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

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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

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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 (IFNγ) and tumor necrosis factor-alpha (TNFα) 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(LeMaoult 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 (CD8α-CD27+), CM (CD8α+CD27+), and EM (CD8α+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 IFNγ 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/DEAD™ 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/Permeablization 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% CO_2) 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, IFNγ, and TNFα (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 TNFα 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 Dickison 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, TNFα, IFNγ, 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 TNFα and IFNγ 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 TNFα+ and IFNγ+ 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 IFNγ expression followed these same trends for both CD4+ and CD8+ T cells (P= 0.004 for both), while a significantly higher MFI for TNFα 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 TNFα 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 TNFα+ 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 TNFα 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 TNFα 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 TNFα production by EM-, compared to CM-like CD4+ T cells was observed, and this difference was statistically significant for TNFα MFI between the 2 subsets (P = 0.022; Fig. 2B). TNFα 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 TNFα+ 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 TNFα+ (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 TNFα 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 TNFα+ 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 TNFα+ 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 TNFα MFI mirrored changes in TNFα+ cell frequencies for both CD28 and CD44 subsets (Suppl. Fig. 8A–B). No significant differences in the proportion of TNFα+ 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. TNFα 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+ CD28mid T cells, compared to young dogs (P= 0.002 for both; Fig. 4C). This finding coincided with higher frequencies of CD4+CD28high 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+ CD44low T cells (P= 0.002 and P= 0.065, respectively), and increased frequencies of CD4+ and CD8+ CD44high 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 IFNγ and TNFα 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-g 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 TNFα 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 TNFα (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 naive 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 TNFα 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 TNFα 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+CD28mid cells, and decrease in the proportion of CD4+CD28high 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, TNFα 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 CD44high CD4+ and CD8+ T cells, compared to corresponding CD44low 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+CD44high 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 TNFα and IFNγ 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 IFNγ 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 IFNγ in our evaluation of functional differences between purposed memory subsets, unfortunately the limited variety of available fluorochrome conjugates prohibited this. TNFα 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, CD45R0 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.

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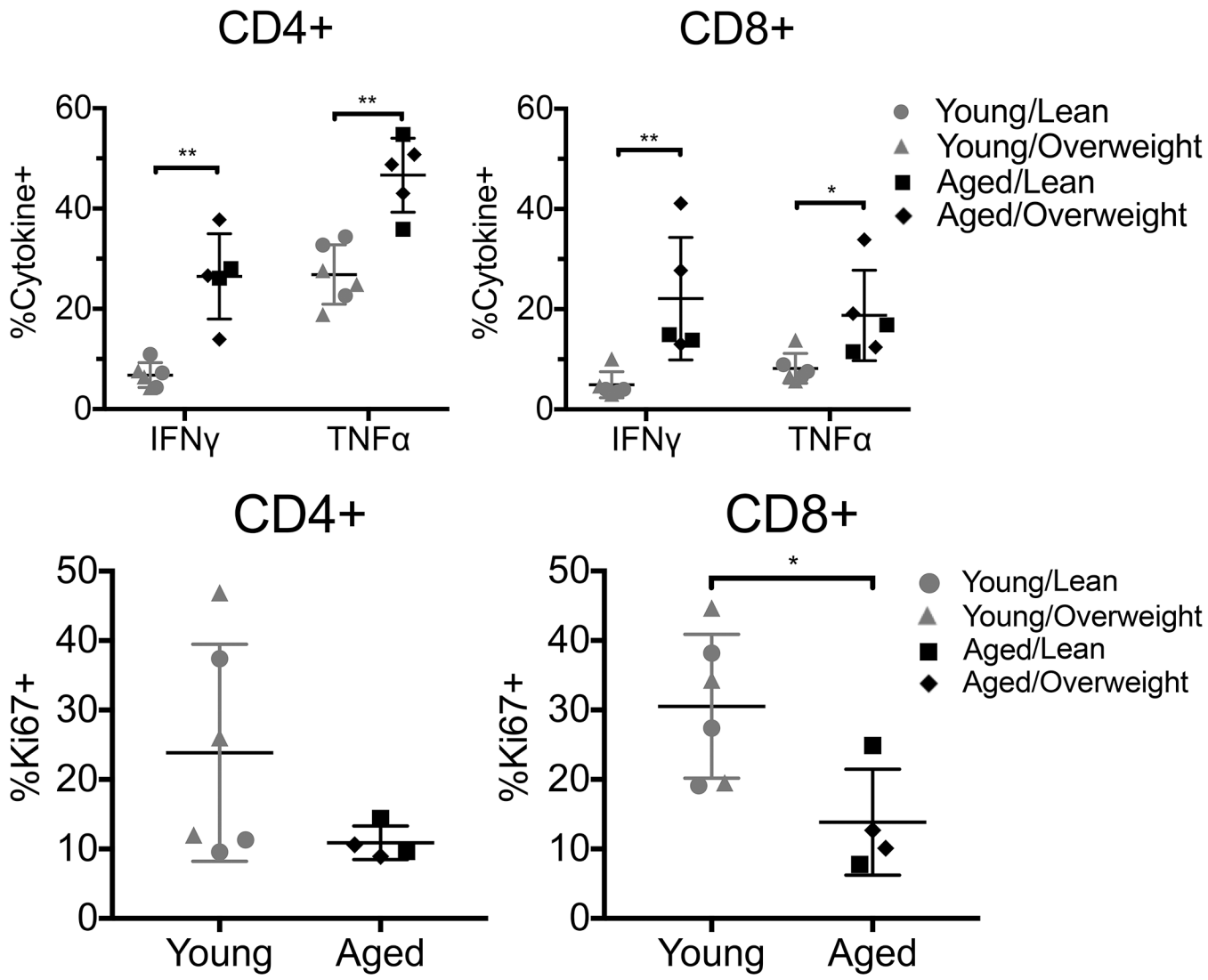
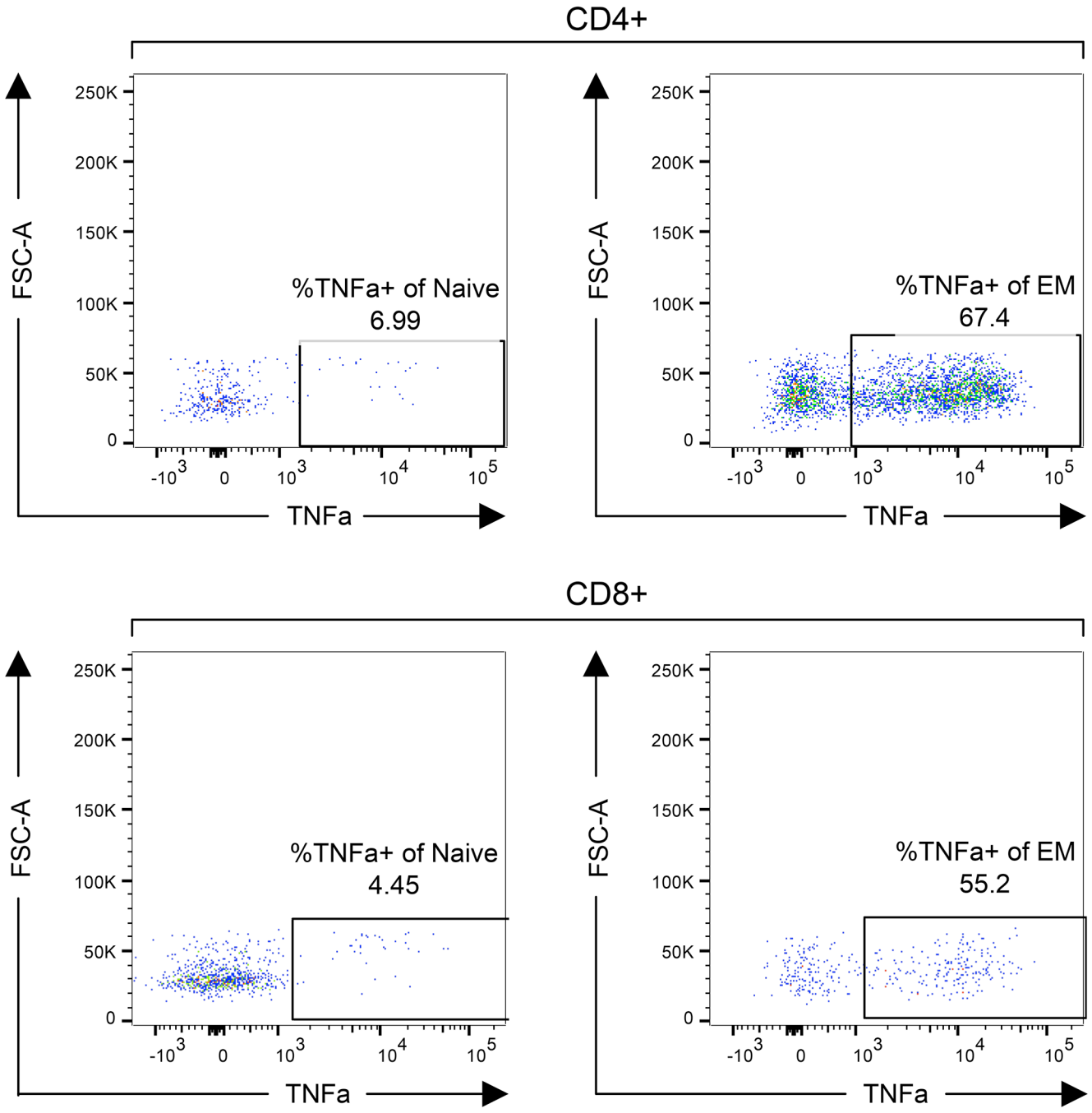
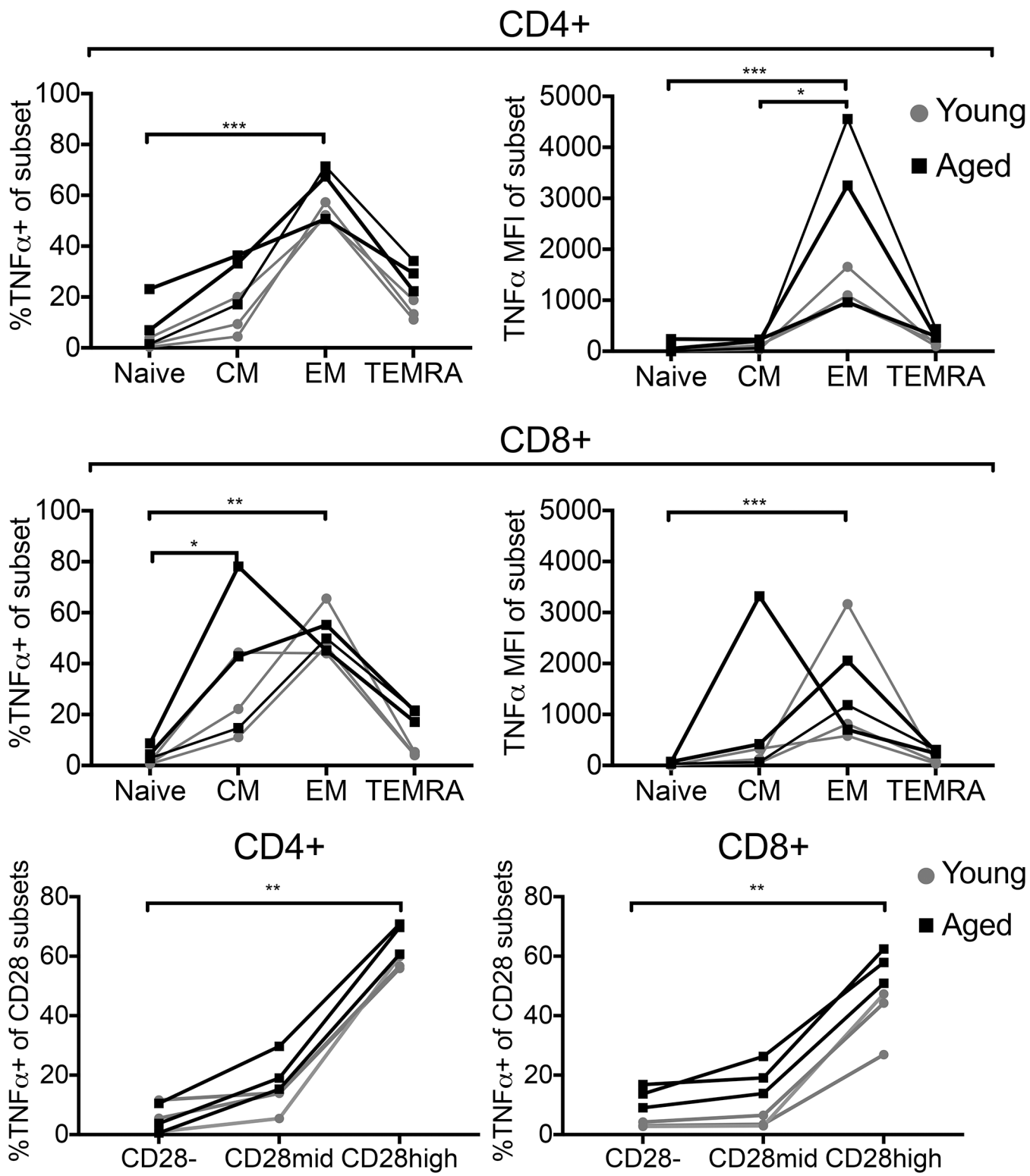


Figure 1-

Functionality of CD4+ and CD8+ T cells in young versus aged dogs. A) Frequencies of IFNγ+ and TNFα+ 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. * = $P<0.05$; ** = $P\leq0.01$; n = 4–6 per age group.





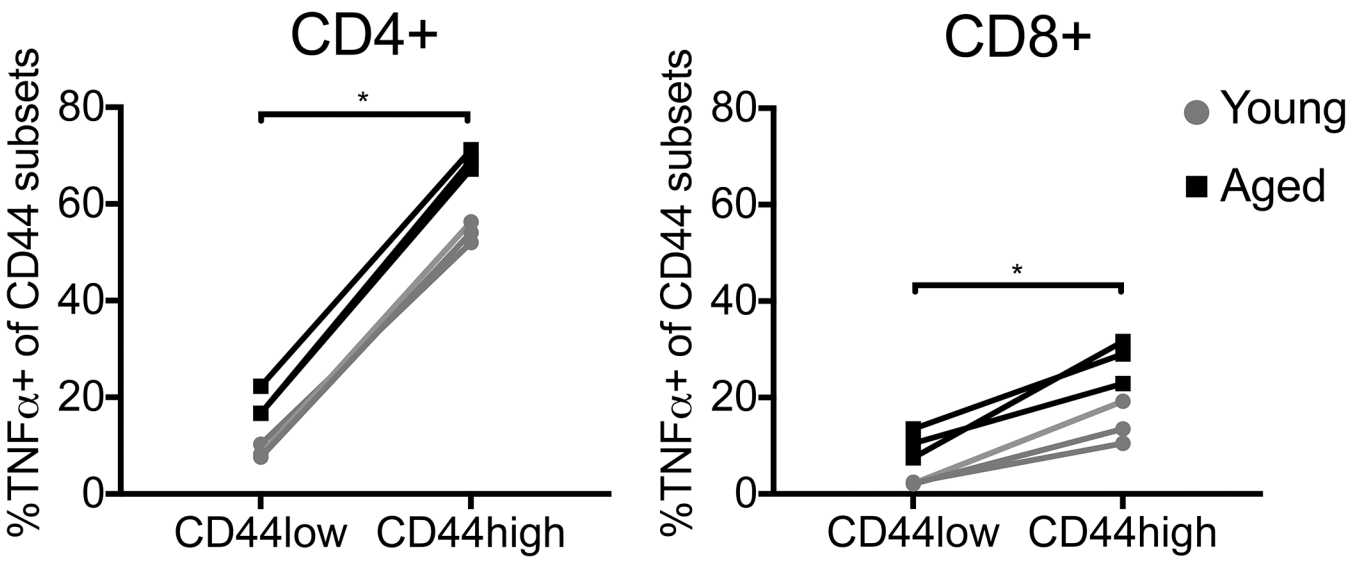
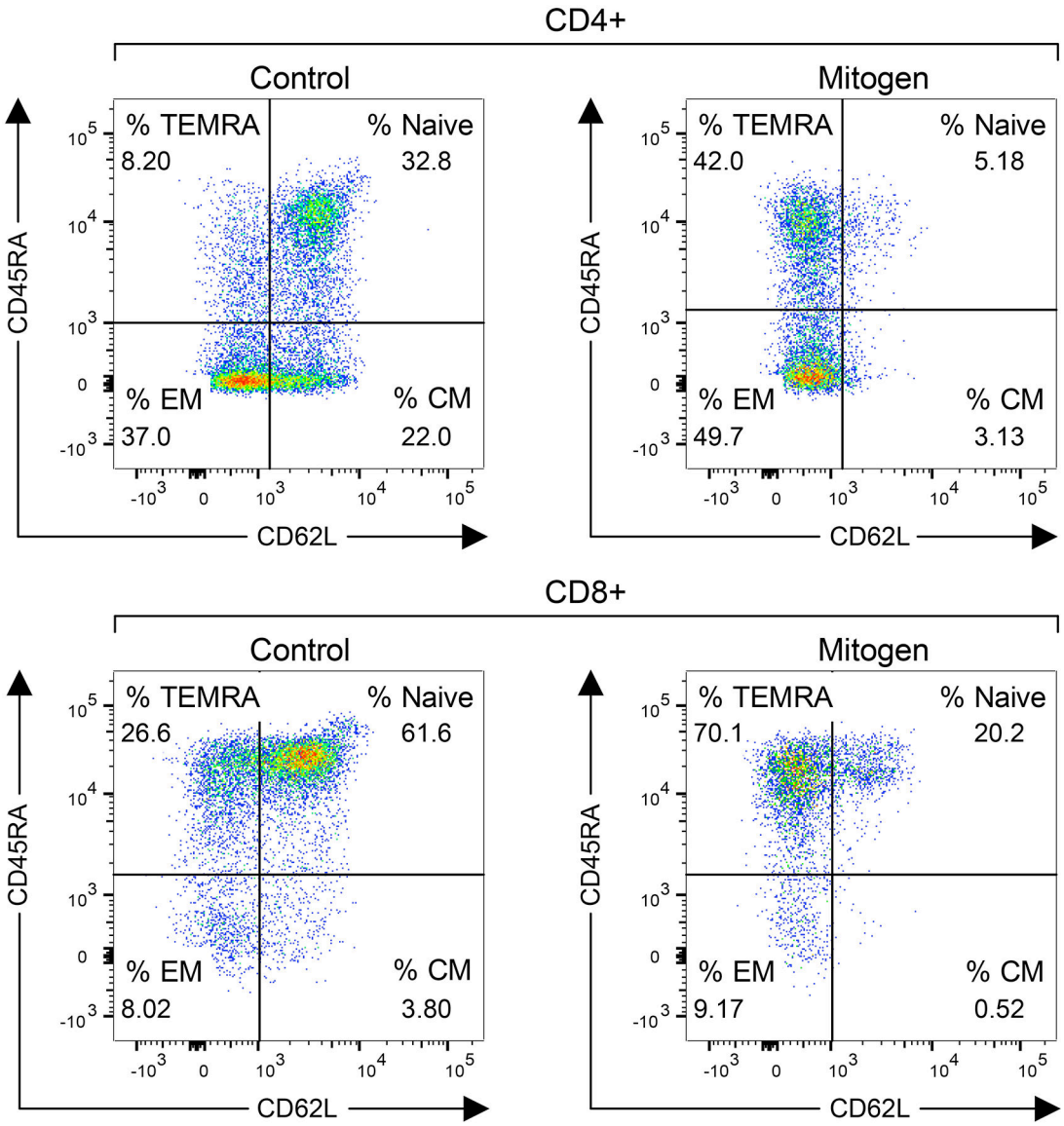


Figure 2-

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. * = $P<0.05$; ** = $P\leq0.01$; *** = $P\leq0.001$; n = 6.



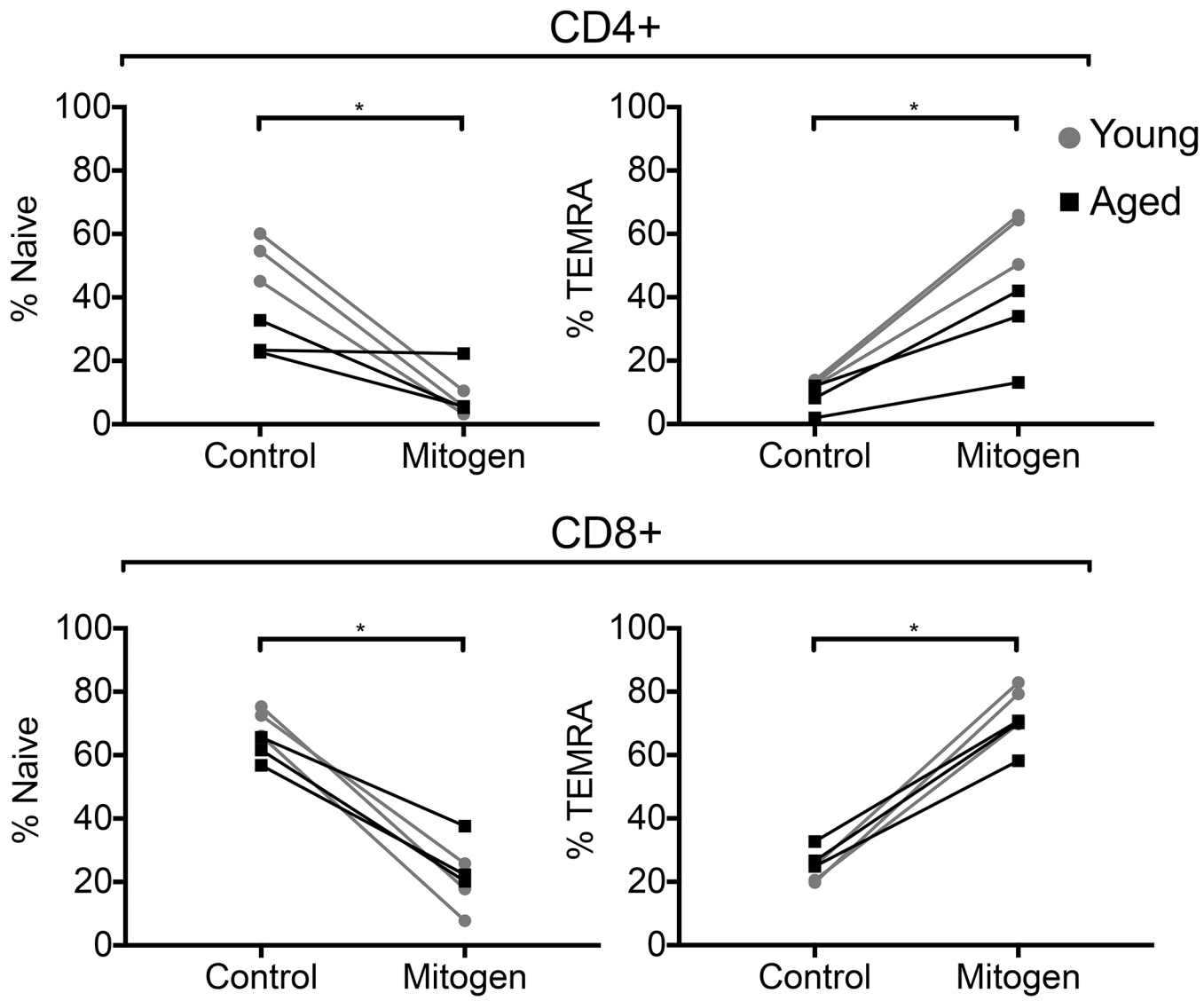
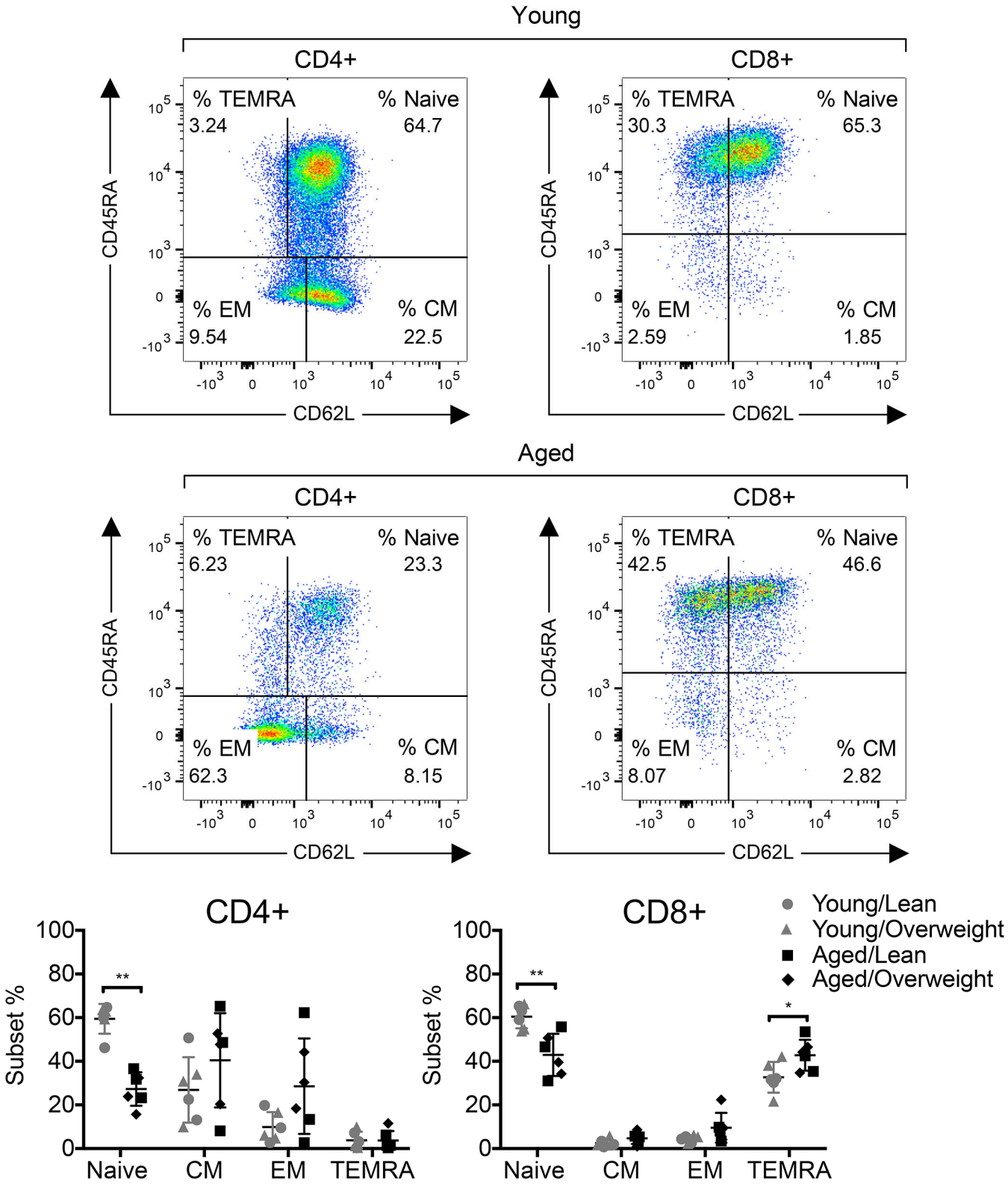


Figure 3-

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.



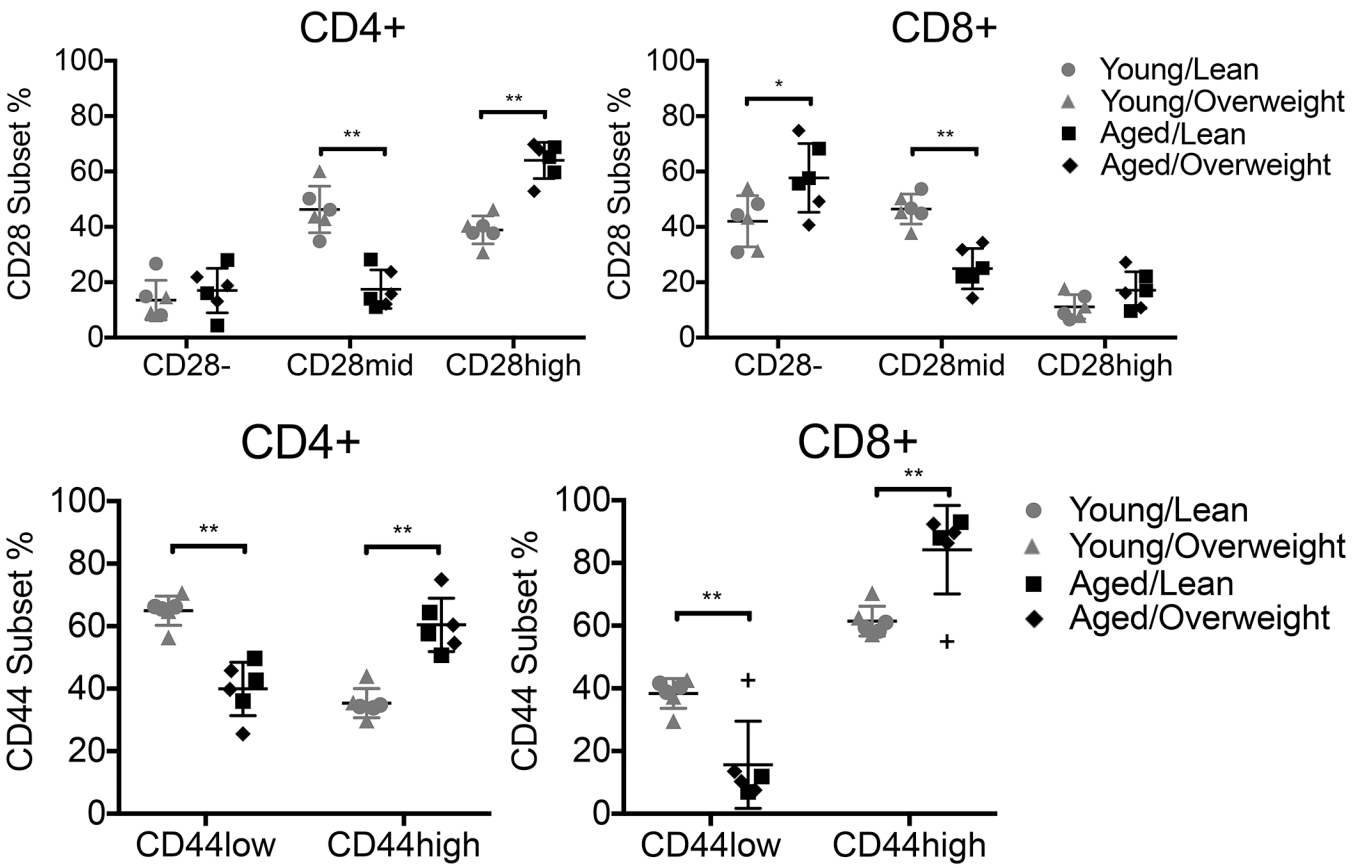


Figure 4-

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)
Streptavadin	BV711	N/A	N/A	Biolegend	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	(Monjazeb et al., 2016)
CD3	FITC	Human	CD3-12	BioRad	MCA1477F	1:50	(Monjazeb et al., 2016)
IFNγ	PE	Bovine	CC302	BioRad	MCA1783PE	1:10	(Hartley and Tarleton, 2015; Pedersen et al., 2002)
TNFα	BV-785	Human	MAB-11	Biolegend	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 chlorphyll protein-cyanine 5.5; PFM lab, Peter F. Moore laboratory; N/A, not applicable.