MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes

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Background: Chronic skin inflammation in atopic dermatitis (AD) is associated with elevated expression of proinflammatory genes and activation of innate immune responses in keratinocytes. microRNAs (miRNAs) are short, single-stranded RNA molecules that silence genes via the degradation of target mRNAs or inhibition of translation. Objective: The aim of this study was to investigate the role of miR-146a in skin inflammation in AD. Methods: RNA and protein expression was analyzed using miRNA and mRNA arrays, RT-quantitative PCR, Western blotting, and immunohistochemistry. Transfection of miR-146a precursors and inhibitors into human primary keratinocytes, luciferase assays, and MC903-dependent mouse model of AD were used to study miR-146a function. Results: We show that miR-146a expression is increased in AD, miR-146a inhibited the expression of numerous proinflammatory factors, including IFN-γ-inducible and AD-associated genes CCL5, CCL8, and ubiquitin D (UBD) in human primary keratinocytes stimulated with IFN-γ, TNF-α, or IL-1β. In a mouse model of AD, miR-146a–deficient mice developed stronger inflammation characterized by increased accumulation of infiltrating cells in the dermis, elevated expression of IFN-γ, CCL5, CCL8, and UBD in the skin, and IFN-γ, IL-1β, and UBD in draining lymph nodes. Both tissue culture and in vivo experiments in mice demonstrated that miR-146a–mediated suppression in allergic skin inflammation partially occurs through direct targeting of upstream nuclear factor kappa B (NF-κB) signal transducers caspase recruitment domain-containing protein 10 and IL-1 receptor–associated kinase 1. In addition, human CCL5 was determined as a novel, direct target of miR-146a. Conclusion: Our data demonstrate that miR-146a controls nuclear factor kappa B–dependent inflammatory responses in keratinocytes and chronic skin inflammation in AD. (J Allergy Clin Immunol 2014;134:836–47.)

Key words: Allergy, noncoding RNA, atopic eczema, gene therapy

Atopic dermatitis (AD) is a common chronic inflammatory skin disease that affects 10% to 20% of children and persists throughout adulthood in 1% to 3% of the cases. Recurrent exacerbations, activation of the immune system, expansion of T(H)2 cells, increased IgE serum levels, production of thymic stromal lymphopoietin (TSLP) by keratinocytes in the early phase, and hyperplasia and keratinocyte apoptosis in the chronic phase represent cardinal features of AD. Skin inflammation during an exacerbation can persist for several weeks and is nearly exclusively complicated with secondary infections, including Staphylococcus aureus (S. aureus), that contribute to the activation of innate immune responses and the nuclear factor kappa B (NF-κB) pathway in keratinocytes.

Recent studies describe novel mediators of NF-κB activation, such as caspase recruitment domain-containing protein 10 (CARD10); also known as CARD recruited membrane associated protein 3) and ubiquitin D (UBD, also known as ubiquitin-like protein FAT10). CARD10 is activated via G protein–coupled receptors and forms a complex with mucosa-associated lymphoid...
tissue lymphoma translocation protein 1, which mediates the degradation of inhibitor of κB kinase, and thereby activates NF-κB. UBD plays a role in TNF-α-dependent NF-κB activation by targeting the inhibitor of κB kinase for degradation in a ubiquitin-independent mode.

The chronic phase of skin inflammation in AD is affected by IL-22, mainly produced by TH22 cells, and TH1-type cytokine IFN-γ. Although IL-22 has been associated with tissue regeneration and hyperplasia, IFN-γ is the main factor responsible for the apoptosis of keratinocytes and intensifies the inflammation through the upregulation of numerous proinflammatory genes in the skin. Two characteristic chemokines that are induced by IFN-γ in keratinocytes and are upregulated in the skin of patients with AD are CCL5 (also known as RANTES) and CCL8 (also known as MCP-2). CCL5 has been shown to attract T cells, macrophages, and eosinophils. CCL5 promoter polymorphisms and enhanced levels have been shown to be associated with AD. CCL8 mediates the recruitment of IL-5–enriched TH2 cells in the inflamed skin in a mouse model of chronic AD.

Regulation of inflammatory responses in diseases is mediated by coordinated control of gene expression, which is also modulated by microRNAs (miRNAs). miRNAs silence genes by triggering the degradation of target miRNAs and inhibition of translation. Several miRNAs, including miR-146a, function in the regulation of various inflammatory processes. miR-146a–deficient mice spontaneously develop autoimmunity later in life because of aberrant activation of NF-κB in T cells and signal transducer and activator of transcription 1 (STAT1) in regulatory T cells. In lung alveolar epithelial cells, miRNA-146a negatively regulates the release of IL-8 and CCL5 in an IL-1β signaling-independent manner. The function of miR-146a in the normal skin and keratinocytes and in AD has not been previously explored.

Here, we demonstrate that the level of miR-146a is increased in keratinocytes from patients with AD and during skin inflammation in AD and that this elevated expression, in turn, controls chronic inflammatory processes that are triggered by IFN-γ and activation of NF-κB in keratinocytes.

Abbreviations used
- AD: Atopic dermatitis
- CARD10: Caspase recruitment domain-containing protein 10
- IRAK1: IL-1 receptor–associated kinase 1
- LN: Lymph node
- miRNA: microRNA
- NF-κB: Nuclear factor kappa B
- siRNA: Small-interfering RNA
- STAT: Signal transducer and activator of transcription
- TSLP: Thymic stromal lymphopoietin
- TLR: Toll-like receptor
- UBD: Ubiquitin D
- 3’UTR: 3’ untranslated region
- WT: Wild type

METHODS

Patients

The study was approved by the Ethical Review Committees on Human Research of the University of Tartu and the University of Szeged. All participants signed a written informed consent form. Detailed description of
FIG 2. miR-146a inhibits the expression of NF-κB–dependent genes in primary keratinocytes (KCs). A-F, KCs were transfected either with control (cont) or pre-miR-146a (miR-146a) for 24 hours and then stimulated with IFN-γ or TNF-α for 48 hours or left unstimulated (us). A and D, Heatmaps of the predicted direct targets (A) and miR-146a–affected genes from the selected signaling pathways (D). Color scale was determined as in
Gene expression analysis and tissue culture experiments

For functional studies, pooled, normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) were used. miRNA and mRNA profiling was performed using Illumina miRNA Universal-16 and HumanHT-12 Expression BeadChips. The array data are available at ArrayExpress as E-MTAB-1740 and E-MTAB-1739. The cytokines and chemokines in keratinocyte cell culture supernatants were measured using the Human Cytokine 27-plex assay according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, Calif). In Western blots, the mean intensities of each protein level were normalized to glyceraldehyde 3-phosphate dehydrogenase. One representative blot of 3 for each studied protein is shown. For detailed description of total RNA isolation, cDNA synthesis, RT-quantitative PCR, array and pathway analysis, transfections, stimulations, Western blot
analysis, and luciferase assays, see the Methods section in this article’s Online Repository.

Mouse experiments

Animal experiments were approved by the Animal Ethics Committee of the University of Tartu. The generation of miR-146a−/− mice in the C57BL/6J background was described earlier.36 For the MC903-dependent mouse model of AD, histopathology, immunohistochemistry, and laser capture microdissection, see the Methods section in this article’s Online Repository.

Statistics

Statistical analysis was performed using the unpaired Student t test. The results were considered significant at P < .05 (*) or #) and highly significant at P < .01 (** or #). Data are presented as scatter blots, with line indicating the mean ± SEM, bar blots indicating the mean ± SEM, or box blots with whiskers showing minimum and maximum.

RESULTS

miR-146a expression is elevated in keratinocytes and skin from patients with AD

To study the role of miRNAs in tissue responses in AD, we performed miRNA expression profiling of cultured primary keratinocytes from healthy individuals and patients with AD. Twelve miRNAs were differentially expressed in keratinocytes from patients with AD (Fig 1, A; see Table E1 in this article’s Online Repository at www.jacionline.org). In this miRNA set, miR-146a was the second most strongly upregulated miRNA after miR-10b, however, with 10-fold higher expression level than that of miR-10b (Table E1). Because miR-146a had been shown to have immune regulatory functions in other cell types, we focused on miR-146a and confirmed its increased expression in nonlesional and chronic lesional skin of patients with AD (Fig 1, B).

To identify AD-related factors and proinflammatory cytokines that affect miR-146a expression, we stimulated normal human primary keratinocytes with different cytokines, heat-killed *S aureus*, or Toll-like receptor (TLR) ligands. miR-146a was strongly upregulated by IL-1β, TNF-α, and IL-17A, with a synergistic effect between the 3 cytokines. IFN-γ did not induce miR146a expression, suppressed the effect of TNF-α, and did not affect the activation by IL-1β and IL-17A (Fig 1, C). Heat-killed *S aureus*, IL-13, TLR2, and TLR4 ligands slightly upregulated and IL-4 and IL-22 did not affect miR-146a expression (Fig 1, D).

Overexpression of miR-146a inhibits NF-κB–dependent genes in human primary keratinocytes

To study miR-146a function in keratinocytes, we overexpressed miR-146a in unstimulated and IFN-γ– or TNF-α–stimulated keratinocytes and performed mRNA array analysis. TNF-α was used as a strong stimulant of miR-146a (Fig 1, C) and IFN-γ as a hallmark cytokine of chronic skin inflammation in AD known to cause global changes in gene expression in keratinocytes.18
transfection of miR-146a resulted in the downregulation of 102, 37, and 410 genes in unstimulated, TNF-α–stimulated, and IFN-γ–stimulated keratinocytes, respectively (see Fig E1, A-C, in this article’s Online Repository at www.jacionline.org). According to Targetscan 6.2,38,39 21 predicted targets were suppressed by miR-146a in both IFN-γ–stimulated and unstimulated conditions, including the previously identified miR-146a direct targets IRAK132 and CARD1034 (Fig 2, A). Suppression of CARD10, IRAK1, CCL5, and IL-8 was confirmed by RT-quantitative PCR (Fig 2, B). Reduced amounts of CCL5 and CXCL10 in IFN-γ–stimulated cells were detected in supernatants of keratinocytes on overexpression of miR-146a (Fig 2, C). Because miR-146a downregulated a large number of genes in IFN-γ–stimulated cells, we next performed pathway analysis, which revealed that genes from several immune system–related pathways, including NF-κB signaling, chemokine signaling (Fig 2, D; see Table E2 in this article’s Online Repository at www.jacionline.org), and cytokine–cytokine receptor interaction pathways (Table E2) were enriched among the downregulated genes. The effect of miR-146a overexpression was similar in TNF-α–stimulated keratinocytes (Fig 2, D). There was no significant overlap between the set of genes affected by miR-146a and genes from the janus kinase-STAT pathway, which is a primary pathway activated by IFN-γ40 (Table E2). Consistent with the pathway analysis, reduced levels of phosphorylated and active NF-κB subunit p65 and total p65 were detected in miR-146a–transfected cells in IFN-γ–stimulated and unstimulated cells. The NF-κB domain p50 was expressed at a higher level in IFN-γ–stimulated cells, and overexpression of miR-146a slightly reduced its levels. The expression of the STAT1 protein was slightly enhanced in response to IFN-γ, and miR-146a decreased its levels by

![FIG 5. Inhibition of miR-146a leads to the upregulation of downstream proinflammatory proteins. A-D, Keratinocytes were transfected with control (cont) LNA or miR-146a inhibitor (LNA-146a) for 24 hours and then stimulated with TNF-α or IL-1β for 48 hours or left unstimulated (us). B, The mean intensities of protein levels are compared with those of us cont. A and C, Relative mRNA expression. D, Quantification of secreted chemokines and cytokines (n = 6). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LNA, locked nucleic acid.](image-url)
approximately 20%. Phosphorylated, active STAT1 appeared only in response to IFN-\(\gamma\) and was not significantly different in the presence of miR-146a (Fig 2, E). Protein levels of the direct targets CARD10 and IRAK1 were suppressed by miR-146a in both conditions (Fig 2, F).

**Proinflammatory genes that are suppressed by miR-146a are dysregulated in AD**

We then investigated whether genes suppressed by miR-146a are differentially expressed in the skin of patients with AD. Comparison of the set of genes that are dysregulated in the skin of patients with AD (82 genes)\(^1\) with the list of genes that are suppressed by miR-146a in IFN-\(\gamma\)-stimulated keratinocytes (410 genes) revealed 7 overlapping genes (Fig 3, A). Interestingly, 3 AD-related genes, CCL5, CCL8, and UBD, were induced more than 10-fold by IFN-\(\gamma\) and suppressed by more than 50% by miR-146a (Fig 3, B). The other tested proinflammatory cytokines and TLR ligands had only a moderate effect on the expression of CCL5, CCL8, and UBD in human primary keratinocytes (Fig 3, C). CCL8 and UBD were strongly suppressed by miR-146a (66.1% and 73.4%, respectively) in IFN-\(\gamma\)-stimulated keratinocytes (Fig 3, D), as was shown for CCL5 (Fig 2, B). Further analysis using Targetscan\(^3\) detected that of these 3 genes, the 3’ untranslated region (3’UTR) of the human CCL5 mRNA contains a potential, nonconserved binding site for miR-146a (Table E3 and Fig 3, E). To study whether this predicted binding site is functional, we inserted the corresponding 3’UTRs of human CCL5, the previously confirmed direct targets IRAK1\(^3\) and CARD10,\(^3\) and the CCL5 3’UTR containing a mutation in the miR-146a binding site (Fig 3, E) into the luciferase reporter vector and performed luciferase assays. As presented in Fig 3, F, the transfection of miR-146a resulted in suppression of the luciferase activity of the reporters containing regions of the 3’UTRs of human IRAK1, CARD10, and CCL5 but not the construct containing the mutated CCL5 3’UTR (Fig 3, F), confirming that human CCL5 is a novel, direct target for miR-146a.

**CARD10 is important for the expression of miR146a-suppressed proinflammatory factors in keratinocytes**

Because the miR-146a-regulated genes CARD10 and IRAK1 are upstream elements in the NF-\(\kappa\)B pathway, we used a small-interfering RNA (siRNA)-silencing approach to test whether they are important for the expression of downstream genes suppressed
by miR-146a. IRAK1 and CARD10 were efficiently inhibited by specific siRNAs (see Fig E2 in this article’s Online Repository at www.jacionline.org). Silencing of CARD10 resulted in strong downregulation of the CCL5 mRNA (71.0%) and protein (58.1%) in IFN-γ-stimulated keratinocytes. Similar to CCL5, the expression of UBD mRNA was downregulated by siRNAs against CARD10 (71.4%), but not by siRNA against IRAK1. The expression of the IFN-γ-independent chemokine, IL-8, was synergistically suppressed by siRNAs against IRAK1 and CARD10 in unstimulated and IFN-γ-stimulated keratinocytes (Fig 4). The siRNAs did not significantly affect the CCL8 mRNA (data not shown) and IL-6 protein (Fig 4, B) levels.

**Inhibition of miR-146a results in the upregulation of proinflammatory factors in keratinocytes**

To study whether endogenous levels of miR-146a are sufficient for its suppressor function in keratinocytes, we used locked
nucleic acid–based miRNA inhibitors and stimulations with TNF-α and IL-1β. The transfection of the miR-146a inhibitor was efficient (see Fig E3 in this article’s Online Repository at www.jacionline.org) and resulted in upregulation or a tendency for upregulation of the direct targets IRAK1 and CARD10 at the mRNA and protein levels (Fig 5, A and B). The mRNA and protein levels of CCL5 and IL-8 showed a significant increase, except for the CCL5 protein in the presence of IL-1β, in all conditions. The levels of secreted IL-6 and CXCL10 were increased on miR-146a inhibition in all conditions (Fig 5, C and D). CCL8 and UBD were not significantly induced by TNF-α and IL-1β (data not shown).

miR-146a, IRAK1, and CARD10 are expressed in the epidermis of the mouse skin

We next analyzed the expression of miR-146a and its targets in the skin of wild-type (WT) and miR-146a−/− mice. The expression of miR-146a was detected in the epidermis, areas around hair follicles, and connective tissue in the dermis using laser capture microdissection (Fig 6, A). Slightly higher expression of miR-146a was observed in the dermis than in the epidermis, when the epidermis was separated using dispase treatment (Fig 6, B). The miR-146a direct targets CARD10 and IRAK1 had significantly higher expression in the epidermis and slightly elevated protein levels in miR-146a−/− mice than in WT mice. No difference was observed in the mRNA expression levels of CARD10, IRAK1, and the downstream indirect targets CCL5, UBD, and CXCL2 (mouse homologue of IL-8) in the epidermis of WT mice compared with that of miR-146a−/− mice (Fig 6, C and D). The expression of CCL8 mRNA was not detectable (data not shown).

miR-146a controls AD-like skin inflammation in mice

To investigate the role of miR-146a during skin inflammation, we used MC903-dependent mouse model of AD that involves repeated topical treatments of mouse ears with the vitamin D3 analog MC903, which leads to increased TSLP production and strong Th2-type inflammation in the skin. Histologic analysis showed that on application of MC903, WT and miR-146a−/− mice developed severe skin inflammation with heavy infiltrations in the dermis, strong hyperplasia, and thickening of the epidermis (Fig 7, A). The epidermal thickness of WT and miR-146a−/− deficient mice was enhanced to a similar extent (Fig 7, B). Increased numbers of skin-infiltrating cells were present in MC903-treated, miR-146a−/− deficient mice than in WT mice (Fig 7, C). The expression of miR-146a itself was enhanced in the skin of MC903-treated WT mice, and this correlated with the downregulation of the mRNA and protein levels of miR-146a direct targets CARD10 and IRAK1. In the resting condition, a slight upregulation of the CARD10 and IRAK1 protein levels was observed in miR-146a−/− mice than in WT mice without a significant difference in the mRNA levels (Fig 7, D and E). Consistent with the results in human keratinocytes, the mRNA levels of the IFN-γ and AD-related genes CCL5, CCL8, and UBD were enhanced in the skin of MC903-treated miR-146a−/− mice compared with WT mice (Fig 7, E and F). The Th2-type cytokines TSLP and IL-4 were equally strongly induced by MC903 in WT and miR-146a−/− mice (see Fig E4, A and B, in this article’s Online Repository at www.jacionline.org). Of the other tested cytokines and chemokines, eosinophil-chemoattractant CCL24 (also known as Eotaxin-2) was significantly elevated in MC903-treated miR-146a−/− mice. IL-6, IL-1β, and CXCL2 were elevated in the skin of MC903-treated mice, but no difference between WT and 146a−/− deficient mice was observed (Fig E4, C). The mRNAs of IL-22 and IL-17A were not detectable in all conditions. The number of CD3-expressing T cells was enhanced in response to MC903 in both WT and miR-146a−/− mice (Fig E4, D). In line with the results of the skin, increased levels of IFN-γ, IL-1β, and UBD were detected in draining lymph nodes (LNs) of miR-146a−/− deficient mice compared with WT mice on MC903 treatment. Enhanced expression levels of CCL5, CCL8, and IL-4 were detected in draining LNs in both WT and miR-146a−/− deficient mice (Fig 8).

DISCUSSION

Information regarding the role of miRNAs in allergic diseases is limited. The present study demonstrates an essential immune
regulatory function of miR-146a in the skin. miR-146a is upregulated in the keratinocytes and skin of patients with AD and inhibits the expression of numerous proinflammatory factors, including IFN-γ-inducible and AD-associated genes CCL5, CCL8, and UBD in human primary keratinocytes and in vivo in mouse model of AD. Both siRNA silencing and in vivo mouse experiments suggest that miR-146a-mediated regulation occurs partially through direct targeting of IRAK1, CARD10, and CCL5 in keratinocytes. Our data together show that the expression of miR-146a is increased during skin inflammation in AD and this, in turn, helps to control chronic inflammatory processes triggered by IFN-γ and activation of NF-κB in keratinocytes (see Fig E5 in this article’s Online Repository at www.jacionline.org).

Chronic skin inflammation in AD is affected by numerous factors that besides T helper 2-type cytokines include secondary infections and activation of keratinocytes by IL-22 and IFN-γ.1–7,17,18,20,45 Remarkably, IFN-γ induces expression changes of thousands of genes in keratinocytes, while only 39 genes are affected by IL-22.45 To study the function of miR-146a in keratinocytes, we used stimulations with IFN-γ and TNF-α. miR-146a downregulated 410 genes in IFN-γ-stimulated keratinocytes, pointing to a strong anti-inflammatory function of miR-146a in chronic AD. Only 37 genes were inhibited in the presence of TNF-α, although a similar set of genes tended to be suppressed. It is possible that the increased endogenous level of miR-146a is sufficient for the suppression in TNF-α-stimulated keratinocytes.

Keratinocytes express numerous pattern-recognition receptors, the stimulation of which leads to the activation of the NF-κB pathway. The enhanced expression of NF-κB–dependent miR-146a in keratinocytes and nonlesional skin of patients with AD could be due to epigenetic changes in the miR-146a promoter or could represent a different activation state of keratinocytes of patients with AD. miR-146a is also upregulated in the skin of patients with psoriasis,46 which indicates that elevated expression of miR-146a is a general characteristic of inflammatory responses in the skin. Enhancement of miR-146a expression by NF-κB appears to have a feedback loop effect, because miR-146a targets several modulators of the NF-κB pathway, including IRAK1, TRAF6, RELB, and CARD10, in different cell types.32,34 In the present study, we observed suppression of CARD10 and IRAK1 by miR-146a, whereas silencing of CARD10, but not of IRAK1, led to robust downregulation of the AD-related genes CCL5 and UBD. Interestingly, human CCL5 seems to be particularly well controlled by miR-146a. First, human CCL5 is a direct target of miR-146a, as we demonstrated using luciferase assays, and second, it is suppressed as soon as miR-146a targets CARD10. The targeting of CARD10 and IRAK1 seems to be crucial, because other miR-146a–affected miRNAs, such as UBD, CCL8, and IL-8, do not contain potential binding sites for miR-146a. As we demonstrated using locked nucleic acid inhibitors, endogenous levels of miR-146a were sufficient for its function in keratinocytes.

Previously, it was shown that autoimmunity in miR-146a−/− mice is partially caused by dysregulated IFN-γ production due to the overactivation of STAT1 in regulatory T cells, which renders them incapable of suppressing T<sub>reg</sub>1-type responses.30 In addition, miR-146a has been shown to suppress STAT1 in HEK293 cells.47 Although we observed slight downregulation of the total STAT1 protein level by miR-146a in IFN-γ-stimulated keratinocytes, there was no difference in the phosphorylated STAT1 level or significant influences on Janus kinase–STAT pathway genes.

IFN-γ plays an important role in the chronic phase of allergic inflammation.16,18,44 Although fewer IFN-γ–producing CD4+ cells are present in AD than in psoriatic skin, the number of IFN-γ–producing CD8+ cytotoxic T cells is similar in these 2 diseases.38,44 Because CCL5 and CCL8, which attract T cells, macrophages, and eosinophils,22,26 are strongly induced by IFN-γ in keratinocytes, they could provide a link into how the activation of keratinocytes by IFN-γ contributes to the development of chronic inflammation. Interestingly, our data suggest that CARD10 and UBD are novel factors that link the IFN-γ and NF-κB pathways. CARD10 is activated by angiotensin II and lysophosphatidic acid via G protein–coupled receptors,11,50,51 and UBD has been shown to be important for TNF-α–dependent NF-κB activation in renal tubular epithelial cells and mouse lymphocytes.52 No effect of IFN-γ on UBD and CARD10 has been described.

Although miR-146a was expressed both in the epidermis and in the dermis, its direct targets IRAK1 and CARD10 were expressed higher in the epidermis, suggesting an important function for them in the regulation of the inflammatory responses in keratinocytes. Previously, it was reported that the protein, but not mRNA, expression of the miR-146a direct targets IRAK1 and TRAF6 was elevated in bone marrow–derived macrophages and B cells in miR-146a−/− mice.48 Similarly, we observed slightly higher protein levels of IRAK1 and CARD10 in miR-146a−/− mice in resting conditions; however, there was no difference in their mRNA expression levels. These and previously published results indicate that the effect of miR-146a is not always evident on the mRNA level.

To study how miR-146a affects AD-related inflammation in vivo, we used MC903-dependent mouse model of AD in which ablation of miRNA-processing factor Dicer-1 resulted in the aggravation of AD.53 We observed increased mRNA expression of IFN-γ, CCL5, CCL8, and UBD in the skin of MC903-treated miR-146a−/− mice and reduced mRNA and protein levels of miR-146a direct targets IRAK1 and CARD10 in MC903-treated WT mice. In addition, IFN-γ and IL-1β were upregulated in draining LNs. These results demonstrate that miR-146a has antiinflammatory function in the skin, which involves targeting of the epidermal NF-κB pathway–activating elements CARD10 and IRAK1 and suppression of the downstream IFN-γ–inducible, AD-related genes CCL5, CCL8, and UBD. In line with gene expression changes, we observed an increased number of skin-infiltrating cells in MC903-treated, miR-146a–deficient mice. Anti-CD3 staining of frozen skin sections from MC903-treated mice demonstrated increased accumulation of CD3 positive cells, particularly in the area of the dermis and epidermis connection both in WT and in miR-146a−/− mice, suggesting that most of the infiltrating cells were other than T cells. This finding opens a window for future studies to further characterize the types of cells and their localization in tissues affected by miR-146a.

In mammals, the miR-146 family consists of 2 members, miR-146a and miR-146b, which are encoded by 2 independent genes located on chromosomes 5 and 10, respectively. Mature miR-146a and miR-146b differ from each other in 2 nucleotides, and they most likely target a similar set of genes because of the same seed sequence.52,54 Although miR-146a function has been intensively studied in immune cells and in relation to many diseases, the function and regulation of miR-146b is less described. Recent studies
note the importance of miR-146b in different cell types. 55,56 In monocytes, miR-146b is upregulated via an IL-10–mediated STAT3-dependent loop on LPS stimulation and directly targets TLR4. 55 We did not observe a difference in miR-146b expression in keratinocytes from patients with AD. Future studies are needed to explore functions of miR-146b in the skin.

We observed increased expression of the eosinophil chemoattractant CCL24 in the skin; however, there was no difference in TSLP and IL-4 expressions in the skin and LNs. It is possible that miR-146a suppresses T2-type immune responses on antigen-specific stimulation because miR-146a has been shown to downregulate T-cell receptor–driven NF-kB activation. 37 A limited number of studies have been published on miRNA functions in inflammatory skin diseases. miR-155 is overexpressed in the skin of patients with AD and apparently affects the development of AD by downregulating cytotaxic T lymphocyte–associated antigen CTLA-4. 48 miR-125b is enhanced in psoriatic skin and functions as a suppressor of keratinocyte proliferation via direct targeting of fibroblast growth factor receptor 2. 47 In the present study, we demonstrated an important role of miR-146a in the suppression of innate immune responses in keratinocytes during chronic skin inflammation in AD. Our results suggest that overexpression of miR-146a may have potential for the treatment of inflammatory skin diseases.

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Clinical implications: Our results demonstrate that overexpression of miR-146a may be a potential treatment of chronic inflammatory conditions in the skin.

REFERENCES

METHODS
Patients, human primary keratinocytes, and skin biopsies

This study was approved by the Ethical Review Committees on Human Research of the University of Tartu and the University of Szeged. All participants signed a written informed consent form. Primary keratinocytes (all passage 4) from the nonlesional skin of 3 patients with AD and 3 healthy participants signed a written informed consent form. Primary keratinocytes were isolated from the nonlesional skin of 3 patients with AD and 3 healthy subjects using the method described earlier.11,12 In Fig 1, B, skin biopsies (diameter, 4 mm) from 9 patients with chronic AD (5 women, 4 men, age, 18-42 years) and 9 healthy subjects (4 men, 5 women, age 19-43 years) were used. All patients had experienced 6- to 14-day-long severe exacerbation of the disease. None of the patients had been treated with systemic antihistamines or topical corticosteroids for at least 1 week before inclusion in the study.

Transfections and stimulations

For functional studies, pooled, normal human epidermal keratinocytes (Promocell) were used. Keratinocytes were stimulated for 48 hours with the following concentrations of cytokines: TNF-α, 25 ng/mL; IL-1β, 10 ng/mL; IL-4, 40 ng/mL; IFN-γ, 10 ng/mL; IL-17A, 10 ng/mL; IL-22, 10 ng/mL; IL-13, 50 ng/mL (R&D Systems, Minneapolis, Minn); LPS, 10 ng/mL; Pam3CSK4, 2.5 ng/mL; and heat killed S. aureus, 106 cells/mL (Invivogen, San Diego, Calif). Transfections were carried out in 24-well plates using 0.8 μL of siPORT NeoFX (Life Technologies, Grand Island, NY) and 5 × 104 keratinocytes in 0.6 mL growth medium, which was for keratinocytes and for HEK293 cells Dulbecco modified Eagle medium (both from Invitrogen, Grand Island, NY). After 24 hours, keratinocytes were stimulated with cytokines as indicated for 48 hours. Transfections in keratinocytes (all passage 4) from the nonlesional skin of 3 patients with AD and 3 healthy participants signed a written informed consent form. Primary keratinocytes were isolated from the nonlesional skin of 3 patients with AD and 3 healthy subjects using the method described earlier.11,12

Isolation of RNA, cDNA synthesis, and RT-quantitative PCR

Total RNA was extracted using miRNAeasy Mini Kit (Qiagen, Valencia, Calif). Homogenization of the human and mouse skin, epidermis, and dermis samples and mouse LNs was done by using Precellys 24 (Precellys, Southampton, United Kingdom) homogenizer or gentleMACS diceriovator (Miltenyi Biotec, Heidelberg, Germany). cDNA was synthesized from 200 to 900 ng of total RNA using oligo-dT and reagents from Thermo Scientific. RNA concentration and quality were assessed with NanoDrop ND-1000 and Agilent 2100 Bioanalyzer. SYBR/Rox master mix (Bio-Rad Laboratories or Solis BioDyne, Tartu, Estonia) and ABI Prism 7900 were used for quantitative PCR (qPCR). The relative gene expression levels were normalized to mouse or human EF1A levels and calculated using the comparative Ct (ΔΔCt) method (Life Technologies). The mean level of control experiments or control group was equalized to 1. miRNA qPCR was carried out by using TaqMan microRNA Assays (Life Technologies) and were normalized to Let-7a. All PCR primers were designed with the assistance of Primer 3 and were ordered from Microsynth (Balgach, Switzerland).

RT-qPCR primers

All primers were designed with the assistance of Primer 3 and were ordered from Microsynth.

Array and pathway analysis

miRNA and mRNA profiling was carried out using Illumina miRNA Universal-16 BeadChips (probes for 858 human mature miRNAs based on miRBase 12.013 and Illumina HumanHT-12 Expression BeadChips, respectively). miRNA and mRNA Illumina arrays were carried out at the Core Facility at the Department of Biotechnology, University of Tartu, using 500 ng of total RNA per each sample. The data were analyzed with GenoStudio 2011.1 Gene Expression Module using average normalization for miRNA data and Illumina’s custom rank invariant method for mRNA arrays. Genes were considered to be expressed at detection P < .05 and differentially expressed at P < .05. Detection P value is a parameter that estimates whether a particular gene is expressed with comparing signal intensities of the genes with the controls and noise of the same array. Further analyses and visualizations were performed using Microsoft Excel and Multi Experiment Viewer 4.6.2. Pathway analysis was performed with gProfiler (http://biit.cs.ut.ee/gprofiler), which retrieves the most significant gene ontology groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and enables one to estimate the significance of the search results by calculating enrichment P value using Fisher 1-tailed test. E4

Gene Forward Reverse
CARD10 AGTGTGCCCAAGCGGAAGGCC GATGGCCCGGAGGCTGCACTGCTG
CCL8 AGGACCTTCGCTAGCAAGTTAGCT GCTTGTAGCTCCAGCAGCTG
CCL5 AGGTCTGCTTGTACCGGAA TCCATGTCCAGTCATCTCCCA
EFIAeX7-8 CAATCTTGGAAGTTGACGGG TTCCGACCTAGCATCTCCCA
IRA1 CACCTTGAGCCTTCTTTCAGG CCAGCTTCCTCAGCTCTCCT
IL8 GCAGCTCTGTGAGTGTTCATTT TCCTGTTGTGGCGCAGTTG
hUBD CAGAGATGGCTCCCAATGTC GCGTGTATAGGTGTCG
mCARD10 GCGAGGTCTACCCCATTTGC CAACAGGCCCCTGATCTCCAC
mCCl24 TCCAAAGGCGGTCTCATCTCAA TTGCCTGAAACCACCAAGCCAC
mCCL5 GCCTCACCATATGCTCCGACAC TTGAGCTGGGCCAGCAGGCA
mCCL8 GGACAGAAAGCTCCACGAC TCAAGCCACCAAGGGGATCT
mCXCL2 TGAACCAAGGCAAAGCTCTC CAGTAGCATACGAGCCTCC
mEFIA1 CGCGTGCTCTGGAGGCTTT CAGGGTTTTTACCAACACCGTGT
miFNg TGCGCAATTTGAGTCAACACCCA ACAGCCTGGTGAGCCTG
miL1b AGCTTCTTTGTGGAGTT GAGGTTCTGCTGCACTCCTAA
miL4 CAGGAGAAGGGCAGGTCGCA CAGGAGAAGGGCAGGTCGCA
miL6 ACTCTCACAAGTGCACTG GAGGTTCTGCTGCACTCCTAA
miRACK1 TGTGAGGACACAAGGTGCA TAAAGCTGGTGCTGCTG
mTSLP ATCGAGGACTGTGAGAGCAAGCCAG TCTTGGTCTGCTG
mUBD TTCTGGCAGCTTCTGTTG TCTTGGTCTGCTG
mLot TCCCTGGTGCCTTCAGGGTG ACGCCTGGTGCTG

RT-qPCR primers

All primers were designed with the assistance of Primer 3 and were ordered from Microsynth.
Western blot analysis
Tissue samples were homogenized using gentleMACS in Luciferase cell lysis buffer and then boiled in 1× Laemmli buffer for either 5 minutes (epidermis and dermis samples) or 60 minutes (formaldehyde cross-linked frozen samples) at 100°C before the loading to the gel. A total of 5 µg of total protein was loaded in each lane of SDS PAGE gel. Antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 and 5% milk or 2.5% BSA (antimouse CCL5). Primary antibodies were used at the following dilutions: mouse monoclonal anti-IRAK1 (clone 3F7, ab119289, Abcam, Cambridge, United Kingdom) 1:1000, mouse monoclonal anti-human glyceraldehyde 3-phosphate dehydrogenase (clone 6C5) 1:5000, goat polyclonal antihuman CARD10 (Sc-32233) 1:1000, goat polyclonal anti-mouse CCL5 (sc-1410, clone C-19) 1:1000, rabbit polyclonal anti-mouse IRAK-1 (sc-7883, H-273) 1:1000 (all from Santa Cruz Biotech, Santa Cruz, Calif); rabbit mAbs recognizing human STAT1 (clone 42H3) and phosho-STAT1 (Ty701, clone 58D6) (both from Cell Signaling Technology, Danvers, Mass) 1:1000, mouse monoclonal anti-human NFKB1 (clone 5D10, Thermo Scientific) 1:1000, and antiphospho-NF-kB p65 (Ser68) (Cell Signaling) 1:1000. Anti-rabbit, -goat and -mouse IgG horseradish peroxidase–conjugated antibodies from Cell Signaling Technology were used as secondary antibodies. Signals were detected with the enhanced chemiluminescence plus substrate (Thermo Scientific, Waltham, Mass) and captured with LAS-1000 (FUJIFILM Europe GmbH, Duesseldorf, Germany) or ImageQuant LAS gel (GE Healthcare, Fairfield, Conn) systems. Western blots were quantified using ImageJ software. The mean intensity of the background value was subtracted from the mean intensity of each protein band followed by normalization against the mean values of glyceraldehyde 3-phosphate dehydrogenase.

Luciferase assay
3′UTR fragments of CCL5, CARD10, and IRAK1 were cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, Wis) using the PCR primers containing NheI and SalI sites. The following primers were used: CARD10 3′UTR FOR 5′-ATTGC TAGCGGCTCTCACCACCGTGCT-3′; CARD10 3′UTR REV 5′-ATTGTCGAACAGCGTGTCAG-3′; IRAK1 3′UTR FOR 5′-ATTGCTAAGCT GTGTTCACCTGGGCAGA TTC-3′; IRAK1 3′UTR REV 5′-ATTGTGCACCTCCAAATGCGCCAGCACC-3′; CCL5 3′UTR FOR for 5′-ATTGTACGCA GTAATCCGA CTAACGGCTAAAGGAGGCTT-3′; CCL5 3′UTR REV 5′-ATTGTACGCACT TG ATG AG ACC TAT AAT G-3′. For the generation of CCL5 3′-UTR, IRAK1 3′-UTR, and TNF-α 3′-UTR, primers were used. Transfections were carried out in 24-well plates using 0.8 µL of siPORT NeoFX (Life Technologies), 30 nM of pre-miRNAs (Pre-miR Precursors Molecules hsa-miR-146a or negative control #1, Life Technologies), 50 ng of the reporter plasmid, and 5 × 10⁴ human embryonic kidney epithelial cells HEK293 in 0.6 mL medium for 24 hours. Firefly and renilla luciferase activities were measured using Promega dual luciferase assay, and firefly luciferase activities were normalized to the values of the renilla luciferase.

Mouse AD model
miR-146a⁻/⁻ mice in C57BL/6J background and C57BL/6J (B6) WT mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Generation of miR-146a⁻/⁻ mice has been described earlier.19 The mice were maintained and bred in the animal facility at the Institute of Molecular and Cell Biology, University of Tartu, in accordance with the institute’s regulations. Nine- to 10-week-old female mice were used for the experiments. Animal experiments were approved by the Animal Ethics Committee of the University of Tartu. MC903 (calcipotriol hydrate, C4369, Sigma-Aldrich, St Louis, Mo) was dissolved in EtOH and topically applied on mouse ears (1 nmol per ear) on every other day 10 times starting at day 0. As vehicle control, the same amount of EtOH was applied. Analyses of the ear biopsies and LNs were performed on day 19.

Laser capture microdissection
Pieces of the skin (5 × 5 mm) from trimmed mouse back were embedded in Tissue-Tek (Thermo Scientific) and quick-frozen using the mixture of ethanol and dry ice. For laser capture microdissection, 10-µm tissue sections were cut, fixed in methanol for 10 minutes, stained with hematoxylin, and stored at −20°C. Laser capture microdissection was performed with Zeiss PALM MicroBeam. RNA from microdissected tissue was purified using miRNAeasy Mini Kit (Qiagen). A total of 0.8 µg of bacteriophage MS2 RNA (Roche Applied Science Switzerland) was added as carrier RNA per each sample.

Separation of the epidermis and the dermis
For separation of the epidermis from the dermis, 1 × 1 cm pieces of the skin from trimmed mouse back were incubated for 15 to 18 hours at 4°C in Keratinocyte-SFM Medium (Invitrogen) in the presence of 5 mg/mL of Dispase II (Life Technologies).

Histopathology and immunohistochemistry
Ear biopsies were fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Five-micrometer sections were stained with hematoxylin/eosin. For immunohistochemistry, heat-induced epitope retrieval method using Ph 9.0 Target Retrieval Solution (Dako Denmark) was applied. DAKO REAL peroxidase blocking was used for 7 to 15 minutes to block endogenous peroxidase. For detection, rabbit polyclonal anti-mouse TSLP (ab115700, Abcam) at 1:200 dilution, EnVision-labeled polymer containing horseradish peroxidase–conjugated antibodies for rabbit IgG antibodies, and DAKO DAB chromogen were used. Zeiss Axiocor 405 M microscope and Axiosview Rel. 4.8 software were used to capture images and to perform measurements. For epidermal thickness, the distance between the epidermal-dermal junction and the epidermal-stratum corneum junction was measured from 80 different randomly selected places for each group. The infiltrating cells were counted from 25 random dermal fields per each group with an area of 2000 to 8000 µm².

Immunofluorescence
Immunofluorescence was performed essentially as described before. Cryosections (10 µm) were fixed for 7 minutes with 4% formaldehyde in PBS followed by the permeabilization/blocking step in 10% donkey serum, 1% BSA, and 0.2% Triton X100 in PBS, pH 7.4, for 30 minutes. Specimens were further incubated for 45 minutes with rat anti-mouse CD3 (clone 17A2, BD Bioscience) with dilution 1:2000. Donkey anti-goat IgG Alexa Fluor546 (data not shown) was used as secondary antibody. Before and after antibody incubations, specimens were washed for 3 × 5 minutes with 0.05% Tween in PBS. Slides were mounted with VectaShield mounting medium containing 4′,6-diamidino-2-phenylindol, dihydrochloride (Vector Laboratories, Burlingame, Calif) and analyzed with a Leica Fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

RESULTS
Overexpression of miR-146a inhibits proinflammatory factors in IFN-γ- and TNF-α-stimulated human primary keratinocytes
As demonstrated previously,16,17 1767 genes were upregulated and 1021 downregulated in IFN-γ-treated cells. Only 233 genes were determined to be upregulated and 61 genes downregulated in TNF-α–stimulated keratinocytes (data not shown). In unstimulated keratinocytes, the transfection of miR-146a resulted in the downregulation of 102 genes and the upregulation of 33 genes; and in IFN-γ–stimulated cells, 410 genes were downregulated and 175 were upregulated by miR-146a (see Fig E1, A). Only 27 genes were downregulated and 12 genes were upregulated in response to miR-146a overexpression in TNF-α–stimulated keratinocytes, which showed higher levels of endogenous miR-146a (data not shown). According to Targetscan 6.2 prediction and the array analysis, miR-146a had 1019 direct targets expressed
In keratinocytes (Fig E1, C). In unstimulated conditions, 39.2% of the downregulated genes (40 of 102) and 18% of the genes in IFN-γ-stimulated keratinocytes (74 of 410) were predicted to be direct targets of miR-146a, suggesting that miR-146a indirectly targets many genes in IFN-γ-stimulated cells (Fig E1, C). Although the downregulating effect of miR-146a was statistically significant only in the case of 37 genes in TNF-α-stimulated keratinocytes, there was a tendency for the suppression of the same set of genes that were suppressed in IFN-γ-stimulated keratinocytes.

In conclusion, these data together demonstrate that miR-146a suppresses the expression of numerous proinflammatory factors, including NF-κB signal transducers and direct miR-146a targets IRAK1 and CARD10 in unstimulated, TNF-α–, and IFN-γ–stimulated keratinocytes.

REFERENCES
miR-146a inhibits the expression of proinflammatory factors in TNF-α- and IFN-γ-stimulated keratinocytes. **A-C**, Keratinocytes were transfected either with control (cont) or with pre-miR-146a (miR-146a) for 24 hours and then stimulated with IFN-γ or TNF-α for 48 hours or left unstimulated (us). **B**, Scatter plots are shown in log10 scale. Black dots designate differentially expressed genes, red lines indicate a fold difference of 2.0, and $r^2$ is the correlation coefficient. **C**, The Venn diagram represents the overlap of predicted miR-146a direct targets expressed in keratinocytes and miR-146a–suppressed genes. Relative expression levels of miR-146a are shown in log10 scale.
FIG E2. Silencing of CARD10 and IRAK1 in human primary keratinocytes. Keratinocytes were transfected either with control (cont) siRNA or with siRNAs silencing IRAK1 or CARD10 or both for 24 hours and then stimulated with IFN-γ for 48 hours or left unstimulated (us). A, Data represent mean ± SEM. Statistical analysis was performed relative to cont. B, Western blot analysis of CARD10 and IRAK1 in primary keratinocytes transfected with indicated siRNAs. Mean intensity of each protein level was normalized to the GAPDH and to cont-transfected and us cells. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
FIG E3. LNA-based inhibition of miR-146a in primary keratinocytes. Keratinocytes were transfected either with control (cont) LNA inhibitor or with miR-146a inhibitor (LNA-146a) for 24 hours and then stimulated with TNF-α or IL-1β for 48 hours or left unstimulated (us). LNA, Locked nucleic acid.
FIG E4. The expression of proinflammatory cytokines and chemokines in the skin. WT and miR-146a−/− (146a−/−) mice were topically treated with ethanol (control [cont]) or MC903 10 times on every other day. Skin samples were collected 24 hours after the last treatment. A, Immunohistochemical staining of TSLP on ear sections (bar = 100 μm). B, Relative mRNA expression of T\textsubscript{H}2-type and proinflammatory cytokines and chemokines. C, Immunofluorescence staining of CD3 on ear sections (bar = 100 μm). DAPI, 4′,6-Diamidino-2-phenylindole, dihydrochloride.
The function of miR-146a in keratinocytes and AD. During chronic skin inflammation in AD, the expression of miR-146a is increased in response to NF-κB activation in keratinocytes. This elevated expression of miR-146a, in turn, contributes to the suppression of proinflammatory factors, including CCL5, UBD, and CCL8, which can be activated by the stimulation of IFN-γ and to a less extent by other AD-related factors. The suppression of IL-8 by miR-146a occurs through the targeting of NF-κB signaling transducers IRAK1 or CARD10, and the repression of UBD and CCL5 occurs through the targeting of CARD10. In human cells, CCL5 is a direct target of miR-146a. Suppression of IRAK1 and CARD10 does not affect the repression of CCL8 by miR-146a, which indicates that miR-146a has more direct targets in keratinocytes. It is not known how IFN-γ activates CARD10 and NF-κB in keratinocytes. The increased AD-related expression of endogenous miR-146a in keratinocytes helps to balance the chronic inflammation in the skin.
<table>
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<th>ILMN_GENE</th>
<th>KC H average signal</th>
<th>KC H array SD</th>
<th>KC AD average signal</th>
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FD, Fold difference; KC, keratinocytes.

*Average expression signals of miRNAs with differential $P < .05$ (differential score $>13$ or $<-13$) and with FD $>1.8$ or $<0.55$. The average signals and array SD values are calculated on the basis of results of 3 different donors from both healthy individuals (H) and patients with AD.
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<th>$P$ value</th>
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<th>No. of overlapped genes</th>
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*The $P$ value from Fisher exact test showing the significance of the overlap between the target list and the indicated functional group.
TABLE E3. Predicted miR-146a binding sites according to Targetscan 6.2\cite{40}

| Target gene | Conserved sites | | Poorly conserved sites | | | Total context score |
|-------------|----------------|----------------|-----------------------|----------------|------------------------|
|             | Total | 8mer | 7mer-m8 | 7mer-1A | Total | 8mer | 7mer-m8 | 7mer-1A | | |
| IRAK1       | 2     | 2    | 0       | 0       | 0     | 0    | 0       | 0       | 0     | 0        | 0        | 0        | -0.70    |
| CARD10      | 1     | 1    | 0       | 0       | 0     | 0    | 0       | 0       | 0     | 0        | 0        | 0        | -0.26    |
| CCL5        | 0     | 0    | 0       | 0       | 1     | 1    | 0       | 0       | 1     | 1        | 0        | 0        | -0.40    |

*Conserved = conserved across most mammals, but usually not beyond placental.
†The context score for a specific site is the sum of the contribution of 6 features (site type, 3’ pairing, local AU, position, target-site abundance, and seed-pairing stability contributions) and is calculated as in Li et al.\cite{41}