

Review article

Regulatory T cells: which role in the pathogenesis and treatment of allergic disorders?

Several recent data suggest the importance of different types of cells in the regulation of T-cell effector-mediated immune responses. However, a unique specific marker for these cells has not yet been identified. Moreover, in addition to a dedicated functional lineage, even a 'plastic' phenotype of regulatory T cells seems to exist. The lack of a unique specific marker for regulatory T cells, as well as their heterogeneity, make it difficult to determine whether a defect of regulatory T cells plays a role in the pathogenesis of common allergic disorders. Novel therapeutic strategies based on the induction or potentiation of regulatory T cells able to hamper allergic inflammatory reactions are desirable, but their possible efficacy and safety are not yet known. At present, therapeutic strategies able to induce an immune deviation of allergen-specific type 2 T-helper responses to a less polarized phenotype rather than a general immune suppression appear more realistic.

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Key words: allergy; Th1; Th2; Treg.

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Accepted for publication 9 September 2005

Over the past few years, a variety of T-cell subsets that can inhibit T-cell responses *in vitro* and *in vivo* and can prevent and ameliorate disease in several animal models of autoimmunity have been described (1, 2). These cells have been named as T regulatory (Treg) cells. This discovery has allowed the publication of an impressive number of reports on the characterization and functions of Treg cells in several human diseases but, despite so many studies, several questions still remain unsolved. This review will mainly focus on the possible role of Treg cells in the pathogenesis of allergy, as well as on the possibility that the induction or potentiation of their activity may be useful for the development of novel therapeutic strategies in allergic disorders.

Phenotypic and functional features of Treg cells

So far, two main categories of Treg cells have been described (3). One category of CD4⁺ Treg cells is emerging from the thymus during ontogeny as a population already planned to suppress the response against self-antigens (natural Treg cells; Fig. 1; 1, 2). A similar subset of CD8⁺ thymocytes has also been described in both humans and mice (4, 5). Another category of Treg cells also derives from the thymus but acquires its suppressive activity in the periphery and is devoted to regulate the response not only against self but also against nonself-antigens (adaptive Treg cells; 3). Among these latter, two apparently distinct subsets of CD4⁺ Treg cells have been reported: (i) type 3 T helper (Th3) cells that

can be induced by the oral administration of antigen (6; Fig. 2), and (ii) T regulatory 1 (Tr1) cells, which are induced by the administration of antigen in the presence of interleukin (IL)-10 (7; Fig. 2). Both natural and adaptive Treg cells are antigen-specific, but they exert their regulatory activity in nonspecific way, which may be based on cell-to-cell contact (natural Treg cells; Fig. 1; 1, 2), or the release of suppressive cytokines, such as transforming growth factor (TGF)- β (Th3 cells; 6) or IL-10 (Tr1 cells; Fig. 2; 7).

Markers of Treg cells

A critical problem hindering the study of Treg cells has been the lack of a unique marker that defines all cells with regulatory activity. So far, no specific markers for either Th3 or Tr1 adaptive Treg cells have been described. Natural Treg cells have been first identified for their constitutive expression of the α -chain of the IL-2 receptor (IL-2R), which is named as CD25 (1, 2). However, it is known for many years that one of the first events following the activation of T cells is the induction of the IL-2R and, therefore, even effector T cells can express CD25. It has been claimed that CD25 expression between regulatory and effector T cells is different, being bright in Treg cells (CD4⁺CD25^{high}) and dull in effector T cells (CD4⁺CD25^{low}), so that the isolation of CD4⁺CD25^{high} cells alone can be a good tool to identify and investigate the role of Treg cells (8; Fig. 1). However, the existence of T cells showing intermediate density in the expression of CD25 (CD4⁺CD25^{int}), makes it difficult, if

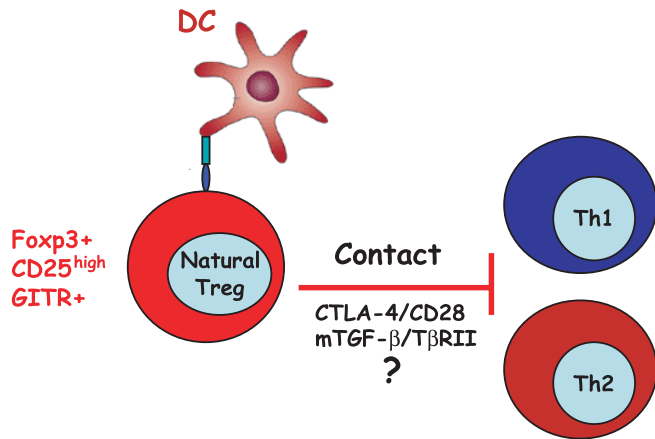


Figure 1. Natural T regulatory (Treg) cells. Natural Treg cells, which originate from the thymus, are characterized by the constitutive expression of Foxp3, glucocorticoid-induced tumor necrosis factor receptor (GITR) and high levels of CD25. Natural Treg cells exert their suppressive effect via a contact-dependent mechanism, probably involving the interaction between cytotoxic T lymphocyte-associated antigen (CTLA)-4 and membrane transforming growth factor (mTGF)- β expressed on their surface following activation and CD28 and TGF- β receptor type II (T β RII) present on the surface of target cells. DC, dendritic cell.

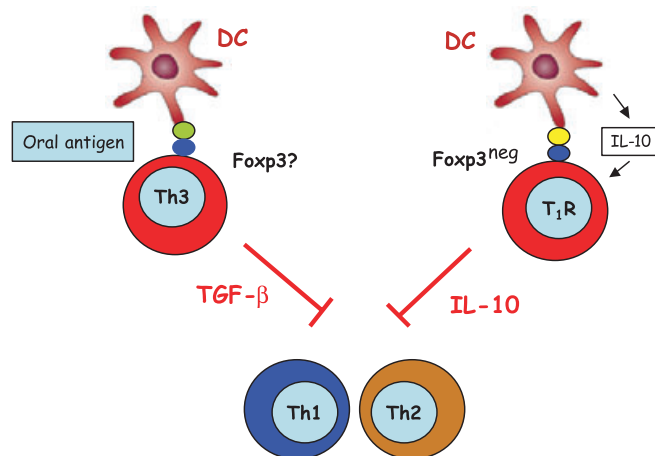


Figure 2. Adaptive T regulatory (Treg) cells, Th3 and T₁R. Th3 cells are induced by the oral administration of antigen and exert their suppressive activity via the production of transforming growth factor (TGF)- β . T₁R cells are induced by antigen administration in presence of interleukin (IL)-10, lack Foxp3, and exert their suppressive activity via the production of IL-10. DC, dendritic cell.

not impossible, to get pure populations of Treg cells, at least from the human peripheral blood. This problem is very critical, because certain functional effects of Treg cells have been certainly observed by using mixtures of regulatory and effector T cells, which may even have led to wrong conclusions. This is probably the reason why in

our previous study performed with CD4+CD25+ thymocytes, which really represent a pure population of Treg cells, as there are no other CD4+CD25+ cells in the thymus, we found partially different mechanisms underlying the suppressive activity in comparison with those reported by studying peripheral blood CD4+CD25+ T cells (9). Other suggested markers of Treg cells, such as cytotoxic T lymphocyte-associated antigen (CTLA)-4 (10), glucocorticoid-induced tumor necrosis factor (TNF) receptor family (GITR; 11), and lymphocyte activation gene (LAG)-3 (12) are not consistently expressed and/or they can also be detected in cell types other than Treg cells.

In more recent years, Foxp3, a member of the *forkhead-winged helix* family of transcription factors, has been reported to be an exclusive marker of natural Treg cells (13; Fig. 1). Retroviral transfer of Foxp3 converts naive T cells into CD25+ Treg. These Foxp3-transduced naive cells have regulatory functions, as demonstrated by their ability to suppress colitis when co-transferred to severe combined immunodeficient mice with naive T cells (13). Even more importantly, mutation of *Foxp3* gene (first described as *JM2*) was found to result in an X-linked autoimmune and allergic dysregulation syndrome, called as XLAAD (14). Mutations in *Foxp3* were subsequently identified as the defect underlying the scurfy mouse and the human X-linked neonatal diabetes mellitus, enteropathy, and endocrinopathy syndrome (IPEX; 15, 16). However, because of its nature of nuclear protein, Foxp3 is of limited value for the isolation of Treg cells. Neuropilin has recently been identified as a potentially useful surface marker of murine CD4+CD25+Foxp3+ Treg cells (17), but it is also expressed by other cell types. More recently, a series of monoclonal antibodies able to react with Foxp3 protein by either flow cytometry or immunohistochemistry was developed (18). Despite these antibodies allowed to confirm that Foxp3 was expressed by the majority of CD4+CD25^{high} and less than half CD4+CD25^{int} T cells in peripheral blood, Foxp3 could also be detected following activation in both CD4+ and CD8+ T-cell clones, thus supporting the view that even if Foxp3 is essential for the development and function of suppressive activity, at least in humans, it is not confined to CD4+CD25+ Treg cells (19).

Treg cells: a dedicated functional lineage, a 'plastic' phenotype or both?

The lack of a unique specific markers for all Treg cells makes it still impossible to solve a fundamental issue regarding the nature of dominant tolerance. Two general models for T cell-mediated immunosuppression have been considered that Treg cells represent a dedicated functional lineage, or that Treg cells represent a 'plastic' phenotype. The first model suggests the existence of a factor responsible for specifying a Treg cell lineage, which

therefore serves as the mediator of genetic mechanisms of dominant tolerance. The second argues that T cell-mediated immunosuppression is not the purview of a dedicated Treg cell lineage but is the consequence of a dynamic balance between cells expressing different amounts of cytokine receptors and different cytokine production profiles (20). Clearly, the identification of mutations in the gene-encoding *Foxp3* as the cause of immune alterations in both scurfy mice (15) and children suffering from IPEX (6) and the subsequent studies of *Foxp3* gene retroviral transfer (13) strongly argue for the idea of Treg cells as a dedicated functional lineage. In addition, analysis of the origin of CD4+CD25+ Treg cells in chimeric mice containing a 1:1 mixture of allelically marked bone marrow stem cells derived from *Foxp3*-deficient and wild-type mice showed that *Foxp3*-deficient bone marrow cannot give rise to CD4+CD25+ Treg cells, thus demonstrating that CD4+CD25+ Treg cell development is critically dependent on *Foxp3* expression (21).

However, there are several issues that remain unresolved before the unique existence of a dedicated functional lineage is accepted. First, in experiments of retroviral transduction with *Foxp3*, a few *Foxp3*+ cells acquired the characteristics of CD4+CD25+ Treg cells, including high expression of CD25 and suppressor activity (13). More importantly, unlike mouse CD4+CD25- T cells, human CD4+CD25- T cells are reported to upregulate *Foxp3* after *in vitro* activation (19). Finally, *Foxp3* expression cannot be detected in Tr1 cells, which represent one of the most important populations of adaptive Treg cells in humans, and CD4+CD25^{high} cells are not required for the development of their suppressive activity (22). These findings would support the possibility that, at least in humans, Treg cells also include a 'plastic' phenotype, which is generated *de novo* from nonregulatory T cells as a consequence of immune activation, and thereby act as negative feedback regulators of conventional immune responses.

Mechanisms of suppressor activity by Treg cells

Even the mechanisms by which Treg cells exert their suppressive activity is still matter of controversies. Most studies agree that although the different types of Treg cells are antigen-specific, all they exert suppressive activity in a nonantigen-specific way. It is also generally accepted that the suppression by Th3 cells is mediated by the release of TGF- β (Fig. 2), whereas Tr1 cells mainly suppress via the release of IL-10 (Fig. 2). By contrast, the majority of reports indicate that suppression by CD4+CD25^{high} *Foxp3*+ cells is due to a cell contact-dependent mechanism in both mice and humans (Fig. 1). However, the surface molecules and the pathways involved in such contact-mediated suppression have not yet been definitively clarified. Initially, an important role

for membrane CTLA-4 and soluble TGF- β in providing the suppressor signal was demonstrated (10). Subsequently, the combined activity of CTLA-4 and membrane TGF- β (mTGF- β) in blocking the proliferation and function of target CD4+ or CD8+CD25- T cells was reported in both mice and humans (9, 23). Of note, in a study performed by using CD4+CD25+ *Foxp3*+ thymocytes, we found that type 1 Th (Th1) cells were more sensitive than type 2 Th (Th2) cells to this CTLA-4/mTGF- β -mediated suppressive mechanisms (24). The hypothesis for an essential role of mTGF- β in inducing the activity of CD4+CD25+ Treg cells was then discounted, because these cells were found to mediate suppressor activity even in the absence of TGF- β 1 production and responsiveness (25). However, an impressive series of more recent studies has definitively proved the role of mTGF- β in the suppressive activity of CD4+CD25+ Treg cells in both humans and mice (Fig. 1). Not only do CD4+CD25+ express latent TGF- β (9, 23), but these cells also bear TGF- β in its active configuration on the cell surface (26). Surprisingly, several studies demonstrated that TGF- β is involved in both the suppressive activity of Treg cells and the expression and/or maintenance of *Foxp3* and of the regulatory activity. In humans, natural and induced CD4+CD25+ T cells can educate CD4+CD25- cells to develop suppressive activity in response to treatment with TGF- β (27). In mice, T cells that cannot respond to TGF- β escape control by CD4+CD25+ Treg cells (28). In an ovalbumin peptide T-cell receptor (TCR) transgenic adoptive model, TGF- β -induced CD4+CD25+ suppressor cells proliferated in response to immunization and inhibited antigen-specific naive CD4+ T-cell expansion (29). Stimulation of both TGF- β and IL-2R were required for *Foxp3* triggering in BALB/c (H-2^d) mice (30). The TGF- β signaling was critical for the *in vivo* expansion and immunosuppressive capacity of CD4+CD25+ Treg cells in mice with dextran sulphate sodium (DSS)-induced colitis (31). In a transfer model of CD4+CD25+ T cells from TGF- β 1-deficient mice into normal recipients untreated or treated with an anti-TGF- β 1 antibody, only untreated animals showed increased levels of *Foxp3* and maintained suppressor function, while TGF- β 1 neutralization resulted in decreased *Foxp3* levels and loss of suppressor function (32).

A mechanism of autocrine/paracrine receptor-ligand interactions has been suggested to explain the suppressive activity exerted by mTGF- β . Treg cells are usually anergic because they express the TGF- β receptor type II (T β RII), which makes these cells susceptible to the activity of the ligand. Likewise, the proliferation of target T cells expressing T β RII (Fig. 1) is also impaired by the interaction with TGF- β -expressing Treg cells, because the primary effect of the T β RII signaling is to block IL-2 production (33). The receptor complex then propagates the signal through the phosphorylation of cytosolic proteins, including the Smad proteins, Smad2 and Smad3

(34), which once activated form a hetero-oligomeric complex, typically including the co-Smad4, and translocate to the nucleus where they modulate transcription of specific genes. However, other intersecting pathways may contribute to the TGF- β signaling cascade (35). The role of CTLA-4 in the suppressive activity of CD4+CD25+ Treg cells (Fig. 1) may be related to the upregulation of TGF- β and T β RII expression on the same cells (26). On the contrary, even the role of GITR, another molecule often associated with CD4+CD25+ Treg cells (11; Fig. 1), can be explained in the context of the TGF- β signaling pathway. It is well known that stimulation of GITR by its ligand or by an anti-GITR antibody results in the impairment of suppressor function by CD4+CD25+ Treg cells, thus providing a counter-regulatory balance for their activity (36). Recent data suggest that GITR stimulation may blunt Smad2/3 phosphorylation, thus interfering with the transduction pathway triggered by the TGF- β /T β RII interaction (26). However, as stated above, the mechanisms by which CD4+CD25+ Treg cells exert their suppressive activity, particular to that dependent upon cell contact, are still a matter of debate.

Possible role of Treg cells in the pathogenesis of allergic disorders

Allergic diseases are chronic inflammatory disorders caused by aberrant Th2-type immune responses against common 'innocuous' environmental antigens (allergens) in susceptible individuals. The discovery of Treg cells has allowed to suggest that genetic and/or environmental factors that blunt the development and/or the suppressor function of these cells may play a role in the pathogenesis of allergic reactions.

There are at least two clear examples of experimentally induced or spontaneous severe genetic alterations which suggest the role of Treg cells in suppressing allergic responses. 17/9 DO11.10 RAG-deficient mice, which only possess monoclonal populations of T and B cells exhibited much stronger immunoglobulin E (IgE) responses than 17/9 DO11/10 RAG+ mice following specific immunization and this response appeared to be strongly down-regulated by transfer of normal CD4+ TCR α / β + T cells, both CD25+ or CD25-, which acted by inhibiting the differentiation of Th2 cells (37). In addition, spontaneous mutations of Foxp3 in both scurfy mice (38) and children affected by IPEX result in the increased production of all cytokines, including IL-4 (14). Most IPEX children suffer not only from severe autoimmune disorders but also exhibit increased levels of IgE, eosinophilia, and food allergy (14). Thus, in both experimental animal models and humans, genetic alterations which heavily hamper the development and/or the function of Treg cells can result in the appearance of multiple and severe inflammatory disorders, including allergy.

A different question is whether a reduced number and/or function of allergen-specific Treg cells may be responsible for the common atopic condition and the consequent appearance of symptoms in allergic individuals. Some recent reports have suggested the existence of defects in the number and/or function of allergen-specific Treg cells in atopic individuals (39–41). Unfortunately, the interpretation of these findings is made difficult by the great heterogeneity of Treg cells and by the lack of specific identification markers (see above). These are probably the reasons why the results of these studies appear to be contradictory. In the first of these studies, CD4+CD25+ T cells from patients with hay fever suppressed allergen-induced proliferation of autologous CD25- cells and their IL-5 production at lower extent than in nonatopic donors, a defect that appeared to be more impressive during the seasonal pollen exposure. The regulatory cells were Foxp3+, but their suppressive activity was not related to CTLA-4, IL-10, or TGF- β (39). In another study, a defect of allergen-specific Tr1-like cells in allergic individuals compared with nonatopic donors was described (40). Surprisingly, however, these Treg cells were CD4+CD25+, like natural Treg cells, which usually do not produce cytokines (1, 2, 9). On the contrary, IL-10-producing Tr1 cells do not belong to the family of CD4+CD25+Foxp3+ cells (7). In addition, the mechanisms of suppression were found to be different from those described by Ling et al. (39), because they involved the activity of IL-10, TGF- β , CTLA-4, and PD-1 (40). In a third study, children who outgrew their cow's milk allergy (tolerant children) had higher frequencies of circulating CD4+CD25+ T cells and decreased *in vitro* proliferative response to β -lactoglobulin compared with children who maintained clinically active allergy. The suppressive activity of these Treg cells was not related to the release of soluble factors, but was due to a cell contact-dependent phenomenon (41). These latter findings are apparently at variance with the results of another study performed in children suffering from peanut allergy (42). Children who outgrew their peanut allergy exhibited increased numbers of allergen-specific interferon (IFN)- γ -producing and reduced levels of IL-4-producing T cells in comparison with those who remained peanut-sensitive, thus becoming nearly similar to peanut nonallergic children. This finding suggests that the lack of peanut allergy or the recovery from this form of hypersensitivity is due to an immune deviation in the specific response from Th2 to Th1 rather than to the enhancement of immune suppression (42).

At present, the more reliable information on the possible role of Treg cells in the control of allergic reactions rests on data obtained in beekeepers and in bee-venom-sensitive subjects (43, 44). Evidence for T-cell production of IL-10 in response to allergen, both in beekeepers who have become tolerant following repeated insect stings and in nonatopic individuals supports the contention that IL-10 regulates tolerance to their

allergen. Moreover, successful allergen-specific immunotherapy (SIT) is associated with a decrease in the allergen-specific Th2 response and the induction of allergen-induced IL-10-secreting cells (43). Surprisingly, however, even IL-10-expressing cells that are detected after allergen SIT have been shown to be CD4+CD25+ (44), arising again the question of the nature of these cells, i.e. if they are natural Treg cells or activated effectors that upregulate CD25 and have deviated from a Th2 phenotype toward an IL-10-secreting phenotype, or a third population that is distinct from either of these. On the contrary, other studies have clearly shown the increase of cells producing both IL-10 and IFN- γ following successful immunotherapy (45), suggesting at least the coexistence of immune suppression and immune deviation in order to explain the induction of clinical improvement. Thus, the existence of a defect of Treg cells as a pathogenic mechanism for the development of atopy, as well as the nature of cells that possibly control the allergen-specific Th2-type immune responses still remain to be elucidated.

Because so far in atopic individuals no alterations of genes controlling the development/function of Treg cells or the production of suppressive cytokines IL-10 and TGF- β have been described, possible defects of Treg cells or of their suppressive activity in these subjects should be related to the interference of environmental factors, and particularly to the effects of microbial infections during early childhood. The concept that the 'hygiene hypothesis' is the most reasonable explanation for the 'allergy epidemics' occurred in the last decades mainly in westernized countries (46) has indeed become stronger after the demonstration that exposure of a pregnant mother and/or her son in the first year of life to microbial products released by farm animals (which is typical of the rural life style) exerts an important protective role against the development of allergy as a consequence of the chronic stimulation of the Toll-like receptors (TLRs) expressed by cells of the innate immunity (47). For some years, the most likely explanation for the increased prevalence of allergy in the last decades has been that the reduction in microbial burden impairs the occurrence of immune deviation from Th2 to Th1, which usually takes places with increasing age. This means that reduced stimulation of TLRs on DCs and natural killer (NK) cells results in decreased production of cytokines, such as IL-12, IFN- α , and IFN- γ , which not only promote the development of Th1 cells, but also antagonize the development of Th2 cells (48). Recently, however, the possibility that the lower microbial burden does not act by inducing a lower production of Th1-polarizing cytokines, but by decreasing the activity of Treg cells has been suggested (49, 50). The view that the lower microbial burden may favor the increased prevalence of allergy by inducing a lower activity of Treg cells is based mainly on emphasis given to some epidemiologic observations, such as the reduced prevalence of allergy in children infested

by parasites (49) and the increased prevalence in developed countries not only of allergy but also of Th1-mediated autoimmune or chronic inflammatory disorders (50). The reasons why I believe that these epidemiologic findings have been misinterpreted or at least oversimplified, while other contrasting epidemiologic observations are usually neglected, have been explicated in a previous review (51). In the same review, the experimental evidence in favor or against the role of reduced immune suppression or reduced immune deviation from Th2 to Th1 in favoring an increased prevalence of allergic disorders has also been extensively discussed (51). As reported there (51) and again discussed here more shortly, the experimental evidence in favor of an important role of Treg cells on dampening allergen-specific Th2 responses is still poor and contradictory, due to the heterogeneity of Treg cells and to the incomplete knowledge of their origin and function. By contrast, an impressive body of data published in the last 10 years has shown that allergen-specific Th2 responses are subjected to the negative control of Th1-polarizing cytokines (IFNs, IL-12), which are produced in response to chronic and repeated stimulation of TLRs present on the cells of the innate immune system. Thus, the whole current knowledge still suggests that IL-12- or IFN- α -producing DCs and IFN- γ -producing NK cells, and Th1 cells themselves, certainly play even more important regulatory effects on allergen-specific Th2 responses than classic Treg cells (Fig. 3). Even very recently it has been reported that lipoprotein I, a TLR2/4 ligand from *Pseudomonas aeruginosa*, is able to suppress allergen-induced airway eosinophilia and to reduce IL-4 and IL-13 production by Th2 cells by inducing a marked increase of the Th1 cytokine IFN- γ

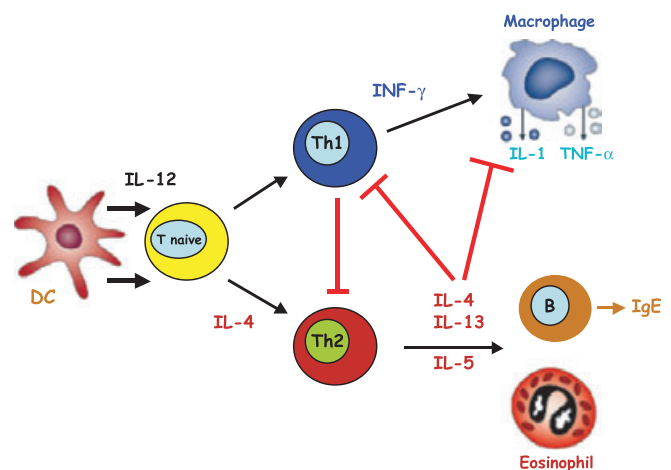


Figure 3. Regulation of effector Th cell responses by immune deviation. Th1 cells produce interferon (IFN)- γ that activates macrophages and inhibits the development of Th2 cells. The Th2 cytokine interleukin (IL)-4 inhibits the development of Th1 cells and both IL-4 and IL-13 exert inhibitory effects on different functions of macrophages.

(52). More importantly, it has been shown that colonization of germ-free animals with *Bacteroides fragilis* results in the correction of systemic T-cell deficiencies and Th1/Th2 imbalances via IL-12-induced IFN- γ production, which was related to the activity of the bacterial polysaccharide PSA (53). This important finding underlines the major role of immune deviation vs immune suppression in modulating the phenotype of Th cell effector function even at level of microflora formation in the gut microenvironment. On the other hand, it is well known that Th2 cytokines can downregulate Th1-mediated responses when they become dangerous for the host, thus behaving themselves as regulatory cells (Fig. 3). In this regard, it is of note that the role of IL-10 in the regulation of Th1 immunity is much less important, whereas the role of IL-4 is much more pronounced, in humans than in the mouse (54). It is also of note that IL-12, in addition to act as the most powerful Th1-polarizing agent, has recently been found capable of allowing CD4+CD25- T cells to escape Treg-mediated suppression to mount physiologic immune responses (55). Finally, a novel subset of adaptive Treg cells able to protect against airway hyperreactivity has been described (56). These cells were induced by activating T cells with CD8 α + DCs, expressed CD25 and Foxp3, and were able to produce both IL-10 and IFN- γ , so they were named as Th1 reg cells (56). In a still unpublished report, we have recently shown that sublingual SIT with a *Dermatophagoides pteronyssinus* monomeric allergoid-induced clinical improvement and downregulated allergen-specific IgE production, which associated with a significant increase of T-cells producing both IFN- γ and IL-10 (L. Cosmi, V. Santarlasci, R. Angeli, F. Liotta, L. Magg, F. Frosali, O. Rossi, P. Falagiani, G. Riva, S. Romagnani, F. Annunziato, E. Maggi, personal communication). Whether these cells induced by sublingual SIT in atopic subjects, which are probably responsible for IgE down-

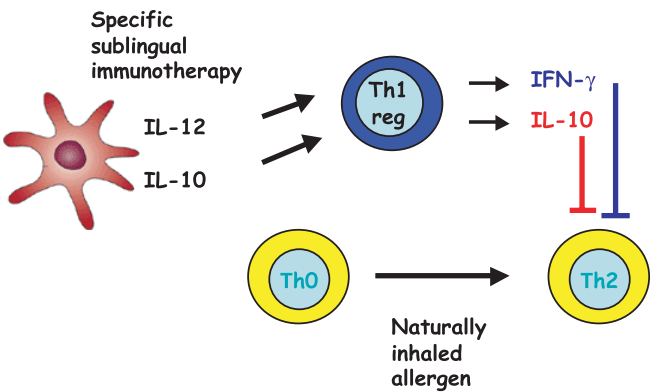


Figure 4. Possible mechanism of action of sublingual allergen-specific immunotherapy (SIT). This type of treatment can induce the activation of Th1 reg that produce both interleukin (IL)-10 and interferon (IFN)- γ , which inhibit the activity of allergen-specific Th2 cells.

Treg type	Origin	Phenotype	Mode of action	Target
Natural	Thymus	CD4+ or CD8+ CD25 ^{high} Foxp3 ⁺	Cell contact (CTLA-4; mTGF β)	Th1/Th2; (mainly Th1)
T ₁ R	Periphery	CD4+ Foxp3 ⁻	IL-10	Th1/Th2
Th3	Periphery	CD4+ Foxp3 (?)	TGF- β	Th1/Th2
Th1	Periphery	CD4+ CD25 ^{low} Foxp3 ^{+/-}	IFN- γ	Th2
Th1 _{reg}	Periphery	CD4+ Foxp3 ⁺	IFN- γ + IL-10	Th2
Th2	Periphery	CD4+ CD25 ^{low} Foxp3 ^{+/-}	IL-4; IL-13	Th1; macrophages

Figure 5. The heterogeneous families of T cells showing regulatory activity.

regulation and improvement of clinical symptoms by producing both IFN- γ and IL-10 (Fig. 4), correspond to the Th1 reg cells described in mice (56) remains to be established. In any case, these findings again support the concept that together with natural Treg cells specifically devoted to the regulation of immune responses specific for autoantigens, a complex panel of cells showing a 'plastic' phenotype, which is generated *de novo* from nonregulatory T cells in the context of a complex cytokine network as a consequence of immune activation is operating in dampening inflammatory reactions, including allergy (Fig. 5).

Possible role of regulatory T cells for the development of new therapeutic strategies in allergy

The emphasis on the importance of the regulatory mechanisms of immune responses has clearly stimulated an intense series of studies devoted to develop new therapeutic strategies for different diseases, including allergic disorders.

Possible regulation of Th2-mediated inflammation by immune deviation

In the last few years in a series of *in vitro* studies, we have clearly shown that cytokines, such as IL-12 and the IFNs, added directly in culture together with the allergen (57–59), or produced by cells of the innate immunity following their stimulation with double-stranded RNA (polyinosinic : polycytidilic acid; 60), microbial CpG-containing oligodeoxynucleotide (ODNs; 61), or synthetic adjuvants, such as imidazoquinolines (62) or a chemically modified adenine (L. Fill., S. Ferri, S. Sampognaro, F. Liotta,

C. Manuelli, A. Mutucci, E. Maggi, S. Romagnani, P. Parronchi, unpublished results), are able to shift the allergen-specific Th2 response to a less polarized, or even to a Th1-polarized phenotype. Of note, IL-12 produced by DCs in response to CpG-containing ODNs was found to be able to induce IFN- γ production even by established allergen-specific Th2 effectors through the upregulation on their surface of the IL-12R β 2 chain (63). Of note, even more pronounced immune deviation from Th2 to Th1 in the allergen-specific response was observed by using allergen : CpG-ODNs conjugates (64). These findings have been confirmed by *in vivo* studies in animal models and more recently even in humans. Experiments using allergens, such as ragweed and house dust mites, demonstrated that allergic asthma could be reduced or eliminated by the administration of CpG-ODNs during or even after the sensitization phase (65). CpG-ODN treatment increased the ratio of IFN- γ - to IL-4-secreting cells by approximately fourfold, decreased the number of allergen-specific IgE-producing cells to an equivalent degree, and diminished the influx of inflammatory cells into the lungs (65). These effects were long lasting; when mice were challenged with allergen some weeks after CpG-ODN treatment, they continued to generate a strong Th1-cell (rather than Th2-cell) memory response, which protected them from lung inflammation (65). This was accompanied by a reduction in allergen-specific IgE and IgG₁ and an increase in IgG_{2a}-secreting cells (65). These outcomes are consistent with the ability of CpG DNA to stimulate the production of Th1-type cytokines that induce isotype switching to IgG_{2a} rather than IgE and IgG₁ (65). The antiallergic effects were observed when CpG-ODNs were administered alone, mixed with allergen or physically linked to allergen, and the greatest success in generating a Th1-dominated response resulted when the CpG-ODN was coupled directly to allergen (66). Conjugation of CpG-ODNs to the short ragweed allergen Amb a 1 enhanced its immunogenicity and reduced its allergenicity (66). At optimal ODN : allergen conjugation ratios, CpG-ODNs not only prevented the development of allergic rhinitis, but also reversed established airway disease (66).

Therefore, clinical trials in allergic patients have been focused on the use of CpG-ODNs conjugated to allergens as an alternative to conventional SIT, because such conjugates could be predictable more potent and the treatment with the conjugate could be devoid of some serious adverse effects. Indeed, it has recently been shown that the administration of Amb a 1 : CpG-ODN in patients with ragweed-induced allergic rhinitis led to a redirection of the allergen-driven immune response from Th2 toward Th1 lymphocytes, being safe and well tolerated (67, 68). Amb a 1 : CpG-ODNs given during winter period resulted in a rapid and persistent downregulation of ragweed allergen-induced production of Th2-type cytokines/chemokines (IL-5, CCL17, CCL22; 66). Conversely, such a therapy caused transient upregulation of the synthesis of ragweed allergen-induced IL-10, as

well as Th1-type cytokines/chemokines (IFN- γ , CXCL9). Cytokine and chemokine responses to unrelated bacterial antigen streptokinase and the global capacity to mount immune responses on polyclonal activation did not change. No clinically significant systemic or local allergic reactions were associated with the administration of the conjugate (67). The reason for the only a transient stimulation of the allergen-specific Th1 response, remaining to some extent in contrast to animal studies, could be the differences in dose of Amb a 1 : CpG-ODNs used (3000-fold lower than in corresponding animal studies; 67). In another clinical trial, Amb a 1 : CpG-ODNs injected in ragweed-sensitive patients six times weekly, immediately before the ragweed season, attenuated an increase in the number of pulmonary eosinophils and IL-4-producing cells. On the contrary, Amb a 1 : CpG-ODN administration increased the number of IFN- γ -producing cells (68). Although no difference between treatment groups was observed in symptom scores or medication use during the first ragweed season, during the second ragweed season, there was a significant decrease in chest symptoms and a trend toward reduced nasal symptoms in the conjugate-treated group (68). Thus, allergen : CpG-ODN conjugates could be considered as promising antiallergy agents influencing the causes of allergy and, therefore, restoring an appropriate immune balance in the allergen-specific T-cell response. Obviously, although the results of these preliminary studies suggest that allergen : CpG-ODN conjugates are apparently safe and well tolerated caution in their use is still desired, as the possibility of long-term side effects especially in the case of systemic administration, cannot be excluded. Perhaps, allergen : CpG-ODNs applied locally may be not only more immunogenic at level of the right site but also safer than classic SIT (69). Thus, a series of Th1-polarizing microbial components or synthetic adjuvants might be used as effective tools for novel types of SIT based on the induction of immune deviation of allergen-specific Th2 responses (Fig. 6).

Possible regulation of Th2 cell-mediated inflammation by immune suppression

The demonstration of the existence of a heterogeneous family of T cells specialized in the control of effector T-cell functions may provide different tools for the control of allergic inflammation and therefore the therapy of allergic disorders (Fig. 7). So far, there is no convincing evidence on the existence of natural allergen-specific CD4⁺CD25⁺ Treg cells. On the contrary, proliferation of Th2 cells seems to be, at least *in vitro*, less susceptible than the proliferation of Th1 cells to the suppressive activity of natural Treg cells, because Th2 cells are more able to escape the suppressive activity, because of their ability to release growth factors other than IL-2, such as IL-4 and IL-9 (24). Moreover, reliable ways for potentiation of the suppressor activity of this type of cells are

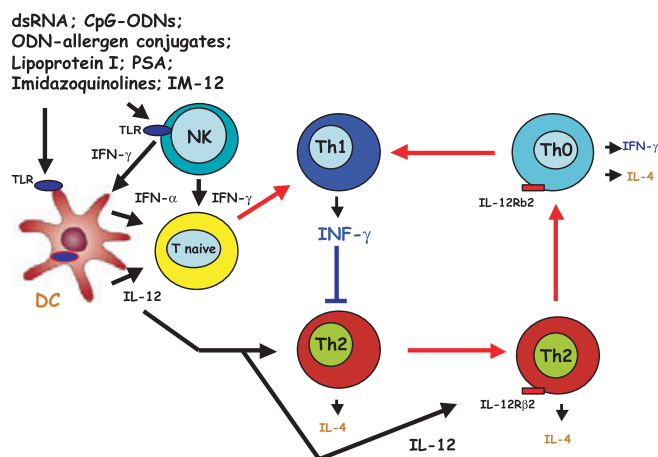


Figure 6. Possible novel therapeutic strategies for the treatment of allergic disorders based on the induction of immune deviation. Different microbial components or synthetic adjuvants injected together with or conjugated to allergen can induce the production of Th1-polarizing cytokines [interferon (IFN)- γ , IFN- α , interleukin (IL)-12] by cells of the innate immunity [DC, natural killer (NK) cells]. IL-12 and IFN- γ promote the polarization of naive Th cells toward the Th1 phenotype. Moreover, IL-12 induces the expression of the IL-12R β 2 chain in established Th2 cell effectors, thus enabling these cells to produce IFN- γ in addition to IL-4 (Th0) or of IFN- γ alone (Th1). IFN- γ produced by Th1 cells in turn inhibits the development of other Th2 cells.

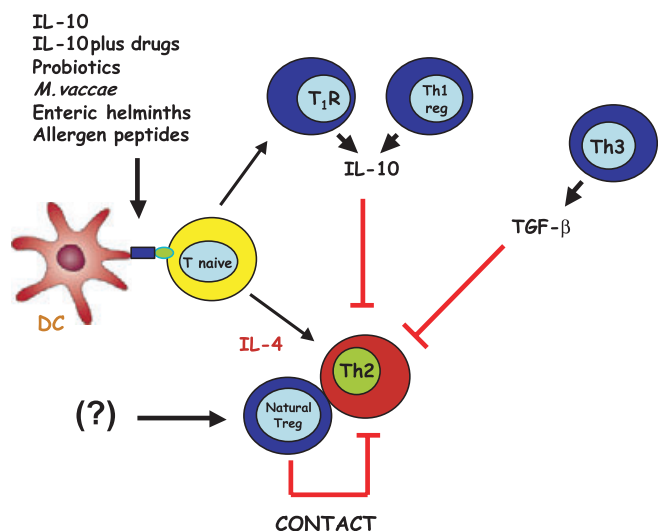


Figure 7. Possible novel therapeutic strategies for the treatment of allergic disorders based on the activation of T regulatory (Treg) cells. While the activation of natural Treg is not desirable, because it may result in pan-immunosuppression, the utilization of some tools able to activate the production of interleukin (IL)-10 and/or transforming growth factor (TGF)- β by allergen-specific T cells has been suggested, but protocols for their use, as well as efficacy and safety, remain to be assessed.

not currently known. Treg cells are refractory to stimulation *in vitro* and are therefore anergic. Treg cells replicate relatively efficiently *in vivo* (70), but protocols that effectively expand Treg populations are not yet available, because in this setting a combination of TCR occupancy, crucial co-stimulatory signals and selective growth factors are essential. Finally and most importantly, natural CD4⁺CD25⁺ + Foxp3⁺ Treg cells exert their suppressive activity by a cell contact-dependent, antigen-independent, mechanism. This means pan-immunosuppression, which makes it potentially very dangerous to stimulate the activity of these cells (provided that allergen-specific natural Treg cells do exist), because immune responses against infectious agents, may temporarily be hampered. Thus, at the present time, both technical difficulties and potential ethical caveats make it unrealistic the use of CD4⁺CD25⁺ Foxp3⁺ Treg cells for the treatment of allergic disorders.

Even the systemic administration of TGF- β or the stimulation of adaptive Treg cells producing soluble TGF- β is certainly not desirable. The TGF- β is known for many years for its oncogenic and immunosuppressive potential, as well as for its ability to induce fibrosis (71). The bronchial remodeling in asthma is mainly due to the fibrogenic activity of TGF- β , just produced locally in exaggerated quantities in the attempt to dampen the inflammatory response (72). At present, IL-10 and adaptive Treg cells producing IL-10 (Tr1 cells; 7) seem to be the most promising candidates for treating allergic disorders (73). Some caveat does also exist for using IL-10 in the treatment of allergic disorders, because in some animal strains IL-10 favors, instead of suppressing, Th2-mediated immune responses (74). Although clinical trials of the treatment of autoimmune disorders using recombinant IL-10 show that, contrary to TGF- β , it is safe and well tolerated (75), the effects exerted by IL-10 on allergen-specific Th2 responses might be different and even opposite according to the genetic background of the allergic recipient. However, a disadvantage of using IL-10 as a therapeutic is that it has a relatively short half-life *in vivo* (75). Moreover, the results of ongoing clinical trials with IL-10 in multiple inflammatory conditions appear so far inferior to the expectations (76). Glucocorticoids have been shown to increase IL-10 synthesis, which associated with an inhibitory effect on CD4+ T-cell activation (77) and this effect can be further enhanced by combining glucocorticoids with vitamin D₃ (78). However, glucocorticoids have been used in clinical practice to treat severe allergic conditions for several decades, so that their side effects are well documented and make it obvious that these drugs cannot be used just as potentiators of endogenous IL-10 production. Other tools capable of inducing, or potentiating the activity of allergen-specific Tr1 cells are desirable. It has been proposed to use oral probiotics, which seem to be able to alleviate clinical symptoms of atopic dermatitis (79). However, the concern is that

whole pathogens might also induce the production of proinflammatory cytokines through TLR ligation, the extent of which will determine whether downregulation of allergic responses or pathology ensues. Indeed, probiotic not only induce IL-10 production, but they also increase the production of IFN- γ (80) or they induce the production of IFN- γ alone (52, 53), thus again supporting the concept that their preventive activity on the development of allergic reactions is mediated by a combined mechanism of immune suppression and immune deviation or perhaps by immune deviation alone. An exception to the rule that bacteria or their components do induce immune deviation rather than immune suppression seems to be *Mycobacterium vaccae*, whose administration during allergen sensitization or challenge suppressed asthmatic features by mediating allergen-specific Treg cells to produce both IL-10 and TGF- β (81). However, the evidence of a possible role of *M. vaccae* as a therapeutic tool for SIT in humans remains to be proven. An enteric helminth infection has also been proposed to protect against an allergic response to dietary antigens (82). However, apart, the current ignorance on the helminthic component(s) possibly responsible for the induction of IL-10 production at gut or systemic level to be used as a preventing or therapeutic tool for allergic disorders, mechanisms other than stimulation of Treg cells can widely explain the reduced prevalence of allergic manifestations in helminth-infested children (51).

Another possible approach to the treatment of allergic disorders by stimulating or potentiating the immunosuppressive mechanisms is the administration of peptides (83). Allergen peptides have the advantage that they do not cross-link adjacent IgE molecules, as the intact allergen does, thus avoiding side effects related to the activation of mast cells and basophils. However, despite evidence from animal models that autoimmune conditions can also be prevented and treated with peptide therapy, the approach has been much slower to translate to the clinic. Administration of a soluble peptide from Fel d 1 allergen to Fel d 1-allergic asthmatic individuals has been shown to result in markedly reduced cutaneous reactions to allergen injection, which associated with decreased Th1 and Th2 cytokines and increased IL-10 production (84). However, future challenges to the development of therapeutic vaccines for allergic disorders based on the use of peptides that can induce allergen-specific suppression require better selection of appropriate molecules, as well as the optimization of peptide dose and route of administration.

Concluding remarks

Over the last years, a heterogeneous family of T cells able to suppress T-cell effector-mediated immune responses has been discovered. They include CD4+CD25^{high}-Foxp3+ cells which mainly act by contact-dependent mechanisms, Th3 cells which act through the release of TGF- β , and Tr1 cells which mainly act via the production of IL-10. Thus, these cells are antigen-specific, but they exert their suppressive activity in nonantigen-specific manner irrespective of whether they act via contact-dependent mechanisms or through the release of soluble cytokines. A unique specific marker for Treg cells has not yet been identified. However, it appears clear that, in addition to natural Treg cells, which represent a dedicated functional lineage, even a 'plastic' regulatory phenotype does exist. It is likely that while natural Treg cells are mainly devoted to suppress immune responses against self-antigens, adaptive Treg cells exploiting their functions even against nonself-antigens represent the result of a plastic phenotype, which can be generated *de novo* from nonregulatory T cells as a consequence of immune activation and thereby act as negative feedback regulators. In this regard, the immune deviation induced by IL-12 and Th1 cells, which downregulate Th2 cytokine production, or by Th2 cells, which dampen the function of Th1 cells and Th1 cell-activated macrophages, can also be considered as a form of regulatory activity.

Although genetic alterations, such as those involving the *forkhead*-winged helix family transcription factor Foxp3, which appears to be critical for the development and maintenance of the suppressor phenotype, result in the development not only of autoimmune disorders but also of allergic inflammatory reactions, the lack of a specific marker for Treg cells makes it difficult to establish whether a reduced number and/or function of allergen-specific Treg cells may be responsible for common atopic conditions and the consequent appearance of symptoms in allergic individuals.

The discovery of Treg cells has also stimulated enthusiasm for the possible development of new therapeutic strategies directed to suppress allergic inflammatory reactions. At present, however, strategies devoted to switch the allergen-specific Th2 response to a less polarized phenotype, such as those based on the administration of allergens conjugated to CpG-ODNs or to appropriate synthetic adjuvants, appear to be more realistic and probably safer than those eventually based on the induction of a general antigen-nonspecific immune suppression.

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