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microRNA cluster 106a~363 is involved in T-Helper 17 (Th17) cell differentiation

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Abbreviations used:

3'UTR	3' untranslated region
AFC	Array fold change
COPD	Chronic obstructive pulmonary disease
EAE	Experimental autoimmune encephalitis
FC	Fold change
Foxp3	forkhead box protein 3
Gatm	glycine aminotransferase
Gimap1	GTPase of the immunity-associated protein family 1
Il	Interleukin
IPA	Ingenuity pathway analysis
miRNA/miR	microRNA
Nfat	Nuclear factor of activated T cells
PMA	phorbol 12-myristate 13-acetate
qRT-PCR	quantitative real-time PCR
RIN	RNA integrity number
Ror	Retinoid-related orphan receptor
SPF	specific pathogen free
Tanc2	*tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2*
Tgf	transforming growth factor

Th cellsT helper cells

Abstract

T-helper cell 17 (Th17) mediated inflammation is associated with various diseases including autoimmune encephalitis, inflammatory bowel disease and lung diseases such as chronic obstructive pulmonary disease and asthma. Differentiation into distinct Th subtypes needs to be tightly regulated to ensure an immunological balance. As microRNAs (miRNAs) are critical regulators of signaling pathways, we aimed to identify specific miRNAs implicated in controlling Th17 differentiation.

We were able to create a regulatory network model of murine Th cell differentiation by combining Affymetrix mRNA and miRNA arrays and in-silico analysis. In this model, the miR-212~132 and miR-182~183 clusters were significantly up-regulated upon Th17 differentiation, while the entire miR-106~363 cluster was down regulated and predicted to target well-known Th17 cell differentiation pathways. In-vitro transfection of miR-18b, miR-106 and miR-363 into primary murine $CD4^+$ lymphocytes decreased expression of retinoid-related orphan receptor c (*Rorc*), *Rora*, *Il17a* and *Il17f*, and abolished secretion of Th17 mediated Il17-a. Moreover, we demonstrated target site-specific regulation of the Th17 transcription factors *Rora* and nuclear factor of activated T-cells (*Nfat) 5* by miR-18b, miR-106a and miR-363-3p through luciferase reporter assays.

Here, we provide evidence that miRNAs are involved in controlling the differentiation and function of T-helper cells, offering useful tools to study and modify Th17 mediated inflammation.

Introduction

Interleukin 17 (Il-17)-secreting T-helper cells (Th17) are essential for protection from bacterial and fungal infections (1,2). Recent literature suggests that Th17 cells are also involved in chronic, non-infectious diseases, such as experimental autoimmune encephalitis (EAE) (3) and inflammatory bowel disease (4), as well as chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and severe asthma (5). The differentiation from naïve Th0 cells to Th17 cells is regulated by Il-6, Il-23 and transforming growth factor beta (Tgf-b). These initiate downstream signaling cascades including activation of T-cell receptor, signal transducer and activator of transcription (Stat) 3, and at last retinoid-related orphan receptor (Ror)γt (6). However the precise regulatory networks of Th17 differentiation in complex diseases are still unknown. Identification thereof may potentially enable to develop novel therapies for Th17-related diseases.

The expression of Rorγt and additional host proteins, such as the aryl hydrocarbon receptor (Ahr) for Th17 cells (7), needs to be carefully controlled to ensure an immunological balance. Such regulatory fine-tuning is often modulated by small, single-stranded non-coding RNAs, the so-called microRNAs (miRNAs) (8,9), the binding of which to the 3' untranslated region (3' UTR) of their target mRNAs either leads to a degradation of the respective mRNA or a translational repression leading to a decreased expression of the target gene (8,9). Hence, miRNAs are able to control gene or protein expression at the post-transcriptional level. Single miRNAs have been shown to play a pivotal role in

many biological systems, also play a pivotal role in regulating the immune system (10). Additionally, several studies have proposed a role for miRNAs in the regulation of Th cell differentiation (11–15), although information on the specific role of single miRNAs during differentiation into distinct Th subtypes is scarce. Therefore, we aimed to decipher the regulatory interactions of miRNAs during Th17 differentiation in order to better understand underlying molecular mechanisms of Th17-driven diseases. We performed mRNA and miRNA microarray analyses of primary, in-vitro differentiated Th17, Th2 and Th0 cells and subsequently validated the candidates on a functional level.

Materials and Methods

Mice

Female wild type Balb/c mice were purchased from Taconic (Silkeborg, Denmark). Mice were maintained under specific pathogen free conditions (SPF) in individually ventilated cages according to the federal guidelines for the use and care of laboratory animals. At the age of 10-14 weeks, mice were sacrificed for spleen collection.

Isolation and in-vitro differentiation of CD4+ T-helper cells

Splenic $CD4^+$ cells were isolated by using the mouse $CD4^+$ Isolation Kit II (Miltenyi Biotech, Teterow, Germany). Isolation was performed according to the manufacturer's guidelines, and yielded at least 96% purity of $CD4^+CD3^+CD8^-$ T-lymphocytes. A total of 200,000 $CD4^+$ cells per well were seeded in a 96-well plate, and were cultured with RPMI-1640 media containing 10 % FCS, 1 % penicillin/streptomycin and antibodies against CD3 (4 µg/ml) and CD28 (30 ng/ml) (both BioLegend, San Diego, US). Th17 cells were also cultured with Il-6 (20 ng/ml), Tgf-b (5 ng/ml), Il-23 (10 ng/ml) (all R&D Systems, Wiesbaden-Nordenstadt, Germany) and an antibody against Ifn-g (10 µg/ml) (BioLegend). Th2 cells were differentiated with Il-4 (100 ng/ml) (R&D Systems) and an antibody against Ifn-g (10 µg/ml) (BioLegend). Th0 cells served as a control and were cultured with antibodies against Cd3 and Cd28 (BioLegend). Primary Th cells were cultivated at 37 °C and 5 % CO_2 in a humidified incubator. After 72 h the media was replaced with fresh differentiation media. After 120 h of culture, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml Ionomycin (both Sigma-Aldrich, St. Louis, US) for 4 h.

Intra-cellular cytokine staining

Prior to intracellular staining, secretion of cytokines during the 4 h stimulation was blocked by Monensin GolgiStop™ (BD, Franklin Lakes, US) according to the manufacturer's recommendations. Cells were washed twice with PBS (containing 2 % FCS and 0.01 M EDTA) and stained with surface antibodies against CD3 (Pacific Blue), CD8 (Fluoresceininisothiocyanat, FITC) and CD4 (Allophycocyanin (APC)-H7) (all 1:100, all BioLegend). Intracellular Il-17a (FITC) and Il-4 (Phycoerythrin, PE) (both Becton Dickinson, Franklin Lakes, US) were stained by using the Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD) according to the manufacturer's instructions. After additional washing, cells were analyzed on a LSRII flow cytometer (BD).

Quantitative real time PCR (qRT-PCR)

RNA containing small RNAs were isolated with the miRNeasy micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations. The quality and quantity of the isolated RNA samples were validated with the Nanodrop ND-1000 Spectrometer (peq Lab Bioscience, Erlangen, Germany) and the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, US). Only RNAs with a RNA integrity number (RIN) > 7.25 were used for further analyses. qRT-PCR was performed using a LC480 instrument (Roche, Basel, Switzerland) and the recommended "Light Cycler DNA master SYBR Green I" Kit (Roche). Primer sequences were self-designed and are listed in the supplementary information (Supplement Table SI). Analysis of all qRT-PCRs was done with the "LC 480 SW1.5" software (Roche) using the second derivative maximum method and fold changes between groups were calculated by the ΔΔCt method (16).

mRNA profiling

For mRNA arrays, total RNA (30 ng) of four independent differentiations was amplified using the Ovation PicoSL WTA System V2 (Nugen, San Carlos, US) in combination with the Encore Biotin Module (Nugen). Amplified cDNA was hybridized on an Affymetrix Mouse Gene 2.0 ST arrays (Affymetrix, Santa Clara, US). Staining and scanning was performed according to the Affymetrix expression protocol, except for minor modifications as suggested in the Encore Biotion protocol (Nugen). Expression console (v.1.3.0.187, Affymetrix) was used for quality control and to obtain annotated normalized RMA data (standard settings including median polish and sketch-quantile normalisation.) Statistical analyses were performed by utilizing the statistical programming environment R (17) implemented in CARMAweb (18). Genewise testing for differential expression was done employing the (limma) *t*-test ($p<0.05$). Heatmaps were generated with CARMAweb. Array data has been submitted to GEO (GSE55013).

In-silico analysis and Ingenuity pathway analysis

Pathway analyses and expression pairing were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) using Fisher`s Exact Test p-values.

miRNA profiling

For miRNA arrays, total RNA (600 ng) was labeled with the FlashTag Biotin HSR kit (Genisphere, Sterling, US) and hybridized on miRNA 3.0 arrays (Affymetrix). Staining and detection of the array was performed as described for mRNA arrays. Expression console (v.1.3.0.187, Affymetrix) was used for quality control and to obtain annotated normalized RMA data (RMA+DABG (Robust Multichip Average + Detected Above BackGround)). Statistical analyses were performed by utilizing the statistical programming environment R (17) implemented in CARMAweb (18). For miRNA analysis a filter for detection in at least n-1 samples in at least one group was used. Heatmaps were generated with CARMAweb. Array data has been submitted to GEO (GSE55013).

miRNA qRT-PCR

For the miRNA analysis, all chemicals were purchased from Exiqon (Vedbaek, Denmark). cDNA of miRNAs were synthesized with the Universal cDNA Synthesis Kit II followed by qPCR, which was done with ExiLENT SYBR®Green Master Mix. Pre-designed primer sets for miRNA qPCR (LNA™ Primer sets, Exiqon) were used for the analysis of following miRNAs: miR-183-5p (204652), miR-212-3p (205589), miR-301a-3p, miR-363-3p (204726), miR-18b-5p (205076), miR-106a-5p (205061). The small nuclear RNA U6 (203907) was used as a reference miRNA for normalization.

Dual-luciferase reporter assays

Potential miRNA binding sites within the genes *Rorc*, *Rora* and *Nfat5* were identified with the Whitehead Institute for Biomedical Research target prediction tool "TargetScan Mouse" (http://www.targetscan.org/mmu_71/). Due to the large size of the 3'UTRs (> 7 kb) with presence of multiple miRNA binding sites, we decided to clone smaller fragments, containing miRNA binding sites of the respective 3'UTR in to reporter plasmids (primer sequences used for cloning are listed in supplementary Table S1). For a more detailed analysis, we used synthetical DNA duplexes spanning ~80 nt regions of the respective 3'UTR each with one single miRNA binding site (or a mutation thereof comprising 7-8 sequential T or A nucleotides, all sequences are listed in Supplementary Table SV) in the reporter assays as described in (19). Synthesized 80 bp DNA fragments within the 3'UTRs of *Rora*, *Rorc* and *Nfat5* with binding sites for either miR-18b, miR-106a or miR-363-3p were ordered as DNA duplexes from Metabion (Planegg, Germany). To ease cloning into vectors, the DNA oligos carried the overhang recognition sites for the restriction enzymes XhoI (TCGAG on the 5' end, C on the 3' end) and NotI (GGCCGC on the 5' end and GC on the 3' end) and are phosphorylated (p) on the 5' ends (Sequences are listed in Supplementary Table SV). All fragments were cloned into individual psiCheck™-2 vectors (Promega, Madison, US) using XhoI and NotI restriction sites. Reporter assays were performed by transfecting 100 ng of the transgene psiCheck™-2 vectors and 5 nM of each miRNA precursor or a scrambled miRNA into A549 human alveolar basal epithelial cells by using peqFECT siRNA transfection reagent (PeqLab Biotech GmbH, Erlangen, Germany). Cells were cultured in DMEM media containing 10 % FCS in 96 well plates (Nunc). After 48 h luciferase activity was measured in a luminescence plate reader (Berthold, Bad Wildbad, Germany) after addition of the respective substrates by using the DualGlo Luciferase Assay System (Promega) according to the manufacture's recommendations. *Renilla* luciferase (fused to 3' UTR) activity was normalized to firefly luciferase activity (transfection control)."

Transfection of miRNAs

Primary $CD4^+$ T cells were transfected with miRNA mimics for miR-106a-5p, miR-18b-5p, miR-363-3p or a scrambled miRNA as control (Ambion, Life Technologies, Carlsbad, US). Freshly isolated cells were cultivated in 75% of normal differentiation media and 25% of transfection media. The latter one contained serum free media, 0.12% transfection reagent (PeqFECT siRNA, PeqLab Biotech GmbH) and the miRNA precursors (6.5 nM final concentration). After 4 h, the medium was exchanged for fresh differentiation media. Cells were harvested after 72 h and analyzed for gene expression, cytokine secretion and viability.

Analysis of cell viability

Viability of primary cells after transfection was measured by a MTT-assay (Thermo Fischer Scientific, Waltham, US). MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the cells and incubated for 4 h. Purple formazan produced by living cells was then analyzed by measuring the absorbance at 500-600 nm with a plate reader (Tecan, Männedorf, Switzerland).

Statistical analysis

Statistical validation of arrays was performed using limma t-test and Benjamini-Hochberg multiple testing correction. For all other experiments, statistical differences between groups were calculated using either one-way ANOVA with Tukey post–test or unpaired students t-test. The calculations and graphs were made with GraphPad Prism 5.01.

Results

Validation of in-vitro Th cell differentiation

In order to study mRNA and miRNA expression in mature Th17 cells, we isolated $CD4^+$ cells from murine spleens, and differentiated them in-vitro into Th17, Th2 and Th0 cells. A successful differentiation into distinct T-helper subtypes was verified and quantified by intracellular cytokine staining for Il-4 for Th2 cells, and Il-17a for Th17 after 120 h of culture. Both cytokines were significantly elevated in the respective T-helper subtype. Compared to Th0 control cells, Th2 cells produced double the amount of Il-4 (Fig. 1A) and Th17 cells showed a three-fold increase of Il-17a production (Fig. 1B).

Next, we analyzed the mRNA expression of distinct Th17 and Th2 markers by quantitative real-time PCR (qRT-PCR). The gene encoding the Th17 transcription factor Rorγt, *Rorc*, and *Il17a* were significantly up-regulated in Th17 cells compared to Th0 and Th2 cells. The two specific Th2 cell markers, *Gata3* and *Il4*, were significantly increased in Th2 cells and unchanged- or lower in expression in Th0- and Th17 cells (Fig. 1C), thus confirming specific differentiation.

mRNA profiling of Th17, Th2 and Th0 cells

In a next step, we aimed to identify regulatory networks in the distinct in-vitro differentiated Th subtypes. We therefore performed mRNA arrays involving more than 35000 transcripts. By comparing the expression values between Th17 and Th0 cells, we identified 2052 significantly regulated genes ($p<0.05$), and 1918 genes that were significantly different between Th17 and Th2 cells (Tables I, II accessible at GEO GSE55013). From these gene sets, 544 genes were similarly regulated in Th17 compared to Th0 cells or compared to Th2 cells (Supplementary Fig. S1). All genes that were increased in expression in Th17 cells compared to both Th0 and Th2 cells were then defined as Th17-specific genes (Table III, accessible at GEO GSE55013).

Next, we used qRT-PCR to confirm the regulation of three Th17 specific and three Th2 specific genes. As Th17 cell validation markers we chose the aryl hydrocarbon receptor (*Ahr*) with an array fold change (AFC) of 5.9x, *Il17f*, the second main cytokine of Th17 cells with an AFC of 29.8, and the highly increased glycine aminotransferase (*Gatm*) with an AFC of 15.0. All three specific Th17 genes were significantly increased (Fig. 2A). For Th2 cells we chose the Th2 cytokines *Il5* (AFC: 1.7), *Il24* (AFC: 2.0) and *tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2* (*Tanc2*) (AFC: 3.0). *Il5* and *Il24* were significantly increased in Th2 cells compared to Th17 and Th0 cells (Fig. 2B). We observed a trend for *Tanc*2 up-regulation in Th2 compared to Th0 and Th17 cells, although this did not reach statistical significance.

In order to determine which genes are involved in the regulation of Th17 cell differentiation and function, we then conducted an in-silico analysis with the 2052 genes differentially regulated in Th17 cells vs. Th0. Ingenuity Pathway Analysis (IPA) revealed 'differentiation of Th17 cells' as the most significantly enriched canonical pathway (Supplementary Table SII). Of note, nearly all Th17 specific genes described in the literature were found to be increased in our Th17 cells (Fig. 2C). In contrast, the Th2 specific genes *Gata3* and *Il4* and the Th1 specific T-box transcription factor TBX21 (*Tbet)* and *Stat4* were all decreased in Th17 cells. Many genes that are connected to Treg were also increased in Th17 cells, including Tgf-beta receptor (*Tgfbr)*, *Rorc* and *Il2*; however, the main transcription factor of Treg, forkhead-box protein 3 (*Foxp3)*, was decreased in expression (Fig. 2C).

Additional prediction of functional properties of Th17 regulated genes revealed a significant enrichment of genes associated with activated lymphocytes and specific Th17 functions with the top four being 'cell movement of T lymphocytes', 'T cell migration', 'recruitment of neutrophils' and 'phosphorylation of protein', and a decrease in 'bacterial infections' and fungal infections' (Supplementary Table SII). Thus, the microarray analysis indicated correct differentiation into Th2 and Th17 cells in our in-vitro system.

microRNA profiling in Th17, Th2 and Th0 cells

To identify regulatory miRNAs involved in Th17 differentiation, we performed Affymetrix miRNA 3.0 arrays with the same RNA samples that were used for the mRNA array. Comparing Th17 and Th0 cells, 92 miRNAs were significantly regulated in Th17 cells ($p<0.05$). In comparison to Th2 cells 162 miRNAs ($p<0.05$) were significantly regulated in Th17 cells (Supplemental Fig. 2, Tables IV & V accessible at GEO GSE55013). We identified 60 miRNAs that were differentially expressed compared to both Th0 and Th2 cells (Table VI accessible at GEO GSE55013), and might thus influence Th17 differentiation or cytokine secretion.

We further compared the significantly regulated mRNAs and miRNAs of all three Th cell subtypes to identify Th17-specific regulatory miRNA and mRNA interactions (Fig. 3A). This expression pairing with IPA predicted a number of miRNAs that are potentially involved in differentiation and induction of Th17 cells, with the top hits listed in Figure 3B+C. The miRNAs showing the highest expression were the miR-212~132 cluster, the miR-182~183 cluster and miR-338-5p (Fig. 3C). The most decreased miRNAs were miR-301a and almost all of the miR-106~363 cluster, including miR-18b, miR-20b and miR-363-3p (Fig. 3D).

The expression of the most prominent up- and down-regulated miRNA candidates was further validated by qRT-PCR. miR-183-5p and miR-212-3p were significantly increased in Th17 cells compared to Th0 and Th2 cells. miR-301-3p, miR-18b and miR-106a were decreased, confirming the array data (Fig. 3D).

Th17 cell differentiation involves miRNA regulation

After identifying distinct Th17 specific miRNAs, we were interested in whether these could play a functional role in the Th17 differentiation process. Thus, we created an in-silico model of T-helper cell differentiation by using the miRNA and mRNA expression pairing data (Fig. 4). According to this in-silico model the increased miRNA clusters 182~183 and 212~132 in Th17 cells might regulate essential genes of Th1 and Treg differentiation. The miRNAs 182 and 183 were predicted to inhibit the expression of GTPase of the immunity-associated protein family 1 (*Gimap1)*, which was decreased in Th17 cells, and which is important for Th1 differentiation. Moreover, the 182~183 cluster may also interfere with *Foxo1/3* and *Foxp3* expression, and thus Treg differentiation. The *Ahr*-induced miRNA cluster 212~132 may inhibit Th1 differentiation by reducing *Stat4* expression. In contrast the down-regulated miR-363-3p, miR-106a and miR-18b are predicted to bind to the Th17 key transcription factors *Rorc, Rora and Nfat5*. All predicted miRNA-mRNA pairings are listed in Table SIII and SIV in this article's online supplement.Changes in expression of these transcription factors could therefore directly affect Il-17a/f secretion (Fig. 4).

Over-expression of miR-106a, miR-18b and miR-363-3p decreases Th17 differentiation and Il-17 secretion

According to TargetScan S (mouse) (http://www.targetscan.org/mmu_71/), the 3'UTRs of *Rora* and *Nfat5* contain three binding sites for miR-18b, one for miR-106a and two for miR-363-3p, while the Rorc 3'UTR only features one binding site for miR-106a. To address this, we performed dual-reporter luciferase-reporter assays, with one specific region of the 3'UTR of the three transcription factors each containing a predicted binding site for the respective miRNAs.

We thus demonstrated a specific binding of miR-18b and miR-106a to the 3'UTR fragment of *Rora* and *Nfat5* via a diminished signal of the reporter luciferase renilla compared to the control luciferase firefly (Fig 5A and Fig S3) and to the scrambled miRNA transfection. miR-363-3p also significantly bound to the 3'UTR of *Rora* and trendwise also to *Nfat*5. Mutating the binding sites for the respective miRNAs abolished the reduction of the renilla luciferase (Fig. 5A), thus indicating a specific regulation by the respective miRNA. The regulation of *Rorc* by miR-106a could not be verified by this assay.

In order to investigate the functional relevance of this miRNA cluster in the differentiation of Th0 to Th17 cells, we transiently overexpressed miR-363-3p, miR-106a and miR-18b in Th0 cells at the beginning of the in-vitro differentiation towards Th17.Compared to Th17 cells transfected with the same concentration of scrambled miRNA, simultaneous transfection of miR-363-3p, miR-106a and miR-18b decreased the mRNA expression of *Rorc*, *Rora*, *Il17a*, and *Il17f* (Fig. 5B). Furthermore, down-regulation of these genes by miRNA overexpression resulted in a significant decrease of Il17a

protein production in Th17 cells compared to the scrambled transfected Th17 cells (Fig. 5C). The viability of transfected cells did not vary between the different conditions; in particular not between miRNA-transfected Th17 cells and scrambled transfected Th17 cells (Fig. 5D).

Discussion

This study aimed to identify regulatory miRNA/mRNA networks that control the differentiation and function of Th17 cells. Thus, we combined mRNA and miRNA microarray data of in-vitro differentiated murine primary Th2 and Th17 cells in an in-silico IPA analysis. The identified Th17 specific miRNA clusters were predicted to be functionally involved in Th17 differentiation and cytokine production. For example, the miR-106a~363 cluster was decreased in expression upon the differentiation of Th17 cells, and we confirmed its direct interference with the specific Th17 transcription factors Rorγt, Rora and nuclear factor of activated T-cells 5 (Nfat5). Further, in-vitro transfections of this cluster into Th17 cells reduced the secretion of Il-17 cytokines. Thus, we suggest that the miR-106a~363 cluster plays an important role in fine-tuning the Th differentiation towards Th17.

A correct in-vitro differentiation of naïve $CD4^+$ T cells into Th17 was confirmed by a) expression of Th17 and Th2 specific genes and production of respective cytokines; b) a unique gene expression signature in the microarray analysis of previously described genes involved in Th17 differentiation and effector function, including *Rorc*, *Il17a*, *Il17f*, *Ahr*, *Il23r*, *Rora* and *Il22* (6,20); and c) strong enrichment of Th17-associated functions such as defending against bacterial or fungal infections, and preventing/hindering recruitment of neutrophilic cells in IPA analysis.

To identify the complex regulatory networks of Th cell differentiation, we combined our mRNA array data with a microRNA microarray of our Th17, Th2 and Th0 cells, creating in-silico a hypothetical Th cell differentiation model. While this model is based on our own expression data of miRNA or mRNA and published data, it has to be emphasized that Ingenuity Pathway Analysis only creates an *in-silico* prediction of potential biological models but does not provide evidentiary facts. Nonetheless, in this theoretical model miR-182 had a central role in Th17 cell function by potentially inhibiting the differentiation pathways of Th1 and Treg cells. Additionally, miR-182 would affect the expression of *Foxo1* and *Foxo3*, indirectly suppressing Foxp3 expression and consequently Treg differentiation. Binding of miR-182 to *Foxo1* and *Foxo3* mRNA has been confirmed (21,22), and the entire miRNA cluster 183~182 has just been shown by Ichiyama *et al*. to promote Th17 pathogenicity through direct repression of *Foxo1* (23). Further, *Foxo1* and *Foxo3* conditional knockout mice are depleted of Treg and have higher $CD4^+$ lymphocyte populations and inflammation (24,25). Thus, *Foxo1* regulation seems to be fine-tuned by miRNAs in Th17 cells, influencing their pathogenicity and function. Taken together, these findings not only support our in-vitro Th17 differentiation, but also the IPA-driven target prediction approach of this study.

In our study, the miR-212~132 cluster was highly increased in Th17 cells. Ahr signaling has been shown to induce the expression of this miRNA cluster and the production of Il-17 in-vitro and in-vivo (11), which is similar to our data where both *Ahr* and the miR-212~132 cluster were increased during

Th17 differentiation. Additionally, we speculate this might prevent Th1 differentiation as the miR-212~132 cluster has been shown to inhibit *Stat4*, indirectly repressing interferon gamma (Ifn-g) (26). miR-10b (27), miR-210 (28), miR-155 (29) and miR-30a (30) have been previously described to be involved in Th17 regulation and function, but are not significantly altered in our arrays.

In our study, the expression of the entire cluster of miR-106~363 was decreased. This cluster is located on the X chromosome (31), but little is known about its role in immune function. miR-17, miR-106a and miR-20a up-regulation has been shown to lead to macrophage activation after LPS stimulation (32) and miR-106a, miR-20a, miR-18b were found to be increased in Th1 cells compared to Th2 cells in a gene array study (33). We observed a decreased expression of these miRNAs in Th17 cells when compared to Th2 cells. Thus, this miRNA cluster seems to be expressed highest in Th1 cells, on intermediate levels in Th2 cells, and lowest in Th17 cells. We speculate that this differential, hierarchical regulation might be a hint for a functional relevance in the differentiation of Th cells into distinct subtypes. In our in-silico model, luciferase reporter assays and in-vitro functional analyses, we demonstrated that miR-106a, miR-18b and miR-363-3p can bind to the 3'-UTR of *Nfat5*, and *Rora*, leading to a consequent decrease of *Il17a/f* gene expression and reduction of Il-17a protein production. This might be further amplified by additional binding of these miRNAs to the transcription factor *Stat3*, as has been shown for miR-106a (34). Stat3 is, next to Rorα and Rorγt, essential for Th17 differentiation and *Il17* gene expression (35). As the miR-106~363 cluster is decreased in Th17 cells, Stat3 might remain at baseline levels, inducing Th17 differentiation and Il-17 production.

As our data suggest a role for miRNA cluster 106a~363 in Th17 differentiation, it will be crucial to analyze its expression in animal models with ongoing Th17 mediated inflammation, such as autoimmune encephalomyelitis, Crohn's disease or chronic respiratory diseases such as severe asthma and COPD (3–5,36). Given our findings, we speculate that therapy approaches using miR-106a, miR-18b and miR-363-3p might potentially ameliorate or prevent Th17 cell mediated inflammation. Along this line, another member of the miR-106a~363 cluster, miR-20b, has been shown to be downregulated in blood of MS patients (37) and in a Th17 driven experimental model for EAE (37). A genetic depletion of the miR-106a~363 cluster resulted in a more severe EAE course and upregulation of the miR-20b target genes Rorgt, and Stat3 (37), while lentiviral overexpression of miR-20b led to decreased Th17 cells and reduced EAE severity (38). Thus, these studies confirm the disease relevance of our in silico model, strengthening the suggestion that the miR-106a~363 cluster might be an interesting target for therapeutic interventions.

In summary, by using microarray profiling we were able to create an in silico miRNA/mRNA regulatory network of Th17 cell differentiation. We observed that the most abundant up- and down-regulated miRNAs are organized in distinct clusters, and the expression of the entire cluster of miR-106a~363 was decreased in Th17 cells. Target prediction and a luciferase reporter assay revealed that this cluster interferes directly with key transcription factors of Th17 differentiation. Over-expression of these miRNAs reduced Th17 differentiation and the secretion of Il-17a. Thus, the present work is a first step towards the identification and understanding of the underlying molecular mechanisms involved in Th17 cell differentiation, which is crucial to develop novel and effective treatment strategies for Th17-mediated inflammatory diseases.

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Authors contributions: S.K.E. conceived the manuscript, designed and supervised the study and had the primary responsibility for writing; M.K. and S.B. performed and analyzed the experiments and wrote the manuscript. K.G-K. performed cloning experiments of the 3'UTRs. M.I. and J.B. performed the mRNA and miRNA arrays and performed initial bioinformatics analysis. B.R. and O.E. participated in critical data interpretation and design of the study. All authors contributed in writing of the manuscript.

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Conflicts of Interest:

The authors declare that there are no conflicts of interests regarding this study.

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FIGURE LEGENDS

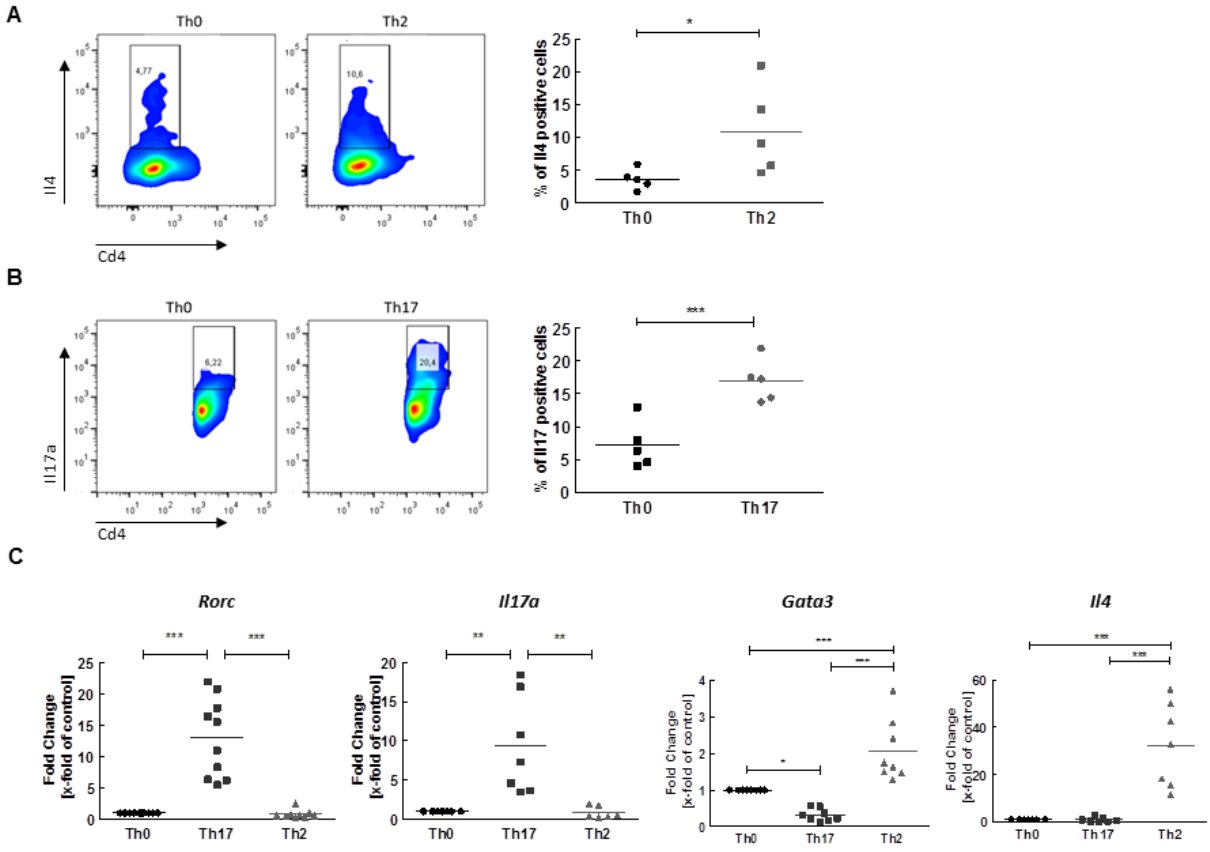
FIGURE 1. Characterization of primary, in-vitro differentiated Th cells. **(A)** Protein expression of the Th2 effector cytokine Il4. Depicted are representative flow cytometry density blots for Il4 and Cd4. Numbers are positive counted cells in %. N=5 independent experiments are summarized in the dot blot on the right column. **(B)** Protein expression of the Th17 effector cytokine Il17a. Depicted are density blots for Il17a and Cd4. N=5 independent experiments are summarized in the dot blot on the right column. **(C)** Gene expression of major transcription factors and effector cytokines of Th17 cells and Th2 cells. Depicted are dot blots of fold changes compared to the control (Th0) and normalized to *Hprt*. Medians in dot blots are represented by a black line. For (A) and (B) significant differences between Th0 control cells and Th17 or Th2 cells were calculated with Student's t-test. For (C) statistical differences were calculated with one–way ANOVA and Tukey post-test. * $p<0.05$, ** $p<0.01$,*** $p<0.001$.

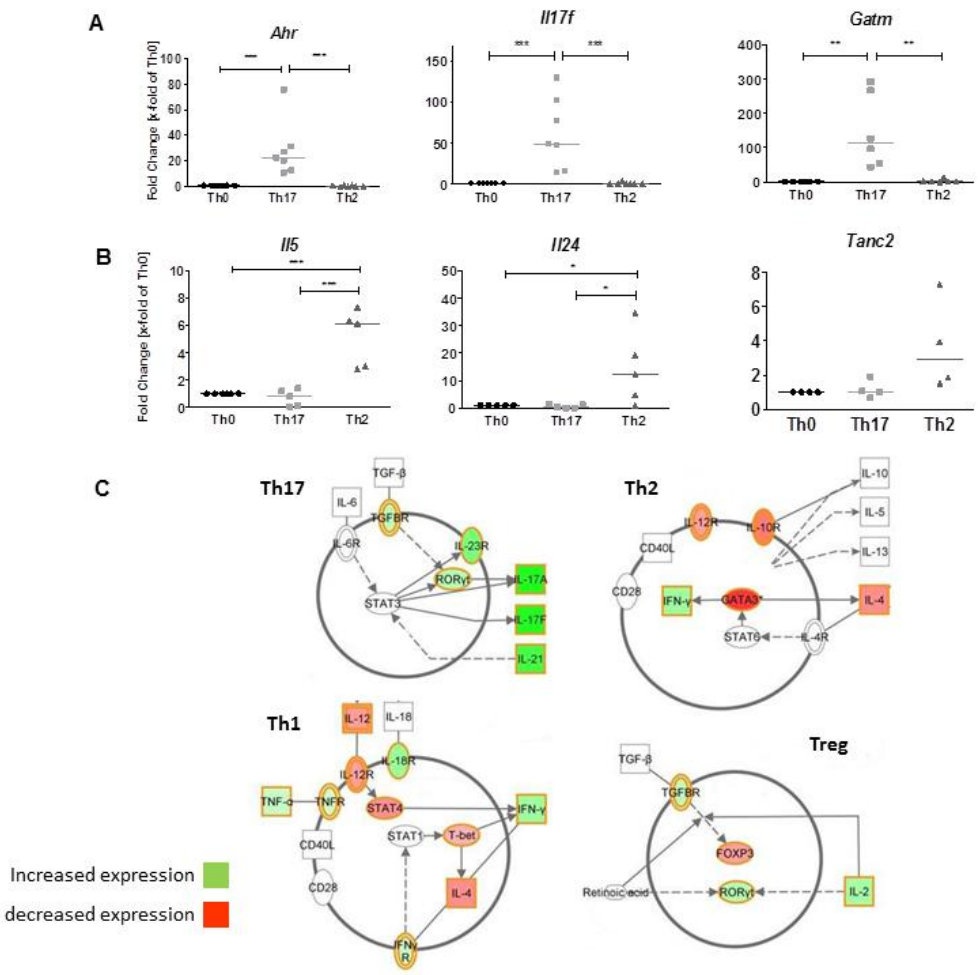
FIGURE 2. mRNA profiling in Helper-T cell subtypes. **(A)** and **(B)** qRT-PCR validation of Th17 and Th2 up-regulated genes. Depicted are dot blots of fold changes compared to the control (Th0) and normalized to *Hprt*. Medians in dot blots are represented by a black line. For (**B**) and (**C**) significant differences between Th0 and Th17 or Th2 cells were calculated of n=4 independent differentiations with one-way ANOVA and Tukey post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (**D**) Regulation of Th17 genes in the context of Th cell differentiation. Depicted are up- and down-regulated genes of Th17 mRNA arrays, which are responsible for the differentiation of Th17, Th2, Th1 and Treg cells. In-silico analysis and schematic figures were conducted with IPA software.

FIGURE 3. miRNA profiling in Helper-T cell subtypes. **(A)** Schematic depiction of the comparison strategy used for the identification of a Th17 miRNAs or mRNAs signature. **(B)** and **(C)** Array data of Th17 cell specific increased (B) or decreased (C) miRNAs. **(D)** Validation of chosen miRNAs. Quantification of miRNAs was performed by qRT-PCR. Shown are the fold changes compared to the Th0 control cells. The miRNA expression levels are normalized to the expression of U6 snRNA. Statistical differences were calculated of n=4-6 independent differentiations with one –way ANOVA and Tukey post-test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$. *FC = Fold change*

FIGURE 4. In-silico pathway prediction of Th17-specific miRNA/mRNA pairs. The depicted pathway was identified with IPA software using Th17-specific regulated miRNA/mRNA pairs. Green colored genes are up-regulated, red colored genes are down-regulated; blue colored genes are not regulated.

FIGURE 5. Target gene validation for miRNA 106a-5p, 18b-5p and 363-3p and function in Th cells. **(A)** Luciferase reporter assay with plasmids containing either wiltype or mutated miRNA binding sites. Depicted are the luciferase values (Renilla/Firefly) relative to the scrambled negative control. **(B)** qRT-PCR analysis of transfected Th- subsets. Cells were simultaneously transfected with 6.5 nM miRNA 106, 18b and 363 (Th0T, Th2T and Th17T) or the same concentration of a scrambled miRNA (Th17scr). Depicted are fold changes compared to the transfected Th0T subset and normalized to the expression of *Hprt*. **(C)** Flow cytometry analysis of Il17 protein production of transfected Th cells. **A-C** medians are depicted of 4-6 independent experiments. (**D)** Viability (MTT assay) of Th cells after transfection. Shown are the mean (±SD) relative amounts of viable cells compared to the control (n=4). Differences are not significant. Statistical differences were calculated with one-way ANOVA and Tukey post test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns = non significant





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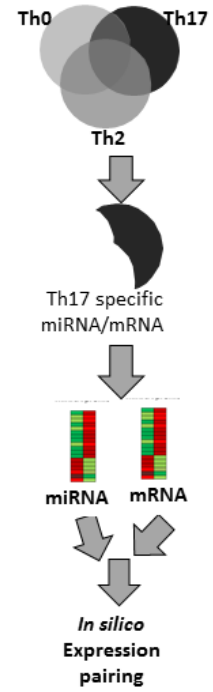

B

miRNA	FC: Th17 vs. Th0	FC: Th17 vs. Th2
miR 212-3p	3.4	3.9
miR 212-5p	3.1	3.2
miR-132	2.4	3.7
miR 147	2.1	2.1
miR 182	2.8	3.5
miR 183	2.2	3.3
miR 338-5p	3.0	3.6

C

miRNA	FC: Th17 vs. Th0	FC: Th17 vs. Th2
miR 301a	-3.1	-3.1
miR 195	-2.7	-6.1
miR-106a	-1.6	-1.6
miR 18b	-1.6	-1.7
miR 20b	-1.6	-1.6
miR 363-3p	-4.9	-6.9

D