Gene transfer to express chemokine receptors on CAR immune cells is considered to be one possible strategy to overcome the trafficking barrier. CXCR1 (interleukin 8 receptor alpha [IL-8RA]) and CXCR2 are the two G protein-coupled receptors that bind IL-8 with high affinity. The expression of the proinflammatory cytokine IL-8, produced by tumor tissues to recruit leukocytes, is significantly higher across many different tumor types as compared to healthy tissues, including solid tumors (brain, breast, cervical, colon, gastric, lung, melanoma, mesothelioma, ovarian, prostate, renal, and thyroid) and hematological malignancies (acute myeloid leukemia [AML], chronic lymphocytic leukemia [CLL], and Hodgkin's lymphoma).[1,2] Previous studies have provided clear evidence that serum IL-8 levels correlate with tumor burden and prognosis in patients with different tumor types.[2] In line with the findings on the increased production of IL-8 in the TME, the involvement of IL-8 in cancer stem cells, angiogenesis, epithelial-mesenchymal transition (EMT), cancer cell invasion, and metastasis has previously been reported.[1,2]

Genetic manipulation to express CXCR1 has been tested in tumor-infiltrating lymphocytes (TILs), which enhance the migration of the transferred T cells toward melanoma or recombinant IL-8.[3] More studies have been performed with gene transfer to express CXCR2. It has been shown that transduction with CXCR2 improves T cell migration to tumor sites, and when tumor-specific T cells were used, the modified T cells display improved antitumor immune responses.[4–6] A clinical phase I/II trial to test adoptive cell transfer with T cells transduced with CXCR2 in patients with metastatic melanoma is currently ongoing (ClinicalTrials.gov: NCT01740557).

Adoptive cell transfer therapy with natural killer (NK) cells is one of the most promising immunotherapeutic modalities for cancer patients. Again, the success of the approach has, so far, been limited to patients with hematological malignancies. To improve the efficacy of NK cell therapy against solid cancers, CXCR2 transduction has been tested to increase the ability of human NK cells to migrate specifically along a chemokine gradient of recombinant CXCR2 ligands or the supernatants of osteosarcoma and renal cell carcinoma cells.[7,8] Genetic engineering of NK cells to improve their homing and tumor penetration has also been explored with genes encoding the chemokine receptor CXCR4 and CCR7.[9,10] Notably, genetic engineering to coexpress CXCR4 and epidermal growth factor receptor (EGFR) vIII-specific CAR on a NK cell line conferred a significantly increased survival of mice carrying CXCL12/stromal cell-derived factor 1a (SDF-1a)-secreting human U87 glioblastoma xenografts when compared with the treatment with NK cells expressing only the EGFRvIII-specific CAR or mock control.[9]

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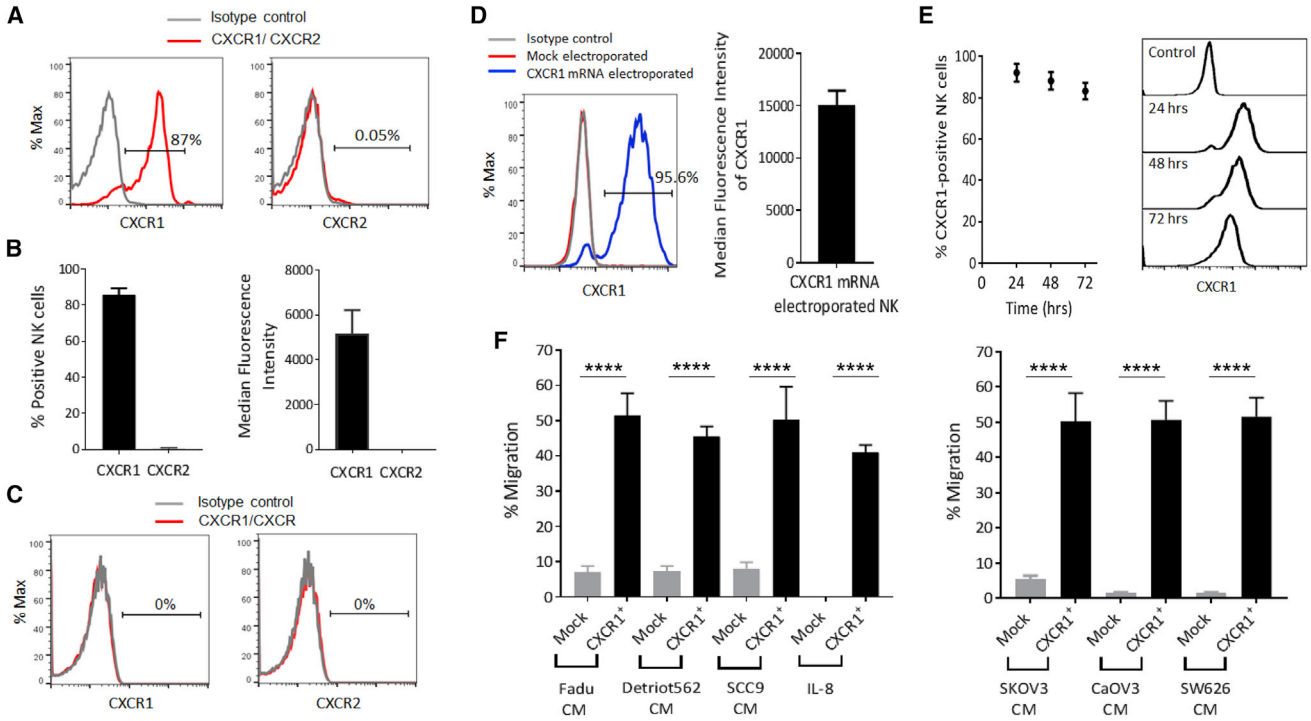

Figure 1. CXCR1 and CXCR2 Expression on Natural Killer (NK) Cells and Overexpression of CXCR1 to Restore NK Cell Migration Ability

(A) Expression of IL-8 receptors CXCR1 and CXCR2 on freshly isolated NK cells. Representative histogram plots are shown. (B) Quantitative analysis of CXCR1 and CXCR2 expression on freshly isolated NK cells. Left: % of positive NK cells. Right: median fluorescence intensity. Data represent the mean ± SD of five independent experiments using five different NK cell samples. (C) Expression of CXCR1 and CXCR2 after *ex vivo* expansion of NK cells, as outlined in Material and Methods. Representative histogram plots are shown. (D) Electroporation to restore CXCR1 expression on NK cells. NK cells were collected 24 h after electroporation for analysis. Left: a representative histogram plot is shown. Right: median fluorescence intensity of CXCR1 expression on NK cells after CXCR1 mRNA electroporation. Data represent the mean (±standard deviation [SD]) of three independent experiments using three different NK cell samples. (E) The persistence of CXCR1 expression on NK cells was maintained for at least 72 h. Left: % change of CXCR1-positive NK cells over time. Data represent the mean (±SD) of three independent experiments. Right: representative histogram plots to show CXCR1 expression peak shifting over time. (F) Overexpression of CXCR1 to restore the NK cell migration ability toward IL-8-secreting tumor cells. *In vitro* migration of CXCR1-overexpressing NK cells toward conditioned media (CM) derived from head and neck cancer cell lines (left) and ovarian cancer cell lines (right). IL-8 (50 ng/mL) was used as a positive control. Data represent the mean ± SD of three independent experiments using three different NK cell samples, each performed in triplicate. ****$p < 0.0001$, statistical significance between CXCR1-overexpressing NK cells and mock NK cells in (F).

Given the observations summarized above that CXCR1 binds to IL-8 with high affinity and that IL-8 is secreted by cancer cells and stroma cells of TME in various cancer types, we reason that genetic engineering to generate CAR-NK cells expressing high levels of the CXCR1 chemokine receptor will result in improved tumor homing/penetration and enhanced tumor-killing efficacy of the modified NK cells across many different cancer types. We tested the hypothesis using electroporation of human NK cells to coexpress CXCR1 and the NKG2D CAR, a unique CAR construct that holds potential to treat multiple tumor types that express NKG2D ligands (NKG2D-L).[11–15]

RESULTS

Gene Transfer to Revert the Expression of CXCR1 on NK Cells Can Augment Their *In Vitro* Migration Ability toward IL-8-Secreting Tumor Cells

Ex vivo expansion of NK cells is a required step to obtain a large number of NK cells prior to adoptive transfer in clinical settings. We started by investigating whether the IL-8 receptors CXCR1 and CXCR2 are expressed before and after NK cell expansion. With the use of flow cytometry analysis, we observed that the majority of freshly isolated NK cells (>80%) expressed a high level of CXCR1, but there was almost no expression of CXCR2 on these cells (Figures 1A and 1B). We adopted a K562 artificial antigen-presenting cell (aAPC)-based method for NK cell expansion.[15] K562 feeder cells expressing membrane-bound (mb)IL-15, mbIL-21, and 4-1BBL were cocultured with peripheral blood mononuclear cells (PBMCs) at a 1:1 ratio for 2 weeks. With this method, the number of NK cells from PBMCs had expanded by approximately 5,000-fold, with a final purity of >90%. When the expression of CXCR1 and CXCR2 on *ex vivo*-expanded NK cells was examined, we observed the downregulation of CXCR1 to a level the same as that provided by the isotype control, whereas expression of CXCR2 remained undetectable (Figure 1C).

We transfected NK cells with mRNA encoding CXCR1 by electroporation to restore its expression. We optimized the electroporation condition, as detailed in Materials and Methods, to achieve

70%–80% NK cell viability yet a satisfactory mRNA transfection efficiency (Figure S1). mRNA electroporation induced the overexpression of CXCR1 on more than 95% of NK cells (Figure 1D). The median fluorescence intensity (MFI) increased from an undetectable level on mock-electroporated NK cells to 15,000 on CXCR1-transfected cells. Compared to freshly isolated NK cells (Figure 1B), CXCR1-electroporated NK cells showed an approximately 3-fold higher expression level of CXCR1. The transgene expression lasted for at least 72 h, the longest time point examined (Figure 1E). We then tested the *in vitro* migration of the transfected NK cells toward the conditioned media collected from a panel of human cancer lines that secretes IL-8 (Figure S2). As shown in Figure 1F, the conditioned media were as effective as, if not more potent than, the chemokine IL-8 (Figure S3) to attract CXCR1-modified NK cells but not those without CXCR1 modification (mock controls). Compared to mock NK cells, CXCR1-modified NK cells displayed an approximately 5-fold increase in migration ability. Mock NK cells showed some migration toward the conditioned media of the head and neck cancer cell lines that secrete CXCL10, probably because of CXCR3 expression after NK cell expansion (Figure S4). These results demonstrated that restoring CXCR1 expression on the *ex vivo*-expanded NK cells is effective in promoting their migration toward IL-8-secreting cancer cells.

CXCR1-Expressing NK Cells Display Improved *In Vivo* Tumor Infiltration

We then investigated whether the enhanced migration of NK cells toward tumor cells via overexpression of CXCR1 could be established *in vivo*. A human head and neck cancer xenograft model was established by inoculating FaDu human hypopharyngeal cancer cells subcutaneously (s.c.) at the left flank of NSG mice. 10 days after tumor inoculation, NK cells were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide or $DiOC_{18}(7)$ (DiR), a lipophilic, near-infrared (NIR) fluorescent cyanine dye ideal for staining cytoplasmic membrane, and DiR-labeled NK cells were intravenously (i.v.) injected through the tail vein. Cell distribution in mice was imaged every 24 h for 3 days. Whereas DiR fluorescence signals remained in the lung region by 24 h post-NK cell injection, enhanced DiR signals in the tumor sites were observed by 48 h in mice injected with CXCR1-expresssing NK cells, which became more pronounced by 72 h (Figure 2A). Whole-body imaging was unable to detect DiR signals in the tumor sites in mice injected with mock NK cells during the 72-h period. The quantitative analysis of DiR fluorescence signal intensity at the tumor sites demonstrated an approximately 10-fold increase in signal intensity in mice injected with CXCR1-expresssing NK cells over those injected with mock NK cells by 48 and 72 h (Figure 2A). Mice were euthanized after 72 h, and the subcutaneous tumors were collected for *ex vivo* imaging. The images of the tumors and the associated flux values are shown in Figure 2B. Similarly, an approximately 10-fold increase in signal intensity was observed in mice injected with CXCR1-expresssing NK cells as compared to those injected with mock NK cells. Thus, overexpression of CXCR1 in NK cells can improve the migration/infiltration of i.v.-injected NK cells toward subcutaneous tumors.

We repeated the mouse experiment in a human SKOV3 ovarian peritoneal carcinoma xenograft model established by intraperitoneal (i.p.) injection of SKOV3-Luc (luciferase) cells, a tumor cell line genetically modified with the firefly Luc gene, in mice. Seven days after tumor inoculation, the mice were randomized, based on bioluminescence imaging (BLI), with similar tumor burden being used as a criterion for randomization. The mice were then i.v. injected through the tail vein with DiR-labeled mock NK cells or CXCR1-expressing NK cells. Figure 2C shows that based on BLI, SKOV3-inoculated NSG mice had similar tumor burdens before injection with either mock NK or CXCR1-expressing NK cells. When we attempted to examine whether there was an increase in the DiR signal at the peritoneal region of the mice inoculated with SKOV3 cancer cells, increased DiR signals over the peritoneal region were observed in the mice receiving CXCR1-expressing NK cells but not in those injected with mock NK cells (Figure 2C). Furthermore, we scarified the mice and isolated the tumors from the peritoneal cavity for *ex vivo* imaging of the tumor tissues. Figure 2D showed that CXCR1 overexpression enhanced the DiR signals of the tumor tissues, resulting in a 3.8-fold increase in signal intensity by 48 h post-NK cell injection compared with the tumors from mice injected with the mock NK cells. The flux value difference was statistically significant between two groups, $p < 0.0001$.

We further used another method to investigate *in vivo* NK cell migration toward tumors in the human SKOV3 ovarian peritoneal carcinoma xenograft model. 7 days after tumor inoculation, human NK cells mock electroporated or CXCR1-modified NK cells were injected through the tail vein into tumor-bearing mice, and 2 days later, the whole-cell population from the i.p. lavage was collected, stained with an antibody specific to human CD56, and analyzed by flow cytometry. The results showed that the percentage of human CD56-positive cells in i.p. lavage increased from 4% in the mock NK cell-injected mice to close to 8% in the mice injected with CXCR1-modified NK cells (Figure 2E). We therefore conclude that the overexpression of CXCR1 on NK cells can improve the migration/infiltration of i.v.-injected NK cells toward peritoneal tumors.

Improved Tumor Infiltration of CXCR1-Expressing NK Cells Is Associated with an Enhanced *In Vivo* Tumor Control

We went on to examine whether the improved tumor migration/infiltration could enhance the *in vivo* killing capacity of CXCR1-overexpressing NK cells against cancer cells. Prior to the animal experiment, we examined whether *in vitro* killing activity of NK cells would be affected by overexpression of CXCR1 on NK cells. Wild-type K562, a human leukemia cell line susceptible to NK cell-mediated cell lysis, was used as a target in a standard cytotoxicity assay. As shown in Figure 3A, CXCR1-expressing NK cells displayed similar cell lysis activity against K562 cells as nonelectroporated NK cells and mock-electroporated NK cells. Moreover, no difference was observed between CXCR1-expressing NK cells and mock-electroporated NK cells when their killing capacities were tested against human SKOV3 ovarian cancer cells.

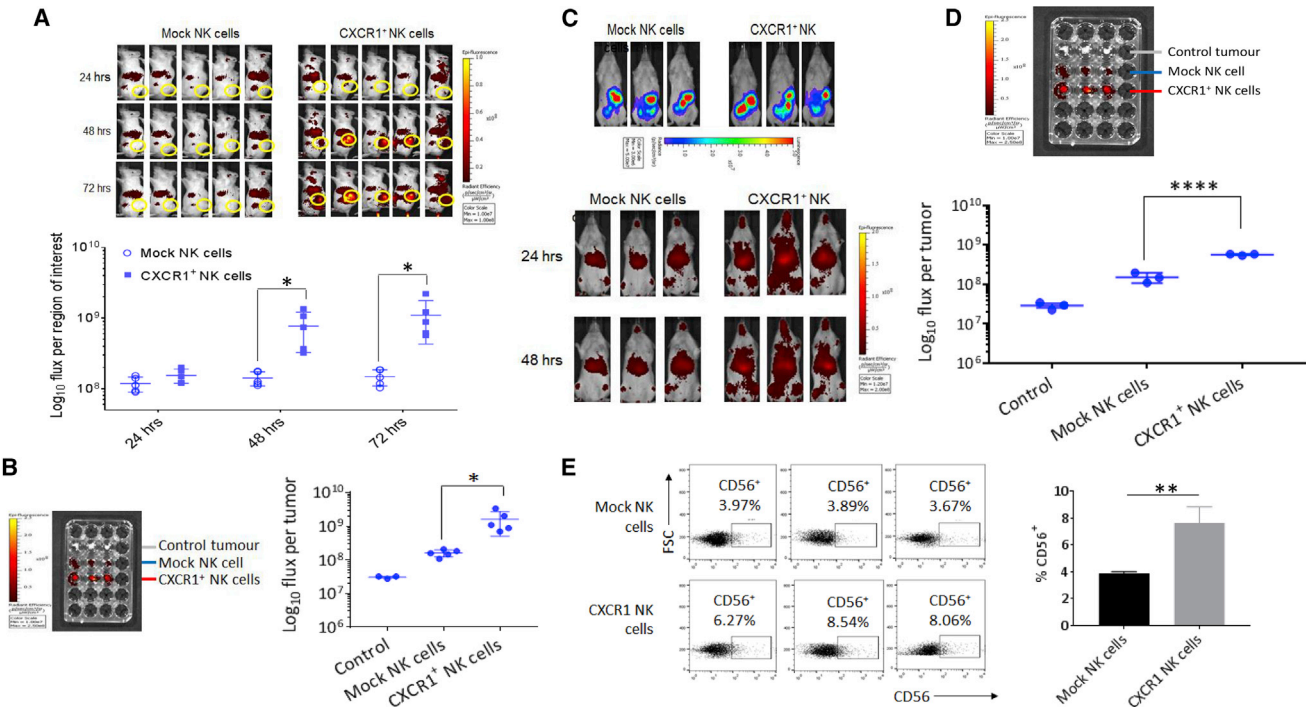

Figure 2. *In Vivo* Migration and Tumor Infiltration of CXCR1-Overexpressing NK Cells

(A and B) A FaDu subcutaneous xenograft model. Mice were inoculated with FaDu human hypopharyngeal cancer cells subcutaneously at the left flank, and 10 days later, DiR-labeled NK cells were intravenously injected through the tail vein. After the NK cell injection, mice were imaged every 24 h up until 72 h. (A) Whole-body imaging of mice and flux values at the tumor sites (circled in yellow in the above whole-body images) were plotted in the bar graph, n = 5. (B) *Ex vivo* imaging of isolated tumors from the right flank, 72 h after NK cell injection. Tumors obtained from mice without NK cell injection served as control. Flux values of the isolated tumors are shown on the right, n = 5. *$p < 0.05$, statistical significance between CXCR1-overexpressing NK cells and mock NK cells in (A) and (B). (C–E) A SKOV3 intraperitoneal (i.p.) xenograft model. The SKOV3 xenograft model was established by i.p. injection of SKOV3-Luc cells. 7 days after tumor inoculation, mice were intravenously injected with NK cells through the tail vein. (C) Top: bioluminescence images to show similar tumor burdens in the two groups of mice on day 7 post-tumor inoculation. Bottom: whole-body imaging to show *in vivo* migration of DiR-labeled, mock NK cells versus CXCR1-overexpressing NK cells toward SKOV3 xenografts, 24 and 48 h after NK cell injection. (D) *Ex vivo* imaging of isolated tumors from the peritoneum, 48 h after NK cell injection. Tumors obtained from mice without NK cell treatment served as controls (control tumor). Flux values of the isolated tumors are shown below, n = 3. ****$p < 0.0001$, statistical significance between CXCR1-overexpressing NK cells and mock NK cells in (D). (E) In another experiment with the same SKOV3 model, two groups of mice (n = 3 per group) were intravenously injected with mock NK cells or CXCR1-modified NK cells through the tail vein. 2 days later, the whole-cell population from the i.p. lavage was collected, stained with an antibody specific to human CD56, and analyzed by flow cytometry. Left: flow cytometry plots to show the percentages of $CD56^+$ NK cells recovered from three mice of each group after peritoneal wash. Right: the percentages of $CD56^+$ NK cells of the two groups were represented in a bar chart. **$p < 0.01$, statistical significance between CXCR1-overexpressing NK cells and mock NK cells.

We then established the SKOV3-Luc tumor model, as described above, and treated the mice with three different ways: PBS, mock NK cells without CXCR1, and NK cells overexpressing CXCR1 (Figure 3B). The treatments via i.v. tail vein injection started 7 days after tumor inoculation, twice a week for 2 weeks. As shown in Figure 3C, tumor burdens were similar among the three groups of mice on day 7. By day 21, we observed a modest effect in the reduction of tumor burden in mice treated with CXCR1-expressing NK cells, as demonstrated by BLI. The flux value differences on day 21 were statistically significant, with the values from mice treated with CXCR1-expressing NK cells lower than those from the PBS control group, as well as the mock NK cell group, $p < 0.05$ (Figure 3D). No statistical significance was observed between the PBS group and the mock NK cell group on day 21. Thus, improved tumor migration/infiltration of CXCR1-expressing NK cells can be helpful to reducing tumor burden *in vivo*.

NK Cells, Cotransfected with CXCR1 and NKG2D CAR, Are Effective in Reducing Tumor Burden

Müller et al.[9] have reported a study using the human YTS NK cell line, derived from a patient with NK cell leukemia and modified with EGFRvIII-specific CAR and CXCR4. Several other papers reported immunotherapy studies using CAR-T cells overexpressing a chemokine receptor to improve therapeutic efficacy.[16–20] However, so far, no such studies are reported in the literature with human primary NK cells from peripheral blood, the most commonly used type of NK cells in the clinical setting.

We were particularly interested in investigating whether combining a chemokine receptor and a CAR may enhance the anti-cancer efficacy of human PBMC-derived primary NK cells. A CAR specific to NKG2D-L, which is highly upregulated

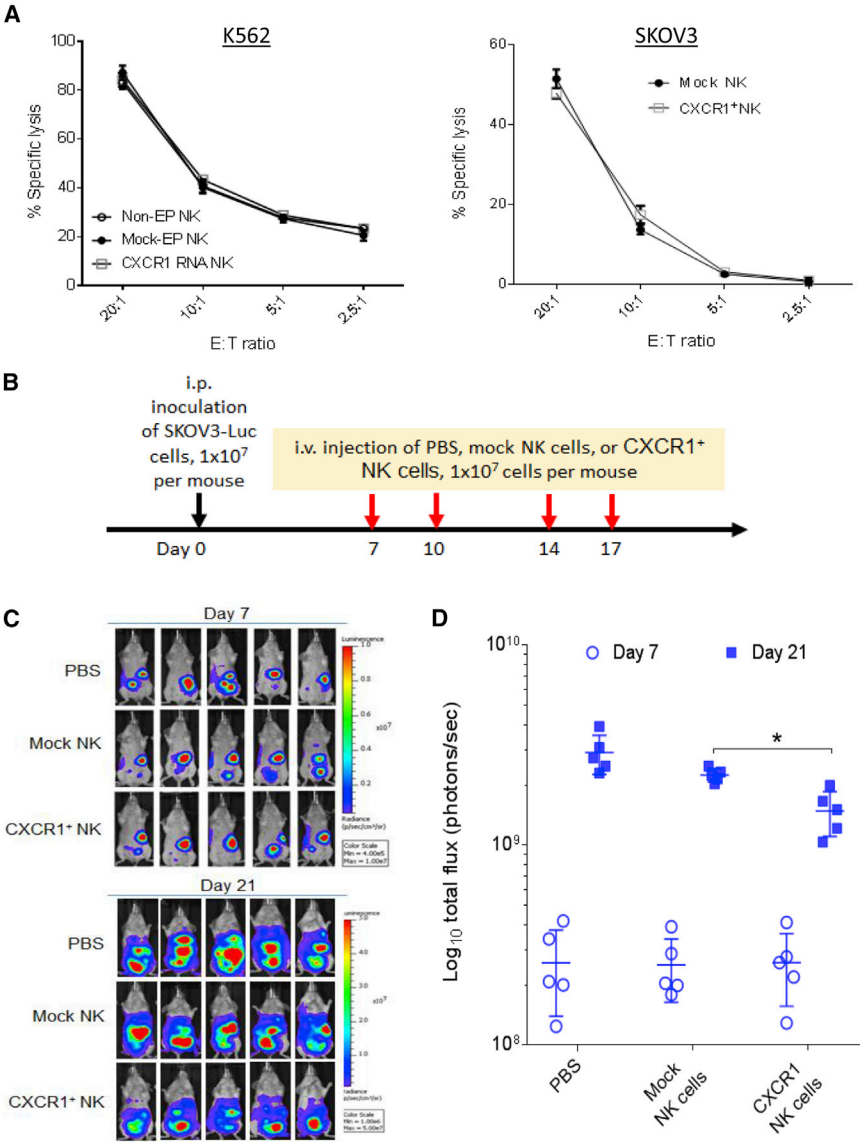

Figure 3. Killing Capacity of CXCR1-Overexpressing NK Cells against Cancer Cells

(A) *In vitro* killing capacity. Left: modification of NK cells to overexpress CXCR1 using RNA electroporation (EP) did not affect their killing capacity against the K562 cell line. CXCR1-overexpressing NK cells, mock-EP NK cells, and non-EP NK cells were used. Right: no difference was observed between CXCR1-overexpressing NK cells and mock-electroporated NK cells when their killing capacities were tested against human SKOV3 ovarian cancer cells. Experiments were performed in triplicate and repeated three times with similar results. (B) Experimental outline for the animal study to examine *in vivo* killing capacity against i.p. xenografts of human SKOV3 ovarian cancer cells. Three groups of NSG mice (5 mice per group) received i.p. injection of SKOV3-Luc cancer cells (day 0), followed by i.v. injection of PBS, mock NK cells, or CXCR1-overexpressing NK cells, as indicated. (C) Bioluminescence images on day 7 and day 21 post-tumor inoculation. (D) Flux values of tumor burden on day 7 and day 21 were plotted in the bar graph, n = 5. *$p < 0.05$, statistical significance between CXCR1-overexpressing NK cells and mock NK cells on day 21 is indicated.

on various types of tumor tissues, was used. Three different NKG2D-L-targeting CAR constructs were prepared initially, which share the same human NKG2D extracellular domain (ED), a CD8α hinge and transmembrane region, and the intracellular signaling domain CD3zeta motifs. These CAR constructs differ in costimulatory domains, varying from no costimulatory domain (1st-generation CAR) to one costimulatory domain CD28 (2nd-generation CAR) to two costimulatory domains CD28 and 4-1BB (3rd-generation CAR) (Figure S5). After mRNA electroporation to introduce the CAR constructs into NK cells, we compared their tumor cell killing activities against two head and neck cancer lines and selected the first-generation NKG2D CAR (NKG2Dz) that showed the highest activity among the 3 tested RNA CARs for following investigations in the current study.

A representative flow cytometry histogram plot of NKG2D CAR expression on NK cells, 24 h after mRNA electroporation, is shown in Figure 4A. western blot analysis, using an anti-CD3zeta antibody, confirmed that NKG2D CAR expression could last for at least 5 days in NK cells (Figure 4A). Coexpression of the NKG2D and CXCR1 was achieved in close to 90% of modified NK cells (Figure 4B). To exclude the possibility of fratricide by the native NKG2D receptor or NKG2D CAR, we examined the expression of NKG2D ligands on fresh, expanded, and mock-electroporated NK cells, and none of these ligands could be detected on NK cells (Figure S6). In the *in vitro* migration assay, CXCR1 was effective in augmenting the migration of the cotransfected NK cells toward the SKOV3 conditioned medium when compared with the migration of NK cells modified with NKG2D CAR alone (Figure 4C), albeit resulting in a lower percentage of migratory cells when compared to the migration of the NK cells transfected with the CXCR1 gene alone in Figure 1F. This is because coelectroporation of two different mRNA molecules led to a lower expression level of CXCR1 on NK cells (Figure S7). Further functional evaluation with the *in vitro* cytotoxicity assay demonstrated that CXCR1 expression did not affect the target tumor cell lysis activity of NKG2D CAR in NK cells (Figure 4D).

Having obtained encouraging *in vitro* results, we moved on to use the SKOV3-Luc tumor model again for *in vivo* evaluation. NKG2D ligand expression was confirmed on SKOV3 cells (Figure S8).

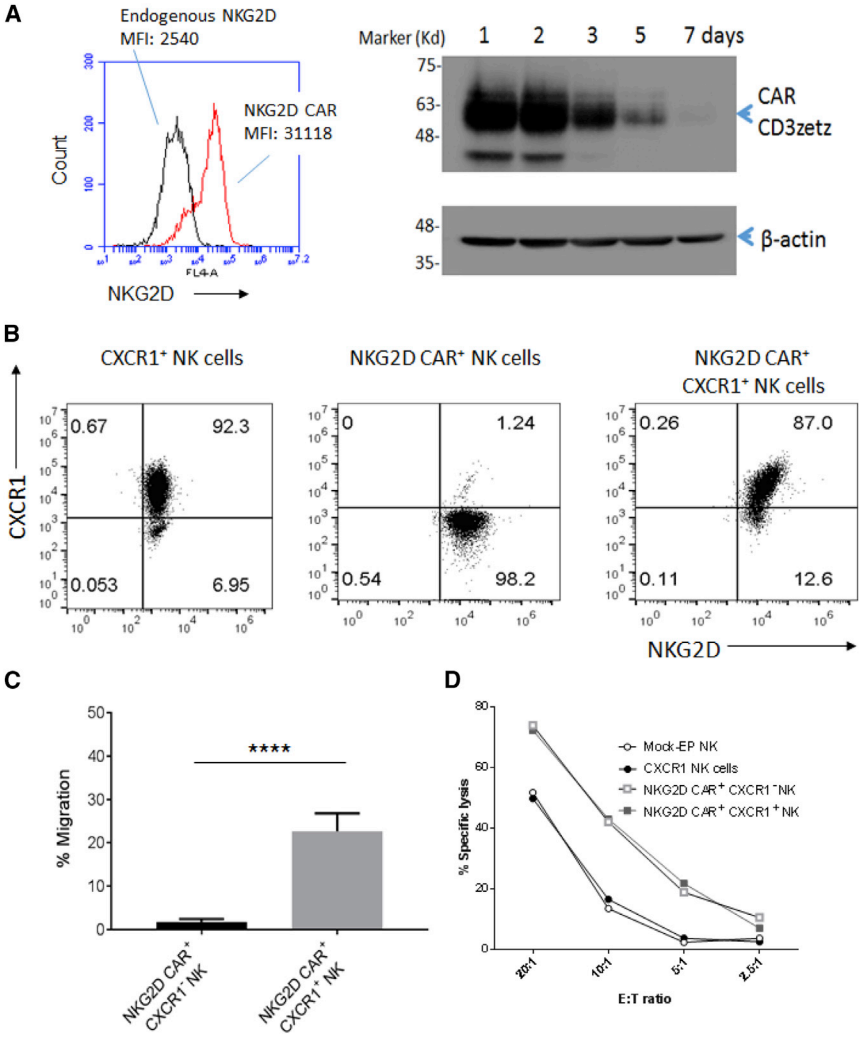

Figure 4. *In Vitro* Killing of Tumor Cells by NKG2D CAR-Modified, CXCR1-Overexpressing NK Cells

(A) NKG2D CAR expression. Left: a representative flow cytometry histogram plot. NK cells were electroporated with an mRNA-encoding NKG2D CAR, and the NKG2D CAR expression was examined 24 h later with an antibody against NKG2D. Right: time-lapse analysis of NKG2D CAR expression in NK cells with anti-CD3zeta antibody western blotting. (B) Modification of NK cells to coexpress NKG2D CAR and CXCR1 using RNA electroporation (EP). Flow cytometry two-parameter density blots to show CXCR1 and endogenous NKG2D expression, NKG2D CAR expression without CXCR1, and the simultaneous expression of CXCR1 and NKG2D CAR on NK cells. (C) CXCR1 expression increases the *in vitro* migration of NKG2D CAR-modified NK cells toward the SKOV3 conditioned medium. ****$p < 0.0001$, statistical significance between NKG2D CAR-modified NK cells and CXCR1/NKG2D CAR-modified NK cells. (D) *In vitro* killing against NKG2D ligand-positive SKOV3 cancer cells. NK cells mock electroporated or electroporated with CXCR1 alone display similar *in vitro* killing activities. Also, NK cells electroporated with NKG2D CAR alone and with both NKG2D CAR and CXCR1 display similar *in vitro* killing activities. Experiments were performed in triplicate and repeated three times with similar results.

Mice inoculated with the tumor cells were treated via intravenous tail-vein injection of three different materials: PBS, NK cells expressing NKG2D CAR, and NK cells coexpressing NKG2D CAR and CXCR1 (Figure 5A). The treatments started 7 days after tumor inoculation, twice a week for 2 weeks. BLI demonstrated that the tumor burdens in mice receiving NKG2D CAR$^+$ CXCR1$^+$ NK cells were obviously reduced relative to the initial tumor burdens during the course of treatment (Figure 5B). On day 21, whereas established tumors continued to grow robustly in PBS-treated mice or remained relatively stable in mice treated with NKG2D CAR$^+$ CXCR1$^-$ NK cells, the tumor burdens in mice receiving NKG2D CAR$^+$ CXCR1$^+$ NK cells were noticeably reduced relative to the initial levels. Quantitative analysis of SKOV3-Luc cell bioluminescence signals at this point demonstrated a statistically significant difference in flux values over the peritoneal region with tumor burden between the group of mice treated with NKG2D CAR$^+$ CXCR1$^-$ NK cells and the group with NKG2D CAR$^+$ CXCR1$^+$ NK cells ($p < 0.01$); virtually all of the tumors in the latter group had regressed (Figure 5C). To evaluate the combinatorial effect of CXCR1 and NKG2D CAR, we compared mean photon flux values of tumor burden on day 21 from 6 groups of mice used in the two experiments shown in Figure 3D and Figure 5C, and the values from mice treated with PBS were used as an internal control for cross-experiment comparison (Table S1). The comparison demonstrated that combinatorial modification of NK cells with CXCR1 and NKG2D CAR exerted synergistic anticancer effects.

Although tumors regrew after termination of the treatment, tumor burdens in the group treated with NKG2D CAR$^+$ CXCR1$^+$ NK cells remained lower than those in the mice treated with NKG2D CAR$^+$ CXCR1$^-$ NK cells until at least day 42 (Figure 5D). Consequently, the treatment with NK cells cotransfected with NKG2D CAR and CXCR1 significantly prolonged the survival of the tumor-bearing mice over the two controls (Figure 5E) ($p < 0.0001$), and the median survival time increased to 60 days, relative to 38 days in the PBS group and 50 days in the group treated with NKG2D CAR-NK cells without CXCR1. Thus, the treatment with NKG2D CAR$^+$ CXCR1$^+$ NK cells prolonged median survival by 20% as compared to the group of mice treated with NKG2D CAR-NK cells without CXCR1, demonstrating the beneficial effects of including chemokine receptor CXCR1 in i.v.-injected CAR-NK cells in treating peritoneal carcinoma. Since NKG2D CAR expression on NK cells lasted for only

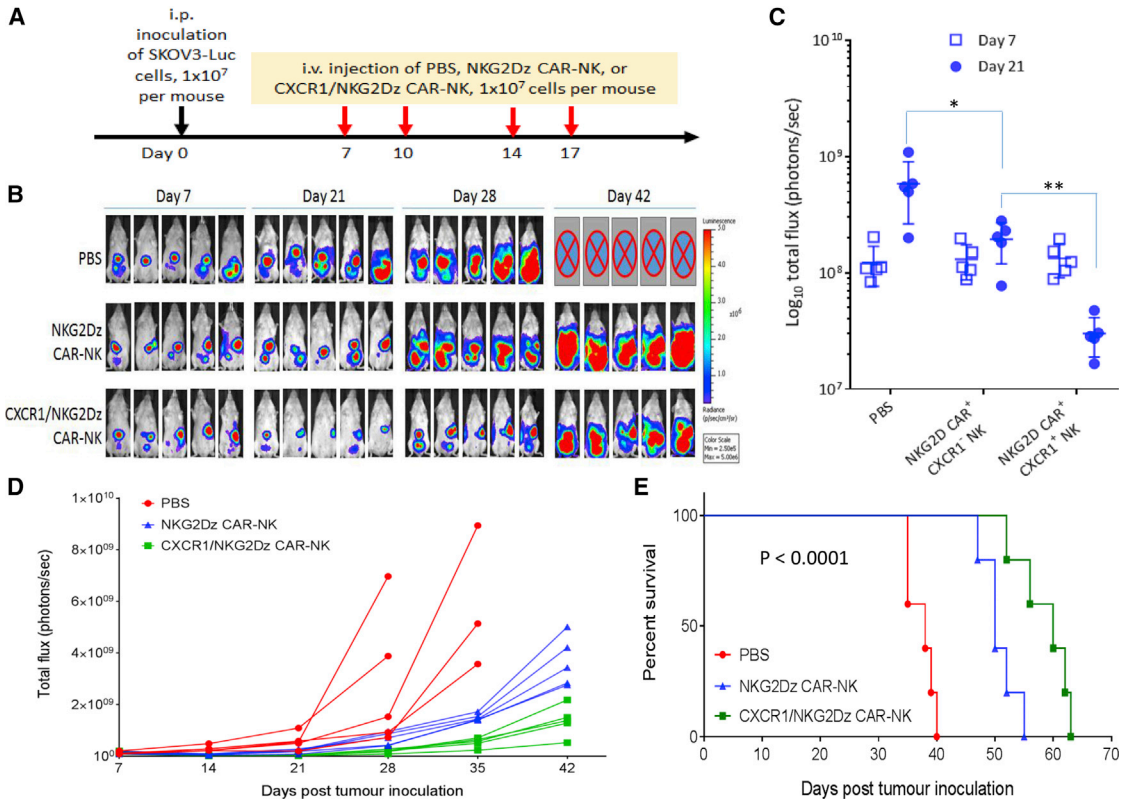

Figure 5. *In Vivo* Killing of Tumor Cells by NKG2D CAR-Modified, CXCR1-Overexpressing NK Cells against SKOV3 i.p. Xenografts

(A) Experimental outline for the animal study. Three groups of NSG mice (5 mice per group) received i.p. injection of SKOV3-Luc cancer cells (Day 0), followed by i.v. injection of PBS, NKG2Dz CAR-NK cells, or CXCR1/NKG2Dz CAR-NK cells, as indicated. (B) Bioluminescence images on days 7, 21, 28, and 42 post-tumor inoculation. All mice died in the PBS group by day 42. (C) Log flux values of tumor burden on day 7 and day 21 were plotted in the bar chart, n = 5. *p < 0.05, statistical significance between the PBS group and the group treated with NKG2D CAR-modified NK cells without CXCR1 on day 21; **p < 0.01, statistical significance between NKG2D CAR-modified, CXCR1-overexpressing NK cells and NKG2D CAR-modified NK cells without CXCR1 on day 21. (D) Flux values of tumor burden from day 7 to day 42 were plotted in the line graph. (E) Kaplan-Meier analysis of survival of the *in vivo* experiment. Statistical analysis of survival between groups was performed using the log-rank test.

5 days, the prolonged mouse survival also could be partially contributed by the persistence of human NK cells in NSG mice, which could be for at least 2 weeks[21] or up to 35–54 weeks when exogenous IL-2 is provided.[22,23]

DISCUSSION

CAR-NK cell therapy is starting to gain traction in recent years. There are at least 11 ongoing clinical trials investigating the effects of CAR-NK cells.[24] A recent paper reported a novel, single-center pilot study using mRNA-electroporated CAR-NK cells with transiently increased specificity and cytotoxicity against NKG2D-L in three cancer patients diagnosed with late-stage, metastatic colorectal cancer.[15] These ligands are an attractive CAR cell therapy target since they are not usually expressed on healthy tissues but commonly detected on carcinomas due to the upregulated expression upon DNA damage and cell transformation.[11–15] The clinical benefits observed in the study include reduced ascites generation, a decrease in number of tumor cells in ascites samples, and a positron emission tomography-computed tomography (PET-CT)-confirmed complete metabolic response in the treated tumor lesions, highlighting the feasibility of using NKG2D CAR-modified NK cells to treat metastatic colorectal cancer. However, the antitumor effects were restricted locally, and all patients died soon after the CAR-NK treatment in the study,[15] indicating a need for further improvement of the efficacy of the NKG2D CAR-NK cells.

The improvement of CAR immune cell trafficking to a tumor site may conceivably affect the efficacy of CAR cell therapy. With the consideration of the possibility that chemokine/receptor mismatch may account for the insufficient trafficking, we tested gene transfer to express CXCR1 on NK cells in the current study and observed that the anticancer efficacy of NKG2D CAR-NK cells could be further improved by the overexpression of CXCR1 in a mouse tumor model. The study was designed based on our observation that whereas CXCR1 was expressed on freshly isolated NK cells, its expression did not persist and was totally abolished after *ex vivo* expansion of NK cells, a process necessary for adoptive therapy of NK cells. This change on NK cells is similar to what was observed in a study using TILs, in which the

expression of CXCR1 on TILs was downregulated during *in vitro* culturing,[3] setting the ground for genetic manipulation with a gene encoding CXCR1. Whereas the study by Sapoznik and colleagues[3] has shown the improved migration ability of infused TILs toward tumors by genetic manipulation to express CXCR1, to the best of our knowledge, this is the first report demonstrating that gene transfer of CXCR1 can improve the migration of another type of immune cells, namely, innate NK cells, toward tumors. More importantly, we extended the investigation to potent CAR-NK cells and are the first to show that improved trafficking of intravenously infused CAR-NK cells to tumor sites augments their antitumor immune response, producing obviously enhanced tumor control in mice bearing peritoneal ovarian carcinoma.

Both CXCR1 and CXCR2 chemokine receptors display high affinity for IL-8. Thus, gene transfer to express CXCR2 on *ex vivo*-expanded NK cells represents another possible strategy to improve antitumor effects of adoptively transferred NK cells.[25] These two receptors, however, differ noticeably in their chemokine-binding specificity: CXCR1 interacts with IL-8 with high affinity and with CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7 with low affinity, whereas CXCR2 is capable of binding all of these CXC chemokines with higher affinity.[1,2] Nasser and colleagues[26] reported that after IL-8 binding, CXCR1 was less sensitive to receptor internalization relative to CXCR2; thus, its surface expression was prolonged. The expression of CXCR1 and CXCR2 on NK cells has been investigated with somewhat divergent results. Campbell and colleagues[27] have found that $CD56^{+}CD16^{+}$ NK cells uniformly express high levels of CXCR1 but low levels of CXCR2. Kremer and colleagues[8] reported that whereas circulating NK cells from healthy donors expressed CXCR2, these cells rapidly lost CXCR2 expression upon *in vitro* culture and expansion, which is similar to what we observed for CXCR1 in the current study. They further noted that when a mixed pool of CXCR2 cognate ligands CXCL1–8 was used, an increased concentration of IL-8 did not improve the migration ability of $CXCR2^{+}$ NK cells.[8] Inngjerdingen and colleagues[28] neither detected CXCR2 expression on fresh nor on *in vitro*-activated NK cells, a finding consistent with our observation for CXCR2. Nevertheless, our finding that whereas CXCR1, but not CXCR2, was physiologically expressed by freshly isolated NK cells, and neither of them was expressed on *ex vivo*-expanded NK cells prompted us to investigate the genetic modification to re-express CXCR1. This modification could be clinically meaningful, since as demonstrated in the TME of melanoma-infiltrated lymph nodes, the release of IL-8 by TME can efficiently recruit CD56 (dim) KIR^{+} $CD57^{+}$ highly cytotoxic NK cells, and the recruitment is associated with improved prognosis for melanoma patients.[29]

It has been reported that *ex vivo*-expanded NK cells display an increased level of CXCR3 expression, which may facilitate their migration toward CXCL10-producing tumor cells and intratumoral infiltration.[30] The increased CXCR3 expression upon NK cell expansion was confirmed in the current study (Figure S4) and might already contribute to the migration of nonmodified NK cells toward head and neck cancer cell lines that show low-level release of CXCL10 (Figure S2). Of note, CXCL10 secretion was observed in 3 of the 3 tested head and neck cancer cell lines, albeit at a level lower than that related to IL-8, whereas none of the 3 tested ovarian cancer cell lines that are IL-8 positive secrets CXCL10. Hence, the ability of cancer cells to secrete CXCL10 might be an important step to attract CXCR3-expressing NK cells. On the other hand, with genetic manipulation to express CXCR1 on our CXCR3-positive NK cells, we observed a significantly increased *in vitro* migration of the NK cells over mock NK cells toward both CXCL10-positive and -negative cancer cell lines that all secrete IL-8, a ligand for CXCR1. Furthermore, improved *in vivo* infiltration of these NK cells into solid tumors, as compared with mock NK cells, was confirmed both in subcutaneous and i.p. xenograft models. These findings clearly indicate a more vital role of CXCR1 transgene expression than CXCR3 upregulation upon cell expansion in directing the migration and infiltration of *ex vivo*-expanded human NK cells toward solid tumors.

Clinically, intravenous delivery of NK cells in patients with peritoneal carcinoma has not been successful in ameliorating disease, possibly because of the difficulty for intravenously injected NK cells to traffic to and infiltrate into tumors in a remote region.[31] There are few preclinical animal studies testing intravenously delivered NK cells in treating peritoneal carcinomas. One previous study has discussed the challenge of using systemically delivered NK cells in treating ovarian peritoneal carcinoma mouse model *in vivo*,[22] suggesting that NK cells administered intravenously are unable to receive proper signals to traffic into the peritoneum. Our data provide a clear demonstration of the actual shrinking of established tumors during the treatment (rather than just slowing down tumor growth) for peritoneal ovarian cancer after intravenous infusion of CAR-NK cells. Inhibition of the growth of peritoneal ovarian cancer, followed by intravenous infusion of NK cells without CAR modification, was also observed. The success of the current study is most likely due to equipping the NK cells with CXCR1, thus allowing the modified cells to respond to IL-8 secreted by the tumors in a remote region. The detailed trafficking process of NK cells to tumor tissues through the interaction of CXCR1 on NK cells and IL-8 released from tumor cells is still unclear. Migration of CXCR1-expressing NK cells through the gradient toward the higher concentration of the chemokine in tumors should play a pivotal role. In view of the fact that IL-8 is overexpressed in a broad range of tumor types, our CXCR1 overexpression approach will have important clinical implications in treating many other types of cancers.

mRNA electroporation is expected to be readily applied in a clinical setting given its inherent advantages of ease of use and high reproducibility. This technology was used in the current study to express CXCR1 and NKG2D CAR for the proof-of-concept purpose, which provided a relatively high percentage of doubly positive cells. As expected, coelectroporation of two individual mRNA constructs might reduce transgene expression when compared to transfection of a single construct at the same dosage, as shown in this study for CXCR1 expression, representing a technical limitation. Also, this nonintegrating gene-transfer technology provides only transient

transgene expression. Whereas short-term expression of CXCR1 is probably sufficient to support the enhanced migration of the adoptively transferred NK cells toward tumor sites, in terms of CAR effects, mRNA electroporation can only generate short-lived CAR-expressing NK cells, and viral transduction approaches will be crucial for long-term CAR expression on infused NK cells across multiple cell divisions, thus achieving functional persistence of CAR-expressing cells. However, with the consideration of a coincident inflammatory/infectious process in which both NKG2D stress ligands and IL-8 are overproduced, and the potential on-target/off-tumor risk for toxicity against nonmalignant cells is caused by a long persistence of NKG2D CAR-modified immune cells, mRNA electroporation may be a valuable approach to mitigate the risk.

In contrast to T cells, NK cells hold the clinical advantage in inducing immune responses against malignancies in an antigen-independent and non-major histocompatibility complex (MHC)-restricted manner and can be used as allogeneic cell therapeutics. The significance of NK cell alloreactivity in improving survival has been well demonstrated in hematopoietic stem cell treatment (HSCT) patients, who after receiving grafts with alloreactive NK cells, display a significantly lower risk of relapse.[32] In a CAR-NK cell therapy regimen, it can be reasonably speculated that NK cell alloreactivity will play important roles in preventing relapse with target-negative tumor cells that cannot be recognized by the CAR construct.

In conclusion, with the consideration of the increased production of IL-8 in a broad range of solid tumor malignancies, we have applied genetic manipulation to express the IL-8 receptor CXCR1 in order to match CAR-NK cells with the tumor-secreted chemokine, enabling augmented migration and infiltration to the tumor and most importantly, improved *in vivo* antitumor responses of the immune effector cells. We believe that this strategy is promising in promoting the success of CAR-NK cell therapies against solid tumors.

MATERIALS AND METHODS

Cells and Cell Culture Conditions

Human PBMCs of healthy donors were isolated by gradient centrifugation using Ficoll-Paque (GE Healthcare, Milwaukee, WI, USA) from buffy coats obtained from Health Sciences Authority (HSA; Singapore) approved by the National University of Singapore Institutional Review Board (NUS-IRB reference code B-14-133E). For *ex vivo* expansion of NK cells, PBMCs were cocultured with γ-irradiated K562 clone 5 cells in stem cell growth medium (SCGM) media (CellGenix, Freiburg, Germany), supplemented with 10% fetal bovine serum (FBS) and 50 IU IL-2 (PeproTech, Rocky Hills, NJ, USA) at a ratio of 1 PBMC:2 K562 cells. Human solid tumor cell lines used in the current study were originally obtained from ATCC and maintained in DMEM or McCoy's 5A medium, supplemented with 10% FBS (HyClone, Logan, UT, USA).

CXCR1 and NKG2D CAR Constructs and NK Cell Electroporation

The CXCR1 open-reading frame, together with a T7 promoter and an alpha-globin 3′ UTR sequence, was cloned into a pFastbac1 expression vector previously developed in the lab.[33] To construct a 1st-generation NKG2D RNA CAR vector, the ED of human NKG2D (UniProt P26718, amino acid residues at positions 83–216) was amplified by PCR from a PBMC cDNA library. The NKG2D CAR construct was generated by fusing the C terminus of NKG2D ED to the N terminus of the CD8α hinge and transmembrane region (UniProt P01732, amino acid residues 128–210) and CD3ζ signaling moiety (UniProt P20963, amino acid residues 52–164). The mMESSAGE mMACHINE T7 ULTRA transcription kit (Invitrogen, Carlsbad, CA, USA) was used to generate capped mRNA molecules encoding the CXCR1 expression vector and the NKG2D CAR.

Electroporation of NK Cells

Electroporation with the NEPA21 electroporator (Nepagene, Chiba, Japan) was used for mRNA transfection of NK cells. During the optimization process, a range of conditions for different transfection parameters (RNA concentration, number of NK cells, pulse settings) was tested. The following optimized conditions were used in the current study: 10^7 expanded NK cells were resuspended in 100 μL of Opti-mem media (GIBCO) and mixed with 5 μg of mRNA. NK cells were then transferred to a 2-mm cuvette (Bio-Rad, Hercules, CA, USA) and electroporated at a voltage of 240 and pulse length of 4 ms. Electroporated NK cells were rested overnight prior to downstream experiments.

Flow Cytometric Analysis, Transwell Migration Assay, and Cytotoxicity Assay

Flow cytometric analysis was performed with Acuri C6 (BD Biosciences). Transwell migration assays were performed using 24-well (6.5 mm diameter, 5 μm pore size) Transwell chambers (Costar Transwell, Corning, NY, USA). The cytolytic activity of modified NK cells was examined with a nonradioactive method (DELFIA EuTDA Cytotoxicity Reagents kit; PerkinElmer, MA, USA).

Animal Experiments

Nonobese diabetic/severe-combined immunodeficiency/IL-2Rγcnull (NSG) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained and used in the current study. Animal experiments were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), the Biological Resource Centre (BRC), and the Agency for Science, Technology and Research (A*STAR), Singapore (IACUC protocol number 181324). Mouse xenograft models were generated by s.c. injection of 5×10^6 FaDu tumor cells in the left flank in male NSG mice (8–10 weeks) or i.p. injection of 1×10^7 SKOV3-Luc tumor cells in female NSG mice (8–10 weeks). For *in vivo* migration experiments, NK cells (5×10^6) were labeled with Xenolight DiR (Perkin Elmer, OH, USA) and injected via the tail vein (i.v.) into tumor-bearing mice. Biodistribution of NK cells was examined by DIR imaging at time points indicated and using the IVIS imaging system (Xenogen). The SKOV3-Luc xenograft model was used to evaluate the *in vivo* antitumor efficacy of CXCR1-modified NK cells or NKG2D CAR$^+$ CXCR1$^+$ NK cells.

Statistical Analysis

All statistics were performed using GraphPad Prism 7 (San Diego, CA, USA). p values < 0.05 were considered significant.

For details, see Supplemental Materials and Methods.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2019.12.006.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments, S.W. and Y.Y.N.; Performed the experiments, Y.Y.N. and J.C.K.T.; Analyzed the data, Y.Y.N. and J.C.K.T.; Wrote the manuscript, Y.Y.N. and S.W.

CONFLICTS OF INTEREST

The authors have filed patent applications related to CAR technology and could potentially receive licensing royalties in future.

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