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TWEAK Functions with TNF and IL-17 on Keratinocytes and is a Potential Target for Psoriasis Therapy

Rinkesh K. Gupta, PhD[1], **Donald T. Gracias, PhD**[1,2], **Daniela Salgado Figueroa, BSc**[1], **Haruka Miki, MD PhD**[1], **Jacqueline Miller, BSc**[1], **Kai Fung, MSc**[3], **Ferhat Ay, PhD**[1], **Linda Burkly, PhD**[4], **Michael Croft, PhD**[1,5,*]

[1]Center for Autoimmunity and Inflammation, La Jolla Institute for Immunology, La Jolla, CA, 92037, USA

[2]Currently Janssen Pharmaceutical Companies of Johnson & Johnson, Spring House, PA, USA

[3]Bioinformatics Core, La Jolla Institute for Immunology, La Jolla, CA, 92037, USA

[4]Biogen Inc, 115 Broadway, Cambridge, MA 02142, USA

[5]Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA

Abstract

TNF and IL-17 are two cytokines that drive dysregulated keratinocyte activity and their targeting is highly efficacious in psoriasis patients, but whether these molecules act with other inflammatory factors is not clear. Here, we show that mice possessing a keratinocyte-specific deletion of Fn14 (*Tnfrsf12a*), the receptor for the TNF superfamily cytokine TWEAK (*Tnfsf12*), displayed reduced imiquimod-induced skin inflammation, including diminished epidermal hyperplasia and less expression of psoriasis signature genes. This corresponded with Fn14 being expressed in keratinocytes in human psoriasis lesions and TWEAK being found in several subsets of skin cells. Transcriptomic studies in human keratinocytes revealed that TWEAK strongly overlaps with IL-17A and TNF in upregulating the expression of CXC chemokines, along with cytokines such as IL-23, inflammation-associated proteins like S100A8/9 and SERPINB1/B9, all previously found to be highly expressed in the lesional skin of psoriasis patients. Importantly, TWEAK displayed strong synergism with TNF or IL-17A in upregulating mRNA for many psoriasis-associated genes in human keratinocytes, including *IL23A*, *IL36G*, and multiple chemokines, implying that TWEAK acts with TNF and IL-17 to enhance feedback inflammatory activity. Correspondingly, therapeutic treatment of mice with anti-TWEAK was equally as effective as antibodies to IL-17A or TNF in reducing clinical and immunological features of psoriasis-like skin inflammation, and combination targeting of TWEAK with either cytokine had no greater inhibitory effect, reinforcing the conclusion that all three cytokines function together. Thus, blocking TWEAK could be

*Corresponding Author: Michael Croft, PhD, Center for Autoimmunity and Inflammation, La Jolla Institute for Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA, mick@lji.org, Tel: 858 752 6731.

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comparable to targeting TNF or IL-17 and might be considered as an alternate therapeutic treatment for psoriasis.

One Sentence Summary:

TWEAK synergizes with TNF and IL-17 in keratinocytes to drive psoriasis-like skin inflammation.

Introduction

Psoriasis is one of the most common immune mediated skin disorders, affecting about 125 million people globally and more than 8 million in the United States (1). Psoriasis lesions may exhibit a variety of clinical manifestations, including acanthosis (an increase in epidermal thickness), hyperkeratosis, parakeratosis, hypervascularity, and a dense dermal immune cell infiltrate (2). Keratinocytes are of central importance to triggering early pathogenic events, as well as amplifying psoriatic inflammation during disease progression (3, 4). In response to external and internal danger stimuli, keratinocytes can be a source of innate immune mediators. These include a variety of proinflammatory cytokines and chemokines, which recruit the cells that are critical to the innate and adaptive immune responses (5, 6). The IL-23/IL-17 axis and TNF were first recognized as central to the pathogenesis of psoriasis-like skin inflammation in animal studies, and their role has now been proven in humans. IL-36γ has also been strongly implicated in human psoriasis. IL-36γ can be made by keratinocytes, and is able to induce IL-23 gene expression in keratinocytes (7). Therefore, it may drive an amplifying loop from IL-23 to IL-17 to IL-36γ and back again to IL-23, thus maintaining the diseased state. All of these cytokines are elevated in psoriasis skin lesions, and correspondingly neutralizing TNF, IL-23 p19, or IL-17A has shown strong therapeutic benefit in patients with psoriasis (2, 8, 9). Although, these current treatments have proven efficacy, some patients fail to respond, or they become resistant to therapy over time, or their disease comes back when treatment is stopped. Therefore, continuing efforts to understanding the pathological mechanisms that might occur in psoriasis are needed, including identifying novel molecules that can be targeted alone or combined with existing therapies.

TNF-like weak inducer of apoptosis (TWEAK, *TNFSF12*) can be expressed similar to TNF (*TNFSF2*) as a membrane bound molecule or soluble cytokine by a variety of cell types including structural and immune cells (10–12). TWEAK binds to Fn14 (fibroblast growth factor-inducible 14, TNFRSF12A) and regulates many cellular activities such as proliferation, migration, differentiation, apoptosis, and angiogenesis (13). TWEAK has been implicated in the pathogenesis of several inflammatory disorders and autoimmune diseases (14, 15). We recently found that TWEAK-deficient mice were protected from displaying strong imiquimod-induced skin inflammation that has some features of psoriasis, with gene set enrichment analysis confirming an association of Fn14 transcripts and its signaling mediators with psoriasis lesions in humans (16). The pathogenic activity of TWEAK was subsequently validated by another group using Fn14-deficient mice in the same experimental model (17). Other literature has found that soluble TWEAK is upregulated in serum of psoriasis patients, and both TWEAK and Fn14 expression at high levels is detected in tissue

sections from psoriatic lesional skin (18, 19), collectively implying that neutralizing the TWEAK/Fn14 pathway could be a novel therapeutic approach to reduce the skin pathology in psoriasis.

The primary cell target of TWEAK in the skin is not clear. We found that subcutaneous injection of a bolus of recombinant TWEAK (rTWEAK) in mice led to cutaneous inflammation, and some histological features reminiscent of human psoriasis. This was associated with the production of a range of chemokines which attract the innate and adaptive immune cells characteristic of psoriasis (16). Many of these chemokines are products of keratinocytes, and Fn14 is expressed on keratinocytes (16), suggesting this cell type could be central to the action of TWEAK. Before considering clinical therapy targeting this pathway, more needs to be understood about the action of TWEAK in the skin, especially on keratinocytes, and its relationship to other pathogenic molecules such as IL-17 and TNF that also have receptors on keratinocytes.

In this study, we investigated if TWEAK signaling specifically in keratinocytes is required to develop psoriasis-like skin lesions after imiquimod treatment using Fn14-conditional knockout mice, and also performed RNA-seq analysis in human epidermal keratinocytes to determine how TWEAK alone or in combination with IL-17 and TNF controls expression of a variety of gene sets found to be upregulated in human psoriasis. Our data demonstrates that Fn14 signaling in keratinocytes is crucial for the development of imiquimod-induced skin inflammation. Furthermore, transcriptomic data establish substantial similarities in the genes induced in keratinocytes by TWEAK, IL-17, and TNF, and notably we found strong synergistic activities of these cytokines acting together on a number of genes associated with psoriasis. Correspondingly, a similar effect of blocking TWEAK therapeutically was observed in reducing skin lesions in mice compared to blocking either TNF or IL-17A, and no greater effect was seen with combination treatments. These results suggest that TWEAK might be as good a target to counter the keratinocyte hyperresponsiveness and dysregulated immune system seen in psoriasis as observed when IL-17 and TNF are neutralized.

Results

Conditional deletion of Fn14 in keratinocytes protects mice from hyperproliferative skin inflammation

To better characterize the cell types and subsets that are major producers of TWEAK and Fn14, we reanalyzed a recently published single-cell RNA-seq dataset from human psoriasis lesions (Fig. S1) (20) This revealed that subsets of fibroblasts, melanocytes and some endothelial cells, as well as a small fraction of T cells and dendritic cells express TWEAK (*TNFSF12)* (Fig. 1A). Its receptor, Fn14 (*TNFRSF12A*), was mainly expressed by several keratinocyte clusters (Fig. 1B, KC-2 and KC-6), marked by high expression of KRT(keratin)14 and KRT5 in the original paper (20) and Fn14 was also found in some fibroblasts, endothelial cells and melanocytes. The former confirmed previous observations of Fn14 expression in human keratinocytes cultured in vitro (16, 17, 21), and suggested that keratinocytes could be a primary skin cell affected by TWEAK activity. To address this in vivo, we used conditional deletion with KRT14-cre-Fn14$^{fl/fl}$ mice (Fn14 cKO). Flow cytometry analysis confirmed the efficient deletion of Fn14 in epidermal keratinocytes

in these mice (Fig. S2). After 7 days of skin exposure to imiquimod that induces some features of psoriasis, control Fn14 flox mice developed severe skin inflammation with marked epidermal hyperplasia, whereas Fn14 cKO mice appeared macroscopically protected with significantly less epidermal thickening and hyperkeratosis, and reduced rete ridge-like structures extending into the dermis (Fig. 1C). Immunofluorescence analysis also revealed a markedly lower number of Ki-67 and Keratin (KRT) 14 positive cells in the epidermis of Fn14 cKO mice (Fig. 1D, 1E). These results demonstrate that Fn14 signaling in epidermal keratinocytes contributes to maximal hyperproliferation of keratinocytes. mRNA analysis of skin biopsies from Fn14 cKO mice further confirmed the histological analysis with strongly reduced expression of a number of keratinocyte responsive transcripts that have been found upregulated in human psoriasis lesions. Expression of several proliferative markers specific for keratinocytes like KRT14, 16 and 6b were reduced, whereas a differentiation marker, loricrin, was not downregulated in the skin of Fn14 cKO mice, compared to control mice (Fig. 1F). Transcripts for the keratinocyte alarmins, S100A8 and S100A9 (22), and antimicrobial peptide beta-defensin 2 (DEFB4), were also lower in Fn14 cKO mice. Similarly, mRNA for the proinflammatory factors IL-23A, IL-17A, IL-36γ, CXCL3 and IL-19 were reduced in expression (Fig. 1F). Together, these results demonstrate that Fn14 signaling in keratinocytes is necessary for the pathogenesis of imiquimod-driven skin inflammation in mice that has several features in common with human psoriasis.

TWEAK, TNF and IL-17A broadly modulate epidermis and immune-related genes in keratinocytes

IL-17 and TNF have been found to regulate a variety of epidermal and immune responsive genes in keratinocytes associated with psoriasis (23–25). Since keratinocytes co-express the TWEAK receptor with those for IL-17 and TNF, we sought to investigate the impact of TWEAK stimulation, alone or in combination with either TNF or IL-17, on keratinocyte transcriptomes, to identify psoriasis-associated genes that might be individually- or co-regulated by these cytokines. Human epidermal keratinocytes were then treated with the human cytokines, and analyzed by RNA-seq. We identified a broad genomic response in keratinocytes with 819, 2422, and 948 gene transcripts upregulated greater than 1.5-fold by TWEAK, TNF, and IL-17, respectively, compared to PBS treated keratinocytes (Fig. S3A). To understand how many of these were relevant to psoriasis, we created a human psoriasis biopsy transcriptome by selecting 1196 genes (Table S1) that have been found to be upregulated (>2-fold) in skin lesions from psoriasis patients compared to non-lesional or healthy skin (26–28). Out of the 819 TWEAK-upregulated transcripts in keratinocytes, ~16% (129 genes) were in the psoriasis transcriptome (Fig. 2A, 2B, 2C; Table S2A). Among the TNF and IL-17 upregulated transcripts, ~9% (224 out of 2422) and 21% (199 out of 948), respectively, were psoriasis-associated (Fig. 2A). 85 psoriasis genes were targeted by TWEAK that were shared with IL-17 and TNF, with an additional 15 common to TWEAK and IL-17 and another 15 common to TWEAK and TNF (Fig. 2A; Table S2A, S2B). TWEAK also upregulated 14 unique psoriasis-associated transcripts compared to 54 for IL-17 and 79 for TNF (Fig. 2A, Table S2B). Differences were noted in the extent of upregulation of the genes induced by TWEAK. Among the shared gene targets, TWEAK had stronger activity on transcription of some of these genes whereas TNF or IL-17 had greater activity on other genes (Fig. 2B, Table S2A). The TWEAK-targeted psoriasis genes

common to both TNF and IL-17 included the proinflammatory cytokines IL-1B, TNF, and IL-23A, implying TWEAK can be upstream of both IL-17 and TNF; the chemoattractants CXCL1, CXCL2, CXCL3 and CXCL5; and keratinocyte responsive molecules KRT6, S100A8, S100A9 and SERPINB1 and B9 (Fig. 2B, Table S2A, S2B). These results show that TWEAK has the potential to induce the expression of key inflammatory mediators in keratinocytes that have been associated with psoriasis pathogenesis, many of which are shared targets of IL-17 and TNF.

We next related this data to RNA-seq results (GSE96957) obtained in our previous in vivo study where mice were injected subcutaneously with recombinant mouse TWEAK, which led to skin inflammation (16). TWEAK upregulated 1506 transcripts in mouse skin including 283 (~19%) that were found in the 1196 genes in the human psoriasis biopsy transcriptome (Fig. 2D, Table S3A). From these 283 gene targets in the mouse skin biopsies, 60 out of 129 (47%) were also upregulated by recombinant human TWEAK in human keratinocytes (Fig. 2E, Table S3B). These included IL-23A, TNF, IL-1B, KRT6, CXCL3, CXCL5, S100A8, S100A9 and SERPINB9 (Table S3B). This data provides a further correlation between studies in the mouse and human, and again supports the strong relevance of TWEAK activity in keratinocytes to psoriatic skin inflammation.

TWEAK synergistically cooperates with both TNF and IL-17A for psoriasis-relevant gene expression

As previous studies found that IL-17 and TNF synergized together to upregulate transcription of a number of psoriasis-related genes in keratinocytes (26), we expanded our RNA-seq transcriptomic analysis to investigate synergism between TWEAK and these two cytokines in keratinocytes. We considered a gene to be synergistically upregulated when the combined stimulation of both cytokines was greater than the addition of their individual effects. The combination of TWEAK with TNF synergistically enhanced the expression of 272 total gene transcripts (Fig. S3B). When this data was analyzed for genes in the human psoriasis biopsy transcriptome, the combination of TWEAK and TNF synergistically upregulated transcripts for 95 genes (Fig. 3A–3C; Table S4A). When TWEAK was combined with IL-17A, 39 total transcripts were synergistically upregulated in keratinocytes (Fig. S3B), and of these, 13 were genes in the human psoriasis biopsy transcriptome (Fig. 4A–4C; Table S4B).

Key pathogenic genes were driven in each combination, highlighting the important cross-talk between TWEAK and both TNF and IL-17. TWEAK together with TNF strongly upregulated transcripts for cytokines and chemokines such as IL-36γ, IL-23A, TNF, CXCL2, and CCL20, alarmins/antimicrobial proteins such as S100A8, S100A9, DEFB4A, and PI3, and molecules involved in keratinization such as SPRR2B and SPRR2D, to levels far in excess of those driven by the individual cytokines (Fig. 3B, 3D). For example, TWEAK had little effect on IL-36γ transcript expression alone (1.2-fold); TNF moderately induced IL-36γ expression (6.3-fold); but the combination of TWEAK and TNF synergistically drove a 97-fold increase (Fig. 3D, and Table S4A). TWEAK combined with IL-17A also synergistically drove transcription of psoriasis-relevant genes including IL23A and several CXCL chemokines (Figure 4C–D). For example, individual increases in CXCL5

transcript expression were 12.1 and 31.1-fold for TWEAK and IL-17A, respectively, but their combination resulted in 147-fold upregulation (Fig. 4D and Table S4B). A number of these effects were further validated by RT-PCR analysis (Fig. S4A–4F). These results strongly suggest that TWEAK may act together with TNF and IL-17A, and that all three cytokines might collectively promote the inflammatory phenotype in keratinocytes that appears in psoriatic skin lesions.

TWEAK blocking attenuates imiquimod-driven skin inflammation similarly to blocking IL-17A and TNF

One prediction based on the conclusion that all three cytokines work in synergy for regulating some key pathogenic gene targets in keratinocytes might be that blocking any one of the cytokines would reduce the transcription of these genes to non-pathogenic levels and would correspondingly suppress psoriasis-like characteristics. To investigate this, we asked in mice whether therapeutically inhibiting TWEAK would then be similar to therapeutically inhibiting TNF or IL-17. Imiquimod-induced skin inflammation has traditionally been performed over 6-7 days (16, 29), limiting a large window for therapeutic manipulation. In order to have a more suitable protocol, we extended the treatment every day for 14 days and analyzed the extent of epidermal thickness (Fig. S5A). This peaked by day 6, was maintained through day 12, and then started to resolve by day 14. Strong induction of pathology by day 6 correlated with upregulated expression of several gene transcripts in the skin that are also upregulated in human psoriasis lesions. These were already elevated on day 4 and did not increase further when analyzed on day 8 (Fig. S5B). We then adopted an 11-day imiquimod treatment protocol for further therapeutic studies, administering blocking antibodies after disease was evident starting at day 6.

We initially compared blocking TWEAK to IL-17, alone and in combination (Fig. 5). Administration of anti-TWEAK or anti-IL-17A resulted in a comparable attenuation in skin lesions when measured at day 12 (Fig. 5A). Immunofluorescence staining of the epidermis with anti-Ki-67 confirmed a similar level of reduced keratinocyte proliferative activity when blocking TWEAK or IL-17 (Fig. 5B). Some differences, however, were observed in cellular infiltrates in the skin. Neutralization of TWEAK caused a strong reduction in the total numbers of $CD45^+$ leukocytes, including neutrophils and macrophages, and a trend toward fewer γδ T cells, whereas a lesser effect was seen with neutralization of IL-17 with only a statistically significant decrease in neutrophils (Fig. 5C). No obvious effect was seen on the number of dendritic cells, NK cells or total αβ T cells. In line with the RNA-seq results suggesting TWEAK can control IL-23 production, we observed a markedly reduced expression of mRNA for IL-23A and IL-17A in the skin of anti-TWEAK treated animals (Fig. 5D). We also tested several other factors that are characteristic of human psoriasis lesions (Table S1) and found therapeutic blocking suppressed levels of the chemokines CXCL3 and CCL5, as well as S100A8, S100A9, SERPINB1, and IL-19 (Fig. 5D). Reduced expression of IL-36γ was seen although it did not reach statistical significance. Further supporting reduced keratinocyte activity, various keratin isoforms (KRT6b, 14, and 16) were also downregulated in the lesional skin of mice receiving anti-TWEAK. Interestingly, blocking IL-17 had similar effects to blocking TWEAK on the expression of all of these molecules.

Based on the conclusion that all three cytokines act in synergy to drive expression of pathogenic genes, and blocking any one can reduce transcription to non-pathogenic levels, another prediction might be that blocking any two of the cytokines together would not further diminish disease compared to targeting only one. Indeed, when TWEAK and IL-17 were inhibited together, although there was a trend toward a greater reduction in epidermal thickening and Ki-67 staining compared to mice treated with the individual antibodies, this was not statistically significant. No greater effect of the combined antibodies was seen on the cellular infiltrates, and no greater reduction in mRNA expression was observed of the factors that were measured in the skin (Fig. 5). These results reinforce the conclusion that TWEAK and IL-17 work together to promote a number of inflammatory features that are similar to those in human psoriasis.

We then blocked TNF versus TWEAK (Fig. 6). Skin histological analysis showed a significant reduction in epidermal thickness after injecting both anti-TWEAK or anti-TNF compared to controls with again no difference evident in the effect between the antibodies (Fig. 6A). A similar lower number of Ki-67-expressing keratinocytes was also found in the epidermis of anti-TWEAK or anti-TNF injected mice (Fig. 6B). The result of blocking TWEAK on skin infiltrates was similar to before with reduced numbers of neutrophils, macrophages, and γδ T cells, and blocking TNF had a slightly milder effect reminiscent of blocking IL-17 (Fig. 6C). Administration of anti-TWEAK again resulted in lower transcriptional levels of KRT6b, KRT14, KRT16, IL-17A, IL-23A, IL-19, CCL5, CXCL3, SERPINB1, and S100 genes, and this was replicated with anti-TNF (Fig. 6D). A trend toward reduced expression of IL-36γ was seen as before when blocking TWEAK, although in this case blocking TNF had a greater and statistically significant effect. Given these collective results, unsurprisingly, combined blockade of TWEAK and TNF did not lead to any additional suppression of epidermal thickness, albeit a statistically lower number of Ki-67 expressing keratinocytes was found (Fig. 6B). Other disease phenotypes assayed, including cellular infiltrates and levels of inflammatory mediators, were similar to those in mice where either of the individual cytokines were neutralized. Taken together, these results suggest that blocking TWEAK has the same therapeutic potential to reduce pathological and immunological features of dermatitis as blocking TNF or IL-17A, and that TWEAK functions together with both TNF and IL-17 to drive dysregulation in keratinocytes.

Discussion

In the present article, we show through specific deletion of Fn14 that TWEAK signaling in keratinocytes is important in driving experimental skin inflammation and epidermal activation in mice that has some features of human psoriasis. We also demonstrate the therapeutic effect of TWEAK inhibition in skin pathophysiology and that it provides a similar level of efficacy as blocking either IL-17 or TNF. Furthermore, studies of human keratinocytes demonstrate that TWEAK is capable of inducing transcription of a variety of genes including inflammatory chemokines and cytokines that are found upregulated in human psoriasis lesions, and that TWEAK has overlapping actions compared to IL-17 and TNF. Most importantly, we identify a high degree of cooperativity between TWEAK and TNF and IL-17 in promoting the activity of a number of genes thought to be central to the dysregulated epidermal reaction that is characteristic of psoriasis lesions. Our data highlight

the potential of TWEAK to be central to psoriatic disease and the potential of clinically ameliorating psoriasis symptoms by targeting TWEAK or Fn14.

Previous studies have implied that the TWEAK/Fn14 pathway may be important in psoriasis development from experiments with imiquimod skin treatment of animals globally deficient in TWEAK (16) or Fn14 (17). While the lesions created by imiquimod are not human psoriasis, and clear differences have been reported and highlighted between skin inflammation observed in the mouse model and that in human psoriasis (30–32), the model system does involve keratinocyte activation and a number of genes are induced in the mouse skin that correspond to genes found upregulated in human psoriasis lesions. Our data now assessing imiquimod-induced dysregulation in mice lacking the Fn14 receptor specifically in keratinocytes clearly demonstrates that keratinocytes are one of the key cells that can receive TWEAK signals in the skin, and that this activity is required for maximal phenotypic changes in these cells. Hyperproliferation and altered differentiation of keratinocytes are part of the pathological diagnosis of psoriatic skin, and keratinocyte-specific Fn14-deficient mice exhibited impairments in these features with lowered expression of keratin 6, keratin 14 and keratin 16 and no defect in loricrin that relates to barrier function. Reduced expression of other inducible keratinocyte genes like S100A8/9 and DEFB4 in the conditional knockouts further substantiated the importance of signals through the Fn14 receptor in keratinocytes.

Over the past few years, the FDA has approved several inhibitors of the IL-23/IL-17 signaling pathway as biologic therapies for psoriasis (33). IL-23 acts upon T cells to drive high levels of IL-17, and IL-17 directly or indirectly via IL-36γ can promote IL-23 production in keratinocytes, creating a self-amplifying, feed-forward response to maintain skin lesions. TNF may also impact this axis, and our new data additionally show that TWEAK can function in this regard as well, upregulating IL-23 expression in keratinocytes and contributing to IL-23, IL-17, and IL-36γ expression in vivo. Both IL-17 and TNF can directly trigger the production of inflammatory features in keratinocytes including keratins, defensins, lipocalin, S100 proteins, and also chemokines and cytokines attracting and acting on innate and adaptive immune cells (34–36). Similarly, TWEAK upregulated many of the same inflammatory products in keratinocytes that are relevant to psoriasis with 85 gene targets out of 129 shared with IL-17 and TNF, and a further 30 targets individually shared with IL-17 or TNF. The RNA-seq data *in vitro* was also supported by results in mice subcutaneously injected with TWEAK, which induced in the skin ~50% of the psoriasis-related genes that were upregulated in human keratinocytes *in vitro*. More important than the individual action of TWEAK, we found a strong synergism between TWEAK and TNF or IL-17 in keratinocytes, resulting in amplified production of a number of key genes known to be upregulated in psoriasis skin lesions. These included IL-23 and IL-36γ that were driven much more strongly by the combination of TWEAK with one or both of these cytokines. Similar results were found for genes reflecting direct keratinocyte dysfunction such as S100 proteins, PI3, SERPINB4, SPRR2 proteins, and several matrix metalloproteinases. Moreover, the synergy between TWEAK and TNF or IL-17 in driving strong production of chemokines such as CXCL2, CXCL3, CXCL5, and CCL20, which can activate and maintain the innate and adaptive immune response by recruitment of macrophages, neutrophils, and Th17 cells in the skin, is also likely to be of major importance to psoriasis progression.

Given the keratinocyte and gene expression studies, we extended our efforts to define the treatment efficacy of TWEAK neutralization in the same imiquimod mouse model, and to relate it to the effects of neutralizing TNF and IL-17 that are approved for psoriasis therapy. Perhaps not surprisingly given these aforementioned *in vitro* results, blocking TWEAK was equally as effective as blocking TNF or IL-17 in attenuating the major features of skin inflammation, including hyperproliferation of keratinocytes. Moreover, dual blockade of TNF or IL-17 with TWEAK was not obviously more effective in reducing skin inflammation compared to treatment with each of the neutralizing antibodies alone. These results again correlate well with our gene expression data that showed that TWEAK synergizes with TNF and IL-17, and others' data that TNF synergizes with IL-17 (26, 37), dramatically amplifying many of the pathways that are thought to be key to psoriasis. The primary implication of these studies is that reducing the activity of any one of the cytokines would then impair the activity of the other cytokines, and this was validated in the dual blockade experiments.

In our prior study, we also found that TWEAK-deficient animals were protected from skin inflammation induced by allergen that had features of human atopic dermatitis, including a biased Th2 response (16). While atopic dermatitis and psoriasis have been considered strongly different skin inflammatory diseases, recent RNA-seq studies have highlighted both similarities and differences in the transcriptomes within the skin lesions (28). A number of the psoriasis-related transcripts that we found upregulated by TWEAK in keratinocytes in the present study are also upregulated in atopic dermatitis. This raises the question of whether TWEAK, similar to its effects with IL-17 and TNF, will also be highly synergistic with more classic Th2 cytokines, such as IL-13 and IL-4, that additionally have receptors on these cells. We provided a small amount of evidence for this in our previous publication with TWEAK and IL-13 cooperating to promote CCL2, CCL5, CCL7, and CCL17 in keratinocytes (16). Further in line with this, in an experimental colitis study, TWEAK was found to work with IL-13 to enhance TSLP expression in colonic epithelial cells (38). Additional studies are then needed to fully explore this idea and the potential that blocking TWEAK in atopic dermatitis could also be effective to treat this skin disease similar to dupilumab that blocks IL-4Rα.

In summary, we conclude that TWEAK can be viewed as a cytokine that might be equally as important to psoriasis as TNF and IL-17, and that neutralizing reagents to TWEAK or Fn14 could be as effective as the current biologics to TNF, IL-17, or IL-23. We acknowledge that our conclusions are limited because of only using one mouse model system of skin inflammation that may not recapitulate all of the features of human psoriasis (31, 32). We also only used one strain of mice (C57BL/6), although this is unlikely to have influenced the results given that our prior study that found that TWEAK-deficient animals were protected from imiquimod-induced skin inflammation in C57BL/6 mice (16) was replicated by another group using Fn14-deficient animals on the BALB/c background (17). Regardless of these caveats, as neutralizing TWEAK produced essentially the same result as neutralizing TNF or IL-17, this suggests that the current study is highly relevant for clinical psoriasis therapy. It provides strong evidence of the cooperativity between these cytokines and indirect support for the idea that TWEAK might be active in human psoriasis and display synergism with these other cytokines in driving the human disease. The inhibitors of TNF,

IL-17, and IL-23 do not provide relief in every psoriasis patient, or do not always achieve 100% improvement in symptoms, and patients can stop responding to prolonged treatment in some cases (39–42), suggesting there is a need for alternative therapeutics. Moreover, TNF and IL-17/IL-23 blockade are associated with a number of adverse health effects, such as increased susceptibility to infections related to their fundamental roles in protective immunity (43–47). Current data does not support such an important role for TWEAK in infectious disease (13, 48), and clinical trials of an antibody to TWEAK in rheumatoid arthritis did not have any outstanding immunosuppressive events (49). This suggests that TWEAK blockade may exhibit a greater safety profile than the current biologics. Future studies targeting TWEAK in clinical trials for psoriasis may then be warranted.

Materials and Methods

Antibodies and reagents

Neutralizing antibodies to mouse TNF (rat anti-mouse; clone XT3.11) and IL-17A (mouse anti-mouse; clone 17F3) were in vivo grade from Bio X Cell (Lebanon, NH, USA). Mouse anti-mouse TWEAK neutralizing antibody (clone mP2D10) was produced by Biogen, Inc. Isotype control antibodies were rat IgG1, mouse IgG1, and mouse IgG2a, all from Bio X Cell (Lebanon, NH, USA) for the TNF, IL-17A and TWEAK neutralizing antibodies, respectively. All recombinant human cytokines, TWEAK, TNF, and IL-17A, were from R&D Systems (Minneapolis, MN, USA).

Mice

6–8-week old male WT and K14-cre mice (Jackson Labs) were bred in-house on the C57BL/6 background. Fn14 (*TNFRSF12A*) flox/flox mice on the C57BL/6 background, provided by Biogen, Inc., were generated by Taconic by inserting loxP sites upstream of exon 2 and downstream of exon 4 of Fn14. K14-creFn14$^{fl/fl}$ (Fn14 cKO) mice were generated by crossing to produce mice lacking the Fn14 receptor specifically in keratinocytes. All experiments were carried out in compliance with the regulations of the La Jolla Institute for Immunology Animal Care Committee (IACUC # AP00001043).

Skin Inflammation Protocol and Treatment

Three days prior to initiation of experiments, mice were shaved along their upper backs using electric clippers. Psoriasis-like skin inflammation was induced by application of an FDA-approved 5% imiquimod cream (Perrigo Company, Michigan), for 7 or 11 consecutive days on the shaved back. This cream is a generic equivalent to Aldara from Graceway Pharmaceuticals, but does not contain isostearic acid, an inflammasome activator (50). Based on other publications, the extent of skin inflammation induced by the Perrigo cream appears comparable to Aldara, and direct comparison with another 5% imiquimod cream that does contain isostearic acid (from Taro Pharmaceuticals) revealed no obvious differences in the extent of inflammation. Control mice received a vehicle cream (Vaseline Lanette cream; Fagron). Clinical phenotypes were evident at day 4 and maintained up to day 12 with continuous imiquimod treatment. Prior reports suggest that mice treated with Aldara can experience some adverse health effects including acute weight loss (51) possibly due to ingestion of this cream. We applied imiquimod cream to the upper portion of the

back, limiting this possibility, and did not observe any visible changes to the mice such as weight loss or diarrhea during the experiments. For therapeutic antibody studies, mice were injected with either 200 μg anti-TWEAK, anti-IL-17A, anti-TNF, or a combination of two therapeutics (200 μg each) on day 6 and 9 via the intraperitoneal route. Control mice received the appropriate individual or mixtures of isotype matched IgGs at the same concentrations.

Histology and Immunofluorescence

Tissue samples from the dorsal skin were harvested, formalin-fixed and paraffin-embedded. 4 micrometer sections were stained with hematoxylin and eosin stain. Images were acquired on a Zeiss Axioscan Z1 slide scanner and epidermal thickness was quantified using Zen2 software. To perform immunofluorescence staining, skin samples were deparaffinized by sequential placement in xylene and ethanol. Skin sections were treated with Fc Block in 5% donkey serum (in PBS) and stained with rabbit anti-mouse Ki67 (clone SP6; Abcam) at 1:200 concentration followed by goat anti-rabbit AF647 (Abcam) at 1:500 concentration. Images were captured using Zeiss Axioscan Z1 slide scanner and positive cells were quantified using QuPath software.

Analysis of skin immune cell infiltrates

Two 10 mm punch skin biopsies from each animal were taken and processed for flow cytometry. Biopsies, including epidermis and dermis, were digested using 3 mg/ml Collagenase Type IV (Worthington Biochemical, Lakewood, NJ), 200 U/ml DNase I (Worthington Biochemical, Lakewood, NJ), 2.5 mg/ml Liberase (Roche) for 1 hr at 37 °C followed by 2 mg/ml Dispase II for another 45 mins. Thereafter, single cells were counted in a Neubauer cell chamber and stained with the following anti mouse antibodies from Biolegend, San Diego, CA: CD45-BV605 (clone 30-F11), CD11b-BV510 (clone M1/70), Ly6G-PerCP/Cy5.5 (clone A1-8), F4/80-PECy7 (clone BM8), Ly6C-FITC (clone HK1.4), CD90.2-PE (clone 53-2.1), from Invitrogen, Carlsbad, CA: CD11c-ef450 (N418), γδTCR-APC (clone eBioGL3), MHC-II-AF700 (clone M5/114.15.2) and from BD bioscience, San Jose, CA: NK1.1- CD711 (clone PK136), CD3ε-PECF594 (clone145-2C11), TCRβ- BV786 (clone H57-597). To avoid non-specific staining, cells were incubated with CD16/CD32 block (clone 2.4G2) for 5 min at RT prior to actual staining. Cellular infiltration was assessed using an LSR-II Flow Cytometer and analyzed using Flow Jo Software. Total numbers of inflammatory cells were calculated using relative frequencies.

Real-time quantitative PCR

Total RNA was isolated from skin biopsies using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy Fibrous Tissue mini kit (74704; Qiagen). 2 μg total RNA was used to prepare cDNA using a High Capacity RT Kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was carried out using SYBR Green Endpoint measurement. All samples were run in triplicate and expression calculated using the delta CT method relative to the house-keeping gene GAPDH. Primer sequences are provided in Table S5.

Keratinocyte Culture and Stimulation

Human epidermal keratinocytes from neonates (nHEK) were purchased from ATCC (NHEK, PS-200-010, ATCC, Manassas, VA) and grown in Dermal Cell Basal Medium (PCS-200-030, ATCC). Cells were cultured (seeding density of $3x10^5$ cells/well) in triplicates using a 6-well format plate and stimulated with predetermined optimal concentrations of either human rTWEAK (100 ng/ml), human rIL-17A (100 ng/ml), or human rTNF (10 ng/ml), or a combination of rTWEAK with rIL-17A or rTNF at the above concentrations. Controls received PBS. After 48 h, cells were harvested in Trizol and processed for RNA-seq and real-time PCR analysis.

RNA-seq Analysis

RNA was isolated from 3 different replicates per group, using a Qiagen kit and further subjected to RNA-seq analysis. In brief, the paired-end reads that passed Illumina filters were filtered for reads aligning to tRNA, rRNA, adapter sequences, and spike-in controls. The reads were then aligned to GRCh38 reference genome and mm10 reference genome for *in vitro* and *in vivo* data set analysis, respectively. DUST scores were calculated with PRINSEQ Lite (v 0.20.3) and low-complexity reads (DUST > 4) were removed from the BAM files. The alignment results were parsed via the SAM tools to generate SAM files. Read counts to each genomic feature were obtained with the feature counts (v 1.6.5) using the default option along with a minimum quality cutoff (Phred > 10). After removing absent features (zero counts in all samples), the raw counts were then imported to R/Bioconductor package DESeq2 (v 1.24.0) to identify differentially expressed genes among samples. P-values for differential expression were calculated using the Wald test for differences between the base means of two conditions. These P-values were then adjusted for multiple test correction using Benjamini-Hochberg algorithm. We considered genes differentially expressed between two groups of samples when the DESeq2 analysis resulted in an adjusted P-value of <0.05 and the difference in gene expression was at least 1.5-fold. The sequencing data generated in this study was submitted to the Gene Expression Omnibus (GSE171170).

Analysis of Published Single-cell RNA-seq Data

The single-cell RNA-seq of psoriasis patients was downloaded from EMBL-EBI repository under the accession number E-MTAB-8142. Reads were aligned to GRCh38 human reference genome and collapsed into Unique Molecular Identifiers (UMI) counts using 10x Genomics' Cell Ranger software (v3.1.0). Samples with >1,000 Median genes per cell and >60% of reads mapped confidently to transcriptome were used in the downstream analyses. To further eliminate cells that are doublets or are low quality, cells expressing <250 or >2500 unique genes or <500 total UMIs or with >15% of reads mapping to mitochondrial transcripts were removed. The transcriptome data was log-transformed and normalized by a factor of 10,000 per cell, using default settings in Seurat R package v4.0.2. Samples were integrated by first using FindIntegrationAnchors function (top 2000 variable genes) with the canonical correlation analysis (CCA) followed by IntegrateData function. Transcriptomic data from each cell was further scaled. Principal component (PC) analysis was performed using the variable genes on scaled counts, and based elbow plots were used to pick the first 22 and 19 PCs for $CD45^+$ and $CD45^-$ populations, respectively. Cells were clustered

using FindNeighbors and FindClusters functions with a resolution of 0.9 ($CD45^+$) and 0.3 ($CD45^-$). Clusters were further annotated using the markers from the original paper.

Statistical analysis

Statistical analysis was performed using Prism5 software (GraphPad, San Diego, CA). Groups were analyzed by t-test or Mann–Whitney U-test where indicated. A P value <0.05 was considered statistically significant (*).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Funding:

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Data and materials availability:

The floxed Fn14 mouse strain and the neutralizing anti-mouse TWEAK antibody used in this study were obtained through a Material Transfer Agreement with Biogen. The RNA-seq dataset generated during the current study is available through the Gene Expression Omnibus (GSE171170). All other data associated with this study are provided in the paper or the Supplementary Materials.

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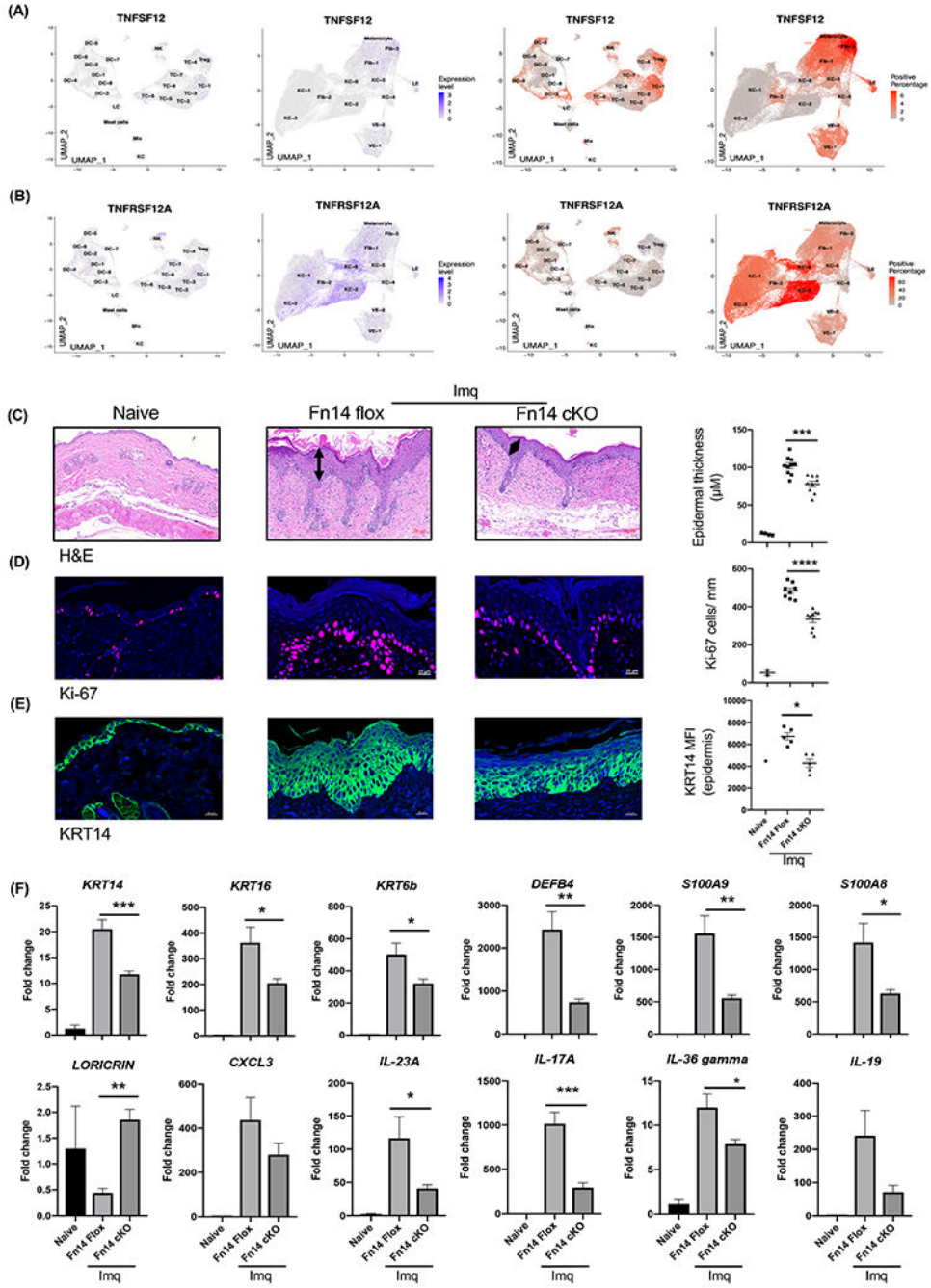

Fig. 1. TWEAK and Fn14 are expressed in cells from psoriasis lesions and Fn14 keratinocyte-specific conditional knockout mice are protected from imiquimod-induced skin inflammation.

(A-B) UMAPs showing Seurat-normalized expression levels (left) and percentage of cells that are positive per cluster (right) for **(A)** TNFSF12 and **(B)** TNFRSF12A in CD45+ (left) and CD45− (right) cells in human psoriasis lesions (see Fig. S1 for cluster annotations). **(C-F)** Fn14 flox and Fn14 cKO mice were treated with imiquimod on the back for 7 days and compared to naive untreated mice. **(C)** Representative H&E stained skin sections. Arrows highlight epidermis thickness. Average epidermal thickness (μM), calculated from 18-20

random measurements, from two experiments with 5 mice/group. Each point represents one mouse. (**D**) IF staining for Ki-67 (pink) in the epidermis and quantification of Ki-67+ cells/mm. (**E**) IF staining for KRT14+ cells (pink) in the epidermis and mean fluorescence intensity measurement. (**F**) mRNA expression of indicated genes in skin biopsies. Data fold change over naïve group. Individual mice or means ± SEM from 3-8 mice/group, combined from 2 experiments. *P <0.05, **P<0.01, ***P<0.001.

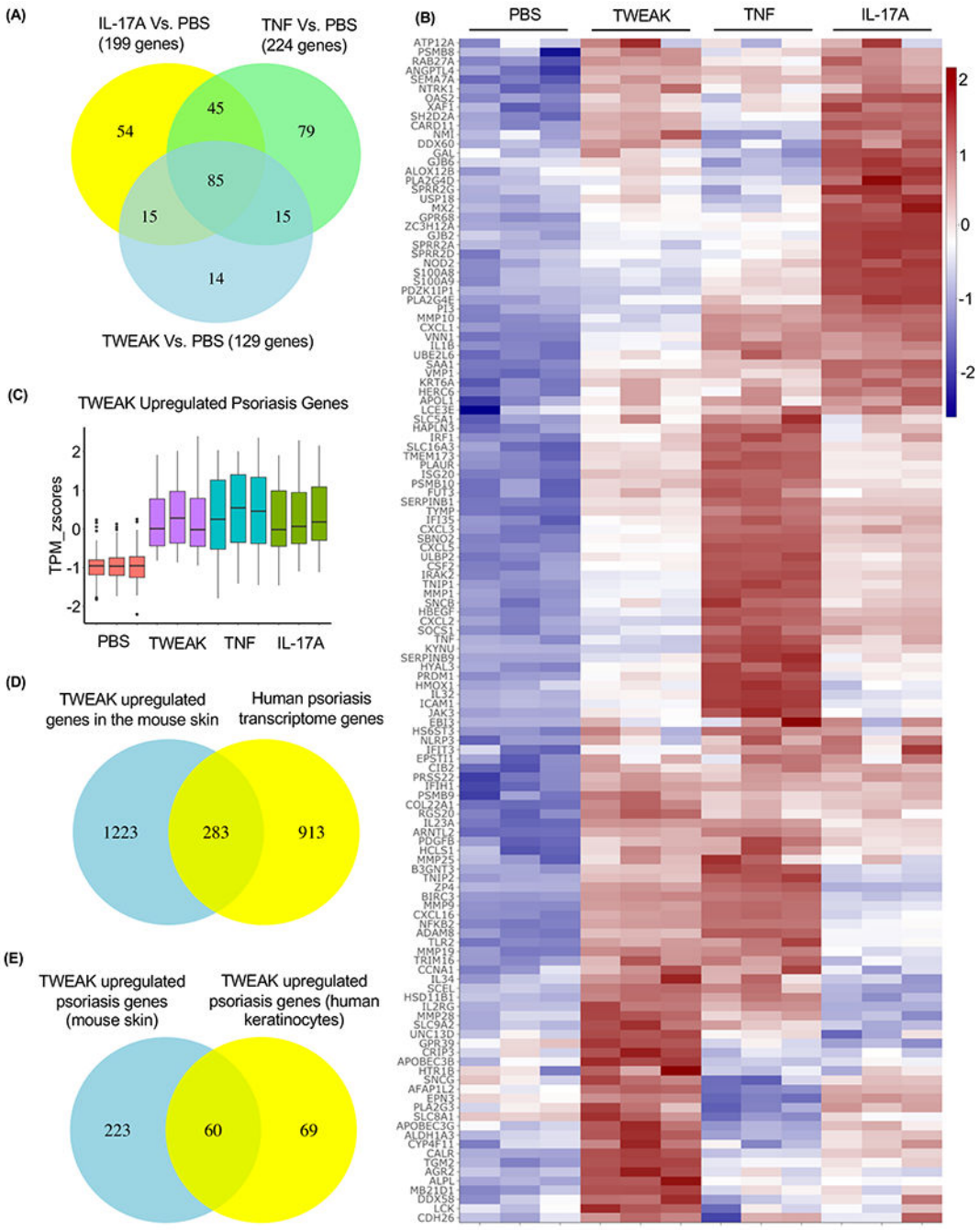

Fig. 2. TWEAK induces inflammatory activity in human keratinocytes overlapping with IL-17 and TNF.

(A-C) RNA-seq of human keratinocytes from triplicates stimulated with human TWEAK, IL-17A, or TNF. **(A)** Venn diagram comparing the numbers of upregulated unique and overlapping transcripts common to the human psoriasis biopsy transcriptome. (**B, C**) Heatmap and boxplot analysis showing the transcript patterns of the 129 psoriatic genes upregulated by TWEAK. (**D-E**) RNA-seq of skin from mice subcutaneously injected with mouse TWEAK. (**D**) Venn diagram showing the number of transcripts upregulated

in the skin common to the human psoriasis biopsy transcriptome. (**E**) Venn diagram showing the number of transcripts upregulated in the skin that were common to the human psoriasis biopsy transcriptome compared to the number upregulated by TWEAK in human keratinocytes.

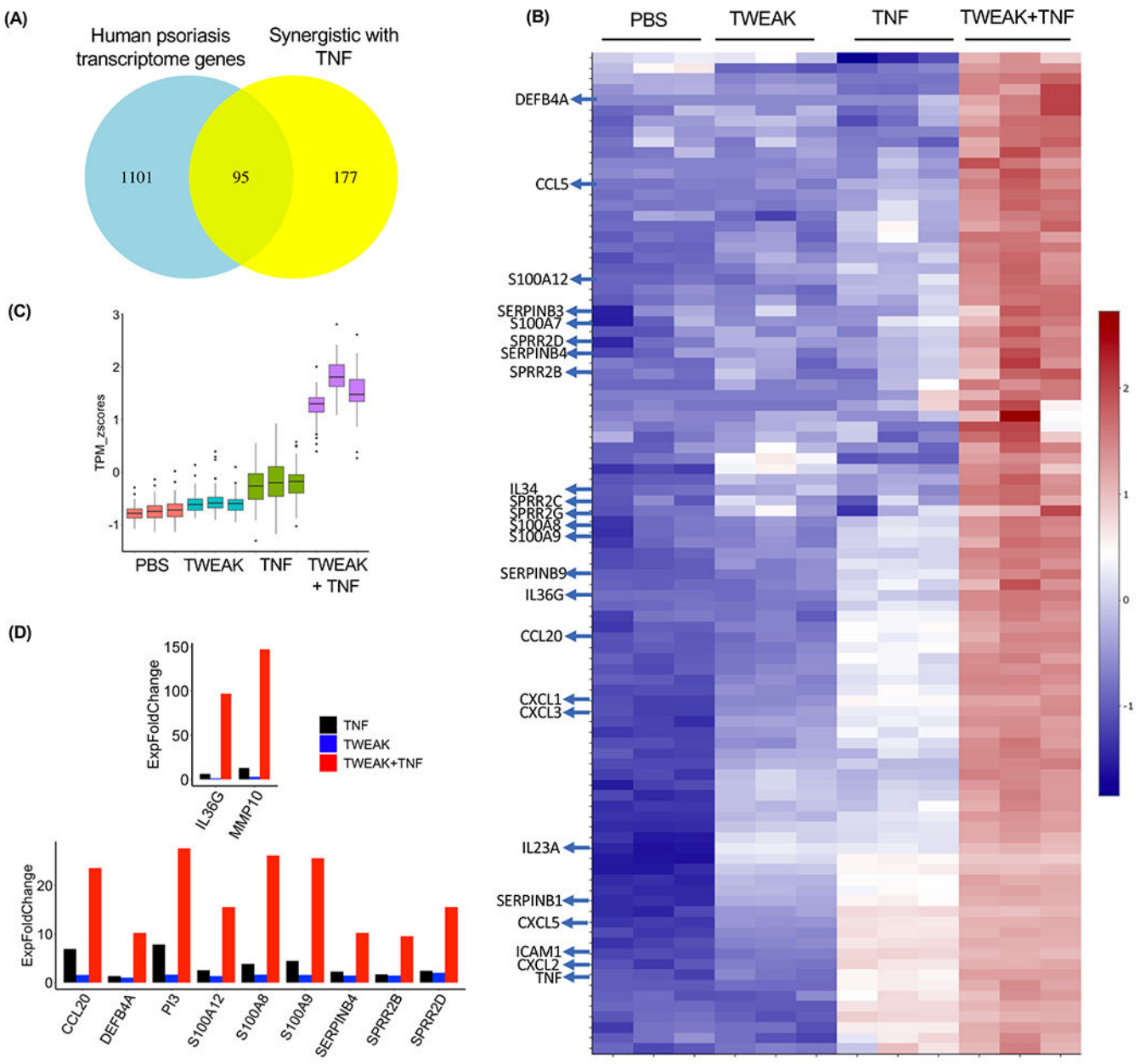

Fig. 3. TWEAK and TNF synergize to upregulate multiple psoriasis-relevant gene transcripts in human keratinocytes.

RNA-seq of human keratinocytes from triplicates stimulated with TWEAK or TNF alone or in combination. (**A**) Venn diagram showing the number of transcripts synergistically upregulated by TWEAK and TNF common to the human psoriasis biopsy transcriptome. (**B, C**) Heatmap and boxplot analysis showing the expression pattern of the 95 synergistically upregulated psoriatic genes. Several key psoriasis genes highlighted. (**D**) Experimental fold change for select psoriasis genes synergistically upregulated by TWEAK and TNF.

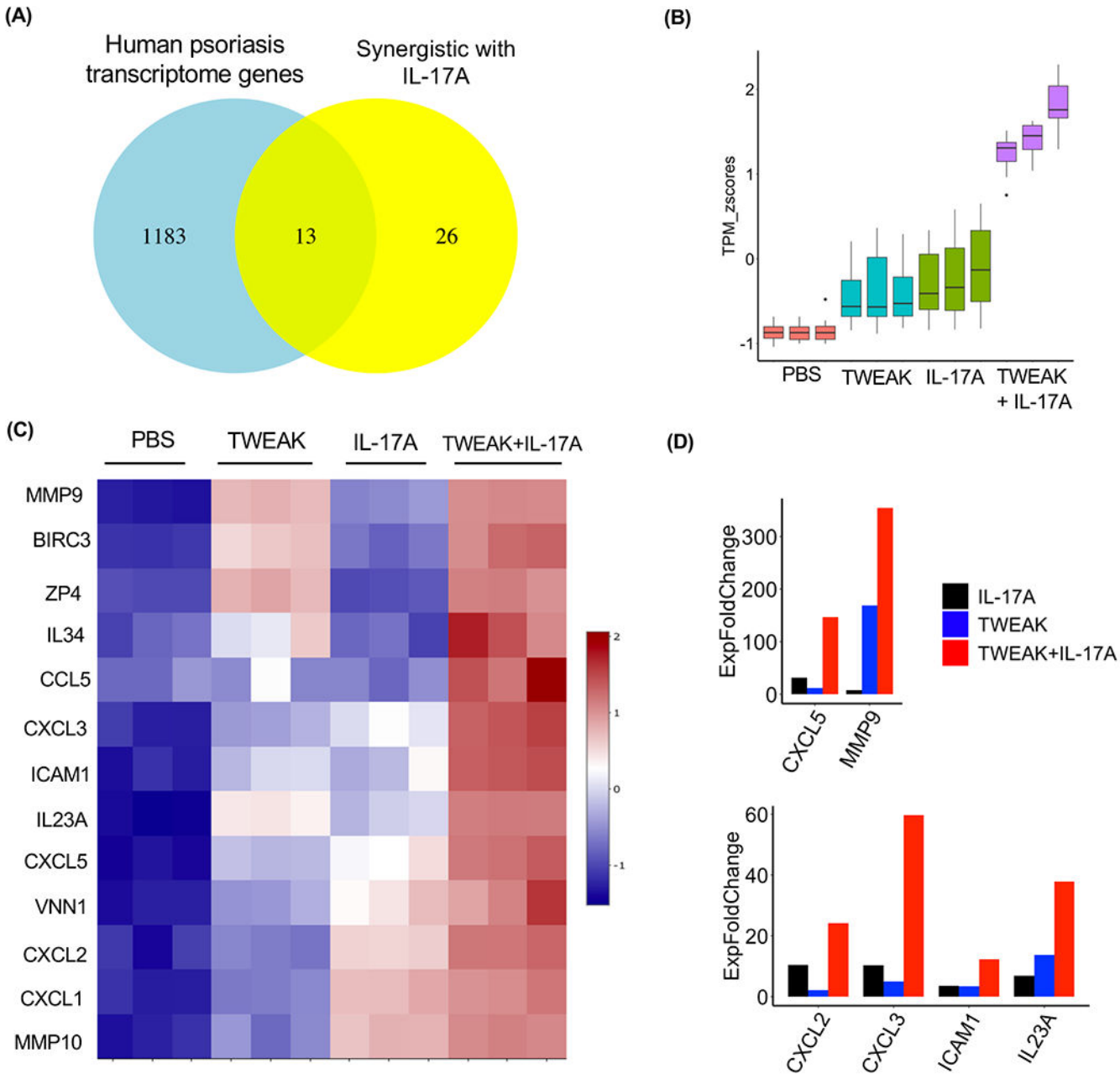

Fig. 4. TWEAK and IL-17A synergize to upregulate multiple psoriasis-relevant gene transcripts in human keratinocytes.

RNA-seq of human keratinocytes from triplicates stimulated with TWEAK or IL-17A alone or in combination. (**A**) Venn diagram showing the number of transcripts synergistically upregulated by TWEAK and IL-17A common to the human psoriasis biopsy transcriptome. (**B, C**) Heatmap and boxplot analysis showing the expression pattern of the 13 synergistically upregulated psoriatic genes. (**D**) Experimental fold change for select psoriasis genes synergistically upregulated by TWEAK and IL-17A.

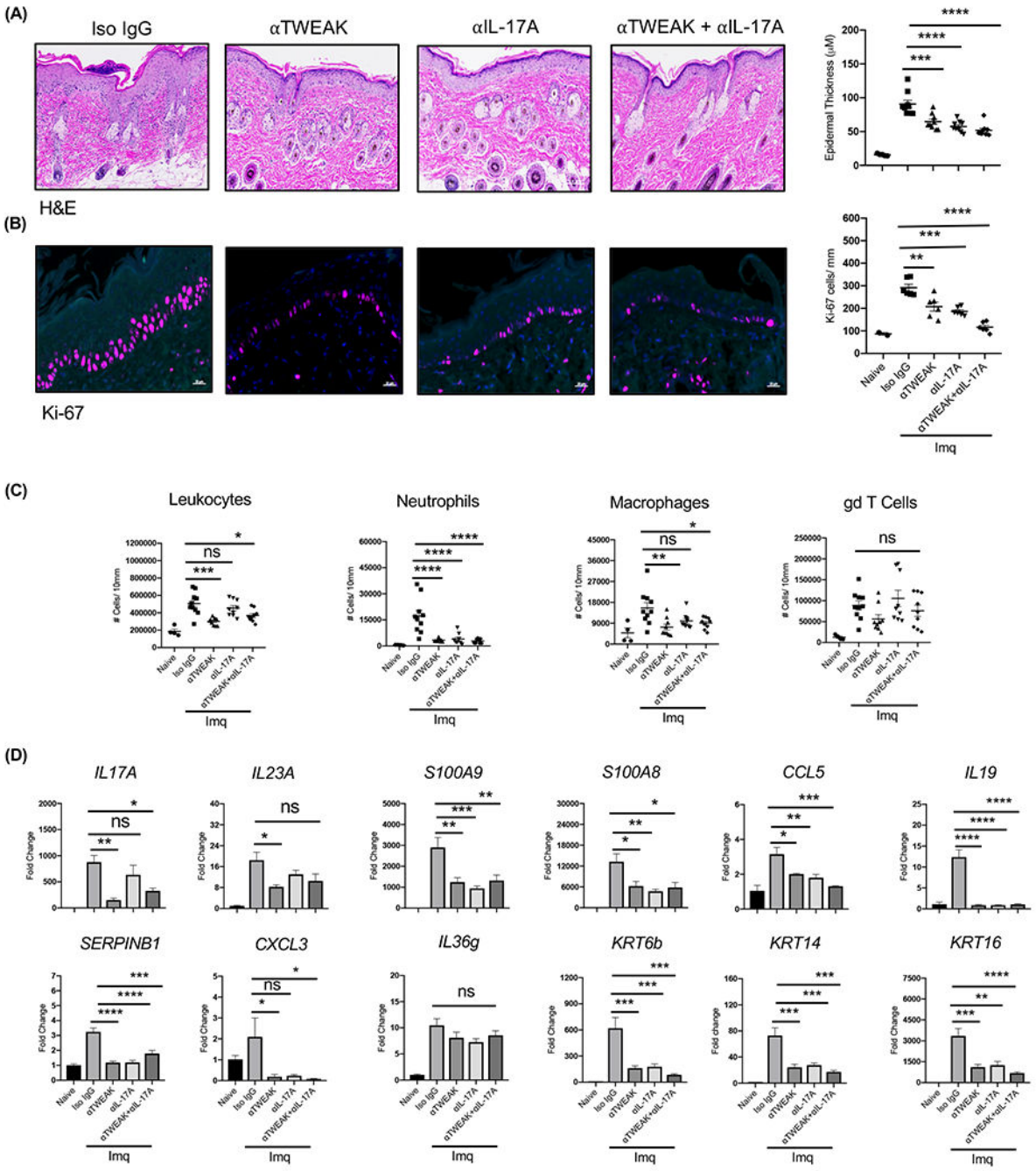

Fig. 5. Therapeutic blockade of TWEAK reduces clinical and immunological features of imiquimod-driven skin inflammation similar to blockade of IL-17A.

WT mice were treated with imiquimod on the dorsal skin for 11 constitutive days. Mice received anti-TWEAK or anti-IL-17A alone or together, or control IgG, on days 6 and 9, with analysis on day 12. (**A)** Representative H&E stained skin tissue sections, and quantitation of epidermal thickness in individual mice. (**B**) IF staining and enumeration of Ki-67+ cells/mm of epidermis in individual mice. (**C**) Immune cell infiltrates in skin biopsies showing the number of leukocytes, neutrophils, macrophages, and γδ T cells.

(**D**) mRNA expression of indicated genes in skin biopsies. GAPDH was considered as an endogenous control. Data mean fold change ± SEM over naïve group. (**A-C**) Data from 6-10 mice/group; (**D**) Data from 4-6 mice/group, combined from 2 experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

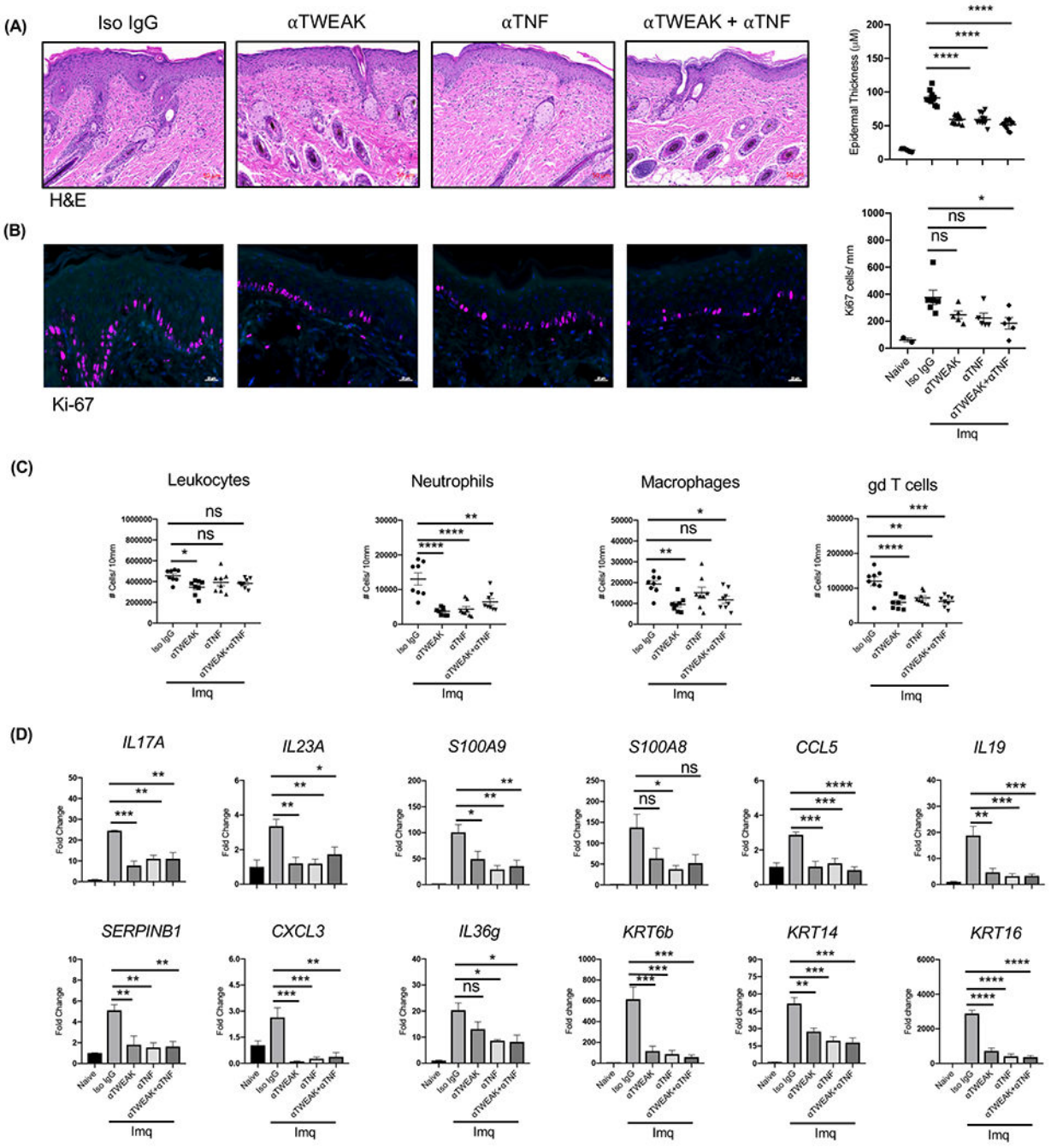

Fig. 6. Therapeutic blockade of TWEAK is comparable to blockade of TNF in reducing features of imiquimod-induced skin inflammation.

WT mice were treated with imiquimod on the dorsal skin for 11constitutive days. Mice received anti-TWEAK or anti-TNF alone or together, or control IgG, on days 6 and 9, with analysis on day 12. (**A)** Representative H&E stained skin tissue sections, and quantitation of epidermal thickness in individual mice. (**B**) IF staining and enumeration of Ki-67+ cells/mm of epidermis in individual mice. (**C**) Immune cell infiltrates in skin biopsies showing the number of leukocytes, neutrophils, macrophages, and γδ T cells. (**D**) mRNA expression of

indicated genes in skin biopsies. GAPDH used as an endogenous control. Data mean fold change ± SEM over naïve group. (**A-C**) Data from 6-10 mice/group; (**D**) Data from 4-6 mice/group, combined from 2 experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.