The Role Of miRNAs In CD8\(^+\) T Cell Differentation

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo
Os linfócitos T CD8⁺ desempenham um papel fundamental na defesa do hospedeiro contra patogénios intracelulares e tumores. A citocina com maior relevância produzida pelas células T CD8⁺ é o interferão-gama (IFN-γ), caracterizada por possuir efeitos pleiotrópicos sobre uma vasta gama de células do sistema imunológico e por ser essencial tanto na imunidade inata como na adaptativa. Em 2003 foi identificada a Eomesodermina, considerada como um factor de transcrição principal, necessário e suficiente para regular a transcrição e a produção de IFN-γ em células T CD8⁺.

Para além da regulação a nível transcricional, a diferenciação de sub-populações de células T efetoras encontra-se também sujeita a mecanismos de regulação pós-transcrição, mediados por microRNAs (miRNAs). Apesar desta função estar claramente demonstrada para células T CD4⁺ helper, os miRNAs que controlam a diferenciação de células CD8⁺ T produtoras de IFN-γ são ainda em grande parte desconhecidos.

Os miRNAs são moléculas de RNA não-codificantes de pequenas dimensões, que inibem pós-transcriacionalmente a expressão genética através da diminuição da estabilidade e/ou bloqueio da tradução de um dado mRNA, o que lhes permite desempenhar um papel relevante na diferenciação e proliferação celular.

A análise de ratinhos deficientes para a produção de miRNAs especificamente em linfócitos T (ratinhos LckCre Dicer), demonstrou que os miRNAs possuem um papel global na diferenciação de células T CD8⁺ e na produção de IFN-γ. Verificou-se um aumento da frequência de células T CD8⁺ produtoras de IFN-γ, tanto na periferia (nódulos linfáticos e baço) como no timo, em ratinhos deficientes para a produção de miRNAs quando comparados com ratinhos controlo.

Com o objectivo de identificar os miRNAs implicados na diferenciação das células T CD8⁺, realizaram-se microarrays que permitiram a identificação de 22 miRNAs diferencialmente expressos entre timócitos CD8⁺ YFP⁺ e CD8⁺YFP⁻ de ratinhos repórter para o IFN-γ (IFN-γ-YFP). Focamos a nossa análise sobre os 3 miRNAs mais expressos nas células T CD8⁺ YFP⁺, miR-139, miR-200a, miR-451a, e os 3 miRNAs mais expressos em células T CD8⁺ YFP⁻, miR-132, miR-181a e miR-322. Curiosamente, a expressão destes miRNAs não se encontra restrita às células T CD8⁺, sendo os mesmos também expressos noutras populações de células T,
sugerindo que possam ter funções pleiotrópicas nas células T. A sua expressão foi influenciada pela ativação do receptor das células T e pela presença de citocinas. É importante salientar que a expressão de dois dos nossos candidatos - miR-132 e miR-451 - foi mais elevada em condições indutoras da produção de IFN-γ, sugerindo que estes miRNAs poderão ser induzidos no decorrer da diferenciação de células T CD8+ em células efetoras produtoras de IFN-γ. Por último, efetuaram-se ensaios funcionais dos nossos candidatos com recurso a vectores retrovirais e verificou-se para um miRNA em particular, o miR-132, uma redução significativa da produção de IFN-γ em células T CD8+ que sobre-expressem este miRNA quando comparadas com as células controlo. No seu conjunto, estes dados sugerem que o miR-132 é um possível regulador da expressão de IFN-γ em células T CD8+. Como tal e para compreender os mecanismos moleculares pelos quais o miR-132 regula a produção de IFN-γ nas células T CD8+, recorreu-se a ferramentas bioinformáticas e pesquisa bibliográfica para encontrar possíveis mRNAs alvo. Foram encontrados vários candidatos promissores envolvidos na regulação do IFN-γ, incluindo Stat4, TWIST1 e RUNX3. A expressão destes candidatos está atualmente a ser analisada em estudos funcionais realizados em células T CD8+. Estes e outros candidatos serão futuramente caracterizados em experiências que elucidarão as redes moleculares de mRNAs controladas pelo miR-132 em células T CD8+ produtoras de IFN-γ. No seu conjunto, os resultados obtidos neste estudo abrem perspectivas de novos mecanismos de regulação da diferenciação de células T CD8+ produtoras de IFN-γ. A sua consolidação em estudos subsequentes dará uma contribuição essencial para a compreensão de respostas imunes contra infecções e tumores mediadas por células T CD8+.

Palavras-chave: Diferenciação de células T, células T CD8+, interferão-gama, microRNAs, regulação pos-transcricional.
Abstract

CD8+ T lymphocytes play a crucial role in host defense against intracellular pathogens and tumors. The key effector cytokine produced by CD8+ T cells is Interferon-gamma (IFN-γ) which has pleiotropic effects on a wide range of immune cells and is essential for both innate and adaptive immunity. In 2003 the “master” regulatory transcription factor Eomesodermin (Eomes) was identified, which is sufficient and necessary to drive IFN-γ production in CD8+ T cells. Besides transcriptional regulation, also post-transcriptional mechanisms mediated by microRNAs (miRNA) impact on the differentiation of effector T cell subsets, as clearly demonstrated for CD4+ T helper cells. However, the miRNAs controlling the differentiation of IFN-γ-producing CD8+ T cells are largely unknown.

miRNAs are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level, repressing gene expression by targeting mRNA stability and/or blocking translation, which enables them to play key roles in cell differentiation and proliferation. Our analysis of T cell-specific miRNA-deficient mice (LckCre Dicer−/− mice) revealed a global role of the miRNA network in the differentiation of IFN-γ producing CD8+ T cells. We observed an increase frequency of IFN-γ-producing CD8+ T cells in both the thymus and periphery (lymph nodes and spleen) of Dicer-deficient mice compared to control mice. To identify individual miRNAs implicated in CD8+ T cell differentiation, we undertook a transcriptome-wide analysis of miRNA expression in YFP+ versus YFP− CD8+ thymocytes from an Ifng-YFP reporter mouse. We identified 22 miRNA differentially expressed between the two cell populations and focused our analysis on the top 3 miRNAs up-regulated in CD8+ IFN-γ+ T cells, i.e. miR-139, miR-200a, miR-451a, and the top 3 miRNAs up-regulated in CD8+ IFN-γ− T cells, miR-132, miR-181a and miR-322.

Interestingly, our candidate miRNAs were expressed in T cell populations other than CD8+ T cells, suggesting that they might have pleiotropic functions in T cells, and their expression was influenced by T cell receptor and cytokine activation. Importantly, the expression levels of two of our candidates – miR-132 and miR-451 - were up-regulated in the presence of IFN-γ driving conditions suggesting that these miRNAs are induced in the course of CD8+ T cell differentiation towards IFN-γ producing effector cells. Finally, when employing a retroviral mediated over-expression strategy to investigate the impact of the candidate miRNAs on IFN-γ production by naïve CD8+ T cells, we detected a significant reduction of IFN-γ
production in CD8\(^+\) T cells over-expressing miR-132 compared to control cells. Collectively, our data suggest that miR-132 is a possible negative regulator of IFN-\(\gamma\) expression in CD8\(^+\) T cells. To address the molecular mechanisms by which miR-132 regulates IFN-\(\gamma\) production by CD8\(^+\) T cells we have initiated a miR-132 target search based on published evidence and bioinformatics analysis. Several promising mRNA candidates involved in IFN-\(\gamma\) regulation, including Stat4, Twist1 and Runx3, are currently being analyzed at the expression level in functional studies on CD8\(^+\) T cells. These and other candidates will be further characterized in future experiments that will elucidate the molecular mRNA networks controlled by miR-132 regulating the differentiation of IFN-\(\gamma\)-producing CD8\(^+\) T cells. Ultimately the results will contribute to a better understanding of CD8\(^+\) T cell differentiation into IFN-\(\gamma\) producing effector cells that make key contributions to immune responses against infections and tumors.

Key-words: T cell differentiation, CD8\(^+\) T cells, interferon-gamma, microRNAs, post-transcriptional regulation.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>Eomes</td>
<td>Eomesodermin</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LN</td>
<td>Lymph node</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>miR/miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
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<tr>
<td>Spl</td>
<td>Spleen</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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Note: List of all abbreviations used at least two times on different paragraphs throughout the manuscript. Additional abbreviations are defined in the text once they are first introduced to the reader.
Introduction
1 Introduction

1.1 CD8$^+$ T Lymphocytes Major Players in Immunity

1.1.1 Adaptive immune system

The adaptive immune system provides specific protection against pathogens, has the ability to form ‘memory’ (the basis of vaccination), that enables a rapid response to previously encountered pathogens, fights nascent cancers and mediates tumor destruction\textsuperscript{1,2}. The major mediators of the adaptive immunity, responsible for providing efficient, specific and long-lasting immunity, are lymphocytes\textsuperscript{3}. Lymphocytes can be subdivided into two separate lineages: thymic-derived (T) lymphocytes (T cells) and bone marrow-derived (B) lymphocytes (B cells) that further differentiate into plasma cells to secrete antibodies\textsuperscript{4}.

T cells are involved in cell-mediated immunity, provide help for B cells to produce antibodies (humoral immunity) and regulate immune responses\textsuperscript{4}. T cells are further classified according to the co-receptor (either CD4 or CD8) that they express at the cell surface, thus marking CD4$^+$ T cells or CD8$^+$ T cells. These markers are important for T cell function, because they help to determine the interactions between the T cell and its T cell receptor (TCR) and other cells expressing major histocompatibility complex (MHC) molecules. The TCR complex of conventional T cells is a transmembrane heterodimer composed of two polypeptide chains α and β chains, which associate with co-receptor CD3 molecules. Each TCR chain consists of a constant (C) and a variable (V) region, and is formed by a process termed somatic recombination which joins variable (V), joining (J), and diversity (D) gene segments to generate combinatorial diversity\textsuperscript{4}. Additionally, the addition or removal of nucleotides at the joining sites increases the repertoire of TCRs.

The TCR recognizes specific peptides presented by MHC molecules: CD4$^+$ T cells recognize MHC class II and CD8$^+$ T cells recognize MHC class I\textsuperscript{5}. After activation upon its first encounter with an antigen, T cells proliferate and differentiate into functional effector T lymphocytes. These include CD8$^+$ cytotoxic T cells, which secrete pro-inflammatory cytokines and kill cells that are infected with viruses or other intracellular pathogens; CD4$^+$ helper T cells, which provide essential additional
signals that influence the behavior and activity of B cells and innate immune cells; and CD4⁺ regulatory T cells that suppress the activity of other lymphocytes and help to control immune responses ⁴.

1.2 CD8⁺ T cells

Activated CD8⁺ T cells differentiate into cytotoxic T cells and secrete high amount of pro-inflammatory cytokines. They are crucial to fight against intracellular pathogens but also to eliminate tumor cells. On the other hand, they are also involved in the rejection of transplants and in the pathogenesis of a number of autoimmune diseases ⁶–¹¹. CD8⁺ T cells recognize peptides derived from proteins present in infected or transformed cells, bound to MHC class I molecules (pMHC). The direct lysis of target cells mediated by CD8⁺ T cells is one of the most powerful actions of T cells and is therefore tightly regulated, for example CD8⁺ T cells require more co-stimulation for their activation compared to CD4⁺ T cells ¹². Additionally CD8⁺ T cells can form memory T cells, contributing to a long-lived immunological protection ¹³. As CD8⁺ T cells are the focus of this thesis, different aspects of CD8⁺ T cell biology will be discussed in detail in the following sections.

1.2.1 CD8⁺ T cell Effector Mechanisms

Naïve CD8⁺ T cells acquire two critical effector functions after antigen activation: secretion of cytokines and direct contact-mediated cytotoxicity ¹⁴. Concurrent with the initiation of proliferation is the establishment of gene expression that arms the CD8⁺ T cell with effector mechanisms to combat infection, such as increased expression of the master transcription factors Eomesodermin and T-bet, encoded by Tbx21 ¹⁵,¹⁶ (that promotes IFN-γ expression), elevated levels of mTOR and CD25 ¹⁷, the high affinity IL-2Rα chain, thereby potentiating IL-2 signals which further support effector differentiation ¹⁸.

The CD8⁺ T cell effector mechanisms include: a) contact-mediated cytotoxicity which proceeds through the release of preformed cytolytic molecules into the synaptic cleft between the CD8⁺ T cell and its target cell; b) triggering of the TNFR family member CD95 (Fas) and c) secretion of effector cytokines which contribute to a broad range of immunological effects and contribute to local inflammatory responses ¹⁹.

CD8⁺ T cells are able to induce cytolysis of infected or abnormal cells by two distinct molecular pathways ²⁰: the granule exocytosis pathway, dependent on the pore-forming molecules, or the upregulation of FasL (CD95L), which can initiate programmed cell death by binding to Fas receptors (CD95) on target cells. Both
pathways, activated in response to signals from the TCR, stimulate the caspase cascade in the target cell, leading to apoptotic death. Efficient lysis by the granule exocytosis pathway requires the coordinated delivery of perforin and granule enzymes, such as granzymes A and B, into the target cell. CD8+ T cells also release the cytokines interferon-gamma (IFN-γ), tumor necrosis factor-a (TNF-α) and lymphotoxin-α (LT-α), as well as chemokines that function to recruit and/or activate the microbicidal activities of effector cells such as macrophages and neutrophils which contribute to host defense. Of note, CD8+ T cells rapidly produce IFN-γ and TNF-α when their TCR is engaged by the pMHC complex of the target cell but will immediately cease IFN-γ production when antigenic contact is broken, presumably until they encounter the next target cell. TNF-α production is even more strictly regulated and stops after a short period even when antigen contact is sustained. Effector cytokines produced by antigen-specific CD8+ T cells are likely to be strictly regulated to minimize the damage to the host. Cytokines may also directly interfere with pathogen attachment or pathogen gene expression, or they may restrict intracellular replication.

In contrast with the on/off cycling of cytokines, expression of the pore-forming cytotoxic protein perforin is constitutively maintained. Another important capacity that effector and subsets of memory CD8+ T cells acquire is the ability to migrate to virtually any extra-lymphoid tissues after both localized and systemic infections.

1.2.1.1 The function of IFN-γ

Activated CD8+ T cells are crucial providers of the pro-inflammatory cytokine IFN-γ. IFN-γ was discovered around five decades ago and is critical for the regulation of the host immune response against viral and intracellular bacterial pathogens. Based on the type of receptor through which they signal, interferons have been classified into three major types, and IFN-γ is the sole type II IFN. It is structurally unrelated to type I IFNs, binds to a different receptor, is encoded by a separate chromosomal locus and it is produced by T cells, Natural killer (NK) cells, macrophages and macrophage-derived dendritic cells (mDCs).

The fundamental role of IFN-γ is clearly demonstrated by the study of IFN-γ receptor 1- (IFNGR1) deficient mice, which failed to appropriately clear mycobacterial and other bacterial, parasitic, and viral infections. Furthermore, IFN-γ is implicated in tumour surveillance and is an important anti-tumoral mediator.
Already in 1986, early clinical trials on IFN-γ began to evaluate the therapeutic potential of its anti-infectious and anti-tumoral functions and until today it has been used in a wide variety of clinical indications (reviewed in 38).

This notwithstanding, the excessive release of IFN-γ has been associated with the pathogenesis of chronic inflammatory and autoimmune sclerosis, and plays a pivotal role in the development and severity of autoimmune diseases such as hashimoto thyroiditis, type I diabetes, lupus, arthritis and colitis 39–42. The mechanism whereby IFN-γ leads to systemic autoimmunity remains unclear; however, the importance of IFN-γ to T cell differentiation and immunoglobulin class switching in B cells underlines a substantial contribution to adaptive immune responses in autoimmunity.

1.2.2 CD8+ T cell Response to Virus infection

Cytotoxic T cells (CTL) are the main effector T cells that act against cells infected with viruses. Antigens derived from the virus multiplying inside the infected cell are displayed on the cells surface, where they are recognized by the antigen receptors of cytotoxic T cells. Hence, MHC class I surface expression is essential for antiviral immunity. During virus infection, viral gene products expressed in the cytosol may be targeted for degradation and presented by class I molecules 43. In this manner, CTL can act early to eliminate the infected cell before viral replication is complete and new viruses are released 44.

After antigenic stimulation, CTL up-regulate the expression of cytotoxic granule
proteins, such as granzymes and perforin, and become cytolytic, and gain the ability to enter non-lymphoid tissues. They also acquire antiviral effector functions, including the ability to rapidly produce cytokines, such as IFN-γ, which inhibits viral replication and is an important inducer of MHC class I molecule expression, macrophage activation, and drives TNF-α expression. Also CD8+ T cell Fas-dependent-mediated cytolysis, is critical for resistance against some non-lytic, such as lymphocytic choriomeningitis virus (LCMV) and at least some lytic viruses such as vesicular stomatitis virus (VSV), influenza virus, Herpes Simplex Virus Type 1 (HSV-1). In addition to changes in the expression of these effector molecules, the overall pattern of gene expression is dramatically altered during this activation phase, and a complex pattern of genetic regulation accompanies T-cell activation and expansion.

Cytotoxic T cells kill infected targets with great precision, sparing adjacent normal cells. This precision is crucial in minimizing tissue damage while allowing the eradication of infected cells.

1.2.3 CD8+ T cell Response to Intracellular Bacteria

Whereas CD8+ T cells are principally associated with defence against viral infections, they also combat intracellular bacterial infections. All intracellular bacteria enter eukaryotic cells in a membrane-bound structure. Organisms such as Mycobacteria, Salmonella, and Chlamydia survive within a membrane-bound structure, whereas Listeria and Shigella escape from the vesicle into the cytosol of the infected cell. As with most pathogens, the immune response to bacterial infection is complex, and CD8+ T cells are frequently but not always major effectors in this process.

While bacterial entry into the cytosol provides direct access to the MHC class I antigen-processing pathway, allowing direct priming of CD8+ T cells, vacuolar pathogens such as Mycobacteria and Salmonella, although not directly, also induce CD8+ T cell protective responses.

CD8+ T cells contribute to resistance against intracellular infections with bacterial pathogens through perforin dependent cytolysis in the case of L. monocytogenes infection, and its action appears to be most potent in the spleen and dispensable in the liver. In contrast, no evidence for perforin dependent immunity against Chlamydia or M. tuberculosis has been reported. In fact, CD8+ T cell-derived production of IFN-γ is an important mediator of resistance to Chlamydia infection, and this issue remains to be addressed in MTB infection.
Although limited, there is evidence suggesting the possibility that the subcellular location of the bacterial pathogen may impact on the relevance of specific CD8+ T cell effector mechanisms.

1.2.4 CD8+ T cell Response against Tumours

The immune system has three primary roles in the prevention of tumors. First, it protects the host from virus-induced tumors through elimination or suppression of viral infections. Second, the timely elimination of pathogens and rapid resolution of inflammation prevents the establishment of an inflammatory environment conducive to tumorigenesis. Third, the immune system can specifically identify and eliminate tumor cells in certain tissues on the basis of their expression of tumor-specific antigens (TSAs). This third process, referred to as cancer immunosurveillance, occurs when immune cells like CD8+ CTLs identify (upon recognition of pMHC class I complexes) transformed cells that have escaped cell-intrinsic tumor-suppressor mechanisms. These transformed cells are directly lysed by CTLs before they can establish malignancy. Much attention has been given to the role of CD8+ CTLs because most tumors are MHC class I positive, but negative for MHC class II.

1.2.5 CD8+ T cells in autoimmunity

Although the principal purpose of CD8+ T cells is to protect the host from “non-self” (i.e. pathogens) and “altered self” (i.e. tumours), there has been an growing evidence implicating CD8+ T cells in the pathogenesis of several autoimmune disorders, such as type 1 diabetes, systemic lupus erythematosus, multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and Psoriasis vulgaris.

Much of what is currently known has been provided by employing animal models of human type 1 diabetes (T1D), such as non-obese diabetic (NOD) mice. In these mice T1D results from selective destruction of the insulin-producing pancreatic β cells by autoreactive CD4+ and CD8+ T cells. This happens due to the cross-presentation of autoantigens by dendritic cells to naïve autoreactive CD8+ T cells in the pancreatic lymph nodes. The quality and quantity of this cross-presentation event is determinant for whether the cognate CD8+ T cells undergo productive (and potentially pathogenic) or non-productive activation (leading to antigenic unresponsiveness or cell death).

Other factors that also influence the outcome of cross-presentation includes the activation state of the DCs, the cytokines present during the cross-presentation, the
total number of autoantigenic peptide–MHC complexes that are presented on the DC surface, and the affinity of the TCR for peptide–MHC complexes. In transgenic models of spontaneous and virus-induced diabetes, for example, the incidence of diabetes clearly correlates with the avidity of the T cell–DC interaction. CD8+ T cell-mediated killing of target cells might also foster autoimmune disease progression, since it might facilitate the access of autoantigens to the cross-presentation pathway.

Upon activation, CD8+ T cells secrete TNF-α and IFN-γ, among other cytokines, and these cytokines have a role in autoimmune disease. TNF-α and IFN-γ can contribute to disease progression by ligating TNF receptor 1 on DCs and promoting the presentation of autoantigens. TNF-α also plays important roles in EAE/MS, inflammatory bowel disease (IBD), experimental myasthenia gravis and rheumatoid arthritis (RA). CTL-mediated lysis of chondrocytes has been reported to require the upregulation of MHC class I molecules by IFN-γ.

1.3 CD8+ T cell development and differentiation

1.3.1 Thymus

T cell development is unique relative to other hematopoietic lineages, as T cells complete the majority of their development in the thymus instead of the bone marrow (BM). The differentiation in the thymus is a complex and tightly controlled process that begins with the immigration of bone marrow-derived progenitor cells, and ends with the generation of self-tolerant, lineage committed T cells capable of performing an array of immune functions upon recognition of their antigen.

Progenitor T cells begin to migrate to the thymus from the early sites of hematopoiesis at about day 11 of gestation in mice and ninth week of gestation in humans. Upon thymic settling, progenitors undergo a series of differentiation events accompanied by migration through the thymic microenvironment where they receive various inductive signals. Progenitors settle the thymus with the potential to generate multiple blood lineages. However, as they differentiate and mature in the thymus, their potential for alternative lineages is constrained, and then irreversibly lost as the cells ultimately become restricted to the T lineage.

The thymus can be divided anatomically into an outer cortex, where most of the differentiation takes place, and an inner medulla, where the newly formed cells undergo final maturation before exiting and seeding peripheral lymphoid organs.
Early intrathymic progenitor cells lack CD4 and CD8 expression and are referred to as double negative (DN) cells \(^77\). The precursors that first enter the thymus do not express the antigen recognition machinery, lacking both the co-receptors CD4 and CD8 that direct MHC recognition by T cells and the T cell receptors for antigen recognition, TCR\(\alpha\beta\) or TCR\(\gamma\delta\) \(^72\). DN progenitors enter the thymus at the cortico-medullary junction (CMJ) and subsequently migrate to the subcapsular zone (SCZ) \(^78\)–\(^80\). This migration is accompanied by a progressive differentiation of these progenitors indicating that differentiation-inducing signals locate to distinct cortical regions \(^81\).

Based on the expression of the surface molecules CD25 and CD44 on lineage-negative cells, four early differentiation stages have been defined, double negative 1 to 4 (DN1-4), \(^82\). Differentiation to the DN1 stage (CD25\(^-\) CD44 high) proceeds in proximity to the site of thymic entry \(^83\), whereas the consecutive differentiation of
stages DN2 (CD25⁺ CD44 high) and DN3 (CD25⁺ CD44 low) occur while cells migrate outwards of this region into the mid and outer cortex, respectively. DN3 cells accumulate in the SCZ where they differentiate to DN4 (CD25⁻ CD44⁻), the pre–double positive (DP; CD4⁺ CD8⁺) stage of development. Transition from the DN3 to the DN4 stage is accompanied by a reversion of the migration polarity, which finally guides the DP thymocytes across the cortex toward the medulla, although only positive selected cells will actually enter the medulla, where the functional maturation is completed ⁸¹(Fig. 2).

The genes encoding the highly diverse TCRs undergo a carefully programmed series of DNA rearrangements triggered within the thymus in a stepwise fashion, beginning in DN stage thymocytes ⁷². For conventional TCRαβ T cells that recognize peptide antigens presented by classical MHC molecules, commitment to the T cell lineage is sealed by rearrangement of the TCRβ gene. The process of β selection tests the accuracy of this rearrangement event, and drives the proliferation and CD4 and CD8 co-receptor expression by those cells expressing a functional TCRβ chain defined as one that pairs with the product of the unrearranged pre-Tα gene ⁸⁴. The result is a large population of CD4⁺CD8⁺ double positive (DP) thymocytes that initiate TCRα rearrangement. Accurate TCRα rearrangement, along with successful TCRαβ chain pairing and surface expression is required for positive selection, the second critical checkpoint for maturing T cells. Positive selection is driven by the successful, low affinity interaction between the expressed TCRαβ receptor on a DP thymocyte and self-peptide in the context of self-MHC ⁸⁴,⁸⁵. Positive selection rescues DP thymocytes from the alternative destiny of programmed cell “death by neglect”, and drives the accurate alignment between co-receptor expression and lineage commitment ⁸⁴–⁸⁶. This process results in a population of CD4⁺CD8⁻ single positive (SP) thymocytes that can differentiate into helper T cells upon further recognition of peptide presented by MHC class II molecules, and CD4⁻CD8⁺ SP thymocytes that can differentiate into cytotoxic T cells upon encounter with antigen presenting cells whose MHC class I molecules carry the appropriate peptides. At the DP or SP stages, thymocytes are subjected to negative selection, the third checkpoint that regulates T cell development. During this process, central to the establishment of self-tolerance among developing T cells, TCRαβ⁺ thymocytes that react with high avidity to self-peptide/MHC complexes are deleted ⁸⁷–⁸⁹. The remaining 1% of thymocytes that successfully transit β selection, positive selection, and negative selection undergo additional maturation that promotes their regulated exit from the thymus ⁷². The naïve T cell population that exits the thymus after this selection expresses a broad array of unique TCRs that are able to detect a wide range of
foreign antigens. In steady state, the survival of naïve peripheral CD8+ T-cell pools depends on interleukin-7 (IL-7) and interaction with MHC class I molecules.

1.3.2 Periphery

Upon exiting the thymus, mature naïve CD8+ T cells circulate in the blood and lymphoid organs and signals from the interleukin-7 (IL-7) and IL-15 receptors promote their survival. These cells lack most of the effector functions characteristic of activated cytotoxic T lymphocytes (CTLs).

Once in the periphery, naïve T cells constantly survey and sample antigen presenting cells (APCs) in secondary lymphoid tissues in search of cognate pMHC molecules. The professional APCs, in particular dendritic cells (DC), collect antigen in the periphery and undergo a maturation process, then travel to secondary lymphoid organs, including the spleen, lymph nodes (LN), Peyer’s patches (PP), tonsils and appendix. Naïve T cells, which have yet to encounter their cognate antigen, are programmed to recirculate continuously between the blood and these organs. If naïve T cells encounter a cognate antigen presented by MHC molecules the response is initiated in the immunologic synapse (IS). The outcome is antigenic stimulation upon engagement of the TCR and CD8, as a co-receptor, that binds to cognate pMHC complexes presented by APCs. TCR-mediated signalling induces phosphorylation of several residues in the CD3 coreceptor chain and activation of ζ-associated protein of 70 kDa (ZAP-70) and the src-family kinases Lck and Fyn, thereby initiating downstream signalling pathways that lead to proliferation and differentiation.

Costimulatory signals augment TCR signals and prevent induction of anergy or apoptosis by TCR signalling alone. The main costimulatory receptor for T cells is the immunoglobulin (Ig) superfamily member CD28, which is constitutively expressed on all naive T cells. If the appropriate signals are present, naïve T cells are programmed to undergo clonal expansion, develop effector functions, and establish a long-lived memory population following clearance of antigen. There is now considerable evidence demonstrating that naïve cells can be stimulated by antigen, referred to as signal 1, and CD28-dependent costimulation, signal 2, to undergo several rounds of cell division. However, programming for survival, effector function, and memory requires a third signal that can be provided by either interleukin 12 (IL-12) or type I interferons (IFNs). IL-12 helps to develop a strong clonal expansion and cytolytic activity by the naïve cells. In the absence of this signal, in the case of steady-state presentation of antigen by immature DCs, the antigen-stimulated cells fail to develop effector functions, and those that survive long term are tolerant by default. Other cytokines (such as TNF
and IL-4) are not secreted in a directional preference and thus can potentially also act on bystander cells. 80.

For both CD4\(^+\) and CD8\(^+\) T cells, transient exposure to antigen is sufficient to induce an antigen-dependent program of proliferation and differentiation (2–5), although the kinetics and efficiency of CD8\(^+\)T cell proliferation differ substantially from those of CD4\(^+\)T cell proliferation. The time of antigen exposure required to launch the proliferative program for naive CD8\(^+\) T cells seems to be less than that required for naive CD4\(^+\) T cells (3,4,8,9). CD8\(^+\) T cells also divide sooner and have a faster rate of cell division than do CD4\(^+\) T cells. 98–101.

Antigen-driven activation of naïve CD8 T cells is a crucial first step in the differentiation process which generates heterogeneous subsets of cells, that vary in their phenotypic attributes, functional capacity, anatomical location, and ability to persist over time (Fig. 3). Following exposure to antigens in an appropriate inflammatory environment, these cells undergo a period of massive expansion, dividing as many as 15–20 times and increasing up to 50,000-fold in number. 52,102,103.

After receiving all of the signals necessary to program a response, the resulting CTL population is limited in its capacity to continue to expand, due to the development of an anergic state. This anergy can be rapidly reversed by IL-2, and possibly by other proliferative signals, to allow continued expansion of the CTL population. 96. IL-2 was initially characterized as a potent T-cell growth factor in vitro, but the function during primary expansion of CD8\(^+\) T cells in vivo is dispensable in lymphoid organs and to some extent required in non-lymphoid tissues. 104–106. IL-2 signals during priming, nonetheless, do contribute to secondary clonal expansion of memory CD8\(^+\) T cells, but it is thus far unknown whether CD4 or DC-derived IL-2 are essential for this phenomenon or if autocrine IL-2 production is sufficient. 105,106. When a CD8\(^+\) T cell response has occurred and antigen is cleared from the system, either because the initial CTL expansion was sufficient or because IL-2-dependent help was available to maintain and expand the CTL population until clearance was achieved, the effector CTLs decline in number as the cells undergo apoptosis, leaving behind a long-lived population of memory cells. 96.
The Role Of miRNAs In CD8⁺ T Cells Differentiation

Fig. 3 - Antigen-driven activation of naïve CD8 T cells. After the encounter with an antigen presenting cell, CD8⁺ T cells go into the differentiation process which generates heterogeneous subsets of cells, that vary in their phenotypic attributes, functional capacity, anatomical location, and ability to persist over time 13.

1.3.3 Memory CD8⁺ T cells

The cytokines required for programming and maintaining a CD8⁺ T cell response that leads to memory are provided, either directly or indirectly, by CD4⁺ T cells, or by alternative ways. The CD4⁺ T cell-mediated help during the primary response or thereafter and during recall is essential for the generation and maintenance of functional memory CD8⁺ T cells to both non-inflammatory and inflammatory agents 107–110.

The maintenance of the memory population is a dynamic process that requires slow proliferation of the cells in response to endogenous IL-15 and/or IL-7. In terms of the cell-intrinsic factors required for CD8⁺ memory T cell generation, it is reported that T-bet deficiency, particularly coupled with Eomesodermin deficiency impairs memory T cells development 96.

Memory CD8⁺ T cells have higher frequencies than naïve T cells and can be maintained for long periods of time without antigenic stimulation. This increment in size, the ability to rapidly reactivate and kill upon antigenic stimulation and the varied tissue distribution makes the memory CD8⁺ T-cell compartment able to protect its host in a better and faster way to recurrent infections when compared to naïve T-cell pools. Depending on the subtype of memory T cell, most but not all cell-surface markers are gradually reversing to baseline during the ensuing development of effector cells into memory T cells. Commonly, memory T cells are subdivided into
two main subsets: first, effector memory cells (TEM) are found in non-lymphoid tissues, and are CD62Llo, CCR7−; and second central memory cells (TCM) that reside in lymphoid organs, and are CD62Lhi, CCR7+. Additionally, other memory subsets defined by markers like CD27, CD28, CD43 exist as well as memory CD8+ T cells with mixed phenotypes, such as CD62Llo, CCR7+. TEM are preferentially localized in non-lymphoid tissues and mucosal sites and have more rapid cytotoxic potential, whereas TCM are mainly present in secondary lymphoid organs and possess superior expansion potential. Thus, protection is essentially linked to the anatomical location of memory T cell subsets and to the route of infection.

Memory CD8+ T cells in tissues such as the liver and lung are not a sessile, tissue-resident population, and studies have shown that memory cells continuously enter non-lymphoid organs from the bloodstream. In contrast, memory CD8+ T cells present in the brain and lamina propria equilibrated very slowly with blood-borne memory cells.

Interestingly, recent studies correlate the protection of vaccines with multifunctional T cells (i.e. simultaneous production of the cytokines IFN-γ, TNF, and IL-2), implying that not only location and quantity of memory CD8+ T cells but also ‘fitness’ should be considered important for protection. The three phases towards memory cell formation (expansion, contraction, and memory development) are found in response to many different types of acute infection and for different epitopes within the same pathogen, indicating a common pathway for memory T cell formation. At the moment, it is still controversial how naïve CD8+ T cells differentiate into effector and central memory T cells and several models have been suggested that regulate this differentiation process. Contrary to the dominant linear model of differentiation, a very recent study based on single-cell PCR suggested pre-commitment of CD8+ T cells to either the effector or memory lineages as early as in the first division after activation.

1.4 microRNAs as Gene Regulators

MicroRNAs (miRNAs) are critical post-transcriptional regulators of gene expression. miRNAs are an abundant class of evolutionarily conserved small non-coding RNAs of approximately ~21-25 nucleotides in length that can regulate gene expression by binding to the 3’UTR of specific mRNAs leading to the degradation or translational block of one or more target mRNAs.

In mammals, miRNAs are predicted to control the activity of ~50% of all protein-coding genes setting this post-transcriptional control pathway within nearly every major gene cascade. Functional studies indicate that miRNAs participate in the
regulation of diverse aspects of biology, including developmental timing, differentiation, proliferation, cell death and metabolism\textsuperscript{120–122}. Changes in their expression levels are associated with several human pathologies, including cancer, heart ailments and neurological dysfunctions\textsuperscript{123}. Since their discovery in \textit{Caenorhabditis elegans} in 1993, thousands of miRNAs have been identified in plants, animals, and viruses by molecular cloning and bioinformatics approaches\textsuperscript{121}. Furthermore miRNAs and their accessory proteins have been shown to be conserved throughout phylogeny\textsuperscript{124}. Interestingly miRNAs are now being used as both targets and therapeutics for a growing industry hoping to tackle the power of RNA-guided gene regulation to combat disease and infection\textsuperscript{119}, highlighting the importance of microRNAs in the gene regulation and therapeutic field.

1.5 MicroRNA Biogenesis

1.5.1 miRNA Transcription

miRNAs are encoded in regions of the genome including both protein coding and non-coding transcription units. It is estimate that 50\% of miRNAs are derived from non-coding RNA transcripts, while approximately \~40\% are located within the introns of protein coding genes\textsuperscript{125,126}. miRNAs are transcribed by RNA polymerase II and bear a 7-methyl guanylate cap at the 5\' end and a poly (A) tail at the 3\' end, similar to mRNAs\textsuperscript{124,127,128}. The nascent transcripts are referred to as primary (pri-) miRNAs. The pri-miRNAs can be long, typically over 1kb and contain one or more secondary structures primarily consisting of extended stem-loop structures\textsuperscript{124,129}. The pri-miRNA is then processed within the nucleus by a multiprotein complex called the microprocessor, of which the core components are the RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha\textsuperscript{121,130–132}. A special subset of miRNA, mirtrons, bypass the Drosha cleavage step\textsuperscript{122}, as the spliced intron itself corresponds exactly to a single, processed miRNA precursor\textsuperscript{133}. After being processed by Drosha or the being excise as a mirtron, the resulting \~70 nucleotide long RNAs, now precursor (pre-)miRNAs, folds into mini-helical structures, allowing for recognition by Exportin 5 (Exp5), the nuclear export factor responsible for trafficking pre-miRNAs from the nucleus to the cytoplasm\textsuperscript{124,134,135} (Fig. 4).
1.5.2 miRNA Maturation

After the translocation to the cytoplasm, the pre-miRNA is cleaved near the terminal loop by the RNaseIII enzyme Dicer and generates a ~22-nt double-stranded miRNA (Fig. 4).

Dicer is highly conserved throughout evolution and it is present in nearly all-eukaryotic organisms; \(^{122}\). The cleavage by Dicer takes place in a complex, that includes the human immunodeficiency virus trans-activating response RNA-binding protein (TRBP or TARBP2, known as loquacious in Drosophila), which contains three dsRNA-binding domains and stabilizes the interaction of Dicer with the pre-miRNA \(^{138-140}\). The resulting 19-24mers double-stranded RNA duplexes contain the mature miRNAs, also known as guide strand, and its antisense strand, also known as the passenger strand or miRNA* strand \(^{141}\).

The antisense miRNA strand can also be found in libraries of cloned miRNAs although in a much lower frequency than the guide strands \(^{142,143}\).

1.5.3 RISC Assembly

The final step in miRNA biogenesis is the subsequent incorporation of the miRNA duplex into the RNA-induced silencing complex (RISC), the effector complex whose diverse functions can include mRNA cleavage, translation suppression, transcriptional silencing and heterochromatin formation \(^{124,144}\) (Fig.4).

The primary component of the RISC complex and the effectors of miRNA-mediated repression are the Argonaute (Ago) proteins \(^{145}\). While all of the Ago proteins have the ability to interact with small RNAs, Ago2 is the only one with RNA cleavage activity and is thought to play a prominent role in miRNA-mediated silencing.

*In vivo*, Ago2 associates with Dicer and the double-stranded RNA binding proteins (TRBP) and also with protein kinase R-activating protein (PACT) to form the RISC Loading Complex (RLC). This allows the tight coupling of Dicer cleavage to the incorporation of miRNA into the RISC complex \(^{140}\). *In vitro* reconstituted RLC composed of recombinant Dicer, TRBP, and Ago2 efficiently catalyses pre-miRNA cleavage \(^{146,147}\). An important role of the RLC is the unwinding of the double-stranded miRNA, which is followed by incorporation of the guide strand into the miRNA-containing ribonucleoprotein (miRNP) complex and degradation of the passenger strand \(^{148}\).
miRNAs are processed by RNA polymerase II as precursors (pri-miRNA) from intronic, intergenic or polycistronic gene regions. In the canonical pathway the primary precursor (pri-miRNA) processing occurs in a two steps cleavage by Drosha together with DGCR8 into 70-nucleotide stem loop known as pre-miRNA, and then by Dicer. In contrast in the non-canonical miRNA pathway mirtrons are processed by the spliceosome. Exportin 5 transports the pre-miRNAs to the cytosol, where they are further processed by Dicer together with TRBP to mature miRNA. The mature miRNA, indicated in red, is incorporated into the RNA-induced silencing complex (RISC) whose core components are the Argonaute family proteins (Ago1-4). The RISC complex either mediates mRNA degradation or translational repression (from 154).

Usually, the strand with the 5’ terminus located at the thermodynamically less-stable end of the duplex is the one selected to function as a mature miRNA, and the other strand is degraded 149–152.

The assembly of RNA into the RISC complex is driven by thermodynamic properties, though may be also subject to additional regulation as the ratio of miRNA:miRNA* can vary dramatically, depending on the specific properties of the miRNA duplex, on the tissue itself and on the developmental stages 142,153. These findings suggest that
differential strand selection could represent a yet unappreciated mechanism of miRNA regulation. After the assembly of the miRNAs into the miRNPs or miRNA-induced silencing complexes (miRISCs) the effector complex is ready for targeting the mRNAs.

1.6 MicroRNA Mechanisms of Action

1.6.1 Target regulation by miRNAs

miRNAs interact with their mRNA targets via sequence-specific base-pairing. With few exceptions, miRNAs base pair with their targets imperfectly, following a combination of rules, that have been formulated, based on experimental and bioinformatics analyses. It is known that an individual miRNA is able to control the expression of more than one target mRNA and that each mRNA may be regulated by multiple miRNAs. The 5' region, termed the "seed" sequence, of the miRNA is used to recognize complementary regions mainly in the 3' UTRs of mRNAs leading to deadenylation or inhibition of translation, ultimately resulting in mRNA decapping and decay. Recent studies based on ribosome profiling have shown that, although there are some contribution of translational inhibition, for most of the proteins (>84 %) regulated by miRNAs, the inhibition was accounted by destabilization of the target mRNA. Perfect pairing of a miRNA with its target sites supports direct endonucleolitic cleavage of the mRNA by Argonaute, both in plants and animals. This is a common mechanism in plants but is very rare in animals.

1.6.2 miRNA-mediated regulation of T cell Differentiation

The initial studies that established the role of miRNAs in T cells were based on the generation of mice in which global miRNA maturation was blocked. Several T cell-specific miRNA-deficient mice were generated through targeting of essential components of the miRNA biogenesis, namely Dicer, Drosha or Dgcr8. Interestingly, early elimination of Dicer in the T cell lineage, mediated by IckCre expression, caused a dramatic (~10-fold) reduction of total thymocyte numbers, most probably due to increased cell death. The relative numbers at the various developmental stages remained intact and only a 4-fold reduction of peripheral CD8+ T cells was detected. In general it was shown, by using a conditional gene ablation approach, that Dicer-sufficient cells outgrow Dicer-deficient cells, implicating miRNA in the
general T cell fitness. Furthermore, Dicer and Dgcr8-deficient T cells have decreased T cell proliferation capacity after activation. Importantly, a striking effect on T cell differentiation was observed in all T cell-specific miRNA (entire miRnome) knockout lines. This data clearly demonstrated that miRNAs are involved in the establishment and/or maintenance of specific T cell identities. Dicer-deficient CD4+ T cells were strongly biased towards IFN-γ-producing (Th1) cells, suggesting a specific role of miRNAs to repress the Th1 program. Other T cell subsets were also affected, as miRNAs are essential for the homeostasis and suppressive function of FoxP3+ regulatory T cells. Dicer and Drosha-deficient mice displayed a scurfy-like disease and their fatal autoimmunity could not be distinguished from FoxP3-deficient mice. Consistent with this, Dicer-deficient Tregs lose the expression of FoxP3. In another mouse model using depletion of AGO2, which is the key AGO protein in haematopoietic cells and crucial for the maintenance of physiological miRNA levels, an increased proportion of IFN-γ and IL-4 double producing CD4+ T cells were detected.

In summary, even if miRNA-deficient T cells are still able to function, the approaches using T cell-specific miRNA-deficient mice, clearly demonstrated that miRNAs are implicated in various aspects of T cell biology, namely proliferation, survival and differentiation. However, the interpretation of the resulting phenotypes is complex, as the question remains which miRNAs are important for the detected dysfunctions. Deletion of two counteracting miRNAs can mask their role and prevent them from establishing a phenotype. Therefore the “second” generation of miRNA research is focusing on the role of individual miRNAs. The starting point is usually the profiling of miRNAs in multiple T cell types. Unlike miR-122 or miR-1 which are exclusively expressed in the liver and the heart, respectively, no individual miRNAs were found, that are uniquely expressed in lymphocytes. miR-181a was identified as an important regulator of positive selection, which may constitute up to half of the total microRNA content of DP cells. It is specifically enriched at the CD4+ CD8+ DP stage of thymocyte development and suppresses the expression of Bcl-2, CD69, and T cell receptor (TCR), all of which are important in positive selection. miR-181a has also been shown to increase sensitivity to peptide antigens by down-regulating multiple phosphatases. These findings have indicated that miR-181a functions as an intrinsic “rheostat” in TCR signalling, which is very important in T cell development. In addition to miR-181a, miR-150 is important in T cell development as its up-regulation inhibits the expression of the target gene NOTCH 3.
Interestingly, the expression of miRNAs changes during activation and differentiation of individual T cell subsets. Most miRNAs expressed in resting T cells are down-regulated after T cell activation, with a few exceptions, such as miR-155 and miR-17-92, that are selectively up-regulated, suggesting a role for these miRNAs in the transition of naïve T cells to specialized effector cells.

1.6.3 miRNA-mediated regulation of T cell effector function

Until now several miRNAs have been identified for regulating the differentiation of the T cells effector function. These are summarized in Table 1.

miRNAs expression rapidly change after activation in CD8+ T cells as well in CD4+ T cells, and miR-155 is an example of those miRNAs. It is induced in CD8+ T cells during activation and rapidly declines to regulate CD8+ memory T cell differentiation. It is also known that miR-155 is required for normal CD8+ T cell responses to lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* infections.

The miR-17-92 cluster, is also induced in viral infections and promotes proliferation. The down-regulation of this cluster after the initial expansion phases is needed for the normal memory CD8+ T cell formation. Therefore miR-17-92 cluster appears to be also involved in T cell differentiation.

Another important role of this cluster, is that in its absence the production of many cytokines including IFN-γ, IL-2, IL-4, IL-5 and TNF-α, was impaired, suggesting that multiple Th subsets and possibly CD8+ T cells were affected.

A microRNA with an important role in cytokine production, especially in the production of IFN-γ is the miR-29. miR-29 suppresses IFN-γ production by indirectly targeting two mRNAs coding for transcription factors that promote Th1 differentiation, Tbx21 (T-bet), and Eomes or by directly targeting IFN-γ. Interestingly mice infected with *Listeria monocytogenes* or *Mycobacterium bovis bacillus Calmette-Guérin* (BCG) exhibit a down-regulated miR-29 expression in IFN-γ-producing natural killer cells, CD4+ T cells, and CD8+ T cells.

Also miR-146a, one of the best studied miRNAs in immune cells, mediates immune suppression in all cell types analyzed so far, namely by inhibiting the production of IFN-γ and IL-17 in CD4+ and CD8+ T cells.

Additionally, numerous miRNAs, including miR-150, miR-155, and the let-7 family were shown to be associated with the development of effector and central memory CD8+ T cells using an *in vitro* system where CD8+ T cells activity was driven by IL-2 or...
IL-15 cytokines. In particular, miR-150 regulates the protein expression of Kv channel interacting protein 1 (KChIP1) in mouse central memory T cells \(^{196}\).

In sum, until today several miRNAs were identified as critical regulators of T cell differentiation. In this thesis we will address the role of miRNAs in the differentiation of IFN-\(\gamma\)-producing CD8 T cells (see section: 2), as the role of miRNAs in this differentiation process is poorly understood.

Table 1 - Function of miRNAs in T cells. Adapted from Kroesen et al. 2014 \(^{197}\).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Subset</th>
<th>Target(s)</th>
<th>Effect</th>
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<tbody>
<tr>
<td>miR-10a</td>
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<td>BCL-6</td>
<td>Differentiation / maintenance</td>
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<td>PTEN</td>
<td>Proliferation</td>
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<td>Cytokine signaling</td>
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<tr>
<td>miR-210</td>
<td>Th17</td>
<td>HIF-1(\alpha)</td>
<td>Differentiation</td>
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<td>miR-301</td>
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<td>miR-326</td>
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Aims of The Thesis
2 Aims of the thesis

The published observations that CD8⁺ T cell survival, activation and migration are compromised in the genetic absence of Dicer, clearly implicate miRNAs in CD8⁺ T cell physiology. This notwithstanding, it remains unknown which specific miRNAs control the development and activation of CD8⁺ T cells, and how they may regulate the production of IFN-γ and the cytotoxic function of CD8⁺ T cells. Such understanding may pave the way to novel clinical interventions in settings of infection and cancer where CD8⁺ T cells are pivotal effectors in immune responses.

In this thesis we set out to investigate the role of miRNAs in the post-transcriptional regulation of IFN-γ producing CD8⁺ T cell differentiation. Preliminary data from the host lab detected increased IFN-γ production in CD8⁺ T cells from miRNA-deficient mice, both in the thymus and in the periphery compared to control mice. Building on these interesting findings we proposed to identify, in this study, specific miRNAs that might control IFN-γ expression in CD8⁺ T cells. We used Ifng-YFP reporter mice to isolate YFP⁺ and YFP⁻ thymic CD8⁺ T cell populations for miRnome profiling. Additionally we proposed to analyze candidate miRNA regulation under TCR and cytokine stimulation, and finally to conduct functional over-expression experiments. The final goal would be to identify and characterize specific miRNAs implicated in IFN-γ-producing CD8⁺ T cell differentiation.
Material and Methods
3 Material and Methods

3.1 Mice

For all experiments adult mice (6 – 12 weeks old) were used. C57BL/6J mice (6 – 12 weeks old) were from Jackson Laboratories (Bar Harbor, ME). IckCre-Dicer\textsuperscript{Δ/Δ} mice were kindly provided by Dr. M. Merkenschlager (London, UK). IFN\textgamma-IRES-YFP-BGHpolyA knockin (YETI) mice were from Jackson Laboratories (Bar Harbor, ME). Both female and male mice were used, however, individual experiments were conducted with either females or males. Mice were maintained within the specific-pathogen-free animal facilities at the Instituto de Medicina Molecular (Lisbon, Portugal). All experiments involving animals were done in compliance with the relevant laws and institutional guidelines and were approved by local and European ethic committees.

3.2 Cell preparations

For all \textit{in vitro} analyses, cells were obtained from spleen, thymus and lymph nodes (axillary, brachial, inguinal, mesenteric and lumbar).

3.2.1 Spleen, lymph nodes and thymus

The lymph nodes and spleen, or thymus, were strained using BD Falcon Cell strainer 70 µM. Red blood cells (RBC) were lysed with RBC Lysis Buffer from Biolegend (#420301) and spun for 5 minutes (min) at 1500 rotations per minute (rpm). The supernatant was discarded and the pellet resuspended in approximately 3 ml of complete RPMI medium (RPMI media 1640, containing 1mM sodium pyruvate #11360-039, 1x non-essential acids amines #11140-050, 10 mM hepes #15630-056, penicillin-streptomycin #15140-122, 50µg/ml gentamycin, 50 µM βmercaptoethanol and 10% fetal calf serum (FCS) al from Gibco\textregistered).

3.2.2 Cell Sorting by Flow-Cytometry

Respective staining antibodies were added to the cells and incubated for at least 15 min at 4ºC in complete RPMI medium. For sorting of T cells subsets the following
antibodies were used: anti-CD3e PerCP-Cy5.5 (145-2C11 e-Bioscience), anti-CD8 APC-eFluor 780 (53-6.7 e-Bioscience), anti-TCRγδ PE (GL3 e-Bioscience), anti-CD27 PE-Cy7 (LG.7F9 e-Bioscience), anti-CD4 eFluor-450 (RM4-5 e-Bioscience) and anti-CD25 APC (PC61 BD Pharmingen) (Fig. 5).

For sorting of CD8+ T cells the following antibodies were used: anti-CD3e PerCP-Cy5.5 (145-2C11 e-Bioscience), and anti-CD4 eFluor-450 (RM4-5 e-Bioscience) and anti-CD8 APC-eFluor 780 (53-6.7 e-Bioscience). Cells were washed by adding an excess of medium to the suspension followed by centrifugation for 5 min at 1500 rpm, after which pellets were resuspended in complete RPMI medium and transferred to 96 well U bottom plates from TPP (#92097) with 200,000 cells in 100µl per well and kept in an incubator at 37°C and 5% CO2. Cell sort was performed with either BD FACSaria I or BD FACSaria III cell sorter.

3.3 In vitro cell stimulation and polarisation

CD8+ T cells were sorted by flow cytometry and subjected to various stimulation conditions for 48h for functional studies or 12h for miRNA expression analysis. Cells were incubated with various cytokines including IL-12 (5 ng/ml PeproTech), IL-4 (10 ng/ml eBiosciences), IL-7 (10 ng/ml; eBiosciences), IL-18 (10 ng/m PeproTech), IL-2 (10 ng/m PeproTech), IL-15 (10 ng/m PeproTech) and or activated with plate-bound monoclonal antibody (mAb) anti-CD3 (5 µg/ml; 145.2C11; eBiosciences) and mAb
anti-CD28 (5 m\(\mu\)g/ml; 37.51; eBiosciences).

3.4 Restimulation and FACS staining

To measure cytokine secretion, cells were restimulated with PMA (50 ng/ml, Sigma; P-8139) and Ionomycin (1 \(\mu\)g/ml, Sigma; I-0634) for 4h at 37°C, with the addition of Brefeldin A (10 \(\mu\)g/ml, Sigma; B-7651). Cells were transferred into Nunc® 96 well V bottom plates and washed once in FACS buffer (PBS, 0.5% FCS, 2 mM EDTA) (5 min, 1500 rpm). For extracellular cell staining, cells were resuspended in 50\(\mu\)l FACS buffer with the respective antibodies and incubated for 30 min at 4°C. For intracellular cell staining the cells were resuspended in 100\(\mu\)l of fixation/permeabilization (BD Cytofix/CytoperTM Fixation/Permeabilization kit; # 554714) and incubated for 30 min at 4°C. Cells were washed twice in Perm/wash (BD Cytofix/CytoperTM Fixation/Permeabilization kit; # 554714) (5 min, 2000 rpm). Cells were resuspended in 40\(\mu\)l of Perm/wash containing Anti-mouse anti-FcR (2.4G2; BD Pharmingen) and incubated for 15 min at RT. Cells were then stained without washing with respective antibodies in an additional 10 \(\mu\)l of the same Perm/wash buffer and incubated for 30 min at RT. For cytokine expression analysis with FACS the following antibodies were used: anti-IL-17A Alexa Fluor 488 (17B7; eBiosciences), anti-IFN-\(\gamma\) APC (XMG1.2 BD Pharmingen) and anti-TNF-\(\alpha\) PE (MP6-XT22 BD Pharmingen).

Cells were washed once in Perm/wash and once in FACS buffer (5 min, 20000 rpm). Cells were finally resuspended in FACS buffer and FACS acquisition was performed on BD LSRFortessa cell analyser.

All the data were analysed using FlowJo v.9.3.3 software.

3.5 Quantitaive RT-PCR

All quantitative RT-PCRs were performed in MicroAmp® Optical 384-Well Reaction Plate (Applied Biosystems® #4343370) using Applied Biosystems ViiATM 7 Real-Time PCR system. Data was analysed using ViiATM 7 software v1.2.1.

For miRNA expression analysis: RNA was isolated from sorted cell populations by flow cytometry using miRNeasy Mini Kit (Qiagen). For cDNA synthesis and real-time PCR amplification the miRCURY LNA™ Universal RT microRNA PCR protocol (Exiqon) was performed. LNA™ PCR primer sets (Exiqon) were used and relative quantification of specific miRNAs to small
RNA reference miR-423-3p was carried out using SYBR on ABI ViiA7 cycler (Applied Biosystems).

3.6 Retroviral transduction for miRNA overexpression

The native precursor stem loop of our miRNAs candidates were cloned into the retroviral vector MSCV-IRES-GFP (pMIG) (Fig. 6) using genomic DNA as a template and the following primers:

miR-451a-F – CGTTTCTGCCTGTAACTCTGG
miR-451a-R – CTCACAAAGGTCTCCCATC
miR-132-F – GCCGCCTTCAGTAACAGTCT
miR-132-R – AGGACTCCTGATCCCCATG
miR-200a-F – CCTAGTGCGCTACTCAAGC
miR-200a-R – GCATCCTCACTAACCCTCACA
miR-322-F – CCGGGGAAATAATGAGAC
miR-322-R – TGCCACTTGTATTCACAC
miR-181a-F – CCCAGCATGTGTTATGGTCTT
miR-181a-R – CCGCAGTCATAACAGTCCT
miR-139-F – AGAGGACTAACAACCCCTGC
miR-139-R – GGAGAGGAGGCATAAGGGT

Viral supernatant was produced by transfecting the plasmids pMIG, pCMV-VSV-G and pCL-Eco with Opti-MEM® (Life Technologies) and X-tremeGENE DNA Transfection Reagent (Roche) in 293T/17 [HEK 293T/17] (ATCC® CRL-11268™) packaging cell line cultured in TPP 100mm cell culture dishes. The efficiency of the viral particles was tested by transducing 200.000 NIH/3T3 (ATCC® CRL-1658™) cells per well, in a Nunc™ 6 well plate, with 8µg/ml of polybrene during a 60 min centrifugation at 37°C, 2200 rpm. After 24h the media was changed for fresh media. And after 120h the cells were washed with FACS buffer (1500 rpm 5 min), and resuspended in FACS buffer for FACS acquisition.

For viral transduction of peripheral and thymic CD8⁺ T cells, the cells were cultured in a TPP 96 well U bottom plate, with 200.000 sorted CD8⁺ T cells per well. The cells were activated for 48h with plate-bound anti-CD3ε mAb (1µg/ml; 145.2C11; eBiosciences) and anti-CD28 mAb (1µg/ml; 37.51; eBiosciences) in presence with IL-2 (10 ng/ml PeproTech) for peripheral CD8⁺ T cells and IL-7 (10 ng/ml; eBiosciences) for thymic CD8⁺ T cells. Per well 200.000 CD8⁺ T cells were transduced with miR-
181a-pMIG, miR-200a-pMIG, miR-132-pMIG, miR-139-pMIG or empty-pMIG vector using 50µl of concentrated viral supernatant. The transduction was performed with 8µg/ml of polybrene during a 60 min centrifugation at 37ºC, 2200 rpm. After 120h GFP⁺ (transduced) CD8⁺ T cells were sorted and restimulated for intracellular cytokine staining for IFN-γ and TNF-α.

Fig. 6 - miRNA overexpression in CD8⁺ T cells. Workflow of the miRNA overexpression in CD8⁺ T cells using retroviral transduction. miRNAs were cloned into the retroviral plasmid pMIG, using the precursor stem loop and genomic DNA as template. Viral particles were produced in 293T/17 [HEK 293T/17] (ATCC® CRL-11268™) packaging cell line and their efficiency tested in the NIH/3T3 (ATCC® CRL-1658™) cell line. CD8⁺ T cells were then transduced with the viral particles for 120h and afterwards sorted for GFP⁺. Later GFP⁺ (transduced) CD8⁺ T cells were restimulated and analyzed for intracellular cytokine staining for IFN-γ and TNF-α.
3.7 Redirected cytotoxicity assay

YFP\(^+\) and YFP\(^-\)CD8\(^+\) T cells were sorted and were either cultured in complete RPMI for 72h in a TPP 96 well U bottom plate, with plate-bound anti-CD3\(\varepsilon\) mAb (1\(\mu\)g/ml; 145.2C11; eBiosciences) and anti-CD28 mAb (1\(\mu\)g/ml; 37.51; eBiosciences), or the cells were incubated directly after sorting in a 96 well U bottom plate for 4h at 37°C with P815 (ATCC® TIB-64™) mouse mastocytoma cell line, label with DDAOse (1\(\mu\)M), with soluble anti-CD3\(\varepsilon\) mAb (1\(\mu\)g/ml; 145.2C11; eBiosciences). After 4h of incubation with P815 (ATCC® TIB-64™) mouse mastocytoma cell line, cells were transferred to a 96 well V bottom plate, and washed with FACS buffer (1500 rpm 5 min).

For Annexin V/Dead Cell Apoptosis staining Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (V13241; Life Technologies) was used. Cells were resuspended in 100\(\mu\)l per well in 5x annexin-binding buffer (Component C), and stained with Alexa Fluor® 488 annexin V (Component A) for 15 min at room temperature. Cells were resuspended in 5x annexin-binding buffer (Component C) for FACS acquisition on BD LSRFortessa cell analyser.

3.8 Statistical analysis

A two-tailed non-parametric Mann-Whitney test was used for statistical analysis. \(P\) values of <0.05 were considered significant and are indicated on the figures.
Result
4 Results

4.1 Increased differentiation of IFN-γ producing CD8+ T cells in pLck-Cre DICER−/− mice

To address the overall role of miRNAs in the regulation of IFN-γ producing CD8+ cells we analyzed T cell-specific miRNA-deficient mice (kindly provided by Dr. M. Merkenschlager, Imperial College, London). Dicer, a crucial enzyme in the miRNA biogenesis pathway, is essential for the generation of mature miRNAs. As Dicer mutation in mice or mouse embryonic stem (ES) cells results in developmental failure 198,199, we used T-cell specific conditional Dicer deletion in mice using the Cre-lox system. The Cre recombinase was expressed under the lck proximal promotor, which is active from the earliest stages of T cell development 200. Therefore in lckCre-Dicer+/− mice, Dicer is deleted from the CD44+CD25+ (DN3) stage onwards, while -importantly for our work- does not prevent CD8+ T cell development in the thymus 164.

Fig. 7 - DICER deficiency in T cells results in increased frequency of INF-γ producing CD8+ T cells. Frequency of IFN-γ+ CD8+ T cells isolated from lckCre-Dicerlox/lox (wild-type control) and lckCre-Dicerlox/− mice (left). Representative intracellular staining of IFN-γ and IL-17 in (A) thymic and (B) peripheral CD8+ T cells isolated from those mice (right). Each symbol (in A, B, left panels) represents an individual mouse. * P ≤ 0.05 (Mann-Whitney two-tailed test).
Interestingly, we observed a ~8-fold higher frequency of IFN-γ producing CD8⁺ T cells in the thymus (Fig. 7A) of Dicer⁻/⁻ compared to Dicer⁺/⁺ mice and a ~3-fold higher frequency of IFN-γ producing CD8⁺ T cells in the lymph nodes. We additionally analyzed the frequency of another pro-inflammatory cytokine, IL-17A, that is expressed in activated CD8⁺ T cells after differentiation in a specific cytokine milieu including TGF-β and IL-6. We did not detect any expression of IL-17A in ex vivo isolated CD8⁺ T cells in Dicer⁻/⁻ mice in the thymus (Fig. 7A) and in the periphery (Fig. 7B). These data suggested an important role of miRNAs in selectively regulating the differentiation of IFN-γ producing CD8⁺ T cells.

4.2 YFP expression encompasses intracellular IFN-γ production in Yeti mice

On the basis of the previous data we hypothesized that mature miRNAs play a role in the differentiation of IFN-γ producing CD8⁺ T cells in the thymus and periphery. To examine the role of specific miRNAs in the differentiation of IFN-γ producing CD8⁺ T cells we relied on a bicistronic reporter mouse strain Ifng-YFP (termed “Yeti” mice), in which transcription of the Ifng gene also results in enhanced yellow fluorescent protein (eYFP) reporter expression. Therefore, immune cells producing IFN-γ mRNA will also be YFP⁺.

To ensure that the expression of YFP fluorescence associated with intracellular IFN-γ production we stimulated CD8⁺ T cells of the Ifng-YFP reporter mice with diverse cytokines cocktails capable of triggering IFN-γ production. Thymic CD8⁺ T cells were cultured for four days with plate bound anti-CD3 and anti-CD28 mAb in the presence of IL-7; IL-7 plus IL-2; IL-7 plus IL-12; IL-7 plus IL-18; and IL-7, IL-12 plus IL-18. The combination of IL-7 plus IL-12 was the most potent cytokine cocktail to induce IFN-γ production in thymic CD8⁺ T cells and resulted in ~ 40% of IFN-γ producing CD8⁺ T cells and ~ 90% YFP positive cells (Fig. 8B).

The peripheral CD8⁺ T cells were cultured for four days with plate bound anti-CD3 and anti-CD28 in the presence of IL-2; IL-2 plusIL-4, IL-2 plus IL-18; and IL2, IL-12 plus IL-18. The combination of IL-2, IL-12 plus IL-18 was the most potent cytokine cocktail to induce IFN-γ production in peripheral CD8⁺ T cells and resulted in ~ 60% of IFN-γ producing CD8⁺ T cells and ~ 90% YFP positive cells (Fig. 8B).

YFP expression encompassed intracellular IFN-γ production (Fig. 8A and B). There are at least two possible reasons with YFP expression is more frequent than the
actual cytokine: faster translation rates; or increased mRNA or protein stability. Importantly, they follow similar tendency in the different conditions tested in thymic and in peripheral CD8⁺ T cells, as increased frequency of IFN-γ⁺ CD8⁺ T cells associated with increased expression of YFP.

These data suggests that Yeti mice can be a reliable tool to study and characterize IFN-γ producing CD8⁺ T cells, without the need of intracellular staining for IFN-γ, since the expression of YFP encompasses IFN-γ production.

Fig. 8 - eYFP expression associates with intracellular expression of IFN-γ in Yeti mice, upon IFN-γ inducing conditions. Representative intracellular staining for IFN-γ and IL-17A in (A) thymic and (B) peripheral CD8⁺ T cells of Ifng-YFP mice stimulated in vitro for four days with plate bound anti-CD3 and anti-CD28 and in the presence of different cytokines (IL-7, IL-2, IL-4, IL-12 and IL-18) (left, top). Representative histograms indicating the eYFP expression in the same conditions (left, bottom). Frequency of IFN-γ⁺ and YFP⁺ (A) thymic and (B) peripheral CD8⁺ T cells (right).

4.3 YFP⁺ (IFN-γ⁺) CD8⁺ T cells display increased cytotoxicity

CD8⁺ T cells are known to mediate protection against infection through the secretion of cytokines, such as IFN-γ and tumor necrosis factor (TNF), and through CTL activity
via the release of cytotoxic granules containing granzymes, granulysins and perforin (Pfn)\(^{203}\). Interestingly, previous studies have shown a strong relationship of IFN-\(\gamma\) or perforin expression and the cytotoxic ability of virus-specific CD8\(^+\) T cells and CD8-mediated cytotoxicity against tumors\(^{204-207}\).

We confirmed the association of the cytotoxic potential and IFN-\(\gamma\) production in CD8\(^+\) T cells isolated from Ifng-YFP reporter mice. We performed a redirected lysis assay against P815 mastocytoma cell line comparing the cytotoxic responses of YFP\(^+\) versus YFP\(^-\) CD8\(^+\) T cells. Interestingly, we observed an increased killing capacity of freshly isolated YFP\(^+\)CD8\(^+\) T cells (IFN-\(\gamma\)^+ cells), when compared with the YFP\(^-\) CD8\(^+\) T (IFN-\(\gamma\)^-) cells at an 1:10 target:effector ratio (40% of lysed cells versus 20% of lysed cells) (Fig. 9 A). This enhanced cytotoxicity of YFP\(^+\)CD8\(^+\) T cells diminished in long term cultures, (60% of lysed cells versus 50% of lysed cells) (Fig. 9 B). This is most possibly due to the induction of differentiation in YFP\(^-\) CD8\(^+\) T cells. Of note, we observed the highest percentage of lysis by both YFP\(^+\) and YFP\(^-\) CD8\(^+\) T cells at day 3 (Fig. 9 B).
These data confirmed an increased CTL activity of IFN-γ producing CD8+ T cells compared to IFN-γ non-producing CD8+ T cells in our redirected lysis assay, pointing to a polyfunctionality of CD8+ T cells.

4.4 YFP+ versus YFP- CD8+ T cells from Ifng-YFP mice have different miRNA repertoires

We next aimed at identifying individual miRNAs that regulate the differentiation of IFN-γ+ CD8+ T cells and could mediate the increased frequency of IFN-γ+ CD8+ T cells in DicerΔ/Δ mice. Following the observations made in sections 2 and 3, we used Yeti reporter mice to profile the individual miRNAs present in YFP+ versus YFP- CD8+ T cells. For this purpose we stimulated CD8+ thymocytes from Yeti mice, for two hours with PMA and ionomycin, and sorted YFP+ and YFP- cells (Fig. 10 A). We then profiled the microRNAs using ready-to-use PCR panels from Exiqon which analyzes the expression of 372 miRNAs. We identified 22 microRNAs differentially expressed in YFP+ versus YFP- CD8+ T cells (Fig. 10 B), 12 of which were higher expressed in YFP+ cells, and 10 were higher expressed in YFP-CD8+ cells.

Fig. 10 - YFP+ versus YFP- CD8+ T cells from Ifng-YFP mice have different miRNAs profiles. Representative FACS staining of YFP expression in thymic CD8+ T cells stimulated for two hours with PMA and ionomycin (A). Differential miRNA expression level in thymic YFP+ versus YFP- CD8+ T cells. The expression levels are represented relative to YFP-CD8+ T cells and converted to LOG2 fold changes (B).

4.5 RT-qPCR validation of miRNA expression in thymic YFP+ versus YFP- CD8+ T cells from Ifng-YFP mice

We chose to concentrate on the six miRNA candidates based on the highest differential expression in thymic YFP+ versus YFP- CD8+ T cells. miR-139, miR-451a
and miR-200a were higher expressed and miR-132, miR-322 and miR-181a were lower expressed in YFP⁺ compared to YFP⁻ CD8⁺ T cells. We validated their differential expression by independent miRNA expression analysis using specific primers for each miRNA (Exiqon).

![Image](https://via.placeholder.com/150)

**Fig. 11** - RT-qPCR validation of thymic miRNA candidates. Quantitative RT-PCR analysis of (A) miR-139-5p, (B) miR-451a, (C) miR-200a, (D) miR-132, (E) miR-181a-5p, (F) miR-322-5p expression in thymic CD8⁺ YFP⁺ (IFN-γ⁺) and CD8⁺ YFP⁻ (IFN-γ⁻) T cells. Expression levels are relative to the reference miRNA, miR-423-3p. The graphs show the geometric mean of the miRNA expression from four independent experiments. The differences were statistically not significant (n=3-4).

The RT-qPCR results confirmed the array data for miR-139, miR-451a, and miR-322 (Fig. 11 A, B, E, F). Although the differences are not significant between samples (due to low sample numbers), the trend observed in the qPCR array is maintained. miR-139 and miR-451a are higher expressed in YFP⁺ (Fig. 11 A and B) and miR-322 is higher expressed in YFP⁻ CD8⁺ T cells (Fig. 11 E and F).

The differentially expression of miR-181a and miR-200a was not confirmed (Fig. 11 C and E). While miR-132 seemed to be more expressed in YFP⁺ cells (Fig. 11 D), in contradiction to the initial screening data (Fig. 10 B).

In sum, we could validate the expression pattern of 3 out of our 6 miRNA candidates, and miR-132 showed an interesting differential expression in YFP⁺ compared to YFP⁻ CD8⁺ T cells which was not detected in the initial screening.
4.6 RT-qPCR analysis of peripheral YFP⁺ versus YFP⁻ CD8⁺ T cells from IフギN-GFP mice

We further analyzed the expression of the six miRNA candidates in peripheral eYFP⁺ versus YFP⁻ CD8⁺ T cells isolated from pooled LNs and spleen and investigated if the differential expression detected in the thymus of the candidate miRNAs was maintained in peripheral CD8⁺ T cell subsets.

miR-139 was significantly higher expressed in YFP⁺ CD8⁺ T cells, P < 0.05, (Fig. 12 A) when compared with their YFP⁻ counterparts, in accordance with the thymic expression pattern. By contrast, miR-451a and miR-200a were similarly expressed in the two subsets (Fig.12 B and C).

miR-132 was significantly higher expressed in YFP⁺ CD8⁺ T cells (Fig.12 D), P < 0.0021, in accordance with the previous RT-qPCR profiling in thymic CD8⁺ T cells. miR-181a showed a non-significant trend to be higher expressed in YFP⁺ CD8⁺ T cells (Fig.12 E). miR-322 was significantly higher expressed in the YFP⁺ CD8⁺ T cells, P < 0.05 (Fig. 12 F), in accordance with the previous results in thymic CD8⁺ T cells.
conclusion, our expression analysis showed various expression patterns in our miRNA candidates. miR-139 and miR-132 are consistently higher expressed in CD8\(^+\) YFP\(^+\) CD8\(^+\) T cells, and miR-322 is consistently higher expressed in YFP\(^-\) CD8\(^+\) T cells in both thymus and periphery, whereas another miRNA, e.g. miR-451, only showed a differential expression in the thymus. Additionally we could not confirm a significant differential expression in miR-200a and miR-181a in neither the thymus nor the periphery.

4.7 Candidate miRNAs expression in T cells subsets

We further analysed the expression of our miRNAs candidates in other T cell subsets such as CD4\(^+\) T cells, Tregs (CD4\(^+\) CD25\(^+\)), \(\gamma\delta\) CD27\(^+\) and \(\gamma\delta\) CD27\(^-\) T cells. All of the miRNAs candidates were expressed in the different T cell subsets (Fig.13). miR-139 was the most abundant miRNA in all subsets (Fig. 13 A), on the other hand the least expressed miRNA in the majority of the subsets was miR-451a, being only slightly more abundant in \(\gamma\delta\) CD27\(^-\) T cells (Fig. 13 B). miR-200a was highest expressed in CD8\(^+\) T cells and in \(\gamma\delta\) CD27\(^+\) T cells (Fig. 13 C), two populations prone to produce IFN-\(\gamma\). Interestingly, miR-132 displayed the opposite expression pattern and was lowest expressed in naïve CD8\(^+\) T cells and in CD27\(^+\) \(\gamma\delta\) T cells. As for miR-132, miR-322, and miR-181a, they were highest expressed in CD4\(^+\) T cells (Fig. 13 D, F and E).

The data shows that our six candidates are expressed in other T cell subsets rather than being exclusive to CD8\(^+\) T cells and we detected distinct expression levels ranging from low abundant e.g. miR-451, to highly abundant e.g. miR-181a. Interestingly, some of the miRNAs seemed to be less expressed in subsets linked to the production of IFN-\(\gamma\), e.g. miR-132 and miR-322; whereas miR-200a was highly expressed in those T cell subsets.
Fig. 13 - Candidate miRNAs expression in T cells subsets. Quantitative RT-PCR analysis of (A) miR-139, (B) miR-451, (C) miR-200a, (D) miR-132, (E) miR-181a, (F) miR-322 expression in peripheral T cell subsets. Expression levels are relative to the reference microRNA, miR-423-3p. The graphs show the geometric mean of the miRNA expression from four independent experiments.

4.8 Candidate miRNAs expression under IFN-γ promoting conditions in vitro

To address potential mechanisms that regulate the miRNA expression of our candidates, we analysed their expression patterns under different IFN-γ promoting conditions. We isolated peripheral CD8⁺ T cells and polarized them overnight in different conditions and subsequently analysed the miRNA expression levels by RT-qPCR. We either activated the CD8⁺ T cells just via TCR stimulation using plate-bound anti-CD3 and anti-CD28 mAb, or activated the cells via TCR plus cytokines. The cells were cultured in the presence of either IL-12 plus IL-18 (a rapid IFN-γ inducing cytokine cocktail) or in the presence of IL-15 plus IL-2. IL-15 induces both IFN-γ production in CD8⁺ T cells and the proliferation of memory-phenotype CD8⁺ T cells. IL-2 supports the growth and survival of naïve T cells. Moreover we showed in section 2 that in vitro stimulation with only IL-2 induces IFN-γ production in CD8⁺ T cells.
miR-139 was the miRNA with the highest expression levels and it was significantly upregulated in the presence of IL-2 plus IL-15 (*P < 0.05) (Fig. 14 A). Of note, TCR/CD28 stimulation plus IL-2 and IL-15 did not induce the expression of miR-139 (Fig. 14 A). miR-322 displayed a similar expression pattern (Fig. 14 F). The combination of the two stimuli (TCR and cytokines) mostly caused a down regulation of the miRNA expression compared to cytokines alone. miR-451a was the only miRNA upregulated in all tested cytokine combinations, including in combination with TCR/CD28 activation (*P < 0.05) (Fig. 14 B). On the contrary, miR-200a and miR-181a expression levels exhibited no significant differences between the control and the different stimuli, although both have the tendency to be higher expressed in the condition of IL-2 plus IL-15 (Fig. 14 C and E).

Fig. 14 - Candidate miRNAs expression under IFN-γ promoting conditions. Quantitative RT-PCR analysis of (A) miR-139, (B) miR-451, (C) miR-200a, (D) miR-132, (E) miR-181a, (F) miR-322 expression in peripheral CD8⁺ T cells under different culture conditions. Peripheral CD8⁺ T cells were stimulated overnight with IL-12 plus IL-18 or IL-2 plus IL-15 with or without TCR activation (plate bound anti-CD3 and anti-CD28). The conditions are indicated below each graph. Expression levels are relative to the reference microRNA, miR-423-3p. The graphs show the geometric mean of the miRNA expression from four independent experiments.*P < 0.05.
Interestingly, the expression of miR-132 was induced in conditions that trigger a rapid production of IFN-γ by CD8⁺ T cells (Fig. 14 D). miR-132 was upregulated upon incubation with IL-12 and IL-18 and, on contrary to the previous described miRNAs, the TCR/CD28 stimulus further increased miR-132 expression, suggesting a synergetic effect of TCR/CD28 plus cytokines (Fig. 14 D). The expression levels were also upregulated in the presence of IL-2 plus IL-15 and TCR stimulation, *P < 0.05 (Fig. 14 D).

In sum, the expression of our six miRNA candidates was influenced by the tested stimuli. With the exception of two miRNAs, miR-200a and miR-181a, the expression of the other miRNAs was upregulated in response to different stimuli. Of note, only miR-132 and miR-451 were significantly upregulated in the condition that leads to rapid IFN-γ production (IL-12 plus IL-18). Interestingly the expression levels of miR-132 were further upregulated upon cytokines plus TCR activation. The expression levels of the others miRNAs were downregulated when TCR stimulation was added to the cytokine cocktails.

4.9 - Overexpression of candidate miRNAs in the 3T3 cell line

To investigate the function of our candidate miRNAs in the differentiation of IFN-γ producing CD8 T⁺ cells we chose an over-expression screening strategy. We used retroviral vectors encoding the native precursor stem-loop to transduce activated CD8⁺ T cells. The precursor stem loop has to be processed by the cellular miRNA machinery, which avoids uncontrolled overexpression levels usually observed upon transfection of mature miR mimics.

The retroviral vectors encoding the precursor stem loops were tested in the easy-to-transduce 3T3 cell line to confirm that the transduction leads to an overexpression of the respective miRNA. Furthermore with this test transduction we could evaluate the produced viral titers. The RT-qPCR analysis of miRNA expression confirmed that transduction with the cloned precursor stem loops induced a substantial increase in the miRNAs expression levels of all our candidate miRNAs, ranging from 5 fold to ~5000 fold when compared to control cells (Fig. 15 A-F).
4.10 miRNA-132-3p over-expression down-regulates IFN-γ production in peripheral CD8⁺ T cells

To identify whether the candidate miRNAs had an impact on the differentiation of IFN-γ producing CD8⁺ T cells we conducted an over-expressing screening with retroviral vectors containing the precursor stem loop of miR-181a, miR-139, miR-200a or miR-132. Total CD8⁺ T cells were sorted from C57BL/6 mice and activated for 48h, followed by retroviral transduction with either a control vector expressing GFP or a miRNA overexpressing vector, containing the native precursor stem loop of the candidate miRNAs and an IRES-GFP site. 3 days after the retroviral transduction, GFP⁺ (transduced) CD8⁺ T cells were sorted and stained for IFN-γ. Additionally we analysed the expression of TNF-α, another cytokine produced by activated CD8⁺ T cells, to investigate if potential effects on cytokine production are IFN-γ specific (Fig. 16 A).
A

Sorted naive CD8⁺ T cells
miRNA transduction
Restimulation for i.c. staining for TNF-α and IFN-γ

0h 48h 120h
Anti-CD3/anti-CD28 + IL-2

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>TNF-α (%)</th>
<th>IFN-γ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>32.3</td>
<td>63.1%</td>
</tr>
<tr>
<td>miR-200a</td>
<td>29.8</td>
<td>59%</td>
</tr>
<tr>
<td>miR-132</td>
<td>16</td>
<td>43.7%</td>
</tr>
<tr>
<td>miR-181a</td>
<td>26.5</td>
<td>60.5%</td>
</tr>
<tr>
<td>miR-139</td>
<td>35.1</td>
<td>54.8%</td>
</tr>
</tbody>
</table>

Counts

IFN-γ
TNF-α

Graphs showing expression levels of IFN-γ and TNF-α under different conditions.
miR-132 overexpression down-regulates IFN-γ production in CD8+ T cells. Workflow of miRNAs overexpression strategy in sorted peripheral naïve CD8+ T cells (A). Representative intracellular staining of peripheral CD8+ T cells expressing either the retroviral GFP-control vector (Ctrl), or the retroviral vector containing the native stem loop of miR-200a, miR-132, miR-181a or miR-139 (B). Histograms and graphs indicate the frequency of IFN-γ and TNF-α producing CD8+ T cells. Validation of miR-132 overexpression experiment in CD8+ T cells (C). The graphs indicate the frequency of IFN-γ and TNF-α producing CD8+ T cells.

We detected no difference in the frequency of IFN-γ and TNF-α producing CD8+ T cells when overexpressing miR-200a, miR-181a and miR-139 (Fig. 16 B). By contrast, the overexpression of miR-132 reduced the frequency of IFN-γ-producing CD8+ T cells (~20% reduction), whereas the frequency of TNF-α-producing CD8+ was unchanged, thus confirming a specific role of miR-132 in the differentiation of IFN-γ-producing CD8+ T cells (Fig. 16 B). As validation we repeated the experiments 4 times and confirmed that miR-132 overexpression significantly decreases the IFN-γ production in peripheral CD8+ T cells (P < 0.05) (Fig. 16 C).

The results obtained so far indicate that miR-132 is implicated in the differentiation of IFN-γ-producing CD8+ T cells, whereas the overexpression of the other miRNA candidates did not influence IFN-γ production by peripheral CD8+ T cells.

4.11 mRNA targets of miR-132

miRNAs mediate their functions by repressing sequence-specific target mRNAs. To further explore how miR-132 is implicated in CD8+ T cell differentiation, we have enquired its mRNA targets based on previous experimental evidence (validated targets) (Table 2), or bioinformatics prediction tools (predicted targets) (Table 3). One interesting validated target is STAT4 which is a critical transcription factor know to drive the differentiation of Th1 (IFN-γ-producing) CD4+ T cells. Additionally, Twist1 and Runx3 (predicted targets), are involved in the differentiation of IFN-γ producing T cells.
Whereas Twist 1 is a negative regulator of IFN-γ differentiation, Runx3 is a positive regulator of the effector CTL program in CD8⁺ T cells and drives IFN-γ expression in CD8⁺ T cells by inducing Eomes. Future experiments, including RT-qPCR analysis (in control CD8⁺ T cells versus miR-132-overexpressing CD8⁺ T cells) will be performed to clarify which targets are important for the role of miR-132 in the differentiation of IFN-γ producing CD8⁺ T cells.

Table 2 - Validated targets for the miR-132. List made from targets previously validated experimentally by reporter assays, western blot or qPCR.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Reporter Assays</th>
<th>Western Blot</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ep300</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>AChE</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jarid1a</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT4</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRAK4</td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 - miR-132 bioinformatic predicted targets. Putative targets were chosen with a score of 3 out of 5 in the bioinformatics programs. The Bioinformatic programs used were MiRanda, MiRTarget2, PicTar, PITA and RNAhybrid

<table>
<thead>
<tr>
<th>Gene</th>
<th>MiRanda</th>
<th>MiRTarget2</th>
<th>PicTar</th>
<th>PITA</th>
<th>RNAhybrid</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twist1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>IRF4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Runx3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>
Discussion
5 Discussion

miRNAs are key regulators of mammalian genome and add another layer of complexity to the regulation of gene expression. During the last several years, mounting evidence has shown that miRNAs are critical not only for the development of immune cells, but also to regulate the function of both the innate and adaptive arms of the immune system. In this study we investigated the role of miRNAs in the differentiation of IFN-γ-producing CD8+ T cells.

Others have previously shown that miRNA-deficient T cells exhibit defects in proliferation and in the regulation of cytokine production. Regarding CD8+ T cells it was also shown that in Dicer-deficient mice there is a decrease in the numbers of CD8+ mature cells and a failure in CD8+ effector T cell expansion in response to an infectious challenge in vivo. Notwithstanding, the ability of miRNA-deficient CD8+ T cells to secrete IFN-γ had not been addressed.

In this study we used CD8+ T cells from pLck-Cre DICERfl/fl mice, as a source of miRNA-deficient CD8+ T cells. In these mice miRNAs are absent from early T cell development onwards. We showed that pLck-Cre DICERfl/fl mice have a significantly increased frequency of IFN-γ producing CD8+ T cells (upon short restimulation in vitro), both in the periphery and in the thymus when compared to wt controls. This ability of rapid production of IFN-γ by naïve CD8+ T cells in pLck-Cre DICERfl/fl mice suggests an important role for DICER/miRNAs in the post-transcriptional regulation of IFN-γ expression and resembles the behavior of innate T cells, which rapidly secrete cytokines after activation.

Conventional naïve CD8+ T cells require activation, expansion, and effector cell differentiation before producing IFN-γ and participating in a protective immune response. Memory CD8+ T cells are, in turn, major direct producers of IFN-γ. Interestingly, a population of CD8+ T cells with “innate-like” function has been described recently, that expresses a memory-like phenotype and can rapidly secrete IFN-γ. These innate-like CD8+ T cells have been identified in the thymus of several gene-deficient mouse strains, including Itk, KLF2, CBP and Id3 knock-out mice, have the CD44hiCD122+ phenotype and function of memory CD8+ T cells, without previous exposure to antigens. These CD8+ T cells can rapidly secrete IFN-γ upon stimulation with IL-12 and IL-18, and play important roles in the innate response against infections such as Listeria monocytogenes, or chronic infections...
with viruses such as Herpes virus\textsuperscript{208,215,219,220}. Also interestingly, BALB/c but not C57BL/6 mice have CD8 SP thymocytes that contain a distinct sub-population of Eomes$^{hi}$-bet$^{lo}$CD44$^{hi}$CD122$^{hi}$ innate phenotype CD8$^+$ T cells that produce IFN-$\gamma$\textsuperscript{221}. The detection of an increased frequency of IFN-$\gamma$ producing CD8$^+$ T cells in the thymus of miRNA-deficient mice raises the possibility that miRNAs are important negative regulators of the differentiation of innate-like CD8$^+$ T cells. This specific question will be addressed in future experiments where the expression of surface markers of miRNA-deficient thymic CD8$^+$T cells will be analysed (further discussed below).

In this work we focused on deciphering which individual miRNA regulate the differentiation of IFN-$\gamma$-producing CD8$^+$ T cells. We performed a miRNA profiling in thymic IFN-$\gamma$-expressing CD8$^+$ T cells versus IFN-$\gamma$ negative CD8$^+$ T cells using Ifng-YFP reporter (Yeti) mice. We reasoned that miRNAs with distinct abundance in YFP$^+$ versus YFP$^-$ CD8$^+$ T cells could be important mediators of IFN-$\gamma$ differentiation in CD8$^+$ T cells. In total we identified 22 differentially expressed miRNAs.

For practical reasons, we narrowed down our initial list to the six microRNA candidates that showed the highest differential expression in YFP$^+$ versus YFP$^-$ CD8$^+$ T cells. miR-139, miR-451a and miR-200a were significantly higher expressed in YFP$^+$ CD8$^+$ thymocytes whereas miR-132, miR-322 and miR-181a were significantly lower expressed in this cell population when compared to their YFP$^-$ counterparts.

We further validated the results of the qPCR profiling in peripheral CD8$^+$ T cells as well as in CD8$^+$ thymocytes. The candidates showed a similar pattern of expression in peripheral CD8$^+$ T cells as in CD8$^+$ thymocytes.

Our six miRNAs candidates were not exclusively expressed in CD8$^+$ T cells, as they were found in other T cells subsets. They had different levels of expression in each subset, reinforcing the idea that miRNAs tend to be highly pleiotropic, with a given miRNA having several functions and most likely different mRNA targets. Of note, miR-200a was the only miRNA significantly higher expressed in CD8$^+$ T cells.

We further investigated the role of IFN-$\gamma$-driving cytokines and TCR signalling on the expression of our candidate miRNAs in CD8$^+$ T cells. Based on previous knowledge that activated murine T cells\textsuperscript{222} and a subset of human CD8$^+$ T cells express more IL-12 and IL-18 receptors\textsuperscript{223}, and these activated CD8$^+$ T cells are prone to produce IFN-$\gamma$, we decided to analyze the influence of these cytokines in the production of our candidate miRNAs. In addition we tested IL-15 and IL-2 conditions, which were previously shown to induce IFN-$\gamma$ production$^{224,225}$. Moreover, IL-15 promotes the proliferation of memory-phenotype CD8+ T cells$^{224}$ and IL-2 is known to support the
growth and survival of naïve T cells. Our results showed that miR-139 and miR-322 were the only miRNAs with higher expression with IL-2 plus IL-15, suggesting that these miRNAs may be involved in memory CD8$^+$ T cell development. This possibility should be investigated in the future.

miR-132 was the most responsive of our miRNA candidates to the cytokines promoting IFN-γ production and TCR signalling. Thus, the highest expression of miR-132 was obtained with the combination of anti-CD3/anti-CD28 plus IL-12 and IL-18. This cytokine combination has been shown to induce a rapid IFN-γ secretion by CD8$^+$ T cells and provide innate protection in a non–antigen-specific manner. In addition, *Listeria monocytogenes* infected macrophages secrete IL-12 and IL-18, which act upon NK cells and primed T cells to induce the production of IFN-γ. Thus our results suggest that miR-132 might be involved in a setting where IFN-γ is rapidly produced and needed.

It was also curious to observe that only when the cells received TCR/CD28 signals there was a significant increase in the miR-132 expression by the IL-2 and IL-15, suggesting that for these cytokines to have a significant impact in the miR-132 expression the cells may have to be previously activated. Since these cytokines promote the differentiation of CD8$^+$ T cells into memory CD8$^+$ T cells, maybe miR-132 is also important in memory CD8$^+$ T cells.

The functional relevance of the all miRNA candidates could not be tested by an overexpression screening due to technical limitations of the viral production. We were not able to produce high viral titers for the overexpression constructs of miR-451 and miR-322. Additionally, we performed our functional screening in peripheral CD8$^+$ T cells but not in CD8$^+$ thymocytes as these cells are more difficult to transduce. We could not obtain a level of transduced thymocytes to allow conclusive results.

Most retroviral vectors (RVs) are based on the molony murine leukemia virus (MoMLV), which has a simple genome encoding the gag, pol, and env, flanked by long terminal repeats (LTR). Upon cell entry, the RNA is copied by the reverse transcriptase enzyme into double-stranded DNA, which becomes stably integrated into one of the host chromosomes, thus allowing long-term expression of inserted genes. Using a stable transduction with the original stem loop of our miRNA contributes to a more physiological result avoiding false positive results introduced by supra-physiological increase in miRNA levels generally achieved after transient transfection.

Despite the fact that the transgene is integrated into the host cell genome, by the retroviral vectors, persistent gene expression is not guaranteed as silencing of
transcriptional units may occur over time. Another major limitation of RVs is their inability to infect non-dividing cells. This is due to the fact that the pre-integration complex cannot cross the intact nuclear membrane, which is only disassembled during mitosis. To overcome this obstacle we had to culture the cells always with plate-bound anti-CD3 and anti-CD28 mAb.

The production of the retroviral vectors is a crucial step for the success of the experiments, and there are some factors that influence the vector production and titers, such as the construction of the vector backbone and the transgene to transfer, extracellular factors, culture conditions of the packaging cells and harvesting the virus. In general, a bigger vector size leads to a lower efficiency of viral particles, which can be neglected in our case as the miRNA cassettes were very small ~400bp. In addition, the expression of the transgene can be toxic for the producer cells or can significantly reduce vector production (e.g. GFP or miRNA itself). This could have influenced the titers and the production of some vectors, such as miR-451 and miR-322.

Our viral vectors were produced in mammalian adherent cell line, HEK 293T (Human embryonic kidney 293 cells with large T antigen) cell line. These cells produce variable quantities of extracellular matrix proteins partially consisting of proteoglycans. These macromolecules are negatively charged, of variable size, and act as inhibitors during cell transduction, thus eventually reducing significantly the transduction efficiency despite high vector titers. To avoid this problem, a suspension cell line could be used to produce the virus.

The replacement of glucose by fructose in the culture medium has also been described as having potential for improving vector production rates and titers, but this would need to be tested in our cultures.

In sum, future experiments are needed to optimize the viral transduction efficiency in CD8+ thymocytes and peripheral T cells. If these technical limitations are solved, retroviral-mediated over-expression or knock-down strategies of individual miRNAs are a powerful tool to study the role of miRNAs in cell differentiation.

The over expression of miR-139, miR-181a and miR-200a had no significant impact on IFN-γ production by CD8+ T cells, but the over-expression of miR-132 resulted in decreased production of IFN-γ in peripheral CD8+ T cells. In line with this is the observation that in human NK cells both the miR-132/miR-212 cluster and miR-200a are negative feedback regulator of IL-12 signalling through targeting of STAT4, an inducer of IFN-γ production. It is possible, thus, that miR-132 may also target STAT4 in CD8+ T cells. Of note is the fact that, in our experiments, the expression of
miR-200a in CD8⁺ T cells was not influenced by the cytokines IL-12 and IL-18 as shown for human NK cells. This suggests that the role of miR-200a might be dissociated from the control of IFN-γ in CD8⁺ T cells.

In vivo, cytokine-mediated T cell activation is in many ways a double-edged sword. In some cases, bystander T cell activation can be beneficial as is the case when CD8⁺ T cells produce IFN-γ in response to cytokines triggered by infection with *Listeria monocytogenes* and provide innate protection. On the other hand, endotoxic shock associated with Gram-negative bacteria can be exacerbated by a cytokine storm that includes IFN-γ-mediated immunopathology due to CD8⁺ T cells and NK cells. Therefore the miR-132-based regulation may constitute an important negative feedback mechanism to prevent the IFN-γ mediated immunopathology in bacterial infection or in autoimmune pathology.

The most striking evidence, so far, for the effect of miR-132 in the immune system comes from a mouse model deficient in the miR-212/132 cluster (where miR-132 and miR-212, which are tandem miRNAs at the same chromosomal location and sharing close sequences, are simultaneously removed). These mice have higher resistance to the development of experimental autoimmune encephalomyelitis (EAE), a prototype for T-cell-mediated autoimmune disease in general, and lower frequencies of Th1 T cells, responsible for IFN-γ production. These results do not go along with our overexpression phenotype in which excess of miR132 instead causes a decrease in IFN-γ production. As the loss of function phenotype comes from a double knockout mouse, we cannot discard the possibility that the role of miR-212 is distinct from that of miR-132. It will be important to clarify these issues in future experiments, where the outcome of overexpression studies with miR-212 alone or both miR-212 and miR-132 should be analyzed.

Finally, we investigated miR-132 potential mRNA targets based on literature search and bioinformatic analyses. Several of the validated targets for miR-132 found in the literature are known players involved in the inflammatory and IFN-γ response, including Stat4 AChE, HB-EGF, p300, MeCP2 and SirT1.

Stat4 is a critical Th1 driving transcription factor and acts together with STAT1 to induce T-bet. It still remains to be established if Stat4 is also implicated in the differentiation of IFN-γ-producing CD8⁺ cells.

Runx3 is one of the predicted targets that came out of our bioinformatics analysis. Runx3 is present in naive CD8⁺ T cells before activation and has a positive role in the induction of Eomesodermin (Eomes), granzyme B, perforin, and IFN-γ and it is also involved in CD8⁺ effector T cell differentiation. This is an interesting
candidate that being targeted by miR-132 could down-regulate the production of IFN-γ, as it directly impacts on the master transcription factor Eomes.

Another predicted target is the transcription factor Twist1. In Th1 cells, NF-kB, NFAT, and IL-12/STAT4 signalling can induce Twist1 expression, and Twist1 limits inflammation by suppressing IFN-γ and TNF-α production by decreasing expression and function of transcription factors, including T-bet, Stat4, and Runx3. The precise role of this transcription factor remains to be established in CD8+ T cells but the fact that miR-132 overexpression decreases IFN-γ+ CD8+ T cell frequency does not go along with the observation that Twist1 represses IFN-γ production in CD4+ T cells. The targeting of this transcription factor may be an example where the miRNA activates the expression rather than represses it, as it has been reported during the last years by several groups. If that were the case, then the overexpression of miR-132 would activate the expression of Twist1, which would decrease T-bet, STAT4 and Runx3, decreasing as well the IFN-γ production.

In addition to analyzing already validated or bioinformatics-predicted mRNA targets, further experimental studies will be needed to identify novel targets that could explain the IFN-γ phenotype in miR-132 overexpressing CD8+T cells. One method to use could be differential high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) analysis. This technique uses ultraviolet irradiation to covalently crosslink RNA–protein (Ago-RNA) complexes that are in direct contact and partial RNA digestion to reduce bound RNA to fragments that can be sequenced by high-throughput methods. Ago HITS-CLIP can simultaneously identify Ago-bound miRNAs and the nearby mRNA sites. This should be complemented by luciferase reporter assay as a final confirmation that a given mRNA is targeted by a specific miRNA.

To characterize a possible role of our candidate miRNAs in IFN-γ producing CD8+ T cells differentiation, it would be interesting to perform in vitro experiments, such as reaggregate thymic organ cultures (RTOC), where our miRNA of interest would be over-expressed in CD8+ thymocytes during their development. With this tool we could characterize the development of CD8+ T cells, and check for surface markers that could indicate if there was a bias towards the development of memory like phenotype CD8+ T cells, the so called “innate-like CD8+ T cells”.

In summary, we identified miR-132 as a negative feedback regulator of IFN-γ production in CD8+ T cells. We additionally showed, that miR-132 expression is modulated by cytokines promoting IFN-γ production. Several promising mRNA targets of miR-132 include the known STAT4 and the novel predicted Twist1 and
Runx3. Future experiments will address if these targets are responsible for the observed phenotype. Collectively, miR-132 is likely to play an important role in the differentiation of IFN-γ producing CD8⁺ T cells, which are crucial in immune responses against intracellular pathogens and tumours.
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6 Bibliography


The Role Of miRNAs In CD8+ T Cells Differentiation


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