Harnessing dendritic cells to promote immune tolerance: Opportunities for allergen-specific immunotherapy

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GENERAL DISCUSSION
TREG-BASED THERAPY OF AUTOIMMUNE AND ALLERGIC DISORDERS

Efficient immune tolerance, carried out by Treg cells, is crucial for maintaining homeostasis. Treg cells prevent self-reactivity as their absence sparks autoimmune diseases (1). In addition to autoimmune responses, Treg cells also suppress excessive immune reactions towards harmless non-self antigens, like environmental substances involved in allergies and commensal microbes involved in inflammatory bowel diseases (2,3). T cell tolerance is mediated by different Treg cell types. Besides the naturally occurring Foxp3+ Treg cells, tolerogenic activities are also carried out by induced Treg cells. Inducible Foxp3+ Treg (iTreg) cells can be raised under certain conditions. Inducible Foxp3+ Tr1 cells, distinct for the immunoregulatory IL-10, also strongly contribute to peripheral tolerance. Interestingly, the Tr1 subset and in contrast to other Treg subsets is characterized by IFN-γ production (4). This is consistent with accumulating evidence pointing out the importance of immunoregulatory cytokines produced by effector T cells. Those cytokines play a critical role in self-regulating the effector functions of T cells and amplifying the activity of Treg cells; eventually limiting the risk of harmful immunopathology as a result of progressing pathogen-induced T cell-mediated immune responses (5). Thus, Treg cells are pivotal regulators of the adaptive arm of immunity.

Since autoimmune and allergic diseases are correlated with the absence or defective functions of Treg cells, the upregulation of these cells form an attractive target for the treatment of these diseases (6). Treg-based therapy is postulated to have a long lasting effect despite the limited lifespan of Treg cells. This effect is attributed to the capacity of Treg cells to convey a tolerant phenotype to other cells; a phenomenon denoted “infectious tolerance”. Thus far, utilizing Treg cells for therapeutic purposes has been developed in three directions: first by isolating fully differentiated Treg cells and expanding them in vitro; second by generating and expanding Treg cells in vitro; and finally by enhancing Treg numbers and functions in vivo (7). Notably, all three approaches depend on DCs to generate Treg cells, corroborating the central role of DCs in immune tolerance.

UNDERSTANDING ALLERGY

It is becoming evident that allergic diseases are the consequence of subverted reactivity of innate immune cells, including epithelial cells and DCs, towards allergens that promote allergic sensitization of adaptive immune cells. Although the allergic diseases are manifested by IgE-mediated reactions with Th2 effector responses, the underlying etiology leading to these responses remains elusive. Allergies are multifaceted disorders that are governed by multiple variables including the age and the genetic makeup of the subject, the timing of exposure to the sensitizing allergen, and the assortment of factors that can influence immunological responses including the diversity of commensal microbes (8). Gaining a deeper insight into the causes of allergies and identifying what makes certain environmental or dietary components allergenic and why certain individuals are more susceptible to sensitization than others, will be crucial in the design of effective therapies.
The functional properties and structural features of allergens are probably key determinants of its allergenicity. In addition to allergenic antigens, comprising T and B cell epitopes, allergenic entities (e.g. house dust mite fecal pellets, pollen, and peanut) include a complex mixture of biologically active constituents such as proteases, glycans, endotoxins and a variety of unidentified molecules. This bioactivity can be intrinsic to the allergen itself. For instance, one of the major allergens of house dust mite (HDM), Der p 2, was reported to be homologous to MD2, the LPS-binding member of the TLR4 signaling complex, implying that Der p 2 promotes TLR4 signaling (9). Moreover, the protease activity of HDM allergens was revealed to mediate NLRP3 inflammasome activation (10). In addition to intrinsic properties of allergens, the induction of danger signals by allergen-associated molecules can be attributed to allergen-associated components. Activating TLR4 of airway epithelia by HDM-associated LPS was found to be central for asthma induction (11). Another example of the role of accompanying molecules is chitin particles, ubiquitously associated with allergens, that were demonstrated to set off a wide array of immune responses in a TLR2 dependent manner (12). Those allergen-associated compounds may account for the allergenicity of allergens devoid of any known bioactivity. More than half of defined major allergens, including Bet v 1, appear to be lipid-binding proteins and it is postulated that the lipid cargo of these allergens underlies their allergenicity (13). Another example of how allergenicity can be endowed by allergen-accompanying compounds is adenosine, which is identified in the water-soluble fractions of birch pollen and other tree pollen. Adenosine was found to be responsible for cyclic AMP accumulation and subsequently blocking IL-12 production by LPS-stimulated DCs. In healthy individuals this resulted in blocking IFN-γ and enhancing IL-10 production by induced T cells. On the other hand, DCs derived from allergic individuals failed to induce IL-10 production in stimulated T cells (14). Moreover, it is speculated that the oxidative activity of pollen grains may contribute to the allergenicity of allergens. Such pollen grains were shown to induce high levels of reactive oxygen species in cultured cells and trigger airway inflammation in experimental animal models (15,16). Finally, it is plausible that the net immunological effect of a given allergen may result from the simultaneous triggering and interplay of several PRRs by the diverse constituents of that allergen.

Apart from allergen-related factors contributing to the development of allergies, individual-related factors also play a role in predisposition for atopy. A genetic component in the development of allergies has been identified since 1916 when about half of asthma and allergic rhinitis patients were recognized for a positive family history (17). Genome-wide linkage studies revealed that allergies are associated with a plethora of chromosomal regions (18). Polymorphisms in one of these regions (17q21) were recently shown to be involved in regulating the expression of IL-17, which is implicated in the development of asthma (19). On the other hand, candidate gene linkage studies established a connection between atopic disorders and a variety of genes including IL4, IL13, IL4RA, TNF, HLA-DRB1, HLA-DQB1, FCER1B, CD14 and ADAM33 (20). However, the strong association of atopic dermatitis and mutations in filaggrin, central for the skin barrier function, remains to date the most robust genotype-phenotype linkages reported for atopic disorders (21). Interestingly and in relation to allergen bioactivity, susceptibility to asthma is associated with polymorphisms in molecular elements through which allergens trigger innate immunity like TLRs and oxidative stress-related genes (22,23).
However, the bioactive nature of allergenic entities or level of exposure to allergens and the genetic make-up of the population, cannot explain why the prevalence of allergies has dramatically increased over the last 50 years. Notably, this increase was concurrent with drastic socioeconomic changes, implying a central role of environmental factors in the etiology of allergy. This was initially addressed by the “hygiene hypothesis”, which suggested that exposure to infections reduces the tendency to develop allergic diseases by altering the Th1/Th2 balance (24). This hypothesis was optimized later on by linking atopy to defects in immune tolerance and its executive arm: Treg cells (25). The effect of surrounding environment starts from early on during the fetal and neonatal period. It has been recently demonstrated that the maternal sensitization status may have an influence on hematopoietic progenitors in utero, influencing the risk of subsequent allergy. Cord blood CD34+ hematopoietic progenitors from high-atopic-risk infants, defined by maternal allergic sensitization, had significantly lower expression of TLR2, TLR4 and TLR9. Interestingly, these progenitors gave rise to significantly more eosinophils and basophils when cultured in vitro (26). Moreover, farm exposure during pregnancy was shown to augment the count and function of cord blood Treg cells, which coincided with lower Th2 responses upon innate exposure to allergens (27). Environmental effects are also exhibited through gastrointestinal microbes. Reduced diversity of enteric flora during infancy was linked to increased risk of allergic disease later on in life (28).

In addition to unraveling the factors surrounding the development of allergic disorders, delineating the immunological mechanisms underlying those disorders is of utmost relevance for innovating therapeutic interventions. Although immunological processes leading to a full-blown Th2-dominated allergic response are well-defined, new players have been emerging. In the initiating events of allergic asthma, airway epithelial cells seem to play a key role in orchestrating immune responses. Expressing a variety of PRRs, these cells can sense allergenic insults and respond by secreting multiple cytokines and endogenous danger signals (GM-CSF, TSLP, IL-25, IL-1 family, IL-33 and uric acid), which collectively drive tissue-resident DCs to induce Th2 responses (29). Additionally, impaired barrier functions of airway epithelia is suggested to promote allergic asthma by allowing close interaction between allergens and immune cells and by launching a wide array of inflammatory and airway remodeling responses (30). In a similar fashion, keratinocytes in barrier-disrupted skin are believed to promote Th2 responses to allergens taken up by skin DCs and LCs. This suggests the skin as a primary site of sensitization and may help explaining the so-called “allergic march” where atopic dermatitis is the earliest atopic disorder followed by the other atopic disorders such as allergic rhinitis, asthma and food allergy (31). At the other end of allergic reactions, and although the inflammatory phase is dominated by Th2 responses, compelling evidence is pointing out the involvement of other types of effector T cells in this phase. Th1 cells are documented to emerge in the chronic phase of atopic dermatitis as a consequence to rising IL-12 levels in the local environment (32). Furthermore, the participation of Th17 effector cells in allergic inflammation was observed. Th17 cells are distinct for secreting IL-17, important for the activation and recruitment of neutrophils to infected tissues (33). Interestingly, airway neutrophilia, observed in severe asthmatic patients, is correlated with elevated IL-17 levels in both lung tissue and serum (34). However,
the role of Th17 cells in developing allergic asthma is undermined by the fact that these cells appear in patients with severe asthma. Additionally, mouse models of allergic asthma failed to establish any functional significance of Th17 cells in allergic airway inflammation. This prompts the suggestion that Th17 cells may arise subsequently to insults other than allergic reactions, such as bacterial infections (35). Another type of effector T cells, involved in atopic reactions, is the Th22 cells characterized by secreting IL-22. This cytokine possesses dual effects as on one hand it induces the expression of antimicrobial peptides, promoting immunity, and on the other it confers protection against tissue damage (36). IL-22 levels were found to be elevated in both chronic asthma and atopic dermatitis. However, and unlike IL-17, IL-22 is less pro-inflammatory and more protective due to its regenerative and wound healing functions that contribute to restoring epithelial cell functions (35). The recently established correlation between the chromosomal region containing the IL9 gene and atopy may implicate another type of effector T cells: Th9 cells. However, the functional relevance of this subset to allergic reactions is still to be defined, especially that IL-9 may be derived from Th2 cells and eosinophils (37). Delineating the mechanisms by which these different effector cells home to the allergic sites is essential for any targeting-based therapeutic strategies. The integrins α4 and β2 were revealed to be crucial for the development of allergic asthma, as mice deficient in either integrins had ablated leukocyte migration into the lungs and airways, resulting in protection against asthma (38). Collectively, optimal therapeutic approaches to treat allergic disorders should take into consideration factors related to the nature of the allergen, the genetic makeup and surrounding milieu of patients and the interplay between different cellular compartments and immunological pathways.

102 YEARS OF SIT: WHERE DO WE STAND?

After a century of the first successful trial of SIT, this treatment remains the only disease-modifying cure of mono-allergies. In addition to the compelling body of evidence regarding its safety and efficacy, SIT is also recognized by the World Health Organization as a well-tolerated treatment indicated for allergic rhinitis, Hymenoptera hypersensitivity and allergic asthma (39). Among the most appealing attributes of SIT is the long lasting effect, as SIT-induced immunological tolerance and thus clinical benefits persist after cessation of therapy (40). However, the widespread use of SIT is hampered by the small but real risk of developing anaphylactic reactions. Although this risk has been further minimized in the recent years by standardizing all procedures related to SIT administration, systemic reactions remain the major drawback of SIT (41). Another concern about SIT is the longevity of treatment regimes, reflecting low efficacy. A recent study, comparing 4-years SIT to 2-years SIT, demonstrated that longer SIT regimes are required to induce substantial increase in IgG4 antibodies, crucial for blocking IgE-mediated effects (42).

Standardizing allergen extracts, used in SIT, in terms of content and activity would contribute to reducing the risk of systemic reactions. Allergen standardization can also be realized by using recombinant allergens, which are currently used for diagnostic purposes. In addition to using recombinant native allergens, which resemble the natural molecule in sequence and conformation, modified allergens are being created to reduce allergenic reactivity and/or increase immunogenicity.
of wild type allergens. Compared to native Bet v 1, recombinant Bet v 1 fragments, trimers or trimers of hypoallergenic fragments were dramatically less allergenic (43,44). A recent study also showed that substituting certain amino acids in the Bet v 1 sequence resulted into a molecule with a lower IgE-binding capacity, elevated T cell reactivity and the capacity to skew immunity into Th1 responses (45). Thus, subtle alterations in the structure of classical allergens may change their immunological properties, rendering them safer and more efficacious for SIT purposes.

Although SIT is typically applied through the subcutaneous route (SCIT), the desire to enhance safety and improve patient compliance motivated an increased interest in alternative delivery modalities of SIT. The most prominent among suggested alternatives is the sublingual route (SLIT), which is gaining clinical acceptance especially in Europe. SLIT is recognized as a safe and clinically efficient for of SIT (46), albeit its late onset of action and treatment benefit that is half what is achieved by SCIT (47). A genuine approach in delivering SIT resulted from combining SCIT for the build-up phase and SLIT for maintenance, which allowed benefiting of the early onset achieved by SCIT and safety and injection avoidance achieved by SLIT (48). Other plausible ways for applying SIT that have been investigated are nasal, bronchial, epicutaneous, oral and intralymphatic routes (49). Further studies are required to confirm the safety and efficacy of these approaches.

Mechanistically, SIT depends on intercepting allergic pathogenesis via inducing allergen-specific Treg cells that would quench Th2 responses, and IgG antibodies that would block the effects of IgE. Further intervention in immunological pathways contributing to allergy may potentiate the effects of SIT. Following that principle, several attempts had been made to modulate innate immunity, namely DCs, to support the SIT-induced adaptive alterations. Linking allergens to TLR agonists like monophosphoryl lipid A (TLR4) and CpG sequences (TLR9) was shown to reduce allergic symptoms and reduce IgE levels (50,51). An emerging strategy to augment the activity of SIT is based on conditioning DCs to drive the induction of allergen-specific Treg cells. Several studies had pointed out that infection with certain pathogens (bacterial or parasitic) lead to the induction of Treg cells that would suppress allergic responses in mouse models of allergic asthma (52). The active components of these pathogens driving this tolerance need to be identified and fully characterized before any potential clinical application. Another bacterial component that is beneficial for SIT is the cholera toxin B subunit, which was shown to stimulate DCs to induce the production of the anti-inflammatory IgA antibodies by B cells (53)..

Furthermore, the active metabolite of vitamin D3, 1,25-dihydroxy vitamin D3 (VitD), got a lot of attention recently as a potent immunosuppressor that can possibly potentiate SIT. This issue will be discussed in details below. Finally, interfering at the other end of immunological processes, like IgE and inflammatory mediators, was also shown to be advantageous for SIT. Blocking IgE by specific antibodies (Omalizumab) or the administration of levocetirizine (antihistamine) during SIT, led to a decrease in the risk of systemic reactions (54,55).

VITAMIN D AND ALLERGY

Evidence suggests that VitD deficiency is associated with increased airway hypersensitivity, lower pulmonary functions, worse asthma control and possibly steroid resistance (56).
is supported by genomic analysis, which unraveled a link between VDR polymorphisms and increased susceptibility to asthma (20). Indeed, VitD effects span the different cell types and pathways involved in the development of allergic asthma. First of all, respiratory epithelium was shown to express CYP27B1, crucial for transforming VitD precursor into the active form, providing a local source of VitD (57). Moreover, VitD was shown to inhibit the proliferation and matrix metalloproteinases production of sensitized bronchial smooth muscle cells, implying the importance of VitD in arresting airway remodeling associated with asthma (58). Angiogenesis, a process associated with airway remodeling, is also influenced by VitD, which was shown to suppress the proliferation of nonquiescent vascular smooth muscle cells (59).

In addition to its effects on airway cells, VitD profoundly modulates the functions of the immune system. VitD was found to repress differentiation and promote apoptosis of precursors of mast cells, which are known for their central role in the effector phase of allergic reactions (60). However, the immunosuppressive properties of VitD are mainly manifested by marked inhibition of adaptive immune responses. This inhibition is exerted through two routes: direct effect on T cells and indirect inhibition via DCs. The direct effect of VitD on T cells is to inhibit T cell proliferation, IL-2 expression and inflammatory cytokines production and to promote their development into Treg cells (61-65). Alternatively, VitD modulate DC functions by reducing their immunostimulatory capacity and inhibiting IL-12 production (66,67). Furthermore, VitD endows DCs with tolerogenic qualities reflected by inducing the expression of IL-10, immunoglobulin-like transcript 3 (ILT3) and programmed death-1 ligand (PD-L1). Those acquired tolerogenic qualities lead eventually to Treg cell priming and inhibiting the induction of other types of effector T cells (68-71). Those findings were corroborated by observations in VDR null mice, which exhibited higher proportions of more mature DCs in lymph nodes (67). Interestingly, we obtained evidence that the type of induced Treg cells completely depends on the type of DCs being primed by VitD. Whereas VitD conditioning drives epidermal LCs to induce Foxp3+ Treg cells, it drives dermal DCs to give rise to IL-10-producing Treg cells (Tr1 cells) (Chapter 2).

Although the aforementioned findings were mainly in vitro observations, resulting sometimes from applying supraphysiological concentrations of VitD, there are strong indications of the role of physiological VitD in maintaining immune tolerance. Many autoimmune diseases are correlated with VitD deficiency, including multiple sclerosis, type I diabetes and Crohn’s disease (72). Another argument linking physiological VitD to immune tolerance is the correlation between areas with insufficient sunlight exposure, meaning low VitD supply, with increased incidence of different autoimmune diseases (73). As mentioned above, VitD-achieved immune tolerance is dependent on the induction of Treg cells. The observation that VitD leads to the induction of different types of Treg cells (Chapter 2), may suggest a mechanism by which VitD assures tolerance. The variable functions and properties of Tr1 and Foxp3+ Treg cells (6), induced by VitD, may complement each other in enforcing tolerance. It is of relevant importance to investigate how VitD endows other types of tissue-resident DCs, like airway and mucosal DCs, with tolerogenic capacities and the type of resulting Treg cells.

In addition for its role in maintaining homeostatic immune tolerance, VitD was also shown to be a potent immunosuppressive drug with beneficial effects in mouse models of autoimmune,
inflammatory and allergic diseases (74–77). Indeed, combined SIT/VitD application enhanced SIT efficacy by inhibiting airway hypersensitivity, augmenting IgE and Th2 cytokines reduction and increasing the Treg-characteristic cytokines, IL-10 and TGF-β (74). Consistent with this study, we found that DC conditioning by VitD in conjunction with Bet v 1d targeting to DCs promoted the development of allergen-specific Treg cells (Chapter 5). Furthermore, performing SIT using VitD-linked allergen markedly reduced eosinophilia and enhanced IgG production in comparison to classical SIT protocol (78).

Despite the clinical potential of VitD, its systemic application is hindered by deleterious side effects manifested mainly by hypercalcemia and hypercalciuria. Factually, VitD clinical application as an immunosuppressor is limited to the topical treatment of psoriasis (79). Interestingly, the VitD precursor calcidiol, had the same effect on DCs and promoted the development of Treg cells, albeit IFN-γ expression by these cells was not altered (Chapter 3). Using VitD precursors may prove even more beneficial than active VitD to potentiate SIT, since T cell expression of IFN-γ is sustained, which may corroborate the effect of Treg cells in overweighing Th2-dominated responses. Moreover, those precursors were proven safe for clinical application as their application does not lead to the calcemic liability usually associated with VitD (80–83). Although the effects of calcidiol are mediated by transforming it into active VitD (Chapter 3), this transformation occurs specifically in DCs that are equipped with the required enzymatic machinery (84). Thus, DCs are specifically targeted with the VitD effects through the application of the VitD precursors, which may dramatically reduce the deleterious side effects reported with systemic application of active VitD.

**IT IS ALL ABOUT LOCATION: SKIN AS AN EXAMPLE**

Equipped with an extensive network of DCs, skin is a major site of interest for vaccination purposes. The concept of exploiting the cutaneous route for vaccination was introduced by Edward Jenner, who managed to raise protection against smallpox by exposing skin DCs through skin scarification. This method, which was later on substituted by intradermal injection (ID), appears to be superior to subcutaneous and intramuscular routes that fail to deliver vaccines to a DC-dense tissue like the skin compartment. Despite being an efficient route of administration, ID vaccines are currently limited to rabies and Bacille Calmette-Guréin (BCG) vaccines (85). In addition to inducing protection against pathogens, the concept of ID vaccination can be extended to induce tolerance as a treatment to allergic disorders. As mentioned earlier, skin is hypothesized to be a primary site of sensitization in case of allergies. Disrupted barrier functions of skin not only exposes skin DCs to allergens, but also induces keratinocytes to release inflammatory mediators that influence DC functions and lead to Th2 responses (31). Therefore, targeting skin DCs, which may be responsible for triggering atopy, may be a sound therapeutic approach. This advocates for ID administration of classical SIT or in combination with an immunomodulatory adjuvant, like VitD. In a skin explant model, ID application of VitD resulted into enhanced migration of CD14⁺ DDCs and primed the whole skin DC population to promote the development of Foxp3⁺ Treg cells (Chapter 4). In contrast,
isolated skin DC subsets induce distinct types of Treg cells. As shown previously (Chapter 2), LCs induce Foxp3+ Treg cells, whereas CD1a+ DDCs induce IL-10-producing Treg cells. Furthermore, CD14+ DDCs were reported to promote the development of IL-10-producing Treg cells (86). However, the collective effect of injecting VitD intradermally was the induction of Foxp3+ Treg cells. This discrepancy may be explained by the different mechanisms used by the separate DC subsets to induce Treg cells. Whereas the induction of Foxp3+ Treg cells by LCs depend on the production of TGF-β by LC, the induction of IL-10 producing Tr1 type of Treg cells by both DDC subsets depend on their production of IL-10. The dominant induction of Foxp3+ Treg cells by the total population of skin DCs may be explained by the fact that the induction of IL-10+ Tr1 cells by IL-10 from VitD-primed DDCs is counteracted by the mere presence of LC-derived TGF-β, which favors the induction of Foxp3+ Treg cells. TGF-β was shown to inhibit IL-10 production by T cells activated in the presence of IL-10 (87), or RA (Chapter 6). Collectively, This implies the importance of utilizing models like the skin explant model, which allows determining how certain interventions affect different cell types and the interactions between these cells leading to the net effect in a close to reality setting.

**DC TARGETING**

Antigen delivery to DCs in vivo can be achieved through the administration of antigens coupled to antibodies specific for particular DC surface molecules. Targeted delivery of allergens may have a great potential in enhancing the quality of SIT. This approach may help limiting allergen interactions to DCs, thereby reducing the possibility of IgE cross-linking on mast cells and basophils and subsequently minimizing the risk of systemic reactions. Moreover, allergen targeting in SIT may trigger antigen-specific responses at lower allergen concentrations, further reducing the chances of developing anaphylaxis.

When DC targeting approaches are applied, certain issues should be considered. First of all, the expression pattern of the targeted molecule should be determined, i.e. to what extent this molecule is specific for DCs or even a certain DC subset. In this way, desired immune responses can be induced by targeting molecules that are solely expressed by functionally well-defined DC subsets. For example, allergen targeting via CD103, which is widely expressed by mucosal DCs that are known for their ability to induce tolerance, was shown to suppress the development of allergic airway inflammation in a mouse model (88). On the other hand, targeting tumor antigens via CLEC9A, specifically expressed by the cross-presenting BDCA3+ DCs, resulted in cross-presenting these antigens to CD8+ T cells (89). Furthermore, targeting DC-surface molecules should take into account that some of these molecules are associated with signaling cascades which may influence DC functions and the ensuing immunological responses. Despite being widely used as targets in animal models and in vitro experiments, C-type lectins like Dectin-1 and DC-SIGN can activate DCs when ligated (90). Interestingly, the C-type lectin DEC205 is not linked to any signaling pathways rendering it an interesting target as it will not interfere with immunological outcome. Another key determinant of efficient DC targeting is the route through which the targeted antigen is administered. Different DC
subsets can be reached depending on the administration modality. Logically, when skin is employed as a site of administration, targeting should be done through molecules expressed on migratory skin DC subsets, assuring that antigen would reach the draining lymph nodes where T cell induction takes place. ID administration of DEC205-coupled antigen was shown to result in rapid antigen uptake and presentation by LCs (91). Finally, the activation state of targeted DCs is crucial in defining the final immune response. Antigen targeting to DCs without the concomitant application of an adjuvant induced immune tolerance towards the antigen (92). Although this is not desired in anti-viral or anti-tumor vaccines, this quality may prove beneficial for treating allergies and autoimmune diseases. This tolerogenic profile can be further potentiated by applying regulatory adjuvants such as VitD (Chapter 5).

Another variable that may play a role in targeting is the method followed in coupling the cargo (antigen) to the antibody. Initial studies depended on chemical conjugation (92,93). Albeit efficient, this method cannot be easily controlled and lacks standardization with varying antigen: antibody binding ratios and variations between different batches of the chemically coupled conjugates. An alternative for chemical coupling is genetic fusion of antigen to the Fc tail of the antibody leading to a more reliable product with comparable activity (94). Antigen delivery can also be achieved by genetic fusion to single chain antibody fragments which are composed of the variable regions of both heavy and light chains of an antibody. This approach neutralizes any possible host reactions towards the antibodies since single chain fragments lack the Fc part of immunoglobulin chains. Furthermore, the small size of these single chain antibodies allows the conjugation of a wide array of antigens (95). Proven to be efficient in triggering immune responses both in vitro and in vivo (96,97), this elegant method bares a lot of promises in the field of targeting. However, the absent capacity to cross link the DC surface target by single chain antibodies may represent a plausible caveat for this approach, as it may lead to reduced binding and antigen uptake by DCs.

RETINOIC ACID: AN OLD PLAYER WITH HIDDEN TALENTS

Similar to VitD, vitamin A is a potent immunomodulator that can be exploited in potentiating SIT. The physiologically active form of vitamin A, retinoic acid (RA), is produced by specific DC subsets at tissues known to be plausible sites of allergic sensitization, such as the airways, skin and gut (98), which may predict a role of RA in protection against or pathogenesis of atopy. Recently, a lot of controversy has been raised about the role of RA in promoting tolerance vs. immunity. The seminal observation that lamina propria CD103+ DCs produce the Treg-driving RA, has framed RA as a tolerance-committed factor. However, the influence of RA on immunological health was first recognized upon the discovery that vitamin A deficiency is associated with dampened immunity and that its replenishment drastically reduced fatality in children suffering from malnutrition (99,100). On one hand this may be explained by the important role of RA in inducing the differentiation and influencing the permeability of gut
epithelia (101). On the other hand, RA contribution to immunity was demonstrated by impaired or dysregulated T cell functions in response to infection during vitamin A or RA receptor deficiency (102-104). This may be attributed to the positive effect of RA, at low concentrations, on IL-2-mediated CD4+ T cell proliferation (105) and its suggested effect on effector CD4+ T cell differentiation. In a mouse model for systemic infection with Toxoplasma Gondii, RA signaling was found to be important for the development of both Th1 and Th17 responses, which were ablated in RA-deficient or RAR-α null mice (104). Notably, the effect on T cell differentiation was found to be secondary to impaired T cell proliferation caused by disrupted RA signaling. In accord with this finding, RA did not induce IFN-γ production in human CD4+ T cells, nor did it influence IL-12-induced differentiation of Th1 cells (Chapter 6). Interestingly, effector T cells generated in the presence of RA and the Th1-skewing IL-12, co-produced IFN-γ and the regulatory cytokine IL-10 (Chapter 6). A similar phenomenon was also observed in the mouse small intestine, where around 30% of IL-10+ T cells coproduced IFN-γ (106). Effector T cell-derived IL-10 is believed to facilitate the suppressor functions of tissue-resident Treg cells by inducing IL-10 production in these cells (106,107). During infections, IL-10 production by CD4+ effector T cells is recognized for preventing immune responses from exacerbation and progression into lethal immunopathology without fully compromising the pathogen-directed effector functions of these cells (5). In addition to T cell responses, RA is of great importance in shaping humoral immunity. This is demonstrated by its role in sustaining IgG responses (108), and by being an essential factor in driving the generation of IgA-secreting B cells (109). Furthermore, RA was shown to augment macrophage activation in response to infection (110). Thus, the role of RA is not only pivotal for development of immune tolerance but also for supporting immunity and protection against pathogens.

DC-derived RA contributes to a lot of the RA-mediated effects on T cells. Although, retinal aldehyde dehydrogenase activity, important for converting retinal into RA, was reported for several DC subsets, including skin and airway resident DCs, lamina propria CD103+ DCs are recognized as the major RA-producing DC subset. This subset produces RA at levels sufficient to leave its signature on primed T cells, manifested by high CCR9 expression (98). In this respect, vitamin A is indispensible to imprint this DC subset with RA-producing capacity. Local vitamin A can come from different sources like dietary intake and bile (111). Furthermore, both gut epithelia and mesenteric lymph node stromal cells were defined as providers of intestinal RA, crucial for DC imprinting (112,113). Although RA is known to be a key inducer of RA production in DCs In vitro, several factors, including IL-4, GM-CSF and IL-13, had been implicated in this process (114). However, human monocyte-derived DCs, generated in the presence of both IL-4 and GM-CSF, were solely dependent on external RA in order to produce RA themselves (Chapter 6). This may be explained by interspecies differences and using different DC progenitors (bone marrow vs. monocytes). Additionally, microbial stimulation seems to influence RA-mediated education of gut DCs, as disrupted TLR signaling resulted into ablated RA production by these DCs (115). This effect might take place indirectly through epithelial cells which are known to express TLRs and contribute to DC conditioning by producing RA. The concept of a critical role of TLR-stimulation of bystander cells and not DCs in RA production is corroborated by our
observations that neither viral nor bacterial stimulation augmented RA production by human monocyte-derived CD103⁺ DCs or the subsequent RA effects on T cells (Chapter 6). Dissecting the interactions between RA and TLR pathways, specifically in DCs, is important to understand the possible effects of gut microbiota on DCs. Similar to airway epithelia; gut epithelial cells play a major role in shaping immune responses in their local environment. Equipped with a series of PRRs, gut epithelial cells are capable of recognizing microbes and transmitting resulting signals to gut DCs. Besides being a major source of RA, epithelial cells secrete other factors like TGF-β and TSLP, which are recognized to promote intestinal tolerance and skew T cells away from Th1 responses (116). Thus local tissue milieu is vital in dictating the immunological outcome, and should be considered in any therapeutic strategies exploiting RA. In an inflammatory environment, RA-driven signals can foster vigorous responses instead of promoting the desired tolerance. A recent study by DePaolo et al. clearly demonstrated that RA collaborates with IL-15, a cytokine generously available in the intestinal milieu of celiac disease patients, in amplifying inflammatory circuits (117). Therefore, it is essential to delineate the immunological outcomes resulting from RA interactions with inflammatory cytokines before applying it clinically as a treatment for inflammatory or autoimmune diseases.

In addition to tailoring immune responses, RA is a key determinant of lymphocyte homing. In an RA-dependent manner, gut CD103⁺ DCs induce the expression of the mucosal homing integrin α4β7 and chemokine receptor CCR9 (see intro) on stimulated effector T cells (Chapter 6), directing them back to the intestine. Gut-homing specificity was confirmed by showing that DC-derived RA did not induce the expression of other tissue homing markers like α4β1 (lung) or CCR10 (skin) (unpublished data). A recent study revealed that α4 expression by murine T cells is preferentially regulated by RA, whereas β7 expression is under the control of TGF-β (118). However, human T cell activation readily triggered α4 expression, whereas β7 levels were under the control of RA (Chapter 6), deepening the discrepancy about the differing RA effects in humans and mice. It is noteworthy that the expression of CCL25, the ligand of CCR9, by gut epithelia is highest at proximal small intestine (duodenum) and is reduced at distal sites (ileum) and it is almost absent in the colon (119). This expression pattern is correlated with a similar pattern of RA synthesis in the aforementioned parts of the bowel, indicating that RA, by inducing CCR9 expression, has the potential to compartmentalize T cell-mediated immune responses within the intestine.

Thus far RA has not been implicated in the pathogenesis of atopy, though a compelling body of evidence indicates that RA may have useful effects in therapy. Being one of the key factors produced by lamina propria CD103⁺ DCs, RA is postulated to be crucial in promoting tolerance to dietary antigens, a process denoted “oral tolerance” (120). In addition to food allergy, RA may prove useful in treating allergic asthma. RA was shown to inhibit eosinophil and basophil differentiation, eosinophil recruitment to airways and smooth muscle migration, the last being important in airway remodeling associated with asthma (121-123). These observations were further corroborated by a rat model of asthma, in which RA application dramatically reduced airway inflammatory cell counts and attenuated pathological changes in lung tissue (124). Considering these effects, in addition to its Treg-inducing capacity, RA can be exploited to potentiate SIT.
CONCLUDING REMARKS

Conclusively, allergies are complex diseases that develop under the control of a wide range of variables. Although SIT is considered the only curative treatment of allergy, its weak efficacy and the accompanying risk of systemic reactions are impeding exploiting it on a large scale. This study was focused on harnessing DCs to enhance the quality of SIT. As shown, allergen targeting to DCs has a high potential in improving the safety of SIT by reducing allergen concentrations used to elicit T cell responses. On the other hand, DC programming by regulatory adjuvants to induce Treg cells is a promising approach for potentiating SIT. VitD proved to be competent in directing DCs for Treg induction. This was confirmed using human ex vivo DCs and in a skin explant model. VitD precursors may provide a safer and efficient substitute for active VitD. Finally, RA can be exploited as a regulatory adjuvant in SIT as it primes T cells to produce IL-10, predicted to be essential for SIT therapeutic effects.
REFERENCE LIST


78. Grundstrom, J., T. Neimert-Andersson, C. Kemi, et al. 2012. Covalent coupling of vitamin D3 to the major cat allergen Fel d 1 improves the


