The possible role of epiregulin from dermal fibroblasts in the keratinocyte hyperproliferation of psoriasis

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Running head: Fibroblast-derived epiregulin in psoriasis

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Abstract

Psoriasis, an immune-mediated inflammatory disease, is characterized by keratinocyte hyperproliferation. Tumor necrosis factor alpha (TNFα), interleukin (IL)-23, and IL-17A play critical roles in the pathogenesis of psoriasis. IL-17A secreted by Th17 acts more directly against keratinocytes than TNFα or IL-23 do. Regarding the receptors of cytokine, fibroblasts also express receptors against IL-17A and TNFα, and induce the production of growth factors. Epiregulin (EREG), an epidermal growth factor receptor (EGFR) ligand, is produced by both keratinocytes and fibroblasts. EREG enhances keratinocyte proliferation and differentiation. We hypothesized that fibroblasts stimulated with IL-17A and/or TNFα may play a role in epidermal hyperproliferation through the production of epidermal growth factors in psoriasis. The mRNA expression of EREG was found to be significantly upregulated by co-stimulation with IL-17A and TNFα (mean 49.2-fold). Furthermore, the stimulation with TNFα alone, but not IL-17A alone, induced significant increases. Immunofluorescent staining demonstrated that the protein expression level of EREG was also increased in fibroblasts stimulated with these cytokines. Stimulation with EREG significantly enhanced keratinocyte proliferation in vitro. In human psoriatic patients' skin, immunofluorescence staining of EREG showed significantly high intensity in the dermis of lesional skin. In conclusion, cytokine stimulations with TNFα and IL-17A induce the overexpression of EREG from dermal fibroblasts in the lesional skin of psoriasis, and plays a role in epidermal hyperproliferation.

Introduction

Psoriasis is an immune-mediated inflammatory disease characterized by keratinocyte hyperproliferation. Among the pathomechanisms of psoriasis, it is well known that tumor necrosis factor alpha (TNFα), interleukin (IL)-23, and IL-17 play critical roles, and biologics block these cytokines or their receptors. Of these cytokines, IL-17A, which is mainly secreted by Th17 cells, is thought to act more directly against keratinocytes than TNFα or IL-23 does, and to induce keratinocyte proliferation(1). In general, growth factors also contribute to cell proliferation. A previous report showed that epiregulin (EREG), an epidermal growth factor receptor (EGFR) ligand, is overexpressed in psoriatic lesions(2). This overexpression is thought to be involved in epidermal hyperproliferation via Erk/JNK, MAP kinase, and stat3 signaling(2,3).

The IL-17 receptor family consists of five members: IL-17RA, RB, RC, RD, and RE. These receptors are composed of a heterodimer, such as IL-17RA/IL-17RC for IL-17A, IL-17F and IL-17A/IL-17F(4,5). IL-17RA is expressed in almost all cell types, and fibroblasts produce inflammatory cytokines and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and EREG via IL-17A signaling(4,5). Of these growth factors, EREG is produced by both fibroblasts and keratinocytes(6,7). EREG enhances the proliferation and differentiation of keratinocytes(7) and of follicular keratinocytes, leading to hair growth(8). In addition, keratinocytes secrete EREG and stimulate keratinocyte growth in an autocrine manner(7). Dermal–epithelial interactions, both direct and indirect, play essential roles in skin biology, such as in skin homeostasis, cancer progression, and wound healing(9,10). Fibroblast-derived EREG is known to promote epidermal tumors(11,12), cancer invasion(13), and wound healing(14,15).

Based on this evidence, we hypothesized that fibroblasts activated by IL-17A may play an important role in epidermal hyperproliferation through the production of epidermal growth factors, especially EREG.

Materials and Methods

Patients

Cryo-skin samples were obtained from 5 patients with psoriasis vulgaris whose diagnosis was confirmed by histological examination. This study was approved by the Hokkaido University Certified Review Board and was performed in accordance with the Declaration of Helsinki.

Cell cultures and cytokine stimulation

Normal human fibroblasts obtained from uninvolved skin of surgical specimens were cultured for 24 hours in Dulbecco's modified Eagle's medium (Life Technologies, Tokyo, Japan) containing 1.8 mM of calcium. An antibiotic-antimycotic solution (Sigma Aldrich, St. Luis, MO) and 10% fetal calf serum were supplied to the medium. Immortalized human keratinocytes (Ker-CT) purchased from ATCC (Manassas, VA) were cultured in keratinocyte growth medium (KGM-Gold, Lonza, Basel, Switzerland).

The recombinant human TNFα, IL-17A and IL-23 were purchased from PeproTech (Cranbury, NJ, 10602HNAE5), from Thermo Fisher Scientific (Waltham, MA, PHC9174) and from abcam (Cambridge, UK, ab106889), respectively.

Primer and quantitative polymerase chain reaction (qPCR)

Fibroblasts were stimulated with PBS or cytokines for 48 hours. mRNA was extracted from fibroblasts. Single-stranded cDNA was synthesized using the SuperScript IV (Thermo Fisher Scientific, Waltham, MA). qPCR assays were performed using RT^2 SYBR GREEN/ROX PCR Master Mix (Qiagen, Valencia, CA) and Step-OnePlus (Applied Biosystems). Relative expression ratios were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each primer sequence is listed below.

EGF F: GTTAGTCTCTCAGCACCGCA R: GTGCAGGACCCACACAAGTA

FGF1	F: AAAGCGTGGGGGAGGTGTAT	R: ATTTGGTGTCTGTGAGCCGT
FGF2	F: GCGACCCTCACATCAAGCTA	R: AGCCAGGTAACGGTTAGCAC
FGF7	F: GAAAGGCTCAAGTTGCACCAG	R: GCTATTTGACTTTTGTTTTGTTGCT
FGF10	F: AGATGTCCGCTGGAGAAAGC	R: TCTCCAGGATGCTGTACGGG
EREG	F: CTCTGCCTGGGTTTCCATCTT	R: ACTGGACTCTCCTGGGATACAT
GAPDH	F: ACCATCTTCCAGGAGCGAGA	R: GACTCCACGACGTACTCAGC

Immunofluorescence staining

For the immunofluorescence staining, the cells were cultured on cover glass and incubated for 48 hours with cytokines (Matsunami Glass, Kishiwada, Japan). The cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 minutes. After permeabilization with 0.25% TritonX-100 for 10 minutes, the cells were incubated with goat anti-epiregulin (R&D Systems, Minneapolis, MN, final concentration: 10 μg/ml) at 4°C overnight. To stain the human cryo-skin samples, 5 μm-thick sections were incubated with goat anti-epiregulin (10 μg/ml), rabbit anti-CD3 (Roche diagnostics, Rotkreuz, Switzerland) and mouse anti-CD68 (Thermo Fisher Scientific, Waltham, MA, 1:100 dilution). After washing with PBS, cells or sections were stained with FITC-conjugated anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:100 dilution), FITC-conjugated anti-rabbit IgG (SouthernBiotech, Birmingham,AL, 1:100 dilution) and FITC-conjugated anti-mouse IgG (SouthernBiotech, 1:100 dilution). The fluorescent intensity was measured by ImageJ (min-max, 0-256).

Keratinocyte proliferation assay

On day 0, 1.0 x 10^4 cells/well were cultured in 96-well plates and then treated with growth factor or cytokine (concentration of 100 ng/ml); recombinant human EREG (PeproTech, Cranbury, NJ, 100-

04), TNFα, IL-17A, and EGF (PeproTech, Cranbury, NJ, AF-100-15). For 72 hours of incubation, cell proliferation was measured by the Premix WST-1 Cell Proliferation Assay System according to the manufacturer's instruction (Takara Bio, Kusatsu, Japan, MK400). The indicated optical density (OD) was the value after the OD value of the control well was subtracted (KGM-gold and Premix WST-1).

Statistical analysis

Statistical calculations were performed using SigmaPlot (Version 14.5, Systat Software, Chicago, IL). To compare relative gene expression levels and cell proliferation, one-way ANOVA was used. A p-value of <0.05 was considered statistically significant.

Results

Co-stimulation with IL-17A and TNFα induced the EREG overexpression of mRNA and proteins in fibroblasts

We investigated the relative gene expressions of six growth factors under cytokine stimulation (final concentration: 100 ng/ml, N=4). EGF, FGF1, FGF7, or FGF10 did not change under any stimulation condition (Figure 1a). FGF2 was significantly upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, EREG, mean 49.2-fold). In addition, TNFα alone, but not IL-17A alone, induced significant increases of EREG (mean 18.2-fold). This indicates that co-stimulation with IL-17A and TNFα has a synergistic effect and not just an additive effect. Based on this result, we focused on EREG expressed by fibroblasts under cytokine stimulation. We next investigated protein expression level. Immunofluorescence staining of EREG clearly showed strong fluorescence intensity under co-stimulation with IL-17A and TNFα (Figure 1b, TNFα+IL-17A). Stimulation with TNFα alone also demonstrated slight intensity compared to those with PBS or IL-17A alone.

EREG enhanced keratinocyte proliferation

Next, we investigated keratinocyte proliferation under cytokine or growth factor stimulation (N=8). The 100 ng/ml concentration significantly enhanced cell proliferation with EREG stimulation, but not with TNFα or IL-17A (Figure 1c).

EREG was highly expressed in the dermis of lesional skins

Finally, we studied EREG staining in psoriasis patients. Representative images are shown in Figure 2. EREG was highly stained in the dermis of psoriasis lesions (Figure 2a, red square, Psoriasis 1 and 2), but not in the dermis of healthy controls. The average fluorescent intensity of lesional dermis was significantly higher than that of normal control (Figure 2b, dermis). The fluorescent intensity of epidermis was not significantly different (Figure 2b, epidermis). Psoriatic lesional skin from three patients (Patients 1, 2 and 3) out of five patients showed positive staining of EREG in the basal layer, with one patient showing a particularly high expression of EREG in the lower epidermis (Psoriasis 2). Interestingly, we found that several round cells in the papillary dermis and in the epidermis were highly expressing EREG (white arrow). To confirm which inflammatory cells express EREG, we performed immunofluorescent staining of CD3 and CD68. Most of infiltrating cells in the papillary dermis and in the epidermis were CD3-positive T cells, but rarely CD68 macrophages (Figure 3c).

Discussion

Fibroblasts express receptors of IL-17A, IL-17C, and TNFα, but not of IL-23 (Human Protein Atlas). Then, we focused on the function of the growth factors produced by fibroblasts stimulated with IL-17A and TNFα. Interestingly, the combination of TNFα and IL-17A induced a synergistic effect on the expression of EREG from fibroblasts. We found no significant effects on EREG expression level by IL-23 stimulation (data not shown). Immunofluorescence staining of EREG demonstrated high-intensity staining in the upper dermis of psoriatic skin. EREG from fibroblasts that are stimulated by TNFα and IL-17A might induce the epidermal hyperproliferation seen in psoriasis. It has been known that the proliferation of keratinocytes and epidermal tumor cells is enhanced by stimulation with EREG(2,8,11,15). In this study, EREG demonstrated significantly high proliferation.

We found EREG expression to be high not only in the dermis but also in the epidermis, especially in the basal layer (Figure 2). Shirakata et al. investigated *EREG* mRNA expression in the epidermis of normal humans and psoriasis patients(2). They found faint expression in the basal layer of normal skin, and found overexpression in the spinous layer, but not in the basal layer, of lesional psoriatic skin. Immunofluorescence staining may not be sensitive enough, compared to *in-situ* hybridization. However, protein expression is generally more directly associated with the phenotype than mRNA expression is. In addition, we found several round EREG-positive cells in the papillary dermis and in the epidermis by immunofluorescence staining. A previous report showed that peripheral blood cells, particularly macrophages, express EREG(16). However, the present study revealed most of the infiltrating cells to be CD3-positive T cells. This suggests that the EREG-positive round cells might be T cells, rather than macrophages.

In conclusion, TNFα and IL-17A, which are essential cytokines in psoriasis pathogenesis, induce the overexpression of EREG from fibroblasts and other cells (Figure 3). Although the systemic blocking of growth factors can have serious side effects, topical application may be a potential therapeutic

option for psoriasis.

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Authorship contributions

HI performed the experiments. HI and NH performed medical examinations on the patients. HI and HU designed the study. HI wrote the manuscript, and all the coauthors had final approval of the submission.

Abbreviations

epiregulin (EREG), epidermal growth factor receptor (EGFR), interleukin (IL), epidermal growth factor (EGF), fibroblast growth factor (FGF), tumor necrosis factor alpha (TNFα), quantitative polymerase chain reaction (qPCR)

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Figure legends

Figure 1 Overexpression of EREG from fibroblasts by co-stimulation with TNFα and IL-17A

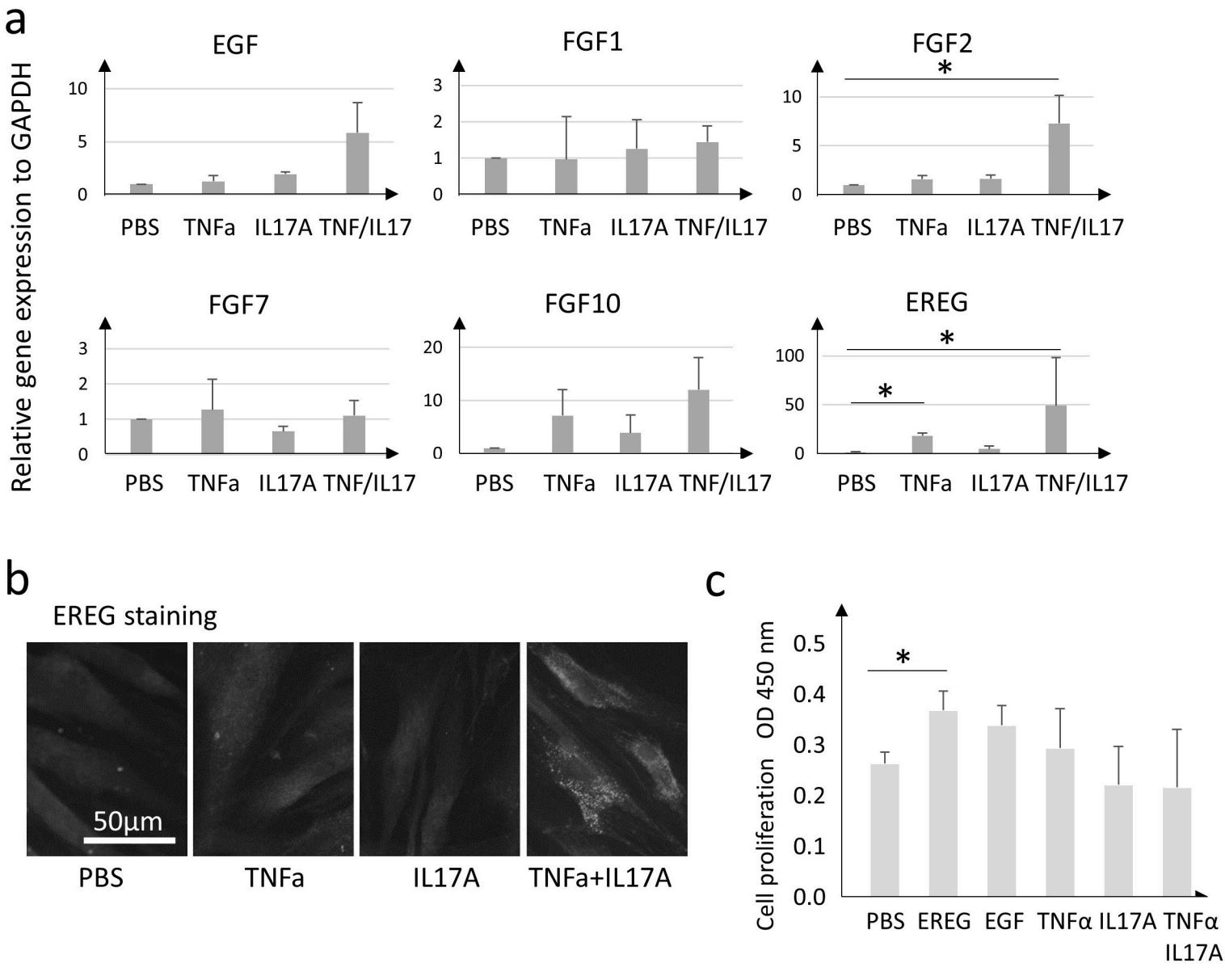
Fibroblasts were stimulated with cytokines for 48 hours. a) qPCR analysis of growth factors under cytokine stimulation. The Y axis is the gene expression level relative to that of GAPDH b) Representative immunofluorescence staining of EREG. Scale bar: 50μm. c) Keratinocytes were stimulated with growth factors or cytokines (100 ng/ml). Asterisk indicate a significant P-value ($P < 0.05$).

Figure 2 Lesional psoriatic skin overexpress EREG in the dermis

a) Immunofluorescence staining demonstrates high intensity in the dermis, especially in the papillary dermis of psoriatic lesions (Psoriasis 1 and 2). High magnification images show that some round cells are highly stained and are found in the papillary dermis and in the epidermis of psoriatic lesions (squared area from psoriatic lesions, white arrow). Negative used PBS instead of primary antibody in normal human skin. Scale bar: 200μm. b) The average of fluorescent intensity in the dermis and epidermis. Asterisk indicate a significant P-value ($P < 0.05$). c) Immunofluorescence staining show CD3-positive cells in the papillary dermis (left) and epidermis (right). Scale bar: 50μm.

Figure 3 Schematic of the hypothesis

Dermal dendritic cells produce TNFα and IL-23. The IL-23 induces IL-17A production from Th17 cells. Both the TNFα and the IL-17A stimulate fibroblasts and generate EREG. The EREG induces the epidermal hyperplasia seen in psoriasis. TNFα and the IL-17A may also enhance epidermal hyperplasia. Black arrows demonstrate known signals, and red arrows indicate the hypothesis based on this study.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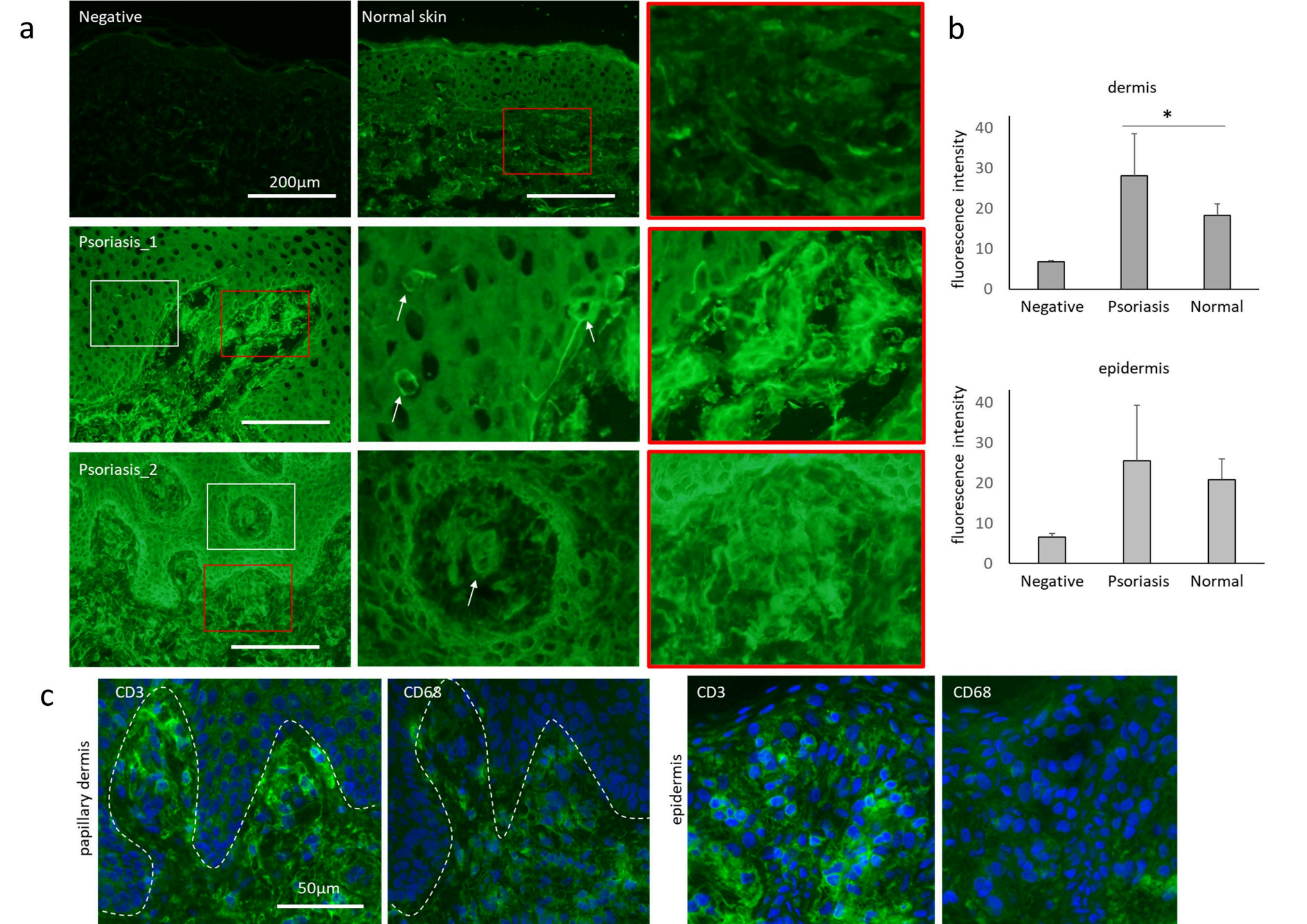
Relative gene expression to GAPDH
EGF
FGF1
FGF2
FGF7
FGF10
EREG
PBS
TNFa
IL17A
TNF/IL17
b
EREG staining
50μm
PBS
TNFa
IL17A
TNFa+IL17A
c
Cell proliferation OD 450 nm
PBS
EREG
EGF
TNFα
IL17A
TNFα IL17A


a
Negative
200μm
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Psoriasis_1
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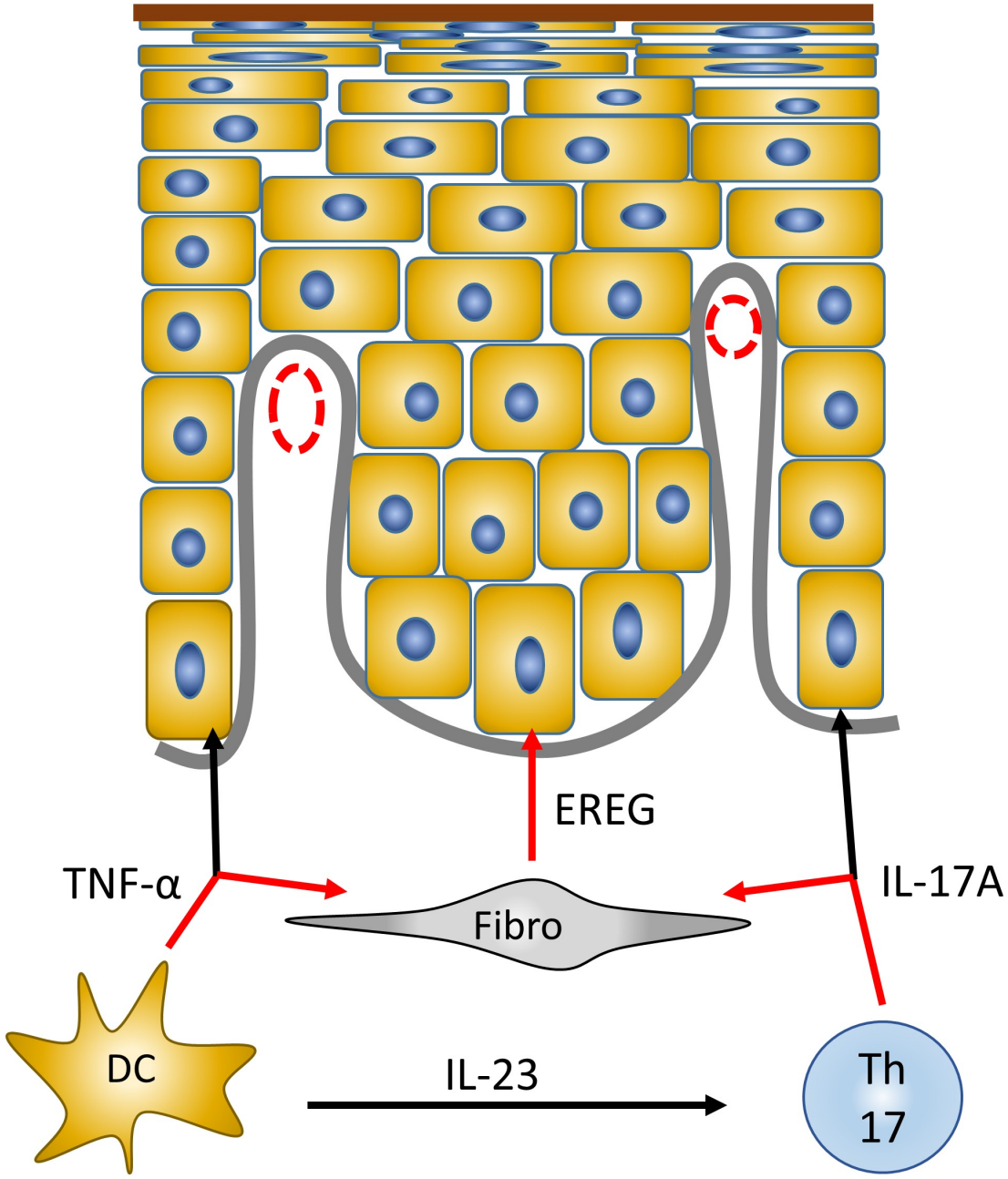
EREG
TNF-α
IL-17A
Fibro
DC
IL-23
Th
17