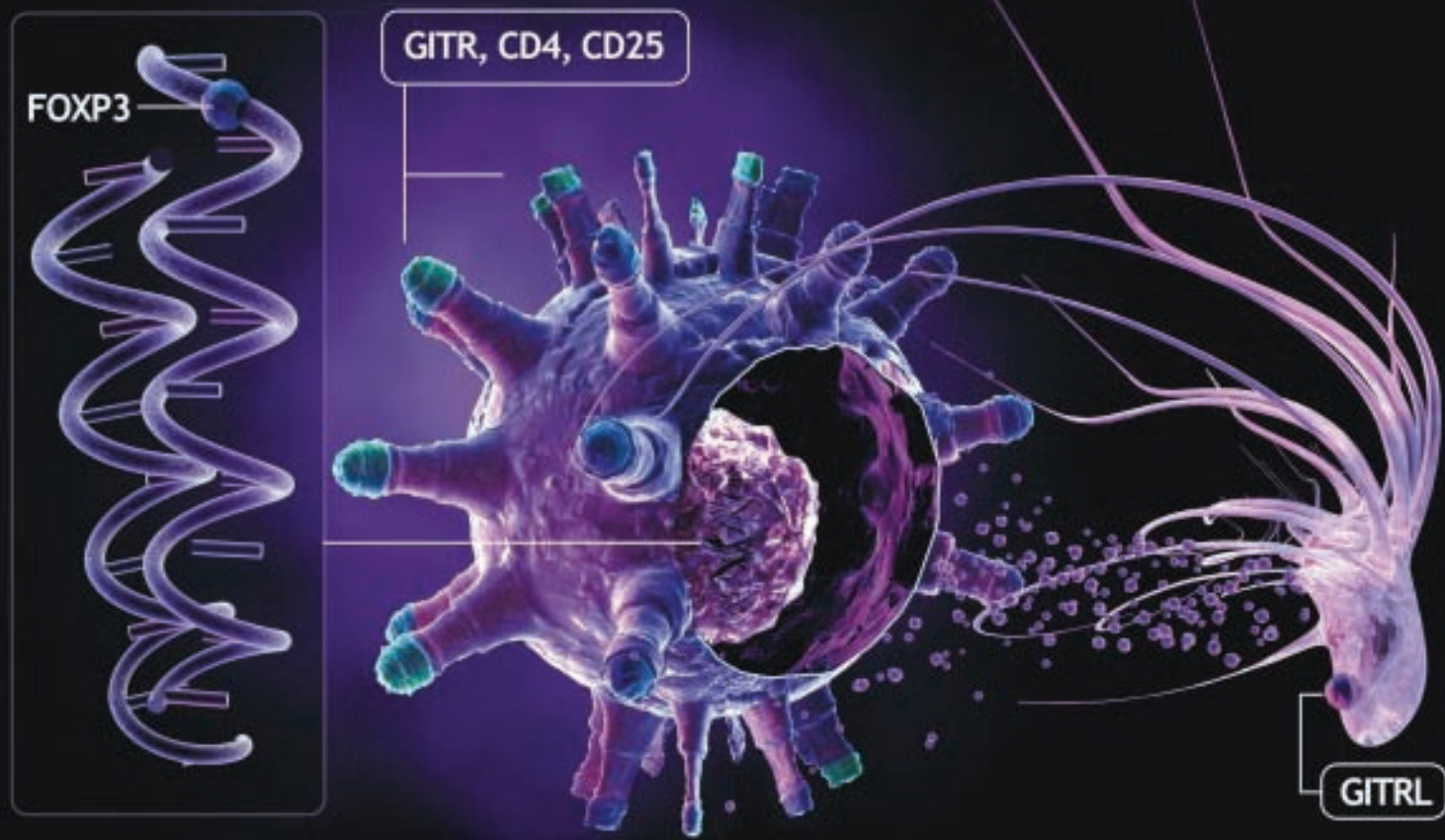


The path to legendary discovery



The Road to Regulation or Ruin: T Regulatory Cells



Table of Contents

Defining T _{reg} by Function, Location, and Activation Requirements	3
FoxP3: The Making of a T _{reg}	5
Regulating the Regulators: Controlling T _{reg} Through Cytokines	6
Regulating the Regulators: Controlling T _{reg} Through Signaling Molecules	7
T _{reg} Related Markers	8
Human and Mouse FoxP3: Multicolor Flow Cytometry	10
Identifying T _{reg} Populations by Phenotype	11
Concluding Remarks	12
BioLegend T _{reg} Related Products	13
References	18

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Defining T_{reg} by Function, Location, & Activation Requirements

Introduction

The existence of T suppressor cells capable of restricting the development, function, and duration of T effector cell responses were first described over 30 years ago by Gershon and Kondo.^{1,2} Although the transfer of such cells could induce antigen-specific tolerance in naïve animals,³ the notion of T cell-mediated suppression rapidly lost credibility due to an absence of reproducible biologic assays and specific phenotypic markers. This was one of the most controversial topics in modern immunology until, in 1995, work by Sakaguchi et al.⁴ reinvigorated the concept of antigen-specific suppression by documenting that depletion of the $CD4^+CD25^+$ fraction in normal Balb/c T cells caused widespread autoimmune disease and multi-organ failure when injected into Balb/c athymic nude mice. $CD4^+$ cells constitutively expressing the IL-2 receptor α -chain (CD25) were popularly renamed “regulatory” T cells or T_{reg} cells to overcome the negative connotations associated with the original moniker. Since that time, T_{reg} cells controlling fundamental immune responses involved in self-tolerance, anti-tumor immunity, and infectious disease have been identified in both mouse and man. The identification and isolation of T_{reg} cells represents a modern renaissance in immunologic theory related to the control of innate and inducible immune responses.

Defining T_{reg} by Function, Location, and Activation Requirements

The fundamental property that defines a T_{reg} is its ability to transfer immunological unresponsiveness from one animal to another (*in vivo*) or one culture to another (*in vitro*). Unresponsiveness *in vitro* is usually measured by the inhibition of either proliferation or cytotoxicity (type and amount of cytokine secretion appears to be less reliable), while *in vivo* measurements include the inhibition of autoimmune disease, graft rejection, allergic reactions, or other immune responses.

Experimental models suggest the existence of two functional types of T_{reg} cells designated naturally occurring and induced. These pathways are graphically illustrated in Figures 1A and 1B. In mice, naturally occurring T_{reg} are $CD4^+CD25^+FoxP3^+$ T cells that differentiate in the thymus and account for between 5–10% of the $CD4^+$ T cell population found in the blood, lymph nodes and spleen.^{5–7} Surprisingly, greater than 25% of the $CD4^+$ T cells in both human and mouse bone marrow have been shown to be T_{reg} cells, suggesting that T_{reg} cells may also be actively recruited and retained at this site.⁸ The recruitment and retention of $FoxP3^+$ cells in the bone marrow requires

Figure 1A

Development and Function of Naturally-Induced T_{reg}

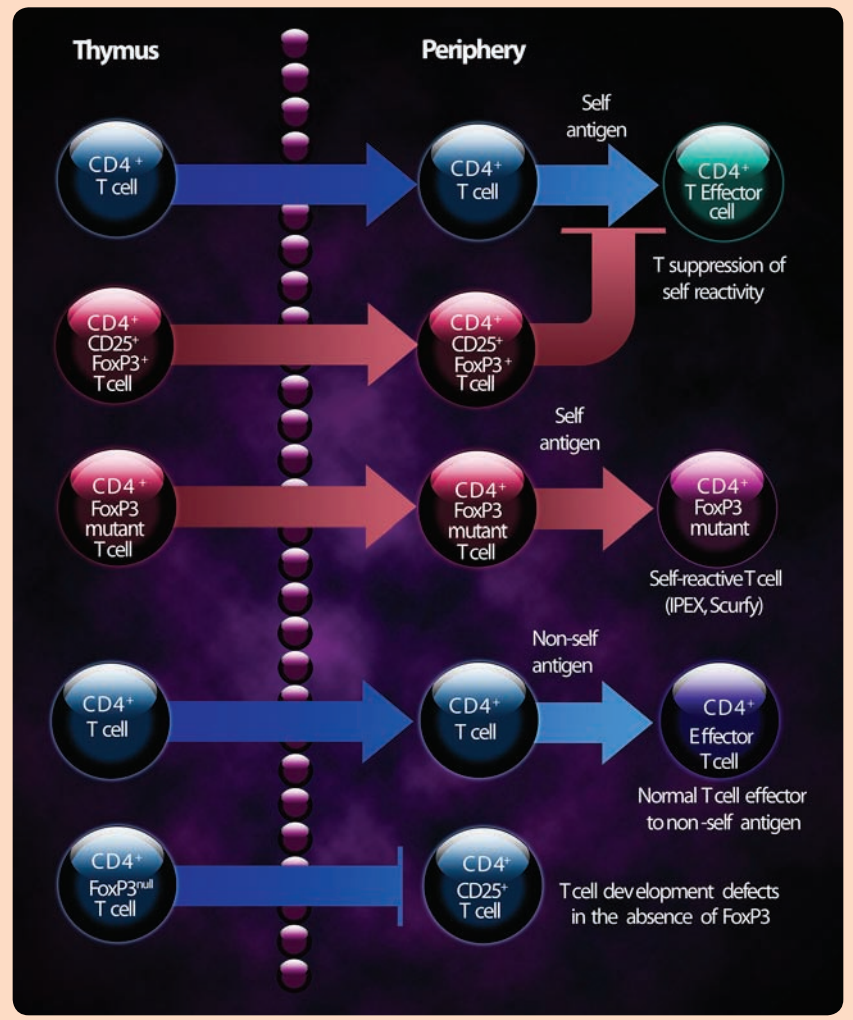
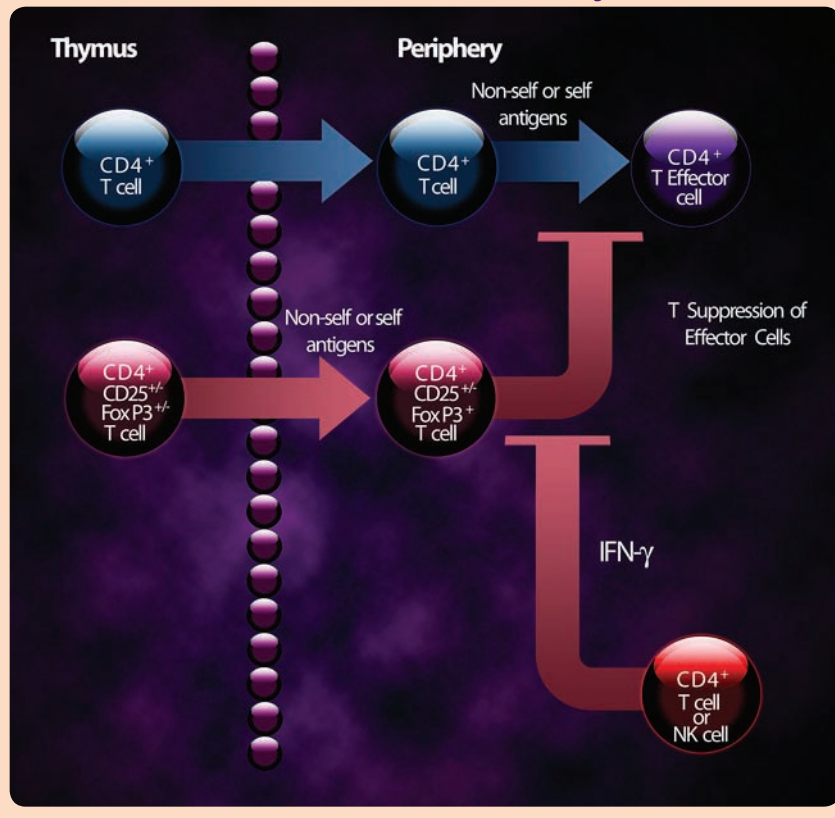


Figure 1B

Development and Function of Induced T_{reg}



after immunization with self-peptides.¹³ The suppressive activity of the CD4⁺CD25⁺ T_{reg} cells (as measured by the inhibition of proliferation of antigen-specific CD4⁺CD25⁻ and CD8⁺ T cells) gradually declines over a period of 8 weeks to almost undetectable levels in the absence of antigenic stimulation.¹³

Induced T_{reg} , on the other hand, are thought to be derived from a thymic CD4⁺ population that is negative or variable for CD25 expression, and negative for FoxP3 expression. In the periphery, these cells can be activated through TCR stimulation to become T_{reg} and acquire FoxP3 expression.¹⁷ Although CD25 levels can remain negative or low on these cells, under some circumstances these cells can become CD25⁺ as well.^{17, 18} As originally described by Walker et al.,¹⁹ human T_{reg} activity isolated from the peripheral blood is exclusively confined to the CD4⁺CD25⁺ T cell population expressing FoxP3. Upon polyclonal activation (anti-CD3 and anti-CD28 stimulation) of these CD4⁺CD25⁻ cells *in vitro*, such cells become

CXCL12/CXCR4 signals^{8, 9} as evidenced by the fact that T_{reg} can be released from bone marrow by the reduced CXCL12 expression following G-CSF treatment.

The “naturally occurring” T_{reg} population requires T cell receptor (TCR) engagement for functional activation, but unlike effector T cells T_{reg} populations show little proliferation or production of mitogenic cytokines after TCR stimulation. Naturally occurring T_{reg} appear to be restricted to self antigens and pathogens and require cell contact and to mediate suppression. The development^{10, 11, 12} maintenance¹³ of this CD4⁺CD25⁺ FoxP3⁺ cell population requires interactions with self-antigens which have only recently begun to be identified.¹³ Naturally occurring T_{reg} are thought to play pivotal roles in the maintenance of immunological homeostasis and have been shown to require cell contact and proteins such as CD152 (CTLA-4) GITR (glucocorticoid-induced tumor necrosis factor receptor) and granzyme B¹³⁻¹⁶ to exert their suppressive effects. Recent studies suggest that FoxP3 expression can be increased in the naturally occurring T_{reg} population (along with suppressive function)

FoxP3⁺ and CD25⁺ and acquire suppressive function that is contact- and cytokine-dependent. Later studies by these investigators documented that specific human T_{reg} can be generated from CD4⁺CD25⁻FoxP3⁻ T cells using cognate antigen,¹⁸ suggesting that *de novo, ex vivo* generated, antigen-specific T_{reg} might be feasible in the treatment of immune-mediated diseases. *De novo* FoxP3 upregulation and acquisition of a contact-dependent T_{reg} phenotype after antigen activation does not appear to occur in mice.

In mice, three types of regulatory T cells have been proposed, a thymic-derived CD4⁺CD25⁺FoxP3⁺ cell that suppresses the *in vitro* proliferation and cytokine production of effector T cells in a contact-dependent manner (so-called naturally occurring T_{reg}), and two other cell types designated Tr1 and Th3. The Tr1 and Th3 cells are generated in the periphery and elicit their suppression via a cytokine-dependent mechanism requiring IL-10 and IFN- γ in the case of Tr1 cells²⁰ and IL-4, IL-10, and TGF- β in the case of Th3 cells.²¹ In mice, naturally occurring CD4⁺CD25⁺FoxP3⁺ T_{reg} have been proposed to be involved in



the protection against self-reactive immune responses, while the Tr1 and Th3 cells have been proposed to limit immune responses to both self and foreign antigens in the periphery. In contrast to humans, stimulation of mouse CD4⁺CD25⁻ cells does not usually result in the upregulation of FoxP3 expression,^{22,23} suggesting that natural T_{reg} activity (as defined by contact-dependent, CD4⁺CD25⁺FoxP3⁺ regulatory cell function) can be uniquely marked through FoxP3 expression. However, it is important to note that the induction of T_{reg} activity by CD8α⁺ dendritic cells has been reported for CD4⁺CD25⁻FoxP3⁻ mouse T cells.²⁴ These cells have been reported to upregulate FoxP3 and acquire potent, IL-10 dependent suppressive activity, suggesting that some induced T_{reg} populations in mice can also express FoxP3.²⁴ In direct contrast, FoxP3 behaves more like an activation antigen in human cells. Stimulation of naïve human CD4⁺ T cells with polyclonal antigen or specific antigen can induce FoxP3 expression and these cells can exert suppressive activities on CD4⁺ effector populations from the same donor.¹⁸ Thus, in humans and mice it appears that FoxP3 can be used to identify populations with potential regulatory T cell function (whether naturally occurring or induced).

FoxP3: The Making of a T_{reg}

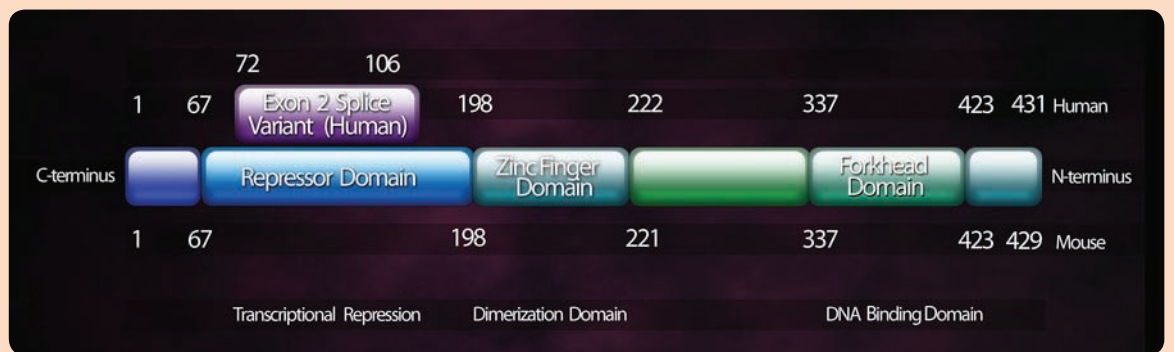
The transcription factor, FoxP3 is a critical component of regulatory T cell development. The mouse and human FoxP3 protein showing the location of the structural domains is shown in Figure 2. The role of FoxP3, a mammalian member of the Forkhead box-winged helix protein family originally discovered in *Drosophila*, in T_{reg} biology was spotlighted as a result of a natural mutation in the X-linked FoxP3 gene in *scurfy* mice.²⁵⁻²⁷ Mice carrying the *scurfy* mutation present with severe autoimmune disease characterized by multiorgan lymphocytic infiltration and CD4⁺ T cells that constitutively express a variety of activation

markers (including CD69 and CD25) and secrete large quantities of cytokines.^{28,29} The *scurfy* mutation in mice was shown to be the result of a 2-bp insertion in the FoxP3 gene that generated a premature stop codon and resulted in a truncated protein product.²⁸

Mutations in FoxP3 in humans have also been linked to a variety of autoimmune diseases including X-linked autoimmunity immunodeficiency syndrome and other X-linked autoimmune and allergic disorders.³¹⁻³² The position of these mutations relative to the functional domains in FoxP3 are shown in Table I (on page 6). The FoxP3 gene was experimentally linked to the development of autoimmune disease through adoptive transfer experiments and introduction of FoxP3 transgene into *scurfy* animals. CD4⁺ T cells from *scurfy* mice can transfer autoimmune disease to normal mice, autoimmune disease could be transferred.^{28,33} Reciprocally, when a wild-type FoxP3 gene was expressed in *scurfy* mutant mice autoimmune disease development was prevented.²⁸ Interestingly, however, FoxP3 transgenic animals were unable to mount antigen-mediated responses *in vivo* and isolated T cells were shown to produce virtually no IL-2 or proliferative response after TCR crosslinking *in vitro*.³⁴ Taken together, these findings suggested that T cells constitutively expressing FoxP3 were functionally inert.

Although enforced expression of FoxP3 in CD4⁺ T cells has deleterious effects on their ability to respond to TCR-mediated signals, the fact that a CD4⁺FoxP3⁺ population exists in normal animals led investigators to look for a role for FoxP3 in immune homeostasis. Several groups had reported that the predominant cell type that expressed FoxP3 was CD4⁺CD25⁺ T cells,

Figure 2
Structural Domains of Human and Mouse FoxP3



the same population that had been reported to suppress proliferation and cytokine production in conventional CD4⁺ T cells.^{18, 22, 23, 35} Further, when FoxP3 was ectopically expressed in conventional T cells, these cells were converted to a regulatory phenotype with the ability to suppress the proliferation of CD4⁺CD25⁻ T cells and prevent autoimmune gastritis and colitis when transferred *in vivo*.^{22, 23}

Bone marrow chimeras between wild type and FoxP3⁻ mice further showed that only bone marrow from wild-type donors could contribute to the development of CD4⁺CD25⁺ T_{reg}.²³ Taken together with the data from the *scurfy* mouse, the adoptive transfer and transgenic models, these data further strengthen the evidence that FoxP3 is both necessary and sufficient for T_{reg} development and function. How might this forkhead/winged helix transcription factor be involved in the control of autoimmune disease? Members of the Fox family can act as both transcriptional activators and repressors. FoxP3 appears to exert its function through the transcriptional repression of many genes including that of the cytokines. Supporting this, ectopic FoxP3 expression in Jurkat has been shown to markedly reduce IL-2 production after stimulation³⁶ and CD4⁺ T cells from FoxP3 transgenic animals were unable to produce IL-2, IL-4 or IFN- γ *in vitro* after TCR stimulation and failed to secrete cytokines *in vivo* following immunization.^{37, 38} Recent evidence, suggests that FoxP3 may specifically repress NFAT-mediated transcription.³⁶

Many studies are being directed toward defining the functional domains within the FoxP3 protein. As shown in Figure 2, FoxP3 contains at least three structural domains including the forkhead domain, a leucine zipper, and a zinc finger. The forkhead domain is critical for both DNA binding and nuclear localization, while the leucine zipper is thought to be critical for dimerization (based on functional studies with other family

members). In addition to the structural domains, functional domains in the N-terminal portion of FoxP3 have been shown to be involved in transcriptional repression.^{39, 40} Humans express two forms of FoxP3 as a result of an mRNA splice variant. The smaller form lacks exon 2 encoded by amino acids 71–105;^{18, 41} this isoform has not been observed in mouse CD4⁺CD25⁺ T_{reg} cells. Functionally, cells containing the FoxP3 Δ exon2 isoform show an intermediate response to TCR signaling with inhibition of proliferation and IL-2 production, albeit not to the same degree as those cells expressing full-length FoxP3.⁴¹

Regulating the Regulators: Controlling T_{reg} through cytokines

Normal immune homeostasis requires a fine balance so that immunologic tolerance is maintained, aberrant and excessive immune responses to invading pathogens or environmental antigens are limited, and effector function is allowed to develop and persist during the course of a normal immune response. In the absence of T_{reg} cells, robust responses to self antigens occur with subsequent autoimmune disease.^{6, 17} On the other hand, if T_{reg} activity predominates, T cell effector activity can be impeded and normal immune responses blocked. How are T_{reg} controlled to achieve the fine balance between regulation and ruin? As mentioned previously, FoxP3 levels are subject to environmental activation cues (especially in the case of human T_{reg})

Table I
FoxP3 Mutations Define Functional Domains

Species	Disease	Amino acids affected (Region)	Functional Consequence
Human	IPEX	250, 251, others (Leucine zipper)	Homodimerization disrupted, autoimmune disease
		363, 371, 384, 397, others (Forkhead domain)	DNA binding domain disrupted, autoimmune disease
		76, others (N-terminal repressor domain)	Repression of transcription disrupted, autoimmune disease
Mouse	Scurfy	276 (2 bp insertion resulting in truncation) GGACAAGAGCTC wild-type GGACAAAAGAGCTC scurfy	Loss of function, autoimmune disease

**Table II****Cytokines Regulating T_{reg} Development, Maintenance, and Function**

	Effect on T _{reg}	Reference
IL-2	Required for homeostasis of CD4 ⁺ CD25 ⁺ T _{reg} . Administration of IL-2 can expand T _{reg} population, IL-2 neutralization <i>in vivo</i> causes T _{reg} loss and autoimmune disease induction	42, 45
IL-4	Potently induces FoxP3 expression and increases T _{reg} activity	47
IL-7	Shown to be a survival factor for T _{reg}	46
IL-10	Can play a role in T _{reg} maintenance, expansion, and FoxP3 expression	48-50
IL-13	Potently induces FoxP3 expression and increases T _{reg} activity	47
TGF-β	Can play a role in T _{reg} maintenance, expansion, and FoxP3 expression	48-50
IFN-γ	Inhibits the generation of T _{reg} , suppresses T _{reg} activity	13
TNF-α	Downmodulates FoxP3 expression, inhibits T _{reg} function	51
G-CSF	Mobilizes T _{reg} from bone marrow compartment to periphery	8, 9

increase T_{reg} activity and/or expansion. The IL-4R α-chain binding cytokines IL-4 and IL-13 were recently documented to induce functional CD4⁺CD25⁺FoxP3⁺T_{reg} cells from peripheral CD4⁺CD25⁻ precursors by potently upregulating FoxP3.⁴⁷ Both IL-10 and TGF-β have been implicated in numerous models to play a role in the maintenance, expansion, and FoxP3 expression of CD4⁺CD25⁺T_{reg} cells.⁴⁸⁻⁵⁰ Just as cytokines have been reported to induce, maintain, and expand T_{reg} populations, others such as TNF-α and

and are upregulated following TCR engagement. In addition, T_{reg} respond to many cytokines in the environmental milieu including IL-2, IL-4, IL-7, IL-10, IL-13, TGF-β, and TNF-α. A summary of the cytokines and their reported effects on T_{reg} are shown in Table II.

Naturally arising T_{reg} express a high affinity IL-2 receptor that includes the CD25 (IL-2Rα chain), the IL-2Rβ (CD122) chain, and IL-2Rγc (CD132) chain in both the thymus and the spleen.⁴² Further, IL-2 is critically required for the maintenance of self-tolerance mediated by T_{reg} as neutralizing IL-2 monoclonal antibody administration can induce autoimmune disease and reduce the number of CD4⁺CD25⁺FoxP3⁺T cells in the thymus and periphery (without affecting the number of CD4⁺CD25⁻T cells in either compartment).⁴² These results suggest that IL-2 is required for the physiological proliferation of T_{reg} and provide a plausible explanation for the lethal autoimmune disease phenotype originally observed in IL-2 deficient animals.^{43,44} On the other hand, IL-2 administration has been shown to increase CD4⁺CD25⁺FoxP3⁺T_{reg} in the peripheral blood of cancer patients suggesting that high levels of IL-2 can expand T_{reg} populations.⁴⁵ IL-7 has also been suggested to be a survival factor for CD4⁺CD25⁺T cells.⁴⁶ Other cytokines such as IL-4, IL-10, IL-13, and TGF-β have been reported to

IFN-γ have been reported to suppress T_{reg} activity. IFN-γ has been reported to inhibit the generation of CD4⁺CD25⁺T_{reg} allowing CD4⁺CD25⁻ effector cells to predominate following immunization with self-antigens.¹³ Likewise, TNF-α has recently been reported to downmodulate FoxP3 expression and function in both naturally induced CD4⁺CD25⁺T_{reg} as well as CD4⁺CD25⁺TGF-β induced T_{reg}.⁵¹

Regulating the Regulators: Controlling T_{reg} through signaling molecules

A variety of signaling molecules have been reported to influence T_{reg}. A number of co-stimulatory receptors as well as receptors involved in innate immunity have been reported to regulate the development, proliferation, and function of T_{reg}. Examples of these T_{reg}-associated markers are listed in Table III. Several of these markers merit additional discussion.

It is well established that engagement of the TCR is required for CD4⁺CD25⁺T_{reg} cell development in the thymus.¹¹ Recent studies have documented that the linker for activated T cell protein (LAT) is required for TCR-induced T_{reg} generation.⁵² Additional studies have provided compelling evidence that CD28 co-stimulation is required for efficient T_{reg} generation

Continues on page 11

Table III

T_{reg} Related Markers

Name	Description/Function	Reference
CD3	T cell antigen receptor complex, found on thymocytes, mature T cells, and NK cells. TCR signaling is required for T _{reg} differentiation and is expressed on CD4 ⁺ CD25 ⁺ T _{reg} cells.	11
CD4	TCR co-receptor involved in T cell activation. Expression on natural and induced T _{reg} cells	4, 6
CD25 (IL-2R α)	Low affinity IL-2 receptor. Forms high affinity IL-2 receptor along with IL-2 R β and γ . Expressed on activated T and B cells and thymocyte subset. Expressed at high levels on natural T _{reg} and upregulated on induced T _{reg}	4, 6
CD27	Transmembrane tumor necrosis factor receptor (TNF-R) superfamily member. Expressed on thymocytes, some T and B cells and NK cells. Binds to CD70. Highly suppressive subset of CD4 ⁺ CD25 ⁺ alloantigen-induced T _{reg} express CD27	69
CD28	Member of the immunoglobulin superfamily expressed on thymocytes, T cells and NK cells. Binds to CD80 and CD86 to costimulate activation and proliferation. Thymic generation of T _{reg} involves CD28 costimulation; CD28superagonists activate CD4 ⁺ CD25 ⁺ T _{reg} in the periphery	53-57
CD45RO	Transmembrane tyrosine phosphatase expressed on activated and memory T cells, a B cell subset and other cell types. Enhances TCR and BCR signaling, binds to CD22.	70
CD62L	L-selectin expressed on the majority of B cells, naïve T cells and a subset of memory T and NK cells and others. Binds to CD34, GlyCam, MAdCAM-1. Involved in leukocyte homing, rolling and tethering. Antibody to CD62L has been reported to block T _{reg} expansion and function <i>in vivo</i>	71
CD95	Type I transmembrane glycoprotein also known as Fas, APO-1, and TNFRSF6. Expressed on T and B lymphocytes, monocytes, neutrophils and fibroblasts; expression is upregulated following activation. Binds to CD178 (Fas ligand) to induce apoptosis; plays a role in the maintenance of peripheral tolerance. Expressed at high levels on CD4 ⁺ CD25 ⁺ T _{reg}	(Unpublished observation BioLegend)
CD103	Type I transmembrane glycoprotein also known as human mucosal lymphocyte antigen 1 (HML-1) and integrin alphaE chain. Expressed on more than 90% of intestinal intraepithelial lymphocytes and 40% of lamina propria T lymphocytes in the intestine. Low expression on peripheral blood lymphocytes (0.5-5%). Thought to be involved in the retention and function of lymphocytes. Expressed on T _{reg} and may be involved in retention of T _{reg} at specific tissue sites as well as function	72, 73
CD120b	Type I transmembrane receptor for TNF also known as TNFRII or p75. Expressed on a variety of cells at low levels; expression is upregulated by activation. Binds both TNF- α and LT- α (also known as TNF- β) and induces signal transduction, leading to apoptosis, NF-kB activation, increased expression of proinflammatory genes or differentiation depending on cell type and differentiation state. Highly expressed on CD4 ⁺ CD25 ⁺ T _{reg}	(Unpublished observation BioLegend)
CD122 (IL-2R β)	Immunoglobulin superfamily member, forms high affinity IL-2 receptor with IL-2 R α and γ . Expressed on activated T and B cells and thymocyte subset. Expressed at high levels on natural T _{reg} and upregulated on induced T _{reg}	42
CD134 (OX40)	TNF receptor superfamily member expressed on activated CD4 ⁺ and CD8 ⁺ T cells and B cells. Provides CD28-independent costimulation. Constitutively expressed on T _{reg} , triggering can inhibit CD4 ⁺ CD25 ⁺ T _{reg} activity	51



Table III *continued*

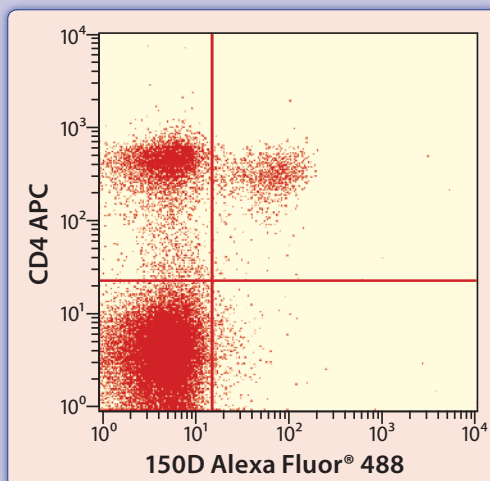
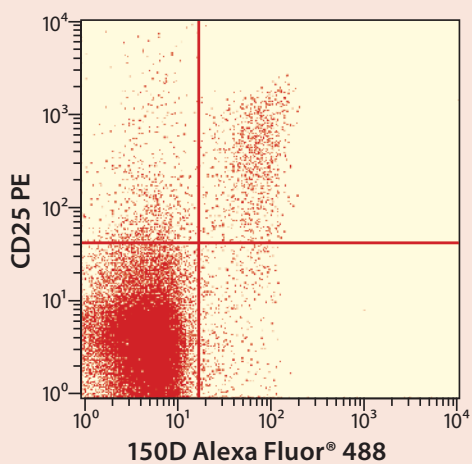
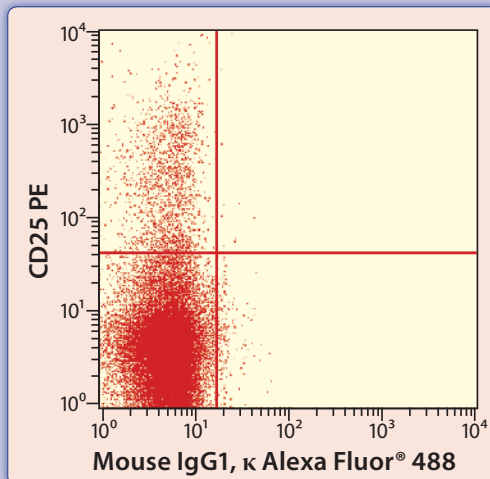
T_{reg} Related Markers

Name	Description/Function	Reference
CD150 (SLAM)	Transmembrane immunoglobulin superfamily member expressed on a T cell subset, B cells and other cell types. Self-ligation induces B cell costimulation, proliferation, and immunoglobulin production. Has been used to identify alloantigen-activated CD4 ⁺ CD25 ⁺ T _{reg}	74
CD152 (CTLA-4)	Transmembrane immunoglobulin superfamily member expressed on activated T cells. Binds to CD80 and CD86 to negatively regulated T cell activation. Constitutively expressed on CD4 ⁺ CD25 ⁺ T _{reg} may play a role in T _{reg} function	60, 61
CD184 (CXCR4)	G-protein linked seven transmembrane glycoprotein expressed on a variety of cells including T and B cells. Receptor for SDF-1. Thought to be involved in migration and retention of T _{reg} in the bone marrow	75
CD197 (CCR7)	G-protein coupled receptor for MIP-3 beta, SLC (6CKine, TCA-4, Exodus-2) plays a role in T cell adhesion, thymic development, and recirculation of memory cells and chemotaxis. Expressed on human T _{reg}	76, 77
CD223 (LAG-3)	CD4 homologue, colocalizes with CD4, CD8, and CD3 in lipid rafts during activation. Plays a role in the down-regulation of T cell responses. Antibodies to LAG-3 inhibit T _{reg} ; ectopic expression of LAG-3 induces T suppressor activity	63, 64
CD278 (ICOS)	Highly expressed on T _{reg} in target organs during autoimmunity. Blockade of ICOS inhibits T _{reg} function and increases autoimmunity	65
TLR-2 (CD282)	TLR-2 stimulation inhibits T _{reg} activity	67
TLR-4 (CD284)	Reported to be expressed on T _{reg} . Activation with LPS increases CD4 ⁺ CD25 ⁺ T _{reg} activity	66
TLR-5	Reported to be expressed on T _{reg} .	66
TLR-7	Reported to be expressed on T _{reg} .	66
TLR-8	Reported to be expressed on T _{reg} . Ligands to TLR-8 inhibit T _{reg} activity	66, 68
FoxP3	Forkhead/winged helix transcription factor considered to be the master switch for T _{reg} development and maintenance. Critically required for immune homeostasis	6, 17–19, 22–42
GITR	TNF receptor superfamily member expressed on resting T cells (low levels), CD4 ⁺ CD25 ⁺ T cells and activated T cells (high levels). Binds to GITR ligand; triggering inhibits the suppressive activities of CD4 ⁺ CD25 ⁺ T _{reg} cells	15, 16, 58, 59
LAT	Linker for activated T cells, phosphor-tyrosine adaptor protein linking TCR signals to Ras-MAPK activation. Required for CD4 ⁺ CD25 ⁺ T _{reg} thymic development	52
PD-1	Immunoglobulin superfamily member expressed on a subset of CD4 ⁺ CD8 ⁻ thymocytes and on activated T and B cells. Involved in lymphocyte clonal selection and peripheral tolerance. Intracellular expression PD-1 (but not cell surface expression) reported for both human and mouse T _{reg}	78
TCR	Immunoglobulin superfamily member expressed on T cells and thymocytes that recognizes peptide bound to MHC. With CD3 forms CD3/TCR α/β complex; involved in antigen recognition and T cell activation. The TCR is expressed on T _{reg} cells.	11



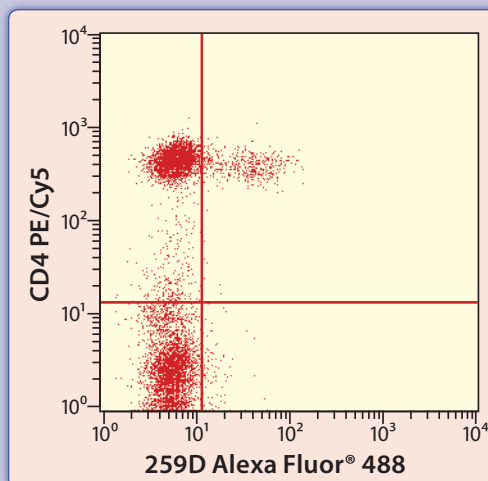
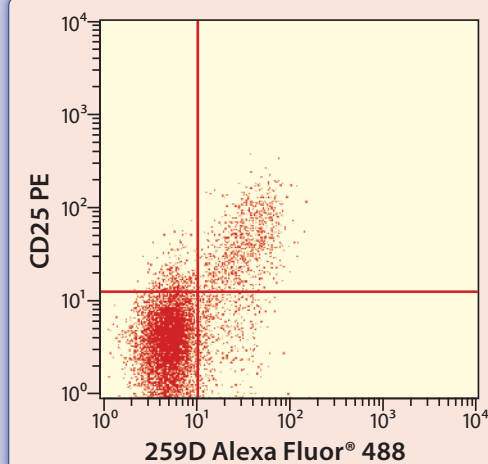
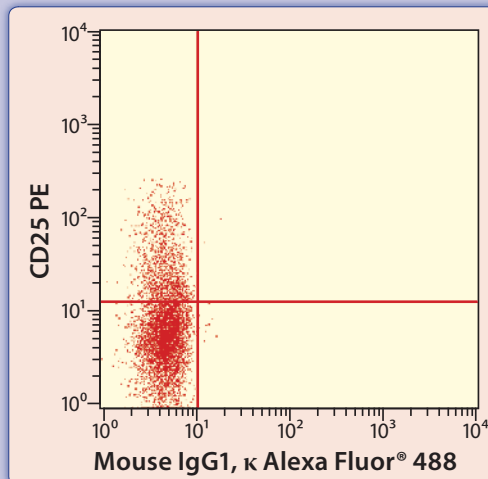
Figure 3

Mouse FoxP3



BALB/c mouse splenocytes stained with
Mouse Treg Flow™ kit
(FOXP3 Alexa Fluor® 488/CD25 PE/CD4 APC)

Human FoxP3



Human peripheral blood mononuclear cells stained with
Human Treg Flow™ kit (FOXP3 Alexa Fluor® 488/CD25 PE/CD4
PE-Cy5) and analyzed on lymphocyte population



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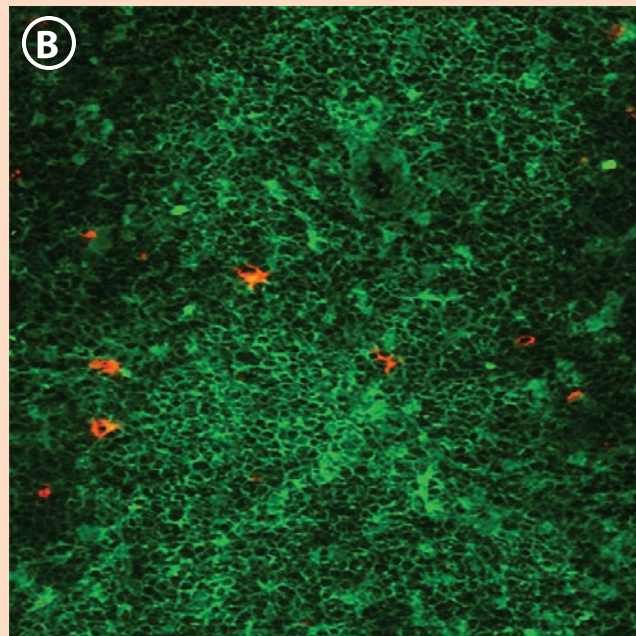
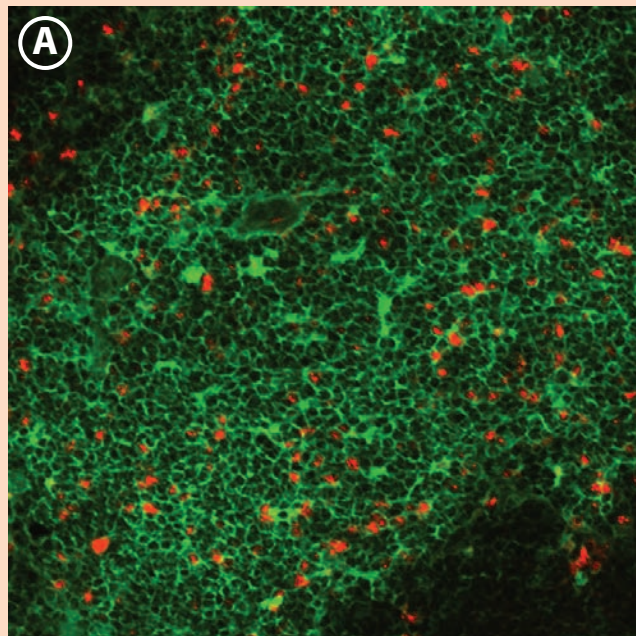
in the thymus⁵³ and maintenance in the periphery.⁵³⁻⁵⁵ CD28 co-stimulation has been shown to induce developing thymocytes to express FoxP3 (as well as GITR and CTLA-4) and initiate the T_{reg} differentiation program independent of IL-2.⁵³ In the periphery, CD28 stimulation has been shown to be essential for the homeostasis of the $CD4^+CD25^+$ T_{reg} population.⁵³⁻⁵⁵ Recent evidence suggests that CD28 superagonists can selectively expand T_{reg} populations in the periphery and allow effective prevention or therapy of various autoimmune diseases.⁵⁵⁻⁵⁷

Similar findings have been reported for the glucocorticoid-induced TNF receptor related protein (GITR) and cytolytic T lymphocyte-associated antigen 4 (CTLA-4) that are constitutively expressed at high levels on the surface of T_{reg} cells.^{15,16,58-61} *In vitro* studies have shown that cross-linking GITR at the same time as TCR stimulation triggers T_{reg} proliferation, but at the same time inhibits T_{reg} activity.^{15,16,58,59} Administration of GITR antibodies to neonatal mice has been shown to break self-tolerance and elicit autoimmune disease.^{15,16} In adult mice,

the administration of crosslinking GITR antibodies (that do not result in GITR blockade) have been shown to costimulate effector T cells, inhibit T_{reg} activity, enhance antigen-specific responses, allograft rejection, and provoke effective tumor immunity.^{15,16,58,59} Similarly, antibody-mediated blockade of CTLA-4 has been shown to abrogate T_{reg} suppression and result in the loss of immunologic self-tolerance, implying a critical role for both GITR and CTLA-4 in the control of autoimmunity through T_{reg} function.^{60,61}

Antibody-mediated triggering of CD134 (OX40), CD233 (LAG-3), or blockade of CD278 (ICOS) has been shown to inhibit T_{reg} activity.⁶²⁻⁶⁵ LAG-3 is particularly interesting in this regard, as ectopic expression of this protein has also been shown to induce T_{reg} activity in T cells,⁶⁴ implying that this protein may be involved in the differentiation of T_{reg} . Additional studies will be required to determine how this protein compares to FoxP3 in the development of T_{reg} populations. Ligand-mediated stimulation of toll-like receptors TLR-4⁶⁶ or TLR2⁶⁷ and TLR-8⁶⁸ have been shown to increase and decrease

Figure 4



A. Frozen 8 μ m spleen section from a naïve Balb/c spleen stained with FITC-CD4 (green) and purified FoxP3 (clone 150D) followed by goat anti-mouse-Cy3 (red).

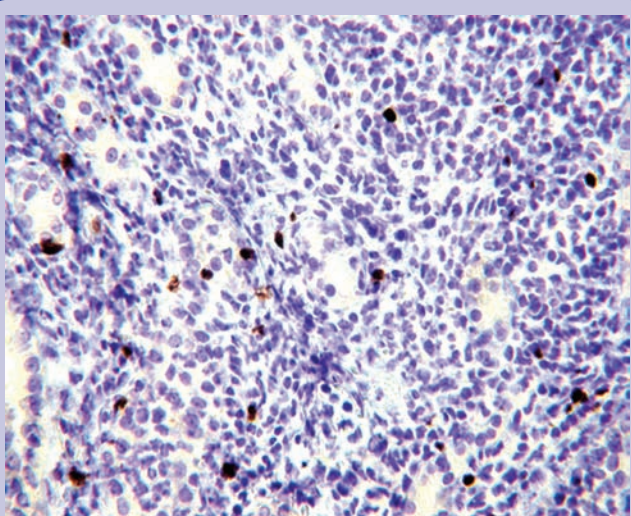
B. Frozen 8 μ m spleen section from a Balb/c mouse treated with anti-CD25 (clone PC-61). Mice received 250 μ g anti-CD25 i.p. on day 1 and 48 hrs later received a second dose of 250 μ g anti-CD25. Spleens were harvested 72 hrs after the first antibody treatment and stained with FITC-CD4 (green) and purified FoxP3 (clone 150D) followed by goat anti-mouse-Cy3 (red). *In vivo* treatment with PC-61 results in the depletion of splenic FoxP3 positive cells.

T_{reg} activity, respectively. Taken together, these data imply that there are a number of cell surface proteins that can regulation the development, proliferation, and function of T_{reg} cells.

Identifying T_{reg} Populations by Phenotype

Isolation of T_{reg} for functional studies requires markers to identify these important cells. CD25 has been recognized as an important marker for the identification of T_{reg} , however many other activated effector lymphocytes also express CD25.^{4,6} The most reliable of all markers for T_{reg} activity remains the “ T_{reg} master switch” transcription factor FoxP3. Additional phenotypic markers associated with the immunosuppressive activities of CD4⁺ lymphocytes have also been reported to include cell surface proteins such as ICOS, GITR, CTLA-4, LAG-3, and a variety of others. A compilation of T_{reg} -associated markers (both intracellular and cell-surface proteins) are shown in Table III. Although no single or dual markers can be used to identify a particular T_{reg} population by flow cytometry, the use of marker sets to define various cell surface proteins (CD4, CD25, CTLA-4, GITR, for example), in combination with the transcription factor FoxP3, and/or intracellular cytokine staining

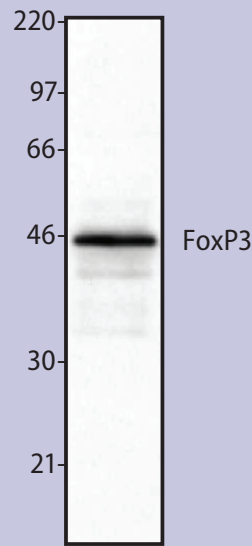
Figure 5



Formalin-fixed, paraffin-embedded Cynomolgus kidney was treated with EDTA pH 8.0 with a high pressure cooker prior to staining with monoclonal anti-FoxP3 (clone 259D) at 10 $\mu\text{g}/\text{ml}$ followed by biotinylated goat anti-mouse and streptavidin HRP. Staining was visualized with DAB substrate and counterstained with hematoxylin.

Figure 6

Cell extract from HEK293T cells transfected with human FoxP3 cDNA was resolved by electrophoresis, transferred to nitrocellulose, and probed with monoclonal anti-FoxP3 antibody (clone 206D). Proteins were visualized using a goat anti-mouse secondary conjugated to HRP and a chemiluminescence detection system.



(IL-10, for example) will aid in the further characterization of these regulatory populations.

Concluding Remarks

Taken together, existing studies suggest that the development and maintenance of T_{reg} are critically required for immune homeostasis. T_{reg} cells express a variety of cell surface markers that can be used for identification. The most reliable markers that track with T_{reg} function are CD4, CD25, and FoxP3. Other markers such as GITR and CTLA-4 are also very useful for the identification of this functional cell population (Please see Table III for a comprehensive guide to T_{reg} associated markers). BioLegend offers a wide array of antibodies for intracellular staining of mouse, human, and rat FoxP3 as well as a reliable and easy to use FoxP3 Fix/Perm buffer set for flow cytometry. An example of mouse and human FoxP3 staining by flow cytometry is shown in Figure 3. Our FoxP3 antibodies have also been verified for immunohistochemistry and Western blotting. Examples of FoxP3 staining on frozen tissue samples is shown in Figure 4; formalin-fixed, paraffin-embedded tissues are shown in Figure 5. A Western blot using anti-FoxP3 is shown in Figure 6. Please visit our website (www.biolegend.com; support section tab, Technical protocols) for a detailed FoxP3 intracellular staining protocol.



BioLegend T_{reg} Related Products

Specificity	Species Reactivity	Clone	Purified	LEAF™	Biotin	FITC	PE	PE/Cy5	PE/Cy5.5	PE/Cy7	APC	APC/Cy5.5	APC/Cy7	Alexa Fluor® 488	Alexa Fluor® 647	Alexa Fluor® 700	Pacific Blue™
Mouse CD3 and Related Molecules																	
CD3	Mouse	17A2	X	X		X	X							X	X	X	X
CD3ε	Mouse	145-2C11	X	X	X	X	X	X	X	X	X	X		X	X		
CD4	Mouse	GK1.5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CD4	Mouse	RM4-5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CD4	Mouse	RM4-4	X			X	X										
CD8a	Mouse	53-6.7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CD8a	Mouse	5H10-1	X			X											
CD8b	Mouse	CD8b	X		X	X	X										
CD11a (Integrin α _L)	Mouse	2D7	X		X	X	X										
CD11a (Integrin α _L)	Mouse	M17/4	X	X	X	X	X							X	X		
CD11b (Integrin α _M)	Mouse	M1/70	X	X	X	X	X	X		X	X			X	X	X	
CD11c (Integrin α _X)	Mouse	N418	X		X	X	X	X		X	X			X	X	X	
CD18 (Integrin β ₂)	Mouse	M18/2	X	X	X	X	X							X	X		
CD25 (IL-2Rα)	Mouse	3C7	X	X			X										
CD25 (IL-2Rα)	Mouse	PC61	X	X	X	X	X	X		X	X			X	X	X	X
CD28	Mouse	37.51	X	X	X		X	X			X						
CD29 (Integrin β ₁)	Mouse	HMB1-1	X	X	X	X	X				X			X	X	X	
CD30	Mouse	mCD30.1	X	X	X		X										
CD44	Mouse	IM7	X	X	X	X	X	X			X			X	X	X	X
CD45RB	Mouse	C363-16A	X	X		X	X										
CD49d (Integrin α ₄ , VLA-4α)	Mouse	R1-2	X	X	X	X	X							X	X		
CD49d (Integrin α ₄ , VLA-4α)	Mouse	9C10(MFR4.B)	X	X	X		X										
CD62L (L-selectin)	Mouse	MEL-14	X	X	X	X	X	X		X	X			X	X	X	X
CD69 (VEA)	Mouse	H1.2F3	X		X	X	X	X		X	X			X	X		
CD80 (B7-1)	Mouse	16-10A1	X	X	X	X	X	X			X			X	X		
CD86 (B7-2)	Mouse	GL-1	X	X	X	X	X	X		X	X			X	X	X	X
CD86 (B7-2)	Mouse	PO3	X	X	X	X	X	X		X	X			X	X	X	
CD120b (TNF R II/p75)	Mouse	TR75-32.4		X	X												
CD120b (TNF R II/p75)	Mouse	TR75-54.7	X	X													
CD120b (TNF R II/p75)	Mouse	TR75-89	X		X		X										
CD122 (IL-2Rβ)	Mouse	5H4	X	X	X		X										
CD126 (IL-6Rα)	Mouse	D7715A7	X	X	X		X										
CD127 (IL-7Rα)	Mouse	SB/199	X		X	X	X							X	X		
CD134 (OX-40)	Mouse	OX-86	X		X												

The Road to Regulation or Ruin: T Regulatory Cells

Specificity	Species Reactivity	Clone	Purified	LEAF™	Biotin	FITC	PE	PE/Cy5	PE/Cy5.5	PE/Cy7	APC	APC/Cy5.5	APC/Cy7	Alexa Fluor® 488	Alexa Fluor® 647	Alexa Fluor® 700	Pacific Blue™
CD150 (SLAM)	Mouse	TC15-12F12.2	X	X	X		X	X		X	X			X	X		
CD152 (CTLA-4)	Mouse	9H10	X	X													
CD152 (CTLA-4)	Mouse	UC10-4B9	X	X	X		X										
CD154 (CD40 Ligand)	Mouse	MR1	X	X	X		X										
CD197 (CCR7)	Mouse	4B12	X		X		X	X			X			X	X		
CD278 (ICOS)	Mouse	15F9	X		X		X	X									
CD278 (ICOS)	Mouse	C398.4A	X	X	X	X	X				X			X	X		
CD279 (PD-1)	Mouse	RMP1-14	X	X													
CD279 (PD-1)	Mouse	RMP1-30	X		X		X										
CD284 (TLR4 /MD2 Complex)	Mouse	MTS510	X	X	X		X										
FOXP3	Mouse	150D	X				X							X	X		
FOXP3 Flow Kit	Mouse	150D					X							X	X		
Mouse Treg Flow Kit	Mouse	150D												X			
GITR	Mouse	YGITR 765	X	X	X	X	X	X		X	X			X	X	X	
GITRL	Mouse	YGL 386	X		X		X										
Integrin β ₇	Mouse	FIB27	X	X													
Integrin β ₇	Mouse	LS722	X		X	X	X										
T-bet	Mouse	Poly6235	X														
Mouse MHC Antigens																	
I-A/I-E	Mouse	M5/114.15.2	X	X	X	X	X	X			X			X	X	X	X
I-A ^b	Mouse	AF6-120.1	X		X	X	X							X	X		
I-A ^b	Mouse	KH74	X		X	X								X	X		
I-A ^b (Aβ ^b)	Mouse	25-9-17	X		X	X								X	X		
I-A ^d	Mouse	39-10-8	X		X	X								X	X		
I-A ^k (Aα ^k)	Mouse	11-5.2	X		X	X	X							X	X		
I-A ^k (Aβ ^k)	Mouse	10-3.6	X		X	X	X							X	X		
I-A ^q	Mouse	KH116	X		X									X	X		
I-E ^k	Mouse	14-4-45	X		X	X	X							X	X		
Mouse T Cell Receptors (TCRs)																	
β T Cell Receptor	Mouse	H57-597	X	X	X	X	X	X			X			X	X		
γ/δ T Cell Receptor	Mouse	UC7-13D5	X	X	X	X	X							X	X		
γ/δ T Cell Receptor	Mouse	GL3	X		X	X	X										
Vβ7 T Cell Receptor	Mouse	TR310	X		X	X	X										
Vβ 8.3 T Cell Receptor	Mouse	8C1	X			X	X										
Mouse Cytokines/Chemokines																	
IL-2	Mouse	JES6-1A12	X	X													X
IL-2	Mouse	JES6-5H4	X	X	X	X	X				X			X	X	X	
IL-4	Mouse	11B11	X	X			X				X			X	X		
IL-4	Mouse	BVD6-24G2			X												



Specificity	Species Reactivity	Clone	Purified	LEAF™	Biotin	FITC	PE	PE/Cy5	PE/Cy5.5	PE/Cy7	APC	APC/Cy5.5	APC/Cy7	Alexa Fluor® 488	Alexa Fluor® 647	Alexa Fluor® 700	Pacific Blue™
IL-10	Mouse	JES5-2A5	X	X	X												
IL-10	Mouse	JES5-16E3	X	X	X	X	X				X			X	X		
IFN-γ	Mouse	R4-6A2	X	X	X												
IFN-γ	Mouse, Rat	DB-1	X	X		X	X										
IFN-γ	Mouse	XMG1.2	X	X	X	X	X				X			X	X	X	
TNF-α	Mouse	MP6-XT22	X	X	X	X	X				X			X	X	X	
TNF-α	Mouse	TN3-19.12	X	X			X										
TNF-α	Mouse	Poly5062			X												
TNF-α	Mouse	6B8	X	X													
Rat CDs and Related Molecules																	
CD3	Rat	1F4				X											
CD4	Rat	W3/25	X		X	X	X				X						
CD8a	Rat	OX-8	X			X	X										
CD8a	Rat	G28	X	X	X	X	X				X						
CD8b	Rat	341	X	X	X	X											
CD11b/c (Integrin α_M/α_X)	Rat	OX-42	X		X	X	X				X						
CD25 (IL-2R α)	Rat	OX-39	X			X	X										
CD28	Rat	JJ319	X	X		X	X				X						
CD29 (Integrin β_1)	Rat	HM β 1-1	X	X	X	X	X				X			X	X	X	
CD45RA	Rat	OX-33	X		X	X	X										
CD49d (Integrin α_4)	Rat	MR α 4-1	X	X		X											
CD80 (B7-1)	Rat	3H5	X	X	X		X										
CD86 (B7-2)	Rat	24F	X	X	X	X	X										
CD126 (IL-6R α)	Rat	D7715A7	X	X	X		X										
CD278 (ICOS)	Rat	C398.4A	X	X	X	X	X				X			X	X		
FOXP3	Rat	150D	X				X							X	X		
FOXP3 Flow Kit	Rat	150D					X							X	X		
Rat T Cell Receptors (TCRs)																	
α/β T Cell Receptor	Rat	R73	X	X	X	X	X				X						
γ/δ T Cell Receptor	Rat	V65	X			X	X										
V β 8.2/8.4 T Cell Receptor	Rat	R78	X			X											
Rat Cytokines/Chemokines																	
IL-2	Rat	BL-7015	X														
IL-2	Rat	BL-7030			X												
IL-4	Rat	BL-7045	X														
IL-4	Rat	BL-7060			X												
IFN-γ	Rat	DB-1	X	X		X	X										
IFN-γ	Rat	Poly5109			X												
TNF-α	Rat	TN3-19.12	X	X			X										

The Road to Regulation or Ruin: T Regulatory Cells

Specificity	Species Reactivity	Clone	Purified	LEAF™	Biotin	FITC	PE	PE/Cy5	PE/Cy5.5	PE/Cy7	APC	APC/Cy5.5	APC/Cy7	Alexa Fluor® 488	Alexa Fluor® 647	Alexa Fluor® 700	Pacific Blue™
TNF-α	Rat	Poly5062			X												
Human CD's and Related Molecules																	
CD3 (T3)	Human	HIT3a	X	X	X	X	X	X		X	X		X	X	X	X	
CD3 (T3)	Human	UCHT1	X	X	X	X	X	X		X	X			X	X	X	X
CD4 (T4)	Human	RPA-T4	X	X	X	X	X	X		X	X		X	X	X	X	X
CD8a (T8)	Human	HIT8a	X		X	X	X	X		X	X			X	X	X	
CD8a (T8)	Human	RPA-T8	X	X	X	X	X	X	X	X	X		X	X	X	X	X
CD11a (Integrin α _L)	Human	HI111	X	X	X	X	X	X			X			X	X		
CD11b (activated) (Integrin α _M , Mac-1)	Human	CBRM1/5	X	X		X	X										
CD11b (Integrin α _M , Mac-1)	Human	ICRF44	X	X	X		X	X			X			X	X		X
CD11c (Integrin α _X)	Human	3.9	X	X	X	X	X	X		X	X			X	X		
CD18 (Integrin β ₂)	Human	TS1/18	X	X	X	X	X	X									
CD25 (IL-2Rα)	Human	BC96	X			X	X	X		X	X		X	X	X	X	X
CD27	Human	O323	X		X	X	X				X				X	X	
CD28 (T44, Tp44)	Human	CD28.2	X	X	X	X	X	X			X			X	X	X	
CD29 (Integrin β ₁)	Human	TS2/16	X	X			X	X			X		X	X	X	X	
CD44 (Hermes, Pgp-1)	Human	IM7	X	X	X	X	X	X			X			X	X	X	X
CD45RA	Human	HI100	X		X	X	X	X			X			X	X	X	X
CD45RB	Human	MEM-55	X				X										
CD45RO	Human	UCHL1	X			X	X	X			X			X	X	X	X
CD49d (Integrin α ₄ , VLA-4α)	Human	9F10	X	X			X	X			X						
CD62L (L-Selectin)	Human	DREG-56	X	X		X	X	X			X		X	X	X	X	
CD69 (VEA)	Human	FN50	X			X	X	X		X	X		X	X	X	X	X
CD80 (B7-1)	Human	2D10	X	X	X	X	X	X						X	X		
CD86 (B7-2)	Human	IT2.2	X	X	X		X	X			X			X	X		X
CD150 (SLAM)	Human	A12 (7D4)	X	X	X	X	X							X	X		
CD154 (CD40 Ligand)	Human	24-31	X	X	X	X	X	X			X			X	X	X	X
CD184 (CXCR4, Fusin)	Human	12G5	X	X	X		X	X			X						
CD278 (ICOS)	Human	C398.4A	X	X	X	X	X				X			X	X		
CD282 (TLR2, Toll Like Receptor 2)	Human	TL2.1	X	X	X	X	X							X	X		
CD284 (TLR4, Toll Like Receptor 4)	Human	HTA125	X	X	X		X										
FOXP3	Human	206D	X			X	X							X	X		X
FOXP3	Human	259D	X				X							X	X		
FOXP3	Human	150D	X				X							X	X		
FOXP3 Flow Kit	Human	150D					X							X	X		
FOXP3 Flow Kit	Human	206D					X							X	X		
FOXP3 Flow Kit	Human	259D					X							X	X		
Human Treg Flow Kit	Human	259D												X			
GITR (AITR)	Human	621	X	X			X										
GITR Ligand (AITRL)	Human	EB11-2	X	X			X										



Specificity	Species Reactivity	Clone	Purified	LEAF™	Biotin	FITC	PE	PE/Cy5	PE/Cy5.5	PE/Cy7	APC	APC/Cy5.5	APC/Cy7	Alexa Fluor® 488	Alexa Fluor® 647	Alexa Fluor® 700	Pacific Blue™
Granzyme A	Human	CB9	X			X	X									X	X
Integrin β ₇	Human	FIB27	X	X													
Integrin β ₇	Human	FIB504	X				X	X			X						
LAT	Human	LAT1111	X														
Perforin	Human	dG9	X			X	X							X	X		
T-bet	Human	Poly6235	X														
Human MHC Antigens																	
HLA-DR (MHC Class II)	Human	L243	X	X	X	X	X	X		X	X		X	X	X	X	X
Human T Cell Receptors (TCRs)																	
α/β T Cell Receptor (α/β TCR)	Human	IP26	X		X	X	X	X						X	X		
α/β T Cell Receptor (α/β TCR)	Human	T10B9	X			X											
TCR Vβ5 related subset	Human	MEM-262	X			X								X	X		
Multicolor Cocktail Reagents																	
CD3 FITC/CD4 PE Cocktail	Human	UCHT1/RPA-T4				X	X										
CD3 FITC/CD8 PE Cocktail	Human	UCHT1/RPA-T8				X	X										
CD3 PE-Cy5/CD4 PE/CD8 FITC Cocktail	Human	UCHT1/RPA-T4/RPA-T8				X	X	X									
CD4 PE-Cy5/CD25 PE Cocktail	Human	RPA-T4/BC96					X	X									
Human Cytokines/Chemokines																	
IL-2	Human	MQ1-17H12	X	X		X	X				X			X	X	X	
IL-2	Human	Poly5111			X												
IL-4	Human	8D4-8	X	X			X										
IL-4	Human	MP4-25D2	X	X	X	X	X				X			X	X		
IL-10	Human	JES3-12G8	X		X												
IL-10	Human	JES3-19F1	X	X			X				X						
IL-10	Human	JES3-9D7	X	X			X				X			X	X		
IL-13	Human	JES10-5A2	X	X			X				X						
IL-13	Human	Poly5020			X												
IFN-γ	Human	NIB42	X	X													
IFN-γ	Human	4S.B3	X		X	X	X				X			X	X	X	
IFN-γ	Human	MD-1	X	X													
IFN-γ	Human	B27	X	X		X	X				X					X	
TNF-α	Human	MAB1	X	X													
TNF-α	Human	MAB11	X		X	X	X				X			X	X		
Related Products																	
FOXP3 Fix/Perm Buffer set	Cat. No. 421403																
Cell Staining Buffer	Cat. No. 420201																
RBC Lysis Buffer	Cat. No. 420301																

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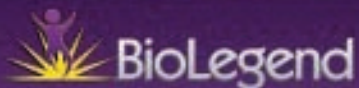
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