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Cellular and humoral immune responsiveness to inactivated *Leptospira interrogans* in dogs vaccinated with a tetravalent *Leptospira* vaccine

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ABSTRACT

Vaccination is commonly used to protect dogs against leptospirosis, however, memory immune responses induced by canine *Leptospira* vaccines have not been studied. In the present study, antibody and T cell mediated responses were assessed in dogs before and 2 weeks after annual revaccination with a commercial tetravalent *Leptospira* vaccine containing serogroups Canicola and Australis. Vaccination significantly increased average $\log_2$ IgG titers from 6.50 to 8.41 in year 1, from 5.99 to 7.32 in year 2, from 5.32 to 8.32 in year 3 and from 5.32 to 7.82 in year 4. The CXCL-10 levels, induced by *in vitro* stimulation of PBMC with Canicola and Australis, respectively, significantly increased from 1039.05 pg/ml and 1037.38 pg/ml before vaccination to 2547.73 pg/ml and 2730.38 pg/ml after vaccination. IFN-γ levels increased from 85.60 pg/ml and 178.13 pg/ml before vaccination to 538.62 pg/ml and 210.97 pg/ml after vaccination. The percentage of proliferating $CD4^+$ T cells in response to respective *Leptospira* strains significantly increased from 1.43 % and 1.25 % before vaccination to 24.11 % and 14.64 % after vaccination. Similar responses were also found in the $CD8^+$ T cell subset. Vaccination also significantly enhanced the percentages of central memory $CD4^+$ T cells from 12 % to 26.97 % and 27.65 %, central memory $CD8^+$ T cells from 3 % to 9.47 % and 7.55 %, and effector $CD8^+$ T cells from 3 % to 7.6 % and 6.42 %, as defined by the expression of CD45RA and CD62L, following stimulation with Canicola and Australis, respectively. Lastly, enhanced expression of the activation marker CD25 on T cells after vaccination was found. Together, our results show that next to IgG responses, also T cell responses are induced in dogs upon annual revaccination with a tetravalent *Leptospira* vaccine, potentially contributing to protection.

1. Introduction

Pathogenic Gram-negative *Leptospira* spirochetes are the causative agents of leptospirosis, an emerging bacterial zoonosis affecting all vertebrate species, including humans and dogs [1]. Numerous pathogenic serovars have been identified based on the carbohydrate structure of leptospiral lipopolysaccharides (LPS) that may belong to different *Leptospira* genomospecies [2]. Leptospirosis symptoms may vary from mild subclinical symptoms and fever to acute kidney and renal damage, reproductive failure, pulmonary haemorrhage, uveitis, myositis and death [1].

Vaccination is widely used to control leptospirosis in domestic and farm animals. All licensed *Leptospira* vaccines consist of chemically or physically inactivated whole bacteria (bacterins) of different pathogenic *Leptospira* strains [3]. Initially, serovars Canicola and Icterohaemorrhagiae have been associated with canine leptospirosis which stimulated the development of bivalent whole-cell *Leptospira* bacterins for vaccination of dogs [4]. Although these vaccines contributed to the reduction of canine leptospirosis, emergence of the disease caused by non-vaccine serovars led to the inclusion of serovars Grippotyphosa and Australis in *Leptospira* bacterins licensed in Europe [5–7]. The immunity induced by *Leptospira* vaccines is considered serovar specific and was shown to prevent or significantly reduce infection and subsequent renal carriage up to one year after vaccination in dogs challenged with different *Leptospira* isolates belonging to the same serovars as the vaccine strains [4,7,8]. To protect against severe canine disease, annual revaccination of dogs is recommended after initial adminis-

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tration of *Leptospira* vaccine to puppies at 8 weeks or older and a booster dose 2 to 4 weeks later [9].

The ultimate goal of vaccination against infectious diseases is the development of a long-lasting effective immunological memory response consisting of antigen-specific memory T (cellular immunity) and B cells (humoral immunity) [10]. Although cell-mediated immunity has been studied [11,12], so far memory T cell responses induced upon *Leptospira* vaccination have not been investigated in dogs. Most efficacy studies of canine *Leptospira* vaccines rely on the presence of agglutinating serum antibodies, protection against clinical signs of disease, renal colonization and mortality, as well as absence of *Leptospira* in blood and urine following challenge [4,6,13]. Antibodies elicited by *Leptospira* vaccines are generally directed against the leptospiral LPS, a *T*-cell independent antigen, and therefore fail to induce a memory response [3]. However, it has been shown that dogs vaccinated against *Leptospira* were protected from clinical disease, infection and urinary shedding one year after vaccination even though antibody titers declined and could no longer be detected [8]. In contrast, cattle vaccinated against serovar Hardjo were susceptible to infection with serovar Hardjo despite the presence of high agglutinating antibody levels [14]. Lack of correlation between the vaccine-induced antibody titers and protection from experimental infection [4,13] suggests the involvement of other immunological memory responses induced by *Leptospira* bacterins. In fact, studies in cattle have demonstrated development of an antigen-specific $CD4^+$, $CD8^+$ and γδ T cell-mediated response after vaccination against serovar Hardjo [15–17].

In the present study, antibody and T cell responses were assessed before and after annual revaccination with a commercial tetravalent *Leptospira* vaccine in dogs with previous history of vaccination against leptospirosis. *Leptospira* specific IgM and IgG antibody titers were measured in plasma of vaccinated dogs. PBMC from these animals were restimulated *ex vivo* with two inactivated *Leptospira* vaccine strains and subsequently T cell proliferation, and expression of T cell surface markers CD62L, CD45RA and CD25, defining different T cell memory subsets, was measured. In addition, T cell functionality, as defined by CXCL-10, IFN-γ and IL-17 cytokine expression, was analyzed. Whereas so far only antibody titers were considered to be involved in *Leptospira* vaccine protection, the present study identified additional immunological parameters which may correlate with protection induced by canine *Leptospira* vaccines.

2. Materials and methods

2.1. Leptospira strains

Chemically inactivated *L. interrogans* serogroup Canicola serovar Portland-vere (strain Ca-12–000) and *L. interrogans* serogroup Australis serovar Bratislava (strain As-05–073) were kindly provided by a pharmaceutical company that is part of the VAC2VAC consortium (https://www.vac2vac.eu/), hereafter referred to as company B. To estimate bacterial cell count, the optical density of these bacterial preparations was measured at 600 nm on an Ultrospec 200 spectrophotometer (Amersham Pharmacia Biotech, Inc.) using Ellinghausen-McCullough-Johnson-Harris (EMJH)-based medium (leptospiral culture medium provided by company B) as a blank. To allow comparison between bacterial preparations, *Leptospira* suspensions were diluted to the same OD_{600} (0.519) in leptospiral culture medium. Subsequently, throughout this study, PBMC were stimulated with a 200-fold dilution of these suspensions with an OD_{600} of 0.519 as this was the highest non-toxic dilution of the inactivated *Leptospira*.

2.2. Experimental design

Blood obtained from 15 privately-owned 2–10 years old mixed-breed dogs that came to the clinic for diagnostic or healthcare assessment and were fully vaccinated against core canine diseases and *Leptospira* was used in this study (**Supplementary Table S1**). Whole blood was collected from the cephalic or jugular vein before and 2 weeks after the annual revaccination with a commercially available non-adjuvanted tetravalent *Leptospira* vaccine containing the inactivated *L. kirschneri* serogroup Grippotyphosa, *L. interrogans* serogroup Canicola, Icterohaemorrhagiae and Australis according to manufacturer's recommendation (Fig. 1). Blood was collected in a heparinized tube and centrifuged 20 min at 1200xg at room temperature. Plasma was collected and stored at −20 °C. In year 4, peripheral blood mononuclear cells (PBMC) were isolated from remaining cells after plasma collection from 6 dogs according to the protocol described below.

2.3. PBMC isolation

PBMC were isolated by density gradient centrifugation at room temperature. Briefly, remaining cells after plasma collection were 2x diluted with 1x Dulbecco's Phosphate-buffered saline without calcium and magnesium ($DPBS^{-/-}$; Corning), layered onto Histopaque-1077 (Sigma Life Science) and centrifuged 30 min at 800xg with slow acceleration and brake. The interphase containing PBMC was transferred to a new tube and washed twice in wash medium consisting of RPMI-1640 GlutaMAX (Gibco) supplemented with 5 % heat inactivated fetal bovine serum (FBS; Bodinco BV), 50 U/ml Penicillin and 50 µg/ml Streptomycin (Gibco). PBMC were counted in the NucleoCounter (ChemoMetec) and either cryopreserved in RPMI supplemented with 10 % dimethyl sulfoxide (Sigma) and 50 % FBS or directly used in the *ex vivo* stimulation experiments.

2.4. Ex vivo lymphocyte culture

Canine PBMC were thawed in a water bath at 37 °C or freshly isolated from whole blood. PBMC were washed in $DPBS^{-/-}$, suspended at 10^7/ml, and stained with 0.2 µM proliferation dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) in $DPBS^{-/-}$ for 5–8 min at 37 °C in the dark. PBMC were washed in wash medium to remove unbound CFSE and centrifuged for 5 min at 400xg and 4 °C. Cells were then cultured in a 24-well plate at 3×10^6/ml in 1 ml culture medium consisting of serum-free X-VIVO (Lonza Group, Switzerland) supplemented with 0.05 mM β-mercaptoethanol (Sigma Life Science), 50 U/ml Penicillin and 50 µg/ml Streptomycin (Gibco). Inactivated *L. interrogans* serogroups Canicola or Australis (OD_{600} = 0.519) or leptospiral culture medium (L-CM) as a negative control were added to cells, resulting in 1:200 dilution of stimulus in well. After 72 h of incubation at 37 °C and 5 % CO_2, the same volume of fresh culture medium supplemented with 20 IU/ml IL-2 (Proleukin, Clinigen Healthcare ltd., UK) was added to each well. Cells were then incubated for additional 72 h before analysis by flow cytometry.

2.5. Flow cytometry

CFSE-labeled PBMC were washed with FACS buffer consisting of $DPBS^{-/-}$ supplemented with 2 % FBS and 2 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen). All centrifugation steps were performed at 300xg and 4 °C for 3 min. To characterize central memory, effector memory and effector memory T cells re-expressing CD45RA (TEMRA) subsets (Withers et al, 2018 [18]), cells were labeled with rat anti-dog CD4-Pacific Blue clone YKIX302.9, rat anti-dog CD8-RPE clone YCATE55.9, mouse anti-

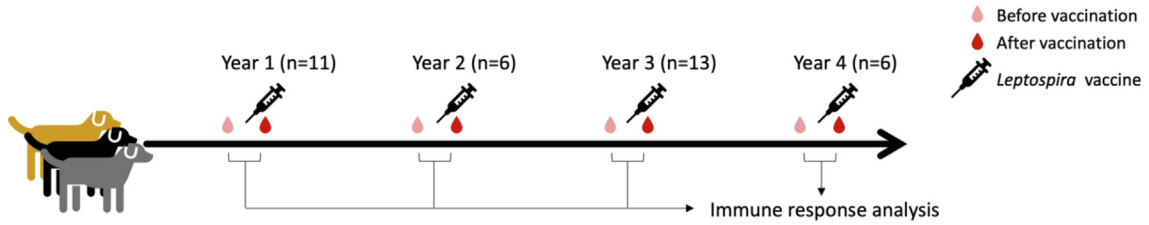

Fig. 1. Schematic overview of the experimental timeline with *Leptospira* vaccination and blood collection for immune response evaluation. In total, 15 mixed breed dogs were used in the study. Dogs were annually immunized (syringe) with the tetravalent *Leptospira* vaccine containing inactivated bacteria of *L. interrogans* serogroup Canicola, Icterohaemorrhagiae and Australis as well as *L. kirschneri* serogroup Grippotyphosa. Over the course of 4 years, blood was collected each year weeks before (light red drop) and after (dark red drop) vaccination for evaluation of immune responses. N = number of dogs sampled each year (out of 15). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human CD62L-Alexa Fluor 647 clone FMC46 (Bio Rad), mouse anti-dog CD25-PE Cy7 clone P4A10 (eBioscience), rat anti-mouse CD44-BV605 clone IM7 (Biolegend), mouse anti-dog CD45RA biotin clone CA21.4B3 (University of California, Davis, USA) and ViaKrome 808 (Beckman Coulter) in 100 µl staining buffer consisting of FACS buffer supplemented with 2 % normal dog plasma for 30 min on ice in the dark. Cells were centrifuged, washed thrice in 100 µl of FACS buffer and centrifuged again before labeling with streptavidin BUV395 (BD Horizon) in 100 µl staining buffer for 15 min on ice in the dark. Cells were analyzed on the CytoFLEX LX Flow Cytometer (Beckman Coulter, Brea, CA, USA) and by FlowJo V10.6 software at the Flow Cytometry and Cell Sorting Facility, Faculty of Veterinary Medicine, Utrecht University.

2.6. Cytokine analysis

For cytokine analyses, culture supernatants of stimulated cells were collected after 24 h or 72 h incubation and stored at −80 °C until analyses. Capture and detection antibodies for canine IFN-γ, IL-17 and CXCL-10 were purchased from Kingfischer Biotech, Inc. and used for multiplex cytokine analyses of supernatants of stimulated PBMC on a Magpix system (Luminex XMAP) according to the manufacturer's instructions. The cytokine concentrations in supernatants of stimulated cells were calculated using the standards provided in the kits. The MFI data were analyzed using a 5-parameter logistic method (xPONENT software, Luminex, USA).

2.7. Leptospira-specific IgM and IgG ELISA

High affinity 96 well ELISA plates (Greiner Bio one BV, the Netherlands) were coated with 2 µg/ml outer envelope antigens isolated from *L. interrogans* serovar Canicola strain Hond Utrecht IV, serovar Icterohaemorrhagiae strain Kantorowicz, and serovar Copenhageni strain Wijnberg (Leptospirosis Reference Centre, Amsterdam, the Netherlands) overnight at room temperature in $DPBS^{-/-}$. Plates were stored at −20 °C until use and rinsed 4 times with a wash buffer consisting of distilled water and 0.05 % Tween 80 before adding the samples as previously described [19]. Serial twofold dilutions of plasma samples were made from 1:20 to 1:2560 in dilution buffer consisting of $DPBS^{-/-}$ supplemented with 1 % protifar (Nutricia Advanced Medical Nutrition, the Netherlands) and 0.05 % Tween 80. Plates were incubated 1 h at 37 °C and rinsed 4 times with a wash buffer. After the last wash, peroxidase conjugated goat anti-dog IgG and goat anti-dog IgM antibodies (Tebu-bio.com, the Netherlands) were added to the corresponding wells. Plates were incubated and washed as described above before adding 100 µl substrate consisting of 10 ml $DPBS^{-/-}$, 5.2 ml Na_2HPO_4, 4.8 ml citric acid, 10 mg 2,2′-azino-bis (3-ethylben zothiazoline-6-sulfonic acid) diammonium salt tablet, and 10 µl H_2O_2 30 % (all Merck KGaA, Germany). After 30 min incubation, the reading of the plates was performed visually at room temperature. The titer was determined as the reciprocal of the highest dilution producing a positive (i.e., clear colour change) result. Positive and negative controls with known antibody titers were included on each ELISA plate.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). Antibody titer values were $\log_2$ transformed before statistical analysis. For comparison of matched data, a paired *t*-test was used to evaluate the differences between before and after vaccination samples unless otherwise stated. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Antibody responses before and after annual vaccination with a tetravalent Leptospira vaccine

To assess humoral responses after vaccination, *Leptospira* specific IgG and IgM antibody titers were measured in plasma collected from 15 adult dogs before and after annual vaccination with a tetravalent *Leptospira* vaccine over the course of 4 years (**Supplementary Tables S2-3**, respectively). In 8 out of 15 dogs, IgG and IgM antibody titers were measured in two or more consecutive years (Fig. S1**a, b**, respectively). In year 1, the mean $\log_2$ IgG titer significantly increased from 6.50 before vaccination to 8.41 after revaccination (1.29-fold increase), in year 2 from 5.99 to 7.32 (1.22-fold increase), in year 3 from 5.32 to 8.32 (1.56-fold increase) and in year 4 from 5.32 to 7.82 (1.47-fold increase) (Fig. 2**a-d**). Although a significant increase in IgM titer was found in year 1 (a 1.15-fold increase from 4.14 before vaccination to 4.78 after vaccination), revaccination did not significantly alter the IgM antibody levels in subsequent years. Mean $\log_2$ IgM titers were respectively 3.82 and 3.66 in year 2, 4.71 and 5.25 in year 3, and 4.99 and 5.66 in year 4 before and after vaccination (Fig. 2**e-h**).

3.2. Induction of cytokine responses in PBMC before and after vaccination

To differentiate between different effector T cell responses to *Leptospira* vaccination in dogs, the production of CXCL-10, IFN-γ and IL-17 was measured in PBMC incubated with inactivated serovars Canicola and Australis, as well as L-CM as control before and after vaccination (Fig. 3). Before vaccination, mean CXCL-10 levels were 1039.05 pg/ml, 1037.38 pg/ml and 2270.37 pg/ml, whereas after vaccination mean CXCL-10 levels were 2547.73 pg/ml, 2730.38 pg/ml and 2120.48 pg/ml in Canicola, Australis and L-CM treated cells, respectively. The CXCL-10 levels significantly increased after vaccination in PBMC incubated with *Leptospira* ser-

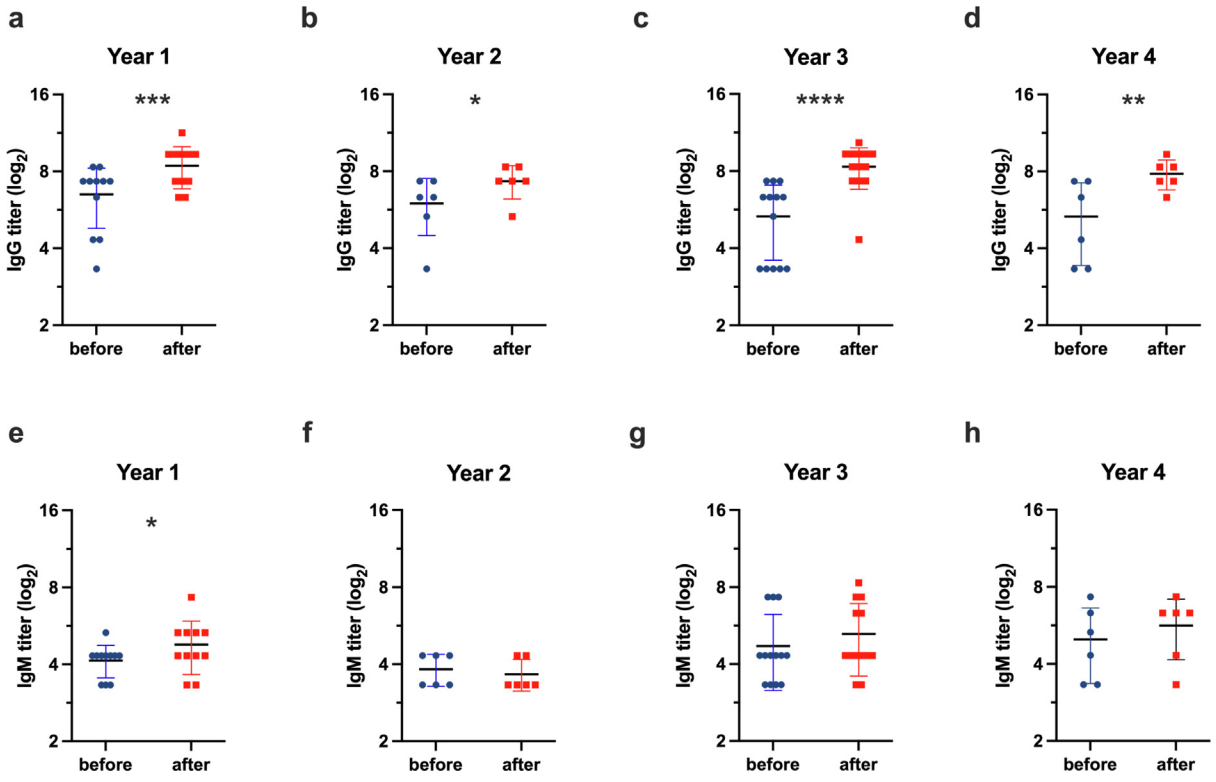

Fig. 2. Antibody responses induced by *Leptospira* vaccine. *Leptospira* specific IgG (**a-d**) and IgM (**e-h**) were measured in plasma before and after annual revaccination with the *Leptospira* vaccine over the course of 4 years. Each dot represents one donor. A paired *t*-test was performed on $\log_2$ transformed antibody titers. The p-value indicates a significant difference in antibody titers before and after vaccination (* P < 0.05, ** P < 0.001, *** P < 0.001, **** P < 0.0001). Mean with standard deviation is shown.

ogroups Canicola and Australis compared to before vaccination (Fig. 3**a, d**), while vaccination did not significantly alter CXCL-10 expression in control cells (Fig. 3**g**). Mean IFN-γ levels before vaccination were respectively 85.60 pg/ml, 178.13 pg/ml and 6.73 pg/ml in Canicola, Australis and L-CM incubated cells. After vaccination, IFN-γ levels increased to 538.62 pg/ml (Canicola), 210.97 pg/ml (Australis) and 71.58 pg/ml (L-CM), however neither increase was statistically significant. Nevertheless, measured IFN-γ levels in *Leptospira* stimulated cells were higher compared to L-CM (Fig. 3**b, e, h**). Mean IL-17 levels before vaccination were 384.45 pg/ml, 436.12 pg/ml and 81.87 pg/ml, while after vaccination mean IL-17 levels were 459.79 pg/ml, 368.26 pg/ml and 139.63 pg/ml in Canicola, Australis and L-CM stimulated cells, respectively. Vaccination did not significantly alter IL-17 levels. However, similar to IFN-γ levels, stimulation with *Leptospira* induced higher IL-17 levels compared to L-CM (Fig. 3**c, f, i**).

3.3. Enhanced T cell responses to inactivated Leptospira serovars after immunization

T cell responses in year 4 were analyzed in CFSE-labeled canine PBMC isolated before and after vaccination with *Leptospira* vaccine following *in vitro* restimulation with the inactivated *L. interrogans* serogroup Canicola or Australis or L-CM as the negative control. On day 6 of *in vitro* culture, proliferation as well as expression of surface markers CD62L, CD45RA and CD25 were measured in the $CD4^+$ and $CD8^+$ lymphocyte populations. The gating strategy and fluorescence minus one (FMO) controls are shown in **Supplementary Fig. S2**. Percentages of total $CD4^+$ and $CD8^+$ lymphocytes in PBMC before and after vaccination did not differ significantly (**Supplementary Fig. S3**). Cell proliferation in response to the inactivated *Leptospira* strains was determined in $CD4^+$ and $CD8^+$ T cells (Figs. 4 and 5, respectively, **Supplementary Fig. S4**).

Mean percentages of proliferating $CD4^+$ T cells before vaccination were 1.43 %, 1.25 % and 0.14 % in PBMC cultured in presence of inactivated serogroup Canicola, Australis or L-CM, respectively. After vaccination, the mean percentages of proliferating $CD4^+$ T cells increased to 24.11 % (Canicola), 14.64 % (Australis) and 2.09 % (L-CM). A significant increase in proliferating $CD4^+$ T cells was found in PBMC stimulated with the inactivated serogroup Canicola or Australis after vaccination compared to before vaccination (Fig. 4**b-d, Supplementary Fig. S4a**). In addition, before vaccination mean percentages of proliferating $CD8^+$ T cells were 0.32 % (Canicola), 0.43 % (Australis) and 0.14 % (L-CM). After vaccination, mean percentage of proliferating $CD8^+$ T cells increased to 16.66 % (Canicola), 10.96 % (Australis) and 1.73 % (L-CM). The increase in the percentage of proliferating $CD8^+$ cells after vaccination was significant in PBMC stimulated with the inactivated serogroup Canicola or Australis (Fig. 5**b-d, Supplementary Fig. S4b**). In summary, our results indicate expansion of *Leptospira* responsive $CD4^+$ and $CD8^+$ T cells after vaccination.

Next, the expression of cell surface markers CD62L and CD45RA was analyzed on $CD4^+$ and $CD8^+$ T lymphocytes stimulated with the inactivated *Leptospira* serogroups Canicola or Australis, or L-CM as a negative control to distinguish between the naïve ($CD62L^+$-$CD45RA^+$), central memory ($CD62L^+CD45RA^-$), effector memory ($CD62L^-CD45RA^-$) and terminally differentiated effector memory (TEMRA; $CD62L^-CD45RA^+$) T cell subsets as previously described [18] (Fig. 6, **Supplementary Fig. S5-6**). On average, 12 % of $CD4^+$ T cells comprised of central memory subset before vaccination. After vaccination, the mean percentages of central memory $CD4^+$ T cells was 26.97 % (Canicola), 27.65 % (Australis) and 14.49 % (L-CM). The increase in the percentage of central memory $CD4^+$ T cells after vaccination was statistically significant in cells stimulated with inactivated *Leptospira* (Fig. 6**b-d, Supplementary Fig. S6a**). The mean percentage of naïve $CD4^+$ T cells was 68.78 % (Canicola),

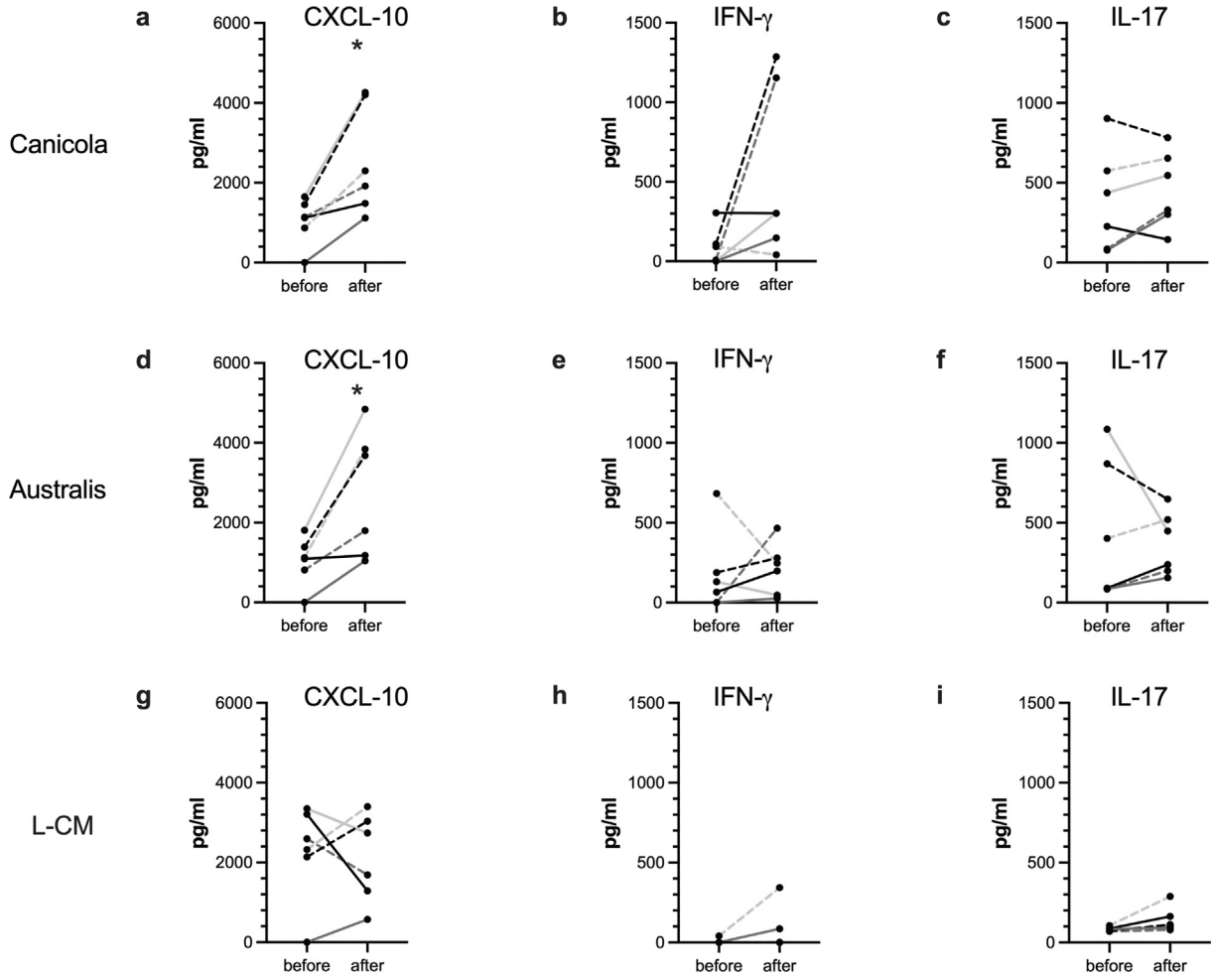

Fig. 3. Cytokine expression in the supernatant of PBMC before and after vaccination. PBMC from 6 dogs (donors 1–6) were obtained before and after vaccination and incubated with inactivated *L. interrogans* serogroup Canicola (**a**-**c**) or Australis (**d**-**f**) or leptospiral culture media as negative control (L-CM; **g**-**i**). The expression of CXCL-10 (**a**, **d**, **g**) was assessed in the supernatant of stimulated cells after 24 h, whereas the expression of IFN-γ (**b**, **e**, **h**) and IL-17 (**c**, **f**, **i**) was assessed after 72 h. The p-value indicates a significant difference in the amount of cytokine expression before and after vaccination (* $P < 0.05$). Each line represents one donor.

67.50 % (Australis) and 70.57 % (L-CM) before vaccination. After vaccination, the percentage of this subset decreased on average to 50.35 % (Canicola), 51.63 % (Australis) and 68 % (L-CM). The decrease in the percentage of naïve $CD4^+$ T cell subset was statistically significant in cells stimulated with *Leptospira* strains (Fig. 6**b-d**). Before vaccination, on average 11 % of $CD4^+$ T cells comprised of effector memory cells. After vaccination, the percentage of effector memory $CD4^+$ T cells was on average 15.29 % (Canicola), 14.3 % (Australis) and 11.06 % (L-CM), however this was not significant (Fig. 6**b-d, Supplementary Fig. S6b**). Vaccination did not alter the percentage of TEMRA $CD4^+$ T cells. The percentage of this subset was on average 7 % before and after vaccination (Fig. 6**b-d**).

In the $CD8^+$ lymphocyte population, the percentage of both central and effector memory $CD8^+$ T cells significantly increased after vaccination in cells stimulated with the inactivated *Leptospira* strains. Before vaccination, on average 3 % of the $CD8^+$ T cells had a central or effector memory phenotype. After vaccination, this increased on average to 9.47 %, 7.55 % and 5.23 % of the $CD8^+$ T cells with a central, and 7.6 %, 6.42 % and 3.53 % with an effector memory phenotype in cells stimulated with Canicola, Australis or L-CM, respectively. Therefore, stimulation with *Leptospira* strains significantly increased the percentage of both central and effector memory $CD8^+$ T cells after vaccination. A significant increase in effector memory cells was also found in L-CM stimulated cells after vaccination (Fig. 6**e-g, Supplementary Fig. S6c, d**). The percentage of naïve $CD8^+$ T cells before vaccination was on average 72.68 % (Canicola), 70.68 % (Australis) and 77.75 % (L-CM). After vaccination, the mean percentage of this subset decreased to 57.38 % (Canicola), 53.62 % (Australis) and 74.87 % (L-CM). The decrease in the percentage of naïve $CD8^+$ T cells was statistically significant in cells stimulated with Canicola and L-CM. In contrast, vaccination did not alter the percentage of TEMRA $CD8^+$ T cells. Before vaccination, on average 20.77 % and 23.32 % of $CD8^+$ T cells demonstrated a TEMRA phenotype, while after vaccination, this increased to 25.58 % and 32.4 % in Canicola and Australis stimulated cells, respectively. The percentage of TEMRA cells in L-CM treated cells was on average 16.3 % before and after vaccination (Fig. 6**f-h**). These results suggest that revaccination with a tetravalent *Leptospira* vaccine mainly promotes the expansion of the *Leptospira*-specific central memory $CD4^+$ T cell subset.

Lastly, to identify the activation status of different $CD4^+$ and $CD8^+$ T cell subsets, the expression of CD25 was analyzed on naïve, central memory, effector memory and TEMRA T cells cultured in presence of inactivated *Leptospira* strains or L-CM as negative control (Fig. 7). On average, 19.25 % and 18.30 % of central memory $CD4^+$ T cells stimulated with Canicola and Australis, respectively expressed CD25 before vaccination, while after vaccination, CD25 expression increased to 41.83 % (Canicola) and 41.01 % (Australis). Of the effector memory $CD4^+$ T cells 16.21 % (Canicola) and 17.09 % (Australis) expressed CD25 before vaccination. After vaccination

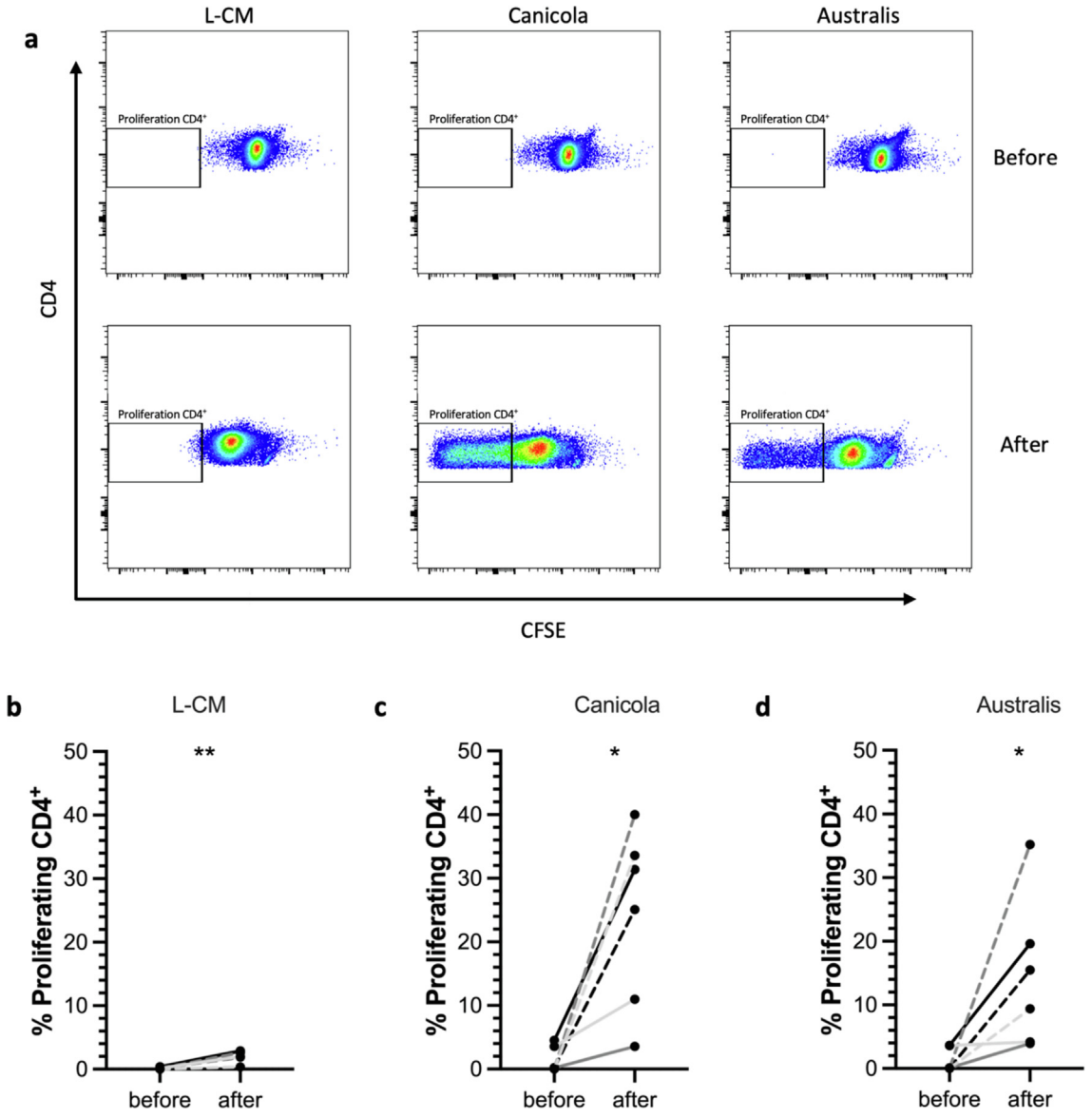

Fig. 4. CD4$^+$ lymphocyte proliferation after *ex vivo* restimulation with inactivated *Leptospira*. PBMC from 6 dogs (donors 1–6) were labeled with the proliferation dye CFSE and cultured for 5 days in presence of the leptospiral culture medium (L-CM) as a negative control, inactivated *L. interrogans* serogroup Canicola or Australis. (**a**) Representative dot plots show proliferation of live, single CD4$^+$ lymphocytes after incubation with L-CM (left), serogroup Canicola (middle), and Australis (right). Top panels show PBMC isolated from dogs before vaccination and bottom panels show PBMC isolated from dogs after vaccination. Percentages of proliferating CD4$^+$ cells before and after vaccination, cultured in presence of (**b**) L-CM, (**c**) Canicola, or (**d**) Australis are shown. The p-value indicates a significant difference in the percentage of proliferating CD4$^+$ lymphocytes before and after vaccination (* $P < 0.05$, ** $P < 0.01$). Each line represents one donor.

the mean percentage of CD25 expression in this subset increased to 53.8 % (Canicola) and 49.05 % (Australis). Furthermore, the mean percentage of TEMRA CD4$^+$ T cells expressing CD25 was 36.37 % (Canicola) and 37.75 % (Australis) before vaccination, while after vaccination this increased to 58.67 % (Canicola) and 51.52 % (Australis). The expression of CD25 on naïve CD4$^+$ T cells also increased from 12.04 % (Canicola) and 11.42 % (Australis) before vaccination to 22.33 % (Canicola) and 22.93 % (Australis) after vaccination. Both strains significantly upregulated CD25 expression on effector memory CD4$^+$ T cells, while Canicola significantly enhanced the expression of this marker also on TEMRA CD4$^+$ T cells after vaccination. Although stimulation with *Leptospira* also enhanced the expression of CD25 in the central memory CD4$^+$ T cell subset after vaccination in 4 out of 6 animals, this was not statistically significant (Fig. 7**b, c**). In L-CM cultured PBMC, 12.81 % and 11.65 % of central and effector memory cells, respectively expressed CD25 before vaccination, while after vaccination CD25 was expressed on 10.35 % and 12.34 % of these two subsets. The CD25 expression on TEMRA CD4$^+$ T cells increased on average from 35.07 % before vaccination to 45.62 % after vaccination, while the expression of CD25 on naïve CD4$^+$ T cells remained 4.5 % before and after vaccination in L-CM cultured cells Fig. 7**a**).

In CD8$^+$ T cells, on average 25.67 % (Canicola) and 24.8 % (Australis) of central memory cells expressed CD25 before vaccination, while after vaccination the CD25 expression increased to 33.97 % (Canicola) and 34.73 % (Australis). In L-CM cultured cells, vaccination reduced CD25 expression in central memory subset from 25.92 % to 12.3 %. A similar trend was found in effector memory CD8$^+$ T cells. Before vaccination, on average 20.83 % (Canicola) and 21.05 % (Australis) of effector memory CD8$^+$ T cells expressed CD25, while after vaccination CD25 expression increased to 41.05 % (Canicola) and 37.18 % (Australis). In effector memory sub-

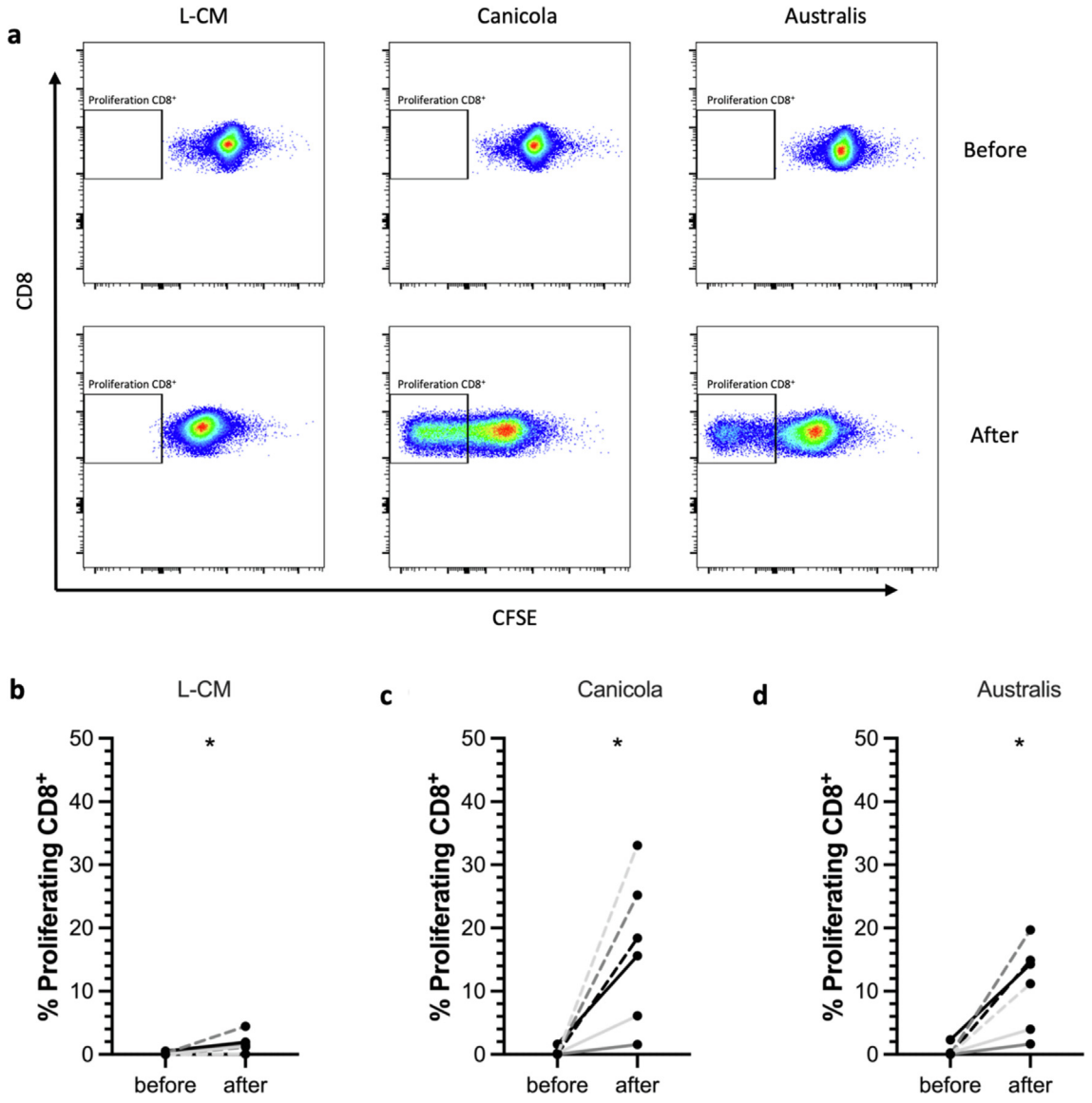

Fig. 5. CD8$^+$ lymphocyte proliferation after *ex vivo* restimulation with inactivated *Leptospira*. PBMC from 6 dogs (donors 1–6) were labelled with the proliferation dye and cultured as described in Fig. 3. (**a**) Representative dot plots show proliferation of live, single CD8$^+$ lymphocytes after incubation with L-CM (left), serogroup Canicola (middle), and Australis (right). Top panels show PBMC isolated from dogs before vaccination and bottom panels show PBMC isolated from dogs after vaccination. Percentages of proliferating CD8$^+$ cells before and after vaccination, following incubation with (**b**) L-CM, (**c**) Canicola, or (**d**) Australis are shown. The p-value indicates a significant difference in the percentage of proliferating CD8$^+$ lymphocytes before and after vaccination (* $P < 0.05$). Each line represents one donor.

set, vaccination reduced CD25 expression from 21.84 % to 14.92 % in L-CM cultured cells (Fig. 7**d-f**). Furthermore, the mean percentage of CD25 expressing CD8$^+$ TEMRA T cells was 21.37 % (Canicola) and 23.98 % (Australis) before vaccination, while after vaccination, on average 38.73 % (Canicola) and 41.4 % (Australis) of these cells expressed CD25. Vaccination induced CD25 expression on naïve CD8$^+$ T cells from 3.5 % (Canicola) and 4.9 % (Australis) to 8.0 % (Canicola) and 9.95 % (Australis) (Fig. 7**e, f**). In contrast, vaccination did not alter CD25 expression in TEMRA and naïve CD8$^+$ T cells treated with L-CM. On average, 13.39 % of TEMRA cells and 1.66 % of naïve CD8$^+$ T cells expressed CD25 in L-CM cultured cells before vaccination, while after vaccination 15.54 % of TEMRA cells and 2.17 % of naïve CD8$^+$ T cells expressed CD25 (Fig. 7**d**). A significant increase in CD25 expression after vaccination in response to stimulation with Canicola and Australis was found on TEMRA and naïve CD8$^+$ T cell subsets (Fig. 7**e, f**). Together, these data show that restimulation with inactivated *Leptospira* vaccine serovars mainly activates the effector memory and TEMRA CD4$^+$ as well as TEMRA CD8$^+$ T cells after vaccination.

4. Discussion

Protection induced by canine *Leptospira* vaccines is so far considered to be mediated mainly by LPS-reactive antibodies [4,20] even though correlation between antibody titers and protection following *Leptospira* vaccination could not be established [21]. This suggests that mechanisms other than anti-LPS antibodies could play a role in protective immune responses induced by *Leptospira* vaccines. In spite of this, T cell and specifically memory responses upon *Leptospira* vaccination have not been investigated in dogs. To get more insight in the adaptive immune responses to *Leptospira* in dogs, in the present study, antibody levels in plasma and T cell responses in canine PBMC were assessed before and after vaccina-

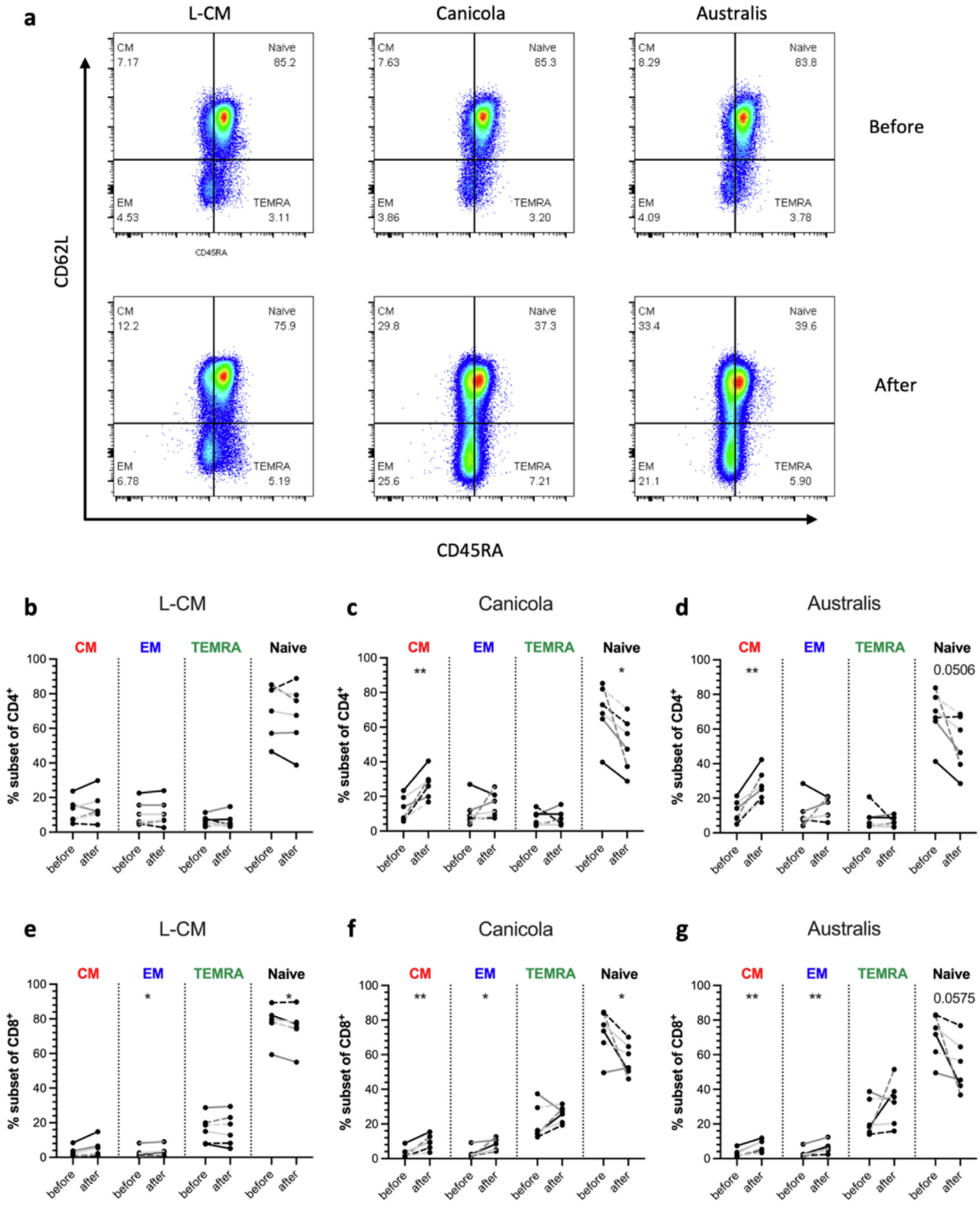

Fig. 6. Effector and central CD4$^+$ and CD8$^+$ memory response before and after vaccination. PBMC from 6 dogs (donors 1–6) were incubated for 5 days with the leptospiral culture medium (L-CM) as a negative control, inactivated *L. interrogans* serogroup Canicola or Australis. (**a**) Representative dot plots show CD45RA and CD62L expression on live, single, CD4$^+$ lymphocytes after incubation with L-CM (left), serogroup Canicola (middle), and Australis (right). Top panels show PBMC isolated from dogs before vaccination and bottom panels show PBMC isolated from dogs after vaccination. (**b**-**g**) Data show percentages of central memory (CM; CD62L$^+$CD45RA$^-$), effector memory (EM; CD62L$^-$CD45RA$^-$), terminally differentiated effector memory (TEMRA; CD62L$^-$CD45RA$^+$) and naïve (CD62L$^+$CD45RA$^+$) CD4$^+$ (**b**-**d**) and CD8$^+$ lymphocytes (**e**-**g**) before and after vaccination, following incubation. The p-value (* $P < 0.05$, ** $P < 0.01$) indicates a significant difference in the percentage of each cell population before and after vaccination. Values 0.0506 and 0.0575 represent non– significant p-values. Each line is one donor.

tion with a commercial tetravalent *Leptospira* vaccine containing inactivated *L. kirschneri* serogroup Grippotyphosa, *L. interrogans* serogroup Canicola, Icterohaemorrhagiae and Australis. Our results demonstrate activation of adaptive immune responses to serovars Canicola and Australis, as measured by increased *Leptospira*-specific IgG in plasma, increased secretion of CXCL-10 and IFN-γ, enhanced proliferation of CD4$^+$ and CD8$^+$ T cells as well as expansion of central memory CD4$^+$ T cell subset in vaccinated dogs.

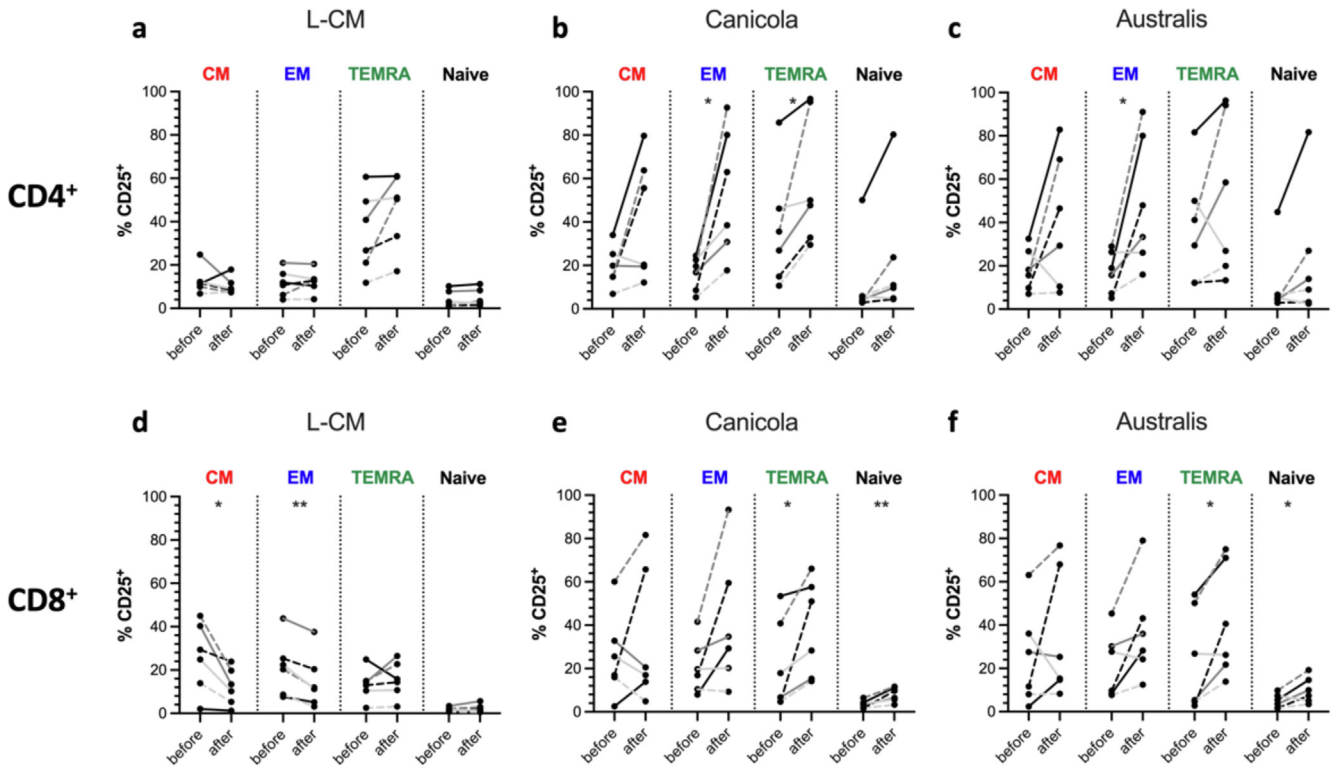

Fig. 7. CD25 expression before and vaccination. PBMC from 6 dogs (donors 1–6) were incubated for 5 days with the leptospiral culture medium (L-CM) as a negative control or inactivated *L. interrogans* serogroup Canicola or Australis. CD25 expression was analyzed on live, single central memory (CM), effector memory (EM), terminally differentiated effector memory (TEMRA) and naïve cells in $CD4^+$ (**a**-**c**) and $CD8^+$ (**d**-**f**) T cell subsets before and after vaccination. The p-value (* $P < 0.05$, ** $P < 0.01$) indicates a significant difference in the percentage of $CD25^+$ cells. Each line is one donor.

Previously, *Leptospira*-specific IgM and IgG antibody levels were assessed in dogs to determine the quality of humoral immunity after vaccination with a *Leptospira* vaccine [22]. This study showed that IgM antibody levels were induced immediately after each vaccination as primary response, while substantial IgG antibody levels were induced after the booster dose, and further enhanced upon annual revaccination [22]. In the present study, significant 1.29, 1.22, 1.56 and 1.47-fold increases in IgG antibody levels were found in plasma following annual revaccination of dogs with the tetravalent *Leptospira* vaccine in year 1, 2, 3 and 4, respectively (Fig. 2**a-d**, **Supplementary Fig. S1a, Supplementary Table S3**), indicating the presence of memory B cells. Vaccination increased IgM antibody levels in some years in donor 2 (year 4), 3 (year 4), 4 (year 4), 5 (year 1 and 3) and 7 (year 3) (**Supplementary Table S2**). This may suggest activation of B cells with novel antigen specificities or the need of multiple vaccinations required for class switch and seroconversion to IgG as donors 3, 4 and 5 were 2 years of age at the first vaccination event (**Supplementary Table S1**). Alternatively, following multiple *Leptospira* vaccine boosters in older dogs, few low affinity IgM B cells present may avoid activation due to affinity maturation and increasing number of memory B cells. Nevertheless, *Leptospira* specific plasma IgM antibody levels were low in most dogs and were no longer affected by annual revaccination (Fig. 2**e-h, Supplementary Fig. S1b, Supplementary Table S2**).

Vaccination induced mean CXCL-10 levels from 1039.05 pg/ml (Canicola) and 1037.38 pg/ml (Australis) to 2547.73 pg/ml (Canicola) and 2730.38 pg/ml (Australis) and IFN-γ levels from 85.60 pg/ml (Canicola) and 178.13 pg/ml (Australis) to 538.62 pg/ml (Canicola) and 210.97 pg/ml (Australis) (Fig. 3). CXCL-10 is secreted in response to IFN-γ by different cell types under pro-inflammatory conditions and promotes migration of Th1 cells[23], suggesting the induction of a Th1- or cell-mediated immune responses[24] in dogs immunized with the *Leptospira* vaccine. IL-17 was also detected in cells stimulated with Canicola and Australis, however IL-17 levels before and after vaccination were comparable (384.45 pg/ml and 436.12 pg/ml before vs 459.79 pg/ml and 368.26 pg/ml after), indicating that Th17 responses were not induced by vaccination (Fig. 3**c, f, i**). The role of Th17-mediated responses in *Leptospira* vaccination remains unclear as IL-17 may be secreted by various innate immune cells in response to IL-1β and IL-23 as well as $CD4^+$ and $CD8^+$ T cells and γδ T cells [25]. It has been shown that specific pathogen-associated molecular patterns (PAMPs) expressed by the extracellular bacteria can skew Th1/Th2 -mediated immune responses [26]. Activation of TLR4, TLR5 and TLR9 by bacterial LPS, flagellin and CpG DNA motifs present in the vaccine, respectively, may act as natural adjuvants promoting a Th1 immune response. On the other hand, bacterial lipoproteins which are recognized by TLR2 are potent inducers of a Th2-mediated response [27]. We have recently shown that both TLR4 and TLR2 are involved in *Leptospira* recognition in dogs [28], therefore both Th1- and Th2-mediated responses could be expected in vaccinated animals. Additional studies are needed to identify which T cell subsets and leptospiral PAMPs specifically are involved in *Leptospira* vaccine-induced immunity in dogs.

Cytokine production coincided with enhanced proliferation of $CD4^+$ and $CD8^+$ T cells after vaccination, following stimulation with the inactivated *Leptospira* serogroups Canicola and Australis. The mean percentage of proliferating $CD4^+$ T cells increased from 1.43 % (Canicola) and 1.25 % (Australis) before vaccination to 24.11 % (Canicola) and 14.64 % (Australis) after vaccination. Similarly, proliferation of $CD8^+$ T cells increased from 0.32 % (Canicola) and 0.43 % (Australis) before vaccination to 16.66 % (Canicola) and

10.96 % (Australis) after vaccination (Figs. 4**c, d and 5c, d**). The observation that proliferation induced by L-CM alone increased significantly after vaccination (0.14 % before vs 2.09 % after) may indicate an immune response to medium components (Figs. 4**b and 5b**). However, the low proliferative response induced by L-CM was not significant when comparing T cell proliferation in all groups after vaccination (**Supplementary Fig. S4**), therefore the importance of L-CM induced T cell proliferation is expected to be minimal. Because PBMC were stimulated with whole inactivated bacteria, we speculate that the $CD8^+$ T cells that proliferated most likely were activated as bystanders to cytokines produced by other cells. The activation of $CD8^+$ T cells through cross-presentation by professional antigen presenting cells in our *in vitro* setting seems less probable as it requires the upregulation of MHC class I molecules and high antigen concentration. However, as exogenous antigens are normally cross-presented to $CD8^+$ T cells in lymphoid tissues [29], $CD8^+$ T cells may contribute to *Leptospira* induced vaccine responses *in vivo*. The observation that no antigen-specific T cell proliferation could be detected prior to revaccination indicates that the number of antigen-specific T cells in blood decreases below detection within a year. Future studies are required to determine whether *Leptospira*-reactive memory T cells persist in secondary lymphoid organs such as lymph nodes.

In the present study, we aimed to differentiate canine effector/memory T cell subsets with anti-CD4, CD8, CD44, CD62L, CD45RA and CD25 antibodies. Similar to Withers *et al.* [18], we distinguished naïve, central and effector memory as well as terminally differentiated effector memory $CD4^+$ and $CD8^+$ T cells based on the expression of CD62L and CD45RA molecules (Fig. 6) while the CD44 marker proved to be not suitable (data not shown). Vaccination significantly increased the percentage of central memory $CD4^+$ T cells from 12 % to 26.97 % (Canicola) and 27.65 % (Australis), central memory $CD8^+$ T cells from 3 % to 9.47 % (Canicola) and 7.55 % (Australis), as well as effector memory $CD8^+$ T cells from 3 % to 7.6 % (Canicola) and 6.42 % (Australis) (Fig. 6). Although CD45RA negative cells indicate existence of antigen-specific central and effector memory T cells in vaccinated dogs, it remains unknown whether these cells are also involved in protective responses during *Leptospira* infection. Interestingly, although *Leptospira* vaccination did not significantly alter the frequencies of TEMRA $CD4^+$ and $CD8^+$ T cells (Fig. 6), a significant increase in CD25 expression was found after vaccination on TEMRA $CD4^+$ and $CD8^+$ in response to *Leptospira* strains (Fig. 7). TEMRA cells have been associated with protective immunity after dengue [30], influenza [31], yellow fever and smallpox [32] vaccination and exhibit rapid effector functions upon stimulation [33]. However, the involvement of TEMRA in immunity to *Leptospira* vaccination in dogs is unclear. Overall, the expression of the cell activation marker CD25 was more prominent in the $CD4^+$ T cell subsets compared to $CD8^+$ T cells. Together, this may indicate involvement of Th-mediated immunity towards a protein component following *Leptospira* restimulation.

In this study, we analyzed antibody and T cell responses raised to serogroups Canicola and Australis after vaccination with a tetravalent *Leptospira* vaccine. Since non-vaccine serovars have been recently found to cause leptospirosis in dogs [19,34,35] and a certain degree of cross-protection has been described following *Leptospira* vaccination [36], future studies, assessing the level of cross-reactivity of *Leptospira*-specific T cells generated after vaccination would be valuable to determine protective immunity against these strains. Although our results demonstrate the presence of *Leptospira*-specific IgG antibodies, a Th1-like immune response and expansion of antigen-specific memory T cells in vaccinated dogs, further studies are needed to investigate which specific *Leptospira* antigens elicit T cell and antibody responses (in addition to anti-LPS antibodies) and what their contribution to protection is. T cell assays described in this study may be used to monitor the efficacy of canine *Leptospira* vaccines in the future. Combined with the currently used serology tests, T cell assays may provide a tool for better characterization of immune responses and protection offered by these vaccines.

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Data availability

The datasets generated for this study are included in the article/Supplementary material.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.11.017.

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