$CD4^+CD25^+$ *FOXP3*$^+$ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses

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Summary

Activation of self-reactive T cells in healthy adults is prevented by the presence of autoantigen-specific $CD4^+CD25^+$ regulatory T cells ($CD25^+$ T_{reg}). To explore the functional development of autoantigen-reactive $CD25^+$ T_{reg} in humans we investigated if thymic $CD25^+$ T_{reg} from children aged 2 months to 11 years and cord blood $CD25^+$ T_{reg} are able to suppress proliferation and cytokine production induced by specific antigens. While $CD4^+CD25^-$ thymocytes proliferated in response to myelin oligodendrocyte glycoprotein (MOG), tetanus toxoid and beta-lactoglobulin, suppression of proliferation was not detected after the addition of thymic $CD25^+$ T_{reg}. However, $CD25^+$ T_{reg} inhibited interferon (IFN)-γ production induced by MOG, which indicates that MOG-reactive $CD25^+$ T_{reg} are present in the thymus. In contrast, cord blood $CD25^+$ T_{reg} suppressed both proliferation and cytokine production induced by MOG. Both cord blood and thymic $CD25^+$ T_{reg} expressed *FOXP3* mRNA. However, *FOXP3* expression was lower in cord blood than in thymic $CD25^+$ T cells. Further characterization of cord blood $CD25^+$ T cells revealed that *FOXP3* was highly expressed by $CD25^+CD45RA^+$ cells while $CD25^+CD45RA^-$ cells contained twofold less *FOXP3*, which may explain the lower expression level of *FOXP3* in cord blood $CD25^+$ T cells compared to thymic $CD25^+$ T cells. In conclusion, our data demonstrate that low numbers of MOG-reactive functional $CD25^+$ T_{reg} are present in normal thymus, but that the suppressive ability of the cells is broader in cord blood. This suggests that the $CD25^+$ T_{reg} may be further matured in the periphery after being exported from the thymus.

Keywords: $CD25^+$ regulatory T cells, cord blood, myelin oligodendrocyte glycoprotein, thymus.

Introduction

In recent years naturally occurring $CD4^+CD25^+$ regulatory T cells ($CD25^+$ T_{reg}) have become established as important contributors to the maintenance of immunological self-tolerance. The majority of $CD25^+$ T_{reg} appear to be produced in the thymus because neonatal thymectomy of mice leads to multi-organ autoimmunity, which can be prevented by adoptive transfer of $CD4^+CD25^+$ T cells from normal mice.[1] In addition, functional $CD25^+$ T_{reg} expressing CTLA-4, GITR and *Foxp3* can be isolated from both the periphery and the thymus in mice as well as in humans.[2–5] Mice lacking interleukin (IL)-2 or IL-2 receptor are deficient in peripheral $CD25^+$ T_{reg}[6] and rats without a thyroid are devoid of thyroid-specific T_{reg}.[7] Thus, after leaving the thymus the survival of $CD25^+$ T_{reg} in the periphery depends on IL-2 and recognition of antigen. $CD25^+$ T_{reg} have a T cell receptor (TCR) repertoire as broad as $CD25^-$ T cells[8,9] and they need antigen-specific stimulation via the T cell receptor to become suppressive, but once activated they suppress in an antigen non-specific fashion.[10] The mechanism(s) by which $CD25^+$ T_{reg} suppress effector T cells remain to be fully determined. In the majority of human and murine studies the inhibition of T cell proliferation and cytokine production *in vitro* has been shown to be cell-contact-dependent.[8,11] In addition, IL-10 and transforming growth factor (TGF)-β contribute to suppression in *in vivo* models of inflammatory bowel disease but not in models of gastritis.[12]

Functional studies of human adult peripheral blood have revealed that only $CD4^+$ T cells with the brightest expression of CD25 are regulatory[13] and the $CD25^+$ T_{reg} are confined to the memory T cell compartment as they express CD45RO.[11] Because $CD25^+$ T_{reg} are not a discrete cell population and activated T cells and T_{reg} could have overlapping CD25 expression levels, it is difficult to obtain pure $CD25^+$ T_{reg} from adult peripheral blood. In order to acquire a more homogeneous population of human $CD25^+$ T_{reg} devoid of recently activated/memory T cells we and others have investigated the potential use of $CD25^+$ T cells derived from cord blood as a source of human $CD25^+$ T_{reg}.[3,14–16] Cord blood $CD25^+$ T cells are different from adult $CD25^+$ T cells as they express markers of naive T cells, including CD45RA, CD38 and CD62L.[14] Freshly isolated adult, cord blood and also thymic $CD25^+$ T cells suppress T cell proliferation induced by polyclonal stimuli.[3,13,16–18] Thus, the memory phenotype of adult $CD25^+$ T_{reg} is a sign of antigen experience rather than a characteristic of T_{reg}. $CD25^+$ T_{reg} from adults have been shown to suppress T cell responses to autoantigens as well as exogenous antigens such as tetanus toxoid and allergens.[3,19–21] However, little is known about how cord blood $CD25^+$ T_{reg} respond to specific antigens and no attempt has been made to study this in thymus. We have shown previously that cord blood $CD25^+$ T_{reg} are less able to suppress T cell proliferation induced by the neural self-antigen myelin oligodendrocyte glycoprotein (MOG) when compared to adult $CD25^+$ T_{reg}.[3] It was unclear whether this observation was due to a lower frequency of antigen-specific $CD25^+$ T_{reg} in cord blood compared to adults or if they were functionally immature in the newborn child. To address this question we have analysed in this study the antigen-specific suppressive ability of human thymic $CD25^+$ T_{reg} compared to peripheral cord blood $CD25^+$ T_{reg}.

Materials and methods

Tissue collection and cell preparation

Thymus and 6 ml of peripheral blood were obtained from 13 children, aged 2 months to 11 years (median age 6 month), undergoing surgery for congenital heart defects. The peripheral blood was used for the preparation of antigen-presenting cells (APC, described below). A single-cell suspension from the thymus was obtained by gently passing the tissue through a 70 μm nylon sieve (Becton Dickinson, Le Pont De Claix, France). Cord blood from 16 healthy full term neonates was collected in heparin-containing tubes immediately after delivery. Blood lymphocytes were separated by Lymphoprep™ (Nycomed, Oslo, Norway) gradient centrifugation. Freshly isolated cells from each sample were examined for the capacity to proliferate and produce cytokines and the expression of *FOXP3* was analysed in four thymic samples and nine cord blood samples. In addition, four samples of cord blood were used solely for the analysis of *FOXP3* expression. Informed consent was obtained from the parents and the study was approved by the human Research Ethics Committee of the Medical Faculty, Göteborg University.

Isolation of cells

Thymic single positive $CD4^+$ cells were obtained after depletion of $CD8^+$ cells. Briefly, cells were incubated with CD8 antibody (Ab) (OKT-8) for 15 min at 4°C. Excess antibody was removed by centrifugation followed by CD8 cell depletion according to the manufacturer's instructions using Pan Mouse IgG (Dynal Biotech ASA, Oslo, Norway). Cord blood, and in some additional control experiments, also thymic $CD4^+$ cells were purified with Dynabead® CD4 Positive Isolation Kit (Dynal Biotech ASA, Oslo, Norway). $CD4^+$ cells were incubated with anti-CD25 beads and $CD25^-$ and $CD25^+$ fractions were recovered using a LS-magnetic column according to the manufacturer's instructions (Miltenyi Biotech, Germany). The percentage of $CD8^+$ thymocytes after CD8 depletion was less than 3% and remaining cells consisted of 70–90% $CD4^+$ cells, with 10–30% $CD4^-CD8^-$ cells. The purity after $CD4^+$ selection were > 95% for both thymus and cord blood $CD4^+$ cells; the purity of thymic and cord blood $CD25^+$ T cells were routinely above 80% and 90%, respectively. $CD25^+CD45RA^+$, $CD25^+CD45RA^-$ and $CD25^-CD45RA^+$ and $CD25^-CD45RA^-$ were obtained from cord blood after initial separation with a Dynabead® CD4 Positive Isolation Kit (Dynal Biotech ASA, Oslo, Norway), followed by cell sorting using a FACSVantage SE (BD, San José, CA) after staining with FITC- anti-CD4 (SK3), APC- anti-CD25 (2A3) and PE-anti-CD45RA (HI100) from Becton Dickinson (Erembodegem, Belgium). Peripheral blood mononuclear cells (PBMC) or cord blood mononuclear cells (CBMC) were depleted of $CD3^+$ cells (Dynabeads®), γ-irradiated (25 Gy) and used as APC. Purity of cell populations was determined by flow cytometry using the following monoclonal antibodies (mAb) obtained from Becton Dickinson (Erembodegem, Belgium): PerCP-anti-CD4 (SK3), APC-anti-CD3 (SK7) and PerCP-, APC- or phycoerythrin (PE)-isotype control IgG_1, and PE-anti-CD25 (clone M-A251) obtained from Pharmingen (San Diego, CA).

Analysis of FOXP3 mRNA levels by quantitative PCR

Total RNA was extracted from 1×10^5 previously purified and frozen $CD25^-$ or $CD25^+$ T cells using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and contaminating genomic DNA was removed by treatment with DNA-free (Ambion, Austin TX). cDNA was prepared in a random hexamer-primed Superscript RT reaction (Invitrogen, Stockholm, Sweden) according to the manufacturer's protocol.

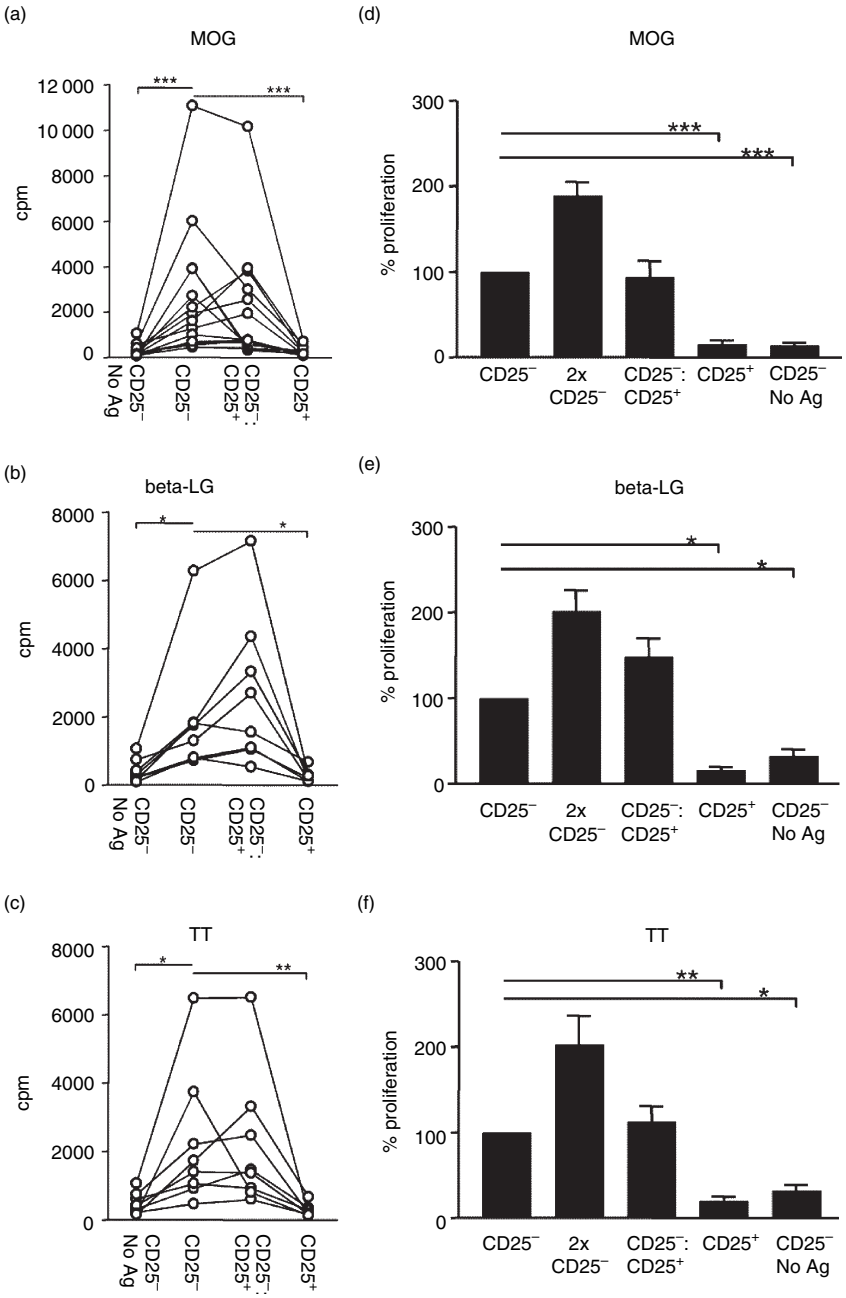

Figure 1. Capacity of thymic $CD4^+CD25^+$ T cells to suppress proliferation of $CD4^+CD25^-$ thymocytes after antigen-specific stimulation. (a–c) Absolute level of proliferation of thymic $CD25^-$ and $CD25^+$ T cells cultured alone or together in a 1:1 ratio after stimulation with MOG (a), beta-LG, (b) and TT (c). Data points from the same individual are connected by lines. (d,e) Percentage of proliferation relative to the level of $CD25^-$ T cells for each individual after stimulation with MOG (D, $n = 13$), beta-LG (E, $n = 8$) and TT (F, $n = 8$). Bars depict mean percentage proliferation ± SEM. Statistical significance between cultures is shown as: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

FOXP3 mRNA levels were measured in duplicate with reagents from the LightCycler FastStart DNA Master SYBR Green I kit using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) and the following primers: 5′-CAG CAC ATT CCC AGA GTT CCT-3′ (forward) and 5′-CG TGT GAA CCA GTG GTA GAT-3′ (reverse). GAPDH was used as endogenous reference gene for relative quantification and detected by the primers: 5′-GGC TGC TTT TAA CTC TGG-3′ (forward) and 5′-GGA GGG ATC TCG CTC C-3′ (reverse). Primers were ordered from Tib Molbiol (Berlin, Germany) and designed not to amplify genomic DNA. A melting curve analysis was performed in each run to ensure specificity of the primers. Data was collected using LightCycler Data Analysis Software and the LightCycler Relative Quantification Software was used for calibrator normalized relative quantification.

Cell culture

The suppressive capacity of the cells was determined by culturing 200×10^3 $CD4^+CD25^-$ T cells with 200×10^3 $CD4^+CD25^+$ cells together with 50×10^3 APC for 6 days in flat-bottomed 96-well plates in X-Vivo 15 medium (BioWhittaker, Walkersville, MD). Cultures were stimulated with 10 μg/ml recombinant human myelin oligo-

dendrocyte glycoprotein extracellular region (MOG^{IgD} abbreviated MOG), 50 μg/ml purified bovine beta-lactoglobulin (beta-LG) (Sigma-Aldrich, St Louis, MO) or 10 μg/ml tetanus toxoid (TT, Seruminstitute Copenhagen, Denmark). In addition, 10×10^3 $CD4^+CD25^-$ responder cells were cultured with 10×10^3 $CD4^+CD25^+$ cells in the presence of 100×10^3 APC in flat-bottomed 96-well plates and stimulated with 0·2 μg/ml staphylococcal enterotoxin B (SEB, Sigma-Aldrich, Stockholm, Sweden) for 4 days. At the end of the culture period cells were pulsed overnight with 1 μCi [^{3}H]-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK). Plates were harvested with a Filtermate 196-cell harvester (Packard) and [^{3}H]-thymidine incorporation was measured with a Matrix 96 Direct Beta Counter (Packard). We accepted only experiments in which there was no overlap between the proliferation counts ± 2 SD from unstimulated and stimulated $CD25^-$ T cells.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from cultures with specific antigens on day 6 and from cultures with SEB on day 4 of culture and stored at −20° until analysis. A standard ELISA protocol was performed as described elsewhere.[22] All antibodies and standards were purchased from BD Pharmingen, San Diego, CA. Costar plates (Invitrogen, San Diego, CA) were coated with the following anti-human capture mAb: interferon (IFN)-γ (NIB42), IL-13 (JES10–5A2) and IL-10 (JES3–9D7). Standard curves were generated using recombinant human IL-13, IFN-γ and IL-10, respectively. The following biotinylated mAb were used: IFN-γ (4S.B3), IL-13 (B69-2) and IL-10 (JES3–12G8). Samples, standards, biotinylated antibodies and streptavidin–horse radish peroxidase (HRP) were diluted in high-performance ELISA dilution buffer (Sanquin, Amsterdam, the Netherlands). Some samples were analysed using the Biocore Multiplex system (Biorad, Hercules, CA) according to the manufacturer's instructions.

Statistics

Data were analysed by Wilcoxon's signed-rank test except for data in Fig. 5, which were analysed by a two-tailed Mann–Whitney *U*-test using GraphPad Prism 3·00 (GraphPad, San Diego, CA). *P*-values $\leq 0{\cdot}05$ were regarded as significant, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

Results

Thymic $CD25^+$ T_{reg} do not suppress antigen-specific T cell proliferation

We first investigated if $CD25^+$ T_{reg} directly isolated from human thymus were able to inhibit antigen-specific proliferation of thymic $CD25^-$ T cells after developing an optimized proliferation assay for thymic cells. Figure 1 shows the level of proliferation of freshly isolated thymic $CD4^+CD25^-$ T cells after stimulation with myelin oligodendrocyte glycoprotein (MOG), beta-lactoglobulin (beta-LG) and tetanus toxoid (TT) in the presence of autologous peripheral antigen-presenting cells (APC). Although the counts are generally low, as expected for a primary antigen-specific response, they are specific as cell culture wells with twice the number of T cells gave a two-fold higher proliferative response (Fig. 1d–f), and cultures without the antigen resulted in only background proliferation. Only experiments in which there was no overlap between the proliferation counts ± 2 SD from unstimulated and stimulated $CD25^-$ T cells were included in the study. Cultures of $CD4^+CD25^+$ T cells alone did not induce proliferation to the chosen antigens. The addition of an equal number of $CD25^+$ T_{reg} to the $CD25^-$ T cell cultures resulted in suppression of proliferation in only a

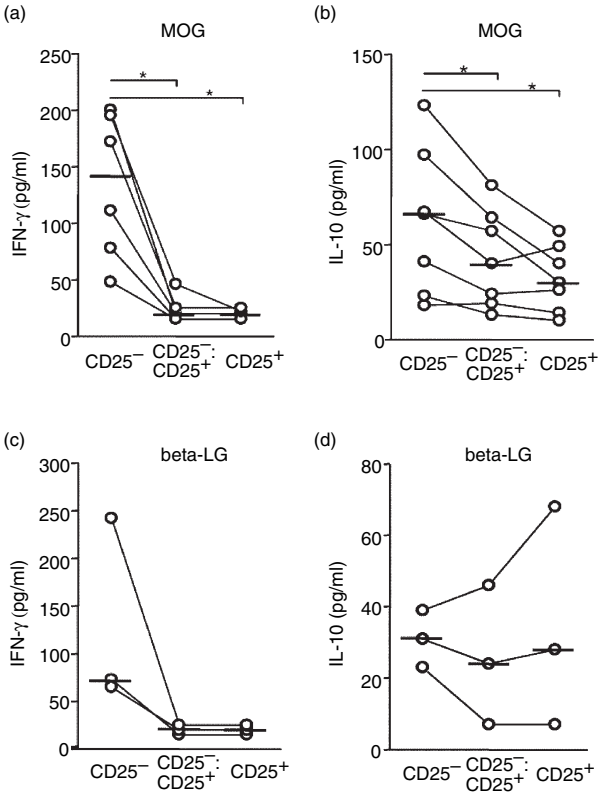

Figure 2. Cytokine levels in cultures of thymic cells after stimulation with specific antigens. (a) Production of IFN-γ from thymic $CD4^+$ $CD25^-$ T cells, $CD4^+CD25^+$ T cells and cocultures thereof stimulated with MOG ($n = 6$). (b) IL-10 production after stimulation with MOG ($n = 7$). (c) IFN-γ production after stimulation with beta-LG ($n = 3$). (d) IL-10 production after stimulation with beta-LG ($n = 3$). Lines connect data points from the same individual and horizontal bars depict median values. Statistical significance between cultures is shown as: $^*P < 0{\cdot}05$.

minority of individuals after MOG (4/13) and TT (1/8) stimulation and no suppression at all was detected after stimulation with beta-LG (Fig. 1a–c). The suppression of proliferation to MOG and TT was detected in different individuals. The level of proliferation or the ability to suppress proliferation was not correlated with the age of the child. In summary, there was no significant suppression of proliferation to any of the antigens when the results of the individuals were summarized (Fig. 1d–f).

Thymic CD25$^+$ T_{reg} suppress antigen-specific IFN-γ production

We measured the production of IFN-γ, IL-13 and IL-10 from the thymic T cell cultures after antigen-specific stimulation with MOG, beta-LG and TT. First, it should be noted that not all thymic cultures responded with cytokine production. IFN-γ was detected in six of 13 thymi after MOG stimulation (Fig. 2a) and in three of eight thymi after beta-LG stimulation (Fig. 2c). The cytokine production was not correlated with the level of proliferation. Furthermore, cytokine production was not detected after TT stimulation despite the occurrence of a proliferative response to this antigen. In Fig. 2 we show the absolute levels of IFN-γ and IL-10 by CD25$^-$ T cells, CD25$^+$ T cells and in the cocultures of the cell fractions after stimulation with MOG and beta-LG. We found that CD25$^-$ T cells produced both IFN-γ and IL-10 but not IL-13 after stimulation with MOG or beta-LG, and that thymic CD25$^+$ T_{reg} produced low levels of IL-10 but no detectable levels of IFN-γ or IL-13 (Fig. 2a–d). Cytokine production was not detected in control wells with APC stimulated alone (data not shown). Interestingly, despite the lack of suppression of proliferation, thymic CD25$^+$ T_{reg} potently suppressed IFN-γ production by CD25$^-$ T cells in the cocultures after stimulation with MOG or beta-LG (Fig. 2a,c). With the exception of one individual stimulated with MOG, suppression of IFN-γ was not reflected by corresponding suppression of proliferation. In five of seven donors, IL-10 was detected at lower levels in the coculture compared to CD25$^-$ T cells cultured alone after MOG stimulation (Fig. 2b). The capacity of CD25$^-$ T cells to produce cytokines in response to the antigens did not improve with increasing age of the children (data not shown). In summary, when MOG and beta-LG were able to induce IFN-γ production from thymic CD25$^-$ T cells, the CD25$^+$ T_{reg} irrespective of the donors' age potently suppressed the response, which suggests that human antigen-specific CD25$^+$ T_{reg} are present and functional in the thymus.

Cord blood CD25$^+$ T_{reg} suppress MOG-induced proliferation and cytokine production

In our earlier work we did not detect suppression of MOG-induced proliferation using cord blood CD25$^+$ T cells. However, we did not analyse cytokine production, which seems to be a more sensitive way of measuring suppression than is proliferation. Therefore, we reinvestigated the capacity of cord blood CD25$^+$ T cells to suppress

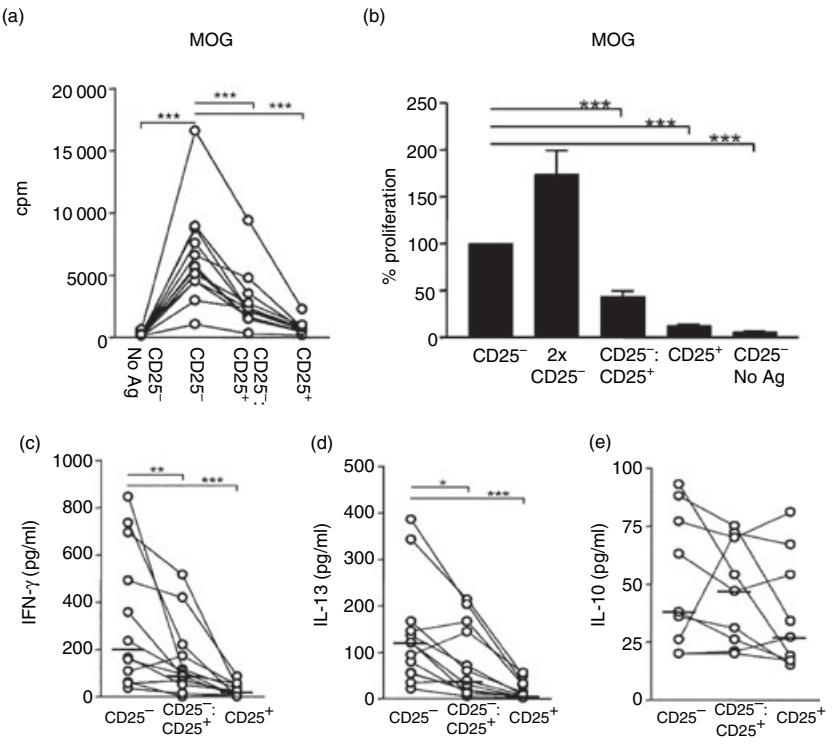

Figure 3. Capacity of CD4$^+$CD25$^+$ T cells to suppress proliferation and cytokine production of CD4$^+$CD25$^-$ T cells in cord blood after stimulation with MOG. (a) Absolute level of proliferation of cord blood CD25$^-$ and CD25$^+$ T cells cultured alone or together in a 1:1 ratio after stimulation with MOG ($n = 12$). (b) Percentage of proliferation relative to the level of CD25$^-$ T cells for each individual after stimulation with MOG ($n = 12$). Bars depict mean percentage proliferation ± SEM. (c,d) Levels of IFN-γ (c, $n = 12$), IL-13 (d, $n = 12$) and IL-10 (e, $n = 9$) in cultures from cord blood CD25$^-$ T cells, CD25$^+$ T cells and cocultures thereof stimulated with MOG. Lines connect data points from the same individual and horizontal bars depict median values. Statistical significance between cultures is shown as, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

responses by cord blood CD25− T cells to MOG. We employed the same suppression assay as for the thymocytes, using 200 000 T cells to increase the chance of obtaining sufficient numbers of MOG-reactive effector T cells and CD25+ T_{reg}. In Fig. 3 we show the proliferation of CD25− T cells, CD25+ T cells and of cocultures of both cell fractions after stimulation with MOG. CD25− T cells proliferated well in response to MOG, while CD25+ T cells where hyporesponsive. In contrast to thymic CD25+ T cells, we found that cord blood-derived CD25+ T_{reg} significantly suppressed the proliferation of CD25− T cells stimulated with MOG (Fig. 3a,b). The analysis of cytokines in the cultures revealed that cord blood CD25− T cells from all individuals produced IFN-γ, IL-13 and nine of 12 also produced detectable amounts of IL-10 (Fig. 3c–e). In contrast, CD25+ T cells did not produce detectable levels of IFN-γ and IL-13, while IL-10 was measurable. Cord blood CD25+ T_{reg} were able to inhibit the production of IFN-γ and IL-13, but not IL-10 in the coculture wells (Fig. 3c–e). In summary, cord blood CD25+ T_{reg} from nine of 12 individuals tested were able to suppress both proliferation and IFN-γ and IL-13 production induced by MOG *in vitro*.

Thymic CD25+ T_{reg} suppress polyclonal responses

Because we did not detect suppression of proliferation to specific antigens in thymus, and others have shown that CD25+ T_{reg} from thymus inhibit proliferation to phytohaemagglutinin (PHA) and alloantigens, we decided to test the suppressive ability of thymic CD25+ T_{reg} after polyclonal stimulation. Indeed, CD25+ T_{reg} from thymus significantly suppressed proliferation induced by staphylococcus enterotoxin B (SEB, Fig. 4a). We also found that the production of IFN-γ, IL-13 and IL-10 after SEB stimulation was significantly suppressed (Fig. 4b–d). In contrast to thymic T cells, stimulation of cord blood T cells with SEB resulted in vigorous proliferation of the CD25− T cells, which was not inhibited by the CD25+ T cells when added in a one-to-one ratio (Fig. 4e). In fact, CD25+ T cells also proliferated, although to a lower extent than CD25− T cells cultured alone. A similar lack of suppression was observed for the production of cytokines in the cord blood cultures. CD25+ T cells did not significantly suppress IFN-γ, IL-13 or IL-10 in cord blood after SEB stimulation (Fig. 4f–h). Interestingly, CD25+ T_{reg} produced similar amounts of IL-13 compared to CD25− T cells, while the levels of IFN-γ and IL-10 remained significantly lower in the cultures of CD25+ T_{reg} than in those of CD25− T cells. In summary, cord blood CD25+ T_{reg} are not able to suppress proliferation or cytokine production induced by SEB, while CD25+ T_{reg} from thymus have this capacity. One possible explanation for the discrepancy in suppressive ability could be that the cord blood CD25+ T cell population is contaminated by effector T cells in contrast to the thymic CD25+ T cells. In order to investigate this we decided to study the expression of *FOXP3*.

CD25+ T_{reg} in cord blood express less FOXP3 than CD25+ T_{reg} from thymus

We analysed the expression of *FOXP3* mRNA by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and compared the levels in CD25+ T cells from thymus and cord blood. Figure 5a shows histograms of the CD4+CD25− and CD4+CD25+ T cell fractions from thymus and cord blood after magnetic bead separation and Fig. 5b depicts their expression of *FOXP3*. We found that CD25+ T cells from cord blood expressed on average half as much *FOXP3* mRNA as thymic CD25+ T cells. This may indicate that the CD25+ T cell fraction in cord blood contains a lower proportion CD25+ T_{reg} than do purified thymic CD25+ T cells. We have shown previously that CD25+ T cells from cord blood can be divided further into one population of cells expressing intracellular CTLA-4 and CD122 and one population of cells not expressing these markers.[14] Because CD25+CTLA-4+ T cells are mainly CD45RA+ and CD25+CTLA-4− T cells are CD45RA− we investigated whether the CD25+ CD45RA+ cells were the major expressors of *FOXP3* mRNA. Cord blood CD4+ T cells where sorted by FACS according to the expression of CD25 and CD45RA (Fig. 5c). We found that CD25+CD45RA+ T cells expressed twofold higher levels of *FOXP3* than the CD25+CD45RA− T cells (Fig. 5d). Of note, the absolute expression levels of *FOXP3* in cord blood in Fig. 5b,d should not be compared, as different purification techniques and individuals were used. Our data indicate that cord blood contains two different populations of CD25+ cells according to cell surface phenotype and *FOXP3* expression. CD25+CD45RA+ cells express high levels of *FOXP3* mRNA and thus most probably qualify as the dominant T_{reg} population, while the CD25+CD45RA− cell fraction contains lower levels of *FOXP3* mRNA.

Discussion

During the last few years several studies have demonstrated the presence of CD4+CD25+ T_{reg} in humans, which share striking similarities with their murine counterparts. Human CD25+ T_{reg} capable of suppressing proliferation and cytokine production after various polyclonal stimuli have been isolated from peripheral blood, cord blood, tonsils and thymus.[3,13,18,23] Despite the fact that CD25+ T_{reg} are activated in an antigen-specific manner, few attempts have been made to investigate responses elicited by specific antigens in humans or normal mice. In this study we demonstrate for the first time that CD25+ T_{reg} directly isolated from human thymus can respond to self-

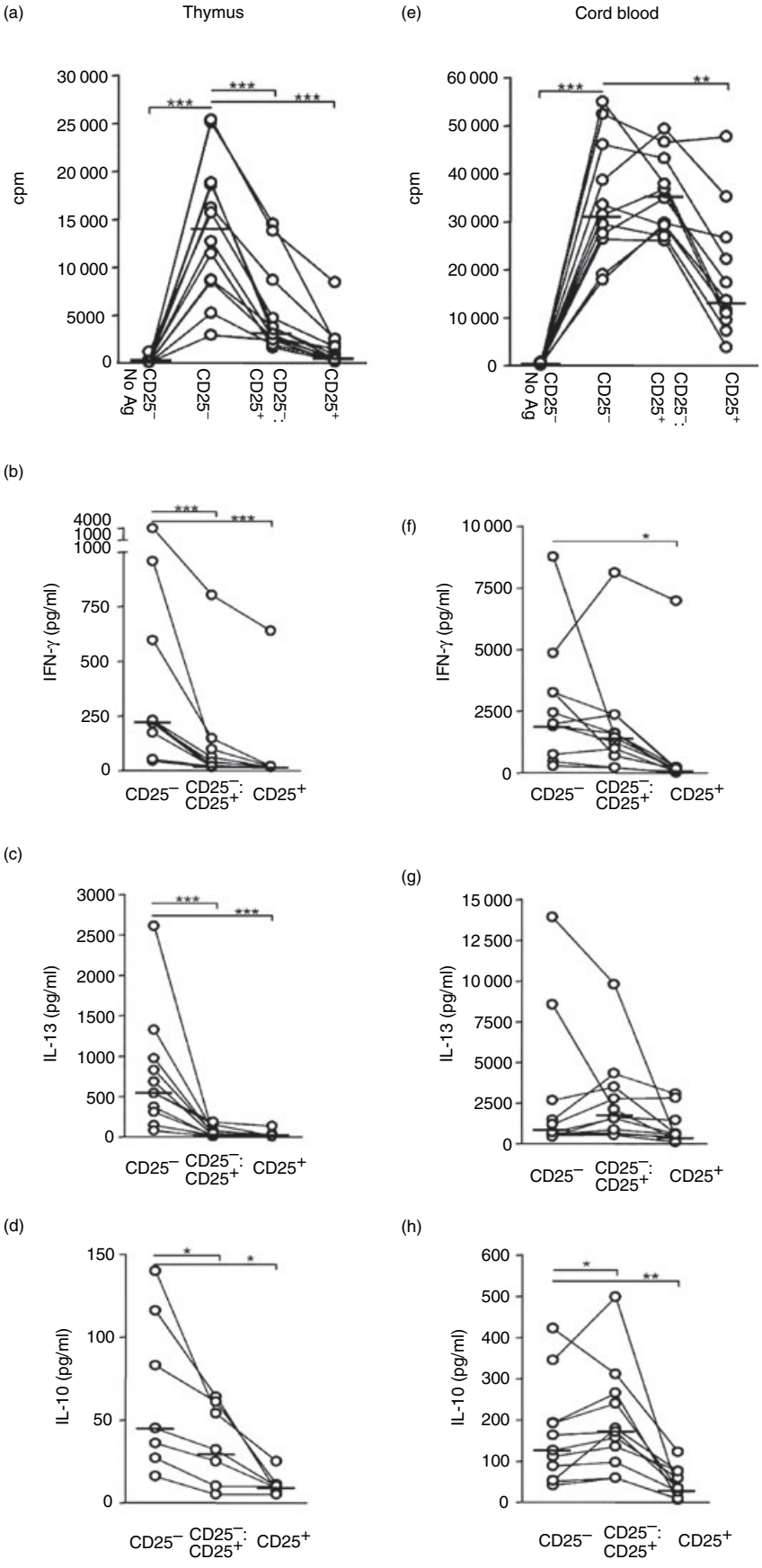

Figure 4. Capacity of $CD4^+CD25^+$ T cells from thymus and cord blood to suppress proliferation and cytokine production of $CD4^+CD25^-$ T cells after SEB stimulation. (a,e) Proliferation of thymus (a, $n = 12$) and cord blood (e, $n = 11$) $CD25^-$ T cells, $CD25^+$ T cells and cocultures thereof after stimulation with SEB. Data points from the same individual are connected by lines. (b–d) Levels of IFN-γ (b, $n = 11$), IL-13 (c, $n = 11$) and IL-10 (d, $n = 7$) in cultures of thymic $CD25^-$ T cells, $CD25^+$ T cells and cocultures thereof. (f–h) Production of IFN-γ (f, $n = 11$), IL-13 (g, $n = 11$) and IL-10 (h, $n = 11$) from cord blood $CD25^-$ T cells, $CD25^+$ T cells and cocultures thereof. Individual levels are shown as dots and horizontal bars depict median values. Statistical significance between cultures is shown as: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

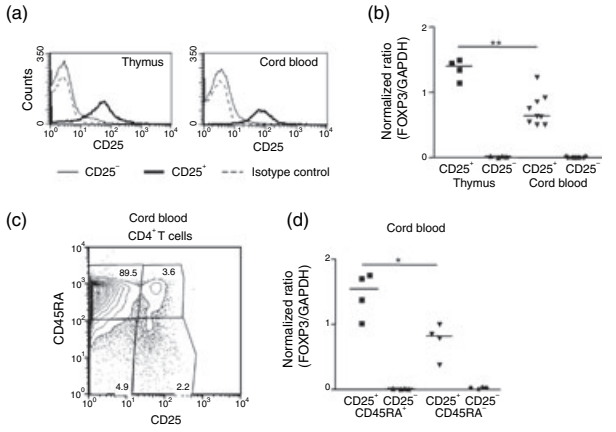

Figure 5. Flow cytometric analysis of CD25 expression and analysis of *FOXP3* expression by quantitative RT-PCR in thymus and cord blood. (a) One representative histogram depicting bead-purified $CD4^+CD25^+$ T cells (thick line), $CD4^+CD25^-$ (thin line) and isotype control (broken line) is shown for thymus and cord blood. (b) Normalized ratios of *FOXP3/GAPDH* in the bead-purified $CD25^+$ and $CD25^-$ T cell fractions of thymus ($n = 4$) and cord blood ($n = 9$). (c) One representative contour plot of $CD4^+$ cord blood T cells used for FACS-sorting depicting $CD25^+CD45RA^+$, $CD25^-CD45RA^+$ and $CD25^+CD45RA^-$, $CD25^-CD45RA^-$ T cells. (d) Normalized ratios of *FOXP3/GAPDH* in the FACS-sorted T cell fractions of cord blood ($n = 4$). Each dot represents one individual and horizontal bars depict median values. Statistical significance between cultures is shown as, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

antigen by inhibiting the IFN-γ production of $CD25^-$ T cells. In addition, we show that this response is enhanced after migration to the periphery because $CD25^+$ T cells in cord blood suppress not only IFN-γ and IL-13 production but also proliferation.

In previous reports, human thymic $CD4^+CD25^+$ T_{reg} have been found to suppress proliferation and cytokine production induced by alloantigens and PHA.[18,24] In accordance with these results, we show that $CD25^+$ T_{reg} derived from thymus are anergic and produce low or undetectable levels of cytokines, while they suppressed the proliferation as well as IFN-γ, IL-13 and IL-10 production by $CD25^-$ thymocytes after stimulation with SEB. In contrast, stimulation with specific antigens resulted only in suppression of proliferation in a minority of the tested individuals. It is conceivable that this is due to a low frequency of MOG-specific $CD25^+$ T_{reg} in the thymus, which makes suppression of proliferation harder to detect. However, thymic $CD25^+$ T_{reg} from individuals who responded with IFN-γ production suppressed the response after simulation with both MOG and beta-LG, which indicates that functional antigen-specific $CD25^+$ T_{reg} are indeed present in the thymus. In our experience suppression of IFN-γ can be more pronounced than suppression of proliferation, also when using T_{reg} and T_{eff} from adult blood.[3] This discrepancy between suppression of proliferation and cytokine production is difficult to explain. Currently little is known about how $CD25^+$ T_{reg} mechanistically inhibit different T cell responses such as proliferation and production of various cytokines. However, it has been observed that $CD25^+$ T_{reg} from birch-allergic patients stimulated with birch allergen lack the ability to suppress Th2 cytokines during season while the ability to suppress proliferation was retained.[21,25] This indicates that in a different system the production of a primary cytokine such as IL-2 may be regulated differently from the production of secondary cytokines such as IL-13 and IL-5.

In contrast to thymus, cord blood $CD25^+$ T_{reg} suppressed proliferation and production of IFN-γ and IL-13 by $CD25^-$ T cells stimulated with MOG but not SEB. Thus, autoantigen-specific T_{reg} are present in both the thymus and cord blood. In a previous study cord blood $CD25^+$ T_{reg} were not found to suppress MOG induced proliferation.[3] However, these experiments were aimed to compare the suppressive ability of cord blood $CD25^+$ T cells and adult T_{reg} and therefore only 50 000 T cells were used in the study. Thus, the lack of suppression of proliferation was due conceivably to the use of fourfold lower T cell numbers than employed in the current study. The increase in cell numbers allowed for a higher proliferation of the cord blood $CD25^-$ T cells as well as the detection of MOG-induced suppression of proliferation and cytokine production by the $CD25^+$ T cells. Despite individual variations in responses, cord blood T cells generally responded with higher proliferation and cytokine production than thymic T cells. Together this suggests that the number of MOG-specific effector and regulatory T cells expands in the periphery of the child after being exported from the thymus.

Because cord blood $CD25^-$ T cells cannot be stimulated to proliferate by anti-CD3 mAb we used SEB for polyclonal stimulation.[26] Interestingly, while thymocytes were suppressed easily by thymic T_{reg} after SEB stimulation, cord blood $CD25^+$ T_{reg} did not suppress $CD25^-$ T cells after SEB stimulation and the $CD25^+$ T_{reg} even proliferated themselves and also produced IL-13 when cultured alone. In this regard cord blood $CD25^+$ T_{reg} resemble adult $CD25^+$ T cells, which have also been reported to have diminished capacity to suppress proliferation induced by SEB.[27] One could argue that SEB provides a very strong TCR signal that makes $CD25^-$ T cells refractory to inhibition by $CD25^+$ T_{reg} even if a low dose of SEB is used.[27] In addition, neither adult nor cord blood $CD25^+$ T cells were anergic when stimulated with SEB. In both studies $CD25^+$ T cells were purified by magnetic bead separation and therefore non-regulatory $CD25^+$ T cells might contaminate the $CD25^+$ T_{reg} fraction. Interestingly, the cord blood $CD25^+$ fraction produced the Th2 cytokine IL-13 but not the Th1 cytokine IFN-γ after SEB stimulation, which would argue against a general contamination by effector

T cells. It is, however, possible that $CD25^+$ T_{reg} from both cord and adult blood are contaminated selectively by Th2 cells as bead-separated $CD25^+$ T_{reg} from adult peripheral blood produce IL-4 when stimulated with PHA as opposed to thymocyte cultures.[18]

Recently FOXP3, which is a negative regulator of T cell activation, has been established as a marker for naturally occurring $CD25^+$ T_{reg}. Mice carrying a mutation in the *Foxp3* gene display multi-organ autoimmune diseases and lack $CD25^+$ T_{reg}.[2] Similarly, children born with a dysfunctional *FOXP3* gene also succumb to organ-specific autoimmune diseases.[28] *Foxp3* mRNA has been shown to be expressed by $CD4^+CD25^+$ T cells and thymocytes in both mice and human adults and newborns.[2,4,5,15,16] We detected lower levels of *FOXP3* mRNA in $CD25^+$ T cells from cord blood than in $CD25^+$ T cells from thymus after magnetic bead-purification. This observation made us ask whether only a subpopulation of the cord blood $CD25^+$ cells might be expressing *FOXP3*. After sorting cord blood $CD25^+$ T cells into either $CD45RA^+$ or $CD45RA^-$ T cells by FACS we found that *FOXP3* was predominantly expressed by $CD25^+CD45RA^+$ T cells. Cord blood $CD25^+CD45RA^+$ T cells constitute about two-thirds of the $CD25^+$ T cells and correspond to cells with a regulatory and naive phenotype that express intracellular CTLA-4 and CD122. The remaining third of the $CD25^+$ T cells are $CD45RA^-$, $CD62L^{low}$, $CD38^{low}$, $CD122^{low}$, CTLA-4^- and might constitute effector T cells.[14] However, they still express *FOXP3* even though at lower levels than the T cells with regulatory phenotype. Therefore, further studies sorting the $CD25^+$ cells into other subpopulations may yield additional information as to where the regulatory cells concentrate. It is also possible that the level of *FOXP3* mRNA does not directly correlate with the suppressive function of the cells.

In summary, these data suggest that autoantigen-specific $CD25^+$ T_{reg} are exported from the thymus in low numbers and in a naive state. In the periphery before birth autoreactive $CD25^+$ T_{reg} encounter their specific antigens in the draining lymph nodes and divide, as has been shown by murine studies of non-lymphopenic animals.[29–31] Conceivably, after birth $CD25^+$ T_{reg} may need to become activated by antigen possibly in combination with other foreign agents for example bacterial antigens[32,33] in order to be expanded further, become fully functional and be converted to a memory phenotype in adults. Further research is needed to provide information on how $CD25^+$ T_{reg} mature and expand in humans and what stimuli might enhance the function of $CD25^+$ T_{reg} in order to decrease the risk of developing autoimmune and allergic disease.

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