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CCR7 expression in CD19 chimeric antigen receptor-engineered natural killer cells improves migration toward CCL19-expressing lymphoma cells and increases tumor control in mice with human lymphoma

Nathan T. Schomer, Ziyue Karen Jiang, Marit I. Lloyd, Hans Klingemann, Laurent Boissel*

ImmunityBio, Inc, Culver City, California, USA

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ABSTRACT

Background aims: Chimeric antigen receptor (CAR) T-cell therapy can be associated with significant toxicities. CAR-engineered natural killer (NK) cells provide a safer alternative while maintaining anti-tumor effects. Activated NK (aNK) cells are a clinical-grade cellular product obtained from the NK-92 cell line that have demonstrated both safety and potent cytotoxicity toward a wide range of cancers in phase 1 trials. Genetically engineered variants of aNK cells expressing a high-affinity Fc receptor (haNK) or co-expressing a CAR (t-haNK) are currently in phase 1/2 clinical trials. A key factor in the efficacy of cellular immunotherapies is biodistribution and tumor infiltration, which affect the local effector:target ratio. The chemokines CCL19 and CCL21 can drive recruitment of CCR7 receptor-expressing immune cells to secondary lymphoid organs.
Methods: Since NK-92 cells do not spontaneously express CCR7, clinical-grade aNK cells were transfected with a non-viral vector containing the CCR7 receptor, an anti-CD19 CAR and a high-affinity CD16 Fc receptor.
Results: CCR7-engineered CD19 t-haNK showed significant migration *in vitro* toward K562 cells engineered to secrete CCL19. This observation was confirmed in a NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mouse model in which subcutaneous tumors of CCL19-expressing K562 cells displayed a higher number of infiltrating CCR7_CD19 t-haNK cells than CCR7-negative CD19 t-haNK cells. In NSG mice inoculated either intravenously or subcutaneously with CCL19-secreting Raji cells, treatment with CCR7_CD19 t-haNK improved survival and tumor control compared with CD19 t-haNK or vehicle.
Conclusions: Expression of CCR7 receptor by off-the-shelf t-haNK cells improves their homing toward lymph node chemokines both *in vitro* and *in vivo*, resulting in superior tumor control.

Introduction

An important component in the efficacy of cellular immunotherapies is the ability of the infused immune cells to reach and recognize their malignant target. Equipping T lymphocytes with a chimeric antigen receptor (CAR) that recognizes and binds to a surface receptor on malignant cells has been shown to improve *in vitro* cytotoxicity. This has translated into disease remissions and improved outcomes, especially in patients with lymphoma and myeloma [1]. It is generally assumed that an increase in effector to target (E:T) ratio at the tumor site will increase malignant cell death, provided that the surrounding tissue does not restrict access to tumor cells.

Immune cells express a variety of adhesion molecules and chemokine receptors that direct their migration toward specific organs or tissues under homeostatic conditions as well as toward sites of inflammation [2]. These mechanisms can be hijacked by tumor cells, and a number of ligands for chemokine receptors of the CXCR and CCR family of molecules have been identified as being involved in tumor metastasis [3,4]. The chemokine receptor CCR7 and its ligands CCL19 and CCL21, which are involved in homing of immune cells to secondary lymphoid tissues [5], are also involved in tumor progression and lymph node metastasis in both solid and liquid cancers [6]. In addition, expression of CCL19 and CCL21 has been shown to be upregulated in secondary lymphoid tissue in follicular lymphoma [7].

Natural killer (NK) cells have potent anti-tumor properties [8], and augmented expression of CCR7 on NK cells has been shown to enhance both homing to lymph nodes and anti-tumor activity in human lymphoma tumor models [9–11]. It is unknown, however, whether NK cells that have been modified to express a CAR targeting lymphoma cells, such as CD19, would exhibit additional anti-tumor efficacy if they have also been genetically engineered to overexpress CCR7.

Continuously growing activated NK (aNK) cells, which are a clinical-grade cellular product obtained from the human NK-92 cell line,

* Correspondence: Laurent Boissel, PhD, ImmunityBio, Inc, 9925 Jefferson Blvd, Culver City, California 90232, USA.
E-mail address: laurent.boissel@immunitybio.com (L. Boissel).

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present an unlimited supply of source material for NK cells that can be genetically engineered, expanded *in vitro* and cryopreserved [12–14]. aNK cells have completed multiple phase 1 studies, and their safety and efficacy in patients with advanced cancer have been well documented [15–18]. Moreover, aNK cells have been engineered to express a high-affinity FcγRIIIa receptor (CD16-158V) as well as ERIL-2 [19,20]. These haNK cells display increased antibody-dependent cellular cytotoxicity (ADCC) properties when combined with tumor-targeted monoclonal antibodies [21,22]. The addition of an intracellular retention sequence to IL-2 (ERIL-2) sequesters the cytokine inside the cell while maintaining autocrine stimulation [23], thereby allowing haNK cells to grow independently of exogenous IL-2. haNK cells, in combination with avelumab, are currently in clinical trials in patients with Merkel cell carcinoma (NCT03853317) [22].

To further drive tumor-targeted specificity, aNK cells have been engineered to co-express various CARs in addition to CD16-158V. These t-haNK cells display dual targeting abilities, either CAR-mediated or via ADCC. A t-haNK expressing PD-L1 CAR [24] is currently in a phase 1/2 trial in patients with advanced pancreatic or breast cancer and is demonstrating its safety as well as showing some initial favorable responses. In addition, Burger *et al.* [25] are currently conducting a study with intra-tumoral HER2/neu-engineered aNK cells in patients post-surgery for glioblastoma.

Here the authors present data suggesting that further engineering of a CD19 CAR t-haNK line to express a CCR7 receptor augments migration toward CCL19-expressing lymphoma cells both *in vitro* and *in vivo*. Systemic injection of CCR7_CD19 t-haNK cells in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} /SzJ (NSG) mouse lymphoma models controlled both subcutaneous tumor growth and systemic disease and resulted in improved survival of animals compared with CD19 t-haNK cells not expressing the CCR7 homing receptor.

Methods

Ethical approval

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee of the Los Angeles Biomedical Research Institute and The Lundquist Institute (protocol 21565-03, approved April 1, 2017).

Cell lines and culture conditions

K562, Raji and SUP-B15 cells were obtained from American Type Culture Collection (Manassas, VA, USA). K562 and Raji cells were cultured in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). SUP-B15 cells were grown in the same medium with the addition of 0.2 mM β-mercaptoethanol (Thermo Fisher Scientific). aNK cells were grown in X-VIVO 10 medium (Lonza, Walkersville, MD, USA) supplemented with 5% human AB serum (Access Biologicals, Vista, CA, USA) and 500 IU/mL rhIL-2 (Proleukin; Prometheus Biosciences, San Diego, CA, USA). CCR7-positive or CCR7-negative CD19 t-haNK cells were generated by electroporation (Neon Transfection System; Thermo Fisher Scientific) of aNK cells with a linear DNA fragment encoding tricistronic (CD19CAR/CD16_158V/ERIL-2) or quadricistronic (CCR7/CD19CAR/CD16_158V/ERIL-2) expression cassettes under the control of an *EF1*α promoter. Electroporated cells were immediately transferred into X-VIVO 10 supplemented with 5% human AB serum without IL-2. CCL19-expressing K562 and Raji cell lines were generated by transduction of the corresponding parental cells with a lentiviral vector (System Biosciences, Palo Alto, CA, USA) encoding full-length human CCL19. The resulting cell lines are referred to as K562^{CCL19} and Raji^{CCL19} in this article. The SUP-B15$^{CD19KO/CD20+}$ cell line was generated by transduction of SUP-B15 cells with a lentiviral vector (System Biosciences) encoding full-length human CD20 followed by purification of CD20+ SUP-B15 cells by magnetic bead column separation (MACS; Miltenyi Biotec, Auburn, CA, USA). Purified cells (>90%) were then transduced with a lentiviral vector encoding the Cas9 nuclease (PerkinElmer, Waltham, MA, USA) and electroporated with a guide RNA targeting CD19. Cells then underwent negative separation of CD19− cells by magnetic bead column separation (Miltenyi Biotec).

Antibodies and reagents

The following antibodies were used for immunophenotyping: anti-CCR7 (BioLegend, San Diego, CA, USA), anti-CD16 (BD Biosciences), anti-Fab (Jackson ImmunoResearch, West Grove, PA, USA) and streptavidin–allophycocyanin (Invitrogen, Waltham, MA, USA). Rituximab (Rituxan [anti-CD20]) and trastuzumab (Herceptin [anti-HER2/neu]) were purchased from Genentech, Inc. (South San Francisco, CA, USA). Stained cells were analyzed using an IntelliCyt iQue Screener PLUS (Sartorius, Cambridge, MA, USA).

Cytotoxicity and ADCC assays

In vitro cytotoxicity assays were performed as previously published [26]. Briefly, target cells (K562, SUP-B15 and SUP-B15$^{CD19KO/CD20+}$) were stained with 0.1 μM carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific) and co-incubated with effector cells (aNK, CD19 t-haNK or CCR7_CD19 t-haNK) at various E:T ratios for 4 h at 37°C in a 5% carbon dioxide (CO_2) incubator. Cells were then stained with 10 mM propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) for 10 min and analyzed using an IntelliCyt iQue Screener PLUS. Percentage killing of target cells was calculated using the following formula: [(%PI+ CFSE+ cells in sample) − (%PI+ CFSE+ cells in targets alone)] / [100 − (%PI+ CFSE+ cells in targets alone)] * 100. For ADCC assay, CFSE-stained target cells were first incubated with 2 μg/mL antibody (rituximab or trastuzumab) for 20 min at room temperature before being co-incubated with CD19 t-haNK or CCR7_CD19 t-haNK effector cells at various E:T ratios for 4 h at 37°C in a 5% CO_2 incubator in the presence of antibody.

In vitro *migration assay*

A modified Boyden chamber assay was performed using a Matrigel-coated, 8-μm-pore Transwell (Corning BioCoat Matrigel Invasion Chamber; Thermo Fisher Scientific). K562 or K562^{CCL19} cells were resuspended in reduced serum medium (X-VIVO 10 + 1% human AB serum) and seeded in triplicate in the bottom chamber at a density of 2.5×10^4 cells per well. CFSE-labeled aNK or CCR7_CD19 t-haNK cells were resuspended in the same medium and seeded in the upper chamber at a cell density of 1×10^4 cells per well. The plate was incubated overnight at 37°C in a 5% CO_2 incubator. Cells in the bottom chamber were then collected, and CFSE+ cells were counted using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec). Migration was determined by calculating the ratio: (#CFSE+ cells in well with K562^{CCL19}) / (#CFSE+ cells in well with K562).

In vivo *migration assay*

NSG mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in accordance with Los Angeles Biomedical Research Institute standard operating procedures and AAALAC guidelines. A cohort of 16 mice was injected subcutaneously with 1×10^6 K562^{CCL19} cells resuspended in a 1:1 mix of serum-free Roswell Park Memorial Institute 1640 medium and Matrigel prior to injection. When tumors became measurable (approximately 100 mm^3), half of the cohort (n = 8) was injected via the tail vein with 1×10^7 CFSE-

labeled CD19 t-haNK cells, whereas the other half (n = 8) was injected with 1×10^7 CSFE-labeled CCR7_CD19 t-haNK cells. At 3 h and 24 h post-intravenous infusion, four mice per time point were euthanized, and tumors from both sides were resected and dissociated using a collagenase/hyaluronidase mix (STEMCELL Technologies, Vancouver, Canada) for 2 h at 37°C followed by ammonium chloride, trypsin–ethylenediaminetetraacetic acid, dispase (5 mg/mL) and DNase I (1 mg/mL) treatment (STEMCELL Technologies). The cell suspension was passed through a 40-μm cell strainer to remove large debris and analyzed by flow cytometry to quantify the number of CSFE+ cells. One processed tumor sample from the 3-h post-infusion group in the CD19 t-haNK arm yielded too few cells for analysis and was therefore removed from the study.

In vivo *lymphoma model*

In order to create a model of systemic lymphoma disease, NSG mice were injected intravenously with 1×10^6 Raji^{CCL19} cells. After 3 days, mice were randomly assigned to three cohorts and treated with twice-weekly intravenous injection of 1×10^7 CCR7_CD19 t-haNK cells (n = 10), CD19 t-haNK cells (n = 10) or vehicle control (n = 10) for up to four consecutive weeks. Mice were monitored daily for external signs of disease over a period of 32 days. In order to create a model of localized lymphoma, NSG mice were injected subcutaneously on one flank with 2.5×10^5 Raji^{CCL19} cells. When tumor size reached an average of approximately 190 mm^3, mice were randomly assigned to three cohorts x003Dand treated with twice-weekly intravenous injection of 1×10^7 CCR7_CD19 t-haNK cells (n = 10), CD19 t-haNK cells (n = 10) or vehicle control (n = 10) for up to two consecutive weeks. Tumor size was measured every 3–4 days, and tumor volume was calculated using the formula $(L \times W^2)/2$. Mice were euthanized when tumor size exceeded 2000 mm^3. Tumor growth inhibition (TGI) was calculated using the formula $(TC - Tt) / \Delta TC \times 100\%$, where TC and Tt are average tumor volume for the control and treatment groups, respectively, at a specific time point and ΔTC is the change in average tumor volume in the control group.

Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA, USA). *P* values for cytotoxicity, ADCC, *in vitro* migration assays and subcutaneous tumor size at day 9 were calculated using a Student's *t*-test. *P* values for *in vivo* migration assays were calculated using a one-way analysis of variance followed by multiple comparison by Tukey test. *P* values for subcutaneous tumor size were calculated using a two-way analysis of variance followed by multiple comparison by Tukey test. *P* values for survival assay were calculated using a log-rank (Mantel–Cox) test.

Results

CCR7_CD19 t-haNK cells co-express CCR7, CD19CAR and CD16(158V) receptors

The authors generated a quadricistronic non-viral expression vector containing the human CCR7 chemokine receptor, a CAR targeting CD19, the 158V high-affinity variant of CD16 and an endoplasmic-retained IL-2. The four genes were separated by 2A ribosomal skip sequences or by an internal ribosome entry site, which allowed translation of all four proteins from a single messenger RNA transcript under the control of the *EF1*α promoter. The CD19 CAR is a first-generation construct comprising an immunoglobulin heavy chain signal peptide, a single-chain variable fragment domain derived from CD19-specific antibody FCM63 [27], a CD8 hinge region, the transmembrane domain of CD28 and the intracellular region of the signaling protein FcεRIγ. A tricistronic version of the expression vector without CCR7 was also generated. The linearized expression vectors were transfected into aNK cells by electroporation, and the cells were subsequently cultured in the absence of exogenous IL-2 for selective expansion of the transfected cells. As shown in Figure 1, over 85% of the expanded transfected cells co-expressed all three surface receptors (CCR7, CD19CAR and CD16).

CCR7_CD19 t-haNK cells display efficient CAR-directed killing and ADCC against NK-resistant cell lines

In order to verify whether the expressed CD19 CAR and CD16 surface receptors were functional and whether co-expression of the various transgenes had altered the cytolytic properties of aNK cells, *in vitro* cytotoxicity assays were performed with the NK-sensitive CD19– K562 cell line or the NK-resistant CD19+ SUP-B15 cell line. After incubation for 4 h, aNK, CD19 t-haNK and CCR7_CD19 t-haNK cells displayed efficient and comparable killing of K562 target cells ($88.5 \pm 6.8\%$, $75.7 \pm 5.3\%$ and $85.1 \pm 3.4\%$, respectively, at an E:T ratio of 10:1) (Figure 2A). CCR7_CD19 t-haNK and CD19 t-haNK cells were equally able to efficiently kill SUP-B15 target cells ($89.0 \pm 6.4\%$ and $88.7 \pm 3.3\%$, respectively, at an E:T ratio of 10:1 compared with $13.4 \pm 6.5\%$ for aNK cells) (Figure 2B), indicating that co-expression of CCR7 did not affect CAR-mediated killing activity. *In vitro* ADCC assays were performed by co-incubating effector cells with a HER2/neu–, CD19–, CD20+ variant of the SUP-B15 cell line in the presence of rituximab (anti-CD20) or trastuzumab (anti-HER2/neu). As shown in Figure 2C and D, CD19 t-haNK and CCR7_CD19 t-haNK cells were able to specifically kill SUP-B15$^{CD19KO/CD20+}$ target cells in combination with rituximab ($82.1 \pm 2.7\%$ and $71.4 \pm 4.9\%$, respectively, at an E:T ratio of 10:1), but not in combination with trastuzumab ($<17.8 \pm 5.0\%$), indicating that co-expression of CCR7 did not affect the ability of CD16 to mediate ADCC.

CCR7_CD19 t-haNK cells show increased migration toward CCL19-secreting K562 cells in vitro *and* in vivo

In order to ascertain whether expression of CCR7 on the surface of CD19 t-haNK cells enabled a chemotactic response to a gradient of the chemokine CCL19 generated by K562^{CCL19} cells, an *in vitro* Transwell migration assay was performed, where the Transwell was coated with a layer of Matrigel. After an overnight incubation, CCR7_CD19 t-haNK cells displayed a significant increase in migration toward CCL19-expressing K562 cells compared with wild-type K562 cells (4.8 ± 0.6-fold, $P < 0.001$), whereas parental aNK cells did not (Figure 3).

For *in vivo* studies, a cohort of NSG mice was injected subcutaneously with K562^{CCL19} cells. When the tumors became measurable, half of the cohort was injected intravenously via the tail vein with CFSE-labeled CD19 t-haNK cells, whereas the other half was injected with CSFE-labeled CCR7_CD19 t-haNK cells. The presence of tumor-infiltrating CSFE-labeled cells was detected by flow cytometry at 3 h and 24 h post-intravenous infusion. At 3 h, K562^{CCL19} tumors harbored significantly more infiltrating CCR7_CD19 t-haNK cells than CD19 t-haNK cells (Figure 4A). The difference in numbers of tumor-infiltrating cells between the two arms was no longer significant at 24 h, although mice in the CD19 t-haNK arm displayed a much higher variability between individual tumors than those in the CCR7_CD19 t-haNK arm (Figure 4B).

Treatment with CCR7_CD19 t-haNK cells increases survival in a mouse model of systemic lymphoma

The authors next evaluated whether CCR7_CD19 t-haNK cells could increase localized killing of tumor cells in an *in vivo* model of lymphoma. NSG mice were injected intravenously with CCL19-expressing Raji cells (which are CD19+). Three days later, mice were

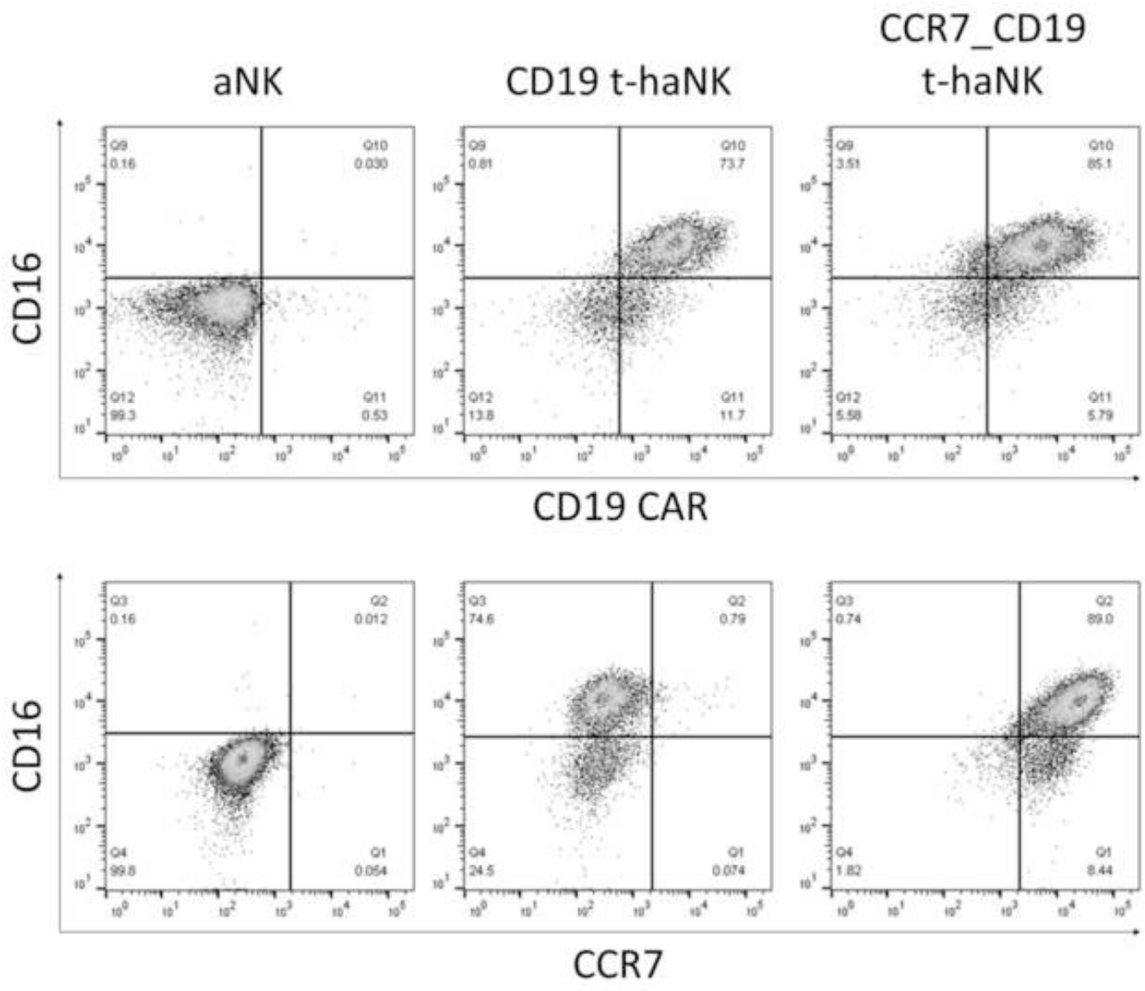

Fig. 1. CCR7_CD19 t-haNK cells co-express all three CCR7, CD19CAR and CD16(158V) receptors.

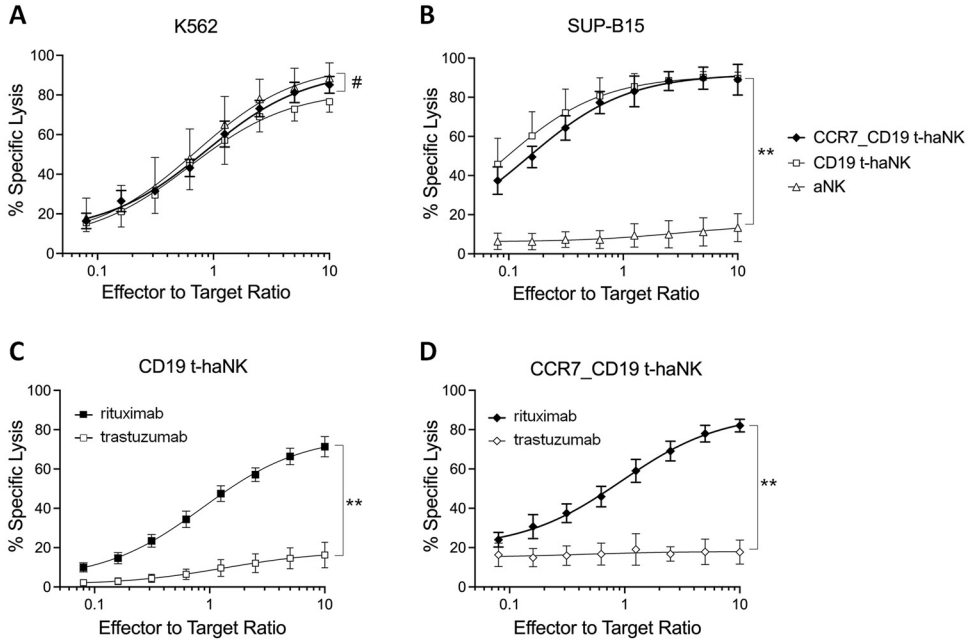

Fig. 2. CCR7_CD19 t-haNK cells demonstrate efficient killing of NK-resistant SUP-B15 targets *in vitro*. (A) Parental NK-92 cells (aNK), CD19 t-haNK cells and CCR7_CD19 t-haNK cells all efficiently lyse K562. (B) The CCR7_CD19 t-haNK and CD19 t-haNK cell lines both show efficient targeted killing of the CD19+ SUP-B15 cell line, which is resistant to non-CAR-expressing aNK cells. (C) CD19 t-haNK cells and (D) CCR7_CD19 t-haNK cells both mediate specific and robust ADCC against a SUP-B15$^{CD19KO/CD20+}$ variant line when combined with rituximab but not with a trastuzumab control. $^{\#}P > 0.05$, $^{**}P < 0.01$.

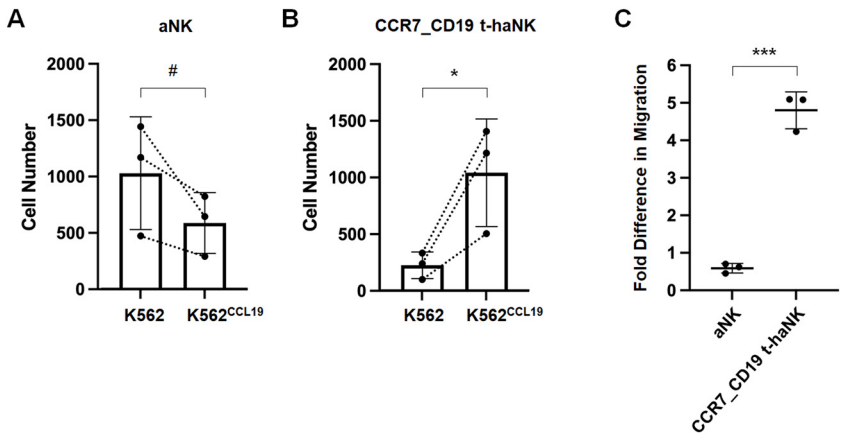

Fig. 3. CCR7_CD19 t-haNK cells demonstrate specific increased migration toward CCL19-secreting cells *in vitro*. K562 cells and CSFE-stained NK cells were placed in the bottom and top wells, respectively, of a Boyden chamber coated with a layer of Matrigel. Unlike aNK cells (A), CCR7_CD19 t-haNK cells displayed significantly greater (4.8-fold) migration through the Matrigel layer toward CCL19-secreting K562^{CCL19} cells than toward wild-type K562 cells (B and C). $^{\#}P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$.

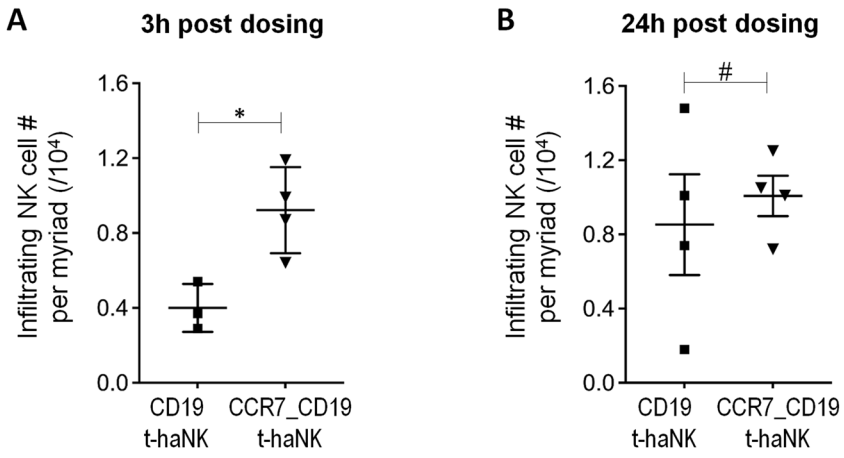

Fig. 4. CCR7_CD19 t-haNK cells demonstrate increased homing and infiltration of CCL19-secreting solid tumors *in vivo*. NSG mice inoculated subcutaneously with CCL19-expressing K562^{CCL19} cells were injected intravenously with either CCR7_CD19 t-haNK or CD19 t-haNK cells. (A) At 3 h post-intravenous injection, K562^{CCL19} tumors showed significantly higher infiltration of CCR7_CD19 t-haNK cells than CD19 t-haNK cells. (B) The difference was no longer significant at 24 h. Results are presented in cells per myriad (1/10^4) units. $^{\#}P > 0.05$, $^{*}P < 0.05$.

treated with a biweekly intravenous injection regimen of CCR7_CD19 CAR t-haNK cells, CD19 t-haNK cells or vehicle control for four consecutive weeks. The vehicle-treated group had to be euthanised between 21 days and 22 days post-inoculation, displaying hind leg paralysis and other signs consistent with high tumor burden or tumor invasion of the spinal cord. The CD19 t-haNK-treated group showed a moderate (but significant) increase in survival (+2.5 days, $P < 0.001$), although almost all mice had to be euthanised at day 24. The CCR7_CD19 t-haNK-treated group showed significantly increased survival (+6.5 days, $P < 0.0001$) and delayed morbidity compared with the vehicle and CD19 t-haNK groups (Figure 5).

Systemic treatment with CCR7_CD19 t-haNK cells delays growth of local CCL19-expressing Raji tumors

NSG mice were injected subcutaneously in one flank with CCL19-expressing Raji^{CCL19} cells. Three weeks after inoculation, when the average tumor volume reached approximately 190 mm^3, mice were treated with a biweekly intravenous injection regimen of CCR7_CD19 t-haNK cells, CD19 t-haNK cells or vehicle control for the duration of the experiment. The CCR7_CD19 t-haNK-treated cohort displayed a clear trend toward better control of tumor progression (mean TGI, 30.6%) compared with the CD19 t-haNK and vehicle groups (Figure 6A,B); however, this did not reach statistical significance. Surprisingly, stratification of the data according to tumor volume at the time of randomization (i.e., 3 weeks post-tumor inoculation, day 0) showed that mice with a tumor size above the median value (>150 mm^3) that were treated with CCR7_CD19 t-haNK cells displayed significantly slower tumor progression than the vehicle-treated group compared with mice with a tumor size under the median (901 ± 328 mm^3 in CCR7_CD19 t-haNK arm versus 1778 ± 628 mm^3 in vehicle arm at day 9, $P = 0.038$) (Figure 6C).

Discussion

Although CAR T cells have been relatively successful in the treatment of lymphoma, late relapse of the disease and safety issues (especially cytokine release syndrome and neurotoxicity) continue to be a concern and are currently guiding the development of the next generation of engineered CAR T cells [28]. CAR-engineered NK cells are considered safer, but obtaining sufficient numbers of NK cells from peripheral blood continues to be a challenge. Moreover, like CAR T cells, blood NK cells require viral vectors for efficient transfection of CAR transgenes. The NK-92 cell line (aNK) can be grown in large numbers under Good Manufacturing Practice conditions, and more than 50 patients with advanced cancers have received aNK cell infusions [15–18]. No adverse events greater than grade 2 were observed in these patients, and a significant tumor response was seen in some patients.

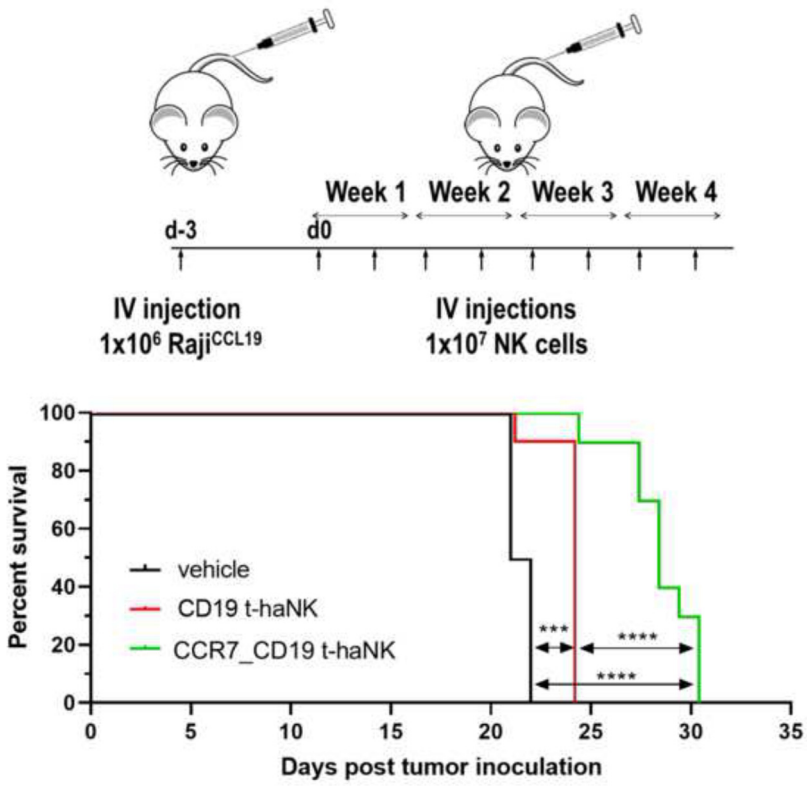

Fig. 5. CCR7_CD19 t-haNK cells significantly increase survival in a systemic lymphoma mouse model. NSG mice were injected intravenously with 1×10^6 Raji^{CCL19} cells and 3 days later were treated with biweekly intravenous injections of 1×10^7 CCR7_CD19 t-haNK cells (n = 10) (green), CD19 t-haNK cells (n = 10) (red) or vehicle control (n = 10) (black). $^{***}P < 0.001$, $^{****}P < 0.0001$. IV, intravenous.

Clinical-grade NK-92 cells (aNK) can be easily and reproducibly engineered genetically through electroporation with non-viral plasmids. The first generation of engineered aNK cells express a high-affinity Fc receptor (CD16-158V) variant together with a non-secreted ERIL-2, and these haNK cells are currently being tested in clinical trials in combination with avelumab as treatment for patients with Merkel cell cancer (NCT03853317). The second generation of engineered aNK cells co-express a CAR in addition to CD16-158V and ERIL-2 and are referred to as t-haNK cells. Several clinical-grade t-haNK cell lines have been generated, with PD-L1 t-haNK cells now in clinical trials for indications such as advanced pancreatic cancer (NCT04390399), triple-negative breast cancer and non-small cell lung cancer.

To augment the homing properties of CD19 t-haNK cells and further increase their clinical efficacy, the authors have engineered a third generation of clinical-grade aNK cells with a quadricistronic plasmid to make them co-express the chemokine receptor CCR7 in addition to CD19 CAR, CD16-158V and ERIL-2. Expression of CCR7 enables recognition of CCL19 and CCL21 chemokine gradients and induction of migration toward their sources. In a Transwell assay, these CCR7_CD19 t-haNK cells showed increased migration through extracellular matrix toward CCL19-expressing target cells. This increased migration toward CCL19-expressing K562 cells was also observed *in vivo*, although only at less than 24 h post-infusion. This could suggest a difference in the kinetics of tumor infiltration, where CCR7-expressing cells would undergo an active and rapid migration to the tumor site, whereas non-CCR7-expressing cells would infiltrate tumors more slowly and in a more stochastic fashion. Moreover, when CCR7_CD19 t-haNK cells were injected into NSG mice that had been inoculated with CCL19-secreting Raji lymphoma cells, increased tumor control and survival were seen in both local and systemic tumor models compared with mice treated with CD19 t-haNK cells not expressing the CCR7 receptor and control mice. A surprising observation was that larger subcutaneous tumors (>150 mm^3 at the beginning of treatment) appeared to be more sensitive to CCR7_CD19 t-haNK injections than their smaller counterparts. Since larger tumors are likely to be more vascularized, it is possible that they also allow infiltration of higher numbers of circulating t-haNK cells. Alternatively, larger CCL19-secreting Raji tumors may produce a stronger gradient of CCL19, which would drive more efficient tumor homing and infiltration of CCR7-expressing t-haNK cells. Further *in vivo* work using labeled t-haNK cells would be warranted to explore these hypotheses.

CAR-expressing t-haNK cells have significant advantages over CAR T cells. In addition to the lower occurrence of adverse events associated with NK cells, the quadricistronic t-haNK cells presented here not only have increased targeted homing to lymphoma tissue but also provide specific CAR-directed killing and ADCC. These cells also maintain spontaneous cytotoxicity through expression of “natural” NK receptors such as NKG2D, which binds to the family of major histocompatibility complex class I chain-related stress molecules often overexpressed on cancer cells. These multiple modalities of tumor cell recognition can circumvent the loss of effective targeting due to antigen downregulation.

The aNK cells and aNK-derived haNK and t-haNK cell lines can be easily expanded in conventional bioreactors, and large quantities of this powerful cytotoxic cell product can be generated over a relatively short period. The logistics are also simplified by the fact that the cells can be cryopreserved without losing activity upon thawing. Although these cell lines, because of the malignant lymphoma origin of NK-92, require low-dose irradiation for inactivation before infusion, irradiated cells maintain functionality for an additional 24–48 h, which guides the twice-weekly treatment schedule.

Increased expression of homing receptors on CAR T cells has previously been shown to improve anti-tumor activity in a Hodgkin tumor model [29], and there is broad evidence that immune cells

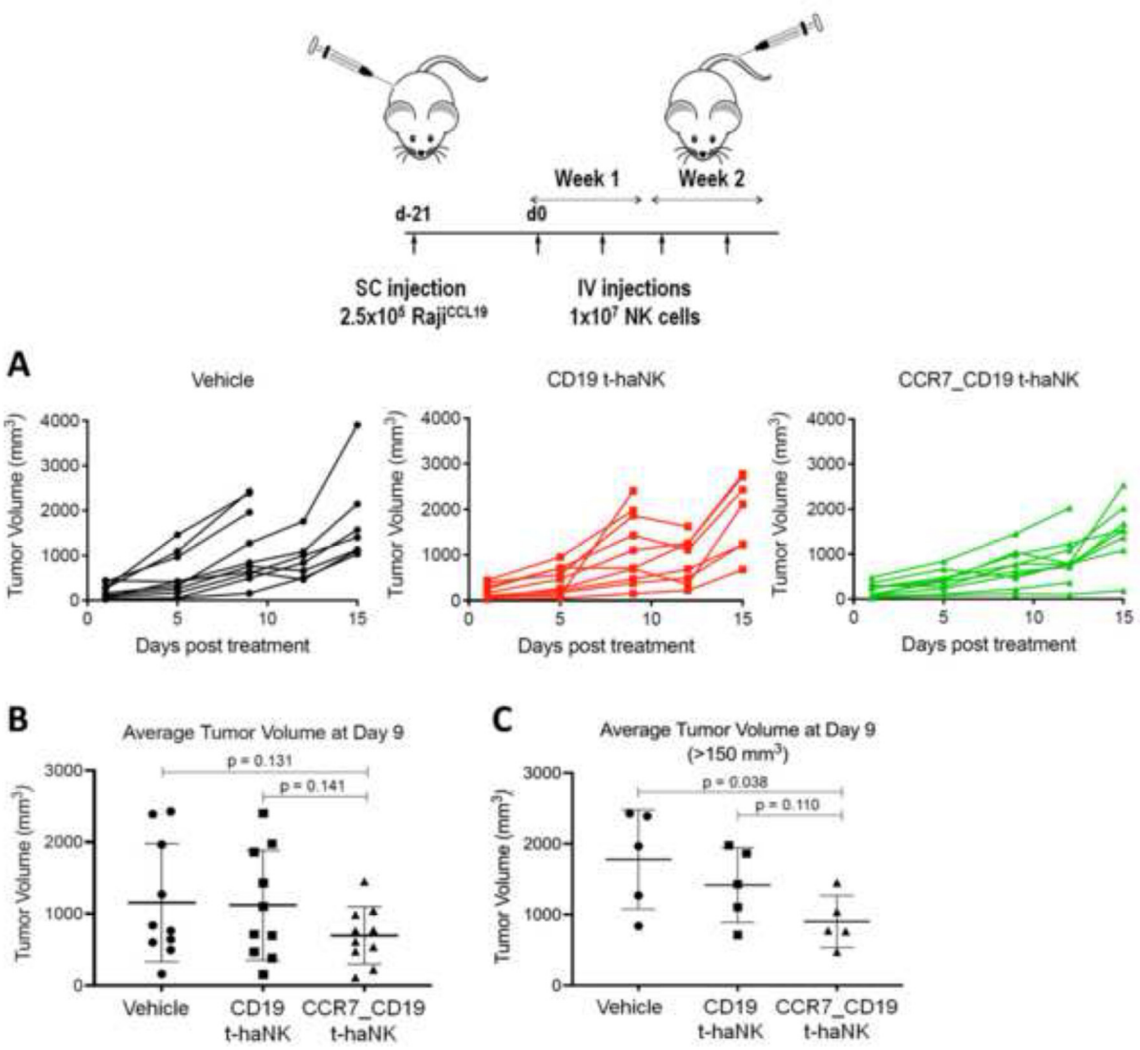

Fig. 6. CCR7_CD19 t-haNK cells significantly increase survival in a subcutaneous lymphoma mouse model. NSG mice were injected subcutaneously with 2×10^5 Raji^{CCL19} cells and treated with biweekly intravenous injections of 1×10^7 CCR7_CD19 t-haNK cells (n = 10) (green), CD19 t-haNK cells (n = 10) (red) or vehicle control (n = 10) (black) for up to two consecutive weeks. (A) Tumor volume progression for all cohorts. (B) Tumor volume at day 9 post-treatment. (C) Tumor volume at day 9 post-treatment for mice with initial tumor size >150 mm^3. IV, intravenous; SC, subcutaneous.

expressing or overexpressing the CCR7 chemokine receptor preferentially home to lymphoma sites that express the CCL19 and CCL21 ligands. Earlier studies had confirmed that CCR7 was expressed by almost all CD56^{bright} NK cells but was not detected on CD56^{dim} NK cells [30]. NK cell reprogramming using electroporation of messenger RNA coding for the chemokine receptor CCR7 resulted in augmented NK cell migration toward the lymph node-associated chemokine CCL19 [11]. Loss of CCR7 on CD56^{bright} NK cells correlates negatively with HIV viral load in *in vitro* models, suggesting the relevance of this chemokine receptor–ligand interaction for virally infected cells [31,32]. It has also been demonstrated that NK cells exposed to a microenvironment rich in IL-18 can *de novo* express/upregulate CCR7 and acquire the ability to respond to the lymph node chemokines CCL19 and CCL21 [10]. Conversely, a recent study showed that NK cells expanded *ex vivo* on feeder layers downregulate CCR7 expression, which could impair their ability to home toward secondary lymphoid organs [33]. In addition to the CCR7/CCL19 homing pathway in lymphoma, other tumors may benefit from the inclusion of a specific homing receptor in CAR NK cells to augment the cytotoxic CAR effect [9].

Efficient homing of immune cells to primary and metastatic tumor locations is still a challenge for current adoptive cellular immunotherapy. NK-92-derived t-haNK cells engineered to co-express a CD19-targeted CAR and the CCR7 chemokine receptor display enhanced migration toward CCL19-expressing tumor sites, improved tumor control and increased survival in a lymphoma mouse model compared with t-haNK cells that express only CD19 CAR. The present study demonstrates the feasibility and potential therapeutic benefit of an “off-the-shelf” cellular immunotherapy specifically engineered to improve both homing and targeted cytotoxicity in a single therapeutic.

Author Contributions

Conception and design of the study: NTS, ZKJ, MIL and LB. Acquisition of data: NTS and ZKJ. Analysis and interpretation of data: NTS and ZKJ. Drafting or revising the manuscript: NTS, MIL, HK and LB. All authors have approved the final article.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Declaration of Competing Interest

MIL, HK and LB are employed by ImmunityBio, Inc (formerly NantKwest, Inc). All authors own stocks and/or stock options in ImmunityBio, Inc. NTS, HK and LB are inventors of PCT/US2018/044842 (patent pending).

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