Peripheral Generation of $CD4^+CD25^+Foxp3^+$ Regulatory T Cells

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ABSTRACT

$CD4^+CD25^+$ regulatory T cells (Tregs) expressing the lineage-specific marker Foxp3 represent an important regulatory T cell that is essential for maintaining peripheral tolerance. Although it was believed that Treg development is solely dependent on the thymus, accumulating evidence demonstrates that Tregs can also be induced in the periphery. Considering the various origins of peripherally developed $CD4^+CD25^+Foxp3^+$ regulatory T cells, it seems likely that multiple factors are involved in the peripheral generation of Tregs. **(Immune Network 2007;7(1):1-9)**

Key Words: $CD4^+CD25^+Foxp3^+$ regulatory T cell, development, generation

Introduction

Since Sakaguchi and colleagues identified a subpopulation of $CD4^+$ T cells constitutively expressing the IL-2 receptor α-chain (CD25) in 1995 (1), $CD4^+CD25^+$ regulatory T cells have been a main theme of immunological studies over the past few years. Due to the arduous efforts of many researchers to unravel the underlying mechanisms of development and function of Tregs, there have been tremendous advances in our knowledge about biology of the Tregs (2). In this review, we will briefly discuss the current understanding of developmental characteristics of Tregs, especially in the context of peripheral generation of Tregs.

Role of $CD4^+CD25^+$ regulatory T cells for maintaining the peripheral tolerance

One of the major roles of the immune system is to distinguish between self and non-self (2). In the thymus, potentially self-reactive T cells are almost completely deleted and thus T cells that egress to the periphery are mostly self-tolerant (2,3). This highly sophisticated process to maintain self-tolerance is so-called central tolerance. However, because not all peripheral tissue antigens are expressed in the thymus and thymic selection of self-reactive T cells is not perfect, some self-reactive repertoires of T cells are still present in the periphery (2). Therefore, to prevent the possible deleterious effects of remaining peripheral self-reactive T cells, another tolerance mechanism that can effectively control self-reactivity is also required. Fortunately, our immune system has evolved to develop another means of control, namely peripheral tolerance (2,4-7).

Peripheral tolerance consists of several mechanisms including deletion (4,5), anergy (6,7) and immunological ignorance (8). In addition to these passive mechanisms, an active process mediated by cells with regulatory activity is also well-known (9,10). So far, a number of T cell populations with regulatory function have been reported (1,11-13) and among them $CD4^+CD25^+$ regulatory T cells have been most intensively studied (14,15). Although the existence of suppressor T cells was first described by Sakakura and colleagues more than 30 years ago (16), it was not until Sakaguchi and colleagues identified $CD4^+CD25^+$ regulatory T cells that regulatory T cells made their way into the main stream of immunological research (1).

$CD4^+CD25^+$ regulatory T cells comprise 5～10% of peripheral $CD4^+$ T cells in naïve mice and 1～3% of peripheral $CD4^+$ T cells in normal humans (17). $CD4^+CD25^+$ regulatory T cells constitutively express several cell surface markers such as CD25, CTLA-4 and GITR (17). These cell surface molecules, however, can also be up-regulated by activated T cells

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so it is impossible to discriminate between regulatory T cells and activated T cells using these molecules (2,17). However, the advent of transcription factor Foxp3, which is the most Treg-specific marker at present and does not induced to express in activated T cells at least in mice, makes it possible to distinguish $CD4^+CD25^+$ regulatory T cells (which are $Foxp3^+$) from activated $CD4^+CD25^+$ T cells (which are $Foxp3^-$) (18,19). In general, Tregs are anergic to T cell receptor (TCR) stimulation in vitro and upon TCR stimulation they can suppress the proliferation of $CD4^+$ and $CD8^+$ T cell (20). Although Tregs were originally thought to primarily control autoimmune diseases mediated by self-reactive immune cells, recent studies suggest that they also regulate other immune responses to non-self antigens such as microbial antigens (21), alloantigens (22) and tumor antigens (13).

Thymic development of $CD4^+CD25^+$ regulatory T cells

Several findings indicated that $CD4^+CD25^+$ regulatory T cells as well as other conventional T cells can develop in the thymus (23,24). As thymic development of Tregs is not the main focus of this review, we will briefly summarize the evidence that demonstrates the critical role of the thymus in the generation of Tregs.

In the mid 1990s, Sakaguchi and colleagues reported that neonatal thymectomy 3 days after birth in mice can cause spontaneous organ-specific autoimmunity which correlates with reduced numbers of naturally occurring $CD4^+CD25^+$ regulatory T cells (nTregs) in the peripheral lymphoid pool (25). On the other hand, mice thymectomized prior to day 3 showed a marginal autoimmune phenotype most likely due to the decreased development of self-reactive T cells. Furthermore, mice thymectomized even later (day 7 and day 14) also manifested an alleviated autoimmune phenotype presumably because at these time points a sufficient number of functional nTregs have already emerged to the periphery. Collectively, these results suggest that functional $CD4^+CD25^+$ regulatory T cells begin to develop in the thymus about 3 days after birth and egress to the periphery repeatedly until complete thymic involution occurs.

In line with this speculation, Sakaguchi and colleagues also reported that $CD4^+CD8^-$ thymocytes depleted of $CD25^+$ cells induce autoimmune diseases when transferred into athymic nu/nu mice (26). In addition, a number of studies demonstrate that both human and mouse $CD4^+CD8^-CD25^+$ thymocytes are phenotypically and functionally similar to peripheral $CD4^+CD25^+$ regulatory T cells in that they express Foxp3 as well as Treg-specific cell surface molecules and suppress both mitogen- and antigen-induced T cell proliferation in vitro (19,27,28).

Therefore, it seems likely that the thymus plays a critical role not only in the development of conventional T cells but also in the development of naturally occurring $CD4^+CD25^+$ regulatory T cells.

Evidence for peripheral generation of $CD4^+CD25^+$ regulatory T cells

Although $CD4^+CD25^+$ regulatory T cells have been proved to develop in the thymus (24,29), accumulating evidence, mostly obtained from animal in vivo studies, suggest that these cells may also develop in the periphery (30-46). Most of them took advantage of either the precursor T cell adoptive transfer system (35-38,40-44) or the Ab-mediated in vivo depletion of $CD4^+CD25^+$ regulatory T cells following thymectomy (39,45) or both (46). Furthermore, several in vitro studies demonstrating the de novo generation of $CD4^+CD25^+$ regulatory T cells using various experimental protocols strengthen the relevance of the in vivo studies and explain the possible mechanisms of peripheral generation of Tregs (18,47-58).

Soluble foreign antigen-induced Tregs. To our knowledge, Thorstenson and Khoruts reported indisputable evidence for the peripheral de novo generation of $CD4^+CD25^+$ regulatory T cells for the first time (35). They adoptively transferred ovalbumin (OVA)-specific $CD4^+CD25^-$ T cells from DO11.10-$RAG2^{-/-}$ mice into syngenic BALB/c mice and induced peripheral tolerance with soluble intravenous or oral OVA. Within 1 week after Ag exposure, significant numbers of OVA-specific $CD4^+CD25^+$ T cells with immunoregulatory properties emerged in the periphery regardless of antigen delivery routes, although the researchers didn't check the Foxp3 expression level of these induced-Tregs (iTregs). As TCR-transgenic mice on a $RAG^{-/-}$ background actually have no $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells due to the lack of endogenous TCR (26) it was not the result of expansion of contaminant $CD4^+CD25^+$ regulatory T cells but the consequence of de novo generation of $CD4^+CD25^+$ regulatory T cells from $CD4^+CD25^-$ precursors. Lafaille and colleagues reached the same conclusion using a similar but a little different system (59). They used T/B monoclonal mice which have monoclonal na ve T (OVA-specific) and B (Influenza HA-specific) cells and are devoid of Tregs. Oral administration of soluble cognate antigen into T/B monoclonal mice induced antigen specific $CD4^+CD25^+$ $Foxp3^+$ T cells with anergic and suppressive properties.

Boehmer and colleagues also generated antigen-specific Tregs in the periphery with noble techniques (36,38). In an earlier study, they described that prolonged subcutaneous infusion of low dose antigenic peptides by means of osmotic pumps generated antigen-specific $CD4^+CD25^+$ regulatory T cells from na ve $CD4^+CD25^-$ precursors. Moreover, in a more recent study, they also generated antigen-specific $CD4^+CD25^+$ regulatory T cells in the periphery by targeting antigens to the immature dendritic cells.

Alloantigen-induced Tregs. Antigen-specific Tregs against alloantigen can also be induced in the periphery (45). According to the report by Wood and colleagues, alloantigen-specific $CD4^+CD25^+$ regulatory T cells can develop in vivo from $CD4^+CD25^-$ precursors in a thymus independent process (45). They intravenously administered alloantigen into mice that had previously been thymectomized and depleted of $CD4^+CD25^+$ regulatory T cells, and then confirmed the emergence of $CD4^+CD25^+$ T cells that have the ability to suppress skin allograft rejection mediated by $CD4^+CD45RB^{high}$ effector T cells.

Tumor microenvironment-induced Tregs. Performing thymectomy and anti-CD25 depleting Ab treatment followed by tumor inoculation, Colombo and colleagues have shown that a tumor microenvironment can induce conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells (46). They also conducted the experiment adoptively transferring precursor $CD4^+CD25^-$ T cells into tumor bearing mice and came to the same conclusion. In addition, a recent study by Levitsky and colleagues demonstrated that the tumor microenvironment is necessary but not sufficient to generate Tregs and nominal tumor antigen encounter is also essential in inducing tumor antigen-specific Tregs (44).

Peripheral generation of Tregs under homeostatic proliferation and steady state. In addition to antigen-induced peripheral generation of Tregs, lymphopenia induced homeostatic proliferation of polyclonal $CD4^+CD25^-$ T cells can also lead to the generation of polyclonal $CD4^+CD25^+$ regulatory T cells. Indeed, there are more than five reports that have shown the peripheral generation of polyclonal $CD4^+CD25^+$ regulatory T cells under lymphopenic conditions (37,39,41-43). Most of them adoptively transferred polyclonal $CD4^+CD25^-$ T cells into lymphopenic mice such as $CD3\varepsilon^{-/-}$, $RAG^{-/-}$ and T/B monoclonal mice (37,42,43), whereas one of them used sublethally irradiated mice as a lymphopenic host (41). In these studies, the $CD4^+CD25^+$ regulatory T cells generated through homeostatic proliferation of $CD4^+CD25^-$ T cells are almost completely identical to naturally occurring $CD4^+CD25^+$ regulatory T cells in their phenotypic and functional characteristics. However, it is noteworthy that these studies used polyclonal $CD4^+CD25^-$ T cells derived from $RAG^{+/+}$ mice as adoptively transferred precursor cells. Although some of the studies proved that peripheral $CD4^+CD25^+$ regulatory T cells are induced by homeostatic proliferation of $CD4^+CD25^-$ T cells rather than by expansion of pre-existing $CD4^+CD25^+$ contaminant using splendid numerical methods (37,41), it seems unlikely that it was the sole result of de novo generation of $CD4^+CD25^+Foxp3^+$ Tregs from $CD4^+CD25^-Foxp3^-$ T cells as these polyclonal $CD4^+CD25^-$ precursors also contain few $CD4^+CD25^-Foxp3^+$ T cells which is reported to constitute a reservoir of committed Tregs (42).

One of the studies also reported that conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells can occur spontaneously under natural conditions in a thymus-independent manner (41). These Tregs converted in steady state may be induced by natural endogenous antigen and also exhibit characteristics of the nTregs.

Experimental generation of Tregs in vitro. It has been shown that in vitro treatment of TGF-β can generate $CD4^+CD25^+$ regulatory T cells from $CD4^+CD25^-$ T cells in the presence of TCR stimulation in both mouse and human systems (47,49,50,55,56, 58). Furthermore, cytokines other than TGF-β, such as IL-4 and IFN-γ, can also induce the de novo generation of Tregs (52,54). It is important to note that activated human $CD4^+CD25^-$ T cells can develop into $CD4^+CD25^+Foxp3^+$ regulatory T cells even in the absence of exogenous regulatory cytokines (48,53), although stable Foxp3 expression and suppressive activity of these cells have some discrepancies between experimental systems (48,53, 60,61).

Factors involved in the peripheral generation of $CD4^+CD25^+$ regulatory T cells

At present, it is unclear which factors are involved in the peripheral generation of Tregs. Considering the combined outcomes from several in vitro and in vivo studies, there may be multiple conditions that can induce peripheral generation of Tregs (33,34). We will discuss in detail about the factors involved in the peripheral development of Tregs.

TGF-β. As mentioned earlier, several lines of evidence suggests that TGF-β is important in generating $CD4^+CD25^+$ regulatory T cells from $CD4^+CD25^-$ T cells in the periphery (33,38,47,49, 51,56-59). However, it is unlikely that TGF-β is indispensable for the peripheral generation of Tregs. When Lafaille and colleagues investigated the role of

TGF-β in the peripheral generation of $CD4^+CD25^+$ regulatory T cells using oral tolerance model, TGF-β neutralization has a marginal effect on the generation of $CD4^+CD25^+$ regulatory T cells although early Foxp3 expression levels of MLN $CD4^+$ T cells are somewhat decreased (59). Moreover, several in vitro studies demonstrated that $CD4^+CD25^-$ T cells can develop into $CD4^+CD25^+$ regulatory T cells in the absence of exogenous TGF-β (48,52-54) and most of them ruled out the possibility of endogenous TGF-β involvement as well (52-54). Therefore, it appears that TGF-β is critical but not necessary for the peripheral generation of Tregs.

IL-4/IL-13 (IL-4 receptor α-chain (IL-4Rα) cytokine). Th2 cytokines such as IL-4 and IL-13 have been related to amelioration of several autoimmune diseases but precise mechanisms of action remains elusive (62-64). A recent study by Skapenko and colleagues proposed induction of Tregs as one of the mechanisms of autoimmune regulation mediated by IL-4 and IL-13 (52). They cocultured human PBMC $CD4^+CD25^-$ T cells with irradiated autologous APC in the presence of IL-4 and confirmed the development of $CD4^+CD25^+$ regulatory T cells. In contrast to other Th2-type cytokines including IL-5 and IL-9, IL-13 also have the ability to induce $CD4^+CD25^+$ regulatory T cells. In that experimental system, neutralization of endogenous IL-10 or TGF-β has little effect on the Treg generation. Because IL-4Rα is a high affinity receptor for IL-4 and also involved in IL-13 signaling, it seems likely that signaling through the IL-4Rα may play a critical role in the generation of Tregs in this condition.

IFN-γ. IFN-γ is an important Th1 proinflammatory cytokine but also has a paradoxical regulatory effect on Th1-mediated autoimmune disease in that EAE is unexpectedly exacerbated in IFN-γ-deficient mice (65-67). A recent study by Zhang and colleagues suggests one explanation for this enigma by showing that IFN-γ is critical for the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells (54). In this study, IFN-γ was sufficient to convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells in the absence of TCR stimulation although

Table I. Evidence for peripheral generation of $CD4^+CD25^+$ regulatory T cells (mouse *in vivo* studies)

Driving factor for Treg generation	Precursor T cell source	Presence of $CD4^+CD25^-Foxp3^+$ T cells within precursor T cells	TCR stimulator	Foxp3 expression	References
IV or oral soluble antigen	OVA-specific $CD4^+CD25^-$ $Foxp3^-$ T cells	No	Foreign antigen	ND	35
Oral soluble antigen	OVA-specific $CD4^+CD25^-$ $Foxp3^-$ T cells	No	Foreign antigen	mRNA	59
Prolonged release of low dose antigen	HA-specific $CD4^+CD25^-$ $Foxp3^-$ T cells	No	Foreign antigen	mRNA	36
Targeting antigen to immature DC	HA-specific $CD4^+CD25^-$ $Foxp3^-$ T cells	No	Foreign antigen	Protein	38
Alloantigen	Alloantigen specific $CD4^+$ $CD25^-$ T cells	Yes	Alloantigen	ND	45
Tumor microenvironment	Tumor antigen-specific $CD4^+CD25^-$ T cells	Yes	Tumor antigen	Protein	44, 46
Endogenous systemic antigen	OVA-specific $CD4^+CD25^-$ $Foxp3^-$ T cells	No	Endogenous-like antigen	mRNA, protein	40
Partial lymphopenia	Polyclonal $CD4^+$ $CD25^-$ T cells	Yes	Endogenous antigen	ND	39
Complete lymphopenia	Polyclonal $CD4^+CD25^-$ T cells ($CD45RB^{high}$ or $CD45RB^{low}$)	Yes	Endogenou antigen	mRNA, protein	37, 41-43
Natural condition	Polyclonal $CD4^+CD25^-$ T cells	Yes	Endogenous antigen	mRNA	41

Treg: $CD4^+CD25^+$ regulatory T cell, Foxp3: Forkhead box P3, TCR: T cell receptor, IV: Intravenous, OVA: Ovalbumin, HA: Hemagglutinin, ND: Not determined, DC: Dendritic cell.

Table II. Experimental generation of $CD4^+CD25^+$ regulatory T cells *in vitro*

Treg-inducer	Precursor T cell source	Presence of $CD4^+CD25^-Foxp3^+$ T cells within precursor T cells	TCR stimulator	Foxp3 expression (reference)	References
Mouse in vitro studies					
TGF-β	Polyclonal $CD4^+CD25^-$ T cells	Yes	aCD3 and aCD28, aCD3 and irradiated APC	mRNA	47, 49
TGF-β	Polyclonal $CD4^+CD25^-$ T cells ($CD62L^+$ or $CD62L^-$)	Yes	aCD3 and aCD28, aCD3 and irradiated APC	Protein	55, 58
TGF-β	5CC7-transgenic $CD4^+CD25^-Foxp3^-$ T cells	No	aCD3 and/or aCD28	Protein	56
IFN-γ	Polyclonal $CD4^+CD25^-$ T cells	Yes	Not required*	Protein	54
Tumor-derived TGF-β	Polyclonal $CD4^+CD25^-$ T cells	Yes	aCD3 and irradiated APC	mRNA, protein	57
Human in vitro studies					
TCR stimulation	Polyclonal $CD4^+CD25^-$ T cells	Yes	aCD3 and/or aCD28	mRNA, protein	48, 53
TGF-β	Polyclonal $CD4^+CD25^-$ CD45RA+ T cells	Yes	aCD3 and aCD28, aCD3 and irradiated APC	mRNA	49
IFN-γ	Polyclonal $CD4^+CD25^-$ T cells	Yes	Not required*	Protein	54
IL-4/IL-13	Polyclonal $CD4^+CD25^-$ CD45RA+ T cells	Yes	Irradiated autologous APC	mRNA	52

Treg: $CD4^+CD25^+$ regulatory T cell, Foxp3: Forkhead box P3, TCR: T cell receptor, APC: Antigen-presenting cell. *The rate of conversion was enhanced by the addition of anti-CD3 Ab.

Foxp3 expression level and suppressive activity of converted Tregs was inferior to those of naturally occurring Tregs. And the rate of conversion induced by IFN-γ was enhanced by TCR stimulation. These findings indicated that IFN-γ may contribute to the peripheral generation of Tregs in some conditions such as inflammation.

IL-2. Because $CD4^+CD25^+$ regulatory T cells constitutively express high affinity IL-2 receptor CD25, many researchers have investigated the role of IL-2 in the development and function of Tregs (40,68-71). According to the recent reports, IL-2 is not required for the thymic development of $CD4^+CD25^+$ regulatory T cells but plays a critical role in the peripheral maintenance and function of Tregs (68-71). However, Abbas and colleagues announced that IL-2 is required for the peripheral generation of Tregs (40). Furthermore, two recent reports extended the conclusion deduced by Abbas and colleagues to the point that IL-2 has a non-redundant role for TGF-β ~mediated induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells and also enables induced-Tregs to expand (56,58). The essential role of IL-2 in the generation and expansion of iTregs cannot be compensated by other common γ-chain (γc) cytokines such as IL-4, IL-7 and IL-15 (56,58).

TCR stimulation and co-stimulatory molecules. Most of the above mentioned studies indicated that TCR stimulation is required for the peripheral generation of Tregs, except when IFN-γ was sufficient for the de novo generation of Tregs without TCR stimulation (54). TCR stimulators, in this context, include mitogen (47-50,53,55,56,58), soluble foreign antigen (35,36,38,59), autologous antigen (37,41-43,52), alloantigen (45,51), tumor antigen (44,46,57) and so on.

Whereas CD28/B7 co-stimulation but not CTLA4/B7 co-stimulation is essential for the generation of

nTregs (23), whether B7-mediated co- stimulation is required for the peripheral generation of Tregs remains unclear. There have been a few studies exploring the role of B7 co-stimulation in the de novo generation of $CD4^+CD25^+$ regulatory T cells (41,52,55). For example, Kosiewicz and colleagues reported that B7 co-stimulation is required for the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells in steady state (41). Schulze-Koops and colleagues have also shown that IL-4-induced generation of $CD4^+CD25^+$ regulatory T cells in vitro is dependent upon B7 co-stimulation (52). Moreover, a recent study by Horwitz and colleagues described that CTLA-4 ligation of CD80 (B7-1) is required for TGF-β-mediated generation of Tregs and CD28/B7 co-stimulation, which is critical for the development of nTregs, is dispensable for generating TGF-β-induced Tregs (55). Distinct developmental requirements for B7 co-stimulation of nTregs and iTregs may have implications for defining the intrinsic differences between the two cell populations.

Thymus. As mentioned earlier, the thymus is a site of nTreg development and a few mature peripheral T cells can re-circulate into the thymus (72). So, there is a possibility that conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells occurs in the thymus. However, several studies have shown that thymectomized mice can efficiently generate Tregs in the periphery (36,39,41,45,46). Therefore, it looks like peripheral generation of Tregs is independent of the thymus.

Relative contribution of nTregs and iTregs to the total peripheral Treg pool

There is much debate about whether induced-Tregs substantially contribute to the total peripheral Treg pool. It has been suggested that a small number of nTregs may maintain peripheral tolerance by increasing total Treg number through converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Tregs (51,73). This phenomenon is so-called infectious tolerance. However, recent reports using Foxp3 knock-in mice contradicted this possibility by showing that contribution of iTregs to the peripheral Treg pool is little, if any, during either immune response (29) or lymphopenia-induced proliferation (74). And a recent report by Freitas and colleagues consolidated this view using lymphopenia model (43).

Nevertheless, considering the differences in life span between mice and humans, iTregs may play a pivotal role for maintaining total peripheral Treg pool as the thymus begin to involute at least in humans (34). In line with this perspective, Akbar and colleagues proved that human $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells are derived by rapid turnover of memory $CD4^+CD25^-$ T cells especially in older subjects (75). Therefore, although nTregs make up a major part of the peripheral Treg pool, iTregs may also substantially contribute to the peripheral Treg pool throughout the life time.

Conclusion

Due to their potent suppressive activity and convenience of manipulation, $CD4^+CD25^+$ regulatory T cells have been proved useful for the treatment of various diseases such as autoimmune disease (76,77), graft rejection (22) and allergy (78-80). However, it is difficult to obtain a sufficient number of $CD4^+CD25^+$ regulatory T cells from human blood as $CD4^+CD25^+$ regulatory T cell is a minor population in human PBMC (53). Therefore, generation and expansion of $CD4^+CD25^+$ regulatory T cells from relatively abundant $CD4^+CD25^-$ T cells, using above-mentioned Treg-inducing stimulus, would be an alternative candidate for clinical application. To this end, it is important to establish standard methods for efficient generation and expansion of $CD4^+CD25^+$ regulatory T cells from $CD4^+CD25^-$ T cells in vitro.

There has been remarkable progress in the peripherally induced $CD4^+CD25^+Foxp3^+$ regulatory T cell research field, but complexity has also increased as different researchers used different experimental systems. So, it is necessary to uncover the intrinsic differences between the systems and form a more precise definition of induced-Treg to resolve this problem. Furthermore, the physiological role of peripherally generated $CD4^+CD25^+Foxp3^+$ regulatory T cells for maintaining peripheral tolerance would be an important open question in the future.

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