Published in final edited form as:

Dev Comp Immunol. 2018 October ; 87: 64–74. doi:10.1016/j.dci.2018.05.022.

Multi-color Flow Cytometry for Evaluating Age-Related Changes in Memory Lymphocyte Subsets in Dogs

Sita S. Withers^a, Peter F. Moore^b, Hong Chang^a, Jin W. Choi^c, Stephen J. McSorley^c, Michael S. Kent^a, Arta M. Monjaze^d, Robert Canter^e, William J. Murphy^f, Ellen E. Sparger^g, and Robert B. Rebhun^a

^aCenter for Companion Animal Health, Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California-Davis, One Shields Avenue, Davis, CA 95616 USA.

^bDepartment of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California-Davis, One Shields Avenue, Davis, CA 95616 USA.

^cCenter for Comparative Medicine, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California-Davis, County Road 98 & Hutchison Drive, Davis, CA 95616 USA.

^dComprehensive Cancer Center, Department of Radiation Oncology, School of Medicine, University of California-Davis, 4501 X Street, G-140, Sacramento, CA 95817 USA.

^eComprehensive Cancer Center, Department of Surgery, School of Medicine, University of California-Davis, 4501 X Street, G-140, Sacramento, CA 95817 USA.

^fDepartment of Dermatology, School of Medicine, University of California-Davis, 2921 Stockton Blvd, Sacramento, CA 95716 USA.

^gDepartment of Medicine and Epidemiology, School of Veterinary Medicine, University of California-Davis, One Shields Avenue, Davis, CA 95616 USA.

Abstract

While dogs are increasingly being utilized as large-animal models of disease, important features of age-related immunosenescence in the dog have yet to be evaluated due to the lack of defined naïve vs. memory T lymphocyte phenotypes. We therefore performed multi-color flow cytometry on peripheral blood mononuclear cells from young and aged beagles, and determined the differential cytokine production by proposed memory subsets. CD4⁺ and CD8⁺ T lymphocytes in aged dogs displayed increased cytokine production, and decreased proliferative capacity. Antibodies targeting CD45RA and CD62L, but less so CD28 or CD44, defined canine cells that consistently exhibited properties of naïve-, central memory-, effector memory-, and terminal effector-like CD4⁺ and CD8⁺ T lymphocyte subsets. Older dogs demonstrated decreased frequencies of naïve-like CD4⁺ and CD8⁺ T lymphocytes, and an increased frequency of terminal effector-like CD8⁺ T

Co-Corresponding authors: Robert Rebhun, rrbrehun@ucdavis.edu; Ellen Sparger, eesparger@ucdavis.edu.

Declarations of interest:

None

lymphocytes. Overall findings revealed that aged dogs displayed features of immunosenescence similar to those reported in other species.

Keywords

T lymphocyte; immunosenescence; inflammaging; canine; CD45RA

1. Introduction

Naturally occurring cancers, as well as infectious, inflammatory and autoimmune diseases in dogs are increasingly being investigated as large-animal models for comparable human diseases(Alvarez, 2014; Day, 2009; Ho et al., 2015; LeBlanc et al., 2016; Marx et al., 2015). Immune responses are inherently involved in disease pathogenesis, necessitating a thorough understanding of the comparative immunology across species. Since ageing is correlated to many of these disease processes, it is particularly important to characterize species differences that might occur in the shifting function of the immune system with age, a process often referred to as “immunosenescence”(Pawelec, 2018).

Age-related changes in lymphocyte function have been observed for decades in humans and mice. For example, aged CD4+ and CD8+ T lymphocytes (T cells) have consistently demonstrated decreased proliferative capacity(Effros and Walford, 1983; Jiang et al., 2007). More recently, aging has been associated with increased expression of intracellular pro-inflammatory cytokines such as interferon-gamma (IFN γ) and tumor necrosis factor-alpha (TNF α) by mitogen-stimulated CD4+ and CD8+ T cells(Bandres et al., 2000; Hoffmann et al., 2005; Macaulay et al., 2013). Subsequently, accumulating evidence suggests that T cells may play a role in the proinflammatory state that develops with advancing age, known as “inflammaging”(Macaulay et al., 2013).

Additionally, clinical and subclinical antigen exposure throughout life results in the selection of naïve T cell clones that expand and differentiate into memory and effector T cells(Pawelec, 2018). In humans, differentiation from naïve, to central memory (CM), effector memory (EM), and finally terminally differentiated effector memory (TEMRA) T cells is thought to occur in a linear progression(Mahnke et al., 2013). As cells gain effector functions such as cytokine production, they lose proliferative capacity and lymph node homing ability(Mahnke et al., 2013). By the TEMRA stage, T cells become generally senescent(Geginat et al., 2003). This process enables development of an improved adaptive immune response to previously encountered pathogens. The immunologic cost of this is a decrease in the naïve T cell pool, which is exacerbated by decreasing output of naïve T cells from the involuting thymus(LeMaout et al., 2000; Schwab et al., 1997). Consistent hallmarks of immunosenescence in humans is the predictable decrease in naïve CD4+ and CD8+ T cell pools, and the increase in CD8+ TEMRA T cells(Fagnoni et al., 2000; Kang et al., 2004; Koch et al., 2008; Pawelec, 2018; Thome et al., 2014). Concurrent increases in specific memory subsets such as CM and EM subsets are somewhat inconsistent across studies(Fagnoni et al., 2000; Kang et al., 2004; Koch et al., 2008). Proportional and absolute decreases in naïve T cells, in conjunction with expansion of selected T cell clones over time,

lead to the development of dominant clones and a relative loss of T cell repertoire diversity(LeMaoult et al., 2000; Schwab et al., 1997). These age-related changes have the potential to diminish immune responses to novel antigens, including certain pathogens and vaccinations, in mouse models and humans, and therefore warrant investigation in dogs(Messaoudi et al., 2004; Schulz et al., 2015; Yager et al., 2008).

In humans, naïve and memory T cell subsets are generally differentiated using a combination of phenotypic markers that represent antigen-experience and lymph node homing(Mahnke et al., 2013). Exposure to antigen is signified by alternative splicing of the CD45 gene, such that the gain of CD45R0 and the loss of CD45RA expression occurs during the transition from a naïve to memory T cell(Johannisson and Festin, 1995; Sallusto et al., 1999). Lymph node homing markers such as CCR7 and CD62L enable further characterization of memory T cells into EM (CD45RA-CD45R0+CCR7-CD62L-) and CM (CD45RA-CD45R0+CCR7+CD62L+) subsets, and distinguish naïve (CD45RA+CD45R0-CCR7+CD62L+) from TEMRA (CD45RA+CD45R0-CCR7-CD62L-) subsets(Hengel et al., 2003; Sallusto et al., 1999). Alternatively, a combination of CD28 and CD95 are routinely used to define naïve and memory T cells in primates(Pitcher et al., 2002), while CD44 and CD62L are typically used for phenotyping murine memory T cell subsets(Gerberick et al., 1997; Sprent, 1997). In pigs, another relevant large animal model, T helper cell subsets have been proposed to consist of naïve (CD8 α -CD27+), CM (CD8 α +CD27+), and EM (CD8 α +CD27-), with CD45RC and swine leukocyte antigen-DR (SLA-DR) also useful in enriching for functionally distinct subsets(Reutner et al., 2013). In dogs, combinations of Ig-CCL19, anti-CD62L and anti-CTL2.58, as well as anti-CD44 with anti-CD62L, have been used to identify naïve, CM, EM and TEMRA T cells(Hartley et al., 2014; Rothe et al., 2017), although the function of these cell subsets has not yet been verified and unfortunately many of these antibodies are not conjugated, or commercially available.

Canine studies have determined that aged dogs display certain clinical characteristics of immunosenescence such as decreased antibody responses to novel pathogens and increased risk of cancers(Day, 2010; Fleming et al., 2011; Kearns et al., 1999; Kennedy et al., 2007). Corresponding evidence of decreasing immune function includes decreased proliferation of peripheral blood mononuclear cells (PBMCs), increased IFN γ expression by CD4+ T cells, and decreased proportions of CD45RA+ lymphocytes(Greeley et al., 2001; HogenEsch et al., 2004; Horiuchi et al., 2007; Massimino et al., 2003; Reis et al., 2005). Furthermore, a recent study showed that aged Labradors displayed a significant decrease in the number of clonal peaks in the locus encoding the T cell receptor (TCR) β chain variable region (TCRB V gene families) compared to young Labradors(Holder et al., 2018). This suggests that, similar to humans, dogs exhibit decreasing TCR repertoire diversity as they age. Thus far however, phenotypic evaluation of T cell subsets and details of T cell function changes in older dogs has been limited, with the study of subsets utilizing only 1–3 phenotypic markers(HogenEsch et al., 2004; Reis et al., 2005). With advancements in flow cytometric capabilities and availability of a larger array of conjugated anti-canine and cross-reactive antibodies, we have been able to develop panels of antibodies that can stain for up to 9 different markers on each cell. In this study, we applied these panels to investigate possible memory T cell phenotypes in dogs, in addition to age-related changes in the function of T cell subsets and the proportions of T cells with memory subset phenotypes.

We therefore employed multi-color flow cytometry, utilizing markers of cell viability, basic T cell subsets, antigen experience, lymph node homing, intracellular cytokines, and proliferative fraction, to detail the process of immunosenescence in canine T cells. We evaluated age-dependent differences in mitogen-induced cytokine production and proliferation by CD4⁺ and CD8⁺ T cells. We then evaluated phenotypic markers used to define T cell memory subsets in other species, such as CD45RA, CD62L, CD44 and CD28, for their ability to delineate canine T cell subsets with different functionality. Finally, we evaluated changes in the proportions of lymphocytes with memory subset phenotypes between young and aged dogs. Our findings illustrate many similarities in the process of immunosenescence between dogs and humans. Furthermore, we propose that flow cytometry panels described herein will serve to guide future studies characterizing immune-related diseases and responses to immunotherapeutics in canine patients.

2. Methods:

2.1. Biological samples

Whole blood from purpose-bred Beagle dogs was purchased from a USDA Class A dog breeder and shipped overnight on ice. All dogs were vaccinated as puppies for parvovirus, adenovirus type 2, distemper, parainfluenza, *Bordetella bronchiseptica*, papillomavirus, and rabies, and did not receive additional vaccinations in adulthood. Blood was collected and transported in vacutainer tubes containing EDTA anticoagulant (Midwest Veterinary Supply, Lakeville, MN, USA). A total of 6 young (15–18 months old), and 6 aged (8–10 year old) uncastrated male dogs were used for these experiments. Due to known proinflammatory effects of obesity, groups were stratified by body condition scores (BCS). Three lean and 3 overweight dogs were included in each age group, with BCS of 4 and 6, respectively. All experiments were completed within 3 months of each other and the same group of dogs was used for each experiment.

Whole blood was diluted in Hank's Buffered Salt Solution (HBSS; Corning, Corning, NY, USA) at a ratio of 1:1, and then layered over Histopaque 1077 (Sigma-Aldrich, Saint Louis, MO, USA). The samples were centrifuged at 650 rcf for 30 minutes at room temperature with the brake turned off. The layer of PBMCs was extracted, transferred to a clean tube, and washed with HBSS. Cell preparations were centrifuged again at 650 rcf for 10 minutes to pellet the cells. Red blood cells were next eliminated from cell preparations by incubating with 1x RBC lysis buffer (420301, Biolegend, San Diego, CA, USA) for 3 minutes on ice. PBMCs were washed again with HBSS, centrifuged at 650 rcf for 10 minutes, and counted.

2.2. Reagents

Fixable viability dye (LIVE/DEADTM Fixable Near-IR Dead Cell Stain Kit, Invitrogen, Eugene, OR, USA) was used to identify live cells for each experiment at a dilution of 1:1000 in Dulbecco's phosphate buffered saline (DPBS; Gibco, Carlsbad, CA, USA). Surface antibody staining of PBMCs was performed on cells suspended in staining buffer, consisting of 3% fetal bovine serum (FBS, heat-inactivated; Omega Scientific, Tarzana, CA, USA) and 1 mM of EDTA (Thermo Fisher Scientific, Carlsbad, CA, USA) in DPBS. Intracellular staining was performed using Fixation/Permeabilization concentrate and diluent (00-5123-43

and 00-5223-56, eBioscience, Carlsbad, CA, USA), and Permeablization buffer (00-8333-56, eBioscience, Carlsbad, CA, USA). The details of antibodies used in these studies, and references justifying non-commercial antibodies and antibodies directed against non-canine antigens are listed in Table 1. PBMCs were cultured *in vitro* in complete media, which included 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA, USA), 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), and 2-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA) in Roswell Park Memorial Institute media (RPMI 1640; Gibco, Carlsbad, CA, USA). Concanavalin A (ConA; C5275, Sigma-Aldrich, Saint Louis, MO, USA) was used to stimulate cells at a concentration of 5 µg/ml for a duration of either 6 hours or 2 days. For intracellular cytokine staining experiments, both mitogen-stimulated and control cells were concurrently incubated with Brefeldin A (B7651, Sigma-Aldrich, Saint Louis, MO, USA) at a dilution of 1 µl/ml in complete media during the 6-hour stimulation period.

2.3. Staining procedure

Firstly, cells were washed in PBS, and stained with viability dye for 30 minutes in a total volume of 100 µl. Samples were then washed again in PBS before staining with a cocktail of surface antibodies diluted in staining buffer for 20 minutes. Samples were next washed in staining buffer. For experiments requiring biotinylated anti-CD45RA, subsequent incubation with streptavidin conjugated to BV711 was required for 20 minutes, followed by another wash with staining buffer. Cells were then incubated in 250 µl of Fixation/Permeablization solution for 20 minutes. Permeablization buffer was then used to wash cells before incubation with a cocktail of intracellular antibodies diluted in Permeablization buffer. Finally, all samples were washed again in Permeablization Buffer and then suspended in 1% paraformaldehyde (Affymetrix) while awaiting flow cytometer acquisition. All antibody-staining steps were performed in a total volume of 50 µl per sample. All incubation steps were performed at 4°C.

2.4. Mitogen-induced cytokine production

After isolation, PBMCs were incubated in complete media overnight (37°C, 5% CO₂) at a density of 2.0×10^6 cells/well, in a 48 well plate. The following morning, Brefeldin A was added to all wells, and ConA was added only to wells labeled “mitogen”. After incubation for 6 hours, cells were retrieved from wells and counted (Ritt et al., 2015). Unstimulated control and mitogen-stimulated cells (1×10^6 cells) from each dog were then stained for subsequent analysis by flow cytometry.

Experiments evaluating cytokine production by CD4⁺ and CD8⁺ T cell subsets from young and aged dogs utilized antibodies targeting CD3 (PB), CD4 (PE-Cy7), CD8, IFN γ , and TNF α (Table 1). Samples from 6 young and 5 aged dogs were used in this experiment, and a minimum of 2.5×10^5 events were recorded per sample.

Alternatively, for evaluation of cytokine production by the purposed T cell memory subsets, PBMCs from 6 beagle dogs were assessed, including 3 young and 3 aged dogs. Antibodies

against CD3 (PB), CD4 (PE-Cy7), CD8, CD28, CD44, CD62L, CD45RA, and TNF α were used (Table 1). A minimum of 5×10^5 events were recorded per sample.

2.5. Mitogen-induced proliferation

Isolated PBMCs from 6 young and 4 aged beagles were incubated in either complete media or complete media containing ConA at a density of 1.5×10^6 cells/well, in a 48 well plate for 2 days. These mitogen stimulation conditions induce sub-maximal lymphoproliferative effects in prior canine and human studies, and are maintained for a shorter duration compared to previous studies utilizing ConA stimulated lymphocytes as a positive control (Boggiatto et al., 2010; Molaee et al., 2017; Wagner et al., 1999). Next, mitogen-stimulated and unstimulated control cells (1×10^6 cells) from each dog were stained for analysis by flow cytometry. Antibodies against CD3 (FITC), CD4 (PB), CD8, CD25, and Ki67 were used (Table 1), and a minimum of 3×10^5 events were recorded per sample.

2.6. Lymphocytes with memory subset phenotypes in young and aged dogs

Freshly isolated PBMCs (1×10^6) from each dog (6 young, 6 aged) were stained with a panel of antibodies against CD3 (PB), CD4 (PE-Cy7), CD8, CD28, CD44, CD62L, and CD45RA (Table 1). A minimum of 7.5×10^5 events were recorded per sample.

2.7. Data acquisition and analysis

Controls included a fluorescence minus one (FMO) or staining with an isotype control for antibodies that did not produce a bimodal distribution of cell staining. Mitogen-activated cells also served as a biological control to establish appropriate gating for cytokine and proliferation markers. Flow cytometry was performed on a Becton Dickinson LSRII flow cytometer, utilizing blue (488nm), violet (405nm) and red (633nm) lasers. Compensation settings were conducted using BD CompBeads (BD Biosciences).

Flow cytometry data was analyzed using FlowJo software (Version 10; TreeStar Inc.). The gating strategy used is illustrated in Suppl. Fig. 1A. CD4 $^+$ and CD8 $^+$ T cells were then evaluated for expression of CD45RA, CD62L, CD44, CD28, TNF α , IFN γ , CD25, and/or Ki67. Memory subsets were defined by their CD45RA and CD62L expression. Naïve-like T cells were defined as CD45RA $^+$ CD62L $^+$, CM-like T cells were defined as CD45RA $^+$ CD62L $^+$, EM-like T cells were defined as CD45RA $^+$ CD62L $^-$, and TEMRA-like T cells were defined as CD45RA $^+$ CD62L $^-$ (Suppl. Fig. 1B). CD28 and CD44 were also evaluated for their ability to define canine T cells with different effector functions, as has been shown for other species (Gerberick et al., 1997; Pitcher et al., 2002; Sprent, 1997). Both cytokine positive frequencies and the median fluorescence intensity (MFI) of cytokine expression were recorded when evaluating TNF α and IFN γ expression.

2.9. Statistics

Prism 7 was used for statistical analysis of data. For comparison of independent groups, a Mann Whitney test was used to determine differences between 2 groups, while a Kruskal-Wallis test with a post hoc Dunn's multiple comparisons test analyzed differences between 3 or more groups. For matched data, a Wilcoxon matched-pairs signed rank test evaluated differences between 2 groups, while a Friedman test with a post hoc Dunn's multiple

comparisons test were used to compare 3 or more groups. A P value of <0.05 was considered significant.

3. Results

Firstly, we investigated the effects of aging on canine CD4⁺ and CD8⁺ T cell function. PBMCs from 6 young and 5 aged dogs were stimulated with ConA for 6 hours and 2 days prior to evaluation of cytokine production and proliferative fraction, respectively. We observed significantly higher frequencies of TNF α ⁺ and IFN γ ⁺ CD4⁺ T cells ($P = 0.004$ for both) and CD8⁺ T cells ($P = 0.017$ and $P = 0.004$, respectively) in aged dogs compared to young dogs after mitogen stimulation (Fig. 1A; Suppl. Fig. 2A–B). MFI for IFN γ expression followed these same trends for both CD4⁺ and CD8⁺ T cells ($P = 0.004$ for both), while a significantly higher MFI for TNF α expression was only observed for CD4⁺ T cells in aged dogs ($P = 0.009$; Suppl. Fig. 3). In addition, we detected decreased proliferative fractions of CD4⁺ and CD8⁺ T cells in aged dogs, compared to young dogs, after mitogen stimulation (Fig. 1B; Suppl. Fig. 4). However, this difference was only significant for the CD8⁺ subset ($P = 0.038$). The average frequency of CD25⁺ CD4⁺ T cells was 87% (70%–94%), and the average frequency of CD25⁺ CD8⁺ T cells was 88% (74%–98%), indicating that a 2-day stimulation condition was sufficient to activate most cells (data not shown).

We then sought to identify phenotypic markers of canine T cell memory subsets by comparing cytokine production between cell subsets defined by their expression of CD28, CD44, and those defined by dual expression of CD45RA and CD62L. CD4⁺ and CD8⁺ T cells were designated as displaying a naïve-, CM-, EM- or TEMRA-like phenotype based on their CD45RA and CD62L expression levels, as outlined in the Methods subsection 2.7 and Suppl. Fig. 1B. Given that a feature of EM T cells is enhanced cytokine production, we compared the proportion of TNF α expressing T cells after 6 hours of mitogen stimulation between phenotypic groups of cells defined by markers of antigen exposure (e.g. CD45RA) and lymph node homing (e.g. CD62L)(Sallusto et al., 1999). We observed significantly higher frequencies of TNF α ⁺ cells in both CD4⁺ and CD8⁺ T cells with an EM-like phenotype, compared to T cells with a naïve-like phenotype after mitogen stimulation ($P < 0.001$ and $P = 0.002$, respectively; Fig. 2A–B), suggesting these phenotypic markers may highlight similar lymphocyte subsets in dogs, as in humans. Corresponding increases in the MFI of TNF α expression were detected between T cells with naïve- and EM-like phenotypes for both CD4⁺ and CD8⁺ subsets ($P < 0.001$ for both) (Fig. 2B). While there were too few CD8⁺ T cells with a CM-like phenotype after mitogen stimulation (mean = 0.5%; range = 0.1–1.2%) to accurately evaluate their relative capacity for TNF α expression, the proportion of CD4⁺ CM-like T cells was deemed adequate for further analysis (mean = 9.4%; range = 0.7% - 43%; Suppl. Fig. 5). A clear trend towards increased TNF α production by EM-, compared to CM-like CD4⁺ T cells was observed, and this difference was statistically significant for TNF α MFI between the 2 subsets ($P = 0.022$; Fig. 2B). TNF α expression was consistently less in the CD4⁺ and CD8⁺ T cells with a TEMRA-like phenotype compared to those with an EM-like phenotype, although this difference was not statistically significant. The frequency of TNF α ⁺ CD4⁺ T cells in each memory subset appeared to be higher in aged dogs compared to young dogs, however this difference was

not significant. 1% or less of unstimulated control cells in each subset were TNF α + (Suppl. Fig. 6).

Given the utility of CD28 and CD44 as phenotypic markers of memory and effector function in other species, we also tested CD28 and CD44 T cell subsets for TNF α expression upon mitogen stimulation (Gerberick et al., 1997; Pitcher et al., 2002; Sprent, 1997). Examination of unstimulated canine CD4+ and CD8+ T cells for CD28 expression revealed 3 populations that we defined as CD28-, CD28mid, and CD28high (Suppl. Fig. 7A). CD28mid and CD28high T cells combined were thought to have been previously described as CD28 positive by the group that created this antibody (Graves et al., 2011; Rosinski et al., 2015). After stimulation with ConA, increased frequencies of TNF α + T cells were observed in CD4+ and CD8+ CD28high subsets when compared to CD28- subsets ($P=0.002$ for both; Fig. 2C). This finding is in contrast to those reported by Pitcher et al. that describe EM subset functional characteristics of CD28- T cells in primates, as evidenced by their greater cytokine production (Pitcher et al., 2002). CD44 staining was detected in virtually all CD4+ and CD8+ T cells for most dogs, and revealed 2 distinct populations defined as CD44low and CD44high (Suppl. Fig. 7B). ConA stimulation of PBMCs resulted in significantly greater frequencies of TNF α + cells within the CD44high population compared to CD44low cells for both CD4+ and CD8+ subsets, consistent with findings in mice, and supporting the role of CD44 in identifying memory T cells in both species ($P=0.031$ for both; Fig. 2D) (Pihlgren et al., 1999). Significant changes in TNF α MFI mirrored changes in TNF α + cell frequencies for both CD28 and CD44 subsets (Suppl. Fig. 8A–B). No significant differences in the proportion of TNF α + cells in each of the CD28 and CD44 subsets were detected between the 2 age groups, although values clearly tended to be higher in aged dogs. TNF α expression was detected in 2% or less of unstimulated control cells for each subset (data not shown).

Mitogen stimulation elicited a decrease in the frequency of CD4+ and CD8+ T cells with a naïve-like phenotype ($P=0.031$ for both), and an increased frequency of those with a TEMRA-like phenotype ($P=0.031$ for both; Fig. 3A–B). Young dogs tended to have greater proportions of CD4+ T cells with a naïve-like phenotype in the absence of mitogen stimulation, in addition to greater proportions of CD4+ T cells with a TEMRA- phenotype after mitogen stimulation, although again these differences were not significant. Age differences in subset frequencies with and without stimulation were less consistent in CD8+ T cells. A decreased frequency of CD4+ and CD8+ T cells with a CM-like phenotype was observed with mitogen stimulation as well ($P=0.031$ for both; Suppl. Fig. 5). However, an increase in T cells with an EM-like phenotype was observed for only CD4+ T cells ($P=0.031$; Suppl. Fig. 8). Mitogen stimulation induced a significant increase in the frequency of CD28mid CD4+ T cells, and a significant decrease in frequency of CD28high CD4+ T cells ($P=0.031$ for both; Suppl. Fig. 9A). No differences in frequencies of CD28 subsets for CD8+ T cells were observed with stimulation (Suppl. Fig. 9A). Significant changes in CD44 subset frequencies induced by mitogen stimulation were only observed in CD8+ cells, with frequencies of CD44low CD8+ T cells increasing, and CD44high CD8+ T cell frequencies decreasing with stimulation ($P=0.031$ for both; Suppl. Fig. 9B).

Finally, we utilized our data suggesting the validity of CD45RA and CD62L for distinguishing canine memory T cell subsets, to determine changes in memory subset proportions with age. We observed significant differences in the proportion of T cells with various memory subset phenotypes in aged dogs compared to young dogs (Fig. 4A–B). Similar to what is reported in humans (Thome et al., 2014), old dogs demonstrated lower frequencies of CD4⁺ and CD8⁺ T cells with a naïve-like phenotype, compared to young dogs ($P = 0.002$ and $P = 0.009$, respectively). Furthermore, CD8⁺ T cells with a TEMRA-like phenotype were detected at a significantly higher frequency in aged dogs, compared to young dogs ($P = 0.026$). Numerical increases in the frequencies of CD4⁺ and CD8⁺ T cells with CM- and EM-like phenotypes were observed in aged dogs compared to young dogs, although these differences were not significant (CD4⁺: CM $P = 0.394$, EM $P = 0.180$; CD8⁺: CM $P = 0.240$, EM $P = 0.132$). Aged dogs also demonstrated lower frequencies of CD4⁺ and CD8⁺ CD28^{mid} T cells, compared to young dogs ($P = 0.002$ for both; Fig. 4C). This finding coincided with higher frequencies of CD4⁺CD28^{high} T cells ($P = 0.002$), and CD8⁺CD28⁻ T cells ($P = 0.04$) in aged dogs. Meanwhile, compared to young dogs, aged dogs exhibited lower frequencies of CD4⁺ and CD8⁺ CD44^{low} T cells ($P = 0.002$ and $P = 0.065$, respectively), and increased frequencies of CD4⁺ and CD8⁺ CD44^{high} T cells ($P = 0.002$ and $P = 0.065$, respectively; Fig. 4D), although these differences were only statistically significant for CD4⁺ T cells due to one CD8⁺ data point being an extreme outlier. The consistency of our findings with those reported for memory T cell subsets in humans of different ages, further supports the use of CD45RA and CD62L in identifying canine memory T cell subsets.

4. Discussion

Despite growing enthusiasm for utilization of the canine model in comparative clinical studies, and increased recognition of the immune system's role in modulating disease risk and progression, our understanding of the impact of age on the phenotype and function of T cell subsets in dogs has remained insufficient. Furthermore, historically a lack of validated tools to evaluate canine memory T cell subsets has prevented a comprehensive evaluation of age-related changes in the dog. Herein, we report elevated IFN γ and TNF α production, as well as decreased proliferation by CD4⁺ and CD8⁺ T cell subsets in aged dogs, compared to young dogs. In addition, we have shown that antibodies against CD45RA and CD62L differentiate T cell subsets in dogs with distinct functional characteristics consistent with those of T cells with similar memory phenotypes in other species. Aged dogs in this cohort displayed decreased proportions of CD4⁺ and CD8⁺ T cells with a naïve phenotype, consistent with the features of immunosenescence described in other species (Fagnoni et al., 2000; Kang et al., 2004; Koch et al., 2008; Thome et al., 2014). Overall, these results highlight many similarities between dogs and humans in the effects of aging on the immune system, providing further support for utilization of the canine model in comparative studies of disease.

Evaluation of canine naïve and memory T cell subsets is rarely reported. Findings by Hartley et al. determined that CD62L distinguished naïve T cells based on down-regulation of this marker after ConA stimulation. Also, CTL2.58 positivity associated with activated T cells as it increased with stimulation (Hartley and Tarleton, 2015). Subsequently, these investigators

reported decreased CCR7⁺ and CD62L⁺ T cells in Argentinean dogs exposed to *Trypanosoma cruzi* compared to dogs from the United States, although no differences in CCR7, CD62L and CTL2.58 expression were detected between *T. cruzi* seropositive and seronegative dogs (Hartley et al., 2014). Findings by Rothe, et al. indirectly suggested that canine CD44⁺CD62L⁻ T cells might have functional characteristics consistent with that of human EM T cells based on mitogen-induced IFN- γ and CD25 expression observed for a highly activated canine CD4⁺CD8⁺ T cell subset that predominantly express this EM-like phenotype (Rothe et al., 2017). Collectively, these studies suggested that these phenotypic markers may warranted further examination as markers for canine memory T cell subsets.

Functional differences between canine T cells with naïve and memory phenotypes were assessed by quantifying cytokine production after mitogen stimulation as one approach to validate phenotypic markers for canine T cells. In order to do so, we optimized a 9-color flow cytometry panel that enabled detection of intracellular TNF α by CD4⁺ and CD8⁺ T cell memory subsets defined by CD45RA and CD62L. Anti-canine CD45RA identified clear populations of positive and negative cells, and distinguished T cell subsets with differing function. We observed that CD4⁺ and CD8⁺ T cells with a naïve-like phenotype produced less TNF α (lower MFI and frequency) after mitogen stimulation, compared to those with an EM-like phenotype. Furthermore, we noted a significant decrease in the proportions of T cells with a naïve-like phenotype, and a significant increase in the proportions of those with a TEMRA-like phenotype after mitogen stimulation. Similar findings for naïve and EM T cell subsets using the same phenotypic markers exist in the human and mouse literature (Jiang et al., 2007; Mahnke et al., 2013; Mascher et al., 1999; Sallusto et al., 1999). In addition, a significant decrease in TNF α MFI was detected for CD4⁺ CM-like T cells compared to CD4⁺ EM-like T cells, supporting the use of the lymph node homing marker CD62L to distinguish CM cells. These functional differences are consistent with those reported between CM and EM T cell subsets in mice (Sallusto et al., 1999). However, while our observation of a trend towards decreased TNF α production by canine TEMRA-like T cells, compared to EM-like T cells, is consistent with TEMRA T cells in other species, evaluation of additional functional characteristics is necessary to confirm that this is the appropriate classification for canine CD45RA⁺CD62L⁻ T cells. Notwithstanding, overall our results support the use of both anti-canine CD45RA and anti-human CD62L to phenotype functionally distinct canine T cell memory subsets. Further studies are necessary however to investigate differences in the many other functional properties of T cells, between the phenotypic subsets described in this study.

We also evaluated functional differences between T cell subsets defined by CD28 or CD44 expression since these markers identify memory subsets in other species, and anti-canine antibodies are commercially available. Mitogen stimulation induced a significant increase in the proportion of CD4⁺CD28^{mid} cells, and decrease in the proportion of CD4⁺CD28^{high} cells, which is somewhat consistent with findings in primates in which CD28 downregulation is a feature of effector T cells (Pitcher et al., 2002). Contrary to primate studies however, TNF α was expressed by a greater proportion of canine T cells with high CD28 expression after mitogen stimulation when compared to negative or mid CD28 expression (Pitcher et al., 2002). Therefore, our results predominantly conflict with findings in primates but are consistent with studies from Rosinski, et al. that show CD28 expression

on T cells in dogs more closely mirrors that of humans, in which CD28 expression is retained by effector memory T cells and potentially only lost in the final stages of T cell differentiation (Eastwood et al., 2010; Rosinski et al., 2015).

The *in vitro* characteristics of canine CD44 T cell subsets showed similarities with findings previously reported in mice (Jiang et al., 2007; Pennock et al., 2013). CD44 was expressed at least at low levels in all canine T cells. Furthermore, we noted significantly increased cytokine production by CD44^{high} CD4⁺ and CD8⁺ T cells, compared to corresponding CD44^{low} subsets. In contrast to murine T cells however, which demonstrate upregulation of CD44 after 24 hours of ConA stimulation, we observed a significant decrease in CD8⁺CD44^{high} T cell frequency and no significant alterations to CD4⁺ CD44 subsets with stimulation (Jiang et al., 2007). Further investigation is needed to determine whether the effect of mitogen stimulation on murine CD44 T cell subsets can be recapitulated in canine CD44 subsets after a longer period of stimulation or stimulation with alternative mitogens. Overall, we found CD28 and CD44 subsets to be less reliable in delineating memory T cell subsets with distinct functions, although further investigation into the utility of combining these markers with others is necessary.

We confirmed similar age-related changes occur in the function of canine CD4⁺ and CD8⁺ T cell subsets, compared to those observed in human T cells. In concordance with human studies, our data reveal elevated mitogen-induced TNF α and IFN γ production by both CD4⁺ and CD8⁺ T cell subsets in aged dogs, compared to young dogs (Bandres et al., 2000; Hoffmann et al., 2005). These findings also agree with a prior beagle study that reported advancing age was associated with increasing frequencies of CD4⁺ T cells expressing IFN γ with stimulation (Horiuchi et al., 2007). Furthermore, 2 days of mitogen stimulation elicited decreased proliferative fractions in CD4⁺ and CD8⁺ T cells from aged dogs, relative to young dogs, although this difference was only significant for the CD8⁺ subset. In humans, decreased T cell proliferation in the elderly is well documented, and has been associated with all-cause mortality (Effros and Walford, 1983; Ferguson et al., 1995; Jiang et al., 2007; Wayne et al., 1990). Several canine studies have also previously detected decreased lymphocyte proliferation in aged dogs although specific lymphocyte subsets were not assessed (Greeley et al., 2001; HogenEsch et al., 2004; Kearns et al., 1999; Massimino et al., 2003; Strasser et al., 2000). Notably, it has been suggested that these age-associated differences in lymphocyte function are a consequence of changes to the proportions of naïve and memory subsets that occur with age in humans; an explanation that would also explain our results in dogs (Ginaldi et al., 2001; Hoffmann et al., 2005). Overall, our findings concur with, and expand on, previous literature on canine immunosenescence, highlighting comparable age-related functional changes to T cell subsets between this canine population and humans.

Using our findings of functional differences between T cell subsets defined by CD45RA and CD62L expression, we utilized these flow panels to further characterize immunosenescence in dogs. We detected lower proportions of CD4⁺ and CD8⁺ T cells with a naïve-like phenotype, and an increased proportion of CD8⁺ T cells with a TEMRA-like phenotype in aged dogs compared to young dogs. Both the trend and proportions of T cell subsets that we observed in this population of beagles were remarkably similar to that previously reported in

young and aged people(Fagnoni et al., 2000; Kang et al., 2004; Koch et al., 2008; Thome et al., 2014). A combination of decreased thymic output and increased antigen exposure over time is thought to result in decreasing naïve T cell frequencies, and consequently maintenance of the peripheral T cell pool occurs by expansion of existing T cell clones(LeMaoult et al., 2000; Schwab et al., 1997). While the precise mechanism is unclear, ultimately a decrease in TCR diversity is observed(Goronzy et al., 2015; Naylor et al., 2005). Our data showing a decreased frequency of T cells with a naïve-like phenotype in aged dogs, therefore agrees with a recent publication in which a diminished TCR repertoire diversity in aged dogs was suggested by a drop in the number of clonal peaks detected across CDR3 spectratypes(Holder et al., 2018). Furthermore, our results also concur with prior canine reports describing decreased CD45RA expression on either the whole lymphocyte fraction (single-stained), or CD4+ and/or CD8+ lymphocytes (double-stained) (Fujiwara et al., 2012; HogenEsch et al., 2004; Reis et al., 2005). The ability of CD45RA and CD62L to define T cell populations that shift with age as would be expected for naïve and memory T cells, further supports the utility of these markers in classifying these cell subsets.

Significant differences in the proportions of CD28 and CD44 T cell subsets were also observed in aged, compared to young dogs. We found that aged dogs exhibited increased proportions of CD28- CD8+ and CD28high CD4+ T cells while proportions of CD28mid CD4+ and CD8+ T cells were decreased compared to young dogs. Interestingly, the association of aging with an increase in circulating CD28- T cells, particularly from the CD8+ lineage, is well documented in both primates and humans(Boucher et al., 1998; Dennett et al., 2002; Weng et al., 2009). Our findings of age-associated changes in CD8+ CD28 subsets align with our functional data, and reports by Rosinski et al. Overall, these findings suggest CD28 expression may only be lost in terminal stages of T cell differentiation (i.e. in TEMRA-like T cells) in dogs, which occurs at a much higher frequency in CD8+ T cells(Rosinski et al., 2015). Canine CD4+ and CD8+ CD44 subsets appear to vary with age in a similar way to those of mice, with CD44low T cells decreasing with advancing age(Bloom et al., 1994). These findings are also consistent with our functional data and together indicate that CD44low T cells may represent a naïve-like T cell subset.

Further studies are needed to investigate a range of additional phenotypic and functional parameters in canine T cell memory subsets. While we ideally would have included the antibody against IFN γ in our evaluation of functional differences between purposed memory subsets, unfortunately the limited variety of available fluorochrome conjugates prohibited this. TNF α does however appear to be differentially expressed by human naïve and memory T cells after a similar brief *in vitro* stimulation period, although expression in murine memory T cell subsets is somewhat less discriminating(von Fliedner et al., 1992; Yu et al., 2014). In addition, further functional characterization of these subsets by evaluation of their proliferative capacity, cytotoxic potential, and production of cytokines such as IL2, are also necessary in the future(Brenchley et al., 2002; Geginat et al., 2001; Ohshima et al., 1999; Seder et al., 2008). In depth, phenotypic characterization of these cell subsets using antibodies targeting CD57, CD27, KLRG1, CD45RO and PD1 is also necessary, although

currently hindered by the limited availability of cross-reactive antibodies(Brenchley et al., 2002; Mahnke et al., 2013; Romero et al., 2007).

Many human studies currently utilize CCR7 as a marker of lymph node homing instead of CD62L due to its greater expression stability under various conditions(Mahnke et al., 2013). In the dog however, no commercially available, cross-reactive antibodies targeting CCR7 exist. Importantly, it should be acknowledged that TCR activation during *in vitro* ConA stimulation can cause direct cleavage of CD62L off the surface of cells(Jabbari and Harty, 2006). This event can therefore cloud the distinction of post-stimulation naïve and CM T cells, from TEMRA and EM T cells, respectively. Our findings however in fresh PBMCs from aged dogs of a decreased frequency of T cells with a naïve-like phenotype and increased frequency of CD8+ T cells with a TEMRA-like phenotype, compared to young dogs, closely recapitulates observations in humans, and supports the utility of CD62L to define memory T cell populations in dogs(Thome et al., 2014).

It remains to be determined how breed variation and sex could impact the results reported here. In humans, differing rates of autoimmune disease in women compared to men, and the divergent effects of estrogen and testosterone on immune function suggest sex differences cannot be ruled out(Brodin and Davis, 2017; Cutolo et al., 2004). In one study evaluating age-associated changes to immune parameters in German Shepherd dogs, lymphocyte proliferation and α -globulin concentrations were significant higher in female dogs(Strasser et al., 2000). Furthermore, breed-related differences in lymphocyte proportions and function have previously been observed in canine studies(Faldyna et al., 2001; Kearns et al., 1999). Finally, differing antigen exposure of purpose-bred beagles, compared to client-owned pet dogs, may be expected due to their dissimilar housing and environment. Environmental factors may potentiate divergent immune responses and features of immunological aging(Beura et al., 2016). Therefore, some variation of the values reported here in male purpose-bred beagles is to be expected when utilizing these phenotypic markers in a clinical context that typically would include a mixture of breeds, and males and females.

5. Conclusions

Phenotypic characterization of antigen-experienced T cell subsets in humans and mice has long been used to characterize immune competency and response to immunotherapeutics. The absence of established equivalent phenotypic markers in dogs has been a major limitation to utilizing naturally-occurring canine models to study human disease. In this study, we show that functionally distinct T cell subsets can be defined by their expression of CD45RA and CD62L using multicolor flow cytometry. Using these and other optimized flow cytometry panels, we have also detailed features of canine immunosenescence, such as increased cytokine production and decreased proliferative responses by CD4+ and CD8+ T cells, and decreased proportions of T cells with a naïve phenotype in aged dogs. Our hope is that these findings will help to bridge the gap between human and canine clinical studies by providing validated biomarkers of immune response, while also highlighting comparable age-related changes in immune function across species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding sources:

UC Davis Center for Companion Animal Health; NIH-NCI 1R01CA195904-01; NIH-NCI P30CA093373; NIH-NCIU01 CA224166-01

References:

- Alvarez CE, 2014 Naturally occurring cancers in dogs: insights for translational genetics and medicine. *ILAR J* 55, 16–45. [PubMed: 24936028]
- Bandres E, Merino J, Vazquez B, Inoges S, Moreno C, Subira ML, Sanchez-Ibarrola A, 2000 The increase of IFN-gamma production through aging correlates with the expanded CD8(+high)CD28(-)CD57(+) subpopulation. *Clin Immunol* 96, 230–235. [PubMed: 10964541]
- Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, Thompson EA, Fraser KA, Rosato PC, Filali-Mouhim A, Sekaly RP, Jenkins MK, Vezys V, Haining WN, Jameson SC, Masopust D, 2016 Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* 532, 512–516. [PubMed: 27096360]
- Bismarck D, Schutze N, Moore P, Buttner M, Alber G, Buttlar H, 2012 Canine CD4+CD8+ double positive T cells in peripheral blood have features of activated T cells. *Vet Immunol Immunopathol* 149, 157–166. [PubMed: 22789871]
- Bloom ET, Mostowski HS, Horvath JA, 1994 Does the age-related change in CD44-defined T-cell subsets have functional significance for cytotoxic T lymphocyte generation? *Immunol Lett* 40, 251–258. [PubMed: 7525465]
- Boggiatto PM, Ramer-Tait AE, Metz K, Kramer EE, Gibson-Corley K, Mullin K, Hostetter JM, Gallup JM, Jones DE, Petersen CA, 2010 Immunologic indicators of clinical progression during canine *Leishmania infantum* infection. *Clin Vaccine Immunol* 17, 267–273. [PubMed: 20032217]
- Boucher N, Dufeu-Duchesne T, Vicaute E, Farge D, Effros RB, Schachter F, 1998 CD28 expression in T cell aging and human longevity. *Exp Gerontol* 33, 267–282. [PubMed: 9615924]
- Brenchley JM, Douek DC, Ambrozak DR, Chatterji M, Betts MR, Davis LS, Koup RA, 2002 Expansion of activated human naive T-cells precedes effector function. *Clin Exp Immunol* 130, 432–440. [PubMed: 12452833]
- Brodin P, Davis MM, 2017 Human immune system variation. *Nat Rev Immunol* 17, 21–29. [PubMed: 27916977]
- Cobbold S, Metcalfe S, 1994 Monoclonal antibodies that define canine homologues of human CD antigens: summary of the First International Canine Leukocyte Antigen Workshop (CLAW). *Tissue Antigens* 43, 137–154. [PubMed: 8091414]
- Cutolo M, Sulli A, Capellino S, Villaggio B, Montagna P, Seriolo B, Straub RH, 2004 Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. *Lupus* 13, 635–638. [PubMed: 15485092]
- Day MJ, 2009 Canine sino-nasal aspergillosis: parallels with human disease. *Med Mycol* 47 Suppl 1, S315–S323. [PubMed: 18608893]
- Day MJ, 2010 Ageing, immunosenescence and inflammageing in the dog and cat. *J Comp Pathol* 142 Suppl 1, S60–69. [PubMed: 20005526]
- Dennett NS, Barcia RN, McLeod JD, 2002 Age associated decline in CD25 and CD28 expression correlate with an increased susceptibility to CD95 mediated apoptosis in T cells. *Exp Gerontol* 37, 271–283. [PubMed: 11772513]
- Eastwood D, Findlay L, Poole S, Bird C, Wadhwa M, Moore M, Burns C, Thorpe R, Stebbings R, 2010 Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. *Br J Pharmacol* 161, 512–526. [PubMed: 20880392]

- Effros RB, Walford RL, 1983 The immune response of aged mice to influenza: diminished T-cell proliferation, interleukin 2 production and cytotoxicity. *Cell Immunol* 81, 298–305. [PubMed: 6605806]
- Fagnoni FF, Vescovini R, Passeri G, Bologna G, Pedrazzoni M, Lavagetto G, Casti A, Franceschi C, Passeri M, Sansoni P, 2000 Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging. *Blood* 95, 2860–2868. [PubMed: 10779432]
- Faldyna M, Leva L, Knotigova P, Toman M, 2001 Lymphocyte subsets in peripheral blood of dogs--a flow cytometric study. *Vet Immunol Immunopathol* 82, 23–37. [PubMed: 11557292]
- Ferguson FG, Wikby A, Maxson P, Olsson J, Johansson B, 1995 Immune parameters in a longitudinal study of a very old population of Swedish people: a comparison between survivors and nonsurvivors. *J Gerontol A Biol Sci Med Sci* 50, B378–382. [PubMed: 7583794]
- Fleming JM, Creevy KE, Promislow DE, 2011 Mortality in north american dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *J Vet Intern Med* 25, 187–198. [PubMed: 21352376]
- Fujiwara M, Yonezawa T, Arai T, Yamamoto I, Ohtsuka H, 2012 Alterations with age in peripheral blood lymphocyte subpopulations and cytokine synthesis in beagles. *Vet Med Res Rep* 3, 79–84.
- Galkowska H, Waldemar LO, Wojewodzka U, 1996 Reactivity of antibodies directed against human antigens with surface markers on canine leukocytes. *Vet Immunol Immunopathol* 53, 329–334. [PubMed: 8969052]
- Geginat J, Lanzavecchia A, Sallusto F, 2003 Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* 101, 4260–4266. [PubMed: 12576317]
- Geginat J, Sallusto F, Lanzavecchia A, 2001 Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* 194, 1711–1719. [PubMed: 11748273]
- Gerberick GF, Cruse LW, Miller CM, Sikorski EE, Ridder GM, 1997 Selective modulation of T cell memory markers CD62L and CD44 on murine draining lymph node cells following allergen and irritant treatment. *Toxicol Appl Pharmacol* 146, 1–10. [PubMed: 9299591]
- Ginaldi L, De Martinis M, D'Ostilio A, Marini L, Loreto F, Modesti M, Quagliano D, 2001 Changes in the expression of surface receptors on lymphocyte subsets in the elderly: quantitative flow cytometric analysis. *Am J Hematol* 67, 63–72. [PubMed: 11343377]
- Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM, 2015 Naive T cell maintenance and function in human aging. *J Immunol* 194, 4073–4080. [PubMed: 25888703]
- Graves SS, Stone DM, Loretz C, Peterson LJ, Lesnikova M, Hwang B, Georges GE, Nash R, Storb R, 2011 Antagonistic and agonistic anti-canine CD28 monoclonal antibodies: tools for allogeneic transplantation. *Transplantation* 91, 833–840. [PubMed: 21343872]
- Greeley EH, Ballam JM, Harrison JM, Kealy RD, Lawler DF, Segre M, 2001 The influence of age and gender on the immune system: a longitudinal study in Labrador Retriever dogs. *Vet Immunol Immunopathol* 82, 57–71. [PubMed: 11557294]
- Hartley AN, Cooley G, Gwyn S, Orozco MM, Tarleton RL, 2014 Frequency of IFN γ -producing T cells correlates with seroreactivity and activated T cells during canine *Trypanosoma cruzi* infection. *Vet Res* 45, 6. [PubMed: 24456537]
- Hartley AN, Tarleton RL, 2015 Chemokine receptor 7 (CCR7)-expression and IFN γ production define vaccine-specific canine T-cell subsets. *Vet Immunol Immunopathol* 164, 127–136. [PubMed: 25758065]
- Hengel RL, Thaker V, Pavlick MV, Metcalf JA, Dennis G, Jr., Yang J, Lempicki RA, Sereti I, Lane HC, 2003 Cutting edge: L-selectin (CD62L) expression distinguishes small resting memory CD4+ T cells that preferentially respond to recall antigen. *J Immunol* 170, 28–32. [PubMed: 12496379]
- Ho KK, Campbell KL, Lavergne SN, 2015 Contact dermatitis: a comparative and translational review of the literature. *Vet Dermatol* 26, 314–327, e366–317. [PubMed: 26184842]
- Hoffmann F, Albert MH, Arenz S, Bidlingmaier C, Berkowicz N, Sedlacek S, Till H, Pawlita I, Renner ED, Weiss M, Belohradsky BH, 2005 Intracellular T-cell cytokine levels are age-dependent in healthy children and adults. *Eur Cytokine Netw* 16, 283–288. [PubMed: 16464742]

- HogenEsch H, Thompson S, Dunham A, Ceddia M, Hayek M, 2004 Effect of age on immune parameters and the immune response of dogs to vaccines: a cross-sectional study. *Vet Immunol Immunopathol* 97, 77–85. [PubMed: 14700539]
- Holder A, Mirczuk SM, Fowkes RC, Palmer DB, Aspinall R, Catchpole B, 2018 Perturbation of the T cell receptor repertoire occurs with increasing age in dogs. *Dev Comp Immunol* 79, 150–157. [PubMed: 29103899]
- Horiuchi Y, Nakajima Y, Nariai Y, Asanuma H, Kuwabara M, Yukawa M, 2007 Th1/Th2 balance in canine peripheral blood lymphocytes--a flow cytometric study. *Vet Immunol Immunopathol* 118, 179–185. [PubMed: 17604844]
- Jabbari A, Harty JT, 2006 Simultaneous assessment of antigen-stimulated cytokine production and memory subset composition of memory CD8 T cells. *J Immunol Methods* 313, 161–168. [PubMed: 16762359]
- Jiang J, Gross D, Elbaum P, Murasko DM, 2007 Aging affects initiation and continuation of T cell proliferation. *Mech Ageing Dev* 128, 332–339. [PubMed: 17383712]
- Johannisson A, Festin R, 1995 Phenotype transition of CD4+ T cells from CD45RA to CD45RO is accompanied by cell activation and proliferation. *Cytometry* 19, 343–352. [PubMed: 7796699]
- Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi JY, Craft J, 2004 Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. *J Immunol* 173, 673–681. [PubMed: 15210831]
- Kearns RJ, Hayek MG, Turek JJ, Meydani M, Burr JR, Greene RJ, Marshall CA, Adams SM, Borgert RC, Reinhart GA, 1999 Effect of age, breed and dietary omega-6 (n-6): omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. *Vet Immunol Immunopathol* 69, 165–183. [PubMed: 10507303]
- Kennedy LJ, Lunt M, Barnes A, McElhinney L, Fooks AR, Baxter DN, Ollier WE, 2007 Factors influencing the antibody response of dogs vaccinated against rabies. *Vaccine* 25, 8500–8507. [PubMed: 18006120]
- Koch S, Larbi A, Derhovanessian E, Ozelik D, Naumova E, Pawelec G, 2008 Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. *Immun Ageing* 5, 6. [PubMed: 18657274]
- LeBlanc AK, Mazcko CN, Khanna C, 2016 Defining the Value of a Comparative Approach to Cancer Drug Development. *Clin Cancer Res* 22, 2133–2138. [PubMed: 26712689]
- LeMaout J, Messaoudi I, Manavalan JS, Potvin H, Nikolich-Zugich D, Dyal R, Szabo P, Weksler ME, Nikolich-Zugich J, 2000 Age-related dysregulation in CD8 T cell homeostasis: kinetics of a diversity loss. *J Immunol* 165, 2367–2373. [PubMed: 10946259]
- Macauley R, Akbar AN, Henson SM, 2013 The role of the T cell in age-related inflammation. *Age (Dordr)* 35, 563–572. [PubMed: 22252437]
- Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E, 2013 The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol* 43, 2797–2809. [PubMed: 24258910]
- Marx A, Porubsky S, Belharazem D, Saruhan-Direskeneli G, Schalke B, Strobel P, Weis CA, 2015 Thymoma related myasthenia gravis in humans and potential animal models. *Exp Neurol* 270, 55–65. [PubMed: 25700911]
- Mascher B, Schlenke P, Seyfarth M, 1999 Expression and kinetics of cytokines determined by intracellular staining using flow cytometry. *J Immunol Methods* 223, 115–121. [PubMed: 10037239]
- Massimino S, Kearns RJ, Loos KM, Burr J, Park JS, Chew B, Adams S, Hayek MG, 2003 Effects of age and dietary beta-carotene on immunological variables in dogs. *J Vet Intern Med* 17, 835–842. [PubMed: 14658721]
- Messaoudi I, Lemaout J, Guevara-Patino JA, Metzner BM, Nikolich-Zugich J, 2004 Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *J Exp Med* 200, 1347–1358. [PubMed: 15545358]
- Molae M, Mosayebi G, Pishdadian A, Ejehadifar M, Ganji A, 2017 Evaluating the Proliferation of Human Peripheral Blood Mononuclear Cells Using MTT Assay. *Int J Basic Sci Med* 2, 25–28.

- Monjazebe AM, Kent MS, Grossenbacher SK, Mall C, Zamora AE, Mirsoian A, Chen M, Kol A, Shiao SL, Reddy A, Perks JR, W TNC, Sparger EE, Canter RJ, Sckisel GD, Murphy WJ, 2016 Blocking Indolamine-2,3-Dioxygenase Rebound Immune Suppression Boosts Antitumor Effects of Radio-Immunotherapy in Murine Models and Spontaneous Canine Malignancies. *Clin Cancer Res* 22, 4328–4340. [PubMed: 26979392]
- Moreira ML, Dorneles EM, Soares RP, Magalhaes CP, Costa-Pereira C, Lage AP, Teixeira-Carvalho A, Martins-Filho OA, Araujo MS, 2015 Cross-reactivity of commercially available anti-human monoclonal antibodies with canine cytokines: establishment of a reliable panel to detect the functional profile of peripheral blood lymphocytes by intracytoplasmic staining. *Acta Vet Scand* 57, 51. [PubMed: 26362860]
- Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, Witkowski J, Fulbright J, Weyand CM, Goronzy JJ, 2005 The influence of age on T cell generation and TCR diversity. *J Immunol* 174, 7446–7452. [PubMed: 15905594]
- Ohshima Y, Yang LP, Avic MN, Kurimoto M, Nakajima T, Sergerie M, Demeure CE, Sarfati M, Delespesse G, 1999 Naive human CD4+ T cells are a major source of lymphotoxin alpha. *J Immunol* 162, 3790–3794. [PubMed: 10201895]
- Pawelec G, 2018 Age and immunity: What is “immunosenescence”? *Exp Gerontol* 105, 4–9. [PubMed: 29111233]
- Pedersen LG, Castelruiz Y, Jacobsen S, Aasted B, 2002 Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Vet Immunol Immunopathol* 88, 111–122. [PubMed: 12127410]
- Pennock ND, White JT, Cross EW, Cheney EE, Tamburini BA, Kedi RM, 2013 T cell responses: naive to memory and everything in between. *Adv Physiol Educ* 37, 273–283. [PubMed: 24292902]
- Pihlgren M, Arpin C, Walzer T, Tomkowiak M, Thomas A, Marvel J, Dubois PM, 1999 Memory CD44(int) CD8 T cells show increased proliferative responses and IFN-gamma production following antigenic challenge in vitro. *Int Immunol* 11, 699–706. [PubMed: 10330275]
- Pitcher CJ, Hagen SI, Walker JM, Lum R, Mitchell BL, Maino VC, Axthelm MK, Picker LJ, 2002 Development and homeostasis of T cell memory in rhesus macaque. *J Immunol* 168, 29–43. [PubMed: 11751943]
- Reis AB, Carneiro CM, Carvalho M, Teixeira-Carvalho A, Giunchetti RC, Mayrink W, Genaro O, Correa-Oliveira R, Martins-Filho OA, 2005 Establishment of a microplate assay for flow cytometric assessment and its use for the evaluation of age-related phenotypic changes in canine whole blood leukocytes. *Vet Immunol Immunopathol* 103, 173–185. [PubMed: 15621304]
- Reutner K, Leitner J, Müllebnner A, Ladinig A, Essler SE, Duvigneau JC, Ritzmann M, Steinberger P, Saalmüller A, Gerner W, 2013 CD27 expression discriminates porcine T helper cells with functionally distinct properties. *Vet Res* 44, 18. [PubMed: 23497134]
- Ritt MG, Lindborg BA, O'Brien TD, Bisignano J, Modiano JF, 2015 Stimulation with Concanavalin-A Induces IL-17 Production by Canine Peripheral T Cells. *Vet Sci* 2, 43–51. [PubMed: 29061930]
- Romero P, Zippelius A, Kurth I, Pittet MJ, Touvrey C, Iancu EM, Cortes P, Devedre E, Speiser DE, Rufer N, 2007 Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J Immunol* 178, 4112–4119. [PubMed: 17371966]
- Rosinski SL, Storb R, Strong RK, Sale GE, Stone DM, Gewe MM, Friend DJ, Abrams VK, Randolph-Habecker J, Graves SS, 2015 Anti-CD28 Antibody-Initiated Cytokine Storm in Canines. *Transplant Direct* 1.
- Rothe K, Bismarck D, Buttner M, Alber G, von Buttlar H, 2017 Canine peripheral blood CD4(+)CD8(+) double-positive T cell subpopulations exhibit distinct T cell phenotypes and effector functions. *Vet Immunol Immunopathol* 185, 48–56. [PubMed: 28242002]
- Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A, 1999 Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708–712. [PubMed: 10537110]
- Schuberth HJ, Kucinskiene G, Chu RM, Faldyna M, 2007 Reactivity of cross-reacting monoclonal antibodies with canine leukocytes, platelets and erythrocytes. *Vet Immunol Immunopathol* 119, 47–55. [PubMed: 17643496]
- Schulz AR, Malzer JN, Domingo C, Jurchott K, Grutzkau A, Babel N, Nienen M, Jelinek T, Niedrig M, Thiel A, 2015 Low Thymic Activity and Dendritic Cell Numbers Are Associated with the

- Immune Response to Primary Viral Infection in Elderly Humans. *J Immunol* 195, 4699–4711. [PubMed: 26459351]
- Schwab R, Szabo P, Manavalan JS, Weksler ME, Posnett DN, Pannetier C, Kourilsky P, Even J, 1997 Expanded CD4+ and CD8+ T cell clones in elderly humans. *J Immunol* 158, 4493–4499. [PubMed: 9127016]
- Seder RA, Darrah PA, Roederer M, 2008 T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 8, 247–258. [PubMed: 18323851]
- Sprent J, 1997 Immunological memory. *Curr Opin Immunol* 9, 371–379. [PubMed: 9203411]
- Strasser A, Teltscher A, May B, Sanders C, Niedermuller H, 2000 Age-associated changes in the immune system of German shepherd dogs. *J Vet Med A Physiol Pathol Clin Med* 47, 181–192. [PubMed: 10842468]
- Thome JJ, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, Kato T, Lerner H, Shen Y, Farber DL, 2014 Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell* 159, 814–828. [PubMed: 25417158]
- von Fliedner V, Miescher S, Gerain J, Gallati H, Barras C, Heumann D, Cerottini JC, 1992 Production of tumor necrosis factor- α by naive or memory T lymphocytes activated via CD28. *Cell Immunol* 139, 198–207. [PubMed: 1309488]
- Wagner U, Burkhardt E, Failing K, 1999 Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the 3H-thymidine incorporation assay. *Vet Immunol Immunopathol* 70, 151–159. [PubMed: 10507358]
- Wayne SJ, Rhyne RL, Garry PJ, Goodwin JS, 1990 Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. *J Gerontol* 45, M45–48. [PubMed: 2313042]
- Weng NP, Akbar AN, Goronzy J, 2009 CD28(–) T cells: their role in the age-associated decline of immune function. *Trends Immunol* 30, 306–312. [PubMed: 19540809]
- Yager EJ, Ahmed M, Lanzer K, Randall TD, Woodland DL, Blackman MA, 2008 Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J Exp Med* 205, 711–723. [PubMed: 18332179]
- Yu SF, Zhang YN, Yang BY, Wu CY, 2014 Human memory, but not naive, CD4+ T cells expressing transcription factor T-bet might drive rapid cytokine production. *J Biol Chem* 289, 35561–35569. [PubMed: 25378399]

Figure 1-
Functionality of CD4+ and CD8+ T cells in young versus aged dogs. A) Frequencies of IFN γ + and TNF α + CD4+ and CD8+ T cells in young and aged dogs after a 6 hour ConA stimulation. B) Proliferative fraction of CD4+ and CD8+ T cells in young and aged dogs, determined by Ki67 expression after 2 days of mitogen stimulation. Means and standard deviations are shown. * = P<0.05; ** = P 0.01; n = 4–6 per age group.

Figure 2-

Functionality of memory T cell subsets after ConA stimulation of PBMCs from young and aged dogs. A) Representative scatter plots illustrating TNFα production by CD4+ and CD8+ T cells with EM- and naïve-like phenotypes after 6 hours of ConA stimulation. The example shown is from an aged, overweight male beagle. B) TNFα+ frequencies and TNFα MFI of CD4+ and CD8+ memory subsets. C) TNFα+ frequencies of CD4+ and CD8+ CD28 subsets. D) TNFα+ frequencies of CD4+ and CD8+ CD44+ T cell subsets. Lines connect data points from each individual dog. * = P<0.05; ** = P 0.01; *** = P 0.001; n = 6.

Figure 3-

Changes in frequencies of canine memory T cell subsets between mitogen-stimulated and unstimulated PBMCs. A) Representative scatter plot illustrating the frequencies of CD4+ and CD8+ T cells with various memory phenotypes with and without 6 hour ConA stimulation. The example shown is from an aged, overweight male beagle. B) Summary of changes to the frequencies of CD4+ and CD8+ T cells with naïve- and TEMRA-like phenotypes after mitogen stimulation in young and aged dogs. Control PBMCs were incubated in complete media concurrently with stimulated PBMCs. Lines connect data points from each individual dog. * = $P < 0.05$; $n = 6$.

Figure 4-

Alterations to T cell memory, CD28 and CD44 T cell subset frequencies in fresh PBMCs of young vs. aged dogs. A) Representative scatter plots illustrating memory subset frequencies of CD4+ and CD8+ T cells in a young/lean dog and an aged/lean dog. B) Frequencies of T cells with various memory phenotypes in young vs. aged dogs. C) Frequencies of CD28 subsets in young vs. aged dogs. D) Frequencies of CD44 subsets in young vs. aged dogs. Means and standard deviations are shown. ns = not significant; ** = $P < 0.01$; $n = 6$ per age group.

Table 1:

Cell surface and intracellular antibodies and markers used in flow cytometry studies

Antigen/ marker	Conjugated fluorochrome	Antigen species	Antibody clone #	Manufacturer	Catalogue #	Dilution	Reference
Cell Surface							
Viability dye	Near infra-red	N/A	N/A	Thermo Fisher	L34975	1:1000	N/A
CD4	PE-Cy7	Canine	YKIX302.9	eBioscience	25-5040-41	1:10	N/A
CD4	PB	Canine	YKIX302.9	Biorad	MCA1038PB	1:10	N/A
CD8	PerCp-Cy5.5	Canine	YCATE55.9	eBioscience	46-5080-41	1:25	N/A
CD28	APC	Canine	5B8	eBioscience	17-0282-41	1:10	N/A
CD44	FITC	Canine	YKIX337.8	Thermo Fisher	11-5440-41	1:10	N/A
CD62L	PE	Human	FMC46	BioRad	MCA1076PET	1:10	(Bismarck et al., 2012; Hartley and Tarleton, 2015; Rothe et al., 2017; Schuberth et al., 2007)
CD45RA	Biotin	Canine	CA4.1D3	PFM Lab	N/A	1:50	(Cobbold and Metcalfe, 1994; Reis et al., 2005)
Streptavidin	BV711	N/A	N/A	Biologend	405241	1:50	N/A
CD25	PE	Canine	P4A10	eBioscience	12-0250-42	1:10	N/A
Intracellular							
CD3	PB	Human	CD3-12	BioRad	MCA1477PB	1:50	(Monjazez et al., 2016)
CD3	FITC	Human	CD3-12	BioRad	MCA1477F	1:50	(Monjazez et al., 2016)
IFN γ	PE	Bovine	CC302	BioRad	MCA1783PE	1:10	(Hartley and Tarleton, 2015; Pedersen et al., 2002)
TNF α	BV-785	Human	MAB-11	Biologend	502947	1:25	(Moreira et al., 2015)
Ki67	PE-Cy7	Human	20Raj1	eBioscience	25-5699-42	1:50	(Galkowska et al., 1996)

APC, allophycocyanin; BV-711, brilliant violet™711; BV-785, brilliant violet™785; FITC, fluorescein; PB, pacific blue; PE, phycoerythrin; PE-Cy7, PE-cyanine 7; PE-TR, PE-texas red; PerCPCy5.5, peridinin chlorophyll protein-cyanine 5.5; PFM lab, Peter F. Moore laboratory; N/A, not applicable.