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Research paper

Canine CD8 T cells showing NK cytotoxic activity express mRNAs for NK cell-associated surface molecules

Yi-Chun Lin [a], Yi-Chun Huang [a], Yu-Shan Wang [a,c], Rong-Huay Juang [b], Kuang-Wen Liao [d], Rea-Min Chu [a,*]

[a] Animal Cancer Center, School of Veterinary Medicine, National Taiwan University, 1, Roosevelt Road, Section 4, Taipei 106, Taiwan, ROC
[b] Department of Biochemistry and Technology, College of Biological Sciences, National Taiwan University, Taiwan, ROC
[c] Department of Radiation Therapy and Oncology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, ROC
[d] Department of Biological Science and Technology, National Chiao-Tung University, Hsin-Chu, Taiwan, ROC

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ABSTRACT

Natural killer (NK) cells have been considered to be a group of lymphocytes lacking clonally distributed receptors for antigens typical of T cells and B cells. In some mammalian species, including humans, a subpopulation of $CD8^+$ peripheral blood lymphocytes (PBLs) exhibits NK activity. This NK subpopulation has not been well characterized in mammals and its characterization is particularly poor in the dog. In this study, we demonstrated that a subset of canine $CD8^+$ cells derived from PBLs and lymphokine (IL-2)-activated killers (LAKs) of PBLs that was $CD3^+$, $CD4^-$, $CD21^-$, $CD5^{lo}$, $\alpha/\beta TCR^+$, and $\gamma/\delta TCR^-$ contained substantially higher levels of mRNAs for NK cell-related receptors (NKp30, NKp44, NKG2D, 2B4, and CD16 for PBL, and NKG2D and CD56 for LAK) than the corresponding $CD8^-$ cells. This subset of $CD8^+$ lymphocytes derived from LAKs also displayed significantly higher NK cytotoxic activity than the corresponding $CD8^-$ cells. In contrast, $CD8^+$ cells derived from nonstimulated PBLs showed very low levels of NK cytotoxic activity. Our results indicate that, in IL-2-stimulated PBLs, canine $CD8^+$ cells are an important subset associated with NK cytotoxic activity.

1. Introduction

Natural killer (NK) cells are important effector cells involved in innate immune responses. They have been described on a functional basis as capable of killing tumors or virally infected cells without previous stimulation (Cerwenka and Lanier, 2001; Kiessling et al., 1976). The function of NK cells is regulated by a balance between signals transmitted via activating receptors, which recognize ligands on tumors and virus-infected cells, and signals transmitted via inhibitory receptors, which recognize major histocompatibility complex (MHC) class I molecules (Backstrom et al., 2004). Thus, NK cells preferentially kill target cells lacking, or with low levels of, surface MHC class I antigens, the so-called missing self-hypothesis (Ljunggren and Karre, 1990), which shifts the balance towards activation. Many types of inhibitory and activating NK cell receptors, such as KIR, NCRs, ILT/ILR, 2B4, CD94/NKG2, NKG2D, Ly49, and NKR-P1, have been identified in mammals (Lanier et al., 1998; McQueen and Parham, 2002; Sawicki et al., 2001), but there is only limited information on such receptors in the dog. In 1993, it was reported that CD18 and CD44 might be associated with canine NK cell activity (Ishiyama et al., 1993; Loughran et al., 1993). Pende et al. (1999) discovered that a canine genomic DNA segment was similar to that coding for human NKp30. The genes for the NK cell-related molecules Ly49 and CD56 have been

* Corresponding author. Tel.: +886 2 2368 6570; fax: +886 2 2365 5147.
E-mail address: redman@ntu.edu.tw (R.-M. Chu).

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reported in the dog (Bonkobara et al., 2005; Gagnier et al., 2003).

NK cells are large granular lymphocytes and represent about 5–15% of the lymphocyte population (Timonen et al., 1981). They are generally defined as a lymphocyte population lacking T or B cell-specific surface antigens and were designated as non-T non-B cells before NK cell-specific cell markers were well-defined. More recently, the phenotype of canine NK cells has been reported to be $CD4^-$ $CD6^-$ $CD8^-$ (Guenther et al., 1994; Knapp et al., 1995). Leukemic cells isolated from a dog which were able to kill CTAC cells (canine NK target cells) carried no CD4 or CD8 (Helfand et al., 1995). Our recent study showed that, after IL-2 stimulation of PBLs, strong NK activity is seen in the $CD5^{lo}$ cells, which also express CD8 and CD3 (Huang et al., 2008). This supports the notion that one of the canine NK cell subpopulations may be of the T cell lineage. In addition, CD8 has been shown to be expressed on a subpopulation of NK cells in other mammals. Human NK cells express CD8α (Moretta et al., 2002). In cattle, 4–15% of the NKp46+ cells express CD8 and the proportion of $CD8^+$ cells increases after IL-2 culture (Storset et al., 2004). In swine, $CD8^+$ cells show NK cytotoxicity (Yang and Parkhouse, 1997). CD8 T cells from rat lymph nodes or spleen almost exclusively express the alpha/beta isoform, while NK cells only carry the CD8α chain (Torres-Nagel et al., 1992).

In this study, we carried out an in-depth study on a canine $CD8^+$ subpopulation in the peripheral blood that showed NK cytotoxic activity. We demonstrated that this subpopulation of cells expressed mostly TCR α/β and contained much higher levels of mRNAs for NKp30 and other NK cell-related molecules than the $CD8^-$ cells. When purified from IL-2 stimulated PBLs (LAKs), this subset showed significantly higher NK cell cytotoxicity than the corresponding purified $CD8^-$ cells. The phenotypic and morphologic characteristics of this subset were also studied.

2. Materials and methods

2.1. Preparation of canine peripheral blood lymphocytes (PBLs)

Peripheral blood was obtained from six healthy 1- to 2-year-old beagles. All the dogs had been dewormed and vaccinated on a regular basis. All experiments were performed according to the University Animal Experimental Ethics Committee guidelines. Peripheral blood mononuclear cells (PBMCs) were purified from sterile heparinized whole blood as described previously (Hsiao et al., 2004). Briefly, the whole blood was layered onto Ficoll-Hypaque (density: 1.077) (Amersham Pharmacia Biotech, Piscataway, NJ) and the PBMCs were harvested from the interface after centrifugation and re-suspended in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin (GIBCO, Grand Island, NY, USA), and 10% fetal bovine serum (FBS) (PERBIO, USA). To prepare PBLs, the PBMCs were allowed to adhere to a 25-cm^2 flask ($2–4 \times 10^6$ cells/ml) for 2 h at 37 °C and the non-adherent cells were collected by gentle pipetting and washed with phosphate-buffered saline, pH 7.4.

2.2. Generation of canine lymphokine-activated killer cells (LAKs)

To generate LAKs, canine PBLs (10^6 cells/ml) were cultured for 6 days in LAK medium [RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, 50 mM 2-mercaptoethanol (Sigma), 2500 U/ml of recombinant human interleukin-2 (IL-2) (Aldesleukin, Chiron B.V., Amsterdam, The Netherlands), and 10% FBS]. Fresh IL-2 was added every 3 days (Hsiao et al., 2002).

2.3. Flow cytometry analysis

Commercial mouse and rat monoclonal antibodies were used to analyze canine PBL and LAK surface antigens (Table 1). The cells were incubated for 30 min at room temperature with R-phycoerythrin (RPE)-conjugated rat antibodies against canine CD5 or CD8α or mouse isotype control or monoclonal antibodies against canine CD3, CD4, CD8β, CD21, α/βTCR, or γ/δTCR, washed, stained for 30 min at room temperature with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibodies (Becton Dickinson, Mountain View, CA, USA), then washed, and stained again with 5 μg/ml of propidium iodide (Sigma, Steinheim, Germany). The surface immunofluorescence of 1×10^4 viable cells was examined using a FACS Caliber flow cytometer and fluorescence intensities analyzed using Cell Quest software (both from Becton Dickinson, Mountain View, CA). The cell diameters of each subpopulation were measured using SPOT advanced software (Cell Quest). The number of cells used in the flow cytometry was counted using a hemocytometer. Cell numbers positive for each antibody were calculated by multiplying the original number by the percentage of antibody-positive cells determined by flow cytometry. The percentage of triple negative cells was obtained by subtracting the percentage of positive cells from 100%. The granularity of the $CD8^+$ and $CD8^-$ cells in PBL and in LAK populations was analyzed by measuring the mean fluorescence intensity of SSC.

2.4. Isolation of $CD8^+$ and $CD8^-$ cells and light microscopic examination

Canine PBLs were incubated with RPE-conjugated rat anti-canine CD8α antibody as described above and the

Table 1
List of antibodies used for surface phenotype assays and other tests.

Specificity	Clone	Ig class	Source
Canine CD3	CA17.2A12	Mouse IgG1	Dr. PF Moore[a]
Canine CD5-RPE	YKIX322.3	Rat IgG2a	Serotec[b]
Canine CD4	CA13.1E4	Mouse IgG1	Serotec
Canine CD21	CA2.1D6	Mouse IgG1	Serotec
Canine α/βTCR	CA15.8G7	Mouse IgG1	Dr. PF Moore
Canine γ/δTCR	CA20.8H1	Mouse IgG2a	Dr. PF Moore
Canine CD8α-RPE[c]	YCATE 55.9	Rat IgG1	Serotec

[a] Veterinary Medicine, University of California at Davis, California, USA.
[b] Kidlington, UK.
[c] RPE: R-Phycoerythrin-conjugated.

$CD8^+$ and $CD8^-$ cells selected according to the fluorescence intensities using a FACSAria™ flow cytometer (Becton Dickinson) in the Cell Sorting Core Facility (National Taiwan University College of Medicine, Taipei, Taiwan). The isolated $CD8^+$ and $CD8^-$ cells were stained with Diff-Quik and examined under a light microscope to calculate the size of the cells. The granularity of the cells was also examined.

2.5. Cytotoxicity assay

The canine thyroid adenocarcinoma cell line (CTAC), purchased from the European Collection of Cell Cultures organization (ECACC, Salisbury, Wiltshire, UK), was used as the target cell in the canine NK cytotoxic activity test performed using a CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) (Odeberg et al., 2003). Briefly, 50 μl of CTAC cells (2×10^3 cells) in RPMI 1640 containing 10% FCS, 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 2 mM L-glutamine was placed in the wells of a 96 well flat-bottom plate, then 50 μl of various concentrations of effector cells (PBLs, LAKs, or $CD8^+$ or $CD8^-$ PBLs or LAKs) was added to make mixtures with different effector/target (E/T) cell ratios (50/1, 25/1, 12.5/1, and 6.25/1). After 16 h incubation at 37 °C, the culture medium was harvested to assess lactate dehydrogenase (LDH) release, measured by reading the absorbance at 490 nm of the forazion formed in the reaction mix. The percentage cytotoxicity was calculated as: (experimental LDH release − effector spontaneous LDH release − target spontaneous LDH release)/(target maximum LDH release − target spontaneous LDH release) × 100%.

2.6. Real-time RT-PCR

Total RNA was extracted from canine PBLs and LAKs using TRIzol (Invitrogen, Grand Island, NY, USA) (Wang et al., 2007) and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT primers. The primers are listed in Table 2 and were designed using Primer Express software (Applied Biosystems) based on the sequence predictions on the Ensembl website (http://www.ensembl.org/index.html). β-Actin primers were used as the positive control. PCR was performed on a Mastercycler Autorisierter thermocycler (Eppendorf, Hamburg, Germany). Real-time RT-PCR was performed as described previously (Wang et al., 2007) using an ABI Prism 7000 (Applied Biosystems) and an AmpliQ Universal Real-Time PCR Master Mix kit (Ampliqon), according to the manufacturer's instructions. A single product at a specific melting temperature was found for each target. The purity of the amplified product was determined from the single peak of the dissociation curve. The specificity of the PCR product, based on the predicted size of the product, was also confirmed by gel electrophoresis. All samples were tested in duplicate and the mean was used for further calculations. Each run included a no-template control to test for contamination of assay reagents. The purity of the real-time RT-PCR products was determined by looking for primer contamination after electrophoresis on a 2% agarose gel and staining with ethidium bromide. PCR was performed in 96-well optical reaction plates. The relative amount of mRNA in each sample was calculated based on its threshold cycle (Ct) compared to the Ct for the β-actin housekeeping gene. The results are presented as $2^{-(\text{Ct of target gene} - \text{Ct of housekeeping gene})}$ ($2^{-\Delta CT}$). This experiment was performed four times, each in triplicate.

Table 2
cDNA sequences of the primers used for real-time RT-PCR.

Target gene	Primer	Sequence (5′ → 3′)
β-Actin	Sense	GAC CCT GAA GTA CCC CAT TGA G
	Anti-sense	TTG TAG AAG GTG TGG TGC CAG AT
NKp30	Sense	ACT GCT GCC TTC TTG CCG TGT T
	Anti-sense	ACT CCA GCA TCA CAG TCT TGG ATG T
NKp44	Sense	ATC GAG TGG CAG GGC AGA CA
	Anti-sense	TTC CTC CTT CAG ACC AAT CAT GGT
NKG2D	Sense	ACG AAG GCA AAA GAG AAA GCC
	Anti-sense	TGA TGA TTA TGG CAC CGC AT
2B4	Sense	TGG TAA CGT GAG CTA TGC TTG GTA
	Anti-sense	ACG ATC ACC AGA AAG TAC ACG AAG T
CD16	Sense	ACA TTC CAG CAG CAA CAA GTG A
	Anti-sense	AGC AAA ATA CAG CCC AGT GTC CA
CD56	Sense	TTG TCC CCA GCC AAG GAG AAA T
	Anti-sense	TAG ATG GTG AGC GTG GAG GAA GA
Ly49	Sense	TTT CCA GTG CCA AGA CAT CTC A
	Anti-sense	CCC AGG TGA TGG TTT TCT TGA A

2.7. Statistical analysis

All results are expressed as the mean ± SD and were analyzed using the two-tailed Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

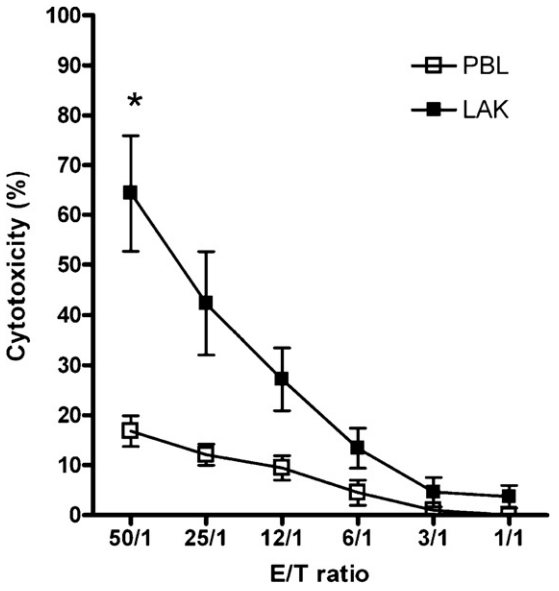

Fig. 1. NK cytotoxic activity mediated by PBLs and LAKs. The NK cytotoxic activity of PBLs and IL-2-activated PBLs (LAKs) was measured using CTAC target cells as described in Section 2. The data were averaged for samples collected from four different dogs and were analyzed using the *t*-test ($^*p < 0.05$).

3. Results

3.1. Cytotoxicity of PBLs and LAKs

We first examined differences in the NK cytotoxic activity of PBLs and LAKs. As anticipated, LAKs were significantly more effective than PBLs in killing CTAC cells ($p < 0.01$) (Fig. 1). We then analyzed phenotypic subpopulations in the PBLs and LAKs. The percentage of non-T/non-B cells ($CD3^-$ $CD21^-$ or $CD4^-$ $CD8^-$ $CD21^-$ cells) was not higher in the LAKs cells than in the PBLs. Instead, the percentage (Fig. 2) of $CD8^+$ cells were increased in the LAK cells ($p < 0.01$). This led us to further explore the differences between the $CD8^+$ and $CD8^-$ lymphocytes.

3.2. Morphology and phenotypes of the $CD8^+$ and $CD8^-$ cells

Morphological differences between the $CD8^+$ and $CD8^-$ cells in the PBLs and LAKs were studied using a FACS Caliber flow cytometer (Fig. 3a) and light microscopy (Fig. 3b). The data for size [shown by the forward scatter (FSC)], CD8 positivity, and cell count were combined to compile the three-dimensional plot (Fig. 3a). The results showed that, after IL-2 stimulation (LAK cells), the number of $CD8^+$ cells increased. The cell diameters of each subpopulation were measured using SPOT advanced software (Cell Quest) and the $CD8^+$ cells (6.56 ± 1.73 μm) were found to be significantly larger than the $CD8^-$ cells (4.96 ± 1.1 μm) ($p < 0.01$) in the LAK population, but not in the PBL population ($CD8^+$ cells, 4.15 ± 0.34 μm; $CD8^-$ cells, 4.26 ± 0.35 μm). The cell granularity of each population was analyzed using side scatter light (SSC) and similarly, only in LAK population, the $CD8^+$ cells were significantly more granular than the $CD8^-$ cells (Fig. 3c). On examining the LAK cells under light microscopy, although large granular lymphocytes (one of the morphological criteria of NK cells) were seen in both the $CD8^+$ (Fig. 3b) and $CD8^-$ cells (data not shown), the $CD8^+$ population contained many more larger cells with numerous granules in the cytoplasm. We then determined the phenotypes of the isolated $CD8^+$ and $CD8^-$ cells by staining for surface antigens using commercial monoclonal antibodies and flow cytometry. The lymphocytes in the PBL and LAK populations were gated to analyze the expression of the surface markers. In both PBLs (Fig. 4a) and LAKs (Fig. 4b), the phenotype of the $CD8^+$ cells was $CD3^+$, $CD4^-$, $CD21^-$, $\alpha/\beta TCR^+$, and $\gamma/\delta TCR^-$ (Table 3).

3.3. NK cytotoxic activity of the $CD8^+$ and $CD8^-$ cells

We then compared the NK cytotoxic activity of the $CD8^+$ and $CD8^-$ cells isolated from PBLs using a FACSAria™ flow cytometer (Becton Dickinson). The purity of each subpopulation was >97%. There was no significant difference between these $CD8^+$ and $CD8^-$ cells in NK cytotoxic activity in the absence of IL-2 stimulation (Fig. 5a). We then tested for differences between the two cell populations after 6 days' incubation with IL-2 (LAK cells) and found that the $CD8^+$ cells had consistently higher cytotoxicity than the $CD8^-$ cells (Fig. 5b) ($p < 0.05$). These results indicated that, after IL-2 stimulation, NK cell cytotoxicity of dog PBLs was closely associated with $CD8^+$ lymphocytes.

3.4. Levels of mRNAs for NK cell-associated receptors and markers in $CD8^+$ and $CD8^-$ cells

To verify the association of NK cytotoxic activity with the $CD8^+$ population, we used real-time RT-PCR to measure levels of mRNAs for a number of NK cell receptors and markers that have been identified in other mammals. The nucleotide primers for canine NKp30, NKp44, NKG2D, 2B4, and CD16 were designed according to the sequences predicted on the Ensembl website and the CD56 primers were designed according to a previous publication (Bonkobara et al., 2005). β-Actin mRNA was also amplified in all samples. In the $CD8^+$ cells from the PBL population, relative to that of β-actin, levels of the mRNA for all the tested markers except CD56 and Ly49 were significantly higher ($p < 0.01$ or $p < 0.05$) than in the corresponding $CD8^-$ cells (Fig. 6a). After generation of LAK cells by IL-2 stimulation, the pattern of expression in $CD8^+$ cells was

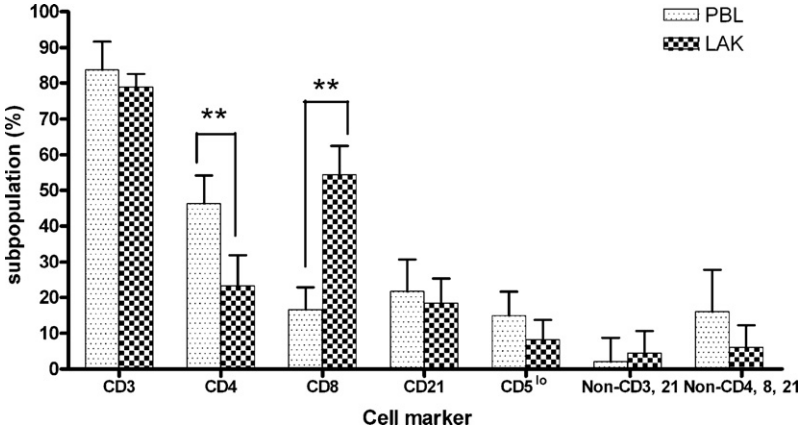

Fig. 2. Phenotypes in the PBL and LAK populations and number of cells with the different phenotypes. The phenotypes of PBLs and LAKs was investigated by staining with various antibodies and FACS Caliber flow cytometry. The experiments were performed using samples from six different dogs. The data were analyzed using the *t*-test (**$p < 0.01$).

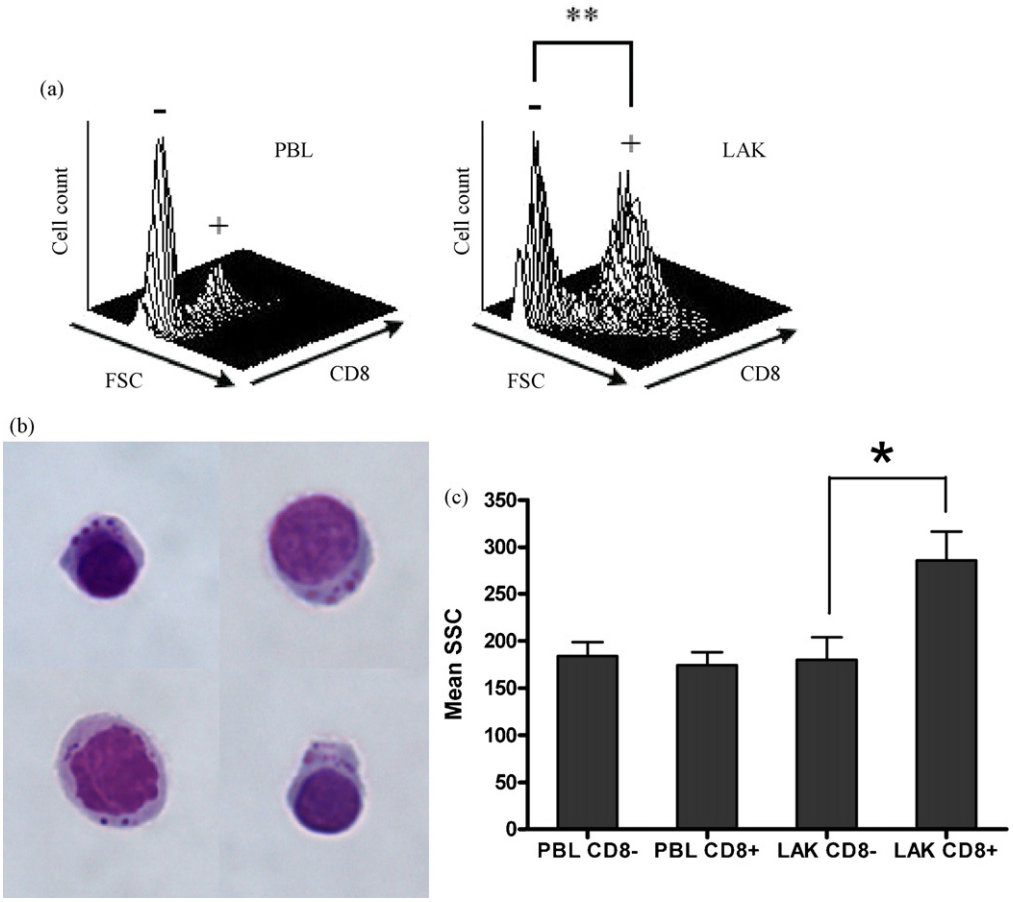

Fig. 3. Size differences between the $CD8^+$ and $CD8^-$ cells isolated from PBL and LAK cells. (a) Sizes of $CD8^+$ and $CD8^-$ cells isolated from PBLs and LAK cells studied using flow cytometry. The data for the size, shown by the forward scatter (FSC), CD8 positivity, and cell count were combined to compile the three-dimensional plots. The data were analyzed using the *t*-test ($^{**}p < 0.01$). (b) $CD8^+$ and $CD8^-$ cells isolated from PBLs and LAKs by FACSAria™ flow cytometry were stained with Diff-Quik. Large granular lymphocytes were observed by light microscopy (1000×) in the $CD8^+$ LAK cell population. The figure shows 4 typical cells. (c) The granularity of the isolated cells was also analyzed by flow cytometry.

generally the same; However, the mRNA levels for most of the receptors, except NKG2D, were no longer significantly higher in the $CD8^+$ cells than in the $CD8^-$ cells, and CD56 mRNA levels showed a slight increase after IL-2 stimulation (Fig. 6B). These results showed that canine $CD8^+$ cells in the peripheral blood express significantly higher levels of NK-associated receptors and markers than $CD8^-$ cells.

4. Discussion

Although canine NK cells have not been defined as $CD8^+$ cells, the first study suggesting that canine NK cells express cell surface antigen of the T cell lineage was performed in 1985 (Loughran et al., 1985). These authors found that NK cytotoxic activity was decreased when canine PBMCs were treated with complement and a monoclonal antibody functionally equivalent to anti-human $CD8^+$ antibody. In the present study, we provided several lines of evidence indicating that an IL-2-activated population of $CD8^+$ cells ($CD8^+$ LAK cells) from canine peripheral blood was closely associated with NK cytotoxic activity against CTAC cells.

The majority of the canine LAK cells showing NK cytotoxic activity were not $CD8^-$ cells, as shown by the facts that (i) the proportion and number of $CD8^+$ cells were greatly increased in the LAK cells, but not in the population of non-T non-B cells ($CD3^-CD21^-$ and $CD4^-CD8^-CD21^-$), (ii) marked cytotoxicity was seen in the $CD8^+$LAK cells, but not the untreated PBLs, and (iii) the $CD8^-$ cell population isolated from LAK cells was almost devoid of NK cytotoxic activity. In addition, the significantly higher levels of mRNAs for NK cell receptors and markers seen in the $CD8^+$ cells compared to the $CD8^-$ cells also suggested that the $CD8^+$ cells had a closer relationship than the $CD8^-$ cells to NK activity. This $CD8^+$ subpopulation was also found to be $CD4^-$. This last result is in agreement with a previous report (Loughran et al., 1985) that treatment of canine PBLs with an antibody recognizing CD4 plus complement did not cause a decrease in NK cytotoxic activity. CD8 has been identified on NK cells in a number of mammals, such as humans, primates, rats, pigs, and cows (Boysen et al., 2006; Helfand et al., 1995; Moebius et al., 1991; Torres-Nagel et al., 1992; Yang and Parkhouse, 1997). Further, we recently found that canine NK cell cytotoxicity in the

peripheral blood heavily relies on $CD5^{lo}$ cells, which are of the T cell lineage and strongly express CD8 (Huang et al., 2008). These data all support the idea that $CD8^+$ PBLs are an important NK population in the dog.

However, there is also evidence that canine NK cells are $CD3^-$ $CD4^-$ $CD8^-$ large granular lymphocytes (Guenther et al., 1994; Jardine et al., 1989; Knapp et al., 1995). In these studies, the authors used PBLs without IL-2 stimulation to obtain cells with increased NK activity that were a non-CD8 phenotype. This procedure may explain, at least in part, the discrepancy between their findings and those of our present study, in which the cells were isolated from IL-2-activated PBLs. In addition to the different isolation techniques and cytokine treatment, other factors may markedly influence NK cytotoxic activity, including the method used and the purity of the isolated NK cells. The LDH method we used and the increased purity of over 97% obtained by FACSAria™ flow cytometer compared to the reported purities of 36–51% in previous studies (Knapp et al., 1995) might also account for the discrepancy.

Taking together our present results and those of previous studies (Guenther et al., 1994; Knapp et al., 1995), it is clear that at least two subpopulations of canine PBLs have NK cytotoxic activity, and that, according to our previous report (Huang et al., 2008) and the present study, $CD8^+$ cells in IL-2-activated PBLs play a significant role in NK cytotoxic activity.

As presented in the results, the levels of most (5 out of 7) mRNAs coding for NK receptors (NKp30, NKp44, NKG2D, CD16, and 2B4) were increased in the $CD8^+$ population of

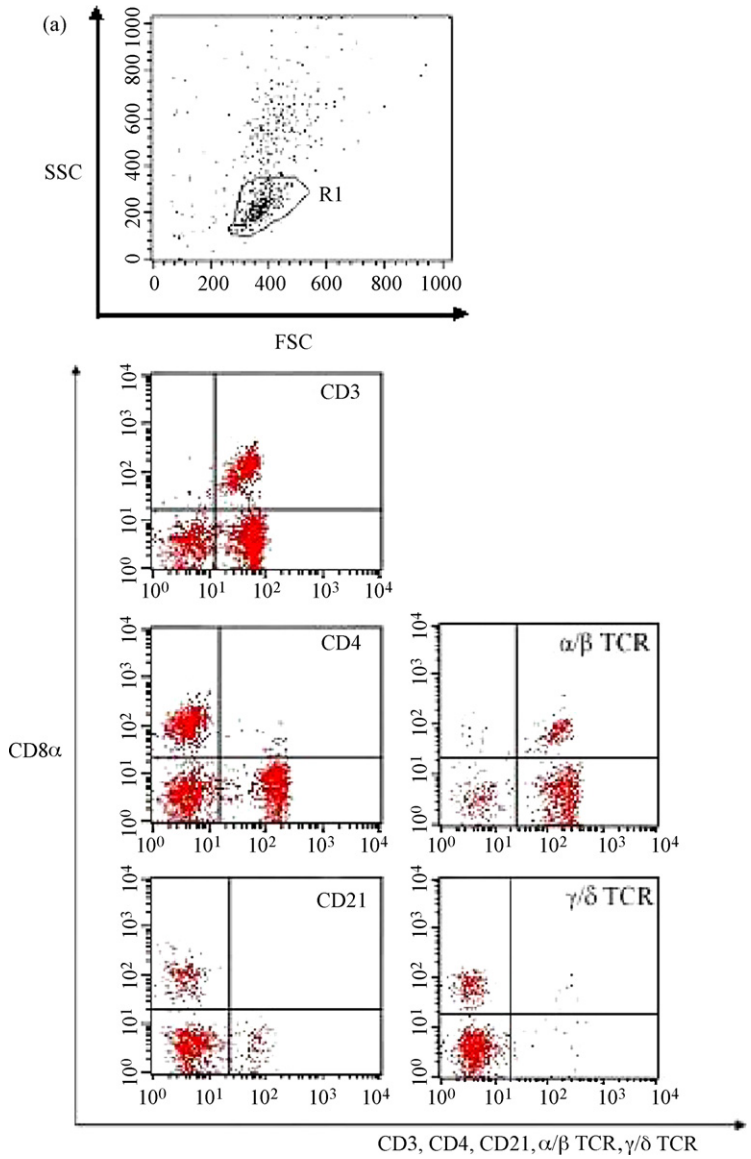

Fig. 4. Phenotypes of the $CD8^+$ population isolated from PBLs and LAK cells. The phenotypes of the $CD8^+$ cells isolated from PBLs (a) and LAKs (b) using a FACSAria™ flow cytometer were analyzed by dual-staining FACS Caliber flow cytometry using the antibodies shown in figure. The result shown is representative of those obtained for four different dogs.

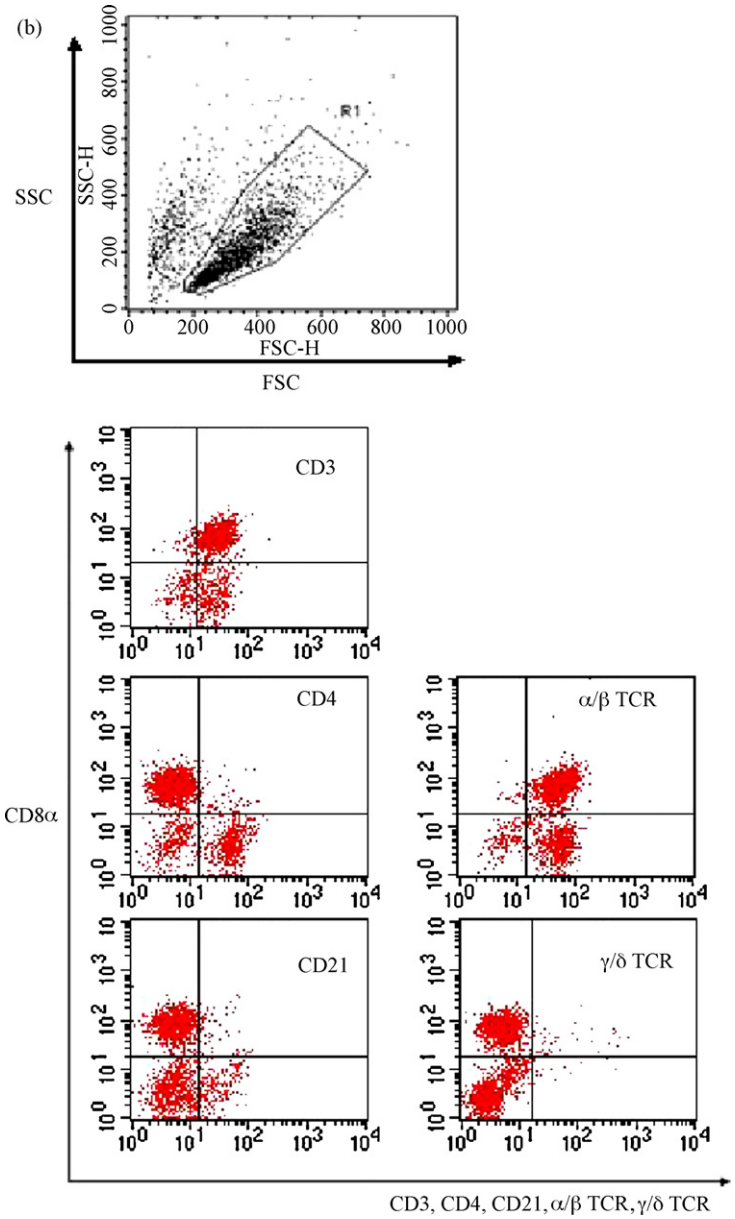

Fig. 4. (*Continued*).

PBLs. It is interesting to note that, although canine $CD8^+$ NK cytotoxic activity was IL-2-dependent, mRNA levels for these five except NKG2D were no longer higher in the $CD8^+$ population after the PBLs were incubated with IL-2. We do not know the reasons for this. However, it is an important area that needs to be explored to further understand canine NK cell activation.

Many inhibitory and activating receptors on NK cells have been identified in humans and rodents. NKp30 was identified on human NK cells in 1999 (Pende et al., 1999), then on NK cells of several other mammalian species (De Maria et al., 2001; Hsieh et al., 2004). Because NKp30 is selectively expressed by all NK cells in humans and rats (Biassoni et al., 2001; Hsieh et al., 2006; Pende et al., 1999), it is one of the specific markers for NK cells in mammals. However, in all 13 strains of mice tested except Mus calori, NKp30 was found to exist as a pseudogene (Hollyoake et al., 2005). We showed that canine cells with NK cytotoxic activity expressed high levels of NKp30 mRNA and that $CD8^+$ cells derived from canine PBLs and LAKs expressed significantly higher levels of NKp30 mRNA than the corresponding $CD8^-$ cells. However, the lack of antibodies recognizing canine NKp30 has hampered the characterization of this molecule and the delineation of its relationship with canine NK cells. In addition, NKp44 and NKG2D are activating receptors of NK cells in other species (Biassoni et al., 2001; Cantoni et al., 1999; Hsieh et al., 2006; Vitale et al., 1998). NKG2D mRNA levels were

Table 3
Percentage of $CD8^+$ and $CD8^-$ cells derived from PBLs or LAK cells expressing different surface markers (average for four dogs).

	Surface marker expression percentage (PBL)			
	$CD8^+$		$CD8^-$	
	+	–	+	–
CD3	24.49 ± 3.14	0.46 ± 0.23	61.60 ± 6.64	13.45 ± 5.21
CD4	1.19 ± 1.72	23.53 ± 3.74	47.41 ± 5.06	27.87 ± 2.15
CD21	0.11 ± 0.09	24.91 ± 3.48	10.76 ± 4.23	64.22 ± 5.14
TCRαβ	24.10 ± 4.01	0.86 ± 0.28	58.64 ± 7.13	16.41 ± 5.54
TCRγδ	0.57 ± 0.37	24.07 ± 3.22	1.80 ± 0.53	73.56 ± 2.96
	Surface marker expression percentage (LAK)			
	$CD8^+$		$CD8^-$	
	+	–	+	–
CD3	41.90 ± 13.30	3.00 ± 1.69	42.50 ± 13.28	12.60 ± 9.23
CD4	3.05 ± 0.35	40.08 ± 14.52	32.88 ± 11.72	23.99 ± 8.09
CD21	2.10 ± 0.60	39.37 ± 12.27	15.70 ± 4.84	42.83 ± 7.99
TCRαβ	39.99 ± 14.09	2.21 ± 0.64	41.92 ± 12.04	15.89 ± 6.92
TCRγδ	2.49 ± 0.80	3.00 ± 1.69	3.48 ± 2.20	55.47 ± 10.42

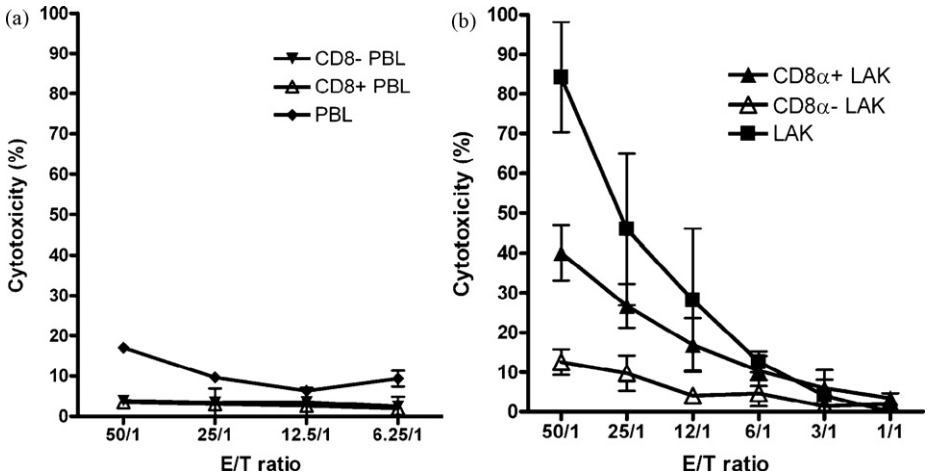

Fig. 5. NK cytotoxic activity of the $CD8^+$ and $CD8^-$ cells isolated from PBLs and LAKs. The NK cytotoxic activity of $CD8^+$ and $CD8^-$ cells isolated from PBLs (a) and LAKs (b) using a FACSAria™ flow cytometer was assayed as described in the materials and methods. The data are averaged from the results for samples from four different dogs and were analyzed using the *t*-test ($^*p < 0.05$).

significantly higher in $CD8^+$ cells from the PBL and IL-2-activated population in the present study than in the $CD8^-$ cells. It is possible that NKG2D might be involved in canine IL-2-dependent NK cytotoxic activity. CD56 has been identified in NK cells of humans and higher primates, but has rarely been found in other mammals. Bonkobara et al. (2005) reported that the CD56 gene after adding phytohemagglutinin and culturing the cells for 3 days, CD56 mRNA levels are increased in dog peripheral lymphocytes. Although our data showed that CD56 mRNA levels increased in the $CD8^+$ population after culture with IL-2, there is no direct evidence for an association of CD56 and NK cells in the dog.

We demonstrated that levels of mRNAs for NK receptors and markers were higher in the $CD8^+$ population than in the $CD8^-$ population. However, the $CD8^+$ population in LAK cells contains both NK cells and cytotoxic T cells. Many of the NK cell receptors, such as CD16, CD56, NKG2D, and KIR, are also expressed by some T cells (Abedin et al., 2005). Thus, we do not know the extent of the contribution of the mRNAs from $CD8^+$ cytotoxic T cells. On the other hand, the NK cells in the $CD8^-$ population should also be enriched in the LAK cell population. This latter increase, along with the activated T cells produced by culturing with IL-2, could significantly increase mRNA levels for the tested receptors and markers. This might be one of the reasons why the levels of the examined mRNAs in the LAK cells were not significantly different in the $CD8^+$ and $CD8^-$ populations. In conclusion, we have demonstrated that a subpopulation of canine PBLs of the CD8 phenotype show significant NK cytotoxic activity after activation with IL-2. These $CD8^+$ cells are also $CD3^+$, $CD4^-$, $CD21^-$, $CD5^{lo}$, $\alpha/\beta TCR^+$, and $\gamma/\delta TCR^-$ and contain substantially higher levels of mRNAs for NK cell-related receptors (NKp30, NKp44, NKG2D, 2B4,

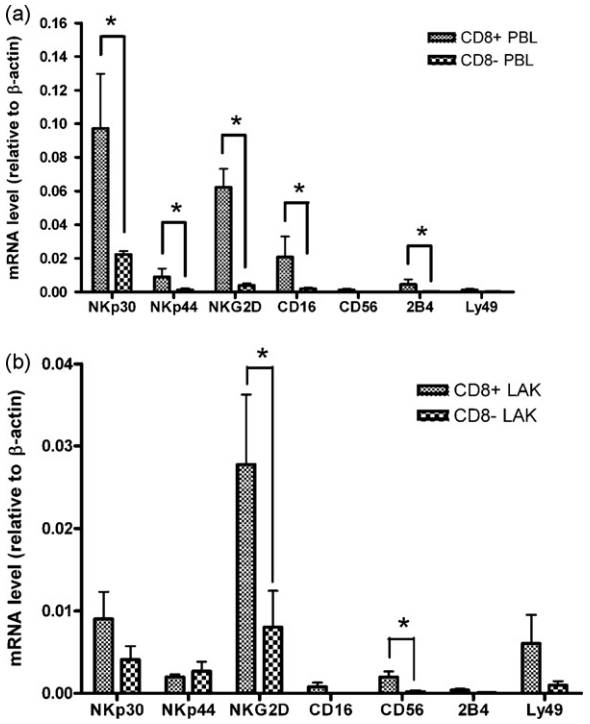

Fig. 6. Real-time RT-PCR analysis of NK cell receptor-related gene expression in $CD8^+$ and $CD8^-$ cells isolated from PBLs and LAKs. Real-time RT-PCR was used to determine the levels of mRNAs for NKp30, NKp44, NKG2D, 2B4, CD16, or CD56 in the $CD8^+$ and $CD8^-$ cells isolated from PBLs (a) and LAKs (b) using a FACSAria™ flow cytometer. The amount of mRNA is expressed relative to the amount of β-actin mRNA in each sample. The results are shown as the mean ± SD for four different dogs and were analyzed using the *t*-test ($^*p < 0.05$).

and CD16 for PBL, and NKG2D and CD56 for LAK) than the $CD8^-$ cells. We believe this $CD8^+$ subpopulation to be an important NK cell population in canine PBLs after IL-2 stimulation.

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