

Regulatory T Cells and Allergic Disease

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Abstract: Allergic diseases such as asthma, rhinitis, and eczema are increasing in prevalence and affect about 15% of the population in countries such as the UK or USA. Regulatory T cells (T_{Regs}) have been shown to be critical in the maintenance of immune responses and T cell homeostasis. For example, depletion of $CD4^+CD25^+$ T_{Regs} from mice resulted in the development of multiorgan autoimmune diseases. So-called 'natural' $CD4^+CD25^+$ T_{Regs} and/or IL-10-producing Tr-1 cells are capable of suppressing Th2 responses to allergens in health, whereas such inhibition is attenuated in allergic conditions. In this context, both cell-cell contact-dependent (either through membrane bound TGF- β or *via* suppressive molecules such as CLTA-4) and soluble cytokine- (TGF- β and IL-10) dependent mechanisms have been shown to contribute to the function of T_{Regs} . Moreover, adoptive transfer of $CD4^+CD25^+$ T_{Regs} from healthy to diseased animals resulted in the prevention or cure of certain autoimmune diseases, and was able to induce transplantation tolerance. Clinical improvement seen after allergen immunotherapy for allergic diseases such as rhinitis and asthma is associated with the induction of IL-10 & TGF- β producing Tr-1 cells as well as Foxp3 expressing IL-10 T cells, with resulting suppression of the Th2 cytokine milieu. Activation and expansion of antigen-specific $CD4^+CD25^+$ T_{Regs} *in vivo* using adjuvants or pharmacological agents such as low dose steroids or vitamin D3 could represent novel approaches to induce antigen-specific tolerance in human diseases including allergic asthma, autoimmune disease and the rejection of transplanted organs.

INTRODUCTION

Allergic diseases are immunological disorders that have increased in prevalence over the past three decades and affect ~15% of people in industrial countries [1]. Consequently, the current healthcare expenditures for conditions such as allergic rhinitis and asthma are enormous in the western countries. Allergic rhinitis and asthma are manifestation of inflammatory immune responses in the respiratory mucosa to environmental antigens in genetically susceptible individuals. These complex genetic disorders are also influenced by environmental factors and characterized by an inflammatory cellular influx composed of T lymphocytes, basophils and eosinophils resulting in exaggerated T-helper 2 (Th2) responses [2, 3]. The activation of $CD4^+$ T cells plays a major role in the development of allergic disorders. T-helper subsets include T helper 1 (Th1), Th2, Th3, Th17 and T_{Regs} and their transcriptional regulation elements [4-6] are shown in Fig. (1). Th1 cells characteristically interact with foreign antigens/pathogens (bacteria, viruses) and secrete interleukin 2 (IL-2), tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ) and under certain circumstances IL-10. Th2 cells on the other hand, are the hallmark of allergic diseases and have been shown to be involved in the induction of humoral immunity and in particular in immunoglobulin switching in B-cells in favour of IgE with IL-4 and/or IL-13 as determining switch factors. Other Th2 cytokines involved during allergic reaction include IL-5 (facilitates eosinophilic inflammation and induces nasal symptoms and airway inflammation), IL-9 (engages in mast cell maturation, and mucus hyper-secretion and airway hyperreactiv-

ity), and IL-10 (with both inflammatory and anti-inflammatory activities) [7]. The over production of Th2 cytokines (IL-4, IL-5, IL-9 & IL-13) is deemed to play a prominent role in the target organ, resulting in the upregulation of adhesion molecules like vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells facilitating the infiltration of mast cells, eosinophils, T cells and basophils into the respiratory mucosa [8,9]. Chemokines such as RANTES, eotaxin, monocyte chemoattractant protein-4 (MCP-4), thymus and activation regulated chemokine (TARC) and macrophage derived chemokine (MDC) released from epithelial cells all serve as chemoattractants for eosinophils, basophils and T lymphocytes [10,11]. In addition eosinophils are major players in the late phase responses release an array of proinflammatory mediators including cysteinyl leukotrienes, cationic proteins, eosinophil peroxidase, and major basic protein, and serve as a major cell source of IL-3, IL-4, IL-5, GM-CSF, and IL-13 [12].

T helper 17 (Th17) cells, a recently identified T helper subset, preferentially produce interleukin-17A (IL-17A), IL-17F, IL-21, and IL-22. Th17 cells and their cytokines mediate host defense mechanisms to various infections, and are regarded as effector candidates involved in the immunopathogenesis of many autoimmune diseases [13, 14].

The role of Th2-driven immune responses has been decisively established in the development of allergic diseases. Nevertheless environmental factors and the genetic predisposition of allergic individuals are also believed to contribute, as co-factors, in the severity of these Th2 diseases. Conversely, the lack of response to allergens in nonatopic healthy individuals and the mechanisms by which such immune tolerance is induced and regulated are poorly understood. This article summarises *in vitro* & *in vivo* evidence for the functional abilities of regulatory T cells (T_{Regs}) in controlling cell proliferation and cytokine production in health

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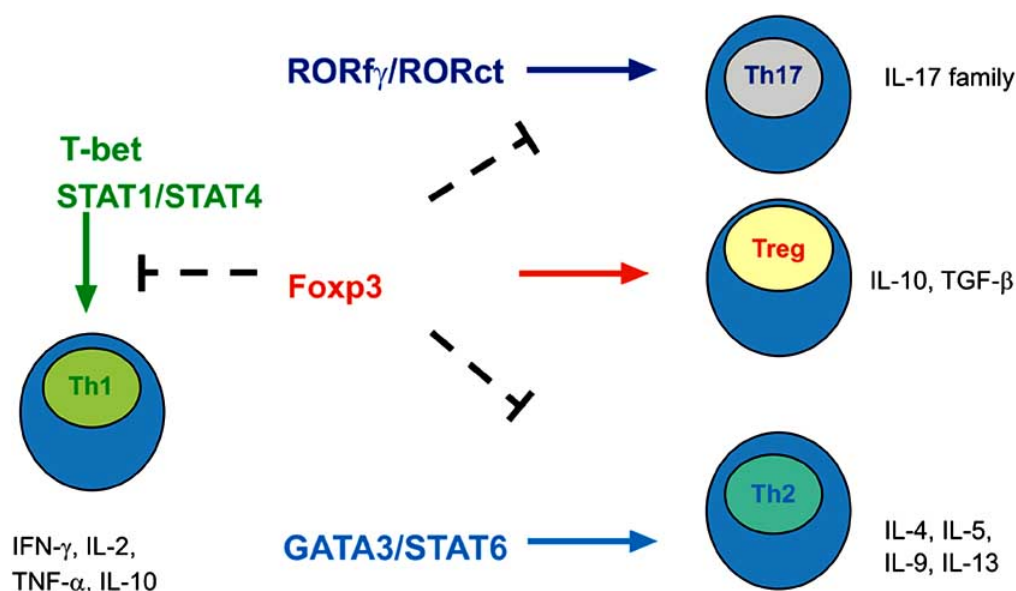


Fig. (1). Schematic representation of transcriptional regulation of Th1, Th2, Th17 and T_{Reg} cells by transcription factors T-bet/STAT1/STAT4, GATA3/STAT6, ROR γ t and Foxp3 respectively. Foxp3⁺ T_{Regs} regulate the function of Th1, Th2 and Th17 cells.

and in a number of immunological related disorders including allergic diseases and discusses the possibility of manipulating T_{Reg} for treatment of allergic conditions such as allergic rhinitis.

REGULATORY T CELLS (T_{REGS})

The interest in regulatory T cells (T_{Regs}), formerly known as suppressor cells, was revived in the 1990s following the report that athymic nude (nu/nu) mice when injected with CD4⁺ T cells depleted of CD25⁺ subset, developed multi-organ autoimmune diseases [15, 16] and that these autoimmune phenomena could be reversed by the adoptive transfer of CD4⁺CD25⁺ T cells from naïve mice to the athymic mice. This landmark observation provided compelling evidence for the putative regulatory role of CD4⁺CD25⁺ T cells in the control of immune responses and generated considerable interest in all aspects of basic and clinical immunology. Further observations confirmed the existence of CD4⁺CD25⁺ T_{Regs} in the peripheral blood and lymphoid tissues in man [17-20]. Numerous studies on CD4⁺CD25⁺ T cells revealed a critical role for T_{Regs} as the controller of immune responses in a number of clinical settings such as autoimmunity, tumour and microbial infection, transplantation, and allergy. Thus the level of T_{Reg} mediated suppression is a critical factor in determining the development of immunity or progression to disease. For example the lack of adequate suppression in immune responses to self antigens or allografts result in autoimmunity or graft rejection which contrasts to over suppression of immune responses to tumour antigens in cancer.

CD4⁺CD25⁺ T_{Reg} cells constitutively express the high-affinity interleukin-2 (IL-2) receptor α -chain (CD25), a receptor which is critical for IL-2 signalling and events leading to maintenance, homeostasis and the function of T_{Reg} cells *in vivo*. However, the main source of IL-2 responsible for the *in vivo* maintenance and activation of CD4⁺CD25⁺ T_{Reg} cells seems to be T cells other than the T_{Regs} themselves, present in close proximity with the CD4⁺CD25⁺ T_{Regs} within the

lymphoid organ [21,22]. Freshly isolated CD4⁺CD25⁺ T cells were anergic, as they failed to proliferate after T cell receptor (TCR) stimulation *in vitro*, whereas the addition of exogenous IL-2 reversed and generated potent T cell responses [15, 23-25]. From these observations it would appear that the initial process of suppression by CD4⁺CD25⁺ T_{Reg} cells is antigen dependent.

In the thymus CD4⁺CD25⁺ T_{Regs} are positively selected following interaction of CD4⁺CD25⁺ T cells with intrathymic cortical epithelium expressing MHC class II molecules. Athymic mice grafted at birth with allogeneic thymic epithelium sustain life-long tolerance to skin grafts of donor haplotype [26]. Mice lacking the expression of MHC class II on thymic cortical epithelium were also devoid of CD4⁺CD25⁺ T cells, implying the importance of MHC Class II molecules in the induction of CD4⁺CD25⁺ T_{Regs} [26]. The intrathymic generation of CD4⁺CD25⁺ T cells requires TCR that have higher affinity for self-peptide MHC class II complexes than those expressed by effector CD4⁺CD25⁺ T cells [27].

Thymic selection of CD4⁺CD25⁺ T_{Regs} is controlled by transcription factor Foxp3 [28, 29], a master switch gene in the development and function of T_{Regs}. Foxp3 is a member of the forkhead-winged helix family of transcription regulators located on chromosome Xp11.23 and has been mainly regarded as a repressor of T_{Regs}. Foxp3 full-length protein is encoded by 11 exons and contains a forkhead DNA-binding domain at the C terminus which can bind to the IL-2 promoter and repress IL-2 mRNA transcription [30]. Foxp3 is constitutively expressed at high levels on natural T_{Regs} in both man and mice and is essential for the T_{Regs} lineage commitment in the thymus as shown by Lin *et al.* [31]. Foxp3 can activate or stifle other transcription factors, or signalling pathways or membrane expression of certain molecules in the periphery. Scurfy mice lacking Foxp3 are deficient in CD4⁺CD25⁺ T_{Regs} and develop severe lymphoproliferative autoimmune disease [32]. Mutations in the Foxp3 gene in man also gave rise to severe immune dysregu-

lation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX), a condition with the spontaneous development of allergic airways inflammation, hyper IgE, and eosinophilia [33]. The majority of affected males die during the first year of life of either due to metabolic derangements or sepsis. Conversely, the ectopic expression of Foxp3 can phenotypically and functionally convert effector T cells (Teffs) to T_{Regs} [34]. In mice, Foxp3 expression has been shown to be both necessary and sufficient for T_{Regs} development [35]. Whereas in man, there is increasing evidence demonstrating that, Foxp3 is transiently expressed in activated T cells [34, 35]. The observation of abundant Foxp3 messenger RNA (mRNA) in recently activated $CD4^+CD25^+$ cells lacking regulatory function [36] may suggest that in humans, Foxp3 expression alone is not sufficient to indicate regulatory activity of $CD4^+CD25^+$ cells.

The molecular mechanisms involved in the regulation of Foxp3 expression remain poorly understood. A recent characterization of the human Foxp3 promoter indicated that its transcriptional activity is dependent on TCR signalling, which is consistent with the presence of several AP1- and NFAT-binding sites within the promoter [37]. In addition, the binding of signal transducer and activator of transcription (STAT) 5 and STAT3 to conserved binding sites within the Foxp3 locus have been implicated in the regulation of Foxp3 expression in human T_{Regs} [38]. It was concluded that the conversion of $CD4^+CD25^-$ T cells into $Foxp3^+T_{\text{Regs}}$ can only occur through demethylation of DNA. Activation of thymic dendritic cells (DCs) by thymic stromal lymphopoietin (TSLP) has been shown to be able to induce $CD4^+CD25^+T_{\text{Regs}}$ in man [39]. In the peripheral blood and in peripheral tissues $CD4^+CD25^+Foxp3^+T_{\text{Regs}}$ can be induced from $CD4^+CD25^-$ T cells in the presence of TGF- β as a principle cytokine [40, 41] and interleukin-2 (IL-2), a critical factor involved in cell signalling and in the maintenance of $CD4^+CD25^+T_{\text{Regs}}$ [42, 43]. The down-regulation of $CD4^+CD25^+T_{\text{Regs}}$ cell function (in the case of cytokine withdrawal or neutralization) might have beneficial effects to enhance immunogenicity during viral infection or in the breakdown of immunotolerance in tumours. The mechanism of suppression used by T cells remains controversial, nonetheless it has been postulated that TGF- β 1 may mediate the immunosuppressive activity of T_{Regs} .

MARKERS OF T_{REGS}

Naturally occurring $CD4^+T_{\text{Regs}}$ express various cell surface molecules commonly associated with activated and/or memory T cells. These include CD25, CD45RB^{low}, CD62L, cytotoxic T-lymphocyte antigen-4 (CTLA-4, or CD152) and glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40 and programmed death-1 (PD-1) antigen [44-46]. The expression of chemokine receptors CCR4 & CCR6 has also been reported in T_{Regs} [47]. The lymphoid homing receptors CD103 and CD62L and molecules such as perforin and granzyme A are also in the list of markers expressed by T_{Regs} [48]. Although none of these surface markers are uniquely expressed by natural T_{Regs} , the high level of expression and their constitutive nature have made them useful surrogate makers for the functional T_{Regs} . The search for molecules, which exclusively identify cell surface markers that define T_{Regs} , has led to the identification of an additional marker with potential specificity for T_{Regs} .

Two studies have reported an inverse correlation between the expression of the IL-7 receptor α chain (CD127) and the suppressive function of the $CD4^+Foxp3^+$ expressing T cells [49, 50]. CD127 molecule is downregulated in all human T cells after activation, however it is re-expressed on the majority of effector and memory T cells [51- 53]. Whereas the expression of this molecule remains low or even undetectable in $CD4^+Foxp3^+$ expressing T cells [49, 50]. Liu *et al.* demonstrated that cells separated based on $CD4^{\text{high}}$ and $CD127^{\text{low}}$ expression were anergic and had comparable suppressive capabilities to the 'classic' $CD4^+CD25^{\text{high}}$ T-cell subset, suggesting that CD127 could be used as a potential distinct marker in identifying T_{Regs} [50].

CTLA-4

In contrast to CD28 (a costimulatory molecule constitutively expressed on T cells), CTLA-4 is transiently expressed on activated T cells, initially within the cytoplasm, followed by expression on the cell surface membrane. Ligation of CD28 with its counter parts (B7 family molecules) is critical for the induction of immune responses of naïve $CD4^+$ cells, whereas CTLA-4 ligation plays a crucial role in downregulating T cell responses by inhibiting IL-2 production and other components involved in the machinery cell cycle progression. In experimental models, the blockade of CTLA-4 on T cells with anti-CTLA-4 antibodies induced rejection of several types of established tumours, including colon carcinoma, fibrosarcoma, prostatic carcinoma, lymphoma, and renal carcinoma [54,55], confirming the involvement of CTLA-4 molecule in suppression of immune responses against tumour antigens. Interestingly and in accord with these observations, therapeutic intervention with Gvax/anti-CTLA4 (a vaccine expressing GM-CSF) increased the number of $CD8^+IFN-\gamma^+$ effector T cells and $CD4^+Foxp3^-$ T cells within the tumour, with a significant decrease in the percentage of infiltrating $Foxp3^+$ T cells. Instead the Foxp3 expressing $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells migrated from the tumour regions into the lymph nodes. The migration of Foxp3 from the tumour site into the lymph nodes was probably in response to the blockade of CTLA-4 molecule resulting in cell-signalling and the expansion specific T cell responses to tumour antigens [54].

GITR

GITR, a member of the tumour necrosis factor (TNF) receptor family, is expressed on CD4 T cells after TCR ligation and plays a major role in the regulation of TCR-driven cell activation and cell death [56, 57]. GITR is constitutively expressed on T_{Regs} in mice, but only ~10% of human T_{Regs} are GITR positive [58]. The ligation of GITR with its ligand on APC results in inhibition of immune reactions through toll-like receptors (TLRs). Agonistic anti-GITR seems to function by intervening with the immunosuppressive effects of T_{Regs} on the effector T cells, but the exact molecular mechanisms have not yet been elucidated.

OX-40

OX-40 (CD134), another member of the TNF family, is also constitutively expressed by T_{Regs} . Similarly to GITR, OX40L ligand (CD134L) is expressed by APCs (B cell, DC, and macrophages), and activated endothelial cells [59]. Triggering of OX40 on T_{Regs} by administration of OX40L has

been shown to block the inhibitory activity of T_{Regs} in mice [60] as well as inhibiting the induction of IL-10-producing Tr-1 cells from naïve CD4 T cells in man [61]. OX40L has the ability to enhance immunity and break immunological tolerance in certain types of tumours.

SUBTYPES OF T_{REGS}

Naturally occurring T_{Regs} : T_{Regs} represent a highly heterogeneous populations of $CD4^+$ T cells (Fig. 2). Naturally occurring $CD4^+CD25^{\text{high}}$ T_{Reg} cells are a distinct lineage of T_{Regs} that originate in the thymus and are probably committed to debilitate immune responses against self-antigens [62, 63]. Naturally occurring T_{Regs} account for 5-10% of peripheral $CD4^+$ T cells and have the ability to inhibit the activation and proliferation of effector T cells [64]. The foremost phenotypic markers of T_{Regs} are the surface expression CD4 and CD25 and the nuclear expression of the transcription factor Foxp3, tools that natural T_{Regs} are equipped with before leaving the thymus [65]. Foxp3 $^+$ $CD4^+$ T_{Regs} can also be generated *de novo* from naïve T cells in a TGF- β -dependent environment by TCR triggering. It has been reported that naturally occurring, but not *in vitro* TGF- β -induced T_{Regs} display stable Foxp3 that is associated with selective demethylation of an evolutionarily conserved element within the Foxp3 locus named TSDR (T_{Reg} -specific demethylated region). Naturally occurring regulatory T cells that co-express high levels of CD25 and Foxp3 exert strong suppressor activity following TCR stimulation and have been reported both in mice and humans [66, 67]. While these cells are thought to originate predominantly from the thymus [68, 69], a number of *in vitro* studies have demonstrated that cells with similar characteristics can be generated from peripheral T cells under appropriate conditions that use TCR signalling

pathways [70, 71]. Naturally occurring T_{Regs} are, in particular, involved in the maintenance of normal homeostasis, prevention of autoimmunity and graft rejection and over expression of Foxp3 by $CD4^+CD25^+$ T_{Regs} has been demonstrated to play a critical part in tumour growth. Experimental models of inflammatory bowel disease [72] and clinical conditions such as atopic dermatitis [73], asthma [74] and a number of autoimmune conditions [75, 76] have been associated with impaired expression of Foxp3 $^+$ $CD4^+CD25^+$, natural T_{Regs} .

The presence of T_{Regs} in inflamed tissues such as pancreatic islets, synovia of arthritic joints, colitis lesions, asthmatic lung, skin, intestinal tissue of graft-versus-host disease (GVHD), cardiac and renal allografts, various tumours, virus and parasite-infected tissues, are all suggestive of the potential role of T_{Regs} at the site of inflammation [77]. Some studies have demonstrated that the tissue-resident T_{Regs} have functional ability to regulate local immune responses. For example, in the skin allograft setting, the T_{Reg} -infiltrated allograft was capable of transferring antigen-specific tolerance when the tissue was re-transplanted to a new host, suggesting that the tolerant skin graft harbored functional T_{Regs} [78]. In one study, depletion of T_{Regs} within the tumour was sufficient to promote a productive anti-tumour response and induce tumour regression [79]. More recently, Foxp3 mRNA levels in urine samples inversely correlated with serum creatinine levels in patients with acute renal graft rejection. In fact, higher levels of Foxp3 mRNA in urine predicted reversal of acute rejection, while lower levels of Foxp3 mRNA level identified as a marker of graft failure [80]. Collectively, these results suggest that T_{Regs} can function within the peripheral tissue to suppress inflammation and to prevent tissue damage.

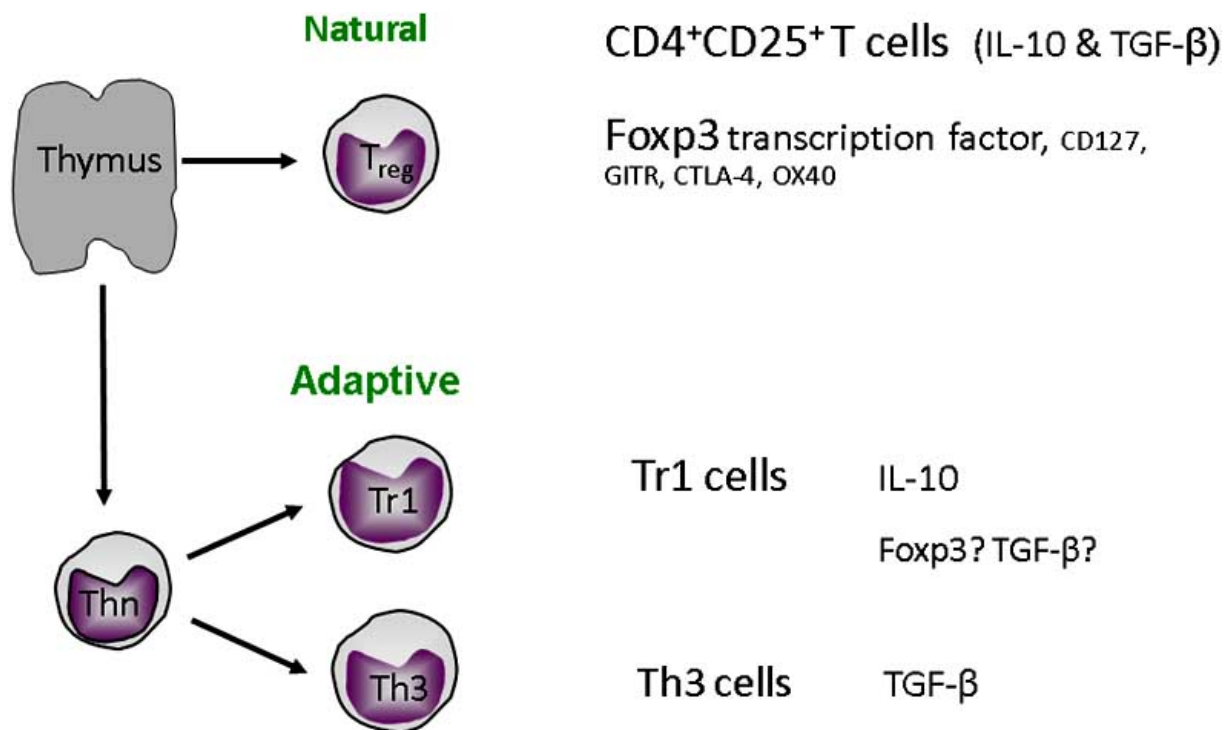


Fig. (2). Regulatory T cell subsets: During T cell development, a small proportion of $CD4^+$ express CD25 and Foxp3 before leaving the thymus (Natural T_{Regs}). In contrast, Adaptive T_{Regs} are developed in the periphery following antigen exposure. Adaptive T_{Regs} include of IL-10 producing Tr-1 cells, and TGF- β producing Th3 cells.

Adaptive/inducible T_{Regs} : In contrast to naturally occurring T_{Regs} , adaptive or inducible T_{Regs} are generated from naïve T cells ($CD4^+CD25^-Foxp3^-$ populations) in the periphery under low-dose, but possibly repetitive, exogenous antigenic stimulation [81] or by immunosuppressive cytokines such as TGF- β [82]. TGF- β has been shown to convert peripheral $CD4^+CD25^-$ naïve T cells into regulatory $CD4^+CD25^+$ T cells, presumably through the induction of Foxp3 expression and upregulation of suppressive molecules such as CTLA-4 [40, 82]. The inducible T_{Regs} do not however, have the classical $CD4^+CD25^+$ T-cell phenotype and is defined on the basis of their cytokine profile. The relationship between the inducible- T_{Regs} and the natural $CD4^+CD25^{\text{high}}Foxp3^+$ T cells is still unclear, nor is clear the antigen specificity of the human adaptive $CD4^+CD25^{\text{high}}Foxp3^+$ T cells. The adaptive $CD4^+CD25^{\text{high}}Foxp3^+$ T cells have been described in several experimental and clinical conditions [83, 84]. The inducible/adaptive subset of T_{regs} are $Foxp3^-$, do not express high CD25 or Foxp3 and include type 1 regulatory T cells and Th3 cells [85, 86].

TYPE 1 REGULATORY T CELLS (Tr-1)

Tr-1 cells are defined by their ability to produce high levels of IL-10 upon repetitive exposure with antigens. IL-10 production by Tr-1 cells may be associated with or without the production of TGF- β [87]. A subset of Tr-1 cells can also be generated *in vitro* by stimulation naïve $CD4^+$ T cells in the presence of IL-10, IFN- α , or a combination of IL-4 and IL-10 [87, 88]. Addition of Dexamethasone (Dex) and vitamin D₃ have been shown to induce the differentiation of naïve $CD4^+$ T cells to IL-10 secreting Tr-1 cells both in man and mice [89, 90].

Antigen specific $CD4^+$ T cell clones when stimulated *in vitro* in the presence of IL-10, mature to a distinct cytokine profile that is different from those of Th1 or Th2 cells [91]. These $CD4^+$ clones develop large capacity to release high levels of cytokines such as IL-10 and also TGF- β . This inducible cell type of T_{Reg} has been named Tr-1 cells. The adaptive transfer studies using the IL-10 producing T cells in SCID mice inhibited the development of colitis when these cells were co-administered with $CD4^+CD45RB^{\text{hi}}$ splenic T cells [91]. Stimulation of $CD4^+$ T cells derived from cord blood with allogeneic immature DCs gave rise to IL-10 producing Tr-1 cells [92]. In another study, *in vivo* generation of Tr-1 cells was achieved by administration of IL-10 and rapamycin in diabetic mice after pancreatic islet transplantation. However, when the IL-10 activity was blocked using anti-IL-10R, mice rapidly induced graft rejection. Thus the combination of rapamycin plus IL-10 not only prevented allograft rejection but also induced Tr-1 cells that mediated stable antigen-specific, long-term tolerance *in vivo* [93].

The inducible $CD4^+T_{\text{Regs}}$ (iT_{Regs}) comprise not only IL-10 producing Tr-1 cells, but also another distinct population of T_{Regs} , so called TGF- β 1 secreting T-helper cell type- 3, "Th3 cells" which can be antigen-specific or antigen non-self-specific, and can develop after antigen encounter in the periphery. The expression of Foxp3 by Tr-1 and Th3 remain controversial.

TH3 CELLS

Administration of antigens *via* oral route has long been recognized to induce immune tolerance in several animal models of autoimmune diseases including experimental autoimmune encephalitis, uveitis, thyroiditis, myasthenia, arthritis, colitis, and diabetes in NOD mouse. In these experimental models TGF- β -producing Th3 cells are considered to be responsible for provoking oral tolerance and are assumed as the main regulator of antigen immune responses [94]. Nonetheless, the role of Th3 in the development and involvement of T_{Regs} is unclear, despite its influence in the induction of oral tolerance through a mechanism which possibly entails TGF- β [95, 96]. An increasing body of evidence however suggests that Th3 cells may overlap with naturally occurring $CD4^+CD25^+$ T_{Regs} because of the established influence of TGF- β in the induction and development of T_{Regs} . Further evidence for this argument is provided by the observation that oral administration of autoantigen in mice suffering from autoimmune conditions resulted in attenuation of the disease, the mechanism of which is believed to be through Th3⁺ cells [96].

MECHANISMS OF SUPPRESSION

Cytokines: The suppressive cytokines TGF- β and IL-10 have been implicated as active players in the effector function of T_{Regs} [85, 97, 98]. Original studies unravelling the immunosuppressive mechanisms of $CD4^+CD25^+$ T_{Regs} using neutralization antibodies against IL-10 and TGF- β 1 revealed unexpected findings i.e. IL-10 and TGF- β were not engaged in the suppression of cell proliferation *in vitro*. The findings also confirmed the requirement of physical contact between $CD4^+CD25^+$ T_{Regs} and $CD4^+CD25^-$ effector T cells *in vitro*. Moreover, $CD4^+CD25^+$ T_{Regs} isolated from IL-10^{-/-} or TGF- β 1^{-/-} mice were as effective as T_{Regs} from wild type mice in suppressing T cell proliferation *in vitro* or preventing the development of colitis in a murine model [99]. In contrast to these observations, when $CD4^+CD25^+$ T_{Regs} were stimulated with anti-CD3 *in vitro* they developed high expression of membrane-bound latent TGF- β (latency-associated peptide of TGF- β 1- LAP) and the extent of TGF- β 1 LAP expression correlated with the levels of suppression enforced by T_{Regs} . In accord with these findings were $CD4^+CD25^+$ TGF- β 1LAP⁺ T cells that appeared to be responsible for the control of colitis induced by $CD4^+CD45RB^{\text{high}}$ T cells in mice [100,101]. Further studies using administration of anti-TGF- β and anti-IL-4 antibodies neutralised the suppressive activity of $CD4^+CD45RC^{\text{low}}$ T cells in rat type-1 diabetes and thyroiditis [102,103]. TGF- β has also been shown to indirectly enhance the expression of Foxp3 in ~ 10 - 30% of $CD4^+CD25^-$ T cells upon TCR stimulation under *in vitro* and *in vivo* conditions. The induced Foxp3 expression in these effector cells generated an anergic response in a TGF- β dose dependent fashion [104]. These observations collectively advocate a role for the membrane-bound latent TGF- β 1 (but not for the soluble form of TGF- β) which was accountable for the suppression caused by T_{Regs} in this system.

One mechanism of suppression involving TGF- β by $CD4^+CD25^+$ T_{Regs} was described to be Notch1 dependent.

Notch 1, a transmembrane molecule required by T cells during their developmental process is controlled by TGF- β . Notch 1 is reported to bind to its ligands Jagged 1 and Jagged 2, inducing the expression of a transcription repressor HES-1 [105].

In vivo evidence against cell-cell contact-dependent suppression by T_{Regs} was provided by the administration of suppressive cytokines, IL-10 and TGF- β , controlling autoimmune responses and prevention of graft versus host reactions in experimental models of autoimmunity and transplantation. Blocking of IL-10 receptor with appropriate antibodies, but not the adoptive transfer of CD4⁺CD25⁺ T_{Regs}, into mice grafted with allogeneic skin graft markedly accelerated transplant rejection [106]. The monitoring role of IL-10 in CD4⁺CD25⁺ T_{Reg}-mediated control of murine model of transplantation tolerance [107], chronic parasite infection [108], and type 1 model of diabetes [109] have also been reported. IL-10 was shown to be indispensable for the regulation of mouse model of colitis as IL-10-deficient mice developed spontaneous colitis mediated by Th1 CD4⁺ T cells [110]. In one study IL-10-producing CD4⁺CD25⁺Foxp3⁺ T_{Regs} were found to be selectively present within the colonic lamina propria of both colitis mice and wild-type mice [75, 111]. The inflamed intestine of patients with inflammatory bowel disease also showed accumulation of CD4⁺CD25⁺Foxp3⁺ T_{Regs}, suggesting impaired function, but not physical absence of T_{Regs} in the development of the disease. Suppression of T_{Regs} *via* IL-10 has also been reported in a number of *in vivo* experimental models of T_{Regs} to control inflammation and homeostatic expansion of effector cells [112, 113].

Discrepancies between mechanisms used in suppression *in vitro* and *in vivo* might be explained by differences in the stage of T-cell activation in each setting. Powrie and colleagues [114] highlighted that while IL-10 was a key player in T_{Reg} inhibition of colitis- mediated by antigen-activated T cells, it was not essential for the regulation of colitis induced by naive T cells, i.e. IL-10 was relevant in the T_{Regs} control of effector function within inflamed tissue but not for T-cell activation and/or differentiation events in lymph nodes.

The occurrence of Foxp3⁺ T_{Regs} in the intestinal lamina propria or its presence in the central nervous system could be accountable for regulation of colitis and experimental allergic encephalomyelitis (EAE), through IL-10 locally secreted by T_{Regs} [115,116]. Interestingly splenic or tonsillar T_{Regs} do not appear to synthesize or secrete IL-10 *in situ* [117], endorsing that “naturally occurring” T_{Regs} in primary lymphoid organs do not express IL-10 *de novo* in the absence of stimulation.

Considerable evidence supports a fundamental role for IL-2 in the maintenance and survival of natural T_{Regs} since most Foxp3⁺ T_{Regs} express CD25 molecule, the high affinity IL-2 receptor. In agreement with these findings was a substantial reduction in Foxp3⁺ T cells in IL-2-deficient mice [118]. Furthermore, neutralization of circulating IL-2 by anti-IL-2 antibody treatment specifically diminished the number of CD25⁺CD4⁺ T cells resulting in the induction of organ-specific autoimmune diseases [119]. These observations may reinforce the requirement of IL-2 for the survival of natural T_{Regs}, although the main cell source of IL-2 seems to be T cells other than T_{Reg} populations themselves [120]. IL-2 has also been shown to augment the expression of

Foxp3 in human T_{Regs} through a signal transducer and activator of transcription 3 & 5 (STAT3 & STAT5)-dependent pathways [121]. Thus, IL-2 emerges as a critical cytokine for T_{Regs} survival and function, and can be a key therapeutic target to control immune responses involving T_{Regs}.

The newly discovered inhibitory cytokine, IL-35, also appears to contribute to T_{Reg} suppression [122]. EBV-induced gene 3 was identified to be overrepresented in mouse CD4⁺ CD25⁺ T cells in a genomic screen. EBV-3 gene encoded the IL-27 β -chain which pairs with IL-27- α to form IL-27 or with IL-12- α to form the new cytokine IL-35. Unlike IL-10 and TGF- β , IL-35 seems to be involved in suppressive function both *in vitro* and *in vivo*. Moreover, the ectopic expression of IL-35 in T cells confers the regulatory property of IL-35 for T_{Regs} [122]. Although IL-35 and IL-12 α are expressed by several cells other than T_{Regs} within the immune system, IL-35 may nevertheless represent an important therapeutic target for the modulation of immunity.

The modulation of cyclic adenosine monophosphate (cAMP) levels in the target cells by T_{Regs} has also been reported as a further mechanism that initiates an inhibitory pathway proceeding to restrain cellular proliferation and differentiation in effector cells. This will result in the reduction in IL-2 and IFN- γ gene expression through protein kinase A (PKA) which consequently blocks the nuclear factor- κ B (NF- κ B) activity [123].

Cell-cell contact: The use of transwell membrane experiments which allows the exchange of soluble factors between the wells but prevents a direct physical contact between CD4⁺CD25⁺ T_{Regs} and CD4⁺CD25⁻ effector T cells provided convincing evidence for cell-cell contact dependent suppression by T_{Regs} *in vitro* [124].

The suppression of cell proliferation *in vitro* involves contribution from several T cell accessory molecules such as CTLA-4 (CD152), Lymphocyte-activation gene (LAG)-3 and possibly other molecules such as GITR, OX40L expressed on T_{Regs} in a contact-dependent fashion [125, 126]. Upon stimulation, CTLA-4 expression is upregulated on CD4⁺CD25⁺ cells more than on CD4⁺CD25⁻ T cells, supporting a possible role for CTLA-4 in suppression induced by T_{Regs}. In concurrence with this hypothesis was the administration of anti-CTLA-4 mAb into normal mice over a period of time that elicited autoimmune gastritis and a murine model of colitis, two clinical conditions similar to those observed in mice following the depletion of CD4⁺CD25⁺ T cells [127, 128]. In addition, ectopic expression of Foxp3 in naïve T cells enhanced the expression of CTLA-4 and increased suppressive ability of T_{Regs}, entailing the involvement of both Foxp3 and CTLA-4 molecules in the inhibitory function of CD4⁺CD25⁺ T cells [129]. One possible mechanism of suppression induced by CTLA-4 is the competition between CTLA-4 and CD28 with co-stimulatory molecule for B7-1/B7-2 present on APCs instigating cell signalling. An alternative suppressive mechanism(s) engaged by CTLA-4 has been proposed to include the induction of indoleamine 2,3 dioxygenase (IDO) in dendritic cells possibly through activation of B7-1 & B7-2 antigens expressed on these cells and consequently triggering T_{Reg} activation [130]. IDO is responsible for the metabolism of an essential amino acid, tryptophan, required for the activation of CD4⁺CD25⁺ T cells. Conversely, T_{Regs} from CTLA4^{-/-} mice showed fully suppress-

sive capacity *in vitro* and the observations that anti-CTLA-4 mAb failed to eliminate the suppressive function of T_{Reg} *in vitro*, challenge the concept that CTLA-4 *per se* is responsible for the suppression mechanisms induced by T_{Regs} [131]. These findings may instead support the involvement of immune suppressive cytokines such as membrane bound TGF- β in this model [132].

$CD4^+CD25^+ T_{Regs}$ also express high levels of LAG-3 (a CD4-related molecule that binds MHC class II) upon activation. $CD4^+CD25^+ T_{Regs}$ from LAG3 $^{-/-}$ mice exhibited reduced regulatory activity and an antibody to LAG-3 inhibits suppression of T_{Regs} both *in vitro* and *in vivo*. In contrast, ectopic expression of LAG-3 on normal $CD4^+$ T cells resulted in reduction of proliferative responses and rendered the cells towards suppressive $CD4^+CD25^+$ T cells, supporting an inhibitory role for LAG-3 antigen in T_{Regs} [133].

The contact-dependent suppression by $CD4^+CD25^+$ cells was not, however, due to the killing of $CD4^+CD25^-$ T cells via TNF/TNFR- or Fas/FasL dependent pathways, since blocking antibodies to TNF- α or FasL had no effects on the number of $CD4^+CD25^-$ responder cells. Other suppressive mechanisms reported by T_{Regs} include Granzyme-B-dependent and perforin-dependent killing of $CD4^+CD25^-$ T cells, as Granzyme-B-deficient mice showed less efficient ability to suppress than wild type mice [134].

These observations collectively support the proposition that different mechanisms of suppression are employed by T_{Regs} in *in vitro* versus *in vivo* conditions. While a contact-dependent mechanism dominates during *in vitro* suppression, soluble factors such as IL-10, TGF- β and possibly IL-35 seem to be imperative in the induction of suppression *in vivo*.

T_{REGS} IN ALLERGIC DISEASES

Impairment in the function of regulatory T cells has been postulated to be responsible in the evolution of autoimmune diseases, rejection of solid organ transplants, and uncontrolled tumour growth. In the last three decades allergic diseases such as asthma, atopic dermatitis and rhinitis have been rising in prevalence worldwide. The well-recognized role of natural T_{Regs} in preventing autoimmune diseases suggests $CD4^+CD25^+ T_{Regs}$ may also play a critical role in hampering allergic diseases and implies a low frequency and/or a weakened function of allergen specific T_{Regs} may be accountable for the increase in the number of cases with allergy seen in the past 30 years. Thus, better understanding of mechanisms of T-cell regulation of Th2 responses seen in allergic diseases may help in developing more effective therapeutic strategies for treatment of allergic conditions.

The regulatory influence of Tr-1 cells in controlling Th2 type responses was established by the adoptive transfer of IL-10 producing $CD4^+CD25^+$ cells from unchallenged mice to OVA sensitized mice resulting in the resolution of inflammatory responses in the bronchial mucosa [135]. In man, the influence of $CD4^+CD25^+ T_{Regs}$ in regulating immune reactions to allergens has been identified [136] by the observation that the proliferative responses to allergens was completely absent in healthy volunteers in contrast to those of atopic subjects where T cell responses to allergens were shifted towards Th2 producing cytokines (IL-4, IL-5 & IL-13) [137]. These observations insinuate an active immune

surveillance by T_{Reg} populations operates in the healthy non-atopic individuals. The majority of allergen-specific T cells in these healthy individuals were of IL-10-secreting Tr-1 type. This report also supports the concept that rather than an imbalance between Th1:Th2 responses, the regulation of Th2 cell function may be hampered in atopy. It would also provide a plausible explanation as to why no immunological responses seen in non-atopic healthy volunteers following exposure to allergens.

In concordance with these findings was the lack of proliferative T cell responses to cows' milk antigen in healthy children. Depletion of $CD4^+CD25^+$ T cells from PBMCs however reversed T cell responses to cow's milk antigen, implying that the food tolerance to dietary antigens is also imposed by $CD4^+CD25^+ T_{Regs}$ [138]. Contrary to these data, children who had outgrown cows' milk allergy showed increased frequencies of circulating $CD4^+CD25^+$ T cells and decreased *in vitro* proliferative response to bovine β -lactoglobulin (a milk protein) when compared with children who remained clinically milk sensitive [139]. A similar state of IL-10-associated T cell induced anergy has been reported in hyperimmune individuals i.e. bee keepers, who had received multiple bee stings. The intracytoplasmic IL-10 $^+$ cells from these individuals were co-localized to activated $CD4^+CD25^+$ T cells with specificity for bee venom antigens [140].

A reduction in absolute numbers and proportions of the Foxp3 $^+CD4^+CD25^+$ have been described in the blood and in the nasal mucosa of allergic rhinitics. In several *in vitro* studies, $CD4^+CD25^+$ T cells from aeroallergen sensitive individuals (e.g. grass & birch pollens, cat and HDM) were found to be defective resulting in high proliferative responses and IL-5 secretion when $CD4^+CD25^+$ T cells were co-cultured with autologous allergen-stimulated $CD4^+CD25^-$ T cells [140 - 142]. The dysregulation of $CD4^+CD25^+ T_{Regs}$ in the atopics controlling IL-13 and IL-5 production [143] was most pronounced during the peak of birch and grass pollen season. In contrast, $CD4^+CD25^+$ T cells from both allergic and non-allergic individuals were potentially suppressive of T-cell proliferation and Th2 cytokine production to allergens outside of the pollen season.

The antigen specificity of T_{Reg} and its role in the induction of tolerance remains controversial. However, the observations that the adoptively transferred polyclonal T_{Reg} cells from healthy donors into myasthenic rats inhibited the progression of experimental autoimmune MG and led to down-regulation of humoral acetylcholine receptor-specific responses, endorsing antigen specific T_{Regs} may exist systemically and locally in these rats. Although the number of $CD4^+CD25^+$ cells in the spleen of treated rats remained unchanged, the proportion of $CD4^+CD25^+$ cells expressing Foxp3 was significantly elevated [144]. In another study T_{Regs} from non-allergic healthy subjects suppressed proliferation of influenza antigen as well as birch pollen stimulated effector cells, whereas $CD4^+CD25^+$ cells from allergic patients were only capable of dampening proliferative responses of T cells to influenza antigen, but not birch pollen stimulated cells. Similarly, the regulation of Th2, but not Th1 cytokine production by $CD4^+CD25^+$ cells was impaired in allergic patients, upon stimulation with birch pollen extract. Neutralization of IL-10 led to increased production of

IFN- γ and TNF- α in the atopics and non-atopic controls, substantiating a dysregulation of allergen-specific CD4⁺CD25⁺ T cells in atopic subjects. The increased in TNF- α production following neutralization of IL-10, however suggests counter-acting properties of IL-10 and TNF- α , and that IL-10 producing T_{Regs} are possibly involved in promoting tolerance. In contrast, the elevated levels of TNF- α may be involved in the ongoing process of inflammatory responses [145].

Further evidence for the co-existence of allergen (Der p 1)- specific and streptokinase (SK) specific-CD4⁺CD25⁺ Foxp3⁺ T_{Regs} in the peripheral blood of atopic individuals was reported by Maggi L. *et al.* [146]. These investigators demonstrated that CD4⁺CD25⁺Foxp3⁺T cells from Der p 1-sensitive atopic individuals when cultured with Der p 1-loaded DCs, but not those cultured with either unloaded or SK-loaded DCs, suppressed the proliferative responses of autologous CD4⁺CD25⁺Foxp3⁺ phenotype to Der p 1 [146]. These findings also suggest that the pool of human circulating CD4⁺CD25^{high}Foxp3⁺ T cells may consist of T_{Regs} specialised in the recognition of antigens with different specificities. Whilst natural T_{Regs} have a large repertoire for self-specific T-cell receptors and suppress immune responses *via* contact-dependent mechanisms, the inducible T_{Regs} consist of self- and non-self-specific cells that recognise autoantigens as well as foreign antigens, and are capable of suppressing a wide range of immune cells *via* high concentrations of TGF β 1 (possibly Th3 cells) or IL-10/TGF β 1 producing Tr-1 cells [146, 147]. Although antigen exposure is required by T_{Regs} to initiate suppressive activity, *in vitro* studies revealed that once they are activated, T_{Regs} inhibit immune responses in an antigen-nonspecific manner [147].

The role of TGF- β in the control of atopic diseases has been recently unveiled. TGF- β -deficient mice have been demonstrated to show a higher susceptibility to asthma-like lung inflammation than their healthy counterparts [148]. A mouse model of food allergy showed a significant reduction of secreted IgA antibodies, a class of immunoglobulin which is closely regulated by TGF- β , in the gut. Interestingly, recovery from food allergy in these mice was correlated with local production of TGF- β [149].

The expression of Foxp3 and GITR in normal skin and in a panel of different inflammatory dermatoses revealed that most Foxp3⁺ T cells co-expressed with the suppressive molecule, GITR. In atopic dermatitis and psoriatic skin, a dysregulation of effector T cells has also been reported by the absence of CD4⁺CD25⁺FoxP3⁺ T cells of in the lesional region and in the atopy patch test areas of the skin, despite the abundant expression of IL-10 and TGF- β as well as receptors for these cytokines in the dermis [150]. In normal skin Foxp3⁺ T cells were abundant, their frequency was, however, significantly lower than in patients with spongiotic dermatitis, psoriasis, and lichen planus, implying a role for the transcription factor Foxp3 in regulation and control of these skin conditions.

MODULATION OF T_{REGS} WITH TREATMENT

Corticosteroid: The administration of glucocorticoids, inhaled or systemic, in patients with severe asthma showed increases in the transcription factor Foxp3 and IL-10 mRNA expression in the bronchial mucosa. The expression of

Foxp3 mRNA was tightly correlated with IL-10⁺ cells (possibly Tr-1 cells), but not with the levels of TGF- β 1 mRNA expression. The increase in the frequency of CD4⁺CD25⁺ T cells in circulation and the expression of Foxp3 mRNA by CD4⁺ T cells was transient, but significantly greater in patients who received systemic glucocorticoid treatment. Moreover, addition of corticosteroids to *in vitro* cultures of CD4⁺ T cells induced upregulation of IL-10 and Foxp3 expression on these cells [74, 151, 152], suggesting that glucocorticoids are not only potent immunosuppressor agents with anti-inflammatory effects, they also endorse the differentiation of CD4⁺ T cells toward Tr-1 cells by a Foxp3-dependent mechanism. These observations further underline a distinct role of TGF- β 1, in T_{Regs} controlling inflammatory responses and provide some evidence on a diverse regulatory pathway of this suppressive cytokine.

In a myelin oligodendrocyte glycoprotein-induced experimental model of autoimmune encephalomyelitis (EAE), splenic CD4⁺CD25⁺ T cells failed to suppress the proliferation of CD4⁺CD25⁻ T cells. Pre-treatment of CD4⁺ T cells with Dex/IL-2 strikingly increased the proportion of CD4⁺FoxP3⁺ cells and partially restored the function of splenic CD4⁺CD25⁺ T cells which consequently inhibited the development of EAE [153]. Short exposure and simultaneous administration of Dex and IL-2 markedly developed suppressive ability of Foxp3⁺CD4⁺CD25⁺ T cells in murine lymphoid tissues. Thus, the combination of glucocorticoids with or without IL-2 may provide a novel approach for the treatment of autoimmunity as well as allergic diseases [154].

Evaluation of CD4⁺CD25^{hi} T cells expressing Foxp3 in peripheral blood and bronchoalveolar lavage fluid of asthmatic children compared with children with cough or healthy children showed a reduction in the numbers of regulatory T cells in asthmatic children [155]. In asthmatic children, inhaled corticosteroid treatment was associated with an increased proportion of CD4⁺CD25^{hi} T cells in both peripheral blood and BALF and an improvement in suppression of proliferation and cytokine/chemokine production by CD4⁺CD25⁻ responder T cells. The lower level of IL-10 in the bronchoalveolar lavage fluid of adult asthmatic patients compared with healthy controls has previously been reported [156]. Neutralization studies using anti-IL-10 almost abolished the suppressive effect of Tr-1 cells *in vitro* and further strengthened the role of this immunosuppressive cytokine in the control of Th2 responses and in the maintenance of T cell homeostasis [157].

Strategies that convert transient glucocorticoid-induced T_{Reg} activity into a stable phenotype should probably be considered to improve the treatment strategy in allergy and asthma.

Specific allergen immunotherapy (SIT) is highly effective in selected patients with IgE-mediated disease who are monoallergic [158, 159]. SIT was first described almost 100 years ago [160] and involves subcutaneous administration of small but increasing doses of allergen using a relatively crude extract.

Allergen immunotherapy is the only treatment to date that can affect the natural course of allergic rhinitis, conjunctivitis, allergic reactions to stinging insects and allergic asthma [161, 162]. Conventional allergen-desensitization

immunotherapy prevents further allergen sensitisations and the development of asthma in patients with allergic rhinitis [163]. The clinical improvement following allergen immunotherapy is sustained for years after cessation of treatment, thus SIT is believed to modify the underlying immunological mechanisms of allergic responses [164, 165]. The specific mechanisms by which allergen immunotherapy reduces allergic symptoms have been studied for decades. Induction of blocking antibody i.e. IgG4, a shift from Th2 to IFN- γ producing Th1 cytokine profile [166] and reductions in the numbers of effectors cells such as mast cell, eosinophils and basophils in the target organ are amongst the immunological changes observed following successful SIT.

Recent studies also demonstrated improvement in numerical and/or functional CD4⁺CD25⁺ T_{Regs} [167, 168] attenuating allergen-induced proliferation of Th2 cells and their cytokine pattern are associated with successful allergen IT. The intracellular IL-10 positive T cells from patients undergone immunotherapy was almost exclusively colocalized to CD4⁺CD25⁺ cells, implying that IL-10-producing CD4⁺CD25⁺ regulatory T cells have emerged as potential mediators of immune tolerance not only in numerous murine models of immunopathology, but also following grass pollen immunotherapy in man [167].

In house dust mite-sensitive mice, peptide IT increased the number of CD4⁺CD25⁺ T_{Regs} in the peripheral blood and the adoptive transfer of CD4⁺CD25⁺ T_{Regs} prevented the induction of experimental allergic encephalomyelitis [169]. A marked increase was found in the number of FoxP3⁺CD4⁺CD25⁺ T_{Regs} in the peripheral blood of patients with rheumatoid arthritis who were successfully treated with peptide IT, dnaJP1 derived from the bacterial heat shock protein) [170]. Although these CD4⁺CD25⁺ T_{Regs} had suppressive capacities *in vitro*, their effects *in vivo* depended on the induction of antigen-specific IL-10 producing Tr-1 cells [171]. As shown above, Tr-1 cells are likely to play a crucial role in the control of allergic disease. The major effector molecules of Tr-1 cells in this setting are the suppressive cytokines IL-10 and TGF- β .

The suppressive mechanisms of Tr-1 cells not only involve cytokines IL-10 and TGF- β , but also involve molecules such as CTLA-4 and PD-1 antigens. IL-10 inhibits CD28 tyrosine phosphorylation and prevents the binding of phosphatidylinositol 3-kinase p85, hence reducing the co-stimulatory CD28 signalling pathway [172]. Induction of antigen-specific Tr-1 cells can thus re-direct inappropriate immune responses against allergens using a broad range of suppressor mechanisms. Wasp venom- or phospholipase A2-pulsed dendritic cell stimulation of CD4⁺CD25⁺ T cells from healthy donors resulted in inhibition of proliferation and Th2 cytokine production by T_{Regs} at 10-fold lower than the optimal concentration. Whereas IFN- γ production was inhibited at all concentrations, suggesting that the threshold of response is different between allergic and non-allergic individuals [173].

The autocrine action of IL-10 and TGF- β includes initiation and the induction of peripheral T cell tolerance and plays a crucial role in the mechanisms of allergen-SIT [174, 175]. Reactivation of T cells tolerized by IL-10 and TGF- β can result in the distinct pattern of either Th1 or Th2 cytokine profiles depending on the cytokine milieu in the target

organ [176]. Antigen presentation to the anergic T cells was, however, fully restored in the presence of IL-2 or IL-15 as documented by the secretion of IFN- γ , but not IL-4 [177]. IL-10 and TGF- β expressing cells were increased significantly in the nasal mucosa of GP-IT patients, suggesting the immunological changes are not only systemic, but also occur in the target organ, i.e. nasal mucosa.

Another crucial change seen after successful allergen-SIT is a shift in the balance from IgE to IgG, in particular of the IgG4 subclass which is under the control of IL-10. Although peripheral tolerance has been demonstrated specifically in T cells, the capacity of B cells to produce specific IgE and IgG4 antibodies is not eliminated during allergen-SIT. GP-IT resulted in blunting of seasonal increases in serum allergen Phl p 5-specific IgE, increases in Phl p 5-specific IgG, and in particular Phl p 5-specific IgG4, the latter requires IL-10 for class switching [174]. Phl p 5-specific IgA rose modestly and it was predominantly of mucosal IgA2 subclass and correlated with the local expression of TGF- β [175]. These two independent studies have shown that IL-10- and TGF- β producing T cells in humans are generated *in vivo* during the course of allergen-SIT, implicating that high and increasing doses of allergens administered during the course of GP-IT induce the proliferation and activation of Tr-1 cells [142]. Jutel *et al.* reported the suppression induced by CD4⁺CD25⁺ Tr-1⁺ cells was partially blocked by neutralization of antibodies against secreted forms or membrane-bound IL-10 and TGF- β [157]. Thus, in addition to their cellular inhibitory influence, IL-10 and TGF- β secreted by Tr-1 cells can skew the antibody production from immunoglobulin E (IgE) towards the non-inflammatory isotypes IgG4 and IgA respectively.

In a recent article, striking numbers of Foxp3 expressing CD25⁺ and CD4⁺ cells were identified in allergic rhinitics who completed a successful course of GP-IT. Seasonal elevations in dual-positive Foxp3⁺CD4⁺ and Foxp3⁺CD25⁺ cells in these patients were accompanied by suppression of local allergic inflammation, indicative of a regulatory phenotype post immunotherapy (Fig. 3). Foxp3-positive and Foxp3-negative IL-10⁺CD25⁺ T cells co-existed in the nasal mucosa supporting the emergence of phenotypically distinct populations of regulatory cells i.e. Foxp3 expressing adaptive T_{Regs} and IL-10 producing Tr-1 cells after GP-IT [168, 174]. These findings are in agreement with those reported in murine models of colitis and in patients with ulcerative colitis and/or Crohn's disease, in whom IL-10 producing Foxp3⁺CD4⁺CD25⁺ cells were present at increased density in the colon and the presence of IL-10 was associated with amelioration of colitis [168,178]. These findings support strategies targeting increases in local T_{Regs} numerically and/or functionally might be beneficial in the treatment of allergic rhinitis. Similar observations using venom-specific IT inducing a significant progressive increase in the frequency of regulatory T cells as defined by CD25^{bright} and/or Foxp3⁺CD4⁺ T cells was reported by Pereira-Santos. The increased number of circulating regulatory T cells correlated with the venom-specific IgG4/IgE ratio shift, confirming the findings in GP-IT treatment in allergic rhinitis [179].

Less is known about the molecular mechanisms behind TGF- β -mediated suppression in allergen-SIT. In a murine model of allergic inflammation, blocking of CTLA-4 was

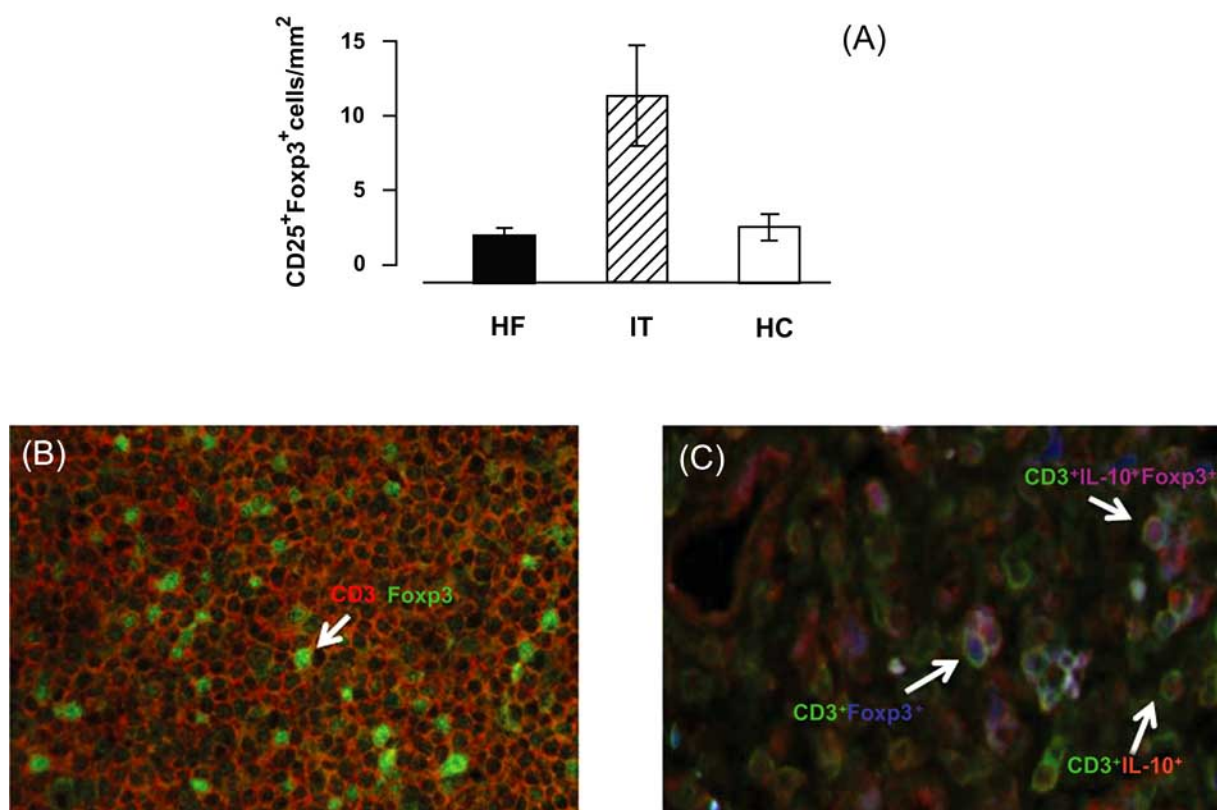


Fig. (3). (A) CD25⁺Foxp3⁺ T cells/mm² in the nasal mucosa from HF (hay fever sufferers), IT (grass pollen immunotherapy treated) and HC (healthy controls), (B) Foxp3⁺CD3⁺ T cells in the tonsillar section, and (C) Foxp3⁺IL-10⁺ T cells in the nasal mucosa following GP-IT. Arrows show cells expressing Foxp3⁺ cells.

associated with decreased TGF- β levels within the bronchoalveolar lavage fluid [180]. TGF- β has been shown to deviate antibody response against allergen from an IgE to an IgA-dominated response in mice and in man post IT [181].

Based on these data, and given the fact that decreases in T_{Reg} cell populations are possibly one of the major causes of allergic diseases, the upregulation of T_{Reg} numerically or functionally holds promising therapeutic potential in the treatment of allergic diseases. Novel approaches should be adopted to improve the clinical efficacy of IT using adjuvant(s) such as vitamin D3 or CpG or cytokines e.g. IL-10. Treatment of mice with mycobacterium induced allergen-specific T_{Reg} producing IL-10 and TGF- β protected against airway inflammation [182]. The application of adenoviral vectors encoding IL-10 also resulted in a longer suppressive effect than injected IL-10, with a very limited half-life [183]. Alternatively immunostimulatory CpG motifs, an agonist of TLR9 [184] has been demonstrated to reduce allergenicity and improve clinical efficacy of treatment when combined with pollen immunotherapy.

CONCLUSIONS

Naturally occurring CD4⁺CD25⁺ regulatory T cells (T_{Reg}) play a critical role in the control of periphery tolerance to self-antigens and the prevention of autoimmunity. Evidence from experimental models and studies in man have also demonstrated a key role for T_{Reg} in allergic diseases including rhinitis, atopic dermatitis, and asthma. Significant

progress has been made in understanding specific mechanisms resulting in allergic inflammation. Foxp3 expressing CD4⁺CD25⁺ T_{Reg}s and IL-10 producing Tr-1 cells capable of suppressing Th2 responses to allergens seem to be defective in those who develop allergic sensitization. The exact mechanism of suppression also remains controversial and may vary for different regulatory populations. Better understanding of regulatory mechanisms involved in the development of allergic sensitization and the manipulation of these cells holds the promise of effective treatment strategies to prevent allergic disease. Allergen immunotherapy modifies T cell responses to allergen and may do so through induction of adaptive T_{Reg}s, e.g. IL-10-producing Tr-1 cells contributing to the clinical efficacy of the treatment in aeroallergen sensitive individuals. Adoptive transfer of T_{Reg}s may represent an effective, donor-specific therapeutic approach, although it can also be cost effective. *In vivo* induction or expansion of T_{Reg}s in patients remains an attractive option and may present a more realistic approach to improve allergen-specific T_{Reg} induction and provide significant benefits to allergic and asthmatic patients.

Allergen immunotherapy may enhance the development of allergen-specific T_{Reg}s and provides safe, specific, and long-term control of allergic diseases and asthma. Co-administration of specific allergen immunotherapy with drugs such as corticosteroids and agents, e.g. vitamin D₃, adjuvants for example CpG or cytokine IL-10 are strong candidates for enhancing and generating antigen specific regulatory responses.

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ABBREVIATIONS

RANTES	= Regulated upon activation normal T cell expressed & secreted
TGF- β	= Transforming growth factor
CTLA-4	= Cytotoxic T-lymphocyte antigen-4
TNF- α	= Tumour necrosis factor
IFN- γ	= Interferon-gamma
TCR	= T cell receptor
MHC	= Major histocompatibility complex
IPEX	= Immune dysregulation, polyendocrinopathy and enteropathy, X-linked syndrome
STAT	= Signal transducer and activator of transcription
GITR	= Glucocorticoid-induced tumor necrosis factor receptor
PD-1	= Programmed death-1 (PD-1)
CCR	= Chemokine receptors
APC	= Antigen presenting cells
TSDR	= T _{Reg} -specific demethylated region
GVHD	= Graft-versus-host disease
Tr-1	= Regulatory T cells type 1
SCID	= Severe combined immunodeficiency syndrome
DC	= Dendritic cells
LAP	= Latency associated peptide
EBV	= Epstein bar virus
EAE	= Experimental autoimmune encephalomyelitis
cAMP	= Cyclic adenosine monophosphate
IDO	= Indoleamine 2,3 dioxygenase
LAG-3	= Lymphocyte-activation gene (LAG)-3
OVA	= Ovalbumin
HDM	= House dust mite
Der-p 1	= <i>Dermatophagoides Pteronyssinus</i>
Phl p	= <i>Phleum Pratense</i>
SK	= Streptokinase
Dex	= Dexamethasone
GP-IT	= Grass pollen immunotherapy
TLR	= Toll like receptor
NF- κ B	= Nuclear factor- κ B

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