Regulation of T cell activation by miRNAs

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Abstract

$CD4^+$ T-cells play crucial roles in the immune response. Triggering of the TCR results in the activation and differentiation of a T cell into different T-cell subsets so that the T cells can participate in different immune responses. Therefore, T-cell activation must be tightly regulated to avoid excessive immune responses that can lead to immunopathological conditions. microRNAsare a class of small regulatory RNAs that regulate different signalling and functional events in a variety of cell types including T cells. However, it is not clearly known how miRNAs regulate primary human naive $CD4^+$ T-cell activation and immune function. Modulation of miRNAs expression in T cells may represent a novel therapeutic tool to control T-cell activation and T-cell-mediated diseases.

In my work, I have specifically focused my investigations on miR-20a. I have shown that miR-20a is rapidly upregulated upon TCR triggering. In addition, I have shown that the expression of miR-20a is primarily dependent on the activity of AP-1 and NFAT upon TCR triggering. To investigate the role of miR-20a in T-cell activation, I have overexpressed miR-20a in primary naïve human $CD4^+$ T cells. I have found that overexpression of miR-20a negatively regulates TCR-mediated signalling and decreased the surface expression of CD69. To further confirm the negative regulatory effect of miR-20a, I have downregulated its expression in human naïve $CD4^+$ T cells using miR-20a decoy. In line with the overexpression data, downregulation of miR-20a showed an increase of TCR-mediated signalling. Further functional analysis on the role of miR-20a in $CD4^+$ T cells revealed that miR-20a mildly decreases the production of IL-2 and IL-8, but strongly decreases the production of IL-6 and IL-10. In conclusion, I have shown that miR-20a inhibits primary human naive $CD4^+$ T-cell activation by negatively regulating TCR-mediated signalling, CD69 expression and cytokine production.

1. Introduction

1.1 The immune system

Complex biological systems such as vertebrates are protected against pathogenic and environmental insults by a variety of defence mechanisms collectively known as immune system. The immune system can be divided into two sub-systems according to the mode of protection it provides. The innate (inborn) immune system ensures a first line of defence against infections. Cells of the innate immune system recognise pathogens non-specifically via germline encoded pattern recognition receptors (PRRs). PRR binds to the pathogen associated molecular patterns (PAMPs) such as LPS, nucleic acids etc. On the other hand, the adaptive immune system provides a second line of defence which is characterized by the recognition of molecules (antigens) specific for a given pathogen. T and B lymphocytes, the cells of the adaptive immune system, express antigen receptors, T-cell receptor (TCR) and B-cell receptor (BCR) for T and B cells, respectively which recognise antigens. Adaptive immunity also provides long-term protection (memory) against reinfection with the same pathogen. Although the innate immune system has only limited capacity to eliminate pathogens, it is indispensable for the activation of an effective specific adaptive immune response (Iwasaki A. et al. 2010).

1.2. Components of the innate and adaptive immune system

All cellular elements of the immune system are derived from pluripotent haematopoietic stem cells (HSC) in the bone marrow (BM). Both the innate and adaptive immune systems are composed of white blood cells, or leukocytes. The pluripotent HSCs give rise to two types of stem cells (Figure 1):

- a common myeloid progenitor (CMP) that develops into different types of cells including granulocytes, monocytes/macrophages, dendritic cells, erythrocytes (red blood cells), and platelets that are important for blood clotting
- a common lymphoid progenitor (CLP) that generates natural killer (NK) cells, T and B lymphocytes.

Granulocytes, monocytes and NK cells participate in the defence mechanisms of the innate immune system, whereas T and B lymphocytes mediate adaptive immune defenses.

The cells of the innate immune system: Granulocytes are characterized by distinct cytoplasmic granules and irregularly shaped nuclei. They are subdivided into three main subsets - neutrophils, eosinophils and basophils - which perform various important functions in the immune response ranging from phagocytosis to the release of inflammatory and

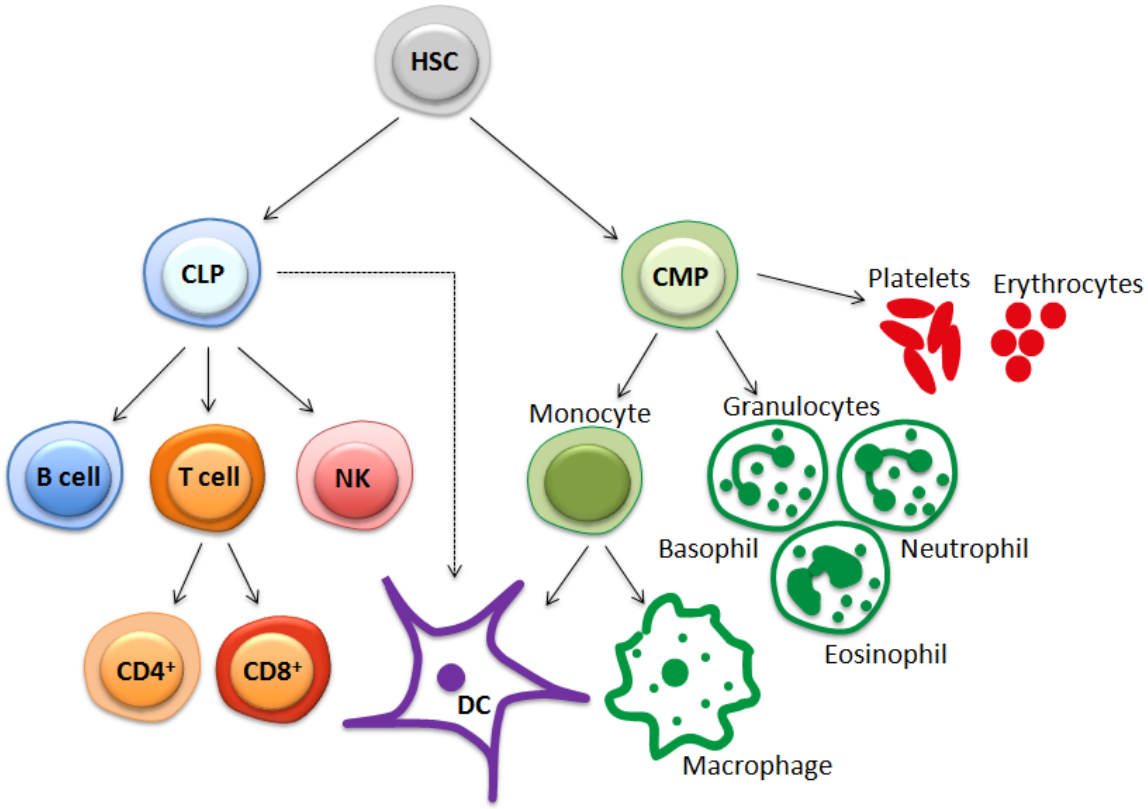

Figure 1.1. Schematic representation of heamatopoiesis. Cellular components of both innate and adaptive immune system derive from haematopoietic stem cells (HSCs). HSCs give rise to common myeloid progenitors (CMP) and common lymphoid progenitors (CLPs). CMPs further give rise to granulocytes, monocytes and platelets, whereas CLPs differentiate into T, B, NK cells, and dendritic cells *(adapted from Murphy K. et al., 2007).*

antimicrobial agents. Monocytes circulate in the blood and when they migrate into tissues, they differentiate into macrophages. Monocytes and macrophages engulf and kill the invading microorganisms. In addition, macrophages act as general scavengers by clearing apoptotic cells and also participate in tissue repair and in the maintenance of tissue homoeostasis.

NK cells are a type of cytotoxic lymphocyte derived from the CLP. Because they lack antigen-specific receptors, they are considered component of the innate immune system. NK cells recognize stressed and abnormal cells through various inhibitory and activating receptors and induce cell lysis or apoptosis by secreting cytotoxic molecules.

Dendritic cells (DC) develop from both lymphoid and myeloid precursors into the most efficient antigen presenting cells (APC) and thus create a link between the innate and adaptive immunity. When a DC takes up a pathogen in infected tissue, it becomes activated and migrates to the nearby lymph nodes. Activated DCs express and secrete effector molecules that influence both innate and adaptive immune responses and determine whether and how the immune system responds to certain infectious agents. In particular DCs process and present antigenic peptides or antigens derived from pathogens in association with major histocompatibility complex molecules to T cells and also express co-stimulatory molecules that are needed for the activation of naive T lymphocytes (Chaplin DD, 2010).

The cells of the adaptive immune system:T and B lymphocytes develop in the thymus and bone marrow, respectively. They are distinguished by the expression of antigen-specific receptors which recognize practically any foreign antigen. B cells secrete antibodies against extracellular pathogens and mediate the humoral immune response. T cells, on the other hand, are further subdivided into two main subsets according to their coreceptor (i.e. CD4 and CD8) expression. $CD4^+$ helper T cells regulate the immune response mainly by secreting cytokines or providing costimulation to activate macrophages, B- and $CD8^+$ T cells. $CD8^+$ T cells mainly recognize and kill cells infected by intracellular pathogens and tumour cells by pro-apoptotic signals and the secretion of cytotoxic molecules (Iwasaki A. et al. 2010; Chaplin DD. 2010).

1.3. The course of the immune response

The type, route and mode of transmission and the resistance of an infectious agent determine its infectivity and the course of the triggered immune response. When a pathogen trespasses physical barriers of the body, it induces inflammatory responses within the first hours of infection by activating innate immune cells residing in the damaged tissue. Phagocytes such as macrophages and neutrophils provide the first line of defense against many common microorganisms and are essential for the control of infections. Furthermore,

inflammatory responses induce the production of chemokines and adhesion molecules by myeloid and endothelial cells, which attract further cells of the immune system to the site of infection. Pathogens can also activate DCs by triggering multiple innate receptors leading to enhanced antigen presentation, costimulation, and production of polarizing cytokines. When the first line of immune defense fails to control an infection, then the adaptive immune response is initiated. Uptake and presentation of an antigen by tissue-residing APCs is the first step for the induction of the adaptive immunity. APCs, such as DCs, recognize foreign conserved molecules and molecular patterns via PRRs. Activation of these receptors stimulates internalization of the pathogen (phagocytosis) and maturation of the DC into professional antigen-presenting cells. Mature APCs containing antigens of the pathogen migrate through lymphatic vessels to regional lymph nodes. There, they encounter naïve lymphocytes that continuously circulate through the lymphoid organs. Naïve T cells recognize antigens bound on major histocompatibility complexes (MHC) on the surface of APCs. Antigen-specific effector T cells and antibody-secreting B cells are generated by clonal expansion and differentiation over the course of several days. Activated effector T cells either leave the lymphoid organ to induce cell-mediated immunity at sites of infection or, in the case of $CD4^+$ T cells, remain in the lymphoid organ to participate in humoral immunity by activating antigen-specific B cells. Eventually, activated T cells and antibody-secreting B cells are recruited to the site of infection. There, extracellular pathogens are cleared by antibodies and intracellular pathogens are eliminated through the actions of effector T cells. Once the pathogen has been cleared, most of the effector cells die by apoptosis, while few of them survive as memory cells. These memory cells form the basis for protective immunity against recurrent infections and diseases (Iwasaki A. et al. 2015).

1.4. Adaptive immunity: T cell development

As discussed above, adaptive immunity provides specific protection against pathogens and $CD4^+$ T lymphocytes play central roles in orchestrating adaptive immune response by providing help to other immune cells. Therefore, I describe in this paragraph their ontogeny and the molecular mechanisms involved in their activation. T lymphocytes develop from the hematopoietic stem cells in the bone marrow. As shown in Figure 1, T cell progenitors (common lymphocyte progenitors, CLPs) are generated in the bone marrow from the HSCs. CLPs then migrate to the thymus where they are committed to the T cell lineage and further develop into mature T cells (Figure 2). Within the thymus, developing T cells (also referred as to thymocytes) pass through a series of developmental phases which are defined by the expression of distinct markers. The earliest T cell precursors derived from CLPs do not express any surface markers (i.e. CD4 and CD8) that are characteristic of mature T cells. Because of the absence of CD4 and CD8, these cells are called double negative (DN) thymocytes. DN thymocytes are located in the cortical region of the thymus and can

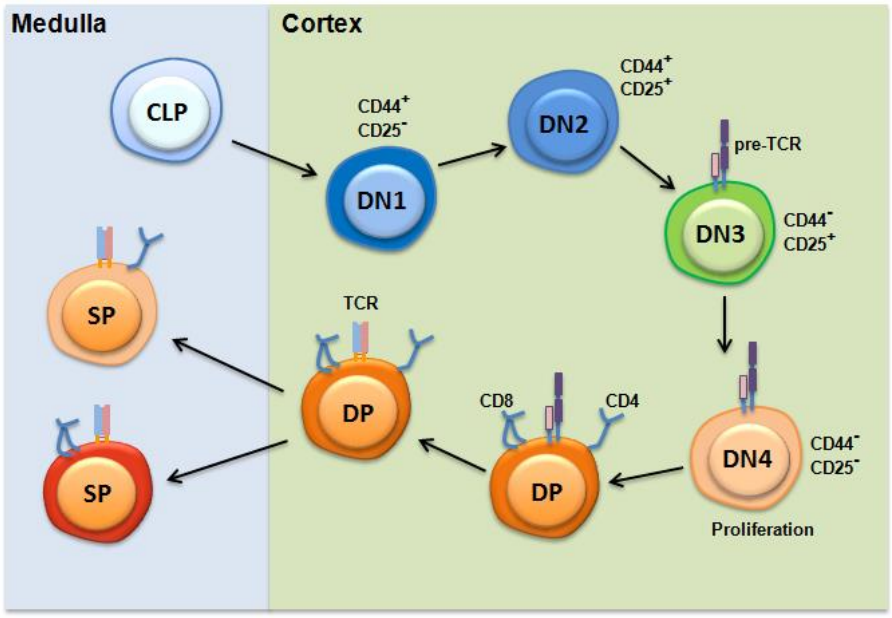

Figure 1.2. Schematic representation of T-cell development in the thymus.T-cell progenitors enter the thymus and undergo several maturation stages. CLPs enter the cortex and undergo further differentiation. Four double negative (DN) stages, characterised by the differential expression of CD44 or CD25 can be distinguished. At the DN3 stage, T-cell precursors express the pre-TCR which is required to drive further differentiation to the DN4 stage. Subsequently, DN4 thymocytes strongly proliferate and develop into double positive (DP) cells. DP cells undergo selection processes which are required for the establishment of self-tolerance. Only DP cells expressing a non-self reactive functional TCR will finally develop into either $CD4^+$ or $CD8^+$ mature T cells. Finally, fully mature single positive (SP) $CD4^+$ and $CD8^+$ T cells leave the thymus and migrate into the periphery *(adapted from Murphy K. et al., 2007).*

be further subdivided into four stages based on the expression of CD25 and CD44. The most immature DN subset is characterized by the expression of CD44 but not of CD25 and are called DN1 ($CD25^-$ $CD44^+$) cells. As the development progresses, DN1 cells express CD25 and become DN2 (CD25+ CD44+) cells. DN2 cells eventually lose CD44 and enter into the DN3 stage ($CD25^+$ $CD44^-$). DN3 thymocytes express the T cell receptor (TCR) β-chain which associates with a surrogate pre-Tα chain (pTα) and also with CD3γ, CD3δ, CD3ε and ζ chains to form the pre-T cell receptor (pre-TCR). DN3 cells that made a successful rearrangement of the β-chain and were able to express the pre-TCR will undergo proliferation. Additionally, signals transduced through the pre-TCR lead to the arrest of further β-chain rearrangement, a process called allelic exclusion, thus ensuring that each T cell express one kind of TCRβ-chain. Subsequently, DN3 cells lose CD25 and become DN4 ($CD25^-$ $CD44^-$) cells. Differentiating DN4 cells will express CD4 and CD8 and become double positive (DP) thymocytes. Extensive TCRα chain rearrangement takes place in DP thymocytes and, upon successful rearrangement of TCRα, a unique TCR will be expressed on each DP thymocytes. To test the functionality of the TCR, DP thymocytes are exposed to self-peptide:MHC complexes. DP thymocytes that recognize, through their TCRs, these complexes with a weak affinity will be positively selected. On the other hand, DP thymocytes that strongly recognize self-peptide:MHC complexes will be eliminated from the T-cell pool to avoid auto-reactivity. This process is called negative selection. Finally, also DP clones that fail to recognize self-peptide:MHC complexes will die by apoptosis. Positively selected DP thymocytes will mature into single positive T cells bearing either CD4 or CD8 and leave the thymus to populate the periphery as naïve T cells (Germain RN. 2002; Zúñiga-Pflücker JC. 2004; Rothenberg EV. et al. 2008).

1.5. Molecular mechanism involved in T-cell activation

In the periphery, T cells are exposed to a variety of both foreign and self antigens. Activation begins when T cells recognize foreign antigens in complex with MHC molecules expressed on APCs via the TCR. In fact, TCR engagement by Ag/MHC complexes initiates intracellular signalling events that ultimately culminate into transcriptional activation, proliferation, differentiation, and in the generation of the immune response (Weiss A. et al. 1994). The TCR is a heterodimeric transmembrane surface receptor consisting of anα and β glycoprotein chains linked by a disulphide bond. In the majority of T cells (95%), the TCR is composed of a TCRα and a TCRβ chain, whereas a minor fraction of T cells express an alternative pair of chains termed TCRγ and TCRδ. Both chains of the TCR contains an amino-terminal variable (V) region and a constant (C) region which are extracellular, a short positively charged hydrophobic transmembrane region and a short cytoplasmic tail (Figure 3). The V regions confer specificity for antigen recognition. However, the TCR alone cannot

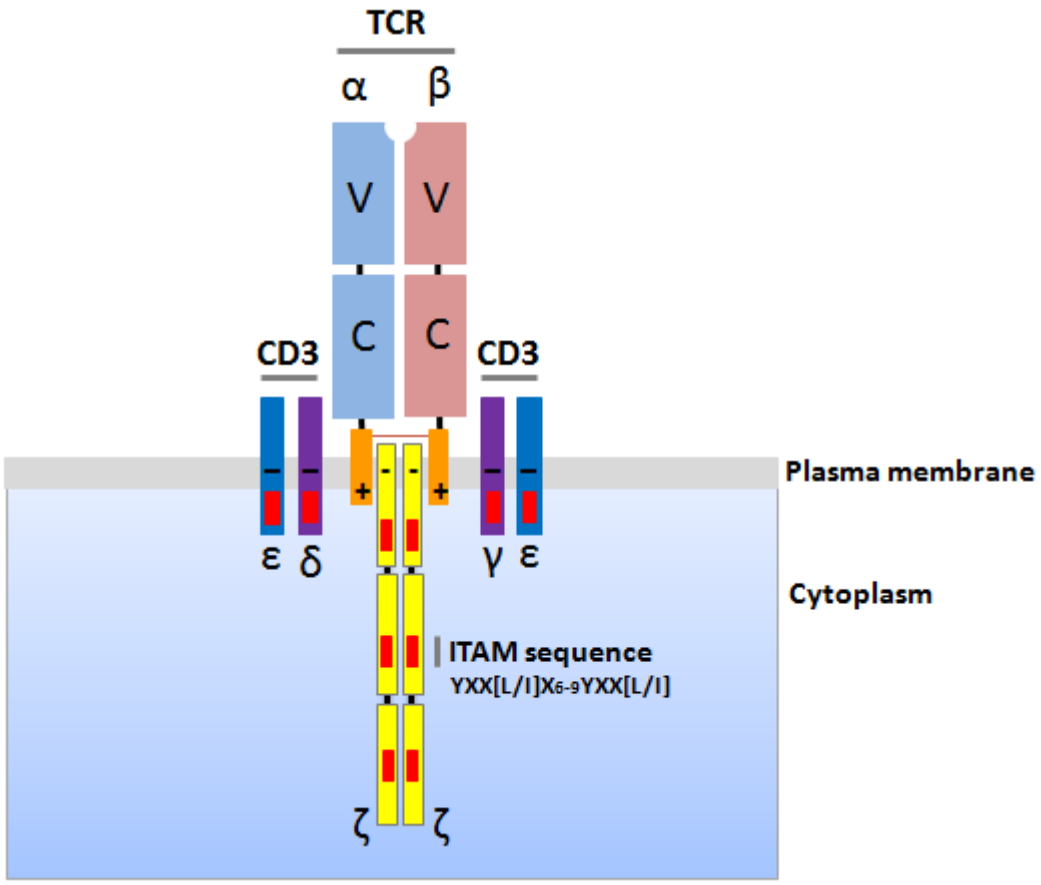

Figure 1.3. Schematic representation of the TCR/CD3 complex: The TCR is heterodimer consisting of two chains TCRα and TCRβ. Each TCR chain contains a variable (V), a constant (C), and a positively charged (+) transmembrane region. A functional TCR/CD3 complex is formed from the association of positively charged TCR with negatively charged (-) CD3δε and CD3γε heterodimers, and a homodimer of ζ chains. The red segment in CD3 and ζ chains indicates one ITAM signalling motif. Each CD3 chain contains one ITAM, whereas each ζ chain contains three ITAMs. Each ITAM is composed of two YXX[L/I] motifs separated by about six to nine amino acids. X in the ITAM sequence represents any amino acid *(adapted from Murphy K. et al., 2007).*

transduce signals in the cytoplasm because of the lack of intrinsic enzymatic activity. Therefore, the TCR relies on CD3 ζ-chain chains. The negatively charged transmembrane residues of the CD3 subunits and the ζ chains assemble with the positively charged transmembrane residues of TCRαβ chains to form the TCR/CD3 complex. The distinct feature of CD3 molecules and ζ chains is the presence of immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic part. Each ITAM consists of two conserved tyrosine motifs YXXL/I (where X represents any amino acid) interspaced by 6-9 amino acids. Each ζ chain contains 3 ITAMs, whereas CD3 subunits contain one ITAM each. Thus, the TCR/CD3 complex contains 10 ITAMs in total (figure 3). TCR triggering brings the ITAMs in close proximity of Lck, a crucial tyrosine kinase that phosphorylates the ITAMs (Figure 4). Phosphorylated ITAMs provide signalling platforms and recruit another tyrosine kinase the zeta chain-associated protein of 70 kDa (ZAP-70). ZAP-70 contains two tandem SH2 domains that can bind to the phosphorylated tyrosines in the ITAMs. Binding of ZAP-70 to the ITAMs induces conformational changes in ZAP-70 which becomes in turn ready for Lck-mediated phosphorylation. ZAP-70 phosphorylation results in its activation. Activated ZAP-70 phosphorylates two scaffold molecules the Linker for Activation of T cells (LAT) and the SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP-76) (Figure 4) (Smith-Garvin JE. et al. 2010).

LAT is a transmembrane adaptor protein containing nine tyrosine residues, which are phosphorylated by ZAP-70 upon TCR triggering. Phosphorylated LAT directly interacts with signalling molecules such as Grb-2 related adapter protein downstream of Shc (GADS), Phospholipase C gamma 1 (PLCγ1), and Growth factor receptor-bound protein-2 (Grb-2). Via these molecules, LAT assembles a signalling complex also including SLP-76, SOS, Itk, Vav1, etc. The LAT signalosome mediates the activation of different cellular signalling pathways leading to gene transcription and cytoskeletal reorganization. Activation of PLCγ1 is one of the key events mediated by the LAT signalosome. Activated PLCγ1 hydrolizes the membrane lipid PIP2 (phosphatidylinositol-4,5-bisphosphate), thus generating the second messengers IP3 (inositol 1,4,5-trisphosphate) and diacylglycerol (DAG). IP3 and DAG will in turn mediate the activation of three different transcription factors NF-κB, NFAT and AP1 (Figure 4).

1.5.1 Activation of NF-κB

DAG is localised at the plasma membrane and is crucial for NF-κB activation. It recruits PKCθ to the plasma membrane, an event required for its activation. In turn, PKCθ phosphorylates a scaffold protein CARMA1. CARMA1 recruits two other proteins Bcl10 and MALT1 to the plasma membrane to form the membrane-associated CBM (CARMA1/Bcl10/MALT1) complex. Formation of the CBM complex recruits and activates IκB

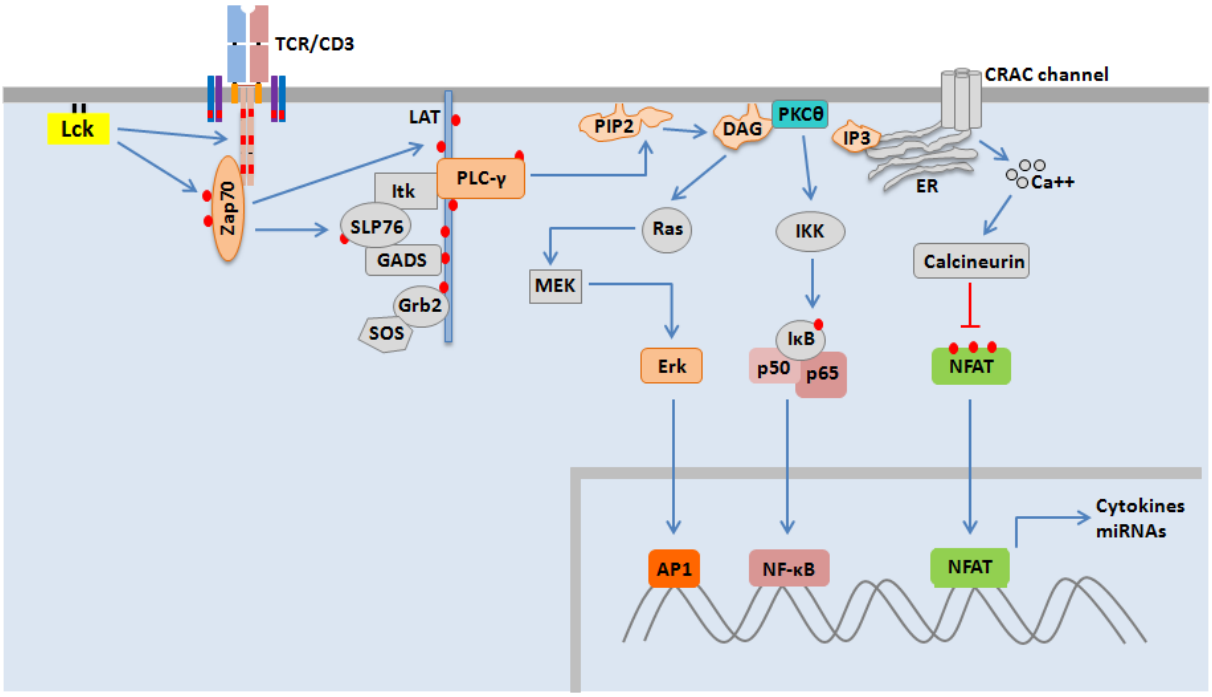

Figure 1.4. Organization of TCR-mediated signalling events. Triggering the TCR leads to phosphorylation of Lck. Lck in turn phosphorylates the ITAMs of the TCR/CD3 complex, allowing recruitment and Lck-mediated activation of ZAP70. Subsequently, activated ZAP70 phosphorylates the adaptor molecules LAT and SLP76, thus facilitating the formation of the LAT signalosome. The assembled LAT signalosome consists of LAT, Grb2, SOS, Gads, SLP76, PLCγ1, Itk etc. Within the LAT signalosome, Itk phosphorylates PLCγ1, which subsequently hydrolyzes PIP2 to generate two second messengers DAG and IP3. DAG mediates the activation of transcription factor AP1 via the activation of Ras-Erk pathway. In addition, DAG also recruits PKC-θ to the plasma membrane in which is required for the activation of NF-κB (p50 and p65 heterodimer). PKC-θ activates the IKK complex. IKK phosphorylates and promotes the degradation of IκB thereby enabling the translocation of NF-κB into the nucleus. On the other hand, IP3 participate in the activation of NFAT by increasing the intracellular Ca^{++} flux by triggering the release of Ca^{++} from endoplasmic reticulum (ER) Ca^{++} stores. In addition, the opening of CRAC channels further increase the influx of Ca^{++} inside the cells. Ca++ signalling activates the phosphatase calcineurin which dephosphorylates NFAT, thus allowing NFAT translocation into the nucleus. Activated AP1, NFκB, and NFAT drive the synthesis of the cytokines and miRNAs to further support T-cell activation, proliferation and differentiation. Phosphorylation of crucial molecules is indicated by red dots *(adapted from Smith-Garvin JE. et al. 2009).*

kinase (IKK). This results in the phosphorylation of IκB and its degradation. This leads to the release of NF-κB, thus resulting in its translocation to the nucleus where NF-κB stimulates the transcription of its target genes. NF-κB exists as a dimer and it is typically formed of p65 and p50, which belong to the Rel family of transcription factors.

1.5.2 Activation of NFAT

In resting cells, NFAT exists in a phosphorylated state that is kept in the cytoplasm in the inactive state. IP3, which is generated by PLC-γ1, diffuses in the cytoplasm and binds to the IP3 receptors on the endoplasmic reticulum, thus stimulating the release of stored calcium into the cytosol. This leads to the opening of calcium channels known as CRAC (calcium release-activated calcium) at the plasma membrane, thus allowing the entry of extracellular calcium into the cell. Increase in intracellular free Ca^{++} levels induces conformational changes in calmodulin which in turn activates calcineurin. Ultimately, calcineurin dephosphorylates NFAT, thus allowing its translocation into the nucleus.

1.5.3 Activation of AP1

AP1 is a heterodimer composed of Fos and Jun, whose formation depends on Ras-Erk signalling. Ras is a small GTPase that hydrolyses GTP to GDP. In the resting state, Ras is bound to GDP and becomes active when it is loaded with GTP. Upon T cell activation, PLC-γ1-generated DAG activates the GTP-exchange factor RasGRP1 that specifically activates Ras by favouring the association of Ras with GTP. GTP-bound active Ras triggers the activation of the mitogen activated protein (MAP) kinase cascade which is a three-step kinase system. First, active Ras binds MAP kinase kinase kinase (Raf), a serine/threonine kinase, through its Ras binding domain (RBD). This allows Raf to dimerize and to activate another kinase in the cascade called MAP kinase kinase (MEK), a dual specificity kinase. Finally, activated MEK phosphorylates the MAP kinase, also called Extracellular signal-regulated kinase (Erk). Activated Erk is essential for the generation of the AP1 transcription complex.

All three transcription factors are indispensable for T-cell activation and functional responses. One of the first events that occur upon T-cell activation is the transactivation of the CD69 gene, which is exclusively carried out by AP1. Thus CD69 serves as one of the earliest markers of T-cell transcriptional activation. On the other hand, the combinatorial effect of all these transcription factors drives IL-2 production, which is essential for T cell clonal expansion.

1.5.4. Additional signals required for T-cell activation

Although TCR triggering is essential for T-cell activation, additional signals are provided by costimulatory molecules which are required for full T-cell activation. Among these, CD28 is the most well-known costimulatory molecules. CD28 interacts with its ligands CD80 or CD86 expressed on APC. Ligation of CD28 activates phosphoinositide 3-kinase (PI3K), thus leading to the activation ofAk thymoma(Akt). Akt is involved in enhancing NF-κB nuclear translocation by associating with CARMA1 and facilitating the formation of CBM complex. Akt is also involved in NFAT activation by inactivating the negative regulators of NFAT nuclear translocation such as GSK3 and Homer. On the other hand, CD28 signalling is also important for the membrane localization of Itk. Once T cells are fully activated, their proliferation and further differentiation into effector cells is mediated by the cytokine milieu of the microenvironment (Chen L, 2013).

1.6. Proliferation and differentiation of T cells

1.6.1. Proliferation:

IL-2 is one of the important cytokines that is principally secreted by T cells upon triggering of the TCR. IL-2 is required for T-cell proliferation/expansion and for the acquisition of effector functions. It binds to the IL-2 receptor (IL-2R) which is composed of IL-2Rα (CD25), IL-2β (CD122), and the common γ chains (CD132). Among different T-cell subsets, Tregs express high levels of CD25 and they consume much of IL-2 that is produced during the course of immune response. By limiting the availability of IL-2, Tregs suppress clonal expansion of effector T cells. Deficiency of IL-2 signalling leads to wide spectrum of abnormalities including severe autoimmunity, lymphadenopathy and persistent viral infections (Malek TR. 2008).

1.6.2. Differentiation:

T-helper cells can be further subdivided into different Th-cell subsets based on their cytokine secretion profile (Zhu J. et al., 2010). I will briefly discuss the importance of a few well-defined Th-cell subsets and their secreted cytokines in the course of immune response (see also table 1).

Th1 cells secrete IFN-γ and activate macrophages, thus enhancing their ability to kill ingested pathogens. Th-1 cells secrete also several other proinflammatory cytokines such as TNF-α, lymphotoxin, and Granulocyte-Macrophage Colony-stimulating factor (GM-CSF) and are characterized by the expression of the transcription factor T-bet. IFN-γ is a signature cytokine of Th1 cells. It is produced in response to both bacterial and viral infections. IFN-γ also inhibits proliferation and induces apoptosis of cells. Because of these potent immunomodulatory effects, IFN-γ can be considered as a potent pro-inflammatory cytokine

and its increased production is frequently associated with autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) and autoimmune type 1 diabetes. IFN-γ production is negatively regulated by IL-4, IL-10 and TGF-β (Romagnani S. 2000).

Th2 cells are characterized by the production of IL-4, IL-5, IL-9, and IL-13. They participate in the proliferation and differentiation of B cells into antibody secreting plasma cells. One of the immunopathological roles of Th2 cells is their involvement in allergies. By inducing IgE production, Th2 cells also participate in the elimination of multicellular parasites and other environmental antigens. IL-4 is a signature cytokine of Th2 cells. It is mainly involved in type 2 immunity to helminths or allergic airways disease. IL-4 promotes the secretion of IgE antibody by B-cells, eosinophilia, smooth muscle contraction and mucus over production in response to helminthic infections, asthma and allergic inflammation. On the other hand, IL-4 antagonizes Th1 differentiation and also Th17 differentiation and suppresses the secretion of IL-1, IFN-γ, TNF-α, reactive oxygen species (ROS), and reactive nitrogen species (RNS), thereby acting as anti-inflammatory cytokine. However, because of their role in the activation of B cells, Th2 cells may also contribute to autoimmune disorders by enhancing the production of autoantibodies (Romagnani S. 2010; Pulendran B. et al., 2012).

Th17 cells secrete IL-17 and participate in inflammatory responses against extracellular bacteria, fungi and parasites. In addition to IL-17, Th17 cells also secrete IL-6, IL-21, IL-22 and IL-26. However, much of the pathogenic functions of Th17 cells are attributed to IL-17. IL-17 induces the production of pro-inflammatory mediators such as IL-1β, TNF-α, GM-CSF and recruits neutrophils and monocytes to the site of inflammation. It also induces the expression of other inflammatory mediators such as intracellular adhesion molecule 1 (ICAM1), prostaglandin E2 (PGE2) which further promote the production of IL-17 through positive-feedback mechanism and thereby sustain a proinflammatory environment and may cause tissue pathology. Additionally, IL-17 secreting T cells were shown to play a key role in the induction of organ specific autoimmune disorders in murine models such as Systemic Lupus erythematosus (SLE), experimental autoimmune encephalomyelitis (EAE), type 1 diabetes in NOD mice, antibody-independent RA (Korn T. et al., 2009).

Treg cells are Th cells with regulatory functions. Based on the expression of forkhead box protein 3 (FoxP3), Tregs can be distinguished in two subsets: natural (constitutive FoxP3 expression) or induced (FoxP3 is induced upon stimulation) Tregs. They are actively involved in the peripheral tolerance and participate in the suppression of immune responses that lead to autoimmune diseases, allergies, infection-induced immunopathology etc., They also play a role in the prevention of transplant rejection and in the maintenance of immune homeostasis. Tregs secrete IL-10 and TGF-β. Both these cytokines suppress inflammation and

Th subsets	Cytokine	Functions
Th1	IFN-γ, TNF-α, IL-2, GM-CSF, Fas ligand, CD40 ligand	-Activation of macrophages - Inhibition of Th2 differentiation - upregulation of MHC class I and class II molecules -activation of NK cells - induction of B-cell proliferation and isotype switching (IgG) - induction of reactive oxygen intermediates - activation of anti-bacterial, anti-viral and anti-tumour responses.
Th2	IL-4, IL-5, IL-9, IL-10, IL-13, CD40 ligand	-Activation and proliferation of B cells, isotype switching - induction of MHC class II molecules - inhibition of macrophage activation - supporting the survival of mast cells and eosinophils, - anti-parasitic immune responses - asthma and other allergic responses
Th17	IL-17, IL-6, IL-21, IL-22,	- Stimulation of neutrophil recruitment to the sites of inflammation - induction of pro-inflammatory cytokines such as IL-1β, GM-CSF, TNF-α - involvement in autoimmune disorders
Treg	IL-10, TGF-β	- Inhibition of T-cell proliferation - inhibition of the activation of macrophages, - induction of IgA isotype switching - participateion in immune suppressive mechanisms - induction of peripheral tolerance
T_{FH}	IL-21, IL-10, IL-4, IL-12, IFN-γ	- Regulateion of the formation and maintenance of germinal centers - augmentation of B-cell activation and proliferation - maintenance and regulation of humoral immune responses - development of long-term antibody responses - participation in anti-parasitic immune responses and some autoimmune disorders including Sjogren's syndrome and systemic lupus erythematosus

Table 1. Main features of Th cell subsets.

autoimmune disorders. IL-10 also inhibits the activity of Th1 cells, NK cells and macrophages. In addition to its anti-inflammatory properties, IL-10 has also been shown to promote inflammatory responses in humans by enhancing LPS-induced IFN-γ release and by increasing the activation of cytotoxic T cells and NK cells. Furthermore, Tregs compete with other Th substes for the binding of IL-2. In this way Tregs also inhibit the proliferation of other Th cell subsets and indirectly suppress excessive immune responses that might cause collateral tissue damage (Josefowicz SZ. 2012).

Tfh cells are essential in the development of humoral immune responses. Tfh cells are characterised by the secretion of IL-21, IL-10, IL-4, and IL-12. Upon stimulation, they migrate to the follicle regions of secondary lymphoid organs and participate in the formation and maintenance of germinal centres and the activation, proliferation, and differentiation of B cells into antibody secreting plasma and memory cells. They are implicated in autoimmune diseases such as systemic lupus erythematosus and Sjogren's syndrome (Crotty S. 2011).

1.6.3. Other cytokines involved in the immune response

IL-6

IL-6 is one of the pro-inflammatory cytokines produced by T cells. It acts as a growth factor of T and B cells. It promotes the differentiation of Th cells into Th17 and Tfh subsets and inhibits Treg differentiation. It induces IgA production in B cells. It has pathological roles in inflammatory, automimune and malignant diseases (Rincon M. 2012).

IL-8

IL-8 is known not as a typical cytokine produced by T cells. it acts as a pro-inflammatory cytokine mainly by attracting neutrophils to the site of infection and inflammation. It has been shown that the production of IL-8 is enhanced under Th17 differentiation conditions. During the course of immune response IL-8 also promotes phagocytosis and respiratory burst, and it increases the release of histamine (Bickel M. 1993).

1.7. Regulation of T cell activation

In the previous section, I have discussed about how T-cell proliferation and differentiation is regulated by cytokines and the role of different Th subsets in inflammatory responses. All these events are initiated upon TCR triggering. Therefore, to ensure appropriate immune response and to avoid unwanted immune reactions, which may lead to autoimmunity or immunodeficiency, TCR-mediated signalling must be tightly controlled. Cells can employ different strategies to regulate signalling. One level of regulation is represented by post-translational modifications (e.g. phosphorylation, ubiquitination etc.) of signalling molecules, which in turn results in their activation, deactivation or relocalization within the cell. These mechanisms of regulation of signalling events at biochemical level have been studied very

well during the last several years. Apart from biochemical modifications, cells can also modulate signalling by either increasing or decreasing the expression levels of signalling molecules. This may occur at both transcriptional and post-transcriptional levels. However, very little is known about the role of post-transcriptional regulation in TCR-mediated signalling. A newly discovered class of small regulatory molecules named microRNAs has been shown to play a major role in the post-transcriptional regulation of protein expression (Lee, R.C. and Ambrose, V. 2001). Here, I will discuss about what miRNAs are, how they regulate signalling events, and their functions in the immune system.

1.8. microRNAs

Over the past years, one of the most significant advances in cell biology has been the discovery of small (typically ~20-30 nucleotides long), non-coding RNAs that regulate gene expression. In general, these small RNAs act as inhibitors of gene expression and their corresponding regulatory mechanisms are collectively referred to as RNA silencing or RNA interference (RNAi). Small RNAs can be divided in three main classes which include microRNAs (miRNAs), short interfering RNAs (siRNAs), piwi interacting RNAs (piwiRNAs). miRNAs and siRNAs are approximately 21 nucleotides long, which associate with ARGO proteins to mediate post-transcriptional regulation of mRNAs. piwiRNAs, on the other hand, are 24-30 nucleotides long that bind to PIWI proteins and are involved in silencing transposable elements in germline cells. Here, I will focus on miRNAs because of their emerging role as the master regulators of gene expression due to their ability to target several mRNAs at post-transcriptional level.

1.8.1. Biogenesis of mammalian miRNAs

miRNAs represent an abundant class of non-coding small RNAs of approximately 22 nucleotides in length that function as regulators of protein expression by inducing degradation of target messenger RNAs (mRNAs) (Bartel, D.P. 2004). The mammalian genome encodes hundreds of miRNA genes. For example, in the human genome more than 1000 miRNA genes have been identified. miRNAs are transcribed both as individual and in clustered form. Individual miRNAs are transcribed from their own promoters and the clustered miRNAs are transcribed from a single polycistronic unit as polycistronic primary transcripts. A simplified view of miRNA biogenesis is depicted below (figure 5). Transcription of miRNA genes is mediated by RNA polymerase II (Pol II), and sometimes by RNA polymerase III (Pol III) to produce the primary miRNA transcripts or pri-miRNAs. In general, pri-miRNAs are generated from the intronic sequences of the genome. Pri-miRNAs are usually several kilobases long and contain internal uridine stretches. Pri-miRNAs also contain both cap structures at their 5’ end and poly (A) tail at their 3’ end. One of the

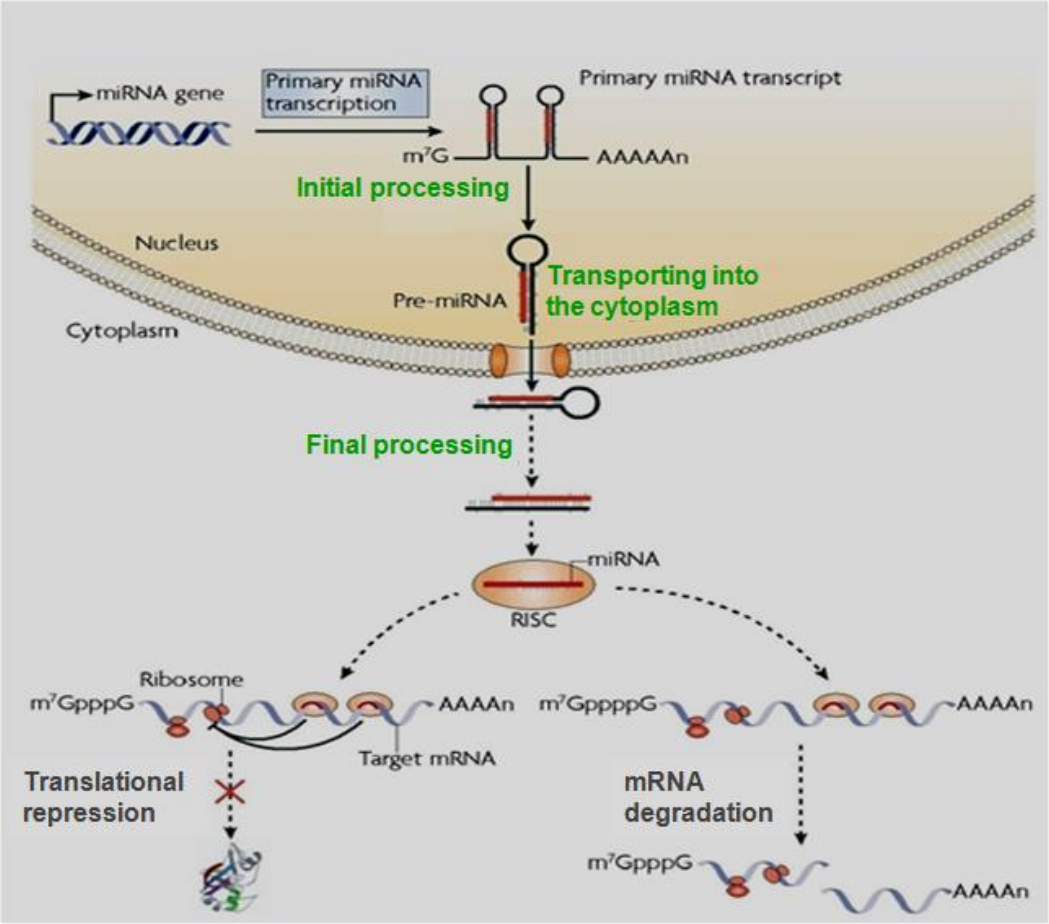

Figure 1.5. miRNA biogenesis and function. miRNAs are transcribed as long primary transcripts (pri-miRNAs) in the nucleus. These primary transcripts are initially processed into pre-miRNAs by the ribonuclease III enzyme Drosha. Pre-miRNAs are then transported into the cytoplasm by exportins where they are finally processed into miRNA duplexes by Dicer. One of the strands from the duplex is eventually loaded into RNA-induced silencing complex (RISC) and binds target mRNAs. A partial complementarity between miRNA and mRNA leads to translational repression, whereas the prefect complementarity leads to mRNA degradation. *(modified from Lodish H.F. et al. 2008)*

characteristic features of pri-miRNAs is the presence of hairpin structures which represent the miRNA stem-loops. pri-miRNAs are processed by the RNase III family of enzymes into pre-miRNAs (Ambrose V, 2004; Bartel DP, 2004; Kim VN, 2005; Carthew RW, 2009; Graves P, 2012).

The RNase III family:

The RNase III family includes endoribonucleases that specifically cleave double stranded RNA. All RNase III family members contain a characteristic ribonuclease domain (RBD), which is commonly called as the 'RNase III domain'. These enzymes cleave and produce a characteristic terminal dsRNA structure consisting of a 5' phosphate group and a two base over hang at the 3' end. RNase III enzymes are subdivided into 3 groups based on the domain composition. These are:

1) Class I RNase III enzymes
2) Class II RNase III enzymes
3) Class III RNase III enzymes

Class I RNase III enzymes are the simplest and smallest ones which contain a single ribonuclear domain. Class II RNase III enzymes have a dsRBD and two ribonuclease domains, which are commonly referred to as RNase IIIa and RNase IIIb. A typical example for class II RNase III enzyme is Drosha (Figure 6a). Class III RNase III enzymes are largest and typically contain two ribonuclease domains; a dsRBD and an N-terminal DExD/H-Box helicase domain. They also contain two other domains; a small domain (DUF283) of unknown function and a PAZ domain. Class III RNase III enzymes are also known as Dicer family of enzymes (Figure 6b).

1.8.2 Nuclear processing by Drosha:

Drosha is a Class II RNase III enzyme. It contains two ribonuclease domains namely, RNase IIIDa and RNase IIIDb domain and a dsRNA binding domain (dsRBD). Drosha is involved in the nuclear processing of pri-miRNAs and also plays a major role in the maturation of miRNAs. The specificity of Drosha in recognising its substrates comes from a Drosha-associated protein called DiGeorge syndrome critical region gene 8 (DGCR8) or Pasha. Drosha associates with DGCR8 to form a microprocessor complex in which it cleaves long pri-miRNA transcripts and liberates stem-loop intermediates (Figure 6A). These stem-loop intermediates are typically ~60-70 nt in length that often contain a 5' phosphate and 2-nt 3' overhang, known as the pre-miRNAs. Once formed the pre-miRNA duplexes are exported to the cytoplasm for further processing and maturation (Han J, 2004).

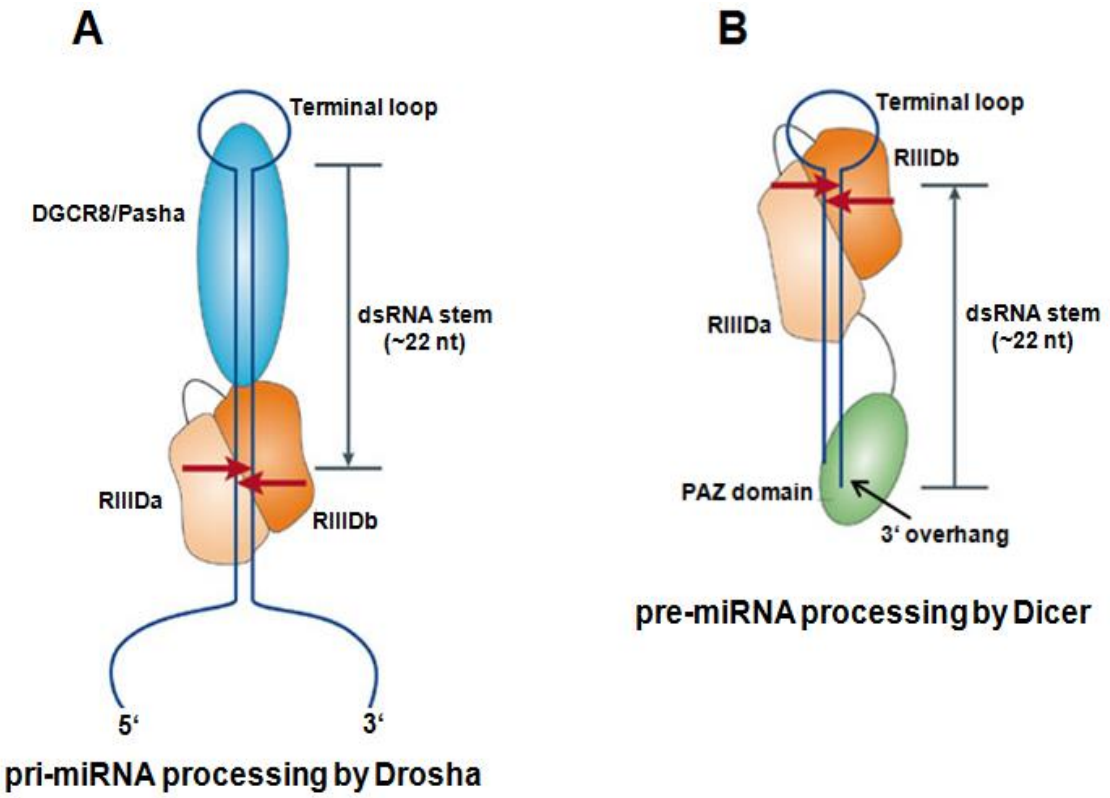

Figure 1.6. Processing of pri-miRNA and pre-miRNA by Drosha and Dicer. (A) Drosha mediates the generation of pre-miRNAs from pri-miRNAs. The two RNase III domains (RIIIDa and RIIIDb) of Drosha together form a processing centre. After recognising a pre-miRNA, the processing centre of Drosha cleaves two nearby phosphodiester bonds on opposite RNA strands. DGCR8/Pasha might help binding of and orienting Drosha on pri-miRNAs. Drosha mediated cuts (indicated by red arrows) are introduced at approximately two helical turns (~22 nucleotides) from the terminal loop to generate pre-miRNAs. (B) Dicer participates in the cytoplasmic processing of pre-miRNAs to generate mature miRNA duplexes. Using its RIIIDa and RIIIDb domains Dicer cleaves (indicated by red arrows) the pre-miRNA at approximately two helical turns (~22 nucleotides) from the 3’ end of the pre-miRNA. The PAZ domain of Dicer is believed to hold the 3’ end of pre-miRNA *(modified from Kim VN. 2005).*

1.8.3 Nuclear export of pre-miRNAs

Nuclear export of pre-miRNAs to the cytoplasm is mediated by a nuclear transport receptor known as exportin-5. Exportin-5 binds to the pre-miRNA cargo and also associates with its cofactor Ran coupled to GTP in the nucleus. Transportation occurs through nuclear pore complexes. Once transported, GTP of Ran is replaced by GDP in the cytoplasm, thus resulting in the release of pre-miRNA cargo from Exportin-5. In the cytoplasm, pre-miRNAs are subjected to final processing by Dicer to generate a mature miRNA duplex (Yi R, 2003).

1.8.4 Cytoplasmic processing by Dicer

Dicer is a class III RNase III ribonuclease which cleaves pre-miRNAs into miRNAs. It contains a N-terminal DExH-box RNA helicase domain, a Paz domain, two RNase III domains and a dsRNA binding domain. The two RNase III domains together form the dsRNA processing centre of Dicer and cleave phosphodiester bonds on each of the two strands. In addition, Dicer also associates with three cooperative-partner Argonaute proteins Ago2, PACT, TRBP and other accessory proteins to perform its function. Once pre-miRNAs are exported to the cytoplasm, they are engaged by Dicer's cooperative partners and the dsRNA processing centre of Dicer cuts the pre-miRNA at the bottom of the stem loop at about two helical turns away from the step loop. This generates a miRNA duplex of ~22 nt in length. The precise activity of the two RNase III domains generates the miRNA duplex with ~2 nt 3' overhangs. One of the strand in the miRNA duplex is called miRNA and its complementary strand is called miRNA*. The miRNA:miRNA* duplex is always associated with Ago2 proteins. The role of Dicer in the generation of mature miRNA is indispensable as the deletion of Dicer in mice resulted in the accumulation of pre-miRNAs and the diminished levels of mature miRNAs in the cells. The dicer-processed miRNA duplex resembles siRNA in structure and participates in mRNA silencing (Koscianska E, 2011; Ha M, 2014).

Once formed, the miRNA:miRNA* duplex is separated by an unknown mechanism into two separate strands. The mature miRNA strand is loaded into a ribonucleoprotein complex known as miRNA induced silencing complex (miRISC). The miRNA* is eventually degraded in the cytoplasm. However, it is still not yet clear which strand of the miRNA duplex will become miRNA or miRNA*. The loaded miRNA can bind the 3' untranslated regions (3' UTRs) of the target mRNA, thus downregulating gene expression (Bartel DP, 2004; Ha M, 2014). This can be achieved by either of two posttranscriptional mechanisms: mRNA degradation or translational repression of the mRNA. This is determined by the complementarity between the nucleotides of miRNA and mRNA. A partial complementarity promotes translational repression, whereas a perfect complementarity allows Ago2-mediated degradation of mRNA (Lodish HF, 2008).

1.8.5. Posttranscriptional repression by miRNAs

Because of their ability to form imperfect base pairing with the target mRNAs, virtually miRNAs can target several hundred of mRNAs. However, how the specificity of miRNA-mediated translational repression is regulated is still not clear. Currently, three models have been proposed to explain how miRNAs function. Before going through these models, I will briefly explain the mRNA translation process in eukaryotes to understand better about miRNA-mediated translational repression.

mRNA translation:

mRNAs have a 5' cap and a poly A sequence at their 3' end. Translation of mRNAs initiates when eukaryotic initiation factor (eIF) 4F binds the 5' cap region of mRNA. eIF4F forms a complex with the eIF4A and eIF4G subunits. Once recruited to 5' cap, eIF4F in the eIF4F complex interacts with eIF3, another elongation factor, and recruits 40S ribosomal subunit to the 5' end of the mRNA, thus forming the 40S pre-initiation complex. Eventually, this complex joins with the 60S ribosomal unit, which is recruited by eIF6, at the AUG start codon, thus initiating translation. eIF4F also interacts with another protein called PABP1 that binds the poly A sequence of mRNA at its 3' end. The interaction between eIF4F, PABP1, and mRNAs results in the circularization of the mRNAs. Circularization of mRNAs enhances the translation efficiency of mRNA. Any of these steps involved in the translation of mRNA can be blocked by miRNA resulting in translation repression (Carthew RW, 2009).

Models of miRNA-mediated target repression:

According to one of the models, the miRISC complex competes with eIF4F for binding to 5' cap region of mRNA, thus blocking the initiation of translation. Another model proposes that miRISC, through Ago2, inhibits the association of the 60S ribosomal subunit to the 40S preinitiation complex. Ago2 can also bind to eIF6, which is involved in the maturation of 60S ribosomal subunits. Thus, according to this third model, miRISC represses translation of mRNAs by inhibiting the binding of ribosomal units at the start codon on mRNAs. The third model proposes that miRISC stimulates deadenylation and prevents the binding of PABP1 to the 3' end of mRNA. Binding of miRISC to miRNA recruits deadenylases including CCR4, NOT and CAF1 to the 3' region of the mRNA. This event blocks the circularization of mRNA resulting in translational repression. Simultaneously, miRISC also promotes the association of decapping molecule DCP1 to the 5' region of mRNA and induce degradation of mRNA. (Figure 7) (Carthew RW, 2009).

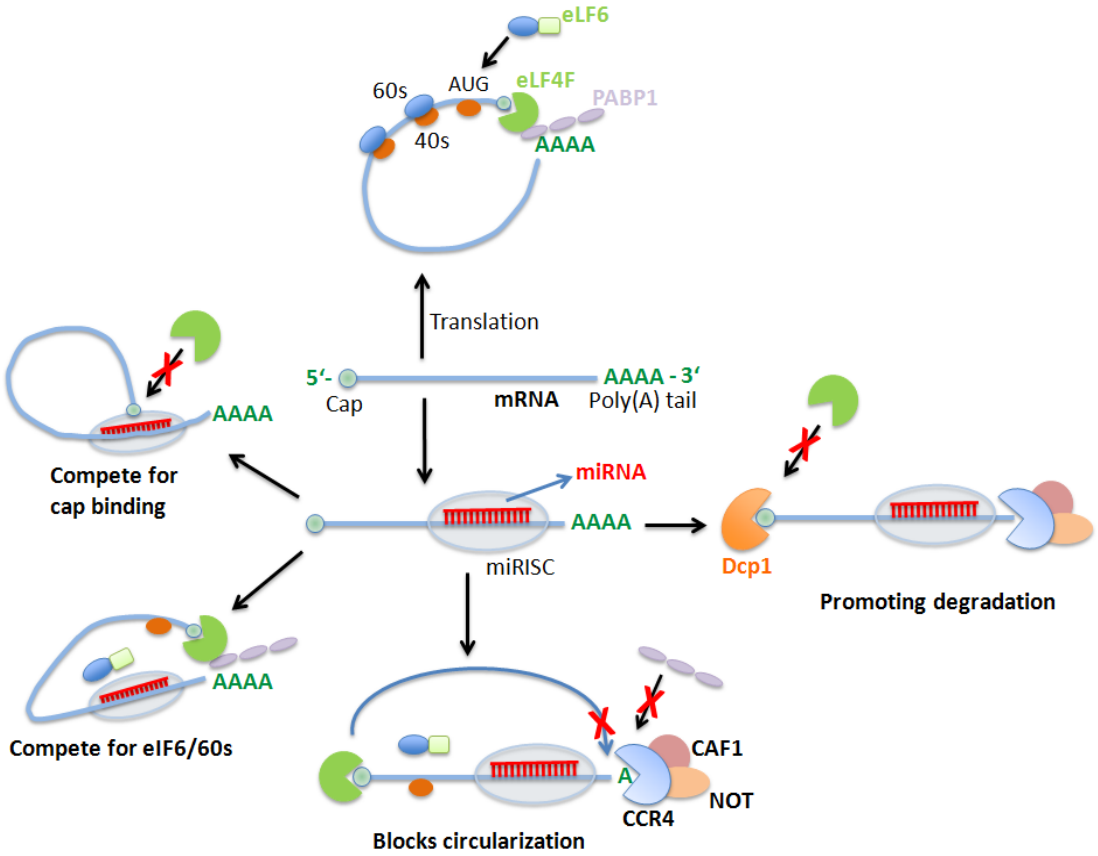

Figure 1.7. Possible mechanisms of miRNA-mediated trananslational repression. Once transcribed, usually a mRNA recruits ribosomal subunits and initiation factors to form circularised structures that leads to translation (top). However, when the mRNA is targeted by a miRNA, the mRNA is pulled into the miRISC resulting in either translation repression (left and bottom) or degradation of mRNA (right) *(modified from Carthew RW and Sontheimer EJ. 2009).*

1.8.6. Degradation of mRNAs by miRNAs

Although it is thought that the perfect complementarity between miRNA and mRNA will result in Ago2-mediated mRNA degradation, it is not yet clear how exactly this occurs. Some studies showed that mRNA degradation is due to deadenylation or decapping and not because of Ago2-mediated mRNA cleavage. Nevertheless, it has also been shown that Ago-2 in the miRISC catalyses the cleavage of target mRNA at ~10 nt from the 5' end of the miRNA. The cleaved mRNA is then degraded and the miRISC is recycled to cleave additional target mRNAs (Rana TM, 2007). Because of their ability to repress multiple targets, miRNAs can act as an important cellular machinery to regulate a wide range of biological processes. There is growing body of evidence which suggests that miRNAs play crucial roles in the development and function of the immune system.

1.9. miRNAs in the immune system

miRNAs have been shown to modulate the vertebrate immune system by regulating hematopoietic stem cell development. Several miRNAs have been shown to suppress the expression of genes that affect the production of haematopoietic progenitor cells. For example, miR-221 and miR-222 modulate stem cell homeostasis by regulating Kit or c-Kit expression. miR-126 has been shown to promote the expansion of progenitor cells by targeting tumour suppressor Polo-like Kinase 2 (PLK2) and Homeobox preotein A-9 (HOXA9), which regulates HSC homeostasis. In the innate immune system, miRNAs modulate the development of the myeloid cell lineage by regulating the expression of lineage-specific transcription factors. Overexpression of the miR-17~92 cluster and its paralogs has been shown to control monocytopoiesis by negatively regulating the expression of RUNT-related transcription factor 1 (RUNX1; also known as AML-1). RUNX1 regulates the development of HSCs into different hematopoietic lineages and also increases M-CSFR expression on monocytes. M-CSFR promotes the differentiation of monocytes into macrophages. On the other hand, miR-223 promotes monocyte differentiation by targeting nuclear factor I/A (NFI-A), which negatively regulates myeloid cell progenitors differentiation into monocytes. Likewise, miR-424 promote neutrophil differentiation and proliferation by repressing NFI-A. miR-155 has been identified as a crucial regulator of myeloid cell differentiation as it targets SH2-domain-containing inositol-5-phosphatase 1 (SHIP1) (which a negative regulator of phosphoinositide 3-kinase (PI3K)/Akt pathway)) and transcription factor PU.1 (which is specifically involved in the differentiation of macrophages and B cells). In addition to the role in the development of innate immune cells, miRNAs also regulate signalling in innate immune cells downstream of Toll-Like Receptors (TLRs). miR-155 positively regulates TLR signalling by targeting Suppressor of cytokine signalling 1 (SOCS1) and SHIP1, which are negative regulators of TLR signalling. miR-146a, whose expression

depends on NF-κB, inhibits aberrant activation of innate immune cells by targeting IL-1 Receptor-associated Kinase 1 (IRAK1) and TNF-Receptor-Associated Factor 6 (TRAF6), which are TLR-signalling molecules (O'Connell RM, 2010).

1.10. Physiological roles of miRNAs in T cells

Besides their role in the regulation of development and function of heamatopoietic stem cells of the innate immune system, miRNAs also play an indispensable role in T cell biology. Expression profiling of miRNAs in different T-cell subsets and in different stages of T-cell development has revealed that miRNA-mediated regulation of signalling networks is dynamic and highly regulated. T-cell specific deletion of Dicer in mice resulted in decreased numbers of T cells and abnormality in T-cell differentiation. This finding suggests that miRNAs are required for both T-cell development and function (Mehta A, 2016). Regarding the role of miRNAs during T-cell development, it has been shown that the deletion or the overexpression of certain miRNAs impairs developmental progression at various stages (Figure 8A). For example, the miR-17-92 cluster suppresses the expression of Bcl-2-interacting mediator of death (BIM) and phosphatise and tensin homologue (PTEN) and influences T-cell survival at the DN2 stage (Xiao, C. et al,. 2008). Another work shows that miR-181a, which is highly expressed in thymocytes, can modulate TCR signalling strength and T-cell development by directly targeting certain phosphatases (Li, Q.J. et al.,). Recently, it has been demonstrated that the heamatopoietic cell-specific loss of miR-17-92 cluster resulted in profound defects in the transition of DN thymocytes to DP stage, which is due to reduced expression of IL-7 receptor and concomitant limited responsiveness to IL-7 signalling, which is crucial for thymic development (Regelin M, 2015). miRNAs can also modulate the differentiation of mature T cells in the periphery (Figure 8B). For example, it has been shown that miR-155 promotes the differentiation of T cells into the Th2 subset (Thai TH, 2007). Moreover, miR-155 is also important for the survival of regulatory T cells (Treg cells) by targeting SOCS1 (Lu LF,2009). It has been shown that miR-326 plays an exclusive role in Th17 differentiation by directly targeting Ets-1 (Du, C. et al,. 2009). I have listed targets and functions of miRNAs in T-cell development, proliferation, differentiationand other function in the table below (Table 2). In addition, I will discuss about the functions of a few selected miRNAs, which are relevant to my work, in more detail.

1.10.1. miR-21

miR-21 was identified as an oncomir (miRNA that promotes tumours). It is significantly overexpressed in a wide range of solid tumours affecting breast, lung, colon, stomach and pancreas. miR-21 is also upregulated in leukemia (CLL, AML) and lymphomas (DLBCL,

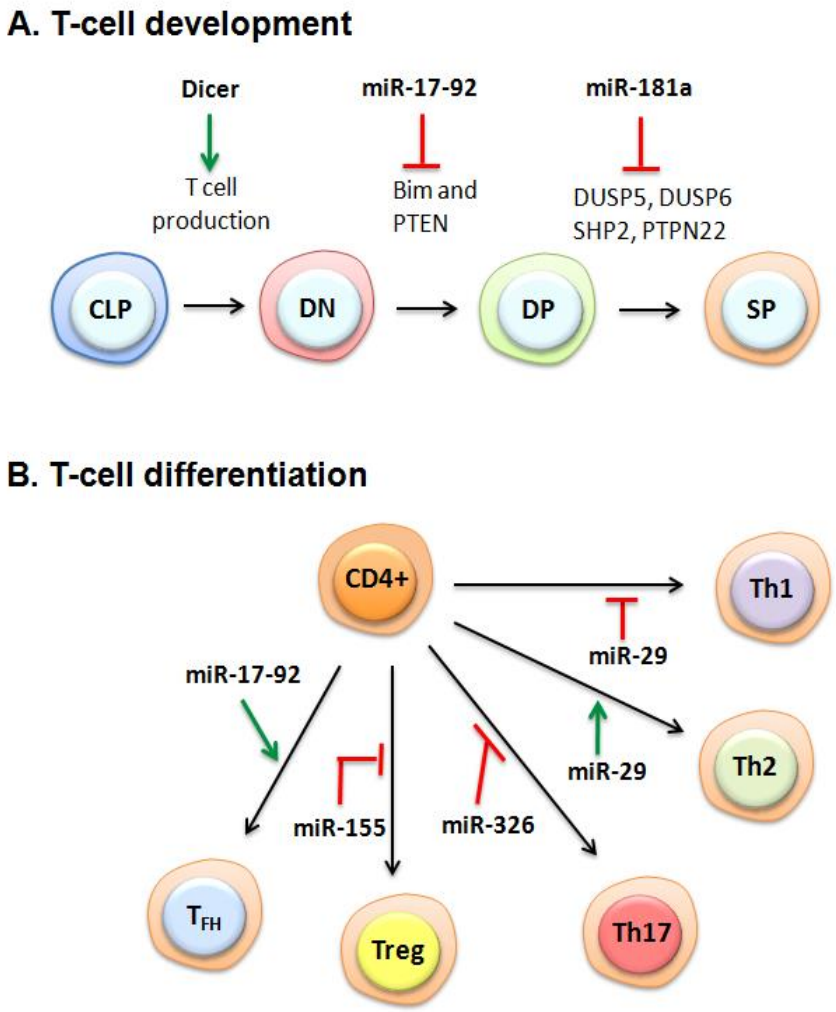

Figure 1.8. Schematic representation of the function of crucial miRNAs in T-cell development and differentiation. A) miRNAs tightly regulate T-cell development. Deletion of Dicer results in the block of T-cell development from CLPs. On the other hand, different miRNAs have been show to fine tune different stages of T-cell development in the thymus. miR-17~92 cluster promotes the progression of thymocytes from DN to DP stage by regulating the expression of Bim and PTEN. miR-181a has been shown to play roles in thymic selection during DP to SP progression by modulating the expression of DUSP5, DUSP6, SHP2 and PTPN22. B) miRNAs also regulate differentiation of T cells in the periphery. Different miRNAs have been shown to regulate the differentiation of $CD4^+$ T cells to different T-cell subsets as depicted in the figure *(adapted from Sethi A. et al. 2013).*

Hodgkins) (Musilova K, 2015). The tumor promoting ability of miR-21 is because of its ability to target PTEN, which is a known tumor suppressor (Pezzolesi MG, 2008).This observation indicates that activation of the PI3K pathway, due to loss of PTEN, may contribute to the increased proliferation of cellsoverexpressing miR-21.A recent work suggests that in the HL60 cellline, which is derived from Human promyeolcytic leukemic cells, miR-21 overexpression suppressed PMA-mediated differentiation into macrophages and led to increasedproliferation of these cells,suggesting a role for miR-21 in driving cell division. Other studies have found that overexpression of miR-21 may result in a decrease of Programmed Cell Death 4 (PDCD4), which is also a tumour suppressor gene associated with poor prognosis in lung and colorectal cancers(Asangani IA. et al., 2008). In addition, miR-21 expression is controlled by oncogenic Ras both *in vitro* and *in vivo*(Frezzetti D, 2011).

During the last few years, much work has been done to further elucidate the role of miR-21 in T cells. Analysis of the miRNA signature in human Tregs has shown that miR-21 increases the levels of FoxP3, a transcription factor and master regulator for the development of regulatory T cells (Rouas R. 2009). It has been additionally shown that miR-21 positively promotes Treg cells by inhibiting signal transducer and activator of transcription 3 (STAT3), a master transcription factor of Th-17 differentiation that antagonizes Treg cell differentiation (Shaowen W, 2016). However, a recent study in patients suffering from coronary heart disease revealed that miR-21 negatively regulates Treg differentiation (Li S, 2015). In addition to its role in Tregs, miR-21 also inhibits the inflammatory response in macrophages. More specifically, miR-21 regulates this process by targeting the expression of the tumour suppressor PDCD4, which is a negative regulator of IL-10 production. Following LPS stimulation, induction of miR-21 in macrophages decreased the levels of PDCD4 and this inhibition was abrogated upon treatment with a miR-21 antagomir (Sheedy FJ, 2010). Another study has shown that miR-21 expression is positively regulated via an Akt-dependent pathway and this mechanism supports the survival of the cells as miR-21 targets PTEN and PDCD4 (Syed D, 2010). miR-21 was also shown to play a positive role in autoimmune diseases. Inhibition of miR-21 in T cells from psoriasis and SLE patients reversed the severity of the disease by increasing the expression of IL-10, CD40L and PDCD4 (Stagakis E, 2011). When conditionally overexpressed in the heamatopoietic system in mice, miR-21 induced hematologic malignancies characterized by massive lymphadenopathy and splenomegaly due to the development of B-cell lymphoblastic lymphoma/leukemia. In contrast, deletion of miR-21 resulted in massive apoptosis of tumour cells and complete tumour regression (Medina PP, 2010). These data demonstrate the importance of miR-21 not only in the induction but also in the progression of this malignancy. In addition to its functions in tumour progression and cell survival, recent works show that

Stage	miRNAs	Targets	Function
Development	miR-181a	DUSP6, SHP2, PTPN22	Promotes positive selection
	miR-150	NOTCH3	Promotes T cell maturation
	miR-17-92	BIM and PTEN	Promotes T cell maturation
Activation Proliferation Apoptosis	miR-9	PRDM1	Inhibits proliferation
	miR-15	Bcl-2	Promotes apoptosis
	miR-17-92	Bim	Promotes apoptosis
	miR-31	KSR-2	Inhibits activation
	miR-122	Activates Akt pathway	Inhibits Apoptosis
	miR-130	CD69	Inhibits activation
	miR-155	CTLA4	promotes proliferation
	miR-182	FOXO1	Inhibits proliferation
	miR-214	PTEN	Promotes proliferation
Differentiation	miR-29	T-bet and IFN-γ	Inhibits Th1
	miR-17-92	TGFβRII, CREB1	Induces Th1
		PTEN, Phlpp2	Induces Tfh
	miR-21	IL-12	Inhibits Th1, Promotes Th2
	miR-146a	TRAF6, IRAK1	Inhibits Th1
	miR-155	SOCS1, SHIP1	Inhibits Th1 Promotes Treg
	miR-326	Ets1	Inhibits Th17
	miR-10	Bcl6, Ncor1	Inhibits Th17
	Let-7e	IL-4	Inhibits Th2

Table 2. Role of different miRNAs in the immune system.

miR-21 plays also a role in the regulation of T-cell activation. In primary mouse $CD4^+$ T cells miR-21 promotes Erk and Janus kinase (JNK) signalling by targeting Sprouty1, a negative regulator of Erk activation, upon TCR stimulation. However, another recent study reports that miR-21 acts as negative modulator of signal transduction downstream of the TCR. This study shows that miR-21 targets Guanine nucleotide-binding protein G(q) subunit alpha (GNAQ), upon T-cell activation (Carissimi C, 2014). GNAQ has been shown to positively regulate T-cell activation by inducing the activation of Lck. Collectively, miR-21 displays a diverse set of functions in the immune system, ranging from the regulation of Treg development and the modulation of the inflammatory response to the development of heamatopoietic malignancies.

1.10.2. miR-142

miR-142 plays different roles in the immune system. Unlike undergoing degradation, the passenger strand of miR-142 duplex (miR-142* or miR-142-3p) can also assemble in RISC complex and mediate the repression of target mRNAs. It has been shown that miR-142-3p acts as a positive modulator of hematopoiesis by repressing interferon regulatory factor 7 (IRF7)-mediated signalling (Lu X, 2013). In the periphery, miR-142-3p attenuates the migration of human $CD4^+$ T cells by targeting RAC1 (Ras-related C3 botulinum toxin substrate 1)and ROCK2 (Rho-associated, coiled-coil containing protein kinase 2), two known regulators of actin cytoskeleton (Liu J, 2014). Furthermore, miR-142-3p inhibits the expansion of regulatory T cells by targeting GARP (Glycoprotein A repetitions predominant), which is involved in the uncontrolled proliferation of tumours (Zhou Q, 2013). It has been shown that Tregs exert their suppressive function in part by transferring cAMP, which is a potent inhibitor of proliferation and IL-2 production in T cells, to responder T cells. Interestingly, Huang B. et al., showed that miR-142-3p restricts cAMP levels in cells by targeting AC9 mRNA, which regulates the production of cAMP. However, they have also observed that FoxP3 downregulates miR-142-3p in Treg cells. The authors therefore report that downregulation of miR-142-3p is needed to keep the AC9/cAMP pathway active in Treg cells (Huang B, 2009). miR-142 has also been shown to regulate the activation of T and B cells in SLE. Both miR-142-3p and miR-142-5p have been downregulated in SLE and enforced expression of these miRNAs increased the severity of the disease. Both miRNAs directly inhibited the expression of IL-10, CD84 and signaling lymphocytic activation molecule-associated protein (SAP), which are SLE-related targets. Furthermore, suppression of both miRNAs in $CD4^+$ T cells and B cells caused increased T-cell and B-cell activation (Ding S, 2012). In addition to its roles in lymphocytes, miR-142-3p has been shown to act as tumour suppressor by preventing the differentiation of macrophages during cancer-induced myelopoiesis. Myeloid-derived cells show immunosuppressive functions. During malignancies, tumour progression is accompanied by an altered myelopoiesis causing

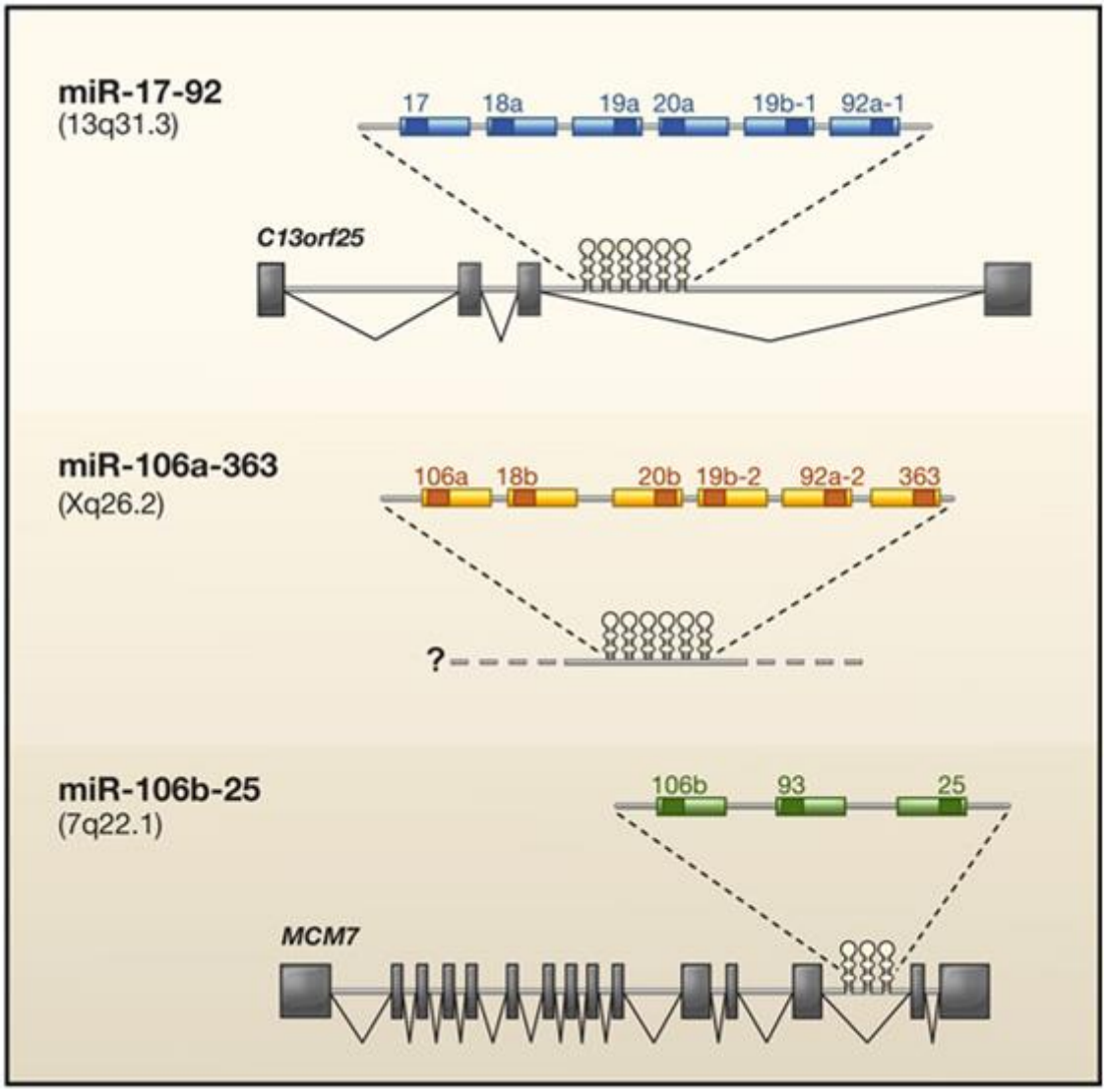

Figure 1.9. Schematic representation of the organisation of the miR-17~92 cluster and its paralogs. In humans, the miR-17~92 cluster is located on chromosome 13 in the third intron of a ~7kb primary transcript known as *C13orf25.* This primary transcript gives rise to six miRNAs (miR-17, 18a, 19a, 20a, 19b-1 and miR-92-1). Because of gene duplications, two paralogs of the miR-17~92 cluster have been formed in mammals. In humans, the miR-106b~25 cluster is located on chromosome 7, whereas the miR-106a~363 cluster is located on the X chromosome. The miR-106b~25 cluster is encoded from MCM7 primary transcript, whereas the primary transcript that encodes the miR-106a~363 cluster is currently not known. They give rise to there and six miRNAs, respectively *(adapted from Mendell JT. 2008).*

accumulation of immunosuppressive cells. Enforced expression of miR-142-3p inhibited the immunosuppression ability of macrophages by targeting gp130 and CCAAT-enhancer-binding protein (C/EBPβ) which are required for macrophage differentiation (Sonda N, 2013). In mice, deletion of miR-142 gene caused decreased proliferation and apoptosis in T cells. In addition T cells lacking miR-142 also showed decreased production of IFN-γ and IL-17 upon stimulation (Sun Y, 2015).

1.10.3. miR-17-92 cluster

Some miRNAs are transcribed as one common primary transcript giving rise to polycistronic miRNA clusters. One of these is the miRNA-17~92 cluster, which is located in an 800bp region within the third intron of the primary transcript *C13orf25* on human chromosome 13q31 (Ota A. et al., 2004). The genomic organization of this cluster is highly conserved among all vertebrates. This cluster encodes 7 miRNAs, miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a. It is homologous to two additional paralogs, miR-106a~363 and miR- 106b~25, likely originating from a series of duplications and deletions of the miR-17~92 cluster during evolution. All these clusters together can give rise up to 15 mature miRNAs (Figure 9).

Functional importance of the miR-17~92 cluster and its paralogs:

The miR-17~92 cluster is frequently overexpressed in tumours. The first observation came from studies in B-cell lymphoma (Ota A. et al., 2004). An additional work further demonstrated that overexpression of a truncated miR-17~92 cluster comprising miR-17~19b promoted disease progression of Eμ-myc-induced B-cell lymphoma (He L. et al., 2005). Subsequent studies demonstrated that the cluster is activated directly by the proto-oncogene c-myc or by a combination of c-myc and Ras (O´Donnell KA. et al., 2005; Dews M. et al., 2006). The miR-17~92 cluster acts together with the transcription factor *c-myc,* thus accelerating tumour growth and higher mortality rates in B-cell lymphoma in mouse models. Tumours isolated from these mice were also characterized by the lack of apoptosis that is normally present in *c-myc*-induced lymphomas. Further analysis of the miR-17~92 cluster has revealed that miR-19 is a key oncogenic component of the miR-17~92 cluster and that the oncogenic activity of miR-19 is due in part to the repression of the tumour suppressor *Pten* (Olive V. et al., 2009). In addition to its role in suppressing tumour cell apoptosis, the miR-17~92 cluster has recently been found to inhibit *Ras*-induced senescence via miR-17- and miR-20a-mediated suppression of p21, which is an inhibitor of cell proliferation and a promoter of senescence (Hong L. et al., 2010). Also several E2F family members, which are essential regulators of the cell cycle and apoptosis, have been shown to be direct targets of the miR-17~92 cluster. Furthermore, E2F1, E2F2, and E2F3 directly bind to the promoter of

the miR-17~92 cluster and activate its transcription, creating an autoregulatory feedback loop between miR-17~92 cluster members and E2F proteins (Sylvestre Y. Et al., 2007). It has additionally been shown that this cluster regulates cell cycle progression in various cell type by targeting p21 and Rb12 (Wang Q. 2008). In addition to its tumour promoting properties, a recent transgenic overexpression of the miR-17~92 cluster in mice resulted in the development of a fatal lymphoproliferative disease and autoimmunity. The same study also showed that the cluster targets two tumour suppressors, Bim and PTEN, thus favouring increased lymphocyte proliferation and reduced activation-induced cell death in transgenic mice (Xiao C. et al., 2008). In contrast, targeted deletion of the miR-17~92 cluster resulted in the death of mice shortly after birth due to lung hypoplasia and defects in the development of heart. Furthermore, ablation of the miR-17~92 cluster in mice resulted in increased levels of the pro-apoptotic protein Bim and an inhibition of pro-B to pre-B cell transition during B-cell development, due to excessive cell death (Ventura A. et al., 2008). Furthermore, it has also been shown that three miRNAs, miR-17, miR-20a of the miR-17~92 cluster and miR-106a of the miR-106a~363 cluster are involved in monocyte differentiation and maturation and their reduced expression results in increased levels of their target protein AML-1 (acute myeloid leukaemia-1) (Fontana L. et al., 2007). Recently, it has been found that the miR-17~92 cluster plays a role not only in cell-autonomous tumour survival, but also in tumour evasion from the immune system. Myeloid-derived suppressor cells (MDSCs) are a major component of the immune suppressive network, and expression of the transcription factor STAT3 induces expansion of MDSCs and suppresses activation of $CD4^+$ and $CD8^+$ T cells in tumour-bearing mice. STAT3 is a direct target of both miR-17-5p and miR-20a, and tumour-associated factors can downregulate the expression of these miRNAs in MDSCs. Thus, tumour-associated factors serve as indirect activators of STAT3 by inhibiting their repressor miRNA, which in turn leads to proliferation of MDSCs and to the suppression of T-cell activation (Zhang M. et al., 2011). This pathway could offer an attractive target in the development of cancer immunotherapies.

Very recently, two studies have shown that the miR-17~92 cluster plays indispensable roles in the differentiation of Tfhcells. In one study, Baumjohann *et al.* observed a striking increase in the expression of Tfh cell negative regulators including Ccr6, Il1r2, Ilr1, Rora, and Il22 in miR-17~92 deleted T cells during T_{FH} differentiation. Among these genes, the authors have identified RORα as a direct target of the miR-17~92 cluster that functions as an inhibitor of Tfh differentiation. Signalling via RORα is sufficient to induce IL-R1 and CCR6 which antagonizes Tfh differentiation (Baumjohann D. et al., 2013). In addition, they also observed that miR-17~92-deficient mouse T cells displayed impaired upregulation of Bcl-6 and CXCR5 which are hall marks of Tfh differentiation. Also the study from Kang SG *et al.* showed that

the miR-17~92 cluster plays essential roles in Tfh differentiation. Using a conventional knock-out mouse model for this cluster, they have observed that the mice deficient in miR-17~92 expression exhibited severely compromised Tfh differentiation and failed to control chronic viral infection. In contrast, they observed spontaneous accumulation of Tfh cells and severe immunopathology in T-cell specific miR-17~92 transgenic mice. The authors further showed that the miRNAs of this cluster control the migration of $CD4^+$ T cells into B-cell follicles in the secondary lymphoid organs by negatively regulating PI3K signalling by suppressing the expression of the AKT phosphatase PHLPP2 (Kang SG. et al., 2013).

So far, studies analysing the function of the miR-17~92 cluster in the immune system have been mainly focused on T-cell development and differentiation. However, not much is known about the contribution of the miR-17~92 cluster in T-cell activation. Although they originate from a single polycistronic unit and embedded in a single miRNA cluster, the seed sequence, which plays a key role in recognising target mRNAs, is not shared among the individual miRNAs of this cluster (Mendell JT. 2008). This observation raises the question whether individual members of this cluster have a specific, non-shared function with the other members of the cluster. For example, it has been shown that miR-20a but not other members of the cluster inhibits TGF-β signalling and may regulate the cross talk between the T-cell receptor and other surface receptors such as BMPR2 and TGFβR2 (Brock M. et al., 2009). Similarly, miR-19 has been shown to promote the production of Th2 cytokines during asthma (Simpson LJ. et al., 2015). These results suggest that there is selective expression of the individual members of this cluster in different cellular conditions.

1.11. Aims of the Study

In this study, I have investigated the role of miRNAs in T-cell activation. T-cell activation can be divided into two phases. An early phase usually lasting up to 2-4 hours after activation which is driven by TCR-mediated signalling and a second phase which is largely mediated by the cytokines produced during the initial wave of activation. To avoid the unwanted immune responses, both phases of T-cell activation must be tightly regulated. So far, work has been done on the role of miRNAs in the late phase of activation and little is known about the role of miRNAs in the early phase of activation. Since TCR-mediated signalling initiates all the molecular events leading to T-cell activation, I wanted to investigate whether miRNAs also play a role during early TCR signalling.

To address this question, I have firstly screened for new miRNAs that might regulate T-cell activation. To this aim, I have done a microarray analysis to fish out new miRNAs that are rapidly upregulated in T cells upon TCR triggering. I have found that several miRNAs are

upregulated within 2 h upon TCR triggering. Among them, I have selected three miRNAs – miR-20a, miR-21 and miR-142-5p to investigate their role in T cell activation.

Initially, I focused on miR-20a and investigated its role in T-cell activation. I have found that miR-20a negatively regulates TCR-mediated signaling by inhibiting ZAP-70. Accordingly, also the expression of CD69, which is an earlier marker of T cell activation, was inhibited. Furthermore, I have also found that miR-20a negatively regulates the expression of cytokines such as IL-2, IL-6, IL-8 and IL-10, which can regulate inflammatory responses. In addition, I have also found that the transactivation of miR-20a is strongly depends on AP1 and NFAT and to a lesser extent on NF-κB. Conversely to miR-20a, I have found that miR-21 and miR-142-5p positively regulates T-cell activation.

The results of my study are presented in the section below.

2. Results

2.1. Expression profiling of miRNAs upon T-cell stimulation

In order to investigate whether miRNAs are rapidly expressed at early time points upon T-cell activation, I have purified human peripheral blood $CD3^+$ T cells from a healthy donor. I have stimulated them with CD3/CD28 for a short period of time, and the expression pattern of miRNAs was analyzed using a miRXplore Microarray from Miltenyi. Since the stimulation conditions may strongly influence T-cell activation, I have used a stimulation protocol that is well established in our lab (Arndt B. et al., 2013). This method is based on agonistic CD3xCD28 Abs coated on microbeads (iAbs) as an artificial antigen presenting system. This is one of the best available systems that mimic physiological human T-cell activation *in vitro*.After stimulation for 120min, T cells were immediately frozen at –80°C and subsequently assayed for miRNA expression. The analysis included up to 700 miRNAs. The results show that the expression of different miRNAs was rapidly either increased or decreased upon T-cell stimulation. I have next selected six miRNAs whose expression was increased more than 2 folds compared to the unstimulated control T cells. The selected miRNA are 20a, 21, 29a, 29c, 30e, and 142-5p (Fig. 2.1A).

I have next confirmed the upregulation of the selected miRNAs by real-time PCR (Figure 2.1B). For these experiments, I used naïve human $CD4^+$ T cells. Purified naïve human $CD4^+$ T-cells were stimulated with microbeads coated with anti-CD3 and anti-CD28 antibodies (iAbs) for 2, 24, 48 and 72 hours. Total RNA, including small RNAs, was purified and cDNA was prepared by reverse transcription. Reverse transcription was done using miScript Reverse Transcription kit from Qiagene. miRNAs are not polyadenylated. Therefore, to allow their conversion into cDNA by reverse transcriptase, I have included in the reaction mixture a poly (A) polymerase that can polyadenylate the miRNAs. As shown in Figure 2.1B, real-time PCR analysis has revealed that not all the selected miRNAs were upregulated upon 2h TCR stimulation. I have confirmed that miR-20a, miR-29a, and miR-142-5p were indeed rapidly upregulated. Interestingly, the upregulation of miR-20a was sustained and lasted up to 48h upon T-cell activation. On the other hand, the expression of miR-29a and miR-142-5p were transient and was detected only 2h upon TCR stimulation. Conversely to the micro array data, we could not confirm the rapid upregulation of miR-21, miR-29c, and miR-30e, despite the fact that the expression of miR-21 inceased 24h after stimulation and lasted up to 72h. The different expression pattern of the selected miRNAs in the micro array versus real-time PCR analysis could be due in part to inter-individual differences among different donors and different T-cell populations used in these expression analysis experiments.

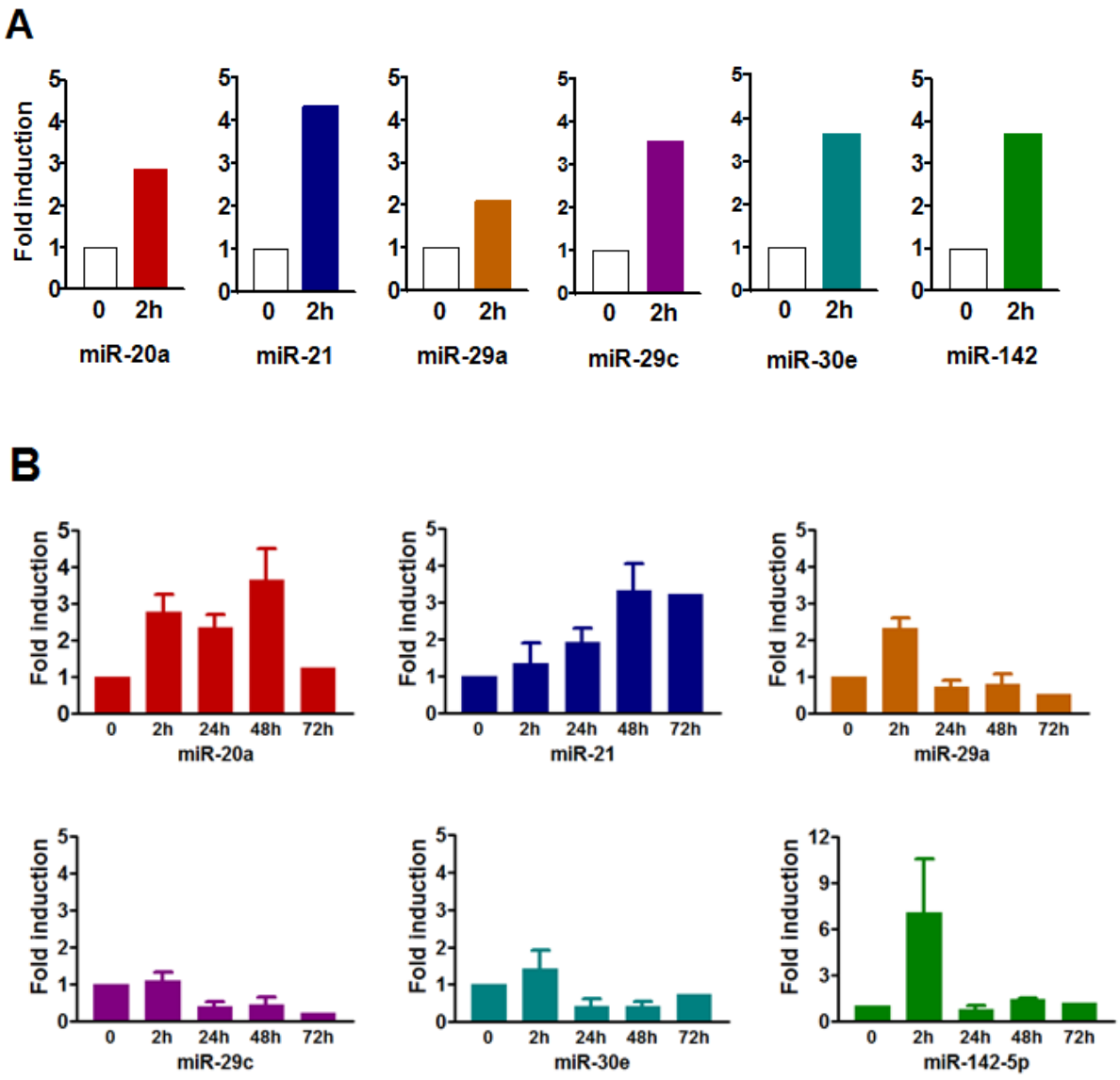

Figure 2.1. Expression profiling of miRNAs upon TCR stimulation. (A) Primary human T-cells were either left unstimulated or stimulated with CD3xCD28 antibodies immobilized on microbeads (iAbs). After 2 hours cells were immediatelyfrozen and sent for microarray analysis. Graphs shows the expression of selected miRNAs whose expression is >2 folds compared to unstimulated controls (n=1). **(B)** Primary human naïve $CD4^+$ T cells were either left unstimulated or stimulated with iAbs for the indicated time points. After each time point, cells were immediately lysed to isolate RNA. Subsequently, expression of the indicated miRNAs was quantified using RT-qPCR (n=2).

Having confirmed that some of the selected miRNAs are indeed upregulated upon TCR stimulation, I have next investigated whether they play a role in the regulation of TCR-mediated signalling. I have initially focused on the function of miR-20a. The reason for this choice is that miR-20a appears to inhibit gene transcription in the Jurkat T-cell line and also because miR-20a expression is downregulated in whole blood samples from patients suffering from multiple sclerosis (MS) (Cox M.B et al). Thus, from these observations it is possible that miR-20a play a role in T-cell activation.

2.2. miR-20a is *de novo* synthesized following TCR stimulation and its transactivation depends on AP1, NFAT and NF-κB

miRNA gene transcription results in the generation of pre- and pre-miRNAs, collectively called as miRNA precursors, that are finally processed into mature miRNAs. As shown in Fig. 2.1 A and B, miR-20a is rapidly expressed upon T-cell activation compared to unstimulated control samples. Therefore, I wanted to investigate whether the rapid upregulation of miRNA-20a is due to a *de novo*expression or to the maturation of a pool of stored miR-20a precursors. To test this, I have analyzed the expression of miRNA-20a precursors. Real-time quantitative PCR (RT qPCR) analysis showed that the expression of miR-20a precursors was induced very rapidly already after 30 min upon TCR triggering. In addition to the rapid induction, stimulated T-cells showed a gradual increase in the expression of miR-20a precursors upto 48 hours (Figure 2.2A). These data suggest that miR-20a is rapidly transcribed upon T-cell activation and indicate that it may play a role in early activation events. Signals transduced via the TCR result in the activation of AP1, NF-κB and NFAT, three key transcription factors required for the induction of crucial genes (e.g. IL-2) and hence for T-cell proliferation. To investigate whether these transcription factors are also involved in the transactivation of miR-20a in response to TCR stimulation, I have measured the levels of miR-20a precursors in activated $CD4^+$ T cells in the presence or absence of U0126, IKK VII, and EGTA, which selectively inhibit the activation of AP1, NF-κB, and NFAT, respectively. Here, I have stimulated T cells using an anti-CD3 antibody alone to make sure that the signals are exclusively driven by TCR and thereby minimizing the involvement of other transcription factors whose activation can be triggered by signals emanating from CD28. As shown in figure 2.2B, treatment of T cells with IIK VII and U0126 inhibited transcription of miR-20a about 50% and 85% respectively, upon TCR triggering, compared to DMSO+CD3 positive control cells. Strikingly, treatment of cells with EGTA, which blocks extracellular Calcium signaling and hence NFAT activation, completely abolished the expression of miR-20a precursors compared to DMSO+CD3 positive control cells. Collectively, these results suggest that miR-20a transcription strongly depends on NFAT and AP1 and to a lesser extent on NF-κB activity.

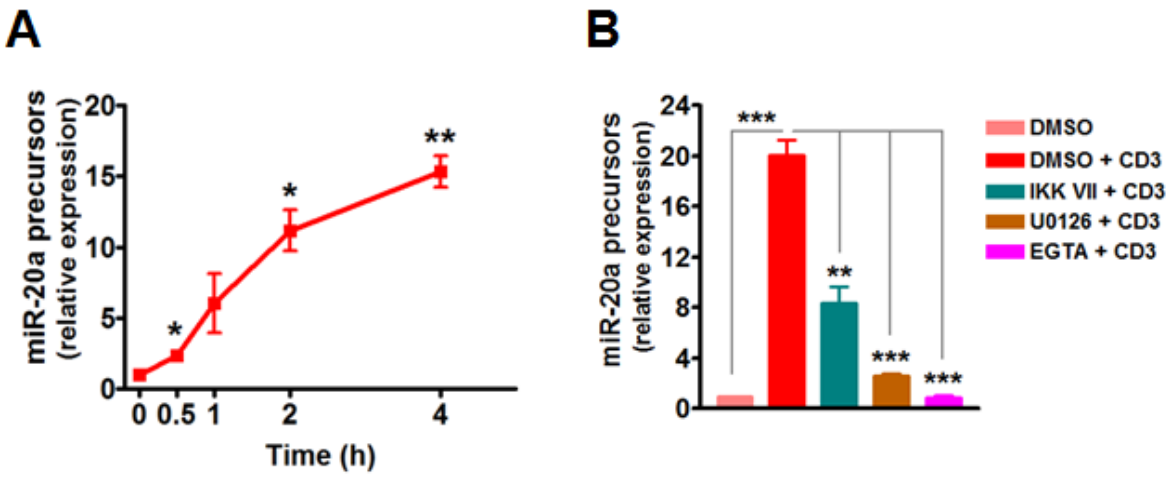

Figure 2.2. miR-20a is *de novo* induced and its expression is regulated by Erk and NFAT activity upon T-cell stimulation. Human naïve $CD4^+$ T cells were **(A)**stimulated with plate-bound CD3 and CD28 mAbs and the expression of miR-20a precursors (both pri- and pre-miRNAs)was quantified using RT qPCR.Values obtained for each time point were normalized to unstimulated (0h) controls (n=4). **(B)** Human naïve $CD4^+$ T cells were treated with U0126 (10 µM), IKK VII (200 nM) and EGTA (10 mM). DMSO was used as a negative control. All samples were preincubated with indicated reagents for 30 minutes prior to stimulation. Samples were either left unstimulated or stimulated with plate-bound CD3 mAb for 4 hours at 37˚C. Expression levels of miR-20a precursors were then quantified using RT qPCR. Values in DMSO+CD3 positive control were normalized to unstimulated DMSO negative control, where as samples treated with inhibitors were normalized to DMSO+CD3 positive control sample (n=4).*P* values in (A) and (B) were calculated by using Student's paired *t* Test. (*, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$).

Given that the levels of miR-20a were decreased in blood samples from MS patients (Cox M.B. et al., 2010), I have also compared the levels of miR-20a in experimentally induced autoimmune encephalomyelitis (EAE) in mice. EAE mimics the pathological features of multiple sclerosis. To induce EAE, mice were immunized with MOG peptide andpertusis toxin in the presense of Freuend's complete adjuvant.Lymph nodes were isolated when the EAE clinical score, which represents the disease severity, reached 2.5-3. RNA was isolated from total lymphnodesand assayed for miR-20a expression by real-time PCR. As shown in Figure 2.3 A and B, the levels of miR-20a were indeed decreased in mice with active EAE compared to control mice.

The rapid TCR-mediated induction of miR-20a and the indispensable role of TCR-induced transcription factors in the expression of miR-20a prompted me to investigate whether miR-20a is involved in the regulation of T-cell activation. To gain insight into the function of miR-20a in TCR-mediated signalling, I have used the following strategies:

i) Overexpression of miR-20a, and
ii) Knockdown of miR-20a.

2.3. Effect of the overexpression of miR-20a on TCR-mediated signalling

I have initially established a miR-20a overexpression system. To characterise the role of miR-20a, it was necessary to clone the miR-20a into a suitable miRNA expression vector for transient or stable expression. I have chosen the pcDNA 6.2-G.W/EmGFP-miR vector from Invitrogen, which constitutively expresses the miRNA at a high level, using the human cytomegalovirus (CMV) immediate early promoter and allow proper processing of the miRNA. Because of the presence of co-cistronic expression of GFP, this vector also allows the selection of T cells overexpressing the miR-20a (Figure 2.4A). I have designed the sequence coding for pre-miR-20a which was successfully cloned into the vector. I have also cloned a scrambled sequence, which works as a miR-negative control. This sequence is predicted not to target any known vertebrate gene. Primary human T cells are difficult to transfect with plasmids. However, using this miRNA overexpression system, I have achieved 70% transfection efficiency as measured by flow cytometry (Figure 2.4B). Next, I have measured the levels of miR-20a in T-cells transfected with the miR-20a overexpressing plasmid. As measured by real-time PCR, compared to control plasmid transfected cells, miR-20a plasmid transfected cells showed a moderate overexpression of miR-20a (Figure 2.4C). In addition, T-cells transfected with the miR-20a plasmid also responded to TCR triggering and showed an efficient induction of miR-20a (Figure 2.4C).

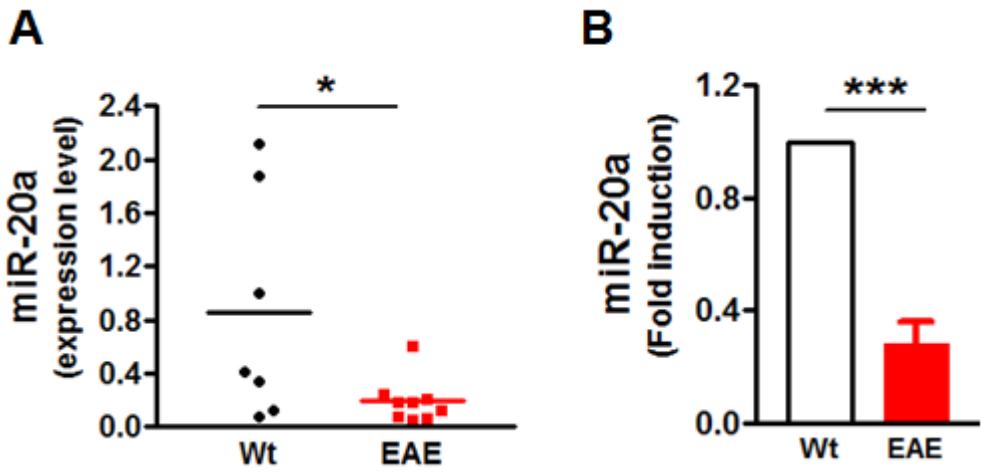

Figure 2.3. miR-20a expression is decreased during EAE. Mice were injected with MOG peptide to induce EAE or with PBS. After 14 days lymphnodes from both EAE and Control mice were taken for the isolation of RNA. Expression of miR-20a was quantified by using RT qPCR. **(A)** Expression levels of miR-20a during EAE compared to control mice. **(B)** Expression levels of miR-20a during EAE normalized to control mice expression levels (n=7). Significant *P* values were done by using Student's paired *t* Test. (*, $P< 0.05$; ***, $P< 0.001$).

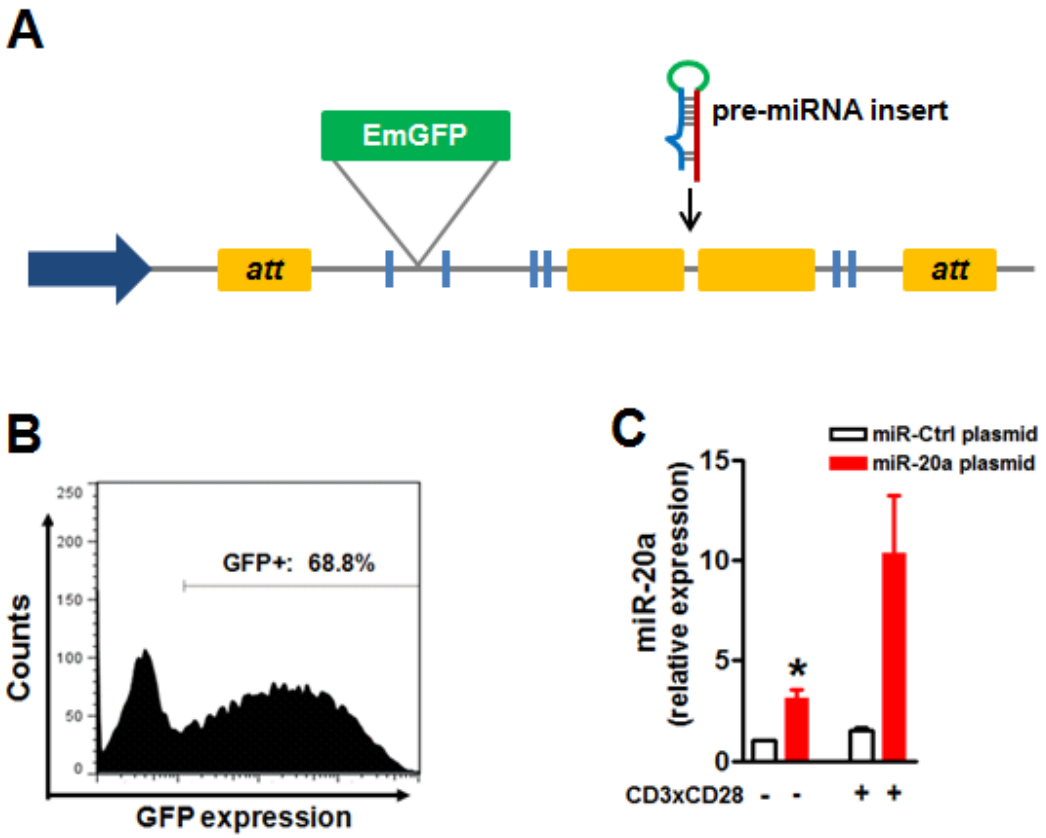

Figure 2.4. Scheme and efficiency of the miRNA overexpressing system. (A) Schematic picture of miR-expression plasmid. Human naïve $CD4^+$ T cells were transfected with plasmids encoding either miR-20a or miR-control (miR-Ctrl). After 16 hours **(B)** cells were briefly centrifuged and resuspended in PBS to measure the transfection efficiency of miR-plasmid construct by gating on GFP+ cells on flow cytometry or **(C)** cells were either left unstimulated or stimulated for 1 hour with CD3xCD28 mAbs. Expression levels of miR-20a in both resting and activated cells were quantified by RT-qPCR. Data represent arbitrary units ± SEM of at least 2 independent experiments. Significant *P* values were done by using Student's *t* Test. (*, $P< 0.05$).

Triggering of the TCR results in the activation of the key tyrosine kinase Lck. Lck phosphorylates the ITAMs in CD3/ζ chains and also activates another tyrosine kinase, Zap-70, thus initiating downstream signaling events leading to gene transcription and T-cell functional responses. Lck and Zap-70 activation is required for the formation of a signaling complex organized by the transmembrane adaptor protein LAT, which in turn will orchestrate the activation of PLC-γ1. PLC-γ1 generates two second messengers DAG and IP3 that mediate the activation of the Ras-Erk cascade, the activation of IKK and Ca^{++}-Calcineurin pathways, which in turn result in the activation of AP-1, NF-κB, and NFAT, respectively. Firstly, I wanted to analyze whether overexpression of miR-20a had any effect on the activation of the Ras-Erk cascade, which is crucial for T-cell activation and cell-fate specifications. To test whether miR-20a regulates the Ras-Erk cascade, I have first transfected primary human naïve $CD4^{+}$ T cells with a plasmid encoding either miR-20a or miR-control. Second, I have stimulated the cells with anti-CD3 and anti-CD28 antibodies in suspension for 2 minutes. Subsequently, biochemical analysis of the Ras-Erk cascade was performed using Western blotting. As shown in figure 2.5A, overexpression of miR-20a inhibits the phosphorylation and hence the activation of signalling molecules of the canonical Ras pathway such as PLCγ-1, Raf, MEK and Erk. However, there was no change in the phosphorylation of LAT which is required for the activation of PLC-γ-1. Given that activated PLCγ-1 mediates Ca^{++} influx by generating IP3, a decrease in the phosphorylation of PLCγ-1 upon miR-20a overexpression should also result in the reduction of intracellular Ca^{++} concentration in response to TCR stimulation. To test this, I have measured Ca^{++} influx in miR-20a transfected cells upon TCR triggering. As shown in Figure 2.5B and 2.5C, in agreement with the decreased PLCγ-1 activity, miR-20a transfected cells indeed showed a reduction in the intracellular Ca^{++} flux compared to cells expressing miR-control. This result suggests that miR-20a inhibits the TCR-mediated Ras-Erk pathway at the level of PLCγ-1 by decreasing its activation and also decreases Ca^{++} flux.

The experimental setting I have used to study the effect of miR-20a overexpression on TCR-mediated signalling was based on soluble antibody (sAbs) approach. Although soluble antibody approach is widely used to study signalling events that take place during transient activation of T cells, it has been previously shown that this approach is not suitable to study sustained activation and functional responses of T cells (Arndt B. et al., 2013). Therefore, I have used immobilized CD3xCD28 Abs on beads (iAbs) to induce sustained activation of T-cells that mimics physiological activation of T cells. Both iAbs and sAbs systems trigger completely different signalling profile in T-cells and our lab elegantly showed the mechanistic basis for the different signalling profile that can be triggered by sAbs or iAbs approach

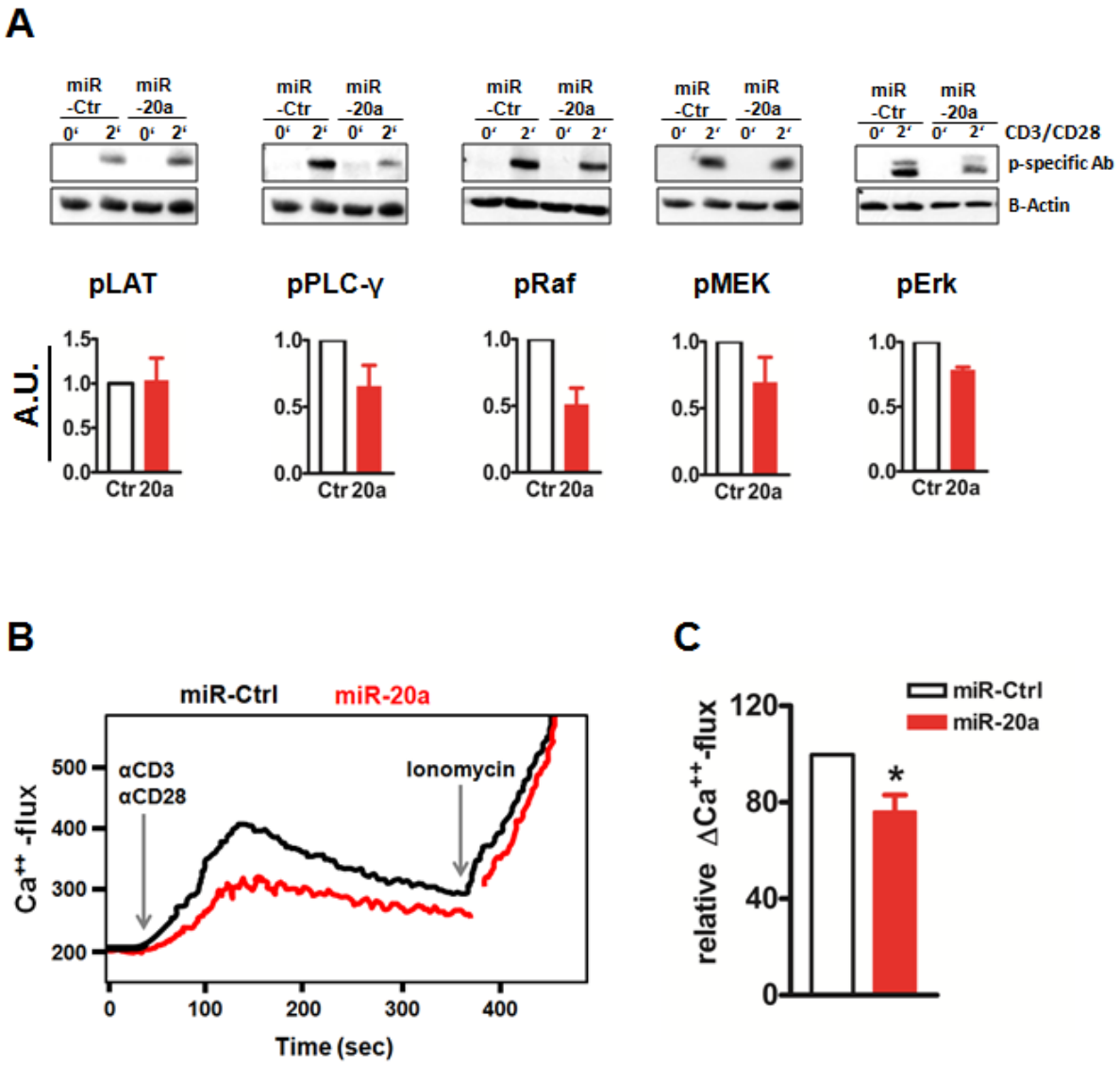

Figure 2.5. miR-20a inhibits transient TCR signaling. Human naïve CD4⁺ T cells were transfected with plasmids encoding either miR-20a or miR-control (miR-Ctrl) and cultured for 16 hours. **(A)** Cells were stimulated with anti-CD3 and CD28 mAbs for the indicated time periods.Subsequently, lysates were analyzed by immunoblotting using the indicated Abs. Bands in **(A)** were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs show the phosphorylation levels of the indicated molecules as arbitrary units ± SEM of at least 4 independent experiments. **(B)** CD4⁺ T cells were incubated with Indo-1AM, stimulated with CD3 and CD28 mAbs, and Ca^{++} flux was measured by flow cytometry. Ionomycin is used to induce maximum Ca^{++} flux. Graph in **(C)** shows quantification of Ca^{++} flux expressed as arbitrary units ± SEM of at least 3 independent experiments. Significant *P* values were done by using Student's *t* Test. (*, $P< 0.05$).

(Poltorak M. et al., 2013). Therefore, I decided to investigate how overexpression of miR-20a influence the TCR-mediated signalling upon sustained activation of T cells triggered by iAbs. To test this, freshly purified naïve $CD4^+$ T cells were transfected with plasmid encoding either miR-20a or miR-control. Subsequently, cells were stimulated with iAbs. With this approach the result was much clearer compared to sAb approach and miR-20a affected Ras-Erk cascade very proximally. As shown in Figure 2.6A and 2.6B, overexpression of miR-20a inhibits the TCR-mediated phosphorylation of Zap-70, LAT, PLC-γ, and Erk1/2. I have next assessed whether miR-20a affects the activation of signaling molecules upstream of Zap-70. However, I did not observe any decrease in the phosphorylation of CD3ζ (Figure 2.6A and 2.6B). Therefore, I have proposed that the decrease in the phosphorylation of Zap-70 could be due to the reduction in its recruitment to CD3ζ chains upon T-cell stimulation. To test this hypothesis I have performed immunoprecipiations of CD3ζ under mild detergent conditions to pull down intact CD3ζ chains to quantify how much Zap-70 is associated with the ζ chain. As shown in figure 2.6C and 2.6D, I have observed a slight decrease in the recruitment of ZAP70 to CD3ζ upon miR-20a overexpression compared to miR-control. Collectively, these data indicate that miR-20a regulates TCR-mediated signaling at the level of Zap-70 activation during sustained TCR signaling. In addition to investigating the molecular mechanisms that results in defective Ras-Erk signaling upon miR-20a overexpression, I have also checked the activation of p38 MAPK, which is another crucial molecule that is activated upon TCR triggering and regulates T-cell functional responses. In $CD4^+$ T cells activation of p38 is essential for IL-17 production and contributes to the development of EAE (Noubade R et al, 2011). Furthermore, it has been shown that miR-20a decreases p38 activation in endothelial cells VEGF (Pin AL. et al, 2012). To test whether miR-20a also targets p38 activation, I have stimulated transfected T-cells with CD3xCD28 iAbs. In contrast to the previous observation(Pin AL. et al, 2012), p38 is constitutively activated upon miR20a overexpression (Figure 2.6B).

To investigate how overexpression of miR20a inhibits TCR signaling, I have analysed whether miR20a suppresses the expression of molecules which are crucial for TCR signaling. However, I have found that overexpression of miR-20a does not affect the expression of Lck, Zap-70, LAT, PLC-γ, SLP-76, GADS, Sos1, Erk in resting $CD4^+$ T-cells (Figure 2.7A). Several studies have previously showed that BIM, PTEN, and STAT3 are validated targets of miR-20a and other members of the miR-17~92 cluster (Xiao C. et al., 2008; Zhang Y. et al., Zhang M. et al., 2011). Therefore, I have checked whether miR-20a decreases the expression levels of these three validated targets in resting T-cells. Surprisingly, I have not observed any difference in the expression levels these three molecules in cells transfected with miR-20a overexpression plasmid compared to control

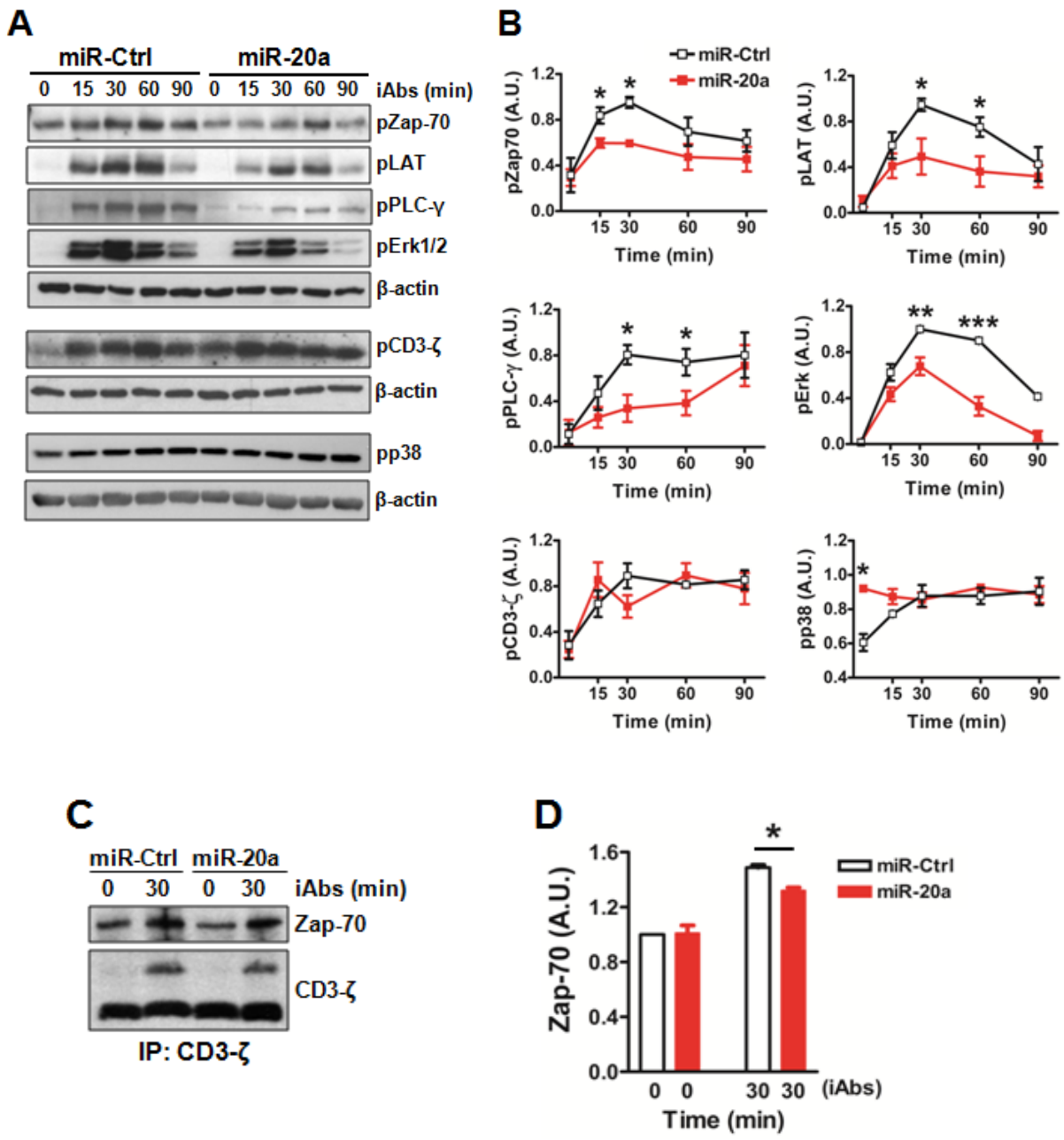

Figure 2.6. miR-20a inhibits sustained TCR signaling. Human naïve CD4⁺ T cells were transfected with plasmids encoding either miR-20a or miR-Ctrl and cultured for 16 hours. **(A)** Cells were stimulated with CD3 and CD28 mAbs immobilized on microbeads (iAbs) for the indicated time periods. Lysates were used to analyze the phosphorylation of indicated signaling molecules by immunoblotting using the indicated Abs. Bands in **(A)** were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in **(B)** show the phosphorylation levels of the indicated molecules as arbitrary units ± SEM of at least 4 independent experiments. **(C)** CD4⁺ T cells were stimulated with iAbs for the indicated time periods. Subsequently, cell lysates were prepared and CD3ζ immunoprecipitations were analyzed by immunoblotting using the indicated Abs. Bands in **(C)** were quantified as described above. Graph in **(D)** shows the levels of CD3ζ-associated Zap-70 as arbitrary units ± SEM of 2 independent experiments. Significant *P* values were done by using Student's *t* Test. (*, *P*< 0.05; **, *P*< 0.01; ***, *P*< 0.001).

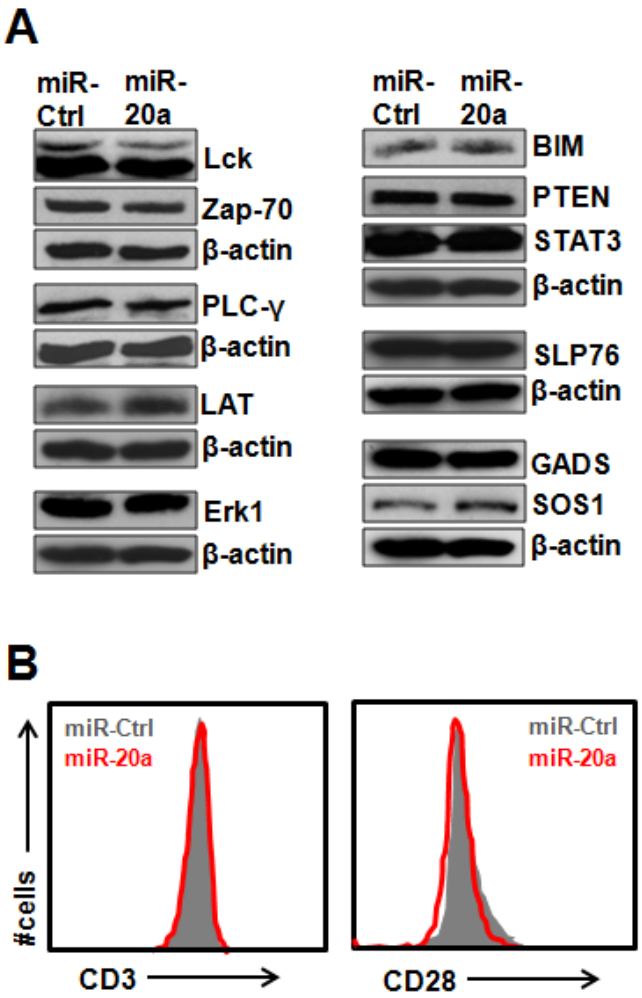

Figure 2.7. miR-20a does not affect the expression of signaling molecules and CD3 and CD28 receptors. Human naïve CD4+ T cells were transfected with miR-20a overexpression plasmid or control plasmid. 16 h after transfection cells were either **(A)** lysed or **(B)** used for flow cytometric analysis. Bands in **(A)** show the expression levels of different signaling molecules after transfection as analyzed by immunoblotting using the indicated Abs. Histograms in **(B)** show the surface expression of CD3 (left) and CD28 (right) in $CD4^+$ T cells overexpressing either miR-20a or miR-control.Figures in (A) and (B) show one representativefrom at least 3 individual experiments.

cells (Figure 2.7A). In addition, I have also checked whether the defective TCR signaling in miR-20a overexpressing cells is due to reduced CD3 and CD28 levels.Flow cytometry analyses revealed that miR-20a overexpression has no effect on the expression of these two receptors (Figure 2.7B).

2.4. miR-20a overexpression inhibits CD69, but does not affect CD25 and proliferation.

Next, in order to evaluate whether miR-20a also affects functional responses in T-cells, I have analyzed the expression of CD69, a well-characterized activation marker of T cells whose upregulation depends on the Ras cascade (Cebrián M. et al., 1988; López-Cabrera M. et al., 1993). Primary naïve human $CD4^+$ T cells were transfected by electroporation with either miR-20a or with miR-control plasmids. 16h after transfection, cells were stimulated for 6 hours with plate bound CD3xCD28 mAbs. Analysis of CD69 expression on GFP^+-gated (transfected) cells showed that overexpression of miR-20a significantly decreased CD69 expression (about 40%) compared to cells transfected with miRNA control plasmid (Figure 2.8A and 2.8B). However, there was no difference in the expression of CD25 upon miR-20a overexpression compared to control cells (Figure 2.8C and 2.8D). To further evaluate the functional role of miR-20a, I have investigated its role in T-cell proliferation. To this aim, I have stimulated transfected cells with plate bound CD3xCD28 mAbs for only 60 hours. However, as shown in figure 2.8E and 2.8F, miR-20a transfected cells show no significant differences in the number of proliferated cells compared to control cells. This result suggests that miR-20a has no effect on T-cell proliferation. Because of the transient approach to overexpress miR-20a using plasmid it is very like that the transfected cells lose the plasmid after a few divisions and thereby also lose the suppressive effect of miR-20a that additionally coming from the overexpression. This could be reason why there is no difference in proliferation between cells transfected with miR-20a and controls.

2.5. miR-20a decreases cytokine production.

To further address the role of miR-20a in $CD4^+$ T cell functional responses, I have next investigated whether miR-20a is involved in the regulation of cytokine production. I have measured cytokines such as IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TGF-β and TNF-α. As shown in Figure 2.9A and 2.9B, overexpression of miR-20a significantly decreased the production of IL-6, IL-8, IL-10 and mildly decreased IL-2 compared to miR-control. On the other hand, overexpression of miR-20a did not affect the release of other cytokines such as IL-4, IFN-γ, TGF-β and TNF-α. Collectively, the data indicate that miR-20a play an important role in the differentiation of $CD4^+$ T cells.

Having shown that overexpression of miR-20a inhibits TCR-mediated signalling and there by decreases functional responses of T cells I have hypothesized that suppression of miR-20a

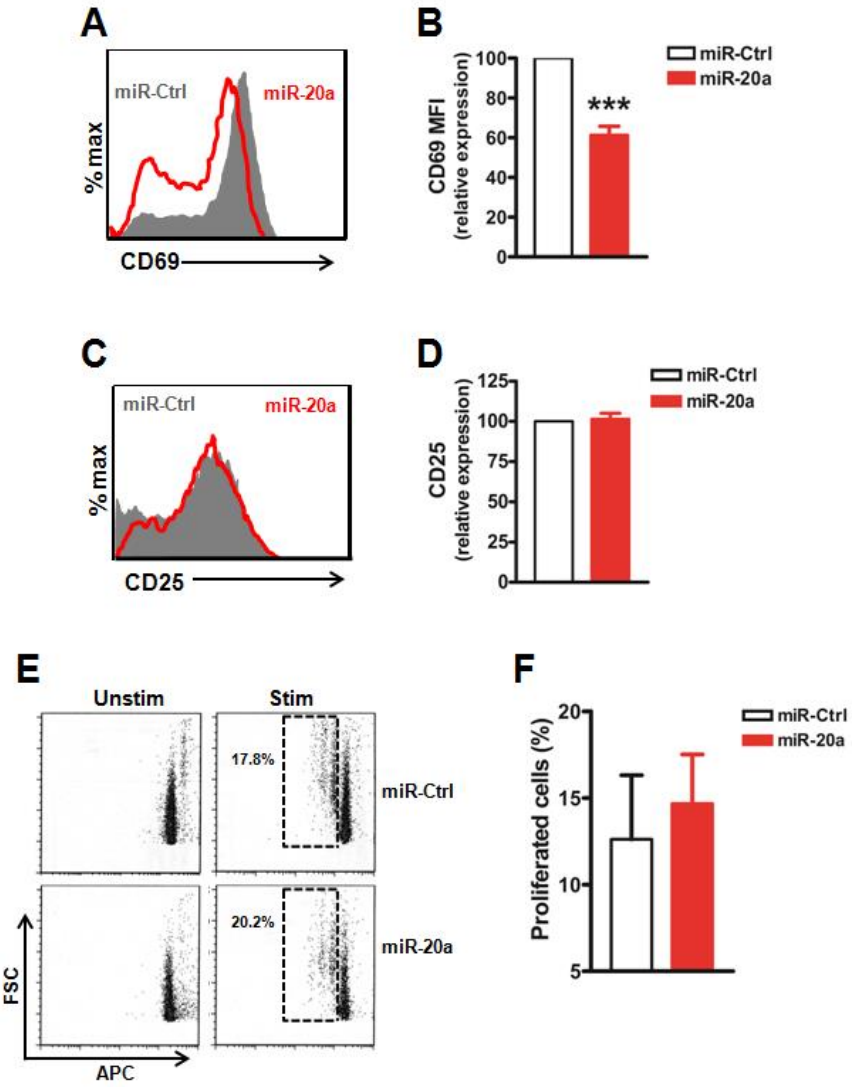

Figure 2.8. miR-20a inhibits early T-cell activation events but does not affect late activation events and proliferation. Human naïve CD4⁺T cells were transfected with either miR-20a or miR-control expression plasmids. Cells were stimulated with plate bound CD3xCD28 mAbs after 16 hours of transfection. **(A)** 6 hours after stimulation cells were washed and stained with CD69 mAb conjugated with APC to measure the surface expression of CD69 by flow cytometry. Histogram shows one representative figure of 4 independent experiments. Graph in (**B**) shows the analysis of relative mean fluorescent intensity (MFI) of CD69 expression in cells transfected with miR-20a overexpression plasmid compared to miR-control.**(C) 1**6 hours after stimulation cells were stained with an APC-conjugated CD25 mAb. Surface expression of CD25 was measured by flow cytometry. One representative experiment of 2 is shown. Graph in **(D)**MFI of CD25 expression of miR-20a overexpressing cells compared to miR-control. **(E)** Cells were stained with Alexa Flour 700 proliferation dye and left either stimulated or unstimulated for 60 hours. Proliferation was then measured by flow cytometry by gating on GFP⁺ cells. Histograms in **(E)** shows the percentages of divided cells from one representative experiment. Graph in **(F)** shows the percentages of divided cells from 4 individual experiments.Data represent arbitrary units ± SEM ofat least 4 independent experiments. Significant *P* values were done by using Student's *t* Test. (***, *P*< 0.001).

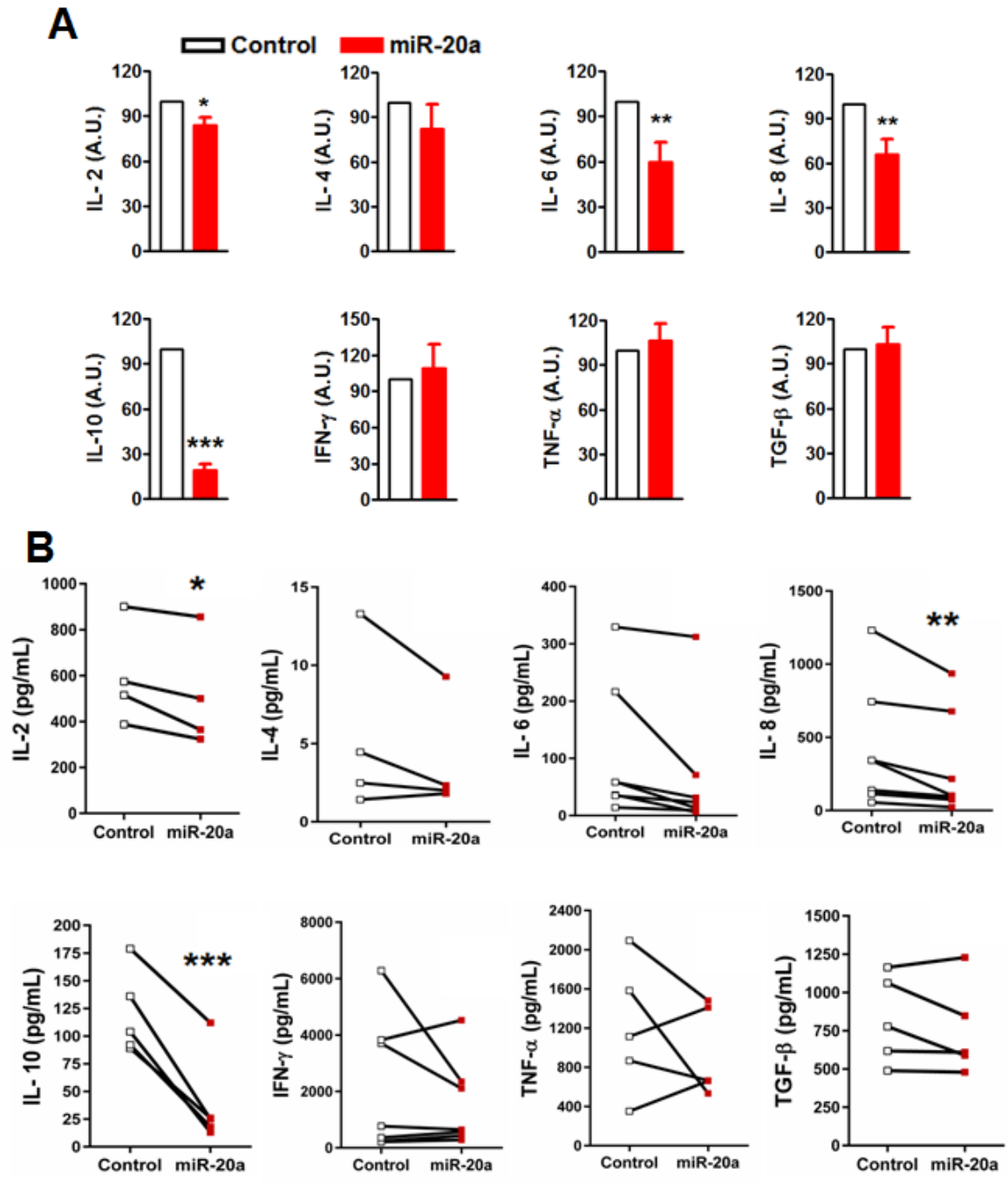

Figure 2.9. Analysis of cytokine production in T cells upon miR-20a overexpression. Human naïve $CD4^+$T cells were transfected with either miR-20a or miR- control expression plasmids. 16h after transfection, cells were stimulated with plate bound CD3 (MEM92) and CD28 mAbs for 48 h after stimulation. At the end of 48 hours, supernatants were collected and the concentration of secreted cytokines was measured by ELISA. Graphs in **(A)** show relative cytokine levels from miR-20a overexpressing cells compared to miR-control. Graphs in **(B)** show the absolute values of cytokine levels from individual experiments. Data expressed as arbitrary units ± SEM of at least 4 independent experiments. Significant *P* values were done by using Student's *t* Test. (*, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$).

should result in enhanced TCR-mediated signalling and T cell functional responses. Therefore, I next decided to suppress miR-20a in T-cells.

2.6. Downregulation of miR-20a results in a modest increase of T-cell activation.

An efficient and long lasting downregulation is necessary in order to investigate the role of miRNAs upon their silencing. There are different chemically modified, single stranded antisense nucleic acids that are commercially available to downregulate miRNAs *in vitro*. To achieve efficient miR-20a suppression, I have tested several commercially available reagents such as miR-inhibitors and antagomirs. I have first used a miRNA-20a inhibitor to suppress miR-20a in human naive $CD4^+$ T cells. However, with this approach I have achieved only up to 40% downregulation of miR-20a. Therefore, I have next used miR-20a antagomir. Unlike miR-inhibitors, antagomirs have cholesterol modification at the 3' end of the sequence. This modification allows the entry of antagomirs into the cells without the need for electroporation. I have found out that antagomirs are also not effective to suppress miR-20a in primary human T cells. Therefore, I have next tested miR-decoys as an alternative approach to efficiently downregulate miR-20a expression. miR-decoys are DNA sequences complimentary to the sequence of mature miRNA. I have designed a miR-20a decoy and also a miR-Scramble as negative control. Using this approach, I was able to achieve a very good suppression (about 80%) of miR-20a (Figure 2.10A). To test the specificity of miR-20a decoy, I also measured the levels of miR-21, which has no sequence similarity to that of miR-20a. Real-time PCR analysis showed that the expression level of miR-21 was unaltered in cells after miR-20a decoy transfection (Figure 2.10B). This result indicates that miR-20a decoy is not targeting other miRNAs which do not have sequence similarity to miR-20a.

Next, I have analysed the effect of downregulation of miR-20a on TCR-mediated signalling. To this end, primary naïve human $CD4^+$ T cells were transfected with either miR-20a decoy or miR-Scramble. 16h after transfection, T cells were stimulated with immobilized CD3xCD28 mAbs. In agreement with the overexpression experiments described above, biochemical analyses of stimulated T cells expressing low levels of miR-20a show an increase in TCR-mediated signalling. However, this effect was modest (Figure 2.10C and 2.10D). I have thought that the cells must possess certain mechanism to overcome the suppressive effect of miR-20a upon TCR stimulation. I have proposed following three hypotheses:

(i) intracellular stores of miR-20a precursors
(ii) a redundancy among the members of miR-17~92 cluster
(iii) a rapid reexpression of miR-20a upon T-cell stimulation

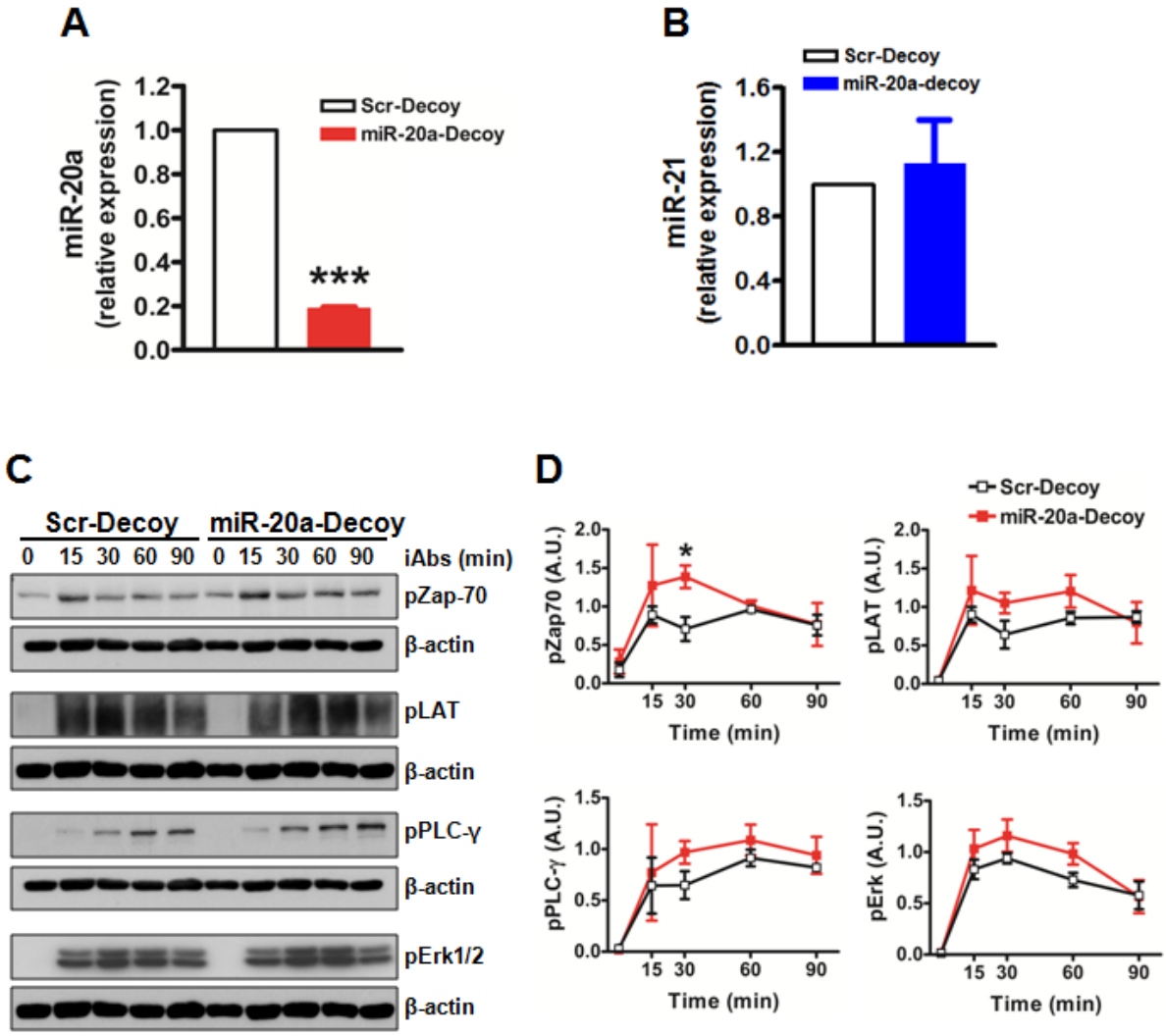

Figure 2.10. Efficiency and specificity of miR-20a decoy and its effect on TCR-mediated signaling. Human naïve CD4⁺ T cells were transfected with either miR-20a or scramble decoys. 24 hours after transfection cells were used to **(A)** measure the levels of miR-20a and **(B)** miR-21 levels using RT-qPCR. In addition, **(C)**cells were stimulated with iAbs for the indicated time periods. Lysates were prepared after stimulation and analyzed by immunoblotting using the indicated Abs. Bands showing the phosphorylation of indicated molecules in **(C)** were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in **(D)** show the phosphorylation levels of the indicated molecules as arbitrary units ± SEM of 3 independent experiments.Data represent arbitrary units ± SEM of at least 2 independent experiments. Significant *P* values were done by using Student's *t* Test. (*, *P*< 0.05; **, *P*< 0.01; ***, *P*< 0.001).

I have assessed which of these three hypotheses may explain the mild effect of miR-20a suppression.

2.6.1. No intracellular stores of miR-20a precursors

Triggering of the TCR induces several intracellular signalling events leading to different functional responses. At this point cell must actively employ all possible regulatory mechanisms to fine tune the T-cell signalling and functional responses. In order to meet the sudden onset of increased regulatory demands we assumed that there might be intracellular store of miR-20a precursors which will be rapidly processed into mature miR-20a upon T-cell stimulation. To test this I have measured the levels of miR-20a precursors in resting and activated cells. As shown in figure 2.2A, miR-20a precursors are rapidly upregulated upon T-cell stimulation. However, a closure look at the Ct values of pre-miR-20a in resting cells revealed that it took more than 30 cycles to detect the signal that indicates the presence of miR-20a precursors (Fig. 2.11A). This observation suggests that there are little or no intracellular stores of miR-20a precursors in unstimulated T-cells that can be available to T-cell regulatory machinery upon T-cell stimulation.

2.6.2. No redundancy among the members of miR-17~92 cluster upon downregulation of miR-20a

The miR-17~92 cluster contains at least five other miRNAs sharing the same seed sequence, which is important for the recognition of target mRNA and hence they may have common targets. These are: miR-17, miR-20b, miR-93, miR-106a and miR-106b. A redundancy among members of this cluster has already been shown. In fact, it appears that miR-17, miR-20a and miR-106a, play redundant roles in the differentiation and maturation of monocytes (Fontana L. et al., 2007), macrophages (Zhu D. et al., 2013; Zhang M. et al., 2011), neuronal cells (Trompeter HI et al., 2011). Therefore, I have analysed their expression levels upon downregulation of miR-20a. Real-time PCR analysis revealed that the expression of all the tested miRNAs is also downregulated upon miR-20a suppression (Fig. 2.11B). This observation suggests that it is unlikely that they may compensate for the loss of miR-20a.

2.6.3. T-cell stimulation rapidly re-induces miR-20a expression in miR-20a suppressed cells

My previous results showed that the expression of miR-20a was induced in T cells upon stimulation. Therefore, I hypothesized that miR-20a may be rapidly re-expressed after T-cellstimulation, thus abolishing the suppressive effect of the decoy on miR-20a expression.

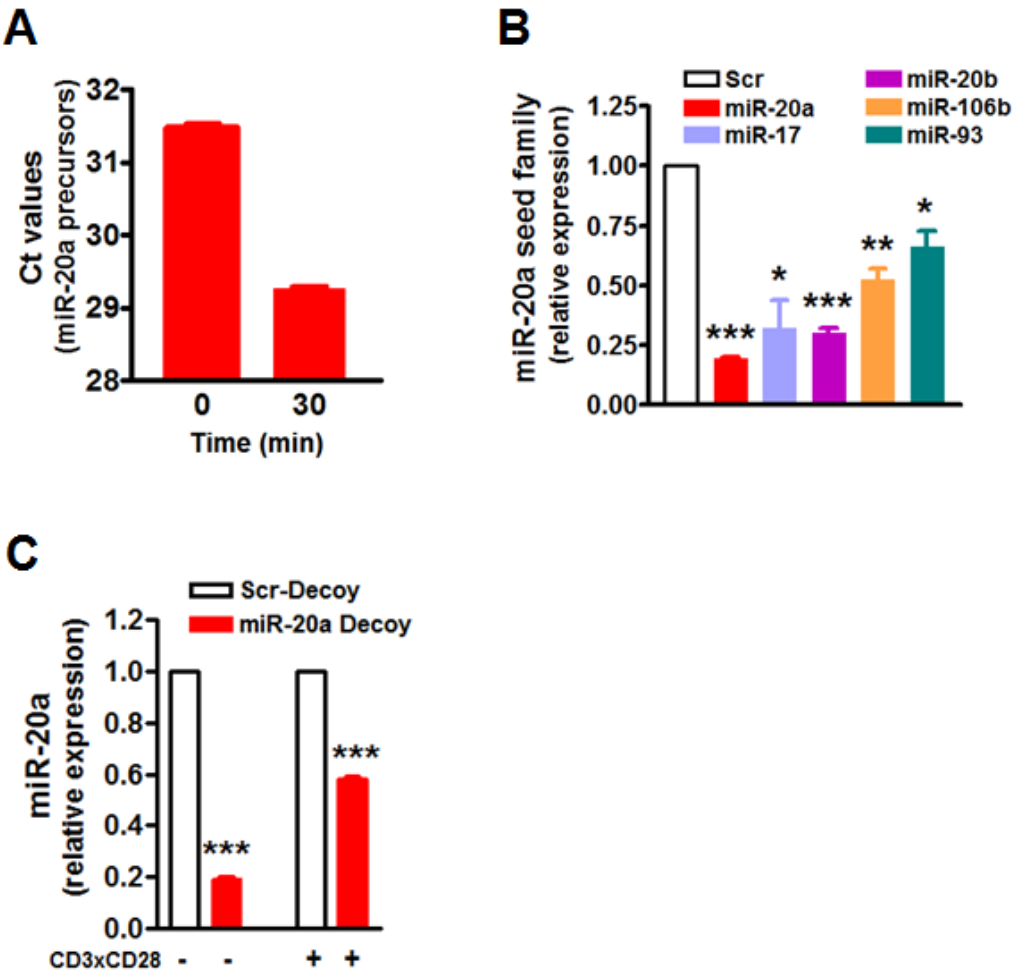

Figure 2.11. Expression profiling of miR-20a precursors, and miR-19-72 cluster upon miR-20a suppression. Primary human naïve $CD4^+$ T cells were transfected with miR-20a decoy or miR-negative control. **(A)** and **(B):** 16h after transfection, expression of **(A)** miR-20a precursors **(B)** miR-20a seed family memberswere quantified using RT qPCR upon transfection with miR-20a decoy.**(C):** 16 hours after transfection, cells were either left unstimulated or stimulated with CD3xCD28 antibodies for 45 minutes and the expression of miR-20a was measured by RT qPCR. In (C) values of Scr-Decoy in each condition (either unstimulated or stimulated), were set to 1 and the results of miR-20a decoy were then normalized to the values of Scr-Decoy in each condition.Data represent arbitrary units ± SEM of at least 2 independent experiments. Significant *P* values were done by using Student's *t* Test. (*, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$).

To test this, I have measured the level of miR-20a 30 minutes after TCR stimulation in cells that were treated with the miR-20a decoy. I have found that the expression of miR-20a is rapidly induced even in the presence of the decoy (Fig. 2.11C). This induced re-expression may be enough to overcome the suppressive effect of miR-20a decoy in the cells. This may explain why there is only a mild effect on TCR-mediated signaling upon suppression of miR-20a.

2.7. Analysis of the function of miR-20a in T-cell migration

Since triggering the TCR results in the rapid expression of miR-20a in T-cells, I decided to investigate how the suppressive effect of miR-20a would influence the signalling emanating from the receptors other than TCR. It has been shown that miR-20a represses endothelial cell migration by targeting MKK3 and p38 MAPK activation in response to VEGF (Pin AL. et al, 2012). Migration of T cells between secondary lymphoid tissue and inflamed tissues is a crucial event during immune responses. Therefore, I have performed migration assay to check whether miR-20a can also repress the migration of naïve human $CD4^+$ T cells in response to chemokines. To this aim, I have transfected naïve human $CD4^+$ T cells with miR-20a decoy or scramble control and exposed them to chemokines in a transwell migration chambers containing either SDF-1α or CCL21. Control chambers were also included without any chemokines. This assay facilitates the chemotactic migration of T-cells from the upper chamber to the lower chamber that contains the chemokines. At the end of the experiment I have counted in the lower chamber the cells that have been migrated from the upper chamber. As shown in Figure 2.12, downregulation of miR-20a has no effect on the chemotactic migration of T cells in response to either SDF-1α or CCL21 compared to the number of migrated cells transfected with scramble decoy.

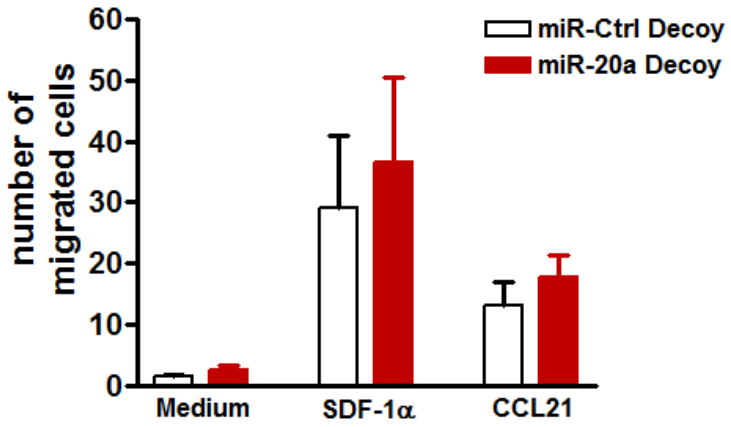

Figure 2.12. miR-20a suppression on T-cell migration. Human naïve CD4⁺ T cells were transfected with miR-20a or scramble decoys. 16 hours after transfection, chemotaxis assay was performed by transwell migration system in the presense or absence of indicated chemokines. After 2h number of T cells in the lower chamber was counted. Data represent arbitrary units ± SEM of at least 2 independent experiments.

2.8. Role of miR-21 and miR-142-5p on T-cell stimulation

Given that also the expression of miR-21 and miR-142-5p were upregulated upon stimulation of T cells, I have investigated the role of these two miRNAs on TCR-mediated signalling. I have cloned the pre-miRNA sequences of both these miRNAs into a miRNA expression plasmid vector as described above (see 2.3).

2.8.1. miR-21

First, I have investigated the effect of the overexpression of miR-21 on TCR-mediated signalling using sAb which results in the transient activation of T-cells. Conversely to miR-20a, overexpression of miR-21 increased T-cell activation as evidenced by the increase in the phosphorylation of Raf, MEK and Erk molecules. As shown in Figure 2.13A and 2.13B, miR-21 appears to increase TCR-mediated T-cell signalling at the level of Raf. Next, I have investigated how miR-21 influences TCR-mediated T-cell activation upon induction of sustained signaling using iAbs. However, as shown in Figure 2.13C, I have not observed any effect on sustained TCR signalling upon overexpression of miR-21. Indeed, miR-21 triggered the activation of signalling molecules of the canonical Ras-Erk pathway in a comparable fashion to negative control (Figure 2.13C). However, surprisingly, cells transfected with miR-21 overexpression plasmid showed increased CD69 expression, which is the earliest marker of T-cell activation (Figure 2.13D). These results suggest that miR-21 increases TCR-mediated signalling during transient activation of T cells and do not affect the signalling events upon sustained activation. Although CD69 expression in T-cell exclusively depends on Ras-Erk pathway miR-21 seem to increase its expression upon long term activation of T cells. The molecular mechanism by which miR-21 increases CD69 is currently not known and should be explored further. Some contradictory results have been observed by others regarding the role of miR-21 in different cell systems. For example, a previous study showed that miR-21 overexpression in Jurkat T cell line dampened Erk phosphorylation followed by decreased AP1 activation and CD69 expression (Carissimi C. et al., 2014). On the other hand, transgenic overexpression of miR-21 in mouse models of non-small cell lung cancer (NSCLC) resulted in increased Ras-Erk pathway and thereby promoted tumorigenesis (Hatley ME. et al., 2010). A transgenic miR-21 system would offer better resolution over the role of miR-21 in primary T-cell activation and functional responses.

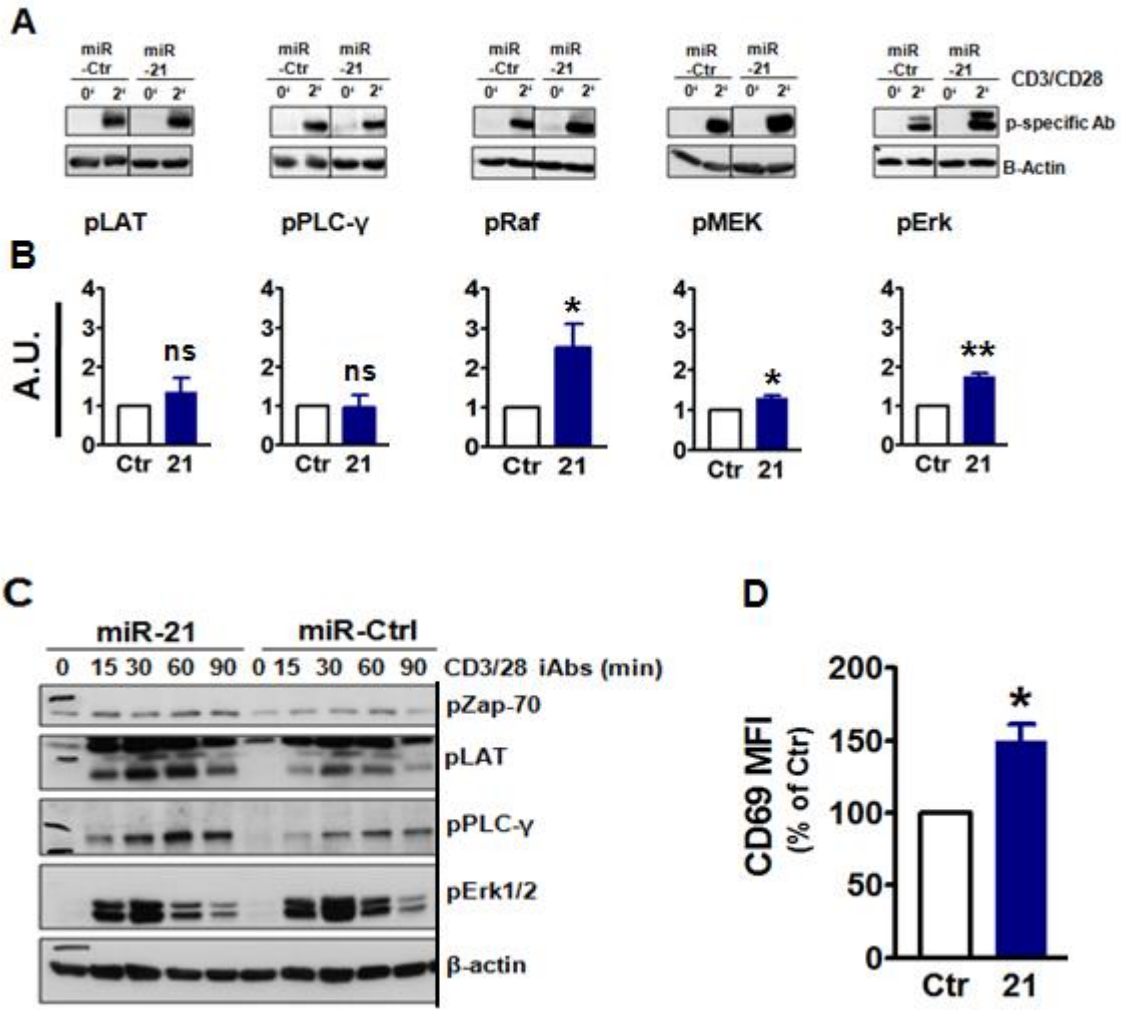

Figure 2.13. Effect of miR-21 overexpression on T cell activation. Human naïve CD4⁺ T cells were transfected with plasmids encoding miR-21 or miR-control (miR-Ctrl) and cultured for 16 hours. **(A)** Cells were stimulated with CD3 (MEM92) and CD28 (CD28.2) antibodies (sAb) for the indicated time periods. Subsequently, lysates were analyzed by immunoblotting using the indicated Abs. Bands in **(A)** were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graph **(B)** show the phosphorylation levels of the indicated molecules as arbitrary units ± SEM of 3 independent experiments. **(C)** Cells were stimulated with microbeads coated with CD3 and CD28 mAbs (iAbs) for the indicated time periods. Subsequently, lysates were analyzed by immunoblotting using the indicated Abs. **(D)** 6 hours after stimulation cells were washed and stained with an APC-conjugated CD69 mAb. Surface expression of CD69 was measured by flow cytometry. Graphs represents relative mean fluorescent intensity (MFI) of CD69 expression of miR-21 overexpressing cells compared to miR-control. Data in (A), (B), and (C) represent arbitrary units ± SEM of at least 3 independent experiments.*P* values were done by using Student's paired *t* Test. (ns, *P*> 0.05, **P*< 0.05, ** *P*< 0.005).

2.8.2. miR-142

Second, I have investigated the effect of the overexpression of miR-142 on TCR-mediated signalling using sAb which results in the transient activation of T-cells. Overexpression of miR-21 increased T-cell activation by the increasing the phosphorylation of Raf, MEK and Erk molecules. As shown in Figure 2.14A and 2.14B, miR-142 appears to increase TCR-mediated T-cell signalling at the level of Raf. Next, I have investigated how miR-142 influences TCR-mediated T-cell activation upon induction of sustained signaling using iAbs. However, as shown in Figure 2.14C, I have not observed any effect on sustained TCR signalling upon overexpression of miR-142. Indeed, miR-142 triggered the activation of signalling molecules of the canonical Ras-Erk pathway in a comparable fashion to negative control (Figure 2.14C). However, surprisingly, cells transfected with miR-142 overexpression plasmid showed increased CD69 expression, which is the earliest marker of T-cell activation (Figure 2.14D). These results suggest that miR-142 increases TCR-mediated signalling during transient activation of T cells and do not affect the signalling events upon sustained activation. Although CD69 expression in T-cell exclusively depends on Ras-Erk pathway miR-142 seem to increase its expression upon long term activation of T cells. Further studies are required to dissect the molecular mechanism by which miR-142 increases CD69 and its role in T cell functional responses.

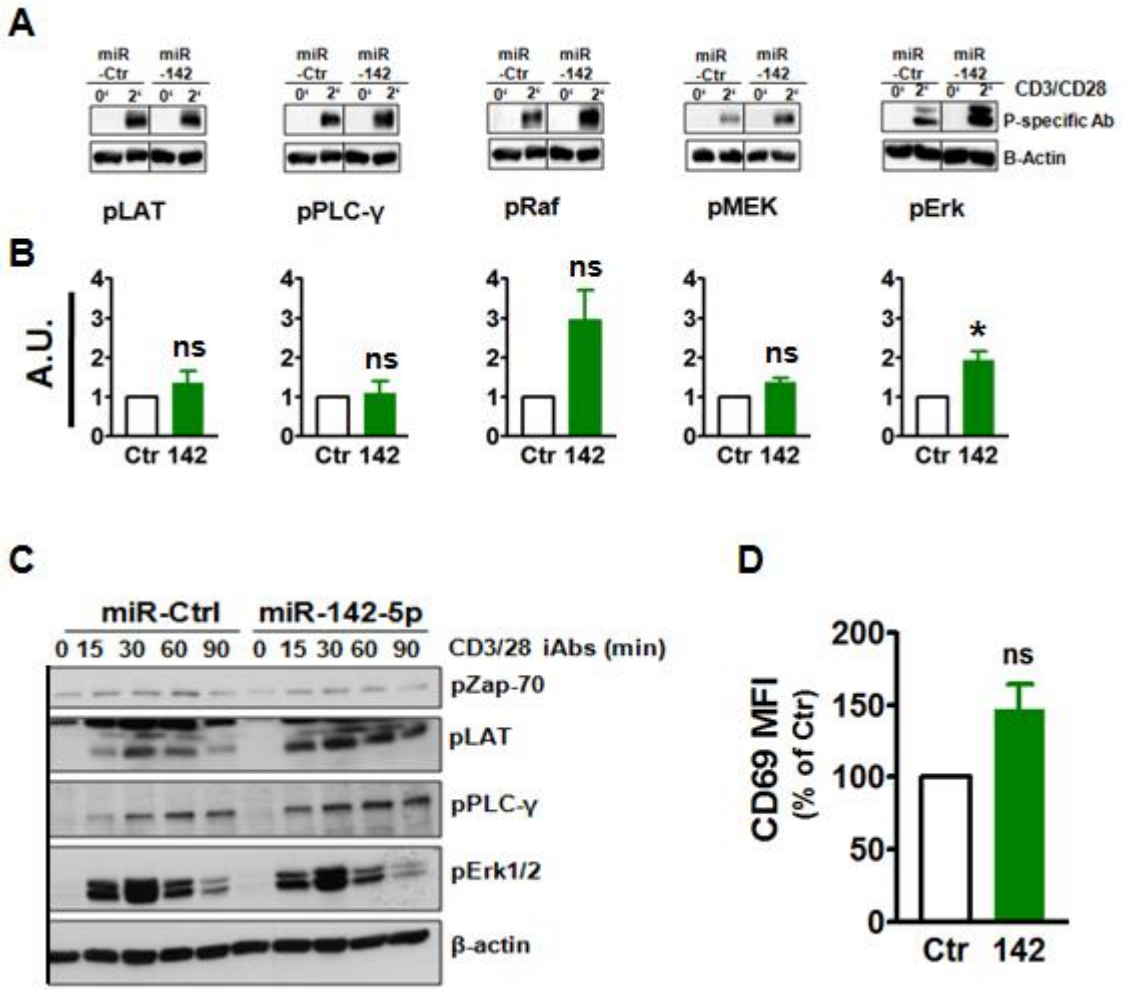

Figure 2.14. Analysis of miR-142-5p overexpression in T cell activation. Human naïve $CD4^+$ T cells were transfected with plasmids overexpressing miR-142-5p or miR-control (miR-Ctrl) and cultured for 16 hours. **(A)** Cells were stimulated with CD3 (MEM92) and CD28 antibodies (sAb) for the indicated time periods. Subsequently, lysates were analyzed by immunoblotting using the indicated Abs. Bands in **(A)** were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in **(B)** show the phosphorylation levels of the indicated molecules as arbitrary units ± SEM of 3 independent experiments. **(C)** Cells were stimulated with microbeads coated with CD3 and CD28 mAbs (iAbs) for the indicated time periods. Subsequently, lysates were analyzed by immunoblotting using the indicated Abs. **(D)** 6 hours after stimulation cells were washed and stained with an APC-conjugated CD69 mAb. Surface expression of CD69 was measured by flow cytometry. Graph in **(D)** represents relative mean fluorescent intensity (MFI) of CD69 expression of miR-142-5p overexpressing cells compared to miR-control. Data represent arbitrary units ± SEM of at least 3 independent experiments. Significant *P* values were done by using Student's paired *t* Test. (ns, $P > 0.05$; * $P < 0.05$).

3. Discussion

$CD4^+$ T cells play crucial roles in the immune response. However, abnormal activation of T-cells can lead to excessive immune responses and chronic inflammation. Therefore, T-cell activation must be tightly regulated to prevent immunopathological conditions. During the last years, miRNAs have emerged as key regulators of T-cell development and functional responses (Xiao C. et al 2009). For example,it has been shown that miR-17~92 cluster is frequently overexpressed in hematopoietic malignancies (van Haaften G. et al., 2010). In agreement with this observation, transgenic overexpression of this cluster in mice resulted in spontaneous activation of the lymphocyte compartment associated with severe lymphoproliferative disorders (Xiao C. et al., 2008). In contrast, deletion of this cluster leads to defects in B-cell development and increased B-cell apoptosis (Ventura A. et al. 2008). It has additionally been shown in a recent study that the deletion of this cluster in the hematopoietic compartment resulted in profound developmental defects at DN4 stage during thymic development. Interestingly, the miR-17~92 cluster has been shown to regulate also T-cell responses. Indeed, it promotes the differentiation of $CD4^+$ T cells into Tfh subset (Baumjohann D. et al., 2013; Kang SG. et al., 2013) and is required for the fitness of regulatory Tcells (Skinner JPJ. Et al., 2014). In $CD8^+$ Tcells, this cluster regulates effector and memory differentiation by targeting multiple negative regulators of the PI3K-Akt-mTOR axis, such as PTEN, PD1 and BTLA (Wu T. et al. 2012). Recently, the functional analysis of individual members of this cluster has gained much attention. It has been shown, in mouse $CD4^+$ T cells, that miR-17 and miR-19b regulates Th1 differentiation and prevents inducible Treg differentiation by targeting PTEN, TGFβRII, and CREB1 (Jiang S. et al., 2011), and also promotes Th17-mediated inflammation by targeting PTEN and IKZF4 (Liu SQ. et al., 2014).

Here, I have mainly investigated the role of miR-20a in the activation of human naïve $CD4^+$ T cells. In fact, it has been shown that miR-20a represses transcriptional activation in the Jurkat T-cell line and miR-20a was found to be decreased in Multiple Sclerotic patients' whole blood samples. I have initially assessed the role of miR-20a in the initial phase of naïve $CD4^+$ T-cell activation. TCR triggering resulted in the rapid upregulation of miR-20a and its expression is sustained up to 48 hours. It has been previously showed that c-myc in P493-6 cells (O'Donnell KA. 2005), E2F transcription factors in HeLa cells (Sylvestre Y. 2007), NF-κB in LPS treated biliary epithelial cells, STAT3 in human hepatocellular carcinoma cells (Brock M. 2009)regulate the transactivation ofmiR-20a and the other members of miR-17~92 cluster. I have found that NF-κB is only partially involved in the transcription of miR20a. Conversely, my data suggest that the expression of miR-20a is strongly dependent on AP-1 and NFAT activation at least in the early phase of naïve $CD4^+$ T cell activation which is driven by the TCR-mediated signalling.

In other cell systems, for example in B-cells, miR-17~92 cluster has been shown to stimulate BCR signaling. This study has demonstrated that the cluster targets CD22 and FCGR2B, two immunoreceptor tyrosine inhibitory motif (ITIM)-containing proteins and thereby increases the phosphorylation of Syk and Ca^{++} flux upon BCR triggering (Psathas JN. et al., 2013). Therefore, it is plausible that the members of this cluster may also function as positive regulators of TCR-mediated signaling in T cells. Interestingly, when I assessed the role of miR-20a in T-cell activation by overexpressing miR-20a in human naïve $CD4^+$ T-cells, I have observed that miR-20a inhibits TCR-mediated signaling by decreasing the phosphorylation of ZAP70, LAT, PLC-γ and Erk. Furthremore, as a functional consequence, overexpression of miR-20a also reduced the surface expression of CD69. Additionally, I have observed that miR-20a decreases the recruitment of Zap-70 to CD3ζ. These results are in agreement with previous observations suggesting that miR-17 and miR-20a repress transcriptional activation in the Jurkat T-cells. One of the important findings of this result is that miR-20a inhibits TCR signaling at a very proximal level. Several studies have shown PTEN as a validated target of miR-17~92 cluster. PTEN is a crucial regulator of proximal signaling also in T cells and hence it could represent an ideal target candidate for the regulation of TCR signaling mediated by miR-20a. However, I did not observe any change in PTEN levels upon miR-20a overexpression in T cells. This result corroborates a recent study which showed that only miR-17 and miR-19b could target PTEN in mouse mouse $CD4^+$ T cells. In addition to PTEN, also STAT3 has been shown to be a target of miR-20a although in HEK 293 and RAW 264.7 cell lines. Similarly to PTEN, I did not find alterations in STAT3 expression upon miR-20a overexpression. One possible reason to explain this inconsistency between my results and those of other groups could be the different experimental systems used. Many studies that have successfully demonstrated the silencing of the specified or predicted miRNA targets involved the use of miRNA mimics. miRNA mimics are mature miRNA sequences with several chemical modifications, such as addition of 2´-O-methyl group on their 3´end for instance, which are required in order to stabilize the miRNA mimics and also to render them not accessible to ribonucleases. In addition, they have other modifications to promote their loading into RISC complex. Therefore, these miRNAmimics can better recognize the targets than natural miRNAs and thereby they can silence the target genes more efficiently. In my experimental setup, I have used a plasmid that overexpressespre-miR-20a. The mature miR-20a that is generated with this system is completely natural and, unlike the commercially available miRNA mimics, does not possess any chemical modifications.

In contrast to overexpression, suppression of miR-20a using miR-20a decoy resulted in an increased phosphorylation of different signaling molecules downstream of the TCR. These results are in line with the overexpression data and support the hypothesis that miR-20a is a

negative regulator of TCR-mediated signaling. Nevertheless, the observed differences between miR-20a decoy- and Scrambled-treated $CD4^+$ T cells were only minor. This discrepancy between overexpression and suppression experiments could either be due to the rapid re-expression of miR-20a upon TCR stimulation or intracellular stores of miR-20a precursors or even to redundancy among members of the miR-17~92 cluster and its paralogs. Indeed, a functional overlap has been shown between miR-20a and a three other members of miR-17~92 cluster namely miR-17, miR-106a, and miR-106b in different systems including macrophages, myeloid, and neuronal cells. Interestingly, the seed region, which is crucial for the recognition of the mRNA target, is highly conserved among miR-17, miR-20a, miR-20b, miR-93, miR-106a, and miR-106b. However, I have observed that miR-20a is rapidly expressed despite the suppressive capacity of the decoy and the levels of miR-20a expressionincreased from 20% to 60% upon TCR triggering. Redundancy was ruled out as the other members of the miR-17~92 cluster were also suppressed by miR-20a decoy. In addition, there were no intracellular stores of miR-20a precursors as the precursors were only expressed upon TCR triggering. Therefore, I favor the hypothesis that the rapid expression of miR-20a in miR-20a suppressed cells upon TCR triggering could be a reason for the observed minor differences in signaling profiles between miR-20a decoy and control cells.

Given that the expression of miR-20a is upregulated upon TCR triggering, I have analyzed the effect of miR-20a downregulation by triggering other receptors. To this aim, I have tested T-cell migration that is exclusively dependent on chemokine receptors. It has been shown that miR-20a represses migration of endothelial cells in response to VEGF by targeting MKK and inhibiting p38 MAP kinase activation. In contrast to this observation, my overexpression results showed that miR-20a increases p38 MAP kinase activation in resting $CD4^+$ T-cells. In Tcells CXCR4 and CCR7 are important for T-cell migration. When I stimulated these two receptors, I have not observed any difference in the migration of T cells transfected with miR-20a decoy compared to control cells. It has been shown that signals generated via these receptors result in the activation of Ras-Erk and other signaling pathways. Thus, it is plausible that chemokine receptor triggering might also result in the transactivation of miR-20a which could override the suppressive effect of miR-20a decoy.

Despite defective TCR-mediated signaling and CD69 expression, overexpression of miR-20a did not affect CD25 expression and T-cell proliferation. The latter observation is in line with almost comparable levels of IL-2 production in miR-20a overexpressing vs. control cells. Moreover, these observations also corroborate previous data showing that miR-19b, but not miR-20a, regulates T-cell proliferation in mouse $CD4^+$ T cells upon antigen stimulation (Jiang

S. etal., 2011). On the other hand, I have found that miR-20a strongly inhibits the production of cytokines such as IL-6 and IL-10. Therefore, I favor the hypothesis that miR-20a is not involved in the regulation of T-cell proliferation but rather in the differentiation of $CD4^+$ T cells. A number of studies have demonstrated that TCR-dependent signal strength regulates T-cell polarization (Milner JD. et al., 2010; Yamane H. et al., 2013; van Panhuys N. et al., 2014). The regulation of the magnitude or the duration of signaling is used in many biological systems to control cell fate decisions (Ebysuya M. et al., 2005). Thus, I hypothesize that miR-20a may be a part of the cell machinery that translates quantitative differences in TCR signaling into qualitative regulation of $CD4^+$ T-cell differentiation.

In addition to IL-2, I have observed that miR-20a also moderately decreased the production of IL-8, which function as a chemotactic factor. Therefore, I suspect that miR-20a may limit the recruitment of inflammatory cells at the site of inflammation by decreasing the production of IL-8. Interestingly, a previous study showed that miR-17 and miR-20a targets IL-8 in breast cancer cell lines and controls cellular invasion and tumour metastasis (Yu Z. et al,. 2010). Thus, it is also plausible that miR-20a might directly target *IL-8* in $CD4^+$ T-cells and might play a role in the amelioration of inflammatory condition.

In contrast to the mild effect on IL-2 and IL-8 production, miR-20a appears to strongly suppress IL-6 and in particular IL-10 production in primary human $CD4^+$ T cells. Importantly, in agreement with my data, a previous study has shown that the concentration IL-10 is decreased upon ectopic expression of miR-17 and miR-20a in breast cancer cell lines. IL-6 and IL-10 play crucial roles in the regulation of inflammatory responses. IL-6 is produced upon both sterile and pathogen-induced inflammatory conditions and strongly contributes to host defense. However, elevated levels of IL-6 are associated with the development of chronic autoimmune and inflammatory diseases such as rheumatoid arthritis and MS. Several effects of IL-6 on $CD4^+$ T cells have been described including the induction of differentiation of Th17 and Tfh cells and the triggering of antibody production by B cells. On the contrary IL-6 inhibits TGF-β induced Treg differentiation and thereby also potentially promotes immunopathological conditions. Conversely to the pro-inflammatory effects of IL-6, IL-10 possesses anti-inflammatory properties. IL-10 is the principle cytokine produced by Treg cells and plays critical role in the suppression of immune response. IL-10-deficient mice exhibit prolonged inflammatory responses under a variety of experimental conditions. In humans the levels of IL-10 inversely correlate with the severity of autoimmune diseases. IL-10 enhances the suppressiveness of Treg subsets, attenuates the pathogenicity of Th17 cells, and inhibits both Th1 and Th2 responses. Another important finding from my cytokine analysis is that there are no differences in the levels of IFN-γ and TNF-α production between miR-20a overexpressed cells and control cells. Therefore, it appears that miR-20a may play

strong roles in regulating immune response by mainly modulating the expression of IL-6 and IL-10. Hence, it will be interesting to check how miR-20a tips the balance between pro- and anti-inflammatory states in tissues by strongly decreasing both IL-6, which is a pro-inflammatory cytokine, and IL-10, which is considered as an anti-inflammatory cytokine, at the same time during the course of immune response.

The results I have obtained suggest that miR-20a alone is a regulator of cytokine production in $CD4^+$ human T cells and hence may play an important role in fine turning the adaptive immune responses. The molecular mechanism by which miR-20a regulates cytokine production is not yet clear. It has been shown that the TCR-mediated production of IL-2 and IL-6 depends on Erk1/2 and Ca^{++} signaling (Dumont FJ. et al., 1998). Similarly, IL-10 secretion in $CD4^+$ T cells also depends on TCR-mediated Erk1/2 activity (Saraiva M. 2010). Furthermore, it has been shown that sustained phosphorylation of Erk1/2 upon TCR triggering is required for the differentiation of Th17 cells and in the development of autoimmune pathologies (Mele F. et al., 2015). Importantly, my results show both Erk1/2 and Ca^{++} signaling are reduced in $CD4^+$ T cells overexpressing miR-20a. Thus, miR-20a may regulate cytokine production at least in part via TCR-mediated activation of cellular signaling pathways. However, at the same time, it is also possible that miR-20a may also directly target different cytokine gene transcripts and modulates the production of cytokines in response to T cell stimulation.

IL-17 is a signature cytokine secreted by Th17 cells that plays pivotal roles in promoting autoimmune disorders (Bartlett HS. et al., 2015). As mentioned above, miR-20a levels were decreased in multiple sclerosis. In agreement with this observation, I have observed that miR-20a levels were also reduced during EAE. Furthermore IL-6 production was significantly decreased upon miR-20a overexpression, and IL-6 is indispensable for Th17 differentiation. Therefore, investigating the role of miR-20a in Th17 differentiation would be an interesting approach to check its possible role in suppressing autoimmune disorders. However, differentiation of human naïve $CD4^+$ T cells into Th17 is often tricky and typically requires cells to be stimulated for 5-7 days. Furthermore, cells transfected with miR-20a plasmid loseit upon long term stimulation.It was not possible to achieve a constant overexpression of miR-20a during long term stimulation. Because of this technical limitation, I could not study the role of miR-20a in Th17 differentiation. This is also the reason why it was not possible to measure IL-17 levels, in my cytokine experiments, upon 48 hours of stimulation. A transgenic approach would offer a better opportunity to analyze the role of miR-20a in Th17 differentiation. In contrast to IL-17, IL-10, which is produced by regulatory subsets of T cells, mediates suppression of immune responses. Conversely, it would also be interesting to

investigate whether miR-20a promotes Treg differentiation and ameliorates inflammation and autoimmune disorders.

In conclusion, my results indicate that miR-20a is rapidly induced upon TCR-triggering, which in turn negatively regulates TCR-mediating signaling and decreases the activation of primary human naïve $CD4^+$ T cells. In addition to this, I have shown for the first time that a single member of the miR-17~92 cluster inhibits cytokine production in primary human $CD4^+$ T cells. Thus, miR-20a represents a novel potential target for the regulation of inflammatory conditions, for the treatment of autoimmune diseases. Further studies using *in vivo* models to identify the molecular targets of miR-20a are required to further reveal the physiological function of miR-20a and the mechanism of its action.

4. Materials and Methods

4.1. Ethics

Approval for these studies involving the analysis of TCR-mediated signaling in primary human T cells was obtained from the Ethics Committee of the Medical Faculty at the Ottovon-Guericke University, Magdeburg, Germany with the permission number [107/09]. Informed consent was obtained in writing in accordance with the Declaration of Helsinki.

4.2. Materials

4.2.1. Reagents and recipes

Human T-cell culture medium

1) RPMI 1640 liquid medium with NaHCO3 and stable glutamine (Biochrom) + 10% FCS (PAN Biotech) + 2 µg/ml Ciprobay (Bayer Schering Pharma)
2) X-VIVO 15 Serum free medium w/ Gentamycin (Lonza)

Lysis buffer (1 mL)

ddH_2O – 657 µL

10% lauryl maltoside – 100 µL (Calbiochem)

10% IGEPAL CA-630 – 100 µL (Sigma Aldrich)

1M tris(hydroxymethyl)-aminomethane (pH 7.5) – 50µL (Carl Roth GmbH)

5M NaCl (Carl Roth GmbH) – 33 µL

0.5M ethylenediaminetetraacetic acid (pH 7.5) – 20 µL (Carl Roth GmbH)

0.5M NaF – 20 µL (Sigma-Aldrich)

0.1M Na_3VO_4– 10 µL (Sigma-Aldrich)

0.1M phenylmethanesulfonyl fluoride (PMSF) – 10µL (Sigma-Aldrich)

5 x Reducing sample buffer

50% glycerol (Merck)

330 mM Tris pH 6.8 (Roth)

10% SDS (Serva)

0.01% bromphenolblue (Roth)

10% 2-mercaptoethanol (Merck)

SDS-PAGE resolving 7.5% gel

ddH_2O- 7,2 ml

30% Acrylamid (Bio-Rad) - 3,8 ml

1,5 M Tris pH 8.8 (Roth) - 3,8 ml

10% SDS (Serva) - 150 µl

10% APS (Roth) - 150 µl
TEMED (Roth) - 15 µl

SDS-PAGE resolving 10% gel
ddH_2O - 4,5 ml
30% Acrylamid (Bio-Rad) - 3,8 ml
1,5 M Tris pH 8.8 (Roth) - 2,9 ml
10% SDS (Serva) - 113 µl
10% APS (Roth) - 113 µl
TEMED (Roth) - 11 µl

SDS-PAGE resolving 12% gel
ddH_2O - 3,5 ml
30% Acrylamid (Bio-Rad) - 3,8 ml
1,5 M Tris pH 8.8 (Roth) - 2,5 ml
10% SDS (Serva) - 100 µl
10% APS (Roth) - 50 µl
TEMED (Roth) - 5 µl

SDS-PAGE stacking gel
ddH_2O - 2,1 ml
30% Acrylamid (Bio-Rad) - 0,6 ml
1,5 M Tris pH 8.8 (Roth) - 0,9 ml
10% SDS (Serva) - 38 µl
10% APS (Roth) - 38 µl
TEMED (Roth) - 4 µl

Running Buffer
25 mM Tris pH 8.3 (Roth)
192 mM glycine (Roth)
0.1% SDS (Serva)

Transfer Buffer
39 mM glycine (Roth)
48 mM Tris (Roth)
0.037% SDS (Serva)
20% methanol (Merck)

Blocking Buffer

10 mM Tris pH 7.5 (Roth)

150 mM NaCl (Roth)

5% Milk (Roth)

TBS Buffer

10 mM Tris pH 7.5 (Roth)

150 mM NaCl (Roth)

Washing Buffer

20 mM Tris pH 7.5 (Roth)

150 mM NaCl (Roth)

0.02% Tween 20 (Roth)

IP Washing Buffer

0.1% lauryl maltoside (N-dodecyl β-maltoside) (Calbiochem)

0.1% IGEPAL CA-630 (Sigma Aldrich)

1 mM PMSF (Sigma Aldrich)

50 mM Tris pH 7.4 (Roth)

10 nM NaF (Sigma Aldrich)

0.16 M NaCl (Roth)

4.2.2. Antibodies

Antibodies used for stimulation

Biotin conjugated mouse anti-human CD28 (clone CD28.2, eBioscience)

Biotin conjugated mouse anti-human CD3ε (clone UCHT1, eBioscience)

Mouse anti-human CD3ε MEM92 hybridoma supernatants (kindly provided by Vaclav Horejsi, Academy of Sciences of the Czech Republic, Prague, Czech Republic)

Antibodies used for western blotting

anti-pY_{493}ZAP70 (#2704. Cell Signaling Technology)

anti-pY_{171}LAT (#3584, Cell Signaling Technology)

anti-pY_{783}PLCγ1 (#2821, Cell Signaling Technology)

anti-pT_{202}/Y_{204}ERK1/2 (#9101, Cell Signaling Technology)

anti-pS_{338}cRaf (clone 56A6, Cell Signaling Technology)

anti-phospho(p)$S_{217/221}$ MEK1/2 (clone 41G9, Cell Signaling Technology)

anti-ZAP70 (clone 29/ZAP70, BD Biosciences)

anti-LAT (#06-807, Milipore)

anti-ERK1/2 (#V1141, Promega)

anti-Lck (clone 28/Lck, BD Biosciences)

anti-Sos1 (clone C-23, Santa Cruz Biotechnology)

anti-β-actin (clone AC-15, Sigma-Aldrich)

Each antibody was diluted in TBS buffer (see 4.2.1) supplemented with 5% BSA (Sigma Aldrich) or 5% Milk (Roth) according to manufacturer's recommendation.

Antibodies used for immunoprecipitation

agarose-conjugated anti-CD3ζ (clone 6B10.2, Santa Cruz Biotechnology)

normal goat control IgG (Santa Cruz Biotechnology)

normal rabbit control IgG (Santa Cruz Biotechnology)

Each antibody was added into cell lysate in the presence of Protein A or G agarose beads (Santa Cruz Biotechnology) and 2% of BSA (Sigma Aldrich) in the concentration as recommended by manufacturer.

Antibodies used for flow cytometry

APC conjugated anti-human CD69 (clone FN50, BD Biosciences)

APC conjugated anti-human CD25 (clone M-A25, BD Biosciences)

APC conjugated anti-human CD3ε (BD Biosciences)

APC conjugated anti-human CD28 (BD Bioscineces)

For cell staining antibodies were diluted in PBS (Biochrom) in the concentration recommended by the manufacturer.

4.3. Sequences used for overexpression and suppression of miRNAs

4.3.1. Overexpression of miR-20a:

Top strand: 5'-GTAGCACTAAAGTGCTTATAGTGCAAGTAGTGTTTAGTTATCTACTGCATTATGAGCACTTAAAGTACTGC-3'.

Bottom strand: 3'–GCAGTACTTTAAGTGCTCATAATGCAGTAGATAACTAAACACTACCTGCACTATAAGCACTTTAGTGCTA-5'.

Both sequences were derived from the miRNA database miRBase (http://microrna.sanger.ac.uk/) and overhangs were added to aid efficient cloning into a vector plasmid. Sequences were chemically synthesized from biomers.net. The sequences were then cloned into pcDNA6.2-GW/EmGFP miRNA expression plasmid provided with BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen) according to the manufacturer's instructions. Negative control plasmid used in overexpression study was

provided with BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen).

4.3.2. Suppression of miR-20a:

miR-20a Decoy: 5'-CTAAACACTACCTGCACTATAAGCACTTTAGTGCTAC-3'

Scramble decoy: 5'-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGAC-3'

Complimentary sequence to mature miR-20a sequence was used to design miR-20a decoy. miR-20a decoy in addition contains a 5 nucleotide stretch at both the ends and the base 'U' is replaced with base 'T' to design the DNA decoy of miR-20a. The decoys were chemically synthesized from biomers.net.

4.4. Methods

4.4.1. Human T-cell purification and culture

Peripheral blood mononuclear cells were isolated by Ficoll gradient (Biochrom) centrifugation of heparinized blood collected from healthy volunteers. Naive human $CD4^+$T-cells were further purified by non-T-cell depletion using T-cell isolation kits and the AutoMacs magnetic separation system (all from Miltenyi Biotec). The purity of naive $CD4^+$T-cells, as determined by flow cytometry, was usually more than 96 %. After isolation, T cells were usually cultured at 37°C and 5% CO2 in RPMI medium supplemented with FCS and antibiotics. For cytokine analysis, T cells were cultured in X-VIVO 15 serum free medium.

4.4.2. T-cell transfection

For miRNA overexpression, T-cells were washed with PBS w/o Mg^{++} and Ca^{++} (Biochrom), and approximately $5x10^6$ cells were resuspended in 100 µl of AMAXA nucleofect transfection reagent (Lonza), and transferred into transfection cuvette (Lonza). Cells were added with miR-overexpression plasmid or negative control plasmid at the concentration of 1 µg DNA/10^6cells and mixed gently.Subsequently, cells were electroporated using the AMAXA nucleofector apparatus and transferred to 6 well plates containing medium. Cells were collected after 16 h and used accordingly.

To achieve efficient downregulation, designed miR-20a decoy, along with Scramble-Controls nucleotides were purchased from biomers.net. Primary human naive $CD4^+$T-cells or total T-cell were washed as mentioned above and resuspended in in 200 µl of Opti-MEM transfection medium (Gibco), and transferred into transfection cuvette (Bio-Rad). Subsequently, $1x10^6$ cells were treated with 1 µM of miR-20a decoy or Scramble, mixed

gently and incubated for 3 min at room temperature before transfection. Cells were then electroporated using the Gene Pulser Xcell (Bio-Rad) (one 500V pulse for 3 ms). Cells were collected 24 h after electroporation and stimulated accordingly.

4.4.3. miRNA expression analysis

Primary human naive $CD4^+$T-cells were either stimulated or left unstimulated. Approximately $1x10^6$ were lysed using lysis buffer (Qiagen) and total RNA from approximately 18 nucleotides upwards was isolated using miRNeasy kit (Qiagen), according to the manufacturer's instructions. The quality of the isolated RNA was then evaluated by measuring the OD at 260 and 280 nm with Ultrospec 3000 (Pharmacia Biotech) and then used for the reverse transcription of miRNAs using the miScript II RT kit supplied by Qiagen. Real-time PCR analysis was done by using miScript SYBR Green PCR kit (Qiagen) and the ABI Prism 7000 Sequence Detection System (Applied Biosystem). 3 ng (for mature miRNA) and 15 ng (for precursor miRNAs) from each sample were used for the Real-time PCR analysis. The small RNA, RNU6B was used as reference gene and the primers for the detection of mature miRNAs, miRNA precursors (both pri- and pre-miRNAs), and the reference gene (RNU6B) were purchased from Qiagen (miScript Primer Assays). The ΔΔCt method was used to calculate the differences in gene expression and the data were normalized to the reference gene RNU6B.

4.4.4. T-cell stimulation

T cells were stimulated with either soluble or immobilized mAbs as follows:for soluble Ab stimulation, $1x10^6$ cells were loaded with 100 µL of MEM92, whereasfor microbead stimulation, SuperAvidin-coated polystyrene microspheres (Ø~10 µm, Bangs Laboratories) were coated with biotinylated CD3 in combination with CD28 and CD4 mAbs as indicated (5 µg/ml each) for 30 min at 37°C in PBS (Biochrom). Antibody-coated microbeads were washed twice with PBS, resuspended in RPMI 1640 medium and incubated with T cells in a 1:1 ratio. Stimulation with plate-bound antibodies was performed as follows: 48-well plates were initially coated with goat anti-mouse IgG – IgM (H+L) at a concentration of 4.5 µg/mL and incubated for 4 h at 37°C. After washing, wells were coated with mouseanti-human CD3ε (clone MEM92) or mouse anti-human CD28 hybridoma supernatents (clone 248.23.2; Nunes J. et al. 1991) and incubated for 12 h at 37°C. After washing 1 x 10^6 cells were added to the plates incubated at 37°C for the indicated time periods for different experiments. Stimulations in the presence of either the MEK inhibitor I, U0126 (10 µM; Cell Signaling), IκB inhibitor, IKK VII (200 nM; Calbiochem) or calcium chelator, EGTA (10 mM; Sigma-Aldrich) or DMSO (10 µM; Sigma Aldrich) were performed by pre-incubating T cells for 30 min before stimulation.

4.4.5. Immunoblotting

Stimulation of T cells was stopped by the addition of 1 ml ice-cold TBS and brief centrifugation at 10,000xg. Subsequently, cells were lysed in 30 µl of lysis buffer for 30 min on ice. Afterwards, nuclear content was separated from protein suspension by 10 min centrifugation at 16,000xg. To unwind proteins, 7,5µl of 5x reducing sample buffer was added and samples were heated for 5 min at 95°C. Post-nuclear lysates were separated by SDS-PAGE electrophoresis system (Bio-Rad) and transferred using semi-dry Western blotting onto nitrocellulose membranes (Amersham). Membranes were probed with the indicated primary antibodies and the appropriate HRP-conjugated secondary antibodies (Dianova) and developed using the ECL detection system on the Hyperfilm MP (Amersham).
For quantifications of the immunoblots, the intensity of the detected bands was acquired using the Perfection V700 Photo Scanner (Epson) and analysis was performed using 1D ImageQuant software (Kodak). Unless indicated otherwise, β-actin was used as a loading control (typical loading error in the experiment: ±13%).

4.5.6. Immunoprecipitation

Primary human naive $CD4^+$T-cells ($3x10^7$) were either left untreated or stimulated with sAbs or iAbs for the indicated periods of time. Cells were processed as for immunoblotting, lysed in standard or mild lysis buffer and cleared by centrifugation. Proteins of interest were immunoprecipitated from lysates with specific antibodies conjugated with recombinant 40 µl of protein A or G agarose beads (Santa Cruz Biotechnology) in the presence of 2% BSA (Sigma Aldrich) at 4°C overnight. After washing thoroughly with IP washing buffer, immunoprecipitates were resolved by SDSPAGE, transferred to a nitrocellulose membrane (Amersham), and analyzed by immunoblotting with the indicated antibodies.

4.5.7. Flow cytometric measurements

To determine the efficiency of T-cell activation, T cells were stimulated with plate-bound antibodies as described above (see 4.4.4). After 24 h, T cells were stained with specific antibodies against activation markers (see 4.2.3) for 15 min at 4°C and analyzed by flow cytometric analysis using a BD LSRFortessa, FACSDiva Software 6.1.3 (BD Biosciences), and FlowJo 7.6.5 (Tree Star).

Proliferation experiments were carried out in 24-well plates (Costar). Purified human T cells were labeled with 5 µM Alexa Fluor 700 (eBiosciences) for 10 min at 37°C. After washing, $1.5x10^6$ cells were seeded in 24-well plates coated with antibodies as described above in a total volume of 1000 µl RPMI T-cell culture medium (see 4.2.1). T-cells were either left stimulated or unstimulated and were cultured for 60 h at 37°C and 5% CO2. Proliferation was

assessed by the dilution of proliferation dye using a BD LSRFortessa, FACSDiva Software 6.1.3 (BD Biosciences), and FlowJo 7.6.5 (Tree Star).

For cytokine measurements, T cells were cultured for 48 h in X-VIVO 15 serum free medium (Lonza). At the end of stimulation, supernatants were collected, centrifuged at high speed for 10 min. to remove cell debris. Supernatants were then used to measure cytokine concentrations by ELISA (R&D Systems) or by Multiplex Cytokine Assay (Bio-Rad) according to manufacturer's instructions.

4.5.8. Ca^{++} flux measurement

To measure Ca^{++} flux, human naive $CD4^{+}$ T cells cells were transfected with miR-20a overexpression plasmid or negative control asdescribed above. 16h posttransfection, $1.5x10^{6}$ cells were resuspended in 2 mL of indicator free RPMImedium supplemented with 5% BSA and incubated with 4 µM Indo-1 AM (Invitrogen, MolecularProbes) at 37°C for 45 min in a water bath. Cells were then added with 10x more volume of indicatorfree RPMI medium with 5% BSA and incubated for 45 min at 37 o C in water bath. At the end ofincubation cells were washed and resuspended in 5% indicator free RPMI+BSA solution. Ca++ flux ineach cell sample was measured by stimulating the cells by using with soluble CD3 mAb (Clone MEM92;kindly provided by V. Horejsi, Academy of Sciences of the Czech Republic, Czech Republic) and CD28 (clone 248.23.2) mAb. Subsequently, cells were illuminated at 325 nm with a Helium-Cadmium laser (BD Biosciences) on aLSRII flow cytometer (BD Biosciences). Ionomycin (10 µg/mL; Sigma Aldrich) was added to thesamples at the end of the measurement as a positive control to measure maximum Ca++ flux. Rawdata were transferred to FlowJo for the analysis.

4.5.9. Statistical analysis

Graphical representation of the data and statistical analysis were performed using GraphPad Prism 3.02 (GraphPad Software Inc). Significance was calculated by paired two-tailed student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns $P > 0.005$).

5. Used abbreviations

Abs	antibodies
ADAP	adhesion and degranulation promoting adapter protein
Ago	argonaute protein
Akt	Ak thymoma
AP1	activator protein 1
APC	antigen presenting cell
Bcl10	B-cell lymphoma 10
BCR	B-cell receptor
BM	bone marrow
BIM	Bcl-2-interacting mediator of death
BMPR	bone morphogenetic protein receptor
C	constant region
CARMA1	CARD-containing MAGUK protein 1
CD	cluster of differentiation
C/EBPβ	CCAAT-enhancer-binding protein
CLP	common lymphoic progenitor
CMP	common myeloid progenitor
CRAC	calcium release activated channels
DAG	diacylglycerol
DC	dendritic cell
DN	double negative
DP	double positive
EGF	epidermal growth factor
eIF	elongation initiation factor
ELK	ETS domain-containing protein
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase
GADS	Grb2-related adapter protein downstream of Shc
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GM-CSF	granulocyte monocyte-colony stimulating factor
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HSC	hematopoietic stem cell
iAbs	immobilized anitbodies
IFNγ	interferon gamma
Ig	immunoglobulin
IκB	inhibitor of kappa B
IKK	IκB kinase
IL	interleukin
IL-2R	IL-2 receptor
ICAM1	intracellular adhesion molecule 1
IP_3	inositol 1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
Itk	IL-2-inducible T-cell kinase
JNK	c-Jun N-terminal kinase
LAT	linker of activated T cells
Lck	lymphocyte-specific protein tyrosine kinase
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
MAP3K	mitogen-activated protein kinase kinase kinase
MAPK	mitogen-activated protein kinases

MEK	mitogen-activated protein kinase kinase
MEKK1	mitogen-activated protein kinase kinase kinase 1
MHC	major histocompatibility complex
miRNA	microRNA
miRISC	microRNA-induced silencing complex
MKK	mitogen-activated protein kinase kinase
mTOR	mammalian target of rapamycin
MW	molecular weight
NK	natural killer
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor kappa light chain enhancer of activated B cells
PAMP	pathogen-associated molecular pattern
PDCD4	programmed cell death 4
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLCγ1	phospholipase C gamma 1
PRR	pattern recognition receptor
PTEN	phosphotase and tensin homologue
PTPN22	protein tyrosine phosphatase, non-receptor type 22
Raf	proto-oncogene serine/threonine-protein kinase
Ras	rat sarcoma
RasGAP	Ras GTPase activating protein
RasGRP1	Ras guanyl-releasing protein 1
RBD	Ras binding domain
RNAi	RNA interference
ROS	reactive oxygen species
RNS	reactive nitrogen species
sAbs	soluble antibodies
SAP	signaling lymphocytic activation molecule-associated protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	SH2 domain-containing transforming protein
SHP1	Src homology region 2 domain-containing phosphatase 1
SHIP1	SH2 domain-containing inositol-5-phosphatase
siRNA	short interfering RNA
SLE	systemic lupus erythematosus
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
Sos	son of sevenless
SP	single positive
Src	sarcoma tyrosine kinase
STAT	signal transducer and activator of transcription
TCR	T-cell receptor
TGF	transforming growth factor
TGFβR	Transforming growth factor beta receptor
TNF	tumor necrosis factor
Th	T helper cell
Tfh	follicular T helper cells
Treg	regulatory T cell
TT	tetanus
V	variable region
Vav1	proto-oncogene vav 1
ZAP70	zeta-chain-associated protein kinase 70 kDa

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Curriculum Vitae

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Publication record

1) De novo phosphorylation and opening of Lck act in concert to initiate TCR signaling. Philipsen L*, **Reddycherla AV***, Hartig R, Gumz J, Kästle M, Kritikos A, Mateusz P, Prokazov Y, Turbin E, Weber A, Zuschratter W, Simeoni L,Schraven B, Mueller AJ. (under publication in***Science Signaling.*** 2016) (***- Co-First author**).

2) miR-20a inhibits TCR-mediated signaling and cytokine production in human naïve $CD4^+$ T cells.**Reddycherla AV**, Ines Meinert, Annegret Reinhold, Dirk Reinhold, Burkhart Schraven, Luca Simeoni. ***PLoS One***. 2015 Apr 17;10(4)

3) TCR activation kinetics and feedback regulation in primary human T cells.Poltorak M, Arndt B, Kowtharapu BS, **Reddycherla AV**, Witte V, Lindquist JA, Schraven B, Simeoni L. ***Cell Commun Signal***. 2013, Jan 14;11(1):4

Declaration

I, Amarendra Varma Reddycherla, hereby declare that the work contained herein has been created independently and has not been submitted elsewhere for any other degree or qualification. The research work was carried out from February 2010 to May 2014 at the institute of Molecular and Clinical Immunology, Otto-von-Guericke University, Magdeburg. All sources of information are clearly marked, including my own publications.

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