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Human CD8$^+$CD25$^+$CD127^{low} regulatory T cells: microRNA signature and impact on TGF-β and IL-10 expression

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Abstract

Regulatory T cells (Tregs) are central for maintaining immune balance and their dysfunction drives the expansion of critical immunologic disorders. During the past decade, microRNAs (miRNAs) have emerged as potent regulators of gene expression among which immune-related genes and their immunomodulatory properties have been associated with different immune-based diseases. The miRNA signature of human peripheral blood (PB) CD8$^+$CD25$^+$CD127^{low} Tregs has not been described yet. We thus identified, using TaqMan low-density array (TLDA) technique followed by individual quantitative real-time polymerase chain reaction (qRT-PCR) confirmation, 14 miRNAs, among which 12 were downregulated whereas two were upregulated in CD8$^+$CD25$^+$CD127^{low} Tregs in comparison to CD8$^+$CD25$^-$ T cells. In the next step, microRNA Data Integration Portal (mirDIP) was used to identify potential miRNA target sites in the 3′-untranslated region (3′-UTR) of key Treg cell-immunomodulatory genes with a special focus on interleukin 10 (IL-10) and transforming growth factor β (TGF-β). Having identified potential miR target sites in the 3′-UTR of IL-10 (miR-27b-3p and miR-340-5p) and TGF-β (miR-330-3p), we showed through transfection and transduction assays that the overexpression of two underexpressed miRNAs, miR-27b-3p and miR-340-5p, downregulated IL-10 expression upon targeting its 3′-UTR. Similarly, overexpression of miR-330-3p negatively regulated TGF-β expression. These results highlighted an important impact of the CD8$^+$ Treg mirnome on the expression of genes with significant implication on immunosuppression. These observations could help in better understanding the mechanism(s) orchestrating Treg immunosuppressive function toward unraveling new targets for treating autoimmune pathologies and cancer.

KEYWORDS
human Tregs, IL-10, micro-RNA, TGF-β

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1 | BACKGROUND

Regulatory T cells (Tregs) are a subgroup of T cells with inhibitory properties affecting the activation of the immune system. Tregs can be divided into natural Tregs (nTregs), which are thymus-derived and adaptive Tregs, which can be induced in the periphery following activation by many stimuli, including pathogens, interleukin 10 (IL-10), and transforming growth factor-β (TGF-β; Bluestone & Tang, 2005; Raghavan & Holmgren, 2005; Schramm et al., 2004; Shevach, 2006; Zheng, Wang, Gray, Soucier, & Horwitz, 2004). Different Treg subpopulations exist, including, IL-10 producing Tr1, TGF-β secreting Th3 cells, $CD8^+$ suppressor cells, $CD4^-CD8^-$ T cells, and γδT cells (Brusko, Putnam, & Bluestone, 2008).

Treg cells were initially characterized by the expression of CD25 (the α-chain of the IL-2 receptor) and forkhead box P3 (FOXP3), a forkhead/winged helix transcription factor 3, which is essential for Treg development and function (de la Rosa, Rutz, Dorninger, & Scheffold, 2004; Fontenot, Gavin, & Rudensky, 2003; Pandiyan, Zheng, Ishihara, Reed, & Lenardo, 2007; Thornton & Shevach, 1998; Vignali, Collison, & Workman, 2008). FOXP3 was shown to be indispensable for Treg function as revealed by studies, which showed that mutations in human lead to an X-linked immunodeficiency syndrome termed IPEX (immunodysregulation, polyendocrinopathy enteropathy, X-linked syndrome; Sakaguchi, 2004; Sakaguchi, Miyara, Costantino, & Hafler, 2010; Sakaguchi, Yamaguchi, Nomura, & Ono, 2008).

The discovery of FOXP3, indispensable for Treg development and function, was very important for studying mouse and human Tregs (Fontenot et al., 2003; Gambineri, Torgerson, & Ochs, 2003; Hori, Nomura, & Sakaguchi, 2003; Khattri, Cox, Yasayko, & Ramsdell, 2003). However, despite remaining an important specific marker of Tregs, its usage for isolating Tregs is limited because of its intracellular expression. Recent reports have shown that the combination of low surface expression of CD127 (the α-chain of the IL-7 receptor) with high CD25 expression allows distinguishing between human regulatory and conventional T cells in the thymus, cord blood, adult peripheral blood (PB), and lymph nodes (Hartigan-O'Connor, Poon, Sinclair, & McCune, 2007; W. Liu et al., 2006; Seddiki et al., 2006).

$CD8^+$ T cells are an important adaptive effectors in several immunopathological conditions, such as autoimmune disease (Daniele et al., 2011; Huseby et al., 2001; Liblau, Wong, Mars, & Santamaria, 2002; A. Miller, Lider, Roberts, Sporn, & Weiner, 1992; G. X. Zhang et al., 1995), transplantation (Fowler, Breglio, Nagel, Eckhaus, & Gress, 1996; P. J. Martin, 1993), host defense, and cancer (Fowler et al., 1996; Prezzi et al., 2001). Despite that $CD8^+$ Tregs could exhibit a suppressive function, the regulatory properties of $CD4^+$ Treg subsets were focused on by most reports while only a few studies have shown $CD8^+$ Tregs' mediated immune regulation due to the absence of markers that allow their identification. Recently, several $CD8^+$ Treg cells' subsets have been identified (Billerbeck, Blum, & Thimme, 2007; Bisikirska, Colgan, Luban, Bluestone, & Herold, 2005; Cosmi et al., 2003; Hu, Weiner, & Ritz, 2013; Joosten et al., 2007; Uss et al., 2006; Xystrakis et al., 2004) including $CD8^+CD25^+$ Treg cells, with similar functional and phenotypic properties with $CD4^+CD25^+$ Treg cells, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and intracellular FOXP3 expression (Churlaud et al., 2015). These $CD8^+CD25^+$ Treg cells were shown to suppress $CD4^+CD25^-$ T cells in a membrane-bound TGF-β and CTLA-4-mediated contact-dependent manner that induced IL-2Rα downregulation on target T cells (Cosmi et al., 2003). They can also produce immunosuppressive cytokines, such as TGF-β and IL-10 (Gilliet & Liu, 2002) or by inactivating dendritic cells (Chang et al., 2002).

MicroRNAs (miRNAs) are short noncoding single-stranded RNA molecules that are derived from hairpin-structured precursors (Bartel, 2004). These miRNAs regulate gene expression following their binding to the potential target site in the 3′-untranslated regions (3′-UTRs) of specific target messenger RNA (mRNA), where they can lead to mRNA degradation and/or repression of protein translation. A large number (more than 700) of miRNAs have been identified in mammalian cells and have been shown to be implicated in different biological processes including human development, cellular differentiation and homeostasis, adaptation to the environment, oncogenesis, and host cell interactions with pathogens (Calin et al., 2002; Michael, O'Connor, van Holst Pellekaan, Young, & James, 2003; Navarro & Lieberman, 2010; O'Connell, Rao, Chaudhuri, & Baltimore, 2010; Slezak-Prochazka, Durmus, Kroesen, & van den Berg, 2010; Waki et al., 2015). Despite being important regulators of many biological processes, their abnormal expression is nowadays considered a common feature of various diseases (Haramati et al., 2010; B. Miller & Wahlestedt, 2010; Pallante, Visone, Croce, & Fusco, 2010; Schetter, Heegaard, & Harris, 2010). Recently, more miRNAs have been shown to be implicated in regulating many aspects of immune responses, such as differentiation, proliferation, cell fate determination, function of immune cells, cytokine responses as well as intracellular signaling pathways (Baltimore, Boldin, O'Connell, Rao, & Taganov, 2008; Lindsay, 2008; Lodish, Zhou, Liu, & Chen, 2008; Xiao & Rajewsky, 2009).

As an immunoregulatory function of Treg cells may hinder the induction of immune responses against cancer and infectious agents (Ha, 2009), counteracting Treg activity can evoke effective antitumor immunity (Beyer & Schultze, 2006; Curiel, 2007, 2008; Ha, 2009). Therefore, modulating cancer patients' Treg function will be very important to improve the efficacy of antitumor therapies, especially those based on immunotherapeutic approaches (Qin, 2009; Sakaguchi et al., 2010). Moreover, inducing or enhancing the Treg function can be desirable in autoimmune disease and in transplantation.

We, therefore, started to study these Tregs at the molecular level. Having identified human $CD8^+$ nTregs miR signature and its functional impact (Jebbawi et al., 2014), we proceeded to study the signature of $CD8^+CD25^+CD127^{low}$ Tregs, isolated from the PB of adult healthy volunteers, which were recently characterized by Churlaud et al. (2015) who showed their in vitro suppressive activity in addition to the differential expression of several markers, including, FOXP3 and CTLA4. Here, we show a Treg miRNA signature composed of 14 differentially expressed miRNAs among which miR-27b-3p, miR-330-3p, and miR-340-5p expression were downregulated in this regulatory population. miR-27b-3p

and miR-340-5p were shown to negatively regulate IL-10 expression by binding directly to two target sites in the IL-10 3′-UTR. Finally, we showed that miR-330-3p negatively regulated TGF-β expression in $CD8^+$ PB Tregs by binding directly to its target site in the 3′-UTR of its transcript.

2 | MATERIALS AND METHODS

2.1 | Purification of PB $CD8^+CD25^+CD127^{low}$ Tregs

After informed consent, $CD8^+CD25^+CD127^{low}$ regulatory cells were purified from PBMCs, isolated from about 60 ml of heparinized whole blood by Ficoll-Paque™plus density gradient, in two-steps. The first steps involves negative selection of $CD8^+$ T cells using a cocktail of biotin-conjugated antibodies (CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, glycophorin A, and CD127) and anti-biotin microbeads and then passing through magnetic separation columns, whereas the second step involves positive selection of these $CD8^+$ T cells for CD25 expression using anti-CD25-coated microbeads and then passing through magnetic separation columns. Two $CD8^+$ T cell populations will be obtained at the end and will be referred to as $CD8^+CD25^+CD127^{low}$ Tregs and $CD8^+CD25^-$ T cells.

2.2 | TaqMan low-density array (TLDA) and data analysis

Trizol total RNA isolation reagent (Roche Diagnostics, Vilvoorde, Belgium) was used to extract total RNA from cells. NanoDrop Spectrophotometer was used to quantify the concentration. First, to synthesize complementary DNA (cDNA) from miRNAs, a TaqMan microRNA Reverse Transcription Kit (#4366596; Applied Biosystems, Gent, Belgium) and Megaplex RT Primers (Human Pool A; #4399966; Applied Biosystems, Gent, Belgium) were used following the manufacturer's protocol allowing simultaneous reverse-transcription of 380 mature human miRNAs. Reverse-transcription was carried out using Mastercycler Ep Gradient Thermocycler (VWR International, Leuven, Belgium) with the following parameters: 40 cycles at 16°C for 2 min, 42°C for 1 min, 50°C for 1 s, and a final step of 80°C for 5 min to inactivate the reverse-transcriptase. Preamplification was thereafter carried out using Megaplex PreAmp Primer (Humam Pool A; #4399233; Applied Biosystems) and PreAmp Master Mix (#4384266; Applied Biosystems) following the manufacturer's instructions to generate enough miRNA cDNA template. The preamplification cycling conditions were as follows: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min followed by 12 cycles at 95°C for 30 s, and 60°C for 4 min; the samples were then held at 99.9°C for 10 min. The products were then diluted with RNase-free water, mixed with TaqMan Gene Expression Master Mix and then charged into TaqMan Human MicroRNA Array A (#4398965; Applied Biosystems), corresponding to a 384-well formatted plate and real-time polymerase chain reaction (qPCR)-based microfluidic card containing embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs. Quantitative PCR (qPCR) was carried out following the manufacturer's instructions. Real-time PCR was carried out using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with the following parameters: 50°C for 2 min, 94.5°C for 10 min followed by 40 cycles at 95°C for 30 s and 59.7°C for 1 min. RNU48 embedded in the TaqMan Human MicroRNA Arrays was considered as an endogenous control.

The relative expression levels of miRNAs were calculated using the comparative $\Delta\Delta C_t$ method as described previously (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). The fold changes in miRNAs were calculated by equation $2^{-\Delta\Delta C_t}$.

2.3 | qPCR for individual miRNAs

For each miRNA, reverse-transcription was carried out using 10 ng of purified total RNA, 100 mM dNTPs, 50 U MutliScribe Reverse Transcriptase, 20 U RNase inhibitor, and 50 nM of RT primer samples using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Reactions (15 μl) were incubated: 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real Time-PCR reactions (5 μl of RT product, 10 μl TaqMan 2X Universal PCR Master Mix [Applied Biosystems], and 1 μl TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes) were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems) at the following parameters: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The expression levels ($2^{-\Delta\Delta C_t}$) of miRNAs were calculated as described previously (Schmittgen & Livak, 2008).

2.4 | Bioinformatics

MicroRNA Data Integration Portal (mirDIP; http://ophid.utoronto.ca/mirDIP/) was searched for potential miRNA target sites. Using "IL-10" and "TGF-β" as search terms, "IL-10" was identified as having potential miR-27b-3p and miR-340-5p target sites (TargetScan) whereas "TGF-β" was identified as having miR-330-3p potential target site (RNAhybrid).

2.5 | Construction of plasmids

A 470-bp fragment of IL-10 3′-UTR containing the miR-27b-3p and miR-340-5p potential target sites, and a 302-bp fragment of TGF-β containing the miR-330-3p potential target site were cloned in the psiCHECK-1 plasmid (Promega, Mannheim, Germany) downstream of the Renilla luciferase gene (*Eco*RI/*Xho*I sites) and designated as psiCHECK 3′-UTR WT. PCR primers used for amplification of the IL-10 and TGF-β 3′-UTRs were as follows:

IL-10:
Forward primer: 5′-CAACCCCCATTTCTATTTATTTACTG-3′
Reverse primer: 5′-CCCGGCCTAGAACCAAAT-3′

TGF-β:
Forward primer: 5′-GGGACTCTGATAACACCCATTT-3′
Reverse primer: 5′-CCTTAGCCTCCAGAGTAGCTG-3′

Deletion of miR-27b-3p, miR-340-5p, and miR-330-3p target sites in psiCHECK 3′-UTR WT was performed by QuikChange site-directed mutagenesis according to the manufacturer's protocols (StrateGene, La Jolla, CA) and designated as psiCHECK-UTRdel. QuikChange site-directed mutagenesis was performed using the following primers:

IL-10 (miR-27b-3p deleted):
Forward primer: 5′-CTATTTATTTACTGAGCTTCTAACGATTTAGAAAG AAGCCC -3′
Reverse primer: 5′-GGGCTTCTTTCTAAATCGTTAGAAGCTCAGTAAA TAAATAG -3′

IL-10 (miR-340-5p deleted):
Forward primer: 5′-GGCCAGCTTGTTAACAACCTAAATTTG-3′
Reverse primer: 5′-CAAATTTAGGTTGTTAACAAGCTGGCC-3′

TGF-β (miR-330-3p deleted):
Forward primer: 5-GTGGTCCCAGCTATGGAGGCTAAGG-3′
Reverse primer: 5′-CCTTAGCCTCCATAGCTGGGACCAC-3′
Sequencing was performed to verify the constructs.

2.6 | Cell culture

The 293 T and Hela cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Europe, Paisley, UK), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin (all from Lonza).

2.7 | Luciferase assays

Assays were performed in a 24-well format. Hela and 293 T cells were cotransfected, using Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) according to the manufacturer's guidelines, with reporter plasmids (psiCHECK/psiCHECK 3′-UTR WT/psiCHECK 3′-UTR deleted; 100 ng) along with miR-27b-3p, miR-330-3p, and miR-340-5p-mimic/miR-negative control-mimic at final concentration of 10 μM (miRIDIAN mimic; Dharmacon, Geel, Belgium) and control firefly plasmid pGL3-CMV (100 ng). The cells were first assessed for their expression levels of miRNAs of interest using quantitative RT-PCR, as described below. Forty hours posttransfection, cells were collected and luciferase levels were determined using the Dual-luciferase reporter assay system (Promega) following the manufacturer's guidelines. Relative protein levels corresponded to Renilla/Firefly luciferase ratios.

2.8 | RT-PCR for FOXP3, CTLA4, CD25, IL-10, and TGF-β expression

Quantitative mRNA expression was determined by real-time PCR, with PRISM 7900 Sequence Detection System (Applied Biosystems), and the TaqMan Master Mix Kit with EF1-α mRNA was used as an internal control. Human Taqman gene expression assays for IL-10 (Hs00961622_m1), TGF-β (Hs00998133_m1), FOXP3 (Hs01085834_m1), CTLA4 (Hs00175480_m1), CD25 (Hs00907777_m1), and EF1-α (Hs00951278_m1) were purchased from Applied Biosystems. The program used for amplification was 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

2.9 | Lentiviral vector production

293 T cells were used to generate VSV-G pseudotyped lentiviral particles following polyethylene imine (PEI; Sigma, St. Louis, MO) cotransfection with three plasmids, pMIRNA, pCMVΔR8.91, and pMD.G (Naldini et al., 1996).

pCMVΔR8.91 is a HIV-derived packaging construct, which encodes the HIV-1 Gag and Pol precursors as well as the regulatory proteins Tat and Rev (Zufferey, Nagy, Mandel, Naldini, & Trono, 1997). VSV-G was expressed from pMD.G (Yeung, Bennasser, LE, & Jeang, 2005). pMIRNA is a lentiviral-based vector containing the miRNA precursor and copGFP (ppluGFP2) as a reporter gene encoding for green fluorescent protein (GFP) characterized by super bright green fluorescence and fast maturation rate at a wide range of temperatures (Shagin et al., 2004). Viral supernatants were collected 24 and 48 hours posttransfection, filtered through 0.45 μm low protein-binding filters (Nalgene, Rochester, NY), and concentrated as previously described (Johnston et al., 1999). Lentiviral vector preparations collected 24 and 48 hr posttransfection displayed, respectively, titers of 10^8–10^9 and 5×10^7–5×10^8 transducing units (TU) per ml in HeLa cells.

2.10 | Lentiviral transduction of CD8$^+$ Tregs

Human PB CD8$^+$ Tregs, 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 AB serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza Europe), in the presence of 5 μg/ml phytohemagglutinin (PHA-L, Sigma-Aldrich, Bornem, Belgium) and 20 U/ml IL-2, were exposed 24 hr postpurification to lentiviral vector preparations with multiplicity of infection = 5, in a volume of 500 μl in the presence of 8 μg/ml polybrene (Sigma Aldrich). Flow cytometry was used to sort GFP-positive cells 7 days after transduction.

2.11 | TaqMan real-time PCR of mature miR-27b-3p, miR-330-3p, and miR-340-5p

TaqMan miRNA assays (Applied Biosystems) used the stem-loop method (Abbas-Terki, Blanco-Bose, Déglon, Pralong, & Aebischer, 2002; Cobb et al., 2006) to detect the expression level of mature miR-27-3p, miR-330-3p, and miR-340-5p. For each miRNA, reverse-transcription was carried out using 10 ng of purified total RNA, 100 mM dNTPs, 50 U MutliScribe Reverse Transcriptase, 20 U RNase inhibitor, and 50 nM of RT primer samples using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Reactions (15 μl) were incubated: 30 min at 16°C, 30 min at 42°C, and 5 min at

85°C. Real Time-PCR reactions (5 μl of RT product, 10 μl TaqMan 2X Universal PCR Master Mix, [Applied Biosystems], and 1 μl TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes) were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems) at the following parameters: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. RNU48 was used as an endogenous control.

2.12 | Detection of IL-10 and TGF-β in cell culture supernatant using ELISA kit

The IL-10 and TGF-β levels in the supernatant of lenti-miR-27b-3p, lenti-miR-330-3p, and lenti-miR-340-5p transduced $CD8^+$ Tregs was performed by ELISA in IL-10/TGF-β assay plate supplied by the R&D System.

2.13 | Statistical analysis

Data are presented as mean ± standard error of the mean of at least three independent experiments and analyzed using Student's *t* test. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered significant.

3 | RESULTS

3.1 | Assessment of Treg cell-related gene expression and miR signature in $CD8^+CD25^+CD127^{low}$ T cells

The expression level of Tregs' specific genes like FOXP3, CD25, and CTLA4 were examined following the RNA extraction from immunomagnetically purified human $CD8^+CD25^+CD127^{low}$ T cells and $CD8^+CD25^-$ T cell subsets. qPCR results revealed upregulation of FOXP3, CD25, and CTLA4 in $CD8^+CD25^+CD127^{low}$ versus $CD8^+CD25^-$ T cells as shown in Figure 1. Next, using the TLDA technique, we assessed the miR expression profile in $CD8^+$ Tregs versus $CD8^+$ conventional T cells. Relative miRNA expression was normalized against the RNU48 endogenous control. Our TLDA analysis revealed differential expression of 29 miRNAs in $CD8^+CD25^+CD127^{low}$ Treg cells versus $CD8^+CD25^-$ T cells. Among these, 24 miRNAs (miR-103, -106b, -127-5p, -145, -184, -18b, -27b-3p, -223, -301, -30c, -324-3p, -330-3p, -331-5p, -340-5p, -362, -365, -424, -450a, -484, -579, -616, -655, -708, and -758) were downregulated whereas five miRNAs (miR-130a, -199a, -299-3p, -34a, and -489) were upregulated. Details of the results are shown in Table 1. A clustergram of the samples as well as the significantly differentially expressed miRNAs in Tregs and $CD8^+CD25^-$ T cells is illustrated in Figure 2 in the form of a heat map generated using ΔC_t values.

Second, miRNAs that appeared to be differentially expressed in Tregs versus $CD8^+CD25^-$ T cells were further examined by qRT-PCR.

A miR signature for $CD8^+$ PB Tregs was identified consisting of 14 miRs, among which 12 miRNAs (miR-103, -106b, -27b-3p, -301, -30c, -324-3p, -330-3p, -331-5p, -340-5p, -362, -484, and -579) were downregulated whereas two miRNAs (miR-130a and miR-34a) were upregulated (Figure 3).

3.2 | IL-10 is directly regulated by miR-27b-3p and miR-340-5p

As IL-10 is known to be indispensable for Treg function (Bjarnadottir, Lemarquis, Halldorsdottir, Freysdottir, & Ludviksson, 2014; Gilliet & Liu, 2002) we, therefore, proceeded to investigate the effect of miR-27b-3p and miR-340-5p, which have potential target sites in IL-10 3′-UTR, on its expression.

A Renilla luciferase reporter gene-based vector was designed in which 470 bp fragment of the 3′-UTR of IL-10 containing the miR-27b-3p and the miR-340-5p target sequences (Figure 4a) was cloned downstream of the reporter gene. This vector was referred to as psiCHECK-UTRwt. In parallel, the same procedure was applied to clone this IL-10 3′-UTR fragment with deleted target sites (seed region) of miR-27b-3p and miR-340-5p (psiCHECK-UTRdel). Before

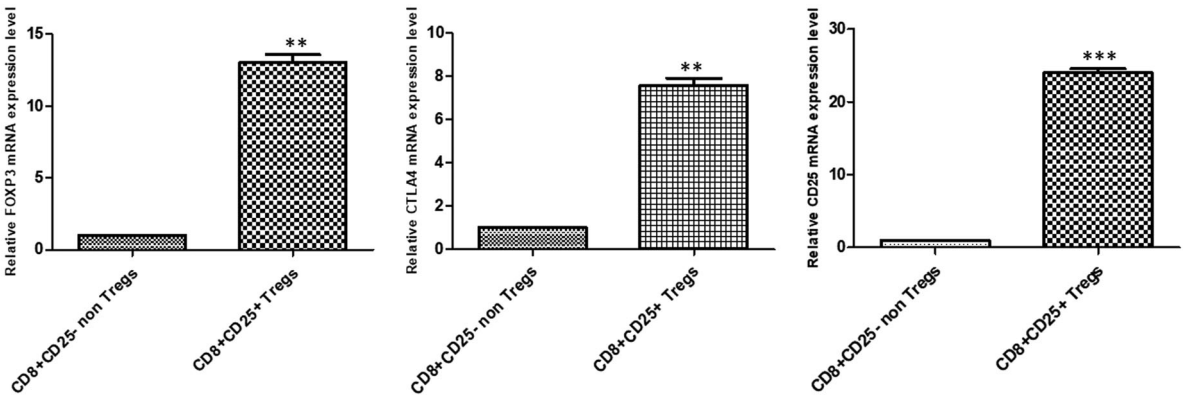

FIGURE 1 Relative Tregs-associated gene expression for $CD8^+CD25^+CD127^{low}$ T cells/$CD8^+CD25^-$ T cells. Gene mRNA levels were evaluated using qRT-PCR of $CD8^+CD25^+CD127^{low}$/$CD8^+CD25^-$ T cells. Data represent the mean ± standard error of the mean of five independent experiments each done in triplicate. The statistical significance was determined using unpaired Student's *t* test ($^{**}p < 0.01$, $^{***}p < 0.001$ vs. $CD8^+CD25^-$ T cells). CTLA4: cytotoxic T-lymphocyte-associated antigen 4; FOXP3: forkhead box P3; mRNA: messenger RNA; qRT-PCR: quantitative real-time polymerase chain reaction; Tregs: regulatory T cells

TABLE 1 Differentially expressed miRs identified by the TLDA Technique

MicroRNA	Tregs vs. non-Tregs	*p* Value
miR-103	0.3	0.03
miR-106b	0.044	0.01
miR-127-5p	0.22	0.032
miR-145	0.23	0.045
miR-184	0.34	0.037
miR-18b	0.18	0.046
miR-27b-3p	0.31	0.022
miR-223	0.29	0.046
miR-301	0.23	0.021
miR-30c	0.19	0.032
miR-324-3p	0.31	0.018
miR-330-3p	0.21	0.006
miR-331-5p	0.15	0.022
miR-340-5p	0.06	0.022
miR-362	0.29	0.039
miR-365	0.35	0.042
miR-424	0.45	0.038
miR-450a	0.39	0.041
miR-484	0.33	0.005
miR-579	0.23	0.031
miR-616	0.01	0.046
miR-655	0.3	0.043
miR-708	0.18	0.039
miR-758	0.01	0.04
miR-130a	6.5	0.02
miR-199a	4.7	0.042
miR-299-3p	3.8	0.048
miR-34a	5.8	0.03
miR-489	4.3	0.043

Note. The TLDA technique identified microRNAs differentially expressed between $CD8^+$ Tregs and $CD8^+CD25^-$ T cells with a $p < 0.05$.The expression of each of these miRs was then subjected to individual quantitative polymerase chain reaction assay.
miR: microRNA; TLDA: TaqMan low-density array.

the transfections with the reporter constructs, we examined the expression level of miR-27b-3p and miR-340-5p, which was shown by qPCR to be similar in Hela and $CD8^+CD25^-$ T cells (data not shown). Transient transfection of psiCHECK-UTRwt in Hela cells led to a 48% decrease in reporter luciferase activity when compared with the psiCHECK control vector (Figure 4b). However, no decrease in reporter luciferase activity was observed in cells transfected with psiCHECK-UTRdel (Figure 4b). Notably, a more substantial reduction in reporter luciferase activity was observed in Hela cells being cotransfected with either miR-27b-3p and psiCHECK-UTRwt (~46%) or miR-340-5p and psiCHECK-UTRwt (~43%) in comparison with the cells being transfected with psiCHECK-UTRwt alone (Figure 4b). Further, the negative effect of either miR-27b-3p or miR-340-5p was bypassed in cells cotransfected with psiCHECK-UTRdel and miR-27b-3p and/or miR-340-5p (Figure 4b). Remarkably, cotransfection of Hela cells with psiCHECK-UTRwt and both miR-27b-3p and miR-340-5p resulted in more striking negative effect (~49%) in comparison with the cells cotransfected with psiCHECK-UTRwt and either miR-27b-3p or miR-340-5p (Figure 4b). Altogether, these data indicate that IL-10 expression could be negatively regulated by miR-27b-3p and miR-340-5p.

3.3 | miR-330-3p negatively regulates TGF-β expression upon targeting the 3′-UTR

Using the computational mirDIP, TGF-β was identified as a potential target for miR-330-3p. We, therefore, checked whether TGF-β expression could be directly regulated by miR-330-3p.

A Renilla luciferase reporter gene-based vector was designed in which the 3′-UTR of TGF-β containing the miR-330-3p target sequence (Figure 5a) was cloned downstream of the reporter gene. This vector was referred to as psiCHECK-UTRwt. In parallel, the same procedure was applied to clone this TGF-β 3′-UTR fragment with deleted potential target sites of miR-330-3p (psiCHECK-UTRdel). Before the transfections with the reporter constructs, we examined the expression level of miR-330-3p, which was shown by qPCR to be similar in 293 T and $CD8^+CD25^-$ T cells (data not shown). Transient transfection of psiCHECK-UTRwt in 293 T cells led to a 47% decrease in reporter luciferase activity, when compared with the psiCHECK control vector (Figure 5b). However, no decrease in reporter luciferase activity was observed in cells transfected with psiCHECK-UTRdel (Figure 5b). Notably, a more substantial reduction in reporter luciferase activity was observed in 293 T cells being cotransfected with miR-330-3p and psiCHECK-UTRwt (~50%) in comparison with the cells being transfected with psiCHECK-UTRwt alone or its cotransfection with miR-ctrl (Figure 5b). In contrast, the mutant reporter (psiCHECKUTRdel) luciferase activity was not repressed by miR-330-3p, which indicated that the target site directly mediated the repression (Figure 5b). Taken together, these data indicate that TGF-β expression could be negatively regulated by miR-330-3p.

3.4 | Overexpression of miR-27b-3p and miR-340-5p in primary human $CD8^+$ Tregs leads to diminished IL-10 production

Lentiviral-based systems have been developed to generate replication incompetent lentiviral vectors that efficiently transduce both dividing and nondividing mammalian cells and provide stable, long-term expression of RNA of interest. MiRNAs can downregulate gene expression by two posttranscriptional mechanisms: mRNA cleavage or translational repression (Bartel, 2004). Because luciferase assay does not distinguish the two mechanisms well, the effect of miR-27b-3p and miR-340-5p on $CD8^+$ Tregs, and more specifically on IL-10 expression, was investigated following $CD8^+$ Tregs transduction with lenti-miR-27b-3p

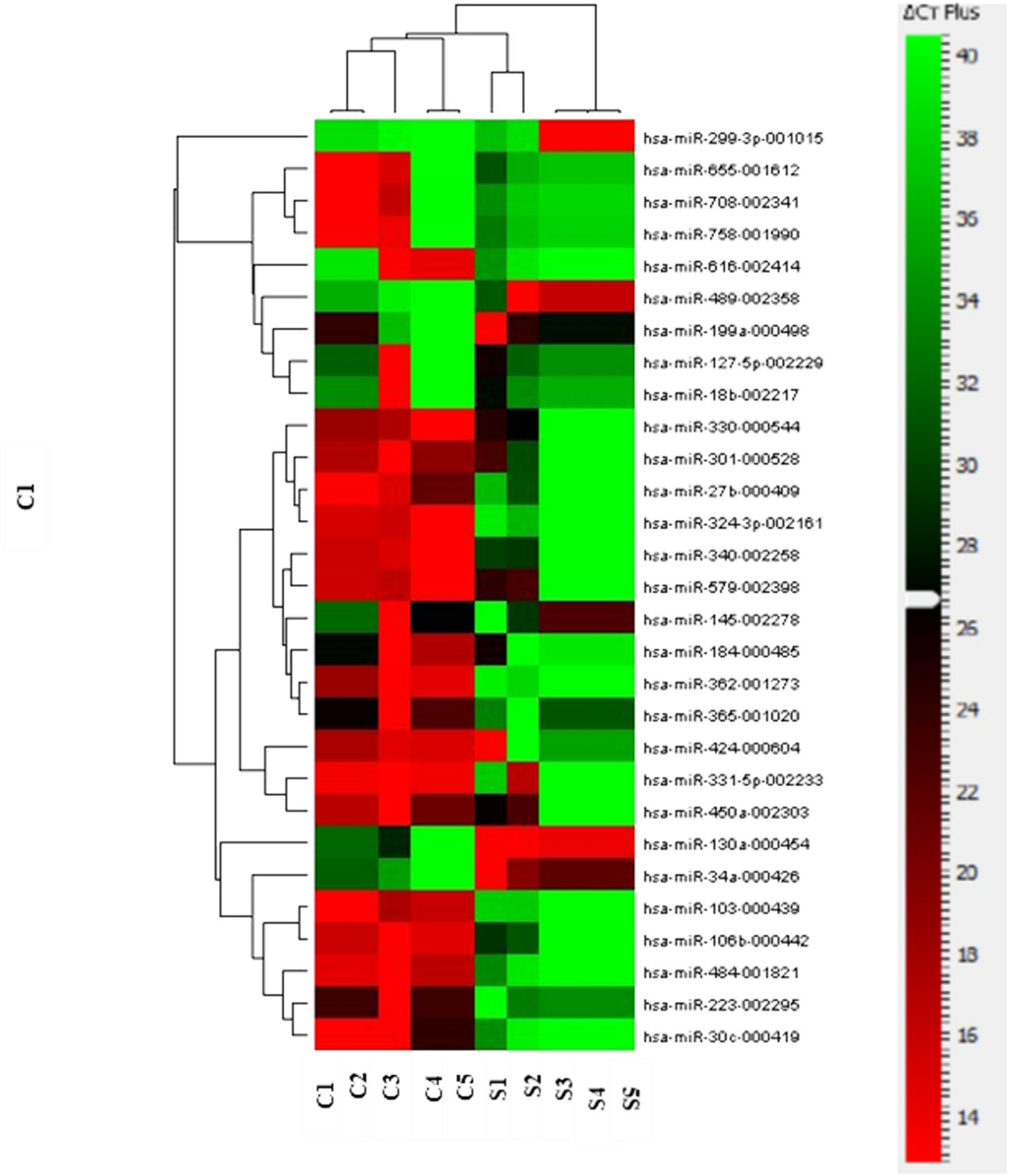

FIGURE 2 Unsupervised hierarchical cluster analysis of miRs differentially expressed in human CD8$^+$ Tregs vs. CD8$^+$CD25$^-$ T cells based on their relative expression levels. miR profiles of Tregs and CD8$^+$CD5$^-$ T cells were visualized with agglomerative hierarchical clustering using Euclidean distance from TaqMan low-density arrays. Columns correspond to samples and are labeled to indicate whether a column represents a Treg sample group (S) or the CD8$^+$CD25$^-$ T cell control group (C). Each row corresponds to an individual miR sequence. The miR names and the dendrogram for miR clustering are displayed on the right. The colors display miR expression variance: red indicates a higher gene expression and green indicates lower expression. miR: microRNA; Tregs: regulatory T cells [Color figure can be viewed at wileyonlinelibrary.com]

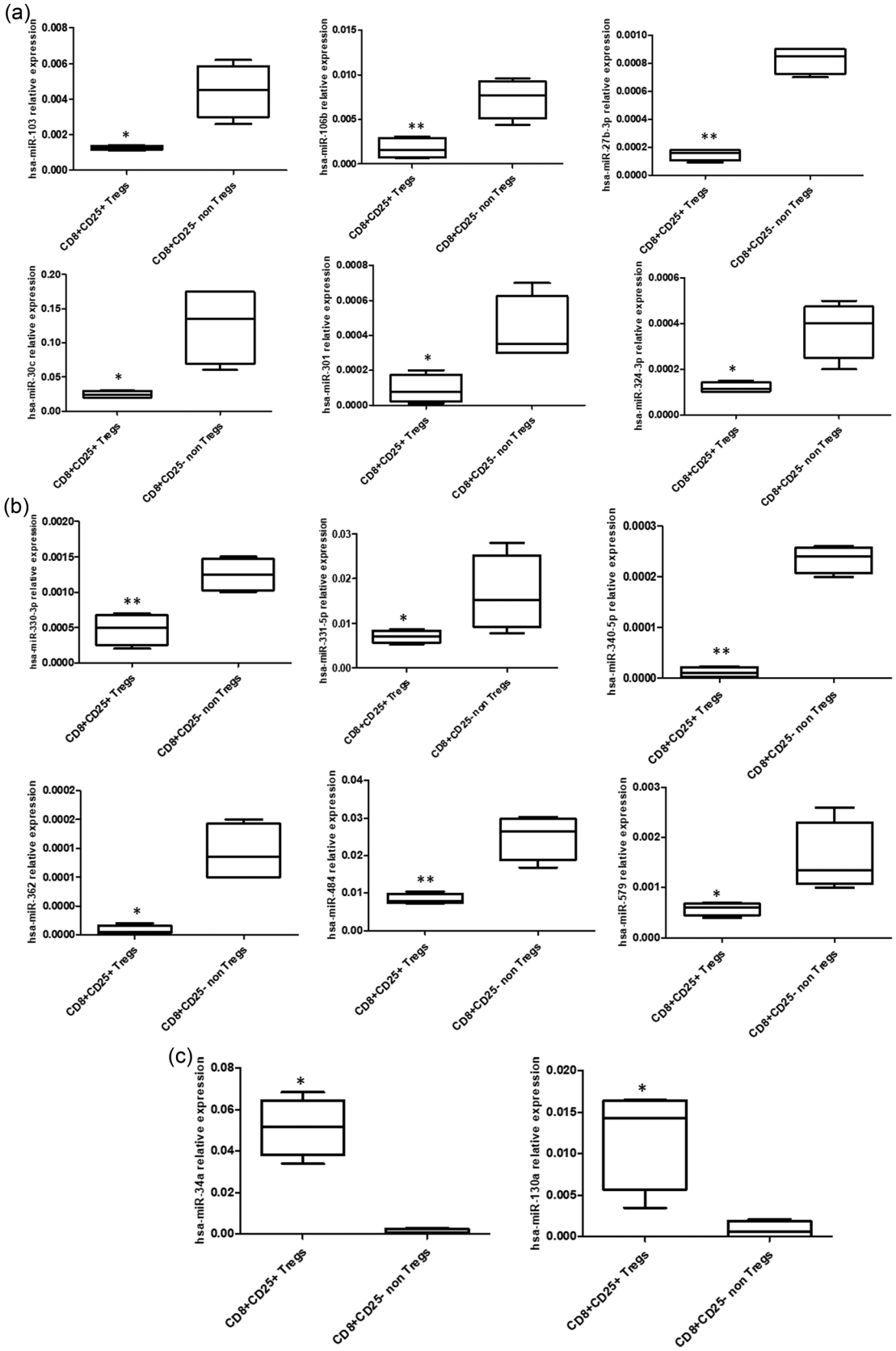

FIGURE 3 Differential expression of the 14 miRs in $CD8^+$ Tregs vs. $CD8^+CD25^-$ T cells. Data obtained by qRT-PCR amplification of miRs were normalized to RNU48 and plotted as Box plots. The statistical significance was determined using unpaired Student's *t* test (*$p < 0.05$ and **$p < 0.01$ vs. $CD8^+CD25^-$ T cells). miRNA: microRNA; qRT-PCR: quantitative real-time polymerase chain reaction; Tregs: regulatory T cells

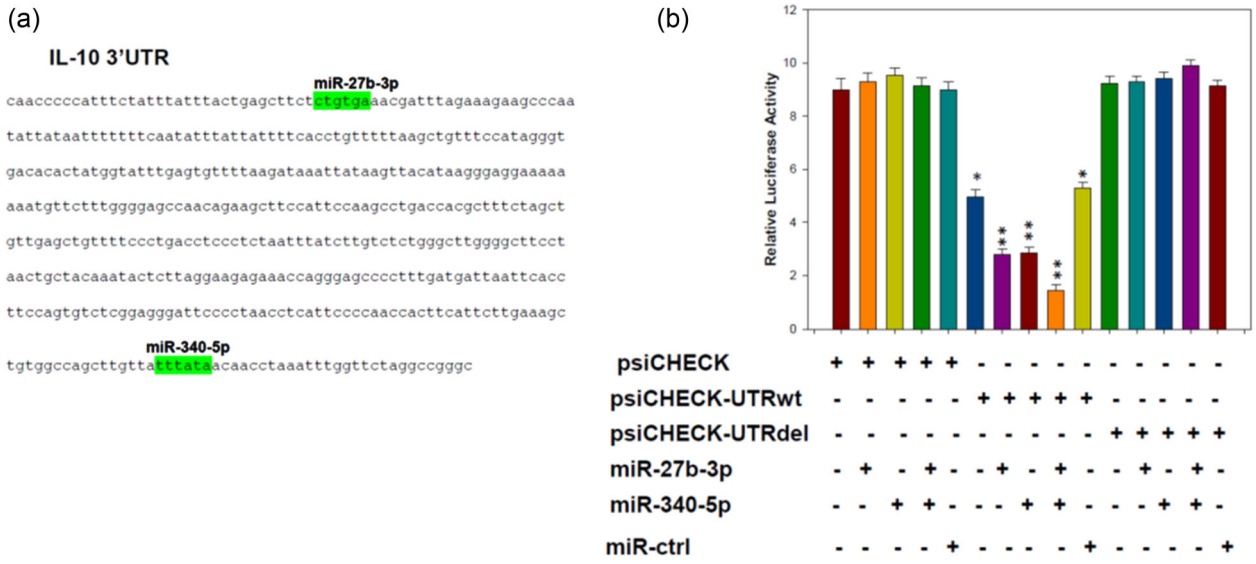

FIGURE 4 miR-27b-3p and miR-340-5p negatively regulate IL-10 expression. (a) Representation of IL-10 3′-UTR nucleotide sequence containing miR-27b-3p (green color) and miR-340-5p (green color) potential target sites as revealed by miRDIP (Targetscan). (b) Renilla luciferase reporter assays with constructs holding IL-10 3′-UTR sequences were cotransfected into Hela cells along with a firefly luciferase transfection control plasmid either alone or together with miR-27b-3p and miR-340-5p. Shown are relative luciferase values and the data represents mean ± standard error of the mean of three independent experiments, each performed in triplicate. *$p < 0.05$ and **$p < 0.01$ vs. psiCHECK transfected cells; Student's *t* test. 3′-UTR: 3′-untranslated region; IL-10: interleukin 10; miR: microRNA [Color figure can be viewed at wileyonlinelibrary.com]

and lenti-miR-340-5p that contain copGFP as a reporter gene. Flow cytometry measurement of GFP expression revealed 82–89% transduction efficiency for lenti-miR-ctrl, lenti-miR-27b-3p, and lenti-miR-340-5p. In contrast, miR-27b-3p and miR-340-5p expression levels, in respective lenti-miR-27b-3p and lenti-miR-340-5p transduced cells were significantly higher than in lenti-miR-ctrl transduced cells as shown by qPCR results. The IL-10 mRNA expression level was twofold lower in lenti-miR-27b-3p and lenti-miR-340-5p transduced cells compared with lenti-miR-ctrl and nontransduced cells (Figures 6a,b). Figure 6c shows that IL-10 secretion was significantly reduced in lenti-miR-27b-3p and lenti-miR-340-5p transduced cells compared with lenti-miR-ctrl and nontransduced cells. These results demonstrate that IL-10 mRNA and protein levels are regulated by miR-27b-3p and miR-340-5p.

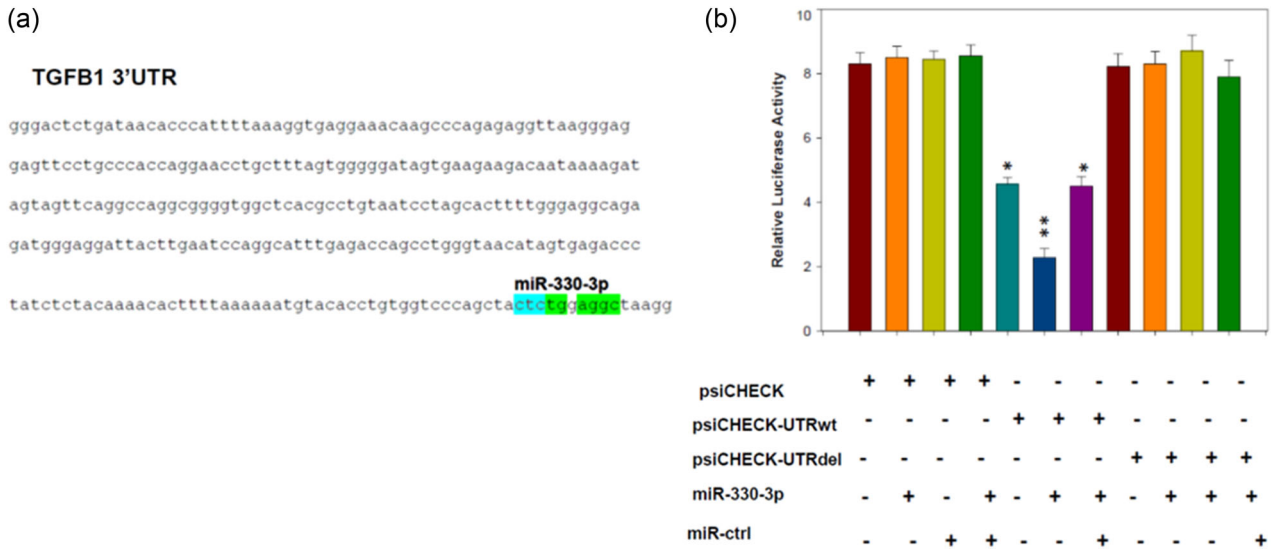

FIGURE 5 miR-330-3p negatively regulates TGF-β expression. (a) Representation of TGF-β 3′-UTR nucleotide sequence containing miR-330-3p potential target site (green color) as revealed by miRDIP (RNAhybrid). The blue color refers to the deleted part of the miR-330-3p potential target sequence. (b) Renilla luciferase reporter assays with constructs holding TGF-β 3′-UTR sequences were cotransfected into 293 T cells along with a firefly luciferase transfection control plasmid either alone or together with miR-330-3p. Shown are relative luciferase values and the data represents mean ± standard error of the mean of three independent experiments, each performed in triplicate. *$p < 0.05$ and **$p < 0.01$ vs. psiCHECK transfected cells; Student's *t* test. 3′-UTR: 3′-untranslated region; miR: microRNA; TGF-β: transforming growth factor β [Color figure can be viewed at wileyonlinelibrary.com]

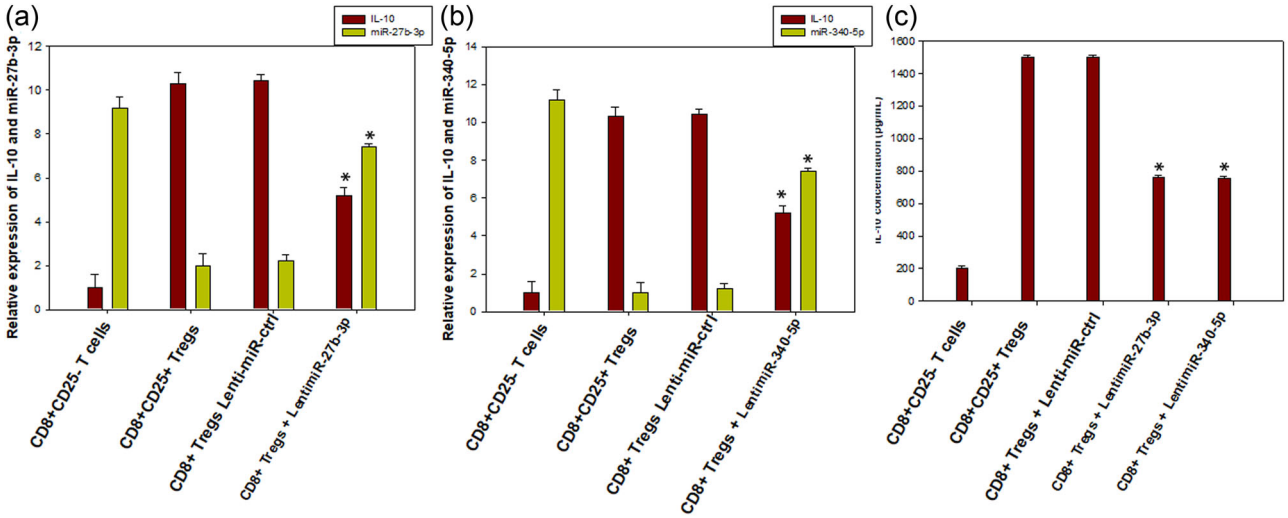

FIGURE 6 (a,b) Lentiviral-mediated miR-27b-3p and miR-340-5p expression and their effect on IL-10 in human CD8$^+$ Tregs. miR-27b-3p, miR-340-5p, and IL-10 expression were determined by qRT-PCR in Tregs, miR-27b-3p, miR-340-5p, and miR-ctrl transduced Tregs, and in CD8$^+$CD25$^-$ T cells. Mean of three independent experiments are shown. (c) ELISA assay of IL-10 protein level after lenti-miR-27b-3p, lenti-miR-340-5p, and lenti-miR-ctrl transduction. Cells were collected 7 days after transduction. Data represent mean ± standard error of the mean of three independent experiments, each performed in triplicate. *$p < 0.05$ (lenti-miR-27b and lenti-miR-340 vs. lenti-miR-ctrl transduced Tregs; Student's *t* test). IL-10: interleukin 10; miR: microRNA; qRT-PCR: quantitative real-time polymerase chain reaction; Tregs: regulatory T cells [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Lentiviral transduction of miR-330-3p in Tregs and the negative effect on TGF-β expression

Next, we examined the effect of miR-330-3p on the endogenous TGF-β expression in Tregs. Seven days after lenti-miR-330-3p and lenti-miR-ctrl CD8+ Treg transduction the GFP-positive ("GFP+") Treg population were isolated by flow cytometry. Quantitative real-time RT-PCR and ELISA assay results revealed that the mRNA (Figure 7a) and protein levels (Figure 7b) of this gene level decreased (twofolds) in the lenti-miR-330-3p transduced Tregs compared with the lenti-miR-ctrl transduced cells, thus indicating the negative effect of miR-330-3p on TGF-β expression.

4 | DISCUSSION

CD8$^+$ Tregs, initially described by Gershon and Kondo (1970), have emerged as a putatively important subset of Tregs. Diverse studies have indicated an important role for CD8$^+$ Tregs in modulating immune responses during cancer (Alvarez Arias et al., 2014; Kiniwa et al., 2007; Li et al., 2011), autoimmune pathologies (Notley, McCann, Inglis, & Williams, 2010; Tardito et al., 2013), and infectious diseases (Holderried, Lang, Kim, & Cantor, 2013). Compared with CD4$^+$ Tregs, our understanding of the molecular mechanisms regulating CD8$^+$ Treg cells function is much less. miRNAs that modulate protein expression upon binding to the 3′-UTR of target genes (Bartel, 2004) play a key role in regulating physiological processes where deregulated miRNA expression could lead to pathological conditions (Sayed & Abdellatif, 2011; Tüfekci, Öner, Meuwissen, & Genç, 2014). In a previous study, we defined the miRNA signature of CD8$^+$ nTregs from human umbilical cord blood and characterized the impact of this miRNA profile on the expression status of different genes (FOXP3, CTLA4, and GARP) implicated in CD8+ nTreg biology (Jebbawi et al., 2014). A recent study had characterized the molecular profiling and functional properties of human PB CD8$^+$CD25$^+$ Tregs (Churlaud et al., 2015). In our present study, we proceeded to the molecular profiling of CD8$^+$ Tregs at the miRNA level. Thus, using miRNA TLDA analysis and toward a better understanding of the biology of these CD8$^+$ Treg cells, we have identified the miR signature of these CD8$^+$CD25$^+$CD127^{low} Tregs. In total, 14 miRNAs were unraveled by the TLDA technique and validated by individual qRT-PCR to be differentially expressed in CD8$^+$CD25$^+$ Tregs in comparison with their negative counterpart CD8$^+$CD25$^-$ T cells. Among these, 12 miRNAs (miR-103, -106b, -27b-3p, -301, -30c, -324-3p, -330-3p, -331-5p, -340-5p, -362, -484, and miR-579) were downregulated while two miRNAs (miR-130a and miR-34a) were upregulated. Next, we aimed at gaining further insight into the impact of these differentially expressed miRs on the expression of genes relevant to CD8$^+$ Tregs function and survival. Interestingly, we found that the 3′-UTR of IL-10 have potential target sites for miR-27b-3p and miR-340-5p, which are being under-expressed in CD8$^+$ Tregs. Moreover, TGF-β 3′-UTR appeared to contain a target site for miR-330-3p.

IL-10, being produced by different immune cells among which are CD25$^+$Foxp3$^+$ Tregs (Maloy et al., 2003; Maynard et al., 2007), is a key immune suppressive cytokine required for tolerance establishment. In fact, IL-10 can inhibit the proliferation and function of different effector immune cells (Moore, De waal malefyt, Coffman, & O'Garra, 2001). Increased production of IL-10 has been associated with impaired antitumor responses (De Santo et al., 2010; Kim et al., 1995; Steinbrink et al., 1999) whereas blockade of IL-10 has been accompanied with the

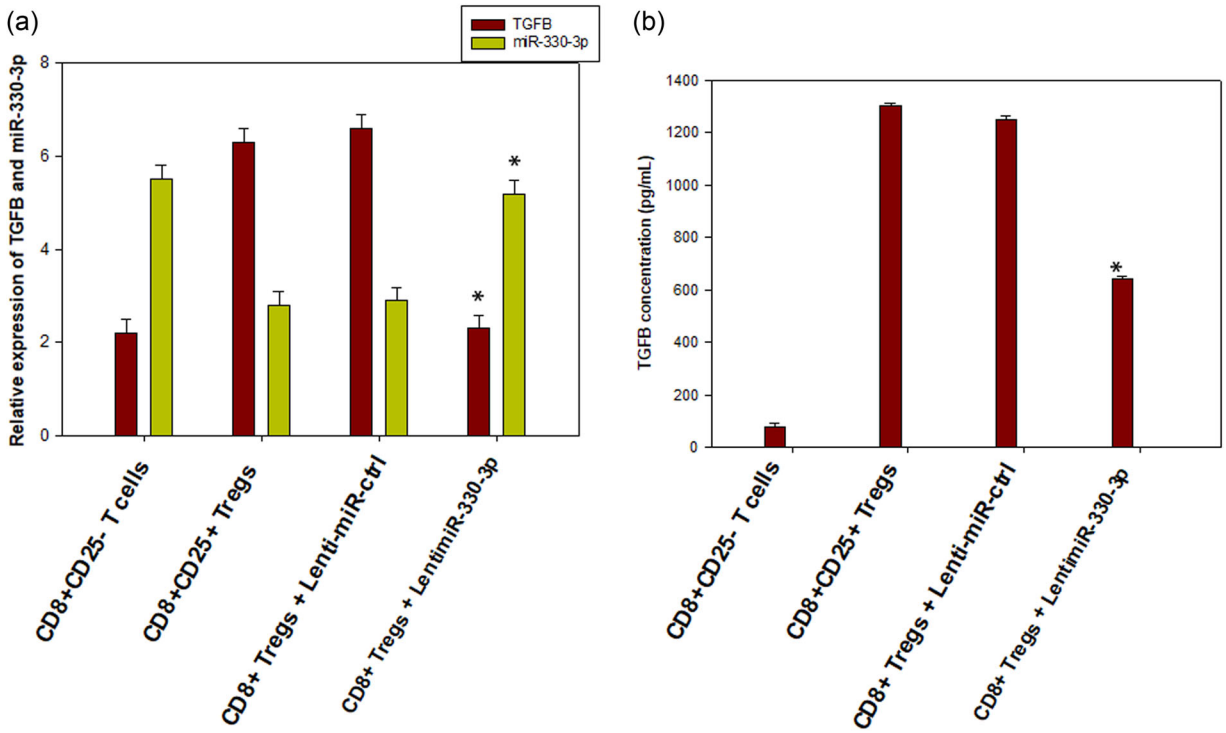

FIGURE 7 (a) Lentiviral-mediated miR-330-3p expression and its effect on TGF-β in Treg cells. miR-330-3p and TGF-β expression were determined by qRT-PCR in Tregs, miR-330-3p, miR-ctrl transduced Tregs, and in $CD8^+CD25^-$ T cells. Mean of three independent experiments are shown. (b) ELISA assay of TGF-β protein level after lenti-miR-330-3p and lenti-miR-ctrl transduction. Cells were collected 7 days after transduction. Data represent mean ± standard error of the mean of three independent experiments, each performed in triplicate. $^*p < 0.05$ (lenti-miR-330 vs. lenti-miR-Ctrl transduced Tregs; Student's *t* test). miR: microRNA; qRT-PCR: quantitative real-time polymerase chain reaction; TGF-β: transforming growth factor β; Tregs: regulatory T cells [Color figure can be viewed at wileyonlinelibrary.com]

increased rejection of organ transplantation (Vicari et al., 2002). Further, IL-10 plays a major role in inhibiting inflammatory pathologies (Asadullah, Sterry, & Volk, 2003). Interestingly, it has been reported that during H5N1 viral infection, $CD8^+$ Tregs can suppress $CD8^+$ T cells-responses via IL-10-dependent mechanisms (Zou et al., 2014). IL-10 expression could be regulated at the transcriptional level with Sp1 and Sp2 playing a major role as well as at the translational level via mechanisms involving the adenylate-uridylate (AU)-rich elements in the 3′-UTR of IL-10 mRNA (Saraiva & O'Garra, 2010). Additional molecular mechanisms regulating IL-10 expression remains to be unraveled. In this study, we investigated the effect of miR-27b-3p and miR-340-5p on IL-10 expression. Following 3′-UTR cloning, site-directed mutagenesis, and miRNA cotransfection procedures, we showed that miR-27b-3p alone, as well as miR-340-5p alone, could downregulate IL-10 expression. Moreover, miR-27b-3p and miR-340-5p appeared to synergistically affect the IL-10 expression level. Consistently, transduction of lenti-miR-27b-3p and lenti-miR-340-5p in primary $CD8^+$ Tregs resulted in reduced expression and secretion of IL-10 in comparison with lenti-miR-ctrl and nontransduced cells. In agreement with a role for miRNAs in regulating IL-10 expression, a previous study revealed that miR-27a modulates the inflammatory responses of macrophages upon altering IL-10 expression (Xie et al., 2014). Indeed, it has been shown that upregulating miR-27a expression reduced IL-10 expression in activated macrophages while downregulation of miR-27a expression had an opposite effect (Xie et al., 2014). Moreover, miR-98 has been described to inhibit IL-10 production, upon targeting its 3′-UTR, in macrophages following LPS stimulation (Y. Liu et al., 2011). Our observations highlight miR-27b-3p and miR-340-5p posttranscriptional control as one mechanism accounting for the different expression pattern of IL-10 in $CD8^+CD25^+$ Tregs versus $CD8^+CD25^-$ T cells.

TGF-β1 is a pleiotropic cytokine that is of critical immunologic importance. In fact, TGF-β is of central importance during inflammation, immune suppression, antibody production, and immune tolerance (Tran, 2012). Moreover, TGF-β1 is important for T cell lineage commitment and specifically for the development and maintenance of $Foxp3^+$ Tregs (Tran, 2012). TGF-β1 suppresses immune responses either via inhibiting the function of inflammatory cells or upon inducing the function of Tregs (Wan & Flavell, 2007). TGF-β1 is also considered as a pathogenic factor involved in both tumor suppression and promotion (Wan & Flavell, 2007). TGF-β1 expression has been well described to be subjected to a posttranscriptional control (Allison, Mumy, & Wakefield, 1998; Fraser, Brunskill, Ito, & Phillips, 2003; Fraser et al., 2008; Jenkins et al., 2010; Romeo, Park, Roberts, Sporn, & Kim, 1993; Tang et al., 1998; M. Zhang, Fraser, & Phillips, 2006). However, the underlying mechanisms are yet to be fully characterized. In this report, and upon following the same procedure as in the case of IL-10, TGF-β1 3′-UTR cloning, site-directed mutagenesis, miRNA cotransfection, and transduction experiments, we showed that miR-330-3p (downregulated in $CD8^+$

Tregs), when overexpressed, can significantly inhibit TGF-β1 expression as evidenced by reduced TGF-β1 mRNA and protein levels. miRNA-mediated regulation of TGF-β1 expression has already been described as miR-744 was shown to downregulate TGF-β1 expression upon targeting its 3′-UTR (J. Martin et al., 2011).

In conclusion, we provided here, for the first time the miRNA signature of peripheral $CD8^+CD25^+$ Tregs. We identified 14 miRNAs to be differentially regulated in $CD8^+CD25^+$ versus $CD8^+CD25^-$ T cells and showed that miR-27b-3p and miR-330-3p could be implicated in the posttranscriptional control of the immune suppressive cytokines IL-10 and TGF-β. This, in turn, highlights the impact of the mirnome on the immunoregulatory function of $CD8^+$ Tregs. Although a complete understanding of the mechanisms regulating $CD8^+$ Tregs function is still required, our observations suggest an important role for miRNAs in modulating $CD8^+$ Treg-mediated immunosuppressive capacity and thus highlights miRNAs as a potential therapeutic tool/target toward treating different immune-based disorders. In-depth physiological experiments run in inflamed and noninflamed in vivo conditions should definitely clarify the behavior of circulating CD8 Tregs and the biochemical mechanisms at work.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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