Hitting the Mucosal Road in Tolerance Induction

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Abstract
Within the last decades a dramatic increase in allergic diseases has been recognized in the Westernized societies, leading to the fact that meanwhile 25–30% of the population is afflicted by allergic disorders. Besides a hereditary disposition, other factors, including a reduced microbial contact early in life or changes in nutrition, might also have influenced this epidemiological development. So far the only causative treatment against type-I allergies is specific immunotherapy. In young and monosensitized patients this treatment is highly efficacious, while there are clear limitations in older or multisensitized patients. Allergy research therefore aims at establishing new and more efficacious treatment strategies in prophylactic as well as therapeutic settings. Our research programs focus on the development of novel allergy vaccines based on the induction of mucosal tolerance. In different mouse models of respiratory allergy mucosal treatment with genetically engineered allergen constructs proved to prevent the development of allergic mono- and multisensitivities. The additional use of mucosal adjuvants seems particularly important to improve therapeutic treatment approaches. Recent studies on the inverse relation of certain parasite infections and the development of allergy prompted us to search for selected parasitic molecules with immunosuppressive properties as potential adjuvant systems for novel allergy vaccines. An overview of our recent studies will be given.

Type-I Allergy and Current Treatment Protocols

Allergic diseases, such as atopic dermatitis, allergic rhinoconjunctivitis and allergic asthma meanwhile affect more than 25–30% of the population in industrialized countries, thereby causing a substantial healthcare problem in these societies. Sensitization to normally harmless antigens with the induction
of exaggerated Th2 responses is the initiating event in the inflammatory cascade of type-I allergy. The high levels of IL-4, IL-5 and IL-13 produced by Th2 cells trigger IgE production by B cells. Once allergen-specific IgE is bound to basophils or tissue mast cells, a new allergen contact leads to cross-linkage of IgE and release of histamine, leukotrienes and other mediators of acute allergic inflammation.

The expression of an allergic phenotype depends on various factors, such as genetic predisposition [1], the nature of the allergenic proteins [2] or environmental factors, including changes in lifestyle and nutrition [3]. There is a strong body of evidence suggesting that the lack of certain infections during early life due to ‘sterile living conditions’ may contribute to the significant increase in allergy over the last decades. This phenomenon, described by the hygiene hypothesis [4, 5], is supported by several epidemiological studies showing a higher prevalence of atopic diseases in urban than rural areas of the industrialized as well as developed countries [6]. Furthermore, lack of exposure to certain orofecal infections, such as hepatitis A, Toxoplasma gondii or Helicobacter pylori [7] or certain helminth infections [8], as well as the composition of the intestinal flora [9] have been linked to increased allergy development. Reasonable explanations for this development include a lack of counter-regulatory mechanisms by Th1 cells. More recently, the importance of regulatory T cells and the production of immunosuppressive cytokines, such as IL-10 and TGF-β, for the prevention of allergic immune responses and hyperreactivity reactions have been implicated [10, 11]. Whether finally an appropriate or an allergic immune response develops in response to allergen contact may not depend solely on one particular cell type but rather on a complex network with a fine balance between Th2, Th1 and T regulatory cells.

Currently, specific immunotherapy (SIT) is the only causative treatment performed by injection of increasing amounts of allergen extracts in order to induce hyporesponsiveness to the respective allergen [12, 13]. Even though used for more than a century, the underlying mechanisms of this treatment are not fully solved. The clinical improvement has been linked to: (i) a rise in allergen-specific IgG4 antibodies acting as possible blocking antibodies; (ii) a reduction in the number of mast cells, eosinophils and release of mediators, and (iii) a modulation of allergen-specific Th2 cells by the induction of counter-regulatory Th1 cells as well as the generation of regulatory T cells which produce suppressive cytokines [14, 15]. Very recently, sublingual immunotherapy has become an attractive alternative to systemic immunotherapy, which might in part also operate via immune deviation and induction of IL-10-producing regulatory T cells [16–18].

Even though these treatment approaches have been shown to be effective in many cases, some disadvantages, such as long treatment duration, anaphylactic side reactions or, in terms of SIT, frequent injections, may limit the patients’ compliance to the treatment. Furthermore, the use of allergen
extracts, also containing several other allergenic molecules not represented in the patients sensitization profile, may include the risk of de novo sensitization during treatment. The currently used aluminum salts as adjuvant systems may also have some limiting effects on SIT as they are known to promote Th2-biased immune responses.

Approaches to improve treatment have therefore focused mainly on three aspects: (i) the use of recombinant allergens instead of allergen extracts; (ii) the change from the systemic to the mucosal route of allergen application, and (iii) the use of adjuvant systems with immunomodulatory and/or immunosuppressive properties [19].

**Animal Models of Type-I Allergy**

Among the numerous inhalant allergens, tree pollen of the white birch *Betula verrucosa* is one of the most important sources responsible for eliciting allergic symptoms. The major birch pollen allergen, Bet v 1, a 17-kD molecule, to which 95% of birch pollen allergic patients (and 60% exclusively) display IgE-binding reactivity, was one of the first allergens to be cloned, sequenced, and produced as a recombinant protein in *E. coli* [20]. We have previously established an animal model of allergic sensitization to birch pollen and its major allergen Bet v 1, presenting immunological features comparable to human type-I allergy [21]. The established standard sensitization scheme is based on systemic immunization with recombinant Bet v 1 followed by an aerosol challenge with a natural birch pollen extract. This sensitization procedure leads to high allergen-specific IgE/IgG1 antibody levels, positive immediate type skin reactions, eosinophilic infiltration within the airways along with airway hyperresponsiveness. These manifestations are reflected in vitro by the secretion of Th2 cytokines from allergen-stimulated splenocytes [21].

There is substantial clinical evidence that many allergic patients become co-sensitized to several other airborne allergens, and particularly birch pollen allergy is frequently associated with sensitization to grass pollen allergens [22, 23]. We therefore established a mouse model of polysensitization to Bet v 1 and the non-cross-reactive major grass pollen allergens, Phl p 1 and Phl p 5. Immunization with the optimal doses of Bet v 1, Phl p 1 and Phl p 5 and subsequent aerosol challenge with birch and grass pollen extracts led to comparable humoral and cellular immune responses to all three allergens/antigens and airway inflammation [24]. The fact that the immunodominant epitopes recognized by T cells from polysensitized mice were identical to some of the T-cell epitopes in birch and grass pollen-allergic patients [25–27] indicated that our murine model of multiple allergen sensitivity shows similar immunological characteristics to those of human pollinosis.
Mucosal Tolerance Induction with Recombinant Allergens and New Allergen Constructs

As early as 1911 the first experimental model was described showing that oral application of soluble antigens leads to antigen-specific immunological unresponsiveness following systemic antigen re-exposure [28]. This phenomenon was termed ‘oral tolerance’ but is nowadays referred to as ‘mucosal tolerance’, since tolerance can be induced equally well by the nasal, bronchial, vaginal or rectal mucosa. Several experimental studies have by now demonstrated the efficacy of mucosal tolerance in suppressing Th1- and/or Th2-biased immune responses [29, 30]. The fact that sublingual immunotherapy, a meanwhile well-accepted alternative treatment to conventional immunotherapy, leads to a reduction in allergic immune responses indicates that the concept of mucosal tolerance induction is indeed applicable in humans. In contrast to the current treatment protocols with whole allergen extracts, novel approaches aim at using only the disease-eliciting allergens or novel allergen constructs in recombinant form for mucosal application thereby representing a noninvasive, patient-tailored prophylactic or therapeutic treatment approach.

Mucosal Tolerance Induction against Monosensitization

Using our mouse model of birch pollen allergy/asthma we demonstrated that intranasal and oral application of Bet v 1 suppressed allergic immune responses in naïve and in already sensitized mice [31, 32]. Immunosuppression after prophylactic and therapeutic treatment with the recombinant allergen was long-lasting, i.e. up to 1 year in the prophylactic set up and 6 months in the therapeutic setting. When using a hypoallergenic fragment of Bet v 1, harboring the immunodominant T-cell epitope, immunosuppression was equally well established in naïve and sensitized mice. However, with respect to the long-term efficacy of tolerance only the prophylactic, but not the therapeutic, treatment with the Bet v 1 fragment was long-lasting. The underlying mechanisms differed in dependence on the structure of the molecules: tolerance induction with Bet v 1 was associated with a simultaneous increase in TGF-β, IL-10 and Foxp3 mRNA levels in CD4+ T cells along with enhanced TGF-β levels also in CD8+ T cells in both the prophylactic and therapeutic setting. In contrast, tolerance induction with the fragment in naïve mice resulted in upregulation of Foxp3 in CD4+ cells, whereas in sensitized mice only elevated IL-10 mRNA levels were found. From these data we concluded that overall immunosuppression depends on the conformation of the tolerogen via simultaneous induction of TGF-β and IL-10, while expression of Foxp3 seems mandatory for maintenance of tolerance [33].

Mucosal Tolerance Induction against Multisensitization

Based on the fact that patients with multisensitivities are difficult to treat with conventional immunotherapy, we focused our research on the
development of new treatment approaches for this particular patient collective. We recently established a mouse model of polysensitization to the major birch and grass pollen allergens Bet v 1, Ph l p 1 and Ph l p 5. In contrast to mucosal tolerance induction with single allergens in monosensitized mice, it was not possible to induce immunosuppression by multi-allergen application but only by application of the immunodominant T-cell epitopes of the selected allergens [24]. Intranasal application of these polypeptides led to a reduction in the systemic allergic immune responses and airway inflammation [24, 34]. The immunosuppressive effects were not associated with induction of the suppressive cytokines IL-10 or TGF-β, nor was tolerance transferable by splenocytes to recipient mice, indicating that polypeptide-induced tolerance was not mediated by cytokine-dependent regulatory mechanisms.

Even though peptide treatment has been shown to be effective in humans [35], there are certain limitations due to major histocompatibility complex and T-cell receptor restrictions [36]. To overcome such limitations we decided to genetically engineer an allergen chimer consisting of the whole Bet v 1 molecule as a backbone for linkage of the immunodominant peptides of Phl p 1 and Phl p 5 [37]. Intranasal tolerance induction with this allergen chimer led to a reduction in systemic allergic immune responses and induction of allergen-specific IgA antibody responses within the airways. This phenomenon of suppressed systemic immune responses along with induction of IgA at mucosal sites is known as split tolerance and in this setting IgA might function as blocking antibodies at the site of allergen encounter. In contrast to tolerance induction with polypeptides, intranasal pretreatment with the allergen chimer was associated with induction of regulatory cytokines and tolerance was transferable with splenocytes of tolerized mice. To further evaluate the concept of polytolerance induction new allergen chimeras against house dust mite and animal dander allergens as well as against the oral allergy syndrome against pollen-related food allergy are currently under investigation.

New Adjuvant Systems with Immunomodulatory Properties

For improvement of mucosal tolerance induction, in particular in therapeutic settings, the additional use of adjuvant systems with immunomodulatory properties might be of importance.

Use of Cholera Toxin B Subunit as Mucosal Adjuvant

Among the different mucosal adjuvants, the B subunit of cholera toxin has been shown to improve the tolerogenic properties of an antigen when coupled to it [38]. We previously demonstrated that these properties of cholera toxin B (CTB) are influenced by the nature of the coupled antigen as well as by the mode of conjugation, as ovalbumin chemically coupled to CTB led to a reduction, while Bet v 1 chemically coupled to CTB enhanced allergic
immune responses [39]. In order to improve the immunomodulatory capacity of such a mucosal delivery system we recently genetically engineered a Bet v 1-CTB fusion molecule [40]. The clear advantage of a recombinant fusion molecule over a chemical conjugate is its homogeneity, since the molecular composition and position of the antigen do not vary and the amount of antigen is increased to five molecules per one molecule CTB pentamer. Indeed, intranasal treatment with this fusion molecule prior to sensitization with Bet v 1 led to a significant reduction in allergen-specific IgE and in vitro IL-4 and IL-5 production, while humoral and cellular Th1-like responses were markedly enhanced. In the lung compartment a significant rise in IgA was detected after pretreatment with the fusion molecule or CTB alone. Whether the immunomodulatory effects are only due to counter-regulatory Th1-like immune responses and protective IgA or due to induction of regulatory cells and their products, as described by others [41], is currently under investigation.

Use of Lactic Acid Bacteria for Immunomodulation

Within recent years lactic acid bacteria (LAB) have gained interest as mucosal adjuvants and vaccine vehicles with immunomodulatory properties. Due to their non-pathogenic status and their capacity to induce dendritic cell-derived regulatory properties, they are suggested as potent antigen delivery systems for humans [42]. In particular, active delivery of recombinant molecules to mucosal surfaces by genetically modified LAB represents a novel vaccination approach. With respect to allergy treatment we recently evaluated the immunomodulatory properties of two LAB strains, Lactococcus lactis and Lactobacillus plantarum, showing that both strains are effective in shifting immune responses towards a Th1 profile in vitro [43]. To evaluate their potential for modulation of allergic immune responses in vivo, recombinant strains producing the Bet v 1 allergen were constructed. Intranasal or intragastric pretreatment with the Bet v 1-producing LAB led to significantly reduced allergen-specific IgE and increased IgG2a levels, indicating a shift to non-allergic Th1 responses [44, 45]. With respect to local immune responses and airway inflammation, pretreatments with Bet v 1-producing LAB and the control strains led to a reduction in eosinophils and IL-5 in lung lavages, suggesting that the LAB strains themselves induce a counter-regulatory milieu. In already sensitized mice mucosal application of these recombinant strains did not sufficiently reduce allergic immune responses, which indicated that LAB-inducing immunosuppressive cytokines, such as TGF-β or IL-10, rather than Th1-like cytokines, might be more beneficial in therapeutic settings.

Regarding the optimal time point for intervention with probiotics, the implementation of primary prevention early in infancy is increasingly being discussed. A recent meta-analysis of several clinical trials suggests that pre- and postnatal probiotic intervention is effective in preventing the development of pediatric dermatitis [46], however the effects on allergy development are less clear. In our recent murine study on primary prevention of birch pol-
Mucosal Tolerance for Allergy Treatment

len allergy, we compared different treatment windows in pregnant mothers during gestation and/or lactation and in the offspring during the neonatal phase [Schabussova and Wiedermann et al., unpublished data]. Preliminary data indicate that oral application of live probiotic bacteria to pregnant mice before allergic sensitization and aerosol challenge suppressed eosinophilic airway inflammation. Consistent with this, allergen-induced cytokine production was suppressed in re-stimulated cultures of spleens, lungs and mediastinal lymph nodes. Moreover, administration of the probiotic strains to mice during gestation and lactation had a suppressive effect on allergen-specific immune responses in the sensitized offspring. Further studies will be needed to elucidate the underlying mechanisms of immunosuppression by the selected probiotic strains and to evaluate whether intervention with probiotics might also have unwanted suppressive effects on co-applied antigens including common pediatric vaccines.

Immunomodulation by Parasites

According to the hygiene hypothesis, recent epidemiological studies have demonstrated an inverse relationship of certain orofecal and food-borne infections, such as infection with hepatitis A, *H. pylori* or *T. gondii*, and the development or manifestation of respiratory allergy [7, 47]. Based on the fact that *T. gondii* is a worldwide prevalent, intracellular protozoan parasite [48], we recently established a mouse model of toxoplasma infection in order to study the effects of this parasitic infection on allergy development. We demonstrated that infection with *T. gondii* before or after allergic sensitization with the major birch pollen allergen Bet v 1 reduces systemic and respiratory allergy. Our data argue for an important role of innate defense mechanisms induced by *T. gondii* leading to Th1-biased responses preferentially in the beginning of infection [49]. During the chronic stage of *T. gondii* infection regulatory cells seem to be involved, eliciting their immunosuppressive properties and thereby downregulating the allergic symptoms. Further studies are planned to analyze in depth the immunomodulatory properties of *T. gondii*, as well as selected parasitic molecules in order to establish new preventive and therapeutic strategies against allergic diseases, including novel adjuvant systems for anti-allergy vaccines [50].

Conclusions

Improvement in conventional immunotherapy by changing the route of application has already been realized with the concept of local/sublingual immunotherapy.

Further development of this strategy concentrates on the use of novel allergen molecules for allergy treatment in a component-resolved patient-tailored manner. The construction of new chimeric molecules, which may allow simul-
taneous treatment of different allergies, could be a tremendous improvement in the treatment of multisensitized individuals. Novel treatment approaches for mucosal immunotherapy also include the use of new adjuvants, ranging from bacterial components to parasitic molecules that have the properties to counteract immune dysregulations via induction of Th1-like and/or immuno-suppressive responses.

Most recently the implementation of primary prevention strategies for programming the immune system during ontogeny is increasingly being discussed. In this respect the role of selected probiotics for intervention early in life against allergy development is being studied in clinical and experimental studies, representing an exciting field in allergy research.

The translation of all these promising new prophylactic and therapeutic concepts into clinical trials will be a major challenge of forthcoming allergy research.

References

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Mucosal Tolerance for Allergy Treatment

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Discussion

Dr. Renz: Your toxoplasma data are fascinating and this opens a new field in using parasites or parasitic components to boost allergy prevention. More and more data are coming out, more from basic science and parasitic immunology, indicating that at least some of the parasites actually actively modulate the immune response in the host by secreting various components and molecules and so on. Could that also be a mechanism, in terms of the toxoplasma response, by which the parasite is actively modulating the immune response?

Dr. Wiedermann: So far, we have only studied the immunomodulatory effects of Toxoplasma gondii during infections. We do not know yet, if the modulation of the immune responses only occurs during infection or if it can be also induced after application of killed Toxoplasma gondii or of only certain fractions of the parasite. These are the issue of our next studies, because we aim at using only parasitic molecules with immunomodulating properties rather than the whole parasite as new adjuvant systems for allergy vaccines.

Dr. Renz: Do you know whether this is a soluble factor or just something which is bound to the membrane or is it an intrinsic molecule?

Dr. Wiedermann: For some parasites, such as certain helminths, secreted antigens have been associated with immunomodulation, for others non-secreted surface molecules were identified. With respect to Toxoplasma gondii we have not yet identified, which fractions (sugars, lipids or proteins) or molecules might be responsible for the immunomodulating properties of this parasite. Certain non-secreted surface molecules, such as SAG1, Mag1 or Gra7, are thought to be important for the induction of protective immunity against the parasite. These proteins are the first candidate antigens which will be further studied for their immunomodulating properties.
**Dr. Cerf-Bensussan:** What is the difference between acute versus chronic immunization?

**Dr. Wiedermann:** Inoculation of the parasite to BALB/c mice always leads to a biphasic infection, as it also occurs in humans. After an acute phase, which peaks about 8 days post inoculation and is associated with acute phase proteins, the infection turns into a chronic phase, which is characterized by the occurrence of cysts within the brain.

**Dr. Cerf-Bensussan:** So it is related to the strain of mice that you use; Balb/c is used for chronic infection which is not the case for B6.

**Dr. Wiedermann:** Yes, only in BALB/c mice the infections develops into a chronic state and thereby immunomodulation can be studied. In other strains, such as Black/6 mice, the infection is lethal.

**Dr. Cerf-Bensussan:** How do you translate this into the human situation given the fact that the nasal lymphoid tissue is quite different in humans and rodents?

**Dr. Wiedermann:** With respect to mucosal tolerance we have compared the oral and the nasal route of tolerance induction and found quite similar results in mice. The intranasal route might not be the preferred route for human application in allergic patients due to local inflammation etc; however, in experimental studies on mechanistic pathways of tolerance induction, we realized that it is easier to achieve robust immunosuppressive effects with lower doses of antigen given via the nasal than via the oral route. Apart from that, eventhough the NALT of mice might differ from that of humans, several immunization trials in human have shown that immune responses are well induced via the nasal route, indicating that this mucosal route of application should not be underestimated for human use. Additionally, we are now also testing the sublingual route for tolerance induction.

**Dr. Brandtzaeg:** My question more or less regards the problem pointed out by Dr. Cerf-Bensussan, which is the variable structure of NALT. The NALT of rodents is a smooth organ in the floor of the nose; there are no crypts, so it's more like Peyer's patches in the gut with no mechanical retention mechanism. In contrast, NALT of humans is represented by the tonsils and adenoids where we have crypts. I just wondered why you actually stressed that IL-10 was produced mainly when you have colonization of lactic acid bacilli; did they actually colonize the airways?

**Dr. Wiedermann:** We have not looked for the persistence of lactic acid bacteria within the NALT after nasal application. It might however be important for the quality and quantity of local cytokine production how long the bacteria will persist within the NALT. Within the gut it has been shown that these bacteria do not colonized and are only transiently present.

**Dr. Brandtzaeg:** You said you have predominant IL-10 from the NALT?

**Dr. Wiedermann:** Yes, after nasal application of lactic acid bacteria we looked for induction of suppressive cytokines, such as IL-10 in the NALT as well as within the draining lymph nodes. We preferentially detected IL-10 by PCR within the NALT.

**Dr. Brandtzaeg:** But when you use soluble peptides for tolerance induction, was that applied as nasal drops under general anesthesia in the mice?

**Dr. Wiedermann:** Yes.

**Dr. Brandtzaeg:** So then most of it could probably go down into the lungs?

**Dr. Wiedermann:** No, we have been controlling for this by initially using a dye when applying the proteins via the nose. By giving only small drops alternating into both nostrils will prevent that the solution is either swallowed or will go down into the deep respiratory tract.

**Dr. Brandtzaeg:** Even under general anesthesia?

**Dr. Wiedermann:** Yes, with an anesthesia that induces easy and soft breathing.
Dr. Brandtzaeg: Do you think that the NALT structure is important for tolerance induction, or the cervical lymph node, or the mediastinal lymph nodes. Where do you think it was induced?

Dr. Wiedermann: According to the induction of IL-10 in the NALT, but not in the sublingual or cervical lymph node at the end of the experiment, we believe that the NALT is an important inductive site after nasal application. Within the mediastinal lymph nodes we measured the effects of immunomodulation (suppression of Th2 cytokines) rather than induction of regulatory cytokines. Nevertheless to know better, kinetic studies on the induction of regulatory cytokines in different lymphoid organs at different times points are still pending.

Dr. Brandtzaeg: How do you handle sublingual application in mice where there is a keratinized epithelium covering the floor of the mouth, whereas in humans the sublingual squamous epithelium is not keratinized?

Dr. Wiedermann: Sublingual application is trickier than intranasal application and unfortunately mice are less cooperative than humans in this matter; so it need to de done during anesthesia as well.

Dr. Smith: In trying to teach an old immune system new tricks, have you found an age at which you cannot get the immune system to respond in the animal models? Do you have to do this in suckling mice? Are you able to retrain the immune system to develop better tolerance responses in young or older mice?

Dr. Wiedermann: Most of the studies on tolerance induction I presented today have been performed in adult mice. As it is most desirable to prevent allergic development rather than to treat established allergic diseases, the question of the right time point of intervention is crucial. In particular, in our studies on immunomodulation with probiotics we are currently performing several experiments on the window of opportunity for early intervention, using pre- peri- and postnatal intervention schedules. I hope I will be able to give more concrete answers to this question in the next meeting.