CC16 in allergy and allergic inflammation

Sofi Johansson

UNIVERSITY OF GOTHENBURG

Department of Paediatrics
The Sahlgrenska Academy, University of Gothenburg, Sweden, 2008
Abstract

Clara cell 16-kDa (CC16) is an anti-inflammatory protein mainly produced in the lung epithelium by Clara cells. Patients with asthma have lower levels of CC16 in bronchoalveolar lavage fluid and serum compared with healthy controls. In OVA-sensitised and challenged CC16-deficient mice, eosinophilia and the production of Th2 cytokines in the lung is higher compared with wild-type mice. Moreover, CC16 has been shown to inhibit cytokine production from a murine Th2 cell line and to inhibit the migration of rabbit neutrophils. CC16 also binds to the mast-cell derivative PGD₂ and inhibits the stimulation of the DP1 receptor.

For this reason, the first aim was to investigate whether CC16 levels in nasal lavage would be lower in children with allergic rhinitis compared with healthy controls. Our second aim was to evaluate whether a low level of CC16 in plasma early in life is involved in the development of asthma, eczema and allergic rhinitis (ARC). Our third aim was to examine whether CC16 would inhibit Th2 differentiation and if CC16 would inhibit PGD₂ and fMLF-induced eosinophil and neutrophil migration.

CC16 was measured in nasal lavage samples from children with and without birch-pollen induced allergic rhinitis and serum samples from Icelandic children with or without RSV bronchiolitis. CC16 levels were also measured in plasma samples from a prospective birth cohort study at birth, and at four, 18 and 36 months. Clinical evaluations regarding the development of asthma, eczema and ARC were made at 36 months of age. Moreover, the effect of CC16 on Th2 differentiation was measured with an in vitro model for allergic T-cell sensitisation using human autologous neonatal mononuclear cells. The migration of eosinophils and neutrophils was assessed in a microplate migration system using specific ligands and receptor antagonists.

We found that the levels of CC16 were significantly lower in nasal lavage fluid in children with birch-pollen-induced rhinitis compared with healthy controls both during and after the pollen season. Plasma levels of CC16 in children peaked at four months but we found no relationship between low levels of CC16 at any of the time points and the development of asthma, eczema or ARC. However, the CC16 serum levels were higher in children with RSV compared with healthy controls and we noted that the healthy Swedish children had significantly higher levels of CC16 in plasma compared with healthy Icelandic infants. CC16 did not inhibit cytokine production of human Th2 cells. However, CC16 was able to inhibit Th2 differentiation induced by birch pollen allergen via the dendritic cell. CC16 did not inhibit PGD₂-induced eosinophil migration but CC16 inhibited the migration of both neutrophils and eosinophils towards fMLF.

To conclude, levels of CC16 in plasma during the first years of life do not appear to be related to the development of asthma, eczema or allergic rhinitis. Instead, low levels of CC16 in asthmatic and allergic patients may be due to epithelial damage and the reduced re-growth of Clara cells. Reduced CC16 production may cause an increase in the allergic inflammatory response and thus lead to more severe asthma or allergy.

Key words: CC16, CC10, uteroglobin, Clara cell, allergy, asthma, children, allergen, respiratory syncytial virus, T cell, eosinophil, neutrophil, fMLF, PGD₂
This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. Sofi Johansson, Christina Keen, Arne Ståhl, Göran Wennergren and Mikael Benson.  
Low levels of CC16 in nasal fluid of children with birch pollen-induced rhinitis.  
*Allergy*. 2005 May; 60(5):638-42

II. Sofi Johansson, Bill Hesselmar, Sigurdur Kristjánsson, Nils Åberg, Ingegerd Adlerberth, Agnes E. Wold, Göran Wennergren and Anna Rudin.  
CC16 levels in infants in relation to allergy and respiratory syncytial virus infection.  
*Submitted for publication*

III. Sofi Johansson, Göran Wennergren, Nils Åberg and Anna Rudin.  
Clara cell 16-kd protein downregulates T\(\hbox{h}2\) differentiation of human naive neonatal T cells.  

IV. Sofi Johansson, Kerstin Andersson, Göran Wennergren, Christine Wennerås and Anna Rudin.  
Clara cell 16-kDa (CC16) protein inhibits the migration of human eosinophils towards fMLF but not towards PGD2.  
*Submitted for publication*
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<td>PAMP</td>
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Introduction

The main function of the immune system is to protect the host against pathogens. There are several barriers a pathogen must overcome in order actually to injure its host. The epithelial cells lining the body have traditionally not been included as members of the immune system but have instead been regarded as simply a mechanical barrier. However, current research shows that the epithelial lining of the gut mucosa and airways, for example, is an active part of the immune system.

Innate immunity

Innate immunity is regarded as the first line of defence. It detects and destroys foreign microorganisms that enter the body. This is done by stimulating the pattern-recognition receptors (PRR) which react with pathogen-associated molecular structures (PAMPs). Examples of these PAMPs include lipopolysaccharide (LPS), other cell wall components and formylated peptides. Granulocytes (neutrophils, eosinophils, basophils), mast cells, macrophages, dendritic cells (DC) and natural killer cells (NK cells) are classical innate immune cells. Epithelial cells, which are able both to secrete anti-microbial agents and to recruit leukocytes are more unconventional innate immune cells (1).

Eosinophils

Eosinophils are multifunctional leukocytes which are involved in the pathogenesis of numerous inflammatory processes, including parasitic helminth infections and allergic diseases (2). In healthy individuals, the eosinophil has a bi-lobed nucleus and contains four different types of granule called primary, secondary and small granules and secretory vesicles. The secondary (also called specific) granules contain the cationic proteins that are stained by negatively charged dyes and an electron-dense angular core visible by electron microscope, which is the hallmark of the eosinophil. The granules contain major basic protein (MBP), eosinophil peroxidase (EPO), eosinophilic cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), also called
eosinophil protein X (EPX). Eosinophils express most of the surface proteins expressed by other leukocytes. It is therefore the lack of the low-affinity IgG receptors (FcγIII, CD16) on eosinophils that makes it possible to isolate them from blood (3). The recruitment of eosinophils to the gastrointestinal tract, thymus and mammary glands is regulated by eotaxin-1 in homeostatic conditions (2). The trafficking of eosinophils into inflammatory sites involves IL-4, IL-5 and IL-13, eotaxins, RANTES and several adhesion molecules. Eosinophils have also been shown to migrate towards prostaglandin D2 (PGD₂) (4), which is found in high levels in BAL fluid during allergen-induced airway inflammation (5). Moreover, environmental allergens can directly activate eosinophils which have been isolated from healthy non-allergic individuals(6).

**Dendritic cells**

The dendritic cell (DC) is considered to be the principal antigen-presenting cell (APC) and its Main function is to recognise and present microbial structures to naïve T cells in the lymph nodes (7). The DCs are distributed along sites in the body that are possible pathogen entry sites, such as the lung epithelium and the gastrointestinal tract (8, 9). DCs are also present in the circulation. There are two distinct subsets of DCs in human blood, myeloid and plasmacytoid dendritic cells. Because of the rarity of circulating DCs, peripheral blood monocytes are frequently used as precursors generating human DCs in cell culture. The CD14-positive monocytes are cultured in the presence of granulocyte-macrophage colony-stimulation factor (GM-CSF) for about seven days where they differentiate into immature DCs (10). For the DCs to mature into activated APCs, they have to be stimulated via pattern-recognition receptors and/or with cytokines. During maturation, DCs start to secrete cytokines, such as IL-12, IL-10 and TNF, and up-regulate co-stimulatory molecules. The DCs also up-regulate the chemokine receptor CCR7 that directs the migration of the DCs to the lymph nodes, where a primary T-cell response is initiated (11, 12).

**Adaptive immunity**

Innate immunity is efficient at eradicating pathogens. However, it is important that the immune system can be more specific and is able to distinguish the molecules that are dangerous from
those that are harmless. The adaptive immunity responds to specific antigens to which the immune system has been taught to react. To do this, the adaptive immunity creates cells that remember the pathogens. T and B lymphocytes are the main cells of adaptive immunity. They respond to specific antigens which can lead to proliferation and differentiation into effector or memory cells.

**T cells**

T cells originate from the bone marrow and are educated in the thymus before they enter the circulation. The thymus teaches the T cells to recognise foreign antigens but not self-antigens. T cells are activated by recognising antigens that are presented with MHC molecules on APCs. When the T cells are activated, they recruit or kill other cells, through the expression of cytokines or chemokines secreted into the extracellular milieu or through cell-membrane-associated molecules. T cells can be divided into two main subsets, the T helper cell, which is CD4 positive (a co-receptor to MHC class II), and cytotoxic T cells, which are CD8 positive (a co-receptor to MHC class I). The CD4-positive T cells activate, enhance or suppress other cells of both the innate and adaptive immunity. The CD8-positive T cells are able to kill cells that are infected with viruses or bacteria.

The CD4-positive T cells can differentiate into different cell types, such as T-helper 1 (Th1) and T-helper 2 (Th2), Th17 or regulatory T cells. Both cytokines produced by APCs and co-stimulatory molecules on APCs play an important role in the direction in which the naïve T cells polarise. The theory of Th1 and Th2 is popular as it simplifies immune response. However, it is not that simple, most naïve T cells differentiate into a cell producing both Th1 and Th2 cytokines to different degrees. The Th1 response is associated with the reaction to infectious and autoimmune diseases. Th1 cell polarisation may be caused by the release of IL-12 by the APC. The Th1 cell produces large amounts of interferon gamma (IFN-γ), which stimulates phagocytosis, oxidative burst and the killing of intracellular microbes. Furthermore, IFN-γ upregulates the expression of MHC molecules on a variety of cells and thereby enhances their ability to present antigens to T cells (13).
The Th2 cells are involved in the immune defence against parasitic infections and in allergic disorders. These cells are defined by the increased production of IL-4, IL-5 and IL-13, which recruit and activate eosinophils and mast cells and stimulate B cells to produce antibodies. It is not fully understood how naïve T cells differentiate into Th2 cells, although IL-4 is considered important. The cells that have been found to produce IL-4 are Th2 cells, eosinophils and basophils, but it is unclear where the initial signal for Th2 differentiation originates. What is clear is that Th1 cells and Th1-promoting cytokines inhibit Th2 differentiation (14).

One important factor in T-cell differentiation is the activation of the APC that stimulates the naïve T cell. The APC is believed to differentiate depending on the PRR, or toll-like receptor (TLR), that is stimulated. For example, the stimulation of TLR-4 with LPS on DCs in vitro reduces Th2 and enhances Th1 responses in vivo after transfer (15). On the other hand, when DCs are stimulated with Pam3Cys (a synthetic ligand for TLR2), they promote the Th2 response both in vitro and in vivo (16, 17), (18). The activation via TLRs may have an effect on the up-regulation of different cell surface molecules on the APCs that selectively stimulate Th1 and Th2 differentiation. One example of this is the Notch family of receptors on T cells. The Notch1 receptor and its ligand Jagged 1 on APCs have been shown to induce early IL-4 production in naïve T cells (19).

Not only PAMPs but also substances from other cells surrounding the DCs are believed to change the DCs into Th2-skewing DCs. Mast cells release prostaglandin D2, which has been shown to mature the DCs into differentiating naïve T cells into Th2 cells (20). The protein thymic stromal lymphopoietin (TSLP) is secreted by epithelial cells and mature DCs to induce a Th2 response(21). These Th2 cells are called inflammatory Th2 cells and, apart from the traditional Th2 cytokines, they also secrete tumour necrosis factor (TNF) (21).

Inflammation

Inflammation performs different roles in an infection or tissue injury, such as recruiting effector molecules and cells to the infectious site to kill the invading micro-organisms, to provide a physical barrier to prevent the spread of the infection in the form of microvascular coagulation and to promote the repair of injured tissue. The inflammatory response is characterised by pain,
redness, heat and swelling at the site of infection. These signs are due to changes in the local blood vessels, such as an increase in vascular diameter, which leads to increased blood flow and activated endothelial cells with up-regulated adhesion molecules. The adhesion molecules allow leukocytes to attach to the endothelium and migrate into the tissue. All these changes are initiated by cytokines and chemokines produced by activated macrophages. The first cells that arrive at the infected site are monocytes, which differentiate into additional tissue macrophages. The leukocytes, lymphocytes and eosinophils then arrive and they can pass the endothelium more easily because of the increase in vascular permeability.

These changes are caused in part by macrophages recognising PAMPs and releasing lipid mediators like prostaglandins and leukotrienes. Macrophages also release cytokines in response to PAMP; they include TNF, which is a potent activator of endothelial cells. Both prostaglandins and leukotrienes are products of arachidonic acid formed in what is called the eicosanoid cascade.

Figure 1. The eicosanoid cascade.
**Prostaglandin D2**

PGD\(_2\) is mainly generated by cyclo-oxygenase and PGD synthases in activated mast cells. PGD\(_2\) exhibits a wide range of biological activities, such as vasodilation, bronchoconstriction and the inhibition of platelet aggregation (22, 23). Other cell types that can synthesise small amounts of PGD\(_2\) include macrophages, Th2 cells and DCs (23). It has been shown that monocyte-derived DC that mature in the presence of PGD\(_2\) skew the immune response toward a Th2 response (20). High levels of PGD\(_2\) are found in bronchoalveolar lavage fluid during allergen-induced airway inflammation (5).

PGD\(_2\) acts through two receptors, the prostanoid D1 (DP1) and the prostanoid D2 (DP2) receptor, also called CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells). The DP1 receptor is present on haematological and non-haematological cells of various types and the DP2 receptor has been identified on cells such as Th2 cells, eosinophils, basophils, mast cells and a subset of monocytes (24). Both receptors are expressed on airway DCs and monocyte-derived DCs, but the expression density of DP1 is considerably higher than that of the DP2 receptor (20). Eosinophils have been shown to migrate towards prostaglandin D2 (PGD\(_2\)) (4). The eosinophil may express both receptors and they induce different responses in the cell (25). Stimulation of the DP2 receptor evokes cell shape change, degranulation and chemotaxis, while stimulation of the DP1 receptor appears to counteract apoptotic cell death (24).

**Airway epithelium**

The pulmonary epithelium consists of a well-structured layer of a heterogeneous population of cells. These cells provide a mechanical barrier to the surface of the airway lumen, contribute to host defence via mucociliary clearance and modulate the pulmonary inflammatory response of the airways (26). Ciliated cells, characterised by prominent cilia, predominate on the surface of the epithelium of the proximal lower respiratory tract where they form a pseudostratified epithelium together with basal cells and a small percentage of goblet cells. Goblet cells contain large granules of varying electron density and make up 15-25% of the surface epithelium in the proximal airways, decreasing in number distally. The surface epithelium extends into the ducts of the mucosal glands where the specialised secretory epithelium is composed of serous cells.
and mucous cells. Clara cells (described in the next section) and basal cells predominate in the
distal airways, where there are no ciliated cells, where goblet cells are less numerous and where
the epithelium has a more columnar appearance.

Figure 2. The lung and airway epithelium.

**Clara cells**

Clara cells are predominantly found in the bronchioles and to some extent also in the terminal
bronchioles of humans. They are non-mucous, non-serous, columnar to cuboidal, secretory cells.
Apically, the cell contains membrane-bound, electron-dense secretory granules (26). The Clara
cells were first described by Kolliker in 1881-1882 based on morphology and histochemistry.
However, it was Max Clara, who described the same cells in 1937, after whom the Clara cell was named (27). Subsequent studies of these cells in various species found considerable structural and morphological variations (28, 29). The cells are, however, united by their secretory nature and their lack of mucus. The physiological role of the Clara cells is not fully known. They have been ascribed different roles, such as secreting proteins into the liquid layer lining the alveoli and epithelium, functioning as reparative cells in the bronchial epithelium and degrading foreign substances through abundant P450 cytochrome-dependent mixed-function oxygenases (27). The Clara cell secretes several proteins such as CC16, Clara cell 55-kDa protein, surfactant protein A, B and D, Clara cell tryptase and β-galactoside binding lectine (27).

Airway defence

The nasal mucosa is the first line of defence against pathogen since the nose and upper airway filter most particles in the air. However, the lower respiratory tract is still exposed to large amounts of environmental agents on a daily basis. The function of the pulmonary epithelium can be grouped into three categories, which are 1) the barrier function 2) mucociliary clearance and 3) the secretion of substances.

The barrier function comprises junctional complexes within the airway and alveolar epithelia. The junctional complexes are composed of three parts i.e. the zonula adherence, desmosomes and tight junctions (26). The luminal cell membrane forms an impermeable barrier to macromolecules and infectious agents and ionic diffusion is greatly limited by the junctional complexes. The airway epithelial barrier also hinders the exposure of potential allergens to DCs and B cells, for example. However, some allergens contain protease activity that is able to loosen the tight junctions, allowing the allergens to access the underlying tissue (30). Der p1 (cystein protease) from house dust mites, fungal serine proteases (Pen ch13) from penicillin and Bet v1 from birch pollen are all found to cleave the tight junction protein called occludin (31-33). The airway epithelium also provides an effective barrier to invasion by microbes (34). However, injury of the airway epithelium by infection with viruses, particularly the influenza virus, has been shown to permit bacterial attachment (35).

The mucociliary clearance means the clearance of aspirated particles, including viruses and bacteria. This is done by catching the particle in mucus and clearing the mucus by coughing and
ciliary activity. As previously mentioned, cilia are not found in the distal airways. Instead, surfactant produced by Type II epithelial cells and Clara cells assists in the clearance by changing the surface charge properties and making the particles less adhesive and these may then be cleared by coughing. Macrophages are also active in clearing foreign particles in the distal airways.

Several substances with antimicrobial properties are secreted by the airway epithelium (26). Some substances have direct antibacterial activities, such as lysozyme and lactoferrin (36). The complement factors C3 and C5 act as opsonins and facilitate phagocytosis (37).

The airway epithelial cells can recruit inflammatory cells to the airways through the release of chemoattractants and cytokines. The epithelial cells also up-regulate adhesion molecules to direct inflammatory cell migration across the epithelium in order to attract cells that kill or destroy the pathogen.

**Asthma and allergy**

Hypersensitive reactions mediated by immunological mechanisms that cause tissue damage have been divided into four types. Types I through III are antibody mediated and type IV is T-cell mediated. Type I hypersensitivity is mediated by IgE, which induces mast-cell activation. The IgE may be specific to allergens such as pollen, house dust mites and animal dander.

The asthmatic and allergic response is characterised by mucosal infiltration and the presence of plasma cells, mast cells and eosinophils at the inflammatory site. The inflammatory response occurs in two phases, an early and a late phase. The early phase results from the action of IgE-coated mast cells, which recognise the mucosally deposited allergen and consequently degranulate. These granules contain histamine, tryptase, chymase, kininogenase, heparin and other enzymes. Mast cells also secrete several inflammatory mediators *de novo* including prostaglandin PGD₂ and the sulphidopeptidyl leukotrienes, LTD₄, LTE₄ and LTC₄. The mediators cause the typical symptoms of allergic rhinitis, such as congestion of the nasal airways, itching and sneezing, or an acute asthma exacerbation, such as inflammation,
bronchospasm and increased mucus production. The responses develop within minutes of allergen exposure.

Allergic diseases are the most common chronic childhood diseases in almost all industrialised countries. In the first year of life, eczema and food allergy are the most common allergic manifestations. This usually peters out with age. More than 30% of all children will have at least one episode of wheezing, as a rule triggered by virus infection, before the age of three years (38). However, in many of these children, the wheezing is transient. Allergic rhinoconjunctivitis usually appears later during childhood. Most schoolchildren with asthma have an acute immediate hypersensitivity response to allergens. Very small amounts of these allergens trigger IgE-dependent mast-cell degranulation, which leads to reversible airway obstructions. In adults, a large percentage of patients with asthma have a negative skin-prick test and a normal serum concentration of IgE. Even though the non-allergic patients do not have an apparent allergy, the asthmatic symptoms are very similar. The main difference is stronger macrophage and neutrophil infiltration in non-allergic asthma (39, 40).

The mediators released in the early phase are believed to stimulate the adhesion of circulating leukocytes to the endothelial cells, which initiates the late phase. Chemoattractant cytokines such as IL-5 promote the infiltration of the mucosa with eosinophils, T-lymphocytes and macrophages. These cells become activated and release inflammatory mediators, which in turn re-activate many of the pro-inflammatory reactions in the immediate response (41). The primary inflammatory lesion of allergic asthma consists of an accumulation of Th2 cells and eosinophils in the airway mucosa. The Th2 cytokines IL-4 and IL-13 are essential for the first signalling step in isotype switching to IgE in B cells. This is followed by other signals, such as that from CD40/CD40L interaction (42). IL-4, IL-13 and IL-9 are important in mast cell development, mucus over production and asthmatic hyper-responsiveness and IL-5 is important in eosinophil accumulation (41, 43).

**Allergens**

An allergen has the ability to induce the immune system to produce high-affinity allergen-specific IgE antibodies. This makes it possible for the allergen to trigger an allergic reaction in sensitised subjects. The most common airborne allergens are those derived from the pollen of
Introduction

various plants, from pets and from house dust mites. There are few features of the various allergens that connect them structurally to each other. The most common sensitisation in Sweden is to birch pollen, more specifically the allergen protein Bet v1. As described earlier, several allergens have enzymatic activity. Recombinant hypoallergenic derivatives of Bet v1 have been used successfully in a trial immunotherapy study to treat birch-pollen allergic patients (44). On the other hand, more interest is also being shown in the whole allergen extract when investigating the development of an allergic immune response. It has been shown that pollen also releases bioactive lipids, pollen-associated lipid mediators (PALMs), that are able to recruit and activate neutrophils and eosinophils in vitro (45, 46). PALMs include phytoprostanes, which structurally resemble prostaglandins and isoprostanes in humans. The E1-phytoprostanes in aqueous birch-pollen extract has been shown to suppress LPS- or CD40-induced IL-12p70 production by human DCs and thereby induce a Th2-polarising capacity in the DCs (47). The same aqueous birch-pollen extract also has a Th2-polarising capacity in vivo (48). However, intranasal E1- and F1-phytoprostanes downregulate both Th1 and Th2 cytokine production in vivo (48).

Clara cell 16-kDa

Clara cell 16-kDa (CC16) was first discovered in the rabbit uterus, which is the reason for the name uteroglobin (49). Thereafter, the protein was isolated from other organs and it was named after the different production sites or after its physiological properties. Eventually, the lung was shown to be the major production site, more precisely the Clara cells. A combination of the main producer of the protein and an electrophoretic mobility consistent with a protein of 10-kDa gave the protein the name CC10. When the structure and mass was determined by X-ray crystallography and mass spectrometry, it was found to be 16-kDa (15.8) (49), which assigned it the name CC16. Today, uteroglobin (UG), CC10 and CC16 are the names most commonly used and, in the year 2000, a nomenclature committee recognised the protein as the founding member of a new and growing superfamily of proteins called secretoglobin (Scgb). CC16 is a homodimeric protein with identical 70-amino acid subunits, joined in an anti-parallel orientation by two sulphide bridges. The disulphide bridges facilitate the stabilisation of the CC16 dimer and help the protein to form a central hydrophobic cavity. This cavity is large enough to fit small
hydrophobic molecules such as progesterone, polychlorinated biphenyls or retinol. However, the physiological function of this hydrophobic binding has not yet been elucidated.

**CC16 levels in serum**

CC16 is secreted from Clara cells into the lungs and the amount of this protein in different conditions can be investigated by bronchoalveolar lavage (BAL) yielding bronchoalveolar lavage fluid (BALF). CC16 is also believed to be small enough to diffuse into serum from the airways along a concentration gradient (50). Normal serum levels of CC16 are high enough to be measured by enzyme-linked immunosorbent assay (ELISA) and the production of CC16 in the lung is much higher compared with other organs. Three factors can affect the CC16 levels in serum an increase in serum of the CC16 levels could be due to increased permeability in the airway epithelium, the increased production of CC16 in the lung or reduced renal clearance.

**Increased permeability in the airway epithelium**

To elucidate whether CC16 is a good marker for temporary epithelial damage, several studies with airway irritants such as ozone, tobacco smoke, lipopolysaccharide (LPS) and chlorine have been conducted.

There are conflicting results regarding the influence of ozone on the CC16 levels in serum. Some researchers have found an increase in CC16 levels and some have found no effect (50-52). LPS given to rats intratracheally result in an increase in the CC16 level in serum and a subsequent decrease in BALF (53). This result suggests that the increase in CC16 in serum is due to an increase in epithelial permeability. However, LPS is known to cause pulmonary inflammation without causing epithelial cell damage (54, 55). An increase in CC16 in serum was found six hours after LPS inhalation in healthy volunteers (56).
There is an increase in CC16 serum levels after one hour of cigarette smoke exposure in rats (57). However, when investigating the long-term effect of tobacco smoke in humans, lower levels of CC16 in serum are found in smokers compared with non-smokers (58, 59). In addition, CC16-positive bronchiolar cells were reduced in smokers compared with life-long non-smokers (58, 60). The long-term effect of tobacco smoke may thus be due to its Clara cell toxicity.

Viral infections can cause damage to the epithelial barrier and thus an increase in the epithelial permeability. The most common pathogen of severe lower respiratory tract infections in infants and young children worldwide is the respiratory syncytial virus (RSV). RSV infection with wheezing in early childhood is strongly associated with the subsequent development of asthma (61, 62). It is not clearly understood whether the children that are predisposed to the development of asthma are more prone to develop RSV bronchiolitis with wheezing or whether the RSV infection itself increases the risk of developing asthma. However, infection with RSV

*Figure 3.* Fluctuation of CC16 levels in serum.
during early infancy appear to promote a Th2-like response in the nose with the local production of IL-4, IL-5 and the infiltration of eosinophils (63). Mice that are deficient in CC16 show an increase in lung inflammation and an increase in the Th2 cytokines IL-5 and IL-13 compared with wild-type mice (64). The lung histopathological scores are abrogated by the reconstitution of CC16 in the airways of RSV-infected, CC16-deficient mice (64).

**Induction of CC16 production**

The regulation of protein production involves several steps and can be influenced in many ways. One epithelial cell line that produces CC16 is the BEAS-2B, which, after stimulation with TNF or IFN-γ, increased its production of CC16 (65, 66). There was no induction of CC16 production when the cell line was stimulated with the Th2 cytokines, IL-4 or IL-13 (65, 66). Several studies suggest that glucocorticoids may induce CC16 production both *in vitro* (67) and *in vivo* (68) and estradiol-17 beta and progesterone induce the transcription of CC16 production in epithelial cells from the rabbit uterus (69).

**Renal clearance of CC16**

Serum CC16 has a half-life of approximately two to three hours due to rapid clearance through the kidney (70) and it has been shown that serum CC16 levels are strongly predicted by creatinine clearance (71). The glomerular filtration rate is relatively low in newborns but increases rapidly during the first weeks and is almost at the level of adults at one year of age. No studies have been conducted on the relationship of serum levels of CC16 to creatinine clearance in infants. However, in adults, CC16 levels in serum become higher with age through adulthood (70-72). Thus, age-related changes in CC16 levels in serum can be explained by either increased alveolar capillary leakage and non-specific deterioration of the lung (71) or an age-related decline in glomerular filtration rate (70).

**CC16 in allergy**

In a gene profiling study, it was found that adult patients with intermittent allergic rhinitis, from birch and grass pollen, had a decrease in CC16 gene expression and lower amounts of CC16 in nasal lavage compared with healthy controls (73). Furthermore, a murine model of allergic rhinitis revealed lower levels of CC16 in both nasal and bronchial epithelium compared with
healthy mice (74). The group of mice that was treated with dexamethasone displayed no decrease in CC16 mRNA in nasal mucosa (74). Which may be due to the CC16-inducing effect by glucocorticoids (67, 68).

Mice that are deficient in CC16 display a significant increase in Th2 cytokines IL-4, IL-5 and IL-13 as well as altered pulmonary eosinophilic inflammation when sensitised and challenged with ovalbumin (OVA) compared with wild-type mice (75). The eosinophil infiltration into the airways was abrogated in CC16-deficient mice treated with recombinant CC16 prior to OVA challenge (76).

**CC16 in asthma**

CC16 levels in both BALF and serum have been shown to be lower in adult patients with asthma and in children compared with healthy controls (58, 77, 78). Moreover, children aged 18 months with frequent wheezing (≥3 times) have lower levels of CC16 in serum (79). The lower amount of CC16 in serum and BALF may be due to a reduced number of CC16-positive cells in the airways of asthmatics compared with healthy controls (80). Allergic and non-allergic asthma patients have similar CC16 levels in serum (81). Asthmatics with a disease duration of more than 10 years have lower levels of CC16 in serum compared with asthmatics with a duration of less than 10 years (81). A positive correlation between lung function (FEV1/FVC) and serum CC16 levels has been observed in asthmatics, which indicates that levels of CC16 somehow may be linked to lung function (77).

**CC16 as an anti-inflammatory protein**

CC16 is described as an anti-inflammatory protein in the literature. This is mainly due to its inhibitory effect on phospholipase A2 (sPLA2) *in vitro*, notably the pancreatic or type 1b enzyme and the macrophage-derived or type 2a enzyme (82). PLA2s are a large group of acylesterases that catalyse the hydrolysis of the ester bond at the Sn-2 position in glycerophospholipids releasing a free fatty acid (83) and thereby starting the eicosanoid cascade. However, when CC16 was administered intratracheally to piglets with meconium aspiration syndrome, no effect on PLA2 activity was seen (84). CC16 has also been shown to bind to PGD2 and inhibit stimulation through the DP1 receptor, thereby reducing the expression of the COX-2 enzyme *in vitro* (76).
When CC16-deficient mice were given *Pseudomonas aeruginosa* intratracheally, the mice developed an enhanced pulmonary inflammation, a modest increase in IL-1β and TNF and an improved killing of bacteria compared with wild-type mice. The improved killing is believed to be due to an increase in inflammatory response in the CC16-deficient mice compared with wild-type mice (85). Similarly, CC16-deficient mice infected by an adenoviral vector obtained an increase in lung inflammation with neutrophilic infiltration and an increase in the inflammatory cytokines IL-1β, IL-6 and TNF compared with wild-type mice (86). As in the study with bacteria, there was also a reduction in pathogen survival compared with wild-type mice (86). It therefore appears that CC16 has some anti-inflammatory effect also *in vivo*.

**CC16 as an immunomodulatory protein**

The effects of CC16 on the immune system have not been widely explored. CC16 has been shown to have a direct effect on the cytokine production of murine Th2 cells (87). The production of IL-4, IL-5 and IL-13 was reduced when the Th2 cells were incubated with CC16 and no effect was seen on Th1 cells and IFN-γ production (87).

**CC16 and cell migration**

CC16 has been shown to affect the motility of human monocytes and rabbit neutrophils towards formyl-met-leu-phe (fMLF) (88), a formylated tripeptide of bacterial and mitochondrial origin. These results were confirmed by Camussi *et al.* using CC16-derived synthetic peptides (89). CC16 has been found to be a natural inhibitor of neutrophil function, as CC16 reduces neutrophil-mediated lung damage in patients with ARDS (90).
Aims of the study

The specific aims of this study were:

- To investigate whether the levels of CC16 in nasal lavage fluid in children with birch-pollen-induced allergic rhinitis would be lower than in healthy controls
- To investigate whether low levels of CC16 in the first three years would be related to an increased risk of developing asthma or allergic rhinoconjunctivitis at three years of age
- To investigate whether infants with RSV bronchiolitis would have lower levels of CC16 in serum compared with healthy controls
- To investigate whether CC16 would have a direct inhibitory effect on the Th2 differentiation of birch-allergen-stimulated neonatal cells
- To investigate whether CC16 would be able to inhibit the induced migration of eosinophils and neutrophils
Material and methods

The purpose of this section is to provide an overview of the materials and methods that were used in the work on this thesis. Detailed descriptions are available in the papers and manuscripts in the thesis.

Clinical studies

Nasal lavage study (I)

Patients
The nasal lavage study was performed to investigate the cytokine levels in the nasal mucosa of children with birch pollen-induced allergic rhinitis and healthy controls. Thirty children with allergic rhinitis and 30 healthy controls were recruited from the Catholic School in Göteborg. Allergic rhinitis was defined as a positive seasonal history and a positive skin-prick test for birch. Healthy controls were defined by a lack of history of perennial rhinitis and a negative skin-prick test for birch.

Study design
Nasal lavage samples were collected before the birch pollen season in January and February 2003, when all the subjects were asymptomatic, and during pollen season, April-May 2003, for both allergic and healthy children. The allergic children were examined after a few days of symptoms of allergic rhinitis and also after a period of treatment for seven to 10 days with local steroids (budesonide nasal spray). The healthy controls were examined during the same period. The study was approved by the Human Research Ethics Committee at the Medical Faculty, University of Gothenburg.

Recovery of nasal lavage fluids
Nasal lavage sampling techniques are much debated. This is due to the possibility that the samples will be diluted because of excess mucus production and/or an increase in epithelial permeability. We therefore measured ECP and albumin in the nasal lavage.
Material and methods

The children were first asked to blow their nose to clear excess mucus. The nasal lavage fluid samples were then collected using sterile saline (5-8 ml), which was delivered by aerosol into each nostril and collected in a test tube through passive dripping until 5 ml had been recovered. The collected fluid was kept on ice until it was centrifuged at 4°C and the supernatant was put in a freezer (-80°C) within three hours. The samples were prepared for a differential count before centrifugation. Slides were prepared and stained according to the May-Grünwald-Giemsa method to measure the percentage of neutrophil, eosinophil and epithelial cells in the samples.

Nasal symptom scores

At each time point, the nasal symptoms were scored from 0 to 3 in the categories of nasal secretion, itching and blockage (0 = no, 1 = mild, 2 = moderate, 3 = severe symptoms). The number of sneezes during the hour prior to the nasal lavage were counted and transformed into a score (0 = 0 sneezes, 1 = 1–4 sneezes, 2 = 5–9 sneezes and 3 = 10 or more sneezes). A total symptom score was calculated by adding the four scores. The maximum score was 12. Nasal symptoms were recorded before recovering secretion fluids. As symptom scores are subjective data, we aimed to reduce the variation in symptom scores between subjects by having all the children examined by the same paediatrician.

IMMUNOFLORA study (II, III)

Patients

Sixty-four healthy Swedish infants born in 2001-2003 at the Sahlgrenska University Hospital (Göteborg, Sweden) were involved in the study and formed part of a prospective birth-cohort study (IMMUNOFLORA). The study was originally designed to investigate the colonisation pattern of the gastrointestinal flora with respect to the maturation of immunoregulatory factors during the first years of life. Blood samples were collected from cord (n=49) at 4 (n=48), 18 (n=54) and 36 (n=54) months of age and plasma was prepared. Plasma samples were also prepared from 20 adult subjects of whom 10 were birch-pollen allergic and 10 non-allergic. Informed consent was obtained from the parents and from the 20 adult individuals and the studies were approved by the Human Research Ethics Committee at the Medical Faculty, University of Gothenburg, Sweden.

Whenever a child had symptoms suggestive of allergy or asthma, a clinical examination was performed by a paediatric allergy specialist in the study team. In addition, an interview with the
parents was conducted when the children were six and 12 months of age to investigate feeding practice, family and living conditions, infections and other types of disease. The children were examined for food allergy, eczema, asthma and/or allergic rhinoconjunctivitis by a paediatric allergy specialist at the age of 18 and 36 months. The diagnosis was based on a structured interview relating to medical history and on clinical signs of allergic manifestations and the child was scored from 0-3. For the comparison with healthy children, we only included children with a symptom score of 3. The criteria for a disease score of 3 or for a healthy control were as follows:

**Asthma:**

a) At least three episodes of viral wheezing of which at least one of the episodes took place during the last year, together with symptoms occurring between infections

b) Persistent wheeze with heavy breathing or cough for at least one month during the last year

c) At least three episodes of viral wheezing of which at least one of the episodes took place during the last year in children with at least one allergic disease, i.e. eczema, ARC or food allergy.

**Allergic rhinoconjunctivitis (ARC):** Symptoms from the eyes and/or nose on exposure to pollen or animals, together with a positive allergen-specific IgE test to that specific allergen

**Eczema:** Symptoms either fulfilling Williams criteria (91) or the presence of an itchy rash during the past six months at a typical location.

**Healthy children:** No wheeze or other respiratory symptoms suggestive of asthma, no symptoms suggestive of ARC, no skin manifestations suggestive of eczema, no adverse reactions to foodstuffs compatible with food allergy and no antigen-specific IgE

**RSV study (II)**

The Icelandic RSV study was originally designed to investigate the effect of RSV bronchiolitis early in life on cytokine levels in serum (63). The study comprised 49 children ≤ 7 months of age with respiratory tract infections caused by RSV, influenza or parainfluenza virus and admitted
Material and methods

to the emergency department at the Landhospitali-University Hospital in Reykjavik. Fifty
gender-matched healthy children of seven months of age with no history of infection were
included as a control group. Viral infection was diagnosed in infected infants and excluded in
controls by the direct and indirect immunofluorescent staining (RSV, adeno, parainfluenza 1, 2
and 3, influenza A and B viruses) of nasopharyngeal aspirates. Viral culture was also performed.
Venous blood was obtained and serum was isolated and stored at -20°C until measurements of
CC16 were made.

In the Immunoflora study, CC16 is measured in plasma but in the RSV study, CC16 was
measured in serum. The difference between plasma and serum is that plasma is treated with
heparin, which is an anti-coagulant. As a result, plasma still contains clotting factors like
fibrinogen. Plasma is also diluted with phosphate-buffered saline (PBS), which we correct for
after analysing the samples. Serum, on the other hand, is the liquid part left after the blood clots
and it therefore contains no clotting factors. To exclude the fact that protein content differs in
serum and plasma, we measured the CC16 content from two different persons and compared
CC16 levels in serum and plasma. We found no difference in CC16 levels between serum and
plasma.

**CC16 measurement (I-III)**

CC16 was measured using a sandwich ELISA. In the first study, the analysis of nasal lavage was
made with an ELISA that had already been developed by Arne Ståhl at the Allergy laboratory,
Sahlgrenska University hospital in Gothenburg. For Studies II and III, we attempted to set up
the ELISA using the same protocol at the Department of Rheumatology and Inflammation
Research, University of Gothenburg, where it failed to work. Therefore, an alternative ELISA
was developed for Studies II and III.

In all three studies in which CC16 was measured, plates were coated with Mab 788 over night at
room temperature. In Studies II and III, ½ area plates were used. Wells were blocked with
bovine serum albumin (1% in I and 5% in II and III) in PBS. In the first study, samples were
added to each well together with 6% diluent (6% PEG 6000) and, in Studies II and III, high-
performance ELISA dilution buffer (HPE) was used to dilute samples and antibodies. Human
recombinant CC16 was used as standard. The wells were subsequently incubated with the anti-
CC16 antibody rabbit polyclonal anti-human urine protein 1. This was followed by a secondary monoclonal horseradish peroxidase-conjugated goat anti-rabbit IgG antibody in the first Study I or a mouse anti-rabbit antibody in Studies II and III. In Studies II and III, this step was followed by incubation with streptavidine-conjugated HRP. The plate was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) for approximately 20 minutes in darkness. The reaction was then stopped with 1 M H₂SO₄. Enzyme activity was measured at an absorbance of 450 nm with a 650 nm correction on a Vmax Kinetic microplate analyser. Titration curves for standard and unknowns were fitted from a four-parameter logistic model using the SoftMax™ software package and the samples were calculated from the linear part of the standard curve.

Immunomodulatory effect of CC16

Reagents

Birch (Betula verrucosa) allergen extract was kindly provided by ALK-Abelló (Hørsholm, Denmark). The percentage of protein in the allergen extract was 67.3% and the endotoxin content was 0.5 pg of LPS/µg, as assessed by using the chromogenic Limulus amoebocyte lysate end-point test. Recombinant human CC16, kindly provided by Claragen, Inc. (Bethesda, Md, USA), contained less than 0.5 pg LPS/ng. Human serum albumin (HSA) was purchased from Octapharma (Stockholm, Sweden). The PGD₂, DP1 agonist BW 245C, DP2 agonist DK-PGD₂, DP1 antagonist BW868c and the DP2 antagonist CAY 10471 were acquired from Cayman Chemical, Ann Arbor, MI, USA, and fMLF from Sigma Chemical Co, St Louis, MO, USA.

Cell separation and monocyte differentiation (III and IV)

Monocytes and T cells in Study III were isolated from umbilical cord blood obtained from healthy vaginally delivered babies (atopic status of the parents unknown) born at the Sahlgrenska University Hospital (Göteborg, Sweden). The study was approved by the Human Research Ethics Committee at the Medical Faculty, University of Gothenburg, Sweden. Blood samples were collected in heparin-containing tubes. The blood was diluted in PBS to enhance the cell separation and was placed on Ficoll-Hypaque for density gradient centrifugation. The cord blood mononuclear cells formed the middle layer, which was suctioned up and washed with cold PBS. The upper layer was collected and kept as plasma. CD14⁺ monocytes and CD4⁺ T
cells were isolated by using positive selection with magnetic beads coated with mouse anti-human antibodies against CD14 or CD4 respectively. CD14+ monocytes were differentiated into monocyte-derived DCs by culturing them with IL-4 and GM-CSF for six to seven days in RPMI supplemented with 4% autologous plasma. The CD4+ naïve T cells were either differentiated into Th2 cells or gently suspended in fetal-calf serum containing 7.5% dimethyl sulphoxide and gradually cooled to -70°C. After 24 hours, the cells were transferred to -143°C pending coculture with DCs.

In Study IV, peripheral blood granulocytes were isolated from heparinised blood from healthy volunteers. The blood was mixed with dextran (2%) and left to sediment by gravitation to remove red blood cells. The top layer was then loaded on Ficoll-Hypaque and density gradient centrifuged. All the liquid down to about 1 ml was gently suctioned up and discarded to make sure there were no lymphocytes among the granulocytes. The remaining red blood cells were removed using hypotonic lysis. The recovered neutrophils were tested for chemotaxis. To obtain eosinophils, the neutrophils were removed by using anti-human CD16 magnetic beads as previously described (6, 92). Cells were diluted in Krebs-Ringer glucose buffer (KRG) and kept on ice until use.

**DC-T cell co-cultures (III)**

It had previously been shown that the stimulation of DCs with birch pollen extract during the maturation process differentiated naïve T cells into Th2 cells (93) and we wanted to investigate whether CC16 could inhibit this effect. The monocyte-derived DCs used for DC/T-cell co-cultures were either frozen or matured with TNF, IL-1β and prostaglandin E2 under serum-free conditions for 24 hours. The DCs were stimulated with birch allergen extract with or without different concentrations of CC16 (3, 30, or 100 ng/mL respectively) during the maturation. To investigate whether CC16 alone or an unspecific protein has an effect on DCs, we also added CC16 alone, human serum albumin (HSA; 30 or 100 ng/mL) alone and birch allergen with HSA.
Material and methods

Figure 4. DC/T cell co-culture.

PGD₂ has been shown to skew T-cell differentiation toward Th2 (20) and we found PGD₂ in supernatants from mature monocyte-derived DCs. As a result, we wanted to investigate whether the inhibitory effect seen by CC16 on Th2 differentiation could be mimicked by blocking the PGD₂ receptors DP1 and DP2. We repeated the above experiment, but we added a DP1 or a DP2 antagonist instead of CC16. The concentrations of antagonists were chosen according to the respective dissociation constants (dissociation constant value of BW A868C, 1.7 nmol/L; dissociation constant value of CAY10471, 0.6 nmol/L) and a concentration level ten times above and below the dissociation constant. The antagonists were added about five minutes before birch pollen extract to block their respective receptor before the stimulation.

For all DC/T cell co-cultures, DCs were washed after 24 hours of stimulation and co-cultured with naive autologous CD4⁺ T cells in serum-free X-Vivo15 medium. After three days of culture, the cells were re-stimulated with IL-2 in fresh medium. After six days, frozen autologous DCs were thawed, stimulated and washed as described above and were then used on day 7 for the
re-stimulation of the T cells. After 10 days, the secretion of cytokines was analysed by means of ELISA.

**T-cell differentiation (III)**

There are several published methods describing ways of differentiating naïve T cells into Th2 cells (94, 95). Most studies are performed using human peripheral blood cells and, in our experiments, we differentiated naïve cord blood T cells into IL-13- and IL-5-producing cells. Other studies regarding Th2 differentiation have been conducted using cord blood naïve T cells (96). However, neither these studies nor we were able completely to quench the IFN-γ production, as was found in several publications regarding adult Th2 cell lines (94, 95).

T cells were purified as described above and were cultured in wells coated with anti-CD3 mAb (0.4 μg/mL) and anti-CD28 (0.2 μg/mL). The antibody concentrations used in other studies were considerably higher, but we found that the intracellular IFN-γ production was somewhat reduced when we decreased the strength of stimulation, which is well known (97, 98). Moreover, the survival of the cells increased.

*Figure 5.* Th2 cell differentiation.

The culture medium RPMI 1640 was supplemented with 10% heat-inactivated autologous plasma, IL-2, IL-4 and anti-IFN-γ antibody (complete medium). The effect of using a lower percentage of plasma in the medium was investigated, but 10% was the minimum concentration for a good differentiation. Several studies use anti-IL-12, but, as we did not add any APCs producing IL-12 we did not think it necessary. The cells were cultured for seven days and were
Material and methods

re-fed with complete medium every second day. After four days, the cells were transferred to uncoated wells for a resting period of three days. On day 7, the cells were washed and re-suspended in serum-free medium with the same cytokines and antibodies as above and again cultured in wells coated with anti-CD3 and anti-CD28. The cells were stimulated with or without CC16 (1, 10 and 30 ng/mL) for 48 hours and the intracellular expression and secretion of cytokines were analysed using flow cytometry and ELISA. For the analysis of intracellular cytokines, cells were fixed with paraformaldehyde (2%) and permeabilised with saponin (0.5%). Intracellular cytokines were detected by means of flow cytometry with fluorescein isothiocyanate–anti–IFN-γ (B27) and phycoerythrin–anti–IL-13 (JES10-5A2) mAb.

Eosinophil and neutrophil migration (IV)

The migration of cells is usually measured by placing a fine net, with pores much smaller than the cell diameter, between the cells and the chemoattractant. The pore size in Study IV was 3 µm and the eosinophil diameter was about 10-12 µm, which ensures that the migration is not only due to osmosis. When blocking the cell migration, it is important to ensure that the cells do not migrate along a concentration gradient. The way the antagonist blocks the effect of the stimulant, e.g. by the direct binding of the receptor or by binding the stimulant and thereby inhibiting signalling through the receptor, is also important.

Eosinophil and neutrophil migration towards PGD₂ or fMLF was measured using a microwell migration system. PGD₂, fMLF or control buffer KRG-BSA were added to the lower wells in the microplate and then covered with the filter. The lowest concentration of CC16 in the experiments was the highest concentration that we used in the DC/T cell co-culture experiment. The highest concentration of CC16 in the experiments was the concentration, which was used by Vasanthakumar et al. that inhibited the migration of rabbit neutrophils toward fMLF (88).
Material and methods

Figure 6. Eosinophil and neutrophil migration.

(1) CC16 has been shown to block the stimulatory effect of PGD₂ by binding to it (76). In the first experiments, CC16 was therefore incubated for 15 minutes together with PGD₂ before it was added to the lower chamber. The cells (30,000 cells/30 μl) were incubated in medium only and then added on top of the filter.

(2) To mimic the effect seen by Vasanthakumar et al. on monocytes and rabbit neutrophils, the eosinophil or neutrophil suspensions (60,000 cells/30 μl) were pre-treated with CC16 in KRG-BSA or in medium alone for 15 minutes before they were loaded on top of the filter.

(3) To investigate which DP receptor is involved in PGD₂ migration, eosinophils were incubated with either the DP1 antagonist BW868c or the DP2 antagonist CAY 10471 or medium. To ensure that any effect seen or not seen was due to migration along a concentration gradient, in three of the experiments, CC16, DP1 and DP2 inhibitors were added both to the lower well and on top of the filter.
Migration was allowed to proceed for two hours at 37°C in a humidified atmosphere with 5% CO₂. As a positive control, 30,000 or 60,000 eosinophils or neutrophils were added to a bottom well to mimic maximum migration (100%). All the experiments were conducted in triplicate. To analyse the percentage of neutrophils that had migrated, the neutrophils in the lower wells were pooled into one well and counted in a Bürker chamber and divided by the number of cells counted in the well mimicking 100% migration. To calculate the number of eosinophils that had migrated, the cells in the lower wells were lysed and their EPO contents were determined with a reaction solution composed of 30% H₂O₂ and o-phenylendiamine in citrate buffer with EDTA and hexadecyl-trimethylammonium-bromide. The reaction was stopped by the addition of 1M H₂SO₄ and the optical density was analysed at 490 nm with an ELISA reader, SpectraMax Plus. The percentage of migrated cells was estimated by dividing the median sample absorbance in the wells containing chemoattractants by that of control wells mimicking 100% migration. Eosinophils and neutrophils were checked for viability using Comassie blue staining.

**Determination of changes in intracellular Ca²⁺**

To investigate the effect of the different DP receptors on the eosinophils, intracellular Ca²⁺ transients were determined as described earlier (6). Eosinophils were suspended in cell loading medium (CLM) together with Fura-Red and Fluo-3. The cells were then washed twice in CLM to remove any excess of Fura-Red and Fluo-3 outside the cells and then re-suspended and put on ice. Eosinophils were pre-warmed at 37°C for five minutes before the DP1 agonist BW 245c or the DP2 agonist DK-PGD₂ was added. If the agonist signals through a receptor, that receptor releases Ca²⁺ intracellularly and Fura-Red and Fluo-3 will bind to it. When Fura-Red binds Ca²⁺, its fluorescence emission wavelength changes and it cannot be detected in the flow cytometer. As a result, the signal that is detected is reduced when Ca²⁺ is released inside the cell. When Fluo-3 binds to calcium, it starts to emit fluorescent light. As a result, the signal that is detected rises when Ca²⁺ is released inside the cell. The fluorescence emission of Fluo-3 and Fura-Red was measured in the FL-1 and FL-3 channels respectively. The release of intracellular Ca²⁺ is magnified when the mean Fluo-3 emission is divided by the mean Fura-3. Data were analysed using FlowJo 5.7.1 software.
Material and methods

Cytokine determination

Concentrations of IL-5, IL-13 and IFN-γ in cell-culture supernatants were determined using ELISA. Briefly, ½ area Costar plates were coated with the respective capture mAbs. Standard curves were generated with recombinant human IL-5, IL-13, or IFN-γ respectively. All the antibodies and standards were purchased from PharMingen. Biotinylated detection antibodies of each individual cytokine were used. Samples, standards, biotinylated antibodies and streptavidin-horseradish peroxidase were diluted in high-performance ELISA buffer. PGD₂ concentrations in DC culture supernatants were measured using a commercial EIA kit according to the manufacturer’s instructions.

Statistical analysis

(I) In this thesis we use Wilcoxon’s matched-pairs test to investigate differences in CC16, ECP and albumin levels between sampling times and Mann-Whitney U test to investigate differences in protein levels between groups of healthy and allergic children. Data were analysed using Software GraphPad prism 4 software and a p-value of < 0.05 was considered to be statistically significant.

In article I however, a statistician was involved in the statistical analysis of the CC16 levels. To investigate the change over time in the levels of CC16 in allergic children and healthy controls, respectively, the differences in concentration between two consecutive occasions (before the season and during the season) were calculated in each individual. The median, range, and IQR of the differences were calculated for each group.

To compare the overall CC16 levels between the groups of allergic children and healthy controls, a cut-off between high and low concentrations of CC16 was defined by the median concentration of the total data set (allergic children and healthy controls) before and during the season, respectively. These medians were used to identify high and low producers of CC16 on both occasions. Subjects who had levels below both the two median levels were considered to be overall low producers of CC16, while subjects with levels above the medians on both occasions were regarded as overall high producers of CC16. The proportion of allergic children and healthy controls defined as low producers was calculated in this way. The difference in proportions of low producers between allergic children and healthy controls and the 95% confidence interval (CI) of this proportion were calculated. The sign test with correction of
continuity was used to analyze the hypothesis that there was no difference of proportions between the groups (99). A p-value <0.05 was considered significant.

(II-IV) All the data were analysed using Software GraphPad prism 4 software and a p-value of < 0.05 was considered to be statistically significant. Wilcoxon’s signed-rank test was used to compare paired data in (II-IV). The Mann-Whitney U test was used for unpaired data (III). The Friedman test followed by Dunn’s post test were used to investigate the effect of CC16 on birch allergen-induced T-cell differentiation.
Results and discussion

The nasal lavage study (I)

*Children with birch-pollen-induced allergic rhinitis have lower levels of CC16 in nasal lavage*

Levels of CC16 have been shown to be lower in both broncholalveolar lavage fluid (BALF) and serum in patients with asthma compared with healthy controls (58, 77, 78). Moreover, CC16 mRNA in nasal epithelium is down-regulated in adult patients with seasonal allergic rhinitis (73). We wanted to investigate whether the levels of CC16 in nasal lavage differ between children with birch-pollen-induced allergic rhinitis and healthy children. Samples were taken before the birch pollen season and after a few days of allergic symptoms in the birch pollen season to ensure a late-phase allergic inflammation.

We analysed the levels of CC16, ECP and albumin in the samples. Levels of ECP were measured to confirm the presence of an allergic inflammation. Levels of albumin were measured to account for a possible increase in epithelial leakage from plasma in the nasal lavage fluid.

We found that levels of CC16 in nasal lavage are lower in children with birch-pollen-induced allergic rhinitis compared with healthy controls both before and during the birch pollen season. The levels of CC16 were constant before the season and during the season in allergic children as well as in healthy controls (Fig. 7A). The levels of ECP increased in allergic children during the season compared with before the season. There was no change in ECP levels in healthy children before and during the season (Fig. 7B). Albumin levels were constant before and during the birch pollen season for both groups (Fig. 7C).
Results and discussion

Figure 7. (A) Levels of CC16, (B) ECP and (C) albumin before and during the birch pollen season, in children with birch-pollen-induced allergic rhinitis and healthy controls. * p<0.5, ** p<0.01, ***p<0.001 (between allergic children Wilcoxon’s matched-pairs test, between allergic children and healthy controls Mann-Whitney U test).

When analysing mediators in nasal fluids, it is important to take into account of the possibility of dilution of the lavage fluids and by the transudation of mediators and fluid from plasma, which is indicated by an increase in albumin. Owing to the increase in ECP levels during the pollen season we conclude that allergic inflammation was present at the mucosa of the allergic children. Since ECP increased during the season and the albumin levels were constant, we conclude that the CC16 levels in the nasal lavages were not diluted due to epithelial leakage. CC16 levels are therefore lower in children with birch-pollen-induced allergic rhinitis compared with healthy controls. Moreover, the low levels of CC16 are constant before and during the pollen season.

The IMMUNOFLORA and RSV Studies (II)

The prospective study, IMMUNOFLORA, originally aimed to investigate the relationship between the maturation of the immune system and bacterial colonisation. Blood samples were taken at birth, at four and 18 months of age and at three years of age. We measured the levels of CC16 in the plasma samples and found that CC16 levels in plasma peak at four months of age.
Results and discussion

Figure 8. Plasma levels of CC16 in children at birth, at four and 18 months and at three years of age.

***p<0.001 (Wilcoxon’s matched-pairs test).

The level of CC16 was higher at four months of age compared with the level in cord blood. The CC16 levels are then lower at 18 months and three years of age (Fig 8). The CC16 levels in serum at 3 years of age are below adult levels (data not shown). We found only small amounts of CC16 up to 200 pg/ml in breast milk and therefore concluded that the high plasma levels of CC16 at four months of age are not due to the direct up-take of CC16 through the intestine.

Colonisation of gut-flora bacteria and upper airway infections does not affect levels of CC16 in serum

The high levels of CC16 at the age of 4 months may be due to different environmental factors. Bacterial colonisation of the intestine commences directly after birth and may play an important role in immune stimulation. We therefore investigated whether the levels of CC16 in plasma at 4 months of age might be due to the colonisation of Staphylococcus aureus, Eschirichia coli, enterococci, Bacteroides, bifidobacteria and lactobaccili in the gut, at birth, at three days and at one, two four and eight weeks of age.

We did not find any relationship between bacterial colonisation of S.aureus and E.coli early in life and plasma levels of CC16 at four months of age (Fig. 9). Neither did we find any relationship between bacterial colonisation of the other bacteria sampled and levels of CC16 at four months of age (data not shown).
Results and discussion

Figure 9. Plasma levels of CC16 at four months of age relative to time of intestinal colonisation of S. aureus or E. coli with samples collected at multiple time-points.

As no relationship was found between plasma levels of CC16 at four months of age and gut-flora colonisation early in life is not surprising, as CC16 is mainly produced in the airways and not in the intestine. We therefore investigated whether the number of reported upper airway infections before the age of four months affected the levels of CC16 in plasma at the age of four months, but again we found no association (data not shown).

Another aspect that may explain the variations in the CC16 levels is the kidney function of infants, as serum CC16 levels in adults are strongly correlated with creatinine clearance (71). The glomerular filtration rate (GFR) is low in newborn infants but increases relatively quickly. In a full term baby the GFR is 15-20 ml/min per 1.73 square meters of body surface, 35-45 at 2 weeks of age, 63-80 at 8 weeks of age and, at one year of age, it is 90-110 ml/min. An adult has a GFR of 120 ml/min (100). This may partly explain the high levels of CC16 at four months of age. However, the levels of CC16 in plasma are still elevated at 18 months of age, when the GFR equals that of adults, suggesting that the observed elevation could be explained by other factors such as the increased production of CC16 to protect the small airways of infants from inflammation. Moreover, the difference between levels of CC16 at 4 and 18 months of age is too large to be explained by lower GFR early in life.
Results and discussion

**RSV bronchiolitis increases CC16 serum levels**

In the IMMUNOFLORA study, the upper airway infections were mild and reported by the parents, making this data material somewhat uncertain for comparison analysis with the levels of CC16. Only a few percent of infants with an RSV infection are hospitalised and only the presence of RSV bronchiolitis with subsequent wheezing is closely related to the development of asthma (61, 101, 102). Moreover, mice deficient in CC16 display a stronger inflammatory response in both viral and bacterial infections in the lungs. This leads not only to the increased eradication of the pathogen but also to increased damage to the airway epithelium (85, 86). We therefore hypothesized that the infants admitted to hospital with a RSV bronchiolitis might have lower levels of CC16 in serum compared with healthy controls. Thus, we collaborated with Sigurdur Kristjánsson at the Children’s Hospital Iceland in Reykjavik.

We measured CC16 in the serum from infants with RSV bronchiolitis and compared it with serum levels of CC16 in Icelandic age-matched healthy controls. We found significantly higher levels of CC16 in serum from infants with RSV infection compared with CC16 serum levels in healthy controls (Fig. 10A). The increase in CC16 levels in serum may be due to epithelial damage and the subsequent leakage of protein content from lungs to serum. The elevation may also be due to an increase in the production of CC16, as it has been shown previously that the pro-inflammatory cytokines TNF and IFN-γ induce CC16 production in vitro (65, 66).

![Figure 10](image)

**Figure 10.** (A) Levels of CC16 in serum in children with RSV bronchiolitis compared with healthy controls. (B) Serum levels of CC16 in Swedish children at four months of age and healthy Icelandic children from three to seven months of age. **p<0.01 (Mann-Whitney U test).**
When the circulating levels of CC16 from the four-month-old infants in the IMMUNOFLORA study were compared with the healthy controls in the RSV study, we found that infants from Sweden had much higher levels of CC16 in plasma compared with infants from Iceland (Fig. 10B). When we examine the group of healthy children from Iceland, there was a greater difference in ages in the Icelandic group compared with the Swedish four-month-old group. However, when the healthy children from Iceland were divided into their respective age groups, we found no difference in CC16 serum levels between the different ages (Fig. 10B). This indicates that something other than the age difference between Swedish and Icelandic children is responsible for the difference in CC16 levels in plasma in the Swedish infants and serum from the Icelandic infants.

Several irritants have been shown to elevate the levels of CC16 in serum which is believed to be due to increased epithelial permeability (103). The discrepancies in plasma and serum levels between Swedish and Icelandic infants may thus be due to different degrees of exposure to airway irritants in these children. However, the levels of CC16 in plasma in the Swedish children decrease with age and reaches adult levels of CC16 at the age of three years.

Another explanation for the difference in CC16 levels between Swedish children and Icelandic children may be a genetic difference. Genetic studies suggest that a polymorphism in the CC16 gene (A+38G) is associated with lower levels of CC16 and an increased risk of developing asthma (79, 104), although other studies have not found this association (105, 106). We believe that the differences between the Swedish and Icelandic children are too large to be explained by genetic variations, since the CC16 plasma levels in adult patients with different genetic polymorphisms differ only slightly (107).

We conclude that CC16 levels in serum are higher in children with severe RSV infection compared with healthy controls. However, further studies are required to explain the differences in circulating CC16 levels between Swedish and Icelandic children at four months of age.


**Results and discussion**

Low levels of CC16 at four months of age do not affect the development of asthma, ARC, eczema or allergic sensitisation

We have shown that children with allergic rhinitis have lower levels of CC16 in nasal lavage compared with healthy controls. It has also been demonstrated that asthmatic children display lower levels of CC16 in serum compared with healthy controls (78). We wanted to examine whether the low levels of CC16 in the IMMUNOFLORA study could be related to the development of asthma, ARC, eczema or antigen-specific IgE at three years of age.

At three years of age, few children had developed asthma or ARC. The levels of CC16 at the different time points in the children with asthma or ARC, eczema and allergen-specific IgE were compared with those of healthy controls at three years of age.

![Figure 11](image.png)

*Figure 11.* Levels of CC16 at (A) four months of age and (B) three years of age, in children with no allergies, asthma or ARC and allergen-specific IgE at the age of three years.

We found no relationship between low levels of CC16 at any time point and the development of allergic sensitisation and ARC or asthma at three years of age (Fig. 11). However, we realise that at 3 years of age few children had developed allergic disease. We cannot exclude that some relationship between low levels of CC16 and allergic disease could develop later during childhood. When comparing plasma levels of CC16 in children with allergic sensitisation and asthma or ARC with those of healthy children, the allergic children did not have lower levels of
CC16 in plasma after the development of allergic sensitisation and asthma or ARC at three years of age. This may indicate that the reduction in CC16 levels observed in nasal lavage in children with allergic rhinitis and children with asthma is a result of the progression of the disease (78).

**Effect of CC16 on Th2 cell cytokine production and Th2 cell differentiation (III)**

*CC16 inhibits Th2 differentiation via dendritic cells but not cytokine production from human Th2 cells*

We have not found that the plasma levels of CC16 early in life affected the development of allergy or asthma at three years of age. However, mice that are deficient in CC16 show an increase in Th2 cytokines in the lungs compared with wild-type mice following OVA-sensitisation and the induction of allergy (75). When CC16 is administered to CC16-deficient mice after OVA-sensitisation, the allergic reaction is abrogated (76). Moreover, it has previously been shown that CC16 also has direct inhibitory effects on murine Th2 cells (87).

We therefore wanted to investigate whether CC16 inhibits Th2 cytokine production in human Th2 cells. We differentiated naïve cord-blood-derived CD4+ T cells into Th2 cells, as defined by their increased production of IL-13 and IL-5. When the cells were cultured for 48 hours with CC16 under serum-free conditions, there was no reduction in IL-5, IL-10 or IL-13 production using either of the CC16 concentrations (Fig. 12).
Results and discussion

Figure 12. Naïve cord-blood-derived T cells were differentiated into Th2 cells. The Th2 cells were cultured with CC16 at different concentrations for 48 hours in serum-free media. Levels of IL-13, IL-5 and IL-10 were measured by ELISA.

We also investigated whether CC16 inhibited the levels of intracellular IFN-γ, but we found no such inhibition (data not shown).

Since we found no effect of CC16 on differentiated Th2 cells we wanted to investigate if CC16 might have an effect on Th2 differentiation via DCs. For this purpose, naïve cord derived CD4+ T cells were isolated and cultured with monocyte-derived DCs, which had been matured in the presence of birch pollen extract. We found that CC16 is capable of inhibiting pollen-induced Th2 differentiation (Fig. 13).
Results and discussion

Figure 13. Cord blood monocyte-derived DCs were stimulated with birch allergen extract or birch allergen in combination with CC16. DCs were then washed and co-cultured with naïve autologous CD4+ T cells. IL-13 and IL-5 levels were determined by means of ELISA. * p < 0.05 (Friedman test followed by the Dunn multiple comparison test).

It is not fully understood how the birch pollen extract used in our in vitro method affects the DCs and it is therefore difficult to investigate the mechanism of the effect of CC16 in this regard. Aqueous birch pollen extract has previously been shown to contain E1-phytoprostanes, which inhibit the LPS-induced DC maturation and augment the Th2 polarising capacity of the DCs (47). Data also demonstrate that CC16 inhibits the effect of PGF2α and CC16 binds to PGD2 and inhibits signalling through the DP1 receptor (76, 108). It is therefore possible to speculate that CC16 binds or otherwise inhibits prostaglandins or prostaglandin-like substances such as the phytoprostanes, thereby inhibiting the effect of E1-phytoprostane in the maturation of DCs.

PGD2-matured monocyte-derived DCs have been shown to induce Th2 differentiation and this effect can be mimicked by a selective DP1 agonist (109). As CC16 has been shown to inhibit the effect of stimulation through the DP1 receptor (76), we wanted to explore whether mature DCs produced PGD2, which affects the properties of the DCs. We therefore measured PGD2 in the cell-culture media after maturation with PGE2, TNF, IL-1β and birch and found that mature DCs produce PGD2. However, when we attempted to block the DP1 and the DP2 receptors
respectively, we did not see an effect resembling that of CC16 (Table 1; data not shown). We therefore conclude that the inhibitory effect of CC16 on Th2 differentiation is not exerted through the inhibition of PGD₂.

**Table 1.** An overview of receptors, their ligands and cells that express the receptors investigated in this thesis.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP1</td>
<td>PGD₂</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td></td>
<td>BW245c (agonist)</td>
<td>Eosinophils?</td>
</tr>
<tr>
<td></td>
<td>BW868c (antagonist)</td>
<td></td>
</tr>
<tr>
<td>DP2</td>
<td>PGD₂</td>
<td>Eosinophils</td>
</tr>
<tr>
<td></td>
<td>PGD₂-DK (agonist)</td>
<td>Th2 cell</td>
</tr>
<tr>
<td></td>
<td>CAY 10471 (antagonist)</td>
<td>Dendritic cells?</td>
</tr>
<tr>
<td>FPR</td>
<td>fMLF</td>
<td>Monocytes</td>
</tr>
<tr>
<td></td>
<td>VKYMVm</td>
<td>Neutrophils</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eosinophils</td>
</tr>
<tr>
<td>FPRL1</td>
<td>fMLF</td>
<td>Monocytes</td>
</tr>
<tr>
<td></td>
<td>VKYMVm</td>
<td>Neutrophils</td>
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<tr>
<td></td>
<td></td>
<td>Eosinophils</td>
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<tr>
<td></td>
<td></td>
<td>Immature dendritic cells</td>
</tr>
<tr>
<td>FPRL2</td>
<td>VKYMVm</td>
<td>Monocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mature dendritic cells</td>
</tr>
</tbody>
</table>

In future studies, it would be interesting to examine whether receptors other than the PGD₂ receptors on DCs could be involved in the inhibitory effect of CC16 on Th2 differentiation. One receptor family that may be involved is the formyl peptide receptors (FPR). CC16 has been found to bind with high affinity to the murine formyl-peptide receptors (fpr)2-transfected cells and hinder their migration towards serum amyloid A (SAA), which is a ligand of fpr2 (110). Unfortunately, discrepancies exist between murine and human FPR. Although fpr1 in mice has been described as the orthologue for human FPR and murine fpr2 appears to share many ligands with human FPRL1, there are no clear orthologues between human and mice (111).

However, monocytes express all three human FPRs: FPR, FPRL1 and FPRL2 (Table 1). The differentiation of monocytes into immature DCs leads to the selective loss of expression of FPRL1, while the maturation of the DCs results in the loss of FPR (112). As a result, FPRL2 is the
Results and discussion

only receptor expressed by mature monocyte-derived DCs (Table 1; 112). The synthetic peptide VKYMVM, which is a ligand for all three FPRs, has been shown to inhibit LPS-induced IL-12 production from monocyte-derived dendritic cells (113). It is therefore possible that the inhibition of Th2 differentiation is due to an interaction between CC16 and the FPR receptors. It would therefore be interesting to investigate the effect of FPR ligands on birch pollen-induced Th2 differentiation.

**CC16 does not affect IL-6 and TNF production from LPS-matured monocyte-derived DCs**

As CC16 inhibits birch pollen-induced Th2 differentiation via the DC, we wanted to investigate whether CC16 had an effect on cytokine production from LPS-matured DCs. We matured the DCs with a low concentration of LPS in order not to fail to notice a weak effect. However, we found no effect by CC16 on TNF or IL-6 production (Fig. 14).

![Graph A](image1.png)
![Graph B](image2.png)

**Figure 14.** Cord blood monocyte-derived dendritic cells were matured with LPS (10 ng/ml) with or without CC16 (10-500ng/ml) for 24 hours in serum-free conditions. TNF and IL-6 were measured by means of ELISA. Control was calculated as 100% and TNF and IL-6 levels were calculated as the percentage increase or decrease from control (100%).
Results and discussion

This indicates that CC16 may primarily be involved in inhibiting the Th2 response and not general danger signals or Th0-mediated immune responses.

CC16 in neutrophil and eosinophil migration (IV)

CC16 inhibits fMLF-induced migration of neutrophils and eosinophils

CC16 deficient, OVA-sensitised and challenged mice exhibit an increase in eosinophil numbers in lungs compared with OVA-sensitised and challenged wild-type mice (75). In humans, CC16 has been found to be a natural inhibitor of neutrophil function as high concentrations of CC16 reduce neutrophil-mediated lung damage in patients with acute respiratory distress syndrome (90). In vitro, CC16 has been shown to affect the motility of human monocytes and rabbit neutrophils towards a formylated tripeptide of bacterial and mitochondrial origin (fMLF) (88). These results were confirmed by using CC16-derived synthetic peptides (89). fMLF binds to the human FPR and FPRL1, but not FPRL2 (Table 1). We wanted to investigate whether CC16 inhibits the fMLF-induced migration of human neutrophils and eosinophils. We pretreated neutrophils and eosinophils with CC16 and allowed them to migrate towards fMLF through a fine net. We found that CC16 has an inhibitory effect on the fMLF-induced migration of human eosinophils (Fig 15A).
Results and discussion

Figure 15. CC16 inhibited the migration of eosinophils (A) and neutrophils (B) towards fMLF. Neutrophil and eosinophil chemotaxis was investigated using a micro-migration system. The cells were pre-incubated with CC16 before the addition of fMLF. Each dot represents data derived from one individual run in triplicate. Horizontal bars denote medians for the group of individuals. Spontaneous migration indicated the percentage of cells that migrated towards the buffer. * p<0.05; ** p<0.01 (Wilcoxon’s signed-rank test).

We have also demonstrated that CC16 inhibited the migration of neutrophils towards fMLF (Fig. 15B). The inhibitory effect of CC16 on eosinophil migration was only seen when low concentrations of fMLF were used. The inhibition of migration towards fMLF may operate through interaction by CC16 and one or both of the FPR receptors on neutrophils or eosinophils, FPR and FPRL1 (Fig. 16).
Results and discussion

Figure 16. Schematic diagram of (A) eosinophil migration towards PGD<sub>2</sub> and (B) eosinophil and neutrophil migration towards fMLF and all the receptors possibly involved.

The mast-cell product, PGD<sub>2</sub> is increased in broncholaveolar lavage fluid in asthmatics compared with controls (5). As previously described, CC16 binds to PGD<sub>2</sub> and inhibits signalling through the DP1 receptor (76). PGD<sub>2</sub> is a potent migration inducer of eosinophils and it is the DP2 receptor which is primarily involved in eosinophil migration (4). However, it was recently demonstrated that the inhibition of the DP1 receptor on the eosinophil cell surface can inhibit eosinophil migration (114). We attempted to inhibit PGD<sub>2</sub>-induced eosinophil migration with CC16, DP1 and DP2 antagonists. However, only the DP2 antagonist was able to block eosinophil migration in our experiments (Fig. 16 and Fig. 17).
Figure 17. DP2 antagonist, but not DP1 antagonist and CC16 inhibited eosinophil migration towards PGD₂. The cells were pre-incubated with CC16 before addition of PGD₂. Each dot represents data derived from one individual run in triplicate. Horizontal bars denote medians for the group of individuals. Spontaneous migration indicated the percent of cells that migrated towards buffer. * p<0.05 (Wilcoxon’s signed-rank test).

As we did not find any active DP1 receptors on our eosinophils, the lack of inhibitory effect by CC16 on PGD₂-induced eosinophil migration may be explained by the ability of CC16 selectively only to inhibit signalling through the DP1 receptor and not through the DP2 receptor.
Asthma and allergy are the most common inflammatory disorders among children in industrialised countries. CC16 is an anti-inflammatory protein which is abundantly expressed primarily by the epithelium in the airways. Although several studies have been conducted to measure CC16 in both serum and BALF in different immunological diseases, the physiological function of CC16 is not fully understood. However, it does appear to be closely linked to the immunological response in asthma and allergy, as CC16-deficient mice compared with wild-type mice sensitised and challenged with OVA display a more severe allergic inflammation with increased Th2 cytokines and increased eosinophilia in the lungs (75). Moreover, patients with asthma have been shown to have lower levels of CC16 in both BALF and serum compared with healthy controls (58).

This thesis shows that children with allergic rhinitis have lower levels of CC16 in nasal lavage samples compared with healthy controls. We also show that infants with RSV infection have higher levels of CC16 in serum compared with healthy controls. When we studied the plasma levels of CC16 prospectively during the first years of life we found no relationship between low levels of CC16 and the development of asthma or ARC. Furthermore, the three-year-old children who had developed asthma or ARC did not have lower levels of CC16 in plasma compared with healthy controls.

Different hypothesis as to why asthmatics have lower levels of CC16 in BALF and serum have been proposed. Several studies have found that a genetic polymorphism in the CC16 gene is associated with the development of asthma and also with lower levels of CC16 in serum (79, 104, 115). Other studies have been unable to establish this association (105, 106). Instead of low production of CC16 in early life that would cause the development of asthma or allergy, the low levels of CC16 in patients with asthma and with allergic rhinitis may be caused by epithelial damage at the inflammation or at exposure to airway irritants. The damage to the epithelium may cause a loss of CC16 producing epithelial cells (Fig. 18). Biopsies from bronchial epithelium
in asthmatic patients show fewer CC16-producing cells compared with healthy controls (80). In
addition, biopsies from nasal epithelium in patients with allergic rhinitis show lower amounts of
CC16 in epithelial cells compared with healthy controls (68). This could be a defect from birth,
but it may rather be a change in the epithelium due to the inflammatory process. In fact, CC16
levels in serum from patients with asthma of short duration, (<10 years) have higher
concentrations of CC16 compared with patients with longer disease duration (≥ 10 years) (81).
This thesis shows that low levels of CC16 early in life do not appear to be related to the
development of asthma or ARC at three years of age. However, we must be cautious with our
conclusions as our material is limited and in many children allergic disease has not yet
developed by age 3 years. As a result, the reduction of CC16 in serum over time may be due to a
loss of Clara cells. However, there is a difference in the duration of the inflammation in
asthmatic patients and patients with ARC. Asthma is a chronic inflammatory disorder and
pollen-induced allergic rhinitis is an intermittent inflammatory disorder. Mice sensitised and
challenged with OVA display a large reduction in Clara cells in the airways and CC16 levels in
BALF compared with PBS-treated mice (116). Clara cells may therefore be damaged by the
allergic inflammation in ARC although it is unclear whether this damage is permanent.
However, as the children with allergic rhinitis in our study have low levels of CC16 in nasal
lavage before the pollen season, the reduction in CC16 in nasal epithelium appears to be long
lasting. It remains to be seen whether the low levels of CC16 in nasal epithelium indicate low
levels of CC16 in lung epithelium.
General discussion

Figure 18. Damage to the airway epithelium may cause loss of Clara cells and thus a reduction in CC16 levels in the circulation.

The destruction of Clara cells and subsequent reduction of CC16 may be due to different inflammatory agents in the airways, such as the RS virus (103). We found an increase in serum levels of CC16 in infants with RSV compared with healthy controls, possibly due to increased epithelial permeability. When CC16 levels are measured in serum, there is always uncertainty about whether a change in CC16 is due to increased production in the lung or a change in lung epithelial permeability. It would therefore be of interest to examine the levels of CC16 in both BALF and serum from the children. However, as BAL is an invasive method, it is difficult to use. So, safe non-invasive methods for measuring levels of CC16 in the airways, such as induced sputum and nasal lavage, may give proxy levels of these in BALF. As severe RSV infections with subsequent wheezing are closely related to the development of asthma, it is tempting to speculate that the epithelial damage of an RSV infection is followed by the reduced production of CC16 and a subsequent increase in the risk of inflammation in the airways. It would be very interesting to follow up and re-examine the children in the study at seven years of age, when
asthma symptoms may have occurred, to assess the development of asthma in relationship to the levels of CC16.

Several studies suggest that glucocorticoids are effective in stimulating in the re-growth of Clara cells and up-regulate CC16 production (67, 68, 117). In future studies it would be interesting to study what stimulus affects CC16 production in the Clara cells and how the re-growth of Clara cells is affected following epithelial damage in the airways.

Mice deficient in CC16 show an increase in Th2-derived cytokines and PGD₂ and an increase in eosinophils in the lung compared with wild-type mice after OVA sensitisation and challenge (76). The administration of CC16 to the lung of the CC16-deficient, OVA-sensitised and challenged mice dampened the allergic inflammatory response (76, 87). Several different effects of CC16 in vitro have been found. These include an inhibition of PLA₂ and of PGD₂, an inhibitory effect on murine Th2 cytokine production and an inhibition of neutrophil migration (76, 82, 87). We have been unable to find a direct inhibitory effect on Th2 cytokine production by CC16 in human Th2 cells in vitro. However, CC16 inhibits birch-pollen-extract induced Th2 differentiation in naïve T cells via the DCs. As a result, CC16 may be of importance for the control of allergic inflammation. The inhibition of Th2 differentiation leads to a subsequent reduction in the production of IL-5, which is a cytokine that is important for inducing the migration of eosinophils towards the airways. Instead of low levels of CC16 early in life predisposing an individual to developing asthma or allergic rhinitis. Low levels of CC16 in the lung in an individual may cause a more severe allergic inflammation compared with an individual with high levels of CC16 in the lungs.

PGD₂ is also a potent chemoattractant for eosinophils and has been shown to skew monocyte-derived DCs to Th2 differentiating DCs (4, 109). We concluded that the maturation of the DCs in our study was not dependent on PGD₂. Nor was the PGD₂-induced migration of eosinophils affected by CC16. It is therefore more likely that in asthmatics, the lack of CC16 promotes Th2 differentiation and a subsequent increase in IL-5, which may be responsible for a possible increase in pulmonary eosinophilia (Fig. 19).
Another factor involved in the reduction of allergic inflammation may be a direct anti-chemotactic effect on neutrophils by CC16. CC16 has previously been shown to bind to the murine fpr2 and thereby to inhibit migration towards SAA (110). The FPRs in humans are activated by a very diverse set of natural ligands, ranging from exogenous bacterial or viral products, to endogenous peptides, proteins and lipids (111). The FPR receptors are located on a number of haematological cells and the receptors can promote either the stimulation or the inhibition of the immune response, depending on the ligand, concentrations and the cell type involved (111). We show here that the stimulation of the receptors FPR and FPRL1 by the bacterially derived ligand, fMLF, on neutrophils and eosinophils and the subsequent migration of these cells are inhibited by CC16 (Fig. 16). This indicates that CC16 may have more direct involvement in inhibiting eosinophil and neutrophil migration than merely the reduction of IL-5 production.

Figure 19. Reduction in CC16 levels may cause an up-regulation of Th2 cytokines, such as IL-5. The increase of IL-5 and reduced inhibition of eosinophil migration may increase eosinophilia in the lung. This taken together may cause an increase in the airway epithelial permeability, further reduce the levels of CC16 and therefore cause more severe allergic inflammation.
In conclusion, the development of asthma, ARC and eczema does not appear to be related to the levels of CC16 in plasma during the first years of life. Instead, low levels of CC16 in asthmatic and allergic patients may be due to epithelial damage and the reduced re-growth of Clara cells. Consequently, the reduced CC16 levels may cause an increase in eosinophilia and Th2 cytokine production at the allergy inflammatory site. This in turn can lead to a negative spiral and more severe asthma or allergy.
Förekomsten av allergiska sjukdomar, vilka inkluderar astma hos barn och unga har ökat markant i hela västvärlden och är idag de vanligaste kroniska sjukdomarna hos svenska barn och ungdomar. Immunförsvar består av flera olika celler, bland annat vita blodkroppar som på olika sätt medverkar till att försvara kroppen mot mikroorganismer som bakterier, virus och parasiter. Ett bra immunförsvar är förutsättningen för vårt välbefinnande, men det är samtidigt viktigt att det inte reagerar på ofarliga och kroppsegna ämnen. Allergiska reaktioner uppstår när immunförsvar svarar på ofarliga ämnen, allergener i omgivningen vilka kroppen tror att den behöver skyddas från. Allergener kan komma från t.ex. pollen, kvalster och pälsdjur. Både astma och hösnuva involverar flera olika typer av celler i immunförsvar. På senare tid har det även framkommit att epitcelceller, vilka klär insidan av luftvägarna, spelar en betydelsefull roll i immunförsvar och således även vid allergisk inflammation.

Clara cell 16 (CC16) är ett protein som kan hämma inflammation. Detta protein tillverkas i stora mängder av speciella epitcelceller i lungan som kallas Clara celler. CC16 produceras även i mindre mängd av andra epitcelceller i kroppen. Eftersom den stora produktionen av CC16 sker i lungan och proteinet är så litet att det kan ta sig ut i blodomloppet, gör att nivåerna av CC16 i blod följer mängden CC16 i lungan. Detta gäller så länge som lagret av epitcelceller i lungan är intact. Nivåer av CC16 har uppmätts vara lägre i både lungsköljvätska och blod hos astmatiker jämfört med friska. Vi ville därför undersöka om CC16 var lägre i näsenslemhinnan hos barn med hösnuva jämfört med friska barn, eftersom hösnuva i första hand påverkar epitcelcellerna i näsan. Vi sköljde näsorna på barnen med koksaltlösning och mätte sedan CC16 före och under björkpollensäsongen. Vi fann att CC16 var lägre i nässköljvätska hos barn med hösnuva jämfört med friska barn såväl före som under björkpollensäsongen.

Eftersom nivåerna av CC16 i cirkulationen under tidiga barnålder inte var känt mätte vi CC16 i blod från födsel upp till tre års ålder och fann att nivåerna av CC16 varierade mycket mellan olika barn. De högsta halterna av CC16 uppmättes vid 4 månaders ålder för att sedan sjunka fram till tre års ålder. Därefter var vi intresserade av vad som kunde bidra till att nivåerna av CC16 i blodet vid 4 månaders ålder var så höga. Tre aspekter har visats påverka nivåerna av
CC16 i blod. Dessa är epitelcellernas produktion av CC16, hur intakt lagret av epitelceller är i lungan och hur snabbt CC16 i blod filtreras ut i urin genom njurarna. Epitelcellernas produktion av CC16 har visats öka efter stimulering med olika signalproteiner (cytokiner) som förkommer vid virus- eller bakterieinfektion. Lagret av epitelceller i lungan kan påverkas av flera yttre faktorer t.ex. irriterande faktorer i luften och olika virusinfektioner. Tyvärr är det ofta svårt att avgöra vilken av dessa faktorer som påverkar nivåerna av CC16 i blod om man inte även mäter CC16 på platsen där det produceras. Vi jämförde antalet förkylningar hos barnen innan fyra månader och nivåerna av CC16 vid fyra månaders ålder men fann inget samband. Filtreringen av blod i njurarna hos nyfödda är lägre än hos vuxna men vid ca ett års ålder har barnens njurar uppnått nästan samma funktion som hos vuxna. En låg hastighet av utsöndringen av CC16 i urin kan ha påverkat så CC16 att ansamlas till viss grad i blodet, men eftersom nivåerna av CC16 i blod fortfarande är relativt höga vid 18 månadars ålder så tror vi inte att det står för hela förklaringen. Hög nivåer av CC16 skulle även kunna vara gynnsamt för spädbarnet genom att ge ökat skydd mot inflammation i luftvägarna.

Därefter undersökte vi om låga nivåer av CC16 i blod var relaterade till utvecklingen av astma och allergi. De barn som utvecklade astma och allergi vid 3 års ålder hade emellertid inte lägre halter än friska barn under de tre första åren. Tyvärr är antalet barn i studien begränsat och långt ifrån alla barn som utvecklar allergisk sjukdom har hunnit göra det redan vid 3 års ålder. Det går därför inte att utesluta helt att låga CC16 nivåer tidigt i livet skulle kunna påverka utvecklingen astma och allergi senare i livet.

RS-virus är ett av de vanligaste virus i världen som orsakar inflammation i nedre delen av lungan. De flesta barn märker knappt av infektionen medan andra tvingas bli inlagda på sjukhus. RS-virus har en stor inverkan på luftvägsepitélet och en kraftig RSV infektion med pipande och väsande hos små barn är förknippat med ökad risk för utveckling av astma senare i livet. Vi ville nu undersöka om barn med RS-virus infektion hade lägre halt av CC16 än friska barn. I ett samarbete med isländska forskare fann vi att barn med svår RS-virus infektion istället hade högre halter av CC16 i blod jämfört med friska kontroller. Detta beror sannolikt på att RS-viruset skadar lagret av epitelceller i lungan och på så sätt ökar CC16-nivåerna i blodet.
Populärvetenskaplig sammanfattning

I djurförsök har det visats att möss som saknar CC16 får en kraftigare allergireaktion än möss som har CC16, när de i en allergimodell exponerades för allergen. Den kraftiga allergiska inflammationen bestod av ett ökat antal inflammatoriska celler i luftvägarna och ökade halter av inflammatoriska cytokiner som är förknippade med allergi och astma. I provrör har det även visats att CC16 dämpar muscellers produktion av inflammatoriska cytokiner från vita blodkroppar som är involverade i den allergiska inflammationen. Dessa celler kallas Th2-celler. Därför undersökte vi om även CC16 kunde dämpa produktionen av Th2-cytokiner från nyfödda barns Th2-celler. Vi undersökte även om CC16 kunde dämpa utvecklingen av outvecklade T-celler från nyfödda barn till att bli Th2-celler. Vi fann ingen hämning av produktionen av de inflammatoriska cytokiner, men vi fann att CC16 hämmade utvecklingen av Th2-celler.


De låga nivåerna av CC16 hos patienter med astma och allergi är troligen en långvarig effekt av inflammationen vid astma och allergi som orsakar en skada på lungepitelet. Detta ger i initialskedet en högre halt CC16 i blod men den långvariga effekten är troligtvis en sänkning av CC16 på grund av förstörda celler som producerar CC16. De lägre halterna av CC16 vid astma och allergi kan innebära att inflammationen förvärras. Även om nivåerna av CC16 vid tidig ålder inte var relaterade till astma- och allergiutveckling så kan CC16 spela en viktig roll för att dämpa den allergiska inflammationen när den väl har etablerats.
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