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Factors regulating apoptosis and homeostasis of CD4+CD25highFOXP3+ regulatory T cells are new therapeutic targets

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1. ABSTRACT

$CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Tregs), as an active mechanism of immune suppression, have been targeted due to their tremendous therapeutic potentials to prevent autoimmune diseases, transplant rejection, and to inhibit progression of tumors and chronic viral diseases. In last twelve years, substantial molecular differences between homeostasis of Tregs and that of other subsets of T cells and some factors specific in regulation of Treg survival have been characterized. In this overview we focus on panoramic reviewing of 91 factors, pathways and drugs, both well-characterized and newly defined, regarding the survival and homeostasis of Tregs in the following sections: 2: Tregs, an essential mechanism of immune tolerance; 3: nTregs, aTregs and other regulatory T cells; 4: co-stimulation receptor signaling; 5: innate immunity and Toll-like receptor (TLR) signaling; 6: effects of cytokines and hormones; 7: transcription factors in regulation of Tregs; 8: Treg intracellular signaling

pathways; 9: drugs and potential therapeutics; and 10: Treg cell survival and death. We recently reported that removal of Tregs via a Bax-dependent apoptotic pathway significantly enhances anti-self antigen immune responses, which demonstrated for the first time the proof of principle that apoptosis of Tregs is a new therapeutic target. Therefore, continued characterization of Treg apoptosis and homeostasis pathways would further improve our understanding in regulation of immune responses by cytokines, hormones and drugs, and would also lead to development of new therapeutics.

2. Tregs, AN ESSENTIAL MECHANISM OF IMMUNE TOLERANCE

About 5-7% of people in developed countries are affected with one or another autoimmune disease (1). In addition, for the diseases traditionally considered to be caused by other etiology, autoimmune mechanisms are found to significantly contribute to the pathogenesis of these diseases including insulin-dependent type I diabetes mellitus (2-4), atherosclerosis (5) and other vascular diseases (6-10). In physiological conditions, the adaptive immune system recognizes a multitude of antigens and then expands as effector cell populations that can recognize molecules derived from the pathogens. To function properly, the system must develop a mechanism of self-tolerance that can recognize self antigens (11). On one hand, to improve our understanding on selection of autoantigens/tumor antigens, we recently proposed a new model of stimulation-responsive alternative splicing for extra-thymically expressed, untolerized self-protein isoforms to become immunogenic tumor antigens and autoantigens (12-15). On the other hand, we now realize that autoimmune cellular mechanisms against self-antigens occur as a consequence of self-tolerance breakdown, presumably resulting from a combination of inherited DNA variations, gene polymorphisms, environmental triggers, and stochastic events (16). Further determination of cellular mechanisms underlying self-tolerance and the pathogenesis of autoimmune diseases in humans and animal models (17) holds promise for future development of novel therapeutics for these diseases.

Self-tolerance breakdown results from the disruption of T cell tolerance (18-20). Several primary mechanisms relative to the generation and maintenance of T cell tolerance have been proposed: *I)* Clonal deletion in thymus (21), by which self-reactive thymocytes can be eliminated via apoptosis in various stages of their maturation (22). In addition, thymus also generates naturally occurring Tregs for maintaining peripheral tolerance (23), which is facilitated via expression of transcription factors FOXP3 in thymocytes and autoimmune regulator (AIRE) in stromal cells (24-26); *II)* peripheral T cell anergy (27), in which T cells cease to proliferate or secrete interleukin-2 (IL-2) in the absence of T cell co-stimulation; *III)* Regulatory T cells in periphery: As an active mechanism of immune suppression, distinct subsets of Tregs suppress the activation of autoreactive T cells that have escaped the mechanisms of thymic clonal deletion and T cell anergy (19). Historically, approaches to treat autoimmune diseases have been focused on immunosuppressive drugs that block the activation of antigen-specific T cells by altering early signaling pathways. However, these interventions have several problems: (*a)* less effective on activated T cells to abort established autoimmune responses; (*b)* require continuous use; and (*c)* suppress immune surveillance for tumor growth (28, 29) and protective immunity targeted at microbial pathogens (30). The enthusiasm in exploration of therapeutic potentials of Tregs has been tremendously increased, which is driven by the need to develop strategies that prevent autoimmune diseases, transplant rejection, and inhibit progression of tumors and chronic viral diseases (31, 32). New vaccination using antigen-specific Tregs makes enhancement of antigen-specific therapy possible (30). In addition, since development of Tregs in thymus and homeostasis of Tregs in the periphery are regulated by the mechanisms distinct from those of CD4+CD25- T cells (33), therefore, continuous characterization of homeostatic differences between Tregs and other subsets of T cells and factors in regulation of Tregs suppression (34) would provide better immune therapies (35). As outline in Figure 1, in this overview, we will focus on reviewing reported molecular mechanisms including 91 factors and pathways (Table 1), both well-characterized and newly defined, regarding the homeostasis of Tregs including factors governing the generation, suppression (35) and cell death/survival of Treg cells. We apologize for failing to cover many invaluable reports, and reviews due to the space limit of this review. Several excellent reviews have recently been published on the function of Tregs (36-41), which are different from the focus of this review. Of note, since numerous cited papers did not specify nTregs or aTregs in their studies, thus we use Tregs unless specified.

3. nTregs, aTregs AND OTHER REGULATORY T CELLS

Several regulatory T cell subsets (42-48) including CD4+CD25^{high} T cells, CD4+CD25-PD-1+ T cells (49), CD8+ cells (50), CD8+CD25+ T cells (51, 52), CD8+CD28- T cells (31), TCR$\gamma\delta$+ T cells (50), NKT cells (50), CD3+CD4-CD8-$\alpha\beta$TCR+ T cells (53) have been reported, among which CD4+CD25^{high} Tregs are the best characterized (11, 38, 39, 54, 55). Comprising 5-10% of peripheral CD4+ T cells (56-59), Tregs exhibit potent immunosuppressive functions (60), and play an important role in the regulation of autoimmunity (18, 61), anti-tumor immunity (62-64), pathogenesis of atherosclerosis (65), allergy and asthma (66), transplantation immunity (67, 68), and anti-microbial immunity (69-72). There are two subsets of Tregs, (*a)* peripherally adaptive Tregs (aTreg cells, transforming growth factor-β (TGF-β) secreting, and IL-10 secreting) (73, 74), and (*b)* naturally occurring Tregs (thymic generated, nTregs). After encounter with foreign antigens (75), aTregs can be generated (35) with a standardized protocol (76) and are defined by their cytokine profile, including two subsets: Treg type 1 (T_R 1) cells that secrete high levels of IL-10, and Th3 cells that secrete high levels of TGF-β (77). nTregs are primarily engaged in the maintenance of self-tolerance and down-regulation of various immune responses via a cell-cell contact manner

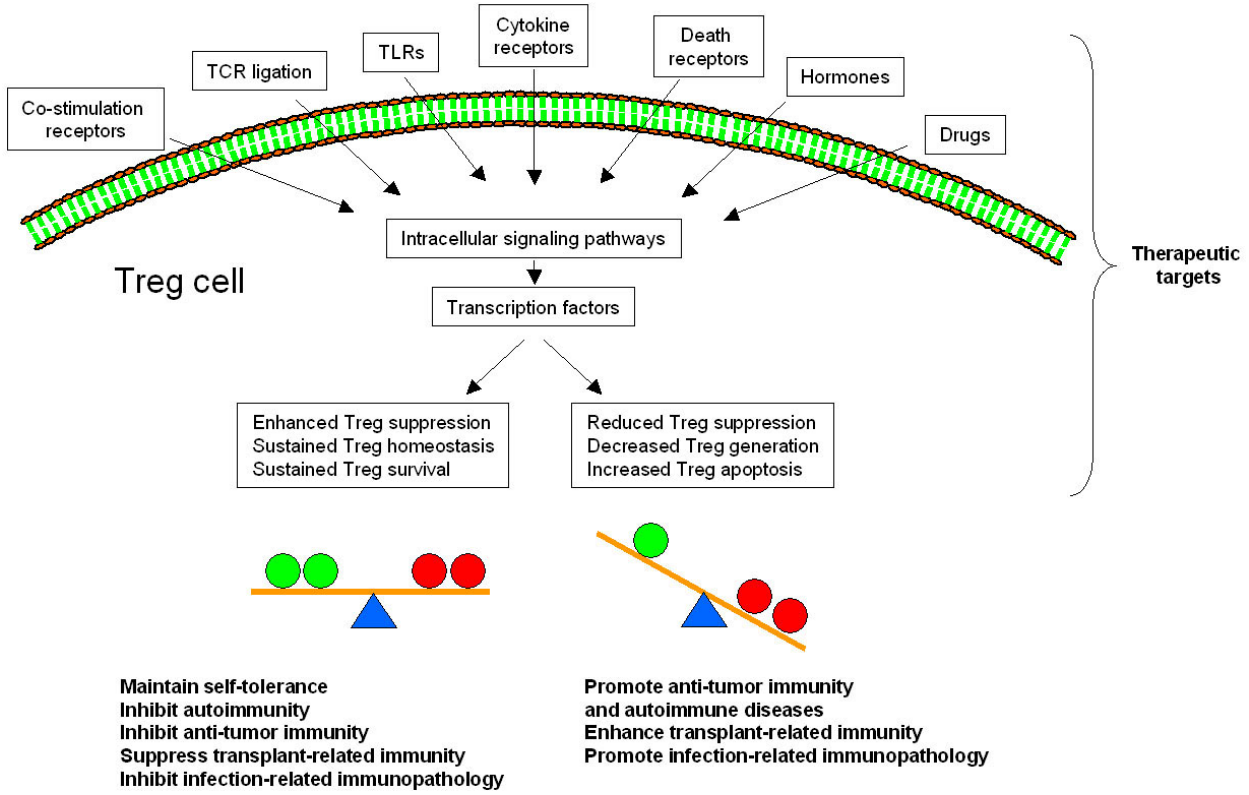

Figure 1. The schematic representation of our working model entitled "factors regulating Treg survival and homeostasis are new therapeutic targets". Various factors, including T cell antigen receptor ligation, T cell co-stimulation, toll-like receptor ligation, cytokine stimulation, hormone stimulation, death receptor ligation, and drug effects, may trigger Treg intracellular signaling pathways, which further regulate expression and function of Treg specific transcription factors. In consequence, Treg survival and homeostasis are affected either positively or negatively. Treg survival and homeostasis lead to maintaining immune tolerance, inhibiting autoimmunity and anti-tumor immunity, and suppressing transplant-related immunity and infection-related immunopathology. In contrast, increased Treg apoptosis and Treg malfunction would lead to promoting autoimmune diseases and anti-tumor immunity, and enhancing transplant-related immunity and infection-related immunopathology. Therefore, factors regulating Treg survival and homeostasis become new therapeutic targets.

(78), and aTregs are concerned with ablating an ongoing immune response (79). nTregs can be defined by cell surface and intracellular marker profile (CD4+, $CD25^{high}$, intracellular FOXP3+ (80), GITR+ (81), $CD62L^{+/high}$ (82), CD5+, CD27+ (83), CD38+, CD39+ (84), $CD45RB^{low}$ (82), $CD45RA^{low}$, $CD45RO^{high}$, CD73+ (84), CD103 (integrin αEβ7)+ (85), intracellular $CTLA4^{high}$, surface $CTLA4^{low}$ (86, 87), HLA-DR^{high}, $CD122^{high}$, $CD127^{low/-}$ (88), CD130- (89), CD134 $(OX40)^{high}$, LFA-3^{medium}, CCR4+ (90), CCR7+ (91), CCR8+ (92), TNFR2+ (93)) (also see excellent reviews (94, 95)). In a recently published review, Roncarolo and Battaglia wrote a very good summary on the difference between mouse and human Tregs (96). Of note, $CD25^{high}$ has proved to be by far the most useful surface marker for nTregs (97, 98). Moreover, CD25 is a key component (IL-2 receptor α chain, IL-2Rα) of the high affinity IL-2R so that it is not a mere marker of nTregs but is also functionally essential for nTregs since nTregs are highly dependent on exogenous IL-2 for their survival (99). nTregs can be activated by self-antigens and non-self-antigens (100). Once activated, nTregs can suppress T cells in antigen-specific and antigen-nonspecific manners. Interestingly, the suppressive effects of these cells are not restricted to the adaptive immune system including CD4+ T cells, CD8+ T cells (101), NK cells (102), and B cells (103), but can also affect the activation and function of innate immune cells (monocytes, macrophages, dendritic cells) (73) and neutrophils (104). nTreg suppression *in vitro* is dependent on cell contact with target cells as shown by experiments utilizing transwells (79). Blocking antibodies to IL-10 and TGF-β do not affect the nTreg suppression, and nTregs isolated from IL-10- or TGF-β-deficient mice are also functional *in vitro* (79). nTregs suppress target cells by four mechanisms: nTregs may kill target cells via a granzyme B-dependent pathway (105), perforin pathway (106), programmed death-1/programmed death-1 ligand pathway (107), or Fas-Fas ligand interaction in an epitope-specific manner (103). In addition, direct contact of Tregs with their targets leads to increased secretion of regulatory cytokines IL-4, IL-10, and TGF-β, suggesting a mechanism of linked immunosuppression (108). Furthermore, CD73 (5'-

Table 1. Factors that enhance or inhibit Treg homeostasis and/or apoptosis

	Enhancement	Inhibition		Enhancement	Inhibition
Co-stimulation			**Intracellular signaling**		
CD28	+		SOCS3		+
CTLA-4 blocking		+	Galectins		+
CD7 + CD28	+		PIM1	+	
GITR blocking		+	PI-3K	+	
OX40 blocking		+	Ras-ERK	+	
4-1BB Ab binding	+/-	+/-	HO-1/NO		+
PD-1 blocking		+	Cyclin E/A		
4C8 antigen	+		p27kip1		+
CD38	+				
ART2		+	**Drugs**		
LAT	+		Anti-CD25		+
GPCR83	+		Denileukin		+
Anti-CD200R	+		Nonmitogenic anti-CD3	+	
			SEB		+
TLR signaling			Anti-TNF-alpha	+	
BLP-TLR2	+		Anti-IL-10		+
LPS-TLR4	+		Anti-IL-17		+
Flagellin-TLR5	+		S1p-FTY720	+	
TLR8-ligand		+	Corticosteroids	+	
CpG-ODN-TLR9		+	Rapamycin	+	
HSP60	+		Cyclosporine A		+
			Tacrolimus	+	
Cytokines/hormones			Mycophenolate Mofetil	+	
IL-2	+		Cyclophosphamide		+
IL-7	+		Flutarabine		+
IL-15	+		FK778	+	
IL-4	+		B:9-23	+	
TGF-beta	+		AutoAb peptide	+	
IL-13	+		FOXP3 siRNA		+
IL-12		+	DC exosomes		+
IL-6		+	15-deoxyspergualin	+	
IFN-alpha	+		Pentoxifylline	+	
IFN-beta	+		Vitamin D R-L	+	
TSLP	+		Vitamin C	+	
VIP	+		Vitamin E	+	
VEGF blockade		+	Glatiramer acetate	+	
Estrogen	+		a-galactosylceramide	+	
Leptin		+	Listeriolysin O		+
Prostaglandin E2	+		Inhibitor of APC proteosome	+	
AhR-TCDD	+				
IDO	+		**Apoptosis**		
			Fas		+
Transcription factors			Bax		+
FOXP3	+		NAD/ATP		+
NFAT	+		Bcl-2	+	
NF-kB		+	Bcl-xL	+	
p53		+			
CREB	+				
STAT1	+				
STAT3		+			
STAT4		+			
STAT5	+				
T-bet		+			
GATA3	+				
IRF-1		+			

ctonucleotidase) expressed on both Tregs and CD25- uncommitted primed precursor Th (Thpp) cells converts extracellular 5'-AMP to adenosine. Adenosine suppresses proliferation and cytokine secretion of Th1 and Th2 effector cells. This mechanism represents an additional suppressive manner of Tregs and a previously unrecognized suppressive activity of Thpp cells (109). It is unknown whether these mechanisms work in collaborative or independent manner.

4. CO-STIMULATION RECEPTOR SIGNALING

Since Tregs are functional in suppression of T cell activation where T cell co-stimulation play an essential role, several questions need to be solved regarding effects of T cell co-stimulation on Tregs: (*a)* whether T cell co-stimulation is needed for selection of Tregs in the thymus; (*b)* whether co-stimulation is required for Treg suppression function; (*c)* whether co-stimulation is needed for Tregs to survive. The following results are organized along these lines.

4.1. CD28 T cell co-stimulation

CD28 is one of the molecules expressed on T cells that provide co-stimulatory signals, which are required for T cell activation. CD28 is the receptor for B7.1 (CD80) and B7.2 (CD86). Both B7-deficient mice and CD28-

deficient mice have decreased nTregs in spleen, as low as 10% in B7 deficient mice and 25% in CD28 deficient mice in comparison to that of wild-type mice (61). Thus, B7/CD28 T cell co-stimulation plays an essential role in the generation and survival of nTregs (61, 110-116). Nevertheless, CD28 costimulation provides more than IL-2 to developing Tregs, as CD28 costimulation of TCR-signaled double-positive thymocytes induces expression of FOXP3, as well as GITR and CTLA-4, two proteins highly expressed on Tregs (117). Some investigators showed that the CD28-mediated homeostasis of nTregs is independent of IL-2, OX40, CD40L, and survival factor Bcl-xL (118). However, others have found that IL-2 plays a critical role in the maintenance of nTregs *in vivo* by regulating FOXP3 expression through a STAT-dependent mechanism and inducing the expansion of these cells *in vivo* (119).

4.2. Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4)

CTLA4 is a CD28-family receptor expressed on mainly CD4+ T cells. It binds the same ligands as CD28 (CD80 and CD86 on B cells and dendritic cells), but with higher affinity than CD28. However, in contrast to CD28 which enhances cell function when bound at the same time as the T cell receptor, CTLA4 inhibits the T cell and prevents it from functioning. CTLA-4 expression is not required for nTreg development or function since in CTLA-4-deficient mice Treg development and homeostasis is normal (120). However, non-activating anti-CTLA-4 antibodies block the suppressor activity of Tregs *in vitro*. Of note, clinical application of co-stimulatory blockade using agents such as CTLA-4Ig in the treatment of autoimmune disease results in complicated outcomes (118).

4.3. Synergy of CD7 and CD28 T cell co-stimulation

CD7 and CD28 are T cell Ig superfamily molecules that share common signaling mechanisms. CD28-deficient mice have decreased nTregs in spleen compared with wild-type mice, and CD7/CD28-double-deficient mice have decreased numbers of nTreg cells in both thymus and spleen compared with both wild-type and CD28-deficient mice (113). Tregs from CD28-deficient mice and CD7/CD28-double-deficient mice could mediate suppression of CD3 mAb activation of CD4+CD25- wild-type T cells, but were less potent than wild-type Tregs (113). These results suggest that there is a synergy of CD7 and CD28 T cell co-stimulation in regulating the generation and suppressive function of Tregs (113).

4.4. GITR and other TNF superfamily members

Glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein (TNF superfamily member 18, TNFSF18, GITR) and its ligand (GITR-L) play an important role in the control of nTreg activity. GITR is a constitutively expressed marker for nTregs, but it also is upregulated on activated CD4+ T cells (81). Blocking GITR with an anti-mouse GITR mAb inhibits nTreg suppression function *in vitro* (121, 122) and leads to the induction of autoimmunity (122). Furthermore, GITR-L blocks *in vitro* suppression mediated by nTregs (123, 124). These results suggest that interaction of GITR-GITR-L is important for Treg suppression.

4.5. OX40 (CD134)

OX40 (CD134) is a member of the TNF receptor family that is transiently expressed on T cells after TCR ligation. Both naive and activated Tregs express OX40. Triggering of OX40 on CD4+CD25+ Treg cells with agonist antibodies blocks their inhibitory activity (125).

4.6. 4-1BB (CD137)-4-1BB ligand

4-1BB (CDw 137), a member of the TNFR superfamily, is a costimulatory receptor primarily expressed on activated T cells. The administration of agonistic anti-4-1BB mAb enhances tumor immunity and allogeneic immune responses. nTregs from 4-1BB-deficient mice are able to prevent naive CD4+ T cell-induced colitis (126). Paradoxically, others reported that administration of agonistic anti-4-1BB monoclonal antibody leads to the increased number of splenic Treg cells and amelioration of TNBS-induced inflammatory bowel disease (colitis) (127). Taken together, TNF superfamily members play critical roles in regulation of Treg survival as well as suppressive function of Tregs.

4.7. Programmed death-1 (PD-1)

Programmed death-1 (PD-1), an inhibitory receptor up-regulated on activated T cells plays a critical immunoregulatory role in peripheral tolerance and in allo-immune responses. PD-L1, but not PD-1 or PD-L2, blockade accelerates the rejection of MHC class II-mismatched skin graft (bm12 (I-Abm12) into B6 (I-Ab)) in a similar manner to CTLA-4 blockade. The effect of PD-L1 blockade is dependent on the inhibition of Tregs *in vivo* (128), suggesting that PD-L1 blockage may inhibit Treg suppression function.

4.8. 4C8 antigen

A novel 4C8 antigen costimulation pathway plays an important role for the induction of Tregs (129). The costimulation induces full activation of CD4+ T cells with high levels of IL-2 production and cellular expansion, which are comparable to those obtained on costimulation by CD28. Tregs are induced by costimulation of CD4+ T cells with anti-CD3 plus anti-4C8 antibodies (129).

4.9. CD38 and ART2

Ubiquitously expressed CD38 and T cell-expressed ADP-ribosyltransferase 2 (ART2) are ectoenzymes competing for NAD substrate. Transfer of a genetically disrupted CD38 allele into the autoimmune diabetes-prone NOD/Lt background accelerates diabetes onset in both sexes, whereas transfer of a disrupted ART2 complex has no effect. This correlates with impaired Treg development (10-fold reduction in FOXP3 mRNA expression) in CD38-/- mice. Both ART2-deficient and CD38/ART2 combined deficient T cells are resistant to NAD-induced killing *in vitro*, whereas CD38-deficient but ART2-intact T cells show increased sensitivity, particularly the Tregs to NAD-induced T cell death (130).

4.10. Linker for activation of T cells (LAT)

TCR engagement induces formation of signaling complexes mediated through the transmembrane adaptor protein, the linker for activation of T cells (LAT). LAT

plays an important role in T cell development, activation, and homeostasis. nTregs are nearly absent in both thymus and peripheral lymphoid organs of LAT (Y136F) mutant mice (131). Ectopic expression of FOXP3 confers a suppressive function in LAT (Y136F) T cells. Thus, the LAT-PLC-γ1 interaction plays a critical role in FOXP3 expression and the development of nTregs (131).

4.11. G protein-coupled receptor 83

The heptahelical G protein-coupled receptors (GPCRs) constitute the most diverse forms of transmembrane signaling proteins (132), which are targeted by 40% of all current therapeutics (133). The G protein-coupled receptor (GPR) 83 is selectively up-regulated by Tregs in contrast to naive CD4+CD25- or recently activated T cells. GPR83-transduced T cells are able to inhibit the effector phase of a severe contact hypersensitivity reaction of the skin. This *in vivo* acquisition of suppressive activity is associated with the induction of FOXP3 expression in GPR83-transduced CD4+ T cells under inflammatory conditions. These results suggest that GPR83 may be critically involved in the peripheral generation of Tregs *in vivo* (134).

4.12. Anti-CD200R

CD200 is a transmembrane protein delivering immunoregulatory signals after engagement of CD200R. A family of CD200Rs exist (CD200R1-4) with different tissue expression and functional activity. Anti-CD200R2, but not anti-R1, augments induction of Tregs from anti-CD3/CD28 activated thymocytes by promoting development of dendritic cells (DCs) (135).

5. INNATE IMMUNITY AND TOLL-LIKE RECEPTOR (TLR) SIGNALING

Toll-like receptors (TLRs) represent a primary line of defense against invading pathogens in mammals, plants and insects. TLRs recognize the structures of lipids, carbohydrates, nucleic acids and various proteins, which are collectively referred to as pathogen associated molecular patters (PAMPs) (136). Eleven TLRs have been identified in humans while 13 TLRs can be encoded by mouse genome. TLRs 1-9 are conserved between humans and mouse (137). TLRs are primary sensors of these two systems in response against PAMPs (138). TLRs are also important for induction of adaptive immune responses, but misguided responses can lead to autoimmune pathology (138). The knock-out (KO) mice of MyD88, a key component of TLR signaling, have significantly diminished numbers of nTregs. Together with the following reports regarding the roles of TLRs, the results suggest an important role for TLR signaling in Treg homeostasis as well as Treg suppression (139). Of note, TLRs 1, 2, 4, 5, and 6 are located in the plasma membrane and recognize bacterial cell wall components. In comparison, TLRs 3, 7, 8, and 9 are preferentially expressed in intracellular compartments, such as endosomes, and recognize nucleic acid structures (137). Both types of TLRs are participated in regulation of Tregs. Linking TLR signaling to the functional control of Tregs may offer new opportunities to improve the outcome of cancer immunotherapy by coadminstration of certain TLR ligands (140).

5.1. TLR2

Synthetic bacterial lipoprotein (BLP) Pam3Cys-SK4 is a TLR2 agonist that is capable of modulating T cell immune responses. BLP, together with anti-CD3 antibody for TCR activation, induces proliferation of both Tregs and CD4+CD25- effector T cells in the absence of antigen-presenting cells. BLP also expands the Tregs, which recover their suppressive activity when the infection has subsided, in time to limit potential autoimmunity that might result from the overactivated effectors (141).

5.2. TLR4 and MPD-1

CD4+CD25+CD45RB^{low} Treg cells selectively express Toll-like receptors TLR-4, -5, -7, and -8. Exposure of nTregs to the TLR-4 ligand LPS induces up-regulation of several activation markers and enhances their survival/proliferation. This proliferative response does not require antigen-presenting cells and is augmented by TCR triggering and IL-2 stimulation. Most importantly, LPS treatment increases Treg suppressor efficiency by 10-fold. Moreover, LPS-activated Tregs efficiently control naive CD4 T cell-dependent wasting disease, suggesting that Tregs respond directly to proinflammatory bacterial products, a mechanism that likely contributes to the control of inflammatory responses (142). However, Treg induction and function in transplant recipients after CD154 blockade is TLR4 independent (143). MD1, a molecule known to be important in regulation of expression of RP105, also is important in regulating alloimmunity. Blockade of MD1 functional activity in dendritic cells (using anti-MD1 mAbs, MD1 antisense deoxyoligonucleotides, or responder cells from mice with deletion of the MD1 gene), results in elevated Treg induction in response to allogeneic stimulation (*in vivo* or *in vitro*) in the presence of LPS, which offer one mechanistic explanation for the augmented immunosuppression described following anti-MD1 treatment (144).

5.3. TLR5

Both human Tregs and CD4+CD25- T cells express TLR5 at levels comparable to those on monocytes and dendritic cells. Costimulation of effector T cells with anti-CD3 and TLR5 ligand flagellin results in enhanced proliferation and production of IL-2, at levels equivalent to those achieved by costimulation with CD28. In contrast, costimulation of TLR5 with flagellin does not break the hyporesponsiveness of Tregs, but rather, potently increases their suppressive capacity and enhances expression of FOXP3 (145).

5.4. TLR8

Synthetic and natural ligands for human TLR8 can reverse Treg cell function, which is independent of dendritic cells but requires functional TLR8-MyD88-IL-1 receptor-associated kinase 4 signaling in Treg cells (146). Adoptive transfer of TLR8 ligand-stimulated Treg cells into tumor-bearing mice enhances anti-tumor immunity.

5.5. TLR9

Cytosin-guanosin dinucleotide (CpG) motifs of bacterial DNA as ligands for TLR9 are known to be potent activators of innate immunity. Administration of CpG containing oligodeoxynucleotide (CpG-ODN) to mice before the onset of dextran sodium sulphate-induced colitis ameliorates colitis, and inhibits induction of proinflammatory cytokines. Also, Tregs from TLR9-deficient animals induce a significantly more severe colitis in SCID recipients than cells from wild-type littermate controls, suggesting that a similar protective role of "endogenous" bacterial DNA by binding to TLR9 leads to a less "aggressive" phenotype of Tregs. Therefore, bacterial DNA derived from the normal gut flora may contribute essentially to the homeostasis between effector and regulatory immune mechanisms in healthy individuals to protect them from chronic intestinal inflammation (147). In contrast to the Treg protection roles, stimulation of TLR9 with CpG ODN enhances apoptosis of glioma and prolongs the survival of mice with experimental brain tumors (148).

5.6. Heat shock protein 60

The immunodominant self-Heat shock protein 60 (Hsp60), abundantly present in joints of patients with juvenile idiopathic arthritis, can induce Treg in the periphery and contribute in regulation of inflammation. Heat-shock protein 60 is identified as a tool for novel therapeutic strategies that target the induction of Tregs in human arthritis (149).

6. EFFECTS OF CYTOKINES AND HORMONES ON Tregs

The capacity of T cells to orchestrate humoral and cellular immune responses is exerted by selective secretion of unique cytokines, which also applies to Tregs. However, despite of progress, the questions of how the major T cell cytokines affect homeostasis and suppressive function of Tregs remain to be incompletely defined.

6.1. Common γ chain cytokines

6.1.1. IL-2

Differential expression of IL-2R-α (CD25) and IL-2Rβ (CD122) (150) in nTregs suggests that nTregs are more dependent on IL-2 for survival (151, 152) and function (153) than CD4+CD25- T cells. nTregs are poor IL-2 producers (150). There are no nTregs in IL-2-deficient mice (151, 154, 155), IL-2Rα (CD25)-deficient mice (156) and IL-2Rβ-deficient mice (155, 157). These results suggest that IL-2, IL-2Rα (CD25) and IL-2Rβ are required for the generation and survival of nTregs (158). In addition, Tregs express higher levels of glucocorticoid receptor and Bcl-2 (159), and are therefore more resistant to dexamethasone (Dex)-mediated cell death than CD4+CD25- T cells. Furthermore, IL-2 selectively protects Tregs from Dex-induced cell death, while IL-7 and IL-15 did not exert preferential protective effects. In addition, IL-2 regulates FOXP3 expression in human Tregs and induces the expansion of these cells *in vivo* (119). This regulation involves the binding of STAT3 and STAT5 proteins to a highly conserved STAT binding site located in the first intron of the FOXP3 gene. Overall IL-2 treatment results in a 1.9 fold increase in the frequency of Tregs in peripheral blood as well as a 9.7 fold increase in FOXP3 expression in CD3+ T cells. On the other hand, it was reported that hypoproliferative response of Tregs to IL-2 is associated with defective downstream PI-3 kinase signaling (158), which acts through the inhibitory effect of PTEN (phosphatase and tensin homolog deleted on chromosome 10) on IL-2 receptor-mediated expansion of Tregs (160). The results suggest that IL-2R-mediated Treg expansion is normally acted by PI-3 kinase signaling.

6.1.2. IL-7

There is growing evidence favoring IL-7 as a master regulator of T cell homeostasis, based on its essential role in the homeostatic expansion of naïve T cells in response to low affinity antigens and its capacity to enhance expansion of peripheral T cells dramatically in response to high affinity antigens. The naïve T cell proliferation behavior reflects the lower IL-7 receptor (IL-7R) expression levels in Tregs compared with that in naïve T cells. Self-antigen presentation in combination with IL-7 expression promotes Treg cell proliferation, and contributes to the specific accumulation of Tregs at sites where their self-antigen is presented (161). Recent studies demonstrate that immunoregulatory dendritic cells (iDCs) confer immune hyporesponsiveness in part through Tregs. Unlike control dendritic cells, these dendritic cells express significant levels of IL-7. A novel mechanism by which iDCs delay autoimmunity through the Treg pathway and suggest IL-7 as a survival factor for these putative Tregs, which express the alpha-chain of its receptor at considerably higher levels than CD4+CD25- T-cells (162).

6.1.3. IL-15

IL-15, like IL-2, is a T cell growth factor presumably due to that fact IL-2 and IL-15 share IL-2Rβ chain for signaling. IL-15 results in enhanced survival via increased expression of Bcl-2. Moreover, IL-15 induces a distinct type of anergy characterized by hyperreactivity to IL-15, resulting in improved Treg expansion, which is likely attributed to increased propensity of these cells to up-regulate both α- and γ-chains of the IL-2 and IL-15 receptor. Notably, IL-15-expanded Tregs suppress both naive and memory T cells in a superior way that appears γ-irradiation resistant and independent of IL-10, TGF-β, or CTLA-4 interactions. Therefore, IL-15-expanded, *de novo*-induced human anergic Tregs are of interest in Ag-specific immunotherapy (163). Taken together, the common γ chain cytokines IL-2, IL-4 and IL-7 and IL-15 maintain the optimal suppression function of human Tregs in a PI-3 kinase dependent manner (164, 165). Of note, a recent report showed that in contrast to the promotion roles for Tregs, IL-7 and IL-15 may interfere with Treg function in inflamed synovia (83).

6.2. TGF-β

TGF-β induces a regulatory phenotype in CD4+CD25- T cells through FOXP3 induction and down-regulation of inhibitory Smad7 protein (166). Blocking CTLA-4 on wild-type Tregs abrogates nTreg suppressive activity *in vitro*, whereas neutralizing TGF-β has no effect, supporting a TGF- β-independent role for CTLA-4 in

nTreg-mediated suppression *in vitro* (120). The CTLA-4-deficient Tregs express increased levels of the suppressive cytokines IL-10 and TGF-β, and *in vitro* suppression mediated by CTLA-4-/- Treg is markedly reduced by neutralizing TGF-β, suggesting that CTLA-4-deficient Tregs develop a compensatory suppressive mechanism presumably via Th3 cells that secrete high levels of TGF-β but not via nTregs (77). Of note, TGF-β does not always induce aTregs. The range of identified effector CD4 T cell lineages has recently expanded with description of an IL-17-producing subset, called Th17, which develops via cytokine signals distinct from, and antagonized by, cytokines produced by the Th1 and Th2 lineages. Remarkably, Th17 development depends on the pleiotropic cytokine TGF-β, which is also linked to Treg development and function, providing a unique mechanism for matching CD4 T cell effector and Treg lineage specification (167).

6.3. Th1 and Th2 cytokines

6.3.1. IL-4 receptor α chain binding cytokines IL-4 and IL-13

IL-4 promotes Type 2 helper T cell (Th2) differentiation. The IL-4Rα binding cytokines, IL-4 and IL-13, induce FOXP3-expressing Tregs from CD4+CD25- precursors extrathymically in an antigen-dependent manner, which therefore provide an intriguing link between the well-established immunoregulatory capacity of Th2 cells and Tregs (168).

6.3.2. IL-12

IL-12 promotes Th1 cell differentiation and cell-mediated immunity. IL-12 plays an important role in the induction of mucosal inflammation and abrogation of Treg function in chronic experimental colitis (169). In an experimental colitis model in SCID mice reconstituted with syngeneic CD4+CD45RB^{high} T cells, splenic CD4+ T cells are unable to induce disease as a result of the presence of CD4+CD25+CD45RB^{low} Tregs (82). However, splenic CD4+ T cells, preactivated by IL-12 and anti-CD3 *in vitro*, are highly pathogenic in inducing severe mucosal inflammation, suggesting that IL-12 and anti-CD3 abrogate Treg suppression function (169).

6.4. Proinflammatory cytokines

6.4.1. IL-6

Proinflammatory cytokine IL-6 has been found to be an essential factor, induced by toll-like receptors (TLRs) upon recognition of microbial products, in blocking the suppressive effect of Tregs, allowing activation of pathogen-specific adaptive immune responses (170) (also see the STAT3 section). In support of the IL-6 function, local blockade of IL-6R signaling induces lung CD4+ T cell apoptosis via Tregs (171).

6.4.2. Tumor necrosis factor (TNF)-α

Nonobese diabetic (NOD) mice have a relative deficiency of Tregs in thymus and spleen. Administration of TNF-α or anti-TNF-α to NOD mice can modulate levels of this population consistent with their observed differential age-dependent effects on diabetes in the NOD mouse. Tregs from NOD mice treated neonatally with TNF-α show compromised effector function in a transfer system, whereas those treated neonatally with anti-TNF-α show no alteration in ability to prevent diabetes. Repeated injection of Tregs into neonatal NOD mice delays diabetes onset for as long as supplementation occurred. These data suggest that alterations in the number and function of Tregs may be one mechanism by which TNF-α and anti-TNF-α modulate type I diabetes mellitus in NOD mice (172).

6.5. Type I interferons

6.5.1. Interferon-α (IFN-α)

IFN-α induces expression of FOXP3 (173), irrespective of the presence of IL-12, but not that for GATA-3, in anti-CD3-stimulated CD4+ T cells. IFN-α enhances the induction of Th1 responses through upregulation of T-bet mRNA expression, as well as the induction of Th2 responses through upregulation of c-Maf mRNA expression, followed by IL-4 expression. Therefore, upregulation of FOXP3 by IFN-α may not be specific (173).

6.5.2. IFN-β

Similar to IL-2, IFN-β can promote survival of Tregs, which are susceptible to apoptosis that is associated with low Bcl-2 expression. The anergic/suppressive state of these cells is maintained after being rescued by cytokine (59), suggesting that promotion of Treg survival has therapeutic potential. It is not clear how effects of type I interferons on support of Treg suppression and survival contribute to anti-viral and anti-tumor immune responses.

6.6. Thymic stromal lymphopoietin (TSLP)

Human TSLP activates thymic CD11c-positive dendritic cells to express high levels of CD80 (B7.1) and CD86 (B7.2). These TSLP-conditioned dendritic cells are then able to induce the proliferation and differentiation of CD4+CD8-CD25- thymic T cells into Tregs. This induction depends on peptide-major MHC class II interactions, and the presence of CD80 and CD86, as well as IL-2. CTLA-4+ Tregs associate in the thymic medulla with activated or mature dendritic cells and TSLP-expressing Hassall's corpuscles, which suggests that Hassall's corpuscles and TSLP have a critical role in the generation of nTreg cells within the thymus (174).

6.7. Vasoactive intestinal peptide (VIP)

Vasoactive intestinal peptide (VIP) promotes generation of Tregs *in vivo* (175). The administration of VIP together with specific antigen to TCR-transgenic (Tg) mice results in the expansion of the FOXP3/neuropilin 1-expressing Tregs, which inhibit responder T cell proliferation through direct cellular contact. In addition, VIP induces more efficient suppressors on a per-cell basis. The VIP-generated Tregs transfer suppression inhibits delayed-type hypersensitivity in TCR-Tg hosts, and prevent graft-versus-host disease in irradiated hosts reconstituted with allogeneic bone marrow.

6.8. Vascular endothelial growth factor (VEGF)

VEGF blockade reduces intratumoral Tregs and enhances the efficacy of a GM-CSF-secreting cancer immunotherapy. Enhanced anti-tumor protection

correlated with an increased number of activated CD4+ and CD8+ tumor-infiltrating T cells and a pronounced decrease in the number of suppressive regulatory T cells residing in the tumor. Conversely, overexpression of VEGF from tumors resulted in elevated numbers of regulatory T cells in the tumor, suggesting a novel mechanism of VEGF-mediated immune suppression at the tumor site. This therapeutic combination may prove to be an effective strategy for the treatment of patients with cancer (176).

6.9. Indoleamine 2,3-dioxygenase (IDO)

Numerous immunosuppressive factors are produced by tumor cells, such as VEGFs, TGF-β, colony-stimulating factor (CSF)-1, IL-10, etc (177). IDO is a novel immunosuppressive enzyme expressed in some subsets of normal and neoplastic cells (178), which catalyzes the rate-limiting step of tryptophan degradation along the kynurenine pathway (179). Interestingly, IDO correlates with increased circulating Tregs in patients with acute myeloid leukemia (AML) (180).

6.10. Other hormones

6.10.1. Estrogen

Estrogen drives expansion of Tregs (181). Estrogen (E2)-mediated immunomodulation also reduces activation of effector T cells. Potentiation of Treg cells by estrogen is presumably via enhanced expression of the PD-1 costimulatory pathway (182).

6.10.2. Leptin

Leptin production is significantly increased in both serum and cerebrospinal fluid (CSF) of naive-to-therapy relapsing-remitting multiple sclerosis (RRMS) patients and correlated with IFN-γ secretion in the CSF. In leptin-deficient (ob/ob) and leptin-receptor-deficient (db/db) mice, the significant increase in Tregs is observed. Moreover, treatment of wild-type mice with soluble ObR fusion protein (ObR:Fc) increases the percentage of Tregs and ameliorates the clinical course and progression of disease in proteolipid protein peptide (PLP (139-151))-induced relapsing-experimental autoimmune encephalomyelitis (R-EAE), an animal model of RRMS. These findings show an inverse relationship between leptin secretion and the frequency of Tregs in RRMS and may have implications for the pathogenesis of and therapy for multiple sclerosis (183).

6.10.3. Prostaglandin E2

Prostaglandin E2 induces FOXP3 gene expression and enhances Treg function in human CD4+ T cells (184).

6.10.4. Aryl hydrocarbon receptor (AhR) by its ligand TCDD

AhR is a basic helix-loop-helix (bHLH) transcription factor that binds to heat shock protein 90 in the cytoplasm while inactivated. Activation of the AhR by its most potent ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), leads to immune suppression in mice via induction of Tregs. These findings suggest a novel role for AhR in the induction of Tregs and provide a new perspective on the mechanisms that underlie the profound immune suppression induced by exposure to TCDD (185).

7. TRANSCRIPTION FACTORS IN REGULATION OF Tregs

Initiation of T lymphocyte development depends on dynamically balanced regulatory inputs from multiple essential transcription factors (186). A set of transcription factors has been found to be involved in regulation of Treg homeostasis:

7.1. FOXP3

Tregs express high levels of FOXP3 (16), which is a forkhead box P3 transcription factor, Scurfin (187, 188). FOXP3 belongs to the forkhead family of transcription factors defined by the presence of a winged helix, forkhead, DNA binding domain (189). FOXP3 suppresses transcription of genes from nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB) response elements (190, 191). T cells from FOXP3 transgenic mice, and primary T cells transduced with FOXP3, acquire Treg cell features: (*a)* increased surface expression of Treg markers CD25, CTLA-4, CD103, and GITR; (*b)* decreased production of IL-2, IFN-γ, and IL-4; and (*c)* ability to suppress effector CD4+ T cells (192). Three groups have demonstrated that induced knock-out or spontaneous mutation of the mouse FOXP3 gene ('scurfy' mice) leads to a systemic autoimmune disease associated with absence of Tregs (187, 193, 194), emphasizing that FOXP3 is a master switch gene for Tregs (189). FOXP3 mutations also underlie a homologous autoimmune lymphoproliferative disorder in human subjects, termed immune dysregulation polyendocrinopathy enteropathy-X-linked (IPEX) syndrome and X-linked autoimmunity-allergic disregulation syndrome (XLAAD) (195-197). Increased FOXP3 expression may not be always associated with expansion of nTregs (198). The FOXP3 gene and its protein product are preferentially expressed in peripheral Tregs, in particular CD45RO+ Tregs in normal individuals. TCR stimulation of CD4+CD25-CD45RO- naive T cells fails to elicit FOXP3 expression at the gene or protein level (198). In addition to its essential roles in generation of suppressive Treg cells, FOXP3 has other functions: *i)* CD4+ T cells from FOXP3 transgenic (Tg) mice express elevated levels of mRNA for pro-apoptotic genes and undergo rapid apoptosis following stimulation (199). These results correspond well with previous reports, suggesting that suppressive Tregs are a highly apoptosis-prone population (59, 200); *ii)* These Tregs also display slower cell cycle transit following activation, suggesting that FOXP3 is capable of regulating the ability of T cells to respond to TCR-mediated activation; *iii)* Lastly, contrary to expected results, under Th1 or Th2 driving conditions, CD4+ T cells from FOXP3 Tg mice differentiate into effector cells (199). Concomitant with differentiation is a loss of FOXP3 mRNA and protein. These data demonstrate that FOXP3 levels regulate T cell function, and that FOXP3 itself is dynamically regulated during effector T cell differentiation (199). A recent report from Shevach's team suggests that TCR stimulation alone is insufficient to induce FOXP3 expression in the absence of TGF-β, while high levels of FOXP3 expression could be

induced in the presence of TGF-β. Although FOXP3 expression is stable, the TGF-β-induced FOXP3+ T cells are neither anergic nor suppressive and produce high levels of effector cytokines. These results suggest that even high levels of FOXP3 expression are insufficient to define a human CD4+ T cell as a T regulatory cell (201). A most recent report showed that FOXP3 expression is strongly associated with hyporesponsiveness of activated T cells, but is not directly correlated with their suppressive capabilities, as FOXP3 is also expressed in activated nonsuppressive T cells, suggesting that expression of endogenous FOXP3, in humans, is not sufficient to induce regulatory T cell activity or to identify Tregs (202).

7.2. Nuclear factor of activated T-cells (NFAT)

Antigen stimulation of immune cells activates the transcription factor NFAT, a key regulator of T cell activation and anergy. NFAT forms cooperative complexes with the AP-1 family of transcription factors and regulates T cell activation-associated genes. In addition, NFAT cooperates with FOXP3 in controlling Treg Cell function (192). Structure-guided mutations of FOXP3, which are designed to progressively disrupt its interaction with NFAT, interfere in a graded manner with the ability of FOXP3 to repress expression of the cytokine IL-2, upregulate expression of the Treg markers CTLA4 and CD25, and confer suppressor function (192). Thus, by switching transcriptional partners, NFAT converts the acute T cell activation program into the suppressor program of Tregs.

7.3. Nuclear factor-kappa B (NF-κB)

NF-κB promotes inflammation and inhibits the generation and homeostasis of Tregs. NF-κB-inducing kinase deficiency results in the development of Tregs (203), suggesting a novel role of NIK in controlling the development and expansion of Tregs. However, IκBαΔN (an inhibitor of NF-κB)-transgenic mice do not have increased numbers or function of Tregs (204). Enhancement of Tregs by IκBαΔN function may be compensated by the downstream regulatory mechanisms. Moreover, FOXP3 suppresses the transcription of two human retroviral promoters (HIV-1 and human T cell lymphotropic virus type I (HTLV-I)) utilizing NF-κB-dependent and NF-κB-independent mechanisms, suggesting an expanded role for FOXP3 in regulating NF-κB-dependent and independent cellular and viral gene expression (205).

7.4. Tumor suppressor p53

nTreg cells survive clonal deletion during their development in the thymus by escape from activation-induced cell death (161). This protective mechanism appears to be maintained in Tregs encountered in the periphery because a signaling module that counteracts apoptosis and mediates the release of survival factors could be identified in microarray experiments. Tumor necrosis factor receptor superfamily, member 1B (TNFRSF1B, TNF-RII) is upregulated in the Tregs from individual healthy donors (161). Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. To date, four distinct groups of MAPKs have been characterized, including extracellular signal-regulated kinases (ERKs), C-Jun N-terminal kinases (JNKs), p38 kinase isoforms and ERK5. Activation of TNFRSF1B leads to the recruitment of TRAF family members and subsequent activation of signal transduction pathways such as NF-κB, JNK, p38 kinase, ERK, and phosphoinositol-3-kinase (PI-3K), which in turn influence immune responses and increase the expression of survival factors. This mechanism is also linked to additional molecules that control the activity of p53 (tumor protein, TP53), a tumor suppressor gene that induces cell growth arrest or apoptosis. These data suggest that destabilization and thereby inactivation of TP53 provokes a shift in Treg cells from apoptotic sensitivity to protection and survival. This apoptotic process eliminates the expanded pool of effector lymphocytes during the contraction phase of the immune response and maintains lymphocyte homeostasis. In accordance with the findings, murine Treg cells are found to be more resistant to certain apoptosis stimuli, including treatment with dexamethasone or anti-CD95 antibody than CD4+ total or CD4+CD25- effector T cells. Moreover, human Treg cells are less sensitive to activation-induced cell death than their naïve counterparts (161).

7.5. cAMP-responsive element binding protein (CREB)

In addition to targeting NF-κB pathway, FOXP3 also represses retroviral promoter (HIV-1 and human T cell lymphotropic virus type I (HTLV-I)) transcription, and possibly cellular gene transcription by targeting CREB pathways (205). Therefore, FOXP3 may promote Treg apoptosis (199) by suppressing NF-κB- and CREB-dependent transcription of survival proteins, such as Bcl-xL (206-208) and TCTP (209, 210).

7.6. Signal Transducers and Activators of Transcription 1 (STAT1)

The STAT proteins, as transcription factors, are critical in mediating all cytokine driven signaling. These proteins are latent in the cytoplasm and become activated through tyrosine phosphorylation, which typically occurs through cytokine receptor associated kinases (JAKs), growth factor receptor tyrosine kinases, or a number of non-receptor tyrosine kinases (for example, Src and Abl). Phosphorylated STATs form homo- or hetero-dimers, enter the nucleus, work coordinately with other transcriptional co-activators or transcription factors, and lead to increased transcriptional initiation (211). STAT1 plays an important role in growth arrest in promoting apoptosis and is also implicated as a tumor suppressor; while in contrast, STATs 3 and 5 are involved in promoting cell cycle progression and cellular transformation and preventing apoptosis (211) through dysregulation of gene expression, including cyclin D1, c-Myc, Bcl-xL, Mcl-1 and survivin genes (212). CD28-upregulated STAT1 plays a critical role in the generation of Tregs (213). The heightened susceptibility to autoimmune disease (214)/or experimental autoimmune encephalomyelitis (EAE) (215) appears to be triggered by a reduced number as well as a functional impairment of Tregs in STAT1-deficient animals. Adoptive transfer of

wild-type Tregs into STAT1-deficient hosts is sufficient to prevent the development of autoimmune disease, suggesting an essential role of STAT1 in the maintenance of Treg homeostasis (214).

7.7. STAT3

STAT3 participates in anti-apoptosis signaling initiated by IL-6-IL-6R interaction. Following IL-6 binding, the soluble IL-6R (CD126)-IL-6 complex can directly activate cells that express the signal-transducing gp130 molecule (CD130), which mediates two distinct signals, mitogenesis by mitogen-activated protein kinase (MAPK) activation and anti-apoptosis by STAT3 activation. CD4+CD25- T cells are strongly CD126+ and CD130+, whereas $CD25^{high}$ Tregs express CD126 but little CD130 (89). The differential expression of CD126 and CD130 and subsequent STAT3 phosphorylation might be relevant for the recently described role of IL-6 in the control of Treg activity (89).

7.8. STAT4

In mice deficient for STAT4, because of reduced levels of IL-12 and interferon (IFN)-γ (216), rheumatoid arthritis (216), diabetes (217), and induction of experimental allergic encephalomyelitis (218) are suppressed, which underlie the inhibitory roles on Tregs.

7.9. STAT5

In conventional CD4+ T cells, IL-2 triggers signaling pathways that promote proliferation and survival by activating the STAT5 and by increasing the expression of the antiapoptotic proteins, Bcl-2 and Bcl-xL (219). Transient activation of STAT5 is sufficient to increase Treg numbers in IL-2-deficient mice, suggesting an essential role for STAT5 in maintaining Treg survival (220). Similarly, in human STAT5b deficiency, accumulation and regulatory function of Tregs are decreased (221).

7.10. Th1 and Th2 specific transcription factors

The differentiation of naïve T cells is induced by TCR activation and either IL-12/STAT4 or IL-4/STAT6 signaling pathways leading to a Th1/Th2 lineage specification that is further directed by the transcription factors T-bet and GATA3, respectively. STAT4 and STAT6 are both downregulated in the peripheral Tregs, indicating a potential inability to be transformed into Th cells upon restimulation via their TCR. Coexpression of Th2-specific transcription factor GATA3 and FOXP3, but the lack of Th1-specific transcription factor T-bet, suggests similarities in the gene expression profiles of Th2 and Tregs in humans. Like their murine homologs, human Tregs represent a separate lineage. They are undergoing a unique differentiation pathway distinct from those committing Th1 or Th2 cells, and are therefore equipped with a tightly regulated set of transcription factors acting in addition to FOXP3 (161).

7.11. Interferon regulatory factor-1 (IRF-1)

IRF-1-/- mice show less aggravated dermatitis compared to the wild-type mice. Mesangial cells cultured from IRF-1-/- mice produce significantly lower levels of nitric oxide and IL-12 but not TNF-α when stimulated with lipopolysaccharide (LPS) + IFN-γ. Splenic CD4-CD8-CD44+ T cells are decreased while Tregs are increased in the IRF-1-/- mice when compared to IRF-1+/+ mice (222), suggesting the inhibitory role of IRF-1 on Tregs.

8. Treg INTRACELLULAR SIGNALING PATHWAYS

The dysregulation of intracellular signaling pathways affects Treg homeostasis and function in terms of survival/apoptosis, differentiation, proliferation, and suppressor function, thereby promoting breakdown of self-tolerance and eventually leading to autoimmunity. The intracellular signaling pathways are not functional independently and are connected to upstream cell surface receptors that are the signal initiators and downstream transcription factors that are the signal effectors.

8.1. SOCS

McHugh *et al* (121) analyzed Tregs and CD4+CD25- T cells by DNA microarray, identifying 29 genes differentially expressed in the resting subpopulations, suggesting that Tregs have specific transcription signature. There are 77 activation-associated genes that are differentially expressed in Tregs, suggesting a previously activated phenotype of Tregs. Among these are a number of genes that antagonize signaling, including members of the suppressors of cytokine signaling (SOCS) family (223), which completes a negative feedback loop to attenuate signal transduction from cytokines that act through the janus kinase/STAT (JAK/STAT) pathway. In accordance with this finding, a recent report showed that FOXP+ Tregs are selectively expanded by SOCS3-/- dendritic cells (224).

8.2. Galectins and PIM1

In another study using microarray, genes controlling T cell receptor signaling, activation, and proliferation of Tregs (161) have been identified. Galectin-1 (LGALS1) antagonizes T cell activation by partial phosphorylation of the TCR-ζ chain, blocks secretion of IL-2, and skews the balance towards a Th2-type cytokine profile. Dimeric LGALS1 triggers immunosuppressive IL-10 production in T cells, contributing to their immune regulatory function. Galectin-3 (LGALS3) can potentially form complexes on the TCR with N-glycans, thereby limiting the lateral mobility of the TCR and resulting in restricted TCR-mediated signaling on T cells. In addition, this set of genes also includes CTLA4, TNFRSF1B, and PIM1, that controls proliferation. The serine/threonine kinase PIM1 directly transactivates NFAT at the end of the Ras signaling cascade to facilitate IL-2 dependent proliferation and/or survival of lymphoid cells. Because PIM1 is downregulated in Tregs from individual healthy donors, NFAT signaling is reduced to respond to IL-2, resulting in lower proliferation of Tregs.

8.3. Phosphoinositol-3-kinase (PI-3K)-Akt pathway

Engagement of IL-2R on Tregs on Tregs results in the activation of JAK/STAT signaling pathway, but fails to activate downstream targets of PI-3K signaling pathway, such as Akt or $p70^{s6kinase}$ (158). In correlated with this report, another report showed that the ability of CD28 to

promote efficient Treg generation requires neither an intact PI-3K-binding motif nor an intact Itk kinase-binding motif but does require an intact Lck-binding motif in the CD28 cytosolic tail (117). Thus, Treg survival is dependent on IL-2R-STAT5 pathway but not PI-3K pathway (158). In contrary, it was also reported that TCR/CD28-mediated activation of Rap1 and Akt is retained in nTregs (200). Moreover, most recently, PI-3K p110δ is found to be critical for the function of Tregs (225).

8.4. Ras-ERK pathway

Activation of Ras, mitogen activated protein kinase 1/2 (MEK1/2), and ERK1/2, are impaired in nTregs (200). However, TCR/CD28-mediated and IL-2 receptor-mediated signals converge at the level of MEK-ERK kinases to regulate Treg survival and expansion, which suggests that manipulation of the MEK-ERK axis, may represent a novel strategy for Treg expansion for immunotherapy (200).

8.5. Heme oxygenase-1 (HO-1) and nitric oxide (NO)

The heme oxygenases, which consist of constitutive and inducible isozymes (HO-1, HO-2), catalyze the rate-limiting step in the metabolic conversion of heme to the bile pigments and thus constitute a major intracellular source of iron and carbon monoxide (CO) (226). Similar to another gaseous molecule nitric oxide (NO), endogenously produced CO has been shown to possess intriguing signaling properties affecting numerous critical cellular functions including but not limited to inflammation, cellular proliferation, and apoptotic cell death. Tregs constitutively express HO-1, which suppresses proliferation and can be reversed by zinc protoporphyrin, a HO-1 competitive inhibitor (227). In addition, significantly lower levels of nitric oxide and IL-12 found in interferon regulatory factor-1 (IRF-1)-/- mice lead to increased Tregs and less aggravated autoimmune dermatitis (222). Collectively, these results suggest that NO may inhibit the homeostasis of Tregs.

8.6. Cyclins and cell cycle

nTregs are blocked from cell cycle progression due to decrease of cyclin E and cyclin A and increase of p27kip1 (p27kip cyclin-dependent kinase inhibitor). IL-2 induces and sustains increase of cyclin E and cyclin A, and prevents up-regulation of p27kip1 (200).

9. DRUGS AND POTENTIAL THERAPEUTICS ON Tregs

Definition of the roles of drugs and therapeutics on Treg apoptosis and homeostasis is significant in two aspects: (1) to explore future therapies for suppression of autoimmune responses, transplantation-related immune responses, and enhancement of anti-tumor immune responses; (2) to define how other drugs and therapies affect immune responses by modulating Treg function and survival.

9.1. Antibodies and binding ligands to cell surface receptors

9.1.1. Anti-CD25 monoclonal antibodies (mAbs)

Two mAb preparations to IL-2 receptor α-chain (IL-2Rα) are available for use, basiliximab and daclizumab (228). These antibodies specifically bind to and block the IL-2Rα, result in the depletion of Tregs, which might be of value for the selective depletion of immunosuppressive Tregs (229) to promote anti-tumor immune responses (29).

9.1.2. Denileukin (DAB (486) IL-2)

The human IL-2 receptor exists in three forms, low affinity (CD25), intermediate affinity (CD122/CD132) and high affinity (CD25/CD122/CD132). DAB (486) IL-2 is a fusion protein which targets the diphtheria toxin to the high affinity IL-2 receptor. After internalization, the diphtheria toxin inhibits cellular protein synthesis, resulting in cell death. Thus, application of DAB (486) IL-2 may deplete Tregs (229).

9.2. TCR binding

9.2.1. Nonmitogenic anti-CD3 mAb

Treatment with nonmitogenic anti-CD3 mAb induces CD4+ T cell unresponsiveness and functional reversal of established experimental autoimmune encephalomyelitis (230). Interestingly, this protection correlates with an increase in the frequency of Tregs.

9.2.2. Superantigen-staphylococcal enterotoxin B

In contrast to nonmitogenic anti-CD3 mAb, stimulation of nTregs with the superantigen staphylococcal enterotoxin B (SEB) results in abrogation of nTreg function, breaking their anergy and inhibiting their suppressor activity (231), which may underlie the mechanism of how superantigens are superior over conventional antigens in activation of T cells. It is unclear that whether effect of SEB stimulation on Tregs results from the binding of SEB to TCRVβ8 (232) or TCRVβ8+ Treg rapid proliferation after SEB stimulation.

9.3. Anti-TNF-α mAb infliximab

Increased secretion of TNF-α, along with IL-1 and IL-6, is important in the pathogenesis of rheumatoid arthritis (RA). Increased apoptosis of Tregs in patients with active RA is reduced by anti-TNF-α mAb infliximab (233). In addition, the function of nTregs has been found to be altered in active RA, whereas anti-TNF-α mAb has been found to improve the suppressive abilities of Tregs.

9.4. Neutralizing mAbs to IL-10, or IL-17

Antibody neutralization of IL-10 or IL-17 completely reverses Treg-mediated suppression. These results suggest that similar to blockage of CTLA-4, GITR (121, 122) or OX40 (125), neutralization of IL-10 and IL-17 attenuates Treg function (124). Sphingosine 1-phosphate (S1P) receptor agonist FTY720: The sphingosine 1-phosphate (S1P) receptor agonist FTY720 is well known for its immunomodulatory activity. An increased number of Tregs are found in blood and spleens of FTY720-treated mice, but not lymph nodes after treatment, suggesting that this compound differentially affects the homing properties of Tregs compared with other T cell subsets. Moreover, analysis of the functional response of FTY720-treated Tregs reveals an increased suppressive activity in an *in vitro* Ag-specific proliferation assay, suggesting that it may have disease-modifying potential for inflammatory disorders (234).

9.5. Immunosuppressive drugs

9.5.1. Corticosteroids

Fluticasone propionate increases nTreg suppression of allergen-stimulated T effector cells by means of IL-10-dependent mechanism (235). In addition, inhaled or systemic glucocorticoids have been found to induce FOXP3 and IL-10 expression and generation of aTregs (236). The number of Treg cells in the blood is significantly lower in untreated myasthenia gravis patients than in age-matched healthy subjects, whereas it is normal or elevated in patients on immunosuppressive therapy (prednisone frequently associated with azathioprine) (237). The following mouse studies have demonstrated that murine Tregs show differential response to dexamethasone-induced cell death in comparison to CD4+CD25- T cells (159). Administration of dexamethasone into BALB/c mice enhances the proportion of Tregs and the ratio of Tregs to CD4+CD25- cells in the lymphoid organs, especially in the thymus. This correlates with the *in vitro* observation that Tregs express higher levels of glucocorticoid receptor (159), and are therefore more resistant to dexamethasone-mediated cell death than CD4+CD25- T cells. Rapamycin: Rapamycin is an immunosuppressive compound that is currently used to prevent acute graft rejection in humans. Rapamycin selectively expands Tregs *in vitro* (238). These expanded nTregs suppress proliferation of syngeneic T cells *in vitro* and prevent allograft rejection *in vivo*.

9.5.2. Cyclosporine A, mycophenolate mofetil, and tacrolimus

Cyclosporine A, but not rapamycin and mycophenolate mofetil, inhibits Treg function by reducing IL-2 production (239). Immunosuppression with tacrolimus is superior to cyclosporine A after pulmonary allotransplantation, which is associated with induction of Tregs (240).

9.5.3. Cyclophosphamide

Cancer chemotherapy and immunosuppressive drug cyclophosphamide could have the following effects on Tregs: *1)* enhance apoptosis and decreases homeostatic proliferation of Tregs; *2)* abolish the suppressive function of Tregs (241); *3)* markedly enhance the magnitude of secondary but not primary CTL responses induced by dendritic cell-derived exosomes vaccines; *4)* synergize with dendritic cell-derived exosomes in therapy but not prophylaxis tumor models (229).

9.5.4. Fludarabine

Fludarabine is a cytotoxic analog of deoxyadenosine monophosphate and has high efficacy in the treatment of chronic lymphocytic leukemia (CLL). In CLL patients receiving fludarabine therapy, the inhibitory function of Tregs is decreased or even abrogated (242).

9.5.5. FK778

The immunosuppressive drug FK778, an analogue of the active metabolite A77 1726 in leflunomide (243), induces regulatory activity in stimulated human CD4+CD25- T cells (244). This anergic state is reversible by exogenous IL- 2 and is induced independent of nTregs. Taken together, it is realized that promotion of Treg survival and Treg function are underlying the pharmacological mechanisms of those immunosuppressive drugs.

9.6. Experimental therapeutics and vitamins

9.6.1. Insulin B:9-23 peptide

NOD mice have a relative deficiency of Tregs that could result in an inability to maintain peripheral tolerance. Immunization of NOD mice with insulin B-chain peptide B:9-23 followed by 72 hours *in vitro* culture with B:9-23 peptide induces generation of Tregs. Adoptive transfer of these Tregs into NOD-SCID mice completely prevents the adoptive transfer of disease by diabetogenic T cells. Our results suggest the possibility of using autoantigens to induce antigen-specific Tregs to prevent and regulate autoimmune diabetes (245).

9.6.2. Autoantibody peptide

A peptide based on the complementarity-determining region 1 (hCDR1) of an autoantibody ameliorates lupus by upregulating Tregs and TGF-β (246, 247).

9.6.3. FOXP3 targeting siRNA

RNA interference (RNAi) describes the sequence specific degradation of mRNA in animals and plants initiated by double-strand RNA molecules (dsRNA), which consist of two 21 to 22 nucleotide long short-interfering RNA strands (siRNA) (248). To overcome the instability and relatively poor delivery of unmodified siRNA into mammalian cells *in vivo*, noncovalent complexation of synthetic siRNAs with low molecular weight polyethylenimine (PEI) efficiently stabilizes siRNA. The systemic (intraperitoneal, i.p.) administration of PEI-complexed siRNA targeting the c-erbB2/neu (HER-2) receptor results in a marked reduction of tumor growth through siRNA-mediated downregulation of HER-2 (249). It has been proposed that application of siRNA targeting the critical transcription factor FOXP3 might abrogate the Tregs *in vivo*, thereby facilitating the generation of an efficient anti-tumor immune response (229).

9.6.4. Dendritic cell (DC)-derived exosomes

Dendritic cell-derived exosomes are nanomeric vesicles harboring MHC/peptide complexes capable of promoting primary T cell responses and tumor rejection in the presence of adjuvants. Therapeutic vaccines such as dendritic cell-derived exosomes aimed at boosting tumor-primed effector T cells could benefit procedures that minimize the effects of Tregs (241).

9.6.5. 15-deoxyspergualin

15-deoxyspergualin-treated DCs, cultured with naive T cells, could promote the formation of Tregs (250) and the production of higher levels of IL-10. The mechanism of 15-deoxyspergualin-induced tolerance is inhibition of maturation and function of DC and induction of the formation of Tregs by "suppressor DCs" to achieve a new immune balance.

9.6.6. Pentoxifylline

Pentoxifylline, a general inhibitor of phosphodiesterase, does not expand nTregs but it increases FOXP3 expression (198), suggesting that FOXP3 expression is not always associated with the suppression activity of Tregs. Vitamin D receptor ligand: A short treatment with 1,25-dihydroxyvitamin D3, a vitamin D receptor ligand, and mycophenolate mofetil, a selective inhibitor of T and B cell proliferation, enhances Treg function (251).

9.6.7. Vitamins C and E

Vitamins C and E are both antioxidants. Allogeneic T cells are energized following exposure to vitamin-treated DCs, and secreted higher levels of Th2 cytokines and IL-10 than controls. These anergic T cells act as Tregs in a contact-dependent manner, suggesting that vitamin C- and E-treated DC might be useful for the induction of tolerance to allo- or autoantigens (252).

9.6.8. Glatiramer acetate (Copaxone)

Glatiramer acetate (GA; Copaxone) is approved as a disease-modulating agent that ameliorates the course of multiple sclerosis (MS). *In vitro* exposure to GA results in elevated levels of IL-10-producing Tregs in all MS patient groups irrespective of receiving treatment as well as in healthy controls (253).

9.6.9. α-galactosylceramide (alpha-GalCer)

Treatment with alpha-galactosylceramide (alpha-GalCer) at the time of the experimental allergic conjunctivitis (EC)-priming immunization (ragweed followed by challenge with ragweed in eye drops) significantly increases Th2 responses and markedly upregulats the severity of the EC. However, treatment with alpha-GalCer just before the Ag challenge significantly suppresses the disease. This is associated with an increased frequency of Tregs, which express FOXP3, in the spleen (254).

9.6.10. Listeriolysin O

Listeriolysin O (LLO)[4] from *Listeria* is a member of the pore-forming cytolysins capable of binding and perforating phagosomal membranes at low pH. Listeriolysin O expressed in a bacterial vaccine suppresses CD4+CD25high Treg function *in vivo* (255).

9.6.11. Inhibition of APC proteosome function

Antigen-specific, Foxp3-expressing CD4+ regulatory T cells can be generated by inhibition of APC proteosome function. Immature APC, whose NF-kappaB-signaling pathway and thus maturation is blocked by the proteosome inhibitor benzyloxycarbonyl-isoleucyl-glutamyl (O-tert-butyl)-alanyl-leucinal (PSI), could be a source of Ag-specific Tregs. DO11.10 CD4 (+) T cells that are incubated with Ag- and PSI-pulsed APC proliferated poorly, produced less IL-2, IFN-γ, and IL-10 in secondary cultures, and inhibit the response of both naive and memory CD4+ T cells stimulated by Ag-pulsed APC. The generation of PSI-APC Treg cells required IL-10 production by APC. PSI-APC Treg inhibition required cell-cell contact but not IL-10 or TGF-β. Addition of IL-2 does not reverse, but Ab to CTLA-4 does reverse partially the inhibitory effect. Depletion of CD25+ T cells before initial culture with PSI-APC does not affect Treg generation. PSI-APC Tregs express high levels of FOXP3, inhibit proliferation of naive DO11.10 T cells *in vivo*, and abrogated colitis driven by a memory Th1 response to bacterial-associated Ag. Thus, NF-kappaB-blocked, immature APC are able to induce the differentiation of Treg cells that can function *in vitro* and *in vivo* in an Ag-specific manner (256).

10. Treg CELL SURVIVAL AND DEATH

Programmed cell death (PCD) plays a critical role in the development of T cells, including Tregs. Six forms of cell death have been identified in various types of cells, including two established forms (apoptosis and autophagy (257, 258)), and four atopical forms, such as paraptosis, calcium-mediated cell death, apoptosis-inducing factor (AIF)/poly- (ADP-ribose) polymerase (PARA)-dependent cell death, and oncosis (259). It is unclear whether all of the forms of cell death play a role in regulation of Treg development and homeostasis. Our and others' reports showed that T cell apoptosis pathway involves members of the Bcl-2 family, in particular Bcl-2, Bcl-xL (260, 261), TCTP (209), Bcl-xγ (208, 262, 263), Bim, and probably Bak. In addition to roles of apoptosis in regulation of T cell maturation and function, autophagy has also found to play an important role in T cell death (264). A recent report showed that in the autophagy gene Atg5 deficient mice, CD4+ and CD8+ T cells fail to undergo efficient proliferation after TCR stimulation, suggesting a critical role for Atg5 in T cell proliferation and survival (265). Apoptosis-induced decrease of Tregs has been associated with autoimmune thyroid diseases (266), autoimmune diabetes (267), atopic dermatitis and asthma (268), suggesting that Treg cells have cell death programs are different from other T cell subsets (161, 269) and are regulated by autoimmune disease status. However, cell death types (apoptosis, necrosis, or autophagy) and the mechanisms underlying the Treg death are poorly defined. Our recent report showed that pro-apoptotic protein Bax is highly expressed in Tregs in comparison to that in CD4+CD25- T cells. Removal of Tregs via a Bax-dependent apoptosis pathway significantly enhances of anti-self antigen (15, 29, 270-277) immune responses (278). This report has demonstrated for the first time that Treg apoptosis pathway can be therapeutically targeted. In addition, in 2005, 15 pharmacological inhibitors of apoptosis and 23 pharmacological inducers of apoptosis have been developed (FDA approved or in clinical trials) (279). Future characterization of potential roles of these apoptosis-regulatory drugs on Treg homeostasis will lead to definition of the drug-targeted apoptosis pathways in Treg homeostasis and development of new Treg apoptosis-targeted therapeutics. In the following sections, I have summarized the results on the roles of several important factors and pathways in regulating Treg apoptosis.

10.1. Extrinsic receptor-mediated apoptosis pathways

Twelve cell death receptors including TNF-R1, CD95 (Fas), DR3, TRAIL-R1 (tumor necrosis factor

(TNF)-related apoptosis-inducing ligand receptor 1), TRAIL-R2, DR6 (death receptor 6), DcR2 (decoy receptor 2), DcR3, osteoprotegrin (OPG), oectodysplasin receptor (EDA-R), and nerve growth factor receptor (NGF-R), six death ligands TNF, CD95L (FasL), TL1A, TRAIL, EDA, and NGF have been characterized in various cellular systems (280). However, the results regarding these death receptors/ligands are limited to Fas-FasL. nTregs are resistant to clonal deletion induced by viral superantigen *in vivo*. Isolated Tregs activated *in vitro* by anti-CD3 Ab are resistant to Fas-induced apoptosis, in contrast to their CD4+CD25- counterparts (281). However, recent reports do not agree with this conclusion. In contrast to effector T cells, Tregs are highly susceptible to CD95 ligand- but not to TCR-mediated cell death (282). Freshly isolated FACS-sorted Tregs are highly sensitive toward CD95-mediated apoptosis, whereas other T cell populations are resistant to CD95-induced apoptosis shortly after isolation. In contrast, TCR restimulation of Treg *in vitro* reveals a reduced sensitivity toward activation-induced cell death compared with CD4+CD25- T cells. This conclusion is further complicated by a recent report showing that naïve Tregs have a novel subpopulation defined by resistance towards CD95L-mediated cell death. The majority of Tregs from adult peripheral blood expresses high levels of CD45RO and CD95 and is prone to CD95L-mediated apoptosis in contrast to conventional T cells. However, a Treg subpopulation remains consistently apoptosis-resistant. Gene micro-array and 6-color flow cytometry analysis including FOXP3 showed that in contrast to Tregs found in adults, most Tregs found in cord blood are naive and exhibit low CD95 expression. Furthermore, the great majority of these newborn Tregs is not sensitive towards CD95L similar to naive Tregs from adults. After short-time stimulation with anti-CD3/28 mAbs cord blood Tregs strongly upregulated CD95 and are sensitized towards CD95L. This change is paralleled by a rapid upregulation of memory T cell markers on cord blood Treg that are frequently found on adult memory Tregs. Thus, there is a clear difference between naive and memory Tregs that could result in different survival rates of those two cell populations *in vivo*, which is of significance for the planning of therapeutic application of Tregs (283). In both the Fas-deficient *lpr* mice and IL-2Rα KO mice bearing the *lpr* mutation (double mutant mice); the phenotypes are observed including (*a)* spontaneous lymphocyte activation, (*b)* infiltration of leukocytes in multiple organs, and (*c)* B cell lymphopoiesis in bone marrow. These results indicate that Treg function and the phenotypes attributed directly to Treg abnormality are largely Fas-independent (284). The results from the studies with generalized lymphoproliferative disease (*gld*, defects in the Fas system) mutant mice suggest that Treg apoptosis requires Fas function. In *gld* mice, Tregs are disproportionately increased in the pool of CD4 T cells perhaps due to their unique apoptosis phenotype. Freshly isolated Tregs, unlike CD4+CD25- T cells, are highly sensitive to FasL-induced apoptosis in the steady state, suggesting that FasL-deficient Tregs have a functional Fas-mediated apoptosis pathway. Tregs that accumulate in *gld* mice express similar levels of FOXP3, and have suppression potency and Treg gene expression profile as wild-type Tregs (285). Similar to our results on the Bax-dependent apoptosis pathway of Tregs (278), Chen *et al* showed that depleting intratumoral Tregs via FasL protein transfer enhances the therapeutic efficacy of adoptive cell transfer (286).

In addition to the role of Fas/FasL pathway in Treg apoptosis, recent reports showed that Tregs suppress target cells via a Fas/FasL dependent mechanism (103). A subpopulation of human Tregs carrying a protein Tag7 (aka PGRP-S) and FasL on their surface can indeed kill HLA-negative tumor-derived cells K562 and MOLT-4 that expose Hsp70 (heat shock protein 70) and Fas. Tag7 is capable of tight and specific interaction with cognate Hsp70. The primary binding of lymphocyte Tag7 to target-cell Hsp70 is very specific (e.g., it is blocked by preincubating either cell with minimal peptides from the "partner" protein) and secures cell contact indispensable for subsequent FasL/Fas-triggered apoptosis (287).

10.2. Nicotinamide adenine dinucleotide (NAD) and ATP

Treg cells are highly sensitive to cell death induced by extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors (269). Micromolar concentrations of the common cell metabolite NAD induce death in FOXP3-expressing Treg cells with high efficiency and within minutes. Similar, but less dramatic, effects are demonstrable with ATP and its nonhydrolysable derivative, benzoylbenzoyl-ATP. Other T cell subsets are more resistant, with CD8 cells being the least sensitive and CD4 cells expressing intermediate sensitivity. The higher sensitivity of Tregs is demonstrable *in vivo*. Injection of NAD or benzoylbenzoyl-ATP causes preferential induction of a cell death signal in Tregs. Transmission of the death signal requires functional P2X7 receptors, pointing to a role for these receptors in regulation and homeostasis of Tregs. Consistent with this, P2X7R gene-deleted mice possess increased levels of FOXP3-expressing Treg cells.

10.3. Intrinsic apoptosis pathways:

10.3.1. Bcl-2

In contrast to what are discussed regarding the resistance of certain Tregs to certain apoptotic stimuli (the section of transcription factor p53), human anergic/suppressive nTregs are a highly differentiated and apoptosis-prone population (59). Isolated Tregs are susceptible to apoptosis that is associated with low Bcl-2 expression, but this death can be prevented by IL-2 or fibroblast-secreted IFN-β. The anergic/suppressive state of Tregs is maintained after cytokine rescue (59). However, another report suggests that Bcl-2 deficiency does not affect Treg homeostasis, since ectopic expression of Bcl-2 fails to rescue Treg numbers and prevent the development of autoimmunity in IL-2-deficient mice (220). Previous reports suggest that regulation of Bcl-2 is inversely correlated with that of pro-apoptotic protein Bax (288). However, the engagement of CD4 separately from TCR influences the expression of the proapoptotic protein Bax independently of the anti-apoptotic protein Bcl-2, whereas Ag activation coordinately modulates both Bax and Bcl-2. Furthermore, analysis of the mechanisms, by which IL-2

and IL-4 cytokines exert their protective function on CD4+ T cells in the presence of soluble CD4 ligands, shows that they are able to reduce susceptibility to Bax-mediated apoptosis but not to CD95-dependent apoptotic pathways (289). A recent report from our laboratory showed that Tregs express higher levels of Bax than CD4+CD25- T cells (278). Our results further demonstrated for the first time that removal of Tregs via a Bax-dependent apoptosis pathway significantly enhances anti-self-tumor antigen immune responses (278), which points out that apoptosis pathway of Tregs is a new therapeutic target for treatment of immune related diseases.

10.3.2. Bcl-xL

nTregs have high susceptibility to apoptosis that is reversed by IL-2, which correlates with activation of ERK1/2, up-regulation of Bcl-xL, and phosphorylation of pro-apoptotic protein Bad at Ser112 (200). In addition, since nTregs require CD28 co-stimulation for their homeostasis (61), and CD28 co-stimulation significantly upregulates anti-apoptotic molecules Bcl-xL (261), Bcl-xγ (208, 262), TCTP (209), therefore, these pro-survival proteins may play critical roles in maintaining homeostasis of nTregs.

10.4. Treg proliferation rates

There is another concern that if a smaller population of Tregs composing 5-10% of CD4+ T cells is highly susceptible to apoptosis, then the a key question remains poorly defined that how the homeostasis of Treg population is maintained. A recent report shows that using a technique of deuterium labeling of cycling cells *in vivo*, CD45RO+ Tregs are highly proliferative, with a doubling time of 8 days, compared with memory CD45RO+FOXP3-CD25- (24 days) or naive CD4+CD45RA+FOXP3-CD25- populations (199 days). However, the regulatory population is susceptible to apoptosis and have critically short telomeres and low telomerase activity. It is therefore unlikely to be self regenerating. These data are consistent with continuous production from another population source. We found extremely close TCR clonal homology between Tregs and memory CD4+ T cells. Furthermore, antigen-related expansions within certain TCR Vβ families are associated with parallel numerical increases of CD45RO+ Tregs with the same Vβ usage. It is therefore unlikely that all human Tregs are generated as a separate functional lineage in the thymus. Instead, the data suggest that a proportion of this Treg population is generated from rapidly dividing, highly differentiated memory CD4+ T cells; this has considerable implications for the therapeutic manipulation of these cells *in vivo* (290).

11. CONCLUSION

Although significant progress has been made, many aspects regarding regulation of Tregs remain to be further defined. No doubt, continuous characterization of the molecular mechanisms underlying Treg survival and homeostasis and the regulatory factors would eventually lead to development of novel therapeutics for autoimmune diseases (14, 15, 274), tumor immunotherapy (13, 29, 270, 272, 273, 275-277), graft-versus-host-disease (271), stem cell therapy (291), and chronic infectious diseases.

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Abbreviations: Ab, antibody; Ag, antigen; AhR, aryl hydrocarbon receptor; AIRE, an autoimmune regulator transcription factor; Akt, a serine/threonine-specific protein kinase; APC, antigen presenting cells; ART-2, ADP-ribosyltransferase 2; ATP, adenosine triphosphate; aTregs, adaptive Tregs; BLP, a synthetic bacterial lipoprotein; CO, carbon monoxide; CpG, cytosine-guanosin dinucleotide; CREB, cAMP-responsive element binding protein; CSF, cerebrospinal fluid; CTLA-4, cytotoxic T-lymphocyte antigen-4; CTLA-4Ig, fusion proteins of CTLA-4 and antibodies; DC, dendritic cells; DR3, death receptor 3; DcR2, decoy receptor 2; ERK, extracellular signal-regulated kinase; FOXP3, forkhead box P3 transcription factor; GITR, glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein; *gld* mice, Fas ligand deficient mice; GPCRs, G protein-coupled receptors; HO-1, heme oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL-2, interleukin-2; IRF-1, interferon regulatory factor-1; KO mice, gene knock-out mice; LGALS1, galectin-1; *lpr* mice, Fas deficient mice; LPS, lipopolysaccharide; mAb, monoclonal antibody; NAD, nicotinamide adenine dinucleotide; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-kappa B; NK cells, natural killer cells; NKT cells, natural killer T cells; NO, nitric oxide; NOD mice, nonobese diabetic mice; nTregs, natural occurring Tregs; ODN,

oligodeoxynucleotide; PAMPs, pathogen associated molecular patterns; PD-1, programmed death-1; PI-3K, phosphoinositol-3-kinase; PIM1, a human serine/threonine kinase; PSI, a proteosome inhibitor, benzyloxycarbonyl-isoleucyl-glutamyl (O-ter-butyl)-alanyl-leucinal; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RRMS, relapsing-remitting multiple sclerosis; SCID mice, severe combined immunodeficiency mice; SOCS, suppressors of cytokine signaling; STAT, signal transducers and activators of transcription; TCR, T cell antigen receptor; TCTP, translationally controlled tumor protein; Tg, transgenic mice; TGF-β, transforming growth factor-β; Th1, type I T helper cells; Th17, IL-17 producing T helper cell subset; TLR, toll-like receptor; TNF, tumor necrosis factor; TNF-R1, tumor necrosis factor receptor 1; TRAIL-R1, tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor 1; Tregs, CD4+CD25highFOXP3+ regulatory T cells; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor; VIP, vascular intestinal peptide

Key Words: $CD4^{+}$, $CD25^{high}$, $FOXP3^{+}$, Regulatory T cells, Immunosuppression, Homeostasis, Cell Survival, Death, Review

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