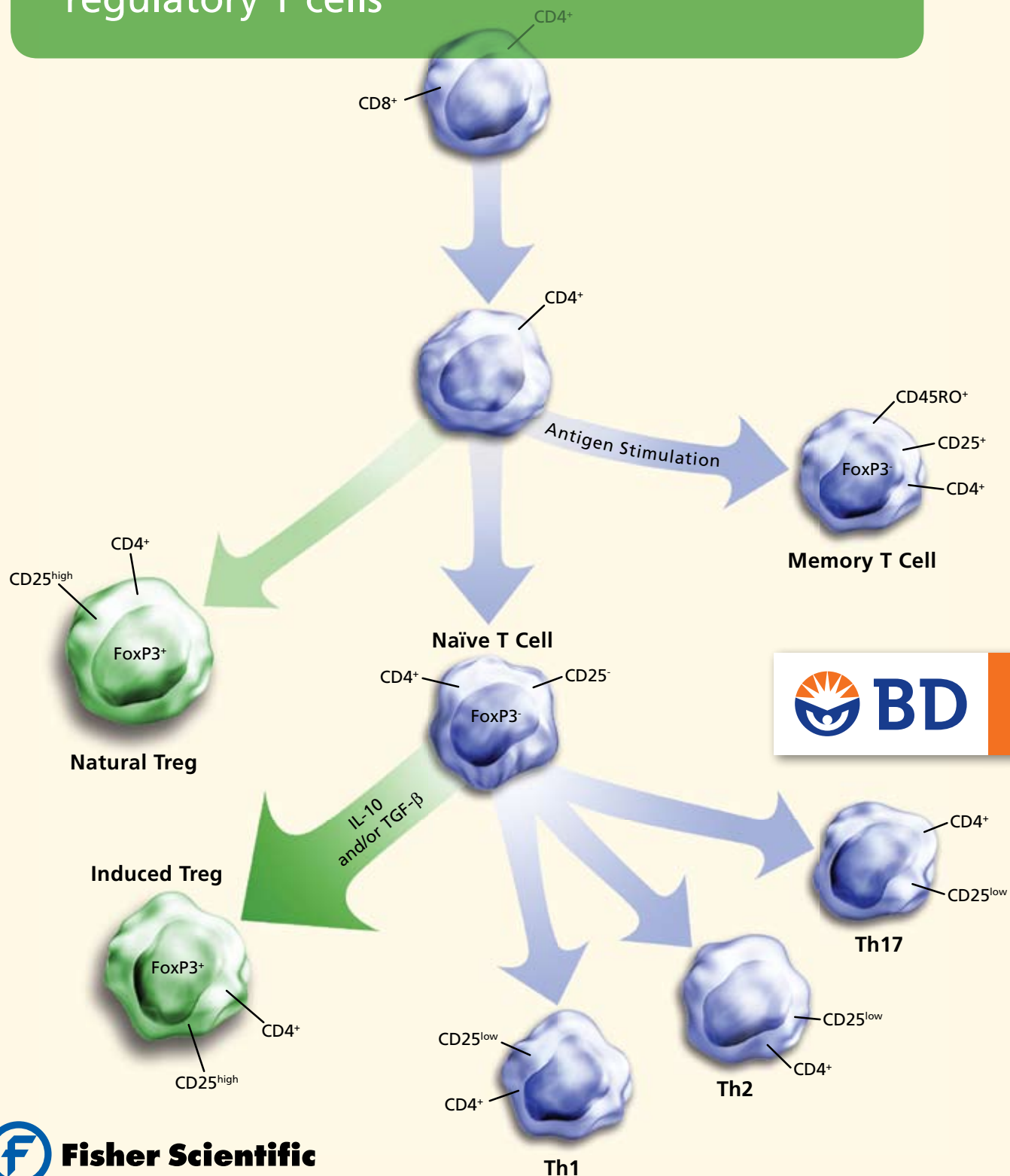# Regulatory T Cells: Essential Regulators of the Immune System

Tools for the identification, isolation, and multicolor analysis of human regulatory T cells

$CD4^+$

$CD8^+$

$CD4^+$

Antigen Stimulation

$CD45RO^+$

$CD25^+$

$FoxP3^-$

$CD4^+$

Memory T Cell

$CD4^+$

$CD25^{high}$

$FoxP3^+$

Natural Treg

Naïve T Cell

$CD4^+$ $CD25^-$

$FoxP3^-$

IL-10 and/or TGF-β

Induced Treg

$FoxP3^+$

$CD4^+$

$CD25^{high}$

$CD25^{low}$

$CD4^+$

Th1

$CD25^{low}$

$CD4^+$

Th2

$CD4^+$

$CD25^{low}$

Th17

Fisher Scientific

A proven commitment to regulatory T cell research

As evidence of the immunosuppressive potential of T cells has developed in recent years, interest in Regulatory T cells (Tregs) and enthusiasm for their potential therapeutic application has intensified. Thus Treg research is very active, and new publications emerge almost daily. Today the most commonly used markers for Treg identification, isolation, and characterization are CD4, CD25, CD127, and FoxP3. However, new targets with functional significance such as CD39, CD45RA, CTLA-4, and others are rapidly emerging.

For over 20 years, BD Biosciences has actively supported groundbreaking research in the field. With a rich portfolio of high quality immunology products, BD Pharmingen™ brand reagents support both established markers as well as emerging trends in this dynamic environment. With new discoveries about the role of proteins in Tregs, many existing markers gain new utility. This proven commitment to help advance discovery in Treg research is the foundation of BD Biosciences ongoing efforts to provide a full range of tools to simplify the identification, isolation and characterization of Treg cells and their interacting partners.

BD Biosciences reagents are backed by a world-class service and support organization to help customers take full advantage of our products to advance their research. Comprehensive services include technical application support and custom assay services provided by experienced scientific and technical experts.

Regulatory T cells

Different subsets, similar functions

Regulatory T cells (Tregs) play an important role in maintaining immune homeostasis. Tregs suppress the function of other T cells to limit the immune response. Alterations in the number and function of Tregs has been implicated in several autoimmune diseases including multiple sclerosis, active rheumatoid arthritis, and type 1 diabetes. High levels of Tregs have been found in many malignant disorders including lung, pancreas, and breast cancers. Tregs may also prevent antitumor immune responses, leading to increased mortality.[1]

CD4 and CD8 Tregs

Two major classes of Tregs have been identified to date: CD4 and CD8 Tregs. CD4 Tregs consist of two types, "natural" Tregs (nTregs) that constitutively express CD25 and FoxP3, and so-called adaptive or inducible Tregs (iTregs).
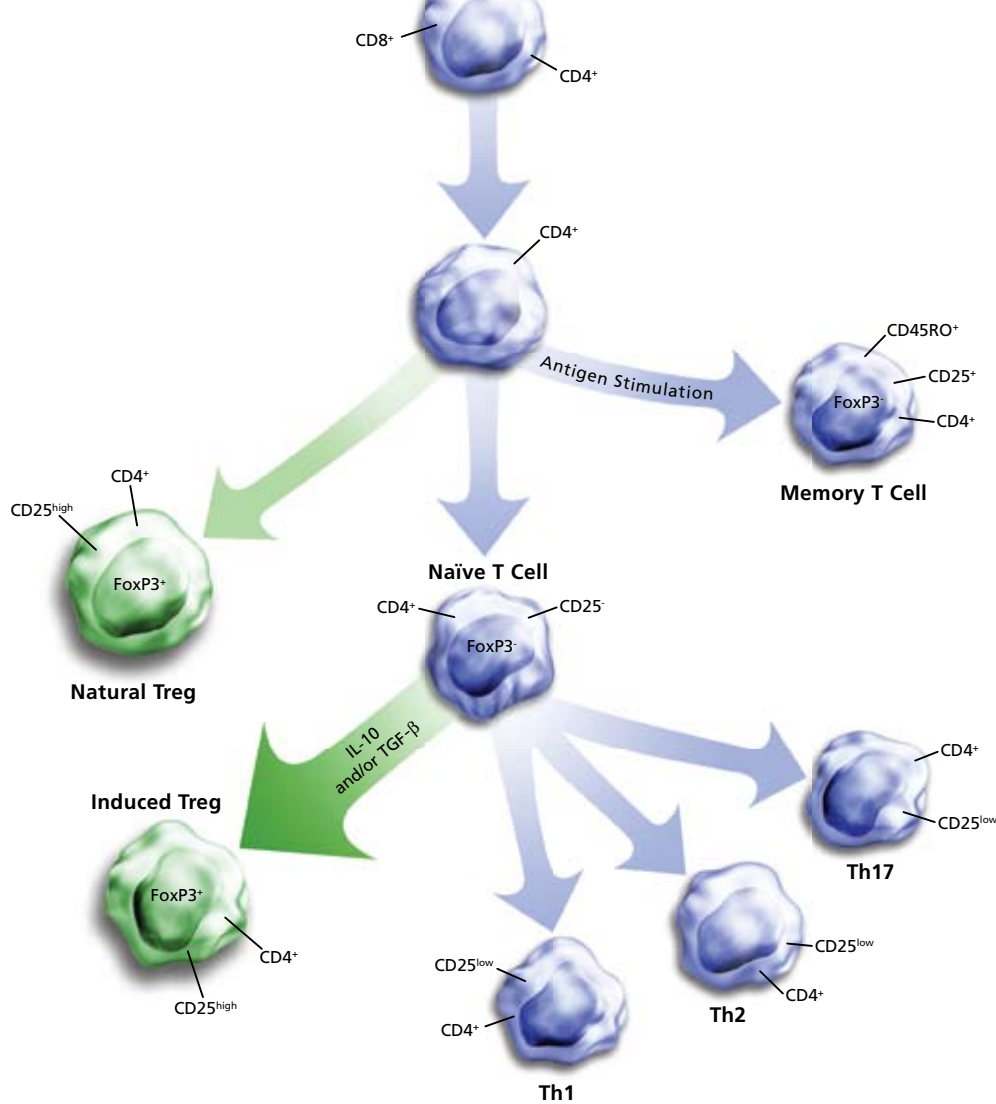
Natural Tregs originate from the thymus as CD4^{+} cells expressing high levels of CD25 together with the transcription factor (and lineage marker) FoxP3. nTregs represent approximately 5–10% of the total CD4^{+} T cell population, and can first be seen at the single-positive stage of T lymphocyte development.[2] They are positively selected thymocytes with a relatively high avidity for self-antigens. The signal to develop into Treg cells is thought to come from interactions between the T cell receptor and the complex of MHC II with self peptide expressed on the thymic stroma.[3] nTregs are essentially cytokine independent.

Adaptive or inducible Tregs originate from the thymus as single-positive CD4 cells. They differentiate into CD25 and FoxP3 expressing Tregs (iTregs) following adequate antigenic stimulation in the presence of cognate antigen and specialized immunoregulatory cytokines such as TGF-β, IL-10, and IL-4.[4]

FoxP3 is currently the most accepted marker for Tregs, although there have been reports of small populations of FoxP3^{-} Tregs. The discovery of transcription factor FoxP3 as a marker for Tregs has allowed scientists to better define Treg populations leading to the discovery of additional Treg markers including CD127.

Treg subsets

	Natural Treg (nTregs)	Induced Tregs (iTregs)	
	nTregs	Tr1	Th3
Phenotype	CD4^{+}CD25[int/high], CD127^{low}	CD4^{+}CD25^{-}	CD4^{+}CD25^{+} from CD25^{-} precursors
Other associated markers	CTLA4^{+}, GITR^{+}, FoxP3^{+}, CD127^{low}	CD25[low-variable], CD45RB^{low}, FoxP3^{-}	CD25[low-variable], CD45RB^{low}, FoxP3^{+}
Suppression	Contact dependent, granzyme B-dependent, makes TGF-β	Through cytokines, produces IL-10	Through cytokines, produces TGF-β
Target cells	APC and T effector cells	T effector cells	Not yet Identified
CD28 Involvement	Thymic development and maintenance in periphery	Not for development or function	Not involved
In vivo Role	Suppression of autoreactive T cells	Mucosal immunity, inflammatory response	Mucosal immunity, inflammatory response
In vitro Expansion	Expandable using TCR/CD28 stimulation and IL-2	CD3, IL-10, rehnoic acid	CD3, TGF-β

CD8+
CD4+

CD4+

Antigen Stimulation

CD45RO+
CD25+
FoxP3-
CD4+

Memory T Cell

CD4+
CD25high
FoxP3+

Natural Treg

Naïve T Cell
CD4+   CD25-
FoxP3-

IL-10 and/or TGF-β

Induced Treg

CD4+
CD25low

Th17

FoxP3+
CD4+
CD25high

CD25low
CD4+

CD25low
CD4+

Th2

CD4+

Th1

CD127 discovery

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, USA) and confirmed in laboratories at BD Biosciences demonstrated that CD127 expression is down-modulated on the Treg cells, inversely correlating with the expression of Treg marker FoxP3. It was also demonstrated that Tregs are also present (at varying levels) in populations of cells expressing high and low levels of CD25. These findings provided a new cell surface marker for Tregs,[5,6] enabling isolation of viable cells by flow cytometric sorting for further downstream analysis.

CD127 is part of the heterodimeric IL-7 receptor that is composed of CD127 and the common γ chain, which is shared by other cytokine receptors (IL-2R, IL-4R, IL-9R, IL-15R, and IL-21R). CD127 is expressed on thymocytes, T- and B-cell progenitors, mature T cells, monocytes, and some other lymphoid and myeloid cells. Studies have shown that IL-7R plays an important role in the proliferation and differentiation of mature T cells, and in vitro experiments show that the expression of CD127 is down-regulated following T cell activation.[7-9] It is believed that FoxP3 interacts with the CD127 promoter and might contribute to reduced expression of CD127 in Tregs.[6]

Correlation and regression analysis

Correlation and regression of responses, CD4+CD25(int/high)FoxP3+ vs. CD4+CD25(int/high)CD127low predictors in a PBMC assay.

Scatterplot of CD4+CD25+Foxp3+ vs. CD4+CD25+CD127low

CD4+CD25+CD127dim

CD4+CD25+Foxp3+
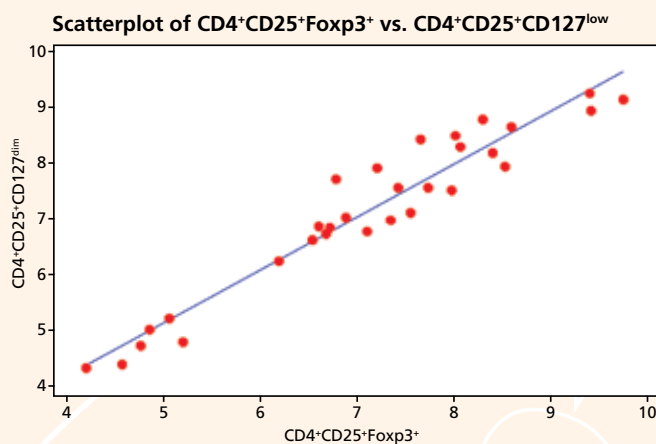
Leading tools to support and streamline T cell research

Enrichment of Tregs with CD4, CD25, and CD127

Tregs represent a small population of cells; enrichment is often necessary for downstream analysis. Several methods exist for the enrichment of whole or subpopulations of Tregs. While FoxP3 currently is considered the most accepted marker for Tregs, its intracellular localization prohibits its use for the isolation of viable Tregs. Other markers used for enrichment are either negative, positive, or used in combination. One reported method of negative selection is the removal of cells expressing CD127 and CD49d.[10] Cells expressing CD4+ and the highest levels of CD25 are used for positive selection. Combination methods can include the use of magnetic beads to remove contaminating populations prior to cell sorting. One of the best characterized methods enriches for CD4+, CD25+, CD127- cells.

### Enrichment of Tregs with CD4 and CD25

In humans, initial analysis of Treg populations revealed that only those ex vivo cells that express the highest levels of CD25, which represent approximately 2-3% of total CD4 T cells, demonstrate an in vitro suppressive activity[11,12] in contrast to mouse cells in which all CD25 cells are considered Tregs.[4,5] Furthermore, cells expressing low to intermediate levels of CD25 were thought not to exhibit any suppressive activity directly ex vivo.

Additionally, the definition of high and low levels of CD25 expression lacks consensus and has limited its use for obtaining viable human Tregs via flow cytometric cell sorting. As a result, many researchers only select cells with the highest expression of CD25, dramatically reducing the yield of isolated Tregs. These results intensified research to identify cells surface markers other than CD4 and CD25 that are exclusive to human Tregs.

Cell surface characterization and isolation of viable human Tregs using CD127

Staining human CD4 T lymphocytes with CD4, CD25, and CD127 enables the enrichment of viable regulatory T cell fractions for further culture and/or use in other in vitro assays. Using this method, viable cells can be quickly isolated through cell sorting. The BD Pharmingen brand Human Regulatory T Cell Cocktail, a pre-mixed three-color reagent, simplifies enrichment of viable T cells using a gating strategy that relies on CD4+CD25$^{int/high}$ CD127^{low} cells. This strategy enhances the recovery of CD25$^{int/high}$ sorted Treg cells by 2 to 4 times compared to gating on CD25^{high} cells alone, while eliminating contaminating CD25$^{-/int}$ effector cells.
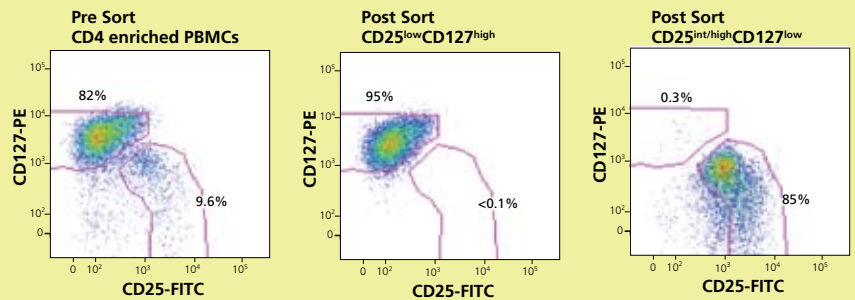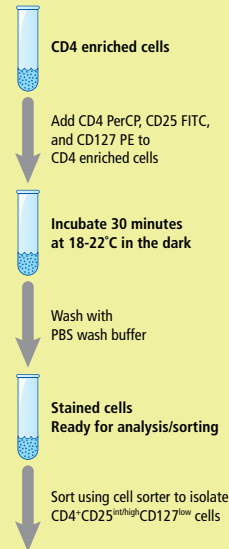
PBMCs were stained with anti-human CD4, CD25, and CD127 as well as FoxP3. Lymphocytes were determined using the light scatter properties (FSC and SSC) and then gated on CD4 vs. CD25 as shown. Percentages of Tregs (FoxP3+) are shown for each of the CD25 populations, CD25^{high}, CD25^{int}, CD25^{low}, and CD25$^-$.

Magnetic based pre-enrichment of CD4⁺ T cells to increase yield of human Tregs
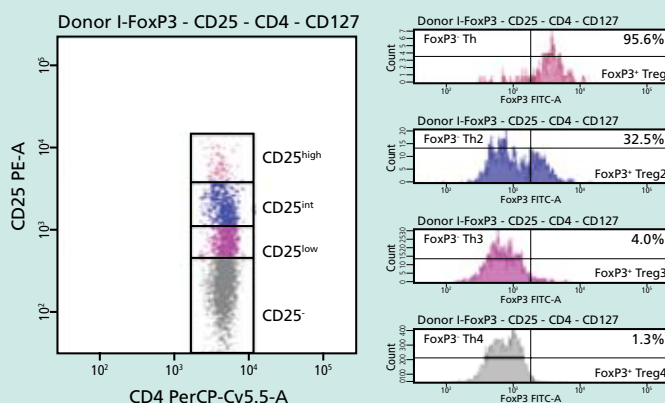
Magnetic based pre-enrichment of CD4$^+$ T cells to increase yield of human Tregs

Human Tregs represent a small population of total cells. One method to increase the yield of highly enriched human Treg cells combines magnetic pre-enrichment of CD4$^+$ cells with BD IMag™ technology with CD127-based cell sorting.

The BD IMag Human CD4 T Lymphocyte Enrichment Set (Cat. No. 557939) offers a convenient way to pre-enrich CD4$^+$ T cells. Purities of greater than 90% were routinely attained for the enrichment. The procedure captures erythrocytes, platelets, and peripheral leukocytes that are not CD4 T lymphocytes, by magnetically removing them from the sample to create an enriched CD4 population. Enriched cells are then stained and shown in the figure on the right.

CD4 enriched cells

Add CD4 PerCP, CD25 FITC, and CD127 PE to CD4 enriched cells

Incubate 30 minutes at 18-22°C in the dark

Wash with PBS wash buffer

Stained cells Ready for analysis/sorting

Sort using cell sorter to isolate CD4$^+$CD25$^{int/high}$CD127^{low} cells

Pre Sort
CD4 enriched PBMCs

82%
9.6%
CD127-PE
CD25-FITC

Post Sort
CD25^{low}CD127^{high}

95%
<0.1%
CD127-PE
CD25-FITC

Post Sort
CD25$^{int/high}$CD127^{low}

0.3%
85%
CD127-PE
CD25-FITC

Cells were stained with anti-human CD4 PerCP (Cat. No. 345770), anti-human CD25 FITC (Cat. No. 555431), and anti-human CD127 PE (Cat. No. 557938). The enriched cells were sorted using a BD FACSAria™ cell sorter. A detailed protocol and recommended gating strategy can be found at bdbiosciences.com/treg.

The BD Pharmingen brand Human Regulatory T Cell Cocktail

PBMCs were stained with either an isotype control (Cat. No. 557872/555909: data not shown) or Human Regulatory T Cell Cocktail (Cat. No. 560249). The PBMCs were then fixed, lysed, and permeabilized using BD Pharmingen Human FoxP3 Buffer Set (Cat. No. 560098) and stained with BD Horizon V450 conjugated anti-human FoxP3 monoclonal antibody (Cat. No. 560459). During data analysis, lymphocytes were identified by light scatter profile and CD4 positive expression.

A Data representing the CD25 and CD127 expression profile of the CD4 positive cells.

B Data showing hFoxP3 expression on CD25^{high}/CD127^{low} T regulatory cells (solid line) and other T cells (dashed line). Flow cytometry was performed on a BD™ LSR II system.

4.43
CD127
CD25

A

Relative Cell Number
BD Horizon™ V450 hFoxP3

B
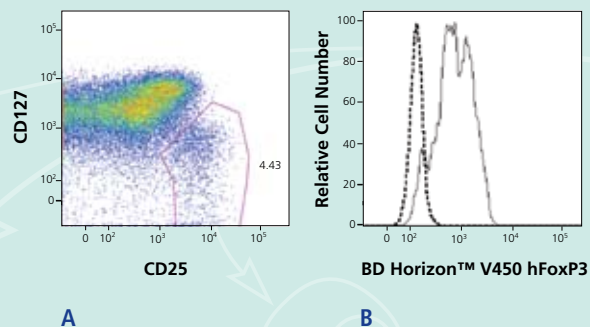
FoxP3: The classic Treg marker

Although several surface markers were defined for Treg identification, a classic marker specific and unique to Tregs remained undiscovered until FoxP3 was identified as a Treg marker in mice in simultaneously reported studies by Sakaguchi and Rudensky.

FoxP3 (also known as Scurfin, IPEX and JM2) is a transcriptional repression factor of the forkhead or winged helix family of transcription factors.[13] FoxP3 has been found to be expressed in all CD4+ Treg cells that have regulatory activity. Mutations in FoxP3 are associated with the inherited auto-immune diseases Scurfy in mice and its human counterpart, IPEX (immune dysregulation, polyendocrinopathy and enteropathy, X-linked syndrome).[14]

FoxP3 is useful for confirming purity and yield of isolated Tregs or for characterizing fixed Treg cells. However, it is not suitable for use in isolating viable Treg cells as FoxP3 staining requires fixation and permeablization of the cells. In these cases CD127- is a better solution.

FoxP3 staining

Human FoxP3 monoclonal antibody clone 259D/C7 from BD Biosciences reacts with all currently identified isoforms of the human FoxP3 transcription factor and is cross-reactive with cynomolgus, rhesus, and baboon.

The BD Pharmingen human FoxP3 antibody and buffer kit is a high performance reagent system for the detection of FoxP3 positive Tregs. An easy-to-use buffer system allows researchers to fix and permeabilize cells in just a few simple steps, with the option of freezing samples up to 72 hours.

Multiple conjugates and test sizes offer flexibility

Available FoxP3 fluorescent conjugates include Alexa Fluor® 488, Alexa Fluor® 647, and PE formats to enable maximum flexibility for the design of multicolor panels in combination with our family of BD FACS™ brand flow cytometers. For flow cytometers equipped with violet lasers, BD offers FoxP3 conjugated to BD Horizon V450 dye. Human FoxP3 detection kits containing all necessary reagents for identification of Tregs using FoxP3 are also available in 100-test sizes.

Mouse Foxp3

Foxp3 is the currently accepted marker for mouse Tregs. Foxp3 was originally identified as the defective gene in the mouse strain Scurfy. Scurfy mice develop a lymphoproliferative disorder that is typically fatal within one month after birth.

In mice, like humans, Foxp3 is a transcription factor that alters the expression of genes necessary for Treg function.

Detection of CD4+ and Foxp3+ Treg cells in BALB/c mouse splenocytes

BALB/c mouse splenocytes were surface-stained with Rat Anti-Mouse CD4 (APC, PE, or FITC), clone RM4-5 (Cat. Nos. 553051, 553048, or 553047). They were then fixed and permeabilized using the BD Pharmingen™ Mouse Foxp3 Buffer Set (Cat. No. 560409), followed by intracellular staining with PE Rat Anti-Mouse Foxp3 (Cat. No. 560406, 0.25 µg/test), Alexa Fluor® 488 Rat Anti-Mouse Foxp3 (Cat. No. 560403, 0.12 µg/test), or Alexa Fluor® 647 Rat Anti-Mouse Foxp3 (Cat. No. 560401, 0.03 µg/test). The dot plots were derived from the gated events based on light scattering characteristics of lymphocytes. Flow cytometry was performed on a BD FACSCalibur™ system.
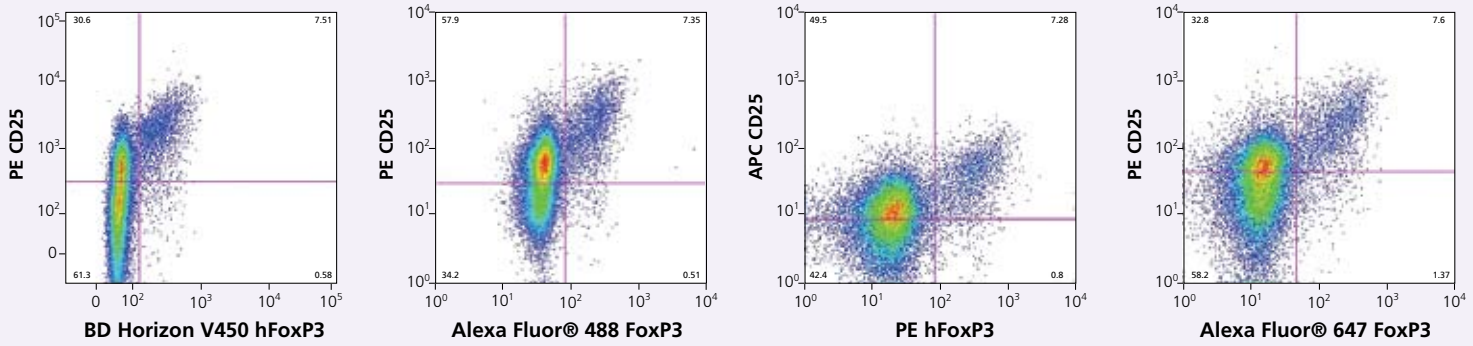
Detection of CD4+ and CD25+ FoxP3+ Treg cells in peripheral blood lymphocytes

Fresh human PBMCs from three separate donors were surface stained with CD4 (FITC or PE) clone RPA-T4 (Cat. Nos. 555346, 555347) and CD25 (PE or APC) clone 2A3 or M-A251 (Cat. Nos. 555432, 340939) antibodies (data not shown). Cells were then fixed for 10 minutes and permeabilized for 30 minutes using the BD Pharmingen Human FoxP3 Buffer Set (Cat. No. 560098), then stained with 20 μL/test of conjugated human FoxP3 (clone 259D/C7)

antibody (Cat. Nos. 560045, 560047, 560046, and 560460). The data shown are derived from an acquisition of 50,000 events in a lymphocyte gate, followed by CD4+ gating by fluorescence. A compound gating strategy by morphology, then side scatter vs. fluorescence, was used to identify FoxP3+ Treg cells shown in a final plot representing CD25 vs. FoxP3.
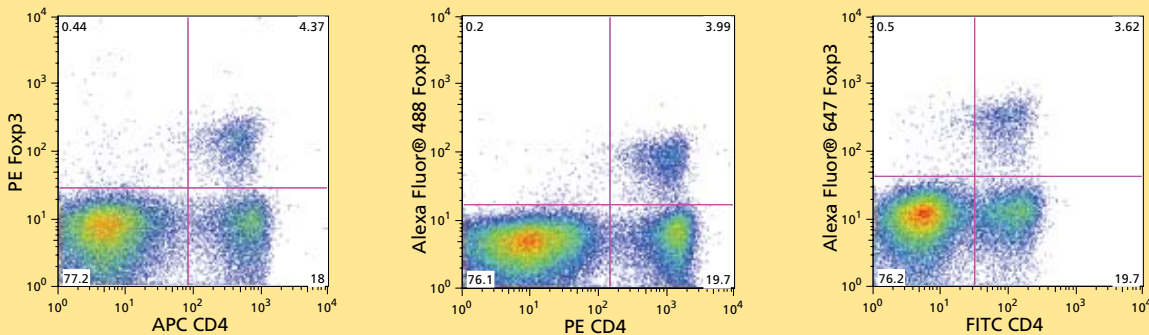
Supporting an emerging list of target markers

While CD4, CD25, FoxP3, and CD127 are commonly used markers for Treg identification, isolation, and characterization, Tregs are a very active area of research and an emerging list of targets has been published in the literature.

To support these emerging discoveries, the BD Biosciences portfolio of new high quality reagents and solutions continues to grow.

CD39: enhanced characterization of Tregs

Previously localized primarily on B cells, dendritic cells, and certain subsets of T cells, CD39 has recently been shown to be coexpressed with FoxP3 in CD4+ Tregs in humans and mice.[15] This discovery is adding to the growing list of cell surface markers such as CD25, CD45RA, HLA-DR, and CTLA-4, that are important in the identification and functional characterization of CD4+ Tregs.

Extracellular ATP and its metabolites are potent regulatory molecules modulating a broad range of cell and organ functions. Cellular ATP release is an indicator of tissue destruction and a "danger signal" that activates the immune response. CD39 hydrolyzes extracellular ATP (or other triphosphates) into its respective nucleotides such as AMP. Extracellular nucleoside monophosphates are, in turn, rapidly degraded to nucleosides (eg, adenosine) by soluble or membrane bound ecto-5'-nucleotidases (CD73). Pericellular adenosine then mediates anti-inflammatory T cell responses. Coexpression of CD39 and CD73 is thought to be one of the key mechanisms of immunosuppression mediated by Tregs.[16,17]

Reported markers of human Tregs

This table highlights research reagents that are most relevant for human Regulatory T cell research. Our reagent portfolio is constantly expanding. Please visit **bdbiosciences.com/ treg** for the most recent product information.

Antigen	Human Expression	Reference Number
CCR4	positive	21, 22
CCR6	positive	21
CCR7	positive	23
CD4	positive	12, 24, 25
CD11a	high	26, 27
CD25	positive	28
CD39	positive and negative subsets	29
CD45RA	positive	30
CD45R0	positive	31
CD46	positive	10
CD54 (ICAM-1)	high	
CD62L	low and high subsets	32
CD69	positive	33
CD103	positive	34
CD122 (IL-2Rβ)	positive	
CD127	low	5, 6
CD134 (OX-40)	positive and negative subsets	35

Antigen	Human Expression	Reference Number
CD152 (CTLA-4)	high	36, 37
CD154 (CD40L)	positive	38
CD223/LAG-3	positive	39, 40, 46
CXCR4	positive	33
CXCR5	positive	33
CXCR6	positive	33
FoxP3	positive	41, 42
GITR	high	43
GPR83	positive	44
Granzyme B	positive	45
IL-10	positive	46
IL-35	positive	47
MHCII	positive	48
Neuropilin 1	positive	49
Perforin	positive	50
TGFβ	positive	51

The BD Pharmingen brand anti-human CD39 (clone TÜ66) monoclonal antibody is a novel marker for human Tregs and is available as PE and APC conjugates in ready-to-use reagent kits for flow cytometry. TÜ66 recognizes ENTPD1, an ectoenzyme that belongs to the family of ectonucleoside triphosphate diphospho-hydrolases (E-NTPDases). The members of this family are involved in extracellular nucleotide catabolism, controlling the extracellular nucleoside triphosphate pool (NTPs).

CD152 (CTLA-4)

Cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) is considered to be critical for Treg suppressive function.[18] In a study by Zheng et al, CD152 was transfected into CD25⁻FoxP3⁻ T cells. The resulting cells had suppressive activity but did not express FoxP3.[19]

Studies from other groups have demonstrated that blockage of CD152 impairs the suppressive activities of Tregs. Abnormalities in CD152 expression have been reported to play a role in autoimmune diseases such as rheumatoid arthritis.[19]

CD152 may mediate suppressive activities through the down-regulation of CD80 and CD86 expression on dendritic cells, affecting the potency of antigen-presenting cells to activate other T cells.[20]

Studies such as those described for CD152 further our understanding of Treg function. The existence of defined populations, existing markers, and emerging markers will greatly contribute to exciting new discoveries in Treg biology.

Profile of anti-CD152 (CTLA-4) staining on fixed PBMCs

PBMCs were surface stained with anti-CD4 PerCP Cy™5.5 (clone SK3, Cat. No. 341654) and anti-CD25 PE (clone 2A3, Cat. No. 341010). Cells were washed with staining buffer and fixed with BD Cytofix™ buffer (Cat. No. 554655) per the protocol. The cells were then permeabilized with BD Perm/Wash™ buffer (Cat. No. 554723), stained with anti-CD152 APC (clone BNI3, Cat. No. 555855), and acquired on a BD FACSCalibur™ system.

A shows the gated events based on light scattering profile of lymphocytes and fluorescence characteristics of CD4 and CD25. The cells are differentiated as CD25high, CD25int, and CD25⁻ based on their CD25 expression.

B shows the overlay of the CD152 expression on these three subsets.

A

B

- CD25⁻ subset
- CD25int subset
- CD25high subset

SERVICES AND SUPPORT
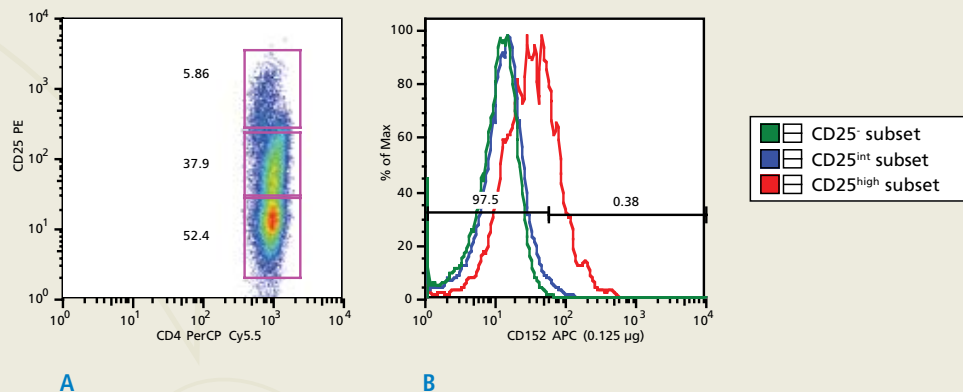
Committed to customer success

BD Biosciences is fully committed to the success and satisfaction of its customers. To help customers take full advantage of our offerings, BD Biosciences products are backed by a world-class service and support organization with unmatched experience in flow cytometry, cell biology, and antibody reagent development.

Technical application support

BD Biosciences technical application support specialists are available to provide field- or phone-based assistance and advice. Expert in a diverse array of topics, BD technical application support specialists are well equipped to address your needs in both instrument and applications support.

Custom services

Mobilizing technology for research applications requires close collaboration. The Custom Technology Team (CTT) at BD Biosciences works with customers to provide solutions through custom reagents, panels, or assay protocols.

Staffed by leading scientists with the breadth and depth of scientific and technical expertise, the CTT team will coordinate with researchers to study the problem at hand, make recommendations, and help implement the solutions. In this way, BD Biosciences technical know-how is translated into practical solutions that allow customers to focus on research.
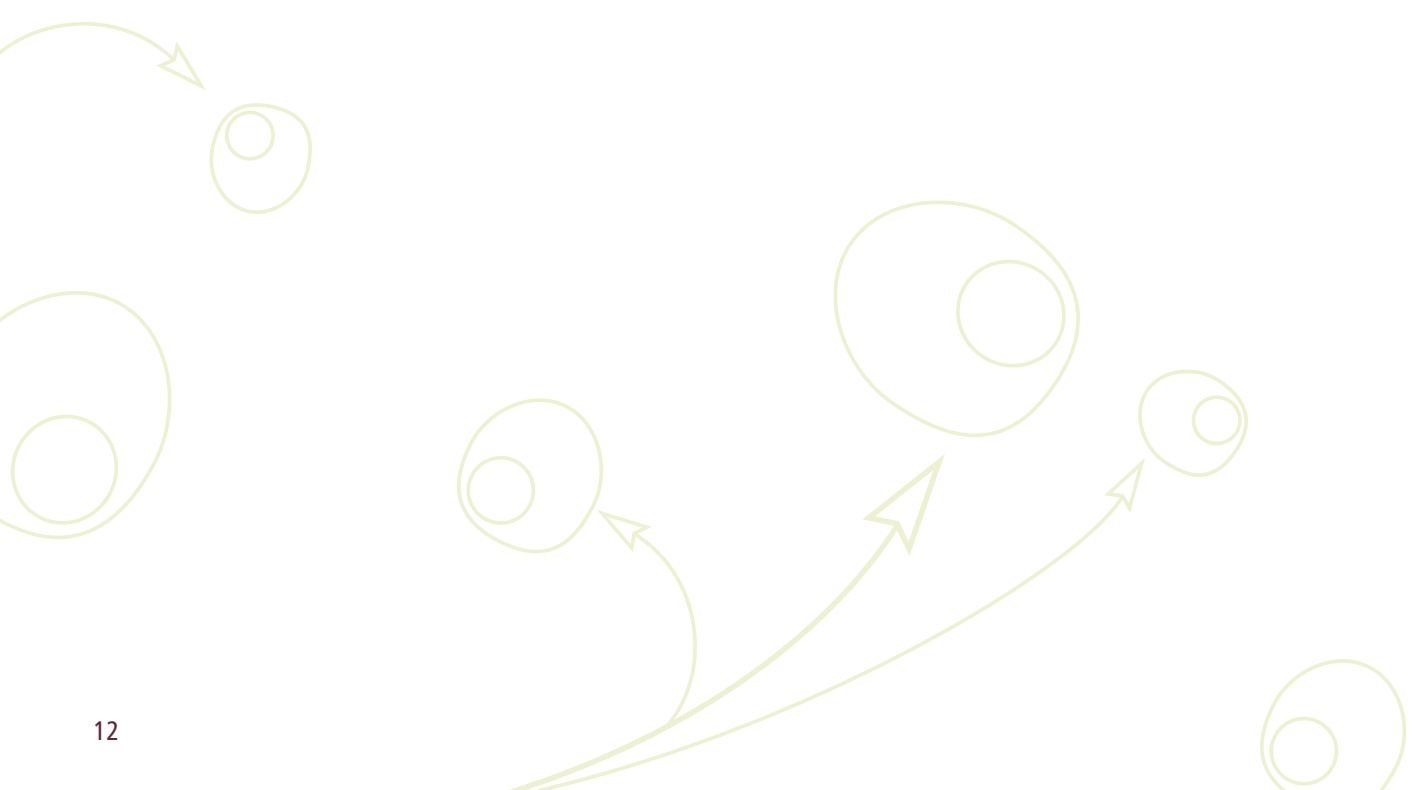
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