

ORIGINAL ARTICLE

Reduced maternal regulatory T cell numbers and increased T helper type 2 cytokine production are associated with elevated levels of immunoglobulin E in cord blood

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Summary

Background There is evidence that the basis of an atopic-skewed immune response is acquired early in life, perhaps at the fetal stage. Thus, we hypothesized that the development of the fetal immune system might be influenced by maternal regulatory T cells (Treg) and maternal T cell cytokine production during pregnancy. The aim of the present study was to assess the influence of maternal Treg and cytokine production during pregnancy on Treg and atopy at birth.

Methods Within the mother–child study LINA (Lifestyle and Environmental factors and their Influence on Newborns Allergy risk), we determined the frequency and function of Treg and the total IgE concentration in pregnant women in the 34th week of gestation and in corresponding cord bloods at birth ($n = 24$). Furthermore, we assessed how maternal mitogen-induced T-helper type 1/T-helper type 2 and inflammatory cytokines influence the level of cord blood Treg and IgE.

Results Frequencies of CD4⁺CD25^{high} T cells were higher ($P = 0.001$), whereas percentages of FOXP3⁺ T cells were lower ($P < 0.001$) in cord blood cells compared with maternal blood.

Reduced maternal CD4⁺CD25^{high} Treg frequencies correlated with increased total IgE concentrations at the 34th week of gestation ($r = -0.32$, $P = 0.028$) and with increased IgE concentrations in cord blood ($r = -0.50$, $P < 0.001$). Elevated maternal mitogen-induced Th2 cytokine production was related to increased total IgE levels in the serum of corresponding cord bloods (IL-4, $r = 0.53$; IL-5, $r = 0.43$; IL-13, $r = 0.52$).

Conclusions Because cord blood IgE has been shown to be predictive for allergic diseases in early childhood, our results indicate that reduced maternal Treg numbers and increased Th2 cytokine production during pregnancy might influence the allergy risk of the child.

Keywords cord blood, maternal regulatory T cells, neonates, Th2 cytokines

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Introduction

The onset of an allergic reactivity seems to occur very early in life, and probably occurs sometime before birth. It has been shown that T-helper type 1/T-helper type 2 cytokine response profiles in cord blood have a predictive value for the development of allergic diseases later in life [1–3]. Specifically, reduced IFN- γ levels or increased IL-4 concentrations following phytohaemagglutinin (PHA)

stimulation in cord blood were associated with the development of atopic dermatitis and other allergic manifestations in early childhood [4, 5]. Furthermore, elevated IgE concentrations in cord blood may predict subsequent atopy [6, 7]. Thus, the immunological bias towards atopy or a protection against allergic diseases later in life might be established during *in utero* development of the fetal immune system. It has also been suggested that, in addition to genetic predisposition, the neonatal immune

response is primed by the environment *in utero* and that cytokines from the mother may have an impact on the development of children's T cell subsets [8–11]. Indeed, elevated maternal cytokine production during pregnancy has been related to the development of childhood allergic diseases. A stronger maternal Th2 immune response during the first trimester of pregnancy was associated with childhood wheezing and atopy at 3 years of age [12]. Moreover, a reduced maternal production of the Th2 antagonist IFN- γ was related to elevated production of the Th2 cytokine IL-13 in the child [9]. Recent findings demonstrate that maternal influence on priming of fetal immune system is also mediated at the cellular level. According to Mold et al. [13], maternal cells can cross the placenta and influence the regulation of immune responses after birth. It is also conceivable that regulatory T (Treg) cells that control immune balance [14, 15] may have a role in this process and that a reduced frequency or a perturbed function of Treg might be associated with the development or progression of atopic diseases [16]. The question that arises is whether Treg function and frequency in pregnancy influences the development of newborns' Treg state and the development of childhood atopic features. Few studies in humans showed quantitative and qualitative differences in the Treg cell population of adults and neonates [17, 18]. The novelty of our study lies in the fact that until now no other study has specifically investigated the relationship between Treg phenotype and function in pregnancy and corresponding cord bloods. Here, we report for the first time that maternal Treg and cytokine profile during pregnancy are involved in priming of the fetal immune system towards atopy.

Methods

For details, please see the methods section of the supplementary data.

Study population

The mother–child study LINA (Lifestyle and Environmental factors and their Influence on Newborns Allergy risk) is a birth cohort study designed to investigate the influence of prenatal life style and environmental factors on newborn allergic risk. Six hundred and twenty-two mother–child pairs were recruited from May 2006 to December 2008 in Leipzig, Germany. Maternal blood samples were collected during pregnancy at week 34 of gestation. Venous umbilical cord blood was obtained at delivery. Blood samples were analysed for cytokine and IgE concentration. In a randomly selected group (women $n = 54$, cord blood $n = 24$), the function and phenotype of Treg were tested.

Pregnant mothers gave informed consent at gestation week 34 and completed detailed questionnaires regarding housing conditions, family atopy history, smoking and

exposure to environmental tobacco smoke, exposure to household pets, professional life and lifestyle factors. The study was approved by the Ethics Committees of the University of Leipzig (file reference 046-2006).

Blood sampling

Heparinized peripheral (7 mL) and cord blood (maximum 7 mL) samples were obtained at the times indicated above and processed within 6 h for further analysis. For cytokine measurement, whole-blood samples (500 μ L) were stimulated *ex vivo* with PHA (50 μ g/mL; Sigma Aldrich, Munich, Germany) for 4 h at 37 °C. Thereafter, samples were diluted in a ratio of 1 : 1 with RPMI-1640 medium without supplements. After centrifugation, the cell-free supernatant was collected and stored at –80 °C until further analysis.

Functional analysis of regulatory T cells

The functional capacity of Treg was measured using *in vitro* proliferation assays. Peripheral or cord blood mononuclear cells (PBMC/CBMC) were isolated from heparinized whole blood by density gradient centrifugation using Ficoll-PaqueTM (Pharmacia, Uppsala, Sweden). CD4⁺CD25[–] T cells and CD4⁺CD25⁺ T cells were isolated using MACS technology (Miltenyi Biotech, Cologne, Germany) in a two-step procedure. Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labelled CD4⁺CD25[–] T cells with/without CD4⁺CD25⁺ T cells was analysed using flow cytometry (FACSCalibur; BD Bioscience, Heidelberg, Germany). Analysis was performed as shown in Fig. S2 of supporting information. Supernatants of all cultures were analysed for levels of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, MCP-1 (CCL2), IFN- γ and TNF- α by flow cytometry using the BD CBA Human Soluble Flex Set system (BD Bioscience).

Phenotype analysis of regulatory T cells

For measurement of Treg frequency, the phenotype of freshly isolated PBMC/CBMC was analysed by four-colour immunostaining. Immediately after isolation, 5×10^5 cells were washed [1 $\times$ phosphate-buffered saline (PBS) buffer, 2% fetal calf serum (FCS)] and incubated for 15 min at room temperature protected from light with anti-CD4-PC5 (BD Bioscience), anti-CD25-PE (Miltenyi Biotech) and anti-CD127-FITC (eBioscience, San Diego, CA, USA), antibodies. For intracellular staining of FOXP3, cells were fixed and permeabilized (Human FOXP3 Buffer Set; BD Bioscience) and incubated with anti-human FOXP3 monoclonal antibodies (BD Bioscience) for 30 min protected from light. After two washing steps, cells were analysed by flow cytometry using BD CellQuest (BD Bioscience) and Flow Jo software (Tree Star Inc., Ashland, OR, USA).

Cytometric bead array

Concentrations of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, MCP-1 (CCL2), IFN- γ and TNF- α in the supernatant from stimulated whole blood were measured by flow cytometry using the BD CBA Human Soluble Flex Set system (BD Bioscience), according to the manufacturer's instructions and as described elsewhere [19]. The detection limits were 3 pg/mL for each cytokine.

Immunoglobulin E and A measurement

Total IgE in the sera of mothers and children was determined by the Pharmacia CAP System (Pharmacia Diagnostics, Freiburg, Germany). Samples with a total IgE concentration of >0.7 or >20 kU/L (cut-off values) in cord blood and maternal blood, respectively, were regarded as enhanced [20]. IgA concentrations in cord bloods were measured using a NANORID™ kit (The Binding Site Group Ltd., Birmingham, UK) according to the manufacturer's instructions.

Statistical analysis

Data were generally not normally distributed and are presented as medians with 25th and 75th percentiles. For comparison of the phenotype and functional capacity of Treg between pregnant women and cord bloods, the non-parametric Wilcoxon's rank sum test was used. The χ^2 -test for cross relationship was used to compare the analysed sub cohort with the entire LINA cohort. IgE concentrations were dichotomized using the cut-off values for enhanced concentrations. The relation between Treg frequency/function in these groups was analysed using the Mann-Whitney *U*-test. Using a linear regression model, adjusted means ratios were calculated to determine the relation between maternal Treg frequency/function and maternal and cord blood IgE concentrations. Means ratios were adjusted for maternal atopy history, maternal education and cat ownership. Spearman's rank correlation was used to assess the association between maternal cytokine production and the frequency of Treg and IgE concentrations in maternal and cord blood. Cytokine concentrations below the detection limit were assigned a value of one half of the detection limit and were taken into account for calculation. Statistical analysis was performed with Statistica for Windows Version 8.0 [Statsoft Inc. (Europe), Hamburg, Germany].

Results

Characteristics of the study population

Six hundred and twenty-two mother-child pairs participated in the LINA study. We undertook analyses of Treg frequency and function in a sub cohort of 54 pregnant

women and 24 corresponding cord bloods. Complete data from 20 mother-child pairs were available for the functional analysis of Treg, and data from 24 mother-child pairs were available for phenotypic characterization of the Treg population. Measurements of IgA concentrations revealed that none of the cord bloods were contaminated with maternal blood (supporting information). The basic characteristics of the sub cohort are summarized in Table 1. There was no difference between the analysed sub cohort ($n = 24$) and the entire LINA cohort ($n = 622$) when the considered parameters were compared (Table 1). However, there were more participants with a positive maternal history of atopy ($P = 0.051$) in the analysed subgroup. Therefore, this potential confounding factor was considered in further analyses.

Comparison of the regulatory T cells phenotype from maternal and cord blood cells

To compare the phenotype of Treg cells from cord and maternal blood, we performed four-colour immunostaining in unstimulated, freshly isolated lymphocytes using anti-CD4, -CD25, -CD127 and -FOXP3 antibodies. The gating strategy used is presented in Fig. S1 of supporting information. The proportion of CD4⁺CD25^{high} T cells in cord blood was significantly higher compared with maternal cells (Table 2). However, the frequency of FOXP3⁺ T cells within the CD4⁺CD25^{high} population was significantly lower in cord blood compared with maternal blood (Table 2) and the proportion of CD127⁻ cells within the CD4⁺CD25^{high} population was at the same level in maternal and corresponding cord bloods. In trend, the frequency of maternal CD4⁺CD25^{high} Treg correlated with the frequency of CD4⁺CD25^{high} Treg cells in the corresponding cord bloods ($r = 0.37$, $P = 0.069$, from the Spearman's rank test).

Comparison of regulatory T cells functional capacity from maternal and cord blood cells

We next determined the *in vitro* regulatory function of the CD4⁺CD25⁺ population derived from maternal and cord blood samples by measuring the suppression of the proliferation of effector T cells (CD4⁺CD25⁻) and down-regulation of cytokine production in these cells after incubation with CD4⁺CD25⁺ T cells. The proliferation of CD4⁺CD25⁻ effector T cells induced by anti-CD2/CD3/CD28 mAb-coated beads was significantly higher in maternal samples (Table 2). In coculture with autologous CD4⁺CD25⁺ T cells, we measured on average 86% inhibition of proliferation in maternal samples. The inhibitory capacity was slightly decreased in cord blood cells (Table 2). The suppressive effect of CD4⁺CD25⁺ T cells on cytokine production by CD4⁺CD25⁻ effector cells was analysed in the supernatant after 5 days of incubation. In

maternal samples, significantly reduced concentrations of IL-5, IL-8, IL-10 and IL-13 were detected in coculture experiments compared with the supernatants of effector T cells (Table 3). In contrast, in maternal as well as in cord blood samples, IL-6 was slightly increased in coculture experiments compared with the supernatants of effector cells. Because of the low amount of cytokine production by CD4⁺CD25⁺ effector cells isolated from cord blood, the suppressive capacity of CD4⁺CD25⁺ T cells was determined only for IL-6 and IL-8 concentrations (Table 4).

Table 1. Characteristics of analysed subcohort ($n = 24$) and entire LINA cohort ($n = 622$)

Parameters	Analysed subcohort n (%) $N = 24$	Entire LINA cohort n (%) $N = 622$	P -value*
Gender child			
Male	11 (45.8)	316 (50.2)	0.778
Female	9 (37.5)	294 (46.7)	
Maternal school education [†]			
Low	1 (4.1)	24 (3.8)	0.581
Intermediate	5 (20.8)	189 (30.0)	
High	18 (75.0)	401 (63.7)	
Maternal history of atopy			
Yes	16 (66.6)	292 (46.4)	0.051
No	8 (33.3)	337 (53.6)	
IgE (kU/mL) in serum, median (IQR)			
Maternal peripheral blood	17.55 (5.10–83.70)	25.0 (10.0–84.8)	0.977
Cord blood	0.12 (0.04–0.39)	0.19 (0.08–0.45)	
Pet keeping			
None	14 (58.3)	358 (56.9)	0.507
Cat	3 (12.5)	113 (18.0)	
Dog	5 (20.8)	70 (11.1)	
Others	2 (8.3)	68 (10.8)	

* P -value from the χ^2 test for cross table relationship.

[†]Low, 9 years of schooling or less 'Hauptschulabschluss'; intermediate, 10 years of schooling 'Mittlere Reife'; high, 12 years of schooling or more 'Fach-/Hochschulreife'; Significant values ($P < 0.05$) are in bold.

Because of missing information for some characteristics number of subjects varies.

LINA, Lifestyle and Environmental factors and their Influence on Newborns Allergy risk.

Relationship between frequency and functional capacity of maternal CD4⁺CD25^{high} regulatory T cells and immunoglobulin E concentration in maternal and cord blood

Allergies are frequently characterized by an activation of the immune system and the production of IgE antibodies. We aimed to investigate the influence of maternal Treg during pregnancy on IgE levels in maternal and in corresponding cord bloods. Reduced maternal CD4⁺CD25^{high} Treg frequencies correlated with increased total IgE concentrations at the 34th week of gestation ($r = -0.32$, $P = 0.028$, from the Spearman's rank test) and with increased IgE concentrations in cord blood ($r = -0.50$, $P < 0.001$, from the Spearman's rank test). When IgE concentrations were dichotomized using the cut-off values of 20 kU/L for adults and 0.7 kU/L for cord bloods [20], reduced frequencies of maternal Treg were found in the groups with increased maternal IgE concentrations (> 20 kU/L) and increased IgE cord blood concentrations (> 0.7 kU/L) (Fig. 1). In order to consider factors known to influence the allergic sensitization, we calculated mean ratios adjusted for maternal history of atopy, maternal education and cat ownership. The results from this model showed that high maternal frequencies of Treg may have a protective effect on IgE production for themselves and in offspring (adjusted means ratios 0.65, 95%CI 0.46–0.93 and 0.55, 95%CI 0.31–0.97, respectively). In contrast, analysis of raw data level and after adjustment revealed that the functional capacity of maternal Treg had no effect on IgE concentrations in mothers and corresponding cord bloods (data not shown).

Relationship between maternal cytokine production and frequencies of regulatory T cells and immunoglobulin E concentrations in maternal and cord blood

Maternal cytokine concentrations in mitogen-stimulated whole blood are shown in Table S1. Pregnant women with a high *ex vivo* production of PHA-induced IL-5 and MCP-1 (CCL2) had significantly reduced frequencies of CD4⁺CD25^{high} Treg cells (Table 5). Furthermore, increased concentrations of MCP-1 (CCL2) were related to higher IgE

Table 2. Comparison of Treg function and phenotype of pregnant women (PBMC) and cord blood (CBMC)

Parameters	Maternal blood	Cord blood	P -value*
Function	$n = 20$	$n = 20$	
Proliferation of effector T cells (%)	43.56 (16.89–56.40)	13.64 (3.57–26.50)	0.003
Suppression of effector T cell proliferation (%)	85.89 (66.66–91.13)	73.08 (67.26–92.23)	
Phenotype	$n = 24$	$n = 24$	
CD4 ⁺ CD25 ^{high} of CD4 (%)	2.49 (1.60–4.05)	3.91 (3.14–4.91)	0.001
CD127 ⁺ of CD4 ⁺ CD25 ^{high} (%)	92.81 (90.11–95.56)	94.10 (89.89–95.62)	
FOXP3 ⁺ of CD4 ⁺ CD25 ^{high} (%)	45.01 (34.25–56.57)	27.05 (18.25–30.80)	0.008

* P -value from Wilcoxon's test. Significant values ($P < 0.05$) are in bold.

Shown are medians and the 25th and 75th percentile.

Table 3. Cytokine concentration in supernatant of CD4⁺CD25⁻ T cell culture compared with coculture of CD4⁺CD25⁻/CD4⁺CD25⁺ T cells isolated from PBMC of pregnant women (*n* = 42)

Cytokine	CD4 ⁺ CD25 ⁻ T cells (<i>n</i> = 42)	CD4 ⁺ CD25 ⁻ /CD4 ⁺ CD25 ⁺ (<i>n</i> = 42)	<i>P</i> -value*
IL-4	3.59 (2.20–6.45)	3.95 (2.29–5.71)	0.171
IL-5	7.61 (1.95–13.06)	3.53 (1.50–9.79)	0.005
IL-6	2.24 (1.57–4.05)	3.03 (1.50–11.67)	0.006
IL-8	66.97 (27.73–174.26)	41.36 (12.25–77.14)	<0.001
IL-10	6.15 (1.50–17.32)	5.44 (1.63–9.43)	0.015
IL-13	55.61 (13.80–78.18)	28.97 (14.31–57.93)	0.003
IFN- γ	43.41 (30.59–70.52)	46.40 (27.80–74.26)	0.935
TNF- α	3.75 (2.53–5.79)	3.96 (2.75–6.00)	0.701
MCP-1 (CCL2)	nd [#]	nd	

**P*-value from Wilcoxon's test. Significant values (*P* < 0.05) are in bold. Shown are medians and the 25th and 75th percentile. nd, not defined.

Table 4. Cytokine concentration in supernatant of CD4⁺CD25⁻ T cell culture compared with coculture of CD4⁺CD25⁻/CD4⁺CD25⁺ T cells isolated from cord blood (*n* = 20)

Cytokine	CD4 ⁺ CD25 ⁻ T cells (<i>n</i> = 20)	CD4 ⁺ CD25 ⁻ /CD4 ⁺ CD25 ⁺ (<i>n</i> = 20)	<i>P</i> -value*
IL-6	1.50 (1.50–2.20)	2.56 (1.50–7.28)	0.009
IL-8	48.47 (24.85–104.68)	55.90 (26.02–70.22)	0.178

**P*-value from Wilcoxon's test. Significant values (*P* < 0.05) are in bold. Shown are medians and the 25th and 75th percentile.

concentrations in maternal blood. Children from mothers with high PHA-induced IL-4, IL-5, IL-13 and TNF- α production had increased IgE concentrations in cord blood (Table 5). Treg frequencies in cord blood were not related to maternal cytokine production (Table 5). Furthermore, the maternal cytokine production was neither associated with the functional capacity of maternal Treg nor with the Treg from cord blood.

Discussion

There is evidence that the imprinting of a child's immune system is affected during the entire prenatal period by the mother's immune system [13]. Based on our study, we speculate that maternal Treg and Th2 cytokines are involved in the development of an IgE response in newborn infants. Treg are very important in controlling allergy, maternal tolerance to the fetus, autoimmunity and tumour immunity [14]. To investigate differences of function and phenotype of maternal Treg and Treg in the cord blood of their neonates, we compared effector T cell proliferation, functional capacity of Treg and the expression of CD4, CD25, CD127 and FOXP3 in PBMC and corresponding CBMC. Recently published data have shown that the amount of CD4⁺CD25^{high} Treg cells in

cord blood is higher than in adults' peripheral blood [17, 21, 22]. This was confirmed by our data in the present study. In contrast, the frequency of FOXP3⁺ cells in the CD4⁺CD25^{high} Treg population was lower in cord blood compared with corresponding maternal blood. This is in line with the finding that at the protein level, unstimulated CD4⁺CD25⁺ cord blood cells have a low expression of FOXP3 compared with adult cells [17]. Similarly, it has been shown that FOXP3 mRNA expression is lower in CBMC in comparison with adults PBMC following innate stimulation [18]. Thus, our results from mother-child pairs indicating a reduced frequency of FOXP3 expressing cells in CBMC support the data from these previous findings.

Naturally occurring regulatory T cells are CD4⁺CD25⁺. However, as CD25 is also expressed on activated T cells, it is not a specific marker for Treg. FOXP3 has a pivotal role in Treg development and function [23], and at present, is thought to be the most but not entirely specific marker for human Treg. Recently, the IL-7 receptor α chain (CD127) was identified as a marker to distinguish Treg from activated T cells. In contrast to activated T cells, Treg display a low level of expression of CD127 [24–26]. In the present study, there was no difference between maternal and cord blood Treg frequencies. In addition, we found no relationship between the frequency of FOXP3⁺ cells or the frequency of CD127^{low}-expressing cells in maternal and cord blood and the functional capacity of Treg. The functional capacity of cord blood Treg was not significantly reduced compared with maternal blood as determined by the suppression of effector T cell proliferation. This can be explained by the fact that effector T cells from cord blood had a low capacity to proliferate (only 13.64%). The suppression of these low proliferating cells perhaps does not necessitate Treg with a strong inhibitory capacity, resulting in similar suppression indexes as in adults. Using different stimuli for the induction of effector T cell proliferation, Schaub et al. [18] demonstrated an impairment of Treg function at birth.

Maternal Treg had a strong capacity to inhibit effector T cell proliferation and the production of IL-5, IL-8, IL-10 and IL-13. Interestingly, IFN- γ and TNF- α production was not affected, whereas IL-6 concentrations were slightly increased in coculture experiments. Furthermore, the suppressive capacity of Treg did not correlate with IgE serum levels and it was not related to maternal atopy reported by questionnaires (see supporting information). A possible explanation for these results is the fact that the suppressive capacity of Treg clearly depends on the stimulation conditions of effector T cells [27]. In other publications, the suppressive capacity of Treg correlated with atopy under Staphylococcal endotoxin B or PHA stimulation conditions [28, 29].

In contrast, in our analyses, IgE levels were related to the proportion of CD4⁺CD25^{high} Treg cells, i.e. mothers

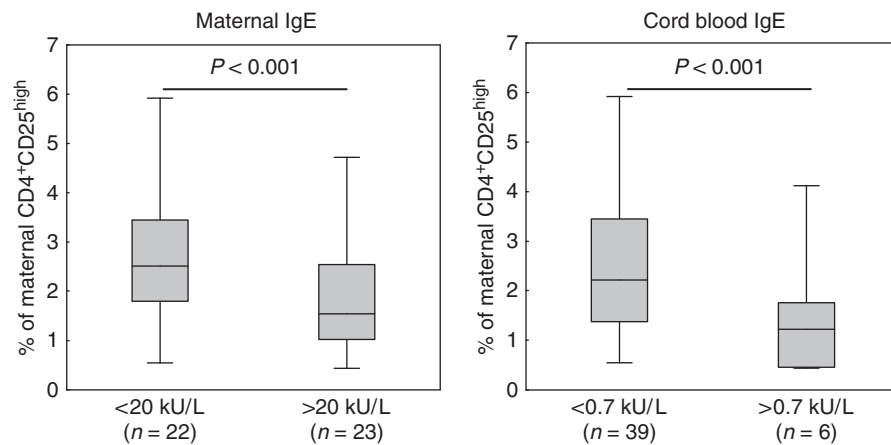


Fig. 1. Relationship between frequency of maternal CD4⁺CD25^{high} Treg and IgE concentration in maternal serum and in corresponding cord bloods. Box plots represent medians and the 25th and 75th percentile. *P*-values were calculated using Mann–Whitney rank sum test.

Table 5. Relation between maternal cytokines and frequency and function of T_{reg} and IgE concentration in blood samples of pregnant women and corresponding cord bloods

Maternal cytokine [†]	<i>r</i> , <i>P</i> -value*	CD4 ⁺ CD25 ^{high} (%) (<i>n</i> = 24)		Suppression of effector T cell proliferation (%) (<i>n</i> = 20)		IgE concentration (kU/L) (<i>n</i> = 24)	
		Cord blood	Maternal blood	Cord blood	Maternal blood	Cord blood	Maternal blood
IL-4	<i>r</i>	0.10	−0.10	0.08	0.39	0.53	0.30
	<i>P</i>	0.632	0.634	0.733	0.087	0.007	0.144
IL-5	<i>r</i>	−0.26	−0.50	0.31	−0.18	0.43	0.28
	<i>P</i>	0.209	0.012	0.169	0.441	0.034	0.175
IL-6	<i>r</i>	−0.05	−0.20	−0.10	−0.03	0.16	0.09
	<i>P</i>	0.799	0.328	0.655	0.882	0.434	0.647
IL-8	<i>r</i>	−0.02	−0.006	−0.004	−0.18	0.16	0.10
	<i>P</i>	0.910	0.977	0.984	0.423	0.435	0.613
IL-10	<i>r</i>	0.02	−0.10	0.13	0.17	0.03	−0.06
	<i>P</i>	0.916	0.618	0.566	0.467	0.859	0.766
IL-13	<i>r</i>	0.22	−0.14	0.05	0.01	0.52	0.36
	<i>P</i>	0.280	0.513	0.822	0.934	0.009	0.083
IFN-γ	<i>r</i>	−0.18	−0.22	0.02	−0.15	0.34	0.25
	<i>P</i>	0.394	0.288	0.909	0.522	0.100	0.231
TNF-α	<i>r</i>	−0.23	−0.30	−0.20	−0.21	0.42	0.27
	<i>P</i>	0.260	0.148	0.394	0.379	0.039	0.186
MCP-1 (CCL2)	<i>r</i>	−0.31	−0.41	−0.24	0.38	0.34	0.43
	<i>P</i>	0.134	0.042	0.089	0.291	0.103	0.031

**r*, *P*-value from Spearman's rank correlation.

[†]Analysis performed in phytohaemagglutinin (PHA)-stimulated blood samples.

Significant values (*P* < 0.05) are in bold.

and children from mothers with high numbers of Treg had reduced IgE concentrations. *In vitro*, maternal Treg reduced the production of Th2 cytokines IL-5 and IL-13 by effector T cells. It is conceivable that *in vivo*, these cells may act in the same way, thereby inhibiting the production of IgE antibodies. Moreover, the direct influence of Treg on B cells and the induction of IgG4 and suppression of IgE have been demonstrated in cell cultures of peripheral blood cells of healthy individuals [30]. Thus, there is strong evidence that Treg can dampen the development of IgE-mediated allergic diseases. The mechanism by which

maternal Treg might regulate IgE concentrations found in fetal serum is not clear. It is known that maternal Treg can migrate from the peripheral blood to the decidua in human pregnancy. In addition, a significantly higher percentage of CD4⁺CD25^{high} T cells is present in decidual tissue compared with maternal blood [31]. Certainly, the main role of these cells here is to provide tolerance to fetal alloantigens. Maternal cells can cross the placenta to reside in fetal lymph nodes, inducing the development of Treg that suppress fetal anti-maternal immunity [13]. It is also possible that maternal Treg may migrate into the

fetus and exert regulative activities in the process of allergic sensitization. Finally, the IgE concentrations in cord blood might reflect the IgE concentrations in maternal blood. It has been shown that allergen-specific IgE in cord blood does not reflect intrauterine sensitization but seems to result from transfer of maternal IgE to the fetus [32]. Thus, in our study, mothers with high numbers of Treg and reduced total IgE blood concentrations might transfer only low amounts of IgE to the fetus. This is supported by the fact that in our study maternal IgE concentrations highly correlated with cord blood IgE levels ($r = 0.72$, $P < 0.001$, from Spearman's rank test).

In addition, mothers with high percentages of Treg had lower blood concentrations of IL-5 and MCP-1 (CCL2) after PHA stimulation. Thus, a protective cytokine milieu with lower pro-allergic cytokines may also lead to reduced IgE blood concentrations in mother and child. Obviously, maternal cytokines can influence fetal immune regulation [9]. It has been shown that decidual cytokine production influences the cord blood cytokine production [33]. We aimed to analyse the relation between maternal blood cytokine production and Treg and total IgE production in mother and cord blood. Reduced blood IL-5 and MCP-1 (CCL2) levels after PHA stimulation were related to higher maternal Treg percentages. The levels of maternal blood cytokines were neither related to frequency nor to the functional capacity of neonates' Treg. On the contrary, we found a significant correlation between PHA-induced maternal IL-4, IL-5 and IL-13 production and elevated IgE concentrations in cord, but not in maternal blood. In mothers, only the chemokine MCP-1 (CCL2), which is known to contribute to allergic inflammation [34, 35], was related to higher IgE concentrations. Thus, children from mothers with a Th2-skewed immune response are possibly at a higher risk of developing atopic diseases in later life, indicating that maternal cytokines, besides Treg, may play instructive roles in the development of the fetal immune system.

The strength of this study is that for the first time, we analysed Treg in pregnant women and corresponding cord bloods after delivery in a well-documented cohort. We emphasize that the Treg phenotype was analysed in freshly isolated blood cells without further *in vitro* manipulation and that maternal cytokine production was determined *ex vivo* as it more closely reflects the *in vivo* situation. Our study has the limitation that Treg were only analysed in a small subgroup of mothers and corresponding cord bloods. Furthermore, the number of newborns with enhanced levels of total IgE within this group was low. Our results therefore have to be interpreted with caution, but nevertheless they confer direction and facilitate further studies.

In summary, we demonstrate that children from mothers with low percentages of Treg and a Th2-skewed immune response are born with elevated IgE levels. Long-

itudinal follow-up studies into childhood are underway to monitor the development of IgE levels (total and specific IgE), cytokine production, Treg marker expression and other relevant parameters for the development of allergic diseases.

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Supporting Information

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

Fig. S1. Four colour flowcytometric Treg analysis of maternal blood (PBMC) and cord blood (CBMC). After lymphocyte gating, cells were gated for the expression of CD4 and CD25^{high}. Within this population frequency of CD127^{low} and FOXP3 expressing cells was analysed. Two representative plots from maternal and cord blood are shown.

Fig. S2. Functional capacity of Treg cells was measured by *in vitro* proliferation assay using CFSE labelling. CD4⁺CD25[–] effector T cells and CD4⁺CD25⁺ Treg cells were isolated from PBMC/CBMC in a two step procedure as described in the methods and cocultured at a ratio of 1 : 1. Proliferation of CD4⁺CD25[–] effector T cells without and in coculture with CD4⁺CD25⁺ Treg cells was assessed flowcytometrically. One representative experiment of 20 is shown (maternal sample).

Fig. S3. Purity of isolated CD4⁺CD25⁺ T cells. CD4⁺CD25⁺ T cells were isolated from human PBMC/CBMC by a two step procedure as described in the methods. To control isolation purity of CD4⁺CD25⁺ T cells, cells were stained with anti-CD4-PC5 and anti-CD25-PE antibodies.

Table S1. Comparison of cytokine production in unstimulated and PHA stimulated samples of maternal peripheral blood. Shown are medians and 25th and 75th percentile.

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Supplementary data

Methods

Study population

The mother-child study LINA (Lifestyle and Environmental factors and their Influence on Newborns Allergy risk) is a birth cohort study designed to investigate the influence of prenatal life style and environmental factors on newborn allergy risk. Six hundred and twenty-two mother-child pairs were recruited from May 2006 to December 2008 in Leipzig, Germany. Maternal blood samples were collected during pregnancy at week 34 of gestation. Venous umbilical cord blood was obtained at delivery. Blood samples were analysed for cytokine and IgE concentration. In a randomly selected group (pregnant women n = 54, cord blood n = 24) function and phenotype of regulatory T cells was tested. For reasons of standardisation Treg analysis have been performed from freshly drawn human blood within 6 hours. Analysis of blood samples from the cord within 6 hours was not possible in all cases. To gain comparable results cord blood samples older than 6 hours were excluded from Treg analysis and data of only 24 mother-child-pairs could be analysed.

Pregnant women gave informed consent at gestation week 34 and completed detailed questionnaires regarding housing conditions, family atopy history, smoking and exposure to environmental tobacco smoke, pet keeping, professional life and life style factors. The study was approved by the Ethics Committees of the University of Leipzig (file reference 046-2006).

Blood Sampling

Heparinized peripheral (7 ml) and cord blood (max. 7 ml) samples were obtained at times indicated above and processed within 6 hours to further analysis. Detection of cytokine concentrations was performed in heparinised whole blood (500 µl), stimulated with PHA (50 µg/ml, Sigma Aldrich, Munich, Germany). After 4h of incubation, blood samples were

diluted 1:1 with RPMI1640 without supplements and centrifugated. Cell free supernatant was stored at -80°C until analysis. Table 1 of the supplementary includes cytokine concentration values in the maternal PHA and unstimulated samples. The medium only control was not subtracted from the PHA stimulated values.

Functional analysis of Treg cells

Functional capacity of Treg was measured by *in vitro* proliferation assays. Peripheral or cord blood mononuclear cells (PBMC/CBMC) were isolated from heparinized whole blood by density gradient centrifugation using Ficoll-Paque™ (Pharmacia, Uppsala, Sweden). CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells were isolated using MACS technology (Miltenyi Biotech, Cologne, Germany) in a two step procedure. After depletion of non-CD4⁺ T cells, a positive selection of the CD4⁺CD25⁺ cell fraction from pre-enriched CD4⁺ T cells followed. The purity of isolated CD4⁺CD25⁺ T cells was above 95% (Figure 2 supplementary). CD4⁺CD25⁻ (5x10⁴/well) T cells were labelled with 5µmol/L Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Karlsruhe, Germany) and cultured using RPMI medium supplemented with 5% heat-inactivated FCS in 96well U-bottom plates without or in co-culture with CD4⁺CD25⁺ Treg cells in a ratio of 1:1 (2.5x10⁴/well, respectively) for 5 days at 37°C. Cells were stimulated by MACSiBead Particles coated with anti-CD2,-CD3,-CD28 antibodies (Miltenyi Biotech, Cologne, Germany). Proliferation of CD4⁺CD25⁻ T cells with/without CD4⁺CD25⁺ T cells was analysed using flow cytometry (FACSCalibur, BD Bioscience, Heidelberg, Germany). Analysis was performed as shown in Figure 2 of the supplementary data. Supernatants of all cultures were analysed for IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, MCP-1 (CCL2), IFNγ and TNFα by flow cytometry using the BD CBA Human Soluble Flex Set system (BD Bioscience, Heidelberg, Germany).

Phenotype analysis of Treg

For analysis of Treg frequency, the phenotype of freshly isolated PBMC/CBMC was analysed cytometrically by 4 colour staining. 5×10^5 cells were washed (1xPBS buffer, 2% heat-inactivated FCS) and incubated for 15 min at room temperature protected from light with 2 μ L antihuman CD4-PC5 (clone 13B8.2, BD Bioscience, Heidelberg, Germany), antihuman CD25-PE (clone 4E3, Miltenyi Biotech, Cologne, Germany) and antihuman CD127-FITC (clone eBio RDR5, eBioscience, San Diego, USA), antibodies, respectively. For intracellular staining of FOXP3, cells were fixed and permeabilized (Human FOXP3 Buffer Set, BD Bioscience, Heidelberg, Germany) and incubated with 15 μ L antihuman FOXP3-Alexa647 monoclonal antibodies (clone 259D/C7, BD Bioscience, Heidelberg, Germany) or 2 μ L mouse IgG₁ κ Alexa647 isotype control (clone MOPC-21, BD Bioscience, Heidelberg, Germany) 30 min protected from light. After two washing steps, cells were analysed by flow cytometry using BD CellQuest (BD Bioscience, Heidelberg, Germany) and Flow Jo software (Tree Star, Inc. Ashland OR, USA).

For T reg phenotype analysis a sequential gating was performed as follows: after lymphocyte gating, first gate was set on CD4⁺CD25^{high} cells. The gate for CD4⁺CD25^{high} cells was set in relation to CD127 expression in the way that this population encompasses no or only few cells expressing the CD127 marker. As shown in table 2 this population was 93% CD127 negative in maternal and 94% negative in cord blood samples. The CD4⁺CD25^{high}CD127^{-/low} population was then analysed for FOXP3 expression. These cells were considered as T reg.

Cytometric bead array

Concentrations of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, MCP-1 (CCL2), IFN- γ and TNF- α in supernatant of stimulated whole blood were measured by flow cytometry using the BD CBA Human Soluble Flex Set system (BD Bioscience, Heidelberg, Germany), according to the manufacturer's instructions and as described elsewhere (18). In brief, cytokine specific

antibody-coated beads were incubated for one hour with 25µl of blood samples or standard solution. Thereafter, samples were incubated with the corresponding phycoerythrin (PE)-labelled detection antibodies for 2 h. After one washing step, samples were measured by flow cytometry. Analysis of data and quantification of cytokines were performed using the FCAP Array™ software (BD Bioscience, Heidelberg, Germany) on the basis of corresponding standard curves. Finally plasma dilution factor was determined. The detection limits were 3pg/ml for each cytokine.

IgA measurement in cord bloods

IgA concentrations in cord blood were measured using a NANORID™ kit (The Binding Site Group Ltd., Birmingham, UK) according to manufactures instruction (Procedure One). This is a radial immunodiffusion (RID) assay which is based on the complexation of antigen and antibody to produce a visible precipitin ring and enables accurate quantitation of very low concentrations of protein in serum (sensitivities as low as 0.15 mg/ml). In brief, 20 µl of serum cord blood samples, controls and calibrator were added to the RID plates and incubated for 96 h at room temperature. After completion, ring diameter was measured and protein concentrations were calculated using a reference table.

Results

Influence of maternal atopy on Treg numbers/function and cytokine production

As IgE concentrations and % of suppression are not normally distributed we performed a Spearman Rank correlation which revealed that neither in maternal samples nor in cord blood samples the maternal atopy was related to suppression of proliferation ($r = 0.18$, $P = 0.454$ for mothers, $r = 0.09$, $P = 0.698$ in cord bloods). Using the dichotomised maternal IgE data, we analysed whether high maternal IgE levels (>20 kU/l IgE) had an influence on T reg suppression capacity from themselves and their offspring. We found no relation neither to

maternal nor to cord blood Treg suppressive capacity $P = 0.229$ and $P = 0.260$, respectively, from Mann Whitney U Test).

Maternal history of atopy (information from questionnaire) was associated with reduced maternal Treg numbers ($P = 0.03$), increased PHA-induced IL-4 ($P < 0.001$) and IL-6 concentrations ($P = 0.019$) in maternal blood. There was no correlation between maternal history of atopy and Treg numbers in cord blood ($P = 0.88$). (P -values from Mann-Whitney U Test).

IgA concentrations in cord bloods

On average the mean concentrations in our cord blood samples were 4.07 (SD 1.57) mg/l. According to Pfefferle et al, 2008, values higher than 32 mg/l were classed as contamination by maternal blood. Ownby et al. 1996, considered IgA values above 10 mg/l as contamination by maternal blood. Thus, in any case our cord bloods IgA concentrations are below these values indicating that the samples were not contaminated by maternal blood.

Table 1 supplementary. Comparison of cytokine production in unstimulated and PHA stimulated samples of maternal peripheral blood. Shown are medians and 25th and 75th percentile.

Maternal Cytokines	Stimuli	Cytokine concentration (pg/ml) (n = 24)
IL-4	U* PHA [#]	4.68 (2.23-8.81) 10.63 (7.22-12.55)
IL-5	U PHA	n. d. ^π 1.50 (1.50-3.22)
IL-6	U PHA	11.18 (8.12-16.54) 13141.29 (11640.94-15333.86)
IL-8	U PHA	68.67 (47.17-106.03) 5874.06 (4750.27-7759.87)
IL-10	U PHA	3.80 (2.89-4.38) 43.14 (34.16-57.18)
IL-13	U PHA	1.50 (1.50-5.95) 16.55 (10.68-20.34)
IFN-γ	U PHA	5.88 (1.50-8.64) 327.39 (199.87-558.73)
TNF-α	U PHA	7.71 (6.94-11.73) 3641.28 (2964.10-4501.22)
MCP-1 (CCL2)	U PHA	82.41 (71.60-93.67) 1692.75 (1248.42-2073.94)

*U, unstimulated

[#]PHA, phytohemagglutinin

^π n. d., not detectable

Figure 1 supplementary. Four colour flowcytometric Treg analysis of maternal blood (PBMC) and cord blood (CBMC). After lymphocyte gating, cells were gated for the expression of CD4 and CD25^{high}. Within this population frequency of CD127^{low} and FOXP3 expressing cells was analysed. Two representative plots from maternal and cord blood are shown.

Figure 2 supplementary. Functional capacity of Treg cells was measured by *in vitro* proliferation assay using CFSE labelling. CD4⁺CD25⁻ effector T cells and CD4⁺CD25⁺ Treg cells were isolated from PBMC/CBMC in a two step procedure as described in the methods and cocultured at a ratio of 1:1. Proliferation of CD4⁺CD25⁻ effector T cells without and in coculture with CD4⁺CD25⁺ Treg cells was assessed flowcytometrically. One representative experiment of 20 is shown (maternal sample).

Figure 3 supplementary: Purity of isolated CD4⁺CD25⁺ T cells. CD4⁺CD25⁺ T cells were isolated from human PBMC/CBMC by a two step procedure as described in the methods. To control isolation purity of CD4⁺CD25⁺ T cells, cells were stained with anti-CD4-PC5 and anti-CD25-PE antibodies.

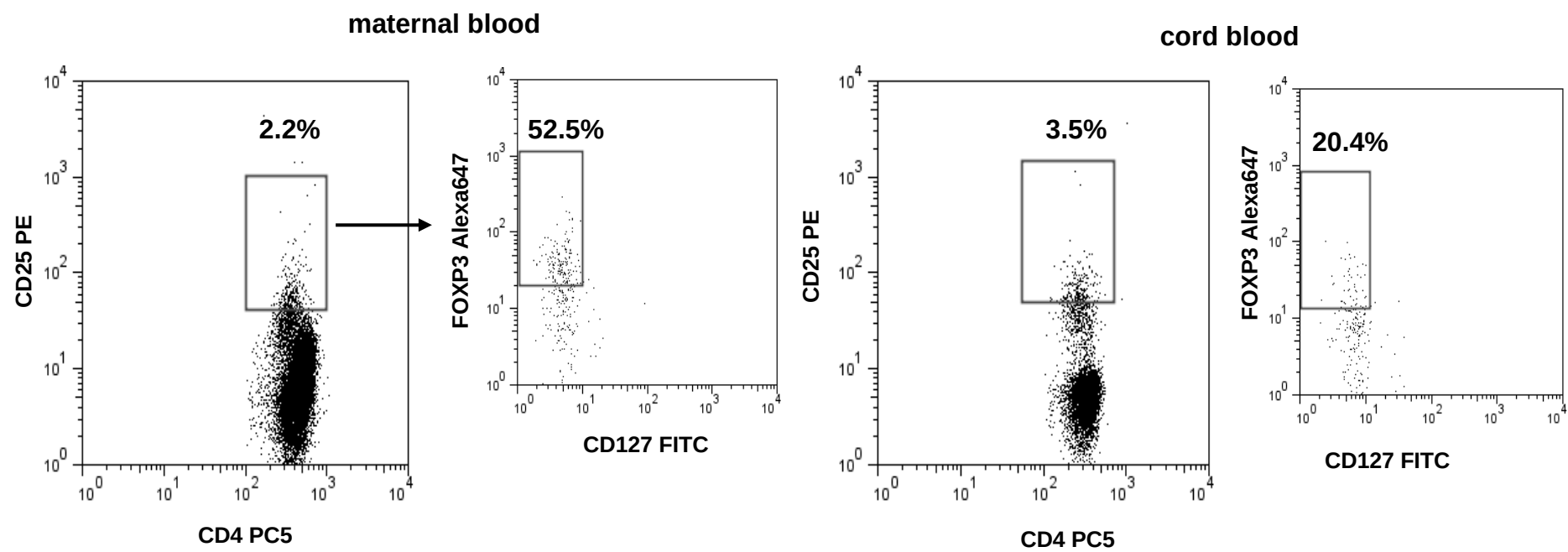


Figure 1 supplementary

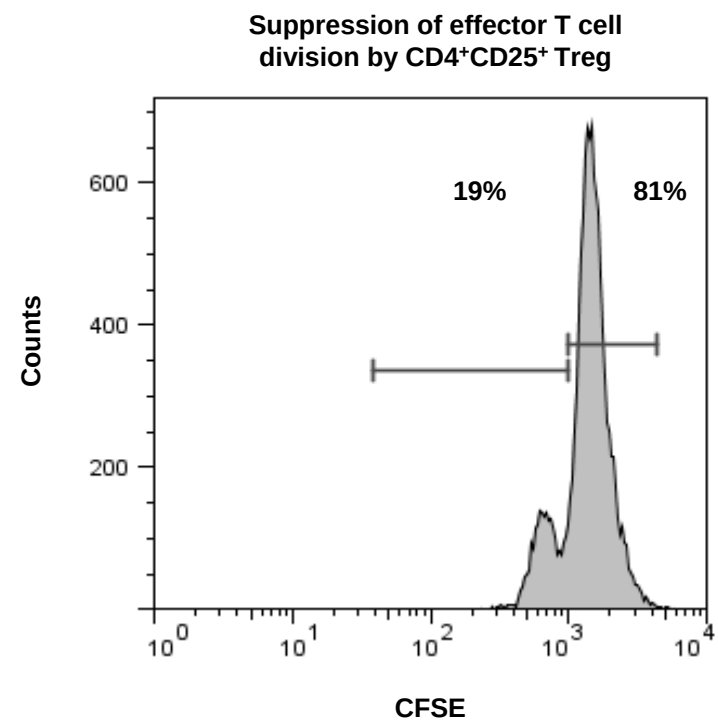
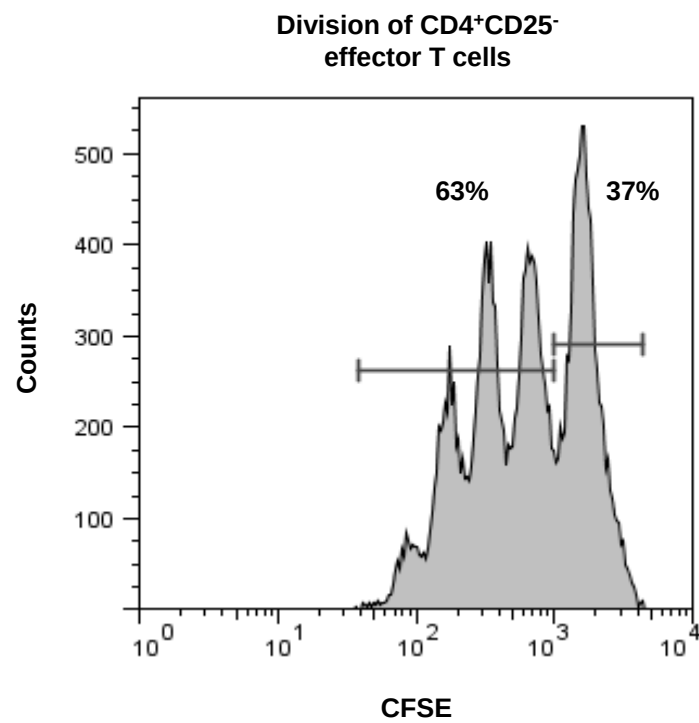


Figure 2 supplementary

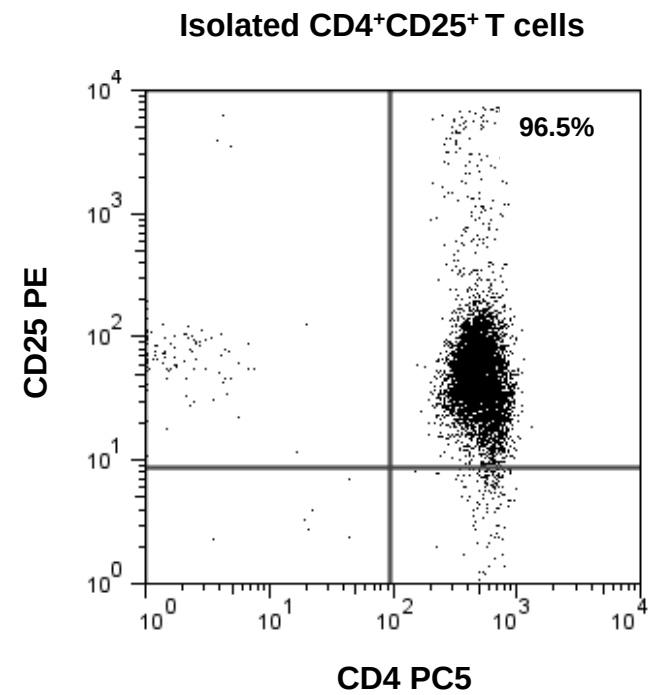
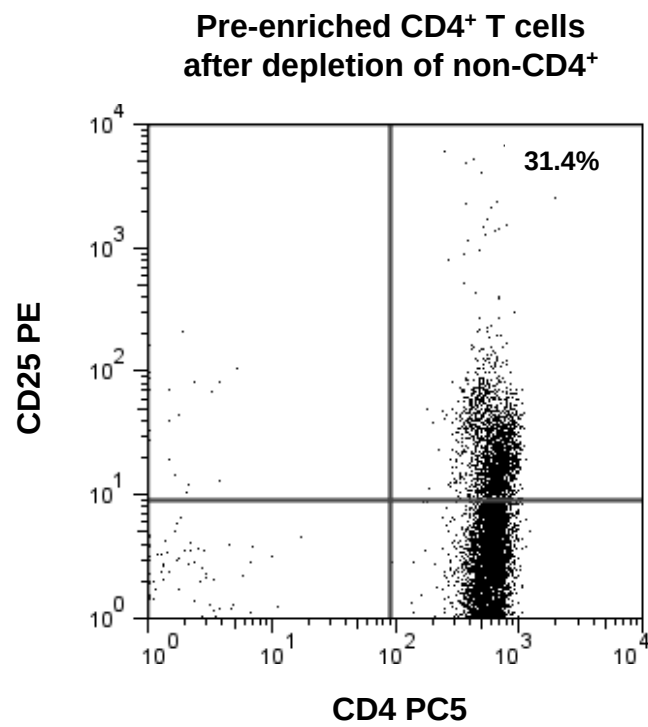


Figure 3 supplementary