Micromanagement of lupus autoimmunity by microRNAs
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Chapter 9

Summary and general discussion
General Discussion

Background and focus

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by chronic immune activation in multiple organs, resulting in damage and organ failure. In spite of intensive research efforts our insight into the pathogenesis of this disease is limited, and our therapeutic armamentarium is restricted. Consequently there is a strong need for more effective and safer therapies. To identify novel molecular targets in this disease, potentially also serving as biomarker, the research described in this thesis focuses on regulation of major immunological pathways, since dysregulation of key molecular pathways is thought to contribute to the pathogenesis of SLE. More specifically this thesis focuses on delineating the role of microRNAs, in the immunopathogenesis of systemic lupus erythematosus.

MicroRNAs represent a large class of small non-coding RNAs that ubiquitously exist in a broad range of species from worms to mammals (1, 2), which function as a critical repressor of gene expression. Although our whole genome has been computationally predicted to encode approximately 1000 miRNAs, thus constituting a small fraction of the human genes only (3), they are capable of targeting about 30% of the human mRNA pool. Interestingly, almost half the immune genes are targeted (4). Previous studies have indicated that miRNAs act as a novel class of immune regulators, and play an essential role in multiple physiopathological processes such as immune cell differentiation, establishment of immunological tolerance and involvement in the pathogenesis of multiple autoimmune diseases (1, 5-8).

Main findings
Chapter 2 provides a comprehensive review on the current insights into miRNA biogenesis. It also discusses novel functions of miRNA in the regulation of immune responses and the development of autoimmune disease. Moreover, the role of miRNAs in the pathogenesis of SLE is described, including the effects of miRNAs on one of the key pathogenic pathways in SLE, the interferon type I pathway. The data underscore the potential of miRNAs as novel biomarkers and therapeutic targets for the future management of patients with SLE.

In chapter 3 cutting edge technology is used to study miRNA global expression profiling in our lupus cohort. This showed differential expression of multiple microRNAs, including miR-146a, a negative regulator of innate immunity. Further analysis showed that expression of miR-146a negatively correlated with clinical disease activity and with interferon scores in lupus patients. Of note, overexpression of miR-146a reduced, while inhibition of endogenous miR-146a increased, the induction of type I IFNs in PBMCs. Furthermore, miR-146a directly repressed the transactivation downstream of type I IFN. At the molecular level, miR-146a could target several key signaling molecules, including IRF5 and STAT-1. More importantly, introduction of miR-146a into the patients’ PBMCs alleviated the coordinate activation of the type I IFN pathway. Our findings suggest that manipulation of miR-146a levels may constitute a useful therapeutic intervention in SLE.

Chapter 4 explores why the expression of miR-146a is reduced in SLE patients. To this end we conducted short parallel sequencing of potential regulatory regions of miR-146a, and identified a genetic variant, rs57095329, in the promoter region exhibiting evidence for association with SLE. This was replicated in an independent cohort of 7,182 Asians (P meta = 2.74*10^{-8}, odds ratio = 1.29 [1.18 – 1.40]). Compared to the A allele the risk-associated G allele was associated with significantly lower expression of miR-146a in peripheral blood
leukocytes in healthy controls. Combined functional assays showed that the risk-associated G allele reduced the protein-binding affinity and activity of the promoter compared with that of a promoter containing the protective A allele. Transcription factor Ets-1, encoded by the gene ETS1, identified as a lupus-susceptibility gene in recent genome wide association studies, binds near this variant. The manipulation of Ets-1 levels strongly affected miR-146a promoter activity in vitro; and the knockdown of Ets-1, mimicking its reduced expression in SLE, directly impaired the induction of miR-146a. We also observed additive effects of the risk alleles of miR-146a and ETS1. Our data identified and confirmed an association between a functional promoter variant of miR-146a and SLE. This risk allele had decreased binding to transcription factor Ets-1, contributing to reduced levels of miR-146a in SLE patients.

Chapter 5 studies the effects of miRNAs on another route of gene regulation. DNA methylation of CpG islands plays an important role in gene silencing. Previous research showed that in lupus DNA extracted from T cells is hypomethylated compared to that from healthy controls. Other studies showed that DNA methylation can also be regulated by miRNAs that target the DNA methylation machinery. While dysregulation of miRNAs has been described in human lupus, the role of miRNAs in the aberrant CD4+ T cell DNA hypomethylation of lupus is unclear. In this study we performed high-throughput microRNA profiling, and identified two miRNAs (miR-21 and miR-148a) that were overexpressed in CD4+ T cells sampled in patients with lupus and in lupus-prone MRL/lpr. These miRNAs promoted cell hypomethylation by repressing DNA methyltransferase 1 (DNMT1) expression. This in turn led to overexpression of autoimmune-associated methylation-sensitive genes, such as CD70 and LFA-1. Bisulphite sequencing showed concurrent hypomethylation of the LFA-1 promoter. Further experiments revealed that miR-21 indirectly downregulated DNMT1 expression by targeting an
important autoimmune gene, \textit{RASGRP1}, which mediates the Ras–MAPK pathway upstream of DNMT1. In contrast, miR-148a downregulated DNMT1 expression by directly targeting the protein coding region of its transcript. Additionally, inhibition of miR-21 and miR-148a expression in CD4+ T cells from patients with lupus could increase DNMT1 expression and attenuate DNA hypomethylation. Together, these data demonstrated a critical functional link between miRNAs and the aberrant DNA hypomethylation in lupus CD4+ T cells and could help to develop novel therapeutic approaches.

Chapter 6 describes our studies on the role of MiR-125a in SLE. In preliminary experiments we showed a lower expression of MiR-125a in PBMCs from SLE patients compared to controls. We confirmed this in a second independent cohort of SLE patients. Bioinformatic algorithms predicted that \textit{KLF13} could be a target of miR-125a. KLF13 belongs to a family of transcription factors that regulates the expression of the inflammatory chemokine RANTES in T lymphocytes. Given that RANTES expression level in T cells from SLE patients was significantly higher than that in T-cells from healthy individuals, we hypothesized that miR-125a regulates RANTES. We used in vitro mitogen (PHA-P)-activated T-cells to induce RANTES production in T cells. Ectopic expression of miR-125a following transfection significantly reduced RANTES and KLF13 expression. Bioinformatic analysis predicted that the \textit{KLF13} 3'UTR harbors at least two putative binding sites for miR-125a. Using a Dual-Luciferase Reporter Assay System, we demonstrated that miR-125a directly targets the \textit{KLF13} 3'UTR.

We isolated CD3+ cells from peripheral blood samples in 10 SLE patients and in 8 healthy donors, and observed that activated T cells from SLE patients showed significantly reduced miR-125a levels and elevated RANTES and KLF13 mRNA levels compared to those in T-cells from the healthy controls. We explored whether manipulation of miR-125a levels could attenuate the elevated expression of RANTES in T cells from SLE patients. These cells were transfected
with a miR-125a mimic or a control mimic, followed by PHA-P activation for seven days. In this experiment miR-125a reduced RANTES expression in activated T cells on the protein level, as determined by Enzyme Linked Immunosorbent Assay (ELISA). This suggests that manipulation of miR-125a levels holds therapeutic promise for lupus patients.

Chapter 7 describes studies analyzing the contribution of microRNAs to the relatively low production of interleukin 2 by lupus T cells. Our data show that both IL-2 and MiR-31 are markedly under-expressed in lupus T cells. Over-expression of miR-31 in T cells increased IL-2 production by altering nuclear NF-AT expression and IL-2 promoter activity, while knockdown of endogenous miR-31 reduced it. MiR-31 repressed RhoA in T cells. Knockdown of RhoA using small interfering RNAs enhanced IL-2 promoter activity and consequently up-regulated IL-2 production. Consistent with these results, in primary T cells from lupus patients RhoA expression was up-regulated and correlated negatively correlated with miR-31 levels. Transfection of lupus T cells with miR-31 mimics restored IL-2 expression at both the mRNA and protein levels. We conclude that miR-31 is a novel enhancer of IL-2 production during T cell activation. Dysregulation of miR-31 and its target RhoA could be a novel molecular mechanism underlying the IL-2 deficiency in lupus patients. Overall, this study provides new mechanistic insights into IL-2 defect in SLE and suggests a promising therapeutic utility of miR-31.

In Chapter 8 we studied the role of miRNAs in the regulation of type I interferon production (type I IFN) by plasmacytoid dendritic cells (pDCs). These cells are distinct dendritic cells specialized in rapid secretion of type I interferons (type I IFN) in response to viruses. These cells are also involved in autoimmune diseases. To identify miRNAs relevant for type I IFN production we analyzed miRNA changes in pDCs during TLR7 stimulation. We observed that 5
miRNAs were significantly increased, whereas 14 miRNAs were significantly reduced. MiR-155* and miR-155 were the most highly induced miRNAs. To investigate the roles of miR-155* and miR-155 in type I IFN production by pDCs stimulated with TLR7, we transfected pDCs with miR-155* mimic, as-miR-155*, miR-155 mimic, or as-miR-155, and measured IFN-α expression. Compared to the controls miR-155* augmented IFN-α expression, whereas miR-155 suppressed it. Bioinformatics analysis indicated that IRAKM is a potential target for miR-155* while TAB2 is a potential target for miR-155. IRAKM negatively regulates the TLR pathways. TAB2, as an adaptor linking TAK1 to TRAF6, has been reported to enhance type I IFN production in pDCs upon TLR stimulation. Western blot, and reporter gene assay demonstrated IRAKM and TAB2 were the direct targets of miR-155* and miR-155, respectively. We monitored the kinetics of both miR-155* and miR-155 when induced by TLR7 over a 24 h time course and found that miR-155* was rapidly induced, reached its peak level at the 4 h time point, and then decreased. However, miR-155 increased much more slowly and reached its peak level at the 12 h time point. IFN-α mRNA was strongly induced, had increased more than 100-fold at the 4 h time point, and then declined rapidly. From the results discussed above, we infer that the more substantial increase in miR-155* expression in the early stage of pDC activation is part of the positive feedback that facilitates type I IFN production, whereas the rapid decline in miR-155* and the increase in miR-155 at a relatively later stage forms part of the negative feedback regulation that circumvents excessive type I IFN production.

Conclusions and Future Research
MicroRNAs are a group of recently discovered regulators that can fine tune cellular gene expression programs to control diverse biological functions of immune cells. Since disturbances in the fine regulation of gene expression can cause complex disease phenotypes, it is not unexpected that a series of recent papers link
miRNAs with several common human diseases.

This thesis focuses on testing the hypothesis that expression of some miRNAs is altered in SLE, and may have unrecognized impact on key lupus disease pathways. Using cutting edge technology to study the roles of microRNAs in lupus development we reveal how changes in the expression of multiple microRNAs, including miR-146a, miR-125a, miR-21, and miR-148a, contributes to the pathogenesis of lupus by regulating the type I IFN pathway, expression of the inflammatory chemokine RANTES and changes in DNA methylation patterns respectively. Furthermore, we show that the expression levels of these disease-related miRNAs are also correlated with disease activity and organ involvement. More importantly, we demonstrate that in vitro manipulation of these miRNA levels in the patients’ immune cells can alleviate the coordinate activation of the type I IFN pathway; reduce inflammatory chemokine RANTES expression, and correct the DNA methylation state of lupus T cells, respectively. We also are the first to reveal that a functional variant in the microRNA-146a promoter modulates the expression of this gene and confers disease risk for systemic lupus erythematosus. This indicates that dysregulation of miRNAs caused by genetic defects could be novel players involved in the multiple pathways of lupus and other diseases.

The clinical implication of our published data is that abnormal expressed lupus related miRNAs could be not only used as novel biomarkers for disease assessment, but could also be manipulated to provide us with useful therapeutic interventions in SLE. However, several aspects should be researched and clarified before such interventions may reach the clinics. Information is needed on the effects of genetic polymorphisms on variation in miRNA expression and phenotype. Concurrently, we should evaluate the role of these miRNAs in the diverse autoimmune phenotypes of lupus, probably in vivo systems using genetically modified mice. More information is also needed from in vivo models regarding the effects of interventions targeting these miRNAs (e.g. using synthetic miRNA
mimics or inhibitors) on lupus pathways and phenotypes. However, because the interactions between miRNAs and their targets are highly complicated, we need to validate the targets, and link cell-signaling pathways regulated by these miRNAs in in vivo systems, exploring novel undefined cellular and molecular mechanisms accounting for miRNA mediated regulation. We can use genetically modified mice, bone marrow chimeric mice and a systems biology approach to identify the function of lupus relevant miRNAs in their targeted immune cell type(s) or resident cells of target tissues, and define the signaling pathways and molecular regulatory networks regulated by these miRNAs in vivo. We expect that this will provide us with novel insights into the cellular and molecular mechanisms of miRNAs involved in the progression of systemic autoimmune diseases, and promote the development of novel targeted approaches for immune intervention in lupus.
References