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REVIEW ARTICLE

Antigen-specific regulatory T-cell responses against aeroantigens and their role in allergy

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The mucosal immune system of the respiratory tract is specialized to continuously monitor the external environment and to protect against invading pathogens, while maintaining tolerance to innocuous inhaled particles. Allergies result from a loss of tolerance against harmless antigens characterized by formation of allergen-specific Th2 cells and IgE. Tolerance is often described as a balance between harmful Th2 cells and various types of protective "regulatory" T cells. However, the identity of the protective T cells in healthy vs. allergic individuals or following successful allergen-specific therapy is controversially discussed. Recent technological progress enabling the identification of antigen-specific effector and regulatory T cells has significantly contributed to our understanding of tolerance. Here we discuss the experimental evidence for the various tolerance mechanisms described. We try to integrate the partially contradictory data into a new model proposing different mechanism of tolerance depending on the quality and quantity of the antigens as well as the way of antigen exposure. Understanding the basis of tolerance is essential for the rational design of novel and more efficient immunotherapies.

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INTRODUCTION

The intestine and the respiratory tract are the two largest mucosal surfaces of the human body, which are optimized for interacting with the environment to allow gas and nutrient exchange. This renders these barrier organs vulnerable for invading pathogens. Thus, the mucosal immune system has the important task to protect against pathogens but to maintain a state of unresponsiveness, termed immunological tolerance, against a large number of harmless environmental substances, such as food or commensal microbiota, as well as inhaled particles, such as plant pollen, pet dander, or insect excretions.

Loss of tolerance results in immune pathology, such as inflammatory bowel diseases (IBDs), which is thought to be driven by exaggerated immune responses against the intestinal microbiota[1] or allergic reactions against innocuous substances such as food or inhaled particles.[2,3] Within the immune system CD4+ T helper cells seem to play a central role for maintenance of the immunological balance. Effector T cells keep potentially pathogenic microbes at bay and various types of regulatory T cells (Tregs) have been described, which suppress or balance unwanted inflammatory responses. In the gastrointestinal tract, especially Foxp3+ Tregs have been convincingly shown to maintain the intestinal homeostasis (reviewed in ref.[4]). However, due to the complexity of the intestinal microbiota, we are only beginning to understand which antigens induce effector T cells and Treg responses, and how the balance between both is regulated on the antigen-specific level in health and disease. The regulation of intestinal immune reactions by antigen-specific Tregs has comprehensively been reviewed recently.[4]

In the airways, allergic diseases against harmless inhaled particles represent a considerable and strongly increasing health problem in industrialized countries.[5,6] In contrast to the largely undefined target antigens in IBD, allergies against airborne particles represent highly specific, IgE-mediated immediate-type hypersensitivities against a restricted subset of proteins (allergens) released from inhaled particles. Allergen-specific IgE is the central mediator of the allergic reactions. The formation of IgE-secreting plasma cells requires help from allergen-specific Th2 cells. Thus, loss of tolerance requires uptake of the allergen by antigen-presenting cells (APCs) and activation and formation of allergen-specific Th2 cells as the initial event underlying allergy development.[5,7,8] However, even in highly atopic donors, allergy only develops against a limited number of "allergenic proteins" and typically only after several years of exposure, while against the majority of proteins constituting inhaled particles, tolerance is maintained. This highlights that tolerance against aeroantigens is a highly efficient process for most proteins, and that evasion from this tolerance mechanism by allergenic proteins is actually a rather rare event even in susceptible donors. Thus, allergies against inhaled particles represent unique examples for antigen-specific loss of T-cell tolerance, which can also be reversed by antigen-specific immunotherapy (SIT) with allergen extracts.[9]

However, despite the fact that the disease-inducing antigens are well-characterized and that even successful tolerance induction protocols exist, the basic mechanisms mediating tolerance against inhaled antigens are controversially discussed, such as the role of various types of T cells with protective capacity.[10,11] This is primarily due to technical problems since antigen-specific T cells are rare and therefore their direct identification and characterization is challenging.[12,13] Most information on antigen-specific lymphocytes during tolerance development is therefore derived from experimental animal models, although they do often not

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recapitulate critical aspects of natural allergen exposure in humans. Especially T-cell receptor (TCR) transgenic systems and recently peptide-major histocompatibility complex (pMHC) class II multimers allow to follow the reaction to defined inhaled antigens at the level of antigen-specific T cells. In humans the results are highly variable and largely influenced by the applied analysis technique, namely direct analysis using tetramers and short-term antigen activation vs. prolonged in vitro cultures [reviewed in refs. [12–15] (details see below)]. Furthermore, almost all studies in humans and murine models concentrate on the few well-characterized IgE-binding allergenic proteins, although additional proteins may be recognized by Th2 cells, independently of IgE.[16] In addition, specific IgG to aeroantigen proteins seems to precede or accompany an IgE response to the same antigen and may therefore predict the onset of an allergic response.[17] Importantly, the non-allergenic proteins derived from inhaled particles are so far largely ignored, although they represent actually the vast majority of inhaled proteins and do not elicit Th2 responses. These non-allergenic proteins may in fact be important targets for Foxp3 + Tregs in the humans as recently shown by our group.[18]

Here we review the current experimental evidences for the various models how aeroantigen-specific tolerance is established and discuss which airborne antigens are actually encountered by the immune system. In particular, we focus on the available data about antigen-specific Tregs, which have the capacity to actively prevent or control Th2 reactions and therefore represent an attractive therapeutic target. The molecular mechanisms for induction of Th2 responses and their downstream effector functions are not part of this review but have been extensively reviewed elsewhere.[5,7,8]

The main questions we address are:

1. Which antigen-specific tolerance mechanisms against inhaled antigens are described?
2. How does the technological approach for antigen-specific T-cell analysis impact on the result?
3. Which inhaled antigens actually get into contact with the immune system as a pre-requisite for active tolerance induction?
4. What is the experimental evidence for the different types of T-cell responses against inhaled antigens?
5. What are the effects of antigen-SIT on the specific T-cell subsets?

WHICH ANTIGEN-SPECIFIC TOLERANCE MECHANISMS AGAINST INHALED ANTIGENS ARE DESCRIBED?

Mechanisms of tolerance

Tolerance against exogenous, non-self-antigens can be achieved by various not mutually exclusive mechanisms. These comprise passive and instable forms, such as ignorance or antigen-specific T-cell deletion or anergy and deviation into non-allergenic T helper cell subsets. These mechanisms bear the intrinsic risk of remaining or newly generated naive T cells, which are still susceptible to develop into allergenic Th2 cells. In contrast, the induction of T cells with immunosuppressive function represents the most efficient way to generate a potentially stable state of tolerance, due to suppression of Th2 formation or effector functions. However, many different types of "regulatory" or "suppressor" T cells have been described, which significantly differ with regard to their origin, inducing conditions, long-term survival, lineage stability, and effector functions. To better understand the mechanisms of tolerance induction and maintenance it is essential to differentiate between the various "regulatory" T-cell subtypes and discuss their contribution to tolerance against different types of antigens. The two major groups discussed here are the Foxp3 + Treg lineage vs. various types of interleukin (IL)-10-producing T cells, which can so far not be clearly assigned to a defined lineage as outlined below.

In any case, the differentiation between these forms of tolerance requires direct quantitative and qualitative analysis of all subsets contributing to the antigen-specific T-cell compartment, which can be technically challenging, especially in humans.[12,13]

Foxp3+ Tregs

The best-characterized lineage of immunosuppressive T cells is the Foxp3+CD25+ Tregs.[19] Tregs are essential for immune homeostasis, mucosal tolerance, and in particular for the prevention of broad allergic responses to environmental antigens,[20] also demonstrated by the fact that patients with *Foxp3* deficiency develop hyper IgE syndrome already early after birth.[21] Tregs are generated as a separate T-cell lineage in the thymus (tTregs) and directed by the lineage-defining master transcription factor Foxp3. They are selected for high affinity to autoantigens, but they can also recognize exogenous antigens[18,22–26] most likely due to their broad TCR repertoire.[27] Importantly, the Treg lineage identity is centrally regulated by Foxp3 and Tregs also show a distinct and stably imprinted epigenetic landscape for essential Treg genes.[28–32] Tregs may also be generated in the periphery from naive Tcon (pTreg), which seems to be particularly relevant for the generation of Treg against food or members of the intestinal microbiota.[4,20] The relative contribution of tTregs and pTregs to airway tolerance and in particular to the human Treg repertoire is currently not known, due to the lack of appropriate markers to distinguish both populations (see discussion below).

IL-10-producing T cells

IL-10 is a major immunosuppressive cytokine especially relevant for immune homeostasis at mucosal surfaces. IL-10-producing T cells, often termed "Tr1 cells", to subsume IL-10-producing T cells with mainly anti-inflammatory capacity, have frequently been described to mediate tolerance or to restrain effector T-cell responses against mucosal antigens. They originate from naive Tcons and thus belong to the group of peripherally induced suppressive T cells. However, whether "Tr1 cells" represent a separate and stable T-cell lineage similar to Foxp3 + Tregs or effector T-cell lineages like Th1, Th2, or Th17 is still not clear.[33] IL-10 can be produced by basically any T-cell subset, including all inflammatory T-cell subsets as well as Tregs.[34] In most situations the capacity to produce IL-10 is not stably imprinted but rather seems to be regulated on demand by external signals, such as tissue-derived signals for Foxp3+ Tregs[35] or strong TCR signals[36,37] as well as persistent antigens and chronic antigen stimulation for Foxp3-negative IL-10-producing T cells.[38] For example, high-dose intradermal allergen application resulted in the transient induction of IL-10 presumably by Th1 or Th2 effector T cells.[39] So far no lineage-defining master transcription factor or epigenetic imprinting at the IL-10 gene locus has been described[40] that would secure a stable Tr1 phenotype in vivo similar to Foxp3 in Tregs. However, recently, alterations of histone H3 acetylation and methylation at the IL-10 promotor region following in vitro IL-10 induction have been reported although the relevance for phenotype stabilization is not clear.[41,42] Taken together, regulation of IL-10 production might rather reflect a dynamic, self-limiting state of inflammatory and Treg differentiation modulated on demand by external signals. However, the molecular regulation and stability of IL-10 production by the various T-cell subsets requires more detailed analyses. Moreover, although IL-10 has been described in many experimental systems to downregulate Th2 responses and allergies, the association between genetic variants of the IL-10 or IL-10 receptor genes and allergic diseases or asthma are not as evident as for *Foxp3*.[43] Indeed, patients with IL-10 or IL-10 receptor deficiency suffer from severe IBD but not overt allergies.[44,45] These data suggest that even if IL-10 can modulate allergic reactions, it may not be considered as an essential

player for induction and maintenance of global tolerance against airborne antigens in humans.

HOW DOES THE TECHNOLOGICAL APPROACH FOR ANTIGEN-SPECIFIC T-CELL ANALYSIS IMPACT ON THE RESULT?

Technological aspects regarding the characterization of antigen-specific T cells

Much of the uncertainty with regard to the identity of T cells mediating tolerance against self-antigens as well as against exogenous proteins may result from the various technological approaches used to identify the relevant cells in mice and humans. In fact, all technologies used to identify antigen-specific T cells introduce a certain bias (Fig. 1), which needs to be taken into consideration for interpretation of the results (reviewed in ref. [13]). The analysis of allergen-specific T cells is technically challenging in particular in humans, due to the low frequencies of specific T cells. Studies that evaluated the frequencies of specific CD4+ T cell directly ex vivo by enrichment approaches, reported frequencies in human blood ranging from 10^{-5} to 10^{-3} for whole protein extracts,[18] or 10^{-6} to 10^{-3} for single allergen proteins or epitopes.[46–52] Strongly increased frequencies of allergen-specific CD4+ Tcons in allergic (~10 to 1000 cells within 10^6) compared to healthy donors (~1 to 100 cells within 10^6) have been identified, indicating allergen-induced clonal T-cell expansion. However, even in allergic donors the overall frequencies are still very low.

In vitro expansion of antigen-reactive T cells

Due to these low frequencies many studies used pre-expansion protocols to increase the number of antigen-specific T cells before functional or phenotypic analyses.[53–69] However, this approach has significant limitations, since in vitro cultures may introduce substantial phenotypic and functional modifications of the antigen-specific T cells, such as acquisition of an antigen-experienced phenotype or changes in cytokine production. Furthermore, Tcons may also acquire Treg-specific makers, such as CD25 and Foxp3 and lack of CD127,[70,71] which makes it difficult to discriminate Tregs and Tcons in expansion cultures.

Furthermore, it is almost impossible to draw conclusions on the original ex vivo frequencies and clonal composition of the antigen-specific T cells, since the expansion induces an unpredictable bias on the outgrowth of single clones, as well as bystander proliferation.[72] Also Tregs and Tcons have different proliferative capacity, which might lead to an under- or over-estimation of their respective frequencies. In addition, prolonged in vitro culture may also expand T cells from unprimed donors either from the naive or the antigen-experienced T-cell pool.

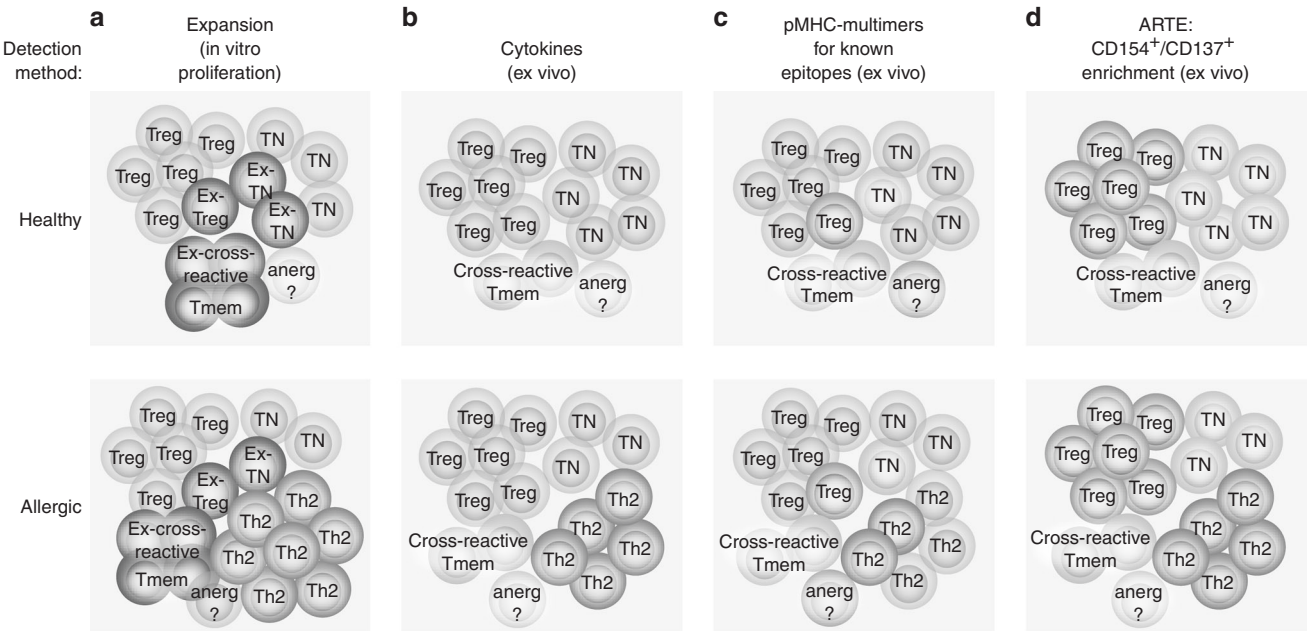

Fig. 1 Detection tools for antigen-specific CD4+ T-cell analysis and how they reveal different aspects of antigen-specific responses. The cartoon depicts the total repertoire of antigen-specific T cells. Colored cells indicate the T-cell subset(s), which can be detected by the respective detection methods in healthy (upper rows) or allergic donors (lower rows). All available detection methods lead to a certain bias by favoring or missing different T-cell populations. **a** Long-term (days–weeks) in vitro activation protocols detect proliferating antigen-reactive T cells but modulate the ex vivo phenotype or frequencies. Thus, antigen-reactive T cells may not derive from in vivo antigen-primed memory T cells but instead from naive or cross-reactive memory T cells naturally present human memory pool due to its highly diverse TCR repertoire. **b** Analysis of T cells expressing particular effector cytokines after short-term stimulation (e.g. ELISPOT, ELISA, and cytokine staining) may be useful to detect functionally important T-cell subsets. However, other subsets, such as naive or anergic T cells as well as Tregs or effector T cells producing other or no cytokines are missed. Cross-reactive memory T cells [see under **a**)] can also generate false-positive signals. **c** pMHC-multimers allow in principal the detection of all antigen-specific T-cell subsets, independent of their phenotypic or functional properties. Since they require prior knowledge of antigenic epitopes and MHC alleles, pMHC-multimers may be less suitable for screening of unknown antigen-specificities from complex antigens, e.g. like allergen extracts. Target specificities and affinity may differ between antigen-specific subsets, such as Tcons and Tregs or cross-reactive T cells. This may influence the composition of the T-cell pool detected by pMHC-multimers. **d** Detection of antigen-reactive T cells based on universal CD4+ T-cell activation markers such CD154 (CD40L) and CD137 (4-1BB) being expressed after only a few hours (5–7 h) of activation allows detection of naive and all antigen-experienced antigen-specific Tcons (CD154+CD137−) and Tregs (CD154−CD137+). This marker combination comprises all antigen-reactive CD4+ T-cell subsets, but per definition misses anergic cells. Importantly, it can be used with any type of antigen, since proteins and naturally processed by autologous APCs, thus providing a global view on the antigen-reactive T-cell response, even for complex antigens

Indeed "neo"-antigen-specific T cells have convincingly been demonstrated to be a common component of the human memory T-cell pool.[73–75] Thus, the detection of antigen-specific T cells following in vitro expansion may grossly overestimate and misinterpret the presence and the phenotype of antigen-primed T cells in vivo. Due to these limitations, the interpretation of results derived from long-term antigen-specific T-cell expansion cultures has to be critically revisited.

Direct analysis without prolonged in vitro expansion

MHC multimers. To overcome the limitations of prolonged in vitro cultures, several technological advances have been made in the recent years. In particular the development of pMHC-multimers,[76] which directly bind to the TCR, has significantly advanced the detection and characterization of allergen-specific T cells in humans. In combination with magnetic enrichment of rare tetramer positive cells, they enable the direct ex vivo detection, quantitation, and characterization of rare allergen-specific T cells from blood and tissues.[46–52,77,78] However, the use of tetramers is limited to previously defined antigenic epitopes and MHC restriction. Although technical advances like tetramer-guided epitope mapping now facilitate the rapid identification of allergenic epitopes,[79] the approach is not easy to adapt for multiple target proteins and MHC alleles as they occur in humans. Although in many allergies, allergenic proteins are known, and several immune-dominant allergenic epitopes have been defined,[80] unknown peptides and proteins may be missed by pMHC-multimers. For example, we showed that the majority of Tregs specific for birch pollen recognize mainly particle-associated, non-allergenic proteins[18] and are therefore not identified by the available pMHC-multimers directed against major allergens, i.e., Th2 peptide epitopes.

Short-term activation assays. Due to the limited availability of pMHC-multimer reagents, functional assays, such as cytokine secretion or expression of activation markers in response to antigen stimulation have widely been be used to detect allergen-specific T cells. The detection of antigen-specific T cells by functional parameters requires only the prior in vitro stimulation with the specific antigen. Thus whole proteins or whole antigen extracts can be used, which are subjected to processing and presentation by autologous APCs. In combination with magnetic enrichment, functional read-outs enable the same sensitivity as tetramer enrichment approaches. For example, enrichment of cytokine-producing T cells enabled by the cytokine secretion assay[81] has been used to isolate allergen-specific IL-4, IL-10 and interferon (IFN)-γ producers from allergic patients and healthy individuals.[82] An obvious limitation of this approach is that especially CD4+ T cells are heterogeneous and cells producing another or no cytokines at all, such as certain memory subsets, naive T cells, or Tregs, are being missed. Several activation markers have been proposed, which are not restricted to a certain subset but allow the identification of the total repertoire of specific T cells, including naive, all antigen-experienced Tcons, as well as Tregs to generate a comprehensive picture about the allergen-specific immune response (reviewed in refs.[12,13]). We recently introduced antigen-reactive T-cell enrichment (ARTE), which in our view allows comprehensive analysis of all relevant Th cell subsets specific for a certain antigen.[18,22] ARTE is based on the two activation markers CD154 (CD40L) and CD137 (4-1BB), which are differentially expressed on antigen-reactive Tcons (CD154+) and Tregs (CD137+) following short (5–7 h) in vitro stimulation with an antigen.[83] Magnetic enrichment of the few CD154- and CD137-expressing cells enables detection of 1 cell out of 10^5–10^6 and thus provides even access to the naive repertoire.[22,73] Several studies have used the CD154 enrichment approach to detect allergen-specific Tcons in healthy and allergic donors.[18,46,48,49,84] In combination with CD137 as activation marker for Tregs, a first comprehensive analysis of Tregs specific for several tolerogenic and pathogenic exogenous antigens in humans was possible.[18] However, per definition, stimulation-dependent analyses miss the detection of anergic antigen-specific T cells, which might be induced by SIT.[85]

The challenge of a broad TCR repertoire: how T-cell cross-reactivity impacts on antigen-specific T-cell analysis

In humans, who are naturally exposed to many different antigens throughout life, the accumulating broad TCR repertoire within the memory pool facilitates cross-reactivity, since it is well-known that a particular TCR is able to recognize multiple ligands.[86] Indeed cross-reactive CD4+ memory T cells recognizing neo-antigens in non-exposed subjects have recently been demonstrated in several studies.[73–75] These findings significantly impact on the interpretation of antigen-specific T-cell analyses in humans,[86] since this implicates that the detection of antigen-specific memory T cells per se is not a reliable indicator for a true antigen-induced in vivo reaction. The quantitative impact of cross-reactive memory responses is also influenced by donor-specific factors such as aging or infection history, which broaden the memory repertoire. Thus, to resolve in vivo-expanded antigen-specific memory responses, additional parameters, such as TCR repertoire and avidity measurements of antigen-specific T cells are required.[18,60,61,65,84]

Animal models for analysis of T-cell responses against inhaled antigens in vivo

Animal experiments contributed significantly to our knowledge about priming and differentiation of T cells against inhaled antigens and allergy development. Induction of various Treg subsets has been described, including CD25+ cells[87] or Foxp3+ Tregs,[88–91] IL-10[36,92,93], or transforming growth factor-β/latency-associated peptide-expressing T cells.[94] In particular, the adoptive transfer of TCR transgenic T cells helped to resolve antigen-specific T-cell responses in vivo.[87,92,94] Likewise, the adoptive transfer of ovalbumin (OVA)-specific, as well as of polyclonal Tregs into naive mice was used to analyze their Th2 suppressive activity.[89,90,95–97] However, the precise conditions and the exact definition of the induced T-cell phenotypes are variable and not completely resolved. In any case, relatively high doses (»1 µg per intranasal application round) are used in all experimental models of intranasal tolerance induction. This is several orders of magnitude higher than the typical allergen doses taken up via the breathing air (see below). Such high doses are probably required since usually soluble antigens were used rather than particles, which are taken up upon natural exposure. Finally, experiments using adoptive T-cell transfer of naive allergen-specific T cells focused on the conditions for peripheral induction of a regulatory phenotype rather than on the possibility that tTregs with specificity for exogenous antigens might be expanded. However, recent tetramer-based analysis of Treg responses within a physiological TCR repertoire demonstrated selective tTreg expansion against antigens ectopically expressed in the lung and intestinal mucosa rather than induction of pTregs.[98] As further discussed below, it is a central question whether aeroantigens induce pTreg or tTreg, including potentially different mechanisms for intestinal (microbial, food) vs. airborne antigens, as well as potential differences between mice and humans. To precisely analyze this it might be necessary to adapt animal models to resemble more physiological (human) ways of antigen dose, formulation, and exposure to determine the conditions leading to the induction of either Foxp3+ or IL-10 responses from naive Tcon or Treg precursors and to determine the actual role of these pathways for the maintenance of tolerance in the airways.

WHICH INHALED ANTIGENS ACTUALLY GET INTO CONTACT WITH THE IMMUNE SYSTEM AND MAY ACTIVELY INDUCE TOLERANCE?

The nature of airborne antigens: inhaled particles vs. soluble allergens

As discussed above for a better understanding how tolerance against airborne antigens is maintained, it is important to consider at first the quantitative and qualitative parameters of the naturally inhaled antigens. For active induction of tolerance, the antigen needs to be taken up by APCs and presented to antigen-specific T cells in sufficient quantity to activate them. Although allergic reactions are in the majority of cases directed against few specific proteins targeted by specific IgE, it has to be emphasized that individual proteins are not naturally present in our breathing air. Physical constraints prevent the existence of airborne protein molecules. Thus, natural exposure to inhaled antigen typically occurs through uptake of antigen-carrying particles, which are present within the breathing air and are small enough to reach the mucosal surfaces. It is assumed that particles with a diameter of 1–5 µm can reach the upper and lower respiratory tracts and are efficiently phagocytosed.[99,100] Indeed, the majority of known inhalation allergens are proteins derived from particles, such as plant pollen, spores, animal dander, or insect excretions.[2] However, these particles are composed of hundreds or thousands of different proteins and only a tiny fraction thereof are actually allergens targeted by IgE. IgE binding has in fact been used to define the clinically relevant allergens based on the assumption that only allergens represent the immune-dominant antigens derived from inhaled particles. This has also been supported by the fact that all allergens represent rather small and stable, highly soluble molecules, which in contrast to particles might easily cross the epithelial barrier and allow improved interaction with the immune system.[2] As a consequence the majority of studies on tolerance against airborne proteins actually focused on the known clinically relevant allergenic proteins, rather than on all proteins contained within the inhaled particles. However, we could recently demonstrate high frequencies of Foxp3+ Tregs specific for the non-soluble particle-associated protein fraction of birch pollen in peripheral blood of both, healthy and in equal quantity and quality also in allergic donors.[18] This suggests that particle-derived proteins are not immunologically inert but rather represent potent inducers of tolerogenic Treg responses. In principle particles are excellent immunogenes compared to soluble proteins since many phagocytes are specialized on uptake of particulate antigens. Furthermore, upon uptake of an individual particle, a relatively high dose of antigen can be delivered to a single APC, even when the total amount of antigen is very low. For example, the protein content of a single plant pollen or mite fecal particle is very low (~0.2 ng/particle) but within the particles this corresponds to a high concentration of about 2 mg/ml for a 20 µm particle.[2] Therefore, antigen-loaded particles are the basis of pulmonary vaccines.[100] The quantitative aspect of particulate vs. soluble antigen uptake is highly relevant when considering to the natural exposure to inhaled antigens. For example, although it is estimated that we inhale approximately 1000 *Aspergillus fumigatus* spores per day, but the weight of a single spore is just around 3 pg,[101] with a protein content of approximately 15%.[102] This results in a total *A. fumigatus* protein inhalation of 0.45 ng/day or 0.16 µg/year. Similarly, estimates on the quantity of mite- or plant pollen-derived proteins range from 5 to 50 ng/day.[2,103] The amount of a single allergenic protein is even lower, e.g., the uptake of Betv1 has been calculated to be <1 µg/year.[104] At such low doses soluble proteins that are released following particle uptake have in fact little potential to induce immune responses and may rather be ignored by the immune system. Indeed, most experimental allergy models repeatedly apply soluble antigen with »1 µg/dose, which is obviously far beyond the quantities taken up upon natural exposure.

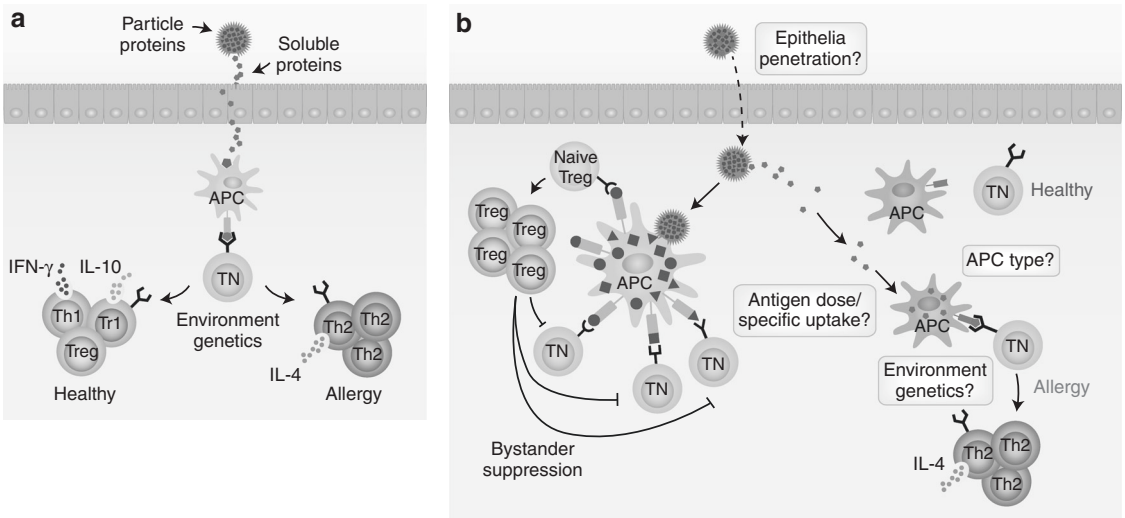

Fig. 2 Two alternative models for uptake of aeroantigens and induction of protective and pathogenic T-cell responses: **a** classical model: allergenic proteins are rapidly released from inhaled particles and thus have preferential access to the immune system, e.g., their small size and protease activity facilitate transfer across the epithelial barrier, uptake by antigen-presenting cells (APCs), and activation of T cells. They either induce tolerance (Tr1 and Treg) or protective Th1 responses in healthy donors vs. Th2 responses in allergic donors. **b** New model: particle-associated proteins represent the immmunodominant antigens generating preferentially Treg responses that suppress the activation of naive Tcons. Thus, particles may cross the epithelial barrier and can be taken up by APCs. A single particle contains enough antigen to generate a T-cell responses. Bystander suppression protects all particle-associated antigens that are taken up (group tolerance). In contrast, soluble antigens are released before particle uptake and due to their extremely low dose cannot be efficiently taken up by APCs and fail to induce Treg or Tcon responses in healthy donors (ignorance). Under conditions favoring Th2 development, such as the genetic background, environmental factors, antigen dose, or a different APC type only those soluble antigens not protected by specific Tregs represent potential Th2 targets. T-cell priming typically occurs in the draining lymph node rather than in the mucosal tissue. However, where and by which APCs the antigens are taken up and are presented is currently not clear

Thus, there are two alternative scenarios, which aeroantigens are actually taken up, presented to the immune system and what types of T-cell responses they induce (Fig. 2). Uptake of particles would lead to preferential presentation of particle-associated proteins. Proteins that are released before particle uptake are less likely to generate local protein concentrations high enough for APC uptake and for the induction of a T-cell response. Thus, they are rather ignored by the immune system. Alternatively, particles may not cross the epithelial barrier and thus mainly the soluble proteins cross the epithelium and get in contact with the immune system. Depending on the scenario, tolerance has to be maintained against the particle-associated and/or the soluble fraction of the proteins.

WHAT IS THE EXPERIMENTAL EVIDENCE FOR THE DIFFERENT TYPES OF T-CELL RESPONSES AGAINST INHALED ANTIGENS?

In the following we provide an overview on the experimental evidence for the various types of mechanisms, which have been proposed for tolerance against airborne antigens. We discuss how the outcome may be influenced by the experimental system used and integrate the various results into a unified model for tolerance to allergens.

Passive tolerization: ignorance, deletion, anergy

A major tolerance mechanism against aeroantigens could be that T cells specific for harmless are either deleted or functionally inactivated. This has been demonstrated in many animal models following intravenous or oral application of high doses of antigens.[105] However, such high antigen doses do certainly not apply for most airborne antigens, which are typically encountered in rather minute quantities, as discussed before. Alternatively, antigens may be simply ignored by the healthy immune system, because they do not reach high enough concentrations to activate an immune response. However, mainly animal studies have shown that repeated exposure to proteins or peptides via the respiratory mucosa induces immunologic tolerance.[36,90,92,93,106–113] Very early studies already showed that the induction of inhalation tolerance is specific for the inhaled antigen,[113] long-lasting (at least 6 month),[108] and requires only microgram quantities of inhaled antigen if applied repeatedly.[109,113] Furthermore, activation of adoptively transferred antigen-specific naive CD4+ T cells has directly been demonstrated following inhalation of aerosolized antigens or their intranasal and intratracheal application.[36,90,92,93] Under these conditions the antigens are rapidly taken up locally by lung dendritic cells, which migrate to the local lymph nodes to prime T-cell responses (reviewed in refs.[114,115]). However, doses of <1 µg of antigen failed to downregulate T-cell responsiveness,[113] Thus, inhaled antigens, if applied repeatedly and in sufficient doses (>1 µg) trigger naive T-cell responses in regional lymph nodes of mice. However, as discussed above the natural doses of airborne antigens are probably much lower.

Different tolerance mechanisms against particle-bound and released proteins

These minute quantities of inhaled antigens actually suggest that the mechanism of tolerance may depend on their physicochemical properties, in particular whether antigens are compacted and remain associated within particles, or whether they are released and diluted upon uptake into body fluids. In fact, given the low overall antigen dose it is quite likely that allergenic proteins, which are typically rapidly released from inhaled particles, may not regularly reach the critical threshold for T-cell activation when taken up by APCs. Indeed analyses of human T-cell responses against major allergens provide significant evidence for ignorance as an important tolerance mechanism. Short-term activation assays as well as pMHC-multimer enrichment revealed that healthy and allergic donors do not only differ in the quality of T-cell responses (Th2 vs. non-Th2) but in fact displayed 1–2 log differences in the frequencies of allergen-specific T cells.[18,22,51,52,116] Indeed, the frequencies of allergen-specific T cells in healthy donors were in the range of 1–10/10e6 CD4+ T cells, similar to the frequencies of T cells specific for neoantigens.[73,74] TCR sequencing[18] and avidity measurement[18,60,61,65,84] also revealed clonally expanded, high-avidity memory T cells only in allergic donors. In healthy donors allergen-reactive T cells mainly displayed a naive phenotype, low avidity, and lack of clonal expansions.[18,60,65,84] Allergen-specific Tcons from healthy donors did also not express Ki-67 during allergen season, a marker of recent in vivo activation,[18] which can clearly be detected in allergic donors.[18,51] Interestingly, the direct ex vivo analysis of birch pollen-reactive Tcons and Tregs demonstrated a clonally expanded, high-affinity Ki-67+ Treg population, specifically against particle-associated proteins but not against the soluble fraction, containing the main allergens such as Betv1.[18] Other studies detected tetramer positive CD4+ T cells in healthy donors predominantly in the memory compartment albeit at low frequencies.[51,52,116] However, the clonal composition or antigen affinity of these cells has not been investigated. Thus, it cannot be excluded that these rare memory T cells are not induced by in vivo antigen activation but actually represent natural cross-reactivity within the memory repertoire[73–75] as discussed above. Taken together, the lack of a clearly detectable antigen-experienced effector T-cell response in healthy donors suggests that upon natural exposure ignorance of allergenic proteins released at very low dose from inhaled particles might be a major tolerance mechanism in healthy donors. In contrast particle-associated proteins seem to be able to induce Treg responses even at low dose (see below) pointing toward an important role for Tregs in mucosal tolerance to airborne antigens in general.

Evidence for Foxp3+ Treg-mediated tolerance against aeroantigens

Global impairment of Tregs in allergy. It is generally accepted that Tregs play a fundamental role for the prevention of allergies, as demonstrated by the neonatal hyper IgE syndrome of patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome patients carrying a functionally impaired *Foxp3* gene.[117] Genome-wide association studies have, besides many Th2-related genes, identified the gene coding for the IL-2 receptor, a cytokine central for Treg survival and function, to be associated with allergy and asthma.[118] Inappropriately controlled Th2 responses have also been demonstrated in a variety of animal models with diminished or functionally impaired Tregs. Defective or instable Tregs result in increased Th2 inflammation.[20,119–122] Reduction of Foxp3 expression in genetically modified mice[121,122] or loss of Foxp3 expression in repeatedly stimulated human memory Tregs[123] results in preferential conversion of Tregs into Th2 cells. These Treg-derived Th2 cytokines contributed to the Th2 differentiation of naive CD4+ T cells in murine models.[121,122] A series of human studies have addressed the question whether impaired Treg functionality may be related to allergic or asthmatic diseases. Studies have been conducted in adults[66,124–129] and pediatric patients,[130–135] and total Treg frequencies were analyzed in peripheral blood,[66,124–130,132,133,135] as well as in bronchoalveolar lavage,[130] nasal,[129] and bronchial tissues.[131] The obtained results are inconsistent, describing a reduction of total Tregs in patients with allergy or asthma,[128–130,132,135] no difference between patients and healthy people,[124,125,134] or reduced levels of Foxp3 expression in asthmatic donors.[126,133] Several reasons for these discrepancies in human studies have been discussed, including the clinical form of diseases and the therapeutic regimen, age of patients, natural exposure to allergen, and the source of analyzed cells, such as peripheral blood vs. tissue-derived cells. However, a major technical issue for the analysis of human Tregs is the lack of markers allowing precise definition of

Tregs and discrimination from activated Tcons.[136] These technical difficulties also explain, why similarly inconsistent results were obtained regarding the suppressive capacity of Tregs from allergic donors. Several studies reporting no differences of the Treg suppressive capacity in in vitro proliferation assays,[64,127,137] while others suggested that Tregs from allergic patients have reduced potential to suppress proliferation of effector cells, especially during the allergen season.[53,62,66,133] This may be due to increased proportions of contaminating effector T cells within the CD25+ subset.[53] As outlined above, the affinities and frequencies of allergen-specific Tcons significantly differ between healthy and allergic donors.[18,22,46,48,49,51,52,60,61,65,84,116] Thus, the reduced suppressive activity observed in some studies may also reflect a stronger Th2 activation in patients. Indeed, in an antigen-specific in vitro suppression assay CD25+ Tregs during birch pollen season were unable to suppress birch-specific Th2 responses, whereas Th1 cells were readily suppressed.[57,66,125]

Antigen-specific Tregs against allergens and airborne particles. Despite the demonstrated importance of Tregs for the overall maintenance of tolerance, there is currently no good evidence that human allergies are associated with a general numerical or functional loss of Tregs. This is also supported by the clinical appearance of most allergic patients, resembling highly selective antigen-specific loss of tolerance. This is in sharp contrast to IPEX patients and animal models with impaired Tregs, which develop broad allergies against a variety, mostly undefined exogenous antigens at the mucosal surfaces of the lung and the intestine, already early after birth.[21] Thus, it is an intriguing question whether allergen-specific Tregs contribute to the prevention of specific allergies in healthy donors and whether their loss or functional impairment underlies allergy development.

Repeated inhalation of OVA has been shown to make mice tolerant against subsequent antigenic re-challenge, which correlated with the expansion of CD25+ cells[87] or Foxp3+ Tregs.[88–91] Depletion of CD4+CD25+ Tregs prior to antigen challenge in OVA-sensitized mice or a house-dust mite induced asthma model, resulted in increased allergic airway disease.[88,89,138] OVA-specific Tregs efficiently suppress the development of Th2 from naive T cells[89,90,95–97] or development of a pathogenic Th2 subset[139] but may fail to control already established Th2 responses.[95,140] Tregs also suppressed Th1-mediated lung inflammation better than that mediated by Th2 cells[141] and it was suggested that Th2 cytokines per se limit the potential of human Tregs to control Th2 cells in vitro[142] or may require pre-activation.[143] Pre-activated OVA-specific and even polyclonal Tregs inhibited established Th2-mediated lung inflammation.[97,144–147] Overall, there is clear evidence that antigen-specific Tregs can efficiently prevent specific Th2 development and may also ameliorate established disease although their exact therapeutic potential is still debated.

However, only very few studies directly identify airborne antigen-specific Tregs in humans and mice. In particular, most pMHC-multimer studies failed to detect allergen-specific Tregs in healthy or in allergic donors, while effector T cells were readily detected.[46–52,78,148] In one study by Palomares et al.,[77] Betv1-specific Tregs have been detected ex vivo at increased frequencies in human tonsils (~0.4%) as compared to blood (~0.1%). However, differences between healthy and allergic donors have not been investigated. van Overtwelt et al.[67] detected tetramer+ Tregs against house-dust mite following 2–3 weeks' expansion cultures and suggested that tetramer+ Foxp3+ Tregs were present in healthy but not in allergic donors. However, even after expansion the overall frequencies of tetramer+ antigen-specific T cells were still very low (<1%), especially in healthy donors, and no further data on the Treg identity of the cells following in vitro expansion are provided. Finally Maggi et al.[64] reported the generation of Der p 1-specific Treg clones from peripheral blood, but they did not directly compare their frequencies in healthy and allergic individuals.

Direct enumeration of Tregs reactive against major allergenic proteins derived from birch and grass pollen or *A. fumigatus* showed that Tregs specific for allergenic proteins can be detected but only in a minority of healthy donors.[18] Taken together all direct ex vivo analyses demonstrated that human Tregs specific for major airborne allergens can only be found in a fraction of healthy donors, suggesting that the presence of specific Tregs is not an absolute requirement for the maintenance of tolerance. This is supported by the findings that CD25 depletion does not enhance proliferative responses against allergenic proteins in healthy donors.[65,149] The lack of Tregs and strong effector T-cell responses against allergenic proteins in healthy donors further supports the idea that ignorance against allergens is a major tolerance mechanism probably due to their low dose.

However, the unexpected finding that robust Treg responses are generated in all donors against the particle-associated fraction of

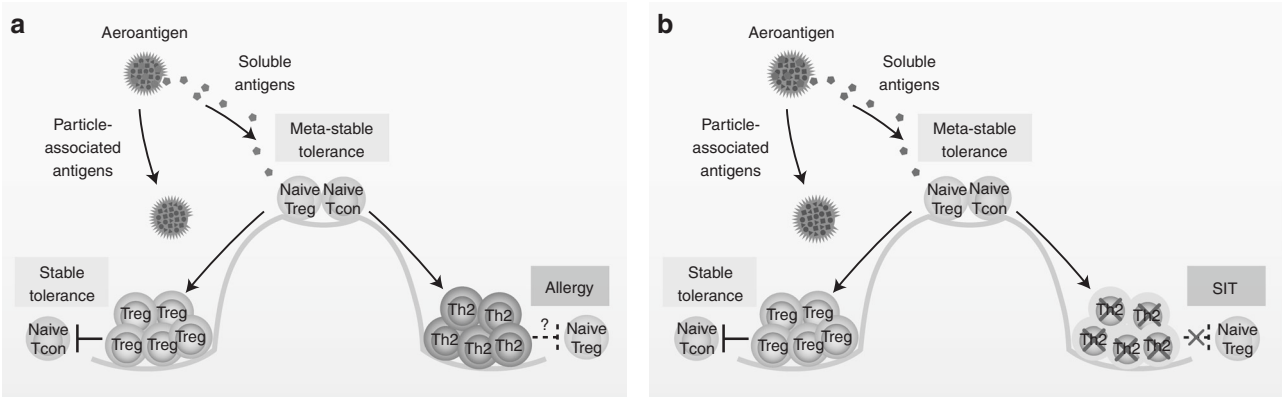

Fig. 3 Stable and metastable states of tolerance against aeroantigens. **a** Particle-associated antigens generate a robust Treg response. This is a stable state of tolerance since Tregs actively prevent generation of Th2 responses. Soluble allergens are typically ignored by the immune system, due to their low antigen concentration in solution. Therefore, allergen-reactive cells remain in a naive state (ignorance). This represents a metastable state of tolerance, since naive T cells may still deviate into Th2 cells. Once primed, Th2 differentiation also represents a stable status. Whether established Th2 cells also prevent activation/expansion of naive Tregs is unclear. **b** SIT can delete pathogenic Th2 cells without de novo induction of Tregs. Depending on the availability of allergen-specific naive T cells, e.g., by thymic generation, this post-SIT status is also metastable. Thus, to achieve stable induction of tolerance in healthy or post-SIT allergics the generation of specific Treg responses against allergenic proteins should be a therapeutic target

inhaled proteins[18] highlights the importance of Tregs for tolerance against inhaled antigens. Since most of these proteins have not been described as allergens it is tempting to speculate that the specific Treg response effectively suppresses development of Th2 responses. This is further corroborated by the exclusive presence of either Tregs or Th2 cells against individual soluble proteins observed in allergic donors,[18] suggesting that individual soluble proteins released from particles can only generate Th2 responses in the absence of specific Tregs. For all particle-associated proteins the Treg-mediated protection may be particularly effective because the entire particles are taken up by one APC, facilitating bystander suppression or "group tolerance".

Taken together, Treg responses are efficiently induced against those inhaled proteins, which reach a critical threshold for T-cell activation in vivo and these Tregs provide active tolerance. Treg induction and tolerance occurs preferentially for particle-associated proteins and the Treg protection is enforced by the "group tolerance" effect. In contrast, those proteins, which fail to efficiently generate Treg responses and which escape "group tolerance" due to rapid release from particles before uptake by APC, i.e., the majority of allergens, are not actively protected but rather ignored by the immune system. However, ignorance of individual allergens represents a metastable state of tolerance since it bears the intrinsic risk of Th2 and allergy development under appropriate genetic or environmental conditions. Such metastable tolerance can actually explain why allergies against individual proteins can (a) develop independently of each other and (b) typically develop even after many years of previous allergen exposure without induction of any allergic symptoms. A hybrid model of stable Treg-mediated tolerance against the particle-associated proteins and metastable tolerance against a limited number of soluble proteins, which escape from Treg protection provides a logical explanation why Tregs are essential for the prevention of broad-spectrum IgE against environmental antigens as observed in IPEX patients, whereas at the same time allergies in immune competent patients may develop in the absence of any overt Treg defect (Fig. 3). When integrating these observations, a new model of tolerance to airborne antigens emerges (Fig. 4), based on two individual checkpoints (Treg-dependent and -independent). The first and dominant checkpoint 1: the capacity of individual proteins to generate antigen-specific Treg responses provides a stable tolerance mechanism. This is enforced by a low "Treg escape potential", e.g., "group tolerance" for particle-associated proteins. Checkpoint 2: proteins that do not generate Treg responses and with high Treg escape potential (e.g., soluble proteins escaping group tolerance) generate metastable tolerance ("ignorance") but may potentionally switch to Th2. The parameters defining checkpoint 1 + 2 are currently not fully understood but comprise protein-intrinsic features, e.g., association with inhaled particles, enzymatic activity, etc., as well as patient-specific features, e.g. genetic predisposition or environmental factors supporting Th2 formation. In particular, factors influencing checkpoint 1 may be suitable therapeutic candidates to achieve long-term protection against allergies.

Thymic or peripherally induced pTreg against aeroantigens?. The dominant role of antigen-specific Treg to protect from Th2 development postulated in our model highlights the importance to understand the mechanisms of their generation. In that respect it is an eminent and still unanswered question, whether Treg responses are generated from naïve conventional (pTreg) or regulatory precursors (tTreg). As discussed above, despite the fact that mice deficient in pTreg formation have increased susceptibility to mucosal Th2 responses[20] there is little direct evidence that antigen-specific pTregs are formed against inhaled allergens, whereas tTreg expansion has so far not been carefully addressed in mice. This is surprisingly different from animal models of intestinal tolerance to food or intestinal microbiota, which clearly suggest the generation of pTreg as important tolerance mechanism.[4] Microbiota colonization of the airways has also been shown to reduce neonatal allergy susceptibility against house dust mite via generation of pTreg, although their specificity has not ben analyzed.[150,151] Unfortunately, it is not possible to distinguish between pTregs and tTregs in humans. However, as discussed above the few data on human Treg against airborne antigens, reveal classical tTreg features, based on TCR sequence comparison to Tcon as well as epigenetic and transcriptional signatures.[18]

Thus, it has yet to be clarified whether intestinal and airway tolerance may be based on different mechanisms of Treg induction and whether additional differences may exist between human and mice.

Evidence for IL-10 induction against aeroantigens

The induction of IL-10-producing T cells is often considered as a major tolerance mechanism against allergens. Indeed, a technically challenging study using direct isolation and analysis of IL-10-secreting allergen-specific T cells from blood of healthy donors revealed rather high frequencies of IL-10 producers even

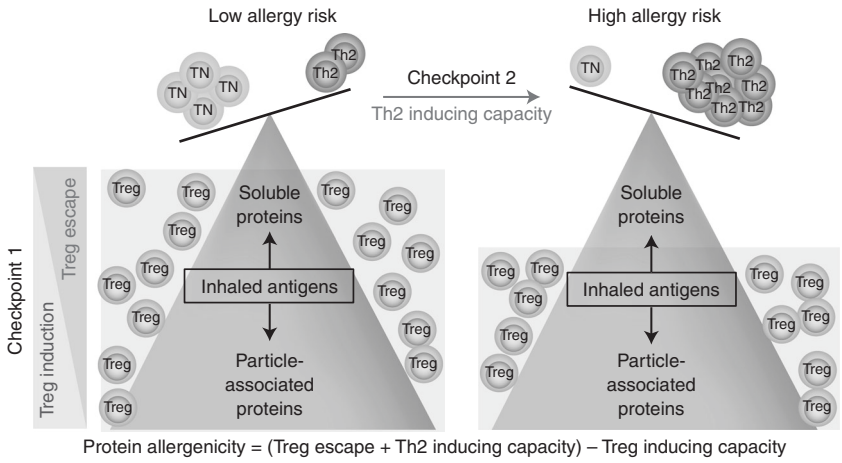

Fig. 4 Dual checkpoints for tolerance to airborne antigens. A new model of two connected but mechanistically independent checkpoints regulating tolerance and the susceptibility to allergies: the first and dominant checkpoint is set by the proteins' capacity to induce Treg or escape Treg control. The second checkpoint is determined by the Th2-forming capacity of those proteins escaping Treg control. Although both checkpoints have a different mechanistic basis they are both susceptible to protein-intrinsic as well as patient-specific factors (see main text for details). Treg-inducing and Th2-inducing capacity may vary between individuals, increasing the risk for allergy development

exceeding those of IL-4-producing T cells in allergic donors.[82] Furthermore, an altered ratio of allergen-specific IL-10- vs. IL-4-producing T cells in healthy vs. allergic has been demonstrated. However, as outlined above the direct analyses of allergen-specific T cells, using pMHC-multimers or short-term activation assays did not support the idea of strong or IL-10 biased allergen-specific T-cell responses in healthy donors.[18,22,46–52,65,78,84,148] Interestingly, not even Foxp3+ Tregs specific for various airborne antigens did produce IL-10 mRNA or protein.[18] We could recently also confirm the absence of IL-10 production by Foxp3+ Tregs isolated from lung tissues (Bacher and Scheffold, unpublished data). Furthermore, also in the nasal mucosa of healthy volunteers only low levels of IL-10 mRNA were observed.[152] Taken together there is accumulating evidence that IL-10-producing T cells do not represent an essential tolerance mechanism against natural exposure to aeroantigens in healthy donors, which is also in agreement with genetic data from human patients, as outlined above.[43–45]

In contrast to the natural low-dose exposure to aeroantigens, high-dose antigen challenge in vivo seems to induce IL-10 in antigen-specific T cells. Increased levels of IL-10 have been described following antigen-SIT with subcutaneous or sublingual antigen application[152–163] or upon natural high-dose exposure to bee venom.[39] However, the T-cell capacity to produce IL-10 seems to be transient.[40] IL-10-producing allergen-specific T cells were mainly found during the allergen exposure in blood[39] and in mucosal tissues.[152] Under these IL-10-inducing conditions, the allergen was applied locally in concentrated form, e.g., by intra- or subcutaneous injection. In contrast, data about IL-10 induction in human T cells following inhalation of antigens are scarce. In murine models, IL-10 production was induced after high-dose intranasal antigen application.[36,90,92,93] Another form of high-dose tolerance against aeroantigens has been reported from cat owners. High-dose cat allergen exposure can induce tolerance accompanied by high allergen-specific IgG4 serum levels, which have been suggested to be induced by IL-10-producing T cells.[156,164] However, a recent short-term activation and pMHC-multimer-based study identified cat allergen-specific Th2 cells to directly correlate with the plasma IgG4 levels, whereas no qualitative differences with regard to IL-10 or Th2 cytokine production were observed in allergic vs. tolerant allergen-exposed donors. Thus, the cellular identity and molecular requirements of IgG4-inducing T cells is not finally clarified and it is presently not known whether the protective phenotype in asymptomatic allergics with high exposure to allergen and high IgG4 levels is associated with a potentially "tolerogenic" form of Th2 response.

Taken together the majority of data indicate that IL-10 production by allergen-specific T cells seems not to be the main tolerance mechanism upon natural allergen exposure of healthy individuals. However, IL-10 is induced transiently in antigen-specific T cells following high-dose antigen exposure, e.g., upon sub-/intracutaneous injection, which may represent a natural feedback mechanism to restrain hyper-activation. In contrast, the proof for induction of an IL-10-producing Treg subset by airborne antigen in humans is still missing.

WHAT ARE THE EFFECTS OF ANTIGEN-SIT ON THE SPECIFIC T-CELL SUBSETS?

SIT is currently the only applied therapy with a clinical long-term effect against allergies. The aim of SIT is the therapeutic reestablishment of allergen-specific immune tolerance. In particular it is assumed that the modulation of pathogenic Th2 responses and re-induction of tolerogenic T-cell responses is a critical step (reviewed in ref.[11]). However, SIT bears the risk of side effects, shows variable treatment success, and requires several years of treatment. Finally, although the clinical effects can last for several years[165–170] it is so far not clear whether stable remission can be achieved. Related to this the mechanisms underlying successful SIT are so far not resolved, which would be essential for the optimization of clinical protocols to improve (long-term) efficacy and safety. In this context, it is interesting to compare the allergen-specific T-cell response as a basis of tolerance in healthy subjects and patients after successful SIT. The same mechanisms as discussed above for natural tolerance against inhaled antigens, i.e., anergy, deletion, immune deviation, IL-10-induction, and Treg responses, have been suggested for the successful outcome of SIT. The discrepancies between these studies may in part be due to different modes of antigen administration, formulation, and dose as well as the time point of analysis. However, it may also arise from technical limitations of early studies to directly assess immunological changes within the rare allergen-specific CD4+ T cells.[11] Importantly, successful SIT involves repeated subcutaneous or sublingual administration of increasing and relatively high doses of the allergen over months or years, whereas peptide inhalation failed.[171] Thus, neither dose nor the site of exposure mimic natural airborne allergen exposition. Therefore, the mechanisms induced by SIT may significantly differ from "natural" tolerance mechanisms against inhaled antigens. Whether the immunologic response induced via the two major application routes subcutaneously vs. sublingual administered antigen is mediated by the same mechanisms is also not clear, although similar processes have been suggested.[163,172]

Initially, SIT has been proposed to redirect Th2 cells into a protective Th1 response or restore the Th2/Th1 balance. This idea is based on the findings of an increased Th1/Th2 ratio following SIT.[51,154,161,173–175] However, direct quantitation of allergen-specific T cells suggested that this shift resulted from a decrease in Th2 cells, rather than an increase in Th1 cells.[51,52,160,174,176,177] In a study using TCR tracking in humans, conversion of Th2 cells into IL-10 producers has been suggested.[178] In contrast, ex vivo tetramer enrichment showed that Th2 and Th1 cells are directed against different epitopes of the same allergen[52] and SIT induced preferential Th2 deletion[52] or anergy[85] of Th2 cells and no increase of Th1/Tr1 cells. The deletion of Th2 cells as a critical step for SIT also supports a new model suggesting a "pathological" population of Th2 cells as the disease driving entity in allergic donors, rather than a disturbed Th2/protective T-cell balance.[8] The specific deletion of these pathologic Th2 cells would then be sufficient for the therapeutic reversal of clinical symptoms without restoration of a protective T-cell subset. Indeed, such a pathogenic Th2 population being specifically depleted during SIT has recently been characterized.[50,51]

Several studies demonstrated an increase of IL-10-producing CD4+ T cells following subcutaneous as well as sublingual SIT[152,154–163] and the suppressive role of IL-10 was confirmed by in vitro assays.[158,161,179] SIT might also induce linked suppression as shown by SIT with a selected epitope from a cat protein, which induces IL-10+ T cells with additional specificities.[180] However, as discussed above the increase in IL-10 production is transient or requires the continuous presence of high doses of allergen,[39,154,160,163,172] despite the long-lasting reduction of allergic symptoms for several years.[165–170] This suggests that IL-10 induction might play a role in the early phase of SIT, but that other or additional mechanisms to IL-10 induction, such as deletion of pathogenic Th2 cells may be more relevant in the stable induction of tolerance following SIT.[11]

How SIT impacts on allergen-specific Tregs is currently also unclear. Only a limited number of studies addressed the role of Foxp3+ Tregs during SIT and almost no data on antigen-specific Tregs are available. Increased Treg frequencies or elevated allergen-specific suppressive activity of CD25+ T cells,[157,158,162,181,182] as well as increased numbers of Foxp3+ Tregs with epigenetically stabilized phenotype[183,184] were described in blood as well as in the nasal mucosa either following SIT[185] or after local challenge with grass allergen.[186] Other human

studies observed no difference in the frequencies of Foxp3+ Tregs during SIT.[54,159,161,179] Animal models suggest that thymic Tregs may also contribute to the effectiveness of SIT by promoting IL-10 production in Foxp3-negative T cells[187] although depletion of Tregs only partially abrogates the suppressive effects induced by SIT.[188] However, direct analysis using pMHC-multimer enrichment did not detect an increase of allergen-specific Foxp3+ Tregs,[51] indicating that the generation of allergen-specific Tregs may not be the primary mechanism of SIT. This corresponds very well with the absence of allergen-specific Tregs in pMHC-multimer studies in healthy donors discussed above and our own recent finding that specific Tregs against allergenic proteins are found only in a fraction of healthy donors.[18] Our preliminary data from patients allergic against grass pollen actually suggest that during SIT with whole grass pollen lysate, which contains Treg as well Th2 target antigens, specific Tregs decrease similar to Th2 cells (Bacher and Scheffold, unpublished).

Taken together, the current data suggest that depletion of pathogenic Th2 cells is the main therapeutic effect of SIT, which seems to be preceded by a transient increase of IL-10 production by allergen-specific T cells. In contrast, there is so far no evidence that SIT actually induces a protective population of allergen-specific Tregs. Thus, by depletion of pathogenic Th2 cells, SIT induces a hypo-reactive state reminiscent of the metastable tolerance state established due to ignorance of low-dose inhaled antigens in healthy donors described above (Fig. 4). Both situations bear the intrinsic risk of the recurrence of pathological Th2 responses. Therefore, long-term tolerance induction may require novel therapeutic combination strategies, aiming at specific expansion of allergen-specific Tregs, following the successful elimination of pathogenic T cells. In that respect, the remarkable capacity of even minute quantities of naturally inhaled airborne particles to induce strong Treg responses may point in the right direction.

CONCLUSIONS

In this review, we tried to summarize and to re-evaluate the experimental evidence for the various proposed mechanisms of T-cell-mediated tolerance against aeroantigens in healthy donors and during antigen-SIT. The recent technological advances allowing for direct identification and characterization of antigen-specific effector and Tregs have actually revealed different types of T-cell responses and modes of tolerance, depending on antigen formulation and dose as well as the antigen application route. The analysis of antigen-specific human Tregs has highlighted their fundamental role for active maintenance of tolerance against airborne antigens. However, Tregs seem to target mainly particle-associated non-allergenic proteins. Therefore, the escape from direct or bystander Treg suppression due to rapid release from inhaled particles seems to be an essential pre-requisite for classical airborne allergenic proteins. Thus, the allergenicity of a protein is the sum of two independent features, its capacity to escape Treg control plus its Th2-inducing capacity (Fig. 4). Both features however may be affected by host and environmental factors.

It is actually an intriguing question, which APCs allow efficient Th2 priming, requiring either selective uptake of minute amounts of soluble allergenic proteins or mechanisms to escape from bystander suppression? Furthermore, reestablishment of tolerance in allergen-primed donors via high-dose antigen injection, e.g., during SIT, may induce IL-10 production transiently but eventually leads to the deletion of pathogenic effector T cells rather than to the generation of a durable protective Treg response. Thus, natural as well as therapy-induced tolerance to airborne allergenic proteins appears to be metastable. To generate stable active tolerance it remains a major task to expand the physiological Treg responses to therapeutically relevant allergenic proteins. To achieve this goal, several open questions remain: 1. does interindividual variation exist regarding the capacity to generate antigen-specific Treg responses and how does this contribute to allergy susceptibility? 2. Which parameters, such as antigen dose, formulation, exposition time, APC type, and co-stimulatory signals are relevant for induction of Treg vs. Tcon responses against inhaled antigens? 3. Are Tregs expanded from thymic Tregs or induced from naive Tcon precursors? 4. Is it possible to induce allergen-specific Treg responses in allergic patients and are they therapeutically active? 5. Does antigen-specific Treg memory exist, i.e., are Tregs long-lived? To answer these questions it may also be necessary to adapt current animal models to better recapitulate the human situation, with regard to natural antigen exposure. In any case, the new possibilities to monitor human antigen-specific Treg and Tcon responses provide an ideal basis for systematic development of improved antigen-specific therapies.

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AUTHOR CONTRIBUTIONS

P.B. and A.S. contributed equally to the writing and editing of this review.

ADDITIONAL INFORMATION

Competing interests: A.S. works as a consultant for Miltenyi Biotec who owns IP rights concerning the ARTE technology. P.B. declares no competing interests.

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