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Early tissue responses in psoriasis to the antitumour necrosis factor-α biologic etanercept suggest reduced interleukin-17 receptor expression and signalling

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Conflicts of interest
None declared.

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Summary

Background Antitumour necrosis factor (anti-TNF)-α therapy has made a significant impact on the treatment of psoriasis. Despite these agents being designed to neutralize TNF-α activity, their mechanism of action in the resolution of psoriasis remains unclear.

Objectives To understand better the mechanism of action of etanercept by examining very early changes in the lesional skin of patients with psoriasis responding to etanercept.

Methods Twenty patients with chronic plaque psoriasis were enrolled and received etanercept 50 mg twice weekly. Skin biopsies were obtained before treatment and on days 1, 3, 7 and 14 post-treatment. Skin mRNA expression was analysed by quantitative reverse-transcription polymerase chain reaction and microarray; cytokine and phosphoprotein levels were assessed using multiplexed bead arrays.

Results In etanercept responders, we observed no significant changes in interleukin (IL)-17A, IL-22 or interferon-γ mRNA or protein in the first week of treatment; however, there was a 2·5-fold downregulation of IL-17 receptor C (IL-17RC) mRNA ($P < 0·05$) after day 1, accompanied by decreased extracellular signal-regulated kinase-1/2 phosphorylation. Transcriptional analysis revealed that genes suppressed by etanercept significantly overlapped with IL-17A-induced genes, and a marked overlap was also observed between the genes suppressed by etanercept and by the anti-IL-17A agent ixekizumab. Finally we show that TNF-α enhances the expression of IL-17RC, and short hairpin RNA inhibition of IL-17R expression abrogates synergistic gene induction by TNF and IL-17A.

Conclusions These results suggest that the early responses of psoriasis plaques to etanercept may be due to decreased tissue responsiveness to IL-17A due to suppressed IL-17RC expression in keratinocytes, blunting the strong synergy between TNF-α and IL-17, which contributes to the maintenance of psoriasis lesions.

What's already known about this topic?

- Tumour necrosis factor (TNF)-α inhibitors are effective for the treatment of psoriasis, yet their mode of action appears to involve more than just inhibition of TNF-α responses.

What does this study add?

- This study provides evidence that the early responses of psoriasis plaques to etanercept may be due to diminished tissue responsiveness to interleukin (IL)-17A as a result of the decreased expression of IL-17 receptor C.
- This effect blunts responses to T helper 17 cytokines and breaks a potentially self-sustaining cycle that contributes to the maintenance of psoriasis lesions.

Biologic agents targeting tumour necrosis factor (TNF)-α have made a significant impact on the treatment of psoriasis, arthritis and Crohn disease.[1–3] Despite these agents being designed to neutralize TNF-α activity, their mechanism of action in the resolution of disease remains unclear. Psoriasis vulgaris is a common inflammatory and hyperproliferative skin disease, affecting over 4 million individuals.[4] The most characteristic feature of psoriasis is the marked hyperproliferation and altered differentiation of epidermal keratinocytes. This epidermal hyperproliferation is now thought to be driven largely by interleukin (IL)-17A, IL-22, interferon (IFN)-γ and TNF-α-secreting T cells in the skin.[5–8] It has been shown that the IL-17 cytokine family members IL-17A, C and F are significantly elevated in lesional psoriasis skin.[9] These cytokines utilize a family of five receptor subunits (IL-17RA to IL-17RE), all of which have been detected in skin,[9] with IL-17A and IL-17F using a combination of IL-17RA and IL-17RC for signalling.[9,10] The synergistic pro-inflammatory activity of cytokines has recently become a focus of attention,[11–14] with an understanding that rather than acting alone, each cytokine is participating in an inflammatory network[15–17] with the possibility that sequestration of one key member of this network could result in the collapse of the network and resolution of inflammation.

We propose that the mechanism of action of etanercept involves dismantling the powerful synergy between TNF-α and IL-17A, reducing IL-17A signalling and the expression of IL-17A-induced chemokines prior to changes in T-cell numbers, keratinocyte differentiation and proliferation. To test our hypothesis, we analysed the expression of mRNA, cytokines and phosphoproteins in the lesional skin of patients with chronic plaque psoriasis being treated with the anti-TNF agent etanercept twice weekly, focusing on the first 2 weeks of treatment before improvements in disease severity were clinically evident.

Materials and methods

Study population

Twenty individuals with chronic plaque psoriasis were enrolled (age range 18–75 years). Entry criteria included age ≥ 18 years and stable plaque-type psoriasis involving at least 10% body surface area (BSA). Exclusion criteria included use of systemic psoriasis therapy within 4 weeks or topical therapy within 2 weeks, or severe comorbid diseases. For 12 weeks, subjects received etanercept (Enbrel; Wyeth, Madison, NJ, U.S.A.) 50 mg twice weekly subcutaneously. At baseline, 6-mm punch biopsies were obtained under local anaesthesia (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 at least 1 cm away from previous biopsy sites. Disease severity was recorded on enrolment and at weeks 1, 2, 4 and 12 using percentage BSA and Physician's Global Assessment (PGA) scores. Informed consent was obtained from all subjects, under protocols approved by the Institutional Review Board of the University of Michigan Medical School (HUM00037994). This study was conducted in compliance with good clinical practice and according to the Declaration of Helsinki principles.

Real-time quantitative reverse-transcription polymerase chain reaction analysis

After removal from the skin, biopsies were bisected and snap frozen in liquid nitrogen and stored at −80 °C until use. Biopsies were pulverized with a hammer while still frozen, dissolved in RLT buffer (Qiagen, Chatsworth, CA, U.S.A.), homogenized using glass beads (Biospec Products Inc, Bartlesville, OK, U.S.A.) and total RNA was isolated (RNeasy Mini Kit; Qiagen). A sample of 200 ng RNA was reverse transcribed (High Capacity cDNA Transcription Kit; Applied Biosystems Inc., Foster City, CA, U.S.A.) and transcripts quantified using a 7900HT Fast Real-Time polymerase chain reaction (PCR) system (Applied Biosystems) using Taqman primer sets purchased from Applied Biosystems (IL-17A, Hs00174383_m1; IL-22, Hs00220924_m1; IL-17RA, Hs01064648_m1; IL-17RC, Hs00994305_m1; IL-10RB, Hs00175123_m1; IL-22RA1, Hs00222035_m1). All values were normalized to the expression of the housekeeping gene ribosomal protein, large, P0 (*RPLP0*, Hs99999902_m1).

RNA processing and microarray hybridization

Total RNA was isolated as above from biopsies from six individuals, quality checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.) and transcribed for probe biotinylation using 5 μg RNA according to the manufacturer's protocols (Ambion, Austin, TX, U.S.A.). Six samples were obtained from each individual, including two baseline samples (psoriatic, PP and nonlesional, PN), as well as four samples obtained following 1, 3, 7 and 14 days of etanercept therapy, respectively (36 samples in total). Samples were run on Affymetrix Human Genome U133 Plus 2.0 arrays, which include 54 675 probe sets corresponding to 20 184 human genes (Affymetrix, Foster City, CA, U.S.A.). CEL image files were obtained for each sample, and posthybridization quality control checks were performed using metrics appropriate for Affymetrix gene chips [i.e. average background, intensity scale factor, percentage probe sets called present, RNA degradation score, relative log expression (RLE) median, RLE interquartile range (IQR), normalized unscaled standard errors (NUSE) median and NUSE IQR].[18,19] Based on these analyses, a day-3 PP sample was removed for one patient, and a day-14 PP sample was removed for another, yielding a total of 34 samples upon which further analyses were based. Normalization of these 34 samples was then performed using the Robust Multi-chip Average (RMA) method.[20] The Wilcoxon signed-rank test was also used to calculate the presence/absence of each of the 54 675 probe set features represented on the Affymetrix array platform.[21] Prior to further analyses, we removed 19 258 probe sets not expressed above background in at least 5% of the 34 samples. This yielded 35 417 probe sets upon which subsequent analyses were based. Raw microarray data have

been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO series accession number GSE47751.

Statistics and gene set analyses

Data were tested for normality, and statistical significance was calculated using the Student's *t*-test, Mann–Whitney test or Friedman test as appropriate, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, U.S.A.). To evaluate the effects of etanercept on gene expression, paired moderated t-tests were carried out using the 'limma' package developed for the R statistical software.[22] Four separate analyses were performed in which lesional expression in treated patients was compared with the baseline lesional sample (i.e. day 1 vs. day 0, day 3 vs. day 0, day 7 vs. day 0, day 14 vs. day 0; $n = 5–6$ for each comparison). To generate ranked gene lists, results from these analyses were filtered to remove redundant probe sets corresponding to the same human gene.[23] This was done by choosing a representative probe set for each gene, corresponding to the probe set for which the lowest P-value was obtained in the aforementioned test for differential expression. Following this filtering, ranked lists of genes were generated for each comparison, based on the evidence for differential expression provided by raw P-values. Overlap between these lists and genes decreased in the skin of ixekizumab-treated patients or genes induced by cytokines *in vitro* was assessed using the hypergeometric distribution[24] as recently described.[25] For each analysis, the number of overlapping genes between two ranked gene lists was compared at different depths, with etanercept-decreased genes compared with genes induced by IFN-γ, IL-17, TNF-α or IL-22 in cultured keratinocytes (GEO accessions GSE2737 and GSE36287) or suppressed by ixekizumab (GSE31652) (Fig. S1; see Supporting Information). This provided an idea of how the number of genes belonging to the intersection region of a Venn diagram would change when the top N genes were selected and compared from two gene lists (with $10 < N < 2000$). In addition to such comparisons, we identified Gene Ontology (GO) biological process terms enriched among the 100 genes most strongly decreased by etanercept following 14 days of therapy (i.e. day 14 vs. day 0; Fig. S2; see Supporting Information), and we performed the same analyses with respect to the 100 genes most strongly increased by etanercept (Fig. S3; see Supporting Information). These analyses were generated using the conditional hypergeometric enrichment test procedure implemented in the GOstats package designed for the R statistical software.[26]

Multiplex bead assays

Biopsies were pulverized while still frozen, then suspended in 500 μL ice-cold lysis buffer (Bio-Rad, Hercules, CA, U.S.A.) and ground in a glass tissue grinder. After one freeze–thaw cycle, samples were centrifuged and supernatants collected and diluted to 1 mg mL^{-1} total protein in phosphate-buffered saline. Bead assays were conducted as directed by the manufacturer (Bio-Plex; Bio-Rad) using duplicate 50-μL samples. Concentrations were determined by four- or five-parameter fitting of standard curves.

Keratinocyte culture

Normal human keratinocyte (NHK) cultures were established from sun-protected adult human skin, as described,[27] in serum-free medium optimized for high-density keratinocyte growth (Medium 154; Invitrogen, Portland, OR, U.S.A.). NHKs were used for experiments in the second or third passage. All cells were plated at 5000 cells cm^{-2} and maintained

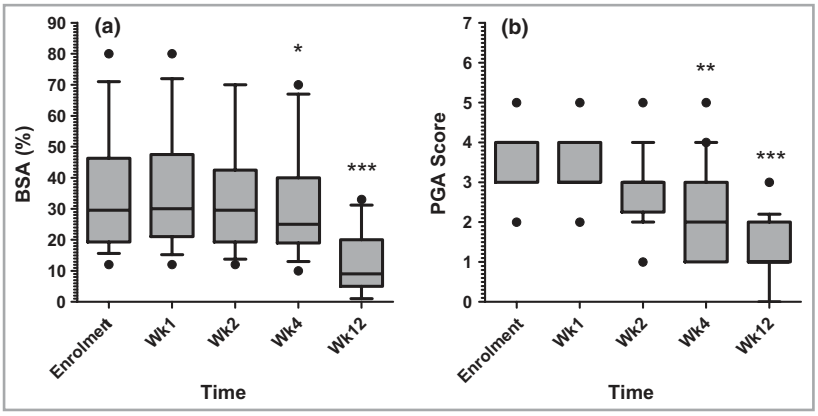

Fig 1. Clinical changes in disease severity were evident after 4 weeks of etanercept treatment. (a) On recruitment, the median body surface area (BSA) was recorded as 35%. Overall 18/20 subjects responded to treatment with at least a 25% decrease in BSA by week 12 (Wk12), with a mean decrease of 64% (range 26–99%). The two subjects with poor responses (0% and 17% reduction in BSA at week 12) were excluded from further analysis as they were considered nonresponders. The decrease in disease severity was significant at week 4 and week 12 with respect to BSA and Physician's Global Assessment (PGA) scores (b). Boxes extend from the 25th to 75th percentiles showing the median as a line; whiskers denote the 10th and 90th percentiles and dots show minimum and maximum values if outside this range. Statistical significance was determined using the Friedman test ($P < 0·001$, both series) with Dunn's post-test on individual time points. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, $n = 18$.

to 4 days postconfluence. Cultures were then starved of growth factors in unsupplemented Medium 154 for 24 h before use. NHKs were stimulated with recombinant human TNF-α and IL-17A (R&D Systems, Minneapolis, MN, U.S.A.) under low calcium (0·1 mmol L^{-1}) conditions.

Interleukin-17 receptor knock-down

shIL-17RA (shIL17RA-53; Sigma, St Louis, MO, U.S.A.) and shIL17RC (shIL17RC-10; Sigma) pLKO.1-puro vectors (Sigma) were grown in 293FT cells, and the constructs were tested for knock-down of IL-17RA and IL-17RC expression, respectively, as assessed by quantitative reverse-transcription (qRT)-PCR and Western blotting using antibodies against IL-17RC (NBP1-32481, 1 : 1000 dilution; Novus, Littleton, CO, U.S.A.) and β-actin, to control for protein loading (clone AC-74, 1 : 2500 dilution; Sigma). The shIL17RA-53 and shIL17RC-10 constructs were selected for use in keratinocyte stimulation experiments on day 2 postinfection, when knock-down was most effective.

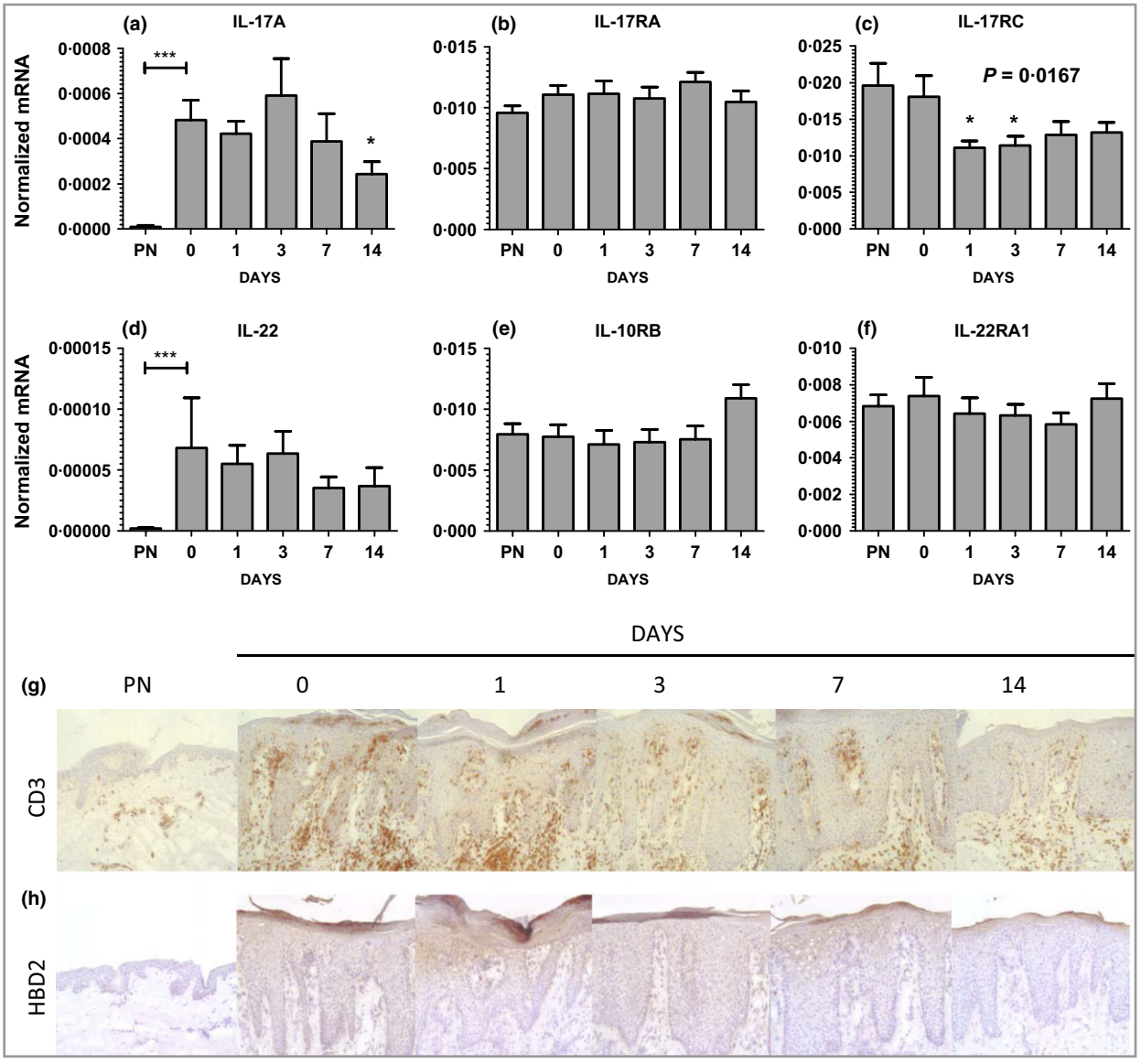

Fig 2. Etanercept alters tissue cytokine and receptor mRNA expression in the first 2 weeks of treatment. Total RNA was extracted from snap-frozen 6-mm punch biopsies of responding patients and analysed with quantitative reverse-transcription polymerase chain reaction. Transcripts for interleukin (IL)-17A (a) and IL-22 (d) were significantly more abundant in lesional (day 0) than nonlesional skin (PN). During treatment, mRNA for IL-17RC (c) but not IL-17RA (b) was significantly decreased. Neither IL-22 (d) nor its receptor subunits (e, f) were downregulated in the first 2 weeks of treatment. The decrease in IL-17RC preceded any changes in CD3+ T-cell infiltration (g) or human β-defensin 2 (HBD2) expression (h). Statistical significance was determined with the Mann–Whitney test (PN vs. day 0) or Friedman test with Dunn's multiple comparison test (time course). Bars, mean + SEM, n = 18. *P < 0·05, **P < 0·01, ***P < 0·001.

Results

Clinical results

On recruitment, disease severity was recorded as a median BSA of 35% and median PGA score of 3·6. We determined that 18 of 20 subjects were responders to treatment, defined as at least a 25% decrease in BSA by week 12. The mean decrease in BSA score was 64%, which was in accordance with our previous study where 25 of 30 patients responded to etanercept with a mean BSA decrease of 62%.[28] The decrease in disease severity was significant at weeks 4 and 12 with respect to both BSA and PGA scores (Fig. 1a, b). Analyses of the two nonresponders were underpowered to detect differences due to small sample size, thus only responder data are presented in this report.

Etanercept alters tissue cytokine and receptor expression

Given that our hypothesis is that the mechanism of action of etanercept involves early tissue responses that precede changes in T-cell numbers, keratinocyte differentiation and proliferation, we first analysed cytokine mRNA expression by qRT-PCR in the 18 responding patients. In the first 2 weeks of treatment both IL-17A and IL-22 remained significantly elevated compared with uninvolved skin, with IL-17A significantly decreased only at day 14 ($P < 0{\cdot}05$, Fig. 2a). Expression of the IL-17A receptor subunit IL-17RA remained unchanged (Fig. 2b); however, IL-17RC expression declined rapidly, with significant decrease in expression observed already at day 1 ($P = 0{\cdot}0167$ overall, Fig. 2c). This preceded any changes in T-cell infiltration or human β-defensin 2 expression (Fig. 2g, h). In contrast, neither of the receptor subunits for IL-22 were altered in expression (Fig. 2e, f).

We also analysed tissue cytokine protein levels in the skin biopsies using multiplex bead assays. Levels of IL-12p70, IL-17A, IL-18, IFN-γ, CXCL10 (induced protein-10), chemokine (C–C motif) ligand (CCL)2 (monocyte chemoattractant protein-1), CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β) and CCL5 (RANTES) were all significantly higher in lesional psoriasis (PP) biopsies than nonlesional skin

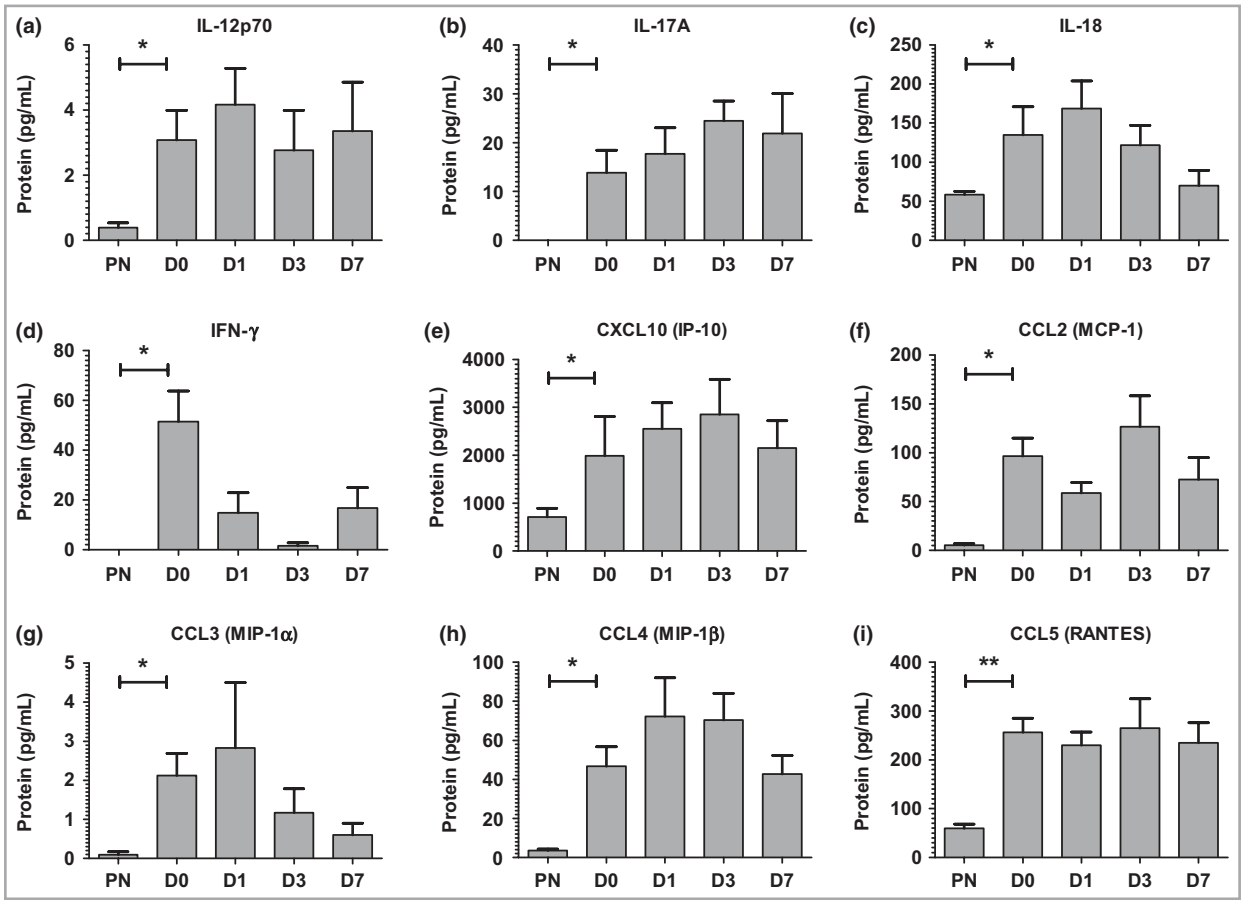

Fig 3. Etanercept alters tissue cytokine protein expression in the first 2 weeks of treatment. Snap-frozen 6-mm punch skin biopsies were pulverized and diluted to 1 mg mL^{-1} total protein before analysis with Bio-Rad human cytokine multiplex kits. (a) Interleukin (IL)-12p70, (b) IL-17A, (c) IL-18, (d) interferon (IFN)-γ, (e) CXCL-10, (f) chemokine (C–C motif) ligand (CCL)2, (g) CCL3, (h) CCL4 and (i) CCL5 protein levels were all determined to be significantly elevated in psoriasis plaques at day 0 (D0) compared with uninvolved skin (PN) from the same individuals (Mann–Whitney test). During the first 7 days of treatment, CCL3 (g, $P = 0{\cdot}039$) showed a significant decline in protein levels (Friedman test with Dunn's multiple comparison test). Bars, mean + SEM, $n = 5$. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.

(PN) at day 0 (Fig. 3). However, of these only IFN-γ (Fig. 3d, P = 0·078, day 7) and CCL3 (Fig. 3g, P = 0·039, overall) showed a substantial decline in protein expression in the first week of treatment.

Etanercept treatment results in decreased interleukin-17A signalling

Given that we saw suppressed IL-17RC mRNA expression in the psoriatic lesions, we examined events immediately downstream of cytokine receptors, using multiplex bead array assays to examine the expression of the activated (phosphorylated) forms of several important signal transduction molecules downstream of the IL-17 receptor. We observed a 64% decrease in the level of phospho-ERK1/2 on day 1 compared with pretreatment; however, we detected no changes in the levels of phospho-STAT3 (signal transducer and activator of transcription 3) or phospho-Tyk-2 in skin (Fig. 4). This suggests that in the first week of treatment there are no alterations in the activity of type 1 interferons (via phospho-STAT2) or IL-22 and IL-6 (and other cytokines that signal via phospho-STAT3) in the skin.

Etanercept treatment is accompanied by decreased interleukin-17A-induced gene expression in skin

Following RNA extraction, microarray analysis was performed on skin biopsies taken from six individuals on days 1, 3, 7 and 14 of etanercept treatment. Using publicly available datasets archived on GEO, specifically GEO series GSE31652, GSE36287 and GSE7216 (Table S1; see Supporting Information), we compared lists of genes most strongly downregulated by etanercept against lists of genes induced both by treatment of NHKs with IL-17A, TNF-α, IFN-γ and IL-22, and by treatment of patients with psoriasis with the IL-17 inhibitor ixekizumab (LY2439821). We found a significantly larger than expected overlap between etanercept-decreased genes and those genes induced by IL-17A on days 1, 7 and 14 (Fig. 5a). Next, we compared a list of genes downregulated by etanercept against those genes decreased by the IL-17 inhibitor ixekizumab. We found a strong association between the genes suppressed by etanercept and ixekizumab, which was evident as early as day 1 of treatment and remained significant at each time point tested (Fig. 5b). We also examined the overlap between etanercept-decreased genes and lists of keratinocyte genes induced by TNF-α (Fig. 5c), IFN-γ (Fig. 5d) and IL-22 (Fig. 5e), with significant gene list overlaps only on day 1 of treatment for TNF-α and IFN-γ, and days 1, 7 and 14 for IL-22-induced genes. This overlap between the top 1000 genes most strongly decreased by etanercept and the top 1000 genes most strongly altered by IL-17A, TNF-α, IFN-γ, IL-22 and ixekizumab is also depicted in an alternative graphic (Fig. S1; see Supporting Information). Furthermore, we analysed the genes most strongly altered by etanercept with respect to GO biological processes, i.e. those genes increased or decreased in day-14 lesions vs. those lesions obtained at baseline (Figs S2 and S3; see Supporting Information). Among the top 100 genes most strongly decreased in the day-14 lesions, we

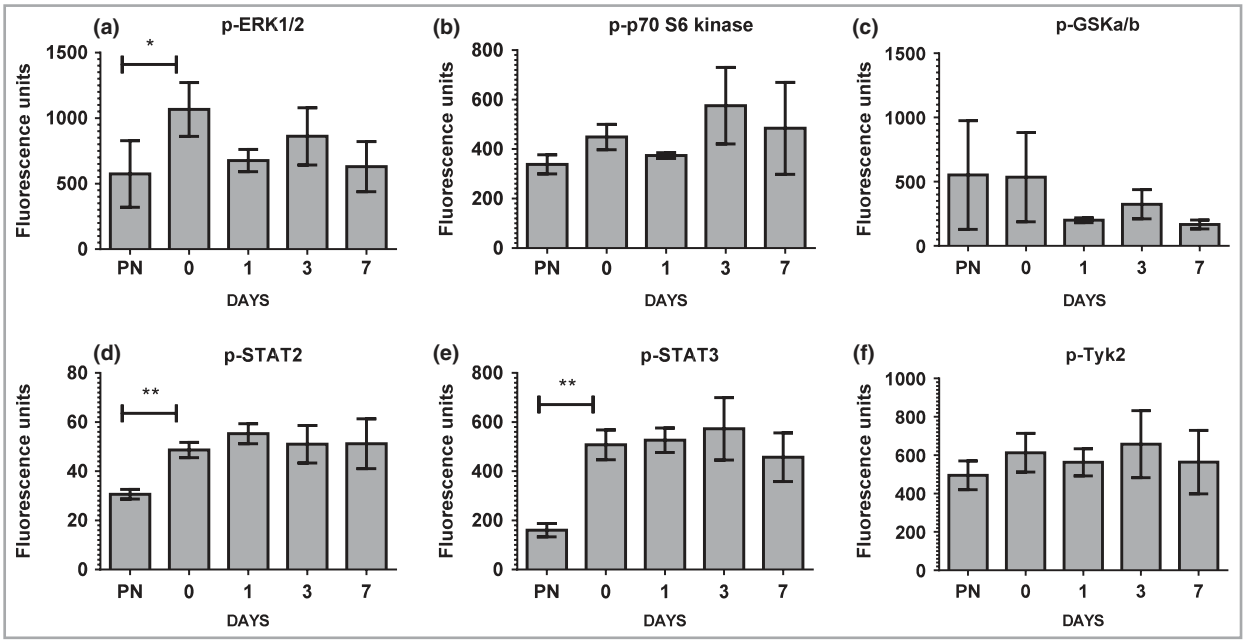

Fig 4. Extracellular signal-regulated kinase (ERK)1/2 (a), signal transducer and activator of transcription (STAT)2 (d) and, STAT3 (e), but not p70 S6 kinase (b) or Tyk2 (f) are significantly activated in lesional skin compared with uninvolved skin. Snap-frozen 6-mm punch skin biopsies were pulverized and diluted to 1 mg mL^{-1} total protein before analysis with Bio-Rad human phosphoprotein multiplex kits. Tissue levels of phospho-ERK1/2 (a) and phosphoglycogen synthase kinase (p-GSK)3a/b (c) were suppressed on days 1 and 7 after treatment with etanercept. Bars, mean fluorescence ± SEM, n = 5. Statistical significance determined with paired t-test. *P < 0·05, **P < 0·01.

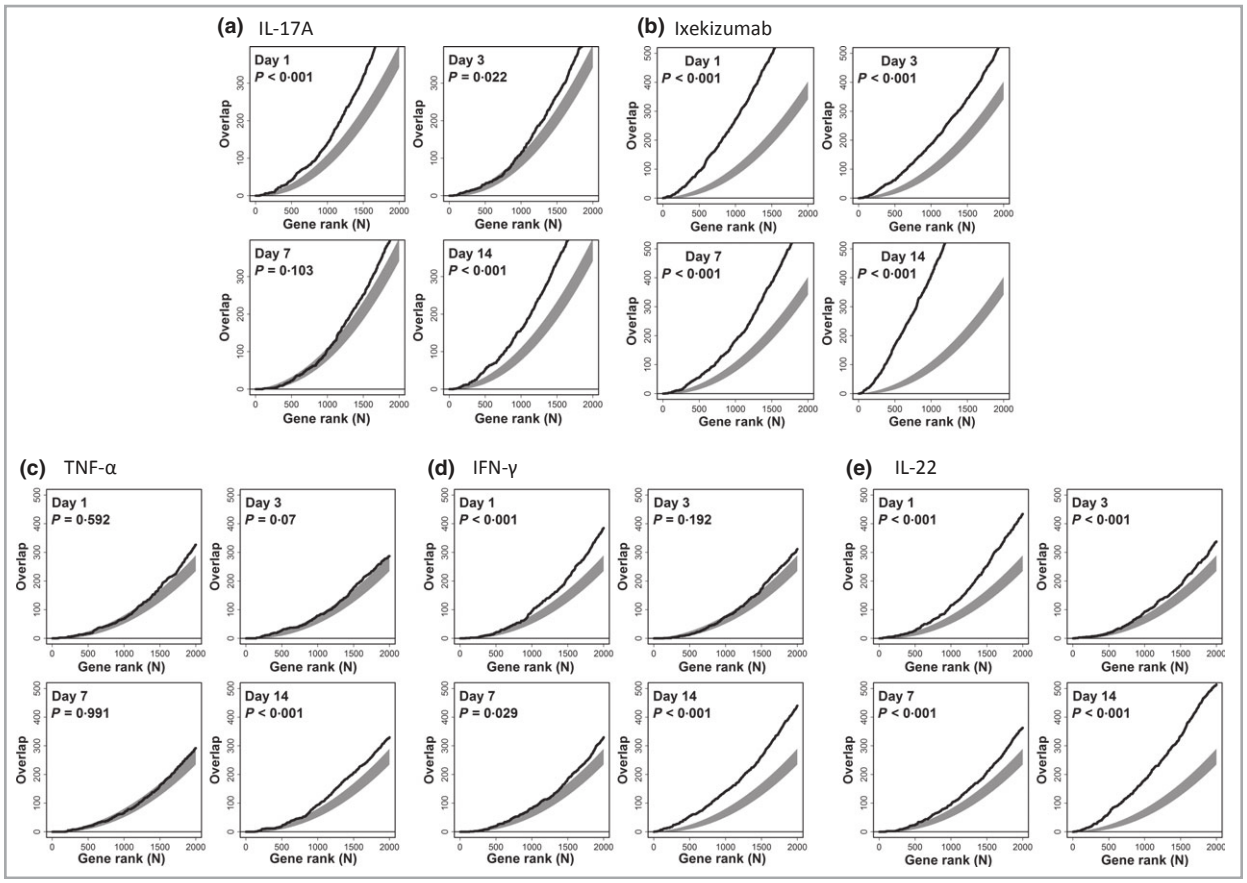

Fig 5. Etanercept treatment leads to suppression of interleukin (IL)-17-induced genes, and its effect overlaps with that of the anti-IL-17A biologic ixekizumab. Following RNA extraction, microarray analysis was performed on skin biopsies taken from six individuals on days 1, 3, 7 and 14 of etanercept treatment. We compared lists of genes downregulated by etanercept against a list of IL-17A-induced genes (a) or genes strongly decreased by the IL-17 inhibitor ixekizumab (b) or genes induced by either tumour necrosis factor (TNF)-α (c) or interferon (IFN)-γ (d). The overlap between etanercept-decreased genes and IL-17A-induced genes (a) was significantly larger than expected at days 1, 7 and 14. There is a strong association between etanercept-decreased genes and the genes most strongly decreased by the IL-17A inhibitor ixekizumab, even after only 1 day of treatment (b). The overlap between etanercept-decreased genes and those induced by TNF-α (c), IFN-γ (d) or IL-22 (e) was much less pronounced. In each box, the black line shows the level of overlap among the top n genes taken from each list, whereas the grey region outlines the central 95% of the null distribution under a random sampling model (hypergeometric distribution). Larger than expected overlap (for given n) is present if the line lies above the grey region, whereas lower than expected overlap (for given n) is present if the line lies below the grey region. P-values are based on the overlap between the top 1000 genes from each gene list.

detected significant enrichment for GO terms such as response to 'type I interferon', 'leucocyte migration' and 'regulation of T-cell apoptosis' (false discovery rate < 0·05). These functional terms are consistent with those associated with genes most strongly elevated in psoriasis lesions (as compared with normal uninvolved skin), suggesting that the effect of etanercept is to shift gene expression patterns towards those in normal skin.

Keratinocyte interleukin (IL)-17RA and IL-17RC are induced by tumour necrosis factor (TNF)-α, and short hairpin RNA knock-down of IL-17RA or IL-17RC abrogates TNF-α–IL-17A synergism

Given that we observed a decrease in the expression of IL-17RC in the skin of responders to etanercept, we tested the effects of TNF-α on IL-17R expression by keratinocytes. NHKs were treated with 0–10 ng mL^{-1} TNF-α for 24 h and the mRNA expression of the two receptor subunits IL-17RA and IL-17RC was quantified by qRT-PCR. We found that while TNF-α increased the expression of IL-17RA, the effect was more pronounced for IL-17RC (Fig. 6a, b). The increased expression of IL-17RC was confirmed using immunohistochemical detection of IL-17RC in resting and TNF-α-treated NHKs. After 24 h of TNF-α treatment, strikingly increased immunofluorescence for IL-17RC could be seen (Fig. 6c).

To show how sequestering TNF-α by etanercept, leading to a decrease in IL-17R expression, could lead to a decrease in the expression of keratinocyte genes induced by the synergistic activity of TNF-α and IL-17A, we selectively knocked down IL-17RA or IL-17RC expression in NHKs using shRNA (Fig. 6d, e). IL-17RA and IL-17RC mRNA expression was

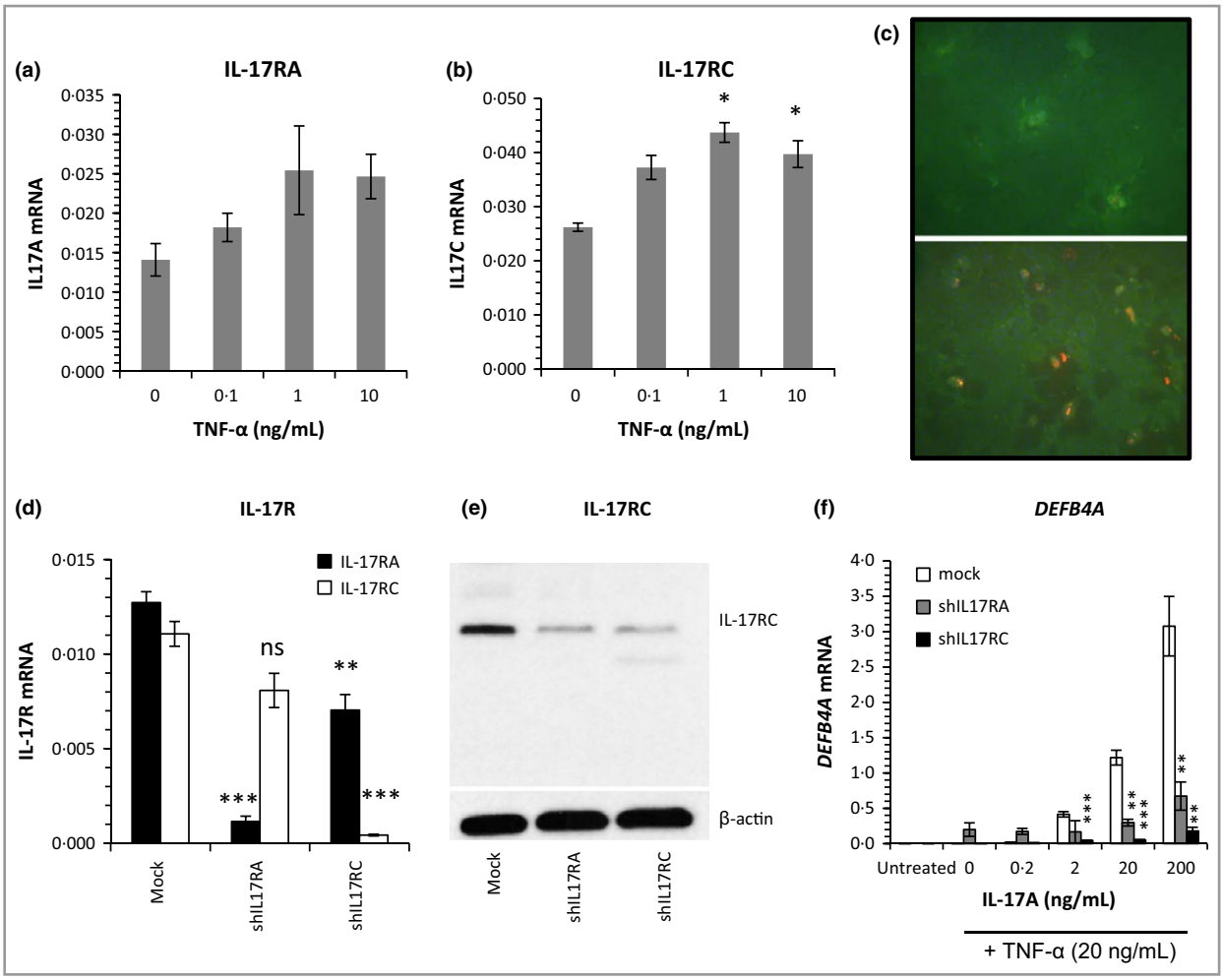

Fig 6. Tumour necrosis factor (TNF)-α induces interleukin (IL)-17R expression by keratinocytes, and short hairpin (sh)RNA knock-down of IL-17R expression abolishes synergistic TNF-α–IL-17A gene induction. (a, b) Normal human keratinocytes (NHKs) treated with TNF-α for 24 h significantly increased IL-17RC but not IL-17RA mRNA expression. (c) Immunocytochemical detection of IL-17RA (green) and IL-17RC (red) protein expression in resting (top) and TNF-α-stimulated (bottom, 10 ng mL^{-1}, 24 h) NHKs showing increased IL-17RC protein expression after TNF-α treatment. Targeted knock-down of IL-17RA and IL-17RC expression (d, reverse-transcription polymerase chain reaction; e, Western blot) leads to significantly decreased *DEFB4A* responses to IL-17A + TNF-α in cells deficient in IL-17RA (grey bars) and IL-17RC (black bars) compared with mock transfected cells (open bars) (f). All bars, mean ± SEM (n = 3), statistical significance determined by ANOVA (a, b) or Student's t-test (d–f). *P < 0·05, **P < 0·01, ***P < 0·001; ns, not significant.

suppressed to 9% and 4% of control levels, respectively (Fig. 6d), and Western blotting revealed a strong suppression in IL-17RC protein expression in shRNA-treated cells (Fig. 6e). Keratinocytes with suppressed IL-17RA or IL-17RC expression were stimulated with IL-17A in the presence of TNF-α to invoke the synergistic induction of human β-defensin 2 (encoded by *DEFB4A*), one of the most upregulated proteins in lesional psoriasis skin. Knock-down of either receptor subunit, but IL-17RC in particular, led to a significant suppression of TNF/IL-17A-induced *DEFB4A* mRNA expression (Fig. 6f).

Discussion

The anti-TNF biologics etanercept, infliximab and adalimumab have revolutionized the treatment of chronic skin disease over the last 13 years, with disease remission rates (75% improvement in Psoriasis Area and Severity Index) up to 80%,[29–32] demonstrating that specific cytokine targeting can be extremely effective in managing this disease. As such etanercept is considered a first-line systemic biologic for chronic plaque psoriasis. The anti-TNF biologics typically require a few weeks before significant clinical improvement is noted,[29–32] but early studies on the mechanism of action of etanercept in psoriasis have identified a number of changes in immune cell frequencies[33] and cytokine and chemokine mRNAs[34] as early as 1 month into treatment, before any meaningful clinical improvement has occurred, and more recent studies have revealed biochemical and histological changes as early as 1 week into treatment.[35,36] IL-1β and IL-8 expression was shown to be decreased after 1 week,[36] and interestingly this

occurred in patients who both did and did not respond to etanercept treatment (about 30% of patients with psoriasis do not respond well clinically).[29] Using gene set enrichment analysis it was further shown that both responders and nonresponders to etanercept had decreases in TNF-α-induced genes, but only responders had decreases in IL-17A-induced gene sets.[36] This suggests that control of IL-17A activity is a crucial part of the mechanism of action of etanercept. In that respect, etanercept has been shown to block TNF-α activation of myeloid dendritic cells leading to IL-23-driven Th17 responses.[35] Moreover, IL-17A and TNF-α have been shown to have additive and synergistic effects directly on keratinocytes,[11,28] which may be driven by the stabilization of TNF-α-induced mRNA transcripts by IL-17A.[37] Interestingly, the biology behind the poor responses of the group of patients unresponsive to etanercept is unknown, and there are still no reliable predictors of clinical response.

IL-17 has been implicated in psoriasis for more than 15 years,[14] and interest in this cytokine is now very strong given the promising data from trials of anti-IL-17 biologics in psoriasis.[38–40] It is now widely appreciated that cytokines can have strongly synergistic activities,[11–14] working in a complex inflammatory network[15–17,41] promoting immune activation, proliferation and inflammation. Thus we hypothesized that etanercept acts by dismantling the powerful synergy between TNF-α and IL-17, reducing IL-17 signalling and the expression of IL-17-induced chemokines prior to later changes in T-cell numbers, keratinocyte differentiation and proliferation (Fig. 7). To test this idea, we took the approach of examining very early time points in the treatment protocol to detect some of the earliest biochemical changes that precipitate the collapse of the inflammatory circuits that drive the psoriatic hyperplasia.

We found that IL-17A, IL-22 and IFN-γ mRNA and protein showed no significant change in the first week of treatment (Figs 2 and 3); however, there was a 2·5-fold downregulation of IL-17RC mRNA after day 1 (Fig. 2c). In contrast, IL-17RA, and subunits of the IL-22 receptor (IL-22RA and IL-10RB), were unchanged (Fig. 2b, e, f). Examining the activation of pathways downstream of these receptors showed reduction in the phosphorylation of glycogen synthase kinase 3a/b and ERK1/2 by 50% and 64%, respectively, on day 1, suggesting reduced IL-17R signalling (Fig. 4). When we examined global gene expression by microarray, we found that etanercept induced a suppression of IL-17A-induced genes (Figs 5a and S1), which is consistent with the findings of Zaba *et al.*,[42] who found that IL-17 pathway genes were strongly downregulated in etanercept responders. Moreover, this etanercept-suppressed gene set overlapped with those genes suppressed by the anti-IL-17A drug ixekizumab (Fig. 5b). Moreover, TNF-α was shown to increase the expression of IL-17RC mRNA and protein by keratinocytes (Fig. 6a–c), and shRNA inhibition of IL-17RC expression curtailed synergistic TNF–IL-17A responses (Fig. 6d–f). As IL-17A utilizes the IL-17RA–IL-17RC complex for signalling, the decreased expression of IL-17RC induced by etanercept is likely to lead to decreased keratinocyte activation

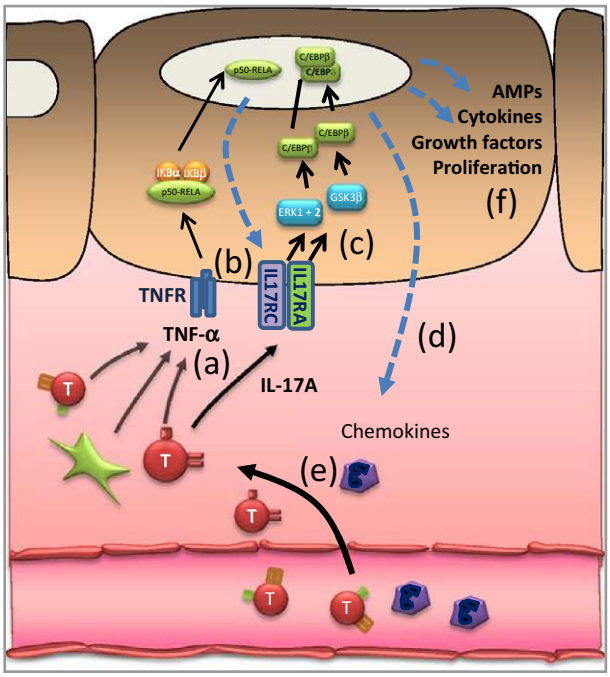

Fig 7. A proposed model of action of etanercept in psoriasis lesions. Tumour necrosis factor (TNF)-α and interleukin (IL)-17A are integral parts of a self-sustaining proinflammatory cytokine network that maintains psoriatic lesions. We hypothesized that etanercept acts by dismantling the powerful synergy between TNF-α and IL-17 (a):[11,28,37] by reducing keratinocyte IL-17 receptor expression (b); by decreasing IL-17 signalling (c) and by decreasing the induction of IL-17-induced chemokines (d), all prior to changes in T-cell (red), antigen presenting cell (APC, green) and neutrophil (purple) numbers in skin (e), and keratinocyte differentiation and proliferation (f). EBP, enhancer-binding protein; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; IKB, inhibitor of κB; TNFR, TNF receptor.

by IL-17A, with subsequent decreases in T-cell and antigen-presenting-cell activity,[28,35,36] and an eventual decrease in disease activity resulting in resolution of skin inflammation (Fig. 7). Along these lines, the recent data on the efficacy of biologics targeting both IL-17A[38,39] and its IL-17RA receptor chain[40] highlight the crucial role of IL-17A in psoriatic inflammation. Moreover, given the need for IL-17RC in the IL-17R complex, agents targeting IL-17RC may also hold the promise of efficacy for psoriasis and Th17-driven inflammatory diseases.

In conclusion, these results support the view that the early responses of psoriasis plaques to etanercept are due to decreased IL-17A activity, not from lowered Th17 cytokine expression, as the lesions are still rich in IL-17A, IL-22 and activated STAT3. Instead, response to etanercept is due to diminished tissue responsiveness to IL-17A as a result of decreased expression of an essential subunit of the IL-17A receptor (IL-17RC), thus blunting responses to Th17 cytokines and breaking a potentially self-sustaining cycle that contributes to the maintenance of psoriasis lesions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. The association between etanercept-altered gene expression and the genes altered by ixekizumab, tumour necrosis factor-α, interferon-γ and interleukin-22.

Fig S2. Gene ontology biological process terms associated with etanercept-repressed genes.

Fig S3. Gene ontology biological process terms associated with etanercept-induced genes.

Table S1 Description of additional microarray datasets.

Table S2 Polymerase chain reaction vs. microarray.