Feline Glycoprotein A Repetitions Predominant Anchors Transforming Growth Factor Beta on the Surface of Activated CD4⁺CD25⁺ Regulatory T Cells and Mediates AIDS Lentivirus-Induced T Cell Immunodeficiency

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Abstract

Using the feline immunodeficiency virus (FIV) model for AIDS-lentivirus infection, our laboratory has previously demonstrated that T regulatory (Treg) cell-mediated immune T and B cell dysfunction contributes to lentivirus persistence and chronic disease through membrane bound transforming growth factor beta (mTGFb). Studying Treg cells in the context of infection has been problematic as no inducible marker for activated Treg cells had been identified. However, recent reports in human Treg studies have described a novel protein, glycoprotein A repetitions predominant (GARP), as a unique marker of activated human Treg cells that anchors mTGFb. Herein we extend these studies to the feline Treg system, identifying feline GARP and demonstrating that human and feline GARP proteins are homologous in structure, expression pattern, and ability to form a complex with TGFb. We further demonstrate that GARP and TGFb form a complex on the surface of activated Treg cells and that these GARP⁺TGFb⁺ Treg cells are highly efficient suppressor cells. Analysis of expression of this Treg activation marker in the FIV-AIDS model reveals an up-regulation of GARP expressing Treg cells during chronic FIV infection. We demonstrate that the GARP⁺ Treg cells from FIV-infected cats suppress T helper cells *in vivo* and that blocking GARP or TGFb eliminates this suppression. These data suggest that GARP is expressed in complex with TGFb on the surface of activated Treg cells and plays an important role in TGFb⁺ Treg-mediated T cell immune suppression during lentivirus infection.

Introduction

CD4⁺ REGULATORY T CELLS (Treg cells), currently defined by constitutive expression of the high affinity interleukin (IL)-2 receptor CD25 and the transcription factor Foxp3, play an important role in controlling autoimmune disease.^{1,2} Treg cells also shape the pathogenesis of viral infections by controlling inflammation from excessive activation of T and B effector subsets.^{3–7} The study of Treg population dynamics and function has thus become important for an array of diseases. However, their purification has been problematic as there has been no marker unique to Treg cells. Historically, the transcription factor Foxp3 has been used as a ubiquitous marker for CD4⁺CD25⁺ Treg populations, but the transient expression of Foxp3 has since been demonstrated in non-

regulatory, activated T helper cells in both the human and feline immune systems, limiting the use of this marker in identifying pure populations of Treg cells^{8,9} (M.B. Tompkins, unpublished observations). Additionally, Foxp3 and CD25 are considered constitutive markers of Treg cells and cannot be used to evaluate activation status. The novel protein GARP, or glycoprotein A repetitions predominant (LRRC32), has only recently been described as a unique activation marker of human Treg cells and has been shown to correlate with suppressor function.^{10–12} This surface marker thus presents a method for isolating pure Treg populations and for evaluating activation status.

Importantly, human GARP has been shown to bind transforming growth factor beta (TGFb) within the Treg cell before being targeted for membrane expression.^{11,13} The

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GARP:TGF β complex is then displayed on the Treg cell surface with GARP anchoring the complex via its transmembrane region, leaving the majority of the protein exposed on the extracellular surface with TGF β .^{10,11,13} Human GARP (hGARP) has a short cytoplasmic tail devoid of overt signaling residues, indicating that the dominant role for this protein is to display surface TGF β .^{10,14} While numerous mechanisms for Treg cell-mediated suppression have been proposed, studies on murine, human, and feline Treg cells have identified TGF β signaling to be important.^{5,15–18} In the case of autoimmune disease, it has been reported that membrane bound TGF β (mTGF β) mediates T cell suppression by ligation of the TGF β receptor (TGF β RII) expressed on the surface of activated target Th cells.^{16–19} We have demonstrated that engagement of the TGF β RII on target cells activates the SMAD pathway,⁵ which may in turn induce the expression of Foxp3, a transcription repressor of IL-2. Using the well-established feline immunodeficiency virus (FIV) model for HIV infection, we have demonstrated an important role for mTGF β in Treg cell-mediated suppression of CD4⁺CD25[–] Th cells in a contact-dependent manner.^{5,18} This suppression can be abrogated by the addition of blocking antibodies to TGF β on the Treg cell or TGF β RII on the target cell,¹⁸ providing evidence that AIDS lentiviruses may induce T cell immunodeficiency by activating mTGF β ⁺ immunosuppressive Treg cells. When taken together, these studies suggest that GARP is not only a marker of activated Treg cells but, by anchoring TGF β on the cell surface, represents an important component of Treg cell-mediated immune suppression.

Here, we are the first to identify GARP in the *Felis catus* genome and evaluate expression of this protein on feline Treg cells. We isolate and sequence feline GARP (fGARP) mRNA and identify expression of two fGARP protein isoforms in Treg cells. We extend our findings for mTGF β expression on the surfaces of feline CD4⁺CD25⁺ T cells in association with fGARP by surface phenotyping of this novel protein and show that feline Treg but not Th cells display fGARP-bound TGF β complexes on their surfaces. We also demonstrate that the fGARP–TGF β complex on Treg cells is capable of mediating suppression of Th cells and report a significantly higher level of fGARP:TGF β complex expression on the Treg cell surface from chronic FIV-infected cats when compared to noninfected cats. We further demonstrate that GARP⁺ Treg cells isolated from FIV-infected cats are functionally suppressors of Th cells *in vitro* and that this suppression is abrogated by the addition of GARP or TGF β blocking antibodies. These data suggest that the GARP–TGF β complex plays a role in FIV-induced immunodeficiency. We propose that GARP⁺TGF β ⁺ Treg cells represent a dedicated regulatory population activated in response to lentivirus infection, mediating suppression and contributing to the immune dysfunction in AIDS lentivirus infections.

Materials and Methods

Cats and *in vivo* FIV infection

Specific pathogen-free (SPF) cats were obtained from Liberty Research, Inc. (Waverly, NY) at 6 months of age, and housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. FIV⁺ cats had been previously inoculated with the NCSU₁

isolate of FIV²⁰ and FIV-infected cats were housed separately from FIV-negative cats. All chronic cats had been infected in excess of 2 years at the time of this study.

Blood and lymph node cell collection

Whole blood was collected by jugular venipuncture into EDTA container tubes. Where indicated, peripheral blood mononuclear cells (PBMCs) were isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient as previously described.²¹ Peripheral lymph node (PLN) cells were obtained by lymph node biopsy and single cell suspensions were prepared by repeatedly injecting sterile phosphate-buffered saline (PBS) into the tissue with an 18-gauge needle. After isolation, cell counts and viability were obtained using Trypan Blue dye exclusion. Cell viability was >90% in all experiments.

Real time polymerase chain reaction (PCR)

FACS purified populations of CD4⁺CD25⁺ and CD4⁺CD25[–] T cells from PLN and peripheral blood were used for quantification of GARP, FoxP3, and GAPDH mRNA by reverse transcription and real-time PCR. Total RNA extraction was carried out using Qiagen's RNeasy plus mini kits (Qiagen, Valencia, CA). RNA was used in reverse transcription reactions using oligo(dT) primers and the Promega Reverse transcription system according to the manufacturer's instructions (Promega, Madison, WI). Products were assessed in triplicate for the specific mRNA of interest. Development and use of FoxP3 and GAPDH primers have been described elsewhere.¹⁸ fGARP primers were designed to amplify a 155-base pair product within the 1,431-base pair (bp) open reading frame (ORF) sequence (f 5'-GCCGGAAGTCCAGCACCCA-3', r 5'-CCGCACTCAAAGGCTCGCA-3'). fGARP RT-PCR amplification product was separated on a 1% agarose gel and analyzed with the VersaDoc Imaging System (BioRad, Hercules, CA) to confirm uniform amplification of a single product. The SYBR Green Taqman PCR master mix (Applied Biosystems, Life Technologies, New York) was used for quantification according to the manufacturer's guidelines. All three primer sets were optimized to an annealing temperature of 61°C and reactions were carried out in an ICycler PCR machine. Quantification was performed with the delta delta CT method for fold change in expression or the delta CT method for relative expression with GAPDH as the housekeeping gene.

Amplification and cloning of GARP ORFs

For sequencing analysis of fGARP, primers were designed to span the predicted 1,431 (f 5'-CCACAGCAACGTGCTCATGG-3', r 5'-GGCCGGGCGCTTCTACAGTC-3') or 1,851-bp (f 5'-GTCCCCCTCAATGCTCCCGCT-3', r 5'-GGCCGGGCGCTTCTACAGTC-3') ORF. cDNA was synthesized from feline CD4⁺CD25⁺ T cells using the Promega Reverse Transcription System with an Oligo(dT) Primer (Promega, Madison, WI). PCR amplification of the 1,431 or 1,851-bp ORF product was performed using the HotStar HiFidelity PCR Kit (Qiagen, Valencia, CA) on a Mastercycler Pro Thermocycler (Eppendorf, Hauppauge, NY) programmed with the following cycling conditions: initial denaturation/hot start at 95°C for 15 min; 40 cycles with denaturation at 95°C for 15s, annealing at 60.4°C for 45s, and extension at 72°C for 2 min; final

elongation step at 72°C for 10 min. The 1,431-bp product was analyzed by electrophoresis on a 1.0% Agarose gel; the appropriate weight band was visualized with GelRed (Biotium, Hayward, CA), then excised and purified using the QIAquick Gel Extraction Kit following the supplied manufacturer's protocol. TA cloning and ligation performed using the pGEM T Easy Vector system (Promega, Madison, WI) and chemically competent *Escherichia coli* (GC10; Genesee Scientific, San Diego, CA) were transformed with recombinant plasmids. Samples were sent to Eurofins MWG Operon for sequencing. Results were analyzed using Geneious Pro software.²²

Flow cytometry analysis

At least 5×10^5 PBMCs or PLN cells were stained for surface expression of various markers using specific antibodies. Mouse antifeline CD25 [monoclonal antibody (mAb) 9F23] was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan) and conjugated to fluorescein isothiocyanate (FITC). Mouse antifeline CD4 (mAb 30A) and CD8 (mAb 3.357) were developed in our laboratory.²³ Anti-TGFb (MAB240) was purchased from R&D systems and conjugated to allophycocyanin (APC). Phycoerythrin (PE)-conjugated anti-TGFbRII (FAB241P) was purchased from R&D. Unconjugated and FITC-conjugated anti-GARP IgG2b monoclonal antibodies (LRRC32, Plato-1) were purchased from Enzo Life Sciences (Ann Arbor, MI). Isotype specific, PE-conjugated antimurine IgG2b was purchased from Jackson ImmunoResearch Laboratories. Three or four color flow cytometry was performed on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA). Each antibody combination was analyzed in duplicate and lymphocytes were gated based on forward versus side scatter. Gated events were analyzed using CellQuest software and gates were determined by isotype controls.

Western blot and coimmunoprecipitation

PLN cells were collected from FIV-negative cats, and CD4⁺CD25⁺ cells were FACS sorted using anti-CD4 and anti-CD25 antibodies. A total of $10\text{--}15 \times 10^6$ sorted cells was used for preparation of sample lysate. Gels were transferred to PVDF membranes and analyzed using either anti-GARP or anti-TGFb followed by HRP-conjugated goat anti-rabbit IgG and detected by chemiluminescence. Where indicated, an IgG1-specific HRP-conjugated rabbit antimouse IgG was used to detect TGFb as anti-GARP was IgG2b. The Pierce Co-Immunoprecipitation (Co-IP) Kit was used for isolation of GARP from the CD4⁺CD25⁺ cell lysate following the supplied protocol. Negative controls consisted of a column with inactive resin or with activated resin bound to anti-GARP with PBS in place of sample lysate.

Cell culture and stimulation

Unfractionated PBMCs, PLN cells, or various purified T cell subsets were cultured in RPMI medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, and 1 mM HEPES buffer. For suppression assays, cells were maintained in AIM V Serum-Free Medium (Invitrogen Life Technologies, New York) to eliminate potential TGFb contamination from FBS. All cells were cultured at 2×10^6 total cells per ml of culture media. For Con A stimulation, 5 µg/ml Con A was

added to the media for the indicated length of time. For LPS/IL-2 stimulation, cells were cultured with 10 µg/ml LPS for 2 days, washed, and cultured for 2 more days in fresh media supplemented with 100 U/ml recombinant human IL-2 (rhIL-2) (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD).

T regulatory cell suppression assay

Purified CD4⁺CD25⁺ cells from FIV-negative cat PLNs were stimulated with LPS/rhIL-2 for 4 days then FACS sorted into CD4⁺CD25⁺ or CD4⁺CD25⁺GARP[−] effector populations. In the case of FIV-infected cats, freshly isolated CD4⁺CD25⁺ and CD4⁺CD25⁺GARP[−] cells from PLNs, in the absence of *in vitro* stimulation, were used as effectors. For initial suppression assays using FIV-negative Treg cells stimulated for 4 days as effector cells (Fig. 4), PBMCs were collected from the same cats on day 4 and CD4⁺CD25[−] cells were purified by FACS and used as target cells. The remaining PBMCs were depleted of CD4⁺ and CD8⁺ cells and used as antigen presenting cells (APCs) in the assay. For all studies using CD4⁺CD25⁺ cells from chronically FIV-infected cats (Figs. 6 and 7), PLNs were used as a source of CD4⁺CD25[−] target cells and APCs as well.

The sorted cells were combined at a 1:1 APC to target cell ratio and stimulated with 5 µg/ml of Con A for 1 h, washed, then cultured in fresh media. Activated CD4⁺CD25⁺ or CD4⁺CD25⁺GARP[−] effector cells were then added to the cultures to give a final effector:target:APC ratio of 1:2:2. Controls consisted of Con A-stimulated CD4⁺CD25[−] cells plus APCs without effector cells or unstimulated CD4⁺CD25[−] cells as effector cells. After 24 h, 100 µl of the supernatant from each well was analyzed in triplicate by IL-2 ELISA using the Feline IL-2 DuoSet DY1890 (R&D Systems, Minneapolis, MN) as per the manufacturer's protocol. In blocking studies, unconjugated anti-GARP (Plato-1, Enzo Life Science, Ann Arbor, MI), neutralizing anti-TGFbRII (AF-241-NA, R&D Systems, Minneapolis, MN), and/or anti-TGFb (MAB240 R&D Systems, Minneapolis, MN) were added at 100 µg/ml to the effector cells 30 min prior to addition of APCs and target cells to the coculture. For analysis of proliferation, CD4⁺CD25[−] target cells were labeled with the Cell-Trace CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen Life Technologies, New York) according to the manufacturer's instructions and then cocultured as described for 72 h prior to analysis. The proliferation index (PI) was determined using ModFit LT Software (Verity Software House) and the percent suppression was calculated as follows: $\{[(PI \text{ stimulated } CD4^+CD25^- \text{ alone}) - (PI \text{ with effector cells})] / (PI \text{ stimulated } CD4^+CD25^- \text{ alone})\} \times 100$.

Statistical analysis

The Mann-Whitney *U* test (*t* test for nonparametric data) was used for pairwise comparison of parameters (e.g., surface molecule expression). Differences were considered to be significant at $p < 0.05$.

Results

Sequence and predicted structure of fGARP in CD4⁺CD25⁺ lymphocytes

The hGARP gene resides at chromosome locus 11q14 and encodes a transmembrane protein.²⁴ The full length mRNA

sequence for hGARP (NCBI Reference Sequence: NM_005512.2) was obtained and a blast against the whole genome shotgun (WGS) sequence for *Felis catus* revealed a region with 87% nucleotide (nt) and 85% amino acid (aa) sequence homology (accession number ACBE01342003.1). Using the GARFIELD Genome Browser²⁵ this region was mapped to feline chromosome D1. The human genes adjacent to hGARP (LRRC32) on chromosome 11 are displayed in Fig. 1A. Homologous regions in the feline genome also mapped to feline chromosome D1, supporting the identification of fGARP at this location (Fig. 1A).

The identified fGARP region encodes two potential ORFs of 1,431 and 1,851 bp for which primers were designed. RT-PCR was performed on RNA isolated from FACS sorted CD4⁺CD25⁺ or CD4⁺CD25⁻ lymphocytes from FIV-negative cats. Both ORFs were successfully amplified from CD4⁺CD25⁺ cells, but neither product was amplified from CD4⁺CD25⁻ lymphocytes, consistent with the known restriction of hGARP gene expression to Treg cells (Fig. 1B). The 1,431-bp product was then cloned and sequenced (GenBank accession number JX297379). In Fig. 1C, the corresponding amino acid translation is aligned with the hGARP protein sequence and identical residues are highlighted. As predicted, the two proteins are homologous with fGARP representing a truncated version of hGARP. The transmembrane domain resides at the C-terminus and is underlined in Fig. 1C. The differences between fGARP and hGARP are at the N-terminus.

To create an *in silico* model for the fGARP protein, the aa translation of the 1,431-bp ORF was submitted to the Swiss-Model server²⁶ using the solved crystal structure of human TLR-3 ectodomain, 2AOZ chain A, as has been done previously for hGARP.¹⁰ The protein models differ in overall length and density. Specifically, two looped regions, indicated by asterisks in Fig. 1D, are more disordered in the fGARP model than in the hGARP model and may highlight functional differences between these two proteins.

TGFb is found in complex with GARP on feline lymphocytes

hGARP:TGFb complexes localize to the surface of Treg cells after intracellular association.^{13,27} To confirm surface expression of fGARP:TGFb, PBMCs were isolated from FIV-negative cats and analyzed by flow cytometry for GARP and TGFb expression. We observed, however, that dual staining for fGARP and TGFb resulted in a decrease in percentage of positive cells for each protein compared to single color analysis, suggesting the close association of these molecules results in steric hindrance (data not shown). Therefore, percentages of gated CD4⁺CD25⁺ cells stained for fGARP or TGFb were compared. As shown in Fig. 2A, the CD4⁺CD25⁺ population expressed comparable numbers of fGARP⁺ and mTGFb⁺ cells while CD4⁺CD25⁻ cells were negative. Dual staining with fGARP and Foxp3 antibodies confirmed that fGARP⁺ cells also expressed Foxp3, supporting the classification of these cells as Treg cells (Fig. 2B).

As hGARP has been found in close association with and thought to provide an anchor for TGFb on the surface of human Treg cells^{11,13} and demonstration of dual expression by flow cytometry was problematic, the association of these two proteins in feline cells was further analyzed by immunoprecipitation (IP). Western blot analysis of whole cell

FIG. 1. Predicted feline glycoprotein A repetitions predominant (fGARP) gene locus, mRNA expression dynamics, and protein composition are homologous to human GARP (hGARP). (A) The predicted feline GARP (LRRC32) gene is located on feline chromosome D1 and is flanked by genes homologous with those adjacent to hGARP. (B) The mRNA product corresponding to the 1,431 or 1,851 GARP open reading frames (ORFs) was amplified from purified feline CD4⁺CD25⁺ or CD4⁺CD25⁻ cells, products separated on 1% agarose gel, and imaged. Both gene products were detected only in CD4⁺CD25⁺ cells. (C) The sequenced fGARP gene product was translated into the corresponding amino acid (aa) sequence (F) and aligned to the hGARP aa sequence (H). Shaded boxes identify matching aa residues; asterisks indicate changes in which the aa are not of similar composition. Categories used for composition were acidic, basic, aliphatic, aromatic, hydroxylic, sulfur-containing, and amidic. The C-terminal transmembrane domain is underlined. (D) Ribbon diagram of the predicted fGARP protein structure modeled by comparison to human TLR-3. Asterisks highlight disordered loops. (E) The corresponding space-filling model. Arrows identify the C-terminus where the transmembrane domain has been deleted for this image.

FIG. 2. fGARP and transforming growth factor beta (TGFb) form a complex that is expressed on the surface of CD4⁺CD25⁺FoxP3⁺ cells, consistent with a regulatory phenotype. **(A)** Lymphocytes were analyzed for GARP and TGFb expression by flow cytometry. Representative gating for GARP expression within the CD4⁺CD25⁺ (top) and CD4⁺CD25⁻ (bottom) populations is shown on the left. The total percentage of cells expressing GARP or TGFb in each population is shown on the right with lines connecting each cat ($N=10$). Elevated levels of GARP and TGFb are seen only on CD4⁺CD25⁺ cells. **(B)** Lymphocytes were analyzed for GARP surface expression and intracellular FoxP3 expression. The population shown is gated on CD4⁺CD25⁺ cells. GARP-expressing CD4⁺CD25⁺ cells were found to coexpress FoxP3. **(C)** Whole cell lysate from purified feline CD4⁺CD25⁺ cells was analyzed by western blot for GARP and TGFb. **(D)** Purified CD4⁺ cells from two different cats were analyzed by coimmunoprecipitation. Lysates were immunoprecipitated using anti-GARP and then blotted with anti-TGFb. Negative control 1 corresponds to sample immunoprecipitated with anti-GARP and blotted with only secondary HRP-conjugated antibody. Negative control 2 corresponds to sample run through a nonactivated resin and blotted with anti-TGFb.

lysates from purified feline CD4⁺CD25⁺ cells confirmed the presence of fGARP and TGFb proteins (Fig. 2C). Interestingly, two bands were identified for fGARP at approximately 70 kDa and 55 kDa. Previous hGARP publications have described this protein as 80 kDa in size²⁴ but two hGARP isoforms of 552 aa and 662 aa are available through GenBank (accession numbers AAH52210.2 and AAH70079.1), consistent with our discovery of two molecular weights for fGARP. TGFb was detected at 25 kDa, consistent with the mature form of this protein.²⁸ Anti-GARP was used to IP lysate from purified CD4⁺ T cells for analysis of TGFb. As shown in Fig. 2D, the fGARP protein immunoprecipitated from CD4⁺ cells was complexed with mature TGFb, confirming the association of these two proteins *in vivo*.

Surface staining identifies upregulation of fGARP, TGFb on activated CD4⁺CD25⁺FoxP3⁺ Treg cells

hGARP:TGFb is reported to be up-regulated on the surface of Treg cells following activation.^{13,27} As we and others have demonstrated phenotypic and functional activation of naive Treg cells by LPS/IL-2 stimulation,^{29,30} FACS purified CD4⁺CD25⁺ cells from FIV-negative cats and activated by LPS/rhIL-2 were analyzed by flow cytometry for expression of fGARP, FoxP3, and TGFb. A significant increase in the percentage of fGARP⁺ cells, as well as mean fluorescence intensity (MFI), was observed in the stimulated group (Fig. 3A and B). The percentage of FoxP3⁺ cells was not significantly altered following CD4⁺CD25⁺ activation, but the expression level of this protein as measured by MFI was increased

FIG. 3. GARP, FoxP3, and TGFb protein and message are up-regulated in CD4⁺CD25⁺ lymphocytes following *in vitro* activation. CD4⁺CD25⁺ lymphocytes were sorted from peripheral lymph nodes (PLN) of feline immunodeficiency virus (FIV)-negative cats and activated *in vitro* by LPS/rhIL-2 stimulation and analyzed for surface expression of GARP or TGFb, or for intracellular expression of FoxP3 by flow cytometry. (A) Percent of cells expressing GARP. (B) Mean fluorescence intensity (MFI) of GARP expression. (C) Percent of cells expressing FoxP3. (D) MFI of FoxP3 expression. (E) Percent of cells expressing TGFb. (F) Sorted CD4⁺CD25⁺ lymphocytes were analyzed for GARP and FoxP3 message before and after stimulation by real-time RT-PCR and results are shown as fold change over GAPDH ($2^{-\Delta\Delta CT}$). (Error bars represent standard error of the mean of four independent experiments, * $p < 0.05$ Mann-Whitney.)

following stimulation in cells that were constitutively Foxp3⁺ (Fig. 3C and D). mTGFb was also expressed on a significantly larger proportion of stimulated CD4⁺CD25⁺ cells over control cells (Fig. 3E). fGARP and FoxP3 mRNA levels were also up-regulated after stimulation (Fig. 3F). These data demonstrate that activation of feline CD4⁺CD25⁺ Treg cells results in up-regulation of surface fGARP and TGFb, as well as intracellular FoxP3 message and protein.

fGARP⁺ CD4⁺ CD25⁺ Treg cells are potent suppressors of CD4⁺ CD25⁻ Th cells

As we have previously demonstrated an important role for mTGFb in Treg cell-mediated suppression,⁵ demonstration of fGARP-anchored mTGFb on activated Treg cells by Co-IP (Fig. 2D) is consistent with a TGFb-mediated suppressor function. To demonstrate the suppressor activity of these cells, IL-2 production from Con A-stimulated CD4⁺CD25⁻ Th cells was measured by ELISA following coculture with LPS/rhIL-2-activated CD4⁺CD25⁺ Treg cells or activated CD4⁺CD25⁺ Treg cells depleted of fGARP expressing cells. As shown in Fig. 4, Con A-stimulated CD4⁺CD25⁻ cells produced high levels of IL-2, whereas nonstimulated CD4⁺CD25⁻ cells produced little IL-2. In contrast, coculture with LPS/rhIL-2-stimulated Treg cells decreased IL-2 production, confirming the suppressor function of these Treg cells. Depletion of fGARP⁺ cells from the activated Treg cell population resulted

in an increase in IL-2 production (Fig. 4), demonstrating that fGARP⁺ cells function as suppressor cells. These data provide evidence that the fGARP:TGFb complex is a contributing factor to Treg-mediated suppression of CD4⁺CD25⁻ T helper cells and further indicate that fGARP⁺ Treg cells represent a highly efficient Treg suppressor subset.

fGARP mRNA levels and surface fGARP:TGFb expression on Treg cells is elevated during chronic FIV infection

We have reported that immunosuppressive Treg cells are chronically activated during long-term FIV infection.³⁰ As shown in Fig. 3, *in vitro* activation of feline CD4⁺CD25⁺ T cells by LPS/rhIL-2 resulted in the up-regulation of surface fGARP and TGFb. To determine whether *in vivo* activation of immunosuppressive Treg cells by FIV infection correlated with the up-regulation of fGARP:mTGFb expression, mRNA was extracted from freshly isolated CD4⁺CD25⁺ or CD4⁺CD25⁻ lymphocytes from either FIV-negative or chronically FIV-infected cats and mRNA levels were analyzed by real time RT-PCR. As expected, the CD4⁺CD25⁺ population from chronically FIV-infected cats expressed increased fGARP mRNA as compared to FIV-negative cats (Fig. 5A). PBMCs from chronically infected cats were then analyzed for surface fGARP and TGFb expression by flow cytometry. As shown in Fig. 5B, CD4⁺CD25⁺ cells from FIV-infected cats had

FIG. 4. Depletion of GARP⁺ Treg cells decreases Treg-mediated suppression of CD4⁺CD25⁻ Th cells. CD4⁺CD25⁺ effector cells were obtained by FACS purification from specific pathogen-free (SPF) cat PLNs and activated with LPS/IL-2 treatment for 4 days as described. CD4⁺CD25⁻ Th cells were used as targets and were obtained from freshly isolated peripheral blood mononuclear cells (PBMCs) on the day of coculture addition. Antigen presenting cells (APCs) were prepared by FACS depletion of CD4⁺ and CD8⁺ PBMCs at this time as well and added at a 1:1 ratio with Th cells. Where indicated, these cells were stimulated for 1 h with 5 μ g/ μ l of Con A, washed and then plated. CD4⁺CD25⁻, LPS/rhIL-2 activated CD4⁺CD25⁺, or activated CD4⁺CD25⁺GARP⁻ populations were used as effector cells at a 1:2 effector:target ratio. Supernatant was collected after 24 h of coculture and IL-2 analyzed by ELISA in triplicate. (Data shown are representative of two separate experiments; error bars represent standard error of the mean, * p < 0.05 Mann-Whitney.)

significantly higher fGARP expression as compared to FIV-negative PBMCs. fGARP was not detected on the surface of CD4⁺CD25⁻ cells regardless of infection status, further supporting data that fGARP is a Treg cell-specific protein. Similar to fGARP expression, TGF β expression was higher on the CD4⁺CD25⁺ cells than on CD4⁺CD25⁻ cells. As the contact-dependent suppression by fGARP⁺ Treg cells is mediated by ligation to TGF β receptor (TGF β RII) on the surface of target cells, TGF β RII expression on freshly isolated CD4⁺CD25⁻ targets was evaluated. The percentage of CD4⁺CD25⁻ cells expressing TGF β RII is significantly greater in FIV-infected cats than FIV-negative cats (Fig. 5C).

CD4⁺CD25⁺ GARP⁺ Treg cells from chronic FIV-infected cats are capable of suppressing CD4⁺CD25⁻ Th cells

To confirm the activation of CD4⁺CD25⁺ Treg cells during chronic FIV infection and to further evaluate the contribution of fGARP⁺ Treg cells to CD4⁺CD25⁻ Th suppression, a suppression assay was performed utilizing PLN cells from chronically FIV-infected cats. Purified CD4⁺CD25⁻ target cells and APCs were stimulated for 1 h with Con A, washed, and freshly isolated, purified CD4⁺CD25⁺ cells were added to the culture. After 24 h of coculture, supernatant was collected and assayed for IL-2 by ELISA. As shown in Fig. 6,

CD4⁺CD25⁺ Treg cells from chronically FIV-infected cats suppressed IL-2 production by Th cells. Additionally, depletion of fGARP⁺ cells from the effector pool resulted in an increase in Th cell IL-2 production (Fig. 6).

To further confirm that the presence of GARP:TGF β complexes on these effector cells was responsible for the suppression, PLN cells from chronically FIV-infected cats were used in a blocking assay. Briefly, FACS purified CD4⁺CD25⁺ cells were cultured for 30 min with blocking antibodies for GARP, blocking antibodies for TGF β and TGF β RII, or with a combination of all three antibodies. APCs and CFSE-labeled CD4⁺CD25⁻ target cells were then added to these cultures. After 24 h, supernatants were analyzed by IL-2 ELISA and, as shown in Fig. 7A, the addition of anti-GARP antibodies to these cultures abrogated the suppressor capacity of the CD4⁺CD25⁺ cells as measured by Th cell IL-2 production. Addition of TGF β /TGF β RII blocking antibodies reduced the suppressor capacity to a similar degree (Fig. 7A), and treatment with all three blocking antibodies also had this effect, consistent with our model for the GARP:TGF β complex mediating suppression of Th cells. After 72 h in coculture, the CFSE-labeled target cells were analyzed for proliferation and percent suppression was determined (Fig. 7B.) As shown in Fig. 7B, the IL-2 suppression measured in Fig. 7A was correlated with a decrease in proliferation when CD4⁺CD25⁻ target cells were cocultured with activated CD4⁺CD25⁺ effectors but not when they were cocultured with nonsuppressive CD4⁺CD25⁻ Th cells. Importantly, the addition of GARP or TGF β blocking antibodies to the CD4⁺CD25⁺ effector cells prior to coculture resulted in a decrease in percent suppression (Fig. 7B). These data support the hypothesis that chronic lentivirus infection induces GARP⁺:mTGF β -activated Treg cells that suppress Th cells and mediate lentivirus-induced immune dysfunction in a TGF β -dependent manner.

Discussion

The GARP gene product, first described in human breast carcinoma cells,²⁴ was subsequently shown to be expressed in lymphocytes but restricted to CD4⁺CD25⁺ Treg cells activated through the TCR.³¹ As mTGF β has been implicated in Treg-mediated immune suppression,^{5,16,18} Stockis *et al.*¹¹ examined the possible relationship between GARP and mTGF β and reported that latent TGF β was bound to GARP on the surface of Treg cell clones. While a number of studies have documented the role of activated FoxP3⁺ Treg cells in mediating T cell suppression and immune deficiency in AIDS lentivirus infections,^{6,32,33} the role of mTGF β , and in particular the GARP:TGF β complex, is largely unknown. We have reported that mTGF β may be an important mediator of Treg-induced T cell immune suppression in AIDS lentivirus infections.^{5,18} Here, we are the first to identify GARP in the feline species and its role in Treg cell-mediated suppression in FIV infection. Moreover, we present evidence that fGARP complexes with mTGF β on the surface of Treg cells and may play a major role in TGF β -mediated T cell immune suppression.

Two fGARP ORFs were identified and mRNA corresponding to the two different length fGARP transcripts was amplified from feline CD4⁺CD25⁺ T cells but not from CD4⁺CD25⁻ Th cells. The 1,651-bp fGARP mRNA product differs from the 1,431-bp fGARP product only at the three prime end, as these ORFs are within the same frame and differ only in the location of the start codon. This additional region

FIG. 5. GARP expression on CD4⁺CD25⁺ and TGFbRII expression on CD4⁺CD25⁻ cells are up-regulated during chronic FIV infection. **(A)** Lymphocyte populations from chronic FIV-infected cats or FIV⁻ cat PLNs were FACS purified into CD4⁺CD25⁺ or CD4⁺CD25⁻ populations. RNA was extracted from each population and analyzed for GARP mRNA levels by real time RT-PCR ($n=3$). Data shown as fold expression over GAPDH (error bars represent the standard error of the mean). **(B)** Freshly isolated PBMCs from chronically FIV-infected cats ($n=11$) or FIV⁻ cats ($n=10$) were analyzed by flow cytometry for the expression of GARP and TGFb by gating on CD4⁺CD25⁺ or CD4⁺CD25⁻ populations. Data are expressed as percentage of gated cells, gating determined by isotype controls. **(C)** FIV⁻ and chronic FIV⁺ PBMCs ($n=5$) were analyzed by flow cytometry for the expression of TGFb receptor (TGFbRII). (* $p<0.05$ Mann-Whitney, mean shown for each group.)

of the mRNA when translated represents the N terminus or extracellular tail of the fGARP protein. Translating the 1,431-bp region and modeling this amino acid sequence to human TLR-3 reveals that fGARP may retain the ability to fold into the same horseshoe-shaped tertiary structure proposed for hGARP.¹⁰ Two isoforms of fGARP were identified by western blot analysis, consistent with the two hGARP proteins previously identified and submitted to GenBank (accession numbers AAH52210.2 and AAH70079.1). Whatever differences between hGARP and fGARP may exist, the data

reported herein suggest that they are structurally similar and that they perform the same function as accessory proteins in mediating the suppressor activity of Treg cells.

In support of the association of fGARP and TGFb on feline Treg cells, flow cytometric analysis revealed that these surface proteins were expressed on similar numbers of CD4⁺CD25⁺ lymphocytes but not on CD4⁺CD25⁻ Th. These TGFb⁺ fGARP⁺CD4⁺CD25⁺ were shown to express FoxP3, consistent with the established phenotype of Treg cells. While dual staining for GARP and TGFb on activated Treg cells proved to

FIG. 6. CD4⁺CD25⁺ Tregs from chronic FIV-infected cats suppress CD4⁺CD25⁻ Th and this is partially dependent upon GARP expression. Lymphocyte populations from chronic FIV-infected cat PLNs were FACS purified. CD4⁺CD25⁻ cells were used as target cells. APCs were prepared by FACS depletion of CD4⁺ and CD8⁺ lymphocytes and added at a 1:1 ratio with Th cells. Where indicated, these cells were stimulated for 1 h with 5 μ g/ml of Con A, washed, and then plated. Freshly isolated CD4⁺CD25⁻, CD4⁺CD25⁺, or CD4⁺CD25⁺GARP⁻ populations were used as effector cells with an effector:target ratio of 1:2. Supernatant was collected after 24 h of coculture and IL-2 was analyzed by ELISA in triplicate. (Data shown are representative of three independent experiments; error bars represent the standard error of the mean, * p < 0.05 Mann-Whitney.)

be problematic, likely due to steric hindrance, supporting the close association of the two proteins, analysis of CD4⁺CD25⁺ cells by Co-IP revealed that the fGARP protein was complexed with mature TGF β in CD4⁺CD25⁺ lymphocytes. These data support previous findings by Stockis *et al.*¹¹ who reported that hGARP formed a complex with TGF β . However, Stockis *et al.*¹¹ focused on soluble TGF β rather than membrane-bound TGF β in suppressor signaling and they did not investigate the contact-dependent suppressor capacity of these cells. Other studies have found that Th cells transduced with GARP are capable of suppressing CD4 T cell proliferation in a contact-dependent manner and that silencing the expression of GARP in CD4⁺CD25⁺ Treg cells reduces suppressor function.^{10,14} The role of TGF β was not evaluated in these later studies. These results suggest that activation and suppressor function of GARP-bound TGF β should be evaluated within the context of the Treg cell membrane.

Studies have demonstrated that the hGARP surface protein is expressed only on activated Treg cells^{10,31} while FoxP3 is constitutively expressed on resting Treg cells³⁴ but is up-regulated after activation.^{7,35} In agreement with these findings, LPS/rhIL-2 stimulation of feline CD4⁺CD25⁺ lymphocytes resulted in the up-regulation of fGARP and FoxP3 message and protein as seen by real-time RT-PCR and flow cytometry. Flow cytometric analysis also revealed up-regulation of mTGF β protein following LPS/rhIL-2 activation of CD4⁺CD25⁺ Treg cells. These results support our conclusion that fGARP:TGF β complexes are expressed on the

FIG. 7. Blocking GARP and/or TGF β signaling abrogates suppressor capacity of CD4⁺CD25⁺ Treg cells isolated from FIV-infected cats. Lymphocyte populations from chronic FIV-infected cat PLNs were FACS purified. Freshly isolated CD4⁺CD25⁻ or CD4⁺CD25⁺ cells were used as effector cells. Where indicated, CD4⁺CD25⁺ cells were plated with 100 μ g/ml each anti-GARP, anti-TGF β , and/or anti-TGF β RII neutralizing antibodies for 30 min. CD4⁺CD25⁻ cells were used as target cells and were labeled with CFSE immediately after purification. APCs were prepared by FACS depletion of CD4⁺ and CD8⁺ lymphocytes and added at a 1:1 ratio with already labeled Th cells. Where indicated, these cells were stimulated for 1 h with 5 μ g/ml of Con A, washed, and then plated with the pretreated CD4⁺CD25⁺ cells at an effector:target ratio of 1:2. (A) Supernatant was collected after 24 h of coculture and IL-2 was analyzed by ELISA in triplicate. (Data shown are pooled from two independent experiments; error bars represent the standard error of the mean, * p < 0.05 Mann-Whitney.) (B) CFSE proliferation was analyzed using ModFit LT and the percent inhibition of proliferation was calculated for each of the effector cell groups as follows: [(PI stimulated CD4⁺CD25⁻ alone) - (PI with effector cells)] / [(PI stimulated CD4⁺CD25⁻ alone)] $\times$ 100. (Data are representative of two independent experiments.)

surface of feline Treg cells and that these complexes are up-regulated following activation.

Assay of IL-2 production by stimulated CD4⁺CD25⁻ target cells cocultured with *in vitro*-activated CD4⁺CD25⁺ Treg cells demonstrated reduced suppressor capability in fGARP-depleted CD4⁺CD25⁺ Treg cells, indicating that fGARP⁺ Treg cells are potent immune suppressors. Similar results were obtained from hGARP studies in which GARP⁺ Treg

cells were found to be more efficient suppressors than GARP⁺ Treg cells.³¹ Studies by Wang *et al.*³¹ on hGARP and the studies here show that while GARP-expressing Treg cells do not mediate total Th cell suppression, they are major mediators of T cell immune suppression. The redundancy and overlap of Treg suppressor mechanisms have been widely accepted^{36–38} and the results reported herein do not provide a definitive model for Treg cell-mediated suppression but do identify the GARP:TGFb complex as an important cell surface regulatory mechanism utilized by Treg cells in the suppression of Th cells. Collectively, these studies support the proposal that GARP-bound TGFb on the surface of activated Treg cells mediates Treg suppression of Th cells.

To further pursue this question, with the understanding that AIDS lentivirus infections, including FIV, activate the Treg cells *in vivo*,^{4,30,33} we evaluated Treg expression of GARP and TGFb in FIV-infected cats. A significant increase in fGARP and TGFb expression was observed on freshly isolated CD4⁺CD25⁺ T cells from cats with chronic FIV infection. While Wang *et al.*¹⁴ did not examine freshly isolated Treg cells from HIV⁺ donors, they did report GARP expression on stimulated Treg cells from HIV patients and found no significant difference in Treg cell expression between HIV⁺ and HIV[−] donors poststimulation. However, there was a significantly larger population of CD4⁺CD25⁺FoxP3⁺ cells in the HIV⁺ patients, suggesting that HIV infection may not significantly alter GARP⁺ Treg cell numbers but may expand nonregulatory T cells expressing FoxP3. It is unclear whether the HIV⁺ donors in this study were receiving chemotherapeutic treatments that could further complicate comparisons to HIV[−] donors as antiretroviral therapy has been shown to alter the numbers and phenotypes of Treg cells.³⁹ Our findings suggest that lentiviral infection is capable of activating GARP⁺ Treg cells *in vivo*.

We have previously demonstrated that FIV preferentially and productively infects Treg cells^{7,40,41} and that freshly isolated Treg cells from FIV-infected cats are not only productively infected, but are activated and capable of suppressing Th cells.^{30,41} We have also demonstrated that the Th cells express higher levels of TGFbRII during chronic FIV infection.⁵ As we propose that TGFb⁺ Treg cells engage TGFbRII on Th target cells, expression of TGFbRII on the target cell would promote Treg cell-mediated suppression. In this study, we extend these findings, demonstrating that elimination of fGARP⁺ cells from the CD4⁺CD25⁺ Treg population from chronically FIV-infected cats reduced Treg-mediated suppressor activity. Blocking assays further demonstrated that pretreatment of the *in vivo*-activated CD4⁺CD25⁺ Treg cells from FIV-infected cats with neutralizing antibodies to GARP, TGFb, and/or TGFbRII eliminated this suppressor capacity as measured by both Th target cell IL-2 production and target cell proliferation. These data suggest that FIV infection activates Treg cells, up-regulating fGARP:TGFb complexes on their surface, inducing their immunosuppressive function against TGFbRII⁺ Th cells via TGFb signaling. The up-regulation of GARP and TGFb on Treg cells may not be unique to FIV infection but may occur with other chronic virus infections known to activate immunosuppressive Treg cells such as hepatitis and herpes viruses.^{3,42–44} Further research will be necessary to evaluate the existence of a GARP⁺ Treg cell population in these additional models of disease.

Sequence Data

The feline GARP cDNA sequence is listed in GenBank under accession number JX297379.

Acknowledgments

The authors thank Beth Thompson, Deb Anderson, Janet Dow, and Linda English for their excellent technical assistance. This work was funded by National Institute of Health grants 1K08AI074445 and R01-A1080288.

Author Disclosure Statement

No competing financial interests exist.

References

1. Adeegbe D, Bayer AL, Levy RB, and Malek TR: Cutting edge: Allogeneic CD4+CD25+Foxp3+ T regulatory cells suppress autoimmunity while establishing transplantation tolerance. *J Immunol* 2006;176(12):7149–7153.
2. Bettini M and Vignali DA: Regulatory T cells and inhibitory cytokines in autoimmunity. *Curr Opin Immunol* 2009;21(6):612–618.
3. Bellou AL, Sarantopoulos A, Gigi E, *et al.*: CD4+CD25+FOXP3+ TREGS: Do they inhibit HCV clearance in chronic HCV genotype 1 infection? *J Hepatol* 2011;54:S111.
4. Kinter AL, Horak R, Sion M, *et al.*: CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells *in vitro*. *AIDS Res Hum Retroviruses* 2007;23(3):438–450.
5. Fogle JE, Mexas AM, Tompkins WA, and Tompkins MB: CD4(+)CD25(+) T regulatory cells inhibit CD8(+) IFN- γ production during acute and chronic FIV infection utilizing a membrane TGF- β -dependent mechanism. *AIDS Res Hum Retroviruses* 2010;26(2):201–216.
6. Holmes D, Jiang Q, Zhang L, and Su L: Foxp3 and Treg cells in HIV-1 infection and immuno-pathogenesis. *Immunol Res* 2008;41(3):248–266.
7. Mexas AM, Fogle JE, Tompkins WA, and Tompkins MB: CD4+CD25+ regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol* 2008;126(3–4):263–272.
8. Walker M, Kaspruwicz DJ, Gersuk VH, *et al.*: Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25[−] T cells. *J Clin Invest* 2003;112(9):1437–1443.
9. Morgan ME, van Bilsen JH, Bakker AM, *et al.*: Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* 2005;66(1):13–20.
10. Probst-Kepper M, Geffers R, Kroger A, *et al.*: GARP: A key receptor controlling FOXP3 in human regulatory T cells. *J Cell Mol Med* 2009;13(9B):3343–3357.
11. Stockis J, Colau D, Coulie PG, and Lucas S: Membrane protein GARP is a receptor for latent TGF- β on the surface of activated human Treg. *Eur J Immunol* 2009;39(12):3315–3322.
12. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, and Shevach EM: GARP (LRRC32) is essential for the surface expression of latent TGF- β on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci USA* 2009;106(32):13445–13450.
13. Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, and Shevach EM: Selective expression of latency-associated

- peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3⁺ regulatory T cells allows for their purification from expansion cultures. *Blood* 2009;113(21):5125–5133.
14. Wang R, Wan Q, Kozhaya L, Fujii H, and Unutmaz D: Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. *PLoS ONE* 2008;3(7):e2705.
 15. Gorelik L and Flavell RA: Abrogation of TGFβ signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12(2):171–181.
 16. Nakamura K, Kitani A, and Strober W: Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194(5):629–644.
 17. Gregg RK, Jain R, Schoenleber SJ, *et al.*: A sudden decline in active membrane-bound TGF-β impairs both T regulatory cell function and protection against autoimmune diabetes. *J Immunol* 2004;173(12):7308–7316.
 18. Petty CS, Tompkins MB, and Tompkins WA: Transforming growth factor-beta/transforming growth factor-betaRII signaling may regulate CD4+CD25+ T-regulatory cell homeostasis and suppressor function in feline AIDS lentivirus infection. *J Acquir Immune Defic Syndr* 2008;47(2):148–160.
 19. Shi Y and Massague J: Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* 2003;113(6):685–700.
 20. English RV, Johnson CM, Gebhard DH, and Tompkins MB: In vivo lymphocyte tropism of feline immunodeficiency virus. *J Virol* 1993;67(9):5175–5186.
 21. Tompkins MB, Ogilvie GK, Franklin RA, Kelley KW, and Tompkins WA: Induction of IL-2 and lymphokine activated killer cells in the cat. *Vet Immunol Immunopathol* 1987;16(1–2):1–10.
 22. Geneious v5.5. Available from www.geneious.com [computer program]. Version, 2011.
 23. Tompkins MB, Gebhard DH, Bingham HR, Hamilton MJ, Davis WC, and Tompkins WAF: Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. *Vet Immunol Immunopathol* 1990;26(4):305–317.
 24. Ollendorff V, Noguchi T, deLapeyriere O, and Birnbaum D: The GARP gene encodes a new member of the family of leucine-rich repeat-containing proteins. *Cell Growth Differ* 1994;5(2):213–219.
 25. Pontius JU and O'Brien SJ: Genome Annotation Resource Fields—GARFIELD: A genome browser for *Felis catus*. *J Hered* 2007;98(5):386–389.
 26. Arnold K, Bordoli L, Kopp J, and Schwede T: The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* 2006;22(2):195–201.
 27. Stockis J, Fink W, Francois V, *et al.*: Comparison of stable human Treg and Th clones by transcriptional profiling. *Eur J Immunol* 2009;39(3):869–882.
 28. Annes JP, Munger JS, and Rifkin DB: Making sense of latent TGFβ activation. *J Cell Sci* 2003;116(Pt 2):217–224.
 29. Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, and Demengeot J: Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* 2003;197(4):403–411.
 30. Vahlenkamp TW, Tompkins MB, and Tompkins WAF: Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol* 2004;172(8):4752–4761.
 31. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, and Unutmaz D: Expression of GARP selectively identifies activated human FOXP3⁺ regulatory T cells. *Proc Natl Acad Sci USA* 2009;106(32):13439–13444.
 32. Kinter AL, Hennessey M, Bell A, *et al.*: CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* 2004;200(3):331–343.
 33. Aandahl EM, Michaëlsson J, Moretto WJ, Hecht FM, and Nixon DF: Human CD4+CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 2004;78(5):2454–2459.
 34. Ziegler SF: FOXP3: Of mice and men. *Annu Rev Immunol* 2006;24:209–226.
 35. Milkova L, Voelcker V, Forstreuter I, *et al.*: The NF-κB signalling pathway is involved in the LPS/IL-2-induced upregulation of FoxP3 expression in human CD4+CD25 high regulatory T cells. *Exp Dermatol* 2010;19(1):29–37.
 36. Miyara M and Sakaguchi S: Natural regulatory T cells: Mechanisms of suppression. *Trends Mol Med* 2007;13(3):108–116.
 37. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, and Yamaguchi T: Regulatory T cells: How do they suppress immune responses? *Int Immunol* 2009;21(10):1105–1111.
 38. Sojka DK, Huang YH, and Fowell DJ: Mechanisms of regulatory T-cell suppression—a diverse arsenal for a moving target. *Immunology* 2008;124(1):13–22.
 39. Presicce P, Orsborn K, King E, Pratt J, Fichtenbaum CJ, and Choungnet CA: Frequency of circulating regulatory T cells increases during chronic HIV infection and is largely controlled by highly active antiretroviral therapy. *PLoS ONE* 2011;6(12):e28118.
 40. Joshi A, Garg H, Tompkins MB, and Tompkins WA: Preferential feline immunodeficiency virus (FIV) infection of CD4+CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. *J Virol* 2005;79(8):4965–4976.
 41. Joshi A, Vahlenkamp TW, Garg H, Tompkins WAF, and Tompkins MB: Preferential replication of FIV in activated CD4+CD25+ T cells independent of cellular proliferation. *Virology* 2004;321(2):307–322.
 42. Ebinuma H, Nakamoto N, Li Y, *et al.*: Identification and in vitro expansion of functional antigen-specific CD25+ FoxP3⁺ regulatory T cells in hepatitis C virus infection. *J Virol* 2008;82(10):5043–5053.
 43. Suvas S, Kumaraguru U, Pack CD, Lee S, and Rouse BT: CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 2003;198(6):889–901.
 44. Suvas S, Azkur AK, Kim BS, Kumaraguru U, and Rouse BT: CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* 2004;172(7):4123–4132.

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