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Review

The Physiological Role of Regulatory T Cells in the Prevention of Autoimmunity: Generation, Specificity and Mode of Action

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Abstract. Until recently, the traditional view was that tolerance to self antigens was maintained by a combination of physical deletion, in the thymus, and functional deletion, in the periphery, of autoreactive T cells. There is now, however, abundant evidence that the normal T cell repertoire contains overtly autoreactive T cells whose pathogenic potential is held in check by the activity of a distinct subset of peripheral T cells, the so-called regulatory or suppressor T cells. This article examines data from one model of rodent autoimmunity, where autoimmune pathology develops following thymectomy and irradiation of normal laboratory rats, which characterize the development and function of these so-called regulatory T cells.

Key words: regulatory T cells; autoimmunity; prevention.

Introduction – Passive and Active Mechanisms of Self Tolerance

The immune system has evolved a number of potent mechanisms for the elimination of foreign pathogens. Given that these effector mechanisms are potentially damaging to the host, an essential feature of the adaptive immune system is its ability to distinguish self antigens from non-self and respond only to the latter. For the most part, the immune system successfully makes this distinction and remains self-tolerant. However, under certain circumstances and in genetically susceptible individuals, failures do occur and the resulting immunopathology can affect a wide variety of tissues, causing a number of different diseases. Diabetes, arthritis and multiple sclerosis are examples of diseases in which an autoimmune mechanism has been implicated in their pathogenesis.

With regard to T cell tolerance, the immune system has evolved a number of strategies for preventing autoreactive T cells from making potentially pathogenic responses against host tissues. The traditional view holds that T cell self tolerance is maintained by a combination of T cell deletion intrathymically and anergy or indifference extrathymically. During T cell development in the thymus, T cells bearing receptors which recognise complexes of major histocompatibility antigens and self peptides with a high affinity are induced to die[13]. In the periphery, foreign antigen is presented to specific T cells by antigen-presenting cells which can provide important co-stimulatory signals for T cell activation, while it is considered that self antigens are presented by cells lacking the ability to provide such signals[20, 22], with the result that potentially autoreactive T cells are either rendered anergic[20] or simply do not respond to antigen[23]. These are passive

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mechanisms, because they rely upon a functional absence of autoreactive cells, either through their physical deletion intrathymically or their functional inability peripherally. However, it is now clear that these mechansims do not completely account for immunological self tolerance and that other active mechanisms operate, called so because they rely on the regulatory action of T cells upon overtly autoreactive T cells which have neither been deleted nor are anergic. This article reviews data largely from one rodent model of autoimmunity which characterizes the function of these regulatory T cells (T_{reg}). A more detailed analysis of evidence for the existence of regulatory T cells in different models of autoimmunity can be found elsewhere[34].

Evidence for the Existence of T_{reg} in the Normal T Cell Repertoire

The fact that the normal T cell repertoire contains overtly autoreactive T cells which have not been deleted intrathymically or are anergic has been demonstrated in a variety of rat and mouse models. Rodents that are either congenitally lymphopoenic, such as the BB rat[4], or have been induced to be so by neonatal thymectomy[32] or adult thymectomy and irradiation[25] have been shown to spontaneously develop organ-specific autoimmunity in a strain-dependent manner. Were self tolerance maintained exclusively by a combination of physical and functional deletion of autoreactive cells, a reduction in the size of the T cell pool would not be expected to result in the overt autoimmunity observed in these cases.

In one of these models, normal laboratory rats, with no predisposition to development of spontaneous autoimmunity, could be induced to develop a range of organ-specific autoimmune pathologies when thymectomized between 3 and 6 weeks of age and given 4 doses of irradiation at two-week intervals starting two weeks after thymectomy (TxX protocol hereon)[24]. Shortly after the final irradiation, PVG-strain rats developed thyroiditis characterized by high titres of anti-thyroglobulin autoantibody and extensive infiltrates of the thyroid glands[24]. PVG.RT1^u rats, which share the same major histocompatibility complex (MHC) haplotype as the spontaneously diabetic BB rat[4], similarly thymectomised and irradiated spontaneously developed a fatal diabetes shortly after the final irradiation[7]. In a more extreme example, Lewis rats treated in the same way developed a fatal autoimmune pathology involving multiple organs so severe that many rats failed to survive the complete thymectomy and irradiation protocol[25].

Evidence that autoimmunity developed as a result of a failure of T_{reg} to control autoreactive T cells in these rats first came from the observation that disease development could be prevented in some TxX rats following adoptive transfer, shortly after the last irradiation, of splenocytes from normal syngeneic donors[26]. Later studies purified and tested the ability of different lymphocyte subsets to prevent autoimmune diabetes in TxX PVG. RT1^u rats and determined that only a specific subset of $CD4^+$ T lymphocytes was capable of preventing disease development upon adoptive transfer. The regulatory T cell population was found amongst $CD4^+$ $TCR\alpha\beta^+$ T cells that express the RT6 antigen but fail to express the CD45 isoform that utilises the C exon (CD45RC), and they were long-lived cells, since they were present in long-term thymectomised rats[7]. While adoptive transfer of 10^7 $CD4^+$ T cells could only prevent diabetes in 50% of TxX PVG. RT1^u recipients, all recipients of 5×10^6 $CD4^+TCR\alpha\beta^+CD45RC^-$ T cells were protected from disease development, whereas no recipients of $CD4^+TCR\alpha\beta^+CD45RC^+$ T cells were protected[7]. These data demonstrated that the normal T cell repertoire contains both overtly autoreactive T cells and a phenotypically distinct population of T_{reg} that could control their pathogenic activity.

The Role of the Thymus in the Generation of T_{reg}

The phenotype of the peripheral T cell subset that contains the T_{reg} ($CD4^+RT6^+CD45RC^-$) is also the phenotype of a resting antigen-experienced cell[28]. The implication of this result was that T_{reg} are generated in the periphery by clonal expansion of naive cells following encounter with a specific antigen. It was therefore significant to find that $CD4^+CD8^-$ thymocytes, a naive population, are capable of protecting a significant proportion of TxX recipients from development of autoimmunity. This protection was shown to be mediated by mature L-selectin$^+$ $CD4^+CD8^-$ thymocytes and not by a peripheral cell that had recirculated to the thymus[37]. Furthermore, the relative potency of $CD4^+CD8^-$ thymocytes in preventing development of autoimmunity is far greater than that of peripheral $CD4^+CD45RC^-$ cells. In thyroiditis development in TxX PVG rats[36] and diabetes in TxX PVG. RT1^u rats[33], thymocytes were shown in both cases to be effective at cell doses approximately a tenth of the number of peripheral T cells required. Significantly, this functional potency of $CD4^+CD8^-$ thymocytes appears to be restricted only to their ability

to prevent diabetes upon adoptive transfer. When assayed for their ability to provide primed B cells with help for antigen-specific secondary antibody responses, $CD4^+CD8^-$ thymocytes were relatively poor at providing such help in contrast to either naive or primed peripheral $CD4^+$ T cells[33].

Together, these data suggest that, rather than being generated by clonal expansion in the periphery, the cells responsible for protection of TxX rats from development of diabetes are already generated at a high frequency in the thymus, and it is for this reason that $CD4^+CD8^-$ thymocytes are such a potent source of T_{reg}. That the thymus is implicated in the generation of T_{reg} that prevent autoimmunity is also suggested by studies of transplantation tolerance in chickens that revealed a potent ability of xenogeneic thymic epithelium to induce tolerance to donor-type grafts. Chicken embryos grafted with quail thymic epithelium (TE) were found to be tolerant to quail limb-bud grafts[15]. Tolerance was not a consequence of clonal deletion on quail TE, since it was found even in chicks in which some of the thymic lobes were entirely of chick origin. The implication of these data is that the selection of T cells on foreign TE generates T_{reg} that maintain dominant tolerance to grafts of the donor genotype. It is therefore significant that expression of mRNA for many so-called tissue-specific antigens can be detected in the thymus, including insulin, thyroglobulin and myelin basic protein, all of which have been implicated as autoantigens[1, 10, 19]. Taken together, these data support the view that selection of the T cell repertoire on self peptides may be important in generating T_{reg}.

Development of T_{reg} Requires Relevent Extrathymic Self Antigen

While there is strong evidence that the thymus plays a vital role in generating T_{reg}, the observation that the T_{reg} in the periphery shares the phenotype of an antigen-experienced cell suggests that the development of functional T_{reg} from thymic precursors requires encounter with specific antigen which drives maturation. Evidence that extrathymic antigen is required for this process comes from studies of rats whose thyroid glands were ablated *in utero*.

In the first series of studies, athyroid rats were generated by exposure of rats *in utero* to radioactive ^{131}I at day 17–18 of gestation, just as the embryonic thyroids become able to concentrate iodine and, significantly, prior to population of the periphery with mature T cells. Syngeneic thyroid grafts in athyroid rats underwent rapid rejection with extensive leukocytic infiltration, whereas third-party endocrine organ grafts were unaffected[5]. Significantly, when athyroid rats were parabiosed with euthyroid partners, the athyroid rats accepted thyroid grafts[16], suggesting that euthyroid rats provided T_{reg}, lacking in the athyroid partner, that could prevent thyroid graft rejection.

A second study provides further evidence that athyroid rats specifically lack T_{reg} that prevent thyroid autoimmunity. When $CD4^+$ T cells were obtained from athyroid PVG rats, it was found that these cells, unlike those from euthyroid donors, were unable to prevent thyroiditis upon adoptive transfer into TxX recipient rats[35]. The deficit in T_{reg} activity is specific for thyroid antigens, since $CD4^+$ T cells from athyroid PVG.RT1^u donors retain the ability to prevent diabetes upon adoptive transfer into prediabetic TxX PVG.RT1^u rats. Significantly, $CD4^+$ single positive thymocytes from athyroid donors are still able to prevent disease upon adoptive transfer. This result implies that intrathymic development of T_{reg} precursors is normal in these rats and that the failure to develop specific T_{reg} in the periphery is due entirely to the absence of the specific peripheral autoantigen.

The precise role of peripheral autoantigen in generating T_{reg} is not clear from these studies. It is not possible from these data to determine whether athyroid rats fail to expand thyroid-specific T_{reg} to a sufficient frequency required for activity or whether these cells simply fail to survive in the absence of relevant autoantigen. The dependence on peripheral antigen for the development of T_{reg} may explain the finding that the balance between autoreactive and regulatory T cells is a very fine one[33], with the number of T_{reg} found in the periphery only just sufficient to prevent autoimmunity. The implication of this result is that generation of T_{reg} in the periphery is under homeostatic control, such that only as many T_{reg} are generated as are required following encounter with relevant peripheral self antigen. This could also explain the relative differences in the potency of thymocytes and peripheral T cells in preventing autoimmunity. Thymocytes contain a high frequency of T_{reg} precursors which, under conditions of T_{reg} depravition found in lymphopoenic TxX rats, can all be recruited to the T_{reg} pool, whereas peripheral T cells only contain a fixed number of terminally differentiated T_{reg}, with no further recruitment possible. This difference in the plasticity of the two populations could explain the differences in their potency.

It is not known what might regulate this homeostasis; transient activation of autoreactive T cells may drive maturation of T_{reg}, and it is also possible that T_{reg}

themselves regulate their own development by inhibiting further T_{reg} generation when they are at a sufficiently high frequency. The advantage of such mechanisms is that they ensure that the immune system is not overly populated by T_{reg}, which could interfere with the ability of the immune system to mount a normal response to a foreign pathogen.

Mode of Action of T_{reg}

Data that contribute to an understanding of how T_{reg} function in this system comes from both *in vitro* and *in vivo* studies. Studies of subsets of $CD4^+$ T cells as defined by their expression of CD45RC have provided some clues. $CD4^+CD45RC^+$ T cells bear some of the characteristics of Th1-like cells, since they secrete high levels of IFN-γ and IL-2 following activation *in vitro*[2, 17], but, more significantly, there is evidence that they contain cells with an autoreactive potential, since their adoptive transfer into nude recipients results in a wasting disease characterized by mononuclear infiltrates in many organs[29]. In contrast, $CD4^+CD45RC^-$ cells, which contain the T_{reg} population, are a potent source of IL-4 following activation *in vitro*[17] and their co-transfer with $CD4^+CD45RC^+$ cells into nude recipients prevents the induction of wasting disease by the latter population[29]. The most direct interpretation of these data are that Th2 cytokine-secreting T_{reg} counteract the activity of Th1-like autoreactive T cells. While, for many years, this had been an attractive model for the function of T_{reg} in several systems, data from more recent studies suggest this interpretation is at best an oversimplification.

As described earlier, TxX PVG.$RT1^u$ rats spontaneously developed insulin-dependent diabetes mellitus, while PVG rats subjected to the same protocol developed autoimmune thyroiditis. Diabetes in PVG.$RT1^u$ rats is characterized by a cell-mediated immunopathology, dependent on the cytolytic activity of $CD8^+$ T cells[7]. In contrast, the thyroiditis that develops in PVG rats occurs independently of $CD8^+$ T cells and is characterised by extensive plasma cell infiltration of the thyroids and high titres of anti-thyroglobulin antibodies of the Th2-associated IgG1 but little of the Th1-associated IgG2b isotype[36]. That autoimmune thyroiditis represents a Th2-mediated immunopathology is further supported by the observation that PVG rats immunized with homologous thyroglobulin in complete Freunds adjuvant, an adjuvant that strongly promotes Th1-responses, developed high titres of Th1-associated IgG isotype anti-thyroglobulin antibodies[36], but failed to develop thyroiditis[6]. Given the stark contrast in the pathogenesis of diabetes and thyroiditis in these TxX rats, it is highly significant that both diseases were prevented by their reconstitution with the same subset of T cells. In both instances, rats reconstituted with either $CD4^+CD45RC^-$ peripheral T cells or $CD4^+CD8^-$ thymocytes were protected from development of disease[33, 36]. These data do not, therefore, support the view that the mechanism of regulation involves a switch from a Th1- to Th2-like response, but rather depends on the specific suppression of autoreactive T cells.

Studies using neutralising antibodies against cytokines provide data implicating specific cytokines in the mechanism of regulation by T_{reg} in TxX rats. IL-4 and TGF-β both appear to be essential for the prevention of thyroiditis by thymocytes and peripheral cells, since neutralization of one or the other cytokine is sufficent to abrogate protection[36]. While these data indicate the requirement for these cytokines, no conclusion can be drawn with regard to where and by what cell type they are made or the target of their activity. However, the immuno-suppressive effect of TGF-β has been reported in a number of systems[9, 14, 18, 39, 40] and has been implicated in suppression of both cell-mediated responses, as in models of inflammatory bowel disease[27] and in humoral autoimmunity induced by mercuric chloride[3]. The role of IL-4 in T_{reg} function is less clear. The ability of IL-4 to modulate immune responses from pro-inflammatory Th1 to anti-inflammatory Th2 responses may explain its protective effects in some autoimmune systems[21, 30]. However, data from other studies implicate a role for IL-4 in the generation of T_{reg}, since IL-4 has been shown to promote TGF-β production *in vitro*[38], while administration of IL-4 *in vivo* synergises with sub-optimal doses of orally administered antigen to induce oral tolerance, a process also shown to be TGF-β dependent[11, 12]. Taken together, these data suggest that IL-4 may have a role as a growth factor in the generation of T_{reg}, while the role of TGF-β is as an effector cytokine, effecting both T cells and modulating antigen-presenting cell function[31, 38].

A Model for T_{reg} Development and Function

While the data reviewed in this article still leave many questions unanswered, it is possible to propose a model of T_{reg} development and function (Fig. 1). In this model, selection of the T cell repertoire in the thymus on self determinants not only generates the precursors of naïve T cells that will recognise and respond to foreign determinants, but also programs cells with the potential to become regulatory T cells. The signal re-

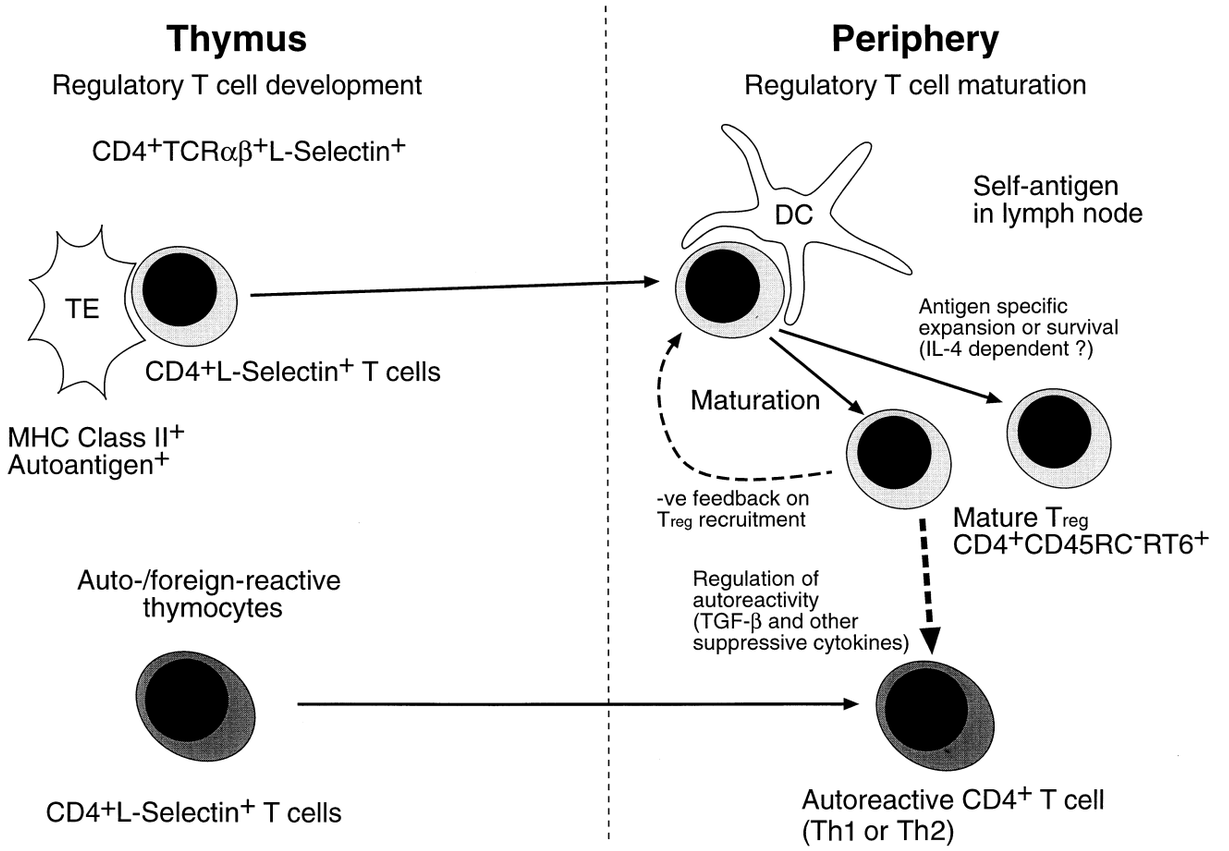

Fig. 1. Model of T_{reg} development in rats. The figure summarizes current knowledge of the developmental pathway of T_{reg} in rats, from positive selection in the thymus to final maturation into an effector state in the periphery. Solid arrows indicate T cell development, while dashed arrows indicate the action of T_{reg} either on autoreactive T cells or as part of a homeostatic feedback regulation to prevent excessive generation of T_{reg}. Abbreviations: TE – thymic epithelium, DC – dendritic cell, MHC – majorhistocompatibility complex, T_{reg} – regulatory T cells, -ve – negative

quired for completion of maturation comes in the periphery only if appropriate self antigen is encountered by T_{reg} precursors. This peripheral selection event is either permissive for T_{reg} survival or may drive expansion of T_{reg}, a process possibly dependent on IL-4. Finally, T_{reg} function to specifically suppress Th1 and Th2 autoreactive T cells through the secretion of TGF-β and, most likely, other cytokines, such as IL-10, which have been implicated in T_{reg} function in other systems[8].

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