REVIEW

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Mechanisms regulating the development and function of natural regulatory T cells

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Abstract

The key of the immune system is to protect the host from foreign threat posed by pathogens and from the internal threat posed by self-attacking lymphocytes. The ability to discriminate self versus non-self ensures that only "non-self" pathogens, but not the self antigens, are attacked. Such tolerance to "self" arises from the central tolerance mechanisms that include the deletion of thymocytes with high reactivity to self antigens and also the induction of unresponsiveness of autoreactive T cells in the periphery. Natural regulatory T cells (nTregs) directly inhibit effector T cells, and keep their proliferation in control. Apart from preventing autoimmune reactions, Tregs also contribute to peripheral immune homeostasis as evidenced by the excessive lymphocyte accumulation in peripheral lymphoid organs and intestinal inflammation in the absence of nTregs. Here we discuss the molecular aspects of the development and suppressive function of naturally occurring Tregs. Accumulating evidence shows the importance of these Tregs in autoimmunity, tumor immunity, organ transplantation, allergy, and microbial immunity.

Key words: development, Treg, T cell activation, signaling, PKC-θ.

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PHENOTYPIC CHARACTERIZATION OF NATURAL T REGULATORY CELLS

The existence of regulatory T cells (Tregs) was reported in the early 1970s, as a "shut-off" substance of the thymus [51], and later as "suppressor T cells" [50]. Thymectomy performed on the third day after birth induced autoimmune reactions in various organs, causing gastritis, thyroiditis, oophoritis, and orchitis [79, 159]. Adoptive transfer of $CD5^{low}$ ($Lyt.1^{low}$) into *nu*/*nu* mice induced autoimmunity which was prevented by $CD5^{high}$ cells [125]. In non-obese diabetic (NOD) mice, which spontaneously develop type 1 diabetes, adoptive transfer of $CD4^+$ T cells from syngeneic normal mice delayed onset of diabetes [21]. Injection of splenic $CD45RB^{high}$ ($OX\text{-}22^{high}$) $CD4^+$ T cells into congenitally athymic mice induced a severe wasting disease with inflammatory infiltration observed in liver, lung, stomach, thyroid, and pancreas [121]. In addition, $CD45RB^{high}$ $CD4^+$ T cells adoptively transferred into severe combined immuno-deficiency (SCID) mice induced a wasting disease characterized by intestinal inflammation along with increased numbers of T cells in the colon [103, 120]. Co-transfer of total $CD4^+$ T cells or enriched $CD45RB^{low}$ $CD4^+$ T cells with $CD45RB^{high}$ $CD4^+$ T cells prevented the onset of the wasting disease. However, owing to a lack of specific markers for such regulatory cells, the nature of the Treg cells have remained controversial for a long period of time. Subsequently, high-affinity α-subunit of the interleukin (IL)-2 receptor, IL-2Rα (CD25), was identified as the marker for the $CD4^+$ Tregs [126]. $CD4^+CD25^+$ T cells are present in both $CD5^{high}$ and $CD45RB^{low}$ populations and were shown to be more effective in preventing the onset of autoimmune disease caused by adoptive transfer of $CD4^+CD25^-$ T cells. When $CD4^+$ T cells from normal mice were depleted of the $CD25^+$ population (~10% of total $CD4^+$ T cells) and inoculated into athymic *nu*/*nu* mice, they caused multiple organ-specific autoimmune diseases which were prevented by inoculation of $CD4^+CD25^+$ T cells. Thymectomy of a 3-day-old mouse resulted in elimination of the $CD4^+CD25^+$ T cell the sub-population, causing autoimmune gastritis, orchitis, oophoritis, and thyroiditis, whereas reconstitution of neonatally thymectomized mice with $CD4^+CD25^+$ T cells prevented the development of the

above autoimmune diseases [10]. The use of the CD25 marker enabled the identification of human Tregs with phenotype and function similar to those in mice [7, 87]. The primary causes for many autoimmune diseases in human were found to be genetic mutations affecting the generation and function of naturally arising Tregs [19, 164]. In the mouse, CD25 is also expressed by activated T cells, although the expression of CD25 is transient and eventually lost once the stimulation is removed. Naturally occurring $CD4^+CD25^+$ T cells express higher levels of CD25 upon activation. CD25 expression remains high even when the stimulation is removed [83]. Furthermore, *in vitro* and *in vivo* suppression of T cell activation is mediated only by naturally occurring $CD4^+CD25^+$ T cells, but not by $CD4^+CD25^+$ arising from activation [83]. In contrast to mouse, human peripheral blood represents a heterogeneous population of $CD4^+$ T cells in terms of CD25 expression, consisting of activated and memory T cells along with unprimed T cells. Treg's properties are mainly attributed to the $CD25^{high}$ population [15].

In addition to CD25, CD4 Tregs also express several other surface molecules that are not unique to Tregs, but facilitate the identification of Treg cells. Cytotoxic T lymphocyte-associated antigen (CTLA)-4 is generally not expressed on naïve $CD4^+$ T cells, but is upregulated following T cell activation. In contrast, Read et al. [123] and Takahashi et al. [149] independently reported that CTLA-4 was constitutively expressed on $CD4^+CD25^+$ Tregs in normal naïve mice. Furthermore, treatment with anti-CTLA-4 monoclonal antibody (mAb) abrogated the function of Tregs, resulting in spontaneous organ-specific autoimmune diseases in normal BALB/c mice and wasting disease in SCID mice [123, 149]. Consistent with this result, CTLA-4-deficient mice develop a severe lymphoproliferative disorder and die from autoimmune-like disease within one month after birth [158, 172]. This result also explains why anti-CTLA-4 antibody exacerbated autoimmune responses in the murine model of multiple sclerosis or type 1 diabetes [72, 92, 115], enhanced anti-tumor responses [84], and prevented the induction of immunologic tolerance to concurrently administered non-self antigens [114]. Using DNA microarray to analyze the differential expression of genes in $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells, glucocorticoid-induced TNFR-related (GITR) was found to be upregulated on the surface of $CD4^+CD25^+$ T cells [100]. Shimizu et al. [139] also showed that GITR expression is elevated on peripheral $CD4^+CD25^+$ T cells and $CD4^+CD8^-CD25^+$ thymocytes. Depletion of $GITR^+$ T cells in normal mice or adoptive transfer of a $CD4^+GITR^-$ T cell population into SCID mice resulted in multiple organ-specific autoimmune diseases [139]. $GITR^+CD4^+$ T cells, regardless of CD25 expression, prevented wasting disease and colitis [161]. $GITR^+CD4^+$ T cells express intracellular CTLA-4, suppress T cell proliferation, and are themselves anergic. T cell stimulation through GITR increased anti-tumor immunity by decreasing Treg infiltration of tumors [78]. Adoptive transfer of $GITR^{high}$-depleted T cells (both $CD25^-$ and $CD25^+$) induced autoimmune diseases which were more severe than those induced by T cells depleted of $CD4^+CD25^+$ Treg [108]. Though, it is clear from multiple reports that a portion of $CD4^+CD25^-$ ($CTLA\text{-}4^+CD45RB^{low}GITR^+$) T cells also have potent suppressive capacity, this population is only about 5% of the $CD4^+CD25^-$ T cell population. $CD103^+$ (integrin $\alpha_E\beta_7^+$) T cells were reported to be more suppressive than $CD103^-$ T cells *in vitro* and *in vivo* and comprised both the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations [86]. Also, $CD62L^{high}CD4^+CD25^+$ Tregs were found to be more potent in preventing type 1 diabetes in NOD mice, presumably because of the different homing specificities of these cells [146]. Neuropilin-1, a receptor involved in axon guidance, angiogenesis, and the activation of T cells, is constitutively expressed on the surface of $CD4^+CD25^+$ Tregs independently of their activation status. In contrast, neuropilin-1 expression is down-regulated in naive $CD4^+CD25^-$ T cells in response to T cell receptor (TCR) stimulation. Furthermore, $CD4^+$ neuropilin-1^{high} T cells express high levels of Foxp3 and suppress $CD4^+CD25^-$ T cell activation [22]. In general, natural $CD4^+CD25^+$ Tregs express lower levels of TCR and CD4 co-receptor than effector T cells, as normally is the case with anergic T cells. Treg cells expresses higher Fas, CD27, and CD62L, similarly to activated memory cells. However, Treg cells are still a distinct population of $CD4^+$ T cells. Human $CD4^+CD25^+$ Tregs in both the peripheral blood and thymus express surface GITR, CTLA-4, tumor necrosis factor receptor (TNFR)2, and CCR8 [16]. Unfortunately, none of these markers are truly specific to Tregs since their expression also reflects the activation state of T cells.

Foxp3

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which displays multiple autoimmune disorders including diarrhea, type 1 diabetes along with auto-destruction of endocrine glands, insulitis, and thyroiditis in human males, was attributed to the dysfunction of an immune-response locus on X chromosome [119]. Similar immune dysfunction was found in "scurfy" mice marked by severe lymphocyte infiltration of various organs and hyperactivation of $CD4^+$ T cells [53]. Furthermore, it was shown that T cells from scurfy mice exhibited spontaneous proliferation and over-expression of pro-inflammatory cytokines. IPEX was later found to be the human equivalent of scurfy mouse, as the gene responsible for both IPEX and scurfy mice was identified as *Foxp3*, which encodes a nuclear protein, Scurfin or Foxp3, a member of the forkhead/winged-helix family of transcriptional factors [19, 23]. Foxp3 has very high sequence homology across humans, mice, and rats [45]. Three independent groups showed that Foxp3 is specifically expressed in $CD4^+CD25^+$ Tregs in the periphery and $CD4^+CD8^-CD25^+$ thymocytes and is required for their

development and function [42, 60, 74]. Scurfy mice had few $CD4^+CD25^+$ T cells, although they developed many chronically activated T cells [74]. $CD4^+CD25^+$ T cells from scurfy mice did not have suppressive activity [74]. Ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells convert them *in vitro* and *in vivo* to a suppressive activity similar to that of $CD4^+C25^+$ Tregs. Retroviral transduction of Foxp3 suppressed cytokine gene transcription and the upregulated Treg-associated markers, CD25, GITR, CD103, and intracellular CTLA-4 [60, 74]. Foxp3-transgenic mice had increased numbers of $CD4^+CD25^+$ Tregs. The fatality of *CTLA-4*$^{-/-}$ mice resulting from autoimmunity was delayed by 20 weeks when crossed with Foxp3-transgenic mice [74]. Mice deficient in Foxp3 specifically in T cells displayed a phenotype identical to that of scurfy mice, confirming that the autoimmunity observed in scurfy mice was due to deficiency of $CD4^+CD25^+Foxp3^+$ Tregs [44]. These results demonstrated that Foxp3 expression is both necessary and sufficient for the suppressive function of natural Tregs (nTregs). Foxp3 deficiency neither affected the development of conventional $CD4^+$ T cells nor did it affect their antigen sensitivity, co-stimulation requirement, or proliferative capacity, suggesting that Foxp3 does not play a role in conventional T cell function [44]. Foxp3 expression is highly restricted to Tregs, as it was mostly detected in $CD4^+CD8^-CD25^+$ thymocytes and $CD4^+CD25^+$ peripheral T cells. Foxp3 is virtually absent in thymic and peripheral $CD4^+CD25^-$ T cells, $CD8^+$ T cells, B cells, and brain, heart, kidney, liver, testes, and lung tissues [23, 60, 74]. Using transgenic mice with green fluorescent protein (GFP) fused to Foxp3 ($Foxp3^{GFP}$mice), Foxp3 expression was showed to be highly restricted to $TCR\alpha\beta^+$ cells, as also revealed by a total lack of $Foxp3^+$ cells in *Rag*$^{-/-}$ mice which lack $\alpha\beta^+$ T and B cells [44]. Foxp3 expression was also absent in $TCR\gamma\delta^+$, natural killer (NK), NKT, macrophages, and dendritic cells (DCs). In lymph nodes, more than 97% of Foxp3-expressing cells are $CD4^+$ T cells [44], which is confirmed by $Foxp3^{RFP}$-transgenic mice [169]. In these mice, Foxp3 was also detected in >30% of bone-marrow-derived $CD4^+TCR^+$ cells, 1% of single-positive (SN) CD4 thymocytes, and 0.37% of double-positive (DP) thymocytes. The *in situ* distribution of $Foxp3^+$ cells was localized in the $CD4^+CD25^+$ subset of both lymphoid (spleen, mesenteric lymph nodes) and non-lymphoid organs (colon) [160]. Furthermore, 82% of $Foxp3^+$ cells were $CD25^{high}CD4^+$. However, $CD25^{low}Foxp3^+$ cells were equally suppressive as $CD25^{high}Foxp3^+$ T cells, which is in clear contrast to $CD25^+Foxp3^-$ cells, which were not suppressive, and proliferated faster than $CD25^-Foxp^-$ or $Foxp3^-CD4^+$ T cells [44]. Moreover, $CD25^{high}Foxp3^-$ cells showed a gene expression profile characteristic of activated/effector T cells, with increased expression of cytokines IL-2, IL-4, IL-17, T-bet, and Edg3 and decreased CD62L expression. This confirms that Foxp3, rather than CD25, directly correlates with Treg function. In the mouse, Foxp3 expression in CD4 T cells is not associated with the activation state of these cells, as stimulation of $CD4^+CD25^-$ T cells does not induce Foxp3 expression [60, 74]. Human Tregs also express high levels of Foxp3, which correlated with $CD25^{high}$, GITR, and CTLA-4 surface expression [168, 175]. In contrast to mice, humans express two different splice variants of Foxp3. In addition, human $CD4^+CD25^-$ T cells upregulate Foxp3 upon activation [168]. Reduced expression levels of Foxp3 protein and mRNA have been shown to be associated with impaired function of $CD4^+CD25^+$ T cells in patients with graft-versus-host disease [102], multiple sclerosis [64], and myasthenia gravis [17]. Transgenic mice that have endogenous Foxp3 gene expression attenuated in Tregs were established [170]. Decreased Foxp3 expression in these mice led to a severe autoimmune condition similar to that observed in scurfy mice but did not affect thymic development, homeostatic expansion, and transforming growth factor (TGF)-β-induced *de novo* generation of $Foxp3^+$ cells. The attenuated Foxp3-expressing cells obtained from transgenic mice were not suppressive both *in vitro* and *in vivo* although they were still hypoproliferative. These results confirm that true Treg activity resides in $Foxp3^+$ T cells. Since Foxp3 is a nuclear protein, it is unsuitable for live cell sorting, and thus $CD4^+CD25^+$ cells are isolated as Tregs cells in most functional studies.

THYMIC DEVELOPMENT OF NATURAL TREGs

Prior to the third day after birth in mice, only the thymus contains $CD4^+CD25^+$ Tregs with full suppressive potential, which are totally absent in the periphery at this time [68]. In addition, $CD4^-CD8^-$ double-negative immature thymocytes, when adoptively transferred into the thymus of the recipient mice, gave rise to donor-specific $CD4^+CD25^+$ T cells one week after adoptve transfer [68]. This result supports the notion that Treg cells are developed in the thymus and migrate to the periphery to carry out their functions.

Role of TCR signaling

Natural Tregs were present in normal numbers in TCR-transgenic mice expressing TCRs specific for a peptide, but were absent in TCR-transgenic mice on a *Rag2*$^{-/-}$ background. The higher percentage of $CD4^+CD25^+$ T cells expressing endogenous TCR α--chain than transgenic β-chain and the total absence in TCR α-β-rearrangement-defective *Rag2*$^{-/-}$ mice suggests that Tregs are subject to a thymic selection process [68]. In other words, endogenous rearrangement of TCRs in the thymus is required for the development of Tregs. Furthermore, the proportion of Tregs that develop in TCR-transgenic mice is increased when the cognate antigen is expressed by the thymic antigen-presenting cells (APCs) in the same mice [70, 167]. Such generation of neo-self antigen-specific Tregs was shown to be

dependent on radio-resistant thymic epithelium cells, but not on bone marrow (BM)-derived DCs [70]. Using a similar double-transgenic mouse model it was shown that promiscuous expression of the hemagglutinin (HA) peptide in thymic epithelial cells (TECs) is involved in the selective induction and expansion of HA-specific thymic Treg precursors at as early as the DP stage [25]. Thus, the self antigen seems to drive the positive selection of self-reactive $CD4^+CD25^+$ Tregs in the thymus. This "positive-selection" process requires a TCR with moderately high affinity for the self peptide, since thymocytes with low affinity did not get selected to become Tregs. However, thymocytes presented with very high--affinity self antigens were clonally deleted, similar to the negative selection of conventional thymocytes. Therefore, Tregs development in the thymus directly correlates with TCR signaling strength [70, 144]. Attenuating the efficiency of TCR-ligand interactions by one or two orders of magnitude abolished the increase in the proportion of thymic Tregs, again suggesting the requirement of high-affinity TCR-peptide--MHC interactions for Treg development [29]. When Foxp3 expression was examined among CD4 SP thymocytes and compared with the levels of TCR, TCR levels positively correlated with Foxp3 expression. Most of the $Foxp3^+$ cells are also TCR^{high}, whereas almost no Foxp3 expression was defected in the $TCR^{low}CD4^+$ thymocytes [169]. Induction of Foxp3 is thus believed to depend on the strength of TCR signals. It has also been suggested that thymic $CD4^+CD25^+$ Tregs are more resistant than $CD4^+CD25^-$ T cells, by two to three orders of magnitude, to activation-induced cell death, resulting in increasing their frequency relative to the $CD4^+CD25^-$ cells regardless of the dose of the agonist ligand [163]. The role of Foxp3 in Treg development was evident from BM chimeras with a mixture of BM cells from wild-type and Foxp3-deficient mice. Foxp3-deficient BM cells failed to give rise to $CD4^+CD25^+$ T cells, and all the $CD4^+CD25^+$ T cells were derived from Foxp3--intact BM cells [42]. The expression of Foxp3 was restricted mostly to $CD4^+CD8^-$ (CD4 SP) thymocytes, with only a few $Foxp3^+$ cells in the $CD8^+$ SP and DP^{dull} (double-positive but lower expression of CD4 and CD8) population. Using $Foxp3^{GFP}$ mice on *MHCI*$^{-/-}$, *MHCII*$^{-/-}$, and *MHCI*$^{-/-}$*MHCII*$^{-/-}$ double-deficient backgrounds, it has been shown that the expression of MHCII is absolutely required for Foxp3 expression in SP $CD4^+$ thymocytes, and MHCI for Foxp3 expression in the remaining SP $CD8^+$ and DP^{dull} thymocytes [44]. This result also implies that Foxp3 expression in the thymus is strictly dependent on TCR/MHC interactions and independent of commitment CD4 or CD8 lineage. The $Foxp3^+$ CD4 SP cells do not arise from the DP subset, as evident by concomitant expression of Foxp3 in both the CD4 SP and DP subsets. Furthermore, there is no difference in the percentage of SP and DP $Foxp3^+$ thymocytes expressing cell cycle-associated nuclear protein Ki67 [41]. Antigen-independent interactions via accessory or co-stimulatory molecules expressed on developing thymocytes and TECs also contribute to the thymic generation of Tregs, presumably by enhancing the avidity of the interactions between thymocytes and stromal cells. The major co-stimulatory signal is provided by the interaction of CD28 on the T cell surface with either B7-1 or B7-2 ligands on the surface of the APC [57]. This is evident from the substantial reduction in numbers of thymic Tregs from *B7-1*$^{-/-}$/*B7-2*$^{-/-}$ and *CD28*$^{-/-}$ mice [128, 147], *CD40*$^{-/-}$ mice [56], and *LFA*$^{-/-}$ [99]. CD28 co-stimulation along with TCR stimulation induced Foxp3 expression along with GITR and CTLA-4 in DP thymocytes undergoing *in vitro* differentiation [147]. This *in vitro* induction of Foxp3 expression required stimulation from both TCR and CD28 simultaneously. The requirement for CD28 could not be replaced by stronger TCR stimulation. Generally, CD28 co-stimulation induces immature (HSA^{high}) SP $CD4^+$ thymocytes to undergo negative selection [76, 122], whereas it provides survival signals to mature (HSA^{low}) SP $CD4^+$ thymocytes. Since most of the $Foxp3^+$ thymocytes are mature CD4 SP (HSA^{low}), their survival could be mediated by CD28 co--stimulation.

All the current evidence echoes the belief that TCR coupled with co-stimulatory signals induces Foxp3 expression in developing thymocytes. However, the molecular events underlying Treg development are unclear. It was suggested that NF-κB signaling might be responsible for the thymic development of $CD4^+CD25^+$ T cells [130]. It has been widely reported that the activation of TCR and CD28 activates protein kinase C (PKC)-θ, an isoform of the PKC family of proteins expressed specifically in lymphoid cells [116, 142]. PKC-θ is believed to mediate the TCR signals required for the activation of NF-κB, NFAT, and AP-1 in T cells [32, 90, 142]. We have recently observed that PKC-θ is required for the development of thymic $CD4^+Foxp3^+$ Tregs (Gupta et al., unpublished data). The frequency of $Foxp3^+$ cells was drastically reduced among CD4 SP thymocytes in *PKC-θ*$^{-/-}$ mice. However, in contrast to a previous report [130], we did not see a major difference in the population of $CD25^+$ CD4 SP thymocytes between *PKC-θ*$^{-/-}$ and wild-type mice. This is consistent with the otherwise normal thymic development of T cells in *PKC-θ*$^{-/-}$ mice. Since, $CD25^+$ cells appear prior to $Foxp3^+$ cells in the thymus during development [41], PKC-θ might be responsible for specific upregulation of Foxp3 in these $CD25^+$ CD4 SP thymocytes. Similarly, mice expressing a mutant linker of activated T (LAT) cells incapable of binding phospholipase C (PLC)γ1 displayed drastically reduced $Foxp3^+$ Tregs in the thymus and periphery, despite the presence of $CD4^+CD25^+$ thymocytes [80]. LAT is a transmembrane adaptor molecule essential for T cell activation and for normal thymocyte development [180]. Considering that PKC-θ mediates the survival of CD4 and CD8 T cells by upregulating the anti-apoptotic protein Bcl-x_L, which is important for thymocyte survival, one might predict that the reduced numbers of Tregs in the thymus of *PKC-θ*$^{-/-}$ mice are due to defective survival. We eliminated this possibility by breeding

PKC-θ$^{-/-}$ mice to transgenic mice over-expressing Bcl-x_L. Over-expression of Bcl-x_L restored survival to conventional T cells, but did not restore the numbers of Foxp3$^+$ Tregs in *PKC-θ*$^{-/-}$ mice (Gupta et al., unpublished data), thus ruling out the role of Bcl-x_L-mediated survival in Treg development. Moreover, thymic Tregs have been shown to be moderately resistant to apoptosis via TCR comparison with the conventional thymocytes [153]. PKC-θ seems to be directly responsible for Foxp3 expression, as *PKC-θ*$^{-/-}$ CD4 SP thymocytes expressed less Foxp3 protein per cell compared with normal CD4 SP thymocytes. Furthermore, PKC-θ could directly upregulate Foxp3 promotor activity, likely via NFAT activation, since dominant-negative NFAT inhibited PKC-θ-mediated activation of Foxp3 promoter activity. This result is in agreement with another study reporting that Foxp3 promoter activity is induced with TCR stimulation via NFAT activation [97]. This is particularly interesting considering that PKC-θ is otherwise dispensable for the development of conventional CD4 and CD8 T cells [142]. While TCR-MHCII interaction is a common requirement in the development of conventional T cells and Tregs, different downstream signaling events might be responsible for their lineage commitment. As noted earlier, the strength of TCR signaling is crucial for Treg positive selection in the thymus. Increasing the TCR signaling intensity or duration in polyclonal developing thymocytes promotes Treg lineage commitment. This is evident by the increase in thymic Treg development in mice lacking the negative regulators of TCR signaling SHP-1 (Src homology domain 2-containing phosphotyrosine phosphatase) or Dok-1 or both [28, 73]. In *SHP-1*$^{-/-}$ mice, the percentages of CD4$^+$CD25$^+$ Tregs was increased 3-fold within CD4$^+$ T cells. The percentage of Tregs was also increased in fetal thymic organ cultures (FTOCs) derived from *SHP-1*$^{-/-}$ mice, establishing the thymic origin of these Tregs. Exposure of increasing doses of cognate ovalbumin peptide to FTOCs from DO11.10 TCR-transgenic mice favored the appearance of Tregs [28]. In SHIP/Dok-1 double knock-out mice, there was a huge decrease in total CD4$^+$ and CD8$^+$ T cells. Interestingly, all the remaining CD4$^+$ T cells in the SHIP/Dok-1 double knock-out mice had a Treg phenotype in terms of surface markers, hypoproliferation, no secretion of IL-2 or interferon (IFN)-γ, secretion of TGF-β1, intracellular Foxp3 expression, and suppression of T cell activation [73]. Using glucocorticoid receptor knock-out mice and pharmacological inhibitor of glucocorticoid synthesis, it was shown that large proportions of conventional CD4$^+$ thymocytes are biased towards commitment to the Treg lineage by reducing the threshold of thymocyte activation [141]. PKC-θ is believed to lower the threshold of T cell activation, i.e. higher antigenic concentration or stronger TCR stimulation is required to compensate for PKC-θ deficiency in T cell activation. Thus, PKC-θ, through its role in the activation of NFAT and NF-κB, seems to be one of the key elements in the Foxp3 expression and lineage commitment for Treg cells.

Role of cytokines

IL-2 is not essential for the thymic production of Tregs in terms of frequency, cell number, and suppressive function [34, 43]. Foxp3 mRNA in Tregs was comparable in *IL-2*$^{-/-}$ and *IL-2*$^{+/+}$ mice. Similarly, there was no defect in the thymic generation of Foxp3$^+$ Tregs in *IL-2Rα*$^{-/-}$ mice, showing that CD25 has no function in thymic development of Tregs [34, 43, 111, 140]. However, the frequency of Tregs is reduced in the thymus and periphery of *IL-2Rβ*$^{-/-}$ mice [111, 140]. This defect in functional CD4$^+$CD25$^+$ Treg development is restored by the thymic expression of IL-2Rβ in *IL-2Rβ*$^{-/-}$ mice [93]. Interestingly, Foxp3$^+$ Tregs were not present in the thymus or periphery of *IL-2Rγ*$^{-/-}$ or *γc*$^{-/-}$ mice [43]. IL-2Rβ is the common subunit between IL-2 and IL-15 cytokine receptors, while γc is the common subunit among IL-2, IL-15, and IL-7 receptors. Thus, it is possible that IL-15 and IL-7 may compensate for IL-2 required for the development of Foxp3$^+$ Tregs in the thymus.

TCR specificity

The requirement of TCR stimulation for their suppressive function along with the fact that they protect mice from multiple organ-specific autoimmunity suggested the self-reactive specificity of their TCR. As noted before, the increased frequency of neo-self antigen-specific Tregs in double-transgenic mice reinforces the idea of the self-reactive nature of Tregs [9, 70]. More recent data showed that Tregs utilize a diverse repertoire of TCRs to recognize a wide spectrum of antigens. The TCR diversity of peripheral CD4$^+$CD25$^+$ Tregs was comparable to that of conventional CD4$^+$CD25$^-$ T cells, but their TCR repertoires were distinct, although with some overlap [62]. Similar results were obtained when the TCR specificities of Tregs were compared [63] between the thymus and spleen. These results were also confirmed by another report showing that peripheral Foxp3$^+$ Tregs share TCR specificities with thymic Foxp3$^+$ Tregs, but not with thymic and peripheral Foxp3$^-$ conventional T cells [110], implying the thymic origin of peripheral Foxp3$^+$ Tregs rather than peripheral conversion from Foxp3$^-$ precursors. Moreover, the TCR diversity on Tregs was higher compared with Foxp3$^-$ conventional T cells [110]. Activated CD4$^+$CD25$^+$ T cells from *Foxp3*$^{-/-}$ mice lacking Tregs and suffering from massive lympho-proliferative autoimmunity displayed TCRs common with Tregs from normal mice [63]. It is believed that T cells expressing TCRs with higher affinity to self antigens show better expansion in lymphopenic mice [49]. Retroviral expression of TCRs cloned from Tregs made the naïve T cells proliferate much better in TCR-transgenic lymphopenic mice than the T cells with TCRs from conventional T cells [62]. However, in the normal non-lymphopenic mice, T cells bearing TCRs from both naïve and Tregs expanded equally [111]. Also, the Treg

repertoire of TCRs was shown to be similar to the conventional T cells in terms of self-specificity. This was supported by the observation that T cells expressing Treg-derived TCRs caused wasting disease in lymphopenic mice [111]. An intriguing aspect of Treg development is when and where (cortex-early or medulla-late) in the thymus certain thymocytes differentiate into Tregs. Immunohistochemical analysis showed that most of the Foxp3$^+$ thymocytes were localized to the medullary region of the adult thymus [44]. Expression of Foxp3 paralleled the development of the medullary region of the thymus during ontogeny [41], i.e. low expression of Foxp3 was mirrored by smaller and disorganized medullary regions of day-1 thymus relative to the cortical regions. This is also supported by the observation that mice with disrupted thymic medullar architecture, such as NF-κB-inducing kinase-deficient (*Nik*$^{aly/aly}$) and TNF-associated receptor 6-deficient (*Traf6*$^{-/-}$) mice, showed drastically reduced numbers of CD4$^+$CD25$^+$ thymocytes and relatively lower levels of Foxp3 mRNA [1, 71]. This coupled with an observation that medullary TCEs (mTECs) express a diverse range of tissue-specific self antigens [35] explains the generation of self-specific Tregs in the thymic medulla. mTECs exclusively express transcriptional factor autoimmune regulator (Aire), whose mutation in humans leads to multi-organ autoimmune diseases. Furthermore, Aire-deficient mice displayed general autoimmunity [5]. *Aire*$^{-/-}$ mTECs showed a specific reduction in ectopic transcription of genes encoding peripheral antigens, thus showing a requirement for Aire for peripheral self-antigen expression on mTECs. Since Aire$^+$ mTECs are more effective in presenting antigens to developing thymocytes, Aire-induced self-peptide expression by mTECs was shown to be responsible for the negative deletion of non-regulatory autoreactive thymocytes [4, 91]. Thus it is likely that Aire-mediated expression of self antigen on mTECs directs the positive selection of nTregs in the thymic medulla via TCR-MHC and CD28$^-$CD80/CD86 interactions. It has been shown that agonist peptide presented by thymic epithelia prevents the deletion and promotes the positive selection of mature Tregs with diverse specificities [124]. It has been suggested that thymic stromal lymphopoietin (TSLP) DCs in the central part of the medulla may be critical for the positive selection of high-affinity autoreactive T cells to differentiate into CD4$^+$CD25$^+$ Tregs [171]. Human Hassall's corpuscles expressing TSLP activate thymic CD11c$^+$ DCs to express high levels of CD80 and CD86. These TSLP-conditioned DCs can induce the proliferation and differentiation of CD4$^+$CD8$^-$CD25$^-$ thymocytes into CD4$^+$CD25$^+$Foxp3$^+$ Tregs [171]. This induction depended on the peptide-MHC II interactions, and the presence of CD80 and CD86 as well as IL-2. Immunohistochemical analysis revealed the association of these Tregs with activated or mature DCs and TSLP--expressing Hassall's corpuscles in the thymic medulla. More support comes from the recent finding that self antigen expressed on mTECs efficiently induces the differentiation of Foxp3$^+$CD25$^+$ nTregs [11]. Antigens exclusively expressed by mTECs can be presented concomitantly by mTECs as well as DCs [11, 165]. However, differentiation of antigen-specific Tregs resulting from exclusive expression of antigen by mTECs was shown to be autonomously mediated by mTECs, but not by thymic DCs. Thymic DCs that cross-present mTEC-derived self antigens negatively select both CD4$^+$ and CD8$^+$ T cells, whereas mTECs efficiently eliminate CD8$^+$ but not CD4$^+$ T cells [47]. This could explain the development of Tregs in the medullary region of the thymus where other self-specific CD4 and CD8 thymocytes are deleted by negative selection. In addition, since Treg development requires CD28 signaling, expression of its cognate ligand B7 by mTECs could provide the necessary survival signals for developing Tregs [41].

PERIPHERAL MAINTENANCE OF TREGS

Natural Tregs maintain their homeostatic numbers in the periphery by expansion so as to prevent the onset of autoimmunity [40, 59]. Adult thymectomy does not reduce the peripheral levels of nTregs, suggesting they can be maintained in the absence of thymic output [112]. In addition, CD4$^+$CD25$^+$ Tregs could expand *in vivo* when CD4$^+$ cells were injected into athymic nude mice [112]. For a given age and genetic background (BALB/c versus C57B/6), the percentage of Foxp3$^+$ Tregs is constant among peripheral CD4$^+$ T cells. This suggests that there are homeostatic mechanisms in place to control the population of Tregs. When transferred into lymphopenic mice, CD4$^+$CD25$^+$CD45RB^{low} Tregs expanded and reached equilibrium at 10-fold lower numbers than similarly transferred CD4$^+$CD25$^-$ CD45RB^{high} T cells [2]. This number represents the normal percentage of Tregs among the total CD4$^+$ T cells. Peripheral homeostasis of conventional CD4 T cells depends primarily on TCR-self-antigen-MHCII interactions and IL-7 availability [135]. When polyclonal CD4$^+$CD25$^+$ Tregs (CD62L^{high}) were adoptively transferred into normal mice, they formed two distinct populations, long-lived quiescent cells (CD62L^{high}) and rapidly dividing cells (CD62L^{low}) with activation markers [40]. The rapidly dividing population is believed to be the auto-reactive Tregs which colonize the cognate tissue-draining lymph nodes and become activated continuously, while the quiescent population represents the non-self-specific Tregs. Peripheral CD4$^+$ T cells from rat lacking a thyroid could not prevent thyroiditis when adoptively transferred into thymectomized and irradiated recipients, but they could prevent diabetes in the recipients. In contrast, CD4$^+$CD8$^-$ thymocytes from rats lacking a thyroid could prevent thyroiditis, suggesting the requirement of the presence of self antigen for the peripheral expansion of self-reactive Tregs [133]. HA--specific CD4$^+$CD25$^+$ Tregs proliferate only upon transfer into irradiated HA-transgenic mice, but not in

irradiated normal mice, suggesting an essential role of TCR-MHCII interactions [33]. Polyclonal Tregs, when transferred into lymphopenic *Rag-1*$^{-/-}$ mice, proliferated extensively, but not when transferred into *MHC class II I-Ab*$^{+/-}$ *Rag-1*$^{-/-}$ mice, again suggesting TCR-MHC class II interactions are important for the homeostatic proliferation of Tregs [48]. Co-stimulation via B7 is also important for the peripheral homeostasis of Tregs. *B7-1/B7-2*$^{-/-}$ and *CD28*$^{-/-}$ NOD mice have reduced numbers of CD4$^+$CD25$^+$ Tregs in the periphery, implying a defective maintenance of Tregs [128]. Administration of murine CTLA-4-Ig antibody, which blocks B7 signaling, into wild-type NOD mice leads to a 5-fold reduction in the numbers of CD4$^+$CD25$^+$ Tregs. In addition, CTLA-4-Ig treated mice had increased incidence and rapid onset of autoimmune diabetes compared with control mice [128]. The peripheral expansion of nTregs *in vivo* is believed to be dependent on weak stimulation provided by the interaction of its TCR with self-peptide-MHC on peripheral tissues. PKC-θ, with its ability to lower the threshold of activation, might play an important role in the peripheral activation and expansion of nTregs. We also found that mice deficient in PKC-θ may have defective maintenance of Tregs in the periphery, as evident by reduced numbers of CD4$^+$CD25$^+$Foxp3$^+$, CD4$^+$CD25$^+$ GITR$^+$, and CD4$^+$CTLA-4$^+$ T cells in the spleen and lymph nodes (Gupta et al., unpublished data). The homeostasis of Tregs was also shown to be regulated by CD40/CD40L interactions [56]. *CD40*$^{-/-}$ mice or normal mice treated with CD40/CD40L-blocking antibodies have reduced numbers of Tregs in the periphery. In addition, Tregs injected into *CD40*$^{-/-}$ mice showed poor survival and homeostatic expansion [56]. It has been suggested that B cells can affect the peripheral homeostasis of nTregs without affecting their thymic development [144]. In μ-chain-deficient mice lacking mature B cells, the percentage of CD4$^+$CD25$^+$ Tregs was reduced to half compared with the spleens of wild-type mice, but the percentage of Tregs in the thymus was not affected. TGF-β may also contribute to the peripheral homeostasis of CD4$^+$CD25$^+$ Tregs, as mice deficient in TGF-β [98] and mice defective in TGF-βRII have reduced numbers of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the periphery [66]. However, the thymic development of nTregs is not dependent on TGF-β, as indicated by the normal numbers of thymic Tregs in *TGF-β*$^{-/-}$ mice [98].

Since CD4$^+$CD25$^+$ Tregs express all the subunits of a functional high-affinity IL-2 receptor (IL-2Rα, IL-2Rβ, and γc), it makes sense to believe that IL-2 is important for Treg maintenance and function. IL-2-deficient mice had reduced numbers of CD4$^+$CD25$^+$ Tregs in the thymus as well as in the spleen and lymph nodes, although the composition of CD4$^+$ and CD8$^+$ T cells was normal [2, 132]. A similar phenotype was observed in CD25 (IL-2Rα)-deficient mice, which also suffered lymphocyte accumulation and autoimmune disorders [173]. The frequency of CD4$^+$Foxp3$^+$ Tregs was reduced in the periphery of both IL-2- and IL-2Rα-deficient mice [34, 43], even though Treg numbers were normal in the thymus [34, 43]. In addition, *in vivo* neutralization of IL-2 using anti-IL-2 mAb significantly and specifically reduced the CD4$^+$CD25$^+$ T cell numbers and consequently caused autoimmune disease [137]. Adoptive transfer of polyclonal CD4$^+$CD25$^+$ Tregs into *IL-2Rβ*$^{-/-}$ mice which lack endogenous Tregs caused their expansion, sustenance, and subsequent prevention of autoimmune pathology in the recipient mice [93]. In contrast, Tregs, when transferred into IL-2-deficient mice, disappeared in the recipients after four weeks and the recipients eventually developed autoimmunity [93]. Similarly, when both conventional CD4 T cells and Tregs were adoptively transferred into an *IL-2*$^{-/-}$ host, survival of Tregs, but not of conventional CD4 T cells, was impaired [34]. Likewise, IL-2 therapy resulted in the expansion of Tregs in the blood of normal individuals, and even more extensive peripheral expansion in lymphopenic cancer patients [179]. These data collectively highlight the requirement of IL-2 in the peripheral maintenance of Tregs. IL-2 signaling activates two major signaling pathways in T cells for their survival, proliferation, and differentiation [162]. One leads to the activation of the serine/threonine kinase Akt and consequent upregulation of the anti-apoptotic molecules Bcl-2 and Bcl-x_L. The other pathway leads to the activation of the transcription factor STAT5 required for proliferation and differentiation. Using *IL-2*$^{-/-}$ mice over-expressing Bcl-2 transgene it was shown that reduced Treg numbers in the periphery of *IL-2*$^{-/-}$ mice were not due to defective survival [8]. However, transient over-expression of STAT5 rescued the reduction in peripheral Treg numbers in *IL-2*$^{-/-}$ mice, suggesting a role of STAT5 in the homeostasis of Tregs in the periphery [8]. Consistent with this observation, deletion of STAT5 resulted in a dramatic reduction in CD4$^+$Foxp3$^+$ cells [178]. STAT5$^{-/-}$ fetal liver reconstituted mice still displayed reduced Tregs, suggesting the intrinsic requirement of STAT5 for the development of Tregs. Furthermore, STAT5 binds to the target DNA sequences on the Foxp3 promoter, suggesting that STAT5 is likely to regulate Treg development by transcriptional regulation of Foxp3 expression. Interestingly, reduction of STAT3 does not affect Tregs. Therefore, members of the STAT family differentially regulate the development of Tregs. Reduced levels of IL-2 in the periphery of *PKC-θ*$^{-/-}$ mice [3] could be responsible for the low frequency of CD4$^+$CD25$^+$Foxp3 Tregs in the spleen and lymph nodes of *PKC-θ*$^{-/-}$ mice (Gupta et al., unpublished data). The source of IL-2 required for Treg peripheral maintenance is believed to be DCs [56]. Accordingly, DCs from *CD40*$^{-/-}$ mice produce lower levels of IL-2 and could not support Treg expansion. These defects were reversed by adding IL-2 to the cultures or injecting it into *CD40*$^{-/-}$ mice [56]. Some studies also addressed the role of apoptosis in the maintenance of peripheral Tregs. *In vitro* TCR re-stimulation of naïve T cells and Tregs showed reduced sensitivity of Tregs towards AICD compared with CD4$^+$CD25$^-$ T cells [46]. Moreover, murine Tregs were

shown to be more resistant to clonal deletion induced by viral superantigen *in vivo* and by ligation of Fas [18]. In contrast, freshly isolated naïve human Tregs were shown to be highly sensitive towards Fas-mediated apoptosis, unlike conventional $CD4^+$ T cells [46]. Since *PKC-θ*$^{-/-}$ T cells are defective in FasL upregulation upon stimulation *in vivo* and *in vitro* [96], increased AICD of *PKC-θ*$^{-/-}$ Tregs in the periphery is less likely. The resistance of Tregs to TCR-mediated apoptosis [153] also suggests that poor maintenance of Tregs in *PKC-θ*$^{-/-}$ mice may be independent of TCR-mediated apoptosis [95].

FUNCTIONAL FEATURES OF NATURAL TREGs

While most of the self-specific T cells are deleted in the thymus by negative selection, some of these self-destructive T cells come out in circulation, but their activation and proliferation are kept in check by nTregs. The suppressive function of Tregs was explored using an *in vitro* assay in which $CD4^+CD25^-$ conventional T cells isolated from naïve mice were stimulated in the presence of $CD4^+CD25^+$ T cells and irradiated APCs. Inhibition of $CD4^+CD25^-$ T-cell proliferation and cytokine secretion was measured as the suppressive potential of $CD25^+$ Tregs [148, 156]. This is still the most widely used method to assess the suppressive potential of Tregs *in vitro*. The following were the main functional attributes of Tregs as deciphered by these *in vitro* assays [148, 156]: $CD4^+CD25^+$ Treg cells from normal naïve mice potently suppressed proliferation and cytokine production by $CD4^+$ and $CD8^+$ effector T cells in a dose-dependent manner. This suppression required the activation of $CD4^+CD25^+$ T cells by stimulation via their TCR either by polyclonal anti-CD3 antibody or antigen-specific stimulation of the T cells. Co-stimulation was provided by APCs. $CD4^+CD25^+$ T cells stimulated with plate-bound anti-CD3 antibody alone did not cause suppression of $CD4^+CD25^-$ T cells. However, Tregs can suppress the proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells even in the absence of APCs when stimulated by immobilized anti-CD3 and anti-CD28 antibodies. Cross-linking with anti-CD3 and anti-CD28 antibodies mimics stimulation through the TCR and CD28 receptor on T cells. Nonspecific antigenic stimulation of $CD4^+CD25^+$ T cells does not evoke suppression by these cells, highlighting the relevance of appropriate TCR stimulation. However, once $CD4^+CD25^+$ T cells are appropriately stimulated, they can suppress the activation and proliferation of both $CD4^+$ and $CD8^+$ T cells in an antigen nonspecific manner, i.e. they suppress the proliferation of not only cells with the same antigen specificity, but also suppress T cells with different antigen specificities. In addition, MHC histocompatibility between $CD4^+CD25^+$ Tregs and non-Tregs is not required for suppression. This was evident in the allogeneic mixed lymphocyte reaction where $CD4^+CD25^+$ Tregs activated by allogeneic APCs suppressed the proliferation of $CD4^+CD25^-$ T cells of a third-party strain [127]. The suppression by $CD4^+CD25^+$ Tregs is highly sensitive to antigenic stimulation. About a 10- to 100-fold lower concentration of antigen is required to activate $CD4^+CD25^+$ T cells for suppression than that required for the activation and proliferation of $CD4^+CD25^-$ T cells from the same TCR-transgenic mice [156]. Although they themselves do not secrete IL-2, $CD4^+CD25^+$ Tregs suppress IL-2 production in $CD4^+CD25^-$ T cells at the mRNA level [148, 156] and inhibit IFN-γ production by $CD8^+$ T cells [118]. Tregs also suppress B cell proliferation, immunoglobulin production, and class switch [89, 181]. Studies have also shown that they can inhibit the cytotoxic functions of NK cells [52] and NKT cells [14], as well as the function and maturation of DCs [101]. Tregs can also inhibit memory T cells to some extent [87, 145].

Though TCR stimulation is required for their suppressive function, $CD4^+CD25^+$ Tregs are themselves hypo-proliferative or anergic to *in vitro* antigenic stimulation. They were unable to flux Ca^{2+} upon TCR engagement [48]. Studies exploring the molecular basis of the Treg anergic phenotype found defective Ras, MEK1/2, and ERK1/2 activation in $CD4^+CD25^+$ Tregs [88]. Examination of TCR proximal and distal signaling events revealed impaired amplitude and duration of tyrosine phosphorylation compared with $CD4^+CD25^-$ T cells [58]. However, the duration of such tyrosine phosphorylation was increased by strong CD28 co-stimulation in Tregs, explaining the transient loss of anergy upon strong CD28 ligation. Activation of PLCγ and downstream events of calcium mobilization, NFAT, NF-κB, and Ras-ERK-AP-1 activation were also impaired. This anergic phenotype was not lost even upon lymphopenia-driven proliferation of Tregs *in vivo*. Stimulation of Tregs with PMA+ionomycin or TCR stimulation along with PMA treatment can break their anergy, resulting in subsequent proliferation and IL-2 production due to enhanced NF-κB and AP-1 activation [58]. The anergic phenotype of Tregs can be explained by the finding that Foxp3 represses the gene transcription of IL-2, IL-4, and IFN-γ through direct physical interactions with the transcription factors NF-κB and NFAT [20]. Also, the NFAT-Foxp3 complex upregulates the expressions of CD25 and CTLA-4 and is required for the suppressive function of Tregs [174]. This hypo-proliferative state is closely linked to their suppressive potential, as breaking their anergy by TCR stimulation along with a high dose of IL-2 and CD28 ligation results in their proliferation and simultaneous loss of suppression. Such proliferating and non-suppressive Tregs revert to their "default" anergic and suppressive state once the IL-2 and CD28 ligation are removed. Tregs expanded in such a way are more suppressive than unexpanded Tregs. Antigen-specific Treg expansion can also be achieved *in vitro* when Tregs are exposed to alloantigen-loaded DCs in the presence of IL-2 [107] or by mature DCs in the absence of added IL-2 [177]. Human Tregs from both the thymus and the peripheral blood display similar properties as murine nTregs. They

are anergic to TCR stimulation *in vitro* and are capable of suppressing the proliferation and cytokine production by $CD4^+CD25^-$ T cells *in vitro* [7, 36, 69, 87]. Human Tregs also do not produce IL-2, IL-4, IFN-γ, IL-5, or IL-13 upon activation [7]. Human Tregs that are expanded in the presence of high IL-2, IL-4, or IL-15 maintain their suppressive potential. In contrast to the *in vitro* situation, Tregs that are proliferated in normal or lymphopenic mice still maintain their suppressive phenotype [2, 48, 77, 137, 167]. In T cell-deficient mice, exposure of alloantigen to transferred Tregs elicited spontaneous expansion of alloantigen-specific $CD4^+CD25^+$ Tregs, which provided long-term graft tolerance against subsequently transferred naïve T cells [107]. Besides *in vitro* suppression, Tregs are shown to regulate various *in vivo* immune responses mediated by $CD4^+$ T cells, $CD8^+$ T cells, B cells, and others, highlighting the essence of Treg function in preventing autoimmune diseases, allograft transplant rejection, allergic responses, etc. Antigen-specific Tregs prevented graft rejection or autoimmunity by directly inhibiting effector cells *in vivo* [85, 150]. Tregs were found to prevent various *in vivo* pathologies by inhibiting the differentiation of effector T cells in terms of cytokine production and chemokine receptor expression, but not much by inhibiting their proliferation [37, 129, 150]. *In vivo* depletion of $CD4^+CD25^+$ T cells or injection of $CD4^+CD25^-$ T cells into immunodeficient mice leads to various pathologies (gastritis, colitis, etc.) which can be both prevented [2] and cured by introduction of enriched $CD4^+CD25^+$ Tregs from normal mice [104]. By using TCR-transgenic mice that either harbor or lack nTregs as recipients, proliferation of injected $CD4^+$ T cells was shown to be restricted only in recipients with nTregs [138].

MECHANISM OF SUPPRESSION

The mechanisms by which Tregs exert their suppressive function are still controversial, owing to disparate *in vivo* and *in vitro* studies. It seems highly possible that Tregs employ multiple mechanisms to suppress various kinds of effector cells.

Role of cytokines

Initial studies explored the immunosuppressive cytokines IL-4, TGF-β1, and IL-10 in the suppression mediated by Tregs since their transcripts were more actively transcribed in $CD4^+CD25^+$ T cells than in $CD4^+CD25^-$ T cells during an *in vitro* suppression assay [148]. Neutralization of these cytokines with corresponding monoclonal antibodies, alone or in combination, was unable to abrogate the suppression of $CD4^+CD25^-$ T cells, *in vitro*. The supernatant from the suppression co-culture also failed to suppress anti-CD3-stimulated $CD4^+CD25^-$ T cells, ruling out the possibility that soluble factors such as cytokines are responsible for suppression [148, 156]. Moreover, $CD4^+CD25^+$ Tregs isolated from *IL-4*$^{-/-}$, *TGF-β1*$^{-/-}$, or *IL-10*$^{-/-}$ mice were as effective as wild-type Tregs in suppressing T cell proliferation *in vitro* [117, 156]. When stimulated with soluble anti-CD3 in the presence of APCs, Tregs highly expressed membrane-bound latent TGF-β [106]. The suppressive role of TGF-β1 was attributed to this membrane-bound TGFβ-1, but not to the soluble factors present in the supernatant. Another TGF-β1-blocking agent, recombinant latency-associated peptide of TGF-β1 (rLAP), is also shown to inhibit Treg-mediated suppression *in vitro* in a dose-dependent manner, confirming the importance of membrane-bound TGF-β1 in suppressor function [105]. Membrane-bound TGF-β on Tregs is also involved in NK-cell suppression [52]. Another mechanism of suppression involves TGF-β and Notch1, a transmembrane molecule required for commitment to the T cell lineage during development [166]. Intracellular domain of Notch1, which splits after the binding to its ligands jagged 1 and jagged 2, acts as a transcription factor to induce the expression of hairy and enhancer of split 1 (HES1), a transcription repressor. It has been demonstrated that membrane expression of TGF-β is required for the expression of Notch1 ligands on Tregs [109]. Tregs that express membrane-bound TGF-β can activate the Notch1-HES1 axis in activated T cells. Inhibition of the Notch1 pathway abrogates Treg-mediated suppression of allergic airway inflammation. Because of the constitutively high expression of CD25 on Tregs, it was suggested that Tregs suppress by depriving the available IL-2 required for effect or T cell function. However, $CD4^+CD25^+$ Tregs inhibit IL-2 transcription in $CD4^+CD25^-$ T cells even in the presence of exogenous IL-2 [154]. This observation challenges the IL-2 competition theory. Furthermore, Tregs isolated from *IL-2Rα*$^{-/-}$ (CD25-deficient mice) and *IL-2*$^{-/-}$ mice are fully capable of suppressing the proliferation of normal $CD4^+CD25^-$ T cells *in vitro* [45].

Cell-cell contact

The *in vitro* suppression is dependent upon contact between $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ effector T cells, since their separation by a Transwell™ membrane, which permits soluble factor exchange but not direct physical contact, abrogates the suppression [148, 156]. Such contact-dependent suppression is not because of direct killing of $CD4^+CD25^-$ T cells by $CD4^+CD25^+$ cells via the Fas/FasL or TNF/TNFR-dependent pathway, since blocking antibodies to FasL or TNF-α had no effects on the number of $CD4^+CD25^-$ responders [148]. Another way of suppression by Tregs is Granzyme-B-dependent perforin-independent killing of $CD4^+CD25^-$ T cells [54]. When co-cultured with $CD4^+CD25^+$ in the presence of stimulation, $CD4^+CD25^-$ T cells were shown to be non-proliferating and dying. $CD4^+CD25^+$ Tregs from Granzyme-B-deficient mice were less efficient than normal Tregs for suppression *in vitro*. Also, mRNA and protein levels of

Granzyme-B were shown to be elevated in $CD4^+CD25^+$ Tregs by stimulation with anti-CD3 antibody. More recently, Tregs were also shown to inhibit B cell proliferation by direct killing in a Granzyme B and perforin-dependent, but Fas/FasL-independent fashion [181]. Tregs were also shown to inhibit NK and $CD8^+$ T cell-mediated tumor-immunity by direct killing of these cells in a Granzyme B- and perforin-dependent way at tumor sites *in vivo* [26]. The contribution of several T cell accessory molecules such as CTLA-4 (CD152) and lymphocyte-activation gene (LAG)-3 expressed by Tregs and B7-1/B7-2 co-stimulatory molecules expressed by APCs have also been tested in contact-dependent suppression. $CD4^+CD25^+$ Tregs highly express LAG-3 (a CD4-related molecule that binds MHC class II) upon activation. An antibody to LAG-3 inhibits suppression by Tregs both *in vitro* and *in vivo* [65]. $CD4^+CD25^+$ Tregs from *LAG-3*$^{-/-}$ mice exhibited reduced regulatory activity. In addition, ectopic expression of LAG-3 on normal $CD4^+$ T cells reduced their proliferative ability and rendered them suppressor activity towards $CD4^+CD25^-$ T cells [65]. CTLA-4 is constitutively expressed on the surface of $CD4^+CD25^+$ Tregs, in contrast to naïve $CD4^+CD25^-$ T cells, which express CTLA-4 only after activation [123, 149]. Upon stimulation, CTLA-4 expression is upregulated on Tregs more than on $CD4^+CD25^-$ T cells [149]. This suggested a possible role of CTLA-4 in contact-dependent suppression. Tregs from *CTLA-4*$^{-/-}$ mice were fully capable of *in vitro* suppression although they produced more TGF-β1 than normal Tregs [151]. Injecting anti-CTLA-4 mAb into normal mice over a limited period of time elicited autoimmune gastritis similar to that produced by depletion of $CD4^+CD25^+$ Tregs without reducing the number of total $CD4^+CD25^+$ Tregs in the treated mice [149]. Administration of anti-CTLA-4 mAb also neutralized the protection mediated by $CD4^+CD25^+$ Tregs in the murine model of colitis [123]. $CD4^+CD25^+$ T cells from normal mice suppressed $CD4^+CD25^-$ T cells from CTLA-4-deficient mice, but not when treated with antigen-binding (Fab) fragments of anti-CTLA-4 mAb [149]. Furthermore, ectopic expression of Foxp3 in naive T cells upregulated the expression of CTLA-4 in a Foxp3-dependent way [60]. Another direct action of CTLA-4 is believed to be the induction of indoleamine 2, 3-dioxygenase (IDO) in DCs by interaction with B7-1 and B7-2 on DCs. IDO is responsible for the metabolism of an essential amino acid, tryptophan, required for the activation of $CD4^+CD25^-$ T cells [38, 39]. The soluble fusion protein CTLA-4-immunoglobulin (CTLA-4-Ig) induced IDO in DCs, resulting in the induction of tolerance *in vivo* [55]. This tolerance was abrogated by the IDO inhibitor 1-MT. Some reports suggested that Tregs may also down-modulate APC functions in Tcell activation by inhibiting the expression levels of B7-1 and B7-2 on DCs [30, 101]. It is very well documented that CTLA-4 has a higher affinity than CD28 for ligands B7-1 and B7-2. CTLA-4 on the surface of Tregs may compete for B7-1/B7-2 required for activating CD28 co-stimulatory signals. This could also explain why strong stimulation with anti-CD28 mAb can break the suppression by Tregs. However, use of lipopolysaccharide (LPS)-activated APCs which expressed B7-2 at high levels did not decrease the suppression by $CD4^+CD25^+$ Tregs. Furthermore, suppression was not affected when varying amounts of APCs were used in an *in vitro* suppression assay, ruling out the notion that competition for co-stimulatory molecules is the major mechanism responsible for Treg-mediated suppression [157]. On the contrary, some reports showed that anti-CTLA-4 mAb or anti-CTLA-4 Fab fragments did not abrogate the suppressive activity of Tregs *in vitro* [87, 155, 156], and thus challenged the requirement of CTLA-4 for suppression. A possible explanation could be a compensatory increased production of TGF-β1 with anti-CTLA ligation in these cultures [106], as also seen with the $CD4^+CD25^+$ Tregs from CTLA-4-deficient mice [151]. Therefore, cell-to-cell cognate interaction-dependent suppression seems to be the dominant mechanism of suppression by Tregs *in vitro*, but the molecular basis of this interaction remains unknown.

Quite different from contact-dependent suppression *in vitro* is the requirement of cytokines IL-10 and TGF-β1 for *in vivo* suppression. Adoptive transfer of $CD4^+CD25^+$ Tregs into mice with allogeneic skin graft induces graft tolerance, but the administration of IL-10-receptor blocking antibody notably accelerated transplant rejection [75]. The role of IL-10 in the $CD4^+CD25^+$ Treg-mediated control of murine transplantation tolerance, chronic parasite infection, and a rat model of type 1 diabetes has also been reported [61]. IL-10 is required for the control of the mouse model of colitis and the homeostatic maintenance of T cell numbers by Tregs. IL-10-deficient mice developed spontaneous colitis [81] mediated by Th1 $CD4^+$ T cells and triggered by intestinal *Helicobacter hepaticus* [82, 136]. $CD4^+CD25^+CD45RB^{low}$ Tregs, which normally can prevent colitis and homeostatic proliferation of $CD4^+$ T cells in immunodeficient $Rag^{-/-}$ mice, failed to do so when isolated from IL-10-deficient mice [6]. Treatment with anti-IL-10 receptor mAb abrogated the inhibition of colitis mediated by normal $CD4^+CD45RB^{low}$ T cells, suggesting a role for IL-10 in Treg-mediated suppression [12]. Furthermore, injection of anti-IL-10 receptor mAb to wild-type mice induces colitis [13]. It was concluded that IL-10 is required for Treg-mediated suppression of colitis induced by antigen-experienced colitogenic Th1 cells, but is not absolutely required for $CD4^+CD25^+$ T cell-mediated inhibition of colitis induced by the transfer of naïve $CD4^+CD45RB^{high}$ cells. It is intriguing that *IL-10*$^{-/-}$ Tregs can effectively prevent autoimmune gastritis produced by depletion of Tregs in BALB/C mice, but are not sufficient to suppress bacterial-driven colitis in the same recipient mice [143]. $IL\text{-}10^{-/-}$ mice spontaneously develop colitis, but not gastritis. Recently it was shown that IL-10-producing $CD4^+CD25^+Foxp3^+$ Tregs are selectively enriched within the colonic lamina propria of

colitic mice and wild-type mice [160]. Also, inflamed intestine of human patients with inflammatory bowel disease showed an accumulation of $CD4^+CD25^+$ $Foxp3^+$ Tregs, thus suggesting impaired function, but not physical absence of Tregs in the development of the disease [160]. The partial requirement of IL-10 in Tregs from colon, but not from secondary lymphoid organs explains the discrepancies found in the above studies. In contrast to *in vitro* suppression, $CD4^+CD25^+$ Tregs from *TGF-β1*$^{-/-}$ mice failed to prevent the development of colitis in the murine model [105]. $CD4^+CD25^+LAP^+$ T cells expressing a membrane-bound form of TGF-β1 were suggested to be responsible for the control of colitis induced by $CD4^+CD45RB^{high}$ T cells [105]. Administration of anti-TGF-β and anti-IL-4 antibody neutralized the suppressive activity of $CD4^+CD45RC^{low}$ T cells in rat type-1 diabetes and thyroiditis [134]. TGF-β also contributes to the suppression by Tregs in an indirect way. TGF-β1 (presumably from Tregs) induces Foxp3 expression in about 10–30% of $CD4^+CD25^-$ T cells upon TCR stimulation under *in vitro* and *in vivo* conditions [31, 113]. Induced Foxp3 expression in these effector cells made them anergic and they could suppress IFN-γ cytokine production by other $CD4^+CD25^-$ T cells [169]. Such an increase in $Foxp3^+$ cells from a $Foxp3^-$ population upon anti-CD3/CD28 stimulation in the presence of TGF-β is dose dependent. The highest dose of TGF-β (10 ng/ml) results in the production of 30% $Foxp3^+$ cells [169]. TGF-β1 is a well-characterized immunosuppressive cytokine known to be produced by a variety of cells other than Tregs or activated $CD4^+CD25^-$ T cells. Thus there are doubts about a direct requirement of TGF-β1 production by nTregs. All the studies so far collectively imply that the mechanisms of suppression employed by Tregs are different in *in vitro* and *in vivo* situations. While a contact-dependent mechanism dominates during *in vitro* suppression, soluble factors such as IL-10 and TGF-β are important in *in vivo* suppression. These results might reflect the different conditions prevailing during *in vitro* and *in vivo* set-ups. *In vitro* conditions provide higher proximity among Tregs and effector cells and normally stronger than physiological TCR stimulation. In contrast, during *in vivo* suppression, other long-range factors come into play, such as the migration of Tregs to the appropriate sites of suppression, which might involve the upregulation of certain receptors on Tregs. Moreover, it is possibly that more than one mechanism exists during *in vitro* and *in vivo* suppression, with the choice lying in the nature and magnitude of the immune responses and the tissue involved.

ACTIVATION OF TREGs

The requirement of co-stimulation provided either by APCs or CD28 cross-linking along with TCR stimulation during *in vitro* activation of suppressor function reflects the contribution of various co-stimulatory molecules highly expressed on the Treg surface. A strong stimulation of $CD4^+CD25^+$ Tregs through CD28 abrogates their anergic and suppressive state [148, 156]. In contrast, they are not dependent on CD28 for their activation, since Tregs from CD28-deficient mice exhibited an equally potent *in vitro* suppressive activity [149]. CTLA-4 ligation increases the receptor clustering and the interaction of lymphocyte function-associated antigen (LFA)-1 on T cells with ICAM-1/2 on APCs. This can possibly augment the physical interactions between Tregs and APCs, enhancing their activation [131]. Co-stimulation through CTLA-4 enhances the proliferation of and TGF-β1 production by $CD4^+CD25^+$ Tregs [106]. Stimulation of GITR on Tregs abrogated their suppressive function *in vitro* without changing their anergic state [139]. Administration of anti-GITR mAb produced organ-specific autoimmune disease in otherwise normal mice. This suggests that the signal through GITR attenuates the ability of Tregs to exert suppression. $CD4^+CD25^+$ Tregs selectively express several members of the Toll-like receptor (TLR) family, such as TLR4 [27]. *In vitro* stimulation of Tregs with a high concentration of LPS through TLR4 elicited their proliferation, prolonged their survival, and augmented their *in vitro* suppressive activity even in the absence of APCs, indicating that LPS directly acts on TLR4 molecules expressed by Tregs [27]. TLR4-mediated stimulation of Treg cells, as a result of infection by Gram-negative bacteria, may lead to inhibit T cell-mediated immune responses against bacteria. The expression pattern of other accessory molecules on Tregs, such as $CD45RB^{low}$, $CD44^{high}$, $CD5^{high}$, CD54 $(ICAM\text{-}1)^{high}$, CD11a/CD18 $(LFA\text{-}1)^{high}$, and $CD62L^{low}$, is somewhat similar to that of primed, activated effector or memory T cells [68, 83, 126, 156]. This suggests that Tregs may be continuously stimulated by self antigens in the normal internal environment. Human Tregs express the chemokine receptors CCR4 and CCR8 on their surface [67]. Consequently, mature DCs preferentially attract Tregs among circulating $CD4^+$ T cells by secreting the CCR4 ligands, CCL17 and CCL22 [67]. Similarly, it was shown that activated B cells and professional APCs upregulated the expression of CCL4, which acts as a potent chemoattractant for Tregs [24]. The expression of such receptors may enable Tregs to preferentially migrate to the sites of antigen presentation in the secondary lymphoid tissues and recruit to the sites of inflammation and tissue damage to control physiological and pathological immune responses. Several cytokines are also shown to play important roles in the activation of Tregs. TGF-β activation induced the upregulation of CD25 and CTLA-4 expression on naïve $CD4^+$ T cells in peripheral human blood, leading to suppressive phenotype *in vitro* and *in vivo*, and also induced the expansion of Tregs [176]. Cell surface TGF-β1, from autocrine or paracrine sources, may mediate its effects by activating $CD4^+CD25^+$ Tregs themselves, possibly by maintaining their survival, differentiation, expansion, or suppressive functions [31, 94]. Moreover, TGF-β1 sig-

naling may enhance the suppressive function of Tregs by stimulating Foxp3 expression [98]. IL-2 and IL-4 are showed to activate nTregs *in vitro* along with TCR stimulation, leading to Treg expansion [155]. IL-4 can substitute for IL-2 both in terms of *in vitro* expansion of Tregs and activation of inhibitory function of Tregs [155].

CONCLUSION

Although nTregs share few of their markers with other cells, the identification of Foxp3 as a specific and lineage-determining factor in Tregs has helped immensely in the understanding of the thymic development, peripheral maintenance, and function of Tregs. The only handicap in the field of Tregs is the reliance on CD25 as the marker for Treg isolation and for functional studies. However, CD25 expression on naïve $CD4^+$ T cells correlates well with Foxp3 expression and thus Treg phenotype. There are chances of contamination with activated T cells, especially when studying Tregs from human peripheral blood. Significant observations have been made regarding Treg development in the thymus, but there are still some questions remaining to be answered. No doubt exists regarding the requirement of high-avidity TCR-MHC class II interactions in the thymic medullary region, along with additional co-factors which might provide a survival advantage to the developing Tregs. CD28 is one of the most important cofactors known to upregulate anti-apoptotic genes in the cells such as Bcl-x_L. Thus it is not surprising that mice lacking CD28 do not support the development and peripheral maintenance of Tregs. Similar is the case with mice deficient in LAT, which is an adaptor molecule transmitting signals from TCR to the nucleus. Besides TCR and co-stimulatory signals, signals from cytokine receptors are equally important for the thymic generation of Tregs. This is exemplified by the absolute requirement of the γc subunit of the receptor common to IL-2, IL-4, IL-7, and IL-15. It is not very clear how these important factors coordinate during Treg development to induce Foxp3 expression. PKC-θ is one such protein which integrates TCR and CD28 signaling to lower the threshold of T cell activation, and is thus important for the activation, survival, and differentiation of T cells. Not surprisingly, *PKC-θ*$^{-/-}$ mice have drastically reduced $Foxp3^+$ Tregs in the thymus. We have shown recently that PKC-θ, through coupling of TCR and CD28 signaling, is responsible for Foxp3 gene expression via stimulating NFAT. Once Foxp3 is expressed, it can associate with NFAT to act as a transcriptional complex to regulate various genes involved in Treg differentiation. The presence of some $Foxp3^+$ Tregs in the periphery of *PKC-θ*$^{-/-}$ mice suggests that Treg development is not completely abrogated. Factors that govern the thymic development of Tregs seem to be different from those which govern its function. Co-stimulation provided by CD28 signaling is important for the thymic development and peripheral homeostasis of Tregs, but not essential for its suppressive function [147, 149, 152]. In contrast, CTLA-4 signaling is required for Treg function, but not for their development and maintenance [151]. The expression of B7-2 is important for thymic generation and *in vivo* function of Tregs, but the *in vitro* activation of Tregs with B7-1/B7-2-double-deficient APCs or in the presence of an antagonist of B7/CD28 interaction (CTLA-4-Ig) does not affect their suppressive function [155]. We also observed that PKC-θ is important for Treg development, but not for their activation and suppressive function. The mechanism of suppression by Tregs has been studied extensively. Factors important for *in vitro* and *in vivo* suppression appear to be different. These discrepancies about the roles of various factors involved in suppression may reflect different experimental conditions between *ex vivo* and *in vivo*, and also between various laboratories. It could have serious implications when transferring such experimental observations to clinic applications. It is truly remarkable that Tregs, which appear to be just a subset, have such distinct properties to be qualified as a separate lineage of T cells. Equally remarkable is the fact that one molecule like PKC-θ can highlight the difference between nTregs and conventional CD4 T cells in terms of their thymic development, activation, survival, and effector function.

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