Video Article

New Tools to Expand Regulatory T Cells from HIV-1-infected Individuals

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Abstract

CD4+ Regulatory T cells (Tregs) are potent immune modulators and serve an important function in human immune homeostasis. Depletion of Tregs has led to measurable increases in antigen-specific T cell responses in vaccine settings for cancer and infectious pathogens. However, their role in HIV-1 immuno-pathogenesis remains controversial, as they could either serve to suppress deleterious HIV-1-associated immune activation and thus slow HIV-1 disease progression or alternatively suppress HIV-1-specific immunity and thereby promote virus spread. Understanding and modulating Treg function in the context of HIV-1 could lead to potential new strategies for immunotherapy or HIV vaccines. However, important open questions remain on their role in the context of HIV-1 infection, which needs to be carefully studied.

Representing roughly 5% of human CD4+ T cells in the peripheral blood, studying the Treg population has proven to be difficult, especially in HIV-1 infected individuals where HIV-1-associated CD4 T cell and with that Treg depletion occurs. The characterization of regulatory T cells in individuals with advanced HIV-1 disease or tissue samples, for which only very small biological samples can be obtained, is therefore extremely challenging. We propose a technical solution to overcome these limitations using isolation and expansion of Tregs from HIV-1-positive individuals.

Here we describe an easy and robust method to successfully expand Tregs isolated from HIV-1-infected individuals *in vitro*. Flow-sorted $CD3^{+}CD4^{+}CD25^{+}CD127^{low}$ Tregs were stimulated with anti-CD3/anti-CD28 coated beads and cultured in the presence of IL-2. The expanded Tregs expressed high levels of FOXP3, CTLA4 and HELIOS compared to conventional T cells and were shown to be highly suppressive. Easier access to large numbers of Tregs will allow researchers to address important questions concerning their role in HIV-1 immunopathogenesis. We believe answering these questions may provide useful insight for the development of an effective HIV-1 vaccine.

Video Link

The video component of this article can be found at http://www.jove.com/video/50244/

Introduction

With more than 34 million individuals living with HIV/AIDS worldwide and an estimated 2.5 million people newly infected in 2011, the need for an effective HIV vaccine to curb the worldwide HIV epidemic remains paramount. However, despite three decades of intense research efforts, the HIV-1 vaccine efficacy trials to date have resulted in only modest protection [1-3] and the correlates of protective immunity remain poorly understood. Elucidating the nature of the immune response needed for protection is essential for the strategic design of an effective HIV-1 vaccine and other immunotherapeutic strategies targeting HIV-1 infection.

Natural CD4+ regulatory T cells (Tregs) are critical to the maintenance of immune cell homeostasis by controlling excessive immune activation, thus limiting immune-mediated tissue damage. However, they can also suppress immune responses against pathogens and prevent their clearance. Cancer and Hepatitis B vaccine studies have demonstrated that decreasing the activity of Tregs can enhance vaccine response and antigen-specific immunity against viruses [4-7]. However, in the context of HIV-1 infection, the exact impact of regulatory T cells remains incompletely understood. Tregs were shown to decrease virus replication in activated T cells [8] and possibly impact immune activation [9]. They were also shown to suppress HIV-1-specific immune responses, which could have negative outcomes for disease progression [10,11]. Thus, before being able to modulate Treg activity to enhance the efficacy of an HIV-1 vaccine, it is important to gain further insight into their function in this disease context.

Human CD4+ regulatory T cells are a relatively scarce cell population, representing about 5% of CD4+ T cells in the peripheral blood, and their absolute numbers further decrease with HIV-associated CD4+ T cell depletion [12]. Current assays to assess Treg function, such as T cell

proliferation assays with Treg co-culture, use relatively large cell numbers [12]. Therefore, characterizing function and specificity of regulatory T cells in individuals with advanced HIV-1 disease has been challenging, despite their importance for HIV pathogenesis.

The *ex vivo* isolation and expansion of Tregs from HIV-1 patients could represent a solution to overcome some of these limitations. Here we describe an easy and robust protocol to expand functional Tregs derived from HIV-1 infected individuals *in vitro*; we further explain how to phenotype them and test their suppressive function using flow cytometric assays. We believe this protocol will facilitate access to Tregs and help understanding their role in HIV-1 disease progression.

Protocol

1. Regulatory T cell isolation from HIV-1 Positive Blood

1. Carefully transfer blood, collected in ACD tubes, into a 50 ml conical tube for a final volume of 15 ml blood per tube.
2. Add 25 µl/ml of blood of RosetteSep Human CD4+ T Cell Enrichment Cocktail, mix carefully and incubate 20 min at room temperature.
3. Add 15 ml of PBS/2% FBS to the blood and mix carefully. Layer the diluted blood sample on top of 15 ml of Histopaque at room temperature in a 50 ml conical tube. Spin the conical tube for 20 min at 1,200 x g with a slow start and no brakes.
4. Transfer the $CD4^+$ T cell enriched PBMC layer in a new 50 ml conical tube, wash the cells by adding PBS/2% FBS and spin them down for 10 min at 1,200 x g. Then count the cells, wash again and resuspend the cells at about 20 x 10^6/200 µl.
5. Add the following antibodies (concentration):

   anti-CD3-Phycoerythrin-Cyanine 7 (PE-Cy7) (1/100)

   anti-CD4-Fluorescein Isothiocyanate (FITC) (1/40)

   anti-CD25-Allophycocyanin (APC) (1/40)

   anti-CD127-Phycoerythrin (PE) (1/20)

   Incubate 30 min in the dark at 4 °C
6. Wash the cells with PBS/2% FBS. Resuspend the cells at 20 x 10^6/ml in PBS/2% FBS and filter them on a 35 µm nylon mesh.
7. Using a FACS Aria cell sorter equipped for handling biohazardous material, sort the $CD3^+CD4^+CD25^+CD127^{low}$ Treg in X-VIVO 15 media (see gating strategy in **Figure 1**). Conventional T cells ($CD3^+CD4^+CD25^-CD127^+$) can be isolated and expanded as negative controls.

2. Cell Culture

1. After isolation, wash the Treg with X-VIVO 15 media.
2. Resuspend the cells at 250 x 10^3/ml in X-VIVO 15 media complemented with 10% Human Serum and Penicillin-Streptomycin (50 U/ml).
3. Wash Human T-Activator CD3/CD28 beads according to manufacturer's protocol. Add beads to isolated Tregs at a ratio of 1:1 bead per cell.
4. After two days of culture, double the media volume and add IL-2 (300 U/ml).
5. Culture the Tregs for 2 weeks. Change media (X-VIVO 15/Human serum/P/S/IL-2) at days 5, 7, 9, 12. Add beads at a 1:1 ratio at day 9. When changing media, keep cells at 250 x 10^3/ml.

3. Phenotyping

At the end of the expansion culture, expanded $CD3^+CD4^+CD25^+CD127^{low}$ Treg can be phenotyped by flow cytometry and compared to expanded $CD3^+CD4^+CD25^-CD127^+$ conventional T cells as a control.

1. Harvest expanded Tregs/Tconvs and wash them in PBS. Label dead cells using the LIVE/DEAD Fixable Violet Dead Cell Stain Kit according to manufacturer's protocol. Wash the cells in PBS/2% FBS.
2. Add the following antibodies (concentration):

   anti-CD3-PECy7 (1/100)

   anti-CD4-Qdot-655 (1/200)

   anti-CD25-PECy5 (1/100)

   Incubate 30 min in the dark at 4 °C
3. Wash the cells and perform the intracellular staining using the Foxp3/ Transcription Factor Staining Buffer Set according to manufacturer's protocol and the following antibodies:

   anti-FOXP3-PE (1/50)

   anti-HELIOS-FITC (1/40)

anti-CTLA4-APC (1/20)

Acquire the data on a flow cytometer.

4. Suppression Assay

At the end of the expansion culture, the suppressive function *i.e.* the capacity of the expanded Treg isolated from HIV-1 positive individuals to suppress the proliferation of activated T cells can be assessed *in vitro*.

1. Thaw autologous cryopreserved *ex vivo* PBMCs. Leave them for about 3 hr in a 37 °C incubator in RPMI 1640 medium containing penicillin/streptomycin, L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (=R+ media), and 10% FBS (=R10 media).
2. Label the dead cells using the LIVE/DEAD Fixable Violet Dead Cell Stain Kit according to the manufacturer's protocol. Wash the cells in PBS/2% FBS.
3. Incubate the cells with anti-CD3-PECy7 for 30 min in the dark at 4 °C. Wash the cells with PBS/2% FBS. Resuspend the cells in PBS/2% FBS and filter them on a 35 µm nylon mesh.
4. Using a FACS Aria cell sorter equipped for handling biohazardous material, sort the viable CD3+ T cells in R10 media.
5. Label the T cells with a cell tracing reagent such as CellTrace Violet or Vybrant CFDA SE Cell Tracer at 5 µM diluted in PBS for 7 min at 37 °C according to the manufacturer's protocol. Resuspend cells in R+ media supplemented with 10% human serum (=hR10 media) at 1 x 10^6/ml.
6. Harvest the expanded Tregs, resuspend the cells at 0.5 x 10^6/ml in hR10 and prepare dilutions at 0.25 x 10^6/ml and 0.125 x 10^6/ml.
7. Prepare anti-CD2/anti-CD3/anti-CD28 microbeads according to the manufacturer's protocol, resuspend the microbeads at 0.75 x 10^6/ml and prepare dilutions at 0.625, 0.562, 0.5 x 10^6/ml in hR10 media.
8. In a 96 wells round bottom plate, transfer cells and beads according to the following plan:

T cells:Treg ratio	**1:0**	**1:1/2**	**1:1/4**	**1:1/8**
T cells (50 µl)	1 x 10^6/ml	1 x 10^6/ml	1 x 10^6/ml	1 x 10^6/ml
Tregs (50 µl)	no	0.5 x 10^6/ml	0.25 x 10^6/ml	0.125 x 10^6/ml
Beads (100 µl)	0.5 x 10^6/ml	0.75 x 10^6/ml	0.625 x 10^6/ml	0.562 x 10^6/ml
hR10 (50 µl)	yes	no	no	no
i.e.				
T cells	50 x 10^3	50 x 10^3	50 x 10^3	50 x 10^3
Tregs	0	25 x 10^3	12.5 x 10^3	6.25 x 10^3
Beads	50 x 10^3	75 x 10^3	62.5 x 10^3	56.25 x 10^3

9. After 4 days of culture, wash the cells and incubate them for 30 min at 4 °C with the following antibodies:

   anti-CD3-PECy7 (1/100)

   anti-CD4-APC (1/100)

   anti-CD8-AF700 (1/100)

Acquire the data on a flow cytometer. Use the FlowJo proliferation platform to calculate the percentage of divided cells.

Representative Results

The expression of interleukin 2 receptor (CD25) and the interleukin 7 receptor (CD127) have been described as reliable surface markers to identify functional Treg populations [13] and have been shown to correlate with $CD4^+CD25^+FOXP3^+$ Tregs [9,12]. **Figure 1** represents the gating strategy used to flow-sort single $CD3^+CD4^+CD25^+CD127^{low}$ Tregs from PBMC isolated from an HIV-1-positive individual. The CD25/CD127 antibody clones and their conjugate are crucial to reach a good separation of the Treg population; we found the CD25 APC (eBioscience)/CD127 PE (BD Pharmingen) combination to work well in our hands.

In a representative example (**Figure 2**), the Treg expansion fold change reached 765 after 14 days of culture. The expansion fold change can vary from one individual to another and can be as low as *ca.* 100 or reach *ca.* 2500. The human serum lot used in the media can also impact the expansion fold change; it is suggested to screen different lots of human serum to achieve optimal expansion capacity.

Examples of histograms staining for selected markers of Tregs (CTLA4, FOXP3 and HELIOS) are represented in **Figure 3**. Since most of the Treg markers are also expressed by activated conventional T cells, displaying the mean fluorescence intensity (MFI) may be more informative than the frequency of the positive population in this context.

A fundamental characteristic of regulatory T cells is their capacity to suppress cellular immune responses. Using a standardized flow-based T cell proliferation assay (**Figure 4**) expanded Tregs can suppress the proliferation of activated autologous T cells. Proliferation of T cells

are assayed by a membrane-permeant reactive tracer (CellTrace Violet) after 4 days of co-culture with different anti-CD2/anti-CD3/antiCD28 microbeads activated T cells to Tregs ratios. The numbers of divided cells were calculated using the FlowJo Proliferation platform. CD2 stimulation was shown to up-regulate FOXP3 expression and lead to enhanced Treg function [14]. In our hands the use of microbeads gives a stronger and more consistent proliferation than plate-bound anti-CD3 with or without the addition of anti-CD28. For the suppression assay, media is complemented with human serum, we suggest to screen different serum lots to achieve optimal and constant suppressive capacity.

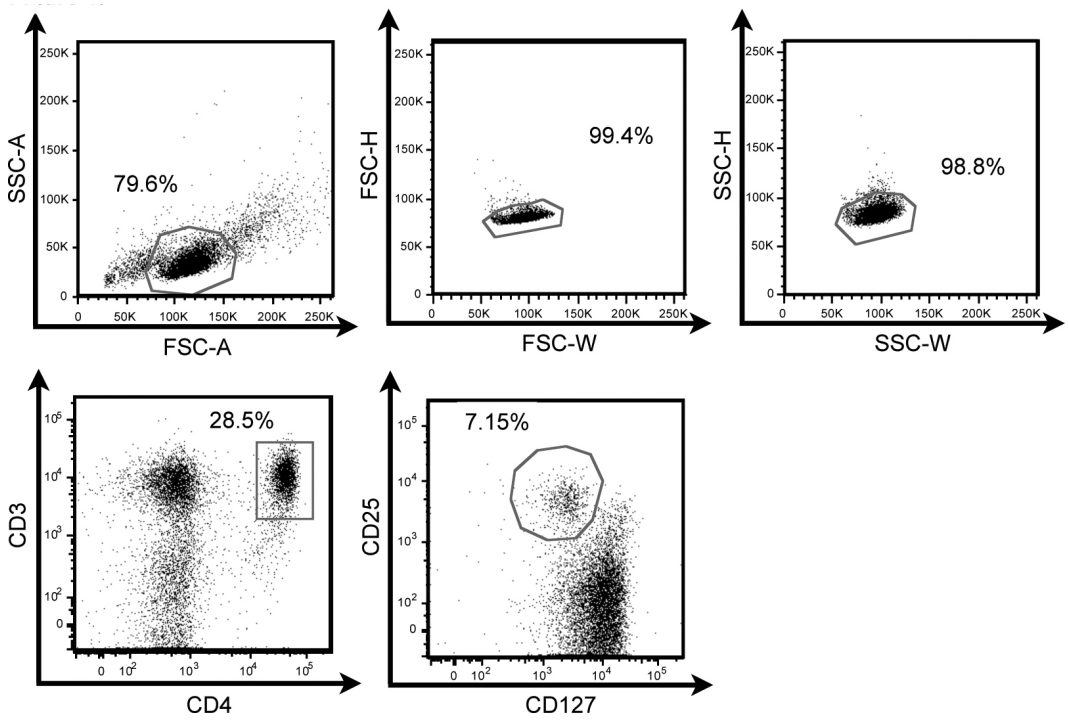

Figure 1. Flow cytometry gating strategy to sort singles $CD3^{+}CD4^{+}CD25^{+}CD127^{low}$ Tregs from PBMC isolated from an HIV-1-positive individual. Click here to view larger figure.

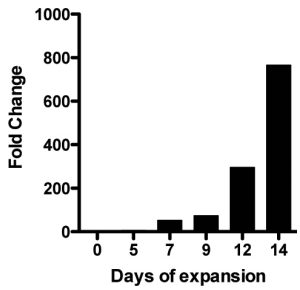

Figure 2. Representative example of the expansion fold change of Tregs isolated from an HIV-1-infected individual cultured during 14 days.

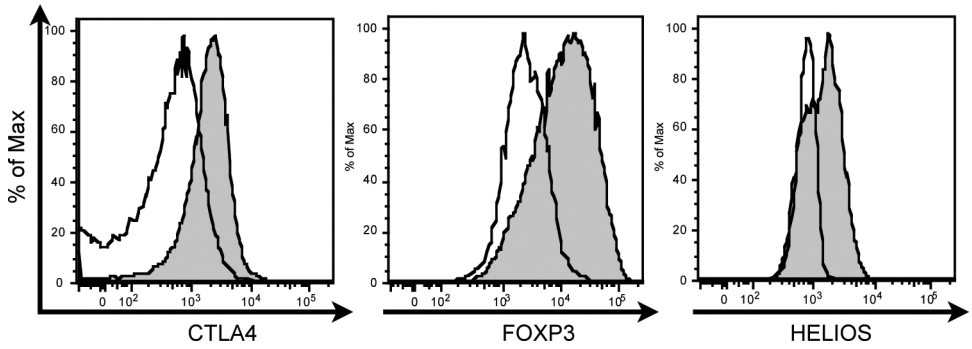

Figure 3. Examples of flow cytometry histograms staining of expanded Tregs isolated from an HIV-1-infected Treg using different Tregs markers. Expanded Tregs are figured in grey and expanded Tconvs in white. Click here to view larger figure.

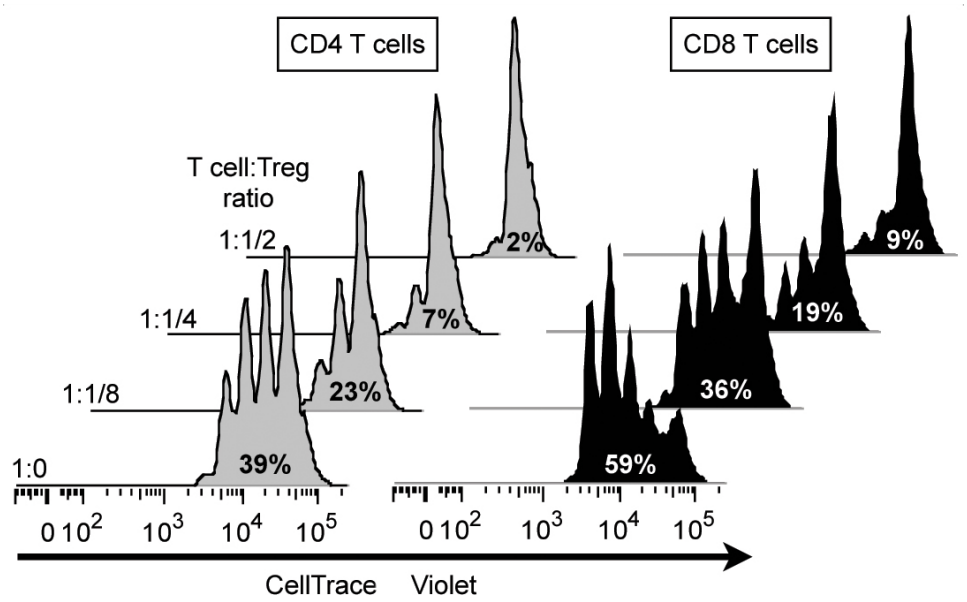

Figure 4. Example of flow-based T cell proliferation assay using a membrane-permeant reactive tracer and activated T cells at different responder T cell to Treg ratios. $CD4^+$ T cells are figured in grey and $CD8^+$ T cells in black. The numbers of divided cells are figured in the histograms.

Discussion

Using the protocol described above, Tregs can be successfully isolated and expanded from HIV-1-infected individuals *in vitro*. Expanded Tregs express high levels of FOXP3, CTLA4 and HELIOS, are highly suppressive and display a highly demethylated Treg-Specific Demethylation Region (TSDR) locus of the FOXP3 gene (data not shown) [15], suggesting true origin from the regulatory T cell lineage, as opposed to activation-induced transient FOXP3 upregulation. Deep sequencing demonstrated that the TCR repertoire of the Tregs is not skewed during the expansion (data not shown).

Regulatory T cells are naturally anergic and need strong stimuli to proliferate; here we used beads coated with anti-CD3 and anti-CD28 [16] antibodies in the presence of exogenous IL-2 [17]. Other groups have successfully used similar methods to expand polyclonal Tregs from healthy donors [18], diabetic patients [19], transplant recipients [20] or antigen-specific Tregs from HCV patients [21] but, to our knowledge, this is the first description of a successful protocol to expand functional Tregs from HIV-1 positive donors.

We chose to use flow-cytometry over immunomagnetic cell sorting in order to isolate the cell population with the best purity possible and to avoid contamination with $CD3^+CD4^+CD25^-CD127^+$ conventional T cells (Tconvs) during expansion. Avoiding contamination with Tconvs is crucial since we found that under the same conditions Tconvs proliferate more rapidly than regulatory T cells, and thus could overgrow Tregs during a prolonged expansion culture. Rapamycin was previously used to inhibit Tconvs growth while favoring expansion and survival of Tregs [22]. However rapamycin was found to downregulate CCR5 expression *in vitro*, an important co-factor of R5 HIV virus entry, which would have made expanded cells less usable to study HIV-1 infection of regulatory T cells by this HIV-1 strain [23].

In the present protocol, Tregs are expanded for 14 days. Although we found expanded Tregs to be still actively suppressive after 21 days, we chose not to prolong our cell culture and preserve the suppressive activity of our cells. Indeed, the group of M. Edinger reported a decrease in FOXP3 when Tregs were expanded more than two weeks and this was associated with moderate Treg suppression [24,25].

Most markers used to characterize regulatory T cells are shared by activated T cells (*e.g.* FOXP3 [26], CTLA4 [27]). In our hands, even though expanded Tregs expressed higher levels of FOXP3 and CTLA4 compared to expanded conventional T cells (Tconvs), HELIOS was the only marker that was almost exclusively expressed by the expanded Tregs. This protein is mostly expressed by thymic-derived but not by peripherally induced FOXP3+ regulatory T cells [28]. Thus, the Tregs isolated and expanded by our protocol could be used to understand the role of thymic-derived Tregs in HIV-immunopathogenesis. The expansion of other Tregs subsets, such as induced antigen-specific Tr1 or Th3 suppressor cells from HIV-1 positive individuals would need alternative adapted protocols [29,30].

In summary, using the described protocol we successfully expanded Tregs from frozen adult and pediatric PBMC as well as GALT (gut associated lymphoid tissue), the major site of HIV-1 replication. Easier access to large numbers of Tregs will help to address important questions concerning their role in HIV-1 immunopathogenesis and to better understandthe role of this T cell subpopulation in HIV-1 immune evasion. We believe that answering these questions may have a strong impact in the development of an effective HIV-1 vaccine and potential new strategies for immunotherapeutic intervention in HIV-1 infection.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Rerks-Ngarm, S., *et al.* Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* **361**, 2209-2220, doi:10.1056/NEJMoa0908492 (2009).
2. Buchbinder, S.P., *et al.* Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet.* **372**, 1881-1893, doi:10.1016/S0140-6736(08)61591-3 (2008).
3. Pitisuttithum, P., *et al.* Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J. Infect. Dis.* **194**, 1661-1671, doi:10.1086/508748 (2006).
4. Morse, M.A., *et al.* Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood.* **112**, 610-618, doi:10.1182/blood-2008-01-135319 (2008).
5. Furuichi, Y., *et al.* Depletion of CD25+CD4+T cells (Tregs) enhances the HBV-specific CD8+ T cell response primed by DNA immunization. *World J. Gastroenterol.* **11**, 3772-3777 (2005).
6. Rech, A.J. & Vonderheide, R.H. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. *Ann. N.Y. Acad. Sci.* **1174**, 99-106, doi:10.1111/j.1749-6632.2009.04939.x (2009).
7. Ruter, J., *et al.* Altering regulatory T cell function in cancer immunotherapy: a novel means to boost the efficacy of cancer vaccines. *Front Biosci.* **14**, 1761-1770 (2009).
8. Moreno-Fernandez, M.E., Rueda, C.M., Rusie, L.K., & Chougnet, C.A. Regulatory T cells control HIV replication in activated T cells through a cAMP-dependent mechanism. *Blood.* **117**, 5372-5380, doi:10.1182/blood-2010-12-323162 (2011).
9. Schulze Zur Wiesch, J., *et al.* Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. *J. Virol.* **85**, 1287-1297, doi:10.1128/JVI.01758-10 (2011).
10. Kinter, A., *et al.* Suppression of HIV-specific T cell activity by lymph node CD25+ regulatory T cells from HIV-infected individuals. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3390-3395, doi:10.1073/pnas.0611423104 (2007).
11. Moreno-Fernandez, M.E., Presicce, P., & Chougnet, C.A. Homeostasis and function of regulatory T cells in HIV/SIV infection. *J. Virol.* doi:10.1128/JVI.00993-12 (2012).
12. Angin, M., *et al.* Preserved Function of Regulatory T Cells in Chronic HIV-1 Infection Despite Decreased Numbers in Blood and Tissue. *J. Infect. Dis.* **205**, 1495-1500, doi:10.1093/infdis/jis236 (2012).
13. Seddiki, N., *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* **203**, 1693-1700, doi:10.1084/jem.20060468 (2006).
14. De Jager, P.L., *et al.* The role of the CD58 locus in multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5264-5269, doi:10.1073/pnas.0813310106 (2009).
15. Baron, U., *et al.* DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur. J. Immunol.* **37**, 2378-2389, doi:10.1002/eji.200737594 (2007).
16. Salomon, B., *et al.* B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity.* **12**, 431-440 (2000).
17. Malek, T.R. & Bayer, A.L. Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* **4**, 665-674, doi:10.1038/nri1435 (2004).
18. Hoffmann, P., Eder, R., Kunz-Schughart, L.A., Andreesen, R., & Edinger, M. Large-scale *in vitro* expansion of polyclonal human CD4(+)CD25high regulatory T cells. *Blood.* **104**, 895-903, doi:10.1182/blood-2004-01-0086 (2004).

19. Putnam, A.L., *et al.* Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* **58**, 652-662, doi:10.2337/db08-1168 (2009).
20. Kreijveld, E., Koenen, H.J., Hilbrands, L.B., & Joosten, I. *Ex vivo* expansion of human CD4+ CD25high regulatory T cells from transplant recipients permits functional analysis of small blood samples. *J. Immunol. Methods.* **314**, 103-113, doi:10.1016/j.jim.2006.06.001 (2006).
21. Ebinuma, H., *et al.* Identification and *in vitro* expansion of functional antigen-specific CD25+ FoxP3+ regulatory T cells in hepatitis C virus infection. *J Virol.* **82**, 5043-5053, doi:10.1128/JVI.01548-07 (2008).
22. Strauss, L., Czystowska, M., Szajnik, M., Mandapathil, M., & Whiteside, T.L. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. *PLoS ONE.* **4**, e5994, doi:10.1371/journal.pone.0005994 (2009).
23. Heredia, A., *et al.* Rapamycin causes down-regulation of CCR5 and accumulation of anti-HIV beta-chemokines: an approach to suppress R5 strains of HIV-1. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 10411-10416, doi:10.1073/pnas.1834278100 (2003).
24. Hoffmann, P., *et al.* Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon *in vitro* expansion. *Blood.* **108**, 4260-4267, doi:10.1182/blood-2006-06-027409 (2006).
25. Hoffmann, P., *et al.* Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive *in vitro* stimulation. *Eur. J. Immunol.* **39**, 1088-1097, doi:10.1002/eji.200838904 (2009).
26. Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W., & Toes, R.E. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur. J. Immunol.* **37**, 129-138, doi:10.1002/eji.200636435 (2007).
27. Takahashi, T., *et al.* Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* **192**, 303-310 (2000).
28. Thornton, A.M., *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J. Immunol.* **184**, 3433-3441, doi:10.4049/jimmunol.0904028 (2010).
29. Zheng, S.G., Gray, J.D., Ohtsuka, K., Yamagiwa, S., & Horwitz, D.A. Generation *ex vivo* of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. *J. Immunol.* **169**, 4183-4189 (2002).
30. Gregori, S., Roncarolo, M.G., & Bacchetta, R. Methods for *in vitro* generation of human type 1 regulatory T cells. *Methods Mol. Biol.* **677**, 31-46, doi:10.1007/978-1-60761-869-0_3 (2011).

