Exploring immunological mechanisms in cow’s milk allergy
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GENERAL DISCUSSION AND PERSPECTIVES
INTRODUCTION

CMA is the most common food allergy in infancy. To date the gold standard for the diagnosis of CMA is the DBPCFC which has its limitations, including the risk of a severe anaphylactic reaction.\textsuperscript{(1)} Treatment of CMA is based on strict elimination of CMP from the infant’s diet, which may have major impact on the quality of life of patients and their families.\textsuperscript{(2)} Fortunately, the majority of CMA infants will regain tolerance to CMP before the age of three years.\textsuperscript{(3)} However, infants with CMA are more prone to the development of other allergic disorders later in childhood; a process also termed the allergic march.\textsuperscript{[4,5]} Currently, the pathogenesis of CMA and the development of tolerance is poorly understood. Whereas the prevalence of food allergy is increasing worldwide,\textsuperscript{(6,7)} improvement of the current knowledge on the pathogenesis underlying CMA is urgently needed. A better understanding of the pathophysiology of CMA may provide clues for the development of preventive, diagnostic and therapeutic strategies.

The studies in this thesis have attempted to extend our insight of the pathogenesis of CMA by focusing on the role of B- and T-cells in CMA in infancy and the development of tolerance to CMP and the development of other allergic disorders later in childhood. In the next paragraphs, the most relevant findings of this thesis are discussed. In addition, suggestions for future research are provided.

CMP-SPECIFIC T-CELL RESPONSES

CMP-specific T-cells are presumed to play a major role in CMA. Several studies have shown that CMA is characterized by a Th2-skewed CMP-specific T-cell response,\textsuperscript{(8,9)} while the absence of CMA has been associated with enhanced production of regulatory cytokines, such as IL-10.\textsuperscript{(10,11)} Important limitations of previous studies are that a DBPCFC was used to diagnose CMA only in a limited number of studies and that most studies have been performed in older children with CMA. To our knowledge, there are no studies which have investigated the CMP-specific T-cell response in infants in association with the development of tolerance to CMP or persistency of CMA later in childhood.

In Chapter III we presented the first controlled prospective follow-up study on the role of T-cells in the pathogenesis of CMA in infancy and the development of tolerance later in childhood. Both at diagnosis and follow-up a DBPCFC was performed to assess clinical hypersensitivity to CMP. In this thesis we demonstrated for the first time that the CMP-specific T-cell response of children with persistent CMA beyond the first year of life is characterized by a combination of enhanced CMP-specific CD4+ T-cell proliferation, IL-10 production and a Th2-skewed cytokine pattern in infancy. These data support the role of Th2-cytokines in IgE-mediated disease by regulating isotype switching and differentiation of B-cells into plasma cells that produce and secrete IgE. In addition, we found that IL-10 production was found significantly decreased in the group of infants with CMA in
comparison to NA-controls. These data emphasize the presumed importance of regulatory cytokines such as IL-10 in suppressing the Th2-skewed response and thereby preventing clinical disease.

The presumed important role of cytokines in regulating the immune response and thereby preventing or inducing clinical reactivity to food allergens was further investigated in Chapter VI. In this chapter we compared plasma cytokine levels in food allergic with food tolerant children to attain more insight in the contribution of cytokines to a food allergic response and the development of tolerance. We found that plasma levels of IL-25 (or IL-17E), a recently identified member of the IL-17 family of cytokines, were highly elevated in children with a proven clinical peanut allergy in comparison to NA-controls. Furthermore, we found low levels of IL-25 (1.5-14 pg/ml) in 42% of CMA-infants (n=12). Interestingly, further analysis of the data showed that 4 of the 5 infants with elevated IL-25 in the CMA group had elevated levels of food-specific IgE later in life. Together these data may indicate that elevated plasma IL-25 is a sign of chronic immune activation that is not induced by the provoking allergen itself and reflects a persistent or severe allergic subtype.

In conclusion, our results suggest that CMP-specific T-cell cytokine production plays an important role in the presence or absence of clinical reactivity to CMP in infancy and persistency of CMA later in childhood. Long term follow-up of our study population may reveal if a Th2-skewed CMP-specific T-cell response in CMA-infants is associated with the development of other allergic disorders later in childhood. However, because our data were established in a relatively small study population, it is of great importance that our results will be confirmed in a large prospective controlled, randomized study. In our opinion future research should be aimed at the development of immunological strategies to modulate the CMP-specific T-cell immune response in order to prevent or to overcome clinical reactivity to CMP. We feel that future studies should be focused on the development of immunotherapies which are based on utilizing engineered proteins, for example by immunomodulation of CMP-specific T-cell epitopes as discussed in the next paragraph.

**CMP-EPITOPE-SPECIFIC T-CELL RESPONSES**

As described in Chapter III, no difference in CMP-specific T-cell proliferation was found between CMA-infants and NA-controls. Consecutively we proposed that CMA-infants recognize different CMP-specific T-cell epitopes than NA-controls. Previously four studies have been reported on the identification of T cell epitopes on CMPs, which were either focused on αs1-casein or β-lactoglobulin, of which three were performed in low numbers of subjects without comparing T cell epitope recognition with a control group. Ruiter et al. identified a region on αs1-casein recognized by T cells from children tolerant to CMPs and not by T
cells of children with IgE mediated CMA. In the present study we used a novel matrix-based computer algorithm designed to identify pan-DR-binding T cell epitopes. The selected potential CMP-specific T-cell epitopes were synthesized as peptides and next we compared the peptide specific T cell responses of older CMA-children and NA-controls in a pilot study. The results of this pilot study were encouraging in that way that four peptides were recognized solely by CMA children, of which two were recognized by 67% and two epitopes by 44% of the subjects. However, when we tested the proliferative response of these peptides to CMP-specific T cell lines from 11 infants with CMA and 9 NA controls, we observed no difference in proliferative responses between infants with and without CMA. The observed difference in recognition of peptides between the pilot study and the study in infants may reflect differences in age, CMP-specific IgE status and test-analyses. For example, in the pilot study PBMCs were used to test epitope-specific proliferative responses, while in the second study CMP-specific T-cell lines needed to be created, because of the limited amount of blood which could be collected of the infants due to ethical considerations. We conclude that identification of CMP-specific T cell epitopes in infants is difficult, which may be explained by the facts that the frequency of allergen specific T cells in peripheral blood is very low (in the order of 1:10^4 – 5:10^4) and CMPs are commonly known as weakly stimulating antigens. However, we feel that the results of both previous studies and our pilot study have shown that CMP-specific T-cell epitopes should be considered as potential targets for the development of diagnostic, preventive and therapeutic strategies. Therefore, in our opinion future research in this field is warranted.

**CMP-SPECIFIC B-CELL RESPONSES**

The role of CMP-specific B-cells in CMA has been studied extensively. CMP-specific IgE has been shown to play a major role in the CMP-specific B-cell mediated immune response. CMP-specific IgE has been shown to elicit immediate type clinical allergic reactions by binding to Fc receptors on mast cells and thereby inducing mast cell degranulation and release of mediators such as histamine.

Several studies have shown that more than 50% of children with CMA have detectable CMP-specific IgE levels in serum. In contrast, we found detectable CMP-specific IgE levels in only 30.8% of CMA-infants (Chapter II). However at age 1 and 2 years the percentage of detectable CMP-specific IgE levels in CMA-children increased to more than 50% (Chapter III). Differences in our observations and previous reported CMP-specific IgE levels in CMA-children may be explained by differences in age and the fact a DBPCFC was only performed in a limited number of previous studies.

Numerous studies have related the presence and levels of serum CMP-specific IgE in children with CMA with the development of tolerance or persistency of CMA
It has been shown that infants with non-IgE mediated CMA develop tolerance to CMP earlier in childhood than infants with IgE mediated CMA. In line with these studies we found that CMA-infants with elevated serum CMP-specific IgE-levels are likely more prone to persistent CMA than CMA-infants with no detectable CMP-specific IgE-levels in infancy. Taken together, our results subscribe the major contribution of CMP-specific IgE in CMA and the persistency of CMA beyond the first year of life.

Previous studies have shown that immediate type allergic reactions can occur in CMA-children who do not have detectable CMP-specific-IgE levels. In our study population we found that only 50% of infants with immediate type allergic reactions to CMP had raised serum CMP-specific IgE levels (Chapter III). Therefore, we hypothesized that immediate type allergic reactions may be caused by other mediators which can elicit mast cell degranulation, for example immunoglobulin free light chains (Ig-fLC). Ig-fLC have been shown to possess antigen specific binding activity and elicit mast cells degranulation in mice. Up till today, data on Ig-fLC in human models of allergy are scarce. Concentrations of total Ig-fLC have been demonstrated to be significant higher in the sera of patients with allergic asthma and allergic rhinitis as compared to healthy non-allergic controls.

In Chapter V we presented the first clinical study on the contribution of Ig-fLC in CMA. We showed that plasma Ig-fLC levels were significantly higher in a group of CMA- infants in comparison to NA-controls. In addition, we showed in a mice model that sensitization with CMP can lead to both IgE- and Ig-fLC dependent clinical allergic reactions. Our results emphasize the presumed role of Ig-fLC in CMA and the possible diagnostic and therapeutic value of Ig-fLC in CMA and other food allergies. To date, an important restriction in the utility of Ig-fLC as a diagnostic tool is the fact that detection of CMP-specific Ig-fLC is not possible. Therefore, studies aimed at the development of methods to detect CMP-specific Ig-fLC are necessary to further explore the diagnostic properties of Ig-fLC in CMA and are currently under analysis. Next, other efforts need to be addressed to develop reference data and predicted values of CMP-specific Ig-fLC in CMA-subjects.

CONCLUSION

The studies performed in this thesis have resulted in a better understanding of the immunological mechanism underlying the pathogenesis of CMA in infancy and the development of tolerance to CMP or persistency of CMA beyond the first year of life. It was found that the CMP-specific T-cell response of children with persistent CMA beyond the first year of life is characterized by a combination of enhanced CMP-T cell reactivity, IL-10 production and a Th2-skewed cytokine pattern in infancy.

Follow-up of our study population may reveal if a Th2-skewed cytokine pattern in CMA-Infants is associated with the development of other allergic disorders.
later in childhood. Because our data were established in a relatively small study population we feel that future research should initially be aimed at confirmation of our results, for example in a large controlled, randomized prospective study. Consecutively, studies should be aimed at the development of new diagnostic methods and immunological strategies to modulate the CMP-specific T-cell immune response in order to prevent or overcome clinical reactivity to CMP and possibly to prevent the development of other allergic disorders later in childhood.
REFERENCES


