

REVIEW

Animal models of allergen-induced tolerance in asthma: are T-regulatory-1 cells (Tr-1) the solution for T-helper-2 cells (Th-2) in asthma?

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Clinical and Experimental Allergy

Summary

Non-specific anti-inflammatory medication is actually the treatment of choice for controlling the T-helper type 2 (Th-2) cell-driven airway inflammation in asthma. The induction of counterbalancing Th-1 cell clones, long considered a promising approach for immunotherapy, has failed to fulfil its promise because of potentially detrimental side-effects. This is therefore probably not a valid option for the treatment of asthma. With the increasing awareness that active immune mechanisms exist to control inflammatory responses, interest rises to investigate whether these can be exploited to control allergen-induced airway disease. The induction of antigen-specific T cells with suppressive characteristics (regulatory T cells) is therefore a potentially interesting approach. These regulatory T cells mediate tolerance in healthy, non-atopic individuals and have the potential of becoming an effective means of preventing allergen-induced airway inflammation and possibly of suppressing ongoing allergic immune responses. Here we review the available knowledge about allergen-induced suppressive immunity obtained from animal models taking into account the different developmental stages of allergic airway disease.

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Introduction: time for a new paradigm

Basic and clinical research has shown that inflamed airways represent the pivotal hallmark of the immune biology underlying asthma. There is compelling evidence that allergen-induced airway inflammation is orchestrated by T-helper type 2 (Th-2) cells that are activated by matured antigen-presenting dendritic cells (DCs) [1]. These Th-2 cells produce pro-inflammatory cytokines, which in turn activate and recruit a number of inflammatory cells infiltrating the asthmatic airways.

As Th-2 cells and cytokines (mainly IL-4, IL-5 and IL-13) are activated in patients with asthma, their natural counterpart, Th-1 cells, appeared to be suppressed, although this could not be demonstrated invariably. Nevertheless, a paradigm emerged in which an imbalance in the Th-1/Th-2 balance was thought to be the fundamental problem. A number of factors influence this balance including the nature and dose of the allergen, DC and T cell-dependent factors (cytokines and co-stimulatory molecules) and infectious agents, all taking into account the particular genetic backgrounds (for a review, see [2]).

As a consequence, influencing the Th-1/Th-2 balance by interfering with either the Th-1 or Th-2 activity was an

attractive means of controlling the allergen-induced airway inflammation. Induction of Th-1 activity using the cytokines IFN- γ or IL-12 or by administration of infectious agents was tested for counterbalancing the Th-2 activity. These strategies all proved to be efficient in suppressing the eosinophilic airway inflammation, but had the major drawback of causing massive Th-1 cell recruitment and pulmonary inflammation. Treatment of Th-2-driven allergic airway disease with immunostimulatory sequences of DNA containing CpG motifs (also known as ISS DNA, or CpG DNA conjugated with allergen) in animal models of asthma have shown the potential to cause a tolerable Th-1 shift, and some of these ISS DNA-based therapies have entered clinical trials [3]. Selective interferences with protagonists of the Th-2 response including anti-cytokine therapy, blocking the involved co-stimulatory molecules or immunoglobulin (Ig)E are probably too specific and have variable success in controlling the allergen-induced airway inflammation [2, 4].

An increasing body of evidence is now accumulating, suggesting that active immune mechanisms are available for the control and regulation of the immune system to prevent or minimize reactivity to self-antigens or harmless exogenous antigens [5, 6]. Regulatory CD4⁺ T cell

populations have a predominant role in the process, as they constitute a powerful mechanism in controlling immunity-induced damage to the host. However, other cells, including immature DC and plasmacytoid DC [7, 8], macrophages [9], B cells [10], CD8⁺ T cells and $\gamma\delta$ T cells [11], all have also been claimed to have a role in immune control [12].

As allergen-induced airway disease constitutes an unwanted and unnecessary Th-2-driven inflammatory reaction against harmless inhaled antigens, a new concept has emerged where these active tolerance mechanisms fail in patients with atopic diseases such as hayfever or asthma [13]. The balance between the regulatory T cell and the inflammatory Th-2 cell therefore might be decisive in the development of allergic diseases. As animal models proved useful to study the immune biology of allergen-induced airway disease, they may also contribute to new insights into tolerance (failure) in asthma.

Animal models of asthma: limitations for studying tolerance

Mouse models of allergic asthma mimic the human disease and have been mainly used for the study of those factors governing the acute allergic immune response until now. These models are now also being used to study the immune biology of tolerance (failure) in asthma. We therefore thought it important to highlight some characteristics of the animal models, relevant for the interpretation of the results obtained on tolerance.

There is no mouse model that completely reproduces all the features of the human disease, but mice develop a clinical syndrome resembling asthma after sensitization

and respiratory challenges [14, 15]. In the majority of the acute asthma models, systemic priming is necessary and is achieved by intraperitoneal (i.p.) immunization with antigen [mainly ovalbumin (OVA)] precipitated in aluminium hydroxide (alum), the latter also providing a protracted antigen presentation to the immune system. Sensitization via the i.p. route without alum has been reported [16, 17], while intratracheal (i.t.) or intranasal (i.n.) immunization with or without alum is also possible. The i.t. delivery of antigen-pulsed antigen-presenting cells (APCs) equally results in Th-2 sensitization [18]. Although protocols 'without adjuvant' suggested that mice can become atopic by exposure to pure, inert antigens [19, 20], strong evidence indicates that an additional danger signal is necessary to achieve the critical threshold of activated allergen-specific Th-2 cells, called sensitization (Fig. 1). For inert allergens such as the model allergen OVA, it is known that even grade V contains substantial amounts of lipopolysaccharide (LPS), providing such a danger signal. Accordingly, OVA preparations lacking LPS contamination will induce tolerance upon inhalation, whereas minor amounts of LPS promote sensitization. Moreover, TLR-4 knock-out mice, lacking the LPS receptor, appeared unable to mount Th-2 responses in the absence of alum [21]. Other allergens containing intrinsic enzymatic activity, e.g. protease activity in house dust mite (HDM) extracts, are thought to raise endogenous danger signals through this enzymatic activity. This explains why allergic sensitization can be achieved in these models without the aid of alum [22]. It is tempting to speculate that, also in humans, there must be corresponding alarm signals (i.e. adjuvant) that finally induce unexpected DC maturation upon allergen exposure [23].

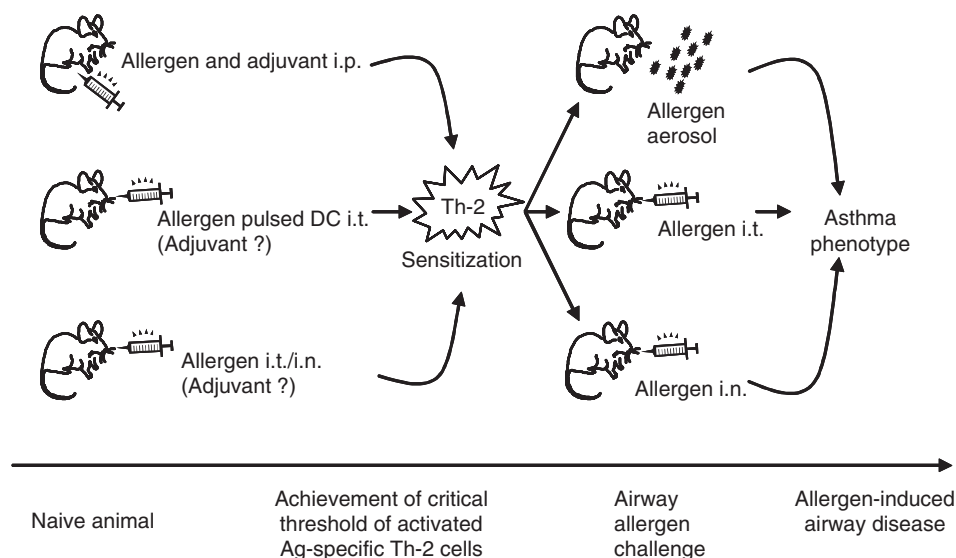


Fig. 1. Schematic representation of the most common animal models of allergen-induced airway disease. An additional danger signal is needed to achieve the critical threshold of activated T-helper type 2 cells (see text for discussion). Not only differences in the protocol of allergen sensitization and challenges but also other factors such as mouse strain, type of allergen, role of adjuvant and other concurrent modifying elements need to be considered.

Subsequent repeated challenge for 2–7 days of the airways either by aerosols, by i.t. or by i.n. allergen administration then results in the Th-2-driven eosinophilic lung inflammation with allergen-specific serum IgE, mucus hypersecretion, airway hyper-responsiveness (AHR) and remodelling.

Besides differences in the protocols of sensitization and airway challenge, other factors need to be considered when analysing these models. These encompass the mouse strain, the nature and allergenicity of the antigen (mostly OVA, but also hen egg lysozyme, HDM allergens, birch and ragweed pollen antigens, cockroach antigens, sheep red blood cells, *Aspergillus fumigatus* extracts and others), the role of adjuvants or danger signals (mostly alum, but also endotoxin present in OVA, enzymes present in HDM extracts, ozone, diesel exhaust particles, cigarette smoke, concurrent viral infections), the way in which the allergen is administered and the presence or absence of some down-regulating factors (e.g. gender, concurrent infections) [15, 24, 25].

We should also consider differences between mouse and human immune biology that might further limit the value of these mouse models. Mast cells, allergen-specific IgE and eosinophils, mediating the early and late allergic response in humans, have shown to be unnecessary for the generation of allergic asthma in mice. Whereas patients with asthma have AHR upon cholinergic challenges, even if they have no symptoms, most of the mouse models exhibit AHR, only very transiently shortly after allergen exposure. And important, in the context of this review, chronic allergen exposure in patients induces chronic symptoms, whereas variable outcomes have been reported in the available mouse models. Although persisting airway inflammation has been reported in an HDM [22] and an *Aspergillus* model [26] and although some reports describe a weak but persisting inflammation after chronic OVA exposure [27, 28], several studies now report that chronic OVA exposure (i.e. weeks) of sensitized mice can cause fading away of the allergen-induced airway disease [29–31], in parallel with progressive airway remodelling.

By consequence, the observations not only from the mouse models of allergic asthma but also from the mouse models of tolerance discussed below always have to take the above-mentioned limitations into account. Extrapolation and interpretation with respect to the human condition based on the mouse data should always be confirmed in the human situation.

New players to the allergy game: focus on the regulatory T cell

CD4⁺ T cells able to suppress active immunity constitute a heterogeneous cell population (Fig. 2).

The naturally occurring T-regulatory cells (T-reg) originate in the thymus as a functionally distinct and mature

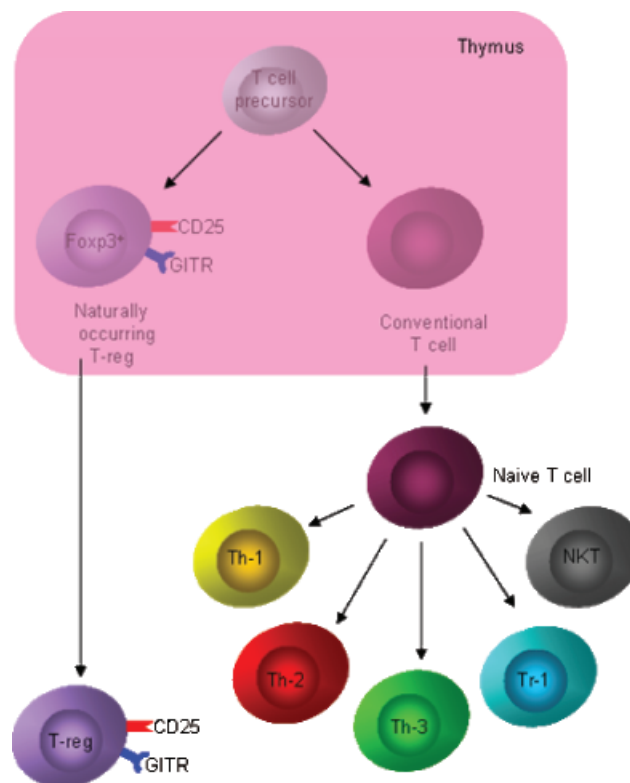


Fig. 2. Schematic representation of the different T cells in the allergy condition. All T cells originate in the thymus; however, the naturally occurring T-regulatory cells represent a separate lineage. The conventional T cells develop into a range of T cells with either pro-inflammatory [T-helper type 1 (Th-1) and (Th-2)] or anti-inflammatory characteristics (Th-3, Tr-1 and NKT).

subpopulation persisting in the periphery [32]. These cells carry out their regulatory function during normal surveillance of self-antigens [5]. T-reg cells constitutively express IL-2R α (CD25), cytotoxic T-lymphocyte-associated molecule 4 (CTLA-4) (CD152) and glucocorticoid-induced tumour necrosis factor receptor (GITR) on their surface. These markers are also up-regulated on effector/memory T cells and are therefore not T-reg specific. For the IL-2 pathway, a critical role in the development of T-reg cells was shown [33, 34]. LAG-3, a CD4-related cell surface molecule, is expressed on the CD4⁺ C25⁺ T-reg cells, but is also involved in induced T-regulatory cell activity [35]. It has to be further investigated whether LAG-3 is a universal T-regulatory cell marker or whether it selectively marks only certain subsets of regulatory T cells [35]. The development and peripheral maintenance of T-reg cells also depend on the presence of CD28/CD80–86 co-stimulation [36], although the regulatory action does not depend on this pathway [37]. The 4-1 BB co-stimulator receptor is constitutively expressed on the naturally occurring T-reg [38]. Signalling after 4-1 BB ligand engagement induces a marked peripheral expansion of the T-reg population [38]. The transcription factor Foxp3

appears to be a master control gene for T-reg cell development and is considered as a marker for this population with good but, as for LAG-3, not absolute specificity [39–42]. Neuropilin-1, a factor involved in T cell activation, has also been suggested as a surface marker for the T-reg cells, but conflicting data have been reported [35, 43].

The naturally occurring T-reg cells suppress the development of Th-2 cells [44]. Although they have the potential to inhibit allergen-induced airway inflammation but not AHR [45], and although there is circumstantial evidence of the involvement of these cells in patients with asthma (for a review, see [46]), it remains to be fully established whether a defective naturally occurring T-reg cell function has a role in the natural history of allergic airway diseases. Moreover, the fact that only small cell numbers can be obtained and that, once activated, their regulatory function occurs in an antigen non-specific manner [47] reduces the potential of these $CD4^+ CD25^+$ T-reg for therapeutic applications.

The adaptive regulatory T cells, on the other hand, do not represent a separate cell lineage, but are derived from conventional T cells. Their generation, expansion and activation occur under certain conditions of (suboptimal) antigen stimulation, and hence, these cells are sometimes termed inducible regulatory T cells. These regulatory T cells are heterogeneous as different phenotypes have been described, among them Th-3, Tr-1 and natural killer cell (NKT) cells. To date, no good selective phenotypic or genotypic markers are available. Th-3 cells have been isolated from mesenteric lymph nodes after tolerance induction with orally administered antigens [48] and are characterized by the production of transforming growth factor- β (TGF- β). NKT cells express surface markers of both NK and T cells, depend on the presence of IL-15 and express a very limited T cell receptor (TCR) repertoire. Both Th-3 and NKT cells appear to be important in the control of certain autoimmune diseases [48, 49].

Tr-1 cells are characterized by the production of IL-10 and TGF- β [50] and were initially generated *in vitro* upon repetitive antigen stimulation with non-professional APCs in the presence of IL-10 [51]. These Tr-1 cells have also been shown to be inducible *in vivo* [52, 53]. The surface marker LAG-3 is expressed on induced regulatory T cells, but also on the naturally occurring T-regs [35]. Signalling through the inducible costimulator (ICOS)–ICOS ligand pathway and also through the IL-6R α chain has been shown to be critically involved in the induction of induced Tr-1 cells and respiratory tolerance [54, 55]. At this stage, it is not clear whether these molecules are good markers for induced T-regulatory cells. Although it was shown that the generation of the induced regulatory T cells does not depend on Foxp3 expression [56], there are an increasing number of reports showing that this marker can be induced equally [42, 57]. As suggested before, Foxp3 is probably not a selective marker. Umetsu et al.

[42] demonstrated that in the presence of an ongoing Th-1 response, a particular population of Tr-1 cells could be generated that not only produced IL-10 but also IFN- γ and even Foxp3. Therefore, it was recently suggested that the Tr-1 cells represent a larger family of IL-10-secreting T cells with regulatory capacities, although this issue remains a matter of intense debate [58]. Once activated by APCs, the inducible T-reg cells exert their immune modulation activity via cell/cell interactions and through the secretion of regulatory cytokines such as IL-10 and TGF- β [50].

The major problem in the study of the inducible regulatory T cells is the lack of good phenotypic and even genotypic descriptors for a consequent identification. To date, identification has been indirect and mainly based on functional (i.e. cell transfer) tests.

Induction and mechanisms of antigen-specific tolerance in mouse models

Antigen-specific tolerance can be generated in naïve mice in numerous ways by taking into account several variables of antigen presentation and immune activation. The three players necessary to induce antigen-specific tolerance are the presence of an antigen, an APC and a T cell (Fig. 3).

A number of antigen-delivery protocols have been shown to be capable of inducing T cells with a regulatory character. As for the animal models of acute asthma, model-dependent factors have to be taken into account when comparing data and making conclusions. Not only the mouse strain, the type of allergen and adjuvant but also the route of antigen administration (oral vs. i.n. vs. subcutaneously) and the duration and the dose all appear to be important in this respect [59].

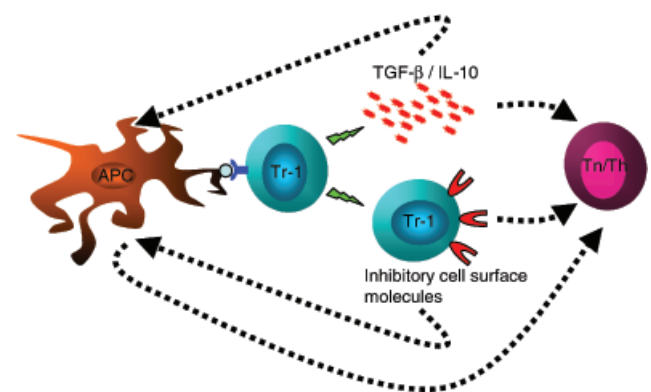


Fig. 3. Antigen-presenting cell (APC), the T-regulatory cell (Tr-1) and the T-helper (Th)/naïve T cell (Tn) in close interplay. When APCs engage the Tr-1, the latter not only up-regulate cell surface molecules but also release immunomodulatory cytokines (IL-10/transforming growth factor- β). The Tr-1 cells inhibit (dotted arrows) the Tn/Th cells either directly or indirectly by affecting the APC.

Several cellular and molecular mechanisms have been proposed to explain how exposure to inhalational allergens in naïve animals can induce a state of allergen-specific tolerance, with a prominent role for the DC at close interplay with the T cell. This 'regulatory' DC population is at least partially decisive whether tolerance is induced or not [8]. Naïve T cells can die, because of a suboptimal costimulatory process by immature DC [60]. This antigen-induced tolerance by deletion of T cells probably represents a coinciding mechanism of antigen-specific tolerance or ignorance. Immature DC, characterized by a low-level expression of the co stimulatory molecules CD40, CD80 and CD86, can directly induce the formation of T cells with regulatory capacities [61]. Interference with the ancillary signals of the antigen-presenting process, including CD4, CD8 [62], CD3 [63], CD154 (CD40L) [64, 65] and ICAM-1/lymphocyte function associated antigen (LFA)-1 [66], but also with more downstream signal-mediating molecules such as NF κ B [67] at the time of TCR engagement, induces regulatory T cells. A third alternate concept indicates plasmacytoid dendritic cells (pDC) as a critical mediator codeciding tolerance.

Whether these pDC induce T-regulatory cells or suppress the generation of T-effector cells directly or indirectly by competing with the stimulatory mature myeloid DC is not clear at this moment. It has been shown that myeloid, but not pDC, are able to induce Th-2 sensitization [68].

Besides the lack of some costimulatory signals (immature DC), the presence of other signals including CD2 and ICOS engaged by, respectively, LFA-3 (CD58) and ICOS-ligand expressed on DC is supportive for the induction of regulatory T cells [54, 69]. Similar findings were reported for CD152 (CTLA-4) [70] and programmed death-1 (PD-1), the latter engaging PD-ligand 2 on the DC [71]. Signalling through NOTCH receptors, implicated in T-cell differentiation, has also been suggested as being relevant for the development of T cells with regulatory capacities [72].

Not only the surface-located receptor-ligand system, but also the soluble mediators IL-10 and TGF- β contribute to the induction of regulatory T cells [73, 74].

Although beyond the scope of this review, it is obvious that manipulation of DC represents a possible gateway for the induction of tolerance. In particular, therapeutic strategies with gene-modified DC lacking costimulatory molecules or DC transduced with retroviruses to produce disease-modifying cytokines or molecules interfering with clonal deletion, are currently under investigation as a potential gateway for the promotion of tolerance. The same holds for selective interference with the allergen-specific Th-2 cell population and its molecular mediators (e.g. IL-4 and IL-5) for which a number of approaches have been tested clinically. Although regulatory T cells will be involved in the above-mentioned strategies of tolerance induction, in this review, we focus exclusively

on the emergence of regulatory T cells upon allergen administration.

As the new concept of tolerance failure has emerged to be important nowadays for the understanding of immune deregulation, we thought it important to place the current available knowledge from the animal models of antigen-specific tolerance in the context of the natural history of the disease (Fig. 4). To summarize the data comprehensively, we systematically present and discuss the available mouse models. For each particular phase, we first consider the effects of oral antigen administration (oral tolerance) and then the effects of antigen administration in the airways (inhalational tolerance) as the two most common routes to induce or restore tolerance, always taking into account the limitations inherent in these models (discussed in paragraph 2).

Animal models of allergen-induced tolerance induction before sensitization took place

The normal response to inhaled allergens is tolerance, characterized by the absence of Th-2 cytokine production or antigen-specific IgE. In subjects who have allergen-induced airway disease, the primary contact with an allergen results in the development of Th-2 cells. The emergence of Th-2 cells is seen as the primary derailment of the immune system and could be the consequence of a defective tolerance mechanism. And thus, several investigators seek to induce regulatory T cells in order to prevent this primary immune derailment (Fig. 4a).

Holt *et al.* showed that feeding OVA before *i.p.* adjuvant-aided sensitization is a simple method to prevent the development of serum IgE [75]. This phenomenon was transferable and mediated by CD4⁺ T cells with suppressive characteristics [75]. Using OVA T cell receptor transgenic mice, it was shown that OVA feeding induced a CD25⁺ CD4⁺ T cell subset. It were these cells that, in contrast to CD25⁻ CD4⁺ T cells, proliferated only minimally to OVA or anti-CD3 and secreted IL-10 and TGF- β [76]. Although the authors proposed that these cells were naturally occurring T-regs, it is likely that they were faced with *in vivo*-induced Tr-1, especially taking into account the cytokine profiles of these cells.

Not only the formation of serum IgE but also allergen-induced airway changes were prevented by feeding antigen before sensitization [77]. This model was characterized by a suppression of Th-2 and Th-1 cytokines; however, administration of anti-TGF- β did not abolish the induction of tolerance [77]. The dose of OVA feeding in these preventive models does not appear critical [78]. The concept of oral tolerance induction to prevent sensitization was confirmed and further elaborated by Russo *et al.* [79], who showed that mice fed with OVA before *i.p.* sensitization were unable to mount airway eosinophilia, the Th-2 cytokines IL-4 and IL-5 or AHR. Interestingly, no

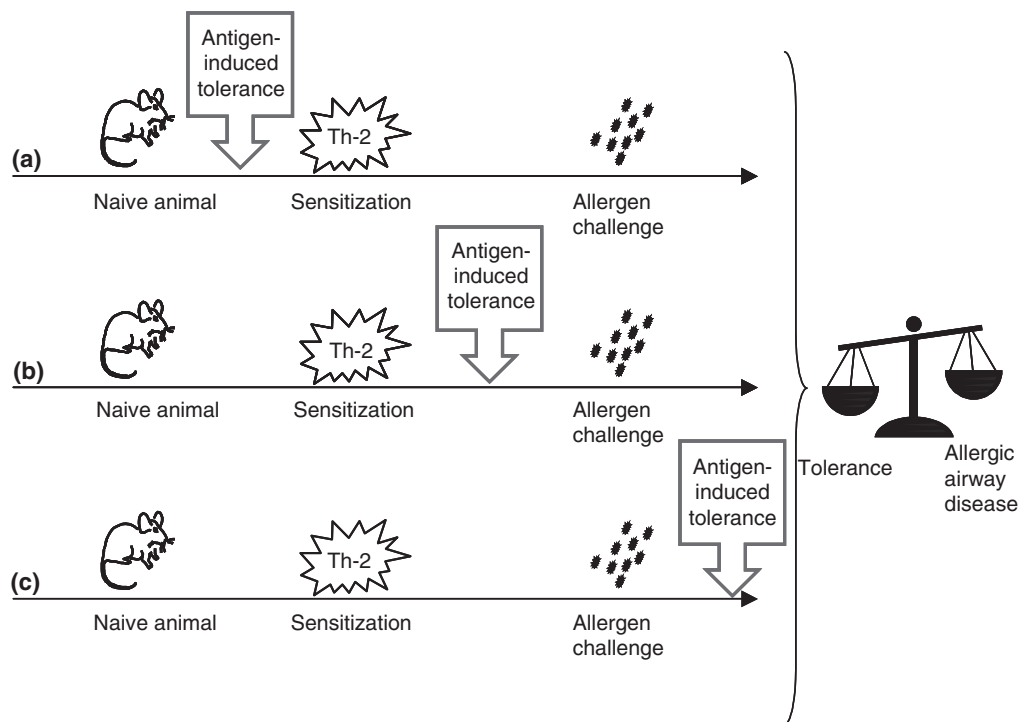


Fig. 4. In the pathogenesis of asthma, several stages can be distinguished. The first is the sensitization phase in which the primary allergen contact induces a Th-2 cell development and activation. The second is the effector phase in which sensitized atopic subjects develop allergen-induced airway changes upon allergen exposure. Chronic airway disease is then characterized by airway remodeling, representing the third phase. In this review, we summarize the data available on the potential of antigen-induced tolerance in controlling allergen-induced airway disease, taking into account the different stages of the disease.

changes in Th-1 cytokines (IFN- γ) were observed, while IL-10 and TGF- β also did not change in the bronchoalveolar lavage fluid (BALF) compartment upon this oral tolerization protocol. Although the absence of effects on using antibodies against presumed important mediators or the absence of changes in the mediator concentration in BALF might be indicative, these findings certainly do not exclude the crucial involvement of these mediators.

Very recently, it has been shown that oral antigen administration prior to i.p. alum-aided immunization induced antigen-specific CD4⁺ CD25⁺ CD45RB^{low} Foxp3⁺ T-regulatory cells different from the naturally occurring T-reg cells. The antigen-induced regulatory T cells had a suppressive activity effective in preventing Th-2-driven allergen-induced airway disease, which was independent of IL-10 and partially dependent on TGF- β [57].

Almost 25 years ago, it was shown that inhalational OVA in naïve animals induces a barely detectable level of IgG1 but no IgE. Inhalational OVA, furthermore, prevents the formation of IgE upon subsequent i.p. allergen (with or without adjuvant) administration [80, 81]. Seymour et al. [82] confirmed this finding and showed that the effectiveness of aerosolized OVA as a pre-treatment before alum aided sensitization and airway challenge does not depend on CD8⁺ T cells or TCR- γ/δ ⁺ T cells. Tsitoura et al. [83] showed that i.n.-delivered OVA before i.p. sensitization

and airway challenge was effective in the prevention of airway eosinophilia and hyper-responsiveness. Here, treatment with anti-IFN- γ , but also with anti-IL-10 and anti-TGF- β did not abrogate the preventive effects of this tolerization protocol [83, 84]. Also, using IFN- γ knock-out mice (KO) and IL-10 KO mice, the protective effects were suggested to be independent of both cytokines [85]. These findings do not exclude a role for these cytokines with certainty. The authors only measured total cell recruitment into the BALF as a parameter; moreover, the possibility of the involvement of compensatory mechanisms was not considered. The finding that the regional lymph node system constitutes a unique and necessary micro-environment enabling the rapid induction of regulatory T cells was convincingly shown because lymph node removal abrogated the induction of tolerance [86, 87].

Although based on *in vitro* generated Tr-1 cells (exposure of naïve Ag-specific T cells to OVA in the presence of IL-10), Groux et al. [88] showed that it was possible to inhibit IgE synthesis and Th-2 cell development *in vivo*. These experiments serve as the proof of concept for the functionality of T-cell-mediated tolerance vs. Th-2 immunity during the primary antigen contact. It is not surprising that the effects were IL-10 dependent as the *in vitro* Tr-1 cells not only produced IL-10, but they were also generated in the presence of IL-10. The direct

evidence showing that suppressive CD4⁺ T cells are induced *in vivo* after OVA aerosol for 10 days in naïve animals was provided recently by Ostroukhova et al. [89]. CD4⁺ T cells from spleens of tolerized mice were adoptively transferred to naïve animals. Although these mice were immunized with OVA-alum and challenged with OVA aerosols, they were unable to mount allergic airway disease. As for oral tolerance induction [57], an up-regulation of Foxp3 was noted in the activated T cell population, again suggesting that this marker is not unique for the naturally occurring T-regs. Neutralization of TGF- β prevented this inhibitory effect, which was not seen with neutralizing IL-10 antibodies [89].

Not only the T cell but also the DC can transfer a state of unresponsiveness. Transfer of DC from mice that entered a state of tolerance after exposure to i.n. OVA equally prevented the development of allergic sensitization with alum-OVA as shown by a defective OVA-induced proliferation of spleen cells [74]. Whether the airways were protected against allergen-induced airway disease was not investigated. Interestingly, DC from IL-10 KO mice were not able to transfer tolerance suggesting that IL-10 is more critical at the level of antigen presentation and communication with the T cell, than it is at the effector stage of the regulatory T cell. Also, at the intercross of DC and T cell, a role of B7RP-1, one of the ligands of ICOS, has been demonstrated in nasal tolerance [90]. Using B7RP-1 KO mice, it was shown that the ICOS/B7RP-1 pathway is redundant for the generation of Th-2 responses but essential for the induction of inhalation tolerance.

Although OVA has been used in most of the animal models, this antigen has no clinical significance. Investigations with clinically relevant allergens are sparse. HDM allergens deserve special attention in this respect. It has been shown that continued (7 weeks) exposure of the airways to HDM extracts overcomes tolerance and elicits chronic airway inflammation and structural remodelling [22]. This represents a fundamental difference from OVA. It is well known that the main antigens of HDM, Der p 1, 3, 5 and 9, are cysteine and/or serine proteases, and *in vitro* studies demonstrated that these antigens are able to stimulate airway epithelial cells to produce cytokines, including granulocyte-macrophage colony-stimulating factor, likely through a protease-activated receptor-2-mediated mechanism. *In vivo*, Gough et al. [91] demonstrated that the proteolytic activity of Der p 1 enhances the allergenicity of this antigen in a conventional (i.e. peritoneal sensitization) acute murine model of airway inflammation. However, in a model of HDM-induced allergic airway disease, the administration of allergen with TGF- β *in vitro* generated suppressor CD4⁺ CD25⁺ CD45⁺ RB^{low}-Foxp3⁺ T cells from naïve peripheral T cells. These cells prevented the development of airway eosinophilia [92], supporting the findings of the OVA models.

Whether tolerance could be induced by administration of allergen fragments or modulated allergens has also been investigated in this preventive setting. In contrast with findings for OVA epitopes [93], it was shown that mice exposed i.n. to a major epitope of the HDM allergen Der p1 before i.p. allergen-adjuvant administration had a suppressed IL-2 production by the lymph node T cells next to a suppression of IgE synthesis [94]. In line with this, Bet v 1 and micro-encapsulated Der p1 immunodominant epitopes were capable of inducing features of linked tolerance [95–99] with important roles for both IL-10 [98] and TGF- β [96]. Whether these models of linked tolerance are actively mediated through the induction of regulatory T cells has not yet been proven.

In summary, there is an increasing body of evidence supporting the new paradigm that allergic sensitization in mice is related to failing tolerance mechanisms. It is very difficult to prove that a single cell, *in casu* the induced regulatory T cell, has an important role in the complex multicellular immunopathology underlying allergic sensitization and tolerance failure. However, animal models prove suitable for this purpose. These animal models show that it is feasible using both oral and i.n./airway antigen delivery to promote a tolerogenic immune response, so allergic sensitization with subsequent Th-2 cell development becomes inhibited. They also show that this tolerogenic immune response involves DC and T cells. The role for the antigen-induced regulatory T cell was proven by means of transfer experiments where these cells have been shown to be capable of preventing subsequent allergic immunization. The demonstration that sensitization is associated with a selective depletion of these cells and inversely that a selective depletion causes sensitization would be very valuable. However, these experiments are still hampered by the lack of specific surface markers and thus depleting antibodies. The immunomodulatory cytokine IL-10 appears crucial in the tolerogenic activity of DC, while this has not been demonstrated unequivocally for the induced T-regulatory cell. TGF- β emerges as a key mediator for the suppressive effector function of the induced T-regulatory cell in preventing allergic sensitization.

Animal models of allergen-induced tolerance repair after sensitization took place

Once subjects show raised Th-2 cell clones and produce IgE, they become prone to the development of allergic diseases. As a consequence, it is interesting to analyse whether tolerance can be re-established by administering antigens in order to control and thus to prevent allergen-induced airway disease (Fig. 4b).

Haneda et al. [100] demonstrated that high-dose OVA feeding (100 mg/days) of sensitized OVA TCR transgenic mice inhibited the development of airway eosinophilic inflammation induced by i.t.-administered antigen. CD4⁺

T cells mediated this antigen-specific process, and the authors elegantly demonstrated a critical role for TGF- β but not IFN- γ . Low-dose OVA feeding (100 μ g/days) had very similar effects resulting in the suppression of Ag-specific IgE along with a down-regulation of costimulatory molecules on the APCs [101]. This concept was further elaborated in a model where mice received allergen dissolved in drinking water during 5 days showing a preventive effect on the development of IL-5-driven airway eosinophilia and IL-4-driven serum IgE [79]. There is one report where analogous findings were found for mice sensitized with the recombinant HDM allergen Der f 2. Feeding these mice with crude mite extracts before allergen challenge had effects similar to those described in the OVA models of tolerance [102]. These findings suggest that the observations are conceptual and not restricted to one allergen.

Although there is a clinical concern that the local i.n. delivery of allergens in the airways of sensitized subjects may induce an asthmatic response, animal models exist where repeated (weekly doses) i.n. low-dose (10 μ g) OVA had inhibitory effects on the subsequent development of allergen-induced airway changes [103]. Absence of IL-5-induced airway eosinophilia and AHR was documented upon weekly (four times) i.n. administration of allergen. The local administration of allergen-specific IgG before OVA challenge also prevents allergic airway responses. This protective effect was mediated through the Fc γ -R on the macrophage and was critically dependent on the presence of IFN- γ [104]. It remains unclear whether the latter finding indicated immune deviation (role of IFN- γ) rather than T cell-mediated tolerance. Van Oosterhout et al. [105] demonstrated that subcutaneous OVA in sensitized mice prevents the development of Th-2-driven allergen-induced airway disease and provided evidence for the critical role of IL-10. Discrete molecular changes of the immune-dominant epitope (OVA 323–339) are decisive for the protective effects [106].

Although the models described above suggested the involvement of a specific regulatory T cell population, Umetsu et al. [54] provided proof for the concept that, despite allergic sensitization, airway disease could still be prevented with regulatory T cells. *In vitro*-generated Tr-1 cells transferred after OVA sensitization were capable of inhibiting the development of Th-2-driven allergen-induced airway inflammation, serum IgE and AHR. This protection was abolished by the administration of anti-IL-10 and was critically mediated through ICOS/ICOS-ligand communication between regulatory T cells and DC [54].

As for the findings with OVA, findings with clinically more relevant allergens have been reported in mice that were sensitized. The i.n. administration of recombinant major birch pollen allergen Bet v1 in animals sensitized three times with a 4-day interval before the allergen aerosol was effective in suppressing allergen-induced air-

way inflammation [107]. Lower levels of Th-2 cytokines and IgE underscored the immune regulatory effects of this treatment. The i.n. administration of microencapsulated immune-dominant epitopes of Der p1 allergen in sensitized mice prevented HDM-induced eosinophilic airway inflammation. This was not observed with the Der p1 protein, indicating that established Th-2 activity can only be controlled under specific conditions of epitope engagement [98].

Taken together, these data provide evidence that administration of allergen under certain conditions can restore a state of tolerance, protecting the sensitized subject from developing subsequent allergen-induced airway disease. Both local (airways) and systemic (oral or subcutaneous) administration appear effective, although one report suggests the superiority of local treatment strategies [103]. Only in OVA models thus far have *in vitro*-generated Tr-1 cells been shown to be capable of mediating this immune modulation along with the critical interference of IL-10. The evidence is still based on very sparse reports. There are no data on the role of DC or TGF- β in this regard.

Animal models of allergen-induced tolerance repair following allergen-induced airway inflammation

Models of tolerance induction/repair have all been developed to gain an insight into the underlying cellular and molecular mechanisms. Preventive medicine for allergic airway disease is only possible when we understand these mechanisms. Unfortunately, we come across asthma patients who had already developed allergen-induced airway disease. The most relevant question is whether tolerance can be restored in these patients in order to control the Th-2-driven airway disease. In line with this, it will be intriguing to determine whether this concept could be applied to cure the disease (Fig. 4c).

Contrasting with the findings of tolerance induction when allergens are administered orally before the systemic sensitization or before airway challenge, oral administration of allergens after mice developed allergen-induced airway disease was not unequivocally effective for the induction or restoration of tolerance. Chung et al. [78] showed a moderate but significant suppression of allergen-induced airway eosinophilia, Th-2 cytokine production and AHR in a model of 'mild' asthma, whereas no effects were seen in a model of more pronounced allergen-induced airway disease. Even more dramatically, an exacerbation of asthmatic features was documented with increased eosinophilic airway inflammation but without effects on the cytokines IL-4 and IL-5 measured in the BALF [79]. In these models, the observations were limited to a phenotypical characterization. There is no evidence at all concerning the possible involvement of regulatory T cells.

It is well documented that inhalation of allergens in sensitized mice (or atopic individuals) quickly induces a Th-2-driven eosinophilic airway disease. However, some inhalation protocols have been developed that were apparently successful in reversing the established airway inflammation back to the normal condition. These protocols were, until now, invariably based on the experimental allergen OVA as chronic exposure to HDM extracts or to *Aspergillus fumigatus* can induce chronic airway inflammation [22, 26]. Whether LPS contamination plays a role in these OVA protocols that restore tolerance is not yet known. Swirski *et al.* [29] reported complete resolution of allergen-induced airway eosinophilia after prolonged (4 weeks) exposure to OVA. The duration of exposure determined the response of the memory T cells to a subsequent re-challenge. Mice exposed to OVA for 2 weeks displayed an extensive inflammatory reaction upon re-exposure, while mice that were initially exposed to OVA for 4 weeks had dramatically reduced tissue inflammation. This suppression could be reversed by i.n. delivery of rGM-CSF. Sakai *et al.* [31] showed that a prolonged (6 weeks) exposure to OVA results in a marked suppression of OVA specific IgE, eosinophilia, Th-2 cytokines and AHR. In this model, there was no change in INF- γ , although an increase in IFN- γ -induced IL-12 production was noted. Schramm *et al.* [30] extended this protocol and showed that not only the duration but also the sustained character of airway OVA challenge is an important factor for the restoration of allergen-induced tolerance. Daily OVA-exposed mice (4 weeks or 8 weeks) had attenuated allergen-induced airway disease while re-challenging these mice after another 4 weeks induced a renewal of the airway inflammation. Interestingly, this way of tolerance induction promoted bystander tolerance against unrelated allergens. In this model, there was also no role for the $\gamma\delta$ -T cell as continuous allergen exposure still induced tolerance in the $\gamma\delta$ -TCR KO mice. The immune modulating cytokine IL-10 was up-regulated in the mice that were continuously challenged. However, there was no direct proof of Tr-1 involvement [30]. In an analogue model, we show that in animals sensitized with OVA aerosols for 6–8 weeks, administered only three times a week, equally resulted in a gradual disappearance of the Th-2-driven airway inflammatory changes. These mice were equally protected against secondary immunization with the same or with other allergens (our data). There are no data available for the other allergens such as those derived from HDM. No data are available so far on the role of Tr-1, DC or TGF- β .

Collectively, few reports exist suggesting that established allergen-induced airway disease can be controlled under certain conditions of allergen administration. Especially, the continued local delivery of allergens in to the airways might induce a tolerance restoration. Here, we are confronted with the limitations of the animal models.

Whereas the mice restore their natural tolerance, asthma patients just become worse upon continued allergen inhalation.

From the few animal models to date, there is no direct evidence for the involvement of regulatory T cells in the restoration of tolerance upon continued allergen exposure. The reports, nevertheless, demonstrate the flexibility of the immune system to control established allergen-induced airway disease, and the need for further investigations.

Conclusion

The immune system not only serves to protect against harmful pathogens but it is also equipped to prevent active immunity against harmless antigens. These mechanisms explain why the normal functional outcome after inhaling harmless allergens is tolerance. Based on the animal models summarized above, there is an increasing amount of evidence supporting the new paradigm where a failure of tolerance instead of a failure in Th-1 cell development plays a prominent role in the immune biology of allergic airway disease.

Although several mechanisms of failing tolerance have been suggested [8], here we reviewed the data available from those animal models where the investigators also tried to induce or to restore allergen-specific tolerance (Fig. 4). Antigen-specific tolerance implies that allergens are applied to induce T cell populations characterized by immune suppressive effects.

From the reviewed data, it was found that the majority of the reports investigated the effects of tolerance induction in a preventive setting, i.e. before adjuvant-aided allergen sensitization and challenge took place. There is abundant evidence indicating that OVA administration in naïve animals induces tolerance that protects against manipulations that normally induce allergic sensitization and Th-2 cell development. This concept also works for other allergens, some of which have clinical relevance. The protective effect is mediated through a regulatory T cell population in close interplay with the DC. Here, both IL-10 and TGF- β represent the critical mediating cytokines, although their relative contribution is unclear and sometimes conflicting, possibly because of model-related factors.

Few reports have investigated the effects of tolerance induction in atopic subjects prone to developing allergic airway disease. Nevertheless, also in this setting, it appears possible to induce/restore tolerance by administering (oral/subcutaneous and even i.n.) allergens in order to inhibit allergen-induced airway disease. With transfer experiments, it was shown that induced regulatory T cells can reverse the Th-2-dominated immune deviation, although additional experiments underscoring this concept are warranted.

Very sparse reports exist where antigen-specific tolerance is induced or restored in mice already suffering from allergen-induced airway disease. Nevertheless, OVA-sensitized mice that continuously inhale OVA appear to restore their tolerant state. This might indicate that mice that normally do not develop asthma in the absence of a 'forced immunization' can overcome the immune deviation and restore their normal tolerance mechanisms. No direct proof of the involvement of antigen-induced regulatory T cells has been provided so far in this context.

As we have strong arguments from these animal models for the plasticity of the immune system to switch between allergen-induced T cell-mediated inflammation and tolerance, the main challenge is now to further elaborate the underlying mechanisms of tolerance failure and to investigate the possibilities to induce or restore tolerance against allergens, which is the normal situation.

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