miR-10b-5p is a novel Th17 regulator upregulated in Th17 cells from Ankylosing Spondylitis.

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Objective

To determine the microRNA (miR) signature in ankylosing spondylitis (AS) Th17 cells.

Methods

IL-17A-producing CD4 T cells from AS patients and healthy controls were FACS-sorted for miR sequencing and qPCR validation. miR-10b function was determined by miR mimic expression followed by cytokine measurement, transcriptome analysis, qPCR and luciferase assays.

Results

AS Th17 cells exhibited a miR signature characterized by upregulation of miR-155-5p, miR-210-3p and miR-10b. miR-10b has not been described previously in Th17 cells and was selected for further characterization. miR-10b is transiently induced in in-vitro differentiated Th17 cells. Transcriptome, qPCR and luciferase assays suggest that MAP3K7 is targeted by miR-10b. Both miR-10b over-expression and MAP3K7 silencing inhibited production of IL-17A by both total CD4 and differentiating Th17 cells.

Conclusions

AS Th17 cells have a specific miR signature and upregulate miR-10b in vitro. Our data suggest that miR-10b is upregulated by pro-inflammatory cytokines and may act as a feedback loop to suppress IL-17A by targeting MAP3K7. miR-10b is a potential therapeutic candidate to suppress pathogenic Th17 cell function in AS patients.

INTRODUCTION

Ankylosing Spondylitis (AS) is a common inflammatory rheumatic disease with predilection for the sacroiliac joints and axial skeleton[1]. Several lines of evidence implicate interleukin (IL)-17A-secreting T helper (Th)-17 cells in AS pathogenesis. Multiple type 17 immune pathway genes have been identified in AS in genome wide association studies, [2] Th17 cells are increased in frequency in the blood of AS patients,[3] and IL-17A neutralization has been shown to be an effective therapy in AS [4]

microRNAs (miRs) are noncoding RNA oligonucleotides that are potent posttranscriptional regulators of gene expression, targeting messenger RNAs (mRNA) for degradation upon binding to complementary sequences predominantly in the 3' untranslated region (3'UTR) of mRNAs.[5] Emerging data suggest that single miR species can profoundly alter the phenotype and outcome of immune responses, offering the prospect of therapeutic use.[6 7 8]

Studies of AS blood and serum have implicated miRs in AS.[9] However, the miR profile of AS Th17 cells has not been studied previously. We hypothesized that aberrant expression of miRs in AS Th17 cells might alter the expression of downstream target molecules contributing to the pathogenesis of AS.

In this study we characterize the miR signature of AS Th17 cells, showing specific upregulation of miR-10b in AS Th17 cells in vitro. miR-10b is transiently induced during Th17 differentiation, can be driven by TNF-α and IL-6, and acts to dampen IL-17A production, at least in part through direct inhibition of MAP3K7 gene expression.

RESULTS

miR-10b is increased in Th17 cells cultured from AS patients

We used an IL-17A secretion and capture assay followed by FACS sorting to study the miR profile of Th17 cells grown from the blood of AS patients. We obtained genome-wide miR expression profiles using next generation RNA sequencing from equal numbers of $CD4^+IL\text{-}17A^+$ and $CD4^+IL\text{-}17A^-$ from 4 AS patients and 4 healthy controls. Figure 1A compares the miR expression profiles of AS Th17 cells with those from healthy controls. Among novel miRs that appeared differentially expressed in AS Th17 cells (miR-10b-5p, miR-144-3p, miR-127-3p, and miR-409-3p), miR-10b-5p (hereafter shortened to miR-10b) was studied further, since it was only miR significantly increased in AS Th17 cells compared to HC Th17 cells after correction for multiple comparison (adjusted $p = 0.013$, normalized gene expression counts are AS Th17: 86.944, HC Th17: 0.196, AS non-Th17: 21.92, HC non-Th17: 1.187). miR-10b and some previously described Th17-associated miRs (shown in red),[10-13] were selected for qPCR validation in an extended cohort comprising 15 AS patients and 10 age and sex-matatched controls (demographic data shown in Supplementary Table). miR-10b, miR-155-5p and miR-210-3p were all increased in AS Th17 cells compared to AS non-Th17 cells, and miR-10b was also higher than healthy Th17 cells (Figure 1B, C and D). miR-21-5p was significantly higher in healthy Th17 cells than AS Th17 cells, miR-146a-5p did not differ significantly between populations (Figure 1E-F). Thus AS Th17 cells have a unique miR signature characterized by upregulation of multiple Th17-specific miRs (including miR-155 and miR-210) together with AS-Th17-specific miRs, such as miR-10b.

Expression of miR-10b in AS is inversely correlated with the frequency of in-vitro

enriched Th17 cells.

Next, we carried out a correlation analysis between clinical and immunological parameters and expression levels of miR-10b. We found an inverse correlation between Th17 percent and miR-10b expression levels ($r = -0.58$, $p = 0.01$, see online supplementary Figure S1). miR-10b expression did not correlate with age, disease activity or CRP (see online supplementary Figure S1). Since miR-10b was not elevated in directly isolated Th17 cells from a small additional cohort of AS patients (see online supplementary Figure S2), these results suggested that miR10b is upregulated in AS as part of a possible feedback mechanism.

miR-10b inhibits Th17 responses and is transiently induced during Th17 differentation

We then investigated the effects of miR-10b on Th17 cell function. Efficient transfection of CD4+ T cells was confirmed using FAM-labeled siRNA and miR-10b qPCR (see online supplementary Figure S3). Over-expression of miR-10b reduced both Th17 frequencies and IL-17A production in CD4+ T cells from both AS patients and HC (Figure 2A). Neither interferon (IFN)-γ nor interleukin (IL)-4 was affected by miR-10b (see online supplementary Figure S4). Notably miR-10b did not significantly affect CD4+ T cell proliferation or viability (see online supplementary Figure S5).

We next asked if miR-10b expression in CD4+ T cells could be increased by pro-inflammatory cytokines known to be upregulated in AS.[14] TNF-α and IL-6, but not IL-1β, IL-23 or transforming growth factor (TGF)-β, enhanced miR-10b expression (see online supplementary Figure S6).

We next examined the expression of miR-10b in different T helper cell subsets. Figure 2B shows efficient differentiation of Th17, Th1 and Th2 cells from naïve CD4+ T cells, and the transient upregulation of miR-10b in Th17 cells on day 3. Figure 2C shows that transfection of miR10b into developing Th17 cells, but not Th1 or Th2 cells, selectively inhibited their lineage-specific cytokine.

miR-10b inhibits MAP3K7 through 3′ UTR binding; MAP3K7 silencing in Th17 cells inhibits IL-17A and IL-22 production.

To identify the cellular targets of miR-10b, we performed RNA-sequencing of CD4+ T cells transfected with miR-10b together with *in silico* TargetScan analysis (Figure 3A and data not shown). Of potential targets that were down-regulated upon miR-10b overexpression and were predicted to be regulated by miR-10b by TargetScan, MAP3K7 was selected for further study because of its known role in cytokine regulation. We used qPCR to confirm that MAP3K7 gene expression was indeed decreased in primary CD4 cells by miR-10b over-expression (Figure 3B). Moreover, an inverse correlation between expression of miR-10b and MAP3K7 was found in Th17 cells of patients with AS ($r = -0.58$, $p = 0.02$) (Figure 3C). In luciferase assays using HEK-293T cells, miR-10b only inhibited the reporter activity of the 3′ untranslated regions (UTRs) of wild type MAP3K7, confirming that MAP3K7 was a direct target of miR-10b (Figure 3D).

We then silenced MAP3K7 in CD4+ T cells using siRNA and found the selective suppression

of Th17 response, mimicking the effect of miR-10b over-expression (Figure 3E). Silencing of MAP3K7 in naïve CD4+ T cells inhibited IL-17A and IFN-γ in Th17 and Th1 cells respectively (Figure 3F), without affecting IL-4 production in Th2 cells (see online supplementary Figure S7).

DISCUSSION

In the present study, we show that AS Th17 cells upregulate micro RNAs miR-10b-5p, miR-155-5p and miR-210-3p. We further describe the function of one of these, miR-10b, in the regulation of cytokine production by Th17 cells. IL-6 and TNF-α upregulated miR-10b, which in turn suppressed IL-17A production. We suggest that miR-10b constitutes part of a negative feedback loop attempting to limit the enhanced inflammatory Th17 responses in AS, since miR-10b was not elevated in directly ex vivo isolated AS Th17 cells and miR-10b expression in vitro correlated inversely with Th17 cell frequency.

A function for miR-10b in immune cells has not been described previously, although a role in cancer has been suggested. [15] [16] We here show that miR-10b can inhibit MAP3K7 expression in CD4 T cells and that this can lead to inhibition of IL-17A production. Our transcriptomic and TargetScan analysis suggest that, in addition to MAP3K7, miR-10b almost certainly targets other genes with additional downstream effects. Coordinated action on multiple target genes provides a powerful mechanism by which a single miRNA can impact on a complex regulatory network and ultimately a physiological process or disease.[17]

Increased frequencies of Th17 cells are a hallmark in AS patients, and appear to be functionally important as evidenced by therapeutic response to IL-17 blockade.[3,] Our data suggest that miR-10b may act as a negative feedback mechanism which, albeit only partially, suppresses the pathogenicity of AS Th17 cells. This is supported by the inverse correlation of miR-10b expression with Th17 cell number in AS.

We also found that miR-155-5p and miR-210-3p were significantly upregulated in AS Th17 cells. This confirms previous data showing an important role of these two miRs in regulating Th17 responses in mice and humans. [9, 11] Further work will be needed to assess the roles of additional miRs, including miR-144-3p, miR-127-3p, and miR-409-3p, that did not reach statistical significance in our RNAseq analysis.

In summary, we here describe the miR signature of AS Th17 cells. Highly expressed miRs include miR-155-5p and 210-3p, which have well characterized roles in Th17 cells. Our data show for the first time a role for in suppressing Th17 function via targeting MAP3K7, and suggest that miR-10b expression in AS Th17 cells constitutes a negative feedback loop. Since we have demonstrated that miR10b transfection suppresses Th17 cell cytokine production by naïve and memory AS and healthy CD4 T cells, miR-10b mimics might be used therapeutically to suppress the function of pathologic Th17 cell in AS patients.

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