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Murine models for mucosal tolerance in allergy

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Abstract

Immunity is established by a fine balance to discriminate between self and non-self. In addition, mucosal surfaces have the unique ability to establish and maintain a state of tolerance also against non-self constituents such as those represented by the large numbers of commensals populating mucosal surfaces and food-derived or air-borne antigens. Recent years have seen a dramatic expansion in our understanding of the basic mechanisms and the involved cellular and molecular players orchestrating mucosal tolerance. As a direct outgrowth, promising prophylactic and therapeutic models for mucosal tolerance induction against usually innocuous antigens (derived from food and aeroallergen sources) have been developed. A major theme in the past years was the introduction of improved formulations and novel adjuvants into such allergy vaccines. This review article describes basic mechanisms of mucosal tolerance induction and contrasts the peculiarities but also the interdependence of the gut and respiratory tract associated lymphoid tissues in that context. Particular emphasis is put on delineating the current prophylactic and therapeutic strategies to study and improve mucosal tolerance induction in allergy.

Keywords

mucosal tolerance; regulatory T cells; commensals; allergy; animal models; GALT; NALT; BALT

Introduction

Induction of immunological tolerance is one of the key mechanisms to ensure that immunity is directed exclusively against pathogens but not against innocuous ingested antigens in food or inhaled antigens thereby guaranteeing immunological homeostasis. In this review, we focus on established and novel approaches to induce tolerance with a special focus on the use of mucosal tolerance induction as prophylactic and therapeutic interventions in allergic diseases.

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General principles of mucosal tolerance

The mucosal surfaces lining the airways, gut, and urogenital tract are constantly exposed to a multitude of environmental antigens and microbes [1]. Immune-surveillance at mucosal surfaces includes the action of immunological mechanisms that overlap with those that help maintain tolerance to self [2, 3]. In healthy individuals, inhalation and/or ingestion of innocuous environmental antigens but also beneficial commensal-host interactions in the gut, the respiratory tract and other mucosal surfaces result in immunological tolerance and maintenance of mucosal homeostasis [4]. This is an active process during which induction of regulatory T cells in the periphery (pTreg), but also other suppressive cells, cytokines and regulatory antigen presenting cells (APCs) are the key factors, which are required to avoid pathological responses in the airways and the gut [5–7]. An imbalance in tolerance control mechanisms may result in the development of different forms of allergies, culminating in the generation of asthma or severe anaphylactic reactions, but also other inflammatory diseases with a disease-associated bias in T helper cell responses [2, 8]. However, especially the immune pathways that trigger the initial induction of tolerance but also those which lead to a possible later break in tolerance are still largely unknown [9–11].

Basic mechanisms of the induction of mucosal tolerance

The phenomenon of tolerance to mucosally delivered allergens has first been described by *Dakin* who reported about tolerance induction as a strategy to avoid allergic reactions. He observed that Native Americans prevented contact hypersensitivity reactions to urushiole, found *e.g.* in plants of the genus *Toxicododendron*, by eating poisonous ivy leaves [12, 13]. In 1911, *Wells* could demonstrate that continuous feeding of inert protein antigens could lead to a state of antigen-specific unresponsiveness [14, 15]. Later on, *Bienenstock and co-workers* introduced the concept of a common mucosal immune system in which immunization within one mucosal compartment could confer protection at distant mucosal sites through the movement of cells and humoral factors (antibodies) [16, 17]. Mucosal tolerance induction has since been exploited and shown to be dependent on factors such as the antigen itself, the dose of the antigen (high versus low), the route of application, the sensitization schemes and the host factors (*e.g.* genetic, epigenetic, demographic and immunological factors, as well as the local microbiome) [18, 19]. These interventions, whether via the nasal, sublingual, or oral route aim at the induction of specific pTreg cells or blocking antibodies that migrate/diffuse and control established systemic and local allergic responses also at distant mucosal sites such as the lung [20]. Models of oral and nasal allergen application using T cell receptor (TCR) transgenic or RAG-deficient mice have greatly helped to demonstrate that mucosal tolerance is impaired when the conversion of peripheral T cells into pTregs was impaired [21–23]. In that context, exact dosing and immunization schedules for mucosally administered antigens were shown to play a pivotal role. In mice, two forms of tolerance, *i.e.* high-dose and low-dose, can be discriminated. Administration of a single high dose of antigen (>20 mg) results in lymphocyte deletion via CD95 (FAS)-dependent caspase activation and apoptosis or lymphocyte anergy as a result of TCR ligation in the absence of adequate co-stimulation or by CLTA-4 mediated feed-back regulation [19, 24–28]. In contrast, low dose tolerance is induced by repeated exposure to lower doses of antigen (100 ng – 1 mg), and is dependent on pTregs and active suppression

[19, 29–31]. Importantly, these two forms of tolerance induction (anergy and active suppression) are not mutually exclusive but may occur simultaneously. Tolerance induction depends also on the formulation of the antigen. While soluble antigens favor systemic tolerance induction, particulate antigens are generally more immunogenic and may prime immune responses [27, 32, 33].

The variation of application regimen, but also modifications in the formulation of mucosally, especially of orally, applied antigens provide possibilities to favor desired immunomodulation/-deviation. In this respect antigens prone to enzymatic degradation in the upper gastrointestinal tract can be protected by differential coating and/or containment strategies (*e.g.* enteric coating) and might thus have a better chance to reach the more distal mucosal surfaces in comparison to unmodified forms [34]. An important distinction based on the induced effector mechanisms should be made between long-lasting tolerance induction and short-lived down-regulation of effector cell function [35]. The goal of clinically induced tolerance is to reach a state of unresponsiveness that involves an immunologic deviation away from pro-allergic Th2 immunity that persists regardless of further allergen exposure [35, 36].

How the microbiome shapes mucosal tolerance

It is generally believed that mucosal tolerance to environmental allergens requires microbial colonization early in life [37]. More than 10^{14} microorganisms of more than 500 different species make the intestine the major source of commensal microbes. The local microbiota of the airways and the gut have profound and long-term effects on the hosts' mucosal immunity [38–40]. The number of bacteria largely vary from the proximal to the distal end of the gastrointestinal tract, ranging from $10^2 – 10^3$ per milliliter in the stomach to up to 10^{12} bacteria per gram (dry weight) of colonic contents [41, 42]. Commensals also have the capacity to actively suppress inflammatory pathways in epithelial cells by *e.g.* blocking NF-κB activity [43, 44], resulting in the release of transforming growth factor beta (TGF-β), retinoic acid (RA) and thymic stromal lymphopoietin (TSLP) from the epithelium that instruct the differentiation of tolerogenic dendritic cells (DCs) [45, 46] (Figure 1). Additionally, retinoid-related orphan receptor γt (RORγt) positive innate lymphoid cells (ILCs) can selectively suppress pathological $CD4^+$ T cell responses to commensal bacteria through MHCII engagement, limiting pathological immune cell responses to commensals and ensuring intestinal homeostasis [47]. It is hence conceivable that an intact response to the commensal bacteria and its components is essential for mucosal tolerance and that commensal dysbiosis is associated with biased type 2 immunity and susceptibility to allergic diseases [37, 38, 48, 49]. The importance of the microbiome for intact immune tolerance has been shown in germ-free (GF) as well as antibiotic-treated mice. Mice lacking a normal microbiome show increased allergic responses in models of both allergic airway disease and food allergy [38, 50–53].

Likewise, establishment of oral tolerance depends on the presence of the gut microbiota [54, 55]. There is, however, also data showing that tolerance, particularly nasal tolerance, can be established in the absence of commensal bacteria [56, 57].

Mechanisms underlying mucosal tolerance induction

a) Oral tolerance: Function of the gut associated-lymphoid tissue (GALT)

IL-10 is the master regulator of intestinal mucosal homeostasis [58], which has been shown by the development of spontaneous enterocolitis upon its genetic deletion in mice [59]. However, no such pathology becomes evident in IL-10 knock-out (KO) mice under GF conditions [60], indicating that under physiological conditions, the microbiome shapes mucosal immunity against potential pathogens in the intestines in an IL-10 dependent manner. At mucosal surfaces, critical factors influencing immunity and tolerance are determined by i) antigen size and ii) particularity, which dictate the mode of antigen uptake. The inductive sites, the so called Peyer's patches (PP), are constituted by lymphoid follicles that are covered by epithelium densely populated with microfold cells (M cells) (Figure 1). Microfold cells represent Siglec-F^+ enterocytes [61], which are able to engulf particulate material and shuttle it to the lamina propria (LP), the mucosal effector site in the gut [62]. In contrast, soluble molecules are taken up by different modalities depending on the molecule size. Small molecules are able to diffuse freely from the gut lumen via tight junctions and reach the LP through a process called paracellular permeability [63]. Larger molecules reach the LP by active transcellular transport. In addition, exosomal-based pathways operated by MHC class II^+ enterocytes and transporting cargo within vesicles to the basolateral site of enterocytes seem to be operative as well [64, 65]. Additionally, DCs as well as $CX3CR1^+$ macrophages sample soluble antigens by virtue of their cellular processes, which extend from the LP through the epithelium into the gut lumen [18, 66].

Using *ex vivo* confocal imaging of intestinal lymphatics and sampling of intestinal draining lymph it has been shown that (soluble and particulate) antigenic material becomes transported within $CD103^+$ LP-derived DCs from the LP via afferent lymphatic vessels to the mesenteric lymph nodes (mLN) [67]. Before they migrate towards the mLN, DCs are located in special regions of the GALT where they function as sentinels and are forming an interconnected mesh within the LP of intestine. Once arrived in the mLN, $CD103^+$ DCs are believed to play a critical role in the initiation of oral tolerance. Under steady-state conditions, more than three quarters of the $CD11c^+MHCII^+$ DC found in the intestinal lymph are constituted by $CD103^+$ cDCs [68]. This is consistent with the observation that antigen-specific proliferation of $CD4^+$ and $CD8^+$ T cells can be induced by $CD103^+$ DCs, which had been isolated from mLN shortly after oral challenge [69, 70]. Migration of $CD103^+$ LP DCs is a highly ordered, CCR7-dependent process. In fact, it has been clearly shown that CCR7 deficiency is incompatible with the induction of oral tolerance [71, 72], establishing the importance of constitutive antigen transport from the LP to the mLN to maintain mucosal integrity. The continuous flow of DCs towards the mLN might be a major mechanism how tolerance to food-derived antigens and the commensal flora [73] is initiated and maintained [74]. Epithelial cells heavily imprint the gut-resident $CD103^+$ DC by their production of instructive factors such as TSLP, TGF-β1 and RA [75, 76]. Accordingly, the imprinted phenotype of the gut-derived $CD103^+$ DCs arriving at mLN strongly favors the induction and generation of pTregs. Once arrived within the mLN, the non-hematopoietic stromal cells contribute to final maturation of the migratory $CD103^+$ DCs since they are also

a rich source for RA-inducing enzymes [77]. In that respect, stromal cells together with the mLN synergize with $CD103^+$ DCs for RA production [69].

Upon arrival in the T cell-rich areas of mLN, the $CD103^+$ gut-derived DCs differentiated under the influence of RA start to induce gut-homing receptors on paracortical T cells residing in mLN [69, 78]. The majority of them induces a pTreg program by virtue of their production of RA from retinoids (vitamin A). In fact, different forms of pTregs are formed and can be purified from mLN, ones which are TGF-$\beta1^+Foxp3^+$ but also IL-10^+Foxp3^+ and IL-10^+Foxp3^- populations. Mesenteric LN seem to be the primary site for oral tolerance induction, which has been convincingly shown by surgical removal of mLN [72], genetic manipulations eliminating the generation of mLN [79], or pharmacological deletion of the mLN that completely inhibited the induction oral tolerance.

Peripheral Tregs have obtained the capability to circulate back to the LP with the help of their newly acquired homing receptors CCR9 and $\alpha4\beta7$ integrin [80–84]. Upon arrival in the LP, they encounter a milieu rich in IL-10 provided by $CX3CR1^+$ macrophages [85] residing in the LP and communicating with the gut lumen, which guarantees the pTregs' secondary expansion and their long-term maintenance locally [82]. Of note, the pool of pTregs that homes to and gets expanded in the LP is, however, not fully confined to this anatomical site. Indeed, some of the pTregs generated and expanded within this compartment acquire the capability to escape the local environment and to exert their functional program at distant mucosal effector sites or even systemically, thus leading to systemic tolerance by oral intake of antigen. A less well recognized organ involved in tolerance induction is certainly the liver, which might not only harbor gut-derived pTregs but also represent a primary site for oral tolerance induction by liver-resident APCs, such as Kupffer cells, myeloid or plasmacytoid DCs [86, 87].

b) Respiratory tolerance: Function of nasal (NALT) and bronchus (BALT) associated lymphoid tissue

The respiratory tract and its associated lymphoid tissues seem to be similarly efficient in inducing mucosal tolerance and/or a shift in pathologic T cell populations in experimental animals [88–92]. In the upper airways, rodents present with a NALT that consists of paired, bell-shaped lymphoid cell accumulations at the entrance of the nasopharyngeal tract, considered to be equivalents of the pharyngeal lymphoid ring of *Waldeyer* in humans [93–95]. Apart from the various forms of tonsils making up the *Waldeyer* ring there are no data on organized lymphoid tissues in the ventral parts of the nose of adult humans [96]. Nevertheless, there are immune cells found disseminated in the nasal mucosa [97]. The antigen-presenting environment of nose-draining cervical lymph nodes is pivotal for the proper development of nasal pTreg. In fact, ablation of nose draining lymph nodes in rodents results not only in loss of tolerance but also makes subsequent attempts to induce nasal tolerance impossible such as transplantation of non-mucosa-associated lymph nodes [98].

In the lower airways, *i.e.* the lungs, BALT seems to be dispensable for T cell priming since mediastinal lymph nodes draining the lower respiratory tract represent the major sites for T cell priming [99]. Under homeostatic conditions, many mammalian species, among them also mice and humans, lack a *bona fide* BALT [100, 101], although it has been reported in

the fetal lung and in adolescents [102, 103]. However, specific circumstances of infection or chronic inflammation can induce formation of neolymphoid tissues that participate in immune response initiation and control. Along those lines, respiratory syncytial virus infection has been shown to induce BALT (iBALT) [4, 104], thus belonging to the ectopic or tertiary lymphoid tissues that form in the lungs only after chronic inflammation or infection [104, 105]. Once formed, the iBALT represents a highly ordered lymphoid tissue consisting of separate B and T cell areas, follicular and resident DCs, high endothelial venules and lymphatics, which promote rapid and efficient immune responses. In contrast to mediastinal lymph nodes that are the major T cell priming sites, BALT seems to offer a site for the expansion of memory B cells with a preponderance of IgA precursor cells and as such is a major local contributor of mucosal IgA [99]. In fact, secretory IgA is thought to play an important role in the prevention and/or downregulation of pro-inflammatory responses associated with the inhalation or ingestion of potentially allergenic antigens [106]. The recognition that tolerogenic responses can be generated locally in the lungs dates back as far as 1971 [107]. Similarly to the gut, lung RA-producing $CD103^+$ DCs have the ability to promote the induction of pTregs, although pDCs might contribute in that respect as well [91, 108–111]. In addition, several studies have associated lung γδ T cells with tolerance induction in rodent models of airway exposure to inhaled antigens through their production of IFN-γ that resulted in a suppression of Th2 immune responses and IgE antibody production [112, 113].

Despite their distinct lymphoid organogenesis and adaptation to the particular anatomic location, NALT and BALT share many structural and some functional features with PP, in terms of their role as mucosal inductive sites [99]. Both NALT and PP of the intestine share the presence of a specific follicle associated epithelium containing M and goblet cells along with intraepithelial lymphocytes [114]. However, there are also several differences such as the ratio of $CD4^+$ to $CD8^+$ T cells, the mode of antigen presentation and the provision of secondary signals as well as the cytokine milieu [115]. These are most likely caused by variations in high-endothelial venules determining the lymphoid migration pattern as well as the specific configuration of the nasal tissues with regards to antigen-presenting cells [116]. In accordance with the importance for priming but also silencing of primary immune reactions, several populations of DCs patrolling the nasal mucosa have been described, which are, in fact, rapidly mobilized upon exposure of the nasal mucosa to gram-negative bacteria or their outer membrane component LPS [116]. Besides a number of different classical DC subsets, characterized by the phenotype $CD11c^+MHCII^+CD103^+CD11b^-$ and $CD11c^+MHCII^+CD11b^+CD64^-F4/80^-$, populations of nasal $CD11b^+CD64^+F4/80^+$ macrophages and of $CD103^+CD11b^+$ migratory DCs, similar to those observed in the intestinal mLP, have been detected [116]. Irrespective of the diverse APC populations in the NALT, upon antigen challenge, only few germinal centers form. Notably, tolerance induction seems to become organized in superficial cervical lymph nodes draining the nasal mucosa of rats [117], while specific Ig(A)-secreting cells are induced in the posterior cervical lymph nodes of mice [95]. Nasal tolerance induction seems to rely on active suppression and the generation of pTreg cells with the capability to produce TGF-β, as proven by the abrogation of tolerance by anti-TGF-β blocking antibodies [90]. Comparisons of gene expression pattern in nasal-draining lymph nodes and peripheral lymph nodes identified regulatory

factors intrinsic to the nasal-draining lymph node environment, among them secretory leukoprotease inhibitor (SLP1), which was found to be differentially expressed in DCs [118].

Constitutively, DC-expressed SLP1 was identified to be a rheostat controlling the level of anti-microbial stimuli owing to its capability *e.g.* to suppress LPS responses. More recently, SLP1 has also been shown to be induced in a TSLP-1-dependent fashion in the intestines, during wound repair after intestinal injury in a DSS colitis model [119].

General strategies for oral/nasal/sublingual tolerance induction

Even though oral/mucosal tolerance refers to physiological induction of tolerance to innocuous antigen that occurs in the GALT and other mucosal surfaces such as the respiratory tract, the majority of initial experimental studies in the past concentrated on the effects of orally applied antigens [19], influencing Th1-driven autoimmunity and inflammatory diseases [19, 120–122]. These studies identified TGF-β and IL-10 secreting Tregs as major players in the development of bystander tolerance. For IgE-associated type 2 immune responses, allergen-specific immunotherapy (SIT) also intends to induce Tregs as well as blocking IgG antibodies against the culprit antigen(s) and aims at reestablishing a state of (mucosal) tolerance. Besides allergen source avoidance, conventional treatment regimen for allergic diseases use symptom-relieving drugs that target end-organ manifestations, temporarily suppressing inflammatory mediators and immune cells [123]. However, the disease causing Th2 bias remains largely uncontrolled. To date, SIT is the only rationale-based and long-term curative treatment for allergies [5].

Holt et al. were the first to describe that aerosolized or intranasally (i.n.) applied antigen, such as ovalbumin (OVA), prevented formation of antigen-specific IgE antibodies upon intraperitoneal challenge of alum-adsorbed OVA [124]. Subsequent adoptive cell transfer experiments identified suppressor cells in such treated mice paving the way for respiratory tolerance induction (nasal or aerosol antigen exposure) as treatment concept for allergy and allergic asthma [125]. While both oral and nasal tolerance induction with OVA reduced airway eosinophilia, airway hyperresponsiveness (AHR), mucus secretion and cytokine production, OVA-specific IgE antibodies were only modestly reduced. That the degree of tolerance induction is, however, not only dependent on the route of antigen application but also on the nature of the antigen *per se*, was shown by us recently. While both nasal and oral application of low doses of the major birch pollen allergen Bet v 1 prevented allergen-specific T and B cell responses robustly [126, 127] (Table 1), major grass pollen or latex allergens applied in a similar manner were clearly less effective in preventing allergic sensitization and airways inflammation [128, 129]. Moreover, sublingual allergen administration had been identified and established as an effective treatment modality for tolerance induction and is clinically applied nowadays. The underlying mechanisms of tolerance induction were, however, only recently elucidated [130, 131]. In mice, sublingual tolerance induction with antigen or antigen-conjugated to cholera toxin B subunit (CTB) was shown to effectively reduce Th2 responses by effector cell depletion or active regulation through pTreg [132, 133]. The similarities of the induced immune mechanisms that lead to nasal, sublingual and oral tolerance induction are currently poorly defined. In addition, the

translational aspects allowing the comparison between murine models and human disease need to be taken into account.

For nearly a century SIT in allergic patients has been performed by subcutaneous (s.c.) or intradermal injection of allergen extracts in conjunction with alum as adjuvant. Allergens in natural extracts can show great variations in amounts and immunogenicity [134]. Therefore, standardized production processes are difficult to control and molecular approaches to produce recombinant allergens have been successfully introduced for immunotherapy [135, 136]. In order to reduce potential side effects during therapy, genetically engineered hypoallergenic derivatives, such as allergen fragments or mutated allergens, which lack IgE reactivity but retain T cell epitopes, have been introduced [134, 137]. However, injection-based SIT requires frequent applications along with up-dosing schedules and thus poses a rather inconvenient treatment option for patients. Accordingly, testing the mucosal route for tolerance induction in respective animal models gained considerable attractiveness. Mouse models aim to mimic the complex character of human allergic disorders as close as possible in order to use them as a tool for (i) the development of novel treatment strategies, *e.g.* mucosal tolerance induction; (ii) studies on mechanistic pathways of intervention; and (iii) evaluation of both safety and efficacy in preclinical settings prior to translation into clinical application in humans [138].

Mouse models of allergic asthma

While the acute phase of asthma can be easily monitored experimentally, the typical pathophysiological correlates of the chronicity of allergic asthma are much more difficult to achieve in mouse models. They critically depend on the antigen which upon repeated application over a longer period of time can either lead to tolerizing [139, 140] or inflammatory effects [141]. In the past, low, intermediate, and high responder strains were identified according to parameters such as the levels of allergen-specific IgE and the degree of airway inflammation upon allergen challenge. BALB/c mice are high responders reacting with many allergens and are thus widely used; A/J and AKR/J mice respond with pronounced AHR upon methacholine exposure. In contrast, DBA/2 or C3H/HeJ are largely resistant to AHR challenges. C57BL/6 have several limitations since they tend to mount Th1-biased immune responses and are thus only intermediate IgE responders. However, they are easy to bread and the regular availability of genetically modified lines on this background makes them a commonly used strain for allergy models [142, 143]. A variety of allergens have been used for allergic sensitization, of which OVA is one of the most commonly used model allergens for studying pathophysiological events and mechanisms governing immunomodulation and tolerance induction [144]. In particular, the availability of OVA-specific TCR transgenic mice has proven to be a great advantage for studies on the inductive and effector sites of allergic sensitization and tolerance induction [145]. However, with respect to models of allergic airway inflammation, the use of *bona fide* respiratory allergens rather than a dietary antigen such as OVA, is certainly closer related to the human situation. In fact, the extracts from house dust mite (HDM), cockroach or *Alternaria alternata* can be used for sensitization via the respiratory route even in the absence of any adjuvant [146]. Of interest, antigens/allergens can vary not only in their immunogenic but also in their tolerogenic properties. Along those lines, OVA, applied under identical

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experimental conditions as the major birch pollen allergen Bet v 1, was less immunogenic and tolerogenic than the inhalant allergen [147]. Furthermore, OVA models fail to recapitulate important aspects of allergic sensitization, e.g. the necessity of TLR4 triggering on epithelial cells which is otherwise important for sensitization to HDM allergens [148]. Furthermore, several newly developed drugs targeting key-players in allergy that showed efficacy in murine OVA models subsequently failed in clinical trials. This indicates that differences in the underlying mechanisms between the OVA allergy model and the human disease might be responsible for the poor success in clinical translation [149–151]. Therefore, it is important that each allergenic molecule is examined individually in a suitable model of IgE-associated allergy when considered as a therapeutic agent. Along these lines, the recently developed humanized major mugwort pollen allergen-specific mouse model based on a human TCR and the human-relevant restriction element (HLA-DR1) seems to be very promising (*Neunkirchner A*, in preparation).

Mouse models of mono- and poly-sensitization with aeroallergens

Seasonal respiratory allergies afflict a large number of atopic individuals, with tree and grass pollen allergies being very prominent particularly in Northern and Central Europe. We previously established a mouse model of birch pollen allergy and its major allergen Bet v 1 [147, 152] (Table 1). The standard sensitization protocol based on systemic (s.c.) injection of Bet v 1 adsorbed to alum followed by aerosol challenge with birch pollen extract, leads to classical features of allergy, *i.e.* systemic allergen-specific Th2 responses, airway inflammation, and AHR [127, 153]. A similar model for grass pollen allergy was established in BALB/c mice [154]. In contrast to birch pollen, grass pollen harbors several immunodominant allergens, of which particularly Phl p 1 and Phl p 5 have strong sensitizing properties in BALB/c mice [154]. Similarly, murine models of olive pollen allergy with both Ole e 1 or Ole e 9 for systemic sensitization followed by aerosol challenge with the whole pollen extract have been developed [155–157]. With respect to perennial allergies, murine models of HDM allergy have been established, either using the dominant HDM allergens Der p 1 [158–160] and Der p 2 [161–163] or whole HDM extracts [164–166]. Similar models were established to study cat allergy using the major cat dander allergen Fel d 1 for systemic sensitization followed by challenge with the whole protein extract [167]. In this model it was first shown that subcutaneous injection of the immunodominant peptide of Fel d 1 led to non-responsiveness not only to the injected peptide but also to the whole extract upon subsequent challenge, a phenomenon termed “linked suppression” [167, 168]. The importance of immunodominant T cell epitopes for successful suppression was thereafter demonstrated also in other mouse models [169] including the Bet v 1 allergy model [170] which paved the way for further treatment strategies based on mucosal tolerance induction.

There is substantial evidence that sensitization to one allergen can favor sensitization to further, unrelated allergens [171]. In a mouse model of HDM-induced airway inflammation subsequent sensitization to OVA was facilitated by IL-4 and the presence of inflammatory cells in the airways [172]. Based on the clinical observation that birch pollen allergy is frequently associated with co-sensitization to grass pollen we established a mouse model of poly-sensitization to Bet v 1 and two of the major grass pollen allergens, Phl p 1 and Phl p 5 [154]. The poly-sensitized BALB/c mice displayed comparably strong humoral and cellular

Th2-responses to all three allergens and the immunodominant T cell epitopes in these mice were identical to some of the T cell epitopes in birch and grass pollen allergic patients, indicating that this model has similar immunological characteristics as that of *bona fide* human pollinosis. Further, we established a model to study the oral allergy syndrome (OAS) induced by Bet v 1 homologues in food. OAS is frequently associated with birch pollen allergy, and as such, hampers the compliance for birch pollen-specific sublingual immunotherapy due to adverse reactions experienced in the oral cavity during vaccine application [173, 174]. In this model, BALB/c mice were sensitized against Bet v 1 and the homologous food allergens of carrot and celery, Dau c 1 and Api g 1, followed by a sublingual challenge with carrot, celery, and birch pollen extracts [175]. These models, aiming at closely resembling clinical features of allergic patients, serve as tools for testing novel constructs for mucosal tolerance induction as outlined below in more detail.

Mucosal tolerance induction with recombinant allergens and allergen constructs against respiratory allergy

In contrast to the difficulties associated with allergen preparation/purification from natural sources, recombinant allergens and hypoallergenic variants thereof offer the possibility to work with defined antigenic material. Along those lines, nasal application of recombinant Bet v 1 successfully prevented allergic sensitization and airway inflammation in BALB/c mice, which was independent of the conformation of the allergen but strictly depend on the presence of the immunodominant T cell epitope [126, 176, 177] (Table 1). Our results confirmed previous studies obtained with immunodominant peptides from HDM [178–180] or bee venom allergens [181]. Moreover, they further corroborated the fact that tolerance was inducible in prophylactic and therapeutic settings with either the folded or non-folded recombinant allergen (including the immunodominant T cell peptide), but showed that the underlying mechanisms differed due to the conformational changes. While the folded Bet v 1 version led to the induction of $Foxp3^{+}TGF\text{-}\beta^{+}IL\text{-}10^{+}$ Th cells accompanied with maintenance of tolerance over a prolonged time period, the fragment including the immunodominant peptide led to exclusive upregulation of IL-10 resulting in immediate but only short-term immunosuppression [152]. Our findings are in line with clinical studies using allergen-peptide therapy in which IL-10 was required for peptide-induced suppression [169]. Further studies on the uptake of i.n. applied Bet v 1 and Bet v 1 peptides revealed that both allergen molecules were preferably taken up by alveolar epithelial type II cells, ATII-like cells. However, peptide was taken up faster and more efficiently and induced transcription of IL-10 and MCP1 mRNA. It was therefore assumed that this cell type might contribute to the polarization processes of the respiratory immune system in dependence on the structure and size of the encountered antigen confirming the *in vivo* findings with the two allergen molecules [182].

The clinical observation that poly-sensitized patients are by far more difficult to treat by conventional immunotherapy than mono-sensitized patients gave rise to the concept of poly-tolerance induction with several allergens. Despite the fact that bystander tolerance to unrelated antigens was described as one key feature in oral tolerance [183], it was ineffective to use only one grass pollen allergen, *e.g.* Phl p 1, to induce nasal tolerance against other,

unrelated grass pollen allergens used for co-sensitization [154]. A mixture or a hybrid of the immunodominant peptides of the major grass pollen allergens, however, facilitated the prevention of allergic poly-sensitization. Moreover, only mucosal but not systemic application of the polypeptides prevented allergic airway inflammation upon challenge with grass pollen extracts, which was associated with increased IL-10 levels in the lungs, similarly as seen with tolerance induction by single peptides [128]. In humans, HLA restriction of selected peptides would, however, demand the use of a broader range of allergen T cell epitopes across the different HLA subtypes as has been described for different peptide immunotherapy approaches [179, 184, 185]. Alternatively, chimeric molecules including whole proteins and immunodominant peptides from different allergens could overcome the problem of HLA restriction. Based on the strong and long lasting tolerizing properties of Bet v 1, a recombinant allergen chimer was constructed consisting of Bet v 1 as backbone for linkage of the immunodominant peptides of the major grass pollen allergens Phl p 1 and Phl p 5. Comparison of the tolerogenic properties of this allergen chimer with the polypeptides showed that both constructs led to suppression of allergen-specific Th2 responses and airway inflammation in poly-sensitized mice, but only the chimer additionally induced allergen-specific IgA within the airways [186]. The latter finding was also obtained in a model of oral allergy syndrome using a recombinant chimer between Bet v 1 and Bet v 1-homologues food allergens [175]. Induction of IgA along with suppression of systemic immune responses has been previously described and termed 'split tolerance' in relation with oral immunization [187] and has been also described after intrapulmonary instillation of CTB in mice [188]. A possible protective role of IgA is further supported by studies in infants showing that high intestinal IgA levels were associated with reduced allergic diseases [189].

Murine models of food allergy

Egg, milk, nuts, soy, wheat, fish and shellfish are allergen sources that commonly cause food allergy in humans. The majority of mouse models is performed with OVA, peanut or milk allergens. Many of these models have concentrated on revealing molecular mechanisms underlying food allergic reactions and have proven to be useful tools in evaluating mucosal prophylactic or therapeutic strategies. The different sensitization routes in use for the establishment of food allergy models include the intraperitoneal, subcutaneous, epicutaneous, intragastric and intranasal route. To overcome physiological tolerance to ingested antigens in experimental settings, the mucosal adjuvant cholera toxin (CT), known to enhance Th2 responses, or staphylococcal enterotoxin B (SEB) are often co-applied with the food allergens [190]. Only very few food allergens, such as rice seed allergens, possess allergenic properties that lead to classical symptoms of food allergy upon frequent intragastric application even without any mucosal adjuvant [191]. Another approach leading to allergic sensitization to food allergens in an adjuvant-free setting has been shown by repeated epidermal/transdermal exposure to hazelnuts [192], cashew nuts [193] and milk whey proteins [194].

Mechanisms of long-term hypersensitivity with long lasting memory of antigen-specific IgE and IL-4 production were discovered in these models [195]. As discussed for airway hyperreactivity (see above), susceptibility of mice to experimental food allergy is strictly

strain-dependent [196]. Comparing different mouse strains, BALB/c mice exhibited more pronounced symptoms of food allergy after ingestion of OVA such as higher incidence of diarrhea, hypothermia or mastocytosis as opposed to A/J, C3H/HeJ or C57BL/6 strains. Hence, the BALB/c strain is suitable for the establishment of most models of experimental food allergy [197]. Nevertheless, even in these cases tolerance can be overcome by systemic sensitization in conjunction with alum or oral application together with CT [198]. On a similar note, AKR/J mice can be successfully used for peanut but not milk allergen-induced anaphylaxis [199], highlighting the role of the genetic background governing susceptibility to food allergens.

Oral tolerance induction against food allergy

In a mouse model of casein food allergy based on oral sensitization with casein and CT followed by challenge with casein, *Kim et al.* could show that oral pre-treatment with casein reduced allergic responses including hypothermia, diarrhea, or mastocytosis [200] (Table 2). Notably, the treatment induced IL-10-producing $CD5^+$ B cells in mLN, which could transfer the protection to recipient mice in an IL-10 and $Foxp3^+$ Treg-dependent manner [200].

To evaluate the impact of the allergen dosage on tolerance induction, OVA sensitized mice were fed daily with either low dose or high dose hen egg proteins followed by a challenge with egg protein. Of note, only high dose treatment led to successful desensitization [201]. Differences in the tolerogenic properties of food allergens were shown in a model of peanut allergy in which higher doses of whole peanut protein extract in comparison to OVA were required to induce similar suppressive effects [202]. Furthermore, chemical manipulation of the fed antigen can positively influence its tolerogenic properties as demonstrated with mannosylated egg white, signaling through C-type lectin receptor SIGNR-1 on intestinal DC [203], which reduced allergy development via promotion of $CD25^+Foxp3^+$ cells [204]. Tropomyosin is a major allergen for patients suffering from shellfish allergy [205]. *Wai et al.* identified six major T cell epitopes of shrimp tropomyosin allergen Met e 1 and tested them in a mouse model of shrimp allergy [206]. This novel peptide-based oral immunotherapeutic strategy alters the function of both T and B cells as well as the recruitment and function of effector cells and led to the reduction of allergic symptoms.

Similarly, the feeding of three immunodominant peptides of OVA reduced the allergic symptoms and was associated with increased Foxp3- and TGF-β-producing $CD4^+$ T cells in the gut [207]. Using immunodominant peptides of ovomucoid, another major allergen of egg, provided similar results [208]. Furthermore, human immunodominant T cell epitopes of the peanut allergen Ara h 2 were tested for their potential to treat peanut allergy [209]. In a therapeutic model of peanut allergy three routes of allergen application, *i.e.* oral, sublingual and epicutaneous, were compared for the potential to reduce allergic responses. All three routes led to comparable immunosuppression mediated by Treg, which however differed in their phenotypic characteristics [210] (Table 2). In order to overcome potential limitations in translating the immunological finding of food allergy and tolerance induction to the human system, a humanized mouse model of anaphylactic peanut allergy was generated very recently [211]. This model opens new possibilities to study therapeutic intervention on a humanized adaptive immune system of allergic patients.

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Established and novel adjuvants for mucosal tolerance induction

Soluble and surface-expressed pattern recognition receptors (PRR) at epithelial surfaces, that integrate signals from allergen sources but also respond to infectious agents, might represent the crucial rheostat 'setting the tone' of the immune responses and thus deciding on health or disease in the respective target organs. Since not all protocols of mucosal tolerance, particularly in therapeutic settings, have been successful [199, 212], several of these mucosal PRR-pathways have been exploited to redirect or even prevent allergen-triggered Th2 immune responses. The combination of established and novel forms of adjuvants along with engineered, non-allergenic forms of the allergens, the type of allergen-adjuvant conjugation as well as the route of application (nasal, oral or sublingual) are all critical factors to improve future mucosal vaccines. Bacterial products/toxins, toll-like receptors (TLR) agonists, nanoparticles, but also probiotics or parasites, have been used for immunomodulation in mouse models of respiratory or food allergy.

Bacterial toxins

Bacterial toxins, such as CT or the closely related *E. coli* heat-labile toxin (LT) are very potent mucosal adjuvants. Although wildtype CT is commonly used in experimental models of anaphylaxis to break food tolerance, its detoxified form retaining adjuvanticity but almost lacking enterotoxicity can improve tolerance induction when co-applied with antigen [213–215].

a) CTB—The genetically detoxified cholera toxin B subunit (CTB) represents a transmucosal carrier-delivery system, which can be co-administered with soluble allergen as a mixture or conjugate. Already a single oral dose of antigen conjugated to CTB could suppress T cell responses at doses 15- to 500-fold lower than what was needed for induction of tolerance with unconjugated antigen [216]. Similarly, CTB-conjugated OVA suppressed the induction of allergen-specific IgE in prophylactic and therapeutic settings, which was associated with suppression of both Th1 and Th2 responses [217, 218]. Of note, OVA-CTB treatment increased numbers of $Foxp3^{+}CD25^{+}$ and $Foxp3^{-}CD25^{-}CD4^{+}$ Treg cells and elevated levels of TGF-β. We have shown that i.n. administration of OVA chemically coupled to CTB prior to systemic sensitization decreased levels of IL-4 and IL-5 in both lungs and spleens, reduced levels of antigen-specific serum IgE, while increasing IgG2a levels [147]. In contrast, a similar treatment with Bet v 1 chemically conjugated to CTB increased both specific serum IgE and IL-4 and IL-5 levels in spleens and lungs. This suggests that the potential of CTB to induce peripheral tolerance or immunity depends on the nature of the coupled antigen and most probably also on the method of conjugation. Accordingly, application of a genetically engineered fusion molecule of CTB and Bet v 1, led to a decrease of specific serum IgE and IL-5 levels in the lungs [219]. A similarly successful strategy was followed by *Takagi et al.* who fused CTB with three peptides of major Japanese cedar pollen allergens and expressed this construct in rice [220]. Feeding BALB/c mice with such rice seeds suppressed allergen-specific IgE, histamine release as well as clinical symptoms such as the number of sneezes. The advantages of using such an expression system for mucosal delivery comprise low-cost production and facile application, no requirement for purification and easiness of upscaling of antigen production [221].

Smits et al. have shown that CTB-allergen conjugates lead to the production of specific secretory IgA in the airways [188], which was dependent on a RA and TGF-β rich milieu [222]. Thus, the promotion of IgA synthesis by adjuvants (or as we previously reported by newly created allergen-chimers) might be beneficial for induction of tolerance against allergy [106].

b) LTB—Similar to CTB, genetically detoxified lymphotoxin (LTB) strongly induces mucosal and systemic IgA. In early work, *Tamura et al.* showed that nasal application of a single dose of OVA covalently conjugated to LTB suppressed the induction of OVA-specific serum IgE in sensitized mice [223]. More recently, *Lin et al.* tested the LT-detoxified mutant LTS61K for its effects in prophylactic and therapeutic settings in a mouse model of HDM allergy [224]. Intranasal application of Der p 1 together with LTS61K or only LTS61K prior to sensitization and challenge reduced methacholine-induced AHR, the production of Th2 cytokines in the lungs and was associated with high levels of specific IgA (when supplemented with Der p 1) in both serum and lungs. Importantly, LTS61K showed beneficial effects also in therapeutic settings. On a cellular level, LTS61K may act on DCs as revealed by adoptive transfer of bone marrow-derived DCs pulsed with LTS61K or LTS61K and Der p 1 which reduced airway inflammation in sensitized mice [224].

CpG motif-containing oligonucleotides

Apart from bacterial toxins, non-methylated CpG DNA represents another typical microbial component that can target innate immunity through interaction with TLR9. Triggering of TLR9 expressed in endosomal compartments of antigen presenting cells such as B cells and pDC, results in the induction of inflammatory immune responses [225]. Several animal models have shown the efficacy of CpG-oligodeoxynucleotides (ODN) in prophylaxis or therapy of experimental allergy. Neonatal sublingual vaccination with denatured OVA admixed to CpG stimulated mucosal production of IgA along with increased systemic Th1 responses in newborn BALB/c mice [226]. In a mouse model of OVA-induced allergic airway inflammation, oral application of OVA and CpG ODN to already sensitized and challenged mice prevented eosinophilia and suppressed specific IgE [227, 228]. Similar effects were observed in a mouse model of HDM-induced airway inflammation [229] where intratracheal application of CpG ODN mixed with *Dermatophagoides farinae* extract inhibited eosinophilia and Th2 cytokines in the lungs in a dose-dependent manner. For the efficacy of CpG ODN, the route of mucosal application does not seem to play a major role as both intranasal and intratracheal routes are comparable, redirecting the immune response towards a Th1 type. Such treatment not only affected the numbers of eosinophils in the airway and lung parenchyma but also in blood and bone marrow [230]. In a mouse model of ragweed allergy, pre-treatment with CpG reduced AHR, lung eosinophilia and serum levels of specific IgE [231] up to 6 weeks. The critical role of IFN-γ in the beneficial effect was demonstrated in IFN-γ–deficient mice. *Campbell at al.* established a chronic mouse model of ragweed allergy and tested the effect of i.n. administration of free CpG ODN [232]. Intranasal CpG ODN suppressed eosinophilia and production of lung Th2 cytokines but increased the expression of regulatory markers, such as Foxp3, IL-10 or TGF-β. Importantly, the suppression sustained several weeks after the last CpG ODN treatment, despite

continuous ragweed exposures, demonstrating the disease-modifying effect of mucosal CpG ODN application.

Chitin/Chitosan

Chitin, a natural polymer of N-acetyl–D-glucosamine, is a characteristic component of fungi, helminth parasites and arthropods such as crustaceans [233]. Recognition trough the innate receptors TLR2, Dectin-1 and mannose receptor leads to induction of immune responses [234]. Oral application of chitin to ragweed-sensitized mice decreased levels of serum IgE and lung eosinophilia [235]. *In vitro* stimulation of splenocytes from ragweed-sensitized mice with the combination of ragweed extract and chitin decreased the production of Th2-type cytokines associated with induction of IFN-γ, suggesting a redirection towards Th1 immunity. Similarly, i.n. chitin administration to HDM-sensitized mice redirected the immune response towards a Th1 phenotype [236]. In contrast, *Reese et al.* showed that i.n. application of chitin increased the accumulation of eosinophils and basophils in tissue [237]. These contradictory outcomes might likely result from the size of chitin particles used as well as the dose and/or route of application, as discussed previously [238].

Chitosan is a natural, biodegradable, and well-tolerated muco-adhesive polysaccharide formed by deacetylation of chitin [239]. Chitosan promotes the mucosal absorption of proteins, which can be released slowly and effectively at mucosal surfaces [240].

Intranasal application of chitosan during sensitization and challenge reduced *D. farinae*–induced airway inflammation, manifested by reduced levels of infiltrating inflammatory cells, epithelial damage and reduced goblet cell hyperplasia [241]. Similar to chitin, chitosan induced a Th1-polarized phenotype, which was also observed in mice i.n. treated with *D. farinae* entrapped in chitosan microparticles [242]. *Saint-Lu et al.* compared medium and high molecular weight chitosan microparticles and found that only high molecular weight chitosan increased uptake, processing and presentation of OVA by APC [243]. Sublingual application of high molecular weight chitosan to OVA-sensitized mice with established asthma enhanced tolerance induction, with reduced AHR, lung inflammation, and eosinophilia. Moreover, oral vaccination of mice with chitosan nanoparticles containing plasmid DNA encoding the HDM allergen Der p 1 [244] or Der p 2 [245] led to Th1 immune responses against the respective allergen. Additionally, chitosan-Der p 2 nanoparticles induced the expression of Der p 2 in epithelial cells of the gastrointestinal tract and reduced the levels of sensitization.

Probiotics

Mice lacking a normal flora showed increased serum IgE levels and higher numbers of circulating basophils in the blood that contribute to exaggerated Th2 cell responses in an experimental model of HDM-induced asthma, indicating that commensal bacterial–derived signals might limit allergic lung inflammation [51, 246]. Similarly, oral exposure of GF or antibiotic-treated mice to OVA resulted in Th2-predominated inflammation with exaggerated IgE/IgG1 responses and increased IL-4 production [247, 248]. Moreover, antibiotic treatment during early life altered microbial communities in the gut, leading to significantly increased total IgE/IgG1 levels and enhanced asthma severity in a murine model of OVA-

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induced AHR [249, 250]. Consequently, the intentional introduction of non-pathogenic bacterial strains, commonly referred to as probiotics is an exciting possibility to modulate systemic immunity. In fact, probiotic bacteria can trigger diverse PRR receptors, such as TLR2, TLR4 or DC-SIGN and have shown to induce Th1 and/or Treg responses.

In a model of type I allergy, we could show that i.n. co-application of *L. lactis* or *L. plantarum* with Bet v 1 led to reduced IgE-dependent basophil degranulation and shifted immune responses towards a non-allergic Th1 phenotype with increased IFN-γ production in both therapeutic and prophylactic settings [251]. Similarly, a recent study by *van Esch et al.* showed that oral administration of *B. breve* together with short-chain fructooligosaccharides reduced allergic immune responses such as mast cell degranulation and anaphylactic shock along with increased Th1 responses in sensitized mice [252]. Along these lines, *Trompette et al.* showed that diet changes the metabolic output of gut bacteria, such as the levels of short-chain fatty acids (SCFA), that protect from allergic airway inflammation [253].Taking advantage of a GF mouse model, which allows to study the impact of single bacterial strains or a defined mixture of strains on tolerance induction, we demonstrated that neonatal mother-to-offspring mono-colonization of GF mice with *B. longum* reduced the development of allergic responses to Bet v 1 [254]. Furthermore, we showed that colonization of GF mice with a mixture of three lactobacillus strains ameliorated allergic sensitization to Bet v 1. This effect was associated with increased levels of IgA and TGF-β and increased integrity of the gut mucosa [255]. *Van Overtvelt et al.* compared the potential of several probiotic bacteria to induce pro- and anti-inflammatory cytokines by DCs and identified two different types of immune responses induced by probiotics [256]. The first group of probiotics induced both IL-12 and IL-10 cytokines, such as *L. helveticus* while the second group contained exclusive Th1-inducers, such as *L. casei*. Interestingly, sublingual application of *L. helveticus*, but not *L. casei* reduced symptoms of airway inflammation, suggesting, that strains potentiating Th1/Treg responses might be preferable over exclusive Th1-inducers. Similarly, in a mouse model of poly-sensitization to major birch and grass pollen allergens, we could show that i.n. application of *B. longum*, a strain inducing high IL-10/IFN-γ or IL-10/IL-12p70 ratios, but not of *L. paracasei*, a potent Th1-inducer, had long lasting protective effects against the development of allergy [257]. Recently, we have investigated the impact of i.n. exposure to the probiotic *E. coli O83* (Colinfant Newborn®) strain on the development of allergic airway inflammation in mice. We could show that i.n. treatment with *E. coli O83* during sensitization and challenge completely abrogated allergen-induced AHR, which correlated well with decreased IL-13 levels and eosinophil numbers in broncho-alveolar lavage fluid. Local as well as systemic Th2 cytokine production was decreased, whereas allergen-specific serum IgE was not affected (*Zwicker C*, unpublished data). Very recently, *Gollwitzer* and co-workers elegantly demonstrated that immune tolerance to allergens applied to the lungs was influenced by the resident lung microbiota and associated with the occurrence of microbial-induced Treg cells that required interaction with PD-L1 for their development [38]. Probiotic bacteria have been shown to act as antigen delivery system and vaccine vehicle with immunomodulatory properties. *L. plantarum* producing Bet v 1 led to reduction of humoral and cellular Th2 responses along with increased levels of allergen-specific secretory IgA in both lungs and gut [258], suggesting that the lung-gut axis, in fact, works in both directions. Neonatal

mono-colonization of mice with the same recombinant bacteria revealed that the prevention of allergic responses was accompanied by upregulation of regulatory markers, such as Foxp3 [259]. Moreover, we compared the efficacy of prophylactic treatment with two recombinant *L. plantarum* strains, one secreting Bet v 1 extracellularly, the other producing intracellular Bet v 1 and demonstrated that secretion of the allergen offered better protection against sensitization than intracellular expression of the allergen [260]. A higher immunomodulatory effectiveness of allergen-secreting recombinant probiotics was also demonstrated for other allergens such as Ara h 2 [261].

Due to its important role in mucosal tolerance induction, therapeutic strategies aiming to increase levels of IL-10 at mucosal surfaces might be similarly promising. Accordingly, *Frossard et al.* have shown that the administration of IL-10-expressing *L. lactis* before sensitization to β-lactoglobulin prevented anaphylaxis in young mice along with reduced antigen-specific serum IgE and IgG1 levels [262]. Recently, we have tested the effect of i.n. application of recombinant *E. coli Nissle 1917* expressing chimers of birch and grass pollen allergens in adult mice to suppress allergic poly-sensitization. Mice fed with this strain exhibited significant reduction of airway eosinophilia, Th2 responses in the lungs and reduced IgE but elevated mucosal IgA levels. Whole body and organ specific live imaging technology showed that the recombinant bacteria did not persist longer than 7 days after feeding, an important finding regarding the safety of such a potential intervention strategy (*Sarate P*, unpublished data).

Regarding the underlying mechanisms of immunomodulation via probiotic bacteria studies in GF mice raised and bred on an elemental diet devoid of dietary antigens *Kim et al.* could elegantly demonstrat that pTregs found in the colon were driven by microbiota while those found in the small intestine mostly relied on dietary antigen exposure [52]. These two cell subsets where not only different in location but also with regard to their expression of the transcription factor RORγt, which is primarily expressed by lymphocytes upon exposure to microbes. Furthermore, a skewed composition in mucosal $CD103^+$ DCs, that promote pTreg generation and differentiation and which are thought to represent master regulators of immune tolerance, was found in the LP of the small intestine of mice lacking dietary antigen exposure. Somewhat surprising and despite the paucity of food-induced pTregs, such mice did not show any adverse reactions when switched onto a normal chow diet during adulthood [52]. The exact mechanisms that underlie the maintenance of immune homeostasis and consequently oral tolerance induction to environmental antigens remain unclear as of yet. An experimental setting not addressed in the above study, but which could provide a possible answer to that question, would be based on mice harboring an intact microbiome but being fed on an elemental diet. Overall, it seems plausible that both proximal and distal intestinal compartments contribute to food tolerance [18, 52, 263, 264].

Parasites

Epidemiological and experimental studies have provided an important link between certain infections and reduced allergic responses [265]. In mouse models, symptoms of both airway inflammation or food allergy were decreased by certain parasitic infections [266–268]. In this context, we demonstrated that oral application of *Toxoplasma gondii* oocysts before or

during sensitization reduced Bet v 1-induced allergic airway inflammation [269]. Importantly, the immunomodulatory effects also were inducible by inactivated *T. gondii* extracts omitting the potential risks associated with a live infection. The reduced allergic immune responses were accompanied by increased numbers of $CD4^{+}CD25^{high}Foxp3^{+}$ pTregs in spleens and increased granulocyte myeloid-derived suppressor cells in the lungs [270]. These effects were linked to the presence of distinct, heat-stable components such as immunomodulatory sugars (*Drinic M*, unpublished data). Similarly, heat-stable factors in the extract of the nematode *Oesophagostomum dentatum* prevented the development of birch pollen allergy in mice [271]. Identification of parasite-derived molecules, which can recapitulate the effect of live infections and confer protection against allergy, may offer a safe concept for disease prevention or treatment.

Infection with the gastrointestinal nematode *H. polygyrus* reduced OVA- as well as Der p 1-induced airway inflammation, presumably due to the expansion of $CD4^{+}CD25^{+}Foxp3^{+}$ pTregs [272], B cells [273] or suppressive DC [274]. Infection with another gut-restricted parasite, *Trichuris muris*, induced modulation of immune responses in the lung [275]. The cross-mucosal interaction reduced papain-induced allergic airway inflammation, highlighting the importance of the gut-lung immune axis. Indeed, i.n. application of soluble excretory/secretory products of *H. polygyrus* could supress the production of Th2 cytokines by lymphoid cells as well as eosinophilia in a mouse model of allergy to the fungus *A. alternata* [276].

Imprinting by perinatal maternal signals has been shown to influence the maturation of the immune system in the offspring, which might be important for the development of allergy later in life [277]. Along these lines, *Straubinger et al.* have shown that established *Schistosoma mansoni* infection during pregnancy can reduce allergy in offspring depending on the maternal immune responses to the helminth [278]. Future intervention strategies on mucosal immunomodulation/tolerance may particularly focus on the window of opportunity during the neonatal period for primary prevention of allergy development.

Concluding remarks

Mouse models of IgE-associated allergies share important hallmarks and clinical features with human allergic diseases and have proven useful to study the pathophysiological mechanisms underlying the initiation and maintenance of allergy. Moreover, mouse models of respiratory and food allergy have been widely used for the development and validation of novel treatment strategies. As a result, an increasing number of studies concentrate on concepts to re-establish mucosal tolerance using recombinant allergens, hypoallergenic molecules or allergen peptides with or without the combination of novel adjuvants. The microbiome, in particular, its diversity and dysbiosis, has been recognized as a major factor influencing health and disease, including also IgE-associated allergies. In this respect, GF or gnotobiotic mice, harboring selected bacterial species, significantly contributed to our better understanding of the crosstalk between the host immune system and commensals. Interventions involving the microbiome or factors and metabolites thereof opened a number of new perspectives for allergy prophylaxis and therapy.

It is now essential to translate these findings made in the mouse models into treatment modalities for the human disease. Although tolerance induction in mouse models of autoimmunity could only be recapitulated partially in clinical trials, the outcomes have been more promising with allergic diseases. In fact, sublingual immunotherapy is nowadays considered an efficacious alternative treatment to s.c. immunotherapy of grass or mite allergen-triggered respiratory allergies. As current allergen formulations may bear a risk for adverse events or *de novo* sensitization, many novel recombianant allergen constructs that were successful in mice are now being tested in clinical studies. Importantly, treatment parameters such as schedules, doses, or treatment duration cannot, or only rarely, be directly translated from mouse studies into humans.

In recent years, concepts to prevent allergy, especially in high-risk populations, have gained interest. Many preclinical studies have indicated that tolerance is easier to induce in naïve mice than in mice with an already established allergic disease status. In this respect, both mouse models and clinical data on probiotic substitution postulate that pregnancy and the neonatal period constitute a window of opportunity for immunomodulation and immune-imprinting, particularly for individuals with high risk of allergy development in their children. Future treatment concepts will include patient-tailored interventions based on patient characteristics to predict which treatment modality can provide the most beneficial outcome. This includes either tolerance induction with selected allergens, allergen-nonspecific immunomodulatory treatment approaches, or a combination of both.

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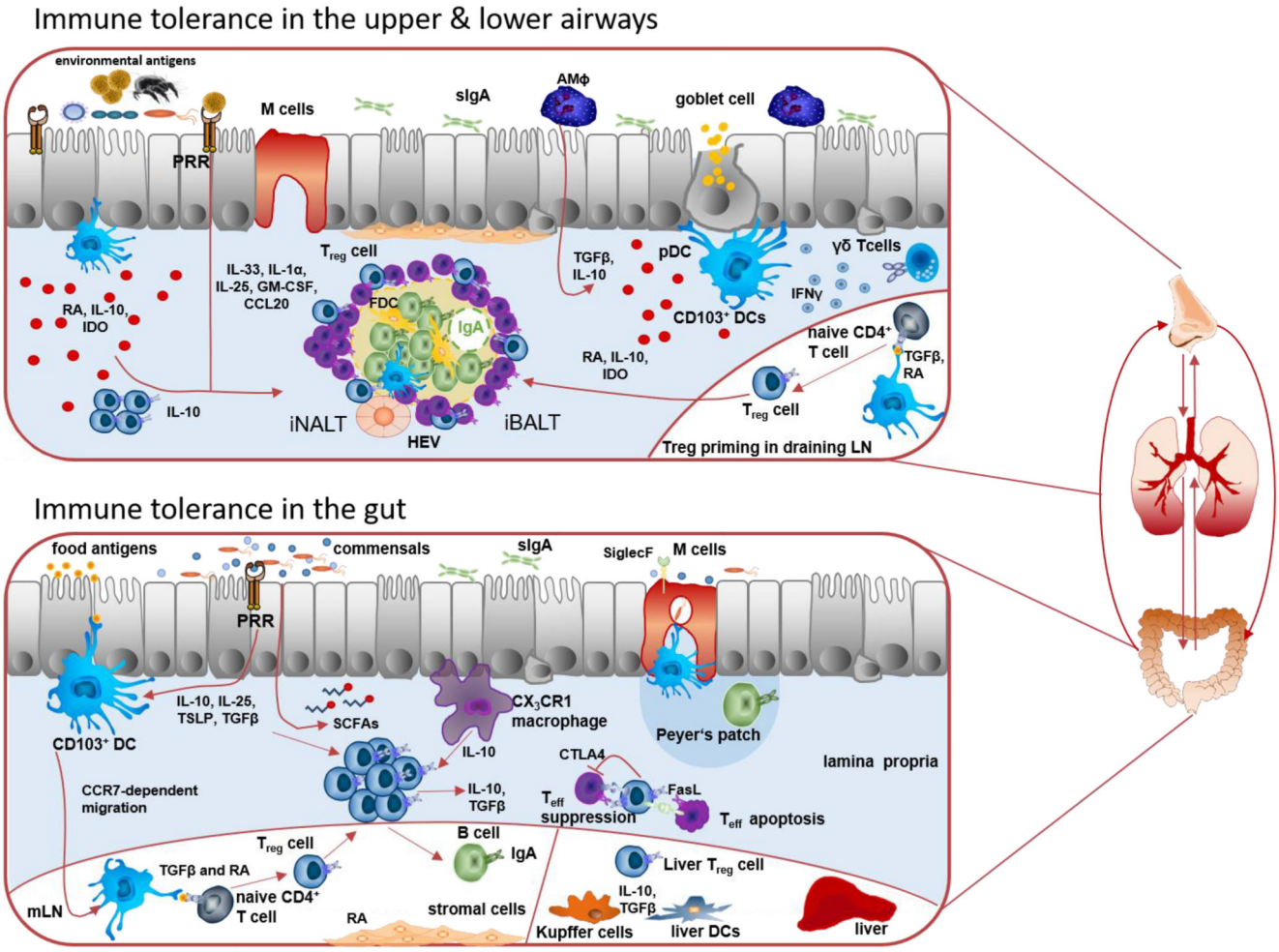

Fig. 1. Maintenance of immune tolerance in the airways (upper panel) and the gut (lower panel).

Under homeostatic conditions, tolerogenic immune responses are created by factors released from airway and gut epithelial cells (cytokines, chemokines, vitamin metabolites, etc) upon triggering of pattern recognition receptors (PRR) by environmental antigens, food and commensals. Shown are the critical mediators that contribute to the priming and differentiation of antigen presenting cells and lymphocytes. Apart from epithelial-derived mediators, these also include metabolites directly derived from commensals (e.g., SCFAs). Moreover, pathways of transepithelial antigen transfer, which include uptake by $CD103^+$ DC, macrophages and M cells but also diffusion through tight junctions and transcellular routes, are depicted. Upon CCR7-dependent migration to draining lymph nodes, $CD103^+$ DC instruct naïve $CD4^+$ T cells to differentiate into Treg, which is dependent on a TGF-β and RA rich milieu created by both DC and LN resident stromal cells. In the periphery, Tregs get further expanded under the influence of IL-10, IDO and RA, and exert their regulatory function (T_{eff} suppression and apoptosis). In addition, gut-derived antigens might also reach the liver, where they become presented (liver DCs, Kupffer cells) and lead to Treg priming. A fraction of gut-resident antigens is directly bound (neutralized) by secretory IgA elaborated by the large number of gut-resident B cells, many of them organized in Peyer's

patches found in the small intestine. Similarly organized structures can be found in the upper and lower airways (iNALT and iBALT). Once induced, those can be recognized as highly organized structures consisting of B cell- and T cell-rich zones, interspersed with follicular (FDC) and conventional DCs and typically associated with HEV. The airways are protected by sIgA and in addition the lower airways are patrolled by alveolar macrophages (AMϕ). FDC, follicular dendritic cells; HEV, high endothelial venules; iBALT, induced bronchial associated lymphoid tissue; IDO, indoleamine 2,3-dioxygenase; iNALT, induced nasal associated lymphoid tissue; PRR, pattern recognition receptor; RA, retinoic acid; SCFA, short chain fatty acid; sIgA, secretory IgA; T_{eff}, T effector cells; T_{reg}, T regulatory cells.

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Table 1
Mouse models of mono- and poly-sensitization with aeroallergens

Sensitizing allergen	Strain	Sensitization	Tolerance induction	Evaluation	Reference
Monosensitization					
Bet v 1	BALB/c	Bet v 1/Alum; i.p. and BP pollen extract; aerosol	Bet v 1 or Bet v 1 fragments; i.n. Bet v 1; oral Bet v 1; i.n., Bet v 1 fragments; i.n., or Bet v 1 hypoallergenic derivative	Specific IgE, IgG1, IgG2a; Th1, Th2, and Treg cytokines; eosinophils; lung histopathology,; AHR IgG2a Skin test, Th1, Th2, Treg cytokines eosinophils BAL, AHR	[126, 152] [127, 147, 153]
Der p 1	C57BL/6	Der p 1/Alum, i.p and Der p 1 i.t.	Microencapsulated Der p 1 peptides; i.n.	Eosinophils; Th1 and Th2 cytokines; specific IgG1, IgG2a and IgE	[178]
Der f 2	A/J	Der f 2/Alum; i.p.	Der f 2 peptides; i.n.	PCA; Th1 cytokines; airway constriction; specific IgG1 and IgG2a, total IgE, peripheral blood leukocytes	[180]
PLA2	CBA/J	PLA2/Alum; s.c.	PLA2 and three PLA2 peptides; i.n.	Specific IgG1, IgG2a and IgE, Th1 andTh2 cytokines	[181]
Ole e 1	BALB/c	Ole e 1/Alum; i.p. and Ole e 1; i.n.	Ole e 1 or Ole e 1 peptides; i.n.	Specific IgG1, IgG2a and IgE; Th1, Th2 and Treg cytokines; total IgE; lung inflammation	[155]
Ole e 9	BALB/c	Ole e 9/Alum; i.p. and Ole e 1; i.n.	Ole e 9 fragments; i.n.	Specific IgG1, IgG2a, IgG2b and IgE; total IgE; lung inflammation	[156]
Hev b 1	BALB/c	Hev 1/Alum; i.p.	Hev b 1; i.n.	Specific IgG1 and IgE; Th1, Th2 and Treg cytokines	[129]
Hev b 3	BALB/c	Hev b 3/Alum; i.p.	Hev b 3; i.n.	Specific IgG1and IgE; Th1, Th2 and Treg cytokines	[129]
Polysensitization					
Bet v 1, Phl p 1, Phl p 5	BALB/c	Bet v 1, Phl p 1; Phl p 5/ Alum; i.p. and BP and PH extract; aerosol	Bet v 1, Phl p 1 and Phl p 5 proteins or peptides as a mixture or hybrid peptide; i.n.	Specific IgG1, IgG2a and IgE; eosinophils, Th2, Th1 and Treg cytokines	[154, 128]
Bet v 1, Phl p 1, Phl p 5	BALB/c	Bet v 1, Phl p 1; Phl p 5/ Alum; i.p. and BP and PH extract; aerosol	Bet v 1, Phl p 1 and Phl p 5 chimer; i.n.	Specific IgG2a, IgE and IgA; eosinophils, Th1 and Th2 cytokines	[186]
Bet v 1, Dau c 1, Api g 1	BALB/c	Bet v 1, Dau c 1, Api g 1/Alum; i.p. and BP, carrot and celery extract; s.l.	Bet v1/Dau c 1/Api g1 chimer¸ i.n.	Specific IgG2a and IgE; total IgA; Th1, Th2 and Treg cytokines	[175]

AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BP, birch pollen; CFA, complete Freud´s adjuvant; DTH, Delayed-type Hypersesitivity; i.n., intranasal; PCA, passive cutaneous anaphylaxis; PH, phleum; s.c., subcutaneous. Examples for murine models of sensitization and tolerance induction using clinically relevant inhalant or food allergens

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Table 2
Mouse models for oral tolerance induction against food allergy

Sensitizing allergen	Strain	Sensitization	Tolerance induction	Evaluation	Reference
Casein	BALB/c	Casein/CT; oral and casein; oral	Casein; oral	Rectal temperature; allergic symptom score; diarrhea; degranulation of mast cells; eosinophils; Th1 and Th2 cytokines; histamine; specific IgG1, IgG2a, IgE and IgA; induction of Treg	[200]
OVA	BALB/c	OVA /CFA; i.p.	OVA; oral	Splenocytes proliferation; Th1 and Th2 cytokines, IgG1, IgG2a, IgG	[201]
OVA	BALB/c	OVA/CT; oral and OVA oral	Peptides; s.c.	Systemic anaphylaxis; histamine; specific IgG, IgE and IgA; Th1, Th2 and Treg cytokines	[207]
Ovomucoid	BALB/c	Ovcomucoid/CT; oral and Ovomucoid; oral	Ovomucoid T cell peptides; oral	Clinical symptoms; histamine; specific IgG, IgG1, IgG2a, IgE and IgA; Th1, Th2 and Treg cytokines	[208]
Peanut proteins and OVA	BALB/c	Peanut protein extract/CFA or OVA/CFA; s.c. and peanut protein extract or OVA s.c. in footpad	Peanut protein extract or OVA ; i.g.	T cell proliferation; Th1, Th2 and Treg cytokines; specific IgG, IgG1, IgG2a; total IgE	[202]
Peanut protein	BALB/c	Peanut protein extract/CT; oral	Peanut protein extract; i.g. or s.l.	Specific IgG2a and IgE; Th1, Th2 and Treg cytokines	[210]
Met e 1	BALB/c	Met e 1/CT; i.g. and Met e 1	Mixture o six T cell epitopes; i.g.	Systemic allergic symptoms; goblet cells; eosinophils; mMCP-1; specific IgG1, IgG2a and IgE; Th1, Th2 and Tregs cytokines	[206]

CFA, complete Freud´s adjuvant; CT, cholera toxin; i.g., intragastric; i.p., intraperitoneal; s.c., subcutaneous; s.l., sublingual