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# Evaluating miR-34c-5p impact in naïve CD4 T cell differentiation using logical modelling

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# Abstract and key words

Key Words: miR-34c-5p; miR-155-5p; Logical modelling; CD4+ T cell differentiation

Micro RNAs (miRNAs) are small non-coding single stranded RNAs who act as post-transcriptional silencers of gene expression through the translational inhibition or degradation of mRNA, being involved in the regulation of most cellular processes, including haematopoiesis, the differentiation of multipotential haemotopoietic stem cells into blood cellular components, such as T lymphocyte cells.

CD4+ T helper cells (Th cells), one of two main classes of T lymphocytes, coordinate other immune system cells, and are further classified in several subtypes, which express different transcription factors and cytokines and are produced preferentially in different cellular environments.

Logical models (Bornholdt, 2008; Wang et al., 2012) are defined by Logical Regulatory Networks (LRG), a series of nodes and edges corresponding to the regulatory components of a system (example: proteins), and the interactions between nodes (example: activations and inhibitions). Nodes are also associated with discrete variables, which represent their activity level, and logical rules, whose role is to determine the activity level of a node.

Through the adaptation of a previously published model (Abou-Jaoudé et al., 2015), with new nodes and interactions, we studied the impact of miR-34c-5p on naïve CD4 T cell differentiation, along with miR-155-5p (which we use as a control). We found a set of logical rules able to produce all Th cell subtypes in their preferred environment and analyzed the steady states of the model across thirty-six different initial conditions, representing different cellular environments.

Thus, we conclude that miR-34c-5p may play an inhibitory role in naïve CD4 T cell differentiation into Th9 and Th17 cells, a stimulatory role in the formation of Th2, Th22, Tfh and Treg cells, while playing no role in the formation of Th1 cells. We also found some evidence that miR-34c-5p can inhibit the effect of miR-155-5p in naïve CD4 T cell differentiation.

# Resumo e palavras-chave

Palavras-chave: miR-34c-5p; miR-155-5p; Logical modelling; CD4+ T cell differentiation

Micro RNAs (miRNAs) são pequenos RNAs não-codificantes, com 22 a 23 nucleotídeos de comprimento, que atuam como silenciadores pós-transcricionais da expressão génica através da sua ligação a mRNAs, que resulta na inibição ou degradação do mRNA. Como um único miRNA pode ter múltiplos mRNAs como alvo, estima-se que pelo menos 50% dos genes codificadores de proteínas estão sob a regulação de um miRNA, e a maioria, ou talvez até mesmo todos, os processos celulares estão sob a influência de pelo menos um miRNA. De entre os processos celulares sob a regulação dos miRNAs encontra-se a hematopoiese, o processo de diferenciação de células estaminais hematopoiéticas pluripotentes em componentes celulares do sangue. É este o processo celular que dá origem a muitas das células do sistema imunológico.

O sistema imunológico é um sistema de defesa em profundidade com três camadas. Desta forma, a primeira camada, que raramente é pensada como sendo parte do sistema imunológico, refere-se às barreiras físicas entre o organismo e seu ambiente circundante, tais como a pele e o epitélio gástrico. Por sua vez, a segunda camada, o sistema imunológico inato, fornece uma resposta imune inespecífica contra patógenos estranhos e inclui uma grande variedade de tipos de células, tais como os macrófagos, basófilos e neutrófilos. Finalmente, a última camada do sistema imunológico, o sistema imunológico adaptativo é responsável pelas respostas imunes específicas contra patógenos estranhos específicos, e é composto principalmente por linfócitos B e T.

Os linfócitos T são classificados principalmente em duas classes: células citotóxicas CD8+ e células T auxiliares CD4+ (células Th). Estas últimas actuam como os "maestros" do sistema imunológico, servindo de coordenador de funções das restantes células do sistema imunológico. As células Th também podem ser classificadas em vários subtipos, de acordo com a expressão de conjuntos específicos de fatores de transcrição e citocinas. Por sua vez, os diferentes subtipos de células Th são produzidas preferencialmente em ambientes celulares específicos.

Esta tese pretende avaliar qual o impacto que o miR-34c-5p tem sobre a diferenciação de células T CD4 imaturas através do uso de modelação lógica. Esta tese tem também um interesse especial no miR-155-5p, que é utilizado como controlo, uma vez que se trata de um miRNA extremamente bem estudado cujo potencial oncogénico e papel no sistema imunológico está muito bem documentado na literatura. Por outro lado, a maioria do conhecimento acerca do miR-34c-5p deve-se ao estudo deste miRNA num contexto oncológico. Embora tenham ocorrido sugestões prévias acerca da capacidade deste miRNA de regular genes associados à sinalização de TCR, foi o laboratório hospedeiro que relatou pela primeira vez a expressão de miR-34c-5p em células T CD4+ (Amaral et al., 2017).

Como mencionado previamente, o estudo do impacto do miR-34-c-5p na diferenciação de células T CD4 imaturas é realizado através do uso de modelos lógicos, cuja natureza qualitativa e capacidade de modelar sistemas com até algumas centenas de componentes, permite o estudo de grandes redes regulatórias de sinalização e transcrição nas quais dados quantitativos detalhados estão total ou parcialmente ausentes, tal como no caso de redes regulatórias Th.

Os modelos lógicos (Bornholdt, 2008; Wang et al., 2012) são definidos por Redes Regulatórias Lógicas (LRG), que são constituídas por uma série de nós e arestas, sendo que os primeiros correspondem aos componentes regulatórios de um sistema (tais como proteínas ou genes), enquanto que os últimos referem-se às interações entre os nós (como ativações e inibições). Por sua vez, os nós estão também associados a variáveis discretas, que representam o seu nível de atividade, e a regras

lógicas, cuja função é determinar qual o valor de um nó, e que são escritas com uma série de operadores lógicos tais como "!", "|" e "&" (cujo significado é, respectivamente, "não", "ou" e "e").

Nesta tese, adaptamos um modelo publicado anteriormente por (Abou-Jaoudé et al., 2015), com a adição de novos nós e interações, que correspondem a miR-34c-5p, miR-155-5p, e aos seus alvos e fatores de transcrição, desde que tais alvos e fatores de transcrição sirvam como alvos e origens de interações aos nós que estavam presentes no modelo original. Usando este modelo adaptado como a base, procedemos a criar uma nova série de regras lógicas, devido a necessidade de criar regras lógicas para os nós que estão a ser adicionados, e a necessidade de adaptar as regras lógicas dos nós que estavam presentes no modelo original, mas que devido as nossas adições, passaram agora a interagir com os nós que passaram a estar nesta versão do modelo. Após realizar esta tarefa, procedemos à obtenção dos estados estacionários dessa versão do nosso modelo modificado para um conjunto de trinta e seis condições iniciais diferentes (correspondendo a nove ambientes de interesse especial e as quatro combinações possíveis de ausência ou expressão dos nossos dois miRNAs de interesse), que são depois analisados de forma a avaliar o impacto do miR-34c-5p na diferenciação de células T CD4 imaturas. Infelizmente, a análise dos estados estacionários obtidos mostrou que vários subtipos das células Th não eram obtidos nos ambientes em que esses subtipos de células Th deviam ser produzidos preferencialmente. De forma a resolver este problema, prosseguiu-se a criação de novos conjuntos de regras lógicas. Estes novos conjuntos contrastavam com as regras anteriores por serem mais "leves", isto é menos restritivas, e por procurarem evitar cenários nos quais o modelo adaptado poderia mostrar um nó como estando inactivo, enquanto que o modelo original mostraria o mesmo nó como estando activo. Após vários conjuntos de regras lógicas, finalmente obtivemos um conjunto de regras lógicas que levaram a obtenção de estados estacionários nos quais os diferentes subtipos de células Th eram obtidos nos ambientes em que esses subtipos de células Th são preferencialmente produzidos.

Neste ponto, podíamos simplesmente prosseguir à análise dos estados estacionários de forma a estudar qual o impacto que miR-34c-5p tem sobre a diferenciação das células Th. No entanto, decidimos tomar um caminho diferente. Dessa forma, procedemos a criação de um último modelo adaptado. Nos modelos anteriores, nunca tivemos em conta a existência de provas que provassem se um nó era ou não expresso em células Th. Desta forma, tendo em conta os dados obtidos em (Cano-Gamez et al., 2020), procedemos à identificação dos nós que sabemos que são expressos em células Th. Por sua vez, se um nó não tem provas da expressão em células Th, procedemos à sua eliminação. Desta forma, procedemos à eliminação dos seguintes nós: FLI1, ETS1, TCF4, NR3C1, EGR1, AR, ELF3, FOXO3, FOS, HIF1A and SNAI1. É de ter em conta, que é perfeitamente possível que estes nós sejam na realidade expressos e simplesmente tal informação não esteja atualmente disponível. De qualquer forma, a eliminação destes nós tornou necessário a alteração de algumas das regras lógicas.

Após tais alterações procedemos à obtenção dos estados estacionários do último modelo e sua subsequente análise, a partir do qual podemos concluir que o miR-34c-5p desempenha um papel de inibição da diferenciação de células T CD4 imaturas em células Th9 e Th17, ao mesmo tempo que desempenha um papel estimulador na formação de células Th2, Th22, Tfh e Treg, enquanto que no caso da formação de células Th1 parece não ter nenhum envolvimento. Da mesma forma, obtivemos algumas provas de que miR-34c-5p pode inibir o efeito de miR-155-5p na diferenciação de células T CD4 imaturas.

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# List of abbreviations, acronyms and symbols

**Terms related to Macromolecules and cells**

RNA=Ribonucleic acid

mRNA=Messenger RNA

miRNAs= Micro RNAs

Pre-miR=Precursor miRNA

DGCR8=DiGeorge syndrome critical region 8

RISC=RNA-Induced silencing complex

HSPC=Haematopoiesis stem and progenitor cells

MHC-II=Major histocompatibility complex class II protein complex

Anti CD-28=CD-28 antibody

Anti CD-3=CD-3 antibody

**Pathologies**

MS=Multiple sclerosis

EAE=Experimental autoimmune encephalomyelitis

IBD=Inflammatory bowel disease

**Logical Model Terminology**

LRG=Logical Regulatory Network

STG=State Transition Graph

SCC=Strongly Connected Components

HTG=Hierarchical Transition Graph

**Software used in Logical Modelling**

GINsim=Gene Interaction Network simulation

SBML-qual=Systems Biology Markup Language Level 3 standard

MaBoSS=Markovian Boolean Stochastic Simulator

GNA=Genetic Network Analyzer

INA=Integrated Net Analyzer

Graphviz=Graph visualization

SVG=Scalable Vector Graphics

**CD4+ T cell subtypes**

Th0=T helper 0

Th1=T helper 1

Th2=T helper 2

Th9=T helper 9

Th17=T helper 17

Th22=T helper 22

Treg=Regulatory T cells

iTreg = “Induced” regulatory T cells

Tfh=Follicular helper T cells

**Logical Operators**

“!” = “not”

“|” = “or”

“&” = “and”

**Miscellaneous**

CoLoMoTo=Consortium for Logical Models and Tools

TFs=Transcription factors

KO= Knockout

**Logical Model Nodes**

IL1B_e=Environmental Interleukin 1 beta

IFNG_e=Environmental Interferon gamma

IL2_e=Environmental Interleukin 2

IL4_e= Environmental Interleukin 4

IL6_e= Environmental Interleukin 6

IL10_e= Environmental Interleukin 10

IL12_e= Environmental Interleukin 12

IL15_e= Environmental Interleukin 15

IL21_e= Environmental Interleukin 21

IL23_e= Environmental Interleukin 23

IL27_e= Environmental Interleukin 27

TGFB_e= Environmental Transforming growth factor beta

IL36_e= Environmental Interleukin 36

IL33_e= Environmental Interleukin 33

IL18_e= Environmental Interleukin 18

IL25_e= Environmental Interleukin 25

IFNB_e= Environmental Interferon beta

IFNA_e= Environmental Interferon alpha

IL1A_e= Environmental Interleukin 1 alpha

IL29_e= Environmental Interleukin 29

APC= Antigen Presenting cells

TCR= T-cell receptor

CD28=Cluster of Differentiation 28

IFNGR=Interferon gamma receptor

IL36R=Interleukin 36 receptor

IL1R= Interleukin 1 receptor

IL2R= Interleukin 2 receptor

IL4R= Interleukin 4 receptor

IL6R= Interleukin 6 receptor

IL10R= Interleukin 10 receptor

IL12R= Interleukin 12 receptor

IL15R= Interleukin 15 receptor

IL21R= Interleukin 21 receptor

IL23R= Interleukin 23 receptor

IL27R= Interleukin 27 receptor

IFNAR=Interferon alpha and beta receptor

IFNAR1=Interferon alpha and beta receptor subunit 1

IFNAR2= Interferon alpha and beta receptor subunit 2

TGFBR=Transforming growth factor beta receptor

IFNGR1=Interferon gamma receptor 1

IL1RAP=Interleukin 1 Receptor Accessory Protein

IFNGR2=Interferon gamma receptor 2

GP130=Glycoprotein 130

IL6RA=Interleukin 6 receptor alpha

IL12RB1=Interleukin 12 receptor subunit beta 1

CGC=Common gamma chain

IL12RB2=Interleukin 12 receptor subunit beta 2

IL10RB=Interleukin 10 receptor subunit beta

IL10RA=Interleukin 10 receptor subunit alpha

IL4RA=Interleukin 4 receptor subunit alpha

IL15RA=Interleukin 15 receptor subunit alpha

IL2RB=Interleukin 2 receptor subunit beta

IL2RA=Interleukin 2 receptor subunit alpha

IL27RA=Interleukin 27 receptor subunit alpha

IL1R1=Interleukin 1 receptor type 1

IL29R= Interleukin 29 receptor

IL17RB= Interleukin 17 receptor subunit beta

IL18RAP=Interleukin 18 receptor accessory protein

IL18RA=Interleukin 18 receptor 1

IL18R=Interleukin 18 receptor

ST2=Suppression of Tumorigenicity 2

IL25R=Interleukin 25 receptor

IL33R=Interleukin 33 receptor

IL1RL2=Interleukin 1 receptor like 2

IL28RA=Interleukin 28 receptor subunit alpha

TBET=T-box expressed in T cells

GATA3=GATA Binding Protein 3

RORGT=RAR-related orphan receptor gamma t

FOXP3=Forkhead box P3

BCL6=B-cell lymphoma 6

IFN-γ /IFNG=Interferon gamma

IL4=Interleukin 4

IL2=Interleukin 2

IL17=Interleukin 17

IL22=Interleukin 22

IL9=Interleukin 9

IL10=Interleukin 10

IL3=Interleukin 3

IL21=Interleukin 21

IL5=Interleukin 5

IL13=Interleukin 13

IL6=Interleukin 6

STAT1=Signal transducer and activator of transcription 1

STAT3=Signal transducer and activator of transcription 3

STAT4=Signal transcription and activator of transcription 4

STAT5=Signal transcription and activator of transcription 5

STAT6=Signal transcription and activator of transcription 6

cMAF=MAF BZIP transcription factor

PU1=SPI1 proto-oncogene

TGFB=Transforming growth factor beta

SMAD3=SMAD Family Member 3

IRF1=Interferon regulatory factor 1

RUNX3=RUNX Family Transcription Factor 3

NFKB=Nuclear factor kappa B

NFAT=Nuclear factor of activated T-cells

IKB=Inhibitor of kappa B

IL31=Interleukin 31

IL25=Interleukin 25

IL35=Interleukin 35

IL24=Interleukin 24

MYC=MYC proto-oncogene

SNAI1=Snail Family Transcriptional Repressor 1

E2F1=E2F Transcription Factor 1

RUNX1=Runt-related transcription factor 1

TP53=Tumor Protein 53

ZEB1=Zinc Finger E-box Binding Homeobox 1

JUN=JUN proto-oncigene

FOS=FOS proto-oncogene

HIF1A=Hypoxia inducible factor 1 subunit alpha

AKT1=RAC-alpha serine/threonine-protein kinase

JUNB=JunB proto-oncogene

TCF4=Transcription factor 4

EGR1=Early Growth Response 1

AR=Androgen receptor

IRF4=Interferon Regulatory Factor 4

ELF3=E74 like ETS Transcription Factor 3

YY1=YY1 Transcription Factor

SP1=Sp1 Transcription Factor

FOXO3=Forkhead Box O3

ETS1=ETS proto-oncogene 1

NR3C1=Nuclear Receptor subfamily 3 Group C member 1

RBPJ=Recombination signal binding protein for immunoglobulin kappa J region

FLI1=Fli1 proto-oncogene

C/EBPβ/CEBPB=CCAAT Enhancer Binding Protein Beta

SOCS1=Suppressor of cytokine signaling 1
S1PR1=Sphingosine-1-Phosphate receptor 1

SIRT1=Sirtuin (silent mating type information regulation 2 homolog) 1

JARID2=Jumonji and AT-Rich Interaction Domain Containing 2

BACH1= BTB Domain and CNC Homolog 1

FANCJ=BRCA1 associated C-terminal helicase

CSF1R=Colony stimulating factor 1 receptor

CTLA-4=Cluster of differentiation 152

EMT-TFs=Epithelial to mesenchymal transition transcription factors

SHIP1=Inositol polyphosphate-5-phosphatase D

PI3K=Phosphoinisitide 3-kinases

PELI1=Pellino E3 Ubiquitin Protein Ligase 1

c-Rel=Proto-oncogene c-Rel

# 1 Introduction

## 1.1 MicroRNAs

Micro RNAs (miRNAs) are small non-coding single stranded RNAs, with an average length of 22 to 23 nucleotides, whose role is to regulate gene expression by acting as post-transcriptional silencers of gene expression.

Currently half of the known miRNAs have their origin in introns, with only a small part being coded by exons, and the remaining being intergenic (de Rie et al., 2017; Kim & Kim, 2007). MiRNAs undergo transcription by either RNA polymerase II or III, and proceed to fold into a secondary structure with one or more hairpin loops (Yoontae Lee et al., 2002), which are recognized and cleaved by the microprocessor complex, which is comprised of DiGeorge syndrome critical region 8 (DGCR8) and Drosha, giving rise to a stem loop precursor miRNA (pre-miR) with a length of 60 to 70 nucleotides (Y. Lee et al., 2003). This pre-miR is then transported to the cytoplasm where it undergoes a secondary processing step by Dicer, which results in an RNA duplex (Y. Lee et al., 2003; Yoontae Lee et al., 2002), which is incorporated into the RNA-induced silencing complex (RISC), where one strand undergoes degradation while the other forms mature miRNA (Guo et al., 2010).

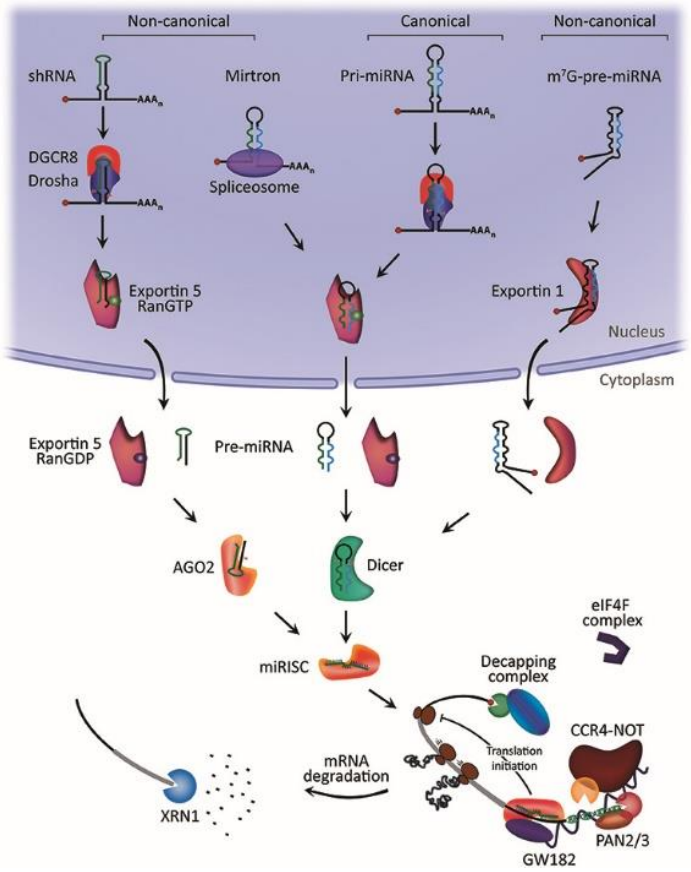


*Figure 1.1.1- General illustration of four different miRNA biogenesis pathways, including both the canonical pathway and three non-canonical pathways. Image taken from* (O'Brien et al., 2018)

Once the mature miRNA is formed, it guides RISC to its target transcript through the use of the partial complementarity between the miRNA and its target mRNA (Filipowicz et al., 2008). The complementarity between miRNA and mRNA is usually high in the seed sequence of the miRNA, which usually corresponds to the second to seventh nucleotides of the 5'-end of the miRNA, and weak on the 3'-end. As such, in general the binding of miRNA and mRNA occurs usually at the 3' untranslated

region of the target mRNA, although it can also occur less frequently at the 5' untranslated region of its target mRNA transcript (Bartel, 2009). This binding leads to the translational inhibition or degradation of mRNA (Filipowicz et al., 2008; Guo et al., 2010). Although, this canonical method of miRNA biogenesis is the one that occurs most of the times, there have been reports of alternative pathways (Figure 1.1.1), as mentioned in (O'Brien et al., 2018).

Given the multi-specificity of a single miRNA that allows it to be able to regulate the expression of multiple genes, and that simultaneously a single gene may be under the regulation of several miRNAs, at least 50% of protein coding genes are estimated to be under the regulation of a miRNA, and therefore most, if not all, cellular processes are under the influence of at least one miRNA.

## 1.2 T lymphocytes

Haematopoiesis, the process of differentiation of multipotential haemotopoietic stem cells into the blood cellular components (Figure 1.2.1), such as erythrocytes and lymphocytes, is one of the many cellular processes under the regulation of miRNAs, as evidenced by the fact that upon Dicer deletion in embryonic stem cells and haematopoiesis stem and progenitor cells (HSPC), there occurs an increase of apoptosis that severely impairs the maturation of HSPC. The existence of distinct miRNA expression patterns in HSPC, during the differentiation and also in mature hematopoietic cell lineages in both mouse and human (Allantaz et al., 2012; Liao et al., 2008; Merkerova et al., 2008; Monticelli et al., 2005; Raghavachari et al., 2014), provides further evidence of the involvement of miRNAs in this process.

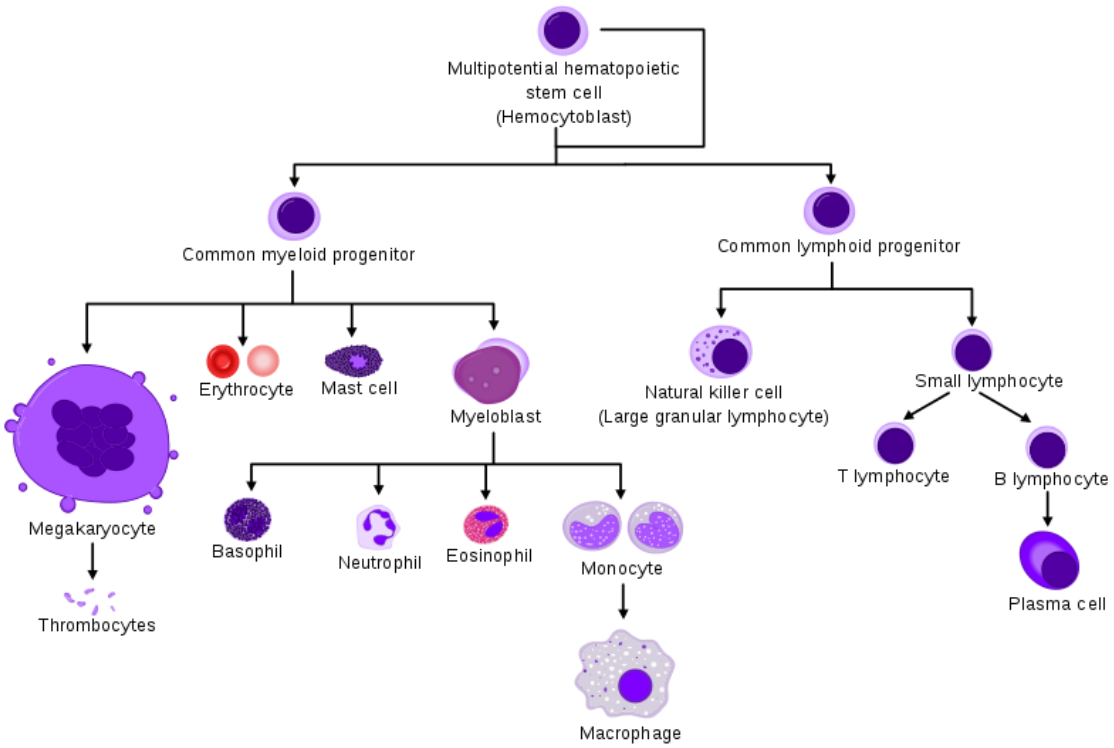


*Figure 1.2.1- Graphical representation of Haematopoiesis, the differentiation of multipotential hematopoietic stem cells in the different cellular components of blood. Figure by A.Rad and M.Häggström. (CC-BY-SA 3.0 license https://creativecommons.org/licenses/by-sa/3.0/deed.en_US).*

Since this is the process that gives origin to the many different cell types of the immune system, and that these cell types are also found to be under the regulation of miRNA, the fact that the dysregulation of specific miRNAs is linked with a myriad of pathological conditions such as

autoimmune diseases, cancers and leukemia, shouldn't come as a surprise (Boldin et al., 2011; Curtale et al., 2010; O'Connell et al., 2008a; Sekuklu et al., 2009; Xiao et al., 2008; Xue et al., 2013; X. Zhou et al., 2008).

The immune system may be seen as a defense in depth system with three layers. The first layer is rarely thought as part of the immune system and refers to the physical barriers between the organism and its surrounding environment, such as the skin. The second layer, the innate immune system provides a non-specific immune response against foreign pathogens and includes a great variety of cell types such as macrophages. The last layer of the immune system, the adaptative immune system is responsible for the specific immune responses against specific foreign pathogens, and is comprised mostly by B and T lymphocytes.

The role of T lymphocytes in the adaptative immune system is to recognize foreign pathogens and initiate a specific immune response to neutralize the danger that they present to the host organism. In order to accomplish this goal, after their production in the bone marrow, T lymphocytes proceed to migrate to the thymus, where they acquire their T-cell receptor (TCR), which then goes through a rearrangement process. Thanks to the differential expression of CD4 and CD8 co-receptors, the TCR rearrangement process can easily be classified into different stages. Those states correspond to four double-negative stages, which are followed by four double-positive phases. These stages of development correspond to positive and negative selection processes whose purpose is to generate T cells whose somatic rearrangements are productive while simultaneously avoiding self-reactivity. The last stage of TCR rearrangement results in either CD4 or CD8 single positive cells, which correspond to mature naïve T lymphocytes (Gameiro et al., 2010). In accordance with which of these co-receptors is expressed, T lymphocytes are classified as either CD8+ cytotoxic cells, whose role is to destroy cells infected by viruses and malignant cells, or CD4+ T helper cells, which act as the conductors of the immune system, by coordinating the functions of other cells of the immune system.

The activation of naïve CD4+ T lymphocytes occurs upon the presentation of antigens by antigen presenting cells (APC cells) with the major histocompatibility complex class II protein complex (MHC-II) to the T cell receptors (TCR) of naïve T cell lymphocytes, an event that results in the initiation of a series of signaling cascades which leads to the activation of several transcription factors that are responsible for whether or not T lymphocytes undergo differentiation (Huang & Wange, 2004). In accordance to the cytokine environment in which it is present, naïve CD4+ lymphocytes will differentiate preferentially into a different subtype of CD4+ T cell. The different preferential environments are presented in Table 1.1.

Although the first two subtypes of CD4+ T lymphocytes, T helper 1 (Th1) and T helper 2 (Th2) cells, where identified in early immune response studies (Mosmann et al., 1986), it has not until recently that studies such as those of (Dardalhon et al., 2008; Soroosh & Doherty, 2009; Veldhoen et al., 2008) lead to the discovery of additional subtypes, such as T helper 9 (Th9) cells, which according to (Kaplan, 2013) are involved in pathogen immunity and inflammatory diseases. The different subtypes of Th lymphocytes are characterized by the production of a specific set of cytokines, which leads to each subtype having different functions in the hosts immune system.

*Table 1.1- Table containing the cytokine environments that result into preferential differentiation of naive T CD4+ lymphocytes into specific CD4+ T cell subtypes, alongisde two addional environments in which naive CD4+ cells undergo no stimulation or only APC presence as the only stimulus.The green spaces correspond to the elements that are present in each of the cytokine environments, while blank spaces correspond to the elements that are absent. As such, an environmnet in which APC and environmnetal IL12 are present leads to the preferential production of the Th1 subtype.*

| | APC | IL12 | IL4 | IL6 | TFGB | IL1B | IL23 | IL21 | IL2 |
|---|---|---|---|---|---|---|---|---|---|
| No stimulation | | | | | | | | | |
| APC only | | | | | | | | | |
| Pro Th1 | | | | | | | | | |
| Pro Th2 | | | | | | | | | |
| Pro Th17 | | | | | | | | | |
| Pro Treg | | | | | | | | | |
| Pro Tfh | | | | | | | | | |
| Pro Th9 | | | | | | | | | |
| Pro Th22 | | | | | | | | | |

Another recent set of discoveries showed, that under certain conditions, some Th cells are able to transform into another subtype. One example of such occurrence are the reports of Th17 cells being able to produce cytokines specific to Th1 cell (Harbour et al., 2015; Y. K. Lee et al., 2009; Nindl et al., 2012; Shi et al., 2008). In this case these Th17 cells went from belonging to the Th17 subtype to being part of a Th1-Th17 hybrid population (Kullberg et al., 2006; Morrison et al., 2013). The existence of these hybrid populations further reinforces the high degree of heterogenicity of CD4+ T cells. It should be noted that even studies performed with polarized CD4+ T cell populations under controlled *in vitro* conditions lead to the creation of hybrid populations (Assenmacher et al., 1994; Bucy et al., 1994; Eizenberg-Magar et al., 2017; Kelso et al., 1999; Openshaw et al., 1995), with segregation of signaling proteins during asymmetric cell division having been proposed as an explanation for such observations (Chang et al., 2007; Verbist et al., 2016).

Regardless, many miRNAs have a regulatory role in the proliferation, activation, development, differentiation and function of T lymphocyte cells, and upon antigen binding to the TCR of a naïve CD4+ T lymphocyte, the miRNA repertoire is altered, as a consequence of signaling and downstream changes in expression of transcription factors. Previous work realized by the host lab (Amaral et al., 2017) shows that two specific miRNAs, miR-155-5p and miR-34c-5p are differentially upregulated after TCR engagement with anti CD-28, anti CD-3 and Interleukin-2 (IL2). This regulatory role of miRNAs doesn't apply only to T lymphocytes, with miRNAs regulatory influence also spreading to the rest immune system. For the sake of brevity, and given the context of this thesis, we shall only mention the roles played by miR-155-5p and miR-34c-5p.

## 1.3 miR-155-5p and miR-34c-5p

In this thesis, we are interested in two particular miRNAs, miR-155-5p and miR-34c-5p.

Given its oncogenic potential and the crucial role that it plays in the immune system, miR-155-5p is extremely well studied. As such, the literature shows that in the innate immune system, in conjunction with miR-146a, miR-155-5p is one of the key players of macrophage development (O'connell et al., 2007). Additionally, several regulators of myeloid cell differentiation, such as PU.1, BACH1/FANCJ, CSF1R and C/EBPβ, are downregulated by miR-155-5p (O'Connell et al., 2008a).

Likewise, during T cell activation, miR-155-5p has also been reported to regulate some transcription factors such as AP1 (Yin et al., 2008) and STAT5 (Kopp et al., 2013). It should be noted that these transcription factors aren't the only ones under the regulatory activity of miR-155-5p. IL-2, an essential autocrine cytokine for T cell activation, has its signaling pathway regulated by several miRNAs, including miR-155-5p. The literature shows that miR-155-5p is induced upon T-cell activation and enhances T cell proliferation, through the inhibition of the suppressor of cytokine signaling 1 (SOCS1) (Dudda et al., 2013), and through the inhibition of CTLA-4 (Sonkoly et al., 2010), a negative regulator of T-cell activation.

The literature also shows that miR-155-5p is one of the miRNAs that is induced early upon activation of both B and T lymphocytes (Rodriguez et al., 2007; Thai et al., 2007), and that it is involved in T-cell differentiation (Banerjee et al., 2010), where it plays a crucial role in the function of Th1, Treg and Th17.

On the other hand, unlike miR-155-5p whose impact on CD4+ T cells was been reported, miR-34c-5p was studied mostly in an oncological context, where its expression is dysregulated, and where it functions as an inducer of apoptosis and a repressor of cell proliferation (Hermeking, 2010). Although, previous works suggests that miR-34c-5p is able to regulate genes associated with TCR signaling, it was the host lab that first reported miR-34c-5p expression on CD4+ T cells (Amaral et al., 2017).

MiR-34c-5p is part of the miR-34 family, which includes two other members, miR-34a and miR-34b. The three members of this family are distinguished by their chromosomal location, with miR-34a being located in chromosome 1 while miR-34b and miR-34c are located in chromosome 11, and by their promoters having slightly different affinity to different transcription factors (TFs).

The main activating TFs of miR-34 family are TP53 (Rokavec et al., 2014), SP1 (X. Xu et al., 2012), and FOXOs (Masui et al., 2013), specially FOXO3 for miR34b and c. On the other hand, in the particular case of miR-34c-5p, the main inhibitor TFs are STAT3 and EMT-TFs (Rokavec et al., 2014).

As previously mentioned, previous work at the host lab, showed that upon naïve CD4 T cell stimulation with anti-CD3, anti-CD28 and IL-2, miR-34c-5p is up-regulated, with its expression peaking at 72 to 96 hours after stimulation. It should be noted that this observation only occurred in naïve CD4 T cells, and when given the same stimulus, memory CD4 T cells showed no up-regulation of this miRNA.

## 1.4 Th subtypes

MiRNA regulation is essential for proper immune function of CD4+ T helper cells. CD4+ T cells are grouped into several subtypes, each one with a different role in the host immune system. The different subtypes can be identified in accordance with the transcription factors and cytokines that a cell expresses, with such patterns being nicknamed as "cytokine expression profile" for the sake of simplicity (Table 1.2). It should be noted that each different Th cell subtype is associated with a different cytokine expression profile.

The dysregulation of miRNA may lead to malfunction of CD4+ T cells, and such malfunctions affect not only how the subtypes of CD4+ T cells play their role in the host immune system, and thus can contribute or be the cause of certain pathologies.

As such, the dysregulation of miRNA has been associated with a myriad of pathological conditions, such as haematological cancers (Calin et al., 2004; Calin & Croce, 2006; de Leeuw et al., 2013; Esquela-Kerscher & Slack, 2006; Garzon et al., 2008; J. Lu et al., 2005; Schotte et al., 2012) and

*Table 1.2- Table of the "cytokine expression profiles" used to identify to which Th subtype a differentiated Th cell belongs. Red spaces indicate nodes that must be absent. Green spaces indicates nodes that must be expressed. Yellow spaces indicate nodes that can be expressed but whose presence is not necessary for a cell to belong to a given Th cell subtype.*

| | | **Transcription Factors** | | | | | | | **Secreted cytokines** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TBET | GATA3 | RORGT | FOXP3 | BCL6 | PU.1 | STAT3 | IFNG | IL4 | IL17 | IL21 | IL22 | IL5 | IL13 | IL9 | TGFB |
| **Th cell subtypes** | Th0 | | | | | | | | | | | | | | | | |
| | Th1 | | | | | | | | | | | | | | | | |
| | Th2 | | | | | | | | | | | | | | | | |
| | Th17 | | | | | | | | | | | | | | | | |
| | Treg | | | | | | | | | | | | | | | | |
| | Tfh | | | | | | | | | | | | | | | | |
| | Th9 | | | | | | | | | | | | | | | | |
| | Th22 | | | | | | | | | | | | | | | | |

autoimmune diseases (Simpson & Ansel, 2015; Singh et al., 2013), such as multiple sclerosis, Sjögren syndrome and autoimmune gastritis.

In haematological cancers, the inhibition of the negative signaling regulators SHIP1 and SOCS1 by miR-155-5p, as lead to the association of miR-155-5p with myeloid and lymphoid leukemia (O'connell et al., n.d., 2007; O'Connell et al., 2008b).

By the same token, it is of the utmost importance for the Th subtypes to be kept in balance, with such balance being kept in accordance with the impact of the different subtypes upon the regulation of autoimmunity and inflammation. This is illustrated by the fact that pro-inflammatory T cells such as Th1 and Th17 are beneficial in cases of infection, but in cases of chronic inflammation and autoimmunity may cause more harm than good. With situations such as these in mind, the understanding of how miRNAs are involved in differentiation of T cells become of the upmost importance, since such knowledge can result in the development of new diagnostic tools and immune therapies. A specific case of such instance can be found in the imbalance of Th17/Treg ratio, which is found to be a major driver of various autoimmune and inflammatory diseases such as Multiple sclerosis (MS) /Experimental autoimmune encephalomyelitis (EAE) (Jamshidian et al., 2013; Naghavian et al., 2015; Tzartos et al., 2008), where miR-155-5p is found to be elevated in CD4+ T cells of EAE mice, and when absent, these cells develop a milder form of EAE and possess fewer Th17 cells (Murugaiyan et al., 2015; O'Connell et al., 2010).

As such, in the following sections we shall focus on the effect of miRNAs, specially miR-155-5p, on the differentiation and function of the different subtypes of CD4+ T cells. For a more in-depth discussion of the effect of miRNAs on CD4+ cell subtype consult (Inácio et al., 2018; Kroesen et al., 2015; Mehta & Baltimore, 2016).

### 1.4.1 miR-155-5p impact on Th1 cells differentiation and function

Together with Th2, Th1 is one of the two "classical" subsets of CD4+ T cells, with Th1 cells activating macrophages, cytotoxic T cells and natural killer cells, and playing a key role in the host defense against intracellular pathogens and tumors, although it can contribute to autoimmune pathologies when its regulation is impaired (Wilson et al., 2009). Th1 are preferentially produced in an environment where APC and environmental IL12 are present and express T-bet and IFNγ.

The effect of miRNAs in Th1 cell can be seen in experiments in which specific genetic inactivation of Drosha, DGCR8 or Dicer, result in miRNA-deficient T cells with an increase in T-bet expression and IFN-γ production (Chong et al., 2008; Muljo et al., 2005; Steiner et al., 2011).

MiR-155 favors Th1 differentiation (O'Connell et al., 2010) through inhibition of SHIP1, a negative regulator of PI3K signaling pathway (Soond et al., 2010). MiR-155-5p plays a crucial role in determining whether a naïve Th0 cell differentiates into a Th1 or a Th2 cell, being rapidly induced upon T cell activation. MiR-155-5p overexpression leads to the downregulation of IFN-γ α-chain receptor (IFNGR1) by direct targeting in activated CD4+ T cells and thus promoting Th1 differentiation (Banerjee et al., 2010).

### 1.4.2 miR-155-5p impact on Th2 cells differentiation and function

The second classical CD4+ T cell subtype, Th2, has its cytokine expression profile characterized by the expression GATA3, IL4, IL5, and IL13 and is preferentially produced in an environment in which

APC is present in conjunction with environmental IL4 and IL2. These cells are responsible for the promotion of the survival of basophil, eosinophil and mast cells, and play a role in the host immune defense against parasitic infections, being responsible for the secretion of cytokines that stimulate B cells and for inflammation. Unfortunately, upon malfunctioning, Th2 cells may contribute to chronic inflammatory diseases, such as allergy and asthma (Fahy, 2015).

Once again, the effect of miRNAs upon Th2 cell differentiation can be observed through the deletion of DGCR8 in CD4+ T cells, in an environment containing IFN-γ blocking antibodies and the conditions for Th2 cell polarization, which results in an increase of IL4 and IL13 expressing cells, as observed in miRNA-sufficient control cells (Pua et al., 2016).

The role that miR-155 plays in the decision of whether naïve Th0 cells becomes a Th1 or Th2 cell, should be noted again with, the differentiation of Th0 cells to Th2 cells being favored in miR-155 deficient cells (Banerjee et al., 2010; Rodriguez et al., 2007). Additionally, during Th2 differentiation, miR155 targets S1PR1 mRNA and consequently promotes Th2 cell migration and Th2-mediated airway disease (Okoye et al., 2014; Simpson et al., 2014).

### 1.4.3 miR-155-5p impact on Th9 cells differentiation and function

The cytokine profile expression of Th9 subtype (Dardalhon et al., 2008; Soroosh & Doherty, 2009; Veldhoen et al., 2008) is characterized by the production of APC in conjunction with environmental IL4 and TGFB. This subset is preferentially expressed in an environment where PU1 and IL9 are present. Th9 cells have been found to be involved in pathogen immunity and inflammatory diseases (Kaplan, 2013).

An example of the influence of miR-155-5p is the recent discovery that this miRNA regulates the differentiation of Th9 cells in children with methicillin-resistant Staphylococcus aureus pneumonia, through the targeting of SIRT1, as described in (Tian & Xu, 2021).

### 1.4.4 miR-155-5p impact on Th17 cells differentiation and function

Th17 cells (Harrington et al., 2005; Mangan et al., 2006) play a key role in the host defense against opportunistic fungi and extracellular bacteria through the enhancement of mucosal barrier functions. The cytokine expression profile of Th17 cells is characterized by the expression of RORGT and IL17 and is produced preferentially in an environment in which APC is present in conjunction with environmental IL6, TGFB, IL1B and IL23. The production of the cytokine IL22 is optional.

Cells that are Drosha deficient result in an increase of CD4+ T cells that produce IL-17A in mice spleen and lymph nodes (Chong et al., 2008; Muljo et al., 2005; Steiner et al., 2011). The impact of miRNA in Th17 cell differentiation can also be seen in dextran sulfate sodium treated mice where miR-155 is highly expressed, affecting Th17 cell differentiation through the targeting of Jarid2, which leads to an increase of IL-22 levels, which is implied with an increase in the number of Th17 cells since IL22 is one of the optional cytokines that it can produce (M. Xu et al., 2017). Likewise, mice that are miR-155 deficient are associated with a decrease of both Th1 and Th17 cells (O'Connell et al., 2010).

When Th17 cell response is dysregulated, these cells can play a pathological role in several inflammatory disorders, such as psoriasis, inflammatory bowel disease (IBD) and MS (Wilson et al., 2009). It can be observed that, miR-155-5p KO cells, present a decrease in the number of Th17 cells

and the antibodies and cytokines associated with it, which can lead to collagen-induced arthritis (Kurowska-Stolarska et al., 2011; L. F. Lu et al., 2015; O'Connell et al., 2010).

### 1.4.5 miR-155-5p impact on Th22 cells differentiation and function

Th22 subtype is preferentially produced in an environment where APC is present in conjunction with environmental IL6 and its cytokine expression profile is characterized by the production of STAT3 and IL22.

In the literature that we consulted there was no link between miR-155-5p and Th22 subtype.

### 1.4.6 miR-155-5p impact on Treg cells differentiation and function

Regulatory T cells (Treg) are responsible for regulating effector T cell responses through the limitation of inflammatory responses and prevention of autoimmunity (Tang & Bluestone, 2006). After their development in the thymus, Treg cells migrate to the periphery where they are critical to prevent tissue autoimmunity. Treg cells can also differentiate from naïve Th0 cells into specific inflammatory niches, like TGF-β rich gut environment. Such "induced" Treg (iTreg) (Chen et al., 2003; Groux et al., 1997; Schmitt & Williams, 2013) play important roles in limiting local inflammation, which can exacerbate to pathologies such as Inflammatory bowel disease (IBD) (Naghavian et al., 2015).

Treg cells are preferentially produced in an environment on which APC is present together with environmental TGFB and IL2 and their cytokine expression profile is characterized by the production of FOXP3 and TGFB.

MiRNA's regulatory role is essential for both kinds of Treg cells, as seen by the fact that Dicer-deficient T cells are unable to maintain their expression profile, upregulating IL-4 and IFN-γ expression while downregulating FOXP3. As a consequence, Treg cells lose their regulatory abilities and possibly lead to a fatal autoimmune phenotype (Cobb et al., 2006; L. Zhou et al., 2011). Under normal circumstances, the upregulation of FOXP3 during thymic differentiation would induce miR-155 which would promote the maintenance of Treg cytokine expression profile through the inhibition of SOCS1, a negative regulator of the IL-2 signaling cascade (which a main transcription factor of Treg cells), which results in increased activation of STAT5, further promoting FOXP3 expression through a positive feedback loop motif (L. F. Lu et al., 2009). Finally, mice that are miR-155 KO present a reduced number of Treg cells even though there appears to be no effect on the Treg immunosuppressive capabilities.

### 1.4.7 miR impact on Tfh cells differentiation and function

Follicular helper T cells (Tfh) (Breitfeld et al., 2000; Schaerli et al., 2000) are yet another of the non-classical CD4+ T cell subtypes, being formed from the CD4+ T cells that remain in lymphoid organs to help the differentiation of B lymphocytes into antibody secreting cells. Their cytokine expression profile is characterized by the production of BCL6 and IL21 and their preferential production environment has APC present in conjunction with environmental IL12 and IL21. Tfh cells have a critical role in germinal centre formation and germinal centre B-cell maturation, and as such are essential for the development of antigen-specific B cell immunity (Crotty, 2011; Schaerli et al., 2000).

Tfh cell differentiation is compromised by the absence of certain miRNAs, with miR-146a and miR-155 being described in recent studies as particularly important post-transcriptional regulators of Tfh cell differentiation, given their respective opposing roles of restricting the expansion of Tfh cells

(Hu et al., 2014), and of being required for both the correct development and expansion of Tfh cells, leads their ratio to be fundamental for the proper regulation of the immune response. MiR-155 has twenty-one direct target genes identified in Tfh cells, with special attention being given to Peli1 which has been validated as a regulator of cellular proliferation, in addition to the miR-155-Peli1-c-Rel pathway being regulated during Tfh cell generation and function (Liu et al., 2016).

## 1.5 Logical modelling

In recent years, the use of computational models has become more common for the study of complex cellular networks, and although different formalisms have been used to model complex biological networks, the logical formalism has been of particular value for the study of large signaling and transcriptional regulatory networks in which detailed quantitative data is either completely or partially absent, such as in the case of Th regulatory networks.

The qualitative nature of logical models is its greatest advantage, since it enables the modelling of biological systems for which detailed quantitative information is either partially or completely absent (Bornholdt, 2008; Naldi et al., 2015; Wang et al., 2012). Other advantages of the modelling formalism are its scalability, with models that have a few hundred components having been successfully simulated. A third advantage, is that the logical formalism allows for the construction of logical models through a modular approach, that is, it allows for the construction, definition and study of smaller models which can then be merged into a single larger model. (Naldi et al., 2010) is one example of a model that was built in such a way. Finally, it should be noted that logical models can be used as a way to obtain a "first glance" at the dynamical properties of complex models, since they are well-suited for the capture of most important dynamical properties of a regulatory network.

*Table 1.3- Examples of Logical rules and their Reading.*

| Node | Logical Rule | Reading |
|---|---|---|
| STAT5 | 2->IL2R:2<br>1->!IL2R:2 &<br>(IL2R:1\|IL15R) | STAT5 is a non-Boolean node which can take three values, "2", "1", and "0". STAT5 takes the value of "2" when the activity level of the node IL2R is "2". When such a thing doesn't occur and IL2R takes only the value of "1" and/or IL15R is present, then STAT5 takes the value of "1". Otherwise STAT5 takes the value "0". |
| IL2 | 1->(NFAT\|NFKB) &<br>!TBET & !FOXP3 &<br>!(STAT5 & STAT6)<br>1->NFAT & TBET &<br>!FOXP3 & !(STAT5 &<br>STAT6) | IL2 is a Boolean node, whose value "1" can be taken on two different circumstances. In the first circumstance, FOXP3 and TBET are absent, STAT5 and STAT6 cannot be expressed simultaneously, and either NFAT or NFKB must be expressed. In the second circumstance, NFAT and TBET must be expressed while FOXP3 is absent, and STAT5 and STAT6 must not be simultaneously expressed. |
| TBET | (TBET\| STAT1\| IL36R)<br>& !BCL6 & !RORGT | TBET is a Boolean node which is expressed when at least one of the TBET, STAT1 and TL36R is present and BCL6 and RORGT are absent. |

In this thesis, we use models that follow upon the work of R.Thomas and colleagues, which allows for the presence of multivalued nodes and the use of sophisticated logical rules and parameters (Thomas, 1991; Thomas & Thieffry, 1995).

In accordance with the work of R.Thomas, a model network is defined in terms of a logical regulatory network (LRG), which is comprised of a series of nodes and edges, with nodes being representative of regulatory components and edges representing interactions between the nodes, such as activations and inhibitions. Additionally, all nodes are associated with a discrete variable which denotes its current functional level of activity, and a logical rule which describes the evolution of such level, in accordance with the values of the regulators of the node. These logical rules are written using a series of operators such as "!", "|" and "&" whose meaning are respectively, "not", "or" and "and" (Table 1.3).

The number of activity levels that a node can take depend on whether the node is Boolean or non-Boolean. When a node is Boolean, it can only take two values, either "0" or "1", with each of these values referring to either the absence or the presence of said node. On the other hand, a non-Boolean node can take three or more values (Thomas, 1991; Thomas & Thieffry, 1995). As such, a hypothetic node A, could be able to take 4 different values, "0", "1", "2", "3". In such a case, if the value of node A was "0", such node would be absent, while if it has had the value "1" it would be present in "small" quantities that depending on the logical rules of the system could not be enough to influence any of the other nodes. When the node was present in "medium" quantities and influence the values of some of the other nodes it would take the value "2", and finally when present in "high" quantities that allowed it to perform all of its functions, it would take the value "3". It should be noted that all of the labels ("absent", "present", "low", "medium", "high"), further reinforce the qualitative nature of the logical formalism. This thesis adapts a model (Abou-Jaoudé et al., 2015) which contains non-Boolean nodes.

Even though, when compared with kinetic models based on differential equations, the logical formalism is unable to perform quantitative analysis and is limited when it comes to studying the dynamical aspects of a model, even a greatly simplified Boolean model can reproduce many of the qualitative features of models that use differential equations. Furthermore, the use of logical models permits the analysis of important functional aspects, that lead to the creation of hypothesis that can be tested in a laboratorial context, for example, through the realization of knock-out and perturbation experiments.

Regardless, it is the activity level of the nodes that define the qualitative state of a logical model, and the value of the activity value of a node is determined in accordance with its logical rule. If the current level of a node is different from its target level, then the node changes its value and the logical model transitions to its successor state. There are several different strategies that are used for updating the state of a logical model, but the two main strategies that are used are the synchronous and asynchronous updating schemes.

The main difference between the two updating schemes can be illustrated with a hypothetic model containing 10 nodes. In such a model, in a scenario where 7 of the nodes may change values, when using a synchronous update, a single successor state is obtained where all of the seven nodes change their value. But, if the model is running under the asynchronous updating scheme, seven different successor states are generated, which one varying the value of only one of the seven nodes. This creates a situation in which, while an asynchronous updating scheme is far more realistic, it is also prone to create situations in which the analysis of the resulting non-deterministic concurrent dynamics is difficulted, especially in larger models, given how many successor states may originate from a single original state.

Additionally, other methods such as the introduction of priority classes allows for the definition of subtler updating schemes (Fauré et al., 2006).

These state transitions lead to the definition of the so-called state transition graph (STG), which represents the dynamical behavior of the logical regulatory graph. In this graph, nodes correspond to logical states, and arcs represent transitions between states. In GINsim, the software that we use in this thesis for the study of logical models (more information in section 2.7 of Methods), STG greatly ease the finding of the so-called model attractors. Given that in logical models, cell fates can be associated with model attractors, the identification and reachability properties of the latter are of the utmost importance. Model attractors correspond to long term stable equilibria, either cyclic attractors or stable states. Cyclic attractors denote stable oscillations as observed in cell cycle or circadian rhythms (Chaves & Preto, 2013; Fauré et al., 2006, 2009), while stable states are associated with cell lineages or other cellular responses to external cues or perturbations (Calzone et al., 2010; Collombet et al., 2017; Naldi et al., 2010; Sánchez et al., 2008). In GINsim, stable states correspond to nodes with no outgoing arcs in the STG graph.

A frequent line of inquiry is finding which model attractors can be reached from a specific initial condition, a question that can be addressed by verifying the trajectories from a certain initial state to the attractor states. This task becomes increasingly difficult in models with a larger number of nodes, and in such cases, STG is usually further compressed into a Strongly Connected Components graph (SCC). A particularity of SCC is that by lumping states that belong to the same strongly connected component (SCC), an acyclic graph is obtained. Frequently, SCC also preserve the reachability properties of the original graph. Unfortunately, it is possible for the SCC graph to result in only a moderate STC compression. In such cases, SCC graphs can be further compressed into yet another acyclic graph called Hierarchical Transition graph (HTG), which further merges linear chains of states (in addition to cycles) into single nodes (Bérenguier et al., 2013). The resulting graph preserves the attractors and other important dynamical properties, but does not fully conserve reachability properties.

Additionally, algorithms such as FIREFRONT and AVATAR may be used to ease the identification of model attractors, especially those that correspond to cyclic attractors, which tend to be more difficult to identify (Mendes et al., 2018).

The increasing popularity of the logical formalism to model regulatory and signaling networks has prompted several groups to develop a variety of tools and methods for the definition and analysis of such models. This leads to standardization issues, which CoLoMoTo (for more information visit http://www.colomoto.org), an informal consortium where those who develop logical models, and the methods and tools used to analyze them, gather with the intent to solve those problems.

The logical formalism has been used in a variety of regulatory networks such as organ differentiation control in the flowers of *Arabidopsis thaliana* (Mendoza et al., 1999); haematopoeisis (Bérenguier et al., 2013); cell differentiation in developmental processes of *Drosophila melanogaster* (Fauré et al., 2014; González et al., 2008; Sánchez et al., 2008); cell cycle control in yeast and mammals (Fauré et al., 2006, 2009; Traynard et al., 2016); neuronal differentiation (Coolen et al., 2012); and T lymphocyte activation and differentiation (Abou-Jaoudé et al., 2015; Mendoza, 2006; Naldi et al., 2010).

Discrete qualitative frameworks, such as the logical formalism, are obviously not the only type of approach used for the modelling of Th cell differentiation, and several models using quantitative modeling approaches have been previously published (Mendoza & Pardo, 2010; van den Ham & de Boer, 2008, 2012).

Regardless, in the context of this thesis, there is one model of particular interest (Abou-Jaoudé et al., 2015), which is one of the Th cell differentiation models, which expands upon the previous model of (Mendoza, 2006). (Mendoza, 2006) proposed a logical model which accounted for the dichotomous differentiation of naïve Th0 cells into Th1 and Th2 cells, in which he was able to obtain four basins of attraction that he was able to associate with the different cell types. Using GINsim to expand this initial model so as to cover additional pathways and the Th17 and Treg subtypes, (Naldi et al., 2010), created a model with 34 components and proceeded to identify all the stable states and group them in accordance with the relevant phenotypic Th markers. This model also identified hybrid Th subtypes that expressed combinations of markers from the Th1, Th2, Th17 and Treg subtypes. Finally, (Abou-Jaoudé et al., 2015), further developed Naldi's model into a multi-valued model, which accounted for three new Th subtypes (Th9, Th22 and Tfh), through the integration of additional transcription factors and cytokine pathways involved in Th cell commitment.

The goal of this thesis was to further expand the model of (Abou-Jaoudé et al., 2015) by introducing as new nodes our two miRNAs of interest, miR-155-5p and miR-34c-5p, in conjunction with their transcription factors and targets. This expansion implied the addition of new interactions, not only the interactions between the added nodes but also the interactions between the added nodes and the nodes that were previously present. New logical rules had to be created for the new nodes and some of the pre-existent logical rules had to be modified to accommodate the added interactions.

Once these tasks were accomplished, we proceeded to obtain the stable states, under a variety of environments that can be classified under four scenarios. The four scenarios correspond to when both miRNAs are expressed, to when both are absent, to when only miR-155-5p is expressed and finally to when only miR-34c-5p is expressed. We then proceeded to use a R script, which was written during this thesis, to identify whether a stable state can be associated with a given cytokine expression profile, thus enabling the correlation between a stable state and a CD4+ T cell subtype. In this way we evaluated the impact of miR-34c-5p and miR-155-5p on upon naïve CD4+ T cell differentiation.

Finally, it should be noted that the construction of these computational models is not a replacement for experimental work. By the contrary, not only is the data obtained through experimental work the basis that allows for the construction of these computational models, but the analysis of such models frequently give rise to hypothesis that lead to further experimental work. As such, both methods of studies are complementary and their combined use leads to a much better understanding of complex biological processes, whose study without the use of computational modelling would otherwise be greatly difficulted.

# 2 Software and Databases

## 2.1 R Studio

Available for Windows, Mac and Linux operative systems, R Studio is an integrated development environment for the R programming language that includes a console, syntax-highlighting editor that supports direct code execution, in addition to tools for planning, history, debugging and workshop management.

## 2.2 TransmiR v2.0 database

TransmiR is the database for finding regulatory relations between TFs and miRNAs, freely available for academic use, which allows to obtain data related to a single miRNA, TF or disease and even visualize such data through the use of the "Search" and "Network" pages. Additionally, it also contains the "Enrichment analysis" modules and "Predict" modules, which allow for the identification of which TFs are likely to regulate miRNA list of interests and the prediction of TF-miRNA regulations based on binding motif matrices of human TFs, respectively. The data available in TransmiR 2.0 can also be downloaded for further analysis. In this thesis TransmiR 2.0 was used to obtain the TFs of miR-155-5p and miR-34c-5p. The specific data used for this thesis was obtained at the 27$^{th}$ November 2019.

## 2.3 miRTarBase

| Features | miRTarBase 8.0 | miRTarBase 9.0 |
|---|---|---|
| **Release date** | 2019/09/15 | 2021/9/15 |
| **Known miRNA entry** | miRBase v22 | miRBase v22 |
| **Known Gene entry** | Entrez 2019 | Entrez 2021 |
| **Species** | 32 | 37 |
| **Curated articles** | 11,021 | 13,389 |
| **miRNAs** | 4,312 | 4,630 |
| **Target genes** | 23,426 | 27,172 |
| **CLIP-seq datasets** | 331 | 440 |
| **Curated miRNA-target interactions** | 479,340 | 2,200,449 |
| **Text-mining technique to prescreen literature** | Enhanced NLP+Scoring system | Enhanced NLP+Scoring system |
| **Download by validated miRNA-target sites** | Yes | Yes |
| **Browse by miRNA, gene, and disease** | Yes | Yes |
| **Regulation of microRNAs** | Yes | Yes |
| **Cell-free miRNA expression** | Yes | Yes |
| **miRNAs in extracellular vesicles** | No | Yes |
| **Human miRNA tissue atlas** | No | Yes |
| **Editing events in miRNAs** | No | Yes |
| **SNPs and disease-related variants** | No | Yes |
| **MTIs Supported by strong experimental evidence** | | |
| **Number of MTIs validated by 'Reporter assay'** | 13,922 | 16,257 |
| **Number of MTIs validated by 'Western blot'** | 12,179 | 14,665 |
| **Number of MTIs validated by 'qPCR'** | 13,263 | 16,483 |
| **Number of MTIs validated by 'Reporter assay and Western blot'** | 10,257 | 12,171 |
| **Number of MTIs validated by 'Reporter assay or Western blot'** | 15,710 | 18,751 |

*Figure 2.3.1- Table pertaining to the versions 8.0 and 9.0 of miRTarBase.*

A database with more than 470000 miRNA-target interactions, miRTarBase, collected these interactions by surveying pertinent literature related to functional studies of miRNAs. These interactions are validated experimentally through the use of reporter assay, western blot, microarray and next generation sequencing experiments. In this thesis, miRTarBase 8.0 (Figure 2.3.1) was used for obtaining the Targets of miR-155-5p and miR-34c-5p with such data having been obtained at 28th November 2019. It should thus be noted that by the date of publication of this thesis we are working with data which is already outdated.

## 2.4 GeneCards

GeneCards is a database that provides information on annotated and predicted human genes, integrating information from about 150 web sources which include genomic, transcriptomic, proteomic, genetic, clinical and functional information. GeneCards is used to manually identify the UNIPROTs of nodes which our scripts were unable to obtain.

## 2.5 HGNC

The HUGO Gene Nomenclature Committee (HGNC) is the committee responsible for setting the standards for human gene nomenclature, and its database allows for the search of the approved symbols for human genes, and their information associated to them (such as their UNIPROT identifier). In conjunction with GeneCards, HGNC was used to identify the UNIPROTs of nodes that our scripts were unable to obtain. An UNIPROT identifier is, according to UNIPROT, the "the unique identifier assigned to the set of proteins that constitute the proteome" in the UNIPROT database.

## 2.6 OMNIPath

Developed by Saez Lab and Korcsmaros Lab, OMNIPath is a database that contains information about protein-protein and gene regulatory interactions, enzyme-PTM relationships, protein complexes, protein annotations and intercellular communication. In this thesis, it was used for finding interactions between miR-155-5p, miR-34c-5p, their respective TFs and Targets, and the nodes of the logical models that we were using. The specific data that we used was obtained at the 18th of December of 2019.

## 2.7 GINSim

GINsim is a software developed in Java that provides a user interface for building, simulating and analyzing logical models. The simulation of such logical systems can be performed under several updating schemes such as the synchronous, asynchronous and priority updating schemes. GINsim includes an algorithm that allows to efficiently obtain all stable states of a logical model, and allows for the use of the reduction method for the reduction of the size of an LRG. GINsim also permits to define genetic perturbations, such as gene knock-out and gene knock-in, by forcing a model to take a specific value, of either 0 in the case of knock-outs, or the maximal activity level in the case of ectopic expression. Additionally, GINsim also allows model exports to a variety of other formats, which allows for the use of the model in other softwares, such as, SBML-qual, MaBoSS, BoolSim, GNA, NuSMV, Integrated Net Analyzer (INA), Snoopy, Graphviz, Cytoscape and Scalable Vector Graphics (SVG). In

this thesis, GINsim is used for the creation of our modified models and subsequently to their simulation and obtention of their stable states.

Finally, GINsim can be downloaded for free from its dedicated website (http://ginsim.org), where a model repository can be found alongside the software's documentation.

# 3 Model Construction

The goal of this thesis was to study the effect of miR-34c-5p on the differentiation of naïve CD4+ T cell differentiation. To reach this goal we intended to expand a pre-existent model (Abou-Jaoudé et al., 2015) (Figure 3.1) by introducing new nodes and interactions relative to two miRNAs of interest, miR-155-5p and miR-34c-5p, in conjunction with their transcription factors (TFs) and targets.

Consequently, the first step of this process was the identification of the TFs and targets of miR-155-5p and miR-34c-5p, through the use of the TransmiR and miRTarBase databases. We used the search function of the TransmiR database to separately search information regarding the TFs of our two miRNAs. On the other hand, the information regarding the targets of the two miRNAs was obtained by consulting the miRTarBase database.

The second step of this thesis was to obtain a list of UNIPROT identifiers of all elements involved. In this case, by all elements involved we mean, all the nodes of the model that is going to be altered, plus the TFs and targets of the two miRNAs of interest.

Unfortunately, some of these elements didn't have a UNIPROT associated with them according to the automatic search using the UNIPROT R package. This led us to use the GeneCards and HGNC databases to manually identify the missing UNIPROTs.

With this information in hand, we proceeded to the third step, the identification of the nodes and interactions which should be added in the modified version of the model. To this end, we identified which nodes of our models were also TFs or targets of our miRNAs of interest. We name these direct interactions as "intercepts". Additionally, we also identified "indirect interactions", that is, the interactions between nodes of our models and the identified TFs and targets of the two miRNAs of interest. We used the OMNIPath database for the identification of the "indirect interactions". We then proceeded to expand the model by introducing not only the two miRNAs of interest but also their TFs and targets which interact with nodes of the models, in conjunction with the interactions that were previously obtained. While the original sources (OMNIPath and Transmir) contain information about whether or not a given interaction is an activation or an inhibition, this information had to be searched manually to check for possible inconsistencies, with the exception of the interactions from a miRNA to its targets, which are always presumed to be inhibitions. Finally, we proceed to remove the TFs and targets who serve only as either targets or sources of interactions. The former would not influence other model variables while the latter would be model inputs whose value would have to be set and would not be influenced by the other variables in the model. An image of the resulting modified model can be consulted in Figure 3.2.

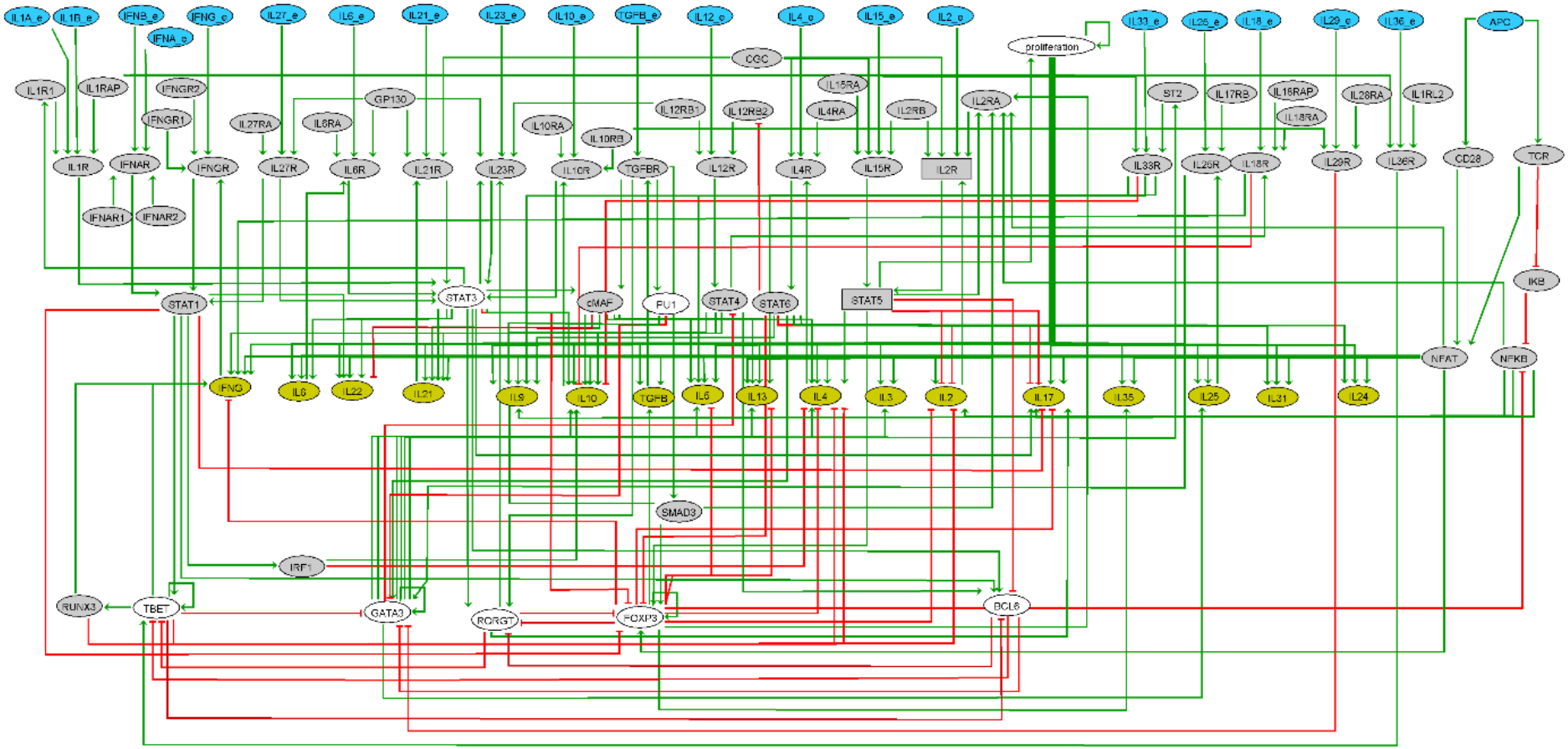


*Figure 3.1- Logical model of CD4+ T cell differentiation from (Abou-Jaoudé et al., 2015) The blue nodes correspond to the inputs (nodes without incoming regulatory interactions), while the yellow nodes correspond to the cytokines secreted by the different Th cell subtypes. The green edges represent activations, while the red edges represent inhibitions. Rectangular nodes refer to non-Boolean nodes, while Boolean nodes are indicated by elyptic nodes.*

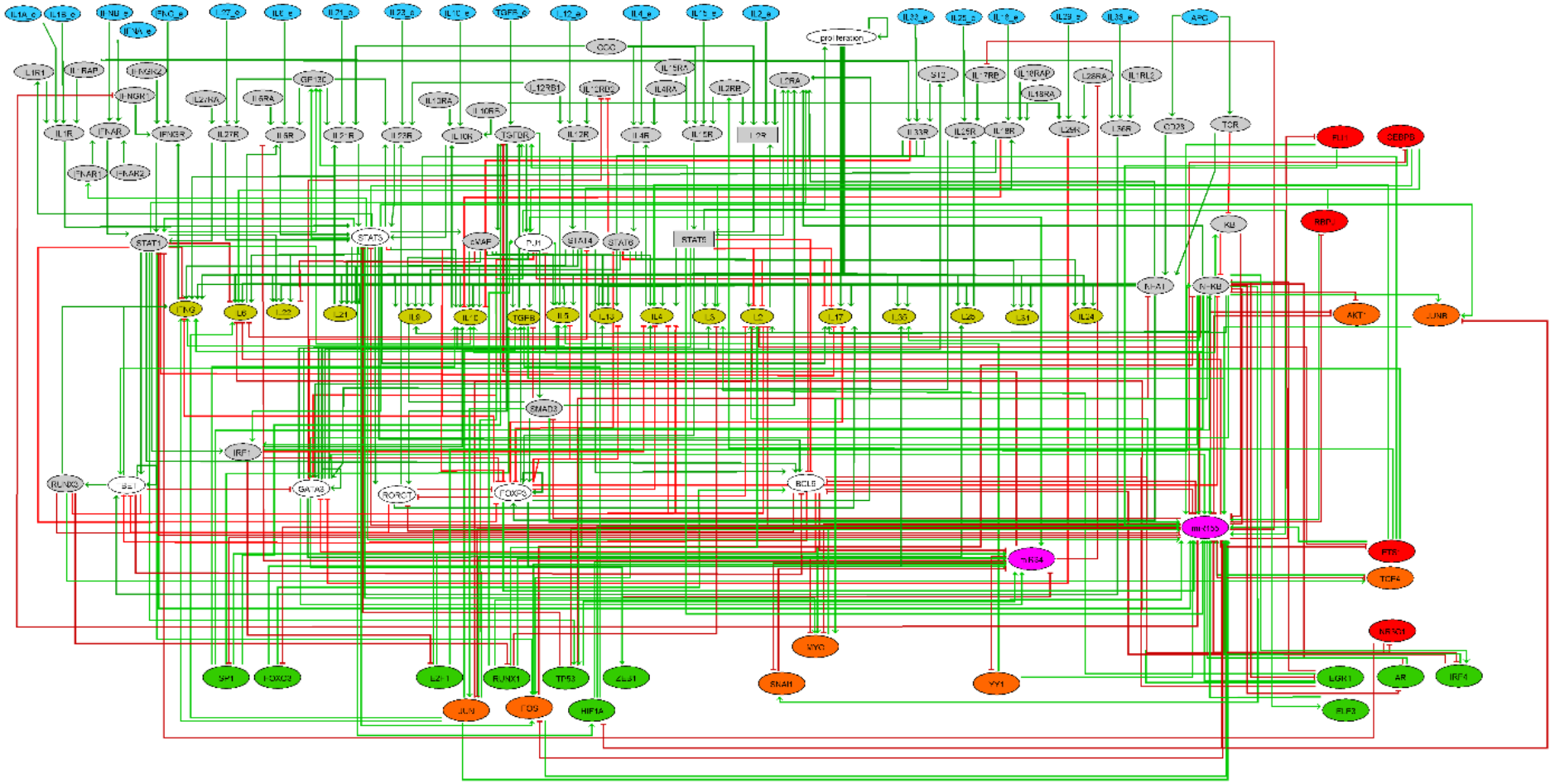


*Figure 3.2- Modified version of the (Abou-Jaoudé et al., 2015) upon addition of the miRNAs of interest and the TFs and targets and respective interactions upon removal of nodes that serve only as either the origin or target of interaction.*

At this stage we proceeded to the fourth step, in which we wrote new logical rules for the nodes that we added, and rewrote the logical rules for nodes already present, in accordance with the interactions that had been added. Before any further advance, we used GINsim to create a series of perturbations to serve as conditions to be used in stable state analysis. Those perturbations referred to nine different cytokine environments that were previously mentioned and can be consulted in Table 1.1. While in the case of the original models these nine environmental conditions are enough, when it comes to the modified models, those nine environments must be multiplied by four, given the four possible miRNA expression scenarios, that is the four possible combinations of whether our two miRNAs of interest are expressed or absent. More specifically the four scenarios refer to the absence of both miRNAs, the expression of both miRNAs, the expression of only miR-34c-5p, and the expression of only miR-155-5p. Consequently, while there are only nine conditions for the original model, the modified models have thirty-six conditions for which we want to find stable states. If our modified model is able to produce all specific Th subtypes in their corresponding favored environment in at least one of the four scenarios, we consider the modified model to be valid and proceed to analyze the effect of miR-34c-5p on Th cell differentiation. After creating all the necessary perturbations, we proceeded to obtain the stable states of the original models in their nine different initial conditions, with the intention of using these results as benchmark for the results of the modified models. Upon obtaining the stable states, we proceeded to identify to which subtype, if any, the different stable states correspond to, and how many stable states correspond to the different Th cell subtypes in the different environmental conditions.

*Table 3.1- List containing the original logical rules of the nodes present in the model of Abou-Jaoude and collaborators, in addition to the strict, loose and intermediate logical rules of respective versions of the modified model. The presence of a "-" indicates that a node either lacks a logical rule or its logical rule doesn't undergo any alteration, and therefore the logical rule of that node should be consulted at the closest written column at the left of the "-" signal.*

| Node | Original Model | Strict Model | Loose Model | Intermediate |
|---|---|---|---|---|
| **IL1B_e** | - | - | - | - |
| **IFNG_e** | - | - | - | - |
| **IL2_e** | - | - | - | - |
| **IL4_e** | - | - | - | - |
| **IL6_e** | - | - | - | - |
| **IL10_e** | - | - | - | - |
| **IL12_e** | - | - | - | - |
| **IL15_e** | - | - | - | - |
| **IL21_e** | - | - | - | - |
| **IL23_e** | - | - | - | - |
| **IL27_e** | - | - | - | - |
| **TGFB_e** | - | - | - | - |
| **IL36_e** | - | - | - | - |
| **IL33_e** | - | - | - | - |
| **IL18_e** | - | - | - | - |
| **IL25_e** | - | - | - | - |
| **IFNB_e** | - | - | - | - |
| **IFNA_e** | - | - | - | - |
| **IL1A_e** | - | - | - | - |
| **IL29_e** | - | - | - | - |
| **APC** | - | - | - | - |
| **TCR** | APC | - | - | - |
| **CD28** | APC | - | - | - |

| | | | | |
|---|---|---|---|---|
| **IFNGR** | IFNGR1 & IFNGR2 & (IFNG\|IFNG_e) | - | - | - |
| **IL36R** | IL36_e & IL1RL2 & IL1RAP | - | - | - |
| **IL1R** | (IL1B_e\|IL1A_e) & IL1RAP & IL1R1 | - | - | - |
| **IL2R** | 2->CGC & IL2RB & IL2RA & (IL2\|IL2_e)<br>1->CGC & IL2RB & !IL2RA & (IL2\|IL2_e) | - | - | - |
| **IL4R** | CGC & IL4RA & (IL4\|IL4_e) | - | - | - |
| **IL6R** | GP130 & IL6RA & (IL6_e\|IL6) | GP130 & IL6RA & (IL6_e\|IL6) & !miR34 | - | - |
| **IL10R** | IL10RA & IL10RB & (IL10\|IL10_e) | - | - | - |
| **IL12R** | IL12RB1 & IL12RB2 & IL12_e | - | - | - |
| **IL15R** | CGC & IL15RA & IL2RB & IL15_e | - | - | - |
| **IL21R** | GP130 & CGC & (IL21\|IL21_e) | - | - | - |
| **IL23R** | GP130 & IL12RB1 & IL23_e & STAT3 & RORGT | - | - | - |
| **IL27R** | GP130 & IL27RA & IL27_e | - | - | - |
| **IFNAR** | (IFNA_e\|IFNB_e) & IFNAR1 & IFNAR2 | - | - | - |
| **IFNAR1** | - | STAT3 | - | - |
| **IFNAR2** | - | - | - | - |
| **TGFBR** | TGFB\|TGFB_e | (TGFB\|TGFB_e) & SP1 & ETS1 & FLI1 & !miR34 | (TGFB\|TGFB_e \| SP1 \| ETS1 \| FLI1) & !miR34 | - |
| **IFNGR1** | - | !miR155 | - | - |
| **IL1RAP** | - | - | - | - |
| **IFNGR2** | - | - | - | - |

| | | | | |
|---|---|---|---|---|
| **GP130** | - | STAT1 & STAT3 & STAT5 | STAT1 \| STAT3 \| STAT5 | - |
| **IL6RA** | - | - | - | - |
| **IL12RB1** | - | - | - | - |
| **CGC** | - | - | - | - |
| **IL12RB2** | !STAT6 | !STAT6 & !GATA3 | - | - |
| **IL10RB** | - | - | - | - |
| **IL10RA** | - | - | - | - |
| **IL4RA** | - | - | - | - |
| **IL15RA** | - | - | - | - |
| **IL2RB** | - | ETS1 | - | - |
| **IL2RA** | (SMAD3\|FOXP3\|STAT5\|NFKB) & NFAT | (SMAD3\|FOXP3\|STAT5\|NFKB) & NFAT & STAT1 & STAT3 | (SMAD3\|FOXP3\|STAT5\| STAT1\| STAT3\| NFKB) & NFAT | - |
| **IL27RA** | - | - | - | - |
| **IL1R1** | STAT3 | - | - | - |
| **IL29R** | IL29_e & IL28RA & IL10RB | - | - | - |
| **IL17RB** | - | !miR155 | - | - |
| **IL18RAP** | - | - | - | - |
| **IL18RA** | - | - | - | - |
| **IL18R** | IL18_e & IL18RAP & IL18RA & STAT4 | - | - | - |
| **ST2** | GATA3 | - | - | - |
| **IL25R** | IL17RB & (IL25_e\|IL25) | - | - | - |
| **IL33R** | IL33_e & ST2 & IL1RAP | - | - | - |
| **IL1RL2** | - | - | - | - |
| **IL28RA** | - | !miR34 | - | - |
| **TBET** | (TBET\|STAT1\|IL36R) & !BCL6 & !RORGT | (TBET\|STAT1\|IL36R) & !BCL6 & !RORGT & NFKB | (TBET\|STAT1\|IL36R\|NFKB) & !BCL6 & !RORGT | (TBET\|STAT1\|IL36R) & !BCL6 & !RORGT |

| | | | | |
|---|---|---|---|---|
| **GATA3** | 1->!GATA3 & !TBET & (STAT6\|IL25R) & !BCL6 & !PU1 & !IL29R<br>1->GATA3 & !BCL6 & !PU1 & !IL29R | 1->!GATA3 & !TBET & (STAT6\|IL25R) & !BCL6 & !PU1 & !IL29R & NFKB<br>1->GATA3 & !BCL6 & !PU1 & !IL29R & NFKB | 1->!GATA3 & !TBET & (STAT6\|IL25R\| NFKB) & !BCL6 & !PU1 & !IL29R<br>1->(GATA3\|NFKB) & !BCL6 & !PU1 & !IL29R | 1->!GATA3 & !TBET & (STAT6\|IL25R) & !BCL6 & !PU1 & !IL29R<br>1->GATA3 & !BCL6 & !PU1 & !IL29R |
| **RORGT** | TGFBR & STAT3 & !BCL6 & !FOXP3 | - | - | - |
| **FOXP3** | 1->STAT5 & NFAT & FOXP3 & !STAT6<br>1->STAT5 & NFAT & !FOXP3 & SMAD3 & !STAT1 & ! (STAT3 & RORGT) & !STAT6 | 1->STAT5 & NFAT & FOXP3 & !STAT6 & !IRF1<br>1->STAT5 & NFAT & !FOXP3 & SMAD3 & !STAT1 & ! (STAT3 & RORGT) & !STAT6 & !IRF1 | - | - |
| **BCL6** | 1->(STAT1\|STAT3\|STAT4) & !TBET & !STAT5<br>1->STAT3 & STAT4 & !TBET | 1->(STAT1\|STAT3\|STAT4) & !TBET & !STAT5 & FOXO3 & !PU1 & !miR155 & !IRF4<br>1->STAT3 & STAT4 & !TBET & FOXO3 & !PU1 & !miR155 & !IRF4 | 1->(STAT1\|STAT3\|STAT4\| FOXO3) & !TBET & !STAT5 & !PU1 & !miR155 & !IRF4<br>1->STAT3 & STAT4 & !TBET & !PU1 & !miR155 & !IRF4 | 1->(STAT1\|STAT3\|STAT4) & !TBET & !STAT5 & !PU1 & !miR155 & !IRF4<br>1->STAT3 & STAT4 & !TBET & !PU1 & !miR155 & !IRF4 |
| **IFNG** | Proliferation & !FOXP3 & NFAT & ((TBET & RUNX3)\|STAT4\|IL18R) | Proliferation & !FOXP3 & NFAT & ((TBET & RUNX3)\|STAT4\|IL18R) & !STAT1 & JUN & STAT5 & NFKB | Proliferation & !FOXP3 & NFAT & ((TBET & RUNX3)\|STAT4\|IL18R\| JUN \| STAT5 \| NFKB) & !STAT1 | Proliferation & !FOXP3 & NFAT & ((TBET & RUNX3)\|STAT4\|IL18R) & !STAT1 |
| **IL4** | NFAT & proliferation & GATA3 & (STAT5\|cMAF) & !FOXP3 & !((TBET & RUNX3)\|IRF1) | NFAT & proliferation & GATA3 & (STAT5\|cMAF) & !FOXP3 & !((TBET & RUNX3)\|IRF1) & CEBPB | NFAT & proliferation & GATA3 & (STAT5\|cMAF\| CEBPB) & !FOXP3 & !((TBET & RUNX3)\|IRF1) | NFAT & proliferation & GATA3 & (STAT5\|cMAF) & !FOXP3 & !((TBET & RUNX3)\|IRF1) |

| | | | | |
|---|---|---|---|---|
| **IL2** | 1->(NFAT\|NFKB) & !TBET & !FOXP3 & !(STAT5 & STAT6)<br>1->NFAT & TBET & !FOXP3 & !(STAT5 & STAT6) | 1->(NFAT\|NFKB) & !TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN & !ETS1 & EGR1 & RUNX1<br>1->NFAT & TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN & !ETS1 & EGR1 & RUNX1 | 1->(NFAT\|NFKB\| EGR1\| RUNX1) & !TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN & !ETS1<br>1->NFAT & TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN & !ETS1 | 1->(NFAT\|NFKB) & !TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN & !ETS1<br>1->NFAT & TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN & !ETS1 |
| **IL17** | NFAT & proliferation & RORGT & NFKB & STAT3 & !(FOXP3 & STAT1 & STAT5 & STAT6) | - | - | - |
| **IL22** | Proliferation & NFAT & (STAT3\|STAT1) & !cMAF | - | - | - |
| **IL9** | (NFKB\|NFAT) & proliferation & (SMAD3\|PU1\|IL33R) & STAT6 | - | - | - |
| **IL10** | (GATA3\|STAT3\|STAT4\|cMAF\|IRF1) & NFAT & proliferation & !IL18R & !IL33R | (GATA3\|STAT3\|STAT4\|cMAF\|IRF1) & NFAT & proliferation & !IL18R & !IL33R & STAT1 & SP1 & NFKB | (GATA3\|STAT3\|STAT4\|cMAF\|IRF1\| STAT1 \| SP1 \| NFKB) & NFAT & proliferation & !IL18R & !IL33R | (GATA3\|STAT3\|STAT4\|cMAF\|IRF1) & NFAT & proliferation & !IL18R & !IL33R |
| **IL3** | GATA3 & proliferation & NFAT | GATA3 & proliferation & NFAT & ETS1 & EGR1 & !RUNX1 | (GATA3\| ETS1 & EGR1) & proliferation & NFAT & !RUNX1 | GATA3 & proliferation & NFAT & !RUNX1 |
| **IL21** | NFAT & proliferation & (STAT3\|cMAF\|STAT4) | - | - | - |
| **IL5** | Proliferation & NFAT & (GATA3\|cMAF\|IL33R) & !FOXP3 | Proliferation & NFAT & (GATA3\|cMAF\|IL33R) & !FOXP3 & PU1 & YY1 & ETS1 | Proliferation & NFAT & (GATA3\|cMAF\|IL33R\| PU1 \| YY1 \| ETS1) & !FOXP3 | Proliferation & NFAT & (GATA3\|cMAF\|IL33R) & !FOXP3 |

| | | | | |
|---|---|---|---|---|
| **IL13** | Proliferation & NFAT & (GATA3\|cMAF\|IL33R) & !FOXP3 | - | - | - |
| **IL6** | Proliferation & NFAT & STAT3 | Proliferation & NFAT & STAT3 & !STAT1 & !miR155 & JUN & !EGR1 & CEBPB & !NFKB | Proliferation & NFAT & (STAT3\| JUN\| CEBPB) & !STAT1 & !miR155 & !EGR1 & !NFKB | Proliferation & NFAT & STAT3 & !STAT1 & !miR155 & !EGR1 & !NFKB |
| **STAT1** | IFNAR\|IFNGR\|IL27R | (IFNAR\|IFNGR\|IL27R) & STAT3 & !NR3C1 & !miR155 | (IFNAR\|IFNGR\|IL27R\| STAT3) & !NR3C1 & !miR155 | (IFNAR\|IFNGR\|IL27R) & !NR3C1 & !miR155 |
| **STAT3** | IL6R\|IL23R\|IL1R\|IL21R\|IL27R | (IL6R\|IL23R\|IL1R\|IL21R\|IL27R ) & IL10R & !miR155 & !TP53 | (IL6R\|IL23R\|IL1R\|IL21R\|IL27R\| IL10R) & !miR155 & !TP53 | (IL6R\|IL23R\|IL1R\|IL21R\|IL27R ) & !miR155 & !TP53 |
| **STAT4** | IL12R & !GATA3 | - | - | - |
| **STAT5** | 2->IL2R:2<br>1->!IL2R:2 & (IL2R:1\|IL15R) | - | - | - |
| **STAT6** | IL4R | - | - | - |
| **cMAF** | TGFBR & STAT3 | - | - | - |
| **PU1** | TGFBR | TGFBR & RBPJ & JUN & RUNX1 & !miR155 | (TGFBR \| RBPJ \| JUN \| RUNX1) & !miR155 | TGFBR & !miR155 |
| **TGFB** | NFAT & proliferation & FOXP3 | NFAT & proliferation & FOXP3 & SP1 & JUN & HIF1A & !SMAD3 | NFAT & proliferation & (FOXP3 \| SP1 \| JUN \| HIF1A) & !SMAD3 | NFAT & proliferation & FOXP3 & !SMAD3 |
| **SMAD3** | TGFBR | TGFBR & !miR155 | - | - |
| **IRF1** | STAT1 | STAT1 & STAT3 & NFKB | (STAT1 \| STAT3 \| NFKB) | STAT1 |
| **RUNX3** | TBET | - | - | - |
| **proliferation** | STAT5:2\|proliferation | - | - | - |
| **NFKB** | !IKB & !FOXP3 | !IKB & !FOXP3 & SP1 & !FOS & !miR155 & E2F1 & !EGR1 & !AR | !IKB & !FOXP3 & (SP1\| E2F1) & !FOS & !miR155 & !EGR1 & !AR | !IKB & !FOXP3 & !FOS & !miR155 & !EGR1 & !AR & (SP1\|E2F1) |
| **NFAT** | TCR & CD28 | TCR & CD28 & !TP53 | - | - |

| | | | | |
|---|---|---|---|---|
| **IKB** | !TCR | !TCR & NFKB | - | - |
| **IL31** | NFAT & proliferation & STAT6 | - | - | - |
| **IL25** | NFAT & proliferation & GATA3 | - | - | - |
| **IL35** | NFAT & proliferation & FOXP3 | NFAT & proliferation & FOXP3 & NFKB | NFAT & proliferation & (FOXP3 \| NFKB) | NFAT & proliferation & FOXP3 |
| **IL24** | NFAT & proliferation & STAT6 | - | - | - |

*Table 3.2- List of the strict and loose logical rules of the nodes added to our modified versions of the model, and the versions of the model in which each of the two rule sets are used. The presence of a "-" indicates that a node either lacks a logical rule or its logical rule is doesn't undergo any alteration, and therefore the logical rule of that node should be consulted at the column at the left of the "-" signal.*

| Nodes | Strict Logical Rules (Strict, Perfected 2,4,6,7) | Loose Logical Rules (Loose, Intermediate, Perfected 1,3,5) |
|---|---|---|
| **miR34** | FOXO3 & JUN & HIF1A & GATA3 & RUNX1 & TP53 & PU1 & SP1 & FOS & E2F1 & !SNAI1 & !ZEB1 & !BCL6 | (FOXO3 \| JUN \| HIF1A \| GATA3 \| RUNX1 \| TP53 \| PU1 \| SP1 \| FOS \| E2F1) & !SNAI1 & !ZEB1 & !BCL6 |
| **miR155** | TGFB & PU1 & STAT1 & RBPJ & YY1 & FOS & STAT3 & E2F1 & IRF4 & JUN & ETS1 & IRF1 & STAT5 & JUNB & NFKB & AR & SMAD3 & FOXP3 & HIF1A & FLI1 & MYC & EGR1 & CEBPB & GATA3 & RUNX1 & TCF4 & !RUNX3 & !IKB & !AKT1 & !NR3C1 & !BCL6 & !TP53 & ELF3 | (TGFB \| PU1 \| STAT1 \| RBPJ \| YY1 \| FOS \| STAT3 \| E2F1 \| IRF4 \| JUN \| ETS1 \| IRF1 \| STAT5 \| JUNB \| NFKB \| AR \| SMAD3 \| FOXP3 \| HIF1A \| FLI1 \| MYC \| EGR1 \| CEBPB \| GATA3 \| RUNX1 \| TCF4 \| ELF3) & !RUNX3 & !IKB & !AKT1 & !NR3C1 & !BCL6 & !TP53 |
| **MYC** | STAT4 & STAT3 & NFKB & !miR34 & !miR155 | (STAT4 \| STAT3 \| NFKB) & !miR34 & !miR155 |
| **SNAI1** | NFKB & !miR34 | - |
| **E2F1** | !IRF1 | - |
| **RUNX1** | !RUNX3 | - |
| **TP53** | STAT1 & NFKB | STAT1 \| NFKB |
| **ZEB1** | GATA3 | - |
| **JUN** | SMAD3 & !miR155 | - |
| **FOS** | STAT1 & STAT6 & STAT3 & !miR155 | (STAT1 \| STAT6 \| STAT3) & !miR155 |
| **HIF1A** | STAT3 & !miR155 | - |
| **AKT1** | !NKFB & !miR155 | - |
| **JUNB** | STAT3 & NFKB & !miR155 | (STAT3 \| NFKB) & !miR155 |
| **TCF4** | RUNX3 & !miR155 | - |
| **EGR1** | !NFKB | - |
| **AR** | !NFKB | - |
| **IRF4** | NFKB & !BCL6 | - |
| **ELF3** | NFKB | - |
| **YY1** | !miR34 | - |
| **SP1** | !miR155 | - |
| **FOXO3** | !miR155 | - |

| | | |
|---|---|---|
| **ETS1** | !miR155 | - |
| **NR3C1** | !miR155 | - |
| **RBPJ** | !miR155 | - |
| **FLI1** | !miR155 | - |
| **CEBPB** | !miR155 | - |

Initially, we considered a given stable state as part of a Th subtype only when both all the nodes that had to be present are expressed and none of the nodes that had to be absent are expressed. This classification criteria resulted in an incredibly low number of identified stable states in the first two sets of logical rules that we used. We hypothesized that such a result was a consequence of most stable states being hybrid populations of two or more Th cell subtypes, which lead us to adopt a new classification in which only the nodes that have to be present are used to identify the Th subtype of a stable state. As a result, the number of identifiable steady states increased, showing that most of the stable states that we obtain refer to hybrid populations of Th subtypes.

Unfortunately, even though the previous alteration resulted in an improvement of the obtained results, those results were still invalid, since Th1, Th2 and Treg subtypes couldn't be produced in the environments that favored their production, under any of the four scenarios. We ascribed these results to the strict approach that we used in the modification and creation of the logical rules of our modified model. This strict approach can easily be seen in the logical rules of the new nodes, which resulted in such nodes only being expressed when all their activators were expressed, while a single inhibitor was sufficient for its inactivation. With this in mind, we proceeded to create a new set of logical rules which took a loose approach. This set of logical rules, which we named Loose rules, allowed the nodes to be expressed when only a single of their activators was expressed, although the presence of a single inhibitor still resulted in their inactivation. Although this approach worked perfectly for the added nodes, the nodes of the original models could be expressed in circumstances that didn't act in accordance with the original logical rules. Two examples of this would be the IRF1 and IL35 nodes, in which, in the first case, the loose rules allowed, for example, for it to be expressed when STAT3 was present, which goes against the original rule which states that the node is expressed when STAT1 is expressed; by the same token, in the second example, IL35 could be expressed when the nodes NFAT, proliferation and NFKB were active, which contradicts the original rule in which expression occurs when NFAT, proliferation and FOXP3 were the active nodes. To remedy this situation a new set of logical rules, named Intermediate rules, was created. With this process done, two different versions of the modified model are created, one for the loose rules set and another for the intermediate rules set, and we proceed to the computation of the stable states of those two new versions. The logical rules of the nodes of the original model can be consulted in Table 3.1, where the strict, loose and intermediate rule sets of those nodes are also present. The strict, loose and intermediate rules of the nodes added in the modified versions of our model can be consulted in Table 3.2.

While the two new rules sets were able to obtain Th1, Th2 and Treg subtypes, a new set of Th subtypes were not obtained. Specifically, the loose model was unable to produce Th17 and Tfh cells, while the intermediate model was unable to produce Th17 cells. Given that the intermediate model was successful at producing a wider set of Th subtypes, future versions of our modified model were based on it.

With the failure of the intermediate model to produce Th17 cells we asked ourselves why this specific Th cell subtype wasn't produced, which lead us to consult the obtained stable states, thus finding that IL 17 wasn't being expressed as a consequence of the NFAT and NFKB not being simultaneously expressed under any situation. Given that under the strict model this Th subtype was produced we wondered whether the changing of the logical rules of the new nodes (miR-34c-5p, miR-155-5p, MYC, TP53, FOS, JUNB) back to the rules used in the strict model would solve the problem. To that end we created 7 so-called "Perfected" models who differ from each other in accordance with whether miR-34c-5p, miR-155-5p and the other remaining new nodes (MYC, TP53, FOS, JUNB) follow the strict or loose versions of their logical rules. The only combination that is not considered is the one in which all these rules are the loose rules for in such case refers to a perfect copy of the intermediate model. The

combination of logical rules used in the different Perfected models can be seen in Table 3.3. Surprisingly, the only nodes who appear to have any bearing in the results are the (MYC, TP53, FOS, JUNB) nodes, with the results of the seven perfected models being categorized in accordance with whether these nodes follow the strict or the loose rules. The perfected model 1, 3 and 5 followed the loose rules and, unfortunately continued to be unable to produce the Th17 subtype. On the other side, the models in which these nodes adapted the strict rules (Perfected 2/4/6/7), are finally able to produce Th17 cells. The seventh perfected model is the one that we consider to be best since it maintains the loose rules of the two miRNAs as they exist in the intermediate model, while changing the other nodes logical rules to their strict versions, thus being able to produce all Th cell subtypes.

*Table 3.3- Table containing which sets of strict or loose rules are used by miR-34c-5p, miR-155-5p and four other nodes of interest (MYC, TP53, FOS, JUNB) in the seven Perfected versions of the modified model.*

| | **miR-34c-5p** | **miR-155-5p** | **(MYC, TP53, FOS, JUNB)** |
|---|---|---|---|
| **Perfected 1** | Strict | Strict | Loose |
| **Perfected 2** | Strict | Strict | Strict |
| **Perfected 3** | Strict | Loose | Loose |
| **Perfected 4** | Strict | Loose | Strict |
| **Perfected 5** | Loose | Strict | Loose |
| **Perfected 6** | Loose | Strict | Strict |
| **Perfected 7** | Loose | Loose | Strict |

At this point of the thesis, we could opt to proceed to the analysis of the impact of miR-34c-5p on the differentiation of Th cells. Instead, we opted to create a final modified model, aptly named “Last Model”. The reason behind the creation of this model was the elimination of added nodes that aren’t expressed on Th cells. In the previous models, through the use of several different databases, we added a series of nodes to our modified models, regardless of whether or not there was prove that they were present in Th cells. In this model, we use data obtained from (Cano-Gamez et al., 2020) to obtain a model whose added nodes have a confirmed expression in Th cells. This resulted in the elimination of the following nodes: FLI1, ETS1, TCF4, NR3C1, EGR1, AR, ELF3, FOXO3, FOS, HIF1A and SNAI1. Obviously given that there is a lack of information for some subtypes, it is perfectly possible that some of the nodes that are now eliminated should be included in the Last Model, and if future discoveries indicate that such is the case, we encourage the future inheritors of this work or any other interested parties to reintroduce such nodes. It also stands to reason that with the elimination of some of the nodes, certain logical rules will have to be altered.

The elimination of nodes from the Last model was done through the creation of perturbations in which the nodes to be eliminated were knocked out. The Last Model logical rules can be consulted in Table 3.4 and 3.5.

*Table 3.4- List of logical rules of the nodes present in the original model for The Last Model version of the modified model. The presence of a "-"indicates that a node lacks a logical rule.*

| Node | The Last Model |
|---|---|
| **IL1B_e** | - |
| **IFNG_e** | - |
| **IL2_e** | - |
| **IL4_e** | - |
| **IL6_e** | - |
| **IL10_e** | - |
| **IL12_e** | - |
| **IL15_e** | - |
| **IL21_e** | - |
| **IL23_e** | - |
| **IL27_e** | - |
| **TGFB_e** | - |
| **IL36_e** | - |
| **IL33_e** | - |
| **IL18_e** | - |
| **IL25_e** | - |
| **IFNB_e** | - |
| **IFNA_e** | - |
| **IL1A_e** | - |
| **IL29_e** | - |
| **APC** | - |
| **TCR** | APC |
| **CD28** | APC |
| **IFNGR** | IFNGR1 & IFNGR2 & (IFNG\|IFNG_e) |
| **IL36R** | IL36_e & IL1RL2 & IL1RAP |
| **IL1R** | (IL1B_e\|IL1A_e) & IL1RAP & IL1R1 |
| **IL2R** | 2->CGC & IL2RB & IL2RA & (IL2\|IL2_e)<br>1->CGC & IL2RB & !IL2RA & (IL2\|IL2_e) |
| **IL4R** | CGC & IL4RA & (IL4\|IL4_e) |
| **IL6R** | GP130 & IL6RA & (IL6_e\|IL6) & !miR34 |
| **IL10R** | IL10RA & IL10RB & (IL10\|IL10_e) |
| **IL12R** | IL12RB1 & IL12RB2 & IL12_e |
| **IL15R** | CGC & IL15RA & IL2RB & IL15_e |
| **IL21R** | GP130 & CGC & (IL21\|IL21_e) |
| **IL23R** | GP130 & IL12RB1 & IL23_e & STAT3 & RORGT |
| **IL27R** | GP130 & IL27RA & IL27_e |
| **IFNAR** | (IFNA_e\|IFNB_e) & IFNAR1 & IFNAR2 |
| **IFNAR1** | STAT3 |
| **IFNAR2** | - |
| **TGFBR** | (TGFB\|TGFB_e) & !miR34 |
| **IFNGR1** | !miR155 |
| **IL1RAP** | - |

| | |
|---|---|
| **IFNGR2** | - |
| **GP130** | STAT1 \| STAT3 \| STAT5 |
| **IL6RA** | - |
| **IL12RB1** | - |
| **CGC** | - |
| **IL12RB2** | !STAT6 & !GATA3 |
| **IL10RB** | - |
| **IL10RA** | - |
| **IL4RA** | - |
| **IL15RA** | - |
| **IL2RB** | - |
| **IL2RA** | (SMAD3\|FOXP3\|STAT5\| NFKB) & NFAT |
| **IL27RA** | - |
| **IL1R1** | STAT3 |
| **IL29R** | IL29_e & IL28RA & IL10RB |
| **IL17RB** | !miR155 |
| **IL18RAP** | - |
| **IL18RA** | - |
| **IL18R** | IL18_e & IL18RAP & IL18RA & STAT4 |
| **ST2** | GATA3 |
| **IL25R** | IL17RB & (IL25_e\|IL25) |
| **IL33R** | IL33_e & ST2 & IL1RAP |
| **IL1RL2** | - |
| **IL28RA** | !miR34 |
| **TBET** | (TBET\|STAT1\|IL36R) & !BCL6 & !RORGT |
| **GATA3** | 1->!GATA3 & !TBET & (STAT6\|IL25R) & !BCL6 & !PU1 & !IL29R<br>1->GATA3 & !BCL6 & !PU1 & !IL29R |
| **RORGT** | TGFBR & STAT3 & !BCL6 & !FOXP3 |
| **FOXP3** | 1->STAT5 & NFAT & FOXP3 & !STAT6 & !IRF1<br>1->STAT5 & NFAT & !FOXP3 & SMAD3 & !STAT1 & ! (STAT3 & RORGT) & !STAT6 & !IRF1 |
| **BCL6** | 1->(STAT1\|STAT3\|STAT4) & !TBET & !STAT5 & !PU1 & !miR155 & !IRF4<br>1->STAT3 & STAT4 & !TBET & !PU1 & !miR155 & !IRF4 |
| **IFNG** | Proliferation & !FOXP3 & NFAT & ((TBET & RUNX3)\|STAT4\|IL18R) & !STAT1 |
| **IL4** | NFAT & proliferation & GATA3 & (STAT5\|cMAF) & !FOXP3 & !((TBET & RUNX3)\|IRF1) |
| **IL2** | 1->(NFAT\|NFKB) & !TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN |

| | |
|---|---|
| | 1->NFAT & TBET & !FOXP3 & !(STAT5 & STAT6) & !miR155 & !JUN |
| **IL17** | NFAT & proliferation & RORGT & NFKB & STAT3 & !(FOXP3 & STAT1 & STAT5 & STAT6) |
| **IL22** | Proliferation & NFAT & (STAT3\|STAT1) & !cMAF |
| **IL9** | (NFKB\|NFAT) & proliferation & (SMAD3\|PU1\|IL33R) & STAT6 |
| **IL10** | (GATA3\|STAT3\|STAT4\|cMAF\|IRF1) & NFAT & proliferation & !IL18R & !IL33R |
| **IL3** | GATA3 & proliferation & NFAT & !RUNX1 |
| **IL21** | NFAT & proliferation & (STAT3\|cMAF\|STAT4) |
| **IL5** | Proliferation & NFAT & (GATA3\|cMAF\|IL33R) & !FOXP3 |
| **IL13** | Proliferation & NFAT & (GATA3\|cMAF\|IL33R) & !FOXP3 |
| **IL6** | Proliferation & NFAT & STAT3 & !STAT1 & !miR155 & !NFKB |
| **STAT1** | (IFNAR\|IFNGR\|IL27R) & !miR155 |
| **STAT3** | (IL6R\|IL23R\|IL1R\|IL21R\|IL27R) & !miR155 & !TP53 |
| **STAT4** | IL12R & !GATA3 |
| **STAT5** | 2->IL2R:2<br>1->!IL2R:2 & (IL2R:1\|IL15R) |
| **STAT6** | IL4R |
| **cMAF** | TGFBR & STAT3 |
| **PU1** | TGFBR & !miR155 |
| **TGFB** | NFAT & proliferation & FOXP3 & !SMAD3 |
| **SMAD3** | TGFBR & !miR155 |
| **IRF1** | STAT1 |
| **RUNX3** | TBET |
| **proliferation** | STAT5:2\|proliferation |
| **NFKB** | !IKB & !FOXP3 & !miR155 & (SP1\|E2F1) |
| **NFAT** | TCR & CD28 & !TP53 |
| **IKB** | !TCR & NFKB |
| **IL31** | NFAT & proliferation & STAT6 |
| **IL25** | NFAT & proliferation & GATA3 |
| **IL35** | NFAT & proliferation & FOXP3 |
| **IL24** | NFAT & proliferation & STAT6 |

*Table 3.5- Logical rules of the added nodes to the Last Model version of the modified model.*

| Nodes | The Last Model Logical Rules |
|---|---|
| **miR34** | (JUN\| GATA3 \| RUNX1 \| TP53 \| PU1 \| SP1 \| E2F1) & !ZEB1 & !BCL6 |
| **miR155** | (TGFB \| PU1 \| STAT1 \| RBPJ \| YY1 \| STAT3 \| E2F1 \| IRF4 \| JUN \| IRF1 \| STAT5 \| JUNB \| NFKB \| SMAD3 \| FOXP3 \| MYC \| CEBPB \| GATA3 \| RUNX1) & !RUNX3 & !IKB & !AKT1 & !BCL6 & !TP53 |
| **MYC** | STAT4 & STAT3 & NFKB & !miR34 & !miR155 |
| **E2F1** | !IRF1 |
| **RUNX1** | !RUNX3 |
| **TP53** | STAT1 & NFKB |
| **ZEB1** | GATA3 |
| **JUN** | SMAD3 & !miR155 |
| **AKT1** | !NKFB & !miR155 |
| **JUNB** | STAT3 & NFKB & !miR155 |
| **IRF4** | NFKB & !BCL6 |
| **YY1** | !miR34 |
| **SP1** | !miR155 |
| **RBPJ** | !miR155 |
| **CEBPB** | !miR155 |

Upon altering the logical rules previously mentioned, we proceeded to obtain the results for the Last Model of the modified model, and observed that those results showed an increase in the fractions of Th1, Th2, Th9 and Treg while showing a decrease in the fractions of Th17, Th22 and Tfh, when compared with the results of the Perfected 2,4,6 and 7 versions of our modified model.

Finally, a comparison of the results obtained in the different sets of logical rules can be consulted in Table 3.6.

*Table 3.6- Comparing the results obtained by the different versions of modified model when it comes to the fraction of obtained Th subtype in the environment that preferentially leads to the formation of said Th subtype. In this table green corresponds to the situations in which the fraction is 0,5 or more, while red indicates the absence of the intended Th subtype. The yellow color corresponds to situations in which the Th subtype in question is the one present in highest numbers but with a fraction inferior to 0,5. Finally the orange color indicates cases in which the intended subtype is present but is not the Th subtype present in highest numbers.*

| | Th0 | Th1 | Th2 | Th9 | Th17 | Th22 | Tfh | Treg |
|---|---|---|---|---|---|---|---|---|
| **Original** | 1 | 0,26 | 0,01 | 0,51 | 0,66 | 0,43 | 0,23 | 0,52 |
| **Strict** | 1 | 0 | 0 | 0,59 | 0,08 | 0,53 | 0,40 | 0 |
| **Loose** | 1 | 0,45 | 0,08 | 0,47 | 0 | 0,29 | 0 | 0,32 |
| **Intermediate** | 1 | 0,24 | 0,10 | 0,18 | 0 | 0,36 | 0,12 | 0,32 |
| **Perfected 1/3/5** | 1 | 0,24 | 0,09 | 0,18 | 0 | 0,36 | 0,12 | 0,32 |
| **Perfected 2/4/6/7** | 1 | 0,26 | 0,09 | 0,18 | 0,05 | 0,38 | 0,13 | 0,22 |
| **Last Model** | 1 | 0,35 | 0,25 | 0,38 | 0,03 | 0,13 | 0,02 | 0,50 |

# 4 Results

Having obtained a version of our modified model in which every Pro Th environment gives rise to the Th cell subtype whose production they favor, we now proceed to present the results analyzing miRNA effects.

## 4.1 No Stimulation Environment Results

The no stimulation environment of the Last Model results (Table 4.1) show the expected lack of naïve Th cell differentiation with the sole obtention of Th0 cells, event that occurs only when miR-155-5p is expressed, although with a higher percentage in the "Just miR155" scenario. In the remaining scenarios there were no classifiable steady states.

*Table 4.1- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which there is no stimulation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **No Stimulation Environment (0/432/0/288)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | **0 (0)** | **96 (22,22)** | **0 (0)** | **48 (16,67)** | **144** |
| **Th1** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th2** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th22** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |

## 4.2 APC only Environment results

The results of the APC only environment (Table 4.2) present three observable patterns of Th subtype obtention, with the scenario where both miRNAs are absent resulting in the production of small quantities of Th0, Th2 and Th22 cells, the scenarios where miR-155-5p is expressed resulting in the obtention of Th0 and Th2 in small quantities, in addition to significative quantities of Treg cells and finally large quantities of Th1 cells. The remaining scenario, "Just miR34c", results in the production of small quantities of Th0 and Th2, in conjunction with moderate quantities of Th22 and Treg cells.

When it comes to the scenarios which express miR-155-5p, we should take into account two observations that shall become common occurrence in the remaining environments. Both of these occurrences refer to the ratios of the percentages obtained between the "Both" and the "Just miR155" scenarios. The first observation is that the percentages of Th1 and Treg cells in the two scenarios tend to be very similar, with ratios around 1. On the other hand, the ratio of the percentages of Th0 and Th2 cells obtained between the "Both" scenario and the "Just miR155" scenario tend to be around 0,75 and in the range of 1,45 to 1,50, respectively.

*Table 4.2- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which the presence of antigen presenting cells (APC) is the only stimulation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **APC only Environment (1388/1164/1076/798)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 48 (3,46) | 96 (8,25) | 16 (1,49) | 48 (6,02) | 208 |
| **Th1** | 0 (0) | 288 (24,74) | 0 (0) | 192 (24,06) | 480 |
| **Th2** | 32 (2,31) | 24 (2,06) | 32 (2,97) | 24 (3,01) | 112 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th22** | 64 (4,61) | 0 (0) | 120 (11,15) | 0 (0) | 184 |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | 0 (0) | 216 (18,56) | 176 (16,36) | 144 (18,05) | 536 |

## 4.3 Pro Th1 environment results

In the Pro Th1 cell environment (Table 4.3), the scenarios where miR-155-5p is expressed result in the production of small quantities of Th0 and Th2 cells, while producing moderate quantities of Treg cells, and large percentages of Th1 cells. On the other hand, the scenario where the only miRNA expressed is miR-34c-5p there is the production of small quantities of Th2 and Tfh cells, and moderate quantities of Th22 and Treg cells. Lastly, the scenario in which none of the miRNAs is expressed results in the production of Th2 and Th22 cells although in small quantities. Finally, the ratios that have been previously mentioned in the APC only environment, also occur in the Pro Th1 environment.

*Table 4.3- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Th1 differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Th1 environment (2624/2232/2080/1548)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 (0) | 96 (4,30) | 0 (0) | 48 (3,10) | 144 |
| **Th1** | **0 (0)** | **576 (25,81)** | **0 (0)** | **384 (24,81)** | **960** |
| **Th2** | 64 (2,44) | 48 (2,15) | 64 (3,08) | 48 (3,10) | 224 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th22** | 32 (1,22) | 0 (0) | 256 (12,31) | 0 (0) | 288 |
| **Tfh** | 0 (0) | 0 (0) | 64 (3,08) | 0 (0) | 64 |
| **Treg** | 0 (0) | 432 (19,35) | 320 (15,38) | 288 (18,60) | 1040 |

## 4.4 Pro Th2 environment results

The Pro Th2 environment results shown the expected Th2 cells being produced in all scenarios (Table 4.4). The four scenarios all present distinct Th subtype production patterns, with Th0, Th2 and Th22 cells being produced in small percentages when both miRNAs are absent, while in the scenario where miR-155-5p is the only expressed miRNA, the Th cells obtained belong in an enormous quantity

of Th1 cells and are accompanied by moderate quantities of Th0 and Th2 cell subtypes. On the other hand, when only miR-34c-5p is expressed, Th cells differentiate into small quantities of Th2 and Th22 cells and finally, the scenario where both miRNAs are expressed, results in the production of huge quantities of Th1 cells and great quantities of Th2 cells.

*Table 4.4- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Th2 differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Th2 environment (1360/384/944/240)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 32 (2,35) | 48 (12,50) | 0 (0) | 0 (0) | 80 |
| **Th1** | 0 (0) | 288 (75,00) | 0 (0) | 192 (80,00) | 480 |
| **Th2** | **64 (4,71)** | **48 (12,50)** | **64 (6,78)** | **48 (20,00)** | **224** |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th22** | 64 (4,71) | 0 (0) | 32 (3,39) | 0 (0) | 96 |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |

While, as previously mentioned, Th2 cells are obtained under all miRNA expression scenario, the obtained percentages vary greatly between the different expression scenarios, with the “Just miR155” and “Both” scenarios obtaining much higher percentages than the other scenarios. Finally, the ratio that has mentioned previously in the APC only environment, only applies to Th1 cells in this environment.

## 4.5 Pro Th9 environment results

The Pro Th9 environment results in the production of Th9 cells in the scenario where none of the miRNAs is expressed. In such scenario, Th9 cells are produced in large percentages in conjunction with a small percentage of Th17 cells. On the other hand, the “just miR-155-5p” scenario leads to the obtention of small quantities of Th2 cells, in addition to a significant population of Th0 cells and a large quantity of Th1 cells. The “Just miR34” scenario results in the production of small numbers of Th2 and Th22 cells. Meanwhile, the last scenario results in the obtention of large quantities of Th1 and small percentages of Th2 cells. These results can be consulted in Table 4.5.

*Table 4.5- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Th9 differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Th9 environment (928/768/1032/480)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 (0) | 96 (12,50) | 0 (0) | 0 (0) | 96 |
| **Th1** | 0 (0) | 288 (37,50) | 0 (0) | 192 (40,00) | 480 |
| **Th2** | 0 (0) | 24 (3,13) | 32 (3,10) | 24 (5,00) | 80 |
| **Th9** | **448 (48,28)** | **0 (0)** | **0 (0)** | **0 (0)** | **448** |
| **Th17** | 64 (6,90) | 0 (0) | 0 (0) | 0 (0) | 64 |
| **Th22** | 0 (0) | 0 (0) | 16 (1,55) | 0 (0) | 16 |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |

Once again, just like in the previous environment, the ratios mentioned in APC only environment only apply to the Th1 cell subtype.

## 4.6 Pro Th17 environment results

The Pro Th17 environment results (Table 4.6), show the production of the expected Th17 cells in the scenario where none of the miRNAs are expressed, which results in the production a small percentage of Th17 steady states. The scenarios where miR-155-5p are expressed result in the production of large quantities of Th1 cells, medium quantities of Treg cells and small quantities of Th0 and Th2 cells. The scenario where miR-34c-5p is the only miRNA present, results in the production of small quantities of Th0, Th2, Th22 and Treg cells. The ratio that was observed in the APC only environment is once again fully appliable to this environment.

*Table 4.6- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Th17 differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Th17 environment (464/1182/912/798)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 (0) | 96 (8,12) | 8 (0,88) | 48 (6,02) | 152 |
| **Th1** | 0 (0) | 288 (24,37) | 0 (0) | 192 (24,06) | 480 |
| **Th2** | 0 (0) | 24 (2,03) | 16 (1,75) | 24 (3,01) | 64 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | **32 (6,90)** | **0 (0)** | **0 (0)** | **0 (0)** | **32** |
| **Th22** | 0 (0) | 0 (0) | 60 (6,58) | 0 (0) | 60 |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | 0 (0) | 216 (18,27) | 88 (9,65) | 144 (18,05) | 448 |

## 4.7 Pro Th22 environment results

As shown in Table 4.7, the Pro Th22 environment results in the production of Th22 cells in the scenarios where miR-155-5p is not expressed. The scenarios where miR-155-5p is expressed result in the production of small percentages of Th0 and Th2, together with moderate percentages of Treg and a large percentage of Th1.

On the other hand, the scenarios where none of the miRNAs are expressed results in the production of small quantities of Th2 and Th22 cells. The last scenario, the "Just miR34c" scenario results in the production of small percentages of Th0 and Th2 cells and moderate quantities of Th22 and Treg cells.

Although the Th22 cells can be obtained in two different scenarios it should be noted that when comparing the results obtained in the "None" scenario with the "Just miR34c" scenarios, the latter results take nearly the double and more than the double of the absolute and relative values that are observed in the former scenario. Finally, the ratios mentioned in the APC only environment occur in this environment.

*Table 4.7- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Th22 differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Th22 environment (1256/1164/1076/798)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 (0) | 96 (8,25) | 16 (1,49) | 48 (6,02) | 160 |
| **Th1** | 0 (0) | 288 (24,76) | 0 (0) | 192 (24,06) | 480 |
| **Th2** | 16 (1,27) | 24 (2,06) | 32 (2,97) | 24 (3,01) | 96 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th22** | **64 (5,10)** | **0 (0)** | **120 (11,15)** | **0 (0)** | **184** |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | 0 (0) | 216 (18,56) | 176 (16,36) | 144 (18,05) | 536 |

## 4.8 Pro Tfh environments results

The results of the Pro Tfh environment (Table 4.8) show three different patterns of Th cell differentiation, with the scenarios where miR-155-5p is present resulting in the production of small quantities of Th0 and Th2 cells, moderate quantities of Treg cells and large numbers of Th1 cells, and the ratios that have been previously described once again occurring in this environment.

The "None" scenario results in the production of small quantities of both Th2 and Th22 cells. Finally, "Just miR34c" scenario results in the production of significative quantities of Th22 and Treg cells and minor quantities of Th2 and Tfh cells.

*Table 4.8- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Tfh differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Tfh environment (2496/2448/1888/1656)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 (0) | 168 (6,86) | 0 (0) | 84 (5,07) | 252 |
| **Th1** | 0 (0) | 576 (23,53) | 0 (0) | 384 (23,19) | 960 |
| **Th2** | 32 (1,28) | 48 (1,96) | 32 (1,69) | 48 (2,90) | 160 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th22** | 32 (1,28) | 0 (0) | 256 (13,56) | 0 (0) | 288 |
| **Tfh** | **0 (0)** | **0 (0)** | **64 (3,39)** | **0 (0)** | **64** |
| **Treg** | 0 (0) | 432 (17,65) | 224 (11,86) | 288 (17,39) | 944 |

## 4.9 Pro Treg environment results

Finally, Treg cells are also obtained in the ProTreg environment, being produced by every scenario where at least one of the miRNAs is expressed. In this environment, when none of the miRNAs are expressed Th17 is the only Th cell subtype that naïve CD4+ T cells differentiate into, which results in a significative Th17 cell population. When it comes to the scenarios where miR-155-5p is the only miRNA present naïve Th cell differentiation results in obtaining a minor percentage of Th2 cells, a significative quantity of Th0 cells and large quantities of Th1 and Treg cells. The "Both" scenario results in the obtention of the same Th cell subtypes, with the only difference being that Th0 is present in small quantities. Finally, the "Just miR34c" scenario results in the obtention of small quantities of Th0 and Th2 cells, a significative percentage of Th22 cells and a large quantity of Treg cells.

It should be noted that the percentages of Treg cells obtained in the scenarios where miR-155-5p is expressed are much higher than the percentage of Treg cells obtained in the "Just miR34c" scenario. finally, the ratio that was so frequently mentioned so far, occurs once again in the Pro Treg environment. More detailed results can be consulted in Table 4.9.

*Table 4.9- Results of the number of steady states identified as being part of a given Th subtype, under the four different combinations of miRNA expression, in the environment in which promotes Treg differentiation, for the Last model version of our modified model. The four numbers under parenthesis following the mentioned environment refer to the number of steady states that the modified model produces in the four different miRNA expression scenarios. The number under parenthesis following the number of steady states classified as being part of a certain Th cell subtype, refers to percentage of that result compared with the total number of steady states.*

| **Pro Treg environment (608/864/1152/576)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 (0) | 96 (11,11) | 16 (1,39) | 48 (8,33) | 160 |
| **Th1** | 0 (0) | 288 (33,33) | 0 (0) | 192 (33,33) | 480 |
| **Th2** | 0 (0) | 48 (5,56) | 64 (5,56) | 48 (8,33) | 160 |
| **Th9** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Th17** | 64 (10,53) | 0 (0) | 0 (0) | 0 (0) | 64 |
| **Th22** | 0 (0) | 0 (0) | 224 (19,44) | 0 (0) | 224 |
| **Tfh** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 |
| **Treg** | **0 (0)** | **432 (50,00)** | **352 (30,56)** | **288 (50,00)** | **1072** |

## 4.10 Results by Th cell subtype

Following the previous description of the results obtained by the Last Model version of our modified model, we proceed to analyze those results per Th cell subtype.

### 4.10.1 Th0

To begin with, our results show that although Th0 cells are obtained in all environments, the miRNA expression scenarios in which they are obtained differ between environments.

As such, in the no stimulation, Pro Th1 and Pro Tfh environments, Th0 cells are produced in the scenarios which express miR-155-5p, with higher percentages being obtained in the "Just miR155" scenario, as seen in Figure 4.10.1.1.

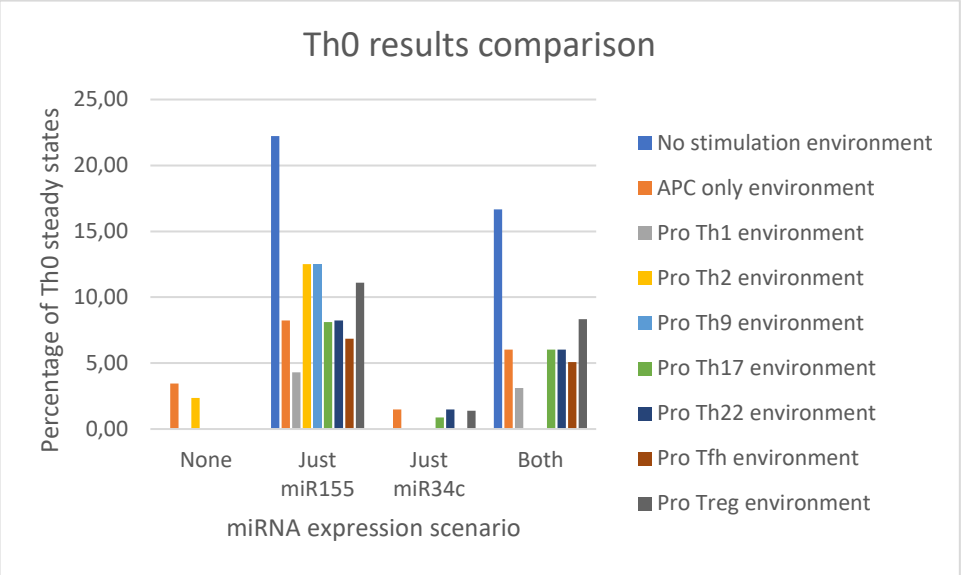


*Figure 4.10.1.1-Comparison of the percentages of steady states classified as belonging to the Th0 cell subtype in the nine different environments of the four miRNA expression scenarios.*

In the Pro Th2 environment, Th0 cells are produced whenever miR-34c-5p is absent, and the "Just miR155" scenario is the one with a higher percentage of obtained Th0 cells.

In the Pro Th9 environment, Th0 cells are obtained only in the scenario where miR-155-5p is the only miRNA present.

In the Pro Th17, Pro Th22 and Pro Treg environments, Th0 cells are formed in the scenarios where at least one of the miRNAs are expressed. With the scenario where miR-155-5p is the only miRNA expressed favoring the obtention of Th0 cells while the presence of miR-34c-5p, especially when alone, being detrimental for the obtention of Th0 cells.

In the APC only environment, Th0 cells are produced in every scenario, with a higher percentage of Th0 cells being obtained in the "Just miR155" scenario and lower in the "Just miR34c" scenario.

In conclusion, Th0 cells are obtainable under every miRNA expression scenario, and its highest percentages are obtained in the "Just miR155" scenario, while its lowest are obtained in the "Just miR34c" scenario. While the expression of miR-155-5p appears to increase the percentage of obtained Th0 cells, miR-34c-5p appears to have the opposite effect, with the "Just miR34c" presenting a lower

percentage of Th0 cells than the scenario where none of the miRs are present. Additionally, given that the percentages obtained in the "Both" scenario tends to be 75% of the percentages obtained in the "Just miR155" scenario, it appears that in this environment the presence of miR-34c-5p is able to inhibit the effects of miR-155-5p.

### 4.10.2 Th1

The Th1 CD4+ T cell subtype is obtained in all environments except the No Stimulation environment, and in all these cases its expression occurs in the scenarios where miR-155-5p is expressed, with the percentages obtained for the scenario pair "Both" and "Just miR155" being similar for each environment (Figure 4.10.2.1).

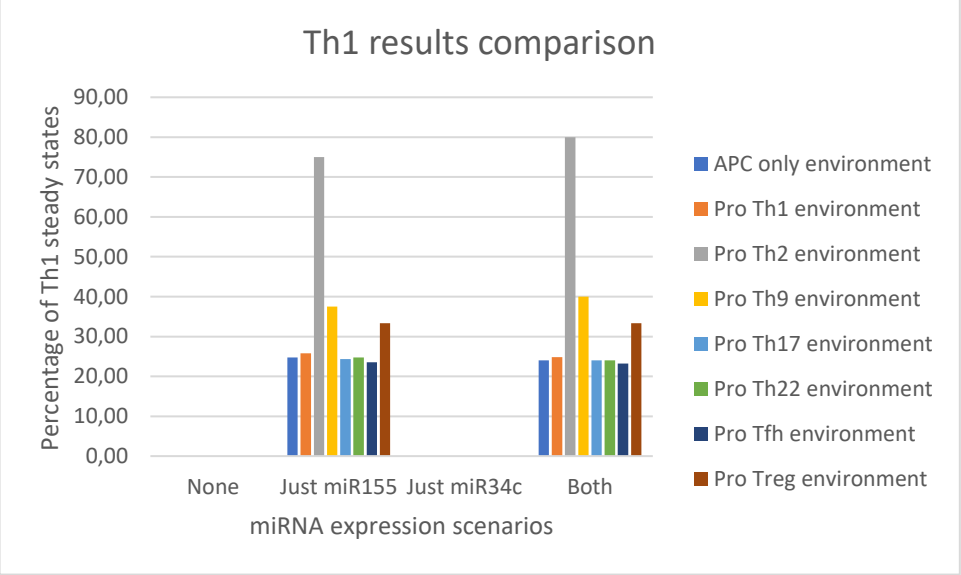


*Figure 4.10.2.1- Comparison of the percentages of steady states classified as belonging to the Th1 cell subtype in the nine different environments of the four miRNA expression scenarios.*

The fact that the production of Th1 cells only occurs upon the presence of miR-155-5p establishes a clear link between this miRNA and the production of this Th cell subtype, thus acting in accordance with the literature, which states that miR-155-5p is essential for the development and function of Th1 cells (Banerjee et al., 2010).

### 4.10.3 Th2

In its turn, Th2 cells are obtained in every environment except the No stimulation environment (Figure 4.10.3.1).

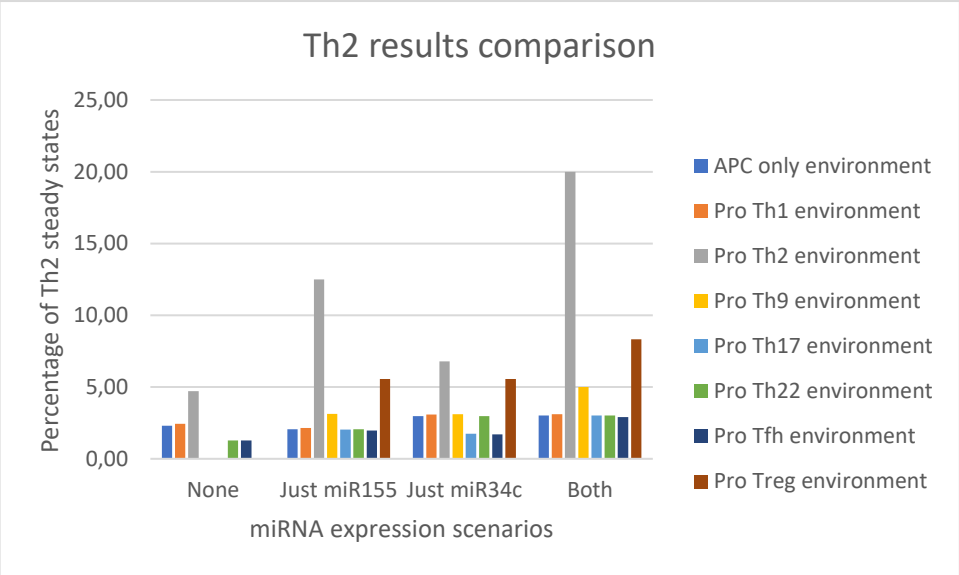


*Figure 4.10.3.1- Comparison of the percentages of steady states classified as belonging to the Th2 cell subtype in the nine different environments of the four miRNA expression scenarios.*

In the APC only and Pro Th1 environments, Th2 cells are produced in every scenario, with the higher percentages being obtained in the scenario where both miRNAs are expressed, and the lowest percentage corresponding to the scenario in which only miR-155-5p is expressed. The comparison of the scenarios where only one of the miRNAs are present with the scenario where none of the miRNAs is expressed, appears to suggest that miR-155-5p has an inhibitory effect on the production of Th2 cells, while the miR-34c-5p has a stimulatory effect in their production. Curiously, the scenario in which both miRNAs are present is the one with the higher percentage of Th2 cells, which appears to go against the previous assessment that miR-155-5p served as an inhibitor of Th2 cell differentiation.

In the Pro Th2 and Pro Tfh environments, all miRNA expression scenarios result in the production of the Th2 cell subtype, but unlike in the environments in the previous paragraph, both miRNAs appear to have a stimulatory effect in the production of Th2 cells, although the miR-155-5p appears to have a stronger effect on the Th2 cell production. Just like in the previous environments, the "Both" scenario is the one which results in a higher percentage of Th2 cells. The exact same situation occurs with the Pro Th22 environment, with the exception that in this environment, the miR-34c-5p is the miRNA with the highest stimulatory effect on Th2 cell differentiation.

In the Pro Th9 and Th17 environments, only the scenarios with no miRNAs don't produce Th2 cells, and, while the "Both" scenario is the one with the higher percentage of Th2 cells, the results of the two remaining scenarios differ between the two environments. As such, while in the Pro Th9 environment, those two scenarios show results with similar percentages, in the Pro Th17 environment, the percentage of Th2 cells obtained in the "Just miR34c" scenario is significantly lower than the percentage obtained in the "Just miR155" scenario.

Finally, the Pro Treg environment results in the production of Th2 cells in every scenario in which at least one of the miRNAs is expressed. With the same percentage of Th2 cells being obtained in the two scenarios where one of the miRNAs is expressed, while the "Both" scenario once again presents the highest percentage of Th2 cells.

In summary, the results of our modified model don't allow to identify a clear role for the two miRNAs of interest in the differentiation of Th2 cells, given that these cells are obtainable in every single scenario, and that the behavior of the two miRNAs differs between different environments, although as a general rule, in most cases both miRNAs stimulate the differentiation of naïve CD4+ T cells into Th2 cells.

### 4.10.4 Th9

Th9 cells are obtained only in the Th9 environment, where they are produced in great quantities in the scenario where none of the miRNAs of interest are expressed (Figure 4.10.4.1). These results thus allow us to establish a clear inhibitory role for the miR-34c-5p and the miR-155-5p in Th9 cell differentiation.

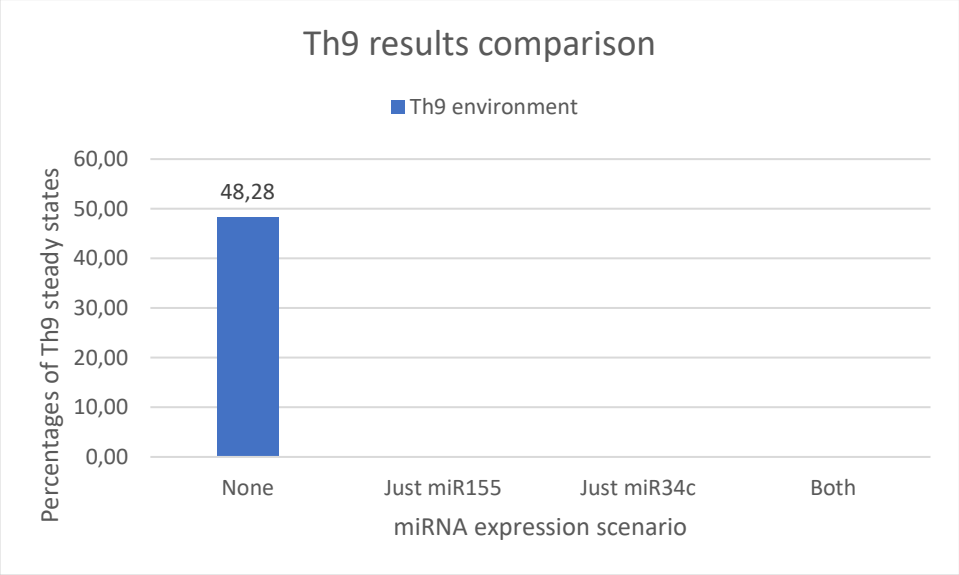


*Figure 4.10.4.1- Comparison of the percentages of steady states classified as belonging to the Th9 cell subtype in the nine different environments of the four miRNA expression scenarios.*

### 4.10.5 Th17

In our modified model, the Th17 CD4+ T cell subtype is obtained under three different environments: the Pro Th9, Pro Th17 and Pro Treg environments. In all of these environments, Th17 cells are produced only in the environment in which none of the miRNAs are expressed, taking a percentage of 6,90% in the Pro Th9 and Pro Th17 environments and a percentage of 10,53% in the Pro Treg environment (Figure 4.10.5.1).

Once again, like in the case of the Th9 subtype, the production of Th9 cells only when none of the miRNAs of interest is expressed, appear to point to both miR-155-5p and miR-34c-5p playing an inhibitory role in the differentiation of naïve CD4+ T cells into Th17 cells. Unfortunately, these results contradict the literature according to which the expression of miR-155-5p is essential for the production of Th17 cells (O'Connell et al., 2010).

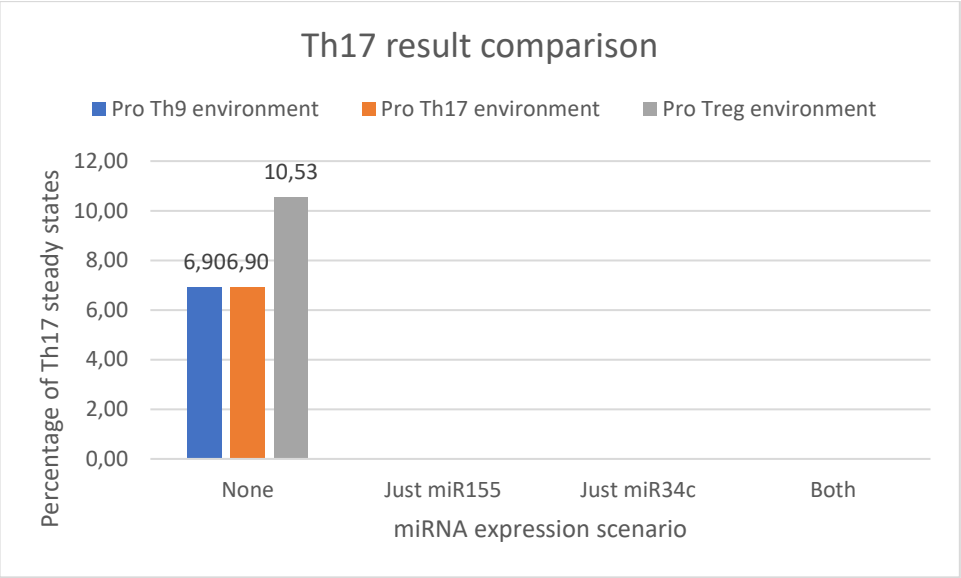


*Figure 4.10.5.1- Comparison of the percentages of steady states classified as belonging to the Th17 cell subtype in the nine different environments of the four miRNA expression scenarios.*

### 4.10.6 Th22

The Th22 cell subtype is obtained in every environment except the No stimulation environment (Figure 4.10.6.1).

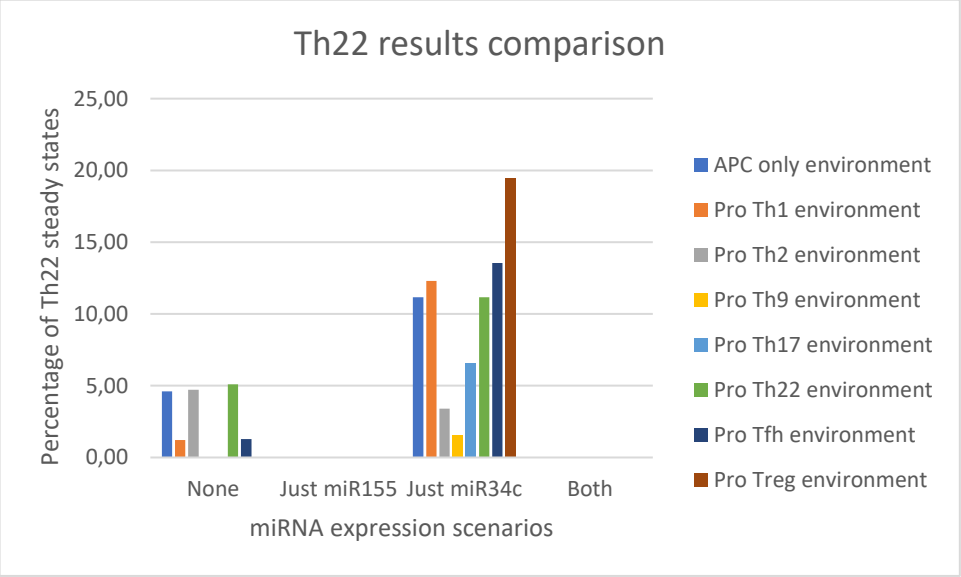


*Figure 4.10.6.1- Comparison of the percentages of steady states classified as belonging to the Th22 cell subtype in the nine different environments of the four miRNA expression scenarios.*

In the APC only, Pro Th1 and Pro Th22 and Pro Tfh environments, Th22 cells are obtained in the scenarios where miR-155-5p is not expressed, with higher values in the "Just miR34c" scenario indicating that miR-34c-5p plays a role in stimulating in the differentiation of naïve CD4+ T cells into Th22 cells.

In the Pro Th2 environment, the scenarios in which miR-155-5p is absent also results in the production of Th22 cells, but in this case the results appear to show that Th22 the miR-34c-5p has an inhibitory role in the differentiation of Th22 cells.

Finally, in the Pro Th9, Pro Th17 and Pro Treg environments, Th22 cells are produced in the scenario where miR-34c-5p is the only miRNA that is expressed.

The fact that our results show that Th22 cells are produced in any environments where miR-155-5p is not present clearly shows us that this miRNA has an inhibitory role in the differentiation of Th22 cells. On the other hand, the results appear to indicate that miR-34c-5p stimulates the differentiation of Th22 cells, with the results of "Just miR34c" scenario increasing of more than 200% when compared with the results of the "None" scenario in the APC only and Pro Th22 environments, and tenfold in the Pro Th1 and Pro Tfh environments. There is an exception to these increases in the Pro Th2 environment where, where the percentage in the "Just miR34c" scenario is about 71% of that in the "None" scenario, but this exception nonetheless, there appears to be more than enough evidence to propose that miR-34c-5p plays a stimulatory role for the production of Th22 cells although in some environments its presence is not necessary for the formation of Th22 cells.

### 4.10.7 Tfh

Tfh cells are produced in the Pro Th1 and Pro Tfh environments, in both cases only in the scenario where miR-34c-5p is the only miRNA that is expressed, with a slightly higher percentage of cells beings produced in the Pro Tfh environment (Figure 4.10.7.1). As such, it is quite easy to conclude that our results show that miR-34c-5p is essential for the differentiation of naïve CD4+ T cells into Tfh cells, even though its stimulatory effect is nullified when miR-155-5p is expressed, as visible by our results in the scenario where both miRNAs are expressed.

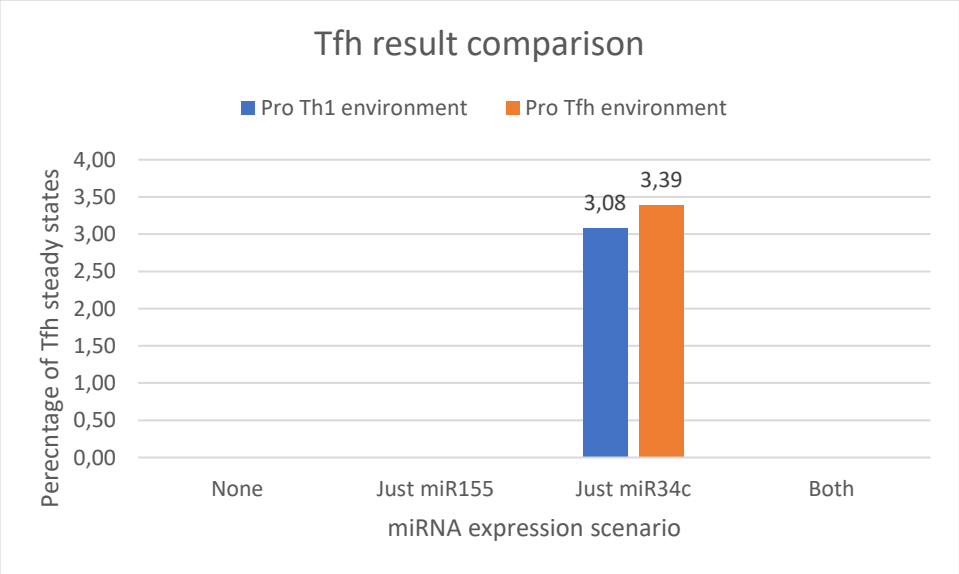


*Figure 4.10.7.1- Comparison of the percentages of steady states classified as belonging to the Tfh cell subtype in the nine different environments of the four miRNA expression scenarios.*

### 4.10.8 Treg

Finally, Treg cells are obtained in the following environments: APC only, Pro Th1, Pro Th17, Pro Th22, Pro Tfh and Pro Treg. In all of these environments, production of Treg cells occurs in the scenarios where at least one of the miRNAs of interest is expressed (Figure 4.10.8.1). In the same way, in all environments, the scenario where the higher percentage of Treg cells occurs is the scenario where miR-155-5p is the only miRNA expressed, and the scenario with the lower percentage is the "Just

miR34c" scenario, with the scenario where both miRNAs are expressed having a percentage of Treg cells close but slightly lower than the percentage of Treg cells in the "Just miR155" scenario. As such, the author is of the opinion that although both miRNAs lead to the stimulation of Treg cell differentiation, the stimulatory role of miR-155-5p is somewhat stronger than that of miR-34c-5p. It is of interest that, once again, there appear to be indications of miR-34c-5p inhibiting to a certain degree the stimulatory effects of miR-155-5p in Th cell differentiation.

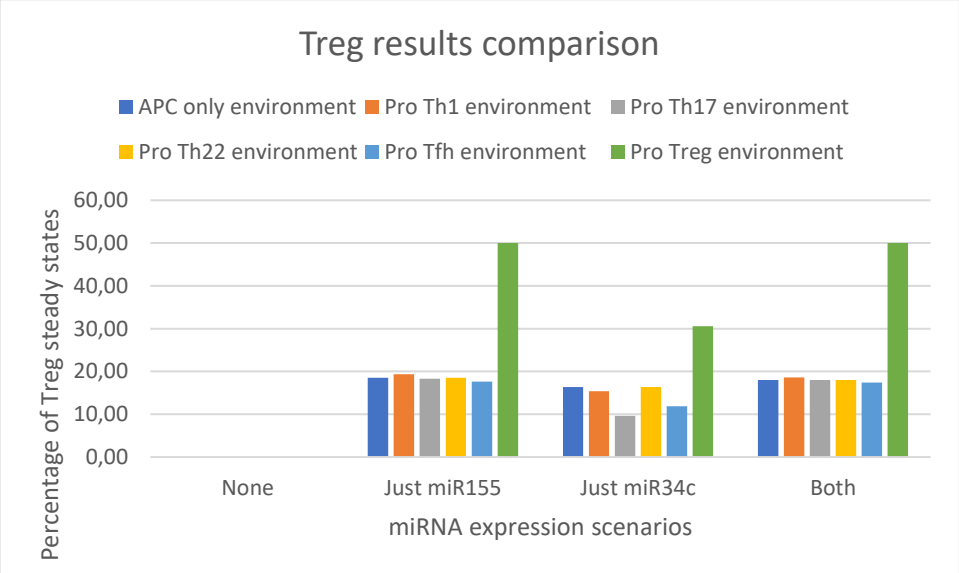


*Figure 4.10.8.1- Comparison of the percentages of steady states classified as belonging to the Treg cell subtype in the nine different environments of the four miRNA expression scenarios.*

# 5 Discussion and Conclusion

Now that we have gone through the description of the results followed by their analysis per Th cell subtype, we proceed to evaluate the role of miR-155-5p and miR-34c-5p in the differentiation of naïve CD4+ T cells into their different subtypes.

Taking into account the previous results, it is clear to the author that miR-155-5p is essential for the formation of Th1 cells, given that the production of Th1 cells only occurs in the environments in which this miRNA is expressed. By the same token, miR-155-5p appears to stimulate the differentiation of naïve Th cells into Treg cell. We arrived to this conclusion through the observation that although Treg cells can be produced in any scenario where at least one of the miRNAs is present, it is in the scenarios in which miR-155-5p is expressed, that the highest percentages of Treg cells are observed. These results are in accordance with the literature (L. F. Lu et al., 2009), in which miR-155-5p is fundamental for the development and function of Th1 and Treg cells.

On the other hand, miR-155-5p appears to play an inhibitory role in the differentiation of Th9 and Th17 cells, given that these cells are only produced in the scenario in which none of the miRNAs are expressed. In the same way, miR-155-5p also appears to be an inhibitor of Th22 cell differentiation, which only is produced when miR-155-5p is not present. Seeing how Tfh cells are produced when miR-34c-5p is present but aren't produced in the presence of both miRNAs, miR-155-5p also appears to be an inhibitor of Tfh cell differentiation and at least in this environment, is able to inhibit the stimulatory effects of miR-34c-5p. In the case of Th17 cells, our result contradicts the literature (O'Connell et al., 2010) which states that miR-155-5p is necessary for the Th17 development and function. It should be

noted that our work only analyses the activity of the miRs at the steady states. It is plausible that miR-155-5p activity could be necessary in the dynamic trajectory of the system before reaching the steady state, and not necessary anymore once steady state is reached. To clarify this situation, future work should simulate the dynamical trajectories of the system that lead to each type of steady state. If these simulations show that miR-155-5p activity is necessary to reach Th17 steady states, but is absent in the final state, the model agrees with previous observations. In any case, experimental detection of activity of miR-155-5p in stable Th17 cells can prove that our model is not valid.

Finally, when it comes to the impact of miR-34c-5p on naïve CD4+ T cell differentiation, we can easily say that it plays no role in the differentiation of naïve Th cells into Th1. We arrive to this conclusion by observing that no Th1 cells are obtained in the "Just miR34c" scenario and that there are significative differences between the percentages obtained in the "Just miR155" scenario and the "Both" scenario, which eliminates the possibility of miR-34c-5p having an inhibitory role in this process.

By the same token, miR-34c-5p plays an inhibitory role in Th9 and Th17 cell differentiation, given that these cells are only produced in the scenario where none of the miRNA are expressed.

On the other hand, miR-34c-5p has a stimulatory role in the differentiation of naïve Th0 cells into Th22 cells. Th22 cells are formed in the scenarios in which miR-155-5p is not present, and as such are not dependent upon the expression of miRNAs, but the scenario in which miR-34c-5p is expressed results in the increase of environments in which Th22 cells are obtained, in addition to an increase of the percentages of Th22 cells obtained that ranges from two to tenfold.

It is on Tfh cells though, that the influence of miR-34c-5p is the clearer, with this cell subtype only being produced when miR-34c-5p is the only miRNA expressed, thus making miR-34c-5p essential for the production of Tfh cells.

MiR-34c-5p has once again a stimulatory role in the production of Treg cells, which are produced in any scenario where at least one of the miRNAs are present. Curiously, even though both of the miRNAs of interest play a stimulatory effect upon the formation of Treg cells, not only does the effect of miR-34c-5p appears to be lesser than that of miR-155-5p but it also appears that in this scenario miR-34c-5p has an inhibitory effect upon miR-155-5p, given that in the scenario where both miRNAs are present, the modified model usually gives rises to percentages of Treg cells that are slightly lower than when only miR-155-5p is expressed.

This is not the first instance in which miR-34c-5p appeared capable to inhibit miR-155-5p effects.

When it comes to the percentages of obtained Th0 cells, the results show that these cells can be obtained in every single scenario. However, higher percentages of Th0 cells are clearly associated with the presence of miR-155-5p, while miR-34c-5p plays an inhibitory role with the "Just miR34c" scenario resulting in lower percentages than the "None" scenario. It is also in this scenario, that once more the inhibitory effect of miR-34c-5p on miR-155-5p is laid bare, with a the "Both" scenario presenting significantly lower percentages of Th0 cells than the "Just miR155" scenario, possibly due miR-34c-5p inhibiting the stimulatory effect of miR-155-5p.

Finally, when it comes to the Th2 cells, our results don't allow us to find any discernible rules, other than that in most environments both miRNAs act as stimulators of the differentiation of naïve Th cells into Th2 cells.

In summary, during this thesis, we proceeded to adopt a previously published CD4+ T cell differentiation model (Abou-Jaoudé et al., 2015) by adding information regarding miR-34c-5p and miR-

155-5p, their targets and transcription factors, and obtaining the steady states of this modified model, aiming to evaluate the impact of these miRNAs upon CD4+ T cell differentiation.

During this process we created several versions of our modified model, with different logical rules for the nodes of our system, until we reached a version of the modified model in which all environments which favored the production of a certain Th cell subtype gave rise to such cell subtype. Upon the obtention of a version of our modified model which managed to succeed in such task we proceeded to the analysis of the model steady states, which permitted to determine the impact of our two miRNAs of interest upon the differentiation of naïve CD4+ T cells.

As such, the analysis of our results showed that miRNA-155-5p plays an inhibitory role in the formation of Th9, Th17, Th22 and Tfh cells, while playing a stimulatory role in the differentiation of naïve Th0 cells into Th1 and Treg cells. Finally, its role in the production of Th2 cells couldn't be inferred although in general it appears to have a stimulatory effect.

When it comes to miRNA-155-5p, there appear to be some discrepancies between the literature and the results obtained by our model. These discrepancies may be explained through the absence of nodes such as SOCS1, that interact with miRNA-155-5p. In this work we only included interactions present in OMNIPath. Although it covers a large number of curated interactions, many real interactions are not yet included. To address these discrepancies, a manual curation of the modified model and a literature search for additional interactions are needed. Regardless, for the most part our results act in accordance with the available literature.

On the other hand, miR-34c-5p appears to have no impact on Th1 cell differentiation, while being an inhibitor of naïve Th cell differentiation into Th9 and Th17 cells, and playing a stimulatory role in the differentiation of naïve Th0 cells into Th22, Tfh and Treg cells. In fact, miR-34c-5p appears to be fundamental for the obtention of Tfh cells. The role of miR-34c-5p in the production of Th2 cells couldn't be inferred with certainty, but in most cases, it would appear that this miRNA has a stimulatory effect in the production of Th2 cells.

It should also be noted that according to our results, miR-34c-5p appears to play an inhibitory role upon the effects of miR-155-5p at least when it comes to the number of Th0 and Treg cells obtained, while on the other hand, miR-155-5p appears to inhibit the effects of miR-34c-5p when it comes to the number of Tfh cells obtained.

Finally, we would like to say that in the future, further laboratorial studies are necessary to confirm whether the removal of FLI1, ETS1, TCF4, NR3C1, EGR1, AR, ELF3, FOXO3, FOS, HIF1A and SNAI1 are or not justified. It is crucially important to study whether any of these is expressed during Th cell differentiation as their reintroduction into our model may have an effect upon the observed results.

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# 7 Supplemental Information

*Supplemental Table 7.1-Table containing the number of steady states obtained by which of the different versions of ethe modified model in the nine different initial environmental conditions when none of the miRNAs is expressed.*

| Steady States Number Comparison (No miRNA is expressed) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **No stimulation** | **APC only** | **Pro Th1** | **Pro Th2** | **Pro Th9** | **Pro Th17** | **Pro Th22** | **Pro Tfh** | **Pro Treg** |
| **Strict** | 480 | 4968 | 14208 | 4608 | 4992 | 1248 | 3432 | 10752 | 864 |
| **Loose** | 960 | 2496 | 4992 | 192 | 1728 | 1152 | 2304 | 4608 | 1536 |
| **Intermediate** | 1152 | 4560 | 8160 | 1344 | 2496 | 1920 | 3840 | 7680 | 3648 |
| **Perfected 1/3/5** | 1152 | 4560 | 8160 | 1344 | 2496 | 1920 | 3840 | 7680 | 3648 |
| **Perfected 2/4/6/7** | 1152 | 5232 | 9120 | 2688 | 4992 | 2112 | 4224 | 8448 | 3264 |
| **The Last Model** | 0 | 1388 | 2624 | 1360 | 928 | 464 | 1256 | 2496 | 608 |

*Supplemental Table 7.2- Table containing the number of steady states obtained by which of the different versions of ethe modified model in the nine different initial environmental conditions when miRNA-155-5p is expressed.*

| Steady States Number Comparison (miR-155-5p expressed) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **No stimulation** | **APC only** | **Pro Th1** | **Pro Th2** | **Pro Th9** | **Pro Th17** | **Pro Th22** | **Pro Tfh** | **Pro Treg** |
| **Strict** | 72 | 72 | 281 | 96 | 144 | 72 | 72 | 288 | 48 |
| **Loose** | 432 | 1260 | 2232 | 384 | 768 | 1278 | 1260 | 2448 | 864 |
| **Intermediate** | 432 | 1164 | 2232 | 384 | 768 | 1182 | 1164 | 2448 | 864 |
| **Perfected 1/3/5** | 432 | 1164 | 2232 | 384 | 768 | 1182 | 1164 | 2448 | 864 |
| **Perfected 2/4/6/7** | 432 | 1164 | 2232 | 384 | 768 | 1182 | 1164 | 2448 | 864 |
| **The Last Model** | 432 | 1164 | 2232 | 384 | 768 | 1182 | 1164 | 2448 | 864 |

*Supplemental Table 7.3- Table containing the number of steady states obtained by which of the different versions of ethe modified model in the nine different initial environmental conditions when miRNA-34c-5p is expressed.*

| Steady States Number Comparison (miR-34c-5p expressed) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **No stimulation** | **APC only** | **Pro Th1** | **Pro Th2** | **Pro Th9** | **Pro Th17** | **Pro Th22** | **Pro Tfh** | **Pro Treg** |
| **Strict** | 240 | 3096 | 8256 | 2688 | 144 | 2580 | 3096 | 288 | 48 |
| **Loose** | 648 | 1608 | 3216 | 144 | 1224 | 840 | 1608 | 3072 | 960 |
| **Intermediate** | 2712 | 10404 | 22104 | 2880 | 5616 | 5478 | 10404 | 19248 | 8448 |
| **Perfected 1/3/5** | 2712 | 10404 | 22104 | 2880 | 5616 | 5478 | 10404 | 19248 | 8448 |
| **Perfected 2/4/6/7** | 2712 | 15168 | 32352 | 5760 | 11568 | 8304 | 15168 | 26880 | 9504 |
| **The Last Model** | 0 | 1076 | 2080 | 944 | 1032 | 912 | 1076 | 1888 | 1152 |

*Supplemental Table 7.4- Table containing the number of steady states obtained by which of the different versions of ethe modified model in the nine different initial environmental conditions when both miRNAs are expressed.*

| Steady States Number Comparison (Both miRNAs are expressed) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **No stimulation** | **APC only** | **Pro Th1** | **Pro Th2** | **Pro Th9** | **Pro Th17** | **Pro Th22** | **Pro Tfh** | **Pro Treg** |
| **Strict** | 36 | 36 | 144 | 48 | 72 | 36 | 36 | 144 | 24 |
| **Loose** | 288 | 822 | 1548 | 240 | 480 | 822 | 822 | 1656 | 576 |
| **Intermediate** | 288 | 798 | 1548 | 240 | 480 | 798 | 798 | 1656 | 576 |
| **Perfected 1/3/5** | 288 | 798 | 1548 | 240 | 480 | 798 | 798 | 1656 | 576 |
| **Perfected 2/4/6/7** | 288 | 798 | 1548 | 240 | 480 | 798 | 798 | 1656 | 576 |
| **The Last Model** | 288 | 798 | 1548 | 240 | 480 | 798 | 798 | 1656 | 576 |

*Supplemental Table 7.5-Comparison of the number of steady states that belong to the Th0 cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Th0 cell subtype, and are thus referred as "Pure".*

| **Pure Th0 Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Th0 Results Comparison when no miRNA is expressed** | | | **Pure Th0 Results Comparison when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 336 | 0 | **0-stimulus** | 168 | 0 |
| **APC only** | 744 | 0 | **APC only** | 312 | 0 |
| **Pro Th1** | 720 | 0 | **Pro Th1** | 240 | 0 |
| **Pro Th2** | 1440 | 0 | **Pro Th2** | 576 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 228 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 576 | 0 | **Pro Tfh** | 96 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 144 | 0 |
| **Pro Th22** | 480 | 0 | **Pro Th22** | 312 | 0 |
| **Pure Th0 Results Comparison when miR34 is absent** | | | **Pure Th0 Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 72 | 96 | **0-stimulus** | 36 | 48 |
| **APC only** | 48 | 96 | **APC only** | 24 | 48 |
| **Pro Th1** | 96 | 96 | **Pro Th1** | 48 | 48 |
| **Pro Th2** | 96 | 0 | **Pro Th2** | 48 | 0 |
| **Pro Th17** | 48 | 96 | **Pro Th17** | 24 | 48 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 96 | 168 | **Pro Tfh** | 48 | 84 |
| **Pro Th9** | 144 | 48 | **Pro Th9** | 72 | 0 |
| **Pro Th22** | 48 | 96 | **Pro Th22** | 24 | 48 |

*Supplemental Table 7.6- Comparison of the number of steady states that belong to the Th1 cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Th1 cell subtype, and are thus referred as "Pure".*

| **Pure Th1 Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Th1 Results Comparison when no miRNA is expressed** | | | **Pure Th1 Results Comparison when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 384 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 768 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 96 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 192 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 384 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 768 |
| **Pro Th9** | 288 | 0 | **Pro Th9** | 0 | 384 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 384 |
| **Pure Th1 Results Comparison when miR34 is absent** | | | **Pure Th1 Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 192 | **APC only** | 0 | 96 |
| **Pro Th1** | 0 | 384 | **Pro Th1** | 0 | 192 |
| **Pro Th2** | 0 | 192 | **Pro Th2** | 0 | 96 |
| **Pro Th17** | 0 | 192 | **Pro Th17** | 0 | 96 |
| **Pro Treg** | 0 | 192 | **Pro Treg** | 0 | 96 |
| **Pro Tfh** | 0 | 384 | **Pro Tfh** | 0 | 192 |
| **Pro Th9** | 0 | 192 | **Pro Th9** | 0 | 96 |
| **Pro Th22** | 0 | 192 | **Pro Th22** | 0 | 96 |

*Supplemental Table 7.7- Comparison of the number of steady states that belong to the Th2 cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Th2 cell subtype, and are thus referred as "Pure".*

| **Pure Th2 Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Th2 Results Comparison when no miRNA is expressed** | | | **Pure Th2 Results Comparison when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |
| **Pure Th2 Results Comparison when miR34 is absent** | | | **Pure Th2 Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |

*Supplemental Table 7.8- Comparison of the number of steady states that belong to the Th9 cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Th9 cell subtype, and are thus referred as "Pure".*

| **Pure Th9 Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Th9 Results Comparison when no miRNA is expressed** | | | **Pure Th9 Result Comparition when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 1008 | 0 | **Pro Th9** | 0 | 96 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |
| **Pure Th9 Results Comparison when miR34 is absent** | | | **Pure Th9 Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |

*Supplemental Table 7.9- Comparison of the number of steady states that belong to the Th17 cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Th17 cell subtype, and are thus referred as "Pure".*

| **Pure Th17 Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Th17 Results Comparison when no miRNA is expressed** | | | **Pure Th17 Results Comparison when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 96 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 96 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 240 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |
| **Pure Th17 Results Comparison when miR34 is absent** | | | **Pure Th17 Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |

*Supplemental Table 7.10- Comparison of the number of steady states that belong to the Th22 cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Th22 cell subtype, and are thus referred as "Pure".*

| **Pure Th22 Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Th22 Results Comparison when no miRNA is expressed** | | | **Pure Th22 Results Comparation when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 144 | 0 | **APC only** | 48 | 96 |
| **Pro Th1** | 216 | 0 | **Pro Th1** | 72 | 0 |
| **Pro Th2** | 240 | 0 | **Pro Th2** | 96 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 48 | 48 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 144 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 96 |
| **Pro Th22** | 108 | 0 | **Pro Th22** | 48 | 96 |
| **Pure Th22 Results Comparison when miR34 is absent** | | | **Pure Th22 Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |

*Supplemental Table 7.11- Comparison of the number of steady states that belong to the Treg cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Treg cell subtype, and are thus referred as "Pure".*

| **Pure Treg Results Comparison** | | | | | |
|---|---|---|---|---|---|
| **Pure Treg Results Comparison when no miRNA is expressed** | | | **Pure Treg Result Comparation when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |
| **Pure Treg Results Comparison when miR34 is absent** | | | **Pure Treg Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 48 | **APC only** | 0 | 24 |
| **Pro Th1** | 0 | 96 | **Pro Th1** | 0 | 48 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 48 | **Pro Th17** | 0 | 24 |
| **Pro Treg** | 0 | 96 | **Pro Treg** | 0 | 48 |
| **Pro Tfh** | 0 | 96 | **Pro Tfh** | 0 | 48 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 48 | **Pro Th22** | 0 | 24 |

*Supplemental Table 7.12- Comparison of the number of steady states that belong to the Tfh cell subtype when using the first version of the R script written for the purpose of identifying to which Th subtype a stable state belongs, under strict, loose and intermediate sets of logical rules, under all thirty-six initial environmental conditions. As a result of the being obtained through the use of the first version of the R script, these results refer to the steady states who present all the characteristics associated with Tfh cell subtype, and are thus referred as "Pure".*

| **Pure Tfh Results** | | | | | |
|---|---|---|---|---|---|
| **Pure Tfh Results Comparison when no miRNA is expressed** | | | **Pure Tfh Result Comparation when miR155 is absent** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 384 | 0 | **APC only** | 192 | 0 |
| **Pro Th1** | 1920 | 0 | **Pro Th1** | 960 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 192 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 1536 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 192 | 0 | **Pro Th22** | 192 | 0 |
| **Pure Tfh Results Comparison when miR34 is absent** | | | **Pure Tfh Results Comparation when both miRs are expressed** | | |
| **Model** | **Strict** | **Loose** | **Model** | **Strict** | **Loose** |
| **0-stimulus** | 0 | 0 | **0-stimulus** | 0 | 0 |
| **APC only** | 0 | 0 | **APC only** | 0 | 0 |
| **Pro Th1** | 0 | 0 | **Pro Th1** | 0 | 0 |
| **Pro Th2** | 0 | 0 | **Pro Th2** | 0 | 0 |
| **Pro Th17** | 0 | 0 | **Pro Th17** | 0 | 0 |
| **Pro Treg** | 0 | 0 | **Pro Treg** | 0 | 0 |
| **Pro Tfh** | 0 | 0 | **Pro Tfh** | 0 | 0 |
| **Pro Th9** | 0 | 0 | **Pro Th9** | 0 | 0 |
| **Pro Th22** | 0 | 0 | **Pro Th22** | 0 | 0 |

*Supplemental Table 7.13-Number of steady states obtained as belonging to a certain Th cell subtypes as a result of the use of the final version of the R script written to such purpose, under all nine initial environmental conditions that are appliable to the Original Model.*

| | **Original Model Results** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **No Stimulation** | **APC only** | **Pro Th1** | **Pro Th2** | **Pro Th9** | **Pro Th17** | **Pro Th22** | **Pro Tfh** | **Pro Treg** |
| **Th0** | 48 | 48 | 0 | 32 | 0 | 0 | 0 | 0 | 0 |
| **Th1** | 0 | 3456 | 5376 | 3456 | 192 | 0 | 2304 | 4608 | 288 |
| **Th2** | 0 | 32 | 64 | 64 | 0 | 0 | 16 | 32 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 592 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 384 | 384 | 0 | 0 | 576 |
| **Th22** | 0 | 2368 | 7712 | 2368 | 0 | 0 | 2368 | 7712 | 0 |
| **Tfh** | 0 | 0 | 4608 | 0 | 0 | 0 | 0 | 4608 | 0 |
| **Treg** | 0 | 1216 | 3072 | 0 | 0 | 200 | 800 | 3072 | 928 |

*Supplemental Table 7.14-Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where there is no environmental stimulation.*

| **No Stimulation Environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | **336** | **72** | **168** | **36** | **612** |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.15- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental stimulus is the presence of APC.*

| **APC only environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 744 | 48 | 312 | 24 | 1128 |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 1128 | 0 | 648 | 0 | 1776 |
| **Tfh** | 384 | 0 | 192 | 0 | 576 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.16- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th1 cells..*

| **Pro Th1 environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 720 | 96 | 240 | 48 | 1104 |
| **Th1** | **0** | **0** | **0** | **0** | **0** |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 2568 | 0 | 1464 | 0 | 4032 |
| **Tfh** | 2112 | 0 | 1056 | 0 | 3168 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.17- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th2 cells.*

| **Pro Th2 environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 1440 | 96 | 576 | 48 | 2160 |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | **0** | **0** | **0** | **0** | **0** |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 576 | 0 | 336 | 0 | 912 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.18- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th9 cells.*

| **Pro Th9 environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 144 | 144 | 72 | 360 |
| **Th1** | 432 | 0 | 0 | 0 | 432 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | **1488** | **0** | **0** | **0** | **1488** |
| **Th17** | 240 | 0 | 0 | 0 | 240 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.19- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th17 cells.*

| **Pro Th17 environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 48 | 228 | 24 | 300 |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | **96** | **0** | **0** | **0** | **96** |
| **Th22** | 0 | 0 | 648 | 0 | 648 |
| **Tfh** | 0 | 0 | 192 | 0 | 192 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.20- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th22 cells.*

| **Pro Th22 environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 480 | 48 | 312 | 24 | 864 |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | **732** | **0** | **648** | **0** | **1380** |
| **Tfh** | 192 | 0 | 192 | 0 | 384 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.21- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Tfh cells.*

| **Pro Tfh environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 576 | 96 | 96 | 48 | 816 |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 1776 | 0 | 0 | 0 | 1776 |
| **Tfh** | **1728** | **0** | **0** | **0** | **1728** |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.22- Number of steady states obtained with the strict version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Treg cells.*

| **Pro Treg environment (Strict version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 0 | 0 | 0 | 0 |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 96 | 0 | 0 | 0 | 96 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | **0** | **0** | **0** | **0** | **0** |

*Supplemental Table 7.23- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where there is no environmental stimulation.*

| **No stimulation environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | **0** | **96** | **0** | **48** | **144** |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.24- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental stimulus is the presence of APC.*

| **APC only environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 48 | 144 |
| **Th1** | 0 | 288 | 384 | 192 | 864 |
| **Th2** | 0 | 24 | 0 | 24 | 48 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 576 | 0 | 576 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 216 | 0 | 144 | 360 |

*Supplemental Table 7.25- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th1 cells.*

| **Pro Th1 environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 48 | 144 |
| **Th1** | **0** | **576** | **768** | **384** | **1728** |
| **Th2** | 0 | 48 | 0 | 48 | 96 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 1152 | 0 | 1152 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 432 | 0 | 288 | 720 |

*Supplemental Table 7.26- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th2 cells.*

| **Pro Th2 environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 0 | 0 | 0 | 0 |
| **Th1** | 0 | 288 | 96 | 192 | 576 |
| **Th2** | **0** | **48** | **0** | **48** | **96** |
| **Th9** | 192 | 0 | 144 | 0 | 336 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 144 | 0 | 144 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.27- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th9 cells.*

| **Pro Th9 environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 48 | 0 | 0 | 48 |
| **Th1** | 0 | 288 | 384 | 192 | 864 |
| **Th2** | 0 | 24 | 0 | 24 | 48 |
| **Th9** | **768** | **0** | **576** | **0** | **1344** |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 576 | 0 | 576 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.28- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th17 cells.*

| **Pro Th17 environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 48 | 144 |
| **Th1** | 0 | 288 | 192 | 192 | 672 |
| **Th2** | 0 | 24 | 0 | 24 | 48 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | **0** | **0** | **0** | **0** | **0** |
| **Th22** | 0 | 0 | 288 | 0 | 288 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 216 | 0 | 144 | 360 |

*Supplemental Table 7.29- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th22 cells.*

| **Pro Th22 environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 48 | 144 |
| **Th1** | 0 | 288 | 384 | 192 | 864 |
| **Th2** | 0 | 24 | 0 | 24 | 48 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | **0** | **0** | **576** | **0** | **576** |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 216 | 0 | 144 | 360 |

*Supplemental Table 7.30- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Tfh cells.*

| **Pro Tfh environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 168 | 0 | 84 | 252 |
| **Th1** | 0 | 576 | 768 | 384 | 1728 |
| **Th2** | 0 | 48 | 0 | 48 | 96 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 1152 | 0 | 1152 |
| **Tfh** | **0** | **0** | **0** | **0** | **0** |
| **Treg** | 0 | 432 | 0 | 288 | 720 |

*Supplemental Table 7.31- Number of steady states obtained with the loose version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Treg cells.*

| **Pro Treg environment (Loose version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 0 | 0 | 0 | 0 |
| **Th1** | 0 | 288 | 384 | 192 | 864 |
| **Th2** | 0 | 48 | 0 | 48 | 96 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 576 | 0 | 576 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | **0** | **432** | **0** | **288** | **720** |

*Supplemental Table 7.32- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where there is no environmental stimulation.*

| **No stimulation environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | **0** | **96** | **96** | **48** | **240** |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.33- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental stimulus is the presence of APC.*

| **APC only environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 98 | 288 | 48 | 432 |
| **Th1** | 192 | 288 | 2304 | 192 | 2976 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 3264 | 0 | 3264 |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 216 | 1584 | 144 | 1944 |

*Supplemental Table 7.34- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th1 cells.*

| **Pro Th1 environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 384 | 48 | 528 |
| **Th1** | **0** | **576** | **3840** | **384** | **4800** |
| **Th2** | 0 | 48 | 576 | 48 | 672 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 7680 | 0 | 7680 |
| **Tfh** | 0 | 0 | 2304 | 0 | 2304 |
| **Treg** | 0 | 432 | 3264 | 288 | 3984 |

*Supplemental Table 7.35- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th2 cells.*

| **Pro Th2 environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 48 | 0 | 0 | 48 |
| **Th1** | 384 | 288 | 2304 | 192 | 3168 |
| **Th2** | **0** | **48** | **576** | **48** | **672** |
| **Th9** | 0 | 48 | 576 | 48 | 672 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 1920 | 0 | 1920 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.36- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th9 cells.*

| **Pro Th9 environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 0 | 96 |
| **Th1** | 384 | 288 | 2304 | 192 | 3168 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | **1344** | **0** | **0** | **0** | **1344** |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 2112 | 0 | 2112 |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.37- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th17 cells.*

| **Pro Th17 environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 144 | 48 | 288 |
| **Th1** | 0 | 288 | 1152 | 192 | 1632 |
| **Th2** | 0 | 24 | 144 | 24 | 192 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | **0** | **0** | **0** | **0** | **0** |
| **Th22** | 0 | 0 | 1632 | 0 | 1632 |
| **Tfh** | 0 | 0 | 192 | 0 | 192 |
| **Treg** | 0 | 216 | 792 | 144 | 1152 |

*Supplemental Table 7.38- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th22 cells.*

| **Pro Th22 environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 288 | 48 | 432 |
| **Th1** | 0 | 288 | 2304 | 192 | 2784 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | **0** | **0** | **3264** | **0** | **3264** |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 216 | 1584 | 144 | 1944 |

*Supplemental Table 7.39- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Tfh cells.*

| **Pro Tfh environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 168 | 384 | 84 | 636 |
| **Th1** | 0 | 576 | 3072 | 384 | 4032 |
| **Th2** | 0 | 48 | 384 | 48 | 480 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 7680 | 0 | 7680 |
| **Tfh** | **0** | **0** | **2304** | **0** | **2304** |
| **Treg** | 0 | 432 | 2688 | 288 | 3408 |

*Supplemental Table 7.40- Number of steady states obtained with the intermediate version of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Treg cells.*

| **Pro Treg environment (Intermediate version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 288 | 48 | 432 |
| **Th1** | 0 | 288 | 2304 | 192 | 2784 |
| **Th2** | 0 | 48 | 576 | 48 | 672 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 4224 | 0 | 4224 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | **0** | **432** | **3168** | **288** | **3888** |

*Supplemental Table 7.41- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where there is no environmental stimulation.*

| **No stimulation environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | **0** | **96** | **96** | **48** | **240** |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.42- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental stimulus is the presence of APC.*

| **APC only environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 288 | 48 | 432 |
| **Th1** | 192 | 288 | 2304 | 192 | 2976 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 3264 | 0 | 3264 |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 216 | 1584 | 144 | 1944 |

*Supplemental Table 7.43- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th1 cells.*

| **Pro Th1 environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 384 | 48 | 528 |
| **Th1** | **0** | **576** | **3840** | **384** | **4800** |
| **Th2** | 0 | 48 | 576 | 48 | 672 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 7680 | 0 | 7680 |
| **Tfh** | 0 | 0 | 2304 | 0 | 2304 |
| **Treg** | 0 | 432 | 3264 | 288 | 3984 |

*Supplemental Table 7.44- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th2 cells.*

| **Pro Th2 environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 48 | 0 | 0 | 48 |
| **Th1** | 384 | 288 | 2304 | 192 | 3168 |
| **Th2** | **0** | **48** | **576** | **48** | **672** |
| **Th9** | 1344 | 0 | 0 | 0 | 1344 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 1920 | 0 | 1920 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.45- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th9 cells.*

| **Pro Th9 environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 0 | 96 |
| **Th1** | 384 | 288 | 2304 | 192 | 3168 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | **1344** | **0** | **0** | **0** | **1344** |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 2112 | 0 | 2112 |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.46- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th17 cells.*

| **Pro Th17 environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 144 | 48 | 288 |
| **Th1** | 0 | 288 | 1152 | 192 | 1632 |
| **Th2** | 0 | 24 | 144 | 24 | 192 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | **0** | **0** | **0** | **0** | **0** |
| **Th22** | 0 | 0 | 1632 | 0 | 1632 |
| **Tfh** | 0 | 0 | 192 | 0 | 192 |
| **Treg** | 0 | 216 | 792 | 144 | 1152 |

*Supplemental Table 7.47- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th22 cells.*

| **Pro Th22 environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 288 | 48 | 432 |
| **Th1** | 0 | 288 | 2304 | 192 | 2784 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | **0** | **0** | **3264** | **0** | **3264** |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 216 | 1584 | 144 | 1944 |

*Supplemental Table 7.48- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Tfh cells.*

| **Pro Tfh environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 168 | 384 | 84 | 636 |
| **Th1** | 0 | 576 | 3072 | 384 | 4032 |
| **Th2** | 0 | 48 | 384 | 48 | 480 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 7680 | 0 | 7680 |
| **Tfh** | **0** | **0** | **2304** | **0** | **2304** |
| **Treg** | 0 | 432 | 2688 | 288 | 3408 |

*Supplemental Table 7.49- Number of steady states obtained with the first, third and fifth perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Treg cells.*

| **Pro Treg environment (Perfected 1/3/5 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 288 | 48 | 432 |
| **Th1** | 0 | 288 | 2304 | 192 | 2784 |
| **Th2** | 0 | 48 | 576 | 48 | 672 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 4224 | 0 | 4224 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | **0** | **432** | **3168** | **288** | **3888** |

*Supplemental Table 7.50- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where there is no environmental stimulation.*

| **No stimulation environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | **0** | **96** | **96** | **48** | **240** |
| **Th1** | 0 | 0 | 0 | 0 | 0 |
| **Th2** | 0 | 0 | 0 | 0 | 0 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 0 | 0 | 0 | 0 | 0 |
| **Th22** | 0 | 0 | 0 | 0 | 0 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.51- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental stimulus is the presence of APC.*

| **APC only environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 192 | 48 | 336 |
| **Th1** | 384 | 288 | 4608 | 192 | 5472 |
| **Th2** | 0 | 24 | 576 | 24 | 624 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 768 | 0 | 0 | 0 | 768 |
| **Th22** | 0 | 0 | 5760 | 0 | 5760 |
| **Tfh** | 0 | 0 | 768 | 0 | 768 |
| **Treg** | 0 | 216 | 1584 | 144 | 1944 |

*Supplemental Table 7.52- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th1 cells.*

| **Pro Th1 environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 288 | 48 | 432 |
| **Th1** | **0** | **576** | **7680** | **384** | **8640** |
| **Th2** | 0 | 48 | 1152 | 48 | 1248 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 1536 | 0 | 0 | 0 | 1536 |
| **Th22** | 0 | 0 | 13440 | 0 | 13440 |
| **Tfh** | 0 | 0 | 3840 | 0 | 3840 |
| **Treg** | 0 | 432 | 3264 | 288 | 3984 |

*Supplemental Table 7.53- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th2 cells.*

| **Pro Th2 environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 48 | 0 | 0 | 48 |
| **Th1** | 768 | 288 | 4608 | 192 | 5856 |
| **Th2** | **0** | **48** | **1152** | **48** | **1248** |
| **Th9** | 2688 | 0 | 0 | 0 | 2688 |
| **Th17** | 768 | 0 | 0 | 0 | 768 |
| **Th22** | 0 | 0 | 3840 | 0 | 3840 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.54- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th9 cells.*

| **Pro Th9 environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 0 | 0 | 96 |
| **Th1** | 768 | 288 | 4608 | 192 | 5856 |
| **Th2** | 0 | 24 | 576 | 24 | 624 |
| **Th9** | **2688** | **0** | **0** | **0** | **2688** |
| **Th17** | 768 | 0 | 0 | 0 | 768 |
| **Th22** | 0 | 0 | 4416 | 0 | 4416 |
| **Tfh** | 0 | 0 | 768 | 0 | 768 |
| **Treg** | 0 | 0 | 0 | 0 | 0 |

*Supplemental Table 7.55- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th17 cells.*

| **Pro Th17 environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 96 | 48 | 240 |
| **Th1** | 0 | 288 | 2304 | 192 | 2784 |
| **Th2** | 0 | 24 | 288 | 24 | 336 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | **384** | **0** | **0** | **0** | **384** |
| **Th22** | 0 | 0 | 2880 | 0 | 2880 |
| **Tfh** | 0 | 0 | 384 | 0 | 384 |
| **Treg** | 0 | 216 | 792 | 144 | 1152 |

*Supplemental Table 7.56- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Th22 cells.*

| **Pro Th22 environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 192 | 48 | 336 |
| **Th1** | 0 | 288 | 4608 | 192 | 5088 |
| **Th2** | 0 | 24 | 576 | 24 | 624 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 768 | 0 | 0 | 0 | 768 |
| **Th22** | **0** | **0** | **5760** | **0** | **5760** |
| **Tfh** | 0 | 0 | 768 | 0 | 768 |
| **Treg** | 0 | 216 | 1584 | 144 | 1944 |

*Supplemental Table 7.57- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Tfh cells.*

| **Pro Tfh environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 168 | 0 | 84 | 252 |
| **Th1** | 0 | 576 | 6144 | 384 | 7104 |
| **Th2** | 0 | 48 | 768 | 48 | 864 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 1536 | 0 | 0 | 0 | 1536 |
| **Th22** | 0 | 0 | 13440 | 0 | 13440 |
| **Tfh** | **0** | **0** | **3840** | **0** | **3840** |
| **Treg** | 0 | 432 | 2688 | 288 | 3408 |

*Supplemental Table 7.58- Number of steady states obtained with the second, fourth, sixth and seventh perfected versions of the modified model that are identified as belonging to the different Th cell subtypes, through the use of the final version of the R script written for this purpose, thus leading such results to refer to both "Pure" and hybrid cell populations, under all miRNA expression scenarios where the only environmental favors the differentiation of Th0 cells into Treg cells.*

| **Pro Treg environment (Perfected 2/4/6/7 version)** | | | | | |
|---|---|---|---|---|---|
| | **None** | **Just miR155** | **Just miR34c** | **Both** | **Total** |
| **Th0** | 0 | 96 | 192 | 48 | 336 |
| **Th1** | 0 | 288 | 4608 | 192 | 5088 |
| **Th2** | 0 | 48 | 1152 | 48 | 1248 |
| **Th9** | 0 | 0 | 0 | 0 | 0 |
| **Th17** | 768 | 0 | 0 | 0 | 768 |
| **Th22** | 0 | 0 | 6336 | 0 | 6336 |
| **Tfh** | 0 | 0 | 0 | 0 | 0 |
| **Treg** | **0** | **432** | **3168** | **288** | **3888** |