

Impaired interleukin (IL)-4-associated generation of CCR4-expressing T cells in neonates with hereditary allergy risk

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Summary

Reduced microbial exposure in early life may contribute to the increase of atopic diseases in 'westernized' societies but the underlying mechanisms remain elusive. The objective of this study was to examine how exposure to bacterial lipopolysaccharide (LPS) during early antigen encounter might influence the maturation of neonatal lymphoid cells, and to define possible differences in this respect between neonates with high risk of allergy due to a family history (FH⁺) and controls with no apparent hereditary risk (FH⁻). Cord blood mononuclear cells from the FH⁺ or FH⁻ group were stimulated with pure LPS or β -lactoglobulin (β -LG) in the presence of LPS. T cell expression of chemokine receptors CCR4 and CXCR3 was determined by flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR). Cellular expression of interleukin (IL)-4 was analysed by quantitative RT-PCR, whereas interferon (IFN)- γ was analysed by both quantitative RT-PCR and immunoassay. Stimulation with LPS, or β -LG together with LPS, induced up-regulation of CCR4 ($P < 0.05$) and CXCR3 ($P < 0.05$). For CCR4, such up-regulation was related to the level of IL-4 produced by the same T cells ($r_s = 0.49$, $P = 0.03$), while CXCR3 expression was negatively correlated with the IL-4 levels ($r_s = -0.56$, $P = 0.02$). Compared with the FH⁻ group, the FH⁺ group showed a significantly lower capacity for generation of CCR4⁺ T cells (mean percentage of total T cells: FH⁺, 2.42% versus FH⁻, 5.74%; $P < 0.01$), whereas induction of CXCR3 and IFN- γ did not differ significantly between the two groups. When the immune system in early life encounters antigen together with LPS, the T cell potential for compartmentalized interaction with other immune cells might be increased by elevated CCR4- and CXCR3-expression levels. In neonates at hereditary allergy risk, this putative homeostatic mechanism could theoretically be jeopardized due to decreased up-regulation of CCR4. Conversely, Th1 responses to antigen in the presence of LPS did not appear to be reduced compared with controls.

Keywords: CCR4, cord blood, CXCR3, lipopolysaccharide, Th2 cytokines

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Introduction

The environmental conditions in which exposure to dietary or inhalent allergens takes place immediately after birth may have lifelong consequences for the clinical manifestation of immunological sensitization [1]. For instance, early life exposure to farm animals and unpasteurized milk [2], cats and dogs [3,4], a large number of older siblings [5] or a crowded living condition [6] appears to protect against atopic diseases. Also notably, changing to a

'westernized' lifestyle for people from the former East Germany has apparently led to an increase of such disorders [7,8]. Different degrees of early life exposure to microbes and their products most probably largely explains these variations in allergy prevalence [9–12]. Thus, the impact of microbial components on the immune system may 'educate' it to respond appropriately to innocuous soluble antigens such as allergens. However, the mechanism(s) behind such homeostatic immune regulation remains elusive.

The aim of the present study was to examine the potential impact of early life exposure to bacterial lipopolysaccharide (LPS) or endotoxin, together with the abundantly available cow's milk antigen β -lactoglobulin (β -LG), on T cell expression of the chemokine receptors CCR4 and CXCR3. These receptors are essential in the induction of adaptive immunity by mediating interaction between antigen-presenting dendritic cells and T cells, as well as by directing T cells to distant sites [13–15]. Also, because T cell competence results from interactions between environmental variables and genetic 'makeup', we aimed to analyse whether possible LPS effects are different in neonates with or without a family history of allergic disease. The obtained *in vitro* results suggested that this indeed could be the case.

Materials and methods

Study groups of neonates

Pregnant women were recruited consecutively at the Department of Obstetrics and Gynaecology at Ullevål University Hospital and Rikshospitalet University Hospital, Oslo. They filled in a checkbox questionnaire concerning family history (FH) of eczema, asthma or inhalant allergy, also including a physician's diagnosis of allergic disease and previous laboratory results of allergic diagnostic testing. Informed consent was given after the parents had received further information from the study paediatricians. Blood tests were drawn for total IgE and Phadiatop analysis (Pharmacia Diagnostics AB, Uppsala, Sweden), which is a screening test for IgE antibodies against some of the most important inhalant allergens in Norway, with a lower detection limit of 0.35 IU/l. Women eligible for the FH⁺ group had symptoms and/or a history of allergic disease, they were Phadiatop-positive and had levels of total IgE above 150 IU/l. Of the 11 families belonging to the FH⁺ group, seven also reported eczema and/or food or inhalant allergy in the father and/or sibling(s) of the newborn. The FH⁻ group comprised 11 families in which no allergic symptoms were reported in either parent. All neonates included in the study were born after 37 gestational weeks, and none had dysmorphism or birth asphyxia. The study protocol was approved by the Regional Ethics Committee.

Cell isolation and culture conditions

Immediately after birth, umbilical cord blood was drawn in sodium heparin VACUTAINER® CPT™ tubes and cord blood mononuclear cells (CBMCs) were isolated as outlined by the manufacturer (Becton Dickinson, Franklin Lakes, NJ, USA). Fresh cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated pooled human serum, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine (GibcoBRL, Paisley, UK). Cells were seeded at a density of 1.0×10^6 cells/ml and 1.5 ml per well in 24-well

flat-bottomed tissue culture trays (Corning Life Sciences Inc., NY, USA). Cell stimulation was performed with 0.45 µg/ml of LPS from *Escherichia coli* (L-8274; Sigma Aldrich, St Louis, MO, USA), 125 µg/ml of bovine β -LG (L0130; Sigma) or medium alone as a negative control. Only the first 13 samples were subjected to separate stimulation with both β -LG and purified LPS (in addition to medium control) because at that stage of the investigation we could observe a statistically significant difference between the two stimulatory conditions (see Fig. 2). Therefore, we subsequently focused our study on possible differences between the risk group (FH⁺) and the control group (FH⁻), limiting the stimulation of CBMCs to β -LG in the presence of its inherent LPS (see below), which showed the strongest effect on chemokine receptor up-regulation.

After 4 days of culture, CD3⁺ cells were isolated for RNA extraction (see below) from CBMCs stimulated with β -LG and inherent LPS or unstimulated controls. The cells were obtained from three wells by means of Dynabeads® M-450 CD3 (Dynal, Oslo, Norway), as recommended by the manufacturer. However, the culture times varied for the different analyses performed. Preliminary flow-cytometric analysis for chemokine receptor up-regulation and cell viability showed that day 5 in culture was preferable. Thus, for confirmation of receptor protein expression, chemokine receptor mRNA was analysed in isolated CD3⁺ cells on day 4 because the delay time between expression of protein and mRNA is in the range of 24 h. According to previous experience in our laboratory, the delay time between cytokine secretion and corresponding mRNA expression is also in the range of 24 h. Therefore, for determination of the effects that cytokines present in the medium during the last 24 h might have on up-regulation of chemokine receptors, cytokine mRNA in CBMC cultures was recorded on day 3, some 48 h before analysing chemokine receptor expression.

Endotoxin test

Endotoxin activity in the LPS and the β -LG preparation was determined as endotoxin units (EU)/ml based on the limulus amoebocyte lysate assay (BioWhittaker, Verviers, Belgium), according to the manufacturer's instructions.

Flow cytometry

The primary murine monoclonal antibodies used were anti-CCR4 (IgG1, produced as described in [16]) or anti-CXCR3 (IgG1, clone Ls77 1C6) (courtesy of D. P. Andrew and C. Mackay, Millennium Pharmaceuticals, Cambridge, MA, USA), or a concentration-matched irrelevant mouse IgG1 (clone MOPC-21; Sigma), followed by a secondary goat anti-mouse IgG1 antibody conjugated with PE (Southern Biotechnology Associates, Birmingham, AL, USA) and anti-CD3-Cy-Chrome (clone HIT3a, IgG2a; BD Pharmingen, San Jose, CA, USA).

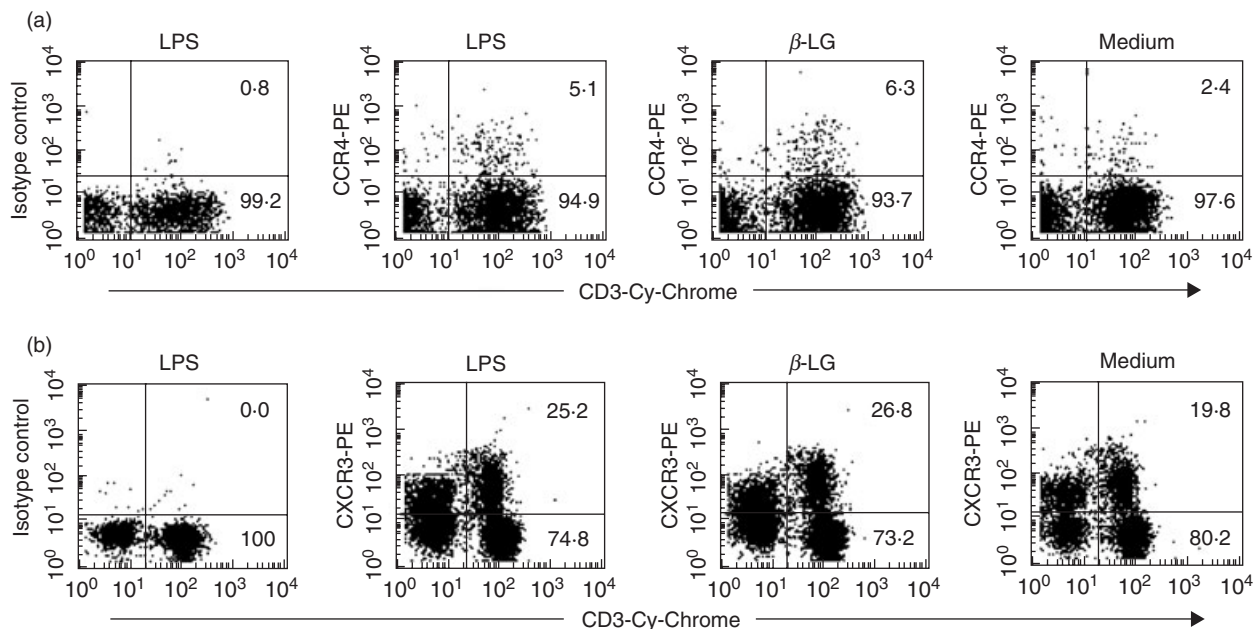


Fig. 1. Flow-cytometric analysis of T cells in cord blood mononuclear cells (CBMCs) cultured for 5 days with purified lipopolysaccharide (LPS, 0.45 μ g/ml) alone, β -lactoglobulin (β -LG, 125 μ g/ml) in the presence of its inherent LPS (0.45 μ g/ml), or in medium only. After gating on lymphocytes via their forward- and side-scatter properties, CD3⁺ cells were analysed for the expression of (a) CCR4 or (b) CXCR3 (isotype controls for chemokine receptors are displayed in the left panel). Numbers in upper right quadrants indicate the percentage of CD3 cells positive for the actual chemokine receptor (● = outlier).

Preparation of sample and standard RNA

RNA was extracted from isolated CD3⁺ T cells by means of the RNAwizTM (Ambion, Cambridge, UK) according to the manufacturer's instructions, and 300 μ l of reagent was used for cells isolated from three CBMC culture wells. After 3 days of culture, cytoplasmic RNA was extracted from bulk CBMC cultures by use of RNeasy Mini Kit (Qiagen, Hilden, Germany), as outlined by the manufacturer, and 175 μ l of the lysis buffer was used for the total cell pool collected from three wells.

Standard mRNA was generated from the plasmid pHQC1 (courtesy M. F. Kagnoff, University of California, San Diego, CA, USA) which carries interleukin (IL)-4 and interferon (IFN)- γ primer sites identical to those used to amplify target RNA. The construction of this plasmid [17] and the generation of standard mRNA [18] have been described previously.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Sample RNA (1 μ g determined spectrophotometrically at 260 nm) or standard mRNA (20 pg) was reverse transcribed in a 20 μ l reaction with first strand buffer, 100 U Superscript II, 0.01 M DTT (all Invitrogen, Taastrup, Denmark), 1 μ M oligo(dT) (5'-CTGAATTCTTTTTTTTTTTTTTTT-3'), 20 U RNasin (Promega, Southampton, UK) and 0.5 mM dNTP (Amersham Biosciences, Oslo, Norway).

Real-time PCR was performed with the Light Cycler[®] and FastStart DNA Master SYBR Green I system according to a standardized procedure (Roche Diagnostics GmbH, Mannheim, Germany). Analyses of the number of transcript copies/ml cDNA for CCR4 and the housekeeping gene human cyclophilin B (HCB) was performed with LightCyclerTM Primer Set kits and included standard (Search LC, Heidelberg, Germany).

For quantitative analysis of CXCR3 message (see Table 1 for primers), a standard curve was generated from a series of fourfold dilutions of a positive control cDNA, which was cDNA from CBMCs stimulated with β -LG in the presence of LPS, aliquoted and stored at -70°C . However, the number of CXCR3 transcript copies/ml of this cDNA was unknown and therefore defined as containing 400 000 arbitrary units/ml. Before analysing a series of 10-, 30- and 100-fold dilutions of sample cDNA, the concentration of cDNA in these samples was adjusted to normalize it according to the housekeeping gene HCB. CCR4 mRNA and CXCR3 mRNA were analysed to see whether the chemokine receptor up-regulation revealed by flow cytometry was accompanied by increased transcription. Because the flow-cytometric data separated satisfactorily β -LG-stimulated from medium control cultures, we considered it sufficient to analyse mRNA expression in only three samples.

The primer sequences for IL-4 and IFN- γ mRNA were derived from separate exons, thus spanning one or more introns and therefore minimizing amplification of genomic DNA. A series of 10-fold dilutions (10^3 – 10^6 transcripts) of

Table 1. Primers and cycle parameters for real-time PCR.

Gene	Amplicon length (bp)	Primer sequence (5'–3')	Annealing temp. (°C)	Measuring temp. (°C)	MgCl ₂ conc. (mM)
IL-4 target	276	AACACAACCTGAGAAGGAAACCTTC	65	87	3
IL-4 standard	347	GCTCGAACACTTTGAATATTTCTC			
IFN- γ target	501	ATGAAATATACAAGTTATATCTTGGCTTT	63	80	3
IFN- γ standard	384	GATGCTCTTCGACCTCGAAACAGCAT			
CXCR3	71	AACTGTGGCCGAGAAAGCAG	59	84	4
		GCAGTGCATCTAGCCCAGG			

the reverse transcribed standard was used for the standard curve, while the sample cDNA was diluted 10-, 30- and 100-fold. Analysis was carried out with the Light Cycler 3.5 software (Roche) according to the Fit Points method.

Enzyme-linked immunosorbent assay (ELISA) for IFN- γ quantification

After 3 days of culture, IFN- γ protein was quantified by a sandwich ELISA in cell supernatants with a capture monoclonal antibody (clone 350B10G6), biotinylated detection antibody (clone 67F12A8), and recombinant human IFN- γ as standard (all BioSource Europe, Nivelles, Belgium). Costar flat-bottomed polystyrene plates (Corning Life Sciences Inc., New York, USA) were coated overnight with 100 μ l/well of capture antibody at 2 μ g/ml in phosphate buffer, pH 7.4. Plates were washed with ion-free water, blocked with 300 μ l/well of the phosphate buffer containing 0.5% (w/v) bovine serum albumin (BSA; Sigma) for 2 h, and washed with water. Serial dilutions of the standard in RPMI medium and undiluted culture supernatants (100 μ l/well), together with 50 μ l/well of the detection antibody at 0.4 μ g/ml, were incubated for 2 h. After washing, 100 μ l of streptavidin horseradish peroxidase (R&D Systems Europe, Abingdon, UK) was added to each well and incubated for 30 min, whereafter the

plates were washed and developed with 100 μ l/well of TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD, USA) for 30 min. The reaction was stopped with 2 M HCl, 50 μ l/well.

Statistical analysis

Comparison of the frequencies of cells expressing cell surface receptors after various stimulations was performed with the two-tailed Wilcoxon's signed-rank test. Non-parametric correlations were tested with the two-tailed Spearman's correlation analysis. Analysis for significant differences between the FH⁺ and FH⁻ groups was performed with the two-tailed Mann–Whitney *U*-test. SPSS 11.0 software was used, and *P*-values < 0.05 were considered significant.

Results

Neonatal T cells up-regulate their CCR4 and CXCR3 expression during stimulation

Stimulation of CBMCs with 0.45 μ g/ml of LPS induced up-regulation of CCR4 and CXCR3 expression on CD3⁺ T cells (Figs 1 and 2). We then compared this effect with that induced by a dietary antigen in the presence of its inherent

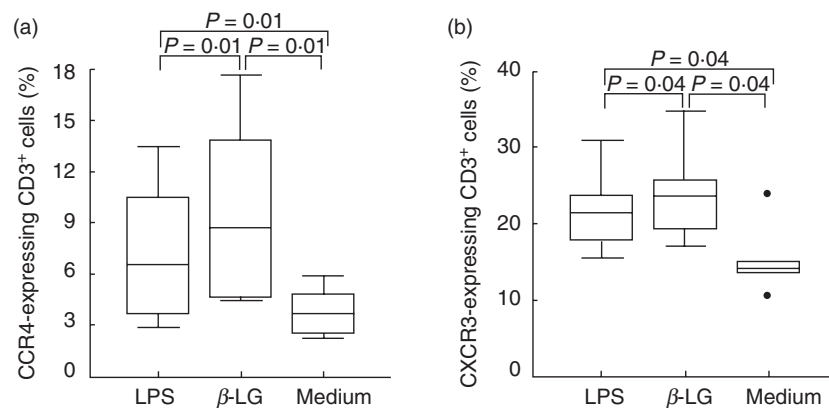


Fig. 2. Up-regulation of CCR4 and CXCR3 expression on T cells in cord blood mononuclear cells (CBMCs) cultured for 5 days with purified lipopolysaccharide (LPS) alone, β -lactoglobulin (β -LG) in the presence of its inherent LPS, or in medium only. The percentage of CD3⁺ cells expressing CCR4 (*n* = 8) or CXCR3 (*n* = 5) was analysed by flow cytometry as exemplified in Fig. 1. The boxes cover the middle 50% of the data values (between the 25th and 75th percentiles), the horizontal lines representing the medians and the vertical lines extending to non-outlier maximum and minimum values (● = outlier). Statistically significant differences between the culture conditions are indicated.

LPS. The limulus amoebocyte lysate assay showed that the endotoxin activity of 125 µg/ml β -LG matched 0.45 µg/ml of purified LPS (i.e. 125 EU/ml). Stimulation with this β -LG preparation up-regulated CCR4 and CXCR3 expression on T cells to a significantly higher level than LPS alone (Figs 1 and

2). All subsequent analyses were therefore performed after stimulation of CBMCs with β -LG in the presence of its inherent LPS.

The up-regulated cell surface expression of chemokine receptors was substantiated by increased mRNA levels in

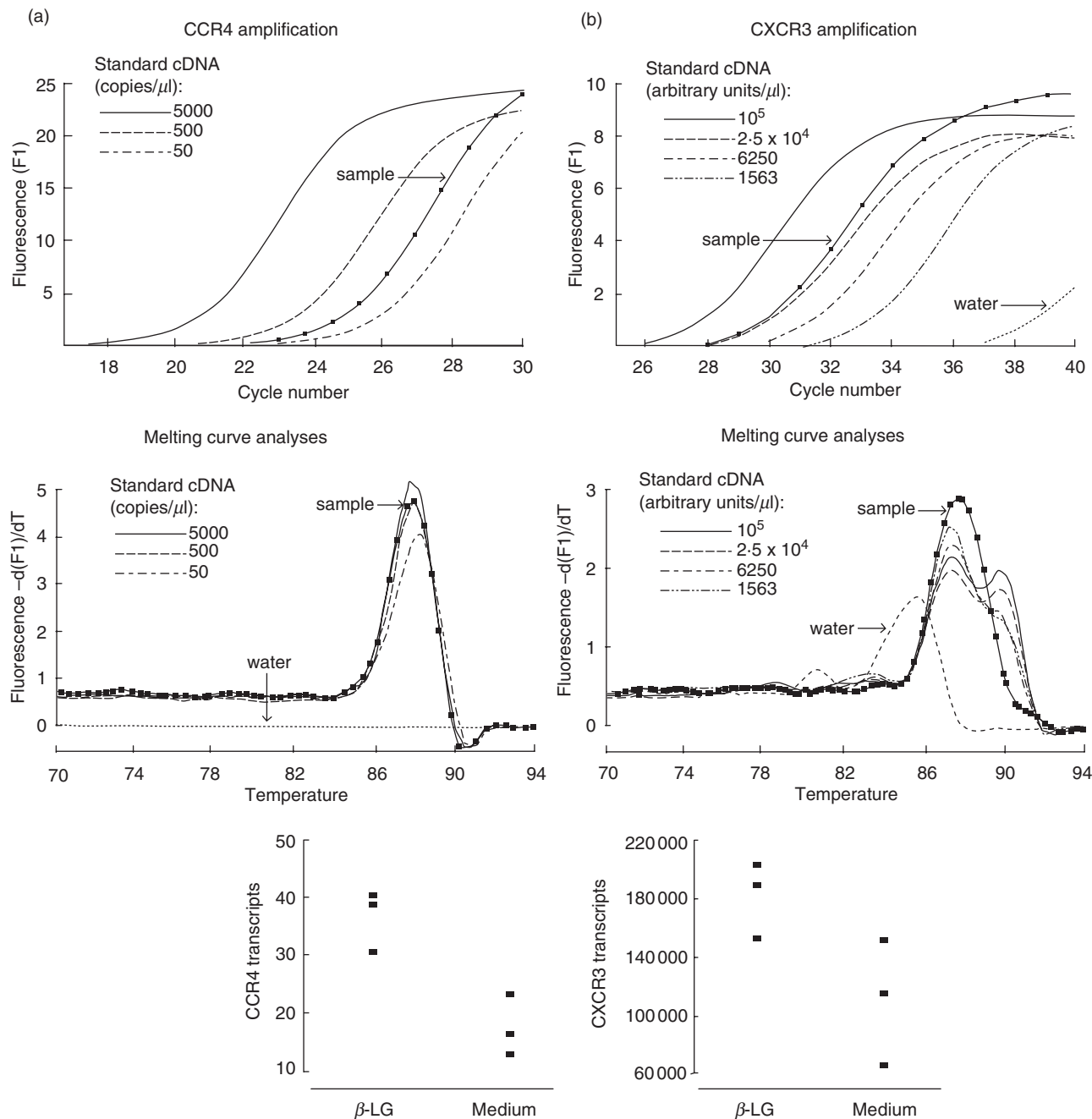


Fig. 3. Light cycle quantification of CCR4 or CXCR3 mRNA in extracts from isolated CD3⁺ T cells after stimulation of cord blood mononuclear cells (CBMCs) cultured for 4 days with β -lactoglobulin (β -LG) in the presence of its inherent lipopolysaccharide (LPS), or in medium only. Top panels show amplifications obtained by serial dilutions as indicated of standard cDNA and one sample cDNA amplified with (a) CCR4-specific primers and (b) CXCR3-specific primers; middle panels show the corresponding melting curves together with water controls; and bottom panels show the results from three different experiments of stimulated *versus* control (medium) cultures, each point representing the mean of two parallel tests. CCR4 values represent the number of transcripts per 10⁶ transcripts of a housekeeping gene (HCB), whereas CXCR3 values represent the number of arbitrary units of transcripts in cDNA containing a fixed HCB copy number (for details, see Methods).

isolated T cells as shown by quantitative RT-PCR (Fig. 3). The melting curve analyses revealed that during amplification with the CXCR3-specific primers, non-specific products or primer dimers were detected occasionally in the water control (Fig. 3b). In the test sample and positive control cDNAs, however, only specific products were produced as demonstrated both by the melting curve analyses (Fig. 3b) and by size verification in agarose gels (results not shown). Control samples containing RNA without reverse transcription were always negative for the specific PCR products (results not shown).

CCR4 and CXCR3 levels on neonatal T cells are differentially related to concurrent IL-4 induction in stimulated CBMCs

We next analysed a possible relationship between the production of IL-4 or IFN- γ and the up-regulation of CCR4 or CXCR3 expression in a series of CBMC samples. For quantification of IL-4 and IFN- γ mRNA in stimulated CBMCs, the reverse-transcribed plasmid pHQC1 was used as stan-

dard cDNA (Fig. 4). This construct carries IL-4 and IFN- γ primer sites identical to those used to amplify sample RNA, but the distance between specific 5' and 3' primer sequences is different from that of sample RNA [17]. Therefore, the size of the PCR amplification products differs for standard and sample cDNAs, which is demonstrated by the respective shifts in melting peak positions (Fig. 4, lower panel).

After CBMC stimulation with β -LG in the presence of its inherent LPS, the induced IL-4 mRNA levels (day 3) were positively correlated with the surface expression of CCR4 (day 5) on T cells (Fig. 5a) but negatively related to that of CXCR3 (Fig. 5b). Results obtained for CCR4 and CXCR3 on day 4 also tended to be positively or negatively associated with IL-4 transcript numbers (day 3), respectively, but these correlations did not reach the significance level (CCR4: $r_s = 0.40$, $P = 0.08$; CXCR3: $r_s = -0.43$, $P = 0.07$; data not shown). The IFN- γ mRNA levels were well correlated with the IFN- γ protein levels determined by ELISA in CBMC culture supernatants ($r_s = 0.76$, $P < 0.0001$), but a relationship was detected neither to CCR4 nor to CXCR3 surface expression on T cells (data not shown).

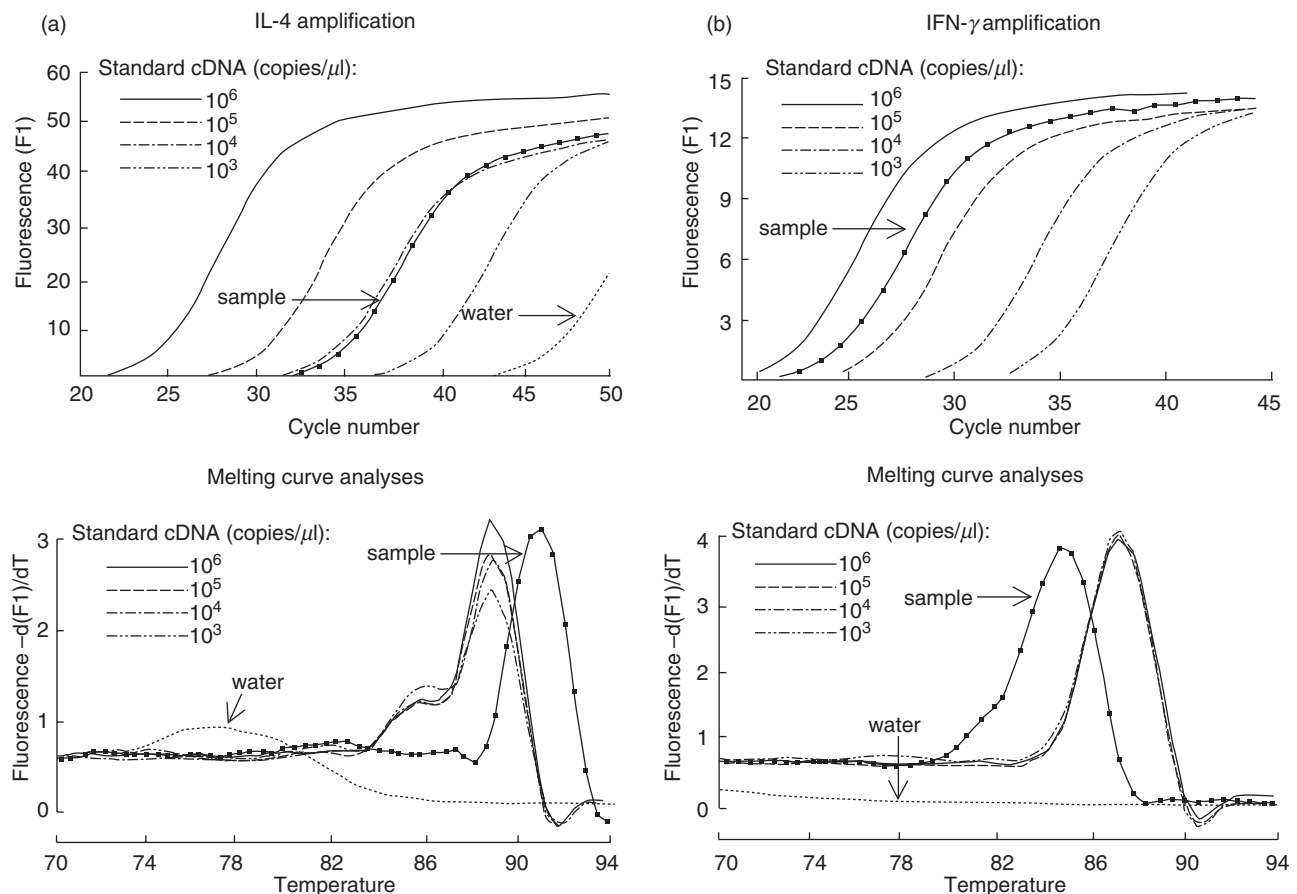
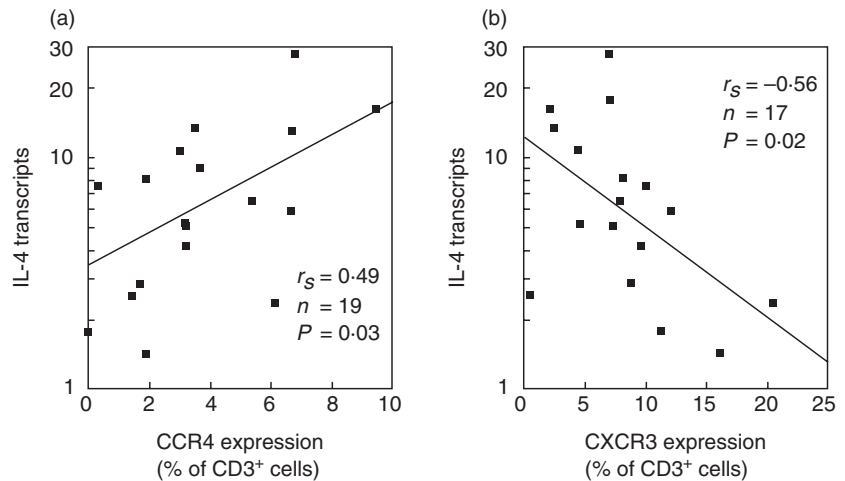


Fig. 4. Light cycler quantification of IL-4 or IFN- γ mRNA of cord blood mononuclear cells (CBMCs) after stimulation for 3 days with β -lactoglobulin (β -LG) in the presence of its inherent lipopolysaccharide (LPS). Top panels show amplifications obtained by 10-fold serial dilutions (10^3 – 10^6 transcripts) of the reverse transcribed plasmid pHQC1 as a standard and one sample of cDNA amplified with (a) IL-4-specific primers and (b) IFN- γ -specific primers; bottom panels show the respective melting curves together with water controls.

Fig. 5. Scatter diagrams depicting the relation between IL-4 transcript copy numbers determined by light cycler, and T cell expression of (a) CCR4 or (b) CXCR3 determined by flow cytometry. IL-4 transcript copy numbers were determined in bulk extracts of cord blood mononuclear cells (CBMCs) after stimulation for 3 days with β -lactoglobulin (β -LG) in the presence of its inherent lipopolysaccharide (LPS), values representing IL-4 copies per 1000 copies of a housekeeping gene (HCB). Expression of chemokine receptors was determined as the percentage of positive CD3 T cells after stimulation of CBMCs for 5 days with β -LG in the presence of LPS subtracted for the percentage CCR4 or CXCR3 expression after culturing in medium alone.



Responses obtained for neonates with hereditary allergy risk *versus* controls

After stimulation with β -LG in the presence of its inherent LPS, CBMCs from neonates with an atopic family history (FH⁺, allergy risk group) tended to show less IL-4 mRNA expression than CBMCs from the control group (FH⁻), the mean values being 1.30 ($\pm$ s.d. 1.89) and 5.62 ($\pm$ s.d. 2.61), respectively (Fig. 6a). Moreover, a difference between the FH⁺ and the FH⁻ group was highly significant when we compared surface expression (positive fraction, %) of CCR4 on T cells after 5 days of stimulation, the mean values being 2.42 ($\pm$ s.d. 0.61) and 5.74 ($\pm$ s.d. 0.88), respectively (Fig. 6b). We also analysed CCR4 up-regulation after 4 days of stimulation, and the difference between the risk group and the controls was similarly significant ($P = 0.014$)

at this time-point (data not shown). Conversely, neither the IFN- γ nor the CXCR3 levels were significantly different between the two groups (median IFN- γ mRNA, transcripts/1000 HCB transcripts: FH⁺, 20.7 *versus* FH⁻, 16.5; median IFN- γ protein, pg/ μ l: FH⁺, 19.5 *versus* FH⁻, 0.0; median CXCR3⁺ cells, % of CD3⁺ T cells: FH⁺, 8.8 *versus* FH⁻, 7.2).

Discussion

CCR4 and CXCR3 are involved in the migration of T cells and their interaction with other immune cells within lymphoid organs and at sites of inflammation [13–15]. By exploiting CBMCs as an *in vitro* model for the immature immune system of neonates, our results suggested that the T cell potential for CCR4- and CXCR3-dependent functions is enhanced by

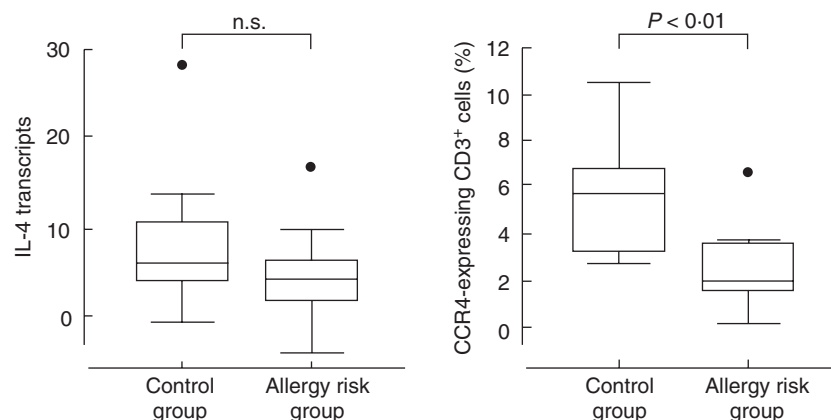


Fig. 6. (a) Light cycler quantification of IL-4 mRNA in cord blood mononuclear cells (CBMCs). Values represent IL-4 copies per 1000 copies of a housekeeping gene (HCB) after stimulation for 3 days with β -lactoglobulin (β -LG) in the presence of its inherent lipopolysaccharide (LPS), subtracted for the copies in CBMCs cultured in medium alone (control group, $n = 11$; allergy risk group, $n = 11$). n.s., non-significant ($P = 0.21$). (b) Flow-cytometric analyses of CCR4 on T cells. Values represent the percentage of CD3⁺ cells positive after stimulation of CBMCs for 5 days with β -LG in the presence of LPS, subtracted for the percentage of CCR4-expressing T cells in CBMCs cultured in medium alone (control group, $n = 10$; allergy risk group, $n = 10$). The boxes cover the middle 50% of the data values (between the 25th and 75th percentiles), the horizontal lines representing the medians and the vertical lines extending to non-outlier maximum and minimum values (● = outlier).

stimulation with bacterial LPS. This effect was even more evident when LPS was present during stimulation with the dietary antigen β -LG. Analyses of mRNA, moreover, showed that LPS together with β -LG enhanced the gene transcription levels for CCR4 and CXCR3. Therefore, increased production rather than translocation to the cell surface of intracellular protein stores, most probably explained the elevated expression level of CCR4 and CXCR3 on T cells.

Because Th1 and Th2 cells can exert countersuppression, and therefore may require spatial separation for maximal responses, it has been postulated that these T cell subsets express different homing receptors [19]. Previous studies have shown that *in vitro* activation preferentially induces up-regulation of CXCR3 on Th1 cells but CCR4 on Th2 cells [20–22], and it has been suggested that these chemokine receptors may determine the trafficking of the two subsets [13]. However, the biological significance of these studies has been questioned, as they were based on cell lines or clones already polarized for Th1 or Th2 cytokine production [20–23]. We aimed to analyse possible associations between Th1 or Th2 cytokines and up-regulation of CCR4 and CXCR3 in an *in vitro*-model system which we consider more relevant to physiological conditions. Therefore, short-term cultures of fresh CBMCs were used without the addition of Th1- or Th2-polarizing cytokines or cytokine-neutralizing antibodies, thus ensuring that the endogenous stimulatory conditions were influenced directly by the genetic background of the neonate. After stimulation with β -LG in the presence of LPS, the levels of IL-4 produced by T cells in the CBMC cultures varied markedly among cord blood samples. The expression levels of CCR4 and CXCR3 on T cells also varied, and we found that the expression of CCR4 was positively associated with the IL-4 levels, while the reverse was true for CXCR3. Our findings thus supported the notion that CCR4-dependent functions of T cells may be enhanced in a Th2-skewed cytokine microenvironment, while the potential for CXCR3-dependent functions appears to be suppressed under such conditions.

A Th2 cytokine profile induced by allergen is involved in the pathogenesis of allergic diseases, and impaired capacity for production of Th1 cytokines can be detected even at birth in individuals who later develop allergy [24–27]. The Th1-enhancing effect of LPS has been suggested to explain why exposure to LPS/endotoxin in early childhood protects against the development of IgE-dependent allergy and asthma [10,28]. Our study showed that, in addition to increased IFN- γ production, LPS stimulation of neonatal lymphoid cells might also enhance the capacity for inducing Th1-skewed immune responses via ligand-engagement of CXCR3 [29]. In this recently revealed mechanism, binding of chemokine(s) to CXCR3 on T cells reportedly enhanced antigen-induced IFN- γ production, and it was concluded that the number of circulating CXCR3⁺ T cells largely determines the intensity of the Th1 response induced via this mechanism [29]. Consequently, the up-regulation of CXCR3

expression by LPS and antigen seen in our study could reflect an enhanced capacity for suppression of Th2 responses. Interestingly, we did not detect differences between the FH⁺ and FH⁻ group as regards LPS- and β -LG-induced IFN- γ production or up-regulation of CXCR3, which suggested that antigen encounter together with LPS stimulation in early life might skew similarly the immune response towards a Th1 profile in the two groups.

Conversely, we found a significantly reduced effect of LPS together with β -LG on the up-regulation of CCR4 in the FH⁺ group compared with the FH⁻ group, demonstrating an inherent immaturity of the immune system in the former. Such immunological immaturity of neonates with a hereditary allergy risk has been revealed previously by a surprisingly low production level of both IL-4 [30] and IFN- γ [24,26]. Because CCR4 expression on T cells enhances their contact with antigen-presenting dendritic cells [13–15], we speculate whether the impaired up-regulation of CCR4 observed in the FH⁺ group might imply reduced potency for TCR engagement by antigenic peptides, which is reported to be a determining factor in Th-cell differentiation towards a Th2 profile [31,32].

Exposure to bacterial products such as LPS from the environment, and/or appropriate colonization of a normal commensal microflora, at the time of early allergen contact has been reported to decrease the prevalence of atopic diseases, but the underlying immunoregulatory mechanisms remain elusive [9,12,33]. The results obtained in our CBMC model suggested that LPS stimulation concomitantly with early antigen (or allergen) encounter might increase the T cell potential for CCR4- and CXCR3-dependent functions. Clinical follow-up of the infants donating CBMCs in this study could help to determine whether an impaired T cell response to bacterial products in children with a familial atopy history predicts later expression of allergic diseases.

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References

- 1 Björkstén B. The environment and sensitisation to allergens in early childhood. *Pediatric Allergy Immunol* 1997; **8** (Suppl. 10):32–9.
- 2 Riedler J, Braun-Fahrlander C, Eder W *et al.* Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001; **358**:1129–33.
- 3 Nafstad P, Magnus P, Gaarder PI, Jaakkola JJ. Exposure to pets and atopy-related diseases in the first 4 years of life. *Allergy* 2001; **56**:307–12.

- 4 Hesselmar B, Aberg N, Aberg B, Eriksson B, Björkstén B. Does early exposure to cat or dog protect against later allergy development? *Clin Exp Allergy* 1999; **29**:611–17.
- 5 Strachan D. Hay fever, hygiene and household size. *Br Med J* 1989; **289**:1259–60.
- 6 Bråbäck L, Breborowicz A, Julge K *et al.* Risk factors for respiratory symptoms and atopic sensitization in the Baltic area. *Arch Dis Child* 1995; **72**:487–93.
- 7 von Mutius E, Weiland SK, Fritzsche C, Duhme H, Keil U. Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. *Lancet* 1998; **351**:862–6.
- 8 Weiland SK, von Mutius E, Hirsh T *et al.* Prevalence of respiratory and atopic disorders among children in east and west Germany five years after unification. *Eur Respir J* 1999; **14**:862–70.
- 9 Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma. *Lancet* 1999; **354** (Suppl. 2):SII 12–15.
- 10 Gereda JE, Leung DYM, Thatayatikom A *et al.* Relation between house-dust endotoxin exposure, type 1 T cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 2000; **355**:1680–3.
- 11 Gehring U, Bolte G, Borte M *et al.* Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. *J Allergy Clin Immunol* 2001; **108**:847–54.
- 12 Baldini M, Vercelli D, Martinez FD. CD14: an example of gene by environment interaction in allergic disease. *Allergy* 2002; **57**:188–92.
- 13 Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Ann Rev Immunol* 2000; **18**:593–620.
- 14 Lieberam I, Forster I. The murine beta-chemokine TARC is expressed by a subset of dendritic cells and attracts primed CD4⁺ T cells. *Eur J Immunol* 1999; **29**:2684–94.
- 15 Wu M, Fang H, Hwang ST. CCR4 mediates antigen-primed T cell binding to activated dendritic cells. *J Immunol* 2001; **167**:4791–5.
- 16 Campbell JJ, Haraldsen G, Pan J *et al.* The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 1999; **400**:776–80.
- 17 Jung HC, Eckmann L, Yang SK *et al.* A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* 1995; **95**:55–65.
- 18 Nilsen EM, Jahnsen FL, Lundin KE *et al.* Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* 1998; **115**:551–63.
- 19 Syrbe U, Sivek J, Hamann A. Th1/Th2 subsets: distinct differences in homing and chemokine receptor expression? *Springer Semin Immunopathol* 1999; **21**:263–85.
- 20 Kim CH, Nagata K, Butcher EC. Dendritic cells support sequential reprogramming of chemoattractant receptor profiles during naive to effector T cell differentiation. *J Immunol* 2003; **171**:152–8.
- 21 Ambrosio DD, Iellem A, Bonecchi R *et al.* Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J Immunol* 1998; **161**:5111–15.
- 22 Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998; **187**:875–83.
- 23 Sallusto F, Kremmer E, Palermo B *et al.* Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur J Immunol* 1999; **29**:2037–45.
- 24 Rinas U, Horneff G, Wahn V. Interferon- γ production by cord-blood mononuclear cells is reduced in newborns with a family history of atopic disease and is independent from cord blood IgE-levels. *Pediatr Allergy Immunol* 1993; **4**:60–4.
- 25 Warner JA, Miles EA, Jones AC, Quint DJ, Colwell BM, Warner JO. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clin Exp Allergy* 1994; **24**:423–30.
- 26 Liao SY, Liao TN, Chiang BL *et al.* Decreased production of IFN- γ and increased production of IL-6 by cord blood mononuclear cells of newborns with a high risk of allergy. *Clin Exp Allergy* 1996; **26**:397–405.
- 27 van der Velden VHJ, Laan MP, Baert MRM, de Waal Malefyt R, Neijens HJ, Savelkoul HFJ. Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN- γ , IL-4 and IL-10. *Clin Exp Allergy* 2001; **31**:997–1006.
- 28 Douwes J, Pearce N, Heederik D. Does environmental endotoxin exposure prevent asthma? *Thorax* 2002; **57**:86–90.
- 29 Campbell JD, Gangur V, Simons FER, Hayglass KT. Allergic humans are hyporesponsive to a CXCR3 ligand-mediated Th1 immunity-promoting loop. *FASEB J* 2004; **18**:329–31.
- 30 Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T cell memory in atopic and normal children. *Lancet* 1999; **353**:196–200.
- 31 Secrist H, DeKruyff RH, Umetsu DT. Interleukin 4 production by CD4⁺ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J Exp Med* 1995; **181**:1081–9.
- 32 Tao X, Grant C, Constant S, Bottomly K. Induction of IL-4-producing CD4⁺ T cells by antigenic peptides altered for TCR binding. *J Immunol* 1997; **158**:4237–44.
- 33 von Mutius E, Braun-Fahrlander C, Schierl R *et al.* Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 2000; **30**:1230–4.