Reduced soluble CD14 levels in amniotic fluid and breast milk are associated with the subsequent development of atopy, eczema, or both

Catherine A. Jones, PhD,[a] Judith A. Holloway, PhD,[a] Eleanor J. Popplewell, BSc,[a] Norma D. Diaper,[a] John W. Holloway, PhD,[b] Gillian H. S. Vance, MRCP,[a] Jill A. Warner, PhD,[a] and John O. Warner, MD[a] *Southampton, United Kingdom*

Background: Exposure to various microbial products in early life reduces the risk of atopy. Such exposure induces downregulation of T_H2 allergy-biased responses by means of pattern recognition molecules, such as CD14, an LPS receptor.
Objective: We sought to determine whether infant and maternal levels of soluble CD14 (sCD14) are associated with the atopic outcomes of infants.
Methods: Levels of sCD14 in plasma, amniotic fluid, and breast milk were measured with a specific ELISA in different cohorts. Expression of toll-like receptors in the fetal gut was examined by using RT-PCR.
Results: Soluble CD14 levels increased during fetal development and postnatally, attaining adult levels by around 4 months of age, with an overshoot of adult levels from 6 months of age. There was no difference in plasma sCD14 levels at birth of children with a high compared with those with a low risk of development of atopy. Amniotic fluid sCD14 levels at midgestation (16-17 weeks) were significantly lower when the child was subsequently atopic ($P < .05$). Soluble CD14 levels in breast milk collected 3 months postpartum were significantly lower in children with eczema at 6 months of age, irrespective of whether they were atopic ($P = .003$). Transcripts for toll-like receptor 4, which would enable transmembrane signaling for LPS/sCD14 complexes, were expressed within fetal gut and skin.
Conclusion: Exposure to reduced levels of sCD14 in the fetal and neonatal gastrointestinal tract is associated with the development of atopy, eczema, or both. Thus the exogenous supply of sCD14 might influence immunologic reactivity both locally and systemically in early life and thereby influence disease outcome. (J Allergy Clin Immunol 2002;109:858-66.)

Key words: *Soluble CD14, amniotic fluid, breast milk, eczema*

Abbreviations used
sCD14: Soluble CD14
TLR4: Toll-like receptor 4

One explanation for the increasing prevalence of atopic diseases in the developed world is the hygiene hypothesis. Not only is there a credible immune mechanism to explain the hypothesis, but diverse influences on hygiene have been associated with a higher prevalence of atopy.[1] Most studies have focused on the inverse relationship between the prevalence of infections and atopy, but gastrointestinal flora might be more important.[2] Differences have been shown in the microflora from the feces of allergic compared with nonallergic infants,[3,4] and alterations in gut microbial flora might explain both the inverse relationship between exposure to farm animals[5-7] and the positive association with antibiotic use in early life.[8,9] A recently published intervention study showed that use of a probiotic lactobacillus in newborns to prevent atopy and atopic disease reduced the prevalence of atopic eczema, but skin test response positivity and total and specific IgE levels remained unchanged.[10] Thus the probiotic affected the manifestation of atopic disease but not atopy itself.

Animal models indicate that antigen exposure in a germ-free environment favors the development of T_H2 responses,[11] which might explain why the newborn shows T_H2-skewed reactivity.[12] The acquisition of commensal flora might affect upregulation of T_H1 reactivity and thereby affect downregulation of T_H2 allergy-promoting responses. This immune switching is critical during early postnatal life, when exposure to high levels of antigen first occurs, because the response that evolves is likely to determine life-long reactivity to that antigen. Indeed, lack of rapid postnatal maturation of T_H1 reactivity (IFN-γ production) and concomitant consolidation of T_H2 reactivity (IL-4 and IL-13) occurs in infants who later become atopic.[13]

The interaction of cells with bacterial products is mediated by evolutionary conserved molecules termed

From the Divisions of [a]Infection, Inflammation and Repair and [b]Human Genetics, School of Medicine, University of Southampton, Southampton.
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Reprint requests: Catherine A. Jones, PhD, Allergy and Inflammation Sciences, Level G Mailpoint 803, Southampton General Hospital, Southampton, UK, SO16 6YD.

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TABLE I. Cohorts used and sources of all samples analyzed in the study

Study number*	Samples	Age at clinical assessment†	Complete or ongoing
Study 1	Serial plasma at birth (cord blood), 6 mo, 1 y, and 5 y of age; high-risk cohort	6 mo and 1, 2, 3, 4, and 5 y	Complete
Study 2	Amniotic fluid at diagnostic amniocentesis (16-18 wk of gestation); not selected on family history of atopy	1 y	Ongoing
Study 3	Breast milk at 3 mo postpartum; high-risk cohort	6 mo	Ongoing
Study 4	Plasma at approximately 1, 2, 4, 8, or 16 wk of age; high-risk cohort	Not part of this study	Ongoing
Study 5	Archived fetal plasma and tissue samples; term amniotic fluid (>37 wk gestation); adult plasma; not selected on the basis of family history of atopy	Not done	Not done

*Study number as referred to in the text and Figure legends.
†Clinically assessed for atopy (skin prick test), eczema, and asthma (as described in the "Methods" section).

pattern-recognition receptors.[14] CD14 (an LPS receptor) is one of the best characterized. CD14 lacks an intracytoplasmic signaling domain,[15] and toll-like receptor (TLR) 4 enables transmembrane signaling in response to LPS.[16,17] Soluble CD14 (sCD14) facilitates the interaction of membrane $CD14^-$ cell populations, such as epithelial cells and dendritic cells, with LPS.[18] A polymorphism in the promoter region of the DNA encoding CD14 has been described to be associated with reduced levels of circulating sCD14, which, in turn, are inversely correlated with total IgE levels.[19] This polymorphism has also been associated with a more severe allergic phenotype in Dutch adults.[20] We investigated the hypothesis that the newborn is ill-equipped to respond to microbial products and relies on an exogenous supply of factors, such as CD14, to compensate for this shortcoming and that an inadequate supply of such factors is associated with the development of disease.

METHODS

Source of samples

Samples from discrete cohorts were used, and these are summarized in Table I. All samples and data were selected on the basis of availability, together with complete outcome information from the subjects when this was required.

Blood, amniotic fluid, breast milk, and tissue samples

Fetal blood was collected by means of umbilical vein or cardiac puncture from fetuses delivered for medical reasons by using prostin (study 5). Umbilical cord blood was collected by means of umbilical vein puncture from neonates delivered preterm or at term (study 5) and by means of venipuncture at approximately 1, 2, 4, 8, and 16 weeks of age (study 4) and longitudinally at 6 months, 1 year, and 5 years of age (study 1). Blood was also collected from adults (healthy laboratory control subjects with no evidence of infection). All blood was collected into lithium heparin, and plasma was prepared, filtered, placed in aliquots, and stored at –80°C until analysis. Amniotic fluid and peripheral blood were collected from women undergoing diagnostic amniocentesis at 16 to 17 weeks' gestation (midgestation, study 2) who were found to have a normal fetus and continued the pregnancy to term, as well as from women undergoing elective cesarean section at term (late gestation). Breast milk was collected by means of expression at 3 months postpartum (study 3). Defatted, cell-free breast milk was prepared by means of centrifugation (at 900*g* for 20 minutes at 4°C). Samples of human fetal gut and skin were obtained from suction or dilatation-evacuation terminations through the MRC tissue bank (study 5). Gestational age (last menstrual period) was determined on the basis of foot length. Tissue was snap-frozen in liquid nitrogen and stored until use. The studies were approved by the Southampton and S.W. Hants Joint Research Ethics Committee and fulfilled the requirements of the Polkinghorne Committee report on the research use of fetuses and fetal tissues.

Soluble CD14 ELISA

Soluble CD14 was measured according to the manufacturer's instructions (R&D Systems). Plasma samples were assayed at a dilution of 1:200, amniotic fluid at 1:2, and breast milk at 1:4000 in the dilution buffer provided.

Clinical evaluation (studies 1, 2, and 3)

Newborns with one or more atopic first-degree relatives validated by means of positive skin prick test responses to one or more common allergen were defined as high-risk babies, whereas low-risk babies lacked any such family history. The subjects in study groups 1, 3, and 4 came from high-risk families only, whereas those in group 2 were recruited because the mother was undergoing diagnostic amniocentesis (the main indication being maternal age). At each clinical visit, the children were weighed and measured, and a physical examination was performed. Skin prick testing to *Dermatophagoides pteronyssinus*, *Felix domesticus*, grass pollen mix, tree pollen mix, cow's milk, hen's egg, a negative control (allergen diluent), and a positive control (10 mg/mL histamine, ALK) was conducted on each child. A child was considered atopic if a wheal of 3 mm or larger was observed in response to any of the allergens in the presence of an appropriate response to the positive and negative controls. Eczema was defined as an erythematous papulovesicular chronic skin eruption with dry skin, itching, and typical distribution (cheeks, torso, and extensor surfaces of limbs in infants <6 months of age and skin flexures in older infants) and was considered atopic eczema if the child had a positive skin prick test response. Asthma was defined as 3 separate episodes of nocturnal cough causing sleep disturbance lasting for at least 3 consecutive nights, 3 discreet episodes of wheezing separated by at least 7 days in which other respiratory conditions had been excluded, or both.[21]

RT-PCR

RNA was extracted by using RNase-free DNase treatment with the RNeasy total RNA isolation system, as directed by the manufacturer (Qiagen Ltd). First-strand cDNA synthesis was performed

with 0.5 μg of total RNA in a 20-μL reaction by using the Omniscript reverse transcriptase kit (Qiagen Ltd) primed with oligo T_{17} (AGC, Sigma Genosys), as recommended by the manufacturer.

PCR reactions were performed in a total reaction volume of 25 μL with 1 μL of cDNA in 1× reaction buffer, with 1.5 mmol/L $MgCl_2$, 200 mmol/L deoxyribonucleoside triphosphate, 0.2 μmol/L of each primer, and 0.625 U of Taq DNA polymerase (Sigma). Amplification was performed for 40 cycles at an annealing temperature of 59°C with the primers for TLR2 forward 5′-AACAGGCTGCATTCCCAAGAC-3′ and reverse 5′-GACATTCCGACACCGAGAGG-3′ and for TLR4 forward 5′-AGCCCTGCGTGGAGACT-3′ and reverse 5′-GCTCTGATATGCCCCATCTT-3′. For β-actin, amplification was performed as above but for 30 cycles at an annealing temperature of 54°C with the primers for β-actin forward 5′-TGATATCGCCGCGCTCGT-3′ and reverse 5′-CTCGGCCGTGGTGGTGAA-3′. Products were resolved on a 2% agarose gel, stained with ethidium bromide, and imaged with a fluorimager 595 (Molecular Dynamics).

Statistical analysis

Differences between groups were compared nonparametrically by using Kruskall-Wallis and Mann-Whitney *U* tests (SPSS, Version 8.0).

RESULTS

Maturation of sCD14 levels

Samples from studies 4 and 5 were used to examine the natural maturation of circulating sCD14 levels. Fetal-neonatal plasma (study 5) sCD14 levels increased significantly with gestational age but remained significantly lower at term than those in adults (Fig 1, *A*). Postnatal plasma samples were only available from high-risk children (studies 1 and 4). Maturation of sCD14 levels continued postnatally, with adult levels achieved by 4 months of age. However, at 6 months, 1 year, and 5 years of age, levels were significantly greater than those in adults (Fig 1, *B*). These children are undergoing ongoing clinical assessment.

Circulating sCD14 and atopic outcome

A comparison of plasma sCD14 levels in high-risk verus low-risk children was undertaken with the birth samples collected from the children from study 1. Levels of sCD14 in cord plasma from newborns at risk for atopic disease did not differ significantly from those at low risk (Fig 2, *A*). The high-risk children have undergone clinical assessment up to 5 years of age, and therefore disease outcome was available for this cohort. The high-risk children from study 1 were divided into 3 groups on the basis of clinical symptoms and skin prick test responses to age 5 years: (1) no symptoms of asthma and negative skin prick test response at all time points tested (atopy never); (2) clinician-diagnosed asthma and positive skin prick test response (to inhalant allergens, food allergens, or both) at age 2 and 5 years (persistent atopy); and (3) positive skin prick test response at 2 years of age but negative skin prick test response at 5 years of age and without asthma (transient atopy). Cord blood (birth) levels of sCD14 were significantly higher ($P < .05$) in the children with transient atopy when compared with those in the never atopic group or the group with persistent atopy (Fig 2, *B*). There was no significant difference between any of the clinical groups in plasma sCD14 levels at 6 months, 1 year, and 5 years of age (data not shown).

Alternative sources of sCD14

Amniotic fluid sCD14 levels were significantly higher in midgestation (16-17 weeks of gestation) compared with those in late gestation (>37 weeks; $P < .01$, Mann-Whitney *U* test; Fig 3, *A*). Levels of sCD14 in midgestation amniotic fluid were significantly lower in infants who were subsequently atopic (positive skin prick test response at 1 year of age and eczema) compared with those in infants who were nonatopic (negative skin prick test response and no eczema; $P < .05$, Mann-Whitney *U* test; Fig 3, *B*; study 2). We have not included a group with eczema and negative skin prick test responses because few subjects fit these criteria at the time of analysis. Clinical data on this cohort were only available at 1 year of age, and therefore this cohort could not be further subdivided on the basis of asthma phenotype, as we had done for study 1. There was no correlation between maternal plasma and amniotic fluid levels of sCD14 in matched samples (n = 20, $r = –0.165$, $P = .376$).

Levels of sCD14 in defatted and cell-free breast milk from subjects in study 3 had extremely high levels of sCD14 (4.74-43.56 μg/mL). Clinical outcome at 6 months of age was available for the children born into this cohort. Initially, we compared atopic (positive skin prick test response) and nonatopic (negative skin prick test response) children but did not find a difference. However, on comparing children with eczema with those without eczema, we found that levels were significantly lower for infants who had eczema at 6 months of age, irrespective of whether they had positive skin prick test responses (Fig 4). Because this cohort is subject to ongoing clinical assessment, we were not able to undertake an analysis on the basis of asthma phenotype. These data will be obtained at ongoing clinical evaluations as the cohort ages through the first 5 years of life.

Toll-like receptors

Transcripts for TLR4 and TLR2 were detected in whole tissue extracts of fetal gut (n = 4) and skin (n = 3) at all the gestational ages examined (Fig 5).

DISCUSSION

To study the potential contribution of sCD14 to the development of atopic diseases in infancy, we took advantage of various samples from a number of cohorts collected by our group over the past few years. One of these cohorts (study 1) has completed clinical assessment, whereas the remainder (studies 2, 3, and 4) are ongoing. Differences in the timing of clinical assessment referred to throughout reflect the different ages of the cohort being analyzed. Soluble CD14 was the focus of this study because it enables responsiveness to microbial

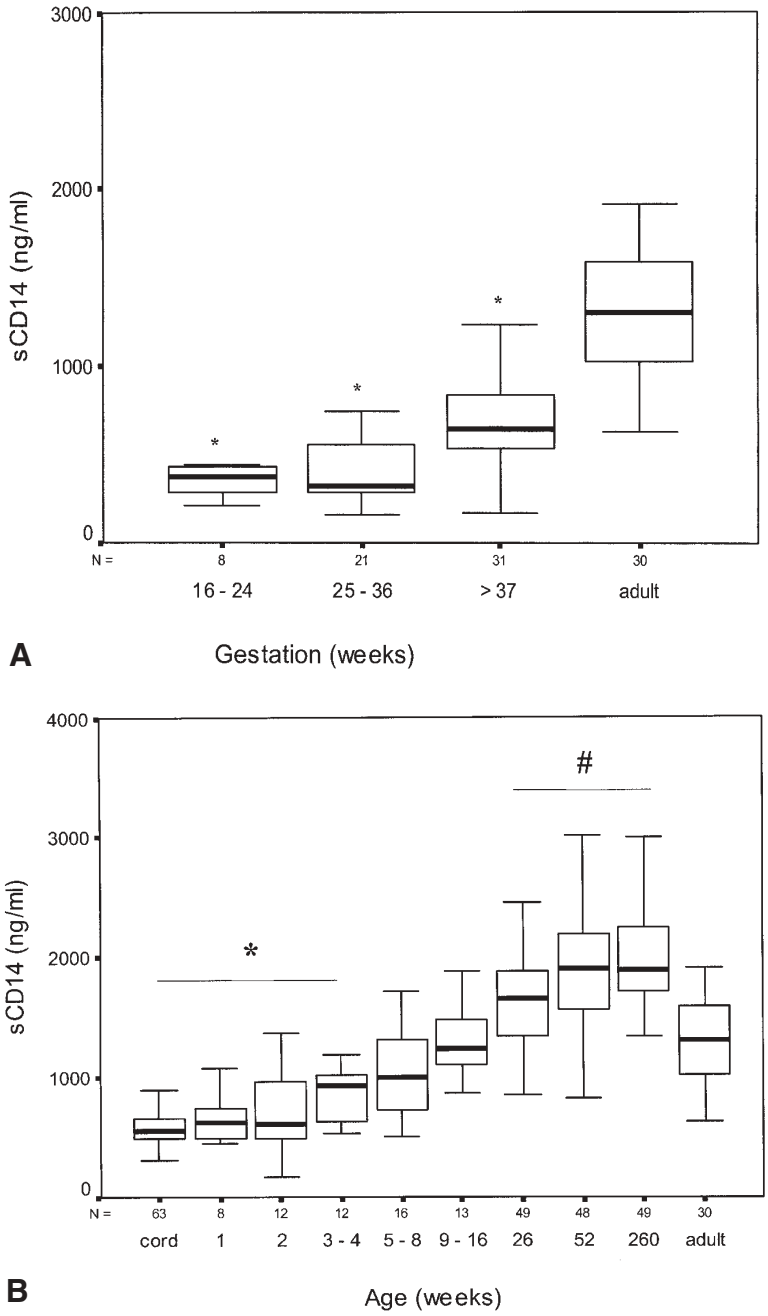

FIG 1. Maturation of plasma sCD14 levels. Plasma sCD14 was measured by means of specific ELISA in samples collected prenatally (study 5; **A**) and postnatally (study 4; **B**) and compared with levels in the adult (study 5). *Significantly lower than adult values; #significantly higher than adult values.

products, and birth is associated with transition from the germ-free to the conventional environment.

Circulating sCD14 (plasma)

First, maturation of circulating levels during prenatal and postnatal development was determined. Adult-like levels of plasma sCD14 were attained by 4 months of age, but there was an overshoot thereafter to 5 years of age (the oldest age studied). This phenomenon is seen for other immune parameters (eg, soluble IL-2 receptor[22]) and might relate to microbial exposure. For ethical reasons, the postnatal samples were from high-risk children, and a comparison of maturation with low-risk children is needed to clarify the natural ontogeny of changes in sCD14 levels and especially how this affects the response to bacterial products, particularly LPS.

The inverse relationship between circulating sCD14 levels and total IgE levels, which reached significance in

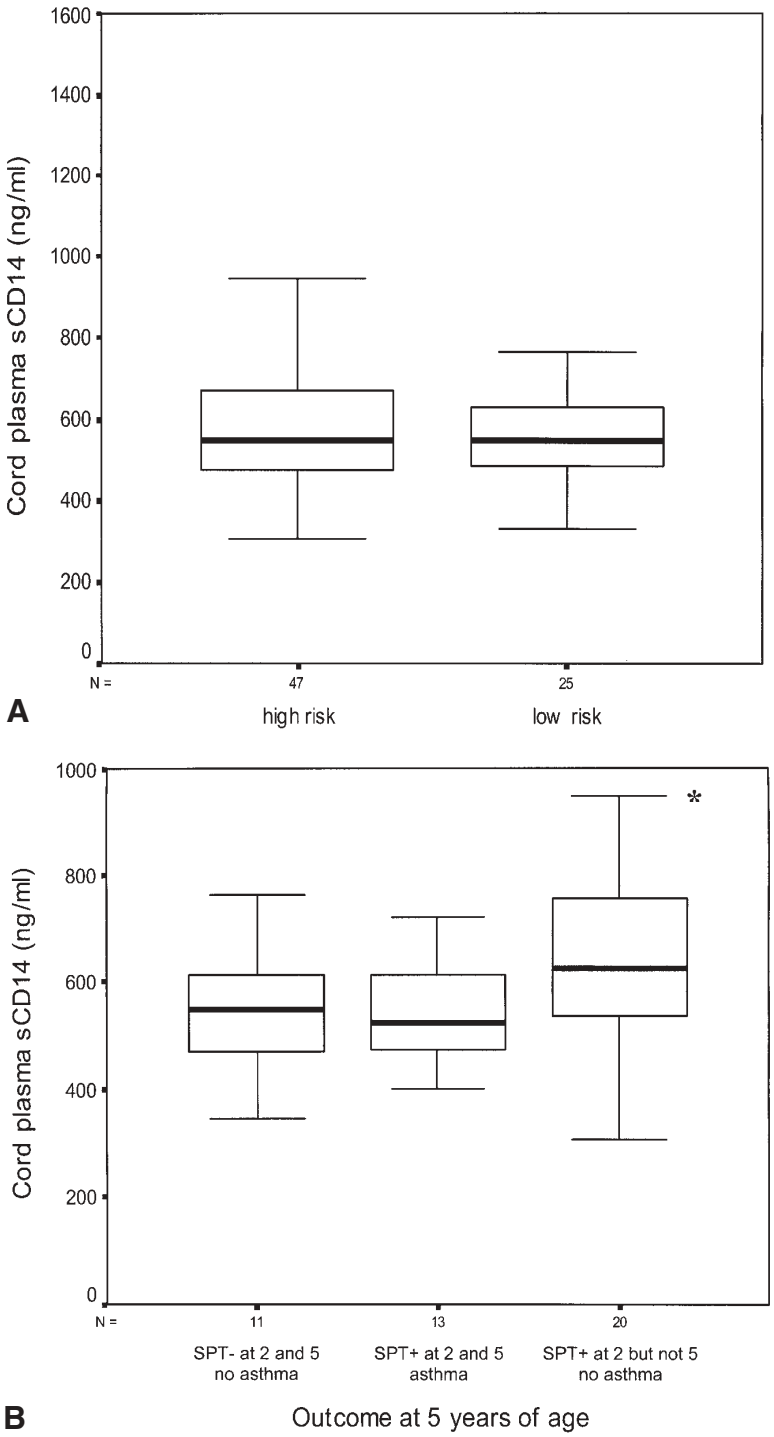

FIG 2. Plasma sCD14 levels and atopic disease outcome at 5 years of age. Soluble CD14 levels were measured at birth in cord plasma of high- and low-risk children **(A)** and children with clinically assessed outcome to age 5 (study 1; **B**). *SPT,* Skin prick test.

children with positive skin prick test responses to aeroallergens,[19] prompted us to hypothesize that circulating sCD14 levels would be reduced at birth in children who later become atopic. An initial analysis was conducted comparing umbilical cord blood plasma sCD14 levels of high- and low-risk infants, but there was no significant difference. Soluble CD14 levels at 6 months, 1 year, and 5 years of age were not associated with disease outcome at 5 years of age, and therefore we do not believe that infant circulating sCD14 is likely to contribute signifi-

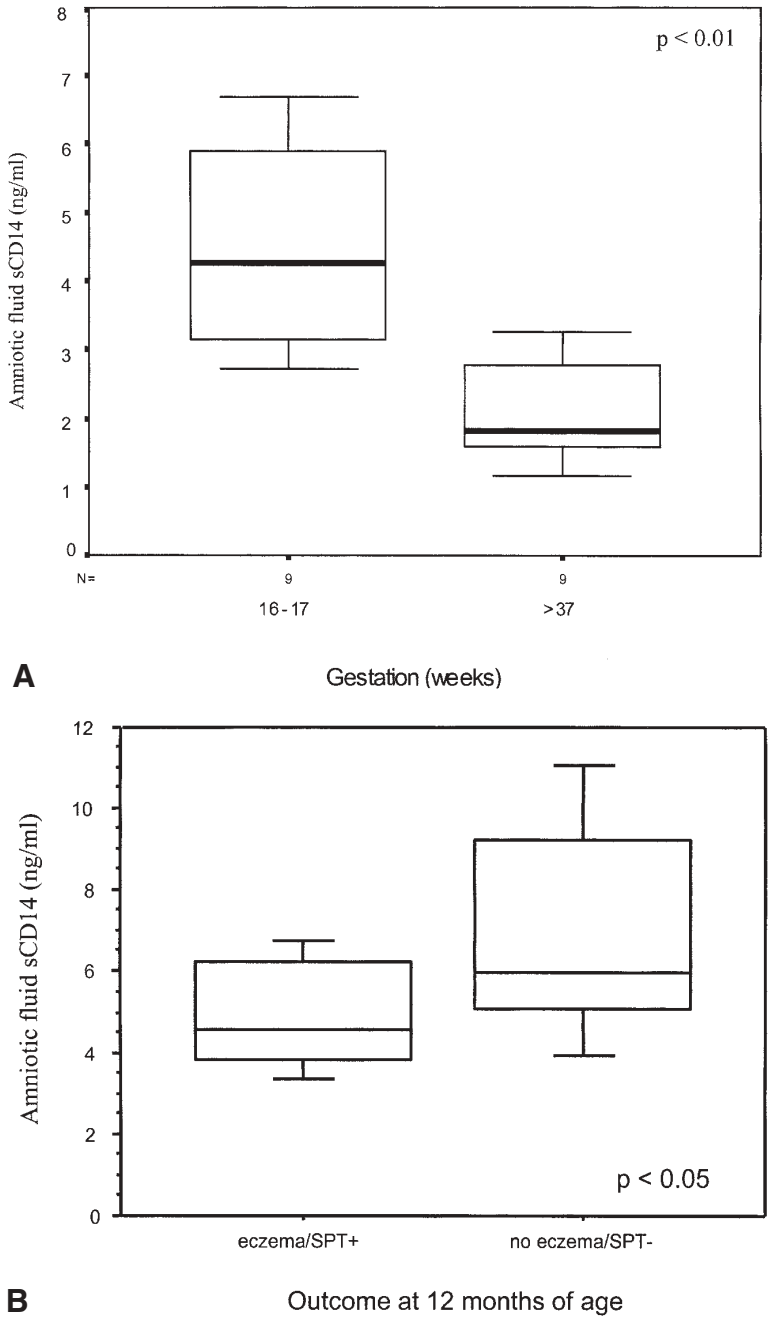

FIG 3. Alternative sources of sCD14. Levels of sCD14 were measured in amniotic fluid at 16 to 17 weeks of gestation and at term (>37 weeks of gestation; studies 2 and 5, respectively; **A**), and levels in amniotic fluid at 16 to 17 weeks of gestation were compared for children who were either atopic or nonatopic at 1 year of age (study 2; **B**). *SPT,* Skin prick test.

cantly to T_H2 to T_H1 switching at these time points. However, elevated sCD14 levels at birth in children with transient atopy might indicate that independent factors affected early sensitization in this subset of children and that higher sCD14 levels at birth facilitated earlier switching from T_H2 to T_H1 responses. The lack of difference in sCD14 levels at birth in children who will have atopic asthma corresponds with the increasing evidence that atopy and asthma have independent risk factors.[23]

Exogenous sCD14 (amniotic fluid and breast milk)

The initial observation of sCD14 and IgE/disease was made in 11-year-old children.[19] Any association between plasma sCD14 levels and atopy in early childhood might be masked by different rates of maturation. Because the gut flora are established in early childhood, when the child is first encountering numerous environmentally

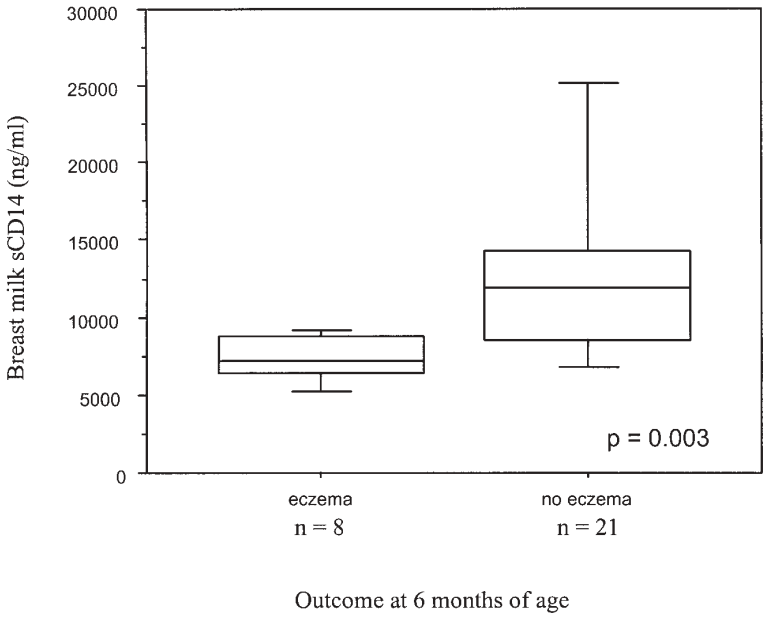

FIG 4. Breast milk sCD14 levels and disease outcome. Soluble CD14 levels in breast milk collected at 3 months postpartum by means of expression were compared on the basis of the development of eczema by the child, irrespective of skin prick test results at 6 months of age (study 3).

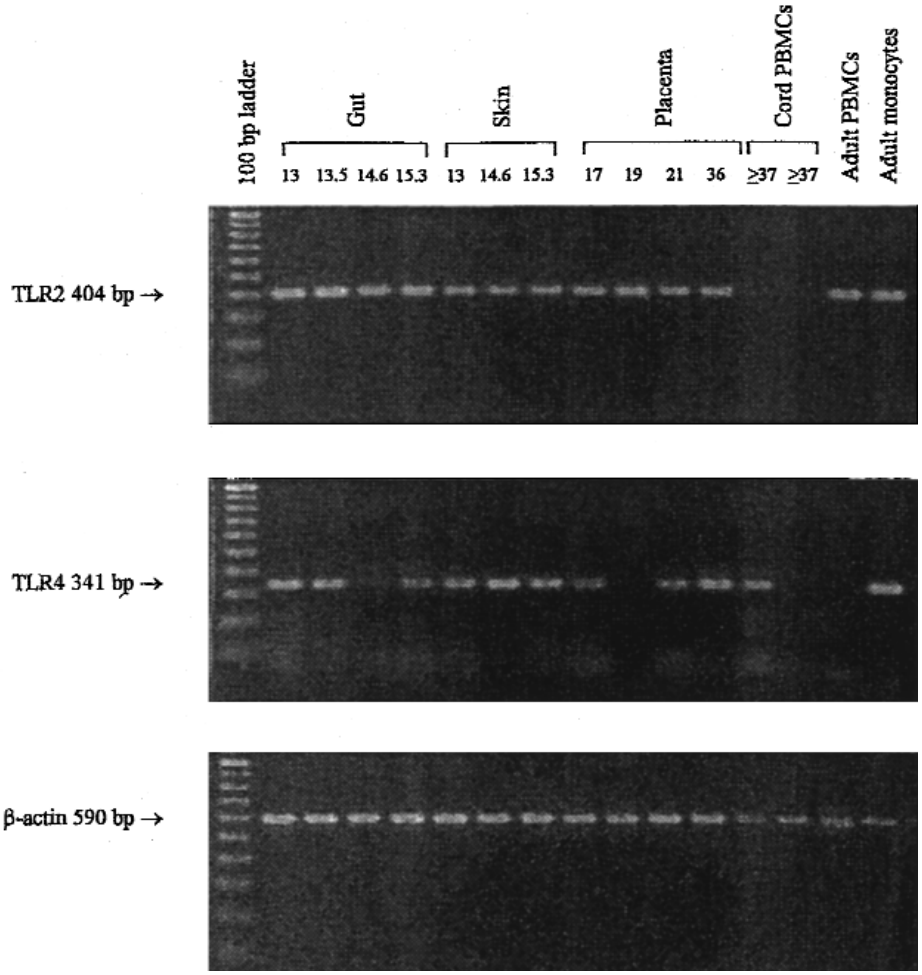

FIG 5. Expression of mRNA for TLR2 and TLR4 in fetal tissues. Whole tissue extracts of fetal gut and skin and placenta contained transcripts for TLR2 and TLR4 at all the gestational ages examined.

derived signals, altered sCD14 levels at mucosa surfaces would be more relevant at this time. Therefore we considered alternative sources of sCD14 to which the fetus-newborn would be exposed.

Soluble CD14 in term amniotic fluid has already been described,[24] and we found that levels were significantly higher in midgestation (16-18 weeks) than at term (>37 weeks). However, because the midgestation cohort of women were significantly older, the effects of maternal age on amniotic fluid levels at midgestation compared with those at late gestation should be considered. This will only become clear with a larger cohort of samples from women at midgestation. Using only those samples collected from women who had diagnostic amniocentesis at midgestation (study 2), we revealed that sCD14 levels were lower in the amniotic fluid of subsequently atopic children (positive skin prick test responses with eczema). There was no correlation with maternal circulating levels, implying that the fetal-placental unit might itself be the source. We speculate that because sCD14 can down-regulate IgE production in an LPS-independent fashion,[25] reduced sCD14 levels in amniotic fluid at this time might lead to higher IgE production by fetal gut B cells (present as early as 14 weeks of gestation)[26] and facilitate the development of atopy. However, to mediate its effects, sCD14 must deliver a complex of LPS/LPS-binding protein to the cell surface for transmembrane signaling through TLR4,[16,17] requiring a physical interaction between CD14 and TLR4.[27] Presumably, LPS-independent effects also require a physical interaction between sCD14 and TLR4. Therefore if amniotic fluid sCD14 is to mediate any effect in utero, TLR4 must be expressed. TLR4 and TLR2 (shown to be important in the response to products derived from gram-positive bacteria[28]) transcripts were found in all of the samples of fetal gut and skin examined. The expression of mRNA for TLR4 within fetal gut and skin at the same time as sCD14 is present in the amniotic fluid indicates that sCD14 could have bioactivity during fetal development, although only further investigation will clarify this. Although TLR4 is expressed in fetal gut tissue, there is very little expression of CD14,[29] and therefore the fetus might rely on exogenous sCD14 from the fetoplacental unit to facilitate normal immune development.

Breast milk contains very high levels of sCD14, which has been postulated to have a sentinel role enabling LPS-induced activation of membrane $CD14^-$ cells, such as intestinal epithelial cells, in the neonatal gut.[30] This would be important in controlling intestinal homeostasis on first encounter with a germ-laden environment. In an initial analysis there was no difference in sCD14 levels in breast milk at 3 months and atopic status of the child at 6 months of age. However, there was a significant association between reduced breast milk sCD14 levels and a diagnosis of eczema by 6 months of age, irrespective of skin prick test results. Thus a reduced ability to respond to the commensal flora might be associated with the IgE-independent development of eczema. In addition, the contribution of sCD14 to circulatory and transcellular phospholipid transport[31] makes it tempting to speculate that the differences in eczema outcome might reflect differing nutritional (phospholipid) provision to infants fed high versus low levels of sCD14 in breast milk. The association of reduced n-6 essential fatty acid metabolism with inflammatory skin diseases[32] supports such a postulate. Furthermore, a recent study that provided probiotics to the mother in late pregnancy and the newborn for the first few months of life noted a dramatic reduction in the incidence of eczema among the probiotic-fed group of infants, without any difference in IgE levels (total or specific) or skin prick test results.[10] This suggests that probiotics exert IgE-independent effects, perhaps by enhancing sCD14 production in breast milk or locally in the gut.

Despite the lack of correlation between circulating sCD14 levels in the first 5 years of life and the development of atopic disease, this study highlights that an exogenous supply of this molecule could affect disease outcome, although the IgE- and non–IgE-dependent consequences of this require elucidation. The lack of microbial exposure before birth might delay the response of the neonate, especially in the gastrointestinal tract, to microbial products from commensal and pathogenic microorganisms. From an evolutionary perspective, the potentially detrimental result of this would be overcome by providing the necessary effector molecules through amniotic fluid and breast milk. However, suboptimal levels, possibly reflecting CD14 promoter polymorphisms in the mother, which we are investigating currently, could influence the risk of development of various diseases in infancy.

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