

Clara cell 16-kd protein downregulates T_H2 differentiation of human naive neonatal T cells

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Background: Levels of the Clara cell 16-kd protein (CC16) are lower in plasma and bronchoalveolar lavage fluid from adults with asthma relative to those seen in healthy control subjects, and CC16 inhibits the T_H2 cytokine production from murine T cells.

Objective: We sought to determine the plasma levels of CC16 in infants and to investigate whether CC16 might inhibit the T_H2 cytokine production from neonatal T cells.

Methods: Cord blood and blood samples at 4, 18, and 36 months of age were taken from 64 children prospectively, and CC16 levels were analyzed in plasma. Cord monocyte-derived dendritic cells (DCs) were pulsed with birch allergen extract alone or together with CC16 or prostaglandin D₂ receptor inhibitors, after which autologous naive CD4⁺ T cells were added to the DCs. The production of IL-5, IL-13, and IFN- γ was measured by means of ELISA and flow cytometry.

Results: The plasma levels of CC16 in children peaked at 4 months. CC16 did not directly affect the cytokine production from human T_H2 cells. However, CC16 inhibited birch pollen extract-stimulated T_H2 differentiation of naive T cells through the DC. Inhibition of the prostaglandin D₂ receptors did not consistently result in suppressed T_H2 differentiation.

Conclusion: The production of CC16 seems to peak early in life, and CC16 has an inhibitory effect on T_H2 cell differentiation from human infants by affecting DCs.

Clinical implications: CC16 is an immunoregulatory protein, and its inhibitory effect on T_H2 cell differentiation might be of importance in the pathogenesis of allergy in infants. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Allergy, children, CC16, CC10, uteroglobin, T_H2

The prevalence of allergic sensitization and disease has tripled in the Western world during the last decades.¹ It is unclear which environmental factors are responsible for

Abbreviations used

CC16: Clara cell 16-kd protein
DC: Dendritic cell
DP1: Prostanoid D1
DP2: Prostanoid D2
HSA: Human serum albumin
PGD₂: Prostaglandin D₂

this course of events, but a defective immunoregulation of allergic inflammation is the result. An immunoregulatory protein that has been ascribed anti-inflammatory properties is the Clara cell 16-kd protein (CC16), also known as *CC10* or *uteroglobin*. CC16 is produced by mucosal epithelial cells lining all organs that encounter the outer environment, but the largest amounts are produced in the lung from the Clara cells, which are nonserous cells mainly located in the bronchioles.^{2,3} Patients with asthma have lower levels of CC16 in both bronchoalveolar lavage fluid and in serum compared with levels seen in healthy control subjects.^{4,5} In addition, we have previously shown that children with allergic rhinitis express lower levels of CC16 in nasal lavage fluid than healthy age-matched control subjects.⁶ However, it is not known whether low CC16 production is an effect caused by the respiratory allergic inflammation or whether the low levels of CC16 precede the development of allergic inflammation.

Studies of sensitized CC16-deficient mice indicate the functional relevance of CC16 in allergic inflammation. These mice produce increased levels of IL-4, IL-5, and IL-13 relative to wild-type mice after sensitization and challenge with ovalbumin.⁷ CC16-deficient mice also have a strong inflammatory response in the lungs after ovalbumin sensitization and challenge.⁸ In addition, CC16 has been shown to downregulate the production of IL-4, IL-5, and IL-13 when added to a murine T_H2 cell line.⁹ CC16 also has direct anti-inflammatory properties because it inhibits the enzyme phospholipase A₂ and the protein prostaglandin D₂ (PGD₂), which are both involved in the arachidonic acid inflammatory cascade.^{10,11} PGD₂ is mainly generated by COX in activated mast cells and exhibits a wide range of biologic activities, such as vasodilatation, bronchoconstriction, and inhibition of platelet aggregation.^{12,13} Other cell types that can synthesize small amounts of PGD₂ include macrophages, T_H2 cells, and

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dendritic cells (DCs).¹² High levels of PGD₂ are found in bronchoalveolar lavage fluid during allergen-induced airway inflammation.¹⁴ PGD₂ acts through 2 receptors: the prostanoid D1 (DP1) and prostanoid D2 (DP2) receptors, also called chemoattractant receptor homologous molecule expressed on T_H2 cells. Both receptors are expressed on airway DCs and monocyte-derived DCs, although the expression density of DP1 receptors is considerably higher than that of DP2 receptors.¹⁵ Monocyte-derived DCs matured in the presence of PGD₂ skew the immune response toward an increased T_H2 response.¹⁵

In this study we measured the levels of CC16 in children at several time points from birth to 3 years of age in a prospective birth cohort study. We also investigated whether CC16 regulates human T_H2 cell differentiation and whether CC16 exerts its effect directly on T cells or through autologous monocyte-derived DCs. Moreover, we explored whether the downregulatory mechanism of CC16 could be explained by inhibition of PGD₂.

METHODS

Subjects

Sixty-four healthy Swedish infants born in 2001–2003 at the Sahlgrenska University Hospital (Göteborg, Sweden) were involved in the study. These children formed part of a prospective birth cohort study (IMMUNOFLORA). Inclusion criteria were normal pregnancy without expected complications and expected vaginal delivery at term (at least 38 weeks' gestational age). The parental history of allergy was recorded. Blood samples were collected from the umbilical cord and at 4, 18, and 36 months of age, and plasma was prepared. We obtained 49 samples at birth, 48 samples at age 4 months, 54 samples at age 18 months, and 54 samples at age 3 years. Plasma samples were also prepared from 20 adult subjects, of whom 10 were allergic to birch pollen and 10 were nonallergic. Informed consent was obtained from the parents and from the 20 adult individuals, and the study was approved by the Human Research Ethics Committee of the Medical Faculty, Göteborg University, Sweden.

Reagents

Birch allergen (*Betula verrucosa*) extract was kindly provided by ALK-Abelló (Hørsholm, Denmark). The proportion 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), contained less than 0.5 pg LPS/ng. Human serum albumin (HAS) was purchased from Octapharma (Stockholm, Sweden).

CC16 ELISA

The levels of CC16 were determined by using a sandwich ELISA. Plates (½ area; Corning Costar, Acton, Mass) were coated with a CC16-specific mAb, mAb 788 (0.59 μg/mL; kindly provided by Pharmacia Diagnostics AB, Uppsala, Sweden), and wells were blocked with BSA (5%). Samples and the standard human recombinant CC16 were diluted 100-fold in high-performance ELISA buffer and added to the wells. Subsequently, the wells were incubated with the anti-CC16 antibody rabbit polyclonal antihuman urine protein 1 (A 0257; Dako, Glostrup, Denmark) followed by a secondary monoclonal mouse antirabbit antibody (B 5283; Sigma-Aldrich, Munich, Germany) and streptavidin-conjugated horseradish peroxidase. The plate was developed with 3,3',5,5'-tetramethylbenzidine

(Sigma-Aldrich), and the reaction was stopped with 1 mol/L H₂SO₄. Enzyme activity was measured at an absorbance of 450 nm on a SpectraMax Plus (Molecular Devices, Sunnyvale, Calif). Titration curves for standard were fitted to a 4-parameter logistic model by using DeltaSoft (BioMetallics, Inc, Princeton, NJ), and the samples were calculated from the linear part of the standard curve.

Cell separation

Monocytes and T cells 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 of the Medical Faculty, Göteborg University, Sweden. Blood samples were collected in heparin-containing tubes, and cord blood mononuclear cells were isolated by means of density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). CD14⁺ monocytes were isolated by using a positive selection technique according to the manufacturer's instructions (MACS System; Miltenyi Biotech, Bergisch Gladbach, Germany). T cells were purified with the Dynabead CD4 Positive Isolation Kit (Dynal Biotech ASA, Oslo, Norway), and flow cytometry showed that more than 95% of the T-cell fraction coexpressed CD4 and CD45RA. T cells were either differentiated into T_H2 cells or gently suspended (11–25 × 10⁶ cells/mL) in FCS containing 7.5% dimethyl sulfoxide and gradually cooled to –70°C, and after 24 hours, the cells were transferred to –143°C until coculture with DCs.

Differentiation of T cells

T cells were purified as described above and were cultured at a concentration of 5 × 10⁵ cells/mL in wells coated with anti-CD3 mAb (0.4 μg/mL; OKT-3; Ortho McNeil Pharmaceutical, Raritan, NJ) and anti-CD28 (0.2 μg/mL; 555725; BD, Franklin Lakes, NJ). The culture medium RPMI 1640 (BioWhittaker, Cambrex Company, Belgium) was supplemented with 10% heat-inactivated autologous plasma, IL-2 (50 U/mL), IL-4 (20 ng/mL), and anti-IFN-γ antibody (5 μg/mL; Nordic BioSite, Täby, Sweden). The cells were cultured in 5% CO₂ at 37°C for 7 days and were refed every second day and transferred on day 4 to uncoated wells for a resting period of 3 days. On day 7, cells were washed and resuspended (5 × 10⁵ cells/mL) in X-Vivo15 (BioWhittaker) supplemented with IL-2, IL-4, and anti-IFN-γ at the same concentrations 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 intracellular expression and secretion of cytokines were analyzed by means of flow cytometry and ELISA. For analysis of intracellular cytokines, cells were fixed with paraformaldehyde (2%) and permeabilized 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 (PharMingen, San Diego, Calif).

Generation of monocyte-derived DCs

For generation of immature DCs, monocytes were cultured in RPMI 1640 medium (BioWhittaker) supplemented with 4% heat-inactivated autologous plasma, 1 mmol/L L-glutamine, and 50 μg/mL gentamicin with the addition of 500 U/mL rIL-4 and 800 U/mL rGM-CSF (R&D Systems, Abingdon, United Kingdom). The cells were cultured in 5% CO₂ at 37°C for 6 to 7 days and were refed with IL-4 and GM-CSF containing medium every second day. This resulted in generation of DCs positive for CD11c but negative for CD14.

DC/T-cell cultures

Monocyte-derived DCs used for DC/T-cell cocultures were either frozen or matured with TNF (20 ng/mL, R&D Systems),

IL-1 β (10 ng/mL, R&D Systems), and prostaglandin E₂ (1 μ g/mL; Sigma-Aldrich, Stockholm, Sweden) under serum-free conditions for 24 hours. The cells were stimulated with birch allergen extract alone (50 μ g/mL) and with birch allergen extract combined with CC16 to study the effect of CC16 on T-cell differentiation. As controls, we also added CC16 alone (3, 30, or 100 ng/mL, respectively), HSA alone (30 or 100 ng/mL), and birch allergen with HSA. To study the effect of the DP1 and DP2 antagonists, the DCs were stimulated with birch allergen extract alone or birch allergen extract combined with DP1 antagonist (BW A868C; 17 nmol/L; Cayman chemical, Ann Arbor, Mich) or DP2 antagonist (CAY10471; 6 nmol/L; Cayman Chemical) in different concentrations. The concentrations of the 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). After 24 hours of stimulation, DCs (10–15 $\times$ 10³ cells per well) were washed and cocultured with naive autologous CD4⁺ T cells (1.5 $\times$ 10⁵ cells per well) in serum-free X-Vivo15 medium. After 3 days of culture, the cells were fed complete medium containing IL-2 (50 U/mL, Nordic BioSite). On day 6, frozen autologous DCs were thawed, stimulated, and washed as described above and were thereafter used on day 7 for restimulation of the T cells. On day 10, secretion of cytokines was analyzed by means of ELISA.

Cytokine and PGD₂ ELISAs

Concentrations of IL-5, IL-13, and IFN- γ in cell-culture supernatants were determined by using ELISAs. Briefly, a standard ELISA procedure was performed, as previously described.¹⁶ Costar plates (Invitrogen, San Diego, Calif) were coated with the following capture mAbs: anti-IL-5 (TRFK5), anti-IL-13 (JES10-5A2), and anti-IFN- γ (NIB42). Standard curves were generated with recombinant human IL-5, IL-13, or IFN- γ , respectively. All antibodies and standards were purchased from PharMingen. The following biotinylated detection antibodies were used: anti-IL-5 (JES1-5A10), anti-IL-13 (B69-2), and anti-IFN- γ (4S.B3). Samples, standards, biotinylated antibodies, and streptavidin–horseradish peroxidase were diluted in high-performance ELISA buffer (Sanquin, Amsterdam, The Netherlands). PGD₂ concentrations in DC culture supernatants were measured by using a commercial EIA kit according to the manufacturer's instructions (Cayman Chemical).

Statistical analysis

The data were analyzed by using the Friedman test, followed by the Dunn multiple comparison test, or by using the Mann-Whitney *U* test or the Wilcoxon signed-rank test, as described in the figure legends (GraphPad Prism, San Diego, Calif).

RESULTS

Levels of CC16 peaked in infants at 4 months of age

To investigate the levels of CC16 in children during the first years of life, we measured the concentrations of CC16 in plasma in cord blood and at 4 and 18 months and 3 years from a cohort of children. The levels of CC16 increased significantly from a median level of 15 ng/mL at birth to a level of 96 ng/mL at the age of 4 months (Fig 1). Thereafter, the levels of CC16 decreased significantly to 20 ng/mL at 18 months and continued to decrease to 7 ng/mL at the age of 3 years. The CC16 levels at 3 years were even significantly lower than those of healthy adults and adults with birch allergy (15 ng/mL). No difference

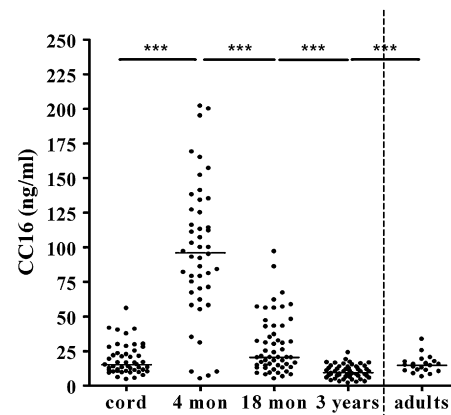


FIG 1. Plasma concentrations of CC16 in children in cord blood (n = 49) and at 4 (n = 48), 18 (n = 54), and 36 (n = 54) months and in 20 adults (10 individuals with birch pollen allergy and 10 healthy control subjects). ****P* < .0001 (Wilcoxon signed-rank test and Mann-Whitney *U* test).

was seen in allergic patients compared with nonallergic control subjects. To ensure that the plasma levels of CC16 were not simply a result of uptake from breast milk, we measured the concentration of CC16 in milk and found very low levels, ranging from 70 to 200 pg/mL. Four-month-old children with a parental history of allergy had similar levels of CC16 as the children with no parental history of allergy (median levels, 94 and 100 ng/mL, respectively).

CC16 does not directly suppress T_H2 cytokine production by differentiated T_H2 cells

Experiments with murine T_H2 cells have shown that concentrations ranging from 3 to 300 ng/mL CC16 directly inhibit T_H2 cytokine production.⁹ Because we found that the median concentration of CC16 in plasma at birth and in children varies between 10 and 100 ng/mL, we decided to test the concentrations of 1, 10, 30, and 100 ng/mL CC16 to investigate whether CC16 would inhibit cytokine production from human differentiated T_H2 cells. CD4⁺ T cells isolated from cord blood mononuclear cells were differentiated toward a T_H2 profile and then cultured together with different concentrations of CC16 for 48 hours. The T cells produced IL-13, IL-5, and IL-10, but we found no inhibitory effect of CC16 on the production of these cytokines, which was also confirmed by using flow cytometry (Fig 2). The amount of IFN- γ produced by the T cells could not be measured because of added anti-IFN- γ in the medium. However, intracellular staining showed that IFN- γ was expressed by 9% and IL-13 by 8.5% of T cells. The number of cells expressing IFN- γ or IL-13 intracellularly was not reduced by CC16 (Fig 2, D).

CC16 suppressed the production of IL-13 and IL-5 from neonatal allergen-stimulated T cells by affecting DCs

Because we found no direct effect by CC16 on the cytokine production of human T_H2-deviated cells, we

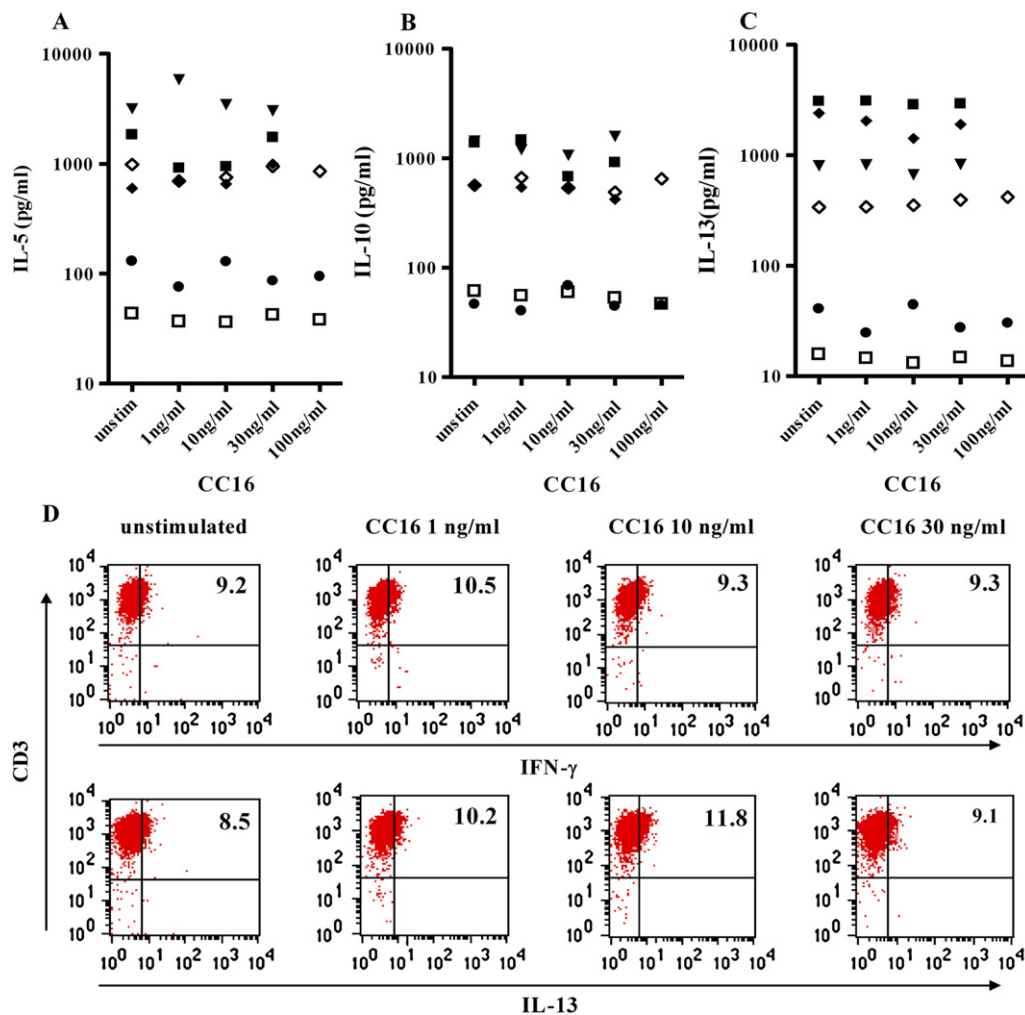


FIG 2. Naive T cells from cord blood were differentiated into TH2-deviated cells before stimulation with 1, 10, 30, and 100 ng/mL CC16 for 48 hours. The levels of IL-13 (A), IL-5 (B), and IL-10 (C) were measured in the supernatants by means of ELISA. Individual data from 6 different experiments are shown, and each individual is represented by a specific symbol. D, One representative flow cytometric experiment out of 6 showing results from intracellular staining of IFN- γ and IL-13 on day 10.

examined whether CC16 would be able to suppress allergen-specific TH2 differentiation of human neonatal T cells through DCs. To this end, DCs were pulsed with birch allergen together with different concentrations of CC16 and then washed before they were added to the autologous naive T cells without CC16. We found that a concentration of 30 ng/mL CC16 significantly suppressed the IL-5 and IL-13 production relative to birch allergen alone. A non-significant trend for inhibition was seen when 3 ng/mL was added, which suggests a dose-dependent effect (Fig 3, A). However, 1 ng/mL CC16 in the culture inhibited neither IL-13 nor IL-5 production, and CC16 alone did not affect the production of IL-13 or IL-5 when compared with that seen in unstimulated cells (data not shown). Addition of the control protein HSA to the allergen-pulsed DCs did not suppress the cytokine production. Here the IL-13 and IL-5 production tended to be increased

compared with both birch allergen alone and HSA alone (Fig 3, C).

The production of IFN- γ was also analyzed, but the cytokine was detected at low levels in only a few experiments (data not shown).

Blocking the DP1 receptor on DCs suppressed production of IL-13 only from neonatal allergen-stimulated T cells that produced high levels of IL-13

Next we investigated whether the inhibitory effect on T-cell differentiation by CC16 was an effect of blocking PGD₂. PGD₂ levels were measured in the cell media from both immature and mature DCs from one experiment to ensure that PGD₂ was produced by our monocyte-derived DCs. Immature DCs did not produce PGD₂, but mature DCs produced PGD₂ (42.2 ng/mL and 36.2 ng/mL,

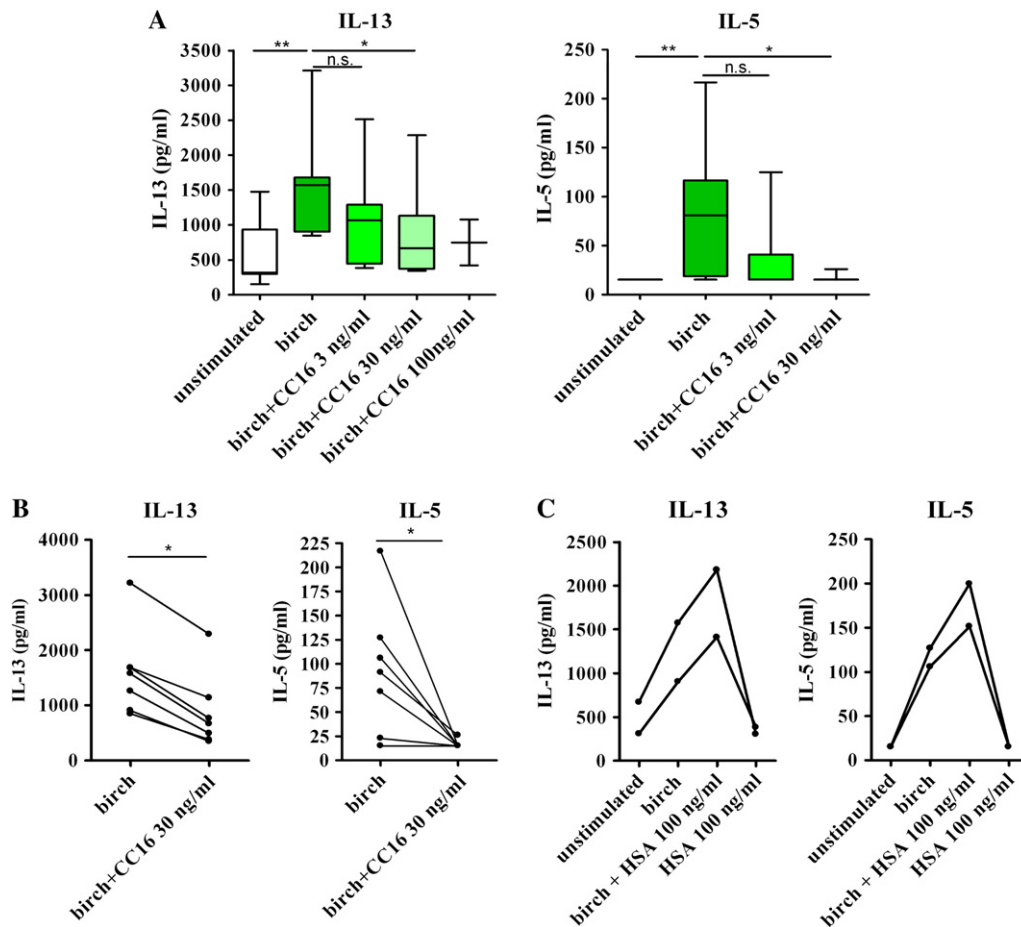


FIG 3. Monocyte-derived DCs were stimulated with birch allergen extract or birch allergen together with CC16. Thereafter, DCs were washed and cocultured with naive autologous CD4⁺ T cells. **A**, IL-13 and IL-5 levels determined by means of ELISA are summarized as box-and-whiskers graphs, with the box indicating median levels and interquartile range and the whiskers indicating the range ($n = 7$). **B**, Individual values of IL-13 and IL-5 from 7 individuals after stimulation with birch allergen alone or together with CC16. **C**, Effect of the control protein HSA on IL-13 and IL-5 production. * $P < .05$, ** $P < .01$ (Friedman test followed by the Dunn multiple comparison test).

respectively) irrespective of whether the cells were unstimulated or stimulated with birch pollen extract. Because the expression density on DCs of the PGD₂ receptor DP1 has been shown to be higher than that of DP2, it was of interest to investigate whether DP1 antagonists would be more effective than DP2 antagonists in blocking the T_H2 differentiation of human neonatal T cells. We therefore pulsed DCs with birch together with different concentrations of DP1 antagonist (BW A868C) and DP2 antagonist (CAY10471), and the DCs were then washed and added to autologous T cells. In the experiments in which the T cells produced high levels of IL-13, we found that the DP1 antagonist, but not the DP2 antagonist, seemed to suppress the IL-13 production (Fig 4, A and B, respectively). However, no inhibitory effect was found when including also those experiments with low production of IL-13 (Fig 4), and no effect on IL-5 production was seen with either antagonist (data not shown).

DISCUSSION

Previous studies show that the levels of CC16 are lower in asthmatic patients compared with those seen in healthy control subjects. However, it is still not clear whether these differences in CC16 levels have an effect on the immunopathogenic mechanisms of allergic inflammation. In the present study we investigated the levels of CC16 in blood during the first 3 years of life and whether CC16 might have an opportunity to affect T-cell development of children *in vitro*. We unexpectedly found that the levels of CC16 peaked as early as 4 months of age. We also show that CC16 does not have a direct effect on differentiated human neonatal T_H2 cells but inhibits T_H2 cell differentiation through neonatal monocyte-derived DCs.

Because CC16 is an abundantly expressed anti-inflammatory protein in the lungs, we were interested in determining the amounts of CC16 in infants and whether

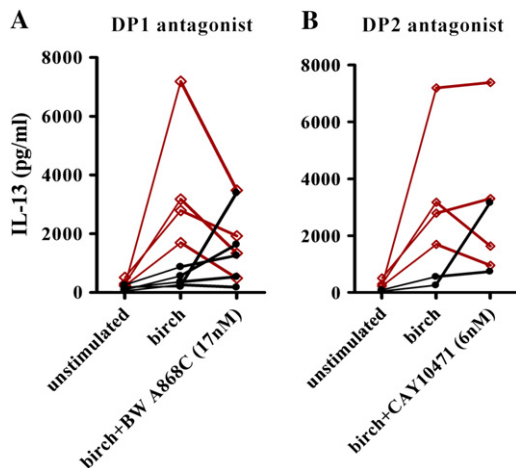


FIG 4. Monocyte-derived DCs were stimulated with birch allergen extract together with DP1 (A) or DP2 (B) antagonist for 24 hours. Thereafter, DCs were washed and cocultured with naive autologous CD4⁺ T cells. On day 10, production of IL-13 was analyzed by means of ELISA. Nine (Fig 4, A) and 6 (Fig 4, B) independent experiments are shown, and red lines in diamonds indicate individuals producing high levels of IL-13.

naturally occurring levels of the protein would affect the development of the immune response to allergens. We found a peak of CC16 levels at 4 months of age, which decreased gradually to 3 years of age. The respiratory mucosa is the main production site of CC16, which indicates that the protein is primarily involved in the regulation of inflammation in the respiratory tract. However, it has been shown that the levels of CC16 in plasma correspond well with the levels of CC16 in bronchoalveolar lavage fluid as long as the lung epithelium permeability is intact.¹⁷ There is a possibility that the high levels seen in plasma in infants might be caused by respiratory infections, resulting in damage of the epithelia. However, the vast majority of our study children did not show any signs of lower respiratory tract infection before 4 months of age because only 3 children had a record of bronchitis and bronchiolitis. The reason for the high levels of CC16 produced in infants might be a natural immunoregulatory response when the child encounters upper respiratory tract viral infections during the first year of life. However, if that is the explanation, perhaps even higher levels could have been expected at 18 months of age than at 4 months. The levels of CC16 in adults in our study agree well with those presented by a study of patients with asthma and healthy control subjects. In the latter study, an ELISA method was also used.¹⁸

It has previously been shown that CC16 has a direct effect on differentiated murine T_H2 cells and furthermore that reconstitution of the CC16 gene in allergen-challenged CC16-deficient mice reduced the levels of T_H2 cytokines.⁹ In this study we were not able to inhibit the production of IL-13, IL-5, or IL-10 when adding CC16 directly to human T cells. On the other hand, we observed that CC16 inhibited the birch allergen-induced differentiation of naive neonatal T cells toward IL-5- and IL-13-producing T cells when we added CC16 during the

maturation of the DCs. We found a significant inhibition by using 30 ng/mL CC16, which is lower than the median levels found in plasma of 4-month-old children and similar to the median levels found in 18-month-old children. These results might implicate that a high level of CC16 at an early age could modulate the immune response because it inhibits T_H2 differentiation that might result in allergy and asthma development later in life. We were not able to investigate whether the birch allergen-specific T_H2 cells would be directly affected by CC16 because the T cells need to be restimulated with allergen-pulsed DCs and addition of CC16 to the T cells might concomitantly have an effect on the DCs in the culture.

It might be argued that DCs are immature during early life and that the effect of CC16 might be different *in vivo* from that observed in an *in vitro* experimental system using matured neonatal DCs. However, it has previously been shown that monocyte-derived DCs from cord blood and adult blood produce similar amounts of IL-8, IL-6, and TNF, and only the production of IL-12p70 is markedly lower in cord blood monocyte-derived DCs compared with that from adult blood-derived DCs.^{16,19} Moreover, when LPS-stimulated neonatal DCs are cultured together with IFN- γ , the levels of IL-12 from cord blood monocyte-derived DCs and adult blood monocyte-derived DCs do not differ.¹⁹ We therefore believe that in whole blood or tissue, DCs from infants are comparable with the *in vitro* matured DCs from cord blood because various bystander cells produce the maturation factor IFN- γ .

It is not known by which molecular mechanism CC16 affects the DCs in the regulatory process. However, CC16 binds to PGD₂ and thus inhibits signaling through the DP receptor, which results in reduced transcription of the enzyme Cox-IL.¹⁰ This in turn causes a reduced production of PGD₂ because Cox-II is the main enzyme in the generation of PGD₂. In our experiments the DCs produced PGD₂ after addition of maturation factors. Thus it is conceivable that CC16 might inhibit the T_H2-polarizing effect of DCs by blocking PGD₂. Our data show that blocking the DP1 receptor resulted in reduced IL-13 production only in individuals who produced high amounts of IL-13 and had no effect on IL-5 production by T cells. Therefore this partial inhibitory effect of the DP1 antagonist might not fully explain the inhibitory effect on T_H2 cell differentiation by CC16. The DP2 receptor antagonist showed no effect on either IL-13 or IL-5 production, which is not surprising because the DP2 receptor is not expressed to the same extent on DCs.¹⁵ We cannot exclude that the DP1 and DP2 antagonists together would block the T_H2 cytokine production. However, because the effect of the DP1 antagonist is only partial and the DP2 antagonist was not effective, we find it unlikely that that would be the case. It has previously been shown that addition of PGD₂ to monocyte-derived DCs skews differentiation of naive T cells into T_H2 cells.²⁰ It has also been demonstrated that PGD₂ suppresses DC activation through the DP1 receptor and thus prevents migration into the T-cell lymph nodes.²¹ These findings do not, however, exclude

that the effect of PGD₂ on human monocyte-derived DCs *in vitro* is stimulatory and involved in T_H2 cell differentiation.

In conclusion, we show that the level of CC16 in plasma peaks at 4 months of age, which might be important in the development of the immune system because we also demonstrate that CC16 inhibits differentiation of birch allergen-induced T_H2 cytokine production through DCs. We therefore propose that CC16 is an immunoregulatory protein that might influence the immunopathogenesis of allergy in infants. It remains to be shown how the production of CC16 could be stimulated *in vivo*.

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REFERENCES

1. Åberg N, Hesselmar B, Åberg B, Eriksson B. Increase of asthma, allergic rhinitis and eczema in Swedish schoolchildren between 1979 and 1991. *Clin Exp Allergy* 1995;25:815-9.
2. Mukherjee AB, Kundu GC, Mantile-Selvaggi G, Yuan CJ, Mandal AK, Chattopadhyay S, et al. Uteroglobin: a novel cytokine? *Cell Mol Life Sci* 1999;55:771-87.
3. Peri A, Cordella-Miele E, Miele L, Mukherjee AB. Tissue-specific expression of the gene coding for human Clara cell 10-kD protein, a phospholipase A2-inhibitory protein. *J Clin Invest* 1993;92:2099-109.
4. Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T, et al. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. *Lung* 1999;177:45-52.
5. Van Vyve T, Chanez P, Bernard A, Bousquet J, Godard P, Lauwerijs R, et al. Protein content in bronchoalveolar lavage fluid of patients with asthma and control subjects. *J Allergy Clin Immunol* 1995;95:60-8.
6. Johansson S, Keen C, Ståhl A, Wennergren G, Benson M. Low levels of CC16 in nasal fluid of children with birch pollen-induced rhinitis. *Allergy* 2005;60:638-42.
7. Wang SZ, Rosenberger CL, Espindola TM, Barrett EG, Tesfaigzi Y, Bice DE, et al. CCSP modulates airway dysfunction and host responses in an Ova-challenged mouse model. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L1303-11.
8. Chen LC, Zhang Z, Myers AC, Huang SK. Cutting edge: altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-kDa protein. *J Immunol* 2001;167:3025-8.
9. Hung CH, Chen LC, Zhang Z, Chowdhury B, Lee WL, Plunkett B, et al. Regulation of TH2 responses by the pulmonary Clara cell secretory 10-kd protein. *J Allergy Clin Immunol* 2004;114:664-70.
10. Mandal AK, Zhang Z, Ray R, Choi MS, Chowdhury B, Pattabiraman N, et al. Uteroglobin represses allergen-induced inflammatory response by blocking PGD₂ receptor-mediated functions. *J Exp Med* 2004;199:1317-30.
11. Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY. Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. *J Clin Invest* 2003;111:121-8.
12. Luster AD, Tager AM. T-cell trafficking in asthma: lipid mediators grease the way. *Nat Rev Immunol* 2004;4:711-24.
13. Lewis RA, Austen KF. Mediation of local homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. *Nature* 1981;293:103-8.
14. Liu MC, Bleecker ER, Lichtenstein LM, Kagey-Sobotka A, Niv Y, McLemore TL, et al. Evidence for elevated levels of histamine, prostaglandin D₂, and other bronchoconstricting prostaglandins in the airways of subjects with mild asthma. *Am Rev Respir Dis* 1990;142:126-32.
15. Gosset P, Bureau F, Angeli V, Pichavant M, Faveeuw C, Tonnel AB, et al. Prostaglandin D₂ affects the maturation of human monocyte-derived dendritic cells: consequence on the polarization of naive Th cells. *J Immunol* 2003;170:4943-52.
16. Karlsson H, Hessle C, Rudin A. Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. *Infect Immun* 2002;70:6688-96.
17. Broeckaert F, Arsalane K, Hermans C, Bergamaschi E, Brustolin A, Mutti A, et al. Serum Clara cell protein: a sensitive biomarker of increased lung epithelium permeability caused by ambient ozone. *Environ Health Perspect* 2000;108:533-7.
18. Shijubo N, Itoh Y, Yamaguchi T, Shibuya Y, Morita Y, Hirasawa M, et al. Serum and BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers. *Eur Respir J* 1997;10:1108-14.
19. Goriely S, Vincart B, Stordeur P, Vekemans J, Willems F, Goldman M, et al. Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes. *J Immunol* 2001;166:2141-6.
20. Gosset P, Pichavant M, Faveeuw C, Bureau F, Tonnel AB, Trottein F. Prostaglandin D₂ affects the differentiation and functions of human dendritic cells: impact on the T cell response. *Eur J Immunol* 2005;35:1491-500.
21. Hammad H, de Heer HJ, Soullie T, Hoogsteden HC, Trottein F, Lambrecht BN. Prostaglandin D₂ inhibits airway dendritic cell migration and function in steady state conditions by selective activation of the D prostanoid receptor 1. *J Immunol* 2003;171:3936-40.