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## Regulatory T Cells (Tregs): Frequently Asked Questions

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### What are the differences between natural Tregs and inducible Tregs?

Natural Tregs (nTregs) originate from the thymus as CD4<sup>+</sup>CD25<sup>+</sup> cells together with expression of the transcription factor FoxP3 and aren't really influenced by the cytokine milieu. Natural Tregs represent approximately 5–10% of the total CD4<sup>+</sup> T-cell population. They are thought to be positively selected during thymic selection, with a relatively high avidity for self-antigens. The signal to develop into Tregs is thought to come from interactions between the T-cell receptor and the complexes of MHC II with self-peptide expressed on the thymic stroma and is observed at the single-positive stage of T-lymphocyte development.

On the other hand, inducible Tregs (iTregs) originate as CD4 single-positive cells from the thymus, and following adequate antigenic stimulation in the presence of cognate antigen and specialized immunoregulatory cytokines such as TGF- $\beta$ , IL-10, and IL-4, differentiate into CD25<sup>+</sup> and FoxP3<sup>+</sup> expressing Tregs—hence called “adaptive” or “inducible” Tregs. Adaptive or inducible T cells are further categorized into Tr1, Th2, or Tr3 depending on the cytokines that modulate them.<sup>1,2</sup>

### Are there any differences between mouse and human CD4<sup>+</sup> Tregs?

Yes there are. In mice, CD4<sup>+</sup> Tregs are a homogenous population, in which all CD4<sup>+</sup> and CD25<sup>+</sup> cells are regulatory T cells. In humans, the Tregs are a heterogeneous population, in which not all CD25<sup>+</sup> cells are Tregs. This was first observed by a detailed analysis of human CD4<sup>+</sup>CD25<sup>+</sup> populations by groups at Harvard and the Royal Free and University College Medical School in London.<sup>3,4</sup> Studies revealed that only those CD4<sup>+</sup> cells that expressed very high levels of CD25, representing approximately 2–3% of total CD4 T cells, demonstrate the in vitro suppressive activity similar to that described in murine cells. Furthermore, the cells expressing low-to-intermediate levels of CD25 do not exhibit suppressive activity directly in in vitro experiments.<sup>3</sup>

### What percentage of CD4<sup>+</sup> cells are Tregs? Does the percentage of CD4 Tregs in humans change with age?

The typical range is 4–9% (unpublished in-house results). There is a fair amount of donor variability even among healthy donors. Mean percentages of CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup> Tregs increase only slightly throughout life, from 6.10% in cord blood to 7.22% in PBMCs from adults between 20 and 25 years, and to 7.50% in PBMCs from adults over the age of 60. Studies show that in both healthy humans and mice, there is a slight increase in CD4<sup>+</sup>/CD25<sup>+</sup> Tregs with age. However, the exact correlation of the increased Treg numbers with immune senescence is not yet clear.<sup>5-7</sup>

### Why is it critical to include CD127 as a marker for human Tregs?

Research from the laboratories of Barbara Fazekas de St. Groth (Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia) and Jeffrey Bluestone (UCSF Diabetes Center, San Francisco, CA) showed that human Tregs can be identified by their low expression of CD127. The expression pattern closely follows that of FoxP3, and indicates that CD4<sup>+</sup> T lymphocytes with low expression of CD127 and intermediate-to-high CD25 expression are Tregs. The advantage of using this marker over intracellular expression of FoxP3 is that fixation/permeabilization is not required, so the cells are still alive and can subsequently be used in culture and other in vitro assays. Using high expression of CD25 to identify Tregs, other researchers have obtained a frequency of 0.6% to 7.9% of Tregs from normal individuals. Using low CD127 expression (in addition to CD25) as a gating strategy increases the recovery of Tregs often missed by gating on the CD25<sup>hi</sup>-expressing cells alone. It can result in a greater than 2–4 fold increase in the recovery of the Tregs while eliminating the contaminating CD25 intermediate T-effector cells.<sup>8</sup>

### What are some different surface markers that can be used to identify human and mouse CD4<sup>+</sup> Tregs?

Suggested surface markers for identification of human CD4 Tregs include GITR, CTLA-4 (cytotoxic T-lymphocyte-associated protein), CD39, CD62L, CD127, CD103, and LAG3 (lymphocyte activation gene) together with CD45RA<sup>+</sup> expression. In mice, nTregs can be identified as (FR4<sup>hi</sup>CD25<sup>+</sup>), effector T cells (FR<sup>lo</sup>CD25<sup>+</sup>), memory-like T cells (FR4<sup>hi</sup>CD25<sup>-</sup>), and naïve T cells (CD25<sup>-</sup>CD4<sup>+</sup>).<sup>9-11</sup>



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### What is FoxP3 and how does it correlate to CD4<sup>+</sup> regulatory T cells?

FoxP3 (forkhead box P3) is an intracellular transcription factor. It is a specific marker for Tregs. It was discovered in mice during research of lymphoproliferative diseases and was also observed in the spontaneous genetic mouse mutant “scurfy.” In humans, it was identified during efforts to understand the fatal autoimmune disease IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked), since genetic defects in FoxP3 cause IPEX.<sup>12</sup>

### Are there cell types other than CD4 that also express FoxP3?

Yes. Studies have shown that CD8 cells also express FoxP3 and are also observed to be regulatory in nature.<sup>13</sup>

### Do various isoforms of human FoxP3 exist? Which isoform does FoxP3 clone 259D/C7 from BD Biosciences recognize?

Yes. There are two isoforms of FoxP3: a full-length form and a splice variant that lacks exon 2. Both of these forms are present in CD4<sup>+</sup>/CD25<sup>+</sup> cells. Human FoxP3 monoclonal antibody clone 259D/C7 from BD Biosciences reacts with both isoforms of FoxP3 and is also cross-reactive with cynomolgus, rhesus, and baboon.<sup>14</sup>

### What are the differences between BD Biosciences 259D/C7 and 236A/E7 human FoxP3 clones?

Both 259D/C7 and 236A/E7 recognize epitopes within the first 235 aa of the FoxP3 protein, specifically within the N-terminus AA 105-235. In-house comparison by flow cytometry between 259D/C7 and 236A/E7 indicates only minor qualitative differences in the staining profile. Choice of conjugate and/or buffer system plays a role in the staining performance of clone 236A/E7. During in-house testing, clone 236A/E7 performed comparably or better than clone 259D/C7.

### How long can samples be stored before proceeding with anti-human FoxP3 staining?

We have been able to store human PBMCs in human FoxP3 Buffer A for 24–72 hours at –80°C before proceeding to stain the samples. For further information, please refer to the technical data sheet for the Human Fox P3 Buffer Set (Cat. No. 560098).

### Can I use human FoxP3 buffers with mouse Foxp3 antibody, or vice versa?

No. We do not recommend interchanging the human and mouse Foxp3 buffer sets. To obtain optimal staining with either mouse or human FoxP3 clones, we suggest using the appropriate FoxP3 buffer set recommended for that clone.

### I would like to perform intracellular cytokine staining of human IFN- $\gamma$ , IL-4, and IL-17A together with FoxP3. What are your recommendations for the buffers that I could use?

To perform co-staining of human FoxP3 together with these cytokines, we recommend using the FoxP3 buffer system. The exception is co-staining with IL-4, which doesn't seem to stain well in this buffer system and works better with BD Cytotfix/Cytoperm™ buffer. However, both IL-17A and IFN- $\gamma$  stain well in the FoxP3 buffer system. For more details and data comparing different buffers, view our webinar from April 2009 entitled “Optimizing Intracellular Flow Cytometry: Simultaneous Detection of Cytokines and Transcription Factors” at [bdbiosciences.com/hotlines/webinars/archives/2009.jsp](http://bdbiosciences.com/hotlines/webinars/archives/2009.jsp)

### Can FoxP3 staining be performed with human whole blood samples?

Yes. The primary reason we use PBMCs is the higher cell count, because you would have to lyse/fix/perm a large amount of whole blood to have enough cells for one experiment. For example, 1 mL of whole blood contains approximately  $1 \times 10^6$  lymphocytes, and Tregs make up less than 4–9% of CD4<sup>+</sup> cells.

### How can I assay for Treg functions?

Tregs can be assayed by their ability to suppress target cell function, such as:



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- To suppress proliferation of target cells or
- To suppress cytokine production by target cells
- Inhibition of activation markers

For further details, please refer to the BD Application Note on Tregs, "Human Regulatory T Cell Isolation and Measurement of Function" and the FastImmune Regulatory T Cell Sorting Kit (Cat. No. 648956).

### Can we stain for internalized human CD152 using clone BNI3 as a Treg marker?

Yes, anti-human CD152, clone BNI3, can be used to stain internalized CD152.

### What are some tips for enriching human Tregs prior to sorting?

Tregs can be enriched prior to cell sorting using the BD IMag™ Human Regulatory T lymphocyte separation set (Cat. No. 558142). The amount of BD IMag particles used and the incubation time on the BD™ IMagnet cell separation magnet are critical factors that may affect cell viability during positive selection. We recommend using 5 µL per one million cells and suggest 6-minute incubation for the first round, followed by 2-minute incubation for subsequent rounds.

Other points to consider:

- Fewer washes increase the recovery, but decrease the purity
- Longer time spent on the magnet increases recovery, but decreases purity
- Shorter time spent on the magnet increases purity, but decreases recovery
- Incubation with the particles has to be performed at room temperature for 30 minutes.
- Bring up the volume as instructed and place samples on the BD IMagnet.
- It is important to titrate the magnetic particles to optimal concentration for your cells, since too many magnetic particles will result in increased non-specific binding.
- Not enough magnetic particles will result in low recovery.

For further details about the human regulatory T lymphocyte separation set, please visit [bdbiosciences.com](http://bdbiosciences.com).

### References:

1. Chatenoud L, Bach JF. Adaptive human regulatory T cells: myth or reality? *J Clin Invest*. 2006;116:2325-2327.
2. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 2008;29:44-56.
3. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+CD25+ T cells. *Clin Immunol*. 2005;115:10-18.
4. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+)/CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol*. 2001;31:1122-1131.
5. Santner-Nanan B, Seddiki N, Zhu E, et al. Accelerated age-dependent transition of human regulatory T cells to effector memory phenotype. *Int Immunol*. 2008;20:375-383.
6. Gregg R, Smith CM, Clark FJ, et al. The number of human peripheral blood CD4<sup>+</sup> CD25<sup>high</sup> regulatory T cells increases with age. *Clin Exp Immunol*. 2005;140:540-546.
7. Zhao L, Sun L, Wang H, Ma X, Liu G, Zhao Y. Changes of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory t cells in aged Balb/c mice. *J Leuk Biol*. 2007;81:1386-1394.



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8. Liu W, Putnam AL, Xu-Yu Z, et al. CD27 expression inversely correlates with FoxP3 and suppressive function of human CD4+ Treg cells. *J Exp Med.* 2006;203:1701-1711.
9. Hoffmann P, Eder R, Tina J, et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood.* 2006;108(13):4260-4267.
10. Borsellino G, Kleinewietfeld M, Di Mitri D, et al. Expression of ectonucleotidase CD39 by FoxP3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood.* 2007;110:1225-1232.
11. Yamaguchi T, Hirota K, Nagahama K, et al. Control of immune response by antigen-specific regulatory T cells expressing the folate receptor. *Immunity.* 2007;27:145-159.
12. Sakaguchi S. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J Clin Invest.* 2003;112:1310-1312.
13. Joosten SA, van Meijgaarden KE, Savage ND, et al. Identification of a human CD8+ regulatory T cell subset that mediates suppression through chemokine CC chemokine ligand 4. *Proc Natl Aca Sci.* 2007;104:8029-8034.
14. Allan SE, Passerini L, Bacchetta R, et al. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest.* 2005;115:3275-3284.

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