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Preclinical evaluation and first-in-dog clinical trials of PBMC-expanded natural killer cells for adoptive immunotherapy in dogs with cancer

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ABSTRACT

Background Natural killer (NK) cells are cytotoxic cells capable of recognizing heterogeneous cancer targets without prior sensitization, making them promising prospects for use in cellular immunotherapy. Companion dogs develop spontaneous cancers in the context of an intact immune system, representing a valid cancer immunotherapy model. Previously, CD5 depletion of peripheral blood mononuclear cells (PBMCs) was used in dogs to isolate a $CD5^{dim}$-expressing NK subset prior to co-culture with an irradiated feeder line, but this can limit the yield of the final NK product. This study aimed to assess NK activation, expansion, and preliminary clinical activity in first-in-dog clinical trials using a novel system with unmanipulated PBMCs to generate our NK cell product.

Methods Starting populations of CD5-depleted cells and PBMCs from healthy beagle donors were co-cultured for 14 days, phenotype, cytotoxicity, and cytokine secretion were measured, and samples were sequenced using the 3'-Tag-RNA-Seq protocol. Co-cultured human PBMCs and NK-isolated cells were also sequenced for comparative analysis. In addition, two first-in-dog clinical trials were performed in dogs with melanoma and osteosarcoma using autologous and allogeneic NK cells, respectively, to establish safety and proof-of-concept of this manufacturing approach.

Results Calculated cell counts, viability, killing, and cytokine secretion were equivalent or higher in expanded NK cells from canine PBMCs versus CD5-depleted cells, and immune phenotyping confirmed a CD3-NKp46+ product from PBMC-expanded cells at day 14. Transcriptomic analysis of expanded cell populations confirmed upregulation of NK activation genes and related pathways, and human NK cells using well-characterized NK markers closely mirrored canine gene expression patterns. Autologous and allogeneic PBMC-derived NK cells were successfully expanded for use in first-in-dog clinical trials, resulting in no serious adverse events and preliminary efficacy data. RNA sequencing of PBMCs from dogs receiving allogeneic NK transfer showed patient-unique gene signatures with NK gene expression trends in response to treatment.

Conclusions Overall, the use of unmanipulated PBMCs appears safe and potentially effective for canine NK immunotherapy with equivalent to superior results to CD5 depletion in NK expansion, activation, and cytotoxicity. Our preclinical and clinical data support further evaluation of this technique as a novel platform for optimizing NK immunotherapy in dogs.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Natural killer (NK) cell adoptive transfer has the potential to overcome barriers to success in immunotherapy.
⇒ Spontaneous canine models of cancer provide an avenue to optimize NK cell-based treatments, but further investigation of canine NK cells and their safety and efficacy in clinical trials are needed to improve comparative studies.

WHAT THIS STUDY ADDS

⇒ This detailed preclinical evaluation of peripheral blood mononuclear cell (PBMC)-expanded NK cells supports an improved method for obtaining activated NK cells in quality and quantity required for adoptive cell transfer.
⇒ Two first-in-dog clinical trials were successfully completed implementing intravenous infusion of PBMC-expanded adoptive NK cells with evidence supporting in vivo NK cell activation in response to treatment.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings are a critical foundation on which future canine clinical trials can build, strengthening the canine cancer model and furthering comparative immunotherapy research to improve cancer outcomes across species.

BACKGROUND

The discovery of immune checkpoint inhibitors and other T cell-based immunotherapies has immensely impacted the treatment of an ever-growing number of cancers.[1] Although

successes with these immunotherapies have been significant, there are lingering barriers regarding toxicity and strategies to increase responses. Natural killer (NK) cells are innate cytotoxic and cytokine-producing lymphoid cells with a crucial role in antiviral and antitumor responses. Their capacity to recognize heterogeneous cancer cell targets without prior sensitization or priming makes them exciting prospects for cellular immunotherapy. However, despite evidence for activity in hematological malignancies such as acute myeloid leukemia,[2] NK cell-based immunotherapies have yielded inconsistent responses in solid tumors to date.[3–5]

With only 10% of therapies that show promise in murine studies successfully showing efficacy in human clinical trials, canine models of spontaneous cancer are an important link to study and improve immunotherapies across species.[6–8] Dogs are an outbred species with an intact immune system that develop cancers strikingly similar to humans.[9–11] And, like humans, there is an urgent unmet need for novel cancer therapies in dogs. Despite the clear benefits of dogs as a comparative immune-oncology model, gaps in knowledge concerning canine immune populations have limited canine immunotherapy success, especially for NK biology.[12] These knowledge gaps are further compounded by significant differences in NK biology between mice and humans, making data interpretation challenging. Our group recently completed one of the first detailed analyses of canine NK cell transcriptomics, revealing that canine NK cells share significant homology with their human counterparts and appear closer phylogenetically to human than mouse NK cells.[13 14] Nevertheless, questions remain regarding the optimal characterization of dog NK populations as they expand and their differentiation status prior to adoptive transfer, which is highly relevant to the longevity of NK cells in vivo and their application in the clinic as adoptive immunotherapy.[4 13] Given these ongoing knowledge gaps regarding canine NK immunobiology, additional in-depth characterization is needed to identify factors that impact in vivo efficacy and persistence, especially in the context of clinical trials.

In contrast to humans, canine NK cells lack expression of CD56, a standard marker of human NK cells, but the depletion of CD5, a marker expressed at high levels in canine T cells, enriches for a $CD5^{dim}$ population which has been shown to harbor key features of NK cells.[13 15 16] Multiple groups have used low expression of CD5 or $CD5^{dim}$ to identify dog NK cells by flow cytometry.[15] Previously, CD5 depletion of peripheral blood mononuclear cells (PBMCs) using magnetic separation has also been used in dogs to isolate or enrich for a $CD5^{dim}$-expressing NK subset prior to co-culture and expansion with an irradiated feeder line such as the genetically modified human erythroleukemia line K562 clone 9.[14 17–19] However, this method using CD5 depletion can limit the yield of the final NK product needed for transfer.

To address the limitations of current canine NK expansion approaches, we sought to characterize the phenotype, function, and preliminary clinical activity of canine NK cells in first-in-dog clinical trials using unmanipulated PBMCs to generate our NK cell product. We aimed to compare bulk PBMC-derived NK cells versus NK cells expanded from CD5-depleted PBMCs, and then evaluate clinical and genomic characteristics of PBMC-expanded dog NK cells using both autologous and allogeneic NK products in first-in-dog clinical trials in dogs with cancer.

The results from this study will enable us to understand optimal NK isolation and expansion techniques for adoptive transfer of canine NK cells for further clinical translation. Given the limitations of canine flow cytometry at this time, our findings will also fill critical knowledge gaps in the transcriptional characterization of canine NK cells and provide a foundation for future immunotherapy trials.

METHODS

PBMC isolation and CD5 depletion of canine cells

Whole blood was collected from 11 healthy, farm-bred beagles (Ridglan Farms, Mt. Horeb, Wisconsin, USA) using EDTA tubes diluted with sterile phosphate-buffered saline (PBS). PBMCs were isolated from whole blood using a density gradient centrifugation (Lymphocyte Separation Medium, Corning Life Sciences) and red blood cell lysis with RBC lysis buffer for 5 min at 4°C. A subset of PBMCs underwent CD5 depletion using the Easy Sep PE Positive Selection Kit (Stem Cell Technologies, Vancouver, British Columbia, USA) and PE-conjugated anticanine CD5 (Invitrogen, clone YKIX322.3) to select for $CD5^{bright}$ cells and enrich the $CD5^{dim}$ fraction for further processing and analysis.

PBMC isolation and NK purification of human cells

PBMCs were isolated from whole blood as described previously.[20] A subset of PBMCs underwent NK isolation using the Rosette Sep Human NK Isolation Kit according to the manufacturer's specifications (Stem Cell Technologies).

NK expansion of canine and human cells

Starting populations of CD5-depleted canine cells or NK isolated human cells, and respective PBMCs were co-cultured with K562 human feeder cells transduced with 4-1BBL (CD137L) and membrane-bound rh-IL21 (K562C9IL21, kind gift of Dr Dean Lee, Nationwide Children's Hospital, Columbus, Ohio, USA), and supplemented with rh-IL2. Flasks underwent media changes and addition of fresh feeder cells as previously described.[14 17–19 21] Cell count and viability were assessed on co-culture days 0, 7, and 14.

Flow cytometry and killing assays

Cells were washed with PBS, incubated with Fc receptor blocking solution (Canine Fc Receptor Binding Inhibitor, Invitrogen #14-9162-42), then stained with the following fluorochrome-conjugated monoclonal antibodies: rat anticanine CD5 on PerCP-eFluor 710 (clone YKIX322.3

Thermo Fisher #46-5050-42), mouse antidog CD3-FITC (clone CA17.2A12, BioRad #MCA1774F), unconjugated NKp46 (clone 48A, kind gift of Dr Dean Lee) which was conjugated secondarily with PE, and live/dead staining using Fixable Viability Dye 780 (eBioscience #65-0865-14). Staining of canine γδ T cells was completed using a mouse antidog TCRγδ (clone CA20.8H1, IgG2a, kind gift of Dr Peter F Moore[22]) primary antibody followed by a goat antimouse IgG (H+L) cross-adsorbed secondary antibody-AF647 (cat. A-21235). All flow cytometry results were acquired using a BD Fortessa flow cytometer (Becton Dickinson, San Jose, California, USA) equipped with BD FACSDiva software and analyzed using FlowJo Software (TreeStar, Ashland, Oregon, USA).

For killing assays, canine osteosarcoma (OSA) and melanoma tumor cell lines (OSCA-78 and M5, respectively) were thawed and labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen #C34554) for 5 min at room temperature at a final concentration of 1.25 µM. After overnight incubation of effector and target cells, staining was performed with Fixable Viability Dye 780 prior to analysis by flow cytometry. Cytotoxicity was calculated according to the following formula: [CFSE+FVD780+/(CFSE+FVD780+)+(CFSE+FVD780−)]×100.[20]

Cytokines

Analytes were measured in cell culture supernatant by using the Eve Technologies (Calgary, Alberta, Canada) Canine Cytokine 13-Plex Discovery Assay (MilliporeSigma, Burlington, Massachusetts, USA) performed on the Luminex 200 system (Luminex, Austin, Texas, USA). The 13 included markers were granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-2, IL-6, IL-7, IL-8/C-X-C Motif Chemokine Ligand 8, IL-10, IL-15, IL-18, interferon gamma-induced protein 10 (IP-10)/C-X-C Motif Chemokine Ligand 10, keratinocyte chemotactic-like (KC-like), monocyte chemoattractant protein-1 (MCP-1)/chemokine (CC-motif) ligand 2, and tumor necrosis factor-α.[23]

RNA sequencing

Samples from NK co-culture and PBMCs from four healthy donors and canine patients receiving immunotherapy underwent RNA extraction using RNeasy Mini kits (Qiagen) followed by sequencing using a 3'-Tag-RNA-Seq protocol for gene profiling completed by the UC Davis Genome Center.[24] Data were only included for samples with sufficient RNA quantity and quality for analysis. Publicly available sequencing data for healthy day 0 and day 14 co-culture samples were used from a previous study by our laboratory (BioProject accession number PRJNA987155).[25] Quality assessment of all raw fastq files was performed using *multiqc*, *bbduk_qc* was used to trim the first 12 bases and carry out quality trimming,[26] and *bbduk_find_ribo* was used to identify and select non-ribosomal RNA.[26] Reads were indexed to a canine reference transcriptome (ROS_Cfam_1.0) or human reference transcriptome (GrCh38), and counts were generated by salmon.[27] Count files were transported to R using *tximport*[28] and downstream analyses were completed in R using the *DESeq2* and *ggplot2* packages.

For single-cell RNA sequencing (scRNA-seq), PBMC-expanded NK cells after 14-day co-culture were quality checked to determine adequate cell concentration and viability. Single-cell suspension of 700–1200 cells/µL in at least 40 µL of PBS/0.5% bovine serum albumin suspension buffer was submitted to the UC Davis Genome Center. Library preparation and sequencing using the 10X Chromium Next GEM Single-Cell 3' V.3.1 Gene Expression protocol were completed by the UC Davis Genome Center. Single cell fastq files were processed using the *cellranger count* pipeline with generation of and alignment to a Canis lupis familiaris reference genome (CanFam3.1) using the *cellranger mkgtf* and *cellranger mkref* pipelines. Further preprocessing was completed in R using the *Seurat* package and data integration and visualization using R packages, *Seurat* and *ggplot2*.

Autologous NK immunotherapy in dogs with metastatic osteosarcoma and melanoma

Based on our prior work showing safety and evidence for clinical activity using inhaled IL-15 in dogs with gross pulmonary metastases, we enrolled client-owned pet dogs with naturally occurring metastatic OSA or melanoma in a clinical trial combining intravenous autologous adoptive NK cell transfer with inhaled IL-15. Treatment of inhaled IL-15 was given through a fitted nebulizer twice daily for 14 days at a dose level of 50 µg as previously described.[23] Entry criteria included histological confirmation of malignant melanoma or OSA, documented metastatic disease to the lungs (based on three-view thoracic radiographs), adequate end-organ function, and weight of 10 kg or greater. PBMCs were isolated from the patient's whole blood and co-cultured with irradiated feeder cells and rhIL-2 for 14 days to expand autologous cells for two scheduled NK cell infusions. NK cell preparations were confirmed as endotoxin and mycoplasma negative before injection on days 0 and 7 of a 2-week twice daily inhaled IL-15 regimen. 7.5×10^6 NK cells/kg intravenous (with 5 ng/mL rhIL-15 in 50 mL solution of 0.9% NaCl) were given for each injection by slow bolus through an intravenous catheter using a closed chemotherapy system. Response was assessed based on the Response Evaluation Criteria for Solid Tumors in dogs (RECIST V.1.0) and evaluated by a boarded radiologist (EGJ).[29] Imaging was performed on day 28 and day 42 after treatment initiation followed by every 4 weeks. Patients had to have a minimum of 28 days to imaging to meet criteria for response assessment.

Allogeneic NK immunotherapy in dogs with malignant melanoma

We also performed a first-in-dog trial using allogeneic NK cells in dogs with locally advanced, unresectable oral melanoma receiving palliative radiotherapy (RT).

Patients were included based on a histological diagnosis of malignant melanoma, adequate end-organ function, a weight of 10 kg or greater, and a plan to undergo palliative RT for disease control. Palliative RT is the standard of care for canine malignant melanoma and consists of 4 weekly treatments at a dose of 9 Gy administered to the primary tumor.[18] Blood was obtained from healthy, farm-bred beagles aged 2–8 years of both sexes 2 weeks prior to scheduled NK cell infusion in canine patients. PBMCs were isolated from the healthy donor whole blood and co-cultured with irradiated feeder cells and rhIL-2 for 14 days to expand allogeneic cells. NK cell preparations were confirmed as endotoxin and mycoplasma negative before a single injection at a dose of 7.5×10^6 NK cells/kg intravenous (with 5 ng/mL rhIL-15 in 50 mL solution of 0.9% NaCl) on the day of the fourth and final session of RT. Cells were given by slow bolus through an intravenous catheter using a closed chemotherapy system, and dogs were administered 3 µg/kg rhIL-15 SQ directly after NK cell infusion and then 20–30 hours post-infusion. Blood draws for complete blood counts and genomic analysis were completed at specified timepoints.[14 23]

Statistics

Graphs and statistical analyses for cell counts, viability, cytotoxicity, and cytokine secretion were completed using Prism software (GraphPad Software). Line graphs are expressed as mean and SEM with significant differences between groups determined by mixed-effects analysis. Bar graphs represent the mean with significant differences between groups determined by Mann-Whitney U test. RStudio V.4.3.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis of bulk RNA-seq data, specifically the DESeq2 package in R which employs the Wald test and adjusts for multiple testing using the Benjamini-Hochberg procedure; $p\leq0.05$ was considered statistically significant across all analyses unless otherwise noted.

RESULTS

Canine NK expansions in vitro

Using PBMCs from healthy donor beagles, we compared the yield, viability and functional characteristics of NK cells expanded from unmanipulated bulk PBMCs versus those derived from CD5-depleted starting populations, using irradiated human feeder cells as we and others have done previously[14 18 21] (figure 1A). To validate the CD5-depleted cell population prior to co-culture, we performed flow phenotyping on both the positively and negatively selected populations, demonstrating successful magnetic separation of CD5 bright and $CD5^{dim}$/ negative subsets (figure 1B). Cell count and viability data were collected for five timepoints up to day 14 (figure 1C). A mixed-effects model analysis showed no statistical difference in calculated cell counts and viability between PBMC-derived and CD5-depleted NK cells. Viability decreased and reached a minimum at day 10 before stabilizing at day 14 in both groups.[15] PBMC-derived NK cells reached a higher mean overall expansion than CD5-depleted cells at day 14, peaking at a mean of 677 million cells from 5 million PBMCs at day 0 compared with 537 million from 5 million starting CD5-depleted cells ($p>0.05$, figure 1C). We next assessed NK frequency at multiple timepoints using flow cytometry, demonstrating high percentages of CD3+NKp46- T cells and low percentages of CD3-NKp46+ NK cells in the resting PBMC populations compared with low percentages of CD3+NKp46- T cells and high percentages of CD3-NKp46+ NK cells by day 14 (figure 1D). Due to the similarities between NK cells and gamma-delta (γδ) T lymphocytes as well as existing literature showing the expansion of γδ T cells in a distinct but partially overlapping co-culture protocol,[30] we sought to confirm the purity of the day 14 NK product. The proportion of CD3+γδTCR+ cells remained low, below 4% as assessed by flow cytometry, in the day 14 NK product (figure 1E), aligning with previous reports of minimal TCRγδ+ cells in canine peripheral blood.[31]

Genomic analysis of CD5 depleted and bulk PMBC-expanded canine NK cells

Given our phenotypic data validating the use of unmanipulated PBMCs to expand purified NK cells, we then aimed to characterize the differential gene expression (DGE) profiles of these respective canine NK products using RNA-seq. First, PBMC-expanded NK cells were compared with unmanipulated PBMCs using scRNA-seq to validate our findings and confirm the populations present following expansion. PBMC-expanded NK cells were sequenced and assessed in relation to a previously published single-cell dataset of PBMCs from a healthy donor which served as an unmanipulated control.[25] The two datasets were integrated to enable comparison and the clusters present in unmanipulated PBMCs were visualized in a Uniform Manifold Approximation and Projection (uMAP) plot (figure 2A). DGE testing was used to determine the genes that distinguished each cluster from remaining clusters in order to match the clusters to cell types based on canonical cell markers. A subset of genes used to confirm cell cluster identities is visualized by dot plot (figure 2B). Overlapping of the resting PBMCs and day 14 NK cells in a single uMAP plot shows the overall changes in clustering due to the expansion (figure 2C). The changes in the resting and expanded populations are further explored in a series of uMAPs visualizing the expression of relevant genes along with the proportion of total cells expressing that gene (figure 2D). Expression of CD3E and CD3D at day 0 was seen in 53.8% and 48.0% of cells, respectively, which dropped to <4% of the cells present at day 14. Similar decreases were seen in cells expressing additional T cell markers, CD4, CD5, and CD8A, as well as cells expressing myeloid markers, CD86, and B cell markers, CD22. In contrast, the percent of cells expressing NK cell markers NKp30 (NCR3), NKG2D (KLRK1), and GZMB increased substantially from day 0 to day 14.

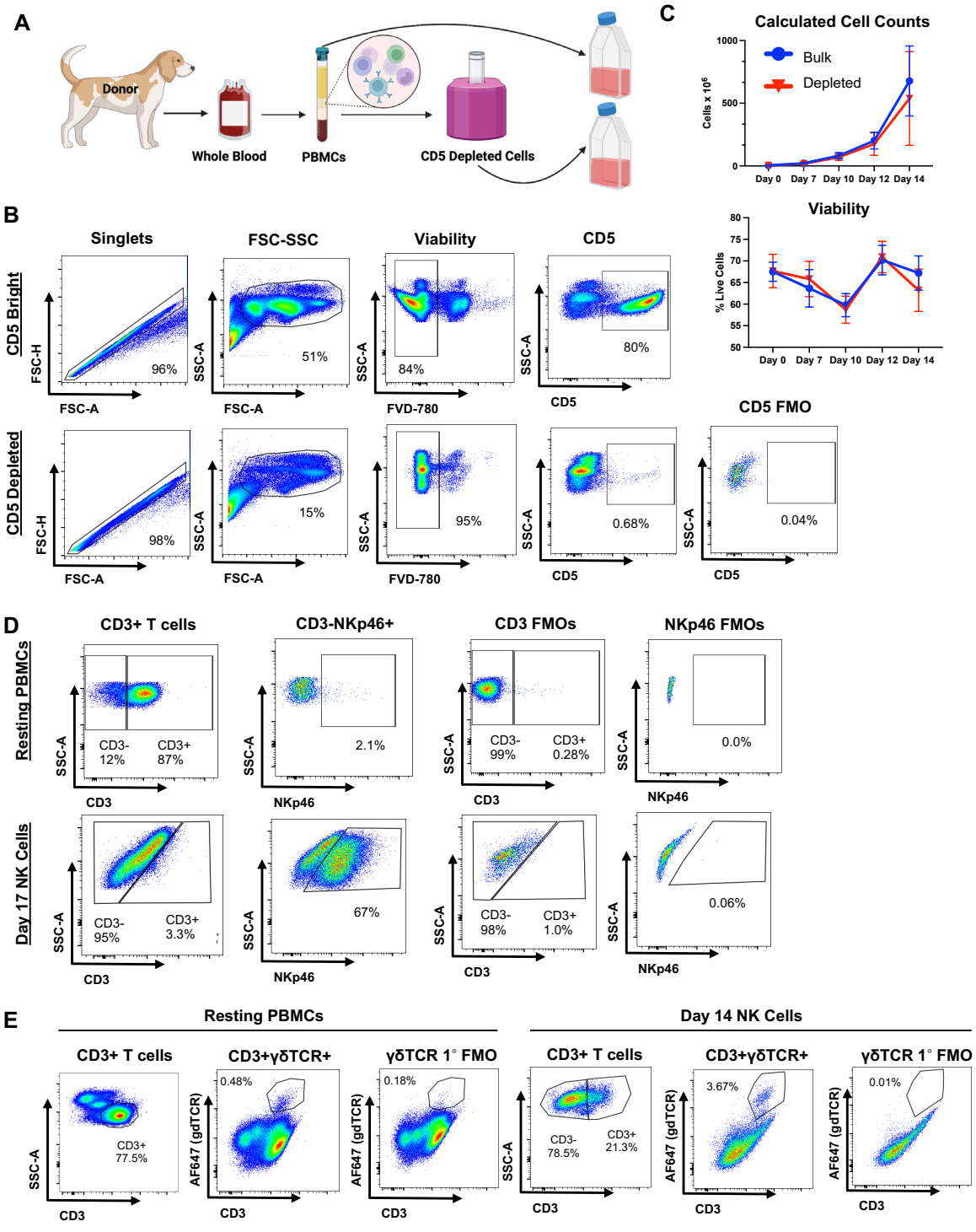


Figure 1 Expansion of canine NK cells in vitro. (A) Schema of experimental strategy expanding NK cells from PBMC and CD5-depleted starting populations. After processing, respective cell populations were co-cultured with irradiated K562 clone 9 feeder cells and 100 mIU/mL rhIL-2 for 14 days. (B) Representative flow cytometry gating of cells prior to co-culture and following magnetic bead separation for CD5 depletion. Depleted cells showed virtually no CD5 expression in contrast to positively selected CD5+ cells, confirming the efficacy of magnetic separation and phenotype of CD5-depleted cells as a starting population. (C) Cell counts and viability were calculated on days 0, 7, 10, 12, and 14 of the 14-day co-culture using bulk PBMCs (blue) and CD5-depleted cells (red) as starting populations. Mean and SEM for 11 healthy donor dogs are plotted against time. (D) Representative flow cytometry gating of bulk PBMCs before and after 14-day co-culture, corroborating the expansion of NKp46+ NK cells from PBMCs without preceding NK-isolation. NK cells were identified as CD3-NKp46+, reaching a majority at day 14 with minimal CD3+ T cell infiltrate. (E) Representative flow cytometry gating of PBMCs at rest and following 14-day co-culture confirming minimal contamination of CD3+γδTCR+ T cells. NK, natural killer; PBMC, peripheral blood mononuclear cell.

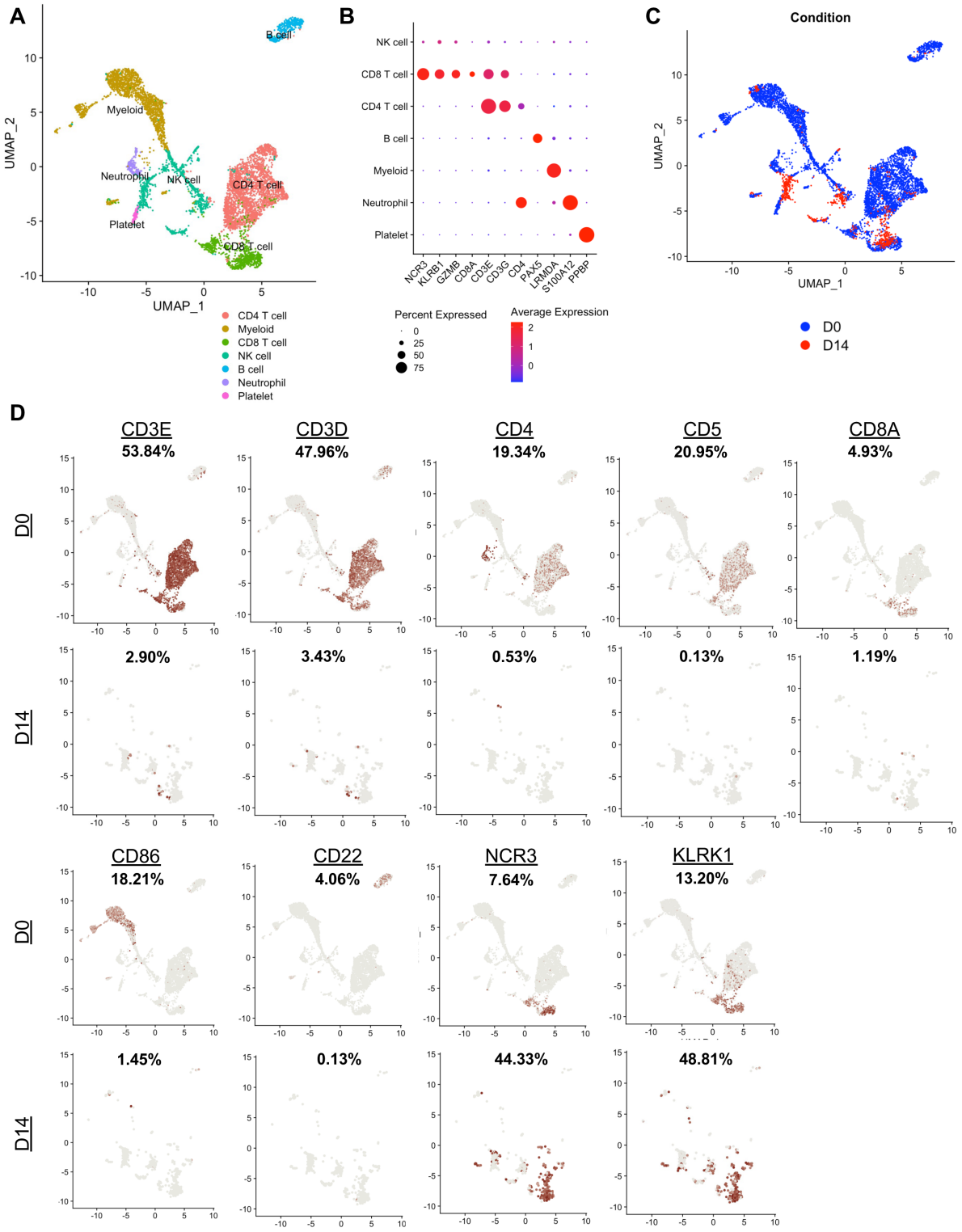


Figure 2 Genomic analysis of expanded NK cells by scRNA-seq. Cells from PBMC-expanded NK cells at D14 were collected and scRNA-seq was completed. The resulting dataset was integrated with a dataset for resting PBMCs. (A) uMAP plot of clusters present in resting PBMCs color-coded by cell type. Cell identities were determined by analysis of differentially expressed genes that distinguished each cluster from all other clusters. (B) Dot plot visualizing a selection of gene markers used to annotate the cells present. Dot size represents the percent of cells expressing the gene while color correlates with average expression within a cell. (C) uMAP of overlapping datasets included within the integration, showing the differences in resting PBMCs (D0, blue) and PBMC-expanded NK cells (D14, red). (D) uMAPs showing distribution and percent of cells expressing various genes associated with cell types in the integrated conditions, D0 (above) and D14 (below). Percentages are calculated as the percent of cells with expression of the specified gene out of the total cells present. The threshold for gene expression is set at 0. D, day; NK, natural killer; PBMC, peripheral blood mononuclear cell; scRNA-seq, single-cell RNA sequencing; uMAP, Uniform Manifold Approximation and Projection.

Next, PBMC-expanded NK cells were compared with NK cells expanded from CD5-depleted PBMCs using bulk RNA-seq of cells from four healthy donors. Principal component analysis (PCA) of PBMC-expanded and expanded CD5-depleted cells at day 0, day 7, and day 14 showed that expansion timepoint was the primary driving force for variance of PC1, with clustering also based on donor. We observed greater variances between bulk and CD5-depleted populations at day 0 than at day 14 where populations were tightly clustered (figure 3A). These results suggest that PBMCs and CD5-depleted populations are distinct at rest, but then converge to form nearly identical NK populations by day 14 of co-culture. This is further substantiated in MA (ratio intensity) plots of PBMC-derived and CD5-depleted cells at day 14 of co-culture versus their respective populations at day 0, showing 3961 and 3107 DGEs, respectively (figure 3B,C), while comparison of both groups at D14 demonstrated high similarity with no DGEs (figure 3D). To ensure that DGEs cannot be solely contributed to the loss of non-NK cells, we then specifically highlighted the log fold changes of genes associated with NK cell signatures at day 14 vs day 0 (figure 3E,F). Notably, IFNG, GZMB, and GZMA (canonical NK functional gene products) had the largest positive fold change, while CD16 showed the largest negative fold change in both groups. There were examples of specific genes, notably KLRB1 and KLRA1, which showed statistically significant DGE at day 14 in PBMC-derived but not CD5-depleted expanded NK cells compared with their resting counterparts. Next, the normalized counts of genes highly associated with NK function, including CD16, KLRB1, NKG2D/KLRK1, and GZMB, were extracted for each group (figure 3G). Interestingly, both bulk PBMCs and CD5-depleted cells had virtually no expression of classic NK genes at rest (timepoint 0), with the exception of CD16. Notably, after co-culture, we observed DGE changes consistent with shedding of CD16 following activation, mirroring the human data for this critical NK marker,[32] as well as increases in other important markers, such as KLRB1 and GZMB.

Functional assessment of expanded NK cells

We then performed killing assays and multiplex ELISA to determine the cytotoxicity and cytokine secretion capabilities of both subsets of NK cells at days 14–17. Representative flow gating demonstrates the effector and target cell populations using CFSE labeling (figure 4A). Overall, we observed a clear dose-response in cytotoxicity for expanded NK cells against both OSA and melanoma targets (figure 4A,B). Although PBMC-expanded NK cells demonstrated increased cytotoxicity compared with expanded CD5-depleted NK cells at all effector-to-target ratios, these differences were not statistically significant (figure 4B). Additionally, we observed donor variability in NK killing against OSA and melanoma targets consistent with other published studies[23] (figure 4C). We then performed multiplex analysis to analyze cytokines in the culture supernatant (figure 4D). Importantly, secretion of GM-CSF and IFN-γ was significantly greater in PBMC-expanded NK cells compared with expanded CD5-depleted NK cells, while MCP-1 was significantly lower ($p<0.05$). No significant differences were observed between NK cells expanded from bulk PBMCs compared with those expanded from CD5-depleted cells in any of the other nine cytokines investigated.

Genomic analysis of magnetic bead purified and bulk PBMC-expanded human NK cells

To provide comparative context to information gleaned from sequencing canine cells, we used RNA-seq to characterize human NK cells expanded from both magnetic bead purified and bulk PBMC starting populations. Studies using human cells have the advantage of well-defined NK markers and well-characterized antibodies and purification protocols, thereby providing a necessary controlled setting to corroborate canine gene expression patterns. At rest, purified human NK cells showed clear differences compared with bulk PBMCs. The normalized counts of genes associated with NK function were higher in purified NK cells than bulk PBMCs, with significantly greater CD56 expression as expected following isolation of the NK population (figure 5A). Genes related to T and B cells similarly followed expected trajectories, with little to no expression in the purified NK population and increased expression in PBMCs (figure 5B). The differences in normalized counts between NK-isolated cells and bulk PBMCs at day 0 are then clearly visualized using an MA plot showing 1739 DGEs between the two groups (figure 5C). The significant differences at day 0 provide the framework to emphasize the lack of differences between the two populations at day 14 co-culture, where an MA plot shows only four DGEs between the two groups (figure 5D). This convergence of gene expression profiles is plainly illustrated using a PCA plot, and the addition of day 7 samples suggests that the majority of the convergence occurs in the first half of the co-culture timeline (figure 5E). To further compare canine and human co-culture expression signatures, we again highlighted the log fold changes of genes associated with NK cell signatures at day 14 vs day 0 (figure 5F). Similar to canine data, IFNG and GZMA had the largest positive fold changes in human cell expansions. However, human cells notably differed in respect to CD16, which had insignificant changes, and KLRG1, which had the largest negative fold change in both groups. The merging of distinct NK and bulk populations into a nearly identical activated NK phenotype, with significant changes in NK functional genes at day 14 vs day 0, aligns biologically with equivalent canine data for CD5-depleted and bulk populations.

First-in-dog clinical trial of adoptive transfer of autologous canine NK cells

Together, these phenotypic, functional and transcriptomic study results aligned with our hypothesis that NK cells expanded from bulk PBMC starting populations produce an equivalent or superior cellular

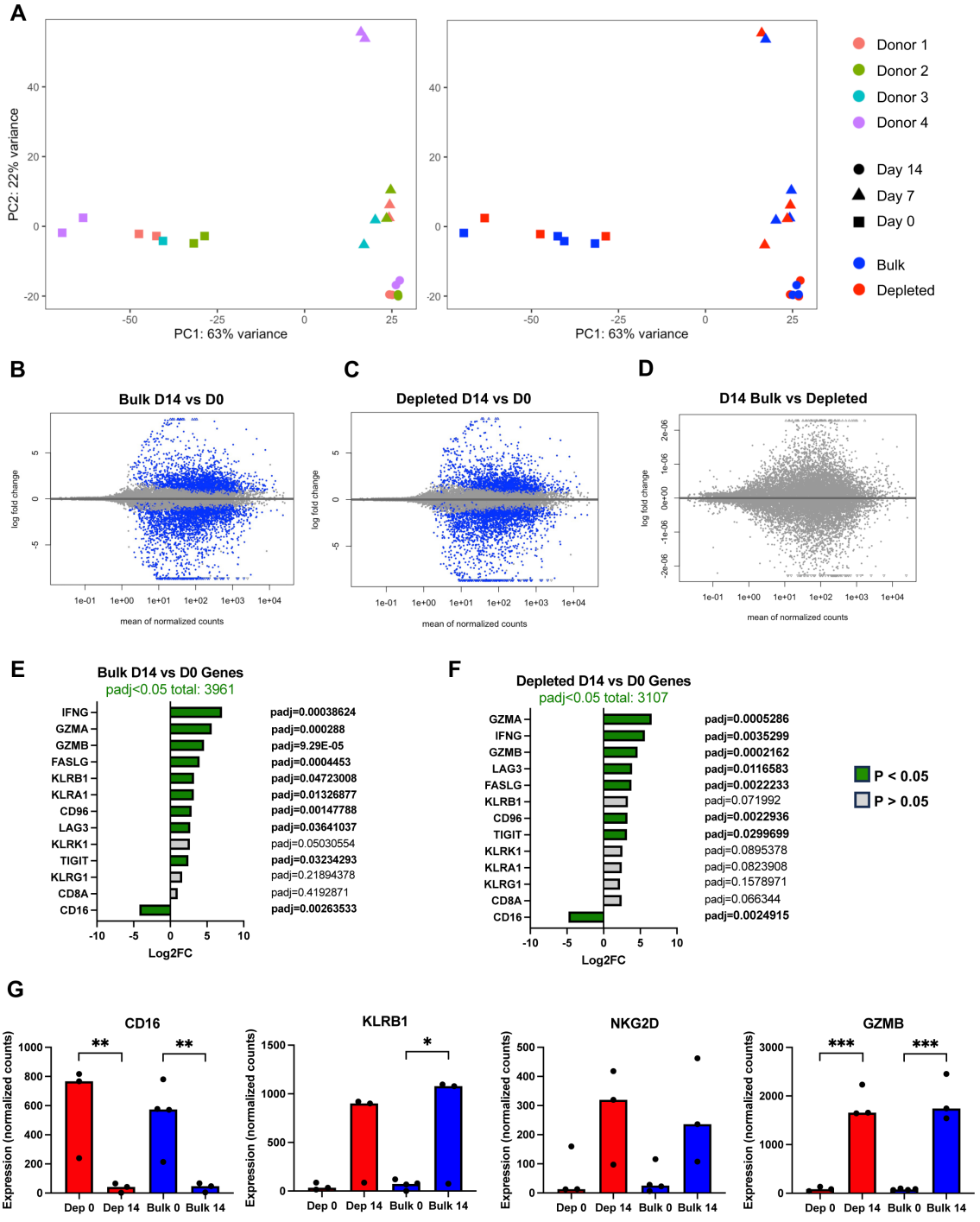


Figure 3 Genomic analysis of expanded NK cells by bulk RNA-seq. Cells from bulk PBMC and CD5-depleted cell starting populations were collected at days 0, 7, and 14 of the 14-day co-culture from four separate healthy beagle donors and 3'-Tag-RNA-seq was performed. (A) Principal component analysis (PCA) of cells color-coded by donor dog (left) or starting population (right) demonstrated variability at days 0 (squares) and 7 (triangles) of co-culture with convergence of cell signatures at day 14 (circles). Certain samples from donor three did not meet RNA quantity standards and were exluded, including day 0 depleted, day 14 depleted, and day 14 bulk. MA plots, using a p<0.05 significance threshold, corroborate PCA plot patterns with (B) 3961 differentially expressed genes (DGEs, blue) between PBMCs at day 14 vs day 0 of co-culture, (C) 3107 DGEs between CD5-depleted cells at day 14 vs day 0 of co-culture and (D) zero DGEs between bulk PBMCs and CD5-depleted cells at day 14 co-culture. The log fold change of NK cell-related gene expression was assessed between day 14 and day 0 co-culture in (E) the bulk PBMC group and (F) the CD5-depleted cell group. Several key genes were significantly different following co-culture compared with day 0 (bold, green). P values were determined using the DESeq2 package in R. (G) Absolute normalized counts for CD16, KLRB1, NKG2D, and GZMB were visualized for CD5-depleted cells at day 0 co-culture (Dep 0) and day 14 co-culture (Dep 14) as well as PBMCs at day 0 co-culture (bulk 0) and day 14 co-culture (bulk 14). Bars show median of normalized counts for donor dogs and p values were determined using the DESeq2 package in R. *P<0.05, **p<0.01, ***p<0.001. NK, natural killer; PBMC, peripheral blood mononuclear cell; RNA-seq, RNA sequencing.

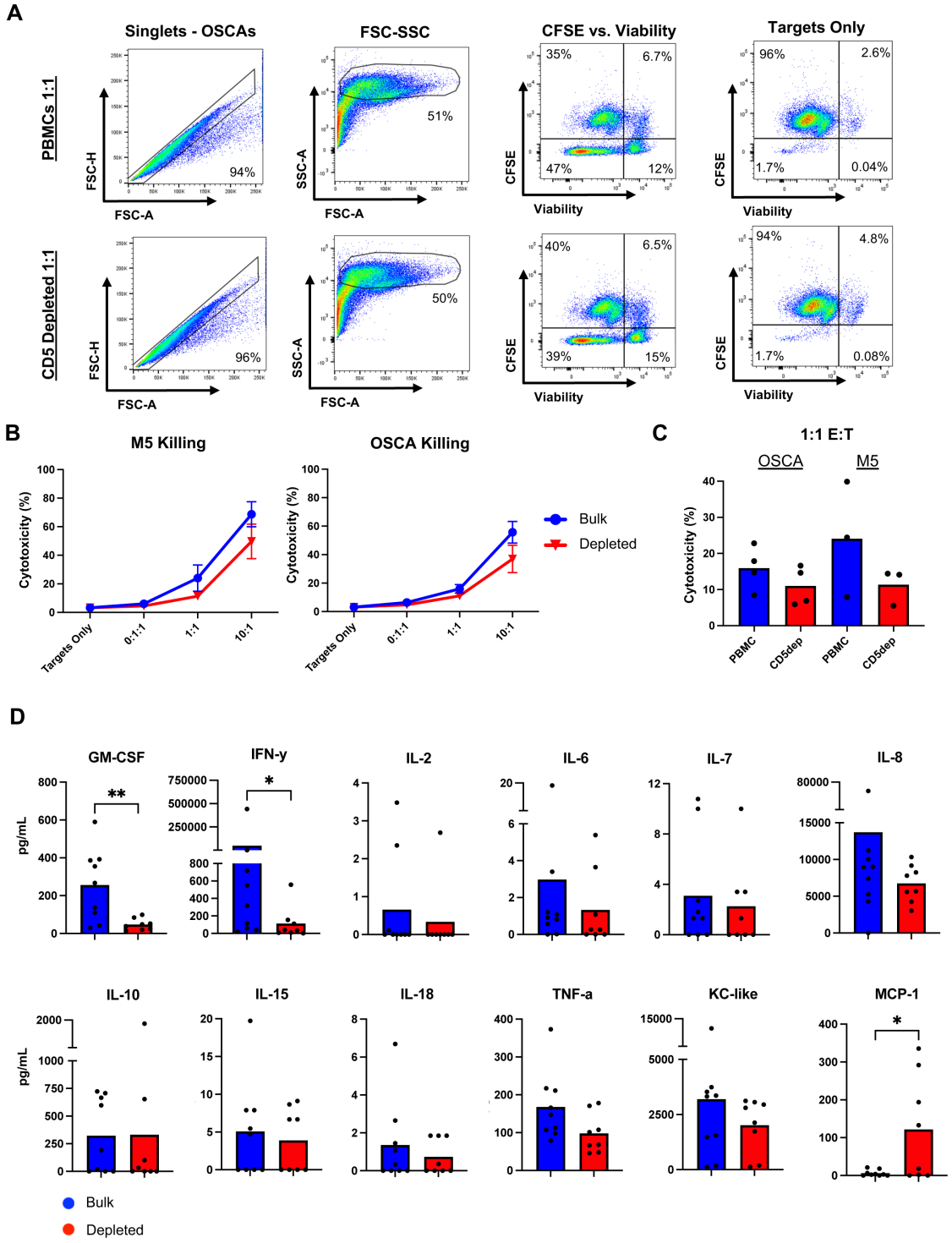


Figure 4 Functional assessment of expanded NK cells. (A) Representative flow cytometry showing gating strategy for NK killing assays using PBMC and CD5-depleted expanded NK cells against osteosarcoma cell line targets (OSCA) at a 1:1 effector-to-target (E:T) ratio. Target cells were identified from effector cells by carboxyfluorescein succinimidyl ester (CFSE)+ labeling with separate viability dye staining to identify dead cells. (B) Mean cytotoxicity (±SEM) of NK cells at day 14 from four donor dogs at increasing E:T ratios from bulk PBMC (blue) and CD5-depleted (red) starting populations against melanoma (M5, left) and osteosarcoma (OSCA, right) targets. (C) At the 1:1 E:T ratio, mean cytotoxicity of expanded NK cell effectors varied against osteosarcoma and melanoma targets, with increased PBMC-expanded NK cell killing against melanoma targets compared with osteosarcoma (p=ns). (D) Supernatant cytokine levels were measured by canine Luminex assay. Bars depict mean values of eight or nine healthy donor dogs for PBMC (blue) and CD5-depleted cells (red) following 14-day co-culture. GM-CSF and IFN-γ concentrations were significantly greater in the PBMC group, while MCP-1 was significantly greater in the CD5-depleted group. P values were determined using the Mann-Whitney U test. *P<0.05, **p<0.01. GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; NK, natural killer; ns, not significant; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor.

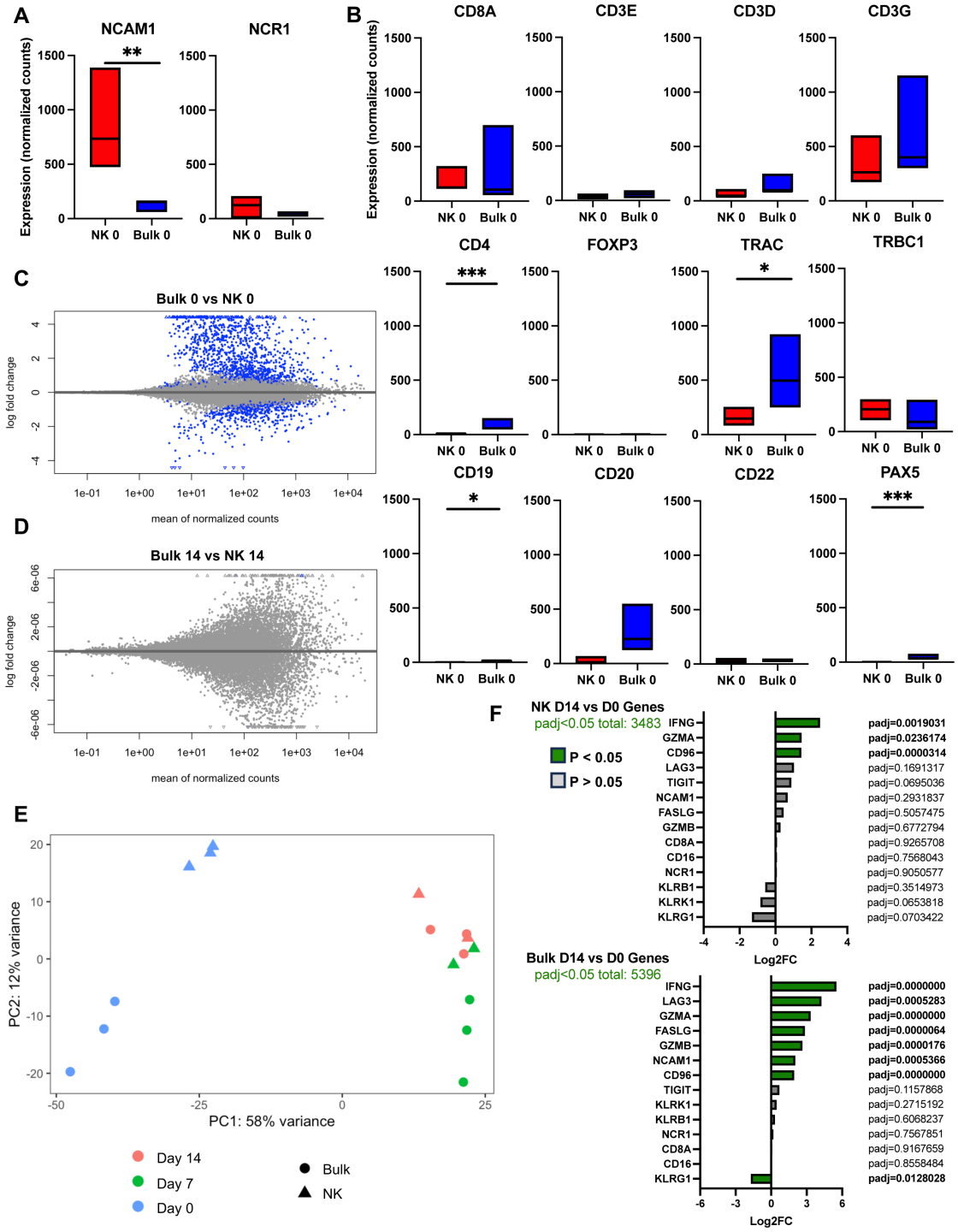
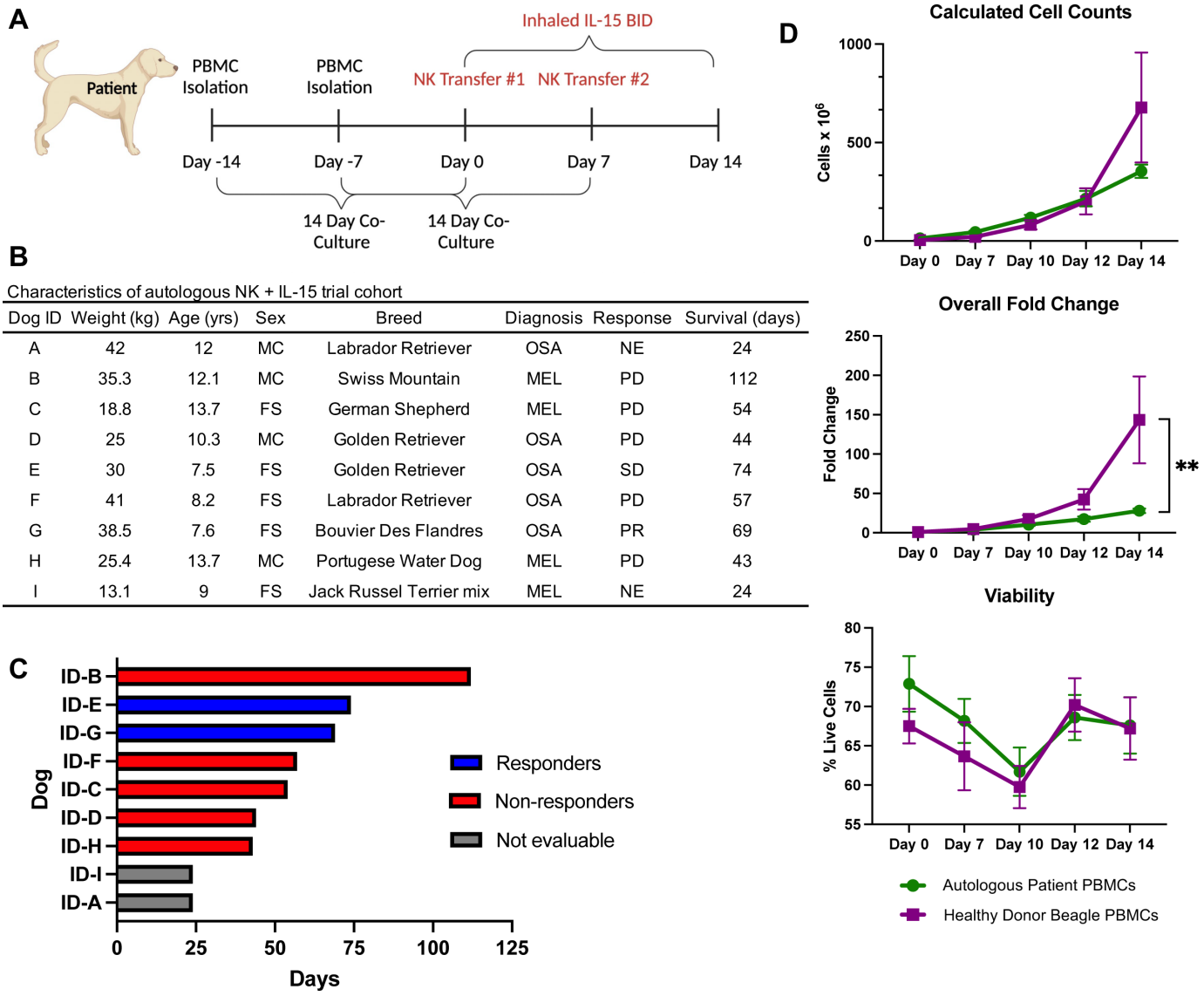


Figure 5 Genomic analysis of human bulk PBMCs versus purified NK cells. Human cells from bulk PBMC and purified NK cell starting populations were collected at days 0, 7, and 14 of 14-day co-culture from three separate human donors and 3'Tag-RNA-seq was performed. Absolute normalized counts for (A) NCAM1/CD56 and NCR1/NKp46 and (B) T and B cell-related genes were visualized for purified NK cells (NK 0) and PBMCs (bulk 0) at day 0 co-culture. Certain samples did not meet RNA quantity standards and were excluded, including day 7 NK isolated and day 14 NK isolated. Floating bars show minimum to maximum of normalized counts for human cells and p values were determined using the DESeq2 package in R. *P<0.05, **p<0.01, ***p<0.001. MA plots, using a p<0.05 significance threshold, reveal starting populations that are distinct at rest, with (C) 1739 DGEs between bulk PBMCs and purified NK cells at day 0, but converge when activated, with (D) only four DGEs between bulk PBMCs and purified NK cells at day 14 co-culture. (E) Principal component analysis (PCA) of cells aligns with MA plot patterns with high variability at day 0 (blue) and concentration of cell signatures at day 7 (green) and furthermore at day 14 (peach) of co-culture. (F) The log fold change of NK cell-related gene expression was assessed between day 14 and day 0 co-culture from purified NK cells (top) and bulk PBMCs (bottom). Several key genes were significantly different following co-culture compared with day 0 (bold, green). P values were determined using the DESeq2 package in R. NK, natural killer; PBMC, peripheral blood mononuclear cell.

product compared with NK cells expanded from CD5-depleted cells. Previously, we completed a first-in-dog trial combining palliative RT and intratumoral autologous NK cell transfer in dogs with unresectable, non-metastatic OSA[18] where we used NK cells expanded from CD5-depleted cells as our starting source material. While intratumoral injections have the advantage of bypassing the constraints of NK homing from the systemic circulation and potentially avoiding toxicity, not all tumors are accessible for injection and the efficacy of intratumoral administration is limited in patients with disseminated disease. We therefore conducted a first-in-dog trial using intravenous injection of autologous NK cells expanded from bulk PBMCs in dogs with pulmonary metastases from OSA and melanoma. This was combined with inhaled IL-15 to support in vivo persistence and activation of endogenous and exogenous NK cells based on our previous work establishing a maximum tolerated dose and suggesting potential clinical activity of inhaled IL-15 as a monotherapy in dogs with lung metastases from OSA and melanoma[23] (figure 6A). Cytokine immunotherapy using IL-15 effectively activates NK cells and the inhaled route appears to stimulate antitumor efficacy against gross metastasis without the toxicities of systemic administration. Although autologous NK cells have been characterized as hypofunctional in human NK trials,[33 34] we designed this trial with primary considerations of safety given that systemic NK immunotherapy had never previously been performed in dogs. Nine dogs with naturally occurring melanoma (n=4) or OSA (n=5) were enrolled and no treatment-related serious adverse events were identified (figure 6B).[29] One dog demonstrated a partial response and one dog demonstrating stable disease based on RECIST criteria (figure 6C). To assess the expansion ability of PBMCs derived from cancer-bearing dogs compared with healthy dogs, we obtained cell count, fold change, and viability data for both expansions from each of the nine patients compared with expansions from beagle donors (figure 6D). Viability was similar between cancer-bearing and healthy donors ($p>0.05$). Absolute cell counts were numerically higher but not statistically significant, while overall fold change was significantly higher in PBMC-expanded NK cells from healthy beagle donors compared with patients with cancer ($p<0.05$). Taken together, this study established the feasibility of obtaining therapeutic amounts of clinical grade dog NK cells from patient derived PBMCs along with the safety of adoptively transferring those cells in dogs with advanced cancer, providing a framework for subsequent trials to improve efficacy.

First-in-dog clinical trial of adoptive transfer of allogeneic canine NK cells

A key advantage of NK cells in cancer immunotherapy centers on the ability to use allogeneic sources for off-the-shelf treatment with decreased risk of AEs.[33 34] NK cells can also be made readily available for patients with canine cancer in the clinic, which is highly valuable in the context of rapidly progressing cancers and in patients experiencing immune suppression, since time delays for cell manufacturing and adequacy of bone marrow reserve can be problematic when using autologous sources for cellular immunotherapy. Therefore, we next evaluated the combination of palliative RT with the first-in-dog use of allogeneic NK cells from unmanipulated PBMC starting populations from healthy beagle donors for dogs with unresectable oral melanoma (figure 7A,B). Standard palliative RT was administered weekly in four fractions followed by infusion of intravenous allogeneic NK cells (7.5×10^6 cells/kg) on the day of the final RT treatment.[35] We observed no serious AEs related to NK cell injections. While the small sample size limits conclusions regarding efficacy, we did observe a median survival of 145 days in this cohort with one dog surviving 445 days, a particularly promising response given the poor prognosis of canine malignant melanoma.[35 36] With standard of care treatment, 85% of dogs diagnosed with malignant oral melanoma will develop metastatic disease within 6 months, and the large majority will die within 1 year of diagnosis.[35 36] We then performed RNA-seq on patient PBMCs using a 3'-Tag-RNA-seq protocol for gene profiling. PCA showed individual dogs as the driving force for variance of PC1, and samples clustered based on patient rather than treatment timepoint (figure 7C). Notably, the dog that lived the longest (ID5) clustered separately from the rest of the dogs. We then analyzed the relative gene expression of patient samples across timepoints and across dogs using a heatmap highlighting genes related to NK activation and function (figure 7D). We observed variability in NK gene expression between dogs, with the exception of CD16 and IFNGR2 which had consistently high relative gene expression in all patients. To interrogate absolute changes in response to treatment, we then analyzed normalized counts of key NK genes as a composite of patient samples for each timepoint (figure 7E). Most striking was a peak in CD16 expression 1 day following NK transfer, while KLRB1 increased moderately from 1 to 14 days after NK transfer. Interestingly, the inhibitory marker TIGIT, a key NK exhaustion marker,[37 38] increased substantially during the course of RT but subsequently decreased following NK adoptive immunotherapy, suggesting marked fluctuations in NK phenotype over the course of multimodality therapy. To address potential gene signatures in relation to outcome, we analyzed NK DGE signatures from the dog with the longest survival (ID5) to those from the dog with the shortest survival (ID1). Genes that were significantly different from dog ID5 to dog ID1 were visualized by Gene Ontology analysis and showed enrichment of several diverse pathways, including cell



Characteristics of autologous NK + IL-15 trial cohort

Dog ID	Weight (kg)	Age (yrs)	Sex	Breed	Diagnosis	Response	Survival (days)
A	42	12	MC	Labrador Retriever	OSA	NE	24
B	35.3	12.1	MC	Swiss Mountain	MEL	PD	112
C	18.8	13.7	FS	German Shepherd	MEL	PD	54
D	25	10.3	MC	Golden Retriever	OSA	PD	44
E	30	7.5	FS	Golden Retriever	OSA	SD	74
F	41	8.2	FS	Labrador Retriever	OSA	PD	57
G	38.5	7.6	FS	Bouvier Des Flandres	OSA	PR	69
H	25.4	13.7	MC	Portugese Water Dog	MEL	PD	43
I	13.1	9	FS	Jack Russel Terrier mix	MEL	NE	24

Figure 6 First-in-dog clinical trial of adoptive transfer of autologous canine NK cells. (A) Schema of trial design combining adoptive transfer of PBMC-expanded autologous NK cells with inhaled IL-15. PBMCs were isolated from whole blood drawn from patient dogs 14 and 7 days before the start of treatment for the 14-day expansion of autologous NK cells. Dogs received two intravenous injections of autologous NK cells at a dose of 7.5 million cells/kg. Additionally, on day 0, dogs began twice daily treatments with inhaled rhIL-15 continuing for 14 days total. (B) Characteristics of the nine total dogs with pulmonary metastatic melanoma (MEL) or osteosarcoma (OSA) that met entry criteria and were enrolled in the trial. Response was determined by RECIST criteria defining partial response (PR), stable disease (SD), progressive disease (PD), and not evaluable (NE). Survival was calculated from initiation of treatment to death or humane euthanasia. (C) Survival plotted as bars color-coded by RECIST criteria defining responders (PR and SD, blue), non-responders (PD, red), and not evaluable (NE, gray). (D) Cell counts, fold change, and viability were calculated on days 0, 7, 10, 12, and 14 of the 14-day co-culture for autologous PBMCs isolated from patient blood (green) compared with PBMCs isolated from healthy beagle blood (purple). Mean (±SEM) for expansions of both injections of PBMC-expanded autologous NK cells were compared with healthy donor PBMC expansions and plotted over time. P values were determined by mixed effects model. **p<0.01. NK, natural killer; PBMC, peripheral blood mononuclear cell; RECIST, Response Evaluation Criteria for Solid Tumors.

cycle and viral-related pathways (figure 7F). Taken together, this proof-of-concept trial provides notable baseline data validating the canine model for investigating allogeneic adoptive NK cell transfer alone or in combination with other therapies and reinforces the value of high-throughput sequencing in hypothesis generation and uncovering mechanisms of therapeutic response and resistance.

DISCUSSION

Our study compared the trajectory of phenotypic, functional, and genomic changes during the 14-day expansion and activation of canine NK cells derived from both bulk PBMC and CD5-depleted starting populations. Overall, we observed that bulk PBMC-expanded canine NK cells were equivalent, if not superior, to CD5-depleted starting populations. Capitalizing on the practical and

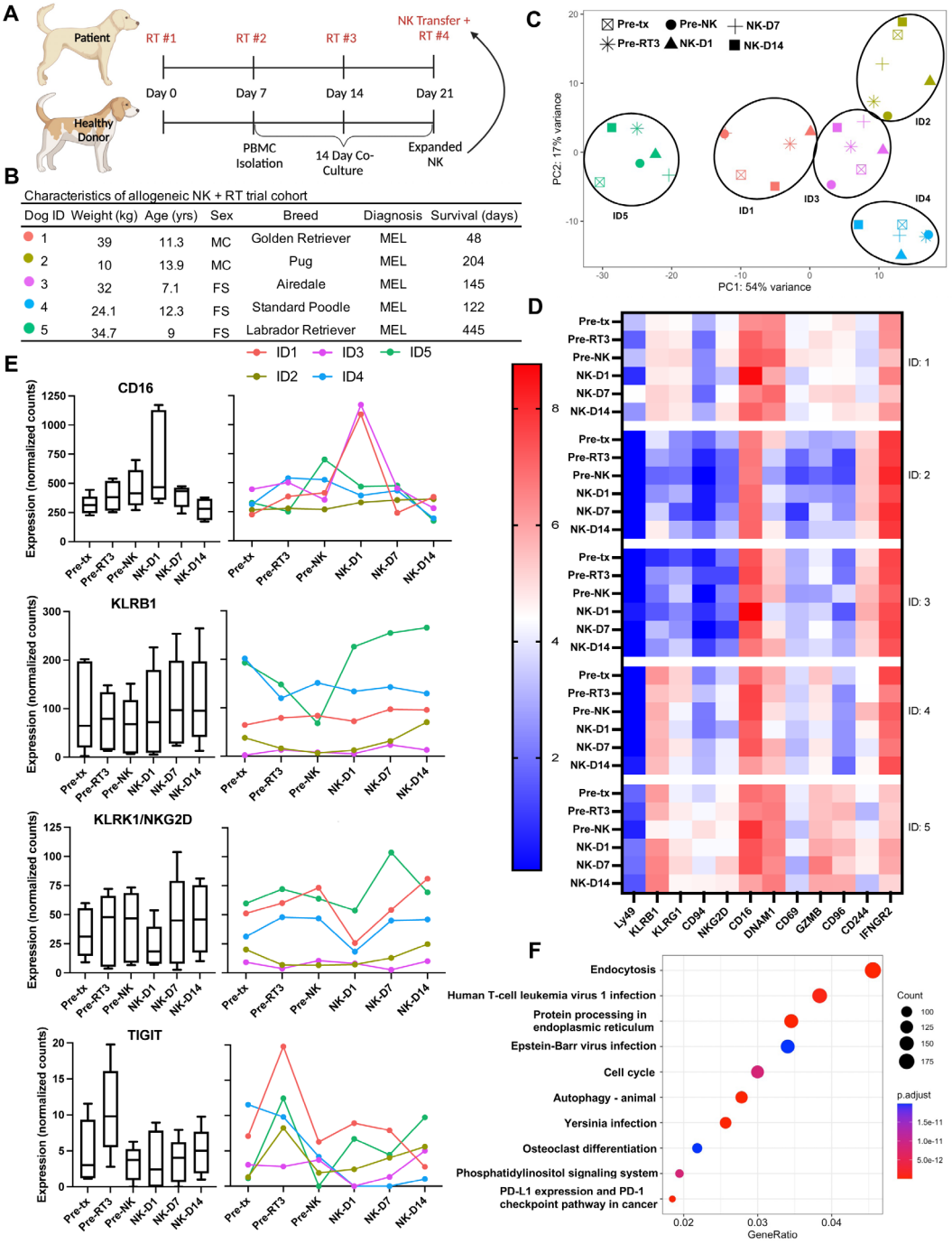


Figure 7 First-in-dog clinical trial of adoptive transfer of allogeneic canine NK cells. (A) Schema of trial design combining adoptive transfer of PBMC-expanded allogeneic NK cells with palliative radiotherapy (RT). PBMCs were isolated from healthy donor beagle blood 14 days before the scheduled NK cell infusion for each patient. Donor PBMC-expanded NK cells at a dose of 7.5 million cells/kg were injected intravenous in patients on completion of RT. (B) Characteristics of the five total dogs with malignant melanoma that met entry criteria and were enrolled in the trial. Dogs ID 1, 4, and 5 had known lymph node metastasis on enrollment and dog ID 4 had pulmonary metastasis on enrollment. Survival was calculated from initiation of RT to death or humane euthanasia. PBMCs were isolated from patients' whole blood at six timepoint and submitted for 3'-Tag-RNA-seq. (C) Principal component analysis (PCA) of cells color-coded by patient and symbols distinguishing timepoint, demonstrated PBMCs clustered based on the dog they originated from rather than timepoint of treatment. (D) A heatmap depicting log counts per million (logCPM) transformed expression of key NK-related genes shows variation across both patients and treatment. (E) Absolute normalized counts for CD16, KLRB1, NKG2D, and TIGIT were visualized as an aggregate of all patients at each timepoint (box and whiskers, left) and individual values by patient (right). While distinct peaks and trends in expression were recognized, changes between timepoints were not significant for the genes assessed. (F) Samples across timepoints were combined based on donor and compared between the dog with the longest (ID5) and the dog with the shortest (ID1) survival. Induced gene pathways in the dog with the highest survival are depicted as a dot plot of gene ontology (GO) analysis. NK, natural killer; PBMC, peripheral blood mononuclear cell.

clinical-trial enabling aspects of this approach, we performed first-in-dog clinical trials using autologous (n=9) and allogeneic (n=5) NK cells for dogs with malignant melanoma and OSA. These first-in-dog clinical trials showed no serious AEs and preliminary efficacy data for canine cancers with extremely poor prognoses.

The ability to achieve sufficient expansions was a fundamental objective in our study. Concerns regarding expanding adequate cell numbers for cellular immunotherapy are not limited to dogs; studies expanding human NK cells have also been restricted by low cell numbers collected after CD3-selection, reducing the cell numbers available to administer to patients.[39] Our results show higher average cell counts at day 14 in expanded PBMCs (677 million) compared with expanded CD5-depleted cells (537 million), which although not statistically significant, remains relevant in the clinic, where doses are based on patient weight and can influence whether dogs receive their full treatment. Previously, major histocompatibility complex (MHC)-low K562 feeder cells with membrane bound IL-21 and cytokine support successfully enriched for NK cells from human PBMCs but had over 21% contamination of T cells at day 21 co-culture.[40] These concerns necessitated our extensive characterization of PBMC-expanded NK cells where we saw minimal CD3+ cells present at day 14 co-culture as assessed by flow cytometry and confirmed by genomic analysis. Flow cytometric analysis of PBMC-expanded day 14 product showed 67% CD3-NKp46+ cells compared with 40.4% in our lab's previous analysis of NK cells expanded from CD5-depleted cells.[18] The variability of CD3+ cells in the day 14 product as assessed by flow cytometry and the potential influence of non-specific staining necessitated the need for thorough validation through both bulk and single-cell RNA sequencing. Potential T cell contamination is particularly relevant in the context of allogeneic transfer, where donor T cells are capable of recognizing healthy recipient tissue as foreign and mounting a response culminating in graft versus host disease. Resting PBMCs analyzed by scRNA-seq were used as a positive control to establish the presence of expected cell types and their proportions prior to expansion. PBMC-expanded NK cells at day 14 confirmed the loss of all cell types except for NK cells. The lack of T cell, B cell, and myeloid gene expression in scRNA-seq and the convergence of distinct PBMC and CD5-depleted resting populations into a near identical NK population at day 14 by bulk RNA-seq, affirmed the ability of the expansion protocol to support the survival of NK cells specifically. This echoes previous work by Gingrich *et al*, where FACS sorted CD3-NKp46+ and $CD5^{dim}$ populations became essentially identical by day 14 of co-culture following the same expansion technique used here.[14] This is especially useful in the context of ongoing efforts for better characterization of the phenotype of canine NK cells. Defining canine NK cells has been notoriously difficult, as neither $CD5^{dim}$ nor CD3-NKp46+ expression appears to satisfactorily identify all canine NK cells at rest.[14 41] This implies that NK selection prior to co-culture could limit cell counts and potentially exclude certain NK subsets altogether, depending on the marker used, with implications for function and engraftment of the NK product. This highlights the utility of using human sequencing data to confirm the convergence of distinct cells at day 14 of co-culture, since human NK cells are reliably identified by a CD3-CD56+ phenotype and can be reproducibly isolated with available protocols. We used several functional assays to confirm the cytotoxic and cytokine secreting capabilities of our expanded NK cell products.

A curious finding was the wide range of cytokine secretion detected by multiplex assay in co-cultured NK cells, particularly IFN-γ. NK cells are known to produce abundant cytokines in response to stimulation. However, RNA-seq results showed significant upregulation of IFNG transcription, aligning with expected functional features of NK cells. RNA-seq also validated this activated phenotype of expanded cells with increased number of cells expression NKG2D and GZMB in scRNA-seq and significant upregulation of KLRB1 and granzyme B along with downregulation of CD16 in bulk RNA-seq. The loss of CD16 in canine NK cells in response to activation is particularly relevant for comparative oncology immunotherapy studies since it is observed in human NK cells following feeder line co-culture, where it is caused by activation of matrix matalloproteases (MMPs) through target cell contact.[42 43] However, cleavage by MMPs is post-translational in nature which would not sufficiently explain the downregulation we observed by RNAseq. Although this transcriptional downregulation was not altogether unexpected since the pattern was similarly reported in a genomic analysis of NK cells expanded from CD5-depleted PBMCs.[14] CD16 in this analysis refers to the low affinity immunoglobulin gamma Fc region receptor III (FCGR3A, Locus 478984), which allows for ADCC in NK cells, and lack of CD16 has been shown to impact NK regulation and cytotoxicity.[32 44] In our study, expanded PBMCs maintained high per cent killing in the context of CD16 transcription downregulation, suggesting that killing is primarily occurring through non-ADCC methods, such as death receptors and perforin and granzyme granules. CD16 is extremely relevant for combinatorial strategies, and our data suggest that CD16 regulation is highly complex in canine NK cells and that dogs with spontaneous cancer may be an informative model interrogating changes in CD16 on NK cells in the future.

While the primary aim in our small cohort using intravenous adoptive transfer of autologous canine NK cells was to establish feasibility, it is important to note that we observed only modest clinical activity, even with the addition of inhaled IL-15 to support NK engraftment where we did previously observe evidence of clinically meaningful responses.[23] Allogeneic NK cells in human

trials have demonstrated superior oncological benefits compared with autologous NK cells, primarily for hematological malignancies.[2 5 45] This is due, at least in part, to the advantage of MHC mismatch in allogeneic donor cells, where the mismatch between donor KIRs and recipient MHC-I ligands contributes an additional activating signal that promotes activation in favor of NK antitumor response.[46 47]

To potentially improve efficacy from autologous transfer, we also piloted a first-in-dog trial using allogeneic NK cells. Since patient-specific immune signatures at baseline and after therapy may ultimately predict outcomes, as has been shown in human immunotherapy trials, we also evaluated DGE signatures from the blood of dogs on therapy.[48] In our canine allogeneic NK recipients, PCA depicted distinct clusters for each patient and reduced variance between patients with similar survival. The intriguing distinctive signatures found within our small patient cohort were further analyzed by comparing gene signatures from PBMCs across treatments between the longest and shortest surviving dogs. The enrichment of pathways associated with response to viral infections suggests an anticipated involvement of NK cells, which are critical in antiviral immunity. However, we observed the highest DGE enrichment for endocytosis pathways, suggesting the influence of non-NK, myeloid populations in shaping differences between gene signatures of these two patients. The assessment of CD16 shedding in NK cells expanded from PBMCs also shapes our interpretation of gene expression patterns in PBMCs from dogs receiving allogeneic NK cells, where CD16 expression peaked 1 day following NK cell transfer. Beyond CD16, we generally did not see clear, consistent NK signature patterns across patients in response to either RT or NK transfer, highlighting the importance of evaluating larger patient cohorts to better define genomic biomarkers of response and resistance to NK immunotherapy, especially given limitations related to longitudinal access to tumor specimens.

NK cells for human immunotherapy have experienced a steep increase in interest with rapid innovation over the past two decades.[49 50] Sources of NK cells for adoptive transfer now include umbilical cord blood stem cells, induced pluripotent stem cells (iPSCs), and NK-92 cell line in addition to peripheral blood.[51] Like T cells, NK cells can also be genetically engineered to increase specificity and longevity.[52] Imai *et al* published one of the first successful applications of chimeric antigen receptor (CAR) NKs, determining the ideal expansion of peripheral blood (PB) NK cells with feeder cells and cytokine support before testing the CAR transduction using the superior expansion method.[53] Similarly, our study is a crucial first step in the forward momentum of immunotherapies in dogs, from which we may begin to develop genetically engineered products and novel NK sources. Dogs are a readily available resource for developing new combinations of therapies, which can then inform the most promising paths for improving immunotherapy across species.[13] Future studies will further contextualize dog NK cells compared with human, and the resulting data can be the framework for advancing NK immunotherapy and improving the prognosis for both dogs and humans with aggressive cancers.

In summary, our findings support the use of unmanipulated PBMCs plus feeder line co-culture for adoptive immunotherapy in dogs with cancer. Overall, our preclinical and clinical data highlight the safety and feasibility of this technique as a novel platform for optimizing NK immunotherapy in dogs. Our work also demonstrates the strength of the dog model to address practical, clinically relevant questions regarding optimization of novel immunotherapy approaches. Ultimately, teaming rapid and innovative clinical trials with robust genomic characterization is anticipated to advance the field of NK immunotherapy across the bench-to-bedside continuum.

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Contributors AMR, RBR, MSK, and RJC designed the studies. AMR, LEF, KRI, MSK, RBR, and RJC conducted the experiments and collected the data. AMR, EGJ, and RJC analyzed the data. RMH and CTB provided bioinformatic assistance. AMR and RJC wrote the manuscript. All authors, including SJJ, ML, CD, and WJM, provided critical reviews of the manuscript. RJC is responsible for the overall content as guarantor.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The collection of whole blood from three human patients undergoing resection of benign lesions was approved by the IRB at the University of California, Davis (Protocol # 218204). Participants gave informed consent to participate in the study before taking part. The autologous NK immunotherapy clinical trial in dogs with metastatic osteosarcoma and melanoma was approved by the UC Davis School of Veterinary Medicine Clinical Trials Review Board and IACUC (protocol #21461). Informed consent was obtained from all owners prior to enrollment. The first-in-dog trial using allogeneic NK cells was approved by the UC Davis School of Veterinary Medicine Clinical Trials Review Board and IACUC (protocol #21620).

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