

Altered expression and editing of miRNA-100 regulates iTreg differentiation

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ABSTRACT

RNA editing of miRNAs, especially in the seed region, adds another layer to miRNA mediated gene regulation which can modify its targets, altering cellular signaling involved in important processes such as differentiation. In this study, we have explored the role of miRNA editing in CD4⁺ T cell differentiation. CD4⁺ T cells are an integral component of the adaptive immune system. Naïve CD4⁺ T cells, on encountering an antigen, get differentiated either into inflammatory subtypes like Th1, Th2 or Th17, or into immunosuppressive subtype Treg, depending on the cytokine milieu. We found C-to-U editing at fifth position of mature miR-100, specifically in Treg. The C-to-U editing of miR-100 is functionally associated with at least one biologically relevant target change, from MTOR to SMAD2. Treg cell polarization by TGFβ1 was reduced by both edited and unedited miR-100 mimics, but percentage of Treg in PBMCs was only reduced by edited miR-100 mimics, suggesting a model in which de-repression of MTOR due to loss of unedited miR-100, promotes tolerogenic signaling, while gain of edited miR-100 represses SMAD2, thereby limiting the Treg. Such delicately counterbalanced systems are a hallmark of immune plasticity and we propose that miR-100 editing is a novel mechanism toward this end.

INTRODUCTION

CD4⁺ T cells are important players of the adaptive immune response; they are helper T cells that provide help to B-cells to generate antibody response, to CD8⁺ T cells to regulate its cytotoxic response and so on. Naïve CD4⁺ T cells upon encountering an antigen, get differentiated into different subsets, depending on the antigen and cytokine milieu

generated by cells of the innate immune system (1). They get differentiated to Th1, in response to intracellular pathogens, into Th2 in response to helminthes infection, and into Th17 against extracellular bacteria and fungi (2). In addition, to these inflammatory effector subsets, it has been found that naïve CD4⁺ T cells can also differentiate into iTreg in the periphery; iTreg (induced regulatory T cells) are immunosuppressive T-helper subset, similar in function to natural Treg (nTreg) generated in the thymus. There are other T-helper subsets like Th9, Th22, Tfh (follicular helper), etc. which are being defined based on the distinct combination of cytokines they secrete (1). Moreover, various reports have suggested that these T-helper subsets also retain the ability to convert into other subsets depending on the cytokine environment (3). This trans-differentiation of Th subsets is important in a disease scenario, where conversion of one subset to another could rescue or exacerbate the disease condition, depending on the subset being formed. For example, conversion of an inflammatory subset to an immunosuppressive one, can subdue an auto-immune like disease condition or vice-versa can be useful in an immune-compromised condition such as in cancer, where inflammatory cells are needed to combat the infection. The inter-conversion of these subsets has been reviewed in peer articles (3,4). Among all the subsets, Treg is considered to be the most flexible one, with the ability to convert to any subset depending on their cytokine milieu. Thus, in order to overcome any infection/pathogen, balance of inflammatory CD4⁺ T cells and immunosuppressive Treg is very crucial.

MicroRNAs (miRNAs) are 20–22 nt long non-coding RNAs that play an important role in the fine tuning of gene expression. miRNAs bind to 3'UTR of the mRNAs and interfere with the gene expression by either degrading the mRNA or inhibiting translation (5). In some cases, they can also increase the gene expression by interacting with RNA binding proteins like HuR, FXR, etc. or by binding to 5'UTR of the gene and activating translation (6). miRNAs have been known to be crucial mediators in regulating T cell

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activation and T cell effector differentiation and function (7). Editing of miRNAs adds another layer to the regulation of miRNA function, with or without change in its expression. miRNA editing refers to the change in the sequence of miRNA without any change in the genome information, as a post-transcriptional processing. There are two known enzymes responsible for canonical miRNA editing; ADAR (adenosine deaminase acting on RNA) causes adenosine (A) to inosine (I) editing and APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide like) that deaminates cytosine (C) to uracil (U). These two editing events are well studied (8). ADAR1 and APOBEC1/3 expression has also been previously shown in CD4⁺ T cells (9,10). A-to-I editing generally occurs on the dsRNA structure formed in the primary (pri)-miRNAs. Depending on the position of editing, it could alter the processing of the given miRNA, thereby affecting the miRNA expression (11,12). In some cases, miRNA editing occurs in seed region of the mature miRNA, which may not affect its expression level but could change its target choice and binding efficiency. Kawahara *et al.* have shown that the brain specific editing of miR-376a-5p in its seed region alters its targets. The edited miR-376a-5p regulates uric acid level in the brain by repressing phosphoribosyl pyrophosphate synthetase 1 which is involved in uric acid synthesis pathway (13). Later, Choudhury *et al.* have shown that a decrease in the level of editing of miR-376a-5p can increase invasiveness and migration of glioblastoma cells and is therefore associated with high grade gliomas (14). miRNA editing thus plays an important role in altering any disease condition by fine tuning the gene expression.

miRNAs being smaller in size, easy to deliver *in vivo* and natural components of gene regulation, can be an important therapeutic tool (15). Studies of miRNAs in T cell differentiation are mainly focused on differentially expressed or unique miRNAs present in a particular subset. To our knowledge, this is the first report focusing on the role of miRNA editing in CD4⁺ T cell differentiation and maintenance. In this study, we have performed miRNA profiling in the *in vitro* differentiated CD4⁺ T cell subsets using next generation sequencing. miRNA profiling data were further analyzed for miRNAs editing. Among various canonically edited miRNAs, miR-100 was found to be edited from C-to-U in its seed region, specifically in Treg as compared to other subsets. The editing alters the miR-100 targets, which further affect Treg differentiation and lineage maintenance. Thus, C-to-U editing in the miR-100 may represent a counterbalancing switch for fine-tuning Treg lineage commitment.

MATERIALS AND METHODS

Sample collection

Cord blood was obtained from St. Stephan's hospital, Delhi, India. A formal ethical clearance certificate, to collect the human cord sample, was obtained from the hospital ethics committee. The cord blood was collected from healthy individuals during both normal and caesarian deliveries. PBMCs (peripheral blood mononuclear cells) were isolated from healthy individuals and cultured in RPMI

with 10% FBS, 1 µg/ml of PHA (Sigma Aldrich) and 20 ng/ml of rhIL-2 (R&D Systems).

Naïve CD4⁺ T isolation and differentiation

The cord blood mononuclear cells (CBMCs) and PBMCs were isolated by layering whole blood on histopaque (Sigma Aldrich) containing tubes, in 1:1 ratio. Tubes were centrifuged at 400g for 30 min and the buffy coat formed at intersection of plasma (top layer) and histopaque (middle layer) was collected. CD4⁺ naïve T cells were isolated from CBMCs using Miltenyi human CD4⁺ naïve T cell isolation kit as per manufacturer's instructions and percentage purity was confirmed by flow cytometry as described below. These CD4⁺ naïve T cells were cultured overnight in X-vivo-15 media (Lonza) with 10% fetal bovine serum (FBS), 1 µg/ml PHA and 20 ng/ml rIL2 (R&D systems); in case of Th17, FBS was not added. The naïve CD4⁺ T cells were polarized in above media with 1 µg/ml soluble anti-CD3 (Sigma Aldrich), 1 µg/ml anti-CD28 (Sigma Aldrich) and in addition other recombinants and antibodies were added specific to the subset being polarized as follows: Th1, 5ng/ml rhIL-12, 1 µg/ml anti-human IL4; Th2, 20 ng/ml rhIL4, anti-human 1 µg/ml IFNγ; Th17, 10ng/ml each of rhIL6, rhIL-1β, rhIL21, rhIL23, rhTNFα, 1ng/ml rhTGFβ1, 1 µg/ml anti-human IL4, 1 µg/ml anti-human IFNγ; Treg, 1ng/ml rhTGFβ1. All the recombinants and antibodies were purchased from BD Biosciences.

Flow cytometry

All the polarized subsets were induced with 20 ng/ml PMA (Sigma Aldrich), 1 µM ionomycin (Sigma Aldrich) and monensin (BD Biosciences) for 4 h after 6 days. A fraction of cells was preserved in Trizol for RNA isolation and other set was used for flow cytometry staining. Cell surface staining was done with anti-human CD4-APC (BD), anti-human CD45RA-FITC (BD) for naïve CD4⁺ T cells. The cells were later permeabilized and intracellular staining was done for anti-human IL4-PE-Cy7 (eBioscience), anti-human IFNγ-Alexa Fluor 488 (BD), anti-human IL17A-PE (eBioscience), anti-human IL17F-Alexa Fluor 647 (eBioscience), anti-human Foxp3-FITC (BD). Flow cytometry was performed on BD FACS calibur and data were analyzed using CellQuest Pro and FlowJo software.

RNA isolation and sequencing

RNA was isolated using Trizol (Life Technologies) according to manufacturer's instructions. Quality of RNA was checked on Bioanalyzer and those with RNA integration number (RIN) above 8 were sent for miRNA profiling. Numbers of biological replicates sent for miRNA sequencing were 3 for Tnaive, Th2, Th17, Treg each and 2 for Th1. miRNA library preparation and sequencing was outsourced to Ocimum Biosolutions, Hyderabad, India. Library preparation was done using TruSeq small RNA sample preparation kit and sequencing was done on Illumina HiSeq 2000 with single end read length of 50bp.

The editing site was validated using ABI SNaPshot multiplex kit as per manufacturer's instructions using reverse

primer for pre-mir-100. Briefly, the pre-mir-100 was amplified and later it was used as template to enrich the edited mir-100 using forward primer whose 3' end was specific for the edited nucleotide in mir-100. The polymerase chain reaction (PCR) product obtained was purified using ExoSAP method and genotyped using ABI SNaPshot multiplex reagents.

miRNA editing analysis pipeline

miRNA editing analysis was done using publicly available pipeline (16), briefly described in Supplementary Figure S1. The single nucleotide polymorphisms (SNP) were filtered by using dbSNP build 138. Among the edited miRNAs, only those miRNAs were studied whose total read count was greater than or equal to 10.

In silico target prediction

In silico target prediction for unedited and edited hsa-miR-100 was done using Target Scan Custom (Release 5.2, June 2011) by putting 7-mer seed sequence from 2 to 8 nucleotides as input (17).

Cloning of MTOR and SMAD2 3'UTRs

The complete 3'UTR (914 nt) of *MTOR* and first 600bp of *SMAD2* 3'UTR (8720 nt), were cloned in *XhoI-NotI* site of psiCHECK-2 vector (Promega). List of primers is given in Supplementary Table S1.

Transfection and luciferase assay

250ng of *MTOR* and *SMAD2* 3'UTR psiCHECK-2 plasmids were transfected with 40nM of scrambled control, unedited or edited miR-100 mimics in HeLa cell line, using Lipofectamine 2000 as per manufacturer's instructions. Luciferase assay was done after 24 h of transfection using Dual-luciferase reporter assay system (Promega) as per manufacturer's instruction (18). To determine the effect of unedited and edited miR-100 on *MTOR* and *SMAD2* RNA and protein levels, 200 nM of both unedited and edited miR-100 mimics were transfected in HeLa cell line. The RNA or total protein extract were prepared 24 h post-transfection. The mimics were purchased from Dharmacon, Thermo Scientific.

CD4⁺ naïve T cells were activated overnight with PHA and rhIL2 and were transfected next day with 500 nM of scrambled control, unedited miR-100 or edited-miR-100 mimics as day 1. The cells were re-transfected on day 4 of Treg polarization and analyzed on day 5 by flow cytometer as done by Simpson *et al.* (19). The transfection was done using Lipofectamine 2000 (Life Technologies) in complete media according to manufacturer's instructions. PBMCs were also activated overnight with PHA and rhIL2 and then transfected similarly and analyzed after 48 hours.

Quantitation by real time PCR

RNA from cell line was isolated using RNeasy Plus mini kit (Qiagen). RNA was quantified on NanoDrop 1000

(Thermo Scientific). cDNA was prepared from 2 µg of RNA using ABI high capacity cDNA synthesis kit. Real-time PCR was done using SYBR FAST qPCR master mix (Kapa) as per manufacturer's instruction on LightCycler 480 II system (Roche). The relative transcripts were calculated using comparative threshold cycle (Ct) method. The primers sequences are given in Supplementary Table S1.

Western blot

Total protein extract was prepared and run on 8% SDS-PAGE, PVDF membrane as described in (20). The membrane was probed with following antibodies: MTOR (1:1000, Cell Signaling), SMAD2 (1:2000, Cell Signaling) and β-actin (1:2000, Sigma Aldrich). The proteins were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer's instruction. Densitometry was done using AlphaEaseFC software.

Mathematical modeling

Differential equation model of CD4⁺ T cell proliferation and differentiation was made. It was assumed that the rate of T cell proliferation is higher than the rate of differentiation (21). In the absence of sufficient evidences it was further assumed that the effect of unedited and edited miR-100 on the kinetics of T cell proliferation and differentiation respectively is same. The differential equations were modeled for three different scenarios as described in Figure 4. All the simulations were performed using Berkley Madonna (22). Runge–Kutta 4 method was used to perform the iterations. The simulation time was set to 1000 time units. The data were recorded in intervals of 0.02 units.

We obtained the following differential equations:

$$\frac{d(T_p)}{dt} = \frac{k}{miR} T_p - kd_1 * T_p$$

$$\frac{d(T_d)}{dt} = \frac{k}{c * emiR} T_p - kd_2 * T_d$$

T_p and T_d are the number of T cells and Treg cells at time t . Their initial count was taken as 100 and zero units, respectively. The rate constant for T cell proliferation (k) was calculated to be 0.05775 a.u taking generation time as 12 h. The dead rate k_{d1} and k_{d2} were estimated by multiple simulations. The amount of unedited miR-100 (miR) and edited miR-100 (emiR) was estimated the same way. The constant c was introduced to take into account the lag between T-cell proliferation and differentiation. The estimated values for each scenario are given in the table.

Conditions	T_p	T_d	K_{d1}	K_{d2}	miR	emiR	c
1	100	0	$10^{-7} * T_p$	$5 * 10^{-8}$	1	1	10
2	100	0	$10^{-7} * T_p$	$5 * 10^{-8}$	1	3.7	10
3	100	0	$10^{-7} * T_p$	$5 * 10^{-8}$	3.7	1	10

RESULTS

Naïve CD4⁺ T cells were polarized *in vitro* into different subsets

To obtain naïve CD4⁺ T cells, cord blood was used, as it is a rich source of immature monocytes and lymphocytes. Naïve CD4⁺ T cells were obtained as described in Materials and Methods. The percentage recovery of naïve CD4⁺ T cells was determined by estimating CD4⁺ CD45RA⁺ T cells by flow cytometry (Figure 1A). The cells obtained were found to be $\geq 97\%$ double positive. Further, naïve CD4⁺ T cells were activated overnight and next day, were either terminated as Th0 or polarized into different subsets namely Th1, Th2, Th17 and Treg using various cytokines, as per the schematic presentation (Figure 1B). The percentage polarization of each subset was validated by flow cytometry at the end of 6 days, before isolating their RNA. The flow cytometry of the representative subsets are shown in Figure 1C. The polarized Th1 were found to be majorly expressing its signature cytokine, i.e. IFN γ ; Th2, IL4; Th17, IL17A and IL17F; and lastly Treg expressed Foxp3. Thus, the CD4⁺ T subsets were successfully polarized. Later, the total RNA was isolated from polarized and Th0 cells and sequenced for small RNA.

miR-100 was found to be specifically edited in Treg

The small RNA sequencing data were analyzed to determine the miRNAs that were edited in each subset. The analysis was done as described in Materials and Methods. We found approximately 2% of the total miRNAs to be edited in most of the subsets (Supplementary Figure S2). We found many non-canonical editing sites, but since their mechanism is not known, we confined this analysis to the canonical editing only (Supplementary Table S2). The percentage of canonical edited sites i.e A-to-I and C-to-U, among the total miRNAs edited, is represented in Figure 2A and B, respectively. Further, we screened for canonical edited miRNAs in their seed sequence, as it can affect its target binding ability and subsequently the signaling pathway in which the target is involved. The percentage of A-to-I and C-to-U edited miRNAs in the seed region in all the subsets is shown in Figure 2C and D, respectively.

Further, we focused on miRNAs with canonical editing in the seed sequence and shortlisted only those where the same event was found in at least two out of three biological replicates for each sample. Among them, four miRNAs were observed to be differentially edited namely, miR-100 (C-to-U), miR-411, miR-381 and miR-589 (A-to-I) (Figure 2E). Among the differentiated subsets, only miR-100 was edited specifically in Treg and thus was selected for further studies.

miR-100 editing in Treg was also validated by SNaP-shot primer extension method as discussed in Materials and Methods. The edited peak was observed at a higher level in Treg as compared to Th0 (Supplementary Figure S3). The edited site was also detectable in Th0, probably due to some non-specific amplification and/or presence of small level of editing in Th0 which was not detectable by our experimental and analysis pipeline.

C-to-U editing in miR-100 changes its target from *MTOR* to *SMAD2*

Target prediction for unedited and edited miR-100 was done using Target Scan Human Custom. We found no overlap among the targets predicted for unedited and edited miR-100, indicating that a single base change in the seed sequence can dramatically affect its target binding ability (Figure 3A). Among the predicted targets, we focused on those genes that are involved in CD4⁺ T cell biology. *MTOR* was found to be a validated target for unedited miR-100 (23) and *SMAD2* was the predicted target of edited miR-100, involved in CD4⁺ T cell differentiation and function. The complementarity binding of both the unedited and edited miR-100 to their respective targets is shown in Figure 3B. The 3'UTR of *MTOR* and *SMAD2* were cloned in psiCHECK-2 plasmid in 3'UTR of *Renilla* luciferase and were transfected along with unedited and edited miR-100 mimics in easily transfectable HeLa cell line to validate the binding. The unedited miR-100 was found to target *MTOR* but not *SMAD2*, while on editing, miR-100 could bind to *SMAD2* and not *MTOR* 3'UTR as depicted by their relative luciferase levels (Figure 3C). Further, unedited and edited miR-100 mimics were transfected in HeLa cells to determine the effect on *MTOR* and *SMAD2* RNA and protein, by real-time PCR and western blot, respectively. The transfection of unedited and edited miR-100 was validated by measuring their expression at the RNA level (Supplementary Figure S4). The expression of *SMAD2* RNA was low in edited miR-100 mimics transfected cells as compared to scrambled control (Figure 3D), whereas there was no effect on *MTOR* transcript in any condition. However, at the protein level, MTOR was decreased upon transfection of unedited miR-100 mimics and the transfection of edited miR-100 mimics led to a decrease in the expression of SMAD2 (Figure 3E and F). This suggests that unedited miR-100 inhibits the *MTOR* gene expression by blocking translation, without affecting the mRNA whereas edited miR-100 degrades the *SMAD2* mRNA. Thus, unedited miR-100 decreases MTOR expression but not SMAD2 expression, which gets reversed when C-to-U editing occurs in its seed region. In conclusion, editing of miR-100 in its seed region at position 5 of mature miRNA, changes its target from *MTOR* to *SMAD2*.

Unedited and edited miR-100 regulate Treg differentiation

Since MTOR and SMAD2 are both important in Treg formation, we simulated the effects of miR-100 in a hypothetical model where naïve T cells proliferate through activation of MTOR but need activation of SMAD2, the effector of TGF β 1, to commit to a Treg lineage. Thus, we divided CD4⁺ T cell differentiation in two phases: proliferative phase and differentiation phase. The change in both proliferation and differentiation due to change in the level of unedited and edited miR-100 was derived mathematically as shown in Figure 4A. The levels of unedited and edited miR-100 were simplistically modeled as either low or high in three different scenarios, where Tp and Td are number of CD4⁺ T cells and Tregs, respectively. In first condition where both unedited and edited miR-100 were low, Tp-1

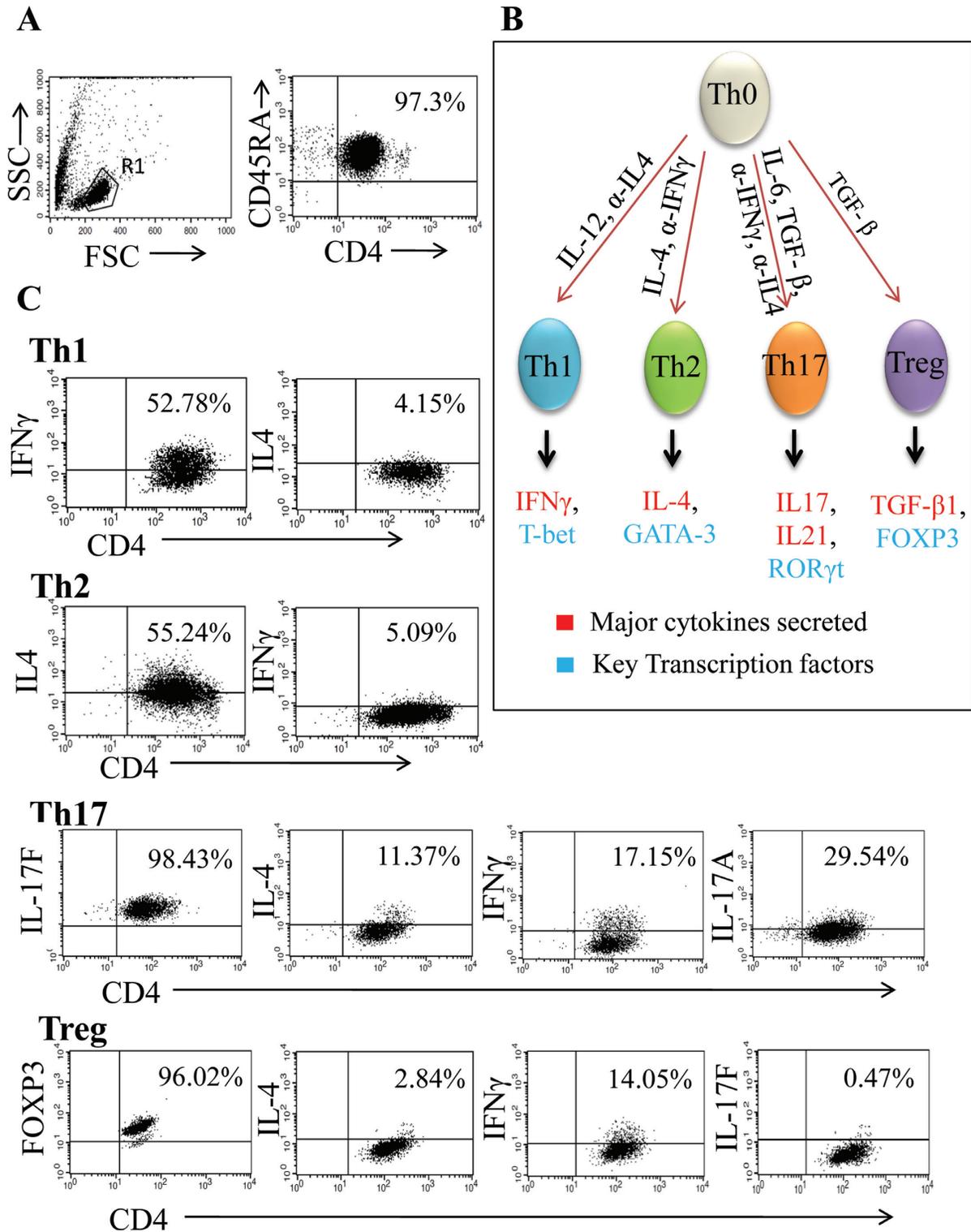


Figure 1. CD4⁺ T cell polarization into Th1, Th2, Th17 and Treg subsets. (A) CD4⁺ T naïve were isolated from cord blood and validated by analyzing naïve T cell marker, i.e. CD4⁺ CD45RA⁺ by flow cytometry. (B) Schematic showing the polarization of CD4⁺ T naïve into different subsets using specific polarizing media and detected by the expression of exclusive cytokines and transcription factors. (C) Percentage polarization of representative Th1, Th2, Th17 and Treg as determined by flow cytometry at the end of sixth day. The cells were gated on CD4⁺ T cells.

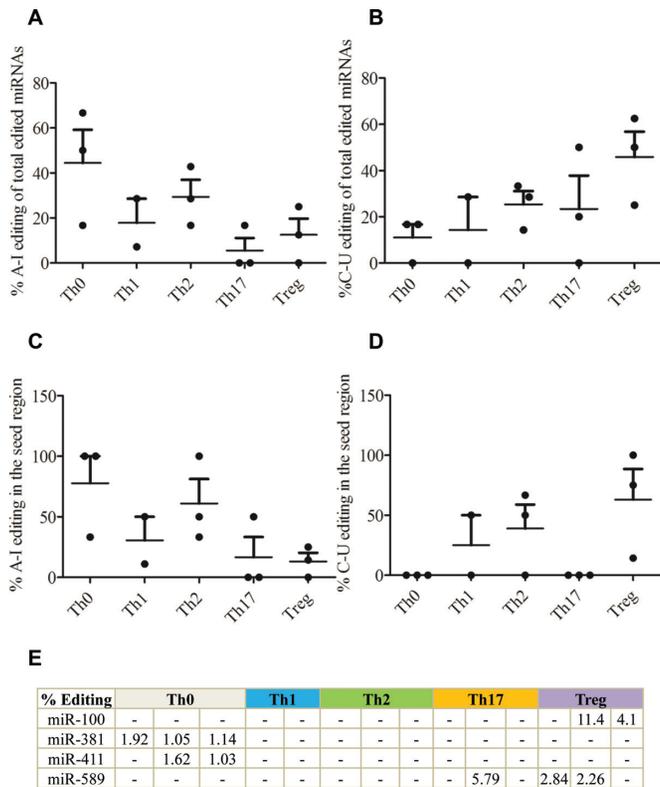


Figure 2. miRNAs edited among different CD4⁺ T cell subsets. (A) Percentage of A-to-I editing among the edited miRNAs in different CD4⁺ T cells. (B) Percentage of C-to-U editing among the edited miRNAs in different CD4⁺ T cells. (C) Percentage of A-to-I editing in the seed region among different CD4⁺ T cells. (D) Percentage of C-to-U editing in seed region among different CD4⁺ T cells. (E) List of miRNAs undergoing A-to-I or C-to-U editing in the seed region of the mature miRNAs.

increases exponentially and so does Td-1 after a few divisions; in second condition where unedited miR-100 was low and edited miR-100 was high, Tp-2 remains same but Td-2 decreases. Lastly, in third condition where unedited miR-100 was high, Tp-3 decreases and so does Td-3, irrespective of the level of edited miR-100. This model can be considered to have two brake points - suppression of MTOR by unedited miR-100, blocking expansion of uncommitted T cells and suppression of SMAD2 by edited miR-100, blocking Treg commitment. The model explains why large reductions in miR-100 would be necessary for lineage commitment, as seen by us (Supplementary Figure S5). In confirmation, transfection of unedited miR-100 mimics at day 1 and day 4 of Treg polarization led to reduced fractions of CD4⁺ Foxp3⁺ Treg cells as compared to scrambled control (Figure 4B). The edited miR-100 led to a greater reduction in Treg formation than unedited miR-100. The transfection efficiency in primary T cell culture was found to be around 95% (Supplementary Figure S6). In differentiated T cells, such as PBMC cultured in proliferating conditions, edited but not unedited miR-100 decreased the Treg fraction (Figure 4C), which is consistent with SMAD2 as the effector for lineage commitment. Moreover, expression of pSMAD2/3 was found to be significantly decreased in PBMCs transfected with edited miR-100 mimics but not in

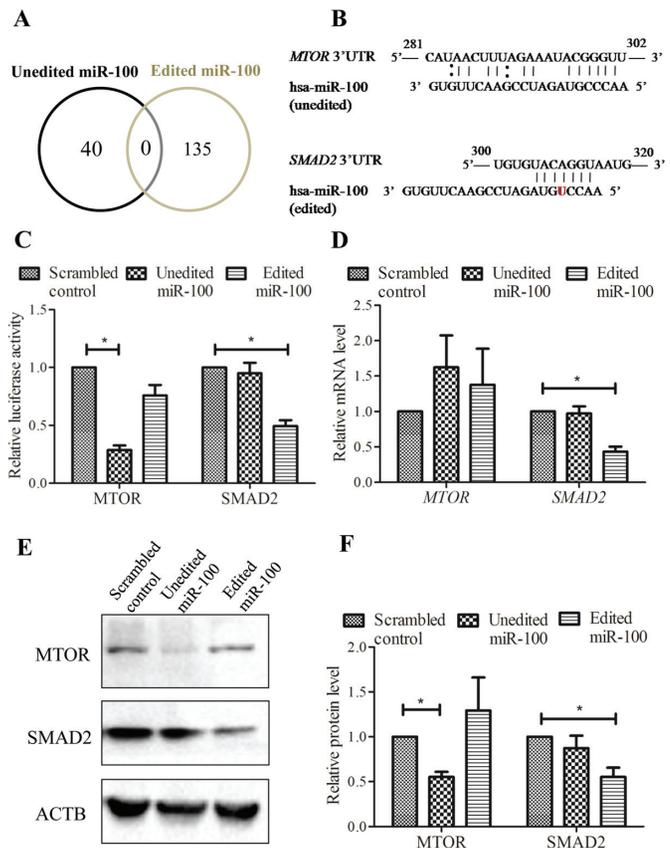


Figure 3. C-to-U editing in miR-100 changes its target from *MTOR* to *SMAD2*. (A) Venn diagram showing the number of predicted targets of unedited and edited miR-100 using Target Scan Custom. (B) Complementarity binding of *MTOR* 3'UTR and *SMAD2* 3'UTR to unedited and edited miR-100, respectively. (C) Relative *Renilla* luciferase activity of *MTOR* or *SMAD2* 3'UTR luciferase construct co-transfected with scrambled or unedited or edited miR-100 mimics normalized to *Firefly* luciferase activity. (D) Relative level of *MTOR* and *SMAD2* transcripts after 24 h of transfection with scrambled or unedited or edited miR-100 mimics, normalized by *GAPDH*. (E) Representative western blot for MTOR, SMAD2 and ACTB protein levels after 24 h of transfection with scrambled, unedited and edited miR-100 mimics. (F) Densitometry analysis of the western blot in (D) was done using AlphaEase FC. Data are representative of four independent experiments, **P* < 0.01, using two tailed *t*-test.

unedited miR-100 mimics transfected cells (Figure 4D), indicating the downregulation of TGFβ signaling.

DISCUSSION

Editing of miRNA has recently been shown to regulate gene expression and thus could involve in cellular differentiation and function. In the present study, we intended to look for the role of miRNA editing in CD4⁺ T cell differentiation or function, as differentially expressed miRNAs are already known to regulate CD4⁺ T cell lineage maintenance (7). This is the first study that has explored the role of miRNA editing in CD4⁺ T cell biology. We found that the level of editing in CD4⁺ T cell subsets is very low as compared to that in the brain. Around 2% of total miRNAs were found to be edited in mostly all of the CD4⁺ T cell subsets. However, since the polarized T cell subsets are heterogeneous (Figure 1C), the actual percentages of edited

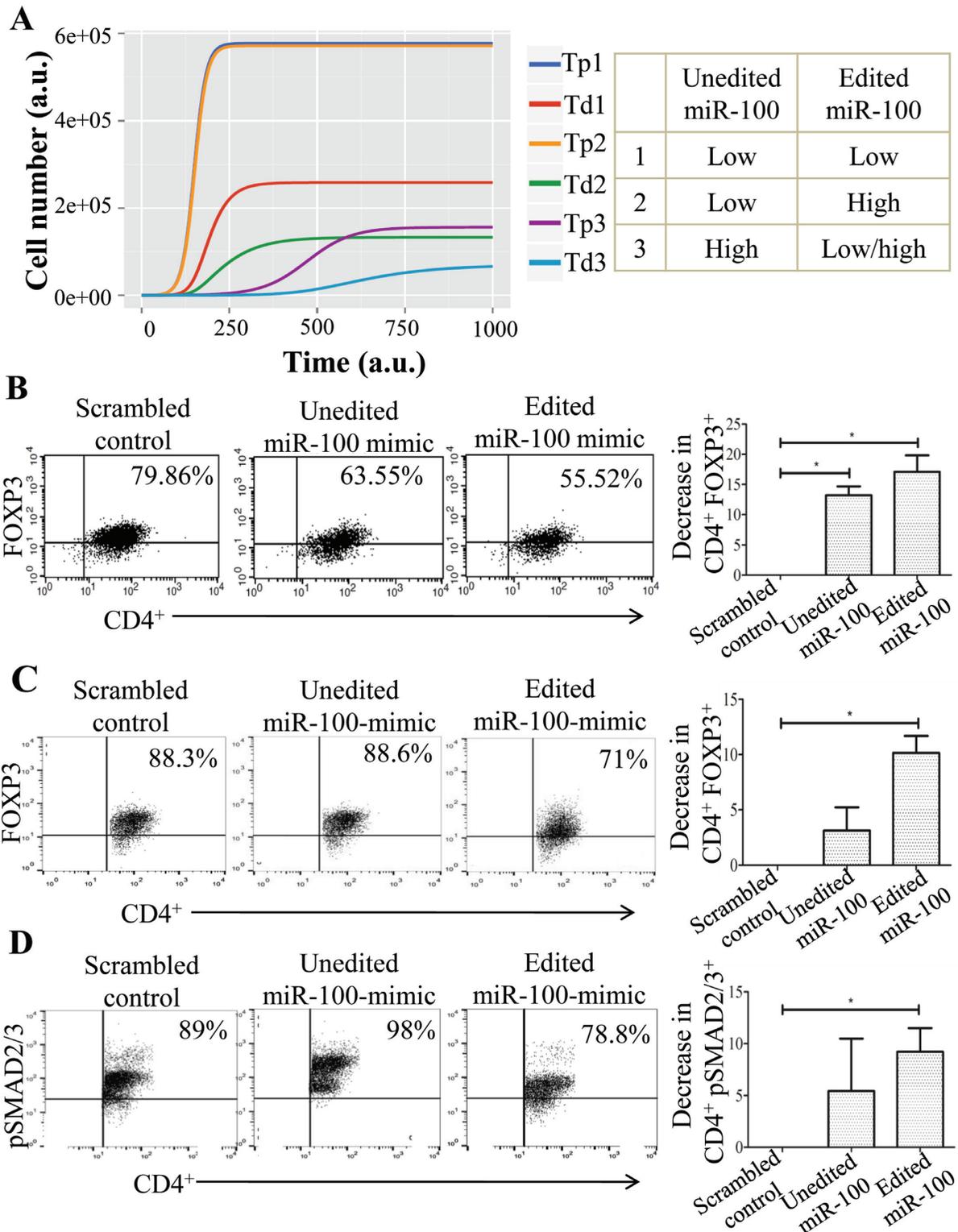


Figure 4. Both unedited and edited miR-100 mimics decrease Treg differentiation. **(A)** Graphical representation of cell number with time using mathematical modeling in different conditions depending on unedited and edited miR-100 levels during Treg differentiation. Tp represents number of CD4⁺ T cells and Td number of Tregs. 1, 2 and 3 represent different levels of unedited and edited miR-100 as shown in the table. **(B)** The representative percentages of CD4⁺FOxp3⁺ T cells transfected with scrambled, unedited and edited miR-100 mimics during *in vitro* Treg differentiation and their respective percentage decrease. **(C)** The representative percentages of CD4⁺FOxp3⁺ T cells in PBMCs transfected with scrambled, unedited and edited miR-100 mimics and their respective percentage decrease. **(D)** The representative percentages of CD4⁺ pSMAD2/3⁺ T cells in PBMCs transfected with scrambled, unedited and edited miR-100 mimics and their respective percentage decrease. The cells were gated on CD4⁺. Data are representative of at least three independent experiments, **P* < 0.01, two tailed *t*-test.

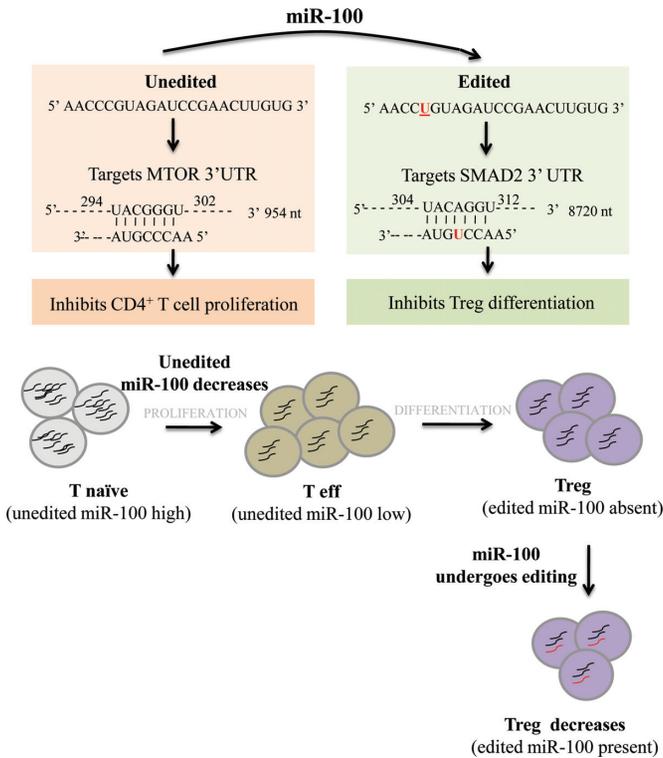


Figure 5. Proposed model for role of miR-100 editing in Treg differentiation and maintenance. miR-100 on C-to-U editing at fifth position result in change of target from *MTOR* to *SMAD2*. We propose a model where unedited miR-100 inhibits MTOR, limiting T cell proliferation and hence its differentiation whereas edited miR-100 inhibits SMAD2 affecting Treg differentiation and lineage commitment. Thus, editing of miR-100 act as a control switch to limit Treg differentiation.

miRNA in a pure population may be higher than estimated by us. Among the edited miRNAs, we focused only on A-to-I or C-to-U editing events that occur in seed region of the mature miRNAs. Interestingly, we found mir-30 family to be edited, non-canonically, at various positions in different subsets. mir-30e was edited at position 35 of pre-miRNA in all the subsets, but this editing position does not lie in the seed region of the miRNA indicating that the editing might affect the processing of the miRNA rather than its target binding ability. In addition, we also observed U-to-C editing in mir-30e at position 33 in two samples, namely Th2 and Treg, indicating that mir-30e is differentially edited with respect to its position and editing type among the subsets. Similarly, mir-30a and mir-30d were among the common miRNAs which had undergone editing. Thus, mir-30 family might be involved in regulating T cell differentiation. In addition, the unique editing event(s) occurring in Th0 is A-to-I in mir-381; in Th17 is U-to-C in mir-30d and U-to-G in mir-27b.

We narrowed down to miR-100 which is specifically edited in Treg because we were interested in miRNA edited in the differentiated subsets only. The editing type found in miR-100 was C-to-U at fifth position of mature miRNA which was validated by SNaPshot primer extension method. Most of the previous studies were focused mainly on the ADAR mediated editing, i.e. A-to-I; while

very few studies discussed the C-to-U editing in miRNAs (24). While APOBEC1 and 3 have both been reported previously to be present in T cells (9,10), further studies will be needed to determine the precise mechanism of miR-100 editing. To determine the effect of miR-100 editing, targets for both unedited and edited miRNAs were determined *in silico*. The predicted targets of unedited and edited miR-100 were found to be completely different, signifying the importance of a single base change in the seed region. One of the targets for unedited miR-100 was MTOR and for edited miR-100 SMAD2, relevant to CD4⁺ T cell biology. MTOR is a known target of unedited miR-100; in T cells it gets activated by PI3K signaling, which in turn gets activated by IL2R or CD28 or other co-stimulatory signaling molecules, in response to environmental cues like antigen, cytokines secreted by innate immunity, availability of nutrients, etc. (25). Thus, on T cell activation, MTOR gets activated and causes T cell proliferation and differentiation. The edited miR-100, present exclusively in Treg, targets SMAD2 which is involved in TGFβ1 signaling required for Treg differentiation (26). Due to limitation in number of cells in human primary culture, we validated the target of unedited and edited miR-100 in easily transfectable HeLa cell lines followed by confirmation of pSMAD2/3 downregulation in PBMCs transfected by edited miR-100.

To determine the biological significance of edited miR-100, we transfected both unedited and edited miR-100 mimics during Treg differentiation and in already differentiated Treg in PBMCs. Transfection of unedited and edited miR-100 mimics during Treg differentiation resulted in a decrease in polarization of Treg as measured by percentage of CD4⁺ Foxp3⁺ cells. The decrease was more in edited miR-100 mimic transfected cells as compared to unedited miR-100 mimic transfected ones. These observations are consistent with a model where there are two brake points in Treg formation; first, inhibition of MTOR by unedited miR-100 limits CD4⁺ T cell proliferation and second, inhibition of SMAD2 by edited miR-100 limits Treg lineage commitment (Figure 5). Both regulatory systems need to be overcome during Treg differentiation from immature T cells while only the second needs to be overcome in differentiated T cells. While this simplistic model functions well in *in vitro* polarization conditions, the full story is likely to be more complex as role of MTOR in Treg is controversial. Powell *et al.* reviewed the role of MTOR in T cell differentiation and mentioned that *in vitro* differentiated Treg, as described in our study, have high levels of MTOR. They further divided Treg into two populations based on the expression level of MTOR; namely, short lived effector Treg, with high MTOR level and long lived memory Treg cells with low MTOR level, depending on their cellular metabolism (27). This contradicts the previous reports which suggest rapamycin, an inhibitor of MTOR promote Treg differentiation while inhibiting other T effectors (28,29). Thus, while we found that unedited miR-100 mimics did not have much effect on the percentage of Tregs in PBMC culture, there may be important changes in their properties. Further, edited miR-100 mimics modestly decreased the Treg fraction despite PBMC representing a differentiated state; possibly indicating that edited miR-100 makes Treg flexible to convert to other subtypes. This effect is likely to be due to the downregulation

of SMAD2 since Treg polarized from *Smad2* cKO mice are shown to be more plastic *in vitro* as compared to control mice (30). We speculate that decrease in miR-100 is an important step in T cell proliferation, since this was seen in all lineages. Further, in cells undergoing Treg transformation, editing of miR-100 leads to new targeting of SMAD2. This is expected to be a counterbalancing process, which restrains unchecked expansion of Tregs and promotes plasticity. This is a potentially important regulatory mechanism for tolerogenic immune responses and miRNA editing merits further exploration in such contexts.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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