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Cow's milk allergy

and the development of tolerance

Hospital for Children and Adolescents

University of Helsinki

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ACADEMIC DISSERTATION

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To my father

Contents

ABSTRACT 7

LIST OF ORIGINAL PUBLICATIONS 9

ABBREVIATIONS 10

REVIEW OF THE LITERATURE 12

Introduction to cow's milk allergy 12

Oral tolerance and cow's milk allergy 12

Clinical aspects of cow's milk allergy 16

Diagnosis 16

Symptoms 17

Prognosis 17

Genetics in cow's milk allergy and other allergies 19

Filaggrin gene 19

Human leukocyte antigen II genes 19

Protein tyrosine phosphatase, non-receptor type 22 (lymphoid) 21

The role of antibodies in cow's milk allergy and tolerance 22

Physiological humoral response to cow's milk 22

Immunoglobulin class E 22

Immunoglobulin isotype class G 23

Immunoglobulin class A 24

Epitope recognition by antibodies in cow's milk allergy 25

Regulatory T cells in allergy 27

Subpopulations of regulatory T cells 27

Markers for regulatory T cells 27

The role of regulatory T cells in allergies 30

A paradigm for the development of oral tolerance and cow's milk allergy 32

AIMS OF THE STUDY 34

MATERIALS AND METHODS 35

Study population 35

DNA extraction and genotyping 37

Measurement of antibodies 37

Peptide microarray based immunoassay 39

Image analysis in microarray based immunoassay 40

PBMC extraction and *in vitro* stimulation 41

RNA extraction and reverse transcription 41

Real time polymerase chain reaction (QRT-PCR) 42

Flow cytometry 42

Statistical analysis 43

Bioinformatic analysis in study III 43

Artificial neural network analysis 44

RESULTS 46

Association of selected genetic factors with humoral responses to early food allergens (I) 46

Development of humoral responses to cow's milk proteins in patients with CMA over time and differences compared to children without CMA at follow-up (II-III) 47

Levels of specific IgE to CM evolved differently in patients with persisting CMA and patients with early recovery (II) 47

Patients with persisting CMA had more intense and stable IgE peptide binding over time than patients with early recovery (III) 48

Patients with persisting CMA had lower IgG4 levels to β-lactoglobulin at follow-up than patients with early recovery (II) 48
IgG4 peptide binding patterns were comparable in persisting and early recovering CMA (III) 49
IgE and IgG4 binding to a panel of peptides at diagnosis discerned patients with persisting CMA from patients recovering early (III) 49
β-lactoglobulin specific IgA levels were higher in patients with early recovery (II) 49
IgA binding to CM epitopes increased over time more in patients with persisting CMA (III) 50
Peptide recognition by IgE had overlap with IgG4, but not with IgA (III) 50

Summary of differences in specific antibody responses between patients with persisting CMA and those with early recovery (II-III) 51

T regulatory cell and Th2 expression profile in children with CMA (IV) 52

DISCUSSION 53

Genetic factors modulating the immune responses to early oral antigens 53

Antibodies in fostering or impeding oral tolerance 54

Balance of T regulatory and T effector cells in CMA and development of tolerance 57

Methodological considerations 59

Implications for CMA diagnostics and prognostics 61

SUMMARY AND CONCLUSIONS 63

ACKNOWLEDGMENTS 64

REFERENCES 66

Abstract

Aims: To gain insight on the immunological processes behind cow's milk allergy (CMA) and the development of oral tolerance. To furthermore investigate the associations of HLA II and filaggrin genotypes with humoral responses to early oral antigens.

Methods: The study population was from a cohort of 6209 healthy, full-term infants who in a double-blind randomized trial received supplementary feeding at maternity hospitals (mean duration 4 days): cow's milk (CM) formula, extensively hydrolyzed whey formula or donor breast milk. Infants who developed CM associated symptoms that subsided during elimination diet (n=223) underwent an open oral CM challenge (at mean age 7 months). The challenge was negative in 112, and in 111 it confirmed CMA, which was IgE-mediated in 83. Patients with CMA were followed until recovery, and 94 of them participated in a follow-up study at age 8-9 years. We investigated serum samples at diagnosis (mean age 7 months, n=111), one year later (19 months, n=101) and at follow-up (8.6 years, n=85). At follow-up, also 76 children randomly selected from the original cohort and without CM associated symptoms were included. We measured CM specific IgE levels with UniCAP (Phadia, Uppsala, Sweden), and β-lactoglobulin, α-casein and ovalbumin specific IgA, IgG1, IgG4 and IgG levels with enzyme-linked immunosorbent assay in sera. We applied a microarray based immunoassay to measure the binding of IgE, IgG4 and IgA serum antibodies to sequential epitopes derived from five major CM proteins at the three time points in 11 patients with active IgE-mediated CMA at age 8-9 years and in 12 patients who had recovered from IgE-mediated CMA by age 3 years. We used bioinformatic methods to analyze the microarray data. We studied T cell expression profile in peripheral blood mononuclear cell (PBMC) samples from 57 children aged 5-12 years (median 8.3): 16 with active CMA, 20 who had recovered from CMA by age 3 years, 21 non-atopic control subjects. Following *in vitro* β-lactoglobulin stimulation, we measured the mRNA expression in PBMCs of 12 T-cell markers (T-bet, GATA-3, IFN-γ, CTLA4, IL-10, IL-16, TGF-β, FOXP3, Nfat-C2, TIM3, TIM4, STIM-1) with real time polymerase chain reaction, and the protein expression of CD4, CD25, CD127, FoxP3 with fluorescence-activated cell sorting. To optimally distinguish the three study groups, we performed artificial neural networks with exhaustive search for all marker combinations. For genetic associations with specific humoral responses, we analyzed 14 HLA class II haplotypes, the PTPN22 1858 SNP (R620W allele) and 5 known filaggrin null mutations from blood samples of 87 patients with CMA and 76 control subjects (age 8.0-9.3 years).

Results: High IgG and IgG4 levels to β-lactoglobulin and α-casein were associated with the HLA (DR15)-DQB1*0602 haplotype in patients with CMA, but not in control subjects. Conversely, (DR1/10)-DQB1*0501 was associated with lower IgG and IgG4 levels to these CM antigens, and to ovalbumin, most significantly among control subjects. Infants with IgE-mediated CMA had lower β -lactoglobulin and α-casein specific IgG1, IgG4 and IgG levels ($p<0.05$) at diagnosis than infants with non-IgE-mediated CMA or control subjects. When

CMA persisted beyond age 8 years, CM specific IgE levels were higher at all three time points investigated and IgE epitope binding pattern remained stable ($p<0.001$) compared with recovery from CMA by age 3 years. Patients with persisting CMA at 8-9 years had lower serum IgA levels to β-lactoglobulin at diagnosis ($p=0.01$), and lower IgG4 levels to β-lactoglobulin ($p=0.04$) and α-casein ($p=0.05$) at follow-up compared with patients who recovered by age 3 years. In early recovery, signal of IgG4 epitope binding increased while that of IgE decreased over time, and binding patterns of IgE and IgG4 overlapped. In T cell expression profile in response to β –lactoglobulin, the combination of markers FoxP3, Nfat-C2, IL-16, GATA-3 distinguished patients with persisting CMA most accurately from patients who had become tolerant and from non-atopic subjects. FoxP3 expression at both RNA and protein level was higher in children with CMA compared with non-atopic children.

Conclusions: Genetic factors (the HLA II genotype) are associated with humoral responses to early food allergens. High CM specific IgE levels predict persistence of CMA. Development of tolerance is associated with higher specific IgA and IgG4 levels and lower specific IgE levels, with decreased CM epitope binding by IgE and concurrent increase in corresponding epitope binding by IgG4. Both Th2 and Treg pathways are activated upon CM antigen stimulation in patients with CMA. In the clinical management of CMA, HLA II or filaggrin genotyping are not applicable, whereas the measurement of CM specific antibodies may assist in estimating the prognosis.

List of original publications

I Emma M. Savilahti, Jorma Ilonen, Minna Kiviniemi, Kristiina M. Saarinen, Outi Vaarala, Erkki Savilahti.

Human Leukocyte Antigen (DR1)-DQB1*0501 and (DR15)-DQB1*0602 Haplotypes Are Associated with Humoral Responses to Early Food Allergens in Children.

Int Arch Allergy Immunol. 2010;152(2):169-77

II Emma M. Savilahti, Kristiina M. Saarinen, Erkki Savilahti.

Duration of clinical reactivity in cow's milk allergy is associated with levels of specific immunoglobulin G4 and immunoglobulin A antibodies to beta-lactoglobulin.

Clin Exp Allergy. 2010;40(2): 251-256.

III Emma M. Savilahti, Ville Rantanen, Jing Lin, Sirkku Karinen, Kristiina M. Saarinen, Marina Goldis, Mika Mäkelä, Sampsa Hautaniemi, Erkki Savilahti, Hugh Sampson.

Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes.

J Allergy Clin Immunol. 2010;125(6):1315-132

IV Emma M. Savilahti, Sirkku Karinen, Harri M. Salo, Paula Klemetti, Kristiina M. Saarinen, Timo Klemola, Mikael Kuitunen, Sampsa Hautaniemi, Erkki Savilahti, Outi Vaarala.

Combined T regulatory cell and Th2 expression profile identifies children with cow's milk allergy.

Clin Immunology. 2010;136(1):16-20.

The publications are referred to in the text by their roman numerals.

Abbreviations

APC antigen presenting cell

AUC area under curve

BSA bovine serum albumin

CLA cutaneous lymphocyte-associated antigen

CM cow's milk

CMA cow's milk allergy

CTLA Cytotoxic T Lymphocyte Antigen

ELISA enzyme-linked immunosorbent assay

FAE follicle-associated epithelium

FACS fluorescent-activated cell sorter

FoxP3 forkhead box protein 3

GATA GATA binding protein

GALT gut associated lymphoid tissue

GLM general linear model

HLA human leukocyte antigen

Ig immunoglobulin

IL interleukin

IFN interferon

NKT natural killer T cell

Nfat-C2 nuclear factor of activated T-cells 2

PBS phosphate buffered saline

PCR polymerase chain reaction

PHA phytohaemaglutinin

PMBC polymorphonuclear blood cell

PBB protein binding buffer

PTPN22 protein tyrosine phosphatase, non-receptor type 22

ROC receiver operating characteristic curve

QRT-PCR quantitative real time polymerase chain reaction

SNP single nucleotide polymorphism

SPT skin prick test

STIM stromal interaction molecule

TGF transforming growth factor

Th1 T-helper cell type 1

Th2 T-helper cell type 2

TIM T cell immunoglobulin mucin

Treg T regulatory cell

Review of the literature

Introduction to cow's milk allergy

In Western societies, the earliest antigen infants are orally exposed to is commonly cow's milk (CM). It elicits an immunological response which in the vast majority of infants leads to tolerance (Tainio et al. 1988, Kemeny et al. 1991). However, usually during the first year of life, 2-3% of children develop cow's milk allergy (CMA) (Host, Halken 1990, Saarinen et al. 1999). CMA is an adverse clinical reaction to ingested CM proteins based on an immunological reaction to the provoking proteins (Sicherer, Sampson 2008). The only therapy currently established is an elimination diet until tolerance develops, and reactive treatment of allergic symptoms with antihistamines, locally or systemically administered corticosteroids and/or injectable epinephrine in case of CM ingestion (Sicherer, Sampson 2008).

Oral tolerance and cow's milk allergy

Oral tolerance can be defined as the antigen-specific suppression of cellular and/or humoral immune responses following preceding oral exposure to the antigen (Faria, Weiner 2005). How and why CMA develops instead of physiological oral tolerance, is not fully understood. Both genetic predisposition and environmental factors have an impact (Sicherer, Sampson 2008). Gut barrier, or its insufficient function, plays a central role in oral tolerance (Sampson 1999, Chehade, Mayer 2005). A controlled inflammation may be a central feature of the development of oral tolerance (Mayer et al. 2001).

The gut is the largest immunological organ of the body, and most of the time it appropriately responds to pathogens, tolerates harmless environmental antigens (such as food proteins and peptides) and maintains commensal bacterial flora (Sicherer, Sampson 2008, Mowat 2003, Tsuji, Kosaka 2008). Exposure to foreign antigens is required for the development of the immune system in the gut (Menezes et al. 2003, Bouskra et al. 2008). The gut barrier consists of numerous components (Sampson 1999). Digestion is initiated already in the mouth by salivary amylases, proceeds further by gastric acid and pepsins, then in the intestine by pancreatic and intestinal enzymes, and finally on the gut surface by intestinal epithelial cell lysozyme activity (Sampson 1999). Digestion breaks proteins down into aminoacids and peptides of various lengths, which are less immunogenic compared with entire proteins that retain both sequential and conformational epitopes (Vickery, Burks 2009). One difference between food allergens and non-allergens is indeed the resistance to digestion (Astwood, Leach & Fuchs 1996). The penetration of ingested antigens is hampered by epithelial cells, the glycocalyx that traps particles, intestinal microvilli, peristaltis and tight junctions between

enterocytes (Sampson 1999, DeMeo et al. 2002). Ingested antigens can also be blocked from penetration by antigen-specific secretory IgA antibodies (DeMeo et al. 2002, Kuitunen, Savilahti & Sarnesto 1994a, Cerutti 2008). If digested by enterocytes, peptides and aminoacids are further digested, and transported as aminoacids to portal circulations. A small proportion (0.001-1%) of intact proteins or large, still antigenic peptides come into contact with dendritic cells beneath the intestinal epithelium, and are transported to the lamina propria of the intestine (Strobel, Mowat 1998). Increased gut permeability may disrupt the achievement of oral tolerance due to increased antigen load (Berin, Shreffler 2008). The gut barrier is immature during neonatal period, and permeability to macromolecules is higher than that of a mature gut (DeMeo et al. 2002, Kuitunen, Savilahti & Sarnesto 1994a, Kuitunen, Savilahti & Sarnesto 1994b). On the other hand, an immature gut and greater permeability, may result in antigen exposure high enough to elicit anergy and diminished immunological responses (Schwartz 2003, Siltanen et al. 2002). The dose of antigen exposure may also affect Th1/Th2-balance: large doses would promote Th1 responses and small doses Th2 responses (Rogers, Croft 1999, Kay 2001a). T regulatory cell function and thus tolerance may be induced by even smaller doses of antigens (Kretschmer et al. 2005).

The gut associated lymphoid tissue (GALT) is where initiative immune responses predominantly take place (Mowat 2003, Cheroutre, Madakamutil 2004). Both innate and adaptive immune cells are active components of GALT (Cheroutre, Madakamutil 2004). Especially important is an area of specialized, single layer epithelium overlying lymphoid follicles called follicle-associated epithelium (FAE) (Mowat 2003), where the microfold (M) cells sample luminal antigens (DeMeo et al. 2002). These specialized M cells internalize and process macromolecules, which subsequently are transported to the cell surface for antigen presentation (Mowat 2003, DeMeo et al. 2002). Antigen sampling can also occur by two alternative means: dendritic cells can extend their processes to the gut lumen through the epithelium, or epithelial cells may transport antigens to the lamina propria (Vickery, Burks 2009, Berin, Shreffler 2008, Cheroutre, Madakamutil 2004). Antigen sampling has been proposed to be a key decisive point in whether oral tolerance is induced successfully (Mayer et al. 2001).

Dendritic cells are concentrated right under FAE (Mowat 2003). Antigen presentation by dendritic cells importantly controls tolerance induction in T cells (Akbari, DeKruyff & Umetsu 2001, Coombes et al. 2007, Sun et al. 2007, Coombes, Powrie 2008). It predominantly takes place in Peyer's patches, which are located beneath FAE and essential, if not required, for oral tolerance to proteins (Fujihashi et al. 2001, Spahn et al. 2001). In addition, epithelial cells may function as antigen presenting cells particularly in maintaining local tolerance to antigens (Mowat 2003), and intraepithelial lymphocytes complement the function of those residing in GALT . Retinoic acid acting in concert with TGF-β induces tolerogenic dendritic cells and subsequently the generation of Tregs as well as class switching to IgA (Coombes et al. 2007, Sun et al. 2007)

Following antigen presentation and costimulatory signals, lymphocytes proliferate and differentiate to immunocompetent cells in secondary lymphoid organs (Cheroutre, Madakamutil 2004, Salmi, Jalkanen 1999). They can then migrate to the effector sites lamina propria and intraepithelial regions (DeMeo et al. 2002, Cheroutre, Madakamutil 2004). Alternatively, they can reside in mesenteric lymph nodes, superior mesenteric duct, and along the thoracic duct (DeMeo et al. 2002). Furthermore, some enter the systemic circulation and migrate to other mucosal surfaces and organs (DeMeo et al. 2002, Eigenmann 2002b). The trafficking of naive lymphocytes to the gut and later antigen-primed memory lymphocytes returning from circulation to the gut are directed by the expression of gut-specific adhesion molecules (such as α4-integrins) as well as local secretion of various chemokines such as TNF-α and IL-8 (Salmi, Jalkanen 1999, Eigenmann 2002b, Denning, Kim & Kronenberg 2005). The expression of CLA (cutaneous lymphocyte-associated antigen), a skin-specific adhesion molecule, in food-antigen primed T memory cells may partly explain the high prevalence of atopic dermatitis in food allergy (Eigenmann 2002b).

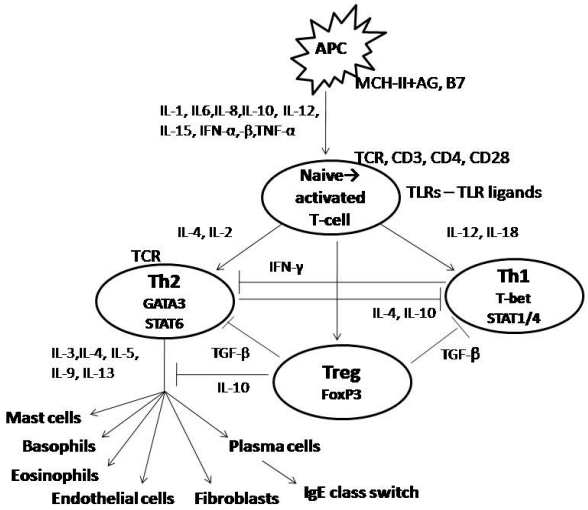
High antigen exposure results predominantly in T cell anergy (Schwartz 2003, Friedman, Weiner 1994, Powell 2006), but may also induce a switch of Th1 and Th2 cells into IL-10 secreting T regulatory type 1 cells (Tr1) (Meiler et al. 2008b). Low doses of antigen usually activate other types of T regulatory cells (Tregs) (Chehade, Mayer 2005, Sun et al. 2006). The de novo generation of Tregs in GALT is a crucial element of oral tolerance (Sun et al. 2007). Although food antigen specific Tregs are induced and reside in the gut, they also circulate in the body, especially at allergen exposure, to maintain systemic tolerance (Tsuji, Kosaka 2008).

During pregnancy, the maternal immune system is Th2-skewed, which diminishes the risk of Th1-mediated rejection of the placenta (Holt, Jones 2000). Transplacental priming of the fetal immune system to environmental antigens results in Th2-deviation (Prescott et al. 1998). The immune system continues to be Th2-dominated and promote IgE production during infancy (Holt, Jones 2000, Prescott et al. 1998). Influenced by genetic and environmental factors, some infants fail to develop a Th1/Th2 balance (Prescott, Sly & Holt 1998, Prescott et al. 1998, Prescott et al. 1999, Neaville et al. 2003), although adequate boost towards Th1 responses may correct the balance with age (Holt et al. 2000). They may further mount an exaggerated Th2-dominated response to an innocuous environmental antigen combined with impaired Th1 responses, which leads to allergy (Kay 2001a, Holt, Jones 2000, van der Velden et al. 2001, Romagnani 2004, Dunstan et al. 2005). The underlying phenomenon is that the balance of transcriptional networks that determine T cell commitment deviates towards Th2 differentiation (Chatila et al. 2008). Environmental factors, such as the gut commensal flora, influence this balance in particular via mechanisms of innate immunity (Chatila et al. 2008, Nigo et al. 2006, Conroy, Shi & Walker 2009). Of note, Macaubas et al reported that higher IL-4 and IFN-γ levels in cord blood were associated with reduced risk of asthma and atopy at the age of six years, which may reflect the failure of transplacental interface to promote the maturation of fetal immune system rather than contradict with the Th1/Th2 imbalance hypothesis (Macaubas et al. 2003).

Dendritic cells trigger the Th2 differentiation by antigen presentation, co-stimulatory molecules and cytokine production profile (Berin, Shreffler 2008, Liu 2007). Differentiation of naive T cells into Th2-cells requires the stimulation of the IL4-receptor and T cell receptor (Romagnani 2004, Chatila et al. 2008). The provenance of IL-4 in this process is unclear. Innate immunity and its reaction to environmental cues, or the lack of them, may be a trigger; innate cells such as mast cells, basophils and NKT cells secrete IL-4 (Berin, Shreffler 2008, Romagnani 2004, Kronenberg 2005, Liu 2008). Activated naive T cells appear to secrete low levels of IL-4, which may act in an autocrine and paracrine manner to drive Th2 differentiation, particularly if TH1-inducing signals are absent(Berin, Shreffler 2008). Intraepithelial and lamina propria lymphocytes have been shown to spontaneously secret IL-4 and IFN-γ in non-inflammed gut (Carol et al. 1998). Furthermore, IL-4 potently inhibits TH1 differentiation (Berin, Shreffler 2008, Romagnani 2004). The combination of inhibition with positive feedback may be the cue for Th2 differentiation (Berin, Shreffler 2008). The concerted stimulation of IL-4 and T cell receptors then leads to the expression of GATA-3, which is required and sufficient to determine the Th2 cell lineage (Zheng, Flavell 1997, Pai, Truitt & Ho 2004). Th2-cytokines such as IL-4, IL-5 and IL-13 induce IgE production and eosonophilic activation (Kay 2001a). Circulating specific IgE antibodies to an antigen, e.g. CM, bind to mast cells and basofils (Kay 2001a). At subsequent CM exposure, the antigen binding leads to the crosslinking of these antibodies and further to the release of inflammatory reagents from effector cells (Herz 2008, Christensen et al. 2008). Lower numbers of activated basophils reportedly associate with development of clinical tolerance to CM (Wanich et al. 2009). Decrease in CM specific IgE levels may predict recovery from CMA (Garcia-Ara et al. 2004). Beyer et al reported that gut-residing lymphocytes from patients with CM-associated gastrointestinal immunological disorders produced more Th2 cytokines and less IL-10 and TGF-β in response to CM stimulation *in vitro* than lymphocytes from healthy individuals (Beyer et al. 2002). Lower levels of TGF-β production were also reported in lymphocytes of duodenal mucosa from children with food allergy, whereas no Th2-skewing was found compared to healthy control subjects (Perez-Machado et al. 2003). In non-IgE-mediated CMA, the immunological reaction is T-cell mediated, and remains poorly characterized (Sicherer, Sampson 2008). Figure 1 presents an overview of the antigen presentation and T cell differentiation leading to clinical manifestations of allergy.

Oral desensitization appears to be a promising treatment for CMA (Staden et al. 2007, Meglio et al. 2008, Skripak et al. 2008). During treatment with increasing doses of oral CM administration, the majority of children with CMA become desensitized to CM. A large proportion of patients achieve longer term tolerance to CM (Staden et al. 2007, Meglio et al. 2008, Skripak et al. 2008, Narisety et al. 2009). Further studies with larger number of patients and longer follow-ups are required to better evaluate the benefits of CM oral immunotherapy.

Figure 1. Schematic overview of cellular mechanisms leading to allergy



Clinical aspects of cow's milk allergy

Diagnosis

The gold-standard for CMA diagnosis is an oral, preferably placebo-controlled and double-blind CM challenge after a successful elimination diet (Niggemann, Beyer 2007). In practice, the diagnosis is at times based on a history of CM-related symptoms combined with high levels of CM specific IgE (Sampson, Ho 1997, Sampson 2001). However, a diagnostic specificity of at least 90% may not be possible in regard to CM (Celik-Bilgili et al. 2005). CM-specific skin prick test is also used for diagnostics (Verstege et al. 2005), but it has the same caveats as specific IgE measurements (Breuer et al. 2004).

Immunologically, CMA can be divided to an IgE-mediated and a non-IgE-mediated form (Sampson 1999, Sabra et al. 2003). The IgE-mediated form is usually defined as the patient having high detectable levels of CM specific IgE antibodies in serum or having a positive skin prick test (SPT) to CM. Threshold for CM specific IgE levels is commonly defined as 0.7 kU/L or for higher specificity, 0.35 kU/L (Vanto et al. 1999). CM specific SPT is considered positive if the wheal diameter is 3 mm or more greater than the negative control (Verstege et al. 2005, Vanto et al. 1999). The reaction to CM in IgE-mediated CMA is characteristically immediate: the symptoms arise within a few hours. They result from the release of proinflammatory mediators from mast cells and other effector cells upon crosslinking of surface-bound IgE antibodies by antigen (Kay 2001a). Later phase reactions arise from the

tissue infiltration of neutrophils and basophils as well as Th2 cells and monocytes (Kay 2001a). Typical symptoms are urticaria, exanthema, angioedema, wheezing and vomiting.

Non-IgE-mediated CMA is more difficult to diagnose than IgE-mediated CMA (Niggemann, Beyer 2007). The reaction probably is T cell mediated and delayed: Symptoms usually appear only several hours or days after allergen exposure (Kay 2001a). Typical symptoms are atopic dermatitis (Darsow et al. 2010), and diarrhea. In combined forms, patients show both immediate and delayed reactions, and meet the criteria for IgE-mediated disease (Sampson 1999).

Symptoms

Cow's milk allergy manifests itself in various symptoms affecting multiple organs, and most patients have more than one symptom (Host, Halken 1990, Sabra et al. 2003). Clinically, CMA can be categorized to immediate and delayed type. No clear consensus exists on the dividing time point: it may be set anytime between one hour and up to 24 hours. This clinical categorization closely associates with the immunologic one: immediate symptoms are caused by IgE-mediated reactions.

Immediate symptoms are often mucosal and cutaneous. They include urticaria, exanthema and angioedema, which usually appear within minutes after allergen exposure (Sampson 1999). Respiratory manifestations also develop rapidly and include allergic rhinitis, cough and wheezing. The most serious symptom in CMA is anaphylaxis (Kay 2001b), which is much rarer than other symptoms (Sampson 1999, Eigenmann 2002a). However, its incidence is more difficult to assess than for other symptoms since patients with a history of possible anaphylactic reaction are usually not challenged in a controlled manner.

Delayed symptoms are mainly gastrointestinal such as vomiting, diarrhea and rarely even haematochezia (Sampson 1999, Rance et al. 1999); these symptoms may also arise in an immediate type reaction. CMA is often associated with infantile atopic eczema, which appears in both immediate and with delayed form (Sampson 1999, Breuer et al. 2004, Rance et al. 1999).

Prognosis

The prognosis for CMA is generally good. CMA persists beyond the age of 3 years in a minority of patients (Host, Halken 1990, Saarinen et al. 2005). High cow's milk (CM) specific IgE levels (Vanto et al. 2004, Skripak et al. 2007, Dias, Santos & Pinheiro 2010) and strong reaction in CM skin prick testing (Saarinen et al. 2005) predict persistence of CMA. Non-IgE-

mediated CMA has a better prognosis than IgE-mediated disease (Saarinen et al. 2005). Moreover, patients who tolerate baked milk appear to recover earlier than those who do not (Nowak-Wegrzyn et al. 2008). Several studies have reported that profiles of IgE binding to CM epitopes differ between patients who recover early and those whose CMA persists (Vila et al. 2001, Cerecedo et al. 2008, Wang et al. 2010). This is discussed more in detail in the chapters "Epitope recognition by antibodies in cow's milk allergy" in the literature review and in "Implications for CMA diagnostics and prognostics" in the discussion section.

Table 1 summarizes results of several studies on the recovery rates in CMA.

Table 1. Summary of recovery from CMA in four independent studies (Garcia-Ara et al. 2004, Saarinen et al. 2005, Vanto et al. 2004, Skripak et al. 2007)

Age (years)	% of patients recovered from CMA	Patient population (n)	IgE-mediated only or both	Reference
2	44	162	both	Vanto et al. 2004
3	69	162	both	Vanto et al. 2004
3	87	39	both	Host, Halken 1990
4	68	66	both	Garcia-Ara et al. 2004
4	19	807	IgE	Skripak et al. 2007
4	77	162	both	Vanto et al. 2004
5	74	86	IgE	Saarinen et al. 2005
8-9	85	86	IgE	Saarinen et al. 2005
10	52	807	IgE	Skripak et al. 2007

The problem is that currently no accurate tools are available for prognostics on an individual level. Thus clinicians face difficulties in deciding on when to start incorporating CM into the diet of patients. Furthermore, specific immunotherapies such as oral desensitisation are being launched in the treatment of persistent CMA. Desensitisation protocols are rather cumbersome for the patient (Skripak, Wood 2009), and clinicians should have better diagnostic and prognostic tools for deciding which patients most benefit from this kind of therapy.

Genetics in cow's milk allergy and other allergies

Allergic disorders result from an interplay between genetic, epigenetic and environmental factors (Cookson 1999, von Mutius 2009, Le Souef 2009) . The genetic component is undoubtedly multifactorial (Cookson 1999, Bosse, Hudson 2007). Numerous genes, some increasing susceptibility and others with a protective effect, together affect the development of e.g. asthma (von Mutius 2009, Weiss, Raby & Rogers 2009). The risk alleles are most probably common variants that alter gene function but do not disrupt it (Cookson 1999). One approach for studying genetic factors in allergy is to investigate candidate genes that have functions related to allergic symptoms or immunopathology (Cookson 1999, Kiyohara, Tanaka & Miyake 2008).

Filaggrin gene

Mutations in the filaggrin gene show associations with atopic eczema (Brown et al. 2008, O'Regan et al. 2009, van den Oord, Sheikh 2009). Dysfunction of the filaggrin protein disrupts the skin barrier, and may thus predispose to sensitization to harmless antigens (O'Regan et al. 2009, Palmer et al. 2006). Indeed, filaggrin gene mutations increase the risk for allergic sensitization (van den Oord, Sheikh 2009). The association of filaggrin mutations and food allergies have not been investigated to date (van den Oord, Sheikh 2009). Since eczema is a common symptom in CMA, the role of filaggrin mutations in CMA is interesting to investigate. Furthermore, a few studies have suggested that children could become sensitized to food allergens via skin (Hsieh et al. 2003). This could be explained by a defective skin barrier linked to filaggrin gene mutations (Weidinger et al. 2006). The hypothesis is of particular interest in the case of CMA since some infants develop CMA even during exclusive breastfeeding.

Human leukocyte antigen II genes

Human leukocyte antigen (HLA) II alleles are associated with the risk of several immunological disorders such as celiac disease and type I diabetes (Ilonen et al. 2002, Hermann et al. 2003, Tollefsen et al. 2006). Functionally HLA molecules are central to the development of an immune response. After endocytosis to antigen presenting cells (APCs), extracellular antigens are processed into peptides of 12-20 amino acids (Monaco 1995). Antigen processing is regulated by synchronized functions of several proteases (Cresswell 2005). Class II molecules bind to a protein called the invariant chain that then directs the HLA molecules to compartments where antigenic peptides are transported (Monaco 1995, Roche 1996). The invariant chain furthermore blocks the binding of class II molecules to peptides prematurely in the endoplasmic reticulum and Golgi apparatus (Roche 1996, Pieters 1997). A proteolytic product of the invariant chain, called CLIP, then binds to the class II molecule, and only after its proteolytic release can the antigenic peptide bind to the HLA molecule (Roche 1996, Pieters

1997). A protein called HLA-DM then facilitates the binding of the antigenic peptide to the binding groove of HLA molecules, which is composed of an α- and β-chain (Cresswell 2005, Roche 1996, Pieters 1997). These HLA-peptide complexes are transported on the cell surface of APCs where they present antigens to T cells for specific immune recognition (Cresswell 2005, Klein, Sato 2000). HLA genotype thus determines the repertoire of presented peptides to lymphocytes (Pieters 1997).

Several studies have reported associations between HLA alleles and atopy and/or specific allergies. Cow's milk allergy was associated with HLA-DQ7 (HLA-DQB1*0301) in an Italian patient sample (Camponeschi et al. 1997). However, a larger Finnish study comparing 100 CMA patients with healthy subjects, did not find any association between CMA and HLA A, B, Bw, C or DR antigens(Verkasalo et al. 1983). Studies on other food allergies have reported associations with HLA haplotypes. Peanut allergy was associated with DRB1*08, DRB1*12 and DQB1*04 in Caucasian subjects (Howell et al. 1998) . Boehncke and coworkers also reported an association between peanut allergy and HLA-DRB1*08 (Boehncke et al. 1998). They found associations between carrot allergy and HLA-DRB1*12, and grass pollen allergy and HLA-DQB*0301, whereas DRB1*01, DQA1*0101 and DQB1*0501 forming a haplotype were decreased among birch pollen allergy associated hazel nut allergy patients (Boehncke et al. 1998) . Several studies have investigated the associations of HLA haplotypes with allergies to aeroallergens. Birch pollen allergy, or possibly atopy more broadly, was reported to associate with HLA-DR7 (Senechal et al. 1999) . Stephan and coworkers detected no significant linkage between grass pollen, birch pollen, or cat dander specific IgEs and sharing of HLA-DPB, -DRB, and -DQB, whereas these loci showed association with house dust mite specific IgE (Stephan et al. 1999) . Another study reported that house dust mite sensitization was associated with HLA DRB1*07, while DRB1*04 conferred a protective effect (Kim et al. 2001) . Allergy to mugwort main allergen Art v1 was found to be restricted to HLA DRB*01 (Jahn-Schmid et al. 2005) . The HLA alleles DRB1*0701 and DQB1*02 were associated with cockroach allergy, and with atopy in general (Kalpaklioglu, Turan 2002) . HLA DQB1*05, especially 0501, but also 0502 and 0503, conferred susceptibility to develop IgE antibodies against organic acid anhydrates (Jones et al. 2004) . Table 2 summarizes these reported associations.

The association of HLA genotypes with humoral responses to allergens has been investigated in few studies, and only rarely the intensity of the response has been studied. Specific IgG response to Ra5 (Amb5), a ragweed allergen, was associated with HLA-Dw2 in a Caucasian population (n=447) (Marsh et al. 1982). Immune response to the grass pollen allergen Lol p III HLA-DR3 was reported to associate with a specific sequence shared by DR3, DR11 and DR6 (Ansari et al. 1991). In a heterogeneous population of allergic patients, HLA-DRB1*1101 and/or 1104 were associated with the presence of specific IgG and IgE antibodies to Par o 1, the major allergen from the pollen of Parietaria (D'Amato et al. 1996).

Table 2. Reported positive associations of HLA II haplotypes with different allergies

Allergy	**Positive HLA association**	**Reference**
Cow's milk	DQ7 (QB1*0301)	Camponeschi et al 1997
Cow's milk	no association	Verkasalo et al 1983
Peanut	DRB1*08, DRB1*12, DQB1*04	Howell et al 1998
Peanut	DRB1*08	Boehncke et al 1998
Carrot	DRB1*12	Boehncke et al 1998
Grass pollen	DQB*0301	Boehncke et al 1998
Birch pollen	DR7	Senechal et al 1999
House dust mite	DRB1*07	Kim et al 2001
Mugwort (Art v1)	DRB*01	Jahn-Schmid et al 2005
Cockroach	DRB1*0701, DQB1*02	Kalpaklioglu et al 2002
Organic acid anhydrates	DQB1*0501, 02 , 0503	Jones et al 2004

Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)

The PTPN22 gene encodes a lymphoid tyrosine phosphatase (LYP) that negatively regulates T-cell responses. This function has raised interest in whether mutations in the gene are associated with immunological disorders, but few studies to date have addressed allergy. A mutation (1858 SNP or R620W allele) in the PTPN22 gene is associated with several autoimmune diseases (Bottini et al. 2004, Michou et al. 2007, Smyth et al. 2008). No association has yet been found with asthma (Majorczyk et al. 2007) or high total serum IgE (Maier et al. 2006).

The role of antibodies in cow's milk allergy and tolerance

Physiological humoral response to cow's milk

Fetal lymphocytes appear to be primed by CM antigens already prenatally (Szepfalusi et al. 1997). At birth, infants have IgG class antibodies to CM acquired from the mother by transplacental transportation (Kemeny et al. 1991, Holt, Jones 2000). Neonates produce immunoglobulins at low levels (Holt, Jones 2000). Later during infancy immune responses such as antibodies to food allergens increase (Cummins, Thompson 1997) and decline by the age of one year (Husby 2000). The physiological response infants develop after CM exposure is dominated by IgG class antibodies (Tainio et al. 1988, Vaarala et al. 1995), IgG1 subclass in particular (Kemeny et al. 1991). The specific IgG levels rise during the first year of life (Tainio et al. 1988, Kemeny et al. 1991). Specific IgA and IgM to CM are absent at birth, but infants produce them even during exclusive breast feeding i.e. during minimal CM exposure (Tainio et al. 1988, Kuitunen, Savilahti 1995). Physiological response entails also the production of specific IgE antibodies, although in lower quantity than other immunoglobulins (Hattevig, Kjellman & Bjorksten 1993).

Immunoglobulin class E

Serum levels of immunoglobulin E are much lower than those of IgG class antibodies, even in sensitized individuals (Corry, Kheradmand 1999). IgE production in B cells is induced by Th2 cytokines IL-4, IL-5, IL-6, IL-9 and IL-13, and inhibited by Th1 cytokines IFN-γ and IL-2 (Corry, Kheradmand 1999). Antigen stimulation of B cell receptor combined with co-stimulatory molecules also promotes immunoglobulin class-switching to IgE (Corry, Kheradmand 1999). When IgE mediates antigen uptake, dendritic cells show augmented antigen presentation and induction of Th2 responses to memory T cells compared with antigen uptake mediated by IgG subclass antibodies (Lundberg et al. 2008).

Already neonates reportedly have antigen-specific IgE antibodies as measured in cord blood (Holt, Jones 2000). In addition to circulating in the blood, IgE antibodies may be secreted to the gut lumen; the function of secretory IgE remains, however, unclear (Corry, Kheradmand 1999, Negrao-Correa, Adams & Bell 1996). Sensitization to food allergens is manifested by heightened production of specific IgE antibody (Jenmalm, Bjorksten 1999, Bottcher et al. 2002). Higher levels of IgE antibodies to CM proteins are associated with persisting CMA compared to patients who recover from CMA early (Garcia-Ara et al. 2004, Vanto et al. 2004, Skripak et al. 2007, Sicherer, Sampson 1999). A slower rate of decrease in specific IgE levels over time may predict persistence of CMA as well as hen's egg allergy (Shek et al. 2004). Several studies on allergen specific immunotherapy have reported decreased levels of specific

IgE (Meglio et al. 2008, Niederberger et al. 2004, Nouri-Aria et al. 2004, Jones et al. 2009) while others have not observed any significant change (Skripak et al. 2008, Francis et al. 2008).

Immunoglobulin isotype class G

Whether IgG class antibodies present purely physiological responses to food allergens or actually play a role in the development of tolerance or allergy, is controversial (Barnes 1995, Keller et al. 1996). Recent research presents several lines of evidence to support the hypothesis that IgG antibodies, IgG4 in particular, do contribute to allergy and tolerance development, but the conclusions remain controversial.

Th2-deviation may be associated with reduced capacity to mount IgG responses (Le Souef 2009). While the Th2-cytokine IL-4 induces class switching in B cells to both IgE and IgG4 (Punnonen et al. 1993), IL-10 up-regulates the secretion of IgG4 and inhibits IgE production (Jeannin et al. 1998, Satoguina et al. 2005, Meiler et al. 2008a). Specific IgG4 functions by blocking the binding of specific IgE to allergen(Nouri-Aria et al. 2004, van Neerven et al. 1999, Wachholz et al. 2003, Ejrnaes et al. 2006). A mechanism crucial to the anti-inflammatory function of IgG4 seems to be the Fab arm exchange (van der Neut Kolfschoten et al. 2007).

A specific IgG4 response to the food antigens may be physiological, the result of continuous exposure to the antigen (Tay et al. 2007, Stapel et al. 2008). On the other hand, higher specific IgG levels to the CM protein β-lactoglobulin (Oldaeus et al. 1999) and hen's egg ovalbumin (Jenmalm, Bjorksten 1999, Eysink et al. 1999) have been associated with increased incidence of atopic diseases, plausibly reflecting a Th2-deviation (Jenmalm, Bjorksten 1999). Lilja et al reported that CM specific IgG and IgE levels correlated positively in infants, and suggested that some individuals are "high responders" and some "low responders" in their humoral response to oral antigens overall (Lilja et al. 1991). In children sensitized to hen's egg, specific IgG1 levels increased during the first year of life more in children whose allergy persisted compared to those who recovered early, whereas specific IgG4 levels did not differ between the groups (Vance et al. 2004). Studies on cat exposure and atopic disorders in adolescents have reported higher specific IgG4 levels and immunological tolerance to cats in individuals with continuous cat exposure at home (Platts-Mills et al. 2001, Hesselmar et al. 2003). The finding may suggest a role of IgG4 in tolerance, although IgG4 levels showed no association with atopic disorders (Platts-Mills et al. 2001, Hesselmar et al. 2003). Higher specific IgG4 levels were also associated with high dose exposure to birch aeroallergen at the postnatal period (Kihlstrom et al. 2005).

Upregulation of allergen specific IgG4 production induced by IL-10 is, however, also related to the development of tolerance (Nouri-Aria et al. 2004, Francis et al. 2008). In allergic disorders,

increased levels of IgG4 antibodies often indicate that anti-inflammatory processes are activated (Aalberse et al. 2009). Several studies on aeroallergen specific immunotherapy have reported increased specific IgG4 levels in patients whose allergy improved clinically (Nouri-Aria et al. 2004, Francis et al. 2008, Mothes et al. 2003, Bussmann et al. 2007), furthermore simultaneously with increasing numbers of circulation Tregs (Pereira-Santos et al. 2008). Results from immunotherapeutic settings in food allergies have reported similar phenomena. A shortcoming of these studies is that they have largely failed to demonstrate long-term recovery, but rather desensitization during and shortly after the active therapy. An exception is the study by Meglio and coworkers, in which children were followed for over four years after oral desensitization. Long-term tolerance was associated with decreased specific IgE levels (Meglio et al. 2008). Contrarily, CM-specific IgE levels remained at baseline levels in another study with shorter follow-up and where only desensitization during immunotherapy to CM could be reported (Skripak et al. 2008). This study also investigated specific IgG, most notably IgG4, and observed an increase in their levels (Skripak et al. 2008). In a study on patients with CMA but asymptomatic to heated CM, similarly an increase in casein specific IgG4 levels was seen after a three-month period of consuming heated CM, whereas specific IgE levels did not change (Nowak-Wegrzyn et al. 2008). Children with peanut allergy who became desensitized during peanut allergy immunotherapy, showed increased specific IgG4 levels and decreased specific IgE levels (Jones et al. 2009).

A few studies have investigated the natural development or maintenance of tolerance to food allergens without therapeutic intervention. Clinical improvement of symptoms in a population of patients with hen's egg allergy was associated with an increase in ovalbumin specific IgG4 and decrease in specific IgE (Lemon-Mule et al. 2008). In children with milk and/or egg allergy, low levels of IgG4 to ovalbumin and/or β-lactoglobulin indicated the need for prolonged elimination diet (Tomicic et al. 2008). A study comparing non-atopic individuals with subjects with CMA reported that the maintenance of tolerance to CM proteins associated with higher levels of CM specific IgG4 levels (Ruiter et al. 2007a).

Immunoglobulin class A

The production of IgA is more abundant than that of any other antibody class (Macpherson et al. 2001). The large majority is secreted to mucosal surfaces, most importantly in the gut (Macpherson et al. 2001).

Serum IgA is produced in small quantities compared with the secretory antibody or with serum IgM and IgG (Macpherson et al. 2001). It may, however, play a role in the development of tolerance. The mechanism differs from IgG4: specific IgA does not inhibit IgE binding (van Neerven et al. 1999, Pilette et al. 2007). The production of IgA seems to be independent of T helper cells (Meiler et al. 2008a), to be associated with local TGF-β expression, and to induce IL-10 production from monocytes (Pilette et al. 2007).

The relatively high prevalence of allergies among patients with IgA deficiency (Aghamohammadi et al. 2009) advocates a role for IgA antibodies in the protection against allergies and/or the development of tolerance. Low total (Savilahti et al. 1991) and CM specific (Jarvinen et al. 2000) IgA in colostrums has been associated with the risk of CMA. High intestinal IgA in infants reportedly associated with reduced the risk for IgE-mediated allergies (Kukkonen et al. 2009). However, another study observed no association of breast milk IgA or cytokine levels with salivary IgA nor development of atopy in children up to the age of two years (Bottcher, Jenmalm & Bjorksten 2003). Studies on grass pollen immunotherapy have reported that specific IgA (Francis et al. 2008) or IgA2 (Pilette et al. 2007) levels in peripheral blood rose as the allergic symptoms improved, and in a study on birch allergen vaccine specific IgA levels reportedly increased during the immunotherapy(Niederberger et al. 2004). Bottcher et al reported that infants who developed allergy had higher levels of total and allergen-specific IgA antibodies in serum (Bottcher et al. 2002). However, they observed that sensitized infants with no allergic symptoms had higher levels of specific IgA than symptomatic, sensitized infants (Bottcher et al. 2002). Their results thus suggest a role for specific IgA in the maintenance of clinical tolerance. Yet, Shek et al observed that in patients with IgE-mediated CMA, cow's milk protein specific IgE levels correlated positively with levels of specific immunoglobulins of other isotypes (IgA, IgG1, IgG4) (Shek et al. 2005).

Epitope recognition by antibodies in cow's milk allergy

Patients with cow's milk allergy typically react to several cow's milk proteins, while the four proteins in the casein fraction (α_{s1}-, α_{s2}-, β- and κ-casein) as well as α- lactalbumin and β-lactoglobulin are considered major allergens (Savilahti, Kuitunen 1992, Wal 2004). Both conformational and sequential epitopes elicit antibody responses (Wal 2004, Sathe, Teuber & Roux 2005, Lin, Sampson 2009). β-lactoglobulin has a globular structure and thus conformational epitopes, whereas the structure of caseins is more linear (Sanchez, Frémont 2002). Nevertheless, β-lactoglobulin also retains much of its immunoreactivity after chemical processing (Selo et al. 1999). Sequential epitopes are thought to be more important in food allergies since proteins are mostly digested in the gut into peptides and aminoacids and thus lose their conformational structures; also heating and other processing reduces conformational epitopes (Sathe, Teuber & Roux 2005, Lin, Sampson 2009). Antigenic peptides are usually at least eight aminoacids long (Herz 2008, Bannon, Ogawa 2006).

Epitope profiling of IgE antibodies has given additional insight into the relation between antibody responses and clinical reactivity in CMA. The pattern of IgE epitope recognition varies remarkably between individual patients with CMA (Wang et al. 2010, Cocco et al. 2007, Han et al. 2008). Patients with persisting CMA tend to recognize a wider variety of sequential IgE epitopes than patients with transient CMA (Vila et al. 2001, Cerecedo et al. 2008, Wang et al. 2010). In peanut allergy (Shreffler et al. 2004) as well as in CMA (Wang et al. 2010), the

diversity of IgE epitope recognition has been linked to the severity of symptoms. Furthermore, some IgE recognition of certain sequential epitopes especially in caseins is associated with persisting CMA (Vila et al. 2001, Cerecedo et al. 2008, Jarvinen et al. 2002). Similar findings have been reported in hen's egg allergy (Jarvinen et al. 2007). Wang et al compared children clinically reactive to all CM-derived foods with those who tolerated heated CM as well as those who had outgrown CMA (Wang et al. 2010). They found that children who tolerated heated CM had similar CM epitope binding patterns to those who had outgrown CMA (Wang et al. 2010). These two groups had lower affinity CM epitope binding by IgE than children who were reactive to all CM forms (Wang et al. 2010).

We do not know much, however, about how epitope recognition in allergy evolves over time and how it changes during tolerance development. A study in children with peanut allergy reported that peanut epitope binding by IgE remained stable over a twenty-month time period (Flinterman et al. 2008).

Little is known about the epitope recognition by IgG4 and IgA antibodies in food allergies. An early study based on enzymatic protein lysis into peptides reported that children with CMA had higher IgG levels to native in particular, but also to pepsin hydrolyzed β-lactoglobulin, than healthy control subjects (Duchateau et al. 1998). According to a recent study, CM epitope binding by IgG4 as measured with a microarray based method was not associated with clinical features of CMA (Wang et al. 2010).

The investigation of epitope recognition by IgE in food allergies began with the generation of epitope with enzymatic cleavage (Selo et al. 1999, Duchateau et al. 1998). This method could screen only a limited variety of epitopes (Lin, Sampson 2009). The next generation technology was based on SPOT membrane (Jarvinen et al. 2002). This method detects IgE binding even at rather low specific IgE levels (Beyer et al. 2003), but has a relatively high signal/noise-ratio and also a limited number of target peptides per assay (Lin, Sampson 2009). It was also labour-intensive, required high allergen specific IgE-levels and consumed large volumes of serum (Lin, Sampson 2009, Shreffler et al. 2005). The next generation peptide microarray based immunoassay has greatly improved possibilities to investigate the subject. It enables a large number of samples to be processed simultaneously, consumes only small amount of serum and is considerably more sensitive than SPOT membrane technology, thus allowing the study of also sera with low specific IgE levels (Shreffler et al. 2005, Beyer et al. 2005, Lin et al. 2009). Furthermore, the microarray-based immunoassay produces quantitative data in contrast to the qualitative or at most semiquantitative data of SPOT membrane technology. Comparison of these two methods has shown that the results are consistent (Beyer et al. 2003).

Regulatory T cells in allergy

Regulatory T-helper cells (Tregs) suppress the functions of other lymphocytes (Annunziato et al. 2002). They represent approximately 5-10% of peripheral CD4+ cells. The dysfunction of Tregs appears to contribute to the immunopathology of allergies (Tang, Bluestone 2008, Akdis, Akdis 2009).

Subpopulations of regulatory T cells

Regulatory T cells are considered to be the primary mediators of peripheral tolerance (Vignali, Collison & Workman 2008). T cells with suppressive function have been observed in experimental settings since the 1970s, but during the last decade their existence has attained substantial and ever-growing evidence both *in vitro* and *in vivo*, and both in animal models and humans (Tang, Bluestone 2008, Chen et al. 1994, Groux et al. 1997). Characterization of Tregs is, nevertheless, still not entirely established. Several subpopulations have been distinguished. "Natural" Tregs originate in the thymus during ontogeny, and according to current knowledge, always express the transcription factor forkhead box protein 3 (FoxP3) (Tang, Bluestone 2008). Other Treg subpopulations are induced from naive T-cells in the periphery and called adaptive Tregs (Kretschmer et al. 2005, Bluestone, Abbas 2003, Walker et al. 2005, Apostolou et al. 2008). These can be either FoxP3 positive or negative (Sun et al. 2006, Chen et al. 2003, Feuerer et al. 2009). Distinct adaptive Treg subpopulations include "Tr1" cells that produce high levels of IL-10 and varying levels of TGF-β,IL-5, low amounts of IFN-γ and IL-2, but no IL-4 (Groux et al. 1997, Levings et al. 2001, Wu et al. 2007). Tr1 cells do not constitutively express Foxp3 (Vieira et al. 2004). They appear to be of special importance in controlling immune responses to environmental antigens at body surfaces such as lungs and the gut (Rubtsov et al. 2008). T helper type 3 cells are characterized by suppressive function and the production of transforming growth factor β (TGF- β)(Levings et al. 2001, Carrier et al. 2007, Wan, Flavell 2008). In addition, inducible T cells with suppressive function have been characterized: CD8+ T suppressor cells, natural killer T cells (Kronenberg 2005), CD4-CD8-T cells (Zhang et al. 2000)and γδ T cells (Hayday, Tigelaar 2003).
Studies have reported have numerous modes of function for Tregs of different subtypes ranging from cell-cell-contact to the secretion of cytokines, but their relative importance *in vivo* remains to be elucidated (Vignali, Collison & Workman 2008).

Markers for regulatory T cells

None of the current markers for identifying Tregs is definitive: effector cells also express these markers, although more transiently and/or at different levels (Feuerer et al. 2009).

The majority of Tregs express the surface marker CD25, a high-affinity receptor for IL-2 (IL-2Rα) (Itoh et al. 1999), and the transcription factor FoxP3 forkhead box protein 3 (FoxP3) (Hori, Nomura & Sakaguchi 2003, Huehn, Polansky & Hamann 2009). Although all T cells express CD25 upon activation (Fontenot et al. 2005), high expression of CD25 seems to stabilize expression and maintenance of a Treg phenotype (Komatsu et al. 2009). Low expression of the surface marker CD127, an IL-7 receptor, distinguishes T cells with suppressive function, and the majority of them are FoxP3 positive (Liu et al. 2006, Seddiki et al. 2006, Bayer et al. 2008).

Mutations in the FoxP3 gene are the cause of IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is manifested as multiorgan autoimmune diseases, allergy and inflammatory bowel disease (Bennett et al. 2001). This finding lead to the identification of FoxP3 as the central regulatory gene of natural Treg commitment (Hori, Nomura & Sakaguchi 2003, Tai et al. 2005). Indeed, forced expression of the FoxP3 gene in CD4+CD25- non-Tregs resulted in a suppressive phenotype and the expression of markers associated with Tregs (Hori, Nomura & Sakaguchi 2003, Fontenot, Gavin & Rudensky 2003). Foxp3 is a transcriptional repressor and activator that interfers with T cell receptor (TCR)-dependent activation of genes. FoxP3 expression is induced by the interplay of relatively short and weak T cell receptor activation, co-stimulatory molecules and cytokines that remain to be fully identified (Huehn, Polansky & Hamann 2009) . Its function is required for Treg cell suppressor activity, but largely it rather amplifies and fixes pre-established molecular features of Tregs, such as anergy and dependence on paracrine IL-2(Gavin et al. 2007). In addition, Foxp3 stabilizes Treg cell lineage through modification of cell surface and signaling molecules so that the cells adapt to the signals required to induce and maintain Tregs (Gavin et al. 2007).

FoxP3 is not, however, the kind of master gene of Treg development in humans as was purported some years ago (Vignali, Collison & Workman 2008, Feuerer et al. 2009). It seems that a higher level of regulation upstream of Foxp3 determines the lineage (Hill et al. 2007). On the other hand, activation induces FoxP3 expression in all T cells, and in T effector cells this transient FoxP3 expression does not necessarily elicit suppressive function (Feuerer et al. 2009, Allan et al. 2007). Indeed, continuous FoxP3 expression is required for the maintenance of Treg phenotype (Williams, Rudensky 2007). FoxP3+ cells seem to be divided into at least two populations: CD4+CD25high cells that have stable FoxP3 expression and are committed to the Treg lineage, and CD4+CD25- cells that are not fully committed, have an unstable FoxP3 expression and may start responding to cytokines directing them to Th effector cells (Komatsu et al. 2009). Transforming growth factor β (TGF-β) seems to be crucial in maintaining the expression of FoxP3 and the Treg phenotype in CD4+CD25- (Komatsu et al. 2009) . In FoxP3+ T cells, epigenetic control of FoxP3 expression appears to determine the stability of the Treg phenotype (Huehn, Polansky & Hamann 2009).

FoxP3 interacts with the transcription factor called nuclear factor of activated T cell (NFAT) to repress expression of the cytokine IL2, and upregulate expression of the Treg markers CD25 and cytotoxic T lymphocyte–associated antigen 4 (CTLA4) (Wu et al. 2006). NFAT either stimulates cytokine expression leading to T cell activation by interacting with the transcription factor AP-1, or suppresses cytokine expression which leads to T cell tolerance by interacting with Foxp3 (Wu et al. 2006, Bettelli, Dastrange & Oukka 2005, Bopp et al. 2005). In knockout mice, double deficiency for NFATc2 and NFATc3 causes massive lymphadenopathy, splenomegaly and a strong increase in serum IgE and IgG1 levels (Bopp et al. 2005). It also renders CD4+ CD25- T cells unresponsive to suppression, although it does not interfere with the development or function of CD4+CD25+ Tregs (Bopp et al. 2005).

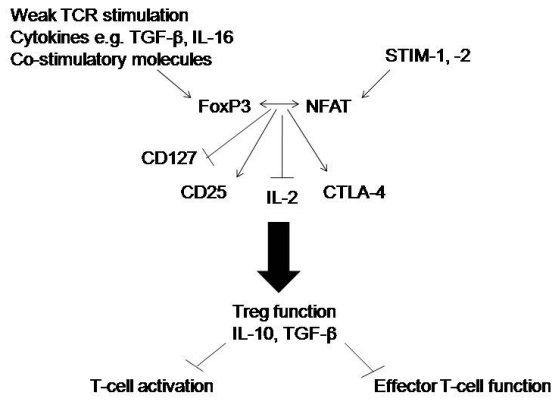
CTLA-4 is constitutively expressed in CD25+CD4+ regulatory T cells in mice (Takahashi et al. 2000) and human in thymocytes (Annunziato et al. 2002). Its blockade in mice results in the spontaneous development of autoimmune disorders(Takahashi et al. 2000). A recent study on mice with CTLA-4 deletion in CD4+FoxP3+ cells reported spontaneous development of systemic lymphoproliferation, fatal T cell–mediated autoimmune disease, and hyperproduction of immunoglobulin E, as well as potent tumor immunity(Wing et al. 2008). Treg-specific CTLA-4 deficiency adversely affected especially the Treg-mediated down-regulation of CD80 and CD86 expression on dendritic cells, and thus CTLA-4 may be of particular importance in the control of T effector cell activation induced by antigen presenting cell (Wing et al. 2008). Transfection of resting human T cells with CTLA-4 conferred suppression, which is facilitated by but not dependent of FoxP3 expression (Zheng et al. 2008). In addition to enabling the suppressive function of natural Tregs (Wing et al. 2008), CTLA4 may also induce suppressive Tregs in the absence of FoxP3 expression (Zheng et al. 2008). Read et al reported that CD4+CD25+ Tregs which suppressed inflammation in the gut, constitutively expressed CTLA-4 (Read, Malmstrom & Powrie 2000).

The number of Tregs as well as the expression of NFAT and cytokines in T cells, are also enhanced by the calcium sensors STIM1 and STIM2 (Oh-Hora et al. 2008). Activated CD4+CD25+ cells preferentially express the T cell immunoglobulin domain, mucin domain (Tim-3), which plausibly reduces Th1-driven immune responses (Sanchez-Fueyo et al. 2003). The migration and possibly *de novo* generation of Tregs suppressing Th2 type cells is induced by IL-16 at inflammation sites (McFadden et al. 2007). In allergic inflammation, Treg function involves IL-10, which further enhances TGF-β secretion by Tregs (Jutel et al. 2003, Joetham et al. 2007). The deviation from IFN-γ secreting Th1 cells, characterized by the transcription factor Tbet (Szabo et al. 2000), and/or of IL-5 and IL-13 secreting Th2 cells, defined by GATA3(Zheng, Flavell 1997, Pai, Truitt & Ho 2004), to IL-10 and TGF-β secreting Tregs is essential for the induction of both tolerance in allergic patients and normal mucosal immunity in nonatopic individuals (Jutel et al. 2003).

Other genes reported to have a characteristic expression profile in Tregs include CD103, G protein-coupled receptor 83 and glucocorticoid-induced tumor necrosis factor receptor (Tang, Bluestone 2008).

Figure 2 summarizes the interaction of central Treg markers.
Recent research further complicates the picture of transcription factors expressed in Tregs. T-bet, which is the master regulator of Th1 cells, and IRF4, which plays a role in Th2 and Th17 differentiation, appear to participate in the suppressive function of Tregs (Feuerer et al. 2009).

Figure 2. Markers for regulatory T cells



The role of regulatory T cells in allergies

Allergies result from an aberrant immunological response to environmental antigens, including a dysfunction of T regulatory cells (Tregs) (Ling et al. 2004, Lin et al. 2005). Natural, inducible as well as unconventional Tregs, especially of the Tr1 type, play an important role in both tolerance induction and allergy (Akdis, Akdis 2009, Cottrez et al. 2000, Akdis 2006, Saurer, Mueller 2009). The balance between Th2 and Treg functions is essential to the development of tolerance (Akdis et al. 2004, Francis, Till & Durham 2003). FoxP3 directly interacts with GATA-3 and thus suppresses the expression of cytokines upregulated byt GATA-3 such as IL-4, IL-5 and IL-13 (Dardalhon et al. 2008). The skewing of effector T cells into Tr1 cells has emerged as a crucial phenomenon in the development of tolerance to harmless environmental antigens (Akdis, Akdis 2009, Taylor et al. 2006). Some evidence suggests that CD4+CD25+ Tregs may not be as efficient in inhibiting Th2 function as they are in inhibiting Th1 function (Cosmi et al. 2004).

Several studies have reported higher numbers of putative Tregs in patients with atopy or allergic symptoms compared with non-atopic subjects (Akdis, Akdis 2009), but also opposite results have been published (Reefer et al. 2008). The caveat in these studies is, however, that

the most widely used marker combination, $CD4^{+}CD25^{high}$ variably combined with FoxP3-positivity, is not necessarily very accurate in identifying Tregs (please see above the chapter on Treg markers) (Romagnani 2006). Suppressive *in vitro* experiments nevertheless add to the reliability of the results.

Patients with atopic dermatitis and/or bronchial asthma had lower FoxP3 expression in CD4+ cells than healthy control subjects, and CD4+FoxP3+ percentage of PBMCs correlated negatively with total IgE levels (Orihara et al. 2007). Lee et al reported that circulating Tregs were fewer in children with bronchial asthma or allergic rhinitis than in healthy control subjects, but on the other hand patients with more severe allergic symptoms had higher Treg numbers than those with milder symptoms (Lee et al. 2007). Increase in circulating Tregs has been reported to associate with the induction of tolerance in specific immunotherapy (Pereira-Santos et al. 2008). PBMCs extracted from patients during aeroallergen immunotherapy showed increased numbers of CD4+CD25+ cells that had allergen specific suppressive function that was dependent on IL-10 and TGF-β signals (Jutel et al. 2003). Patients with active hayfever reportedly had weaker Treg suppressive function to effector T cell proliferation and IL-5 production upon antigen stimulation than atopic patients without symptoms, and non-atopic subjects had the strongest Treg suppressive function (Ling et al. 2004). Contrarily, Jartti et al reported that in pediatric patients with family history of atopy the proportion of CD4+CD25high cells to CD4+ cells correlated positively with pollen-sensitization and IL-5, IL-10 and IL-13 production; however, no functional difference of Tregs was seen between study groups(Jartti et al. 2007). Maggi et al demonstrated that antigen specifc Tregs, both from patients allergic to the antigen and from non-atopic subjects, suppressed cytokine production by effector Tcells upon co-culture with specific antigen loaded dendritic cells (Maggi et al. 2007). A result significantly opposed to many of the afore mentioned studies was reported by Reefer et al (Reefer et al. 2008). They found that in patients with atopic dermatitis and high total IgE levels, CD25 high cells expressing Foxp3, CCR4 and CTLA-4 were more numerous than in subjects with low total IgE levels (Reefer et al. 2008). Furthermore, these cells rather induced than inhibited Th2 function (Reefer et al. 2008).

A few studies have addressed the question of tissue homing and localization of Tregs in atopic disorders. House dust mite immunotherapy was observed to induce IL-10 expressing PBMCs, also positive for CD4+CD25+, that expressed elevated levels of surface molecules related to peripheral tissue homing (Gardner et al. 2004). Grass pollen immunotherapy was associated with increased numbers of CD4+ FoxP3+ and CD25+FoxP3+ cells during pollen season compared with the numbers before immunotherapy (Radulovic et al. 2008). Out of the season numbers of these cells were higher in patients in immunotherapy than in untreated patients with hay fever (Radulovic et al. 2008). Sublingual grass pollen immunotherapy showed association with increased number of FoxP3 expressing cells in oral epithelium (Scadding et al. 2010). In atopic dermatitis, functional Tr1 cells as well as TGF-β and IL-10 were found in the skin lesions, but CD4+CD25+FoxP3 cells were mostly absent (Verhagen et al. 2006).

The role of Tregs in food allergies appers to conform to the observations in other allergies. A genetic defect in Treg function, i.e. a variant of human IPEX caused by a FoxP3 mutation, leads to severe food allergies (Torgerson et al. 2007). A recent study suggests that weaker responses of effector T cells to suppressive Tregs may predispose infants to egg allergy (Smith et al. 2008). Research on CMA has reported that patients with CMA who had become tolerant to CM had higher number of circulating CD4CD25 T cells (Karlsson, Rugtveit & Brandtzaeg 2004) or allergen specific $FoxP3^{+}CD25^{hi}CD27^{+}$, $CTLA4^{+}$, $CD45RO^{+}CD127^{-}$ (Shreffler et al. 2009) than patients with clinical reactivity to CM. In duodenal biopsies, $FoxP3^{+}$ cells were more frequent in patients with food allergy compared with healthy subjects or with patients with Crohn's disease (Westerholm-Ormio et al. 2010). Furthermore, untreated food allergy was associated with higher numbers of $FoxP3^{+}$ cells compared with patients on an elimination diet (Westerholm-Ormio et al. 2010). The lower ratio of FoxP3 mRNA expression to the number of $FoxP3^{+}$ cells in patients with untreated food allergy suggested a lower activity of these cells in comparison with healthy subjects (Westerholm-Ormio et al. 2010).

A paradigm for the development of oral tolerance and cow's milk allergy

The quality and intensity of immune responses an infant mounts to CM antigens partly depend on genetic factors. An essential environmental factor is the timing of first CM exposure: early exposure induces pronounced humoral responses. Gut permeability affects the antigen load that GALT gets in contact with. Increased gut permeability may, furthermore, allow potentially more immunogenic undigested proteins to pass the gut barrier. In physiological conditions, high antigen exposure predominantly results in T cell anergy, whereas low exposure leads to the activation of Tregs. Dendritic cells residing in GALT play a central role in presenting antigens and inducing Treg function. Several different kinds of Tregs are present in the gut, and Tregs that have acquired antigen specificity also circulate in the blood. Tregs suppress cytokine production by effector T cells, such as IL-4 production by Th2 cells. IL-10 producing Tr1 induce IgG4 production while inhibiting IgE production. IgA production is induced by TGF-β-secreting Tr3 cells. Both IgG4 and IgA antibodies promote tolerance rather than sensitization, and indeed physiological humoral responses to CM in infants are dominated by CM specific IgG and IgA antibodies.

Th2 dominated response to CM exposure and failure to induce oral tolerance result in CMA, which usually develops during the first year of life. The majority of patients recover, however, by toddler age. The immunological mechanisms behind the natural development of tolerance in CMA are not fully understood. The trigger for tolerance development is elusive. It might be that during elimination diet CM specific effector T cells are devoid of antigen stimulation and their populations gradually diminish. In contrary, the trigger might as well be unintentional, incremental consumption of CM or controlled oral immunotherapy. In tolerance induction and

recovery from allergy, Tregs, Tr1 cells in particular, become activated. They most importantly suppress Th2 cell proliferation and cytokine production. In addition, activated Tr1 cells secrete IL-10, which shifts IgE production in B-cells towards IgG4 production. Specific IgG4 antibodies block the action of IgE of same specificity, and thus reduce IgE-mediated allergic reactions. Antigen specific IgA, induced by TGF-β, is also related to tolerance development, plausibly in part by inducing IL-10 production. Non-IgE mediated CMA subsides more rapidly than IgE-mediated allergy, which might be due to the fact that Tregs can directly suppress effector Tcells and do not need to additionally dampen antibody production and binding.

Recent research has improved our understanding on how physiological induction of tolerance is disrupted in CMA and how it is regained in clinical recovery from CMA. Still, many controversies and gaps remain to spur further research.

Aims of the study

1. To evaluate the impact of genetic factors, especially HLA II, filaggrin and PTPN22, on the risk of cow's milk allergy and on the immune responses to dietary antigens.

2. To investigate the role of humoral responses to cow's milk in the development of oral tolerance and allergy. To furthermore characterize the profiles and dynamics of circulating specific antibodies binding to cow's milk epitopes.

3. To investigate how expression of genes with immunological functions and specific humoral responses differ between patients with CMA and non-atopic children, and furthermore between patients with persisting CMA and patients with early recovery.

4. To explore ways to improve currently available diagnostic and prognostic tools for CMA.

Materials and methods

Study population

The same population was investigated in all four original publications. For study IV, nine new patients with active CMA were recruited (see below). Informed and written consent was received from infants'/children's parents, and at later age also from children themselves. The Ethics Committee of the Hospital for Children and Adolescents, University of Helsinki, approved of the studies.

The population has been studied, predominantly from the clinical perspective, previously (Saarinen et al. 1999, Saarinen et al. 2005, Saarinen et al. 1999, Saarinen, Savilahti 2000, Saarinen, Savilahti 2000, Saarinen, Suomalainen & Savilahti 2001, Saarinen, Sarnesto & Savilahti 2002, Saarinen, Sarnesto & Savilahti 2002, Savilahti, Saarinen 2009). The population was part of a population-based cohort of 6209 full-term newborn infants who were recruited between August 1994 and November 1995 in three maternity hospitals in the Helsinki metropolitan region. They participated in a prospective, double-blind, randomized trial on early feeding and the emergence of CMA (Saarinen et al. 1999). At all maternity hospitals (mean hospital stay 4 days), exclusive breast feeding was encouraged. The 5385 (87%) infants who required supplementary feeding were randomized to receive in addition to own mother´s breast milk one of three supplements: liquid CM formula (Tutteli, Valio, Finland), extensively hydrolyzed whey formula (Pepti-Junior, Nutricia, The Netherlands) or donor BM (a mixture of BM from multiple donors expressed 1-6 months after delivery) (Saarinen et al. 1999). Of the original cohort, 247 infants reportedly developed cow's milk related symptoms which subsided during elimination diet. These infants underwent at mean age 7 months (+/- SD 2) an open oral CM challenge, which confirmed CMA in 118 and was negative in 129. We obtained serum samples from infants who did not react at CM challenge at this point only, whereas serum samples from children who were diagnosed with CMA were available in addition at time points one year after diagnosis and in most cases, at follow-up at mean age of 8.6 years (see below).

We considered CMA to be IgE-mediated if skin prick test with CM extract resulted in a wheal diameter ≥ 3mm greater the negative control or the level of CM specific IgE antibodies was ≥ 0.7 kU/L (measured with UniCap, Phadia, Uppsala, Sweden) or both were positive at any time point 0-12 months after diagnosis (Saarinen et al. 2005). Of the infants with CMA, 86 (73%) had IgE-mediated and 32 (27%) non-IgE-mediated CMA (Saarinen et al. 2005). All 118 children with CMA were invited to visit an outpatient clinic every 6 months up to age 2 years, and annually thereafter until recovery. Recovery was defined as regular consumption of CM or a negative controlled oral CM challenge performed at outpatient clinic.

Between August 2003 and March 2004, a follow-up study on patients' CMA status and other atopic manifestations was carried out. Of the invited 118 children with a history of CMA, the large majority, 94 (80%) participated. We also invited an equal number of randomly selected subjects without CMA from the same cohort ("control subjects"), and 80 (68%) of them visited the outpatient clinic (Saarinen et al. 2005). At this time, the children in both groups were 8-9 years (mean 8.6 years) old. At the follow-up visit, an investigator carried out a structured interview and a physical examination. Skin prick tests (SPTs) were performed with a panel of 18 allergens, with standard solutions for most allergens and prick-to-prick method for a few food allergens as previously described (Saarinen et al. 2005).

Blood samples were drawn at each visit at the outpatient clinic. Sera were thus available from the majority of patients with CMA at diagnosis, a year later and at follow-up (please see below for numbers of samples in each study). Serum samples from infants with CM-related symptoms but negative CM challenge were available at the time of the diagnostic (negative) CM challenge only. DNA and PBMCs were extracted from whole blood samples drawn at the follow-up visit.

For study I, blood samples were available from 163 children: 87 patients with CMA and 76 control subjects. Of the patients in this study, 11 (13%) had persistent CMA, others had become tolerant to CM by the time of the follow-up visit. Table 1 in the original publication of study I shows the clinical characteristics of the study population.

For study II, serum samples from the patients were available at diagnosis (mean age 7 months, n=111), one year later (mean age 19 months, n= 101) and at follow-up (mean age 8.6 years, n=85); as well as salivary samples (n=107) at diagnosis. In addition, sera from 80 control subjects from the same cohort were available at follow-up (mean age 8.6 years). Clinical characteristics are shown in Tables 1 and 2 of the original publication II.

For study III (table 3), serum samples were available from 11 patients who still had active IgE-mediated CMA at mean age of 8.6 years, and selected 12 patients who had recovered from IgE-mediated CMA by age 3 years. From these patients, we investigated serum samples collected at the time of diagnosis, one year later and at the final follow-up (see table 2 for time points). The selection criteria for the patients in study IV were: active IgE-mediated CMA at age 8.6 years (persisting CMA) or recovery from IgE-mediated CMA by age 3 years (transient CMA), and serum samples available at all three time points. Serum samples drawn at follow-up from six non-atopic control subjects served as reference. Age, the outcome of an open oral cow's milk challenge and cow's milk specific IgE levels at three time points in the study groups are shown in Table I of the original publication III.

In study IV, we examined PBMC samples from 57 children aged 5-12. The population consisted of three groups: 16 had active CMA, 20 had recovered from CMA by age 3 years, and 21 were non-atopic control subjects. The majority of subjects had participated at age 8-9 years to the study described above (Saarinen et al. 2005). In addition, we recruited nine patients with active CMA beyond age 5 years (range 5.4-12.3) in spring 2008 at the Helsinki University Hospital. CMA diagnosis was based on an open CM oral challenge, and the current CMA status on either an oral challenge or regular consumption of CM. The CMA was or had been IgE mediated in all patients. The control subjects were considered non-atopic based on absence of atopic symptoms, total IgE less than 130 kU/l (measured with UniCAP, Phadia, Uppsala, Sweden) and negative skin prick tests (SPTs) that were performed with a panel of 18 allergens, as previously described (Saarinen et al. 2005).

DNA extraction and genotyping

We applied a salting out method for extracting DNA from whole blood samples (Miller, Dykes & Polesky 1988) . We defined the common European HLA class II haplotypes using a low resolution full-house genotyping method that has been described earlier (Hermann et al. 2003).

We genotyped the PTPN22 C1858T (rs2476601) polymorphism with a homogeneous genotyping (Kiviniemi et al. 2003).

For analysing five mutations of filaggrin gene (del22824, 501-C/T, R2447X, S3247, 3702delG) we used direct sequencing with MegaBACE1000 equipment (GE Healthcare, Amersham Biosciences, Piscataway, NJ, USA). The number of analysed samples varied between different filaggrin gene polymorphisms: 113 cases (64 patients; 49 control subjects) were studied for del22824, 153 (83; 70) for 501-C/T, 96 (52; 44) for R2447X, 118 (62; 56) for S3247 and 111 (64; 47) for 3702delG.

All genotyping was performed in professor Ilonen's group, Department of Clinical Microbiology, University of Kuopio, Kuopio, Finland.

Measurement of antibodies

We stored serum samples at -80°C until analyzed.

We measured the serum total IgE concentration and the allergen-specific IgE antibodies to CM, hen's egg ovalbumin and birch with enzymatic UniCap fluoroimmunoassay (Phadia, Uppsala, Sweden) (Saarinen, Savilahti 2000).

We measured bovine-β-lactoglobulin and ovabumin specific IgA and IgG levels with enzyme-linked immunosorbent assays (ELISA) (Savilahti et al. 1993). Microtiter plates (Nunc Maxi Immunoplate, Thermo Fisher Scientific, Roskilde, Denmark) were coated with bovine-β-lactoglobulin (Sigma-Aldrich, St. Louis, MO) at a concentration of 1 μg/mL, or with ovalbumin (Sigma-Aldrich) at a concentration of 2 μg/mL in phosphate buffered saline (PBS). Coated plates were incubated for 3 h at 37 °C. After washing with PBS, unspecific binding was blocked with 2% sheep serum in PBS. Plates were then incubated for 1 h at 37 °C and washed thereafter with PBS-0.05%Tween. Sera were diluted in 1% sheep serum in PBS-0.05% Tween and applied in duplicate to the antigen-coated plates and to control plates (coated only with blocking solution) at dilutions of 1:20 for β-lactoglobulin IgA and IgG, or 1:20 for ovalbumin IgA and 1:400 for ovalbumin IgG. The plates were incubated overnight at 4 °C. Thereafter the plates were washed with 1% PBS-0.05%Tween three times. Alkaline phosphatase-conjugated Affine Pure Rabbit anti-human IgA and IgG (Jackson, Baltimore) in a concentration of 0.3 mg/ml were applied at dilutions of 1:1000 for β-lactoglobulin and ovalbumin IgA, and 1:3000 for β-lactoglobulin and ovalbumin IgG. The plates were incubated for 1 h at 37 °C. After washings, the substrate 1 mg/ml p-nitrophenyl phosphate in Tris buffer (Sigma-Aldrich, St. Louis, MO)was added. After 30 min at 37 °C the reaction was stopped with 1M NaOH. The optical density of the end product was immediately measured at 405 nm with a semiautomatic photometer (Labsystems Multiscan, Frankfurt, Germany).

Protocol for measuring α-casein specific IgA and IgG levels with ELISA has also been described (Savilahti et al. 1993). Microtiter plates (Nunc Maxi Immunoplate, Thermo Fisher Scientific) were coated with bovine-α-casein (Sigma-Aldrich) at a concentration of 2 μg/mL in phosphate buffered saline (PBS). Coated plates were incubated for 3 h at 37 °C. After washing with Tris-buffered saline (TBS), unspecific binding was blocked with 1% gelatin in TBS. Plates were then incubated for 1 h at 37 °C and washed thereafter with TBS- 0,05% Tween. Sera were diluted with 1% gelatin in TBS-0,05%- Tween at dilutions of 1:50 for IgA and 1:200 for IgG, and applied in duplicate to the antigen-coated plates and to control plates. Plates were incubated for 1 h at 37 °C. Alkaline phosphatase-conjugated Affine Pure Rabbit anti-human IgA and IgG (Jackson, Baltimore) in a of concentration 0.3 mg/ml were applied at dilutions of 1:700 for IgA and 1:6000 for IgG. Thereafter, the protocol continued as for β-lactoglobulin and ovalbumin IgA and IgG measurements as described above.

For measuring the levels of IgG1 and IgG4 to β-lactoglobulin and α-casein we adapted ELISA protocols from Ruiter et al (Ruiter et al. 2007a). Microtiter plates (Nunc Maxi Immunoplate, Thermo Fisher Scientific, Roskilde, Denmark) were coated and blocked as described above. Sera were applied on the plates as described above: at dilutions of 1:20 (for α-casein IgG4, BLG IgG4) or 1:100 (for ovalbumin IgG4). The plates were then incubated for 1 h at 37°C. After washing, 100 μL of horseradish peroxidase (HRP) conjugated anti-human IgG4 (Sanquin, Amsterdam, Netherlands) at dilutions of 1:30000 for α-casein and ovalbumin or 1:10000 for β-lactoglobulin , or anti-human IgG1 (Zymed Labotatories, San Fransisco, CA) at

dilution 1:1000 was applied on wells, followed by an incubation of 1 h at 37°C. After washing, 100 µL of tetramethyl-benzidine (TMB) liquid substrate (Sigma-Aldrich) was applied on plates and incubated for 10 min at room temperature, sheltered from light. The reaction was stopped with 1 M H_3PO_4. A wavelength of 450 nm was used to immediately measure the optical density of the end product with a semiautomatic photometer (Labsystems Multiscan, Frankfurt, Germany).

Values of ELISA results were expressed as arbitrary units (AU) deduced from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks.

Peptide microarray based immunoassay

We performed the peptide microassay based immunoassay as previously described (Shreffler et al. 2005, Lin et al. 2009) with minor modifications. Commercially synthesized 289 peptides of 20 amino acids with an offset of 3 amino acids, corresponding to the primary sequences of α_{s1}-, α_{s2}-, β-, and ϰ-caseins, and β-lactoglobulin, (JPT Peptide Technologies GmbH, Berlin, Germany) were resuspended in dimethyl sulfoxide (DMSO) at 1 mg/mL, diluted 1:2 in Protein Printing Buffer (PPB, TeleChem International, Inc., Sunnyvale, CA, USA) with 0.02% Sarkosyl to a final concentration of 0.5 mg/mL. Peptides were printed on epoxy-derivatized glass slides (SuperEpoxy Substrate, TeleChem International, Inc.,) using a NanoPrint™ Microarrayer 60 (TeleChem International, Inc.) equipped with 2×4 ArrayIt Stealth Micro Spotting Pin (SMP3B). Fluorochrome- labelled human serum albumin was printed as a reference for the grid and protein printing buffer as a spot negative control for background normalization. For immunolabeling, an area around the arrays was demarcated with a hydrophobic pen (DakoCytomation Pen, DAKO, Glostrup, Denmark). Incubations were performed in the dark in a humidity chamber (Binding Site, Birmingham, UK) on an orbital rotating platform with gentle agitation. The day after printing, the slides were washed with phosphate-buffered saline containing 0.05% tween 20 (PBS-T) and dH_2O. Non-specific binding sites were blocked with 400 µl of 1% human serum albumin (HSA) in PBS-T (PBS-T/HSA) for 60 minutes at 31 ° C. After aspiration of blocking buffer, 250 µl of patient serum diluted 1:5 in PBS-T/HSA was incubated for 16 hours at 4° C. Slides were then washed with PBS-T.

For IgE and IgG4 detection, slides were incubated for 24 hours at 4° C with a cocktail of four monoclonal antibodies. Three were monoclonal biotinylated anti-human IgE: one from Invitrogen (Carlsbad, CA, USA) and diluted 1:250, one from BD Biosciences (Pharmingen, San Jose, CA, USA) and diluted 1:250, and one as a gift from Phadia (Uppsala, Sweden) biotinylated in our laboratory and diluted 1:1000. The cocktail further included one monoclonal anti-human IgG_4-FITC (Clone: HP6025, Southern Biotechnology Associates Inc.,

Birmingham, AL, USA) diluted 1:1000. Slides were subsequently washed with PBS-T, incubated for 4 minutes with ethylene diamine tetraacetic acid (EDTA) 1 mM in PBS-T, washed again with PBS-T, equilibrated for 1 minute with Dendrimer Buffer (Genisphere, Philadelphia, USA) followed by incubation for 3 hours at 31 ° C with a cocktail of Anti-Biotin-Dendrimer_Oyster 550 (350) (Genisphere) and Anti-FITC_Dendrimer_Oyster 650 (350) (Genisphere) in Dendrimer Buffer both at 0.6 μg/ml with addition of 0.02 μg/ml of salmon sperm DNA. Finally, slides were washed with PBS-T, 15 mM Tris buffer, centrifuge dried, followed by wash with 0.1 X PBS, centrifuge dried, washed again with 0.05 X PBS and centrifuge dried.

For IgA detection, slides were printed a week before immunolabeling in order to reduce background. After serum incubation and washing, slides were incubated for 1 h at 31 ° C with polyclonal goat anti-human IgA diluted 1: 250 (Sigma-Aldrich) which was covalently conjugated with Alexa 546 (Molecular Probes – Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Slides were then washed with PBST, distilled water, and centrifuge dried.

Immunolabeled slides were scanned using a ScanArray®Gx (PerkinElmer, Waltham, MA, USA). Images were saved as TIF format. The results are semiquantitative.

Image analysis in microarray based immunoassay

We analyzed peptide array chip images for quality control, and quantified the spot intensities as the mean of the detected spot area brightness. The local backgrounds of the spots were detected and the final normalized value was expressed as

$$I_n = (I - I_b) / (I_p - I_{pb})$$

Where
I = spot mean intensity
Ib = spot local background intensity
Ip = PBSO spots' median intensity (several on each chip)
Ipb = PBSO spot local background intensity

We calculated a median of the peptide spot intensities on each chip. The intensity was labeled as active if the intensity deviated more than half standard deviations from the normalized zero level (PBSO). We calculated the standard deviation from all of the spot measurements.

PBMC extraction and *in vitro* stimulation

We extracted PBMCs with Ficoll Paque (GE Healthcare Bio Sciences Corp, Piscataway, NJ) centrifugation of heparinized blood samples. The cells were frozen overnight in an isopropanol container at -70°C, and stored at -135°C in a medium with 10% dimethyl sulfoxide ($(CH_3)_2SO$, DMSO ; Sigma Aldrich, St. Louis, MO) and 90% cell culture medium with RPMI 1640 medium with 25 mM Hepes (Gibco Invitrogen, Carlsbad, CA) containing 5% human AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland), 2 mM L-glutamine (Gibco) and 25 μg/ml gentamycin (Sigma Aldrich). Before *in vitro* stimulation, samples were thawn briefly in +37°C water bath, suspended in RPMI 1640 medium with 25 mM Hepes (Gibco) and centrifuged at 1100 g for 10 min to wash out the DMSO. The RPMI medium was removed and cells were suspended in cell culture medium described above. Median cell recovery after thawing was 70-77% in the three study groups.

Immediately after thawing PBMCs were stimulated *in vitro* with bovine β-lactoglobulin (Sigma Aldrich) at a concentration of 200 μg/ml in cell culture medium described above. We confirmed the purity of the β-lactoglobulin with FPLC. Sterile PBS (Biowhittaker Lonza, Basel, Switzerland) served as negative control, and tetanus toxoid (National Public Health Institute, Helsinki, Finland) at a concentration of 16 μg/ml was the positive control whenever cells sufficed for all three stimulations. Cells were cultured at + 37°C, with 5% CO_2. For RNA extraction, PBMCs were cultured for 72 h in 96 well plates (Corning Life Sciences, Corning, NY), 0.2 million cells per well and a total of 1.2 million cells per antigen stimulation. For flow cytometry, PBMCs were cultured for 120h in 48 well plates (Greiner Bio-One, Kremsmuenster, Austria), 0.5 million cells per well and a total of 2-4 million cells per antigen stimulation, depending on the amount of cells available. The culture times for RNA extraction and protein expression had been optimized previously (Marschan et al. 2008), and they are consistent with work by other groups (e.g. (Savolainen et al. 2007).

RNA extraction and reverse transcription

We harvested PBMCs after 72h of stimulation. Wells were washed twice with sterile PBS (Biowhittaker Lonza), and cells spinned at 1100 g for 10 min. We suspended cell pellets in RNeasy Minikit lysis buffer (Qiagen, Hilden, Germany) with 1% mercaptoethanol, and incubated at room temperature for 3-5 min. We stored cell lysates and cell culture supernatants at -70°C. After thawing in room temperature, we spinned cell lysates through QiaShredder columns (Qiagen,) following manufacturer's instructions. We extracted RNA with Qiagen RNeasy Minikit according to manufacturer's instructions with the optional DNAse step added (Qiagen RNAse-free DNAse I set) in order to remove genomic DNA. We measured RNA concentrations with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham,

MA). RNA was reverse transcribed with TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA). RNA was stored at -70°C and cDNA at -20°C.

Real time polymerase chain reaction (QRT-PCR)

We ran complementary-DNA samples in triplicates on StepOne QRT-PCR system (Applied Biosystems) with TaqMan reagents (Applied Biosystems) using FAM labeled probes and ROX as passive reference. We selected 12 gene expression markers based on recent literature on Treg function. Target specific primers for T-bet, GATA-3, IFN-γ, CTLA4, IL-10, IL-16, TGF-β, FoxP3, Nfat-C2, TIM3, TIM4, STIM-1 and 18s were purchased from Applied Biosystems. Ribosomal 18S RNA served as an endogenous control. The sequence detector (Stepone plus real-time PCR systems, Applied Biosystems) was programmed for an initial step of 20 seconds at 95°C, followed by 40 thermal cycles of 1 second at 95°C and 20 seconds at 60°C.

We calculated levels of target gene expression with a comparative threshold cycle (*C*t) method (as recommended by Applied Biosystems). Normalised samples were calibrated with an inter-assay standard: an exogenous cDNA pool generated from PHA stimulated PBMCs. The relative amount of a target gene in a sample was normalized in relation to both the calibrator and to an endogenous control (18S). When ΔCt stands for the difference between *C*t of the marker gene and *C*t of the 18S gene, ΔΔCt denotes the difference between the ΔCt of the analyzed sample and ΔCt of the calibrator. Calculation of $2^{-\Delta\Delta Ct}$ then gives a relative amount of the target gene in analyzed sample compared with the calibrator, both normalized to an endogenous control (18S).

Flow cytometry

We collected PBMCs for flow cytometry after 120h antigen stimulation, and suspended them in 0.5% BSA-PBS. We followed a protocol described earlier (Liu et al. 2006) with a few modifications, and performed intracellular staining with Alexa-488-conjugated anti-FoxP3 kit by BioLegend (San Diego, CA, USA). Cells were first stained with surface markers for 30 min at +4°C. The anti-human antibodies PE-conjugated anti-CD127, APC-conjugated anti-CD25, and PerCP-conjugated anti-CD4 as well as appropriate isotype controls were purchased from Becton Dickinson (BD Biosciences, San Jose, CA, USA). After washing once with 0.5% BSA-PBS, cells were fixed for 30 min at RT using 1 x Fix/Perm buffer provided in the kit. Cells were then washed once with 0.5% BSA-PBS and twice with 1x Perm buffer, and blocked with mouse IgG for 15 min at RT. Anti-human Alexa-488-conjugated FoxP3 was added, and cells were stained for 30 min at +4°C. After washing twice with 1x Perm buffer, cells were suspended in FACS (fluorescent-activated cell sorter) flow solution (BD Biosciences). Stained

cell samples were run on FACS Calibur (BD Biosciences), and analyzed with BD FACSDiva software (BD Biosciences).

Statistical analysis

In study I, the HLA haplotypes as well as PTPN22 and filaggrin alleles were coded dichotomically as present or absent. We tested differences in the distribution of alleles between groups with Fisher's exact test. Logarithmic transformations of antibody levels (IgA, IgG, IgG1, IgG4 to whole cow milk, α-casein, bovine lactoglobulin and ovalbumin, IgE to cow milk, hen's egg and birch) were compared with the t-test of independent variables between allele carriers and non-carriers among all subjects and within subgroups of control subjects, all patients with CMA, and patients with IgE- mediated CMA. We considered a p-value of 0.003 or less to be statistically significant, which was based on dividing 0.05 with the number of HLA haplotypes analyzed (n=14).

In study II, we tested differences in the distribution of clinical characteristics in the two patient groups with Fisher's exact test. Logarithmic transformations of antibody levels (IgA, IgG, IgG4, IgG1 to CM, α-casein, β-lactoglobulin and ovalbumin, IgE to CM, ovalbumin and birch) were compared between the two patient groups ("early tolerant" and "persisting CMA") and between patient groups and control subjects with the t-test of independent variables. We used general linear model for repeated measures to test whether antibody levels changed significantly over time and also to test whether the profiles of development of antibody levels over time differed between different groups. Spearman correlation was calculated for specific IgA levels in serum and salivary samples.

In study IV, we compared data of flow cytometry assays, and of the QRT-PCR markers selected based on neural network analysis (see below) between the three study groups (persisting CMA, recovered CMA and control) with Kruskall-Wallis non-parametric test and pairwise with Mann-Whitney test.

In studies II-II and IV, we considered a p-value of 0.05 or less statistically significant.

We performed the statistical analyses with SPSS 15.0.1.

Bioinformatic analysis in study III

To find active peptide regions within a sample group, the active peptide hits were convoluted with a gaussian curve of <m>\sigma=2</m>. Smoothed activation values were averaged over the sample group. We calculated the differences between groups and time points from the

smooothed averages. A peptide region was labeled active if within it an active peptide was detected in at least half of the patients in a group.

We applied decision tree analysis for peptide microarray data from samples at diagnosis in order to investigate whether a set of peptides could classify patients correctly and thus predict the clinical pace of recovery from CMA. Peptide binding by IgE, IgG4 and IgA was coded dichotomously as active or absent. Since building classifier for 289 (the number of peptides in the microarray) attributes and 23 samples failed to give meaningful classification results, we applied attribute selection algorithm that considers attribute relevance and redundancy (Lei, Huan 2003). Attribute selection was performed with different datasets and each dataset was classified with Random Tree classifier to evaluate which set of attributes best separate cases of the two study groups, and to find a classifier. Random Tree builds a decision tree by considering randomly chosen log2(n)+1 attributes in each node where n is number of attributes. For attribute selection and classification, we used algorithms implemented in Weka-software (Witten, Frank 2005).

Artificial neural network analysis

In study IV for optimal, distinctive profiling of patient groups, we applied artificial neural networks with exhaustive search for all gene marker combinations.

A multi-layer perceptron (MLP) artificial neural network is a supervised machine learning method that mimics biological neural networks. Structurally, MLPs comprise nodes that are ordered into input, hidden and output layers, and arcs that propagated signals from nodes in input layer to nodes hidden layer as well as nodes from hidden layer to output layer (Bishop 1995). Each node in a hidden and output layer has an activation function that transforms the incoming signals to a single value that is further propagated to the connected nodes in the next layer. Arcs are associated with weight values that are modified during the training stage in which the classifier is built.

We evaluated classifier performances with five-fold-cross-validation using three different means to assess the performance. First, we used confusion matrix, which presents the distribution of classification results in matrix form where the columns are real classes and the rows are predicted classes. Second, we used the kappa-value that describes how much the agreement on classification results differs from random guessing (Fleiss 1971). Kappa-statistics is calculated as $(Po - Pc)/(1 - Pc)$, where Po is the observed agreement and Pc is the agreement that would be achieved by chance. For a perfect classification, kappa-value is one and the value of zero means classification expected by chance.

The third statistical validation method we employed is a receiver operating characteristic (ROC) curve that is a graphical presentation of classification sensitivity and specificity where,

for each class, the true-positive rate is plotted against the false-positive rate (Fawcett 2006). . Area under ROC curve (AUC) is the value that describes the size of the area that the curve occupies from the graph. For a perfect classification, the AUC value is one, whereas random guess produces is the AUC value of 0.5.

We created all 4095 marker combinations from the 12 markers (2^{12}-1) and trained 4095 classifiers using the delta Ct transformed β-lactoglobulin stimulated expression marker combinations. Classifiers were built with the MP-algorithm in Weka data mining software (Witten, Frank 2005).Number of layers, neurons and connections were automatically chosen by the MP-algorithm as follows. One input and output neuron for each of three classes and (number of markers + number of classes)/2 neurons in one hidden layer. In Weka, kappa-statistics is calculated from the values in the confusion matrix and the result presents the agreement between true and predicted classes. We compared the validation results using confusion matrices, kappa-statistics and AUC values of categories of the classifiers.

Results

Association of selected genetic factors with humoral responses to early food allergens (I)

We did not find any association between CMA and the genetic factors we investigated. The 14 HLA haplotypes were similarly distributed among control subjects and patients with CMA or with IgE-mediated CMA (study I, Table 2).

Neither did we observe any difference in the distribution of the PTPN22 polymorphism C1858T (rs2476601) between these groups (data not shown). Six subjects were heterozygote carriers of the filaggrin null mutation del22824: 3 patients with CMA (3/87) and 3 control subjects (3/76). All three control subjects carrying the filaggrin del22824 mutation had reported allergic symptoms: eczema (n=3), allergic rhino-conjuctivitis (n=2) or asthma (n=1), and one of them had positive SPT to aeroallergens. The three carriers with IgE-mediated CMA had eczema. The other four filaggrin mutations screened were absent in the study population.

In spite of no association with CMA, humoral responses to CM and hen's egg antigens were associated with two HLA II haplotypes (study I, Table 3, Fig 1 and 2). Data for the entire study population is depicted in Table 1, whereas Figures 1 and 2 show the data divided by subgroups of control subjects, all patients with CMA and patients with IgE-mediated CMA.

Carriers of (DR15)-DQB1*0602 had higher IgG and IgG4 levels to bovine β-lactoglobulin and α-casein than non-carriers (study I, Table 3). In patients with CMA, the association was significant for both bovine β-lactoglobulin and α-casein, whereas no difference was seen in the control group (study I, Fig 1). Specific IgG1 to all antigens tested were on comparable levels among (DR15)-DQB1*0602 carriers and non-carriers (data not shown).

Unlike with (DR15)-DQB1*0602, lower antibody levels to β-lactoglobulin and ovalbumin were associated with (DR1/10)-DQB1*0501 (study I, Table 3, Fig 2). Depending on the antibody subclass and the allergen, the differences were present among either control subjects or patients with CMA (study I, Fig 2). The difference in IgG1 levels to ovalbumin nearly reached significance (t-test p-value 0.005) between control subjects with the DQB1*0501 allele (n=29; geometric mean 12 AU; 95% CI 6.3-22) and without the allele (n=47; geometric mean 36 AU; 95% CI 23-57).

We further compared carriers of (DR15)-DQB1*0602 with those of either of (DR1/10)-DQB1*0501 (study I, Table 3). Carriers of (DR15)-DQB1*0602 had higher IgG and IgG4 levels to β-lactoglobulin as well as higher IgG, IgG4 and IgG1 levels to ovalbumin than carriers of (DR1/10)-DQB1*0501 (study I, Table 3). In the comparison within subgroups, the differences were significant among CMA patients and only suggestive among control subjects (data not shown).

None of the genetic factors investigated was associated with IgA and IgE levels to the early food allergens (data not shown). A suggestive association was found between haplotype (DR9)-DQA1*03-DQB1*0303 and higher IgE levels to birch (allele carriers n=6; geometric mean 4.038 kU A/l; 95% CI 0.488-33.395 vs. non-carriers n=132; geometric mean 0.182; 95% CI 0.116-0.286; t-test p-value 0.006). Filaggrin gene deletion at 22824 was associated with lower IgE levels to cow's milk but the association did not, however, reach statistical significance (allele carriers n=6; geometric mean 0.048 kU A/l; 95% CI 0.026-0.091 vs. non-carriers n=113; geometric mean 0.161 AU; 95% CI 0.125-0.209; t-test p-value 0.039).

Development of humoral responses to cow's milk proteins in patients with CMA over time and differences compared to children without CMA at follow-up (II-III)

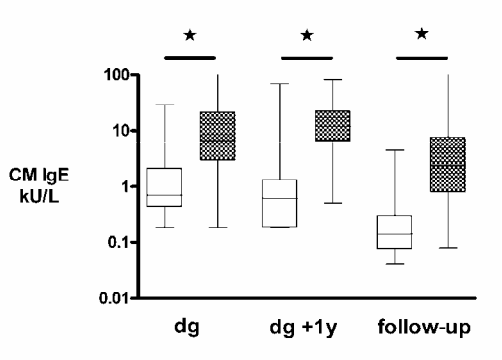
We investigated differences in specific antibody levels to CM proteins and in CM epitope binding by antibodies between patients who had recovered early and those with persisting CMA. Please refer to method section "study population" for characteristics and number of subjects in the study groups these data are based on.

Levels of specific IgE to CM evolved differently in patients with persisting CMA and patients with early recovery (II)

Children with persisting CMA at age 8 years had higher levels of CM specific IgE at all three time points compared with those who became tolerant by age 3 years (Fig 3 and study II, Table 3,). General linear model (GLM) of repeated measures indicated that both time ($p<0.001$) and group (II, Table 4; $p=0.005$) were significant factors. At follow-up, levels of CM specific IgE were higher in all children who had a history of IgE-mediated CMA, including those with early recovery, compared with control subjects (II, Table 3).

Figure 3. Levels of cow's milk (CM) specific IgE in 2 groups: patients who recovered from CM allergy by age 3 years (blank boxes), and those who still had persistent CM allergy at 8

years (checkered boxes). The y-axis represents IgE levels to CM in kU/L. Measurements are shown on a log10 scale; lines mark (from bottom up) the 5th percentile, lower quartile, median, upper quartile and the 95th percentile. The x-axis depicts the 3 time points under investigation: at diagnosis (dg), one year after (dg +1y) and at follow-up. An asterix denotes a p-value < 0.0001 based on the t-test of independent variables.



Patients with persisting CMA had more intense and stable IgE peptide binding over time than patients with early recovery (III)

At diagnosis, IgE binding pattern to CM peptides in both patient groups differed little (III, Fig 1A). The persisting group had more intense IgE binding than early tolerant to one region on β-casein, three regions in β –lactoglobulin and one wide region on κ-casein (III, Fig 1A). The recognition profile of children with persisting CMA did not change over time (study III, Fig 1). The signal overall was strongest at the time of diagnosis, except for a region in αs2-casein which gave a stronger signal at follow-up than at earlier time points (study III, Fig 1E). In contrast, IgE from patients developing early tolerance recognized fewer peptides over time (III, Fig 1A-D, Table II) except for an increased signal at follow-up in a region of κ-casein (III, Fig 1C-D). At follow-up, IgE from patients with persisting CMA bound to a large region within all five proteins (III, Fig 1C).

Comparing the differences in IgE binding between the time of diagnosis and one year later or at final follow-up, the binding increased in regions of αs1- and αs2-caseins more in patients with persisting CMA than in patients with early recovery (III, Fig 1D-E). The opposite was observed for β-casein, β-lactoglobulin and κ-casein (III, Fig 1D-E).

Patients with persisting CMA had lower IgG4 levels to β-lactoglobulin at follow-up than patients with early recovery (II)

In contrast to CM specific IgE levels, serum IgG4 levels to β-lactoglobulin were lower at follow-up among patients with persistent CMA at age 8 years than among patients tolerating CM by 3 (II, Fig 1a) or 3-8 years, and also in comparison to control subjects (II, Table 4).

Comparison of serum IgG4 levels to α-casein resulted in similar, although less pronounced, differences between groups (II, Table 4). Time and group were both significant factors to differences in temporal profiles of β-lactoglobulin IgG4 levels (GLM of repeated measures: $p<0.01$): the levels increased more over time among patients who became tolerant by age 3 years than among those with persistent CMA at age 8 years (II, Fig 1a). Levels of IgG1 to β-lactoglobulin and α-casein did not differ between the groups (data not shown).

IgG4 peptide binding patterns were comparable in persisting and early recovering CMA (III)

Patients with persisting CMA and those with early recovery had similar IgG4 binding patterns at diagnosis, whereas the signal in persisting CMA was more intense (III, Fig 2A). Wide regions especially in αs1-casein and β-casein remained unrecognized (III, Fig 2A). Differences emerged mostly from the two groups recognizing different regions located very close to each other on the same protein (III, Fig 2A). There was, however, a region in β-casein that patients with persisting CMA recognized but those with early recovery did not (III, Fig 2A); the difference was similar with IgE recognition in the same region (III, Fig 1A). The IgG4 binding profiles changed little over time, whereas the binding signal increased (III, Fig 2A-E, Table II). At follow-up, patients with persisting CMA bound more peptides in αs1-casein and β-casein, and patients with early recovery in one region of β—lactoglobulin and one of κ-casein; binding decreased at the terminal end of κ -casein (III, Fig 2C). Comparing the changes in IgG4 binding between time of diagnosis and one year after or follow-up, no clear pattern could be observed in differences between the groups (III, Fig 2E-D).

IgE and IgG4 binding to a panel of peptides at diagnosis discerned patients with persisting CMA from patients recovering early (III)

Random decision tree analysis showed that IgE and IgG4 binding to a panel of regions in αs1-, αs2-, β-and κ-casein (III, Table III) categorized the two patient groups at the time of diagnosis with significant accuracy (area under curve AUC 92%, κ-statistic 0.87). This classification for separating patients with persistent CMA from patients that recovered early from CMA had a sensitivity of 96% and specificity of 91%. We included CM IgE level (measured with UniCAP) as a variable to the dataset before and after the feature selection step, but the prediction accuracy did not improve (data not shown).

β-lactoglobulin specific IgA levels were higher in patients with early recovery (II)

Serum levels of IgA to β-lactoglobulin were higher at the time of diagnosis in patients who recovered from CMA by 3 years compared with both groups where recovery was delayed until either 3-8 years or beyond 8 years (II, Fig 1b, Table 4). Analysis with general linear model (GLM) of repeated measures showed time to be a significant factor ($p=0.04$), with a downward

trend over time (II, Fig 1b), whereas the temporal profiles of specific IgA levels did not differ significantly between the groups.

In patients who recovered from CMA by age 3 years, serum β-lactoglobulin specific IgA levels correlated positively with salivary CM specific IgA levels at diagnosis (Spearman correlation coefficient 0.4; n=53; $p<0.005$) and a year later (Spearman correlation coefficient 0.4; n=52; $p<0.03$). Patients who recovered by age 8 years showed the same association (Spearman correlation coefficient at diagnosis 0.4; n=63, and a year later 0.3; n=61; for both $p<0.001$), but not patients with persistent CMA at age 8 years (Spearman correlation coefficient at diagnosis 0.4; n=16; p=0.2 and a year later -0.04; n=14; p=0.9).

IgA binding to CM epitopes increased over time more in patients with persisting CMA (III)

IgA binding to CM epitopes measured with the microarray based immunoassay was overall low. It increased, however, at follow-up especially in patients with persisting CMA compared with earlier time points (III, Fig 3B). At follow-up, both patient groups had binding with high signal intensity at the terminal end of αs2-casein (III, Fig 3A). The recognition profiles were similar in the two groups, except for two regions in β-casein that persisting CMA group showed more signal intensity in IgA binding than the group with early recovery (III, Fig 3). Comparing the changes in IgA binding between time of diagnosis and one year after, signal intensity in the two groups did not differ. By follow-up, however, the intensity of IgA binding increased more in patients with persisting CMA in several regions across all five proteins (III, Fig 3).

Peptide recognition by IgE had overlap with IgG4, but not with IgA (III)

Peptide binding pattern of IgG4 antibodies was similar to that of IgE in both groups (III, Fig 1, Fig 2, Fig E6, Fig E7).

Among patients with early recovery, IgE binding decreased over time while IgG4 binding remained at same level, or increased in some regions by follow-up (III, Table 2). Among patients with persisting CMA, the signal intensity of IgE binding remained as strong as or became stronger than that of IgG4 binding, apart from regions in β-casein (III, Fig 4A). The difference between IgE binding intensity and that of IgG4 was stronger in the patient with persisting CMA compared than with patients with early recovery at time points after diagnosis, primarily in regions of αs1- and αs2-caseins (III, Fig 4A).

The peptide binding profiles of IgE and IgA had little overlap in αs1- and αs2-caseins in either group, a little more in β-casein, β-lactoglobulin and κ-casein (III, Fig 1C, Fig 3A, Fig 4B). The differences between IgE binding intensity and that of IgA were larger in patients with persisting CMA compared with patients with early recovery at diagnosis and one year after in regions of αs1- and αs2-caseins (III, Fig 4B). On the other hand, little overlap of IgE and IgA

binding existed in these regions (data not shown). At follow-up, the differences were in contrast smaller in patients with persisting CMA compared with patients with early recovery in regions of αs2- and β-casein, β-lactoglobulin and κ-casein (III, Fig 4B).

Summary of differences in specific antibody responses between patients with persisting CMA and those with early recovery (II-III)

Table 3. Summary of findings in studies II and III. P denotes patients with CMA persisting beyond the age of 8 years and E denotes patients with recovery from CMA by the age of 3 years. CM stands for cow's milk and BLG for β-lactoglobulin

CM specific IgE levels over time	P > E
CM epitope binding by IgE over time	P > E
BLG specific IgG4 levels at age 8-9 years	P < E
CM epitope binding by IgG4	P ~ E
BLG specific IgA levels at diagnosis	P < E
CM epitope binding by IgA over time	P > E
CM epitope binding by IgE in relation to binding by IgG4	P > E

T regulatory cell and Th2 expression profile in children with CMA (IV)

We studied the response to β-lactoglobulin *in vitro* by stimulating PBMCs from patients with persisting CMA and those who had recovered from CMA by age 3 years. We investigated the subsequent expression of protein markers identifying Tregs with flow cytometry, and of RNA markers related to Treg and Th2 functions with QRT-PCR.

In the flow cytometry assay, FoxP3 expression intensity in $CD4^+$ $CD25^{high}CD127^{low}$ cells was higher in β-lactoglobulin stimulated PBMCs from children with persisting or recovered CMA compared with non-atopic children (IV, Table 1, Kruskall-Wallis test p-value <0.05, pairwise testing Mann-Whitney p-values < 0.05). FoxP3 expression intensity in children with persisting or recovered CMA was comparable (IV, Table 1, Mann-Whitney p-value > 0.5). The percentage of $CD25^{high}CD127^{low}$ cells in $CD4^+$ cells or the percentage of FoxP3+ cells in $CD4^+$ $CD25^{high}CD127^{low}$ cells did not differ between the groups (IV, Table 1).

We analyzed the QRT-PCR data of 12 marker (T-bet, GATA-3, IFN-γ, CTLA4, IL-10, IL-16, TGF-β, FoxP3, Nfat-C2, TIM3, TIM4, STIM-1) expression in three study groups with artificial neural network analysis with exhaustive search for all marker combinations. The combination of QRT-PCR markers FoxP3, IL-16,Nfat-C2,GATA-3 distinguished most accurately patients with persisting CMA from patients who recovered early and from non-atopic subjects (IV, Table 2). Patients who had recovered early from CMA and non-atopic subjects were less discernable (IV, Table 2). FoxP3 RNA expression level was higher in β-lactoglobulin stimulated PBMCs from children with persisting CMA than from non-atopic children (IV, Table 3, Kruskall-Wallis test p-value <0.02, pairwise testing Mann-Whitney p-value <0.005). The difference between children with recovered CMA or with persisting CMA was suggestive (IV, Table 3, Mann-Whitney test p-value <0.07); children with recovered CMA and non-atopic controls showed no difference (IV, Table 2, Mann-Whitney p-value >0.4). Expression levels of the other three markers did not differ statistically significantly between groups (IV, Table 3, Kruskall-Wallis test p>0.05).

Discussion

Genetic factors modulating the immune responses to early oral antigens

Atopy has an undisputable hereditary component, but the genetic background is multifactorial and in complex interaction with environmental factors (Steinke, Rich & Borish 2008). Studies have reported, nonetheless, associations between HLA II haplotypes and food allergies (Howell et al. 1998, Howell et al. 1998, Boehncke et al. 1998); also a small study has purported an association of CMA with HLA-DQ7 (HLA-DQB1*0301) (Camponeschi et al. 1997). Considering the complexity of genetics in allergy, it is not surprising that we did not find any association between CMA and HLA haplotypes, which confirmed results from a previous study (Verkasalo et al. 1983). Indeed, immune responses in CMA are usually mounted to several CM proteins (Wal 2004) and further to a large array of peptides of a single antigen (Cerecedo et al. 2008). Studies have demonstrated that antibodies to HLA-DR, HLA-DQ and HLA-DP could restrict the proliferation of CM specific T cell clones from CMA patients (Inoue et al. 2001, Ruiter et al. 2007b). Several antigen presenting HLA molecules thus appear to be of importance in CMA.

Only one (del22824) of the five filaggrin mutations tested was present in three patients with CMA and three control subjects, who all had manifestations of atopy. Since the filaggrin mutation was evenly distributed between patients with CMA and control subjects, our results do not support the proposition that topical exposure to CM antigens through disrupted skin barrier would initiate CMA (Hsieh et al. 2003, Weidinger et al. 2006). Our results did conform to the notion that filaggrin mutations associate with atopic dermatitis and with atopic disorders more broadly (Brown et al. 2008, O'Regan et al. 2009, van den Oord, Sheikh 2009, Palmer et al. 2006). On the other hand, filaggrin has been reported to have a recessive inheritance (Brown et al. 2008), whereas our study subjects were heterozygous carriers of the del22824 mutation. In regard to the R620W allele of the PTPN22, we found no association with CMA, or with antibody levels to CM antigens and ovalbumin. This is in agreement with results that PTPN22 polymorphism is associated with autoimmune diseases, not atopy (Maier et al. 2006) or asthma (Majorczyk et al. 2007).

We found a novel association between HLA II haplotypes and humoral responses to early oral antigens. HLA (DR15)-DQB1*0602 haplotype in patients with CMA was associated with high IgG and IgG4 levels to β-lactoglobulin and α-casein. In contrast, (DR1/10)-DQB1*0501 was associated, in particular among control subjects, with low IgG and IgG4 levels to the CM antigens, and to ovalbumin. Given the tolerogenic nature of specific IgG4 antibodies (Nouri-Aria et al. 2004, Francis et al. 2008, Bussmann et al. 2007, Lemon-Mule et al. 2008, Ruiter et al.

2007a), we speculate that (DR15)-DQB1*0602 carriers could develop tolerance more efficiently in case they are sensitized. Since the association was observed only in patients with CMA, (DR15)-DQB1*0602 appears to have a discernible effect on the antibody response in the context of sensitization. Consequently, (DR15)-DQB1*0602 is likely to assert the modulating effect through other mechanisms than its peptide-binding properties. One hypothetical mechanism would be increase in IFN-γ response, which has been associated with (DR15)-DQB1*0602 in humans (Ovsyannikova et al. 2005, Wallden et al. 2008), and in transgenic mice (Mangalam et al. 2008). A caveat in interpreting the observed phenomena is that since DR and DQ alleles are in strong linkage disequilibriums, the result on (DR15)-DQB1*0602 may as well be associated with DR15 molecule encoded by DRB1*1501 and non-polymorphic DRA1 gene or DR51 molecule encoded by DRB5 and DRA1 genes.

With the HLA DQ molecule containing the DQB1*0501 chain (or DR1 molecule usually found with this DQ specificity), we observed an association nearly opposite to the (DR15)-DQB1*0602 haplotype. Again, the mechanism behind weaker humoral responses to CM antigens and ovalbumin in DQB1*0501 carriers is elusive. Our observations are, nonetheless, supported by the fact that the associations with different levels of IgG class responses to food antigens were present both when comparing separately carriers and non-carriers of either haplotypes, and in the comparison between carriers of these haplotypes.

Antibodies in fostering or impeding oral tolerance

We showed that high CM specific IgE levels predict the persistence of CMA, and in case of persistence, levels remain high over time. This is in agreement with previous studies (Garcia-Ara et al. 2004, Vanto et al. 2004, Skripak et al. 2007, Sicherer, Sampson 1999). The observation is further enforced by our results showing that epitope binding by IgE remains stable over time in patients with persisting CMA. Our results also corroborate reports that a wider IgE epitope profile is associated with persisting CMA (Vila et al. 2001, Cerecedo et al. 2008). This may reflect the maturation of B cell clones, which results in higher specificity and variety of IgE antibodies to CM antigens (Christensen et al. 2008, McHeyzer-Williams, McHeyzer-Williams 2005).

We observed that at diagnosis CM epitope binding patterns by IgE were similar in both patients with persisting CMA and those with early recovery. Our prospective data thus does not support previous reports, based on retrospective settings, which have suggested that IgE epitope profiling clearly distinguishes patients with persistent or transient CMA (Vila et al. 2001, Cerecedo et al. 2008, Wang et al. 2010) . We did identify a few regions in β-casein, β – lactoglobulin and κ-casein that were, however, more intensely bound by IgE of patients with

persisting CMA than of patients with early recovery. A previous study reported that IgE binding of regions in these proteins differentiated patients with active CMA at age 2 years from those who had become tolerant by that age (Cerecedo et al. 2008). Contrary to previous reports (Vila et al. 2001, Cerecedo et al. 2008, Jarvinen et al. 2002), we observed no difference between patient groups in IgE binding of α-casein epitopes at the time of diagnosis. The decision tree analysis, however, revealed that combining IgE and IgG4 binding data on a relatively small number of regions in αs1-, αs2-, β-and κ-casein predicted with significant accuracy, at the time of diagnosis, whether a patient would recover from CMA early or have a persisting allergy. Validation in another patient population is required before the prognostic value of this result can be adequately assessed.

We found several clues to enforce the notion that IgG subclass antibodies promote tolerance rather than allergy. In the comparison of different diagnostic groups at mean age 7 months, children who passed an oral CM challenge without symptoms and those who were diagnosed with non-IgE-mediated CMA had higher levels of IgG, IgG1 and IgG4 antibodies to CM proteins than children with IgE-mediated CMA. Children who recovered early from CMA had higher specific IgG4 and lower specific IgE levels to CM proteins at mean age 8.6 years than children with persisting CMA. The observation is in line with previous studies on both natural tolerance induction in food allergy (Lemon-Mule et al. 2008, Tomicic et al. 2008) and on tolerance induced by specific immunotherapy with aeroallergens (Nouri-Aria et al. 2004, Francis et al. 2008, Bussmann et al. 2007) as well as desensitization during cow's milk immunotherapy (Skripak et al. 2008). Higher specific IgG4 levels in patients with early recovery may also partly result from regular exposure to CM since it is often included in the diet after the patient begins to tolerate CM. Nevertheless, our findings support the role of specific IgG4 antibodies in the maintenance of oral tolerance (Ruiter et al. 2007a) since β-lactoglobulin and α-casein specific IgG4 levels were increased still years after the patients had recovered from CMA, while their CM specific IgE levels remained elevated compared with control subjects.

In addition to higher levels of specific IgG4 to both CM protein fractions studied, early recovery was associated with increasing IgG4 binding and decreasing IgE binding to overlapping CM epitope regions. This conforms to the paradigm that IgG4 acts as a "blocking antibody" (Nouri-Aria et al. 2004, van Neerven et al. 1999, Wachholz et al. 2003).

Interestingly, our study showed higher specific IgG4 levels to CM proteins at mean age 7 months in infants who had received CM formula during the first days after birth and who later developed CMA. This plausibly reflects an intense h2 skewing, since Th2 cytokines induce the production of both IgG4 and IgE (Punnonen et al. 1993). It also exemplifies an earlier observation that some infants are high responders and have overall high levels of specific

antibodies after CM exposure (Lilja et al. 1991). That these infants developed CMA despite relatively high IgG4 levels suggests that the balance between IgG4 and IgE was unfavourable for tolerance induction. Possibly the IgG4 production was only a side-product of Th2 activation, and IL-10 production by T regs was not sufficient to switch IgE production more towards IgG4 production(Nouri-Aria et al. 2004, Francis et al. 2008). Furthermore, children who had received CM at maternity hospital and developed CMA had higher specific IgA levels than other feeding groups within children with CMA. Our data thus suggests that early exposure to CM in children predisposed to develop CMA induces an overall elevated humoral response, where nonetheless IgE response overrides tolerance promoting IgG4 and IgA responses.

The association of specific IgA with tolerance is less established compared with IgG4. Increasing levels of allergen specific IgA or IgA2 have been reported during successful immunotherapy (Francis et al. 2008, Pilette et al. 2007, Scadding et al. 2010). We found that at diagnosis, patients whose CMA persisted beyond age 8 years had lower specific IgA levels to CM proteins than children who recovered by age 3 years. Specific IgA thus appears to have a role in spontaneous development of tolerance. The fact that epitope binding by IgA did not overlap with IgE binding conforms to the notion that IgA does not promote tolerance by blocking IgE binding like IgG4 does (van Neerven et al. 1999, Pilette et al. 2007).

The relationship between circulating and secretory IgA is of crucial importance since the primary function of IgA is on the mucosal surface. We found that β-lactoglobulin specific IgA levels in serum correlated positively with salivary levels of CM specific IgA at diagnosis in patients who became tolerant to CM by age 3 years. Our results suggest that not only are the specific, circulating IgA levels higher in patients who recover early, they also have in correlation more secretory IgA to promote tolerance on mucosal surfaces in the gut. These findings further question previous reports that have suggested differential production of serum and secretory specific IgA antibodies in allergy. In a mouse model of food allergy, tolerance to β-lactoglobulin was associated with higher levels of antigen-specific secretory IgA in feces, but lower serum levels of specific IgA (Frossard, Hauser & Eigenmann 2004). A study on human subjects either with pollen allergy or with no atopy showed difference in the antigen moieties recognized by serum IgA and those recognized by secretory IgA in tears (Aghayan-Ugurluoglu et al. 2000).

Differences in specific IgA and IgG4 levels between persisting CMA and CMA that recedes early appeared at different time points, which may reflect the mechanisms and regulation of IgA and IgG4 in tolerance. The difference between patient groups in specific serum IgA levels was seen at diagnosis (mean age 7 months), whereas the difference in IgG4 levels was not observed until follow-up at mean age 8.6 years. The regulation of specific IgA production appears to be independent of T helper cells (Meiler et al. 2008a), in contrast to IgG4 production which is regulated by Th2 cells and Tregs (Nouri-Aria et al. 2004, Francis et al.

2008, Punnonen et al. 1993). Production of specific IgA appears to be associated with local TGF-β expression and to induce IL-10 production from monocytes (Pilette et al. 2007). Since IL-10 induces IgG4 production (Meiler et al. 2008b), the marching order of IgA increasing before IgG4 levels seems logical. Indeed, IL-10 production reportedly increased during grass pollen immunotherapy earlier than specific IgG4 levels (Francis et al. 2008).

Furthermore, the mechanism of anti-inflammatory function differs between serum IgA and IgG4. Specific IgG4 acts by blocking IgE binding, and thus obviously requires IgE production before it has a tolerogenic function (Nouri-Aria et al. 2004, van Neerven et al. 1999, Wachholz et al. 2003). The anti-inflammatory mechanism of specific IgA is not fully understood, but it does not inhibit IgE binding (van Neerven et al. 1999, Pilette et al. 2007). In mucosal surfaces, particularly in the intestinal tract, higher secretory IgA concentrations may more effectively inhibit absorption of food allergens. It may thus function even before IgE levels have reached levels where inhibition would make a difference. Our data on kinetics of CM specific humoral responses as well as on CM epitope binding support this theory.

Balance of T regulatory and T effector cells in CMA and development of tolerance

Our study shows that the balance of T regs and T effector cells, Th2 in particular, function crucially determines whether CMA persists or tolerance develops. We observed that after β-lactoglobulin stimulation, the combined mRNA expression profile of a Treg-determining (FoxP3), a multifaceted T cell-related (Nfat-C2) and a Th2-determining (GATA-3) transcription factor, and cytokine IL-16 distinguished three groups from each other: patients with persisting CMA, patients who had recovered from CMA and non-atopic children. On the antibody level, the increase of specific IgG4 concomitantly with a decrease in specific IgE in patients who recovered early further emphasizes the importance of Treg/Th2 balance.

Treg activation among children with CMA, in contrast to non-atopic subjects, was inferred by strong FoxP3 intensity in circulating $CD4^{+}CD25^{high}CD127^{low}$ cells and high FoxP3 mRNA expression in PBMCs in response to *in vitro* β-lactoglobulin stimulation. We found no difference in the numbers of $CD4^{+}CD25^{high}CD127^{low}$ cells between the study groups. Our findings thus contradict the study by Karlsson et al where frequencies of circulating Tregs were higher in patients who had recovered from CMA than in patients who remained clinically reactive to CM (Karlsson, Rugtveit & Brandtzaeg 2004). Our results are partly in line with a more recent study, which reported that frequency of polyclonal Tregs in peripheral blood did not differ between patients with active and recovered cow's milk allergy, although the latter had higher frequencies of antigen-specific Tregs(Shreffler et al. 2009); we did not, however, study the frequency of antigen-specific Tregs.

In addition to Treg-related transcription factor FoxP3, the mRNA expression profile distinguishing study groups included markers also related to Th2 activation. GATA-3 is widely used as a Th2 marker since it is the major transcription factor that induces the expression of Th2 polarizing cytokines (Zheng, Flavell 1997, Pai, Truitt & Ho 2004). The interaction of those cytokines with $FoxP3^+$ Treg development appears to be inhibitive (Wei et al. 2007). The transcription factor Nfat-C2 has variable functions: it may induce T effector cell activation, or initiate anergy, or promote Treg function in interaction with FoxP3 (Wu et al. 2006, Bettelli, Dastrange & Oukka 2005, Bopp et al. 2005). Studies have associated IL-16 with promoting either tolerance or allergy. This cytokine induced the migration to inflammation sites of $CD4^+CTLA^+$ cells, which were enriched in FoxP3 (McFadden et al. 2007). It furthermore stimulated FoxP3 expression in the recruited cells (McFadden et al. 2007). In PBMCs from patients sensitive to ragweed, IL-16 reduced allergen induced IL-5 and IL-13 expression (Pinsonneault et al. 2001, El Bassam et al. 2006).In contrast, atopic dermatitis severity correlated positively with IL-16 serum levels (Angelova-Fischer et al. 2006), and clinical improvement was associated with decreasing levels both in an observational study (Angelova-Fischer et al. 2006) and during successful house dust mite specific immunotherapy (Bussmann et al. 2007).

Our data on specific antibodies to CM proteins are partly in agreement with the results from experiments with PBMCs. In patients with persisting CMA, CM specific IgE levels were highest, reflecting Th2 activity. Patients who recovered early had lower specific IgE levels, but still even after recovery the levels were higher than in control subjects. Specific IgG4 levels were higher in patients who recovered early, and on similar levels in control subjects, compared with patients with persisting CMA. Since IL-10 secreted in particular by Tr1 cells promotes B cells to switch from IgE to IgG4 production (Jeannin et al. 1998, Satoguina et al. 2005, Meiler et al. 2008a), our data implies heightened Tr1 activity in tolerance development. When studying PBMCs stimulated with a CM antigen, we showed that Tregs were activated in both patients with persisting and recovered CMA. We did not, however, study subpopulations of Tregs, and thus Tr1 cells may be more activated in tolerance development than in active allergy even if the entire population of Tregs is not. The finding that infants who received CM during first days of life and later developed CMA had higher levels of specific IgG4, and thus plausibly Treg activity, further underscores that it is the balance of Th2 and Treg activity which determines the immunological outcome.

The FoxP3 response to *in vitro* β-lactoglobulin stimulation in non-atopic subjects was weak compared with patients with CMA. The finding implicates that non-allergic long-term tolerance relies more on anergy than up-regulation of FoxP3 in Treg activation. It does not exclude, however, the possibility that tolerance to CM in non-atopic individuals involves activated Tregs in the gut, which is then reflected as unresponsiveness in peripheral blood. On the other hand, a recent study reported higher numbers of $FoxP3^+$ cells in duodenal biopsies of patients with food allergy compared with healthy subjects (Westerholm-Ormio et al. 2010). Our study showed that PBMCs from patients with CMA responded to CM antigen exposure by up-regulation of FoxP3. The Treg activation failed, nevertheless, to control allergic

responses since Th2 pathways became activated as well. Our data thus demonstrate that both Treg and Th2 pathways are active in allergy, and Treg activation in peripheral blood does not necessarily indicate tolerance induction in food allergy.

Methodological considerations

We investigated a relatively large number of patients belonging to a population-based cohort of 6209 infants. The feeding trial was randomized, controlled and double-blind. The study followed prospectively the natural course of CMA up to the median age of 8.6 years. The diagnostic criteria were stringent. What is more, for studies III-V we only included patients with IgE-mediated CMA, which further strengthened the reliability of CMA diagnoses and the comparability of study groups. In studies I-II we stratified the data to IgE- and non-IgE-mediated CMA when it seemed meaningful.

Our principal aim was to compare, at three time points, patients who recovered by age 3 years with patients who had persistent CMA beyond age 8 years. We also had samples of control subjects with no history of CM related symptoms from the same original cohort at follow-up (median age 8.6 years), but not at earlier time points. These control subjects had been meticulously investigated for diverse manifestations of atopy, found to have less atopy than the patient population and had been confirmed to be negative in respect to CMA (Saarinen et al. 2005). The subjects designated as controls in study II had had CM related symptoms that had receded during elimination diet. They may thus have had a very transient form of CMA. The controlled CM challenge was performed within in average 3 months (mean, SD +/-2) after the emergence of CM related symptoms (Saarinen et al. 1999). On the other hand, comparing children with CM related symptoms addresses the issues in CMA diagnostics in a clinically more realistic setting than comparison with asymptomatic children.

For technical reasons, the specific IgA and IgG4 levels were measured in arbitrary units, and values for different antibodies are thus not directly comparable. Another technical weakness was that CM epitope binding by IgA was not performed with a dendrimer-amplification protocol like that by IgE and IgG4 were. The signal was thus lower and less comparable to the one by IgE and IgG4. Studies on epitope binding by IgA in food allergies are rare, and despite the technical shortcoming our study provided novel insight on IgA antibodies in CMA.

We screened the five filaggrin mutations that reportedly are the most common in Caucasian population (Sandilands et al. 2007). Only one of them was present in our study population. In order to claim that no other filaggrin mutations were present, we should have sequenced the entire filaggrin gene from all the individuals. A few rare mutations in filaggrin gene reportedly do contribute to the risk of atopic dermatitis (Sandilands et al. 2007). Due to constrained resources, we were however unable to perform the genetic analysis so thoroughly.

Our results on IgE epitope recognition did not conform to reports from a few previous studies. To our knowledge, the current study was the first to prospectively investigate the prognostic value of epitope profiling in CMA at the time of diagnosis: In later time points, the explanation for discrepancies may lie in different patient characteristics and in stages when CMA was investigated. Our patients were from a prospective study, where patient underwent the diagnostic controlled oral CM challenge at mean age 7 months, which was on average within only four months after the first symptoms (Saarinen et al. 1999). The relatively low CM specific IgE levels in our patients therefore plausibly reflect the short period of CM sensitization. CM specific IgE levels were considerably higher and patients had more severe symptoms, including anaphylaxis, in previous studies, where patients were recruited at specialized referral centers (Vila et al. 2001, Jarvinen et al. 2002), than in the current study. Furthermore, we investigated samples from the early (at diagnosis and one year after) and later (at mean age 8.6 years) stages of CMA, whereas samples in the afore mentioned studies were drawn at school age only (Vila et al. 2001, Jarvinen et al. 2002). Cerecedo and coworkers (Cerecedo et al. 2008) investigated patients with CM specific IgE levels more comparable to those in the current study. Their cut-off for persistent or transient CMA was, however, much lower i.e. median age two years, which implies that many patients categorized to have persistent CMA probably became tolerant before school age since the majority of patients do (Host, Halken 1990, Saarinen et al. 2005, Skripak et al. 2007). Since patients with CMA have the potential of recovery at any age (Skripak et al. 2007), the life-time prognosis of patients with CMA categorized as persistent can be expected to differ from study to study. A factor that is bound to confound any study on epitope recognition is the observation that individual diversity in CM epitope recognition by IgE is considerable (Cocco et al. 2007, Han et al. 2008). Differences in methods, sensitivity in particular, and statistical analysis may also contribute to the variance between studies.

In study V, the amount of PBMC samples obtained from children were insufficient for suppression experiments. We did, however, investigate cell markers which have been shown to identify Tregs and those cells to have suppressive function (Liu et al. 2006, Seddiki et al. 2006).

In study III we used a random decision tree method to find a combination of epitope binding that would predict the clinical course of CMA. Decision tree methods are strong predictors and able to identify interactions between variables. They have indeed been successfully applied in several biomedical applications (Kingsford, Salzberg 2008, Reif et al. 2009). However, our results need to be tested with another patient population.

We applied artificial neural network analysis in study IV to find an optimal marker combination distinguishing the three study groups. This method is able to penetrate the complex data and underlying interactions (Krogh 2008, Khan et al. 2001, Lancashire et al. 2010). Our data set was, however, smaller than would be desirable for such analysis. Ideally,

the result of the analysis should be validated with another data set that the neural network was not trained on (Krogh 2008).

All in all, we are confident that the methodology adequately addressed the aims of the study despite some constraints related to the availability of patient samples, technical issues and at times limited resources. The underlying strength of our study was the relatively large and well-characterized study population.

Implications for CMA diagnostics and prognostics

Our results reinforce the role of CM specific IgE levels in diagnostics and prognostics of CMA. Our study furthermore suggests that in diagnostics, low levels of specific IgG subclass antibodies to CM proteins support a CMA diagnosis. For predicting the natural course of CMA, β-lactoglobulin specific IgA may give additional information. Specific IgG4 levels are not as useful since the difference between patients with transient or persistent CMA was evident only after the time of diagnosis. Our data corroborate, nevertheless, that both IgA and IgG4 subclasses play a role in the development of tolerance in CMA, and may thus serve as markers and vehicles worth investigating in immunotherapeutic settings.

CM epitope binding profile of IgE has previously given some hope of becoming a tool in CMA prognostics. With random decision tree analysis, we did find some IgE and IgG4 peptide recognition patterns that categorized patients with persisting or transient CMA rather accurately. The entire peptide microarray data in our study does not however provide a clear pattern of epitope binding that would differ at the time of diagnosis between patients who recovered early and those whose CMA persisted. The variability of IgE epitope profiles between individuals (Cocco et al. 2007, Han et al. 2008) further complicates the interpretation of epitope profiling in clinical practice. Our results thus provide at most weak support for the application of epitope profiling in CMA prognostics. Previous studies have not evaluated prospectively epitope binding by IgE in patients with different pace of recovery, and they have thus extrapolated the retrospective results to have prognostic value. Overall, IgE binding was more intense and stable over time in patients with persistent CMA. An advantage of epitope profiling with the microarray-based immunoassay is its high sensitivity (Lin et al. 2009). It is, however, questionable whether this kind of analysis would substantially add to the basic measurement of CM specific IgE levels in clinical practice. Our results on epitope profiling rather add to the body of evidence on the role of antibodies in tolerance and active allergy, than provides insights for clinical applications.

Our study did not return genetic associations with CMA, and thus we cannot recommend genetic screening for assessing the risk of CMA. We did, however, observe that humoral

responses to early oral antigens were associated with HLA haplotypes. Further research on the matter may provide clinical applications that could assist in managing the risk and clinical course of CMA.

We found a combination of gene expression markers that distinguished patients with persisting or recovered CMA or non-atopic subjects. The categorization was, however, performed retrospectively. A prospective study must be carried out before the value of T cell profiling in CMA prognostics can be evaluated. Our findings do clearly point out that the frequency or activity of Tregs is not a reliable indication of tolerance in CMA.

In conclusion, specific IgE, IgG4 and IgA antibodies to CM proteins appear to provide useful and complementary information on the immunological status and reflect the clinical activity in CMA. In the development of novel clinical applications for CMA diagnostics and prognostics, simultaneous Th2 and Treg activation should be taken into consideration.

Summary and conclusions

Our study confirms that specific IgG subclass, especially IgG4, play a role in the induction and maintenance of tolerance. Also specific IgA appears to promote tolerance. Our results corroborate that high CM specific IgE levels predict the persistence of CMA. A more novel finding was that specific IgA levels at the time of diagnosis may also have prognostic value. Data from studies on specific antibodies to whole CM proteins and from CM epitope profiling were largely in agreement with each other. CM epitope binding by IgE and IgG4, but not IgA, overlapped, which suggests that IgG4 acts by blocking IgE whereas IgA induces tolerance through another kind of mechanism.

Our results propose that the risk of CMA cannot be assessed with HLA II genotyping. Intriguingly, HLA II genotype modulates, however, the humoral response to CM proteins. The specific humoral response is overall upregulated upon early exposure to CM in the context of sensitization, where tolerogenic IgG4 fails to surpass the effect of allergenic IgE.

Profiling of gene expression markers further emphasized the importance of the balance between Th2 and Treg activity. Expression data on both mRNA and protein level demonstrated that Treg activation upon CM antigen stimulation is stronger in patients with CMA than in non-atopic children. Contrary to the current paradigm, Treg function in peripheral blood appears to be active in allergy and less so in the maintenance of long-term oral tolerance, which plausibly relies on other immunological mechanisms such as T cell anergy.

We investigated the immunological mechanisms behind the emergence and persistence of CMA and the induction of oral tolerance. Our study revealed several phenomena which could be translated into clinical prognostics and immunotherapy in CMA. Validating our results further in a prospective setting would give insight to the possibilities of clinical application.

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