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RELATÓRIO FINAL DE ACTIVIDADES

sncRNA regulatory networks in T cell activation and viral response

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1. Abstract

miRNA canonical function is to inhibit translation of mRNAs by binding to their 3 prime untranslated regions (UTRs), either targeting the mRNA for degradation or blocking progression of the translation machinery. miRNA regulation is essential for proper immune function of CD4 T helper cells. When an antigen binds to the T cell receptor (TCR) of a naïve cell, the miRNA repertoire is altered, as a consequence of signalling and downstream changes in expression of transcription factors. Two specific miRNAs, miR-155-5p and miR-34c-5p were detected as differentially upregulated after TCR engagement with anti-CD28, anti-CD3 and IL2 by the host lab. miR-155-5p is a well-studied regulator of immune activation processes, being also required for Th1, Treg and Th17 function. Expression of miR-34c-5p, on the other hand, has never been reported, to the authors knowledge, in CD4 T cells. Furthermore, miR-34c-5p was not detected when memory cells were activated, suggesting that this miRNA might be involved in memory formation. Also, overexpression of this miRNA in HIV infected CD4 T cells seems to aid HIV replication. To predict the putative role of miR-34c-5p in activated T cells, we adapted previously built models of CD4 T cell activation and differentiation, by Saez-Rodriguez et al. (2007), Naldi et al. (2010) and Abou-Jaoudé et al. (2016). The insertion of the two miRNAs into the model was done based on validated targets, available online and mined with MultimiR algorithm, and also targets predicted by PITA and Miranda with stronger scores. Transcriptional regulators of the miRNAs were obtained from literature, databases and tools such as ENCODE, JASPAR and TRANSFAC. This modelling approach showed that miR-34c-5p expression resulted in a stabilization of the system towards a restriction of Th phenotypes, namely TBX21 expressing Th1 cells and GATA3 expressing Th2 cells, while miR-155-5p also allowed other phenotypes, such as Th17 and Treg, in accordance with what has been reported in the literature. ENCODE, FANTOM5 and IHEC epigenetic data, together with a detailed analysis of the bidirectional promoter region of the BTG4 and miR-34b/c genes, as well as the analysis of an alternative upstream promoter region and a transcription start-site for a miR-34c-only transcript, identified GATA3, MYC and FOS as putative major regulators of transcription of a miR-34c pri-mir, further supported by model predictions. To validate this, we designed reporter gene assays, with the region of binding of GATA3, MYC and FOS (461 bp), the bidirectional BTG4/miR-34b/c promoter (1353 bp), and another putative promoter (promoter C, 588 bp). A bisulphite sequencing PRC assay was also designed in order to determine the methylation status of the CpG islands in the promoter B region. Based on the modelling results, we propose that miR-34c-5p is activated as a differentiation terminator of Th2 cells, possibly involved in formation of an effector memory subtype, which would directly act as a Th2 cell upon reactivation by antigen. The limitations of available models led to the construction of a new model that includes both CD4 T cell activation and differentiation processes, including rules for interactions already described and validated by other models and experimental data, as well as new rules that were not considered or were not described at the time when the other models were built. This model reproduces expected behaviours of T cell differentiation after antigen encounter, as would be expected from TCR and IL2 only stimulation, with stimuli from neighbouring proliferative CD4 T cells, and reveals several distinct features that may be experimentally tested, such as down-regulation of the STAT3/RORC/IL17 axis and PU.1 and IL9 up-regulation. It also supports the idea that miR-34c-5p promotes the quiescent memory state through TP53 and p21 up-regulation. This work leaves the door open for several experiments in isolated naïve and memory CD4 T cells in order to assess the several predictions that were made.

2. State of the Art

The discovery of microRNA (miRNA or miR) regulation of translation brought an extra level of complexity to the understanding of gene expression. miRNAs are generated through a canonical pathway of cleavage of a primary and precursor miRNA by endonucleases Drosha in the nucleus and Dicer in the cytoplasm [12], respectively, forming a miRNA duplex. However, alternative pathways of biogenesis have been identified [345]. One of the chains of the duplex will be led by the RNA induced silencing complex (RISC) to its target mRNA. miRNAs can be intronic, have their own gene and/or be present in clusters [6] and transcription may occur through RNA polymerase II or III promoters. They are thought to target mainly 3' UTR regions of messenger RNAs (mRNAs), preventing its complete translation into protein, or targeting mRNA for cleavage or degradation [7], although they have also been shown to bind 5' UTRs and even coding regions [89]. Furthermore the same miRNA acts on several target mRNAs [10], different miRNAs have redundant targets [11] and they can be secreted, becoming circulating miRNAs and exert their function in other tissues [12], therefore elevating the difficulty in determining the effects of a given miRNA or a set of expressed miRNAs in cell regulation. Their role is essential for proper immune cell development and function (for a review see [13]).

Naïve CD4 T cells rest quiescent in the periphery until their T cell receptors (TCRs) are presented with cognate antigen by an antigen presenting cell (APC). This and costimulatory signals by CD28, and CD4 lead to cell activation by cascades of kinases that will eventually activate transcription of proliferation inducers, such as NFKB, NFAT and IL2. Proliferation starts one to two days after antigen priming, and differentiation into effector or memory cells at 96h post TCR stimulation [14]. Cytokine signalling activates signal transducers and activators of transcription (STATs) factors, which activate specific cytokine and TF genes. The combination of signalling and expressed TFs will result on a different signature that will give rise to a specific T helper (Th) subtype, expressing its master regulator TF, but also cooperative TFs, such as STATs, SMADs, IRFs and many others [15]. The original activated T cell will give rise to differentiated effector and memory cells with diverse functions, depending on the interplay of these signals. During all these phases, different miRNAs are transcribed, modulating the different phenotypical destinies of Th cells. During T cell activation for example, miR-181a has been implicated as a regulator of the TCR signalling sensitivity at the double positive (DP) stage [16] and in resting T cells [17], being also implicated in Th17 differentiation [18]. miR-155 is one of the most studied miRs due to its oncogenic potential and its role in immune function [19], being upregulated after CD4 T cell activation. It down-regulates CTLA-4 (CD152), a T cell surface protein that is responsible for repressing activation when bound to CD80 or CD86 on APCs. In parallel, miR-155 enhances T cell proliferation by targeting suppressor of cytokine signalling 1, SOCS1 [20]. It has been shown to regulate effector Th1 [21], Treg [22], Th17 responses [23 24 25] and Th2 responses directed by dendritic cells (DCs) *in vivo* [26]. There are several other miRNAs that have been identified as relevant in different contexts of T cell physiology, such as let-7 family members, the miR-17~92 cluster or miR-146a [20], with all of them being expressed selectively at key points by different cell phenotypes. These miRNAs are thought to be major players in the plasticity observed in Th cells, partly driven by miRNA modulation of gene expression [27 28 29 30]. Previous work at the host lab described a novel miRNA in the context of naïve activated T cells, miR-34c-5p, which was found to be upregulated, reaching maximum expression at 96h post TCR stimulation by anti-CD28, anti-CD3 and IL2.

The miR-34c family includes miRs-34a/b/c. miR-34a is located in chromosome 1 and its promoter has a slightly different affinity for transcription factors (TF) than the promoter for miR-

34b/c, located in chromosome 11. Their major known activating TFs are TP53 [31], FOXOs [32], most notably FOXO3 for miRs-34b and c, and SP1 [33]. TP53 is the most mutated protein in tumours and is involved in diverse pathways, being referred to as the 'guardian of the genome' and most commonly associated with promoting apoptosis and cell cycle arrest (for a full review on TP53 see [34]. FOXO factors are expressed in response to DNA damage, but also other stress signals such as reactive oxygen species (ROS), being an important mediator of T cell homeostasis [35] [36] and tolerance [37]. SP1 controls cell cycle and growth, differentiation [38], apoptosis, tumorigenesis, immune responses and has been implicated in HIV-1 Tat-dependent transcription [39]. These TFs cooperatively regulate proliferation, differentiation, DNA damage response, the cell cycle and apoptosis [40]. Factors that repress miR-34c transcription include STAT3 and EMT-TFs [31]. miR-34c has been mostly studied in cancer where its expression is dysregulated. It is an inducer of apoptosis and acts as a proliferation repressor, is important in cell cycle arrest and in cell differentiation [41], by targeting anti-apoptotic and pro-proliferation mRNAs. Available data from previous work suggests this miR has the ability to regulate genes associated to TCR signalling, but the functional consequences of this are at present unclear. Furthermore, its specific induction in naive but not memory cells [42] raises the possibility that this miR is linked to the differentiation of memory CD4 Th cells. However, it cannot be discarded that miR-34c-5p is necessary for the differentiation of an effector or an effector memory subtype.

Modelling approaches can be used in biology in multiple ways [43]. The choice of logical modelling to predict a system behaviour comes down to essentially two criteria. The ease of gathering qualitative over quantitative data on molecular interactions and their predictive power. Other types of models are ordinary differential equation (ODE) models, which offer quantitative information and are therefore more precise, and structural networks, which reveal relations between components without describing their specific interactions, and have been described by [44]. Logical models have been thoroughly reviewed elsewhere [45] [46] [47], including with miRNA regulation [48]. They provide us with valuable information about all possible steady-states (called attractors) of a system given a set of logical rules and a specific input, that arise from important features such as feedback-loops (FBLs), feed-forward loops (FFLs) and the strength of each node in the case of non-Boolean models (if the model is multi-valued, i.e., instead of a Boolean model where a state is either 0 or 1, it assumes higher values given a certain condition). These non-Boolean models are required because a Boolean model is not appropriate for complex biochemical pathways, where p.ex. nonlinear feedback loops generate disproportional output [47].

The advent of systems biology and next-generation sequencing technologies brought the possibility of assessing miRNA function *in silico* prior to experimental approaches. miRNA target prediction algorithms, ChIP-seq data, CAGE-seq, TFBS prediction algorithms and network modelling have proven to be powerful tools to better understand how a system might behave. We hereby describe a method for assessing miRNA regulation and function of miR-34c-5p, by using tools available online, to construct a logical model of CD4+ T cell activation and differentiation, predictive of what this miRNA role might be in an activated naïve CD4 T cell and through its differentiation, when stimulated by IL2, plus anti-CD28 and anti-CD3 antibodies (Abs). This methodology was created in order to determine the possible regulation and function of miR-34c-5p in naïve CD4+ T cells after *in vitro* activation. Given the high complexity of processes that miR-34c-5p is predicted and known to modulate, the appropriate chosen model was a discrete multi-valued logical model, where the maximum value can be 2. We set ourselves to analyse the influence of this miRNA on a variety of already built models, in order to build a new one in accordance with the suggestions obtained from those, with the final objective of determining how it is regulated and its function in activated naïve CD4+ T cells.

3. Objectives

The main objective of this work was to understand miR-34c-5p function in CD4 T cell activation using a systems biology/modelling approach. Taking advantage of these methods, we also wanted to assess the ability of the HIV virus to interfere with this process through production of viral miRNAs or the modulation of host-derived miRNAs. For this purpose, we pursued three specific aims:

1. Establish a modelling framework to study CD4+ T cell activation and differentiation with *in vitro* TCR and IL2 stimulation for prediction of miR-34c regulation and function;

2. Validate mechanisms of miR-34c regulation by understanding which factors regulate its transcription and validate miRNA-mRNA interactions, and determine its role in naïve versus memory CD4 T cells in Th1/Th2 polarizing conditions (TCR + Il2 stimulation);

3. Determine whether detected conserved miRNA-like sequences detected in HIV1 and HIV2 infected CD4 T cells are *bona fide* HIV sncRNAs and if so, how they impact these processes.

4. Summary of Activities

This work was developed based on previous work conducted at the host lab, that showed that miR-34c-5p was up-regulated after naïve CD4 T cell stimulation with anti-CD3, anti-CD28 and IL-2, peaking at 72-96 h post-stimulation (Figure 1). At the time it was the first time this miRNA was described in the immune system. Interestingly, this up-regulation did not happen when memory CD4 T cells were given the same stimuli, suggesting a role in memory formation [42].

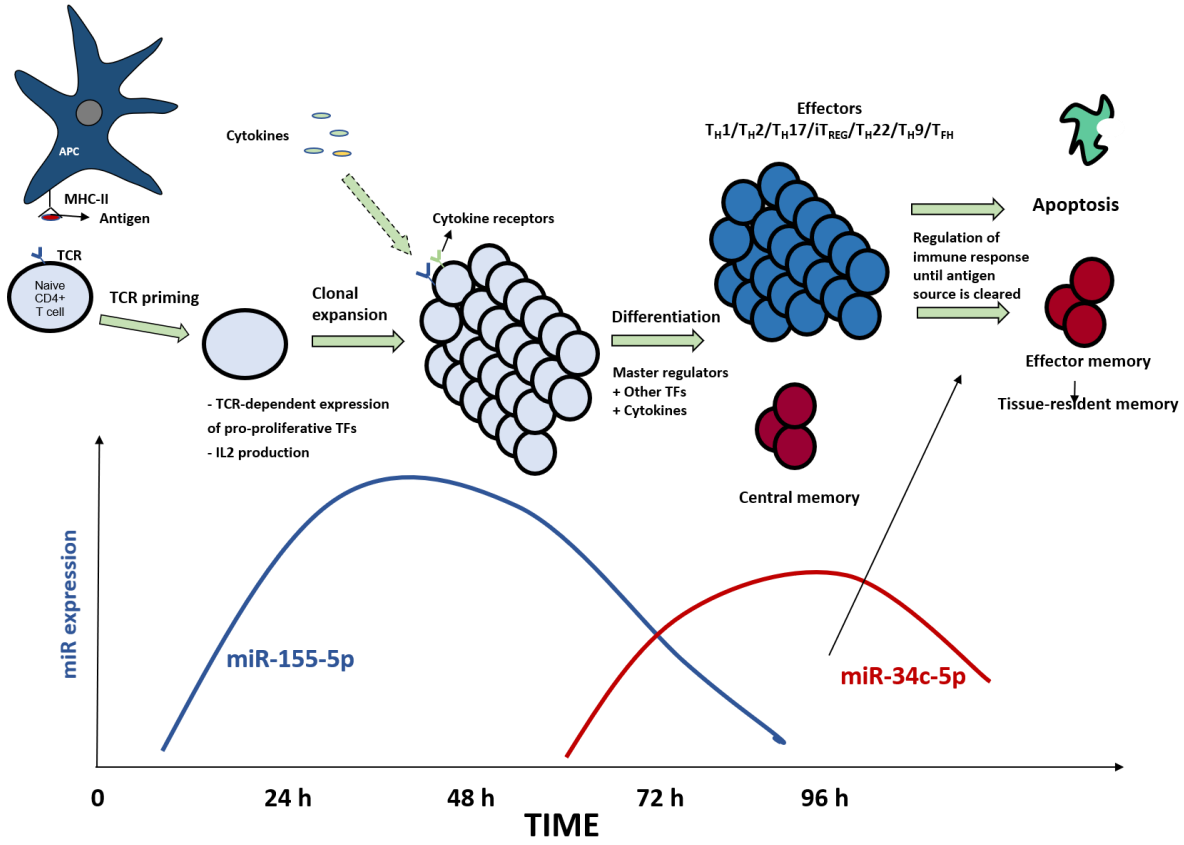


Figure 1 – CD4 T cell adaptive immune response and expression of miR-155-5p and miR-34c-5p during 96h differentiation of naïve CD4 T cells stimulated with anti-CD3, anti-CD28 and IL-2 [42].

Task 1 - Establishment of a modelling framework to explore the impact of miR-34c-5p in activated naïve CD4 T cells.

We started by getting the predicted miR-34c-5p targets using miRanda and PITA, and looking for validated targets, which can be more easily found using the multiMiR R package (Table 1). The targets with better scores and predicted by both algorithms were chosen.

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	CD28; 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	IL10RB; IL6R; 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	MYCN; SMAD2,3,4,7; MYB; RUNX2,3; IRF1,4; PRDM1; NOTCH1,2,3,4
VALIDATED		**ZAP70; NOTCH1,4; MYC; RUNX2; BCL2**

We looked for known regulators of miR-34c transcription, performing a more profound characterization of its promoter region, and therefore new hypotheses for regulation arose (Task 2, Figure 3). We searched for available and published computational logical models of activation and differentiation of CD4 T cells. We expanded the already existing models of activation and differentiation by Saez-Rodriguez et al (2007) [49] (Figure 2), Naldi et al. (2007) [50] (Figure 3) and Abou-Jaoudé et al. (2015) [51] (Figure 4) to include miR-34c-5p and miR-155-5p targets as a preliminary analysis. Posteriorly we added the transcription factors known to be involved in miR-34c transcription as well as newly predicted ones with TFBS prediction tools and validated TFs in databases such as ENCODE and the most relevant predicted and validated targets that were already present in those models.

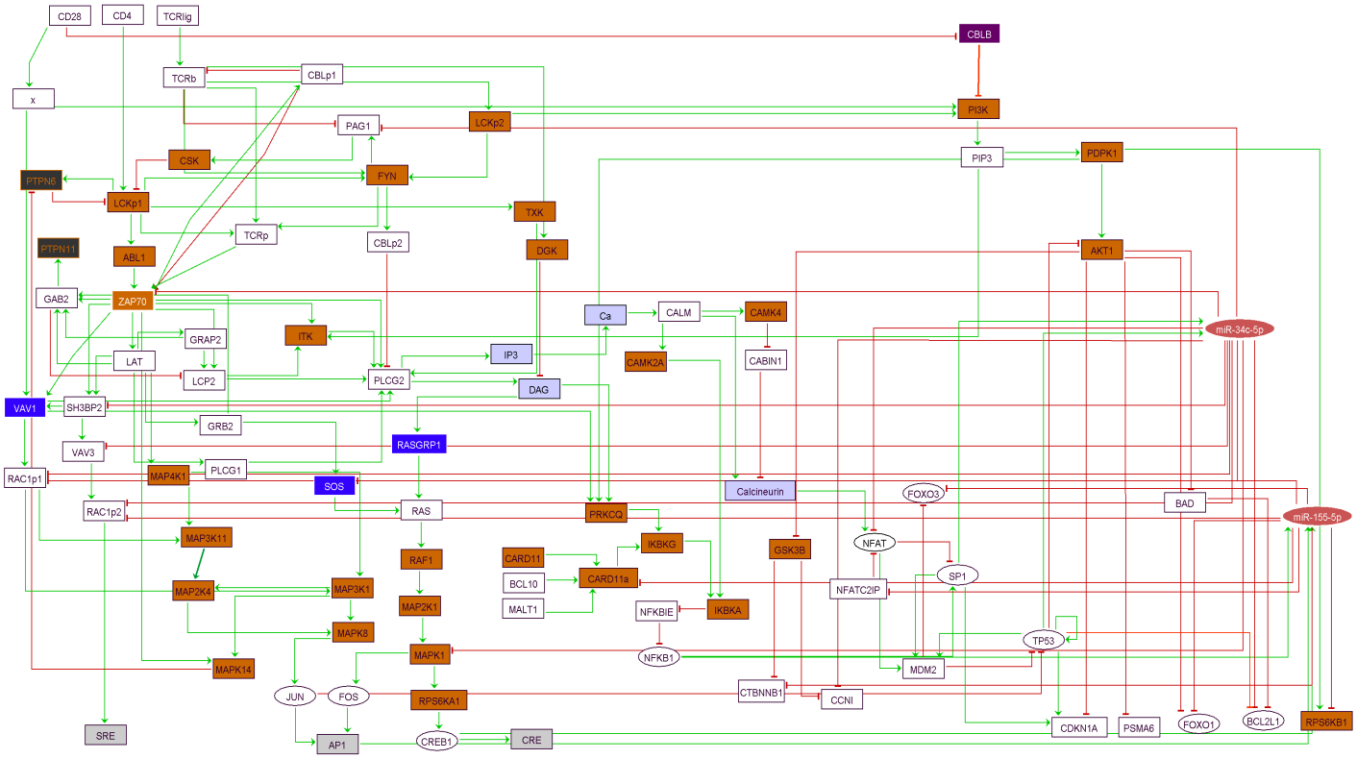


Figure 2 – Logical CD4 T cell activation model diagram built based on Saez-Rodriguez et al. (2007) model. The model has TCR stimulation, CD4 and CD28 as inputs, and signalling cascades activated by TCR activation, leading to expression of proliferation related factors and inhibition of cell cycle arrest and apoptotic factors. Validated and predicted targets of miR-155-5p and miR-34c-5p are represented.

Modelling results suggest miR-34c-5p effects on activation processes seem to be less pronounced than in differentiation, but RAC1 is suggested to be a relevant target when miR-155-5p and miR-34c-5p are up-regulated (Table 2).

Table 2 – Steady-state analysis of the expanded activation model of Saez-Rodriguez et al. (2007). The table shows model steady-state values that change in the presence of miR-155-5p, miR-34c-5p or both miRs for the activation [49] model. Nodes that change from active to inactive when the miRs are turned on 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.

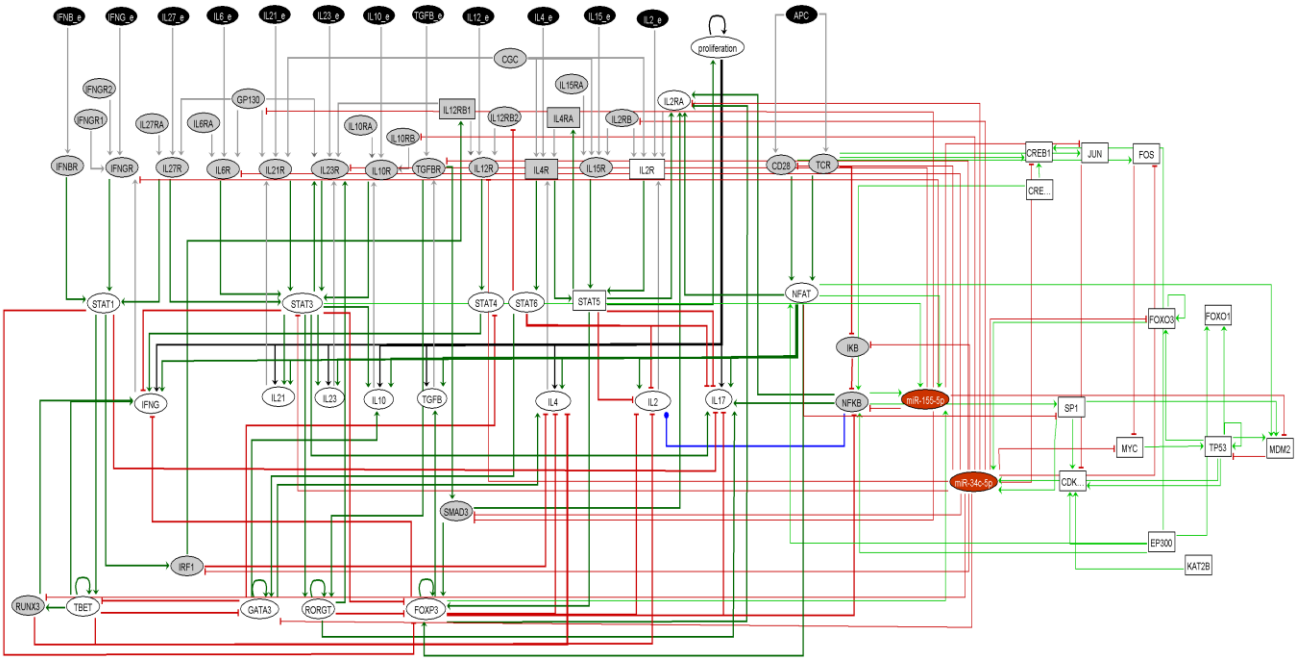


Figure 3 - Logical CD4 T cell differentiation model graph by Naldi et al. (2010), expanded with miR-155-5p and miR-34c-5p. This model has as inputs proliferation, IL2 and environmental cytokines, in order to simulate different responses according to the stimulating interleukins. These activate transcription factors such as STATs that besides activating TFs such as master regulator genes also stimulate signature cytokines. Steady-states represent different Th phenotypes (Th1, Th2, Th17 and iTreg).

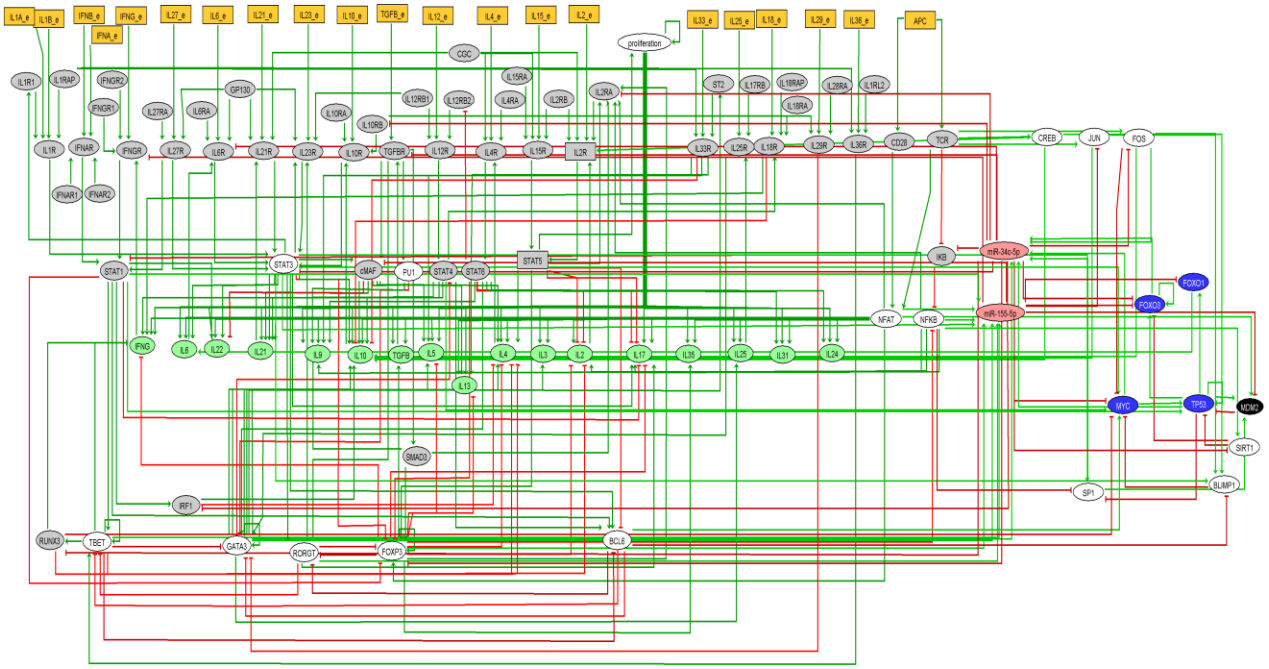


Figure 4 – Logical CD4 T cell differentiation model graph by Abou-Jaoudé et al. (2015), expanded with miR-155-5p and miR-34c-5p. This model has as inputs proliferation, IL2 and environmental cytokines, in order to simulate different responses according to the stimulating interleukines. These

activate transcription factors such as STATs that besides activating TFs such as master regulator genes also stimulate signature cytokines. Steady-states represent different Th phenotypes (Th1, Th2, Th17, Tfh, Th22, Th9 and iTreg).

We obtained *in silico* evidence that miR-34c-5p had an impact in MAPK cascades, NFKB, AP1 (FOS and JUN) and CREB regulation, favoring cell-cycle arrest and suppressing proliferation (Table 2), and also that the miRNA would be active in Th1 or Th2 cells (Table 3).

Table 3 – Percentage of steady-states of Th effector subtypes with the Naldi and Abou-Jaoudé 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.

		Percentage of phenotypes acquired in Naldi model							
Master TFs/ Th subtypes		**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	

However, we could not see any effects with miR regulation in those older models when we conducted dynamic simulations. Therefore, we constructed a new model of CD4 T cell activation and differentiation in Th1/Th2 polarizing conditions, i.e., TCR and IL2 stimulated cells (Figure 5).

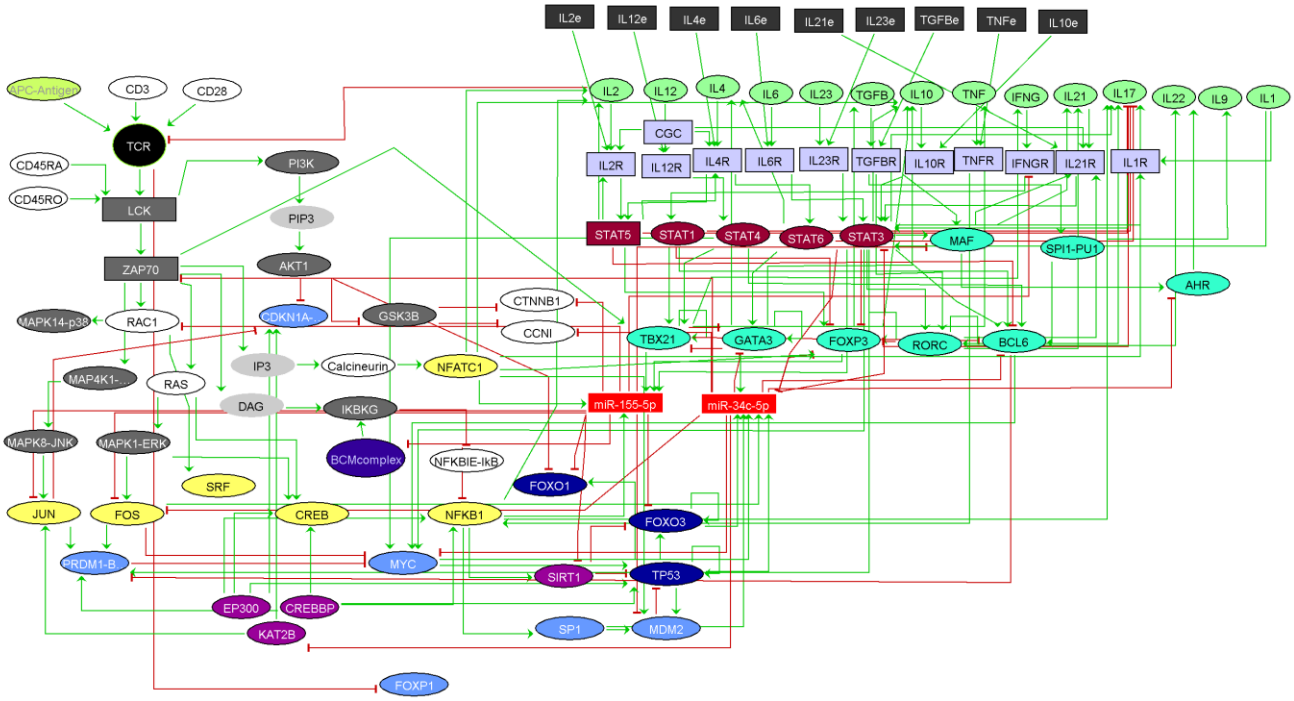


Figure 5 – New model of activation and differentiation of CD4+ T cells (with IL2 and TCR priming) with miR-34c-5p and miR-155-5p regulation, according to literature and data obtained through tools and databases.

The model includes major activation signalling molecules and pro/proliferation transcriptions factors, as well as cytokine and cytokine receptors and their downstream intermediate and master transcription factor. It also has cytokine as inputs in order to simulate *in vitro* polarization of different CD4 T cell subtypes. The directed edges represent the relationships between nodes, that are described in Table 4.

Table 4 – Set of logical rules used in the naïve CD4+ T cell differentiation model after TCR engagement and IL2 stimulation.

Node	Value	Formulae	Description
TCR	1	APC-Antigen & CD3 & CD28 & !IL2:2	T cell activation is dependent on presentation of antigen by MHC-II on APC, CD28 and CD3. Also CD4 in Th.
CD28	1	!IL2:2 & !miR-34c-5p	!IL2:2 represents a state where the cell has been already stimulated by STAT5 for IL2 production, and starts entering a differentiation stage. miR-34c-5p is predicted to target CD28.
LCK	1	TCR & CD45RA	Naïve CD45 receptor, alpha isoform is activated by phosphorylation by LCK [52].
ZAP70	2	LCK & !miR-34c-5p	LCK phosphorylates ABL1, which phosphorylates ZAP70 [53]. miR-34c-5p has been shown to target ZAP70 mRNA [54].
ZAP70	1	LCK	
PI3K	1	LCK & CD28	CD28 and LCK activate PI3K in the absence of CBLB [53].
PIP3	1	PI3K	PIP3 is formed by PI3K transfer of P from PIP2. PIP3 is required for PDK1 activation [55].
AKT1-PKB	1	PIP3	PDK1 phosphorylates PKB(AKT1) leading to its activation [565758]. AKT1 (PKB) inhibits growth arrest and pro-apoptotic proteins.

CDKN1A-p21	1	!AKT1	CDKN1A(p21) promotes growth arrest and is inhibited by phosphorylation by AKT1.
RAC1	1	ZAP70 & !miR-155-5p	RAC1 is activated through cascades downstream of ZAP70, through VAV1 and VAV3 [5960]. RAC1 is a predicted and validated [61] miR-155-5p target.
MAPK14-p38	1	RAC1	GADD45 inhibits the ZAP70 mediated activation of MAPK14(p38) [62].
SRF	1	MAPK14-p38	SRF is activated by VAV3-dependent RAC1 [59].
JUN	1	MAPK8-JNK & !miR-155-5p	MAPK8 (JNK) phosphorylates JUN [63], thereby activating it. JUN is a predicted and validated [64] target of miR-155-5p.
FOS	1	MAPK1-ERK & !miR-34c-5p & !miR-155-5p	ERK activates FOS by phosphorylation [65]. FOS is a predicted target of miR-155-5p and miR-34c-5p.
FOXO1	1	TP53 & !AKT1 & !miR-155-5p	TP53 activates FOXO1 transcription, while AKT1 represses it. miR-155-5p is predicted to inhibit AKT1(PKB).
IP3	1	ZAP70	ZAP70 leads to the formation of IP3 through PLCGA, which splits PIP2 into IP3 and DAG [66].
DAG	1	ZAP70	ZAP70 leads to the formation of DAG through PLCGA, which splits PIP2 into IP3 and DAG [66].
Calcineurin	1	IP3	Binding of IP3 to the IP3 receptor in the endoplasmic reticulum leads to the release of calcium [63]. Calcium binds to calmodulin and this complex to calcineurin [67].
NFAT	1	Calcineurin	Calcium-dependent NFATs (NFATC1,2,3,4) are dephosphorylated by Calcineurin and thus activated after translocation to the nucleus [6668].
BCMcomplex	1	!miR-155-5p	The binding of MALT1 to CARD11 and BCL10 forms the active CARD11 complex [697071]. All BCM complex proteins (BCL10, CARD11 and MALT1) are predicted miR-155-5p targets.
NFKB	2	TNFR & !NFKBIE-IkB	The canonical NFKB pathway is activated after the activated BCM complex [7172]. Activated IKK phosphorylates IKB, releasing NFKB to the nucleus [73].
NFKB	1	!NFKBIE-IkB	TNFR binding to TNF triggers activation through NIK of NFKB complexes [74].
NFKBIE-IkB	1	!IKBKG	IKB is bound to NFKB in the cytoplasm, rendering it inactive. When IKKs phosphorylate IKB, NFKB is released and rendered active [73].
IKBKG	1	DAG & BCMcomplex & !miR-34c-5p	The complex CARD11+BCL10 +MALT1 is required for IKKG activation[697576]. Phosphorylation, probably via PKCth [77], is also required. Several IKBKs are predicted targets of miR-34c-5p.
MAPK8-JNK	1	MAP4K1-MEKKK	GTP-bound RAC1 is able to bind MEKK1, and active MEKK1 leads to JNK activation [7879].
MAPK1-ERK	1	RAS & !miR-34c-5p	RAS signalling cascade leads to ERK activation [8081]. ERK is a predicted and validated target of miR-34c-5p [82].
GSK3B	1	!AKT1	PKB inhibits GSK3[8384].
CTNNB1	1	!GSK3B	GSK3 inhibits CTNNB1(b-cat) [83].
CCNI	1	!GSK3B	GSK3 inhibits CCNI(cyc1) [83].
RAS	1	ZAP70	ZAP70 downstream interactions eventually activate RAS. These were omitted for model simplification.[85]. [83]. [86].
CREB	1	RAS & MAPK1-ERK	RAS signalling cascade eventually leads to ERK activation, which phosphorylates RPS6KA1 (RSK) and FOS. RPS6KA1 activates CREB by phosphorylation [87].
IL2	2	(NFAT & NFKB) | (JUN & FOS) & STAT5 & !(STAT5 & STAT6) & !(NFKB & TBX21)	IL2 production requires NFAT [68]. STAT5 and STAT6 cooperate to inhibit IL2 production [88]. RELA induces IL-2 [89]. TBX21 cooperates with RELA (NFKB subunit) to inhibit IL2 [9091].
IL2	1	(NFAT & NFKB) | (JUN & FOS)	
IL6	1	NFAT & STAT3	IL6 requires NFAT and STAT3 to be produced (at least in Th17 polarizing conditions) - assumed by us and [51].
TGFB	1	FOXP3 & NFAT	TGFB requires NFAT and FOXP3 to be produced (at least in iTreg polarizing conditions) - assumed by us and [51].
IL12	1	NFKB | TBX21	
IL21	1	(RORC | MAF) & !TGFB	IL21 production is activated by either RORC or MAF [92], but not when TGFB is present [93].
IL17	1	NFAT & RORC & !FOXP3 & NFKB & STAT3 & !(STAT5 | STAT1 | STAT6)	IL17 requires TGFB+IL6. STAT3 and RORC activate IL17 cooperatively [94]. The action of RORC can be blocked by FOXP3 [95]. STAT3 and NFKB are required to activate IL17. IL2 inhibits IL17 through STAT5 [9697].
TNF	1	TBX21 | JUN	TNF is trans activated by TBX21 [98] and is induced by JUN [99].
IL23	1	(JUN & FOS) & MAPK14-p38 & NFKB & STAT3	IL23 is up-regulated by AP1, p38 and NFKB/RELA [100] and STAT3 [101].

IL4	1	NFAT & (STAT5 | MAF | GATA3) & !TBX21	IL4 is produced by Th2 cells. Its production requires NFAT (blocked by FOXP3, see [102]), proliferation and GATA3. It is inhibited by TBX21 (cooperating with RUNX3, see [103]) and IRF1 [104].
IL10	1	SP1 & MAF & (GATA3 | STAT3 | TGFB | STAT1)	IL10 production requires SP1, MAF, JUN, GATA3 [105], TGFB [106] STAT1 or STAT3 [107].
IFNG	1	!FOXP3 & !STAT3 & NFAT & (TBX21 | STAT4)	IFNG production is triggered by TBX21 or STAT4 in conjugation with NFAT, in the absence of FOXP3 and STAT3.
FOXO3	1	(FOXO3 | TP53) & !miR-155-5p & !SIRT1	FOXO3 transcription is activated by TP53, and then by itself. miR-155-5p is predicted to target FOXO3 mRNA and SIRT1 deactivates FOXO3 proteins by deacetylation
TP53	2	(MYC | TP53) & !MDM2 & !SIRT1	TP53 is sequestered in the cytoplasm by MDM2 [108109] and is rendered inactive by SIRT1 deacetylation [110]. In the absence of these 2 negative regulators, TP53 is able to upregulate its own transcription [111].
TP53	1	!MDM2 & !SIRT1	
MDM2	2	(NFAT | SP1) & AKT & TP53 & !miR-155-5p	MDM2 transcription is activated by NFAT [112] and TP53 [113]. PI3K/AKT1 phosphorylation is required for its nuclear translocation [114] miR-155-5p is predicted to target MDM2 mRNA.
MDM2	1	NFAT & AKT	
SP1	1	(STAT3 | CDKN1A-p21) & !TP53 & !NFKB	STAT3 binds to the SP1 promoter activating it, while NFKB, CDKN1A and TP53 bind to repress transcription [115].
MYC	1	(STAT4 | STAT3) & !miR-34c-5p & !PRDM1-BLIMP1	MYC requires STAT4 [116], STAT3 [117] to be transcribed, but also the absence of BLIMP1 [118]. MYC is a validated target of miR-34c-5p [119120121].
STAT1	1	IFNGR & !miR-34c-5p	IFNGR activates STAT1 [122], which is predicted to be a miR-155-5p and miR-34c-5p target.
STAT4	1	IL12R & !GATA3	IL12, a Th1 polarizing cytokine activates STAT4, in the absence of GATA3 [123].
STAT5	2	IL2R:2 | IL4R:2	STAT5 is activated by IL-4 and IL-2 receptors [124]. A high level of IL2R promotes a high level of STAT5, required to activate cell proliferation [91].
STAT5	1	!IL2R:2 & !IL4R:2 & (IL4R:1 | IL2R:1)	
STAT6	1	IL4R	IL4R binding to IL4 signals through STAT6 [125] to activate GATA3 transcription.
STAT3	1	(TGFBR | IL6R | IL23R | IL1R) & !SIRT1 & !miR-155-5p & !miR-34c-5p	STAT3 is activated by Th17 polarizing cytokine receptors (TGFBR, IL6, IL23 and IL1). miR-155-5p and miR-34c-5p are predicted to target STAT3 mRNA. SIRT1 deacetylates STAT3 [126].
IL2R	1	IL2 & CGC & STAT5:2	The IL-2 receptor requires the common gamma chain (CGC) and IL2RB sub chains, and activates STAT5 upon binding. The IL2RA sub chain is needed for the high affinity response.
IL2R	1	IL2 & CGC	
IL6R	1	IL6 & !miR-34c-5p	IL6R is activated when ligand IL6 binds. miR-34c-5p is predicted to target IL6R mRNA.
TGFBR	1	TGFB & !miR-34c-5p	TGFBR is activated when ligand TGFB binds. miR-34c-5p is predicted to target TGFBR mRNA.
IL21R	1	MAF | RORC	The IL-21 receptor requires the GP130 and CGC sub chains, and activates STAT3 upon binding.
TNFR	1	TNF	TNFR is activated when ligand TNF binds. miR-34c-5p is predicted to target TNFR mRNA
IL23R	1	(RORC & STAT3) | IL23	STAT3 and RORC activate IL23R transcription [127]. IL23R is activated by IL23.
IL4R	2	CGC & IL4 & STAT5	The IL-4 receptor requires the CGC and IL4RA sub chains and activates STAT6 and STAT5 upon binding. IL4R is activated by bound IL4.
IL4R	1	IL4 | (CGC & IL4)	
IL10R	1	IL10 & !miR-34c-5p	IL10R is activated by bound IL10 and is a predicted target of miR-34c-5p (IL10RB).
IFNGR	1	IFNG & !miR-155-5p	IFNG binds to IFNGR, thus activating it. miR-155-5p has been shown to target IFNGR1 [128].
IL22	1	RORC & TBX21 & AHR & !MAF	AHR together with RORC and TBX21 activate IL22 production [12992]. MAF mediates TGFB dependent suppression of IL22 [130]
IL9	1	SPI1-PU1	PU1 activates transcription of IL9 [131].
TBX21	2	(TBX21 & STAT1) | (TBX21 & STAT4)	STAT1 and/ or STAT4 activate TBX21 which positively regulates itself [132 133].
TBX21	1	(STAT1 | STAT4)	
GATA3	2	(GATA3 | STAT6 | STAT5) & NFKB & !miR-34c-5p	GATA3 is the main transcription factor of the Th2 lineage. It is activated by STAT6 with NFKB and self-maintained [134]. GATA3 is a predicted target of miR-34c-5p.
GATA3	1	(GATA3 | STAT6 | STAT5) & NFKB	
RORC	1	TGFBR & STAT3	RORC is activated by STAT3 [135]. TGFB promotes Th17 differentiation [136].
FOXP3	1	STAT5 & NFAT & FOXP3 & !miR-34c-5p	FOXP3 is the main known marker of natural and induced regulatory T cells. It is activated by NFAT, TGFB (through SMAD3 [137]) and STAT5 [138].

FOXP3	1	STAT5 & NFAT & !FOXP3 & !STAT1 & !(STAT3 & RORC) & !miR-34c-5p	FOXP3 can be inhibited by STAT1 [85], STAT3 [138] and RORC. We assume that FOXP3 can maintain its own expression [85] in the presence of NFAT and STAT5. We also assume that STAT3 and RORC can cooperate to inhibit FOXP3.
BCL6	1	(STAT3 & STAT4) | SPI1-PU1 & !(TBX21 & miR-34c-5p)	BCL6 requires STAT3 or STAT4 to be transcribed, but also the absence of TBX21 [139]. SPI1 (PU.1) also activates the BCL6 promoter [140]. BCL6 is a predicted target of miR-34-5p.
SPI1-PU1	1	TGFBR & AKT & IL1 & !miR-155-5p	TGFBR promotes SPI1 activity [131] as well as PKCd phosphorylation (activated with DAG, PIP3-PDPK1 and SH3BP2) [141] and IL1 [142] and miR-155-5p inhibits its translation [143].
MAF	1	(TGFBR & STAT3) & !miR-155-5p	MAF requires IL6, stimulates IL21 and IL4 promoters [144] and is inhibited by TGFB signalling [93] and STAT3 to be transcribed [145]. Required for Tfh and Th17 differentiation. It's a predicted target of miR-155-5p.
AHR	1	SP1 & NFKB | (MYC & GATA3) | (FOS & JUN) & !miR-34c-5p	Induced by SP1 [146]. GATA3, NFKB(RELA), MYC, SPI1, FOS, JUN, CREB1, EP300, STAT3 and JUND have validated binding sites on ENCODE. miR-34c-5p is a predicted inhibitor of AHR mRNA translation.
IL12R	1	IL12	IL12R is activated by bound IL12.
IL1R	1	STAT3	STAT3 is assumed to account for IL1R differential expression in naive Th and Th17 cells [147].
PRDM1-BLIMP1	1	(JUN & FOS & CREBBP & !BCL6) | (STAT3 & !BCL6)	BLIMP1 requires AP1 (JUN+FOS) [148] or STAT3 [149] and absence of BCL6 to be expressed [150151].
SIRT1	1	FOXO3 | (FOXO3 & CREB) & !TP53 & !miR-155-5p	CREB and FOXO3 activate SIRT1 transcription, while TP53 inhibits it [152].
miR-34c-5p	2	GATA3 & MYC & (TP53 | FOXO3 | SP1)	miR-34c has been shown to be regulated by TP53, SP1 [153] and FOXO3. ENCODE data for ChIP-Seq reveals bound GATA3, MYC and FOS. We assumed the that for transcription of miR-34c to occur, either TP53 or FOXO3 should be available, together with GATA3 and MYC. GATA3 and MYC have been shown to control and maintain T cell proliferation [154]. If TP53, FOXO3 or SP1 are available, miR-34c expression was considered to be enhanced.
miR-34c-5p	1	GATA3 & MYC	
miR-155-5p	1	NFKB & NFAT & (TBX21 | GATA3 | RORC | FOXP3)	miR-155 starts being expressed shortly after activation, requiring NFAT and NFKB [155156157]. It is furthermore important for differentiation of Th1, Th17 [25] and iTreg [158] cells, so we assumed it also required one of the master transcription factors for each subtype.
miR-155-5p	1	NFKB & NFAT	
MAP4K1-MEKKK	1	ZAP70	MEKKK is activated downstream of ZAP70 (via LAT) and leads to JNK activation [159].

& = and ; | = or ; ! = NOT

For the dynamic simulations a script was done in R, to calculate node evolution, as an average of its value (which can be 0, 1 or 2) across multiple parallel simulations for corresponding discrete time points using an asynchronous updating scheme. This results in time-course graphics as can be seen in Figures 6 and 7.

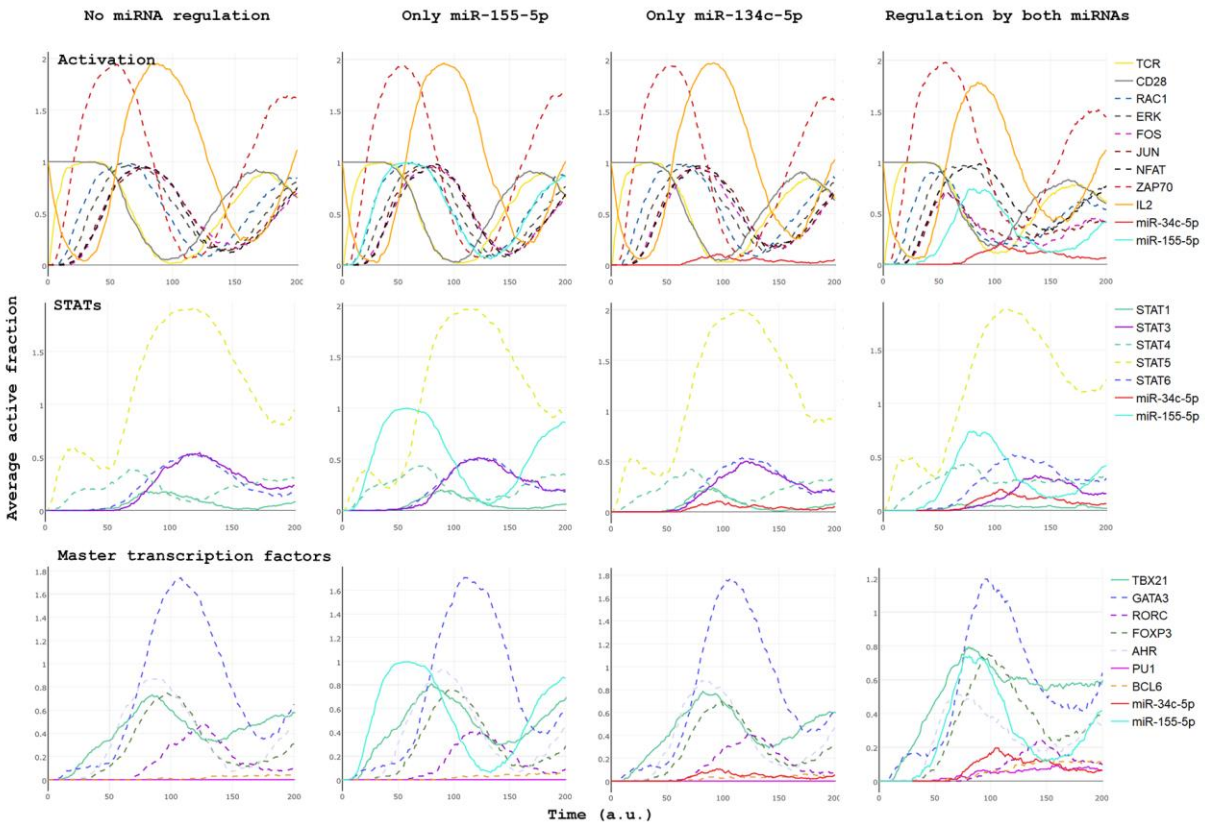


Figure 6 - Node behaviour/evolution (average active fraction) obtained through asynchronous simulation, relative to CD4 T cell activation components, and CD4 subtype associated STAT proteins and master transcription factors, with no miRNA regulation, with both miR-155-5p and miR-34c-5p regulation, and with each miRNA separate. This represents relative activities of each node in the system at a given point in time, a snapshot of the behaviour of the expanding TCR + IL2 stimulated T cell population.

In models of differentiation of more T_H subtypes, steady-sates are obtained only for T_H1 and T_H2 phenotypes, with TCR and IL2 stimulation. Therefore, it is likely that it is in one of these cell types that miR-34c expression is activated, particularly in T_H2 cells. Moreover, only when both miR-155-5p and miR-34c-5p are modulating the system, is their pattern of expression similar to what was observed experimentally in primary CD4 T cells, and with TP53, FOXO3, SP1, GATA3 and MYC controlling miR-34c expression (Figure 6). Also, only with both miRNAs, PU.1 (SPI1) activity is up-regulated, and a T_H9 reprogramming from T_H2 is suggested. The IL6/STAT3/RORC axis seems to be the most down-regulated. Finally, quiescence seems to be promoted by miR-34c-5p (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. These GATA3 T_H2 cells may be down-regulating GATA3, directing the cells to a quiescent state. Whether this may be into a T_H9 effector cell or a memory phenotype needs to be assessed experimentally. A visual representation of the predicted effects of miR-34c-5p in naïve CD4 T cells can be observed in Figure 8.

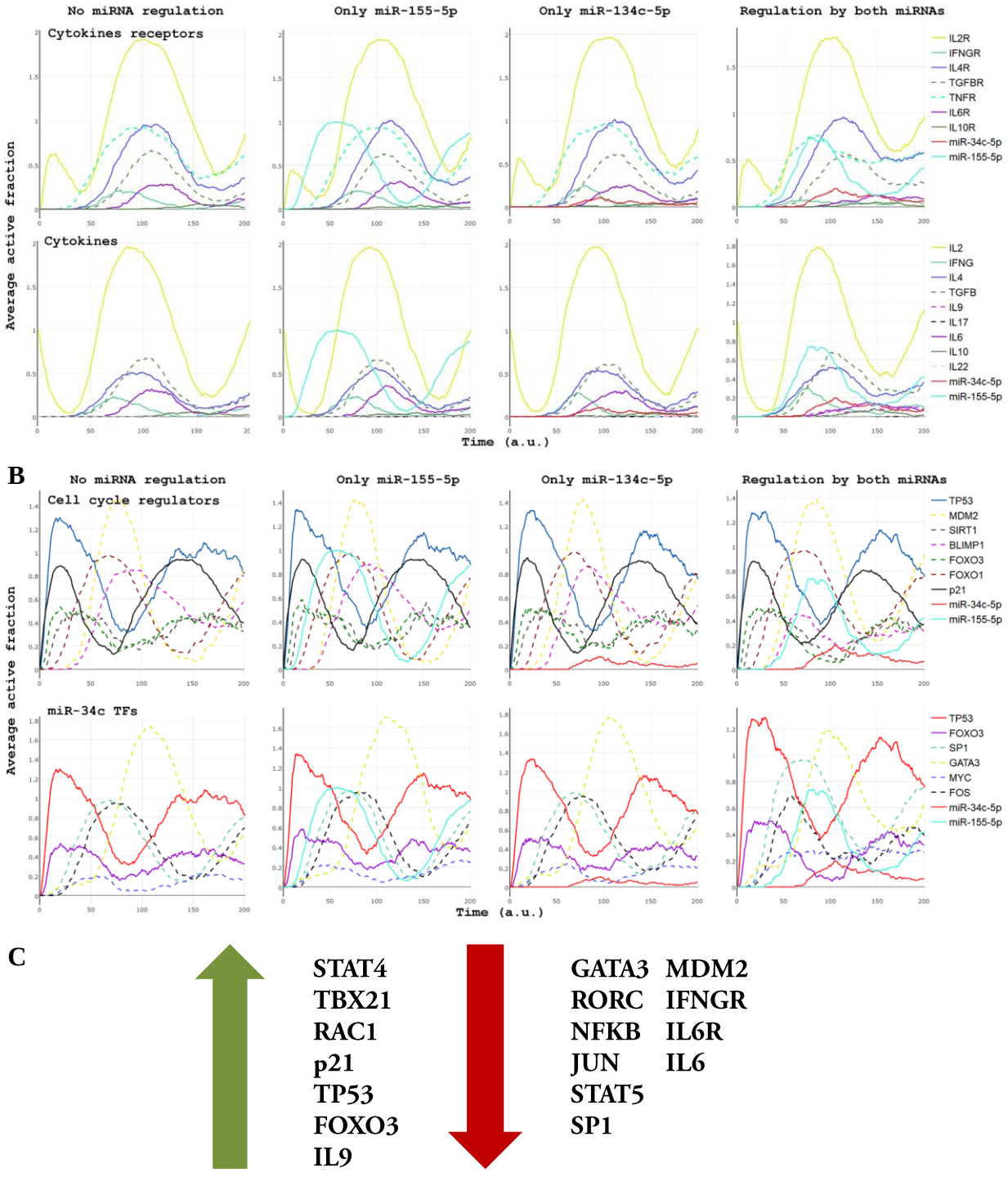


Figure 7 – Node behaviour/evolution (average active fraction) obtained through asynchronous simulation, relative to CD4 subtype associated A) intrinsic and secreted cytokines, as well as production and membrane integration of their receptors B) cell cycle regulation and activity of miR-34c transcriptions factors considered, with no miRNA regulation, with both miR-155-5p and miR-34c-5p regulation, and with each miRNA separate. C) Summary of relevant direct on indirectly up and down-regulated genes/proteins after miR-34c-5p maximum expression.

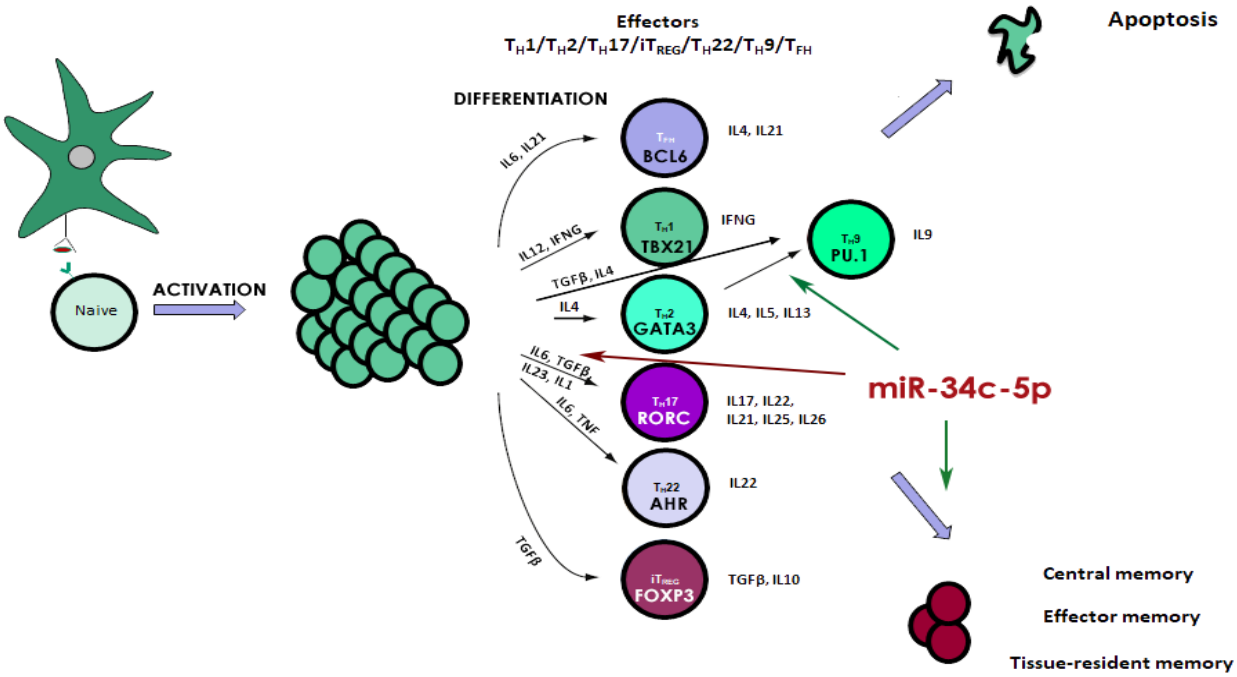


Figure 8 – A summary of the predictions made. Known *in vitro* stimuli to differentiation of CD4 T cells and each subtype major secreting cytokines. TGFβ and IL4 combined give rise to PU.1 expressing T_H9 cells. T_H9 can arise from T_H2 cells. At the end of an immune response, cells suffer apoptosis, 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. 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).

This work leaves the door open to further experimentation, in order to validate the predictions obtained by the analysis of all models. The next suggested step is testing for the levels of expression of the relevant genes by qPCR and the protein levels by western blotting. For such, a proliferation assay is conducted to freshly isolated naïve CD4 T cells, using the EasySep kits (StemCell Technologies). A schematic of the assay to be conducted can be observed in Figure 11 (Task 2), since these assays are intertwined with Task 2.

Task 2 - Elucidation of the mechanisms of transcriptional regulation of miR-34c-5p and the underlying differences between naïve and memory CD4 T cells.

Transcription factors known to regulate the expression of miR-34c and miR-155 were chosen from literature mining and also from the databases TRRUST [160], TransmiR [161] and TRANSFAC [162]. Prediction of transcription factor binding sites (TFBS) was done using JASPAR [163], using the available matrixes for relevant TFs, for three promoter sequences A, B and C that are thought to be regulating miR-34c transcription (Figure 3). TFs were chosen considering experimentally validated interactions found on databases and literature, taking into account that some of them might act both as an activator and/or a repressor. Validated TFBS and DNAseI

hypersensitivity sites (DHS) were obtained with ENCODE [164] annotated ChIP-seq and DNAse-seq data in the UCSC genome browser [165], as well as a CpG island sequence in the BTG4/miR-34b/c bidirectional promoter and conserved TFBS for major miR-34 known TFs TP53, FOXO3 and SP1. TSS (arrows in Figure 3) were retrieved from FANTOM5 CAGE-seq data [166] [167] in the ZENBU browser [168]. Promoter and enhancer regions were also obtained from epigenomic data in the international human epigenome consortium (IHEC) data portal [169], ENCODE and/or the NIH Epigenome Roadmap [170]. This includes chromatin states given by the combined signature of histone marks, with both the core 15-state model and the 18-state model, and individual activating and inhibitory histone marks for naïve and memory CD4+ T cells, Th17 and Treg cells.

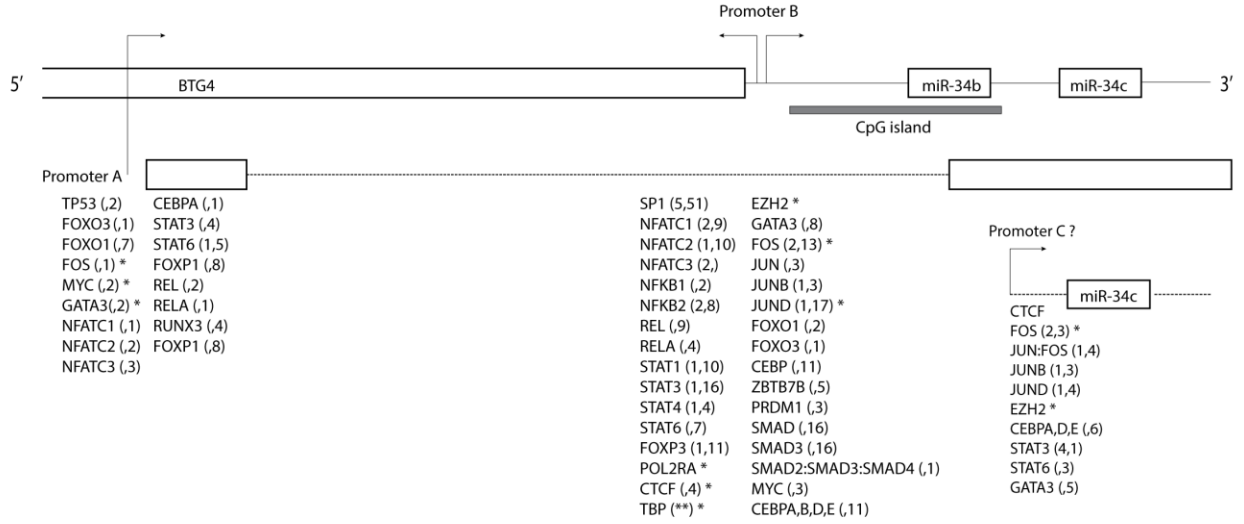


Figure 9 – BTG4 and miR-34-b/c region in chr11:111,380,000-111,387,000 (hg19). An active promoter responsible for an alternative transcript (promoter A, 461 bp, chr11:111,381,003-111,381,522) has been described in hippocampus cells, with ChIP-seq revealing bound GATA3, MYC and FOS. The bidirectional BTG4 miR-34b/c promoter (promoter B, 1353 bp, chr11:111,382,651-111,384,072) is the one described regulating transcription of an initial miR34b/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 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, 588 bp, chr11: 113,835,191-111,384,315), encoding a putative direct pre-mir-34c. * ENCODE validated TFs in Th0 and Th1 cells. ** Only TATA binding protein site detected bound to DNA.

Based on this analysis, we performed bisulfite sequencing PCR to cell lines that have been shown to be either methylated (CaCO2) or unmethylated (NCM or normal colon mucosa), to detect methylation status of the CpG islands in the promoter B region. Only islands 2 and 3 were successfully amplified. Contrarily to what was described, the CpG islands in both cell lines tested, after analyzing the sequencing results with MethylViewer [171], were found to be methylated, which might be explained by the high variability observed even in different cultures of the same cell line,

probably owing to the time of sampling. To solve this problem in the proper experiments to primary naïve and memory CD4 T cells, an additional control using azacytidine, a demethylating agent, may be used as a positive demethylation control in order to compare the results.

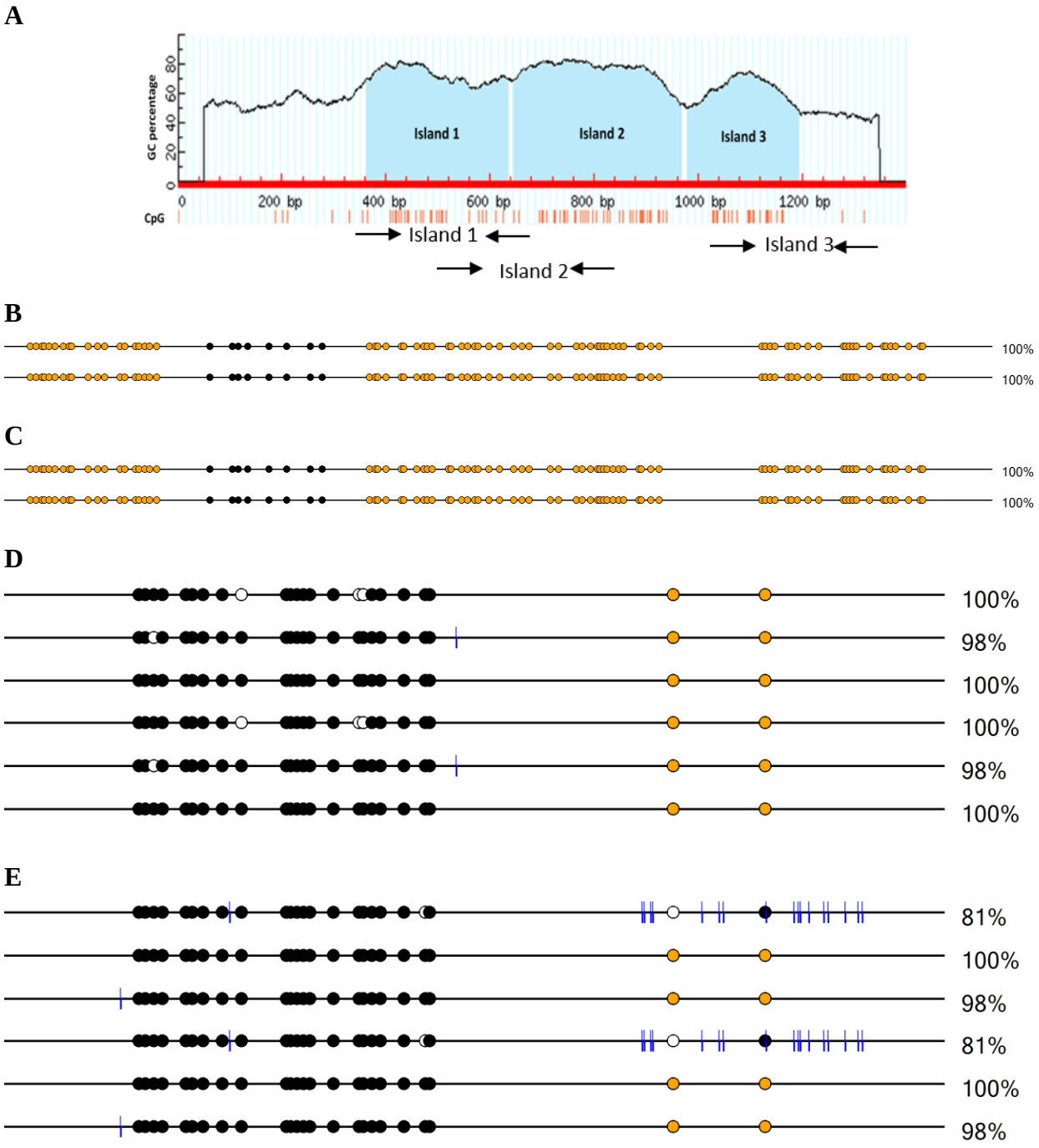


Figure 10 – A) CpG islands and primers designed for bisulfite sequencing PCR of the 3 islands to be amplified and; Methylation status of CpG islands 2 and 3 after bisulfite conversion of genomic DNA and bisulfite sequencing PCR and analysis with MethylViewer [171] for: B) CaCO2, island 2;

C) NCM, island 2; D) CaCO2, island 3; E) NCM, island 3. Black dots indicate methylated cytosines, white dots represent demethylated cytosines that were converted to uracil by bisulfite.

There was no time for the conclusion of this task, since we could only start performing the isolation of naïve and memory CD4 T cells from buffy coats in January 2019. But the data obtained opens the door for experiments (Figure 11) that may be performed by colleagues still working at the lab. They will be able to perform the proliferation assays up to 120 h post TCR stimulation with IL2, check purity of isolated cells and their activation status by flow cytometry, collect DNA for the bisulfite conversion of genomic DNA from both naïve and memory CD4 T cells and therefore test the methylation status of the CpG islands. RNA and protein extracted from naïve cells can be used to test the expression levels of the predicted miR-34c-5p targets and the TFs, as well as the protein levels at each time point and validate the model. As for the reporter assays, vectors containing GATA3, TP53, FOS and MYC, recently acquired, can be used to determine whether these are *de facto* binding to the promoters A, B and C, cloned into the vector psiCHECK2 (Promega), based on downstream luciferase activity measurement.

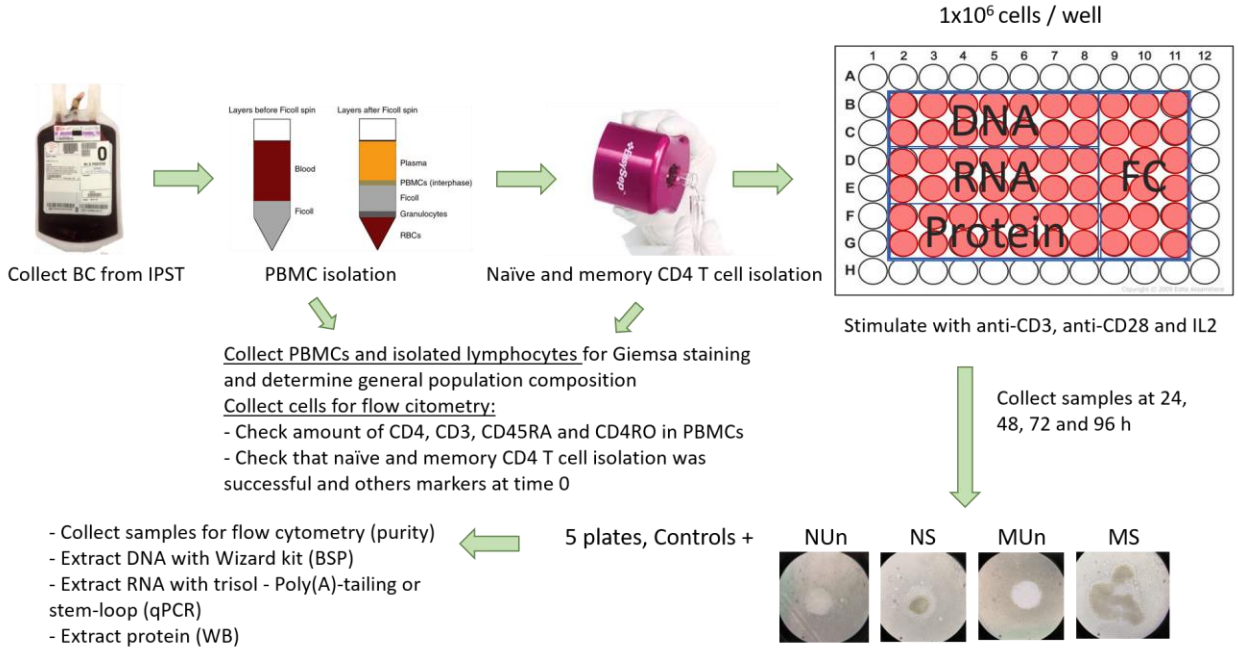


Figure 11 – Diagram of experiments to be conducted in the future. BC – buffy coat; IPST – Portuguese Blood and Transplantation Institute; PBMC – peripheral blood mononuclear cells; NUn – Naïve unstimulated cells; NS – Naïve stimulated cells; MUn – Memory unstimulated cells; MS – Memory stimulated cells.

Task 3 - Characterization of predicted viral encoded sncRNAs and of their functional impact in CD4 T cells.

We aimed to demonstrate whether 2 HIV1 and 1 HIV2 conserved small non-coding RNAs, detected in an analysis of small-RNA sequencing dataset from infected CD4 T cells, and predicted *in silico* to be contained in a genomic region presenting pre-miRNA-like characteristics were *bona fide* miRs. To verify if the putative sncRNAs are processed by the miRNA machinery, we transfected HEK293 cells with pHTP0 vectors with the sncRNA sequences immediately upstream of the H1 Pol III promoter (vector map in Figure 12). We relied on standard PCR procedures in the beginning, for detection of transcripts and precursor miRNAs. Unfortunately, the results obtained with this methodology were inconclusive (data not shown), since there were specificity problems likely due to genomic and/or vector sequences. Therefore, we designed stem-loop primers to synthesize sncRNA specific cDNA (Figure 13). We successfully detected miRNA amplification through real-time PCR using a specific reverse transcription stem-loop and forward primer for each sncRNA. We also tested the non-transfected cells and used as controls samples where no reverse transcriptase (RT) was added to the RT reaction and a no template control. While there is a weak amplification signal in 2 of the non-transfected samples, this is most likely residual amplification of unspecific binding of the primers to another residual DNA or cDNA. We can see that when the PCR products are separated by electrophoresis, the non-transfected sample are the only ones that have unspecific amplification of several fragments (Figure 13), most likely endogenous retroviral elements.

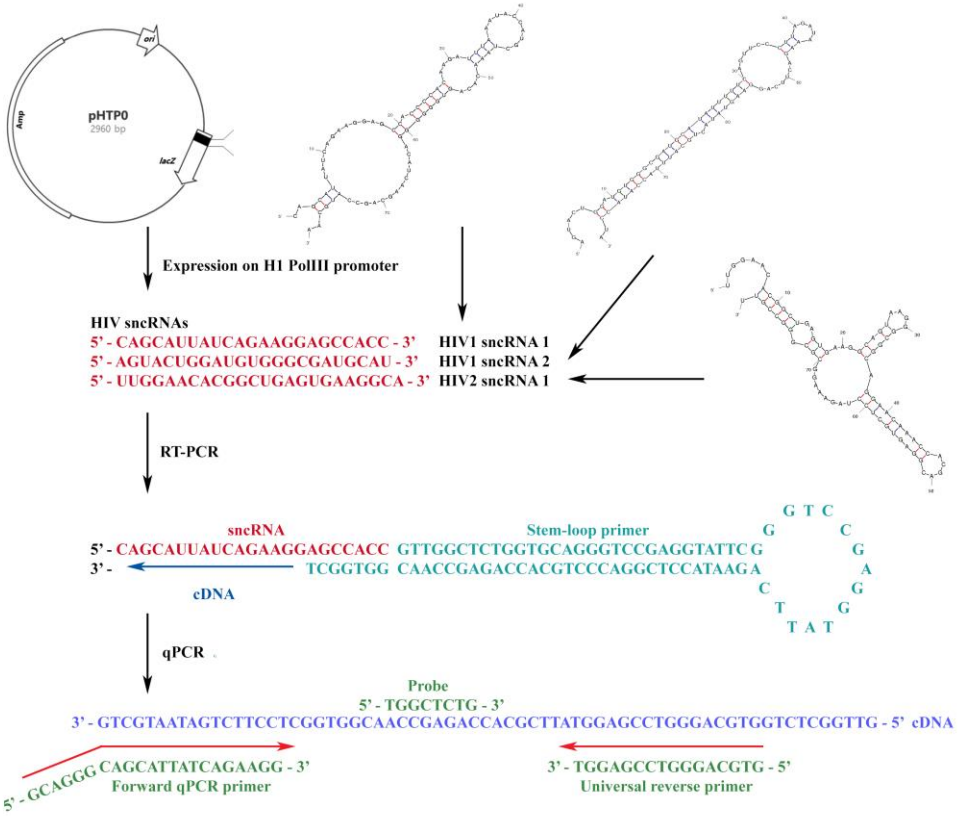


Figure 12 – Protocol for stem-loop cDNA synthesis and sncRNA detection by real-time PCR of the putative HIV sncRNAs. Secondary structures were obtained with *mfold*.

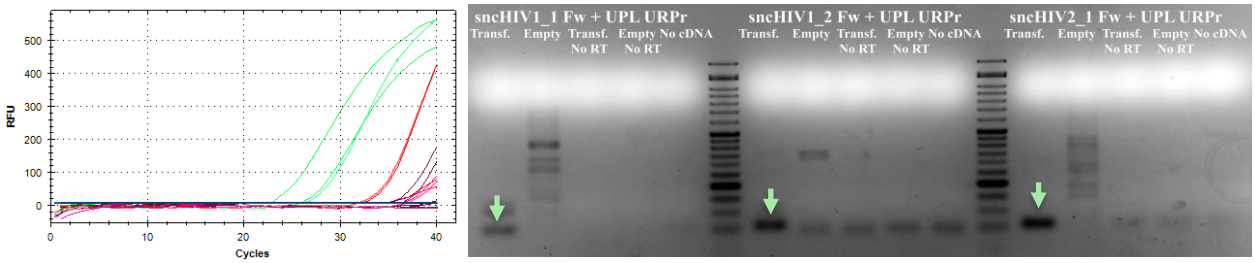
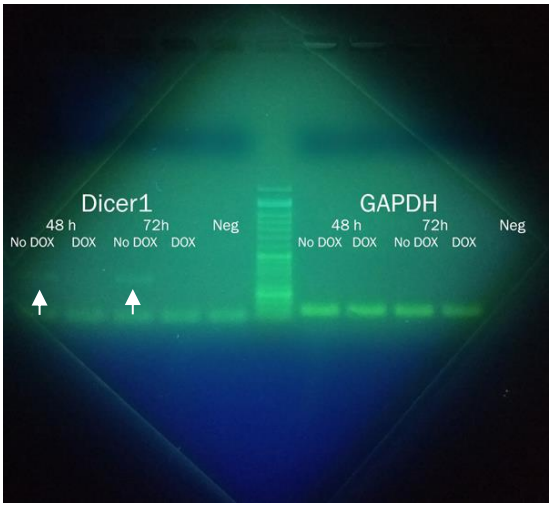


Figure 13 – Stem-loop real-time PCR for detection of the 3 putative HIV sncRNAs. Approximately 60 bp size fragments, as expected. HIV1 sncRNA 1 and 2, HIV2 sncRNA 1; Not Transfected cells; No RT/template controls.

Facing these results, we decided to test whether these sncRNAs are being processed by Dicer, using a Tet inducible Dicer RNAi HEK293T cell line available in the lab. We tested if these cells lost the ability to accumulate the seRNAs when treated with doxycycline, analogous to tetracyclin to confirm that the amplified products result from Dicer processed RNAs.

A



B

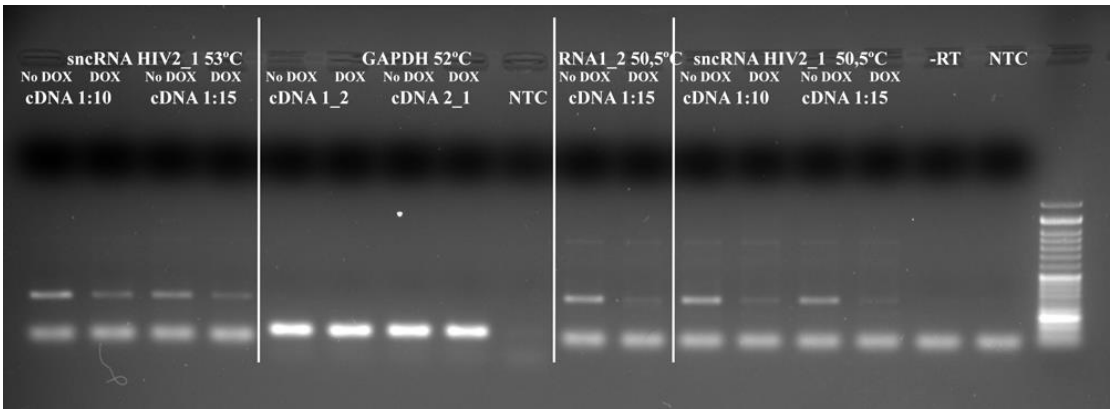
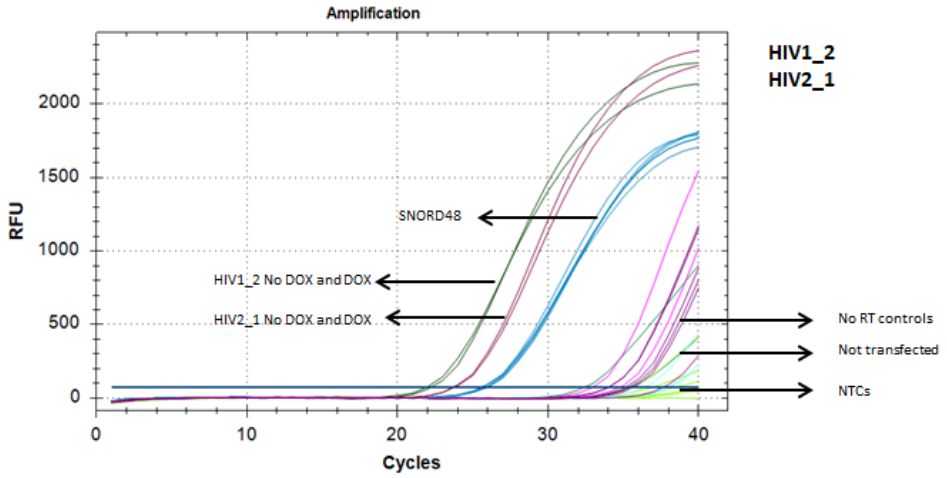


Figure 14 – A) PCR to Poly(A)-tailed cDNA of HEK293T-DicerKD cells to determine *de facto* knockdown of Dicer and **B)** PCR to Poly(A)-tailed cDNA of HEK293T-DicerKD cells transfected with pHTP0 + putative sncRNAs HIV1_2 and HIV2_1. GAPDH was used as reference gene.

A



B

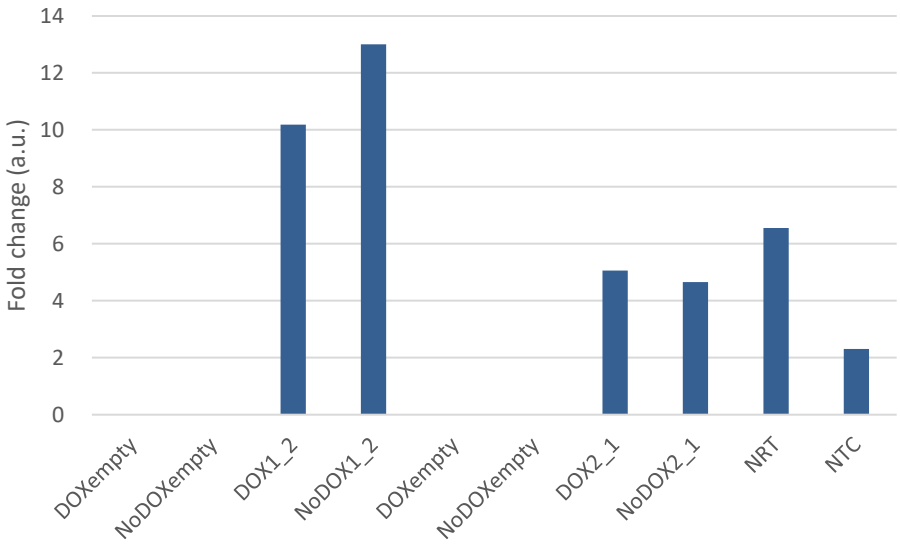


Figure 15 – Stem-loop qPCR to putative HIV1_2 and HIV2_1 sncRNAs. SNORD48 was used as the reference gene. A) Real-Time quantitative PCR curves for all loaded samples and B) Relative quantitation using the $2^{-\Delta CT}$ method [ΔCt = Ct(sncRNA_n) − Ct(SNORD48_n)].

We observed that there was some degree of knockdown by semi-quantitative PCR (Figure 14A) and that the putative HIV1-sncRNA2 and HIV2-sncRNA1 seem to be down-regulated in the Dicer knockdown extracts (Figure 14B). Stem-loop qPCR performed to those sncRNAs revealed that at least HIV1_sncRNA1 seems to depend on Dicer for its biogenesis (Figure 15B).

5. Publications and Conference Communications

5.1. Papers in scientific peer-reviewed journals

One manuscript draft is partially written, with the proposed title 'A methodology for assessing miR-34c-5p regulation and function in naïve CD4 T cell activation and differentiation networks using logical modelling' and will be finalized hopefully until the thesis is delivered, but if not possible, in the following months.

5.2. Communications at scientific conferences

Oral communications:

1. **Domingues, N.**, Pinto, F. and Gama-Carvalho, M. (2017) sncRNAregulatory networks in Th cell activation, differentiation and HIV infection. *2nd BioSys summer retreat*, Beja, Portugal, 6-7 October 2017.

Poster presentations:

1. **Domingues, N.**, Pinto, F. and Gama-Carvalho, M. (2017) sncRNA regulatory networks in T cell activation and viral response, 1st BioSys/BioISI summer retreat, Santa Cruz, Portugal, 23-24 July 2016.
2. **Domingues, N**., Pinto, F. and Gama-Carvalho, M. (2017) sncRNAregulatory networks in Th cell activation, differentiation and HIV infection. *2nd BioSys summer retreat*, Beja (Portugal). 6-7 October 2017.
3. **Domingues N.**, Pinto F., Espada de Sousa A., Gama-Carvalho M. "Exploring miR-34c-5p modulation of effector CD4 T cell differentiation through logical modelling", at the 44th Portuguese Society of Immunology Meeting, IMM, Lisbon, Portugal, 25-27 June 2018.
4. **Domingues N.**, Pinto F., Espada de Sousa A., Gama-Carvalho M. "Exploring miR-34c-5p modulation of effector CD4 T cell differentiation through logical modelling", at the 5th European Congress of Immunology, RAI, Amsterdam, Netherlands, 2-5 September 2018.
5. **Domingues N.**, Pinto F., Espada de Sousa A., Gama-Carvalho M. "Exploring miR-34c-5p modulation of effector CD4 T cell differentiation through logical modelling", at the 8th ptRNA meeting, i3S, Porto, Portugal, 24-25 January 2019.

6. Attendance at Scientific Meetings, Courses and Training Visits

6.1. Conferences

1. 2nd Computational Biology Meeting, Porto, Portugal, 15-17 June 2016.
2. 1st BioSys/BioISI summer retreat, Santa Cruz, Portugal, 23-24 July 2016.
3. Encontro com a ciência e tecnologia em Portugal, Centro de Congressos de Lisboa, Lisbon, Portugal, 3-5 July, 2017.

4. Chem&BioChem postgraduate students meeting, Lisbon, Portugal, 9 February 2017.
5. 2nd BioSys/BioISI summer retreat, Beja, Portugal, 6-7 October 2017.
6. 44th Portuguese Society of Immunology Meeting, Instituto de Medicina Molecular, Lisbon, Portugal, 27-29 June 2018.
7. Travel grant awarded for the 5th European Congress of Immunology, RAI, Amsterdam, Netherlands, 2-5 September 2018.
8. 8th ptRNA meeting, i3S, Porto, Portugal, 24-25 January 2019.

6.2. Courses and Training Visits

1. Career development, organised by *BioISI*, Department of Plant Biology, Lisbon, Portugal, 28-29 April 2016.
2. Introduction to Entrepreneurship, TecLabs – Centro de Inovação, Lisbon, Portugal, 22 February 2017.
3. Bio and scientific ethics, organised by *BioISI*, Department of Plant Biology, Lisbon, Portugal, 27-28 April 2017.
4. Innovation legal warfare - Industrial and intellectual property, TecLabs, Lisbon, Portugal, 24 May 2017.
5. Training visit at Universidade de Trás os Montes e Alto Douro (UTAD), Cytogenetics Lab, Vila Real, Portugal, May 2018.
6. Workshop: Integrative Approaches in Neurodegeneration, University of Lisbon, Portugal, 21-23 June 2018.

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