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Exploring miR-34c-5p modulation of effector CD4 T cell differentiation through logical modelling

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Background and objectives

miRNAs act post-transcriptionally to inhibit protein synthesis, by binding to 3'UTRs of mRNAs. miR-34c-5p was found to be up-regulated in activated naïve CD4 T cells 72 to 96 h post TCR priming with antigen, suggesting a role for this miRNA in CD4 T cell differentiation.

miR-155-5p is a well known immune system specific miRNA, up-regulated post activation. miR-34c-5p regulates differentiation, growth arrest and apoptosis, and its transcription is induced by TP53, FOXO3 and SP1. Its expression in the immune system has not been previously reported.

To understand any given miRNA function in a cell is a complex endeavor, thus *in silico* approaches can be of great help. Logical models are relatively easy to build and provide us with great predictive power. With this in mind, we intend to:

1. Establish a modelling framework to study CD4+ T cell activation and differentiation for prediction of miR-34c regulation and function;

2. Validate the mechanisms predicted by the model for miR-34c upstream/downstream regulation, namely focusing on transcriptional control and critical miRNA-mRNA interactions;

3. Determine the role of miR-34c-5p in naïve CD4 T cell differentiation.

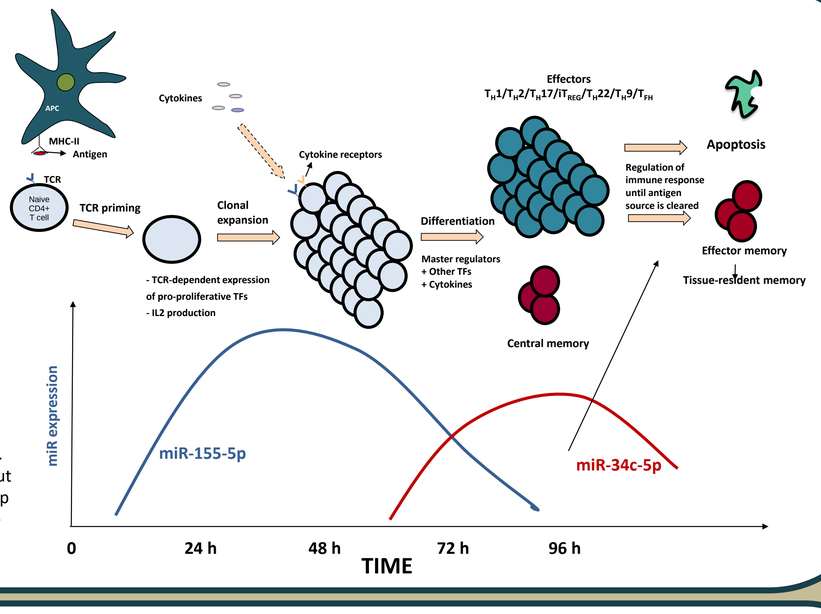

Figure 1 – Naïve T cells are activated by antigen presentation through APCs and differentiate into several effector subtypes. Memory is acquired somewhere throughout the adaptive immune response. miR-155-5p and miR-34c-5p expression detected in the lab is represented.

I. Model development

Target prediction

Table 1 – miR-34c-5p target output for good scoring targets involved in activation and differentiation of CD4+ T cells with miRanda (A) and PITA (B) and predicted by both algorithms (C) even if the score was outside the defined thresholds.

		Relevant predicted miR-34c-5p targets
Activation	A	CD28 CALM3 PRKCQ NFATC4 DGKH DGKB IKBKE PTEN
	B	CD28 CALM3 CBL PRKCQ MAPK1 FOSB REL RELN NFATC4 DGKH IKBKE SH3BP2 PAG1
	C	RELT; PRKCA,B,E,H,Q; AKT2; PTEN; NFKBIA,E; IKBKAB,E,G,AP; CALM3; CREB5; FOS; MAPK1; MAP2K1; KRAS; PTPN11; CDKN1B,C; ABL1; GRAP2
Receptors	A	IL10RB IL6ST IL9R IL6R TGFBR1,2,3
	B	IL10RB IL7R IL21R IL6R IL17RD IL31RA IL1RN IL21R TGFBR1,2,3
	C	IL9R; IL21R; IL2RB; TGFBR1,2,3; IL1R1; IL22RA2; IL31RA
Cytokines	A	-
	B	-
	C	TGFB3
STATs	A	STAT3
	B	STAT1 STAT3
	C	STAT3
SOCS	A	SOCS7 SOCS5 SOCS2
	B	SOCS4 SOCS7
	C	SOCS2,3,5,6,7
Master TFs	A	GATA3 FOXP3
	B	FOXP3
	C	GATA3, FOXP3, BCL6
Others	A	MYCN RUNX3 FOXP1 IRF1 IRF4 PRDM1 NOTCH1,2,4
	B	MYCN MAFF FOXP1 SMAD2 SMAD7 SMAD4 RUNX2 IRF4 PRDM1 NOTCH1,2
	C	SMAD2,3,4,7; MYB; RUNX2,3; IRF1,4; PRDM1; NOTCH1,2,3,4
VALIDATED		ZAP70; NOTCH1,4; MYC; RUNX2; BCL2

Promoter analysis

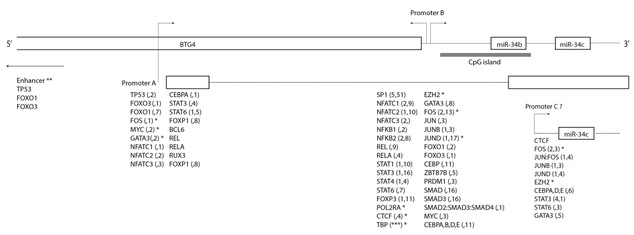

Figure 2 – The BTG4 and miR-34-b/c region in chr11:111,380,000-111,387,000 (hg19). An active promoter responsible for an alternative transcript (promoter A) has been described in hippocampus cells, with ChIP-seq revealing bound GATA3, MYC and FOS. The bidirectional BTG4 miR-34b/c promoter (promoter B) is the one described regulating transcription of an initial miR-34b/c ncRNA. The remnant TFs identified are predicted to bind to those promoters (JASPAR), with values between brackets (x,y) indicating the number of TFBSs for each factor, predicted with a 90% (x) or 80% (y) score, respectively. A 5' upstream enhancer includes HMR conserved binding sites for TP53, FOXO3 and FOXO1, known major regulators of miR-34c transcription. Arrows indicate TSSs obtained through FANTOM5 CAGE-Seq data. TP53 has also been shown to bind to promoter A (Oreganno and binding site detected with JAPSPAR at position 165-182). An additional TSS detected right upstream of miR-34c is suggested as a third promoter (promoter C), encoding a putative direct pre-mir-34c. * ENCODE validated TFs in T_H0 and T_H1 cells. ** Putative enhancer at 5' region of all promoters (note that enhancers change depending on chromatin state). *** Only TATA binding protein detected bound to DNA.

TOOLS: PITA, MiRanda, JASPAR, multiMiR, GINsim, Cytoscape, String, GeneMANIA, Rstudio, Linux shell. UCSC, Ensembl and Zenbu genome browsers.
DATABASES: FANTOM5, ENCODE, Expression Atlas, Protein Atlas, NCBI, TarBase, miRTarBase , Roadmap Epigenomics Mapping, IHEC.

Inferences from existing models of activation (Saez-Rodriguez et al., 2007) and differentiation (Naldi et al., 2010 and Abou-Jaoudé et al., 2016) with added miRNA regulation

STEADY-STATES

Table 2 – Steady-state analysis of the expanded activation model of Saez-Rodriguez et al. **[4]**. Table shows model steady-state values that change in the presence of miR-155-5p, miR-34c-5p or both miRs for the activation model. Nodes that change from active to inactive when the miRs are turned on (=1) are presented.

miRs	Activation model changes in state values
miR-155-5p	RAC1*, MAP3K11, MAPK14, CTNBB1, RPS6KB1, MAP3K1, MAP2K4, MAPK8, JUN**, NFKB
miR-34c-5p	ZAP70**, RAC1 *, MAP3K11, MAPK14, CCNI, RPS6KB1, MAP3K1, MAP2K4, MAPK8, JUN, CREB, MAPK1**
miR-155-5p + miR-34c-5p	ZAP70, RAC1 *, MAP3K11, MAPK14, CCNI, RPS6KB1, MAP3K1, MAP2K4, MAPK8, JUN, CREB, MAPK1, CTNBB1

*Necessary and sufficient target for the remaining changes of state value.
** Validated targets.

Critical target during activation with miRNA regulation:

RAC1

miR-34c-5p active in:

T_H1 - TBX21
T_H2 - GATA3

Table 3 – Percentage of steady-states of T_H effector subtypes with the Naldi **[5]** and Abou-Jaoudé **[6]** differentiation models, with TCR + IL2 stimulation. A) original model with miRs as input; B) expanded model with miR-34c and miR-155 transcriptional regulators. With the Abou-Jaoudé extended model, miRNAs were only used as inputs.

Master TFs/ Th subtypes		Percentage of phenotypes acquired in Naldi model							
		No miRs		miR-155-5p		miR-34c-5p		miR-34c + miR-155	
		A	B	A	B	A	B	A	B
TBX21	T_H1	42	42	44	44	50	50	50	50
GATA3	T_H2	17	17	22	11	50	50	50	50
RORC	T_H17	42	42	78	56				
FOXP3	iT_{REG}	33	33	44	44				
Mixed		33	42	89	56				
SSs (n)		12		9		2		2	
		Percentage of phenotypes acquired in Abou-Jaoudé model							
TBX21	T_H1	50		50		50		50	
GATA3	T_H2	33		50		50		50	
RORC STAT3+	T_H17	8							
SPI1	T_H9	33							
FOXP3	iT_{REG}	33							
STAT3 IL22	T_H22	8							
Mixed		33							
SSs (n)		12		4		4		4	

II. Inferences from new model

Construction of new integrative model

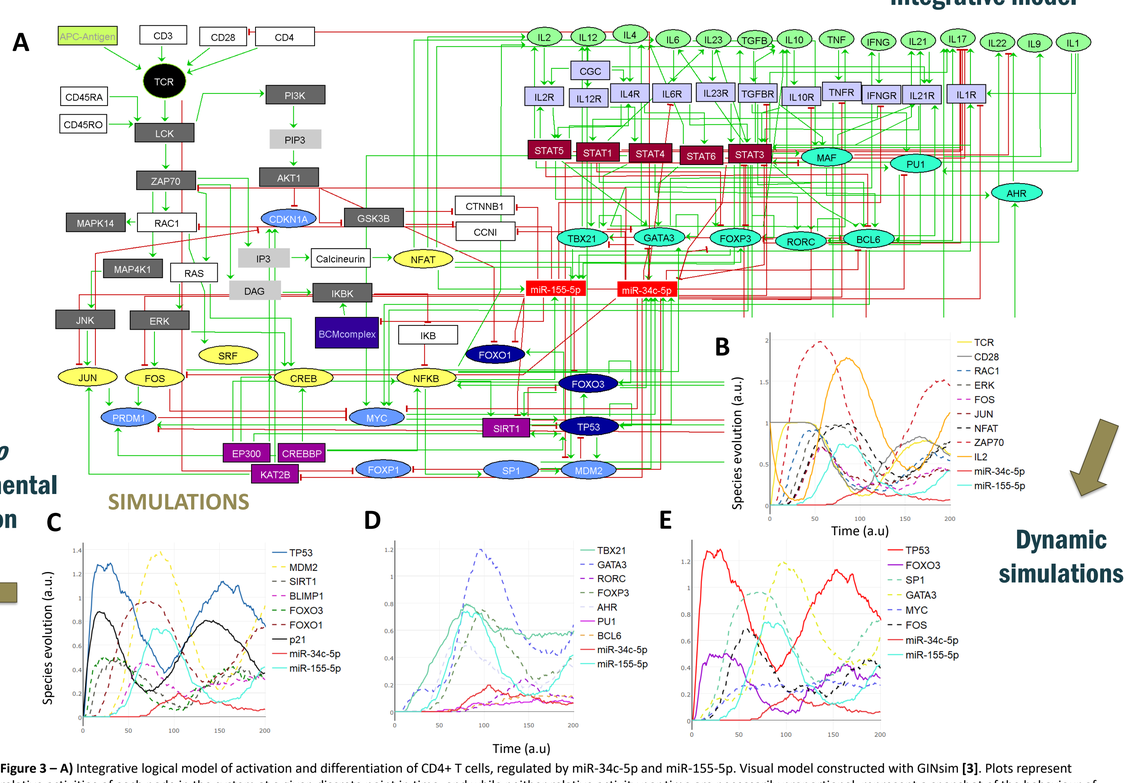

Figure 3 – A) Integrative logical model of activation and differentiation of CD4+ T cells, regulated by miR-34c-5p and miR-155-5p. Visual model constructed with GINsim **[3]**. Plots represent relative activities of each node in the system at a given discrete point in time, and while neither relative activity nor time are necessarily proportional, represent a snapshot of the behaviour of the expanding TCR + IL2 stimulated T cell population. Node behaviour/evolution (a.u.) was obtained through asynchronous simulation (with an R script), which considers the randomness of the cell environment: **B)** relative to CD4 T cell activation; **C)** cell cycle regulators; **D)** CD4 subtype associated master transcription factors and **E)** activity of miR-34c putative transcriptions factors.

Dynamic simulations

III. Validation of predicted transcriptional regulation

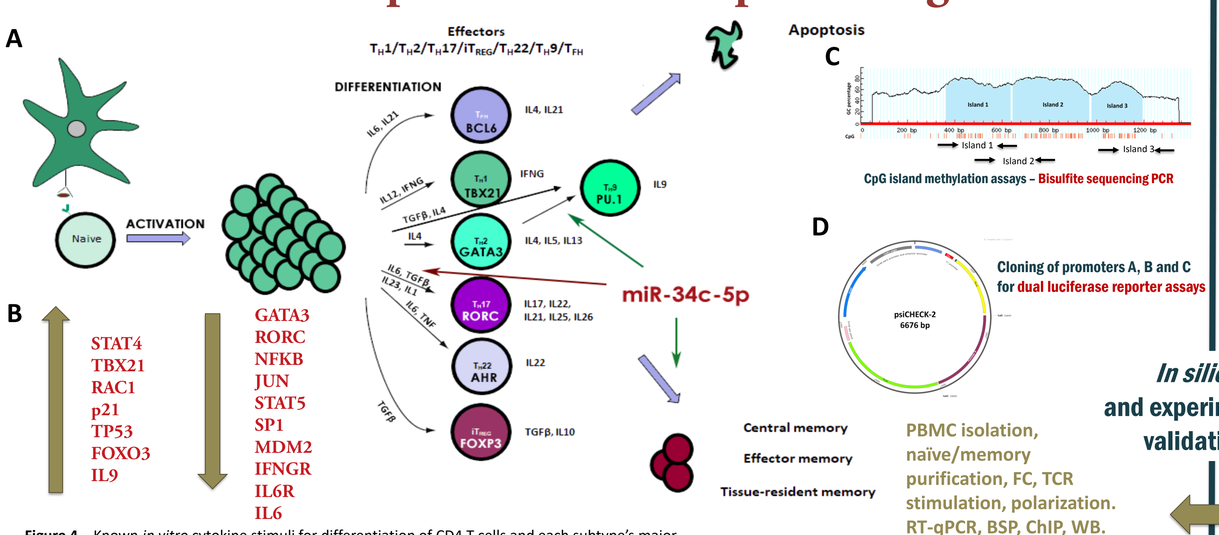

Figure 4 – Known *in vitro* cytokine stimuli for differentiation of CD4 T cells and each subtype's major secreting cytokines. TGFβ and IL4 combined give rise to PU.1 expressing T_H9 cells. T_H9 can arise from T_H2 cells **[7,8]**. At the end of an immune response, cells suffer apoptosis or they turn into memory cells. The latter's origin is still highly debated, but it is thought that at different stages of differentiation cells can give rise to different types of memory cells **[9]**. Green arrows represent miR-34c-5p positive reinforcement of those differentiation pathways (T_H9 and memory), while the red arrow represents pathway repression (T_H17). Below **(B)** are the up and down-regulated genes after miR-34c-5p is expressed, with miR-155-5p regulation. **C)** CpG islands in the promoter B region and amplicons for bisulfite sequencing PCR to determine methylation status of islands and **D)** Construct for transcription factor reporter assays with psiCHECK-2 vector from Promega. Naïve and memory cells will be purified from PBMCs of healthy donors, obtained through the Portuguese Institute of Blood and Transplantations. After TCR stimulation, miR-34c expression and its effect on targets will be explored.

***In silico* and experimental validation**

PREDICTIONS AND VALIDATION

- TF combinations -> GATA3 and MYC presence results in modulation of otherwise high levels of TP53 and FOXO3- only positively regulated miR-34c transcription;
- Polarization with cytokines -> Validated patterns for T_H1, T_H2, T_H9 and T_H17;
- Detection of miR-34c expression mainly in T_H2, less pronounced in T_H1, lower in T_H17.

Conclusions

The potential impact of miR-34c-5p on activation processes seems to be less pronounced than on differentiation. In the context of activation, RAC1 is suggested to be a relevant target when both miR-155-5p and miR-34c-5p are up-regulated. Regarding differentiation of T_H subtypes, steady-sates were obtained only for T_H1 and T_H2 phenotypes. Therefore, it is proposed that miR-34c expression is activated in T_H2 cells. This is further supported by the results obtained with dynamic asynchronous simulations performed using the new integrative model. Those simulations revealed that it is only when both miR-155-5p and miR-34c-5p are modulating the system, that the pattern of expression similar to the observed in primary CD4 T cells emerges. This requires the joint regulation of miR-34c expression by a specific combination of TP53, FOXO3, SP1, GATA3 and MYC. The model predicts that PU.1 (SPI1) activity can be up-regulated in the presence of both miRs. This suggests that miR-34c expression in T_H2 cells could lead to T_H9 reprogramming, proposing a role in plasticity. The IL6/STAT3/RORC axis seems to be the most down-regulated in the presence of miR-155 and miR-34c. Interestingly, previous functional enrichment analysis of differentially expressed genes in T cells over-expressing miR-34c-5p identified T_H17 associated GO terms as significantly enriched **[2]**.

The model predicts miR-34c-5p may promote quiescence because there is a concurrent CDKN1A, FOXO3 and TP53 up-regulation, probably in a GATA3^{hi} TBX21^{me} PU.1^{lo} cell state of mostly IL4 and TGFB secreting CD4 T cells. Interestingly this miRNA was originally identified as part of the TP53 response **[10]**. Thus, the model we have implemented is able to reproduce previous experimental observations.

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