Title: PI16 is expressed by a subset of human memory Tregs with enhanced migration to CCL17 and CCL20.

Ian C. Nicholson[1,2,3], Christos Mavrangelos[1,2], Daniel R.G. Bird[1,2], Suzanne Bresatz-Atkins[1,2,3], Nicola G. Eastaff-Leung[1,2,3], Randall H. Grose[1,2], Batjargal Gundsambuu[1,2], Danika Hill[1,2,3], Deborah J Millard[1,2], Timothy J. Sadlon[1,2,3], Sarah To[1,2], Heddy Zola[1,2,3], Simon C. Barry[1,2,3], Doreen Krumbiegel[1,2,3]**Affiliations:**

[1]. Women's and Children's Health Research Institute, 72 King William Road, North Adelaide, South Australia, 5006, Australia.

[2]. Co-operative Research Centre for Biomarker Translation, Melbourne, Australia

[3]. Discipline of Pediatrics, University of Adelaide, Adelaide. Australia

Running Title: Characterisation of PI16-positive memory Treg.

Corresponding Author:

Doreen Krumbiegel, Leukocyte Biology Laboratory, Women's and Children's Health Research Institute, 72 King William Road, North Adelaide, South Australia, 5006, Australia.

Phone: +61 8 8161 7071 Fax: +61 8 8239 0267

Email: doreen.krumbiegel@adelaide.edu.au

Abstract:

The peptidase inhibitor PI16 was identified previously using microarray analysis as being over-expressed by CD4-positive/CD25-positive Treg compared with CD4-positive/CD25-negative Th cells. Using a monoclonal antibody to the human PI16 protein, we found that the PI16-positive Treg have a memory (CD45RO-positive) phenotype, and express higher levels of FOXP3 than PI16-negative Treg. The PI16-positive Treg are functional in in vitro suppressor assays and have similar potency to PI16-negative Treg. The chemokine receptors CCR4 and CCR6 are expressed by more of the PI16-positive CD45RO-positive Treg compared to PI16-negative CD45RO-positive Treg or other PI16-positive Th cells. The PI16-positive Treg showed enhanced in vitro migration towards the chemokines CCL17 and CCL20, suggesting they can migrate to sites of inflammation. We conclude that PI16 identifies a distinct subset of functional memory Treg which can migrate to the sites of inflammation and regulate the pro-inflammatory response at those sites.

Keywords:

Regulatory T cells

Lymphocyte migration

Memory Treg

Peptidase inhibitor 16

1. Introduction:

Regulatory T cells (Treg) are critical for maintaining tolerance and immune homeostasis [1], and the manipulation of Treg numbers or function is seen as a potential strategy for the treatment of autoimmune diseases and graft-versus-host disease [2; 3]. The expression of the transcription factor FOXP3 is a defining characteristic of Treg [4; 5], but viable Treg cannot be isolated based on their expression of FOXP3, as detection of the protein requires the permeabilisation of the cells [6; 7].

The expression of high levels of the interleukin 2 receptor alpha chain (CD25) by CD4-positive lymphocytes is widely used as a surrogate marker for FOXP3 expression to identify Treg [8; 9]. The effectiveness of approach is, however, limited as CD25 expression is also upregulated during T cell activation [10; 11]. More recently, the expression of high levels of CD25 and low levels of the interleukin 7 receptor alpha chain (CD127) has also been used to isolate functional Treg [12; 13]. Again, this approach may be limited, as the expression of CD127 is also reduced following T cell activation [14]. Neither of these combinations is able to define a population of CD4-positive lymphocytes in which all the cells express FOXP3. As FOXP3 is expressed by several functionally distinct subsets of CD4-positive lymphocytes [15; 16], additional cell surface markers are necessary to identify, isolate and characterise pure populations of viable Treg.

In the course of genome wide expression profiling to identify the genes regulated by FOXP3 in human Treg, and identify novel candidate surface proteins specific to human Treg, we identified the peptidase inhibitor PI16 (PI16) as being over-expressed by in vitro expanded Treg derived from cord blood, compared to T helper (Th) cells from the same source [17].

The peptidase inhibitor 16 (PI16; also called CRISP-9 or PSPBP) is a member of the cysteine-rich secretory protein (CRISP) family. The mature PII6 protein is a 436 amino acid polypeptide with three N-linked glycosylation motifs, which is predicted to localize to the plasma membrane through a GPI anchor [18]. PI16 has been identified as a binding protein for "prostate secretory protein of 94 amino acids" (PSP94), and serum levels of PSP94 and PI16 have been identified as biomarkers of recurrence risk in prostate cancer [19], but the expression of PI16 by immune cells has not been reported previously.

The differential expression of PI16 by peripheral blood Treg compared to Th cells was confirmed by qRT-PCR, and cell surface expression was demonstrated by flow cytometry using polyclonal antibodies to human PI16 [17]. To evaluate the potential of PI16 as a marker for circulating Treg, we generated a mouse monoclonal antibody to the human PI16 protein. Here we describe the phenotype and function of the PI16-expressing Treg in adult peripheral blood.

2. Materials and Methods:

2.1 PBMC Isolation

Peripheral blood was collected using lithium-heparin anticoagulant from healthy adult volunteers who gave informed consent, under ethics clearance from the Research Ethics Committee of the Children's, Youth and Women's Health Service, South Australia (REC 2007/11/2010). Mononuclear cells (PBMC) were isolated by density centrifugation over Lymphoprep (Axis-Shield, Oslo, Norway) using standard protocols.

2.2 Antibodies and Chemokines

A mouse polyclonal antibody raised to full-length recombinant PI16 protein was purchased from Abnova (Taipei City, Taiwan). Monoclonal antibodies to CD3, CD4, CD8, CD19, CD25, CD127, CD154, CD45RA, CD45RO, CCR4, CCR6, the Treg cocktail (an optimised mixture of CD4-APC, CD127-FITC and CD25-PE/Cy7) and fluorophore-streptavidin conjugates were obtained from BD Biosciences (San Jose, CA). Monoclonal antibody to FOXP3 (clone PCH101), and FOXP3 staining buffers were purchased from eBioscience (San Diego, CA), biotinylated horse anti-mouse Ig from VECTOR Laboratories (Burlingame, CA), and normal mouse serum from Dako (Glostrup, Denmark). The recombinant chemokines CCL17 (TARC; cat 228-11481-2) and CCL20 (MIP3-alpha; cat 228-11121-2) were obtained from RayBiotech (Norcross, GA).

2.3 Generation of anti-PI16 Monoclonal Antibody

To generate PI16-expressing cells for immunization, the cloned full-length human PI16 coding sequence from Open Biosystems (Thermo Scientific, Waltham, MA) was recloned into a Gateway pDEST40 vector (Invitrogen, Carlsbad, CA). Sub-confluent mouse L929 fibroblast cells (ATCC, Manassas, VA) were transiently transfected with the pDEST40/PI16 vector using Lipofectamine LTX (Invitrogen).

Six-to-8 week old female Balb/c mice were initially immunised by the sub-cutaneous injection of 50 million pDEST40/PI16 transfected L929 cells in 500ul of PBS. The mice were boosted by sub-cutaneous injection of the same number of cells at four weeks and six weeks after the initial immunization. The mice were sacrificed four days after the final boost, and the spleen and a blood sample were collected. Procedures using mice were approved by the Animal Ethics Committee of the Children's, Youth and Women's Health Service, South Australia (AE667).

Hybridomas were generated by fusing splenocytes with SP2/0 myeloma cells (ATCC) at a 10:1 ratio, essentially as described by Brooks et al. [20]. Colonies expressing antibody with similar reactivity to the PI16 polyclonal antibody were identified by flow cytometry and were expanded and twice-cloned by single-cell sorting using a FACSAria II cell sorter equipped with an Automated Cell Deposition Unit (BD Biosciences). By this process, we derived the hybridoma clone P1G5-P2B3-P1H4, which was designated "CRCBT-02-001".

2.4Flow Cytometry

Flow cytometry experiments using an unconjugated antibody were performed using a three-step "high sensitivity" staining protocol [21]. Briefly, PBMC were incubated with

the unconjugated primary antibody for 30min on melting ice, and then washed twice with PBS-azide. Biotinylated horse anti-mouse Ig reagent was added, incubated for 30min on wet ice, and the cells were washed twice with PBS-azide. Normal mouse serum was added (to block free Ig-binding sites on the anti-mouse Ig reagent) and incubated for 10min, prior to the addition of the fluorophore-streptavidin conjugate detection reagent and any directly conjugated antibodies. No wash step was performed between the incubation of the mouse Ig and the addition of the final reagents. The cells were incubated as above and washed twice with PBS-azide

In experiments where antibodies directed against intracellular antigens were used, the surface staining as described above was performed first, and the cells were then permeabilised and stained according to the manufacturer's instructions.

Stained cells were analysed using a FACSCanto flow cytometer (BD Biosciences). The resulting data were analysed using FlowJo software (TreeStar, Ashland, OR).

2.5 Characterisation of Cytokine Secretion

To identify the profile of cytokines secreted by subsets of CD4-positive cells defined using PI16 and CD25, we used a TH1/TH2/TH17 Cytometric Bead Array (CBA; BD Biosciences) to measure the levels of the cytokines IL2, IL4, IL6, IL10, IL17A, TNFalpha and IFNgamma in the supernatant of cells following in vitro stimulation. CD4-positive lymphocytes were isolated from a peripheral blood buffy coat using the RosetteSep CD4+ T cell enrichment kit and were stained with antibodies to CD25 and PI16. The four CD4-positive subsets defined by CD25 and PI16 were sorted under aseptic conditions using a FACSAria II cell sorter.

To stimulate the cells, 5.0 x10^5 of a population of sorted cells were incubated with CD3/CD28 beads (Dynal T cell expander kit; Invitrogen) for 3 days. Unstimulated control cells were incubated without having the beads added. After incubation, the supernatant was collected and assayed for the cytokines using the TH1/TH2/TH17 CBA kit, according to the manufacturer's instructions. For each subset, the cytokine concentrations were calculated by subtracting the concentration value for unstimulated cells from the value for stimulated cells.

2.6 Assay of Lymphocyte Migration

A Transwell-based chemotaxis assay was used to characterize the ability of subsets of CD4-positive cells defined using PI16 and CD25 to migrate in response to chemotactic signals. PBMC (10^6 cells in 100ul of media) were loaded into the upper chamber of Transwell plates with 5um pores (Corning), and chemokines (100ng/ml CCL17, 100ng/ml CCL20 or 100ng/ml of each of CCL17 and CCL20 in a volume of 600ul) were added to the lower chamber. The cells were allowed to migrate for 2 hours at 37C. After the incubation, the cells from the upper and lower chambers were collected and stained with antibodies to CD4, CD25 and PI16. The proportion of cells in each of the four CD4-positive lymphocyte subsets defined by CD25 and PI16 was determined by flow cytometry.

2.7 Assay for Suppression of Effector Cell Proliferation

To evaluate the ability of a candidate population to suppress the proliferation of an effector population, we used a mixed lymphocyte reaction (MLR), in which the cell

division of the Responder cells was analysed by CFSE dilution [22]. The PI16-positive and PI16-negative fractions of the CD4-positive/CD25-bright subset were isolated as Treg sub-populations by enriching CD4-positive lymphocytes from a peripheral blood buffy coat using the RosetteSep CD4+ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada), staining with antibodies to CD25 and PI16, and sorting under aseptic conditions using a FACSAria II cell sorter. CD25-negative Th cells were also collected to act as a control population with no regulatory activity. PBMC from this donor were irradiated (30Gy) for use as the MLR stimulator cells. CD4-positive/CD25-negative "Effectors" from a second unmatched donor were used as the responder population in the MLR. These cells were isolated using magnetic-activated cell sorting (MACS; Miltenyi Biotech, Germany), and labelled with 1mM CFSE in PBS for 10mins at 37°C.

For the suppression assay, various numbers of the PI16-positive Treg, PI16-negative Treg or CD25-negative Th cells were mixed with $1x10^5$ stimulator cells and $2x10^4$ CFSE-labelled Responder cells at Treg:Responder ratios of 1:1 ($2x10^4$ Treg), 1:2 ($1x10^4$ Treg), 1:4 ($5x10^3$ Treg) and 1:8 ($2.5x10^3$ Treg), in a total volume of 200ul of complete RPMI medium. The cell mixtures were stimulated for 5 days using 100ng/ml anti-CD3 monoclonal antibody (OKT3; eBioscience). Control wells comprised stimulated labelled Responder cells alone or unstimulated labelled Responder cells alone. The proliferation of the Responder cells was visualised by the dilution of the CFSE fluorescence with cell division. The suppression of Responder cell proliferation by a Treg population was calculated by determining the relative suppression as described by Venken et al. [22]: 1-

(%CFSE-low Responder cells in the presence of Treg / % CFSE-low Stimulated Responder cells in the absence of Treg), expressed as a percentage.

2.8 Statistical Analysis

The statistical significance of differences between the cell subsets were determined using the Wilcoxon matched-pairs test, using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA)

3. Results:

3.1 Distribution of PI16 expression by mononuclear cells.

The monoclonal antibody CRCBT-02-001 was confirmed to have reactivity to PI16-transfected NIH3T3 mouse fibroblasts, but not to untransfected NIH3T3 cells (Supplementary Figure 1). To determine the cellular distribution of PI16 by the major peripheral blood lymphocyte subsets, we stained PBMC with the CRCBT-002-01 monoclonal antibody and antibodies to CD4, CD8 and CD19 (Supplementary Figure 2). This staining indicated that the majority of PI16-expressing cells were CD4-positive lymphocytes (typically approximately 20% of CD4-positive subset), with the remaining cells being CD8-positive T cells (approximately 10% of the CD8-bright subset). Almost no CD19-positive B cells were stained with the antibody to PI16.

3.2 PI16 expression by CD4-positive T cells

To characterise the expression of PI16 by Treg and other Th cells, we stained peripheral blood mononuclear cells with the CRCBT-002-01 monoclonal antibody and antibodies to CD4, CD25, CD127 and FOXP3. Using the gating strategy of Seddiki et al [13], we found that PI16 is expressed by a higher proportion the CD25-bright/CD127-dim Treg (average 28% PI16-positive) compared to the CD127-positive Th cell subset (average 18.7% PI16-positive) (Figure 1A; n=15, p=0.006). Similar results are seen when PI16 expression by the FOXP3-positive and FOXP3-negative subsets of CD4-positive lymphocytes is compared (Figure 1B; n=8; p=0.008). These results support the initial microarray data [20], but also indicate that the expression of PI16 is not confined to Treg.

3.3 Phenotype of PI16-positive Treg

To further define the phenotype of the PI16-expressing subset of Treg, we stained lymphocytes with antibodies to CD4, CD25, PI16, CD45RA and FOXP3. Following the gating strategy described by Miyara [16], we found that the majority of cells in the FOXP3-positive "activated Treg" (Fraction 2) population expressed PI16, while few "resting Treg" (Fraction 1) expressed PI16 (Figure 2). We also examined the expression by PI16-positive Treg of other T cell markers, including CD45RO, CD45RA, CCR4 and CCR6 (Figure 3). The differential staining with suggests that the CD25-positive/PI16-positive cells comprise a subset of memory Treg which is phenotypically distinct from both the PI16-negative Treg population and from the other PI16-positive Th cells.

3.4 Correlation between PI16 and FOXP3 expression

We next used antibodies to CD4, CD25, PI16 and FOXP3 to compare the expression of FOXP3 by the PI16-positive and PI16-negative Treg subsets. The mean fluorescence intensity (MFI) of FOXP3 staining was higher in the CD25-positive/PI16-positive fraction (average population MFI of 2759,) compared to the CD25-positive/PI16-negative fraction (average MFI of 1908) (Figure 4; n=6, p=0.03). The level of FOXP3 in the CD25-negative/PI16-positive fraction (average MFI of 884) was similar to the background levels of the CD25-negative/PI16-negative Effector cell fraction (average MFI of 659). The higher expression of FOXP3 suggests that the co-expression of PI16 and CD25 may identify a specific subset of Treg in peripheral blood.

3.5 Chemotaxis by PI16-positive Treg

To characterise the ability of PI16-positive memory Treg to respond to signals from a site of inflammation, we used a Transwell-based chemotaxis assay to examine the migration of PBMC towards CCL17 and CCL20. We found that the proportion of PI16-positive memory Treg in the lower Transwell chamber was increased after incubation in the presence of these chemokines either alone or in combination (Figure 5).

3.6 Cytokine expression by PI16-positive Treg

We utilised a TH1-Th2-TH17 Cytometric Bead Array (CBA) to determine which cytokines were expressed by PI16-expressing Treg following stimulation through the T cell receptor. These experiments showed that the cytokine expression profile of the PI16-positive Treg cells was the same as the PI16-negative Treg cells, and that the PI16-positive Th cells expressed the same cytokines as their PI16-negative counterparts (data not shown).

3.7 Suppressor function of PI16-positive Treg

We utilised a CFSE-based MLR assay to determine whether the PI16-expressing Treg were able to suppress the proliferation of CD3-stimulated Responder Th cells. Over several Treg:Thelper ratios, we found that freshly-isolated PI16-expressing Treg from peripheral blood were able to suppress the proliferation of stimulated CD25-negative Th cells to a similar extent as PI16-negative Treg from the same source (Figure 6). These data confirm that the PI16-positive/CD25-positive cells are functional Treg, but also indicate that their suppressive activity is equivalent to their PI16-negative counterparts.

4. Discussion:

Imbalances in the frequency and/or function of Treg have been reported in many diseases [23; 24; 25; 26]. The difficulty of using FOXP3 to isolate viable Treg for use in functional assays has lead to many candidate cell surface molecules being evaluated as surrogates for FOXP3 expression, but with limited success. As part of a gene discovery program, we identified PI16 as being over-expressed by cord-blood-derived Treg compared to Th cells [17]. We generated a monoclonal antibody to PI16, and using this we have determined that PI16 is expressed by 15-60% of CD25-bright CD4-positive lymphocytes in adult peripheral blood, all of which express high levels of the transcription factor FOXP3. Further analysis suggests that the PI16-positive memory Treg are part of the FOXP3-bright "activated Treg" (aTreg) population described by Miyara et al.[16].

In vitro analysis of sorted cells stimulated through CD3 and CD28 showed that the PI16-positive Treg secrete a profile of cytokines which is similar to the PI16-negative Treg. Furthermore, the PI16-positive/CD25-bright CD4-positive cells were able to suppress the activation and proliferation of CD4-positive "Effector" cells, indicating that this population contains functional Treg. These results suggest that the ability of the Treg subset identified by the co-expression of PI16 and CD25 to regulate an immune response is equivalent to that of the PI16-negative Treg.

The differential expression of chemokine receptors is indicative of the migration capability of subsets of Th cells [27; 28], and can be used to identify functional subsets of Th cells [15; 29; 30]. We found that the profile of chemokine receptors expressed by the PI16-positive memory Treg was different to both the PI16-negative memory Treg and the other PI16-positive Th cells. The expression of chemokine receptors CCR4 and CCR6, and their ability to migrate toward CCL17 and CCL20 suggest that the PI16-positive memory Treg are able to respond to the same inflammation signals as Th17 cells. The co-migration of Th17 cells and PI16-expressing memory Treg provides a means for the immune system to regulate the pro-inflammatory activity of the Th17 cells. Defects which prevent the migration of PI16-positive memory Treg to inflamed sites may exacerbate the development of autoimmune pathologies.

In conclusion, we have determined that the co-expression of PI16 and CD25 identifies a distinct subset of functional FOXP3-positive memory Treg, which demonstrate an enhanced migration in response to the chemokines CCL17 and CCL20. This suggests that PI16-positive memory Treg are capable of migrating toward the same sites of inflammation as other Th cells which express these receptors, including pro-inflammatory Th17 cells, and that the PI16-positive Treg may be preferentially recruited in order regulate the immune response at those sites.

Acknowledgements:

This work was funded by the Co-operative Research Centre for Biomarker Translation.

References:

[1] K. Wing, and S. Sakaguchi, Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol 11 (2010) 7-13.

[2] P. Hoffmann, J. Ermann, and M. Edinger, CD4+CD25+ regulatory T cells in hematopoietic stem cell transplantation. Curr Top Microbiol Immunol 293 (2005) 265-85.

[3] A.L. Putnam, T.M. Brusko, M.R. Lee, W. Liu, G.L. Szot, T. Ghosh, M.A. Atkinson, and J.A. Bluestone, Expansion of human regulatory T-cells from patients with type 1 diabetes. Diabetes 58 (2009) 652-62.

[4] C.L. Bennett, J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs, The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 27 (2001) 20-1.

[5] J.D. Fontenot, M.A. Gavin, and A.Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4 (2003) 330-6.

[6] G. Roncador, P.J. Brown, L. Maestre, S. Hue, J.L. Martinez-Torrecuadrada, K.L. Ling, S. Pratap, C. Toms, B.C. Fox, V. Cerundolo, F. Powrie, and A.H. Banham, Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. Eur J Immunol 35 (2005) 1681-91.

[7] P. Presicce, M.E. Moreno-Fernandez, C.S. Lages, K.I. Orsborn, and C.A. Chougnet, Association of two clones allows for optimal detection of human FOXP3. Cytometry A 77 (2010) 571-9.

[8] C. Baecher-Allan, J.A. Brown, G.J. Freeman, and D.A. Hafler, CD4+CD25high regulatory cells in human peripheral blood. J Immunol 167 (2001) 1245-53.

[9] P. Hoffmann, R. Eder, T.J. Boeld, K. Doser, B. Piseshka, R. Andreesen, and M. Edinger, Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. Blood 108 (2006) 4260-7.

[10] D.A. Cantrell, and K.A. Smith, Transient expression of interleukin 2 receptors. Consequences for T cell growth. J Exp Med 158 (1983) 1895-911.

[11] T.A. Poulton, A. Gallagher, R.C. Potts, and J.S. Beck, Changes in activation markers and cell membrane receptors on human peripheral blood T lymphocytes during cell cycle progression after PHA stimulation. Immunology 64 (1988) 419-25.

[12] W. Liu, A.L. Putnam, Z. Xu-Yu, G.L. Szot, M.R. Lee, S. Zhu, P.A. Gottlieb, P. Kapranov, T.R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D.M. Soper, S.F. Ziegler, and J.A. Bluestone, CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 203 (2006) 1701-11.

[13] N. Seddiki, B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S.I. Alexander, R. Nanan, A. Kelleher, and B. Fazekas de St

Groth, Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 203 (2006) 1693-700.
[14] N.L. Alves, E.M. van Leeuwen, I.A. Derks, and R.A. van Lier, Differential regulation of human IL-7 receptor alpha expression by IL-7 and TCR signaling. J Immunol 180 (2008) 5201-10.
[15] F. Sallusto, and A. Lanzavecchia, Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. Eur J Immunol 39 (2009) 2076-82.
[16] M. Miyara, Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, and S. Sakaguchi, Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 30 (2009) 899-911.
[17] T.J. Sadlon, B.G. Wilkinson, S. Pederson, C.Y. Brown, S. Bresatz, T. Gargett, E.L. Melville, K. Peng, R.J. D'Andrea, G.G. Glonek, G.J. Goodall, H. Zola, M.F. Shannon, and S.C. Barry, Genome wide identification of human FOXP3 target genes in natural regulatory T cells. J Immunol 185 (2010) 1071-1081.
[18] J.R. Reeves, J.W. Xuan, K. Arfanis, C. Morin, S.V. Garde, M.T. Ruiz, J. Wisniewski, C. Panchal, and J.E. Tanner, Identification, purification and characterization of a novel human blood protein with binding affinity for prostate secretory protein of 94 amino acids. Biochem J 385 (2005) 105-14.
[19] J.R. Reeves, H. Dulude, C. Panchal, L. Daigneault, and D.M. Ramnani, Prognostic value of prostate secretory protein of 94 amino acids and its binding protein after radical prostatectomy. Clin Cancer Res 12 (2006) 6018-22.
[20] D.A. Brooks, I. Beckman, J. Bradley, P.J. McNamara, M.E. Thomas, and H. Zola, Human lymphocyte markers defined by antibodies derived from somatic cell hybrids. I. A hybridoma secreting antibody against a marker specific for human B lymphocytes. Clin Exp Immunol 39 (1980) 477-85.
[21] H. Zola, S.H. Neoh, B.X. Mantzioris, J. Webster, and M.S. Loughnan, Detection by immunofluorescence of surface molecules present in low copy numbers. High sensitivity staining and calibration of flow cytometer. J Immunol Methods 135 (1990) 247-55.
[22] K. Venken, M. Thewissen, N. Hellings, V. Somers, K. Hensen, J.L. Rummens, and P. Stinissen, A CFSE based assay for measuring CD4+CD25+ regulatory T cell mediated suppression of auto-antigen specific and polyclonal T cell responses. J Immunol Methods 322 (2007) 1-11.
[23] N. Eastaff-Leung, N. Mabarrack, A. Barbour, A. Cummins, and S. Barry, Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. J Clin Immunol 30 (2010) 80-9.
[24] M. Miyara, Z. Amoura, C. Parizot, C. Badoual, K. Dorgham, S. Trad, D. Nochy, P. Debre, J.C. Piette, and G. Gorochov, Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 175 (2005) 8392-400.
[25] A.M. Wolf, D. Wolf, M. Steurer, G. Gastl, E. Gunsilius, and B. Grubeck-Loebenstein, Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 9 (2003) 606-12.

[26] S. Lindley, C.M. Dayan, A. Bishop, B.O. Roep, M. Peakman, and T.I. Tree, Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 54 (2005) 92-9.
[27] S.K. Bromley, T.R. Mempel, and A.D. Luster, Orchestrating the orchestrators: chemokines in control of T cell traffic. Nat Immunol 9 (2008) 970-80.
[28] H.W. Lim, J. Lee, P. Hillsamer, and C.H. Kim, Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. J Immunol 180 (2008) 122-9.
[29] S.P. Singh, H.H. Zhang, J.F. Foley, M.N. Hedrick, and J.M. Farber, Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol 180 (2008) 214-21.
[30] J. Yamamoto, Y. Adachi, Y. Onoue, Y.S. Adachi, Y. Okabe, T. Itazawa, M. Toyoda, T. Seki, M. Morohashi, K. Matsushima, and T. Miyawaki, Differential expression of the chemokine receptors by the Th1- and Th2-type effector populations within circulating CD4+ T cells. J Leukoc Biol 68 (2000) 568-74.

Figure Legends:

Figure 1: Expression of cell surface PI16 by Treg and Th cell subsets of CD4-positive lymphocytes, demonstrated using by the anti-PI16 monoclonal antibody CRCBT-02-001. Only the CD4-positive lymphocytes are shown. a. (A) Cell surface PI16 is present on a higher proportion of CD25-bright/CD127-dim Treg (upper row, centre panel) than CD127-positive Th cells (upper row, right panel). The values shown in each gate are the mean and SD of the percent in that gate for n=15 different donors. (B) Cell surface PI16 is present on a higher proportion of FOXP3-positive CD4-positive lymphocytes (lower, centre panel) than FOXP3-positive CD4-positive lymphocytes (lower row, right panel). The values shown in each gate are the mean and SD of the percent in that gate for n=8 different donors.

Figure 2: Expression of PI16 and FOXP3 by six fractions of naive and memory Treg defined using the gating strategy described by Miyara [16]. Only the CD4-positive lymphocytes are shown. The values shown are the means and SD for n=5 different donors. The fractions of CD4-positive lymphocytes (Fraction 1 (Fr1) to Fraction 6) were defined using the expression of CD45RA and CD25. The expression of PI16 (CRCBT-02-001) and FOXP3 by the cells in each of these fractions is shown. In each fraction, the PI16-positive and PI16-negative cells express similar levels of FOXP3, with the highest levels of FOXP3 expression seen in the memory aTreg fraction (Fraction 2).

Figure 3: Phenotype of CD4-positive lymphocytes defined using the co-expression of CD25 and PI16 (CRCBT-02-001). Only the CD4-positive lymphocytes are shown. The values shown are the means and SD for n=7 different donors. The four subsets of CD4-positive lymphocytes were defined as shown in Figure 2. (A) Co-expression of CD45RA and CD45RO by the four subsets defined using the co-expression of CD25 and PI16, showing that the cells in the CD25-positive/PI16-positive subset have a memory (CD45RO-positive/CD45RA-negative) phenotype. (B) Expression of CD45RO and CCR4 by the four subsets, showing that CCR4 is expressed by a higher proportion of memory CD25-positive/PI16-positive cells, compared to the memory component of the other subsets. (C) Expression of CD45RO and CCR6 by the four subsets, showing that CCR6 is expressed by a higher proportion of memory CD25-positive/PI16-positive cells, compared to the memory component of the other subsets.

Figure 4: Expression of FOXP3 by subsets of CD4-positive lymphocytes defined using the co-expression of CD25 and PI16 (CRCBT-02-001). Only the CD4-positive lymphocytes are shown. The values shown are the means and SD for n=6 different donors. The cells in the CD25-positive/PI16-positive Treg fraction (heavy solid line; average population MFI of 2759) express higher levels of FOXP3 than cells in the CD25-positive/PI16-negative Treg fraction (dashed line; average MFI of 1908). FOXP3 staining was lowest in the CD25-negative/PI16-positive (light solid line; average MFI of 884) and CD25-negative/PI16-negative (dotted line; average MFI of 659) populations.

Figure 5: Migration of subsets of CD4-positive lymphocytes defined using the co-expression of CD25 and PI16 in response to the chemokines CCL17 and CCL20. The graphs show the proportion of CD4-positive cells in each subset in the upper chambers (light bars) and lower chambers (dark bars) after the incubation, and the error bars show the SEM for six experiments using cells from different donors. The data indicate that there is a preferential migration of the CD25-positive/PI16-positive cells in response to each of the chemokines.

Figure 6: Suppression of CD3 stimulation-induced proliferation of CD4-positive/CD25-negative Responder cells by freshly isolated PI16-positive and PI16-negative Treg. The PI16-positive and PI16-negative Treg were sorted using the gates shown in Figure 2. (A) CFSE fluorescence of the Responder Th cells in the presence of Suppressor cells comprising PI16-positive Treg (upper row), PI16-negative Treg (middle row) or control CD25-negative Th cells (bottom row) at Suppressor to Responder ratios from 1:1 (left column) to 1:8 (right column). Data representative of 5 experiments are shown. The region marker and percentages indicate the proportion of cells which did not proliferate (right side region) and cells that have divided (left side region). The far right panels show the CFSE staining of the unstimulated Responder Th cells (left-side plot) and CD3-stimulated Responder Th cells in the absence of other cells (right-side plot). (B) Relative suppression Responder Th proliferation by PI16-positive and PI16-negative Treg. The values shown are the mean and SEM for 4 different donors. The results suggest that fresh adult PI16-positive and PI16-negative Teg have the same capacity to suppress the proliferation of the Responder cells.

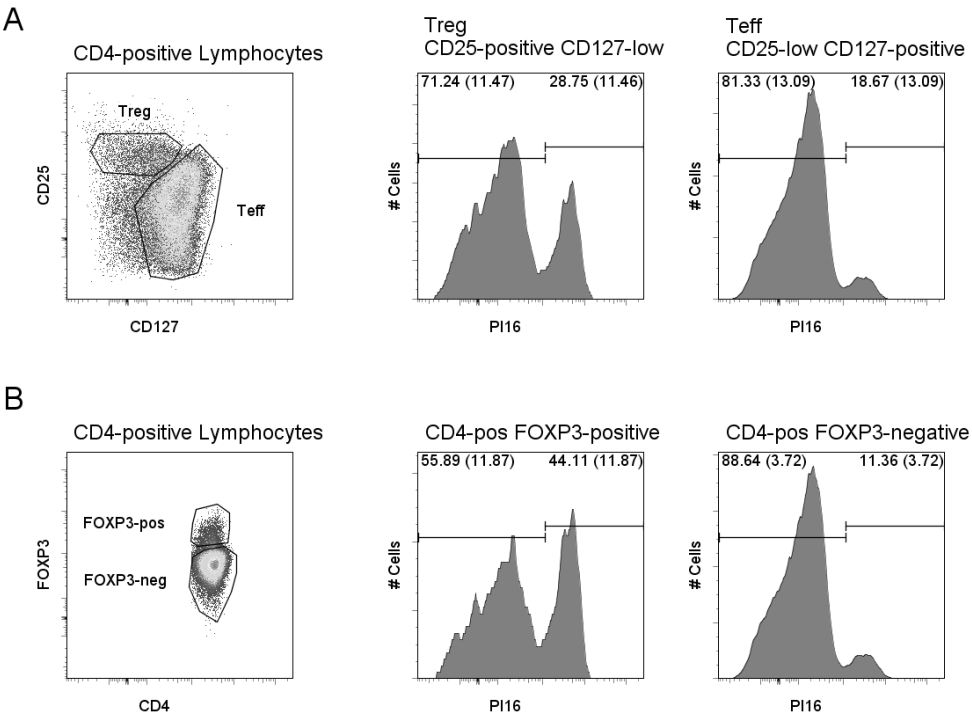

A
CD4-positive Lymphocytes
Treg
Teff
CD25
CD127
Treg
CD25-positive CD127-low
71.24 (11.47)
28.75 (11.46)
Cells
PI16
Teff
CD25-low CD127-positive
81.33 (13.09)
18.67 (13.09)
Cells
PI16
B
CD4-positive Lymphocytes
FOXP3-pos
FOXP3-neg
FOXP3
CD4
CD4-pos FOXP3-positive
55.89 (11.87)
44.11 (11.87)
Cells
PI16
CD4-pos FOXP3-negative
88.64 (3.72)
11.36 (3.72)
Cells
PI16

Fig 1

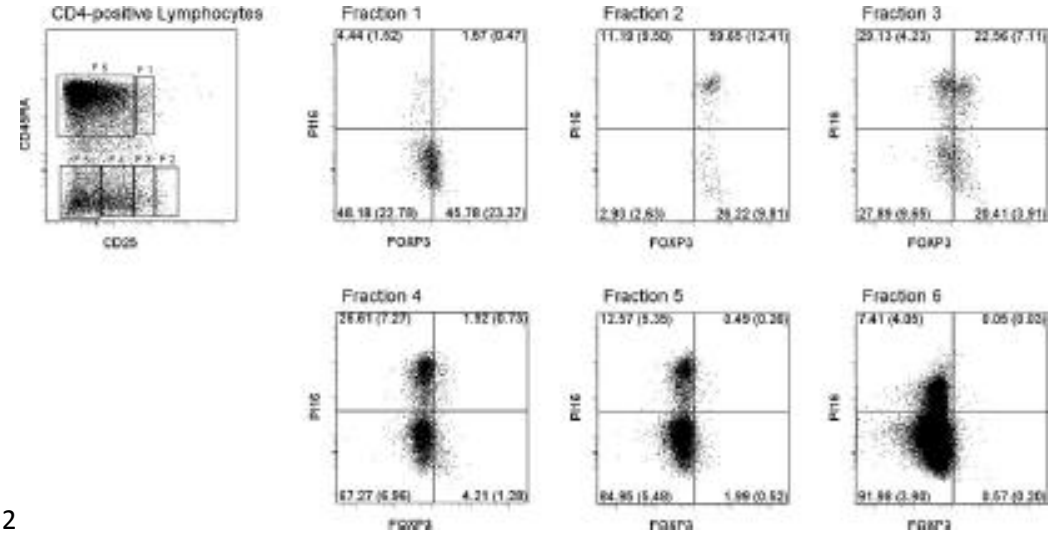

CD4-positive Lymphocytes
CD45RA
CD25
Fraction 1
4.44 (1.52)
1.57 (0.47)
46.19 (22.79)
45.79 (23.37)
FOXP3
Fraction 2
11.19 (5.50)
59.65 (12.41)
2.93 (2.63)
26.22 (9.81)
FOXP3
Fraction 3
29.13 (4.23)
22.56 (7.11)
27.89 (9.55)
20.41 (3.91)
FOXP3
Fraction 4
26.61 (7.27)
1.92 (0.73)
67.27 (6.96)
4.21 (1.28)
Fraction 5
12.37 (5.35)
0.45 (0.26)
84.95 (5.48)
1.99 (0.52)
Fraction 6
7.41 (4.05)
0.05 (0.02)
91.88 (3.90)
0.67 (0.26)

Fig 2

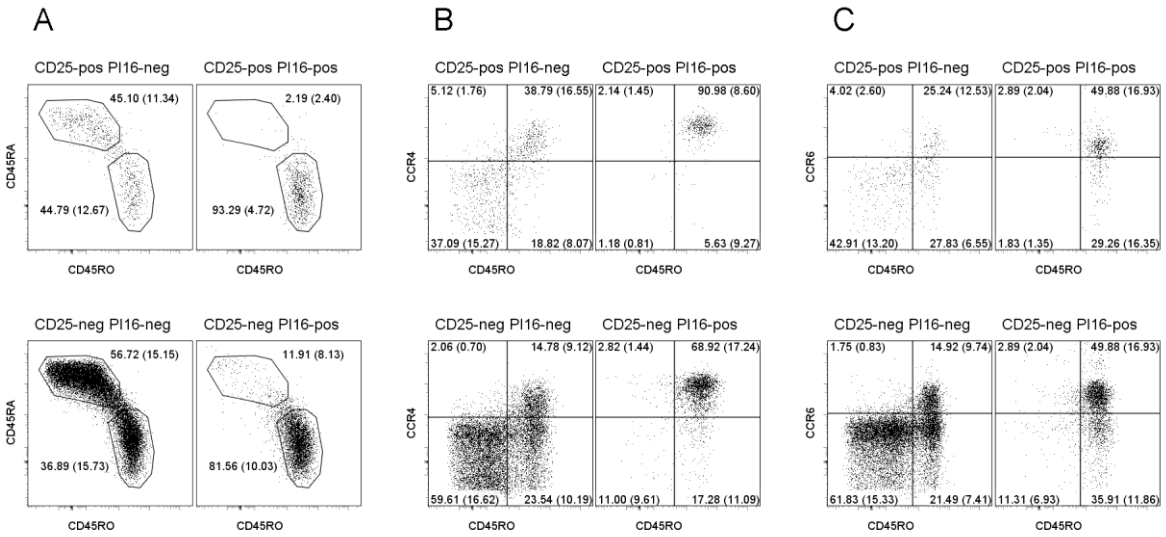
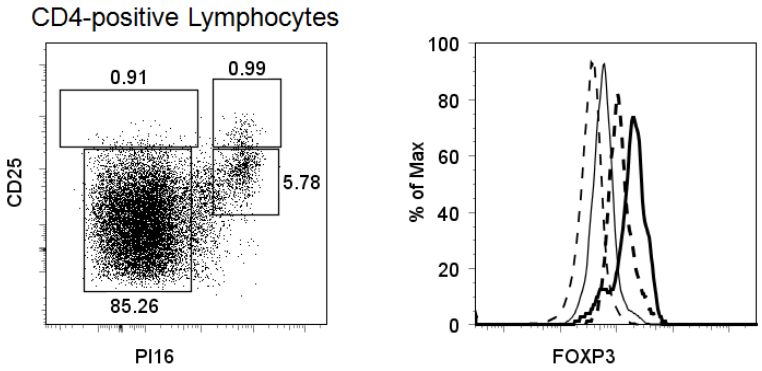

A
CD25-pos PI16-neg
CD25-pos PI16-pos
45.10 (11.34)
44.79 (12.67)
2.19 (2.40)
93.29 (4.72)
CD45RA
CD45RO
CD25-neg PI16-neg
CD25-neg PI16-pos
56.72 (15.15)
36.89 (15.73)
11.91 (8.13)
81.56 (10.03)
B
CD25-pos PI16-neg
CD25-pos PI16-pos
5.12 (1.76)
38.79 (16.55)
37.09 (15.27)
18.82 (8.07)
2.14 (1.45)
90.98 (8.60)
1.18 (0.81)
5.63 (9.27)
CCR4
CD25-neg PI16-neg
CD25-neg PI16-pos
2.06 (0.70)
14.78 (9.12)
59.61 (16.62)
23.54 (10.19)
2.82 (1.44)
68.92 (17.24)
11.00 (9.61)
17.28 (11.09)
C
CD25-pos PI16-neg
CD25-pos PI16-pos
4.02 (2.60)
25.24 (12.53)
42.91 (13.20)
27.83 (6.55)
2.89 (2.04)
49.88 (16.93)
1.83 (1.35)
29.26 (16.35)
CCR6
CD25-neg PI16-neg
CD25-neg PI16-pos
1.75 (0.83)
14.92 (9.74)
61.83 (15.33)
21.49 (7.41)
2.89 (2.04)
49.88 (16.93)
11.31 (6.93)
35.91 (11.86)

Fig 3



Subset	Line	mean MFI (SD)
CD25-pos PI16-neg	– – – – – –	1908 (564)
CD25-pos PI16-pos	———	2759 (661)
CD25-neg PI16-neg	- - - - - - - - - - - -	659 (281)
CD25-neg PI16-pos	———	884 (334)

Fig 4

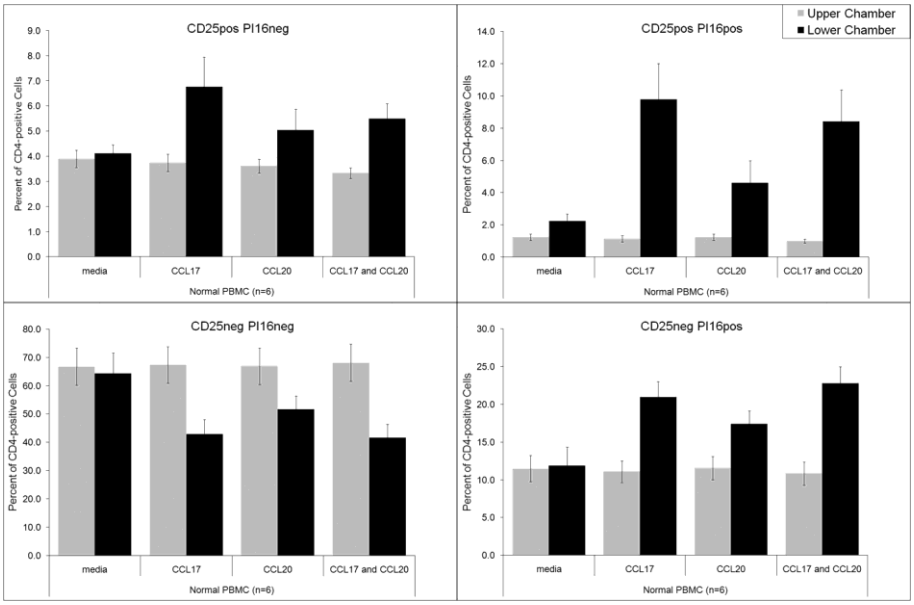

CD25pos PI16neg
CD25pos PI16pos
CD25neg PI16neg
CD25neg PI16pos
Upper Chamber
Lower Chamber
Percent of CD4-positive Cells
media
CCL17
CCL20
CCL17 and CCL20
Normal PBMC (n=6)

Fig 5

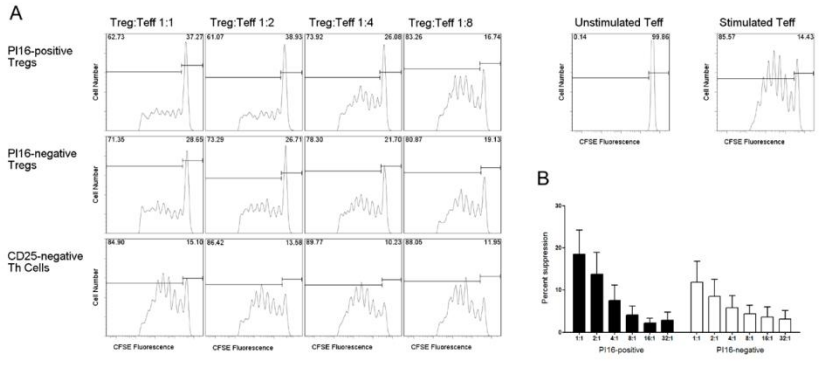

A
Treg:Teff 1:1
Treg:Teff 1:2
Treg:Teff 1:4
Treg:Teff 1:8
Unstimulated Teff
Stimulated Teff
PI16-positive Tregs
PI16-negative Tregs
CD25-negative Th Cells
Cell Number
CFSE Fluorescence
62.73
37.27
61.07
38.93
73.92
26.08
83.26
16.74
0.14
99.86
85.57
14.43
71.35
28.65
73.29
26.71
78.30
21.70
80.87
19.13
84.90
15.10
86.42
13.58
89.77
10.23
88.05
11.95
B
Percent suppression
1:1
2:1
4:1
8:1
16:1
32:1
PI16-positive
PI16-negative

Fig 6