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T cell-expressed microRNAs critically regulate T follicular helper and Th17 cells

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List of Abbreviations

APC	Antigen-presenting cell
Ascl2	Achaete-scute homologue 2
BATF	Basic leucine zipper transcription factor ATF-like
Bcl6	B cell lymphoma 6
CCR6	C-C chemokine receptor type 6
CD40L	CD40 ligand
CXCR5	C-X-C chemokine receptor type 5
DC	Dendritic cell
Dgcr8	DiGeorge syndrome critical region gene 8
Foxp3	Forkhead box protein P3
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICOS	Inducible co-stimulator
IFNγ	Interferon gamma (γ)
IL	Interleukin
IRF4	Interferon regulatory factor 4
KLH	Keyhole Limpet Hemocyanin
LCMV	Lymphocytic choriomeningitis virus
LEF-1	Lymphoid enhancer-binding factor 1
miRNA	microRNA
NP	4-Hydroxy-3-nitrophenylacetyl
PC	Plasma cell
PD-1	Programmed cell death protein 1
Pre-miRNA	Precursor miRNA
Pre-Tfh	Precursor Tfh

Pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RORγt	Retinoic acid receptor-related orphan receptor gamma t
SAP	SLAM-associated protein
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCF-1	Transcription factor 1
TCR	T cell receptor
TF	Transcription Factor
Tfh	T follicular helper (cell)
Tfr	T follicular regulatory (cell)
TG	Transgenic, transgene
TRBP	Transactivation response element RNA-binding protein
Treg	Regulatory T cell
UTR	Untranslated region

List of Publications

Paper I

Zeiträg J., Dahlström F., Chang Y., Alterauge D., Richter D., Niemietz J., Baumjohann D.

T cell-expressed microRNAs critically regulate germinal center T follicular helper cell function and maintenance in acute viral infection in mice

Eur J Immunol. 2021, ***51****(2):* 408*–413.*

Paper II

Montoya M. M., Maul J., Singh P. B., Pua H. H., Dahlström F., Wu N., Huang X., Ansel K. M., Baumjohann D.

A Distinct Inhibitory Function for miR-18a in Th17 Cell Differentiation

J Immunol, 2017, ***199****(2): 559-569.*

Additional Publications:

Zeiträg J., Alterauge D., Dahlström F., Baumjohann D.

Gene dose matters: Considerations for the use of inducible CD4-CreER^{T2} mouse lines

Eur J Immunol. 2020, ***50****(4): 603-605.*

Maul J., Baumjohann D.

Emerging Roles for MicroRNAs in T Follicular Helper Cell Differentiation

Trends Immunol. 2016, ***37****(5): 297-309.*

Maul J., Alterauge D., Baumjohann D.

MicroRNA-mediated regulation of T follicular helper and T follicular regulatory cell identity

Immunol Rev. 2019, ***288****(1): 97-111.*

1. Zusammenfassung

Follikuläre T-Helferzellen (Tfh) sind wichtig für die Entwicklung der humoralen Immunität, da sie als B-Zell-Helfer fungieren und die Entstehung von Keimzentren kontrollieren, wodurch sie die Produktion von Antikörpern hoher Affinität ermöglichen. Th17-Zellen werden für die erfolgreiche Beseitigung von Pilzen und extrazellulären Bakterien sowie für den Aufbau einer widerstandsfähigen Schleimhautbarriere benötigt. Abweichende Tfh- und Th17-Immunantworten werden mit Autoimmunität, Allergien und Entzündungen in Verbindung gebracht.

MikroRNAs (miRNAs) sind kleine RNA Moleküle, die an der post-transkriptionellen Regulation fast aller biologischer Prozesse beteiligt sind. Obwohl man bereits weiß, dass Tfh-Zellen und Th17-Zellen von miRNAs kontrolliert werden und einige an der Tfh-Zell- und Th17-Zell-Differenzierung beteiligte regulatorische Mechanismen einzelner miRNAs identifiziert wurden, bestehen immer noch viele offene Fragen bezüglich der Rolle von miRNAs in T-Helferzellen.

Tfh-Zellen werden aus miRNA-defizienten naiven $CD4^+$ T-Zellen gar nicht erst gebildet, was zeigt, dass miRNAs bereits für die ersten Schritte der Tfh-Zell-Differenzierung unverzichtbar sind. Um zu untersuchen, ob miRNAs die Differenzierung und Funktion von Tfh-Zellen auch zu späteren Zeitpunkten während einer bestehenden Immunantwort kontrollieren, wurde in dieser Thesis zunächst ein *Cd4-CreER*^{T2} Mausmodell entwickelt, welches erlaubte, einen zeitlich kontrollierten Verlust aller miRNAs exklusiv in $CD4^+$ T-Zellen zu induzieren. Damit wurde dann untersucht, welchen Einfluss der induzierte Verlust von miRNAs auf die Tfh-Zellfrequenzen und -zahlen zu verschiedenen Zeitpunkten nach akuter LCMV Infektion hatte und wie diese Unterschiede die Keimzentrumsreaktion beeinflussten. Mithilfe dieser Methodik konnte gezeigt werden, dass miRNAs nicht nur für die früheste Tfh-Zell-Differenzierung notwendig sind, sondern auch spätere Differenzierungsschritte und die Funktion von Keimzentrums-Tfh-Zellen während einer aktiven Immunantwort regulieren. Zusätzlich führte das Fehlen von T-Zell-exprimierten miRNAs auch zu einer reduzierten Immunantwort der Antigen-spezifischen $CD4^+$ T-Zellen sowie von Th1- und regulatorischen T-Zellen.

Um einen besseren Einblick in die Regulation von Th17 Zellen durch miRNAs zu erhalten, untersuchten wir außerdem die Rolle des miR-17~92 Clusters in Th17 Zellen, mit besonderem Fokus auf der Beteiligung der einzelnen Cluster miRNAs. Interessanterweise konnten wir zeigen, dass miR-18a eine einzigartige Rolle innerhalb des Clusters in der Regulation der Th17-Zell-Differenzierung einnahm, da diese die Differenzierung der Th17-Zellen wirksam hemmte, während andere Cluster miRNAs die Differenzierung förderten. Wir identifizierten und validierten außerdem *Smad4*, *Hif1α* und *Rorα* als funktionelle Targets von miR-18a, welche als wichtige Transkriptionsfaktoren die Differenzierung von Th17-Zellen kontrollieren.

Zusammengefasst heben die Ergebnisse dieser Thesis die Rolle von miRNAs als wichtige Regulatoren von Tfh- und Th17-Zellen hervor und tragen so zum besseren Verständnis der komplexen molekularen Prozesse bei, die Interaktionen zwischen T- und B-Zellen sowie die Plastizität von T-Helferzellen kontrollieren. Dies könnte letztendlich dabei helfen, spezifische Angriffspunkte für die Entwicklung wirksamer Therapien gegen Autoimmunerkrankungen oder Entzündungen zu identifizieren.

2. Summary

T follicular helper (Tfh) cells are important for the development of humoral immunity, as they serve as B cell helpers and control the formation of germinal centers (GCs), thus enabling the generation of antibodies with high affinity. Th17 cells are required for the successful clearance of fungi and extracellular bacteria and the establishment of mucosal barrier integrity. Aberrant Tfh and Th17 cell responses are associated with autoimmunity, allergy, and inflammation.

MicroRNAs (miRNAs) are small RNA molecules that are implicated in the post-transcriptional regulation of almost all biological processes and among these, they also critically control immunological processes. Previous studies showed that both Tfh and Th17 cells are controlled by miRNAs and revealed mechanisms by which individual miRNAs regulate Tfh and Th17 cell differentiation. However, there are still many open questions concerning the function of miRNAs in T helper cells.

Tfh cells do not differentiate from miRNA-deficient naïve $CD4^+$ T cells *in vivo*, thus highlighting that miRNAs are indispensable for the first steps of Tfh cell differentiation. To investigate whether miRNAs also control Tfh cell differentiation and function later during ongoing immune responses, we first developed a *Cd4-CreER*^{T2} mouse model that allowed temporally controlled ablation of global miRNAs exclusively in $CD4^+$ T cells. We characterized alterations in Tfh cell frequencies and numbers in response to induced miRNA loss at different time points after acute LCMV infection and assessed how these alterations affected the GC response. Using this approach, we found that miRNAs not only controlled earliest Tfh cell differentiation, but also regulated later differentiation steps and the function of GC Tfh cells during an ongoing immune response. Notably, lack of T cell-expressed miRNAs also affected the antigen-specific $CD4^+$ T cell compartment, the Th1 response, and the regulatory T cell response.

To obtain further knowledge concerning the regulation of Th17 cell differentiation by miRNAs, we further investigated the function of the miR-17~92 cluster in Th17 cells, with special focus on the contribution of the individual cluster miRNAs. Interestingly, we were able to show that miR-18a had a unique role within the cluster during Th17 cell differentiation, as it potently inhibited Th17 cell differentiation, whereas other cluster

members promoted the formation of Th17 cells. We further identified and validated *Smad4*, *Hif1α*, and *Rorα* as functional targets of miR-18a, which encode for key TFs that control Th17 differentiation.

In conclusion, the findings of this thesis further stress the role of miRNAs as crucial regulators of Tfh and Th17 cells and add to the understanding of the complex molecular mechanisms that control the differentiation and plasticity of T helper cells and regulate T-B cell interactions. This might ultimately help to identify specific targets for the generation of potent therapies against autoimmune diseases and inflammation.

3. Introduction

3.1 The role of CD4$^+$ T cells in adaptive immunity

CD4$^+$ T helper cells represent a fundamental part of the adaptive immune system, as they are important for the clearance of different pathogens, such as viruses, fungi, and bacteria (1, 2). Besides helping B cells to establish potent antibody responses, they communicate with other immune cells via cell-cell interactions and secretion of cytokines. Several subsets of CD4$^+$ T helper cells are known to date, and each of them has specialized roles in orchestrating immune responses against specific pathogens (1-3). Th1 cells, which are identified by expression of the master transcriptional regulator *T-box expressed in T cells* (T-bet) and secretion of the cytokine interferon γ (IFNγ), are particularly important to fight intracellular pathogens. In contrast, Th2 cells express the transcription factor (TF) GATA-3, produce interleukin (IL)-4, IL-5, and IL-13, and elicit immune responses against helminths and parasites. Another T helper cell subset, Th17, drives the elimination of extracellular bacteria and fungi. Th17 cells are regulated by the TF *retinoic acid receptor-related orphan receptor gamma t* (RORγt) and produce IL-17 and IL-22. Regulatory T (Treg) cells represent an additional subset of CD4$^+$ T helper cells. These cells, which express the transcriptional regulator *Forkhead box protein P3* (FoxP3) and produce IL-10, are important to dampen ongoing immune reactions to prevent overshooting immune responses and autoimmunity. Dependent on the type of pathogen, predominant differentiation of Th1, Th2 or Th17 cells is elicited (1-3). In addition to the differentiation of either Th1, Th2 or Th17 cells, T follicular helper (Tfh) cells develop during an immune response. These cells represent a unique CD4$^+$ T helper cell subset as they are the main subset that provides B cell help, which was initially believed to be mainly driven by Th2 cells (2, 4). Recently, T follicular regulatory (Tfr) cells were characterized as another discrete T helper cell subset. Tfr cells have characteristics of both Treg and Tfh cells and act as important suppressors of ongoing GC reactions (5).

As my research was mainly focused on Th17 and Tfh cells, the characteristics and functions of these two important T helper cell subsets will be discussed in detail in the next sections.

3.1.1 Tfh cells

Tfh cells are required for the establishment of effective humoral immunity, as they mediate the formation and maintenance of germinal centers (GCs), microanatomical structures in the B cell follicle, where proliferating antigen-specific B cells experience affinity maturation, class-switch recombination, and differentiation into memory B cells and long-lived plasma cells (PCs) (6-10). In addition to mediating GC formation, Tfh cells also control the cellular mechanisms in GCs that are crucial for survival, proliferation, and differentiation of GC B cells as well as for the production of protective antibodies. Tfh cells provide B cell help via different mechanisms, such as direct cell–cell contacts, cytokine secretion or chemoattraction (6-9). Notably, GC B cells not only require help by Tfh cells, but Tfh cells also depend on interaction with GC B cells for differentiation into GC Tfh cells (11, 12). Unlike other T helper cell subsets, the differentiation of Tfh cells is not restricted to one specific pathogenic stimulus, indicating that Tfh cells exert their B cell helper function in a variety of immunological settings (9). Nevertheless, Tfh cells have been shown to be highly plastic and can adapt to the type of pathogen by producing signature cytokines of the corresponding T effector cells in addition to Tfh cell factors, thus directing Ig production that matches the type of infection (9).

Tfh cells are mainly identified by the expression of their master transcriptional regulator *B cell lymphoma 6* (Bcl6), the chemokine receptor *C-X-C chemokine receptor type 5* (CXCR5), and the inhibitory receptor *programmed cell death protein 1* (PD-1) (6-10). In addition, they express *CD40 ligand* (CD40L), *inducible co-stimulator* (ICOS), and OX40, which are required for direct cell-cell interactions with dendritic cells (DCs) and B cells (6-9). Tfh cells further express the intracellular *signaling adaptor SLAM-associated protein* (SAP), which is needed to maintain durable synapses and adhesion between antigen-specific B cells and T cells (7, 13). Dependent on their differentiation stage, Tfh cells progressively increase expression of classical Tfh cell molecules such as CXCR5, Bcl6, PD-1, ICOS, and SAP, with highest expression in fully matured GC Tfh cells, whereas they downregulate others such as CCR7 and PSGL-1, which is crucial for directed migration and selective interaction with other immune cells (7, 13). The main interactions of Tfh cells with DCs and B cells are presented in **Figure 1**.

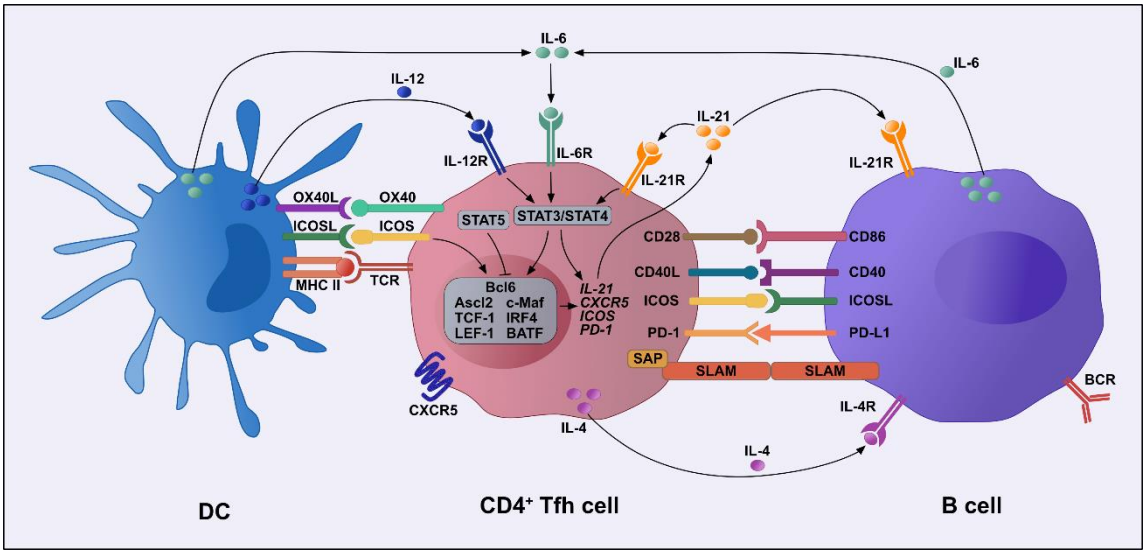

Figure 1. Main interactions between Tfh cells and APCs. Tfh cell differentiation is initiated by DC priming of naïve CD4$^+$ T cells, which involves antigen presentation via peptide-MHCII complexes, leading to T cell receptor (TCR) stimulation. DCs also provide costimulatory signals via ICOSL and OX40L and secrete IL-6 and IL-12 cytokines, thus initiating the Tfh cell differentiation program. TCR signaling, cytokine signals sensed via IL-6R and IL-12R and transduced via STAT3 and STAT4, as well as ICOS signaling induce the expression of characteristic Tfh cell TFs, such as Bcl6, *Achaete-scute homologue 2* (Ascl2), c-Maf, *Interferon regulatory factor 4* (IRF4), *Basic leucine zipper transcription factor ATF-like* (BATF), *Transcription factor 1* (TCF-1), and *Lymphoid enhancer-binding factor 1* (LEF-1). The Tfh transcriptional network then promotes the expression of molecules characteristic for the Tfh cell phenotype and function, such as IL-21, CXCR5, ICOS, and PD-1. Surface expression of CXCR5 on differentiating pre-Tfh cells supports their migration towards the border between the T cell zone and the B cell zone, where they undergo first cognate interactions with B cells, involving ligation via CD28–CD86, CD40L–CD40, ICOS–ICOSL, PD-1–PD-L1, and SAP–SLAM. In addition, B cells receive IL-21 and IL-4 cytokine signals from Tfh cells, whereas they provide IL-6 to Tfh cells. The signals received from B cells enforce expression of Tfh cell molecules such as Bcl6, CXCR5, ICOS, PD-1, and IL-21, thus stabilizing the Tfh cell phenotype. Then again, signals provided by Tfh cells lead to increased proliferation and survival of B cells, support their differentiation towards GC B cells or PCs, and direct affinity maturation and somatic hypermutation within GCs.

The differentiation of Tfh cells is a complex, tightly-regulated process that comprises several differentiation steps and requires constant interactions with antigen presenting cells (APCs), cytokine signals, and chemokine-directed migration towards different microanatomical structures (10, 13). It is regulated by a wide network of TFs and post-transcriptional regulators. More than 20 different TFs, RNA-binding proteins, and miRNAs, have been shown to work in concert to achieve successful Tfh cell differentiation (7, 14). Consecutive antigen presentation, first by DCs and subsequently by B cells, is requisite for optimal Tfh

cell differentiation and GC responses (7, 13, 15). The three-step differentiation process of Tfh cells is presented in **Figure 2**.

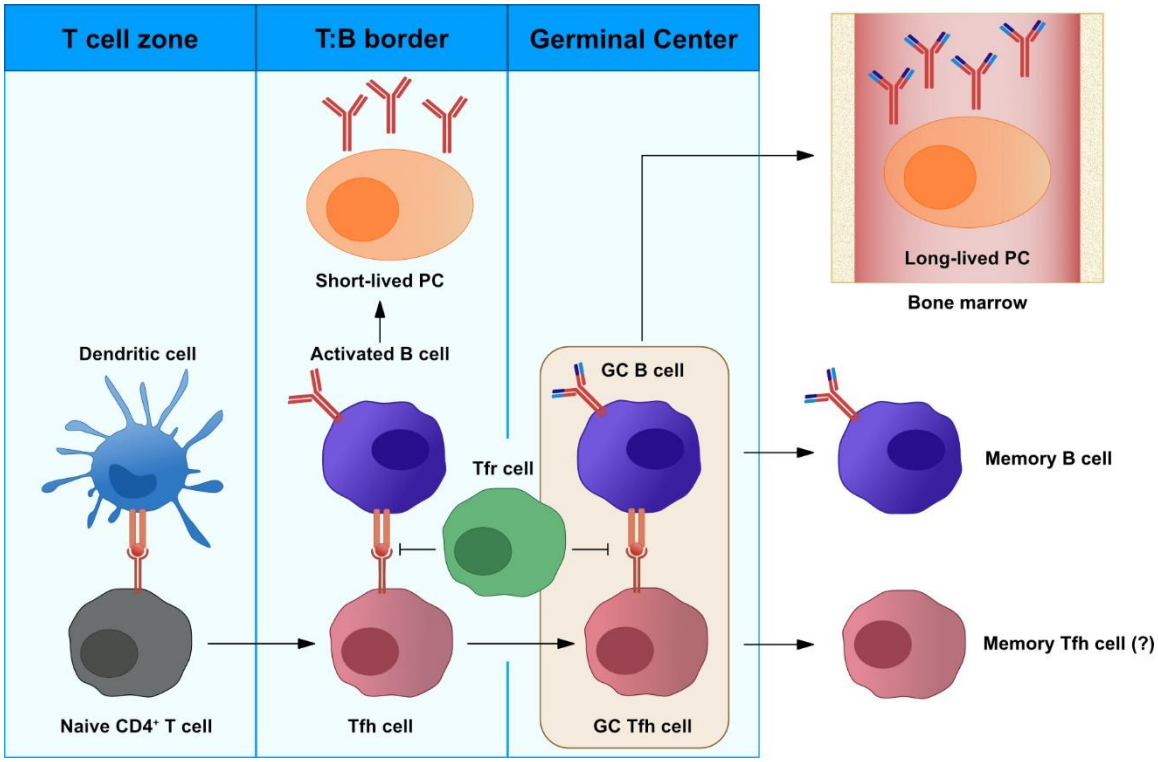

Figure 2. The multistep process of Tfh cell differentiation. Tfh cell differentiation from naïve $CD4^+$ T cell precursors depends on continuous interactions with APCs. First, DCs prime naive $CD4^+$ T cells in the T cell zone of secondary lymphoid organs. This leads to alterations in the expression of chemokine receptors, leading to a migration of differentiating pre-Tfh cells to the border between the T cell zone and the B cell zone. Here, Tfh cells interact with activated B cells, leading to the generation of short-lived PCs. Some Tfh-B cell conjugates migrate towards interfollicular regions where they act as precursors for the formation of GCs inside of follicles. During a GC reaction, GC Tfh cells mediate the generation of long-lived PCs that produce antibodies of high affinity. In addition, memory B and T cells develop during the GC reaction. A formation of memory Tfh cells is also possible, but the exact mechanisms are still unexplained. Tfr cells negatively regulate the humoral immune response and are important to dampen ongoing GC reactions. Adapted from: Maul, Alterauge, Baumjohann, *Immunol Rev* 2019.

Tfh cells are crucial for the development of humoral immunity, and dysregulation of Tfh cells can lead to severe immune pathology (16-18). Increased Tfh cell frequencies can lead to unrestrained GC responses, which are associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus or diabetes type I, whereas defects in Tfh cells elicit defective GC reactions, leading to impaired humoral immune responses (16-18). The implication of Tfh cells in various immune-related diseases

renders them an appropriate target for drug design. Deciphering the exact mechanisms that govern the differentiation, function, and maintenance of Tfh cells should therefore be valuable for the development of new therapies.

3.1.2 Th17 cells

Th17 cells are a heterogenous and highly plastic $CD4^+$ T cell subset (19). They are generally distinguished by the expression of their lineage-defining TF RORγt, *C-C chemokine receptor type 6* (CCR6), and production of IL-17 (1, 19-22). Main tasks of Th17 cells are the elimination of extracellular bacteria and fungi through recruitment of neutrophils, and the establishment of mucosal barrier integrity (1, 20). However, Th17 cells are also implicated in autoimmune diseases and inflammation.

In steady state, Th17 cells are almost exclusively located in the intestinal lamina propria, where they are induced by microbial particles (19, 20, 23). Here, they control the expansion of intestinal bacteria by secretion of IL-17 and IL-22, which drive the production of the antimicrobial proteins RegIIIβ and RegIIIγ (19, 20, 23). Th17 cells also mediate the maintenance of epithelial cell tight junctions and drive the regeneration of epithelial cells (19, 20), with similar functions in lung airways and the skin (22, 24).

Th17 cell differentiation from naïve $CD4^+$ T cells can be induced by activation in the presence of different combinations of the cytokines TGFβ, IL-6, IL-23, and IL-1β (21). Activation of *signal transducer and activator of transcription* (STAT)-3, mainly by IL-6, is necessary for induction of RORγt (19, 20). In addition, Th17 lineage commitment is regulated by other TFs, such as ROR-α, IRF-4, BATF, HIF-1α, and the aryl hydrocarbon receptor (19, 20).

Depending on the combination of inducing factors, Th17 cells can either acquire a pathogenic or an anti-inflammatory phenotype. The differentiation of anti-inflammatory Th17 cells from naïve $CD4^+$ T cells is TGFβ-dependent (25, 26). They produce IL-10 in addition to IL-17, which mediates regulatory and anti-inflammatory processes (19, 25, 26). Interestingly, there are also cells with shared characteristics of Th17 and Treg cells which contain inflammation at mucosal surfaces (27-29). In humans, infection with *Staphylococcus aureus* selectively promotes the differentiation of Th17 cells that produce both IL-17A and IL-10 (30). On the

other hand, the differentiation of pathogenic Th17 cells has been shown to be driven by IL-23 (19, 20). Pathogenic Th17 cells produce IFNγ and *granulocyte-macrophage colony-stimulating factor* (GM-CSF) in addition to Th17 hallmark cytokines, and are associated with the induction of autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis or psoriasis (19, 20, 31). In addition, pathogenic Th17 cells are involved in intestinal inflammation or in inflammatory diseases affecting the respiratory tract, for example asthma or chronic obstructive pulmonary disease (22, 24). Infection of human $CD4^+$ T cells with *Candida albicans* selectively promotes IL-$17A^+IFN\gamma^+$ Th17 cells in an IL-1β-dependent manner (32). Interestingly, several environmental factors, such as environmental pollutants, salt, oxygen, diet, or the circadian clock, also regulate whether Th17 cells differentiate into pro- versus anti-inflammatory cells (19, 20, 33).

Although Th17 cells were extensively studied in the last years, the precise drivers that lead to a transformation from beneficial to pathogenic Th17 cells are still unresolved (19). A precise understanding of the factors that control Th17 cell differentiation and plasticity will help to develop powerful therapies to fight inflammatory and autoimmune diseases.

3.2 miRNAs

miRNAs, non-coding RNA molecules with a length of around 22 nucleotides, are a group of important post-transcriptional regulators (34, 35). In humans, hundreds of different miRNAs have been discovered, and many of these are highly conserved in other animal species as well (34, 36).

Importantly, miRNAs regulate almost all developmental processes (34, 37). The mechanisms by which miRNAs repress their mRNA targets resembles that of RNA interference. It depends on (incomplete) miRNA-mRNA base pairing, which is followed by the accumulation of an *RNA-induced silencing complex* (RISC) that mediates mRNA repression via destabilization or degradation (34). The process of miRNA biogenesis and miRNA functions are presented in **Figure 3**.

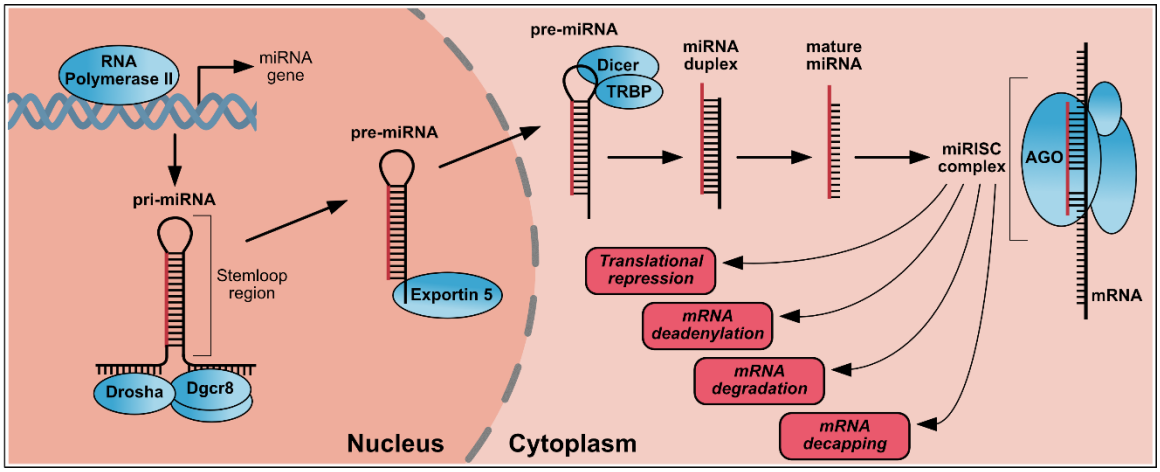

Figure 3. miRNA biogenesis and function. miRNAs are encoded by genomic DNA and transcribed by RNA polymerase II, resulting in the *primary miRNA* (pri-miRNA) transcript. Next, the Microprocessor complex, which comprises the RNAse III Drosha and two proteins of Dgcr8, cleaves the pri-miRNA into the ~60 nt long *precursor miRNA* (pre-miRNA). The pre-miRNA is then carried to the cytoplasm by Exportin 5. Here, the endonuclease Dicer and the *transactivation response element RNA-binding protein* (TRBP) further process the pre-miRNA into a miRNA duplex structure. Upon segregation of the miRNA duplex, the passenger strand (black) is degraded, whereas the guide strand (red), which represents the mature miRNA, is loaded into the miRISC complex and thereby stabilized. The miRISC complex comprises the mature miRNA, proteins from the *Argonaute* (AGO) family, and additional proteins. Based on sequence complementarity between the 3' UTR of the target mRNA and the miRNA seed region located at the 5' end of the miRNA, the mRNA is bound within the miRISC complex. This can either lead to translational repression of the mRNA, or to mRNA deadenylation, decapping or degradation, finally resulting in an inhibition of target protein expression. Modified from: Baumjohann and Ansel, *Nat Rev Immunol* 2013

A variety of studies have demonstrated that miRNAs are powerful regulators of the immune system, and dysregulated miRNAs are associated with autoimmunity or immunodeficiency (36). miRNAs have been shown to control various immune cell types, for example by mediating proliferation, migration, differentiation or lineage commitment (38-40).

3.2.1 miRNAs are crucial regulators of Tfh cells

Previous studies revealed that miRNAs are critically involved in the regulation of T helper cell differentiation and function (39). Whereas Th1, Th2, and Th17 cells can be generated *in vitro* from naïve $CD4^+$ T cells in spite of miRNA deficiency (41, 42), the *in vivo* differentiation of Tfh cells is completely abolished in the absence of mature miRNAs (43). This indicates that Tfh cells are particularly susceptible to miRNA regulation already at the very early differentiation stage. However, there are only a few single miRNAs for which a role in Tfh cells has been described so far. For example, it has been shown that the miR-17~92 cluster positively regulates Tfh cell differentiation (43-46). In addition, several studies showed that miR-146a/b inhibits (47-50) and miR-155 promotes (48, 51-53) Tfh cell differentiation, and one study showed that the proportion of miR-146a and miR-155 defines the degree of Tfh cell differentiation, thus indirectly controlling the magnitude and duration of GC responses (48). There are also individual studies indicating a role for additional miRNAs in Tfh cells. For example, miR-10a (54), miR-31 (55), miR-346 (56), and the miR-23~27~24 family (57) are negative regulators of Tfh cells, whereas miR-181a might promote Tfh cell differentiation (58-62). The so far identified mechanisms by which the aforementioned miRNAs are proposed to regulate Tfh cell differentiation are described in detail in **publication IV and V**.

In contrast to Th1, Th2, and Th17 cells, it is particularly challenging to study the regulation of murine Tfh cells by miRNAs. Appropriate protocols for *in vitro* Tfh differentiation remain unavailable (17), meaning that an assessment of Tfh cell differentiation completely relies on *in vivo* mouse models. So far, mice with a T cell-specific deletion of key factors for miRNA biogenesis (Drosha, Dicer, Dgcr8; **Figure 3**), leading to a loss of mature miRNAs due to an abrogation of miRNA processing, were commonly used to examine the impact of miRNAs in T cells (41, 42, 63, 64). Studies that employed these mice firstly demonstrated that miRNAs were requisite for proper T cell maturation and homeostasis (41, 42, 63, 64). Using

mice with a T cell-specific deletion of *Dgcr8*, it has been shown that miRNA deficiency causes a complete stop of Tfh cell differentiation already at the earliest differentiation stage (43). For this reason, the aforementioned classical mouse models cannot be used to examine the regulatory role of miRNAs at later stages of the multistep Tfh cell differentiation process (**Figure 2**).

The aim of this thesis was therefore to investigate whether miRNAs are also involved in the regulation of Tfh cell differentiation and function later during ongoing immune responses using a newly developed mouse model in which miRNA ablation can be induced at any time point. For this purpose, I first tested two different published *Cd4-CreER*^{T2} mouse lines, which allow for excision of *loxP*-flanked genes upon administration of the synthetic estrogen tamoxifen, concerning their applicability to study Tfh cell responses after immunization. Interestingly, after NP-KLH immunization and subsequent tamoxifen administration, one of the *Cd4-CreER*^{T2} mouse lines displayed severe defects in activated CD4$^+$ T cells, and thus in Tfh cells, and was therefore inapplicable for our study (**publication I** "T cell-expressed microRNAs critically regulate germinal center T follicular helper cell function and maintenance in acute viral infection in mice"). A comparison of both mouse lines and implications for their experimental use are displayed in **publication III** "Gene dose matters: Considerations for the use of inducible CD4-CreER^{T2} mouse lines".

After selection of a suitable *Cd4-CreER*^{T2} mouse line, I developed and characterized a *Cd4-CreER*^{T2}-dependent mouse model in which the miRNA biogenesis factor *Dgcr8* (**Figure 3**) could be deleted in CD4$^+$ T cells by application of tamoxifen, resulting in the loss of mature miRNAs. Thus, this model allowed temporally controlled ablation of global miRNAs exclusively in CD4$^+$ T cells *in vivo*. In **publication I**, I assessed alterations in Tfh cell frequencies and numbers in these mice, and how these affected the GC response after tamoxifen-induced miRNA loss at different time points during acute LCMV infection. Using this approach, I was able to show for the first time that miRNAs, besides being required for earliest Tfh cell differentiation, as has been published before (43), also controlled later differentiation steps. In addition, miRNAs regulated the function of GC Tfh cells during an ongoing immune response, as a loss of T cell-expressed miRNAs during LCMV infection led to an impairment of GC responses. Notably, the lack of T cell-expressed miRNAs also affected antigen-specific CD4$^+$ T cells, the Th1 response, and the regulatory T cell response.

In conclusion, the data presented in **publication I** revealed additional features in the regulation of Tfh cells by miRNAs, thus helping to understand how precisely T-B cell interactions and T helper cell plasticity are regulated on the molecular level. It is anticipated that follow-up studies using similar mouse models that I have established here will help to elucidate the precise mechanisms by which individual miRNAs control Tfh cell differentiation, maintenance, and function, and the identification of relevant target genes implicated in these regulatory processes. This could be relevant for the development of therapeutic approaches against various immune-associated diseases. Also, the discovery of potent miRNA-target networks or specific miRNA-target interactions in the regulation of distinct T helper cell subsets will be of special interest for the development of miRNA-based therapies in the treatment of autoimmune diseases, allergy, and cancer (65, 66).

3.2.2 The function of miRNAs in the regulation of Th17 cells

Activated $CD4^+$ T cells which are deficient in miRNAs due to a knockout of the miRNA biogenesis factor Dicer (**Figure 3**) showed impaired Th17 cell differentiation *in vitro*, indicating that miRNAs are involved in the regulation of Th17 cells (41). Indeed, several miRNAs are implicated in the control of Th17 cell differentiation and function (67-73). For example, miR-326 drives the differentiation of Th17 cell by repression of *ETS1*, a negative regulator of Th17 cells (68). miR-21, miR-301a, and miR-155 are enriched in CNS-infiltrating cells during *experimental autoimmune encephalomyelitis* (EAE), a mouse model of multiple sclerosis that is predominantly dependent on Th1 and Th17 cells (69). Interestingly, these miRNAs all promote Th17 cell differentiation via different mechanisms (69-73).

The miR-17~92 cluster has been proven to be a very potent post-transcriptional regulator in a variety of different biological settings and cell types (74-76). It comprises six mature miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1), which are all processed from a single polycistronic transcript (74, 75). Several studies have demonstrated that miR-17~92 or its individual cluster members support $CD4^+$ T cell survival and proliferation, but also have specific functions in different T helper cell subsets (43, 45, 64, 77-84). For example, it was shown that miR-17~92 stabilizes Tfh cells by inhibition of alternative cell fates (43). miR-17~92-deficient $CD4^+$ T cells differentiated into cells with a

mixed Tfh/Th17 phenotype during acute LCMV infection *in vivo* (43). These cells co-expressed the Tfh cell-specific genes *Bcl6* and *Cxcr5* along with *Ccr6*, *Il1r2*, *Il1r1*, *Rora*, and *Il22*, which are representative for Th17 cells. Importantly, *Rora* was directly targeted by all miR-17~92 miRNAs, and the phenotype of miR-17~92-deficient $CD4^+$ T cells was already rescued by deleting one functional Rora allele (43). These findings suggested that the miR-17~92 cluster is also involved in the regulation of Th17 cells.

In **publication II**, "A Distinct Inhibitory Function for miR-18a in Th17 Cell Differentiation", we therefore assessed the role of miR-17~92 in Th17 cells and especially aimed at deciphering the contribution of the single cluster miRNAs to the regulation of Th17 cells. We could show that miR-17~92 deficiency led to increased Th17 cell differentiation *in vitro*, indicating that miR-17~92 in its entirety inhibits the differentiation of Th17 cells. Interestingly, we found that miR-18a has a distinct function within the miR-17~92 cluster as it is the only miRNA that negatively regulates Th17 cell differentiation. In addition, after $CD4^+$ T cell activation, miR-18a is the most dynamically upregulated cluster miRNA. We showed that *in vitro* activated miR-18a-deficient naïve $CD4^+$ T cells that were cultured under Th17-polarizing conditions displayed increased differentiation into $ROR\gamma t^+CCR6^+$ Th17 cells. miR-18a deficiency also led to elevated numbers of tissue Th17 cells in *in vivo* models of asthma airway inflammation. We further revealed that miR-18a exerted its function by directly targeting genes encoding TFs important for Th17 cell differentiation, namely *Smad4*, *Hif1a*, and *Rora*. In contrast, the other cluster members, especially miR-17 and miR-19 family miRNAs, promoted the Th17 cell phenotype, which is in line with a previous study (79), indicating that miR-18a had a dominant effect in the miR-17~92 cluster´s role in the regulation of Th17 cells.

In summary, **publication II** uncovered that the net effect of a miRNA cluster can be traced back to a single miRNA that overrides the other miRNA´s functions, thus providing new insights in the complex field of miRNA function. Our findings presented in **publication II** further helped to decipher how Th17 cell differentiation is regulated on the molecular level. This could provide new avenues for the development of drugs that aim at targeting Th17 cells, for example in the treatment of autoimmune diseases, inflammation, or, as indicated in our study, for asthma therapy.

4. Contribution to Publications

4.1 Contribution to Paper I

Zeiträg J., Dahlström F., Chang Y., Alterauge D., Richter D., Niemietz J., Baumjohann D.

T cell-expressed microRNAs critically regulate germinal center T follicular helper cell function and maintenance in acute viral infection in mice

Eur J Immunol. 2021, ***51****(2) :* 408*–413*

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As the main author of this publication, I participated in the study design and performed most experimental work and data analysis myself. I also designed all figures shown in the publication. Specifically, I was responsible for breeding, inter-crossing, and maintenance of experimental mice and tested the applicability of the developed mouse models in terms of functionality, specificity, and deletion efficiency. Furthermore, I performed pre-experiments to establish protocols for *in vivo* mouse experiments, involving tamoxifen treatment and infection with lymphocytic choriomeningitis virus (LCMV) Armstrong, and developed protocols for flow cytometric staining and analysis as well as for sorting of cells for subsequent qRT-PCR analysis. Some pre-experiments were performed by Julia Niemietz under my supervision. After the successful establishment of experimental protocols and the selection of appropriate tamoxifen treatment and analysis time points, I performed all *in vivo* mouse experiments presented in the publication and prepared, stained, and analyzed the cells by flow cytometry at the specified time points. I also sorted cells for qRT-PCR, which was performed and analyzed by my colleague Frank Dahlström, and we interpreted the qRT-PCR data together. Frank Dahlström was also responsible for the genotyping of the experimental mice. Yinshui Chang performed an *in vivo* adoptive co-transfer experiment and subsequent cell sorting for RNA-sequencing. Dominik Alterauge sorted cells from two *in vivo* adoptive

co-transfer experiments and further established and performed RNA-sequencing. Daniel Richter analyzed RNA-sequencing data. However, we later decided to exclude RNA-sequencing data from the manuscript. Dirk Baumjohann and I interpreted the data, selected data for publication, and wrote the manuscript. Dirk Baumjohann also conceived the project and provided overall direction of the study.

4.2 Contribution to Paper II

Montoya M. M., Maul J., Singh P. B., Pua H. H., Dahlström F., Wu N., Huang X., Ansel K. M., Baumjohann D.

A Distinct Inhibitory Function for miR-18a in Th17 Cell Differentiation

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As the second author of this publication, I performed some of the experiments, analyzed the data obtained from these experiments, and interpreted it together with Dirk Baumjohann. In addition, I contributed to some of the figures and critically read the manuscript. In our lab, I was responsible for breeding and maintenance of some of the experimental mice used in this study, while genotyping was performed by Frank Dahlström. To verify the reproducibility of the data previously obtained by the main author Misty Montoya, I performed *in vitro* experiments in which I activated naïve $CD4^+$ T cells derived from CD4-Cre miR-17~92$^{fl/fl}$ and control mice, or from miR-18a-deficient and -sufficient mice, and cultured them under Th17-polarizing conditions for 3.5 days. I analyzed these cells by flow cytometry concerning their expression of Th17 markers in response to the presence or absence of miR-17~92 or miR-18a (i.e. replicate experiments of Fig. 2 a, b and Fig. 3 a, b). I also compared the proliferation of miR-18a-deficient and -sufficient $CD4^+$ T cells during the Th17 differentiation process by staining them with Cell Trace Violet prior to activation, and analyzed the cells by flow cytometry at the end of the culture period (Fig. 3 d). In addition, I performed Annexin V staining and subsequent flow cytometric analysis to investigate the viability of miR-18a-deficient $CD4^+$ T cells in comparison to miR-18a-sufficient cells after 3.5 days in culture. I could show that the amount of early-apoptotic and late-apoptotic/dead cells was not affected by a lack of miR-18a, thereby confirming that miR-18a does not affect the survival of Th17 cells *in vitro* (Fig. 3 e). Misty Montoya performed and analyzed most of the experiments. Together with Mark Ansel and Dirk Baumjohann, the supervisors of the

study, she designed the experiments, interpreted the data, and wrote the manuscript. Some experiments were also performed by Priti Singh, Heather Pua, Nanyan Wu, Xiaozhu Huang, and Dirk Baumjohann.

4.3 Contribution to Paper III (Appendix A)

Zeiträg J., Alterauge D., Dahlström F., Baumjohann D.

Gene dose matters: Considerations for the use of inducible CD4-CreER^{T2} mouse lines

Eur J Immunol. 2020, ***50****(4) : 603-605.*

https://doi.org/10.1002/eji.201948461

As the first author of the paper, I was involved in the design of the study, bred, inter-crossed, and maintained the employed experimental mouse lines, and performed most pre-experiments to establish the experimental protocols for the study. The pre-experiments involved assessment of different tamoxifen treatment regimens, determination of suitable *in vivo* tamoxifen treatment and analysis time points after immunization of mice with NP-KLH, and development of staining panels for flow cytometric analysis. Additional pre-experiments were performed by Dominik Alterauge. I further performed all *in vivo* experiments presented in the publication, subsequently analyzed the cells by flow cytometry or I sorted cells for qRT-PCR analysis. Frank Dahlström genotyped the experimental animals and performed and analyzed qRT-PCR experiments. Together with Dirk Baumjohann, I interpreted the data, selected data for publication, and wrote the manuscript. I also designed all figures for the manuscript. As the corresponding author of the paper, Dirk Baumjohann conceived the project and provided overall direction of the study.

4.4 Contribution to Paper IV (Appendix B)

Maul J., Baumjohann D.

Emerging Roles for MicroRNAs in T Follicular Helper Cell Differentiation

*Trends Immunol. 2016, **37**(5): 297-309.*

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As the main author of this review, I did most of the literature research, selected and structured the important information, wrote the initial manuscript, and designed the table and the figures. Dirk Baumjohann designed the first outline of the review. Together, we discussed the final review outline and the content of the figures. Dirk Baumjohann also critically read the initial manuscript, did some corrections, and added missing information. I incorporated the corrections and wrote the final manuscript together with Dirk Baumjohann.

4.5 Contribution to Paper V (Appendix C)

Maul J., Alterauge D., Baumjohann D.

MicroRNA-mediated regulation of T follicular helper and T follicular regulatory cell identity

Immunol Rev. 2019, ***288****(1): 97-111.*

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As the main author of this review, I did most of the literature research, and wrote most of the initial manuscript. Dirk Baumjohann provided the first outline of the review. Together with Dirk Baumjohann and Dominik Alterauge, I discussed the final outline of the review and the content of the figures. Dominik Alterauge wrote the part of the introduction about Tfh and Tfr cells and helped me with literature search concerning the role of miRNAs in Tfr cells. Dirk Baumjohann designed Figure 1, critically read the first drafts of the manuscript, did some corrections, and added missing information. I incorporated the corrections and wrote the final manuscript together with Dominik Alterauge and Dirk Baumjohann. Together with Dominik Alterauge, I also designed Figures 2 and 3.

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Appendix A

Paper III

Zeiträg J., Alterauge D., Dahlström F., Baumjohann D.

Gene dose matters: Considerations for the use of inducible CD4-CreER^{T2} mouse lines

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Appendix B

Paper IV

Maul J., Baumjohann D.

Emerging Roles for MicroRNAs in T Follicular Helper Cell Differentiation

*Trends Immunol. 2016, **37**(5): 297-309.*

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Appendix C

Paper V

Maul J., Alterauge D., Baumjohann D.

MicroRNA-mediated regulation of T follicular helper and T follicular regulatory cell identity

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