Integrative Responses to IL-17 and TNF-α in Human Keratinocytes Account for Key Inflammatory Pathogenic Circuits in Psoriasis

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Psoriasis is a complex inflammatory disease mediated by tumor necrosis factor (TNF)-α and cytokines secreted by specialized T-cell populations, e.g., IL-17, IL-22, and IFN-γ. The mechanisms by which innate and adaptive immune cytokines regulate inflammation in psoriasis are not completely understood. We sought to investigate the effects of TNF-α and IL-17 on keratinocyte (KC) gene profile, to identify genes that might be coregulated by these cytokines and determine how synergistically activated genes relate to the psoriasis transcriptome. Primary KCs were stimulated with IL-17 or TNF-α alone, or in combination. KC responses were assessed by gene array analysis, followed by reverse transcriptase-PCR confirmation for significant genes. We identified 160 genes that were synergistically upregulated by IL-17 and TNF-α, and 196 genes in which the two cytokines had at least an additive effect. Synergistically upregulated genes included some of the highest expressed genes in psoriatic skin with an impressive correlation between IL-17/TNF-α-induced genes and the psoriasis gene signature. KCs may be key drivers of pathogenic inflammation in psoriasis through integrating responses to TNF-α and IL-17. Our data predict that psoriasis therapy with either TNF or IL-17 antagonists will produce greater modulation of the synergistic/additive gene set, which consists of the most highly expressed genes in psoriasis skin lesions.

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INTRODUCTION

Psoriasis is a chronic skin disease characterized by extensive inflammation and altered keratinocyte (KC) differentiation (Krueger and Bowcock, 2005). The dermal infiltrate is a mixture of cells (Lowes *et al.*, 2007) that secrete many proinflammatory mediators, ultimately creating pathogenic inflammation. Although cellular and molecular alterations in psoriatic lesions are extremely complex, with more than 2000 genes with altered expression (Zhou *et al.*, 2003), we are seeking to define how key cytokines of innate or adaptive immunity may interact to create the typical inflammatory pathways of psoriasis vulgaris skin lesions.

One leukocyte subset of extreme interest in psoriasis is the Th17 T-cell subset, with ability to produce IL-17 and IL-22. These cells were shown (*in vitro*) to differentiate from naive $CD4^+$ T cells after IL-6 and TGF-β stimulation, with IL-23 as the major survival factor (Morishima *et al.*, 2009). This T-cell subset and the expression levels of IL-23, IL-17, and IL-22 are highly upregulated in psoriatic skin (Lee *et al.*, 2004; Zaba *et al.*, 2007). Recently, IL-17-producing $CD8^+$ T cells (Tc17) were found to be significantly increased within the psoriatic epidermis (Ortega *et al.*, 2009), suggesting that both Th17/Tc17 subsets contribute to increased IL-17 expression in psoriatic lesions, supporting the role of IL-17 as the most important effector cytokine in psoriasis pathogenesis (Lowes *et al.*, 2008; Ortega *et al.*, 2009). IL-17 receptor antagonism was recently shown to completely reverse the psoriatic phenotype (Russell *et al.*, 2010). This is surprising given that IL-17 regulates only a small list of 35–40 genes in human KCs (Nograles *et al.*, 2008) and human epidermal KCs are the dominant skin cell population expressing the IL-17 receptor, which exerts potent proinflammatory effects (Albanesi *et al.*, 1999; Liang *et al.*, 2006; Wilson *et al.*, 2007; Harper *et al.*, 2009).

Tumor necrosis factor (TNF)-α is another potent inflammatory cytokine that is highly expressed in psoriatic skin. This cytokine has a crucial role in psoriasis pathogenesis, as demonstrated by the efficacy of TNF-α-targeted therapeutics. TNF-α is a powerful inducer of inflammatory gene products

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Abbreviations: AD, atopic dermatitis; FCH, fold change; KC, keratinocyte; RT-PCR, reverse transcriptase-PCR; TNF, tumor necrosis factor

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in human KCs, some of which overlap with IL-17-regulated genes in KCs (Zaba *et al.*, 2007). TNF-α regulates the transcription of target genes mainly through activation of NF-κB, and TNF-α-induced genes in KCs are largely consistent with known NF-κB target genes. Similarly, IL-17 signals in part through the NF-κB pathway, although its major signaling pathway is through CCAAT/enhancer binding proteins (C/EBP) transcription factors. Several IL-17 fingerprint genes (e.g., CXCL1, MIP-2, CXCL5, IL-6, LCN2, and COX2) are transcribed by C/EBP-β and C/EBP-δ (Ruddy *et al.*, 2004b; Shen *et al.*, 2005, 2006).

As either TNF or IL-17 blockade can potentially reverse psoriasis disease (Zaba *et al.*, 2007; Russell *et al.*, 2010), we hypothesized that perhaps unappreciated interactions of TNF and IL-17 regulate gene circuits in KCs as well as in psoriatic lesions. Synergistic activation of some genes by IL-17 and TNF-α has been previously observed in osteoblasts, fibroblasts, and bone marrow stromal cells (Ruddy *et al.*, 2004b; Shen *et al.*, 2005, 2006), possibly reflecting the regulation of common genes by multiple transcription factors. Two previous studies, focusing on a limited number of genes, reported IL-8 (Albanesi *et al.*, 1999) and CCL20 (Homey *et al.*, 2000) to be synergistically upregulated by this cytokine combination in human KCs. A recent study, which used a five-cytokine mixture (including IL-17A, IL-22, oncostatin M, IL-1α, and TNF-α), examined a limited set of preselected genes by complementary DNA microarray analysis. They detected synergistic induction for 14 genes, with additive effects for 6 genes in KCs (Guilloteau *et al.*, 2010). Although IL-17 and TNF-α are clearly an important part of the report, the synergism was demonstrated for a complex five-cytokine interaction, without direct proof of a TNF/IL-17 synergy. Hence, we conducted a more comprehensive analysis of KC responses to IL-17, TNF-α, and the combination of both cytokines using gene arrays. Our work increases the number of genes coregulated by IL-17 and TNF-α to more than 350, with either additive (196) or synergistic (160) effects. Our data predict that *in vivo* treatment of psoriasis with either TNF or IL-17 antagonists will produce greater modulation of the synergistic/additive gene sets, as the dual-regulated genes are among the most highly expressed in psoriasis vulgaris skin lesions.

RESULTS

Previous experiments have shown that KC responses to IL-17 or TNF-α are dose dependent. To define cytokine-regulated gene sets and determine the ''near maximal'' modulation of gene expression, several previous genomic profiling experiments were conducted using relatively high concentrations of these cytokines (Banno *et al.*, 2004). Seeking to define KC responses to IL-17, TNF-α, or a combination of these cytokines, we used IL-17 at 200 ng ml^{-1}, as previously described (Nograles *et al.*, 2008; Harper *et al.*, 2009). As responses to TNF-α show a relatively steep dose–response relationship (Harper *et al.*, 2009), we examined the modulation of two model inflammatory genes (IL-8 and S100A7) after treatment with 0.1, 1, and 10 ng ml^{-1} of TNF-α and IL-17/TNF-α combination across this range of TNF-α concentrations (Figure 1). As expected, IL-17 and TNF-α individually induced KC expression of IL-8. The combination of IL-17 with different concentrations of TNF-α strongly induced the expression of IL-8 mRNA at higher levels than the additive values of the individual cytokines, implying synergistic induction of the target gene. We observed the same phenomenon for S100A7, wherein modest gene induction was observed after individual treatment with IL-17 and TNF-α, but the combination of IL17/TNF-α was synergistic for S100A7 mRNA increases across all TNF-α concentrations.

We further explored whether a broader set of inflammation- or differentiation-related genes in KCs could be regulated by IL-17/TNF-α combination. To answer this question, we treated independently derived ($n=3$) primary KC cultures with individual or a combination of cytokines and evaluated their mRNA levels on whole-genome arrays. Because of the many variables tested and considerably lower cost per array, we used Illumina arrays (Illumina, San Diego,

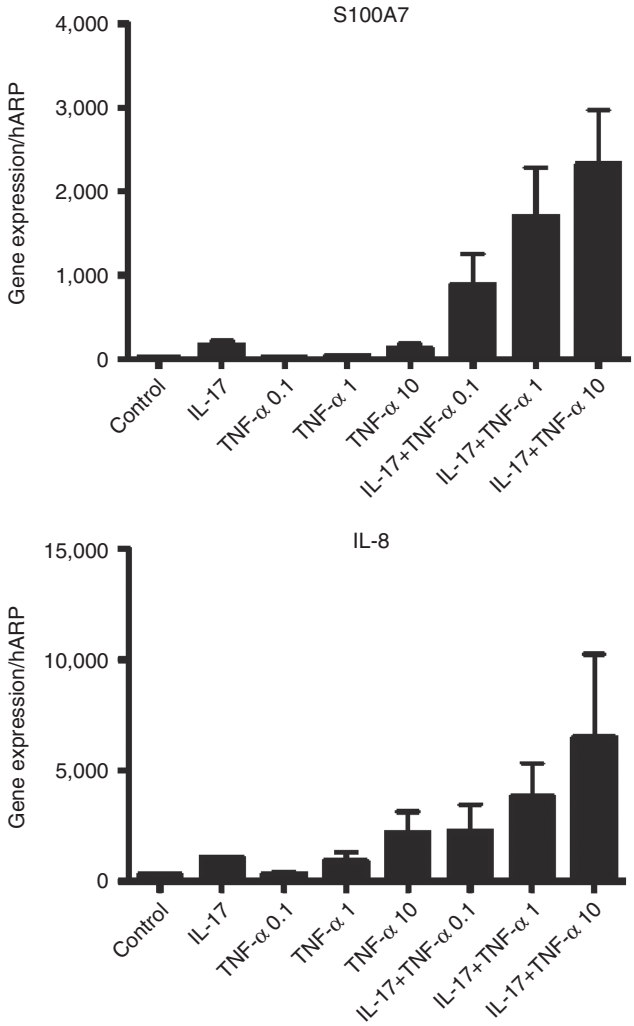


Figure 1. Keratinocyte responses to IL-17, tumor necrosis factor (TNF)-α, or a combination of these cytokines as examined by modulation of two model inflammatory genes, IL-8 and S100A7. The combination of 200 ng ml^{-1} of IL-17 with different TNF-α concentrations (0.1, 1, or 10 ng ml^{-1}) showed synergistic induction of both IL-8 and IL-17 mRNA expression, at much higher levels than the additive values of the individual cytokines.

CA), although most single-cytokine profiling experiments have been previously carried out on an Affymetrix platform (Central Expressway, Santa Clara, CA). However, as Illumina and Affymetrix arrays are different, we needed to first redefine KC responses to individual cytokines on an Illumina platform.

Identification of induced genes by IL-17 in human KC

Using an Illumina platform, we identified 47 upregulated probes (42 unique genes as defined by ENTREZ identifiers) and 4 downregulated probes (4 genes) by IL-17, with a fold change (FCH) >1.3 and a false discovery rate <0.3 (Supplementary Table S1 online). IL-17 induced high mRNA expression levels of (i) the IL-1 family of cytokines, i.e., IL-1F9 and IL-1B; (ii) chemoattractants, i.e., IL-8, CCL20, CXCL1, and CXCL2; and (iii) innate immune mediators, i.e., DEFB4, LCN2, S100A8, and S100A9.

We then compared this IL-17 signature with genes previously identified using Affymetrix arrays (Nograles *et al.*, 2008; Figure 2a). In all, 12 of the 42 upregulated genes in this study are not represented in AffymetrixU133A2, representing IL-17 response genes that, to our knowledge, are previously unreported. Of the 30 genes represented on both platforms and upregulated on Illumina arrays (Figure 2b), 23 (76.7%) were also reported by Nograles *et al.*, (2008), with a significant correlation between FCHs reported by both studies ($r=0.6$; $P=0.0027$). However, we did not detect several previously reported IL-17-induced chemokines, including CXCL3, CXCL5, S100A7, and S100A12 (Figure 2b). The expression level of these genes was highly increased by IL-17, using reverse transcriptase-PCR (RT-PCR) on the same samples (Figure 2c). Hence, different ''whole genome'' arrays show variable sensitivity in detection of IL-17-induced gene products.

TNF-α broadly modulates KC gene expression

TNF-α effects on KCs were also evaluated by Illumina arrays. We determined a broad genomic response, largely consistent with previous results using the Affymetrix platform (Banno *et al.*, 2004). However, FCH induction of inflammatory genes modulated by 10 and 1 ng μl^{-1} concentrations of TNF-α was higher than previous results obtained using a higher TNF-α stimulus (50 ng ml^{-1}; Banno *et al.*, 2004). We identified 459 genes (502 probes) with altered expression after treatment with 10 ng ml^{-1} of TNF-α: 333 upregulated genes (371 probes) and 126 downregulated genes (131 probes; Figure 3a and Supplementary Table S2 online). A smaller number of genes were regulated by 1 ng ml^{-1} of TNF-α: 121 upregulated genes (134 probes) and 45 downregulated genes (45 probes; Figure 3a and Supplementary Table S3 online). A total of 362 probes (320 genes) were uniquely modulated by 10 ng ml^{-1} of TNF-α (250 up- and 112 downregulated) and a small number of 39 probes (37 genes) were induced only by 1 ng ml^{-1} of TNF-α (13 up- and 26 downregulated), possibly as high TNF concentrations inhibit transcription of numerous gene products (Banno *et al.*, 2004; Figure 3a, Supplementary Table S6 online). Most (90%) probes upregulated by 1 ng ml^{-1} were also upregulated by the larger dose (Figure 3a), but the dose–response relationship as measured by FCH was larger for 10 ng ml^{-1} (Supplementary Tables S2, S3 online). Despite the higher number of genes induced by 10 ng ml^{-1} of TNF-α, key genes such as MMP9, IL-8, IL1B, and IL1F9 were highly upregulated by both concentrations, with a larger effect achieved by the higher concentration (Figure 3b).

Synergistic IL-17/TNF-α interaction to induce gene expression modifications

Single cytokines were compared with combined IL-17/TNF-α modulation of KC gene expression. We considered a gene to be synergistically induced by IL-17 and TNF-α when the combined effect of the interacting cytokines was greater than the sum of their separate effects. The synergism could be positive or negative if the combined effect strongly upregulated or downregulated the gene expression levels (Supplementary Figure S1 online). We also measured effects that were additive but not synergistic (see Methods for a formal definition of synergism and additivism). Because of our particular interest in positive IL-17/TNF-α interactions, we refer only to positive synergism as synergistic genes. Overall, the combination of IL-17 with 10 ng ml^{-1} of TNF-α synergistically stimulated the expression of 176 probes (160 genes), compared with 109 probes (99 genes) synergistically stimulated with IL-17 and 1 ng ml^{-1} of TNF-α, and 78 probes (70 genes) synergistically stimulated by either combination (Figure 3c, Supplementary Table S4 online). Table 1a and b lists examples of genes that were synergistically or additively induced by 10 ng ml^{-1} of TNF-α and IL-17. Key inflammatory genes previously implicated in the pathogenesis of psoriasis exist in both lists. An example of the synergistic effect of TNF-α and IL-17 is induction of IL-19. IL-17 induces IL-19 expression by 1.79-fold (0.84 $\log_2$), whereas TNF slightly reduces expression. However, the combination of IL-17/TNF-α synergistically induces IL-19 expression by 54.6-fold (5.77 $\log_2$). Similarly, IL-17 and TNF-α individually induce only small increases in IL-6 expression (1.27- and 1.26-fold, respectively), but the combination of both cytokines induces IL-6 expression by 3.89-fold (1.96 $\log_2$). As an example of an additive effect, IL-1F9 could be considered. The expression of IL-1F9 is induced by IL-17 by 8.75-fold (3.13 $\log_2$), and TNF-α by 7.01-fold (2.81 $\log_2$). The combination of these cytokines induces its expression by 41.4-fold (5.37 $\log_2$). Additive IL-17/TNF-α stimulation is presented in Supplementary Table S5 online.

IL-17 cooperates with TNF-α to significantly enhance KC-derived cytokines and immunomodulating molecules

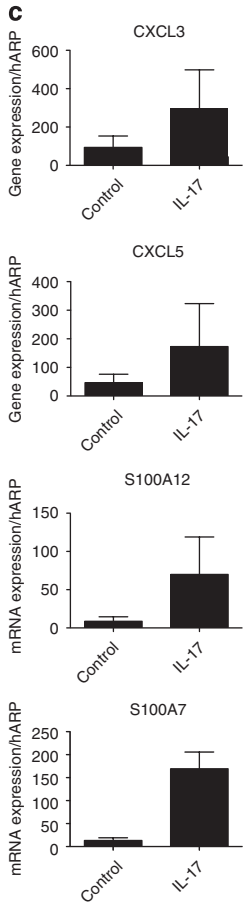
To confirm the microarray results of synergistic and additive genes with IL-17/TNF-α combination, we quantified mRNA expression of biologically significant genes, including IL-8, CCL20, LCN2, S100A7, S100A12, and IL-19, in cytokine-treated KCs by real-time RT-PCR and normalized expression values to the housekeeping gene *hARP* (Figure 4a). We observed a marked synergism in induction of mRNA expression even for primarily IL-17-regulated genes, such as DEFB4, S100A7, and CCL20. LCN2 and S100A12, although genes identified as additive in the microarray experiment, were synergistically upregulated by RT-PCR, demonstrating a higher sensitivity of RT-PCR versus the Illumina platform (Figure 4a).

a

		Illumina			Affymetrix*		
Symbol	Description	Fold change (log_2)	*P*	FDR	Fold change (log_2)	*P*	FDR
DEFB4	Defensin, beta 4	3.46	0	0	7.898	0	0
IL-1F9	Interleukin-1 family, member 9	3.128	0	0	3.913	0	0
SPRR2C	Small proline-rich protein 2C	2.941	0	0	4.256	0	0
ZC3H12A	Zinc finger CCCH-type containing 12A	2.505	0	0	4.253	0	0
PI3	Peptidase inhibitor 3, skin-derived	2.494	0	0	2.162	0	0
LCN2	Lipocalin 2	2.396	0	0	2.179	0	0.01
SERPINB4	Serpin peptidase inhibitor, clade B, member 4	2.082	0	0	4.783	0	0
IL-8	Interleukin-8	1.811	0	0	2.334	0	0
S100A8	S100 calcium-binding protein A8	1.486	0	0	0.655	2.0E–04	0.06
S100A9	S100 calcium-binding protein A9	1.48	0	0	1.104	1.0E–04	0.04
SPRR2D	Small proline-rich protein 2D	1.448	0	0.01	1.784	0	0
NDRG2	NDRG family member 2	1.332	0	0.01	2.468	1.0E–04	0.03
RHCG	Rh family, C glycoprotein	1.257	0	0	2.585	0	0
IL-1B	Interleukin-1, beta	1.189	0	0	1.45	0	0.01
IER3	Immediate early response 3	1.126	0	0.01	0.789	0	0
CCL20	Chemokine (C–C motif) ligand 20	1.048	3.0E–04	0.14	4.823	0	0
PDZK1IP1	PDZK-1 interacting protein 1	1.028	0	0	4.698	0	0
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	1.016	2.0E–04	0.11	0.629	4.0E–04	0.1
CXCL2	Chemokine (C–X–C motif) ligand 2	0.948	3.0E–04	0.14	2.587	0	0
NOD2	Nucleotide-binding oligomerization domain containing 2	0.877	3.0E–04	0.14	2.394	0	0.01
SLC11A2	Solute carrier family 11, member 2	0.807	0	0.01	1.116	0	0
CXCL1	Chemokine (C–X–C motif) ligand 1	0.803	0	0.01	2.943	0	0
SAT1	Spermidine/spermine N1-acetyltransferase 1	0.617	5.0E–04	0.19	0.717	2.0E–04	0.05

* Nograles *et al. Br J Derm 2008.*

c



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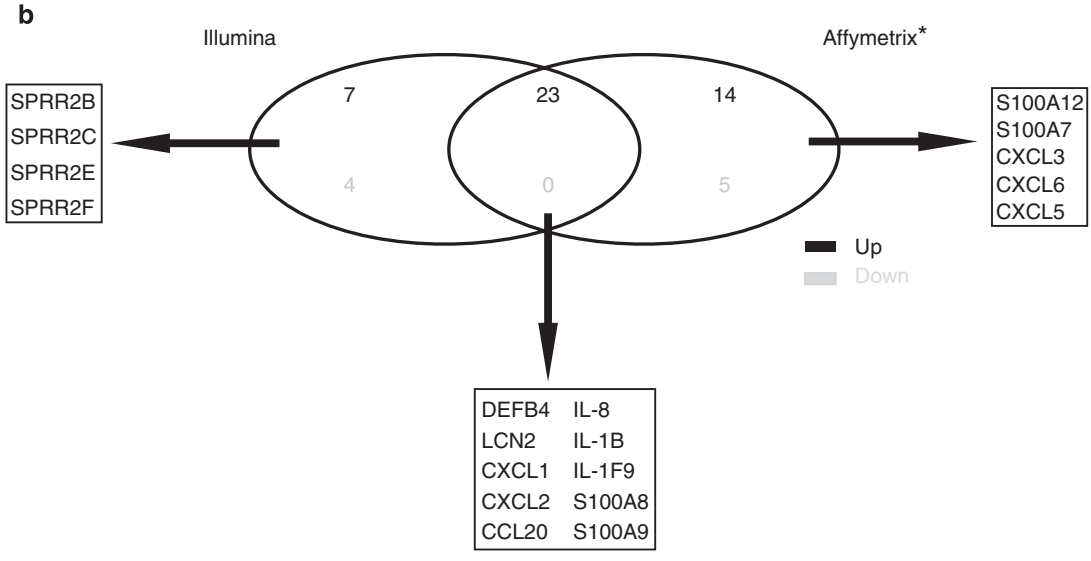


Figure 2. Identification of IL-17-regulated genes in human keratinocytes. (**a**) Comparison of IL-17 signature genes as identified by Illumina and Affymetrix arrays. (**b**) A Venn diagram comparing IL-17 response genes on both array platforms. Arrows indicate biologically relevant genes commonly or uniquely upregulated by each individual platform. (**c**) Mean values of gene expression normalized to hARP ± SD. IL-17 strongly induces mRNA gene expression in the keratinocytes of a few chemokines that were not detected on the Illumina arrays. FDR, false discovery rate.

Kinetics of response

To further investigate IL-17/TNF-α interactions, we harvested KCs after 6-, 12-, and 24-hour cytokine treatment and evaluated mRNA gene expression by RT-PCR (Figure 4b). We observed a rapid response of proinflammatory genes such as CCL20 and IL-8 at as early as 6 hours of treatment with IL-17/TNF-α combination. This indicates that synergistic genes are ''immediate'' response genes, as expected for genes regulated by primary transcription factor circuits.

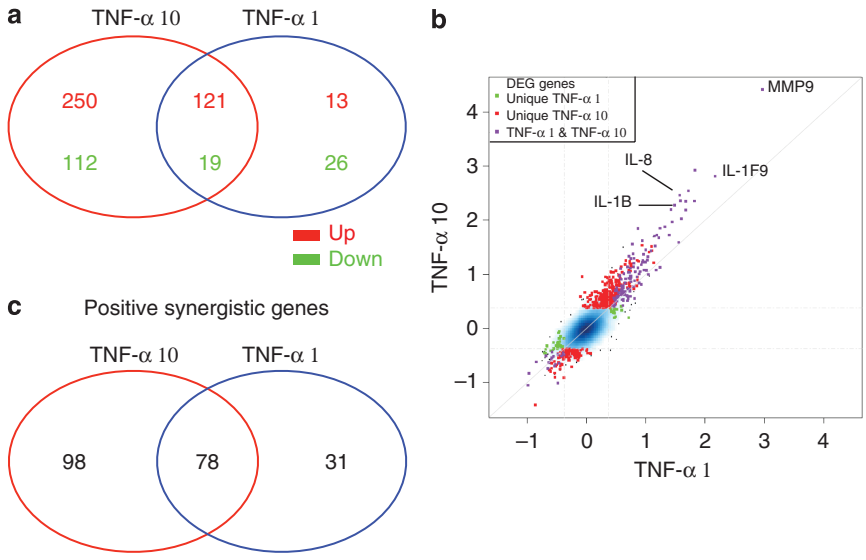


Figure 3. Tumor necrosis factor (TNF)-α broadly modulates keratinocyte gene expression by Illumina arrays. (**a**) A Venn diagram comparing the inflammatory genes modulated by 10 and 1 ng μl^{-1} concentrations of TNF-α. (**b**) A scatter plot of the effect induced in keratinocytes by 1 ng μl^{-1} versus 10 ng μl^{-1} of TNF-α. Inflammatory genes were highly upregulated by both concentrations, although the higher concentration induced larger fold changes. (**c**) IL-17 combination with either TNF-α concentration synergistically stimulated the expression of key psoriasis genes.

Expression of TNF-α/IL-17 synergistic genes in psoriasis skin lesions

To investigate the role of IL-17/TNF-α synergism in psoriasis, we considered two studies that profiled the psoriasis genotype using the Affymetrix platform (Yao *et al.*, 2008; Suarez-Farinas *et al.*, 2010). These studies defined the psoriasis transcriptome as differentially expressed genes (DEGs) between lesional and non-lesional skin of psoriatic patients. Among our synergistic genes, 46 were identified as significantly upregulated in psoriatic skin in the study by Yao *et al.* (2008) and 41 by Suarez-Farinas *et al.* (2010).

Figure 5 identifies multiple synergistic genes that are ranked among the top 100 upregulated genes in the transcriptomes described by either Yao *et al.* (Figure 5a) or Suarez-Farinas *et al.* (Figure 5b). Synergistic IL-17/TNF-α genes in the ''top 100 list'' include key molecules in the psoriasis gene signature, including psoriasin (S100A7), β-defensin (DEFB4), IL-8, CCL20, IL-23 (p19), and CXCL1. Interestingly, the ''top'' three psoriasis-related genes in the study by Yao *et al.* (S100A7, SerpinB4, and DEFB4) are also highly ranked IL-17/TNF-α synergistic genes.

To further investigate the relationship between synergistic genes and the psoriasis gene signature, we used the FCHs reported in the above-mentioned studies. For synergistic genes, an impressive correlation was detected between synergistic effect (defined by synergistic increase) and FCHs of the psoriasis transcriptome ($r=0.785$, $P=6.32\times10^{-11}$ for Yao *et al.*; and $r=0.62$, $P=7.6\times10^{-6}$ for Suarez-Farinas *et al.*; Figure 5d and e).

This correlation was reconfirmed with a gene set enrichment analysis evaluating the enrichment of several psoriatic transcriptomes (Yao *et al.*, 2008; Gudjonsson *et al.*, 2009; Suarez-Farinas *et al.*, 2010) with respect to IL-17/TNF-α synergistic effect (Figure 5c). The normalized enrichment score for all studies was positive (>1.75, false discovery rate <0.0001). This indicates that IL-17/TNF-α synergism induces a strong induction of most characteristic differentially expressed genes in psoriasis, advocating for a crucial role of IL-17/TNF-α combination in the molecular fingerprint of psoriasis (Suarez-Farinas *et al.*, 2010).

We also sought to determine whether our synergistic gene profile was correlated with another common inflammatory skin disease, atopic dermatitis (AD). Unlike the positive enrichment of upregulated psoriasis genes with the ''synergistic phenotype'', no such correlation was identified in AD, as could be appreciated by a negative normalized enrichment score in AD (Figure 5c, Supplementary Figure S2A online). In comparison with psoriasis (mean FCH = 1.8), the IL-17/TNF-α synergistic genes showed significantly (*P*-value $<10^{-6}$) lower FCH induction in AD (mean FCH = 1.2; Supplementary Figure S2B online), with a distribution of most synergistic genes (84/108, 77.8%) within the psoriasis field (Figure 5f). Supplementary Figure S2C online shows that this higher pathological effect in psoriasis is specific to the synergistic gene set.

Rapid attenuation of synergistic genes with etanercept treatment

An absolute proof of synergistic gene regulation of TNF-α/IL-17 combination in psoriatic skin is difficult. However, one would expect that synergistic genes would be more strongly suppressed by TNF inhibition compared with genes regulated by a single cytokine. To test this concept, we reexamined expression data from a published study on the therapeutic mechanism of etanercept, a TNF-α inhibitor (Zaba *et al.*, 2007, 2009). We compared the mean reduction in expression of genes regulated solely by TNF-α versus those by the IL-17/TNF-α synergistic gene set. We

Table 1. Selected genes synergistically or additively induced by TNF-α of 10 ng ml^{-1} and IL-17

			Fold change (log_2)		
	Symbol	**Description**	**IL-17 alone**	**TNF-α alone**	**IL-17+TNF-α**
(a)					
Cytokines and chemokines	IL-19	Interleukin-19	0.84	−0.12	5.77
	IL-8	Interleukin-8	1.39	1.88	4.39
	IL-23A	Interleukin-23, alpha subunit p19	0.74	0.47	3.27
	CCL20	Chemokine (C–C motif) ligand 20	1.05	0.94	3.01
	IL-6	Interleukin-6 (interferon, beta 2)	0.35	0.33	1.96
	CXCL1	Chemokine (C-X-C motif) ligand 1	0.80	0.20	1.65
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	−0.13	0.95	1.43
	IL-17C	Interleukin-17C	0.08	0.15	1.35
	CXCL5	Chemokine (C–X–C motif) ligand 5	0.23	0.15	1.02
Immune response and inflammatory genes	DEFB4	Defensin, beta 4	3.46	0.67	6.26
	CFB	Complement factor B	0.53	1.14	3.35
	S100A7A	S100 calcium-binding protein A7A	0.15	−0.10	3.19
	S100A7	S100 calcium-binding protein A7	0.82	0.28	2.72
	PLAT	Platelet-derived growth factor receptor-like	0.64	−0.04	2.67
	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	0.06	0.25	0.87
	MAP2K3	Mitogen-activated protein kinase kinase 3	0.11	−0.05	0.78
	PTGE2	Prostaglandin E synthase 2	0.12	−0.18	0.62
	TFAP2C	Transcription factor AP-2 gamma	0.11	−0.12	0.57
	MAP2K13	Mitogen-activated protein kinase 13	0.08	0.00	0.56
	CSF3	Colony-stimulating factor 3	0.10	0.17	0.84
(b)					
Cytokines and chemokines	IL-1F9	Interleukin-1 family, member 9	3.13	2.81	5.37
	CXCL6	Chemokine (C–X–C motif) ligand 6	1.09	0.55	2.57
	IL-1B	Interleukin-1, beta	1.19	2.28	3.18
	IL-1F5	Interleukin-1 family, member 5	0.55	0.69	1.74
	TNF-SF18	Tumor necrosis factor (ligand) superfamily, member 18	0.30	0.00	1.01
Immune response and inflammatory genes	PI3	Peptidase inhibitor 3, skin derived (SKALP)	2.49	1.59	4.17
	LCN2	Lipocalin 2	2.40	0.43	3.60
	S100A9	S100 calcium-binding protein A9	1.48	1.73	2.60
	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	0.80	0.06	1.40
	S100P	S100 calcium-binding protein P	0.47	0.32	1.19
	S100A12	S100 calcium-binding protein A12	0.25	0.13	0.92
	S100A8	S100 calcium-binding protein A8	1.49	1.32	1.98
	CEBPA	CCAAT/enhancer-binding protein (C/EBP), alpha	0.62	0.21	0.98

Key inflammatory genes previously implicated in the pathogenesis of psoriasis exist in both lists. (a) Synergistic gene list. (b) Additive gene list. Fold change values are in log_2 scale to simplify the comparison of single versus combined cytokine effects.

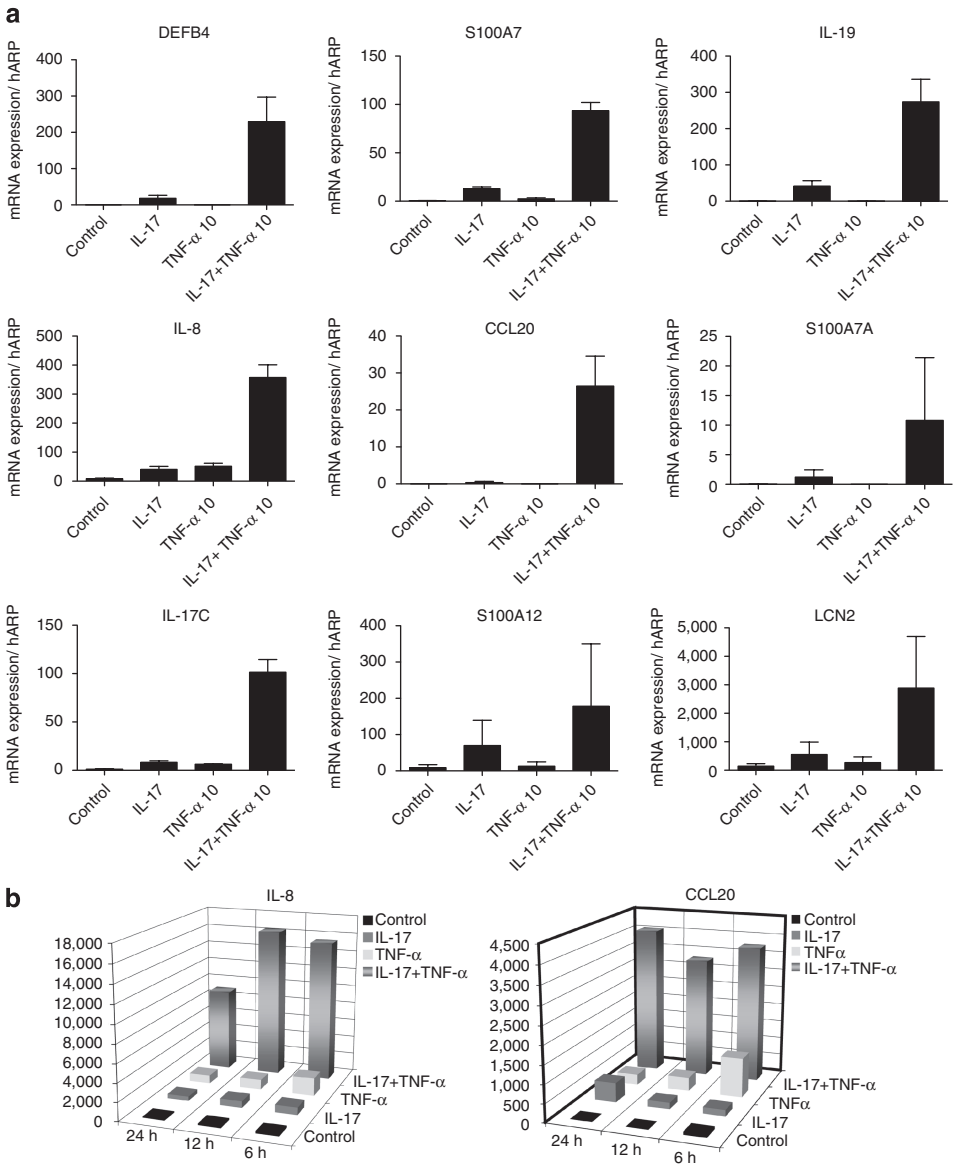


Figure 4. IL-17 cooperates with tumor necrosis factor (TNF)-α to enhance keratinocyte-derived immunomodulating molecules. (**a**) Reverse transcriptase-PCR confirmation of microarray results of synergistic/additive genes with IL-17/TNF-α combination. The mean values of gene expression normalized to hARP ± SD are shown. A marked synergism was observed in induction of primarily IL-17-regulated genes. (**b**) Kinetics of keratinocyte responses after 6, 12, and 24 hours of cytokine treatment. The mean values of gene expression normalized to hARP for each time point are shown. Synergistic genes, such as CCL20 and IL-8, were shown to be ''immediate'' response genes.

found that synergistically regulated genes (for both first and second weeks of etanercept therapy) showed a greater reduction in expression compared with genes regulated by TNF-α alone, with a highly significant difference in response at week 2 ($P = 0.0004$), and approaching significance at week 1 ($P = 0.15$; Figure 5g).

DISCUSSION

Th17 T cells have been implicated in several autoimmune diseases in model systems, as well as in human diseases (Tesmer *et al.*, 2008; Abraham and Cho, 2009; Di Cesare *et al.*, 2009; Gaffen, 2009; Crome *et al.*, 2010). In humans, Th-17 T cells synthesize the inflammatory cytokines IL-17A and IL-17F, which link innate and adaptive immunity (Stockinger *et al.*, 2007; Khader *et al.*, 2009). For example, in human epidermal KCs, IL-17 induces synthesis of antimicrobial peptides (i.e., defensins, lipocalin, and S100 proteins) and also regulates a group of neutrophil-attracting CXCL chemokines (Nograles *et al.*, 2008; Harper *et al.*, 2009; Pelletier *et al.*, 2010). These functions are relevant to skin alterations in psoriasis (Watanabe *et al.*, 2007). The Th17/IL-23 pathway is highly increased in psoriatic skin lesions (Lee *et al.*, 2004; Lowes *et al.*, 2008; Di Cesare *et al.*, 2009) and is central to psoriasis pathogenesis, as genetic

a

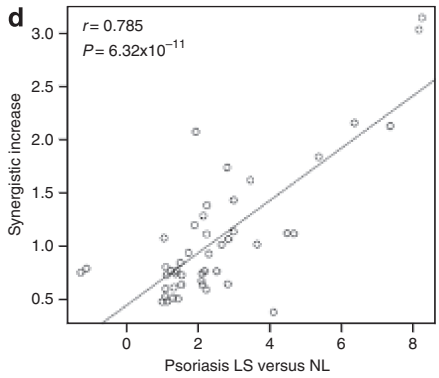
Gene	Description	Top 100 PS upregulated ranked genes	PS upregulated genes fold change (log_2)	Syneristic ranked genes	IL-17 + TNF-α fold change (log_2)
S100A7A	S100 calcium-binding protein A7A	1	8.22	11	3.91
SERPINB4	Serpin peptidase inhibitor, clade B, member 4	2	8.14	3	5.47
DEFB4	Defensin, beta 4	3	7.34	1	6.26
SERPINB3	Serpin peptidase inhibitor, clade B, member 3	8	6.35	8	3.31
KYNU	Kynureninase	14	5.35	12	3.16
IL-8	Interleukin-8	21	4.67	4	4.98
TMPRSS11D	Transmembrane protease, serine 11D	26	4.48	20	2.53
SLC6A14	Solute carrier family 6, member 14	30	4.10	126	0.58
CCL20	Chemokine ligand 20	43	3.64	14	3.00
S100A7	S100 calcium-binding protein A7	48	3.46	16	2.72
VNN3	Vanin 3	76.5	2.99	36	0.66
SOD2	Superoxide dismutase 2, mitochondrial	78	2.99	6	3.19
IL-23A	Interleukin-23, alpha subunit p19	85.5	2.85	9	3.27
PDZK1IP1	PDZK1 interacting protein 1	87	2.85	23	2.13
CXCL1	Chemokine (C–X–C motif) ligand1	89	2.83	33	1.64
C15orf48	Chromosome 15 open reading frame 48	91	2.81	13	1.35

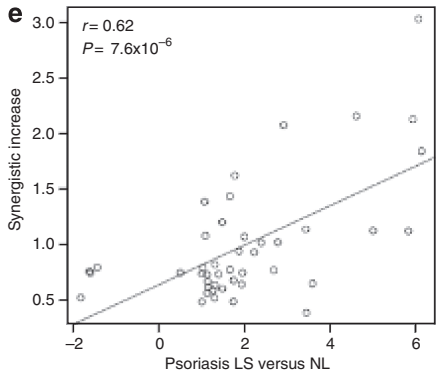
b

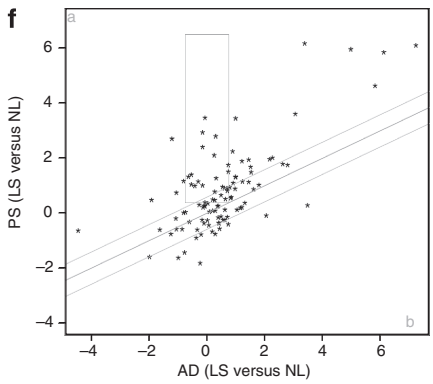
Gene	Description	Top 100 PS upregulated ranked genes	PS upregulated genes fold change (log_2)	Syneristic ranked genes	IL-17 + TNF-α fold change (log_2)
KYNU	Kynureninase	4	6.16	2	3.16
SERPINB4	Serpin peptidase inhibitor, clade B, member 4	5	6.09	3	5.47
DEFB4	Defensin, beta 4	6	5.96	1	6.26
IL-8	Interleukin-8	8	5.85	4	4.98
TMPRSS11D	Transmembrane protease, serine 11D	16	5.02	20	2.53
SERPINB3	Serpin peptidase inhibitor, clade B, member 3	19	4.62	8	3.31
CXCL1	Chemokine (C–X–C motif) ligand 1	36	3.60	33	1.64
SLC6A14	Solute carrier family 6, member 14	38	3.46	126	0.58
SOD2	Superoxide dismutase 2, mitochondrial	39	3.44	6	3.19
PLAT	Plasminogen activator, tissue	66	2.93	17	2.67
CCL20	Chemokine ligand 20	77	2.79	14	3.00
SERPINA3	Serpin peptidase inhibitor, clade A, member 3	81	2.69	45	1.39

c

Transcriptome (LS vs NL)	Size	ES	NES	NOM *P*-val	FDR *q*-val
Psoriasis up (Gudjonsson *et al.*)	406	0.45	2.12	0	0
Psoriasis up (Suarez-Farinas *et al.*)	480	0.41	1.95	0	0
Psoriasis up (Yao *et al.*)	733	0.36	1.77	0	0
Psoriasis down (Yao *et al.*)	521	0.19	0.9	0.748	0.726
Psoriasis down (Suarez-Farinas *et al.*)	434	−0.2	−1.06	0.192	0.342
Psoriasis down (Gudjonsson *et al.*)	124	−0.25	−1.13	0.224	0.29
AD up	153	−0.27	−1.29	0.05	0.129
AD down	88	−0.24	−1.03	0.39	0.349







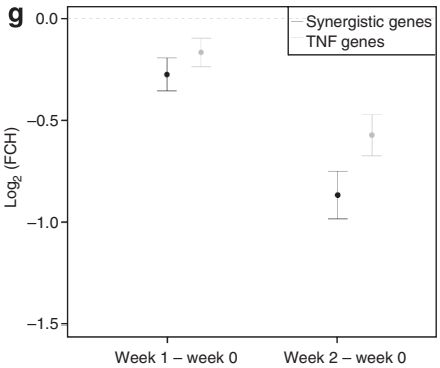


Figure 5. IL-17/tumor necrosis factor (TNF)-α synergistic genes in psoriasis. (**a**, **b**) Synergistic genes ranked among the top upregulated genes in the transcriptomes described in the study by Yao *et al.* (**a**) or that by Suarez-Farinas *et al.* (**b**). (**c**) Gene set enrichment analysis for psoriasis (Yao, Suarez-Farinas, and Gudjonsson) and atopic dermatitis (AD) transcriptomes with respect to the IL-17/TNF-α synergistic phenotype, as defined by the synergistic increase. (**d**–**e**) Correlation between the synergistic increase (*y* axis) and the psoriasis fold changes (*x* axis) for synergistic genes using Yao (**d**) and Suarez-Farinas (**e**) transcriptomes. (**f**) Scatter plot of fold changes of synergistic genes in AD versus psoriasis. Most synergistic genes are distributed mainly within the psoriasis field a. The rectangular area highlights genes unchanged in AD and highly upregulated in psoriasis. (**g**) Mean (with 95% confidence interval) response to etanercept after 1 and 2 weeks of treatment shows a rapid attenuation of synergistic genes with therapy. ES, enrichment score; FCH, fold change; FDR, false discovery rate; LS, lesional; NES, normalized enrichment score; NL, non-lesional.

susceptibility to psoriasis was linked to IL-23p19 and IL-23p40 gene variants and to the IL-23 receptor (Nair *et al.*, 2008, 2009; Elder, 2009).

Numerous clinical trials have now established that antagonism of the p40 subunit, shared between IL-12 and IL-23, can suppress psoriasis in 70–80% of patients (Krueger *et al.*, 2007; Menter, 2009). Emerging studies with IL-17 receptor antagonists showed impressive clinical, histological, and genomic resolution of psoriasis after only 1 week of treatment (Russell *et al.*, 2010). These data strongly implicate IL-17 as the most important pathogenic element in psoriasis. It is thus surprising that IL-17-regulated genes, defined by exposure of skin KCs to IL-17 as a single cytokine, comprise only a small fraction (<1%) of overall transcriptional alterations in this disease (Yao *et al.*, 2008; Suarez-Farinas *et al.*, 2010). Hence, from the standpoint of genomic alterations defining the psoriasis transcriptome, it is most likely that multiple different cytokines and inflammatory elements collaborate to create an interactive network of more than 2000 genes with altered expression (Yao *et al.*, 2008; Suarez-Farinas *et al.*, 2010).

TNF-α cytokine also appears to have a key pathogenic role, as many TNF-α-regulated genes are overexpressed in the psoriatic transcriptome (Yao *et al.*, 2008), and TNF-α antagonists are highly effective therapeutic agents in 60–80% of patients across many clinical trials (Fantuzzi *et al.*, 2008; Menter, 2009; Mossner and Reich, 2009). In fact, the TNF responsiveness of psoriasis is somewhat perplexing, given the strong association of psoriasis with the IL-23/Th17 pathway (Di Cesare *et al.*, 2009) and the general view that TNF-α is a master cytokine of innate immunity (Mizgerd *et al.*, 2001; Gottlieb *et al.*, 2005). We have previously shown that in psoriasis TNF-α is functionally linked to the IL-23/Th17 pathway by its ability to activate myeloid dendritic cells that synthesize IL-23 and other regulators of T-cell development (Gottlieb *et al.*, 2005; Zaba *et al.*, 2007, 2009). Thus, TNF-α may serve as an indirect activator of Th17 responses through activating effects on myeloid DCs (Zaba *et al.*, 2007, 2009; Ortega *et al.*, 2009). We have also associated the therapeutic response of psoriasis patients to the TNF-α inhibitor etanercept with suppression of the IL-23/Th17 pathway, rather than a classic ''sepsis cascade'' pathway of innate immunity that is stimulated by TNF-α (Zaba *et al.*, 2007, 2009).

The results of this study identify an alternative way of interaction between TNF-α and Th17 response pathways to influence skin inflammation. KCs express both TNF-α and IL-17 receptors (Albanesi *et al.*, 1999; Boniface *et al.*, 2007) and, on stimulation by both cytokines, produce many inflammatory products at levels that are additive or synergistic, compared with single-cytokine effects. Thus, a much larger set of significant disease-signature genes in the psoriasis transcriptome can be traced to TNF-α/IL-17 synergism. Furthermore, treatment of psoriasis with etanercept suppresses synergistic TNF-α/IL-17 gene transcripts to a greater extent than TNF-α ''single-regulated'' gene products, as shown here. The *in vivo* significance of TNF-α/IL-17 synergism could be ideally validated when results of a recent clinical trial in psoriasis patients with a potent IL-17 receptor antagonist will become available (Russell *et al.*, 2010). As in this recent study complete response was achieved in psoriasis patients both clinically and genomically, it will be of utmost importance to examine the therapeutic effects against our synergistic/additive gene set. Although a potential TNF-α/IL-17 synergism was suggested by few reports (Albanesi *et al.*, 1999; Homey *et al.*, 2000; Guilloteau *et al.*, 2010), our extensive genomic approach expands the list of IL-17/TNF-α-regulated genes from <20 genes to over 350 genes, many of which are key molecules in psoriasis pathogenesis, with great relevance for future disease reversal with either IL-17 or TNF antagonists. We readily acknowledge that our study identified positive IL-17/TNF-α interactions only in epidermal KCs, whereas other cell types in skin, as well as some leukocytes, also coexpress IL-17 and TNF-α receptors (Maitra *et al.*, 2007; Pelletier *et al.*, 2010). Thus, TNF-α/IL-17 interactions may penetrate deeper into the psoriasis transcriptome than we have demonstrated here and IL-17 interactions with other cell types have been suggested as a basis for rheumatoid arthritis (for example Moran *et al.*, 2009).

Finally, although IL-17 has emerged as a cytokine that links autoimmune or inflammatory reactions in several distinct tissues or organs (Tesmer *et al.*, 2008; Abraham and Cho, 2009; Di Cesare *et al.*, 2009; Gaffen, 2009; Crome *et al.*, 2010), we consider that it is not universally pathogenic in all inflammatory diseases. AD, which shares a number of features with psoriasis (Guttman-Yassky *et al.*, 2007), has lower numbers of Th17 T cells and very little IL-17 mRNA expression or induction of ''classic'' IL-17 genes in KCs (Guttman-Yassky *et al.*, 2008). This study extends cross-disease IL-17 distinctions by showing a much lower expression of IL-17/TNF-α synergistic genes in the transcriptome of AD versus psoriasis skin lesions. Hopefully, future work will help us to define broader roles of IL-17 and interactive TNF-α/IL-17 responses in other immune-mediated skin diseases, and the gene sets that are now defined may be used to explore gene expression signatures of other diseases for these effects.

In summary, to our knowledge, these data provide insight into synergistic interactions between IL-17 and TNF-α that are previously unreported. This synergism appears to have a beneficial effect from a therapeutic perspective, and could potentially lead to identification of novel therapeutic targets to intensify disease suppression.

MATERIALS AND METHODS

Primary KC cultures

Primary KCs ($n=4$) were cultured in Epilife medium (Cascade Biologics, Portland, OR) and, once 80% confluent, the medium was supplemented with or without the following cytokines: recombinant human (rh)-IL-17 (R&D System, Minneapolis, MN) of 200 ng ml^{-1} and TNF-α (Sigma-Aldrich, St Louis, MO; 1 ng ml^{-1} or 10 ng ml^{-1}) for 24 hours before harvesting for other analyses. For the time-course experiment, cells were collected after 6-, 12-, and 24-hour treatment. See Supplementary Methods.

Reverse transcriptase-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and on-column DNAse digestion (RNAse-free DNAse Set,

Qiagen), and used for either RT-PCR or gene array. RT-PCR was performed using EZ PCR core reagents, primers, and probes (Applied Biosystems, Foster City, CA) as previously published (Chamian *et al.*, 2005).

Gene array

Total RNA was amplified and labeled using the Message Amp Premier RNA Amplification Kit (Ambion, Austin, TX) and biotinylted cRNA was hybridized to Illumina human HT-12 Bead Chip (Illumina, San Diego, CA). See Supplementary Methods for details.

Statistical analysis

Statistical analysis was performed using R language (www.R-project.org) and Bioconductor packages (www.bioconductor.org). A mixed-effects linear model was fitted to $\log_2$ expression values and hypotheses were tested using a moderated *t*-test and *P*-values adjusted by the Benjamini–Hochberg procedure. A gene was considered to pass the test if the false discovery rate was <0.3 and FCH was >1.3.

To scan for synergistic genes across the 48,802 probes available in Illumina chips, we needed a formal definition of synergy. We adapted the theory presented by Verrier *et al.* (2001) and designed a series of tests to identify synergistic and additive genes. The formal definition and detailed statistics are presented in Supplementary Methods.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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