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Regulatory T Cells in Inflammation

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Preface

Scientific interest in regulatory T cells has revived during the last decade. Initially described in the early seventies as suppressor T cells, the concept of suppressor/regulatory T cells went through turbulent times during the eighties when molecular analysis failed to identify putative suppressor genes. The constructive and elegant cellular experiments on regulatory T cells during the nineties, initiated by Shimon Sakaguchi and co-workers, however has brought these cells back into the limelight. Nowadays, regulatory T cells are regarded as essential components of the immune system, and several different subsets of regulatory T cells have been described. An important regulatory role has been attributed to the $CD4^+CD25^+$ T cells. They act by suppressing immune reactivity thereby maintaining or restoring the balance between immunity and tolerance. The aim of this book is to bring together recent developments and viewpoints in the field of $CD4^+CD25^+$ regulatory T cells and to discuss the potential use of regulatory T cells as target for immunotherapy of inflammatory diseases.

By linking data on regulatory T cells from experimental models with recent findings from the clinic, this topical book will be of interest to immunologists and other biomedical researchers as well as clinicians that are interested in regulation and manipulation of the immune response during (chronic) inflammatory disease.

October 2004

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Part I
Origin, function and distribution of regulatory T cells

History of $CD25^+CD4^+$ regulatory T cells

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Introduction

It is still a key issue in immunology to understand how the immune system discriminates between self and non-self, inhibiting autoimmune responses while allowing effective immune responses to non-self antigens. In addition to physical elimination of self-reactive T cells (clonal deletion) and their functional inactivation (clonal anergy), there is now substantial evidence that T-cell-mediated suppression of self-reactive T cells is also a key mechanism of immunologic self-tolerance [1–3]. Although the idea of T cells that negatively control immune responses is not a new one, for a long time it was controversial whether they actually constituted a definite cellular entity in the immune system [4]. In recent years, however, we have witnessed resurgent interest in suppressor or regulatory T (T_R) cells in many fields of basic and clinical immunology [5, 6]. This change is partly due to our new understanding that the normal immune system endogenously produces as its normal cellular constituent a T-cell subpopulation which is highly specified for suppressive function; abnormality of this population in number or function can indeed be a cause of immunological diseases – in particular autoimmune disease – and this naturally occurring T_R cell population can be exploited for induction of immunological tolerance (e.g., transplantation tolerance), negative control of pathological immune responses (e.g., allergy), and enhancement of host defense (e.g. tumor immunity and microbial immunity) [5]. In addition to these endogenous T_R cells, there are other types of T_R cell that can be induced by specific methods of antigenic stimulation *in vivo* or *in vivo* [7, 8]. In this chapter, we shall provide a brief historical sketch of how endogenous T_R cells were recognized and characterized, and discuss their roles in immunologic tolerance and immunoregulation.

Regulatory T Cells in Inflammation, edited by Leonie S. Taams, Arne N. Akbar and Marca H.M. Wauben

A historical note on suppressor or regulatory T cells

In 1970, Gershon and Kondo [9] made the seminal finding that T cells not only enhance but also dampen immune responses and that this down-regulation is mediated by T cells that are different from helper T cells. This T-cell population, called suppressor T cells, was intensively studied over the following years in various fields of immunology. The studies showed several types of suppressor T cell interacting in a cascade; some were antigen-specific and others were non-specific; some secreted antigen-specific suppressive factors and others non-specific ones. The phenotype of suppressor T cells was on the most part shown to be Lyt-1$^-$ Lyt-2, 3$^+$, corresponding to CD8$^+$, and they expressed the I-J molecule, which was supposed to be a key suppressor molecule intimately associated with their suppressive function [10]. The research, however, quite abruptly collapsed in the mid-1980s when scrutiny of the mouse MHC gene by molecular biology techniques showed no existence of the I-J region, which was assumed to encode the I-J molecule and locate within the MHC gene complex [11]. With this bewildering I-J episode as a turning point, immunologists' interest in suppressor T cells rapidly waned [4]. There are several reasons for this decline: e.g. failure in finding reliable markers for distinguishing suppressor T cells from other T cells, ambiguity in the molecular basis of suppression, and difficulty in preparing antigen-specific suppressor T-cell clones amenable to fine cellular and molecular analyses. In addition, approaches to immunologic tolerance with reliable molecular tools, such as T-cell receptor (TCR)-specific monoclonal antibodies and transgenic mice, in the late 1980s to the early 1990s unequivocally demonstrated clonal deletion, and also anergy, as key mechanisms of immunologic tolerance [12–14]. Molecular characterization of cytokines clearly revealed pleiotropism, cross-regulation, and redundancy in their function [15]. Collectively these findings generated a climate in which suppressor T cells played little meaningful part in immunologic tolerance. There were even doubts about suppressor T cells as a distinct cellular entity when suppressive phenomena were explicable by T cells secreting particular cytokines. Indeed, interleukin (IL)-10-secreting T cells produced *in vitro* by antigenic stimulation of T cells in the presence of IL-10- or transforming growth factor (TGF)-β-secreting T cells propagated from animals in oral tolerance did not encounter much resistance to being accepted by immunologists in the 1990s [7, 8].

In parallel with the suppressor T-cell research depicted above, there has been a different stream of study on T-cell-mediated suppression. A notable feature of the latter is that it examined from the beginning how the manipulation of the T-cell immune system breaks natural immunologic self-tolerance and causes autoimmune disease, rather than studying experimental immunologic tolerance induced by specific ways of antigen administration [1]. Nishizuka and Sakakura [16] showed in 1969 that neonatal thymectomy (NTx) of normal mice between day 2 and 4 after birth led to the destruction of ovaries, which was first supposed

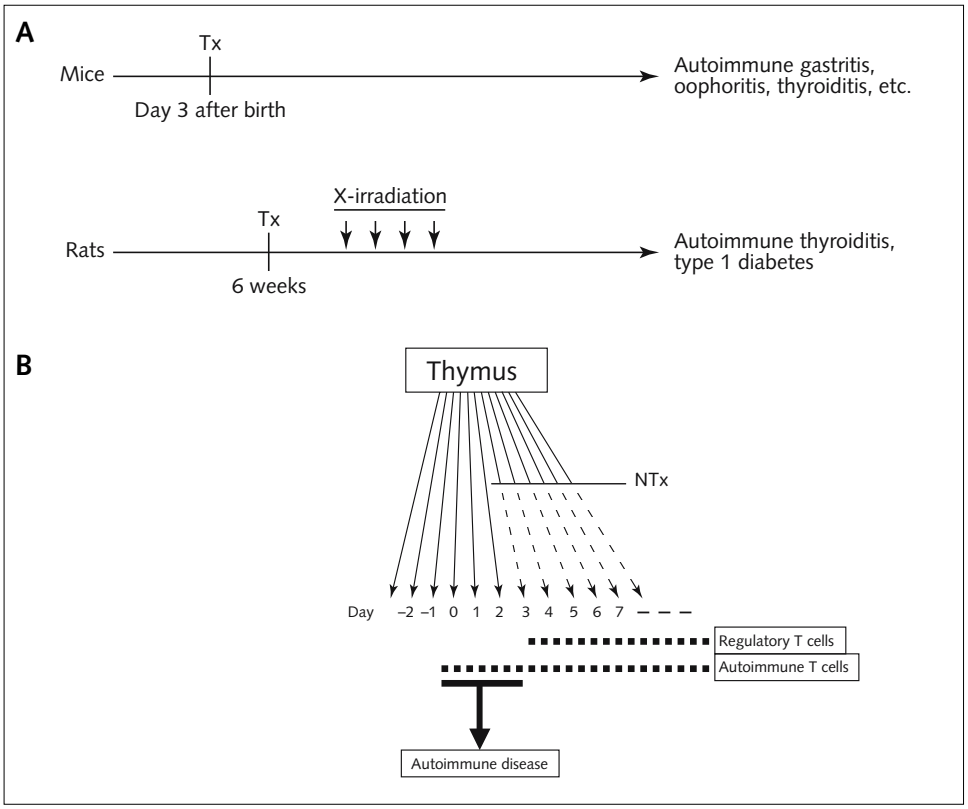

Figure 1
Induction of autoimmune disease by depleting natural T_R cells.
(A) Induction of autoimmune disease in animals by manipulating thymus/T cells. See text for details. Tx, thymectomy. (B). Ontogeny of autoimmune-preventive natural T_R cells. The normal thymus may start to release natural T_R cells around day 3 after birth. NTx on day 3, therefore, abrogates ontogenic development of natural T_R cells in the periphery, allowing self-reactive T cells that have migrated to the periphery before NTx to become activated and cause autoimmune disease.

to be due to deficiency of a certain ovary-tropic hormone secreted by the thymus, and hence was called "ovarian dysgenesis". This ovarian lesion later turned out to be of an autoimmune nature because subsequent investigation demonstrated that NTx also produced inflammatory tissue damage in other organs accompanying the appearance of tissue-specific autoantibodies in the circulation; e.g. thyroiditis, gastritis, orchitis, prostatitis, and sialadenitis of the salivary gland [17] (Fig. 1A). In 1973, Penhale et al. [18] reported that adult thymectomy (ATx) of

rats followed by four sublethal doses of X-irradiation (2–2.5 Gray) every 2 weeks resulted in the development of autoimmune thyroiditis accompanied by anti-thyroglobulin autoantibodies [18]. They and others later showed that the same protocol can produce type 1 diabetes in other strains of rats [19, 20] (Fig. 1A).

A common element between autoimmune disease induction by NTx and by ATx and X-irradiation is thymectomy. A simple interpretation of how these treatments cause autoimmune disease would be that the normal thymus is producing a population of T cells having autoimmune-preventive activity; NTx shortly after birth may abrogate developmentally determined thymic production of autoimmune-preventive T cells, allowing those self-reactive T cells that have been produced before NTx to become activated and cause autoimmune disease because of the paucity of regulatory T cells in the periphery (Fig. 1B); likewise, ATx and X-irradiations may abrogate the thymic supply of regulatory T cells and reduce regulatory T cells in the periphery presumably because they may be relatively radiosensitive (see below). Supporting this notion, inoculation of normal T cells, especially $CD4^+$ T cells, to the treated mice or rats indeed prevented the development of autoimmune diseases [21, 22]. On the other hand, as in other experimental autoimmune diseases, $CD4^+$ helper T cells mediate these autoimmune diseases as helper T cells for autoantibody formation and effectors of cell-mediated immune destruction of the target organs/tissues [23, 24]. These findings led to the idea that there may exist two types of $CD4^+$ T cell in the normal immune system: one potentially capable of mediating autoimmune disease, the other dominantly suppressing it [25] (Fig. 1B).

Thymus-produced natural $CD25^+CD4^+$ T_R cells

The phenotype and function of natural $CD4^+$ T_R cells

The key issue was then to know how these two populations can be dissected out or differentiated from each other if they co-exist in the normal immune system, and to determine whether specific removal of the autoimmune-preventive $CD4^+$ T-cell population can break self-tolerance and cause autoimmune disease in otherwise normal animals. Attempts were made to separate the $CD4^+$ T-cell population in normal naïve mice into an autoimmune-inducing and an autoimmune-preventive population by the expression levels of cell-surface molecules [25–31] (Fig. 2). Our experiments showed in 1985 that, when splenic cell suspensions from normal BALB/c mice were depleted of $CD5^{high}CD4^+$ T cells *ex vivo* (by *in vitro* treatment of the cell suspensions with a mixture of anti-CD8 and anti-CD5 antibodies and complement) and the remaining cells transferred to congenitally T-cell-deficient BALB/c athymic nude mice, the nude mice spontaneously developed autoimmune disease in multiple

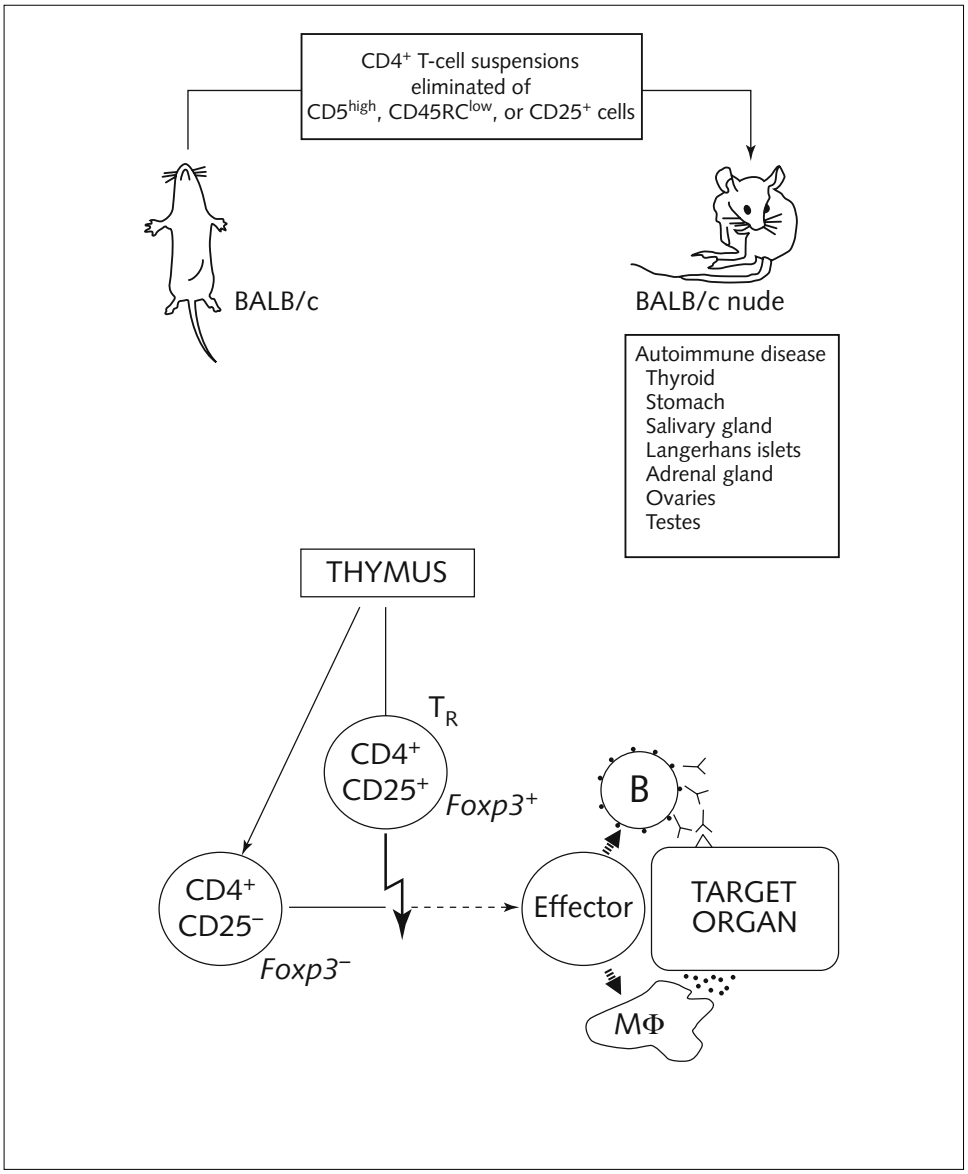

Figure 2

Induction of autoimmune disease by depleting Foxp3$^+$CD25$^+$CD4$^+$ T_R cells.

Thymus-derived natural CD25$^+$CD4$^+$ T_R cells, which specifically express Foxp3, control T cells that mediate autoimmune disease, IBD, and allergy. Genetic defects of Foxp3 cause these immunological diseases by impairing the development or function of CD25$^+$CD4$^+$ T_R cells. MΦ, macrophage.

organs (stomach, thyroid, ovaries, or testes) a few months after cell transfer [25]. Co-transfer of normal untreated $CD4^+$ T cells with $CD5^{low}CD4^+$ T cells inhibited the autoimmunity. Likewise, transfer of $CD5^{low}CD4^+$ T cells from normal C3H mice to T-cell-depleted C3H mice produced autoimmune thyroiditis [26]. Powrie and Mason [28] subsequently reconstituted PVG athymic nude rats with splenic T cells depleted of $CD45RC^{low}CD4^+$ T cells, showing that the transferred $CD45RC^{high}$ $CD4^+$ T cells elicited graft-*versus*-host-disease-like systemic disease and autoimmune tissue damage in multiple organs including thyroid and islets of Langerhans. McKeever et al. [29] similarly showed that transfer of spleen cell suspensions depleted of $RT6.1^+$ T cells was able to produce type 1 diabetes mellitus and thyroiditis in histocompatible athymic nude rats. Powrie et al. [32] and Morrisey et al. [33] then independently showed that transfer of BALB/c $CD45RB^{high}CD4^+$ T cells to T-B-cell-deficient BALB/c SCID mice induced inflammatory bowel disease (IBD). These findings prompted us to search for a cell-surface molecule which would be more specific than CD5 or CD45RB in defining such autoimmunity/inflammation-preventive $CD4^+$ T cells. In 1995, the CD25 molecule (the IL-2 receptor α-chain) was found as a candidate since $CD25^+$ T cells, which constitute 5–10% of peripheral $CD4^+$ T cells and less than 1% of peripheral $CD8^+$ T cells in normal naive mice, are contained in the $CD5^{high}$ and $CD45RB^{low}$ fraction of $CD4^+$ T cells [30, 31]. Transfer of BALB/c splenic cell suspensions depleted of $CD25^+CD4^+$ T cells indeed produced in BALB/c athymic nude mice histologically and serologically evident autoimmune diseases at higher incidences and in a wider spectrum of organs (including stomach, thyroid, ovaries, adrenal glands, and islets of Langerhans) than the transfer of $CD5^{low}$ or $CD45RB^{high}$ T cells prepared from the same number of splenic cell suspensions. Co-transfer of a small number of $CD25^+CD4^+$ T cells with $CD25^-$ T cells inhibited the autoimmunity. Furthermore, transfer of $CD25^-CD4^+$ T cells alone sufficed to mediate the autoimmune disease by giving rise to $CD4^+$ helper T cells for humoral and cell-mediated autoimmunity, although the presence of $CD25^-CD8^+$ T cells also enhanced the autoimmune induction, presumably as a source of self-reactive cytotoxic T lymphocytes. Importantly, these experiments showed that removal of $CD25^+CD4^+$ T cells not only elicited autoimmune disease but also enhanced immune responses to non-self antigens including soluble proteins and allografts [30].

Subsequent *in vitro* characterization of $CD25^+CD4^+$ T cells in normal mice confirmed the CD25 molecule as a highly specific marker for natural T_R cells: $CD25^+CD4^+$ T cells are hyporesponsive to TCR stimulation but, upon stimulation, potently suppress the activation and proliferation of other T cells *in vitro*; this property was confined to the $CD25^+$ fraction of normal $CD4^+$ T cells [34–36]. Their TCR repertoire is as broad as other T cells but appears to have higher affinity for self-peptide/MHC ligands selecting them in the thymus; i.e., they are more self-reactive than other T cells but capable of recognizing a broad spectrum of self and non-self antigens [34, 37, 38]. Unlike *in vitro*, $CD25^+CD4^+$ T_R cells in the *in vivo* physi-

ological state are more vigorously proliferating than CD25–CD4+ T cells, presumably by recognizing self-antigens [38, 39]. This may explain why CD25+CD4+ T_R cells are radiosensitive [18, 40].

Thymic production of natural CD25+CD4+ T_R cells

The above findings, such as the ability of NTx to cause autoimmune disease, requirement of ATx for autoimmune induction by radiation-induced autoimmune disease, and the activity of normal CD4+CD8– thymocytes to prevent autoimmune disease in NTx mice, all indicate that the normal thymus produces CD4+ regulatory T cells. As a direct demonstration of this, transfer of CD4+CD8– mature thymocyte suspensions depleted of CD25+ thymocytes produced various autoimmune diseases in syngeneic nude mice, as shown with the transfer of CD25–CD4+ spleen cells [41]. Thus, the normal thymus is continuously producing both pathogenic self-reactive CD4+ T cells and functionally mature CD25+CD4+ T_R cells, and CD25+CD4+ T_R cells in the thymus and the periphery may constitute a distinct cellular lineage (Fig. 2). Indeed, as discussed below, both CD25+CD4+CD8– thymocytes and CD25+CD4+ T cells express *Foxp3*, a T_R-cell-specific transcription factor, and *Foxp3* deficiency abrogates the development of both populations [42–44]. In addition, both populations are functionally and phenotypically similar; for example, both are naturally anergic to *in vitro* TCR stimulation, exhibiting an equivalent *in vitro* suppressive activity, and constitutively expressing CTLA-4 and a high level of GITR (glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein) [45–49].

Ontogeny of natural CD25+CD4+ T_R cells

Ontogeny of CD25+CD4+ T cells also correlates well with that of natural T_R cells. CD25+CD4+ T cells become detectable in the periphery of normal mice from around day 3 after birth, rapidly increasing to the adult level (i.e. 5–10% of CD4+ T cells) in 3 weeks [31]. NTx on day 3 substantially reduces peripheral CD25+CD4+ T cells; and the inoculation of CD25+CD4+ T cells from normal mice prevents the autoimmune development in NTx mice [31]. These results collectively indicate that the ontogenic time course of the thymic production and the peripheral migration of CD25+CD4+ T_R cells is developmentally determined (i.e. around day 3 after birth in mice); the abrogation of the thymic production of natural T_R cells from the very beginning of their ontogeny, therefore, results in their selective paucity in the periphery, leading to the activation of self-reactive T cells that have migrated to the periphery before NTx, producing autoimmune disease.

The role of IL-2 and CD25 for natural T_R cells

As discussed above, CD25 was found to be a good marker for operationally distinguishing endogenous T_R cells from other T cells in normal naïve animals. The following findings indicate that CD25 is also an indispensable molecule for the generation and maintenance of natural T_R cells. IL-2-deficient mice bear few $CD25^+CD4^+$ T cells, and spontaneously develop severe autoimmunity, although they develop a normal number of other T cells with a normal composition of CD4/CD8 subsets [50, 51]. CD25-deficient or CD122 (the IL-2 receptor β-chain)-deficient mice are also afflicted with similar autoimmunity [52–54]. Besides, *in vivo* neutralization of IL-2 by administration of anti-IL-2 monoclonal antibody substantially reduced $CD25^+CD4^+$ T cells, but not other T cells, in normal mice and consequently produced autoimmune disease [55], and R. Setoguchi et al., unpublished observations). Taken together, CD25 as a component of the high-affinity IL-2 receptor is a key functional molecule for natural $CD4^+$ T_R cells; IL-2 is a key growth/survival factor for them; and its expression, high-level expression in particular, is an excellent marker for natural $CD4^+$ T_R cells in mice and humans [36, 56].

Summary

To summarize, these findings on natural $CD25^+CD4^+$ T_R cells lead to the following notions (Fig. 2B). First, despite thymic negative selection, the normal immune system still harbors self-reactive T cells ($CD4^+$ T cells in particular) that are sufficiently pathogenic in TCR specificity and affinity to mediate various autoimmune diseases similar in immunopathology to their human counterparts, such as autoimmune gastritis/pernicious anemia, premature ovarian failure with autoimmune oophoritis, Hashimoto's thyroiditis, adrenalitis/Adison's disease, and insulitis/type 1 diabetes. Second, the activation/expansion of such self-reactive T cells is normally kept in check by a regulatory $CD4^+$ T-cell population, many if not all of which physiologically express CD25. Third, the normal thymus is continuously producing them, which is another key function of the thymus in self-tolerance, in addition to its role in positive and negative selection of T cells. Furthermore, elimination of this $CD25^+CD4^+$ regulatory population alone, without manipulating the target self-antigens, suffices to break natural self-tolerance and elicit chronic and destructive autoimmune diseases. The appearance of various disease-specific autoantibodies in the T_R-cell-depleted animals implies that the breakdown of this mode of T-cell self-tolerance and development of autoimmune $CD4^+$ helper T cells result in the breakdown of B-cell self-tolerance as well. Thus, one aspect of natural self-tolerance in T cells, and for that matter in B cells, is maintained by a regulatory subpopulation of $CD4^+$ T cells. Furthermore, such naturally present T_R cells also engage in the control of immune responses to non-self antigens as well.

IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) as evidence for the key role of CD25+CD4+ natural T_R cells in natural self-tolerance in humans

The finding that thymectomy during a critical neonatal period induces autoimmune disease suggests that the generation of CD4+ T_R cells may be developmentally controlled. Attempts were therefore made to produce autoimmune disease by genetically altering the normal developmental course of natural T_R cells [57]. An important clue to this developmental control of natural T_R cells came from the human disease called IPEX. IPEX was described in 1982 as an X-linked immunodeficiency syndrome associated with autoimmune disease in multiple endocrine organs (such as type 1 diabetes and thyroiditis), IBD, atopic dermatitis, and fatal infections [58]. The Scurfy strain of mice is an X-linked recessive mutant with lethality in hemizygous males within a month after birth, exhibiting hyperactivation of CD4+ T cells and overproduction of proinflammatory cytokines [59]. The gene defective in Scurfy mice was identified and designated *Foxp3*, which encodes Scurfin, a new member of the forkhead/winged-helix family of transcription factors [60]. Subsequently, mutations of the human gene *FOXP3*, the ortholog of murine *Foxp3*, were found to be the cause of IPEX [61–63].

Recent studies have revealed the specific role of Foxp3 in the development and function of natural CD25+CD4+ T_R cells [42–44]. CD25+CD4+ peripheral T cells and CD25+CD4+CD8− thymocytes predominantly expressed *Foxp3* mRNA, whereas other thymocytes/T cells and B cells did not. Importantly, activation of CD25−CD4+ T cells, Th1 cells or Th2 cells failed to induce *Foxp3* expression [42, 43]. Furthermore, retroviral transduction of Foxp3 to CD25−CD4+ T cells converted them into CD25+CD4+ T_R-like cells with respect to phenotype and function [42, 43]. For example, *Foxp3* transduction induced expression of CD25, CTLA-4, and GITR, which are closely associated with the functions of natural T_R cells [42]. *Foxp3*-transduced CD25−CD4+ T cells were able to suppress proliferation of other T cells *in vitro* and the development of autoimmune disease and IBD *in vivo* [42]. Furthermore, in bone-marrow (BM) chimeric mice with a mixture of BM cells from wild-type and *Foxp3*-deficient mice, F*oxp3*-deficient BM cells failed to give rise to CD25+CD4+ T cells, while *Foxp3*-intact BM cells generated them [43], indicating an essential role of Foxp3 for the development of CD25+CD4+ T_R cells.

Thus, *Foxp3*/*FOXP3* appears to be a master control gene for the development and function of natural CD25+CD4+ T_R cells (Fig. 2B). Given that humans bear natural CD25+CD4+ T_R cells with a common phenotype and function to those found in rodents [64], it is most likely in IPEX that disruption of the *FOXP3* gene abrogates the development of the T_R cells, leading to hyperactivation of T cells reactive with self-antigens, commensal bacteria in the intestine, or innocuous environmental substances, thus causing autoimmune polyendocrinopathy, IBD, and allergy, respec-

tively. This has several implications for self-tolerance and autoimmune/inflammatory disease in humans. First, thus far this is the clearest example that abnormality in naturally arising $CD25^+CD4^+$ T_R cells is a primary cause of human autoimmune disease and for that matter IBD and allergy. Second, the development of natural T_R cells is, at least in part, genetically and developmentally programmed, indicating that autoimmunity is in part a primary T-cell immunodeficiency. Third, females with hemizygous defects of the *FOXP3* gene illustrate that the mechanism of dominant self-tolerance is operating physiologically in humans. Because of random inactivation of the X chromosome (Lyonization) in individual T_R cells, some hemizygous females have a similar number of *FOXP3*-defective T_R cells and *FOXP3*-normal ones as a mosaic, but they are completely normal and do not show intermediate phenotypes [65]. This means that the normal T_R cells dominantly control self-reactive T cells in the presence of defective T_R cells. It also indicates that a partial reconstitution of IPEX patients with normal T_R cells (for example by BM transplantation) or *FOXP3*-transduced autologous T cells may suffice to control the disease dominantly.

Conclusion and perspective

A prominent feature of $CD25^+CD4^+$ T_R cells is that the majority, if not all, of them are endogenously produced by the normal thymus as a functionally distinct and mature subpopulation of T cells and persist in the periphery with stable function, and that their generation is, at least in part, developmentally controlled. Congenital deficiency of this population as in IPEX, therefore, results in serious impairment of self-tolerance and immunoregulation, leading to severe autoimmunity and allergy. Furthermore, as illustrated by IPEX, any genetic abnormality or environmental insult can be a cause or a predisposing factor of autoimmune disease if it would tip the balance between natural $CD25^+CD4^+$ T_R cells and self-reactive T cells towards the dominance of the latter [66]. On the other hand, their natural presence in the immune system as a phenotypically distinct population makes it a good target for designing ways to treat or prevent immunological diseases and to control pathological as well as physiological immune responses. In addition to this naturally arising "professional" T_R cell population there are several other types of T_R cell that can be induced from naïve T cells by antigenic stimulation under specialized conditions in the periphery. Although physiological roles for these inducible or "adaptive" T_R cells need to be fully established, they can still be exploited as a therapeutic tool. Furthermore, suppressive phenomena intensively studied in the 1970s and early 1980s can be re-interpreted from the vantage point of the present.

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