


RESEARCH ARTICLE

Open Access



# Profile of gamma-delta ( $\gamma\delta$ ) T lymphocytes in the peripheral blood of crossbreed dogs during stages of life and implication in aging

Cristina Marchetti, Paolo Borghetti, Antonio Cacchioli, Luca Ferrari<sup>\*</sup> , Federico Armando, Attilio Corradi<sup>†</sup> and Anna Maria Cantoni<sup>†</sup>

## Abstract

**Background:** Data on gamma-delta ( $\gamma\delta$ ) T lymphocytes in the peripheral blood of dogs are scant, related only to healthy pure breed dogs and limited to a restricted age range. The aim of the study was to investigate the modulation of the  $\gamma\delta$  T lymphocyte (TCR $\gamma\delta$ +) subpopulation in peripheral blood of crossbreed healthy dogs according to five identified stages of life: Puppy, Junior, Adult, Mature, Senior and to determine its implication in aging.

A rigorous method of recruitment was used to minimize the influence of internal or external pressure on the immune response. Twenty-three intact female and twenty-four intact male dogs were enrolled. Blood samples were collected and immunophenotyping of peripheral blood T lymphocytes and  $\gamma\delta$  T cell subpopulations was performed.

**Results:** The percentage of  $\gamma\delta$  T cells in peripheral blood lymphocytes was comparable with the value of 2.5% published by Faldyna and co-workers (2001), despite the percentage reported was investigated in less arranged age range groups and coming from four different dog pure breeds, whereas our data were recorded on wider age range groups and coming from crossbreed dogs. Therefore, the  $\gamma\delta$  T cell percentage (2.5%) is consistent and points out that such value is breed-independent. Statistical analysis highlighted differences in both percentage and absolute  $\gamma\delta$  T cells according to the stage of life.  $\gamma\delta$  T cells decreased significantly in the peripheral blood of elder dogs (Senior group) in comparison with previous stages of life (Puppy, Junior, and Adult groups). Differences in  $\gamma\delta$  T cells are significant and they are reported, for the first time, related to dog aging.

**Conclusions:** The study confirms dogs to be among the animals with a low TCR $\gamma\delta$ + cell profile. A decrease of the TCR $\gamma\delta$ + subpopulation percentage was observed in elder dogs. TCR $\gamma\delta$ + cells of group S were different from those of groups P, J, and A. The differences are reported for the first time in dog aging. Identifying the stage of life when the decrease of  $\gamma\delta$  T lymphocytes starts can be useful for providing a rationale for drafting a wellness plan trial to support thymus immune functions and mitigate its functional exhaustion.

**Keywords:**  $\gamma\delta$  T lymphocytes, Dog, Stage of life, Breed, Aging

\* Correspondence: [luca.ferrari@unipr.it](mailto:luca.ferrari@unipr.it)

<sup>†</sup>Attilio Corradi and Anna Maria Cantoni contributed equally to this work.  
Department of Veterinary Science, University of Parma, Strada del Taglio, 10,  
43126 Parma, Italy



© The Author(s). 2020 **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

## Background

Scientific papers on the lymphocyte subpopulation profile in clinically healthy dogs according to age and sex have been published in large numbers, especially in the last 20 years [1–6]. Data are also available on the gamma-delta ( $\gamma\delta$ ) T cell subset ( $\gamma\delta$  T lymphocytes) associated with dog autoimmune diseases [7, 8] or tumors [4, 9–13], but currently, there is a lack of data available on  $\gamma\delta$  T cells in the peripheral blood of healthy dogs. The only data investigating the peripheral blood of healthy pure breed dogs are referred to less arranged age groups [3].

Canine  $\gamma\delta$  T cells were also investigated in lymphoid organs in early post-natal life [14] and the small intestine revealed the presence in the epithelium of villi, being more numerous in the tip of the villous in comparison with the base [15]. Scattered  $\gamma\delta$  cells were also observed in the epithelium of crypts, while not detected either in Peyer's patches or in isolated mucosal lymphoid follicles [15].  $\gamma\delta$  T cells mature mainly in the thymus but may even mature in an extra-thymic microenvironment such as the intestine in the mouse [16].

A study on the distribution of  $\gamma\delta$  cells in the blood circulation defined two different  $\gamma\delta$  cell profiles: animals with a low population (less than 10%) and animals with a high population (more than 10%) [17]. Dogs, like rodents and human beings, have a low  $\gamma\delta$  T cell profile (2.5%) [3]. A higher  $\gamma\delta$  cell fraction (10–90%) is resident within epithelial surfaces, both in the outer epithelial layer (skin) and in the inner luminal epithelium of the body (e.g. gut, and reproductive tract) [18]. An increase of  $\gamma\delta$  T cell number was observed in bitch uterus mucosa affected by pyometra [19].

The mammalian immune system undergoes dysregulation with aging, a process also known as immunosenescence, which involves age-related changes within central lymphoid organs, detectable as lymphocyte production, migration and function. Thymopoiesis during aging is reduced with thymus involution recognized in human beings as well as in animals [20]. In the canine species, a reduction of the thymic output is age-related and associated with longevity. In large sized short-lifespan pure breed dogs, the thymus cell output decreases earlier within the lifetime of the animal in comparison to small sized long-lifespan pure breed dogs [21]. The distress induces acute thymus atrophy, while inflammation, autoimmune diseases, psychogenic distress conditions, immune suppression, immune stimulation, immunonutrition as well as the body score condition (BSC) interact with, involve and influence the immune response. Likewise, the prolonged physical exercise and heavy training are associated with the suppression of cell function within the immune system [22]. Chronic distress, psychogenic distress, (e.g. confinement, long-term hospitalization, trauma, pain)

[23, 24] or immune suppression therapy (e.g. glucocorticoids) can play an important role in determining the efficacy of the canine immune response [25].

Mammals, including the canine species, suffer from the impact of aging, resulting in a decline of the thymic output and reduction of the immune response; moreover, inflammation during aging can impair the immune responses [26]. Having few data currently available investigating the complexity of biology of  $\gamma\delta$  T lymphocytes within the canine species, this topic presents an intriguing opportunity to define the  $\gamma\delta$  T cell profile within the peripheral blood in healthy crossbreed dogs according to the stage of life and implication in aging.

## Results

### Dog recruitment

The study was successful in recruiting 47 (23 intact females and 24 intact males) crossbreed healthy dogs, aged from 4 months to 13 years.

Forty-two dogs were recruited from the VTH and the remaining five were recruited from private veterinary practice VTH partners. These cases were recruited over the course of a 2-year period and singular data of 47 recruited dogs are summarized in Table 1.

Data of recruited dogs according to age, sex, weight and clinical examination are summarized in Table 2. All blood samples collected were suitable for flow cytometry analysis (Fig. 1) and cell viability was assessed as > 95% in all samples. Outlier analysis did not provide extreme values deviating from other observations on data.

### Quantification of WBC, T lymphocytes and $\gamma\delta$ T lymphocytes

Mean values  $\pm$  SD and range values (minimum and maximum) of white blood cells (WBC), total lymphocytes, and CD3+ T lymphocyte counts and percentages in the peripheral blood of dogs in the different stages of life are reported in Table 3 and Fig. 2. Percentage and absolute mean values  $\pm$  SD and ranges of TCR $\gamma\delta$ + cells are summarized in Table 3 and shown in Fig. 2 and Fig. 3.

All-pairs comparison test for normally distributed data with unequal variances stage of life vs. stages of life, via Tamhane's T2 test, are summarized in Table 4. Tamhane's test was also performed for the other immune subpopulations.

The levels of WBC showed a significant increase from Puppy to adulthood and a subsequent decrease in Senior dogs ( $p < 0.05$ ). Both total lymphocytes and CD3+ T lymphocytes showed a significant decrease of cellularity from the young age/adulthood to aged animals ( $p < 0.05$ ) (Fig. 2).

The percentages of TCR $\gamma\delta$ + cells related to the age of dogs independently of the inclusion in one of the five groups identified for classification highlighted a high

**Table 1** Data of recruited dogs according to age, sex, weight and clinical examination

Dog	Age		Sex Female (F) / Male (M)	Weight Kg	Clinical examination VTH or VTH partners
	Years	Months			
1	13	0	F	29.0	Routine check-up; Periodontitis stage 3 *
2	13	2	F	30.0	Routine check-up; Periodontitis stage 2
3	7	2	F	36.0	Routine check-up
4	5	3	F	5.0	Neutering
5	12	6	M	12.0	Orthopedic follow-up; Periodontitis stage 2
6	5	4	F	31.0	Ophthalmic follow-up
7	2	4	M	22.5	Ophthalmic follow-up
8	9	2	F	25.0	Routine check-up; Periodontitis stage 2
9	9	5	F	27.0	Routine check-up; Periodontitis stage 2
10	8	3	M	38.0	Routine check-up; Periodontitis stage 2
11	1	6	M	16.4	Microfilaria and antigen testing
12	6	4	M	18.0	Routine check-up; Periodontitis stage 2
13	0	11	F	18.0	Neutering
14	9	0	M	8.6	Routine check-up; Periodontitis stage 3 *
15	3	8	M	57.0	Orthopedic follow-up
16	5	0	M	43.0	Ophthalmic follow-up
17	5	2	F	44.0	Neutering
18	5	5	M	46.0	Ophthalmic follow-up
19	0	11	F	13.0	Neutering
20	1	6	M	22.0	Ophthalmic follow-up
21	4	6	F	48.5	Orthopedic follow-up
22	1	1	M	36.0	Microfilaria and antigen testing
23	2	11	M	25.0	Microfilaria and antigen testing
24	2	6	F	25.0	Ophthalmic follow-up
25	0	8	F	42.0	Neutering
26	1	4	M	37.6	Ophthalmic follow-up
27	1	0	M	39.5	Ophthalmic follow-up
28	0	8	F	16.5	Neutering
29	12	9	M	35.0	Orthopedic follow-up; Periodontitis stage 3
30	10	1	M	32.0	Routine check-up; Periodontitis stage 3
31	0	9	F	5.7	Neutering
32	0	9	M	8.0	Microfilaria and antigen testing
33	0	9	F	18.5	Neutering
34	2	3	F	31.9	Microfilaria and antigen testing
35	0	7	F	30.0	Neutering
36	0	7	F	17.5	Neutering
37	3	10	F	37.8	Ophthalmic follow-up
38	1	3	M	42.0	Ophthalmic follow-up
39	2	11	M	28.0	Microfilaria and antigen testing
40	0	4	M	17.0	Ophthalmic follow-up
41	0	6	M	31.6	Ophthalmic follow-up
42	2	7	M	18.0	Microfilaria and antigen testing
43	8	0	F	31.0	Routine check-up *

**Table 1** Data of recruited dogs according to age, sex, weight and clinical examination (Continued)

Dog	Age		Sex Female (F) / Male (M)	Weight Kg	Clinical examination VTH or VTH partners
	Years	Months			
44	2	3	F	39.0	Microfilaria and antigen testing
45	1	6	M	17.0	Microfilaria and antigen testing
46	13	3	F	32.0	Routine check-up; Periodontitis stage 2 *
47	10	5	M	23.0	Orthopedic follow-up; Periodontitis stage 3 *

VTH Veterinary Teaching Hospital; \* dogs recruited from private veterinary practice VTH partners

degree of variation during the first 12 months of life. Variation strongly decreased depending on the growth of the animals. Values resulted the lowest and not dispersed in aged dogs between 120 and 160 months. This course was confirmed analyzing dogs according to the stages of life (Fig. 2). Also the absolute values of  $\gamma\delta$  T lymphocytes were subjected to a strong decrease in the M and S groups compared to the other age groups.

Comparison between groups of age for TCR $\gamma\delta$ + cell percentages are as follows:

1. Group P vs. Group J, Group A, Group M, Group S

Specifically, a reduction of TCR $\gamma\delta$ + cell percentages, in a slight but constant fashion, from Puppy (group P) was observed by comparison with Junior (group J) and Adult (group A). Figure 2 shows the decline of the percentage of TCR $\gamma\delta$ + cells in the dog peripheral blood during the first three stages of life in which no statistical differences were found (group P vs. group J,  $P = 1.000$ ; group P vs. group A,  $P = 0.761$ ). Figure 2 shows a decreasing percentage of circulating TCR $\gamma\delta$ + cells from Mature (group M, mean = 1.36%) to Senior (group S, mean = 0.42%). The percentage values of TCR $\gamma\delta$ + cells

in dogs of group P (mean = 2.92%) compared to those of dogs of group S (mean = 0.42%) were different (group P vs. group S,  $P = 0.049$ ). A reduction of percentages of TCR $\gamma\delta$ + cells in the peripheral blood was detectable during the stages of life from Puppy (mean = 2.92%) to Senior (mean = 0.42%).

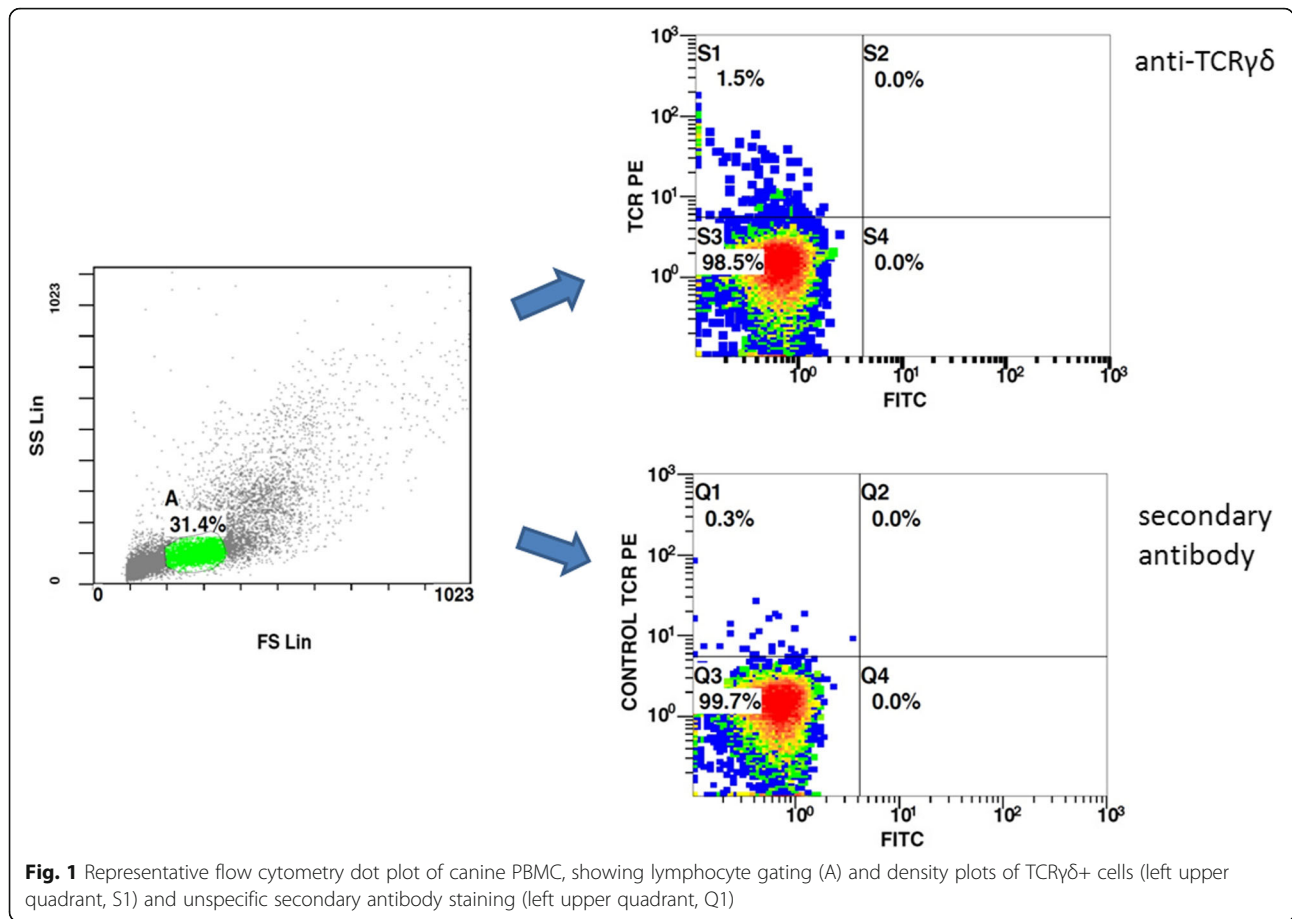
2. Group J vs. Group A, Group M, Group S

The percentage values of TCR $\gamma\delta$ + cells of group J (mean = 2.49%) vs. group A (mean = 2.46%) showed a long steady-state period (years) that matches with adulthood of dogs (Fig. 3), and Tamhane’s test was not found to be significant (group J vs. group A -  $P = 0.679$ ). The percentage values of TCR $\gamma\delta$ + cells of group J (mean = 2.49%) compared to those of group M (mean = 1.36%) were identifiably different and Tamhane’s test was significant (group J vs. group M,  $P = 0.041$ ). The phenomenon of decreasing TCR $\gamma\delta$ + cells between group J (mean = 2.49%) and group S (mean = 0.42%) was remarkable and the Tamhane’s test was relevant (group J vs. group S,  $P = 0.000$ ).

3. Group A vs. Group M, Group S

**Table 2** Definition of dog groups according to stage of life, also indicating sex and describing weight range and clinical features

	N	Sex	Age	Weight	Clinical Examination
Group P (Puppies)	11 healthy dogs	8 Females 3 males	From 4 to 11 months	From 5.7 to 42.0 Kg	2 males ophthalmological follow-up; 1 male microfilaria antigen-testing; all female referred for neutering
Group J (Junior)	14 healthy dogs	3 females 11 males	From 1 to 2 years	From 16.4 to 42.0 Kg	1 female and 5 males ophthalmological follow-up; 2 females and 6 males microfilaria antigen testing
Group A (Adult)	8 healthy dogs	5 females 3 males	From 3 to 5 years	From 5.0 to 57.0 Kg	2 females referred for neutering; 2 females and 2 males ophthalmological follow-up; 1 female orthopedic follow up; 1 male surgical stabilization after Cranial Cruciate Ligament rupture
Group M (Mature)	7 healthy dogs	4 females 3 males	From 6 to 9 years	From 8.6 to 38.0 Kg	All dogs for routine check-up, 6 showed periodontal disease: 3 females and 2 males stage 2 and 1 male stage 3
Group S (Senior)	7 healthy dogs	3 females 4 males	From 10 to 13 years	From 12.0 to 35.0 Kg	3 females and 1 male routine check-up; 3 male follow-up post traumatic arthropathy. All the dogs affected by periodontal disease: 2 females and 1 male stage2; 1 female and 3 males stage 3



The decline of TCRγδ+ cells between dogs in adult-to-senior stages of life was observed (group A, mean = 2.46%; group S, mean = 0.42%) (Fig. 3). Tamhane’s analysis was significant (group A vs. group S,  $P = 0.015$ ). Group A value of TCRγδ+ cells (mean = 2.46%) was highly different with minus 1.10%, compared to group M (mean = 1.36%). In spite of the change of mean data, no differences were detected by the Tamhane’s test (Group A vs. Group M,  $P = 0.632$ ).

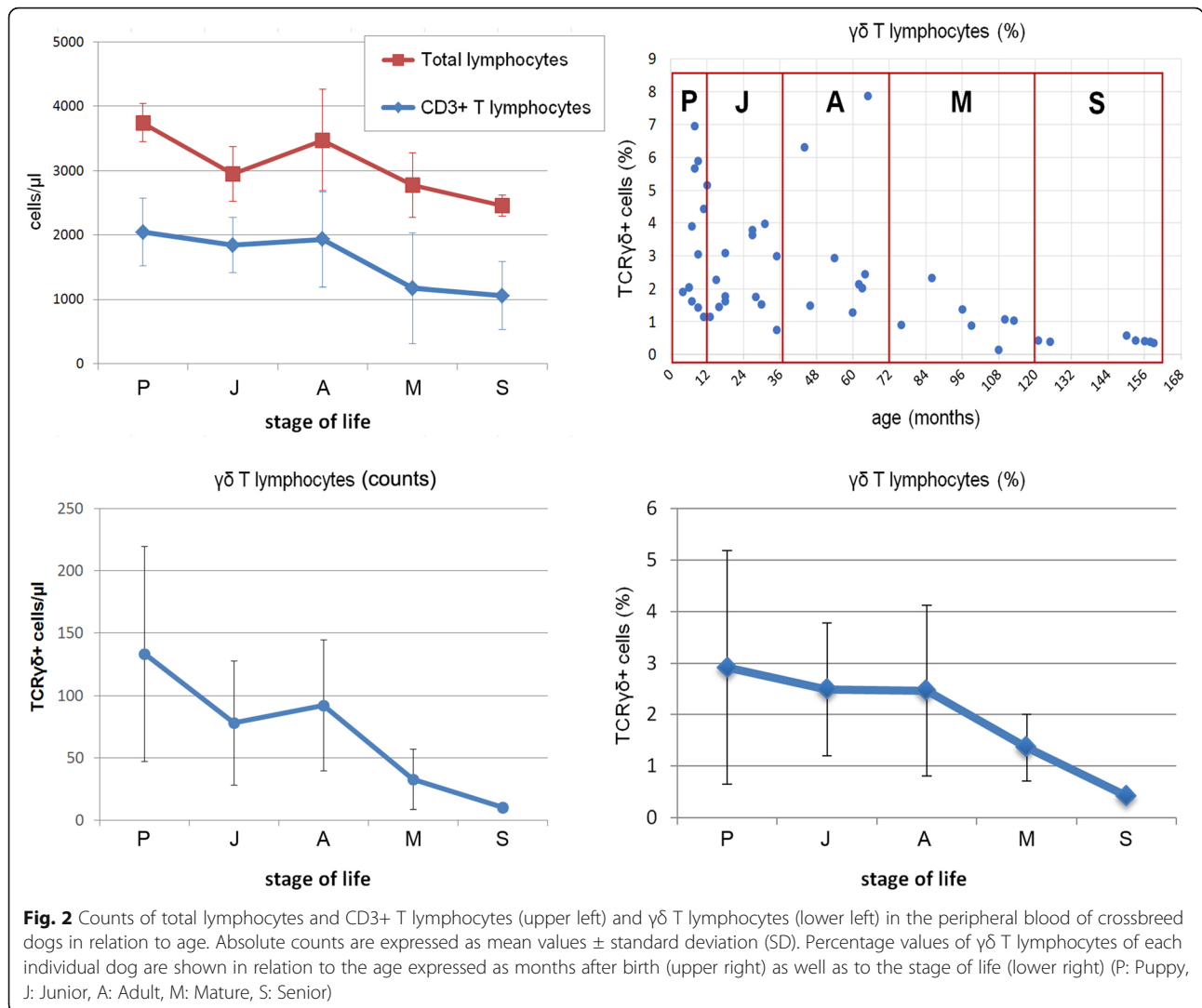
4. Group M vs. Group S

The decline of TCRγδ+ cells in the peripheral blood of dogs of group M (mean = 1.36%) in comparison with Group S (mean = 0.42%) was remarkable (minus 0.94%) but the Tamhane’s test was not significant (group M vs. group S,  $P = 0.297$ ).

5. Group S vs. other Groups (P, J, A, M)

**Table 3** White blood cells (WBC), total lymphocytes, counts and percentages of CD3+ T lymphocytes and TCRγδ+ T lymphocytes in the peripheral blood of dogs expressed as cell count and percentage mean values ± standard deviation (SD) and percentage range values (minimum and maximum) according to the stage of life

Stage of life	WBC		Lymphocytes		T lymphocytes (CD3+)				γδ T lymphocytes (TCRγδ+)			
	mean ± SD (cells/μl)	range	mean ± SD (cells/μl)	range	mean ± SD (%)	range	mean ± SD (cells/μl)	range	mean ± SD (%)	range	mean ± SD (cells/μl)	range
<b>Puppy (P)</b>	6509 ± 2502	1600–11,000	3745 ± 294	3400–4400	55.27 ± 15.92	25.60–84.84	2049 ± 523	964–2944	2.92 ± 2.27	1.15–6.94	133 ± 86	40–278
<b>Junior (J)</b>	9700 ± 3696	4300–18,700	2950 ± 429	2400–3500	62.99 ± 13.69	22.69–83.64	1839 ± 433	775–2630	2.49 ± 1.29	0.74–3.97	78 ± 50	18–180
<b>Adult (A)</b>	10,775 ± 1652	7800–13,600	2968 ± 629	2100–3900	67.35 ± 24.46	14.25–91.60	2037 ± 741	477–2805	2.46 ± 1.66	1.15–6.30	94 ± 53	43–176
<b>Mature (M)</b>	8271 ± 996	7000–9500	2771 ± 502	1800–3500	45.56 ± 34.18	8.62–88.70	1180 ± 860	229–2449	1.36 ± 0.65	0.13–2.32	33 ± 24	2–81
<b>Senior (S)</b>	7514 ± 358	7000–8000	2475 ± 162	2300–2700	43.05 ± 21.88	6.80–65.70	1056 ± 529	159–1707	0.42 ± 0.08	0.34–0.58	10 ± 3	8–16



TCR $\gamma\delta$ + cells of dogs of group S were highly different compared to the values of the other groups of dogs (P, J, A, M). Group S was statistically different in comparison with groups P, J, A (group S vs. group P,  $P = 0.049$ ; group S vs. group J,  $P = 0.000$ ; group S vs. group A,  $P = 0.015$ ), while no statistical difference was detected when group S was tested vs. group M (group S vs. group M,  $P = 0.297$ ). The graph (Fig. 2) shows the TCR $\gamma\delta$ + cell decline in dog aging. The post-hoc power analysis performed 92.3%.

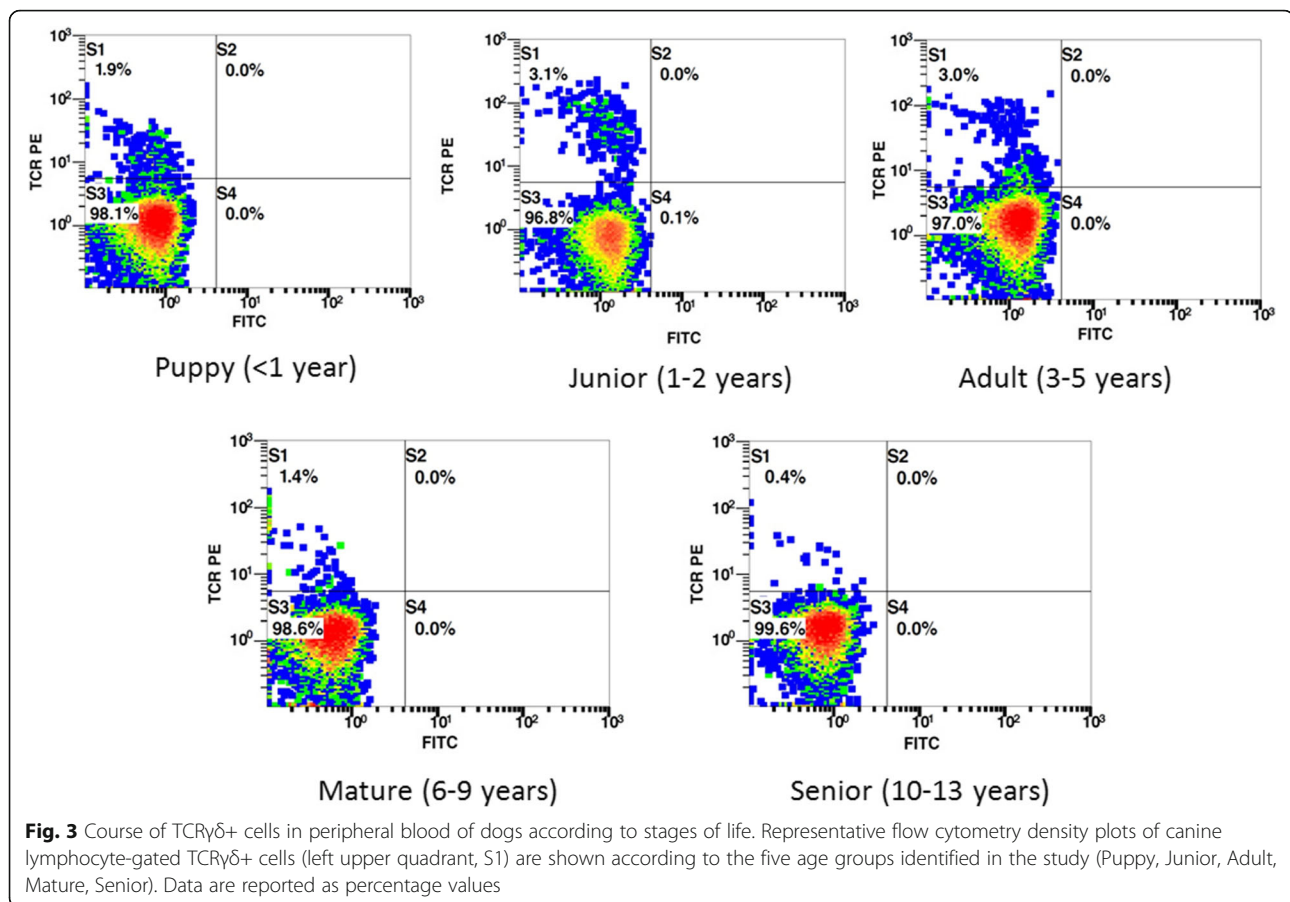
Taking into account the absolute counts of  $\gamma\delta$  T lymphocytes in the stages of life, they confirmed what was observed with percentage values. The slight decrease between Puppy and Adult dogs was followed by a significant decrease of cellularity in the group M and particularly in the groups S ( $P < 0.05$ ) (Fig. 2).

**Discussion**

The present study was carried out to evaluate  $\gamma\delta$  T lymphocytes in the peripheral blood of healthy crossbreed

dogs according to the stage of life. The recruitment of crossbreed healthy dogs, according to age and categorised according to five groups of stages of life (groups P, J, A, M, S) indicated dynamic changes of the TCR $\gamma\delta$ + subpopulation through the dog lifespan. Major significant findings were observed when comparing dogs in the early stages of life (group P and group J) to those in the later stages of life (group M and group S) so that the declining course of the TCR $\gamma\delta$ + subpopulation was significant. These findings were demonstrated in healthy crossbreed dogs in which both WBC and total lymphocytes as well as T lymphocytes subpopulations were within the normality ranges, and particularly WBC showed a course relative to a normal development of the immune cellularity related to bone marrow-derived cells influenced by aging.

It is to underline that the difference in the numbers of dogs recruited in each group is related to the health condition and the decline of aged class demography. The



number of dogs in the Adult, Mature and Senior groups were the most disadvantaged by inclusion and exclusion criteria adopted in the study. The aged dog population (Mature and Senior groups) often did not meet these criteria. Demography of the aged dog population, that spontaneously and naturally decreases, negatively affects group setting up. These conditions can explain first the slowed recruitment speed of dog groups, and secondly, the limited number of dogs, especially, in Mature and Senior groups.

The highest TCRγδ+ cell variation observed in the dogs during the first year of life (Puppy group) can be related to: 1) in general terms, the different extent and course in the development of the immune system after birth after the vanishing of maternal passive immunity; 2) the different development of cells involved in the first lines of defence of the body such as γδ T lymphocytes which exert MHC-unrestricted recognition of antigens; 3) the observation of such aforementioned effects in crossbreed dogs which whole body development and functions can be temporally diverse especially after birth [27].

The thymus reaches its maximum development during puberty and continues to play an immunological role during different stages of life. The decline of TCRγδ+

lymphocytes in the peripheral blood of dogs can be partially associated with the natural aging-dependent thymus involution. A recent study on longevity in the dog performed on thymus lymphocyte output evaluating the recent thymic emigrants (RTE) via signal joint-T cell receptor excision circles (sj-TRECs) concluded that the decline is age-associated when compared to the dog breed [21]. Sj-TRECs participate to VDJ recombination occurring in T cell thymopoiesis after removal of δ gene segments [21]. In order to focus the phenomenon of TCRγδ+ cell drop-off, it is useful to refer to a study performed in aged and centenarian humans [28]. In this paper, a decrease of the TCRγδ+ (δ1 and δ2) cell repertoire related to the increase of TCRγδ+ cell responsiveness to apoptosis stimuli (Fas-α and TNF-α) was recorded in addition to the decrease of the TCRγδ+ cell capability to proliferate. As demonstrated by literature, the complexity of the phenomenon of TCRγδ+ cell decline in the peripheral blood of dogs appears to be complex and influenced by several variables. TCRγδ+ cells in aged and centenarian humans are not involved in the remodelling of the immune system as a result of the T cell decline [28]. Currently, there are no specific investigations on the immune system remodelling in dog

**Table 4** Tamhane's T2 all-pairs comparison test for normally distributed data with unequal variances stage of life vs. stages of life for percentages of TCR $\gamma\delta$ + cells (significant difference when  $P \leq 0.05$ )

Stage of life	Stages of life	P	
Puppy vs.	Junior	1.000	
	Adult	0.761	
	Mature	0.251	
	<b>Senior</b>	<b>0.049</b>	
	Junior vs.	Puppy	1.000
		Adult	0.679
<b>Mature</b>		<b>0.041</b>	
	<b>Senior</b>	<b>0.000</b>	
	Adult vs.	Puppy	0.761
		Junior	0.679
Mature		0.632	
	<b>Senior</b>	<b>0.015</b>	
	Mature vs.	Puppy	0.251
		<b>Junior</b>	<b>0.040</b>
Adult		0.632	
	Senior	0.297	
	Senior vs.	<b>Puppy</b>	<b>0.049</b>
		<b>Junior</b>	<b>0.000</b>
<b>Adult</b>		<b>0.015</b>	
	Mature	0.297	

Stage of life of the dogs associated with a  $P$  value for statistical significance are highlighted in bold characters

aging. Dogs like humans and mice are part of the animal cluster characterized by a low TCR $\gamma\delta$ + cell profile (less than 10%) with genomic similarity for TRG [29]. Therefore, aged dogs may have a remodelling pathway of the immune system in common with aged mice and humans. This pathway could be appropriate for all animal species with a low TCR $\gamma\delta$ + cell profile [30]. Immunonutrients (e.g. arginine, zinc ions) as well as hormones (e.g. melatonin) can play an important role in thymulin biosynthesis and thymus functional restoration in aged mice [31]. Treatment with bovine growth hormone (bGH) in dogs aged 66 months showed to induce histomorphological thymus stimulation but side effects related to anti-bGH immune reaction were observed [32, 33]. Therefore, thymus involution can be considered a consecutive phenomenon related to the plasticity modification of the thymus in the scenario of neuroendocrine-thymus interaction during aging [31, 34]. In elderly humans, chronic low-grade inflammation, also called "inflammaging", is a real risk factor for the onset of even lethal infections [26]. In aged dogs (Mature and Senior), the weakening of the immune system is the result of aging, leading to side effects such as of the onset of

lower-grade infections [6]. Arthritis is one of the most common arthropathies for both species, inducing lameness and consequently reduction of exercise. Less opportunity to exercise combined with a high rich calorie diet increases the risk of obesity in the dog and long-term weight gain induces functional impairment of canine T lymphocytes [35]. Another common effect of aging in the dog is reduced calorie needs related to reduce energy demand (minus 30–40%). Obesity in humans predisposes to the onset of chronic systemic inflammation associated with a decrease of V $\gamma$ 9/V $\delta$ 2 T cells [36]. Therefore, TCR $\gamma\delta$ + cell decrease is inversely related to the body mass index. In obese mice, TCR $\gamma\delta$ + cell reduction was recorded in the skin [36]. Aging, "inflammaging", and obesity are negative organic conditions that impair the preservation of the immune system homeostasis. Aging itself is not a disease but a status, a daily process of molecular and cellular changes that accumulate over time, resulting in damaging effects on biomolecules (e.g. DNA, RNA, and proteins) and structural components of eukaryotic cells (membrane bound-structures and cytoskeletal matrix). Cumulative cell injury evolves to tissue damage and, at the end, to the loss of organ function.

Inflammation is often an unavoidable clinical condition in aged dogs. In our investigation, many aged dogs (M and S groups) that scored ideal BCS (4–5 score) were affected by periodontium chronic inflammation (periodontal disease, stages 2–3: M group, 6 out of 7; S group, 6 out of 7). Dogs of M and S groups showed a significant peripheral blood TCR $\gamma\delta$ + cell decline, with many of these participants affected by chronic periodontitis diagnosed as stage 2 or 3. Significant differences in peripheral blood TCR $\gamma\delta$ + cells were detected in aged dogs (M and S groups) when compared to the youngest animals (J group). The reduction of blood circulating TCR $\gamma\delta$ + cells could be related to an increase of TCR $\gamma\delta$ + cells resident in the epithelium of inflamed gingival mucosa due to bacterial plaque. Persistence of subgingival bacterial plaque induces firstly chronic inflammation, followed by lesions of mucosa around periodontium (e.g. bleeding). TCR $\gamma\delta$ + lymphocytes are activated in the damaged mucosa (gingivitis) in order to repair epithelial wounds and evoke an inflammatory response with release of cytokines and growth factors. Cytokines released by TCR $\gamma\delta$ + cells are crucial for mucosal integrity and functionality preservation while their high-level release can cause the onset of chronic inflammation [36]. In fact,  $\gamma\delta$  T lymphocytes are protectors of the epithelium through a dynamic and precise equilibrium of cytokines and growth factor release [36].

## Conclusions

The study confirms the canine species to be among the animals with a low TCR $\gamma\delta$ + cell profile, less than 10%,



like rodents and humans. The percentage of TCR $\gamma\delta^+$  cells in the peripheral blood of dogs recorded is comparable with the value of 2.5% published by Faldyna and co-workers [3], but the percentage reported by these authors was investigated on less arranged age range groups and coming from four different dog pure breeds (Beagle, German shepherd, Dalmatian, Dachshund), while our data were recorded on wider age range groups and coming from crossbreed dogs. It is important to underline that an identical percentage of TCR $\gamma\delta^+$  cells independently recorded by our research team and Faldyna and co-workers under different experimental conditions, makes the percentage value of 2.5% consistent and point out that such value is breed-independent. The most original data observed in the present investigation is related to the significant drastic decrease of the TCR $\gamma\delta^+$  subpopulation percentage observed in elder dogs (S group). TCR $\gamma\delta^+$  cells of dogs of group S were statistically different in comparison with those of dogs of groups P, J, and A. The differences are significant and reported, for the first time, in dog aging.

Moreover, the common dog and human low TCR $\gamma\delta^+$  cell profile as well as life-longevity and genomic similarity for the T cell receptor gamma locus (TRG) can be intriguing for providing a rationale for drafting a wellness plan in the dog, as “non-invasive animal model”, to support thymus immune functions and mitigate its aging-dependent functional exhaustion (e.g. arginine, zinc ion, or melatonin supplementation in the diet associated with periodical not stressful physical activity) also in the perspective of a multidisciplinary approach based on the concept of “One Health - One Medicine”.

## Methods

### Dog recruitment

The recruitment of dogs followed a rigorous method based on inclusion and exclusion criteria for minimizing the internal-bodily and external-bodily conditions that could influence the immune response. In fact, several conditions negatively modulate immunity or induce a stronger immune response: stressors (eustress or distress), genetics (dog breed), drugs, vaccines, autoimmune diseases, immunostimulants, immunomodulants, immunosuppressive drugs or xenobiotics, immunonutrients as well as body weight gain.

A high degree of accuracy of electronic medical records of candidates was a necessary prerequisite for recruiting. Only the dogs of reliable owners were enlisted in the study. The recruitment was achieved through two sample pools: referred dogs at the Veterinary Teaching Hospital (VTH) of the Department of Veterinary Science, University of Parma (Italy) and patients referred to private veterinary practice VTH partners.

Recruited dogs were referred for neutering, post-surgical orthopedic follow-up or ophthalmological follow-up.

Dog owners had to have a previously established relationship with the clinical staff, reliable and long-term customers of the VTH or VTH partners. Recruited dogs were then divided into five groups of stage of life (Puppy, Junior, Adult, Mature, Senior).

### Inclusion and exclusion criteria

The Fédération Cynologique Internationale (FCI) recognizes 339 distinct pure breeds [27], therefore it was considered appropriate to obtain the  $\gamma\delta$  T cell profile from the peripheral blood of crossbreed dogs because they represent the largest number of patients in a veterinary hospital or private clinic. The choice of recruiting only crossbreed dogs was also related to remove the potential influence on  $\gamma\delta$  T cells in the peripheral blood of pure breeds as an “extra” variable that could affect statistical data (confounding variable).

All blood samples were collected from crossbreed healthy dogs. The inclusion and exclusion criteria for dogs enrolled in the present study are reported in Table 5.

### Stages of life and sample size

Recruited dogs were assigned to one of the following five groups based on the stage of life according to age. Puppy: dogs younger than 1 year (group P,  $N = 11$ ); Junior: dogs aged 1–2 years (group J,  $N = 14$ ); Adult: dogs aged 3–5 years (group A,  $N = 8$ ); Mature: dogs aged 6–9 years (group M,  $N = 7$ );

Senior: dogs aged 10–13 years (group S,  $N = 7$ ).

No power analysis for recruitment was performed and sample size was based on sample availability. The groups were formed including as many subjects as possible according to the inclusion and exclusion criteria. Specifically, the number of dogs was chosen among the clinical cases managed, in 2 years, at the VTH or referred by private veterinary practice VTH partners. The total number of medical care assisted dogs amounted to 7068 patients (crossbreed and pure breed), of which 4806 (67.99%) were crossbreed. The total of potential selected clinical cases amounted to 52 dogs, but in 5 cases (9.61%) the dog owner did not sign the consent form. Three dogs that could not be recruited were classifiable in the Adult group and the remaining two could have belonged to the Mature group and to the Senior group (one in each group). The recruitment ratio was 0.97%: this percentage was calculated on the total of crossbreed patients.

### Physical examination

During physical examination, clinicians identified the suitable dogs and owners were offered the opportunity to participate in the study. The selected owners were asked for permission for recruitment of the dog by

**Table 5** Information relative to the inclusion and exclusion criteria of the variables considered in the dogs enrolled in the study

	INCLUSION CRITERIA	EXCLUSION CRITERIA
<b>Informed consent from the owner</b>	Granted	Not granted
<b>Breed</b>	Mixed breed	Pure breed
<b>Health and Wellness</b>	Healthy life-style in close contact with the owners	Less than 2 h of exercise each day Not required for Puppy
<b>Diet and Feeding Management</b>	Commercial standard pet food specific for each stage of life Regular meal time each day	Immunonutrient supplementation (especially zinc ions) Irregular feeding routine Over- or under-feeding Competitive eating
<b>Nutritional Assessment<sup>a</sup></b>	Junior, Adult, Mature, Senior: BCS 4 or 5 out of 9	Puppy in poor body conditions
<b>Vaccinations</b>	Regularly performed "vaccination plan" <sup>b</sup> (certified only by an electronic medical record) Test performed after 3 weeks post-vaccination	Any dogs with vaccination status not in line with inclusion criteria Data not certified by an electronic medical record
<b>Therapies, Trauma, Stressors or Imaging Diagnostic Techniques</b>	After 2 months from: medications, surgeries, trauma and stressors X-rays exposure or computed tomography (CT)	Data not certified by an electronic medical record Immunomodulant or immunosuppressive therapies Medications, surgeries, trauma and stressors
<b>Sex</b>	Entire female or male	Female: pregnant, in estrus, any pathological status of the reproductive tract Male: cryptorchidism, any pathological status of the reproductive tract
<b>Parasites</b>	Endo/ecto-parasites free	Presence of endo/ecto-parasites or therapies in the last 3 weeks
<b>Periodontal Diseases<sup>c</sup></b>	Stages 1, 2, 3	Stage 4 and/or other inflammatory/ulcerative diseases
<b>Arthritis</b>	Joints had to be free from any pathological status	Acute and chronic pathological conditions

<sup>a</sup> A Body Condition Score (BCS) 9-point scale system according to the 2011 World Small Animal Veterinary Association (WSAVA) Nutritional Assessment Guidelines was adopted [37]: 1, 2, 3 = under ideal; 4, 5 = ideal; 6, 7, 8, 9 = over ideal

<sup>b</sup> According to the 2011 American Animal Hospital Association (AAHA) Canine Vaccination Guidelines [38]

<sup>c</sup> Periodontal disease was scored according to a periodontal 4-point scale system provided by the Merck Veterinary Manual [39]

signature of a consent form. Recruited dogs were individually identified by a microchip.

Blood samples were collected from the brachiocephalic vein for routine clinical biochemistry analyses during physical examination. An aliquot was collected in a vacutainer K<sub>3</sub>EDTA tube (Becton Dickinson, USA) for quantification of CD3+ T lymphocytes and  $\gamma\delta$  T lymphocytes by flow cytometry (FCM).

#### Whole blood cell and lymphocyte quantification

White blood cells (WBC) and total lymphocytes were quantified in whole blood samples by using an automated Cell Dyn 3500 plus hematology analyzer (Abbott Diagnostics, Lake Forest, IL, USA) and from blood smears stained with May Grunwald-Giemsa, respectively.

#### Isolation of canine peripheral blood mononuclear cells (PBMC)

Blood samples were maintained at room temperature and processed within a maximum of 6 h. Blood was diluted 1:1 with sterile Dulbecco's PBS + 5% EDTA (Sigma, St.

Louis, Missouri, USA). The sample was then stratified on an equal volume of Lympholyte<sup>®</sup> (Cedarlane<sup>®</sup>, Burlingtone, NC, USA) and centrifuged at 400 xg for 30 min. PBMC were separated by density gradient, collected and washed twice in 10 ml of sterile PBS + 5% EDTA. The cell pellet was resuspended in PBS + 5% EDTA + 5% heat-inactivated (hi) fetal bovine serum (FBS), quantified and checked for viability by Trypan Blue exclusion (Sigma-Aldrich, St. Louis, Missouri, USA) by using a TC20<sup>™</sup> automated cell counter (BioRad, Hercules, CA, USA) before staining for FCM analysis.

#### Flow cytometry analysis

Quantification of T lymphocytes (CD3+) and  $\gamma\delta$  T lymphocytes within isolated PBMC was carried out by a mouse anti-dog CD3-FITC (clone CA17.2A12, IgG<sub>1</sub>, AbD Serotec, Raleigh, NC, USA) and a mouse anti-dog TCR $\gamma\delta$  (clone CA20.8H1, IgG<sub>2a</sub>, Prof. Peter F. Moore, Leukocyte Antigen Biology Laboratory (LABL), Davis, CA, USA [40]; primary antibodies followed by a goat anti-mouse IgG<sub>2a</sub>-PE secondary antibody (cat. M32204;

Invitrogen™- Molecular Probes® Carlsbad, CA, USA) according to previously established protocols and optimal signal-to-noise ratio concentration [41, 42]. Each incubation step with the anti-CD3 fluorochrome-labelled antibody and the secondary antibody was done in the dark for 15 min. Unstained cells and cells incubated with the secondary antibody were used as negative controls (non-specific binding). All washing steps were performed with 1 ml of PBS + 1% hi FBS. Analysis was carried out using a Cytomics FC500 MCL flow cytometer and Expo32™ ADC software (Beckman Coulter, Indianapolis, IN, USA) based on lymphocyte gating (forward scatter vs. side scatter) after acquisition of at least 10,000 cell events in the gate. The results were expressed as percentage values of CD3+ cells and TCRγδ+ cells gated on lymphocytes as well as absolute values (cells/μl) based on total lymphocyte counts.

### Statistical analysis

The descriptive analysis was performed for WBC, total lymphocytes, T lymphocytes (CD3+ cells) and γδ T lymphocytes (TCRγδ+ cells). The response variables were total lymphocytes, T lymphocytes and γδ T lymphocytes according to the five stages of life. The significance of stages of life was assessed via the Tamhane's T2 all-pairs comparison test for normally distributed data with unequal variances. ANOVA one-way and Tamhane's T2 differences were considered significant if  $P \leq 0.05$ . Outlier analysis was performed before the Tamhane's T2 test. Difference between within-subject and between-subject effects according to stages of life was assessed via post-hoc power with  $\alpha = 0.05$  and significant data were considered if  $> 80\%$ . Values were presented as mean  $\pm$  standard deviation (SD) and range, and individual data only for TCRγδ+ cell percentages. All statistical analyses were performed using the SPSS v.25 program for Windows.

### Ethics approval 12345

Blood sample collection was performed after signing the written consent by the owner in compliance with the requirements of the European Directive 2010/63 of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes transposed by the Italian Parliament with "D.lgs Sperimentazione 04/03/2014 n.26". Chapter 1: General Provisions - Article 1: Subject matter and scope – point 5: this directive shall not apply to the following letter: (f) "practices not likely to cause pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle in accordance with good veterinary practice". The study was approved by the Ethical Committee of University of Parma, Italy (PROT.N. 20/CE/2019).

### Abbreviations

AAHA: American Animal Hospital Association; ANOVA: Analysis of variance; BCs: Body condition score; bGH: Bovine growth hormone; CT: Computed tomography;  $\delta 1$  and  $\delta 2$ : Delta 1 and delta 2; Fas- $\alpha$ : First apoptosis signal alpha; FBS: Fetal bovine serum; FCM: Flow cytometry; FCI: Fédération Cynologique Internationale; hi: Heat-inactivated; K<sub>3</sub>EDTA: Tripotassium ethylenediaminetetraacetic acid; Ig: Immunoglobulin; LABL: Leukocyte Antigen Biology Laboratory; PBMC: Peripheral blood mononuclear cells; PBS: Phosphate buffered solution; PE: Phycoerythrin; RTE: Recent thymic emigrants; SD: Standard deviation; sj-TRECs: Signal joint-T cell receptor excision circles; TCR $\alpha\beta$ : T cell receptor alpha-beta heterodimer; TCR $\gamma\delta$ : T cell receptor gamma-delta heterodimer; TNF- $\alpha$ : Tumor necrosis factor alpha; TRG: T cell receptor gamma locus; VDJ: Variable, diversity and joining genes; Vy9/V $\delta 2$ : Variable gamma 9 / variable delta 2; VTH: Veterinary Teaching Hospital; WSAVA: World Small Animal Veterinary Association

### Acknowledgements

The authors are grateful to Prof. Marco Vitale and Prof. Cecilia Carubbi, Department of Medicine and Surgery, University of Parma (Italy) for collaboration and helpful technical support in flow cytometry and to Dr. Rachel Dalton, Avon Lodge Veterinary Group, Bristol (UK) for support in language revision.

### Authors' contributions

CM conceived the study. CM, AMC and FA contributed to the experimental design. CM, LF and PB contributed to interpretation of data. ACo coordinated the group and drafted the manuscript. CM and LF contributed by performing experimental procedures/data analysis and collaborated to draft the manuscript. ACa performed the statistical analysis. All authors read and approved the final manuscript.

### Funding

No specific funds were used for the present work.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

Blood sample collection was performed after signing the written consent by the owner in compliance with the requirements of the European Directive 2010/63 of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes transposed by the Italian Parliament with "D.lgs Sperimentazione 04/03/2014 n.26". Chapter 1: General Provisions - Article 1: Subject matter and scope – point 5: this directive shall not apply to the following letter: (f) "practices not likely to cause pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle in accordance with good veterinary practice". The study was approved by the Ethical Committee of University of Parma, Italy (PROT.N. 20/CE/2019).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

Received: 3 April 2019 Accepted: 30 July 2020

Published online: 08 August 2020

### References

- Greely EH, Kealy RD, Ballam JM, Lawler DF, Segre M. The influence of age on the canine immune system. *Vet Immunol Immunopathol.* 1996;55:1–10.
- Faldyna M, Toman M. The effect of age on the distribution of lymphocyte and neutrophil subsets in peripheral blood of dog. *Vet Med Czech.* 1998;43:193–9.
- Faldyna M, Levà L, Knotigova P, Toman M. Lymphocyte subsets in peripheral blood of dogs - a flow cytometry study. *Vet Immunol Immunopathol.* 2001;82:23–37.
- Gauthier MJ, Aubert I, Abrams-Ogg A, Woods JP, Bienzle D. The immunophenotype of peripheral blood lymphocytes in clinically healthy

- dogs and dogs with lymphoma in remission. *J Vet Intern Med.* 2005;19:193–9.
5. Watabe A, Fukumoto S, Komatsu T, Endo Y, Kadosawa T. Alterations of lymphocyte subpopulations in healthy dogs with aging and in dogs with cancer. *Vet Immunol Immunopathol.* 2011;142:189–200.
  6. Day MJ. *Clinical immunology of the dog and cat.* London: Manson Publishing Ltd; 2008.
  7. Olivry T, Naydan DK, Moore PF. Characterization of the cutaneous inflammatory infiltrate in canine atopic dermatitis. *Am J Dermatol.* 1997;19:477–86.
  8. Galler A, Rütgen BC, Haas E, Saalmüller A, Hirt RA, Gerner W, Schwendenwein I, Richter B, Thalhammer JG, Luckschander-Zeller N. Immunophenotype of peripheral blood lymphocytes in dogs with inflammatory bowel disease. *J Vet Intern Med.* 2017;31:1730–9.
  9. Donaldson D, Day MJ. Epitheliotropic lymphoma (mycosis fungoides) presenting as blepharconjunctivitis in an Irish setter. *Small Anim Pract.* 2010;41:317–20.
  10. Foglia Manzillo V, Pagano A, Guglielmino R, Gradoni L, Restucci B, Oliva G. Extranodal gammadelta-T-cell lymphoma in a dog with leishmaniasis. *Vet Clin Pathol.* 2008;37:298–301.
  11. Borska P, Faldyna M, Blanty J, Leva L, Vejrostova M, Doubek J, Moore PF. Gamma/delta T-cell lymphoma in a dog. *Can Vet J.* 2009;50:411–6.
  12. Ortiz AL, Carvalho S, Riondato F, Archer J, Clan F. Gamma delta T-cell large granular lymphocyte lymphoma in a dog. *Vet Clin Pathol.* 2015;44:442–7.
  13. Mineshige T, Karawarai S, Yauchi T, Segawa K, Neo S, Sugahara G, Kamile J, Hisasue M, Shirota K. Cutaneous epitheliotropic T-cell lymphoma with systemic dissemination in a dog. *J Vet Diagn Investig.* 2016;28:327–31.
  14. Faldyna M, Sinkora P, Knotigova P, Levà L, Toman M. Lymphatic organ development in dogs: major lymphocyte subsets and activity. *Vet Immunol Immunopathol.* 2005;104:239–47.
  15. German AJ, Hall EJ, Moore PF, Ringler DJ, Newman W, Day MJ. The distribution of lymphocytes expressing  $\alpha\beta$  and  $\gamma\delta$  T-cell receptors, and the expression of mucosal addressin cell adhesion molecule-1 in the canine intestine. *J Comp Pathol.* 1999;121:249–63.
  16. Carding SR, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. *Nature Rev Immunol.* 2002;2:336–45.
  17. Holderness J, Hedges JF, Ramstead A, Jutila MA. Comparative biology of  $\gamma\delta$  T cell function in humans, mice, and domestic animals. *Annu Rev Anim Biosci.* 2013;1:99–124.
  18. Nielsen MM, Witherden DA, Havran WL.  $\gamma\delta$  T cells in homeostasis and host defence of epithelial barrier tissue. *Nat Rev Immunol.* 2017;17:733–45.
  19. Bartoskova A, Turanek-Knotigova P, Matiasovic J.  $\gamma\delta$  T lymphocytes are recruited into the inflamed uterus of bitches suffering from pyometra. *Vet J.* 2012;194:303–8.
  20. Palmer DB. The effect of age on thymic function. *Front Immunol.* 2013;4:316.
  21. Holder A, Mella S, Palmer DB, Aspinall R, Catchpole B. An age-associated decline in thymic output differs in dog breeds according to their longevity. *PLoS One.* 2016;11:e0165968.
  22. Gleeson M. Immune system adaptation in elite athletes. *Curr Opin Clin Nutr Metab Care.* 2006;9:659–65.
  23. Hennessy MB, Williams MT, Miller DD, Douglas CW, Voith VL. Influence of male and female petters on plasma cortisol and behaviour: can human interaction reduce the stress of dogs in a public animal shelter? *Appl Anim Behav Sci.* 1998;61:63–77.
  24. Hekman JP, Karas AZ, Sharp CR. Psychogenic stress in hospitalized dogs: cross species comparisons, implications for health care, and the challenges of evaluation. *Animals.* 2014;4:331–47.
  25. Sataraj E. Emerging paradigms in immunonutrition. *Top Companion Anim Med.* 2011;26:25–32.
  26. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci.* 2014;69:54–9.
  27. Fédération Cynologique Internationale (FCI). For dogs worldwide. <http://www.fci.be/en/>. Accessed 15 Mar 2019.
  28. Colonna-Romano G, Aquino A, Bulati M, Lio D, Candore G, Oddo G, Scialabba G, Vitello S, Caruso G. Impairment of gamma/delta T lymphocytes in elderly: implications for immunosenescence. *Exp Gerontol.* 2004;39:1439–46.
  29. Massari S, Bellahence F, Vaccarelli G, Carelli G, Mineccia M, Lefranc MP, Antonacci R, Ciccarese S. The deduced structure of the T cell receptor gamma locus in *Canis lupus familiaris*. *Mol Immunol.* 2009;46:2728–36.
  30. Kazen AR, Adams EJ. Evolution of the V, D, and J gene segments used in the primate gammadelta T-cell receptor reveals a dichotomy of conservation and diversity. *Proc Natl Acad Sci U S A.* 2011;108:E332–40.
  31. Mocchegiani E, Malavolta M, Costarelli L, Giacconi R, Piacenza F, Lattanzio F, Basso A. Is there a possible single mediator in modulating neuroendocrine-thymus interaction in ageing? *Curr Aging Sci.* 2013;6:99–107.
  32. Goff BL, Roth JA, Arp LH, Incefy GS. Growth hormone treatment stimulates thymulin production in aged dogs. *Clin Exp Immunol.* 1987;68:580–7.
  33. Goff BL. Thymic structure and function in aging dogs. <https://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=12181&context=rttd>. Accessed 15 Mar 2019.
  34. Borghetti P, Saleri R, Mocchegiani E, Corradi A, Martelli P. Infection, immunity and the neuroendocrine response. *Vet Immunol Immunopathol.* 2009;130:141–62.
  35. Van de Velde H, Janssens GPJ, Rochus L, Duchateau L, Sharek-Tedin L, Zentek P, Nguyen P, Cox E, Buysse J, Biourge V, Hesta M. Proliferation capacity of T-lymphocytes is affected transiently after a long-term weight gain in beagle dogs. *Vet Immunol Immunopathol.* 2013;152:237–44.
  36. Fay NS, Larson EC, Jameson JM. Chronic inflammation and  $\gamma\delta$  T cells. *Front Immunol.* 2016;7:210.
  37. Freeman L, Becvarova I, Cave N, MacKay C, Nguyen P, Rama B, Takashima G, Tiffin R, Tsujimoto H, van Beukelen P. WSAVA nutritional assessment guidelines. *J Small Anim Pract.* 2011;52:385–96.
  38. Link WW, DeVries JG, Ford R, Franklin TR, Hurlley KF, McClure KD, Paul MA, Schultz RD. 2011. The American animal hospital association (AAHA), canine vaccination guidelines. [https://www.aaaha.org/public\\_documents/guidelines/vaccination\\_recommendation\\_for\\_general\\_practice\\_table.pdf](https://www.aaaha.org/public_documents/guidelines/vaccination_recommendation_for_general_practice_table.pdf). Accessed: 15 March, 2019. [https://www.aaaha.org/public\\_documents/professional/guidelines/caninevaccineguidelines.pdf](https://www.aaaha.org/public_documents/professional/guidelines/caninevaccineguidelines.pdf). Accessed 15 Mar 2019.
  39. Merck Veterinary Manual. <https://www.msdvetmanual.com/digestive-system/dentistry/periodontal-disease-in-small-animals>. Accessed 15 Mar 2019.
  40. Moore PF, Rossitto PV, Olivry T. Development of monoclonal antibodies to canine T cell receptor-1 (TCR- $\gamma\delta$ ) and their utilization in the diagnosis of epidermotropic cutaneous T cell lymphoma. *Vet Pathol.* 1994;31:597.
  41. Ferrari L, Martelli P, Saleri R, De Angelis E, Cavalli V, Bresola M, Benetti M, Borghetti P. Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after in vitro homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. *Vet Immunol Immunopathol.* 2013;151:193–206.
  42. Ferrari L, Canelli E, De Angelis E, Catella A, Ferrarini G, Ogno G, Bonati L, Nardini R, Borghetti P, Martelli P. A highly pathogenic porcine reproductive and respiratory syndrome virus type 1 (PRRSV-1) strongly modulates cellular innate and adaptive immune subsets upon experimental infection. *Vet Microbiol.* 2018;216:85–92.

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Ready to submit your research? Choose BMC and benefit from:**

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more [biomedcentral.com/submissions](https://biomedcentral.com/submissions)

