Chapter 7

Summary and Discussions

Future perspectives
SUMMARY AND DISCUSSION

The central role played by CD4+ T-cells in the development of the adaptive immunity has been well established in the last decades [1,2]. However, the molecular cues involved in CD4+ T-cell development and function, such as miRNAs, are still a subject of intensive investigation. Studies employing conditional Dicer deletions at defined stages of T-cell development have clearly demonstrated that maturation and function of T-cells, and Tregs in particular, strongly depends on miRNA regulation [3,4]. Indeed, inhibition of miRNA biogenesis in Tregs leads to break of peripheral tolerance and ultimately the development of a lethal autoimmune condition [5,6]. Due to the significant role of Tregs in the prevention of excessive immunity, their malfunction has been suspected to underlie the development of autoimmune conditions such as Rheumatoid Arthritis (RA). At the same time, growing evidence showed involvement of deregulated miRNA expression patterns in T-cells in the pathogenesis of multiple sclerosis and systemic lupus erythematosus (SLE) [7-10]. Based on this knowledge, the overall aim of this thesis was to characterize the miRNA expression patterns of T-cells and Treg cells derived from RA patients (Chapter 2 and 5), and to elucidate the (patho)physiological role of selected miRNAs in T-cell biology (Chapter 3, 4, and 6).

miRNAs in T-cells of RA patients

Several studies have reported altered miRNA expression in T-cells derived from patients suffering from autoimmune conditions such as SLE, ankylosing spondylitis or multiple sclerosis [9,11,12]. However, there are no studies that have assessed the miRNA expression patterns in Tregs derived from RA patients. In Chapter 2 we isolated naive and memory Tregs and Tconvs from RA patients and healthy controls, and assessed the miRNA expression profile using a microarray approach. We found that the general miRNA signature of specific T-cell subsets is not different in RA patients versus healthy controls, nor is it affected by RA treatment. The expression of one miRNA, miR-451, correlated with the disease activity score. Thus, our data indicate that, in contrast to SLE [9,10,13,14], CD4+ T-cells derived from RA patients are not characterized by significantly altered miRNA signatures. Due to the low number of patients used for miRNA profiling in our study (6 RA patients, and 2 healthy controls) we cannot exclude the existence of additional subtle changes between RA patients and healthy controls. Li et al. recently showed an increased expression of miR-146a in CD4+ T-cells isolated from the inflamed joint of RA patients in comparison to CD4+ T-cells isolated from peripheral blood of healthy controls [15]. We found that synovial fluid T-cells are almost exclusively composed
of memory T-cells, and contain significant numbers of memory Tregs (Chapter 5). In Chapter 2 we found that both memory T-cells and memory Tregs are characterized by higher miR-146a levels than naive T-cells [16,17]. Moreover, chronic T cell activation as generally observed in the synovium and synovial fluid leads to upregulation of miR-146a ([16] and Chapter 3). Thus, it is debatable whether an increased expression of miR-146a in synovial fluid T-cells is an intrinsic T-cell defect or a consequence of a local predominance of T-cells with an activated memory phenotype. Thus, the analysis of defined T-cell subsets is essential to reliably study miRNA expression in relation to inflammatory conditions. Another study by Fulci et al. reported elevated levels of miR-223 in peripheral blood CD4+ T-cells of RA patients [18]. We did not confirm this finding in our study, possibly due to inter-donor variation.

**Physiological expression of miRNAs in naive and memory Treg and Tconv cells**

Tregs are particularly dependent on miRNA expression. Disrupted miRNA biogenesis during Treg development has been shown to cause lethal autoimmunity, comparable to lack of the Foxp3 transcription factor expression [6]. Moreover, it has been shown that knockout of miR-146a in mice seriously hampers the capability of Tregs to suppress a Th1 response and results in the development of autoimmunity [19]. Several studies have analyzed Treg specific miRNA expression profiles [4,17,20,21]. However, the isolation of Tregs based on the bright expression of CD25 results in a selective enrichment of memory Tregs (defined by CD25<sup>thick</sup> staining), and depletion of naive Tregs (defined by CD25<sup>int</sup> staining). In Chapter 2 we used a recently described gating strategy (Figure 1A) [22] to isolate naive and memory Tregs separately, followed by an assessment of their miRNA expression profiles. We found that similar to conventional naive and memory T-cells, naive and memory Tregs are characterized by specific miRNA expression patterns. Several miRNAs associated previously with a Treg phenotype, i.e. miR-21, miR-24 and miR-155, were highly expressed in memory Tconv and memory Tregs, but not in naive Tregs, indicating their association with a memory phenotype rather than a Treg phenotype. Importantly, we identified miRNAs specifically expressed in both Treg subsets, and as such associated with a true Treg phenotype. Among these, miR-146a was the most abundantly expressed miRNA, indicating its crucial involvement in human Treg function.

In Chapter 3, we specifically focused on four miRNAs differentially expressed between naive and memory T-cells, i.e. miR-146a, miR-21, miR-155, and miR-31, and examined their expression during activation of naive T-cells. We found that activation of naive T-cells results in pronounced miRNA expression changes. Moreover, we found that the activation induced changes in expression of each of
these miRNAs follow different kinetics. Based on these data, we propose that the differential timing of miRNA induction and/or inhibition during T-cell activation is tightly regulated and programmed to control specific aspects of T-cell activation in time. Importantly, levels of miRNAs upregulated during naive T-cell activation i.e. miR-146a, miR-155, miR-21, remain high in memory T-cells, while miRNAs downregulated during the activation i.e. miR-31 remain low in memory T-cells. Thus it becomes clear that activation of naive T-cells introduces a global re-programming of the miRNA expression profile, which parallels the acquisition of a memory phenotype. Consistent with this, Grigoryev et al. reported that T-cell activation introduces changes in the expression of 71 different miRNAs, of which 51 were upregulated [23]. Bronevetsky et al. reported partly contradictory results, i.e. that T-cell activation introduces a global downregulation of miRNAs due to degradation of Ago2 proteins [24]. Interestingly, we found that a single miRNA may have different functions based on the differentiation state of the T-cell [25]. MiR-21 fine tunes the level of CCR7 protein in activated naive T-cells, and inhibits apoptosis in activated memory T-cells (Chapter 4). This shows that the cellular context affects the outcome of miRNA-based regulation. Therefore, the selection of a biologically relevant cellular system is crucial to reliably study the physiological function of specific miRNAs.

Figure 1. Purification strategy and IRAK1 expression analysis in naive and memory Tregs.
A Gating strategy used to purify naive and memory Tregs. B QRT-PCR analysis of IRAK1 transcript expression in naive and memory Treg and Tconv cells. IRAK1 expression has been normalized to the average of TBP and RPII reference genes. Data derived from 14 donors described in Chapter 2. Wilcoxon matched pairs test was used to determine statistical significance. * p<0.05, ** p<0.01
Identification of new, functionally relevant target genes in a given cell type, is the main challenge in the study of miRNAs. The significant numbers of predicted target genes and the time-consuming luciferase reporter based validation experiments make the identification of new target genes highly inefficient. In order to find miR-21 targets responsible for its anti-apoptotic function observed in primary memory CD4+ T-cells and Jurkat T-cells, we used a high throughput Ribonucleoprotein ImmunoPrecipitation – gene Chip (RIP-Chip) approach (Chapter 6). We used Jurkat T-cells as a model to study miR-21 function, since Jurkat T-cells have been extensively employed to determine molecular cues following T-cell receptor triggering, and apoptosis induction [26]. We used antibodies directed against endogenous Ago2 protein to immunoprecipitate RNA induced silencing complexes from wild type Jurkat T-cells and Jurkat T-cells overexpressing miR-21, followed by target genes analysis using gene expression arrays. This approach allowed us to determine target genes specifically regulated upon miR-21 overexpression. Among those transcripts we found several genes involved in apoptosis regulation as well as immune-related genes. Further work is in progress in order to validate these preliminary findings.

In conclusion, miRNAs are now implicated in virtually all biological processes related to CD4+ T-cell development, differentiation and apoptosis. Moreover, it is evident that specialized T-cell subsets, such as naive, memory, Th1, Th2, Th17 or Treg cells are characterized by distinct miRNA signatures (this thesis [17]). Several studies have underlined the degree of plasticity among T-cell subsets. Specific transcriptional circuits can either promote stability of a given subset or induce plasticity between subsets [27-29]. An example of this is the finding that a single T-cell may share characteristics of two or more functionally distinct subsets, such as Treg and Th17 [30-32]. MiRNAs are emerging as novel regulators of T-cell differentiation. It appears that dynamic expression of miRNAs provides an additional layer regulating the stability and/or plasticity between specific T-cell subsets (reviewed in [33,34]). The data presented in this thesis add to this perspective by showing that miR-146a is the most abundantly expressed miRNA in Tregs irrespective of their maturation state, indicating its involvement in stabilizing the Treg lineage, and by showing that a single miRNA, i.e. miR-21 might provide different functions based on the differentiation state of the T-cell. Moreover, the data presented in this thesis underlies the necessity to analyze defined T-cell subsets when studying miRNA expression in relation to autoimmunity, since the composition of peripheral blood CD4+ T-cells significantly influences miRNA expression readout. Therefore, the fluctuations in the naive:memory T-cell ratio due to infections or disease activity should be taken into account when analyzing miRNA expression in T-cells.
FUTURE PERSPECTIVES

Although the understanding of miRNA function in human T-cells has significantly improved, the majority of the data is still derived from animal studies [19,35,36]. It is important to acknowledge that expression and function of T-cell specific miRNAs might differ between mice and man. An example is the expression of miR-155 in Tregs. Stahl et al. have shown that, in contrary to mice Tregs, human resting Tregs do not express higher miR-155 levels than Tconv cells [37]. Moreover, in this thesis we have experimentally proven that CCR7 is a direct target of miR-21 in primary human naive T-cells [25], whereas the miR-21 binding site is not present in the 3'UTR of the mice CCR7 gene, and as such CCR7 is not a miR-21 target gene in mice. These examples indicate that a direct extrapolation of results obtained in mice models to human cells is not always appropriate, and additional studies in human T-cells are needed to validate findings from mice models.

It is especially important to determine miR-146a function in human Tregs. Lu et al. have shown that miR-146a regulates suppressive capacity of mice Tregs by direct repression of STAT1 [19]. We have observed that miR-146a is the most abundant Treg-specific miRNA (Chapter 2), confirming its involvement in human Treg functioning. Moreover, miR-146a has been shown to target proteins involved in the positive regulation of the NF-κB pathway in mice T-cells, i.e. IL-1 receptor associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) [38,39]. IRAK1 and STAT1 are interesting candidates for further analysis, due to their involvement in regulation of Treg function. IRAK1 negative mice are characterized by higher numbers of Tregs, and decreased expression of IL-17 upon stimulation with IL-6 and TGFβ, which together leads to dampened inflammatory responses in acute and chronic inflammatory mice models [40]. Similarly, STAT1 deficiency enhanced proliferation and reduced the apoptosis of mice Tregs [41]. Using qRT-PCR, we determined that both naive and memory Tregs express lower IRAK1 transcript level than their respective Tconv counterparts (Figure 1B). Whether the same trend is observed at the protein level, and whether the observed difference is a consequence of the high miR-146a expression in Tregs remains to be investigated. Therefore, a functional study, validating miR-146a function in the maintenance of human Tregs lineage is warranted.

Identification of functional, T-cell specific, miRNA target genes is crucial to determine miRNA function in T-cell biology. In Chapter 6 we have applied a high throughput technique to determine miR-21 target genes in the human Jurkat T-cell line. Performing such high throughput identification of miRNA target genes in primary human T-cells would be ideal. Such an approach, however, is technically challenging. Successful Ago2 immunoprecipitation requires a large
number of cells, which is a limiting factor in the study involving specific primary human T-cell subsets. Moreover, lentiviral modulation of miRNA expression, as performed in Chapters 4 and 6, requires activation of naive T-cells in order to achieve sufficiently high transduction efficiency. To study miRNA target genes in resting T-cells, a different strategy to modulate miRNA expression, e.g. transfection with miRNA precursors, could be used. In chapter 6, we used a gene expression array to determine miRNA target genes co-immunoprecipitated with Ago2 protein. Application of RNA-sequencing, instead of gene expression arrays, would allow for a more effective identification of miRNA target genes, including alternative splice isoforms. Application of RNA sequencing could be also considered in future miRNA profiling studies. In this thesis, we have used microarray and qRT-PCR approach to study miRNA expression. The microarray technique allows the analysis of a broad range of miRNAs, while qRT-PCR enables assessment of miRNA in minute RNA samples, derived from FACS isolated T-cell subsets. However, these approaches do not allow identification of novel T-cell subset-specific miRNAs species. The value of small-RNA sequencing lies in the fact that it allows detection of both known and novel miRNAs. In addition, it also provides information on the exact miRNA length and sequence [42]. The drawback of this technique, however, is that it still requires a relatively high amount of RNA which cannot be obtained from less abundant, specific T cell subsets. However, improvements in the sample preparation, allowing the analysis of minute amounts of RNA [43], will make small RNA sequencing the method of choice to study miRNA expression in defined T-cell subsets in the future. An alternative to RNA sequencing, allowing miRNA and mRNA profiling analysis of a single cell, is the high-throughput microfluidic qRT-PCR technique [44].

Differentiation of effector T-cells is regulated by multiple transcriptional and epigenetic factors. Changes in epigenetic makeup, introduced by polarizing cytokines, include chromatin structure changes, histone and DNA modifications and expression of miRNAs, which together enable the acquisition of T-cell subset-specific gene expression programs [33,45]. In addition to these molecular mechanisms, long non-coding RNAs (lncRNAs) are emerging as a new epigenetic layer controlling T-cell development and differentiation. Pang et al. has demonstrated that CD8+ T-cells express a defined set of lncRNAs, which are dynamically regulated during T-cell activation and the transition from a naive to a memory phenotype [46]. LncRNAs might function in multiple ways, including cis or trans regulation of expression, epigenetic regulators, transcriptional regulators, or endogenous miRNA sponges [47], adding another layer to the epigenetic landscape shaping the T-cell phenotype. Therefore the global analysis of protein coding as well as non-coding miRNAs and LncRNAs is crucial to comprehensively study T-cell biology.
Deregulated expression of miRNAs has recently also been found in the T-cell compartment of elderly people ([48] and Teteloshvili et.al manuscript in preparation). Taking into account the important role played by miRNAs, such as miR-21 or miR-146a in T-cell function, their (de)regulated expression might significantly influence functioning of aged T-cells, leading to changes in T-cell homeostasis and autoimmunity. Therefore, proper understanding of physiological miRNA function in aging T-cells and analysis of age-matched controls is essential to find disease-associated changes.
REFERENCES


