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Positive-Negative Feedback Loop between miR-197 and IL-17A Signaling in Human Keratinocytes

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Abstract

Psoriasis is a chronic inflammatory skin disorder which results from pathological interactions between activated immunocytes and keratinocytes. Recent studies implicated the role of IL-17 and IL-22, secreted from Th17 and Th22 in the generation and propagation of the psoriatic plaque. Previously, we and others have shown that the expression of miR-197 is significantly decreased in psoriatic lesions. We further showed that miR-197 targets IL-22RA1 and that ectopic expression of miR-197 prevent IL-22 induced proliferation and migration of keratinocytes.

Since the 3'UTR of the IL17RA subunit mRNA contains a putative binding site for miR-197, our aim was to expand our understanding of the miRNA-mediated crosstalk between immunocytes and keratinocytes by studying the effect of miR-197 expression on IL-17A signaling pathway. Luciferase reporter assays along with Western blot analysis revealed that miR-197 directly targets the 3'UTR of IL17RA. Furthermore, ectopic expression of miR-197 led to a significant decrease in IL-17A-induced expression of CCL20, a known downstream effector of IL-17A. Interestingly, the addition of IL-17A to keratinocytes led to a rapid and transient increase in the expression of miR-197. Chromatin immuno-precipitation assays showed that keratinocytes' treatment with IL-17 leads to C/EBP binding to the promoter region of miR-197, and that the expression level of miR-197 is directly proportional to the extent of C/EBP binding to the promoter. Moreover, following treatment with IL-17A, the histone acetylation pattern at the miR-197 promoter turns to become characteristic of transcribed chromatin.

Taken together, our results suggest that a positive-negative feedback loop exists between IL-17A and miR-197 in keratinocytes; the cytokine induces the binding of C/EBPα to miR-197 promoter sequences, enhances miR-197 expression that negatively attenuates IL-17 receptor and decreases the input along the IL-17A pathway. Our work suggests that in psoriasis, decreased expression of miR-197 may prevent the miR-197-induced attenuation of the IL-17 cascade, leading to its over-activity.

Introduction

Psoriasis is a chronic inflammatory skin disorder. It is characterized by hyperplasia of the epidermis, infiltration of leucocytes into both the dermis and the epidermis, and proliferation of blood vessels [1-3]. One of the characteristics of psoriasis is the cross talk between activated immunocytes and keratinocytes (KC) that begins early upon lesion formation and culminates in the mature psoriatic plaque [4]. Pathogenic T cells, releasing a cascade of cytokines, infiltrate the skin and trigger a hyper-proliferative response of KC [5]. Recent studies suggest a central role for IL-23 and IL-17A, along with TNFα, in the pathogenesis of psoriasis [6,7]. The current pathogenic model of psoriasis suggests that activated dendritic cells (DC) produce IL-23 and IL-12, which stimulate the three populations of resident T cells, namely Th17, Th22, and Th1. These cells produce IL-17A and IL-17F, IL-22 and IFNγ respectively. T cell derived cytokines act on epidermal KC as proximal inducers of these inflammatory circuits, which drive KC responses. Once activated, the epidermis can produce abundant cytokines, chemokines and inflammatory mediators. These chemokines attract leukocytes such as neutrophils, DC, and Th17 cells. Other chemokines recruit additional circulating Th1 cells into the dermis and epidermis [7].

An increased number of Th17 cells have been identified in the dermis and epidermis of psoriatic skin compared with normal skin, which supports the central role that IL-17A plays in the pathogenesis of psoriasis [7-9]. Results from recent clinical trials have confirmed the central contribution of IL-17A to psoriasis. Those include both a correlation between alterations in IL-17A pathway and successful therapy as well as the demonstration of the direct effects of inhibition of both IL-17A and IL-17RA on psoriasis in humans [10,11].

IL-17A was first identified as a product of rodent activated T cells in 1993 [12,13]. IL-17A acts on a variety of cell types such as epithelial cells, KC, macrophages, DC, neutrophils, fibroblasts, endothelial cells, and lymphocytes [14]. In these cells it enhances the production of various pro inflammatory molecules including cytokines (such as TNF and IL-6), chemokines (such as CCL20, CXCL2 and MCP-1), mucins, acute phase proteins, and matrix metalloproteinases [15,16].

The ligands of IL-17 cytokines family act as dimers that bind to a set of functional receptors for IL-17 (IL-17R). Five molecules subunits have been identified as IL-17 receptors; IL-17RA to IL-17RE. [17]. The signaling that IL-17A, IL-17F and IL-17E activate are transcribed via one of three transcriptions factors; NFkB, C/EBP or AP1[18-20].

Recent studies have revealed that interactions between micro-RNAs (miRNAs) and key molecules play critical roles in regulating distinct signaling pathways during skin differentiation [21,22]. Deregulation of miRNAs and their regulated targets has been implicated in the pathogenesis of psoriasis [23,24]. In an earlier study, we and other have shown that miR-197 expression is down regulated in the psoriatic lesions compared to normal or noninvolved psoriatic skin [25,26]. In the present study we show that IL-17A signaling activates the expression of miR-197, and miR-197 can directly repress the expression of the IL17RA subunit of the IL-17 receptor, suggesting the existence of a positive-negative feedback loop between IL-17A and miR-197.

Materials and Methods

The research involved with human skin tissue has been approved by the Sheba medical center Institutional Review Board (IRB) committee according to the principles expressed in the Declaration of Helsinki. The approved application number is SMC-9776-12.

Cells cultures

HaCaT and primary human keratinocytes (PHK) were grown as previously described [25,27] .

Quantitative real time PCR (qPCR)

Total RNA of cells was extracted using Norgen total RNA purification Kit (Norgenbioteccorp #17200). Quantification of miRNA was performed as previously described [25].

Plasmids pMSCV-miR-197 and pMSCV-HTR plasmids were kindly provided by Agami, R [28]. Luciferase-IL17RA-3'UTR (HmiT016091-MT01), and the control no-3'UTR (CmiT000001-MT01) plasmids were purchased from GeneCopoeia, (GeneCopoeiaInc, Rockville, MD 20850USA). The WT psiCHECK-Luciferase-IL17RA-3'UTR was generated by amplifying by PCR reaction fragment of, 1100 bp containing the 3'UTR of IL17RA mRNA from the HmiT070362-MT06 plasmid. The primers used are the following primers:

Forward primer with XhoI-

5' - GCGCCTCGAGCCAGCTTTGAGAGAGGAGTG-3'

Reverse primer with NotI -

5' - ATGCGGCCGCGAGGCTCATCAGACGAAAGG-3'

The fragment was cut with XhoI and NotI and ligated into psiCHECK-2 that was cut with the same enzymes.

The IL17RA-3'UTR mutant for the hsa-miR-197 seed sequence was created using the Megaprimer Mutagenesis assay [29] using primer forward and mutant primer:

Mutant primer -

5'-
GTGGAGATGGGGTATGTGGATGAAGGGGAGGATCGCTCAAAC
TCC-3'

For the first amplification generated a fragment of ~100 bp that was used for the second amplification with the reverse primer. The ~1000 fragment was cut with XhoI and NotI and ligated into psiCHECK-2 that was cut with the same enzymes.

Transfections

HaCaT cells were transfected as previously described [25].

PHK cells were transfected using X-tremeGENE Transfection Reagent. (Roche, CH-4070, Basel, Switzerland). Stably transfected HaCaT cells were generated by transfecting pMSCVmiR-197 or pMSCV-HTR [28]. Lines were achieved after 4 weeks selection with Blasticidin at a final concentration of 16 μg/ml.

Luciferase assay

Luciferase assays were performed using the Dual-Luciferase Reporter (DLR) Assay System (Promega Corporation Madison,WI 53711 USA),

Determination of proteins expression by Western blot analysis (WB)

WB was performed as described previously [27] using monoclonal Mouse IgG clone #ab134086, anti-Human IL17RA antibody (Abcam Cambridge, CB4 OFW, UK) and alpha-Tubulin antibody (ab4074) (Abcam Cambridge, CB4 OFW, UK).

Chromatin immunoprecipitation (ChIP) assay

Epigenetic modifications ChIP was performed as a modified protocol from the one previously described in [30] see detailed in supplementary data, antibodies were coupled to Dynabeads Protein G cat. #10004D from (Invitrogen, Thermo Fisher Scientific Waltham, MA USA 02451). Antibodies used for Epigenetics ChIP; Acetyl-Histone H3 (Lys9) #17-609 and Trimethyl-Histone H3 (Lys27) #17-622 from (Merck Millipore Corporation Massachusetts 01821 USA). Transcription factors ChIP was performed as modified protocol from previously described in [31], see detailed in supplementary data (Figure S1), anti C/EBPα #sc-61, anti C/EBPδ #sc-151 and anti NFκB #sc-372 from (Santa Cruz Biotechnology, Dallas, Texas 75220).

Primers that were used in the ChIP assay:

For miR-197 promoter region

Forward primer –

5' - GTAGTGGGTGGTCTTTTACAGC-3'

Reverse primer -

5' - ACCCTGCTTCACGAATTTAGAG-3'

For GAPDH promoter region promoter

Forward primer –

5'-AC TAG CGG TTT TAC GGG CG-3'

Reverse primer -

5'- TCG AAC AGG AGG AGC AGA GAG CGA-3'

For α-satellite promoter region promoter

Forward primer –

5'- CTG CAC TAC CTG AAG AGG AC-3'

Reverse primer -

5'- GAT GGT TCA ACA CTC TTA CA-3'

Statistical analysis

Statistical significance was done using one-way ANOVAs followed by Tukey's post-hoc teat or by Two-way ANOVAs followed by Bonferroni's post-hoc test or by paired two-sample Student's *t-test*. A probability value of $p<0.05$ was considered significant.

Results

IL17RA is a direct target of miR-197

Recently we showed that miR-197 expression is low in the psoriatic lesion compared to normal skin [25]. Moreover, we found that miR-197 is part of the IL-22 signaling cascade and regulates the expression of subunit IL22RA1 of the IL22 receptor [32]. *In vivo*, Th17 cells co-express IL-22 and IL-17A [16]. We therefore hypothesized that miR-197 may be part of the IL-17A signaling as well. Bioinformatics analysis using 'target scan' (www.targetscan.org) [33,34] suggest that miR-197 can target the IL17RA subunit of the IL-17 receptor with a 7mer-1A seed binding site (Figure 1A). Using the bioinformatics tool miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/generetsys.php) [35] which provides lists of miRNA-predicted targets according to 12 different bioinformatics softwares, IL17RA is a potential target of miR-197 according to six. To determine whether IL17RA is indeed a target of miR-197, we cloned the first 1500 bp of the 3'UTR of the IL17RA (IL17RA-WT-3'UTR) mRNA into a psiCHECK luciferase reporter plasmid. This plasmid was transfected into the immortalized KC cell line HaCaT over expressing control vector (HaCaT-HTR) or to HaCaT cells over expressing miR-197 (HaCaT-miR-197). A luciferase reporter assay was performed 72 h after transfection. As can be seen in Figure 1B, when control cells were transfected by psiCHECK vector or the IL17RA-WT-3'UTR vector the relative expression of luciferase was the same (Figure 1B left graph). However, when these plasmids were transfected into HaCaT-miR-197, the relative expression of luciferase in cells transfected with IL17RA-WT-3'UTR, was significantly lower compared to HaCaT-miR-197 cells transfected with a plasmid lacking the 3'UTR of IL17RA1 (Figure 1B right graph).

To verify that miR-197 directly targets the IL17RA 3'UTR, we generated a mutated vector, in which six nucleotides in the seed response sequence were changed from ATGGTGAA to ATCCACAT (IL17RA-mut-3'UTR). The mutant was transfected to HaCaT-HTR or HaCaT-miR-197 cells and the relative luciferase activity was assessed. Figure 1B clearly demonstrates that mutation in the seed binding site of miR-197 completely abolished the miR-197 WT suppressive effect in the luciferase reporter assay. These experiments prove that miR-197 seed binding sequence at the IL17RA 3'UTR is essential for the regulation of IL17RA by miR-197.

Next we asked whether miR-197 affects the endogenous IL17RA protein expression. To address this, primary human KC (PHK) were transfected with mimic of pre-miR-197. 48 h after transfection the cell proteins were extracted and subjected to WB analysis using anti-IL17RA antibodies. As can be seen in Figure 1C, over expression of pre-miR-197 in PHK cells led to a dramatic decrease in the level of IL17RA protein. These results are confirmed by a densitometry analysis of the blots from 4 different experiments (Figure 1D). Taken together, these results prove that IL17RA is a direct biochemical target of miR-197.

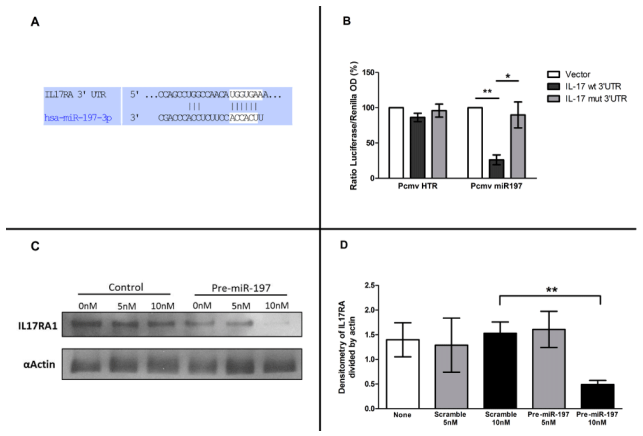

Figure 1: IL17RA is a direct target of miR-197. A) The putative binding of miR-197 to the 3'UTR of IL17RA (adopted from TargetScan) [34]; B) HaCaT-HTR or HaCaT-miR-197 cells were transfected with psiCHECK2 vector encoding luciferase vector), or luciferase fused to the wild type IL17RA-3'UTR (IL17RA-wt-3'UTR), or luciferase fused to a mutated IL17RA 3'UTR (IL17RA-mut-3'UTR). Cells transfected only with vector lacking the IL17RA 3'UTR were taken as 100%. The error bars are calculated as standard error of at least 4 independent experiments. Statistic were performed using- One way ANOVA: $F5,9=7.314$, $p<0.01$; Turkey's post hoc: $^{*}p<0.05$, $^{**}p<0.01$; C) WB analysis of the IL17RA protein 48 h after transfection with 5 or 10 nM of scrambled control pre-miR or with 5 or 10 nM of pre-miR-197; D) Densitometry analysis of 4 WBs analyses of IL17RA protein 48 h after transfection with 5 nM or 10 nM of scrambled control RNA or 5 nM or 10 nM pre-miR-197. The mean -/+ SD was calculated from 4 independent experiments. Statistic were performed using *t-test* ($^{**}p<0.01$).

miR-197 inhibits IL-17A induced enhancement of CCL20 expression

In order to explore whether there is crosstalk between miR-197 affects and the IL-17A signalling cascade we wanted to find out whether over expression of miR-197 affects the expression of its known downstream targets. One such well documented target of IL-17A is the CCL20 [10]. CCL20 can be induced in a variety of cell types by lipopolysaccharides and by pro-inflammatory cytokines such as TNF-α and IL-17A [36]. We anticipated that over expressing miR-197 will affect the expression of CCL20 in KC treated with IL-17A.

The levels of CCL20 mRNA increased significantly in HaCaT cells treated with 5 and 10 ng/ml of IL-17A for 1 h and for 4 h (Figure 2A and 2B). The effect was markedly attenuated in HaCaT cells over

expressing miR-197 at 1 h (treated with 10 ng/ml of IL-17A) or when treating HaCaT cells with 5 ng/ml at 4 h. (Figure 2A and 2B). These results suggest that miR-197 can inhibit the signal transduction pathway induced by IL-17A. The inhibitory effect of miR-197 on CCL20 expression induced by IL-17, faded when PHK were exposed to higher (10 ng/ml) of IL-17 for longer times (4 h), possibly due to overcoming the inhibitory effects of miR-197 at these conditions.

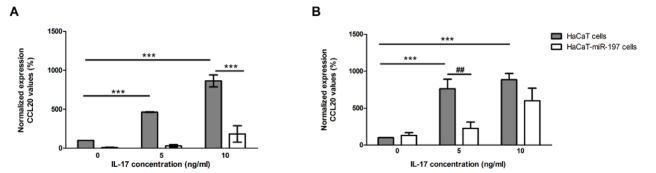

Figure 2: The enhancement of CCL20 mRNA expression by IL-17A is attenuated by miR-197. IL-17A was added to HaCaT and to HaCaT-miR-197 transfected cells at the indicated concentrations. RNA was extracted from the harvested cells and qPCR was performed with CCL20 specific primers. A) 1 h post IL-17A addition. Statistics were performed using- One way ANOVA: $F_{5,9}$= 33.47, p<0.0001; Tukey's post hoc; ***p<0.001. B) 4 h post IL-17A addition. Statistic were performed using One way ANOVA: $F_{5,11}$ =19.39, p<0.0001; Tukey's post hoc: ***p<0.001; ##p<0.01. (The mean -/+ SD was calculated from 3 independent experiments).

IL-17A transiently enhances the expression of miR-197

To study the cross-talk between miR-197 and the IL-17A pathway, we monitored the expression of miR-197 in PHK treated with different concentrations of IL-17A. We performed a dose-response at 2h to choose the IL-17A concentration and treatment. IL-17A signalling transiently enhances the expression of miR-197: The levels of miR-197 increase significantly in cells treated with 20 ng/ml and 200 ng/ml (2 h) of IL-17A as compared to untreated cells (Figure 3). The effect of IL-17A on the expression of miR-197 disappears after 4 h of treatment (Data not shown).

IL-17A induced the binding of C/EBPα to the miR-197 promoter

The IL-17A signalling pathway activates transcription via one of three transcriptions factors: NFkB, C/EBP or AP1 [37]. We focused on NFκB and C/EBP as these factors seem to be the major mediators of IL-17 response [18-20]. The miR-197 gene is located on human chromosome 1p13.3, in a region distinct from other known transcription units. The bioinformatics tools Tfsitescan [38] and TFSEARCH [39] mapped C/EBP binding sites within the 2000 bases upstream of the miR-197 gene. There were no classical NFkB binding sites in this region.

In order to find whether NFkB or C/EBP bind to the promoter of miR-197 subsequent to IL-17A treatment, PHK were subjected to ChIP assay using C/EBPδ or C/EBPα or NFκB specific antibodies. As can be seen in Figure 4, treatment with IL-17A did not change the binding of NFκB or C/EBPδ to this promoter. However, IL-17A enriches the binding of C/EBPα to this region. This suggests that upon exposure of KC to IL-17A the activation of its signalling cascade results in the binding of C/EBPα to the miR-197 promoter. It should be noted that because we used PHK from different human sources and at different passages the activation of miR-197 upon treatment with IL-17A was variable in its extent.

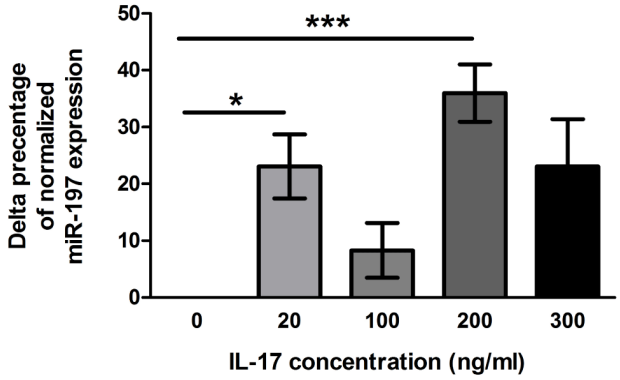

Figure 3: IL-17A enhances miR-197 expression at short time exposures. IL-17A was added to PHK cells at the indicated concentrations. Cells were harvested and then a qPCR was performed with miR-197 specific primers. One way ANOVA: $F_{4,54}$=6.605, p<0.001; Tukey's post hoc: *p<0.05; ***p<0.001. (The mean -/+ SD was calculated from 12 independent experiments).

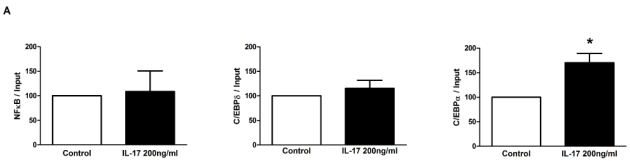

Figure 4: IL-17A induced the binding of C/EBPα to miR-197 promoter. PHK cells were treated with 200 ng/ml IL-17A for 1.5 h then were subjected to ChIP assay using anti NFkB, anti C/EBPδ or C/EBPα antibodies, respectively. The results represent the miR-197 promoter measured by qPCR in the immune precipitated DNA, divided by its amount when measured by qPCR of input DNA. All qPCR were done with qPCR SYBR Green dye. The mean -/+ SD was calculated from 3 independent experiments. Statistic were performed using *t-test* (*p<0.05).

IL-17A treatment affects the acetylation and methylation of histones at the miR-197 promoter region

To further explore the mechanism of miR-197 up-regulation by IL-17A, we asked whether IL-17A treatment affects the epigenetic structure of miR-197 promoter. Histone modification may be divided into three groups; those indicating transcriptionally active genes, transcriptionally paused genes and transcriptionally silenced genes [40-42]. Since miR-197 transcription fluctuates quickly in response to cytokines, we assume that it is not packed as heterochromatin but as euchromatin. Therefore it is expected that its epigenetic markers would shift from active to paused states [41]. We decided to assay for histone modifications which distinguish between these two situations. Actively transcribed genes are characterized by high levels of H3K9 acetyl at the transcription start site while paused genes show high levels of H3K27me2/3 at the transcription start site [41]. As shown in Figure 3,

the expression levels of miR-197 are increased upon treatment of PHK with IL-17A. We analyzed the epigenetic structure of the miR-197 promoter using two modifications, H3K9 acetyl as a marker for transcriptional active genes and H3K27me2/3 as a marker for transcriptional paused genes. As can be seen in Figure 5, following IL-17A treatment of PHK cells, there is a significant enrichment of H3K9 acetyl at the miR-197 promoter region (Figure 5A; left panel). The effect is specific since in the promoter region of GAPDH (A consistently active gene) there is no change in the H3K9 acetyl modification before or after treatment (Figure 5A; right panel). When we measure the effect of IL-17A treatment on the H3K27me3 modification at the same experiments, we found that there is a significant decrease of this modification in the treated cells compared to untreated cells (Figure 5B; left panel). The effect is specific, as there is no change in the amount of H3K27me3 modification at the promoter region of α-satellite gene which is usually paused (Figure 5B; right panel).

To further correlate the acetylation upon IL-17 treatment with the expression of miR-197, we pre-treated PHK with anacardic acid, a histone acetyltransferase (HAT) inhibitor 24 h before adding IL-17. As can be seen in supplementary (Figure S1) indeed pre-treatment of KC with HAT inhibitor completely eliminated the increase in expression of miR-197 after treatment with IL-17.

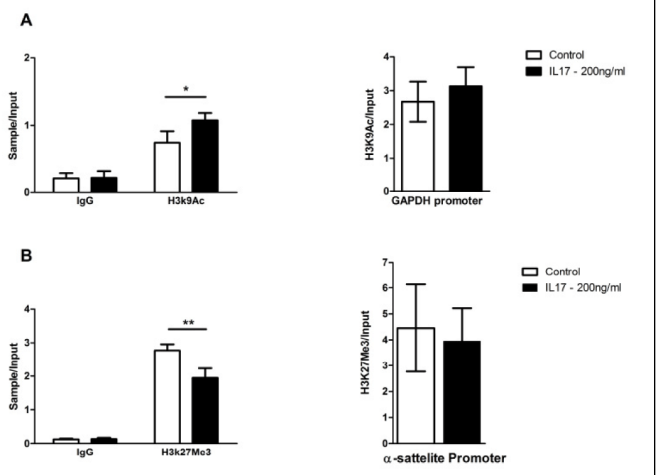

Figure 5: Up-regulation of Histone acetylation and down-regulation of H3K27me3 in miR-197 promoter post IL-17A treatment. PHK cells were treated with 200 ng/ml IL-17A for 2 h and then were subjected to ChIP assay using A) H3K 9acetyl antibodies or B) H3K27me3 antibodies. The results represent the amount of miR-197 promoter copies measured by qPCR of immune precipitated DNA with the specific antibodies divided to the amount of measured by qPCR of input DNA. All qPCR were performed with qPCR SYBR Green dye. The mean -/+ SD was calculated from 6 independent experiments. For H3K 9acetyl antibodies the statistic were performed using Two way ANOVA: F1,10=21.27, p<0.001; Bonferroni post hoc: *p<0.05. For H3K27me3 antibodies the statistic were performed using Two way ANOVA: F1,8=11.44, p<0.01; Bonferroni post hoc: **p <0.01.

The binding of C/EBPα to the miR-197 promoter and its epigenetic structure correlate with the induction of miR-197 expression by IL-17A.

Using multiple sources of PHK and after repeated exposure of these cells to variable concentrations of IL-17A, we noticed that the response of miR-197 to IL-17A is quite variable. Due to this observation we decided to evaluate the correlation between C/EBPα or NFκB binding to miR-197 promoter, as well as the correlation between alterations in its epigenetic structure and the magnitude of its enhancement by IL-17A. For this end we used the data from multiple experiments exposing PHK to 5-200 ng/ml of IL-17A for 30-90 min. As depicted in Figure 6A, there is a significant correlation between miR-197 expression levels and C/EBPα binding to miR-197 promoter (r^2=0.8148) (Figure 6A left panel), but no correlation to the binding of NFκB to the same region (r^2=0.3) (Figure 6A right panel). At the same experiments we evaluated the correlation between the magnitude of miR-197 over expression induced by IL-17A and the change in H3K9acetyl or H3K27methyl3 in miR-197 promoter. As can be seen in Figure 6 B, there is a significant correlation (r^2=0.5906), between miR-197 expression and H3K9acetyl enrichment and correlation with decreased H3K27 3-methylation (r^2=0.6656) at miR-197 promoter (Figure 6C).

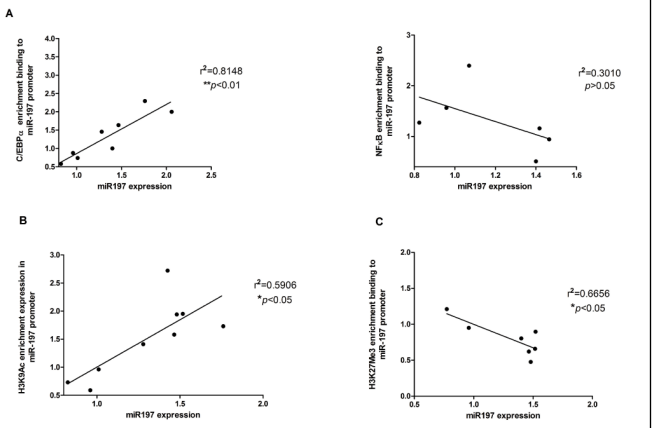

Figure 6: IL-17A induced expression of miR-197 correlates with the binding of C/EBPα, and epigenetic alterations at the miR-197 promoter. A) IL-17A was added to PHK cells at different concentrations (5, 20 or 200 ng/ml) and for variable time points (Between 30 and 90 min). A qPCR was performed with miR-197 specific primers. In the same set of experiments these cells were subjected to ChIP assay using anti C/EBPα (left panel) and NFκB (right panel) antibodies. Scatter gram showing the correlation between the amounts measured by PCR of immune precipitated DNA with the anti C/EBPα (left panel) and NFκB (right panel) divided to the amount of input DNA *vs.* miR-197 expression from 8 independent experiments, as was calculated by Pearson correlation coefficient (r^2=0.8148, r^2=0.3010; respectively; **p<0.01; p>0.05; respectively). The same samples used in Figure 6 A were subjected to ChIP assay using anti H3K9acetyl antibody; (B) or anti H3K27 3-methyl antibody (C). The results represent 9 independent experiments as was calculated by Pearson correlation coefficient (r^2=0.5906; *p<0.05 for B and r^2=0.6656; *p<0.05 in C).

Discussion

Previously, we and others have shown that the expression of miR-197 is significantly decreased in psoriatic lesions [25,26]. We further showed that ectopic expression of miR-197 prevents IL-22-induced proliferation and migration of KC, suggesting that its decreased expression contributes to the pathological pro-proliferative response to IL-22 in psoriatic lesions. Here our aim was to expand our understanding of the miRNA-mediated crosstalk between the immune system and KC by studying the effect of aberrant miR-197 expression on other prototypic signaling pathways, namely the IL-17 signaling pathway.

Both IL-17A and IL-22 orchestrate an innate immune response directed against extracellular pathogens by inducing the expression of antimicrobial peptides and chemokines specific for neutrophil recruitment. However, these cytokines have distinct functions. IL-22 affects KC proliferation, migration and differentiation. IL-17A produces an inflammatory tissue response by inducing KC to produce CCL20 which is probably required for the continuous recruitment of CCR6-positive Th17 cells to the psoriatic lesions [10,43]. Both CCR6 and CCL20 are expressed at high levels in psoriatic lesions as compared to non-involved or healthy skin. Moreover, CCR6 expression is higher on circulating PBMCs from psoriasis patients compared to normal donors [36]. Serum levels of IL-17 and IL-22 are elevated in individuals with chronic plaque psoriasis [44,45]. The Th17 cells, as well as their effector molecules (For example: IL-17A, IL-17F, TNF-α, IL-22) are abundant in psoriatic lesions [46-48]. Their effects on the extra-cellular milieu in psoriasis are thus complementary.

Our results show that the IL-17 receptor subunit, IL17RA, is a bona-fide target of miR-197. Moreover, miR-197 inhibits the flux through the IL-17 signaling pathway, as manifested by its negative effect on the enhancement of CCL20 expression by IL-17. The increase in the expression of miR-197 after treatment with IL-17 was by ~40%. We believe that even this small increase has biological significance. First, miRNA-mediated regulation are best considered as fine-tuners of gene expression and not as complete silencers, therefore even a slight increase would have an effect. Second, the signalling of IL-17 was shown to be regulated by several biochemical ways. It was show that IL-17 triggered phosphorylation of C/EBP beta at two threonine residues which turned C/EBP beta into repressor that inhibited the expression of pro-inflammatory genes induced by IL-17 [49]. The signalling of IL-17 involves the binding of IL-17 to the receptor which recruited the binding of Act1 protein to specific SEFIR domain on IL-17 receptor. Next, TRAF6 protein is binding to the Act1 protein. TRAF6 was shown to be essential for IL-17 induced activation of NF-kB and JNK pathways (review in [37]). In parallel this pathway leads to phosphorylation of Act1. This phosphorylation marks Act1 for degradation through the ubiquitin pathway, suggesting for additional feedback loop regulating IL-17 signalling (review in [37]). Hence, the miR-197 is an additional layer of this complex feedback regulation.

Our results reveal the existence of biochemical positive-negative feedback loop between IL-17A signalling and miR-197; IL-17A binds to its cell-surface receptors on normal KC and initiates a signalling cascade that leads to the binding of C/EBPα to the miR-197 promoter and to the induction of epigenetic changes in the miR-197 promoter region which ultimately result in increased expression of miR-197. miR-197 then represses the expression of the IL-17 receptor subunit IL17RA, leading to attenuated IL-17 signalling and to decreased expression of the CCL20 chemokine. The present results add to the known role of miR-197 in IL-22 signalling [32]. As depicted in Figure 7 our results underscore the critical role played by miR-197 in the fine-tuning of KC response to both IL-17 and IL-22. Taken together, our results suggest that the decreased expression of miR-197 in psoriatic KC possibly propagates the pathological processes within the psoriatic plaque by preventing an important mechanism for dampening the pro-inflammatory IL-17 cascade.

IL-17A increases the expression of miR-197 in KC and activates the binding of C/EBPα to the miR-197 promoter of miR-197 (Figure 4). This binding is associated with activating alterations in the epigenetic structure of miR-197 promoter, namely elevation of H3K9 acetylation and decrease in H3K27 trimethylation (Figure 5). It is tempting to speculate that aberrant epigenetic silencing of the miR-197 promoter underlies its decreased expression in psoriasis. The mechanism leading to miR-197 silencing, as well to additional alterations in miRNAs in the psoriatic lesion [25,26,50,51], should be further investigated. Study of the epigenetic structure of miR-197 and its putative promoter sequences in the psoriatic genome might partially answer this question. Indeed, there are few reviews correlating abnormal epigenetics and the development of psoriasis [46,47]. Abnormal expression of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which regulate the balance of histone acetylation and deacetylation, have been observed in psoriasis. HDAC-1 mRNA is over-expressed in psoriatic skin samples compared to skin specimens from healthy subjects [52]. However, a lot of additional work should be invested in deciphering the links between those alterations and the pathophysiology of psoriasis.

miR-197 is not an evolutionary conserved miRNA. According to the miRBase data, miR-197 gene is present, aside from humans, only in primates, horse, cow and dog [53]. IL-17, IL17RA, IL-22 and IL22RA1 are common across many more species. However, the binding site of miR-197 on the 3'UTR of IL22RA1 exists only in primates, cow and dog and in the 3'UTR of IL17RA exists only in primates. Psoriasis seems also to be a relatively 'new' disease from an evolutionary perspective, occurring only in mammals/primates. It is tempting to speculate that a co-evolutionary process occurred between the mammal-specific miR-197 and the receptors of the two major cytokines IL-22 and IL-17 throughout evolution to allow the former to fine-tune the signalling cascades of the latter as shown in Figure 7. We suggest that an aberration in this miR-197-driven fine-tuning mechanism underscores the pathological process of psoriasis.

Our study focused on the biochemical feedback loop of IL-17 signalling and miR-197 in keratinocytes, because initially we found miR-197 expression to decrease significantly in psoriasis lesions. However, the important role of IL-17 signalling in many autoimmune and chronic inflammatory diseases like; Rheumatoid arthritis, Systemic lupus erythematosus, Crohn's disease, Multiple sclerosis, Allergic rhinitis and Asthma is well known as reviewed in Murdaca et al. [54]. Yet, the expression level and involvement of miR-197 in these diseases is unknown and certainly it would be interesting to study.

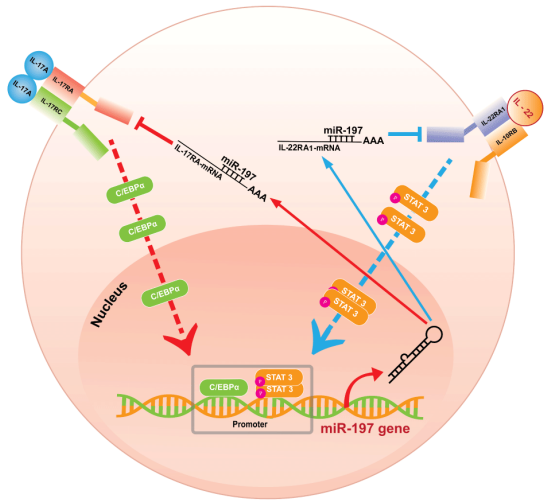

Figure 7: Positive-negative feedback loop between IL-17A and IL-22 signalling and miR-197 model.

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