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

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Abstract

Background: Data on gamma-delta (γδ) 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 γδ T lymphocyte (TCRγδ+) 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 γδ T cell subpopulations was performed.

Results: The percentage of γδ 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 γδ 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 γδ T cells according to the stage of life. γδ 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 γδ 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 TCRγδ+ cell profile. A decrease of the TCRγδ+ subpopulation percentage was observed in elder dogs. TCRγδ+ 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 γδ 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: γδ T lymphocytes, Dog, Stage of life, Breed, Aging
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 (γδ) T cell subset (γδ T lymphocytes) associated with dog autoimmune diseases [7, 8] or tumors [4, 9–13], but currently, there is a lack of data available on γδ 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 γδ 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 γδ cells were also observed in the epithelium of crypts, while not detected either in Peyer’s patches or in isolated mucosal lymphoid follicles [15]. γδ 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 γδ cells in the blood circulation defined two different γδ 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 γδ T cell profile (2.5%) [3]. A higher γδ 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 γδ 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, auto-immune 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 γδ T lymphocytes within the canine species, this topic presents an intriguing opportunity to define the γδ 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 γδ T lymphocytes

Mean values ± 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 ± SD and ranges of TCRγδ+ 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 TCRγδ+ 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 Years | Age Months | Sex Female (F) / Male (M) | Weight Kg | Clinical examination |
|-----|-----------|------------|---------------------------|-----------|----------------------|
| 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 *   |
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 γδ 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 TCRγδ+ cell percentages are as follows:

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

   Specifically, a reduction of TCRγδ+ 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 TCRγδ+ 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 TCRγδ+ cells from Mature (group M, mean = 1.36%) to Senior (group S, mean = 0.42%). The percentage values of TCRγδ+ 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 TCRγδ+ 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 TCRγδ+ 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 TCRγδ+ 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 TCRγδ+ 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 1 Data of recruited dogs according to age, sex, weight and clinical examination (Continued)

| Dog | Age | Sex | Weight | Clinical examination |
|-----|-----|-----|--------|----------------------|
| 44  | 2   | F   | 39.0   | Microfilaria and antigen testing |
| 45  | 1   | M   | 17.0   | Microfilaria and antigen testing |
| 46  | 13  | F   | 32.0   | Routine check-up; Periodontitis stage 2 * |
| 47  | 10  | M   | 23.0   | Orthopedic follow-up; Periodontitis stage 3 * |

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 |
| Group J (Junior) | 14 healthy dogs | 3 females 11 males | From 1 to 2 years | From 16.4 to 42.0 Kg |
| Group A (Adult) | 8 healthy dogs | 5 females 3 males | From 3 to 5 years | From 5.0 to 57.0 Kg |
| Group M (Mature) | 7 healthy dogs | 4 females 3 males | From 6 to 9 years | From 8.6 to 38.0 Kg |
| Group S (Senior) | 7 healthy dogs | 3 females 4 males | From 10 to 13 years | From 12.0 to 35.0 Kg |

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

Fig. 1 Representative flow cytometry dot plot of canine PBMC, showing lymphocyte gating (A) and density plots of TCRγδ+ cells (left upper quadrant, S1) and unspecific secondary antibody staining (left upper quadrant, Q1).

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 (cells/μl) | Lymphocytes (cells/μl) | CD3+ T lymphocytes (mean ± SD) | TCRγδ+ T lymphocytes (mean ± SD) |
|---------------|----------------|------------------------|-------------------------------|----------------------------------|
| Puppy (P)     | 6509 ± 2502    | 3745 ± 294             | 55.27 ± 15.92                 | 2.92 ± 2.27                      |
| Junior (J)    | 9700 ± 3696    | 3290 ± 350             | 52.99 ± 13.69                 | 2.49 ± 1.29                      |
| Adult (A)     | 10,775 ± 1652  | 2698 ± 629             | 67.35 ± 24.64                 | 2.46 ± 1.66                      |
| Mature (M)    | 8271 ± 996     | 2771 ± 502             | 45.56 ± 34.18                 | 1.36 ± 0.65                      |
| Senior (S)    | 7514 ± 358     | 2475 ± 162             | 43.05 ± 21.88                 | 0.42 ± 0.08                      |

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TCRγδ+ 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 TCRγδ+ cell decline in dog aging. The post-hoc power analysis performed 92.3%.

Taking into account the absolute counts of γδ 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 γδ 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 categorized according to five groups of stages of life (groups P, J, A, M, S) indicated dynamic changes of the TCRγδ+ 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 TCRγδ+ 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
aging. Dogs like humans and mice are part of the animal cluster characterized by a low TCRyδ+ 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 TCRyδ+ 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 consequent 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 Vγ9/Vδ2 T cells [36]. Therefore, TCRyδ+ cell decrease is inversely related to the body mass index. In obese mice, TCRyδ+ 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 TCRyδ+ cell decline, with many of these participants affected by chronic periodontitis diagnosed as stage 2 or 3. Significant differences in peripheral blood TCRyδ+ cells were detected in aged dogs (M and S groups) when compared to the youngest animals (J group). The reduction of blood circulating TCRyδ+ cells could be related to an increase of TCRyδ+ 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). TCRyδ+ 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 TCRyδ+ cells are crucial for mucosal integrity and functionality preservation while their high-level release can cause the onset of chronic inflammation [36]. In fact, γδ 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 TCRyδ+ cell profile, less than 10%,
like rodents and humans. The percentage of TCRγδ+ 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 TCRγδ+ 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 TCRγδ+ subpopulation percentage observed in elder dogs (S group). TCRγδ+ cells of dogs of group S were statistically different in percentage observed in elder dogs (S group). TCRγδ+ cells in the peripheral blood of dogs recorded is comparable with the value of 2.5% published by 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 TCRγδ+ subpopulation percentage observed in elder dogs (S group). TCRγδ+ 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 TCRγδ+ 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 profile as well as longevity and genomic similarity of dogs. The Fédération Cynologique Internationale (FCI) recognizes 339 distinct pure breeds [27], therefore it was considered appropriate to obtain the γδ 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 γδ 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...
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 3EDTA tube (Becton Dickinson, USA) for quantification of CD3+ T lymphocytes and γδ 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® (Cedarlane®, Burlington, 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 Tryphan Blue exclusion (Sigma-Aldrich, St. Louis, Missouri, USA) by using a TC20™ automated cell counter (BioRad, Hercules, CA, USA) before staining for FCM analysis.

Flow cytometry analysis
Quantification of T lymphocytes (CD3+) and γδ T lymphocytes within isolated PBMC was carried out by a mouse anti-dog CD3-FITC (clone CA17.2A12, IgG 1, AbD Serotec, Raleigh, NC, USA) and a mouse anti-dog TCRγδ (clone CA20.8H1, IgG2a, Prof. Peter F. Moore, Leukocyte Antigen Biology Laboratory (LABL), Davis, CA, USA [40]; primary antibodies followed by a goat anti-mouse IgG2a-PE secondary antibody (cat. M32204;
performed using the SPSS v.25 program for Windows.

TCR deviation (SD) and range, and individual data only for α power with effects according to stages of life was assessed via post-hoc analysis. In the case of differences in variances, ANOVA one-way and Tamhane comparison tests were used for normally distributed data with unequal variances. The statistical analysis was performed 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 ≤ 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 α = 0.05 and significant data were considered if > 80%. Values were presented as mean ± 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; δ: First apoptosis signal alpha; FBS: Fetal bovine serum; FCVM: Flow cytometry; FCI: Fédération Cynologique Internationale; hi: Heat-inactivated; K3EDTA: Tripotassium ethylenediaminetetraacetic acid; Ig: Immunoglobulin; LABL: Leukocyte Antigen Biology Laboratory; PBMC: Peripheral blood mononuclear cells; PBS: Phosphate buffered solution; PE: Phycocerythrin; RTE: Recent thymic emigrants; SD: Standard deviation; sT1-T3RECs: Signal joint-T cell receptor excision circles; TCRαβ: T cell receptor alpha-beta heterodimer; TCRγδ: T cell receptor gamma-delta heterodimer; TNF-α: Tumor necrosis factor alpha; TRG: T cell receptor gamma locus; VDJ: Variable, diversity and joining genes; Vγ9/Vδ2: 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 Dr. Rachel Dalton, Avon Lodge Veterinary Group, Bristol (UK) for support in language revision.

Authors’ contributions
CM conceived the study. CM, AM, and FA contributed to the experimental design. CM, LF and PB contributed to interpretation of data. ACo contributed to interpretation of data and drafting the manuscript. CM and LF contributed by performing experimental procedures/data analysis and collaborated to draft the manuscript. ACo 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

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