Cross-talk between IFN-γ and TWEAK through miR-149 amplifies skin inflammation in psoriasis

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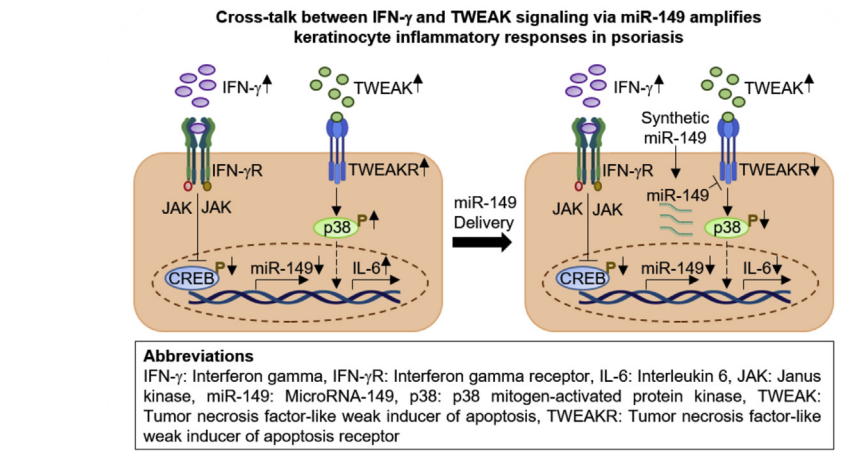
GRAPHICAL ABSTRACT



Background: Psoriasis is a chronic inflammatory skin disease with disturbed interplay between immune cells and keratinocytes. A strong IFN-γ signature is characteristic for psoriasis skin, but the role of IFN-γ has been elusive. MicroRNAs are short RNAs regulating gene expression.
Objective: Our aim was to investigate the role of miR-149 in psoriasis and in the inflammatory responses of keratinocytes.
Methods: miR-149 expression was measured by quantitative RT-PCR in keratinocytes isolated from healthy skin and lesional and nonlesional psoriasis skin. Synthetic miR-149 was injected intradermally into the back skin of mice, and imiquimod was applied to induce psoriasis-like skin inflammation, which was then evaluated at the morphologic, histologic, and molecular levels. miR-149 was transiently overexpressed or inhibited in keratinocytes in combination with IFN-γ- and/or TNF-related weak inducer of apoptosis (TWEAK)-treatment.
Results: Here we report a microRNA-mediated mechanism by which IFN-γ primes keratinocytes to inflammatory stimuli. Treatment with IFN-γ results in a rapid and long-lasting suppression of miR-149 in keratinocytes. Depletion of miR-149 in keratinocytes leads to widespread transcriptomic changes and induction of inflammatory mediators with enrichment of the TWEAK pathway. We show that IFN-γ–mediated suppression of miR-149 leads to amplified inflammatory responses to TWEAK. TWEAK receptor (TWEAKR/Fn14) is identified as a novel direct target of miR-149. The *in vivo* relevance of this pathway is supported by decreased miR-149 expression in psoriasis keratinocytes, as well as by the protective

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effect of synthetic miR-149 in the imiquimod-induced mouse model of psoriasis.
Conclusion: Our data define a new mechanism, in which IFN-γ primes keratinocytes for TWEAK-induced inflammatory responses through suppression of miR-149, promoting skin inflammation. (J Allergy Clin Immunol 2021;147:2225-35.)

Key words: *Psoriasis, skin inflammation, keratinocytes, microRNAs, cytokines, IFN-γ, IFN-γ–mediated priming, TWEAK/TWEAKR pathway*

Psoriasis is a chronic inflammatory skin disease with no available cure and a significant negative effect on patients' quality of life.[1] Skin inflammation in psoriasis is thought to be initiated and maintained by the disturbed communication between infiltrating immune cells and keratinocytes, in which inflammatory cytokines play an important role.[2-4] Today, some of the most effective therapies to treat psoriasis are based on inhibition of key cytokines such as IL-17A, IL-23, and TNF-α.[5] IFN-γ is a proinflammatory cytokine that is mainly produced by T_H1 and T_H17 cells, with high levels in psoriasis skin and in serum of patients with psoriasis.[2,6-10] Despite the presence of a strong IFN-γ-signature in psoriasis skin,[11-14] the potential contribution of this cytokine to psoriatic skin inflammation remains elusive. One key function of IFN-γ is the augmentation of cellular responses to other cytokines or triggers, a process termed *IFN-γ-priming*[15-17]; however, whether such a mechanism is active in keratinocytes and whether it contributes to chronic skin inflammation has remained unclear.

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression at the posttranscriptional level.[18] MiRNAs are potent regulators of inflammatory pathways and often target components of signal transduction pathways, thereby modulating the sensitivity of the target cells to an inflammatory stimulus.[19] Previously, we and others identified miRNAs that regulate cellular processes relevant to psoriasis, and modulation of miRNAs has been shown to affect skin inflammation in preclinical models.[20-27]

In this study, we present experimental evidence that IFN-γ potentiates keratinocyte responses to the inflammatory cytokine TWEAK through suppression of miR-149-5p (hereafter referred to as miR-149). This is mediated by derepression of the TWEAK receptor (TWEAKR [also known as TNFRSF12A and as Fn14]), which we identify as a direct target for miR-149. The *in vivo* relevance of this mechanism is supported by strong downregulation of miR-149 concomitant with induction of TWEAKR in keratinocytes from patients with psoriasis, as well as by suppression of IMQ-induced psoriasis-like skin inflammation in mice following local delivery of synthetic miR-149.

Abbreviations used
CREB-1: cAMP responsive element binding protein 1
IMQ: Imiquimod
JAK: Janus kinase
MAPK: Mitogen-activated protein kinase
miRNA: MicroRNA
qRT-PCR: Quantitative RT-PCR
TWEAK: TNF-related weak inducer of apoptosis
TWEAKR: TNF-related weak inducer of apoptosis receptor
3′UTR: 3′ Untranslated region

METHODS

Human samples

After informed consent had been obtained, 4-mm punch biopsy samples were taken from the nonlesional and lesional skin of patients with plaque psoriasis (n = 20) and from the skin of healthy individuals (n = 19). The study was approved by the regional ethics committee. All procedures concerning human subjects were performed according to the principles of the Declaration of Helsinki.

Cell isolation

Epidermal sheets were separated from whole skin punch biopsy samples collected from patients with psoriasis or from healthy controls by using overnight dispase (5 U/mL) (Thermo Fisher Scientific, Stockholm, Sweden) dissociation at 4°C). $CD45^{neg}$ epidermal cells were sorted as described previously[11,28] and stored in Qiazol (Qiagen, Stockholm, Sweden) at 80°C until RNA isolation.

Mice

Female C57BL/6J mice were obtained from Charles River Laboratories, Écully, France. Synthetic miR-149 or scramble oligonucleotides (5-7 μg [mirVana miRNA Isolation kit, Thermo Fisher Scientific]) packed in *in vivo* transfecting agent (Max Suppressor-*In Vivo* RNA-LANCEr II, Bioo Scientific, Austin, Tex) were injected intradermally into the shaved back skin of the mice. Imiquimod cream was applied on the back skin, and clinical scores and skin thickness were assessed as described.[23] The animal experiments were approved by the local ethics committee (Swedish Board of Agriculture).

Cell culture and treatments

Normal human primary keratinocytes were obtained from Thermo Fisher Scientific and maintained in EpiLife medium (Thermo Fisher Scientific) supplemented with human keratinocyte growth supplements (Thermo Fisher Scientific) and penicillin and streptomycin (Thermo Fisher Scientific) under 5% CO_2 at 37C. Primary keratinocytes were treated with human recombinant IFN-γ (20 ng/mL), IL-1β (10 ng/mL), IL-17A (100 ng/mL), IL-22 (20 ng/mL), TNF-α (50 ng/mL), or IL-36α (10 ng/mL) (R&D Systems, Abingdon, UK) for 1, 3, 6, 24, 48, 72, and 96 hours. Reconstituted human epidermis (3-dimensional epidermal equivalent) cultures were obtained from Mattek, Ashland, Mass, and maintained as recommended by the manufacturer's protocol. Human recombinant IFN-γ/IL-17A/IL-22/TNF-α and IL-1β (20 ng/mL) (R&D Systems) were added to the reconstituted human epidermis cultures for 72 hours.

For cell culture and treatments, RNA isolation and qRT-PCR, *in situ* hybridization, histology and immunohistochemistry, Western blotting and ELISA, 3′untranslated region (3′UTR) luciferase reporter assay, microarray, bioinformatics, and statistical analysis, see the Methods section of the Online Repository (available at www.jacionline.org).

RESULTS

miR-149 is suppressed in keratinocytes of psoriasis skin lesions, and its local delivery alleviates skin inflammation

Recently, we identified a set of miRNAs with altered expression in keratinocytes in psoriasis skin lesions.[28] One of the downregulated miRNAs was miR-149, which is a miRNA that was not previously associated with psoriasis.[28] qRT-PCR analysis on a larger cohort confirmed a significant decrease in miR-149 levels in keratinocytes from psoriasis skin lesions as compared with

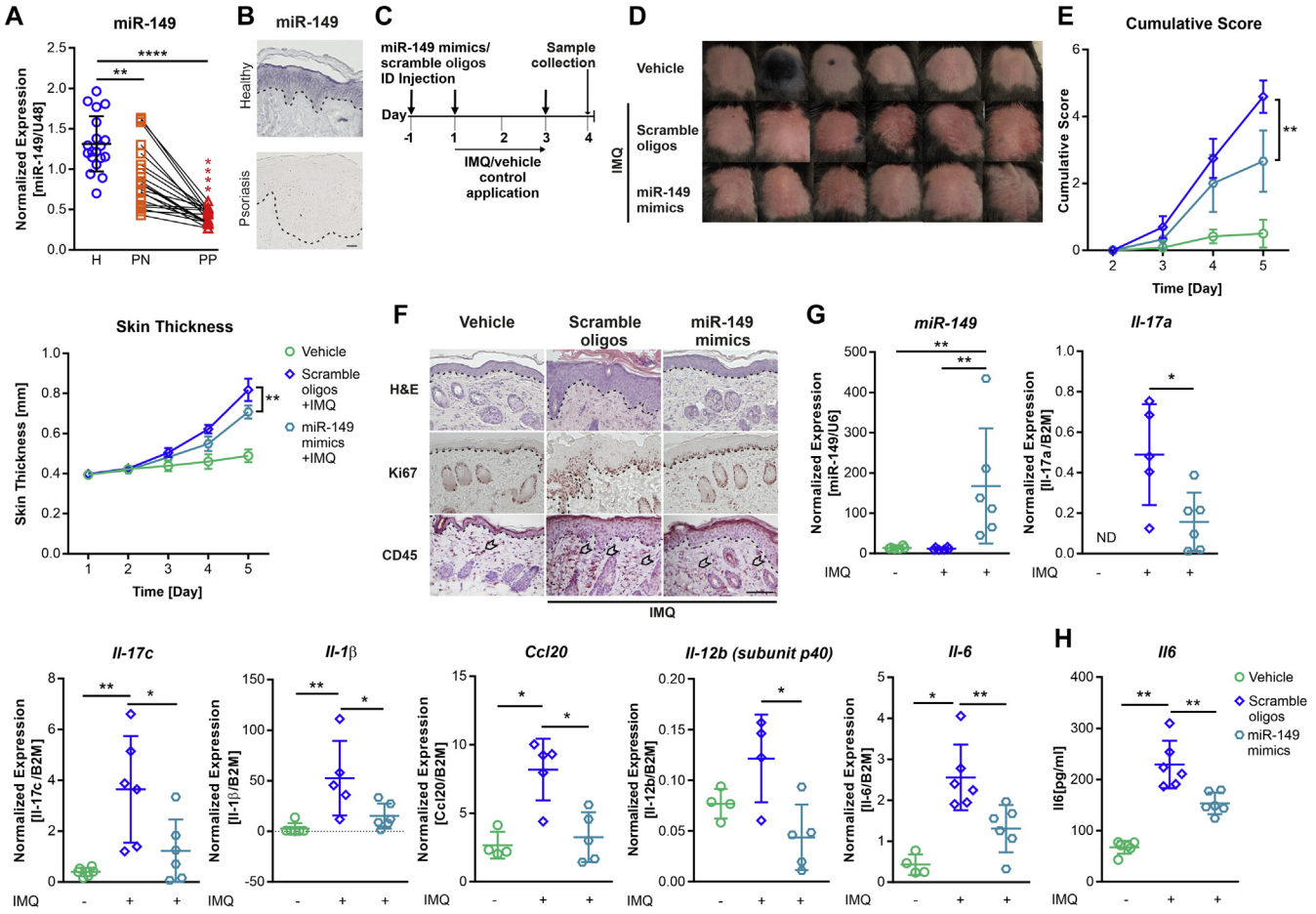

FIG 1. miR-149 is downregulated in psoriatic keratinocytes, and local delivery of miR-149 mimics alleviates psoriasis-associated inflammation. **A,** Total RNA was isolated from keratinocytes sorted from lesional psoriasis (PP) and nonlesional psoriasis (PN) skin (n = 20), as well as from healthy skin (H) (n = 19), and miR-149 expression was measured by qRT-PCR. ****$P < .0001$; **$P < .01$; Mann-Whitney *U* test; Wilcoxon matched pairs signed rank test. **B,** *In situ* hybridization was performed for miR-149 on paraffin-embedded skin sections obtained from healthy donors and lesional skin of patients with psoriasis. Scale bar = 50 μm. **C,** Time line showing intradermal (ID) injection of miR-149 mimics or scramble oligonucleotide (oligos) and topical application of IMQ or vehicle. **D** and **E,** Macroscopic images, cumulative score, and skin thickness for mice either treated with vehicle control or injected with scramble oligos or miR-149 mimics and topically treated with IMQ. **$P < .01$; 2-way ANOVA. **F,** Representative images of hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) for Ki67 and CD45 in mouse skin. Arrows indicate $CD45^+$ cells. Scale bar = 100 μm. **G,** qRT-PCR analysis for miR-149 and key psoriasis-associated inflammatory mediators. **$P < .01$; *$P < .05$; Mann-Whitney *U* test. **H,** Il-6 protein level in skin tissue lysates was measured by ELISA. **$P < .01$; Mann-Whitney *U* test.

the levels in keratinocytes from healthy or nonlesional psoriasis skin (Fig 1, *A*). *In situ* hybridization showed that miR-149 is mainly expressed by keratinocytes in healthy skin and that its expression is decreased in psoriatic epidermis (Fig 1, *B*).

We hypothesized that loss of miR-149 in psoriasis may contribute to skin inflammation. To test this, we investigated whether its modulation affects skin inflammation in the imiquimod (IMQ)-induced mouse model of psoriasis. Synthetic miR-149 or scramble oligonucleotides were injected intradermally into the back skin of mice, 1 day before, on day 1, and on day 3 during topical IMQ application on back skin followed by skin sample collection on day 4 (Fig 1, *C*). Strikingly, local delivery of synthetic miR-149 mimicked attenuated clinical signs of skin inflammation such as erythema, skin thickness, and scaling (Fig 1, *D* and *E*). Histologic analysis demonstrated that delivery of synthetic miR-149 led to significantly reduced epidermal thickening in IMQ-treated mice (Fig 1, *F* and see Fig E1, *A* in this article's Online Repository at www.jacionline.org), as well as to reduced keratinocyte proliferation, as measured by immunohistochemical staining for Ki67 (Fig 1, *F* and see Fig E1, *B*). Immunohistochemical staining for CD45, a surface marker for immune cells, showed that miR-149 significantly reduced the dermal infiltration of immune cells induced by IMQ (Fig 1, *F*). IMQ application significantly decreased the expression of miR-149-5p (see Fig E2, *A* in this article's Online Repository at www.jacionline.org), whereas intradermal delivery of miR-149 mimics robustly increased the expression of miR-149-5p but not expression of miR-149-3p (Fig 1, *G* and see Fig E2, *B*). Moreover, miR-149 significantly suppressed the induction of the

psoriasis-associated cytokines/chemokines *Il-17a*, *Il-17c, Il-1β, Ccl20, Il-12b* (p40 subunit), and *Il-6* in IMQ-treated mouse skin (Fig 1, *G*). Consistent with these results, ELISA analysis demonstrated that IMQ-induced secretion of Il-6 was significantly suppressed by miR-149 (Fig 1, *H*). Altogether, these results demonstrated that miR-149 is a potent suppressor of skin inflammation and suggested that its decreased levels in psoriasis keratinocytes may contribute to chronic skin inflammation.

IFN-γ transcriptionally suppresses miR-149 in keratinocytes via Janus kinase (JAK) and cAMP responsive element binding protein (CREB-1)

Next, we sought to explore the mechanisms underlying decreased miR-149 expression in psoriatic epidermis. To investigate whether its decreased expression is due to the inflammatory cytokine milieu in psoriasis, primary human keratinocytes were treated with IFN-γ, IL-1β, IL-17A, IL-22, TNF-α, and IL-36α or a combination of IL-17A plus IL-22 plus TNF-α, and the expression of miR-149 was analyzed. qRT-PCR analysis was used to examine the known downstream targets of these cytokines that were used as positive controls (Fig 2, *B* and see Fig E3 in this article's Online Repository at www.jacionline.org). Of all the tested cytokines, only IFN-γ regulated miR-149 expression (Fig 2, *A* and *B*). Strikingly, a single treatment with IFN-γ led to significant long-lasting downregulation of miR-149, which remained suppressed even 96 hours after treatment (Fig 2, *B*). Interestingly, the decreased level of miR-149 at 24 hours after IFN-γ treatment coincided with the maximal expression of IL-6 (Fig 2, *B*), suggesting that both miR-149 and IL-6 may be regulated by shared signaling pathways.

To further investigate the regulation of miR-149 in a more *in vivo*–like setting, we analyzed the effects of IFN-γ, IL-17A, IL-22, TNF-α, and IL-1β on miR-149 in 3-dimensional epidermal equivalents. Treatment with these cytokines led to characteristic histologic changes (Fig 2, *C* and see Fig E4, *A* in this article's Online Repository at www.jacionline.org) and induction of known downstream mediators (Fig E4, *B*). Consistent with the results obtained with monolayer cultures, miR-149 was regulated exclusively by IFN-γ (Fig 2, *D*), and its suppression coincided with the induction of the IL-6 (Fig 2, *E*). Altogether, these results identified miR-149 as an IFN-γ target gene and suggested that an elevated IFN-γ level in psoriasis skin may contribute to a decreased level of miR-149 in psoriasis keratinocytes.

Next, we aimed to identify the mechanism of IFN-γ–mediated suppression of miR-149. To investigate whether miR-149 is regulated by IFN-γ at the transcriptional level, we first analyzed the expression of the primary transcript of miR-149 (pri-miR-149) in IFN-γ–treated primary keratinocytes. qRT-PCR analysis showed significant downregulation of the primary miR-149 transcript as early as 1 hour after IFN-γ treatment, indicating that suppression of miR-149 by IFN-γ is a direct, near-immediate transcriptional effect (Fig 3, *A*). Inhibition of transcription with actinomycin D resulted in 50% decrease of the primary transcript of miR-149 by 1 hour (Fig 3, *B*), similar to the effect of IFN-γ, which also reduced pri-MIR149 by 50% 1 hour after treatment, supporting the idea that IFN-γ directly regulates miR-149 expression (Fig 3, *B*).

To test whether IFN-γ–mediated suppression of miR-149 is mediated through the canonical IFN-γ pathway involving JAKs, the JAK inhibitors ruxolitinib (inhibitor of JAK1 and JAK2) and tofacitinib (inhibitor of JAK1, JAK3, and JAK2) were used to treat keratinocytes in combination with IFN-γ. As expected, both ruxolitinib and tofacitinib almost completely blocked phosphorylation of STAT1 at TYR701 and SER727 (Fig 3, *C*), and they inhibited IFN-γ–induced expression of IL-6 (Fig 3, *D* and *E*), confirming the succesful inhibition of the IFN-γ pathway. IFN-γ–mediated suppression of both pri-MIR149 and mature miR-149 was rescued by both JAK inhibitors (Fig 3, *D* and *E*), indicating that IFN-γ suppresses miR-149 through JAKs. Interestingly, ruxolitinib and tofacitinib induced miR-149 expression even in the absence of IFN-γ (Fig 3, *D* and *E*).

To further explore the regulation of miR-149, we interrogated publicly available Encode ChIP data, which revealed multiple binding sites for cAMP responsive element binding protein 1 (CREB-1) upstream of the transcription start site of miR-149 (see Fig E5 in this article's Online Repository at www.jacionline.org). Because IFN-γ has been shown to suppress CREB-1 phosphorylation in primary human macrophages,[29] we hypothesized that this mechanism may be involved in the regulation of miR-149 by IFN-γ. Immunoblotting for phosphorylaetd CREB-1 showed a time-dependent decrease in phosphorylation at the SER133 site of CREB-1 after IFN-γ treatment in keratinocytes (Fig 3, *F*). To investigate the potential involvement of CREB in the regulation of miR-149, keratinocytes were treated with KG-501, an inhibitor of CREB-1 phosphorylation[30] (Fig 3, *G*). Inhibition of CREB-1 phosphorylation by KG-501 alone suppressed the primary and mature transcripts of miR-149 (Fig 3, *H*). In combination with IFN-γ treatment, pretreatment with KG-501 further suppressed the primary as well as the mature transcript of miR-149 (Fig 3, *H*). Conversely, expression of IL-6 was enhanced by suppression of CREB-1 phosphorylation (Fig 3, *H*). Inhibition of CREB-1 by small interfering RNAs (see Fig E6, *A* in this article's Online Repository at www.jacionline.org) led to decreased expression of miR-149 (see Fig E6, *B*), further confirming the involvement of CREB-1 in regulation of miR-149. Collectively, our results indicate that IFN-γ transcriptionally controls miR-149 at least in part through suppression of CREB-1 phosphorylation.

Depletion of miR-149 induces inflammatory responses in keratinocytes

To determine the effects of miR-149 on the gene expression landscape in keratinocytes, we performed transcriptomic analysis after inhibition of miR-149, without any cytokine treatment. A total of 1164 genes were identified to be differentially expressed in miR-149–depleted keratinocytes, of which 446 were upregulated and 718 were downregulated (Fig 4, *A*). Of note, several genes with well-established roles in inflammatory responses and psoriasis (eg, IL-6, CCL5, TNF, JUN, CASP3, MMP9, IL1A, IL1B, IL24, TGFB1, CCL20, TRAF1, and TRAF6) were upregulated in miR-149–depleted keratinocytes (Fig 4, *B*). Pathway enrichment analysis of the upregulated genes revealed an enrichment for terms related to the TNF-related weak inducer of apoptosis (TWEAK) signaling pathway, the TNF signaling pathway, cytokines and inflammatory response, positive regulation of JAK-STAT cascade, cytokine-cytokine receptor interaction, and response to INF-γ (Fig 4, *C* and see Fig E7 in this article's Online Repository at www.jacionline.org). Together with the observation that miR-149 suppressed psoriasis-like skin

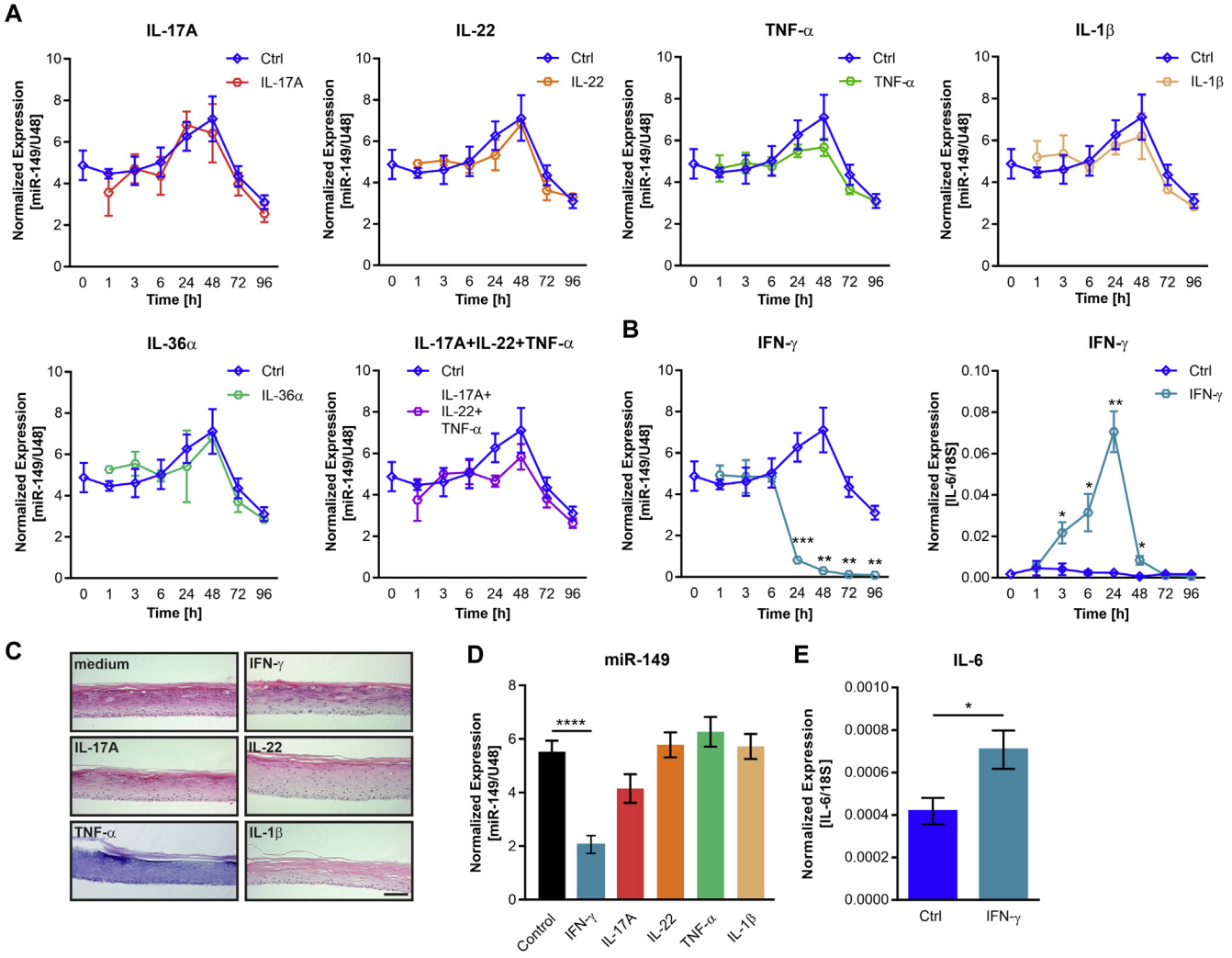

FIG 2. IFN-γ suppresses miR-149-expression in keratinocytes. **A,** Human primary keratinocytes were treated with IFN-γ, IL-1β, IL-17A, IL-22, TNF-α, and IL-36α or a combination of IL-17A plus IL-22 plus TNF-α for the indicated times (0-96 hours), and expression of mature miR-149 was detected by qRT-PCR. **B,** Expression analysis of miR-149 and IL-6 was performed with qRT-PCR after IFN-γ treatment. ****$P < .0001$; ***$P < .001$; **$P < .01$; *$P < .05$; Student *t* test. **C,** Human epidermal equivalents were treated with IFN-γ, IL-17A, IL-22, TNF-α, and IL-1β for 72 hours. Hematoxylin and eosin staining for control and cytokine-treated 3-dimensional epidermal equivalents is shown. Scale bar = 50 μm. Total RNA was isolated after 72 hours of cytokine treatment. **D** and **E,** Expression analysis for miR-149 and IL-6 was performed by qRT-PCR. ****$P < .0001$; *$P < .05$; Student *t* test.

inflammation in mice, these data suggested that miR-149 may act as a brake on the inflammatory responses of keratinocytes.

miR-149 suppresses TWEAK signaling by targeting TWEAKR

The TWEAK signaling pathway was among the most enriched pathway of upregulated genes in miR-149–depleted keratinocytes. TWEAK is an inflammatory cytokine that belongs to the TNF superfamily, which has been shown to be involved in the pathogenesis of psoriasis.[31-33] Analysis of publicly available HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation) data for miR-149[34] revealed direct interaction of miR-149 with the specific receptor for TWEAK, namely, TWEAKR. Immunoblotting showed a robust increase in TWEAKR protein expression in miR-149–depleted keratinocytes, indicating regulation of TWEAKR by miR-149 in keratinocytes (Fig 5, *A*). Next, we performed 3′UTR luciferase assay to experimentally validate direct targeting of TWEAKR by miR-149 in keratinocytes. Synthetic miR-149 suppressed luciferase activity in keratinocytes cotransfected with plasmids containing wild-type 3′UTR of TWEAKR (Fig 5, *B* and see Fig E8 in this article's Online Repository at www.jacionline.org), whereas no suppression was observed in keratinocytes containing plasmids with a mutation in the miR-149 binding site in the 3′UTR of TWEAKR (Fig 5, *B* and see Fig E8), indicating that TWEAKR is a direct target of miR-149 in keratinocytes.

Supporting the *in vivo* relevance of miR-149–mediated targeting of TWEAKR, qRT-PCR showed significantly increased levels

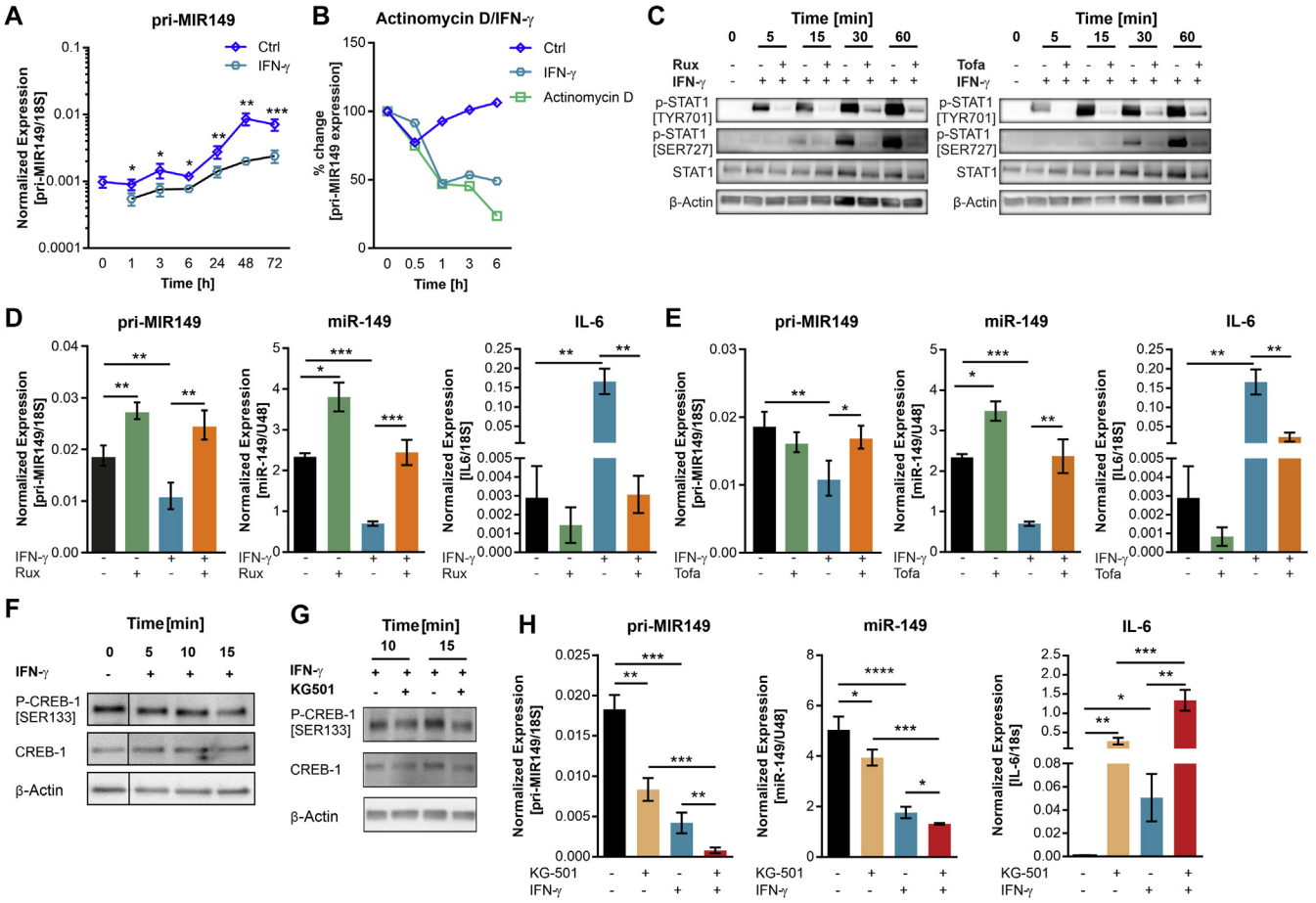

FIG 3. IFN-γ suppresses miR-149 expression via the JAK/STAT/CREB-1 pathway. **A,** Keratinocytes were treated with IFN-γ for the indicated times, and the expression of primary transcript of miR-149 was analyzed with qRT-PCR. ***$P < .001$; **$P < .01$; *$P < .05$; Student *t* test. **B,** The half-life of primary transcript of miR-149 was measured by treating keratinocytes with actinomycin D (an inhibitor of transcription) or IFN-γ. **C,** Keratinocytes were pretreated with JAK inhibitors— ruxolitinib (0.3 μM) or tofacitinib (0.6 μM)—for 1 hour followed by IFN-γ (20 ng/mL) treatment for Western blot and qRT-PCR. Phosphorylation of STAT1 TYR701 and STAT1 SER727 was measured by Western blot. **D** and **E,** Expression of primary transcript of miR-149 (pri-MIR149), mature miR-149, and IL-6 was measured by qRT-PCR. ***$P < .001$; **$P < .01$; *$P < .05$; Student *t* test. Human primary keratinocytes were pretreated with CREB inhibitor (KG-501; 0.1 μM) alone or in combination with IFN-γ. **F** and **G,** Western blot analysis was performed for phosphorylation of CREB-1 at the SER133 site. **H,** Expression analysis of primary and mature transcript of miR-149 was performed along with IL-6 by qRT-PCR. Graphs are representative of 3 independent experiments. ***$P < .01$; **$P < .01$; *$P < .05$; Student *t* test.

of TWEAKR in keratinocytes from psoriasis lesions compared with in keratinocytes from nonlesional skin or from healthy donors (Fig 5, *C*), as opposed to the decreased expression of miR-149 (Fig 1, *A*). In line with this, expression of *Tweakr* was also induced in the IMQ-induced murine skin inflammation model, as judged by qRT-PCR and Western blotting (Fig 5, *D* and *E*). Strikingly, injection of synthetic miR-149 mimics prevented induction of *Tweakr* in the skin after IMQ application, at both the mRNA and protein levels (Fig 5, *D* and *E*), further supporting suppression of *Tweakr* by miR-149 *in vivo*.

Next, we assessed whether suppression of TWEAKR by miR-149 leads to reduced activity of the TWEAK signaling pathway. Because p38 mitogen-activated protein kinase (MAPK) is one of the effector downstream pathways of TWEAK/TWEAKR,[35] we first investigated the effect of TWEAK on phosphorylation of p38 and IL-6 expression. TWEAK treatment induced the phosphorylation of p38 in a time-dependent manner in keratinocytes (Fig 5, *F*) along with induction of IL-6 expression (Fig 5, *F*). The selective p38 MAPK inhibitor SB203580 robustly suppressed both basal and TWEAK-mediated p38 phosphorylation as well as IL-6 expression (Fig 5, *F*). Inhibition of miR-149 led to increased TWEAK-induced phosphorylation of p38 (Fig 5, *G*) whereas its overexpression suppressed the TWEAK-induced phosphorylation of p38 in keratinocytes (see Fig E9 in this article's Online Repository at www.jacionline.org). To investigate whether this mechanism is relevant *in vivo*, the effect of miR-149 delivery on p38 phosphorylation was analyzed in IMQ-treated mouse skin. Western blot analysis showed that phosphorylation of p38 in skin was induced after IMQ application (Fig 5, *H*), which is consistent with increased p38 phosphorylation in human psoriasis lesions.[36-38] In line with the results obtained with human keratinocytes, delivery of synthetic miR-149 suppressed IMQ-induced phosphorylation of p38 in mouse skin (Fig 5, *H*).

FIG 4. Depletion of miR-149 results in inflammatory signaling in keratinocytes. Transcriptome analysis was performed on miR-149–depleted or control-transfected normal human primary keratinocytes. **A,** Heatmap showing the 50 most upregulated and downregulated genes after miR-149 inhibition. **B,** Fold change (mRNA probe fluorescence in microarray) of mRNA expression of inflammatory mediators after miR-149 inhibition in human primary keratinocytes. ***$P < .001$; **$P < .01$; Student *t* test. **C,** Pathway terms enriched among upregulated genes in miR-149–depleted keratinocytes (Wiki pathway terms are shown in the graph). Adjusted *P* value ≤ .001; term overlap ≥ 20%.

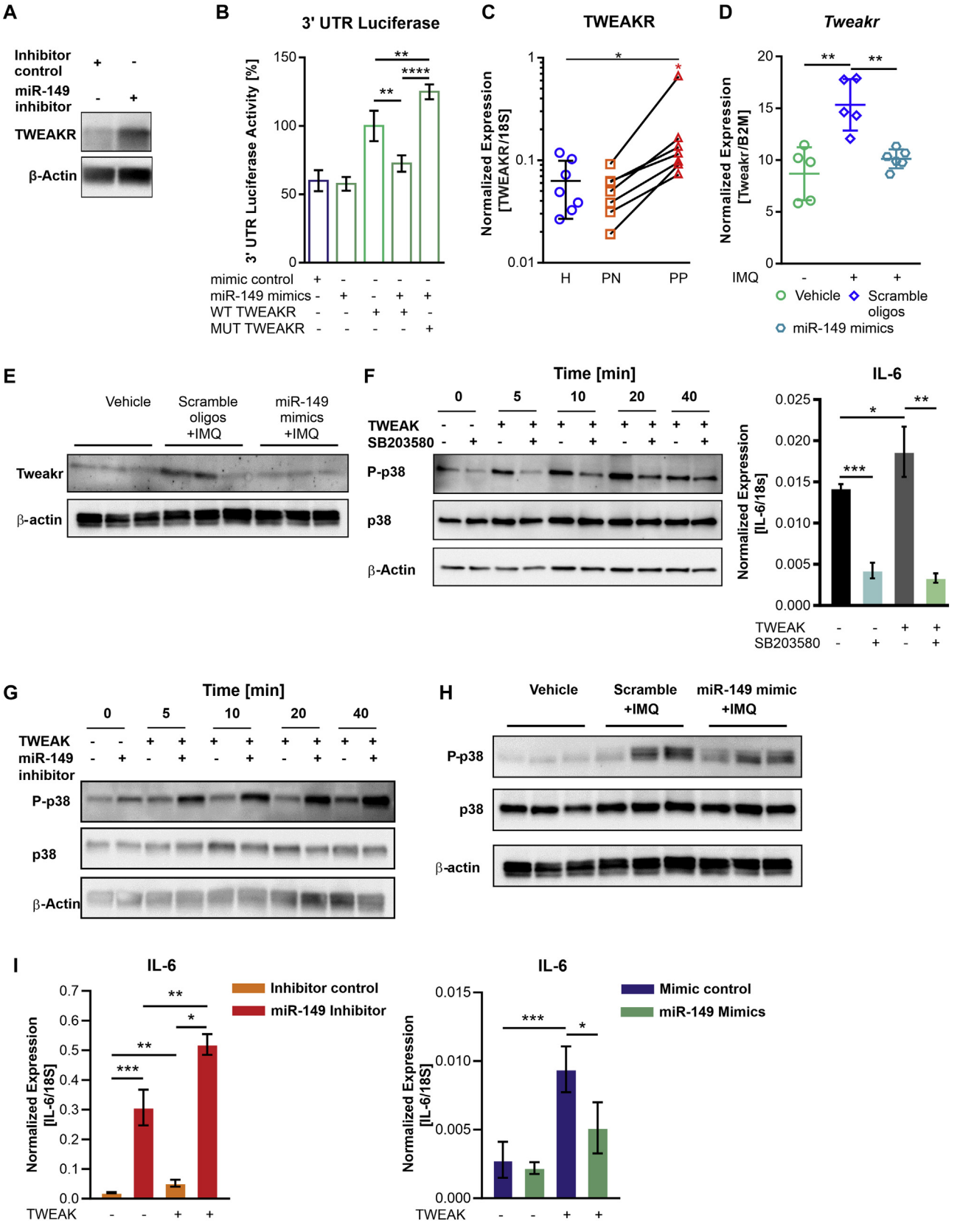

FIG 5. miR-149 regulates the TWEAK/p38-signaling pathway via targeting TWEAKR in keratinocytes. **A,** Immunoblotting for TWEAKR in keratinocytes transfected with miR-149 inhibitor or control. **B,** Keratinocytes were cotransfected with miR-149 mimics and plasmid with wild-type (WT) 3′UTR of TWEAKR or miR-149 mimics and plasmid with mutated (MUT) miR-149 binding site in 3′UTR of TWEAKR, after which

To investigate the effect of miR-149 on TWEAK-mediated cytokine induction, we next inhibited endogenous miR-149 or transiently overexpressed miR-149 in primary human keratinocytes (see Fig E10 in this article's Online Repository at www.jacionline.org) and analyzed expression of TWEAK-induced IL-6 by qRT-PCR. miR-149 inhibition resulted in significantly increased expression of basal as well as TWEAK-induced IL-6 (Fig 5, *I*). Conversely, miR-149 overexpression strongly suppressed TWEAK-induced IL-6 expression (Fig 5, *I*). The selective antagonist of TWEAKR (Fn14), L524-0366, rescued TWEAK-mediated effects on IL-6 in miR-149–overexpressing and/or miR-149–depleted keratinocytes, further supporting the idea that miR-149 regulates TWEAK signaling via TWEAKR (see Fig E11 in this article's Online Repository at www.jacionline.org). These results are also in line with the previously observed suppression of IL-6 expression and/or secretion by miR-149 in the IMQ-induced mouse model of psoriasis (Fig 1, *G* and *H*). Altogether, these results delineate a role for miR-149 in suppression of TWEAK-induced inflammatory responses in keratinocytes by direct suppression of TWEAKR.

IFN-γ primes keratinocytes for TWEAK response by suppressing miR-149

Our findings showing that the TWEAK pathway is regulated by miR-149 and that miR-149 is regulated by IFN-γ suggested possible cross-talk between IFN-γ and TWEAK pathways via miR-149. On the basis of our results, we hypothesized that IFN-γ may augment TWEAK-signaling through suppression of miR-149. To test this, primary human keratinocytes were treated with IFN-γ for 24 hours, followed by treatment with TWEAK for 3 hours (Fig 6, *A*). In line with our previous results, pretreatment with IFN-γ suppressed miR-149 expression in keratinocytes (see Fig E12, *A* in this article's Online Repository at www.jacionline.org). Notably, IFN-γ significantly enhanced TWEAK-induced IL-6 expression (Fig 6, *B*).

To test whether this effect was mediated by miR-149, miR-149 was transiently inhibited or overexpressed before IFN-γ treatment (Fig 6, *C* and *E* and Fig E12, *B* and *C*), followed by treatment with TWEAK. Inhibition of miR-149 enhanced IL-6 induction in keratinocytes with TWEAK alone, and it further enhanced IL-6 induction in IFN-γ–pretreated cells stimulated with TWEAK (Fig 6, *D*). Conversely, overexpression of miR-149 significantly counteracted the effect of IFN-γ pretreatment on TWEAK-induced IL-6 expression (Fig 6, *F*). Interestingly, the immune-regulatory effects of miR-149 were specific for IFN-γ and TWEAK response, as overexpression of miR-149 did not affect either IL-17A– or TNF-α–induced CCL20 expression in keratinocytes (see Fig E13 in this article's Online Repository at www.jacionline.org).

Furthermore, immunoblotting for TWEAKR after IFN-γ treatment showed that TWEAKR was induced after 48 hours of IFN-γ treatment in keratinocytes (see Fig E14, *A* in this article's Online Repository at www.jacionline.org), and this induction was further enhanced by inhibition of endogenous miR-149 (see Fig E14, *B*). Altogether, these results demonstrated that IFN-γ augments TWEAK-induced inflammatory responses in keratinocytes through suppression of miR-149 and induction of TWEAKR.

DISCUSSION

In psoriasis, skin inflammation is initiated and maintained through cross-talk between immune cells and keratinocytes. Keratinocytes are the target cells of multiple inflammatory cytokines in psoriatic plaques, and their response to inflammatory signals is a key element in the disease pathogenesis. In this study, we have demonstrated that IFN-γ treatment leads to a rapid and long-lasting suppression of miR-149 in keratinocytes and miR-149 acts as a mediator of cross-talk between IFN-γ and TWEAK pathway in keratinocytes.

We have demonstrated that miR-149 expression is decreased in psoriasis keratinocytes, suggesting a role for this miRNA in disease pathogenesis. MiR-149 has, to our knowledge, not been implicated in psoriasis; most likely, this can be explained by the analysis of the miRNome in sorted keratinocytes in our study, whereas other studies have used full-depth skin biopsy samples.[28] Previously, miR-149 has been shown to regulate the inflammatory response of chondrocytes in osteoarthritis,[39] mediate cross-talk of tumor and stromal cells,[40] contribute to scarless wound healing,[41] and regulate TLR signaling in macrophages.[42] However, its role in the immune response of epithelial cells, specifically, in the context of skin inflammation has not been addressed. Strikingly, intradermal delivery of synthetic miR-149 had a protective effect against skin inflammation, with fewer infiltrating immune cells, suppressed keratinocyte proliferation, and reduced skin thickness and epidermal hyperplasia in the IMQ-induced mouse model of psoriasis, indicating a pathogenic role for decreased miR-149 in psoriasis.

Consistent with these findings, inhibition of endogenous miR-149 in keratinocytes led to increased expression of multiple inflammatory cytokines and chemokines, many of them with key roles in psoriasis, suggesting a protective role for miR-149 against autoinflammation. An enrichment of genes in the TWEAK signaling pathway suggested that miR-149 may act as a gatekeeper of TWEAK-mediated inflammatory responses. TWEAK is a member of the TNF superfamily, and the TWEAK/TWEAKR pathway has been shown to be pathogenic in various inflammatory skin diseases, including psoriasis, cutaneous lupus erythematosus, wound healing, and atopic dermatitis.[31-33,43,44] Both TWEAK (TNFSF12) and its receptor TWEAKR (TNFRSF12A/Fn14) are

3′UTR luciferase activity was measured. ****$P < .0001$; **$P < .01$; Student *t* test. **C,** Keratinocytes were sorted from healthy skin (n = 7 [H]) and from lesional and nonlesional skin of patients with psoriasis (n = 7 [PN and PP, respectively]), and expression of TWEAKR was detected with qRT-PCR. *$P < .05$; Mann-Whitney *U* test; Wilcoxon matched pairs signed rank test. **D** and **E,** qRT-PCR and immunoblotting for *Tweakr* in murine skin injected with miR-149 or scramble oligos after IMQ treatment. **$P < .01$; Mann-Whitney *U* test. **F,** Immunoblotting for phospho-p38 and total p38 (left) and expression analysis of IL-6 (right) in MAPK inhibitor SB203580 and TWEAK-treated keratinocytes. **G,** Immunoblotting for phospho-p38 and total p38 in miR-149–depleted keratinocytes after TWEAK treatment. **H,** Immunoblotting for phospho-p38 and total p38 in IMQ-treated skin collected from mice injected with miR-149 or scramble oligos. **I,** qRT-PCR analysis of IL-6 in keratinocytes transfected with miR-149 inhibitor/miR-149 mimic or controls followed by treatment with TWEAK. Graphs are representative of 3 independent experiments. ***$P < .001$; **$P < .01$; *$P < .05$; Student *t* test.

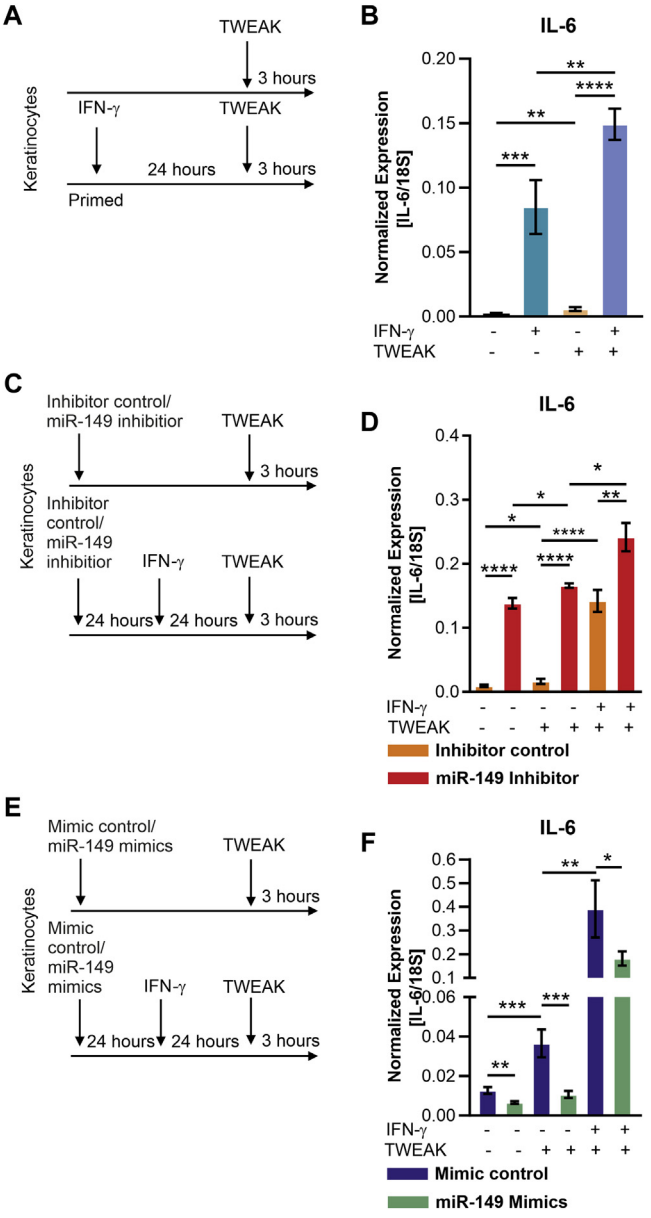

FIG 6. IFN-γ primes keratinocytes for enhanced response to TWEAK by suppressing miR-149. **A,** Schematic for experimental setup. Normal human primary keratinocytes were pretreated with IFN-γ for 24 hours followed by TWEAK treatment for 3 hours. **B,** Expression of IL-6 was analyzed by qRT-PCR. ****$P < .0001$; ***$P < .001$; **$P < .01$;*$P < .05$; Student *t* test. Human primary keratinocytes were transfected with miR-149 inhibitor/miR-149 mimics or the corresponding controls. **C** and **E**, Schematic for experimental setup. Keratinocytes were primed with IFN-γ for 24 hours and then treated with TWEAK for 3 hours. RNA was harvested. **D** and **F,** mRNA expression of IL-6 was analyzed with qRT-PCR. Graphs are representative of 3 independent experiments. ****$P < .0001$; ***$P < .001$; **$P < .01$;*$P < .05$; Student *t* test.

induced in psoriasis skin, and a single injection of recombinant TWEAK can induce psoriasis-like skin inflammation in mice.[31] Moreover, *Fn14/Tweakr* knockout mice are resistant to IMQ-induced psoriasis-like skin inflammation,[33] supporting a pathogenic role of TWEAK in psoriasis.

We have identified TWEAKR as a novel direct target for miR-149 in keratinocytes. Binding of TWEAK to TWEAKR induces chemokine and cytokine production in part via p38 MAPK phosphorylation, and activation of this pathway in keratinocytes results in the recruitment and activation of immune cells in mouse models of skin inflammation.[31,35] We have demonstrated that regulation of TWEAKR by miR-149 leads to modulation of the activity of downstream inflammatory pathways, as evidenced by the increased p38 phosphorylation and increased IL-6 expression in miR-149–depleted keratinocytes after TWEAK treatment. In accordance with these *in vitro* data, delivery of synthetic miR-149 mimics led to decreased p38 phosphorylation and *Il-6* expression in the IMQ-induced mouse model of psoriasis. It has to be noted though, that miR-149 modulated p38 phosphorylation and expression of IL-6 also in the absence of TWEAK stimulation. This suggests that in addition to TWEAKR, other target genes contribute to the anti-inflammatory effects of miR-149 in keratinocytes. Of note, miR-149 has been shown to target several genes related to inflammatory pathways in other cell types, such as MyD88 in macrophages to regulate TLR signaling[42] and TAK1 in chondrocytes to regulate NF-κB signaling.[39] Interestingly, miR-149 has also been shown to negatively regulate IL-6 in cancer-associated fibroblasts to mediate cross-talk of tumor and stromal cells in gastric cancer[40]

IFN-γ was identified as a strong suppressor of miR-149 in keratinocytes, suggesting that high IFN-γ levels in psoriasis may be responsible for the suppression of miR-149 in keratinocytes. Importantly, a single IFN-γ treatment resulted in a rapid, robust, and long-lasting suppression of miR-149 in keratinocytes for up to 4 days, indicating that this miRNA can mediate long-term effects of IFN-γ on keratinocytes. We have shown that this suppression is mediated by the canonical IFN-γ pathway, as blocking JAKs with clinically approved inhibitors in keratinocytes rescued IFN-γ–mediated suppression of miR-149. Of note, JAK inhibitors have been shown to be effective for the treatment of psoriasis, and in addition to immune cells, they also act on keratinocytes.[45] JAK inhibition–mediated induction of miR-149 expression may contribute to their mode of action in keratinocytes.

One of the functions of IFN-γ is the potentiation of cellular responses to other cytokines or triggers, known as IFN-γ priming.[15,16] Although this function of IFN-γ has been known for decades, its mechanisms are not fully understood. One potential mechanism of IFN-γ priming is the chromatin remodeling described in macrophages, which leads to activation of inflammatory pathways.[15] Here we have shown that IFN-γ primes keratinocytes for TWEAK response and that this effect is mediated by miR-149. Given the involvement of IFN-γ and TWEAK/TWEAKR in other inflammatory- and/or immune-mediated conditions,[31,35,43,44,46] this mechanism may have more general implications and should be investigated further in future studies. On the basis of the wide array of inflammatory mediators suppressed by miR-149, it is plausible that exogenous overexpression of miR-149 (eg, by topical delivery of synthetic miR-149) may be beneficial in the treatment of psoriasis and potentially in other inflammatory diseases as well.

In summary, our results delineate a mechanism according to which IFN-γ potentiates TWEAK-induced inflammatory responses and IL-6 expression via the suppression of miR-149 in keratinocytes, promoting skin inflammation. Interfering with the cross-talk between IFN-γ and TWEAK pathways by delivering exogenous miR-149 represents a potential treatment option for psoriasis.

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Key messages

- **miR-149 is suppressed in keratinocytes in psoriasis skin, and synthetic miR-149 alleviates imiquimod-induced psoriasis-like skin inflammation.**
- **Cross-talk between IFN-γ and TWEAK through miR-149 amplifies keratinocyte inflammatory responses.**

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