Award 2003
Immunoregulatory mechanisms
in allergic asthma

Application

Priv. Doz. Dr. med. Gesine Hansen

Martin-Luther-University Halle-Wittenberg
Department of Pediatrics
Division of Allergy and Pneumology
Ernst-Grube-Str. 40
06120 Halle
Germany

Tel: 0049 (345) 552 2948
Fax: 0049 (345) 552 2945
e-mail: gesine.hansen@medizin.uni-halle.de
Submitted Manuscripts:


*The Impact factors are derived from the Institute for Scientific Information (ISI)*, 10.08.2002
I. Introduction

In industrialized countries, the incidence of asthma has nearly doubled since 1980 and asthma is the most common chronic disease in children. As a result, asthma has reached epidemic proportions, and current health care expenditure in industrialized countries is enormous. Asthma is characterized by airway hyperresponsiveness (AHR) to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, mucus secretion, and high serum IgE levels. The pathology in asthma results from excessive production of IL-4, IL-5, and IL-13 by CD4+ Th2 cells [1;2]. These cytokines initiate and sustain the allergic asthmatic inflammatory response by enhancing the production of IgE and the growth, differentiation, and recruitment of mast cells, basophils, and eosinophils.

Although a relatively large amount is known about the immunological processes leading to the development of allergic disorders, thus far no effective prevention measure exists and the specific conditions in industrialized societies that are responsible for driving Th2 biased immune responses are not yet clear. While Th2 cells promote airway inflammation in asthma, it has been proposed that Th1 cells, which secrete interferon (IFN)-γ, protect against allergic disease by dampening the activity of Th2 effector cells. Indirect evidence for this hypothesis was derived from many epidemiological studies, demonstrating a negative correlation between the asthma incidence and a cleaner environment with fewer Th1 promoting childhood infections in westernized countries (“hygiene hypothesis”) [3]. Moreover, many studies have clearly shown that Th1 responses induce the production of IFN-γ, IL-12 and IL 18, and are associated with the inhibition of Th2 cell development and effector functions [4].

Together these studies suggest that methods to enhance IFN-γ production might be clinically useful in the treatment of allergic asthma and indeed, different animal studies including our own work support this idea [5;6]. On the other hand, we and others have shown that Th1 cells may not always be protective in allergic diseases but may in fact sometimes contribute to, rather than inhibit, the pathology in asthma. Particularly for effector T cells this phenomenon has clearly been demonstrated [7;8]. Thus, although Th2 cells play a critical role in the pathogenesis of asthma and Th1 cells seem to have protective capacity in asthma the binary Th1-Th2 paradigm - where Th1 cells balance Th2 cells – cannot explain all the immunological processes that occur in asthma. There is increasing evidence that “unhygienic” environments may protect against asthma by inducing additional non-Th1-Th2 immunological regulatory mechanisms [9]. Lately, increasing interest is drawn towards so called regulatory T cells that are responsible for immune suppression of both, Th1 and Th2 cells [10]. There is first evidence, that regulatory T cells, for instance those that produce IL 10 and TGF-β [11], play an important role in the control of allergic disease and induction of tolerance to inhaled antigens [12-14].

Further studies are needed to investigate the role of the different types of regulatory T cells in allergic asthma and the mechanisms of tolerance induction. These investigations are prerequisites for the development of therapeutic approaches that alter the underlying immune response and convert or deviate detrimental allergic responses toward protective immune responses with the chance for long-lasting therapeutic effects.

In our research, we tested different approaches to improve therapeutic strategies [15] in allergic asthma with the focus on the development of an anti-allergy vaccine utilising Th1-biasing agents like inactivated Listeria monocytogenes [5] and modified bacterial DNA vectors [6]. Moreover, we directly investigated the inhibitory potential of Th1 cells on Th2 cell-induced pro-asthmatic effects in a murine transfer model using well-defined, antigen-specific effector T cells [7]. Finally, we demonstrated the regulatory, protective potential of TGF-β producing cells in allergic asthma [11].
II. Results and Discussion

II.I Modulating the Th2-dominated immune response in allergic asthma by inducing Th1 cells

Asthma is characterized by the overproduction of the Th2 cytokines IL-4, IL-5, and IL-13, which initiate and sustain the allergic asthmatic inflammatory response. The Th2-driven inflammatory process may be a consequence of a relative insufficiency in IFN-γ production because IFN-γ can inhibit the development of Th2 responses [16]. Indeed, immunotherapies and immune modulatory approaches that enhance Th1-dominated responses appear to be beneficial for allergic individuals [17].

One very effective Th1-promoting adjuvant is *Listeria monocytogenes*, a Gram-positive, facultative intracellular bacterium, which elicits a strong classical cell-mediated immune response. Employment of heat-killed *Listeria monocytogenes* (HKL) as an immunotherapeutic adjuvant during antigen (Ag) immunotherapy successfully reversed ongoing Th2-dominated responses and induced an Ag-specific Th1-dominated response in mice [18]. The application of inactivated *Listeria* or their products might therefore be used as a vaccine or as components of a vaccine aimed at inhibiting Th2 responses. In our study we showed that in a murine model of asthma HKL as an adjuvant given once with Ag prevented the development of AHR and airway inflammation in Ovalbumin (OVA) immunized BALB/c mice and significantly reduced airway eosinophilia and mucus production. These effects were accompanied by the conversion of an Ag-specific Th2-dominated immune response into an Ag-specific Th1-like immune response and by a dramatic decrease of Ag-specific IgE (Figure 1). Moreover, when given late after allergen-sensitization, a single dose of HKL with Ag reversed established AHR and reduced airway inflammation. The inhibitory effect on AHR depended on the presence of IL-12 and CD8+ T cells, was associated with an increase of IL 18 mRNA expression, and required close association between HKL and the Ag. Thus, our results demonstrate that HKL as an adjuvant very effectively promotes protective immune responses in the respiratory tract, and down-modulates ongoing Th2-dominated responses, indicating that HKL as an adjuvant for allergen immunotherapy may be clinically effective in the treatment of allergic asthma.

The attractiveness of *Listeria* as adjuvant therapy lies also in the fact that its immunomodulatory effects remain largely Ag specific. *Listeria* had minimal effect on AHR, IgE, and cytokine production unless the *Listeria* was administered in a mixture with the Ag. Thus *Listeria* did not induce a generalized enhancement of IFN-γ production in recipients, but rather induced an Ag-specific protective response. This specificity avoids nonspecific immune augmentation, which could result in the development of autoimmune diseases.

In addition to *Listeria* the application of other bacteria has also been shown to suppress the development of allergic diseases. For example the application of live or heat-killed *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) into the lung of mice strongly suppressed the development of airway eosinophilia with the effect lasting up to two months after application [19,20]. The inhibitory effect was clearly dependent upon IFN-γ signalling and, therefore, Th1 responses. In addition to BCG, *Mycobacterium vaccae* was also shown to mediate protection against the development of allergic Th2 responses [21] and reduction of established allergen induced Th2 responses, with BCG being more effective than *M. vaccae*. Importantly, the authors found that only the treatment with BCG but not *M. vaccae* lead to increased IFN-γ levels in the BAL of the mice suggesting that BCG-induced, but not *M. vaccae*-induced, suppression of allergic responses is mediated by Th1 cells. Recently, it has been suggested that *M. vaccae* mediates the suppression of Th2 responses by the induction of regulatory T cells (Tr) [22] which opens interesting perspectives as far as therapy is concerned.
Another therapeutic approach for asthma that can alter the underlying Th2-biased allergic response in an allergen-specific manner might be the use of DNA-based immunization. DNA vaccination induces Th1-biased responses by unmethylated CpG motifs in the plasmid backbone which act as intrinsic adjuvant. CpG-oligodeoxynucleotides are part of the noncoding sequences of microbial DNA and bind directly to toll-like receptor 9 [23]. Vaccination with allergen in the form of naked plasmid DNA stimulates allergen-specific immune responses with a Th1 bias and amplifies the expansion of CD4+ T cells producing IFN-γ and of cytotoxic CD8+ T cells [24-26]. The key feature of this strategy is that injection of plasmid DNA encoding a specific Ag produces an allergen-specific protective immune response that should be reinforced by natural exposure to the allergen, thus conferring long-lasting protection. Previous studies with DNA immunization strategies demonstrated their success in preventing the development of Ag-specific IgE synthesis and AHR [27;28]. To enhance the effectiveness of DNA vaccination and potentially treat patients with ongoing AHR, we constructed a DNA vaccination plasmid containing cDNA for a prototypic allergen, OVA, fused to the cDNA of a potent immune modulating cytokine, IL-18. This approach is based on the fact that IL-18, a product of activated macrophages and Kupffer cells [29], is very powerful in driving the production of Th1 cytokine synthesis in naïve and memory T cells [30]. We examined the efficacy of the OVA-IL-18 cDNA fusion construct vector in a murine model of asthma and compared its efficacy with that of OVA cDNA, IL-18 cDNA, or a mixture of OVA cDNA and IL-18 cDNA on separate plasmids. The OVA-IL-18 cDNA construct effectively corrected established AHR in an allergen-specific fashion when administered only twice. Although both the OVA-IL-18 and the OVA cDNA constructs, when administered to naïve mice, prevented the subsequent induction of AHR and reduced allergen-specific IgE production, the OVA-IL-18 cDNA construct was unique among the DNA constructs in its capacity to reverse established AHR. The protective effects of the OVA-IL-18 cDNA construct appeared to be mediated by IFN-γ and CD8+ cells, and because the OVA plasmid was not capable of reversing established AHR, we conclude that the addition of IL-18 as a fusion construct greatly enhanced the immunogenicity and effectiveness of plasmid vaccination. The potent inhibitory effects of OVA-IL-18 DNA vaccination on AHR and IgE production was dependent on the fusion of the cytokine and the allergen. Thus, vaccination with the OVA plasmid alone or with the IL-18 plasmid alone or codelivery of nonfused OVA cDNA together with IL-18 cDNA was less effective than the OVA-IL-18 fusion plasmid in inducing IFN-γ production, reducing IgE production, and preventing the development of AHR. This indicated that the presence of the IL-18 cDNA fused to the OVA cDNA was crucial for protection in this model.

Since vaccines that induce very strong Th1 responses may have harmful side effects (see below), we constructed vectors containing the cDNA for the allergen and IL-10 or TGF-β. Thereby, we combined the Th1 inducing effects of unmethylated CpG motifs in the vector backbone with the anti-inflammatory effects of IL-10 or TGF-β. The therapeutic effects of these vectors are currently investigated after mucosal application. So far our results are very promising.

Currently, conventional allergen immunotherapy is the only available therapy that, when successful, alters the underlying pathologic allergenspecific Th2 driven responses, resulting in clinical tolerance to subsequent allergen exposure. However, such therapy is often inefficient in allergic asthma and associated with frequent allergic reactions, including anaphylaxis [31]. DNA vaccination may be a safer form of allergen immunotherapy, particularly because DNA based immunization provides prolonged, endogenous expression of Ag [32]. Plasmids have been found to persist episomally in muscle cells, and gene expression in the skeletal muscle and persistent immunity to the Ag can be detected for more than a year after injection. However, rigorous studies of DNA-based immunization with respect to mechanism of action, safety, and delivery will be crucial before its ultimate application in human atopic disease.
II.II Challenge of the Th1/Th2 paradigm and its implications for therapeutic approaches in the future

Safety may be the most important prerequisite for an anti-allergy vaccine, as allergies are not normally life threatening and may have to be applied at a very early age to be effective. Should vaccines that directly induce allergen-specific Th1 responses be used? There is evidence that Th1 and Th2 cells may not always antagonize each other and that Th1 cells are not always protective in allergic diseases. First, IFN-γ has been identified in BAL fluid and serum of asthmatic patients, suggesting that Th1-like cells may in fact contribute to, rather than inhibit, the pathology in asthma [7;8]. In addition, Th1 cells are not found in large numbers in the lungs or mucus membranes of nonallergic or nonasthmatic individuals after allergen exposure, as would be predicted if Th1 cells reduced airway inflammation. The evidence, demonstrating that Th1 cells actually render salutary effects in allergic disease and asthma, was mainly indirect. Th1 cells inhibit the proliferation, and therefore the development, of Th2 cells, and IFN-γ inhibits IgE synthesis in some instances. Many epidemiological studies demonstrated a negative correlation between the asthma incidence and a cleaner environment with fewer Th1 promoting childhood infections in westernized countries [3]. No data were available, however, that showed that Th1 cells actively suppressed Th2-dominated allergic responses.

Therefore, in our study we directly examined the capacity of well-defined, antigen-specific Th1 CD4+ cells to counterbalance the allergic effects of Th2 cells in a murine model of asthma. Th1 and Th2 CD4+ lines expressing identical OVA-specific T-cell receptors (TCRs) were generated from OVA-specific TCR transgenic mice, and their effects after transfer into lymphocyte-deficient severe combined immunodeficiency (SCID) and immunocompetent BALB/c mice on allergen-induced airway inflammation and AHR were investigated. Our results demonstrated that OVA-specific Th1 cells caused severe airway inflammation and did not reduce the inflammatory effects of OVA-specific Th2 cells (Figure 2). Surprisingly, even though Th1 cells induced severe airway inflammation, they did not cause AHR indicating that inflammation in and of itself is not necessary for the development of AHR, although CD4+ T cells appear to be essential for the development of AHR [33]. While OVA-specific Th1 cells did not induce AHR they were unable to reduce Th2 cell–induced AHR, either in lymphocyte-deficient SCID mice or in immunocompetent recipients (Figure 3). In contrast, Th1 cells were able to reduce the number of airway eosinophils and reduce intrabronchiolar mucus production induced by Th2 cells showing that the transferred cells functioned in vivo (Figure 2). Our studies indicated that the Th1/Th2 paradigm, which predicts that Th1 cells downregulate allergic disease and asthma, may be more complex than initially appreciated and that suppression of allergic inflammation and Th2 activity in vivo may depend on cells other than Th1 lymphocytes. These findings are of particular concern with regard to current therapeutic goals in asthma and allergies, and suggest that conversion of Th2-dominated allergic inflammatory responses into Th1-dominated responses may be problematic. Our results clearly demonstrate that allergen-specific Th1 cells are capable of causing inflammatory responses and tissue destruction in mice and suggest that this may also occur in humans. Further danger associated with vaccines that induce strong Th1 responses is that they might favour the generation of autoimmune disease, as it was reported that the application of CpG–ODN exacerbated autoimmunity in different animal models. Alternatively, other cell types — e.g., transforming growth factor-β–producing CD4+ antigen-specific Th3 cells [34], Tr1 cells [35], γδ T cells [36], or CD8+ cells [37;38] — may be also or even more important than Th1 cells in downregulating allergic inflammation and hyperreactivity. However, it is important to keep in mind that these conclusions are based on
experiments that transfer relatively large amounts of monoclonal TCR transgenic Th1 cells and then challenge with allergen within a short time interval after i.v. application of the T cells. Moreover, the resistance of Th2 cell function to modification by Th1 cells may be due in part to the fact that Th2 effector cells are terminally differentiated with fixed cytokine profiles [39] and cease to express IL-12 and IL-18 receptors [40].

II.III Alternative to Th1-inducing therapeutic approaches in Th2-biased allergic asthma: T regulatory cells

Although Th2 cells play a critical role in the pathogenesis of asthma and Th1 cells seem to have protective capacity the binary Th1-Th2 paradigm - where Th1 cells balance Th2 cells – cannot explain all the immunological processes that occur in asthma. These processes in asthma may be much more complex than is predicted by the Th1-Th2 paradigm [41-43], and "unhygienic" environments may protect against asthma by inducing additional non-Th1-Th2 immunological regulatory mechanisms. Important in this context is the fact that in most individuals, mucosal exposure to allergens leads to CD4+ T cell tolerance, preventing the development of Th2-biased responses and allergen-induced AHR. Although it is not entirely clear how this selective immune suppression is achieved, recent evidence suggests that specific CD4+ T cells in particular are responsible for this effect by shutting down or dampening Th1 or Th2 cell responses. CD4+ T cells responsible for immune suppression have been termed regulatory T cells (Tr). Currently four major Tr cell populations have been described. CD4+CD25+ Tr cells naturally occur both in humans and rodents and suppress T cell responses in a contact-dependent manner. However, not all cells expressing CD4 and CD25 are Tr cells, as activated effector T cells also express CD25, making the identification of CD4+CD25+ Tr cells difficult. T cell clones, which are themselves anergic but can suppress other T cell responses by influencing APC function, are also considered to be Tr cells [12;13]. A further Tr cell type has been named Tr1 cells. They can be generated in vitro by stimulating murine or human CD4+ T cells in the presence of IL-10 [35]. Tr1 cells can inhibit both Th1 and Th2 responses by secreting large amounts of IL-10 and lower amounts of TGF-β. Tr cells induced after oral administration of antigen and secreting TGF-β have been named Th3 cells [12;13]. Currently the relationship between the different Tr populations remains unclear with respect to their development, activation and the mechanisms by which exact immune suppression is achieved.

Our interest was drawn towards Th3 cells or TGF-β, respectively, since TGF-β is one key regulator in the maintenance of immunological homeostasis. It is a pleiotropic cytokine with significant anti-inflammatory and immunosuppressive properties. TGF-β inhibits the production of proinflammatory cytokines from macrophages, B cells, and T cells and is a potent inhibitor of T-cell-mediated immune responses, both in vitro and in vivo. The essential role for TGF-β in the maintenance of immune homeostasis is demonstrated in TGF-β1−/− mice which develop a severe, multifocal inflammatory response in all of the pubs that are born alive [44]. To directly examine the role of T cells producing latent TGF-β in the respiratory mucosa, we generated Th-cell lines secreting TGF-β by transducing established antigen-specific Th1-cell lines with a retrovirus vector containing the cDNA for latent TGF-β [45]. We assessed the capacity of such cells to downmodulate allergic inflammation and AHR in an established adoptive transfer mouse model of asthma. Surprisingly, OVA-specific TGF-β–secreting Th cells, but not OVA-specific Th1 cells, profoundly inhibited AHR and airway inflammation induced by established effector Th2 cells in SCID mice (Figure 3a). The effect of TGF-β–producing T cells was antigen specific and dependent on secretion of TGF-β because a neutralizing mAb to
TGF-β abolished the inhibitory effect, and directly downregulated the function of Th2 cells rather than inhibited homing of Th2 cells into the lungs (Figure 3b). Moreover, the effect was also observed by transfer of the TGF-β-producing T cells into OVA-immunized immunocompetent BALB/c mice, establishing that such cells can not only inhibit the function of effector Th2 cells but can also prevent the development of AHR in immunocompetent animals. Our results indicate that T cells secreting TGF-β in the respiratory mucosa can indeed regulate Th2-induced AHR and inflammation and may be key regulatory cells in asthma. This finding is supported by our current study investigating TGF-β₁⁺/⁻ mice. While TGF-β₁⁻/⁻ mice develop a lethal inflammatory disease, TGF-β₁⁺/⁻ mice seem phenotypically normal. We could demonstrate, that TGF-β₁⁺/⁻ mice express only about 30% of wild type (WT) TGF-β₁ protein levels. The reduced expression of TGF-β₁ is accompanied by a strikingly enhanced inflammatory response to OVA-sensitization with the strongest effect on airway eosinophilia (data submitted for publication). The important role of TGF-β in the control of allergic asthma is also supported by other researchers. For instance the effects of oral tolerization in eosinophilic tracheitis have been attributed to TGF-β producing T cells [46]. Nakao et al. [47] showed that antigen-induced airway inflammation and airway reactivity were enhanced in Smad7 Tg mice, suggesting that TGF-β/Smad signaling in mature T cells was crucial for negative regulation of the inflammatory immune response. Similarly, a recent study identified the T cell as a central effector cell of TGF-β₁-mediated regulation of AHR [48]. Further studies are ongoing to investigate the role of the different types of regulatory T cells, especially IL-10 and TGF-β producing T cells, in allergic asthma and tolerance induction.
III. Future directions

There is increasing evidence that regulatory T cells, like TGF-β or IL-10 producing T cells, play a critical role in downmodulating the Th2 dominated immune response in allergic asthma. These findings open new avenues for the development of immunomodulatory therapies that might not be accompanied by possible risks like inflammation or autoimmunity as Th1-cell inducing approaches. Therefore, further investigations of the role of regulatory T cells and the mechanisms of tolerance induction are important aims of our current research work.

IV. Concluding remarks

The aim of our research in the past years was the investigation of immunoregulatory mechanisms in allergic asthma. We tested different approaches to improve therapeutic strategies [15] with the focus on the development of an anti-allergy vaccine utilising Th1-biasing agents like inactivated Listeria monocytogenes [5] and modified bacterial DNA vectors [6] to counterbalance the Th2-dominated allergic process. In these models we could demonstrate that indeed enhancement of IFN-γ producing cells might be clinically useful in the treatment of allergic asthma. Moreover, we directly investigated in a murine cell transfer model the inhibitory potential of well-defined, antigen-specific effector Th1 cells on effector Th2 cell-induced asthmatic symptoms like airway hyperreactivity and inflammation [7]. We clearly showed, that strong Th1-inducing therapeutic approaches may in fact sometimes contribute to, rather than inhibit, the pathology in asthma by increasing inflammation. This study demonstrated potential risks of Th1-inducing therapeutic strategies and was an important starting point for the search for other immunoregulatory mechanisms in IgE-mediated allergic diseases. Recent evidence suggests that specific CD4+ T cells, the regulatory T cells, are able to inhibit both Th1 and Th2 cell responses. We could demonstrate, for example, that one type of regulatory T cells, the TGF-β producing cell or Th3 cell, is able to inhibit very effectively airway hyperreactivity and inflammation in allergic asthma [11]. This work supported the important role of regulatory T cells in allergic diseases. Based on our findings and the data of many other researchers, currently two approaches seem to have the greatest potential for an efficient and long-lasting treatment or even prevention of allergy: Firstly, vaccines inducing allergen-specific or unspecific Th1 responses during early childhood in atopy-prone children may prove successful in inhibiting Th2 responses. The risk of inducing inflammatory responses and Th1-biased autoimmune responses has to be considered carefully. Secondly, vaccines inducing T regulatory cells that are able to inhibit the development of allergic Th2 responses and inhibit already established Th2 responses seem very attractive for future directions. It might be advantageous when future anti-allergy vaccines aim at inducing Tr and not Th1 responses, as the generation of Tr cells seems to harbour fewer potential negative side effects than the generation of Th1 cells. Finally, learning from nature how we can get tolerant to allergens will be an important challenge for future research in allergic diseases.
References


**Figure 1.** HKL as an adjuvant converts an established Th2- to a Th1- like cytokine response and inhibits the production of OVA-specific IgE in BALB/c mice that were immunized with OVA s.c. and intranasally.
Figure 2. Histologic examination of lungs from SCID mice receiving Th1 or Th2 cells. H&E, x250. 
Inset: Bronchiolar epithelium. H&E, x400.
Figure 3. (a) TGF-β–producing cells, but not Th1 cells, inhibit Th2 cell–induced airway hyperreactivity in SCID mice. (b) Anti–TGF-β mAb abolishes the inhibitory effect of TGF-β–producing cells on Th2 cell–induced airway hyperreactivity.