

THE HYGIENE HYPOTHESIS AND MODULATION OF ALLERGY

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SUMMARY

It has been suggested that a reduced or changed pattern of exposure to certain microorganisms has led to an unbalanced regulation of our immune system with consequently increased development of inflammatory diseases, such as allergic or autoimmune disorders. Studies on the basis of this so called “hygiene hypothesis” have concentrated on identifying microorganisms that may have the potential to re-establish a regulatory network important to prevent or counteract immune overreactions to innocuous antigens leading to immunopathology.

Using mouse models of type I allergy/asthma we tested several approaches to prevent or treat allergic immune responses, either by the use of mucosal adjuvants, the application of certain lactic acid bacteria as antigen delivery systems or by parasite inoculation. The goal of our research is to exploit the underlying mechanisms of immune modulation and to identify some of the microbial components with immunomodulatory properties, which may serve as novel treatment tools against inflammatory disorders, including allergic diseases.

THE HYGIENE HYPOTHESIS

Within the last decades a constant increase in allergic diseases has been recognized within the industrialized countries, leading to the fact that nowadays more than 25% of the population is affected by allergic diseases. This so called new epidemic of the westernized countries may be a result of a concerted action of genetic predisposition to immunological overreactions to innocuous antigens, increased pollution and enhanced exposure to allergenic molecules, as well as changes in nutrition. Furthermore, the hygiene hypothesis postulates that these epidemiological changes might be evoked by a reduced contact to certain

microorganisms particularly early in life. The original description of the hygiene hypothesis by *Strachan* and colleagues (*Strachan*, 1989; *Strachan* et al., 1996), and *Matricardi* et al. (1998) was based on the observation that in families with high numbers of siblings the risk to develop hay fever was less common. Similar observations were made in families living in farming environment (*Riedler* et al., 2001) or in subjects previously exposed to certain oro-faecal infections (*Matricardi* et al., 2000), or after vaccination with *Bacillus Calmette-Guerin* containing attenuated mycobacteria (*Aaby* et al., 2000). Moreover, changes in the composition

and homeostasis of the commensal gut flora have been linked to increased allergy development, as shown by a reduced number of lactic acid bacteria (LAB) and increased numbers of *Clostridia* and *Staphylococci* in the intestinal flora of atopic children (Björkstén et al., 1999; Watanabe et al., 2003).

Initially, it was suggested that a reduced production of particularly IL-12 and IFN- γ by Th1 immune cells due to a lack of bacterial infections may drive the excessive expansion of Th2 cells. However, this assumption was not compatible with the observation that industrialized countries have also experienced an increase in Th1 autoimmunity along with an increase in allergic diseases (Kero et al., 2001; Simpson et al., 2002). Another indication that a simple Th1/Th2 disbalance is insufficient to explain these epidemiological changes came from studies in developing countries, showing that helminth infections, such as with *Schistosoma mansoni*, which classically induces Th2 biased immune responses, are associated with a lower incidence of allergic disorders (Yazdanbakhsh, Kremsner, and van Ree, 2002). Recasting the hygiene hypothesis it was therefore sug-

gested that the activity of suppressive regulatory cells, initiated by a certain pathogen burden, builds up a regulatory network to down regulate both Th1 and Th2 driven immunopathologies. This regulatory network might fail to develop in a high hygienic environment with low pathogen/adjuvant burden (Maizels and Yazdanbakhsh, 2003; Maizels, 2005). However, it does not seem adequate to postulate that active infections are necessary to achieve immunological homeostasis, as it may be sufficient that contact with only microbial products, such as bacterial endotoxins, can drive immunomodulatory responses via stimulation of the innate immune system, such as through Toll like receptors. Thus, exposure rather than live infection, probably at a very early stage in life, may be one of the most determining factors behind the hygiene hypothesis (Rook, 2009).

On the basis of these perceptions, new treatment approaches against allergy, and also autoimmunity, aim at using new adjuvant systems derived from specific microorganisms to counteract immunopathology by induction of regulatory/ immunosuppressive responses (Figure 1).

NEW STRATEGIES FOR TREATMENT AGAINST ALLERGIC DISEASES ON THE BASIS OF THE HYGIENE HYPOTHESIS

Animal models of type I allergy

Based on the fact that birch pollen and its major allergen Bet v1, belong to the most common airborne allergen in Europe we established a mouse model of birch pollen allergy. The established sensitization scheme is based on systemic immunization with recombinant Bet v 1 followed by an aerosol challenge with natural birch pollen extract. This sensitization protocol gives rise to allergen specific IgE and IgG1 antibodies in serum as well as production of

Th-2 like cytokines (IL-4, IL-5, IL-13) in stimulated splenocytes. Among aerosol challenge, eosinophilic infiltration along with IL-5 and IL-13 leads to airway inflammation and airway hyperresponsiveness. These parameters represent similar features of human allergic asthma (Wiedermann et al., 1999a; 2001).

As it is known that the majority of allergic patients are sensitized against several allergens and these patients are particularly difficult to treat, we also

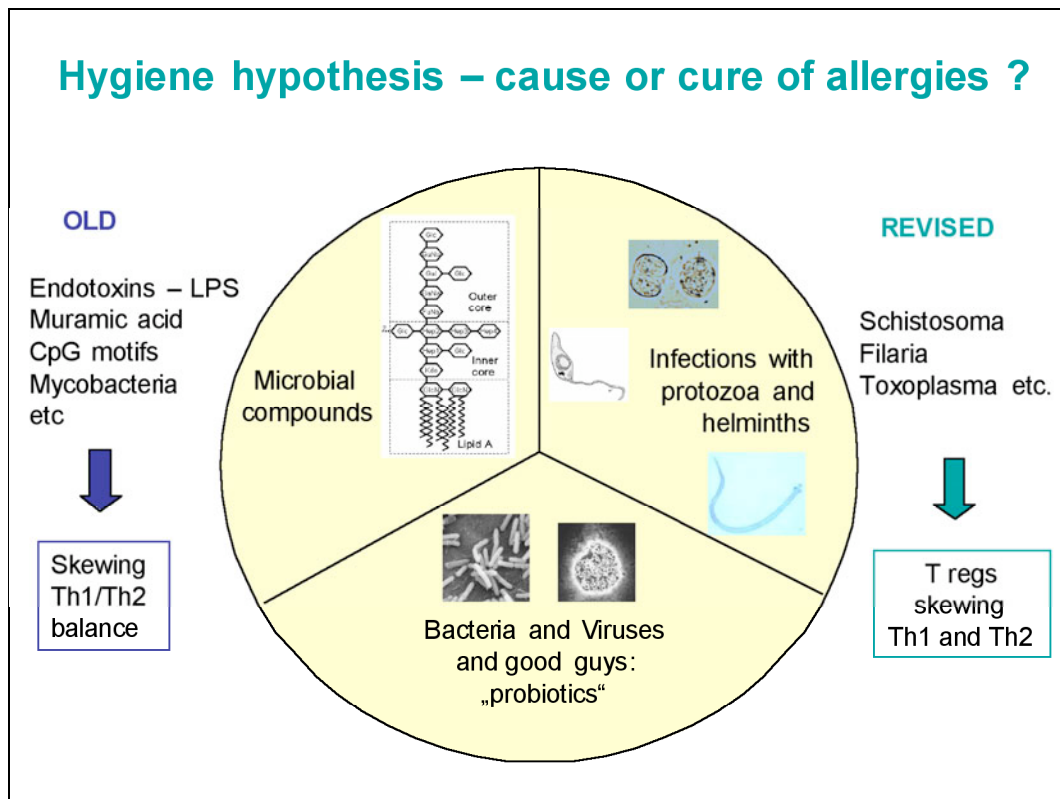


Figure 1: Originally the hygiene hypothesis was explained by induction of Th1 responses by certain bacteria or bacterial compounds to counter balance allergic Th2 responses. Nowadays, the induction of a regulatory network by different infectious agents is suggested to re-balance both Th1 and Th2 responses.

developed a mouse model of polysensitization to the major birch and grass pollen allergens, Bet v 1, Phl p 1 and Phl p 5. Polysensitization with these allergens led to allergic humoral and cellular Th2 immune responses as well as airway inflammation upon allergen-aerosol challenge. The fact that the immunodominant epitopes recognized by T cells from poly-sensitized mice were identical to some of the T cell epitopes in birch and grass pollen allergic patients indicated that our murine model of multiple allergen sensitivity shows similar immunological characteristics to those of human pollinosis (Hufnagl et al., 2005; 2008). These

mouse models were used to study new treatment approaches against allergy on the basis of mucosal tolerance induction with or without the used of certain adjuvant systems.

Use of microbial compounds: Cholera B subunit as mucosal adjuvant/tolerogen

We have previously demonstrated that intranasal application of recombinant allergens (Wiedermann et al., 1999a; Winkler et al., 2002; 2006) or new allergen- constructs (Hufnagl et al., 2005; Wild et al., 2007) prior to sensitization prevents the development of allergic immune responses. However, it

has become obvious that tolerance induction in already sensitized mice, particularly in polysensitized mice, is more difficult to achieve. In order to enhance the tolerogenicity of mucosally applied antigens mucosal antigen delivery systems, such as the B subunit of cholera toxin (CTB), can be used. However, we have shown that the immunosuppressive properties of CTB are influenced by the nature of the coupled antigen as well as by the mode of conjugation: ovalbumin chemically coupled to CTB led to reduction, while Bet v 1 chemically coupled to CTB enhanced allergic immune responses (Wiedermann et al., 1999b). In order to improve the immunomodulatory property of CTB we recently genetically engineered a Bet v 1-CTB fusion molecule (Bublin et al., 2007). 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. Intranasal treatment with this fusion molecule prior to sensitization with Bet v 1 led to significant reduction of 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 pre-treatment with the fusion molecule or CTB alone. Whether the immunomodulatory effects are only due to counter-regulatory Th-1 like immune responses and protective IgA or due to induction of regulatory cells and their products, as described by others (Sun et al., 2006), is currently under investigation. Moreover, the immunomodulatory properties of the Bet v 1-CTB fusion molecule in a therapeutic set up need further exploitation.

Supplementations with probiotics: studies on the right time point of intervention

Lactic acid bacteria (LAB) are non-invasive, non-pathogenic Gram-positive bacteria of which some are within certain foods and important for food processing and preservation, and others are members of the indigenous microflora. Alterations in the composition of the gut flora have been associated with an increase in inflammatory diseases, such as allergies, intestinal bowel diseases or autoimmune diseases (Björkstén et al., 2001; Kalliomaki et al., 2001; Damaskos and Kolios, 2008). Clinical studies indicated that the number of LAB in the gut is different in atopic and non atopic children and that a lack of certain bacterial strains (e.g. lactobacilli, bifidobacteriae) precedes the development of allergic sensitization in infancy (Björkstén et al., 1999; Sjogren et al., 2009). Therefore, there has been increasing interest in supplementing the human diet with certain probiotics strains for allergy prevention (Kalliomaki et al., 2007; Kukkonen et al., 2007; Marschan et al., 2008). The overall finding of the different clinical trials was that the treatment efficacy, leading mainly to reduction of atopic eczema, depended on the respective strain as well as the time point of intervention.

In our recent studies on primary and secondary allergy prevention with probiotics, two strains, *Bifidobacterium longum* and *Lactobacillus paracasei*, were selected to study their immunomodulatory properties *in vivo* and *in vitro*. The two strains, which were shown to induce high levels of IL-10 in splenocytes upon in vitro stimulation, were intranasally applied at the time of sensitization and allergen challenge of adult mice. The treatment with either of the strains reduced allergen specific

humoral and cellular Th2 responses as well as airway inflammation. Moreover, probiotics treatment induced high levels of IgA antibodies in the respiratory mucosa, which might contribute to the local regulatory environment. Our results indicated that probiotic treatment during allergen exposure could constitute a form of seasonal treatment to ameliorate allergic responses during the allergen exposure (*Schabussova et al*, submitted).

With respect to the treatment efficacy, primary prevention, in particular intervention during pregnancy or early childhood, might however have considerable advantages compared to secondary prevention, as existing immune dysregulations are always more difficult to modulate. We have therefore studied different treatment windows in pregnant mothers during gestation and/or lactation and in the offspring during the neonatal phase (*Schabussova et al*, in manuscript). Preliminary data indicate that oral application of live probiotic bacteria to pregnant mice reduced eosinophilic airway inflammation and suppressed Th2 cytokine production in mesenteric lymph nodes and spleens of offspring upon allergic sensitization and allergen challenge. Interestingly, probiotic treatment only in the postnatal phase prior to sensitization did not - or only modestly - reduce allergic responses in the respective infant mice. These results, indicating that the efficacy of probiotics intervention is higher the earlier the intervention starts, are compatible with clinical data showing consistently that pre-and perinatal intervention, but not treatment in the first years of life, prevented allergic manifestations in infancy (*Rautava, Kalliomaki, and Isolauri, 2002; Kopp et al., 2008; Soh et al., 2008*). Further studies will be needed to elucidate the underlying mechanisms of immunosuppression by the selected probiotic

strains during the prenatal period. Another important clinical aspect is to evaluate whether intervention with probiotics might have also unwanted suppressive effects on co-applied antigens, including common paediatric vaccines.

Use of probiotics as antigen delivery system

Within recent years lactic acid bacteria have also evoked interest as mucosal adjuvants and vaccine vehicles with immunomodulatory properties (*Schabussova and Wiedermann, 2008*). 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 (*Wells and Mercenier, 2008*). 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* (*Repa et al., 2003*). 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 pre-treatment 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 (*Daniel et al., 2006; 2007*). With respect to local immune responses and airway inflammation, pre-treatment with Bet v 1-producing LAB and the control strains led to reduction of 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 lactic acid bacteria inducing immunosuppressive cytokines, such as TGF- β or IL-10, rather than Th1-like cytokines might be more beneficial in therapeutic settings. The fact that the first human trial with a recombinant LAB strain has been completed (Braat et al., 2006) indicates that mucosal vaccination with LAB as antigen delivery system could be a successful treatment strategy against allergies in humans.

Infection with *Toxoplasma gondii* and prevention of allergy

According to the hygiene hypothesis, recent epidemiological studies have demonstrated an inverse relationship of certain oro-faecal and food borne infections, such as infection with hepatitis A, *Helicobacter pylori* or *Toxoplasma (T.) gondii*, and the development or manifestation of respiratory allergy (Matricardi et al., 2000). To test the immunomodulatory properties of *T. gondii*, a world wide prevalent, intracellular protozoan parasite (Hill and Dubey, 2002), on allergy development in detail, we recently established a mouse model of toxoplasma infection. *T. gondii* infection in BALB/c mice is somewhat comparable to the infection in humans, exhibiting an acute phase with high serum

TNF- α , IL-6 and IFN- γ levels, followed by a chronic phase characterized by the presence of tissue cysts, particularly within the brain. We demonstrated that infection with *T. gondii* before or after allergic sensitisation with the major birch pollen allergen Bet v 1 reduced systemic and respiratory allergy (Wagner et al., 2009). According to the phase of infection different pathways of immunomodulation were initiated: during the acute infection upregulation of TLR 2, 4, 9 and 11 in splenocytes was associated with Th1 like immune responses along with transient production of IL-10, similarly as described by others (Yarovinsky et al., 2006). In the chronic phase, activation of IL-10 and TGF- β producing Fox p3+ regulatory T cells may have primarily accounted for the suppressive effects against allergic sensitization. Cell transfer experiments of T cells from infected mice supported this notion, as allergic sensitization could be prevented in the recipient mice. Similar findings were also described for other parasites (Wilson et al., 2005). Further studies are planned to analyze in depth the immunomodulatory properties of *T. gondii*, as well as of selected parasitic molecules in order to establish new preventive and therapeutic strategies against allergic diseases, including novel adjuvant systems for anti-allergy vaccines.

CONCLUDING REMARKS

Lessons learned from the hygiene hypothesis are that induction of regulatory T cells and the establishment of a regulatory network by certain microbes and/or infectious agents are essential to prevent or ameliorate immunological overreaction to innocuous antigens, including environmental as well as self-antigens. Exploring in detail the immunomodulatory repertoire of selected

microorganisms at the molecular as well as immunological level may lead to the development of new treatment tools/adjuvant systems for prevention and therapy of different diseases based on immunological hyper-responsiveness, such as allergies and autoimmune diseases, which have nowadays become a great medical and socio-economic problem in westernized societies.

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