The Journal of Immunology

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Supplementary Material	http://www.jimmunol.org/content/suppl/2013/01/28/jimmunol.1201505.DC1
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Keratinocyte Overexpression of IL-17C Promotes Psoriasiform Skin Inflammation

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IL-17C is a functionally distinct member of the IL-17 family that binds IL-17 receptor E/A to promote innate defense in epithelial cells and regulate Th17 cell differentiation. We demonstrate that IL-17C (not IL-17A) is the most abundant IL-17 isoform in lesional psoriasis skin (1058 versus 8 pg/ml; $p < 0.006$) and localizes to keratinocytes (KCs), endothelial cells (ECs), and leukocytes. ECs stimulated with IL-17C produce increased TNF-α and KCs stimulated with IL-17C/TNF-α produce similar inflammatory gene response patterns as those elicited by IL-17A/TNF-α, including increases in IL-17C, TNF-α, IL-8, IL-1α/β, IL-1F5, IL-1F9, IL-6, IL-19, CCL20, S100A7/A8/A9, DEFB4, lipocalin 2, and peptidase inhibitor 3 ($p < 0.05$), indicating a positive proinflammatory feedback loop between the epidermis and ECs. Psoriasis patients treated with etanercept rapidly decrease cutaneous IL-17C levels, suggesting IL-17C/TNF-α–mediated inflammatory signaling is critical for psoriasis pathogenesis. Mice genetically engineered to overexpress IL-17C in KCs develop well-demarcated areas of erythematous, flakey involved skin adjacent to areas of normal-appearing uninvolved skin despite increased IL-17C expression in both areas ($p < 0.05$). Uninvolved skin displays increased angiogenesis and elevated S100A8/A9 expression ($p < 0.05$) but no epidermal hyperplasia, whereas involved skin exhibits robust epidermal hyperplasia, increased angiogenesis and leukocyte infiltration, and upregulated TNF-α, IL-1α/β, IL-17A/F, IL-23p19, vascular endothelial growth factor, IL-6, and CCL20 ($p < 0.05$), suggesting that IL-17C, when coupled with other proinflammatory signals, initiates the development of psoriasiform dermatitis. This skin phenotype was significantly improved following 8 wk of TNF-α inhibition. These findings identify a role for IL-17C in skin inflammation and suggest a pathogenic function for the elevated IL-17C observed in lesional psoriasis skin.   *The Journal of Immunology*, 2013, 190: 2252–2262.

Considerable data have been developed in the past two decades regarding IL-17 cytokine family members and the initiation of inflammation by IL-17, specifically IL-17A and IL-17F, cytokines produced by Th17 cells, mast cells, neutrophils, and γδ-T cells (1). IL-17C is a newly cloned member of the IL-17 family, sharing 23% homology with IL-17A, but maps to a different chromosome location (2). Unlike IL-17A, little is currently known about the biological role or importance of IL-17C in promoting inflammation, although several recent reports identified key roles for IL-17C and its receptors IL-17RE/RA in regulating innate immune function in epithelial cells (3–7), promotion of mucosal barrier maintenance (8), promoting Th17 cell responses (9), and potentially, in the skin disease psoriasis (5, 10).

Johansen et al. (10) initially reported increases in IL-17C mRNA and protein in lesional compared with nonlesional human psoriasis skin. IL-17C was rapidly decreased following TNF-α inhibition (11), possibly as a result of NF-κB signaling downstream of TNF-α (4). More recent reports have demonstrated an amelioration of imiquimod-induced psoriasiform skin inflammation in IL-17C– and IL-17RE/RA–deficient mice (5, 12), suggesting that IL-17C contributes to the initiation and/or maintenance of psoriatic skin. Others have also identified a >50-fold increase in keratinocyte (KC)-derived IL-17C following combined IL-17A/TNF-α stimulation (13) along with increases in ∼160 other genes that respond synergistically and ∼196 genes that respond additively that are also found in the human psoriasis transcriptome. These findings suggest that psoriasis therapies targeting IL-17A, in addition to proven TNF-α–targeted biologics, should lead to a significant downregulation in psoriasis-related genes. Indeed, recent clinical reports demonstrate IL-17A–targeted therapeutics are efficacious in psoriasis patients (using ixekizumab; formerly LY2439821; 75 mg), leading to significant clinical improvement, with 83% of patients achieving a 75% reduction in disease severity (Psoriasis Area and Severity Index score 75) and 38% of patients achieving complete clearance (14) by week 12 of the study. Interestingly, targeting IL-17RA with the Ab brodalumab (formerly AMG827; 210 mg) resulted in 82% of patients achieving a Psoriasis Area and Severity Index score of 75 and 62% of patients achieving complete clearance (15). The observation that IL-17C and IL-17A share IL-17RA as a coreceptor (5, 6, 9) may provide rationale for why patient responses to bro-

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dalumab are closer to optimal and provide contextual relevance for targeting IL-17C–signaling in psoriasis.

Given the shared receptors for IL-17 family proteins, it is likely that IL-17 family members collaborate to enhance inflammation by affecting distinct cellular targets. To further examine the importance of IL-17C in psoriasis pathogenesis, we compared gene and protein expression levels of IL-17 family members in psoriasis and control patient skin, examined primary human KCs stimulated with IL-17C/TNF-α for changes in key psoriasis transcriptome genes, and engineered mice to overexpress KC-specific IL-17C (K5–IL-17C), modeling the epidermal-specific overexpression of IL-17C observed in human lesional psoriasis skin.

Materials and Methods

Study population

Eight individuals with chronic plaque psoriasis and eight normal controls were enrolled (age range 18–75 y). Inclusion criteria included one or more well-demarcated, scaly, erythematous psoriatic plaques that were not limited to the scalp and no systemic antipsoriatic treatments for 2 wk before biopsy. Biopsy sites varied between patients depending on active plaques, whereas biopsies of uninvolved and control skin were from the buttocks.

For the etanercept study, 18 individuals with chronic plaque psoriasis were enrolled (age range 18–75 y). Entry criteria included age >18 y and stable plaque-type psoriasis involving at least 10% body surface area. Exclusion criteria included use of systemic psoriasis therapy within 4 wk, topical therapy within 2 wk, or severe comorbid diseases. For 12 wk, subjects received etanercept (Enbrel; Amgen, Thousand Oaks, CA) 50 mg twice a week s.c. (open label). At baseline, 6-mm punch biopsies were obtained under local anesthesia (lidocaine) from uninvolved skin and a target plaque. Subsequent biopsies were taken on days 1, 3, 7, and 14 of therapy from the same target plaque.

Informed consent was obtained from all subjects under protocols approved by the Institutional Review Board of the University of Michigan or University Hospitals Case Medical Center/Case Western Reserve University. This study was conducted in compliance with good clinical practice and according to the Declaration of Helsinki principles.

Primary human dermal microvascular endothelial cells

Endothelial cells (ECs) were purchased from Lonza (Hopkinton, MA) and grown according to the manufacturer's instructions. Confluent cells were stimulated in triplicate with rIL-17C (200 ng/ml; R&D Systems, Minneapolis, MN) for 6 h. Experiments were reproduced independently at least three times.

Primary human keratinocytes

Normal human KC cultures were established from adult human skin and grown for stimulation experiments as previously described (16–18). Recombinant human TNF-α (2 ng/ml) and IL-17C (200 ng/ml) were obtained from R&D Systems. Experiments were reproduced independently using KCs grown from at least three different patients.

Transgenic mice

To overexpress murine IL-17C, we PCR cloned HindIII and XhoI sites onto IL-17C using pCR4-IL17C as the template (Source BioScience, Nottingham, U.K.) and then subcloned the HindIII and XhoI enzyme digested product into pSecTag2B (Life Technologies, Grand Island, NY) to introduce a secretory signal and a Myc-His epitope. The sequence of this construct was confirmed and a Sal1-digested fragment was then inserted into the pTet^{os} vector using methods previously published (19, 20). Expression was validated in vitro in 293T cells cotransfected with Tet^{os}–IL-17C and CMV-tTA plasmid DNA using electrophoresis and Western blotting on proteins isolated from cells and conditioned media. IL-17C and Myc-His protein expression were confirmed in both cells and supernatants. The backbone of the plasmid was removed using PvuI, and the DNA insert was prepared using standard techniques. The oocyte injection of the construct was completed as described previously (21). One set of injections was completed, with five founder mice being born. These mice were mated to a keratin 5 (K5tTA) keratinocyte-specific driver line (22) (generously provided by Dr. Adam Glick; Pennsylvania State University), which we backcrossed to the C57BL/6 background strain for >10 generations prior to use in these experiments. Offspring were genotyped by PCR using DNA extracted from ear biopsies and tTA transgene primers as previously described (23). Primers for PCR genotyping of the pTet^{os} were: forward 5′-ACC ATG TTC ATG CCT TCT

T-3′ and IL-17C reverse 5′-ACT TCG AGT TAG CAG GTG T-3′. Control littermates were mice that had inherited one or no transgenes. All cloning protocols and animal protocols were approved by the Institutional Biosafety Committee and the Case Western Reserve University Institutional Animal Care and Use Committee and conformed to the American Association for Accreditation of Laboratory Animal Care guidelines.

Systemic TNF-α inhibition

TNF-α inhibition was completed using a rat/mouse chimeric monoclonal IgG2a,κ Ab specific for murine TNF-α (CNTO5048) generously provided by Dr. David Shealy (Johnson & Johnson's Janssen Research and Development). Adult mice with established skin disease were injected with either anti–TNF-α ($n = 4$) or negative control murine IgG2a,κ mAbs ($n = 4$) at 5 mg/kg i.p., once a week for an 8-wk period.

RNA and real-time quantitative RT-PCR

After removal from the skin, human skin biopsies were snap-frozen in liquid nitrogen and stored at $-80°C$ until use. RNA was isolated from human and mouse skin, primary human ECs, and KCs and reverse transcribed as previously described (17, 18) and transcripts amplified and quantitated using a Prism 7700 Sequence Detector (Applied Biosystems, Carlsbad, CA). TaqMan primer sets and probes were purchased from Applied Biosystems by Life Technologies. All values were normalized to the expression of the housekeeping gene RPLP0 (KCs) or 18S (ECs). For mouse experiments, skin mRNA was extracted, reverse transcribed, and transcripts quantified as previously described (23). All values were normalized to GAPDH.

ELISA

Frozen human skin biopsies had protein isolated as previously described (17). Human IL-17A and IL-17C protein expression was quantitated in patient samples using ELISA according to manufacturer's instructions (R&D Systems). Frozen mouse skin from control mice ($n = 4$) and uninvolved and involved skin of K5–IL-17C mice ($n = 4–7$) underwent protein isolation as previously described (23), and murine vascular endothelial growth factor (VEGF)–A, IL-17A, IL-6, and TNF-α protein expression was quantitated using ELISA following the manufacturer's instructions (R&D Systems).

Western blotting and protein expression array

For Western blotting analyses, equal amounts of protein/sample from control ($n = 3$) and K5–IL-17C uninvolved and involved skin ($n = 3$) or from human dermal microvascular ECs stimulated with/without IL-17C (200 ng/ml) was separated on an SDS-PAGE polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (Millipore, Temecula, CA). Using methods published previously by our group (23), membranes were probed using Abs targeting murine S100A8/A9, IL-17A, IL-17C, IL-12/23p40 (R&D Systems), and β-actin (Sigma-Aldrich, St. Louis, MO) or against human TNF-α (R&D Systems) and β-actin (Sigma-Aldrich). Differences in proinflammatory cytokine expression were also examined using a commercially available protein array (R&D Systems; ARY006; $n = 2$ each controls and K5–IL-17C involved skin), following the manufacturer's instructions.

Immunohistochemistry

Human skin biopsies were formalin fixed and paraffin embedded, sectioned at 5 μm, and then immunohistochemically stained using standard approaches (23) with goat anti-human IL-17C or matched goat IgG (1 μg/ml; AF1234; R&D Systems) or anti–IL-17A polyclonal goat IgG or matched goat IgG (2 μg/ml; AF317; R&D Systems). Biopsies from human sigmoid colon diagnosed with Crohn's disease were used as a positive control for IL-17A staining.

Adult mice were euthanized, their hair shaved, and skin from the back and ear was processed as previously described in detail (23). H&E staining and immunohistochemistry using Abs specific for CD4, CD8, CD11c (Becton-Dickinson Biosciences, San Jose, CA), F4/80 (eBioscience, San Diego, CA), mouse endothelial cell Ag-32 (MECA-32; Developmental Studies Hybridoma Bank, Iowa City, IA), Ki67 (DakoCytomation, Carpinteria, CA), Loricrin (Covance, Princeton, NJ), and VEGF-A (A20; Santa Cruz Biotechnology) were also completed as described previously (23).

Epidermal thickness measures and immune cell quantitation were completed as previously described (23).

Statistics

All data are presented as mean $\pm$ SEM. Data were tested for normality and statistical significance calculated using a Student t test, Mann–Whitney

U test, or Friedman's test, as appropriate. Significance was defined as $p \leq 0.05$.

Results

IL-17C is the most abundant IL-17 cytokine in inflamed skin

Expression of IL-17 family genes were compared in lesional (PP) and uninvolved (PN) skin of psoriasis patients as well as skin from healthy controls (NN) using quantitative RT-PCR (qRT-PCR) (Fig. 1A). Consistent with previous reports (10), we identified significant increases in IL-17A ($p < 0.02$), IL-17C ($p < 0.01$), and IL-17F ($p < 0.002$) gene expression in PP compared with NN and PN skin ($n = 8$ each) and significant decreases in IL-17B ($p = 0.05$) and IL-17D ($p = 0.002$) in PP compared with NN skin. IL-17RA/B/E expression was not significantly different among NN, PN, or PP skin, whereas IL-17RC ($p = 0.02$) and IL-17RD ($p = 0.01$) both decreased in PP compared with control skin.

To explore the significance of these changes in gene expression, we measured IL-17A and IL-17C protein by ELISA (Fig. 1B) and determined both to be significantly increased in PP compared with PN and NN skin (p values as indicated). Interestingly, IL-17C protein expression was ∼4-fold greater in PP skin ($p = 0.002$) and ∼125-fold greater than IL-17A protein ($p = 0.006$). These results demonstrate similar mRNA transcript abundance of IL-17A and IL-17C but also illustrate that IL-17C protein is the most highly expressed IL-17 family member found in human involved psoriasis skin (Fig. 1B, 1058 ± 133 pg/mg IL-17C versus 8 ± 2 pg/mg IL-17A protein). Immunohistochemical staining of healthy control and involved and uninvolved psoriasis skin confirmed the increases in IL-17C and IL-17A protein and demonstrated significantly more IL-17C protein present in psoriasis skin than IL-17A (Fig. 1C). IL-17C colocalized with many cells, including KCs, dermal ECs, and skin-resident and infiltrating leukocytes; IL-17A–positive cells were only observed in the dermal papillae (Fig. 1C). No IL-17A staining was observed in control patient skin.

Despite its gross overexpression in lesional psoriasis skin, we observed that IL-17C is one of the most rapidly downregulated transcripts in the skin of psoriasis patients treated with the anti–TNF-α agent etanercept. Seventy-two hours following etanercept treatment, and prior to any changes in clinical disease, cutaneous IL-17C mRNA expression was significantly suppressed ($n = 18$; Fig. 1D; $p = 0.019$).

Endothelial cells and keratinocytes produce proinflammatory molecules in response to IL-17C and IL-17C and TNF-α, respectively

Others have previously demonstrated direct effects of IL-17C on fibroblasts, monocytic cells, and peritoneal exudates (2, 24), and IL-17C has the capacity to elicit antimicrobial peptides from epithelial cells (5, 6); more recent studies have revealed IL-17C regulates Th17 cell differentiation and Th17 cell–derived IL-17A and IL-17F (9). To further identify cell responsiveness to IL-17C, we stimulated human dermal microvascular ECs with IL-17C and observed statistically significant increases in TNF-α and IL-6 mRNA ($p < 0.05$; Fig. 2A), with no significant effect on IL-1α/β, IL-8, or IL-17RE/A/C. The increase in TNF-α was further confirmed at the protein level (Fig. 2B), identifying a novel cellular target for IL-17C promotion of inflammation.
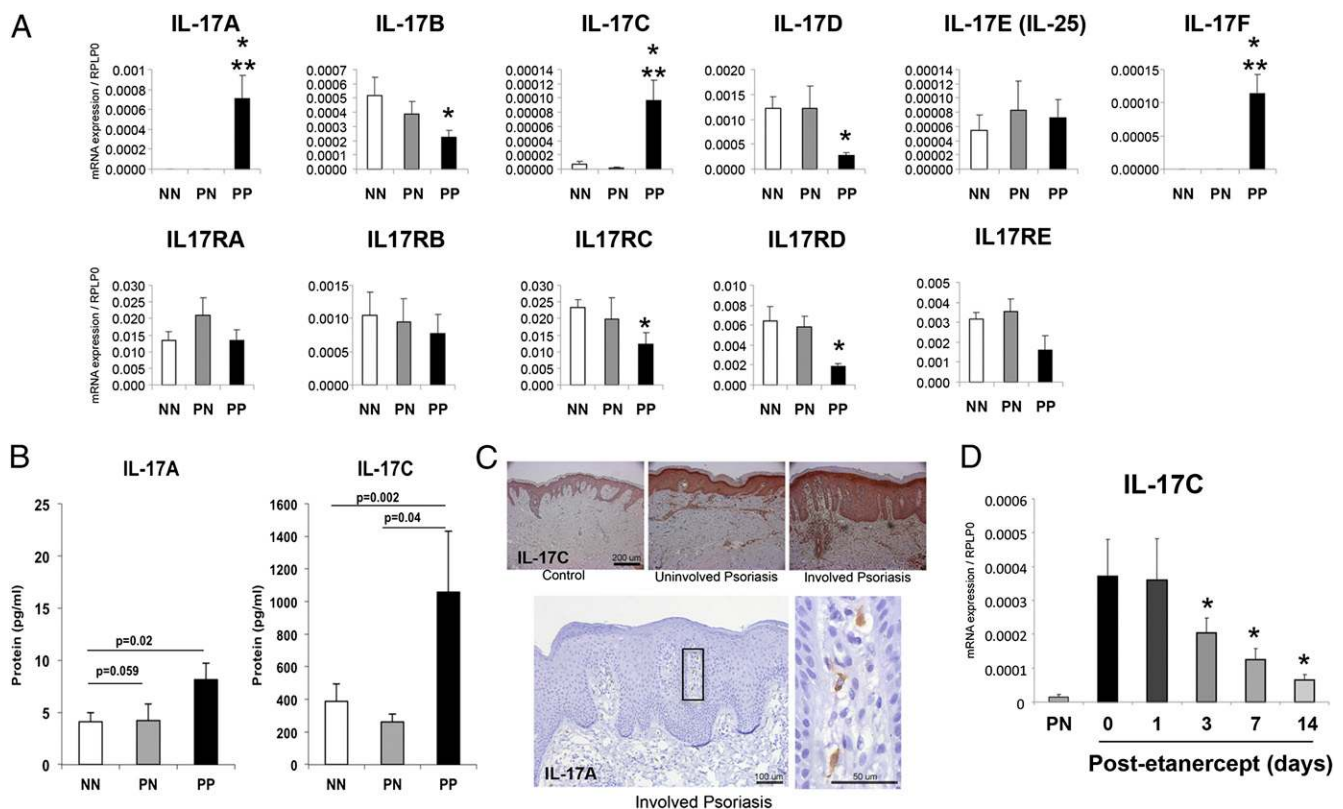
FIGURE 1. IL-17C RNA and protein are increased in lesional psoriasis skin. (**A**) Real-time quantitative PCR analyses of IL-17 ligand and receptor family members in NN and psoriasis patient PN and PP skin. (**B**) ELISA analyses of IL-17A and IL-17C demonstrate significant increases in both cytokines, but IL-17C protein expression is ∼125-fold higher than IL-17A in lesional skin. (**C**) IL-17C and IL-17A immunohistochemistry in control and psoriasis skin shows IL-17C protein localized to epidermal, vasculature, and dermal areas and IL-17A localized to very few cells in the dermal papillae. (**D**) Cutaneous IL-17C mRNA significantly decreases in responding patients 72 h following etanercept treatment. *$p < 0.05$ compared with NN in (A) or day 0 of treatment in (D), **$p < 0.05$ compared with PN in (A) or as indicated in (B). Scale bars, as noted on (C).
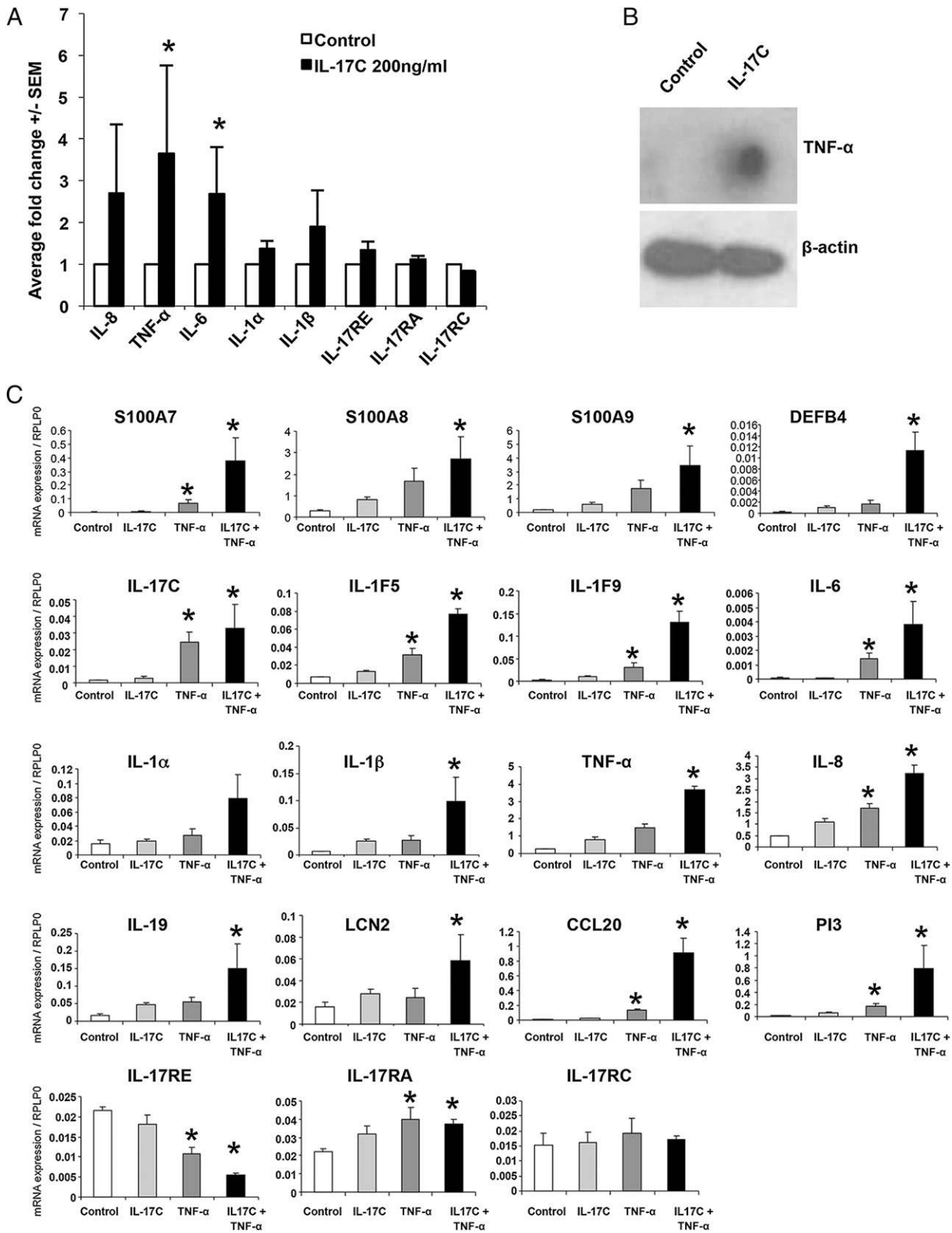
FIGURE 2. IL-17C and TNF-α induce characteristic psoriasis-related transcriptome genes in both additive and synergistic manners. (**A**) Human dermal microvascular ECs stimulated with IL-17C (200 ng/ml) for 6 h increase mRNA expression of TNF-α and IL-6. (**B**) Representative Western blots of cell lysates confirm the increase in TNF-α protein. (**C**) Real-time quantitative PCR analyses of key psoriasis-transcriptome genes in human primary epidermal keratinocytes stimulated with IL-17C (200 ng/ml), TNF-α (2 ng/ml), or both as indicated for 24 h. In vitro experimental data are representative of at least three independent experiments. *$p < 0.05$ compared with control.

Given that KCs are the major source of IL-17C in the skin and that others have recently identified increases in S100A8/A9, RegIIIβ/γ, human β-defensin 2 (hBD2), and hG-CSF in epithe-lial cells following IL-17C exposure (5, 6), we sought to further investigate the role of IL-17C in primary human KCs. Primary human KCs responded poorly to IL-17C alone (Fig. 2C); however,

the addition of suboptimal TNF-α (2 ng/ml) led to a significant induction of candidate genes previously identified to respond either synergistically or additively in response to IL-17A/TNF-α (13) (Fig. 2C) and known to contribute to psoriasis pathogenesis. Additive increases in KC-derived IL-17C, TNF-α, IL-8, IL-1α/β, IL-1F5, IL-6, S100A8/A9, and lipocalin 2 were observed along with synergistic increases in KC-derived IL-1F9, IL-19, CCL20, S100A7, hBD2 (DEFB4), and peptidase inhibitor 3 following stimulation with IL-17C/TNF-α.

K5–IL-17C mice develop a psoriasiform skin phenotype

In psoriasis patients, there is ~125-fold more IL-17C than IL-17A protein in lesional skin (Fig. 1B), and IL-17C is localized principally to activated KCs (Fig. 1C). To model this increase and to test the hypothesis that IL-17C plays a contributing and critical role in psoriasis pathogenesis, we genetically engineered mice to overexpress murine IL-17C in KCs using a conditional tetracycline-repressible binary approach (Fig. 3A) similar to models we have previously published (23). This controlled system allows us to modulate increases in IL-17C and recapitulate levels observed in lesional human psoriasis (Fig. 1C). K5–IL-17C double-transgenic mice appeared normal at birth; however, as early as 8 wk of age, well-demarcated areas of dorsal skin began to develop a thickened appearance with sloughing of epidermis and erythema, whereas adjacent areas of skin appeared relatively normal (Fig. 3B). In severely affected K5–IL-17C animals, alopecia was observed. Almost all mice developed ear involvement by the time they were 12 wk of age. qRT-PCR of uninvolved and involved skin from K5–IL-17C mice revealed ~11- and ~18-fold increases in IL-17C gene expression in K5–IL-17C uninvolved and involved skin, respectively, compared with littermate controls ($n = 9$ to 10 each group; $p = 0.04$ and 0.009; Fig. 3C). Western blotting confirmed the increases in cutaneous IL-17C protein in uninvolved and involved K5–IL-17C skin compared with control mice (Fig. 3D).

Histological examination of uninvolved K5–IL-17C dorsal skin compared with littermate control skin revealed modest increases in epidermal thickness (acanthosis) in uninvolved skin (11.5 ± 0.9 μm control skin versus 16.1 ± 0.6 μm; $p = 0.006$). In contrast, involved skin of K5–IL-17C animals exhibited robust epidermal hyperplasia (70.0 ± 5.2 μm; $p < 0.001$ versus controls and un-
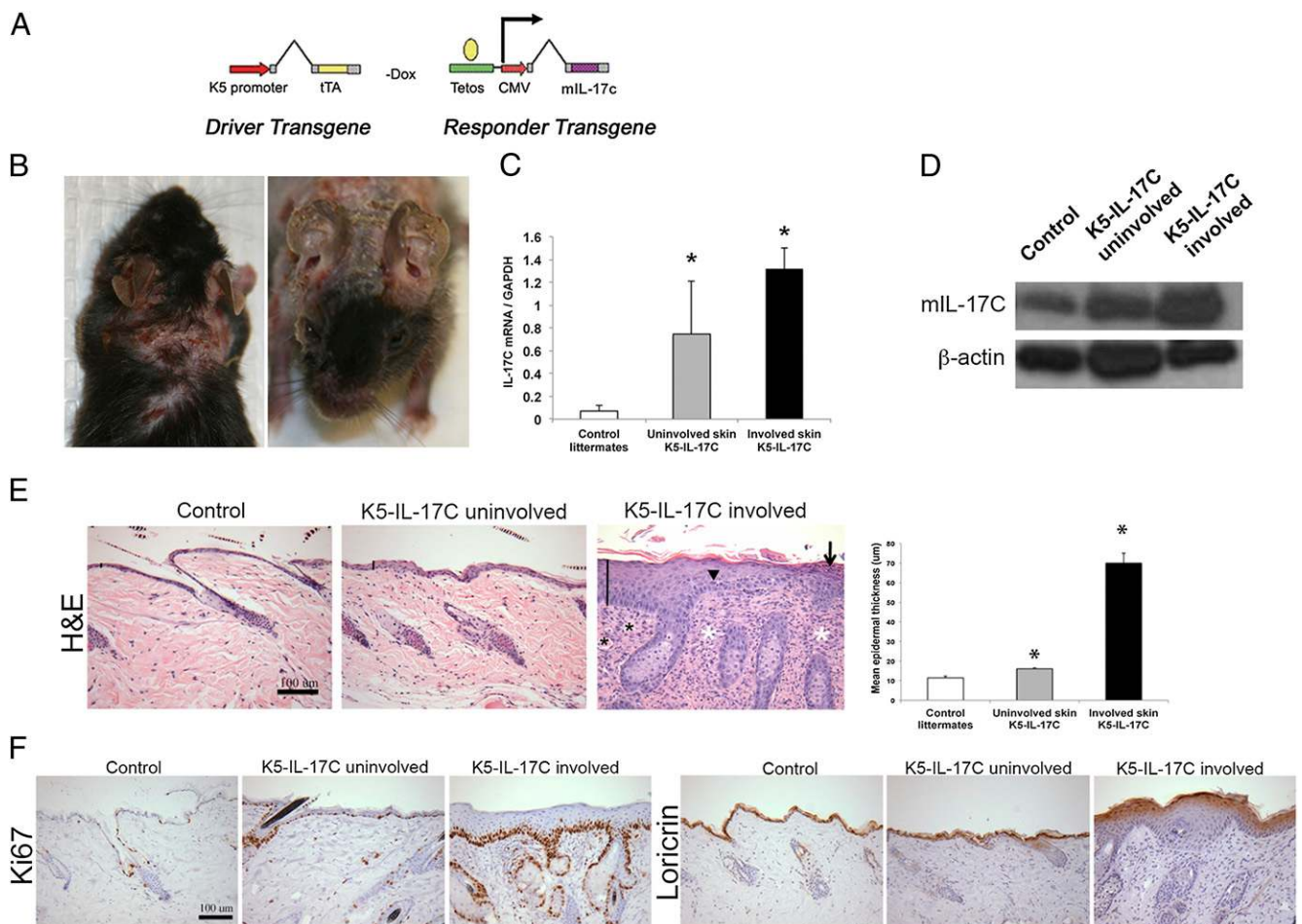
FIGURE 3. K5–IL-17C transgenic mice develop a psoriasiform skin phenotype. (**A**) A tetracycline-repressible binary mouse molecular genetics approach similar to that previously described (23) was used to genetically overexpress IL-17C in a keratinocyte-specific manner using the K5 promoter. (**B**) Mice spontaneously develop regions of affected (involved) and unaffected (uninvolved) skin with involved skin characterized by hyperkeratosis. (**C**) Real-time quantitative PCR analyses of IL-17C gene expression of control mouse and uninvolved and involved skin of K5–IL-17C mice show significant increases in IL-17C gene expression in K5–IL-17C mice. (**D**) Representative Western blot demonstrates increases in IL-17C protein in uninvolved and involved K5–IL-17C skin. (**E**) H&E-stained dorsal skin of control mouse skin and K5–IL-17C mouse uninvolved and involved skin and epidermal thickness quantitation. (**F**) Ki67- and loricrin-immunostained dorsal skin demonstrates increases in cell proliferation and loss of terminal differentiation between control and K5–IL-17C mouse skin. Solid lines in (E) highlight the epidermis, arrow is pointing at a parakeratotic scale, arrowhead is pointing at a mitotic body, black asterisks are placed on dermal blood vessels, and white asterisks are placed on foci of inflammatory infiltrate. Scale bars, 100 μm. $*p < 0.05$ compared with control mouse.

involved K5–IL-17C skin), loss of the granular cell layer, thickening of the interfollicular epidermal layers, and confluent parakeratotic scale (Fig. 3E). This increase in epidermal thickness occurred concurrent with increases in cell proliferation, indicated by Ki67 staining, and decreases in KC terminal differentiation, indicated by loricrin in uninvolved skin and involved K5–IL-17C skin compared with control mouse skin (Fig. 3F). H&E-stained skin sections also revealed an apparent increase in dermal blood vessels and a dense immune cell infiltrate (Fig. 3E).

To further explore these changes, dermal angiogenesis was examined using MECA staining. Increases in MECA-positive staining was observed between control and uninvolved K5–IL-17C skin, which was further amplified in involved skin (Fig. 4A). VEGF protein increased between control and uninvolved K5–IL-17C skin (79.9 ± 6.5 pg/ml control versus 127.9 ± 11.4 pg/ml uninvolved K5–IL-17C; $p = 0.07$) and involved K5–IL-17C skin (258.9 ± 54.1 pg/ml; $p = 0.02$ versus control) and was localized to KCs, infiltrating leukocytes, and cutaneous nerves (Fig. 4B). Primary human KCs and dermal microvascular ECs both directly responded to stimulation with IL-17C by producing 52 ($p = 0.05$)

and 20% more ($p = 0.04$) VEGF mRNA (Fig. 4C), respectively. No differences in angiopoietin or Tie2 expression were observed.

Cell type–specific examination and quantification of the inflammatory infiltrate in K5–IL-17C mouse skin revealed significant increases in CD4^{+} T cells, CD8^{+} T cells, CD11c^{+} myeloid dendritic cells, and F4/80^{+} macrophages in K5–IL-17C involved skin compared with both control mouse skin and K5–IL-17C uninvolved skin (Fig. 5). Modest, but insignificant ($p = 0.09$), increases in CD8^{+} T cells were seen in uninvolved K5–IL-17C skin compared with controls. Similar observations were seen in the affected ear skin of K5–IL-17C animals (Supplemental Fig. 1).

To explore the molecular signature of K5-IL-17C skin, we examined changes in candidate cytokines, chemokines, and innate defense molecules directly related to the histological and immunophenotypic changes observed in the skin (Fig. 5) and that have been proposed to contribute to the pathogenesis of psoriasis (25, 26). Very few significant gene changes were observed between control littermate skin and uninvolved K5–IL-17C skin, but increases in transcript level were observed for S100A8 ($p = 0.04$; $n = 4–6$) and S100A9 ($p = 0.02$) and IL-17F ($p = 0.04$; Fig. 6A),
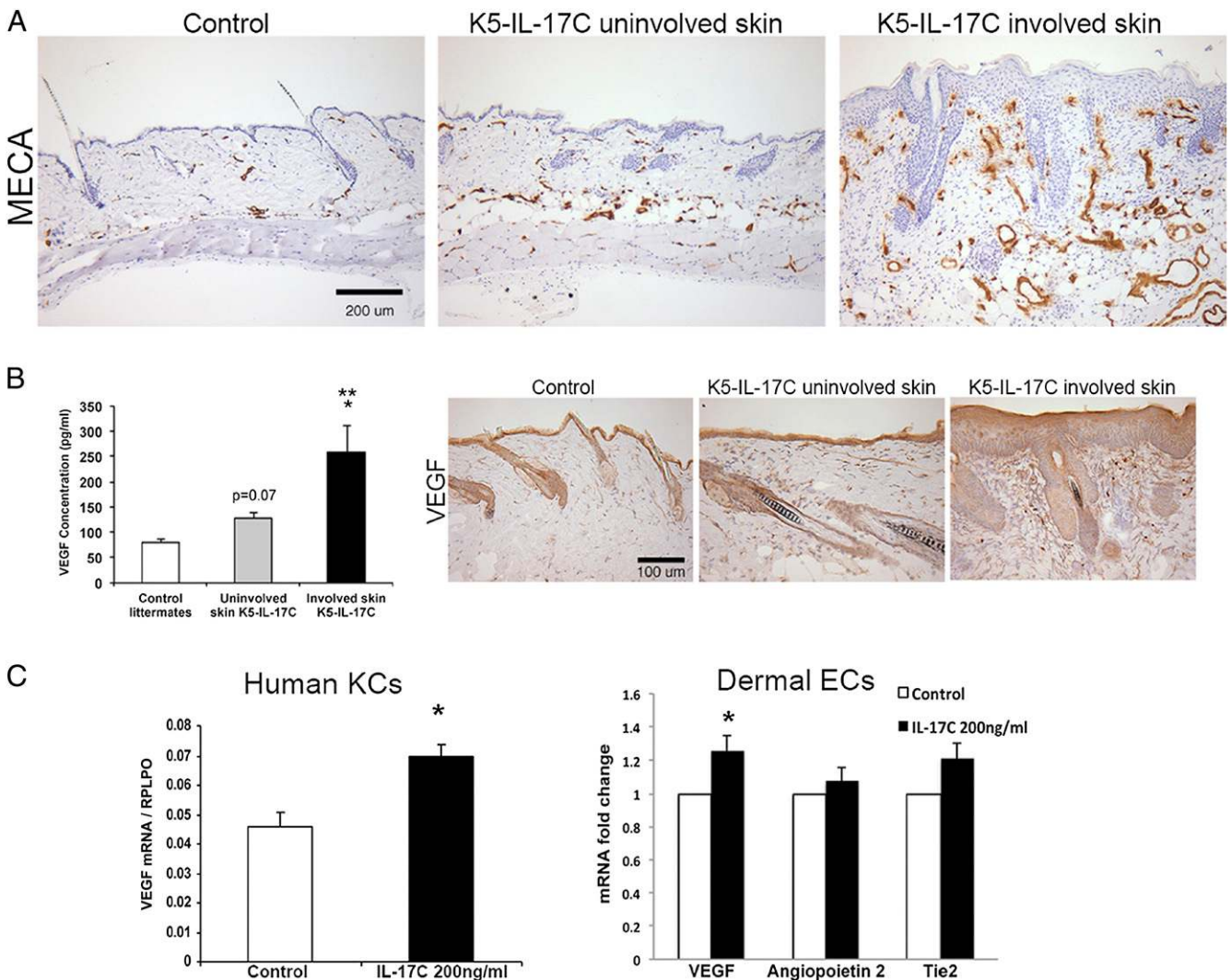
FIGURE 4. K5–IL-17C mouse skin has increases in dermal angiogenesis and VEGF. (**A**) MECA-immunostained dorsal skin of control mouse skin and K5–IL-17C mouse uninvolved and involved skin. (**B**) VEGF ELISA and immunohistochemistry demonstrate increases in VEGF protein in uninvolved and involved K5–IL-17C skin compared with littermate control skin. (**C**) qRT-PCR of primary human KCs and ECs stimulated with IL-17C (200 ng/ml) demonstrates significant increases in VEGF mRNA. Scale bars as noted. *$p < 0.05$ compared with control mouse, **$p < 0.05$ compared with uninvolved K5–IL-17C skin (B). *$p < 0.05$ compared with control stimulated (C).
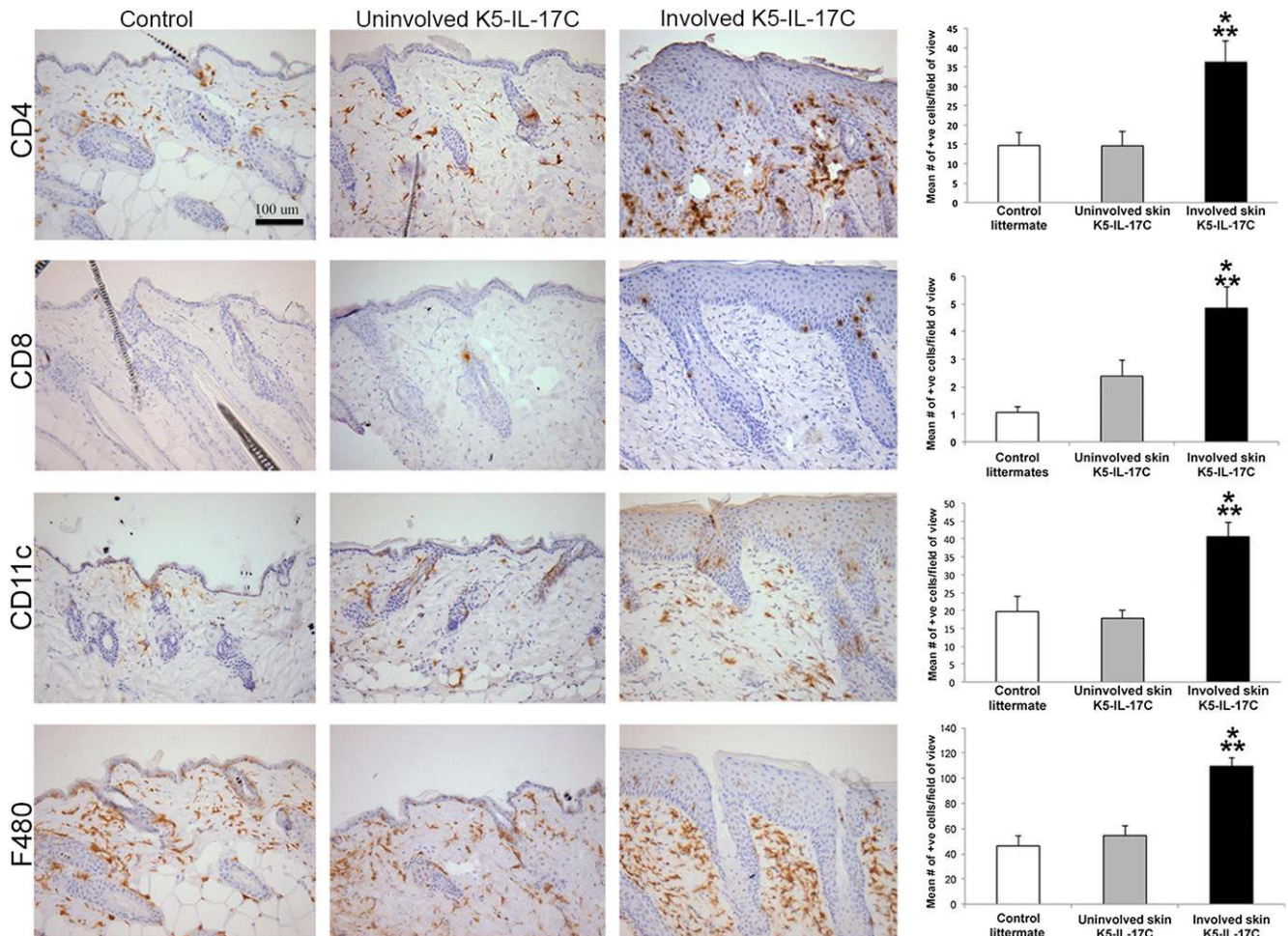
FIGURE 5. Characterization of the immune cell infiltrate in K5–IL-17C mouse skin. Representative photographs of immunohistochemistry and cellular quantitation of CD4-, CD8-, CD11c-, and F4/80-positive cells in control mouse dorsal skin and K5–IL-17C uninvolved and involved dorsal skin. Scale bar, 100 μm. *$p < 0.05$ compared with control mouse, **$p < 0.05$ compared with uninvolved K5–IL-17C skin.

most likely derived from IL-17C direct effects on KCs (5) and T cells (9)(Figs. 3, 5). Comparison between involved K5–IL-17C skin with littermate control skin revealed significant increases in many of the hallmark psoriasis-transcriptome genes, including the proinflammatory cytokines and chemokines, TNF-α, IL-1α, IL-1β, IL-6, and CCL20, Th1- and Th17-derived cytokines IFN-γ and IL-17A, and the myeloid-derived cytokines IL-12 and IL-23 with sustained expression of the innate defense markers DefB3 (the murine homolog of hBD2), S100A8, and S100A9 (Fig. 6A; $p < 0.05$). Genes that changed significantly between uninvolved and involved skin included IL-6 ($p = 0.05$), IL-1β ($p = 0.004$), IL-23p19, DefB3, S100A8, and S100A9 (all $p = 0.03$).

Gene-expression changes between control mouse skin and involved K5–IL-17C skin were further validated at the protein level (Fig. 6B–D). Increases in IL-12/23p40, S100A8, and S100A9 proteins were observed (Fig. 6B) as well as increases in TNF-α, IL-6, and IL-17A proteins (Fig. 6C) between uninvolved K5–IL-17C and control mouse skin. Moreover, additional inflammatory molecules C5/C5A, CD54, IL-16, CXCL1, CCL2, CCL3, CXCL2, TIMP-1, and TREM-1 also increased between involved K5–IL-17C skin and control mouse skin (Fig. 6D).

TNF-α inhibition improves the K5–IL-17C mouse skin phenotype

Psoriasis patients treated with the TNF-α inhibitor etanercept show significant improvement in disease severity (27–29) and have rapid decreases in IL-17C (Fig. 1D). To examine the effects of TNF-α inhibition in K5–IL-17C mice, animals were systemically treated with either anti–TNF-α or negative control murine IgG2a,κ mAbs for 8 wk as described previously (30). Significant improvement in the gross appearance of individual animals treated with TNF-α inhibitors was observed (Fig. 7A), although some level of skin disease was still present. Significant decreases in epidermal thickness and infiltrating CD4$^+$ and CD8$^+$ T cell numbers were identified (Fig. 7B), although these failed to return to control mouse levels. Decreases in neither CD11c$^+$ nor F4/80$^+$ cells were seen (data not shown). Examination of elevated proinflammatory cytokines in treated mice demonstrated sustained levels of IL-17C, as expected based on its genetic overexpression (Fig. 7C), and robust decreases to control levels for IFN-γ, IL-6, and IL-1β and more modest decreases in S100A8 and DefB3. CCL20, IL-17A, and IL-1α levels remained elevated (Fig. 7C).

Discussion

In this study, we have identified IL-17C as a contributing proinflammatory cytokine critical for psoriasis pathogenesis. This conclusion is supported by the following data: 1) IL-17C is the most abundantly expressed IL-17 cytokine at the protein level in lesional psoriasis skin and colocalizes with KCs, ECs, and skin-resident and infiltrating leukocytes; 2) IL-17C elicits increases in TNF-α from human dermal microvascular ECs and, when combined with TNF-α, produces additive and synergistic increases in key psoriasis-related proinflammatory cytokines, chemokines,
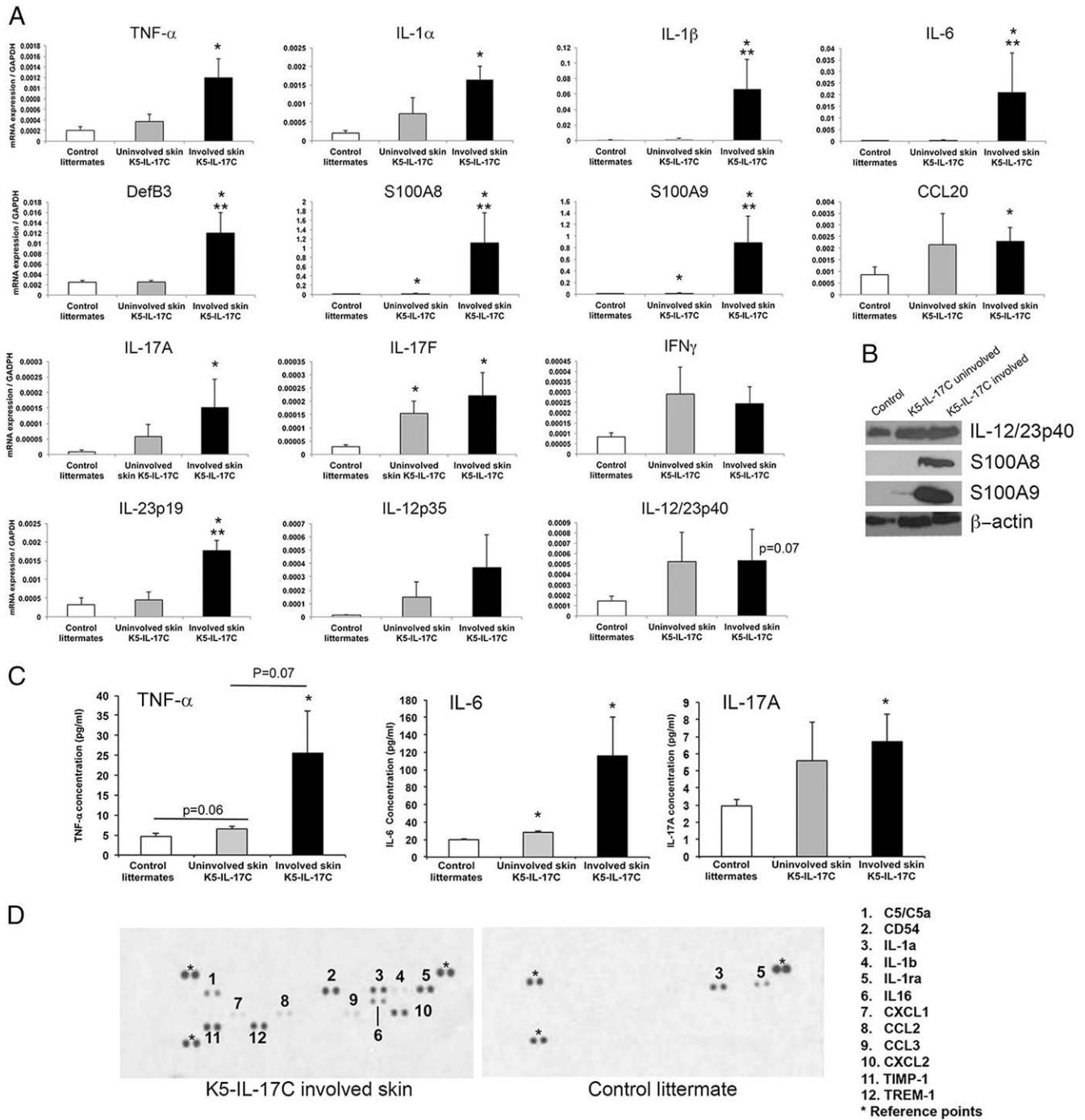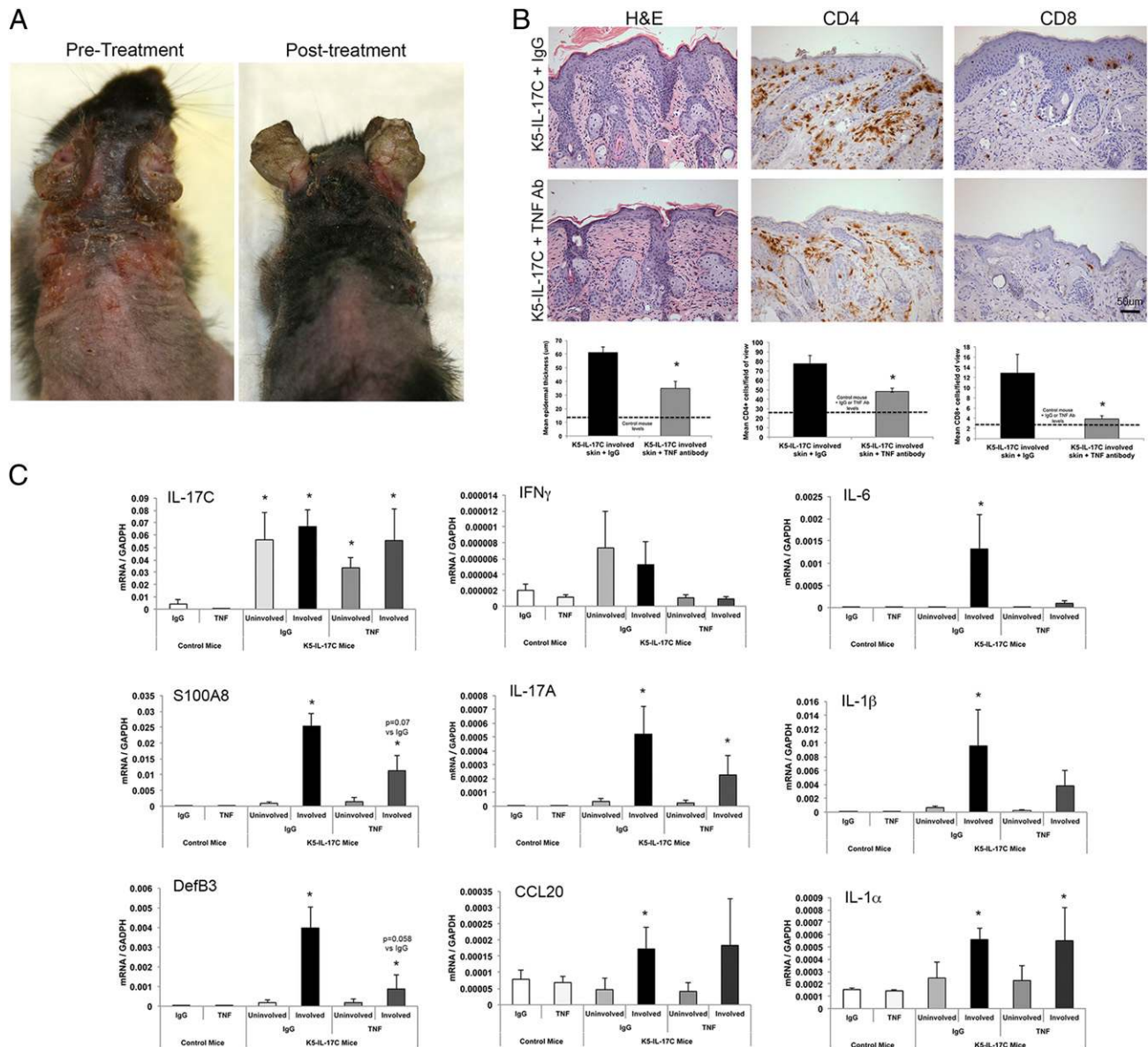
FIGURE 6. Psoriasis-related innate defense and cytokines/chemokines are increased in K5–IL-17C skin. (**A**) Real-time quantitative PCR analyses of key innate defense molecules and psoriasis-related proinflammatory cytokines/chemokines demonstrate significant increases in S100A8/S100A9 and IL-17F in K5–IL-17C uninvolved mouse skin compared with control mouse skin and significant increases in these and other key proinflammatory molecules in involved skin compared with littermate control animals. (**B**) Representative Western blots confirming increases in proinflammatory cytokines and innate defense molecules. (**C**) ELISA analyses of TNF-α, IL-6, and IL-17A confirm the increases in gene expression observed between control and K5–IL-17C animals. (**D**) Representative protein expression array confirms increases in IL-1α and IL-1β protein in involved K5–IL-17C and control mouse skin and identifies increases in other known psoriasis-related molecules. Asterisks in (D) represent reference points. *$p < 0.05$ compared with control littermates, **$p < 0.05$ compared with K5–IL-17C uninvolved skin or as indicated.

and innate defense molecules from human KCs; 3) mice engineered to overexpress IL-17C in KCs develop a psoriasis-like dermatitis containing clinical, histological, cellular, and molecular changes that mimic human psoriasis and that are improved with TNF-α inhibition; and 4) K5–IL-17C mice provide in vivo evidence demonstrating synergistic effects of IL-17C and TNF-α/IL-17A in promoting psoriasiform skin disease.

The biological relevance of increased IL-17C in psoriasis is poorly understood, and the role of IL-17C in psoriasis pathogenesis remains unclear. One potential mechanism by which IL-17C–mediated skin inflammation may occur is by activating host defense pathways in human epidermal KCs, as others have recently identified increases in expression of hBD2, S100A7/8/9, CXCL1/2/3, CCL20, TNFAIP6, and TNIP3 at levels similar to or greater

FIGURE 7. Systemic inhibition of TNF-α in K5–IL-17C mice leads to improvement in disease severity. (**A**) Photographs of the same severely affected K5–IL-17C mouse before and after 8 wk of TNF-α Ab treatment. (**B**) Representative images of H&E-stained and CD4- and CD8-immunostained dorsal skin of K5–IL-17C involved skin treated with either anti–TNF-α Abs or IgG and quantitation of epidermal thickness and CD4$^+$ and CD8$^+$ cell numbers. The dashed line represents control mouse levels. *$p < 0.05$ compared with K5–IL-17C involved plus IgG. (**C**) Real-time quantitative PCR analyses of key innate defense molecules and psoriasis-related proinflammatory cytokines/chemokines demonstrate decreases in IFN-γ, IL-6, and IL-1β in involved skin between K5–IL-17C + IgG and K5–IL-17C + TNF-α–treated mice. *$p < 0.05$ compared with control mice.

than those elicited by IL-17A (5). These gene changes are reminiscent of those previously identified from human KCs following stimulation with IL-17A/TNF-α, as part of the signature psoriasis transcriptome (13, 31). In our experiments, we failed to identify significant changes in these transcripts in primary human KCs following IL-17C stimulation alone (Fig. 2C), perhaps reflective of differences in source of primary KCs (neonate foreskin versus adult buttock). However, increases in the antimicrobial transcripts, S100A8 and S100A9 were observed in uninvolved skin of K5–IL-17C mice (Fig. 6), prior to increases in other proinflammatory cytokines, and models transcriptional gene changes we have observed previously in uninvolved psoriasis patient skin (32). Moreover, IL-17C stimulation lead to increases in EC-derived proinflammatory cytokines, including TNF-α (Fig. 2A, 2B); and primary human KCs stimulated with both IL-17C and subthreshold levels of TNF-α produced significant increases in these as well

as other key innate defense, chemokine, and proinflammatory cytokine transcripts (Fig. 2C). These findings suggest a potential feedback loop occurs between KCs (producing IL-17C) and ECs (responding by producing TNF-α), which may serve to promote self-sustaining inflammation. In vivo support for this feedback loop is evidenced by the increases in dermal angiogenesis in uninvolved skin of K5–IL-17C mice (Fig. 4) that precedes increases in cutaneous TNF-α expression, the development of involved skin lesions, and the upregulation of the these same KC-derived and psoriasis-related transcripts (Fig. 6). Our in vitro data suggest that IL-17C may indirectly promote dermal angiogenesis by eliciting VEGF from both ECs and KCs (Fig. 4), which in turn promotes angiogenesis and additional TNF-α production.

The similarity between genes upregulated by IL-17A and IL-17C suggests they may be functionally redundant on epithelial cells; although transcript levels of IL-17A and IL-17C suggest IL-17A

may be expressed more robustly in lesional psoriasis tissue, our protein data demonstrate there is ~125-fold more IL-17C protein present than IL-17A and differences in signaling strength based solely on the local concentration of ligand, and the number of cells producing and responding to the ligand cannot be overlooked (Fig. 1B, 1C). The discrepancy between cytokine mRNA and protein level correlation is consistent with a recent report (33) and may reflect differences in nontranslational regulation, cytokine storage, and cellular source.

Previous reports have identified the importance of synergistic and additive responses to IL-17A and TNF-α as a hallmark of IL-17A biology and of psoriasis (13). Recently, similar synergies have been identified between IL-17C/TNF-α on hBD2 expression by primary human KCs (5), and IL-17C/IL-22 synergism drives S100A8/A9 production in colonic epithelial cells (6). This phenomenon is recapitulated in this study (Fig. 2C) with 16 gene transcripts being either additively or synergistically induced by IL-17C/TNF-α.

Studies examining IL-17C–IL-17RE interactions have recently revealed that IL-17C regulates Th17 cell differentiation and production of IL-17A and IL-17F (9), cytokines known to promote skin inflammation in the imiquimod model of psoriasiform dermatitis (5) and that are upregulated in psoriasis lesional skin (Fig. 1A). These findings suggest synergy may occur not only between IL-17C and TNF-α but also IL-17C and IL-17A, such that the proinflammatory feedback loop may include the epidermis, the vasculature, and also Th17 cells. In K5–IL-17C skin, increases in IL-17A/F, TNF-α, and IL-6 were found in uninvolved skin compared with control mouse skin (Fig. 6), perhaps reflective of IL-17C direct effects on ECs (Fig. 2A, 2B) and T cells (9). Whether IL-17A/F and IL-6 also synergize with IL-17C, similar to TNF-α, has yet to be explored. Taken together, these data support the idea of a crucial role for IL-17/TNF-α synergism (IL-17A and IL-17C) in the molecular fingerprint of psoriasis and demonstrate the capacity of IL-17C to augment an immune response concurrent with IL-17A/TNF-α. This concept is supported by clinical observations that psoriasis patients treated with the anti–TNF-α agent etanercept exhibit rapid decreases in cutaneous IL-17C expression (within 72 h; Fig. 1D), prior to skin improvement and reported decreases in circulating levels of IL-17A and IL-22 (34). Others have reported similar outcomes in psoriasis patients treated with the TNF-α inhibitor adalimumab in whom IL-17C gene expression decreased within 4 d of the initial treatment, whereas IL-17A/IL-17F and IL-22 failed to decrease significantly until 14 d posttreatment, and IL-23p19 and IFN-γ did not drop significantly until 84 d (11). Moreover, recent clinical trials targeting the common IL-17A/C receptor IL-17RA (using brodalumab; formally AMG841) also demonstrated rapid decreases in IL-17C mRNA (within 2 wk) (35) that occurred prior to clinical disease improvement and preceded the gradual decreases of IL-17A and IL-22, which reached baseline nonlesional levels by 6 wk (36).

K5–IL-17C mice treated with TNF-α inhibitors also showed significant improvement in their disease severity (Fig. 7), although disease was not reversed. This most likely reflects the sustained levels of IL-1α and IL-17A that may synergize with sustained level of IL-17C. TNF-α is known to regulate IL-17C expression via NF-κB (p65/p50) (4); therefore it is possible that efficacy of TNF-α inhibition in psoriasis patients actually reflects decreases in IL-17C; thus, TNF-α inhibition may not be completely effective in K5–IL-17C mice because of the high levels of IL-17C, which are being genetically expressed and cannot be regulated by blockade of TNF-α.

In conclusion, our results suggest that IL-17C is a highly sensitive member of the IL-17 family and that IL-17C may serve as a novel mechanism for amplifying Th17/Th22/TNF-α–mediated inflammatory signaling critical for psoriasis pathogenesis. Support for the sensitivity of IL-17C in other models of inflammatory disease are provided by kinetic studies showing that IL-17C induction precedes that of IL-17A and other Th17/Th22 cytokines (IL-22) in a murine colitis model (5). These data support the concept of a crucial role for both IL-17A and IL-17C synergizing with TNF-α to generate the molecular fingerprint of psoriasis. In this study, we propose that IL-17C serves as a critical cytokine-mediating psoriasis and that psoriasiform skin inflammation is likely to be driven by numerous IL-17C–producing activated KCs in psoriasis tissue rather than the comparatively modest numbers of IL-17A–producing cells that likely initiate the primary response and then feed into the self-sustaining proinflammatory cascade of events. The increases in cytokines are likely reflective of the increased presence of skin-infiltrating immune cells and proliferating KCs and signal a synergistic immune response between IL-17C with TNF-α and IL-17A (Fig. 2) (5). This synergistic increase may also reflect changes associated with IL-1β, which we found elevated compared with control skin, supporting and elaborating recent work illustrating similar IL-17C–IL-1β and IL-17C–TNF-α synergy (5).

Although the cause of psoriasis is unknown, the currently proposed hypothesis is that in patients with a susceptible genetic background, some stimulus, perhaps an infection, leads to a coordinated series of events involving cutaneous cells and cytokines that, once started, initiates a self-sustained vicious proinflammatory signal resulting in KC hyperproliferative cell-cycle response. Recent reports have identified IL-17C as a key innate immune defense cytokine that is rapidly and robustly upregulated following infection or TLR stimulation (5, 6, 9). Our data support the supposition of a crucial role for IL-17/TNF-α synergism (IL-17A and IL-17C) in the molecular fingerprint of psoriasis and, when taken together, provide evidence indicating that IL-17C synergizes with TNF-α as well as IL-17A to initiate and sustain KC activation and promote epidermal hyperplasia. Our results demonstrate a potential inflammatory feedback loop among the endothelium, the epidermis, and Th17 cells that can be amplified by IL-17C. Once triggered, this loop is sufficient to promote chronic skin inflammation and acanthosis, two cutaneous characteristics of psoriasis. Because IL-17C appears to act upstream of many proinflammatory cytokines critical in psoriasis pathogenesis, including IL-1β, IL-17A/F, IL-22, IL-6, IL-8, VEGF, and TNF-α [our data and others (2, 5, 6, 9, 24)], targeting IL-17C as an early upstream regulator of these cytokines may provide a more encompassing therapeutic strategy for the effective treatment of psoriasis. Taken together, our findings support prior work (5) demonstrating a need for IL-17C signaling for imiquimod-elicited psoriasiform skin inflammation and demonstrate a pathogenic role for the elevated IL-17C observed in lesional psoriasis skin.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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