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Cloning of feline *FOXP3* and detection of expression in CD4+CD25 + regulatory T cells

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Abstract

Regulatory T cells (Treg) are increased and directly infected by feline immunodeficiency virus (FIV) and likely play a role in other feline autoimmune, neoplastic, and infectious diseases. Phenotypically, Treg are best characterized by surface expression of CD4 and CD25 and intranuclear expression of the forkhead transcription factor Foxp3. Our objective was to clone and sequence feline *FOXP3* for the purpose of developing assays to enhance studies of feline Treg. We determined the feline *FOXP3* is 1293 nucleotides in length and codes for a protein that shares high homology to other species. A splice variant devoid of exon 2 was also identified. A real-time PCR assay was developed and used to show Foxp3 mRNA expression occurs primarily in CD4+CD25+ T cells. Two cross-reacting antibodies were identified by immunocytochemical staining of HEK293 cells transfected with feline *FOXP3*. The antibody labeling confirmed the nuclear localization of the protein. A flow cytometric assay was also validated and used to correlate the phenotypic and functional characteristics of feline Treg induced by treatment of lymph node lymphocytes with flagellin or LPS in combination with mitogen or IL2. Together, these studies provide useful tools to further investigate Foxp3 and Tregs in cats.

Keywords

Foxp3; feline; Treg; regulatory T cells; Toll-like receptors

Report

Regulatory T cells (Treg) can functionally suppress CD4 and CD8 T cells, B cells, NK cells, NKT cells, monocytes/macrophages, dendritic cells and neutrophils, thereby playing a key role in limiting both innate and adaptive immune responses (Azuma et al., 2003; Fallarino et al., 2003; Lewkowicz et al., 2006; Lim et al., 2005; Piccirillo and Shevach, 2004; Ralainirina et al., 2007; Taams et al., 2005; Wing et al., 2005). On the positive side, Tregs protect against autoimmune disease and destructive inflammatory responses directed at foreign antigens

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including infectious agents and allergens (Ostroukhova and Ray, 2005; Paust and Cantor, 2005; Suri-Payer and Fritzsching, 2006; Xystrakis et al., 2006; Zwar et al., 2006). They also suppress graft versus host disease and transplant rejection (Cohen and Boyer, 2006; Walsh et al., 2004). On the negative side, Tregs may suppress anti-tumor responses and can prevent complete elimination of some infections (Chattopadhyay et al., 2005; Suvas and Rouse, 2006). Phenotypically, Tregs are predominantly CD4+CD25+ but this phenotype is neither all-inclusive nor specific. Although imperfect, coexpression of CD4 and CD25 remains the best way to phenotypically identify Tregs (reviewed in (Banham et al., 2006)).

The forkhead transcription factor Foxp3 has been identified as essential and sufficient to impart Treg function in mice (Ziegler, 2006). This understanding has come from two lines of study. The first involved determination of the genetic basis of autoimmune diseases observed in the scurfy mouse (Brunkow et al., 2001) and human X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy (IPEX) (Bennett et al., 2001). In both cases, mutations in the *FOXP3* gene were shown to be the sole cause of these diseases that result from a lack of Treg. Second, transfection of *FOXP3* into CD4+CD25- cells imparts Treg suppressive function (Fontenot et al., 2003; Hori et al., 2003; Khattry et al., 2003).

Regulatory T cells have been phenotypically and functionally characterized in the cat (Vahlenkamp et al., 2004). Similar to mouse and human, feline Treg are predominantly CD4+CD25+ and when activated, efficiently suppress proliferation of activated T cells. Furthermore, in FIV infected cats, Treg are increased in number and function and are proposed to underlie some aspects of the immune impairment observed with this disease (Vahlenkamp et al., 2004). To date, nothing is known about Foxp3 expression in feline Treg. Our objectives in these studies were to clone the feline *FOXP3* and generate feline-specific tools for the study of Treg as defined by Foxp3 expression.

The feline *FOXP3* cDNA sequence was obtained from feline mesenteric lymph node total RNA. This was accomplished by first amplifying a small segment of the cDNA using primers derived from consensus regions of the predicted canine and known human *FOXP3* sequences. Once the short amplicon (382 bp from nucleotide position 439 to 820) was verified by sequencing, 5'- and 3'-RACE PCR using a GeneRacer™ kit with SuperScript™III RT (Invitrogen, Carlsbad, CA) and Advantage® 2 Polymerase Mix (Clontech, Mountain View, CA) were used until the entire feline *FOXP3* cDNA sequence had been obtained.

Putative exon boundaries were determined by aligning the feline cDNA sequence with feline genomic sequence. Feline *FOXP3* cDNA has a 1293 bp open reading frame that codes for a polypeptide 430 amino acids in length (Figure 1) (Genbank accession EF419427). The protein has the conserved winged helix FKH domain and C₂H₂ zinc finger domain characteristic of the Foxp group of transcription factors. The feline *FOXP3* nucleotide and amino acid sequences have high homology to all known orthologues from other species with the greatest homology seen with the putative sequence from the dog (Table 1). During the cloning process it was noted that some cDNA clones lacked the entire exon 2. A similar Foxp3 splice variant (Foxp3Δ2), lacking exon 2, has been reported in human but not mouse (Allan et al., 2005). Exon 2 is a functionally undefined, proline rich region and Foxp3Δ2 has been shown to maintain transcriptional repression similar to the unspliced Foxp3 in transfected human cells as measured by reduced proliferative capacity and reduced IL2 and IFN-γ expression. Interestingly, the suppressive effects are greater when Foxp3Δ2 and Foxp3 are coexpressed rather than expressed alone, suggesting a potential biological significance for the expression of Foxp3Δ2 (Allan et al., 2005). Similarly, the coexpression of the two isoforms is associated with upregulation of CD25, CD45RO, CCR4, CTLA4, GITR and HLA-DR (Allan et al., 2005). A second human splice variant lacking both exon 2 and exon 7 has also been described (Smith et al., 2006). Exon 7 contains the leucine zipper region but the Foxp3Δ2Δ7 variant still

demonstrates suppressive activity in transfected cells (Smith et al., 2006). We did not identify a similar double deletion variant in the cat. The significance of these Foxp3 splice variants *in vivo* remains to be elucidated, but until then, many PCR- and antibody- based Foxp3 detection methods target exon 2 to ensure only full-length Foxp3 is measured.

Having obtained the feline sequence, a one-step real-time RT-PCR assay that uses HPRT for normalization of RNA quantities was developed. Real-time PCR was performed on a Bio-Rad MyiQ™ PCR Detection System using TaqMan® EZ RT-PCR kit (Applied Biosystems, Foster City, CA) containing *rTth* DNA polymerase and feline Foxp3- and hypoxanthine phosphoribosyltransferase (HPRT)-specific primers and dual-labeled probes (Table 2). Primer pairs were designed to span an exon junction, and the resulting amplicons were confirmed by sequencing. *In vitro* transcribed Foxp3 and HPRT RNA were used to generate standard curves for absolute quantification. 200ng lymphocyte RNA was used in each assay and all samples were run in duplicate. Foxp3 RT-PCR cycling conditions were 60°C for 30 min, 95°C for 5 min, followed by 45 cycles of 95°C for 20 s and 60.5°C for 1 min. HPRT cycling conditions were 60°C for 30 min, 95°C for 5 min, followed by 45 cycles of 95°C for 20 s and 57°C for 1 min.

The level of Foxp3 mRNA was then determined in lymphocyte subpopulations defined by CD4 and CD25 expression. Feline mesenteric lymph node cells were isolated as previously described (K.E. Howard et al., 2005), stained for surface expression of CD25 (9F23; kindly provided by K. Ohno, University of Tokyo, Tokyo, Japan) and CD4 (Southern Biotech, Birmingham, AL) then sorted into CD4–CD25–, CD4+CD25– and CD4+CD25+ populations to a purity of ≥93% using a MoFlo high-speed cell sorter (Dako, Fort Collins, CO). Real-time PCR analysis showed the majority of Foxp3 mRNA expression was in the CD4+CD25+ population and that the level of expression was 12–35 times greater than in the CD4+CD25– population and 122–651 times greater than in the CD4–CD25– population (Table 3).

To identify potential cross-reactive anti-Foxp3 antibodies and to explore the subcellular localization, the feline *FOXP3* sequence was cloned into a eukaryotic expression plasmid (pcDNA™3.1/V5-His TOPO®, Invitrogen, Carlsbad, CA) and the construct was confirmed by sequencing. Human epithelial 293 kidney cells (HEK293, ATCC, Manassas, VA) seeded at 2×10^4 cells per well in an 8-well chamber slide (Nalge Nunc, Rochester, NY) were transfected the following day with pcDNA3.1:*FOXP3*, pCMS-EGFP, or plasmid containing an irrelevant antigen (pND14.DSG1, canine desmoglein-1) (Olivry et al., 2006)) using FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Approximately 48 hours post transfection, cells were fixed for 10 min with 0.4% paraformaldehyde then permeabilized for 5 min with 0.1% Triton X-100. The cells were blocked with normal goat serum, then each well was incubated for 1 hour at 37 C with 10% goat serum/PBS containing Alexa488-conjugated anti-human Foxp3 mAb clone 221D/D3 (AbD Serotec, Raleigh, NC), FITC-conjugated anti-mouse Foxp3 mAb clone FJK-16s (eBioscience, San Diego, CA), or no antibody (for GFP-transfected cells). All wells were then incubated with 2U/well (26.7U/ml) phalloidin-Alexa555 (Invitrogen, Carlsbad, CA) for 20 min at 37 C, and then slides were mounted with VECTASHIELD-DAPI (Vector Laboratories, Burlingame, CA). Cells were examined at 200X magnification and images were captured using SimplePCI (Compix Inc, Sewickley, PA). Both anti-Foxp3 antibodies tested were cross-reactive with the feline Foxp3 (Figure 2). The expression of feline Foxp3 was clearly limited to the nucleus of transfected cells. This was in clear contrast to the GFP-expressing control plasmid that showed GFP only in the cytoplasm (data not shown).

Having demonstrated antibody cross-reactivity, we sought to develop a flow cytometric assay for feline Foxp3. The method was initially validated using HEK293 cells transfected with feline *FOXP3* (data not shown) and then applied to feline lymphocytes. Cells were stained for

intracellular expression of Foxp3 using FJK-16s (eBioscience) according to the manufacturer's protocol with the exception that the cells remained in the fixation/permeabilization buffer no longer than 30 minutes to minimize cell degradation. Samples were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) using FACSDiva™ software (BD Biosciences). The FJK-16s mAb was used because it is known to recognize an epitope within exon 2, thus only the full-length Foxp3 protein should be detected. Staining of intranuclear FoxP3 in feline CD4+CD25+ is similar to that demonstrated in human, and like human, FoxP3 staining was not restricted to CD4+CD25+ cells. Freshly isolated PBMC from five specific-pathogen-free cats <1 year of age were surface labeled for CD4 and CD25, CD8 (clone 3.357, Tompkins et al., 1990), or CD21 (clone CA2-ID6, AbD Serotec, Raleigh, NC) then permeabilized and stained for intra-nuclear Foxp3 using mab FJK-16s. Analysis by flow cytometry revealed very little Foxp3 expression overall and although the greatest expression was seen in CD4+CD25+ cells, Foxp3 was also detected in CD4+CD25-, CD8+, and CD21+ lymphocytes (Figure 3). The specificity of Foxp3 as a Treg marker and the role of Foxp3 in Treg induction is a topic of great interest. Although in the mouse Foxp3 expression appears limited to Treg (Ziegler, 2006), transient expression of Foxp3 in activated human T cells has raised concerns about its specificity (Morgan et al., 2005; Roncador et al., 2005; Walker et al., 2005; Walker et al., 2003). Recent studies have helped to understand these observations. It is now clear that Foxp3 is not simply a transcriptional repressor as initially thought, but instead also functions as a transcriptional activator (Marson et al., 2007; Zheng et al., 2007). Four genes upregulated by Foxp3 (*Il2ra*, *Ctla4*, *Nrp1*, *Icos*) define a surface phenotype, thus providing justification for the use of these surface molecules in combination with Foxp3 as a means to identify Treg. Furthermore, Foxp3 appears to act in a dose-dependent manner rather than as a simple on-off regulator, and it is necessary for a cell to express high Foxp3 levels for an extended period of time to become programmed for Treg function (Gavin et al., 2007; Wan and Flavell, 2007). The ultimate fate of Foxp3+ cells that are not CD4+CD25+ remains to be determined. Upon activation, some cells assume the Treg phenotype but the role of Foxp3 in cells that do not is unknown.

To date there are very few studies of the effects of TLR ligands on Treg. Treg have been reported to express mRNA for TLR1, 2, 4, 5, 7, and 8 and protein for TLR2, 4 and 5 (Sutmoller et al., 2006). For mouse and cat it has been shown that CD4+CD25+ cells treated with IL2 and LPS (TLR4 ligand) are activated, proliferate and are functionally consistent with Treg (Caramalho et al., 2003; Vahlenkamp et al., 2004). Conversely, stimulation of human Treg with flagellin (TLR5 ligand) but not LPS is reported to induce proliferation, Foxp3 expression, and increased suppressor activity (Crellin et al., 2005). As a first step to understand how TLR ligands might be involved in feline Treg control, we incubated feline lymph node lymphocytes (LNL) with mitogen (concanavalin A, 2.5 µg/ml) alone or in combination with flagellin (300 ng/ml, InvivoGen, San Diego, CA) or LPS (2.5 µg/ml, Ultra Pure *E. coli* 0111:B4, InvivoGen). We have previously tested these TLR ligands to confirm purity and specificity using HEK293 cells transfected with individual TLR (Nordone et al., 2007). Foxp3 protein expression was detected by intracellular staining at low levels in untreated LNL and was modestly increased with mitogen stimulation (Figure 4). When mitogen stimulation was combined with flagellin or LPS, the overall percentage of cells expressing Foxp3 was greater. The combination of 100 U/ml recombinant human IL2 (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD) and LPS also resulted in increased Foxp3 expression. In all cases, the vast majority of Foxp3+ cells were also CD4+CD25+. These results correlate increased Foxp3 expression with the increased suppressor activity that has been observed with LPS stimulation of Treg. Numerous questions remain to be answered, including whether the TLR ligands act directly on Treg and whether those cells that are induced to express Foxp3 come exclusively from the CD4+CD25+ pool. The methods described herein will be useful for further studies of regulatory T cells in cats.

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 TGAGCCTACACAAATGCTTTGTGCGGGTGGAGAGTGAGAAGGGGCCGTGTGGACCGTGATGAATTCGAGTTCGCAAGAAGAGGAGCCAGAGGCCAG
 R C S N P T P G P
 CAGGTGTTCACACCCACACCTGGCCCCTAA

Figure 1.

Feline *FOXP3* cDNA sequence. The corresponding Foxp3 amino acid sequence is shown above the cDNA sequence. Vertical bars denote putative exon junctions. Underlined sequence represents Exon 2. The C₂H₂ zinc finger domain is shaded in black and the forkhead domain is shaded in gray.

Figure 2.

Cross-reactivity of Fxp3 antibodies and localization of Fxp3. HEK293 cells were transfected with a plasmid expressing an irrelevant antigen (canine desmoglein-1 in A and C) or feline Fxp3 (B and D). Cells were then stained with FITC-conjugated anti-mouse Fxp3 antibody FJK-16s (A and B) or Alexa488-conjugated anti-human Fxp3 antibody 221D1/D3 (C and D). Cells were counter-stained to identify cytoplasmic actin (Alexa555-conjugated phalloidin, red) and the nucleus (DAPI, blue).

Figure 3.

Foxp3 expression in fresh feline peripheral blood lymphocytes. Freshly isolated PBMC from specific-pathogen-free cats were labeled for surface CD4 and CD25, CD8, or CD21 then permeabilized and stained for intra-nuclear Foxp3 using mab FJK-16s. Samples were analyzed by flow cytometry. Bars show standard error, n=5.

Figure 4.

Foxp3 expression in feline lymph node lymphocytes after *in vitro* stimulation. Lymph node lymphocytes were cultured overnight with mitogen (ConA), TLR ligand (flagellin or LPS) and IL2 as indicated. Dot plots on the left show the Foxp3 expression in the total lymphocyte population (% Foxp3+ is indicated). Dot plots on the right show CD4 and CD25 expression on Foxp3+ cells (% Foxp3+ cells that are also CD4+ and CD25+). Histograms are representative of three experiments.

Table 1Nucleotide and amino acid homology of *FOXP3* between feline and other species.

Species	Nucleotide (%)	Amino acid (%)	Exon 2 amino acid (%)
Canine-predicted ^a	96	95	97
Bovine	91	93	91
Human	91	90	91
Macaque	90	89	91
Murine	86	86	97

^aSequence is predicted from the canine genome

Table 2
Feline-specific primer and probe sequences for real-time PCR.

Primer	Foxp3	HPRT ^a
Forward	GCCTGCCACCTGGAATCAAC ^b	TTACGCTGAGGATTGGAAAAGGT
Reverse	GTGTGCTGGGGCTTGGGA	GCCACCCATCTCCTTCAACAC
Probe	CAGTGCTGGCTCCCTGGACACCCA ^c	CTCGGGCAAGACGCTCGGTCCTGTC

^a Hypoxanthine phosphoribosyltransferase

^b All sequences listed 5'- to -3'

^c Probes labeled at 5' -with 6-FAMTM and -3' with Black Hole QuencherTM1

Table 3

Quantitative Foxp3 mRNA expression in feline lymphocyte subsets normalized to HPRT expression.

Animal	CD4 ⁻ CD25 ⁻	CD4 ⁺ CD25 ⁻	CD4 ⁺ CD25 ⁺
1	< 0.02 (401) ^{ab}	0.38 (21)	8.02
2	< 0.02 (651)	0.28 (35)	9.69
3	< 0.02 (122)	0.14 (17)	2.44

^aValues are expressed as mRNA copies of Foxp3/HPRT^bValues in parentheses is fold difference of Foxp3 expression as compared to CD4⁺CD25⁺ cells