Title: IL-17A plays a central role in the expression of psoriasis signature genes through induction of IκB-ζ in keratinocytes

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Abbreviations: FC, fold change; FDR, false discovery rate; GSEA, gene set enrichment analysis

Abstract

In psoriasis lesions, a diverse mixture of cytokines is upregulated which influence each other generating a complex inflammatory situation. Although this is the case, the inhibition of Interleukin-17A (IL-17A) alone showed unprecedented clinical results in patients, indicating that IL-17A is a critical inducer of psoriasis pathogenesis. To elucidate IL-17A-driven keratinocyte-intrinsic signaling pathways, we treated monolayers of normal human epidermal keratinocytes *in vitro* with a mixture of six cytokines (IL-17A, TNF-α, IL-17C, IL-22, IL-36γ and IFN-γ) involved in psoriasis, to mimic the inflammatory milieu in psoriasis lesions. Microarray and gene set enrichment analysis revealed that this cytokine mixture induced similar gene expression changes with the previous transcriptome studies using psoriasis lesions. Importantly, we identified a set of IL-17A-regulated genes in keratinocytes, which recapitulate typical psoriasis genes exemplified by *DEFB4A*, *S100A7*, *IL19* and *CSF3*, based on differences in the expression profiles of cells stimulated with six cytokines versus cells stimulated with only five cytokines lacking IL-17A. Furthermore a specific IL-17A-induced gene, *NFKBIZ*, which encodes IκB-ζ, a transcriptional regulator for NF-κB, was demonstrated to have a significant role for IL-17A-induced gene

expression. Thus, we present novel *in vitro* data from normal human keratinocytes that would help elucidating the IL-17A-driven keratinocyte activation in psoriasis.

Introduction

Psoriasis, a chronic inflammatory skin disorder characterized by erythematous, scaly, sharply-demarcated plaques, involves excessive keratinocyte proliferation and leukocyte infiltration in thickened epidermal layers (1). Although psoriasis pathogenesis remains unclear, accumulating evidence suggests that immune-mediated inflammatory cytokine overproduction and amplified inflammation by keratinocytes are key abnormalities (2,3).

Within psoriatic lesions, keratinocytes respond to inflammatory cytokines, including interleukin (IL)-17A (2,3). IL-17A is produced by immune cells in psoriatic lesions (4,5) and directly affects the expression of keratinocyte genes involved in innate immune defenses (6-8). By releasing chemokines and cytokines, keratinocytes actively maintain inflammatory microenvironments and sustain plaque development (1,2).

Furthermore, tumor necrosis factor (TNF)-α is highly expressed in psoriatic skin and amplifies IL-17A effects on keratinocytes (7,9) . Interferon-γ (IFN- γ) is also upregulated in psoriatic lesions (10,11) and affects expression of several keratinocyte genes. IL-17A and IFN- γ regulate distinct psoriasis-related gene sets, thus playing distinct roles in disease pathogenesis (6). Psoriatic lesions also exhibit increased IL-17C (12), IL-22 (13), and IL-36

(14) expressions, implicated in psoriasis pathogenesis. Therefore, the interplay between cytokines expressed in psoriatic skin appears to be important for development and/or maintenance of psoriasis clinical features.

In the recent clinical study examining skin lesions from patients treated with anti–IL-17A monoclonal antibody, the set of psoriasis-related genes suppressed at week 2 is much larger and of greater magnitude than genes suppressed with 2 weeks treatment with the TNF antagonist etanercept (15). These observations suggest that IL-17A is the dominant cytokine inducing pathological changes in the expression of psoriasis-associated genes. However, *in vitro* studies of T-cell-derived cytokine-treated human keratinocytes have identified few products induced by IL-17A alone (6,7). Thus, IL-17A effects on inflammatory gene expression differ between *in vitro* results from cultured keratinocytes and clinical results from psoriatic lesions. Understanding IL-17A-specific and keratinocyte-intrinsic responses will require investigation of this discrepancy and development of an *in vitro* experimental condition that can reproduce clinical findings.

The reconstituted human epidermis, a 3D epidermal skin model comprising a keratinocyte multilayer supported by connective tissue, is useful for investigating IL-17A effects (16). This model closely mimics *in vivo* epidermal architecture wherein IL-17A

induces many downstream genes. However, the results obtained from this model are not clear enough to understand keratinocyte responses precisely because model cells are a mixed culture of keratinocytes and fibroblasts.

Here we aimed to reproduce *in vivo* changes in inflammatory gene expressions observed in clinical psoriasis findings, by a simple method which utilizes normal monolayer keratinocyte stimulated with cytokines. Inflammatory gene expressions in this model were compared with those observed in clinical studies of psoriatic skin lesions.

Methods

Cell culture

Primary normal human epidermal keratinocytes were purchased from KURABO (Tokyo, Japan) and cultured in DermaLife K Comp kit (KURABO). The human keratinocyte cell line HaCaT was maintained in DMEM containing 10% FCS. Recombinant human (rh) IL-17A, rhIL-17C, rhIL-17F and rhIL-22 were purchased from R&D Systems (Minneapolis, MN, USA). rhTNF-α was kindly provided by Dainippon Sumitomo Pharmaceutical (Osaka, Japan). rhIFN-γ was purchased from Wako Pure Chemical Industries (Osaka, Japan) and rhIL-36γ was purchased from eBioscience (San Diego, CA, USA). Cytokine concentrations used (IL-17A, 100 ng/mL; TNF-α, 10 ng/mL; IFN-γ, 10 ng/mL; IL-17C, 100 ng/mL; IL-22, 100 ng/mL; IL-36γ, 500 ng/mL) were determined by considering previous reports (6,7,12,14,17). Cytokines were used at half the concentrations used in these reports based on pilot experiments (data not shown).

DNA microarray experiment

RNA was extracted from keratinocytes using RNeasy mini kit (Qiagen, Tokyo, Japan). Complementary RNA was generated and then labeled with cyanine 3 using a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Labeled cRNA was hybridized to Agilent whole human genome oligo microarray ver.2.0 (4 × 44K). Signals were detected with an Agilent DNA Microarray Scanner, and the scanned images were quantified using the Feature Extraction Software (ver. 10.7.3.1). Data were analyzed using GeneSpring GX 13.0 (Agilent Technologies). After data normalization, gene expression profiles of keratinocytes treated with all six cytokines were compared with those of keratinoctyes treated with each combination lacking one cytokine. Genes expressed >2-fold higher with six cytokine treatment than with five cytokine treatment were noted. GSEA was conducted using GSEA software (18). Normalized ESs with an associated FDR ≤ 0.01 were considered to be statistically significant. These data have been deposited in the public repository Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSExxxxxx.

Measurement of cell growth

Keratinocytes proliferation was assessed using a cell counting kit-8 (Dojindo, Kumamoto,

Japan), a modified MTT assay kit which utilizes water-soluble tetrazolium salt, WST-8. Keratinocytes were seeded at 5000 cells/well in 96-well plates with the combinations of cytokines. Two or three days later, the kit reagent WST-8 was added directly to the culture medium, and the culture was incubated for an additional 2 h, after which the absorbance of formazan was measured at 450 nm by a microplate reader (Bio-Rad, Hercules, CA).

Quantitative real-time PCR (qRT-PCR)

Using 1 μg of total RNA, cDNA was synthesized with ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan). qRT-PCR analysis of respective genes was conducted using KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA). PCR amplification and evaluation were performed using an Mx3005P real-time PCR system (Stratagene, Santa Clara, CA, USA). Primers used for qRT-PCR are indicated in supplementary table S1. Data are expressed as mean ± standard deviation. Data were analyzed using Student's two-tailed t-test.

Western Blotting

Western blotting was performed as described previously (19) using

commercially-obtained primary antibodies against IκB-ζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (Sigma-Aldrich). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were purchased from GE Healthcare.

Results

Mixture of six inflammatory cytokines induces gene expression changes in primary keratinocytes

To clarify IL-17A role and its interplay with other cytokines in keratinocyte activation, we treated *in vitro* monolayers of normal human epidermal keratinocytes (NHEKs) with a mixture of IL-17A, TNF-α, IL-17C, IL-22, IL-36γ, and IFN-γ.

Comparison of cells treated with and without this mixture revealed that 1636, 441, and 228 gene probes were upregulated >2-, 5-, and 10-fold, respectively, and 3281, 219, and 49 gene probes were downregulated >2-, 5- and 10-fold, respectively, by this mixture (Figure 1A; full list of differentially-expressed gene probes provided in Supplementary Table S2 online).

Comparison of genes induced by mixture of six cytokines with reported transcriptome by gene set enrichment analysis (GSEA)

We quantified biological similarities between our data and most recently published

comprehensive transcriptome results from psoriatic lesions (20) using GSEA (18).

Previously reported upregulated or downregulated genes were categorized as top 200, top 500, or all according to the fold change (FC) for lesional psoriatic skin vs. normal skin and were assessed for enrichment in our gene expression profiles specific to keratinocytes stimulated with this mixture (Table 1). GSEA showed that each category of genes upregulated in the Li study was significantly biased [false discovery rate (FDR) < 0.01] toward the increased expression in six cytokine mixture-stimulated keratinocytes (Figure 1B; Table 1). Li's top 200 upregulated gene sets gave the highest enrichment score (ES, 0.76) and top 500 upregulated gene sets followed (0.67), indicating that genes upregulated by this mixture corresponded well with genes highly expressed in psoriasis. Conversely, downregulated gene sets reported by Li et al generated low ESs (Table 1), indicating that downregulated gene sets differed between our model and psoriasis lesions. These results indicate that our experimental model at least partially reflects genes upregulated in psoriatic skin.

Microarray data were evaluated for six cytokine-induced expression changes in mRNAs of keratinocyte differentiation markers (Supplementary Table S3 online). Six cytokine mixture reduced the expression of the differentiation marker genes loricrin (*LOR*) and

filaggrin (*FLG*) in cultured NHEKs and these expression changes were similar to that in lesional psoriatic skin (20). However, there were some considerable differences in the expression changes of other differentiation markers (*KRT1*, *IVL*), and proliferation markers (*MKI67*, *KRT16*) between six cytokine-stimulated cells and lesional psoriatic skin (Supplementary Table S3 online). Also, cell proliferation of NHEKs was inhibited by the treatment of six cytokine mixture (Data not shown), while hyperproliferation of keratinocytes is a clinical feature of psoriasis. Therefore, our method with monolayer of normal keratinocytes did not recapitulate some characteristic features of psoriasis, which may be partially due to requirement of a 3D epidermal skin model composed of a keratinocyte multi-layer supported by connective tissue for complete replication of lesional skin.

IL-17A-regulated gene probes are less numerous than TNF-α- or IFN-γ-regulated probes but highly enriched for genes upregulated in skin lesions

Next, we evaluated which cytokine in the six cytokine mixture regulates the induction of the upregulated genes. NHEKs were exposed to all combinations of five of six cytokines (Figure 2A and Supplementary Tables S4-S9 online). Gene expression profiles of

keratinocytes treated with full mixture (six cytokines) were compared with those of keratinoctyes treated with each combination lacking one cytokine. Genes expressed >2-fold higher with six cytokine treatment than with five cytokine treatment were identified as the genes regulated by the cytokine lacking in the five cytokine treatment. Figure 2A shows total number of gene probes induced by six cytokine mixture as well as number of particular gene probes regulated by each cytokine. The data analysis was conducted at three different cutoff values of >2-, 5-, and 10-fold induction by six cytokines. IFN-γ-regulated gene probes were the most abundant followed by TNF-α- and then IL-17A-regulated gene probes. IL-17C, IL-22, and IL-36γ had less influence on keratinocyte gene expression, especially for gene probes with high FC values (>5- or 10-fold). This cytokine ranking by the number of genes affected is similar to that observed in a previous transcriptome study of psoriatic skin (21). These data indicate that IFN-γ and TNF-α affect the expression of more keratinocyte genes than IL-17A.

To estimate the clinical relevance of cytokine-regulated genes in psoriasis pathogenesis, we again compared these genes with those upregulated in psoriasis lesions (20) (Figure 2B and Supplementary Tables S4-S9 online). Of genes upregulated >10-fold by six cytokines in keratinocytes, over 90% of those regulated by IL-17A corresponded to genes upregulated

in psoriasis lesions; TNF-α- and IFN-γ-regulated genes showed a lower degree of overlap with psoriasis genes (Figure 2B and C). Additionally, over 40% (16 of 37) of those IL-17A-regulated genes, such as *DEFB4A*, *IL1F9*, *CXCL1*, *CCL20*, *RHCG*, and *IL19*, overlap with the reported clinical study results with psoriatic skin lesions treated with anti-IL-17A antibody (Figure 2C) (15).

Li et al (20) used weighted gene co-expression network analysis to demonstrate that two of 18 normal skin modules (N10 and N15) and three of 27 psoriatic modules (P20, P23, and P26) have a strong keratinocyte gene expression signature. Importantly, modules P20 and P26 have the highest proportion of genes upregulated in lesional skin and are enriched for genes involved in inflammation, immune responses, and keratinization. We selected three psoriatic modules that have keratinocyte gene signature (P20, P23, and P26) and three psoriatic modules that have immune cell signature with high proportions of upregulated genes (P13, P19, and P21), and compared those modules with the genes upregulated by treatment with six cytokines and with the genes upregulated by IL-17A, TNF-α, or IFN-γ (Figure 3A). Whereas low percentage (ranging from 0.68% to 7.26%) of the total set of 441 upregulated genes contained selected co-expression modules, high percentage of IL-17A-regulated genes contained the modules, in particular module P20 (19 genes, 33.9%)

and module P26 genes (11 genes, 19.6%) with none in other selected module genes. The Venn diagram to analyze each cytokine dependency and their overlaps (Figure 3B) shows that module P20 genes were mainly regulated by both of IL-17A and TNF-α (18 genes), and nine of those IL-17A and TNF-α –coregulated genes were also regulated by IFN-γ and include psoriasis signature genes, such as *IL17C*, *IL1F9*, and *IL23A*. The results demonstrate the co-operative action between IL-17A and TNF-α with a substantial role of IFN-γ in the induction of module P20 genes. Conversely, module P26 genes were mainly IL-17A-unique genes (9 of 17), indicating that TNF-α and IFN-γ are less important for the gene expressions of module P26 genes (Figure 3C). These results show that there are two patterns of gene co-expression networks; keratinocyte gene signature induced in common by IL-17A, TNF-α, and IFN- γ (e.g module 20), and those induced uniquely by IL-17A (e.g. module 26).

Validation of microarray results by qRT-PCR

To verify microarray results, we selected several IL-17A-regulated genes and quantified expression of these genes in cytokine-treated keratinocytes by qRT-PCR. Six cytokine mixture induced expression of selected genes, and the strongest reduction in expression of

these genes was observed with treatment using five cytokines without IL-17A (Figure 4A). Removal of other cytokines from six cytokine mixture had less effect on expression of these genes. Although there are some slight differences between qRT-PCR and microarray data (e.g. the effect of TNF-α removal on *IL19* expression; see Figures 3B and 4A), these data mostly reproduced microarray results and verified that the expression changes of the selected genes are dependent on IL-17A.

We examined whether IL-17F, another cytokine of IL-17 family, can compensate for the absence of IL-17A from the cytokine mixture (Figure 4B). The addition of IL-17F (100 ng/ml; at same concentration as IL-17A) to five cytokines lacking IL-17A failed to restore significantly the reduced expression of the IL-17A-regulated genes in keratinocytes, indicating that, at the same concentration, IL-17A is more potent than IL-17F for the induction of selected genes in keratinocytes.

Next, we evaluated whether each cytokine alone or combination with other cytokine can induce expression of IL-17A-regulated genes. Treatment with each cytokine alone had little or no effect on expression of selected genes; however, these cytokines in combination with IL-17A significantly increased gene expression (Figure 4C). Conversely, TNF-α

combination with each cytokine other than IL-17A did not increase expression of selected genes.

Involvement of IκB-ζ in cytokine-induced keratinocyte activation

Of 37 IL-17A-upregulated genes (Figure 2C), we noticed that only *NFKBIZ*/IκB-ζ is a nuclear transcription cofactor. We hypothesized that *NFKBIZ*/IκB-ζ may regulate IL-17A-induced keratinocyte activation and examined relevance of *NFKBIZ*/IκB-ζ induction with the expression of IL-17A-regulated genes using HaCaT cells. As shown in Figure 5A, increase in *NFKBIZ* expression in HaCaT cells induced by IL-17A peaked at 1.5 h after stimulation. Upregulation of IκB-ζ protein by IL-17A was observed at 3 h after stimulation (Figure 5B). *IL19* and *CSF3* mRNA showed time dependent upregulation, gradually increasing after 3 h (Figure 5C). These data demonstrate that IL-17A-induced upregulation of *NFKBIZ* is relatively rapid and is followed by other IL-17A target genes' upregulation.

Lastly, the role of *NFKBIZ*/IκB-ζ on the expression of IL-17A-regulated genes was examined. Figure 5D shows that IL-17A-induced *DEFB4A*, *IL19*, and *CSF3* expressions were significantly decreased by siRNA knockdown of *NFKBIZ*, even when HaCaT cells

were co-stimulated with IL-17A and TNF-α. In contrast, IFN-γ did not upregulate *NFKBIZ*, and the expression of IFN-γ-regulated genes (*IRF1* and *STAT1*) induced by IFN-γ was not affected by *NFKBIZ* siRNA, suggesting IL-17A-specific IκB-ζ involvement (Figure 5D). These data indicate that *NFKBIZ*/IκB-ζ is essential for IL-17A-induced gene expression in keratinocytes.

Discussion

We investigated the effects of a mixture of 6 well-characterized proinflammatory cytokines of IL-17A, IL-17C, IL-22, IL-36γ, IFN-γ and TNF-α on gene expression in human keratinocytes by microarray analysis. GSEA revealed that the six cytokine mixture induced upregulation of genes similar to those reported in transcriptome studies of psoriasis lesions (20) (Figure 1B). In order to determine how synergistically upregulated genes relate to the psoriasis transcriptome, we compared expression profiles of cells stimulated with six cytokines and those stimulated with five cytokines lacking one of the cytokines, and identified genes regulated by each cytokine. The comparison of the genes regulated by each cytokine with transcriptome results showed that, although the number of the TNF-α- or IFN-γ-regulated genes was higher than the number of IL-17A-regulated genes (Figure 2A), a higher percentage of IL-17A-regulated genes corresponded to those upregulated in psoriatic skin lesions (Figure 2B). Moreover, many of the IL-17A-regulated genes were also reported as the genes strongly suppressed by IL-17A blockade in patients with psoriasis (15)(Figure 2C). The IL-17A-regulated genes include important cytokines

implicated in psoriasis pathogenesis, such as *IL17C* (encoding IL-17C), *IL1F9* (IL-36γ), and *IL19* (IL-19) (12,14,22) (Figure 2C). *IL23A* was also detected as an IL-17A-induced gene in keratinocytes. This cytokine stimulates Th17 cells to produce IL-17A and IL-22, thereby exacerbating psoriasis (2,3). Dominant role of IL-23 in psoriasis has been confirmed by the observation that monoclonal antibody targeting the IL-23 p19 subunit shows remarkable clinical efficacy (23-25). Additionally, IL-17A-regulated genes include inflammatory chemokines (*CXCL1*, *CXCL2*, *CXCL5*, and *CCL20*) that are proposed to be important for leukocyte infiltration (26). High overlaps with the clinical findings of psoriasis suggest that our *in vitro* data successfully mimicked lesional keratinocytes and recapitulated IL-17A-induced response that amplifies and prolongs psoriatic inflammation through immune cell feedback (2).

Li et al reported multiple modules of coordinately expressed genes based on weighted gene co-expression network analysis of the transcriptomes of lesional psoriatic and normal skin. IL-17A-regulated genes in our system were enriched for the specific module sets of P20 and P26, which have the highest proportion of genes upregulated in the lesional skin (20). It was reported by Li et al that module P20 genes are involved in inflammatory and immune responses, and P26 genes are involved in keratinization. Our analysis revealed that

module P20 and P26 genes have different cytokine dependencies. Module P20 genes which are reported to express in lesional skin average of ~9 folds higher than normal skin were mainly regulated by combined actions of IL-17A and TNF-α (Figure 3B), indicating the necessity of synergy by the combination for the vast induction of the genes. On the other hand, IL-17A uniquely regulated many of module P26 genes, especially a number of small proline rich protein (SPRR) genes (6 of 11 in the family), the major components of the cornified keratinocyte cell envelope which are structurally responsible for the barrier properties of skin (27) (Figure 3C). It is interesting to note that the induction of the SPRR genes is solely dependent on IL-17A (Figure 3C and 4A), suggesting that inhibition of IL-17A, but not other cytokine, results in the regression of the expression.

The qRT-PCR experiments on the selected IL-17A-regulated genes showed similar results as the DNA microarray (Figure 4A). Experiments assessing the effect of two cytokine combinations further confirmed IL-17A as the important cytokine regulating expression of the selected genes (Figure 4C). IL-17A in concert with other cytokines (e.g. TNF- α, IL-36γ, IFN-γ) induced high expression of selected genes, whereas TNF-α combined with other cytokines except IL-17A did not, indicating that IL-17A is essential, but TNF-α is dispensable for the expression of these genes in keratinocytes. Nevertheless,

the observation that expression of all IL-17A-induced genes (*DEFB4A*, *IL19*, *SERPINB3*, and *CSF3*) was significantly enhanced by TNF-α indicates a close functional relationship between IL-17A and TNF-α. This observation is consistent with a previous study (7) and with observed clinical effects of anti-TNF treatment (9), confirming the importance of TNF-α as an enhancer of IL-17A action.

Of the IL-17A-induced genes in keratinocytes, expressions of *DEFB4A* and *IL19* were drastically reduced by removal of IL-17A (Figure 4A). Consistently, rapid and strong suppression of *DEFB4A* and *IL19* expressions in lesional skin was observed by IL-17A neutralization in psoriatic patients (15). It was shown that serum level of β-defensin-2 (encoded by *DEFB4A*) and IL-19 increase in psoriasis patients (22,28), indicating that the proteins might serve as serum biomarker for IL-17A-driven skin inflammation like psoriasis.

GSEA results showed low similarity between genes downregulated by six cytokine treatment and those downregulated in psoriatic lesions (Table 1). These results are not surprising because downregulated genes in psoriasis biopsies compared to normal skin include a considerable number of dermis-derived transcripts independently of intracellular changes in gene transcription. This tendency is attributed to the marked expansion of the

epidermis that greatly decreases the proportion of dermal and other types of cells (20). Our experimental model uses epidermal keratinocyte cultures, so the outcome is a direct reflection of changes in keratinocyte-intrinsic gene expression induced by cytokines.

We identified the nuclear transcription cofactor *NFKBIZ*/IκB-ζ as a downstream modulator of IL-17A responses in keratinocytes. While the finding of IκB-ζ involvement in IL-17-induced and IL-17A/TNF-α-combination-induced gene expression in lung epithelial cells has previously been reported (29,30), our data provide evidence for a role of IL-17A-induced IκB-ζ in IL-17A/TNF-α-induced gene expression in keratinocytes. Such function of IκB-ζ is distinct from its known role in $CD4^+$ T cells where IκB-ζ plays a critical role in the development of Th17 cells (31), and thus provides additional insights into pathogenesis of psoriasis. During preparation of our manuscript, a critical involvement of IκB-ζ in psoriasis mouse models was reported through the analyses of IκB-ζ-deficient mice (32). Moreover, the same study demonstrated that in human primary keratinocytes IκB-ζ regulates IL-17A-induced expression of a panel of psoriasis-associated genes (32). Our data together with these findings suggest that the enhanced expression of IkB-ζ in keratinocyte is important for psoriasis pathogenesis.

Intriguingly, disruption of IκB-ζ expression in epithelial cells enhances apoptosis of

epithelial cells that triggers the activation of self-reactive lymphocytes and Sjögren's syndrome-like symptoms (33), suggesting that IκB-ζ in epithelial cells plays a critical homeostatic role in epithelial tissues such as the lacrimal glands. Therefore, expression of IκB-ζ should be tightly regulated at the appropriate level for the control of homeostatic and pathogenic balance. Although IL-17A is shown to increase the expression of IκB-ζ, the exact molecular mechanism for the induction and tight regulation of IκB-ζ expression in cells remains to be clarified in future studies.

In activated B-cell-like diffuse large B-cell lymphoma, physical interaction of IκB-ζ with NF-κB subunits is essential for the expression of large number of known NF-κB target genes (34), indicating that IκB-ζ can act in concert with NF-κB transcription factors to enhance gene expressions. Considering that TNF-α is a strong inducer of NF-κB activation (35), NF-κB-modulating function of IκB-ζ might be essential for previously reported synergistic action of IL-17A and TNF-α in keratinocytes, which is implicated in psoriasis pathogenesis (7). *NFKBIZ* has been recently identified as a psoriasis susceptibility locus in a genomewide association study and as a downstream target of IL-17 receptor signaling in human keratinocytes (36), further suggesting relevance of IL-17A-induced expression of *NFKBIZ* in keratinocytes to the pathogenesis of psoriasis. In summary, we present *in vitro*

keratinocyte gene expression data that may help identify unique molecular targets of IL-17A and IL-17A/TNF-α in keratinocytes. These data should be useful in future studies of IL-17A-induced signaling pathway mechanism in keratinocytes and for investigating the interplay between IL-17A and other cytokines.

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Figure Legends

Figure 1. Six cytokine mixture effectively upregulates psoriasis signature genes in monolayer cultured NHEK

(A) Scatter plot of six cytokine mixture-stimulated keratinocytes. Scatter plots comparing probe signals for unstimulated and six cytokine-treated keratinocytes (concentration of each cytokine was as in material and methods). The inner two parallel red lines represent a 2-fold increase or decrease, whereas the outer two lines reflect a 10-fold change. (B) Enrichment plots for Li's top 200 upregulated genes in our data set sorted by fold change expression. ES: Enrichment Score; NES: Normalized Enrichment Score; FDR: False Discovery Rate.

Figure 2. Identification of IL-17A-regulated genes

(A) Gene probes that were expressed higher with six cytokine treatment than with five cytokine treatment lacking each one cytokine were designated as cytokine-regulated and counted. Shown at different cut-offs (>2-, 5-, or 10-fold change by six mixture). (B) Overlaps between the gene sets shown in (A) and previously published data of genes

upregulated in psoriasis lesion (20). (C) Expression levels of IL-17A-regulated 37 genes with >10-fold change by six mixture. Numbers indicated on the heat map are the $\log_2$ FC values. Symbols by blue text represent genes NOT upregulated in psoriasis (20). Asterisks represent previously described genes that were downregulated greater than 2-fold by IL-17A neutralization (15).

Figure 3. Selective enrichment of psoriatic modules P20 and P26 genes in IL-17A-regulated genes.

(A) Genes belonging to previously annotated gene coexpression modules (P13, P19, P20, P21, P23 and P26; (20)) were counted. The numbers as well as the percentage of total (numbers in parentheses) are shown in the table. The circle graphs show the genes in each module as the percentage of their numbers relative to the total number. (B and C) IL-17A, TNF-α and IFN-γ induce common and unique keratinocyte gene signatures. For module P20 (B) or P26 (C) genes, Venn diagram illustrates the number of genes upregulated by IL-17A, TNF-α or IFN-γ in six cytokine-stimulated keratinocytes. Surrounding tables list genes that are regulated by the cytokines with Agilent ProbeNames.

Figure 4. Validation of microarray results by qRT-PCR

(A) Primary keratinocytes were stimulated with the mixture of six cytokines (IL-17A, TNF-α, IL-22, IL-17C, IL-36γ and IFN-γ) or five cytokine mixtures lacking each one of cytokines for 24 h. The expressions of indicated mRNA were analyzed by qRT-PCR. Data are means ± SD (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with six cytokine mixture-treated keratinocytes. The cytokine concentrations used were as described in material and methods. (B) Primary keratinocytes were stimulated with the mixture of six cytokines, five cytokines lacking IL-17A, or five cytokines lacking IL-17A plus IL-17F (100 ng/ml; the same concentration as IL-17A) for 24 h. The expressions of indicated mRNA were analyzed by qRT-PCR. Data are means ± SD (n=3). (C) Stimulations by each cytokine with IL-17A or TNF-α. Expressions of indicated mRNAs were measured. Data are means ± SD (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with IL-17A alone-treated keratinocytes.

Figure 5. The role of IκB-ζ in IL-17A-induced gene expression

(A and B) Induction time courses of IκB-ζ mRNA and protein levels following stimulation. The amount of IκB-ζ mRNA (A) or protein (B) in HaCaT cells was determined by

qRT-PCR (A) or western blot (B) at the indicated time after stimulation with IL-17A (100 ng/ml), TNF-α (10 ng/ml), or IL-17+TNF-α. (C) Time course analysis of IL-17A-induced mRNA exressions. (D) siRNA knockdown of *NFKBIZ*/IκB-ζ. HaCaT cells transfected with 20 pmol of *NFKBIZ*-siRNA (siNFKBIZ) or control siRNA (siCont) were incubated with or without IL-17A (100 ng/ml) or IL-17A+TNF-α (10 ng/ml) for 3 h. For the specificity control, the effect of IκB-ζ knockdown on the induction of *IRF1* and *STAT1* mRNA by IFN-γ (10 ng/ml, 3 h) was evaluated. The expression of the transcripts is shown as relative values. Data are means ± SD (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 1. Summary of GSEA assessing the enrichment of psoriatic signature gene sets in genes differentially expressed in keratinocyte stimulated with 6 cytokine mixture

Gene Set	FC values	No. of genes	No. of genes overlapping with sorted dataset	ES	NES	FDR
Li_2014_RNA-SEQ_Top200_UP	> 6.75	200	84	0.76	3.95	$< 10^{-4}$
Li_2014_RNA-SEQ_Top500_UP	> 3.10	500	208	0.67	3.9	$< 10^{-4}$
Li_2014_RNA-SEQ_All_UP	> 2.0	1016	431	0.52	3.21	$< 10^{-4}$
Li_2014_RNA-SEQ_Top200_DOWN	< 0.157	200	27	0.15	0.61	0.931
Li_2014_RNA-SEQ_Top500_DOWN	< 0.257	500	74	-0.15	-1.06	0.372
Li_2014_RNA-SEQ_All_DOWN	< 0.50	2415	449	-0.17	-1.81	0.016

FC: Fold Change; ES: Enrichment Score; NES: Normalized Enrichment Score; FDR: False Discovery Rate

Figure 1

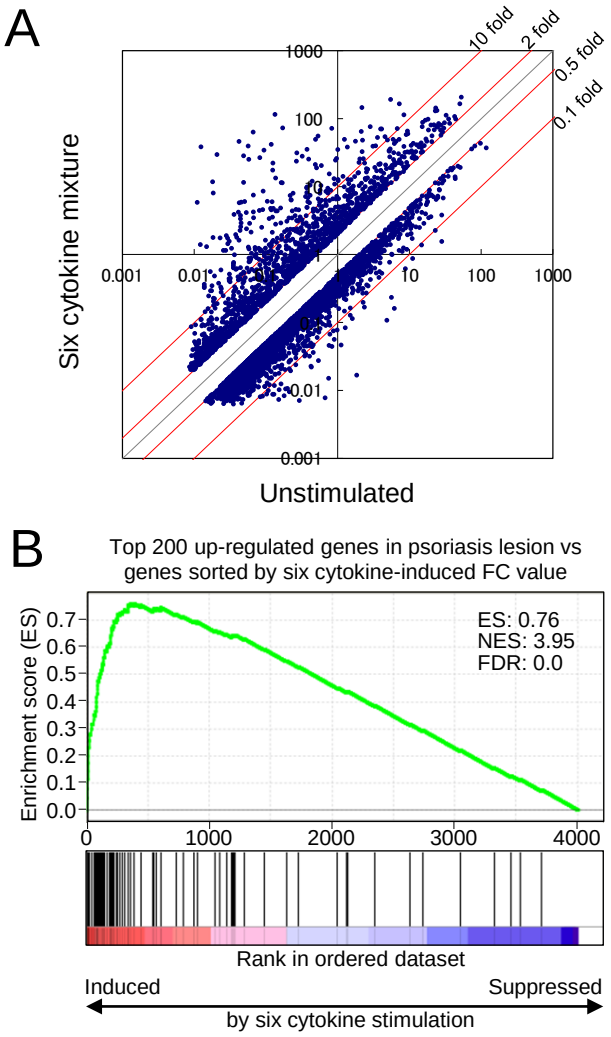
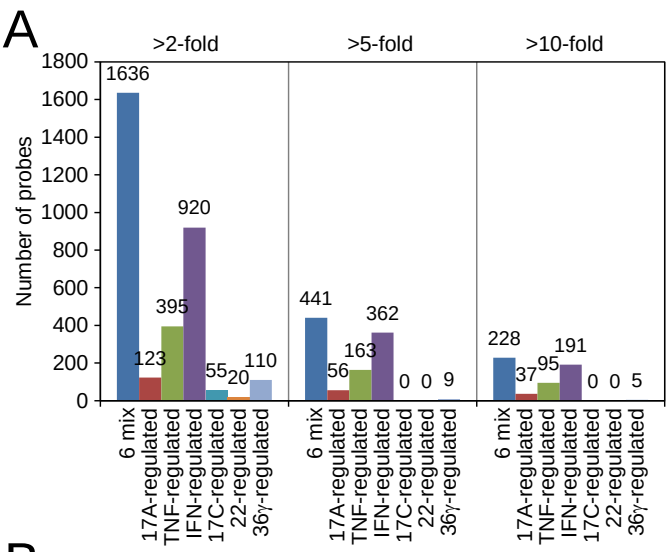
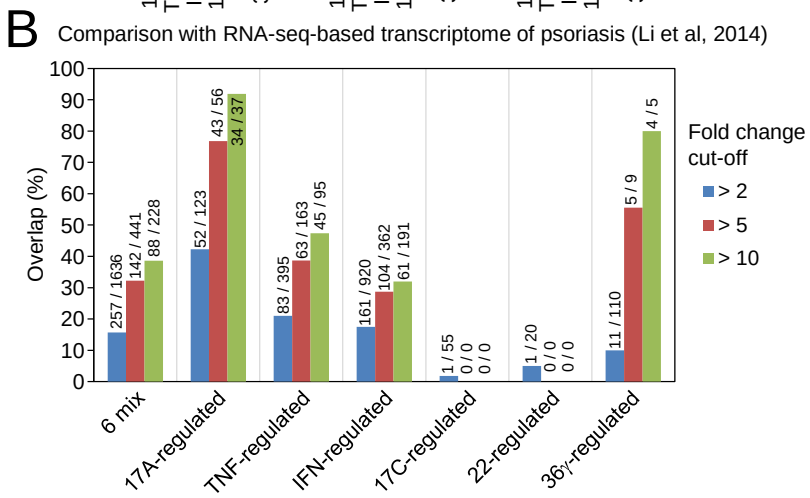

A
10 fold
2 fold
0.5 fold
0.1 fold
1000
100
10
0.001
0.01
10
100
1000
0.01
0.001
Six cytokine mixture
Unstimulated
B
Top 200 up-regulated genes in psoriasis lesion vs
genes sorted by six cytokine-induced FC value
ES: 0.76
NES: 3.95
FDR: 0.0
Enrichment score (ES)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1000
2000
3000
4000
Rank in ordered dataset
Induced
Suppressed
by six cytokine stimulation

Figure 2


A
>2-fold
>5-fold
>10-fold
Number of probes
1800
1600
1400
1200
1000
800
600
400
200
0
1636
123
395
920
55
20
110
441
56
163
362
0 0 9
228
37
95
191
0 0 5
6 mix
17A-regulated
TNF-regulated
IFN-regulated
17C-regulated
22-regulated
36γ-regulated


B
Comparison with RNA-seq-based transcriptome of psoriasis (Li et al, 2014)
Overlap (%)
100
90
80
70
60
50
40
30
20
10
0
257 / 1636
142 / 441
88 / 228
52 / 123
43 / 56
34 / 37
83 / 395
63 / 163
45 / 95
161 / 920
104 / 362
61 / 191
1 / 55
0 / 0
0 / 0
1 / 20
0 / 0
0 / 0
11 / 110
5 / 9
4 / 5
6 mix
17A-regulated
TNF-regulated
IFN-regulated
17C-regulated
22-regulated
36γ-regulated
Fold change cut-off
> 2
> 5
> 10

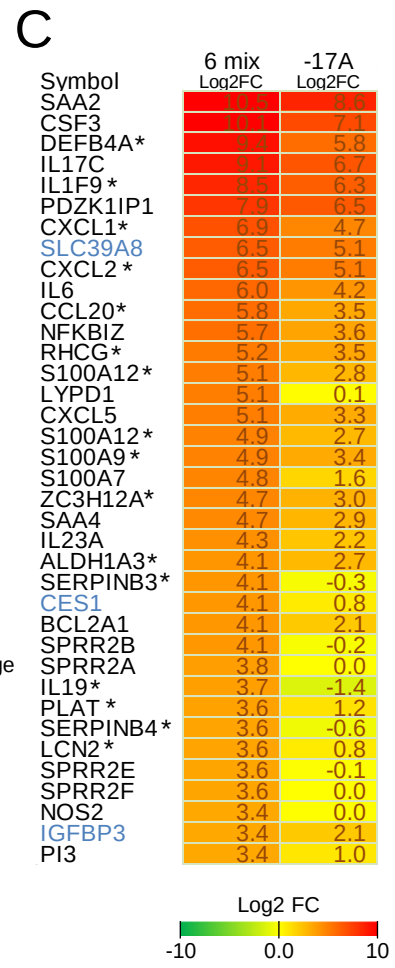
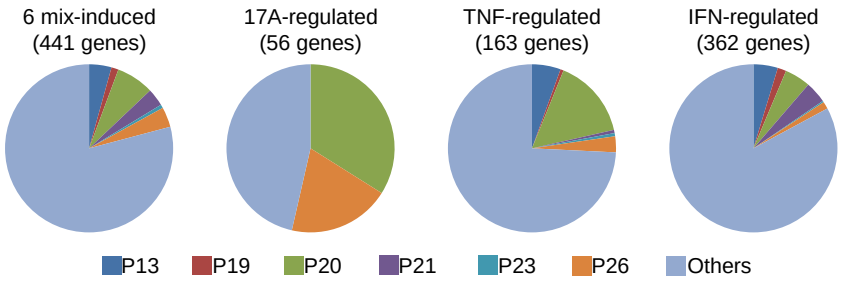

C
Symbol
6 mix Log2FC
-17A Log2FC
SAA2 10.5 8.6
CSF3 10.1 7.1
DEFB4A* 9.4 5.8
IL17C 9.1 6.7
IL1F9* 8.5 6.3
PDZK1IP1 7.9 6.5
CXCL1* 6.9 4.7
SLC39A8 6.5 5.1
CXCL2* 6.5 5.1
IL6 6.0 4.2
CCL20* 5.8 3.5
NFKBIZ 5.7 3.6
RHCG* 5.2 3.5
S100A12* 5.1 2.8
LYPD1 5.1 0.1
CXCL5 5.1 3.3
S100A12* 4.9 2.7
S100A9* 4.9 3.4
S100A7 4.8 1.6
ZC3H12A* 4.7 3.0
SAA4 4.7 2.9
IL23A 4.3 2.2
ALDH1A3* 4.1 2.7
SERPINB3* 4.1 -0.3
CES1 4.1 0.8
BCL2A1 4.1 2.1
SPRR2B 4.1 -0.2
SPRR2A 3.8 0.0
IL19* 3.7 -1.4
PLAT* 3.6 1.2
SERPINB4* 3.6 -0.6
LCN2* 3.6 0.8
SPRR2E 3.6 -0.1
SPRR2F 3.6 0.0
NOS2 3.4 0.0
IGFBP3 3.4 2.1
PI3 3.4 1.0
Log2 FC
-10
0.0
10

Figure 3

A

	Gene coexpression module (Li et al, 2014)					
	P13	P19	P20	P21	P23	P26
6 mixture-induced[1]	19 (4.31)	6 (1.36)	32 (7.26)	15 (3.4)	3 (0.68)	17 (3.85)
17A-regulated	0 (0)	0 (0)	19 (33.9)	0 (0)	0 (0)	11 (19.6)
TNF-regulated	9 (5.52)	1 (0.61)	25 (15.3)	1 (0.61)	1 (0.61)	5 (3.07)
IFN-regulated	17 (4.7)	6 (1.66)	18 (4.97)	15 (4.14)	1 (0.28)	5 (1.38)

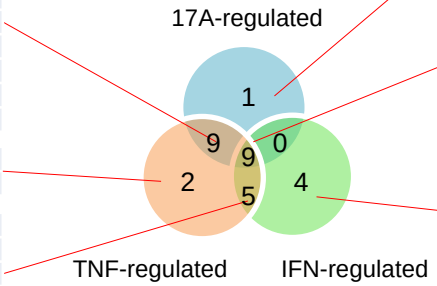
[1] Fold change cut-off: > 5



B

Module_P20 genes

CAMP	A_23_P253791
CXCL1	A_33_P3330264
IL19	A_23_P35092
LCN2	A_23_P169437
PI3	A_23_P210465
S100A12	A_23_P74001
S100A12	A_33_P3385785
SERPINB3	A_23_P55632
SERPINB4	A_23_P502413
IRAK2	A_33_P3352970
SERPINA3	A_23_P2920
IL1B	A_23_P79518
IL1RN	A_33_P3246829
IL1RN	A_33_P3246833
SERPINB1	A_23_P214330
TMEM171	A_32_P110390

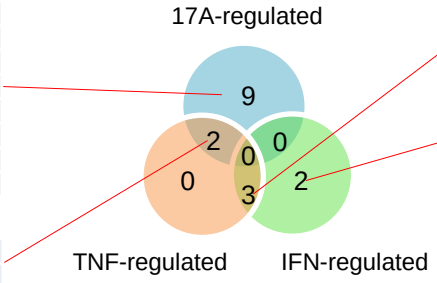


DEFB4A	A_23_P157628
ALDH1A3	A_23_P205959
CXCL2	A_24_P257416
IL17C	A_33_P3339625
IL1F9	A_23_P17053
IL23A	A_23_P76078
LYPD1	A_32_P101031
LYPD1	A_33_P3284129
NOS2	A_23_P502464
SERPINB7	A_23_P141802
APOL1	A_24_P87931
CEACAM1	A_24_P382319
CXCL2	A_23_P315364
ZBP1	A_23_P259141
Others	
DEFB103B	A_23_P169017
SERPINA3	A_33_P3399788

C

Module_P26 genes

PDZK1IP1	A_33_P33938017
S100A9	A_23_P23048
SPRR2A	A_33_P3260426
SPRR2B	A_33_P3247431
SPRR2C	A_23_P126089
SPRR2D	A_23_P11644
SPRR2D	A_33_P3322388
SPRR2E	A_33_P3325704
SPRR2F	A_33_P3274811
NCCRP1	A_33_P3411477
RHCG	A_23_P151975



ELF3	A_23_P104188
SDCBP2	A_23_P131899
SLCO4A1	A_23_P5903
LYPD5	A_24_P323148
PLA2G3	A_23_P17821
Others	
PRSS22	A_23_P400298

Figure 4

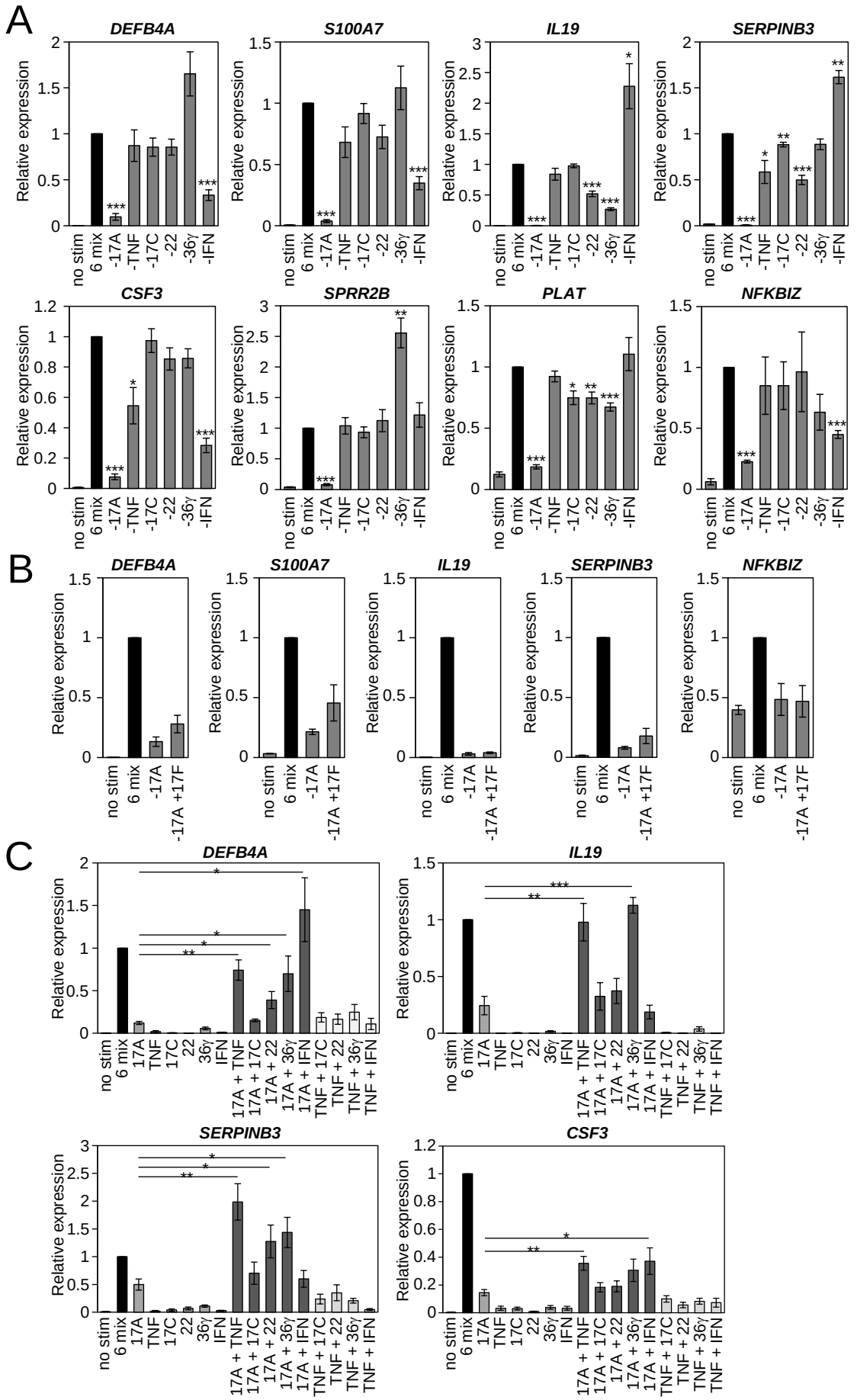
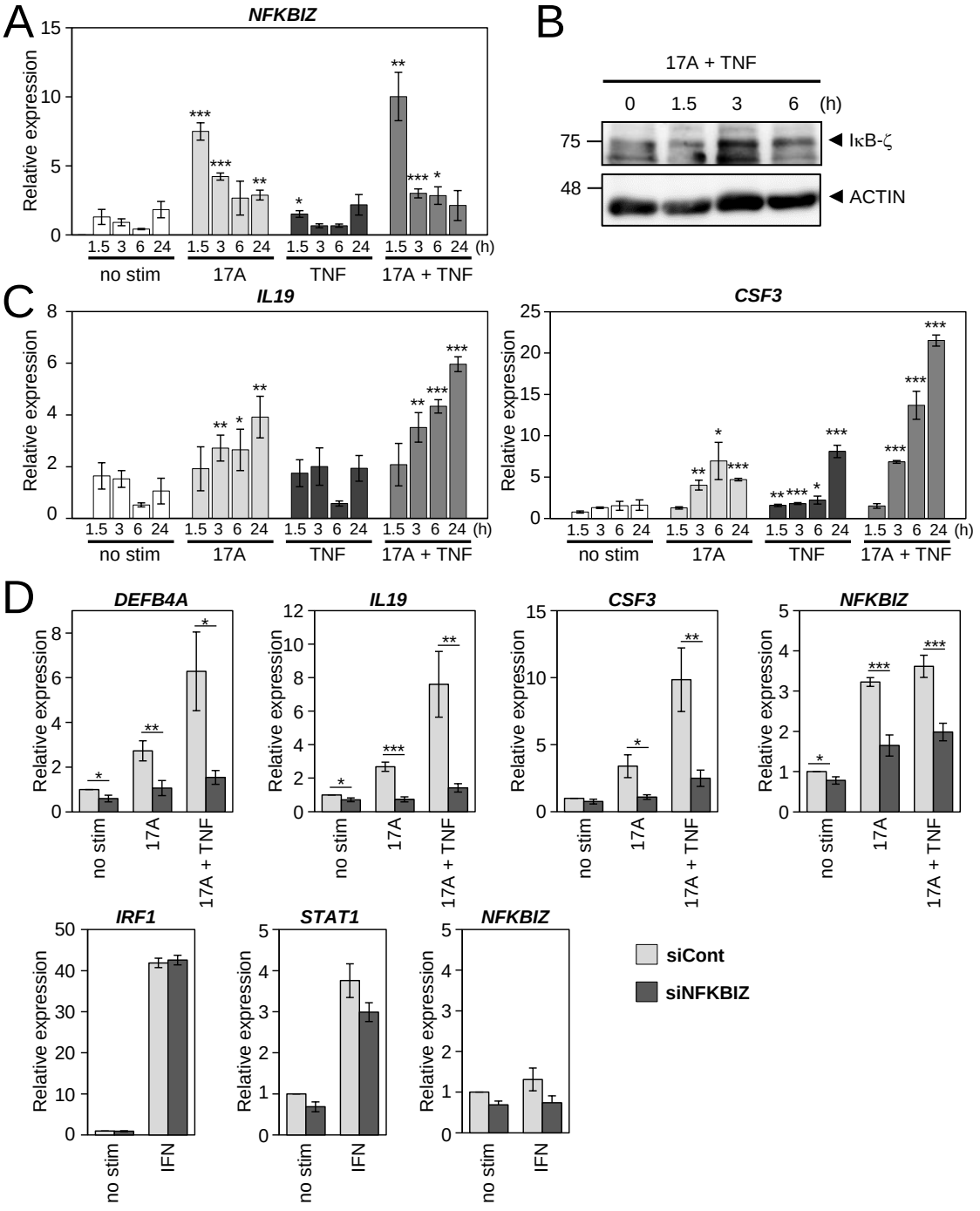

A
DEFB4A
S100A7
IL19
SERPINB3
CSF3
SPRR2B
PLAT
NFKBIZ
Relative expression
no stim
6 mix
-17A
-TNF
-17C
-22
-36γ
-IFN
B
DEFB4A
S100A7
IL19
SERPINB3
NFKBIZ
Relative expression
no stim
6 mix
-17A
-17A +17F
C
DEFB4A
IL19
SERPINB3
CSF3
Relative expression
no stim
6 mix
17A
TNF
17C
22
36γ
IFN
17A + TNF
17A + 17C
17A + 22
17A + 36γ
17A + IFN
TNF + 17C
TNF + 22
TNF + 36γ
TNF + IFN

Figure 5


A
NFKBIZ
Relative expression
1.5 3 6 24 (h)
no stim
17A
TNF
17A + TNF
B
17A + TNF
0 1.5 3 6 (h)
75
48
IκB-ζ
ACTIN
C
IL19
CSF3
D
DEFB4A
IL19
CSF3
NFKBIZ
IRF1
STAT1
NFKBIZ
no stim
17A
17A + TNF
IFN
siCont
siNFKBIZ