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Identification of genes involved in the initiation of human Th1 or Th2 cell commitment

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The differentiation of naïve T helper (Th) cells is induced by TCR activation and IL-12/STAT4 or IL-4/STAT6 signaling pathways, forming Th1 and Th2 cells, respectively. In this study, oligonucleotide arrays were used to identify genes regulated during the initiation of human Th1 and Th2 cell differentiation at 2 and 6 h in presence or absence of immunosuppressive TGF-β. As a result the immediate targets of IL-12, IL-4 and TGF-β were identified. The effects of IL-12 at this early stage were minimal and consistent with the known kinetics of IL-12Rβ2 expression. IL-4, however, was observed to rapidly regulate 63 genes, 26 of which were differentially expressed at both the 2- and 6-h time points. Of these IL-4 regulated genes, one-third have previously been observed to display expression changes in the later phases of the polarization process. Similarly to the key regulators, TBX21 and GATA3, the transcription factors SATB1, TCF7 and BCL6 were differentially regulated at the protein level during early Th1 and Th2 cell polarization. Moreover, the developing Th1 and Th2 cells were demonstrated to be responsive to the immunosuppressive TGF-β and IL-10. In this study, a panel of novel factors that may be important regulators of the differentiation process was identified.

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Introduction

Defects in the polarization of T helper subtypes (Th1 and Th2) may result in the pathogenesis of various immune-mediated diseases such as asthma [1]. To understand the development of these diseases, it is crucial to characterize the polarization process of Th1 and Th2 cells at the molecular level. Th1 and Th2 cells originate from a common Th precursor cell (Thp), the differentiation of which is initiated in response to activation through TCR, costimulatory molecules and cytokine receptors. The main cytokine directing the Th1 commitment is IL-12, whereas IL-4 drives the Th2 polarization [2, 3]. Since the effects of IL-12 and IL-4 are mediated through STAT4 and STAT6, respectively, these are also required for the normal differentiation of Th1 and Th2 cells [4–7]. Other essential factors directing the commitment of Th1 and Th2 subtypes include TBX21 (T-bet) and GATA3, respectively [8–10]. Further to these, additional factors are likely to contribute to the process.

In previous studies large-scale DNA microarray analysis has been used to identify genes involved in the differentiation process in human Th cells after 2 days or later [11–13]. However, to solve the molecular mechanisms leading to the commitment of Th1 and Th2 subsets, it is essential to define also the upstream factors at the very earliest phase of initiation of Th cell commitment. The aim of this study was to identify the immediate genes that are differentially regulated in response to activation and Th1- or Th2-inducing cytokine (IL-12 or IL-4, respectively) at 2 and 6 h after initiation of polarization. Additionally, since earlier studies have indicated that TGF-β is able to inhibit the differentiation of Th1 and Th2 cells [14], the effects of TGF-β on early polarization were determined.

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Abbreviations: CT: threshold cycle · **Thp:** Th precursor cell

Results

Identification of genes involved in the initiation of Th1 and Th2 differentiation

To elucidate how IL-12 and IL-4 induce Th cell differentiation, Affymetrix U95Av2 arrays containing probes for approximately 9,300 human genes were used to study the changes in the gene expression profiles after 2 and 6 h of induction of Th1 and Th2 polarization. To identify genes differentially expressed during early polarization, direct comparison was made of the expression profiles of the cells cultured in Th1 polarizing conditions (anti-CD3 + anti-CD28 + IL-12) to those cultured in Th2 polarizing conditions (anti-CD3 + anti-CD28 + IL-4), at both 2 and 6 h. As a result, 63 genes were determined to be differentially expressed by the cells induced to polarize to Th1 or Th2 direction. To further characterize the genes regulated by IL-12 or IL-4, the expression profiles of cells induced to polarize to a Th1 or Th2 direction were compared to the expression profiles of CD3 + CD28-activated cells (Th0) cultured in "neutral" conditions, *i.e.*, without the polarizing cytokines. This comparison revealed that the early changes in gene expression were mainly driven by IL-4. The only genes that were regulated by IL-12, when compared to the CD3 + CD28-activated cells, were *IFNγ* (1.87-fold) and *GBP1* (1.62-fold) at the 6-h time point (data not shown). Of the 63 genes differentially expressed in Th1 or Th2 conditions, for 26 genes constant regulation by IL-4, or differences between Th1 and Th2 conditions, were seen at both the 2 and 6-h time

Table 1. Genes regulated by IL-4 after both 2 and 6 h of Th2 polarization

GenBank ID	Symbol	Locus	Th1 vs Th2		Th0 vs Thp		Th2 vs Th0	
			2 h	6 h	2 h	6 h	2 h	6 h
A. Transcriptional regulators and other nuclear molecules								
X58072	**GATA3**	10p15	-2.1[a)]	-4.0	.	.	2.3	4.9
X64318	**NFIL3**	9q22	-5.1	-2.8	.	.	4.0	3.4
AL021154	**ID3**	1p36.13-p36.12	2.5	3.0	.	2.1	-2.8	-4.4
Z49194	**POU2AF1**	11q23.1	1.5	2.7	61.8	53.8	-1.7	-2.9
B. Cell adhesion molecules and receptors								
U00672	**IL10RA**	11q23	-4.1	-6.4	-3.5	-5.1	4.6	10.3
L06797	**CXCR4**	2q21	-1.9	-2.1	-10.6	-13.5	2.0	2.3
M31210	**EDG1**	1p21	-4.1	.	.	-1.9	3.4	1.9
U19487	**PTGER2**	14q22	-2.1	.	.	.	2.7	1.6
M63928	**TNFRSF7**	12p13	2.1	2.7	.	4.3	-1.9	-3.1
C. Enzymes and other intracellular signaling molecules								
AB013382	**DUSP6**	12q22-q23	-3.7	-6.7	.	.	4.0	8.6
U78095	**SPINT2**	19q13.1	-2.6	-5.9	.	-1.9	2.1	6.7
AB000734	**SOCS1**	16p13.13	-2.9	-5.3	-2.4	.	3.2	7.5
L08177	**EBI2**	13q32.2	-1.5	-2.9	1.8	.	1.7	2.9
AL049933	**GNAI1**	7q21	-2.9	-1.9	5.9	4.8	2.8	2.0
Y10256	**MAP3K14**	17q21	-5.3	-2.6	.	.	5.1	2.2
AA131149	**S100P**	4p16	-2.1	-2.2	.	.	1.9	3.1
U58334	**TP53BP2**	1q42.1	-2.0	-1.3	.	.	2.1	1.3
D. Transporters								
X83467	**ABCD3**	1p22-p21	-1.5	-2.4	.	1.7	1.5	3.0
X76220	**MAL**	2cen-q13	-2.6	-2.1	-2.9	-2.7	2.7	2.2
AL049963	**SLC39A8**	4q22-q24	-2.8	-1.9	.	.	2.7	1.9
E. Miscellaneous								
AF026941	**cig5**	2p25.2	-35.5	-6.1	.	.	13.5	7.7
D29642	**ARHGAP25**	2p13.2	-2.9	-1.9	-2.4	.	3.2	1.7
AL080177	**UBL3**	13q12-q13	-2.2	-2.4	.	-1.9	2.3	2.7
D63789	**XCL1**	1q23	.	-2.4	.	12.1	1.6	2.4
HG2639-HT2735	**RBMS1**	2q24.3	.	-1.9	.	-1.3	1.5	2.1
M28130	**IL8**	4q13-q21	1.5	2.2	2.2	2.0	.	-1.9

[a)] Average fold-change values for the genes that showed ≥2-fold change in some of the comparisons. For these genes also the significant changes less than 2-fold in parallel comparisons are shown. Missing value indicates that the change was not significant according to the statistical data analysis algorithm MAS5.

points (Table 1). In addition to the genes differentially expressed by Th1 and Th2 cells at both 2 and 6 h, a subset of genes were differentially expressed in Th1 and Th2 conditions after either 2 h (17 genes) or 6 h (20 genes) of polarization (Table 2). From these observations we conclude that the early polarization of Th1 and Th2 cells is primarily driven by IL-4, and, consistent with the known kinetics of IL-12Rβ2, recently activated Th cells are quite unresponsive to IL-12 [15–17].

Table 2. Genes regulated by IL-4 after either 2 or 6 h of Th2 polarization

GenBank ID	Symbol	Locus	Th1 vs Th2		Th0 vs Thp		Th2 vs Th0	
			2 h	6 h	2 h	6 h	2 h	6 h
A. Transcriptional regulators and other nuclear molecules								
M97287	**SATB1**	3p23	-2.5[a)]	.	-2.4	-2.8	2.3	.
AF055376	**MAF**	16q22-q23	-2.1	.	-2.0	.	2.1	.
AB011421	**STK17B**	2q33.1	-2.2	.	.	-4.0	2.1	.
X57985	**HIST2H2BE**	1q21-q23	-2.4	.	.	-13.0	2.1	.
X59871	**TCF7**	5q31.1	.	2.9	-2.4	-5.1	.	-2.6
U00115	**BCL6**	3q27	.	2.8	.	-1.7	.	-2.1
AL120815	**LITAF**	16p13.3-p12	.	2.0	-2.4	-3.0	.	-1.9
B. Cell adhesion molecules and receptors								
AF070594	**CHST10**	2q11.2	-3.0	.	.	.	.	1.9
U91512	**NINJ1**	9q22	2.0	.	.	-1.1	-2.3	.
C. Enzymes and other intracellular signaling molecules								
AF081195	**RASGRP1**	15q15	-2.6	.	-3.0	-2.7	2.5	.
L13857	**SOS1**	2p22-p21	-2.5	.	.	2.3	2.3	.
D78156	**RASA2**	3q22-q23	-2.5	.	.	-3.0	3.2	.
AF117829	**RIPK2**	8q21	-3.9	.	.	.	2.9	.
AF070670	**PPM1A**	14q23.1	-3.0	.	.	-2.6	.	.
X05908	**ANXA1**	9q12-q21.2	.	-2.3	3.5	.	.	2.6
M18737	**GZMA**	5q11-q12	.	3.6	-4.9	-3.9	.	-2.7
M55542	**GBP1**	1p22.2	.	2.4	.	4.6	.	.
D88308	**SLC27A2**	15q21.2	.	2.2	.	3.2	.	.
D. Extracellular molecules and ligands								
AI432401	**FGL2**	7q11.23	-9.2	.	.	.	3.9	.
X52015	**IL1RN**	2q14.2	.	-1.9	.	.	.	2.1
X13274	**IFNG**	12q14	.	2.3	.	.	.	.
U64197	**CCL20**	2q33-q37	.	2.5	26.0	23.4	.	-2.3
M15856	**LPL**	8p22	.	2.1	.	6.5	.	-2.3
M26683 M28225	**CCL2**	17q11.2-q21.1	.	2.8	.	2.3	.	-2.8
J04765 AF052124	**SPP1**	4q21-q25	.	2.0	.	68.6	.	-2.5
E. Structural components								
L34155	**LAMA3**	18q11.2	2.4	.	3.4	.	-1.8	.
M95178	**ACTN1**	14q24	.	-2.5	-2.2	-5.7	.	3.9
F. Miscellaneous								
AC004142	**LRRN3**	7q21.12	-2.6	.	3.2	10.2	3.0	.
AB004857	**SLC11A2**	12q13	-2.1	.	-1.9	-2.0	2.3	.
AI971169	**BCL2L11**		-4.0	.	.	.	2.9	.
S69115	**NKG7**	19q13.33	.	-3.5	.	.	.	.
X13444	**CD8B1**	2p12	.	-3.5	.	.	.	2.5
AB020630	**PPP1R16B**	20q11.23	.	-2.1	.	-1.1	.	2.5
AF070616	**HPCAL1**	2p25.1	.	-2.0	.	.	.	1.9
AA522530	**DDIT4**	10pter-q26.12	2.1	.	.	-9.8	-2.1	.
M38690	**CD9**	12p13.3	.	2.4	2.9	3.1	-1.5	.
X96719	**CLECSF2**	12p13-p12	.	2.2	.	.	.	-1.9

[a)] Average fold-change values for the genes that showed ≥2-fold change in some of the comparisons. For these genes also the significant changes less than 2-fold in parallel comparisons are shown. Missing value indicates that the change was not significant according to the statistical data analysis algorithm MAS5.

To illustrate the putative functional roles of these newly identified immediate targets of IL-4, the genes differentially expressed in Th1 and Th2 conditions were grouped into the functional categories based on Gene Ontology annotations [18]. The dominating functional groups consisted of transcription factors, cell adhesion molecules and receptors, enzymes and other intracellular signaling molecules (Tables. 1, 2). Most of the genes in these groups were already induced by IL-4 already after 2 h of polarization. After 6 h, besides a few additional genes belonging to these functional groups, most of the genes coding for extracellular molecules and ligands became induced or repressed by IL-4.

Validation of the differential regulation of CHST10, NKG7, TCF7 and ID3 during early Th1 and Th2 cell polarization with real-time RT-PCR

The differential regulation of genes coding for *CHST10*, *NKG7, TCF7* and *ID3* during the first week of Th1 and Th2 polarization was further validated with quantitative real-time RT-PCR (Fig. 1). In agreement with the oligonucleotide array results (Table 2F), real-time RT-PCR analysis showed *NKG7* to be induced by IL-4 and to be preferentially expressed in the cells polarized to Th2 direction during the early stage of differentiation (Fig. 1). Interestingly, after 2 days *NKG7* was also induced by IL-12 (4-fold, $p=0.017$) and, as previously described, became preferentially expressed by the polarized Th1 cells (at 7 days) [19]. *CHST10* was also confirmed to be induced by IL-4 and to be preferentially expressed in Th2 conditions after 6 h of polarization (Table 2B and Fig. 1). Similarly, these differences were seen after 7 days of polarization. The observation of *ID3* downregulation by IL-4 and preferential expression in the cells polarized in the Th1 direction at 6 h was found in both the oligonucleotide array and real-time RT-PCR results (Table 1A and Fig. 1). Similarly, the downregulation of *TCF7* by IL-4 at 48 h was confirmed with RT-PCR (Table 2A and Fig. 1). Thus, the RT-PCR confirmed the oligonucleotide array results.

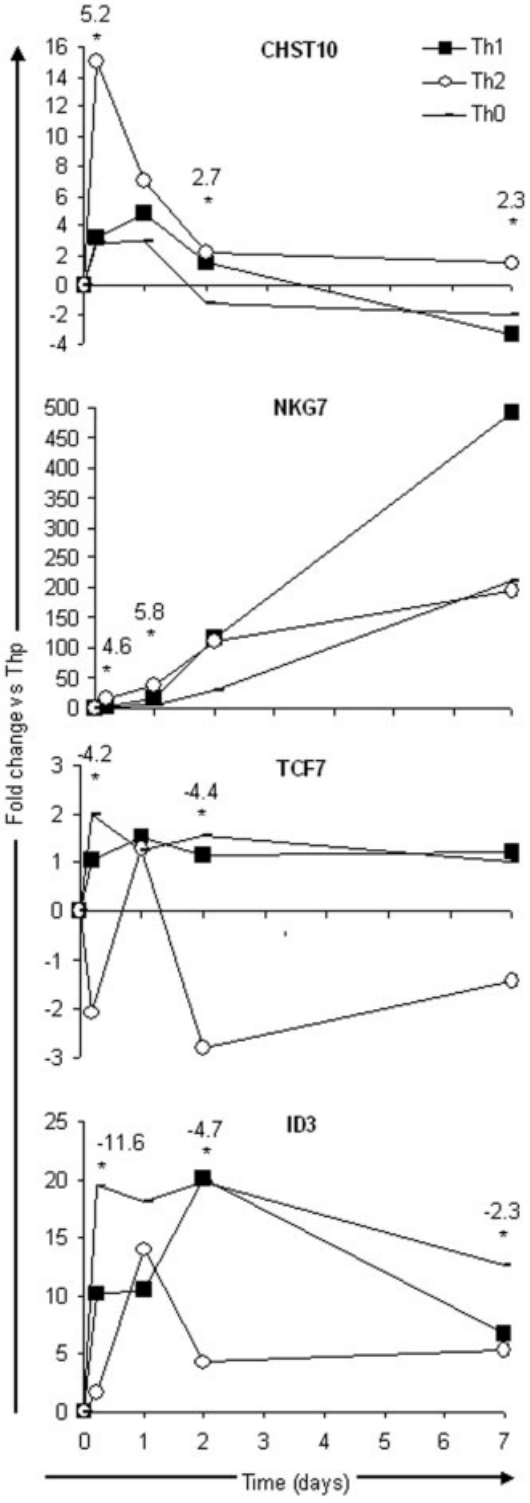

Figure 1. Validation of the differential regulation of *CHST10, NKG7, TCF7* and *ID3* during the early Th1 and Th2 cell differentiation with real-time RT-PCR. To study the expression kinetics of *CHST10, NKG7, TCF7* and *ID3* during 1 week of polarization, long-term Th1 and Th2 cultures were generated from human cord blood $CD4^+$ cells from four individuals. The cells were activated with PHA and irradiated CD32-B7 transfected fibroblasts. Th1 cultures were supplemented with IL-12, whereas Th2 cultures were supplemented with anti-IL-12 and IL-4. After 48 h of priming, IL-2 was added into the cultures. To detect the changes induced by IL-12 or IL-4, a subset of the activated cells were cultured without any polarizing cytokines in the presence of IL-2 alone (Th0). The expression levels of the genes *CHST10, NKG7, TCF7* and *ID3* were measured in cells isolated from four individuals in four technical repeats with real-time RT-PCR at the indicated time points. The significant regulation by IL-4 is displayed in the figure as average fold change in gene expression level between the cells cultured in Th0 and Th2. Paired *t*-test was used to evaluate the statistical significance of the differences between Th2 and Th0 conditions ($^*p \leq 0.05$).

SATB1, BCL6 and TCF7 are differentially regulated during the early Th1 and Th2 polarization at the protein level

In these oligonucleotide array and real-time RT-PCR analyses, it was clearly demonstrated that the transcriptional regulators *ID3*, *SATB1*, *TCF7, BCL6* (Tables. 1A, 2A) and suppressor of cytokine signaling, *SOCS1* (Table 1C), are differentially regulated already at the initiation of Th1 and Th2 differentiation. Similar changes have been previously noted with the known key regulators of Th1 and Th2 differentiation, *TBX21* and *GATA3*, in mouse [8, 20] and human cells (Ylikoski *et al.*, submitted). To validate these results at the protein level, Western blot analysis was carried out to study the expression of these factors after 0, 2, 6, 24 and 48 h of Th1 or Th2 polarization. TBX21 and GATA3 were used as control genes to monitor the polarization of the cells. As expected, TBX21 became preferentially expressed in Th1 and GATA3 in Th2 conditions (Fig. 2). Whereas the SATB1 protein was expressed already in the Thp cells, in keeping with the oligonucleotide array results, SATB1 became expressed at enhanced levels at 6 h in the Th2 polarized cells, and preferential expression was seen in Th2 conditions at 24 and 48 h. Although SATB1 was clearly more expressed in Th2 polarized cells, expression was not absent in the cells polarized to the Th1 direction. At the mRNA level, *BCL6* and *TCF7* were preferentially expressed in the cells induced to polarize in the Th1 direction. At the protein level, the expression of BCL6 was barely detectable in Thp cells. Concordant with the oligonucleotide array results for the Th1 conditions, BCL6 became preferentially expressed within 6 h, with the differences maintained for at least 48 h. A low level of BCL6 expression was seen also in the cells induced to polarize in the Th2 direction. The TCF7 protein was already expressed in the Thp cells. At the 24-h time point the differences in TCF7 protein expression between the cells induced to polarize to the Th1 or Th2 direction vanished temporarily (Fig. 2), in agreement with the expression changes seen with *TCF7* mRNA (Fig. 1). Further, in accordance with the oligonucleotide and RT-PCR results, the TCF7 protein became downregulated by IL-4 and preferentially expressed in the cells polarized to the Th1 direction after 48 h. Low levels of SOCS1 protein were observed in all the samples studied (Fig. 2), although a high level of expression was detected in the Jurkat cells used as positive control (data not shown). In contrast to the results obtained at the mRNA level, no clear difference during the early polarization of Th1 and Th2 cells was detected in the expression of SOCS1 protein. Expression of ID3 protein was not detected in Th cells in any of the samples studied, although cloned ID3 transfected to HeLa cells and used as a positive control confirmed the functionality of the antibody (data not shown). From these observations it was concluded that the transcriptional regulators BCL6, TCF7 and SATB1 are differentially regulated during the early Th1 and Th2 cell polarization both at the protein and mRNA level.

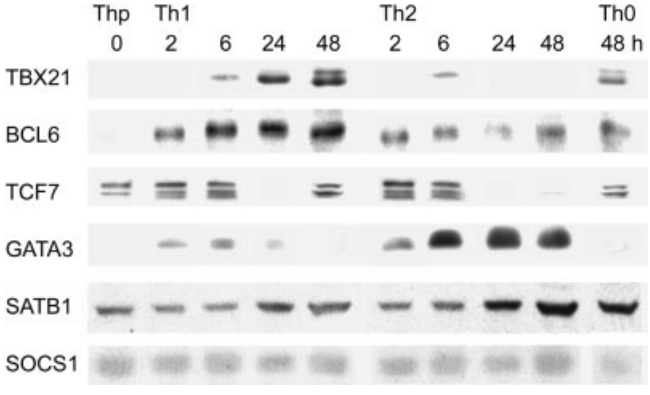

Figure 2. SATB1, TCF7 and BCL6 are differentially and reciprocally regulated during the early Th1 and Th2 cell differentiation. Western blot analysis was performed for TBX21, GATA3, SATB1, TCF7, BCL6 and SOCS1 to study protein levels in cord blood $CD4^+$ cells in response to CD3 + CD28 activation alone (Th0) or during the early polarization of Th1 (anti-CD3 + anti-CD28 + IL-12) and Th2 (anti-CD3 + anti-CD28 + IL-4) cells. The sample amounts in each whole cell lysate loaded on the gel were equalized based on protein amounts.

Effects of TGF-β on gene expression during the early stages of Th polarization

To study the effect of TGF-β on the early Th1 and Th2 polarization, comparison was made between cells cultured under Th1 or Th2 conditions in presence and absence of TGF-β. Altogether 20 genes were regulated by TGF-β in Th1 and/or Th2 conditions (Table 3). Four of these genes, including the transcriptional co-repressor *ID3*, structural components *LAMA3* and *CCL20,* and *DDIT4* with an unknown function, were also clearly regulated by IL-4 (over 2-fold change). Genes *CCL20*, *ID3* and *LAMA3* that were repressed by IL-4 in the absence of TGF-β, were induced by TGF-β in both Th1 and Th2 conditions. However, induction of *ID3* and *LAMA3* by TGF-β in cells cultured in Th2 conditions was less than 2-fold. Moreover, the IL-4-repressed gene *DDIT4* was also induced by TGF-β, but only in cells cultured in Th1 conditions. Gene *C19ORF6* was induced by IL-4 at 6 h by 1.7-fold and 7-fold in two different biological repeats. Interestingly, this gene was strongly repressed in the presence of TGF-β (14.9-fold and 4.3-fold).

Table 3. Genes regulated by TGF-β in the Th1- or Th2-polarizing conditions and their regulation by IL-4 (Th2 *vs.* Th0)

GenBank ID	Symbol	Locus	Th1+TGFbeta vs Th1		Th2+TGFbeta vs Th2		Th2 vs Th0	
			2 h	6 h	2 h	6 h	2 h	6 h
Enzymes and signaling molecules								
U70426	**RGS16**	1q25-q31	2.1[a)]	7.2	1.7	4.3	.	.
U03106	**CDKN1A**	6p21.2	1.7	2.5	2.0	1.7	-1.8	.
X17094	**FURIN**	15q26.1	1.6	3.1	.	2.4	.	.
X12451	**CTSL**	9q21-q22	1.4	3.9	.	3.1	.	1.7
U10550	**GEM**	8q13-q21	.	2.0	1.4	2.1	-1.6	-1.9
AF078077	**GADD45B**	19p13.3	2.0	.	.	.	-1.9	.
J03037	**CA2**	8q22	1.7	.	2.3	.	.	.
U16799	**ATP1B1**	1q22-q25	.	3.0	.	.	.	.
AL050356	**MINPP1**	10q23	.	4.9	.	.	.	.
AL049415	**ADAM19**	5q32-q33	.	2.3	.	.	.	.
M64349	**CCND1**	11q13	.	-2.2	.	.	.	.
Transcriptional regulators								
AL021154	**ID3**[b)]	1p36.13-p36.12	1.4	2.1	1.5	.	-2.8	-4.4
J04111	**JUN**	1p32-p31	.	3.0	1.7	8.3	.	.
J03258	**VDR**	12q12-q14	.	2.9	.	4.4	.	.
AB014569	**KIAA0669**	3q25.1	.	.	.	2.5	.	.
Structural molecules								
L34155	**LAMA3**[b)]	18q11.2	2.2	.	1.7	.	-1.8	.
X52022	**COL6A3**	2q37	.	2.5	.	2.5	.	.
Chemokines, extracellular lignads and cell adhesion molecules								
U64197	**CCL20**[b)]	2q33-q37	.	2.5	.	3.0	.	-2.3
M38449	**TGFB1**	19q13.2	.	2.5	.	-1.3	.	.
M14648	**ITGAV**	2q31-q32	.	2.2	.	.	.	.
U56102	**CD226**	18q22.3	.	-2.1	.	.	.	.
Miscellaneous								
AB004066	**BHLHB2**	3p26	2.0	1.9	1.5	2.1	.	.
AF039656	**BASP1**	5p15.1-p14	.	2.3	.	2.1	.	.
AB023183	**INPP5F**	10q26.13	.	1.7	.	2.3	.	.
AL039458	**LRIG1**		.	1.9	.	2.2	.	.
AB007939	**KAB**	1q44	.	1.6	.	2.1	.	.
AL049923	**OSBPL8**	12q14	.	2.4	.	2.8	.	.
AA522530	**DDIT4**	10pter-q26.12	.	2.1	.	.	-2.1	.
HG3075-HT3236	**PTK2**		.	.	.	2.5	.	.
AC004528	**C19orf6**[b)]	19p13.3	.	.	.	-8.0	.	3.5

[a)] Average fold-change values for the genes that showed ≥2-fold change in some of the comparisons. For these genes also the significant changes less than 2-fold in parallel comparisons are shown. Missing value indicates that the change was not significant according to the statistical data analysis algorithm MAS5.

[b)] Genes differentially expressed in Th1 and Th2 conditions and co-regulated by IL-4 and TGF-β in an opposite manner.

Developing Th1 and Th2 cells are responsive to IL-10 signaling

IL-10R receptor was induced by IL-4 at the mRNA level after both 2 and 6 h of Th2 polarization (Table 1B). *IL-10R* mRNA has previously been reported to be preferentially expressed in Th2 conditions also after 2 and 3 days of polarization [11, 12]. To verify these differences at the protein level, the expression of the receptor was studied with flow cytometry. According to the results, 40–60% of the cells expressed the receptor already at the Thp stage (Fig. 3). The expression was further increased after 2, 6, 24 and 48 h in response to CD3 + CD28 activation alone (data shown only for 0

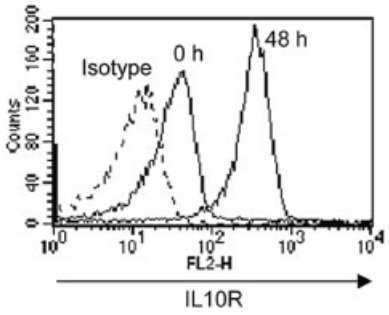

Figure 3. Expression of IL-10R during T cell activation. $CD4^+$ cells were isolated from human cord blood. The cells were activated with anti-CD3 + anti-CD28 and were cultured in "neutral" (Th0) conditions for 0 or 48 h. The expression level of IL-10R on the cells was measured with flow cytometry.

and 48 h). After 24 h of activation nearly all of the cells expressed the IL-10R (73–95%). Although at the mRNA level the expression of *IL-10R* was preferentially induced by IL-4 in Th2 conditions, at the protein level there were no clear reproducible differences between the cells polarized to the Th1 and Th2 direction (data not shown). Thus, the expression of the IL-10R is highly and rapidly induced by CD3 + CD28 activation alone and is similarly induced during differentiation of both Th1 and Th2 cells.

To elucidate whether IL-10 signaling was functional in these developing Th1 and Th2 subtypes, we studied the effects of IL-10 on the expression of the genes *GATA3* and *TBX21*. In addition, the effects of IL-10 on the expression of *SATB1* and *DUSP6*, two genes differentially regulated by Th1- and Th2-inducing cytokines, were studied. Concordant with our previous studies with human cells, in three out of five individuals expression of *TBX21* was increased 3.2-fold ($p \leq 0.05$) at 6 h in the cells induced to polarize to Th1 direction, when compared to the Th2 conditions (Fig. 4) (Ylikoski *et al.*, submitted). In all of these individuals, *TBX21* expression was reduced in the presence of IL-10. At 48 h, *TBX21* was preferentially expressed in the cells induced to polarize in the Th1 direction in each of the five individuals studied (average fold change 14.9, $p \leq 0.005$). At the same time point, there was on average a 2.0-fold decrease ($p \leq 0.05$) in *TBX21* expression by IL-10 in four of five individuals. Concordant to our previous observations with human cells, when compared to the Th1 conditions, *GATA3* was preferentially expressed in the cells induced to polarize in the Th2 direction (average fold change –14.3, $p \leq 0.01$) at the 6-h time point (Fig. 4) (Ylikoski *et al.*, submitted). Similarly, differences in GATA3 expression between Th1 and Th2 cells were detected at 24 h (average fold change –17.9, $p \leq 0.0005$) and 48 h (average fold change –9.8, $p \leq 0.05$). A suppressive effect of IL-10 on *GATA3* expression was detected in two of five individuals (1.65–4.79-fold change) at all of the time points studied (6, 24 and 48 h). However, in three other individuals no suppressive effect by IL-10 on *GATA3* expression was detected during Th2 cell differentiation. Furthermore, IL-10 did not suppress the expression of *SATB1* or *DUSP6* (Fig. 4). These results demonstrated that in four of five individuals studied, IL-10 rapidly decreased the expression of *GATA3* and/or *TBX21*. However, IL-10 had no effect on the expression of the genes *SATB1* or *DUSP6*, which are similarly to *GATA3* immediately induced by IL-4 in the Th2 polarizing conditions.

Discussion

To identify the genes involved in the initiation of human Th1 and Th2 differentiation, oligonucleotide arrays have been used to study the effects of the Th1- or Th2-polarizing cytokines, IL-12 and IL-4, at 2 or 6 h. In agreement with the observations that IL-12R is not expressed by the naïve Thp cells, but is induced in response to TCR activation, the effects of IL-12 were hardly detectable during the early polarization of Th1 cells [15, 17, 21]. Only two genes, *IFNG* and interferon-regulated *GBP1*, were slightly induced by IL-12 during the early polarization of Th1 cells. This indicates that some IL-12R activity is present, possibly due to minute amounts of IL-12R on the cells already at the early stage. Our previous study with cells polarized for 2 days in Th1 or Th2 conditions demonstrated that the effects of IL-12 are clearly seen after 2 days of differentiation [11]. These observations are concordant with results from our recent study of mouse Th1 cell differentiation, where we demonstrated that the earliest gene regulated in a IL-12- and STAT4-dependent manner is *IFNG*, and the effects of STAT4 on the genes, other than *IFNG*, are predominantly seen after 48 h of Th1 polarization [22]. It therefore seems apparent that the developing Th1 cells are quite unresponsive to IL-12 in the early stage of differentiation.

In contrast to IL-12 signaling, the Th2 polarizing cytokine IL-4 had already started to regulate a number of genes within 2 h, by which time a peak in the changes of gene expression was seen. Most of the effects of IL-4 at the 2-h time point were observed as an upregulation of gene expression rather than downregulation. After 6 h several genes became repressed by IL-4. The IL-4-mediated changes led to differences in the gene expression profiles of the cells cultured in Th1 or Th2 conditions. The gene expression profiles varied across the study time course, with only a few genes remaining differentially expressed throughout. According to expectations based on previous studies using human Th cells, among the genes that displayed constant changes throughout the early polarization were well-known

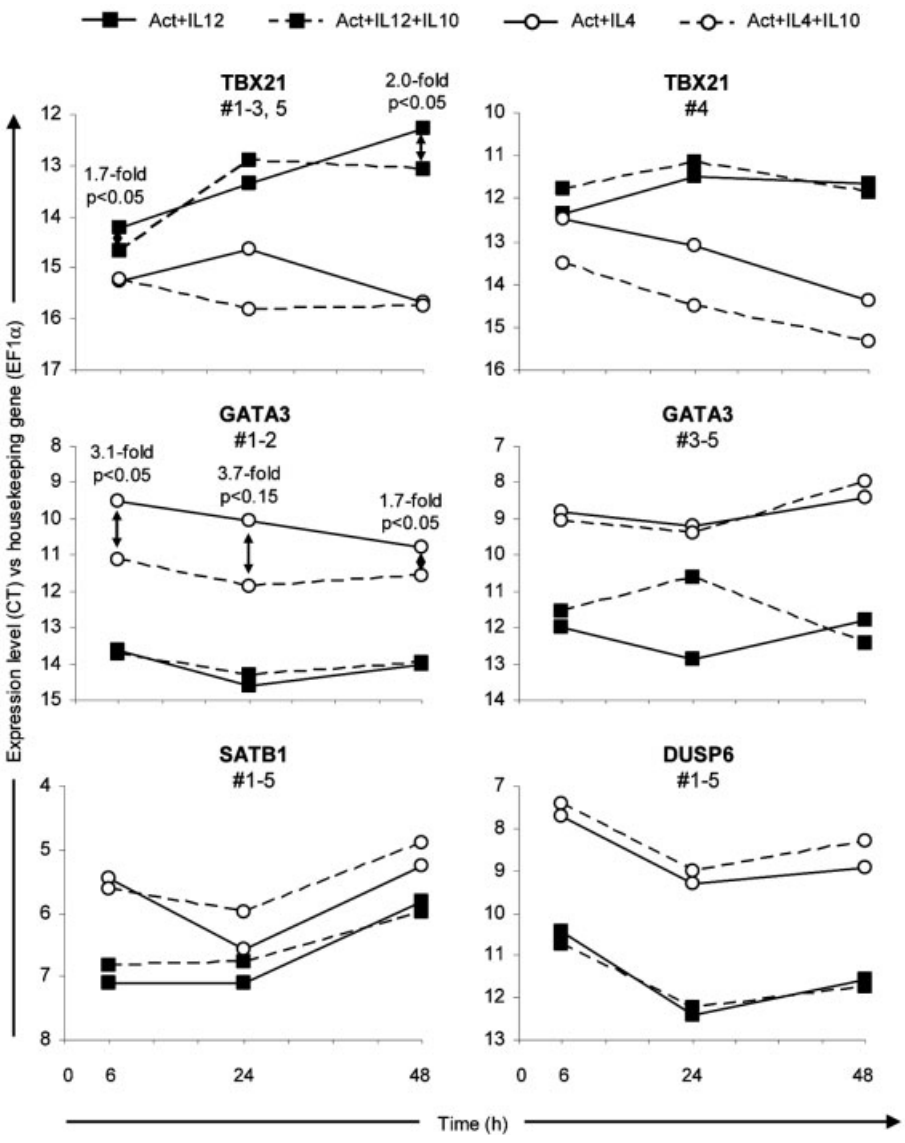

Figure 4. Regulation of gene expression by IL-10. The human cord blood $CD4^+$ cells were cultured in Th1 (anti-CD3 + anti-CD28 + IL-12) or Th2 (anti-CD3 + anti-CD28 + IL-4) conditions in presence or absence of IL-10. Expression of selected genes *TBX21*, *GATA3*, *SATB1* and *DUSP6* was measured at the indicated time points using real-time RT-PCR. The average gene expression levels from five individuals (nos. 1–5) are represented as ΔCT values (see Materials and methods). The reproducible and statistically significant fold differences in gene expression between the samples cultured in the absence and presence of IL-10 are indicated (paired *t*-test).

mediators of Th1 and Th2 differentiation *GATA3*, *MAF* (Tables. 1, 2A) and *IFNG* [23]. In addition, numerous novel genes with similar kinetics were identified to be differentially regulated in Th1 and Th2 conditions at 2 and/or 6 h. Such genes included transcriptional regulators *NFIL3, SATB1, STK17B* and *BCL6* (Tables. 1A, 2A); cell adhesion molecules and receptors *IL-10RA, CXCR4* and *EDG1* (Table 1B); enzymes and intracellular signaling molecules *DUSP6, SPINT2, SOCS1, EBI2, GNAI1* and *GBP1* (Tables. 1C, 2C); transporters *ABCD3* and *SLC39A8* (Table 1D); chemokine *CCL20* (Table 2D) and other genes with miscellaneous functions *XCL1, LRRN3, NKG7* and *HPCAL1* (Tables. 1E, 2G). Similarly to the known key regulators of Th1 and Th2 differentiation, *MAF, GATA3, IFNG* and *TBX21*, differences for these transcripts are maintained throughout the early polarization of human Th1 and Th2 cells [11, 12, 19, 24]. Thus, these genes are likely to play an important role in the regulation of the polarization process.

The majority of the genes involved in the initiation and early Th1 and Th2 cell differentiation code for factors involved in signaling events from the cell surface to nucleus. As regulation at the gene expression level does not necessarily implicate changes in the functional protein products, regulation of a subset of factors was also examined at the protein level. *SATB1, TCF7* and *BCL6* (Table 2A) were selected for further analysis because their expression profiles correlated with the expression profiles of well-known key regulators of Th1 and Th2 differentiation, *GATA3* and *TBX21*. Differential regulation of these genes was detected throughout the early polarization of Th1 and Th2 cells. Furthermore, these factors were among the few genes for which a functional antibody was available. For the transcriptional regulators *SATB1, TCF7* and *BCL6*, the differential regulation in Th1 and Th2 conditions was also confirmed at the protein level. Similarly to the transcription factors *TBX21* and *GATA3*, these genes

are reciprocally regulated during early Th1 and Th2 polarization. The importance of BCL6 in suppression of Th2 differentiation has been previously demonstrated with *BCL6*-knockout mice that display lethal Th2 hyper-response [25, 26]. BCL6 is believed to promote Th1 differentiation by inhibiting expression of GATA3 protein and *IL-5* gene [27, 28]. The role of SATB1 in the regulation of Th cell differentiation has not yet been fully determined, although SATB1 together with another transcriptional regulator TCF7 has functional characteristics similar to GATA3. As GATA3, SATB1 and TCF7 also have important roles in early thymocyte development [29, 30]. Similar to GATA3, SATB1 is a factor involved in chromatin remodeling and it regulates gene expression over long distances [31–33]. Together with GATA3, SATB1 has been suggested to be involved in transcriptional regulation of the *CD8B* gene [34]. Thus, it would be interesting to determine whether these factors together regulate expression of genes involved in Th1 and Th2 cell differentiation. *TCF7* is localized in the Th2 cytokine cluster in chromosomal region 5q31 and, similarly to GATA3, it can bind to the enhancer element of the *TCRα* gene [35]. Both *SATB1* and *TCF7* have been associated with the diseases or phenotypes mediated by Th1 or Th2 responses. *SATB1* was recently demonstrated to be preferentially expressed at both mRNA and protein level in the peripheral blood T cells of patients with atopic dermatitis [36]. Interestingly, a recent study reported a polymorphism in the *TCF7* gene to be associated with type-1 diabetes, which is a disease with dominating Th1 phenotype [37]. As all of these transcription factors have similarly important roles in the regulation of thymocyte maturation, it is possible that they also regulate later differentiation of Th1 and Th2 cells.

Another large functional group among the genes involved in the initiation of Th1 or Th2 differentiation consisted of enzymes and enzyme inhibitors and components of intracellular signaling pathways. Interestingly, many of these genes code for targets or factors involved in RAS and MAPK signaling. Such genes included *NFIL3* (Table 1A); *DUSP6* and *MAP3K14/NIK* (Table 1C); *RASGRP1*, *RASA2*, *SOS1* and *PPM1A/PP2Calpha*, (Table 2C); *LRRN3* (Table 2F) and previously identified *ERK3* [38–47]. This is consistent with the previous observations that RAS/MAPK pathway favors Th2 polarization by enhancing the IL-4R signaling [48].

TGF-β is an immunosuppressive mediator that is able to inhibit the differentiation of Th1 and Th2 cells [14]. Previously, we demonstrated that after 48 h of polarization, TGF-β antagonizes effects of IL-12 or IL-4 on certain genes including *NFIL3* and *SATB1* [11]. However, after 2 and 6 h of polarization, the effects of TGF-β were modest on the genes regulated by IL-4. Only the *ID3*, *LAMA3* and *CCL20* (Table 3) were repressed by IL-4 and were antagonized by TGF-β. In addition, the *C19ORF6* gene was upregulated by IL-4 and strongly antagonized by TGF-β. Positive regulation of *ID3* and *LAMA3* by TGF-β has been previously described in fibroblasts and epidermal keratinocytes, respectively [49, 50]. The chemokine CCL20 is a ligand for CCR6 and is involved in enhancing chemotaxis of T helper cells. Its production by rheumatoid arthritis-derived activated synoviocytes has been shown to be inhibited by Th2 cytokines IL-4 and IL-13 [51–53]. Preferential expression of *CCL20* by Th1 cells, activated and polarized for 12–14 days, has also been previously reported [19]. Gene *C19ORF6* codes for membralin protein that is highly expressed in central nervous system [54]. The function of the *C19ORF6* protein product is poorly known. In conclusion, although TGF-β regulates numerous genes in both Th1 and Th2 conditions during the immediate phase of differentiation, only five of these genes were clearly regulated by IL-4. Opposite regulation of the genes *ID3*, *LAMA3*, *CCL20* and *C19ORF6* by IL-4 and TGF-β may be part of the mechanism of how TGF-β inhibits Th1 and Th2 differentiation.

In addition to TGF-β, IL-10 is a regulatory cytokine involved in suppression of Th1 or Th2 differentiation. IL-10 is produced by a subset of immunosuppressive $CD4^+$ T cells named T regulatory (Treg) cells, which have been shown to inhibit the function of Th cells [55]. Interestingly, the receptor for IL-10 (*IL-10RA*) was among the rare genes that were differentially expressed in Th1 and Th2 conditions immediately after polarizing stimuli and whose differential expression is maintained at least for 3 days (Table 1B) [11, 12]. However, our study demonstrates that at protein level IL-10R is highly induced by CD3 + CD28 activation alone and is expressed at similar levels by the differentiating Th1 and Th2 cells, indicating responsiveness of all these cell types (Th0, Th1, Th2) to IL-10 cytokine. IL-10 signaling was shown to be functional during development of both Th1 and Th2 cells, as the addition of IL-10 to the cell cultures rapidly and selectively decreased expression of the two key regulators of Th1 and Th2 differentiation *GATA3* and/or *TBX21* (Fig. 4). Importantly, this suppressive effect was not exclusively observed in all individuals. Moreover, in some of the individuals the immunosuppressive effect of IL-10 was only observed in either Th1 or Th2 conditions, on *TBX21* or *GATA3* expression, respectively. This variation in the responsiveness of different individuals to the immunosuppressive effects of IL-10 may be important and merits further investigation. The inhibitory effect of IL-10 on *TBX21* expression is consistent with previous reports that have described inhibition of IFN-γ production and STAT1 signaling by IL-10 [56, 57]. The early responsiveness of developing Th cell subtypes to immunosuppressive

effects of IL-10 is likely to be an important mechanism to regulate the magnitude of the Th1- or Th2-mediated responses in different individuals by the IL-10-producing cells.

In summary, the early differentiation of human Th1 and Th2 cells is orchestrated by IL-4 in Th2 conditions. The composition of the gene expression patterns regulated during the early polarization changes rapidly and only a subset of genes is differentially expressed by the polarizing Th1 and Th2 subsets throughout the early polarization. During the immediate response, IL-4 induces the expression of genes that are coding mainly for mediators and components of the intracellular signaling pathways, such as receptors, cell adhesion molecules, signal transducers, enzymes and their inhibitors and transcription factors. Later, additional factors in these groups, as well as extracellular molecules and ligands, become induced or repressed. The presence of TGF-β in Th1- and Th2-inducing conditions leads to changes in the expression of several genes of which only few are regulated also by IL-4, and thus may be implicated in the inhibitory mechanism of TGF-β on Th cell differentiation. In addition to TGF-β, these early developing cells are responsive to immunosuppressive cytokine IL-10, which in most of the individuals studied downregulated the expression of the key regulators of Th1 and Th2 differentiation, *TBX21* and/or *GATA3*.

Materials and methods

Induction of Th1 and Th2 polarization

Induction of human *in vitro* Th1 and Th2 differentiation was performed as previously described [11]. Briefly, $CD4^+$ cells from cord blood (Turku University Central Hospital, Finland) were isolated (Ficoll Isolation Paque, Amersham Pharmacia Biotech, Uppsala, Sweden and Dynal, Oslo, Norway) and activated with plate-bound anti-CD3 (500 ng/μL for coating) and soluble anti-CD28 (500 ng/μl, both from Immunotech, Marseille, France). Th1 polarization was induced with 2.5 ng/mL IL-12, and Th2 differentiation with 10 ng/mL IL-4 (both from R&D Systems, Minneapolis, MN). A fraction of cells were cultured in "neutral conditions" without polarizing cytokines. The cultures were supplemented with 3 ng/mL TGF-β or 17 ng/mL IL-10 (both from R&D Systems), where indicated. This was the most efficient concentration of IL-10 to suppress IFN-γ production (∼50% decrease) by the anti-CD3 + anti-CD28 + IL-12-activated (18 h) PBMC (data not shown). TGF-β-mediated suppression of IFN-γ production by Th1 cells in these conditions has been previously described [11]. The samples were collected after 0, 2, 6, 24 or 48 h of polarization.

For validation of the oligonucleotide array results with real-time RT-PCR, additional Th1 and Th2 primary cultures were generated as previously described [24]. Briefly, cord blood $CD4^+$ T cells were activated with 100 ng/mL PHA (Murex Diagnostics, Chatillon, France) and irradiated CD32-B7 transfected fibroblasts [58]. Th1 cultures were supplemented with 2.5 ng/mL IL-12, whereas Th2 cultures were supplemented with 10 μg/mL anti-IL-12 and 10 ng/mL IL-4 (all from R&D Systems, Minneapolis, MN). After 48 h of priming, 40 U/mL of IL-2 (R&D Systems) was added into the cultures. A fraction of cells were cultured without any polarizing cytokines in the presence of IL-2 alone. The cultures were generated from four individuals. Samples were collected at the time points of 0, 6, 24 and 48 h or 7 days.

Oligonucleotide array studies

The sample preparation and data analysis were performed as previously described according to the instructions and recommendations provided by the manufacturer (Affymetrix, Santa Clara, CA) [11]. Total RNA (5 μg) pooled from different individuals was used as starting material for the Affymetrix sample preparation. The samples were hybridized on human genome U95Av2 arrays containing probes for approximately 9,300 genes. Two biological repetitions for each microarray experiment were performed. After hybridization and scanning, the gene transcript levels were determined from data images with the GeneChip Microarray Suite Software version 5 ($MAS5^3$, Affymetrix). The normalized numerical data was filtered on four consecutive levels according to the statistical classifications performed by the MAS5 software based on 16 probes for each gene. At the detection level, all the genes that were assigned to be "absent" in both samples compared were removed. Similarly, all the genes that were classified as "no change" were removed. Subsequently, the gene expression was considered to be upregulated if the signal log ratio between the reference and the target samples was higher than one (twofold increase) and the target sample was "present". Similarly, a gene was defined as downregulated if the signal log ratio was less than minus one (twofold decrease) and the reference sample was "present". At the fourth level of data analysis, genes that presented a consistent change in two separate biological repeats were considered as differentially expressed. All the genes which fulfilled these criteria in at least one of the comparisons and one of the time points were selected for further analysis where the expression of the genes was explored parallel in different conditions without the threshold of twofold change. The programs GeneSpring 6.0 (Silicon Genetics, Redwood, CA) and Microsoft Access for Windows were utilized in data analysis. The gene annotations were obtained from NetAffx-database [59] and are used throughout this report unless stated otherwise. The data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2770.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed to measure gene expression levels of selected genes using TaqMan ABI Prism 7700 (Applied Biosystems, Foster City, CA) as described elsewhere [24, 60]. The housekeeping gene *EF1α* was used as a reference transcript [61]. Primers and probes (Table 4) used for the quantification of gene expression (MedProbe, Oslo,

Table 4. Primers and probes used in the real-time RT-PCR analysis

GenBank ID	Gene	1) 5′- 6(FAM)-PROBE-(TAMRA)-3′
		2) 5′-PRIMER 1-3′
		3) 5′-PRIMER 2-3′
AB013382	**DUSP6**	**1) 5′-CTCTACGACGAGAGCAGCAGCGACTG-3′**
		2) 5′-GCTGTGGCACCGACACAGT-3′
		3) 5′-ACTCGCCGCCCGTATTCT-3′
J04617	**EF1alpha**	**1) 5′-AGCGCCGGCTATGCCCCTG-3′**
		2) 5′-CTGAACCATCCAGGCCAAAT-3′
		3) 5′-GCCGTGTGGCAATCCAAT-3′
X55122	**GATA3**	**1) 5′-TGCCGGAGGAGGTGGATGTGCT-3′**
		2) 5′-GGACGCGGCGCAGTAC-3′
		3) 5′-TGCCTTGACCGTCGATGTTA-3′
NM_004854	**CHST10**	**1) 5′-CCCGAAAACGTGGTGCACGACC-3′**
		2) 5′-AGCATTTTCTTCCATTGAGGAGAT-3′
		3) 5′-CCGAGGAAGGCCGTTCTT-3′
NM_002167	**ID3**	**1) 5′-ACTGGTACCCGGAGTCCCGAGAGG-3′**
		2) 5′-TGCTGGACGACATGAACCA-3′
		3) 5′-TTTCCACCTGGCTAAGCTGAGT-3′
M13982	**IL4**	**1) 5′-TGCCGGCAACTTTGTCCACGG-3′**
		2) 5′-GGTCTCACCTCCCAACTGCTT-3′
		3) 5′-TCTGCTCTGTGAGGCTGTTCAA-3′
S69115	**NKG7**	**1) 5′-CCTGATTGCTTTGAGCACCGATTTCTG-3′**
		2) 5′-TCCCTGGGCCTGATGTTCT-3′
		3) 5′-TGGGACCCACAGCCTCAA-3′
M97287	**SATB1**	**1) 5′-AACGAGCAGGAATCTCCCAGGCG-3′**
		2) 5′-ACCAGTGGGTACGCGATGA-3′
		3) 5′-TGTTAAAAGCCACACGTGCAA-3′
AF241243	**TBX21**	**1) 5′-TCAGCATGAAGCCTGCATTCTTGCC-3′**
		2) 5′-ACAGCTATGAGGCTGAGTTTCGA-3′
		3) 5′-GGCCTCGGTAGTAGGACATGGT-3′
NM_003202	**TCF7**	**1) 5′-CTCCCTGACCTCAGGCAGCATGG-3′**
		2) 5′-CTGCAGACCCCTGACCTCTCT-3′
		3) 5′-ACACCAGAACCTAGCATCAAGGAT-3′

Norway) were designed using Primer Express software (Applied Biosystems). The quantitative value obtained from TaqMan real-time RT-PCR is a threshold cycle (CT). The fold differences between different conditions can be calculated from the normalized CT values ($CT_{gene\ X} - CT_{housekeeping\ gene}$), ΔCT values, with the formula: fold difference = $2^{(|\Delta CT1 - \Delta CT2|)}$ [13, 24]. The statistical significance of the differences was evaluated with paired *t*-test [24].

Flow cytometric analysis

Cord blood $CD4^+$ T cells cultured for 0, 2, 6, 24 or 48 h in 'neutral' (anti-CD3 + anti-CD28), Th1 (anti-CD3 + anti-CD28 + IL-12) or Th2 (anti-CD3 + anti-CD28 + IL-4) conditions were studied for IL-10R expression. First, the cells were washed with 2% FCS and 0.01% azide in PBS. Subsequently, the cells were stained with anti-human IL-10R-PE (BD Pharmingen, San Diego, CA) for 15 min after which the cells were washed with 2% FCS and 0.01% azide in PBS, and then with 0.01% azide in PBS. The IL-10R expression on the fixed

cells (1% formalin in PBS) was studied with FACScan and CellQuest Software (Becton Dickinson, San Jose, CA).

Western blot

Expression of TBX21, GATA3, SATB1, BCL6, TCF7, SOCS1 and ID3 was studied in the $CD4^+$ cord blood cells polarized to the Th1 or Th2 direction for 0, 2, 6, 24 and 48 h or activated with anti-CD3 + anti-CD28 alone for 48 h (Th0). Briefly, the cells were lysed in SDS buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT). Equal protein amounts of each whole cell lysate sample were loaded on the gel. Bromophenol blue (0.1%) was added in the cell lysates and the samples were run on 5–15% acrylamide gels and were transferred into nitrocellulose membranes (Hybond™-ECL™, Amersham Biosciences, Buckinghamshire, England). The membranes were blocked by overnight incubation in 0.01% TBST with 5% milk at +4°C. For detection of the proteins, the membranes were incubated with the primary antibodies including mouse anti-BCL6 (1.96 μg/mL, DAKO, M7211), mouse anti-TBX21 (2 μg/mL, sc-21749), mouse anti-GATA3 (2 μg/mL, sc-268), goat anti-SATB1 (1 μg/mL, sc-5989), goat anti-TCF7 (0.8 μg/mL, sc-8589), rabbit anti-SOCS1 (2 μg/mL, sc-9021) or rabbit anti-ID3 (2 μg/mL, sc-490) (all from Santa Cruz Biotechnology, Inc, Santa Cruz, CA) in 0.01% TBST with 5% milk for 1 h at room temperature. Subsequently, labeling with HRP-conjugated anti-mouse (M15345, Transduction Laboratories), anti-goat (705–035–003, Jackson ImmunoResearch Laboratories) or anti-rabbit (554021, BD Pharmingen) secondary antibody was performed at 1:10,000 dilution in 0.01% TBST with 5% milk for 1 h at room temperature. The labeled proteins were visualized with ECL reaction (Amersham Biosciences).

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The article cited in the text as Ylikoski *et al.* is currently in press: **Ylikoski, E., Lund, R., Kyläniemi, M., Filén, S., Kilpeläinen, M., Savolainen, J. and Lahesmaa, R.,** IL-12 up-regulates T-bet independently of IFN-γ in human $CD4^+$ T cells *Eur. J. Immunol.* 2005. DOI 10.1002/eji.200526101.

