**IL-4 dysregulates microRNAs involved in inflammation, angiogenesis and apoptosis in epidermal keratinocytes**

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**Abbreviation used**: AD, atopic dermatitis; Tg, transgenic

**Abstract**

IL-4 plays an important role in atopic dermatitis (AD) pathogenesis by dysregulating many key factors at the transcriptional level. In this study, using microRNA array technique and IL-4 transgenic mice, we demonstrated that IL-4 dysregulates microRNAs involved in inflammation, angiogenesis, lymphoangiogenesis and apoptosis. Of all the 372 common microRNAs examined, 26 and 1 microRNAs are up- and down-regulated, respectively. MicroRNA-101-5p, -122-5p, -142-3p, -204-5p, -335-3p, -376a-3p, -378a-5p, -639 and -9-5p are among the most significantly up-regulated microRNAs. MicroRNA-147a, the only down-regulated one in our study, attenuates TLR induced-inflammatory response. These dysregulated microRNAs may provide post-transcriptional regulation of key genes in AD.

Atopic dermatitis (AD) is a common chronic inflammatory skin disease, whose pathogenesis is related to skin barrier defects and dysregulation of the immune response. Th2 cells, Th17 cells, Th9 cells, other inflammatory cells, and their related cytokines may play roles in the development and progression of this disease. We have shown that IL-4 plays an essential role in AD pathogenesis by dysregulating many key factors at the transcriptional level (1-3). In addition, we have developed an AD animal model, IL-4 transgenic (Tg) mice, which spontaneously develop AD-like skin lesions (4). MicroRNAs are small non-coding RNA molecules (about 22 nucleotides) that provide post-transcriptional regulation of gene expression. They can affect cells expressing them as well as neighboring or distant cells. MicroRNAs’ role in AD has begun to gain more and more attention from research community. For the first time, using microRNA PCR array technique (372 most common microRNAs) , we have identified microRNAs regulated by IL-4 in primary human keratinocytes, which may play important roles in AD pathogenesis. We also confirmed the in vivo findings using IL-4 Tg mice.

Primary adult human keratinocytes (Thermo Fisher Scientific, Waltham, MA) were cultured in EpiLife® Medium and Human Keratinocyte Growth Supplement. The cells were transferred to DMEM supplemented with 10% FBS for 3 days to differentiate (high calcium). Then cells were treated with IL-4 (10 ng/ml or 50 ng/ml) in DMEM with 1% charcoal-dextran-treated FBS for 24 hr before harvest. miFinder miRNA PCR Array (Qiagen, Valencia, CA) was performed according to the manufacturer’s instructions. Briefly, 2 μg of total RNA was reversely transcribed to cDNA. 10 μl of master mix with cDNA (total volume 4100 μl) was loaded into each well of the 384-well PCR array. The PCR reaction was run on a ViiA 7 PCR machine (Thermo Fisher Scientific). The cycling conditions were: 95 °C, 15 min followed by 40 cycles of (95 °C, 15 s; 5595 °C, 30s; 70 °C, 30 s). The raw data were analyzed by the web-based statistical Array Analysis Software provided by Qiagen. Genes with at least 1.5-fold dysregulation and with a P-value of less than 0.05 were identified.

IL-4 up-regulates several microRNAs that are known to be involved in inflammation (table 1). MicroRNAs not only affect cells expressing them but also enter other cells to modify cell functions. MicroRNA-223 down-regulates IκBα by targeting FOXO3a (5), leading to activation of the proinflammatory NF-κB signaling pathway. In addition, microRNA-101 that binds to the MKP-1 mRNA 3'-UTR and microRNA-206 can stimulate the production of proinflammation cytokines (6). Furthermore, microRNA-142-3p that is involved in mast cell degranulation (7), microRNA-223 that is up-regulated in the skin of AD patients and is involved in eosinophil development (8), microRNA-155 that is involved in Th2-mediated eosinophil infiltration (9), and microRNA-146b that was reported to suppress Th1 responses (8) are all up-regulated by IL-4 in primary human keratinocytes. Interestingly, microRNA-639 suppresses TGF-β signaling by targeting FOXC1 (10). Since TGF-β is known to suppress IgE synthesis, the up-regulation of microRNA-639 by IL-4 in keratinocytes may provide an explanation for high levels of IgE in AD. On the other hand, microRNA-147a, which attenuates TLR induced-inflammatory response in macrophages (11), is the only microRNA down-regulated by IL-4 in the current study. Suppression of microRNA-147a logically removes this attenuation effect, thus amplifying macrophage-mediated inflammatory responses. Taken together, dysregulation of the above microRNAs by IL-4 may play some roles in the development and progression of inflammatory responses in AD skin lesions.

In addition to direct inflammatory responses, angiogenesis and lymphoangiogenesis are stimulated in AD. Dermal microvascular angiogenesis and lymphangiogenesis paralleled the severity of skin lesions. In the current study, IL-4 up-regulates several microRNAs (table 1), which have been reported to play roles in angiogenesis or lymphoangiogenesis. MicroRNA-10 regulates angiogenesis by directly suppressing mib1 (12); microRNA-30d acts via the MYPT1/c-JUN/VEGFA pathway to promote angiogenesis (13); microRNA-34b-3p affects proliferation, migration and tube formation of endothelial cell (14); microRNA-370 directly targets FOXO1 to promote capillary tube formation (15); microRNA-378a regulates the expression of VEGF, angiopoietin-1 and IL-8 (16); microRNA-9 up-regulates prolymphangiogenic molecules endothelial nitric oxide synthase and VEGF receptor type 3 and enhances lymphatic endothelial cell tube formation (17). Taken together, dysregulation of the above microRNAs by IL-4 in keratinocytes may explain extensive angiogenesis and lymphoangenesis observed in AD.

Keratinocyte apoptosis is an important feature of AD (18, 19). IFN-γ up-regulation of FasR may be one of the most important mechanisms causing apoptosis in keratinocytes. In the current study, we found that IL-4 up-regulates several microRNAs that are known to play roles in apoptosis (table 1). MicroRNA-490-3p induces G1 arrest and apoptosis by directly binding to HMGA2 mRNA 3'UTR and down-regulating its expression (20); microRNA-147a stabilizes and accumulates HIF-1α protein by directly targeting HIF-3α, a dominant negative regulator of HIF-1α (21); microRNA-204-5p suppresses cell proliferation by regulating the expression of SIRT1, BCL2 and IGFBP (22). Other microRNAs involved in apoptosis include microRNA-298, microRNA-335-5p, microRNA-335-3p, microRNA-376a-3p and microRNA-518b. In summary, IL-4 dysregulation of these microRNAs in keratinocytes may be associated with keratinocyte apoptosis in AD.

Also worth mentioning, we have identified several other microRNAs dysregulated by IL-4 (Fig. 1), such as microRNA-302a-3p, microRNA-328-3p and microRNA-382-5p, which may play similar roles in immune responses, angiogenesis and apoptosis. But due to the sample size (n=4), the difference does not reach statistical significance. In the future, we will continue to focus on these microRNAs for their roles in AD pathogenesis.

To further confirm the in vitro findings, we used IL-4 Tg mice, a well established AD animal model. These mice over-express IL-4 driven by a basal keratinocyte-specific keratin-14 promoter/enhancer in epidermal kertinocytes and develop AD-like skin lesions spontaneously (4). Skin tissues on the mouse earlobes were collected from IL-4 Tg mice and wild type mice without separating different layers of the skin. The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago. We studied the expression of several selected microRNAs to determine whether they are similarly dysregulated in vivo. As shown in Fig. 2, the expression of microRNA-142-3p, microRNA-183-3p, microRNA-204-5p, microRNA-223-3p and microRNA-30d-5p is up-regulated in IL-4 Tg mice as compared with control, which suggests that the dysregulation of these microRNAs in IL-4 Tg mice could be due to high levels of IL-4 in the skin.

In this study, using microRNA array analysis, we have identified microRNAs regulated by IL-4 in human primary keratinocytes. These microRNAs may play a role in AD pathogenesis through post-transcriptional regulation of key factors in inflammation, angiogenesis and apoptosis. More detailed examination of these microRNAs is needed to fully delineate their specific roles in the development and progression of AD. This knowledge may be translated into clinical applications in the diagnosis and treatment of AD.

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**Disclosure**

There is no conflict of interest.

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**Table 1. MicroRNAs dysregulated by IL-4 in primary human keratinocytes.**

Primary human keratinocytes (Thermo Fisher Scientific, Waltham, MA) were cultured in EpiLife® Medium and Human Keratinocyte Growth Supplement. The cells were transferred to DMEM supplemented with 10% FBS for 3 days to differentiate (high calcium). Then cells were treated with IL-4 (10 ng/ml or 50 ng/ml) in DMEM with 1% charcoal-dextran-treated FBS for 24 hr. miFinder miRNA PCR Array was performed as described in the paper. All the fold change values are statistically significant at P < 0.05 (n=4).

**Fig. 1. MicroRNAs that could be dysregulated by IL-4 in primary human keratinocytes.**

Primary human keratinocytes were treated with various concentrations of IL-4 for 24 h. miFinder miRNA PCR Array was performed as described in Materials and Methods. These microRNAs play important roles in inflammation, angiogenesis and apoptosis. However, the fold change values are NOT statistically significant (n=4, P>0.05).

**Fig. 2. Dysregulated microRNAs in IL-4 transgenic mice**

The expression of microRNA-142a-3p, microRNA-183-3p, microRNA-204-5p, microRNA-223-3p and microRNA-30d-5p were compared between IL-4 Tg mice and wild type mice. Values are expressed as the mean ± sem (n = 4). \*, P < 0.05 vs. wild type mice.