Valproate Treatment of Human Cord Blood CD4-positive Effector T Cells Confers on Them the Molecular Profile (MicroRNA Signature and FOXP3 Expression) of Natural Regulatory CD4-positive Cells through Inhibition of Histone Deacetylase*

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Regulatory T cells (Tregs) play a key role in immune system homeostasis and tolerance to antigens, thereby preventing autoimmunity, and may be partly responsible for the lack of an appropriate immune response against tumor cells. Although not sufficient, a high expression of forkhead box P3 (FOXP3) is necessary for their suppressive function. Recent reports have shown that histones deacetylase inhibitors increased FOXP3 expression in T cells. We therefore decided to investigate in non-Tregs CD4-positive cells, the mechanisms by which an aspecific opening of the chromatin could lead to an increased FOXP3 expression. We focused on binding of potentially activating transcription factors to the promoter region of FOXP3 and on modifications in the five miRs constituting the Tregs signature. Valproate treatment induced binding of Ets-1 and Ets-2 to the FOXP3 promoter and acted positively on its expression, by increasing the acetylation of histone H4 lysines. Valproate treatment also induced the acquisition of the miRs Tregs signature. To elucidate whether the changes in the miRs expression could be due to the increased FOXP3 expression, we transduced these non-Tregs with a FOXP3 lentiviral expression vector, and found no changes in miRs expression. Therefore, the modification in their miRs expression profile is not due to an increased expression of FOXP3 but directly results from histones deacetylase inhibition. Rather, the increased FOXP3 expression results from the additive effects of Ets factors binding and the change in expression level of miR-21 and miR-31. We conclude that valproate treatment of human non-Tregs confers on them a molecular profile similar to that of their regulatory counterpart.

Regulatory T cells (Tregs)§ are a subpopulation of T cells with suppressive properties. Their deficiency or dysfunction has been linked to several autoimmune and inflammatory diseases, including arthritis, irritable bowel syndrome, atopic dermatitis, and psoriasis (1–9), and to the deleterious graft-versus-host disease occurring post allogeneic stem cell transplant (10).

Tregs exist in two general categories: thymus-derived Tregs, referred to as natural Tregs (nTregs), and adaptive Tregs, which can be induced in the periphery in response to a variety of stimuli, including pathogens, IL-10, and transforming growth factor-β (11–15), or in vitro from naïve CD4+ T cells upon activation of the T cell receptor in combination with transforming growth factor-β treatment (16–18). In addition to a distinct combination of membrane markers, the hallmark of nTregs cells is their high expression level of the transcription factor forkhead box P3 (FOXP3), which is indispensable to their suppressive activity, phenotype, stability, and survival in the periphery (3). Mutations of the FOXP3 gene lead to the lymphoproliferative disease of the Scurfy mouse and the homologous autoimmune lymphoproliferative disorder in man, termed immune deregulation polyendocrinopathy enteropathy X-linked syndrome (19). The importance of FOXP3 for Tregs cell function is supported by the observation that its ectopic expression in effector T cells endows them with regulatory properties and some but not all of the phenotypic markers of regulatory T cells (20, 21). Therefore, the understanding of the mechanisms regulating the expression of FOXP3 is of utmost importance to get an insight into pathological conditions, such

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5 The abbreviations used are: Treg, regulatory T cell; nTreg, natural Treg; IL-10, interleukin-10; FOXP3, forkhead box P3; HDAC, histones deacetylase; UCB, umbilical cord blood; RT, reverse transcription; qRT, quantitative RT; ChiP, chromatin immunoprecipitation; WT, wild type; GFP, green fluorescent protein; ORF, open reading frame.
as certain autoimmune diseases, or the impaired immune anti-tumor response linked with Tregs generation.

Histone acetylation plays a key role in transcriptional regulation (22), probably by altering chromatin structure. Acetylation of core nucleosomal histones is regulated by the balance between the activity of histones acetyltransferases and histones deacetylases (HDACs). Histones acetyltransferases preferentially acetyl specific histone lysine substrates (23, 24) thus neutralizing the lysine residues positive charge and disrupting the nucleosome structure, allowing unfolding of the associated DNA, access by transcription factors, and changes in gene expression. In contrast, HDACs restore the positive charge on lysine residues through the removal of the acetyl group from lysine residues of some histones, causing the repression of gene transcription by compacting the chromatin structure (25).

Pharmacological agents that inhibit HDACs impede the removal of acetyl groups thus facilitating transcription of genes so repressed (26). In particular, HDAC inhibitors induce expression of epigenetically silenced genes that could promote growth arrest (27, 28), cell differentiation (29–31), and death in tumors of various lineages (28, 32–34). In addition, these inhibitors are effective in promoting the transcription of genes that are silenced due to CpG island hypermethylation (35–37). FOXP3 is considered a potential target for the action of HDAC inhibitors, because studies of the promoter and the surrounding chromatin have revealed histone H4 hyperacetylation when the gene was activated (38). In addition, a recent study (39) showed that treatment of human CD4+ T cells with sodium valproate (38), having been used for decades to treat some forms of epilepsy, at doses compatible with the chromatin, and the changes in the expression levels of miR-21 and miR-31.

**Materials and Methods**

Bioinformatics—Genomic sequences spanning the 5′-untranslated region of the FOXP3 gene were analyzed using the alignment software ClustalW (available online) allowing the identification of conserved regions. Transcription factor binding sites were identified using TESS software (available online).

Collection and Preparation of UCB Samples—After informed consent, umbilical cord blood (UCB) was taken from the umbilical vein after normal full-term deliveries. UCB mononuclear cells were isolated by centrifugation over a lymphocyte separation medium (PAA Laboratories, Linz, Austria).

Isolation of T-cell Subpopulations—UCB CD4+ lymphocytes were purified using the CD4+ T cells isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Briefly, UCB mononuclear cells were first incubated in phosphate-buffered saline supplemented with 2% decomplemented fetal bovine serum and saturating amounts of a biotin-conjugated antibodies mixture (CD8, CD14, CD16, CD19, CD36, CD56, CD123, T cell receptor β/δ, and Glycoporphin A). Cells were then incubated with anti-biotin micro-beads, and CD4+ T cells were negatively selected using magnetic separation columns (Miltenyi Biotec). The CD4+ population was then incubated with anti-CD25 micro-beads, and the CD25+ and CD25− cells were sorted using magnetic columns (Miltenyi Biotec).

Cell Culture and Valproate Treatment of Human CD4+, CD25− UCB T Cells—CD4+, CD25− UCB T cells were plated at a density of 2 × 10^6 cells/well in 12-well tissue culture plates in 1 ml of RPMI 1640 supplemented with 10% AB serum, 2 mM l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Lonza Europe, Verviers, Belgium), in the presence of 5 μg/ml phytohemagglutinin (Sigma-Aldrich) and 20 units/ml interleukin-2. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 18–24 h. The next day, the cells were treated with valproate at a concentration of 1 mM and sampled at 2, 4, 6, and 24 h for further analyses.

Quantitative PCR for FOXP3 mRNA Expression—Total RNA was extracted with the TRIzol reagent according to the manufacturer’s guidelines (Invitrogen), and first strand cDNAs were synthesized by reverse transcription using the Superscript First-strand Synthesis System as described for the RT-PCR kit (Invitrogen). Quantitative mRNA expression was measured by real-time PCR, using the Prism 7900 sequence detection system (PE, Applied Biosystems, Foster City, CA), and the TaqMan Master mix kit. EF1-α was used as an internal control. The FOXP3 primers and the internal fluorescence TaqMan probe
were designed as follows: forward, 5’-TTCACCTACGCCA- CGCTCA-3’; reverse, 5’-CCAGCTCATCCACGGTCCA-3’; and probe, 5’-FAM-CCACCTGGAAAGCAGCCCATCAGGCC- TAMRA-3’.

Western Blot Analysis—4 × 10^6 cells were lysed and subjected to SDS-PAGE using 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Amersham Biosciences) using a semidry electrophoretic chamber. Membranes were blocked with TBST containing 5% bovine serum albumin overnight at 4 °C. After blocking, the blots were incubated with a 1/200 dilution of goat anti-FOXP3 antibody diluted in TBST for 1 h at 25 °C. Following 1 h of incubation with anti-goat peroxidase-conjugated antibody (Sigma) at room temperature, proteins were detected by the electrogenerated chemiluminescence method (Amersham Biosciences), according to the manufacturer’s instructions.

To confirm sample loading and transfer, membranes were incubated in stripping buffer and reblocked for 1 h, and then reprobed using anti-Actin (C-2, Santa Cruz Biotechnology). All protein signals were visualized using the LAS-3000 image reader (Fuji), and signals were analyzed with AIDA software (Raytest).

Detection of Mature microRNAs by TaqMan Real-time PCR—TaqMan miR assays (ABI, Forest City, CA) used the stem-loop method (53, 54) to determine the expression level of mature microRNAs. For RT reactions, 10 ng of total RNA was used in each reaction (15 µl) and mixed with the RT primer (3 µl). The RT reaction was carried out in the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then kept at 4 °C. After the RT reaction, the cDNA products were diluted five times, and 9 µl of the diluted cDNA was used for PCR reaction along with TaqMan Microarray assay (1 µl) and PCR master mix (10 µl). The PCR reaction was conducted at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in the ABI 7900 real-time PCR system. The real-time PCR results were analyzed and expressed as relative miR expression of the CT (threshold cycle) value, which was then converted to fold changes. RT primers, PCR primers, and TaqMan probes for miR-21, miR-31, miR-125a, miR-181c, and miR-374 were purchased from ABI. RNU44 was used for normalization.

Plasmid Constructions and Transfection Experiments—A 600-bp fragment encompassing the region upstream of the human FOXP3-translated region was cloned upstream of the Firefly luciferase gene (SacI/HindIII sites) in the PGL3 plasmid (Promega, Madison, WI) and designated as PGL3-F-WT. The PCR primers used for amplification of this region were (5’ ends): forward primer, AAAAAAATTTGGATTATTAGAAGA; reverse primer (F-EtsII mut), ATATGA-CTTAGCATATATAGTTCTTTCTCTCACCACA. The constructs were verified by sequencing.

Transient Transfection—Jurkat cells (3 × 10^6) were transiently transfected using standard DEAE-dextran protocols. Cells were washed once with STBS 1× (25 mM Tris-HCl (pH 7.5), 1.37 mM NaCl, 5 mM KCl, 500 µM CaCl₂, 500 µM MgCl₂, and 600 µM Na₂HPO₄) and resuspended in 410 µl of STBS 1× containing 450 µg/ml DEAE-dextran, 0.5 µg of the reporter plasmid, and 0.06 µg of an internal control plasmid containing the Renilla luciferase gene under control of the herpes simplex virus-1 thymidine kinase promoter (pRL-TK vector, Promega). In cotransfection experiments, an additional 0.085 µg of the p-GL3-F-Ets was added with the reporter plasmid constructs. Luciferase was detected using a dual-luciferase reporter assay system (Promega), according to the manufacturer’s instructions.

ChIP Assay and Quantitative Real-time PCR—The chromatin immunoprecipitation (ChIP) assay was performed using the kit purchased from Upstate Biotechnology following the manufacturer’s protocol. Cells were fixed with 1.5% formaldehyde for 10 min at 37 °C. Chromatin was isolated, sheared using a Bioruptor (Diagenode), and immunoprecipitated with Abs directed to Ets-1 (catalogue no. sc-55581 X), Ets-2 (catalogue no. sc-22803 X), PU.1 (catalogue no. sc-352 X) (all from Santa Cruz Biotechnology), acetylated histone H4 (Upstate Biotechnology, catalogue no. 06-866), HDAC (Upstate Biotechnology, catalogue no. 17-245) or control rabbit IgG (Upstate Biotechnology, catalogue no. 12-370). Cross-linking was reversed by heating, and the proteins were removed subsequently by proteinase K digestion. The presence of selected DNA sequences in the immunoprecipitated DNA was assessed by PCR using the following primer pairs (5’ to 3’ ends): forward, TGAGCCCTATATTCTCATT; and reverse, CGGTT- TAAGTCTCATAATCA.

The amplified 32P-labeled PCR products were separated on a 6% acrylamide gel and detected by autoradiography. The IL-12p35 gene promoter was used as negative control with its own primers sets (not specified here).

Real-time PCR was performed in triplicate using the Prism 7900 sequence detection system (PE Applied Biosystems). Quantitative PCR reactions were performed under conditions standardized for each primer set. Each PCR reaction was carried out in duplicate in a 20-µl reaction mixture: 5 µl of the eluted immunoprecipitated DNA and the SYBR Green Master mix kit (Applied Biosystems).

Dissociation curves were analyzed as a means to ensure the quality of amplicon and to monitor primer dimers. Enrichment was determined based on critical threshold (Ct) measurements (changes in fluorescence per PCR cycle number at a given threshold). The amount of genomic DNA coprecipitated with specific antibody was calculated in comparison to the total input DNA used for each immunoprecipitation in the following way: ΔCt = Ct(genomic input) − Ct(specific antibody), where Ct(genomic input) and Ct(specific antibody) are the mean threshold cycles of PCR performed in duplicates on DNA samples from the genomic input samples and the specific antibody samples, respectively. 2^ΔCt target values were calculated for each antibody.
Lentiviral Particles Production and Transduction of Non-Tregs T Cells—Precision LentiORF pLOC lentiviral vectors purchased from Open Biosystems are lentiviral-based vectors in which the open reading frame (ORF) of the gene of interest has been cloned downstream the CMV promoter and contain tGFP as a reporter gene. The production of VSV-G pseudo-typed lenti-ORF viral particles was performed as previously described in (52).

After the optimization of transduction conditions using CD4$^+$ H11001 T cells, we defined an MOI of five as the one providing the highest transduction rate. This MOI was then chosen to transduce purified CD4$^+$ H11001, CD25$^+$ H11002 UCB T cells.

Non-Tregs purified from cord blood were plated at a density of 10^6 cells/well in 12-well tissue culture plates in 1 ml of RPMI 1640 supplemented with 10% AB serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Lonza Europe, Verviers, Belgium), in the presence of 5 μg/ml phytohemagglutinin (Sigma-Aldrich) and 20 units/ml interleukin-2.

Twenty-four hours after the purification procedure, the cells were exposed to lentiviral vector preparations (multiplicity of infection = 5) in a volume of 500 μl containing 8 μg/ml Polybrene (Sigma). A scrambled lenti-ORF-ctrl was used as a negative control. The efficacy of transduction was measured 1 week after transduction, and subsequent flow cytometry sorting of the GFP-positive cells was performed to have a pure population of GFP-positive cells on which we could perform the measurements.

**Statistical Analysis**—Data are presented as means ± S.D. and analyzed using Student’s t test. p values of <0.05 (*), <0.01 (**), and <0.001 (***) were considered significant.

**RESULTS**

**Identification of Two Evolutionarily Conserved Ets Binding Sites in the FOXP3 Promoter**—The functional human FOXP3 gene sequence has been previously defined (38), and the region preceding the 5'-untranslated region has been studied and found to be highly conserved and to contain important promoter elements such as TATA, GC, and CAAT boxes as well as binding sites for NFAT and AP-1, which are mediators of T-cell activation (55).

The Ets family of transcription factors is evolutionarily conserved and binds to a purine-rich core DNA sequence (GGAA/T), with additional flanking nucleotides often determining specificity (56). Therefore, we investigated the FOXP3 promoter region with respect to GGAA/T nucleotides using ClustalW alignment comparing human, mouse, and rat sequences.

ClustalW alignment determined that, out of several Ets binding sites located just upstream of the transcription start site in the human FOXP3 promoter region, positions 102 and 282 were conserved across species (Fig. 1). These putative Ets binding positions located just downstream of the binding sites for NFAT and AP-1, which are mediators of T-cell activation (55), were of special interest.

**Ets-1 and Ets-2 Bind in Vivo to the FOXP3 Promoter in Natural Tregs**—Given the high expression of FOXP3 in nTregs, we investigated the in vivo binding of Ets-1 and Ets-2 to the human FOXP3 promoter, using a ChiP assay performed on nTregs. In this technique, DNA-binding proteins are covalently linked to the genomic DNA by exposure to a cross-linking agent, and then the DNA-protein complexes are isolated by immunoprecipitation (57). Following reversal cross-linking, the DNA is
amplified with primers encompassing the transcription factor binding sites of interest. Thus, T cells were initially subjected to cross-linking with formaldehyde, followed by lysis and chromatin sonication. The chromatin was then immunoprecipitated with anti-Ets-1, anti-Ets-2, and anti-PU.1 antibodies and normal rabbit IgG as a background control. The resulting DNA was subjected to PCR amplification with primers flanking the region containing the two Ets binding sites. Fig. 2 shows that amplicons were generated from both anti-Ets-1 and anti-Ets-2 immunoprecipitates and not from either anti-PU.1 or control IgG. Furthermore, immunoprecipitated samples were subjected to quantitative real-time PCR (SYBR Green) using primers to specifically amplify the FOXP3 promoter region encompassing the two Ets binding sites. Anti-Ets-1 and anti-Ets-2 immunoprecipitated chromatin showed a highly significant enrichment in the FOXP3 promoter compared with IgG immunoprecipitated chromatin. Hence, these data confirm the binding of both Ets-1 and Ets-2 to the FOXP3 promoter region.

Ets-1 and Ets-2 Stimulate the FOXP3 Promoter—To ascertain whether Ets-1 and Ets-2 binding to the two Ets binding sites had a functional role, we produced two full-length promoter constructs, a wild-type (pGL3-F-wild type) and a mutated one (pGL3-F-Ets-mut) containing the mutation (GGAA/T → AGAA/T) known to abrogate Ets-1 and Ets-2 binding. A transient reporter assay revealed that mutation in both Ets binding sites reduced promoter activity by 60% compared with pGL3-F-WT (Fig. 3A). In addition, cotransfection of Ets-1 and Ets-2 expression vectors with the full-length FOXP3 promoter construct resulted in an increase in promoter activity by 35 and 20%, respectively, compared with pGL3-F-WT cotransfection with pcDNA3.1, whereas cotransfection of both Ets-1 and Ets-2 constructs together with the full-length FOXP3 promoter construct resulted in an increase in promoter activity of 65% compared with pGL3-F-WT cotransfection with pcDNA3.1 (Fig. 3B). These results demonstrate that Ets-1 and Ets-2 play a positive role in the regulation of FOXP3 expression, via their binding to its promoter.

Valproate Treatment Increases FOXP3 mRNA and Protein Levels in CD4+, CD25− T Cells—To approach the epigenetic mechanisms involved in the regulation of FOXP3, we next investigated, in CD4+, CD25− T cells, the impact of valproate treatment on FOXP3 expression and protein level. The effect of valproate on FOXP3 mRNA and protein levels was determined by qRT-PCR and Western blot analysis, respectively. UCB
CD4+, CD25− T lymphocytes were cultured with and without valproate for 2, 4, 6, and 24 h. At each time point, proteins and RNA were prepared from the cells. After culture with valproate, FOXP3 expression increased in a time-dependent manner, compared with FOXP3 mRNA level in cells cultured in the absence of valproate as determined by qRT-PCR. The levels of FOXP3 mRNA increased by 2-, 2.6-, and 3-fold at, respectively, 2, 4, and 6 h after the onset of culture with valproate, compared with control untreated CD4+, CD25− T cells, where no change was observed. By 24 h, the level of FOXP3 mRNA started to decrease but remained elevated slightly above the level detected in cultures without valproate (Fig. 4A). On the other hand, as shown in Fig. 4B, the level of FOXP3 protein increased after 2, 4, and 6 h culture with valproate by 1.8-, 2.2-, and 2.4-fold compared with untreated CD4+, CD25− T cells. By 24 h, FOXP3 protein level started to decline but remained elevated (1.4-fold more) compared with untreated CD4+, CD25− T cells.

Chromatin Studies in CD4+, CD25− T Cells upon Valproate Treatment—Histone H4 hyperacetylation is a typical feature of active transcription (58). We therefore examined by ChIP the effect of HDAC inhibition on the acetylation of H4 associated with the FOXP3 promoter region as well as the binding of HDAC.

Chromatin fragments from cells cultured without and with valproate (1 mM) were immunoprecipitated with antibody to acetylated histone H4 (recognizing histone H4 acetylated at K5, K8, K12, and K16). DNA from the immunoprecipitate was isolated. From this DNA, a 200-bp fragment of the FOXP3 promoter region was amplified. Fig. 5A shows that after 6-h treatment with valproate, FOXP3 promoter DNA was associated with highly acetylated histone, compared with the same region isolated from cells cultured without valproate. By 24 h, the increase in acetylation of histone H4 associated with FOXP3 promoter region observed at 2 h was no longer detectable. Moreover, in vivo binding of HDAC to the FOXP3 core promoter was more abundant in untreated CD4+, CD25− T cells compared with the same cells being treated with valproate for 6 h (Fig. 5A). On the other hand, the in vivo binding of HDAC to FOXP3 core promoter in CD4+, CD25− T cells started to increase after 24-h treatment with valproate. Therefore, valproate induces a transient (24 h) H4 acetylation and inhibition of HDAC binding.

These results were further confirmed by qRT-PCR performed on anti-acetylhistone-H4 and anti-HDAC-immunoprecipitated chromatin from untreated and valproate-treated CD4+, CD25− T cells. Given the above observation that valproate treatment of these cells resulted in an increase in FOXP3
binding of Ets-1 and Ets-2, which in turn increases FOXP3 expression.

**Valproate-treated UCB CD4+, CD25− T Cells Transiently Adopt nTreg miRs Expression Signature**—In our previous report (52), we identified the microRNA signature of human nTregs, which was composed of two down-regulated miRs (miR-31 and miR-125a), and three up-regulated miRs (miR-21, miR-181c, and miR-374). Given these data, and the fact that valproate treatment of CD4+, CD25− T cells resulted in an increase in FOXP3 expression via the binding of Ets-1 and Ets-2, as shown above, we next investigated the effect of valproate on the miR profile of these cells.

This experiment revealed a striking modification of the miR profile of the treated cells. Results are shown as a fold change in miR expression compared with the levels observed in control untreated cells. After 2 h of treatment, the miR profile of these cells began to resemble that of nTreg cells: miR-21 (Fig. 6A), miR-181c (Fig. 6B), and miR-374 (Fig. 6C) showed an increase in their expression by 1.5-fold, 2-fold, and 1.6-fold, respectively, whereas expression of miR-31 (Fig. 6D) and miR-125a (Fig. 6E) decreased by 1.6- and 1.7-fold, respectively. At later time points (4 and 6 h), miR-21 showed 2.2- and 2.6-fold increases in expression, whereas miR-181c showed 2.5- and 3.3-fold increases in expression. Concurrently, miR-374 showed 1.9- and 2.2-fold increases in its expression after 4 and 6 h of culture with valproate, respectively. By 24 h, miR-21, miR-181c, and miR-374 expression levels decreased but remained elevated compared with untreated control cells.

On the contrary, miR-31 and miR-125a relative expression levels continued to decrease, reaching their minimum after 6 h (2.2- and 2.1-fold reductions, respectively). By 24 h, the relative expression levels of both miRs started to increase, but remained below the level of control cells. In summary, CD4+, CD25− UCB T cells transiently adopt an nTreg cell-like miR signature upon valproate treatment (high FOXP3 expression, low miR-31 and miR-125a expression, and high level of miR-21, miR-181c, and miR-374).

**Transduction of Non-Tregs by the Lenti-ORF-FOXP3 Vector Does Not Change Their miRs Expression Profile Despite a Significant Increase in FOXP3 Protein Level**—Following GFP-positive cell sorting, FOXP3 protein level was assessed in non-Tregs after transduction by the lenti-ORFs. Fig. 7 shows that FOXP3 protein level increased by >2-fold in lenti-ORF-FOXP3-transduced cells compared with lenti-ORF-ctrl and non-transduced cells. The faint band observed in non-transduced or mock transduced cells is due to the culture conditions needed for their survival, which leads as we previously showed, to an activation of all the cells. We then assessed the miRs expression levels in lenti-ORF-transduced non-Tregs and found no differences in the levels of the five miRs constituting the signature upon FOXP3 increased levels of expression and protein (Fig. 8, A−E). This indicates that the changes in the miR expression levels observed in valproate-treated non-Tregs cells is not a consequence of the increase in expression of FOXP3.

Rather, these experiments suggest that HDAC inhibition modifies the five miRs expression levels via other mechanisms and support our hypothesis that the increase of FOXP3 expression observed after valproate treatment may be due to the combina-
tion of two additive positive factors, i.e. the accessibility of the Ets transcription factors to their binding sites and the change in the expression of miR-21 and miR-31, which we previously showed to increase FOXP3 expression (52).

**DISCUSSION**

Several reports have demonstrated that HDAC inhibitors could increase FOXP3 expression (39, 59), a case further illustrated in our experiments. We initially focused on mechanisms that could be responsible for this increased expression, in the UCB nTreg model we described previously (52). In that model, we had already demonstrated that two (miR-21 and miR-31) of the five miRs comprising the nTregs signature had an impact (positive and negative, respectively) on FOXP3 expression. It might be of interest to notice that the culture conditions, used both in our previous report for lentiviral transduction and in this report for all the experiments, do increase the expression of FOXP3 and modify the expression of the five miRs but preserve the ratios observed when comparing nTregs and CD4-positive CD25-negative T-lymphocytes. This explains the weak FOXP3 (mRNA and protein) positivity observed in untreated CD4+, CD25− cells. One could wonder why we chose valproate, because there are more potent HDAC inhibitors available. The reason is that we wanted to take advantage of a compound

**FIGURE 6.** Valproate treatment of CD4+, CD25− T cells confers on them the nTreg miRNA signature. CD4+, CD25− T cells were treated with valproate (1 mM) for different periods of time. miR-21 (A), 181c (B), miR-374 (C), miR-31 (D), and miR-125a (E) expression levels were analyzed by qRT-PCR. Shown are the relative levels (mean ± S.D.) of five independent experiments performed on six donors, each done in triplicate. Statistical significance was determined by Student’s t test and is denoted as follows: *, p < 0.05 versus CD4+, CD25− T cells; **, p < 0.01 versus CD4+, CD25− T cells; and ***, p < 0.001 versus CD4+, CD25− T cells.

**FIGURE 7.** Lenti-ORF-FOXP3 transduction in CD4+, CD25− T cells is effective and results in a greater than 2-fold increase in the amount of FOXP3 protein. Western blot analysis of FOXP3 protein level was performed on lenti-ORF-transduced CD4+, CD25− T cells following flow cytometry sorting based on GFP expression. Non-transduced and lenti-ORF-ctrl-transduced CD4+, CD25− T cells were used as negative controls, whereas Tregs were used as positive control. Data are representative of three independent (donors) experiments.
already used in humans to treat medical conditions, at concentrations comparable to the upper limit of the therapeutic ranges. We focused on the mechanisms by which HDAC inhibition by valproate could increase FOX3 expression at the level of the FOX3 promoter and of the two miRs quoted above that could potentially affect the level of FOX3 mRNA and protein, in CD4-positive CD25-negative T cells. First, our experiments demonstrated a profound impact of valproate treatment on miR-21 and miR-31 expression levels that transiently became identical to their levels in nTregs. This led us to investigate the impact of valproate on the three other miRs composing the nTregs signature (125a, 181c, and 374). In our model, the level of expression of these had been shown not to impact on FOX3 expression. Unexpectedly, we observed that these three miRs also acquired levels of expression similar to what was found in nTregs. In other words, upon valproate treatment, the CD4-positive CD25-negative cells acquired the nTregs microRNA signature. We next asked the question whether the opening of the chromatin structure could lead to the binding of yet undescribed transcription factors that could positively affect the expression of the FOX3 gene. The functional human FOX3 promoter has been previously defined (38). The region preceding the 5′-untranslated region is highly conserved across species. This region contains important promoter elements, such as TATA, GC, and CAAT boxes, as well as binding sites for NFAT and AP-1, which are mediators of T-cell activation (55). This region was found also to contain several potential binding sites for the Ets family transcription factors, a purine-rich core sequence with additional flanking nucleotides determining specificity. Therefore we investigated the FOX3 promoter region with respect to GGAA/T nucleotides by comparing human, mouse, and rat sequences. As described under “Results,” we observed that, out of several Ets binding sites located upstream of the transcription start site in the human FOX3 promoter region, two positions, −102 and −282, were well conserved between species. Because these two Ets binding sites are located just downstream of the binding sites for the transcription factors NFAT and AP-1, we per-

FIGURE 8. Lenti-ORF-FOXP3 transduction in CD4+, CD25− T cells does not change their miRs expression profile. miR-21 (A), miR-31 (B), miR-125a (C), miR-181c (D), and miR-374 (E) expression levels were analyzed by qRT-PCR following lenti-ORF transduction. Shown are the relative levels (mean ± S.D.) of three independent experiments performed on three donors, each done in triplicate. There were no statistically significant differences between FOXP3-transduced and control CD4+, CD25− cells for any of the five miRs, as determined by Student’s t test.

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formed a series of experiments in nTregs, using the ChIP technique, showing that these two factors bound to these sites: Ets-1 and Ets-2. Next, using reporter constructs containing the full-length FOXP3 promoter, either wild-type or mutated in these two binding sites, we could then demonstrate, in transfection experiments, that the binding of these two Ets transcription factors up-regulated FOXP3 expression. Next, ChIP experiments were performed in non-Tregs with and without valproate treatment. We could show that the binding of these two factors occurred only when the chromatin was open. This was further confirmed by the demonstration that valproate treatment of non-Tregs strongly increased the acetylation status of histone H4 associated to the FOXP3 promoter region, thereby allowing the access of the two Ets factors to their FOXP3 binding sites. Taken together, these results demonstrate that one way by which HDAC inhibitors regulate FOXP3 expression is by modulating the binding of Ets-1 and Ets-2 transcription factors to the promoter region.

The next question we wanted to ask was whether the two phenomena (the increase in FOXP3 expression and the acquisition by CD4-positive, CD25-negative UCB T cells of a Treg miR profile) were intricately linked or not. To answer this question, we assessed the miR expression levels in non-transduced, lenti-ORF-ctrl- and lenti-ORF-FOXP3-transduced non-Tregs. No difference in the level of the five miRs, constituting the signature, in lenti-ORF-FOXP3-transduced non-Tregs was found, compared with the controls, despite FOXP3 increased levels of mRNA and protein by >2-fold. This indicates that the change of the miR expression levels observed in the valproate-treated non-Tregs is not a consequence of the increase in expression of FOXP3. Rather, these experiments suggest that HDAC inhibition modifies the expression levels of the five miRs via other mechanisms and support our hypothesis that the increase of FOXP3 expression observed after valproate treatment may be due to the combination of two additive positive factors, i.e. the accessibility of the Ets transcription factors to their binding sites and the change in the expression of miR-21 and miR-31, which we previously showed to increase FOXP3 expression (52).

To summarize these observations, one could say that valproate treatment (at concentration similar to the upper limit of the therapeutically window in humans) confers transiently to non-Treg CD4-positive T cells a molecular signature (microRNAs and FOXP3 expression) similar to the one of natural Tregs.

A yet unanswered mechanistic question is how valproate modifies the expression of the five miRs we have tested, in opposite directions. Indeed, HDAC inhibition could account for an increased expression of some miRs, but the mechanism leading to decreased expression has to be indirect. Anyway, it is not due to the increased FOXP3 expression level. Answering this question will require further investigations.

Nevertheless, our findings provide novel insights on the mechanisms by which epigenetic manipulations of non-Treg CD4-positive cells result in the modulation of FOXP3 expression and modification of their miR signature. They also open new ways of research that could lead to novel approaches in the treatment of human diseases linked to a deficit of Tregs.

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