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A Novel Monoclonal Antibody Specific for Canine CD25 (P4A10): Selection and Evaluation of Canine Tregs

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Abstract

A monoclonal antibody (mAb), P4A10, was made to the canine interleukin-2 receptor alpha chain (IL-2R α ; p55; Tac antigen; CD25) to facilitate studies of canine regulatory T-cells (Treg). By non-reduced western blot, P4A10 bound to a 55 kD protein, the expected size of IL-2R α . In flow cytometry assays, it reacted with a minor population of circulating dog CD3⁺CD4⁺ T cells and the majority (>60%) of *in vitro* PMA-Ionomycin (PMA-IO)-activated canine CD3⁺ T-cells. P4A10 recognized a hematopoietic cell population enriched for FoxP3⁺ cells as measured by flow cytometry. The P4A10-selected fractions of T-cells had significantly increased copy numbers of CD25, FoxP3, IL-10, and TGF β as detected by RT-PCR (reverse transcriptase PCR) compared to the negative fractions. The P4A10-selected cells inhibited ³H (tritiated) thymidine incorporation in a mixed leukocyte reaction (MLR) containing responders of the same origin. P4A10-selected T cells from fresh peripheral blood mononuclear cells had less FoxP3 ($p = 0.07$) by qRT-PCR (quantitative RT-PCR) and were less suppressive ($p=0.01$) than *in vitro* alloantigen-activated Treg. The mAb P4A10 is specific for canine CD25 and can be used to facilitate studies of CD25⁺FoxP3⁺ Treg in this clinically relevant large animal model.

Keywords

Monoclonal antibody; CD25; regulatory T cells; dog

1. Introduction

Monoclonal antibodies (mAb) specific to the IL-2R α protein (CD25) have been useful tools for studies of CD4⁺CD25⁺FoxP3⁺ regulatory T-cells (Treg) in mice (Hori *et al*, 2003; Sakaguchi *et al*, 1995), rats (Nolte-'t Hoen *et al*, 2008), cats (Lankford *et al*, 2008), pigs (Kaser

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Conflicts of Interest Statement:

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et al, 2008), monkeys (Gansuud *et al*, 2007) and men (Arias *et al*, 2007; Walker *et al*, 2003; Walker *et al*, 2005). Although Tregs have been identified in dogs via intracellular staining of the Treg-associated protein FoxP3 using FJK-16s (Biller *et al*, 2007), a cross-reactive anti-murine mAb, this technique does not allow for selection of viable cells. Two recent reports (Galkowska *et al*, 1996; Masuda & Yasuda, 2008) of a cross-reactive human CD25-specific mAb, ACT-1, on activated dog T-cells noted that the binding was of intermediate affinity and ACT-1 was not specifically shown to bind to Treg. ACT-1, however, has remained largely uncharacterized (Schweighoffer *et al*, 1993). This antibody had not been clustered by an HLDA Workshop, the immunogen consisted of many possible antigens, the binding was of intermediate affinity on dog cells, and the development and characterization were not published. Therefore, to facilitate studies of Treg in the canine model of hematopoietic cell and organ transplantation, we developed and characterized a mAb specific to canine CD25 (P4A10). The mAb P4A10 bound to an approximately 55 kD protein by non-reduced Western blot and in flow cytometry experiments identified a subset of CD3⁺CD4⁺ T-cells enriched for FoxP3. Positive selection of cells from canine PBMC which bound the P4A10 mAb resulted in a cell population that was enriched with cDNA for CD25, FoxP3, IL-10 and TGFβ. Additionally, we showed that these P4A10-selected cells had a functional phenotype consistent with Treg as they inhibited the uptake of ³H (tritiated) thymidine in MLR.

2. Methods

2.1. Animals

Dogs were male and female beagle and beagle mix (basenji, golden retriever or mini-mongrel) ranging from 7 to 61 months old (median 16.5 months) and were obtained from the Animal Health Resources at the Fred Hutchinson Cancer Research Center (an AAALAC-certified facility). Adult male mice used for immunization were from Jackson Labs (stock number 00726). All blood draws and antibody production were done with IRO/IACUC approval.

2.2. Cloning

Monoclonal P4A10 was obtained via an adaptation of the protocol described by Oi and Herzenberg (Oi & Herzenberg, 1980). Our method makes use of the Robertsonian (Rb [8.12] 5 Bnr) mouse strain (RBF/DnJ) in conjunction with the FOX-NY myeloma cell line described by Taggart and Samloff (Taggart & Samloff, 1983) and has been published (Wayner & Hoffstrom, 2007). In particular, an enriched, stimulated-T-cell immunogen was prepared with PBMC obtained from heparinized, normal dog blood. The buffy coat from a 1.074 Ficoll density gradient was washed twice with Hank's to remove platelets, and then monocytes were removed by adherence to plastic with the remaining T-cell-enriched mononuclear cells cultured in complete dog medium (CDM is 45% Waymouth's, 45% Iscove's, 10% heat inactivated, pooled, normal fasted male dog sera, and supplemented with sodium pyruvate, glutamine, non-essential amino acids, penicillin and streptomycin; Invitrogen-Gibco) containing 10μg/ml ConA (Sigma-Aldrich). After three days, cells were harvested, washed to remove serum, and a whole, live cellular immunogen was injected i.p. into two mice. The mice were boosted after 6 weeks with freshly prepared cells, and three days later, spleens were removed to obtain splenocytes for hybridoma creation. Resultant antibody-producing hybridomas were screened by flow cytometry of CD3-gated PBMC from freshly drawn dog blood compared to that same dog's 3-day ConA-activated CD3-gated PBMC. Cells were first incubated in tissue culture supernatant, washed with HH (Hank's buffered salt solution [Invitrogen] with 2% heat inactivated normal horse serum [Invitrogen] and 0.02% sodium azide [Sigma-Aldrich]), and then incubated with goat-anti-mouse Ig-FITC at 1:500 (Jackson ImmunoResearch) in HH. Cells were washed again and incubated with a biotinylated (BiotinTag; Sigma-Aldrich) CD3 (clone 17.6B3 or 17.6F9 from Peter Moore; UC Davis) in HH followed by another wash step with HH and incubation in HH with 1:100 streptavidin-PE (BD Immunocytometry Systems).

After a last wash step in HH, cells were resuspended in HH and acquired by 2-color flow cytometry (FACScan; BD Immunocytometry Systems). Hybridomas producing antibodies that were reactive with most or all activated, but none or few resting, T-cells were cloned by limiting dilution and re-tested.

2.3. Western blot

Washed canine PBMC obtained via Ficolling 30 cc of dog blood were resuspended in 50 cc of CDM containing PMA and Ionomycin (IO) (at 100 ng/ml and 1 µg/ml, respectively; Sigma-Aldrich). Cells were cultured overnight in an upright T-75 flask (BD-Falcon) in 5% CO₂ at 37°C. Non-adherent cells were decanted and pelleted at 300 rcf for 10 minutes, and then the pellet was resuspended with 50 cc of cold Hank's balanced salt solution and re-spun. Wash solution was aspirated and the remaining pellet was lysed with 500 µl of M-PER Reagent (Pierce) containing 0.1 mg/ml PMSF (Sigma-Aldrich) on ice for 10 minutes. Cells were pelleted at 4°C in a microcentrifuge at 13,000 rpm for 30 minutes, and the supernatant was transferred to a new tube. Protein concentration of the lysate was determined with a BCA Kit (Pierce) and 10 µg/lane was heated for 5 minutes at 95°C in Lane Marker Sample Buffer (Pierce) then used for 10% PAGE (NuPAGE; Invitrogen) with a pre-stained protein marker (BenchMark; Invitrogen). The gel was electroblotted (Sambrook *et al*, 1989) onto a reinforced nitrocellulose membrane (Hybond-N; Amersham). The blot was blocked for 30 minutes in 5% non-fat dried milk (Carnation) in PBS (Invitrogen) then cut in half. One part was incubated with an IgG₁ negative control Mab and the other with P4A10; each antibody was diluted 1:500 (2 µg/ml) in blocking buffer. After washing with 0.1% Tween-20 (Sigma-Aldrich) in PBS (PBST), antibody bound to antigen was detected with a 30-minute incubation with a biotin-conjugated anti-mouse Ig diluted 1:200 in PBST. The blots were washed in PBST, and then a streptavidin-peroxidase complex (ABC Method; Vector Laboratories) was attached for 30 minutes. After a final wash in PBST, visualization was achieved via an enzymatic reaction employing a BCIP/NBT solution (1-Step NBT/BCIP; Pierce) and stopped by dunking the blots in water.

2.4. Flow cytometry

For identifying circulating cells, 100 µl of fresh heparinized dog blood was hemolyzed with 1 ml of ammonium chloride buffer (150 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM Na₂EDTA; Sigma-Aldrich) in 1.7 ml microcentrifuge tubes for ten minutes and then pelleted at 3,500 rcf for 1 minute in a microcentrifuge. Cell pellets were resuspended in 100 µl HH and probed for cell surface markers with ACT-1-PE (DAKO) and/or canine antibodies to CD3, CD4, CD8 (clones 1E4 and JD3, respectively; Peter Moore at UC Davis), and/or P4A10, then allowed to interact for 20 minutes at room temperature. Antibodies were conjugated with FITC (FHCRC Biologics), PE (Prozyme), Alexa dyes 647 or 488 (Invitrogen), or biotin. Cells probed with a biotinylated antibody were washed with HH and incubated with 1:100 streptavidin PerCP (BD Immuno-cytometry Systems) in HH as a second step reaction. After all antibodies and secondary reagents were attached, cells were washed with 600 µl of HH in a microcentrifuge with 3,500 rcf spins for 1 minute, resuspended in 300 µl of HH, and then transferred into flow cytometry tubes (Falcon) for acquisition of up to 6 parameters. For antibody competitions, PBMC were prepared from 10 ml of heparinized dog blood via a 1.074 Ficoll density gradient and plated in CDM onto a 6-well plate (Falcon) at 5 ml per well and one million cells per ml. Cells were activated by adding PMA-IO and used the next day in flow cytometry antibody competition assays. Cells were harvested into microcentrifuge tubes, washed, and resuspended in 100 µl HH at 1 million per ml. After incubating with antibodies, cells were washed and then transferred to flow cytometry tubes (Falcon) and read in HH on a flow cytometer (FACSCalibur) using the manufacturer's acquisition and analysis software (CellQuest; BD Immunocytometry Systems). For intracellular staining, a bulk MLR was prepared with PBMC from two dog leukocyte antigen (DLA) mismatched, unrelated dogs. Responders were incubated with gamma-irradiated stimulators for four days, which was previously found to be

optimal, (Lesnikova *et al*, 2006; Lesnikova *et al*, 2005) and then harvested and surface stained before intracellular staining with cross-species reactive FoxP3 clone 150D (Biolegend) using the manufacturer's kit and method. Specifically, after surface staining, the cells were pelleted in microcentrifuge tubes and resuspended in 500 μ l Fix/Perm solution for 20 minutes. Cells were washed once with 600 μ l HH, then again with 500 μ l Perm Buffer, and then resuspended in 500 μ l Perm Buffer and left at room temperature for 15 minutes. Cells were pelleted, resuspended in 200 μ l of Perm Buffer and redistributed as two aliquots of 100 μ l. One aliquot was stained with isotype control (IgG₁-PE or Alexa 647; BD Immunocytometry Systems) and one was stained with FoxP3 (PE or Alexa 647). After 30 minutes at room temperature cells were washed twice with 600 μ l HH per wash and resuspended in 500 μ l HH for 4-color (6 parameter) flow acquisition (FACSCalibur; BD Immunocytometry Systems).

2.5. Quantitative reverse-transcribed (qRT) PCR

Primers were designed for qRT-PCR of canine FoxP3 from the human and murine FoxP3 consensus sequences and real time qRT-PCR was performed for canine FoxP3 as previously published (Nash *et al*, 2009) <http://www3.interscience.wiley.com/journal/122410096/supinfo>. Primers, probes and amplification criteria for canine G3PDH (control), TGF β and IL10 messages were from (Peeters *et al*, 2006). Quantitative RT-PCR was performed on cDNA from CD3+ T cells using Platinum qPCR Supermix-UDG with Rox kits (Invitrogen, Carlsbad, CA) in a real-time ABI 7900 thermocycler (Applied Biosystems, Foster City, CA). The conditions included two holds at 50°C and 95° for 2 minutes each, 45 cycles of 15 seconds at 95°C, and 1 minute at 60°C during which the fluorescence data was collected. Absolute copy numbers were calculated based on FoxP3, IL-10 or TGF β standard curves, and samples were normalized to a second standard curve for the housekeeping gene G3PDH. Each sample was run in duplicate alongside negative controls to exclude genomic contamination.

2.6. Semi-quantitative reverse-transcribed (RT) PCR

CD25 primers were designed from the canine genome database and contained the following primer set: CD25 Forward 5'-CGCCGCCCATGGACGAAGTT-3' and CD25 Reverse 5'-GACGCAGCTCGCCACTGCTA-3'. RT-PCR for CD25 was done on the same cDNA stocks from the four dogs that had been used to qRT-PCR for FoxP3, TGF β and IL10. Amplification was done for 45 cycles at 58°C for 1 minute, 72°C for 1 minute and 94°C for 1 minute. Products were separated on a 1% agarose gel and stained with ethidium bromide.

2.7. Immunomagnetic selection

Cells were depleted of CD8 and positively selected with P4A10 from freshly prepared PBMC and from day 4 MLR containing irradiated DLA mismatched stimulators. CD8 antibody JD3 was added at 1 μ g/100 million cells/ml, and P4A10 was added at 0.4 μ g/100 million cells/ml in HH. Both antibodies were allowed to incubate for 20 minutes at 4°C on a rocker. After incubation, unbound antibody was washed away with HH and rat-anti-mouse IgG_{2a/2b} beads were added at 500 μ l/500 million cells/ml in HH and allowed to attach at 4°C for 20 minutes. Preps were then sorted on Deplete program on an AutoMACS (Miltenyi) and the negative fractions kept for P4A10 selection. Negative fractions were washed with HH and resuspended at 500 million cells/ml in HH and then anti-mouse-IgG₁ magnetic beads (Miltenyi) were added at 500 μ l/500 million cells/ml in HH and rocked at 4°C for 20 minutes. Immunomagnetically labeled cells were positively selected with program PoselD on an AutoMACS. Selected cells were counted, washed and resuspended at 1 million per ml in CDM for functional analysis and flow phenotyping QC.

2.8. Functional analysis

The suppressive ability of P4A10-selected T cells was measured by ^3H thymidine incorporation in one-way MLR with responders from the same dog as the P4A10-selected T cells were obtained. PBMC collected in heparin from two DLA mismatched dogs were prepared as stimulators from 1.074 Ficoll density gradients. Cells were adjusted to 1 million per ml in CDM, and the stimulators were γ -irradiated with 25 Gray (GammaCell 3000 Elite; Atomic Energy of Canada) to prevent their proliferation. MLRs were plated in triplicates onto a 96-well round bottom plates (Falcon) with 100 μl each to achieve 100,000 responders and stimulators per well in a final volume of 200 μl . P4A10-selected T cells were then placed into the wells at increasing titers from 1:200 to 1:5 (P4A10-selected T cell:Responder) and the plate incubated at 37°C and 5% CO_2 for 6 days. On day 6, 1 μCi of ^3H thymidine (Perkin-Elmer) was added to each well, and cells were incubated for an additional 16 hours. MLRs were harvested onto a porous matrix (Perkin-Elmer), then dried and overlaid with MicroScint-20 scintillation fluid (Perkin-Elmer) for detection on a TopCount NXT automated plate reader (Perkin-Elmer). Suppression of allogeneic proliferation was detected as a decrease in ^3H thymidine signal compared to MLR without added P4A10-selected T cells.

3. Results

3.1. P4A10 binds to an activation antigen

P4A10 was isotypic as IgG₁. P4A10 reacted with $4.9 \pm 2.1\%$ ($n=8$) of CD3-gated cells in hemolyzed whole blood from normal dogs (data not shown), and activation of resting PBMC with PMA-IO resulted in the expression of the P4A10-related antigen on the majority (>60%) of lymphocytes within 24 hours (Figure 1a).

3.2. P4A10 recognizes a unique doublet at approximately 55 kD by Western blot

Since P4A10 was elicited against whole activated cells and then screened against 3-day ConA-activated vs. freshly isolated PBMC containing non-activated canine T-cells, the antigen it recognized was unknown. To help identify the cognate antigen, a lysate from activated canine PBMC was probed with P4A10 using a non-reducing Western blot technique. This revealed a unique doublet with a molecular weight of 55 kD (Figure 1b).

3.3. P4A10 identifies cells expressing FOXP3 by flow cytometry

PBMC from two dogs were immunomagnetically selected with CD3 and surface stained with CD3, CD4 and P4A10 followed by intracellular staining with FoxP3. Total CD4+P4A10+ content was 6.9% and 7.5% with higher density P4A10 cells also staining for FoxP3 (Figure 2a). In a separate experiment, alloantigen activation via MLR resulted in increased expression of both FoxP3 and P4A10 with over 99% of FoxP3 bright cells also positive for P4A10 at day 4 (Figure 2b).

3.4. P4A10-selection of activated T cells are markedly enriched for FoxP3 expression

Eight immunomagnetic selections with P4A10 were done on freshly prepared PBMC or 4-day alloantigen-stimulated PBMC from four normal dogs, and then cDNA was prepared and probed for CD25 and FOXP3. RT-PCR of CD25 showed an increased signal in the P4A10+ sorts of both fresh and stimulated PBMC as compared to the respective P4A10- sorts (Figure 3a). By qRT PCR, the P4A10+ cells had increased copy numbers of FoxP3, TGF β and IL10 compared to the P4A10- cells from fresh PBMC (1429 ± 349 vs. 52 ± 48 ; $p < 0.0001$, 821 ± 230 vs. 100 ± 109 ; $p = 0.029$ and 93 ± 66 vs. 0 ± 0 ; $p = 0.001$ respectively; Figure 3b). P4A10-selected cells from alloantigen-activated cultures had a significantly increased copy number of FoxP3 compared to the P4A10- fractions, however no statistical differences in the TGF or IL-10 copy number were observed between the two fractions of activated T cells possibly due to large

variabilities in the measurements (FoxP3 14413 ± 11795 vs. 4 ± 7 , $p = 0.05$; TGF β 15144 ± 19640 vs. 8 ± 16 , $p = 0.17$; IL-10 182 ± 232 vs. 0 ± 0 , $p = 0.26$). Activated P4A10-selected T cells tended to contain 1 log more FoxP3 copy number than those selected from fresh PBMC (14413 ± 11795 vs. 1429 ± 349 copies, $p = 0.07$; Figure 3c).

3.5. P4A10-selected T cells are inhibitory consistent with a Treg phenotype

As Treg had been demonstrated to inhibit ^3H thymidine incorporation in MLR, P4A10-selected cells from the experiment above were titrated into primary MLRs containing responders of the same origin as the P4A10-selected cells. Inhibition of ^3H thymidine incorporation was observed with P4A10-selected cells from both sources; however, more suppression was seen with the activated P4A10-selected cells than with those selected from fresh PBMC (Figure 3c). At a ratio of 1:10 (Treg:responder), alloantigen-stimulated P4A10-selected cells inhibited ^3H thymidine incorporation in MLR an average of $72.8 \pm 5.5\%$ compared to an average of $44.3 \pm 8.3\%$ suppression observed when adding cells selected from fresh PBMC ($p=0.01$). At a ratio of 1:20, primed cells suppressed the MLRs $81.8 \pm 6.2\%$ vs. $39.5 \pm 11.2\%$ for unprimed cells ($p=0.01$).

3.6. P4A10 competes with human mAb ACT-1 on activated dog T-cells

Since ACT-1 was reported as CD25-specific and had been reported to stain canine ConA- and PHA-activated lymphocytes (Galkowska *et al*, 1996; Masuda & Yasuda, 2008), co-staining experiments with P4A10 and ACT-1 were conducted. As seen in Figure 4a, when added together at appropriate concentrations, P4A10-FITC and mAb ACT-1-PE co-stained PMA-IO-activated canine PBMCs. In order to achieve equivalent co-staining with ACT-1, a 1/400 concentrate of P4A10 was required, and less than twice the concentration of P4A10 as ACT-1 could completely reduce the fluorescent signal of ACT-1-PE to background. In this competitive flow cytometry assay, we were unable to eliminate the fluorescent signal of P4A10-FITC using mAb ACT-1 conjugated to PE. To determine if competition existed for the same epitope, unconjugated P4A10 was added after binding ACT-1-PE to PMA-IO-activated dog T-cells. Figure 4b shows low concentrations of P4A10 successfully competing with PE-conjugated antibody ACT-1.

4. Discussion

A mAb specific for canine CD25 was developed for use in pre-clinical experiments. The antigen to which the mAb binds had a molecular weight of 55kD, the reported molecular weight of the IL2R α protein (CD25) (Leonard *et al*, 1982). The doublet observed in the Western may represent both soluble and membrane-bound IL2R α (Morris & Waldmann, 2000), differential glycosylation of the protein (Leonard *et al*, 1982), or perhaps an alternatively spliced isoform (Leonard *et al*, 1982). The characterization of antibody P4A10 was also done in part by comparing it to the human CD25 mAb (ACT-1) even though ACT-1 had not been well-characterized. In combination with other data that we obtained on the phenotype and function of T-cells selected by P4A10, we concluded that P4A10 is specific for canine CD25.

We have observed that only a small percentage of CD3+ T cells in the peripheral blood of a normal dog are CD25+ and potentially have a Treg phenotype. Most Tregs in normal dogs are found in the lymph nodes and spleen (Nash *et al*, 2009; Biller *et al*, 2007). After activation of canine T-cells from the peripheral blood in MLR, we confirmed that there was a marked increase in the percentage of CD25+ T cells (Masuda & Yasuda, 2008). Activation of T-cells in MLR also resulted in a 10-fold increase of FoxP3 and TGF β copy number but not IL-10. T-cells expressing IL-10 (Tr1) have been described as having significant regulatory functions and appear to be distinct from FoxP3+ regulatory cells that constitutively express CD25 (Battaglia *et al*, 2006; Roncarolo & Gregori, 2008). The lack of increased expression of IL-10

in canine CD25+ T cells activated in MLR would suggest that a canine equivalent of Tr1 did not contribute to the increased regulatory activity we observed in the functional analysis.

CD8-depleted, CD25+-selected canine T-cells have in vitro regulatory activity. Activation of the T-cells in MLR not only increased levels of FoxP3 copy number in CD3+ T cells but also made these cells more suppressive when added back to a MLR (Walker *et al*, 2003; Walker *et al*, 2005). The ability to CD8-deplete and CD25-select to obtain an enriched cell population of Treg will facilitate studies of *ex vivo* expanded Treg in a transplantation model. While P4A10 was being developed, our group also did studies in which ACT-1 was successfully used to phenotype canine T-cells by flow cytometry as well as for immunomagnetic selection of T-cells including Treg after activation in MLR for *ex vivo* Treg expansion with canine artificial APC (Lesnikova *et al*, 2006; Lesnikova *et al*, 2005). We are continuing these studies of the canine Treg with the P4A10 mAb.

P4A10 could also be potentially used as an immunomodulatory agent for treatment of dogs with autoimmune or non-infectious inflammatory diseases or in an experimental model of hematopoietic cell or solid organ transplantation. Two agents, basiliximab and daclizumab, are specific for human CD25 and are used for prevention of acute rejection after solid organ transplantation in humans without increasing the risk for opportunistic infections (Vincenti *et al*, 1998; Nashan *et al*, 1997). Efficacy has also been demonstrated or suggested for some non-infectious inflammatory disorders such as asthma, multiple sclerosis or uveitis (Busse *et al*, 2008; Rose *et al*, 2007; Yeh *et al*, 2008; Bielekova *et al*, 2004). The mechanism of action may involve inhibition of proinflammatory cytokines by the IL-2R blockade of activated T-cells. IL-2R blockade with daclizumab was not effective treatment for ulcerative colitis (Van Assche *et al*, 2006). Elimination of Treg by these agents may potentially compromise the overall effectiveness of this treatment for non-infectious inflammatory diseases. However, even where this was observed, inflammation resulting from the primary disease process may still be substantially reduced (Oh *et al*, 2009). It has also been proposed that CD25-specific mAb could be successfully used as an anti-tumor agent for those hematopoietic malignancies expressing CD25 (Waldmann, 2007).

Competition between P4A10 and ACT-1 for binding a shared epitope on canine CD25 was observed on canine leukocytes, but CD25-specific antibodies recognizing similar epitopes and even of the same isotype (ACT-1 and P4A10 are both mouse IgG₁) may have different biological effects (Wijdenes *et al*, 1992). An important factor in the effectiveness of antibody immunotherapies is that the antibody bioactivity may be decreased in the settings where the target antigen exists in a soluble form (Hagg & Junghans, 1998). The human IL2 α receptor exists as a 45 kDa soluble form (sIL2R α) and is present in serum during allograft rejection (Morris & Waldmann, 2000). Elevated levels of sIL2R α are also biomarkers of tumor load that can be targeted with anti-CD25 antibody therapy (Bien & Balcerska, 2008). Therefore, a high affinity antibody would be needed to overcome this barrier. The humanized CD25 antibody RA8 was being selected for further studies in part because of its low dissociation constant (Arias *et al*, 2007). P4A10 is potentially such an antibody as its affinity was seen to be substantially greater than that of ACT-1.

In summary, using mitogen-activated dog T-cells as an immunogen, we have developed a mouse-derived mAb (P4A10) that recognizes canine CD25. P4A10 can be efficiently used for the identification and selection of CD25+ canine Treg. Selection and evaluation of functional canine Treg is an important step for the understanding of their biological properties in disease or in the canine model of allogeneic hematopoietic cell or solid organ transplantation.

Non-Standard Abbreviations

CDM	Complete Dog Medium
DLA	Dog Leukocyte Antigen
³ H	Tritiated
HH	2% Horse Serum in Hank's Balanced Salt Solution
IO	Ionomycin
qRT-PCR	quantitative Reverse Transcriptase-PCR
RT-PCR	Reverse Transcriptase-PCR
sIL2R α	soluble IL2R α
Treg	regulatory T-cells

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Figure 1.

a) Activation of canine PBMC with PMA-IO results in the appearance of P4A10-antigen on the majority of cells within 24 hours. All samples were stimulated at time zero in individual wells then harvested, stained and fixed for later acquisition. PBMC were gated by FSC and SSC to eliminate debris and back-gated in unstained FL-3 to eliminate auto-fluorescence. Percentages are above that of cells incubated in medium only, and data are representative of three separate experiments. P4A10 reacted with $4.9 \pm 2.1\%$ ($n=8$) of CD3-gated cells in hemolyzed whole blood from normal dogs (data not shown).

b) P4A10 recognizes a unique doublet at approximately 55 kD by western blot. Overnight PMA-IO-activated dog PBMC lysate was probed with either mouse IgG₁ (left lane) or P4A10 (center lane). A non-specific common band is seen at approximately 116 kD in both blot strips.

Figure 2.
a) P4A10^{Hi} T cells in blood are highly enriched for cells expressing FoxP3. PBMC were obtained from fresh blood and immunomagnetically selected with CD3. T cells were not activated before selection. The P4A10-selected cells were then surface stained with CD3, CD4 and P4A10 prior to intracellular staining with FoxP3 clone 150D or isotype control. P4A10^{Hi} T cells were highly enriched for FoxP3 compared to P4A10^{intermediate} or P4A10^{Lo}. This is consistent with observations in humans that CD4+CD25^{Hi} T cells are enriched with cells expressing FoxP3. However, there were 3.4 ± 2.2% (n=4) CD4-CD25+ cells also found in fresh CD3-selected PBMC. Flow phenotyping data from four dogs obtained in two experiments (presented in tabular form) shows that the CD4- cells were also more likely to be FoxP3 positive if they were also expressing the P4A10 antigen. Cells in Figure 2a were gated on CD4+ or CD4- and FoxP3 percentage as a function of P4A10 expression levels (R1, R2 or R3) is above that of the respective isotype control for FoxP3.
b) Emergence of FoxP3^{Hi} cells during alloantigen activation. Reciprocal bulk MLRs were set up between two DLA-mismatched unrelated dogs and followed for four days. Cells were

collected daily, stained for P4A10 then fixed for subsequent intracellular FoxP3 staining with clone 150D in a single acquisition. Cells were gated by FSC and SSC to include cells and blasts and also to back-gate out debris, then quadrants were set to isolate the Day 4 FoxP3 plus P4A10 double positive population vs. the Day 4 isotype control. Over 99% of FoxP3^{Hi} cells were also positive for P4A10 in each of the two MLRs at Day 4 (data shown from one of two dogs).

Figure 3.

a) P4A10⁺ cell fractions are positive for CD25 by PCR. Fresh PBMC from four different dogs were immunomagnetically depleted of CD8 then positively sorted with P4A10 into positive and negative fractions. Resultant sorts were enriched 60-fold in CD3⁺CD25⁺CD4⁺ cells compared to CD3⁺CD25⁺CD8⁺ cells. Copy DNA was prepared and amplified with primers specific for canine CD25 (and canine G3PDH, data not shown) and run out on an agarose gel then stained with ethidium bromide. There was only visible signal in the positive fractions: (left-to-right) MW marker, respective negative and positive P4A10 fractions for dogs G401, G819, G653 and G368. b) Expression of FoxP3, IL-10 and TGFβ in P4A10 positive and negative cell fractions before and after activation in MLR. Copy number (Y-axis), as compared

to the signal from 100,000 copies of G3PDH in a titration curve established by qPCR, of the P4A10⁺ fractions of the same fresh sorts from Figure 3a) had significantly increased copy numbers of FoxP3 ($p<0.0001$), IL-10 ($p=0.001$) and TGF β ($p=0.029$) over the respective P4A10⁻ fractions. T-cells from these same four dogs that had been activated in a mixed leukocyte reaction (MLR) and then P4A10-selected, not only contained more FoxP3 message than the negative activated fractions ($p=0.05$), but tended to have more FoxP3 than the fresh ($p=0.07$) P4A10⁺ fractions when normalized to 100,000 copies of housekeeping gene G3PDH from activated vs. fresh cell sorts. Bars represent normalized copy number means $\pm$ 1 SD.

c) P4A10⁺ T cells inhibit tritiated thymidine uptake in MLR. Functional analysis of P4A10-selected T cells showed that they markedly inhibited uptake of tritiated thymidine and that MLR-activated P4A10⁺ cells inhibited uptake almost twice as much as fresh non-activated P4A10⁺ cells ($p=0.01$ for both 1:10 and 1:20). Bars represent means $\pm$ 1 SD.

Figure 4.

a) P4A10 and ACT-1 recognize the same T cell subset. P4A10 co-staining with cross-reactive human CD25-specific clone ACT-1 on PMA-IO activated canine PBMC. P4A10-FITC (vertical axis) at 0.01 $\mu\text{g/ml}$ and ACT-1-PE (horizontal axis) at 4 $\mu\text{g/ml}$ were added individually (left and middle panels, respectively) or together (right panel). Data are representative of five experiments.

b) Unconjugated P4A10 competes with the PE-conjugated ACT-1 for cell surface CD25 on PMA-Ionomycin-activated dog T-cells. ACT-1-PE (X-axis) at 1.6 $\mu\text{g/ml}$ and CD3-FITC (Y-axis) were added first in all samples and then unconjugated P4A10 was added at 0.5 and 1 $\mu\text{g/ml}$ in the middle and right panels, respectively. The data shown represent one of three dogs from a single experiment.