MicroRNA Profile of Circulating CD4-positive Regulatory T Cells in Human Adults and Impact of Differentially Expressed MicroRNAs on Expression of Two Genes Essential to Their Function*

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Regulatory T cells (Tregs) are characterized by a high expression of IL-2 receptor α chain (CD25) and of forkhead box P3 (FOXP3), the latter being essential for their development and function. Another major player in the regulatory function is the cytotoxic T-lymphocyte associated molecule-4 (CTLA-4) that inhibits cytotoxic responses. However, the regulation of CTLA-4 expression remains less well explored. We therefore studied the microRNA signature of circulating CD4+ Tregs isolated from adult healthy donors and identified a signature composed of 15 differentially expressed microRNAs. Among those, miR-24, miR-145, and miR-210 were down-regulated in Tregs compared with controls and were found to have potential target sites in the 3′-UTR of FOXP3 and CTLA-4. miR-24 and miR-210 negatively regulated FOXP3 expression by directly binding to their two target sites in its 3′-UTR. On the other hand, miR-95, which is highly expressed in adult peripheral blood Tregs, positively regulated FOXP3 expression via an indirect mechanism yet to be identified. Finally, we showed that miR-145 negatively regulated CTLA-4 expression in human CD4+ adult peripheral blood Tregs by binding to its target site in CTLA-4 transcript 3′-UTR. To our knowledge, this is the first identification of a human adult peripheral blood CD4+ Treg microRNA signature. Moreover, unveiling one mechanism regulating CTLA-4 expression is novel and may lead to a better understanding of the regulation of this crucial gene.

Regulatory T cells (Tregs) are a subgroup of T cells endowed with inhibitory properties that affects the activation of the immune system. Tregs exist in two general categories. The first is thymus-derived Tregs, referred to as natural Tregs (nTregs), that play an important role in the maintenance of immune self-tolerance and homeostasis. The process of nTreg selection is determined by the affinity of the interaction with self-peptide MHCs in a thymic microenvironment. A T cell that receives very strong signals will undergo apoptotic death; a cell that receives a weak signal will survive and be selected to become an effector cell. If a T cell receives an intermediate signal, then it will become a natural regulatory cell. They normally remain present during the whole life. The second category is composed of adaptive Tregs, which are induced in the periphery, under diverse tolerogenic microenvironments, from mature T lymphocytes in response to a variety of stimuli, including pathogens, IL-10, and transforming growth factor-β (1–5). They develop during life to suppress excessive reactions against newly encountered antigens (pathogens or newly appearing self-antigens). They include different subpopulations, such as interleukin-10-producing Tr1 cells, transforming growth factor β (TGFβ)-secreting Th3 cells, CD8+ suppressor cells, CD4-CD8-T cells, and γδT cells (6). As a result, circulating Tregs found in adult peripheral blood are a mixture of nTregs, exist-

Background: Regulatory T cells are a subset of T cells with immunosuppressive properties, crucial for immune tolerance, which are also associated with cancer development.

Results: The human circulating CD4+ Treg microRNA signature was identified.

Conclusion: Differentially expressed microRNAs from the Treg miR signature directly and indirectly regulate crucial Treg genes (FOXP3 and CTLA-4).

Significance: Identifying novel regulatory mechanisms of crucial Treg genes expression provides better insight into their biology and offers potential new targets for immunomodulatory therapies.
ing at low frequency, and different adaptive Tregs. CD4-positive Tregs are one of the most important and most studied subpopulations. They exert their suppressive activity by various mechanisms, notably by deprivation of IL-2 (7–9), inhibitory cytokine production (IL-10, TGF-β, and IL-35), cytotoxicity against effector T cells, and metabolic disruption and modulation of APC function leading to anergy (10).

The discovery of a high expression of forkhead box P3 (FOXP3) as a major marker of Treg development and function has been a significant advance in the study of murine and human Tregs (11–14). However, despite remaining the best and most specific marker of Tregs, together with a high expression of a high affinity IL-2 receptor α chain (CD25), it cannot be used to isolate living Tregs because of its intracellular expression. This contrasts with the isolation of nTregs, which is rendered easier by using human cord blood as a source because all CD4+CD25+ cells are natural Tregs in the absence of fetal infection. On the other hand, adult circulating Tregs, because of the presence of CD25-expressing effector T cells, cannot be characterized so simply. However, recent reports have demonstrated that low surface expression of CD127 (the α-chain of the IL-7 receptor) in combination with high CD25 expression can distinguish between human regulatory and conventional CD4+ T cells in adult peripheral blood and cord blood, lymph nodes, and thymus (15–17). In addition, these reports demonstrated that these CD127low FOXP3+ T cells, which accounted for a significant percentage of CD4+ T cells in peripheral blood (PB), could anergize cytotoxic T cells and could suppress effector T cell responses in vitro.

MicroRNAs (miRNAs) are small (19–22-nt) single-stranded noncoding RNA molecules that are derived from hairpin-structured precursors (18). These microRNAs function by directly binding to the potential target site in the 3′-untranslated region (3′-UTRs) of specific target mRNA, leading to the repression of protein expression and the production of target mRNA degradation. More than 700 miRNAs have been identified in mammalian cells and have been shown to play important roles in human development, cellular differentiation and homeostasis, adaptation to the environment, oncogenesis, and host cell interactions with pathogens (19–21). Because miRNAs act as key regulators in a wide variety of biological processes, it is now apparent that abnormal miRNA expression is a common feature of various diseases (22–25). Recently, more miRNAs have been reported to be involved in the regulation of immune systems, demonstrating that miRNAs modulate many aspects of the immune responses, such as differentiation, proliferation, cell fate determination, function of immune cells, cytokine responses, and intracellular signaling pathways (26–29).

Because the immunoregulatory function of Treg cells may hinder the induction of immune responses against cancer, countering their activity can evoke effective antitumor immunity (30–33). Therefore, the modulation of Treg function in cancer patients (a situation where the tumor cells could directly or indirectly induce the host’s suppressive function to protect themselves against immune responses) could be an essential step to improve the efficacy of antitumor therapies, especially those based on immunotherapeutic approaches (34, 35).

Treg cells were initially characterized by the expression of a high affinity IL-2 receptor α chain (CD25) and, later on, by a high level of FOXP3 (forkhead/winged helix transcription factor 3), which is essential for Treg development and function (34, 36–39). The importance of FOXP3 in Treg function was reinforced by studies that showed that mutations in this transcription factor in humans result in an autoimmune syndrome termed IPEX (immune dysregulation, polyendocrinopathy enteropathy, X-linked syndrome), an X-linked immunodeficiency syndrome characterized by insulin-dependent diabetes, thyroiditis, massive T cell infiltration in multiple organs, and chronic wasting (35–37).

In recent years, several Treg gene expression-profiling studies have revealed set of genes that are important for Treg biology, such as cell surface molecules, cytokines, chemokines, killing agents, and transcription factors (40, 41). Some roles of the Treg-associated cell surface molecules have been better identified, such as ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39), which hydrolyzes proinflammatory nucleotides (ATP and ADP) to generate immunosuppressive adenosine in concert with CD73, and tumor necrosis factor receptor superfamily member 18 (TNFRSF18 or GITR), whose engagement in Tregs enhances proliferation while allowing them to retain suppressive function (42). Another major player in the regulatory function is the CTLA-4 (cytotoxic T-lymohocyte antigen-4) surface molecule, which is related to CD28, like inducible co-stimulatory molecule (ICOS) and programmed death molecule 1 (PD-1). CD28 and ICOS provide positive signals that promote and sustain T cell responses, whereas CTLA-4 and PD-1 limit responses. The balance between these stimulatory and inhibitory co-signals determines the nature of T cell responses without excess inflammation and autoimmunity (43–45). Mutations in CTLA-4 have been associated with insulin-dependent diabetes mellitus, Graves’ disease, Hashimoto’s thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, primary biliary cirrhosis, and other autoimmune diseases (46, 47). CTLA-4 up-regulation is associated with increased Treg number and activity in inflammatory bowel disease models (44). CTLA-4 is becoming a target of choice for current immunomodulatory therapies; CTLA-4 up-regulation and signaling prevent organ allograft rejection (48). CTLA-4 blockade as a strategy to boost anti-tumor immune response is being used in clinical trials and for immune enhancement in cancer vaccines (49). CTLA-4 blockade is the first therapy approved by the Food and Drug Administration that clearly demonstrates that patients with metastatic melanoma have a prolonged survival after taking this treatment. Moreover, the B7-binding fusion protein CTLA-4ig (50) is an approved immunosuppressant for the treatment of rheumatoid arthritis (51). Despite its importance for regulatory function, the regulation of CTLA-4 expression is less well explored than that of FOXP3 and therefore deserves further investigation.

We therefore started to study the circulating CD4+CD25+CD127low regulatory T cells, purified from the PB of adult healthy donors, at the molecular level. It is important to note that this population is a heterogeneous population including both the natural Tregs and the different subpopulations of the adaptive Tregs. After having identified the miR signature of
Human circulating CD4+ regulatory T cell microRNA signature

Human natural T cells and its functional impact (52, 53), we set out to study the signature of the peripheral blood CD4+CD25+CD127low Tregs being isolated from adult healthy volunteers. We were able to identify a specific Treg microRNA signature (in comparison with our negative control CD4+CD127lowCD25− T cells) composed of 15 differentially expressed microRNAs. In fact, this signature is different from that of the nTregs published by our group (52) due to the heterogeneity of our studied population in contrast to that of the nTregs. Among the Treg differentially expressed miRs, miR-24, miR-145, and miR-210 expression was down-regulated in this regulatory population. Two miRs (24 and 210) were shown to negatively regulate FOXP3 expression by directly binding to two target sites in the FOXP3 3′-UTR. We also demonstrated that miR-95, whose expression is up-regulated in these Tregs, positively regulated FOXP3 expression. Finally, we could demonstrate that miR-145 negatively regulates CTLA-4 expression in human CD4-positive PB Tregs by binding directly to its target site in the 3′-UTR of its transcript.

EXPERIMENTAL PROCEDURES

Purification of peripheral blood CD4+CD25+CD127low regulatory T cells—After informed consent, PB mononuclear cells were isolated from about 60 ml of heparinized whole blood by Ficoll-PaqueTM plus density gradient. Purification of CD4+CD25+CD127low regulatory cells was performed in a two-step procedure, using the Miltenyi kit for Treg isolation. First, non-CD4+CD127low cells were magnetically labeled with a mixture of biotin-conjugated antibodies (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, glycoporphin A, and CD127) and anti-biotin microbeads. Labeled cells were subsequently depleted by separation using a MACS column. This resulted in the selection of CD4+CD127low T cells. In the second step, these cells were directly labeled with anti-CD25-coated microbeads and isolated by positive selection. The magnetically labeled CD4+CD25+CD127low T cells were retained on the column and eluted after removal of the column from the magnetic field. We thus ended up with two fractions (CD127lowCD25+ and CD127lowCD25−CD4+ T cells). The purity of the two subpopulations was always above 96%, as assessed by flow cytometry. For reasons of clarity and ease of reading, we will call them Tregs and CD4+CD25− T cells, respectively, in this work.

Treg suppressive capacity assessment in mixed leukocyte reaction (MLR) assays—Treg suppressive capacity toward proliferation of allogeneic activated carboxyfluorescein succinimidyl ester (CFSE)-labeled T lymphocytes was assessed by flow cytometry analysis after 5 days of co-culture experiments. Briefly, peripheral blood samples were collected from healthy donors after informed consent had been obtained. T lymphocytes were immunomagnetically purified from mononuclear cells by positive selection using anti-human CD3 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. These T lymphocytes were then labeled by CFDA-SE (CellTrace-CFSE cell proliferation kit, Invitrogen) by using 10 mM CFDA-SE dye to stain ~107 cells.

CD4+CD25+CD127low Tregs were isolated from adult PB mononuclear cells using the Miltenyi Biotec regulatory T cell isolation kit according to the manufacturer’s instructions. The purity of the selected cells was always above 96%, as determined by flow cytometry analysis.

For the allogeneic assay, we performed MLRs using irradiated allogeneic PB mononuclear cells as stimulating cells (2 × 105) to activate CFSE-labeled T lymphocytes responder cells (2 × 105 cells) in a 48-well plate. CD4+CD25+CD127low Tregs were added to MLRs or not added (control MLR) at a 1:1 ratio before culture in RPMI with 10% decomplemented FBS (both from Lonza Europe, Verviers, Belgium). At day 3, 20 IU/ml human recombinant IL-2 (Biotest AG) was added to culture.

After 5 days of co-culture, CFSE fluorescence dilution was measured by flow cytometry. Samples were run on a FACSCalibur (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Treg miRNA profiling and data analysis—Total RNA was extracted from cells using TRIzol® total RNA isolation reagent (Roche Applied Science). The concentration was quantified using a NanoDrop spectrophotometer. A two-step procedure was performed to profile the miRNAs in the purified Tregs and CD4+CD25− T cells. First, for cDNA synthesis from the miRNAs, 1000 ng of total RNA from Tregs and CD4+CD25− T cells was subjected to reverse transcription (RT) using a TaqMan® microRNA reverse transcription kit (catalog no. 4366596, Applied Biosystems, Ghent, Belgium) and Megaplex RT primers (Human Pool A, catalog no. 4399966, Applied Biosystems) following the manufacturer’s protocol, allowing simultaneous reverse transcription of 380 mature human miRNAs. RT was performed on a Mastercycler Eppendorf thermocycler (VWR International, Leuven, Belgium) with the following cycling conditions: 40 cycles of 16 °C for 2 min, 42 °C for 1 min, and 50 °C for 1 s, followed by a final step of 80 °C for 5 min to inactivate the reverse transcriptase.

After the amplification step, the products were diluted with RNase-free water, combined with TaqMan gene expression master mix, and then loaded into TaqMan Human MicroRNA Array A (catalog no. 4398965, Applied Biosystems), which is a 384-well formatted plate and real-time PCR-based microfluidic card with embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs; the RNU48 transcript was used as a normalization signal. Quantitative RT-PCR was performed according to the manufacturer’s instructions. Real-time PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the following cycling conditions: 50 °C for 2 min and 94.5 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 59.7 °C for 1 min. The cycle threshold (Ct) was automatically given by SDS 2.3 software (Applied Biosystems) and is defined as the fractional cycle number at which the fluorescence passes the fixed threshold of 0.2. RNU48 embedded in the TaqMan human microRNA arrays was used as an endogenous control. The relative expression levels of miRNAs were calculated using the comparative ΔΔCt method as described previously (54, 55). The -fold change in miRNAs were calculated by the expression 2−ΔΔCt.

Taqman miRNA assay for individual miRNAs—Gene-specific reverse transcription was performed for each miR using 10 ng of purified total RNA, 100 mM dNTPs, 50 units of MultiScribe reverse transcriptase, 20 units of RNase inhibitor, and 50
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nm gene-specific RT primer samples using the TaqMan microRNA reverse transcription kit (Applied Biosystems). 15-\(\mu\)l reactions were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C to inactivate the reverse transcriptase. Real-time RT-PCRs (5 \(\mu\)l of RT product, 10 \(\mu\)l of TaqMan 2x universal PCR master mix (Applied Biosystems), and 1 \(\mu\)l of TaqMan microRNA assay mix containing PCR primers and TaqMan probes) were carried out on an ABI Prism 7900HT sequence detection system (Applied Biosystems) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All quantitative RT-PCRs were performed in triplicate. The expression levels (2\(^{-\Delta\DeltaCT}\)) of miRNAs were calculated as described previously (54).

**miR-24, miR-145, and miR-210 Target Prediction**—Computer-based programs were used to predict potential targets sites for the down-regulated miRs in FOXP3 and CTLA-4 3'–UTR. We searched miRBase (56) and TargetScan (57). FOXP3 and CTLA-4 3'–UTRs were identified by both programs as having putative miR-24 (FOXP3), miR-210 (FOXP3), and miR-145 (CTLA-4) target sequences.

**Plasmid Construction**—A 249-bp fragment of FOXP3 3'-UTR encompassing the miR-24 and miR-210 potential target sites and a 300-bp fragment of CTLA-4 3'–UTR encompassing the miR-145 potential target site were cloned downstream of the Renilla luciferase gene (EcoRI/Xhol sites) in the psiCHECK-1 plasmid (Promega, Mannheim, Germany) and designated as psiCHECK 3'–UTR WT. PCR primers used for amplification of the FOXP3 and CTLA-4 3’–UTRs were as follows: FOXP3 primers (5’ to 3’), GCGGCTCGAGTTCACCTGTGCTTCACGCTA (forward) and GGCGGAATTCACCTTGTGCTTCAGTCCA (reverse); CTLA-4 primers (5’ to 3’), GGCGCTCGAGGCTGACCTTGTCCTGAGG (forward) and GGCGGAATTCACCTTGTGCTTCAGTCCA (reverse).

QuikChange site-directed mutagenesis (deletion) of miR-24, miR-210, and miR-145 target sites in psiCHECK 3'-UTR WT was performed according to the manufacturer’s protocols (Stratagene, La Jolla, CA) and designated as psiCHECK-UTRdel. QuikChange site-directed mutagenesis was used to change the following primers (5’ to 3’): FOXP3 (miR-34 site deleted), CCTACTTCTCCATCCTTCTCCCACTCCTAGTTGCATCGG (forward) and GCGATTCAGGTCTCGAACGAGG (reverse); FOXP3 (miR-210 site deleted), GCGATTCAGGTCTCGAACGAGG (forward) and CCCACTGGGGGGCGTCGCTGTGCCTAGT (reverse); CTLA-4 (miR-145 site deleted), CTAACTACGCTTGAGTGTGAGG (forward) and CTGCTATGACTACAGTAC (reverse). The constructs were verified by sequencing.

**Cell Culture**—The HEK293T and HeLa cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Europe, Paisley, UK), 2 mM l-glutamine, 50 IU/ml penicillin, and 50 \(\mu\)g/ml streptomycin (all from Lonza).

**Luciferase Assays**—Assays were conducted in a 24-well format. Reporter plasmids (psiCHECK/psiCHECK 3'-UTR WT/psiCHECK 3'-UTR deleted) (100 ng) were co-transfected in HEK293T and HeLa cells along with miR-24, miR-210, and miR-145-mimic/miR-negative control-mimic at a final concentration of 10 \(\mu\)M (miRIDIAN mimic, Dharmacon, Geel, Belgium) and control firefly plasmid pGL3-CMV (100 ng) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guidelines. Before proceeding to the transfection assays, the cell lines were assessed for expression of the miRs of interest using quantitative RT-PCR, as described below. 24 h post-transfection, cells were harvested, and luciferase levels were measured using the Dual-Luciferase reporter assay system (Promega, Mannheim, Germany) according to the manufacturer’s guidelines. Relative protein levels were expressed as Renilla/firefly luciferase ratios.

**Quantitative PCR for FOXP3 and CTLA-4 Expression**—Total RNA was extracted with TRIzol reagent according to the manufacturer’s guidelines (Invitrogen), and first-strand cDNAs were synthesized by reverse transcription (Superscript First-Strand Synthesis System for RTPCR kit, Invitrogen). Quantitative mRNA expression was measured by real-time PCR with the PRISM 7900 sequence detection system (Applied Biosystems), and the TaqMan master mix kit with EF1-\(\alpha\) mRNA was used as an internal control. Human Taqman gene expression assays for FOXP3 (Hs01085834-m1), CTLA-4 (Hs03044418-m1), and EF1-\(\alpha\) (Hs01024875-m1) were purchased from Applied Biosystems. The program used for amplification was as follows: 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 1 min at 60°C.

**Lentiviral Vector Production**—VSV-G pseudotyped lentiviral particles were generated by polyclynethyleneimine (Sigma) co-transfection of HEK293T cells with three plasmids, pMIRNA, pCMVΔR8.91, and pMD.G (58).

PcMVΔR8.91 is an HIV-derived packaging construct that encodes the HIV-1 Gag and Pol precursors as well as the regulatory proteins Tat and Rev (59). VSV-G was expressed from pMD.G (60). pMIRNA, provided by System Biosciences, is a lentivirus-based vector in which a microRNA precursor molecule has been cloned downstream of the CMV promoter and contains copGFP as a reporter gene. 24 h after transient transfection of HEK293T cells, viral supernatants were collected, filtered through 0.45-\(\mu\)m low protein binding filters (Nalgene, Rochester, NY), and concentrated as described previously (61). The viral pellets were then resuspended in \(\frac{1}{100}\) volume of PBS. Viral stocks were stored in aliquots at \(-80°C\), and the titers were determined by transducing HeLa cells in a limiting dilution assay. Lentiviral vector preparations collected 24 h post-transfection displayed titers of 10\(^{6}\)–10\(^{7}\) transducing units/ml in HeLa cells. No replication-competent virus was detected in the concentrated lentiviral stocks. A second production cycle was repeated after 24 h, routinely generating lentiviral vector preparations of \(\sim 5 \times 10^7\) to \(5 \times 10^8\) transducing units/ml.

**Lentiviral Transduction**—Tregs and CD4\(^+\) CD25\(^-\) T cells purified from buffy coats were plated at a density of 10\(^5\) cells/well in 12-well tissue culture plates in 1 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 50 units/ml penicillin, 50 \(\mu\)g/ml streptomycin (Lonza), in the presence of 5 \(\mu\)g/ml phytohemagglutinin (PHA-L, Sigma) and 20 units/ml IL-2.

24 h after Treg and CD4\(^+\) CD25\(^-\) T cell isolation, cells were exposed to lentiviral vector preparations with a multiplicity of infection of 5 in a volume of 500 \(\mu\)l in the presence of 8 \(\mu\)g/ml.
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Polybrene (Sigma). GFP-positive cells were sorted by flow cytometry at day 7 after transduction.

Detection of Mature miR-24, miR-145, miR-210, and miR-95 by TaqMan Real-time PCR—TaqMan miRNA assays (Applied Biosystems) used the stem loop method (62, 63) to detect the expression level of mature miR-24, miR-145, miR-210, and miR-95. 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 under the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then holding at 4 °C. After the RT reaction, the cDNA products were diluted for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then holding at 4 °C. The PCR 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 miRNA expression of mean cycle Ct value, which was then converted to -fold changes. RT primer, PCR primers, and TaqMan probe for miR-24, miR-145, miR-210, and miR-95 were purchased from ABI. RNU48 was used for normalization.

Western Blot Analysis of FOXP3 and CTLA-4 Proteins—4 × 10⁶ cells were lysed and subjected to SDS-PAGE using 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Amersham Biosciences) using a semidy electroblot chamber. Membranes were blocked with TBST containing 5% BSA overnight at 4 °C. After blocking, the blots were incubated with a 1:200 dilution of goat anti-FOXP3 antibody and mouse anti-CTLA-4 antibody (Tebu-Bio, Boechut, Belgium) diluted in TBST for 1 h at 25 °C. Following 1 h of incubation with anti-goat and anti-mouse peroxidase-conjugated antibodies (Sigma) at room temperature, proteins were detected by the electrogenerated chemoluminescence 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 (Tebu-Bio, Boechut, Belgium). All protein signals were visualized using the LAS-3000 image reader (FUJI), and signals were analyzed with AIDA software (Raytest).

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

RESULTS

CD4⁺CD25⁺CD127low T Cells Are Functional and Suppress T Cell Proliferation in Allogeneic MLR—Functional assessment of CD4⁺CD25⁺CD127low Treg suppressive capacity on the proliferation of allogeneically activated CFSE-labeled T lymphocytes was performed after 5 days of co-culture experiments. In Fig. 1, CFSE dilution analysis showed that CD4⁺CD25⁺CD127low Tregs could inhibit allogeneic T cell proliferation.

CD4⁺CD25⁺CD127low Treg miR Signature—RNA, from five independent human CD4⁺CD25⁺CD127low Tregs and CD4⁺CD25⁻ T cell subset samples, was first studied using the T-LDA technique. We could identify several miRs that were differentially expressed between Tregs and CD4⁺CD25⁻ T cells. Differential miR expressions were validated by real-time PCR. A Treg miR signature was identified, consisting of 15 statistically differentially expressed miRs: miR-9, -18a, -24, -27b, -95, -126, -133a, -134, -145, -181b, -181d, -210, -224, -335, and -509. All of these miRs were down-regulated (ratio between 0.1 and 0.004) in Tregs except for two, miR-95 and miR-509, that were overexpressed (ratio greater than 5). For reasons of space and legibility, only the levels of the four miRs that we chose to study further are shown in Fig. 2.

FOXP3 Is Directly Regulated by miR-24 and miR-210—Because FOXP3 is known to be mandatory for Treg cell function (11, 13), we therefore decided to investigate whether underexpressed miR-24 and miR-210, which had potential target sites in FOXP3 3' UTR, might influence the expression of this gene. A 249-bp fragment of the 3' UTR of FOXP3 containing the miR-24 and miR-210 target sequences was cloned in a psiCHECK-1 vector downstream of the Renilla luciferase gene (pSICHECK-UTRwt). In parallel, we also cloned in the same way this fragment with the target sites of miR-24 and miR-210 deleted by site-directed mutagenesis (pSICHECK-UTRdel). Before proceeding to the transfections with the reporter constructs, we screened the HeLa cell line for the expression of miR-24 and miR-210 using quantitative RT-PCR and found that the expression of miR-24 and miR-210 in HeLa cells is...
similar to the level observed in CD4⁺CD25⁻ T cells. Transient transfection of psiCHECK-UTRwt in HeLa cells led to a significant decrease (65%) in reporter luciferase activity compared with transfection with the control vector psiCHECK-UTRdel (Fig. 3). Furthermore, co-transfection of HeLa cells with psiCHECK-UTRwt and miR-24 or miR-210 separately showed a further significant decrease (52 and 51%, respectively) in reporter luciferase activity compared with psiCHECK-UTRwt alone (Fig. 3). Co-transfection of psiCHECK-UTRwt with both miR-24 and miR-210 resulted in a 40% decrease in luciferase activity compared with the co-transfection of the same construct with each microRNA separately. Moreover, the activity of the psiCHECK-UTRdel deleted at the miR-24 and miR-210 target sequence was not affected by co-transfection with these two miRs (Fig. 3). Altogether, these results demonstrate the role of miR-24 and -210 in the regulation of FOXP3 expression through direct binding to their target sites.

miR-145 Directly Targets CTLA-4 3’-UTR Reporter Construct—To investigate whether CTLA-4 can be directly targeted by miR-145, we engineered luciferase reporter plasmids containing either the wild-type 3’-UTR of this gene (psiCHECK-KUTFwt) or the mutant UTR with the miR-145 target site deleted (psiCHECK-KUTrd). Transfection assays were carried out in HEK293T cells that showed a miR-145 expression similar to the level observed in CD4⁺CD25⁻ T cells as revealed by qRT-PCR. A scrambled control miR with no homology to the human genome was used as control for nonspecific effects of the miR transfection procedure. The luciferase measurements revealed that the co-transfection of psiCHECK-KUTFwt with miR-145 significantly reduced the luciferase activity of the wild-type CTLA-4 reporter by 45% compared with the transfection of the same construct alone or compared with the transfection of psiCHECK control plasmid and the control miR. Moreover, the mutant reporter (psiCHECK-KUTrd) luciferase activity was not repressed by miR-145, which indicates that the target site directly mediates the repression (Fig. 4). Taken together, these results show that miR-145 directly targets CTLA-4 3’-UTR.

Lentiviral Transduction of Regulatory and CD4⁺CD25⁻ T Cells—Lentivirus-based systems have been developed to generate replication-incompetent lentiviral vectors that efficiently transduce both dividing and non-dividing mammalian cells and provide stable, long term expression of RNA of interest. To investigate the effect of some microRNAs of this signature (miR-24, miR-95, miR-210, and miR-145) on Tregs and CD4⁺CD25⁻ T lymphocytes and, more specifically, on FOXP3 and CTLA-4 expression, lentiviral vectors (lenti-miR-24, lenti-miR-95, lenti-miR-210, and lenti-miR-145) containing copGFP as a reporter gene were produced and transduced into peripheral blood Tregs and CD4⁺CD25⁻ T cells after an 18-h stimulation period with IL-2 and PHA. A scrambled lenti-miR-ctrl was used as a negative control. After optimization of transduction conditions using adult CD4⁺ T cells, we defined a multiplicity of infection of 5 as the value providing the highest transduction rate. This multiplicity of infection was then chosen to transduce purified Tregs and CD4⁺CD25⁻ T cells. The efficacy of transduction was measured 1 week after transduction. Measurement of GFP expression by flow cytometry showed a
FIGURE 3. miR-24 and miR-210 negatively regulate FOXP3 expression. Renilla luciferase reporter assays with constructs holding FOXP3 3'-UTR sequences from the indicated genes were co-transfected into HeLa cells along with a firefly luciferase transfection control plasmid either alone or together with miR-24 and miR-210. Shown are relative luciferase values normalized to transfections without miRNA. Data represent mean ± S.D. (error bars) of three independent experiments, each performed in triplicate. **, p < 0.01; ***, p < 0.001 versus psiCHECK-transfected cells; Student’s t test.

FIGURE 4. miR-145 negatively regulates CTLA-4 expression. Renilla luciferase reporter assays with constructs holding CTLA-4 3'-UTR sequences from the indicated genes were co-transfected into 293T cells along with a firefly luciferase transfection control plasmid either alone or together with miR-145. Shown are relative luciferase values normalized to transfections without miRNA. Data represent mean ± S.D. (error bars) of three independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01 versus psiCHECK-transfected cells; Student’s t test.
The FOXP3 protein level was significantly reduced (2-fold) in activated cell sorting. Data shown are representative of three independent experiments.}

**FIGURE 5. Lentiviral transduction efficiency in Tregs and CD4^+CD25^- T cells.** GFP expression in Tregs and CD4^+CD25^- T cells was assessed 1 week after transduction with lentiviral vector before (A) and after (B) fluorescence-activated cell sorting. Data shown are representative of three independent experiments.

75–87%, 70–85%, 65–75%, and 60–70% transduction efficiency for lenti-miR-24, lenti-miR-210, lenti-miR-95, and lenti-miR-145, respectively. Subsequent flow cytometry sorting of the GFP-positive cells resulted in a 99% pure population (Fig. 5).

**Lentiviral Expression of miR-95 in CD4^+CD25^- T Lymphocytes Significantly Increases FOXP3 Transcription and Protein Level—**After GFP-positive cell sorting, FOXP3 mRNA and miR-95 expression levels were assessed after transduction by lenti-miR-95 and lenti-miR-ctrl in CD4^+CD25^- T cells. Our results showed that the miR-95 level in lenti-miR-95-transduced CD4^+CD25^- T cells was 4-fold higher than in lenti-miR-ctrl-transduced T cells (Fig. 6A). At the same time, the level of FOXP3 expression was 2-fold higher in lenti-miR-95-transduced lymphocytes compared with lenti-miR-ctrl-transduced and non-transduced cells (Fig. 6A). These results reveal that FOXP3 expression is positively regulated by miR-95. Furthermore, FOXP3 protein level was checked after lenti-miR-95 transduction. As shown in Fig. 6B, FOXP3 protein level increased significantly (2.1-fold) after lenti-miR-95 transduction compared with lenti-miR-ctrl-transduced CD4^+CD25^- T cells. These results reveal that FOXP3 expression is positively regulated by miR-95.

**Lentiviral Expression of miR-24 and miR-210 in Tregs Significantly Reduces FOXP3 Transcription and Protein Level—**In order to study the effect of overexpression of miR-24 and miR-210 in Tregs, and specifically on FOXP3, lenti-miR-24 and lenti-miR-210 were transduced into Tregs, and the GFP-positive cells were sorted. miR-24 and miR-210 levels, in lenti-miR-24- and lenti-miR-210-transduced Tregs, respectively, were significantly higher than in lenti-miR-ctrl-transduced cells. The level of FOXP3 was 2-fold lower in lenti-miR-24- and lenti-miR-210-transduced cells compared with lenti-miR-ctrl-transduced and non-transduced cells (Fig. 7, A and B). These results demonstrate that FOXP3 expression is inversely proportional to miR-24 and miR-210 expression levels. Fig. 7C shows that the FOXP3 protein level was significantly reduced (2-fold) in lenti-miR-24- and lenti-miR-210-transduced cells compared with lenti-miR-ctrl-transduced and non-transduced cells. These results demonstrate that FOXP3 expression and protein levels are inversely proportional to miR-24 and miR-210 expression levels.

**Lentiviral Transduction of miR-145 in Tregs Significantly Decreased CTLA-4 Expression and Protein Level—**miRNAs can down-regulate gene expression by two post-transcriptional mechanisms: mRNA cleavage and translational repression (18). Because luciferase assay does not distinguish the two mechanisms well, we further examined how miR-145 repressed the endogenous CTLA-4 in Tregs. We up-regulated miR-145 by expressing the miR-145 precursor from a lentiviral vector (lenti-miR-145) and studied the effect on the mRNA and protein levels of this gene. Seven days after lenti-miR-145 viral infection, we isolated the GFP-positive (GFP+) Treg population that expressed the viral vector-encoded GFP by fluorescence-activated cell sorting (FACS). To rule out any effect of lentivirus infection, we used the same lentiviral vector expressing a scrambled sequence (lenti-miR-ctrl) with no homology to the human genome in parallel as a control. Quantitative real-time RT-PCR revealed that the miRNA level of CTLA-4 decreased (2-fold) in the lenti-miR-145-transduced Tregs compared with the lenti-miR-ctrl-transduced cells (Fig. 8A). Western blot analysis showed that the protein level of this gene was reduced (1.9-fold) by lenti-miR145 (Fig. 8B). These results indicate that CTLA-4 expression is negatively regulated by miR-145.

**DISCUSSION**

In this report, we have defined the first miR signature for human circulating CD4^+CD25^-CD127low Tregs purified from the peripheral blood of healthy adult donors. This signature consists of 15 statistically differentially expressed miRs; miR-95 and miR-509 are overexpressed, whereas miR-9, -18a, -24, -27b, -126, -133a, -134, -145, -181b, -181d, -210, -224, and -335 are markedly underexpressed in Tregs compared with non-Treg T cells and therefore should be mirrored by up-regulated expression of their target genes. These miRs were detected using the Taqman low density arrays technique and confirmed by quantitative PCR.

To gain further insight into the functional role of this signature, we chose to explore the role of some of the miRs composing the signature: miR-95, which is the most overexpressed in Tregs, and miR-24, miR-145, and miR-210, which are markedly underexpressed and were found by computer screening to have potential target sites in the 3’-UTR of FOXP3 (−24 and −210) and CTLA-4 (−145). The choice of looking at the effect of miRs potentially regulating FOXP3 and CTLA-4 was made given their crucial role in Treg function. We chose miR-95 because it was significantly the most overexpressed miR in PB CD4^+CD25^-CD127low Treg. These four microRNAs (miR-24, −210, −95, and −145) had an observable effect on the expression of two genes, proven by transfection of constructs containing their 3’-UTR wild type or mutated in the miR target sites coupled with a reporter gene and by transduction of fresh primary T cells. As far as the transfection experiments are concerned, it is worth noting that we were first surprised to see a decrease in

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luciferase activity when transfecting the wild type constructs without miR added. It was only when we checked the cell lines for the expression of the miRs of interest using quantitative RT-PCR and we found that these cell lines could express some of the miRs that we were testing at a level comparable with that found in non-Tregs that we could understand our results. As a result, we propose as a general rule that in any such experiment, the expression level of the miR of interest should be checked before proceeding further. Regarding the transduction experiments, it should be noted that activation is required to achieve a highly efficient lentiviral transduction of T cells and to keep them alive in culture. Because activation of human T cells transiently induces FOXP3 (64–67) and CTLA-4 up-regulation (68), it was necessary to verify the impact of stimulation on the expression of the miR signature, FOXP3, and CTLA-4.

The signature (i.e., the ratios of the 15 miRs between the Treg and CD4⁺CD25⁻ T cell populations) did not change, despite a 2–3-fold increase in miR-24, miR-210, miR-95, and miR-145 expression, in both populations, upon activation. The same was true for the level of FOXP3 and CTLA-4 transcripts; although

**FIGURE 6. Lentivirus-mediated miR-95 expression and its effect on FOXP3.** A, miR-95 and FOXP3 relative expression in CD4⁺CD25⁻ T cells, miR-95- or miR-ctrl-transduced CD4⁺CD25⁻ T cells, and untransduced Tregs as determined by qRT-PCR. B, Western blot analysis of FOXP3 protein level after 7 days of transduction with lenti-miR-95 or lenti-miR-ctrl. Data represent mean ± S.D. (error bars) of three independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01 (lenti-miR-95-transduced versus lenti-miR-ctrl-transduced CD4⁺CD25⁻ T cells; Student’s t test).
there was a slight increase in both populations (roughly 2-fold), the ratios remained the same. Several authors (60) have shown that a 50% reduction of the target mRNA seemed to be the maximum achievable, even using a combination of several miRs. Lentiviral transduction of miR-24, miR-210, and miR-145 known to be down-regulated in Tregs versus CD4+CD25+ T cells and to have potential target sites in FOXP3 and CTLA-4 3'-UTR, respectively, resulted in a 2-fold decrease in FOXP3 and CTLA-4 mRNA and protein levels. On the other hand, lentiviral transduction of miR-95 in CD4+CD25- T cells resulted in a 2-fold increase in FOXP3 mRNA and protein level.

It is important to remember that, in this study, we have investigated a population of circulating CD4+ Tregs from healthy adult donors that comprises different subpopulations (natural Tregs and different adaptive Tregs). It is therefore not surprising to find a signature that does not overlap with the signature of pure natural Tregs we described previously (52), given the fact that adaptive Tregs, which represent the major part of the CD4+CD25+CD127low population, may not be induced by the same mechanisms and arise in different organs (periphery versus thymus for nTregs). In addition, it is also not surprising that this signature differs from the murine Treg signature (63). In the latter case, one must also take into account that the comparison was made in a different species and using different T cell source (lymph nodes).

An important point (and this is valid for all experiments, from our group or from others, regarding molecular signatures) is that the choice of the control population is always somehow arbitrary. Had we chosen as a control population CD4+CD25+CD127high T cells, the results probably would have been different.

Thus, our results demonstrate that miR-24 and miR-210 are negative regulators of FOXP3, and their impact is directly
linked to the target sites found in the 3′-UTR of the FOXP3 mRNA, as evidenced by our transfection experiments. Conversely, we found miR-95 (overexpressed in the Treg population and devoid of potential target site in FOXP3 mRNA) to positively regulate FOXP3 expression. Its mechanism of regulation should therefore be indirect, maybe by decreasing a putative inhibitor of FOXP3 expression.

Because the control of CTLA-4 expression in human Tregs remains poorly understood, our finding about the role of miR-145 is an important and novel one. Recent data (45, 69) suggest that TRIM (T cell receptor-interacting molecule) proteins, that are signaling adaptor molecules, could play a role in the regulation of microRNA expression pathways and in the control of CTLA-4 cell surface expression via its phosphorylation by SRC kinases. Phosphorylated TRIM associates with the PI3K regulatory subunit α and could regulate CTLA-4 expression at the promoter level. Interestingly, a rapid screening of the TRIM mRNA 3′-UTR showed that there were numerous sites for several miRs down-regulated in Tregs, among which are miR-9 and miR-145. These data will deserve further intense investigation because they allow speculation about a possible up-regulation of TRIM in Tregs, which in turn could reinforce the up-

**FIGURE 8.** Lentivirus-mediated miR-145 expression and its effect on CTLA-4 in Tregs. A, miR-145 and CTLA-4 expressions were determined by qRT-PCR in Tregs, miR-145- and miR-ctrl-transduced Tregs, and CD4+CD25+ T cells. Means of three independent experiments are shown. B, Western blot analysis of CTLA-4 protein level after lenti-miR-145 and lenti-miR-ctrl transduction. Cells were collected 7 days after transduction. Data represent mean ± S.D. (*error bars*) of three independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01 (lenti-miR-145 versus lenti-miR-ctrl).
regulation of CTLA-4 expression, already turned on by the miR expression level described above.

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