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CD94 Ex Vivo Cultures in a Bone Marrow Transplantation Setting

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Background. Complementary, marrow donor-derived peripheral blood T-lymphocyte infusions enable consistent hematopoietic engraftment in lethally irradiated dog leukocyte antigen (DLA)-haploidentical littermate recipients, but at the cost of severe graft versus host disease (GVHD). Here, we explored whether CD94-selected and in vitro-expanded natural killer (NK) cells could be substituted for T-lymphocytes for enhancing marrow engraftment without causing severe GVHD. **Methods.** Five dogs were conditioned with 700 cGy total body irradiation followed by infusion of DLA-haploidentical donor marrow and CD94-selected, in vitro-expanded NK cells. NK cells were infused at a median of 140 000 (range 78 000–317 000) cells/kg. **Results.** Four dogs rejected their marrow grafts, whereas 1 dog fully engrafted and developed GVHD. We observed an increase in peripheral blood NK cells after infusion of CD94-selected, ex vivo-expanded NK in 2 dogs. Peripheral blood lymphocyte counts peaked at day 7 or 8 posttransplant in the 4 rejecting dogs, whereas in the fully engrafted dog, lymphocyte counts remained stable at suboptimal levels. **Conclusions.** Our study indicates NK cells can be expanded in vitro and safely infused into DLA-haploidentical recipients. Within the range of CD94-selected and expanded cells infused we concluded that they failed to both uniformly promote engraftment and avert GVHD.

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Donor-derived peripheral blood mononuclear cell (PBMC) infusions, which include T-lymphocytes enabled consistent marrow engraftment in lethally irradiated dog leukocyte antigen (DLA)-haploidentical related (littermate or parent) recipients, but at the cost of severe graft versus host disease (GVHD).[1] In contrast, recipients not given supplemental PBMC infusions nearly uniformly rejected marrow grafts from their DLA-mismatched littermates despite conditioning with >1100 cGy total body irradiation (TBI).[2] Host natural killer (NK) cells were responsible for this rejection,[3] and adding donor PBMC to marrow overcame the NK cell barrier and enabled consistent marrow engraftment.[4] T-lymphocytes within the PBMC were responsible for overcoming rejection[5] in a process mediated by both CD4 and CD8 T-cells.[6] However, T-cell infusions caused rapidly fatal GVHD. It is generally believed that NK cells contribute to the graft versus tumor effect without exacerbating GVHD.[7,8] Since radioresistant host NK mediated rejection of DLA-mismatched marrow grafts, we assumed that donor NK cells could be used as a substitute for donor T-cells to enable marrow engraftment without causing severe GVHD. Here, we tested the assumption by infusing CD94-selected and in vitro-expanded NK cells in addition to the marrow graft. Because the canine hematopoietic cell transplantation model has so efficiently predicted outcomes for the treatment of hematological disorders in the patient clinical setting, we felt compelled exploring NK immunotherapy in that model.

MATERIALS AND METHODS

Animals

Recipient dogs (3 females and 2 males) weighed a median of 14 kg (range 13–14.6 kg) and were a median 19 (range 8–22) mo old at transplant. Dogs were housed in the Fred Hutchinson Cancer Research Center Comparative Medicine

Department, an AAALAC-accredited facility. The study was approved by the Institutional Animal Care and Use Committee. DLA-haploidentical pairs were selected based on polymorphic microsatellite markers within DLA class I and class II regions and DLA-DRβ1 sequencing.[9,10]

Hematopoietic Cell Transplantation

Dogs were conditioned for marrow transplantation by 700 cGy TBI delivered by a Clinac 600C/D Linear Accelerator (Varian Medical Systems, Inc.) at 7 cGy/min. Dogs were given an intravenous infusion of DLA-haploidentical marrow on day 0 and a 10-d-expanded CD94-selected cell infusion on day 1 without prophylaxis for GVHD. They were followed for engraftment by daily complete blood counts (CBCs) and GVHD assessments. Blood was drawn twice weekly for chimerism analysis, and cells from marrow and lymph node were also assayed for chimerism at necropsy (median d 13; range 11–15) to assess the degree of donor cell engraftment.[11,12]

CD94+ Selected and Expanded Cell Cultures

Ex vivo cultured NK/NKTs (natural killer-like T-cells) were generated by immunomagnetically selecting CD94 cells from marrow donor peripheral blood mononuclear cells (PBMCs) collected by apheresis (Spectra Optia; Terumo BCT) on day minus 10.[13] Apheresis products were overlaid onto 1.077 Ficoll (GE Healthcare) in 50 mL conical bottom centrifuge tubes and centrifuged for 20 min at 750 rcf without braking. Mononuclear cells were washed at 180 rcf for 10 min then resuspended in Hank's Balanced Salt Solution (Invitrogen) containing 2% horse serum (2% HH; Gibco) at 20 million cells/mL. Anti-canine CD94 clone 8H10 (mouse IgG_1)[14] was added at 10 µg/mL, then incubated on ice for 20 min and washed with 2% HH at 400 rcf for 10 min. CD94-bound cells were labeled for magnetic separation in Miltenyi Running Buffer (Miltenyi Biotech) using either anti-mouse whole-Ig or -IgG_1 coated Miltenyi microbeads according to the manufacturer's instructions, then selected on a CliniMACS (n = 2) or over LS columns (n = 3). Cell culture conditions were based on those previously described for obtaining large granular lymphocyte with NK cell characteristics.[14,15] Cells were cultured in Iscove's Modified Dulbecco's Medium containing 10% FCS, GlutaMAX, and 15 ng/mL recombinant human IL-15 (Shenandoah) plus 100 ng/mL recombinant canine IL-2 (R&D Systems) in upright T-75 tissue culture flasks (Corning, NY) with lethally irradiated (Elite 3000; Atomic Energy of Canada) K562mbIL21[16] at a ratio of 1:2-1:10 (CD94+ cell: K562mbIL21) and at up to 1 million total cells per ml. Cultures were counted daily for live and Trypan positive K562mbIL21 cells and for live, non-K562mbIL21 cells (cultured CD94-selected cells containing NK) and fed with additional medium to maintain total cell density of no more than 2 million total cells per milliliter. K562mbIL21 were added up to 3 times including day 8 and/or day 9 for all cultures (**Figure S2, SDC**, http://links.lww.com/TXD/A295). At day 10, culture cytotoxicity and immunophenotype were obtained from an aliquot of the culture.

Immunophenotyping

NK (CD3-CD94+), NKT (CD3+CD94+), and T-cell (CD3+CD94–) percentages were found using a combination of anti-canine CD3 and CD94 mAb as previously described.[14] Cells per microliter were calculated by multiplying the percentage of immunophenotyped cells found in a lymphocyte ($FSC^{lo}SSC^{lo}$) gate by the absolute lymphocyte count obtained from a respective CBC.

Cytotoxicity Assay

We used a modification of a flow cytometry method employing canine thymic adenocarcinoma cells labeled with 1 µ Molar of fluorescent dye [5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester] and detected cytotoxicity with a Fixable Viability Dye eFlour780 (both reagents from Thermo Fisher Scientific). Cultured, live non-K562 cells were set up with 100 000 canine thymic adenocarcinoma cell (CTACT)/ replicate at ratios of 1:1, 1:2, 1:5, and 1:10.[17]

RESULTS

Marrow Transplantation Outcomes

Table 1 summarizes the results in the 5 dogs given 700 cGy TBI followed by infusion of DLA-haploidentical related marrow (d 0) and an infusion of marrow donor-derived, CD94 selected, in vitro-expanded NK/NKT cells (d 1). Hematologic changes following this protocol are presented in Figure 1. Postirradiation nadirs for lymphocytes occurred between days 2 and 6. The 4 dogs that rejected their grafts failed to recover lymphocyte counts, whereas the engrafting dog H941 achieved almost 10% of normal baseline by day 12. The neutrophil nadirs occurred between days 6 and 11. The engrafting dog's lymphocytes peaked at day 2 (1 d after CD94 prep infusion) at 408 cells/µL. We observed lymphocyte peaks of 255, 410, and 456 cell/µL at day 8 in the 3 rejecting dogs given noncytotoxic preparations, but an earlier (d 7) and higher (1346 cells/µL) peak in the rejecting dog that received the other highly cytotoxic preparation (H981). Neutrophils failed to recover in 4 dogs and returned to >20% of baseline levels in dog H941. The nadirs for platelets occurred between days 8 and 11 and did not recover to normal levels during the study.

Table 1 shows that 2 of the rejecting dogs (H961 and H982) never showed evidence of donor chimerism among PBMC and peripheral blood neutrophils. The other 2 rejecting dogs (H960 and H981) reached peaks of 17% and 25%, respectively, among PBMC and 1 (H960) had a peak neutrophil donor chimerism of 4.3%, whereas the other (H981) did not show donor neutrophil chimerism. Dogs H961 and H982 also had 0% donor chimerism in lymph nodes and bone marrow at necropsy. H981 had >50% chimerism in lymph node, >20% in marrow, and 25% in PBMC at necropsy on day 11, but the total white blood cell count was only 80 cells/µL (100% lymphocytes). In contrast, H941 reached 100% peripheral blood chimerism in both PBMC and neutrophils by day 12 and had 95% donor chimerism in lymph node and 99% in marrow at euthanasia on day 13. The peripheral white blood cell count at the time of death was 1850/µL, platelets were 16 000/µL, and the hematocrit was 41.6%. The differential showed 69% neutrophils and 13% lymphocytes. A longitudinal hematopoietic chimerism analysis of 1 representative rejecting dog, H960, showed a gradual loss of donor chimerism between days 5 and 15 (Figure 1). Day 13 histopathology of engrafting dog H941 showed widespread GVHD in multiple sites (soft palate, ear, skin, lacrimal gland, conjunctiva, liver, jejunum, and ileum). This dog received the greatest number of cultured NK and second greatest number of NKT cells/kg, 317 000

TABLE 1.

Dogs given 700cGy TBI followed by marrow and NK cell infusions from DLA-haploidentical related donors

Dog ID	Weight (kg)	BM TNC, 10^6/kg	Ex vivo TNC, 10^6/kg	% T-cells	T-cells/kg	% NKT	NKT/kg	% NK	NK/kg	Killing = Neg.	% Peak donor chimerism PBMC	Gran.	% Donor marrow chimerism	Outcome	GVHD
H960	13.5	374	0.59	17.4	103 000	64.2	385 000	18.4	109 000	1:1	17	4.3	0	Rejected	No
H961	13.	456	0.54	13.9	75 000	45.8	252 000	40.3	218 000	1:1	0	0	0	Rejected	No
H941	14	540	2.1	2.2	46 000	82.7	1 734 000	15.1	317 000	1:10	100	100	99	Engrafted	Yes
H981	14.6	680	0.82	5.7	47 000	77.2	617 000	17.1	140 000	>1:10	25	0	23	Rejected	No
H982	14	347	2.1	5.1	107 000	91.2	1 915 000	3.7	78 000	1:1	0	0	0	Rejected	No

Bone marrow total nucleated cell (TNC) dose and makeup of ex vivo CD94-selected preparations. The d 10 cultures were counted for total live, non-K562 cells and relative abundance T-cells, NKT, and NK determined by immunophenotyping with a CD3CD94 antibody combination. Marrow chimerism done at end of study is a relative comparison and does not reflect marrow cellularity.
DLA, dog leukocyte antigen; GVHD, graft versus host disease; NK, natural killer; NKT, natural killer-like T-cell; PBMC, peripheral blood mononuclear cell; TBI, total body irradiation.
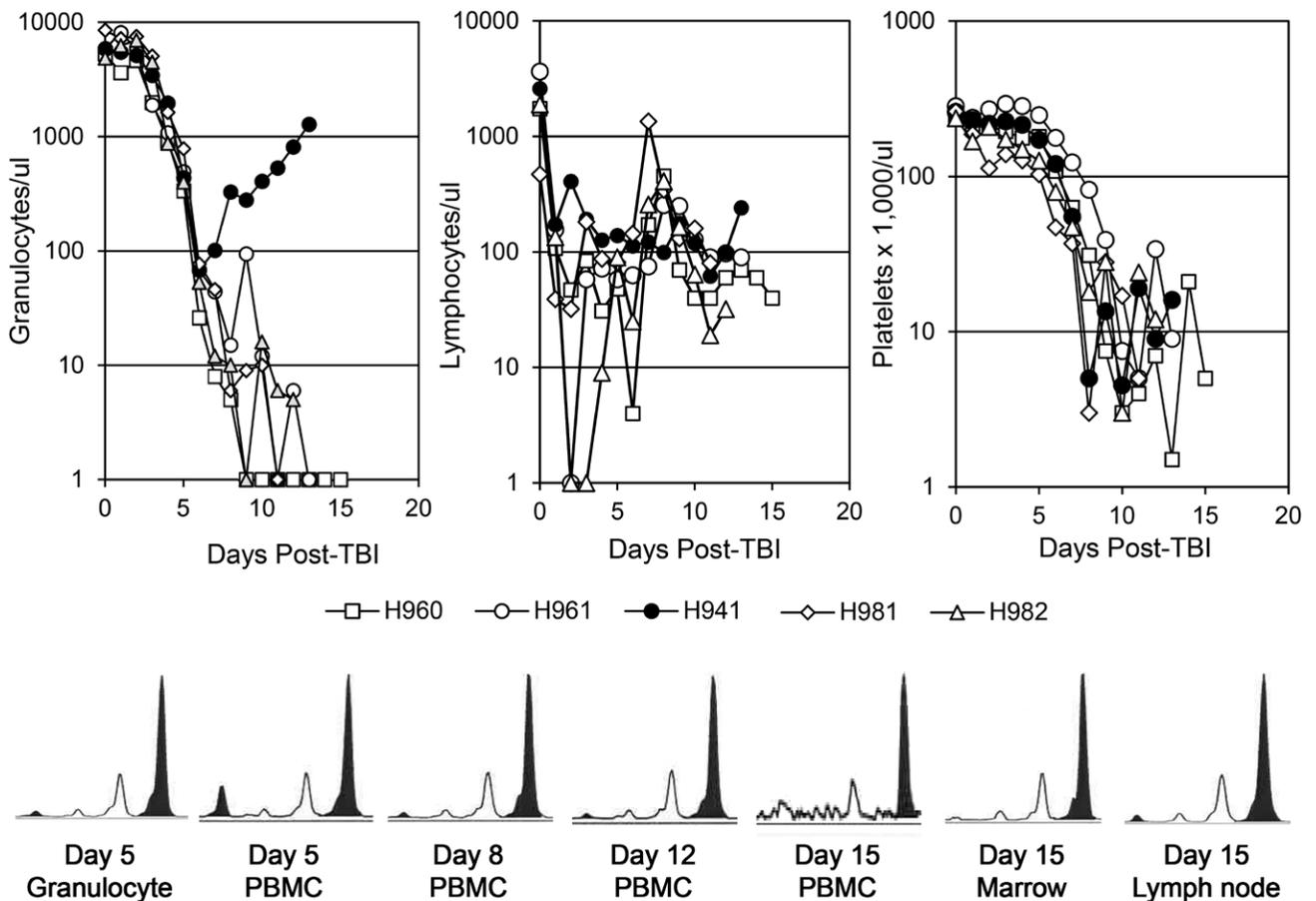

FIGURE 1. Marrow transplant hemodynamics and chimerism. Top, Daily CBC in 5 dogs given DLA-haploidentical related marrow grafts and CD94+ cell preparations in lieu of PBMC. Suggest leaving the following description to the text of the manuscript. At d 7 there was a rise in lymphocytes counts that were associated with acute BMT rejection,[3] whereas granulocytes had dropped to their nadir and did not recover except in H941. Bottom, representative chimerism data of rejecting dog H960 showing informative (black-filled) recipient (right peaks) and donor (left peaks) normalized to recipient signal. CBC, complete blood count; DLA, dog leukocyte antigen; PBMC, peripheral blood mononuclear cell; TBI, total body irradiation.

and 1 734 000, respectively (Table 1). The relative cellularity of donor to recipient cells within the marrow of all 5 dogs at time of euthanasia is shown in Table 1.

In Vivo NK and NKT Cell Counts

Flow cytometry studies combined with CBC (Figure 2, **Figure S1 and Table S1, SDC,** http://links.lww.com/TXD/A295) of recipient PBMC before infusion of cultured NK/NKT cells and on day 1 postinfusion (d 2 post-TBI) showed a postinfusion increase in peripheral blood NK in only 2 dogs. Dog H941's NK cell values increased over 6-fold to 20.2 cells/µL from 3.2 cells/µL, whereas NK cell counts in dog H981 increased about 15-fold from 0.3/µL to 4.6 cells/µL. The 3 other dogs had fewer NK cells 1 d after infusion as H961's cell counts declined from 0.7/µL to 0 NK cells/µL, H982's counts dropped from 1.9/µL to 0 NK/µL, and dog H960's counts decreased from 0.9/µL to 0.2/µL. The engrafting dog was the only subject to present with increased absolute lymphocyte count and NKT cell counts 1 d
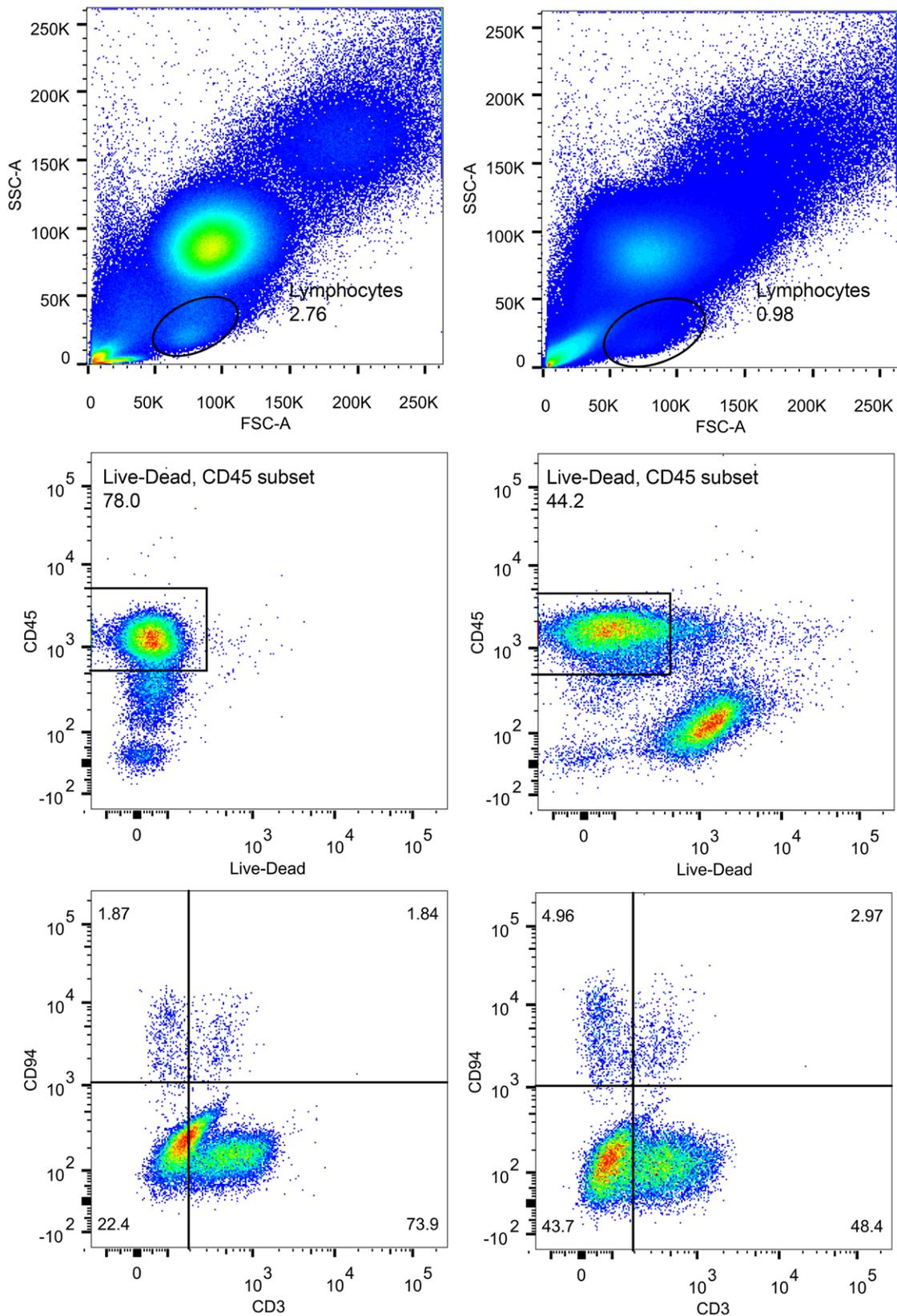
FIGURE 2. Flow data depicting CD94± and CD3± cells within PBMC obtained from H941 before infusion of cultured NK/NKT cells (left panels) and 1 d after infusion (right panels). The lymphocyte gates (upper panels FSC^{lo}SSC^{lo}) were placed into pan white cell (CD45) by viability gates (middle panels) then live, CD45+ cells were placed into CD3 by CD94 plots (lower panels). NK, natural killer; NKT, natural killer-like T-cell; PBMC, peripheral blood mononuclear cell.
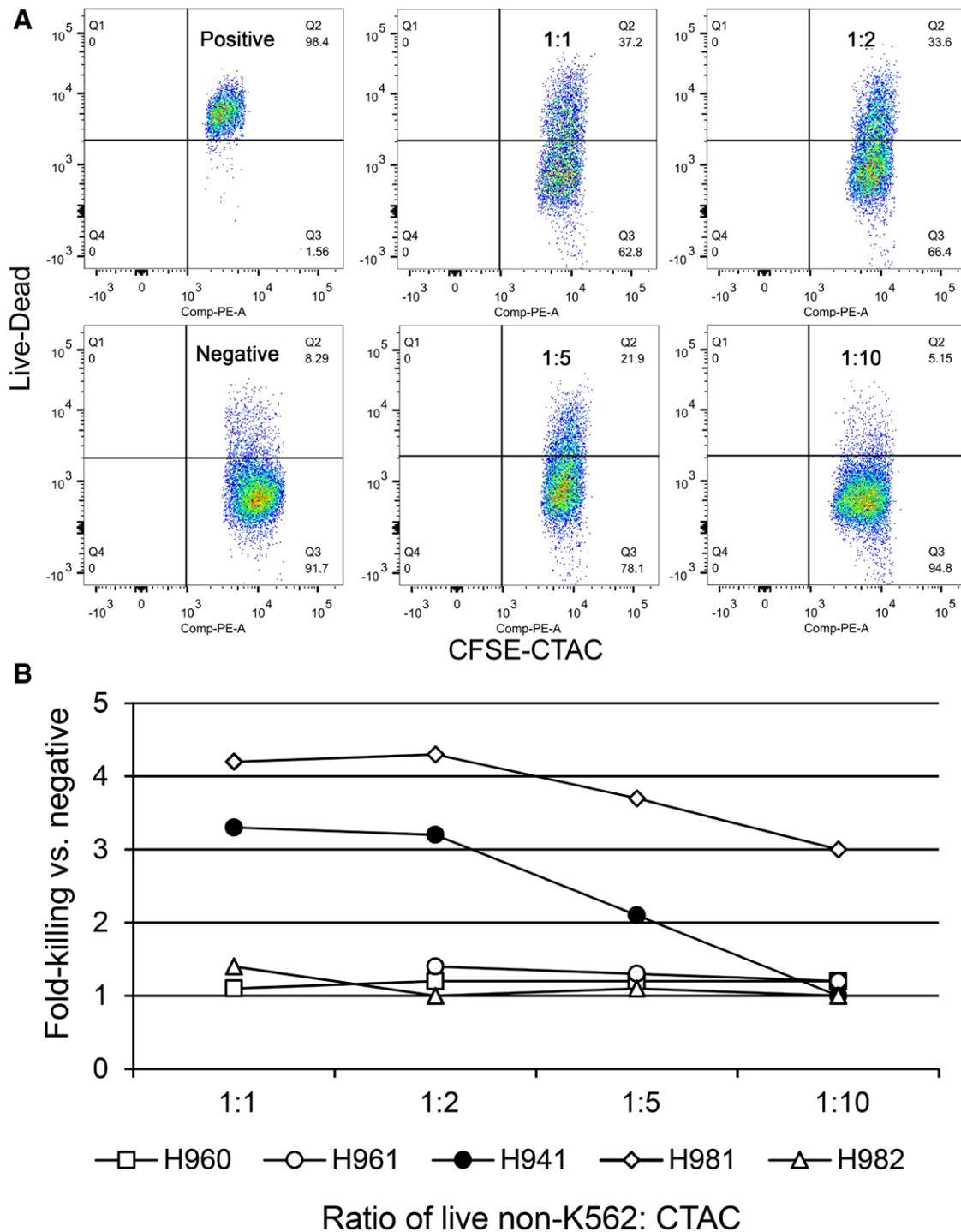
FIGURE 3. Day 10 cytotoxicity assays. A, CFSE-CTAC-based cytotoxicity assay using live, non-K562 cell counts as the effector numbers (E). CTAC targets (T) were first gated on CFSE (FITC channel) by size (FSC) then placed into these CFSE (PE channel) by live-dead (APC Cy7 channel) plots. Percent dead in the upper right quadrants at each titration is compared with the percent of dead targets without cultured cells added (negative). Represented are H941 data with cytotoxicity equal with the negative control at a dilution of 1:10 E:T. B, Fold-killing vs negative at each titration (means for duplicate or medians for triplicate) for all cultures. H941 (black symbols) and H981 received highly cytotoxic preparations; H961 was not tested at 1:1 because of low cell culture numbers. CFSE, 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester; CTAC, canine thymic adenocarcinoma cell.

after ex vivo prep infusion (d 2 post-TBI) (Figure 1 and **Table S1, SDC,** http://links.lww.com/TXD/A295).

Cell Phenotypes Following Expansion

Ten days before transplant, donor dogs underwent leukapheresis for CD94 selection and expansion of NK and NKT cells as described.[14] NK cell content of PBMC was a median of 12 (range 0.87–19.25) million cells per apheresis collection. Following cell sorting, we obtained a median of 63% (range 7–96) CD94-positive cells, with a median yield of 13 (range 3–27) million cells. The 10-d in vitro culture period yielded a median of 2.3 (range 0.3–2.4)-fold expansion of

total collected cells (**Figure S2, SDC,** http://links.lww.com/TXD/A295). Final cell counts after expansion were a median of 12 (range 7–29) million per preparation.

Anti-CD3CD94 mAb immunophenotyping of day 10 cultures showed they were now predominantly CD94 positive (median 94.3%; range 82.6%–97.8%). A median of 0.82 (range 0.54–2.1) million total ex vivo-expanded cells per kg comprised of T-cells, NK, and NKT cells, were infused. T cells were the least abundant lineage and NKT cells were the most prominent (Table 1). NK cells were infused at a median of 140 000 (range 78 000–317 000) cells/kg. At the time of infusion, the CD94 selected and expanded cells were tested for cytotoxicity against CTAC target cells. Three cell preparations failed to kill CTAC target cells above the spontaneous lysis level at a ratio of 1 live non-K562 cell (E) to 1 CTAC target (T), whereas 2 preparations killed at ratios of 1:10 E:T (H941) or greater (H981) (Figure 3).

DISCUSSION

Current results failed to support our assumption that donor-derived NK cells could be substituted for donor T-cells for enabling DLA-haploidentical marrow engraftment. Four of the 5 experimental dogs rejected their marrow grafts, a finding that was similar to earlier observations showing that infusion of marrow alone was insufficient for sustained engraftment in this model.[2] The almost uniform graft rejection seen with the current NK-cell infusions contrasted with results reported earlier in dogs given 700 cGy TBI followed by marrow and PBMC grafts from DLA-identical littermates.[1] These dogs did not receive postgrafting immunosuppression, uniformly developed sustained engraftment, and succumbed to GVHD.[1] Here we infused ex vivo-expanded NK cells at a median of 140 000 (range 78 000–317 000) cells/kg and followed recipients for a median of 13 d (range 11–15). The study's readout was marrow engraftment. The 1 successfully engrafting dog, H941, was injected with greatest number of ex vivo-expanded donor NK cells/kg that had the second-highest degree of in vitro cytolytic activity. The dog developed multilineage engraftment but also succumbed to acute GVHD. Of note is that H941's ex vivo-expanded cell infusion had the least number (46 000 cells/kg) and lowest percentage (2.2%) of T cells, and yet the dog still developed GVHD upon engraftment. Dog H981 developed mixed donor-host lymphocyte chimerism that was most likely temporary since this dog had no evidence of sustained donor myeloid chimerism. Dog H981 experienced an early rise of peripheral blood donor lymphocytes within the first 8 d after transplant. That dog received cultured CD94 positive cells with the highest NK cytolytic activity. We saw no evidence that NKT cells enhanced engraftment as both H941 and H982 received large doses of NKT of 1.7 and 1.9 million cells/kg, respectively, and total NK plus NKT doses were almost equal at 2.1 and 2.0 million cells/kg. Whether further increasing the doses of NK cells while further decreasing contaminating T cells through CD3-depletion after culture results in more consistent engraftment without GVHD remains conjectural.

In conclusion, we showed that CD94+-selected and ex vivo-expanded cells can be safely infused into recipients without toxicity. However, cultured CD94+ NK/NKT cells were a poor substitute for PBMC and failed to enable marrow engraftment without GVHD in this DLA– haploidentical canine marrow transplant model.

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