Novel opportunities for tailor-made immunomodulation in atopic diseases

- Breaking the waves -

Nieuwe mogelijkheden voor immunomodulatie op maat in atopische aandoeningen

- De golven bestrijden -

(met een samenvatting in het Nederlands)

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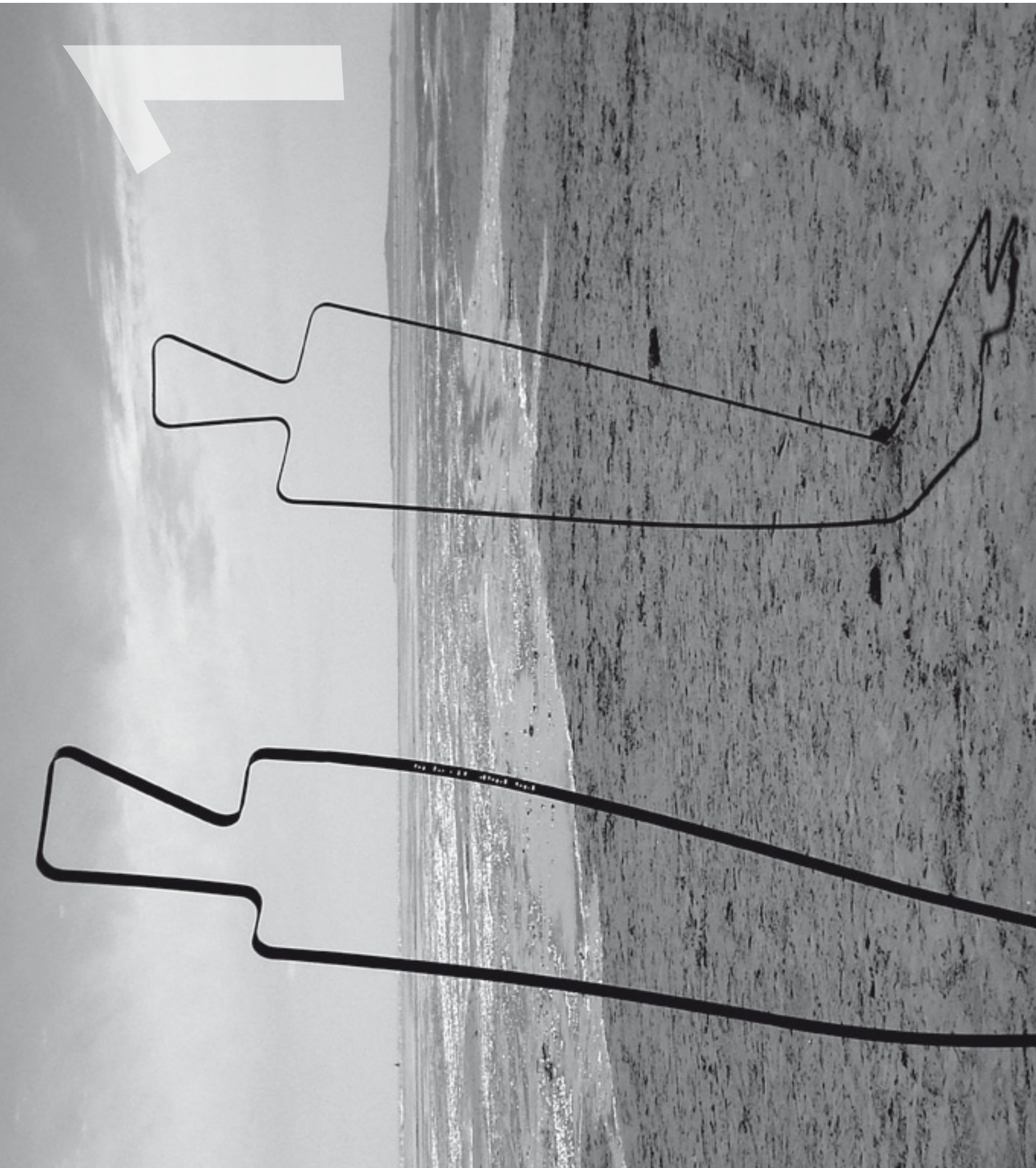
General Introduction

ATOPY: a disease of tides



ATOPY: A DISEASE OF TIDES

Atopy is characterized by the tendency of the immune system of a genetically susceptible individual to respond to antigens, the so-called allergens, in a characteristic fashion[1,2]. After the presentation of the allergen via MHC class II receptors on dendritic cells $CD4^+$ T helper 2 (T_H2) cells are activated. These T cells are named T_H2 cells because this activation results in the excretion of cytokines of type 2, such as interleukin-4 (IL-4), IL-5 and IL-13. Other T helper cells are T helper 1 (T_H1) cells and T helper 17 (T_H17) cells, all secreting a specific set of cytokines and involved in different types of immune responses (Table 1).

Cell type	Transcriptionfactor	Cytokines	Function
T_H1	T-bet, STAT4,	IL-12, IFNγ	Host defence (intracellular pathogens), autoimmunity
T_H2	GATA-3, STAT6, c-Maf	IL-4, IL-5, IL-13	Host defence (parasites), atopy
T_H17	STAT3, RORγt	IL-17A, IL-17F, IL-22	Host defence (extracellular pathogens), inflammation, autoimmunity

Table1.
The different T helper cells, their cytokines and functions.

The cytokines produced by T_H2 cells induce an accumulation of eosinophils in the affected tissue and the production of IgE by B cells, which can bind to specific Fc receptors on mast cells and basophils[3]. The first time this reaction occurs, an individual becomes sensitized. When a new encounter with the allergen occurs, cross-linking of IgE can activate effector cells such as basophils and mast cells which will release histamine, prostaglandins and cysteinyl leukotrienes upon activation. These mediators contribute to an allergic reaction consisting of vasodilatation, bronchial hyperresponsiveness and increased vasopermeability.
Atopy can affect different target organs, like the skin (atopic dermatitis or eczema), the gastro-intestinal tract (food allergy), the lungs (allergic asthma) and/ or the nose and eyes (allergic rhinitis and conjunctivitis or hay fever). Interestingly the difference in atopic diseases expressed in different organs follows an age dependent pattern. Atopic diseases tend to demonstrate a natural history in which sensitization and symptoms occur during a certain age period after which remission is possible. This is referred to as the allergic march (Fig. 1)[4,5].

Figure 1.
The atopic march: age-specific distribution of different atopic diseases. Picture by MP Laan.

PRED: ISLAND OF GENES?

Hygiene hypothesis
"A genotype does not specify a unique outcome of development; rather it specifies a norm of reaction, a pattern of developmental outcomes in different environments"[6].
These words by Richard Lewontin in his book Triple Helix seem to fit for atopic diseases, which are multifactorial in origin with both genetic factors as well as environmental factors playing a role.
Atopic diseases are genetically complex. This means that they are, in part, inheritable, but do not follow a simple Mendelian pattern. Family studies have shown that atopy in a parent predisposes for atopy in a child. However, the different atopic diseases are not necessarily inherited altogether.
Over the last four decades, a dramatic increase in atopic diseases has been observed[7]. This increase is only seen in the industrialized world. In the developing countries, the incidence of atopic diseases remains low[8-10]. These epidemiological observations indicate that environmental factors contribute to development of disease in concert with an individual's genetic susceptibility. As a theoretical model the hygiene hypothesis has been developed[11]. This hypothesis suggests that micro-organisms in the environment of a child skew the immune system from a T_H2 environment at birth towards a Th1 environment. According to this hypothesis, the improvement in general living standards as seen in the westernized world lessens the risk of infections, and thereby increases the risk of T_H2 mediated diseases such as atopy. Indeed, birth order, family size but also life on a farm inversely correlated with the risk of atopy[12]. However, also an increase in T helper 1 (T_H1) mediated diseases, such as diabetes mellitus type 1, Juvenile Idiopathic Arthritis and Crohn's disease, is seen[13-15]. Another conflicting finding is the fact that in non-industrialized

countries certain infections, such as chronic helminth infections, induce a T_H2 skewed reaction, but also seem protective against atopy[16,17]. Another T cell type, the regulatory T cell, turned out to play the key role in this concept, as will be explained later.

Genetics versus gene expression

Since atopic diseases are, at least in part, inheritable, researches have focused on identifying contributing genes for diseases such as asthma. Polymorphisms in candidate genes are believed to play a role in the pathophysiology of the disease. Several candidate genes, such as ADAM33 and RANTES have been found[18]. However, results found with linkage studies tend to be difficult to be reproduced in other populations[19,20]. Firstly phenotypic heterogeneity is seen in the different atopic diseases. Secondly, genetic studies do not take environmental factors into account. Depending on the environment an individual is exposed to, a polymorphism can either be protective or associated with disease[21]. The environment will also influence the expression of a given gene. Therefore, genomic studies in atopic disease need to combine environmental influences on the genetic predisposition in order to give more insight in the pathophysiology of atopy[22-24].

Microarray research in allergy

One of the techniques to analyse gene expression is the microarray. With this technique, which has first been described in 1995, the expression levels of up to 40.000 genes can be determined simultaneously[25]. However, the use of microarrays is hampered by obtaining enough mRNA of high quality. This is a compromising factor especially in diseases like allergy and asthma where large biopsies of the end-organ can not easily be obtained. In diseases where circulating leukocytes play a role it might be assumed that the gene expression profile of those more easily obtained cells reflect a distinct gene expression profile of the diseased organ. Alcorta et al. compared gene expression profiles of peripheral blood mononuclear cells (PBMC) in patients with different forms of renal disease with renal biopsy samples. This study revealed a distinct gene expression profile for each disease[26]. Since lymphocytes play a role in allergy and asthma, PBMC of allergic and asthmatic patients may reflect disease specific changes in involved tissue and therefore be a model for gene expression profiling. Brutsche et al. compared PBMC of asthmatic (allergic and non-allergic) and other allergic patients (without asthma) with healthy controls to see whether a gene-expression based score was discriminating in different atopic phenotypes. This CAGE score (composite gene expression score) proved to be superior to IgE levels in the diagnosis of atopy and could be used as a tool for treatment response in both atopy and asthma[22].

Cord blood

One can expect that a genetic predisposition for atopy may already influence the immune system *in utero*. Interestingly, the immune system *in utero* is skewed towards a T_H2 environment in all pregnancies. This environment is believed to be protective against harmful effects of IFNγ and other pro-inflammatory mediators, capable of causing graft-versus-host (or vice versa) effects[27]. Consequently, T_H2 skewed allergen-specific responses can be seen in virtually all neonates at birth[28]. This by itself does not lead to allergic symptoms. In the following period a deviation in immune responses arises where T_H2 responses are less abundant and mature T_H1 responses develop. This is reflected by the increased capacity to induce IFNγ and T_H1 responses around the age of five. A normal T_H1 immunity exists in non-allergic infants. However, the immune system of infants at risk to develop atopy, fails to deviate towards mature T_H1 responses[29]. In a study by Macaubas et al. higher levels of IFNγ in cord blood serum samples were associated with a lower risk of asthma and atopy at the age of 6. However, paradoxically, also higher levels of IL-4 were found[30]. Still a predisposing difference in the immune system *in utero* seems to exist. This is also reflected in elevated IgE levels in cord blood, and even in allergen specific responses of cord blood derived T cells from fetal origin[28]. Thus, the process of sensitization might start as early as *in utero*. This also means that for modulation of the initiation and course of atopy (and subsequent allergic inflammation) only a narrow window of opportunity in early life is available.

Focus for therapy: providing a dam or re-directing the flow?

Every individual encounters common allergens, such as grass pollen, house dust mite and cat dander. These encounters lead to T cell recognition, as is shown in proliferative responses of PBMC of non-allergic individuals against these allergens[31]. Why the immune system of allergic individuals responds to these allergens by inducing the release of T_H2 cytokines and IgE, and the immune system of non-allergics does not, is still not fully understood. Regulation of the balance between activation and subsequent suppression of T cell responses seems to be critical.
For this matter, the T-cell repertoire of humans also harbours regulatory T cells (T_{regs}). T_{regs} can be divided in the naturally occurring, thymus derived, $CD4^+CD25^+$ T_{regs} and peripherally induced populations, such as T_H3 and T_r1.

Regulatory T cells

As said, T_{regs} are a subset of T cells which play a critical role in the balance between an immune response and the subsequent suppression of this immune response, hence preventing either auto-immune diseases or atopic diseases. The naturally occurring T_{regs} represent a small fraction of the $CD4^+$ T cell population and, like every T cell, express a T-cell receptor (TCR) and other surface molecules among which members of the Toll-like receptor (TLR) family[32]. In addition to signaling via the TCR, TLR and accessory molecules such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and glucocorticoid-inducible tumor necrosis factor receptor-family related receptor (GITR) influence the level of suppression[33,34]. Furthermore, regulatory function of these cells is negatively correlated with CD127 expression[35].

T_{regs} express the transcriptionfactor Foxp3. This transcriptionfactor plays an important role in the development and function of T_{regs}. Mice with a mutation in the gene encoding for Foxp3, Scurfin, display several autoimmune manifestations (such as arthritis, gastritis and insulinitis), caused by hyperactive $CD4^+$ T cells and the production of pro-inflammatory cytokines[36]. Subsequently, humans suffering from the IPEX syndrome (immune dysregulation, polyendocrinopathy, entheropathy, X-linked syndrome) were found to have a mutation in the FOXP3 gene (the gene is named Foxp3 in mice and FOXP3 in humans)[37]. Notably, this syndrome is also characterized by severe forms of eczema and food allergy, indicating that allergic dysregulation is a fundamental attribute of FOXP3 deficiency. This led to the question whether T_{regs} were insufficient in allergy. Indeed several studies have shown a diminished number of T_{regs}, next to a diminished capacity in allergic diseases. Ling et al showed a diminished suppressive capacity of T_{regs} during active allergy, compared to non-active allergy, as seen in allergic rhinitis during hay fever season[38]. In children with non-IgE-mediated food-allergy, both the number of T_{regs} as well as their suppressive function, was diminished compared to children who had outgrown their clinical symptoms of food allergy[39]. That the lack of functioning T_{regs} is both a systemic as a local problem at the target organ was shown in children with asthma. In the bronchoalvelolar compartment of these children, T_{regs} are decreased in number and fail to suppress T_H2 responses[40]. These studies give the impression that a lack of T_{regs}, either in number or functionally contribute to allergic diseases.

Peripherally induced T_{regs}

Next to naturally occurring T_{regs}, another population of antigen-specific, peripherally inducible T cells with suppressive function is known. These are referred to as adaptive or inducible regulatory T cells (iT_{regs})[41]. Activation of these iT_{regs} can occur in a tolerogenic or inflammatory context and is antigen-dependent[42,43]. At least two distinct cell types, different in their cytokine production upon activation, have been demonstrated. T regulatory cells 1 (Tr1) secrete high levels of IL-10 and are capable of secreting IL-5, IL-13 and TGFβ[44]. T_H3 cells secrete high levels of TGFβ with or without IL-4 and IL-10[45]. Another type of inducible T_{regs} which are phenotypically and functionally similar to naturally occurring T_{regs} can be induced *in vitro* from $CD4^+CD25^-$ T cells by TCR stimulation[46,47]. These induced $FOXP3^+$ T_{regs} are highly proliferative and derive from a rapid turnover of memory $CD4^+CD25^-FOXP3^-$ T cells[48]. Recently it has been demonstrated that FOXP3 can also be transiently upregulated in T effector cells upon activation *in vitro.* These cells lack the suppressive function as seen in T_{regs}[49].

Mechanism of regulation

As stated earlier the hygiene hypothesis refers to a dysbalance between T_H1 and T_H2 cells. The higher level of hygiene obtained in the westernized world decreases encounters with pathogens. According to the hygiene hypothesis, this leads to the reduced induction of T_H1 cells and increased T_H2 cells, which would explain the increase of allergic diseases in western countries. However, this hypothesis can not account for the parallel increase of T_H1 mediated diseases. A possible explanation for these conflicting observations was found in the protective effect of the Helminth infection against allergy. Chronic Helminth infections, and as was later shown, also other chronic infections, cause IL-10 production. This cytokine is an inhibitory regulator, not only for T_H2 but also T_H1 mediated immune processes[50-52]. This led to the formulation of the counter-regulatory hypothesis, which states that a lack of IL-10 can lead to either a T_H1 or T_H2 disease, dependent on the immunological activation leading to the initial inflammation and off course a genetic predisposition[53]. Not only IL-10 but also TGFβ can play a regulatory or inhibitory role (discussed below). Among the most important cells producing IL-10 are regulatory T cells. The abovementioned studies also support the fact that T_{regs} may indeed play a role in the pathogenesis of both autoimmunity and allergic diseases.

Regulatory cells function by the secretion of these regulatory cytokines, but can also suppress immunity by direct cell-contact with effector cells, such as $CD4^+$ and $CD8^+$ T cells, natural killer cells, NKT cells, B cells and dendritic cells. Although

activation of regulatory cells themselves is antigen-specific, after activation, the local suppression can be antigen-nonspecific[54].

TGF-β

A cytokine known for its regulatory function is TGF-β[55]. Disruption of the TGF-β signalling pathway in mice results in lymphocyte infiltration into tissues and spontaneous T helper cell activation[56]. Recently it was demonstrated that TGF-β is capable to generate $CD4^+CD25^+$ T cells with suppressive function from naïve $CD4^+CD25^-$ T cells both from cord blood mononuclear cells (CBMC) and from peripheral blood mononuclear cells (PBMC) by induction of FOXP3[57]. The immunomodulating effects of TGF-β on T lymphocytes seem to depend on the maturation status of the lymphocytes. In mice, TGF-β had a suppressive effect on differentiation of naïve T lymphocytes. However, when T lymphocytes were cultured under T_H1 or T_H2 differentiating conditions, this effect was only observed in the T_H1 cells[58,59]. This is of particular interest in T_H2 mediated diseases such as atopic diseases. Indeed a recent study showed that the presence of GATA-3 inhibits the TGF-β induced expression of Foxp3[60]. Thus although TGF-β has a strong regulatory function, its regulatory function in atopic diseases is not clear.

Current treatment options

Treatment options for atopic diseases are still limited. These limitations count for every form of allergy, disrespectfully of the target organ. Avoidance of the allergen is obviously the first "treatment" option. This has been proven to be effective for individuals suffering from food-allergy (in which an encounter with the allergen can lead to symptoms such as diarrhea, vomiting or an exacerbation of eczema, but can also lead to anaphylaxis and thereby be life-threatening). It becomes more difficult in allergic asthma where the most common allergen leading to exacerbations is the house dust mite. Since this mite is extremely abundant in every house, allergic avoidance for individuals suffering from allergic asthma implicates sanitation of the whole house. This is very hard to achieve and has minimal effect[61].
Lastly, in diseases such as eczema the role of specific allergens is less clear and therefore, avoidance is generally not a treatment option.
Therapeutic intervention can also be directed at a local and/ or systemic downregulation of the immune system. In eczema, ointments with corticosteroids or tacrolimus are applied to active eczema lesions. These ointments are not used as maintenance therapy and have to be used with every new flare of eczema. In asthma, inhalants with corticosteroids are available for maintenance therapy. Whenever an exacerbation occurs, an inhalant with β-2 agonists, aimed at reversing the airway narrowing, can be used. In severe asthma, systemic therapy can be used, such as leukotriene receptor antagonists or oral corticosteroids. Thus above mentioned therapies demonstrate that treatment in atopic diseases is aimed at treating the symptoms, not curing the individuals suffering from them. Therefore, both treatment options do not change the essence in atopic diseases.

Immunotherapy in atopic diseases

For a small proportion of the atopic diseases, specific immunotherapy is available, although not yet widely used in the Netherlands. Specific allergen immunotherapy (SIT) consists of the subcutaneous or sublingual administration of the allergen, either naturally derived or artificially processed. SIT with naturally derived proteins has been practiced for over a century and has proven efficacy in the treatment of allergic rhinitis and forms of asthma[62-64]. The effect of SIT is most pronounced in allergies in which the exposure to allergens is rare, e.g. insect venom, or seasonally, e.g. tree pollen. SIT exerts its effect by various mechanisms. For instance, the use of bee venom and grass pollen extract leads to the induction of both IL-10 and IFNγ[65,66]. Subcutaneous injection with the house dust mite allergen Der p1, leads to the induction of TGF-β and a subsequent Ig isotype switch to IgA[67]. Thus, SIT involves reversing the T_H2 response by either inducing a T_H1 response or a regulatory response with direct effects of IL-10 and TGF-β. Furthermore SIT leads to the induction of IgG1 and IgG4.The antibody IgG4 is capable of inhibiting the binding of allergen-IgE complexes to the Fc-receptor on B-cells[66].
Unfortunately difficulties are met in SIT for treating allergic diseases. First of all, the disease-causing antigen has to be known. Second, when using the natural protein cross-linking can still occur due to impurity of the allergen and difficulties in dosing. This cross-linking causes side effects such as skin eruptions, pruritis or asthma. With the use of recombinant grass pollen allergens (artificial proteins) these factors were of a lesser extent[68].

Peptide-based immunotherapy

Although the side effects caused by the allergenic activity of allergens used in SIT are reduced by the use of recombinant allergens, the cross-linking with allergenic specific IgE still causes important side effects. Peptide fragments which correspond to T-cell epitopes may have a reduced ability to crosslink allergen-specific IgE and therefore inflict less IgE-mediated side effects. The first clinical study used a combination of 2 peptides from Fel d 1 (a cat-allergen) with a length 27 aminoacids[69].

Although clinical improvement was seen both in pulmonary function as well as subjective ability to tolerate cats, treatment was associated with late adverse events such as asthmatic reactions. Since an average T-cell epitope is about 14 amino-acids long, the length of the peptides used in this study may still have been too long, thereby capable to elicit and interact with IgE. Late phase responses indicate that T cells are involved in these adverse events as well. More importantly late adverse events seem to be caused by the activation of allergen-specific effector T cells[70].
Since the length of the peptides seems to play a role in eliciting adverse events, studies have been conducted with smaller peptides. In a study in which a combination of 12 smaller peptides was used, dose dependent late asthmatic reactions were also seen but this was associated with subsequent tolerance[71]. Another study demonstrated that this mixture of peptides was associated with an accumulation of both $CD25^+$ cells as well as $CD4^+IFN\gamma^+$ cells and with an increase in TGF-β mRNA in the allergen challenged skin[72]. A pivotal study was performed by Verhoef et al. They administered short synthetic peptides (16-17 aminoacids) derived from the most important cat allergen Fel d 1 to adult patients with a cat-allergy and asthma. PBMC obtained after the treatment showed decreased proliferation and an altered cytokine profile upon stimulation with the whole cat-allergen. Furthermore, $CD4^+$ T cells isolated after peptide-based immunotherapy had suppressive capacity *in vitro*[73]. This study gives evidence that peptide-based immunotherapy is capable of inducing $CD4^+$ T cells with suppressive or regulatory capacities and thus possibly capable of modulating allergen-specific T cell function.

An alternative approach for peptide-based immunotherapy

Bystander activation, a concept described in auto-immunity may hamper the effect of peptide-based immunotherapy. By this mechanism the initiating immune response spreads towards other antigens and expands its response towards these antigens[74]. Therefore, for the development of peptide-based immunotherapy an antigen with the capacity for modulating not only the response towards the initial antigen, but also responses to bystander epitopes (bystander suppression) should be used[75].
Taking the pathophysiology of atopy into account, the appropriate antigen needs certain qualities. First the candidate antigen should encompass activation and/or regulation of inflammatory processes induced by both the innate as well as the adaptive immune system. Furthermore, if searching for a candidate antigen which can also induce bystander activation and/or suppression, the bystander epitope needs to be expressed at the site of inflammation and, obviously, be recognized by immunecompetent cells. Proteins known to have these qualities are heat shock proteins (hsp).

Heat shock protein: bystander in allergic inflammation?
Hsp are expressed in eukaryotic and prokaryotic cellular organisms both constitutively and during various events of cellular stress, such as increased temperature (fever) or exposure to pro-inflammatory mediators. The hsp-families are evolutionary highly conserved as mammalian families of the hsp share a high degree of homology with the microbial hsp, which can result in immunological cross-recognition between these homologues[76,77]. At the site of stress, they act as a "danger" signal, capable of activating both cells of the innate as well as the adaptive system[78]. Thus, hsp are immunodominant antigens and a common target of T-cell recognition in inflammatory responses.
In Juvenile Idiopathic Arthritis (JIA), one of the most common childhood inflammatory diseases, self-hsp60-reactive T cells display an immunoregulatory phenotype, expressing CD30 and producing interleukin 4 (IL-4), interleukin 10 (IL-10) and transforming growth factor (TGF-β)[79].
These observations suggested that hsp can activate immunoregulatory pathways in humans and thus could be candidates for antigen specific immune therapy. Indeed, in several human studies immune reactivity to hsp is associated with downregulation of inflammation, possibly by the induction of regulatory T cells (T_{regs}) leading to first immune therapeutic trials with hsp-derived peptides in DM and RA[80-83].
The role of hsp in atopic diseases has not been clarified yet. In asthma, the expression of hsp70 is increased in both bronchial epithelial cells and alveolar macrophages in comparison to healthy controls or controls with other forms of chronic bronchitis[84,85]. Furthermore, microbial hsp65 suppressed IL-4 and IL-5 secretion and enhanced IL-10 and interferon-gamma (IFNγ) secretion in bronchoalveolar fluid in a murine model of airway hyperresponsiveness[86].
Taken together, hsp could be an appropriate antigen to obtain counter-regulatory immune responses in peptide based immunotherapy in atopy.

Aim of the study

With the increasing incidence of allergic diseases, the economic burden, as well as the impact on the quality of life of affected children, is enormous. Although knowledge about the pathophysiology of allergy is increasing, treatment is nowadays still focused at treating the symptoms, without the possibility of prevention nor curing the disease. In this thesis therefore it was our goal to shed light on crucial mechanisms and risk factors for atopic diseases in children. More specifically, we aimed to:

1. develop tools to help early recognize children at risk for developing atopy
2. study the mechanisms that may determine the chronicity and outcome of children with atopic disease. This is done from two different angles: gene expression profiling of $CD4^+$ T cells and the induction and role of regulatory T cells mechanisms in atopic disease.

Outline of thesis

The design of a tailor-made treatment starts with the recognition of individuals at risk for developing the disease. In atopic diseases, by assessing this risk, either allergen avoidance or immunosuppressive treatment, can be started. By an early start of treatment, the alterations in the end-organ, causing additional morbity in atopic diseases as seen in asthma and eczema, might be diminished. In the first part of this thesis the aim is to recognize children at risk for developing asthma

In the second part of this thesis the aim is to demonstrate whether it is possible to induce regulatory T cells. This is demonstrated by modulation of cord blood mononuclear cells, thought to be the most naïve cells. Furthermore, the naivety of CBMCs is tested to see whether a regulatory feedback mechanism, known as the immunological homunculus, is indeed apparent as early as *in utero* or whether this mechanism is formed later in life.[87] These studies might give more insight in the immunological basis of atopic diseases.

As stated in the introduction, peptide-based immunotherapy might be about modulating the 'right' disease inducing cells and thereby controlling the inflammation specifically without suppressing the whole system but with control of also the bystander effect. Therefore, the third part of this study encompasses the search for the appropriate antigen which is capable of restoring the balance between regulation of immune processes and the inflammation as seen in atopy.

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Early differentiation of infant wheezing phenotypes using differential gene expression in peripheral $CD4^+$ T cells

A technical pilot study

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Abstract

Objective: Childhood wheezing phenotypes are characterised into different phenotypes. Still an early diagnosis of asthma before the age of three is difficult. We hypothesize that gene-expression profiles can differentiate between heterogeneous wheezing phenotypes. This might lead to an early prediction of those children at risk to develop asthma.

Methods: To compare the gene expression pattern in $CD4^+$ T cells from infants with different wheezing phenotypes, we performed microarray analysis on mRNA extracted from *in vitro* non-stimulated peripheral $CD4^+$ T cells from wheezing infants and compared differentially expressed genes. From 5 ml peripheral blood, approximately $20x10^6$ $CD4^+$ T cells were obtained for RNA isolation.

Results: Preliminary results, comparing a persistent wheezer with an early wheezer, indicate the increased expression of 33 genes, including HLA-DPB1 and CCL5 (RANTES) and the decreased expression of 28 genes, including CSF1(colony stimulating factor 1-macrophage) and TNFAIP3 (tumour necrosis factor, alpha-induced protein 3) in the persistent wheezer.

Conclusion: These results indicate that microarray analysis of non-stimulated purified $CD4^+$ T cells from minimally 5 ml blood demonstrate differentially expressed genes. Although several of the differentially expressed genes are associated which inflammation, functional relevance of these genes needs to be further examined.

Introduction

The prevalence of asthma has increased dramatically over the past two decades[1]. In the majority of cases, asthma-like symptoms begin during the first years of life[2]. Of all newborns in the western world, 33% will experience one or more episodes of wheezing, associated with viral respiratory illnesses. Of these infants, 60% do not continue to wheeze between the age of three and six and are therefore called 'Transient Wheezers' (TW). The children who continue to wheeze between the age of three and six, are called 'Persistent Wheezers' (PW) and are regarded as asthmatics[3,4]. Although different predictive factors have been described to identify children who are at risk of developing persistent wheezing, it is still difficult to diagnose asthma before the age of three[5,6]. The importance of an early diagnosis of asthma is suggested by the hypothesis that an early intervention in asthmatic infants by starting inhaled corticosteroids may lead to an increase in lung function, parallel to findings in asthmatic adults. However, inhaled corticosteroids can have long-term side effects, especially when used in a period of rapid lung growth, so treatment in infants needs to be justified[7]. Early diagnosis may be done by differential gene expression patterns in inflammatory cells. In asthma, $CD4^+$T cells orchestrate the pathophysiology, both locally and systemically and it has been shown that circulating lymphocytes can reflect disease specific changes in an organ[8,9]. Therefore, $CD4^+$T cells may be used as a model for characterisation and monitoring of asthma. The microarray technology offers the opportunity to identify disease-specific gene expression patterns and new asthma genes by comparing thousands of messenger RNAs from different tissue or cell samples[10-12].

We hypothesise that heterogeneous wheezing phenotypes are the result of different gene expression profiles, which can be used to diagnose asthma at an early age. Our objective is to compare the 'in vivo' gene expression pattern in circulating non-stimulated $CD4^+$ T cells from wheezing infants.

Materials and Methods

Patients

For the pilot study TW and PW were recruited from another study. Because in this study they were included being EW their history of wheezing was well documented. For a larger, prospective study, EW were recruited by advertisement, asking for infants between the age of 0 and 3, with a paediatrician diagnosis of wheezing. Parents were asked to fill in a standardised questionnaire (based on ISAAC[13]) and

physical examination of the children was performed at the moment of venous blood sampling, to rule out present wheezing. Blood samples were only obtained when the child was not wheezing and had not done so for the past 6 weeks, and had no other signs of infection. Written informed consent was obtained form the parents or legal guardians of all children. The study was approved by the Medical Ethics committee of the University of Utrecht.

CD4⁺ T cells preparation and mRNA hybridisation

Minimally 5 ml venous blood of the patients was collected. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-paque™ (Amersham Biosciences, Uppsala, Sweden) density centrifugation. First PBMCs were depleted of $CD14^+$ cells using antigen-conjugated magnetic CD14 beads (MACS, MiltenyiBiotec, Bergisch-Gladbach, Germany) and then positively selected for $CD4^+$ T cells using antigen-magnetic coated CD4 beads. RNA is isolated from the cells by Trizol® (Invitrogen, Carlsbad, Calofornia, USA) and treated with DNase.
The quality of the RNA was assessed on the basis of demonstration of distinct 23s and 18s ribosomal RNA bands electrophoresed on an agarose gel and spectrophotometric ratio of absorbance at 260 nm to that at 280 nm > 1.8. A total amount 1 µg RNA was reverse transcribed into cDNA and then in vitro transcribed into mRNA by Amino allyl messageAmp™ (Applied Biosystems/Ambion®, Austin, USA), which gave minimally a 10-fold amplification. The mRNA was labelled with either Cy3 or Cy5 (Amersham Biosciences) and hybridised on a micro-array slide containing 25.000 oligonucleotides, fabricated in our in-house genomics centre. Additionally, a dye swap array was performed with each sample.

Analysis

The fluorescence signals on the scanned slides are quantified by Imagene (Biodiscovery, El Segundo, California, USA) and normalised by QQCC (Genomics facility, University Medical Center Utrecht). Data are analysed by Genespring (Agilent technologies, Santa Clara, California, USA). Genes of interest must have a fold increase of 1.5 and be present in at least 75% of all patients.

Results

Non-stimulated $CD4^+$T cells show differential gene expression.

To test whether non-stimulated (that is, *in vitro*) $CD4^+$ T cells of 5 ml of blood would give enough RNA to perform a microarray and consequently show differentially expressed genes, we first performed an experiment in which we did microarray analysis of a PW versus an EW. A total amount of $20x10^6$ $CD4^+$T cells from respectively 10 and 5ml of peripheral blood were obtained, which gave 16.5 and 15 µg total RNA. One microgram of RNA was used for amplification, which gave 7440 and 1666 µg mRNA. An increased expression of 28 genes, i.e. HLA-DPB1 and CCL5 (RANTES) and a decreased expression of 28 genes, i.e. CSF1 and TNFAIP3 was demonstrated. This experiment showed that a microarray could be performed with RNA from $CD4^+$ T cells in 5 ml of blood. Furthermore it showed that *in vitro* non-stimulated $CD4^+$ T cells show differentially expressed genes.

Heterogeneous wheezing phenotypes are reflected in gene expression profiles.

In gene expression profiling age differences can cause different expression patterns Therefore, in a new experiment, wheezing phenotypes which were age-matched were compared. To overcome inter-individual differences a reference pool was used which included 4 samples of TW and 8 samples of PW. Subsequently, a total of 4 TW and 10 PW were analysed versus the reference pool. With the threshold set at differentially expressed genes in 75% of all patients and a 1.5 fold increase, no differences in differentially expressed genes between the TW and the PW were seen. When the threshold was lowered to 50%, 63 differentially expressed were seen in the TW but only 3 in the PW. Amongst the genes with a decreased expression in the TW were HLADP1 and CCL5 (RANTES). Genes with an increased expression were i.e. hspA8 (gene encoding for Heat shock protein 70) and TNFSF1 (Tumour necrosis factor, super family 1). In the PW only three decreased genes were seen, amongst which was also SCYA3 (soluble cytokine A3). Thus in an age-matched pool differentially expressed genes can be demonstrated between different wheezing phenotypes.

Conclusion

In this experiment, the main finding was that *in vitro* non-stimulated CD4⁺T cells derived from 5 ml of blood from patients with different wheezing phenotypes is sufficient to show differentially expressed genes in microarray analysis. Furthermore, several genes, known to be associated with inflammation, were differentially expressed. At this moment, we can only speculate about their functional relevance in the different wheezing phenotypes.

Several limitations in the study may have caused the low number of differentially expressed genes found in the second experiment. First, by using a reference pool of patient samples of non-stimulated CD4⁺T cells, it is not likely to find a high number of differentially expressed genes with a high fold increase or decrease. Second, the cells of interest are CD4⁺T cells. It can be speculated that subpopulations of CD4⁺T cells may show differentially expressed genes. Finally, wheezing phenotypes may need to be defined more precisely. As such, it has been suggested that PW can be divided into sub-populations, which may be characterised by differential expression of genes.

This study demonstrates that non-stimulated CD4⁺ T cells from children with different wheezing phenotypes can be used for gene expression analysis. However the choice for the microarray platform used and the experiment set-up highly influence the outcome of the study. A new retrospective study and a prospective study are now being set up to perform microarray analysis to search for differentiating genes between early wheezing phenotypes.

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Gene expression in CD4$^+$ T cells reflects heterogeneity in infant wheezing phenotypes

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Abbreviations

LRI: Lower Respiratory tract Infection
BHR: Bronchial Hyperresponsiveness
EW: Early Wheezer
TW: Transient Wheezer
PW: Persistent Wheezer
T_H1: T-helper 1 cell
T_H2: T-helper 2 cell
RSV: Respiratory Syncitial Virus
IFNγ: Interferon gamma
IFNα: Interferon alpha
PBMC: Peripheral Blood Mononuclear Cells
HC: Healthy controls
PLIER: Probe Logarithmic Error Intensity Estimate
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

Abstract

Objective: Although a marked increase in reporting of wheezing symptoms in the last few decades has been described, the underlying immunopathology of the different wheezing phenotypes is not clarified. Because differences in gene expression might be involved, our objective was to identify gene expression profiles in $CD4^+$ T cells from two distinct infant wheezing phenotypes.
Methods: We compared gene expression profiles of peripheral $CD4^+$ T cells by means of microarray of 6 transient wheezers, 6 persistent wheezers and 7 healthy controls. The genes differentially expressed were subsequently validated by RT-PCR.
Results: The differential gene expression profiles reflect common immunological pathways involved in apoptosis or proliferation of T cells. Furthermore, both wheezing phenotypes show a decreased expression of complement 5 receptor (C5R1), a gene involved in the regulation of bronchial responsiveness. Moreover, differences in gene expression profiles were found in genes involved in the immune response against respiratory syncitial virus (RSV), such as signal transducer of transcription 1 (STAT1) and an inflammatory mediator with an enhanced production in asthma (prostaglandin E_2 receptor (Ptger2)).
Conclusion: Our findings suggest that clinical symptoms of wheezing are reflected in common immunological pathways, whereas differences between wheezing phenotypes are, partially, reflected in distinct gene expression profiles.

Introduction

It has been reported that the prevalence of wheezing symptoms in preschool children has increased over the past four decades[1]. This increase has not only been described in atopic children, but also in non-atopic children that only wheeze during a lower respiratory tract infection (LRI).[2] In addition, various studies have shown an increase in bronchial hyperresponsiveness (BHR) and airway abnormalities without an associated change in prevalence of atopy[2-4]. In preschool children with wheezing symptoms, several distinct phenotypes have been described. The Tucson birth cohort study showed that of all newborns in the industrialized world, one third will experience one or more episodes of wheezing during the first three years of life, mostly associated with viral respiratory infections (early wheezers-EW). Of these infants, 60% will discontinue wheezing between the age of three and six and are therefore called transient early wheezers (TW). The children who continue to wheeze are called persistent wheezers (PW) and are regarded as asthmatics[5]. Up till now, the change in prevalence of wheezing symptoms has mostly been attributed to environmental factors. However, knowledge of the underlying immunopathological mechanisms is lacking[3,4,6].

An immature immune-system might explain the susceptibility of infants to develop LRI and subsequent wheezing[4]. In the immunopathology of asthma, the disease linked to "persistent" wheezing, an important role has been described for $CD4^+$ T cells. These $CD4^+$ T cells are active at the local inflammatory sites i.e. via the release of T_H2 cytokines[7]. Interestingly, infants have diminished cytokine responses of both the T helper 1 (T_H1) and T helper 2 (T_H2) lineage to nonspecific stimuli (e. g. viruses) compared to adults[8]. The most extensively investigated virus associated with infant wheezing in the first year of life is respiratory syncitial virus (RSV). This virus is the most common cause of acute airway obstruction and subsequent wheezing in infants[9,10]. During RSV infection, children produce low levels of protective antibodies against this virus[11]. Interestingly, the magnitude of IFNγ production during RSV infection seems to be indicative for disease severity and predictive for the subsequent development of persistence of wheezing[12,13]. Another interferon, IFNα also seems to play a role in determining the severity of a RSV infection, and subsequent wheezing, in infants[14].

Combining the immunological profile of an infant with genetic and environmental risk factors might give a good indication of which infant is at risk for developing persistent wheezing and might lead to new intervention strategies. Genetic studies have revealed several asthma susceptibility genes. For instance, ADAM33 has been demonstrated to show linkage to atopic asthma, although its precise function has not yet been revealed[15]. More immunology-related genes were also linked to asthma and atopy, such as RANTES (CCR5) and the gene encoding for complement 5 (C5)[16,17]. Although these studies are promising, the causative role of these genes in the pathophysiology of asthma and especially children wheezing still has to be identified. A promising technique to unravel pathophysiological pathways in complex disease is identifying gene expression profiles by microarrays, or genomics. For this technique, RNA is needed from disease specific tissue. Previous studies have shown that peripheral blood mononuclear cells (PBMC) reflect disease specific changes in an organ and can be used as a model for characterization and monitoring of asthma by microarrays[18-20]. Using unstimulated PBMCs resembles most closely the in-vivo activation state of these cells[21]. Furthermore, the use of microarrays enables one to investigate a disease or process objectively without a stringent hypothesis[18,22,23].

Our aim was to determine the genetic profiles in children with either a transient or a persistent form of wheezing by means of microarray to determine differential expression of genes in various immunological pathways and distinguishing biomarkers in heterogeneous wheezing phenotypes.

Methods

Patients and control subjects

TW and PW were six-year old children who previously participated in another study, because of recurrent or chronic wheeze episodes at the age of 1 to 4 years[24]. Since they were included as EW, their history of wheezing was well documented and therefore, these children could be identified as TW or PW at the age of 6. Since that study represented more PW than TW, another group of TW was recruited from a secondary hospital, using the same characterization and inclusion criteria (n=2). At the time of blood collection, the mean age in years for the healthy controls (HC) (n= 7), the TW (n = 6) and the PW (n = 8) group subjects was 6.0 (SD ± 0.5), 5.7 (SD ± 0.5) and 6.0 (SD ± 0.5), respectively. The sex ratios (male: female) were 4:3 for the HC, 5:3 for the PW and 4:2 for the TW (Table 1). Parents were asked to fill in a standardized questionnaire, based on the ISAAC-questionnaire, to define the phenotype precisely[25]. TW were defined as not having any wheezing complaints after the age of three years and not in need of medication to obtain this clinical state. PW were defined as still having wheezing complaints after the age of three, for which the use of medication and follow-up by a pediatrician or pediatric pulmonologist was needed.

	Healthy Controls (*n*=7)	Persistent wheezers (*n*=8)	Transient wheezers (*n*=6)
Age at inclusion	6.0 (± 0.5)	6.0 (± 0.5)	5.7 (± 0.5)
Wheezing>3 years of age	0/7	8/8	0/6
Medication[1]			
Bronchodilators	0/7	7/8	1/6
Steroids	0/7	6/8	0/6
Atopy	0/7	3/8	1/6
Eczema	0/7	3/8	3/6
Neither	7/7	2/8	3/6
Family history atopy/asthma[2]	0/7	5/7[3]	4/6

Table 1.

Patient characteristics. [1]Use of medication at time of bloodsampling. [2] Family history in first degree relatives. [3] Missing data of 1 patient.

Our aim was to find true differences between TW and PW, which was not attributed to active inflammation. Since both TW and HC were not allowed to have any wheezing complaints, a physical examination was performed at the moment of venous blood sampling to rule out present wheezing. Blood samples were obtained only when the child was not wheezing and had not done so for the past six weeks regarding the PW (indicating proper disease control and no active inflammation), and 3 years regarding the TW. HC were six-year old children who underwent surgery. One HC underwent an orthopedic surgery, whereas the other HC underwent urological interventions. None of them had a history of wheezing, allergy or a recent infection, nor a first-degree family member with an allergy or asthma. Blood sampling took place at the operating room, within 10 minutes after induction of general anesthesia, in order to avoid any effect of anesthetics on parameters of inflammation[26,27]. The study has been approved by the Medical Ethics Committee of the University Medical Center, Utrecht, and the Isala Hospital, Zwolle. Written informed consent of the parents was received.

Study design

Gene expression profiles of unstimulated peripheral CD4$^+$ T cells from either persistent or transient wheezers were compared with those of healthy controls by means of microarray.

CD4$^+$ T cell isolation and RNA extraction

At least 5 ml of venous blood was collected. To diminish differences in gene expression attributable to sample handling, all samples were processed within two hours of collection and handled in exactly the same manner. In the healthy controls, samples were collected within 15 minutes of application of general anesthetics. CD4$^+$ T cells were isolated by Ficoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation and immunomagnetic separation as described previously[21]. CD4$^+$ T cells were ≥ 95% pure as assessed by FACS analysis.

Total RNA was isolated by using Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. The quality of the RNA was assessed on the basis of demonstration of distinct 23s and 18s ribosomal RNA bands electrophoresed on an agarose gel by Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, California, USA) according to the manufacturer's protocol and a spectrophotometric ratio of absorbance at 260 nm to that at 280 nm > 1.8.

Sample handling and microarrays

RNA was submitted to an Affymetrix GeneChip® Core facility (Affymetrix Application support, High Wycombe, UK). Before hybridization, RNA was purified using RNeasy columns (QIAGEN, Hilden, Germany). Consequently, a total of 100 ng of purified RNA per sample was used in the GeneChip® Two-cycle cDNA synthesis kit provided by Affymetrix. Subsequently, cDNA was applied on the HG-U133A GeneChip (Affymetrix, Santa Clara, California), according to Affymetrix guidelines (http://www.affymetrix.com/support/technical). In order to obtain individual expression levels, an individual chip was used per sample and no samples were pooled.

Microarray data analysis

The scanned output files of the Affymetrix data set were analyzed by using the Microarray Suite 5.0 software (Affymetrix). Second stage data-analysis was performed using the software from Array Assist (developed by Lobion, provided by Stratagene, La Jolla, CA, USA), using a PLIER (Probe Logarithmic Error Intensity Estimate) significance analysis, which produces an improved signal by accounting for observed patterns in probe behavior on a chip[28]. The fold-change rate was set at minimally 1.5, with a p-value of ≤ 0.05[29,30]. All the genes within these limits were considered interesting. Expressed sequence tags, hypothetical genes and genes with an unknown function were left out of the list.

RT-PCR analyses

For semi-quantitative RT-PCR (q-RT-PCR) analysis of the genes detected by microarray analysis, the same samples were used as in the microarray analysis. However, RNA samples of 3 HC, 1 TW and 1 PW were no longer available for RT-PCR analysis. Two additional samples of new PW were added, derived from the same local study. Unfortunately, no additional samples of TW nor HC were available. A total of 100 ng of total RNA from 7 PW, 5 TW and 4 HC was used for cDNA synthesis, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California) according to the manufacturer's protocol. Primers (Sigma-Genosys, The Woodlands, TX) were designed using Primer 3 software of the whitehead institute/ MIT center for genome Research[31]. RT-PCR analysis was performed using the MyiQ™ Single-color Real-Time PCR (Bio-Rad, California) as described previously. Data are expressed as expression level: lowest expression level in TW, PW or HC.

Statistical analysis

Basic descriptive statistics were used to describe the patient characteristics. For data analysis of the microarrays, a PLIER logarithm was used, supplied by Array Assist (Stratagene), with a p-value of ≤ 0.05.[28]

Results

Identification of genes differentially regulated in both wheezing phenotypes

Gene expression profiles of 6 PW and 6 TW were compared with the expression profiles of 7 HC. A total of 67 genes were found to be significantly differentially expressed in both wheezing groups by the PLIER method after deleting all genes with unknown function, hypothetical genes and expressed sequence tags. Increased expression of 45 genes and decreased expression of 22 genes was found in the wheezing phenotypes (Table 2 + Table 3).

Description	Symbol	Genbank	TW	PW
Stress response related genes				
MAPK/ERK kinase-ERK pathway				
dual specificity phosphatase 1	DUSP1	NM_004417	-2,1	-2
regulator of G-protein signalling 2, 24kDa	RGS2	NM_002923	-2,1	-2
dual specificity phosphatase 2	DUSP2	NM_004418	-1,5	-1,8
jun B proto-oncogene	JUNB	NM_002229	-1,5	-1,5
tumor necrosis factor (ligand) superfamily, member 13b	TNFSF13B	AF134715	-1,6	-1,6
complement component 5 receptor 1 (C5a ligand)	C5R1	NM_001736	-3,2	-2,4
SNF1-like kinase	SNF1LK	NM_030751	-2,4	-2,1
cAMP responsive element modulator	CREM	AI800640	-2	-1,9
Other				
ferritin, heavy polypeptide 1	FTH1	AA083483	-1,6	-1,5
Rho signaling pathway				
protein tyrosine phosphatase type IVA, member 1	PTP4A1	AL578310	-1,5	-1,5
nuclear receptor subfamily 4, group A, member 2	NR4A2	NM_006186	-3,1	-2,9
NF-kB pathway				
tumor necrosis factor (TNF)-induced protein 3	TNFAIP3	NM_00629	-1,5	-1,8
growth arrest and DNA-damage-inducible, alpha	GADD45A	NM_001924	-1,7	-1,7
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2	LILRB2	AF004231	-2	-2
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	NM_000389	-1,8	-1,6
Leukotriene pathway				
arachidonate 5-lipoxygenase	ALOX5	NM_000698	-1,5	-1,6
Interferon-related				
Interferon inducible	SLC7A7	NM_003982	-2	-1,7
Other proliferation/cell death				
transducer of ERBB2, 1	TOB1	BF240286	-2,1	-2,2
hexokinase 3	HK3	NM_002115	-2,2	-1,7
Other genes				
C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 12	CLECSF12	AF313468	-2,2	-1,8
mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	MGAT1	N40551	-1,5	-1,6
Unknown				
ring finger protein 157	RNF157	BC042501	-1,5	-1,5
TBC1 domain family, member 8 (with GRAM domain)	TBC1D8	NM_007063	-1,9	-1,5

Table 2.
Differentially expressed genes in heterogeneous wheezing phenotypes: genes down-regulated in comparison with HC. Genes depicted in bold were validated by q-RT-PCR.

Validation of genes by q-RT-PCR reveals two immunological pathways

For validation by q-RT-PCR, a total of 7 immune-related genes were randomly selected. With q-RT-PCR, the increased expression of hspA1A and the decreased expression of C5R1, JunB, TNFAIP3, DUSP2, LILRB2 and TNSF13B was confirmed. The group of genes that displayed decreased expression contained a subgroup of genes involved in apoptosis or proliferation of T cells.

Description	Symbol	Genbank	TW	PW
Stress response related genes				
heat shock 70kDa protein 8	HSPA8	AB034951	1,5	1,6
heat shock 90kDa protein 1, beta	HSPCB	AF275719	1,5	1,5
heat shock 90kDa protein 1, alpha	HSPCA	AI962933	1,7	1,7
heat shock 70kDa protein 1A	HSPA1A	NM_005345	1,5	1,5
heat shock 70kDa protein 4	HSPA4	AA043348	1,5	1,6
DnaJ (Hsp40) homolog, subfamily A, member 1	DNAJA1	NM_001539	1,5	1,7
DnaJ (Hsp40) homolog, subfamily C, member 3	DNAJC3	AL119957	1,5	1,5
MAPK/ERK kinase-ERK pathway				
tumor necrosis factor receptor superfamily, member 25	TNFRSF25	U94510	1,6	1,6
homeodomain interacting protein kinase 1	HIPK1	AI393355	1,9	1,9
mitogen-activated protein kinase kinase kinase kinase 5	MAP4K5	Z25426	1,4	1,6
nuclear factor of activated T-cells 5, tonicity-responsive	NFAT5	NM_006599	1,4	1,5
RAS-pathway				
Ras-GTPase-activating protein SH3-domain-binding protein	G3BP	NM_005754	1,5	1,5
son of sevenless homolog 1 (Drosophila)	SOS1	AW241962	1,7	1,7
RAB18, member RAS oncogene family	RAB18	AI769954	1,6	1,7
RAS p21 protein activator 2	RASA2	NM_006506	1,7	2
RAP2A, member of RAS oncogene family	RAP2A	AI302106	1,6	1,8
TGF beta signaling				
Janus kinase 1 (protein tyrosine kinase)	JAK1	AL555086	1,7	1,6
protein kinase, lysine deficient 1	PRKWNK1	AI445745	1,5	1,5
NF-kB pathway				
A kinase (PRKA) anchor protein 13	AKAP13	AI674926	1,7	1,8
butyrate-induced transcript 1	HSPC121	AJ271091	1,5	1,5
Other Proliferation/ cell death				
phosphoinositide 3-kinase/Akt pathway				
serine/threonine kinase 4	STK4	Z25430	1,7	1,8
kinase interacting with leukemia-associated gene (stathmin)	KIS	AW173222	1,7	1,9
pre-B-cell leukemia transcription factor interacting protein 1	PBXIP1	NM_020524	1,6	1,4
Other				
TP53 regulating kinase	TP53RK	BG339450	1,4	1,6
apolipoprotein L, 1	APOL1	AF323540	1,7	1,5
deoxyhypusine synthase	DHPS	NM_001930	1,5	1,5
nuclear receptor interacting protein 1(interacts with glucocorticoid receptor)	NRIP1	AI824012	1,9	2,4
transducer of ERBB2, 2	TOB2	D64109	1,5	1,7
ret finger protein 2, tumor suppressor gene	RFP2	BF939833	1,6	1,9
transducin (beta)-like 1X-linked	TBL1X	AW968555	1,6	1,8
Dicer1, Dcr-1 homolog (Drosophila)	DICER1	NM_030621	1,6	1,6
eukaryotic translation initiation factor 2C, 3	EIF2C3	NM_024852	1,5	1,5
Natural-killer cells				
natural-killer cell receptor;natural killer-tumor recognition sequence	NKTR	AI688640	1,7	1,7
NK cell receptor DNAM-1 (CD226)	CD226	NM_006566	1,4	1,5
integrin alpha L (antigen CD11A (p180)	ITGAL	BC008777	1,6	1,7
Protein degradation				
CCR4-NOT transcriptionfactor; proteasome; mRNA processing complex	CNOT7	NM_013354	1,6	1,6
Proteasome, component of cellular antioxidative system	PSMB5	BC004146	1,5	1,5
hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase	HADHA	BG472176	1,5	1,6
Cytokine related				
Regulator of IL-2 expression	ILF2	NM_004515	1,5	1,5
IL27 receptor, suppressive effect on T cells, especially autoreactive Th17 cells	IL27RA	NM_004843	1,6	1,8
cytokine inducible SH2-containing protein (suppressor of cytokine signalling)	CISH	D83532	1,5	1,5
Transport				
Nuclear protein transport factor	NUP50	AF267865	1,5	1,5
leucocyte transport/binding				
purine-rich element binding protein A	PURA	NM_005859	1,6	1,6
Unknown				
topoisomerase I binding, arginine/serine-rich	TOPORS	NM_005802	1,6	1,7
phenylalanine-tRNA synthetase-like, alpha subunit	FARSLA	AD000092	1,5	1,5

Table 3.

Differentially expressed genes in heterogeneous wheezing phenotypes: genes upregulated in comparison with HC. Genes depicted in bold were validated by q-RT-PCR.

Identification of a distinct gene expression profile in transient wheezers

In the TW group, 6 individual samples were compared with 7 HC and subsequently with 6 PW. In these individual samples of TW, the expression of 34 genes was at least 1.5 fold different from the HC. Of these 38 genes, 6 genes were found to be upregulated, whereas 28 genes were downregulated (Table 4). Among the 10 upregulated genes, 3 genes were immune-related. Furthermore, three different transcripts of one gene, glutathione S-transferase M1 (GSTM1), were consistently upregulated. Among the downregulated genes, 17 genes were immune-related, whereas the other genes were involved in either protein folding and transportation, or regulation of expression of RNA. Amongst the downregulated immune-related genes, two different transcripts of the gene encoding for Signal Transducer and Activator of Transcription 1 (STAT1) were consistently downregulated (Table 4).

Description	Symbol	Genbank	TW/HC	TW/PW
Stressresponse related genes				
p53/ Fas-ligand pathway				
annexin A1; mediator of anti-inflammatory effect glucocorticoids	**ANXA1**	NM_000700	1,5	1,5
protein tyrosine phosphatase	PTPN13	NM_006264	1,3	1,5
MAPK/ERK kinase-ERK pathway				
AHNAK nucleoprotein (desmoyokin)	AHNAK	BG287862	1,6	1,5
Other				
glutathione S-transferase M1	GSTM1	X08020	1,9	1,8
glutathione S-transferase M1	GSTM1	NM_000848	2,0	1,8
glutathione S-transferase M1	GSTM1	NM_000561	2,2	1,9
decapping enzyme hDcp2	DCP2	AI873425	-1,1	-1,5
Interferon related genes				
interferon-induced protein with tetratricopeptide repeats 2	IFIT2	AA131041	-2,0	-1,6
interferon-induced protein with tetratricopeptide repeats 3	IFIT3	AI075407	-5,5	-2,5
interferon-induced protein with tetratricopeptide repeats 3	IFIT3	NM_001549	-2,2	-1,4
interferon -inducible protein viperin	cig5	AW189843	-3,0	-2,0
Toll-like receptor 7, antiviral immunity	**TLR7**	NM_016562	-1,6	-1,5
interferon inducible transcription regulator	**STAT1**	M97935_MB	-1,5	-1,5
component of the IFN type III receptor	IL28RA	AW340139	-1,3	-1,5
Natural Killer cells				
natural killer cell receptor, IL-15 dependent ;inhibitory immune receptor	KLRB1	NM_002258	1,8	2,0
killer cell lectin-like receptor subfamily F, member 1	KLRF1	NM_016523	-2,6	-1,7
phosphoinositide 3-kinase/Akt pathway				
SLAM family member 7	SLAMF7	AL121985	-1,9	-1,9
insulin receptor	INSR	AA485908	-1,7	-1,7
NF-kB pathway				
B cell RAG associated protein	GALNAC4S-6ST	NM_014863	-2,2	-1,5
B-cell CLL/lymphoma 11A (zinc finger protein)	BCL11A	AI912275	-1,5	-1,5
B-cell CLL/lymphoma 11A (zinc finger protein)	BCL11A	NM_022893	-1,6	-1,5
human immunodeficiency virus type I enhancer binding protein 3	HIVEP3	NM_024503	-1,6	-1,6
human immunodeficiency virus type I enhancer binding protein 3	HIVEP3	AB046775	-1,5	0,9
RAS-pathway				
RAB23, member RAS oncogene family	RAB23	AF161486	-1,3	-1,6
Other proliferation/cell death				
ankyrin repeat domain 28	ANKRD28	N32051	1,6	1,6
SH3 multiple domains 4	SH3MD4	AL566989	-1,2	-1,5
Other				
immunoglobulin heavy constant mu	IGHM	X17115	1,4	1,5
tissue integrity/ cytoskelet	EPPK1	AL137725	-1,5	-1,5
CD63 activation marker/ pulmonary type II cells	LAMP3	NM_014398	-2,9	-1,7
transcription activator	ZNF6	AU157017	-1,3	-1,5
Cell membrane transporter (extracellular matrix of epithelial cells)	SLC16A10	N30257	-1,1	-1,6
Zinc finger protein 285	ZNF285	AW513227	-1,3	-1,5
GTP binding protein 5 (putative)	GTPBP5	AI860690	-1,9	-1,7
mRNA expression:polymerase (RNA) II (DNA directed) polypeptide D	POLR2D	BF432147	-1,3	-1,5

Table 4.
Differentially regulated genes in 6 individual samples of TW. Genes show a 1.5-fold in- or decrease compared to either the HC, or to the PW, or both. Genes depicted in bold are validated by q-RT-PCR

Identification of genes differentially regulated in persistent wheezers
In the PW group, also 6 individual samples were compared with 7 HC and subsequently with the 6 abovementioned samples of TW. In these individual samples, 19 genes were at least 1.5 fold different from the HC. In these samples, 13 genes were found to be upregulated, whereas 6 genes were downregulated. Among the upregulated genes, 6 genes were found to be immune-related, whereas the other genes were involved in protein folding and transportation. Among the downregulated genes, three genes were found to be immune related. One of these genes was the gene encoding for the prostaglandin E receptor 2 (PTGER2) (Table 5).

Description	Symbol	Genbank	HC	TW
Stressresponse related genes				
Rho signaling pathway				
Rho guanine nucleotide exchange factor (GEF) 17	ARHGEF17	NM_014786	1,1	1,5
MAPK/ERK kinase-ERK pathway				
scavenger receptor class B, member 1	SCARB1	AV708130	1,3	1,5
Other				
trophoblast-derived noncoding RNA	TncRNA	AI042152	-1,4	-1,8
prostaglandin E receptor 2 (subtype EP2)	**PTGER2**	NM_000956	-1,5	-1,6
RAR-related orphan receptor C	RORC	AI218580	-1,3	-1,5
TGF beta signaling				
SKI-like	SKIL	AW294869	1,6	1,5
dachshund homolog 1 (Drosophila)	DACH1	AI650353	1,4	1,4
Chemokine related				
G protein-coupled receptor 18	**GPR18**	AF261135	1,9	1,5
G protein-coupled receptor 114	**GPR114**	BF057784	1,1	1,5
Natural-killer cells				
granzyme H (cathepsin G-like 2, protein h-CCPX)	**GZMH**	M36118	3	4,6
v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	YES1	NM_005433	1,6	1,6
Other Proliferation/ cell death				
plastin 3 (T isoform)	PLS3	NM_005032	1,3	1,5
LIM and senescent cell antigen-like domains 3	LIMS3	AF288404	-1,9	-1,5
cytochrome b-561	CYB561	AL514271	-1,3	-1,5
Other				
Acetyl co-enzym A/remodeling/endolasmatisch reticulum	ALCAT1	AV717041	1,7	1,7
peroxisomal biogenesis factor 12	PEX12	NM_000286	1,4	1,5
chromosome 21 open reading frame 107	C21orf107	AJ002572	1,5	1,5
SH3 domain containing, Ysc84-like 1 (S. cerevisiae)	SH3YL1	NM_015677	1,3	1,5
Unknown	NY-REN-7	AW514267	-1,5	-1,7

Table 5.
Differentially regulated genes in 6 individual samples of PW. Genes show a 1.5-fold in- or decrease compared to either the HC, or to the TW, or both. Genes depicted in bold are validated by q-RT-PCR

Validation by q-RT-PCR
A total of 6 immune-related genes were validated: 3 genes differentially regulated in the TW, and 3 genes differentially regulated in the PW. These genes are ANXA1, STAT1 and TLR7 in the TW, and GPR18, GZH and PTGER2 in the PW. Q-RT-PCR analysis confirmed the differential expression of these genes.

Discussion

This study is the first to apply the microarray technology to $CD4^+$ T cells of wheezing infants to investigate whether gene expression profiles account for the heterogeneity in wheezing phenotypes. Furthermore, we investigated whether these gene expression profiles might attribute to the understanding of the immunopathology of wheezing. Our data establish that wheezing phenotypes share common gene expression profiles, but also show that the heterogeneity in wheezing phenotypes is, in part, reflected in the gene expression profiles in peripheral blood samples.

When comparing common gene expression profiles of both types of wheezing infants, microarray analysis revealed differential gene expression of several stress-response related genes. For instance, a decreased expression was found for C5R1 of the MAPK/ERK-pathway. Although C5 is a mediator of allergic reactions, an inverse association was seen between C5 and bronchial hyperresponsiveness. Interestingly, blocking the C5R1 inhibits the production of IL-12 by macrophages and monocytes. This cytokine drives type I adaptive immune responses[16,32]. Since it has become clear that part of the increase in symptoms of wheezing is due not merely to atopy, but also due to an increase in bronchial hyperresponsiveness, C5R1 might play a role in this phenomenon[3]. An increased expression was found for hspA1A, a polymorphism of Heat shock protein 70 (hsp70). In adult asthmatics, the expression of hsp70 is increased in BAL samples in both epithelial cells and PBMCs, suggesting a role for hsp70 in asthma[33]. Heat shock proteins are stress proteins, of which the expression is increased at sites of inflammation. Interestingly, the participants of our study were only included when no symptoms of wheezing had been reported for at least 6 weeks (this accounts for the PW) nor other episodes of fever had been reported. This may indicate that a parameter of inflammation still shows an increased expression, even without recent symptoms of wheezing.
Decreased expression levels were also found for JunB, TNFAIP3, DUSP2, LILRB2 and TNSF13B, all of which are involved in stress responses, either via the MAPK/ERK-pathway or the NF-kB-pathway. Of these genes, only JunB has been associated

with asthma before, and has also been described as a transcription factor of T_H2 cells[34,35]. This would implicate that a decreased expression of JunB as found in our analysis would be protective against allergen-induced airway inflammation. However, apart from the fact that this was a murine study, inclusion of our participants was purely based on a history of wheezing, and not on atopic asthma. Interestingly, decreased expression of TNFAIP3 and DUSP2 have also been described in a microarray study of $CD4^+$ T cells in atopic dermatitis (AD)[21]. Taken together, the results of the present study suggest that $CD4^+$ T cells of infants with a reported history of wheezing, but without active disease, show prolonged activated state compared to controls.

In the transient wheezing infants, a decreased expression of several interferon related genes was found. Amongst them was STAT1. STAT1 is known to be the major intracellular response protein for both IFN-γ as well as IFN-α/β. In a murine study by Hashimoto et al. it was shown that the absence of STAT1 resulted in airway dysfunction and an increase in airway mucus production after infection with RSV[36]. During the acute phase of a RSV infection, a suppressed production of IFN-γ from stimulated PBMCs was shown[37]. Subsequently, it was shown that reduced levels of IFN-γ in early life are associated with an increased risk of developing wheezing by 1 year of age[12]. Also a decreased expression was found for Toll-like receptor 7 (TLR7). TLR7 is activated by single stranded RNA-viruses, such as RSV[38,39]. Recently it was shown that the use of a TLR7 ligand could redirect allergen-specific T_H2 responses as well as allergen-induced hyperresponsiveness[40,41]. These findings might give a partial explanation to the subsequent wheezing after viral infections in transient wheezers.
In persistent wheezers a differential expression of stress response related genes was also found. Amongst others, a decreased expression was found of the Prostaglandin E_2 receptor (Ptger2). The ligand for this receptor, prostaglandin E2 (PGE_2) is able to modulate cytokine production of $CD4^+$ T cells towards a T_H2 response[42,43]. Furthermore, PGE_2 induces an increase in IgE production, and is therefore believed to be a mediator in the development of asthma[44]. A protective role for PGE_2 against bronchoconstriction has also been described. This would be an effect of PGE_2 on G-coupled protein receptors, present on airway smooth muscle cells and could explain why a decreased expression of the Ptger2 receptor would give more bronchoconstriction[45]. However, whether the expression of the Ptger2 receptor on $CD4^+$ T cells is of influence on the effect of PGE_2 on smooth muscle cells in the lungs is not known.

One of the shortcomings of this study may be the fact that the $CD4^+$ T cells used in this study are not the only cells playing a role in the pathogenesis of wheezing. Wheezing, and subsequently asthma, are systemic diseases involving several different cell types and tissues. Our objective was to seek in a cell system which is easily obtainable and applicable for diagnostic purposes in the future. Using, for instance, epithelial cells might give different results[22]. Since the gene expression in CD4+ T cells can reflect several immune responses, patients were only included when not having active disease of any type, nor in the previous six weeks before blood sampling. Furthermore, our study did not reveal the increased expression of typical T_H2 genes in the $CD4^+$ T cells. This may support the hypothesis that even persistent wheezing is not always an indication of atopic asthma, but can also be the result of increased lower airway inflammation[4].This is further supported by findings in adult patients with atopic asthma, where genomic linkage analysis of a large population of patients did not reveal any T_H2 involvement[15]. In addition, lack of T_H2 involvement has been described by gene array analysis of $CD4^+$ T cells in atopic dermatitis, a disease with very high serum IgE levels[21].
We have only examined relative small patient numbers. As mentioned previously, the introduction of new techniques such as mRNA amplification in microarray studies has diminished the quantities of RNA needed for microarray analysis. However, the use of peripheral blood of children still makes it difficult to obtain enough RNA to perform both microarray analysis and q-RT-PCR validation. Our patient numbers were further diminished by the stringent inclusion criteria used. This was needed to obtain homogeneous phenotypes. Since we did find significant differences in expression profiles, we are confident that the amounts used were sufficient and differences in phenotyping were not the case. Also because of small sample sizes, we had to make a selection of genes to validate. We chose to validate some immune-related genes. However, we postulate that interesting genes are also among the non-immune-related genes, such as GSTM1[46,47]. Due to these small sample sizes, several possibly interesting subgroup analyses could not be done, such as the influence of atopy in wheezing or the use of inhaled corticosteroids.
Importantly, we did examine differential gene expression levels in clinical resting states. This may implicate that the gene profiles that we demonstrate are a reflection of the intrinsic different phenotype of the $CD4^+$ T cells in the patient groups examined.

In conclusion, the data presented in this study give more insight into the genetic factors in $CD4^+$ T cells contributing to the heterogeneity in wheezing phenotypes and in the immunopathology underlying infant wheezing. Further research to evaluate the predictive quality of these gene expression profiles is currently being performed in a large group of children included prospectively.

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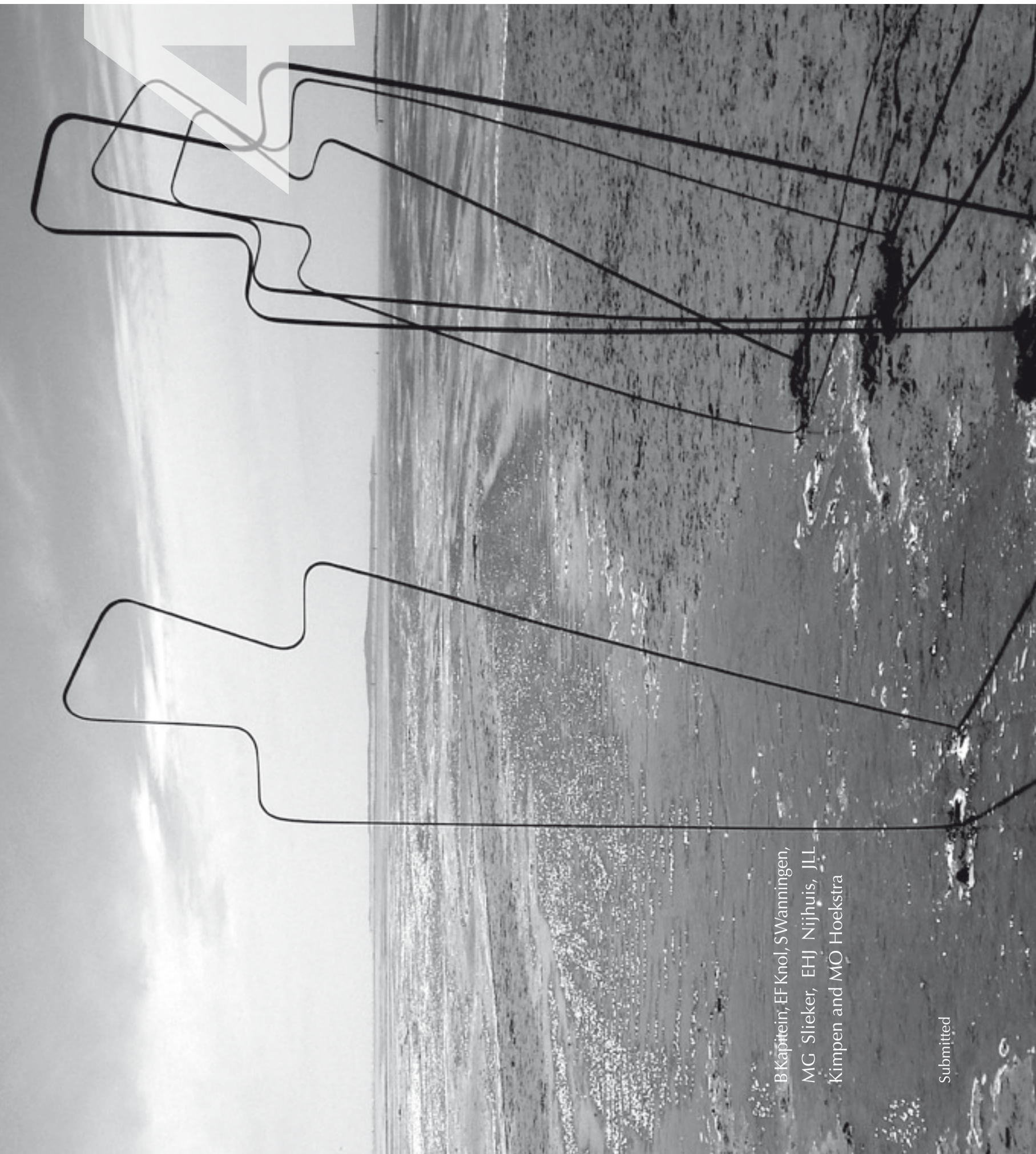
A diagnostic gene-expression score for prediction of persistent wheezing in young children



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Submitted

Abbreviations

LRI: Lower Respiratory tract Infection
BHR: Bronchial Hyperresponsiveness
EW: Early Wheezer
TW: Transient Wheezer
PW: Persistent Wheezer
ICS: inhalation corticosteroids
PPV: positive predictive value
NPV: negative predictive value
RSV: Respiratory Syncitial Virus
CAGE: composite atopy gene expression score
ROC: receiver operator curve
AUC: area under the curve
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
LCI: loose clinical index
SCI: stringent clinical index
GESI: gene expression score index

Abstract

Objective: In asthma, a decrease in lung function can already be detected at the preschool age. Identifying those children at risk of developing asthma could warrant early preventive intervention. In order to predict which infants will develop asthma a clinical score is now being used with a positive predictive value of 59 to 77%. Our aim was to form a composite score of gene expression and the existing clinical indices.

Methods: Quantitative PCR analysis of mRNA of peripheral blood $CD4^+$ T cells of 40 early wheezers (EW) was performed. Gene expression of several genes found in a retrospective cohort was tested. A follow-up of three years was chosen to define whether the EW became a Transient (TW) or Persistent wheezer (PW). This clinical outcome was compared with gene expression profiles and clinical indices.

Results: In 39 children, independent predictors of persistent wheezing were the increased expression of two genes, Toll-like receptor 7 (TLR7), Prostaglandin E2 receptor (Ptger2) and the decreased expression of signal transducer of transcription 1 (STAT1). In comparison with a clinical index alone (area under the curve (AUC): 0.622), the combination with the three genes with a clinical index gave an area under the curve of 0.84 with a positive predictive value of 100%.

Conclusion: A scoring rule based on gene expression of the individual predictors TLR7, Ptger2 and STAT 7 combined with a clinical index classifies EW in young children in a mild, moderate or definitive risk on persistency of wheezing.

Introduction

In the industrialized world an increasing prevalence of atopic diseases such as asthma is seen[1]. A longitudinal study by Martinez et al. showed that up to 33% of all newborns experience one or more wheezing episodes during the first 3 years of life[2]. These children are regarded as early wheezers (EW). Of these children only 40% will continue to wheeze after the age of 3 years. These children are regarded as persistent wheezers (PW) and are more likely to develop asthma[3]. In this group, reduced lung function levels are already seen at the age of 6. Interestingly, these levels of lung function were lower compared to children who begin to wheeze after the age of three (Late-onset wheezers)[2]. Thus, an early onset of asthma is associated with increased functional deterioration of the lungs. Therefore, an early prediction of asthma and consequently an early start with proper treatment may be beneficial in these children on the long term.

However, several studies carried out recently did not show a beneficial effect of early application of inhalation corticosteroids (ICS) in EW on the development of asthma. This may be due to the heterogeneity within the study population of EW[4]. Several studies have addressed the issue of early recognition of asthma by using clinical predictors, sometimes combined with immunological factors[5;6]. Castro-Rodriguez et al. used both a stringent and a loose index which differed with respect to the frequency of wheezing episodes (for further details see Methods section)[6]. They found a mean positive predictive value (PPV) of applying either the loose or the stringent index on the development of asthma between the age of 6 and 13 years of 59 and 77%, respectively. Clough et al combined several clinical indicators with an immunological factor (soluble IL-2 receptor), thereby predicting persistency with an accuracy of 91.8% (PPV between 60 and 76 %)[5]. However, since this test was performed within a selected group (at least one atopic parent and exclusion of RSV-positive children), these findings still have to be replicated in a larger group.

As the aforementioned data indicate, a consensus score has yet to be found to quantify the heterogeneous group of wheezing phenotypes and to predict which child is at risk for developing asthma. The PPV values as reported so far are not yet high enough for clinical use, especially if treatment such as ICS is applied which may cause side-effects.
Another method to study complex diseases, such as asthma, is to determine gene expression patterns by micro-arrays. This technique offers the possibility to observe gene-expression of thousands of genes in cells from a single sample i.e. blood.

Several studies have shown that distinct gene-expression profiles can be found in asthma phenotypes. For instance, Brutsche et al. devised a composite atopy gene expression score (CAGE) which differentiated between atopic and non-atopic asthmatic subjects[7]. Another study showed a clear distinction in gene expression profiles between acute and stable asthma in children[8]. These studies suggest that gene expression profiles can be used to differentiate between heterogeneous phenotypes. Previously, we demonstrated differences in gene-expression profiles in peripheral blood $CD4^+$ T cells between PW and TW in a retrospective design[9]. In the present study, our aim was to use these differences in a prospective cohort of EW to test whether we are able to predict the persistency of wheeze at an early age.

Methods

Patients
Eligible patients were recruited by advertisement in a Dutch magazine about parenting. Parents were asked to participate if their child was younger than three years and had experienced at least one wheezing episode before this age (EW). To exclude false-negatives, wheezing had to be observed at least once by a paediatrician. Exclusion criteria were premature birth and/ or mechanic ventilation in the postnatal period. A total of 80 children were included. At the first visit parents completed a questionnaire based on the ISAAC questionnaire, with questions on wheezing, atopy, parental asthma and atopy, siblings, parental smoking pre- and postnatally and ear-nose-throat infections[10]. During this visit, blood sampling took place. To diminish the risk of finding differences in gene expression profiles due to active disease, physical examination was performed at the moment of venous blood sampling to rule out active wheezing. Of 65 children blood sampling was successful. This high failure rate is mainly caused by the restriction to place one attempt to withdraw blood, placed by the Medical ethics Committee. These children were included for further analysis. Written informed consent of the parents was received. The study has been approved by the Medical Ethics Committee of the University Medical Centre, Utrecht.

Follow up on wheezing and atopy
Follow-up data were collected at year 1, 2 and 3 after the first visit by means of questionnaires based on the ISAAC questionnaires. Parents were asked whether the child had wheezed in the previous year and if so, how often. Furthermore, they were asked if this wheezing had also occurred apart from colds and if a physician

had diagnosed the child with asthma during the previous year.
In all the surveys, parents were asked whether the child had a runny or itchy nose apart from colds, whether a physician had diagnosed hay fever, whether the child had an itchy rash at the extensor surfaces or skin flexures lasting for 6 months and whether a physician had diagnosed eczema. If the child had undergone either atopy blood testing or skin prick testing in the previous year parents were asked for the results.

Outcome measures
Persistent wheezing (PW) was defined as persistence of wheezing complaints after the age of 3, regardless of a physician diagnosis of asthma or use of medication, consistent with previous definition[2].

Blood samples
At least 5 ml of venous blood was collected in sodium-citrate tubes. To diminish differences in gene expression attributable to sample handling, all samples were processed within two hours of collection and handled in the same manner[11].

$CD4^+$ T cell isolation and RNA extraction
$CD4^+$ T cells were isolated by Ficoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation and immunomagnetic separation as described previously[12]. $CD4^+$ T cells were ≥ 95% pure as assessed by FACS analysis.
Total RNA was isolated by using Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. The quality of the RNA was assessed on the basis of demonstration of distinct 23s and 18s ribosomal RNA bands electrophoresed on an agarose gel by Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, California, USA) according to the manufacturer's protocol and a spectrophotometric ratio of absorbance at 260 nm to that at 280 nm > 1.8.

RT-PCR analysis
For semi-quantitative RT-PCR (q-RT-PCR) analysis of the genes, a total of 100 ng of total RNA was used for cDNA synthesis, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California) according to the manufacturer's protocol. Primers (Sigma-Genosys, The Woodlands, TX) were designed using Primer 3 software of the whitehead institute/ MIT center for genome Research. Q-RT-PCR analysis was performed using the MyiQ™ Single-colour Real-Time PCR (Bio-Rad, California). Data are expressed as expression level:lowest expression level in EW and HC.
For the quantification of relative changes in gene expression the $2^{-\Delta\Delta C}{}_T$ method was used[13]. In short, the C_T value of the individual sample is compared with a control value. Both values are normalized to an appropriate endogenous housekeeping gene. In this study [delta][delta]C_t = [delta]$C_{t,sample}$ - [delta]$C_{t,reference}$ is used where [delta]$C_{T,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and [delta]$C_{t,\ reference}$ is the lowest C_t value also normalized to the endogenous housekeeping gene.

Clinical index
The prognostic capacity of the discriminatory genes were compared with the clinical indices as defined by Castro-Rodrígues et al[6]. Two sets of criteria were determined. Major criteria were parental medical doctor (MD) asthma and MD eczema and minor criteria were MD allergic rhinitis, wheezing apart from colds and eosinophilia (≥ 4%). A positive stringent index was obtained when a child was an early frequent wheezer during the first three years of life and fulfilled at least one of the two major criteria or two of three minor criteria. In the loose index the child did not have to be a frequent wheezer, but did have to meet the same criteria[6].

Statistical Methods
Basic descriptive statistics were used to describe the patient population. A non-parametric test (Mann Whitney *U* test, 2-sided) was applied to determine significant differences between both patient groups.
To determine which genes were discriminative between the two groups, frequencies of up- en down-regulated expression was calculated for all genes. The three genes which seemed to be discriminative in the retrospective study were also discriminative in this study and were therefore used for multivariate analysis. A multivariate regression model was formed to evaluate the value of these genes in predicting persistency of wheeze when compared to the loose clinical index. Prior to this analysis a single imputation technique was used for missing gene expression values. The prognostic capacity to discriminate between TW and PW was estimated using the area under the curve (AUC) in a receiver operating characteristics curve (ROC-curve). The difference in discriminatory power between a loose clinical index alone and a loose clinical index combined with the gene expression of the three genes was determined by the difference in AUC (Δ AUC) with 95% confidence intervals (95% CI), taking into account the correlation between the gene expressions as they were based on the same cases[14;15]. The final model was then transformed into a scoring rule by dividing the regression coefficients of the included four predictors by the smallest one and rounding them subsequently to the nearest integer[16]. For each individual patient, a total risk score was computed by assigning

points for each predictor present. Predictive values for each category of the scores were calculated. A Statistical Package for Social Sciences (SSPS Statistical Program, version 15.0.; SPSS inc, Chicago, Ill.) was used.

Results

Patient data

From 38 EW (average age 2.5 years) $CD4^+$ T cells and RNA of good quality were isolated to measure gene expression levels. At the age of 6 years, these EW were defined as PW or TW based on the gold standard used in this study, which was one or more episode of wheezing after the age of three. Based on this gold standard, 22 patients were regarded as PW, whereas 16 patients were regarded as TW. Patient characteristics are shown in Table 1. Interestingly, the male:female ratio was higher in de TW group compared to the PW group (3:1 and 1:1, respectively). A significantly higher percentage of PW used corticosteroids as inhalant at the final follow-up compared to TW (65% and 13% respectively, p=0.007).

	PW (*n=22*)	TW (*n=16*)	Total (*n=38*)
Age at inclusion	2.6 (1.1-4.4)	2.3 (1.1-4.8)	2.5 (1.1-4.6
Male (%)	10 (46%)	13 (75%)	22 (58%)
Early Frequent wheezer	10 (46%)	6 (38%)	16 (42%)
Major Criteria			
Parental MD Asthma	13 (59%)	6 (38%)	19 (50%)
MD Eczema	6 (27%)	4 (25%)	10 (26%)
Minor Criteria			
Allergic Rhinitis	0	0	0
Wheezing apart from colds	11 (50%)	10 (63%)	21 (55%)
Positive IgE/ RAST test	7 (32%)	5 (32%)	12 (32%)
Medication at follow-up			
Corticosteroid inhalant	15 (68%)	2 (13%)*	17 (45%)

Table 1.

Patient characteristics. *: p=<0.01

Retrospectively differentiating genes distinguish between PW and TW.

Given our gold standard being wheezing after the age of three, we next wanted to determine whether retrospectively found genes could differentiate between the two phenotypes. All individuals were ascribed to either TW or PW based on the follow-up data. Next, gene-expression was compared qualitatively, e.g. whether a gene was up- or down-regulated. In PW, 63% had an upregulated level of TLR7, in comparison with 41% in TW. For Ptger2, this was 63% in PW compared to 30% in the TW and downregulation of STAT1 was seen in 70% of the PW compared to 23% of the TW. In Table 2, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), odds ratio (OR) and relative risk (RR) of the individual genes on PW and TW are shown (Table 2).

We next questioned whether six other genes, which seemed to be determinants of wheezing in the retrospective study, could also differentiate between PW and TW. No differences were found for C5R1, JunB, Dusp2, LILRB2, TNSF13B or TNFAIP3 (data not shown).

In conclusion, TLR7, Ptger2 and STAT 1, but not C5R1, JunB, Dusp2, LILRB2, TNSF13B or TNFAIP3 seem to differentiate between PW and TW.

Determinant	n	Sensitivity (%)	Specificity (%)	PPV % (95%CI)	NPV % (95%CI)	OR	RR
TLR7	31	67	62	70 (0.5-0.8)	50 (0.4-0.7)	3.2	1.7
Ptger2	32	61	64	70 (0.5-0.8)	60 (0.4-0.7)	2.8	1.6
STAT1	33	68	71	80 (0.6-0.8)	60 (0.5-0.6)	5.4	2.1
TLR7Ptger2	28	21	86	60 (0.3-0.8)	50 (0.5-0.6)	1.6	1.3
TLR7STAT1	31	31	87	70 (0.4-0.9)	50 (0.5-0.6)	3	1.6
Ptger2STAT1	35	42	94	90 (0.6-1)	60 (0.5-0.6)	10.9	2.1
Loose Index	38	68	56	68 (0.6-0.8)	56 (0.4-0.7)	2.8	1.6

Table 2.

Statistical characteristics of TLR7, Ptger2 and STAT when analysed individually.

For the genes, n is the total number of patients with a gene-expression of the gene outside the 5%-interval of the median expression level of the gene.

Comparison of predictive value of gene expression with clinical indices

We next questioned whether these three genes could contribute to the accuracy of this clinical index. To address to this we first compared our gold standard with the outcomes of the clinical index. When we used the loose clinical index (LCI) in our patient group we found a sensitivity of 68% and a specificity of 56%. For this age-group, a sensitivity of 57% and specificity of 81% was to be expected (Table 2). When we applied the stringent clinical index (SCI) a sensitivity of 27% and a specificity of 69% was found. In this index, Castro et al found a sensitivity for this age-group of 28% and a specificity of 96% (data for SCI not shown).

Considering the small numbers of patients in the SCI (n=7), we decided to compare the prognostic capacity of the three genes with the LCI only. Multivariate regression analysis demonstrated that the loose clinical index and the genes TLR7, Ptger2 and STAT1 could be used as independent predictors of persistency of disease (gene expression score index, GESI, Table 3). An ROC was made comparing the prognostic capacity of the LCI alone with the prognostic capacity of the LCI compared with the GESI (Fig. 1). The AUC changed from 0.62 to 0.84 (95% CI 0.05-0.39; p=0.02) which indicates good discriminatory power of the score.

A gene expression score index (GESI) gives a good positive predictive value on persistency of wheezing

Based on the regression coefficients derived from the multivariate regression model, a risk score was obtained (third column in Table 3). By assigning points for each variable present, a total score was calculated for each patient using the following equation:

Composite gene expression score index (CGESI) = (2 for upregulation of TLR7 + 2 for upregulation of Ptger2 + 2 for downregulation of STAT1) + 1 for LCI.

When a threshold score of 6 (at least 3 positive genes and positive LCI) or higher was used to predict the persistency of wheeze, the positive predictive value of this test was 100% and the negative predictive value was 53% (sensitivity 36%, specificity 100%, Table 4). When a threshold score of 5 (at least 2 genes and a positive loose index) or higher was used, the positive predictive value was still 88% whereas the negative predictive value increased with 67% (sensitivity 68%, specificity 89%, Table 5).

In conclusion, the combination of three wheezing genes with a clinical index was predictive for the persistency of wheeze.

Determinant	Regression coefficient	Contribution to score
Loose index	0.7	1
TLR7 (Upregulated)	1.5	2
Ptger2 (Upregulated)	1.3	2
STAT1 (Downregulated)	1.3	2

Table 3.

Independent determinants of Gene Expression Score Index (GESI). Based on the regression coefficient points were assigned to each individual determinant for contribution to the score. The lowest regression coefficient (LCI) was taken as reference score 1.

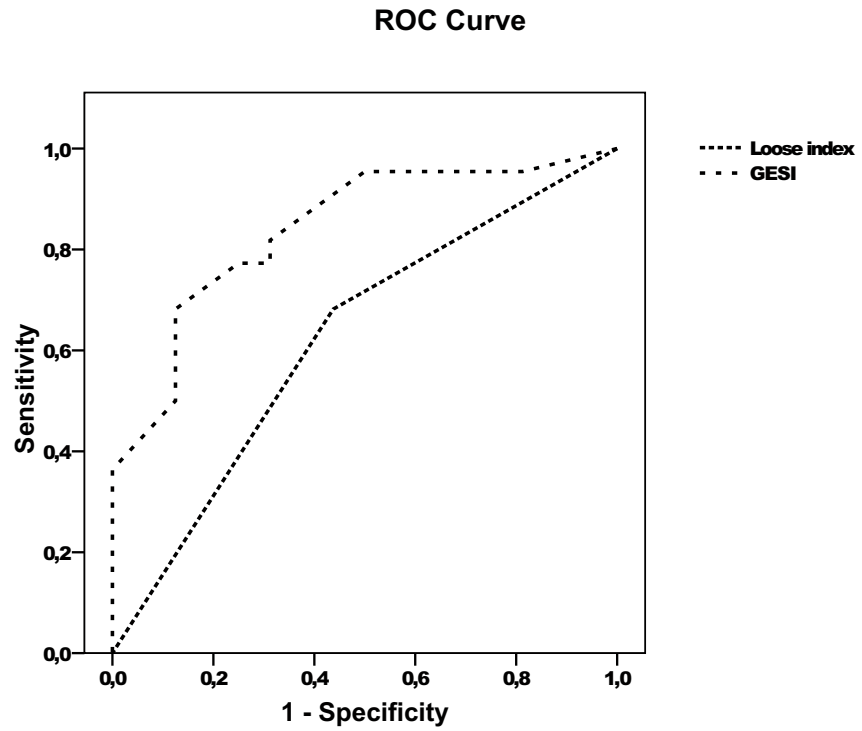

Figure 1.

ROC curve comparing the Loose clinical index (LCI) with the gene expression score index (GESI). The GESI was more able to differentiate between TW and PW (AUC 0.84) compared to the LCI alone (AUC 0.62, p=0.02)

CGESI-1	N	TW	PW
Mild (0-2)	13	10	3
Moderate (3-5)	17	6	11
Severe (6-7)	8	0	8

Table 4.

Number of TW and PW in the different score categories using a strict score. In this score, a total score of at least 6 was needed to have a positive test. The PPV of this score was 100%, but NPV was rather low (53%).

CGESI-2	N	TW	PW
Mild (0-1)	8	7	1
Moderate (2-4)	13	7	6
Severe (5-7)	17	2	15

Table 5.
Number of TW and PW in the different score categories using a loose score. In this score, a total score of at least 5 was needed to have a positive test. With this score, the PPV decreased but was still acceptable (88%), whereas the NPV increased (67%).

Discussion

In this longitudinal cohort study, we used three genes (TLR7, Ptger2 and STAT1) and a loose clinical index as determinants of a composite gene expression score index to predict the persistency of wheeze. Using this score, in the severe scoring group, persistency of wheeze could be predicted with a positive predictive value of 100% and a negative predictive value of 53%. The prognostic capacity of this score was significantly higher compared to the loose clinical index alone (p=0.02).

This is the first study in which gene expression profiles have been combined with clinical symptoms for differentiation between TW and PW in a cohort of EW. When comparing TW and PW in our retrospective cohort microarray analysis revealed differential gene expression of three genes to be differentiating between these two phenotypes[17]. These genes were TLR7, encoding for the Toll like receptor 7 with single stranded RNA viruses such as Respiratory Syncitial Virus (RSV) as ligand[18]. Ptger2 encoding for the prostaglandin E2 receptor which plays a role in the induction of bronchial hyperreactivity and STAT1, also involved in the viral immune response against RSV[19;20]. Despite a lot of debate on the usefulness of microarray analysis, this prospective study replicated the differentiating capacity of these three genes in the recognition between TW and PW. This is also remarkable since analysis of gene expression was done in two different age-groups. The composition of our group of EW is comparable with the cohorts described by Martinez et al[2]. Also the sensitivity and specificity of both the LCI and SCI in our cohort were comparable with their cohort, indicating that the gold standard used in our study is correct[6].

We choose to compare the LCI and not the SCI with our gene expression scores, since Castro-Rodriguez et al. found that only a small proportion of the cohort were frequent wheezers and sensitivity was low. They attributed this to the observation that wheezing symptoms are rather mild and less frequent at an early age. In our cohort also just a small proportion of the PW had a positive stringent index (n=7, 23%). A prerequisite for our test was that it is attributable for the majority of EW.

Using the CGESI, a PPV of 100% was obtained with a NPV of 53%. This means that children with a positive CGESI will develop asthma, but that a negative CGESI not necessarily means that the child will not develop asthma. Thus, this score is capable of identifying those EW who will most likely develop persistent wheezing, but does not rule out persistent wheezing. Further improvement of the score with other determinants might improve the NPV. Whether the clinician decides to use the strict or loose CGESI will further depend on which preventive measures, e.g. medication is recommended for children at-risk for developing asthma. To avoid treating children with only a low risk on developing asthma with a treatment with serious side-effects, the strict index should be used. However if the treatment has little or no side-effects, the loose index is usable.

One of the limitations in this study may be the low patient number. To analyse the effectiveness of a predictive model, a large cohort gives the opportunity to add determinants, such as lung function measurements by the Resistance Interrupter Method (RINT) with a portable interrupter device (MicroRint) to improve the model[21]. However, despite this low number, a good PPV and acceptable NPV were found, at least in the second, less stringent, CGESI. Furthermore, an important purpose in this study was to demonstrate that the genes found retrospectively could be replicated in a prospective cohort with the age group of interest (that is, EW before the age of three) and that these genes were indeed differentiating. Since the replication of microarray studies is technically difficult a pilot study was needed before a large cohort was set up.
In its current form, this test is not yet suitable for the general practice. Gene expression analysis was performed with RNA obtained from peripheral $CD4^+$ T cells. For this technique, a sufficient amount of RNA needs to be obtained and isolation steps should be well controlled for all samples[11;22]. For now this limitation makes the score only applicable in a clinic with diagnostic facilities. Improving the RT-PCR technique with the use of a combination of the primers might reduce the amount of RNA needed and may make the technique more easy and available to other clinical practices.
In this study we demonstrate that gene expression profiles of three distinctive genes

found in a retrospective cohort may be used to predict the persistency of wheezing prospectively in EW. Future studies in a large cohort have to be done to optimise the risk score and to make it useful for the general practice. Furthermore, if we can predict which EW will persist to wheeze by this score, a proper cohort can be defined to measure the effects of early treatment.

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Human heat shock protein 60 induces functional regulatory T-cells in cord blood

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Abbreviations

T_H2: T helper cells type 2
T_H1: T helper cells type 1
T_{regs}: induced regulatory T cells
Hsp60: human heat shock protein 60
TT: Tetanus Toxoid
CBMC: cord blood mononuclear cells
FACs: fluorescence activated cell sorting

Abstract

Background: Though an immune response to self proteins can induce autoimmune disease, most responses towards self-antigens are controlled. It is hypothesized that recognition of a limited set of self proteins such as human heat shock protein 60 (hsp60) is important to regulate inflammation in a healthy immune system. Adaptive and innate immune response towards self hsp60 are found both during autoimmunity and in healthy individuals but little is known on recognition of hsp60 at birth.

Objective: We wanted to investigate whether T cell recognition of hsp60 is present in cord blood, and, if so, whether this recognition may lead to the induction of functionally suppressive regulatory T cells.

Methods: Cord blood mononuclear cells (CBMC) of healthy, full term neonates (n=15), were cultured with hsp60 and TT, and proliferative responses, cytokine secretion, and up-regulation of surface markers were analyzed. The function of hsp60-induced T cells was studied in *in vitro* suppression assays.

Results: Stimulation with hsp60 leads to proliferation of T cells in cord blood, by both $CD4^+CD45RA$ (naïve T-) and $CD4^+CD45RO$ (memory T-) cells. hsp60-induced T cells express FOXP3 and produce various cytokines, among which IL-6, Interferon-gamma, and IL-10. After stimulation with hsp60, T cells can suppress effector T cell responses effectively.

Conclusion: T cell recognition of self hsp60 is already present in cord blood, and may lead to the induction of functional suppressive regulatory cells. Further elucidation of the mechanism of the induction is needed.

Introduction

The immune system harbors various safekeeping mechanisms that prevent the induction of a deleterious response to self antigens. First, through the process of negative thymic selection (central tolerance), T cells with a T cell receptor with a high affinity for self proteins are eliminated[1]. However, some self proteins escape central tolerance. Indeed, although an immune response to self antigens can induce auto immune disease, a healthy immune system also responds to particular self-antigens[2], without leading to auto immune disease. Normally, self-reactive T cells escaping central tolerance are kept under control through processes of peripheral tolerance, e.g. via suppressive regulatory T cells[3].

It is suggested that the response to some self antigens is even an important mechanism in controlling an inflammatory response, and, indeed, regulatory T cells may act through recognition of self-antigens[4]. Cohen has postulated that the response against a vested group of self-antigens is especially important for maintaining peripheral tolerance. He described the response against a limited set of self molecules in a healthy individual, formed by auto reactive T cells and antibodies as the immunological homunculus[5]. This 'homunculus' of self-antigens may share various properties, among which evolutionary conservation between self and the non-self homologue of these proteins. Such proteins may have distinctive capacities to steer immune responses and control peripheral tolerance. One of these self proteins is human heat shock protein 60 (hsp60)[6]. Hsp60 is a remarkably conserved self antigen. Moreover it is a stress protein; it is up regulated at times of cellular stress, such as fever or tissue damage (inflammation)[7]. Since hsp60 can activate both adaptive and innate immune responses this suggests that hsp60 has the potential to be key regulator in any kind of stress response[6]. Previously we and others have shown in both mice and human studies that hsp60-reactive T cells have remarkable immune modulating functions, by up regulating functional regulatory T cells[8,9] This has lead to the set up of various several clinical trials using hsp60 peptides, e.g. in RA and diabetes type I, with promising results[10,11].

If responses to self-antigens such as hsp60 are crucial for maintaining peripheral tolerance in the adult immune system, the question arises whether such self reactivity may play a role in maintaining self-tolerance during pregnancy and, thus, whether it is already present at birth. Indeed, previous studies showed that this may be the case. In 1999 Ramage et al. showed that hsp60 specific T cells are present in the healthy adult population. Subsequently also cord blood cells were successfully stimulated with hsp60 confirming their hypothesis that the response was predominantly a naïve T cell response[12]. In a last year published study Merbl et al. have characterized natural autoantibodies present at birth, together with the antigens they bind[13]. One of the antigens described is hsp60. Again this study suggests that self reactivity, present already at birth, must provide advantages for the healthy immune system. Supported by these previous studies, we now wanted to examine whether the immuno-modulating function of hsp60 is indeed already present at birth. We found that stimulation with self-hsp60 leads to CD4+ T cell proliferation and cytokine production, and induces T cells with an *in vitro* regulatory phenotype.

Methods

Collection of cord blood

Cord blood samples (n=15) were collected from healthy subjects at the department of gynecology and obstetrics. Inclusion criteria were an uncomplicated pregnancy, full-term infant and normal vaginal delivery. No further information about the mother, pregnancy or the delivery was obtained. Written informed consent was obtained. This study was approved by the Medical Ethics Committee, UMC Utrecht.

Isolation of CBMC

Cord blood mononuclear cells (CBMC) were isolated from heparinised venous blood by Ficoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation. CBMC were cultured in RPMI-1640 supplemented with 2mM glutamine, 100u/ml of penicillin/ streptomycin (Gibco BRL, Gaithersburg, MD) and 10% (v/v) AB^{pos} heat-inactivated (60 min at 56° Celsius) human serum (Sanquin, Amsterdam, the Netherlands). For measurement of the proliferative activity, $2x10^5$ cells in 200 µl per well were cultured in triplicate in round bottomed 96-wells plates (Nunc, Roskilde, Denmark) for 96 hours at 37° Celsius in 5% CO_2 with 100% relative humidity. Cells were cultured in the absence or presence of 10 µg/ ml human hsp60 (Faculty of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands). The LPS-content was undetectable (lowest detection level 3 EU/ml). Tetanus-toxoid (TT; 1.5 µg/ ml, RIVM, Bilthoven, The Netherlands) was used as a negative control, assuming that the foetus has not formed a prior response to TT. For the final 16 hours of culture, 1µCi/ well [^{3}H]thymidine (ICN Biomedicals, Amsterdam, The Netherlands) was added to each well. Cells were harvested according to standard procedures and incorporated radioactivity was measured by a liquid scintillation counter and expressed as counts per minute (cpm). The

magnitude of the proliferative response is expressed as stimulation index, which is the mean cpm of cells cultured with antigen divided by the mean cpm of cells cultured without the antigen.
To characterize which cells proliferate to hsp60, a CFSE-assay was also performed. Here fore 5uM CFSE was added to 1*10^6 CBMC which were stimulated with hsp60 for 7 days. Cells were then stained with CD4, CD8 and CD14, as well as CD45RA and CD45RO and analyzed by FACS Calibur (Becton Dickinson Biosciences, San Jose, CA, USA), as described below.

Multiplexed particle-based Immuno assay
Cytokine levels were measured after activation of the lymphocytes *in vitro* as described above. After 96 hours the supernatants of the cell cultures were stored at -80°C. Cytokines levels of IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFNγ and TNFα were measured with the Bio-Plex system and analysed with the Bio-Plex Manager software version 4.0 (Bio-rad Laboratories, Hercules CA, USA) which uses the Luminex xMap technology[14,15]. The specific cytokine production is calculated as the cytokine production of cells cultured with stimulation subtracted with the cytokine production of cells cultured without stimulation.

FACS staining
CBMC were stimulated for 6 days with hsp60 and TT and then washed twice in PBS containing 2% FCS (PBS-FCS) and adjusted to 0.5–1*10^6 cells/ml in staining buffer (PBS-FCS containing 0.1% sodium azide) and blocked with the appropriate normal serum (30 min at 4°C). Subsequently, the cells were incubated in 50 µl of FACS buffer containing four appropriately diluted PE-, FITC-, PerCP-, or allophycocyanin-labeled mAbs against human CD4 (clone SK3) (Becton Dickinson Biosciences) ,CD30 (clone BER-H8)(Becton Dickinson Biosciences), CD45RA (clone JS-83) (Ebioscience, San Diego, CA, USA), CD45RO (clone 4CHL-1) (Ebioscience, San Diego, CA, USA) and FOXP3 (PCH101) (Ebioscience, San Diego, CA, USA). Stained mononuclear cells were diluted in FACs fluid and run on a FACSCalibur (BD Biosciences). CellQuest software (BD Biosciences) was used for analysis.

Cytokine analysis by lymphocyte intracellular staining and flowcytometry
Direct T cell lines were generated as described above. During the last 4 hours of culture, Golgistop (Becton Dickinson Biosciences) was added (final concentration of 2 µM). The cells were harvested and stained for intracellular cytokine and FOXP3 analysis as described previously. For the staining, a predetermined optimal concentration of PE-conjugated anti-IL-10 (clone JES3-19F1), anti-IL-4 (clone MP4-25D2), FITC-conjugated anti-IFNγ (clone 4S.B3) (Becton Dickinson Biosciences) and APC-conjugated Foxp3 (Ebioscience, San Diego, CA, USA) was added to the cells. Cells were analysed as described above.

Suppression Assay
To examine the functionality of induced regulatory T cells, fresh cells were stimulated with medium and hsp60 for 6 days. 12 hours before the Tregs were added to effector cells, a plate was coated with aCD3. In the precoated plate anti-CD28 (clone 15E8: CLB, Amsterdam, The Netherlands) and thawed, negatively selected (by MACS) CD4- cells (functioning as APC) from the same donor as the effector and regulatory cells were added.
With a FACS-sorter we subsequently added 0, 3000 and 3000 CD4+ (effector) cells to respectively 3000, 3000 and 0 induced Tregs. Tregs were sorted on CD4+ CD25+ CD127- cells. The different ratios of cells were incubated for 96 hours and incubated with 3H for 16-18 hours (as described above). Proliferation of effector cells only were set to 100% and proliferation with Tregs was compared to this.

Results

Hsp60 induces CD4+ T cell proliferation in cord blood
The first question we addressed was whether in vitro activation of CBMC with hsp60 induces T cell proliferation. We studies T cell proliferation both with thymidine incorporation and with CFSE dilution. First, using thymidine incorporation we found that five out of ten (50%) samples showed a clear proliferative response (SI>2.0) to hsp60. No proliferation was seen in response to TT (figure 1a). Though this assay suggested T cell proliferation, we next wanted to see whether hsp60 induced not only proliferation of CBMC in general, but also specifically proliferation of cord blood derived $CD4^+$ cells. Therefore in addition to thymidine incorporation, we measured proliferation with CFSE-dilution which allows determining which cells are dividing upon hsp60 stimulation. We found that indeed $CD4^+$ T cells proliferated specifically after hsp60 stimulation in five out of five samples (100%) (fig 1b), but not after stimulation with tetanus. Thus, hsp60 induces proliferation by cord blood derived $CD4^+$ T cells.

Naive and memory cells respond to hsp60

We next questioned whether the responding cord blood cells were naive or memory cells. We stained the cells for CD45RA and CD45RO. Following stimulation with hsp60 we found a lower number of CD45RA and a significantly higher number of CD45RO cells in hsp60 stimulated cells compared to medium and TT stimulated cells (fig 1c). To further elucidate these observations CFSE labeled CBMC were stained with CD45RA and CD45RO to examine which cells had proliferated. As could be expected, the proliferated $CD4^+$ T cells were all CD45RO positive, which may suggest a recall antigen response. To examine whether these CD45RO cells were originally naïve cells that formed into memory cells or if they were already memory cells before birth we sorted the cells prior to the CFSE assay by MACS before activation with hsp60, which showed that both naive cells, and memory cells proliferate in response to hsp60 (Aalberse, personal communication).

Figuur 1 a b c

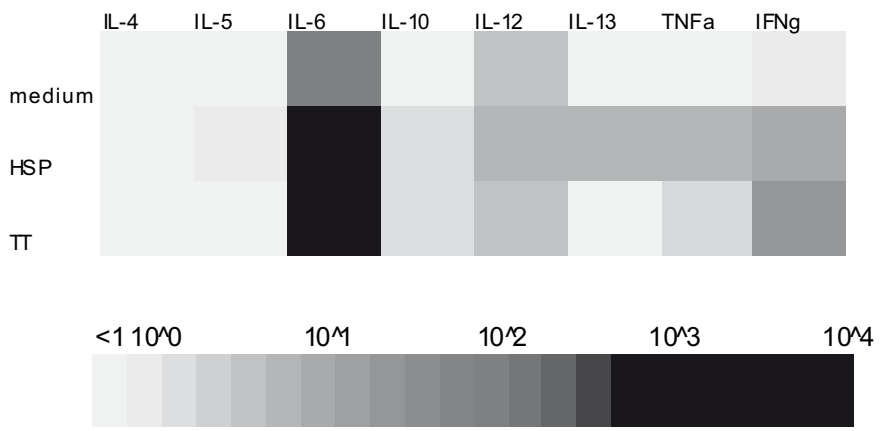
Figure 1.

Human hsp60 is recognized in cord blood. A) Protein-induced proliferation of cord blood mononuclear cells (CBMC). CBMC were cultured for 96 hours in the presence of Human hsp60 or Tetanus Toxoid. One black dot represents one sample, horizontal lines show the median. Significant differences in responses are indicated as follow: * = $p < 0.05$. SI is indicated on a linear scale. **B**) Protein-induced proliferation of cord blood mononuclear cells (CBMC) cultured in the presence of CFSE. CBMC were cultured for 144 hours and afterwards stained for CD4. This figure represents 5 experiments. * = non-proliferated cells; ** = proliferating cells. Proliferating cells, gated on $CD4^+$ Tcells, after medium stimulation: 5%, whereas after stimulation with human hsp60 52% of the $CD4^+$ population proliferated. **C**) Cells were cultured in human hsp60 and TT for 7 days. FACS analysis shows a significant higher level of $CD4^+CD30^+$ T cells and $CD4^+Foxp3^+$ T cells in after stimulation with hsp60. Light gray boxes = medium only; white boxes = Human hsp60; dark grey boxes = Tetanus Toxoid.

Cytokine production in cord blood stimulated cells

We next wanted to know whether stimulation with hsp60 led to cytokine production by CBMC. Therefore, cytokine levels were measured with MIA in culture supernatants after 96 hours of stimulation with either hsp60, TT or medium alone. We found that, compared to stimulation with medium only, antigen induced production of IL-10 was increased both after hsp60 ($p = 0.009$) and TT stimulation ($p = 0.042$). The cytokines IL-5 (p=0,013) IL-6 (p=0.003), IL-13 (p=0.003), TNF-alpha (TNFa) (p=0.003) and IFN gamma (IFNγ) (p=0.005) were significantly increased after activation with hsp60 only (fig. 2a).

To confirm this, and to establish which cells are actually producing these cytokines, we next measured intracellular cytokine production by FACS, again after stimulation in vitro with medium, hsp60 and TT. We found an increased intracellular expression of both IL-10, and interferon-gamma, mainly by $CD4^+$ T cells (fig 2b), after stimulation with hsp60 only (and not with TT). Remarkably, most of the $CD4^+$ T cells expressing IFNγ also expressed IL10 (fig. 2c). Thus, stimulation of CBMC with hsp60 leads to proliferation and cytokine production (IL-10 and IFNγ) of $CD4^+$ T cells.



Figuur 2 b c

Figure 2.

Cytokine secretion induced by Human hsp60 in cord blood. **A**) Cells were cultured in human hsp60 and tetanus toxoid for 4 days. Cytokine profiles in supernatatant of the culture were measured using a multiplex immunoassay. A color profile of the mean levels of cytokines (in pg/ml) was made to show differences

hsp60- and tetanus toxoid stimulated cells and compared to only medium cultured cells. IFNg , interferon gamma; IL, interleukin; TNFa; TNF alpha. **B**) FACS analysis confirmed that $CD4^+$ T cells are also responsible for IFNg and IL10 production after stimulation with human hsp60. Boxes show IQR (interquartile range) for each group of data, horizontal lines show the median. Significant differences in responses are indicated as follow: * = $p < 0.05$. Light grey boxes = medium; white boxes = human hsp60 and dark grey boxes = tetanus toxoid. **C)** A representative FACS-analysis of five experiments remarkably showing that most IFNg producing $CD4^+$ T cells are also producing IL10.

Hsp60 -specific induction of regulatory T cells

There are indications that hsp60, and/or peptides derived from hsp60 may induce or enhance the regulatory T cell population. We therefore wanted to know whether hsp60 may also play a role in T cell regulation at this crucial period at, or even before birth. We therefore stimulated cells again with TT, hsp60 or medium and next stained the cells for expression of CD4, CD30 and FOXP3. FOXP3 is a transcription factor that is characteristic for Tregs. CD30 is an activation marker that has been suggested to be a marker for Th2 cells, whereas we recently found that CD30 is upregulated on antigen induced Tregs (de Kleer et al, submitted). As can be seen in figure 3a following stimulation with hsp60 we found an increase of both $CD4^+CD30^+$ and $CD4^+FOXP3^+$ cells, which is not seen after stimulation with control antigen TT. The $FOXP3^+$ and $CD30^+$ T cells were predominantly memory T cells (figure 3b). Thus, stimulation with hsp60 induces $CD4^+$ T cells that phenotypically have characteristics of regulatory T cells.

Figuur 3

Figure 3.

Human hsp60 induces $CD4^+CD30^+$ T cells and the expression of Foxp3. Cells were cultured in human hsp60 for 7 days. **A**) FACS analysis shows a significant higher level of $CD4+CD30^+$ T cells and $CD4^+FOXP3^+$ T cells after stimulation with human hsp60. Boxes show IQR for each group of data, horizontal lines show the median. Significant differences in responses are indicated as follow: * = $p < 0.05$. Light grey boxes: medium only; white boxes = human hsp60, dark grey boxes = tetanus toxoid. **B**) FACS analysis of $CD4^+$ T cells expressing FOXP3 after stimulation with human hsp60, showing that most FOXP3 positive CD4 T cells also express CD45RO and some express CD30.

Hsp60 induced regulatory T cells are functionally suppressive

We next questioned whether these induced Tregs were indeed functional and thus have suppressive capacity. Therefore we performed suppression assays, in which we added Tregs and effector cells in different numbers. The hsp60 induced Tregs, sorted on $CD4^+CD25^+CD127^-$ cells (which were for 80% FOXP3 positive; data not shown) were able to functionally suppress the effector T cells. The Tregs induced by hsp60 were comparably capable in suppression (67%, figure 4) as the Tregs that are already present (medium-stimulated; which showed 61% suppression). This indicates hsp60 induced Tregs are functional suppressor cells in vitro.

Figuur 4

Figure 4.

Human hsp60 induced $CD4^+CD25^+CD127^-$ T cells are functionally suppressive. Functional assays were performed as described in the methods section. Human hsp60 induced $CD4^+CD25^+CD127^-$ T cells (regulatory T cells) show 65% suppression when added to the effector ($CD4^+CD25^-$) T cell population.

Hsp60 is recognized via the innate as well as the adaptive system

As stated above, hsp60 can induce both innate and adaptive immunity, even simultaneously. To examine whether hsp60 can directly be recognized by T cells, without antigen presenting cells available, we sorted $CD4^+$ cells by MACS before

stimulation with hsp60 in vitro. Although this significantly decreased the proliferation to hsp60, $CD4^+$ T cells were still able to show a positive proliferation response to hsp60 (Aalberse, personal communication). This indicates that although the innate immune system can enhance the response to hsp60, it is plausible that the observed immune response is mediated through TCR stimulation.

Discussion

Here we show that hsp60 induces in cord blood $CD4^+$ T cells that proliferate and produce various cytokines, most notably IL-10 and IFNγ. Moreover such hsp60-induced T cells express CD30 and FOXP3, and act as suppressor T cells in vitro. The results presented in this study provide a new step in understanding the importance of the recognition of self proteins, like hsp60, by the immune system.
A healthy immune system is able to initiate as well regulate inflammation, while maintaining tolerance for self-antigens. In 1991 Cohen hypothesized that immune responses to certain self proteins are important for a healthy immune system[5]. These "immunological homunculus" proteins, among which stress proteins like hsp60, may serve as immune biomarkers to the body educating the immune system on the state of present inflammation. Our results would fit this hypothesis, as it suggests that immune response to self hsp60 are part of an "inborn" mechanism present already at birth that may help to regulate inflammation by up regulating T cells, both in promoting and in suppressing inflammation in vitro. Interestingly, hsp60 has previously been identified as a potent regulator of inflammation in the adult immune system.
In the end of the 80's human hsp60 was found to be recognized by the immune system during inflammation[8,16] .It was then shown that hsp60-specific T cells can dampen experimental autoimmune disorders, while in humans hsp60 is recognized by healthy individuals free of auto immune disease or infection[17] The capacity of hsp60 to induce both innate and adaptive immunity indicates that hsp has a double function: responsiveness to hsp can be related to inflammation, but also to a healthy immune system. Next it was shown in human studies that hsp60-specific immune responses in patients with juvenile arthritis (JIA) and rheumatoid arthritis are associated with a better prognosis and milder symptoms, again suggesting a role in regulating inflammation[18]. In line with these findings we now show that hsp60-specific T cells are already present at birth which again points to the hypothesis that auto-reactive T cells are a principal feature of a healthy immune system.
It is proposed that normally the immune system at birth does not respond specifically to most antigens. Some studies have reported proliferation to allergens (possibly by allergens that are transported over the placenta), but most studies show little responsiveness to recall antigens, which has been contributed to the sterile environment of the fetus[19],[20]. However, a recent study showed that B-cell responses to a large variety of self-antigens is already present at birth[5]. Among the antigens capable of inducing an antibody response are heat shock proteins. This is underscored by an earlier finding that naïve cord blood cells can show reactivity to hsp60[12]. Here we complement the findings from both studies, showing a clear increase in memory T cells after stimulation with hsp60. It is still unclear if the response to hsp60 is indeed only from naïve (CD45RA) cells, developing into CD45RO (memory) T cells or that memory T cells specific for hsp60 are already present at birth. It can be speculated that human hsp60 could be regarded as a recall antigen in the fetus, as self proteins are probably continuously present in the developing fetus due to apoptosis of developing tissues. This may contribute to induction of (tolerogenic) responses to self proteins like hsp60 before birth and may explain why hsp60 in cord blood elicits such evident responses as seen in our study. Further studies should aim to address this and, moreover, elucidate whether the documented T cell reactivity to hsp60 is really the consequence of a TCR driven, antigen specific T cell response or that innate co-activation with hsp60 is responsible. Preliminary experiments seem to speak against the latter as being primarily responsible for the self-reactivity to hsp60.
Thus, we have found that T cell reactivity to hsp60 is a normal feature at birth. Future studies need to further elaborate the mechanism of this reactivity and may help to clarify whether this feature of hsp60 is part of an inborn mechanism of immune regulation.

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The IL-10 inducing effect of TGF-β on human naive $CD4^+$ T cells from cord blood is restricted to the T_H1 subset

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Abbreviations

CBMC: Cord Blood Mononuclear Cell
PBMC: Peripheral Blood Mononuclear Cells
PE: Phycoerythrin
TGF: Transforming Growth Factor
T_{regs}: Regulatory T Cells

Abstract

Objective: Transforming growth factor (TGF-β) seems to play a role in the regulation of immune responses, mainly by its suppressive function towards cells of the immune system. However, both in mice and human, conflicting data are published on the capacity of TGF-β to induce IL-10 secretion in both naive and skewed T cell populations.
Our aim was to test the IL-10 inducing capacity of TGF-β in both naive and skewed cord blood mononuclear cells (CBMCs) and elucidate the mechanism by which TGF-β exerts its effect.
Methods: Naive CBMCs and CBMCs during skewing under T_H1 and T_H2 polarizing conditions were stimulated with CD3 and/or CD28 in the presence or absence of TGF-β. Proliferation, cytokine production and mRNA expression of transcription factors were measured.
Results: TGF-β enhanced the IL-10 production in T_H1 and naive cells only, and suppressed the T_H1 phenotype as demonstrated in cytokine levels and T-bet expression. Interestingly, Foxp3 expression tended to increase in both T_H1 and T_H2 cells.
Conclusion: These data indicate that TGF-β can induce a regulatory phenotype in both naive and T_H1-polarized cells derived from cord blood. The induction of IL-10 was not observed in T_H2-polarized phenotype, indicating that TGF-β might be especially of interest for immunomodulation in T_H1 cells.

Introduction

Atopic diseases are characterized by the production of T_H2 cytokines, such as IL-4, IL-5 and IL-13 by allergen-reactive T cells which induce IgE production and eosinophilia. *In utero* the T_H2-skewed environment prevents rejection of the fetus. After birth the infant's T-cell response is redirected towards a more T_H1-skewed response[1]. In atopic children however a decreased capacity to produce IFN-γ, a T_H1-cytokine, results in the persistence of a T_H2 phenotype. A possible explanation is the absence of a regulatory signal which can inhibit the T_H2 response, leading to early expansion of T_H2 cells and a less pronounced T_H1 cell proliferation[2]. Regulatory T cells (T_{regs}) function as inhibitors of T cell responses. Several studies have shown that in atopic diseases, T_{regs} are less abundant and the T_{regs} available are less potent to suppress immunological reactions[3,4]. The best studied T_{regs} in this context are the naturally occurring $CD4^+CD25^+$ T_{regs}, expressing the transcription factor Foxp3 and producing IL-10. In atopic diseases, ideally, primary prevention should be aimed at triggering these immune responses during early childhood, either via the induction or activation of T_{regs} or by manipulating the immune deviation from T_H2 skewing.

A cytokine known for its regulatory function is TGF-β[5]. Previously, it has been demonstrated in TGF-$β^{-/-}$ mice dying of massive inflammatory responses, that TGF-β is important for attenuating the immune responses[6]. Disruption of the TGF-β signalling pathway in mice results in lymphocyte infiltration into tissues and spontaneous T helper cell activation[7]. Recently it was demonstrated that TGF-β is capable to generate $CD4^+CD25^+$ T cells from naive $CD4^+CD25^-$ T cells both from cord blood mononuclear cells (CBMC) and from peripheral blood mononuclear cells (PBMC) by induction of Foxp3[8]. The immunomodulating effects of TGF-β on T lymphocytes seem to depend on the maturation status of the lymphocytes. In mice, TGF-β had a suppressive effect on differentiation of naive T lymphocytes. However, when T lymphocytes were cultured under T_H1 or T_H2 differentiating conditions, this effect was only observed in the T_H1 cells[9,10]. On the other hand, it was demonstrated in mice that TGF-β abrogated T_H2 development by inhibition of GATA-3[11]. In mature human antigen-specific T cell clones it was shown that TGF-β inhibited antigen-induced proliferation, but had no effect on cytokine release. This was not related to the allergic phenotype of the donor[12].

The capacity of TGF-β to induce T_{regs} in CBMC might be of interest in the primary prevention of atopic diseases, since the immune deviation towards atopy seems to start directly after birth if not *in utero*. However, the finding in the murine model that differentiated T_H2 cells are not susceptible to TGF-β-mediated suppression suggests that TGF-β might only be able to abrogate T_H2 differentiation if present at an early naive state of T cell differentiation present in CBMCs. A previous study has shown that indeed, CBMC harbour a unique naive subtype of T lymphocytes which can be regarded as precursors of the more mature $CD4CD45RA^+$ T lymphocytes[13]. Therefore, we aimed to investigate the immunomodulating potential of TGF-β in the generation of T_{regs} during T_H1 and T_H2 skewing of CBMCs.

Materials and Methods

Cell preparation

Peripheral blood was obtained from healthy volunteers and PBMC were isolated by Ficoll-Isopaque density centrifugation (Amersham Biosciences, Uppsala, Sweden). $CD4^+$ T cells were negatively selected with a CD4 T cell isolation kit obtained from Miltenyi (Miltenyi Biotec, Bergisch Gladbach, Germany). To separate the $CD4^+CD45RA^+$ from the $CD4^+CD45RO^+$ fraction, the $CD4^+$ T cells were incubated with CD45RO beads (Miltenyi Biotec) and run over a negative selection column to ensure the highest depletion efficiency, thereby increasing the purity of the RO-negative fraction ($CD4^+CD45RA^+$ cells). The positive fraction ($CD4^+CD45RO^+$) was further purified by use of a positive selection column.

Umbilical cord blood was obtained from the vein of the umbilical vein of uncomplicated full-term deliveries. Disodium citrate in a concentration of 0.1M was used as an anticoagulant. PBMCs and CBMCs were isolated by Ficoll density gradient centrifugation (Amersham Biosciences) as described previously. FACS analysis showed that a purity of ± 95% $CD4^+$T cells is obtained by this method. The $CD4^+$T cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, NY, USA), supplemented with 2% pooled heat-inactivated human AB serum, 5% Yssel's medium[14], penicillin (100 U/ml), streptomycin (100µg/ml) and glutamin (1mM) (Gibco).

Skewing conditions

Isolated $CD4^+$T cells from CBMCs were stimulated with anti-CD3 and anti-CD28 mAb at 2 µg/ml in the presence of IL-12 (200 U/ml; BD Biosciences, San Jose, USA) and anti-IL-4 (1 µg/ml; Sanquin, Amsterdam, The Netherlands) for T_H1 polarization. For T_H2 polarization, the cells were stimulated with anti-CD3 and anti-CD28 mAb (2 µg/ml) in the presence of IL-4 (1000 U/ml; Novartis Research

Institute, Vienna, Austria) and anti-IL-12 (10 µg/ml) (U-Cytech, Utrecht, The Netherlands). T_H1 and T_H2 polarization was carried out in the presence or absence of TGF-β (1 ng/ml) (R&D systems, Abingdon, UK).

Cytokine measurement by ELISA

At day 2, 4, 5 and/or 7 of culture, samples of culture medium were taken for cytokine measurement by a sandwich ELISA performed according to the manufacturer's protocol (Sanquin, Amsterdam, The Netherlands). The detection limits used were 0.6 pg/ml for IL-4, 5 pg/ml for IL-5, 1.2 pg/ml for IL-10, 0.5 pg/ml for IL-13 and 2 pg/ml for IFN-γ. Absorbance was measured at 450 nm in a Titertek Multiskan Plus (Labsystem, Helsinki, Finland).

RNA isolation and cDNA preparation

RNA was isolated by using Trizol® (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Isolated RNA was subsequently treated with Dnase (Ambion, Austin, Texas USA). The quality of RNA was assessed on the basis of demonstration of distinct 23s and 18s ribosomal RNA bands electrophoresed on an agarose gel and a spectrophotometric ratio of absorbance at 260 nm to that at 280 nm greater than 1.8. Generation of cDNA from RNA was performed by RT-PCR. A total of 400 ng of each RNA sample was mixed with the iScript reaction mix and iScript Reverse Transcriptase according to the manufacturer's manual (Bio-Rad iScript, Bio-Rad Laboratories, Stanford, Ca, USA).

Real-time PCR

For analysis of mRNA levels, a real-time PCR was performed with a MyiQ™ Single-colour Real-Time PCR detection system (Bio-Rad laboratories, Stanford, Ca, USA) using the following protocol: 3 min at 95°C, 45 times 10 sec at 95°C, 20 sec at 61°C and 25 sec at 72°C, 1 min at 95°C, 1 min at 60°C and 70 times 10 sec at 60°C. Results were normalized for PCR efficiency and for the reference genes β-actin and GAPDH. Primer sets are shown in table 1.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
β-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
FoxP3	AAACAGCACATTCCCAGAGTTC	GCGTGGCGTAGGTGAAAG
GAPDH	AGAAGGCTGGGGCTCATTT	GAGGCATTGCTGATGATCTTG
GATA-3	GGCAGGGAGTGTGTGAACTG	CCTTCGCTTGGGCTTAATG
IFN	TTTGGGTTCTCTTGGCTGTTACT	GTTCCATTATCCGCTACATCTGAAT
IL10	GAGAACCAAGACCCAGACATCA	GCATTCTTCACCTGCTCCAC
SMAD3	TGACTGTGGATGGCTTCACC	GCCTCTTCCGATGTGTCTCC
SMAD7	AGAGGCTGTGTTGCTGTGAATC	GCAGAGTCGGCTAAGGTGATG
STAT4	GCAACCGAAGATTTGTACTTTGTG	GACAGCCCTCATTTCCTTTACCT
STAT6	GTGTACCCACCACACTCTCACTC	GGTCAAAGGGCAGGCTCAT
T-Bet	GGGAAACTAAAGCTCACAAACAAC	GCACAATCATCTGGGTCACATT

Table 1.
Primers used in real-time PCR analysis.

Flow cytometry

$CD4^+$T cells were phenotyped using FITC-conjugated antibodies to CD25 and CD45RA, PE-conjugated antibodies to CD69 and CD45RO and a Cy5-conjugated antibody to CD4 (Becton Dickinson biosciences, San Jose, USA) and analysed in a FACSCalibur (BD Biosciences). Intracellular cytokine staining was performed using the method described by Jung et al.[15] Briefly, the cells were stimulated with PMA (10 ng/ml; ICN Biomedicals, Irvine, CA, USA) and ionomycin (1 µg/ml; Sigma, St. Louis, MO, USA) for 6 hrs. During the last 4 hrs of stimulation Brefeldin A (10 µg/ml; Sigma) was added. After fixation with 4% paraformaldehyde (Merck, Darmstadt, Germany), the cells were permeabilized with 0.5% Saponin (ICN biomedicals) and stained with anti-human IFN-γ-FITC, and anti-human IL-10-PE (BD Biosciences) and analysed using a FACScan flow cytometer (BD biosciences).

Statistics

Statistical analysis of the effect of TGF-β on T cell proliferation and cell surface markers expression was performed with use of the non-parametric Wilcoxon test. Prior to the analysis of the cytokine data and mRNA data, all results were normalized by ^{10}log-transformation. Subsequently, differences between the production of the cytokines with and without TGF-β were analyzed using a paired t-test. Differences associated with p values < 0.05 were considered significant.

Results

TGF-β inhibits proliferation in the absence of a costimulatory signal in naive T cells
Stimulation of various T cell subsets was performed with anti-CD3 mAb alone or in combination with anti-CD28 mAb. Upon stimulation with anti-CD3 mAb alone, the proliferation of $CD4^+CD45RA^+$ T cells from PBMC, as well as $CD4^+$ T cells from CBMC was significantly inhibited by TGF-β (48% and 72%, respectively) whereas the proliferation of $CD4^+CD45RO^+$ T cells from PBMC was not affected (data not shown). Addition of exogenous IL-2 (20 U/ml) did not reverse the inhibitory effect of TGF-β on the proliferation of T cells after stimulation with anti-CD3 mAb alone (data not shown). After stimulation of the different T cell subsets with anti-CD3 and anti-CD28 together, the inhibitory effect of TGF-β was abrogated in all T cell subsets.
In conclusion, in the absence of a costimulatory signal, TGF-β inhibits proliferation in naive T cells.

Induction of IL-10 by TGF-β is only observed in $CD4^+$ T cells isolated from CBMC.

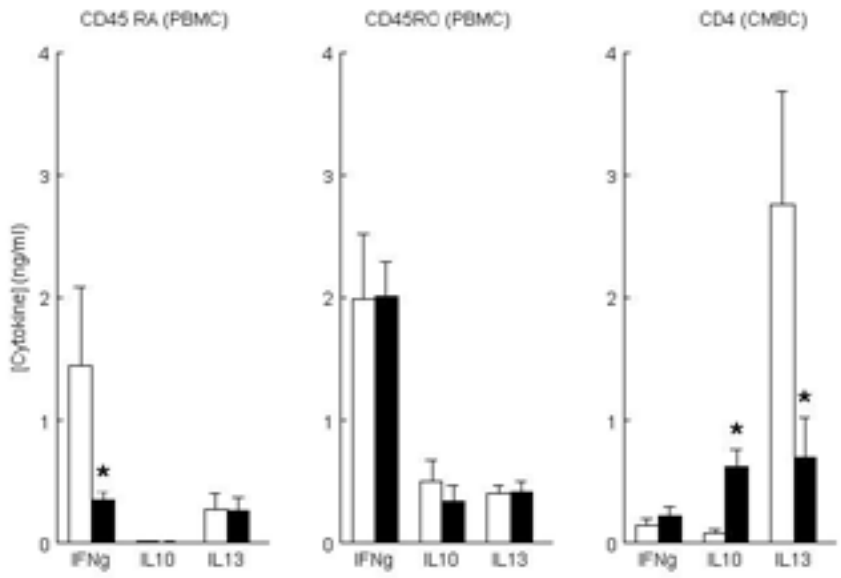

Figure 1.
TGF-β has a differential effect on the cytokine production of various T cell subsets. The production of IFN-γ, IL-10 and IL-13 by $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cells and $CD4^+$ T cells from CBMC was measured in the culture supernatant at 96 hrs after stimulation with anti-CD3 and anti-CD28 mAb in the presence (black bars) or absence (white bars) of TGF-β (1 ng/ml). Results are expressed as mean ± SEM of six independent experiments (* = $p< 0.05$).

Figure 1 shows that addition of TGF-β to CD3/CD28 stimulated $CD4^+$ T cells resulted in a significant decrease of IFN-γ production in naive T cells ($CD4^+CD45RA^+$) from PBMC in comparison with naive T cells cultured without TGF-β, and did not induce IL-10 secretion. However, the IL-10 production by $CD4^+$ T cells isolated from CBMC was significantly enhanced after addition of TGF-β and the production of IL-13 concomitantly decreased. No significant changes were observed in the production of IFN-γ after addition of TGF-β, nor in cytokine production of the memory T cells. In all cell populations the amount of IL-4 produced was very low (< 10 pg/ml) and is therefore not shown. Addition of exogenous IL-2 did not alter the TGF-β-induced enhancement of IL-10 production and the decreased IL-13 production in CBMC-derived $CD4^+$ T cells (data not shown). The production of cytokines by $CD4^+$ T cells from CBMC in the absence of anti-CD28 was very low and was not influenced by addition of TGF-β (data not shown). Thus, TGF-β has the capacity to induce IL-10 secretion exclusively in naive $CD4^+$T cells from CBMC upon full stimulation.

TGF-β does not alter the percentage of IL-10 producing $CD4^+$T cells from CBMC
To investigate whether the increase in IL-10 was due to an increase in the percentage of IL-10 producing cells, an intracellular cytokine staining for IL-10 was performed. The percentage of IL-10 producing T cells varied between 0.3 to 3.2% and was not significantly enhanced after addition of TGF-β (data not shown).

TGF-β enhances the expression of CD25 and CTLA-4 on $CD4^+$ T cells from CBMC
Stimulation of $CD4^+$ T cells from CBMC with anti-CD3 and anti-CD28 mAb in the presence of TGF-β significantly enhanced the expression of the IL-2 receptor α chain (CD25) and CTLA-4 (CD152) (Wilcoxon test, $p<0.05$; Figure 2). The increase in CD25 and CTLA-4 expression occurred in the total T cell population.

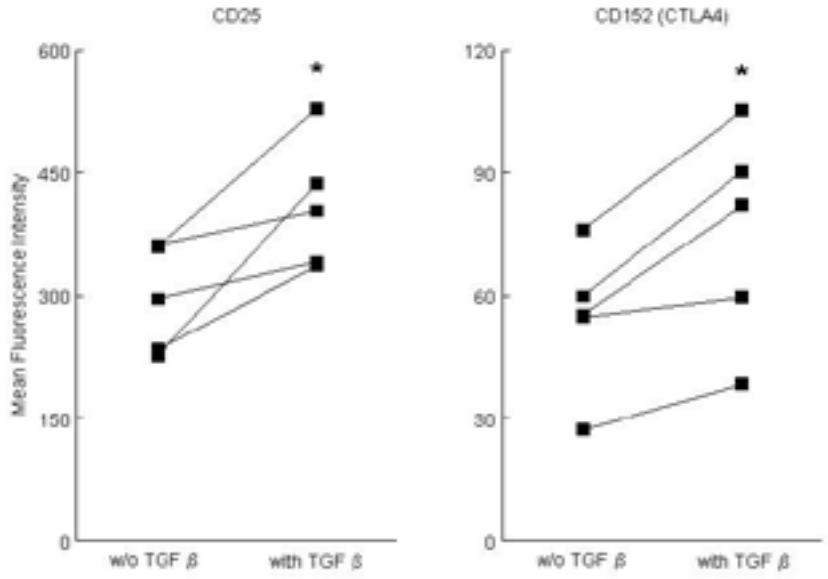

Figure 2.
TGF-β enhances the expression level of CD25 and CTLA-4 on $CD4^+$ T cells from CBMC. $CD4^+$ T cells isolated from CBMC were stimulated with anti-CD3 and anti-CD28 mAb in the presence or absence of TGF-β for 96 hrs. The expression level of CD25 and CTLA-4 is expressed as mean fluorescence intensity (MFI). Each line represents an independent experiment (* = $p< 0.05$).

TGF-β modulates cytokine production in skewed CBMC-derived $CD4^+$.
To analyze whether the IL-10 inducing effect of TGF-β is influenced by polarization of naive $CD4^+$ T cells into T_H1 or T_H2 cells, CBMCs were stimulated with anti-CD3/anti-CD28 mAb (control cells) under T_H1 or T_H2 polarizing conditions, in the presence or absence of TGF-β. As is shown in Figure 3, the production of IFN-γ by T_H1-polarized $CD4^+$T cells was inhibited by the addition of TGF-β. Production of IL-10 was increased in the control cells as well as T_H1-polarized $CD4^+$T cells after addition of TGF-β, but not in the T_H2-polarized $CD4^+$T cells. Thus, the IL-10 inducing effect of TGF-β seems to be restricted to the T_H1 skewed CBMCs (Figure 3).

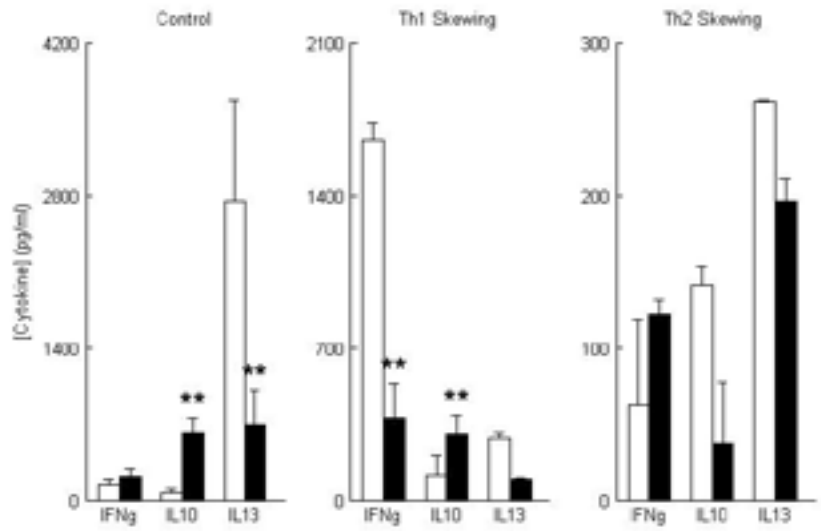

Figure 3.
TGF-β modulates cytokine production in cultured cord blood-derived $CD4^+$ T cells. Cord blood-derived $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb and cultured in the presence (black bars) or absence of TGF-β (white bars) under non-polarizing (control), T_H1 and T_H2 polarizing conditions. Supernatants were collected at 96 hrs post stimulation. Production of the cytokines IFNγ, IL-13 and IL-10 were determined by ELISA. Results are expressed as mean ± SEM of six independent experiments (** = $p< 0.001$).

TGF-β modulates mRNA expression in CBMC-derived $CD4^+$
To examine whether TGF-β modulates mRNA expression of T cell transcription factors, mRNA was isolated 96 hours post-stimulation of CBMCs under T_H1 or T_H2 polarizing conditions in the presence or absence of TGF-β. In the non-skewed cells, only a slight decrease in GATA-3 was observed, whereas expression levels of T-Bet and IFN-γ remained the same (data not shown). In the T_H1-polarized cells, only a minor decrease in T-Bet and IFN-γ and a minor increase in GATA-3 expression were seen, whereas in T_H2 polarizing cells, expression of T-Bet ($p< 0.05$) and IFN-γ decreased and, surprisingly, GATA-3 expression tended to increase. In both conditions, STAT-4 and STAT-6 levels did not change after addition of TGF-β (Fig 4a). Under both T_H1- and T_H2-polarizing conditions, Foxp3 mRNA tended to increase (ns) (Figure 4b). To gain more insight in the signalling cascade via which TGF-β exerts its effect, expression levels of SMAD 3 and SMAD 7 were determined.

Expression levels did not change during the different polarizing conditions, with or without TGF-β addition (data not shown).

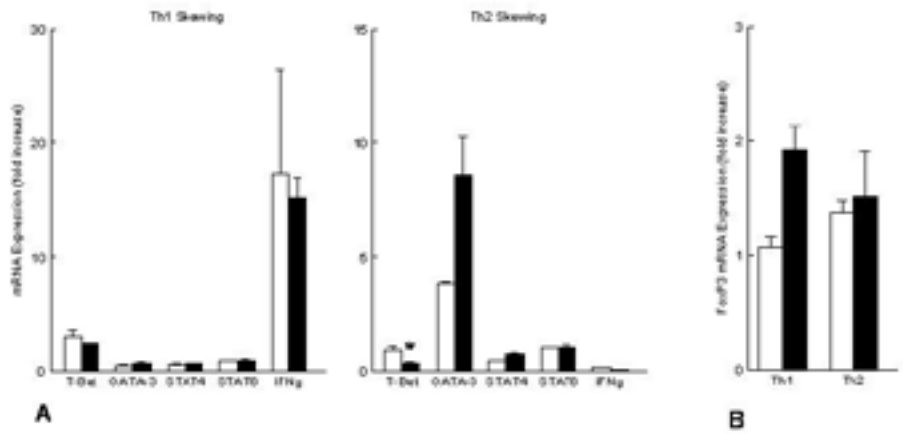

Figure 4.

A) TGF-β modulates mRNA expression levels in cultured cord blood-derived $CD4^+$ T cells.

Cord blood-derived $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb and cultured in the presence (black bars) or absence of TGF-β (white bars) under non-polarizing (control), T_H1 and T_H2 polarizing conditions. Subsequently, mRNA was isolated at 96 hrs after stimulation. The mRNA expression levels of T-bet, GATA-3, STAT4, STAT6 and IFNγ were determined by real-time PCR analysis. The mRNA levels of the control cells are set at a constant value of 1. The mRNA levels of the T_H1 and T_H2 polarized cells are plotted as fold comparison to mRNA levels present in the control cells. Results are expressed as mean ± SEM of 4 independent experiments (* = $p< 0.05$). **B)** Cord blood-derived $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb and cultured in the presence (black bars) or absence of TGF-β (white bars) under non-polarizing (control), T_H1 and T_H2 polarizing conditions. Subsequently, mRNA was isolated at 96 hrs after stimulation. The mRNA expression levels of FoxP3 were determined by real-time PCR analysis. The mRNA levels of the control cells are set at a constant value of 1. The mRNA levels of the T_H1 and T_H2 polarized cells are plotted as fold comparison to mRNA levels present in the control cells. Results are expressed as mean ± SEM of 4 independent experiments.

Discussion

In the present study we analysed the capacity of TGF-β to induce T cells with a regulatory phenotype in human cord-blood derived T cells under T_H1 or T_H2 skewing conditions. We demonstrate that the regulatory phenotype was induced under T_H1 skewed conditions, but not under T_H2 skewed conditions. Although stimulation in the presence of TGF-β enhanced Foxp3 expression in both T_H1 and T_H2 cells, only the T_H1 cells showed an increase in IL-10 production. Furthermore, we demonstrate that the T_H1 phenotype, analyzed by cytokine levels and T-bet expression, was suppressed by TGF-β, whereas the T_H2 phenotype did not seem to be suppressed and even seemed to be enhanced, as shown by an increase of GATA-3. TGF-β did not exert this effect via STAT4 or STAT6.

T_H1 polarization (culturing with IL-12 and anti-IL-4) induced a high production of IFN-γ, which could be down regulated by TGF-β. IFN-γ is known to increase the expression of SMAD7, a TGF-β transcription inhibitor[16]. Hence, inhibition of IFN-γ by TGF-β might lead to lower levels of SMAD7 and thereby an increase in IL-10 production. In our study, although we did see an increase in IL-10 production, no differences in SMAD7 expression levels were detected. However, expression at 96 hours was measured, whereas Kunzmann et al. showed that SMAD7 levels were back at normal expression levels 12 hours after stimulation with TGF-β[17]. The increase in "TGF-β-induced" IL-10 production might also be caused by SMAD4 since Kitani et al. demonstrated that murine $CD4^+$ T cells, transduced with a doxycycline–regulated plasmid which allows for rapid up- and down-regulation of TGF-β1 by in vivo Dox administration, produced large amounts of IL-10, due to TGF-β-induced SMAD4 transcriptional activation of the IL-10 promotor[18]. Unfortunately, we did not measure SMAD4 in our study.

We demonstrate that the IL-10 secretion measured in T cells under T_H2 polarizing conditions is not enhanced after stimulation with TGF-β. This is comparable to what was shown previously in murine $CD4^+$T cells, indicating that T_H2 polarizing conditions make cells less sensitive to the effects of TGF-β[9,18]. A study performed in a HT-2 cell line (producing IL-10 but not IL-4 or IL-5) demonstrated that TGF-β-induced SMAD3 interacts with GATA-3 to form a complex which can up-regulate IL-10 expression[19]. We did not detect a change in SMAD3 expression, and even measured a small increase in GATA-3, next to a further decrease in T-Bet. The different findings between the study from Blokzijl et al. and ours may best be explained by the type of cells used: primary cells used in our study versus the HT-2 cell line. Part of the different responses between T_H1 and T_H2 skewed T cells may be explained by the use of IL-12 and anti-IL-12 in our skewing protocol. IL-12 and

TGF-β share several target genes. Therefore, stimulation of cells with TGF-β might enhance the effect of IL-12 in T_H1 skewing conditions, whereas anti-IL-12 might antagonize with TGF-β in T_H2 skewing conditions[20]. Indeed, induction of IL-10 in $CD4^+CD45RA^+$ T cells, stimulated with anti-CD3 and anti-CD46 was shown to be reduced after addition of IL-12[21]. Our data are comparable with the previously mentioned study in murine T cells in which resembling skewing conditions were used[9]. The inhibitory effect of anti-IL-12 on TGF-β-mediated IL-10 secretion may indicate that IL-12 signalling interferes with the SMAD4 mediated pathway.

Preliminary data indicate that the finding that T_H1 skewed T cells are more sensitive to the differentiating effects of TGF-β compared to T_H2 skewed T cells is also functional, as observed in a MLR assay. The suppression seemed to be consistent in the T_H1 cells, whereas suppression was more fluctuant in the T_H2 cells (data not shown). This was also demonstrated by Godfrey et al.[22]. Indeed, a recent study by Fantini et al. showed that TGF-β-induced $CD4^+CD25^+FoxP3^+$T cells had the functional capacity to suppress T_H1 colitis induced by the adoptive transfer of $CD4^+CD62L^+$T cells[23]. These data suggest that peripherally induced T_{regs} by TGF-β indeed may have a potent anti-inflammatory function in T_H1 mediated diseases.
The data presented here demonstrate that TGF-β in-vitro is able to induce T cells with a regulatory phenotype in naive human CBMC. The immunomodulating effects of TGF-β seem to be most strong and beneficial in T_H1 cells compared to T_H2 cells. In the T_H1 skewed population, the synergistic effects of IL-12 and TGF-β, together with the suppressive effects of IFN-γ seem to lead to cells with regulatory capacities which are able to suppress MLR-induced proliferation. The finding that TGF-β did induce T cells with regulatory capacities in the T_H1 but not in the T_H2 skewed population, and even enhanced the T_H2 phenotype, make TGF-β probably an interesting target for immunomodulating therapies at an early age in T_H1 but not T_H2 mediated diseases

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7

Heat shock protein 60: target of pro-inflammatory autoreactive T cells in children with atopic dermatitis

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Submitted

Abbreviations

T_H2: T helper cells type 2
T_H1: T helper cells type 1
T_{reg}: naturally occurring or induced $CD4^+CD25^+$ regulatory T cells
T_R1: peripherally induced regulatory T cells type 1
T_H3: peripherally induced regulatory T cells type 3
NK T: cells: Natural Killer T cells
Hsp: heat shock protein(s)
PBMC: peripheral blood mononuclear cells
FACS: fluorescence activated cell sorting

Abstract

Background: Atopic diseases can be considered as the result of a disturbance in the balance between T helper cells type 2 (T_H2) and regulatory T cells (T_{regs}). Modulating the regulatory T cell response in an antigen specific fashion could potentially be of therapeutic benefit. Heat shock protein is a stress protein with the capacities to act as bystander peptide in this T cell response.
Objective: Our aim was to investigate whether human heat shock protein 60 (hsp60) is a target for immunomodulation in atopic diseases.
Methods: Peripheral blood mononuclear cells (PBMC) of atopic children were cultured with human hsp60 and proliferative responses, cytokine secretion, surface markers and functional assays were compared to the responses of PBMC of healthy controls.
Results: Human hsp60 was more expressed in skin biopsies of atopic dermatitis. Furthermore, PBMC of atopic children proliferated more strongly in response to self hsp60 compared to healthy controls. Self hsp60-reactive T cells of atopic children produced high levels of IFNγ, and low levels of IL-10. *In vitro* activation with self hsp60 leads to the induction of $CD4^+CD25^{bright}$ T cells expressing FOXP3 in both the healthy controls as well as in the atopic children. However, despite their regulatory phenotype, these hsp60-induced $CD4^+CD25^{bright}$ $FOXP3^+$ T cells were incapable of suppressing effector T cells *in vitro*.
Conclusion: hsp60 is the target of an auto-reactive pro-inflammatory (IFNγ high, IL-10 low) T cell response in atopic children. This suggests that hsp60 specific T cell responses can contribute to local inflammation in children with atopic disease.

Introduction

Atopic diseases are inflammatory disorders caused by the induction of a T_H2 response by an allergen. The immune response is characterized by the production of interleukin-4 (IL-4) and IL-5, IgE production by B-cells and the accumulation of eosinophils[1]. Increasing evidence demonstrates that allergy is not a mere result of an overreactive T_H2 response, but that immune regulatory cells such as NK T cells and regulatory T cells (T_{regs}) play a role in the regulation of this balance[2,3]. T_{regs} can be divided in the naturally occurring, thymus derived, $CD4^+CD25^+$ T_{regs} and peripherally induced populations as T_H3 and T_R1 cells. These cell populations can suppress immunopathological responses, both by cell-cell contact and by the secretion of regulatory cytokines as IL-10 and TGF-β. In active atopic diseases several studies show that the suppressive function of T_{regs} is diminished[4-6]. Specific targeting of these T_{regs} with antigens could provide a novel therapy against inflammation and atopy. Like using synthetic peptide fragments of allergens for antigen specificity, stress antigens, capable to induce bystander activation, might also be beneficial[7]. In autoimmunity and other chronic inflammatory diseases such as atopic dieases heat shock proteins (hsp) are among the most promising antigens for immune modulating therapy aimed at restoration of T_{reg} number and function[8].

Hsp are expressed in eukaryotic and prokaryotic cellular organisms both constitutively and during various events of cellular stress, such as increased temperature (fever) or exposure to pro-inflammatory mediators[9]. The hsp-families are evolutionary highly conserved. Mammalian families of the hsp share a high degree of homology with the microbial hsp, which can result in immunological cross-recognition between these homologues[8,9]. hsp are immunodominant antigens and a common target of T-cell recognition in inflammatory responses. Especially hsp60 seems to be an important regulating antigen in human inflammatory diseases, as it is capable of enhancing the regulatory function of human $CD4^+CD25^+FOXP3^+$ T cells[10]. Furthermore, the expression of hsp is increased specifically at the site of inflammation[8]. In Juvenile Idiopathic Arthritis (JIA), one of the most common childhood inflammatory diseases, self-hsp60 -reactive T cells display an immunoregulatory phenotype, expressing CD30 and producing interleukin 4 (IL-4), interleukin 10 (IL-10) and transforming growth factor (TGF-β)[11].

Altogether, these observations suggest that hsp can activate immunoregulatory pathways in humans and thus could be candidates for antigen specific immune therapy. Indeed, in several human studies immune reactivity to hsp is associated with downregulation of inflammation, possibly by the induction of T_{regs} [12-14] and the first immune therapeutic trials with hsp-derived peptides are now being performed in DM and RA[13,15].

The role of hsp in atopic diseases has not been clarified yet. In asthma, the expression of hsp70 is increased in both bronchial epithelial cells and alveolar macrophages in comparison to healthy controls or controls with other forms of chronic bronchitis[16,17]. An increased expression is also seen in circulating $CD4^+$ T cells of wheezing children[18]. Furthermore, microbial hsp65 (which belongs to the hsp60 family) suppressed IL-4 and IL-5 secretion and enhanced IL-10 and interferon-gamma (IFNγ) secretion in broncho-alveolar fluid in a murine model of airway hyperresponsiveness[19].

In this study, our aim was to investigate whether hsp is a target for T cells in atopic diseases. We found that hsp60 is highly expressed in the skin of atopic dermatitis patients and is recognized by $CD4^+$ T cells secreting high levels of IFNγ and low levels of IL-10.These data suggest that in atopic children, human hsp60 is the target of an auto-reactive pro-inflammatory T cell response in atopic children which may attribute to the chronicity of local inflammation.

Methods

Patients and control subjects

Fifty-two atopic children and 30 healthy controls were included in this study. Eligible patients were children suffering from atopic eczema as defined by Hanifin and Rajka[20]. Underlying atopy was comfirmed by a positive RAST for at least one of the three common food allergens egg, cow's milk or peanut, with a history of a clinical allergic reaction after ingestion of the protein (either parental history or by food challenge). Patient characteristics are given in table 1. Healthy controls were children who underwent a urological or orthopedic surgical procedure. None of them had a history of allergy or a recent infection, nor a first degree relative with a history of allergy or asthma. Both written and oral information about the study was given to the parents and written informed consent from the parents was obtained. The study has been approved by the Medical Ethics Committee of the University Medical Centre, Utrecht, The Netherlands.

	Atopic patients (n=52)	**Healthy controls** (n=30)
Male (%)	73%	78%
Age in years (mean, range)	9,1 (4,9-17,5)	8,6 (1,2-17,3)
Eczema (symptoms before first year of life)	52 (100%)	0
Topical corticosteroid use	52 (100%)	0
Positive food IgE RAST	52 (100%)	0
Egg kU/L (mean,range)	25,6 (0,4-100)	n.a.
Cow's milk kU/l (mean,range)	39,5 (0,4-100)	n.a.
Peanut kU/l (mean, range)	36,6 (0,5-100)	n.a.
Total IgE kU/l (mean, range)	1313,8 (20-5000)	n.a.
Positive skin prick test (egg, cow's milk or peanut)	52 (100%)	0

Table 1.
Patient characteristics. N.a. = non-applicablele

Immunohistochemistry

Biopsy specimens (3 mm) were taken from a healthy and an atopic adult volunteer (for ethical reasons, only adult volunteers were used) under local anaesthesia (Xylocaine) and snap-frozen in liquid nitrogen. The atopic volunteer had chronic atopic dermatitis with a SCORAD of 47. The biopsy was taken from an active lesion and was not treated locally for at least 7 days prior to the biopsy. Subsequently the biopsies were embedded in Tissuetek® (Sakura, Torrance, CA, USA) and stored at -80°C until further handling. The antibody used as marker for immunohistochemical staining of the frozen sections was anti-human hsp60 (LK2, kind gift from P van Kooten, Dept of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands). Single-staining for hsp60 combined with a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc. Burlingame, CA, USA) was performed as described previously[21]. Skin sections were examined by light-microscopy.

Proliferation and direct culture assays with PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by Ficoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation. PBMC were cultured in RPMI-1640 supplemented with 2mM glutamine, 100u/ml of penicillin/ streptomycin (Gibco BRL, Gaithersburg, MD) and 10% (v/v) AB^{pos} heat-inactivated (60 min at 56° Celsius) human serum (Sanquin, Amsterdam, the Netherlands). For measurement of the proliferative activity, $2x10^5$ cells in 200 µl per well were cultured in triplicate in round bottomed 96-wells plates (Nunc, Roskilde, Denmark) for 96 hours at 37° Celsius in 5% CO_2 with 100% relative humidity. For direct cultures, cells were cultured for 7 days. Cells were cultured in the absence or presence of 10 µg/ ml human hsp60 (Faculty of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands). The LPS-content was undetectable (lowest detection level 3 EU/ml). Concavalin A (2.5 µg/ ml, Calbiochem, La Jolla, CA UsA) and tetanus-toxoid (1.5 µg/ ml, RIVM, Bilthoven, The Netherlands) were used as positive controls. A mouse class II restricted peptide (Ova) was used as an irrelevant control. For the final 16 hours of the culture, 1µCi/ well [^{3}H]thymidine (ICN Biomedicals, Amsterdam, The Netherlands) was added to each well. Cells were harvested according to standard procedures and incorporated radioactivity was measured by a liquid scintillation counter and expressed as counts per minute (cpm). The magnitude of the proliferative response is expressed as stimulation index, which is the mean cpm of cells cultured with antigen divided by the mean cpm of cells cultured without the antigen. As additional control on the effect of possible LPS contamination of hsp, proliferation assays were performed in a control group of patients after treatment of hsp (10 µg/ml) or LPS (10 µg/ml; Sigma-Aldrich

Corp, St.Louis,MO,USA), with 10 µg/ml polymyxin B (PmB, Bio-rad Laboratories, Hercules CA, USA) for 1 hour at room temperature or heat inactivation at 95° Celsius for 30 minutes.

Multiplexed particle-based Immuno assay

Cytokine levels were measured after activation of the lymphocytes *in vitro* as described above. After 96 hours the supernatants of the cell cultures stored at -80°C. Cytokines levels of IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13 and IFNγ were measured with the Bio-Plex system and analysed with the Bio-Plex Manager software version 4.0 (Bio-rad) which uses the Luminex xMap technology[22,23]. The peptide specific cytokine production is calculated as the cytokine production of cells cultured with peptide subtracted with the cytokine production of cells cultured without peptide.

Lymphocyte cell surface markers

At day 7 from the direct cell culture assays as described above, PBMC were washed twice in cold fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline [PBS] containing 2% fetal calf serum [FCS] and 0.1% NaN_3). The cells were then incubated in 50 µl FACS buffer containing three appropriately diluted phycoerythrin (PE)-, fluorescencein isothiocyanate (FITC)- or Cy-Chrome (Cy)-labelled monoclonal antibodies (mAb) against human CD4 (clone RPA-T4), CD25 (clones: APC: M-A251; PE: 2A3), CD30 (clone: Ber-H83) and CD69 (clone FN50). After incubation for 20 minutes at 4°C, cells were washed twice in cold FACS buffer and either fixed by incubating for 30 minutes at 4°C in 1% paraformaldehyde or stained for intracellular cytokine levels. Stained mononuclear cells were diluted in sheath fluid and run on a FACSCalibur (Becton Dickinson Biosciences). Cellquest software (Becton Dickinson Biosciences) was used for analysis.

Cytokine analysis by lymphocyte intracellular staining and flowcytometry

Direct T cell lines were generated as described above. During the last 4 hours of culture, Golgistop (Becton Dickinson Biosciences) was added (final concentration of 2 µM). The cells were harvested and stained for intracellular cytokine and FOXP3 analysis as described previously[11]. For the staining, a predetermined optimal concentration of PE-conjugated anti-IL-10 (clone JES3-19F1), anti-IL-4 (clone MP4-25D2), FITC-conjugated anti-IFNγ (clone 4S.B3)(Becton Dickinson Biosciences) and APC-conjugated Foxp3 (Ebioscience, San Diego, CA, USA) was added to the cells. Cells were analysed as described above.

Functional assays

To test the induced $CD4^+CD25^+CD127^-$ for suppressive function, PBMC were cultured for 96 hours as described above in the presence of human hsp60. $CD4^+CD25^+CD127^-$ T cells and $CD4^+CD25^-$ effector T cells from the same donor were directly sorted by FACS (FACS Aria, Becton-Dickinson, San Jose, CA, USA) into plate-bound anti-CD3-coated wells (OKT-3, 1µg/ml) in different ratios (1:0, 1:0.5, 1:1, 1:2). T cell depleted PBMC from the same donor were irradiated (3500 Rad) and were used as APC (1:10). The cells were incubated at 37° C for 96 hours, the last 16 hours of incubation in the presence of [^{3}H]thymidine (1 µCi/well). The suppressive activity was determined by calculating the percentage difference in proliferative response (mean [^{3}H]thymidine incorporation (counts per minute, cpm) of triplicate wells) between $CD4^+CD25^-$T cells cultured alone and $CD4^+CD25^-$ T cells cultured in the presence of induced $CD4^+CD25^+CD127^-$ T cells.

Statistical analysis

Basic descriptive statistics were used to describe the patient population. A non-parametric test (Mann Whitney *U* test, 2-sided) was applied to determine significant differences between the patient and control groups regarding proliferative responses, cytokine production and expression of cell surface markers. Where mentioned, values are noted as mean ± SEM. A p-value < 0.05 was considered significant (SSPS Statistical Program, version 15.0.; SPSS inc, Chicago, Ill.).

Results

Increased expression of hsp60 in skin biopsies of atopic eczema

Hsp are upregulated at sites of inflammation. We therefore expected that the expression of hsp60 was increased in the atopic skin. To demonstrate this, skin biopsies from an adult healthy donor and an adult patient with atopic dermatitis were stained for hsp60 expression. The results are shown in fig.1. Expression of hsp60 was seen in both the healthy skin as in the atopic dermatitis skin, but the pattern of hsp60 expression was strikingly different. The dermis and epidermis of atopic skin was characterized by large cell infiltrates, expressing hsp60, which was not seen to that extent in the healthy skin.

Figuur 1

Figure 1.

Human hsp60 is expressed in the skin. A) Biopsy of skin of a healthy control (HC). Hsp is expressed mostly in the epidermis, and in the cells surrounding small vessels in the dermis. **B**) Biopsy of lesional skin of a patient with chronic atopic dermatitis. Compared to the HC, the thickened stratum corneum and epidermis is typical for atopic dermatitis. In the dermis, infiltration of cells is seen (indicated with arrow). Hsp is mostly expressed in these infiltrates and in the lower layers of the epidermis, whereas coloring grows less dense in the upper areas of the epidermis. x100

Human hsp60 specific proliferation

We next questioned whether this high expression of hsp in the skin leads to an increased recognition of hsp60 by peripheral T cells from atopic individuals. To address this question proliferation of PBMC was determined in response to human hsp60. Proliferative responses of 28 atopic patients were compared with those of 18 healthy controls (HC). Overall, 46% of the atopics had a proliferative response against human hsp60, compared to 22% of the HC (SI>2). In addition, the SI of the PBMC was significantly higher in the patient group compared to the healthy control group (p=0.004). This indicates that PBMC from atopic children proliferate more strongly to human hsp60 compared to PBMC from healthy controls (See Figure 2a).

Human hsp60 specific cytokine induction

Given the increased T cell proliferation to hsp60 by the T cells of atopic children, we next wanted to assess the quality of T cell activation. We first tested which cytokines are produced by hsp60-induced T cells. Hsp-induced cytokine production was measured in culture medium of 26 patients and 22 healthy controls after 96 hours of culture with human hsp60. Figure 2b shows the production of the cytokines tested. Cytokine concentrations are displayed as the mean of the concentration measured after culturing with antigens minus the concentration measured after culturing with medium only. When compared to medium, stimulation with human hsp60 induced a significant higher level of IL-10 in the healthy control group (mean ± SEM: 4.4 ± 1.8, p=0.012), while in the patient group, compared to medium, production of IL-10 was lower after stimulation with human hsp60 (mean ± SEM: -14.77 ± 17.91). A different pattern was seen for IFNγ. Following in vitro stimulation with human hsp60, PBMC from both patients (mean ± SEM: 198.1 ± 140.1) and HC (mean ± SEM: 5.58 ± 4.46) produced higher amounts of IFNγ. Taken together these results demonstrate that stimulating PBMC from atopic patients with human hsp60 leads to the induction of IFNγ but not IL-10. Of the other cytokines tested (IL-4, IL-5, IL-8, IL-12, IL-13) only IL-6 showed an increase after stimulation with human hsp60.

Although monocytes usually do not survive in the conditions used for our T cell cultures, we wanted to be sure that the cytokines measured were indeed secreted by T cells. Therefore, after culturing the cells with or without hsp60, cells were stained for CD4, CD14 and IFNγ. Analysis demonstrated that virtually no monocytes (<1%) were present. Figure 2c shows an overlay histogram of $CD4^{+}CD14^{-}IFN\gamma^{+}$ cells.

To make sure that the effects noticed were not attributable to bacterial contamination, hsp60 with the highest grade of purity was used in our studies (Stressgen, LPS content below the detection level of 3 EU/ml). Moreover, both hsp60-induced proliferation and cytokine production was not influenced by blockage of LPS with either PmB or by heat-inactivation (data not shown) indicating that the observed effects of hsp60 are not attributable to contamination with LPS.

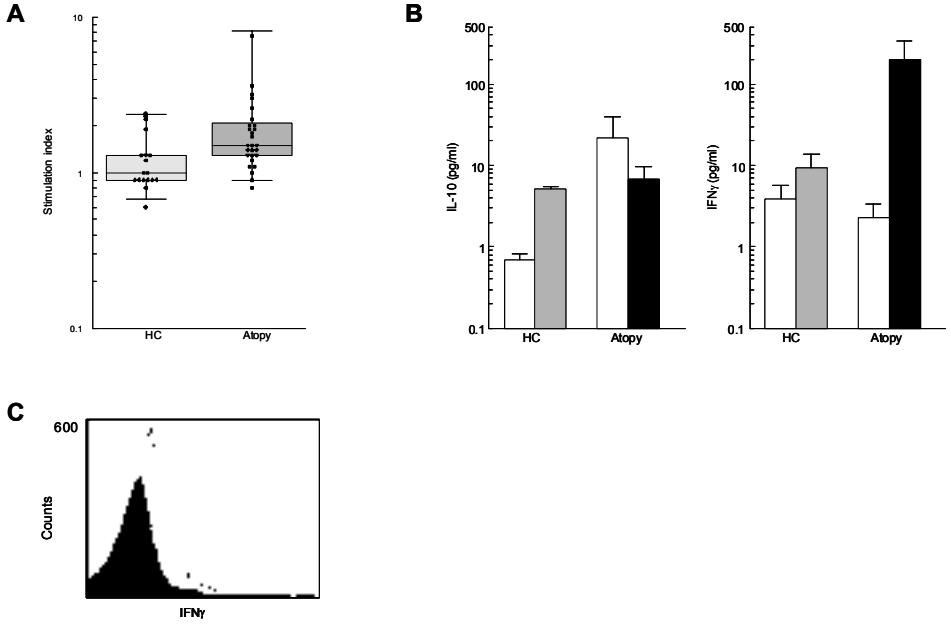
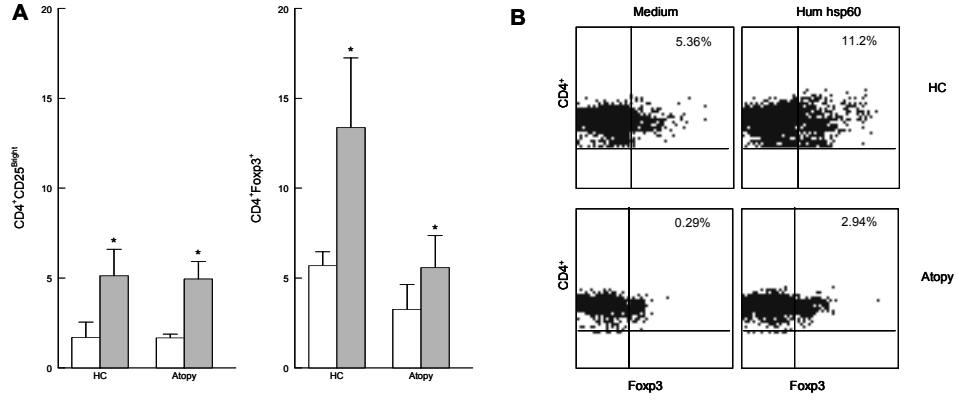


Figure 2.

Human hsp60 is recognized by PBMCs of atopic children and induce cytokine secretion. **A**) Protein-induced T cell proliferation in atopic children and healthy controls. PBMCs were cultured for 96 hours in the presence of Human hsp60. Boxes show IQR (interquartile range) for each group of data, horizontal lines show the median. Significant differences in responses are indicated as follow: * = $p < 0.05$. Light grey boxes = healthy controls, dark grey boxes = atopic children. SI is indicated on a logarithmic scale. **B**) Protein-induced cytokine secretion in atopic children and healthy controls. Boxes show mean levels of cytokine with standard error of mean (SEM). White boxes = medium, grey boxes = healthy controls, black boxes = atopic children. Cytokine levels are indicated on a logarithmic scale **C**) FACS analysis of $CD4^+CD14^-$ T cells from an atopic donor stimulated with hsp60 for 4 days and stained for IFNg. Histogram shows medium level (black) and cytokine level after stimulation with hsp60 (white).

Human hsp60 specific induction of $CD4^+CD25^{Bright}$ T cells expressing the transcription factor FOXP3

The induction of T cells with a regulatory phenotype after *in vitro* activation with hsp60 was previously described by others and us[De Kleer, submitted]. We questioned whether in atopic children *in vitro* activation with hsp60 may lead to the induction of T_{regs}. To address this issue, we first investigated whether the FOXP3 expression in Ag-specific $CD4^+$ T cells was different between HC and atopic children. PBMC of 5 HC and 9 atopic children were cultured for 7 days *in vitro* with human hsp60 (See figure. 3). Stimulation with human hsp60 gave a significant higher percentage of Ag-specific $CD4^+CD25^{Bright}$ T cells and Ag-specific $CD4^+FOXP3^+$ T cells in both the HC and the atopic patients compared to medium only (See Figures 3a, 3b and 3c). Thus, stimulation of T cells with human hsp60 induces T cells with the phenotypical characteristics of T_{regs}, expressing CD25 and FOXP3.



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		Medium	Human hsp60	
Healthy controls	$CD4^+CD25^{Bright}$	1.69±0.72	5.13±3.29	p<0.05
	$CD4^+Foxp3^+$	5.69±1.73	13.37±8.65	p<0.05
Allergic patients	$CD4^+CD25^{Bright}$	1.67±0.21	4.95±0.97	p<0.001
	$CD4^+Foxp3^+$	3.25±1.39	5.58±1.78	p<0.05

Figure 3.

Human hsp60 induces $CD4^+CD25^{bright}$ T cells and the expression of Foxp3. Cells were cultured in human hsp60 for 7 days. **A**) FACS analysis shows a significant higher level of CD4+$CD25^{bright}$ T cells and $CD4^+Foxp3^+$ T cells in both HC as atopic individuals. White boxes = medium only, grey boxes = human hsp60. **B**) FACS analysis of $CD4^+$ T cells expressing Foxp3 after stimulation with human hsp60. **C**) table shows mean percentages ± SEM for the different cell populations after stimulation with human hsp60.

Human hsp60 induced $CD4^+CD25^+CD127^-$ T cells are not suppressive in vitro.

Although $CD4^+$ T cells responding to hsp60 produce IFNγ they also have phenotypical characteristics of T_{regs}. We therefore questioned whether these hsp60 induced $CD4^+CD25^+FOXP3^+$ are functional T_{regs}, thus whether these induced cells

have the capacity to suppress T effector cells. To evaluate the functionality of these induced $CD4^+CD25^+$ T cells, suppression assays were performed on PBMC of 6 atopic patients. Freshly sorted $CD4^+CD25^-$ T effector cells were cocultured with $CD4^+CD25^+CD127^-$ T cells obtained from PBMC precultured for 4 days with human hsp60 (See Figure 4). Despite increased levels of FOXP3 the $CD4^+CD25^+CD127^-$ T cells from these cultures were not suppressive in five of six patients. In only one patient the cells were able to suppress effector cells. In comparison, in HC these cells were indeed suppressive (data not shown). These findings indicate that even though human hsp60 induces T cells that resemble T_{regs} phenotypically, these cells are not capable of suppressing effector T cells in vitro.

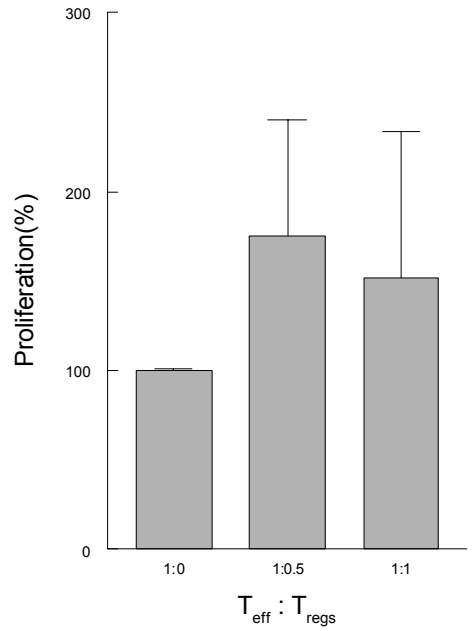

Figure 4.
Human hsp60 induced $CD4^+CD25^+CD127^-$ T cells from atopic patients have no suppressive function. Functional assays were performed as described in the methods section. Only one patient showed suppressive function.

Discussion

In atopic diseases the activity of both allergen-specific IL-10 secreting T_R1-like cells and $CD4^+CD25^+$ regulatory T cells is diminished[5,24,25]. Therefore, it has been speculated whether specific targeting of T_{regs} could provide a novel immunomodulatory pathway for treatment. Up till now, studies have focused on the use of synthetic peptides which contain T cell epitopes derived from known allergens, such as Fel d1 (cat allergy) and Api m1 (bee venom)[26,27]. Another possibility for immunomodulation is the use of bystander antigens, in which the antigen used is not the causative antigen, but an antigen which can alter the reaction by either bystander activation or suppression[28]. Prerequisites for bystander epitopes are expression at the site of inflammation and recognition by immune competent cells such as T cells. For this type of antigen-specific targeting of T_{regs}, hsp60 is a potential candidate[8,10].

An immunomodulatory role for hsp60 has been described in T_H1 diseases such as Diabetes Mellitus (DM) and Juvenile Idiopathic arthritis (JIA) and recent Phase I/II clinical trails with peptides derived from hsp60 and another hsp (dnaJ) had promising results in respectively DM and Rheumatoid Arthritis[15,29]. To date, no information on a potential immunomodulatory role in atopic diseases is available. We hypothesized that in atopic dermatitis due to the chronic inflammation locally, hsp are upregulated and may act as a chronic danger signal. Indeed, as expected the expression of hsp60 is increased in the skin of a patient with atopic dermatitis. In line with this increased local hsp60 expression, we found increased proliferation of T cells from atopic dermatitis patients after stimulation with human hsp60. The phenotypical characteristics of hsp60 induced T cells of children with atopic disease seemed contradictionary at first glance. On the one hand the induction of T_{reg}-like cells was seen, as demonstrated by expression of CD25 and FOXP3. However, these induced T cells proliferated readily, produced IFNγ and could not suppress the activity of T_{eff} cells. This fits with other observations that FOXP3 can be increased in activated effector cells that do not have a suppressive phenotype[30]. Thus, in children with atopic dermatitis hps60 does not induce functional T_{regs} *in vitro* , but, instead induces T cells with a pro-inflammatory phenotype. This suggests that in eczema human hsp60 is a bystander epitope. A bystander antigen is an antigen that is not primarily involved in inflammation, but is upregulated locally and contributes to the ongoing inflammatory process. In this sense we think that hsp60 should not be regarded as the primary trigger in the inflammatory cascade. Instead, during inflammation it is upregulated due to local cellular stress, and becomes the target of a pro-inflammatory T cell response. This is further underlined

when taken the local cytokine profile in the chronically inflamed AD skin into account. This is clearly T_H1-skewed, highlighted with the profoundly expressed levels of IFN-γ both at the level of protein and mRNA[31].

In atopic diseases, immunotherapy is primarily aimed at inducing tolerance towards the allergen, which can be achieved by using peptides that induce a regulatory, IL-10 mediated response towards the allergen[28]. Indeed, IL-10 secreting T cells can reduce allergic inflammation, as for instance, in one study, the adoptive transfer of IL-10 transfected T cells prevented allergen-induced airway hyperreactivity in sensitised BALB/c mice[32].
However, in human atopic diseases, especially in atopic dermatitis, the inducing allergens are not always well defined and/or readily available for immune therapy. In this situation it is still possible to interfere in a local inflammatory process by using a bystander antigen instead of an allergen for immune therapy. Stress proteins, such as hsp60 fit the profile of a bystander antigen and are for that reason now studied extensively in clinical studies in other immune mediated diseases in which disease triggering antigens are not well defined, such as Rheumatoid Arthritis and JIA. Targeting a bystander antigen with antigen specific immune therapy can change the phenotype of these cells from pro-inflammatory to a anti-inflammatory[33]. By altering the immunological environment in which an antigen is presented, e.g. through the induction of mucosal tolerance the cytokine secretion of antigen-specific T cells may be switched from IFNγ towards IL-10. Such change can influence the response not only from the antigen-specific cells but also of neighbouring T cells, thus spreading the IL-10 producing T cell repertoire. This process has been described as infectious tolerance[28,33]. Thus, changing the phenotype of these hsp60-specific T cells with hsp60 (or peptides derived from hsp60) creates an intriguing new immunotherapeutic window of opportunity in atopic dermatitis.
This is the first study to investigate the immunomodulating effects of human hsp60 on PBMC of atopic children. We demonstrated that in PBMC from atopic children hsp60 induced pro-inflammatory T cells which produced high amounts of IFNγ. Further studies are needed to identify the exact peptides from human hsp60 which can modulate the response of the T cells.

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Summary and general discussion

Summary

Treatment and, ultimately prevention of complex diseases such as atopic diseases, should start with the identification of individuals at risk for developing (an) atopic disease(s). But this identification is not easily done since multiple genetic as well as multiple environmental factors are involved, causing the diseases not to follow a simple Mendelian pattern of inheritance. In this respect, gene expression profiles, that is, whether a gene is expressed as mRNA, the first step from gene towards the protein, better reflect both genetic and environmental factors. In the first part of this thesis we explored gene expression profiles in children with wheezing symptoms. Children who persist to wheeze after the age of three are at risk of developing asthma, but recognition of these persistent wheezers is difficult due to several heterogeneous wheezing phenotypes. We found that gene expression profiles not only differentiate between transient and persistent wheezers (Chapter 2 and 3), but that these two distinct wheezing phenotypes also share some common pro-inflammatory genes, such as DUSP2, JunB, TNFAIP3, TNFSF13B and LILRB2 and several genes encoding for heat shock proteins. Three genes, STAT1, TLR7 and PTGER2 which appeared to differentiate between the transient and persistent wheezers proved to distinguish these two phenotypes in a prospective cohort study, thereby recognizing those children at risk to develop asthma (Chapter 4). These genes are involved in the immune response against viral infections (STAT1 and TLR7) and the regulation of bronchial hyperresponsiveness (PTGER2). Interestingly, as stated above, in both the pilot study as well as the large retrospective cohort, the increased expression of several genes encoding for heat shock proteins (hsp) were found when wheezing children were compared with healthy controls. These stress proteins play an important role in the regulation of inflammatory processes and interestingly, their activation secludes both innate as well as adaptive immune responses.

To elucidate the mechanism on if, and if so, how, hsp exert a regulatory role we studied the effects of stimulation of CBMCs with human hsp60 (Chapter 5). The cord blood mononuclear cells are thought to be naive cells, but the immune regulatory capacities of hsp are thought to be innate and might therefore exert certain reactions in CBMCs. Whether certain genetic predispositions influence these effects is of great interest, but first, the exact mechanism by which hsp function should be elucidated. Not only did CBMCs proliferate in response to hsp60 but this recognition led to the induction of suppressive, FOXP3 positive T cells. These suppressive FOXP3 positive T cells are referred to as regulatory T cells. These cells regulate immune responses and evidence is accumulating that a lack of these cells (either in number or function) plays an important role in the immunopathology of atopic diseases.

This induction of suppressive, regulatory T cells is of great interest when exploring new opportunities for therapeutic strategies in atopic diseases. A cytokine known for its immunosuppressive effect is TGF-β. In mice, this cytokine prevents lymphocytic infiltration and spontaneous T helper cell activation. Not only has TGF-β a direct immunosuppressive effect, it is also capable of inducing $CD4^+CD25^+$ T cells with suppressive function. To get more insight in the pathophysiology of the induction of regulatory T cells and the influence of the differentiation status of T cells in this process, a study was performed in which naïve CBMCs of healthy donors were stimulated with TGF-β *in vitro* (Chapter 6). The CBMCs were stimulated either unskewed, or skewed towards a T_H1 or T_H2 type. This skewing process seemed to be of great influence on the effects of TGF-β, since CBMCs skewed towards a T_H2 profile were far less responsive to the influence of TGF-β compared to CBMCs skewed towards a T_H1 profile. This implicates that the mechanisms involved in regulation varies, depending at least in part, to the cell type which is targeted.

As is shown in Chapter 5 hsp are also capable of inducing regulatory T cells in CBMCs of healthy donors. This has also been demonstrated by others in several T_H1-mediated diseases. The role of hsp in atopic diseases is unclear, but a few studies have shown that increased levels of hsp (hsp70 in this particular study) can be detected in asthma. Although its is shown in Chapter 6 that T_H1 and T_H2 cells not necessarily respond in the same manner towards a specific stimulus, because of their capacities hsp are such interesting proteins. We were curious whether hsp were also capable of eliciting a response in PBMCs of atopic children with active allergic symptoms (Chapter 7). The PBMCs of atopic children recognize hsp which induced pro-inflammatory autoreactive T cells *in vitro*. The implication for future perspectives have to be further investigated (see also section future perspectives).

General discussion

Wheezing disorders in children: is it in the genes?

Atopy and consequently atopic diseases are a variety of diseases with the similar immunological background. This immunological background is characterized, at least in the acute phase, by a hyperresponsive T_H2 system towards allergens. The type of allergen, which exerts the reaction, and the target organ in which the inflammatory response occurs, determine the clinical symptoms. Both are dependent on numerous factors. These factors include genetic susceptibility, age and the environment an individual grows up in. They contribute to the large heterogeneity seen in the phenotypes of atopic diseases and make the search for tailor-made therapies even more difficult. Tailor-made therapies are seen as therapies directed at the response towards the allergen, preferably in the target-organ. Ideally, this specific response is modulated in stead of suppressing the immune system a-specifically. The latter is done with local treatment with immune-suppressives.

This phenotypic heterogeneity and the resulting difficulties in choosing proper treatment and (lack of therapeutic) successes become obvious when looking at early infant wheezing disorders. Early wheeze, as seen in a large proportion of children under the age of three, can develop in two separate ways. Either a child has a form of **transient wheeze**, in which the wheezing symptoms will not continue after the age of three, or the child develops **persistent wheeze** and continues to wheeze after the age of three. It is said that the latter group of children is at risk for developing asthma[1]. Several risk-factors for the presence of wheezing symptoms in young children have been described, such as the small airways being narrower compared to healthy children, repetitive viral infections and the influence of the Respiratory Syncitial Virus (RSV) on subsequent wheezing. Whether these factors can also be regarded as risk factors to become a persistent wheezer (PW) is not known. The heterogeneity as seen in this group of wheezing disorders imposes further questions. As has been demonstrated by several studies, early symptoms of asthma result in a subsequent decline in lung function[1]. It has been suggested that this decline is caused by remodeling of lung tissue following chronic inflammation[2]. One can imagine that an early, proper treatment might limit this effect. This poses the first question: which children should be treated as asthmatics? But this heterogeneity makes it also difficult to investigate whether an early treatment of asthma is beneficial in preventing secondary changes in lung function at an older age. Studies investigating this question have demonstrated conflicting results, possibly in a great deal caused by treating not only future asthmatics, but also viral wheezers who are believed to be transient wheezers (TW).

Inflammation at cellular level

Both allergens as well as viral infections can cause a local inflammatory response in the bronchial tissue of the lungs. Several cells of the immune system attribute to this inflammatory response. In general, it is believed that $CD4^+$ T cells orchestrate the process of inflammation both locally as well as systemically as seen in asthma[3]. But other cells, such as mast cells, monocytes, neutrophils, eosinophils and basophils, also contain the inflammation. Obviously, in humans, and especially in children it is virtually impossible to study such cells in depth at the site of inflammation, such as the lungs. However, if these cell types also contribute to a systemic inflammatory response they might form a readout for the inflammatory processes in the lungs. These circulating cells could then be used to search for new insights into the pathophysiology of wheezing and possibly in subsequent development of asthma.

As stated in the introduction of this thesis the microarray technique has introduced the opportunity to study the gene expression of thousands of genes. In the ideal situation, biopsies of the target organ of interest in should be investigated. For obvious ethical reasons this not possible in infant wheezing disorders. The involvement of several cell types in atopic diseases and inflammatory processes such as wheezing makes the selection of the cell type to examine the first challenge in a microarray approach. Guajardo et al. compared samples of nasal mucosa of children with stable versus acute asthma and healthy controls[4]. Although they found gene expression signatures fitting with the diagnosis of acute asthma, the cellular composition showed that the samples of the children experiencing an exacerbation existed of twice the number of neutrophils compared to the other patient group. This heterogeneous cellular composition between different samples highlights the need for a constant cell population to designate a gene expression profile towards a specific disease phenotype. Other microarray experiments did show that circulating cells seem to reflect disease-specific changes in an organ[5]. The role of $CD4^+$ T lymphocytes in the pathophysiology of asthma is known and therefore in our studies these cells were used for gene expression analysis for early recognition of PW[3].

When the gene expression profiles of $CD4^+$ T lymphocytes of TW were compared with those of PW both phenotypes shared the differential expression of several pathways involved in stress-responses (Chapter 2 and 3). Although the children in this study did not have any wheezing complaints for as long as six weeks (PW) or any diseases with fever before blood sampling, an increased expression of hspA1A, a polymorphism in the gene encoding for hsp70 was found in both TW and PW compared to healthy controls. In adult asthmatics a role for hsp70 has been

suggested since the expression of hsp70 is increased in BAL samples in both epithelial cells and PBMCs[6]. The upregulation of RNA of stress proteins, such as heat shock proteins may indicate that even without recent symptoms of wheezing, a parameter of inflammation is still present. This was further demonstrated by the downregulation of JunB, TNFAIP3, DUSP2, LILRB2 and TNSF13B, also in both TW and PW compared to healthy controls. All of these genes are involved in stress responses, either via the MAPK/ERK-pathway or the NF-kB-pathway, which are involved in regulating apoptosis. The decreased expression of these genes suggest that circulating $CD4^+$ T cells in children with present (but not active) wheezing, or a history of wheezing, have an increased and/or prolonged activation state as well as prolonged survival. It can be speculated that these results indicate the presence of chronic inflammation. Interestingly, this seems logic in children with persistent wheezing, as they are regarded to be asthmatics. However, it is remarkable in TW, since these children did not have any wheezing complaints for almost three years, or any other inflammatory disorders. These findings suggest that susceptibility for wheezing, either transient or persistent is still reflected in a prolonged activated state in circulating $CD4^+$ T cells. It remains to be investigated whether an expressed gene is translated into a protein, as is always the case with gene-expression profiling. Furthermore, future studies should aim at discovering the actual function of an altered expression of these genes in human $CD4^+$ T cells. Once these two questions have been addressed, it may become clear what influence these altered gene expressions have in the pathophysiology of wheezing.

Next to the abovementioned uncertainties surrounding the conclusion on the results of this retrospective microarray analysis, another difficulty is encountered when interpreting these retrospectively found gene expression profiles. The study was performed in a small group of children with a history of wheezing, either transient or persistent. To assess the predictive value on persistency of wheezing of these gene expression profiles, a prospective follow-up study in a larger cohort of early wheezers was performed. Children with a doctor's diagnosis of wheezing before the age of three years were included. These children were followed for three years to see whether they had become a TW or PW. In this way, the results found in the retrospective study may both be confirmed and a proper predictive value may be calculated in the right age-group (the young wheezing child).

Viral response or atopic susceptibility?

Currently TW are regarded as viral wheezers, as in these children wheezing is considered a direct (and temporary) consequence of a viral infection. According to this hypothesis, repetitive viral infections cause a form of chronic bronchial inflammation that may result in a response comparable to the allergic inflammatory response in allergic asthma. This has been extensively investigated for RSV, a virus known for causing wheeze after infection[7]. However, the distinction between a viral and an allergic cause of wheezing is difficult to establish since all wheezing infants are likely to respond to respiratory viral infections with an exacerbation of their symptoms. The influence of viral infections and the subsequent inflammatory response was partially reflected in gene expression profiles when comparing the differences between TW and PW (Chapter 3 and 4). In the retrospective study, a decreased expression of STAT1 was found in the TW compared to the PW and healthy controls (Chapter 3). STAT1 is known to be the major intracellular response protein for both IFN-γ as well as IFN-α/β. In a murine study by Hashimoto et al. the absence of STAT1 resulted in airway dysfunction and an increase in airway mucus production after infection with RSV[8]. This would mean that this decreased expression could contribute to increased susceptibility for wheezing after a RSV infection. This could also contribute to the effects seen in asthma, with airway dysfunction and mucus production. In line with this hypothesis, we found that in the prospective study, indeed, a decreased expression of STAT1 was found in the PW, whereas the TW had median levels comparable to healthy controls.

Furthermore, in the retrospective study a decreased expression was also found for Toll-like receptor 7 (TLR7) in TW. TLR7 is activated by single stranded RNA-viruses, such as RSV[9,10]. Recently it was shown that the use of a TLR7 ligand could redirect allergen-specific T_H2 responses as well as allergen-induced hyperresponsiveness[11,12]. Interestingly, an increased expression of TLR7 was found in the PW in the prospective study. What the actual influence of an increased gene expression of TLR7 is on bronchial hyperresponsiveness remains to be investigated. Thus, though viral infections and the immune response against these infections certainly play a role in subsequent wheezing, the immune-pathophysiological mechanisms remain unclear.

The third gene being differentially expressed when comparing TW and PW was the Prostaglandin E_2 receptor (PTGER2). A decreased expression was found in PW in the retrospective study. The ligand for this receptor, prostaglandin E2 (PGE_2) is able to modulate cytokine production of $CD4^+$ T cells towards a T_H2 response[13,14]. Furthermore, PGE_2 induces an increase in IgE production, and is therefore believed to be a mediator in the development of asthma[15]. However, a protective role for PGE_2 against bronchoconstriction has also been described[16]. A decreased expression in PW would therefore not seem logical and indeed in the prospective study, an increased expression was found.

The differences found between the retrospective and prospective study demonstrate

the difficulties in microarray research and in a greater perspective, in human immunological research[17]. Since gene expression is dependent on a great number of factors[18,19], to perform these studies in a human model makes it even a greater challenge. In gene profiling, the behavior of thousands of genes is examined in a relatively small sample size. This is due to the technical restraints encountered in human research. In contrast to research in a specific mouse-strain, in which a genetically identical model with identical environmental factors is used, numerous factors besides the actual disease (in this study TW and PW) which is being investigated can influence gene expression levels in humans. These factors range from differences in diet, life-style and environment to genetic background as a whole[17]. In the microarray studies described in this thesis, the age of the study subjects may also have been of influence of the found differences in gene expression levels. In the retrospective study children with an average age of six years were investigated. These children were either TW or PW, which means that they either did not have had any complaints of wheezing for three years (TW) or were regarded to be asthmatics (PW). On the contrary, in the prospective study early wheezers were investigated, being infants and toddlers with continuous complaints of wheezing. This difference of age and disease stage might be of great influence on gene expression. The prospective design and the more homogeneous study group in the second study make these gene expression levels more reliable. Despite these differences, the recognition of these three genes (STAT1, TLR7 and PTGER2) in relation to wheezing in two separate studies with different, relatively small patient groups may indicate their importance as a potential marker in wheezing phenotypes. This study confirms both the usefulness and the limitations of applying microarrays in patient studies. In order to explain the pathophysiologic mechanisms further research has to be done, but the genes that were discriminative could help to guide such research and to predict the outcome of early wheezers.

For these reasons, the above mentioned three genes were used in a model to predict the persistency of wheezing at an early age. This model was combined with an existing model in which clinical parameters were used (Chapter 3)[20]. The composite score of gene expression profiles with clinical parameters had an increased prognostic capacity above clinical parameters alone and was able to predict persistent wheezing with a positive predictive value of 100%. Although in its current form the score can not be used in general practice (due to technical feasibility), the unique combination of clinical factors with the expression levels of these three genes might provide new possibilities in the recognition and prediction of PW in early wheezers. If it is possible to recognize these children, the effects of early intervention can then be investigated in the right study population.

Figuur 1

Recognition of atopic diseases: the search for biomarkers.

The recognition of STAT1, TLR7 and PTGER2 by microarray analysis and their usefulness as predictors for persistent wheezing indicate that these three genes can be regarded as biomarkers for persistent wheezing. According to Wikipedia, a biomarker can either say something about the risk or progression of a disease, but biomarkers can also be used to tailor treatments for a disease (http://en.wikipedia.org/wiki/Biomarker). According to Cohen, a biomarker is a substance or measurement that indicates important facts about a living organism, usually a patient[21]. These definitions are still rather vague, and a lot of debate is still going on about the right definition. For persistent wheezing, STAT1, TLR7 and PTGER2 seem to be predictive say something about progression of wheezing towards a persistent disease. In the future they may also say something about treatment.

In the search for tailor-made therapies in atopic diseases as a whole, finding the right biomarkers is important. The genes we identified may not only supply a tool to recognize a disease in an early stage, but they may also give new insights into the pathophysiology of wheezing and the progression towards asthma in particular, thereby possibly giving new opportunities for therapies.

In this view, some other genes of which the expression was also differentially upregulated may also be of interest, not only for wheezing and subsequent asthma but also for atopic diseases. These are genes encoding for three members of the heat shock protein (hsp) family, hsp40, hsp70 and hsp90.

As stated earlier in this thesis, hsp are stress proteins with an increased expression at sites of inflammation[22]. Furthermore hsp are immunodominant and capable of exerting adaptive and innate immune reactions. Cohen found hsp to be part of a set of self-antigens which are recognized by the adaptive immune system of healthy individuals, that is, without auto-immune diseases[23]. Cohen termed this natural auto-immunity the “immunological homunculus” and stated that these self-antigens could serve as a set of biomarkers[21].

Are heat shock proteins biomarkers?

Besides a role as biomarker, the role for hsp as stress protein capable of regulating inflammatory events is also seen in several auto-immune diseases, such as Idiopathic Juvenile Arthritis, Diabetes Mellitus and Dermatomyositis[24-26]. Not only could a marked increase be demonstrated at sites of inflammation, but also an immunomodulatory effect towards regulatory T cells was noticed. Interestingly, Cohen states that another role of these self-antigens is to initiate and regulate inflammatory processes to protect and maintain the body whenever damage occurs[21]. How this mechanism works and what the regulatory potential of these self-antigens is has not been fully understood yet. According to Cohen, one of the prerequisites of a biomarker from the immunological homunculus is its recognition by naïve cells, as early as *in utero.* We therefore set out to study whether hsp can be recognized by cells in cord blood, and if so, what the nature of such immune recognition would be. We found that when cord blood mononuclear cells (CBMC) were stimulated with human hsp60 (one of the four hsp found in the study by Cohen et al.[23]) $CD4^+$ T cell proliferation increased, whereas stimulation with tetanus toxoid (TT) did not lead to an increased proliferation (Chapter 5). Interestingly, the responding cells were mainly CD45RO cells. This may indicate that CBMC may have memory T cells capable of responding to a self antigen such as hsp.

Next, we investigated the quality of this response and found that stimulation of CBMC with hsp led to the induction of suppressive regulatory T cells *in vitro*, suggesting that Hsp are self-antigens with an immune-regulatory role.

Thus, CBMC can recognize self-hsp60 and this recognition leads to the induction of cells with possible immunoregulatory cells, pointing at an immunoregulatory mechanism that may be present as early as *in utero*. Since an atopic predisposition may also be present as early as *in utero*[27], it is of interest whether these mechanisms somehow influence each other, which needs to be further investigated.

Regulatory T cell dysfunction in atopic diseases.

The role of transcription factors

The role of regulatory T cells in atopic, T_H2-mediated diseases has been of great interest ever since the discovery of these regulatory T cells. Several studies have shown that in atopic diseases naturally occurring T_{regs} are less abundant and the T_{regs} available are less potent to suppress immunological reactions[28,29]. This is especially of interest in the search for immunotherapeutic options, as redirection of the T_H2 immune response by the induction of a regulatory response seems a logical goal. However, the mechanisms underlying this apparent shortcoming of the regulatory system in atopic diseases are not clear. In atopic diseases, a genetic predisposition is described as early as *in utero* by diminished production of IFNγ after *in vitro* antigen stimulation, compared to healthy controls indicating a persistence of the T_H2 phenotype after birth[30]. Normally, in non-atopic individuals, the immune phenotype is redirected more towards a balanced T_H1 and T_H2 phenotype soon after birth[30P]. Thus, children with an atopic predisposition seem to have a delayed development of a counter regulatory T_H1 response in early life. How this predisposition further influences the regulatory response and vice versa is not known. But it is known that in the absence of functional regulatory T cells several auto-immune and allergic manifestations can occur. This is clear in the IPEX syndrome (immune dysregulation, polyendocrinopathy, entheropathy, X-linked syndrome), where a mutation in the gene of FOXP3 leads to dysfunctional T_{regs}[31]. Next to several auto-immune disorders, patients suffering from this disease also have severe food allergies and eczema.

We questioned whether skewing of CBMCs towards either a T_H1 or T_H2 phenotype may influence the capacity of TGF-β, a cytokine known for its capacity to control T_{regs} development (Chapter 6). We found that CBMC derived $CD4^+$ T cells that were skewed towards a T_H1 phenotype produced high amounts of IL-10 upon stimulation with TGF-β and showed increased expression of FOXP3. These cells seemed to have a suppressive capacity *in vitro*, although this has to be replicated in larger numbers. Stimulation with TGF-β also led to a decrease in the expression of T-Bet, the major T_H1 transcription factor, in both T_H1 and T_H2 skewed cells. Although the increase in expression of FOXP3 was also seen in $CD4^+$ T cells skewed towards the T_H2 phenotype, this increase did not lead to an increase in secretion of IL-10, nor did these cells have suppressive capacity. Furthermore a small increase in GATA-3, the major T_H2 transcription factor was seen.

One explanation for these differences in effect of stimulation of TGF-β on skewed $CD4^+$ T cells is that for the skewing of these cells towards a T_H1 or T_H2 direction, respectively IL-12 and anti-IL 12 was used. This cytokine shares several target genes with TGF-β and especially the use of anti-IL-12 may have antagonized the effects of TGF-β. In addition Mantel et al. demonstrated recently that GATA-3 driven T_H2 responses inhibit the expression of FOXP3 induced by TGF-β and thereby the induction of T_{regs}[32]. After differentiation towards a T_H2 phenotype, these cells become refractory to conversion into $FOXP3^+$ T cells. When they studied the FOXP3 promotor site, a binding site for GATA-3 was also discovered[32]. Experiments showed that GATA-3 binding to this site represses FOXP3 expression. Whether this effect is only of influence in peripheral induction of T_{regs} or also plays a role in the presence of naturally occurring T_{regs} is not known. What is known is that the

transcription factors T-Bet and GATA-3 commit cells towards a T_H1 or T_H2 phenotype respectively. After this commitment, upregulation of the expression of a different "decisive" transcription factor seems more difficult. The atopic predisposition leading to an immunological environment skewed towards a T_H2 phenotype indicates that this skewing decreases the capacity to induce T_{regs}. The counter play between the different transcription factors and the difficulties met when T cells have already been differentiated also illustrate the difficulties encountered in search for new immunomodulatory therapies.

Immunomodulation in atopic diseases: how, what, where?

Immunotherapies for atopic diseases have focused on either redirecting the immune response towards a T_H1 phenotype or targeting T_{regs} towards the specific allergens. The latter studies have focused on the use of synthetic peptides which contain T cell epitopes derived from known allergens, such as Fel d1 (cat allergy) and Api m1 (bee venom)[33,34]. The use of immunostimulatory sequences, consisting of synthetic oligonucleotides containing CpG motives, are also of interest[35]. As agonists of TLR4 and/ or TLR9 these sequences are capable of inducing T_H1-biased immune responses, thereby preventing a T_H2-biased immune deviation[36-38]. Another possibility for immunomodulation is the use of bystander antigens, in which the antigen used is not the causative antigen, but an antigen which can alter the reaction by either bystander activation or suppression[39]. Prerequisites for bystander epitopes are expression at the site of inflammation and recognition by immune competent cells such as T cells. Interestingly, hsp are known for these capacities. Furthermore, as stated above, hsp also have the capacity to induce T_{regs} and are seen as self-antigens which exert a form of autoimmunity to induce regulatory T cell responses. One of the members of this family and a potential candidate for this type of antigen-specific targeting of T_{regs} is human hsp60[22,40]. In several T_H1 mediated diseases hsp60 proved to be a potential candidate for immunotherapy by modulating the immune response towards a more regulatory one.

In atopic diseases such as asthma other members of the hsp family may also play a role, such as hsp70. This was indicated in this thesis (Chapter 1 and 2) as well as previously by Bertorelli et al[6]. Since the immunomodulatory effects of hsp60 are well known in other diseases, we chose hsp60 to investigate whether it could also function as bystander epitope in atopic diseases (Chapter 7). When PBMCs of children with eczema and sensibilisation to one of the three major food allergens egg, cow's milk or peanut, were stimulated with hsp60, these cells recognized hsp. This recognition led to the induction of $CD4^+FOXP3^+$ T cells secreting large amounts of IFNγ. Furthermore, although these cells did express FOXP3, they were not suppressive *in vitro*. This indicates that hsp60 induces autoreactive pro-inflammatory T cells *in vitro* in PBMCs of atopic children, which is in contrast with the effects seen in PBMCs of T_H1 mediated diseases such as Juvenile Idiopathic Arthritis[41]. These findings suggest that in eczema human hsp60 is a bystander epitope that may contribute to the local and systemic inflammation. As a bystander epitope, hsp60 should not be regarded as the primary trigger in the inflammatory cascade. Instead, during inflammation it is upregulated due to local cellular stress. Our data indicate that this upregulation leads to an autoreactive pro-inflammatory T cell response. This effect creates an intriguing possibility to use hsp60 (or peptides derived from hsp60) as targets for immune therapy in atopic diseases. As is shown by Horner et al. the immunomodulatory capacities in immunotherapy are dependent on the site of administration, whereby the mucosal administration cannot only induce mucosal tolerance but also a systemic modulatory response[42]. By altering the immunological environment in which an antigen is presented, e.g. through the induction of mucosal tolerance, cytokine secretion may be switched towards IL-10 producing T cells instead of IFNγ and thereby change the response not only from the antigen-specific cells but also of neighbouring T cells, thus spreading the IL-10 producing T cell repertoire. This process has been described as infectious tolerance[39,42]. Whether the administration of hsp60 can indeed lead to infectious tolerance has to looked at further, but the above mentioned findings indicate that bystander epitopes such as hsp's may lead to novel opportunities for immunotherapy in atopic disease.

Figuur 2

Future perspectives.

This thesis handles two concepts in atopic diseases: the early recognition of individuals at risk and possible mechanisms for immunomodulation. The heterogeneity seen in the different manifestations of atopic diseases make the search for new therapeutic options difficult. When the individuals at risk can be recognized, the effects of tailor-made therapies can be assessed more easily and

more accurately. The studies on gene expression profiles demonstrate that differential expression of genes can be used as a tool for this early recognition. The observation of STAT1, TLR7 and PTGER2 being differentially expressed in two different cohorts of children make this observation very strong. Functional studies should be performed to analyze the immuno pathophysiological role of these three genes in the process of wheezing, and possibly subsequent asthma. Ideally, these functional studies should not be performed in another mouse model in which genetic strain and environment are well controlled. As these microarray studies show, the interplay between all these factors influence the gene expression and exactly this interaction influences the function of the gene in that system.

Thus treatment of atopic diseases starts with the recognition of the right individual who may benefit from the treatment supplied. The next step is then to discover derangements in the immune system of individuals with atopic diseases and to redirect these immunological derangements towards the favored immune response. The question remains what the favored immune response is. Contrary to auto-immune diseases, which are reasonably homogeneous in their hyperreactive T_H1 response, atopic diseases are also heterogeneous in their immune response. It becomes increasingly clear that the chronic phase of allergic inflammation, as seen in chronic eczema for instance, is contained by a T_H1 response, whereas the acute phase is a T_H2 response. Thus, next to recognizing the individual at risk, the second step might be the recognition of the immune response which should be re-educated. Immune regulatory processes could be directed at the allergen-specific T_H2 cells (which may also be done by the induction of IFNγ) or by redirecting the chronic inflammatory response, where, obviously, there is no need for more IFNγ. The epitope used and the site where this epitope will be administered will be of great influence on the effects obtained. Future studies need to further elucidate the specific effects of hsp on different forms of atopic disease and the influence of the site of administration on which effect is exerted.

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