Peripheral leukocyte populations and oxidative stress biomarkers in aged dogs showing impaired cognitive abilities

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Abstract In the present study, the peripheral blood leukocyte phenotypes, lymphocyte subset populations, and oxidative stress parameters were studied in cognitively characterized adult and aged dogs, in order to assess possible relationships between age, cognitive decline, and the immune status. Adult ($N=16$, 2–7 years old) and aged ($N=29$, older than 8 years) dogs underwent two testing procedures, for the assessment of spatial reversal learning and selective social attention abilities, which were shown to be sensitive to aging in pet dogs. Based on age and performance in cognitive testing, dogs were classified as adult not cognitively impaired (ADNI, $N=12$), aged not cognitively impaired (AGNI, $N=19$) and aged cognitively impaired (AGCI, $N=10$). Immunological and oxidative stress parameters were compared across groups with the Kruskal-Wallis test. AGCI dogs displayed lower absolute CD4 cell count ($p<0.05$) than ADNI and higher monocyte absolute count and percentage ($p<0.05$) than AGNI whereas these parameters were not different between AGNI and ADNI. AGNI dogs had higher CD8 cell percentage than ADNI ($p<0.05$). Both AGNI and AGCI dogs showed lower CD4/CD8 and CD21 count and percentage and

higher neutrophil/lymphocyte and CD3/CD21 ratios ($p<0.05$). None of the oxidative parameters showed any statistically significant difference among groups. These observations suggest that alterations in peripheral leukocyte populations may reflect age-related changes occurring within the central nervous system and disclose interesting perspectives for the dog as a model for studying the functional relationship between the nervous and immune systems during aging.

Keywords Aging · Dog · Cognitive abilities · Immunosenescence · Lymphocyte subpopulations · Oxidative stress

Abbreviations

AD Alzheimer's disease
CNS Central nervous system
WBC White blood cells

Introduction

Human aging is accompanied by alterations in the functions of several body components, including the nervous and the immune systems. The most evident consequences of senescence on the nervous system are changes in mental abilities, collectively referred to as cognitive aging. Although changes are not uniform across all cognitive domains or across all older individuals, some functions are more likely to be affected than others. Among the most sensitive to aging are the so-called

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executive functions, a coordinated set of processes that play a key role in many aspects of cognition, for instance, by controlling goal-oriented activities, the allocation of attentional resources, the inhibition of irrelevant information processing, and, more broadly, behavioral flexibility (Glisky 2007). Some decline in cognitive abilities is expected to occur even in the healthy elderly, and, up to some extent, cognitive decline is considered as a physiological consequence of aging (Brayne and Calloway 1988; Buckner 2004; Ownby 2010). In some cases, however, aging impacts severely and progressively on several cognitive functions, eventually leading to impairment in the subject's ability to perform even simple activities, as in the case of Alzheimer's disease (AD).

Various studies support a link between the immune system and the functional alterations of the nervous system. Almost every component of the immune system undergoes age-associated changes, which may include either enhanced or diminished functions (De la Fuente 2008; Yirmiya and Goshen 2011). Age-related alterations of immunological markers, such as IL-6 and C-reactive protein (CRP), were associated with greater decline in cognitive functions (Ownby 2010; Roberts et al. 2009; Yirmiya and Goshen 2011). Inflammatory processes observed in aging were associated with the impaired production of reactive oxygen species (ROS) by neutrophils and monocytes (De la Fuente 2008) and increased mitochondrial decay (Glade 2010). Conditions of acute or chronic inflammation, such as those associated with senescence, can contribute to functional damage of the nervous system through impaired ROS production and oxidative stress (De la Fuente 2008; Glade 2010). Increasing evidences support the hypothesis that reduction of cellular antioxidants and consequent augmentation of oxidative stress are fundamental causes for aging processes and neurodegenerative diseases (Di Bona et al. 2010; Head et al. 2002; Landsberg et al. 2012).

Until recently, it was assumed that interactions between the nervous and immune systems were limited to cases of pathological insults (Kipnis et al. 2008). In the last years, however, it has been widely accepted that the immune system plays a central role in modulating cognitive functions and neuronal plasticity under physiological conditions, through a complex interaction among microglia and astrocytes, peripheral immune cells (mainly T lymphocytes and macrophages), neurons, and neuronal precursor cells. In particular, CD4 lymphocytes have an

important homeostatic role in the modulation of learning, memory, and neuronal plasticity. In several models, depletion of CD4 T cell, but not of CD8 or B cell, resulted in decline in cognitive functions (Huang et al. 2009; Kipnis et al. 2008, 2012; Radjavi et al. 2014a; Wolf et al. 2009; Yirmiya and Goshen 2011).

To the best of our knowledge, only few studies (Bonotis et al. 2008; Magaki et al. 2008; Speciale et al. 2007) dealt with alterations in peripheral lymphocyte subpopulations in human with cognitive impairment. These researches were focused in comparing elderly AD or mild cognitive impaired patients with age-matched healthy subjects while none of them included adult subjects.

In the last couple of decades, the dog has gained increasing reliability as a model of human physiological and pathological brain aging. Aged dogs spontaneously develop neuropathological changes as well as cognitive decline and behavioral alterations that parallel several aspects of human senescence, ranging from that seen in physiological aging to those typical of early-stage AD (Cotman and Head 2008; Head 2013; Landsberg et al. 2012; Waters 2011). A number of diverse neuropsychological tests have been used in dogs to evaluate effects of aging on a specific cognitive domain (Milgram et al. 1994; Mongillo et al. 2013; Studzinski et al. 2006; Tapp et al. 2003). As seen in humans, tasks relying on executive functions are the most age-sensitive in the dog species (Studzinski et al. 2006; Tapp et al. 2003). For instance, aged dogs show decreased abilities to reverse a previously learned stimulus-reward contingency, as shown both in a laboratory canine model of human aging (Studzinski et al. 2006) and in healthy aged pet dogs (Mongillo et al. 2013). Selective attention is another example of executive function, where a decline has been observed in both aged laboratory Beagle dogs (Snigdha et al. 2012) and aged healthy pet dogs (Mongillo et al. 2010; Wallis et al. 2014).

The similarities between canine and human senescence are not limited to brain aging. Some studies have described an age-related decline in the immune system of the pet dog, which parallels that of humans in many respects (Day 2010; Strasser et al. 2000). Although these findings suggest the dog as a good candidate model for studying the functional relationship between the nervous and immune systems during aging, no studies linking cognitive abilities to immunological parameters have been performed in the dog. Thus, assuming that the interaction of the immune cells with brain

structures are reflected in the periphery, we considered as worthwhile to investigate whether dysregulations in the peripheral immune cell populations and oxidative biomarkers are related to cognitive impairment in the pet dog. Overall, this information will contribute both to characterize a spontaneous animal model of human cognitive aging and to improve the care and management of the aged pet dog.

Materials and methods

Animal subjects

Participants were recruited by word of mouth and advertisements among clients of local veterinary clinics and students of the University of Padova. Forty-five healthy pet dogs were enrolled in this study. Inclusion criteria consisted of an age between 2 and 7 years (adult group, $N=16$) or above 8 years (aged group, $N=29$). Since size significantly affects dogs' life expectancy and aging (Kraus et al. 2013), only dogs of similar size (i.e., medium/large size) were selected. Prior to inclusion, all dogs underwent a physical examination, evaluation of historical records and behavioral assessment to exclude medical conditions that could affect the current study. The final sample included 20 intact males, 2 orchidectomised males, 4 intact females, and 19 ovariectomized females of different breeds. Mean ($\pm$ SD) age and height at shoulder of adult dogs were 4.1 ± 1.9 years and 55.6 ± 4.5 cm; aged dogs were 10.1 ± 1.3 years old and 57.7 ± 8.5 cm tall.

All procedures were carried out in accordance with Italian legislation on animal care (DL n.116, 27/01/1992). Owners were informed in advance of the specific aim of the study and took part in the procedure on a voluntary basis.

Cognitive tests

Cognitive testing was conducted in the Laboratory of Applied Ethology (Department Comparative Biomedicine and Food Science, University of Padova; size 5×5 m) where a CCTV system (WV-GP250, Panasonic, Osaka, Japan) allowed real-time monitoring and recording of all procedures for subsequent analysis. The testing assessed attentive and spatial abilities with two protocols already in use in our laboratory and previously proved to be sensitive to cognitive impairment in aged

pet dogs. Details of the experimental procedures to assess social attention and spatial learning can be found in our previous studies (Mongillo et al. 2010, 2013).

As the first evaluation, the dogs underwent social attention tasks (Mongillo et al. 2010). Briefly, the dog was taken into the room and held by an experimenter, while the owner and an unfamiliar person walked simultaneously and in opposite direction across the room, eventually leaving through two different doors. Eight transits were performed, in the last of which the dog's view of the scene was prevented by a curtain. Once the doors were closed after this last transit, the curtain was removed and the dog released. The task ended when the dog approached one of the two doors or after 30 s if the dog did not move. Dogs underwent two variants of the task, with the owner and the unfamiliar person wearing or not a hood that entirely covered their head. The two tasks were administered to each dog on the same day, with a 15-min break in between. The order of administration of the two tasks was balanced within both adults and aged dogs. At the end of both attention tasks, the room floor was sanitized and a T-maze was assembled to evaluate the dog's spatial abilities.

Thirty minutes after the end of the attention tasks, the dog reentered the room for the learning session of the T-maze task (Mongillo et al. 2013). Briefly, the task consisted in continuous consecutive trials, in which the dog was taken into the start compartment of the maze then the owner called the dog, who could freely enter either of the maze's lateral arms. The first trial was used to determine the correct exit arm for all the subsequent learning trials. In learning trials, a correct response was recorded when the dog entered the correct lateral arm first, and learning was considered achieved when the dog choose the correct arm for three consecutive trials within a maximum of 15 trials. Dogs that successfully acquired spatial learning were retested 2 weeks later for long-term retention of the information acquired in the learning phase. Only dogs that passed the retention test underwent a reversal learning session, in which they were evaluated for their ability to learn to exit from the path opposite to that acquired in the learning session. The learning criterion to successfully complete reversal learning was identical to that of the learning session.

Collection of blood samples

At the end of the cognitive testing, the dog underwent a single blood collection using vacuum

tubes with either EDTA or without anticoagulant (VENOJECT, Terumo Europe N.V., Leuven, Belgium). Whole blood samples were immediately refrigerated and transported to the laboratory for hematological analysis. Serum and plasma aliquots were frozen and stored at -20°C until use.

Total and differential leukocyte counts and flow cytometry

Complete hemocromocytometric analyses were performed immediately after blood sample collection. White blood cell (WBC) count and leukocyte formula were analyzed using a CELL-DYN 3700 automatic analyzer with veterinary software (Abbott Laboratories, Abbott Park, Illinois, USA).

Two-color flow cytometry was used to calculate relative percentages of CD3⁺ (T cells), CD21⁺-like (B cells), CD4⁺ (T-helper cells), and CD8⁺ (T-cytotoxic cells) cells using canine-specific monoclonal antibodies (mAbs). Details for all mAbs used for flow cytometry assays are listed in Table 1. Negative control samples were stained with isotype control antibodies suggested by the manufacturer.

Peripheral blood mononuclear cells from dogs were analyzed by whole blood lysis: briefly, 100 μL was incubated with monoclonal antibodies at room temperature for 20 min. Erythrocytes were lysed by adding 2 mL of BD FACS lysing solution 1 $\times$, and tubes were incubated at room temperature for 10 min. The suspension was centrifuged, supernatant removed, and the pellet was finally resuspended in 500 μL of PBS.

Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the BD CellQuest software. Absolute values for each subset were calculated using counts obtained from WBC analysis in combination with flow cytometry data.

Acute phase proteins and oxidative stress indicators

C-reactive protein and haptoglobin analysis

Serum C-reactive protein was determined by a commercial E.I.A. kit (Tridelta PhaseTM CRP kit, Tridelta Development Ltd, Maynooth, Co. Kildare, Ireland) specifically designed for the dog. The intra-assay coefficient of variation was 5.6 %.

Haptoglobin was measured in plasma samples using a commercial enzymatic kit (Tridelta PhaseTM Haptoglobin kit, Tridelta Development Ltd, Maynooth, Co. Kildare, Ireland) following the manufacturer's instructions.

Malondialdehyde, glutathione, and advanced oxidation protein products analysis

Plasma malondialdehyde was measured by the fluorimetric method described by Wasowicz et al. (1993). Readings were performed in a LS-50B fluorimeter (PerkinElmer, Norwalk, USA), using excitation and emission wavelengths of 532 and 553 nm, respectively. Data were expressed as thiobarbituric acid reactive substances.

Total plasma glutathione was measured by an enzymatic method adapted for microtiter plate reader (Baker et al. 1990). Glutathione present in plasma samples and standards was oxidized by dithiobis-2-nitrobenzoic acid. Readings were performed every minute for 10 min until the signal remained constant, indicating that the redox reaction reached equilibrium. Blank was subtracted, and the obtained values of optical density were read against a standard curve (0.339–21.69 $\mu\text{mol/L}$).

The advanced oxidation protein product concentrations were measured spectrophotometrically as described before (Bordignon et al. 2014), using chloramine T (0–100 $\mu\text{mol/L}$, Sigma-Aldrich, Milan, Italy) as

Table 1 Monoclonal antibodies used in the study

Phenotype	Specificity	Dilution	Clone	Conjugate	Manufacturer
CD3	Pan T cells	1:4	CA17.2A12	FITC	Serotec, Oxford, UK
CD4	Helper T cells	1:10	YKIX302.9	RPE	Serotec, Oxford, UK
CD8	Cytotoxic T cells	1:4	YCATE55.9	RPE	Serotec, Oxford, UK
CD21	Pan B cells	1:10	CAD2.1D6	RPE	Serotec, Oxford, UK

the reference. The absorbance of the reaction mixtures was read at 340 nm in a microplate reader (VICTOR X4 2030 Multilabel Reader, PerkinElmer, Norwalk, USA) against blank. The advanced oxidation protein products content was expressed as chloramine T equivalents.

Data collection and statistical analysis

Data of cognitive testing were collected from video recordings by a single observer, as coded variables were unequivocal. In attention tasks, the behavior of dogs at the end of the task was coded as either *responsive*, if they moved toward the doors, or *unresponsive*, if the dog did not move within 30 s after being released. Spatial skills were assessed by the ability of the dogs to successfully reverse a previously learned path. Both criteria were based on previous results with the same protocol where responsiveness (Mongillo et al. 2010) and spatial reversal learning (Mongillo et al. 2013) were found to be impaired by aging. Performances of dogs in cognitive testing were used to classify each subject as cognitively impaired (unresponsive and/or unable to reverse) or not impaired (responsive and able to reverse).

Based on age and performance in cognitive testing, dogs were classified as adult not cognitively impaired (ADNI, $N=12$, mean age $\pm$ SD=3.9 $\pm$ 1.8 years), aged not cognitively impaired (AGNI, $N=19$, mean age $\pm$ SD=10.4 $\pm$ 1.4 years), and aged cognitively impaired (AGCI, $N=10$, mean age $\pm$ SD=9.4 $\pm$ 0.8 years). Given the limited size of the adult cognitively impaired group ($N=4$, mean age $\pm$ SD=4.3 $\pm$ 2.5 years), these subjects were excluded from further analysis.

Immunological parameters, acute phase proteins, and oxidative stress indicators were compared across groups with the Kruskal-Wallis test, followed by pairwise comparisons as appropriate (Dunn 1964). Statistical analyses were performed using SPSS® (SPSS Statistics Version 20.0, IBM Corp., Armonk, NY, USA).

Results

Cognitive performance of adult and aged dogs

All but four adult dogs were classified as cognitively unimpaired; those that were classified as impaired were unresponsive in one of the two attention tasks ($N=2$) or unable to reverse in the spatial task ($N=2$). Of the aged

dogs, ten were classified as cognitively impaired since they behaved unresponsively in one ($N=3$) or both ($N=5$) attention tasks or failed to successfully complete reversal learning ($N=4$). Two of such cognitively impaired aged subjects failed to meet both criteria, staying still at the end of both attention tasks and failing to complete successfully the spatial reversal learning.

Total and differential leukocyte counts and flow cytometry

Absolute differential leukocyte counts are shown in Table 2, and relative leukocyte and immunophenotype populations are reported in Table 3.

The total WBC number did not differ between groups. AGCI dogs displayed a lower absolute CD4 cell count than ADNI and a higher monocyte absolute count and percentage than AGNI whereas these parameters were not different between AGNI and ADNI. AGNI dogs had higher CD8 cell percentage than ADNI. Compared to ADNI, both AGNI and AGCI dogs showed lower total lymphocyte count, CD21 count and percentage and CD4/CD8 ratio, and higher neutrophil/lymphocyte and CD3/CD21 ratios.

Acute phase proteins and oxidative stress indicators

Concentrations of acute phase proteins and oxidative stress indicators in peripheral blood are shown in Table 4. None of them showed any statistically significant difference among groups.

Discussion

To the best of our knowledge, this is the first work documenting modifications of peripheral immune cell populations in association with cognitive impairment in aged dogs. Specifically, a lower peripheral CD4 lymphocyte number and higher monocyte number and percentage were found in aged dogs that did not show an appropriate response in a social attention task and/or were unable to reverse a previously learned response in a spatial task. The classification of some of the adult dogs as cognitively impaired could suggest a low specificity of our cognitive assessment. This may have reduced the possibility to observe other associations between cognition and immune or oxidative stress parameters. However, cognitive aging is a complex

Table 2 Differential leukocyte counts (cells $\times 10^3/\mu\text{L}$) in the peripheral blood of either adult or aged dogs in relation to the response to the cognitive tests

		ADNI ($N=12$)	AGNI ($N=19$)	AGCI ($N=10$)	$\bar{X}$	p value
WBC	Mean $\pm$ SEM	8.26 $\pm$ 0.74	7.99 $\pm$ 0.47	8.73 $\pm$ 0.79	0.513	0.774
	Median (min–max)	7.75 (5.61–13.20)	7.73 (4.90–12.00)	8.29 (4.73–12.90)		
Lymphocytes	Mean $\pm$ SEM	2.01 $\pm$ 0.22	1.43 $\pm$ 0.10	1.34 $\pm$ 0.16	5.916	0.052
	Median (min–max)	1.88 (1.26–3.59)	1.51 (0.67–2.30)	1.14 (0.57–2.46)		
CD3	Mean $\pm$ SEM	1.08 $\pm$ 0.15	0.86 $\pm$ 0.09	0.66 $\pm$ 0.12	4.979	0.083
	Median (min–max)	0.90 (0.61–2.35)	0.86 (0.24–1.51)	0.51 (0.25–1.41)		
CD4	Mean $\pm$ SEM	0.62 $\pm$ 0.08	0.44 $\pm$ 0.05	0.32 $\pm$ 0.07	7.448	0.024
	Median (min–max)	0.57 (0.36–1.24) ^a	0.45 (0.16–0.93) ^{a, b}	0.22 (0.10–0.76) ^b		
CD8	Mean $\pm$ SEM	0.30 $\pm$ 0.05	0.32 $\pm$ 0.04	0.22 $\pm$ 0.04	2.350	0.309
	Median (min–max)	0.24 (0.17–0.71)	0.30 (0.05–0.76)	0.21 (0.09–0.48)		
CD21	Mean $\pm$ SEM	0.50 $\pm$ 0.05	0.25 $\pm$ 0.03	0.21 $\pm$ 0.06	16.730	0.000
	Median (min–max)	0.49 (0.26–0.79) ^a	0.24 (0.11–0.51) ^b	0.16 (0.06–0.71) ^b		
CD4/CD8	Mean $\pm$ SEM	2.16 $\pm$ 0.12	1.98 $\pm$ 0.42	1.46 $\pm$ 0.23	7.726	0.021
	Median (min–max)	2.11 (1.61–3.21) ^a	1.44 (0.54–8.47) ^b	1.34 (0.79–3.06) ^b		
CD3/CD21	Mean $\pm$ SEM	2.22 $\pm$ 0.21	3.72 $\pm$ 0.37	3.97 $\pm$ 0.62	8.050	0.018
	Median (min–max)	2.18 (1.24–3.60) ^a	3.42 (1.19–6.99) ^b	3.92 (1.20–6.80) ^b		
Neutrophils	Mean $\pm$ SEM	5.35 $\pm$ 0.52	5.57 $\pm$ 0.42	6.23 $\pm$ 0.65	1.495	0.474
	Median (min–max)	4.55 (3.57–8.51)	5.54 (2.90–9.56)	5.84 (3.00–8.92)		
NLR	Mean $\pm$ SEM	2.74 $\pm$ 0.13	4.51 $\pm$ 0.68	5.17 $\pm$ 0.67	9.629	0.008
	Median (min–max)	2.78 (1.65–3.36) ^b	4.02 (1.67–14.27) ^a	5.14 (1.99–8.48) ^a		
Monocytes	Mean $\pm$ SEM	0.51 $\pm$ 0.06	0.46 $\pm$ 0.06	0.70 $\pm$ 0.06	8.585	0.014
	Median (min–max)	0.47 (0.23–0.79) ^{a, b}	0.36 (0.17–1.16) ^a	0.69 (0.47–1.08) ^b		
Eosinophils	Mean $\pm$ SEM	0.36 $\pm$ 0.05	0.37 $\pm$ 0.08	0.41 $\pm$ 0.10	0.154	0.926
	Median (min–max)	0.32 (0.08–0.71)	0.30 (0.00–1.23)	0.42 (0.00–1.22)		
Basophils	Mean $\pm$ SEM	0.03 $\pm$ 0.01	0.05 $\pm$ 0.01	0.06 $\pm$ 0.02	1.636	0.441
	Median (min–max)	0.02 (0.00–0.07)	0.05 (0.00–0.14)	0.06 (0.00–0.21)		

Superscript letters are used to indicate significant differences in medians within rows (e.g., CD21 cells $\times 10^3/\mu\text{L}$): groups with the same superscript letter are not significantly different in the row's parameter, groups with different superscript letters are significantly different in the row's parameter, group with two superscript letters are not significantly different in the row's parameter from groups indicated with either of the two superscripts letters. Where no superscripts letters are reported, no significant differences were found among experimental groups for the row's parameter. The level of significance of comparison across groups and pairwise comparisons was $p < 0.05$

ADNI adult not cognitively impaired dogs, AGNI aged not cognitively impaired dogs, ADGI adult cognitively impaired dogs, AGCI aged cognitively impaired dogs, NLR neutrophil/lymphocyte ratio

phenomenon that does not progress in a linear fashion nor at the same rate in all individuals. Even in humans, a certain prevalence of impaired cognitive functions in relatively young subjects is expected (Salthouse 2009) and the limits of cognitive assessment are inherent to the very nature of the phenomenon.

The lower peripheral CD4 lymphocyte count observed in this study in aged cognitively impaired dogs is compatible with the view that the depletion of circulating CD4 cells results in cognitive decline. This hypothesis was tested using different strains of immunocompromised mice and biological or pharmacological T cell depleted mice (Kipnis et al. 2012). Mice deficient in

CD4 T cells or mice with clonally restricted T cell repertoires showed impaired spatial learning and memory tasks, as measured by the Morris water maze (Radjavi et al. 2014a). T cells affecting learning and memory are located in the meningeal space (Kipnis et al. 2012), and peripheral CD4 T cells can migrate from deep cervical lymph nodes to the meningeal space, as the removal of deep cervical lymph nodes interrupts migration and results in cognitive decline (Radjavi et al. 2014b). The impaired spatial performance can be partially rescued by transferring CD4 T cells from wild-type donor mice into immunocompromised mice (Kipnis et al. 2012).

Table 3 Leukocyte populations and immunophenotype percentages (%) in the peripheral blood of either adult or aged dogs in relation to the response to the cognitive tests

		ADNI (<i>N</i> =12)	AGNI (<i>N</i> =19)	AGCI (<i>N</i> =10)	<i>X</i>	<i>p</i> value
Lymphocytes	Mean±SEM	24.02±1.01	18.63±1.95	15.92±2.10	8.389	0.015
	Median (min–max)	23.30 (19.70–33.40) ^a	16.60 (5.40–35.30) ^b	14.55 (9.40–20.10) ^b		
CD3	Mean±SEM	53.16±2.96	58.36±3.30	46.78±3.34	5.116	0.077
	Median (min–max)	52.64 (31.23–66.05)	58.84 (34.72–78.86)	44.55 (27.91–61.53)		
CD4	Mean±SEM	30.81±1.55	29.98±1.89	22.14±2.83	5.320	0.070
	Median (min–max)	29.34 (22.00–41.51)	28.58 (12.43–41.89)	20.18 (9.06–35.18)		
CD8	Mean±SEM	14.81±1.02	21.71±2.48	16.07±1.61	6.258	0.044
	Median (min–max)	15.02 (6.86–19.67) ^a	22.01 (4.25–49.16) ^b	15.62 (9.96–23.64) ^{a, b}		
CD21	Mean±SEM	25.50±2.03	17.75±1.37	15.57±3.20	10.346	0.006
	Median (min–max)	23.49 (14.93–39.62) ^a	16.50 (7.23–29.30) ^b	12.76 (5.22–37.37) ^b		
Neutrophils	Mean±SEM	64.61±1.27	57.69±6.04	70.63±2.11	3.297	0.192
	Median (min–max)	64.60 (55.10–74.00)	64.01 (0.96–81.5)	69.95 (60.10–79.70)		
Monocytes	Mean±SEM	6.46±0.66	5.36±0.68	8.42±0.84	8.428	0.015
	Median (min–max)	6.65 (2.00–9.11) ^{a, b}	5.20 (0.35–12.60) ^a	8.30 (5.20–13.90) ^b		
Eosinophils	Mean±SEM	4.55±0.68	4.53±0.99	4.41±0.85	0.329	0.848
	Median (min–max)	4.90 (1.10–8.30)	4.00 (0.00–18.20)	4.30 (0.00–9.50)		
Basophils	Mean±SEM	0.36±0.09	0.49±0.10	0.61±0.19	0.517	0.772
	Median (min–max)	0.25 (0.00–0.90)	0.40 (0.00–1.30)	0.60 (0.00–1.90)		

Superscript letters are used to indicate significant differences in medians within rows (e.g., CD21 cells×10³/μL): groups with the same superscript letter are not significantly different in the row's parameter, groups with different superscript letters are significantly different in the row's parameter, group with two superscript letters are not significantly different in the row's parameter from groups indicated with either of the two superscripts letters. Where no superscripts letters are reported, no significant differences were found among experimental groups for the row's parameter. The level of significance of comparison across groups and pairwise comparisons was *p*<0.05

ADNI adult not cognitively impaired dogs, *AGNI* aged not cognitively impaired dogs, *ADCI* adult cognitively impaired dogs, *AGCI* aged cognitively impaired dogs

We are not aware of other studies investigating the relationship between leukocyte populations in the peripheral blood and impairment of cognitive tasks in clinically healthy adults and aged animal or human subjects. However, few studies compared elderly AD or mild cognitive impaired human patients with healthy age-matched control subjects, although findings are still controversial. The relative proportion of peripheral CD4 cells was lower, even if not significantly, in mild cognitive impaired subjects than in healthy subjects (Magaki et al. 2008), and a significant decrease in the peripheral CD4 lymphocyte subpopulation was observed in severe but not in mild AD patients (Bonotis et al. 2008). Conversely, in another study, the percentage and absolute number of CD4 lymphocytes did not differ between different stages of AD and healthy subjects (Speciale et al. 2007). It is not fully appropriate to compare our results with the studies reported above, in which aged individuals with a clinical diagnosis of mild or severe cognitive impairment were compared with healthy age-matched counterparts. First of all, our cognitively

impaired aged dogs were clinically healthy, in spite of their inability to express an appropriate response in a social attention task or to reverse a previously learned response in a spatial reversal-learning task. In addition, we compared both normal and cognitively impaired aged dogs with normal adult animals, and this enabled us to detect the significant decline in peripheral CD4 lymphocyte count in cognitively impaired aged dogs. In any case, the whole body of data leads to the hypothesis that alterations in peripheral lymphocyte populations may reflect changes occurring within the CNS parenchyma.

Circulating monocytes interact with T cells and exert protective/healing function in the CNS (see Schwartz et al. 2013); however, we are not aware about studies addressing the relationship between cognitive abilities and peripheral monocyte number. In this study, the monocyte count and percentage were significantly higher in aged cognitively impaired dogs compared with not impaired aged dogs, suggesting that increased monocyte number is associated with cognitive

Table 4 Acute phase protein and oxidative stress indicator concentrations in the peripheral blood of either adult or aged dogs in relation to the response to the cognitive tests

		ADNI (N=12)	AGNI (N=19)	AGCI (N=10)	<i>X</i>	<i>p</i> value
Haptoglobin (mg/mL)	Mean±SEM	0.24±0.07	0.31±0.06	0.44±0.09	5.529	0.063
	Median (min–max)	0.12 (0.03–0.70)	0.28 (0.06–1.09)	0.39 (0.11–0.80)		
C-reactive protein (µg/mL)	Mean±SEM	1.38±0.30	1.47±0.23	1.80±0.30	2.666	0.264
	Median (min–max)	1.01 (0.57–4.28)	1.11 (0.54–4.11)	1.67 (0.47–3.97)		
GSH (µM)	Mean±SEM	0.74±0.04	0.63±0.08	0.63±0.07	3.260	0.196
	Median (min–max)	0.77 (0.45–0.92)	0.57 (0.17–1.65)	0.66 (0.25–1.01)		
MDA (µM)	Mean±SEM	5.26±0.61	4.55±0.45	3.65±0.36	3.626	0.163
	Median (min–max)	4.58 (2.54–9.48)	4.40 (1.76–10.36)	3.42 (1.83–5.26)		
AOPP (µM)	Mean±SEM	88.83±11.56	98.01±12.01	71.52±16.87	2.780	0.249
	Median (min–max)	92.35 (28.02–147.88)	97.36 (34.89–251.68)	46.70 (13.51–180.75)		

ADNI adult not cognitively impaired dogs, AGNI aged not cognitively impaired dogs, ADNI adult cognitively impaired dogs, AGCI aged cognitively impaired dogs, GSH glutathione, MDA malondialdehyde, AOPP advanced oxidation protein products

impairment. An increase in monocyte count indicates an inflammatory process that may explain some functional alterations in the CNS. However, the monocyte counts and percentages observed in all experimental groups in this study are within the physiological range observed in the dog (Blount et al. 2005; Lawrence et al. 2013; Strasser et al. 2000) and are not indicative of an inflammatory status, as it can be also confirmed by the circulating levels of C-reactive protein and haptoglobin. In addition, the dogs included in this study did not show any clinical sign of disease.

In the dog, the effect of aging in the monocyte count is still controversial. In Labrador retriever dogs, peripheral monocyte decreased with age (Blount et al. 2005); while no age-dependent variations in monocyte count and percentage were observed in German Shepherd dog (Strasser et al. 2000). More recently, Lawrence et al. (2013) used an extensive database (more than 6000 dogs) and observed that monocyte count decreased from 1 to 6 years of age and then increased again to values similar to those of dogs younger than 1 year. Thus, it is likely that our age group composition (mean age of adult dogs about 4 years and of aged dogs about 10 years) masked the differences in monocyte number between adult and aged dogs.

In the present study, lymphocyte percentage, CD21, and CD4/CD8 ratio decreased with age, while CD3/CD21 and neutrophil/lymphocyte ratios increased in older dogs. These results are in agreement with those reported in other studies in the dog (Blount et al. 2005; Heaton et al. 2002; Watabe et al. 2011). Indeed, the inversion of the CD4/CD8 ratio has been identified as

a hallmark of immunosenescence not only in dogs (Blount et al. 2005; Heaton et al. 2002; HogenEsch et al. 2004; Watabe et al. 2011) but also in cats, mice, and humans (Campbell et al. 2001; Day 2010; Huppert et al. 1998; Pinchuk and Filipov 2008).

Data on CD21 cells and the CD3/CD21 ratio in dogs are scarce and contradictory, reporting decreasing or stable B cells and CD3/CD21 ratio (Blount et al. 2005; HogenEsch et al. 2004; Watabe et al. 2011). Our results agree with those reported by Watabe et al. (2011). To the best of our knowledge, no studies associating the CD21 or CD3/CD21 ratio and aging are available in humans.

Blood neutrophil/lymphocyte ratio is an inexpensive and easily applicable marker of inflammation and represents a useful diagnostic tool in human and veterinary medicine. It is considered a robust predictor of deleterious outcomes in human cognitive dysfunctions (Halazun et al. 2014; Kuyumcu et al. 2012). In the present study, neutrophil/lymphocyte ratio was higher in aged dogs, but no differences between impaired and not impaired aged dogs were observed. The fact that the animals used in our study were clinically healthy could explain the absence of predictive value of neutrophil/lymphocyte ratio.

We did not observe any significant age-related difference in plasma haptoglobin and C-reactive protein, confirming previous observations in healthy animals (Ceron et al. 2005). In the dog, the measurement of acute-phase proteins is a useful early marker of inflammation, and differences in the time course of response have been detected among these markers. A significant increase in C-reactive protein can be observed in 4 h,

and it reaches peak values in about 1–2 days (major responder) while haptoglobin shows a significant increase in about 1 day and peaks in 3–4 days (moderate responder). Thus, the combined measurement of these two proteins provides information about the temporal evolution of an inflammatory event or disease (Ceron et al. 2005). In all subjects enrolled in this experiment, both C-reactive protein and haptoglobin were in the physiological range (Ceron et al. 2005), suggesting that the animals were free from inflammation.

In our groups of pet dogs, no relationship between markers of oxidative stress and cognitive impairment was observed, and this seems to deny the documented association between cognitive impairment and oxidative stress within the CNS (Glade 2010). However, changes in oxidative stress parameters within the CNS are not necessarily reflected by peripheral changes. In addition, plasma oxidative stress markers did not differ between our adult and aged dogs. Head et al. (2002) reported that serum malondialdehyde increased with age in experimental Beagle dogs. However, no age-related changes were found in erythrocyte glutathione and plasma cysteine concentrations of pet dogs (Moyer and Trepanier 2009). It is likely that dogs' lifestyle, including nutritional and behavioral management, can affect the peripheral oxidative stress indicators, and that results obtained in experimental Beagle dogs may not be reproducible in a heterogeneous population of pet dogs.

Implications and future directions

The dog is a well-established animal model to study age-related phenomena, and the results reported in this article disclose interesting perspectives for using the pet dog also to study the relationship between immunosenescence and cognitive functions. Some of our results suggest that subtler differences may have been undetected due to a relatively small sample size (as it could be the case, for instance, of CD3 lymphocytes), and large-scale studies on pet dog populations are required to deepen the present results. Moreover, it would be crucial to broaden the spectrum of tested cognitive domains, including other paradigms proved to be sensitive to aging in pet dogs. Although at the time of this study there were no other tests applicable to pet dogs with a demonstrated sensitivity to aging, other paradigms became available in more recent times. For instance, Rosado et al. (2012a, 2012b) described an

open field test, which allowed detecting changes in curiosity (Rosado et al. 2012a) and social responsiveness (Rosado et al. 2012b) in healthy aged pet dogs.

More factors potentially able to affect canine immunological status, including cytokines and leukocyte subsets, in relation with aging and cognitive abilities deserve to be studied. Moreover, the infiltration of the different lymphocyte subtypes in the brain parenchyma and in the meningeal space of aged dogs should be investigated in relation to cognitive dysfunctions.

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Authors' contribution PM participated in the design of the study, in the performance of the cognitive tests, performed the statistical analysis and contributed in drafting the manuscript. DB participated in the design of the study and in the manuscript drafting and coordinated the hematological and biochemical analyses. EP looked after and carried out the cognitive tests. AS set up and carried out the hematological and cytofluorimetric assays. LM participated in the design of the study, coordinated the cognitive tests, and looked after the final revision of the manuscript. GG conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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