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Effect of MicroRNA-21 Transfection on In-vitro Differentiation of Human Naive CD4+ T Cells to Regulatory T Cells

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ABSTRACT

Regulatory T cells (Tregs) are important components of the immune system that modulate responses of other cells. These cells are involved in peripheral tolerance mechanisms, so defect in development and function of these cells can result in autoimmune disease. Increasing evidence supports the role of microRNAs-21 (miR-21) in the regulation of forkhead box P3 (Foxp3) expression in Tregs. We aimed to determine whether miR-21 transfection to naive CD4+ T cells can be useful in generation of iTregs in-vitro.

We investigated in-vitro differentiation of miR-21-transfected naive CD4+ T cells to iTregs and compared these iTregs to cytokine-differentiated iTregs and control group.

We showed that expression of Foxp3, transforming growth factor beta (TGF-β), and interleukin-10 (IL-10) are increased in iTregs generated after miR-21 transfection in comparison with cytokine-differentiated iTregs and control group.

Our findings demonstrate that miR-21 has positive role in in-vitro generation of induced regulatory T-cells (iTregs).

Keywords: Foxp3; Mir-21, In-vitro differentiation; Naive CD4-positive T-lymphocytes; Regulatory T lymphocyte

INTRODUCTION

Regulatory T cells (Tregs) play an important role in maintaining of homeostasis, preventing autoimmune diseases, and restraining chronic inflammatory diseases.[1]

It is also believed that these cells stop the activation and spread of autoreactive T-cell clones that have escaped the negative selection in the thymus.[2] Capability to suppress the immune response made these cells as a potential tool that can be utilized in the clinic as a cellular therapy in different settings such as autoimmune diseases and graft rejection.[3] Recent data show that Tregs grow in the thymus during ontogeny and can be distinguished from naive T cells in the periphery.[4] De novo generation of Tregs from conventional effector cells indicates a useful approach to obtain sufficient and effective Tregs appropriate for

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immunotherapy. These cells can be created in-vivo and in-vitro from naive CD4+ T cells using different experimental conditions. In this regard, transforming growth factor beta (TGF-βand interleukin-2 (IL-2) are the most applied factors.[5,6] However, obtaining considerable number of Tregs with powerful and fixed suppressive ability from adult human peripheral blood appears to be difficult. Indeed, the major barriers include lack of specific markers for isolation/characterization, compromised function of isolated Treg populations, and difficulty to convert conventional T cells into Tregs in a consistent manner. Moreover, Tregs have a poor in-vitro expansion potential due to intrinsic anergic phenotype.[7] Although several markers are expressed, the main specific markers are forkhead box P3 (Foxp3), cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF-like receptor (GITR), and CD45RO.[8] Foxp3, as a master regulator of Tregs, play a crucial role in functioning of these cells.[9,10] In humans, Foxp3 mutations were shown to cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX).[11] Attempts have been made by several groups to produce Tregs by forced overexpression of Foxp3 in CD4+CD25-T cells.[12-16] The discovery of microRNAs (miRNAs) in recent years has revolutionized current cell biology and medical science. miRNAs are small (19-25 nucleotide) non-coding RNA molecules that post-transcriptionally regulate gene expression by targeting the 3′ untranslated region (3′ UTR) of specific messenger RNAs (mRNAs) for degradation or translation repression.[17] There is some convincing data indicating that these molecules are highly expressed in Tregs. Furthermore, the expression of Foxp3 is shown to be controled by miRNAs.[18-21] A recent study has demonstrated that non-cell-autonomous gene silencing mediated by miRNA-containing exosomes, is a mechanism employed by Treg cells to suppress T-cell-mediated disease. Treg cells transfer miRNA to various immune cells, suppress cell proliferation, and cytokine secretion.[22] In 2009, Redouane Rouas and his colleagues showed that miR-21 acted as a positive, though indirect, regulator of Foxp3 expression.[23] In line with these findings, in the current study, we aimed to investigate the effect of miR-21 on de-novo generation of CD4+ 25+ Foxp3+ Treg cells from CD4+CD25- conventional T cells, which can be applicable in regulatory T-cell manufacturing industry.

MATERIALS AND METHODS

CD4+ T-cell isolation:

The study was approved by the ethics committee (Ethical code No.911124) of Tehran University of Medical Sciences, and written informed consent was obtained from all subjects before taking blood. Venous blood was collected in ethylene diamine tetra acetic acid (EDTA, Sigma-Aldrich, USA**)** containing tubes from healthy donors. Mononuclear cells were isolated using lymphocyte separation medium (Lymphoprep, Stem Cell Technologies, Vancouver, Canada).

Cells were washed three times with phosphate buffer saline (PBS) and $CD4^+$ naive T cells were separated using human naive CD4+ Cell isolation kit (Miltenyibiotec, Germany) according to manufacturer's instruction briefly as follows: CD45RO+ activated/memory T cells and non-CD4+ T cells were magnetically labeled applying a cocktail of biotin-conjugated antibodies against CD8, CD14,CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56,CD123, TCRγ/δ, HLA-DR, CD235a (Glycophorine A), and anti-biotin micro-beads.

Cell Culture

Human naive CD4+ T cells were activated in vitro with 2μg/mL plate-bound anti-CD3, 1μg/mL soluble anti-CD28 antibodies, with or without 20ng/mL TGF-β (Life Technologies, USA),10 nmol/mL All-trans retinoic Acid (ATRA) (sigma Aldrich, USA), 300 Unit/mL, IL-2, (Life Technologies, USA) and 30 pmoles/mL miR-21 (Ambion–Applied Biosystems, USA) in 24-well culture plates at 10^6 cells per well for 6 days.

The culture medium x-vivo-15 (Invitrogen, USA) was used for all groups. All the treatments and transfection were done once for each group and after 6 days cells were harvested and used for later experiments.

Transfection of Pre-miR-21:

Purified naive CD4+ T cells were seeded in x-vivo-15 (culture medium without serum). Pre-miRNA-21(Ambion–Applied Biosystems, USA) was diluted in x-vivo-15 and transfection reagent (75μL) (Lipofectamine RNAiMAX reagent, USA) was added to the diluted nucleic acid (NA) (30 pmoles/mL at 75μL of x-vivo-15) to produce reagent–NA complexes. For evaluation of transfection efficiency, we also complexed a plasmid expressing green fluorescent

protein (GFP) to transfection reagent, and then the cells were co-transfected with reagent–NA complexes and reagent –plasmid complexes.

Monoclonal Antibodies (mAbs) and Flow cytometery

Naive CD4+ T cells were stained with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD25 antibodies (BD Biosciences, USA). For Foxp3 staining, the cells were fixed and permeabilized using a Fixation/Permeabilization kit according to manufacturer's protocol (leucoperm,Serotec, USA). Foxp3 was stained with PE-conjugated anti-Foxp3Ab (BD Biosciences, USA). For analysis of intracellular cytokine production, CD4+CD25+cells were restimulated on day 6 for 5 hours with 25 ng/mL phorbol 12-myristate 13-acetate (PMA) and 250 ng/mL ionomycin (Sigma Aldrich, USA),along with 3 µg/mL brefeldin A (eBioscience, USA). Afterward, the cells were fixed and permeabilized with an fixation/permeabilization kit (leucoperm,serotec, USA) and stained for IL-10 and TGFβ with anti-IL10-PE and anti-TGFβ-PE antibodies (BD Biosciences, USA) . In all experiments, cells were stained with isotype Control as well. FACScalibur was used for data acquisition, and the data were analyzed with FlowJo software (Tree Star.Inc., Ashland.OR, USA).

Quantitative Real-time PCR Analysis of Foxp3, TGF-β and IL-10 Expression Level

To quantify the expression of Foxp3, TGF-β and IL-10, total RNA was extracted from differentiated T cells using RNX-plus solution (Sinagen, Iran) according to the manufacturer's protocol and checked for quality by measuring OD260/280. All RNA extracts prepared were immediately used as templates for preparation of respective complementary DNA sequences (cDNA). Then cDNA was synthesized by reverse transcriptase using random hexamer in the presence of RNase inhibitor (Vivantis, Malaysia).

Real-time PCR was performed in a final volume of 20 µL containing 2 µL cDNA, 10 µL SYBR Premix Ex Taq II, 1 µL forward primer (10 pM), 1 µL reverse primer (10 pM) and 6 µL H2O. The primers used are indicated in Table 1. The real-time PCR was performed in a StepOne system (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: one cycle at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, IL-10 (60.2°C), TGFβ (60.2°C), FoxP3 (60.3°C) and β-actin (housekeeping gene: 60.2°C) for 18 s, and finally 72°C for 30 s. Fluorescent signal recorded in each cycle by instruments and data analysis were done using SPSS,V.20 (Abaus Concepts, Berkeley, CA, USA).

Relative gene expression levels were quantified using the comparative ΔC_T method as described by Applied Biosystems, USA. This method normalized C_T values of the target mRNA to the average of that of the housekeeping genes in each sample and calculated the relative expression values as relative fold changes (RFC) of the control. Primer designing for Foxp3, TGF-β, IL-10 and β-actin genes was carried out using Primer Express software (Version 3, Applied Biosystems, USA).

Statistical Analysis

All data were expressed as mean ± standard error of at least three independent experiments. Statistical significance was calculated based on paired student's t-test at significance level of $p<0.05$, using SPSS, version.20 (Abaus Concepts, Berkeley, CA, USA) and Prism 5 (Graph Phad, San Diego, CA, USA) softwares.

Table 1. Primers used for real-time PCR analysis for evaluation the effect of microrna-21 transfection on in-vitro differentiation of human naive cd4+ t cells to regulatory t cells

Genes	Primer	Sequence
Foxp3	R reverse	5′-GGCCACTTGCAGACACCAT-3′
	F forward	5′-GCACCTTCCCAAATCCCAGT-3′
TGF-β	R reverse	5′-GTCAATGTACAGCTGCCGCA-3′
	F forward	5′-CCCAGCATCTGCAAAGCTC-3′
IL-10	R reverse	5′-GTCTAGGTCCTGGAGTCCAGCAGACTC-3′
	F forward	5′-CCAGTTTTACCTGGTAGAAGTGATG-3′
β-actin	R reverse	5′-TCGAGGACGCCCTATCATGG-3′
	F forward	5′-GTCTGCCTTGGTAGTGGATAATG-3′

RESULTS

Induction of Foxp3 Expression

To analyze and compare both conventional cytokines and mir-21 in inducing human iTregs, naive CD4+ T cells were isolated from human peripheral blood and the purity of naive CD4+ T cells was confirmed with PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 antibodies (Figure.1A). Naive T cells were cultured in-vitro in the presence of T-cell receptor (TCR) and CD28 stimulation in serum-free medium for 6 days. Stimulated cells were used as $T_{control(con)}$ group. To induce iTregs, mir-21(30 pmoles/mL) and GFP were co-transfected either alone (T_{-21}) or together with IL-2 (300 Unit/mL), TGF-β(20ng/mL) and all trans retinoic acid (ATRA) (10 nmol/mL) ($T_{-cytokines/21}$). In some wells we added just ATRA, TGF-β plus IL-2 ($T_{-cytokines}$) to induce iTreg cells. During 6 days, the expression of GFP in transfected T cells was evaluated with an inverted phase-contrast microscope (Nikon, Melville, NY, USA). On day 2, almost 50% of T cells had expressed GFP protein (data not shown).

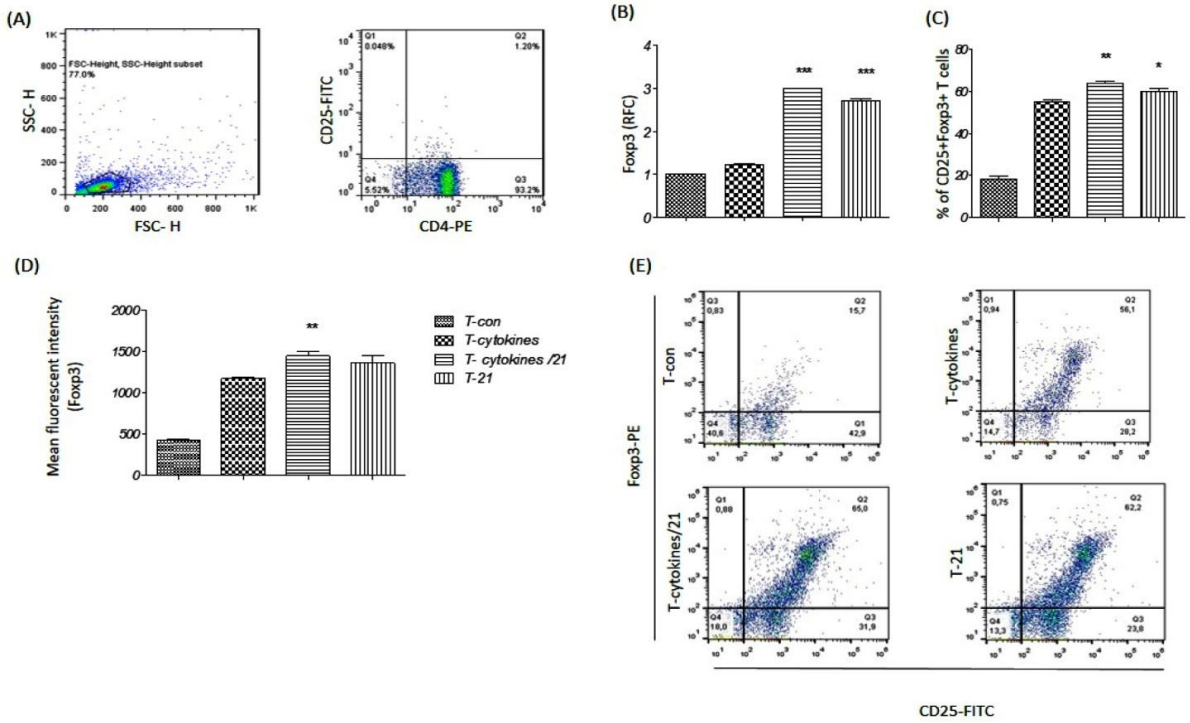

Figure 1. Induction of Forkhead Box P3 (FOXP3) in naive human CD4+ T cells:

Naive CD4+ T cells were isolated from human peripheral blood. (A) Isolation of highly pure naive CD4+ CD25- T cells were confirmed after immune staining with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD25 antibodies. Cells were stimulated by anti-CD3 and anti-CD28 antibodies and cultured in X-Vivo serum-free medium in four groups including: transfected with pre-miR-21 (T_{-21}), treated with IL-2, TGF-β and ATRA ($T_{-cytokines}$), IL-2, TGF-β, ATRA treatment plus pre-miR-21transfection ($T_{-cytokines/21}$). Cells stimulated with only CD3 and CD28 antibodies served as T_{-con}.

After 6 days: (B) Total RNA was prepared. Quantitative PCR was performed on all samples, and Foxp3 expression in 6-day polarization cultures was assessed.

(C) Cells were harvested and stained for Foxp3 and CD25 markers. A representative result of three experiments is shown.

(D) To obtain information about Foxp3 expression levels per cell, the Foxp3 MFI of CD25high Foxp3+ T cell was determined. Data above are representative of 3 independent experiments.

(E) Representative dot plot is one of the three independent experiments showing the expression of CD25 and FoxP3 markers. Statistical significance was calculated based on paired student's t-test. *p<0.05, **p<0.01; * <0.001**

RFC: relative fold change, MFI: median fluorescence intensity

Based on qRT-PCR results, Foxp3 gene expression was found to be induced in $T\text{-}_{cytokines}$, $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups compared to $T\text{-}_{con}$ group ($T\text{-}_{cytokines}$: 1.22±0.01 RFC, $T\text{-}_{cytokines/21}$: 3±0.005 RFC, $T\text{-}_{21}$: 2.7±0.05 RFC vs. $T\text{-}_{con}$: 1±0.00003 RFC, p<0.001).

As shown in Figure. 1B, Foxp3 mRNA expression was significantly increased in the $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups compared to $T\text{-}_{cytokines}$ group ($T\text{-}_{cytokines/21}$: 3±0.005 RFC, $T\text{-}_{21:}$ 2.7±0.05 RFC vs. $T\text{-}_{cytokines}$: 1.22±0.01 RFC, p<0.001).

To further validate these findings, flow cytometry was used to detect Foxp3+CD25+ T cells. All examined groups were observed to induce a high fraction of Foxp3 and CD25 expressing cells, which was significantly higher than $T\text{-}_{con}$ group, where a weak activation-induced Foxp3 expression was observed ($T\text{-}_{cytokines}$: 54.83±0.9%, $T\text{-}_{cytokines/21}$: 63.73±1.1%, $T\text{-}_{21}$: 60.17±1.1% vs. $T\text{-}_{con}$: 18.23±1.53 %, p<0.001).

As indicated in Figure. 1C, the percentage of Foxp3+CD25+ T cells in $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups in comparison with $T\text{-}_{cytokines}$ group was significantly different ($T\text{-}_{cytokines/21}$: 63.73±1.1% vs. $T\text{-}_{cytokines}$: 54.83±0.9 %, p<0.01 and $T\text{-}_{21}$: 60.17±1.1% vs. $T\text{-}_{cytokines}$:54.83±0.9 %, p<0.05).

Consistent to the Treg frequencies, in $Foxp3+CD25^{hi}$ cells, considerable differences were also observed in the median fluorescence intensity (MFI) of Foxp3 level in $T\text{-}_{cytokines}$, $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups in comparison to $T\text{-}_{con}$ group ($T\text{-}_{cytokines}$: 1170±15, $T\text{-}_{cytokines/21}$: 1443±53, $T\text{-}_{21}$: 1359±88 vs. $T\text{-}_{con}$: 430±10, p<0.001).

Foxp3 expression levels induced per cell in Foxp3+CD25hi cells of $T\text{-}_{cytokines/21}$ group were significantly high when compared to $T\text{-}_{cytokines}$ group ($T\text{-}_{cytokines/21}$: 1443±53 vs. $T\text{-}_{cytokines}$: 1170±15, p<0.01) (Figure.1D). Rather, the difference found in MFI of Foxp3 levels of $T\text{-}_{21}$ group did not reach statistically significance when compared to $T\text{-}_{cytokines}$ group ($T\text{-}_{21}$: 1359±88 vs. $T\text{-}_{cytokines}$: 1170±15).

MiR-21 Influences the Cytokine Production of CD4+CD25+Foxp3+T Cells

In order to identify the impact of miR-21 on the production of regulatory T cell cytokines, IL-10 and TGF-β levels were measured in cultured T cells. Looking at the TGF-βmRNA level by qRT-PCR, significantly increased mRNA level was observed in $T\text{-}_{cytokines,}$ $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups than $T\text{-}_{con}$ group ($T\text{-}_{cytokines}$: 13.24±0.6 RFC, $T\text{-}_{cytokines/21}$:19.35 ± 0.1 RFC, $T\text{-}_{21}$: 15.85±0.02 RFC vs. $T\text{-}_{con}$: 1±0.00003 RFC, p<0.001).

Among different groups, the level of TGF-β mRNA expression in $T\text{-}_{cytokines/\ 21}$ and $T\text{-}_{21}$ groups was higher than $T\text{-}_{cytokines}$ group ($T\text{-}_{cytokines/21}$: 19.35 RFC ± 0.1 vs $T\text{-}_{cytokines}$: 13.24±0.6, RFC, p<0.001 and $T\text{-}_{21}$: 15.85±0.02 RFC vs $T\text{-}_{cytokines}$: 13.24±0.6 %, RFC, p<0.05) (Figure. 2. A).

The frequency of TGF-β and CD25 positive cells in $T\text{-}_{cytokines,}$ $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups also showed a significant increase in comparison to $T\text{-}_{con}$ condition ($T\text{-}_{cytokines}$:49.53 ± 0.6%, $T\text{-}_{cytokines/21}$:65.33 ±2.6%, $T\text{-}_{21}$:63.27 ±1.3% vs $T\text{-}_{con}$: 17±1.2 %, p<0.001).

Interestingly, mir-21 either alone or together with cytokines enhanced TGF-βCD25+ T cells compared to cells that were treated with cytokines alone, and the difference reached statistically significant as shown in Figure 2B ($T\text{-}_{cytokines/21}$: 65.33±2.6% vs. $T\text{-}_{cytokines}$: 49.53±0.6%, p<0.01 and $T\text{-}_{21}$: 63.27±1.3% vs. $T\text{-}_{cytokines}$: 49.53 ± 0.6%, p<0.001).

Further looking at the MFI level of TGF-β, in $TGF\text{-}β+CD25^{hi}$ population, a significant difference was found between $T\text{-}_{cytokines}$, $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups compared to $T\text{-}_{con}$ group ($T\text{-}_{cytokines}$: 1147±5.7, $T\text{-}_{cytokines/21}$: 1591±69, $T\text{-}_{21}$: 1584±28 vs $T\text{-}_{con}$: 451±7.5, p<0.001).

As shown in Figure 2C, the expression level of TGF-β, displayed the same pattern as with the percentage of TGF-β+CD25+ T cells. mir-21 either alone or together with cytokines enhanced TGF-β MFI compared to cytokine alone ($T\text{-}_{21}$: 1584±28 vs $T\text{-}_{cytokines}$: 1147±5.7, p<0.001 and $T\text{-}_{cytokines/21}$: 1591±69 vs $T\text{-}_{cytokines}$: 1147 ± 5.7, p<0.01).

Compared to TGF-β, the level of IL-10 mRNA expression was significantly decreased in all three groups compared to $T\text{-}_{con}$ group ($T\text{-}_{cytokines}$: 0.23 ±0.01 RFC, $T\text{-}_{cytokines/21:}$0.35 ± 0.03 RFC, $T\text{-}_{21:}$0.31± 0.01 RFC vs $T\text{-}_{con}$: 1±0.00003 RFC, p<0.001). (Figure 3A). The percentage of IL-10+CD25+ T cells were also evaluated and we observed high frequency of IL-10+CD25+ T cells in $T\text{-}_{cytokines,}$ $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ than control group ($T\text{-}_{cytokines}$: 34.85±2.43%, $T\text{-}_{cytokines/21:}$39.15±1.1%, $T\text{-}_{21:}$35.80±1.22% vs. $T\text{-}_{con}$: 10.37 ± 0.04 %, p <0.001).

No significant differences were observed, when we compared $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups with $T\text{-}_{cytokines}$ group ($T\text{-}_{cytokines/21}$:39.15±1.1% and $T\text{-}_{21}$:35.80±1.22% vs. $T\text{-}_{cytokines}$: 34.85 ±2.43%) (Figure 3 B).

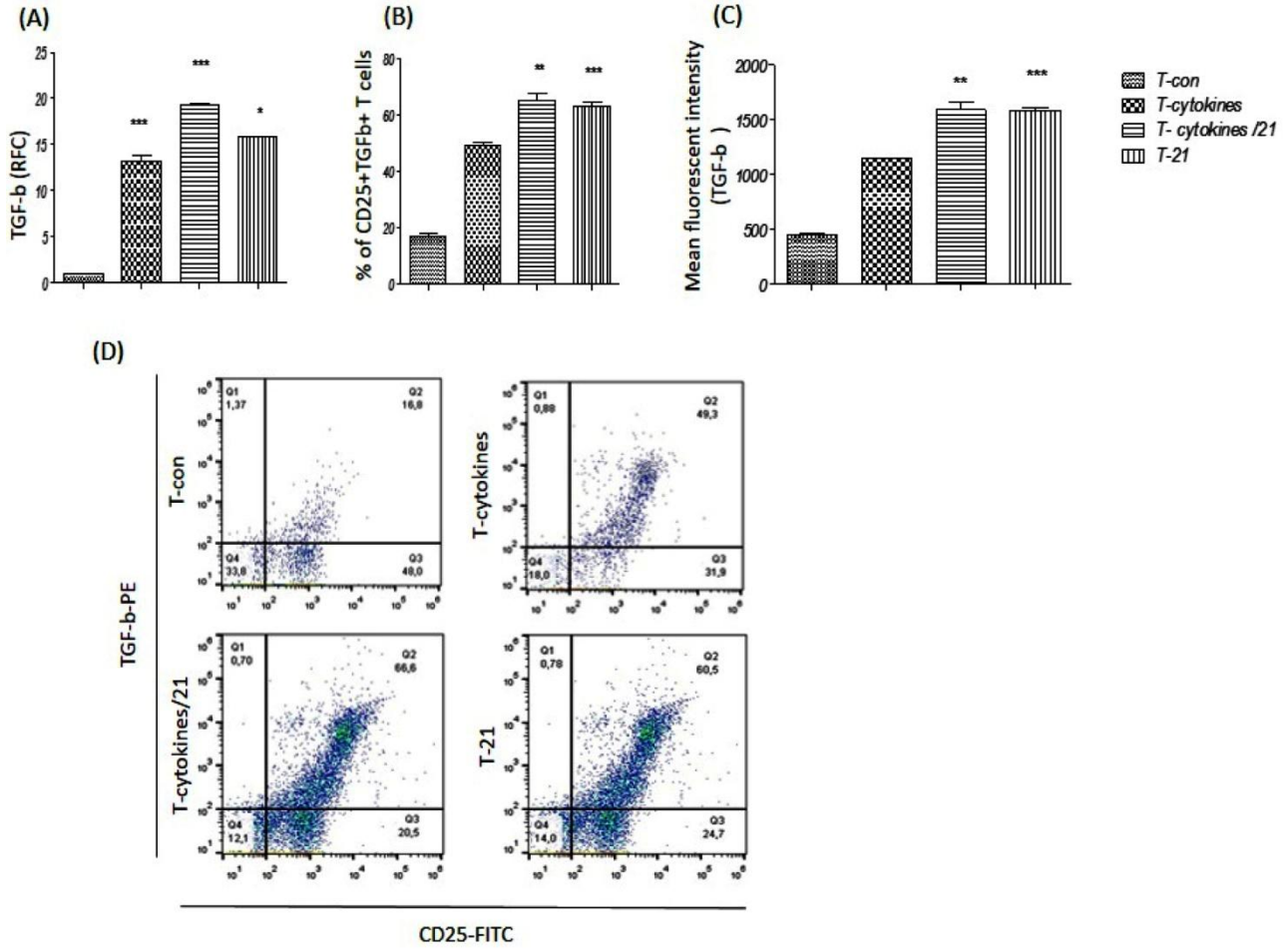

Figure 2. Increased TGF-β levels in miR-21-treated naive T cells:

Naive T cells were activated with anti-CD3 and anti-CD28 antibodies and were cultured in four conditions. Stimulated cells were considered as $T\text{-}_{con}$. Mir-21 was transfected to the cells either alone ($T\text{-}_{21}$) or together with all trans retinoic acid (ATRA), TGF-β plus IL-2 ($T\text{-}_{cytokines/21}$). In the last group, we added just ATRA, TGF-β plus IL-2 ($T\text{-}_{cytokines}$).

After 6 -day incubation:

(A) Total RNA was extracted from all the groups and relative levels of TGF-β in differentiated T cells were measured by real-time quantitative PCR.

(B) On the other hand, after stimulation of rested cells with PMA/ionomycin, and treatment with brefeldin A CD25 and TGF-β markers were stained.

C) Level of TGF-β as defined by median fluorescence intensity (MFI) in TGF-β+ CD25hi Tregs. Data above are representative of 3 independent experiments.

D) The results of dot plots showing the expression of CD25 and TGF-β markers are one representative experiment of three independent experiments.

Statistical significance was calculated based on paired student's t-test. *p<0.05, **p< 0.01; ***p<0.001

Furthermore, there were significant differences in the MFI levels of IL-10 in IL-10+$CD25^{hi}$ population between $T\text{-}_{cytokines}$, $T\text{-}_{cytokines/21}$ and $T\text{-}_{21}$ groups compared to control group ($T\text{-}_{cytokines}$: 1489±42, $T\text{-}_{cytokines/21}$: 1525±17, $T\text{-}_{21}$:1485±30 vs. $T\text{-}_{con}$: 338±5.2 %, p<0.001); however, no significant difference was found between groups ($T\text{-}_{cytokines/21}$: 1525±17 and $T\text{-}_{21}$: 1485±30 vs. $T\text{-}_{cytokines}$: 1489 ±42) (Figure 3 C).

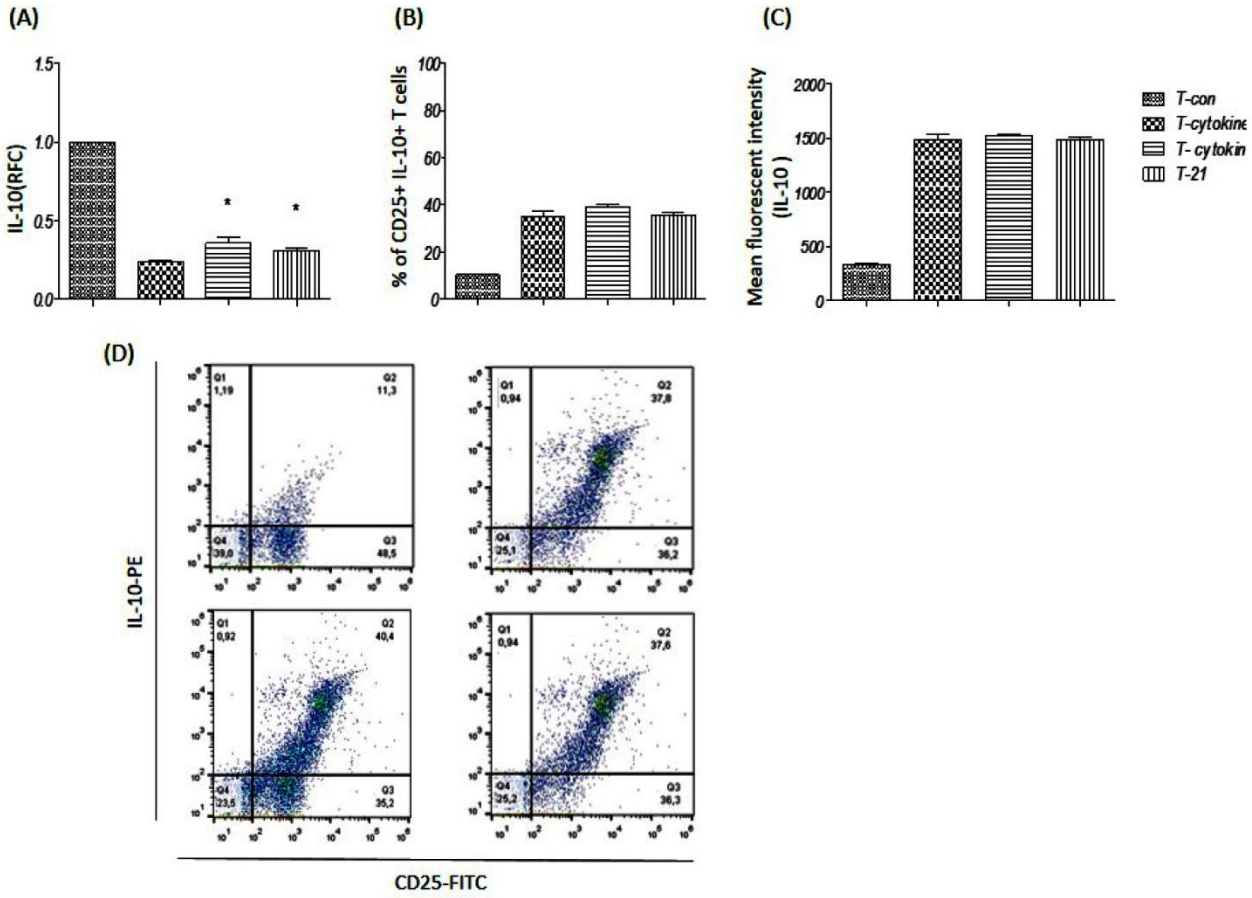

Figure 3. miR-21 induces IL-10 in naive T cells:

Naive CD4+ CD25- T cells were stimulated for 6 d in the presence of anti-CD3 and anti-CD28 antibodies and were transfected with mir-21 alone (T_{-21}) or together with IL-2, TGF-βand all trans retinoic acid (ATRA)($T_{-cytokines/21}$). Some cells just treated with IL-2, TGF-βand ATRA ($T_{-cytokines}$). Cells stimulated with only CD3 and CD28 antibodies considered as T_{-con}.

(A) Total RNA was extracted and the relative expression levels of IL-10 mRNA were determined by quantitative RT-PCR analysis.

(B) In parallel cultures (under the same conditions as described above), after stimulation with phorbol myristate acetate (PMA)/ionomycin, and brefeldin A treatment, cells were harvested and flow cytometry analysis of IL-10 and CD25 expression was performed. Data are representative of at least three independent experiments.

(C) Expression levels based on MFI of IL-10 from IL-10+CD25hi T-cells are shown and compared among the studied groups. Data are representative of 3 independent experiments.

(D) Flow cytometry analysis of IL-10 and CD25 expression. Data show representative dot plots of one out of three independent experiments. Statistical significance was calculated based on paired student's t-test

***p<0.05; **p<0.01; ***p<0.001**

DISCUSSION

Several studies have indicated that defects in the number and function of Treg cells can result in autoimmune diseases.[24-26] miRNAs are shown to change the functions of lymphocytes in particular Tregs., and the role of miRNAs in autoimmune diseases has recently been highlighted by several studies.[27-29]

Cobb et al in 2006 reported that miRNAs expression pattern varies from Tregs to TCD4+ cells. Furthermore, studies indicated that Dicer (the RNase

that is an integral part of miRNA modulation) knockout mice develop severe autoimmune disease, bearing close resemblance to phenotype in mice lacking Tregs or with Foxp3 deficient Treg cells.[29,30]

It has also been revealed that Tregs lacking Dicer in mice lost their suppressive activity in-vivo, showing fatal systemic autoimmune disease identical to the Foxp3 deficient mice. These results indicate that miRNAs can play a pivotal role in maintaining the stability of Tregs and immune system homeostasis.[31,32]

In 2009, Redouane Rouas showed that miR-21 was over-expressed in Treg cells.[23] Moreover, the level of miR-21 together with Foxp3 mRNA levels have been shown to be lower in rheumatoid Arthritis (RA) patients.[33] These findings indicate that miR-21 may expand Foxp3 expression and, therefore, can be regarded as positive regulator of Foxp3.

In line with this view, the present study examined the impact of miR-21 in differentiation of naive T cells to Tregs. Consistent to previous findings, miR-21 induced the Foxp3 expression at both mRNA and protein levels.[23]

Yao Q et al have shown that TGF-β can increase the miR-21 levels in myofibroblast cells.[34] Treatment of 5637 bladder cancer cell line with TGF-β recombinant protein has been also found to cause a significant upregulation of miR-21.[35] In addition, mir-21 was shown to enhance the TGF-β production in EL4 cell line.[36] In a study conducted in breast cancer, a direct correlation has been reported between miR-21 and TGF-β level.[37] These findings suggest a reciprocal effect between TGF-β and miR-21 as documented in our study as well. Our data also suggest that cytokines plus miR-21 can lead to more TGF-β production than using miR-21 alone and cytokine alone.

Looking at the protein levels of IL-10, we found the high level of this cytokine in all three groups compared to control group. Similar to the previous studies,[38-40] miR-21 was found to upregulate the expression of IL-10 although further functional studies are yet to be performed to unveil the mechanisms behind. In spite of elevated protein levels, the gene expression of IL-10 was lower than control group. In this regard, it has been shown that changes in protein level does not necessarily follow the same mRNA expression pattern.[41] Different post-transcriptional mechanisms and various half-lives may also influence the inconsistent patterns seen between protein and mRNA levels.[42,43]

In conclusion, our data indicate that miR-21 may serve as a positive regulator of Tregs, and may provide a new therapeutic target in immune complex disorders such as autoimmune diseases.

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