

Original article

Impairment of T helper and T regulatory cell responses at birth

Background: There is strong evidence that reduced exposures to microbial compounds triggering innate immune responses early in life are critical for the development of allergic illnesses. The underlying mechanisms remain unknown, but will include T-cell responses either along T helper type 1 (Th1)/Th2 pathways or via T regulatory and Th17 cells. Yet, little is known about innate immune responses and the function of T regulatory/Th17 cells at birth. The aim of this study was to investigate T-cell responses to innate (Lipid A/LpA, peptidoglycan/Ppg) and adaptive (phytohemagglutinin) stimuli at birth and to compare these findings with adult immune responses.

Methods: Cord and peripheral blood mononuclear cells including T regulatory and Th17 cells from 25 neonates and 25 adults were examined for proliferation, cytokine secretion, surface, mRNA expression and functional suppression assays.

Results: Proliferation and cytokine responses to innate stimuli were less mature at birth than in adulthood. T regulatory and Th17 cells were less expressed in cord than in adult blood (Ppg-induced Foxp3, $P = 0.001$, LpA-induced CD4⁺CD25⁺ high, $P = 0.02$; Th17 : $P < 0.0001$). Mitogen-induced suppression of T-regulatory cells on T-effector cell function was less efficient in cord than in adult blood ($P = 0.01$). At both ages, Th17 cells were correlated with Th1/Th2 cells ($P < 0.01$), but not with interleukin-10 secretion following innate-stimulation.

Conclusion: Innate immune responses are immature at birth. Furthermore, the function of T regulatory and Th17 cells is impaired. Th17 cells in association with Th1/Th2 cells may be involved in early immuno-modulation. Potent innate immune stimulation early in life can potentially contribute to protection from allergic diseases.

B. Schaub^{1*}, J. Liu^{1,2*}, I. Schleich¹, S. Höppler¹, C. Sattler³, E. von Mutius¹

¹Department of Pulmonary & Allergy, University Children's Hospital Munich, LMU Munich, Germany;

²Department of Respiratory Medicine, The Second Hospital of JI LIN University, Chang Chun, China;

³Department of Gynaecology, University Hospital, LMU Munich, Germany

Key words: allergy; innate; neonatal; T helper type 17/T-regulatory cells; toll-like receptors.

Bianca Schaub
Department of Pulmonary & Allergy
University Children's Hospital Munich
Dr von Haunersches Kinderspital
Lindwurmstr. 4
80337 Munich
Germany

*Both authors contributed equally to the manuscript.

Accepted for publication 27 January 2008

During the maturation of a child's immune system, a range of stimuli may be required at a critical time period for a healthy immune development. In this context, early life exposure to microbial substances was identified to be critical for protection against the development of allergic diseases (1, 2). Thus, healthy immune maturation early in life seems to require specific properties contributing to this protective regulation. Consequently, a disparity in early maturation may potentially shift immune regulation into an unfavorable pathway subsequently resulting in increased incidence of immune-mediated diseases such as allergies.

Abbreviations: CBMC, cord blood mononuclear cells; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN- γ , interferon- γ ; IL, interleukin; LpA, lipid A; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PHA, phytohemagglutinin; Ppg, peptidoglycan; SD, standard deviation; TGF- β , transforming growth factor- β ; Th, T helper; TLR, toll-like receptor; TNF- α , tumor necrosis factor- α ; Tregs, T-regulatory cells.

Human neonates are more susceptible to microbial infections than adults (3). One proposed regulatory specificity of the neonatal immune development is an initially immature immune system contributing to a weak and Th2-polarized immunity, therefore lacking the T helper type 1 (Th1)-capacity to combat infections (4). Another possibility is a quantitative or functional deficit of another T-cell population, namely regulatory T cells (Tregs) in infancy. These cells play an important role in balancing Th1/Th2 effector lineages and in more general terms innate and adaptive immune responses. Tregs, which include mainly natural CD4⁺CD25⁺ Tregs and IL-10-producing Tregs are essential for the downregulation of T-cell responses to both foreign and self antigens (5, 6).

T-regulatory cells with suppressive regulatory function have been detected in cord blood (7), expressing mRNA of the forkhead/winged-helix family transcriptional repressor-p3 (Foxp3) (8). Foxp3, the most but yet not entirely specific marker of Tregs, is necessary for their

function (9, 10). The current knowledge about Treg function early in life as compared to adults is very limited.

Another recently identified T-cell population with ties to Tregs are Th17 cells, potentially involved in allergic diseases (11, 12). Murine and adult studies have demonstrated that Th17 cells represent a unique T-effector cell subset, distinct from Th1/Th2 lineages (11–13). T helper type 17 cells receive signals from Tregs for their development and drive expansion of Tregs thus clearly linking these two T-cell subsets in their regulation. To our knowledge, the role of Th17 cells early in life has not been studied in detail.

This study aimed to characterize T-cell effector responses to innate and adaptive stimuli at birth and to compare these findings with adult immune responses. The contribution of Tregs and Th17 cells was investigated in addition to Th1/Th2 lineages.

Methods

Study population

Healthy adult volunteers ($n = 25$) between the ages of 23 and 51 years (mean 32.6 ± 7.4 years) were recruited at the University Children's Hospital, Munich, Germany. Cord blood ($n = 25$) was sampled in the frame of a cord blood study performed in Munich, Germany. Healthy nonsmoking pregnant women were enrolled in the last trimester. Umbilical cord blood was obtained at the time of delivery from healthy neonates born at term after uncomplicated pregnancies. Exclusion criteria included: (i) signs of infection in the newborn or mother determined through measurement of IL-6 and CrP; (ii) maternal intake of medication; and (iii) severe chronic maternal diseases. There was an equal distribution of atopics and nonatopics in the adults and in the mothers/fathers of the neonates. Informed consent was obtained from the adults and pregnant women for the participation in the study. Approval was obtained from the local review board of the Bayerische Landesärztekammer (Bavarian Ethical Board), Munich, Germany.

Isolation, lymphocyte proliferation and cytokine secretion of PBMC and CBMC

Adult peripheral and cord blood samples were collected by withdrawing blood from a peripheral or umbilical vein after delivery and processed within 24 h, as previously described (14, 15). Cord and peripheral blood mononuclear cells (CBMC/PBMC) were isolated by density-gradient centrifugation with Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) after dilution in phosphate buffer saline (PBS, Gibco; Invitrogen, Karlsruhe, Germany). Cells, washed in RPMI 1640 were diluted in 10% human serum (Sigma Aldrich, Munich, Germany) to a concentration of 5×10^6 cells/ml. For lymphocyte proliferation and cytokine secretion, 0.5×10^6 cells/well were stimulated in replicates with the toll-like receptor (TLR) 2 ligand peptidoglycan (Ppg 10 μ g/ml, *Staphylococcus aureus*), the TLR4 ligand lipid A (LpA 0.1 μ g/ml, *Salmonella minnesota*, Re mutant) or mitogen phytohemagglutinin (PHA 5 μ g/ml; all Sigma Aldrich) for 3 days and compared to unstimulated cells. The doses were established in prior dose- and time-course experiments (16). Endotoxin concentrations in Ppg and PHA preparations, measured by Limulus assay, were very low (< 0.01 EU/ml = 0.002 ng/ml),

and did not significantly change lymphocyte proliferation or cytokine secretion (16). After incubation at 37°C in a humidified 5%CO₂ chamber, samples were pulsed with 1 μ Ci ³H-thymidine for 6–10 h. Cells were harvested using a ComBI cell harvester (Skatron Instrument, Lier, Norway) onto filter plates and analyzed using a β -Counter. Proliferation was either assessed by counts per minute (cpm) or quantified by stimulation index, calculated as ratio of mean cpm of stimulated over unstimulated replicates. Cytokine concentrations were measured in supernatants using the Human Cytokine-Multiplex-Assay Kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany) using LUMINEX technology. The lower limit of detection of the assay (pg/ml) was 1.1 [interleukin-2 (IL-2)]; 1.8 (IL-5); 0.9 (IL-10); 2.1 (IL-13); 0.2 (IL-17); 1.3 [interferon- γ (IFN- γ)] 3.0 [tumor necrosis factor- α (TNF- α)] 1.0 [granulocyte macrophage-colony stimulating factor (GM-CSF)].

Flow cytometry

Cells were analyzed using three-color flow cytometry (FACScan; Becton-Dickinson, Heidelberg, Germany). For surface staining, PBMC/CBMC were incubated in aliquots of 5×10^5 cells (100 μ l buffer PBS 5% FCS, 0.5% Tween), with saturating concentrations of fluorochrome-labeled antibodies (30 min, 4°C). The samples were washed twice with 1 ml buffer and resuspended in 400 μ l PBS-FCS. The following antibodies/corresponding isotype controls were added: 2 μ l anti-human CD4-FITC, anti-human CD25-RPE-Cy5, 1 μ l IgG1-FITC (Dako Cytomation, Glostrup, Denmark), 0.5 μ l IgG2a RPE-Cy5 (BD Biosciences, Pharmingen, Germany).

For intracellular Foxp3 staining, cells were stained with 8 μ l of anti-human CD4-FITC, 4 μ l anti-human CD25-RPE-Cy5 antibodies per tube (1×10^6 /100 ml). After resuspension, 1 ml of freshly prepared fixation/permeabilization working solution buffer (eBioscience NatuTec, Frankfurt, Germany) was added and incubated (4°C, 60 min, dark). After centrifugation, cells were washed with 1 $\times$ permeabilization buffer. After blockade with 2% normal rat serum (2 μ l) in 1 $\times$ permeabilization buffer (4°C, 15 min), 10 μ l anti-human Foxp3-PE antibody or 5 μ l mouse IgG2a-PE isotype control in 1 $\times$ permeabilization buffer was added and incubated (4°C, 30 min, dark). After further washing, cells were resuspended and samples analyzed.

For intracellular IL-17-staining, cells were cultured with 25 μ l beads for 1×10^6 /1 ml cells, anti-human CD3/CD28 T-cell expander beads (DynaL Biotech, Hamburg, Germany), recombinant human IL-1 β (20 ng/ml), IL-6 (100 ng/ml, R&D system, Wiesbaden, Germany). In optimization experiments, transforming growth factor- β (TGF- β) was added in increasing doses (100 ng/ml). After 72 h of culture, washing and removal of beads with the magnet, restimulation for 5 h with PMA/ionomycin (50 ng/ml; 1 μ l/ml, Sigma Aldrich) and monensin was performed. After addition of intracellular anti-human IL-17-antibody/isotype control samples were processed as with Foxp3. Flow cytometry data were analyzed with CELLQUEST software (Becton Dickinson, Mountain View, CA, USA), postacquisition analysis was performed with WINMDI 2.8 software (Becton Dickinson).

Functional analysis of regulatory T cells

After isolation of CD3⁺ cells (CD3 isolation-kit, Miltenyi Biotec, Germany), and irradiation (30 Gy, 3000 rad, 10 min), purity was above 98%. CD4⁺ CD25/CD4⁺ CD25⁺ T cells were isolated in a two-step procedure, using depletion of non-CD4⁺ cells, followed by positive selection of CD4⁺ CD25⁺ T cells (CD4CD25 isolation-kit; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD4⁺ CD25/CD25⁺ T cells was above 95%. CD4⁺ CD25⁺ T cells

(2×10^4 /well), labeled with 5 μ MCFSE (CFSE-Proliferation-Kit C34554; Invitrogen), were incubated with irradiated CD3⁺ cells (4×10^4 /well) in coculture with or without CD4⁺ CD25⁺ T cells (2×10^4 /well). After 3 days of culture, CD4⁺ CD25⁺ T-cell division was analyzed before and after stimulation with 0.8 μ g/ml PHA. Proliferation of CD4⁺ CD25⁺ T cells was assessed by ³H-thymidine incorporation. In supernatants, cytokine concentrations of IFN- γ , IL-13, IL-5, IL-10, TNF- α , IL-17 were measured using the Human Cytokine Multiplex Assay Kit (Bio-Rad).

Quantitative RT-PCR

Total RNA, isolated with TRI Reagent, was processed with reverse transcriptase (Invitrogen). mRNA-specific oligonucleotide primers (FW/RE) were designed with Vector NTI Advance10: 18S rRNA: 5'-AGTCCCTGCCCTTTGTACACA-3', 5'-GATCCGAGGGCC-TCACTAAAC-3'; Foxp3: 5'-ACCTTCCCAAATCCCAGTGC-3', 5'-GAAGATGGTCCGCCTGGC-3'. For real-time RT-PCR, 0.34 μ l cDNA, 7.5 μ l primer, 9.66 μ l DEPC water and 12.5 μ l SYBR Green Master-Mix/well (Applied Biosystems, Darmstadt, Germany) was used. Gene-specific PCR-products were measured continuously by means of iCycler iQ-multicolor Real-Time PCR-Detection-System (Bio-Rad) for 40 cycles. All experiments were run in duplicates with the same thermal-cycling parameters. Nontemplate controls and dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. PCR products were separated on a 3% agarose-gel to control for specificity and expected size of the PCR-fragment. Direct detection of the PCR product (iCycler) was monitored by measuring the increase in fluorescence caused by binding of SYBR Green to dsDNA. For analysis, the determined threshold cycle (CT) was set in relation to the amplification plot of 18S rRNA. The CT is the number of PCR-cycles required for the fluorescence signal to exceed the detection threshold value, which was set to the log-linear range of the amplification curve. The difference in CT values of two genes was used to calculate delta CT (Δ CT). A higher Δ CT to 18S resembles lower mRNA-expression (17). The relative quantitative results were

Table 1. Lymphocyte proliferation, CD4⁺ CD25⁺ T cells and Foxp3 positive cells were lower in CBMC compared with PBMC

Parameters	Stimuli	PBMC (<i>n</i> = 25†)	CBMC (<i>n</i> = 25†)	<i>P</i> -value#
LP (SI) (Median + IQR)	PHA	137.0 (82.6/218.0)	14.9 (10.3/19.1)	<0.0001#
	LpA	2.20 (1.40/3.27)	1.09 (0.73/1.62)	<0.0001#
	Ppg	11.12 (4.87/19.92)	1.90 (1.36/3.33)	<0.0001#
CD4 ⁺ CD25 ⁺ (Mean % $\pm$ SD)‡	PHA	24.3 (7.62)**	30.6 (14.45)**	0.22
	LpA	2.46 (1.24)	1.72 (1.07)	0.11
	Ppg	3.88 (1.94)*	2.45 (1.63)	0.06
CD4 ⁺ CD25 ⁺ high (mean % $\pm$ SD)‡	PHA	2.20 (0.87)**	1.64 (0.89)*	0.13
	LpA	1.11 (0.40)	0.72 (0.41)	0.02#
	Ppg	1.58 (0.67)	1.28 (0.99)	0.39
Foxp3 (Δ CT to 18S)	PHA	13.4 (12.4/15.8)***	12.0 (11.1/13.8)***	0.23
	LpA	14.9 (13.8/15.7)	15.5 (14.8/15.9)	0.27
	Ppg	13.3 (12.7/14.0) *	15.2 (14.6/15.9)	0.001#

LP, lymphocyte proliferation; SI, stimulation index; IQR, interquartile range; SD, standard deviation; Δ CT to 18 S, lower Δ CT represents higher mRNA expression; PHA, phytohemagglutinin; LpA, lipid A; Ppg, peptidoglycan.

#*P*, Comparison between PBMC and CBMC. *Comparison with unstimulated data. **P* $\leq$ 0.05; ***P* $\leq$ 0.001, ****P* $\leq$ 0.0001.

†Because of some missing data not all cells always include 25 participants.

‡Data were normally distributed and analyzed using *t*-test.

used to determine changes in gene expression in stimulated/unstimulated samples (17, 18).

Statistical analysis

Data analysis was performed with SIGMASTAT 3.5 (Jandel Scientific Software, Erkrath, Germany) and SPSS 14.0 (SPSS Inc., Chicago, IL, USA) software. Data for lymphocyte proliferation, cytokine concentrations and mRNA expression were generally not normally distributed and could not be transformed to normality. Nondetectable cytokine concentrations were assigned to a value of 0.01 pg/ml for inclusion into the analysis. Nonparametric tests (Mann-Whitney, Wilcoxon) were used to compare the median of proliferation, cytokine, or gene expression levels between different groups; parametric tests were used for normally distributed data (surface expression). Data were calculated before and after correction for baseline values, and either reported as median $\pm$ IQR or mean $\pm$ SEM depending on the distribution. Spearman's correlation was used to assess the association between cytokine secretions. For suppression assays, mean percent of reduction between conditions was noted. Statistical significance was defined as *P* < 0.05.

Results

Lower lymphocyte proliferation, CD4⁺ CD25⁺ T cells and Foxp3 expression in CBMC after LpA/Ppg activation compared with adults

First, we determined general activation of the neonatal in comparison with the adult immune system by assessing lymphocyte proliferation and the activation marker CD25. We confirmed recent data (19) that lymphocyte proliferation of CBMC was lower compared with that of PBMC after mitogen PHA stimulation (Table 1). Extending these findings, we showed that proliferation to innate LpA/Ppg-stimulation was also reduced in CBMC compared to PBMC (Table 1). Analysing data with cpm including background proliferation showed comparable results (not shown). Interleukin-2 production in CBMC was undetectable before and not significantly elevated following stimulation (not shown). After mitogen-stimulation, CD4⁺ CD25⁺ T cells were significantly upregulated in CBMC and PBMC, while CD4⁺ CD25⁺ T cells were low in unstimulated cells. Following TLR4-stimulation (LpA), CD4⁺ CD25⁺ T cells (%) were not significantly changed (*P* = 0.14). After TLR2-stimulation (Ppg), CD4⁺ CD25⁺ T cells (%) were mildly increased in PBMC (*P* = 0.05), but not in CBMC (*P* = 0.80) compared to unstimulated cells. Comparing CBMC with PBMC, there was no significant difference in CD4⁺ CD25⁺ T cells (%) before and after stimulation (Table 1). CD4⁺ CD25⁺ T cells of Ppg-stimulated CBMC were slightly lower than in PBMC (*P* = 0.06).

CD4⁺ CD25⁺ positive T cells include Tregs (CD25⁺ high) and activated T cells. When assessing CD4⁺ CD25⁺ high populations, the results were similar to the data of CD4⁺ CD25⁺ T cells. However, the lower Ppg-induced difference in CBMC was not significant, whereas LpA-stimulated CD4⁺ CD25⁺ high populations were

now significantly lower in CBMC compared with PBMC ($P = 0.02$) (representative flow cytometry figures available on request).

We next determined whether the transcription factor Foxp3, to date the most specific factor for Tregs, was differently expressed after stimulation and varied between CBMC and PBMC. A higher Δ ct to 18S resembles lower Foxp3-expression (17). Foxp3-expression was upregulated following innate Ppg and particular PHA-stimulation in PBMC ($P = 0.05$, $P < 0.0001$), and for PHA-stimulation in CBMC ($P < 0.0001$, Table 1). Peptidoglycan-induced Foxp3-expression was significantly lower in CBMC compared with PBMC (Table 1). When adjusting for unstimulated Foxp3 expression, data did not change significantly (not shown). Intracellular Foxp3 staining of CD4⁺ CD25⁺ high cells was positive in 89.97% (± 3.30 SEM) for PHA, 86.56% (± 1.45 SEM) for LpA and 92.23% (± 0.75 SEM) for Ppg-stimulated T cells (representative flow cytometry figures available on request). Inclusion of the atopic status of the adults and mothers/fathers of the neonates in the analysis did not influence the comparison between adults and cord blood.

In summary, in CBMC proliferation, CD4⁺ CD25⁺ high and Foxp3-expression were significantly lower compared with the adult mature immune system. This effect was observed after PHA and innate LpA/Ppg stimulation, respectively.

Lower Th1 cytokine secretion in neonates in comparison with adults

Next, we examined whether the effector T-cell populations focusing on Th1/Th2 and Treg cells were different in CBMC and PBMC. Overall, mitogen-induced Th1 (IFN- γ), Th2 (IL-13, IL-5), IL-10 and pro-inflammatory cytokines were significantly upregulated in both ages with a Th2-emphasis in CBMC ($P < 0.001$, Table 2), consistent with previous studies (20). Furthermore, innate-induced Th1/Th2 and IL-10 were also significantly increased ($P < 0.001$). Not shown to date, primarily Th2 and TNF- α secretion were increased after innate stimulation in CBMC, and even significantly higher compared with PBMC regarding IL-5 following LpA/Ppg stimulation (Table 2). Interleukin-10, secreted by Th2 cells and Tregs, was significantly upregulated in both CBMC and PBMC following innate and adaptive stimulation, and significantly higher in PBMC compared with CBMC. Interleukin-4 production was undetectable in cord blood and thus not compared between CBMC and PBMC. Granulocyte macrophage-colony stimulating factor secretion was low, solely Ppg-stimulation induced increased secretion (Median-PBMC 87.7 vs CBMC 150.6 pg/ml) which did not differ significantly between groups ($P = 0.51$, not shown).

In summary, in comparison with the mature adult immune system, CBMC can produce Th2 cytokines,

Table 2. Cytokine concentrations in CBMC compared to PBMC

Cytokines (median + IQR, pg/ml)	Stimuli	PBMC (n = 18)	CBMC (n = 25)	P-value*
IFN- γ	U	1.79 (0.01/4.39)	0.01 (0.01/1.46)	0.04*
	PHA	369.1 (81.2/1381.6)**	29.6 (13.3 /108.3)**	<0.0001*
	LpA	5.07 (0.82/15.8)*	4.50 (2.36/13.2)**	0.98
	Ppg	60.3 (23.05/162.6)**	15.4 (8.72/38.4)**	0.01*
IL-13	U	1.47 (0.87/1.60)	0.32 (0.17/0.53)	<0.0001
	PHA	539.8 (225.6/832.5)**	461.7 (175.9/2374.9)**	0.67
	LpA	2.84 (1.09/7.80)*	3.45 (2.08/7.39)**	0.18
	Ppg	33.8 (19.3/52.8)**	30.2 (18.8/63.9)**	0.92
IL-5	U	0.21 (0.12/0.31)	0.18 (0.01/0.26)	0.14
	PHA	279.1 (172.0/499.2)**	27.9 (16.5/40.5)**	<0.0001*
	LpA	1.61 (0.62/4.09)**	7.9 (3.56/13.82)**	<0.0001*
	Ppg	11.1 (4.19/28.5)**	41.7 (21.27/70.3)**	0.001*
IL-10	U	0.12 (0.05/0.20)	0.16 (0.01/0.24)	0.81
	PHA	340.4 (204.0/601.0)**	57.5 (19.3/110.7)**	<0.0001*
	LpA	363.5 (286.6/440.7)**	166.7 (110.9/201.0)**	<0.0001*
	Ppg	1714.0 (1298.4/2433.0)**	1265.8 (632.8/1779.0)**	0.02*
TNF- α	U	1.58 (0.90/2.08)	0.97 (0.01/1.55)	0.07
	PHA	144.8 (69.2/440.8)**	419.7 (83.6/845.2)**	0.14
	LpA	370.1 (146.7/1243.0)**	865.1 (361.4/1542.8)**	0.21
	Ppg	1457.8 (886.8/2934.5)**	2229.6 (1402.5/3103.7)**	0.17

IQR, interquartile range; U, unstimulated; PHA, phytohemagglutinin; LpA, lipid A; Ppg, peptidoglycan.

P, comparison between PBMC and CBMC. *Comparison between stimulated and unstimulated data. * $P \leq 0.05$; ** $P \leq 0.001$, Mann-Whitney Rank Sum test.

IL-10 and pro-inflammatory cytokines following innate stimulation, but significantly lower Th1 cytokines compared with adults.

Less efficient suppressive capacity of CD4⁺ CD25⁺ Treg cells on CD4⁺ CD25⁻ T-effector cells in neonates compared with adults

To assess the effect of Tregs on the functional capacity of the less mature and Th2-predominant CBMC, we investigated suppression of isolated CD4⁺ CD25⁺ T cells on effector T cells. Parallel to low proliferation following innate stimulation (Table 1), division of effector cells was hardly detectable after Ppg/LpA immune stimulation (not shown). The suppressive capacity of CD4⁺ CD25⁺ T cells on division and proliferation of CD4⁺ CD25⁻ T-effector cells following mitogen-stimulation in neonates was present, but significantly less/half as effective than in adults, respectively (13.4 vs 60.9% reduction; 42.5 vs 83.9% reduction, Fig. 1A,B). Of note, baseline effector cell division and proliferation was higher in CBMC than PBMC.

Regarding Th1/Th2, IL-10 secretion following suppression by CD4⁺ CD25⁺ T cells, IFN- γ , IL-13 were significantly (both $P = 0.03$) and IL-5 nonsignificantly inhibited in adults, while IL-10 was nonsignificantly increased. Furthermore, TNF- α secretion was significantly suppressed in adults (Fig. 1C–G, left 3 panels).

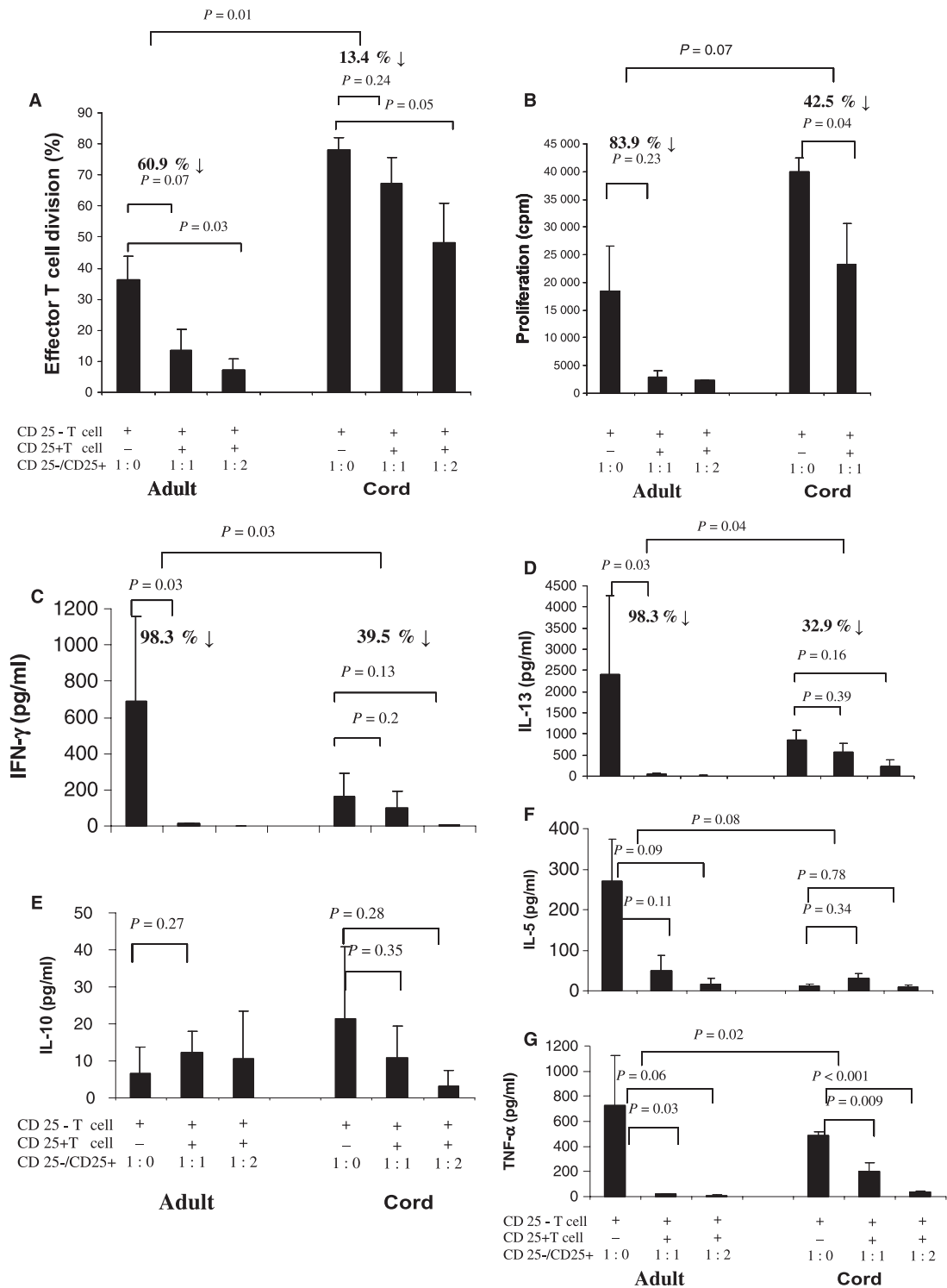


Figure 1. (A–G) The suppressive capacity of CD4⁺ CD25⁺ Tregs on CD4⁺ CD25⁻ T effector cells in PBMC and CBMC. (A/B) Cell division and thymidine proliferation of effector T cells after adding increasing amounts of CD4⁺ CD25⁺ T cells. (C–G) Cytokine secretion after coculture suppression assays with increasing amounts of CD4⁺ CD25⁺ T cells. (A–G) Left 3 panels adult, right 3 panels cord blood. CD4⁺ CD25⁻ T-effector cells (40 000 cells/well) labeled with CFSE, were cultured with irradiated CD3⁻ cells (80 000 cells/well) and stimulated with PHA (0.8 µg/ml), with/without CD4⁺ CD25⁺ Tregs (40 000/80 000 cells/well). After 72-h culture, percentage of new divided CD4⁺ CD25⁻ T-effector cells was measured with FACSscan, proliferation assessed with ³H Thymidin incorporation, cytokine secretion measured with Human Cytokine Multiplex Assay with LUMINEX technology (*n* = 5 PBMC and 5 CBMC).

In CBMC, the suppression of cytokine secretion was less effective for IFN- γ and IL-13 (Fig. 1C–G, right 3 panels). The highest decrease in cytokine secretion through CD4⁺ CD25⁺ T cells was demonstrated for TNF- α (Fig. 1G, right 3 panels). The suppressive function of CD4⁺ CD25⁺ T cells on cell division, proliferation, and cytokine secretion of CD4⁺ CD25⁺ T-effector cells increased with rising amount of CD4⁺ CD25⁺ T cells in CBMC and PBMC (Fig. 1A–G). Regarding the secretion of IL-10, this dose-dependent decrease was only seen in CBMC (Fig. 1E).

In summary, adults express a Th1-shifted phenotype, whereas CBMC express a more Th2-deviated cytokine pattern which is accompanied by lower proliferation, lower CD4⁺ CD25⁺ high expression, and less efficient suppressive function of CBMC.

Next, we examined an additional regulatory T-cell population, namely Th17 cells. It is unknown whether Th17 cells may be modulating Th1/Th2/Treg cells in the early immune system and whether this modulation is retained in the mature immune system.

Lower IL-17 expression in CBMC as compared with PBMC

In both ages, IL-17 secretion was undetectable in unstimulated cells. In adults, IL-17 was markedly increased following mitogen-stimulation and modestly elevated following Ppg-stimulation (Table 3A). In CBMC, IL-17 secretion was undetectable in PHA-stimulated cells, and significantly increased but low following innate stimulation (Table 3A). In parallel to Th1/Th2 (except IL-5) secretion, IL-17 secretion was significantly lower in CBMC compared with adults, after PHA/Ppg-stimulation (Table 3A). Intracellular IL-17-expression was undetectable in CBMC before and after stimulation with anti-human CD3/CD28 beads, IL-6/IL-1 β , and low in adults (up to 2%, Fig. 2A). Addition of TGF- β did not induce significantly higher, but rather decreased IL-17 production (data not shown).

Regarding potential suppression of IL-17 secretion by CD4⁺ CD25⁺ Tregs, IL-17 secretion, in parallel to Th1/Th2 cytokines, was inhibited in PBMC. However, this decrease required a higher dose of CD4⁺ CD25⁺ T cells (Fig. 2B), and was lower compared with IFN- γ /IL-13

suppression (Figs 1C,D and 2B). In CBMC, IL-17 secretion was too low to examine potential suppression.

We further assessed the correlation between IL-17 and Th2/Th1/IL-10 secretion in CBMC to identify a potentially changed cytokine pattern from the early to the mature immune system. In CBMC and PBMC, IL-13 and IFN- γ secretion were both positively correlated with IL-17 secretion following innate but not PHA-stimulation (Table 3B). Regarding IL-10, there were no significant correlations with IL-17 secretion at both ages. However, there were negative, yet nonsignificant correlations in CBMC for IL-17/IL-10 secretion. This may demonstrate a regulatory effect of Th17 cells in combination with Th1/Th2 lineages. In summary, Th17 cells were less expressed in cord than adult blood. At both ages, Th17 cells were correlated with Th1/Th2 cells after innate immune stimulation.

Discussion

This study demonstrates that the early immune system is able to exert suppressive capacities through regulatory T cells affecting T-cell division, proliferation and distinct T-cell cytokine secretion, yet less efficiently than the adult immune system. Both, adaptive and innate stimulation induced generally a less mature response as compared to adults. Furthermore, Th17 cells were expressed at low concentrations following innate stimulation in cord blood. At both ages, Th17 cells were correlated with Th1/Th2 cytokine secretion. Collectively, these data suggest a less mature but functioning early immune system involving primarily regulatory T cells and partly Th17 cells as potential modulators of early immune stimulation.

The hygiene hypothesis proposes that increased exposure to infections or microbial compounds in the environment might confer protection against the development of allergies (21–23) and that such increased exposures early in life may activate innate responses and subsequently influence healthy immune maturation (24, 25). In fact, it has repeatedly been shown that children growing up with high exposures to microbial stimuli, particularly in the first year of life, are at lower risk of allergies (1, 2). These epidemiological findings prompted us to mimic such early life exposures by stimulating CBMC also with innate TLR ligands and investigating their effects on T-cell subpopulations.

After adaptive stimulation, cord blood immune responses have been shown to be less mature than in adult life resulting in impaired defense strategies to combat foreign microorganisms and increased susceptibility to infections early in life (26). Consistent with data from Prescott et al. (20) demonstrating lower lymphocyte proliferation and Th1 cytokines following allergen/mitogen stimulation in cord blood, in this study lower proliferation, lower Th1 and higher Th2

Table 3A. Secretion of IL-17 in CBMC compared to PBMC

Cytokines (median + IQR, pg/ml)	Stimuli	PBMC (n = 18)	CBMC (n = 25)	P-value*
IL-17	PHA	710.01 (487.2/862.2)**	0.33 (0.01/0.84)	<0.0001*
	LpA	0.55 (0.01/5.35)	2.09 (0.73/3.62)**	0.40
	Ppg	53.72 (28.39/75.64)**	2.48 (1.52/9.73)**	<0.0001*

IQR, interquartile range; PHA, phytohemagglutinin; LpA, lipid A; Ppg, peptidoglycan.

*P, Comparison between PBMC and CBMC (Mann–Whitney Rank Sum test).

*Comparison to unstimulated data.

*P $\leq$ 0.05; **P $\leq$ 0.001 (Mann–Whitney Rank Sum test).

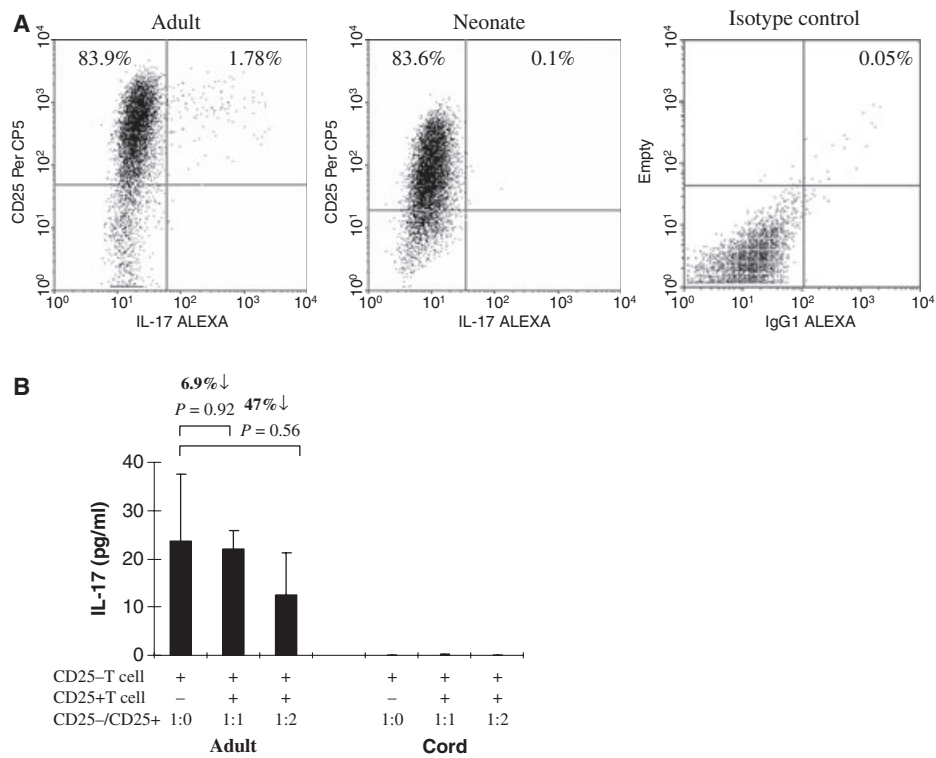


Figure 2. IL-17 protein expression in CBMC and PBMC and the suppressive capacity of CD4⁺ CD25⁺ Tregs on IL-17 secretion of CD4⁺ CD25⁻ T-effector cells. (A) Intracellular expression of IL-17 in PBMC (left), CBMC (middle) and isotype control (right). Cells, cultured with anti-human CD3/CD28 beads, IL-6 and IL-1 β for 72 h, were measured with FACScan. One representative sample out of 6 is shown. (B) IL-17 concentration in suppression assays in peripheral blood; not detectable in cord blood. CD4⁺ CD25⁻ T-effector cells (40 000 cells/well) labeled with CFSE, were cultured with irradiated CD3⁻ cells (80 000 cells/well), stimulated with PHA (0.8 μ g/ml), with/without CD4⁺ CD25⁺ Tregs (40 000/80 000 cells/well). After 72-h culture, IL-17 secretion was measured with the Human Cytokine Multiplex Assay Kit.

Table 3B. Correlation between IL-17 and Th1/Th2, IL-10 secretion

Cytokines	Stimuli	PBMC (n = 18)		CBMC (n = 25)	
		r	P-value	r	P-value
IL-17/IL-13	PHA	-0.003	0.98	-0.13	0.58
	LpA	0.77	<0.0001	0.46	0.02
	Ppg	0.56	0.04	0.62	<0.0001
IL-17/IFN- γ	PHA	0.01	0.95	0.29	0.18
	LpA	0.82	<0.0001	0.50	0.01
	Ppg	0.52	0.06	0.55	0.005
IL-17/IL-10	PHA	0.31	0.24	0.24	0.26
	LpA	0.15	0.54	-0.10	0.62
	Ppg	0.40	0.16	-0.09	0.67

Analysis using Spearman's correlation coefficient *r*.
PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan.

cytokines (IL-13, IL-5) were demonstrated after adaptive stimulation.

While adaptive immune stimulation is important for secondary responses, innate immune activation is particularly relevant not only for immediate protection against infections (27) but also for early life disease modification (2). In this study, not only adaptive but also innate

stimulation resulted in low proliferation and secretion of Th1/Th2 cytokines. Whether present but low T-cell function may still be sufficient for immuno-modulation needs to be further examined. We cannot explain the underlying mechanisms in this study; however, lower innate cord blood responses may be explained by delayed maturation of receptors such as TLR or the adaptor molecule MyD88. Functional consequences of neonatal TLR activation may also be very different to adults. For example, TNF- α and IL-10 secretion were markedly increased in this study, while IL-10 was lower compared with adults. Tumor necrosis factor- α and IL-10 can be produced by other cells including natural killer or B cells. To note, higher TNF- α following both Ppg and LpA stimulation in this study is in contrast to data about greater impairment in TLR2- rather than TLR4-mediated TNF-production (28, 29). One potential explanation may be contamination of Ppg with significant amounts of LPS. This explanation seems however unlikely as we excluded LPS contamination by Limulus assay and previous experiments using TLR2/4 knockout mice have also shown a specificity of the TLR2/4 stimuli (16). Another explanation may be different methods including distinct cell populations (CBMC), dosage, timing

and – though less likely – the use of LpA in comparison to LPS. Also, genetic differences of the study populations such as TLR4/TLR2 mutations cannot be excluded. Higher IL-5 expression in CBMC following innate but not mitogenic stimulation was interesting, and may be explained by an initial Th2-deviated immune system after birth, most prominent following innate immune activation.

Importantly, cytokine secretion beyond the Th1/Th2 lineages in early life such as IL-10, a marker of Th2 and Tregs among others, and expression of additional Treg markers following innate stimulation may point to an involvement of additional T-cell populations. Potential candidates keeping healthy immune maturation in balance are regulatory T cells.

Reduced suppressive capacities of regulatory T cells in CBMC

A significant number of Tregs have been described in the human fetus (19). Their early adequate function may critically influence healthy immune maturation. Thus, assessment at birth contributes to elucidate their functional capacity compared to adult life when disease pathology may be already established. In this study, Tregs were generally present and functional in CBMC following potent mitogen-stimulation, consistent with studies demonstrating functional Tregs in cord blood (19), with suppressor function after culture activation (30).

We have demonstrated two additional features. First, following mitogen-stimulation, Tregs were functioning but less suppressive in cord blood in contrast to adults. One explanation is that a majority of Tregs mature after birth, which is in parallel with data from Wing demonstrating that cord $CD4^+ CD25^+$ cells do not inhibit responses to self-antigens, compared with adult Tregs (31). Furthermore, insufficient Treg purity or additional nonsuppressive T-cell populations may result in reduced suppressive capacity. Because of limited cell numbers, we did not include CD127 as additional Treg marker, but have demonstrated a high percentage of $Foxp3^+ CD4^+ CD25^+$ high cells. In this study, a low percentage of additional $CD4^+$ cells may potentially impact suppressive capacities. However, we do not expect significant bias, as the purity was comparably high in adult and cord blood. Surprisingly, isolated T-effector cell division and proliferation were even higher in cord blood than in adults, in contrast to lower proliferation of total CBMC/PBMC. This may be because of a more active capacity in early life to respond to stimulation, however not quite as efficient in suppressive capacities as in adulthood.

Secondly, we compared the expression of Treg markers in CBMC and adult blood. Again similar results were found demonstrating present but lower expression of $CD4^+ CD25^+$ high cells and $Foxp3$ expression in cord blood compared with adults following innate stimulation. Differences in significance between $CD4^+ CD25^+$ cells

and $CD4^+ CD25^+$ high cells following Ppg and LpA-stimulation may be because of a higher amount of activated T cells ($CD4^+ CD25^+$) following Ppg stimulation. Nevertheless, data all point in the same direction. In summary, lower expression of $CD4^+ CD25^+$ high cells and $Foxp3$ expression may contribute to a less suppressive capacity of Tregs in cord blood compared with adults.

Role of Th17 cells in early life

Adding to the complexity of Th1/Th2/Treg cells, recent studies have defined a previously unknown $CD4^+$ effector T-cell lineage, IL-17-producing Th17 cells which develop via cytokine signals distinct from the Th1/Th2 and Treg lineages. Emerging evidence indicates that Th17 cells probably play a central role in inflammation, autoimmunity and allergy (32). Most knowledge about Th17 cells derives from murine studies, while human IL-17 responses have just been recently recognized (13, 33) and are yet not well defined early in life.

In this study, IL-17 responses were present at low levels in CBMC, and the correlation with Th1/Th2 cytokines was conserved in later life. Interestingly, IL-17 secretion was restricted to innate stimulation in cord blood. This in parallel with low Th1/Th2 cytokine secretion is an essential finding as neonates require adequate innate immune responses for immediate balanced immuno-protection. Thus, Th17 cells in cord blood may be involved in early immune regulation and maturation. Further, efficient innate responses have an impact on the development of subsequent adaptive responses.

One potential explanation for low IL-17 in cord blood is a delayed maturation of Th17 cells after birth as described for Tregs (34). Another possibility is that Th17 cells require additional specific stimulation to facilitate potent activation. Interestingly, murine Th17 development depends on the pleiotropic cytokine TGF- β which is linked to Treg development and function, providing a unique mechanism for matching $CD4^+$ T-cell effector and regulatory lineage specification (11). However, in this study, activation with anti-CD3/CD28 and IL-6 adding TGF- β did not induce significant IL-17 secretion. This is consistent with very recent human data (13, 33). While IL-10 secretion does not quite reflect Tregs, it is interesting that IL-17/IL-10 secretion early in life were negatively, though not significantly correlated after innate stimulation, suggesting that already early in life Th17/IL-10 regulation may be reciprocal as described by Mucida et al. (35).

In summary, the combination of low Th17 and Th1 cells, increased Th2 cytokine secretion in addition to less efficient suppression by Tregs in cord blood may set the stage for a vulnerable immune system early in life. Without additional innate stimulation, early immune responses may be more susceptible for default pathways

leading to disease development. Thus, innate immune stimuli such as high microbial exposures may be potential candidates driving the early immune system at a critical time to a 'protective' immune development. The exact immunological mechanisms probably involving regulatory and Th17 cells interacting in the balance of T-cell responses need to be investigated in longitudinal follow-up studies.

Acknowledgments

We thank the subjects of the study for participation, midwives and doctors of the gynaecology department for support regarding recruitment, Gabriele Sulski for excellent technical support, and Michaela Schedel for critical review of the manuscript. This work was supported by DFG, Friedrich Baur Institute, FoE FoLe (BS).

References

- Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;**347**:869–877.
- Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;**358**:1129–1133.
- Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* 2007;**7**:379–390.
- Adkins B. Peripheral CD4⁺ lymphocytes derived from fetal versus adult thymic precursors differ phenotypically and functionally. *J Immunol* 2003;**171**:5157–5164.
- Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, De Vries JE et al. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997;**389**:737–742.
- Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002;**195**:603–616.
- Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD et al. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 2001;**98**:2736–2744.
- Darrasse-Jeze G, Marodon G, Salomon BL, Catala M, Klatzmann D. Ontogeny of CD4⁺ CD25⁺ regulatory/suppressor T cells in human fetuses. *Blood* 2005;**105**:4715–4721.
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;**299**:1057–1061.
- Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4⁺ CD25⁺ regulatory T cells. *Nat Immunol* 2003;**4**:330–336.
- Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 2006;**24**:677–688.
- Ivanov S, Bozinovski S, Bossios A, Valadi H, Vlahos R, Malmhall C et al. Functional relevance of the IL-23-IL-17 axis in lungs in vivo. *Am J Respir Cell Mol Biol* 2007;**36**:442–451.
- Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007;**8**:950–957.
- Schaub B, Tantisira KG, Gibbons FK, He H, Litonjua AA, Gillman MW et al. Fetal cord blood: aspects of heightened immune responses. *J Clin Immunol* 2005;**25**:329–337.
- Schaub B, Campo M, He H, Perkins D, Gillman MW, Gold DR et al. Neonatal immune responses to TLR2 stimulation: influence of maternal atopy on Foxp3 and IL-10 expression. *Respir Res* 2006;**7**:40.
- Schaub B, Bellou A, Gibbons FK, Velasco G, Campo M, He H et al. TLR2 and TLR4 stimulation differentially induce cytokine secretion in human neonatal, adult, and murine mononuclear cells. *J Interferon Cytokine Res* 2004;**24**:543–552.
- Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Res* 1996;**6**:995–1001.
- Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;**6**:986–994.
- Takahata Y, Nomura A, Takada H, Ohga S, Furuno K, Hikino S et al. CD25⁺ CD4⁺ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene. *Exp Hematol* 2004;**32**:622–629.
- Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998;**160**:4730–4737.
- Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;**299**:1259–1260.
- Liu AH, Leung DY. Renaissance of the hygiene hypothesis. *J Allergy Clin Immunol* 2006;**117**:1063–1066.
- Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;**347**:911–920.
- Lauener RP, Birchler T, Adamski J, Braun-Fahrlander C, Bufe A, Herz U et al. Expression of CD14 and toll-like receptor 2 in farmers' and non-farmers' children. *Lancet* 2002;**360**:465–466.
- Ege MJ, Bieli C, Frei R, Van Strien RT, Riedler J, Ublagger E et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006;**117**:817–823.
- Delespesse G, Yang LP, Ohshima Y, Demeure C, Shu U, Byun DG et al. Maturation of human neonatal CD4⁺ and CD8⁺ T lymphocytes into Th1/Th2 effectors. *Vaccine* 1998;**16**:1415–1419.
- Krishnan S, Craven M, Welliver RC, Ahmad N, Halonen M. Differences in participation of innate and adaptive immunity to respiratory syncytial virus in adults and neonates. *J Infect Dis* 2003;**188**:433–439.

28. LeBouder E, Rey-Nores JE, Raby AC, Affolter M, Vidal K, Thornton CA et al. Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk. *J Immunol* 2006;**176**: 3742–3752.
29. Levy O, Zarembka KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF- α induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J Immunol* 2004;**173**:4627–4634.
30. Godfrey WR, Spoden DJ, Ge YG, Baker SR, Liu B, Levine BL et al. Cord blood CD4(+)CD25(+)-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 2005;**105**:750–758.
31. Wing K, Lindgren S, Kollberg G, Lundgren A, Harris RA, Rudin A et al. CD4 T cell activation by myelin oligodendrocyte glycoprotein is suppressed by adult but not cord blood CD25⁺ T cells. *Eur J Immunol* 2003;**33**:579–587.
32. Schnyder-Candrian S, Togbe D, Couillin I, Mercier I, Brombacher F, Quesniaux V et al. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 2006;**203**:2715–2725.
33. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007;**8**:942–949.
34. Witowski J, Ksiazek K, Jorres A. Interleukin-17: a mediator of inflammatory responses. *Cell Mol Life Sci* 2004;**61**:567–579.
35. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 2007;**317**:256–260.