

Evidence that FcRn mediates the transplacental passage of maternal IgE in the form of IgG anti-IgE/IgE immune complexes

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Clinical & Experimental Allergy

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Cite this as: A. Bundhoo, S. Pavaglio, E. Rafti, A. Dhongade, R. S. Blumberg and A. P. Matson. *Clinical & Experimental Allergy*, 2015 (45) 1085–1098.

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Summary

Background The mechanism(s) responsible for acquisition of maternal antibody isotypes other than IgG are not fully understood. This uncertainty is a major reason underlying the continued controversy regarding whether cord blood (CB) IgE originates in the mother or fetus.

Objective To investigate the capacity of maternal IgE to be transported across the placenta in the form of IgG anti-IgE/IgE immune complexes (ICs) and to determine the role of the neonatal Fc receptor (FcRn) in mediating this process.

Methods Maternal and CB serum concentrations of IgE, IgG anti-IgE, and IgG anti-IgE/IgE ICs were determined in a cohort of allergic and non-allergic mother/infant dyads. Madin–Darby canine kidney (MDCK) cells stably transfected with human FcRn were used to study the binding and transcytosis of IgE in the form of IgG anti-IgE/IgE ICs.

Results Maternal and CB serum concentrations of IgG anti-IgE/IgE ICs were highly correlated, regardless of maternal allergic status. IgG anti-IgE/IgE ICs generated *in vitro* bound strongly to FcRn-expressing MDCK cells and were transcytosed in an FcRn-dependent manner. Conversely, monomeric IgE did not bind to FcRn and was not transcytosed. IgE was detected in solutions of transcytosed IgG anti-IgE/IgE ICs, even though essentially all the IgE remained in complex form. Similarly, the majority of IgE in CB sera was found to be complexed to IgG.

Conclusions and Clinical Relevance These data indicate that human FcRn facilitates the transepithelial transport of IgE in the form of IgG anti-IgE/IgE ICs. They also strongly suggest that the majority of IgE in CB sera is the result of FcRn-mediated transcytosis of maternal-derived IgG anti-IgE/IgE ICs. These findings challenge the widespread perception that maternal IgE does not cross the placenta. Measuring maternal or CB levels of IgG anti-IgE/IgE ICs may be a more accurate predictor of allergic risk.

Keywords allergy, autoantibodies, cord blood, FcRn, IgG anti-IgE, immune complexes, infant, mother, placenta

Submitted 25 September 2014; revised 13 December 2014; accepted 12 January 2015

Introduction

IgG is generally regarded as the only maternal antibody isotype capable of crossing the placental barrier [1]. The widespread belief in the selective transport of maternal IgG is a major reason underlying the continued controversy regarding whether cord blood (CB) IgE originates in the mother or fetus [2]. The receptor mediating placental transmission of maternal IgG is the β 2-microglobulin-associated MHC-class-I-like molecule FcRn,

which in humans localizes to the syncytiotrophoblast of the placental villi [3, 4]. In mice, FcRn also facilitates the acquisition of maternal IgG through expression in the intestinal epithelium during neonatal life [5] and yolk sac placenta [6]. It is well established that maternal passive immunity provides the neonate with protection against a wide range of infectious agents [7]; however, maternal IgG also facilitates the transplacental passage of antigens as IgG/antigen immune complexes (ICs) [8–10]. In a similar process, exogenous insulin, a protein

that by itself does not cross the placenta [11], is transported in the form of IgG anti-insulin/insulin ICs [12].

We recently demonstrated that murine FcRn facilitates the intestinal absorption of IgE in the form of IgG anti-IgE/IgE ICs [13]. In addition, IgE bound by IgG anti-IgE at the Cε4 domain remained biologically active by retaining the ability to bind FcεRI and induce rat basophil leukaemia cell degranulation [13]. Because IgG anti-IgE and IgG anti-IgE/IgE ICs are present in the sera of allergic and non-allergic individuals [14–17], we speculated that human FcRn (hFcRn) localized to the placenta would transport maternal IgE to the fetus in the form of IgG anti-IgE/IgE ICs. In this study, we sought to define the relationship between maternal and CB serum concentrations of IgG anti-IgE/IgE ICs in a cohort of allergic and non-allergic pregnant women and their infants. In addition, we aimed to investigate the capacity of hFcRn to bind and transport IgE in the form of IgG anti-IgE/IgE ICs using a well-validated *in vitro* model system [18, 19]. Our results strongly suggest that maternal IgE crosses the placenta predominantly in the form of IgG anti-IgE/IgE ICs, thereby providing new insight into a pathway for the fetal acquisition of maternal IgE.

Methods

Subject recruitment, sample collection, and measurement of serum IgE levels

The study population consisted of 152 allergic and non-allergic pregnant women and their full-term infants delivered at Hartford Hospital (Hartford, CT, USA) between January 2011 and February 2012. Details of the recruitment strategy and sample collection have been previously reported [20]. In brief, potential participants were screened for eligibility and recruited for participation upon admission to labour and delivery. Pregnant women were eligible if they were English or Spanish speaking, had not received prenatal steroids for the treatment of preterm labour, were not on high dose inhaled steroids (e.g. > 800 mcg/day beclomethasone equivalent), and were delivering an infant ≥ 37 weeks' gestational age with no known major congenital anomaly. Informed written consent was obtained from all prospective mothers and on behalf of all their infants. Maternal blood was collected by venipuncture prior to delivery. Immediately following delivery, CB samples were obtained from the umbilical vein cleansed with alcohol. Pregnant women were divided into two groups based on the absence or presence of allergic disease as defined by a physician's diagnosis of asthma, allergic rhinitis, atopic dermatitis, or food allergy and associated symptoms (e.g. cough, wheeze, skin rash) within the past 12 months. Assignment of study subjects into

these groups was by chart review and personal interview. Total serum IgE concentrations were determined by Phadia (Thermo Fisher Scientific, Portage, MI, USA) using the ImmunoCAP method. The detection limit for IgE in maternal and CB specimens was 2.00 and 0.10 kU/L, respectively. As a surrogate marker for maternal blood contamination, CB samples were analysed for total IgA using a commercial ELISA kit (Mabtech Inc., Cincinnati, OH, USA), and CB samples containing ≥ 10 µg/mL IgA were excluded from the analysis [20, 21]. The study was approved by the Institutional Review Board at Hartford Hospital (IRB# MAT-S003083HU).

Measurement of IgG anti-IgE autoantibodies in maternal and cord blood sera

Thermo Scientific Nunc™ Immunoplates (Thermo Fisher Scientific) were coated with monoclonal human IgE (hIgE) (HE1) (Bioreclamation LLC., Westbury, NY, USA) (10 µg/mL) in 0.1 M carbonate (pH 9.5) for 16 h at 4°C. After blocking non-specific binding, maternal or CB IgG anti-IgE antibodies were captured as twofold serial dilutions of maternal or CB serum. Detection of IgG anti-IgE was with biotin-SP-conjugated mouse IgG (mIgG) anti-hFcγ (Jackson ImmunoResearch, West Grove, PA, USA), followed by avidin-horseradish peroxidase (HRP) (BD Biosciences, San Jose, CA, USA). As a reference standard, adjacent wells on the same plates were coated with ovalbumin (OVA) (grade V; Sigma Chemical Co., St. Louis, MO, USA) (10 µg/mL) in PBS, and predetermined amounts of mIgG₁ anti-OVA (BioX-Cell, West Labanon, NH, USA) were added as twofold serial dilutions in duplicate. Biotin-SP-conjugated goat anti-mFcγ₁ (Jackson ImmunoResearch) was used to detect bound murine IgG, followed by avidin-HRP. Development was with the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and A₄₅₀ measured with a Bio-Rad microplate reader (Hercules, CA, USA).

Measurement of IgG anti-IgE/IgE immune complexes in maternal and cord blood sera

Three sandwich ELISAs were employed using different anti-IgE capture reagents with unique epitope specificities. This strategy enabled the capturing of IgE (and ICs) in which the FcεRI-binding region of IgE remained open or blocked by IgG anti-IgE. Thermo Scientific Nunc™ Immunoplates were coated under the conditions described above with mIgG anti-hIgE (m107) (Mabtech Inc.) (2 µg/mL), mIgG anti-hIgE (HP6029) (SouthernBiotech, Birmingham, AL, USA) (2 µg/mL), or recombinant FcεRIα protein (rFcεRIα) (NBS-C Bioscience, Vienna, Austria) (2 µg/mL). m107 competes for an epitope in

the Cε4 region (A.P. Matson, S. Paveglio, and E. Rafti; unpublished results), HP6029 recognizes an epitope not located in Cε3 or Cε4 [22], and rFcεRIα recognizes the FcεRI-binding region in Cε3 [23]. After blocking non-specific binding, maternal and CB IgE (and ICs) were captured as twofold serial dilutions of serum. Detection of IgG anti-IgE/IgE ICs was with biotin-SP-conjugated mIgG anti-hFcγ, (Jackson ImmunoResearch) followed by avidin-HRP. Development and reading of plates was as above. As a reference standard for the m107 and HP6029 IC assays, IgG anti-IgE/IgE ICs were generated *in vitro* using equal molar amounts of omalizumab (humanized IgG₁ anti-hIgE_{Cε3}) (Genentech, Inc., San Francisco, CA, USA) and hIgE (HE1) (Bioreclamation LLC). Predetermined amounts of ICs were added to adjacent wells on the same plate as twofold serial dilutions in duplicate, and the remainder of the assay was the same. As a reference standard in the rFcεRIα IC assay, IgG anti-IgE/IgE ICs were generated *in vitro* using mIgG₁ anti-hIgE_{Cε4} (Le27) (NBS-C Bioscience) and hIgE (HE1), and predetermined amounts were similarly applied to adjacent wells on the same plate. Biotin-SP-conjugated goat anti-mFcγ₁ was used to detect bound murine IgG, followed by avidin-HRP. In some experiments, serum samples were pre-incubated with omalizumab to exclude the possibility of endogenous IgG directed against the α-chain of FcεRI [24]. To determine the IgG subclasses of ICs captured with rFcεRIα, biotin-SP-conjugated mIgGs directed against hFcγ₁ (6069B), hFcγ₂ (6002B), hFcγ₃ (6047B), or hFcγ₄ (6025B) (kind gifts from Dr. Robert Hamilton, Johns Hopkins Allergy and Asthma Center, Baltimore, MD, USA) were used in place of biotin-SP-conjugated mIgG anti-hFcγ. The remainder of the assay was exactly as described above.

hFcRn-binding and transport studies

As a surrogate model to study the ability of hFcRn to bind and transport IgG anti-IgE/IgE ICs across the placenta, we used hβ2m-positive Madin-Darby canine kidney (MDCK) cells stably transfected with the heavy chain of hFcRn [18]. When grown to confluence on Transwell filters, FcRn-expressing MDCK cells are a well-established *in vitro* model system to study IgG transport [19, 25]. Furthermore, because hFcRn has strong affinity for human and rabbit IgG [19, 26], this system was used to evaluate the binding of humanized or rabbit IgG anti-IgEs to hFcRn, prior to the generation of IgG anti-IgE/IgE ICs. Cells were incubated for 1 h at 4°C with omalizumab (Genentech, Inc.) (1 mg/mL), polyclonal rabbit IgG anti-hIgE (rIgG anti-hIgE) (Dako Inc., Carpinteria, CA, USA) (130 µg/mL), or as a reference standard RhoGAM (polyclonal hIgG anti-RhD) (Ortho-Clinical Diagnostics, Rochester, NY, USA) (1 mg/mL), in PBS containing 0.2% BSA and 0.1% NaN₃, buf-

fered to pH 6.0. After washing to remove unbound antibodies, biotin-conjugated mIgG anti-hFcγ (Jackson ImmunoResearch) was used to detect bound omalizumab or RhoGAM, and biotin-conjugated mIgG anti-rFcγ (Sigma-Aldrich, St Louis, MO, USA) was used to detect bound rIgG anti-hIgE. Streptavidin–allophycocyanin (SA-APC) (Life Technologies, Carlsbad, CA, USA) was subsequently applied, and cell staining was evaluated using a Becton–Dickinson LSR II flow cytometer (BD Biosciences). Resultant data were analysed using FlowJo software (Tree Star Inc., Ashland, OR, USA). The relative fluorescence intensities for cell staining were compared to unstained cells and cells incubated with biotinylated antibodies, followed by SA-APC.

To evaluate the ability of hFcRn to bind IgE in the form of IgG anti-IgE/IgE ICs, MDCK cells were incubated for 1 h at 4°C with rIgG anti-hIgE (10 µg/mL), hIgE (100 µg/mL), (U266B1) (ATCC, Manassas, VA, USA), or rIgG anti-hIgE/hIgE ICs (molar ratio 1 : 10 based on hIgE concentration of 100 µg/mL) in PBS containing 0.2% BSA and 0.1% NaN₃, buffered to pH 6.0 or pH 7.4. Biotin-conjugated mIgG anti-rFcγ (Sigma-Aldrich) was used to detect bound rIgG anti-hIgE, and biotin-conjugated mIgG anti-hIgE_{Cε4} (Le27) (NBS-C Bioscience) was used to detect bound hIgE or rIgG anti-hIgE/hIgE ICs. SA-APC was subsequently applied, and cell staining was examined by flow cytometry. Control conditions were cells incubated with biotinylated antibodies, followed by SA-APC. For blocking experiments, cells were pre-incubated with the hFcRn-blocking antibody DVN 24 [27] (kind gift from Dr. Derry Roopenian, Jackson Labs, Bar Harbor, ME, USA). For immunofluorescence microscopy, hIgE (U266B1) (ATCC) was labelled with Alexa Fluor 555 (AF555) (Molecular Probes Inc., Eugene, OR, USA) and used to generate rIgG anti-hIgE/hIgE^{AF555} ICs. Cells were incubated with mIgG anti-hFcRn (B-8) (Santa Cruz Biotechnology, Dallas, TX, USA) followed by goat anti-mIgG^{FITC} (Santa Cruz Biotechnology) and rIgG anti-hIgE/hIgEAF555 ICs, in PBS containing 0.2% BSA and 0.1% NaN₃, buffered to pH 6.0. Stained cells were adhered to glass slides via cytopins, fixed with methanol, and counterstained with DAPI (Vectashield, Burlingame, CA, USA). Cells were viewed using a fluorescence Olympus DP 73 microscope (Olympus, Center Valley, PA, USA), and CellSens software (Olympus) was used for image acquisition and analysis.

For transport studies, MDCK cells were plated at a density of 800 000 cells/cm² on 12 mm diameter, 0.4 µm pore, semipermeable Transwell filters (Corning Life Sciences, Corning, NY, USA). On day 3, when transepithelial electrical resistance reached 150–200 Ohm/cm², transcytosis assays were performed as described [18, 19], with the input chamber buffered to pH 6.0 and the output chamber buffered to pH 7.4. To the input chamber, rIgG

anti-hIgE (10–50 µg/mL), hIgE (100–500 µg/mL), or rIgG anti-hIgE/hIgE ICs (molar ratio 1 : 10 based on hIgE concentration of 100–500 µg/mL) were added in Hank's balanced salts solution with 10 mM MES, pH 6.0 at 37°C, 5% CO₂ for 2 h. For blocking or competitive inhibition experiments, DVN 24 (0.1–25 µg/mL) or RhoGAM (1 mg/mL) was added to the input chamber 20 min before the antibodies or ICs. After 2 h, the input and output Transwell solutions were collected and the concentrations of antibodies and ICs were determined by ELISA. The percentage of transport was calculated as $[(\text{Concentration}^{\text{output}} \times \text{Volume}^{\text{Output}})/(\text{Concentration}^{\text{input}} \times \text{Volume}^{\text{Input}})] \times 100\%$.

Measurement of antibodies and immune complexes in Transwell solutions and pooled cord blood serum

Because polyclonal rabbit IgG anti-hIgE was used in hFcRn-transport experiments, different assays were required to enumerate antibodies and ICs. To determine concentrations of rIgG anti-hIgE in Transwell solutions, Thermo Scientific Nunc™ Immunoplates were coated with monoclonal hIgE (U266B1) (10 µg/mL) in 0.1 M carbonate (pH 9.5) for 16 h at 4°C. After blocking non-specific binding, rIgG anti-hIgE antibodies were captured as twofold serial dilutions of Transwell solutions. Detection of rIgG anti-hIgE was with biotin-conjugated mIgG anti-rFcγ (Sigma-Aldrich), followed by avidin-HRP. As a reference standard, predetermined amounts of rIgG anti-hIgE were added to the same plate and the remainder of the assay was the same. For the measurement of rIgG anti-hIgE/hIgE ICs, Thermo Scientific Nunc™ Immunoplates were coated under the conditions described above with mIgG anti-hIgE (m107) (Mabtech) (2 µg/mL). After blocking non-specific binding, rIgG anti-hIgE/hIgE ICs were captured as twofold serial dilutions of Transwell solutions. Detection of rIgG anti-hIgE/hIgE ICs was with biotin-conjugated mIgG anti-rFcγ (Sigma-Aldrich), followed by avidin-HRP. As a reference standard, predetermined amounts of rIgG anti-hIgE/hIgE ICs were applied to the same plate and the remainder of the assay was the same. To determine the ability of transcytosed ICs to bind FcεRI, Thermo Scientific Nunc™ Immunoplates were coated with rFcεRIα (NBS-C Bioscience) (2 µg/mL), and the remainder of the assay was as above.

Concentrations of total IgE were determined in pooled Transwell solutions and pooled CB serum, prior to and after absorption of IgG (and IgG anti-IgE/IgE ICs) using protein A coupled agarose (Bio-Rad). Control solutions containing rIgG anti-hIgE or monomeric hIgE (U266B1) were used to assess the selective removal of IgG by protein A. For the measurement of total hIgE, Thermo Scientific Nunc™ Immunoplates were coated under the conditions described above with mIgG anti-

hIgE (m107) (2 µg/mL) or rFcεRIα (2 µg/mL). After blocking non-specific binding, hIgE was captured as twofold serial dilutions of Transwell solutions or CB serum. Detection of hIgE was with a cocktail of biotinylated mIgG anti-hIgEs, including HP6029 (SouthernBiotech), m182 (Mabtech), and Le27 (NBS-C Bioscience), followed by avidin-HRP. The standard in these assays was predetermined amounts of monoclonal hIgE (U266B1).

IgE expression on cord blood basophils

IgE expression on CB basophils isolated from infants included in this cohort has been previously reported [20]. For the current study, CB samples containing ≥ 0.5 kU/L total serum IgE and a minimum of 200 basophils were included in the analysis. The relationship between serum levels of CB IgE and percentages of CB basophils with bound IgE was assessed for infants of allergic and non-allergic mothers as an indicator for the ability of CB IgE to bind FcεRI-expressing fetal cells. CB cells were stained with anti-BDCA-2/FITC (AC144), anti-CD123/PE (AC145), anti-IgE/APC (MB10-5C4), or mouse IgG₁ isotype control/APC (IS5-21F5) (Miltenyi Biotech Inc., Auburn, California, USA). CB basophils were identified as the CD123⁺ high BDCA-2⁺ cells, and surface-bound IgE was quantified relative to the isotype control. Flow cytometry was performed using a FACSCalibur instrument, and resultant data were analysed using FlowJo software (Tree Star Inc.).

Statistical analysis

Comparisons of serum IgE, IgG anti-IgE autoantibodies, and IgG anti-IgE/IgE ICs were performed using the Mann-Whitney *U*-test. Correlation analyses were performed using Pearson's correlation. Transcytosis of antibodies and ICs were compared using ANOVA and Bonferroni's post-test. Concentrations of CB IgE prior to and after protein A chromatography were compared using the paired *t*-test. Chi-square was used to compare the distribution of weight gain between allergic and non-allergic mothers, and the Fisher's exact test was applied to the remaining categorical variables between groups. All statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA, USA).

Results

Study subjects and serum IgE levels

Of the 152 pregnant women included in this study, 62 were allergic and 90 were non-allergic. Of these, two allergic and three non-allergic mother/infant dyads were excluded because of insufficient volumes of blood

collected. One allergic and one non-allergic mother/infant dyad were excluded because of CB serum IgA concentrations $> 10 \mu\text{g/mL}$, and one non-allergic mother/infant dyad was excluded because of a CB serum IgE concentration > 7 SD from the mean. The remaining 59 allergic and 85 non-allergic infant/dyads were included in the analysis. Baseline characteristics of these mothers and infants are shown in Table S1 in the OR. Pregnant women with a history of allergic disease demonstrated higher levels of total serum IgE than pregnant women without a history of allergic disease ($P = 0.03$). CB serum total IgE levels were not significantly different in infants of allergic and non-allergic mothers ($P = 0.31$) (Table 1).

IgG anti-IgE autoantibodies in maternal and cord blood sera

IgG autoantibodies directed against IgE were detected in the serum of allergic and non-allergic pregnant women [median 82 (range 5–1854) vs. 107 (3–4238) ng/mL, $P = 0.14$] (Fig. 1a). In allergic pregnant women, serum concentrations of IgE correlated with serum concentrations of IgG anti-IgE ($r = 0.49$, $P < 0.0001$) (Fig. 1b). In non-allergic pregnant women, serum concentrations of IgE did not correlate with serum concentrations of IgG anti-IgE ($r = 0.03$, $P = 0.77$) (Fig. 1c). For each mother/infant dyad, levels of IgG anti-IgE were similar, regardless of maternal allergic status. A combined analysis of all mother/infant dyads demonstrated that maternal and CB serum concentrations of IgG anti-IgE were highly correlated ($r = 0.90$, $P < 0.0001$) (Fig. 1d).

IgG anti-IgE/IgE immune complexes in maternal and cord blood sera

Sandwich ELISAs utilizing antibodies directed against IgE and IgG are an established method to determine serum concentrations of IgG anti-IgE/IgE ICs [28]. Our initial studies using the rFcεRIα-based assay demonstrated that IgG anti-hIgE_{Cε4}/hIgE ICs could be quanti-

fied using this method (Fig. S1 in the OR). Furthermore, pre-incubation of maternal serum with omalizumab resulted in a significant reduction in the binding of IgG anti-IgE/IgE ICs to rFcεRIα (Fig. S2 in the OR), indicating the detection of ICs in this assay was not due to endogenous IgG directed against FcεRIα [24].

IgG anti-IgE/IgE ICs were detected in the sera of allergic and non-allergic pregnant women using the m107 [median 1950 (range 186–18 355) vs. 2048 (284–8732) ng/mL, $P = 0.40$], HP6029 [median 3472 (range 157–18 486) vs. 4342 (169–15 006) ng/mL, $P = 0.49$], and rFcεRIα [median 6 (range 4–155) vs. 5 (4–171) ng/mL, $P = 0.82$] IC assays (Fig. 2a). In allergic pregnant women, serum concentrations of IgE correlated with serum concentrations of IgG anti-IgE/IgE ICs determined using the rFcεRIα IC assay ($r = 0.83$, $P < 0.0001$) (Fig. 2b). In non-allergic pregnant women, serum concentrations of IgE did not correlate with serum concentrations of IgG anti-IgE/IgE ICs determined using the rFcεRIα IC assay ($r = 0.20$, $P = 0.07$) (Fig. 2c). There were no significant correlations observed between levels of IgE and levels of IgG anti-IgE/IgE ICs determined using the m107 or HP6029 IC assays in allergic or non-allergic pregnant women (data not shown). For each mother/infant dyad, levels of IgG anti-IgE/IgE ICs were similar, regardless of maternal allergic status or the IC assay used. A combined analysis of all mother/infant dyads demonstrated that maternal and CB serum concentrations of IgG anti-IgE/IgE ICs were highly correlated in each of the IC assays [m107 ($r = 0.94$, $P < 0.0001$), HP6029 ($r = 0.85$, $P < 0.0001$), and rFcεRIα ($r = 0.88$, $P < 0.0001$)] (Fig. 2d–f).

To further investigate for differences in IC composition based on maternal history of allergy, serum samples from 10 allergic and 11 non-allergic pregnant women with comparable levels of total IgG anti-IgE/IgE ICs measured using the rFcεRIα IC assay [median 40 (range 18–155) vs. 29 (14–171) ng/mL, $P = 0.50$] (data not shown) were analysed to determine the IgG subclass distribution of IgG anti-IgE/IgE ICs. In these subjects, total IgE levels were significantly higher in the allergic pregnant women as compared to the non-allergic pregnant women [median 289.00 (range 62.08–1259.00) vs. 50.90 (25.40–276.50) kU/L, $P < 0.001$] (Fig. 3a). Despite similar serum concentrations of total IgG anti-IgE/IgE ICs, there were significantly higher levels of IgG₁-containing IgG anti-IgE/IgE ICs in the serum of allergic pregnant women as compared to non-allergic pregnant women [median 34 (range 7–70) vs. 12 (4–66) ng/mL, $P = 0.026$] (Fig. 3b). IgG₃- and IgG₄-containing IgG anti-IgE/IgE ICs were also present; however, differences in serum concentrations between allergic and non-allergic pregnant women were not statistically significant ($P = 0.11$ and $P = 0.15$, respectively) (Fig. 3b). In corresponding CB sera, total IgE

Table 1. Serum total IgE in allergic and non-allergic mother/infant dyads. * $P < 0.05$.

	Allergic mother/ infant dyads ($n = 59$)	Non-allergic mother/infant dyads ($n = 85$)	<i>P</i> -value
Maternal IgE (kU/L)			
Median (range)	62.89 (1.33–1258.90)	34.40 (2.00–1572.52)	*0.03
CB IgE (kU/L)			
Median (range)	0.26 (0.10–2.67)	0.18 (0.10–3.19)	0.31

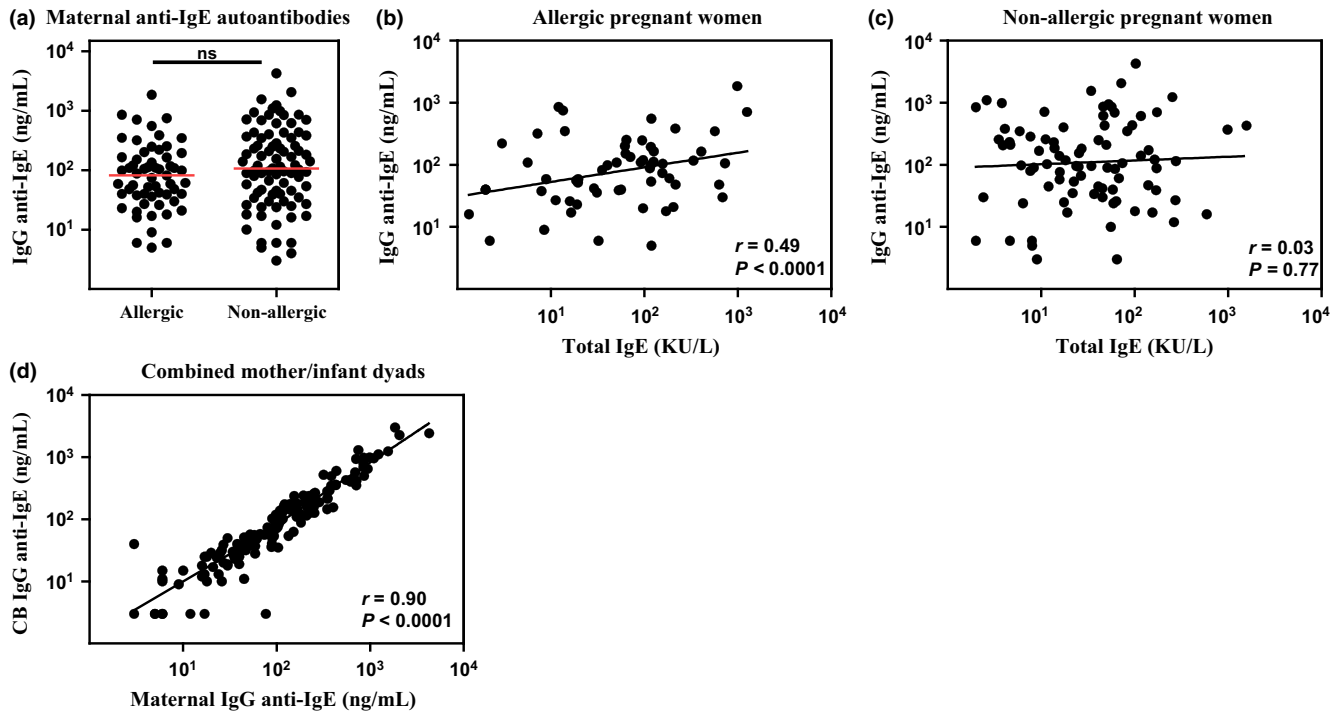


Fig. 1. IgG anti-IgE autoantibodies in maternal and CB sera. Data represent results obtained from 59 allergic and 85 non-allergic mother/infant dyads. (a) Levels of IgG anti-IgE were similar between allergic and non-allergic pregnant women. Total serum IgE correlated with levels of IgG anti-IgE in (b) allergic pregnant women but not in (c) non-allergic pregnant women. (d) A combined analysis of allergic and non-allergic mother/infant dyads demonstrated that maternal and CB levels of IgG anti-IgE were highly correlated. ns, not significant. The horizontal line represents the median.

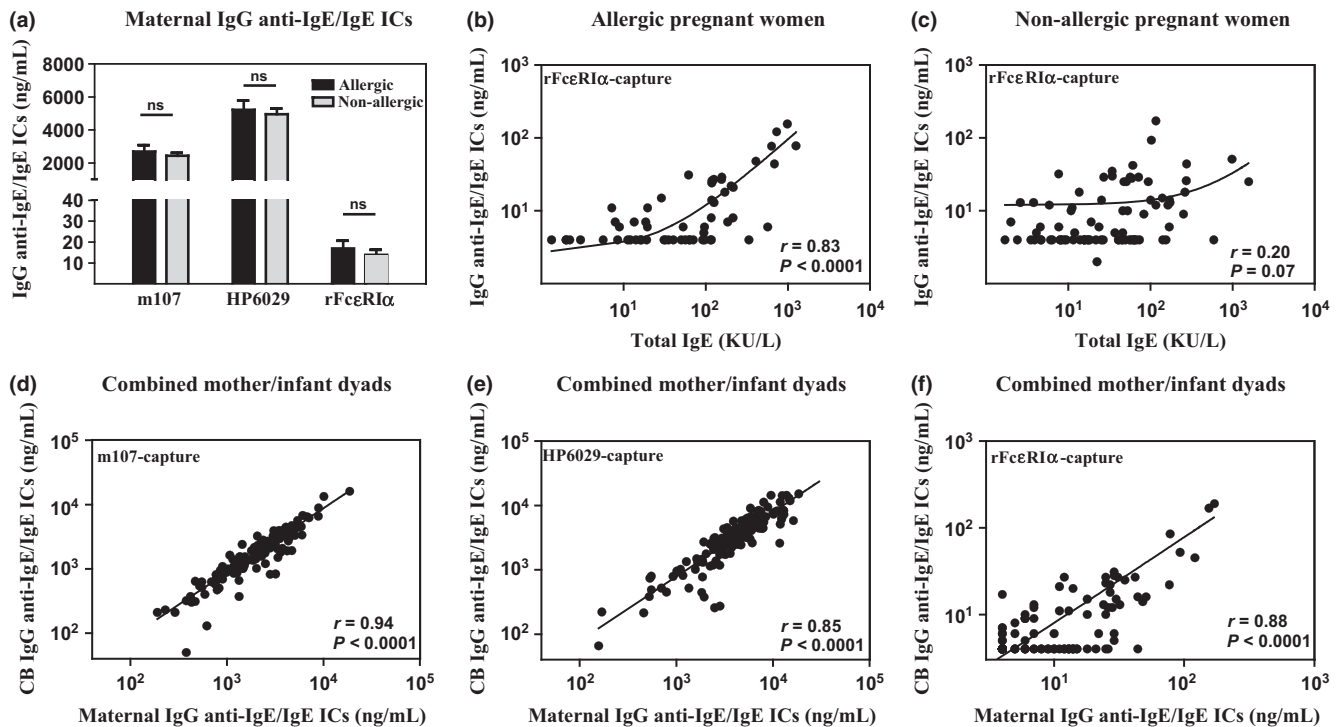


Fig. 2. IgG anti-IgE/IgE ICs in maternal and CB sera. Data represent results obtained from 59 allergic and 85 non-allergic mother/infant dyads. (a) Levels of IgG anti-IgE/IgE ICs were similar between allergic and non-allergic pregnant women, when determined using the m107, HP6029, or rFcεRIα IC assays. Total serum IgE correlated with levels of IgG anti-IgE/IgE ICs in (b) allergic pregnant women but not in (c) non-allergic pregnant women, when ICs were measured using rFcεRIα. (d–f) A combined analysis of allergic and non-allergic mother/infant dyads demonstrated that maternal and CB levels of IgG anti-IgE/IgE ICs were highly correlated when determined using the m107, HP6029, or rFcεRIα IC assays. ns, not significant.

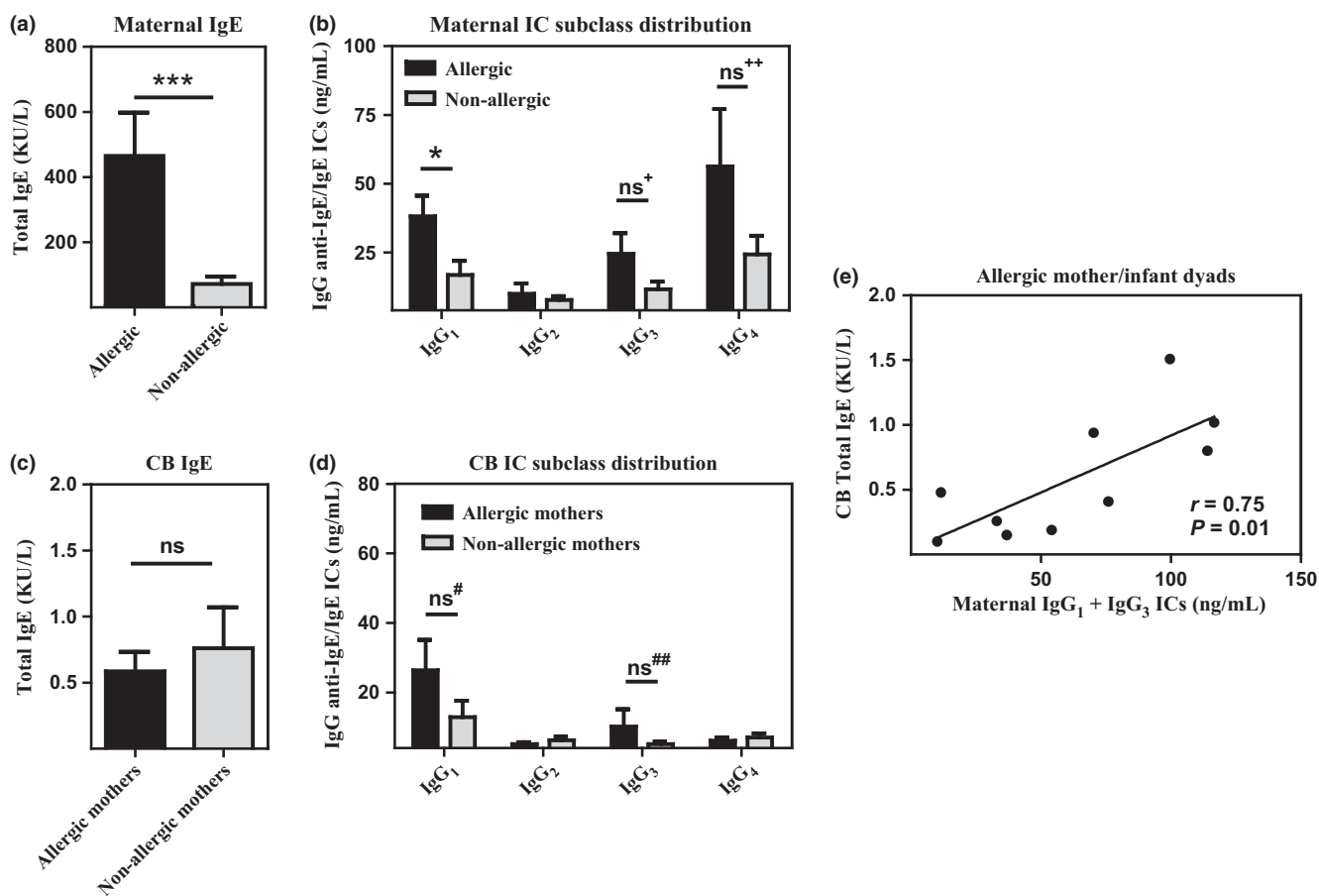


Fig. 3. IgG subclass distribution of IgG anti-IgE/IgE ICs in maternal and CB sera. Data represent results obtained from 10 allergic and 11 non-allergic pregnant women with similar levels of total IgG anti-IgE/IgE ICs, and their infants. (a) Total IgE levels were significantly greater in allergic as compared to non-allergic pregnant women. (b) Levels of IgG₁-containing ICs were significantly greater in allergic as compared to non-allergic pregnant women. In corresponding infants, there were no significant differences in levels of CB IgE (c) or IgG subclass-specific ICs (d) based on maternal history of allergy. (e) In allergic mother/infant dyads, combined levels of maternal IgG₁- and IgG₃-containing ICs correlated significantly with levels of CB IgE. ns, not significant. *** $P < 0.001$, * $P < 0.05$, ns⁺ $P = 0.11$, ns⁺⁺ $P = 0.15$, ns[#] $P = 0.18$, ns^{##} $P = 0.30$.

levels were similar in infants of allergic and non-allergic mothers [median 0.45 (range 0.10–1.51) vs. 0.29 (0.1–3.19) kU/L, $P = 0.67$] (Fig. 3c). IgG₁- and IgG₃-containing IgG anti-IgE/IgE ICs were the most highly represented subclasses in CB sera; however, differences in serum concentrations between infants of allergic and non-allergic mothers did not reach statistical significance ($P = 0.18$ and $P = 0.30$, respectively) (Fig. 3d). In allergic mother/infant dyads, combined levels of maternal IgG₁- and IgG₃-containing IgG anti-IgE/IgE ICs were predictive of CB IgE levels ($r = 0.75$, $P = 0.01$) (Fig. 3e), whereas maternal IgE levels were not ($r = 0.35$, $P = 0.33$) (data not shown). In non-allergic mother/infant dyads, maternal IgE levels were predictive of CB IgE levels ($r = 0.77$, $P = 0.006$), whereas combined levels of maternal IgG₁- and IgG₃-containing IgG anti-IgE/IgE ICs were not ($r = 0.01$, $P = 0.99$) (data not shown).

hFcRn binds and transports IgE in the form of IgG anti-IgE/IgE immune complexes

Initial studies performed to compare the binding of different IgG anti-hIgEs to hFcRn demonstrated that polyclonal rabbit IgG anti-hIgE bound to hFcRn-expressing cells at an equivalent level as RhoGAM, whereas omalizumab demonstrated a much lower level of binding (Fig. S3 in the OR). Thus, polyclonal rIgG anti-hIgE and hIgE were used to generate IgG anti-IgE/IgE ICs for the remaining hFcRn-binding and transport studies.

Flow cytometric analysis of hFcRn-expressing MDCK cells demonstrated no binding of the murine detection antibody biotin-conjugated mIgG anti-hIgE_{Cε4} (Le27) or monomeric hIgE, whereas there was strong binding of polyclonal rIgG anti-hIgE (Fig. 4a–c). These results are consistent with published reports, which demonstrate that mouse IgG and human IgE do not bind hFcRn,

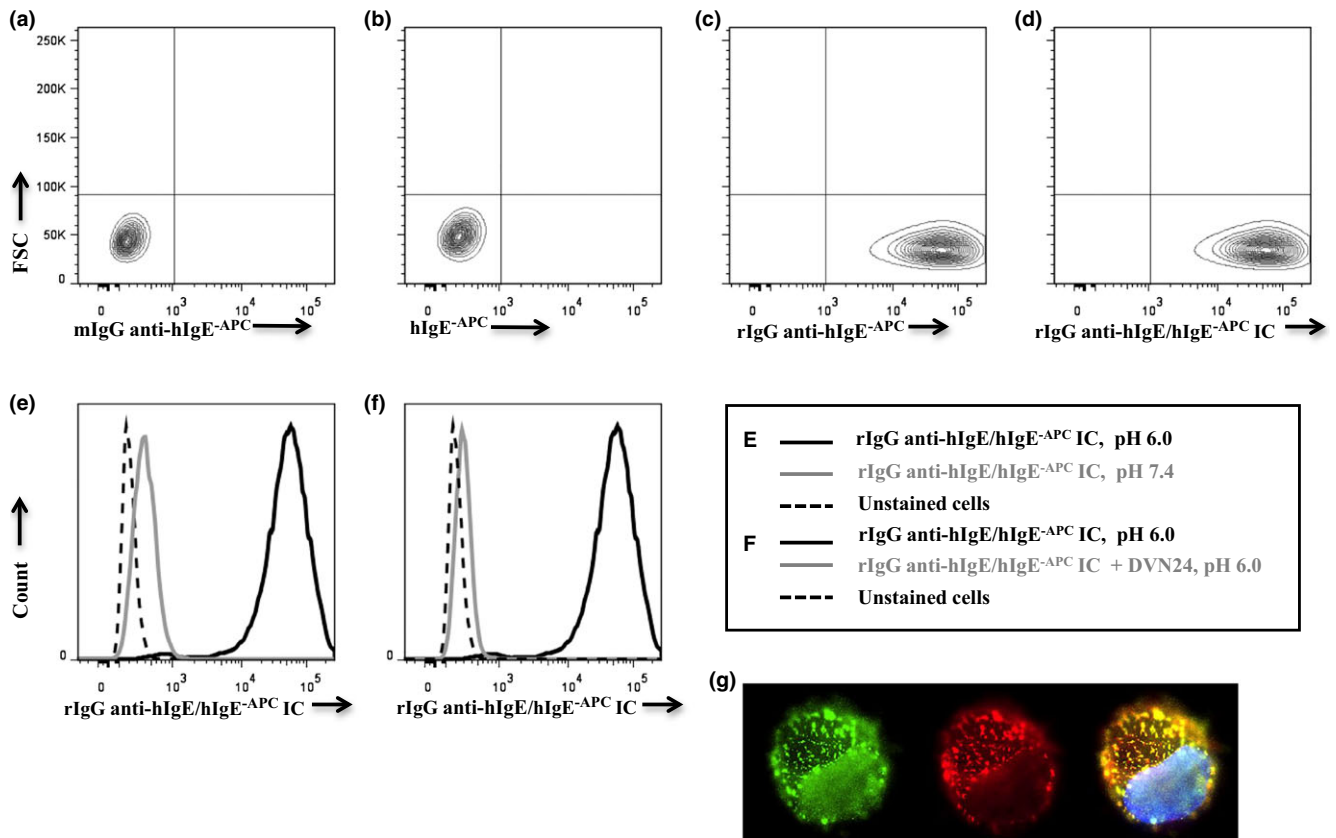


Fig. 4. hFcRn binds IgE in the form of IgG anti-IgE/IgE ICs. hFcRn-expressing MDCK cells were incubated with (a) biotin-conjugated mIgG anti-hIgE_{Cε4}, (b) hIgE, (c) rIgG anti-hIgE, or (d) rIgG anti-hIgE/hIgE ICs, at (e) different pHs, or (f) in the presence of the hFcRn-blocking antibody DVN24. Biotin-conjugated mIgG anti-rFcγ was used to detect bound rIgG anti-hIgE, and biotin-conjugated mIgG anti-hIgE_{Cε4} was used to detect bound hIgE or rIgG anti-hIgE/hIgE ICs. Visualization of bound antibodies was with SA-APC, and the cells were analysed by flow cytometry. (g) Immunofluorescence microscopy of MDCK cells performed at pH 6.0 demonstrated co-localization of hFcRn (green) and hIgE (in the form of rIgG anti-hIgE/hIgE^{AF555} ICs) (red). The blue colour represents nuclear staining by DAPI.

while rabbit IgG binds hFcRn strongly [26, 29]. hIgE, in the form of rIgG anti-hIgE/hIgE ICs, bound strongly to cells (Fig. 4d). The binding of rIgG anti-hIgE/hIgE ICs was pH dependent, inhibited by the hFcRn-blocking antibody DVN24, and co-localized with hFcRn (Fig. 4e–g). pH-dependent binding is a characteristic feature of FcRn which requires an acidic pH to bind IgG with high affinity [27].

In transcytosis experiments, hFcRn-mediated transport was observed for rIgG anti-hIgE and rIgG anti-hIgE/hIgE ICs, but not for monomeric hIgE (Fig. 5a). The transport of rIgG anti-hIgE/hIgE ICs occurred in basolateral to apical and apical to basolateral directions, was competitively inhibited by RhoGAM and by the hFcRn-blocking antibody DVN24 (Figs 5a,b; and data not shown). Following transcytosis, a substantial proportion of rIgG anti-hIgE/hIgE ICs retained the ability to bind rFcεRIα protein (mean % antibody transport = 0.042 ± 0.005; data not shown). In addition, hIgE was detected in output solutions containing transcytosed rIgG anti-hIgE/hIgE ICs, using both the m107 and

rFcεRIα anti-hIgE assays (Fig. 5c, and data not shown). To determine whether the transcytosed hIgE was monomeric or complexed to rIgG anti-hIgE, output solutions from nine separate Transwells were pooled [mean IgE concentration of 7140 (range 2622–26 517) ng/mL, determined using rFcεRIα anti-hIgE assay] and subjected to protein A chromatography. Following exposure to protein A, only 6% of the hIgE was recovered in the primary flow through (Fig. 5c), which coincided with removal of > 99% of the rIgG anti-hIgE/hIgE ICs (data not shown). In control solutions exposed to protein A, > 99% of rIgG anti-hIgE was removed, whereas > 95% of monomeric IgE was recovered (data not shown).

The majority of IgE in cord blood serum exists as IgG anti-IgE/IgE immune complexes

To determine whether CB IgE was monomeric or complexed to IgG anti-IgE, CB sera from 20 different infants were pooled into five separate groups of 4,

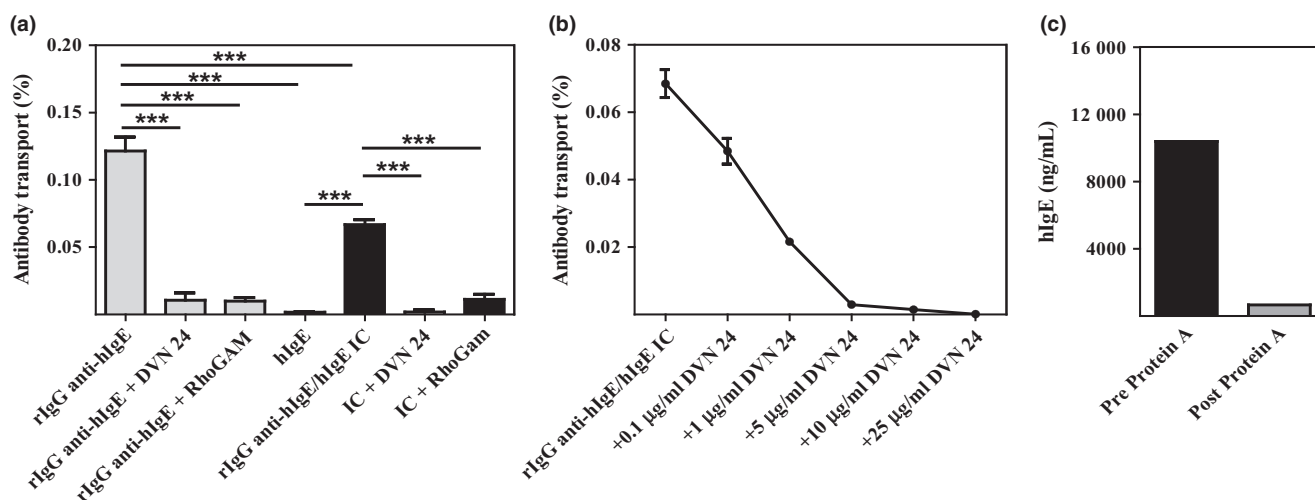


Fig. 5. hFcRn facilitates the transcytosis of hIgE in the form of IgG anti-IgE/IgE ICs. hFcRn-expressing MDCK cells were plated on semipermeable Transwell filters and grown to confluence. Transcytosis assays were performed with the input chamber buffered to pH 6.0 and the output chamber buffered to 7.4. (a) rIgG anti-hIgE and rIgG anti-hIgE/hIgE ICs were transcytosed across MDCK cells, whereas monomeric IgE was not. Transcytosis of rIgG anti-hIgE and rIgG anti-hIgE/hIgE ICs was inhibited by DVN24 and RhoGAM. (b) Increasing concentrations of DVN24 added to the input chambers resulted in greater inhibition in the transport of rIgG anti-hIgE/hIgE ICs. (c) hIgE detected in pooled solutions of transcytosed rIgG anti-hIgE/hIgE ICs was significantly reduced after selective removal of rIgG (and rIgG anti-hIgE/hIgE ICs) using protein A, indicating the majority of the hIgE remained in complex form. Transport data are representative of 4 individual experiments. *** $P < 0.0001$.

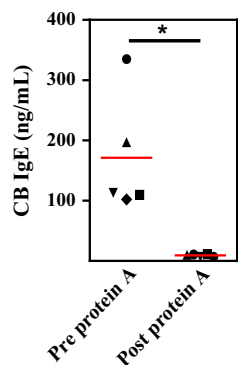


Fig. 6. The majority of IgE in CB sera is bound by IgG. CB sera obtained from 20 different infants were pooled into five separate groups of 4. Symbols represent total IgE levels obtained for each group, before and after selective absorption of IgG (and IgG anti-IgE/IgE ICs) using protein A chromatography. Total IgE in CB sera was significantly reduced after protein A exposure indicating the majority of IgE was bound by IgG. * $P < 0.05$. The horizontal line represents the mean.

subjected to protein A chromatography, and analysed. Prior to protein A, the pooled CB serum contained a mean total IgE level of 171 (range 102–335) ng/mL, determined using the m107 anti-hIgE assay (Fig. 6). Following protein A, only 6% (range 3–10%) of the IgE was recovered ($P = 0.021$) (Fig. 6), which coincided with removal of > 99% of the IgG anti-IgE/IgE ICs determined using the m107 IC assay (data not shown). Total IgE levels determined using the rFcεRIα anti-hIgE assay were also reduced following protein A; however,

several of the pooled specimens were at the lower limit of detection prior to exposure (data not shown).

Binding of cord blood IgE to FcεRI in infants of allergic and non-allergic mothers

To investigate the possibility that CB IgE (in the form of IgG anti-IgE/IgE ICs) could have differential ability to bind FcεRI based on maternal allergic status, we attempted to determine CB IgE levels using the rFcεRIα-based IgE assay. Levels of CB IgE measured using the rFcεRIα-based IgE assay were at the lower limit of detection for the majority of CB samples, precluding a comparison between infants of allergic and non-allergic mothers (data not shown). Thus, we quantified percentages of CB basophils with surface-bound IgE relative to levels of CB IgE as an indicator for the ability of CB IgE to bind FcεRI. Seven CB samples from infants of allergic mothers and 11 CB samples from infants of non-allergic mothers had adequate numbers of basophils and sufficient CB IgE (determined via the ImmunoCAP) to perform this analysis. In these samples, there was no difference in levels of CB IgE between infants of allergic and non-allergic mothers [median 1.56 (range 0.52–2.67) vs. 1.02 (0.54–3.19) kU/L, $P = 0.39$] (data not shown). In infants of allergic mothers, there was a significant correlation between CB IgE levels and percentages of CB basophils with bound IgE ($r = 0.77$, $P = 0.04$) (Fig. 7a). In contrast, there was no significant correlation between CB IgE levels and

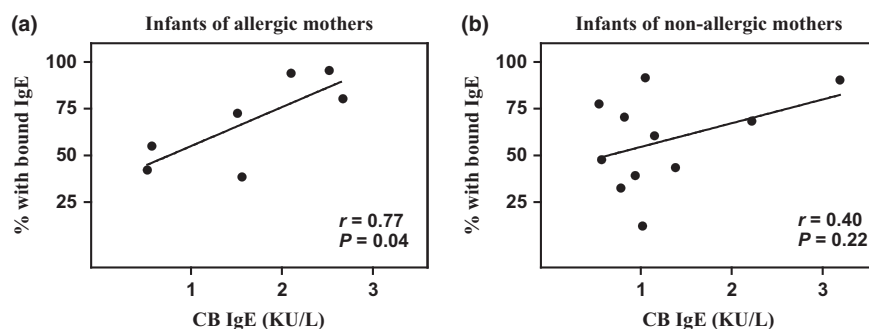


Fig. 7. CB IgE is predictive of basophil-bound IgE in infants of allergic mothers, but not in infants of non-allergic mothers. Data represent CB samples from 7 infants of allergic mothers and 11 infants of non-allergic mothers, containing ≥ 0.5 kU/L total serum IgE (determined by ImmunoCAP) and a minimum of 200 basophils. Flow cytometry was used to calculate the percentages of CB basophils with surface-bound IgE relative to an isotype control.

percentages of CB basophils with bound IgE in infants of non-allergic mothers ($r = 0.40$, $P = 0.22$) (Fig. 7b).

Discussion

Here we report essentially equivalent maternal and CB serum concentrations of IgG anti-IgE/IgE ICs in a cohort of allergic and non-allergic mothers/infant dyads and demonstrate that hFcRn facilitates the transepithelial transport of IgE in the form of IgG anti-IgE/IgE ICs. These data, along with our discovery that the majority of IgE in CB sera is bound by IgG, strongly suggest that maternal IgE is transported across the placenta via FcRn-mediated transcytosis of IgG anti-IgE/IgE ICs. These findings challenge the widespread perception that maternal IgG is the only antibody isotype transported across the placenta and establish a new paradigm to study perinatal aspects of early allergic sensitization. CB IgE existing primarily in the form of IgG anti-IgE/IgE ICs may explain the inconsistent results observed when using CB IgE as a predictor for future allergic disease [30–32] and the variable correlations observed between maternal and CB serum IgE concentrations [33–36].

It is well established that IgG anti-IgE/IgE ICs are present in the sera of allergic and non-allergic individuals [16, 17, 37]. In the present study, we demonstrated that IgG anti-IgE/IgE ICs are also present in the sera of pregnant women and their newborn infants at essentially equivalent concentrations, which is highly suggestive of placental transmission. Because maternal IgG, but not free IgE, is transported across the human placenta via FcRn-mediated transcytosis [29, 38], we investigated the ability of hFcRn to bind and transport IgE in the form of IgG anti-IgE/IgE ICs in a well-validated *in vitro* model system [18, 19]. Our initial studies using omalizumab to generate IgG anti-IgE/IgE ICs demonstrated hFcRn has low affinity for this humanized IgG₁ anti-hIgE_{CE3}. These results are consistent with

the observation that hFcRn binds omalizumab less strongly than other humanized IgGs [39]. Thus, we used polyclonal rIgG anti-hIgE to form rIgG anti-hIgE/hIgE ICs, and in agreement with published literature [19, 26] found that rabbit IgG has strong affinity for hFcRn. Furthermore, using polyclonal rIgG anti-hIgE more closely recapitulates the human situation where multiple IgG anti-IgEs are present, directed against different IgE epitopes with various affinities. hIgE in the form of rIgG anti-hIgE/hIgE ICs, bound to hFcRn and was transcytosed across polarized epithelial cells in an hFcRn-dependent fashion. Taken together, these data are highly suggestive that hFcRn can bind and transfer maternal IgE across the placenta as IgG anti-IgE/IgE ICs.

Interestingly, following FcRn-mediated transcytosis of rIgG anti-hIgE/hIgE ICs, hIgE was detected in output solutions using m107 or rFcεRI α anti-IgE as capture reagents and biotinylated antibodies directed against hIgE. Following protein A chromatography, essentially all of the hIgE was removed, indicating that the majority was in the form of rIgG anti-hIgE/hIgE ICs. The explanation for this may be that the anti-hIgEs were directed against epitopes that differed from those already occupied by rIgG anti-hIgEs. Alternatively, the anti-hIgEs could have displaced rIgG anti-hIgEs because of competing affinities. Regardless of the mechanism, these results suggest that IgE in CB serum might similarly exist as IgG anti-IgE/IgE ICs. Indeed, further supporting this notion was our finding that the bulk of IgE in pooled CB serum was removed following exposure to protein A. To our knowledge, this is the first report demonstrating that the vast majority of IgE in CB sera is complexed to IgG. The low levels of monomeric IgE recovered following exposure to protein A may represent natural IgE produced *in utero* in the absence of classical MHC II cognate help [40].

The ability of maternal IgG anti-IgE/IgE ICs to predispose towards allergy development in the child is of

interest, as the molecular basis for maternal imprinting of allergic risk remains unclear [35, 41–43]. Upon entering the fetal circulation, IgG anti-IgE/IgE ICs may bind to cells expressing FcεRs (e.g. FcεRI or FcεRII), FcγRs, or co-aggregate FcεRs to FcγRs [44]. Factors that influence receptor binding include the epitope specificity of the IgG anti-IgE [13], the ratio of IgG anti-IgE : IgE (unpublished results), or the IgG anti-IgE subclass which can influence binding to activating or inhibitory FcγRs [45, 46]. Our results indicate a fraction of the total pool of IgG anti-IgE/IgE ICs in maternal and CB serum retains the capacity to bind FcεRI. This is further supported by our experiments utilizing FcRn-expressing MDCK cells, which demonstrate that a significant proportion of transcytosed rIgG anti-hIgE/hIgE ICs bind to rFcεRIα. In mice, IgG anti-IgE_{Cε4}/IgE ICs absorbed across the intestine via FcRn retain the capacity to induce rat basophil leukaemia cell degranulation [13]. Thus, it is plausible to hypothesize that maternal IgG anti-IgE/IgE ICs with the ability to bind FcεRs might have yet to be identified functional effects in the fetus. In adults, it is known that allergen-specific IgE localized to the surface of FcεRI-expressing antigen-presenting cells serves as an antigen-focusing agent that optimizes the capacity to elicit T cell responses [47]. Our unpublished work has demonstrated IgG anti-IgE/IgE ICs to be present on the surface and within CB basophils and myeloid dendritic cells (DCs). It remains to be determined whether biologically active IgG anti-IgE/IgE ICs (e.g. IgG bound to IgE at Cε4) localized to FcεRI on fetal or neonatal cells could provide an adjuvant-like stimulus to promote *in utero* or early postnatal T cell priming. The possibility that intracellular IgE recently reported in adult myeloid DCs represents IgG anti-IgE/IgE ICs should also be considered [48].

Our analysis of IgG subclasses is relevant because it demonstrates the potential for differential regulation of neonatal immunity based on maternal history of allergic disease [35, 41, 49]. Despite total levels of IgG anti-IgE/IgE ICs being similar in allergic and non-allergic pregnant women, levels of IgG₁-containing ICs with the capacity to bind FcεRI were significantly greater in the allergic group. In addition, IgG₁- and IgG₃-containing ICs were present at the highest concentrations in CB sera, implying that these subclasses are efficiently transported across the placenta when bound to IgE. IgG₁ and IgG₃ display high affinity binding to Fcγ-activating receptors [50], and IgG₃ antibodies are the most common subclass found bound to IgE on the surface of adult basophils [16]. It remains to be determined whether IgG₃ antibodies are the most abundant subclass bound to IgE on CB basophils [20]. Interestingly, levels of maternal IgG₁- and IgG₃-containing ICs that bind to FcεRI were predictive of CB IgE levels only in infants born to allergic women. Traditional IgE assays such as

the ImmunoCAP do not distinguish IgE that is bound or not bound by IgG anti-IgE at Cε3 [22]. Thus, it is possible that a significant portion of CB IgE found in infants of non-allergic mothers is bound by IgG anti-IgE at Cε3, resulting in reduced binding to FcεRI. Our recent report demonstrating that IgE expression on CB basophils is greater in infants of allergic as compared to non-allergic mothers, despite similar levels of CB IgE, is supportive of this possibility [20]. In the current study, we further established that levels of CB IgE were predictive of basophil-bound IgE in infants of allergic mothers, but not in infants of non-allergic mothers. Collectively, these data suggest that the ability of CB IgE (in the form of IgG anti-IgE/IgE ICs) to bind FcεRI-expressing fetal cells can be influenced by maternal allergy. The low sensitivity of the rFcεRIα-based IgE assay precluded further characterization of CB IgE, perhaps due to natural IgG anti-IgE hindering additional IgE binding sites.

Following transport across the placental syncytiotrophoblast, maternal IgG anti-IgE/IgE ICs may encounter resident Hofbauer cells, the placental macrophages which express all FcγRs [51], and function to trap ICs [52]. This may explain the finding of IgE in Hofbauer cells regardless of maternal allergic status [53]. Our finding of essentially equivalent concentrations of IgG anti-IgE/IgE ICs in CB and maternal serum suggests the majority escape trapping by Hofbauer cells, are recycled back to the surface and released into the fetal circulation or bound to a saturable receptor with the excess being degraded. In the vascular endothelium, FcRn functions to rescue IgG from lysosomal degradation and recycles IgG back to the circulation, thereby prolonging serum half-life [54–56]. FcRn is expressed in human antigen-presenting cells [57, 58], suggesting FcRn might represent the saturable receptor hypothesized above. Although it has not been demonstrated that Hofbauer cells express FcRn, given the broad expression in antigen-presenting cells of dendritic and myeloid origin, this is likely to be the case [59].

The biologic functions of IgG anti-IgE appear diverse, with the potential to induce or suppress IgE-mediated inflammatory responses [60–62]. In our analysis of pregnant women with symptomatic allergic disease, the production of IgG anti-IgE (and generation of ICs) increased in proportion to rising serum IgE levels. Despite this positive correlation, and the finding of higher total IgE in the allergic group, levels of IgG anti-IgE were similar between allergic and non-allergic pregnant women suggesting a potential limit in the quantities of IgG anti-IgE that are produced and thus the regulatory capacity of this pathway. We speculate that as IgE production increases, IgG anti-IgE/IgE ICs are generated that retain the capacity to bind FcεRI [13, 63]. At even higher IgE production rates, the ability of IgG anti-IgE to bind the total pool of IgE becomes sur-

passed, resulting in elevated levels of monomeric IgE and increased risk for IgE-mediated pathology. Additional studies are required to confirm this possibility and to further define structural characteristics that differ between individuals with and without a history of allergy [64]. It should be noted that our IgG anti-IgE assay used adjacent wells on the same ELISA plate coated with OVA and predetermined amounts of mouse IgG₁ anti-OVA as the reference standard. Similar distributions of IgG anti-IgE in maternal and CB sera may be attained using ELISA plates coated with hIgE and predetermined amounts of omalizumab as the reference standard (unpublished results).

As a method of assessing maternal blood contamination of CB samples, we used elevated CB serum IgA. While this is an established method [21], our data suggest maternal autoantibodies directed against IgA could transport maternal IgA into the fetal circulation by a similar mechanism. While we appreciate this limitation, the observed correlation between maternal and CB serum levels of IgG anti-IgE/IgE ICs argue strongly in favour of active transport as opposed to leakage of trace amounts of maternal blood into the fetal circulation. In addition, while significantly more pregnant women smoked tobacco in our allergic group, the strong correlation between maternal and infant serum levels of ICs in both allergic and non-allergic mother/infant dyads implies that smoking tobacco has a negligible effect on maternal IC transmission.

In summary, our study details a novel mechanism for the placental transport of maternal IgE and suggests the

majority of CB IgE originates in the mother as IgG anti-IgE/IgE ICs. The ability of maternal-derived IgG anti-IgE/IgE ICs to modulate fetal or neonatal immune responses and influence risk for future atopy requires additional study.

Acknowledgements

The authors thank Lynn Puddington and Michelle Cloutier for their guidance and mentorship, Justin Radolf for his critical evaluation of the manuscript, and Kristi Baker for her advice in performing the MDCK transcytosis assay. We thank Jennifer Moller and Amanda Augeri for their assistance in recruiting subjects and collecting biologic specimens. We thank Derry Roopeian for suggesting the FcRn blocking experiments using DVN24 and providing the antibody, and Robert Hamilton for suggesting the subclass-specific IC assays and providing the necessary antibodies. We also thank members of the Obstetrical Staff at Hartford Hospital for their support of this project. This work was supported by the National Institutes of Health (NIH): K08AI071918 (to Adam P. Matson), NIH DK53056 (to Richard S. Blumberg), and by funds made available through a Connecticut Children's Medical Center Young Investigator Award (also to Adam P. Matson).

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. The epitope specificity of IgG anti-hIgE impacts the binding of hIgE to rFcεRIα.

Fig S2. Omalizumab inhibits the binding of maternal IgG anti-IgE/IgE ICs to rFcεRIα.

Fig S3. Relative fluorescence intensities for the binding of rIgG anti-hIgE, RhoGAM, and omalizumab to hFcRn-expressing MDCK cells.

Table S1. Maternal and neonatal characteristics based on maternal history of allergic disease.