LIGHT mediates inflammation in psoriasis by up-regulating cellular proliferation and cytokines production of keratinocytes

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Research Article

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Abstract

Background

The pathogenesis of psoriasis is associated with perturbations of immune system. In our previous studies, HVEM served as a LIGHT (homologous to lymphotoxins, inducible, and competes with HSV glycol- protein D), which is also known as receptors for tumor necrosis factor superfamily member 14. HVEM and LTBR were increased in psoriasis compared with health control, revealing a potential correlation in the pathogenesis of psoriasis.

Methods

The objective of this study was to examine LIGHT's effect on keratinocytes proliferation and its therapeutic potential for psoriasis. We added LIGHT protein, anti-HVEM, anti-LTβR, HVEM interference, LTβR interference, NF-κB inhibitor and JNK/AP-1 inhibitor separately or simultaneously in HaCat cells medium in vitro. The expression of NF-κB was stained by immunofluorescence and the expression of inflammatory products was measured with Western blot and ELISA. HaCat cells viability was examined by CCK-8 kit. Flow cytometry was used to detected the expression of HVEM and LTβR on cells and immunohistochemistry in tissues.

Results

We found that NF-κB transferred from cytoplasm into nuclear after HaCat cells stimulated by LIGHT protein. The expression of p-c-Jun, IL-6, IL-8, PGI2 and PTGS2 was increased when adding LIGHT protein, while decreased after blockading LIGHT's receptors or inhibiting NF-κB and JNK/AP-1. HaCat cells viability was consistent with the expression of pro-inflammatory factors. The expression of LIGHT and HVEM was increased in lesions of patients with psoriasis.

Conclusions

These results suggest that JNK/AP-1-HVEM-LIGHT pathway improved the viability and the expression of IL-6, IL-8, PGI2 and PTGS2 in human keratinocytes, revealing the JNK/AP-1-HVEM-LIGHT pathway can be a potential target for the treatment of psoriasis.

Background

Psoriasis is a chronic, lifelong immune disease that remains largely unknown inheriting factors. Skin becomes inflamed and characterized by patches of red and flaky areas(1). The prevalence of condition is estimated to range from 1–5% of the population worldwide (2–5). One of the critical pathogenesis of psoriasis is thought to perturbations of immune system. Although psoriasis is a complex disease

obviously with the disordered proliferation and differentiation of skin cells, recent evidence on the clinical and histological feature changes actually indicates that the epidermal pathogenesis in psoriasis occur in abnormal inflammatory response to cellular immune infiltration in the skin by autoimmune leukocytes(6–10).

The developmental formation of psoriasis is triggered by activating immune cells to produce TNF and IL-23, stimulating Th17 to migrate into epidermis, where they recognized epidermal autoantigens and produced IL-17 and IL-22, characterized by hyper-proliferation of keratinocytes(6). The TNF-IL-23-Th17 axis appears to be crucial in the pathogenesis of chronic psoriasis. Understanding pathogenesis of psoriasis provides the foundation for developing the immune therapies targeting the pathogenic cytokines, recently including anti-TNFs, anti-IL-23, anti-IL-17(11–13). According to TNF-α role in the pathogenesis of psoriasis validly, one selective inhibitor (etanercept) and two antibodies (infliximab, adalimumab) of TNF-α approved for psoriasis clinical treatment(14, 15). Although these biological agents have greatly improved lots of patients with psoriasis, but still lots of patients not be cured even without any response to the drugs(16). Therefore, it is necessary to further investigate the molecular mechanism of psoriasis pathogenesis, which is greatly helpful to discovery new biomarkers and therapeutic targets for psoriasis treatment.

Many members of the TNF superfamily are emerging as mediators and modulators of immune-related disorders. Another family member, a receptor expressed by T lymphocytes, homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycol- protein D for HVEM (LIGHT), also known as tumor necrosis factor superfamily member 14 (TNFSF14), is a new-known secreted member of tumor necrosis factor superfamily(17), and binds to its specific receptors HVEM and LTBR(18, 19). LIGHT plays multiple roles in physiological and pathological processes in multiple tissues and organs(20–23). LIGHT and its receptor on cellular surface, also play a key role in the pathogenesis of inflammatory-response diseases(24–26). In our previous study, we show that the expression of LIGHT and its receptor in the tissue sample of psoriasis showed higher than normal skin. It hinted us that LIGHT and its receptor might be involved in the pathogenesis of psoriasis, and have a crosstalk with classical TNF immune-response pathway.

Therefore, our current study aimed to investigate the role of LIGHT protein on autoimmune-related pathways and cytokine, also the function of the downstream immune-response effectors of LIGHT in human keratinocytes, which were determined by using antibody and gene-silence methods.

Results

Increasing LIGHT protein caused NF-κB transfer into nuclear, and significantly increased the expression of p-c-Jun, IL-6, IL-8, PGI2 and PTGS2

Human keratinocyte HaCat cells were treated with 0.1 and 1 μg/ml recombinant LIGHT protein for 0, 30, and 60 min, respectively. Intracellular NF-κB levels were detected by immunofluorescence. LIGHT treatment can increasing the NF-κB in nuclear localization after LIGHT treatment, less NF-κB was found

in cytoplasm, and more NF-κB (stained in green) was found in nucleus (stained in blue; Figure 1a). Effects of 0.1 and 1 μg/ml LIGHT protein on nuclear localization of NF-κB were similar. In addition, HaCat cells were treated with 0.1 and 1 μg/ml LIGHT protein for 30 min. p-c-Jun expression was detected by western blot analysis. LIGHT (both 0.1 and 1 μg/ml) significantly increased the expression of p-c-Jun (P<0.001, Figure 1b and c). Meanwhile, HaCat cells were treated with 0.03, 0.1 and 1 μg/ml LIGHT protein for 24, 48 and 72 h. The results shows that LIGHT protein at 0.1 and 1 μg/ml markedly increased the viability of HaCat cells at 24, 48, and 72 h (P<0.01, Figure 1f). Moreover, HaCat cells were treated with 0.1 and 1 μg/ml LIGHT protein for 24 h. And the results showed that LIGHT protein at 0.1 and 1 μg/ml can increase the expression of IL-6, IL-8, PGI2, and PTGS2 in HaCat cells at 24 h (P<0.01, Figure 1d and e).

Blockade of LIGHT's receptors significantly decreased cell viability and expression of IL-6, IL-8, PGI2 and PTGS2

The expression of HVEM and LTBR on HaCat cells surface were detected by FCM. HVEM and LTBR normally expressed on HaCat cells (Figure 1g). We used anti-HVEM/LTBR antibodies to determined the effects of LIGHT treatment on human keratinocyte HaCat cells. HaCat cells were divided into 7 groups: (1) LIGHT (0.1 μg/ml); (2) LIGHT (0.1 μg/ml)+anti-HVEM antibody (2 μg/ml); (3) LIGHT (0.1 μg/ml)+anti-HVEM antibody (10 μg/ml); (4) LIGHT (0.1 μg/ml)+anti-LTBR antibody (2 μg/ml); (5) LIGHT (0.1 μg/ml)+anti-LTBR antibody (10 μg/ml); (6) LIGHT (0.1 μg/ml)+anti-HVEM antibody (2 μg/ml)+anti-LTBR antibody (2 μg/ml); (7) LIGHT (0.1 μg/ml)+anti-HVEM antibody (10 μg/ml)+anti-LTBR antibody (10 μg/ml). The viability of HaCat cells was measured by CCK-8. Compared to Group 1, the cell viability of HaCat cells treated with anti-HVEM and anti-LTBR antibodies had significantly decreased at 24, 48, and 72 h (P<0.05, Figure 2a). HaCat cells treated with anti-LTBR antibody had markedly higher cell viability at 48 and 72 h, as compared to HaCat cells treated with anti-HVEM antibody (P<0.01, Figure 2a). In addition, HaCat cells were treated with 0.1 μg/ml LIGHT, 0.1 μg/ml LIGHT+2 μg/ml HVEM, 0.1 μg/ml LIGHT+2 μg/ml LTBR, 0.1 μg/ml LIGHT+2 μg/ml HVEM+2 μg/ml LTBR for 24 h, respectively. Concentration of IL-6, IL-8, and PGI2 in supernatant also was compared to group 1, the expression of IL-6, IL-8, PGI2, and PTGS2 HaCat cells treated with anti-HVEM and anti-LTBR antibodies had significantly decreased at 24 h (P<0.01, Figure 2b). HaCat cells treated with anti-LTBR antibody had dramatically higher expression of IL-6, IL-8, PGI2, and PTGS2 at 24 h, as compared to HaCat cells treated with anti-HVEM antibody (P<0.001, Figure 2c).

Moreover, we suppressed the function of HVEM and LTBR by viruses which can interference their function in HaCat cells. The interference efficiency of vectors listed in Table 3 was detected by qPCR. The interference efficiency was 73%, 44%, 55%, 20%, 70%, and 56% for CL791-1, CL791-2, CL791-3, CL792-1, CL792-2, and CL792-3, respectively. CL791-1 (CL791-1_PL-shRNA-GFP-homo- HVEM-470) and CL792-2 (CL792-2_PL-shRNA-GFP-homo-LT-βR-1402) vectors were screened, and lentiviruses (LV473-1_PL-shRNA-GFP-homo-HVEM-470, and LV473-2_PL-shRNA-GFP-homo- LT-βR-1402) were packed into vectors. HaCat cells were tranfected with LV473-1 and LV473-2 viruses for 48 h respectively, and interference efficiency was detected by qPCR. The interference efficiency was 86% for LV473-1 against HVEM, and 71% for LV473-2 against LTBR. HaCat cells were divided into 4 groups: (1) LIGHT (0.1 μg/ml); (2) LIGHT (0.1

μg/ml)+negative control vector; (3) LIGHT (0.1 μg/ml)+HVEM interference by LV473-1; (4) LIGHT (0.1 μg/ml)+LTBR interference by LV473-2. Compared to group 1, the cell viability of HaCat cells treated with HVEM and LTBR suppressed had markedly decreased at 24, 48, and 72 h (P<0.01, Figure 2d). HaCat cells with HVEM suppressed had markedly lower cell viability at 24, 48 and 72 h, as compared to HaCat cells treated with LTBR suppressed (P<0.01, Figure 2d). Meanwhile, compared to group1, the expression of IL-6, IL-8, PGI2, and PTGS2 HaCat cells with HVEM and LTBR suppressed had significantly decreased at 24 h (P<0.01, Figure 2e). HaCat cells with HVEM suppressed interference had markedly lower expression of IL-6, IL-8, and PGI2 at 24 h, as compared to HaCat cells with LTBR suppressed interference (P<0.05, Figure 2f).

Inhibition of NF-κB and JNK/AP-1 significantly decreased cell viability and expression of IL-6, IL-8, PGI2 and PTGS2 compared to LIGHT treated group.

HaCat cells were divided into 7 groups: (1) LIGHT (0.1 μg/ml); (2) LIGHT (0.1 μg/ml)+NF-κB inhibitor (10 μM); (3) LIGHT (0.1 μg/ml)+NF-κB inhibitor (50 μM); (4) LIGHT (0.1 μg/ml)+NF-κB inhibitor (250 μM); (5) LIGHT (0.1 μg/ml)+JNK/AP-1 inhibitor (2 μM); (6) LIGHT (0.1 μg/ml)+JNK/AP-1 inhibitor (10 μM); (7) LIGHT (0.1 μg/ml)+JNK/AP-1 inhibitor (50 μM). Compared to group 1, HaCat cells treated with inhibitors of NF-κB and JNK/AP-1 had markedly decreased the cell viability at 24, 48, and 72 h (P<0.001, Figure 3a). HaCat cells treated with 50 and 250 μM NF-κB inhibitor had markedly decreased the cell viability at 48 and 72 h compared to HaCat cells treated with 10 μM NF-κB inhibitor (P<0.001, Figure 3b). Meanwhile, HaCat cells treated with 10 and 50 μM JNK/AP-1 inhibitor had markedly decreased the cell viability at 48 and 72 h compared to HaCat cells treated with 2 μM JNK/AP-1 inhibitor (P<0.001, Figure 4). Therefore, 50 μM NF-κB inhibitor and 10 μM JNK/AP-1 inhibitor were used in following experiments.

HaCat cells were divided into 7 groups: (1) LIGHT (0.1 μg/ml); (2) LIGHT (0.1 μg/ml)+NF-κB inhibitor (50 μM); (3) LIGHT (0.1 μg/ml)+JNK/AP-1 inhibitor (10 μM); (4) LIGHT (0.1 μg/ml)+NF-κB inhibitor (50 μM)+anti-HVEM antibody (2 μg/ml); (5) LIGHT (0.1 μg/ml)+JNK/AP-1 inhibitor (10 μM)+anti-HVEM antibody (2 μg/ml); (6) LIGHT (0.1 μg/ml)+NF-κB inhibitor (50 μM)+anti-LTBR antibody (2 μg/ml); (7) LIGHT (0.1 μg/ml)+JNK/AP-1 inhibitor (10 μM)+anti-LTBR antibody (2 μg/ml). Compared to group 1, HaCat cells treated with NF-κB and JNK/AP-1 inhibitors had dramatically decreased cell viability at 24, 48, and 72 h (P<0.001, Figure 4a). HaCat cells treated with JNK/AP-1 inhibitor had markedly decreased cell viability at 48 and 72 h compared to HaCat cells treated with NF-κB inhibitor (P<0.001, Figure 4a). Meanwhile, compared to group 1, HaCat cells treated with NF-κB and JNK/AP-1inhibitors had significantly decreased the expression of IL-6, IL-8, PGI2, and PTGS2 at 24 h (P<0.001, Figure 4b). HaCat cells treated with JNK/AP-1 inhibitor had markedly decreased the expression of PGI2 and PTGS2 at 24 h, as compared to HaCat cells treated with NF-κB inhibitor (P<0.05, Figure 4b).

The expression of LIGHT and HVEM was higher in skin tissues of psoriasis patients

Our previous data had showed that LIGHT treatment can induce pathological process of psoriasis in vitro, and it also hinted us that LIGHT and its receptor might involve in pathological development of psoriasis. To verify this hypothesis, we collected the tissue samples from psoriasis patients and normal skin, and

detected the expression of LIGHT and its receptors HVEM and LTBR by immunohistochemistry. The higher expression pattern of LIGHT and HVEM except LTBR were observed in the skin tissue of psoriasis patients (Figure 5).

Discussion

It is few previous evidence about effects of LIGHT protein on human keratinocytes and psoriasis disease. As we know, keratinocyte proliferation and deregulated cytokine expression are thought to be central in the pathogenesis of psoriasis. In current study, we first provided the evidence that LIGHT stimulated cellular proliferation and production of cytokines and inflammatory mediators in human keratinocytes, which suggested that LIGHT had a key role on in the pathogenesis of psoriasis. The treatment of recombination protein of LIGHT increased the viability in human keratinocytes, and upregulated the induced expression of IL-6, IL-8, PGI2 and PTGS2. Recombinant LIGHT treatment promoted NF-kB downstream transcription by stimulating nuclear translocalization and activated the JNK/AP-1 pathway. Blockade of HVEM and LTBR attenuated LIGHT induced cellular viability and the expression of cytokines and inflammatory mediatorsin human keratinocytes. Our data suggest that it may represent a marker for psoriasis and that blocking LIGHT activity could represent an effective therapy in psoriasis.

Keratinocyte is one of the predominant cell types in the outer layer of the skin. As a fact, aberrant proliferation of keratinocytes breaking skin homeostasis is involved in the formation of chronic inflammatory skin disease including psoriasis. Keratinocytes as innate immune cells in skin can release a cluster of inflammatory molecules, which lead to aggravate inflammatory skin diseases. In our present study, recombinant LIGHT promoting proliferation of keratinocytes suggested the role of LIGHT in psoriasis. Blockade of LIGHT's receptor and downstream molecules, HVEM and JNK/AP-1, suppressing keratinocyte proliferation has also proved that LIGHT pathway is involved in the potential mechanism in the pathogenesis of psoriasis.

NF-kB expressed in most cell types including epithelial cell, and its nuclear translocation induces to produce cytokines and promote inflammation(26, 27). Phosphorylated c-Jun is responsive to inflammation stress stimuli by cytokines(28–32). In the present study, LIGHT treatment caused nuclear localization of NF-kB and expression of phosphorylated c-Jun in human keratinocytes. These data have indicated that the function of LIGHT pathway in keratinocyte also is involved in inflammatory signaling pathways on psoriasis, similar to TNF-α.

IL-6 and IL-8, as both a pro-inflammatory and an anti-inflammatory cytokines are produced by T cells, macrophages and other cell including epithelial cell and stimulate immune response and induce chemotaxis and phagocytosis, especially in inflamed site during infection(33, 34)(31). PGI2 and PTGS2, as effective vasodilators and its precursor, also area prostaglandin members of eicosanoid family, which inhibits platelet activation and prevents platelet plug formation, and are induced during inflammation. PTGS2 involves in the conversion of arachidonic acid to the precursor of PGI2(35). In our present study, recombinant LIGHT protein not only dramatically increased IL-6, IL-8, PGI2, and PTGS2 in human

keratinocytes, but also promoted the proliferation of human keratinocytes. All above data can be concluded that LIGHT involves inflammation responses in human keratinocytes, which suggests LIGHT might have a role in pathogenesis of psoriasis.

HVEM, as a specific cell surface receptor of LIGHT, can activate both NF-kb and JNK/AP-1(26, 36–38). LTBR is the surface receptor of LTB and LIGHT, being two members of TNF superfamily. Both HVEM and LTBR can be detected on the surface of keratinocyte. In the present study, human keratinocyte cells treated with their antibodies or interfered by their RNAi respectively have significantly decreased cell viability, and decreased IL-6, IL-8, PGI2, and PTGS2. Although the blockade of both HVEM and LTBR cellular surface receptors had similar effects on inflammation responses in human keratinocytes, HVEM as the specific receptor of LIGHT had higher efficiency than LTBR. The above results suggest that LIGHT with its specific receptor HVEM, has a potential role in pathogenesis of psoriasis.

The c-Jun NH2-terminal kinase (JNK) is the downstream effector of LIGHT/HVEM, and JNK signaling pathway regulates a number of biological processes through the activation of the AP-1 transcription factor(39–41). Not only AP-1 transcription factors have a critical role in skin homeostasis(42, 43), but also the induction of JNK/AP-1/c-Jun activity leads to tumor progression and development in skin malignant melanoma(44, 45). In our present study, the treatment with inhibitors of NF-kb and JNK/AP-1 respectively had significantly decreased cell viability, expression of IL-6, IL-8, PGI2, and PTGS2. Moreover, JNK/AP-1 might be the specific downstream effector of LIGHT/HVEM in human keratinocytes, with higher efficiency than NF-kb. The above results indicates that JNK/AP-1 not NF-kb, is involved with inflammation responses of psoriasis. It also hints that the pathogenic process of psoriasis may at least partly be due to abnormal LIGHT/HVEM interaction and regulation of JNK/AP-1.

In our present study, we provided new evidence that high expression of LIGHT and its specific receptor HVEM occurred in skin tissues of psoriasis patients. As we also know, TNF-α presents elevated levels in psoriasis, and has multiple actions that could account for the symptoms of psoriasis(46, 47).

TNF-α promisingly has emerged as a target for the clinic treatment of psoriasis, and is a crucial pleiotropic inflammatory cytokine, which is involved in immune-response regulation of inflammation(48). TNF-α also could promote epidermal proliferation(49). Meanwhile, the activation of TNF-α transmembrane receptors on the cell surface induces a multitude of intracellular signaling pathways, including upregulation of the expression and nuclear translocation of NF-kb, and therefore can induce a cascade of events within production of inflammation cytokines and chemokines such as IL-1, IL-8 and IL-6, whose elevated levels are a pathological hallmark of psoriasis. The afore-mentioned immune responses indicates an important pathogenic role of TNF-α in psoriasis. The impressive response of psoriasis to TNF-antagonist drugs blocking its pro-inflammatory effects clinically manifests a remission of psoriasis symptoms. However, it still remains problems about why TNF-α-targeting biological drugs behave differently in patients. Significant proportions of patients do not respond to the anti-TNF-α treatment or develop serious draw-side effects. Mainly adverse drug events in patients with psoriasis are due to their suppressive effects on immune response, that it is risky for these patients to face new or

existed infection, fatal blood disorders and tumors(50). Therefore, it is important to investigate new biomarkers, such as TNF gene polymorphisms, that can help to predict the specific-drug response of patients and develop the new therapeutic targets.

Many members of TNF and its receptor superfamilies including LIGHT and HVEM play a crucial role for manipulation of misguided immune-mediated disorders(23, 51–53). LIGHT can be expressed in skin tissue of psoriasis. LIGHT and its receptor, HVEM, have been involved in the induction of JNK/AP-1 and lead to the expression of several autoimmune-related inflammation cytokines and modulatory molecules. Here in our present study, we have shown a major role of the LIGHT/HVEM/JNK/AP-1 pathway in immune-mediated skin inflammation and proliferation of human keratinocytes. And it has been postulated and proved that the efficacy of neutralizing the Ligand/receptor interaction of LIGHT/HVEM or inhibiting induced transcription by JNK/AP-1 in dampening keratinocyte activity suppresses psoriasis-like disease symptoms in-vitro. Compellingly, the deficiency in LIGHT pathway ameliorated skin cell inflammatory activity characteristic of psoriasis.

According to our present study, we first implied a new possible mechanism of pathogenesis in psoriasis that LIGHT and its pathway might involve in the pathogenic procession of psoriasis. LIGHT alone may not promote full psoriasis phenotypes, but it can promote common immune-response molecules of psoriasis from human keratinocytes so far. Our evidences for the blockade of HVEM, LIGHT receptor (using anti-HVEM antibody or HVEM silence virus) and/or their downstream effector (using JNK/AP-1 inhibitor) suggested the new potential target for psoriasis of TNF-α-no-effect. Moreover, as importantly, further study would be investigated how LIGHT pathway was involved with the disease-specific cytokines IL-13 and IL-17 to distinguishing the pathogenic functions between with TNF-α pathway.

There has already been a report that TNF-like ligand 1A (TL1A) is proved as a key modulator of immune response and critically involved in the pathogenesis of several autoimmune diseases including psoriasis(53, 54). TL1A binds its two cognate receptors, both of which are members of the TNF receptor superfamily(55). TL1A and its two receptors were also reported significantly increased in psoriatic skin lesions, both serum and PBMCs from psoriasis patients. Moreover, it is still another report that the TNF superfamily molecule TWEAK (TNFSF12) was thought to critically contribute to dysregulated expression of cytokines/chemokines and skin inflammation of psoriasis(56–58). All of these studies about the role of TL1A, TWEAK and LIGHT in psoriasis speculated that not only TNF-α but also other members in the same superfamily, being central mediators of inflammatory regulation in autoimmune diseases, could be involved in the pathogenesis of psoriasis.

The activation and nuclear translocation of NF-kb by TNF-α stimulate a major pathogenic immune-response of psoriasis so far(59). Herein we determined that JNK/AP-1 inhibition suppressed more effectively dysregulated immune-responses induced by LIGHT in in-vitro keratinocyte model of psoriasis than NF-kb inhibition, to indicate that JNK/AP-1 not NF-kb did have pivotal contribution to the pathogenesis of LIGHT-related psoriasis. It suggested that JNK/AP-1 might be suitable to be a possible therapeutic target for the treatment to some subtypes of psoriasis. Recent studies also reported that AP-1

was involved in initiation of skin phenotype changes(60, 61), and further TNF-α signaling was not fully sufficient to the aetiology of psoriasis(62). It suggests that both LIGHT/HVEM/JNK/AP-1 and TNF-α signaling pathways are contributors to development and severity of the disease. Interestingly, AP-1 is a pro-inflammation element that directly regulates the expression of the cytokines including TNF-α(63), that it means AP-1 as a therapeutic target will be better than TNF-α in psoriasis. By the way, as we mentioned previously, LIGHT is involved with bone development and increases osteoclastogenesis. One of selective AP-1 inhibitors has proven to prevent osteoclastogenesis in collagen-induced arthritis, an autoimmune disease(64). It indicates that LIGHT/AP-1 signaling pathway has a pivotal role in human autoimmune diseases, and potentially is a highly efficient therapeutic target of such diseases.

Conclusion

We have demonstrated that blockade of HVEM and JNK/AP-1 attenuates LIGHT-induced increase in the viability and expression of IL-6, IL-8, PGI2 and PTGS2 in human keratinocytes. Our results indicated that LIGHT/TNFSF14 axis played a key role on regulating autoimmune cytokines and related pathways in psoriasis. HVEM and JNK/AP-1, as the LIGHT's receptor and downstream effector, can be a potential therapeutic target for the treatment of psoriasis.

Methods

Cells and reagents

Human keratinocyte cell line HaCat cells were kindly donated by Dr. Xinling Bi (Changhai Hospital，Naval Medical University, China). They were cultured in Dulbecco modified Eagle's medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc.) and 1% penicillin and streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Phosphate-buffered saline (PBS) was purchased from Sigma-Aldrich Inc. Trypsin, Lipofectamine 2000, primers and probes, TRIzol reagent, SuperScript III Reverse Transcriptase, SYBR Green I, DEPC H_2O, Platinum Taq DNA polymerase, oligo dT/primer and 100 mM dNTPs were purchased from Invitrogen (Thermo Fisher Scientific Inc.). RNase inhibitor, endonuclease, EcoRI/BamHI, T4 DNA ligase, GeneRuler DNA ladder were purchased from Fermentas (Thermo Fisher Scientific Inc.). Gel extraction kit (AP-GX-50) and plasmid extraction kit (AP-MN-P-50) were perchased from Corning, Inc. Escherichia coli (E. coli) DH5α competent cells were purchased from Takara Biotechnology, Inc.

Flow cytometry (FCM)

Surface proteins HVEM and LTBR on HaCat cells were detected by FCM. Cells were washed with PBS twice, and incubated with trypsin at 37˚C for 1 min, respectively. Following digestion, the cell suspension was centrifuged at 400 g at room temperature for 5 min. The cell pellet was resuspended with PBS and the centrifugation and resuspension steps were repeated a further two times. Cells were blocked with 2%

bovine serum albumin (Sigma-Aldrich Inc.) for 30 min at room temperature. Anti-HVEM antibody (1:500, ab47677, Abcam Inc.) and anti-LTBR (1:500, Abcam Inc.) was added to 100 μl cell suspension respectively, and incubated at 4˚C for 30 min. Cells were centrifuged at 400 g at room temperature for 5 min, and resuspended with PBS three times. Secondary IgG antibody (1:400; goat anti-rabbbit IgG/fluorescein isothiocyanate (FITC) for HVEM; goat anti-mouse IgG/FITC for LTBR) was added into the cell suspension respectively, and incubated at 4˚C for 30 min. Cells were washed with PBS for three times. Cells were resuspended in 500 μl PBS, and detected by FCM. Data was acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software. Experiments were repeated three times.

Immunofluorescence

HaCat cells were treated with 0.1 and 1 μg/ml LIGHT protein for 0, 30, and 60 min, respectively. HaCat cells adhered to cover slips were fixed in 4% paraformaldehyde at room temperature for 10 min, and blocked with 2% bovine serum albumin (Sigma Inc.) for 30 min at room temperature. Cells were incubated with a primary antibody against NF-κB p65 (1:500) at 4˚C overnight. Following overnight incubation, cover slips were washed with PBS, and incubated in the dark with FITC-conjugated goat anti-rabbit secondary antibody (1:1,000) at room temperature for 1 h. Cover slips were washed with PBS and stained with DAPI at room temperature for 5 min. Slides were prepared using an anti-quenching mounting medium. Slides were observed using a fluorescence microscope (100X).

Western blot analysis

HaCat cells were treated with 0.1 and 1 μg/ml LIGHT protein for 30 min at 37 ˚C. Protein expression of phosphorylated (p)-c-Jun was detected by western blot analysis. Cells were lysed in lysis buffer (P0013, Beyotime Institute of Biotechnology) with protease and phosphatase inhibitors (P1045, Beyotime Institute of Biotechnology) at 4˚C. The lysis mixture was centrifuged at 10,000 x g for 10 min at 4˚C, and the supernatant containing cellular proteins was used in following experiments. The protein concentration was determined using a BCA kit. Proteins were separated by 10% SDS-PAGE (120 V) (40 μg per lane). The separated proteins were then transferred to polyvinylidene fluoride membranes (FFP24, Beyotime Institute of Biotechnology; 100 V for 120 min). After being blocked with 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies against p-c-Jun and JNK at 4˚C overnight. Membranes were then washed with Tris-buffered saline containing Tween 20 and incubated with HRP-conjugated goat anti-rabbit secondary antibody at room temperature for 1 h. Membranes were washed and incubated in enhanced chemiluminescence solution (P0018A, Beyotime Institute of Biotechnology), and images were captured on film (FF057, Beyotime Institute of Biotechnology) in a dark room. Experiments were repeated for 3 times. Blot images were quantified in greyscale.

Cell viability assay

The viability of HaCat cells was measured by Cell Counting Kit-8 (CCK-8; CK04, Dojindo Molecular Technologies Inc., Rockville, MD, USA). CCK-8 reagent was added into each well and incubated for 4 h.

The absorbance was measured utilizing a microplate reader at 490 nm. Experiments were repeated for 3 times.

Enzyme-Linked Immunosorbent Assay (ELISA)

HaCat cells were treated with 0.1 and 1 μg/ml LIGHT protein for 24 h, respectively. IL-6, IL-8, and PGI2 concentrations were detected by ELISA according to manufacturer's instructions. The absorbance was measured utilizing a microplate reader. Experiments were repeated for 3 times.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol reagent, following the manufacturer's protocol. A universal cDNA synthesis kit (Invitrogen Inc.) was utilized for reverse transcription. Each reaction contained 0.5 μl random primers (0.2 μg/μl) and 1 μl SuperScript III reverse transcriptase (200 U/μl). The specific primers used were listed in Table 1. PCR was performed by utilizing a SYBR qPCR mix kit (Invitrogen Inc.). PCR conditions were as follows: Predenaturation at 95˚C for 2 min, 40 cycles of denaturation at 95˚C for 10 sec, and annealing and polymerization at 60˚C for 30 sec and 70˚C for 45 sec. PCR was performed using a CFX96 Touch™ Real-Time PCR Detection system (Bio-rad Inc.). Gene expression was determined and normalized to β-actin. Primer for rat β-actin was forward (5' to 3'), AGGGAAATCGTGCGTGAC and reverse (5' to 3'), CGCTCATTGCCGATAGTG. The 2-ΔΔCq method was utilized to measure PCR results. The fluorophore used is included in SYBR qPCR mix.

Construction of recombinant plasmids and lentiviral packaging

According to sequences of HVEM and LTBR, 3 pairs of oligo sequences were designed respectively (Table 2). After annealing, double stranded DNA was inserted into lentivirus vector PDS019_pL_shRNA_F vector and amplified. The recombinant plasmids were extracted. The interference vectors (Table 3) and negative control were transfected into 293 T cells for 48 h. qPCR was utilized to detect interference efficiency, and vectors with high interference efficiency were selected for virus packaging. Packaging mix (9 μg) and recombinant lentiviral plasmids (3 μg) were added into Opti-Minimum Essential Medium (MEM; 1.5 ml; Invitrogen; Thermo Fisher Scientific, Inc.) and mixed. Lipofectamine 2000 (36 μl) was mixed with Opti-MEM (1.5 ml), and incubated at room temperature for 5 min. The plasmid solution and diluted Lipofectamine 2000 were then mixed, and incubated at room temperature for 5 min. The mixture was added into a culture dish with 293 T cells, and cells were cultured for 48 h. Cell supernatant was then collected, centrifuged at 1,500 x g for 10 min at room temperature and filtered. The virus solution was then condensed by centrifuging at 50,000 x g for 2 h at 4˚C, and re-suspended in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.). Interference efficiency was measured by qPCR.

Statistical analysis

Statistical data was analyzed by GraphPad Prism version 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). The results are presented as mean ± standard error. Differences between 2 groups were compared by student's t-test. Differences among ≥3 groups were compared by an one-way analysis of

variance followed by the Bonferroni post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Authors' contributions

Ying Zou: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision; Ting Cao: Writing - Review & Editing, Visualization, Supervision. The author(s) read and approved the final manuscript; Cheng-bin Ye: Software, Resources, Data Curation, Visualization; Yu Shi: Data analysis, Visualization.

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Tables

Table 1. Primers used in qPCR.

Genes	Primers	Sequences (5’ to 3’)
IL6	Forward	CCCCTCAGCAATGTTGTTTGT
	Reverse	CTCCGGGACTGCTAACTGG
IL8	Forward	ACTGAGAGTGATTGAGAGTGGAC
	Reverse	AACCCTCTGCACCCAGTTTTC
PTGS2	Forward	aagatactcaggcagagatgatctaccc
	Reverse	ttaagcacatcgcatactctgttgtgt

Table 2. Oligo sequences.

Primers	Oligo sequences (5’ to 3’)
HVEM-470-F	CACCGCAGTCCAGGTTATCGTGTGACGAATCACACGATAACCTGGACTGC
HVEM-470-R	AAAAGCAGTCCAGGTTATCGTGTGATTCGTCACACGATAACCTGGACTGC
HVEM-924-F	CACCGAGCCTCGTCATCGTCATTGTCGAAACAATGACGATGACGAGGCTC
HVEM-924-R	AAAAGAGCCTCGTCATCGTCATTGTTTCGACAATGACGATGACGAGGCTC
HVEM-947-F	CACCGCTCCACAGTTGGCCTAATCACGAATGATTAGGCCAACTGTGGAGC
HVEM-947-R	AAAAGCTCCACAGTTGGCCTAATCATTCGTGATTAGGCCAACTGTGGAGC
LT-βR-520-F	CACCGCACCTATGTCTCAGCTAAATCGAAATTTAGCTGAGACATAGGTGC
LT-βR-520-R	AAAAGCACCTATGTCTCAGCTAAATTTCGATTTAGCTGAGACATAGGTGC
LT-βR-1402-F	CACCGCAACATCTACATCTACAATGCGAACATTGTAGATGTAGATGTTGC
LT-βR-1402-R	AAAAGCAACATCTACATCTACAATGTTCGCATTGTAGATGTAGATGTTGC
LT-βR-743-F	CACCGTACACACTGCGAGCTACTTTCCGAAGAAAGTAGCTCGCAGTGTGTA
LT-βR-743-R	AAAATACACACTGCGAGCTACTTTCTTCGGAAAGTAGCTCGCAGTGTGTAC

Table 3. Interference vectors.

Vectors	
CL791-1	CL791-1_PL-shRNA-GFP-homo- HVEM-470
CL791-2	CL791-2_PL-shRNA-GFP-homo- HVEM-924
CL791-3	CL791-3_PL-shRNA-GFP-homo- HVEM-947
CL792-1	CL792-1_PL-shRNA-GFP-homo- LT-βR-520
CL792-2	CL792-2_PL-shRNA-GFP-homo- LT-βR-1402
CL792-3	CL792-3_PL-shRNA-GFP-homo- LT-βR-743

Figures

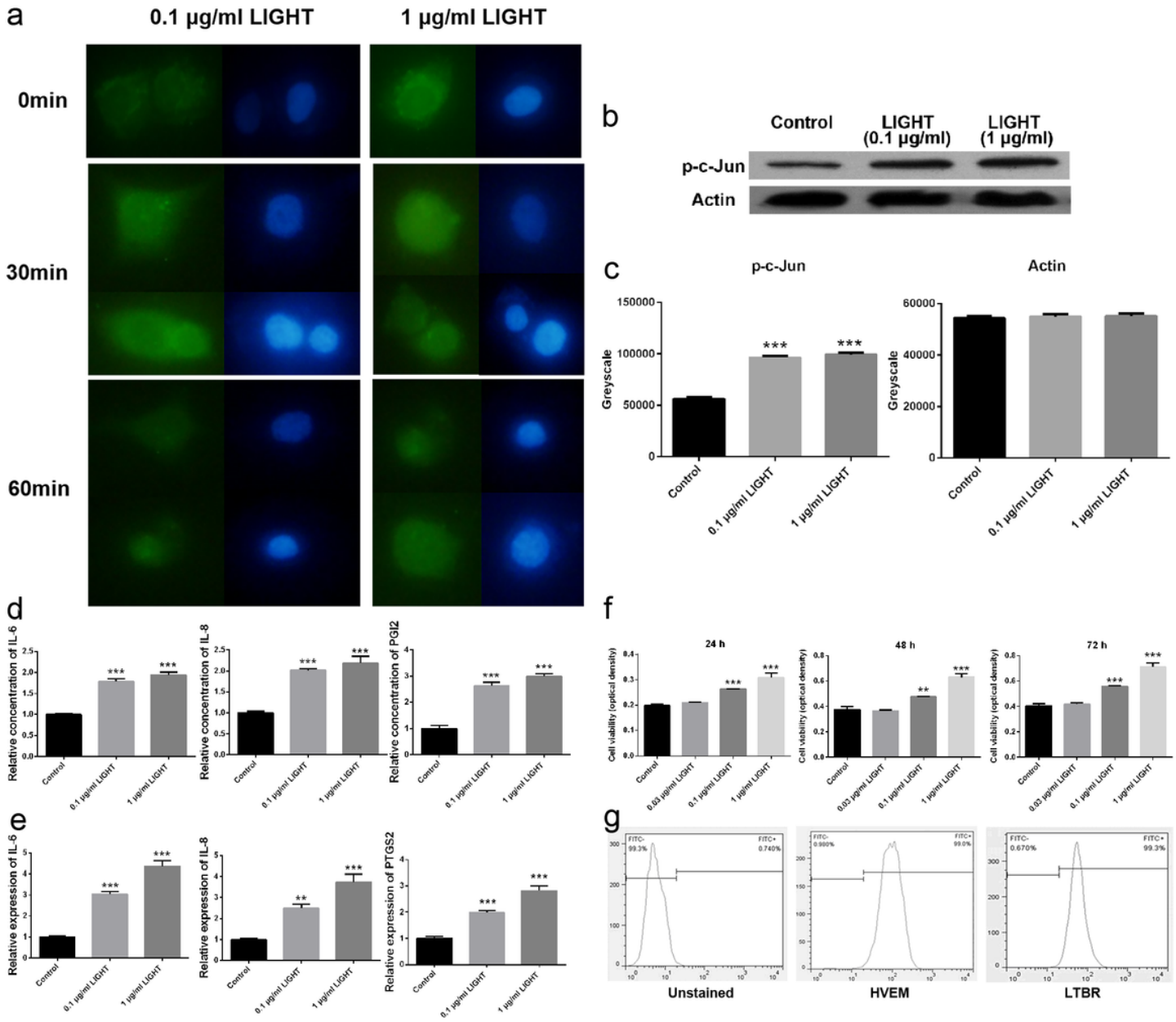

Figure 1

a Immunofluorescence staining of HaCat cells cocultured with LIGHT (0.1μg/ml, left; 1μg/ml, right) after 0 minutes(top),30 minutes (middle),60 minutes (bottom). NF-κB in cytoplasm in green, NF-κB in nucleus in blue. **b** Western blotting showing expression of p-c-Jun and actin (control) proteins in HaCat cells after treatment with PBS(Lane 1) or LIGHT (0.1 μg/ml , Lane2; 1μg/ml, Lane 3), the statistics of stripe greyscale by Image J are shown in (**c**). Bars indicate mean values in each group. ***$P < 0.001$. **d and e** Quantitative real-time results showing levels of mRNA expression of various pro-inflammation factors. **f** CCK-8 kit detecting HaCat cells viability cultured with LIGHT (0.03μg/ml, 0.1μg/ml, 1μg/ml). Bars indicate mean values in each group. **P 0.05, ***$P < 0.001$. **g** The expression of HVEM and LTBR on HaCat cells surface were detected by FCM.

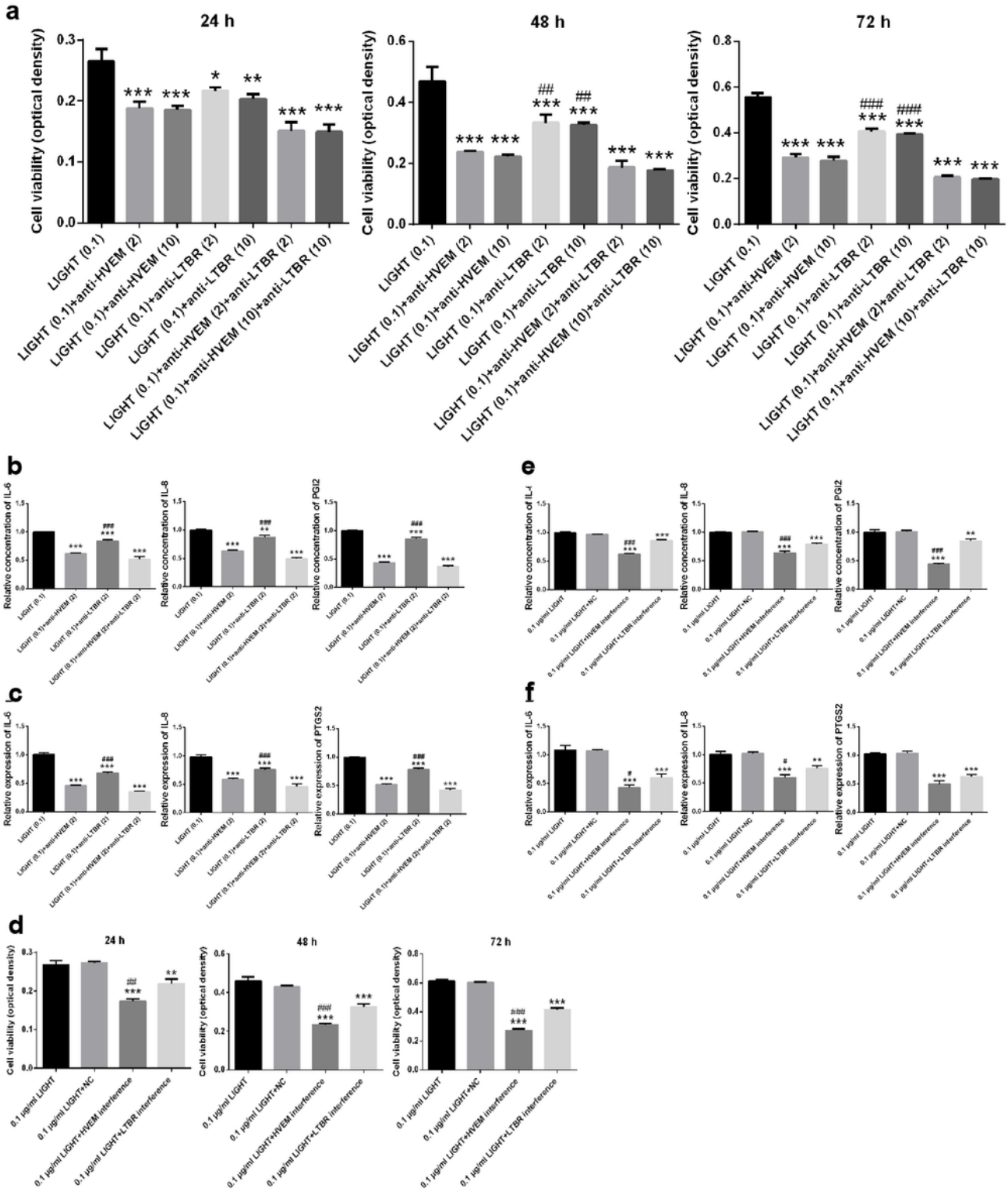

Figure 2

a Optical density was measured of HaCat cells, with or without soluble LIGHT, with or without LIGHT receptor antibodies. Bars indicate mean values in each group. *P 0.05, **P 0.01, ***P < 0.001 versus LIGHT group. ### P < 0.001 versus anti-HVEM group. **b and c:** The expression of IL-6, IL-8, PGI2, and PTGS2 HaCat cells treated with anti-HVEM and anti-LTBR antibodies for 24 hrs were shown in quantitative real-time results. **P 0.01, ***P < 0.001 versus LIGHT group. ### P < 0.001 versus anti-

HVEM group. **d** CCK-8 kit detecting HaCat cells viability cultured with LIGHT, HVEM or LTBR interference. Bars indicate mean values in each group. ** P 0.01, *** P < 0.001 versus LIGHT group. ### P < 0.001 versus HVEM interference group. **e and f** The expression of IL-6, IL-8, PGI2, and PTGS2 of HaCat cells treated with HVEM or LTBR interference for 24 hrs were shown in quantitative real-time results. ** P 0.01, *** P < 0.001 versus LIGHT group. # P 0.05, ### P < 0.001 versus HVEM interference group.

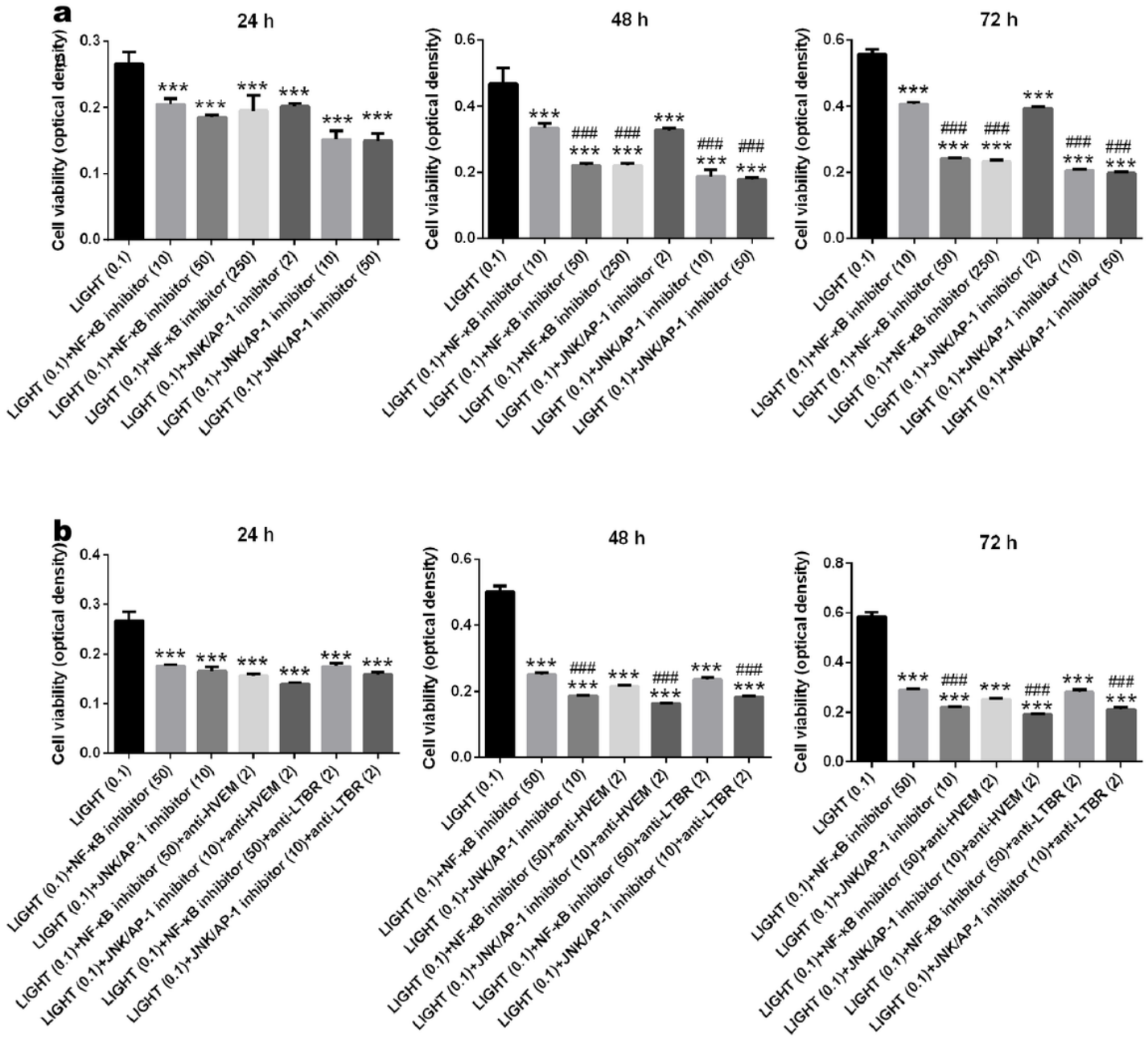

Figure 3

Optical density was measured of HaCat cells, with different treatment for 24 hrs, 48 hrs or 72 hrs. Bars indicate mean values in each group. *** P < 0.001 versus LIGHT group. ### P < 0.001 versus the former group.

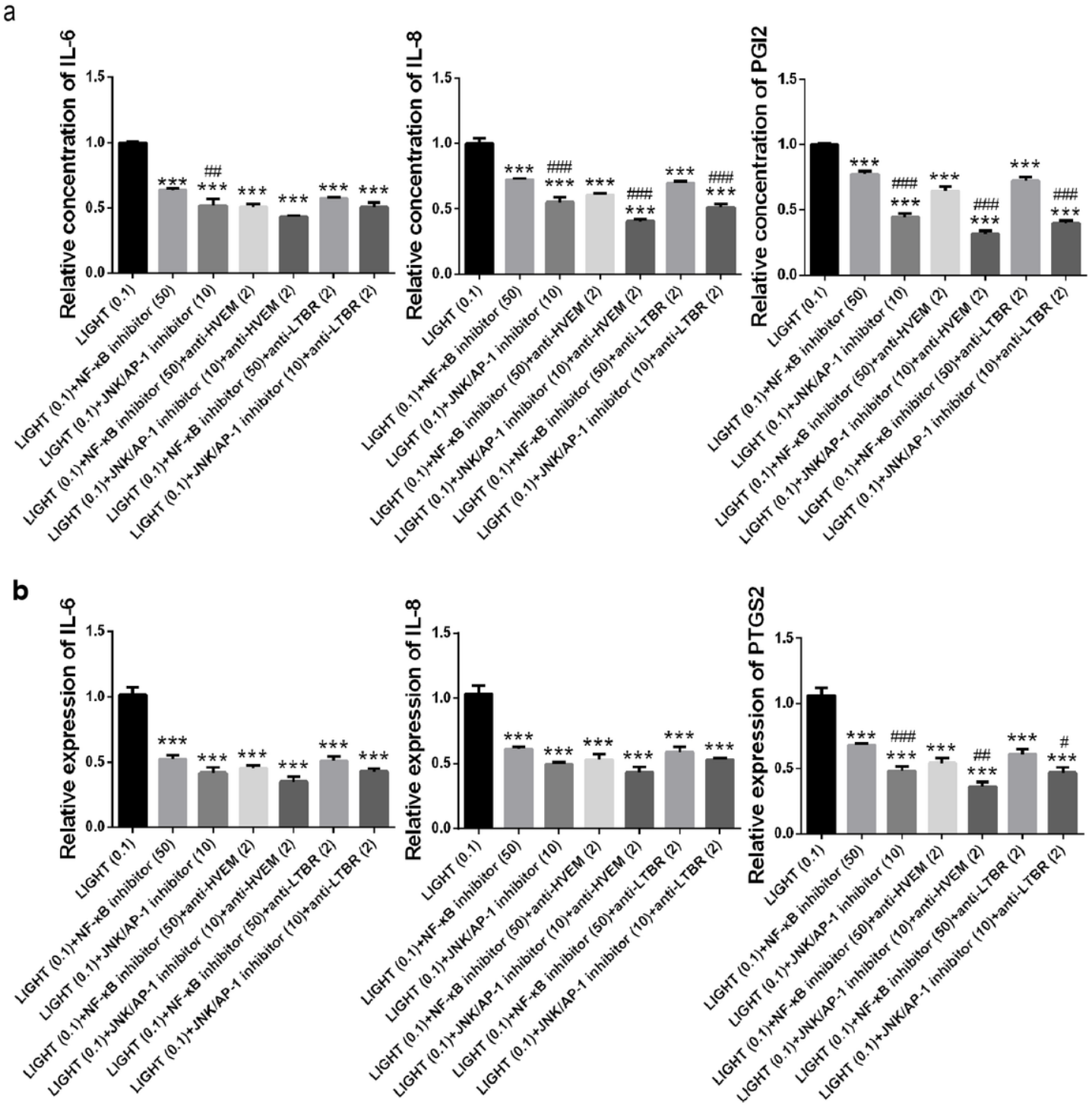

Figure 4

The expression of IL-6, IL-8, PGI2, and PTGS2 of HaCat cells treated with different treatment for 24 hrs were shown in quantitative real-time results. ***P < 0.001 versus LIGHT group. # P□0.05, ##P□0.01,### P < 0.001 versus the former group.

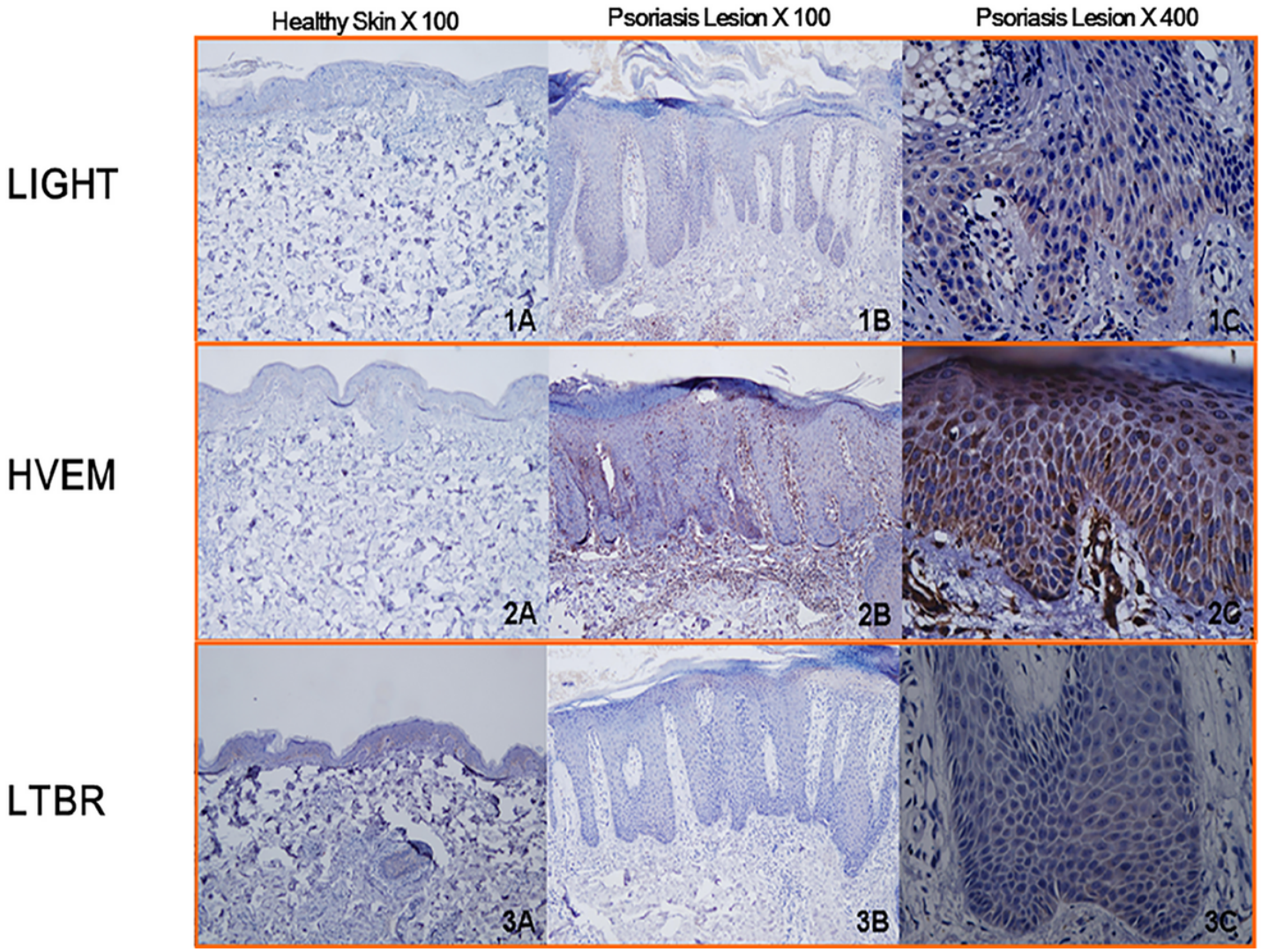

Figure 5

Skin was immunohistochemical stained of LIGHT, HVEM or LT-βR ,Original magnification×100 and×400.