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RESEARCH ARTICLE

A Descriptive Survey of Immune Cell Frequencies and an Evaluation of Pre-analytical Delay Effects on Blood Samples from Healthy Companion Dogs

Eun Young Oh[1†], Yujin Kim[2†], Jun-Yeong Lee[1], Jin Seok[2], Sang-Myeong Lee[1*] and Sungin Lee[2*]

[1]College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea; [2]Department of Veterinary Surgery, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea.

[†]These authors contributed equally to this work.
*Corresponding author: smlee@cbnu.ac.kr; sunginlee@cbnu.ac.kr

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ABSTRACT

Analyzing immune cell profiles in dogs is gaining popularity due to the importance of immune status and its effect on tumors or other infectious and immune-mediated diseases. In this study, the frequencies of immune cell populations in 43 healthy companion dogs were investigated. The impact of pre-analytical delay on the biological integrity of blood samples was evaluated. Blood samples from 43 healthy companion dogs were used to isolate peripheral blood mononuclear cells (PBMCs) and white blood cells (WBCs). Flow cytometry analyzed immune cell frequencies and viability at fresh (~2h), 12h, and 24h after collection. RNA from inflammatory cytokines and apoptosis-associated genes was extracted by TRIzol-based and column-based methods and analyzed at fresh (~2h), 6h, and 12h. The immune cell frequencies in PBMCs were observed as follows: T-cells 30.93±17.62%; helper T-cells (Th) 18.70±12.04%; cytotoxic T-cells (Tc) 7.19±4.20%; B-cells 7.22±5.77%; monocytes 3.10±2.87%, and NK cells 0.60±0.68% of lymphocytes, and regulatory T-cells (Treg) 8.74±4.27% of $CD4^+$ cells. Cell viability was maintained up to 12h in both PBMCs and WBCs, while monocyte, neutrophil, and Treg in WBCs significantly decreased at 24h ($P<0.05$). Cell frequencies remained stable in PBMCs up to 24h, but the frequencies of T-cell subsets were decreased in 12h ($P<0.05$). Although RNA yield was higher with the TRIzol-based extraction compared to the column-based method, mRNA extracted by column-based method was more stable. The mRNA levels varied with each target transcript and storing time, indicating a need for standardized protocols. This study provides preliminary data on immune cell profiles in canine PBMCs from companion dogs and throws light on experimental integrity during transportation of canine blood for multi-center research.

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INTRODUCTION

Analyzing immune cell profiles in dogs is gaining popularity due to the importance of immune status and its effect on tumors (Sfacteria *et al.*, 2021) or other infectious and immune-mediated diseases (Majewska *et al.*, 2016; García-Castro *et al.*, 2022). Despite efforts to find key immunological defects in such diseases, studies on basic canine immune cell profiles in companion dogs remain lacking.

In general, gathering samples from multi-centers offering samples from various dogs can provide more comprehensive results. However, sample transportation itself causes processing delays, and in the laboratory, increased workload on the staff sometimes further delays the analysis of these samples. While several human studies have assured reliability of immune cell viability and RNA expression from transported samples (Savage *et al.*, 2021; Jiang *et al.*, 2023; Yi *et al.*, 2023), similar research is lacking in the veterinary field. Therefore, some basic study on delayed processing effects in canine blood is necessary.

Peripheral blood mononuclear cells (PBMCs) are widely used for analyzing immune properties, but due to the laborious isolation process of these cells, handling whole blood (WB) itself is also reported to be valuable in

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human studies (Bondar *et al.*, 2014; Moris *et al.*, 2021). Furthermore, depending on the molecular target, well-established methods of handling human samples suggest the type of sample to be used and how to prepare it (Müller *et al.*, 2023). In animals, such methodological studies are lacking except one porcine study analyzing WB after red blood cell lysis (Mattoo *et al.*, 2023); thus, evaluation through both PBMC isolation and WB seems necessary.

The influence of processing delay or methodological trials of both sample types (PBMCs and WB) in canine blood might be similar to that reported in humans, but a definitive study is needed for its confirmation. Therefore, aim of the present study was to investigate canine immune cell frequencies in clinically healthy companion dogs before analyzing peripheral blood from pathological cases. Attempts were also made to monitor the effect of processing delay (due to shipment or storage) on immune cell viability and RNA expression in canine peripheral blood.

MATERIALS AND METHODS

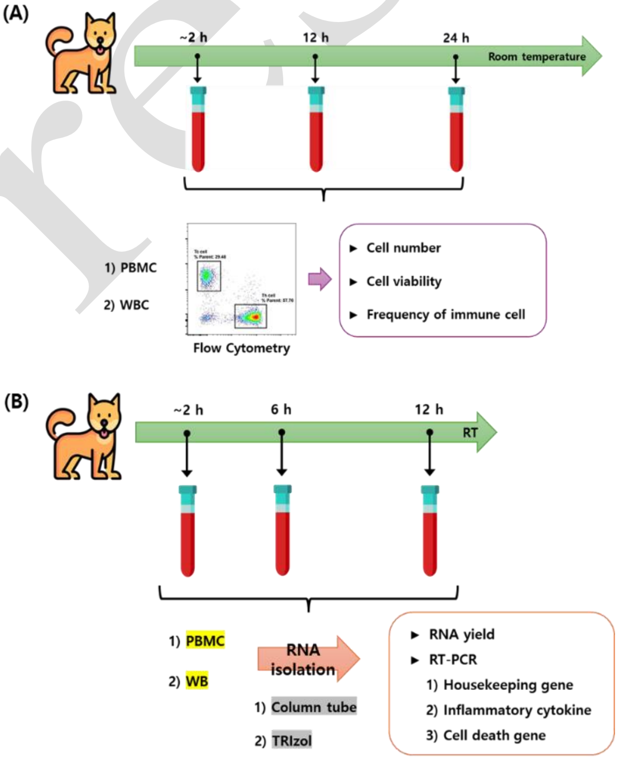
Ethical approval and experimental animals: This study included 43 healthy companion dogs enrolled with written owners' consent during the period from September 2024 to December 2024. Protocol of the study was duly approved by the Chungbuk National University Institutional Animal Care and Use Committee (IACUC). (CBNUA-24-0041-02). The dogs included in the study represented eight different breeds, both sexes, and ranged in age from 1 to 16 years. The detailed demographics of these dogs are shown in Table 1.

Table 1: Demographics of 43 healthy companion dogs included in the study

Category	Companion dogs (n=43)
Breeds	Mixed breed (14) Shih-tzu (10) Pomeranian (6) Maltese (6) Poodle (4) Bichon Frise (1) Welsh Corgi (1) Yorkshire Terrier (1)
Sex	Female intact (4) Female spayed (21) Male intact (0) Male neutered (18)
Age (years) Mean±SD (range)	5.60±3.36 (1-16)
Body weight (kg) Mean±SD (range)	7.11±5.60 (1.63-33.00)

The dogs had received vaccinations against canine distemper virus, infectious hepatitis virus, parvovirus, parainfluenza virus, and Leptospira before 6 months of age, with no boosters were administered during one month prior to the study. All dogs received regular anti-parasitic treatments targeting endoparasite and/or ectoparasite every 1 to 3 months. Furthermore, to confirm clinical health, each dog underwent a comprehensive health assessment which included a physical examination (evaluation of body weight, heart rate, respiratory rate, blood pressure, mucosal membrane color, and capillary refilling time), complete blood count, serum chemistry (evaluation of total protein, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, alkaline phosphatase, blood urea nitrogen, creatinine, electrolytes including sodium, potassium, chloride, calcium, phosphate, blood glucose, total cholesterol, and total bilirubin), and SNAP® 4Dx Plus kit (IDEXX Laboratories, Westbrook, USA) test to confirm clinical health. The health assessments revealed no distinct health issues with any dog.

Blood collection and processing: Blood (3-5mL) was collected via cephalic or jugular venipuncture into EDTA-coated tubes (BD Biosciences, San Jose, USA) from each dog. To assess the impact of delayed processing on sample stability, EDTA-anticoagulated blood was stored at room temperature (RT, 20-25°C) for 12h or 24h prior to downstream procedures; fresh blood samples processed within 2h post-collection served as controls. RT was chosen to reflect common clinical and research handling conditions. For immune cell analysis, samples were stored for 12 or 24h before PBMC and WBC isolation. Stability was evaluated by cell counts per mL of blood, viability, and subset percentages were estimated by flow cytometry. For RNA stability, samples were processed at 6 and 12h post-collection. A schematic overview of workflows is provided in Supplementary Fig. S1.



Supplementary Fig. S1: Experimental schematics for immune cells and RNA analyses. (A): Immune cell workflow: canine whole blood was stored at RT for fresh, 12h, and 24h, followed by isolation of PBMCs and WBCs and flow-cytometric staining. (B): RNA workflow: whole blood was stored at RT for fresh, 6h, and 12h. RNA was extracted from WB and PBMCs using column-based or TRIzol-based methods and analyzed by qRT-PCR. (The dog and blood tube icon image from Flaticon; https://www.flaticon.com/).

Isolation of peripheral blood mononuclear cells (PBMC): At each time point, blood was gently mixed, and PBMCs were isolated by density gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich, St. Louis, USA). Briefly, whole blood was layered over an equal volume of Histopaque and centrifuged at 400×g for 30min at RT with minimal acceleration and no brake (Schildhauer *et al.*, 2006). The mononuclear cell layer was collected and washed twice with PBS. Cells were counted using the Northern Lights™ flow cytometer (Cytek Biosciences, Fremont, USA) and used for immune cell frequency analysis or RNA extraction.

Isolation of white blood cells (WBC): For WBC isolation, peripheral blood was treated with RBC lysis buffer (Invitrogen, Carlsbad, USA) to remove red blood cells (Zhou *et al.,* 2024). Cells were resuspended in PBS and counted using flow cytometer, as described below.

Flow cytometry: The isolated WBCs were pre-treated with the canine Fc receptor binding inhibitor polyclonal antibody (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions (Valle *et al.*, 2023). PBMCs and WBCs were stained at RT with Fixable Viability Stain 620 (BD Biosciences, San Jose, USA) for 15min in the dark, then washed twice with Fluorescence-Activated Cell Sorting (FACS) buffer (1X PBS, 3% heat-inactivated fetal bovine serum, and 0.02% sodium azide). Cells were incubated for 30min on ice with anti-canine CD4, CD5, CD8a, CD21, CD11b, Neutrophil, and NKp46 antibodies in FACS buffer. For Foxp3 staining, cells were fixed, permeabilized (eBioscience™ Foxp3/Transcription Factor Staining Buffer Set; Invitrogen, Carlsbad, USA), and stained for Foxp3 following the manufacturer's protocol. After staining, cells were washed with FACS buffer and analyzed using Cytek Northern Lights™ flow cytometer. The antibody panel was designed based on previously described anti-dog antibody panels and immunophenotyping strategies (Biller *et al.*, 2010; Pantelyushin *et al.*, 2020; Sparger *et al.*, 2021; Parys *et al.*, 2023; McDonald *et al.*, 2025). Gating was performed as shown in Figure 1. Live single cells were gated and classified into T-cells (CD5^{+}CD21^{-}) and B-cells (CD5^{-}CD21^{+}), with remaining CD5^{-}CD21^{-} events designated as other leukocytes. T-cells were subdivided into helper (CD4^{+}CD8^{-}) and cytotoxic (CD4^{-}CD8^{+}) subsets. Within the CD5^{-}CD21^{-} compartment, monocytes and neutrophils were distinguished using CD11b together with a neutrophil-specific antibody. NK cells were defined as CD5$^{-/dim}$ NKp46^{+}, while regulatory T-cells (Treg) were identified as CD4^{+}Foxp3^{+}. Antibody details, including isotype, clone, fluorochrome, and vendor, are summarized in Table 2. For subset-level viability analyses, dead cell was quantified as the absolute number of Fixable Viability Stain 620-positive events within each gated population in PBMCs and WBCs at ~2, 12, and 24h.

Table 2: List of antibodies used for canine immune cell analysis

Antibody	Isotype	Clone (fluorochrome)	Company (Cat#)
CD4	Rat IgG2a	YKIX302.9 (FITC)	Bio-rad (# MCA1038F)
CD5	Rat IgG2a, kappa	YKIX322.3 (PerCP-eF710)	Invitrogen (# 46-5050-42)
CD8a	Rat IgG1, kappa	YCATE55.9 (PE)	Invitrogen (# 12-5080-42)
CD21	Mouse IgG1	CA2.1D6 (PE-Cy5.5)	Invitrogen (# MA5-16610)
CD11b	Rat IgG2b, kappa	M1/70 (PE-Cy7)	Invitrogen (# 25-0112-82)
Neutrophil	Mouse	CADO8A (PerCP)	Kingfisher (# WS0811D)
NKp46	Mouse IgG2a, k	48A (FITC)	Merk (# MABF2109)
Foxp3	Rat IgG2a, kappa	FJK-16s (PE)	Invitrogen (# 12-5773-82)

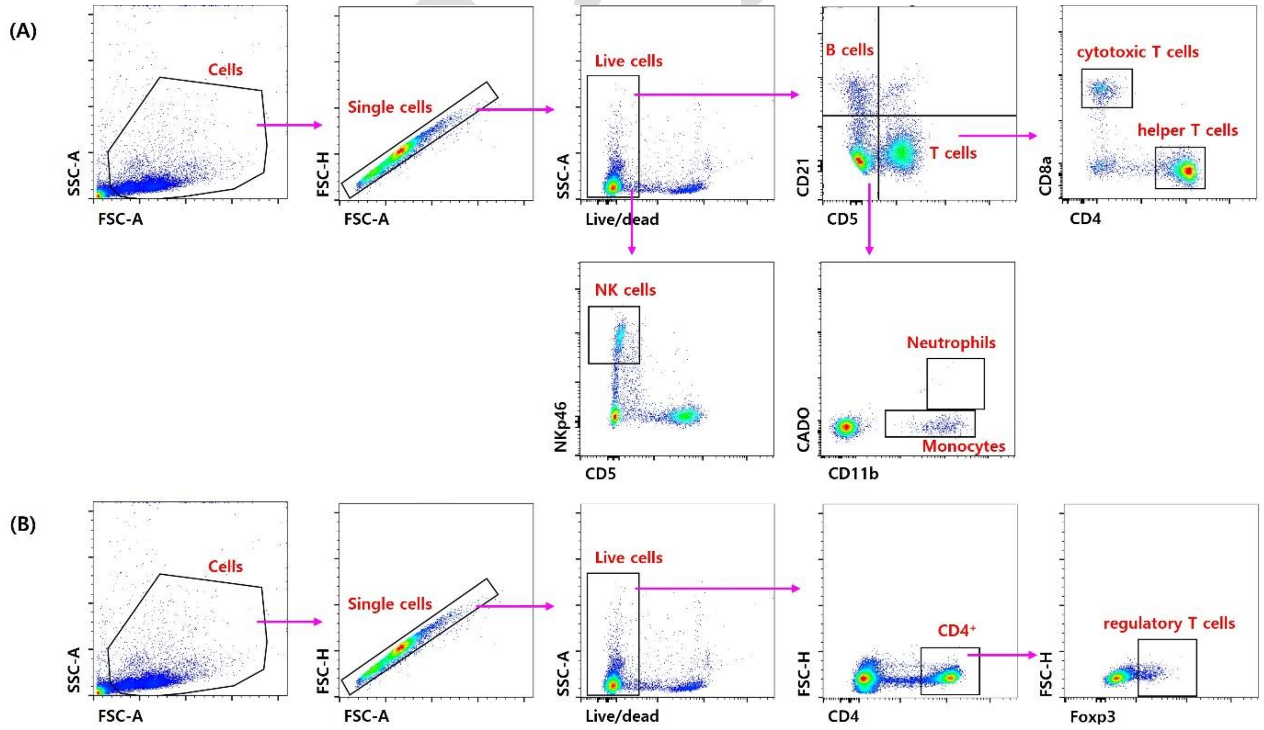


Fig. 1: Gating strategy for canine immune cell populations. Representative dot plots illustrating the gating strategy for identifying major canine immune cell populations. (A): T-cells (CD5^{+}CD21^{-}); B-cells (CD5^{-}CD21^{+}); Th (CD5^{+}CD21^{-} CD4^{+}); Tc (CD5^{+}CD21^{-} CD8a^{+}); Neutrophils (CD5^{-}CD21^{-}CD11b^{+}Neutrophil^{+}); Monocytes (CD5^{-}CD21^{-}CD11b^{+}Neutrophil^{-}); NK cells (CD5$^{-/dim}$NKp46^{+}); (B): Treg (CD4^{+}Foxp3^{+}).

RNA extraction and quantitative RT-PCR (qRT-PCR): Total RNA was extracted from the whole blood and PBMCs using TRIzol-based (Koshy *et al.*, 2025) and column-based methods, as described previously (Kim *et al.*, 2023; Huang *et al.*, 2023). RNAiso Plus (Takara Bio Inc., Shiga, Japan) was used for TRIzol-based extraction. Column-based extraction was performed using the AccuPrep® Blood RNA Extraction Kit (Bioneer, Daejeon, South Korea) for whole blood and the RNeasy Mini Kit (Qiagen, Hilden, Germany) for PBMCs. RNA quantity was measured with a DeNovix DS-11 spectrophotometer, and cDNA was synthesized using the ReverTra Ace™ qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan). Apoptosis-related genes and inflammatory cytokines expression were assessed using AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer, Daejeon, South Korea) with primers shown in Table 3. These genes were selected for their known sensitivity to sample processing delays and their clinically relevance as commonly assessed markers in immunological and pathological evaluations. In accordance with MIQE recommendations, two reference genes including β-actin and TATA-box binding protein (TBP) were selected from distinct functional classes (cytoskeletal and transcriptional, respectively) and were used in canine qRT-PCR studies (Chimura *et al.*, 2011; Rijn *et al.*, 2014). Both genes were empirically evaluated for stability across storage times (~2h, 6h, 12h), sample types (WB, PBMC), and extraction methods (TRIzol-based, column-based) by inspecting Cq dispersion and delta Cq relative to fresh controls. Unless otherwise specified, target gene expression was normalized to the reference genes.

Statistical analysis: Statistical analysis was performed using GraphPad Prism (Version 10.4.1, GraphPad Software). Data were presented as the mean±standard deviation (SD). Paired *t*-test was used to evaluate statistical significance in comparisons between two groups. The P<0.05 was considered statistically significant.

RESULTS

Profiling immune cell frequencies in PBMCs from healthy dogs: Using the predefined gating strategy (Fig. 1) and antibody panel (Table 2), baseline proportions of major leukocyte subsets in PBMCs from healthy dogs were quantified (Table 4). T-cells accounted for 30.93±17.62% of the live single-cell population. Within T-cells, helper T-cells (Th) comprised 18.70±12.04%, and cytotoxic T-cells (Tc) comprised 7.19±4.20%. B-cells constituted 7.22±5.77% of PBMCs. Monocytes made up 3.10±2.87%. Treg comprised 8.74±4.27% of CD4$^+$ cells.

Viability of WBCs and PBMCs during delayed blood processing: Using the delayed-processing design, PBMCs and WBCs maintained stable cell numbers/mL of blood across all time points. However, WBC counts were approximately five-fold higher than PBMC counts at ~2, 12, and 24h (paired two-tailed t tests with Holm–Šidák correction; all P<0.0001; Fig. 2A; Table S1). PBMC viability showed considerable individual variation and tended to decrease at 12h, but the change was not statistically significant. In contrast, WBC viability remained comparable to fresh samples at 12h. At 24h, both PBMC and WBC viability significantly declined (P<0.05) compared to fresh semen (Fig. 2B; Table S1). These findings indicate that while WBC maintains viability better than PBMC during the early period, both cell types show marked viability loss after 24h.

Supplementary Table S1: PBMC and WBC yield and viability across storage times

	~2h (Fresh)		12h		24h	
	PBMC	WBC	PBMC	WBC	PBMC	WBC
Cell per ml blood (x10^6)	0.88±0.37	4.48±1.75	0.98±0.43	5.46±1.30	1.14±0.54	5.49±1.91
Viability (%)	92.91±5.60	91.07±4.58	88.50±9.95	92.11±3.57	88.84±8.16*	87.44±7.03*

*P<0.05 vs ~2h (Fresh) within the same sample type (PBMC or WBC); paired t-test.

Table 3: List of canine-specific primers used for qRT-PCR analysis

Primer		Sequence (5'-3')	Reference
p53	Forward	CGCAAAAGAAGAAGCCACTA	Zayed and Iohara (2020).
	Reverse	TCCACTCTGGGCATCCTT	
BAX	Forward	CCAAGAAGCTGAGCGAATG	Bang *et al.* (2021).
	Reverse	CTGCCACTCGGAAGAAGAC	
Caspase3	Forward	TCATTATTCAGGCCTGCCGAGG	Mili *et al.* (2018).
	Reverse	TTCTGACAGGCCATGTCATCCTCA	
BCL2	Forward	GACAGAGAGGATCATGCTGT	Bang *et al.* (2021).
	Reverse	TGGCATGAGATGCAGGAAAT	
IL-1β	Forward	CAAGTCTCCCACCAGCTCTGTA	Tamura *et al.* (2014).
	Reverse	GGGCTTCTTCAGCTTCTCCAA	
IL-8	Forward	CTCTCTGTGAAGCTGCAGTTCTG	Tamura *et al.* (2014).
	Reverse	GGAAAGGTGTGGAGTGTGTTTTT	
TNF-α	Forward	TCTCGAACCCCAAGTGACAAG	Tamura *et al.* (2014).
	Reverse	CAACCCATCTGACGGCACTA	
β-actin	Forward	CCGCGAGAAGATGACCCAGA	Pandey *et al.* (2018).
	Reverse	GTGAGGATCTTCATGAGGTAGTCGG	

Table 4: Summary of immune cell frequencies in PBMCs from healthy dogs

Immune cell subset (Phenotype)	Frequency (%)
T-cell (CD5$^+$CD21$^-$)	30.93±17.62
Helper T-cell (Th) (CD5$^+$CD21$^-$CD4$^+$)	18.70±12.04
Cytotoxic T-cell (Tc) (CD5$^+$CD21$^-$CD8$^+$)	7.19±4.20
B-cell (CD5$^-$CD21$^+$)	7.22±5.77
Monocyte (CD5$^-$CD21$^-$CD11b$^+$Neutrophil$^-$)	3.10±2.87
Regulatory T-cell (Treg) (CD4$^+$Foxp3$^+$)	8.74±4.27

T-cell, Th, Tc, B-cell, and Monocyte frequencies are presented as a percentage of lymphocytes. Treg frequency is presented as a percentage of CD4$^+$ cells.

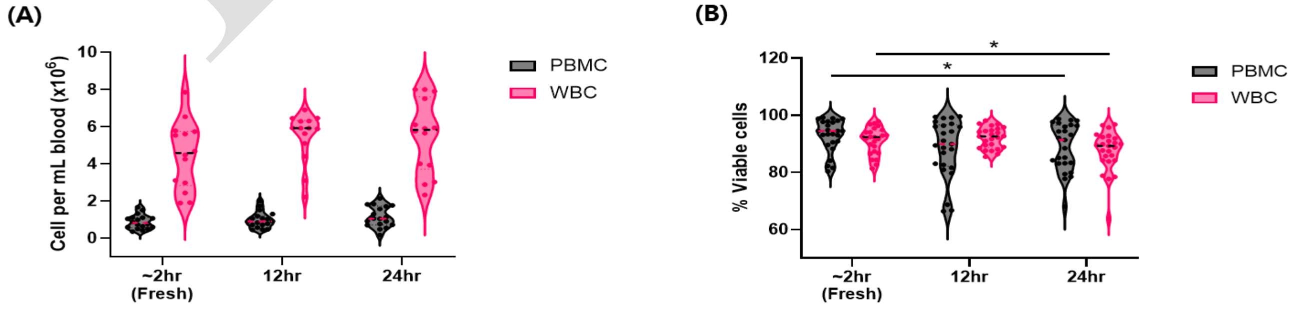


Fig. 2: Effect of room temperature storage of whole blood on subsequent PBMC and WBC isolation. (A): Total cell counts of PBMCs and WBCs at each time point, quantified after isolation. (B): Cell viability of PBMCs and WBCs at fresh, 12h, and 24h, assessed by flow cytometry; *P<0.05.

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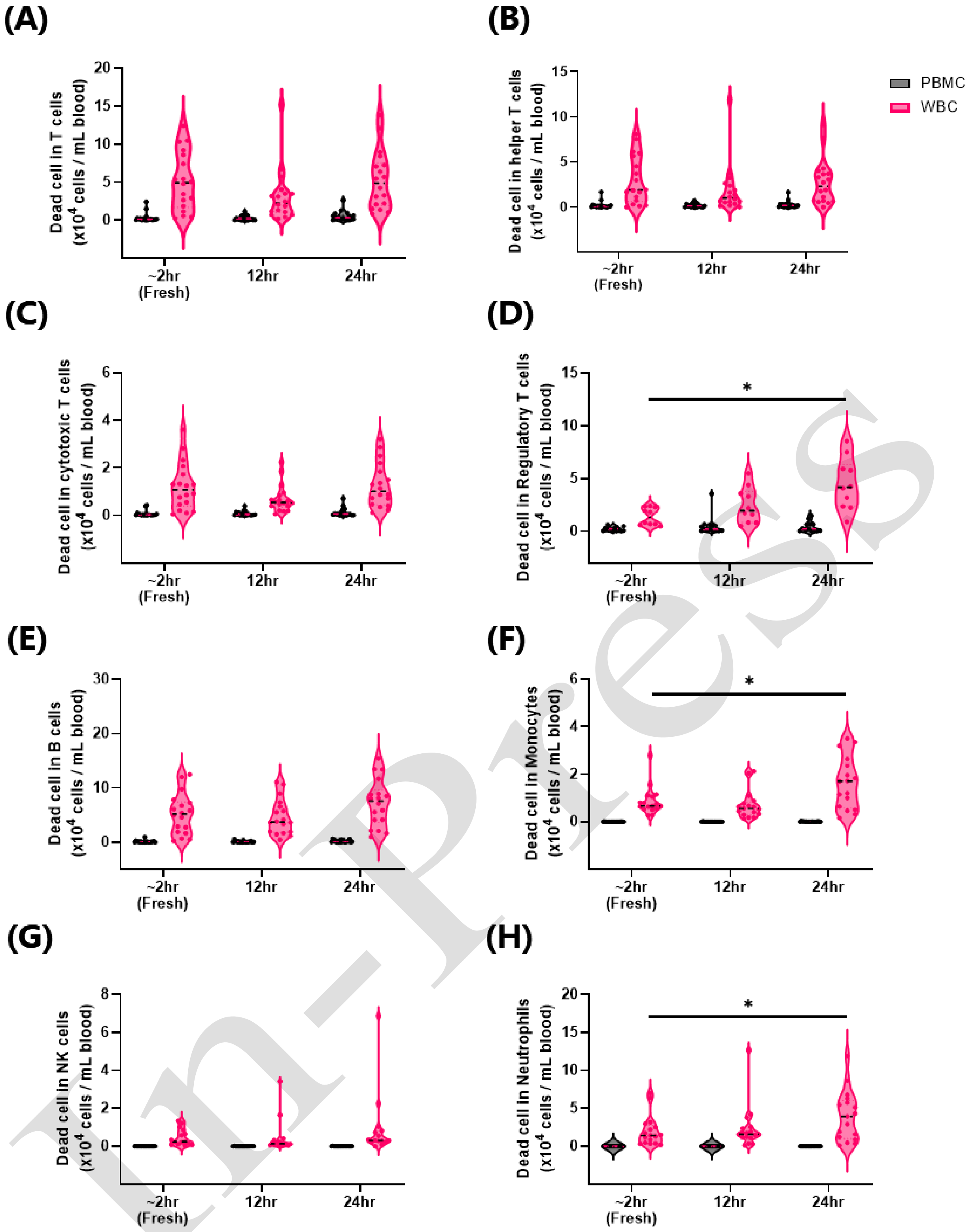

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Fig. 3: Absolute dead cell counts in immune cell subsets of PBMCs and WBCs across processing times. Violin plots show the absolute numbers of viability-dye-positive (dead) cells in PBMCs (black) and WBCs (pink) at fresh, 12h, and 24h post-collection. (A): T-cells, (B): Th, (C): Tc, (D): Treg, (E): B-cells, (F): Monocytes, (G): NK cells, and (H): Neutrophils were analyzed by flow cytometry; *P<0.05.

Viability of immune cell subsets is differentially affected by delayed blood processing: Using dead cell counts (absolute number), PBMCs showed statistically non-significant changes at 12 or 24h across T-cells (Fig. 3A), Th (Fig. 3B), Tc (Fig. 3C), Treg (Fig. 3D), B-cells (Fig. 3E), monocytes (Fig. 3F), NK cells (Fig. 3G), and neutrophils (Fig. 3H), compared with fresh controls. For WBCs, dead cell counts at 12h did not differ significantly from fresh blood across subsets. At 24h, however, regulatory T-cells (Fig. 3D), monocytes (Fig. 3F), and neutrophils (Fig. 3H) showed a marked and statistically significant increase (P<0.05) in dead cell counts, whereas T-cells (Fig. 3A), Th (Fig. 3B), Tc (Fig. 3C), B-cells (Fig. 3E), and NK cells (Fig. 3G) showed non-significant change up to 24h. Numerical summaries for all subsets are provided in Supplementary Table S2.

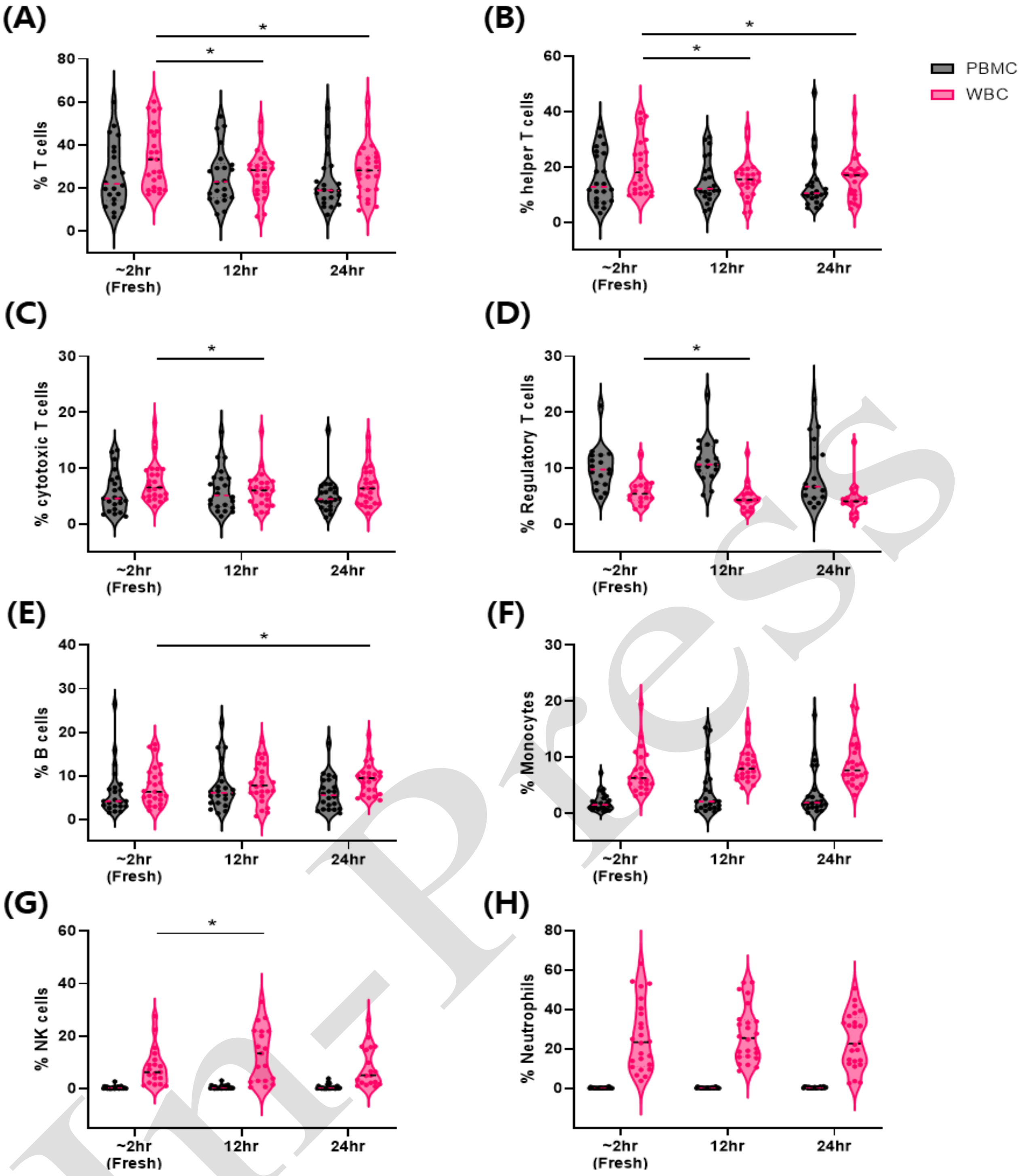

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Fig. 4: Time-dependent changes in immune cell frequency in PBMCs and WBCs stored at room temperature. Violin plots show the percentage of immune cell subsets in PBMCs (black) and WBCs (pink) at fresh, 12h, and 24h post-collection (A): Total T-cell frequency, (B): Th, (C): Tc, (D): Treg, (E): B-cells, (F): Monocytes, (G): NK cells, and (H): Neutrophils were analyzed by flow cytometry; *P<0.05.

Supplementary Table S2: Time-dependent changes in dead cell counts among immune cell in PBMCs and WBCs

Immune cell subset (Phenotype)	Dead cell absolute number (x10^{3} cells/mL blood)					
	~2h (Fresh)		12h		24h	
	PBMC	WBC	PBMC	WBC	PBMC	WBC
T-cell (CD5^{+}CD21^{-})	3.52±6.11	51.45±37.25	3.21±3.38	31.89±34.06	5.28±6.41	52.98±37.13
Th (CD5^{+}CD21^{-}CD4^{+})	2.11±3.98	31.90±25.70	1.93±2.05	19.89±26.76	2.87±3.91	29.08±23.96
Tc (CD5^{+}CD21^{-}CD8^{+})	0.76±1.24	12.15±9.64	0.81±1.03	7.03±5.61	1.29±1.78	12.64±9.01
B-cell (CD5^{-}CD21^{+})	1.29±2.18	52.01±36.38	1.06±1.36	46.12±31.42	1.77±1.83	72.24±42.09
Monocyte (CD5^{-}CD21^{-}CD11b^{+}Neutrophil^{-})	0.02±0.05	8.72±5.76	0.01±0.05	7.04±5.63	0.06±0.10	16.34±10.58*
Neutrophil (CD5^{-}CD21^{-}CD11b^{+}Neutrophil^{+})	0.00±0.00	20.07±19.37	0.00±0.00	23.65±27.84	0.03±0.05	39.93±31.94*
NK cell (CD5$^{-/dim}$NKp46^{+})	0.01±0.03	4.82±3.92	0.02±0.05	4.72±9.04	0.02±0.06	9.61±16.90
Treg (CD4^{+}Foxp3^{+})	2.23±1.89	31.49±54.40	5.14±9.05	24.49±15.96	4.25±4.57	44.83±23.20*

T-cell, Th, Tc, B-cell, Monocyte, Neutrophil and NK cell frequencies are presented as a percentage of lymphocytes. Treg frequency is presented as a percentage of CD4^{+} cells. *P<0.05 vs ~2h (Fresh) within the same sample type (PBMC or WBC); paired t-test.

Immune cell frequency profiles are more stable in PBMCs than in WBCs during prolonged storage: To examine time-dependent changes in immune cell frequency in PBMCs and WBCs, specific immune cell populations were analyzed using flow cytometry. No significant alterations were observed in PBMCs up to 24h in T-cells (Fig. 4A), including Th (Fig. 4B), Tc (Fig. 4C), and Treg (Fig. 4D), as well as in B-cells (Fig. 4E), monocytes (Fig. 4F), NK cells (Fig. 4G) and neutrophils (Fig. 4H). In contrast, WBCs showed a significant decline (P<0.05) in the percentages of T-cells (Fig. 4A) and their subsets including Th (Fig. 4B), Tc (Fig. 4C), and Treg (Fig. 4D) compared to fresh samples from 12h onward. At 24h, the reductions in total T-cells (Fig. 4A) and Th (Fig. 4B) reached statistical significance. While monocytes (Fig. 4F) and neutrophils (Fig. 4H) remained unchanged up to 24h, B-cells (Fig. 4E) and NK cells (Fig. 4G) showed a relative increase at 24h and 12h, respectively. The detailed results of immune cell frequencies are presented in Supplementary Table S3.

RNA yield maintained up to 12h, while column-based extraction ensures greater gene expression consistency: RNA yields from WB and PBMCs were stable at 6 and 12h relative to fresh controls (Fig. 5A). In WB, TRIzol-based extraction yielded approximately two-fold higher yields than the column-based method across time points, whereas PBMC yields were comparable between TRIzol-based and column-based extraction methods (Fig. 5A). These results indicate short-term RT storage does not significantly impact the RNA yield.

Beta-actin and TBP expression were used to assess gene expression stability in WB and PBMCs. The Cq values and delta Cq changes were analyzed at 6 and 12h relative to fresh samples. Although the absolute Cq levels differed between beta-actin (Fig. 5B) and TBP (Fig. 5D), both reference genes exhibited similar profiles. In WB, the column-based method showed more consistent results over time, while the TRIzol-based method exhibited higher inter-sample variability, with inconsistencies evident from 6h. The same pattern was observed in PBMCs, with overall variability larger than in WB. Between the two reference genes, TBP (Fig. 5E) exhibited slightly narrower dispersion than beta-actin (Fig. 5C) under the same conditions.

Impact of sample processing time and RNA extraction methods on gene expression consistency: Table 5 (apoptosis-associated genes: p-53, BAX, Caspase-3, BCL-2) and Table 6 (inflammatory cytokines: IL-1β, IL-8, TNF-α) summarize qRT-PCR results by matrix (WB, PBMC), extraction method (column-based vs TRIzol-based), time (fresh, 6h, 12h), and reference gene (beta-actin and TBP). Across matrices, short-term RT storage produced method- and gene-dependent shifts, but the overall patterns were concordant between beta-actin- and TBP-normalized analyses, which are presented side-by-side. In WB, several targets including p-53 and Caspase-3 decreased significantly (P<0.05) from 6h, whereas IL-8 tended to increase (Table 6). BCL-2 remained largely unchanged (Table 5). In PBMCs, expression patterns differed by method. In the column-based method, BAX, Caspase-3 (Table 5), and IL-8 (Table 6) changed significantly (P<0.05), whereas TRIzol-based method showed non-significant change except for p-53. Variability was consistently higher in the TRIzol-based method (Table 5 and Table 6). Taken together, extraction method and processing time influenced gene expression levels in WB and PBMC samples.

Supplementary Table S3: Time-dependent changes in immune cell frequencies in PBMCs and WBCs from healthy dogs

Immune cell subset (Phenotype)	Frequency (%)					
	~2h (Fresh)		12h		24h	
	PBMC	WBC	PBMC	WBC	PBMC	WBC
T cell (CD5^{+}CD21^{-})	26.05±14.32	34.32±14.06	25.61±12.62	26.37±10.27*	23.08±12.63	28.15±11.63*
Th (CD5^{+}CD21^{-}CD4^{+})	15.94±9.16	20.50±9.86	15.18±7.78	15.78±7.00*	13.83±9.59	16.17±7.99*
Tc (CD5^{+}CD21^{-}CD8^{+})	5.69±3.53	7.63±3.59	6.15±3.75	6.03±3.08*	5.10±3.06	6.60±3.26
B cell (CD5^{-}CD21^{+})	6.45±5.64	7.69±4.40	7.55±5.21	8.36±4.46	6.06±3.80	9.39±3.60*
Monocyte (CD5^{-}CD21^{-}CD11b^{+}Neutrophil^{-})	1.98±1.60	7.30±3.74	3.98±4.48	9.81±6.98	3.80±4.29	8.87±4.03
Neutrophil (CD5^{-}CD21^{-}CD11b^{+}Neutrophil^{+})	0.16±0.22	26.21±17.16	0.20±0.23	27.47±13.79	0.65±1.45	24.31±13.70
NK cell (CD5$^{-/dim}$NKp46^{+})	0.39±0.55	8.27±7.06	0.50±0.65	13.09±10.12*	0.62±0.92	8.24±7.33
Treg (CD4^{+}Foxp3^{+})	10.09±3.73	5.74±2.25	11.42±3.99	4.73±2.43*	9.16±5.61	4.45±2.92

T-cell, Th, Tc, B-cell, Monocyte, Neutrophil and NK cell frequencies are presented as a percentage of lymphocytes. Treg frequency is presented as a percentage of CD4^{+} cells. *P<0.05 vs ~2h (Fresh) within the same sample type (PBMC or WBC); paired t-test.

Table 5: Apoptosis-associated gene expression values by extraction method and reference gene in WB and PBMCs across storage times

Group	Reference gene	p-53			BAX			Caspase-3			BCL-2		
		~2h	6h	12h	~2h	6h	12h	~2h	6h	12h	~2h	6h	12h
WB_Column tube	Beta-actin	0.44±0.08	0.30±0.04*	0.23±0.02*	0.38±0.13	0.40±0.13	0.46±0.1*	0.47±0.12	0.34±0.06*	0.27±0.06*	0.12±0.08	0.18±0.10	0.23±0.19
	TBP	231.04±84.75	114.74±33.64*	98.76±107.43	217.19±95.19	153.89±63.23	196.84±204.45	218.23±70.86	129.62±42.32*	100.83±85.06	66.02±29.68	73.44±56.72	69.30±42.33
WB_TRIzol	Beta-actin	0.8±0.13	0.55±0.17*	0.43±0.04*	0.77±0.20	0.68±0.25	0.62±0.23	1.22±0.12	0.93±0.20*	1.14±039	0.32±0.21	0.27±0.14	0.20±0.09
	TBP	13.07±1.54	8.41±3.53*	4.15±2.83*	12.33±2.94	10.92±6.62	5.89±3.34*	20.03±3.28	14.69±7.26	9.48±5.72*	4.98±2.52	4.62±2.72	2.51±2.35
PBMC_Column tube	Beta-actin	0.96±0.18	0.81±0.23	0.82±0.35	0.67±0.15	1.03±0.44	1.15±0.40*	1.71±0.55	1.55±0.51	1.72±0.71*	0.35±0.30	0.84±0.35	1.14±0.63
	TBP	0.99±0.67	0.74±0.41	0.71±0.43	0.80±0.73	1.02±0.73	1.08±0.89*	1.57±0.78	1.30±0.51	1.37±0.57	0.28±0.13	0.71±0.34*	0.93±0.63*
PBMC_TRIzol	Beta-actin	1.56±0.42	1.18±0.30*	1.00±0.19*	0.87±0.37	0.88±0.43	0.79±0.47	2.25±0.82	2.93±1.59	3.00±1.16	0.94±0.77	0.60±0.30	0.53±0.54
	TBP	49.73±24.81	21.40±10.61	11.75±7.14	33.58±20.13	14.37±10.05	10.59±7.30	62.14±37.58	47.90±37.11	31.72±15.63	28.81±15.22	11.87±8.09	5.96±4.24*

The values represent mRNA expression levels calculated using the $2^{-\Delta\Delta Cq}$ method. *P<0.05 vs ~2h (Fresh) within the same group and the same reference gene; paired t-test.

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Table 6: Inflammatory cytokines gene expression values by extraction method and reference gene in WB and PBMCs across storage times

Group	Reference gene	IL-1β			IL-8			TNF-α		
		~2h	6h	12h	~2h	6h	12h	~2h	6h	12h
WB _ Column tube	Beta-actin	0.22±0.07	0.19±0.08	0.19±0.07	0.24±0.10	0.98±0.42*	1.32±0.59*	0.15±0.06	0.10±0.03	0.11±0.05*
	TBP	126.45±70.40	73.74±38.33	108.24±160.16	128.40±77.81	395.88±187.36*	323.18±163.58*	74.87±28.89	39.07±19.62*	37.98±34.71
WB _ TRIzol	Beta-actin	0.80±0.37	0.54±0.28*	0.67±0.38	0.67±0.32	2.23±0.95*	2.85±1.17*	0.25±0.10	0.19±0.06*	0.18±0.06
	TBP	13.23±6.77	8.66±7.52*	4.53±2.37*	11.05±5.18	37.38±21.00*	29.06±26.11	4.01±1.29	2.81±1.14	1.60±1.00*
PBMC _ Column tube	Beta-actin	0.75±0.39	0.86±0.35	1.37±0.99	0.02±0.01	0.05±0.03*	0.10±0.09	0.44±0.30	0.63±0.39	1.11±1.09
	TBP	0.56±0.05	0.66±0.11	0.94±0.45	0.02±0.01	0.05±0.04	0.07±0.05*	0.35±0.14	0.45±0.14	0.69±0.51
PBMC _ TRIzol	Beta-actin	0.11±0.04	0.32±0.30	0.86±1.13	0.05±0.04	0.13±0.10	0.18±0.14	0.46±0.16	0.36±0.12	0.94±1.07
	TBP	2.71±1.11	3.74±3.10	12.39±14.06	1.10±0.75	1.74±0.98*	1.57±0.90*	15.44±10.93	6.80±5.01	9.27±9.17

The values represent mRNA expression levels calculated using the $2^{-\Delta\Delta Cq}$ method. *P<0.05 vs ~2h (Fresh) within the same group and the same reference gene; paired t-test.

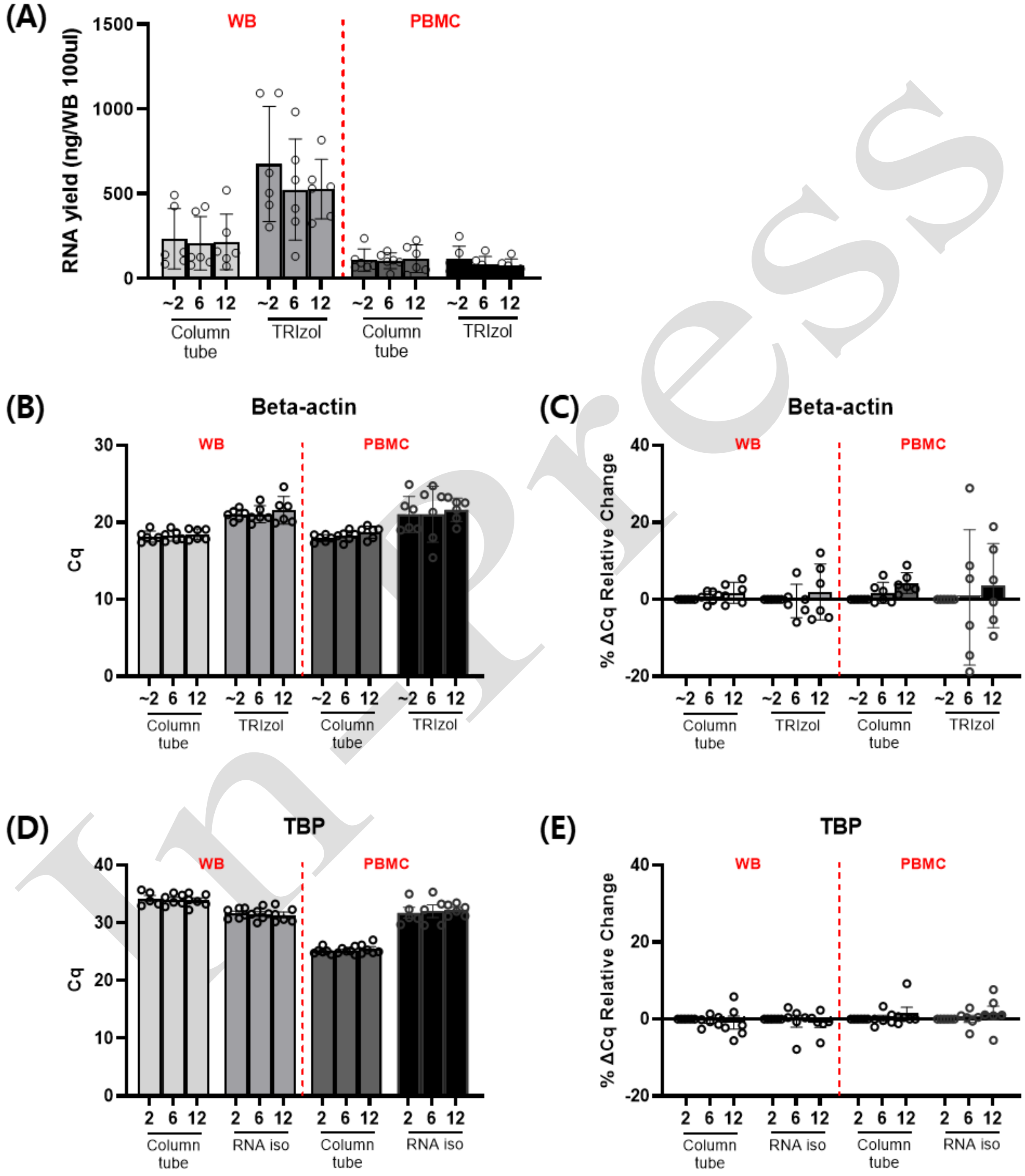

Fig. 5: Time-dependent effect of RT storage on RNA yield and gene expression stability in WB and PBMCs. (A): RNA yield was quantified at each time point. (B): Quantification of beta-actin Cq values to assess gene expression stability over time, comparing RNA extracted using column-based and TRIzol-based methods. (C): Relative ΔCq changes of beta-actin, normalized to fresh samples, to evaluate the impact of extraction methods on gene expression consistency over time. (D): Quantification of TBP Cq values. (E): Relative ΔCq changes of TBP, normalized to fresh samples.

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DISCUSSION

In this study, we identified the normal immune cell frequencies, each immune cell viability and RNA expression levels for up to 24h of blood storage at RT in companion dogs. To date, limited data of normal immune cell profiles in companion dogs have been shown in few studies where the profiles were presented as data of control group against diseased group such as cancer (Karayannopoulou *et al.*, 2017; Sparger *et al.*, 2021), immune-mediated disorders (Majewska *et al.*, 2016), and heart disease (Druzhaeva *et al.*, 2021). These studies provided limited information because small numbers of dogs (8~19 dogs) were involved. Other studies that included larger number of samples primarily focused on Beagles (Fujiwara *et al.*, 2012), or examined only a limited set of markers, such as cytokines (Calvalido *et al.*, 2016; Mavropoulou *et al.*, 2016), CD3, CD4, CD8, or gamma-delta T-cell receptors (Faldyna *et al.*, 2001). Our study attempted to fill this gap by investigating immune cell profiles in canine PBMCs from 43 healthy companion dogs, not experimental dogs, also to provide a preliminary data and methodological trials for future research in canine patients.

Comparing our result to existing data was restricted, though, one study that described immune cell frequencies in eight healthy dogs against atopic dermatitis group, reported T-cells comprising 35.90±2.65% of WBCs, Th accounting for 70.70±1.94% of T-cells, and Tc making up 5.31±0.41% of T-cells. Treg represented 0.48±0.08% of WBCs (Majewska *et al.*, 2016). Although direct comparison is difficult due to differences in sample types and population definitions (WBC vs. PBMC and percentages based on T-cells vs. PBMCs), the frequencies of T-cells, Th, and Tc observed in the previous study fall within the described ranges in our study. Since the present study included a larger number of samples and diverse breeds, the results exhibited a broader range compared to the previous study (Majewska *et al.*, 2016). As this is the preliminary study of immune cell frequency from healthy companion dogs, further research incorporating additional data is necessary to refine the ranges of each immune cell frequency.

Delayed processing of blood led to decreased cell viability, particularly 24h post-venipuncture in both WBCs and PBMC isolation. These findings align with those of the previous human studies, which reported decline in cell viability over 24 to 96h (Savage *et al.*, 2021; Jiang *et al.*, 2023). In human, Zini (2014) found the highest cell viability at room temperature, while Belloni *et al.* (2008) suggested that storage of blood at 4°C is optimum for preserving cell viability. To determine the precise optimal conditions for preserving viable canine immune cells during transportation or storing in laboratories, further experiments are needed using different temperature settings.

In the white blood cell (WBC) samples, a significant increase in the absolute number of dead cells was observed across three phenotypes: monocytes, neutrophils, and Treg. This finding, which especially shows monocytes and neutrophils losing their viability during processing delay at RT, is consistent with the previous human studies (Carter *et al.*, 1992; Tong *et al.*, 2016), indicating a comparable trend for the human and canine blood. Among the cells, Treg exhibited a significant decline in viability. However, no definitive study reporting similar findings in both humans and dogs could be identified in the literature. A previous human cell study investigating the effect of delayed cell processing on immunotyping was conducted but each subset of functional Th, including Treg, could not be verified (Thyagarajan *et al.*, 2018). The viability of Treg is known to be influenced by certain common gamma-chain cytokines, such as IL-2 or IL-15 (Pandiyan and Lenardo, 2008). Therefore, it is possible that cytokine levels in WB might contribute to the decreased viability of Treg. Given the decreased viability of monocytes, which indirectly influences the level of IL-2 (Boring *et al.*, 1997) and is directly involved in the expression or secretion of IL-15 (Armitage *et al.*, 1995; Doherty *et al.*, 1996), there is a possibility that Treg are influenced by such altered environment during storage. As this is the first study to store Treg in WB under RT conditions for up to 24h, further investigation is warranted. Future studies investigating this vulnerability of Treg during delayed processing under various conditions will be crucial to identifying the specific factors that influence their vulnerability in both humans and animals.

Although each cell frequency remained stable for up to 24h in PBMC, a significant decrease in T-cell subset frequency within WBCs was observed at 12h. Previous human studies have shown that granulocytes can affect T-cell viability and function, leading to recommendations for blood dilution before analysis (McKenna *et al.*, 2009; Yi *et al.*, 2023). Additionally, stimulation can prevent apoptosis, as has been reported in a previous human-cell study (Van-Parijs *et al.*, 1996). As our study aimed to define the changes during shipping or storing in laboratories under natural status of immune cells, stimulations to each sample were not applied. To maintain T-cell viability, blood processing should be completed within 12h of venipuncture, or CD3/CD28 stimulation (Kunz *et al.*, 2017) can be performed to prolong the viability of T-cells. Other factors that can be considered to maintain the viability of T-cells include the types of anticoagulants used, temperature, and the PBMC isolation process (Browne *et al.*, 2024).

NK and B-cells in whole blood exhibited an increasing trend in frequency at 12h. This result was supported by a study in humans (Thyagarajan *et al.*, 2018), which found that B-cell and NK cell percentages increased over time. In that study, NK cells were stored in whole blood without stimulation for 72h at room temperature, resulting in a significant increase in NK cell numbers. Meanwhile, B-cells showed a time-dependent decline in naïve B-cells, while IgD-negative memory B-cells increased, leading to an overall rise in B-cell numbers (Thyagarajan *et al.*, 2018). Since our study did not differentiate between B-cell subsets, and there is no previous canine report about changes in NK cell numbers, we were unable to determine the specific factors influencing these changes.

To assess whether RNA expression levels are also maintained in canine blood samples over time, we used two representative extraction methods, including the column extraction method and TRIzol method. As noted in previous studies, the column extraction method provides more consistent RNA yields (Tesfamichael *et al.*, 2020).

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While the TRIzol method resulted in higher RNA quantities, its extraction efficiency was less stable. Consistent with human studies (Savage *et al.*, 2021), our findings indicate that RNA yield in dogs remains relatively unchanged for up to 12h. To better preserve RNA, cryopreservation may have been a more effective approach (Jiang *et al.*, 2023). However, given the challenges of transportation and storing in laboratory conditions, we assessed the stability under natural conditions. The expression levels of inflammatory cytokines and apoptosis-associated genes varied depending on time, sample types and extraction methods in our results. Therefore, further studies on RNA expression of inflammatory cytokines and apoptosis- related genes in canine peripheral blood should maintain consistent experimental conditions for each gene and avoid processing delays. It is traditionally well-known that RNA expression levels vary significantly depending on the specific subject under investigation (Kim *et al.*, 2007; Yang *et al.*, 2016), as well as the storage duration and temperature conditions. Indeed, prior studies have primarily focused on identifying conditions optimized for reliable RNA analysis (Rainen *et al.*, 2002). To ensure the acquisition of reproducible research results using canine blood, it seems essential to adhere to a consistent protocol that is appropriate for the target subject. This meticulous standardization is crucial, as it directly impacts the reliability and scientific rigor of the study.

This study has several limitations. Recruited dogs had various signalments, but the research was conducted at a single institution, primarily involving small breeds. As the animals involved were the companion dogs, the specific anti-parasitic medications (targeting endoparasites and/or ectoparasites) varied among individual dogs. Due to considering situations of transportation or keeping in laboratory, temperature setting was limited to RT.

Conclusions: In conclusion, our study described normal immune cell frequencies in 43 healthy companion dogs and also recorded cell viability in both whole blood and PBMCs over time. Additionally, the stability and expression levels of RNA for inflammatory cytokines and apoptosis-related genes over the course of processing delay were also assessed. These findings suggest standardizing and maintaining consistent protocols for each subject according to the specific factors to be investigated in the further studies. Our findings could serve as an initial framework for future canine immunological studies, providing a sound methodological basis.

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Author's contribution: LSM and LSI conceptualized the idea, OEY and LJY carried out data curation and formal analysis. Funding acquisition was carried out by LSM, while investigation was conducted by KY and SJ. LSM was responsible for methodology. LSM and LSI handled project administration. KY, SJ and LSI provided resources. OEY and LSM developed the software. LSM and LSI performed supervision. KY and LSI were in-charge of validation. OEY and LJY prepared the visualization. KY and OEY wrote the original draft, and LSM and LSI were responsible for review and editing.

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