Induction of Alternative Proinflammatory Cytokines Accounts for Sustained Psoriasiform Skin Inflammation in IL-17C+IL-6KO Mice

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IL-6 inhibition has been unsuccessful in treating psoriasis, despite high levels of tissue and serum IL-6 in patients. In addition, de novo psoriasis onset has been reported after IL-6 blockade in patients with rheumatoid arthritis. To explore mechanisms underlying these clinical observations, we backcrossed an established psoriasiform mouse model (IL-17C+ mice) with IL-6-deficient mice (IL-17C+KO) and examined the cutaneous phenotype. IL-17C+KO mice initially exhibited decreased skin inflammation; however, this decrease was transient and reversed rapidly, concomitant with increases in skin *Tnf*, *Il36α/β/γ*, *Il24*, *Epgn*, and *S100a8/a9* to levels higher than those found in IL-17C+ mice. A comparison of IL-17C+ and IL-17C+KO mouse skin transcriptomes with that of human psoriasis skin revealed significant correlation among transcripts of skin of patients with psoriasis and IL-17C+KO mouse skin, and confirmed an exacerbation of the inflammatory signature in IL-17C+KO mice that aligns closely with human psoriasis. Transcriptional analyses of IL-17C+ and IL-17C+KO primary keratinocytes confirmed increased expression of proinflammatory molecules, suggesting that in the absence of IL-6, keratinocytes increase production of numerous additional proinflammatory cytokines. These preclinical findings may provide insight into why patients with arthritis being treated with IL-6 inhibitors develop new onset psoriasis and why IL-6 blockade for the treatment of psoriasis has not been clinically effective.

Journal of Investigative Dermatology (2016) ■, ■–■; doi:10.1016/j.jid.2016.10.021

INTRODUCTION

The factors responsible for the initiation of a psoriatic skin lesion have yet to be conclusively determined, but involve a tightly orchestrated series of events involving T cells, innate immune cells, dermal fibroblasts, endothelial and epithelial cells, together with chemokines and cytokines that work in concert to exacerbate keratinocyte proliferation giving rise to visible skin plaques (Lowes et al., 2014). The importance of cytokine-mediated inflammation as a driving factor for psoriasis, in particular that of IL-17A and tumor necrosis factor-α (TNFα), is highlighted by the efficacy of TNFα- and IL-17-targeted inhibitors for psoriasis (Crow, 2012; Gordon et al., 2014; Langley et al., 2014; Papp et al., 2012, 2014; Silva et al., 2010).

Patients with psoriasis also exhibit high levels of IL-6 in lesional skin and serum, although IL-6 inhibition has thus far been unsuccessful in mitigating human psoriasis (Castells-Rodellas et al., 1992; Grossman et al., 1989; Mease et al., 2016; Neuner et al., 1991). Paradoxically, psoriasis onset after IL-6 inhibition in patients with rheumatoid arthritis has also been reported (Grasland et al., 2013; Laurent et al., 2010), although the mechanisms underlying this remain unclear.

We recently engineered and characterized a novel transgenic mouse model of psoriasiform skin inflammation that models the cutaneous overexpression of IL-17C observed in human psoriasis lesional skin (K5-promoter-driven IL-17C overexpression, hereafter called IL-17C+) that produced skin-specific elevated levels of TNFα, IL-17A, and IL-6 (Johnston et al., 2013). IL-17C is derived primarily from epithelial cells and exerts context-specific inflammatory effects in the skin and gut and has been hypothesized to regulate T helper17 cell differentiation via Iκβζ (Chang et al., 2011; Johnston et al., 2013; Ramirez-Carrozzi et al., 2011; Song et al., 2011). IL-17C, like IL-17A, synergizes with TNFα to elicit inflammatory responses similar to those induced by IL-17A combined with TNFα (Chang et al., 2011; Johnston

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Abbreviations: KO, knockout; PN, uninvolved human psoriasis skin; PP, involved lesional human psoriasis skin; qRT-PCR, quantitative reverse transcription-PCR; TNFα, tumor necrosis factor-α

Received 1 May 2016; revised 19 September 2016; accepted 11 October 2016; accepted manuscript published online 27 October 2016; corrected proof published online XXX XXXX

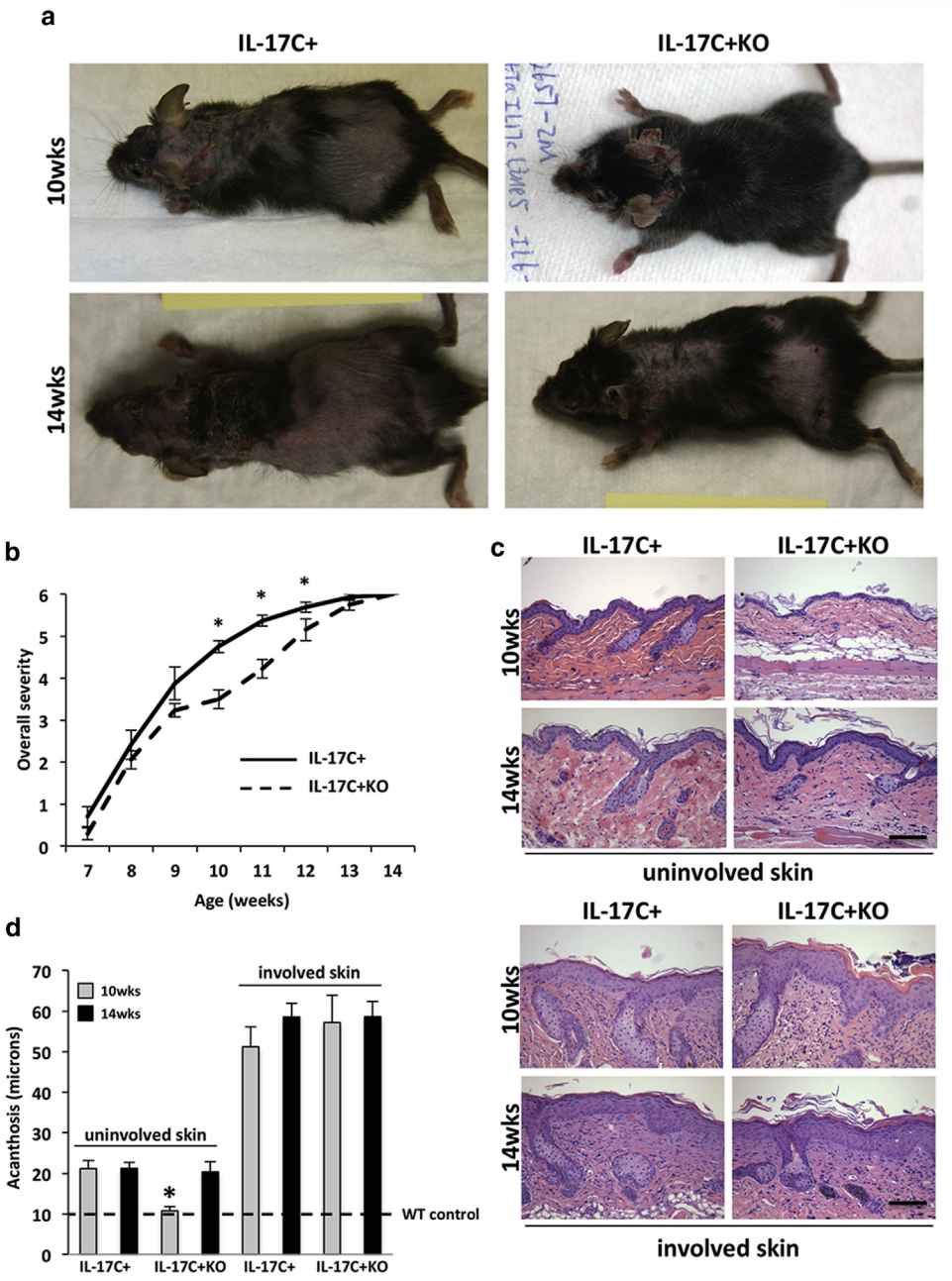

Figure 1. IL-17C+KO mice develop a delayed inflammatory skin phenotype. (**a**) Representative images of IL-17C+ and IL-17C+KO mice at 10 and 14 weeks of age. (**b**) Mouse overall severity scale between 7 and 14 weeks of age. (**c**) Representative images of H&E-stained uninvolved and involved skin from IL-17C+ and IL-17C+KO mice at 10 and 14 weeks of age. (**d**) Mean epidermal thickness (acanthosis; μm) measures (± SEM) of involved and uninvolved dorsal skin of IL-17C+ (n = 8 at 10 weeks; n = 5 at 14 weeks) and IL-17C+KO (n = 8 at 10 weeks, n = 8 at 14 weeks) mice at 10 weeks and 14 weeks of age. The dashed line indicates wild-type mouse levels. Scale bars in (**c**) = 100 μm; **P* < 0.05. H&E, hematoxylin and eosin; KO, knockout; SEM, standard error of the mean.

et al., 2013; Pappu et al., 2011; Ramirez-Carrozzi et al., 2011; Song et al., 2011) including increases in IL-6; and IL-17C+ mice treated with TNFα blocking antibodies have an improved skin phenotype and decreased IL-6.

To determine if IL-6 contributes directly to the skin pathogenesis associated with IL-17C, IL-17C+ mice were backcrossed with IL-6-deficient knockout (KO) animals (IL-17C+KO) and the skin phenotype was examined at the histological, cellular, and molecular levels.

RESULTS

IL-17C+KO mice have delayed skin phenotype

IL-17C+ animals were backcrossed with IL-6KO mice and the skin phenotype was assessed. Initial examination revealed that young adult (approximately 10 weeks of age) IL-17C+KO mice had less skin inflammation than age-matched IL-17C+ animals, determined by a composite severity score based on visual analysis (Figure 1a and b). However, this difference reversed by 14 weeks of age (Figure 1a and b). At the histological level, involved lesional skin from both mouse lines developed similar levels of acanthosis regardless of age (Figure 1c and d), with the epidermal thickness being approximately 5- to 6-fold greater than wild-type C57Bl/6 control animals (indicated by the dashed line), consistent with previous observations (Johnston et al., 2013). Similar levels of dermal angiogenesis, and T-cell and myeloid cell infiltration in lesional skin of IL-17C+ and IL-17C+KO mice were observed regardless of age (data not shown).

In contrast to these findings, acanthosis of uninvolved skin of young adult (approximately 10-week-old) IL-17C+KO mice

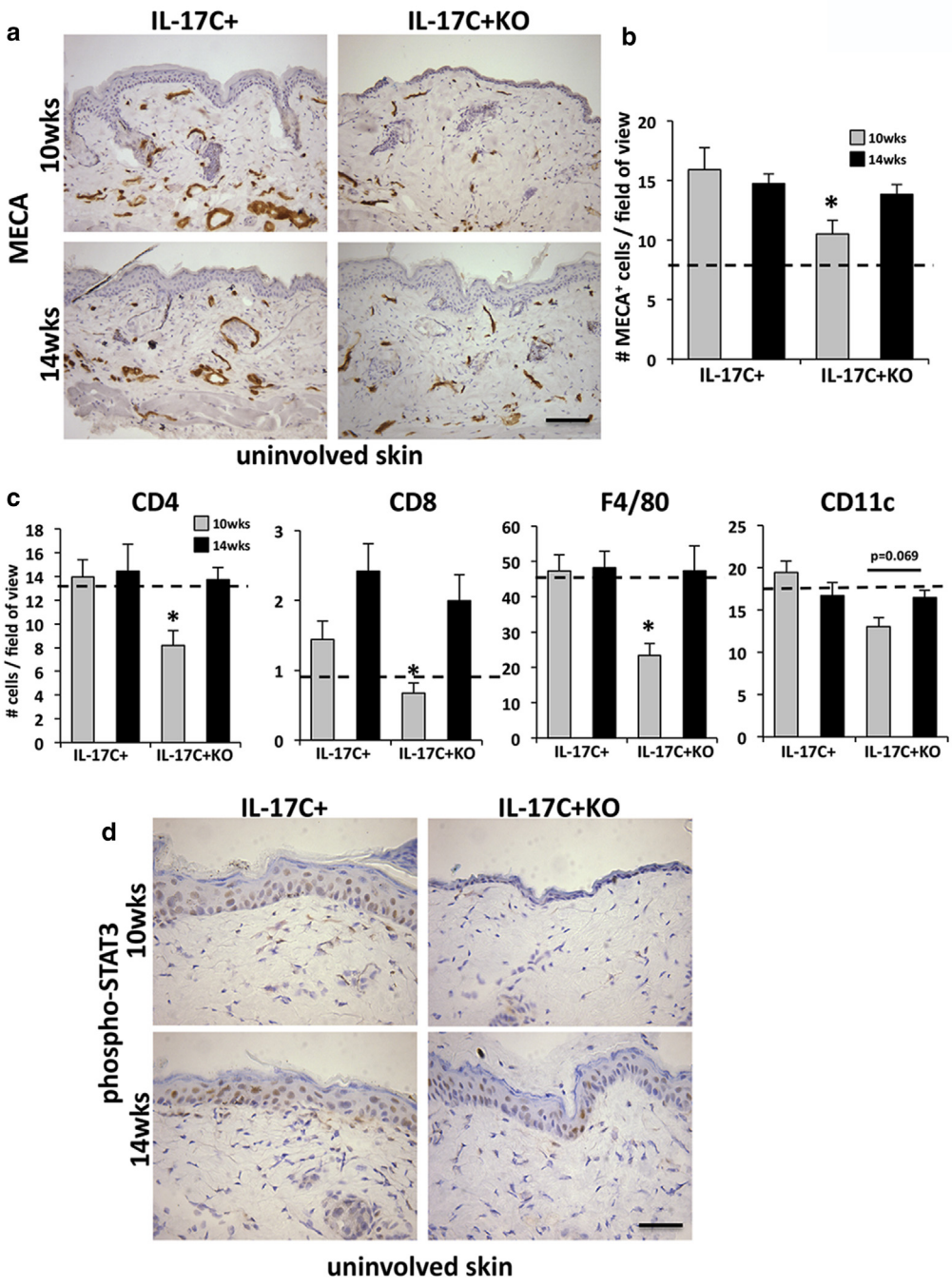

Figure 2. Cutaneous inflammation, angiogenesis, and phospho-STAT3 increase in IL-17C+KO uninvolved mouse skin mice between 10 and 14 weeks. (**a**) MECA-stained uninvolved skin of 10- and 14-week-old IL-17C+ and IL-17C+KO mice. (**b**) Quantification of MECA$^+$ cells (mean cell number/field of view ± SEM) in IL-17C+KO (n = 8) and IL-17C+ mice (n = 7) at 10 and 14 weeks of age (n = 5 each). (**c**) Immune cell quantification (mean number of cells/field of view ± SEM) for CD4$^+$, CD8$^+$ T cells, CD11c$^+$ myeloid cells, and F4/80$^+$ macrophages in IL-17C+KO versus IL-17C+ uninvolved skin at 10 and 14 weeks of age. (**d**) Phospho-STAT3 immunostaining of uninvolved skin from 10- to 14-week-old IL-17C+KO and IL-17C+ mice. The dashed line in (**b**) and (**c**) indicates wild-type mouse levels. Scale bars in (**a**) and (**d**) = 100 μm. *$P < 0.05$ vs. uninvolved IL-17C+ skin at 10 weeks. KO, knockout; MECA, mouse endothelial cell antigen; SEM, standard error of the mean.

was significantly reduced in uninvolved skin compared with age-matched IL-17C+ mice (Figure 1d). Ten-week-old IL-17C+KO mice also had significantly less dermal angiogenesis (Figure 2a and b); fewer skin infiltrating CD4$^+$, CD8$^+$, and F4/80$^+$ cells (Figure 2c; Supplementary Figure S1 online); and decreased expression of phospho-STAT3 (Figure 2d) compared with age-matched IL-17C+ mice. These observations suggest a role for IL-6 in initiating skin inflammation given the differences in nonlesional uninvolved skin. In contrast, once skin inflammation has occurred (in involved lesional skin) deletion of IL-6 does not alter the cutaneous histological phenotype. The differences observed at 10 weeks of age in IL-17C+ and IL-17C+KO mice were eliminated by 14 weeks of age (Figure 1b and c), such that uninvolved skin of IL-17C+KO mice increased in thickness, contained more inflammatory cells and dermal vasculature, and resembled uninvolved skin of IL-17C+ animals. These observations suggest that alternative proinflammatory cells and/or cytokines increase in the absence of IL-6, promoting skin of IL-17C+KO mice to develop a phenotype more similar to that observed for IL-17C+ mice.

IL-6 intradermal injections into IL-17C+KO mouse skin returns the phenotype to that of IL-17C+ mice

Given that IL-6 depletion appeared to decrease the severity of skin lesions early in development, we asked whether exogenous recombinant IL-6 introduced into uninvolved skin of

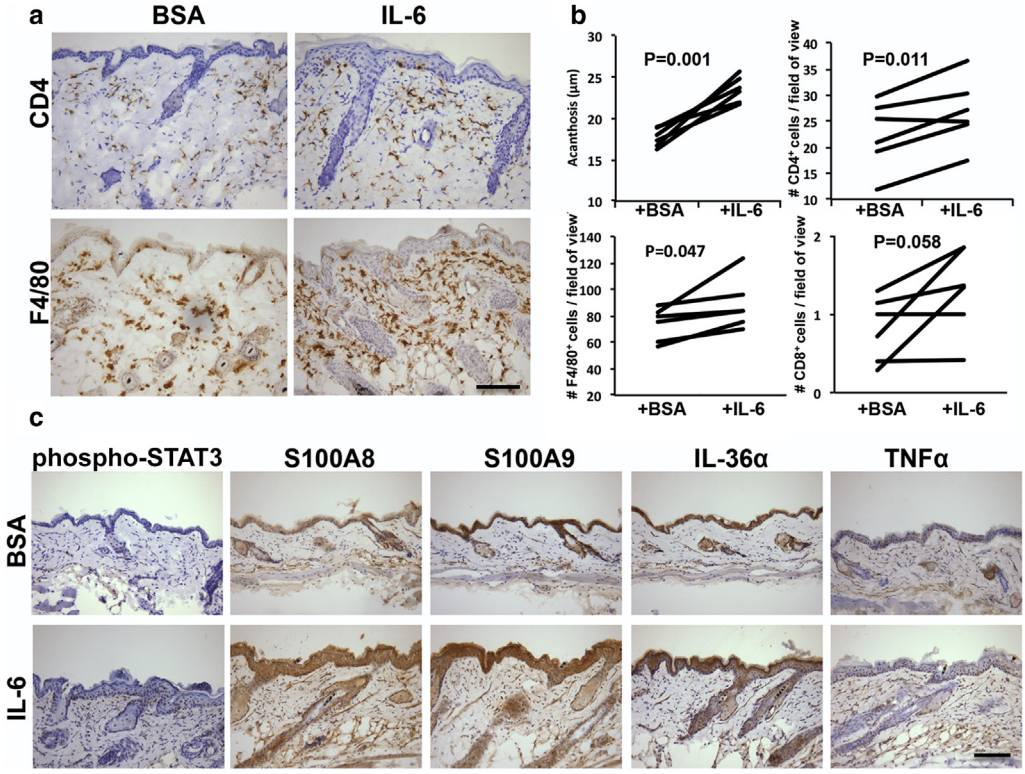

Figure 3. Intradermal IL-6 injection into IL-17C+KO mouse skin elicits an IL-17C+ skin phenotype. (**a**) CD4+ and F4/80+ immunohistochemistry on IL-17C+KO mice skin intradermally injected with BSA or recombinant IL-6 every other day for 16 days. (**b**) Quantification of epidermal thickness (acanthosis, µm) and infiltrating immune cells ($CD4^+$, $CD8^+$, and $F4/80^+$; mean number of cells/field of view) in IL-6- and BSA-injected IL-17C+KO (n = 6). Significance values are as indicated. (**c**) Representative images from IL-17C+KO skin injected with either BSA or IL-6 immunostained for phospho-STAT3, S100A8/A9, IL-36α, and TNFα. Scale bar in (**a**) and (**c**) = 100 µm. KO, knockout; TNFα, tumor necrosis factor-α.

young IL-17C+KO mice (beginning at 8 weeks of age) would result in an enhanced skin phenotype. IL-6-injected IL-17C+KO skin histologically resembled IL-17C+ uninvolved skin, including similar levels of acanthosis and T-cell and macrophage skin infiltration (Figure 3a and b). IL-6 injections also increased phospho-STAT3 activity (Figure 3c). Further analyses of the IL-6- versus BSA-injected skin identified increases in inflammatory proteins including S100A8/A9, IL-36α, and TNFα. These findings suggest that the introduction of exogenous IL-6 into skin genetically overexpressing IL-17C (in the absence of IL-6) promotes immune cell recruitment and increases proinflammatory cytokines and innate defense proteins that potentiate keratinocyte proliferation and promote the development of a skin phenotype that phenocopies uninvolved skin of IL-17C+ mice.

Taking advantage of the KC-Tie2 psoriasis mouse that has elevated cutaneous IL-17C and TNFα (Ward et al., 2011b; Wolfram et al., 2009) (Supplementary Figure S2a online), and when backcrossed with IL-6KO mice has a sustained skin phenotype (Golden et al., 2015), we validated the findings observed in IL-17C+KO mice and determined that intradermal injections of IL-6 or TNFα into the skin of KC-Tie2-IL-6KO mice also lead to an exacerbation of skin inflammation (Supplementary Figure S2b), demonstrating in a second psoriasiform model (one where IL-17C is not genetically manipulated) that similar synergy between IL-17C, IL-6, and TNFα may contribute to skin phenotype. Further evidence of this was seen in primary keratinocytes stimulated with combinations of IL-17C, IL-6, and TNFα where increases in expression of proinflammatory transcripts, including S100A8/A9, IL-36γ, and TNFα, were observed (Supplementary Figures S3 and S4 online).

IL-17C+KO mice have exacerbated inflammation that closely models human psoriasis

We were very interested in the mechanisms underlying the rapid reversal of the delayed phenotype in IL-17C+KO mice. We performed RNAseq on involved and uninvolved skin isolated from IL-17C+ and IL-17C+KO mice and compared changes in identified transcripts with established datasets of psoriasis patient lesional (PP) and nonlesional (PN) skin (n = 44 patients) (Swindell et al., 2016). Genes altered in involved skin of IL-17+KO mice were more similar to those altered in human psoriasis lesions ($r_s = 0.312$), as compared with those genes altered in involved skin of IL-17C+ mice ($r_s = 0.125$; Figure 4a). Genes increased in mouse phenotypes and psoriasis lesions included *Il19, S100a8/a9, Cxcl5, Cxcl1,* and *Klk6/13* (Figure 4b), whereas decreased genes included *Wnt2, Trim55,* and *Myoc* (Figure 4c). To determine why expression shifts in IL17C+KO involved skin were more similar to those in human psoriasis lesions, we evaluated subsets of genes responsive to IL-17C-, TNFα-, IL-22-, IFNγ-, and IL-36-family cytokines. Interestingly, such genes were differentially correlated between mouse phenotypes, with stronger correlations in IL-17C+KO involved skin (PP/PN vs. IL-17C+KO/control, with control = uninvolved skin) compared with IL-17C+ involved skin (PP/PN vs. IL17C+KO/control) (Figure 4d). Taken together, these results suggest that the absence of IL-6 in IL-17C+ animals leads to compensatory increases of proinflammatory transcripts that more closely model human psoriasis.

To further explore the cellular mechanisms mediating these bioinformatics results, we analyzed immune cells isolated from skin draining lymph nodes of IL-17C+ and IL-17C+KO mice and identified increases in $CD3^+$ and $CD4^+$ T-cell

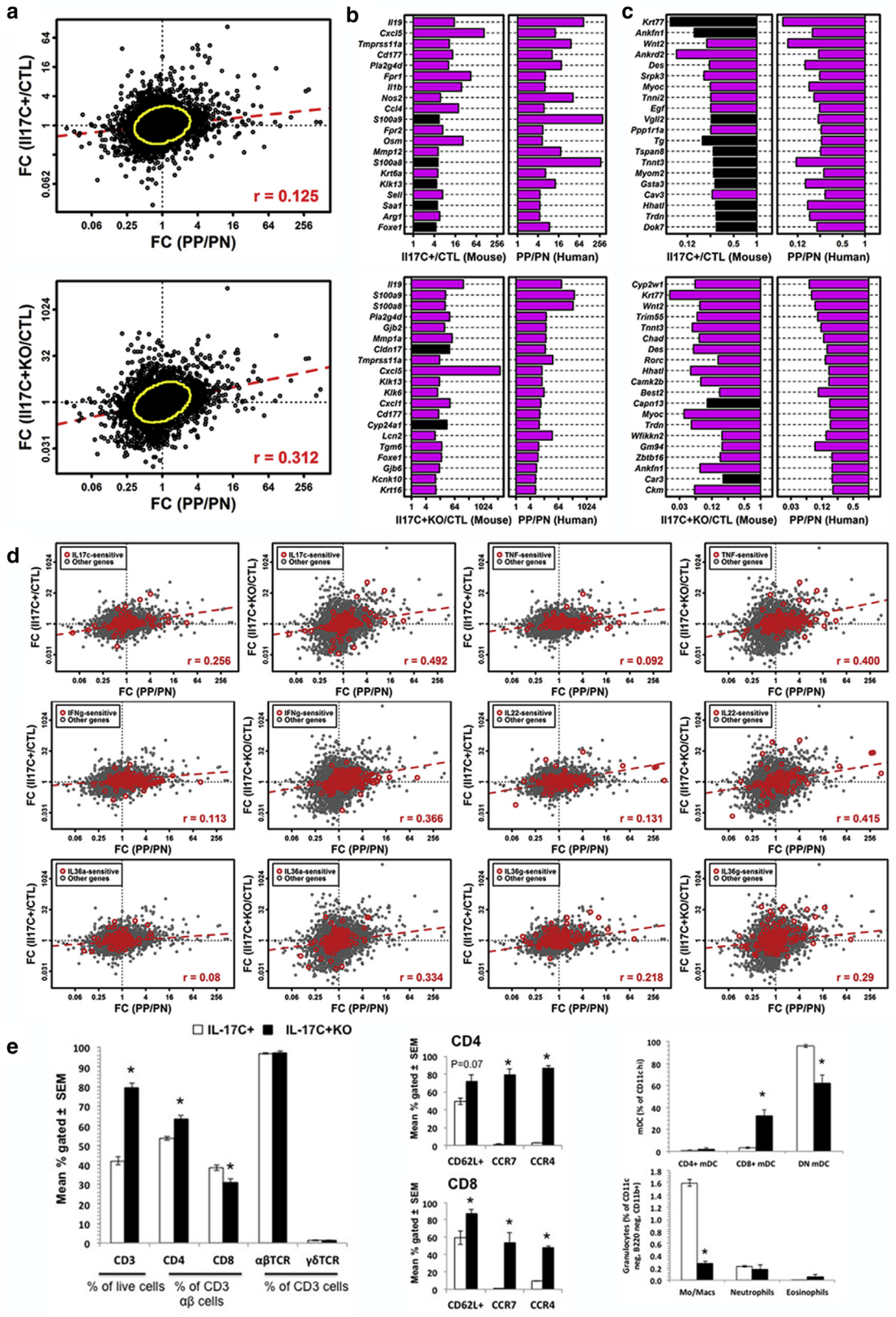

Figure 4. Gene expression changes in IL-17C+ and IL-17C+KO skin and comparison with human psoriasis. (**a**) Comparison of fold changes in IL-17C+ and IL-17C+KO involved and uninvolved skin (CTL; n = 3/group) and human psoriasis (involved [PP]/uninvolved [PN]; n = 44 patients). Yellow circles encompass the 90% of genes closest to the bivariate centroid (Mahalanobis distance). Fold changes for genes (**b**) increased and (**c**) decreased in IL-17C+ and IL-17C+KO mice and human psoriasis (magenta bars, FDR < 0.10). (**d**) Cytokine-sensitive genes and associations among IL-17C+ and IL-17C+KO involved skin and human psoriasis. Red symbols represent cytokine-sensitive genes identified from in vitro experiments performed using cultured keratinocytes (100 cytokine-increased + 100 cytokine-decreased; gray symbols: all other genes). (**e**) Flow cytometric analysis of skin-draining lymph node (SDLN) immune cells from IL-17C+ and IL17C+KO mice examining $CD3^+$ and $CD4^+$ T cell populations and homing molecule expression of CD62L, CCR7, and CCR4 on $CD4^+$ and $CD8^+$ T cells. Analysis of SDLN myeloid cells in IL-17C+ and IL-17C+KO mice. *$P < 0.05$. CTL, control; FC, fold change; FDR, false discovery rate; KO, knockout.

populations in IL-17C+KO mice compared with IL-17C+ mice, and increases in T-cell-derived $CD62L^+$, $CCR7^+$, and $CCR4^+$ expression. $CD8^+$ myeloid dendritic cells increased, but macrophages decreased (Figure 4e). These observations demonstrate an exacerbation of inflammation in IL-17C+KO mice and support the idea that in the absence of IL-6, alternative inflammatory responses occur that can elicit an exacerbated or at the very least, a similar skin phenotype to IL-17C+ mice.

To further identify and validate alternative inflammatory mediators in IL-17C+KO mice, psoriasis-related immune cell-, endothelial cell-, and keratinocyte-derived cytokines were examined. TNFα levels were evaluated by ELISA, as our prior work had identified critical synergy between IL-17C and TNFα (Johnston et al., 2013). Uninvolved skin of 10-week-old IL-17C+KO mice had significantly higher TNFα protein than age-matched IL-17C+ mice (7.3 ± 0.6 vs. 4.8 ± 0.7 pg/ml; $P = 0.01$). Moreover, further increases (approximately 3-fold) in TNFα were seen in uninvolved skin of IL-17C+KO mice as the mice aged (14-week-old), and the skin phenotype worsened toward a phenotype that resembled IL-17C+ mice (7.3 ± 0.6 vs. 21.2 ± 4.9 pg/ml; $P = 0.003$; Figure 5a). Paradoxically, serum TNFα levels were significantly reduced in 10-week-old IL-17C+KO mice (7.07 ± 1.4 vs. 16.7 ± 3.7 pg/ml; $P = 0.03$; Figure 5a), consistent with, and likely reflective of, less overall skin inflammation in IL-17C+KO mice (i.e., less body surface area with lesional skin; Figure 1a). The difference in serum TNFα levels was eliminated by 14 weeks of age, as IL-17C+KO mice exhibited increased skin inflammation.

Next, we used gene expression microarray analyses coupled with Ingenuity Pathway Analysis and quantitative reverse transcription-PCR (qRT-PCR) to validate critical gene expression changes between IL-17C+ and IL-17C+KO mice (Figure 5b and Supplementary Figures S5–S7 online). At 10 weeks of age, when IL-17C+KO mice had a less severe phenotype, a number of key psoriasis signature genes, including *Il36α/β/γ*, *Defb3*, and *Il17c*, were decreased compared with age-matched IL-17C+ mice. However at 14 weeks, these transcripts, as well as *Epgn, S100a8/a9 (Mrp8/14), Il24,* and the IL-6 receptor cytokine, *Osm,* all increased significantly in IL-17C+KO mice (Figure 5b and c). In fact, *Epgn*, *Il36α,* and *S100a8* all increased to levels beyond those observed in IL-17C+ mice at 14 weeks of age; and finally, *Tnf, Il36α/β/γ, Il24, Epgn,* and *S100a8/a9* levels all significantly increased in IL-17C+KO mice at 14 weeks compared with IL-17C+ mice at 10 weeks. These changes suggest that the transient, less severe skin phenotype observed in 10-week-old IL-17C+KO mice may result from delays in elevation of these signaling molecules, and that by 14 weeks of age these transcripts increase to levels beyond that observed in skin of IL-17C+ mice, allowing the skin phenotype to rapidly resemble the skin of IL-17C+ animals and leading to a transcript signature that more closely resembles human psoriasis lesional skin (Figure 4).

Finally, primary keratinocytes isolated and expanded from IL-17C+ and IL-17C+KO mouse skin confirmed increases in *Il17c* (2.3-fold; $P = 0.02$), *Tnf* (1.8-fold; $P = 0.006$), *S100a8* (7-fold; $P < 0.001$), *S100a9* (4.5-fold; $P = 0.002$), *Defb3* (9.4-fold; $P = 0.02$), and IL-36 family members (*Il36α/Il36β/Il36γ*; 2- to 7-fold; $P < 0.001$) in IL-17C+KO keratinocytes compared with IL-17C+ keratinocytes (Figure 5d).

DISCUSSION

We show that in the absence of IL-6, the psoriasiform skin phenotype in IL-17C+KO mice is initially delayed, but this delay is transient and rapidly reversed as a result of increases in compensatory proinflammatory factors. This enhanced inflammatory response was confirmed in primary keratinocytes isolated and expanded from IL-17C+KO mice demonstrating that IL-6-deficient keratinocytes increase production of alternative proinflammatory chemokines and cytokines (Figure 5d). Of particular interest was the higher correlation with human psoriasis transcript changes observed between involved and uninvolved skin for the IL-17C+KO mice, providing additional evidence of an exacerbation of skin inflammation that is relevant to human psoriasis. These preclinical findings provide insight into why patients being treated with IL-6 inhibitors may develop new onset psoriasis and why IL-6 blockade for the treatment of psoriasis has not been clinically effective.

IL-6 inhibitors have failed to improve psoriasis skin manifestation despite efficacy in rheumatoid arthritis and psoriatic arthritis (Hartung et al., 2013, Mease et al., 2016). The paradoxical elicitation of psoriasis in patients undergoing treatment with the IL-6 inhibitor tocilizumab (Grasland et al., 2013; Wendling et al., 2012) suggests that inhibition of IL-6 signaling may not improve skin outcomes. Indeed, inhibition of the IL-6 receptor may allow increases in circulating IL-6. Our data suggest that an alternative reason why IL-6 therapeutic blockade fails to resolve skin inflammation is the ability of the skin to readily and efficiently compensate for the loss of IL-6.

IL-36 family members (*Il36α/β/γ*) along with *S100a8/9, Epgn, Defb3, Osm,* and *Il24* transcripts all increase significantly between 10 and 14 weeks in IL-17C+KO skin, concomitant with increases in angiogenesis, skin infiltrating T cells, and macrophages. Moreover, the transcript changes occurring between involved and uninvolved skin in IL-17C+KO mice more closely correspond with changes identified between human psoriasis lesions and perilesional skin. Significant increases in IL-17C-, TNF-, IFNγ-, IL-22-, and IL-36-sensitive genes were also observed in IL-17C+KO mice when compared with IL-17C+ mice. These occurred at the same time increases in $CD3^+$ and $CD4^+$ T cells in the skin draining lymph nodes of IL-17C+KO mice were observed. Together, these observations support the idea that alternative cellular and molecular changes compensate for the loss of IL-6 in IL-17C+KO mice and allow skin severity to increase to similar levels as IL-17C+ mice.

Paradoxically, IL-17C+KO animals initially showed a delay in the development of the skin phenotype, potentially a result of decreases in skin IL-17C and IL-36. Exogenous introduction of recombinant IL-6 returned the skin phenotype to levels of IL-17C+ mice concomitant with increases in cutaneous phospho-Stat3, TNFα, IL-36α, and S100A8/A9, suggesting a potential capacity of IL-6 to synergize both with IL-17C, TNFα, and IL-36α. IL-17C is known to synergize with TNFα (Johnston et al., 2013; Ramirez-Carrozzi et al., 2011), IL-1β (Ramirez-

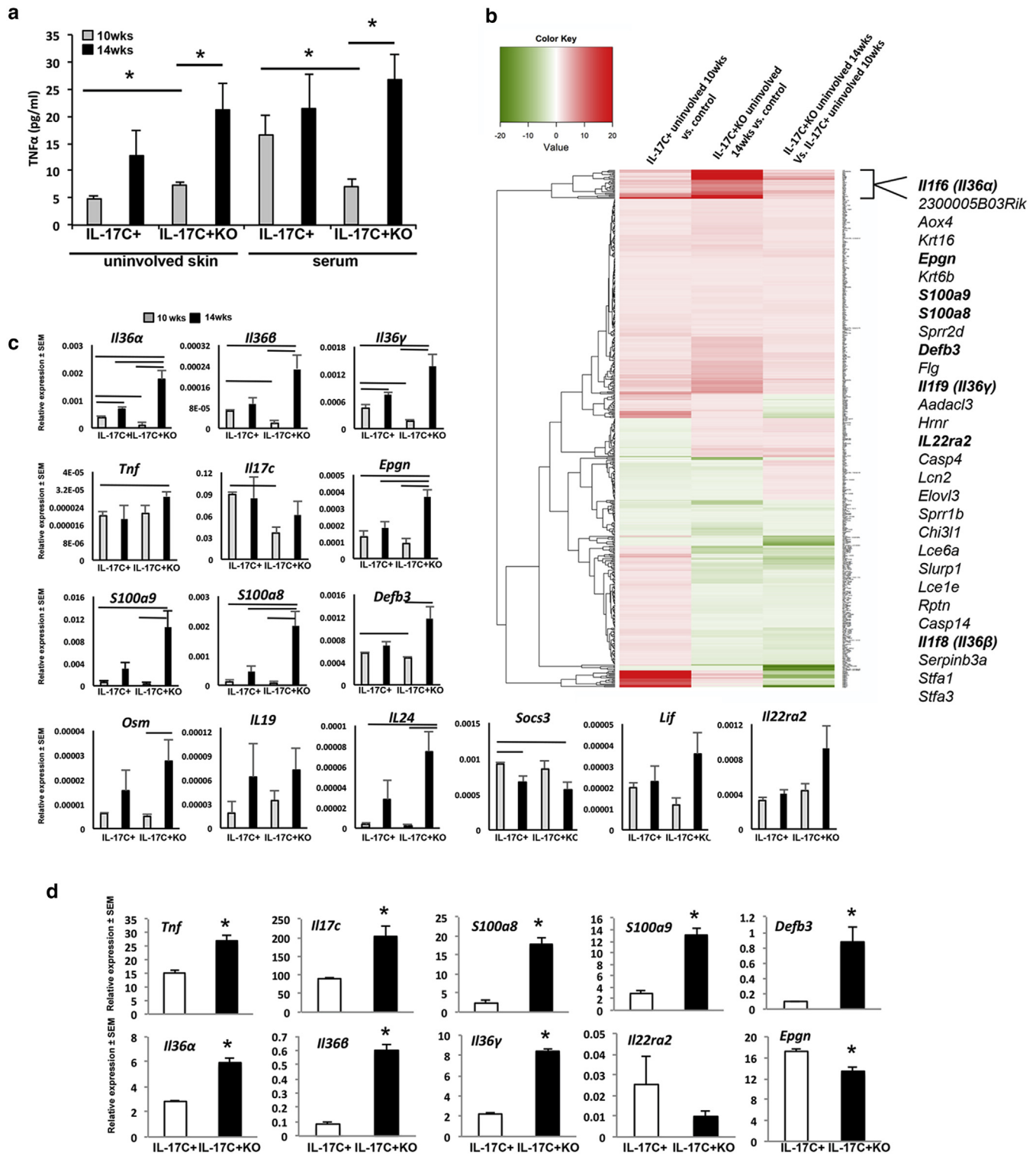

Figure 5. Compensatory proinflammatory molecules increase in uninvolved skin of IL-17C+KO mice between 10 and 14 weeks. (**a**) Uninvolved skin and serum TNFα levels in IL-17C+ and IL-17C+KO mice at 10 and 14 weeks (n = 6 each). (**b**) Heat map of changing transcripts between IL-17C+ and control mice at 10 weeks of age compared with genes altered in 14-week-old IL-17C+KO mice versus controls (n = 4–5/group). (**c**) qRT-PCR validation of transcripts identified in 10- and 14-week-old uninvolved skin of IL-17C+ and IL-17C+KO mice (n = 4/group). Solid lines indicate $P < 0.05$ between groups. (**d**) qRT-PCR on primary neonatal mouse keratinocytes from IL-17C+ and IL-17C+KO mice examining expression levels of *Tnf, Il17c, S100a8/a9, Defb3, Il36α/β/γ, Il22ra2,* and *Epgn* (n = 3/group performed in triplicate). *$P < 0.05$. Lines indicate significant differences between groups with $P < 0.05$. KO, knockout; TNFα, tumor necrosis factor-α; qRT-PCR, quantitative reverse transcriptase-PCR.

Carrozzi et al., 2011), IL-22 (Song et al., 2014), and most recently with IL-36γ (IL-1F9) (Friedrich et al., 2014) to elicit the production of inflammatory signals from epithelial cells. Thus, we also demonstrated that IL-6 synergizes with IL-17C alone and in combination with TNFα to drive a psoriasis-like signature from primary mouse and human keratinocytes, including

increases in *S100a8/a9, Defb3, Il36α/β/γ, Tnf,* and additional *Il17c* (Supplementary Figures S3 and S4). Interestingly, recombinant IL-6 and TNFα, together, but not alone, injected into uninvolved skin of K5-IL-17C mice also significantly increase acanthosis and skin infiltrating $CD4^+$ and $F4/80^+$ immune cells (Supplementary Figure S8 online). These in vitro and in vivo observations support the ability of IL-17C, IL-6 (and TNFα) to promote skin inflammation and identify another IL-17C-synergistic signaling partner in addition to IL-1β, IL-22, and IL-36γ.

The ability of biologic therapies to affect skin changes is largely dependent on direct blockade of signaling molecules and/or their cognate receptors. Despite the success of biologic therapies, patients with recalcitrant disease emerge and show incomplete drug action, or a propensity for disease recurrence despite therapy. In the case of psoriasis, our data suggest that key combinations of signaling molecules, namely IL-6, TNFα, and IL-17C, can combine to elicit skin inflammation and that blocking of any single agent may be temporarily effective, but over time, becomes compensated for by other nontargeted inflammatory molecules. It is likely that combination therapies addressing two or more possible signals may have higher efficacy in psoriasis for patients who become refractory to single-agent therapies. An example of this is the bispecific ABT-122 (AbbVie, North Chicago, IL) antibody currently under development that targets both TNFα and IL-17A. A combined approach may prove useful for patients who fail to respond or stop responding to one targeted drug for multiple reasons: TNFα inhibitors are highly effective for the treatment of skin (psoriasis) and joint inflammation (psoriatic arthritis) and work quickly on targeting joint inflammation; in contrast, IL-17A inhibitors clear skin inflammation rapidly and appear to be effective for psoriatic arthritis, but take more time to reach comparable protection afforded by TNFα inhibitors. Therefore, using TNFα and IL-17A inhibitors simultaneously may offer a unique solution to clearance of skin involvement while protecting the joints from further irreversible damage.

Determining the compensatory pathways upregulated on inhibition of signaling by currently employed therapeutics may inform potential alternative (complementary) targets that must also be inhibited to achieve the optimal efficacy of particular inhibitors. Our data suggest that a combination of anti-IL-6 with either TNF or IL-17 inhibitors may improve the efficacy of IL-6 inhibitors for treating psoriatic skin disease.

METHODS

Mice

K5tTA, Tet^{os}IL-17C, and Tet^{os}Tie2 engineering have been previously described (Diamond et al., 2000; Johnston et al., 2013; Wolfram et al., 2009). Individual mouse lines were backcrossed with IL-6KO mice (JAX cat# 002650; www.Jax.org) to generate either K5tTA-, Tet^{os}-IL-17C-homozygous IL-6KO mice (IL-17C+KO), or Tet^{os}-Tie2-homozygous IL-6KO mice. These were then mated with each other in the presence of doxycycline (200 mg/kg; Bio-Serve, Beltsville, MD) to repress transgene expression until birth (IL-17C+ mice) or for the first week of gestation (KC-Tie2 mice), at which time mice were switched to regular P3000 diet. K5tTA and Tet^{os}IL-17C on a C57Bl/6 background were mated using the same approach to provide K5-IL-17C-C57Bl/6 (IL-6 proficient; IL-17C+) controls. C57Bl/6 and IL-6KO mice served as additional background strain controls. Male and female mice were used for all experimental analyses.

IL-17C+ and IL-17C+KO mice and controls were euthanized at 10 weeks or 14 weeks of age. Involved and uninvolved skin from each mouse were harvested for histology, immunohistochemistry, and protein and RNA analyses as previously described (Johnston et al., 2013). Although alopecia developed in both IL-17C+ and IL-17C+KO mice, this criterion was not used as a measure to identify skin involvement, and was consistent with what we previously reported. Involved and uninvolved areas occur routinely on the mouse body and samples from each were harvested from the same geographic location across mice. Involved skin was defined as skin with well-demarcated scale and erythema, whereas uninvolved demonstrated no phenotypic difference from control skin.

ELISA (R&D, Minneapolis, MN) detected an average approximately 13-fold increase in IL-6 protein in involved IL-17C+ mouse skin (51.3 ± 11.5 pg/ml; range, 20–93 pg/ml; n = 6) and nearly undetectable levels of IL-6 in involved IL-17C+KO mouse skin (4.1 ± 1.9 pg/ml; range, 0–13 pg/ml; n = 8). The assay range of the ELISA was 7.8–500 pg/ml.

Histological and immunostaining analyses

Formalin-fixed paraffin-embedded skin was sectioned and stained with hematoxylin and eosin as described previously (Wolfram et al., 2009). Fresh frozen skin was sectioned and stained using protocols and antibodies previously published (Foster et al., 2014; Johnston et al., 2013; Wolfram et al., 2009) and using antibodies targeting S100A8/A9 and TNFα (all R&D Systems).

Epidermal thickness (acanthosis) measurements and immune cell quantification of skin were performed as previously described (Ward et al., 2011; Wolfram et al., 2009).

ELISA

Sera or protein isolated from skin adjacent to that used for histological and immunostaining analyses using standard protocols as previously published (Wolfram et al., 2009) was used for ELISA according to the manufacturer's instructions.

Intradermal cytokine experiments

Purified recombinant cytokine (IL-6, TNFα, or both; R&D Systems) or BSA (500 ng of each protein in 100 µl volume) was intradermally injected into the dorsum of uninvolved skin of approximately 8-week-old IL-17C+, IL-17C+KO, KC-Tie2-IL-6KO, and/or C57Bl/6 mice every other day for 16 days. Each animal had uninvolved skin injected with both BSA and cytokine at separate sites, and skin from each site was harvested approximately 2 days after the last injection and used for histological and immunostaining analyses.

Primary mouse keratinocyte culture, stimulation, and gene expression analyses

Mouse keratinocytes were isolated and cultured as described (Dlugosz et al., 1995) and stimulated with recombinant mouse IL-17C, TNFα, and IL-6 (all 10 ng/ml, R&D Systems) for 8 hours and then RNA isolated and qRT-PCR performed as above.

Primary human keratinocyte culture, stimulation, and gene expression analyses

Normal human keratinocyte cultures were established from sun-protected adult human skin as described (Elder et al., 1991). Keratinocytes were stimulated with recombinant human IL-17C (100 ng/ml), TNFα (20 ng/ml), and IL-6 (20 ng/ml) or combinations

thereof and then RNA isolated and qRT-PCR performed as described (Johnston et al., 2011).

RNA sequencing and analyses

Total RNA was converted to mRNA by polyA purification and cDNA was generated using random primers. Products were sequenced on a 50-cycle single end HiSeq 2000 system (Illumina; 6 samples/lane; high output mode; version 3 reagents). This generated an average 29.9 million 50 bp reads per sample (range, 23.2−38.7 million). Reads were quality-filtered and mapped to the mouse genome (UCSC, GRCm38/mm10) using a workflow described previously (Swindell et al., 2014, 2016), with reads mapped using tophat2 (Kim et al., 2013) and gene counts and FPKM calculated using HTSeq (Anders et al., 2015) and Cufflinks (Trapnell et al., 2012), respectively. Raw gene counts were normalized using the trimmed mean of the M-values method (Robinson and Oshlack, 2010), and differential expression analyses were performed using edgeR (Robinson et al., 2010). Differential expression trends in IL-17C+ and IL-17C+KO mice were compared with those estimated from lesional (PP) and uninvolved (PN) skin from patients with psoriasis. Psoriasis differential expression statistics were obtained from a recent meta-analysis study that included 44 patients with psoriasis (Swindell et al., 2016) (Gene Expression Omnibus series identifiers GSE41745, GSE54456/GSE63979, and GSE66511). Cytokine-sensitive genes (Figure 4d) were identified from microarray comparisons between cytokine-treated keratinocyte cultures and control-treated keratinocytes (GSE12109, GSE25400, GSE32620, GSE32975, and GSE53751) (Swindell et al., 2016).

RNA, microarray, Ingenuity Pathway Analysis, and real-time qRT-PCR validation on murine skin

RNA was isolated from skin adjacent to that used for histology and immunostaining and was used for microarray, Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA), and qRT-PCR analyses as previously described (Wolfram et al., 2009).

Individual genes of interest were validated using an Applied Biosystems StepOne Plus Real-Time PCR system following our established protocol (Johnston et al., 2013) and using mouse-specific probes and primers obtained from Applied Biosystems (Grand Island, NY). Individual values for each animal and each primer/probe set were normalized to glyceraldehyde-3-phosphate dehydrogenase and then presented as an average/group/time point ± standard error of the mean.

RNA-seq and microarray data have been submitted to Gene Expression Omnibus and are available under the accession GSE86140.

Flow cytometry

Mouse skin draining lymph nodes were minced with sterile razor blades and then disrupted further in GentleMACS C-tubes (Miltenyi) followed by enzymatic digestion with 0.1 mg/ml DNase I, 0.5 mg/ml collagenase, and 0.1 mg/ml hyaluronidase (Sigma) in Hanks' Balanced Salt Solution at 37 °C for 30 minutes with occasional agitation. Cell suspensions were passed through a 40-μm cell strainer (Fisher) and then placed in red blood cell lysis buffer (155 mM NH_4Cl, 0.01 M $KHCO_3$, 0.01% EDTA). After washing, cellular Fc receptors were blocked for 20 minutes at 4 °C with anti-CD16/CD32 antibodies (Biolegend, San Diego, CA) before staining with a viability dye (Invitrogen) and antibodies against CD3e (clone 145-2C11), CD4 (RM4-5), CD8a (5-6.7), TCR-β (H57-597), TCR-γδ (GL3), CD11b (M1/70), CD11c (N418), CD62L (MEL-14), CCR4 (2G12), CCR7 (4B12), or appropriate isotype controls (all FACS antibodies from Biolegend) for 30 minutes. Cells were washed and then analyzed on an LSR2 flow cytometer (BD Bioscience), gating lymphocyte populations guided by isotype control binding.

Statistics

All data are represented as mean ± standard error of the mean. Analysis of between-group comparisons was completed using an unpaired, unequal variance, two-tailed Student's *t*-test, and statistical significance was defined as $P \leq 0.05$. For intradermal injections where BSA and recombinant cytokine were injected into the same animal, a paired two-tailed Student's *t*-test was used and statistical significance remained defined as $P \leq 0.05$.

Study approval

All animal protocols were consistent with guidelines issued by the American Association for Accreditation of Laboratory Animal Care and were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (Cleveland, OH). All human subject protocols were approved by the institutional review boards of the University of Michigan (Ann Arbor, MI) or Case Western Reserve University (Cleveland, OH) and written informed consent was received from participants before inclusion in the study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported in part by the Lozick Discovery Grant, the National Psoriasis Foundation, the American Skin Association, the Babcock Foundation Endowment, the Murdough Family Center for Psoriasis, the Kenneth and Frances Eisenberg Emerging Scholar Award of the A. Alfred Taubman Medical Research Institute, and the following grants from the National Institutes of Health: K08 grant AR060802, R01 AR069071, P30 AR39750, R01 AR063437, R01 AR062546; R21 AR063852; K01 AR064765. Microarray experiments were supported by the Gene Expression and Genotyping Core Facility of the Case Comprehensive Cancer Center (P30 CA043703). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors declare no additional competing financial interests.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.10.021.

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