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CHEMOKINE RECEPTOR 7 (CCR7)-EXPRESSION AND IFNγ PRODUCTION DEFINE VACCINE-SPECIFIC CANINE T CELL SUBSETS

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Abstract

Canines suffer from and serve as strong translational animals models for many immunological disorders and infectious diseases. Routine vaccination has been a mainstay of protecting dogs through the stimulation of robust antibody responses and expansion of memory T cell populations. Commercially available reagents and described techniques are limited for identifying and characterizing canine T cell subsets and evaluating T cell-specific effector function. To define reagents for delineating naïve versus activated T cells and identify antigen-specific T cells, we tested anti-human and anti-bovine T-cell specific cell surface marker reagents for cross-reactivity with canine peripheral blood mononuclear cells (PBMCs. Both $CD4^+$ and $CD8^+$ T cells from healthy canine donors showed reactivity to CCL19-Ig, a CCR7 ligand, and coexpression with CD62L. An *in vitro* stimulation with concanavalin A validated downregulation of CCR7 and CD62L expression on stimulated healthy control PBMCs, consistent with an activated T cell phenotype. Anti-IFNγ antibodies identified antigen-specific IFNγ-producing $CD4^+$ and $CD8^+$ T cells upon *in vitro* vaccine antigen PBMC stimulation. PBMC isolation within 24 hours of sample collection allowed for efficient cell recovery and accurate T cell effector function characterization. These data provide a reagent and techniques platform via flow cytometry for identifying canine T cell subsets and characterizing circulating antigen-specific canine T cells for potential use in diagnostic and field settings.

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Conflict of Interest Statement

The authors declare they have no competing interests.

Keywords

dog; T cell; CCR7; CD62L; flow cytometry; vaccine

2. Introduction

Domestication and tractability have allowed do gs to serve as research subjects for canine-specific diseases as well as models for human disorders. In particular, dogs serve as robust translational models in cardiovascular (Hohnloser et al., 2009), neoplastic (Khanna et al., 2006; Klopfleisch et al., 2010), immunological (Creevy et al., 2003; Marsella and Girolomoni, 2009), neurological (Awano et al., 2009; Selkoe et al., 1987), and genetic (Wilbe et al., 2010) research studies. Canines are also susceptible to and serve as models of zoonotic diseases such as leishmaniasis and American trypanosomiasis and hence used to evaluate anti-parasitic chemotherapeutic regimens (Guedes et al., 2002). Routine vaccination in canines allows an opportunity to assess the development of an appropriate immunological response to foreign antigens. Techniques and commercially available reagents are scarce for studying the canine immune system, especially as compared to those available for humans. As basic research pursues translational applications in animals more physiologically similar to humans, and veterinary medicine strives for more individualized patient therapies, an increasing need exists for identifying, characterizing, and monitoring the canine immune response.

The First International Canine Leukocyte Antigen Workshop (CLAW) was a significant step in identifying canine homologs of human CD antigens that delineated leukocyte populations by monoclonal antibodies (Cobbold and Metcalfe, 1994). Clusters of antibodies collected from several sources identified canine equivalents of CD4, CD8, and Thy1.1 antigens from peripheral blood. Additional antibodies reactive to canine leukocyte antigens including CD45R (Aguiar et al., 2005) CD45RA (Caniatti et al., 1996), CD11/CD18 (Danilenko et al., 1992a; Moore et al., 1990), and CD62L (Crockett-Torabi and Fantone, 1997) and to platelet and erythrocyte antigens (Schuberth et al., 2007) have been described separately from the CLAW workshop. Testing of monoclonal antibodies specific for cytokines in other species have also identified IL-4-, IL-8-, and IFN-γ-producing canine PBMCs and expanded the repertoire of canine specific reagents (Pedersen et al., 2002). However, despite these advances, delineating and characterizing naïve, activated, and memory T cell subsets in canines has remained limited.

The aim of this project was to identify and validate immunological reagents for characterizing canine T cells through phenotypic and effector function evaluation-based assays. Detection of the canine cross-reactive CCL19-hIg, a ligand for CCR7, identified naïve and antigen-experienced but not recently activated canine T cells. CCR7 cell surface expression was consistent with CD62L, an L-selectin expressed by naïve and central memory T cells during homing to secondary lymphoid organs. Decreases in CCR7 and CD62L expression following antigen stimulation or mitogen activation correlated with upregulation of the activation marker, CTL2.58, and delineated activated T cells. IFNγ-production following PBMC whole vaccine stimulation defined antigen-specific T cell

effector function. Extended time between blood collection and PBMC isolation of up to twenty-four hours revealed no significant loss in identifying vaccine-specific IFNγ-producing T cells. These data provide a reagent platform for identifying and characterizing canine T cell populations and assessing antigen-specific effector function.

3. Materials and Methods

3.1. Animals and isolation of mononuclear cells

Approximately 40–50mls of blood from four clinically healthy adult (>3 years of age) mixed breed dogs were drawn into heparinized tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ, USA) by venipuncture. Isolation of peripheral blood mononuclear cells (PBMCs) occurred immediately following collection or as otherwise indicated and as previously described for human subjects (Albareda et al., 2009). PBMCs were washed in Hank's buffered balance salt solution (Mediatech Inc., Manassas, VA, USA) and resuspended in RPMI-1640 (Mediatech Inc.) completed with 50uM 2-β-mercaptoethanol, 2mM L-glutamine, 25μg/mL gentamicin, 200U/mL penicillin (Mediatech Inc), 2μg/mL streptomycin (Mediatech Inc), 1mM sodium pyruvate, and 10% heat-inactivated (30min, 56°C) and aggregate-removed (800gx30min) fetal calf serum (HI-FCS) (HyClone Laboratories, ThermoScientific, Logan, UT, USA). Resuspended cells were frozen in media containing 10% dimethyl sulfoxide (Acros Organics, Fair Lawn, NJ, USA) in liquid nitrogen for long-term storage. Prior to use, PBMCs were recovered, thawed at 37°C, washed and resuspended in complete RPMI-1640 + 10% HI-FCS. These purification, storage, and recovery procedures consistently yielded >95% viability, as determined by microscopic examination of trypan blue dye exclusion. All animal use protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

3.2. PBMC antibody reactivity

For testing canine T cells antibody reactivity, a minimum of 2×10^5 PBMCs, were stained at 1:50 antibody dilution in PBS containing 1% BSA and 0.05% sodium azide (PAB; both from Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 45 min. Antibodies tested were those defined in Table 1. Cells were fixed in 2% formaldehyde prior to flow cytometric collection.

3.3. T cell stimulation and proliferation assessment assays

A total of 4×10^5 PBMCs were stimulated in a 96-well flat-bottom tissue culture plate (Costar, Corning, NY, USA) at 37°C in the presence of media, 15μg/mL anti-canine CD3 (AbD Serotec, Raleigh, NC, USA), or 0.25μg/mL concanavalin A (Sigma-Aldrich) for the indicated days. For assessment of proliferation, PBMCs were washed twice with PBS, incubated with 5μM CFSE (Molecular Probes, Eugene, OR), quenched with FCS, and plated. Two days post-stimulation, centrifuged and washed cells were incubated with or without CCL19-hIg (ELC; (Hargreaves et al., 2001)) supernatant at 4°C for 45 min. Antibodies used were anti-CD8-Pacific Blue, anti-CD62L-PE (AbD Serotec), anti-human IgG-AF488 (Molecular Probes), and a cocktail containing anti-dog Pan T cell-APC, anti-B cell-PE, and anti-dog T cell Activation marker-FITC (Dog Activated T Lymphocyte Cocktail, BD Pharmingen, BD Biosciences, San Jose, CA). 7-amino-actinomycin D (7AAD;

BD Pharmingen) was included for live/dead cell discrimination. Cells were stained in PAB for 45 min at 4°C, washed, and fixed in 2% formaldehyde.

3.4 Intracellular cytokine staining

For assaying IFNγ levels, 4×10^5 PBMCs were stimulated for 5hr in the presence of 2ng/mL phorbol 12-myristate 13-acetate (PMA), 4μg/mL Ca^{2+} ionomycin (Sigma-Aldrich), and brefeldin A (BD GolgiPlug; BD Biosciences) (Pedersen et al., 2002). For polyclonal activation, PBMCs were plated with 15μg/mL anti-CD3 (AbD Serotec) or diluted whole vaccine antigens, incubated overnight at 37°C and brefeldin A added 5hr prior to end of incubation. Canine vaccines were IMRAB 3TF (Rabies; Merial, Athens, GA, USA), Duramune 5 (Canine distemper-Adenovirus Type 2-Parainfluenza-Parvovirus (DAPP); Fort Dodge Animal Health, Fort Dodge, IA, USA), and Leptovax 4 (Leptospirosis bacterial extract; Fort Dodge Animal Health) vaccines. Cells were stained with anti-CD8-Pacific Blue and anti-CD4-FITC (AbD Serotec) followed by intracellular staining with anti-bovine anti-IFN-γ AF647 (AbD Serotec) according to the BD Cytofix/Cytoperm kit (BD Biosciences).

3.5 Flow Cytometry and Analysis

A minimum of 250,000 events were collected for each sample on a CyAn ADP using Summit, version 4.3 (Beckman Coulter, Fullerton, CA, USA). Where appropriate, fluorescence minus one controls were used to establish gating measures. FlowJo flow cytometry analysis software, version 9 (Tree Star, Ashland, OR, USA) was used for analyses.

4. Results

As $CD4^+$ and $CD8^+$ T cells play critical roles in various infections, our primary efforts focused on identifying T cells and discriminatory surface markers for naïve, activated, and memory T cells subsets. A reagent panel used for testing of cross-reactivity with canine PBMCs included antibodies purchased commercially or kindly provided by other investigators (Table 1). A reagent was judged as positive if reactive with >2% of PBMCs. Utilizing this strategy, fifteen of twenty-six reagents were cross-reactive with canine PBMCs as denoted in Table 1, with eleven of twelve (91.7%) previously referenced reactive antibodies confirmed in this study. If a reagent distinguished distinct cell populations individually or following incubation in a T cell stimulation protocol, the reagent was found to be discriminatory, as noted in Table 1.

For antibodies recognizing putative cell surface markers on T cells, we repeated staining to validate marker expression on $CD4^+$ and $CD8^+$ T cells. Canine T cell reactivity was found with CCL19-Ig (ELC), a fusion protein serving as a chemokine ligand of CCR7 (Hargreaves et al., 2001) and previously described to have reactivity with naïve and central memory ovine T cells (Debes et al., 2005). As circulating T cells traffic from the blood and home to lymph nodes, naïve and central memory T cells express CCR7 and other adhesion molecules, like L-selectin (CD62L) and LFA-1, to tether and arrest to endothelium for transmigration across high endothelial venules. To investigate if circulating canine $CD4^+$ and $CD8^+$ T cells express both CCR7 and L-selectin (CD62L), PBMCs were stained with

anti-CD4, anti-CD8, CCL19-Ig, and anti-CD62L. In clinically healthy canine donors, $CD4^+$ and $CD8^+$ T cells expressed measureable levels of CCR7 (Fig 1A). Unlike CCR7 expression in human $CD8^+$ T cells (Campbell et al., 2001), CCR7 expression by canine $CD8^+$ T cells varied greatly between individual dogs. In comparison to humans and sheep where >85% of $CD4^+$ T cells express CCR7 (Campbell et al., 2001; Debes et al., 2005), approximately 40–50% of canine $CD4^+$ T cells from healthy donors expressed CCR7. The observed difference is likely explained by the decreased ability of CCL19-hIg to delineate distinct peaks associated with $CCR7^+$ and $CCR7^-$ canine $CD4^+$ T cells populations (Fig 1A) versus a difference in species or health status of the hosts. Exploration of alternative secondary fluorophore combinations yielded similar $CCR7^+CD4^+$ T cell percentages (not shown). Both canine $CD4^+$ and $CD8^+$ T cells also showed reactivity to the lymph node homing marker CD62L (Fig 1B) and this expression agreed with that of CCR7 (Fig 1C), as the predominance of $CD4^+CD62L^{hi}$ T cells expressed CCR7, results consistent with that observed in human peripheral blood (Campbell et al., 2001). Taken together, the cell surface markers of CCR7 and CD62L identified canine $CD4^+$ and $CD8^+$ T cell populations consistent with the phenotype of naïve or central memory T cells.

To evaluate if CCR7 a nd other cross-reactive antibodies identified in Table 3.1 were discriminatory for naïve versus activated T cells, a method and assay for T cell activation was used. For this purpose, CFSE-labeled canine PBMCs were incubated in media alone or with the T cell mitogen concanavalin A (ConA), and stimulation and proliferation was evaluated by changes in cell size and CFSE dilution. A comparison of forward versus side scatter revealed PBMC stimulated with ConA were on average volumetrically larger than unstimulated cells, consistent with cellular activation (Fig 2A). CFSE dilution indicated cell division of ConA-stimulated $CD8^+$ T cells as early as three days post stimulation (Fig 2B).

As we proposed the CCR7 and CD62L reagents identified naïve and central memory canine T cells, we hypothesized that following T cell activation canine T cells would decrease expression of CCR7 and CD62L. To address this hypothesis, canine PBMCs were cultured in the presence of anti-CD3, ConA, or media alone. Even before detectable proliferation by CFSE dilution two days post stimulation (Fig 3A), $CD8^+$ T cells expressed less CCR7 (Fig 3B) and CD62L (Fig 3C) when activated. Expression levels of these two markers were consistent with the strength of stimulus, as ConAstimulated $CD8^+$ T cells expressed less CCR7 and CD62L than cells stimulated with anti-CD3. Co-expression of both CCR7 and CD62L also decreased in stimulated cells, with ConA-stimulated cells showing greater decreases than cells incubated with anti-CD3 (Fig 3D). Down-regulation of CCR7 and CD62L cell surface expression further correlated with increased expression of the activation marker ($CTL2.58^+$) on stimulated T cells (Fig 3E) and $CD8^+$ T cells (Fig 3F). These experiments identified markers for defining activated $CTL2.58^+CD8^+$ T cells and naïve or central memory-like $CCR7^+CD62L^{hi}CD8^+$ T cells in dogs. Testing of other canine T cell reactive reagents listed in Table 1, including CD45RA, CD45RO, and CD11a, in media versus stimulatory conditions did not alter antibody T cell reactivity in our stimulation assay or were not detectable using secondary antibody reagents and therefore were not investigated further. These results are noted as “not discriminatory” in Table 1.

Cognate antigen recognition through the TCR results in effector cytokine production, and thereby provides a method for evaluating and delineating antigen-specific T cells. To assay the production of the effector cytokine IFNγ by canine T cells in response to previously seen antigens, PBMCs were isolated from three healthy dogs vaccinated three weeks prior with IMRAB 3TF (Rabies), Duramune 5 (Canine distemper-Adenovirus Type 2-Parainfluenza-Parvovirus (DAPP)), and Leptovax 4 (Leptospirosis bacterial extract) vaccines. Following incubation of cells with media or vaccine antigen overnight, intracellular IFNγ staining was performed to identify vaccine-specific $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells from three of three dogs produced IFNγ in response to rabies vaccine, whereas only one of three had IFNγ-producing cells when stimulated with DAPP vaccine antigens (Fig 4A). $CD8^+$ T cells from two of three dogs produced IFNγ in response to rabies vaccine, but all appeared to not be significantly different from media controls when stimulated with the DAPP vaccine (Fig 4B). Despite differences in vaccine sources and stimulus concentrations, the low levels of detectable of IFNγ-producing T cells following canine distemper vaccine (DAPP) stimulation were consistent with previously published ELISAs of stimulated PBMC culture supernatants (Valli et al., 2010). Both $CD4^+$ and $CD8^+$ T cell subsets failed to produce robust IFNγ in response to the Leptovax 4 vaccine, suggesting this vaccine failed to elicit a strong T cell specific immune response three weeks post administration, consistent with the predominant humoral response elicited by the bacterial antigens of the leptospiral vaccine (Adler and Faine, 1977). These results provide a flow cytometric method for identifying antigen-specific $CD4^+$ and $CD8^+$ T cells and evaluating IFNγ production for individual canine T cells.

In fieldwork settings, circumstances may arise where PBMC isolation will occur hours following sample collection, as is the case in field sampling. We asked how this timing would affect sample quality and ability to identify antigen-specific and cytokine-producing T cells. To address this question, we determined recovery and effector function of T cells isolated at various times post-blood collection. Incubation of cells with media or PMA/Ca^{2+} ionomycin followed by intracellular cytokine staining revealed no significant loss in quantities or percentages of IFNγ-producing T cells isolated up to 24 hours post collection (Fig 5). In PBMCs purified 48 hours following venipuncture, a five-fold reduction in recovery of both $CD4^+$ and $CD8^+$ T cell as compared to the 24 hours post collection time point was observed. Additionally, unstimulated $CD4^+$ T cells produced significantly higher levels of IFNγ. These data provide an optimal method for PBMC isolation for application to fieldwork settings and for accurate evaluation of T cell effector function.

5. Discussion and Conclusion

T cells play a central role in the initiation and regulation of the immune response. T cells modulate cell surface expression of ligands and receptors to execute effector function and communicate with the environment. T-cell receptor complex signaling and CD28 co-stimulation results in naïve T cells downregulating lymph node homing receptors (CD62L and CCR7) while upregulating expression of cell adhesion molecules (CD44), T-cell receptor signaling (CD45RO), and early activation (CD69) antigens. Reliable expression of these cell surface molecules following cognate antigen encounter affords T cell monitoring during disease processes. Diagnosis and progression of human T cell lymphoproliferative

disorders have been examine through flow cytometic immunophenotyping. Similar individualized diagnostics are being pursued for veterinary patients, but the paucity of literature describing the detection and characterization of antigen-driven T cell responses limits reaching this goal.

To address the growing need of canine immune system characterization for individualized diagnoses and toward furthering dogs as suitable translational research models, we aimed to identify and validate canine-specific reagents for discriminating naïve versus activated T cells. Novel canine T cell reactivity of CCL19-Ig defined CCR7 expression in $CD4^+$ and $CD8^+$ T cells of healthy donors. CCR7 co-expression with CD62L and decreased expression following stimulation validated these markers for defining naïve or central memory $CD8^+$ T cells. The activation marker CTL2.58 further delineated activated T cells. Combining these reagents into a single panel would allow for identification of naïve or central memory T cells as $CCR7^+CD62L^{hi}CTL2.58^-$ and activated T cells as $CCR7^-CD62L^{lo}CTL2.58^+$. To our knowledge, these results are the first to describe and validate these markers for identifying naïve and activated canine T cell subsets.

With cognate antigen recognition stimulating cytokine-mediated T cell effector function, identification of IFNγ-producing T cells is critical for evaluating appropriate T cell responses. Monitoring antigen-specific T cell responses induction is a critical component for evaluating efficacy of T cell-mediated vaccines, especially in the era of canine oral malignant melanoma DNA vaccines (Grosenbaugh et al., 2011) where cytotoxic T cells would be proposed to mediate protection. Here, we described a method for defining vaccine-specific $CD4^+$ and $CD8^+$ T cells through intracellular cytokine staining following rabies, canine distemper-adenovirus-parainfluenza-parvovirus, and leptospirosis bacterial extract vaccination. Determining induction, kinetics, and effector function of these antigen-specific T cells are ideal for evaluating appropriate and protective vaccine responses and defining goals for future vaccines, where T cells are primary mediators of immune control for the targeted disease.

In instances where immediate PMBC isolation may be unavailable due to geographical location, evaluating appropriate storage conditions, subsequent cell isolation, and cytokine production following extended times post blood sampling is critical. For example, immunophenotyping of lymphoma of a dog living in a rural area or research studies involving characterization of T cell responses of dogs living in an international locale would likely require significant time between blood collection and PBMC isolation and storage. Data herein illustrates these canine subjects can be examined and immune responses characterized, expanding the translational application of this study beyond laboratory settings. These techniques and reagents are powerful in scenarios where human vaccination efforts would be unethical or inaccessible and dogs serve as models of natural infection and appropriate targets for control efforts, such as American trypanosomiasis (Padilla et al., 2010).

In conclusion, limited resources and techniques for phenotyping and characterizing canine T cells have restricted canine adaptive immune system studies. The tools defined here provide a platform for defining T cell subsets and identifying circulating antigen-specific T cells.

The impact of this work for improving canine health and facilitating the translational applications of dogs for human disease modeling is promising. As individualized T cell immunophenotyping becomes more accessible for human patients, application to veterinary medicine and animal patients is encouraging.

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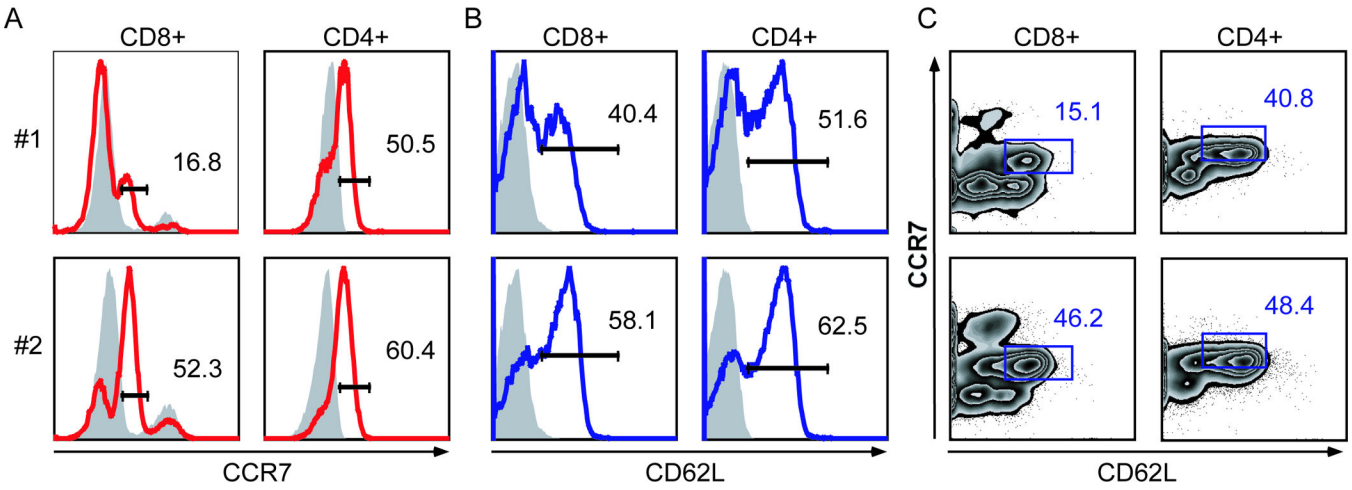

Figure 1. Co-expression of CCR7 and CD62L on canine CD8$^+$ and CD4$^+$ T cells

Canine PBMCs from two clinically healthy dogs (#1 and #2) were incubated for 1 hour at 37°C and stained with or without CCL19-hIg (ELC), a CCR7 ligand. Cells were stained with anti-CD8, anti-CD4, anti-CD62L, anti-human secondary antibody, and 7AAD. Percentages of CD8$^+$7AAD$^-$ and CD4$^+$7AAD$^-$ T cells expressing CCR7 (A) and CD62L (B). Shaded lines are fluorescence-minus-one (FMO) controls for the indicated marker. C, Percentages of CD8$^+$7AAD$^-$ or CD4$^+$7AAD$^-$ T cells co-expressing CCR7 and CD62L. Gating strategies are determined by FMO controls, especially noting the highest CCR7 peak present in experimental and FMO control samples is due to nonspecific secondary antibody binding.

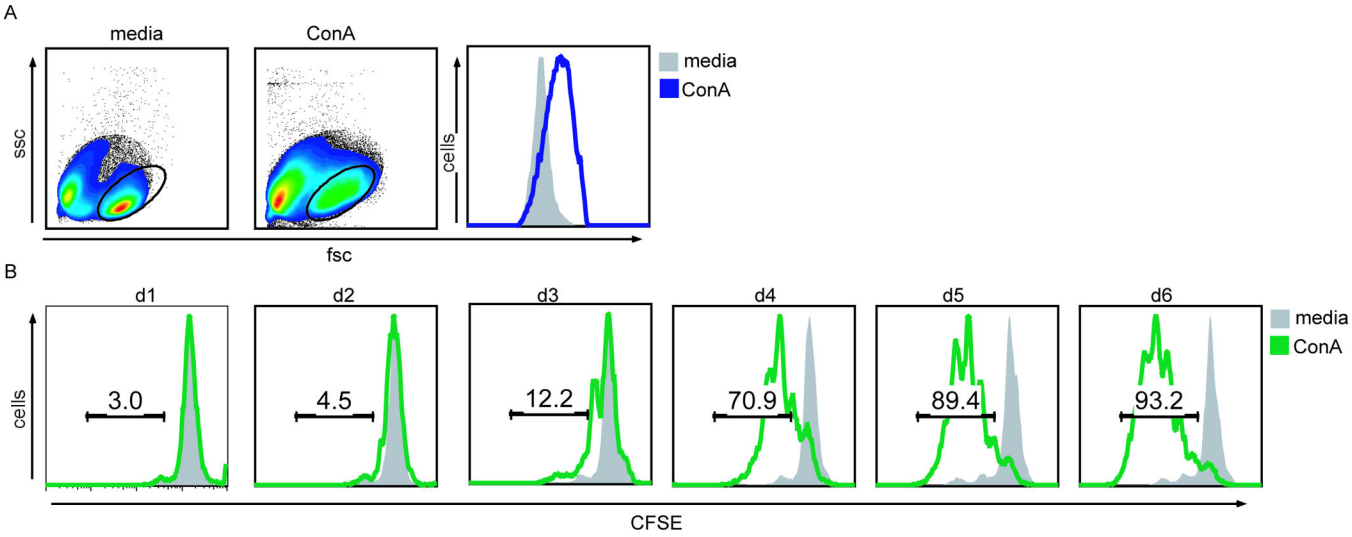

Figure 2. Measuring stimulation and proliferation using CFSE time-series data

A, Forward scatter (fsc) versus side scatter plots (ssc) of CFSE-labeled PBMCs incubated with media or concavalin A (ConA) for 4 days. Gated cells are displayed in the fsc histogram, comparing ConA-stimulation (blue line) to media alone (grey). B, CFSE-labeled PBMCs were harvested on the indicated days and stained with 7AAD and anti-CD8 antibodies. Histograms represent percentages of $CD8^{+}7AAD^{-}$ T cells with CFSE dilution following incubation with media (grey shaded) versus ConA (green line).

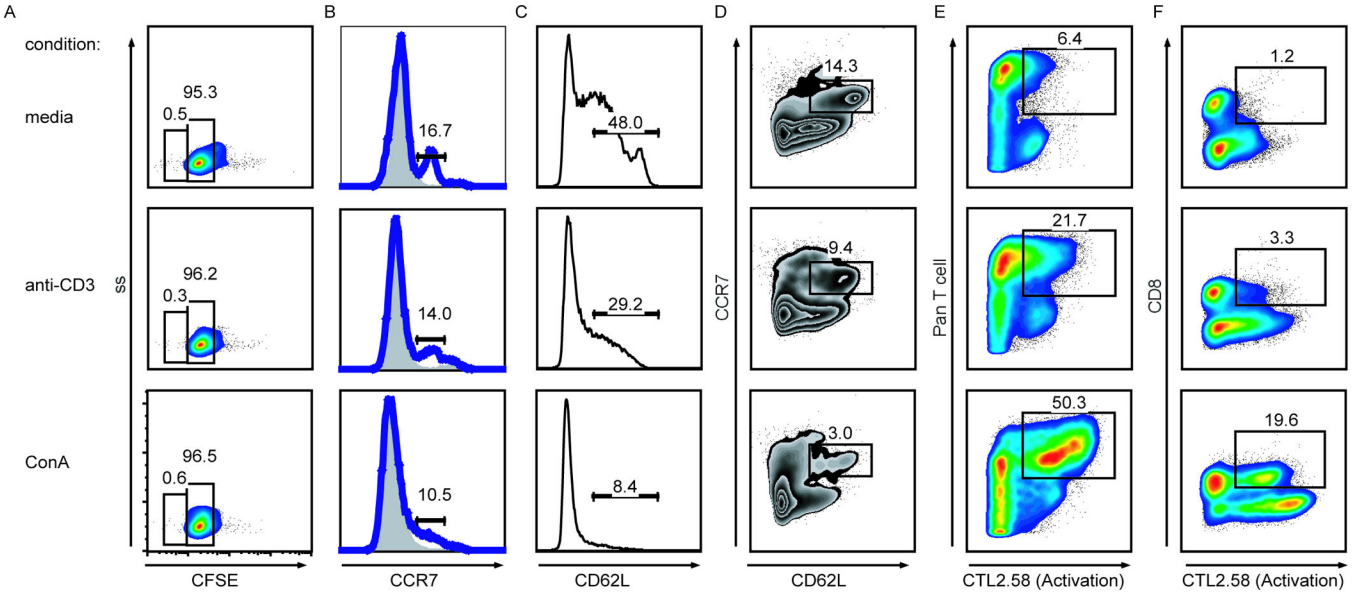

Figure 3. CCR7, CD62L, and CTL2.58 expression of canine PBMCs in stimulation conditions

Canine PBMCs incubated two days with media, anti-CD3, or ConA, harvested, and stained with various antibody combinations. A, Proliferation of CFSE-labeled $CD8^+$ T cells. B–D, PBMCs incubated with (blue line) and without (grey shaded) CCL19-hIg and stained with anti-human secondary antibody and anti-CD62L. Histograms of CCR7 (B) and CD62L (C) expression of $CD8^+$ T cells. D, Percentages of $CD8^+$ T cells co-expressing CCR7 and CD62L. E–F, PBMCs stained with the dog activated T lymphocyte antibody cocktail, 7AAD, and anti-CD8. Percentages of stimulated $PanT^+$ (E) and $CD8^+$ (F) T cells reactive to the CTL2.58 antibody, an activation marker.

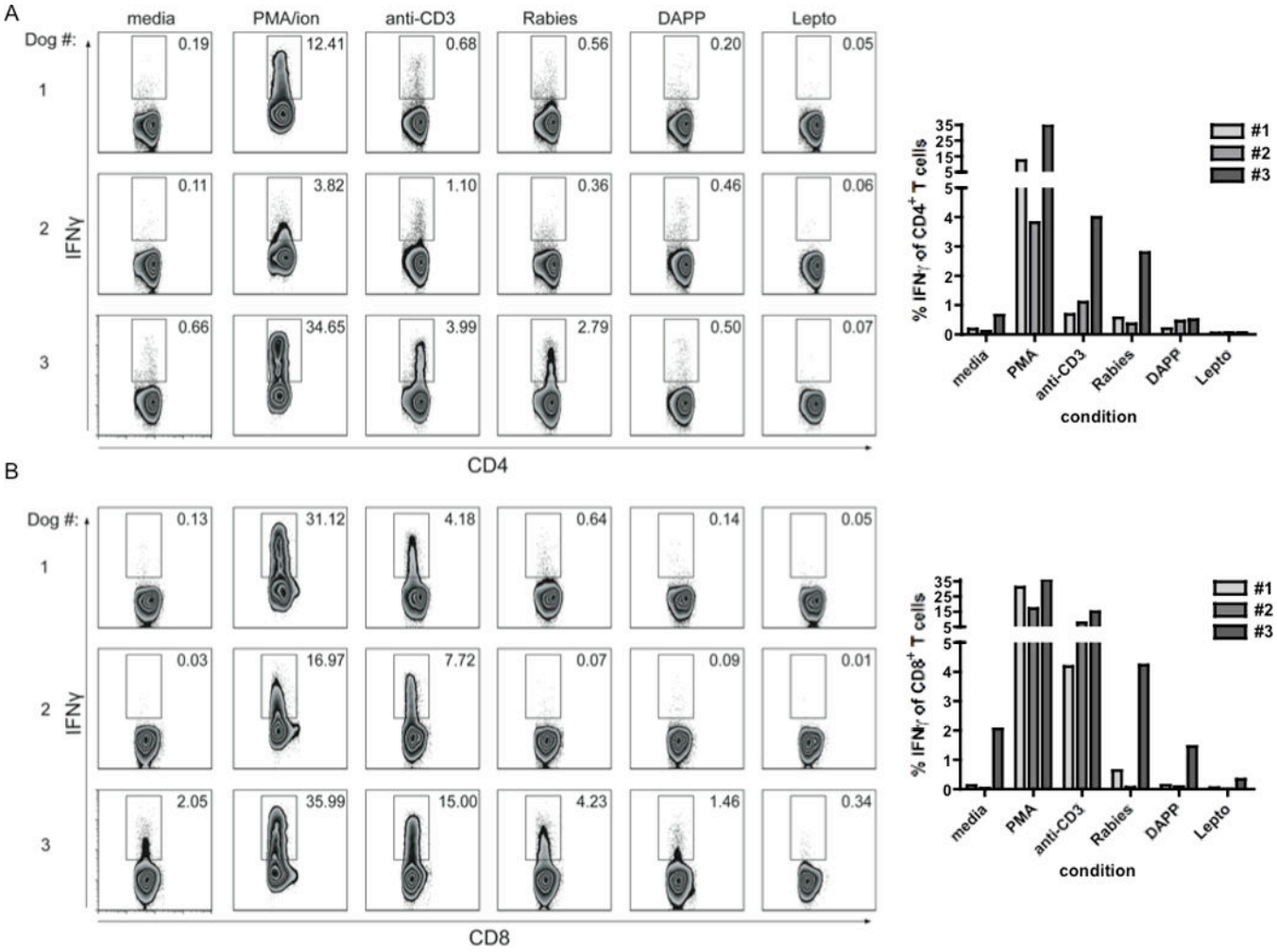

Figure 4. IFNγ production by canine CD4$^+$ and CD8$^+$ T cells upon stimulation with vaccine antigens

PBMCs collected from three dogs three weeks post vaccination with IMRAB 3TF (Rabies), Duramune 5 (DAPP), and Leptovax 4 (Lepto) were incubated with media, PMA/Ca^{2+} ionomycin, and vaccines. GolgiPlug was added 5hr prior to end of incubation. Cells were stained with anti-CD8, anti-CD4, and anti-bovine IFNγ. A–B, Numbers indicate percentages of CD4$^+$ (A) and CD8$^+$ (B) T cells producing cytokine in each condition.

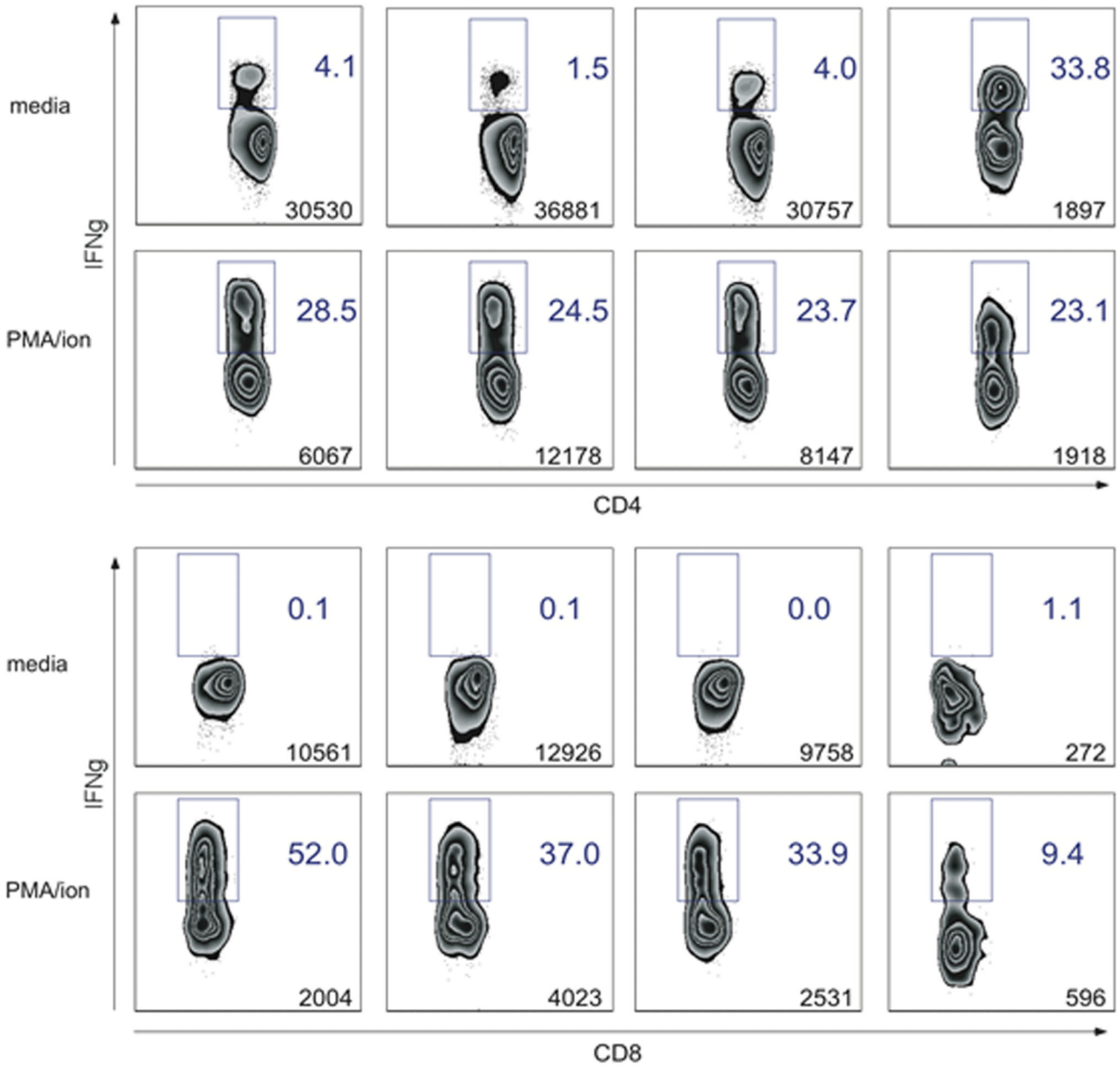

Figure 5. Viability and effector function of canine CD4$^+$ and CD8$^+$ T cells measured over a 48hr period

Canine blood was collected, incubated on ice, and PBMCs isolated at the indicated time points. PBMCs were incubated at 37°C with PMA/ionomycin or media alone in the presence of GolgiPlug for 5 hours. Cells were stained with anti-CD8 and anti-CD4, permeabilized, and stained for IFNγ. Numbers indicate percentage (gated) and count (bottom right) of CD8$^+$ (A) and CD4$^+$ (B) T cells producing IFNγ at each isolation time point.

Table 1

Reagent panel used to study canine peripheral mononuclear cells.

Antigen	Antibody	Host species	Target species	Source (citation)	Reagent specificity	Dog PBMC reactivity	Reactivity in this study	Discriminatory
Leukocyte cell lineages								
CD3	CA17.2A12	mouse	dog	AbD Serotec (Byrne et al., 2000)	T cells	(Byrne et al., 2000)	+	n.d.
CD11a	CA11.4D3	mouse	dog	AbD Serotec (Cobbold and Metcalfe, 1994; Danilenko et al., 1992b)	integrin, all leukocytes	(Cobbold and Metcalfe, 1994)	−	no
CD11c	BU15	mouse	human	AbD Serotec	dendritic cells, monocytes, macrophages, neutrophils	(Trowald-Wigh et al., 1993)	+	n.d.
Lymphocyte subsets								
B cell	LSM11.425	mouse	dog	BD Pharminogen (Cobbold and Metcalfe, 1994; Gebhard and Carter, 1992; Ruslander et al., 1997)	B cells	(Cobbold and Metcalfe, 1994)	+	yes
Pan T cell	LSM8.358	mouse	dog	BD Pharminogen (Cobbold and Metcalfe, 1994)	T cells	(Cobbold and Metcalfe, 1994)	+	yes
CD4	YKIX302.9	rat	dog	AbD Serotec (Cobbold and Metcalfe, 1994)	$CD4^+$ T cells	(Cobbold and Metcalfe, 1994)	+	yes
CD4	RPA-T4	mouse	human	BioLegend (Schlossman et al., 1995)	$CD4^+$ T cells		−	
CD8	YCATE55.9	rat	dog	AbD Serotec (Cobbold and Metcalfe, 1994)	$CD8^+$ T cells	(Cobbold and Metcalfe, 1994)	+	yes
CD8a	RPA-T8	mouse	human	BioLegend (Schlossman et al., 1995)	$CD8^+$ T cells		−	
T cell-specific antigens								
T cell activation	CTL2.58	mouse	dog	BD Pharminogen (Ruslander et al., 1997)	activated T cells		+	yes
CD25	2A3	mouse	human	BD Biosciences (Urdal et al., 1984)	activated & regulatory T cells, activated B cells		−	
CD27	M-T271	mouse	human	BD Pharminogen (Bigler et al., 1988)	tumor necrosis factor R'; T cell costimulatory molecule		−	
CD28	CD28.2	mouse	human	BD Pharminogen (Verwilghen et al., 1993)	T cell costimulatory molecule, required for T cell activation		−	
CD44	IM7	rat	mouse	AbD Serotec (McKallip et al., 2002)	activated effector-memory T cells	(Cobbold and Metcalfe, 1994)	+	no
CD45RA	HI100	mouse	human	BioLegend (Iannello et al., 2010)	naïve T cells		−	
CD45RA	CA4.ID3	mouse	dog	Abd Serotec (Caniatti et al., 1996; Cobbold and Metcalfe, 1994)	naïve T cells	(Moreno et al., 1999)	+	no
CD45RO	UCHL1	mouse	human	Beckman Coulter	memory T cells	(Galkowska et al., 1996)	+	no

Antigen	Antibody	Host species	Target species	Source (citation)	Reagent specificity	Dog PBMC reactivity	Reactivity in this study	Discriminatory
CD62L	FMC46	mouse	human	AbD Serotec (Pilarski et al., 1991)	lymphocyte homing, naïve & memory T cells	(Schuberth et al., 2007)	+	yes
CD62L	DREG-56	mouse	human	BD Pharminogen (Kishimoto et al., 1990)	lymphocyte homing, naïve, & memory T cells		−	
CD127	HIL-7R-M2	mouse	human	BD Pharminogen (Goodwin et al., 1990)	IL-7 R' subunit		−	
CD197 (CCR7)	3D12	rat	human	BD Pharminogen (Sallusto et al., 1999)	secondary lymphoid organ entry R'		−	
CCL19-hIg	ELC		human	(Hargreaves et al., 2001)	chemokine ligand for CCR7 on T cells for secondary lymphoid organ migration		+	yes
Other antigens, receptors, and cytokines								
CD85j	GHI/75	mouse	human	BD Pharminogen (Colonna et al., 1997)	leukocyte R' providing inhibitory signals to APCs		−	
HLA-ABC	09HHLA01E			Chemicon Australia (Murakawa et al., 2000)	human leukocyte antigen MHCI		+	n.d.
CX3CR1		rabbit	human	Torrey Pines Biolab	chemokine R' expressed by activated T cells (T_{H1}), natural killer, epithelial & endothelial cells		+	n.d.
Interferon-γ	CC302	mouse	bovine	AbD Serotec (Fellman et al., 2011; Pedersen et al., 2002)	natural killer & T cell effector cytokine	(Fellman et al., 2011; Pedersen et al., 2002)	+	yes

Abbreviations: MHCI, major histocompatibility complex I; R', receptor; APC, antigen presenting cell.