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Ex vivo expansion of canine cytotoxic large granular lymphocytes exhibiting characteristics of natural killer cells

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Abstract

Canine NK cells still are not well-characterized due to the lack of information concerning specific NK cell markers and the fact that NK cells are not an abundant cell population. In this study, we selectively expanded the canine cytotoxic large granular lymphocytes (CLGLs) that exhibit morphologic, genetic, and functional characteristics of NK cells from normal donor PBMCs. The cultured CLGLs were characterized by a high proportion of CD5(dim) expressing cells, of which the majority of cells co-expressed CD3 and CD8, but did not express TCR α and TCR β . The phenotype of the majority of the CLGLs was CD5(dim)CD3⁺CD8⁺TCR α ⁻TCR β ⁻CD4⁻CD21⁻CD11c⁺/CD11d⁺/CD44⁺. The expression of mRNAs for NK cell-associated receptors (NKG2D, NKp30, NKp44, Ly49, perforin, and granzyme B) were highly upregulated in cultured CLGLs. Specifically, NKp46 was remarkably upregulated in the cultured CLGLs compared to PBMCs. The mRNAs for the NKT-associated iTCR α gene in CLGLs was present at a basal level. The cytotoxic activity of the CLGLs against canine NK cell-sensitive CTAC cells was remarkably elevated in a dose-dependent manner, and the CLGLs produced large amounts of IFN- γ . The antitumor activity of CLGLs extended to different types of canine tumor cells (CF41.Mg and K9TCC-pu-AXC) without specific antigen recognition. These results are consistent with prior reports, and strongly suggest that the selectively expanded CLGLs represent a population of canine NK cells. The results of this study will contribute to future research on canine NK cells as well as NK cell-based immunotherapy.

Keywords

Canine; Cytotoxic large granular lymphocytes; NK cells; Expansion; Cytotoxicity

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Conflicts of interest

The authors declare no financial or commercial conflict of interest exists in relation to the content of this article.

1. Introduction

Natural killer (NK) cells are key components of the innate immune system given that they target and kill cancerous and virally-infected cells (Paust et al., 2010; Terme et al., 2008; Vivier et al., 2008, 2011). Numerous reports on the biology and the function of NK cells over the past decade have reestablished their role in immune defense mechanisms, and NK cells have reemerged as a potentially powerful tool in immunotherapy due to their robust effector functions (Lunemann et al., 2009; Paust et al., 2010; Terme et al., 2008; Vivier et al., 2008, 2011). NK cell-based adoptive immunotherapy is a promising therapeutic approach for many cancers (Ljunggren and Malmberg, 2007). Moreover, adoptive transfer of allogeneic NK cells to cancer patients in the context of haploidentical stem cell transplantation, or following lymphocyte depleting chemotherapy have provided encouraging results without the side effect of graft versus host disease (GVHD) (Iliopoulou et al., 2010; Miller et al., 2005; Ruggeri et al., 2002). However, the development of this NK cell-based immunotherapy has been hampered by the low numbers of NK cells that can be derived from donor blood. Thus, developing *ex vivo* selective expansion and activation systems of highly cytotoxic NK cells is desirable for effective immunotherapy (Carlens et al., 2001; Fujisaki et al., 2009; Somanchi et al., 2011; Spanholtz et al., 2011).

Like humans, dogs develop common types of cancer (Paoloni and Khanna, 2008; Vail and MacEwen, 2000), viral diseases (Dundon et al., 2010), and autoimmune diseases (Halliwell, 1978). These naturally occurring diseases share many features with their human counterparts, including pathophysiological and biological behaviors as well as responses to conventional therapies (Paoloni and Khanna, 2008). Studying dogs exhibiting these diseases will provide a valuable perspective distinct from that provided by studying humans and rodents alone, and new treatments for diseases in dogs could be translated to humans (Paoloni and Khanna, 2008). Recently, several immunotherapeutic techniques for dogs, such as cellular immunotherapy, have been attempted to treat the diseases mentioned above (Gyorffy et al., 2005; Hagglund et al., 2000; Itoh et al., 2003; Mason et al., 2008). Although several studies have reported NK cell activity in both normal healthy dogs (Gondolf et al., 1996; Knapp et al., 1993; Schmitz et al., 2003) and those with advanced tumor disease (Funk et al., 2003; Funk et al., 2005; Raskin et al., 1989), the existence of vastly fewer NK cells in canine peripheral blood mononuclear cells (PBMCs) compared to human PBMCs has complicated the development of NK cell-based immunotherapy for these diseases (Bonkobara et al., 2007; Bonkobara et al., 2005). In addition, the lack of information regarding NK cell-restricted specific markers and NK cell receptors in dogs has limited potential canine research on immunotherapy (Huang et al., 2008; Lin et al., 2010; Loughran et al., 1985).

Canine NK cells remain poorly characterized (Bonkobara et al., 2007; Huang et al., 2008). Early reports describing the phenotypic features of the canine NK cells consistently reported that they express T cell markers (Huang et al., 2008; Lin et al.; Loughran et al., 1985; Ringler and Krakowka, 1985). To date, no NK cell-restricted markers have been described for dogs. Thus, canine NK cells can only be defined as large granular lymphocytes (LGLs) with natural cytotoxic functions in the absence of specific immunization. This represents a morphologically unique population of lymphocytes characterized by intracytoplasmic azurophilic granules (Bonkobara et al., 2007; Funk et al., 2005; Huang et al., 2008; Knapp et al., 1995; Lin et al., 2010). Here, we have successfully expanded the canine cytotoxic large granular lymphocytes (CLGLs) showing characteristics of NK cells which were consistent with prior reports. The results of this study strongly suggest that the selectively expanded canine CLGLs represent a population of NK cells. This is the first report of the successful specific culturing of canine NK cells *ex vivo*, as well as the characterization of cultured

canine NK cells. The results of this study will contribute to future examinations of canine NK cells, as well as to the development of NK cell-based immunotherapies against cancer and viral infections.

2. Materials and methods

2.1. Tumor cell lines

Canine thyroid adenocarcinoma (CTAC) cells (i.e., canine NK cell sensitive cells) were obtained from the European Collection of Cell Cultures (Porton Down, U.K.). CF41.Mg (canine mammary gland tumor cell line) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Dr. Knapp (Purdue University, West Lafayette, IN) kindly provided the canine transitional cell carcinoma cells (K9TCC-pu-AXC). The K562 cell line genetically modified to express membrane-bound IL-15 and the 41BB ligand (K562-mb15–41BBL) was kindly provided by Dr. Campana (St. Jude Children's Research Hospital, Memphis, TN). All cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics.

2.2. Animals and blood collection

Peripheral blood was obtained from 18 normal healthy dogs (6 months to 8 years old) including standard poodles, miniature schnauzers, English cocker spaniels, and golden retrievers. These dogs were undergoing regular health checkups and blood drawing for hematological investigations. All dogs had previously received routine vaccinations against distemper, leptospirosis, parvovirus infection, and hepatitis, and had been dewormed regularly. Blood collection did not exceed 15–20 ml/kg of body weight and the samples were drawn from the jugular vein of each dog by venipuncture into 10 ml of sodium-heparin tubes (BD Vacutainer Systems, Franklin Lakes, NJ) without anesthesia. The use of animals was in accordance with guidelines of the Institutional Animal Care and Use Committee of Kongju National University.

2.3. Expansion of cytotoxic large granular lymphocytes

PBMCs were isolated by discontinuous density gradient centrifugation with Hypaque–Ficoll gradients (Histopaque 1.119, Sigma, St. Louis, MO; Lymphoprep™ 1.077, Axis-Shield PoC AS, Helsingfors, Norway). Heparinized whole blood was diluted 1:2 with phosphate-buffered saline (PBS), carefully layered onto the discontinuous density gradients, and centrifuged at $400 \times g$ for 25 min. PBMCs were then collected and washed twice with PBS. Canine PBMCs (1×10^6) were incubated in a 24-well tissue culture plate with lethally irradiated (125 Gy) K562-mb15–41BBL cells (0.5×10^6) in the presence of 100 IU/ml human IL-2 (PeproTec, Rocky Hill, NJ) and 10 IU/ml human IL-15 (PeproTec) in RPMI-1640 and 10% FBS for 14 days. Fresh medium with IL-2 and IL-15 was provided every other day.

2.4. Morphologic evaluation, rate of cell division, and purity of CLGLs

PBMCs and CLGLs were cultured for 14 days, then isolated from culture and were stained with trypan blue to assess viability, and diluted to appropriate concentrations after counting in a hemocytometer. To evaluate the purity of the CLGLs after 14 days, 5.0×10^4 cells were centrifuged onto microscope slides using a cyctospin centrifuge. Both samples were air-dried and stained with May–Grünwald Giemsa; then 600 cells were counted to obtain differentials. To determine the rate of cell division, total cell number was multiplied by the purity of the CLGLs before (day 0) and after culture (day 14). Canine CLGLs were identified morphologically as lymphocytes containing numerous large cytoplasmic granules.

2.5. Flow cytometric analysis

For double or triple staining of the cell surface molecules, the cells were processed for incubation with fluorescein isothiocyanate (FITC)-conjugated mouse anti-canine monoclonal antibody (mAb), R-phycoerythrin (RPE)-conjugated mouse anti-canine mAbs, and allophycocyanine (APC)-conjugated mouse anti-canine mAb for 20 min on ice. The samples were washed three times with PBS and then analyzed using flow cytometry. Approximately 10,000 cells were analyzed per sample. Fluorescence-activated cell sorting (FACS) analysis was performed using FITC-labeled mAb against CD3, and RPE-labeled mAbs against CD4, CD8, CD21, and APC-labeled mAb against CD5. For CD11b, CD11c, TCR α , TCR β , and CD44, for which directly labeled primary antibodies were not available, 50 μ l of secondary antibody (RPE-conjugated goat anti-mouse IgG against CD11b, CD11c, TCR α , and TCR β , FITC-conjugated goat anti-rat IgG2a against CD44) was added (all from Serotec, Oxford, U.K. except TCR α and TCR β which were from Leukocyte Antigen Biology Laboratory, University of California, CA). Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward- and side-scatter gating. Flow cytometry analyses were performed using the FACSaria flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from both freshly isolated PBMCs (day 0) and cultured CLGLs (day 14) from the same donors using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen) and an oligo-dT primer (Invitrogen) according to the manufacturer's instructions. In brief, 400 ng of total RNAs and 1 μ l of 50 μ M oligo-dT were used to synthesize the first strand of cDNA in a total volume of 20 μ l. The DNA fragments of the beta-actin, iTCR α chain, and NK-associated genes, such as CD16, CD56, NKG2D, NKp30, NKp44, NKp46, Ly49, perforin, and granzyme B, were amplified from 1 μ l of the cDNA reaction with 10 pmole of forward and reverse primers in a final volume of 20 μ l. The thermal cycling conditions of the PCR were as follows: 94 °C for 5 min followed by 30 cycles at 94°C for 30 s, 53 °C for 30 s, and 72°C for 30 s. RT-PCR was performed as described above to detect tissue-specific expression of the CD56 gene. Briefly, brain and liver tissues were dissected from a normal female dog and snap-frozen with liquid nitrogen; then, total RNAs were extracted separately using TRIzol reagent. To examine the differential expression of the canine CD56 gene, 1 μ g of total RNA was reverse-transcribed into cDNA and amplified by 40 cycles of PCR. The primer pairs used are listed in Table 1. All amplified DNA fragments were resolved on a 1.5% agarose gel. RT-PCR products were sequenced using an ABI 3730 (Hitachi, Tokyo, Japan) to verify the specificity of the primer pairs.

2.7. Real-time RT-PCR

Real-time RT-PCR was performed using a QuantiTect SYBR Green PCR Kit and a Rotor-Gene Q (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The cDNAs for NK-related genes were synthesized under the same experimental condition of RT-PCR described above, and 1 μ l of cDNA content was used as a template for real-time PCR in a final volume of 25 μ l. The thermal cycling conditions were 95 °C for 15 min followed by 45 cycles of 94°C for 30 s, 53 °C for 30 s, and 72 °C for 30 s. Each run included no template control (NCR) to test and all samples were tested in duplicate. The specific amplification was verified by a single dissociation curve from the melting temperature analysis. The relative amount of mRNA of target genes was calculated on the basis of its threshold cycle (Ct) compared to the Ct of β -actin. The results are presented as $2^{-(Ct \text{ of target} - Ct \text{ of } \beta\text{-actin})}$ in arbitrary units.

2.8. ELISA for interferon- γ

IFN- γ release was quantified in culture supernatants using an ELISA (BD OptEIA™ Set Canine Kit, BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

2.9. Generation of canine dendritic cells (DCs) and induction of CTAC-specific cytotoxic T lymphocytes (CTLs)

To generate canine DCs, isolated PBMCs were resuspended in RPMI 1640 and incubated in T25 flasks (1.5×10^7 cells/flask) for 1 h at 37°C in 5% CO₂. Non-adherent lymphocytes were removed by repeated washes with PBS. Adherent PBMCs were then cultured with 5 ml culture medium containing 25 ng/ml Flt3L (R&D Systems, Minneapolis, MN), 500 U/ml IL-4 (R&D Systems), and 800 U/ml GM-CSF (PeproTec). Half of the culture medium was replaced with fresh medium containing cytokines every 2–3 days. On day 7, the immature DCs were matured by addition of 10 μ g/ml LPS (Sigma–Aldrich, St. Louis, MO). Then, the maturing DCs were pulsed with the UVB-irradiated CTAC cells at a ratio of 2:1, 2 h after the addition of LPS. CTAC cells were irradiated with UVB (120 mJ/cm²) followed by overnight culture in RPMI1640 without FBS. The irradiated apoptotic cells were tested using annexin-V and propidium (PI) before DC pulsing. DCs pulsed with apoptotic CTAC cells were harvested on day 9. Nonadherent lymphocytes ($1\text{--}1.5 \times 10^6$ cells) were sensitized by autologous DCs (1×10^5 cells) primed with apoptotic CTAC cells. On day 3, rhIL-2 (100 U/ml, R&D Systems, Minneapolis, MN) and rhIL-7 (10 ng/ml, R&D Systems) were added. Half of the culture medium was replaced with fresh medium containing cytokines every 2–3 days. The CTLs were harvested on day 14.

2.10. ⁵¹Cr release assay

Cultured CLGLs were analyzed in a standard 4-h [⁵¹Cr] release assay. The cell lines CTAC, CF41.Mg, and K9TCC-pu-AXC were used as target cells. The target tumor cells were labeled with 200 μ Ci of [⁵¹Cr](Na⁵¹CrO₂) per 10^6 cells for 1 h at 37°C in 5% CO₂. Thereafter, the cells were washed three times with RPMI-1640 containing 10% FBS, and 1×10^4 labeled cells per well were plated in 96-round-bottomed plates with effector cells at effector to target ratios of 20:1, 10:1, 5:1, 2.5:1, and 1.25:1 in 200 μ l of RPMI-1640 containing 10% FBS. Supernatants were collected, and the released [⁵¹Cr] was measured in an automatic gamma counter (WizarD3, PerkinElmer, Waltham, MA). Spontaneous release was determined by incubation of target cells in medium alone, and the maximum release was determined by suspending the cells with 0.5% Triton X-100. The percent cytotoxicity was calculated as follows: specific lysis (%) = (experimental [⁵¹Cr] release – spontaneous [⁵¹Cr] release)/[maximum [⁵¹Cr] release – spontaneous [⁵¹Cr] release] $\times$ 100. In this assay, we used cultured CLGLs that showed more than 70% purity after 14 days of stimulation from seven healthy donors, and the cytotoxic activity of CLGLs was tested without depletion of residual T lymphocytes.

2.11. Statistical analysis

The statistical significance of the differences observed in the expression of the surface antigens, level of gene expression, cytokine production, and the cytotoxicity of cultured CLGLs was determined using the Mann–Whitney *U* test for nonparametric comparisons. The minimal level of significance was $p < 0.05$.

3. Results

3.1. Expansion and morphology of canine CLGLs

The morphology of the cultured CLGLs was investigated under light microscopy. Canine CLGLs had abundant pale-staining cytoplasm with variable numbers of azurophilic cytoplasmic granules, which were generally prominent (Figs. 1B and 5C). A gradual increase in canine CLGLs was observed in the media from 5 to 7 days after the stimulation of the PBMCs with K562-41BB-IL15, and CLGLs dominated in the cultures by 14 days after stimulation (Fig. 1). To evaluate the rate of cell division and purity of CLGLs after 14 days of culture, 600 cells from each stained slide were counted to obtain differentials. The population of CLGLs increased up to 85%, and CLGLs were expanded by approximately 200-fold in the presence of IL-2 and IL-15 after 14 days of culture (Fig. 1D).

3.2. Phenotype of cultured canine CLGLs

The immunophenotypic characteristics of cultured CLGLs (day 14) were investigated by multi-parameter flow cytometric analysis and compared with the phenotypes of peripheral blood lymphocytes (PBLs, day 0) (Fig. 2 and Table 2). The cultured CLGLs were characterized by a high proportion of CD5(dim) expressing cells (72.0–85.1%), of which the majority of cells co-expressed CD3 (88.7–92.7%) (Fig. 2A), but not TCR (2.8–9.5%) or TCR78 (3.1–19.4%) (Fig. 2B). In contrast, CD5(dim) cells comprised just 4.0–19.2% of the total PBLs. (Fig. 2A). The majority of CD5(dim)CD3⁺ CLGLs also co-expressed CD8 (90.7–95.9%) (Fig. 2C). The population of CD5(bright) cells was significantly decreased in CLGLs (7.7–17.9%) compared to that of fresh PBLs (66.9–92.0%) ($p < 0.01$) at day 14 of culture (Fig. 2A). The proportion of CD4⁺ cells (55.1–67.0% vs. 2.1–6.2%) and CD21⁺ cells (5.3–37.1% vs. 0.9–3.2%) was also markedly decreased in the CLGLs compared to the PBLs ($p < 0.01$). Although large variations were recognized between donors, the expression levels of CD11c (30.6–88.6%) and CD11d (33.7–95.8%) were highly upregulated in the CLGLs compared to the resting PBLs (16.2–62.2% and 9.4–64.1%, respectively) in all donors ($p < 0.05$); however, the expression of CD44 on the CLGLs did not change (98.0–98.9% vs. 94.7–99.9%) (Fig. 2C and Table 2). Thus, the phenotype of the majority of the population of CLGLs was CD5(dim) CD3⁺ CD8⁺ TCR[−] TCR78[−] CD4[−] CD21[−] CD11c^{+/−} CD11d^{+/−}. The percentages of each surface molecule expressed on the CLGLs and PBLs are shown in Table 2.

3.3. Expression of mRNAs for NK cell-associated molecules in cultured CLGLs

To examine changes in the levels of mRNAs for NK cell-related molecules that have been identified in other mammals, such as CD16, CD56, NKG2D, NKp30, NKp44, NKp46, Ly49, perforin, and granzyme B, RT-PCR and real-time RT-PCR were performed. As the results from RT-PCR (Fig. 3A and B) and quantitative real-time RT-PCR (Fig. 3C) show in Fig. 3, the mRNA expression of NK-associated genes except for CD16 and CD56 was significantly elevated in CLGLs cultured for 14 days compared to uncultured PBMCs in all donors ($p < 0.05$). The mRNA levels of NKG2D (10.8-fold), NKp30 (13.9-fold), NKp44 (25.4-fold), NKp46 (31.6-fold), Ly49 (25.5-fold), perforin (15.2-fold), and granzyme B (17.1-fold) were greatly elevated in cultured CLGLs compared to freshly isolated PBMCs (Fig. 3C). The expression of mRNA for CD16 was not changed, and little or no expression of the CD56 gene was detected in either freshly isolated PBMCs or cultured CLGLs. We further confirmed that primer pairs precisely amplified the corresponding genes from the nucleotide sequence analysis of amplified DNA fragments (data not shown).

Several tissues, including nervous system components, highly express the CD56 gene, whereas it is undetectable in liver tissues (Bonkobara et al., 2005). Next, we investigated the mRNA expression of the CD56 gene in the brain and liver tissues of a dog to verify the

specificity of our primer pairs for CD56. As expected, the mRNA of CD56 was amplified from brain tissue but not from liver (Fig. 3B). An identical result was obtained using the CD56 primer pairs reported by Huang et al. (2008). We then examined the nucleotide sequences of the CD56 RT-PCR products by sequence analysis and pairwise alignment, and identified that the CD56 fragments were the predicted size and identical to the original CD56 sequence (data not shown). No CD56 mRNA was detected in the freshly isolated PBMCs or cultured CLGLs after 40 cycles of RT-PCR using the reverse-transcribed template from 1 µg of total RNA (data not shown).

3.4. Expression of mRNAs for the NKT-associated iTCR α chain in cultured CLGLs

Given that the majority of the population of CLGLs exhibited the CD5(dim) CD3⁺ CD8⁺ phenotype, determining whether the cultured CLGLs were related to the population of NKT cells was necessary. We determined the mRNA expression of the iTCR gene, a known NKT cell-associated gene, in the PBMCs and cultured CLGLs to indirectly estimate the proportion of the NKT cell population contained in CLGLs. Given that the CD1d gene has not yet been identified in dogs, RT-PCR and real-time RT-PCR were performed using the iV3 and C2 primer pair specific for iTCR (Yasuda et al., 2009). A very low level of iTCR fragments was detected at the predicted size in all donors. Although the expression level of the iTCR gene was very slightly elevated in CLGLs compared to PBMCs, no statistical significance was observed (Fig. 4).

3.5. Production of IFN- γ by CLGLs in response to CTAC

Given that upregulated IFN- γ production is an important characteristic of activated NK cells, we measured the IFN- γ levels in the culture supernatants from the PBMCs or CLGLs after 24 h-stimulation with CTAC. The CTAC cells (5×10^4) were co-cultured with freshly isolated PBMCs (5×10^5) or expanded CLGLs (5×10^5) for 24 h in the absence of cytokine. The concentration of IFN- γ was significantly higher in supernatants from CLGLs cultured with CTAC than from PBMCs stimulated with CTAC ($p < 0.05$) (Fig. 5A). The concentration of IFN- γ from CLGLs and PBMCs after stimulation with CTAC was 2374.6 ± 126.2 pg/ml and 166.5 ± 7.5 pg/ml, respectively. IFN- γ production of PBMCs, CLGLs, and CTAC without stimulation was 34 ± 11.5 pg/ml, 82.1 ± 9.5 pg/ml, and 24 ± 3.4 pg/ml, respectively.

3.6. Cytotoxicity of cultured CLGLs against canine tumor cells

To assess the natural cytotoxicity of cultured canine CLGLs, the cytolytic activity of CLGLs against canine NK-sensitive CTAC cells was analyzed in a standard 4-h [⁵¹Cr] release assay. In this assay, we used cultured CLGLs that showed more than 70% purity after 14 days of stimulation. The cytotoxic activity of CLGLs from seven healthy donors was tested without depletion of residual T lymphocytes. As shown in Fig. 5B, the cytotoxic activity of the cultured CLGLs against CTAC was significant and increased in a dose-dependent manner in all donors ($p < 0.01$). The median cytotoxicity against CTAC continued to exceed 83% at a 20:1 effector to target ratio. Microscope slides stained 1 h after coculture revealed the binding of CLGLs to CTAC target cells and subsequent killing of the CTAC cells (Fig. 5C). Next, we determined whether the antitumor activity of CLGLs extended to the different types of canine tumor cells without specific antigen recognition. The cytotoxicity of CLGLs from four donors was tested against the canine mammary gland tumor cell line CF41.Mg and the canine transitional cell carcinoma cell line K9TCC-pu-AXC. As shown in Fig. 6, CLGLs were consistently cytotoxic against CF41.Mg and K9TCC-pu-AXC in a dose-dependent manner ($p < 0.01$) in all donors. The cytotoxicity of CLGLs after 4 h of coculture at a 20:1 effector to target ratio was $73.6 \pm 2.3\%$ against CF41.Mg, and $66.6 \pm 2.0\%$ against K9TCC-pu-AXC. The cytotoxicity of PBMCs under the same conditions was $2.0 \pm 0.5\%$ against CF41.Mg and $20.1 \pm 3.3\%$ against K9TCC-pu-AXC. We then compare the cytotoxic activity of CLGLs with that of antigen-specific CTLs induced by autologous DCs loaded

with apoptotic CTAC cells against NK cell-sensitive CTAC in order to verify whether cultured CLGLs were associated with CTLs. The results showed that the cytotoxicity of CTLs against CTAC that lack MHC antigen expression was minimal (Fig. 7).

4. Discussion

We successfully cultured canine CLGLs that exhibited morphologic, genetic, and functional characteristics of NK cells from the PBMCs of normal healthy dogs *ex vivo*. The cytotoxic activity of the cultured CLGLs against canine NK cell-sensitive CTAC cells increased remarkably in a dose-dependent manner, and was potently extended to different types of canine tumor cells without specific antigen recognition (Figs. 5 and 6). However, to avoid speculation, we will refer to the cultured cells not as NK cells, but as CLGLs according to their morphologic and functional features until specific NK cell markers for dogs as well as characteristics of canine NK cells are thoroughly identified. This is because no phenotypic characteristics and membrane determinants specific for canine NK cells have yet been described (Bonkobara et al., 2007; Huang et al., 2008; Lin et al., 2010) and because the majority of the cultured CLGLs in the present study were CD5(dim) CD3⁺ CD8⁺ cells, but these cells did not express T cell receptors (TCR α and TCR β) on their surface (Fig. 2).

Although there have been a few reports demonstrating the scientific characteristics of canine NK cells, early reports describing the phenotypic feature of the canine NK cells produced consistent results (Huang et al., 2008; Lin et al., 2010; Loughran et al., 1985; Ringler and Krakowka, 1985), evidence now suggests that canine NK cells share phenotypic characteristics of canine T cells. For example, Ringler and Krakowka (1985) reported that canine NK cells expressed Thy-1, the canine T lymphocyte marker. In addition, canine NK cells were reported to express CD8, suggesting a T cell lineage (Loughran et al., 1985), and a subset of canine CD3⁺CD8⁺ cells from IL-2 stimulated LAK cells contained substantially higher levels of mRNAs for NK cell-related receptors and displayed significantly higher NK cytotoxic activity than the corresponding CD8⁻ cells (Lin et al., 2010). Furthermore, Huang et al. (2008) showed that CD5-low expression lymphocytes in canine peripheral blood had characteristic of NK cells and a high proportion of the CD5-low cell population expressed CD3 and CD8, but the expression of CD4 and CD21 was very low. They reported that CD5-low expression lymphocytes had significantly high NK cytotoxicity but showed very low antigen-specific cytotoxic T cell activity (Huang et al., 2008). In accordance with these findings, the cultured CLGLs described here were characterized by a high proportion of CD5(dim) expressing cells (72.0–85.1%), and the majority of cells co-expressed CD3 (88.7% ~92.7%) and CD8 (90.7–95.9%). The proportions of CD4⁺ (55.1–67.0% vs. 2.1–6.2%), TCR⁺ (83.4–85.4% vs. 2.8–9.5%), and CD21⁺ cells (5.3–37.1% vs. 0.9–3.2%) were also remarkably reduced in CLGLs compared to PBLs ($p < 0.01$). Thus, the majority of the population of CLGLs exhibited the CD5(dim) CD3⁺ CD8⁺ TCR⁻ TCR⁻ CD4⁻ CD21⁻ CD11c^{+/-} CD11d^{+/-} CD44⁺ phenotype (Fig. 2, Table 2).

The effector function of NK cells is regulated by a balance between opposite signals delivered by the MHC class I-specific or nonspecific inhibitory receptors and by the activating receptors responsible for NK cell triggering (Moretta et al., 2001; Terme et al., 2008; Vivier et al., 2008). The inhibitory and activating receptors regulating NK effector function, such as the killer cell Ig-like receptors (KIR), Ly49, NKG2D, CD16, perforin, granzyme B, and the natural cytotoxicity receptors (NKp30, NKp44, NKp46), have been identified in several mammals including humans and rodents (Barten et al., 2001; Gagnier et al., 2003; Ma et al., 2004; Vivier et al., 2008). Some of these receptors are not restricted to NK cells, but natural cytotoxicity receptors (NKp30, NKp44, NKp46) have been identified as NK-specific triggering surface molecules (Moretta et al., 2001). Previous data also suggest that a direct correlation exists between the surface density of natural cytotoxicity

receptors (NCRs) and the ability of NK cells to kill various tumors and viral infected cells (Moretta et al., 2001). Thus, we determined the changes in expression levels of mRNAs for NK cell-related molecules in cultured CLGLs to identify their cellular characteristics. The expression of mRNAs for NK cell-associated receptors, such as NKG2D (10.8-fold), NKp30 (13.9-fold), perforin (15.2-fold), and granzyme B (17.1-fold) were highly upregulated in activated CLGLs compared to PBMCs, and the NKp44 (25.4-fold), the receptor restricted to activated NK cells, as well as Ly49 (25.5-fold) mRNA, were highly induced in cultured CLGLs (Fig. 3C). The expression of mRNAs for NKp46, a receptor selectively expressed by all NK cells in humans and mice, was notably upregulated (31.6-fold) in the CLGLs compared to PBMCs (Fig. 3C). Taken together, these findings suggest that the cultured CLGLs represent a population of highly activated canine NK cells.

Although only one Ly49 gene from the dogs has been isolated, the function of the canine Ly49 gene has not yet been studied and the possibility exists that another Ly49 gene occurs in dogs (Gagnier et al., 2003). Therefore, given that Ly49 mRNA was highly induced in the fully activated CLGLs, further studies are required to characterize the function of Ly49 and to confirm the precise copy number of the Ly49 gene in dogs.

No expression of the CD56 gene was detected in either freshly isolated PBMCs or the cultured CLGLs. CD56 has been identified as a NK cell-specific marker in humans and higher primates. Bonkobara et al. (2005) cloned and analyzed the tissue-specific expression of the canine CD56 gene and reported that CD56 was not detected in canine PBMCs and other tissues including liver and bone marrow, which should exhibit NK cells, although CD56 expression was upregulated in peripheral leukocytes by treatment with phytohemagglutinin (Bombokara et al., 2005). However, other recent reports suggest that no direct evidence exists for an association between CD56 and NK cells in dogs (Huang et al., 2008; Lin et al., 2010). These data suggest that canine NK cells do not express surface CD56.

Unlike conventional T cells with polymorphic TCRs, NKT cells express a single iTCR chain. Yasuda et al. (2009) identified canine NKT cells resembling a CD3⁺ T-lymphocyte population reactive to α -galactosylceramide-loaded mouse CD1d and that preferentially expressed iTCR γ mRNA in the peripheral blood of a dog. They, however, suspected that the mouse CD1d used for the flow cytometric analysis in their study could be less bound to canine iTCR γ chain (Yasuda et al., 2009). As a result of the lack of the suitable antibodies for CD1d determination in dogs, we performed RT-PCR and real-time RT-PCR to determine the expression levels of the iTCR γ gene in the cultured CLGLs using the iV3 and C2 primer pair that is specific to the canine iTCR γ chain. Very low amounts of the iTCR γ fragments were detected at the predicted size in all donors. The expression level of the iTCR γ gene was slightly elevated in CLGLs compared to PBMCs, but no statistical significance was observed (Fig. 4). This suggests that the majority of the population of cultured CLGLs did not express the iTCR γ chain, and that a small fraction of NKT cells was contained in the cultured cells. Human NKT cells were also reported to multiply, when PBMCs were cultured to achieve large-scale multiplication of human NK cells, although no significant enrichment was observed. The cytotoxic activity against tumor cells was reportedly mediated by NK cells and not NKT cells (Voskens et al., 2010).

A recent report suggests that the canine genome is highly similar to that of humans (Paoloni and Khanna, 2008). Additionally, dogs and humans develop common types of spontaneous cancers (Paoloni and Khanna, 2008; Vail and MacEwen, 2000), viral diseases (Dundon et al., 2010), and autoimmune disease (Halliwell, 1978). The current rate of cancer is well recognized to be much higher in dogs than in humans, and naturally occurring cancers in pet dogs and humans share many features, including histological appearance, tumor genetics,

molecular targets, biological behaviors, and response to conventional therapies (Paoloni and Khanna, 2008). Because new treatments for diseases from dogs can be effectively translated to humans, the value of studying diseases in dogs has been increasingly recognized, particularly in terms of the development and evaluation of novel therapeutics for diseases such as cancer and viral infections (Paoloni and Khanna, 2008). Therefore, the dog may be a useful animal model for preclinical studies for the development of new treatment modalities for human diseases.

In conclusion, we successfully established a suitable *ex vivo* culture system for the selective multiplication and activation of canine NK cells, which showed high cytotoxic activity against canine tumor cells. This is the first report of the successful specific culturing of canine NK cells *ex vivo*, as well as the characterization of cultured canine NK cells. The results of this research will contribute to future studies of canine NK cells as well as NK cell-based immunotherapy in dogs, which will provide a valuable perspective distinct from that generated by studying humans and rodents alone (Paoloni and Khanna, 2008).

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Fig. 1.

Light microscopic images of freshly isolated PBMCs (A), cultured canine CLGLs (B), and the morphology of cultured CLGLs in media (C). A gradual increase in canine CLGLs was observed in media with the stimulation of the PBMCs with K562-41BB-IL15, and CLGLs dominated the cultures by day 14 after stimulation. Canine CLGLs exhibited an abundant pale-staining cytoplasm with variable number of azurophilic cytoplasmic granules, which were generally prominent. (D) The fold expansion of CLGLs was determined before (day 0) and after (day 14) CLGLs expansion (n=10).

Fig. 2.

Phenotypic analysis of canine PBMCs (day 0), and cultured CLGLs (day 14). (A) The cultured CLGLs were characterized by a high proportion of CD5(dim) expressing cells, of which the majority co-expressed CD3. (B) The majority of cultured CD5(dim) CD3⁺ CLGLs did not express TCR $\alpha\beta$ and TCR $\gamma\delta$. (C) CD5(dim) cells also co-expressed CD8. The population of CD5(bright) cells and the proportions of CD4⁺ cells and CD21⁺ cells were remarkably lower in CLGLs compared to PBLs ($p < 0.01$). The expression levels of CD11c and CD11d were highly upregulated in the CLGLs ($p < 0.05$), whereas the expression of CD44 on CLGLs did not change. Thus, the phenotype of the majority of the population of CLGLs was CD5(dim) CD3⁺ CD8⁺ TCR $\alpha\beta$ ⁻ TCR $\gamma\delta$ ⁻ CD4⁻ CD21⁻ CD11c^{+/+} CD11d^{+/+}. The results shown represent those obtained from one of nine experiments.

Fig. 3.

Expression of mRNAs for NK cell-associated molecules in freshly isolated PBMCs (day 0), and cultured CLGLs (day 14). (A) The mRNA expression of canine β -actin, CD16, CD56, NKG2D, NKp30, NKp44, NKp46, Ly49, perforin, and the granzyme B gene in PBMCs and cultured CLGLs determined by RT-PCR. These results represent those of three of six experiments. (B) The mRNA expression of the CD56 gene in the brain and liver tissues of a normal dog analyzed by RT-PCR using primer pairs designed in this study (a) and reported by Huang et al. (Huang et al., 2008) (b). (C) The levels of mRNAs for NK cell-associated receptors and markers in PBMCs and CLGLs analyzed by real-time RT-PCR from six different donors. The mRNA levels are expressed relative to the level of β -actin mRNA and

represent the median, first (Q1) and third (Q3) quartile, minimum and maximum. *, $P < 0.05$. The mRNA levels of NKp46 (31.6-fold), Ly49 (25.5-fold), NKp44 (25.4-fold), granzyme B (17.1-fold), perforin (15.2-fold), NKp30 (13.9-fold) and NKG2D (10.8-fold), were elevated in cultured CLGLs compared to freshly isolated PBMCs. The expression of mRNA for CD16 was not changed, and little or no expression of the CD56 gene was detected in either freshly isolated PBMCs or cultured CLGLs (A, C). The mRNA expression of the CD56 gene in the brain and liver tissues of a dog was investigated to verify the specificity of primer pairs for CD56. As expected, the mRNA of CD56 was amplified from brain tissues but not from the liver by both primer pairs (B).

Fig. 4.

Expression of mRNAs for the NKT-associated iTCR- α chain was investigated in freshly isolated PBMCs (day 0) and cultured CLGLs (day 14) to determine whether the cultured CLGLs were related to the population of NKT cells because the phenotype of the majority of the population of CLGLs was CD5(dim) CD3⁺ CD8⁺. RT-PCR (A) and real-time RT-PCR (B) were performed using the iV3 and C2 primer pair specific for iTCR- α . A very low level of iTCR- α fragments was detected at the predicted size in all donors. Although the expression level of the iTCR- α gene was slightly elevated in CLGLs compared to PBMCs, no statistical significance was observed. The mRNA level of iTCR- α is expressed relative to the level of β -actin mRNA and represents the mean $\pm$ SD of six different donors. Error bars represent SD.

Fig. 5.

Production of IFN- γ by CLGLs (A) and the cytotoxic activities of cultured CLGLs against CTAC, which is known as a canine NK cell-sensitive cell line (B), and light microscopic findings of CLGLs bound to CTAC target cells (C). The CTAC cells (5×10^4) were co-cultured with freshly isolated PBMCs (5×10^5) or expanded CLGLs (5×10^5) for 24 h in the absence of cytokine. The IFN- γ production was assessed in the culture supernatants by ELISA. Control conditions consisted of supernatant from CTAC, PBMCs, or CLGLs alone cultured for 24 h without cytokine. The concentration of IFN- γ was significantly higher in supernatants from CLGLs cultured with CTAC than from other conditions ($p < 0.05$) (A). The natural cytotoxicity of cultured canine CLGLs was analyzed in a standard 4-h [51 Cr]

release assay. The cytotoxic activity of the cultured CLGLs against CTAC was markedly elevated in a dose-dependent manner in all donors ($p < 0.01$). The median cytotoxicity against CTAC continued to exceed 83% at a 20:1 effector to target ratio. These represent the results of seven experiments (B). The microscopic slides stained with May-Grünwald Giemsa stain 1 h after coculture revealed the binding of CLGLs to CTAC target cells and the subsequent killing of CTAC cells (C).

Fig. 6.

The cytotoxicity of cultured CLGLs against canine mammary gland tumor cells (CF41.Mg) and urinary bladder transitional cell carcinoma cells (K9TCC-pu-AXC) was investigated to determine whether the antitumor activity of CLGLs extended to different types of canine tumor cells without specific antigen recognition. The cytotoxicity of CLGLs from four donors was tested, and CLGLs were consistently cytotoxic against CF41.Mg and K9TCC-pu-AXC in a dose-dependent manner ($p < 0.01$) in all donors. The cytotoxicity after 4-h coculture at a 20:1 effector to target ratio was $73.6 \pm 2.3\%$ against CF41.Mg and $66.6 \pm 2.0\%$ against K9TCC-pu-AXC.

Fig. 7.

Antigen-specific CTL cytotoxicity against NK cell-sensitive CTAC was compared with that of CLGLs. CTAC-specific CTLs were induced by autologous DCs loaded with apoptotic CTAC cells. The cytotoxicity of CTLs against CTAC that lack MHC antigen expression was minimal, but the natural cytotoxic activity of the cultured CLGLs against CTAC was remarkably elevated in a dose-dependent manner in all donors. These represent the results of three independent experiments.

Table 1

Oligonucleotide primers used for RT-PCR and real-time RT-PCR of canine NK cell-associated genes and the iTCR gene.

Genes (accession number ^a)	Primer	Nucleotide sequence (5'–3')	Region ^b (nt)	Size (bp)	Source or reference
Beta-actin (Z70044)	cActin-F	ACCAACTGGGACGACATGGAGA	165–186	205	This work
	cActin-R	AGGCATACAGGGACAGGACAG	349–369		
CD56 (NM_001010950)	cCD56-F	ATTGTGTGTGATGTGTCAGCTC	457–479	208	This work
	cCD56-R	CCTGAAATGCTCTTGAAGTTGATC	642–664		
	CD56Chu-F	TTGTCCCCAGCCAAAGGAGAAAT	119–140	181	Huang et al
	CD56Chu-R	TAGATGGTGAAGCTGGAGGAAAGA	277–299		Huang et al
CD16 (XM_536141)	cCD16-F	ACAGTGTGACTCTGAAATGCCAG	119–141	188	This work
	cCD16-R	GACTTCTAGCTGCACCGGGTCA	285–306		
NKG2D (XM_849013)	cNKG2D-F	GTTATTGTGTCCTGTCCTAA	188–209	222	This work
	cNKG2D-R	ACTGCCAGGATCCATTGTTGG	388–409		
NKp30 (DQ003484)	cNKp30-F	CTTCTTGCCGTGTTCTTCAA	51–71	236	This work
	cNKp30-R	CCAGAACCTCCACTCTGCACA	266–286		
NKp44 (XM_846203)	cNKp44-F	ACCAAGTTAGTCAGCAGCTCCA	229–250	205	This work
	cNKp44-R	CTGGAGACACTGCCAGATAGAAAT	411–433		
NKp46 (XM_849055)	cNKp46-F	TATGATACGCCCACTCTCTCCGTT	361–384	217	This work
	cNKp46-R	ATCCAAAACATCTGTACGTCCCTC	554–577		
Ly49 (AY191818)	cLy49-F	GATTCTGGGATCCTGTGTTTACT	300–323	236	This work
	cLy49-R	GTAAATCCTGTCTCTTTTCTGCTG	511–535		
Perforin (FJ973622)	cPerforin-F	ACAGGTACAGCTTCAGCACTGAC	494–515	189	This work
	cPerforin-R	CCATGGACCGGATGAAGTGGT	661–682		
Granzyme B (XM_547752)	cGZB-F	GGAGAGATCATCGGGGGACATGA	448–470	195	This work
	cGZB-R	CTCCTGTTCTTGTATGTTGTGG	621–642		
iTCR (DM176745)	iV3	GGACAGGTCTGCTTCTCTG	221–240	338	Yasuda et al
	C2	GGCCCCCTCCGGTTTT	543–558		Yasuda et al

^a GenBank accession number.

^b Positions as counted from the first nucleotide of the canine genes reported.

Table 2

The expression rate of surface molecules on freshly isolated PBMCs and CLGLs cultured for 14 days.

Surface molecules	PBMCs % expression Mean (Min–Max)	CLGLs % expression Mean (Min–Max)
CD3	81.8 (64.7–89.3)	91.2 (88.7–92.7)
CD4	60.3 (55.1–67.0)	3.9 (2.1–6.2)
CD5 ^{dim}	10.5 (4.0–19.2)	78.0 (72.0–85.1)
CD5 ^{bright}	79.8 (66.9–92.0)	13.6 (7.7–17.9)
CD8	18.8 (8.9–33.0)	93.5 (90.7–95.9)
CD11c	40.1 (16.2–62.2)	68.1 (30.6–88.6)
CD11d	26.1 (9.4–64.1)	70.9 (33.7–95.8)
CD21	18.7 (5.3–37.1)	1.7 (0.9–3.2)
CD44	98.5 (98.0–98.9)	97.5 (94.7–99.6)
TCR	84.4 (83.4–85.4)	3.2 (2.8–9.5)
TCR	5.2 (4.5–8.5)	8.1 (3.1–19.4)