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DISSECTING THE ROLE OF SPECIFIC MICRORNAS ON EFFECTOR AND REGULATORY CD4+ T CELL SUBSETS DIFFERENTIATION *IN VITRO*

VOLUME 1

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Dissecting the role of specific microRNAs on effector and regulatory CD4^{+} T cells subsets differentiation *in vitro*

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“If I have seen further it is by standing on the shoulders of giants.”

Isaac Newton

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Abstract

CD4$^+$ T cells orchestrate immune responses to several microorganisms and tumours, help B cells in antibody production, maintain CD8$^+$ T cells' cytotoxicity and suppress inflammation. Upon TCR activation, CD4$^+$ T cells differentiate into functionally distinct T cell subsets with different gene and cytokine expression profiles, that include the pro-inflammatory effector T helper (Th) 1 and Th17 cells, and the anti-inflammatory regulatory T (Treg) cells. An adequate balance between effector and regulatory T cells is crucial for the immunological homeostasis. microRNAs have been demonstrated to regulate gene expression networks, which affects the differentiation or maintenance of Th populations and ultimately impacts the balance between them. Although several specific miRNAs have been implicated in *in vitro* CD4$^+$ T cell differentiation, in this thesis we aimed at further dissecting the miRNA regulation of Teff/Treg balance, based in an *in vivo* holistic approach. Treg, Th1 and Th17 cell populations were isolated from the spleen and lymph nodes of a triple reporter IL-17-GFP:IFN-γ-YFP:Foxp3-hCD2 mouse strain upon EAE induction. The miRNA expression profile of each population was analysed by miRNA-seq and 10 miRNAs were found to be differentially expressed between the 3 subsets. Upon differentiation of naïve CD4$^+$ T cells into Th1, Th17 and Treg cells *in vitro*, the candidate miRNA expression patterns were validated by RT-qPCR. Gain-of-function studies were performed with retroviral transduction of miR-125a, miR-467a, miR-7667 or miR-126a into *in vitro*-differentiated T cell subsets and expression of Foxp3, IFN-γ and IL-17 was analysed. Our data showed that candidate miRNA overexpression had no impact on cell viability or proliferation. We found that despite miR-7667 being upregulated in Th1 cells relative to other subsets, its overexpression decreases the frequency of IFN-γ-producing Th1 cells. Following the same profile, overexpression of miR-126a hinders Th17 polarization as indicated by the decrease in IL-17$^+$ cells. Overall, our data suggest that miR-7667 and miR-126a could play a role in controlling inflammatory responses by Th1 and Th17 cells, respectively and emphasize the importance of epigenetics in the control of immune responses. Further confirmation of physiological relevance of these miRNAs is confirmed and their target identification will highlight the relevance of these miRNAs for the balance of Teffector and Treg cells and ultimately contribute to the development or improvement of immune therapies for autoimmune diseases or cancer.

Keywords: miRNAs, CD4$^+$ T cell differentiation, effector T cell, regulatory T cell, posttranscriptional regulation

Resumo (Portuguese)

O sistema imune, constituído por uma vasta rede de órgãos linfoides, células, fatores humorais e citocinas, é responsável por identificar e erradicar organismos patogénicos potencialmente prejudiciais para o organismo. A imunidade inata, da qual fazem parte células NK, macrófagos e células dendríticas, é a primeira linha de defesa contra os microrganismos. A resposta imunológica adaptativa, por outro lado, tem a particularidade de ser extremamente específica e as células que a executam, nomeadamente os linfócitos B e T, adquirem memória imunológica, ou seja, a capacidade de atuar mais rapidamente em caso de reinfeção com o mesmo microrganismo.

Os linfócitos T, originados na medula óssea, amadurecem no timo, onde adquirem um recetor de célula T (TCR) altamente específico e um recetor de membrana, CD4 ou CD8, do qual depende a sua função. Enquanto que os linfócitos T $CD8^+$ têm a capacidade de destruir células infetadas ou cancerígenas, os linfócitos T $CD4^+$ são responsáveis por orquestrar a defesa imunológica contra diversos patógenios, auxiliar na produção de anticorpos pelos linfócitos B, manter a função dos linfócitos T $CD8^+$ e suprimir respostas pro-inflamatórias. As células T circulam pelo organismo e é através da interação do TCR com as células apresentadoras de antigénios que identificam microrganismos patogénicos e são ativadas. Os linfócitos T $CD4^+$, também designados por linfócitos T auxiliares (T helper ou Th em inglês), podem então diferenciar-se em diversos subtipos celulares funcionalmente diferentes. As células Th1, estimuladas na presença de IL-12, expressam o fator de transcrição T-bet, produzem grandes quantidades de IFN-γ e são necessárias para uma resposta eficiente contra infeções intracelulares e tumores. Os linfócitos Th17, caracterizados pela produção de IL-17 (a citocina que lhes dá o nome) e pela expressão de RORγt, são induzidos na presença de TGF-β, IL-23, IL-21 e IL-6 e intervêm na defesa contra bactérias extracelulares e fungos. Os linfócitos T reguladores surgem na presença de IL-2 e TGF-β e caracterizam-se pela expressão de Foxp3 e elevados níveis de CD25. Estas células detêm a importante função de suprimir respostas pro-inflamatórias, promovendo assim a homeostase e tolerância imunológica.

Uma disfunção dos linfócitos T reguladores ou a excessiva ativação das populações T efetoras leva a que o organismo seja incapaz de controlar a inflamação, podendo originar doenças autoimunes, em que se gera uma resposta imunológica contra o próprio organismo. Por outro lado, caso as células T efetoras não sejam capazes de gerar uma resposta imunológica eficaz, o organismo não conseguirá defender-se, o que resulta numa maior suscetibilidade a infeções oportunistas e certos tipos de tumor.

É, assim, essencial que o balanço entre populações efetoras e reguladoras seja o adequado a cada situação de modo a evitar distúrbios no sistema imunitário.

Os microRNAs (miRNAs) são pequenas moléculas de RNA não codificante capazes de silenciar a expressão de genes-alvo, através da desestabilização ou da inibição da transcrição dos respetivos RNA mensageiros. É cada vez mais evidente a contribuição dos miRNAs, enquanto reguladores pós-transcricionais, para processos celulares fundamentais, tais como a diferenciação de linfócitos T. Ainda que já se conheçam vários miRNAs específicos envolvidos na diferenciação de linfócitos T $CD4^+$ *in vitro*, o papel fisiológico de muitos desses miRNAs permanece ainda por apurar.

O objetivo deste trabalho consistiu em dissecar o papel individual de certos miRNAs na regulação do balanço entre populações de linfócitos T $CD4^+$ efetoras e reguladoras. Para tal, desenvolvemos o nosso trabalho com base numa estratégia holística baseada em dados fisiologicamente relevantes *in vivo*. Isolámos populações de células Treg, Th1 e Th17 do baço e nódulos linfáticos de uma estirpe de murganho repórter tripla para IFN-γ (YFP), IL-17 (GFP) e Foxp3 (hCD2) na qual induzimos encefalomielite autoimune experimental (EAE). Os respetivos repertórios de miRNAs foram analisados por sequenciação de nova geração especialmente desenhada para pequenas moléculas de RNA, tendo-se identificado 10 miRNAs diferencialmente expressos entre as 3 populações analisadas. Seguidamente, a expressão destes miRNAs foi analisada por RT-qPCR em células Treg, Th1 e Th17 diferenciadas *in vitro* a partir de células T $CD4^+$ naïve de murganho. Dos 10 miRNAs identificados *in vivo*, 5 tinham o mesmo perfil de expressão *in vitro*: o miR-125a, o miR-15b e o miR-467a, sobre-expressos em células Treg; o miR-7667, sobre-expresso em Th1 e o miR-126a, sobre-expresso em Th17. De forma a modular a função dos miRNAs que reproduziram os dados de sequenciação, clonámos o miR-125a, o miR-467a, o miR-7667 e o miR-126a em partículas retrovirais com que transduzimos as células Treg, Th1 e Th17 diferenciadas *in vitro*. Para estudar o efeito destes miRNAs na viabilidade e proliferação celular, utilizámos um corante Live/Dead que permite identificar células mortas e avaliámos os níveis de Ki-67, respetivamente. Observámos que a viabilidade das três populações celulares se mantinha e que os níveis de proliferação de Treg e Th1 permaneciam inalterados aquando a incubação com qualquer um dos miRNAs. Para estudar o efeito dos miRNAs no fenótipo de cada uma das populações, analisámos o nível de expressão de Foxp3, IFN-γ e IL-17. O miR-125a e o miR-467a não provocaram qualquer efeito nas populações de interesse. Embora a sobre-expressão do miR-7667 não tenha tido qualquer impacto nas células Treg ou Th17, observámos que diminuía a frequência de células IFN-γ^+ da população Th1. Da mesma forma, a sobre-expressão do miR-126a

apenas interferiu com a diferenciação das células Th17, levando a uma diminuição na frequência de células IL-17^{+}.

Globalmente, os nossos resultados sugerem que o miR-7667 e o miR-126a poderão ter um papel importante no controlo de respostas pro-inflamatórias mediadas, respetivamente, por células Th1 e Th17 e enfatizam a importância da epigenética na modulação da resposta imune. Experiências futuras deverão focar-se na confirmação destes resultados *in vivo* e em perceber que genes poderão estar a ser alvos desta modulação. A identificação de miRNAs serem que modulem o equilíbrio entre linfócitos T efetores e reguladores *in vivo* terá grande impacto, sendo potencialmente útil no desenvolvimento ou melhoria de terapias imunes com vista ao tratamento de doenças do foro imunológico, tais como doenças autoimunes.

Palavras-chave: miRNAs, diferenciação de linfócitos T CD4^{+}, célula T efetora, célula T reguladora, regulação pós-transcricional

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Abbreviations

AGO	Argonaute
Ahr	Aryl hydrocarbon receptor
Amp	Ampicillin
APC	Antigen-presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DGCR8	DiGeorge syndrome critical region gene 8
DMEM	Dulbecco's modified Eagle's medium
DN	Double negative
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
Foxp	Forkhead box P
Fwd	Forward primer
GFP	Green fluorescent protein
HIF-1α	Hypoxia-inducible factor 1α
HEK	Human embryonic kidney
IL	Interleukin
IFN	Interferon
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy X-linked
iTreg	Induced regulatory T cell
IMDM	Iscove's Modified Dulbecco's Medium
LN	Lymph node
MFI	Mean fluorescence intensity
miR	MicroRNA
miRNA	MicroRNA
MHC	Major histocompatibility complex

mRNA	Messenger RNA
MS	Multiple sclerosis
MOG	Myelin oligodendrocyte glycoprotein
mTOR	Mechanistic target of rapamycin (mTOR)
NFAT	Nuclear factor of activated cells
NK	Natural killer
nTreg	Natural regulatory T cell
Opti-MEM	Reduced Serum Media
Pen/Strep	Penicillin-streptomycin
PMA	Phorbol 12-myristate 13-acetate
Rev	Reverse primer
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RORγt	Receptor-related orphan receptor gamma-T
RPMI	Roswell Park Memorial Institute
RT-qPCR	Quantitative real-time polymerase chain reaction
Runx	Runt-related transcription factor
Spl	Spleen
STAT	Signal transducer and activator of transcription
Teff	Effector T cell
Th	T helper
Tfh	T follicular helper
T-bet	T-box transcription factor
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Treg	Regulatory T cell

1. INTRODUCTION

1.1. Overview of T cells in the immune system

The immune system, composed by an extensive network of lymphoid organs, cells, cytokines and other factors, is responsible for identifying and eradicating potentially harmful pathogens. Immune responses are divided, according to the speed and specificity of the reactions, into innate and adaptive responses.[1]

Innate immunity provides immediate host defence and does not require previous exposure to trigger effective responses, despite lacking specificity. The major effectors of these responses are macrophages, natural killer (NK) cells and dendritic cells (DCs).[2] Macrophages are highly specialized tissue-resident phagocytes that remove and digest microorganisms, whereas NK cells rapidly eradicate pathogens and induce apoptosis of ικected cells.[3, 4] DCs, because of their ability to recognize and uptake antigens, are able to bridge innate and adaptive immunity.[5] Adaptive immune responses gradually develop against particular antigens and allow for immunological memory, useful in case of reinfection with previously encountered pathogens. Adaptive immunity relies on the action of T lymphocytes, which mediate the destruction of infected cells or pathogens, and B lymphocytes, which produce antibodies that boost innate immunity and build up T cell-dependent responses.[6, 7] Both T and B lymphocytes differentiate from the common lymphoid progenitor in the bone marrow, where B cells also maturate to express the B-cell receptor (BCR). It is, however, in the thymus that T cells develop and start

expressing the T-cell receptor (TCR), which comprises a heterodimer consisting of either αβ or γδ protein chains that associate with CD3, a T cell co-receptor.[7]

Even though they differ in structure, the BCR and the TCR are assembled by the same mechanism. Both receptors encode a constant and a variable, antigen-recognition domain that is assembled through somatic rearrangement of the scattered germline variable (V), diversity (D) and joining (J) genes.[8, 9] This mechanism is dependent on the recombination activating gene (RAG) proteins and leads to the production of a broad repertoire of B and T cell receptors, further expanded by the insertion of non-templated nucleotides after recombination. Afterwards, lymphocytes are able to trigger an immune response adequate to cover the range of pathogens likely to be encountered in a lifetime.[8, 10]

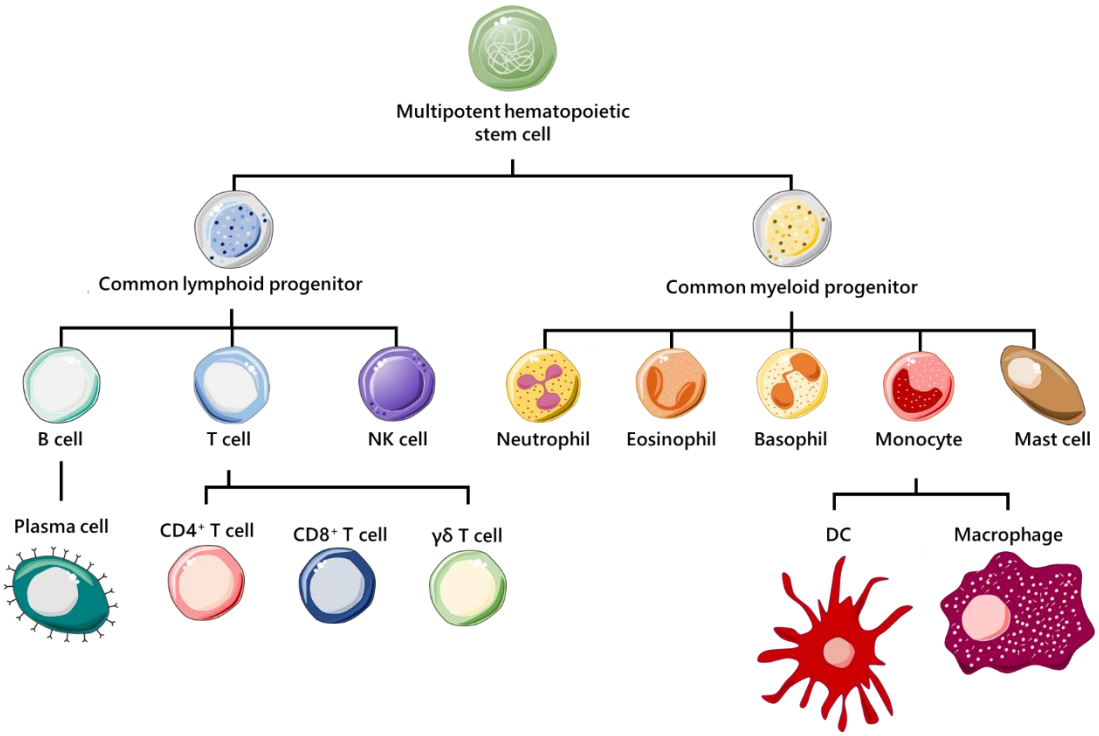


Figure 1.1. – Schematic representation of the myeloid and lymphoid lineage development. Every immune cell arises from a multipotent hematopoietic stem cell that either differentiates into a common lymphoid or myeloid progenitor. The common lymphoid progenitor gives rise to B, T and NK cells. B cells further differentiate into plasma cells, while T cells develop into $CD4^+$, $CD8^+$ or γδ T cells. The common myeloid progenitor gives rise to neutrophils, eosinophils, basophils, mast cells and monocytes, which further differentiates into dendritic cells (DCs) or macrophages.

To ensure that only harmful antigens provoke a reaction, T cells undergo sequential stages of development in the thymus, where they mature to trigger a response upon encounter with foreign antigens while avoiding autoreactivity. For that, thymocytes that functionally recognize the MHC-antigen complex receive survival signals (positive selection) and, subsequently, the ones bind the complex too strongly elicit signals that

lead to their apoptosis (negative selection).[11] Antigen epitopes recognized by the TCR are presented in association with the major histocompatibility complex (MHC) molecules at the surface of various cell types.[12] MHC class I is expressed by virtually all nucleated cells and presents intracellular protein fragments of cytosolic and nuclear origin. Thus, any cell that is infected with an intracellular pathogen or produces tumour proteins might present those antigens and alert T cells. MHC class II, in turn, presents exogenous antigens mainly processed from extracellular microorganisms and is restricted to specialized antigen-presenting cells (APCs), including macrophages, B lymphocytes and dendritic cells. MHC I and II are recognised by T cells bearing either the CD8 or the CD4 co-receptor, respectively.[13]

1.2. $CD4^+$ T cells

Upon entering the thymus, the multipotential lymphoid progenitors irreversibly commit to the T lineage and pass through four $CD4^-CD8^-$ double-negative (DN) stages defined by different expression of the activation markers CD25 and CD44 (DN1-DN4). Herein, cells differentiate into immature thymocytes expressing either αβ or γδ TCR. After a $CD4^+CD8^+$ double positive stage, αβ thymocytes differentiate into naïve, single positive $CD4^+$ or $CD8^+$ T cells.[14]

Naïve T cells exit the thymus and continuously recirculate between secondary lymphoid organs and the bloodstream surveying the organism for the cognate antigen. Carcinogenic or infected cells present foreign epitopes that are recognized by $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs).[15] Upon recognition, $CD8^+$ T cells responses encompass the production of pro-inflammatory cytokines like IFN-γ and tumour necrosis factor α (TNF-α) and the secretion of death-inducing molecules such as perforin, granzymes and Fas-ligand (FasL) towards the target cell, which ultimately leads to its apoptosis.[16] In turn, $CD4^+$ T lymphocytes, also known as T helper (Th) cells, are key mediators of immune adaptive responses in the organism. In addition to controlling the intensity of the immune responses, these cells intervene in antibody production by B cells, mediate immunological memory and contribute to the expansion and survival of $CD8^+$ lymphocytes.[17, 18]

As above mentioned, T cell activation initiates with recognition of the peptide/MHC complex on an APC by the TCR/CD3 complex. However, full activation of the T lymphocyte requires costimulation, for instance, through the engagement of CD28 by CD80 or CD86 proteins on the surface of the APC.[19] TCR engagement recruits LCK kinase that phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3ε chain, which, in turn, phosphorylate ZAP70. The TCR signalling cascade

eventually leads to the activation of nuclear factor-κB (NF-κB) transcription factor.[20] Nuclear factor of activated cells (NFAT) is also activated in response to the TCR signalling and promotes the production of IL-2 and the expression of signal transducer and activator of transcription 5 (STAT5). During TCR activation and according to the surrounding cytokine milieu, $CD4^+$ T cells may further differentiate into one of several lineages mainly characterized by the set of cytokines expressed.[21]

Differentiated $CD4^+$ T cells include at least five well established subsets, namely regulatory T cells (Treg), Th1, Th2, Th17 and T follicular helper (Tfh) cells. Th9 and Th22, as well as other $CD4^+$ phenotypes that have been described, may constitute distinct $CD4^+$ T subsets but are not yet firmly established and currently represent, at the very least, evidence of the diversity and plasticity within the Th lineage.[21, 22] Treg cells express the forkhead transcriptional repressor 3 (Foxp3) and have been shown to be immunosuppressive and key players in the maintenance of the immunological tolerance and homeostasis.[23, 24] Th1 cells produce interferon (IFN)-γ as their signature cytokine and are thus pro-inflammatory whereas Th2 lymphocytes secrete interleukin (IL)-4, IL-5 and IL-13 and are crucial in immune responses directed against helminths and in allergic reactions.[25] The Th17 subset is mainly characterized by the production of IL-17A and IL-17F, two members of the IL-17 pro-inflammatory cytokine family. Tfh express CXCR5 and are located in germinal centres, where they help B cells to produce antibodies.[26] Th9 cells selectively secrete IL-9 and are found to be associated with antitumor immunity, while Th22 only produce IL-22 and were first identified in psoriac lesions.[27, 28]

The commitment of a naïve $CD4^+$ T cell into a specific T cell subset is an intricate process that relies on several environmental co-stimulatory signals, such as the strength of the TCR affinity, the surrounding cytokine milieu, the expression of subset-specific transcription factors and various epigenetic modifications that shape the phenotype of each lineage.[23] Hereafter, throughout the next sections, we will more thoroughly discuss the differentiation of the Th1, Th17 and Treg subsets, which is summarized in a schematic representation (Fig. 1.1.).

1.2.1. Differentiation and function of Th1 cells

There is evidence that a strong TCR signal favours Th1 differentiation over Th2 and Treg, suggesting that the nature or type of antigen itself influence on the response it provokes.[29, 30] TCR-activated $CD4^+$ T cells initiate Th1 cell differentiation in the presence of IL-12, which promotes phosphorylation of STAT4. In turn, phosphorylated STAT4 induces transcription of *Ifng*. In response to IFN-γ, STAT1 is phosphorylated and activates the transcription of *IL12Rβ* and *Tbx21* that further favour Th1 development

through a positive feedback loop. *Tbx21* encodes for the T-box transcription factor (T-bet) which has initially been termed the Th1 master regulator, that is, a gene that dominantly specifies a given cell lineage.[31] T-bet upregulates IFN-γ production, further reinforcing Th1-cell commitment.[19]Other transcription factors have been demonstrated to fine-tune the differentiation of Th1 cells. For example, Runt-related transcription factor (Runx)3-deficient cells produce less IFN-γ and interferon regulatory factor (IRF)1 deletion resulted in decreased levels of IL-12Rα.[21]

Other, less canonical transcription factors have been implicated in Th1 development. That is the case of Hlx, found to enhance IFN-γ production downstream of T-bet.[32] Another, more recent example is Id2, which significantly hampered Th1 differentiation in mice when absent.[33] Interestingly, the *Ifng* locus of naïve $CD4^+$ T cells was found to be in a poised state, that is, to contain several hypersensitive (less compacted) sites that facilitate transcription factor binding, enabling IFN-γ expression.[34]

Recent research has been able to demonstrate the importance of posttranscriptional, posttranslational and epigenetic modifications in shaping the outcome of T cell differentiation, unravelling additional layers of complexity within this context.[25] For example, treatment of Th1 cells with a DNA methylation inhibitor resulted in an increase in the IFN-γ secretion.[31] Mice lacking functional RNA binding proteins Regnase-1 and Roquin in T cells displayed increased levels of *Ifng* mRNA and IFN-γ-producing $CD4^+$ T cells.[35] Furthermore, ablation of a chromatin-modifying enzyme, Ezh2, in mice downregulated gene transcription of *Tbx21* and *Stat4*.[36] Many noncoding RNAs, especially microRNAs, have been implicated in the differentiation of $CD4^+$ T cell subsets as well. This topic will be reviewed on section 1.3.1.

Th1 are the major mediators of immune responses upon infection with intracellular pathogens such as the protozoa *Leishmania*, in which case Th1-mediated IFN-γ production play an essential role.[37] Th1-derived IFN-γ also promotes anti-tumour functions in macrophages.[38] However, Th1 cells can trigger autoimmunity when excessively activated. In fact, it has been demonstrated *in vivo* that the onset of arthritis depends on expansion of autoreactive Th1 cells and that rats treated with anti-IFN-γ-neutralizing antibodies developed significantly milder disease.[39]

1.2.2. Differentiation and function of Th17 cells

RORγt is the so-called master regulator of the Th17 lineage, a subset characterized by the expression of IL-17A and IL-17F as signature cytokines. Th17 also produce IL-22 and IL-21, although the latter might be produced by other Th subsets. IL-6, TGF-β, IL-21

and IL-23 are the major signalling cytokines involved in Th17 differentiation, although IL-1β may synergize with IL-6 in this process.[40]

STAT3 is upregulated in response to IL-6 and promotes the expression of RORγt, IL-22 and IL-23 receptor (IL-23R). IL-21 is expressed by Th17 cells and induces its own expression in an autocrine manner, also activating STAT3.[41] In the presence of IL-6, downstream TGF-β signalling pathway also leads to the activation of RORγt, which induces IL-17A and IL-17F production.[19, 42] Furthermore, IL-17 was undetectable in IL-23-deficient mice, proving the importance of this cytokine for the stabilization of the Th17 lineage.[43]

Ablation of RORγt does not lead to complete abrogation of Th17 cytokines, implying auxiliary transcription factors that collaborate for full generation of Th17 cells.[44] RORα, for instance, participates in the Th17 commitment pathway and acts synergistically with RORγt in such a way that their simultaneous absence completely halts the development of these cells.[45] The importance of the aryl hydrocarbon receptor (AhR) has been highlighted not only by the phenotype of AhR-deficient mice, which show decreased IL-17 production, but also by the impairment of Th17 expansion *in vitro* due to lack of AhR agonists in the medium.[46]

Th17 differentiation is subject to epigenetic modifications as well. Phosphorylation of histone H3 at the *Il17a* locus is required for activation of IL-17A expression and commitment to the Th17 lineage.[47] Moreover, it has been shown that blockade of BET proteins, which are involved in regulation of chromatin structure and gene transcription, suppresses IL-17 production in Th17 cells.[31] On the other hand, the double knockout of Roquin-1 and Roquin-2, which are posttranscriptional repressors of mRNAs, elicited an accumulation of Th17 cells *in vivo*.[48]

Th17 lymphocytes contribute to host defence against extracellular bacterial and fungi. For example, immunization with heat-killed pulmonary *H. influenzae* confers Th17-mediated protection upon infection[49] Although their involvement in cancer is controversial, there is evidence that Th17 cells stimulate CTL-mediated antitumor immunity.[50] Nonetheless, when overly stimulated, these cells are involved in the induction of organ-specific autoimmune diseases including multiple sclerosis (MS), psoriasis, rheumatoid arthritis and inflammatory bowel disease.[51] Indeed, inhibition of Notch1 leads to reduction of Th17 and IL-17A levels, which alleviate severity of psoriasis-like skin inflammation *in vivo*.[52]

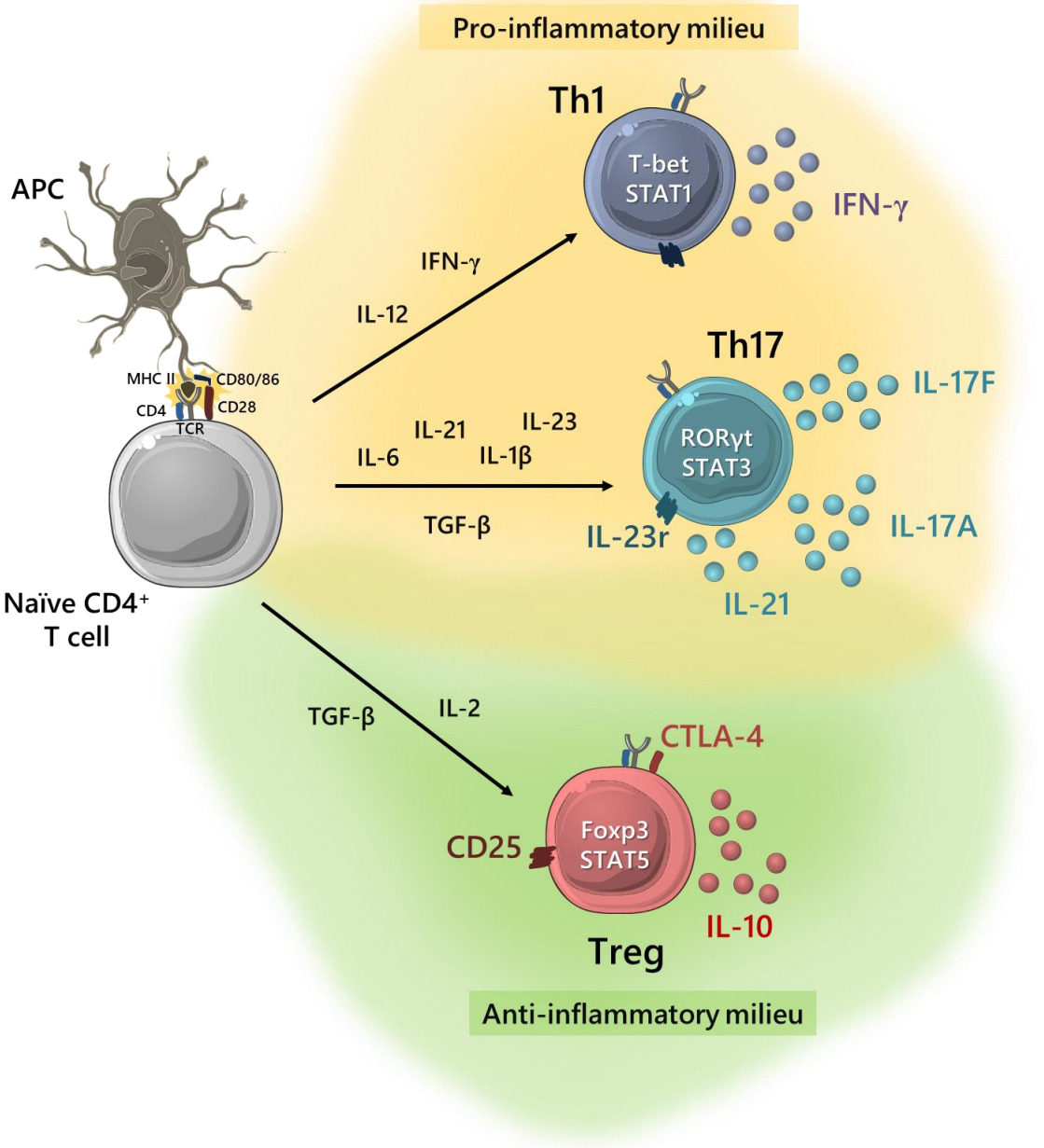


Figure 1.2. – Schematic representation of naïve CD4⁺ T cell activation and differentiation. Naïve CD4⁺ T cells encounter their cognate antigen in the context of MHC II and, following co-stimulation with CD28 and co-stimulatory cytokines present in the surrounding environment, they differentiate into distinct lineages. In the presence of pro-inflammatory IL-12 and IFN-γ, T cells upregulate T-bet and STAT1, generating Th1 cells that further produce IFN-γ. Th17 express retinoic acid receptor (RAR)-related orphan receptor (ROR)γt and STAT3 and produce IL-17A, IL-17F and IL-21 in response to anti-inflammatory TGF-β and pro-inflammatory IL-6, IL-21, IL-1β. IL-23 further stabilizes this subset. Treg cells develop in the presence of TGF-β and IL-2 and express anti-inflammatory molecules such as CTLA-4, IL-10 and CD25 (IL-2R), which stabilize the Treg phenotype in an autocrine manner. The transcription factor Foxp3 is the "master regulator" of Treg differentiation.

1.2.3. Differentiation and function of Treg cells

Treg cells may arise in the periphery, similarly to the other T cell subsets, in which case they are called induced regulatory T cells (iTreg), but there is a sub-group of regulatory T cells, known as natural regulatory T cells (nTreg), which develop in the thymus. Treg cells are absolutely essential for the maintenance of immunological cell tolerance, homeostasis and suppression of immune responses, including against the foetus during pregnancy and commensal bacteria in the gut and severe complications arise if their function is compromised.[53]

Regulatory T cells are characterized by the expression of Foxp3 and of IL-2Rα chain (also known as CD25). TGF-β induces Smad3 binding to the Foxp3 locus and directly promotes Foxp3 transcription.[54] IL-2-induced STAT5 was found to enhance Foxp3 expression and therefore promote iTreg development.[19] It has also been shown that c-Rel regulates the production of endogenous IL-2. Its deficiency severely hampered iTreg differentiation *in vitro* and correlated with reduced numbers of $Foxp3^+$ T cells *in vivo*.[55]

Regarding the epigenetic processes controlling Treg differentiation, there is evidence that expression of Treg-specific genes, such as *Foxp3* and *Il2ra*, correlates with corresponding Treg-specific DNA hypomethylation.[31] Likewise, acetylation has been found to promote Foxp3 stability and improve the activity of Treg cells.[56] It is noteworthy that both Th17 and iTreg share the requirement for TGF-β in their differentiation processes, despite expressing distinct transcriptional regulators (RORγt and Foxp3, respectively) and exhibiting opposing functions (inflammatory versus anti-inflammatory).

As above mentioned, the most outstanding role of Treg cells is the maintenance of immune homeostasis and both self and non-self-tolerance. In fact, due to a mutation in the *Foxp3* gene, which is essential for the immunosuppressive function of this subset, scurfy mice are deficient in functional Treg cells, which results in autoimmunity and premature death.[57] Similar mutations in humans cause the immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, characterized by severe multi-organ autoimmunity[53]. *In vivo*, Treg cells curtail T-cell responses to foreign antigens by various mechanisms, including abundant production of T cell suppressive, anti-inflammatory cytokines such as IL-10, IL-35 and TGF-β.[58] CTLA-4, a co-inhibitory signal, binds to CD80 and CD86 and is also required for Treg-mediated suppression. Blockade of CTLA-4 *in vivo* did not affect activation of Treg cells but impaired their regulatory functions and spontaneously lead to autoimmunity.[59, 60] Treg cells also play other, pleiotropic roles outside de immune system, including mediation of angiogenesis, tissue repair and metabolic regulation.[56, 61]

Hereafter, we will focus on induced Treg lymphocytes and refer to them as Treg, while the term nTreg will be used for natural Treg cells.

1.2.4. Plasticity of CD4$^+$ T cell subsets

Research indicates that CD4$^+$ T cell subsets cross-regulate one another, which means that the expression of lineage-specific factors simultaneously promotes differentiation into that particular subset while suppressing the others. For example, Th17-inducing STAT3 downregulates Foxp3 expression and, in turn, Foxp3 and T-bet both repress RORγt [62, 63] Similarly, following *Mycobacterium avium* complex (MAC) infection, the cytokine expression pattern of T-bet-deficient mice shifted towards a Th17-like profile.[64] Runx3, in coordination with T-bet, binds to the IFN-γ promoter and represses the transcription of *Il4*.[65] On the other hand, T-bet induction results in fewer *Rorc* transcripts and decreased levels of RORγt.[66]

In fact, at first, differentiation of CD4$^+$ T cells into functionally distinct lineages was thought to be an irrevocable process that dictated the final phenotype of a cell. Currently, it is well understood that this is not the case; contrarily, T cell subsets adapt to changing circumstances in response to environmental cues and are able to re-shape their functions.[67] Despite the above-mentioned cross-regulation, recent studies demonstrate that CD4$^+$ T cell subsets might co-express transcription factors and cytokines that were previously thought to be uniquely expressed in one lineage alone.[68]

Treg cells, for instance, have the remarkable ability to acquire expression of transcription factors specifically expressed by other subsets in order to guide themselves to inflammation sites where immune responses are being carried out by those lineages. Specifically, T-bet$^+$ Treg cells accumulate at sites of Th1-mediated inflammation and suppress Th1 responses more efficiently, as opposed to T-bet-deficient Treg cells.[69] In agreement with these findings, loss of function of T-bet-expressing Treg cells elicited Th1-mediated autoimmunity.[70] Similarly, restriction of Th17 responses by Treg cells in mice was lost upon Treg-specific ablation of STAT3.[71] On the other hand, it has been shown that IFN-γ$^+$ Treg cells are an intermediate stage in the conversion of a canonical Foxp3$^+$IFN-γ$^-$ Treg subset into a conventional Foxp3$^-$IFN-γ$^+$ Th1 phenotype.[72] Moreover, once Th17 cells are exposed to IL-12 or IL-23, STAT4 induces the production of IFN-γ and the cells might either become IL-17$^+$IFN-γ$^+$ double producers or fully convert into a Th1 cell.[72] Thus, a transient signal that stimulates the co-expression of opposing lineage-specific factors may ultimately lead to a subset switch.

Together, these findings highlight the diversity of phenotypes and functions of the CD4$^+$ T cell compartment and challenge the perception of all-or-nothing “master regulators”.

Instead, it is now widely accepted that, although some transcription factors are required to define a specific lineage, $CD4^+$ T cell subsets still retain the potential to modify their subset-specific transcriptional programmes.[68] Thereby, T cells of a given subset certainly have greater flexibility to tailor immune responses on the spot and eradicate pathogens more efficiently. Nonetheless, the T cell plasticity coin has another side, with detrimental implications in the context of autoimmune diseases. Conversion of standard Treg cells into an IL-17-producing Treg phenotype in the context of rheumatoid arthritis and colorectal cancer, for example, is associated with pathogenicity.[73, 74] Unexpectedly, T-bet-deficient intestinal Th17 cells still retain the ability to co-express IL-17A and IFN-γ in the context of an IL-23-driven autoimmune pathology.[75] Hence, it may be the case that T cells are reprogrammed into pro-inflammatory phenotypes in response to the microenvironment they are exposed to or, rather, that reprogrammed T cells contribute to the generation of autoimmune responses.[72]

1.3. Importance of the balance between effector and regulatory $CD4^+$ T cell subsets

The outcome of the inflammatory response is determined by an intricate balance between effector (Teff) (for example, Th1 and Th17) and Treg cell subpopulations. This is well demonstrated by the consequences observed in humans or mouse models that are deficient in a particular Teff or Treg subset, including the previously mentioned case of Treg-deficient IPEX patients and scurfy mice. In addition, absence of T-bet in mice challenged with *Leishmania major*, *Mycobacterium tuberculosis, Salmonella enterica* and Herpes simplex virus 2 decreased $CD4^+$ T cell production of IFN-γ and lead to more susceptibility to infection with any of the pathogens.[76]

In general, anti-inflammatory responses, like those mediated by Treg cells, are crucial to prevent autoimmunity but might be strongly detrimental in the context of cancer and infection. On the contrary, pro-inflammatory cytokines produced by Teff subsets play essential roles in immune responses towards tumours and infectious pathogens, however, they have the negative potential to cause chronic inflammation and autoimmune pathologies. In the specific case of MS, reductions and increases in the frequency of Treg cell levels of patients, relatively to Teff subsets, have been correlated with relapse and remission, respectively.[77] In intestinal bowel disease (IBD) patients, it has been demonstrated that the upregulation of pro-inflammatory responses mediated by Th17 is accompanied by decreased levels of Treg immunosuppressive cytokines and that modulating this imbalance could re-establish immune homeostasis.[78] Similarly, in obesity, there is a reduction of Treg cell levels in visceral adipose tissues and chronic

activation of pro-inflammatory processes associated with insulin resistance and type 2 diabetes.[78] Intra-tumoral depletion of Treg cells, which are extremely increased in several cancers, leads to strong antitumor immune responses in mice.[79]

Several factors, ranging from cytokines to transcriptional modulators and even microbiota, play important roles in the (de)regulation of this balance.[78] The role of Foxp3-related transcription factor Foxp1 is one such example. Foxp1-deficient cells showed decreased Foxp3 DNA-binding and reduced expression of functionally important Treg signature genes, as well as increased proliferation of $CD4^+$ T cells and IFN-γ production.[80] The transcriptional regulator TAZ has recently been identified as a co-activator of RORγt required for Th17 differentiation and Th17-mediated inflammation. In addition, Treg cell development was downregulated in the presence of TAZ and induced upon its deficiency.[81] The hypoxia-inducible factor 1α (HIF-1α) is a key metabolic factor activated upon hypoxic conditions that mediates the metabolic switch from oxidative phosphorylation to aerobic glycolysis and was found to regulate the Th17/Treg balance. By inducing RORγt and targeting Foxp3 for degradation, HIF-1α simultaneously promotes Th17 and inhibits Treg cells.[82] Altogether, these examples highlight the role of transcriptional proteins as regulators of the Teff/Treg balance. As mentioned before, lineage-specific transcription factors are able to suppress the other lineages. Nevertheless, some molecules promote the development of more than one lineage simultaneously. For instance, T cell-specific deletion of *Mtor*, which encodes for the mechanistic target of rapamycin (mTOR) protein kinase, hampers the differentiation of Th1, Th2 and Th17 cells, while inducing the accumulation of $Foxp3^+$ Treg cells.[83] Therefore, mTOR has a direct impact on the overall Teff/Treg balance.[83] Interestingly, it has also been found that metabolites produced by commensal microorganisms stimulate differentiation of Treg cells *in vitro* and promote peripheral Treg cell generation *in vivo*, demonstrating the importance of the microbiome in the balance between pro and anti-inflammatory T cells.[84]

Therapeutic approaches designed to correct the imbalance of the Th17/Treg ratio driving autoimmune diseases were shown to be effective and some have been approved for clinical practice.[85] Neutralization of IL-6R in rheumatoid arthritis patients, for instance, increases Treg cell levels while decreasing Th17 and ameliorates clinical symptoms as well.[85]

1.4. Posttranscriptional regulation of gene expression by microRNAs

Small silencing non-coding RNAs mediate posttranscriptional suppression of messenger RNA transcripts (mRNA) and, in animals, are classified into three distinct classes according to their biogenesis, targets and mechanism of action. Silencing RNAs (siRNAs) derive from long double stranded RNAs and mediate antiviral defence. PIWI-interacting RNAs (piRNAs) arise from single-stranded precursors and silence transposons elements in germline cells.[86] MicroRNAs (miRNAs) are generated from hairpin RNAs and regulate the expression of protein-coding genes being the focus of attention henceforth.[87] MicroRNAs comprise a large family of short, non-coding RNAs that are expressed in nearly all eukaryotic organisms and some viruses, constituting a dominating class of small RNAs. Although they are frequently embedded in intronic sequences of protein-coding genes, they can also be encoded as polycistronic single transcripts encoding multiple miRNAs or as unique genes.[88]

In the conventional miRNA biogenesis pathway, as depicted in Figure 1.2, miRNAs are transcribed by RNA polymerase II (Pol II) into long primary miRNAs (pri-miRNAs) containing a stem-loop structure, in which mature microRNA nucleotides are encoded. Pri-miRNAs are recognized by a large protein complex designated Microprocessor, mainly constituted by the RNase III enzyme Drosha and respective cofactor, DiGeorge syndrome critical region gene 8 (DGCR8). Drosha catalyses the cleavage of pri-miRNAs into single hairpins, also known as precursor miRNAs (pre-miRNAs), initiating the maturation process. Exportin-5 (Exp-5) drives the transport of the pre-miRNA from the nucleus to the cytoplasm, where its hairpin loop is cleaved by another RNase III endonuclease called Dicer into an approximately 22-nucleotide long miRNA duplex. Subsequently, in a process termed RNA-induced silencing complex (RISC) loading, the miRNA duplex is loaded onto an Argonaute (AGO) protein that selects one strand to become the functional, mature miRNA, also called guide strand, whereas the other, the passenger strand, is quickly degraded. Typically, the strand with the less stable 5' end is the one retained by the miRISC, although the other strand might also be selected.[86, 89, 90]

The miRNA guides RISC to complementary sequences mainly located in the 3' UTR region of target mRNAs, although the 5' UTR and the coding sequences also contain miRNA binding sites.[91] Nucleotides between positions 2 and 8 from the 5' end of the miRNA, also known as the "seed" sequence, are important for target-site recognition and repression.[91] However, it has also been described that miRNAs can suppress their target genes through 3' end interactions that direct miRNA suppressive function.

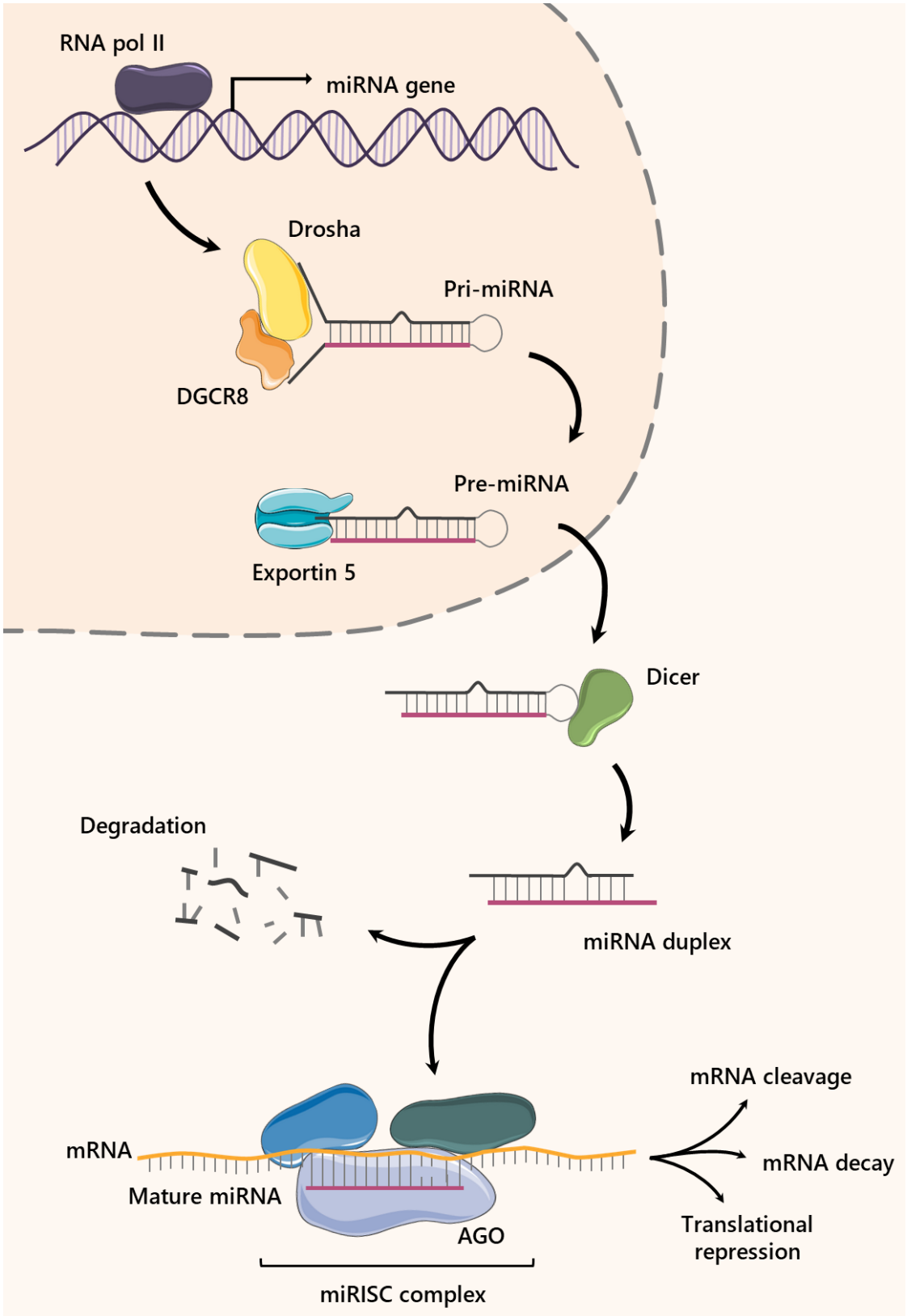


Figure 1.3. – Schematic model of the miRNA biosynthesis pathway. RNA polymerase II transcribes miRNA-encoding genes, forming a pri-miRNA that is further processed by Drosha and DGCR8. The resulting pre-miRNA is then transported to the cytoplasm in association with Exportin 5, where it is cleaved by Dicer to form a miRNA duplex. AGO, together with the RISC complex, selects the guide strand and mediates translational repression, mRNA decay or mRNA degradation of the miRNA targets.

For example, different miRNA family members, which typically possess identical 5' ends but distinct 3' ends, have non-overlapping targets due to the contribution of 3' ends for miRNA-mRNA interaction.[92] Just as a single miRNA can target multiple mRNAs, a single mRNA is a target for several miRNAs. Mechanisms of miRNA-mediated gene silencing include mRNA deadenylation and subsequent decay, translational inhibition and mRNA cleavage.[89] However, ribosome profiling has shown that for over 84% of the proteins regulated by miRNAs, the inhibition was accounted by destabilization of the target mRNAs.[93]

Overall, microRNAs are important regulators of various developmental processes, as demonstrated by Dicer-deficient embryonic stem cells (*in vitro*) and mouse embryos (*in vivo*), which fail to develop.[94] Therefore, it is no surprise that the miRNA expression is altered in numerous pathological contexts, including a large percentage of tumours.[90]

1.4.1. Regulation of $CD4^+$ T cell subset differentiation by miRNAs

The immune system is subject to miRNA-mediated regulation as shown in numerous studies. It was first described that total ablation of the miRNA machinery, achieved by specific deletion of Dicer in the T cell lineage, leads to a severe block in $CD8^+$ T cell development and an intrinsic bias of $CD4^+$ T cells towards Th1 differentiation *in vitro*, along with a reduction in Treg cell numbers and multi-organ autoimmune disease.[95, 96] Later on, it was also shown that T-cell specific ablation of Drosha resulted in spontaneous T cell activation, inflammation and premature death, which corroborated the previous findings.[97] Similarly, Treg-specific Dicer knockout mice rapidly developed systemic autoimmune disease resembling the scurfy phenotype, emphasising the importance of the miRNA compartment for immune regulation and autoimmunity prevention.[97] Upon miRNA depletion due to AGO deficiency, $CD4^+$ T cells are more prone to differentiate into cytokine-producing cells, suggesting that miRNAs are involved in the repression of genes that promote acquisition of effector functions, such as cytokines or cytokine regulators.[98]

Taking into account the effects of entire miRNA machinery depletion in the immune system, the next step was to figure out which individual miRNAs could have an important role in the Teff versus Treg cell balance, which would allow us to better understand the regulatory mechanisms that rule autoimmunity and tolerance.

Over the past decade, dramatic advances have been made in understanding the miRNA network affecting T cell differentiation.[99] A summary of the most relevant findings, with focus on Th1, Th17 and Treg subsets, is presented on Table 1.1.

Table 1.1 – Summary of the roles played by individual miRNAs in CD4⁺ T cell differentiation and/or function and respective mechanism of action

miRNA	Outcome	Mechanism	Targets
miR-21	Th1 [↓]	Modulation of IL-12 production by dendritic cells [100]	*Il12a* [100]
miR-29a/b	Th1 [↓]	Modulation of IFN-γ production [101, 102]	*Ifng* [102] *Tbx21* [101] *Eomes* [101]
miR-17~92	Th1 [↑]	Promotion of Th1 differentiation and IFN-γ production [103]	*Pten* [103]
miR-146a	Th1 [↓] Th17 [↓] Treg [↑]	Modulation of IFN-γ production [100] Inhibition of IL-6 and IL-21 production and Th17 cell differentiation [104] Promotion of Treg functions [105]	*Stat1* [100, 105] *Traf6* [104] *Irak1* [104]
miR-155	Th1 [↑] Th17 [↑] Treg [↑]	Promotion of Th1 differentiation [106, 107] Promotion of Th17 differentiation [99] Maintenance of Treg proliferation [108]	*Ship1* [106] *Ifngr1* [107] *Ets1* [99] *Socs1* [108]
let-7f	Th17 [↓]	Downregulation of IL-23R expression and IL-17 production [106]	*Il23r* [106]
miR-15b	Th17 [↓]	Inhibition of RORγt transcription [109]	*Ogt* [109]
miR-18a	Th17 [↓]	Inhibition of Th17 differentiation [110]	*Hif1a* [110] *Rora* [110]
miR-20b	Th17 [↓]	Modulation of IL-17 production [103]	*Rorc* [103] *Stat3* [103]
miR-210	Th17 [↓]	Modulation of IL-17 production [103]	*Hif1a* [103]
miR-301a	Th17 [↑]	Promotion of Th17 differentiation and function [111]	*Pias3* [111]
miR-326	Th17 [↑]	Promotion of Th17 differentiation [99]	*Ets1* [99]
miR-30a	Treg [↑]	Promotion of Treg differentiation and inhibition of Th17 differentiation [106]	*Il6r* [106]
miR-31	Treg [↓]	Inhibition of Treg differentiation [106]	*Foxp3* [106]
miR-125a	Treg [↑]	Stabilization of the commitment and immunoregulatory capacity of Treg cells [112]	*Stat3* [112] *Ifng* [112]

miR-30a has been shown to increase the percentage of Foxp3⁺ cells within activated CD4⁺ T cells by targeting *Il6r* and *Il6st* mRNA transcripts.[106] miR-155, on the other hand, is maintained in a Foxp3-dependent manner in Treg cells and preserves Treg cell homeostasis by targeting *Socs1*, which reduces phosphorylation of STAT5.[108] miR-29 is the classical example of a miRNA that interferes with IFN-γ production, by directly targeting *Tbx21* and *Eomes*, hence limiting the differentiation of Th1 cells.[101] miR-140,

which has been shown to target *Stat1*, negatively impacts the differentiation of Th1 cells *in vitro* and inversely correlates with IFN-γ and STAT1 expression in MS patients.[113]

It has also been shown that miR-326 promotes Th17 cell differentiation by targeting the *Ets1* transcription factor, which is a negative regulator of this lineage.[106] Overexpression of miR-20b suppresses EAE pathogenesis and Th17 differentiation *in vivo* and *in vitro* by targeting *Rorc* and *Stat3*.[114]

The miR-17~92 cluster, promotes Th1 responses but is required for Treg suppressive function *in vivo*, directly affecting the balance between the two populations.[111] Similarly, miR-21, which upregulates IFN-γ while decreasing IL-4, impacts on the balance between Th1 and Th2.[103] Interestingly, upon Treg generation, TGF-β induces miR-10a, which hampers the conversion of Treg cells into Tfh and differentiation of Th17 and is thus able to fine-tune the fate of T cell subsets, suggesting that cytokines can reciprocally regulate miRNA expression.[98, 115] In addition to the impact on cytokine production (and in part through it), the deregulation and modulation of specific miRNAs has been associated with various immune diseases and respective disease courses.[99] Blockade of miR-21, which is upregulated in $CD4^+$ T cells from systemic lupus erythematosus patients, led to a decrease in IL-10 production.[116] In mice bearing graft-versus-host disease (GVHD), treatment with a miR-142 antagomir significantly improved survival and reduced clinical symptoms.[116] *In vivo*, miR-155 knockout mice are resistant to *Helicobater pylori*-induced colitis.[117] A set of miRNAs overexpressed in naïve $CD4^+$ T cells of MS patients limited differentiation of Treg cells.[116] Also, mice subjected to systemic delivery of lentivirus encoding miR-326 developed more severe experimental autoimmune encephalomyelitis (EAE) compared to controls.[118] Moreover, transfection of PBMCs from patients with MS with a miR-140 mimic reduced Th1 and Th17 polarization *ex vivo*.[113]

As a matter of fact, miRNA regulation depends on target availability, which may direct their functions according to cell type. For example, *Traf6* and *Irak1* are verified targets for miR-146a in $CD4^+$ T cells, in which miR-146a deficiency enhances Th17 responses.[104] Yet, Treg-specific deletion of miR-146a reduced STAT1 expression and IFN-γ production.[105] Recently, it has also been demonstrated that miR-155-mediated repression has distinct, cell-type-dependent functional importance for different types of lymphocytes. These results suggest a mechanism of suppression that relies on biological context, at least for miR-155, but probably for other (if not all) microRNAs and adds another layer of complexity to the field.[119] Therefore, it is necessary to unravel the elaborate networks in which miRNAs take part and frame the mechanistic miRNA regulation in a wider, holistic perspective.

2. PRELIMINARY DATA

Over the past decade, dramatic advances have been made in understanding the miRNA network affecting T cell differentiation. The relevance of these findings both for better understanding the intricate networks of the immune system and developing immune therapies is clear. Therefore, we aimed at further understanding the complex networks of miRNA-mediated regulation of $CD4^+$ T cell biology. Although several individual miRNAs have been implicated in $CD4^+$ T cell *in vitro* differentiation mechanisms, we believe that a holistic approach based on *in vivo* models is required to understand how miRNA networks may control the balance between Teff and Treg subsets in physiological and pathological conditions. Therefore, we intend to dissect the specific contributions of miRNAs for the development of Th1, Th17 and Treg cells during immune responses *in vivo*.

EAE is a common mouse model for the study of the human immune-mediated demyelinating disease multiple sclerosis.[120] This experimental model is characterized by Th1/Th17-induced autoimmunity and subsequent demyelination of neurons in the central nervous system (CNS), which result in a progressive weakness and, ultimately, paralysis.[120] For this project, EAE is a relevant model given the fact that all three populations of interest (Th1, Th17 and Treg cells) play an active role in the initiation, progression and/or recovery of the disease. Both Th1 and Th17 cells, derived from myelin oligodendrocyte (MOG)-specific TCR transgenic mice, were able to induce EAE with similar severity after *in vitro* differentiation, although the pathological phenotypes did not match completely.[121, 122] Treg cells, on the other hand, accumulate in the CNS to

constrain pathogenic T cells, mediating recovery and their depletion hampers resolution of the disease.[77]

EAE was established in a triple reporter mouse for *Ifng*, *Il17a* and *Foxp3* and induced through immunization with MOG in adjuvant (*M. tuberculosis*), and injection of the pertussis toxin on the day of initial immunization and 2 days later. Disease onset typically occurred at day 10 after immunization and mice progressively develop a chronic disease with little variation in severity once a peak has been achieved (Fig. 2.1.). EAE progression was monitored daily using the standard five-point grading system for clinical assessment of the disease.[123]

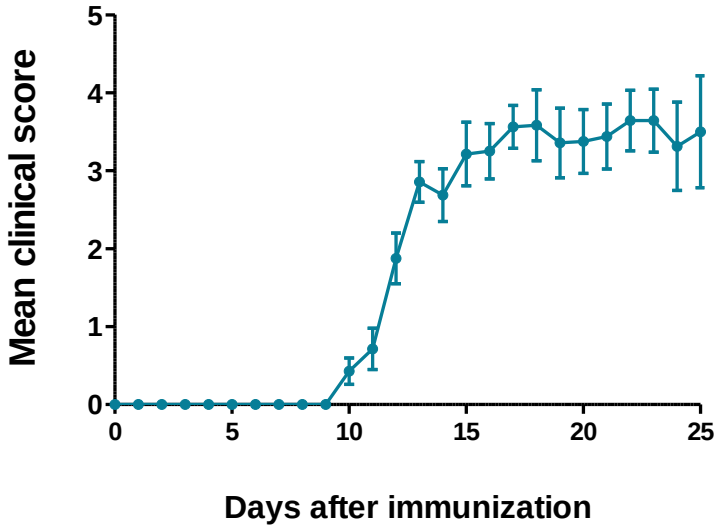


Figure 2.1. – EAE progression in triple reporter mice. Disease onset occurred at day 10 after immunization and mice gradually weaken until a peak is achieved around day 15. Afterwards, mice remained at a peak-plateau stage and rarely recover. Disease was monitored daily using the standard five-point grading system for clinical assessment of the disease: 0, asymptomatic; 1, loss of tail tone or objective weakness of a hind limb; 2, both tail and hindlimb weakness; 3, loss of ability to right self associated with hind limb paralysis; 4, complete hind limb paralysis and forelimb weakness; 5, moribund. When clinical signs were intermediate between two grades of disease, 0.5 was added to the lower score.

The construction of the reporter sequences present in the reporter mice was based on the introduction of an internal ribosome entry site (IRES) sequence followed by the DNA sequence of a fluorescent protein, for *Ifng* and *Il17a* (YFP and GFP, respectively) and the DNA for the human protein CD2 (hCD2) for *Foxp3*.

The triple reporter mouse was established in-house from available reporter mouse strains, as previously described.[42, 124, 125] Th1, Th17 and Treg cells were then isolated from the spleen and lymph nodes of EAE-induced triple reporter mice at peak-plateau stage and the miRNA repertoires of each subset were characterized by small RNA-seq. 110 miRNAs were found to be differentially expressed between effector (Th1 and Th17)

and regulatory T cells, but only 10 were specifically deregulated in each T cell subset when compared to the others (Fig. 2.2). The miRNAs in question were miR-1247-5p, miR-7667-5p (upregulated in Th1), miR-122-5p, miR-126a-5p, miR-5108 (upregulated in Th17), miR-15b-5p, miR-151-3p, miR-211-5p, miR-467a-5p (upregulated in Treg) and miR-125a-5p (downregulated in Th17). Selected miRNAs are highlighted in yellow (Fig. 2.2) and were subject to a more thorough characterization and analysis, which will be the focus of the work presented herein.

Figure 2.2. – Heatmap of the differential miRNA expression identified by miRNA-seq of Th17, Th1 and Treg cell populations of EAE-immunized reporter mice. miRNAs that are deregulated in each T cell population are highlighted in yellow (3 replicates).

3. AIM

From the miRNA-seq analysis of the miRNA repertoires of *in vivo*-generated Th1, Th17 and Treg cell populations, 10 candidate miRNAs (Table 3.1) were found to be specifically up or downregulated in one of the subsets and hypothesised to have a relevant role on the balance between the respective effector and regulatory subsets.

Table 3.1. – Candidate miRNAs and their expression profile in $CD4^+$ T cell subsets

miRNA	Expression profile
miR-1247-5p	Upregulated in Th1
miR-7667-5p	Upregulated in Th1
miR-122-5p	Upregulated in Th17
miR-126a-5p	Upregulated in Th17
miR-5108	Upregulated in Th17
miR-125a-5p	Downregulated in Th17
miR-15b-5p	Upregulated in Treg
miR-151-3p	Upregulated in Treg
miR-211-5p	Upregulated in Treg
miR-467a-5p	Upregulated in Treg

The global purpose of this thesis is to unravel the role of the aforementioned miRNAs in the differentiation of T cell subsets, namely Th1, Th17 and Treg. More specifically, we aim to:

1. Assess the expression of the candidate miRNAs in *in vitro*-differentiated Th1, Th17 and Treg cells;

2. Study the functional impact of the candidate miRNAs in *in vitro*-differentiated Th1, Th17 and Treg cells.

4. MATERIAL AND METHODS

4.1. Mice

C57BL/6J non transgenic, wild-type (WT) female mice were purchased from The Jackson Laboratory (Bar Harbour, ME, USA). All mice were female adults aged 6 to 10 weeks and were maintained in specific pathogen–free rodent facilities of Instituto de Medicina Molecular João Lobo Antunes, Faculty of Medicine, Universidade de Lisboa (Lisbon, Portugal). All experiments involving animals were done in compliance with the relevant laws and institutional guidelines and were approved by the Ethics Committee of the Instituto de Medicina Molecular. Every effort was made to minimize the number of animals used and their suffering.

4.2. Monoclonal antibodies

Anti-mouse purified monoclonal antibodies and fluorescently labelled antibodies against the cell surface proteins, cytokines and transcription factors that were used are stated on Table 4.1.

Table 4.1. – List of antibodies used in cell sorting, flow cytometry and cell culture.

Antibody	Manufacturer	Reference
Purified anti-mouse CD3ε (clone 145-2C11)	BioLegend	100302
Anti-mouse CD3ε (clone 145-2C11) PerCP-Cy5.5	eBioscience	100328
Anti-mouse CD4 (clone RM4-5) Pacific Blue	eBioscience	100544
Anti-mouse CD25 (clone PC61.5) APC-Cy7	eBioscience	47-0251-82
Anti-mouse CD25 (clone PC61.5) APC	eBioscience	17-0251-82
Anti-mouse CD25 (clone PC61) PE	BioLegend	102007
Purified anti-mouse CD28 (clone 37.51)	eBioscience	14-0281-85
Anti-mouse/rat Foxp3 (clone FJK-16s) APC	eBioscience	17-5773-82
Anti-mouse/rat Foxp3 (clone FJK-16s) Pacific Blue	eBioscience	48-5773-80
Purified anti-mouse IFN-γ (clone R4-6A2)	BioLegend	505702
Anti-mouse IFN-γ (clone XMG1.2) PE-Cy7	eBioscience	25-7311-82
Purified anti-mouse IL-4 (clone 11B11)	eBioscience	14-7041-85
Anti-mouse IL-17A (clone TC11-18H10.1) Pacific Blue	BioLegend	506918
Anti-mouse/rat IL-17A (clone eBio17B7) APC	eBioscience	17-7177-81
Anti-mouse IL-17A (clone TC11-18H10.1) PE	BioLegend	506903
Anti-mouse Ki-67 (clone 16A8) BV605	BioLegend	652413
Anti-mouse Ki-67 (clone 16A8) PE	BioLegend	652404
Anti-mouse/human T-bet (clone eBio4B10) PE	eBioscience	12-5825-80

4.3. Cell culture conditions

HEK-293T TAT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose with L-glutamine supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin (Pen/Strep). 3T3 cells were maintained in DMEM supplemented with 10% FBS, 1% Pen/Strep, 1% HEPES and 1% non-essential aminoacids (NEAA). Both cell lines were maintained below total confluence at 37ºC in a humified atmosphere of 5% CO_2 and were passed every 2-4 days. All cell culture reagents were from Gibco.

4.4. Retroviral vector cloning

Retroviral constructs encoding mmu-miR-125a, mmu-miR-467a, mmu-miR-7667 and mmu-miR-126a were generated by inserting the respective native pre-microRNA (pre-miRNA) sequences flanked by about 200 bp into a modified pMIG-PGK-GFP-WPRE retroviral vector (Fig. 4.3). The internal ribosomal entry site (IRES)–GFP sequence was removed and replaced by PGK-GFP-WPRE sequence. The resulting vector encodes GFP under the control of the phosphoglycerate kinase (PGK) promoter

and the respective microRNA under the control of the 5' retroviral long terminal repeat (LTR) promoter. A Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) was inserted for increased viral titers.

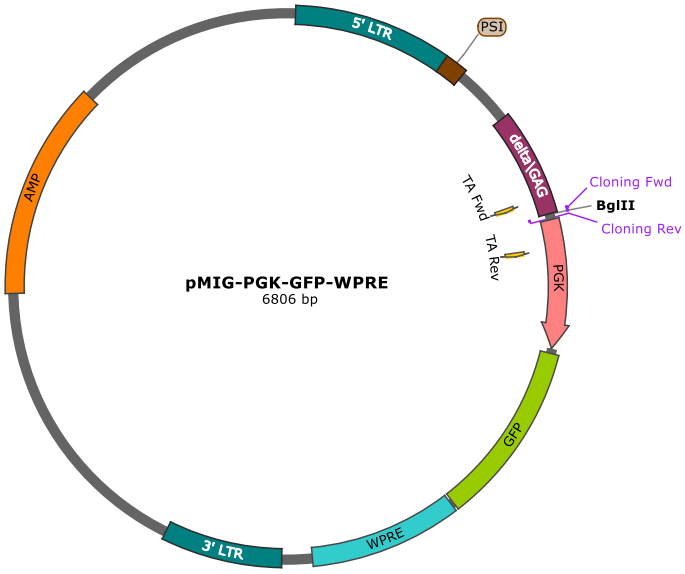


Figure 4.1. – Illustration of the pMIG-PGK-WPRE (pMIG-PGW) retroviral vector. Adapted from Addgene #9044.

4.4.1. Amplification and purification of pre-miRNA sequences

Pre-miRNA sequences were amplified from genomic DNA of C57BL/6J mice by PCR using specifically-designed primers (Sigma-Aldrich) containing a 5' overhang of 15 bp (CGCCGGAATTAGATCT and TAACCTCGAGAGATC for Forward and Reverse primers, respectively) homologous to the pMIG-PGW retroviral vector BglII restriction site. Overlap extension PCR reactions were performed with proof-reading enzyme Phusion® High-Fidelity DNA polymerase (New England Biolabs) and the following primer sequences: 5'-TTGAGGAAGACACCCGAGGA-3' (Fwd) and 5'-CTCCGGGTCTGAGGAGAAGA-3' (Rev) for miR-125a; 5'-ATCACCCTGGATTTGTGGGA-3' (Fwd) and 5'-GGTTTCCCGTGGTTTGTGAG-3' (Rev) for miR-467a; 5'-ACCGTTCCTAGCTGTTAGCC-3' (Fwd) and 5'-CTTCTGGATCCCTGGCTGTG-3' (Rev) for miR-7667; 5'ACCTGGGTAGTCCTTGGGTT-3' (Fwd) and 5'-GCAAGATCCACTCCCAACCA-3' (Rev) for miR-126a. PCR was accomplished under optimized conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 sec, 66 °C for 30 sec and 72 °C for 2 min and another stage at 72 °C for 10 min. Product size was confirmed by 2% agarose gel electrophoresis imaged with Chemidoc XRS^{+} System (Bio-Rad). Positive segments were purified using QIAquick® PCR purification kit (Qiagen).

4.4.2. Insertion of pre-microRNA sequences into pMIG-PGW

The pMIG-PGW retroviral vector was linearized with FastDigest® BglII restriction enzyme (Thermo Scientific) and purified using QIAquick® PCR purification kit (Qiagen). Each pre-miRNA segment was cloned into the pMIG-PGW vector using HD In-Fusion kit (Takara Bio USA) and the resulting plasmid was incorporated into Stellar™ Competent cells (Clontech) by heat-shock transformation according to manufacturer's instructions. Colony PCR reactions were performed using the Xpert directXtract PCR kit (Grisp) under optimized conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min and another stage at 72 °C for 5 min. PCR products were resolved by 2% agarose gel electrophoresis imaged with Chemidoc XRS$^+$ System (Bio-Rad). Positive colonies were purified using GeneJET Plasmid Mini Prep kit (Thermo Scientific) and their respective vector sequences were confirmed by Sanger sequencing (STAB VIDA). Successful plasmids were amplified and purified from transformed One Shot® Stbl3™ *E. coli* competent cells (Invitrogen) as above mentioned. Both colony PCR and sequencing reactions were performed using the following primers: 5'-CCTTGAACCTCCTCGTTC-3' (Fwd) and 5'-GAACGGACGTGAAGAATG-3' (Rev).

4.5. Retroviral particle production

For retroviral particle production, HEK-293T TAT cells were plated at a density of 4×10^6 cells per 10-cm dish in 8 mL of medium. Upon transfection, cell medium was replaced by fresh medium lacking antibiotics. Cells were transfected with fixed amounts of pMIG-PGW-miR-125a, pMIG-PGW-miR-467a, pMIG-PGW-miR-7667, pMIG-PGW-miR-126a or the control vector pMIG-PGW (2.5 µg) together with the viral plasmids pCL-Eco (2 µg) and pCMV-VSV-G (0.5 µg) (both from Addgene). The DNA mixture was incubated with XtremeGene 9 transfection reagent (Sigma-Aldrich) at a ratio of 3:1 (XtremeGene:DNA) in 500 µL of opti-MEM (Gibco) for 20 min. Cells were incubated overnight and standard medium was re-established on the following day. Retroviral particles were harvested at 48 h, 72 h and 96 h post-transfection. After collection of HEK-293T TAT medium, retroviral particles were concentrated through high-speed centrifugation at 25,000 rpm for 2 h at 4 °C in High Speed centrifuge Avanti J-25 (Beckman Coulter Diagnostics). Pellets were resuspended, frozen in liquid nitrogen and stored at -80 °C. The retroviral particle production protocol is depicted on Fig. 4.4.

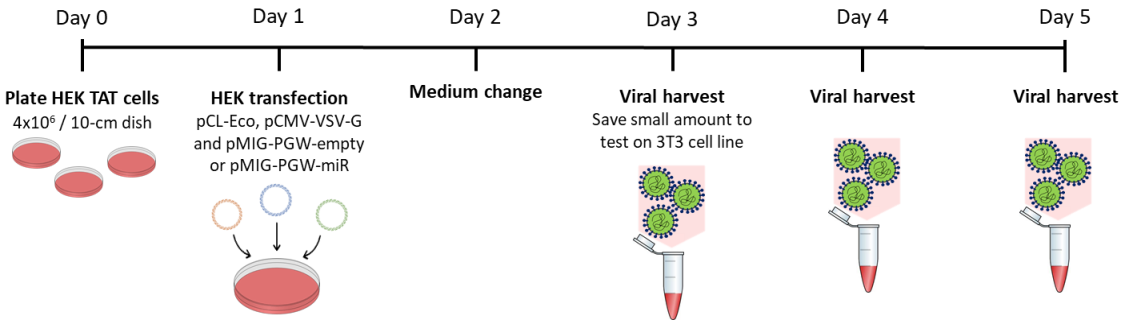


Figure 4.2 – Schematic representation of retroviral production workflow. HEK-293T TAT cells were plated at a density of 4×106 cells per dish. On the following day, cells were transfected with fixed amounts of the retroviral plasmids pCL-Eco, pCMV-VSV-G and pMIG-PGW encoding miR-125a, miR-467a, miR-7667 or miR-126a or control pMIG-PGW (empty vector). Cells were incubated with the plasmids overnight, after which the cell medium was changed. On the following 3 days, retroviral particles were harvested, centrifuged, frozen in liquid nitrogen and stored at -80 ºC.

To test the retroviruses for GFP expression and microRNA overexpression, 24 h prior to transduction, 3T3 cells were plated at a density of 100,000 cells per well of a 6-well plate in 1.5 mL of medium. Upon transduction, cell medium was renewed and 20 µL of retroviral particles were added to the cells. Following overnight incubation, the medium was once again changed and 48 h after transduction, cells were either analysed by flow cytometry or stored at -20 °C in QIAzol® Lysis Reagent (QIAGEN) for quantitative real-time PCR (RT-qPCR). We observed that each retroviral vector was able to overexpress the corresponding miRNA (Fig. 4.5.).

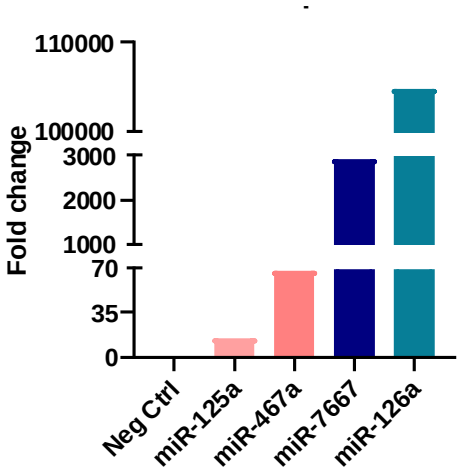


Figure 4.3 – miRNA overexpression in 3T3 cells. RT-qPCR analysis of retrovirally-encoded candidate miRNAs. Relative miRNA quantification was calculated using the ΔΔCT equation normalized to miR-423-3p (endogenous control) and results are represented as fold change.

4.6. *In vitro* CD4$^+$ T cell differentiation and transduction

4.6.1. Cell sorting

For cell sorting, cell suspensions were obtained from lymph nodes (superficial cervical, axillary, brachial, inguinal and lumbar) and the spleen of C57BL/6J mice and erythrocytes from the latter were osmotically lysed in red blood cell lysis buffer (eBioscience). Cells were incubated with fluorochrome-conjugated antibodies for 15 min (surface staining), filtered through 70-μm cell strainers (BD Biosciences) and CD3$^+$ CD4$^+$ CD25$^-$ cells were sorted on FACSAria (BD Biosciences).

4.6.2. *In vitro* CD4$^+$ T cell polarization

FACS-sorted CD3$^+$ CD4$^+$ CD25$^-$ cells from lymph nodes and spleen of C57BL/6J mice were cultured *in vitro* for 4 days under polarizing conditions. For Th1 and Treg, naïve CD4$^+$ T cells were incubated on 96-well with plate-bound anti-CD3ε and soluble anti-CD28 mAbs (both at 2 μg/mL) in the presence of anti-IL-4 (5 μg/mL) and IL-12 (10 ng/mL) or IL-2 (10 ng/mL) and TGF-β (2 ng/mL), respectively. As a control for these conditions (Th0), cells were cultured in basal medium without cytokines and were activated only for CD3ε/CD28. For Th17 polarization conditions, cells were incubated on 48-well with plate-bound anti-CD3ε (1 ug/mL) and anti-CD28 mAbs (10 ug/mL) in the presence of IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-23 (20 ng/mL), anti-IFN-γ (10 μg/mL) and TGF-β (2 ng/mL). Again, a control condition (Th0 IMDM) consisting of cells cultured in basal medium and activated only for CD3ε/CD28 was included. CD4$^+$ T cells in Th0, Th1 and Treg polarization conditions were maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% FBS, 1% Pen/Strep, 1% HEPES, 1% NEAA, 1% Sodium pyruvate, 0.1% β-mercaptoethanol and 0.1% Gentamicin. CD4$^+$ T cells in Th0 IMDM and Th17 polarization conditions were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FBS, 1% Pen/Strep, 0.1% β-mercaptoethanol and 0.1% Gentamicin. All cells were kept at 37 °C in a humified atmosphere of 5% CO_2. All reagents were from Gibco.All cytokines were purchased from Peprotech. Flow cytometry data were acquired using FACSFortessa (BD Biosciences) and analysed using FlowJo software (Tree Star).

4.6.3. Intracellular staining

For flow cytometry analysis of the cytokine profile, $CD4^+$ T cells in culture were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml) (all from SigmaAldrich) for 3.5 h at 37°C. Cells were stained with LIVE/DEAD™ (Life Technologies) or Zombie Aqua™ (BioLegend) for viability and extracellular surface markers for 20 min. For intracellular cytokine staining, cells were fixed with Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent for 30 min at 4 °C, permeabilized with Permeabilization buffer in the presence of anti-mouse CD16/CD32 (all from eBioscience) for 15 min at 4 °C. Lastly, cells were incubated for 45 min at 4 °C with the above identified antibodies in Permeabilization buffer. Flow cytometry data were acquired using FACSFortessa (BD Biosciences) and analysed using FlowJo software (Tree Star).

4.6.4. Retroviral transduction

As represented on Fig. 4.6, several viral transduction conditions were tested and overall the 8h condition was selected the most efficient transduction without inducing the cells into apoptosis. Viral transduction (40 µL per 200 000 cells) was performed on day 1 with polybrene (4 µg/mL; Sigma-Aldrich) after which cells were centrifuged at 31 ºC for 60 min at 2100 rpm. After 8 h of incubation with the viruses at 37 ºC, cell medium was changed to standard polarization conditions and on day 4 cells were activated and collected to intracellular staining and RT-qPCR.

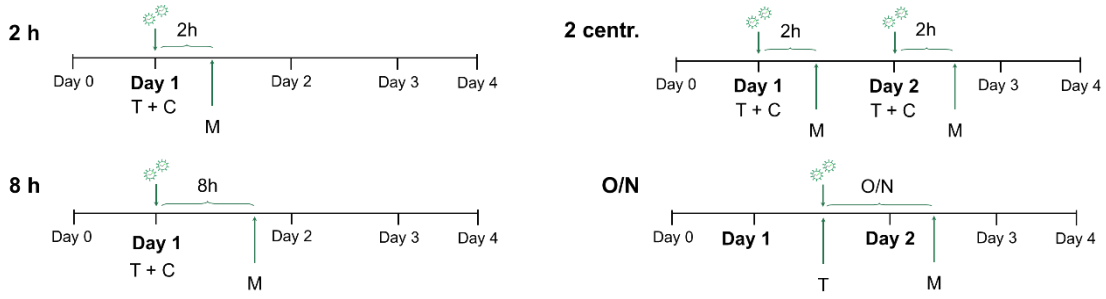


Figure 4.4 – Schematic representation of the retroviral transduction protocols tested. Naïve $CD4^+$ T cells sorted from lymph nodes and spleen of C57BL/6J mice were cultured *in vitro* for 4 days. Several retroviral transduction conditions were tested: centrifugation followed by 2-hour incubation with retroviral particles on day 1 (2 h), centrifugation followed by two 2-hour incubation with retroviral particles both on day 1 and day 2 (2 centr.), centrifugation followed by 8-hour incubation with retroviral particles on day 1 (8 h) and overnight incubation with retroviral particles on day 1 (O/N). T: Transduction; C: Centrifugation; M: medium change.

4.7. RNA isolation, complementary DNA production and quantitative RT-PCR

Total RNA was extracted from 3T3 and $CD4^+$ T cells using the miRNeasy mini kit (QIAGEN) according to manufacturer's instructions and quantified using NanoDrop 2000 (Thermo Scientific). Reverse transcription was performed with the Universal cDNA Synthesis kit II (QIAGEN) using 60-100 ng of RNA according to the following protocol: 42 °C for 60 min followed by heat-inactivation of the reverse transcriptase at 95 °C for 5 min. Quantitative real-time PCR (RT-qPCR) was performed to evaluate miRNA expression and was carried out on a ViiA 7 cycler (Applied Biosystems) using Power SYBR® Green (Thermo Scientific) and the respective miRNA LNA primers (QIAGEN). RT-qPCR reaction conditions consisted of polymerase activation/denaturation and well-factor determination at 95ºC for 10 min followed by 50 amplification cycles of 95 °C for 10 sec and 60 °C for 1 min (ramp-rate of 1.6°/sec). Immediately afterwards, a melting curve analysis was performed in order to verify the specificity of the amplification and non-specific PCR products were excluded. Relative miRNA concentrations were calculated using the ΔΔCT equation normalized to miR-423-3p (endogenous control) and results were represented as fold change.

4.8. Statistical analysis

Data are presented as mean ± s.e.m. The statistical significance of differences between populations was assessed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for the PCR analysis and using the Mann-Whitney *U* test for the retroviral transduction experiments. A difference was considered significant if $p \leq 0.05$.

5. RESULTS

5.1. 5 candidate miRNAs reproduce *in vivo* expression profiles in *in vitro*-differentiated T cell subsets

As previously described, miRNAs are important posttranscriptional regulators of T cell differentiation and modulation of their functions could improve existing immune therapies or help develop new ones. We intend to further understand which specific miRNAs mediate $CD4^+$ T cell differentiation and how they affect the balance between pro and anti-inflammatory subsets. Since many previous studies were based solely *in vitro*, we started by analysing the miRNA repertoires of Treg, Th1 and Th17 *in vivo* using the EAE mouse model, in which the two effector T cell populations – Th1 and Th17 – and the regulatory T cell population – Treg – play important roles for the establishment versus resolution of the disease. As referred in the "Preliminary data" section, we identified 10 differentially expressed miRNAs specifically enriched in either effector or Treg cell populations. To determine whether we could reproduce the sequencing results in an *in vitro* system, we then evaluated the candidate miRNA expression in *in vitro*-differentiated Treg, Th1 and Th17 cells. We sorted naïve $CD4^+$ T cells as $CD3^+$ $CD4^+$ $CD25^-$ from lymph nodes and spleen of WT C57BL/6J mice, cultured them *in vitro* for 4 days in either Treg, Th1 or Th17-polarizing conditions and evaluated the candidate miRNA expression by

RT-qPCR. In all conditions, naïve T cells were activated for CD3ε/CD28 and stimulated with different cytokines to promote a specific T cell subset.

In our system, the Treg-skewing condition consists of stimulation with IL-2 and TGF-β, whereas Th1 polarization is promoted with IL-12 and anti-IL-4. Th17 cells are induced with IL-6, IL-1β, IL-23, TGF- β and anti-IFN-γ. Since Th17 are cultured in IMDM medium, as opposed to the other subsets, two control conditions have been included: one for Treg and Th1, named Th0 and another for Th17, termed Th0 IMDM and both were activated with anti-CD3ε and anti-CD28 only. After 4 days in culture, differentiated T cell subsets were either stained for cytokines (IFN-γ and IL-17) and transcription factors (Foxp3) and analysed by flow cytometry or collected for RT-qPCR analysis upon RNA extraction.

Expression of Foxp3, IFN-γ and IL-17 has been used as a marker for Treg, Th1 and Th17, respectively, for which they provide an indication of the percentage of naïve T cells that have been polarized to each specific lineage. Indeed, our data shows that the expression of these markers is almost exclusive to their specific subset (Fig. 5.1). Foxp3 is expressed by most cells cultured in Treg conditions and expressed only at basal levels by the others, whereas IFN-γ is highly expressed by cells cultured in Th1-inducing conditions. For both Treg and Th1 conditions, the polarization efficiency is approximately 70% (Fig. 5.1 B). Furthermore, Th0 and Th0 IMDM cells express low levels of IFN-γ (around 5%), supporting the idea that the *Ifng* locus is in a poised state.[34] Even though the polarization efficiency of Th17 cells is around 30%, which is lower than the other subsets, this percentage is specific as this is the only condition in which IL-17 expression is detected. (Fig. 3.1 B). Both Th0 and Th0 IMDM control conditions express low or no levels of all lineage markers, behaving as expected for activated, undifferentiated $CD4^+$ T cells[126] and demonstrating that our polarization conditions are specific for each cell population. (Fig. 3.1 B).

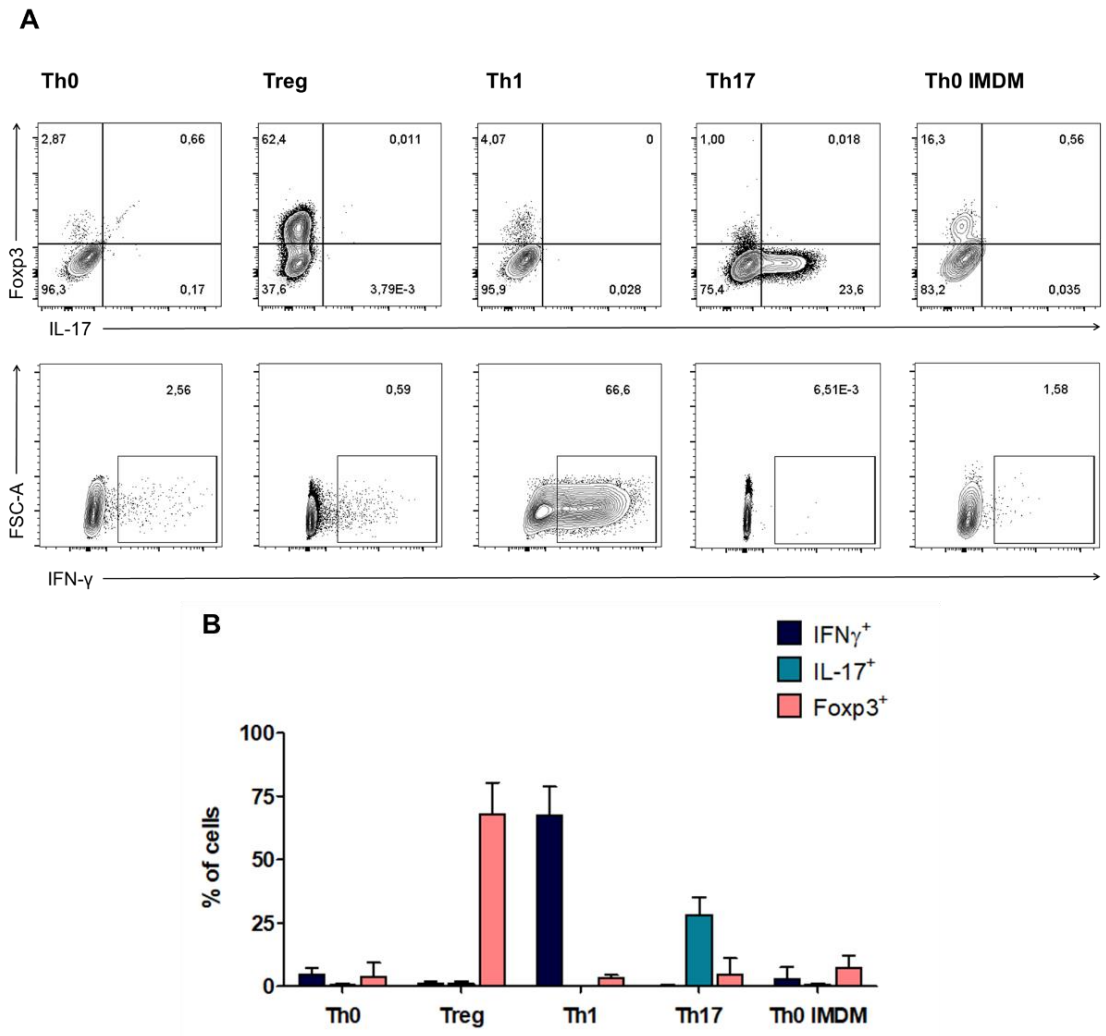


Figure 5.1. – *In vitro*-differentiation of T cell lineages. (A) Flow cytometry analysis of intracellular Foxp3 expression and IFN-γ and IL-17 production from Th0, Treg, Th1, Th17 and Th0 IMDM cells on day 4 of *in vitro* culture in the presence of the corresponding cytokine cocktails. **(B)** Average polarization efficiency of Th0, Treg, Th1, Th17 and Th0 IMDM differentiation conditions analysed by flow cytometry on day 4. Data are representative of 8 independent experiments.

We then evaluated the expression levels of the 10 candidate miRNAs that were found to be differentially expressed between Treg, Th1 and Th17 cells *in vivo* (Table 1.1). As above mentioned, naïve $CD4^+$ T cells were differentiated into T cell subsets *in vitro* and, on day 4, fully polarized cells were collected and their RNA was extracted for RT-qPCR analysis. As shown in Figure 5.2, of the 4 miRNAs that were upregulated in the Treg population analysed *in vivo* (miR-15b-5p, miR-151-3p, miR-211-5p and miR-467a-5p), only miR-467a-5p and miR-15b-5p were significantly overexpressed in *in vitro*-generated Treg cells when compared to both Th1 and Th17 cells. Regarding the miRNAs found to be upregulated in the Th1 subset, miR-1247-5p did not replicate the sequencing data, since it is upregulated in *in vitro*-differentiated Treg cells. On the other hand, miR-7667 reproduced the sequencing data, as it is significantly upregulated *in vitro* in Th1 versus

Th17 and versus Treg (Fig. 5.2). Concerning miR-122-5p and miR-126a-5p, which were upregulated in the Th17 population *in vivo*, only the latter replicated the sequencing data, since it is significantly overexpressed in *in vitro*-generated Th17 versus Th1 and Treg cells (Fig. 5.2).

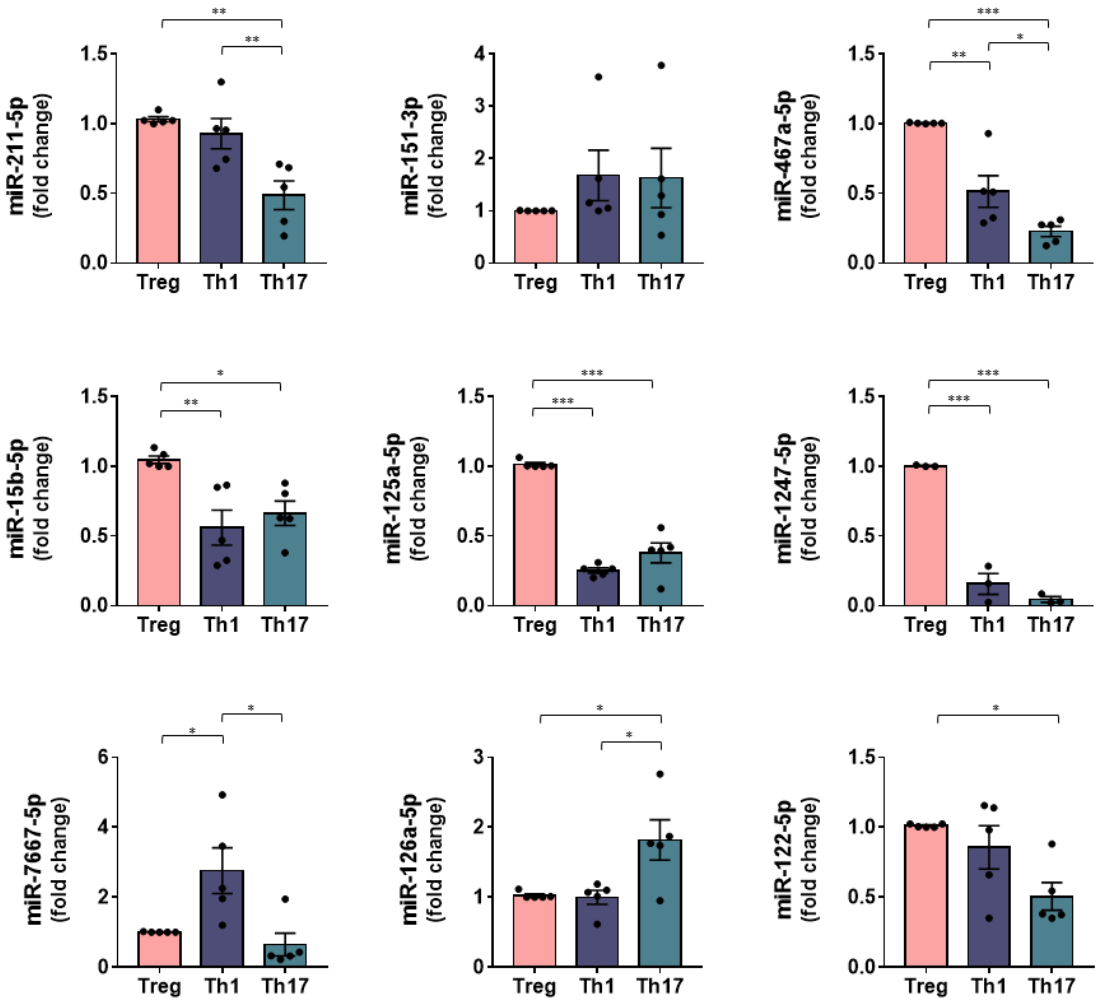


Figure 5.2. – RT-qPCR analysis of candidate miRNA expression in *in vitro*-polarized Treg, Th1 and Th17 cell subsets. Relative miRNA expression between T cell subsets was calculated using the ΔΔCT equation using miR-423-3p as endogenous control to normalize the expression levels. Results are represented as fold change versus Treg. Data are representative of 5 independent experiments.

In the miRNA-seq analysis, miR-125a-5p was found to be downregulated in Th17 cells relatively to the other two subsets, but in the RT-qPCR analysis it is significantly upregulated in *in vitro*-polarized Treg cells when compared to both Th1 and Th17 subsets (Fig. 5.2.). miR-5108 could not be detected in this analysis.

To sum up, the miRNAs whose expression profiles *in vitro* replicated the miRNA-seq analysis were miR-15b-5p, miR-467a-5p, miR-7667-5p and miR-126a-5p. miR-15b-5p has already been demonstrated to suppress Th17 differentiation *in vitro* and *in vivo*, it was shown to alleviate EAE and to be downregulated in $CD4^+$ T cells from MS

patients.[109] Moreover, it has also been shown to enhance the Treg cell lineage.[127] In our *in vitro* analysis, miR-125a-5p was not downregulated in Th17 cells (Fig. 5.2), thus not reproducing our sequencing data. However, the observed upregulation in Treg cells is in agreement with previously reported evidence that miR-125a represses STAT3 and *Ifng*, maintaining Treg functions during EAE.[112] Therefore, both miR-125a and miR-15b have been shown to play a role in Treg differentiation. Since the functions of miR-125a have been thoroughly described in EAE, we decided to keep it as a control in our system. The remaining miRNAs that did not reproduce the sequencing data *in vitro* will be subject of further study *in vivo*.

5.2. Optimization of retroviral transduction conditions

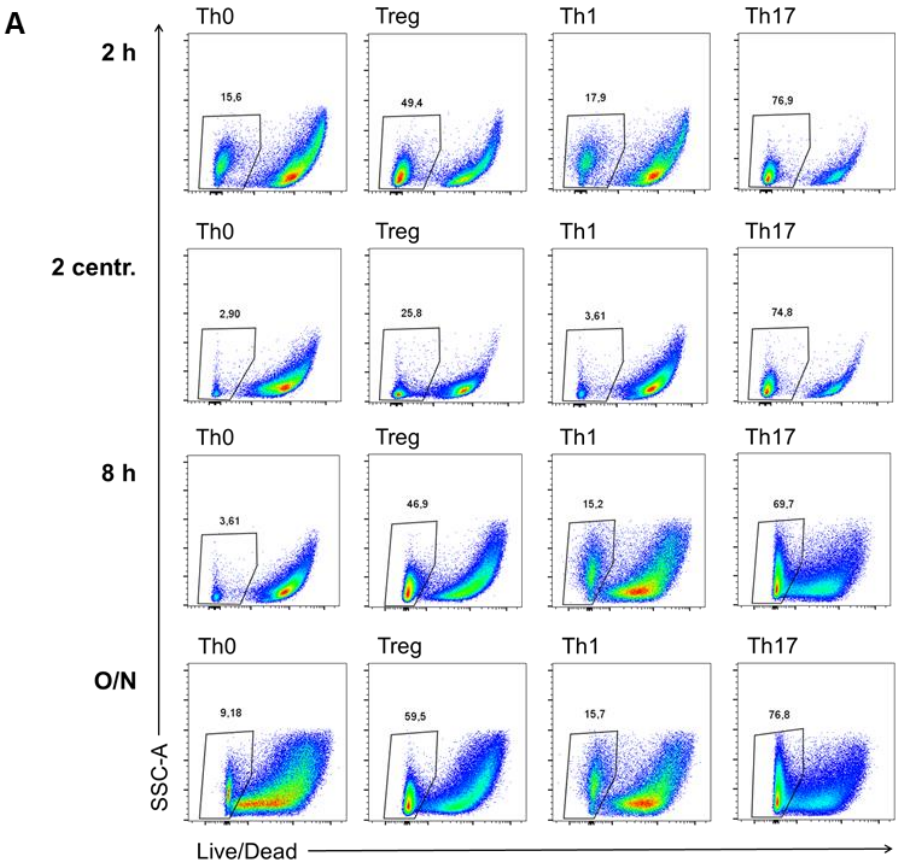
In order to functionally modulate the candidate miRNAs in *in vitro*-differentiated T cell subsets, we cloned the native stem loop of each of the 4 miRNAs (miR-125a-5p, miR-467a-5p, miR-7667-5p and miR-126a-5p) selected from the RT-qPCR analysis together with their adjacent flanking regions onto a retroviral vector with a GFP reporter gene (pMIG-PGW). Such strategy allows miRNA overexpression through the intrinsic miRNA-processing machinery of the cell, simultaneously encoding for GFP. Retroviral particles were assembled in HEK-293T TAT cells by transfection of the necessary viral plasmids and concentrated through high-speed centrifugation to produce a stock. Viral stock was tested in 3T3 cells and GFP expression was analysed by flow cytometry to confirm transduction efficiency.

To include the retroviral transduction step within our $CD4^+$ T cell differentiation protocol interfering as little as possible with the system, we then evaluated the transduction efficiency of different conditions and how they impacted on cell viability and polarization. Herein we present the results of the 4 most representative examples from all the conditions tested. After incubation with the different polarization cocktails (day 0), cells were transduced with retroviral particles corresponding to the empty GFP vector according to different conditions: a) centrifugation followed by 2-hour incubation on day 1 (hereafter referred as 2h), b) centrifugation followed by two 2-hour incubation both on day 1 and day 2 (2 centr.), c) centrifugation followed by 8-hour incubation on day 1 (8h) and d) overnight incubation on day 1 (O/N). After incubation, the medium was always changed. Other conditions for transduction optimization included different time points and viral loads or centrifugation before overnight incubation. Cell viability was assessed with a live/dead dye, to which dead are permeable; and transduction efficiency was directly evaluated by the percentage of GFP-expressing cells, which incorporated the viruses. As shown in Figure 5.3 A, the 2-centrifugation condition has a very clear,

negative impact on cell viability not only of Treg cells, but mostly of Th1, for which the percentage of live cells is below 4% (Fig. 5.3 A). Furthermore, the polarization of Th17 cells is strongly affected in these conditions, as they show increased Foxp3 expression and decreased IL-17 production (data not shown). Regarding the other two conditions (8h-hour and O/N incubation), the best one in terms of cell survival is the O/N incubation (Fig. 5.3 A).

In terms of transduction efficiency, which is depicted in Fig. 5.3 B, the worst condition for Th1 cells is the 2-hour incubation (about 7%), whereas the for Th17 cells it is the O/N condition (around 9%), as well as for Treg cells (around 4%). The condition in which both Treg and Th1 subsets are most effectively transduced is upon 8h incubation (20% and 30%, respectively). It is also the condition in which Th0 are better transduced, with approximately 40% of transduced cells (Fig. 5.3 B) and, in terms of survival, it is similar to the 2 centrifugation one. Regarding Th17 cells, the 2-hour condition is the most efficient (about 50% of transduction) in this aspect, but they are also well transduced in the 8-hour incubation condition (Fig. 5.3 B). Moreover, the 2-hour condition also negatively impacted on the Th17 polarization, leading to increased Foxp3 expression and reduced IL-17 production (data not shown).

Hence, we selected the centrifugation plus 8-hour incubation condition as the final setting for retroviral transduction considering that this condition seemed to most fully accomplish a compromise between transduction efficiency, cell viability and maintenance of all subset polarization.


A
2 h
2 centr.
8 h
O/N
Th0
Treg
Th1
Th17
15,6
49,4
17,9
76,9
2,90
25,8
3,61
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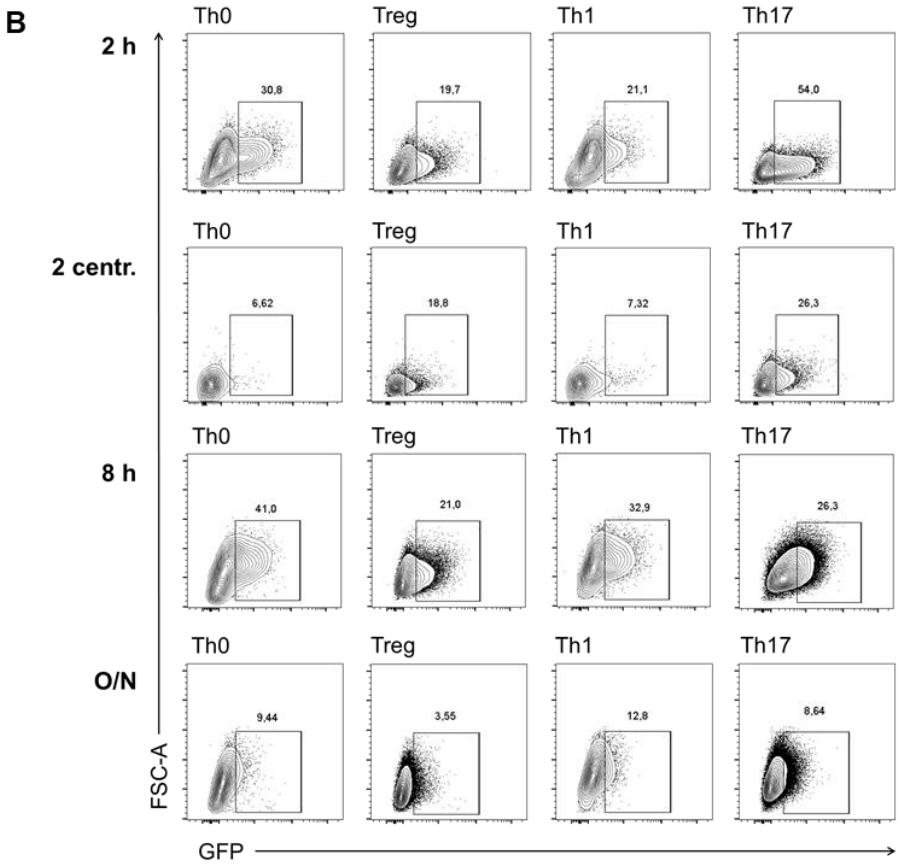
76,8
SSC-A
Live/Dead


B
2 h
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8 h
O/N
Th0
Treg
Th1
Th17
30,8
19,7
21,1
54,0
6,62
18,8
7,32
26,3
41,0
21,0
32,9
26,3
9,44
3,55
12,8
8,64
FSC-A
GFP

Figure 5.3. – Optimization of a retroviral transduction protocol to functionally modulate candidate miRNA expression *in vitro*. Naïve CD4$^+$ T cells were sorted from lymph nodes and spleen of C57BL/6J mice as previously described and cultured *in vitro* for 4 days. Flow cytometry analysis of live/dead staining (indicative of cell viability) **(A)** and of GFP (transduction efficiency) **(B)** in Th0, Treg, Th1 or Th17-polarizing conditions after different transduction conditions. Frequency of live **(A)** or transduced **(B)** cells is indicated in the corresponding gate for each condition and cell type. Several retroviral transduction conditions were tested: centrifugation followed by 2-hour incubation with retroviral particles (10 µL per 200,000 cells) on day 1 (2h), centrifugation followed by two 2-hour incubation with retroviral particles (5 µL per 200,000 cells) both on day 1 and day 2 (2 centr.), centrifugation followed by 8-hour incubation with retroviral particles (10 µL per 200,000 cells) on day 1 (8h) and overnight incubation with retroviral particles (10 µL per 200,000 cells) on day 1 (O/N).

5.3. Overexpression of candidate miRNAs *in vitro* did not impact viability nor proliferation of T cell subsets

Afterwards, we assessed whether the overexpression of candidate miRNAs directly impacted on cell viability or proliferation. We cultured murine naïve CD4$^+$ T cells *in vitro* for 4 days together with the cytokine cocktails needed to generate distinct Treg, Th1 or Th17 cell subsets. On day 1, we transduced each T cell subset with native stem loops of miR-125a, miR-467a, miR-7667 or miR-126a encoded in retroviral particles and, on day 4, we evaluated fully polarized T cell subsets in terms of viability and proliferation. Cell viability was once again assessed with a live/dead dye, to which dead cells are permeable and proliferation was assessed with Ki-67, a cell division protein present in the G1, S, G2 and M cell cycle phases.

For Treg and Th1 cells, no significant differences have been found in the frequency either of live (Fig. 5.4 A) or proliferative (Fig. 5.4 B) cells upon retroviral transduction with any of the candidate miRNAs compared to cells transduced with empty viruses, suggesting that these miRNAs have no direct impact neither on viability nor on proliferation for both of these subsets. For Th17, there is also no impact on viability upon retroviral transduction (Fig. 5.4 A). Preliminary data on Th17 proliferation also suggests that this parameter is not affected by candidate miRNA overexpression (data not shown). Therefore, we inferred that any shift or variation in the phenotype of the T cell subsets that might be observed after transduction with the candidate miRNAs would be due to a biological function of the miRNA on the actual differentiation process and not because of an indirect effect on proliferation or survival.

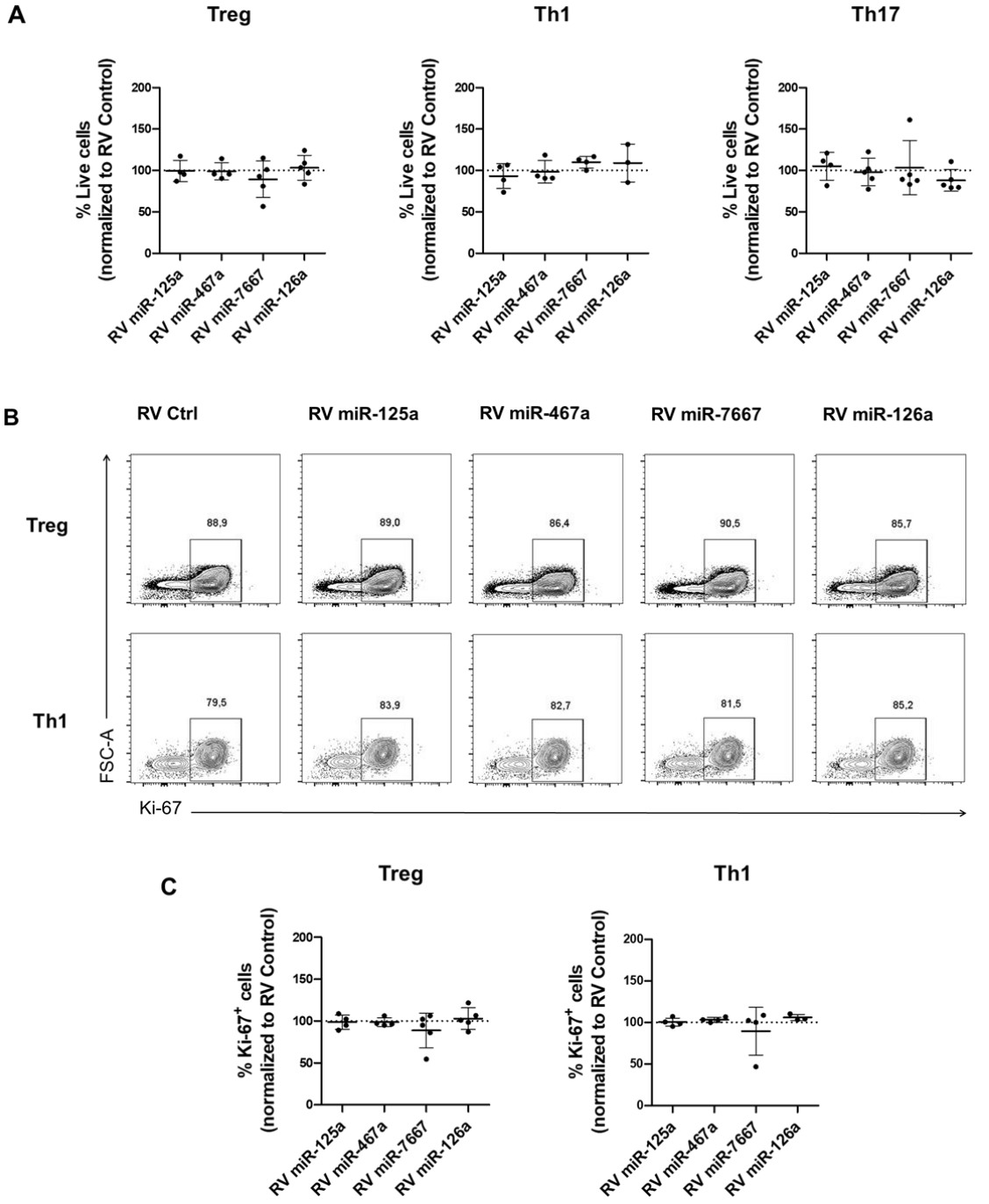

Figure 5.4. – Impact of candidate miRNA overexpression on cell viability and proliferation of *in vitro*-differentiated Treg, Th1 and Th17 cell subsets. Frequency of live cells **(A)**, flow cytometry analysis of Ki-67$^+$ cells **(B)** and average frequency of Ki-67$^+$ cells **(C)** within Treg, Th1 and Th17 cell subsets upon retroviral transduction either with miR-125a, miR-467a, miR-7667 or miR-126a. Data are representative of 4 to 5 independent experiments. Results are normalized to RV Control.

5.4. Impact of candidate miRNAs overexpression on T cell subset differentiation

In order to understand the role played by the candidate miRNAs on $CD4^+$ T cell differentiation, we further analysed their cytokine (IFN-γ and IL-17) and transcription factor (Foxp3) expression profile after transduction, by intracellular staining. Note that, according to the *in vivo* sequencing data and the *in vitro* RT-qPCR analysis, miR-125a and miR-467a were both upregulated in Treg cells, whereas miR-7667 was upregulated in Th1 cells and miR-126a was upregulated in Th17.

As shown in Figure 5.5, we observed that neither the frequency (Fig 1.5 B) nor the mean fluorescence intensity (MFI) (Fig. 5.5 C) of $Foxp3^+$ cells in Treg conditions (gated on GFP^+ cells) change upon incubation with any of the miRNAs, not even with miR-125a, which has already been shown to upregulate the Treg lineage.[112, 128] This may indicate that, in our system, the miRNA is incapable of inducing Treg differentiation at higher levels to those induced by the cytokine cocktail. For Th1 cells (within the GFP^+ population), we observed a significant decrease in the IFN-γ frequency upon incubation with miR-7667 (Fig. 5.5 A and B). This reduction, however, is not accompanied by a decrease of the IFN-γ MFI (Fig. 5.5 C), which suggests that miR-7667 modulates the differentiation process by decreasing the percentage of IFN-γ-expressing cells (Fig. 5.5 B), even though not affecting the amount of cytokine produced by them (Fig. 5.5 C). Of notice, in some of the experiments, we observed a higher percentage of Foxp3 in Th1-polarizing conditions (around 15%), when compared to what we had been observing until then (data not shown). In Th17 cells, the percentage of IL-17-expressing cells significantly reduces after incubation with miR-126a (Fig. 5.5 A and B). Again, similar to what happens in the Th1 population with miR-7667, the MFI of $IL17^+$ Th17 cells (gated on GFP, Fig. 5.5 C) is not altered with miR-126a. These results suggest that miR-126a, while decreasing Th17 cell differentiation, does not fine-tune IL-17 levels within Th17-polarized cells. The remaining miRNAs do not interfere neither with the frequency nor the MFI of IFN-γ and lL17, so they do not seem to modulate Th1 and Th17 differentiation, at least *in vitro*.

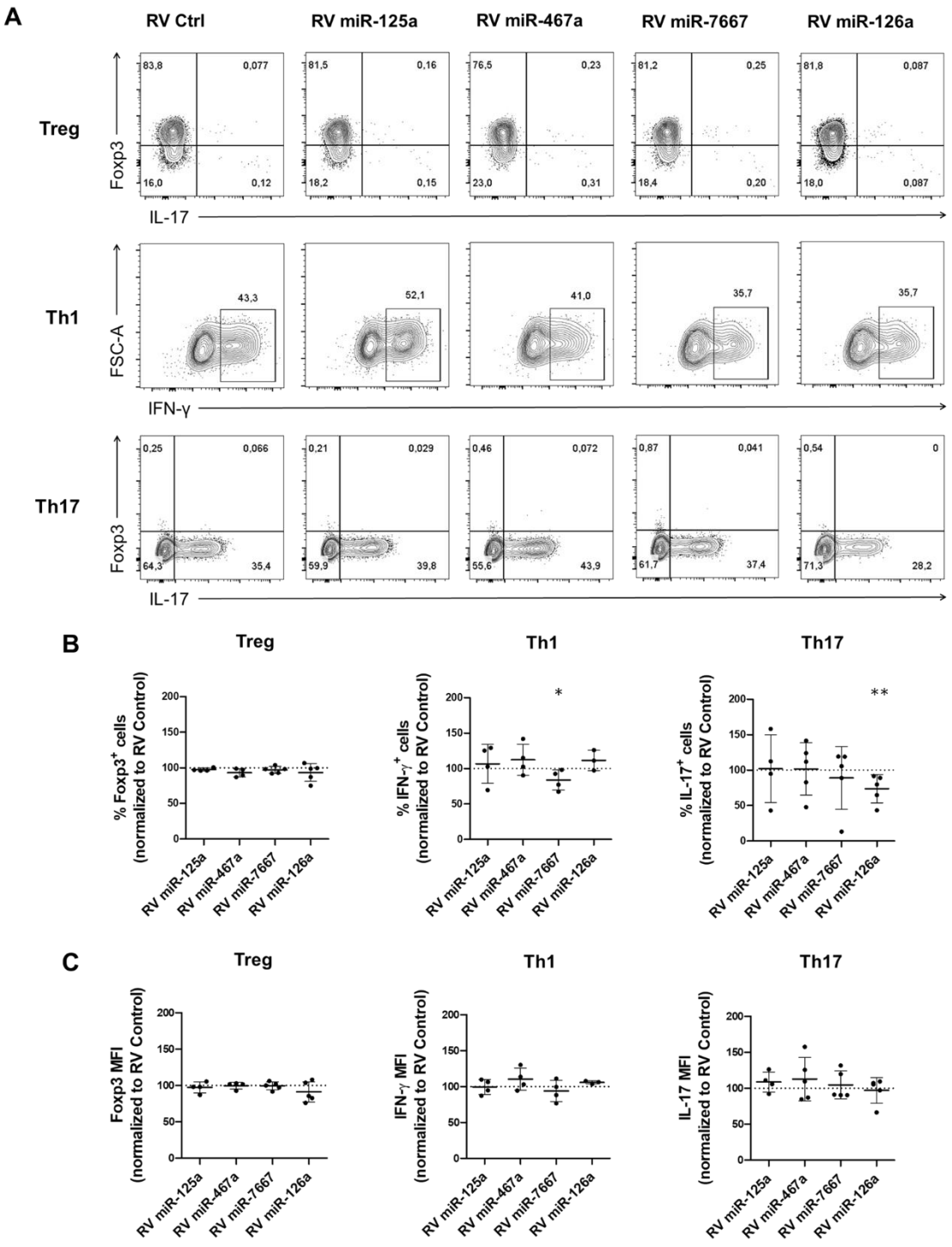

Figure 5.5. – Impact of candidate miRNA overexpression on the polarization of Treg, Th1 and Th17 cell subsets *in vitro*. Flow cytometry analysis **(A)**, average frequency **(B)** and mean fluorescence intensity (MFI) **(C)** of intracellular Foxp3 expression and IFN-γ and IL-17 production by GFP^+ Treg, Th1 and Th17 cells upon retroviral transduction either with miR-125a, miR-467a, miR-7667 or miR-126a. Data are representative of 4 to 5 independent experiments. Results are normalized to RV Control. *$p \leq 0.05$ and **$p \leq 0.01$.

To further understand whether incubation with the miRNAs alone is sufficient to modulate the expression of cytokines or transcription factors, we transduced

in vitro-activated (with anti-CD3ε and anti-CD28) Th0 and Th0 IMDM with retroviral particles encoding either miR-125a, miR-467a, miR-7667 or miR-126a. After 4 days in culture, cells were intracellularly stained for Foxp3, IFN-γ and IL-17. As previously addressed, we used a specific IMDM Th0 control for Th17 cells due to the specific culture conditions required for differentiation of this T cell population. However, we never detected IL-17 in Th0 IMDM cells (data not shown), hence we focused our analysis on Th0 cells. As depicted in Figure 5.6, we observed a significant increase in the Foxp3 MFI within GFP$^+$ Th0 cells upon incubation with miR-125a (Fig. 5.6 C), but the frequency of Foxp3$^+$ cells in the same population remained unchanged (Fig. 5.6 B). Note that, in this case, contrary to what was observed for Treg cells, miR-125a had a positive, although modest, impact in the expression of Foxp3. miR-467a, on the other hand, significantly reduced the frequency of Foxp3$^+$ cells within Th0 cells (gated on GFP, Fig. 5.6 B), although the MFI of this population was not altered (Fig. 5.6 C). Therefore, miR-467a seems to modulate the expression of Foxp3 transcription factor. While the results observed for miR-125a are in accordance with the described role for this miRNA in promoting Treg differentiation,[112] miR-467a seems to play distinct roles in different cell types. This data is in line with the recent advances in the field stating the importance of cell type and biological context,[119] on which miRNA regulation strongly depends.

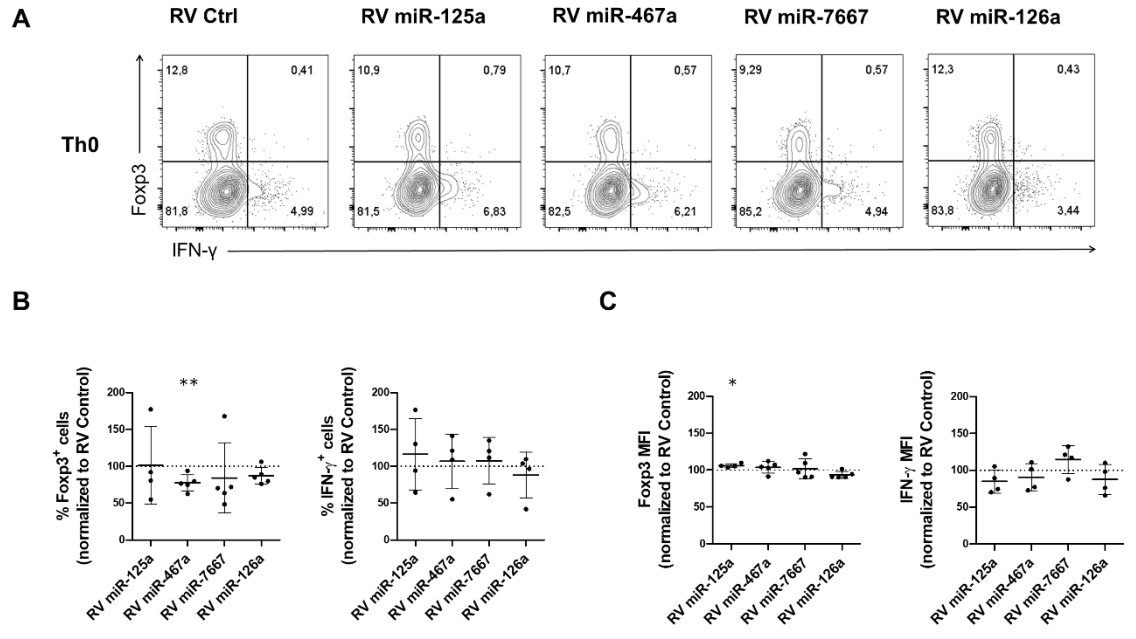

Figure 5.6. – Impact of candidate miRNA overexpression on the phenotype of *in vitro*-activated Th0 cells. Flow cytometry analysis **(A)** and average frequency and mean fluorescence intensity (MFI) **(B)** of intracellular Foxp3 expression and IFN-γ production by GFP$^+$ Th0 and Th0 IMDM cells upon retroviral transduction either with miR-125a, miR-467a, miR-7667 or miR-126a. IL-17 was not detected in any of the conditions. Data are representative of 4 to 5 independent experiments. Results are normalized to RV Control. *p ≤ 0.05 and **p ≤ 0.01.

6. DISCUSSION

miRNAs are a large family of endogenous small noncoding RNAs responsible for gene expression regulation at the posttranscriptional level. Ever since miRNAs were shown to be important mediators of biological processes, particularly within the immune compartment, intensive research efforts have been put to expand the knowledge on miRNA-mediated regulation, for instance, of T cell differentiation.[111] The relevance of miRNAs on $CD4^+$ T cell differentiation has been demonstrated by several studies using miRNA-deficient T cells[105, 110, 117] and several specific miRNAs have been implicated in this process.[104, 129] However, many of the studies were based on *in vitro* systems and the physiological relevance of the mechanisms identified remains to be uncover.[130, 131] Therefore, we aimed at further dissecting miRNA-mediated regulation of T cell differentiation *in vivo*. To address this, we have characterized the miRNomes of *in vivo*-generated Treg, Th1 and Th17 cells, isolated from a triple reporter mouse for *Foxp3*, *Ifng* and *Il17a*, upon EAE induction. Effector and regulatory T cell subsets analysed derive from the same animals, which allowed us to uncover the miRNA repertoires during a well-defined immune response system *in vivo* and to identify specific miRNAs that impacted on $CD4^+$ T cell differentiation.

From the 110 differentially expressed miRNAs between effector and regulatory T cell subsets, we selected 10 candidate miRNAs whose expression levels were either

significantly up or downregulated in one specific T cell subset relative to others for further analysis: miR-15b-5p, miR-151-3p, miR-211-5p, miR-467a-5p (upregulated in Treg cells), miR-122-5p, miR-126a-5p, miR-5108 (upregulated in Th17 cells), miR-125a-5p (downregulated in Th17 cells), miR-1247-5p and miR-7667-5p (upregulated in Th1 cells). The analysis of the expression levels of the 10 candidate miRNAs in *in vitro*-differentiated Treg, Th1 and Th17 cells has revealed that only 5 miRNAs reproduced the sequencing data (Fig. 5.2). Substantial evidence suggests that cells *in vivo* may undergo differentiation pathways that differ from the ones established for *in vitro* conditions.[21] Although it is important to acknowledge the limitations of the *in vitro* differentiation, this system can be a useful tool to study the mechanisms underlying the differentiation of each T cell subset in particular. As such, we have further characterized the miRNA candidates whose *in vitro* expression profiles matched with the ones obtained *in vivo*. The miRNAs that did not match the *in vivo* miRNA profile are out of the scope of this work but will be further evaluated *in vivo* in a future study.

One of the miRNAs that reproduced the *in vivo* profile in T cell subsets differentiated *in vitro* is miR-125a, previously shown to stabilize both commitment and immunoregulatory capacity of Treg cells.[112] Indeed, miRNA-125a overexpression was shown to promote Foxp3 and inhibit RORγt, thus regulating the Treg/Th17 balance in the context of immune thrombocytopenic purpura[132] and also to play a role in Treg polarization upon therapeutic treatment of lupus erythematosus.[128] Moreover, the ability to differentiate into Treg was impaired in miR-125a-deficient T cells.[112] miR-125a-deficient mice had increased levels of IFN-γ and IL-17 and were more susceptible to EAE than WT mice.[112] The miR-15b/16 cluster has also been reported to enhance Treg differentiation and decrease the severity of autoimmune colitis *in vivo*.[127] Furthermore, miR-15b is downregulated in patients with MS, as well as in EAE mice and has been implicated in the suppression of Th17 differentiation both *in vitro* and *in vivo*.[109] Therefore, both miR-125a and miR-15b have been implicated in Treg differentiation. Since the functions of miR-125a have been thoroughly described in EAE, we have used it as a positive control in our system. Contrary to miR-125a and miR-15b, miR-7667 and miR-467a have not yet been reported in the literature as *in vivo* regulators of $CD4^+$ T cell differentiation.

Upon overexpression of miR-7667, we observed that there is a reduction of around 13% in the frequency of IFN-γ^+ cells within the GFP^+ Th1 population when compared with control conditions (Fig. 5.5 B). We also observed a decrease of approximately 10% in the percentage of IL-17 within GFP^+ Th17 cells after transduction with miR-126a in comparison with control conditions (Fig. 5.5 B).

Taken together, our data demonstrate that miR-7667 and miR-126a negatively regulate IFN-γ and IL-17 production, respectively. This is in line with findings of previous studies

showing that, overall, miRNAs act as molecular brakes of cytokine production in $CD4^+$ T cells. In fact, in $CD4^+$ T cells, Dicer deficiency leads to higher levels of IFN-γ [95] and Drosha deficiency results in increased IFN-γ and IL-17 production.[97] The observed miRNA-mediated modulation of cytokine production within the T cell compartment[103] suggests that miRNAs might potentially contribute to the disruption of the Treg/Teff balance.

To have more insight into the molecular mechanisms by which miR-7667 and miR-126a might regulate the production of pro-inflammatory cytokine, we searched for their predicted mRNA targets. We took advantage of the microT-CDS algorithm available online (DIANA tools), which predicted, among others, *Nfat5* as a possible target for miR-7667, with a score of 0.96 (on a scale of 0 to 1). In fact, it has been shown that NFAT5 is associated with a pro-inflammatory profile and promotion of Th1 cell differentiation.[133] *Stat1*, *Il12rb1*, *Eomes* and *Tbx21* are, according to this tool, other possible targets of miR-7667 and, even though the scores were not very high, these predictions corroborate the hypothesis that miR-7667 could be repressing the differentiation of Th1 cells. For miR-126a, the most robust predictions in terms of Th17-related genes were for *Il17a* and *Rora*, with scores of 0.86 and 0.81, respectively. *Csf2*, *Ahr*, *Il22*, *Hif1a* and *Il21* are other genes that, although with lower interacting scores, are also predicted to be targeted by miR-126a and are implicated in Th17 differentiation and function.[41, 82] In addition, there is evidence that mesenchymal stem cell-induced miR-126a regulates the PI3K/Akt pathway, leading to Foxp3 expression and induction of regulatory T cells.[134] The importance of PI3K has also been described for the differentiation of Th17 cells and inhibition of this pathway decreases the expression of *Il17a*, *Il17f* and *Il23r*.[135] Thus, it is possible that miR-126a suppresses the differentiation of Th17 cells through the targeting of some of these proteins.

Further studies testing specific target recognition of miR-7667 and miR-126a, including validation by the luciferase reporter assay, and assessing whether target modulation would rescue the phenotype will be critical to understand the regulatory networks by which miR-7667 and miR-126a regulate IFN-γ and IL-17 production , respectively.

We also evaluated whether each candidate miRNA on its own was sufficient to induce a phenotypic change. To do so, we activated $CD4^+$ T cells for CD3ε and CD28 and cultured them for 4 days *in vitro*. We observed that miR-467a significantly decreases the percentage of Foxp3-expressing cells within GFP in Th0 cells by 4% (Fig. 5.6 B), a result that was not consistent with that obtained upon Treg polarization, in which no differences were observed upon transduction with this miRNA. This suggests that miR-467a could play distinct roles in different cell types. miR-467a has 10 paralogues, from miR-467a-1 to miR-467a-10, some of which are encoded within different stem loop sequences and it

integrates two different clusters in the genome (as described in the miRBase and Ensembl databases). Remarkably, it has recently been observed that the pri-miRNA sequence and structure influence Drosha cleavage and affect the scope of mRNA targets.[136] Indeed, we are not aware of how much each of the miR-467a copies contributes to the differential expression observed by miRNA-seq and RT-qPCR. We also do not have information on the target mRNA repertoire of each of them and we only used one copy of miR-467a to construct the retroviral sequence for the overexpression experiments. This could, at least partially, explain why, despite having the same expression profile *in vivo* and *in vitro*, miR-467a does not impact Treg phenotype. On the other hand, miR-125a increases Foxp3 MFI in GFP^+ Th0 cells (Fig. 5.6 C), suggesting that miR-125a may be able to fine-tune Foxp3 expression according to environmental conditions. In fact, this miRNA has already been reported to have a positive effect in the expression of Foxp3,[112] which supports our result. However, we did not observe any significant effects in Foxp3 expression with this or any other of the miRNAs tested upon *in vitro* differentiation of Treg cells, which may indicate that the incubation with cytokines are stimulating the polarization in a way that miRNAs, particularly miR-125a, are no longer able to modulate this process.

We should bear in mind that transduction of the miRNA native stem loop implies that the other strand is also processed, so the effects we have observed may be due to or affected by the strand we are not interested in. Still, this approach is considered more physiological as it allows for natural processing of miRNA precursors by the RNA machinery of the cell. Nonetheless, confirmation of individual miRNA overexpression after transduction by RT-qPCR, which is ongoing, will be essential to better understand the efficacy of transduction in $CD4^+$ T cells and the specific miRNA strand that is being produced. To further confirm our results, complementary strategies such as incubation with mimics (miRNA-like synthetic RNA molecules), currently ongoing in the lab, will be necessary. Additionally, experiments using antimiRs (antisense oligonucleotides that prevent miRNA binding) or miRNA sponges (constructs with multiple miRNA binding sites) would be interesting to find out whether the effects of miRNA loss-of-function are contrary to the ones observed with the present gain-of-function experiments.

Given the fact that miRNAs participate in a complex regulatory network, the phenotypes that result from genetic manipulation of these regulatory components are often subtle and pleiotropic. It remains a challenge to control the immune response at the system level without causing toxicity to normal tissues. However, albeit in different cell contexts, the therapeutic potential of miRNAs is already being explored.[137] Importantly, EAE, which is the best characterized animal model of human autoimmune disease, was the experimental system used to develop three clinical therapeutic approaches for MS.[120]

Although different induction protocols resemble aspects of MS with varying success, overall the model provides significant insight into clinical efficacy of interventions.[120] Therefore, our data may provide important insight not only on T cell differentiation but also on EAE and, ultimately, multiple sclerosis which, upon further characterization, could be very useful for researchers focused on the development of therapeutic approaches for this pathology.

Overall, our results show that both miR-7667 and miR-126a decrease the frequency of IFN-γ and IL-17-producing T cells (as summarized in Fig. 6.1), respectively, suggesting that they negatively regulate the subsets in which they are produced. This effect may be explained by an attempt to control inflammation, although it needs further validation. Additionally, without the cytokine pressure, miR-467a decreases Foxp3 expression whereas miR-125a upregulates it. Multi-target regulation potential of miRNAs presents opportunities to dissect immune signalling networks by decoding the target-recognition information encoded in the miRNA genes. Thus, it is essential to better understand which genes are being targeted by these miRNAs in the conditions in which we are developing our study.

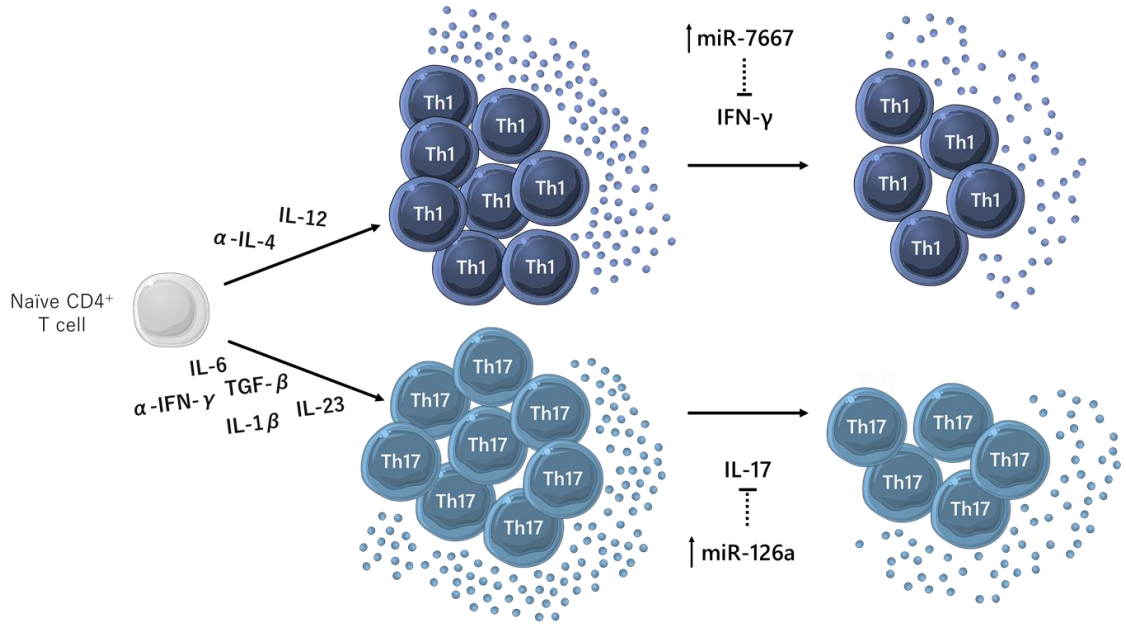

Figure 6.1. – Schematic representation of the main results of this thesis. Naïve CD4⁺ T cells cultured *in vitro* differentiate into Th1 in the presence of IL-12 and anti-IL-4 and into Th17 in response to TGF-β, α-IFN-γ, IL-6, IL-21, IL-23 and IL-1β. Overexpression of retrovirally-encoded miR-7667 and miR-126a upon the differentiation process results in decreased frequency of IFN-γ (within the Th1 population) or IL-17 (within the Th17 population), respectively. The targets by which miR-7667 and miR-126a modulate cytokine production remain to be uncover.

Certainly, miRNAs improve the knowledge of the intricate biological processes that rule our organism and there is a great potential for them to improve current therapies or develop new interventions. We believe this work will help dissect the mechanisms by which $CD4^+$ T cells differentiate and how we can modulate them in order to promote the balance between Treg and Teff cells.

7. FUTURE PERSPECTIVES

I herein summarise some of the future work that can be developed based on the data obtained throughout the project.

First of all, and according to our results, overexpression of candidate miRNAs did not affect cell survival. However, it should be taken into account that the live/dead dye assay is based solely on membrane integrity, hence it only stains for cells that are already dead. Therefore, in the future, other experiments should be performed to understand the percentage of cells that do not stain for live/dead but have already entered apoptosis. For instance, Propidium Iodide (PI) and Annexin V staining allows the distinction between early apoptosis (cells stain for Annexin V only), late apoptosis (cells stain both for PI and Annexin V) and necrotic cells (cells stain for PI only).

As mentioned before, the miRNAs that could not be modulated *in vitro* were not yet excluded and will be evaluated *in vivo*, as they might have relevant functions in the Treg/Teff balance. Those are miR-122, miR-211, miR-151, miR-1247 and miR-5108. miR-122, which accounts for 70% of total liver miRNAs, is widely established as a pivotal player in liver homeostasis and its overexpression resulted in tumour suppression.[138, 139] Though the information on miR-211 is scarce, it has been shown that, in the presence of IL-23, *in vitro* expression of this miRNA was enhanced.[140] Taking into account the essential role of IL-23 in the development of Th17 cells, it would be possible for miR-211

(upregulated in Treg cells, according to our data) to be a negative regulator of Th17 differentiation. In mouse macrophages, stimulation with LPS decreased the levels of miR-151-3p, leading to increased STAT3 protein levels and enhanced production of IL-6.[141] Lastly, miR-1247 has been shown to play a protective role in cancer, for example, by targeting MYCBP2 protein, which reduces colon tumour size *in vivo*.[142] Future *in vivo* experiments will help dissect the mechanisms by which these miRNAs regulate T cell differentiation. Successful *in vivo* modulation of miRNAs has already been achieved with direct intravenous injection of miRNAs mimics or antagomiRs, including for miR-125a[112] and miR-15b[109]. Therefore, we plan to inject antagomiRs for our most promising candidate miRNAs, analyse the effect on cytokine and transcription factor expression by flow cytometry and RT-qPCR and assess the phenotypic outcome on EAE progression. Furthermore, bioinformatic tools will be more thoroughly used to find predicted and validated targets for the candidate(s) that demonstrate the most potential. These data will be crossed with the mRNA-seq analysis that has been obtained for the same set of *in vivo* samples. The cross comparison between mRNA and miRNA profiles will contribute to the establishment of miRNA/mRNA networks based on inversely correlated expression levels between the two types of molecules and on target site predictions. Expression of relevant mRNA targets may then be confirmed *in vitro*, similar to what has been done for the miRNAs. In comparison with other studies on this topic, the results obtained will be more reliable, since our project is based on a holistic approach that primarily relies on *in vivo* responses. Subsequent studies will help validate the miRNA/mRNA networks and potentially find new drug targets or establish miRNAs as a therapeutic approach for immune therapies in the context, for instance, of autoimmune diseases, chronic inflammation or even cancer.

For the development of miRNA-based therapies, in addition to the difficulties of generating an appropriate delivery system and identifying the best miRNA candidates, one of the biggest challenges is the identification of relevant miRNA targets for each disease and this task is further hampered by the regional heterogeneity observed within the same tissue, for instance, in the case of tumours.[137] Therefore, it is of the upmost importance to understand, as accurately as possible, the function of the miRNA in each biological context before moving onto clinical approaches. We believe that the way this project is designed will allow a thorough comprehension of how specifically miRNAs can modulate T cell differentiation.

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