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TITLE:

Establishing Transcription Profile of Psoriasiform Cutaneous In Vitro Using HaCaT Cells Stimulated with Combination of Cytokines

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KEYWORDS:
psoriasis, psoriasiform inflammation, HaCaT, skin inflammation, skin barrier, keratinocyte differentiation, cytokines, chemokines, antimicrobial peptides

SUMMARY:
This paper presents a method of establishing an in vitro psoriasiform cutaneous inflammatory model at the transcription level using a combination of five cytokines (IL-17A, IL-22, IL-1α, TNF-α, OSM) on HaCaT cell line.

ABSTRACT:
Psoriasis is a common chronic inflammatory skin disease mediated by innate and adaptive immune systems, characterized by abnormal proliferation and differentiation of epidermal keratinocytes and infiltration of inflammatory cells. Skin-specific keratinocytes are key participants in innate immunity, responding to immune cells and environmental stimulation, thereby serving an important role in the immunopathogenesis of psoriasis. Here, we present a method for inducing psoriasiform keratinocytes inflammation at transcription level with HaCaT cell line using five proinflammatory cytokines combination (M5 combination), including IL-17A, IL-22, IL-1α, TNF-α, and oncostatin M. Results demonstrate that M5 combination induced HaCaT cells showed increased levels of antimicrobial peptides (*BD2*, *S100A7*, *S100A8*, and *S100A9*), chemokines, and cytokines (*CXCL1*, *CXCL2*, *CXCL8*, *CCL20*, IL-1β, IL-6 and, IL-18). The mRNA levels of keratinocytes differentiation markers (*Keratin1*, *Keratin10*, *Filaggrin*, and *Loricrin*) were down regulated, which was consistent with the transcriptome data derived from psoriasis‐like keratinocytes. The method described here, therefore, establishes an in vitro psoriasiform cutaneous inflammation at transcription level and contributes to the research for molecular pathogenesis of psoriasis.

INTRODUCTION:
Psoriasis is a common non-contagious chronic inflammatory skin disease triggered by a dysregulated immune response, affecting the keratinocytes that predominantly form the epidermis[1], characterized by abnormally rapid multiplication of keratinocytes with hyperkeratosis and parakeratosis. Psoriasis affects about 3% of the world-wild population[2]. Disease burden is further increased by several comorbidities, including cardiovascular diseases and metabolic syndrome caused by the syndrome[3].

Epidermis is composed of five layers of keratinocytes and undergo morphological change with differentiation process: the stratum basal, stratum spinosum, stratum granulosum, stratum lucidum (found on palms and soles) and stratum corneum described here from the inner to outer surface[4]. Change in epidermis differentiation leads to a disturbed skin barrier, which is important for the pathogenesis of skin inflammatory diseases[5-8]. Keratinocytes play a vital role in maintaining an intact epidermal barrier to prevent water loss and against environmental triggers

such as UVB exposure, allergens, and pathogens[9]. Healthy individuals show a balance between basal cells proliferation and stratum corneum desquamation, while multiple skin diseases including psoriasis, are characterized by an imbalances of this complex mechanism[10].

In addition to forming barrier function, keratinocytes are also a critical component of skin's immune system. In the immunopathogeneses of psoriasis, activation of skin-resident Type 1 helper T cells (Th1) and Type 17 helper T cells (Th17), leads to increased production of IFN-γ and IL-17A, respectively. These cytokines induce increased synthesis of chemokines (CCL20, CXCL1/2/8/9/10/11), antimicrobial peptides (BD2, LL37, S100A7/8/9/12), and other inflammatory factors (TNF-α, IL-6, IFN-β) in keratinocytes, leading to the recruitment of more Th1, Th17, and neutrophils into the skin, further amplifying the IL-17/IL23 axis[11]. The crosstalk between keratinocytes and immune cells is responsible for the induction and maintenance of psoriasis[11].

Complex cytokine networks have been described in psoriasis, and the central role of pro-inflammatory cytokines (such as IL-23, IL-22, IL-17, IL-1α, oncostatin M(OSM), and TNF-α) produced by immune cell infiltration has been highlighted[12,13]. Indeed, previous studies have shown that increased levels of IL-17A, IL-22, IL-1α, TNF-α, and OSM induced a profile of psoriasiform on normal human epidermal keratinocytes in vitro[14].

Immortal keratinocyte cell lines (HaCaT) that are more easily obtained and cultured than primary keratinocytes with better reproducibility, have been widely used for the study of psoriasis[15-20]. Different from human papillomavirus16 E6/E7 transformed HEK001 and KerTr cells, HaCaT cell line is capable of expressing differentiation gene products, including *Keratin1* (*KRT1*), *Keratin10* (*KRT10*), *Loricrin*, and *filaggrin*[20-22], thereby providing a promising tool similar to primary keratinocytes to study the regulation of keratinization and proinflammatory.

KRT5/14 is the major type I-type II keratin pair expressed in proliferative basal keratinocytes, whereas differentiated keratinocytes in the suprabasal layers downregulate KRT5/14 and express KRT1/10 as the major keratin pair[23]. Upon comparison of psoriasis lesion with healthy skin, the changes in keratin expression included decreases in KRT1/10[24,25] and increases in KRT5/14 in the psoriatic epidermis[26], characterized by hyperproliferation and parakeratosis[27]. Loricrin is a terminally differentiating structural protein comprising more than 70% of the cornified envelope, contributes to the protective barrier function of the stratum corneum[28], down regulated in the skin of psoriasis patients[29]. Filaggrin is expressed at the final stages of keratinocyte differentiation and is involved in the aggregation of a scaffold-like cornified envelope[30], decreased expression in psoriasis lesion skin[29].

Overall, our goal was to generate inflammatory keratinocytes model in HaCaT cells using a cytokine combination that will be able to synergistically recapitulate some characteristics of

psoriasis skin lesions, including initiating an immune response, keratinocytes proliferation and differentiation.

PROTOCOL:

Perform steps 1 to 3 under sterile condition. All the culture medium contained 0.1 mg/mL penicillin and streptomycin.

1. Cell preparation

1.1. Seed 1 x 10^6 HaCaT cells in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 100 mm cell culture dish. Incubate the culture dish at 37 °C in a humidified 5% CO_2 incubator for 2 days.

1.2. When the culture reaches around 80% confluency, carefully remove the medium from the culture dish and wash the cells with 5 mL of 1x phosphate buffered saline (PBS). Gently rock the culture dish manually.

1.3. Remove PBS and add 2 mL of 0.25% trypsin-EDTA solution to the cells. Gently shake the culture dish to let the solution completely coat the monolayer of cells.

1.4. Incubate the cells at 37 °C in a humidified 5% CO_2 incubator for 5 min until the cells are visibly detached from the surface of the culture dish under phase-contrast microscopy.

NOTE: The morphology of cells should appear round. If the cells are not well detached, incubate for an additional period of 5 min along with manual agitation.

1.5. Once the cells are detached, add 5 mL of DMEM plus 10% FBS to inactivate trypsin and collect the cell suspension in a 50 mL centrifuge tube using a pipette.

1.6. Centrifuge the tube containing cell suspension at 180 x *g* for 5 min. Decant the supernatant.

1.7. Add 10 mL of DMEM plus 10% FBS medium to the pellet and gently pipette the medium up and down to bring the cells into suspension.

2. Cell seeding in the 6-well plate

2.1. Seed the cells at a density of 2.0 x 10^5 cells/well in 6-well culture plate.

2.2. Incubate the 6-well culture plate at 37 °C in a humidified 5% CO_2 incubator overnight before M5 combination stimulation to let the cells adhere to the plate.

3. M5 stimulation of HaCaT cells

3.1. Prepare M5 cytokine combination mix medium containing 10 ng/mL of recombinant IL-17A, IL-22, IL-1α, TNF-α, and oncostatin M protein in DMEM containing 2% FBS.

NOTE: M5 combination consisted of recombination of IL-17A, IL-22, IL-1α, TNF-α, and oncostatin M. Synergistic action of M5 combination recapitulates some features of psoriasis, like upregulation of chemokines and antimicrobial peptides production[31].

3.2. After overnight cell culture, remove the supernatant, and pipette 2 mL of M5 combination mix medium into each well.

3.3. Continue culturing the cells; cell lysates were collected at 24 h for mRNA quantification (step 4) or culture supernatant were collected at 48–72 h for the determination of cytokine levels by ELISA assay[32-37] (step 6).

4. Harvest mRNA of M5 stimulated HaCaT cells

4.1. Aspirate the M5 combination mix medium and wash once with 1–2 mL of cold PBS.

4.2. Aspirate PBS and add 1 mL of commercially available guanidium Isothiocyanate solution directly to the culture dish to lyse the cells.

4.3. Pipette the lysate up and down several times to homogenize and transfer the cell lysate into a 1.5 mL microcentrifuge tube. Leave at room temperature for 5 min.

4.4. Add 200 µL of chloroform, shake the tube vigorously for about 20 s and incubate the sample for 5 min at room temperature.

4.5. Centrifuge at 12,000 x *g* for 10 min at 4 °C.

4.6. Transfer the aqueous phase to a fresh 1.5 mL microcentrifuge tube.

4.7. Add 500 µL of isopropanol to the aqueous phase and mix gently. Leave at room temperature for 5 min.

4.8. Centrifuge at 12,000 x *g* for 15 min at 4 °C.

4.9. Aspirate isopropanol and add 1 mL of 75% ethanol. Wash the pellet once.

4.10. Centrifuge at 7,500 x *g* for 5 min at 4 °C. Pour off the ethanol and let the pellets air-dry.

4.11. Add 30 µL DEPC treated water to the RNA pellet. Prepare for RT-PCR detection.

5. Analysis of mRNA expression by Real-Time PCR

5.1. Perform cDNA synthesis with 1 µg of total RNA using a commercially available kit following the manufacturer's instruction.

NOTE: 1 µg of total RNA for SYBR Green RT-PCR assay.

5.2. Prepare the PCR reaction mixture. 12.5 µL of SYBR premix EX Taq II, 1µL of PCR Forward Primer (10 µM), 1 µL of PCR Reverse Primer (10 µM), 2 µg of cDNA, and 8.5 µL of dH_2O. The final volume is 25 µL per reaction.

NOTE: List of primer sequences of genes used for RT-PCR analysis in this study is shown in **Table 1**.

5.3. Incubate in thermocycler. The cycling conditions included a denaturing step at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72°C for 20s and a melting curve analysis.

5.4. Analyze RT-PCR experiment data by calculating relative differences in gene expression from Ct (threshold cycle) values using $2^{-\Delta\Delta CT}$ equations. Use β-actin as an internal control for RT-PCR relative quantitative gene expression standardization.

6. Harvesting cell culture supernatant for ELISA

6.1 Pipette each cell culture media into a 1.5 mL microcentrifuge tube.

6.2. Centrifuge at 1500 x *g* for 10 min at 4 °C.

6.3. Aliquot the supernatant and store at -80 °C immediately.

7. Analysis of cytokines expression by ELISA

7.1. Follow the manufacturer's instruction to prepare all reagents, working standard, and samples. A three-fold serial dilution of standard with sample diluent ranging from 2,000 to 2.74 pg/mL. Dilute samples 1:2 with sample diluent.

7.2. Add 100 µL of standard and sample per well. Cover wells with lid and seal the plate with paraffin film. Incubate for 2.5 h at room temperature.

7.3. After 2.5 h, discard the solution. Add 300 µL of wash buffer to each well of the plate for 3 min and aspirate. Repeat the process for four additional times. After the last wash, invert the plate and tap on absorbent paper to remove remaining liquid.

7.4. Add 100 µL of the detection antibody solution to each well. Incubate the plate on a shaker for 2 h at 37 °C.

NOTE: Biotinylated detection antibody solution is prepared in each ELISA kits.

7.5. Aspirate and wash each well of plate five times using 300 µL of Wash buffer. After the last wash, invert the plate and tap on absorbent paper to remove the remaining liquid.

7.6. Add 100 µL of HRP-Streptavidin conjugate to each well. Incubate for 45 min at room temperature with gentle shaking.

7.7. Discard the solution by aspirating with a pipette after the incubation. Wash each well of plate five times using 300 µL of Wash buffer. After the last wash, invert the plate and tap on the absorbent paper to remove the remaining liquid.

7.8. Perform detection by adding 100 µL of TMB substrate to each well. Incubate for 30 min at 37 °C in the dark.

7.9. Add 50 µL of stop solution, when color develops, to each well. Gently tap the plate to ensure thorough mixing. Read at 450 nm immediately.

REPRESENTATIVE RESULTS:

M5 combination stimulation induced inflammatory response of HaCaT cells.

HaCaT cells were stimulated with or without M5 cytokines combination for 24 h. mRNA expression of psoriasis-related genes, which are involved in the regulation of the immune and inflammatory chemokines and antimicrobial peptides, were evaluated. Neutrophil chemokines *CXCL1*[38], *CXCL2*[39], *CXCL8*[40], and T-cell chemokines *CCL20*[41,42] were significantly increased in M5 combination stimulated HaCaT cells compared to untreated HaCaT cells in a time-dependent manner (**Figure 1A**). Expression of *BD2*[43], *S100A7*[44,45], *S100A8*[46], *S100A9*[46], and *S100A12*[47,48] , as antimicrobial peptides were barely detected in unstimulated HaCaT cells and were strongly expressed after the M5 combination stimulation with time-dependent expression pattern (**Figure 2A**). Further, microarray expression of psoriasiform cutaneous inflammation in HaCaT was also performed using Human Expr 12x135K Arr Del[49]. Cytokines, chemokines (**Figure 1B**) and antimicrobial peptides (**Figure 2B**) were found to be upregulated in M5 combination stimulated HaCaT. Protein levels of IL-6, IL-1β, IL-18, and CXCL8[20,50-54] were studied via ELISA as a confirmation. Higher protein levels were observed in supernatant of M5 combination stimulated

HaCaT cells compared to control cells (**Figure 1C**). Meanwhile, to evaluate the pathophysiological correlation of M5 combination induced psoriasiform cutaneous inflammation on HaCaT cells, microarray dataset (GDS4602)[55] from lesion skin biopsy sample of normal and patients with psoriasis were downloaded from gene expression omnibus (GEO). We explored the expression levels of several proinflammatory chemokines, cytokines, and antimicrobial peptides. The results showed that proinflammatory chemokines *CXCL1*, *CXCL2*, *CXCL8*, and *CCL20* and antimicrobial peptides *BD2*, *S100A7*, *S100A8*, *S100A9*, and *S100A12* over-expressed in psoriatic skin as compared with normal skin (**Supplementary Table 1**). Taken together, these results indicated that M5 combination stimulation in HaCaT cells contributed to establishing psoriasiform inflammation in vitro, with increased proinflammatory chemokines, and antimicrobial peptides production at the transcription level. However, further studies are needed to establish this as a model for psoriasiform inflammation model.

M5 combination stimulation reduced keratinocyte differentiation level in HaCaT cells

HaCaT as an immortal keratinocyte cell lines, retained functional differentiation proprieties of normal keratinocytes, showed normal keratinization and stratification grown in organotypic cultures[56,57] and normal morphogenesis transformation to differentiation features in high calcium concentration culture medium[57-59]. mRNA levels of *KRT5* and *KRT14*, as cytokeratin associated with proliferating keratinocytes, were increased in M5 stimulated HaCaT (**Figure 3A**). To further explore the effect of M5 combination stimulation on the differentiation level of keratinocytes, mRNA expression of keratinocyte differentiation markers was evaluated. *KRT1* and *KRT10*, which are among the first to be expressed during cornification as early differentiation makers, were significantly down-regulated (**Figure 3A**). Meanwhile, mRNA levels of *Loricrin* and *Filaggrin* as late differentiation markers, which are the main components of the epidermal cornified envelope, were significantly down-regulated (**Figure 3A**). Microarray expression profiling also upregulation of keratinocyte proliferation marker *KRT5* and *KRT6*, while differentiation marker *KRT1*, *KRT10*, *Loricrin* and *Filaggrin* were down-regulated (**Figure 3B**). This is similar to studies performed in human biopsy where compared to normal skin, GEO profile of patients with psoriatic skin lesion (GDS4602)[60] showed decreased KRT10, Filaggrin, and Loricrin expression (**Supplementary Table 1**). In summary, M5 combination decreases keratinocyte differentiation marker expression in HaCaT cell line.

FIGURE AND TABLE LEGENDS:

Figure 1: Chemokines genes sustained increase in HaCaT cells cultured with combination of IL-17A, IL-22, IL-1α, TNF-α, and OSM (M5 combination). HaCaT cells were cultured in M5 combination for 6, 12, and 24 hours. QRT-PCR analysis was carried out and mRNA expression levels for *CXCL1*, *CXCL2*, *CXCL8*, and *CCL20* were normalized using β-actin housekeeping gene and expressed as the fold increased above unstimulated control (**A**), Heatmap analysis of cytokines and chemokines expression profiles between control and M5 stimulated HaCaT (**B**). Levels of IL-6, IL-1β, IL-18, and CXCL8 in the culture supernatant of HaCaT cells stimulated by M5 for 96 h

were measured by ELISA (**C**). Mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. Two-tailed Student's *t*-test. Data represents three independent experiments.

Figure 2: Antimicrobe peptide genes sustained increased in HaCaT cells cultured with M5 combination. HaCaT cells were cultured in M5 combination for 6, 12, and 24 hours. QRT-PCR analysis was carried out and mRNA expression levels for *BD2*, *S100A7*, *S100A8*, *S100A9*, and *S100A12* were normalized using β-actin housekeeping gene and expressed as the fold increased above unstimulated control (**A**), Heatmap analysis of antimicrobial peptides expression profiles between control and M5 stimulated HaCaT (**B**). Mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. Two-tailed Student's *t*-test. Data represents three independent experiments.

Figure 3: Keratinocyte differentiation genes sustained decreased in HaCaT cells cultured with M5 combination. HaCaT cells were cultured in M5 combination for 6, 12, and 24 hours. QRT-PCR analysis was carried out and mRNA expression levels for *KRT1*, *KRT10*, *Filaggrin*, and *Loricrin* were normalized using β-actin housekeeping gene and expressed as the fold increased above unstimulated control (**A**), Heatmap analysis of keratinocytes proliferation and differentiation markers expression profiles between control and M5 stimulated HaCaT (**B**). Mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. Two-tailed Student's *t*-test. Data represents three independent experiments.

Table 1: List of primer sequences of genes used for RT-PCR analysis

Supplementary Table 1: GEO profile of psoriatic lesion skin versus normal skin. GEO profile (GSD4602) data were generated from skin punch biopsies of 58 normal skin and 64 psoriasis lesion skin. Ratio between psoriasis lesion skin and normal skin were calculated using Prism 8 software. The p values were analyzed using the Mann-Whitney U-test.

DISCUSSION:

Described herein is a method using five cytokines combination (IL-17A, IL-22, IL-1α, TNF-α, OSM) into HaCaT cell line to establish an in vitro psoriasiform cutaneous inflammation profile at transcription level. This protocol can be adapted for the study on the mechanism of genes in the pathogenesis of psoriasis as well as the screening of therapeutic drugs for psoriasis. Recent reports have shown that overexpression of IL-17A and IL22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis[61]. IL-1α, IL-22, and IL-17A can induce skin inflammation in animal models[62-65]. Cytokines capable of inducing specific expression patterns associated with antimicrobial peptides produced, such as IL-17 and IL-22[66] or innate immune response, such as IL-17, IL-1α, and TNF-α[11,62,67] or keratinocyte differentiation programs, such as IL-22 or OSM[68,69]. Our results confirmed IL-17A, IL-22, IL-1α, TNF-α, and OSM as major skin inflammation inducers and demonstrated a powerful synergy on the expression of chemokines, cytokines, and antimicrobial peptides in HaCaT cell line.

Notably, M5 combination induced the expression of HaCaT cells neutrophils attracting chemokines CXCL1, CXCL2, CXCL8, and recruitment of $CCR6^+$ Th17 cells chemokines CCL20, which play a decisive role in the initiation and maintenance of psoriasis[70-72]. Antimicrobial peptides are suspected of modifying host inflammatory responses by a variety of mechanisms including action as angiogenic factors, chemotactic agents, and regulators of cell proliferation, have been implicated in the development of psoriasis in recent years[73]. S100A8 and S100A9 are also involved in the recruitment of inflammatory infiltrates[74,75]. S100A8 and S100A9 augments the production of CXCL1, CXCL2, CXCL8, and CCL20 in keratinocytes[76]. BD2 has chemokine activity and may recruit memory T cells and immature dendritic cells to the site of microbial invasion through interaction with CCR6[77]. S100A12 has chemotaxis on mast cells and monocytes[78]. Antimicrobial peptides expressed in M5 stimulated HaCaT psoriasiform in vitro model is essential to the nature defenses of the skin and are also conductive to promoting the infiltration of inflammatory cells.

Our data showed significantly decreased expression of early (*KRT1* and *KRT10*) and late (*Filaggrin* and *Loricrin*) differentiation genes after M5 combination stimulation in HaCaT cells (**Figure 2**). Psoriasis is characterized by uncontrolled increased proliferation rate and poor differentiation[79]. GWAS have identified numerous genetic variations associated with psoriasis, comprising many genes of crucial importance for keratinocyte differentiation[80]. Psoriasis keratinocytes RNA-seq identified decreased differentially expressed genes were enriched for genes associated with epidermal differentiation[81]. Psoriasis keratinocytes showed decreased expression of early (*KRT1*, *KRT10*, *DSC1*) and late (*LOR*, *FLG*, *IVL*) differentiation genes[81]. KRT1 and KRT10 are directly involved in cell cycle control which onsets keratinocytes differentiation[82]. Mutation of KRT1/10 or absence of KRT10 showed greater epidermal proliferation in the basal layer[83,84]. Loss-of-function mutation (p.K4022X) in filaggrin gene is associated with psoriasis[85]. Loricrin, as a candidate for the PSORS4 locus, mutation deranges the keratinocyte differentiation and delays the cell death process[86]. Decreased filaggrin and loricrin expressions by keratinocytes in psoriasis lesion skin were observed[87]. In addition, S100A8/A9 stimulates keratinocytes proliferation through induced phosphorylation of p38 and SAPK/JNK followed by activation of ERK1/2[88]. Together, the mRNA expression pattern of keratinocytes differentiation process stimulated by M5 combination on HaCaT cells is consistent with that of psoriatic skin lesions.

In this protocol, HaCaT cell line was used to establish psoriasiform cutaneous inflammatory models in vitro as they are easy to obtain and culture with good stability and reproducibility. Although HaCaT cells have been widely used in psoriasis studies[15-19], it has been reported that the gene transcriptional profile of cornified envelope-associated proteins in HaCaT cells was generally different from that in primary keratinocytes, suggest HaCaT cells have a limitation as a model to study normal skin barrier development[89,90]. Meanwhile, the keratin profile expressed by HaCaT is much broader than that normally seen in primary keratinocyte cultures, including keratins associated with simple epithelium[19]. In our previous study, Gene ontology (GO) enrichment analysis HaCaT cells stimulated with M5 showed a similar genes enrichment in

transcriptome data with lesional skin and epidermis of psoriasis patients. In the differentially expressed genes (DEGs) of psoriasis-like HaCaT cells, we found DEGs pattern in HaCaT cells stimulated with M5 are correlated with pathogenies of psoriasis (GSE54456, GSE26866)[91]. Meanwhile, immunohistochemistry patterns of chemokines[40,92,93], antimicrobial peptides[46,94-97] and keratinocytes differentiation markers[97-100] in psoriasis patients were consistent with GEO database. Therefore, HaCaT cells stimulated with M5 as an in vitro psoriasiform cutaneous inflammatory model were similar to primary keratinocytes.

Notably, the activity of recombinant cytokines is critical to the success of the experiment. Always make working aliquots of dissolved recombinant cytokines and avoid freeze/thaw cycle are necessary. Meanwhile, we found stronger psoriasiform cutaneous inflammatory in M5 stimulated HaCaT with culture medium plus 2% FBS compared to 10% FBS. In addition, identifying the expression of proinflammatory chemokines is necessary to detect whether the M5 induced psoriasiform model is successful before screening potential drugs or studies on the mechanism of psoriasis.

Here, we have established a M5 combination that stimulates HaCaT cells psoriasiform cutaneous inflammatory model, showing consistent expression of inflammatory mediators and reduced levels of keratinocyte differentiation in patients with psoriasis, demonstrating the advantages of studying the synergistic effects of cytokines on pathophysiological condition in vitro. IL-17A, IL22, IL-1α, TNF-α, and OSM synergistic stimulation could be a novel valuable strategy to identify the key pathogenic tissue-specific molecules for disease and represent potential biomarker or drug target for development of future therapeutics of psoriasis.

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DISCLOSURES:

No potential conflict of interest was reported by the authors.

REFERENCES

1. Lowes, M. A., Bowcock, A. M., Krueger, J. G. Pathogenesis and therapy of psoriasis. *Nature.* **445** (7130), 866-873 (2007).

2. Gupta, R., Debbaneh, M. G., Liao, W. Genetic epidemiology of psoriasis. *Current dermatology reports.* **3** (1), 61-78 (2014).

3. Boehncke, W.-H., Schön, M. P. Psoriasis. *Lancet (London, England).* **386** (9997), 983-994 (2015).

4. Zaidi, Z., Lanigan, S. W. in *Dermatology in Clinical Practice.* Editors: Lanigan, W. S., Zaidi, Z. 1-15. Springer London. (2010).

5. Proksch, E., Brandner, J. M., Jensen, J. M. The skin: An indispensable barrier. *Experimental Dermatology.* **17** (12), 1063-1072 (2008).
6. Van Smeden, J., Janssens, M., Gooris, G., Bouwstra, J. The important role of stratum corneum lipids for the cutaneous barrier function. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids.* **1841** (3), 295-313 (2014).
7. Wolf, R., Orion, E., Ruocco, E., Ruocco, V. Abnormal epidermal barrier in the pathogenesis of psoriasis. *Clinics in Dermatology.* **30** (3), 323-328 (2012).
8. Proksch, E., Brasch, J. Abnormal epidermal barrier in the pathogenesis of contact dermatitis. *Clinics in Dermatology.* **30** (3), 335-344 (2012).
9. Bäsler, K., Brandner, J. M. Tight junctions in skin inflammation. *Pflügers Archiv-European Journal of Physiology.* **469** (1), 3-14 (2017).
10. Hoffjan, S., Stemmler, S. On the role of the epidermal differentiation complex in ichthyosis vulgaris, atopic dermatitis and psoriasis. *British Journal of Dermatology.* **157** (3), 441-449 (2007).
11. Lowes, M. A., Russell, C. B., Martin, D. A., Towne, J. E., Krueger, J. G. The IL-23/T17 pathogenic axis in psoriasis is amplified by keratinocyte responses. *Trends in Immunology.* **34** (4), 174-181 (2013).
12. Saxena, A., Raychaudhuri, S. K., Raychaudhuri, S. P. in *Psoriatic Arthritis and Psoriasis* Springer. 73-82 (2016).
13. Lowes, M. A., Suárez-Fariñas, M., Krueger, J. G. Immunology of psoriasis. *Annual Review of Immunology.* **32**, 227-255 (2014).
14. Guilloteau, K. et al. Skin inflammation induced by the synergistic action of IL-17A, IL-22, oncostatin M, IL-1α, and TNF-α recapitulates some features of psoriasis. *The Journal of Immunology.* **184** (9), 5263-5270 (2010).
15. Wang, Z. et al. Autophagy-based unconventional secretion of HMGB1 by keratinocytes plays a pivotal role in psoriatic skin inflammation. *Autophagy.* 1-24 (2020).
16. Choi, D. H., Hwang, H. S. Anti-inflammation activity of brazilin in TNF-α induced human psoriasis dermatitis skin model. *Applied Biological Chemistry.* **62** (1), 46 (2019).
17. Teng, X. et al. IL-37 ameliorates the inflammatory process in psoriasis by suppressing proinflammatory cytokine production. *Journal of Immunology.* **192** (4), 1815-1823 (2014).
18. Wu, S. et al. The potential of Diosgenin in treating psoriasis: Studies from HaCaT keratinocytes and imiquimod-induced murine model. *Life Sciences.* **241** 117115, (2020).
19. Katarzyna, B., Elwira, S., Marta, M., Joanna, J.-B., Magdalena, G.-C. Models in the Research Process of Psoriasis. *International Journal of Molecular Ences.* **18** (12), 2514 (2017).
20. Auewarakul, P., Gissmann, L., Cid-Arregui, A. Targeted expression of the E6 and E7 oncogenes of human papillomavirus type 16 in the epidermis of transgenic mice elicits generalized epidermal hyperplasia involving autocrine factors. *Molecular and Cellular Biology.* **14** (12), 8250-8258 (1994).
21. Boukamp, P. et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *Journal of Cell Biology.* **106** (3), 761-771 (1988).

22. Irma, C. et al. HaCaT cells as a reliable in vitro differentiation model to dissect the inflammatory/repair response of human keratinocytes. *Mediators of Inflammation.* **2017**, 7435621 (2017).
23. Melino, G. et al. The cornified envelope: a model of cell death in the skin. *Results & Problems in Cell Differentiation.* **24** (4), 175 (1998).
24. Thewes, M., Stadler, R., Mischke, B. K. Normal psoriatic epidermis expression of hyperproliferation-associated keratins. *Archives of Dermatological Research.* (1991).
25. Ishida-Yamamoto, A., Senshu, T., Takahashi, H., Akiyama, K., Iizuka, H. Decreased deiminated keratin K1 in psoriatic hyperproliferative epidermis. *Journal of investigative Dermatology.* **114** (4), 701-705, (2000).
26. Tamilselvi et al. Methotrexate normalized keratinocyte activation cycle by overturning abnormal keratins as well as deregulated inflammatory mediators in psoriatic patients. *Clinica Chimica Acta.* 451B, 329–337 (2015).
27. Ghadially, R., Reed, J. T., Elias, P. M. Stratum corneum structure and function correlates with phenotype in psoriasis. *Journal of investigative dermatology.* **107** (4), 558-564 (1996).
28. Nithya, S., Radhika, T., Jeddy, N. Loricrin - An overview. *Journal of Oral and Maxillofacial Pathology.* **19** (1), (2015).
29. Kim, B. E. et al. TNF-α downregulates filaggrin and loricrin through c-Jun N-terminal Kinase: Role for TNF-α antagonists to improve skin barrier. *Journal of Allergy & Clinical Immunology.* **127** (2), AB148-AB148 (2011).
30. Gutowska-Owsiak, D. et al. IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. *Experimental dermatology.* **21** (2), 104-110 (2012).
31. Guilloteau, K. et al. Skin inflammation induced by the synergistic action of IL-17A, IL-22, oncostatin M, IL-1α, and TNF-α recapitulates some features of psoriasis. *Journal of Immunology.* **184** (9), 5263-5270 (2010).
32. Jeon, B. et al. Optimization and validation of a method to identify skin sensitization hazards using IL-1 α and IL-6 secretion from HaCaT. *Toxicology In Vitro : An International Journal Published in Association with BIBRA.* **61** 104589, (2019).
33. Lee, K.-M., Kang, J. H., Yun, M., Lee, S.-B. Quercetin inhibits the poly(dA:dT)-induced secretion of IL-18 via down-regulation of the expressions of AIM2 and pro-caspase-1 by inhibiting the JAK2/STAT1 pathway in IFN-γ-primed human keratinocytes. *Biochemical and Biophysical Research Communications.* **503** (1), 116-122 (2018).
34. Lee, N., Chung, Y. C., Kang, C. I., Park, S.-M., Hyun, C.-G. 7,8-dimethoxycoumarin attenuates the expression of IL-6, IL-8, and CCL2/MCP-1 in TNF-α-treated HaCaT cells by potentially targeting the NF-κB and MAPK pathways. *Cosmetics.* **6** (3), (2019).
35. Smolińska, E., Moskot, M., Jakóbkiewicz-Banecka, J., Wgrzyn, G., Gabig-Cimińska, M. Molecular action of isoflavone genistein in the human epithelial cell line HaCaT. *PLoS One.* **13** (2), e0192297 (2018).
36. Ying, X., Yan, Y., Li, Y. Tripterine alleviates LPS-induced inflammatory injury by up-regulation of miR-146a in HaCaT cells. *Biomedicine & Pharmacotherapy.* **105**, 798 (2018).

37. Zhang, M. et al. AIM2 inflammasome mediates Arsenic-induced secretion of IL-1 β and IL-18. *Oncoimmunology.* **5** (6) e1160182 (2016).
38. Lowes M. A. et al. Immunology of Psoriasis. *Annual Review of Immunology.* **32**, 227–255 (2014).
39. Nedoszytko , B., Wojdyło, M. -S, Dziurdzińska, K. -S., Roszkiewicz , J., Nowicki, R. J . Chemokines and cytokines network in the pathogenesis of the inflammatory skin diseases: atopic dermatitis, psoriasis and skin mastocytosis. *Postepy Dermatologii i Alergologii*. **31** 84-91, (2014).
40. Glowacka, E., Lewkowicz, P., Rotsztejn, H., Zalewska, A. IL-8, IL-12 and IL-10 cytokines generation by neutrophils, fibroblasts and neutrophils- fibroblasts interaction in psoriasis. *Advances in Medical Sciences.* **55** (2), 254-260 (2010).
41. Li, H. et al. Cyr61/CCN1 induces CCL20 production by keratinocyte via activating p38 and JNK/AP-1 pathway in psoriasis. *Journal of Dermatological Science*. 88 (1), 46–56 (2017).
42. Kim, T. G. et al. Dermal clusters of mature dendritic cells and T cells are associated with the CCL20/CCR6 chemokine system in chronic psoriasis. *Journal of Investigative Dermatology.* **134** (5), 1462-1465 (2014).
43. Kolbinger, F. et al. β-Defensin 2 is a responsive biomarker of IL-17A-driven skin pathology in patients with psoriasis. *The Journal of Allergy and Clinical Immunology.* **139** (3) (2017).
44. Ekman, A., Vegfors, J., Eding, C., Enerbäck, C. Overexpression of psoriasin (S100A7) contributes to dysregulated differentiation in psoriasis. *Acta Dermato-Venereologica.* **97** (4), 441-448 (2016).
45. Peric, M. et al. Vitamin D analog calcipotriol suppresses the Th17 cytokine–induced proinflammatory S100 "Alarmins" psoriasin (S100A7) and Koebnerisin (S100A15) in psoriasis. *Journal of Investigative Dermatology.* **132** (5), 1416-1424 (2012).
46. Aochi, S. et al. Markedly elevated serum levels of calcium-binding S100A8/A9 proteins in psoriatic arthritis are due to activated monocytes/macrophages. *Journal of the American Academy of Dermatology.* **64** (5), 879-887 (2011).
47. Krueger, J. G. et al. IL-17A is essential for cell activation and inflammatory gene circuits in subjects with psoriasis. *Journal of Allergy Clinical Immunology.* **130** (1), 145-154 (2012).
48. Wilsmann-Theis, D. et al. Among the S100 proteins, S100A12 is the most significant marker for psoriasis disease activity. *Journal of the European Academy of Dermatology & Venereology.* **30** (7), 1165-1170 (2016).
49. Wang, Z. et al. Systematic screening and identification of novel psoriasis‐specific genes from the transcriptome of psoriasis‐like keratinocytes. *Molecular Medicine Reports.* **19** (3), 1529–1542 (2018).
50. Mizutani, H., Ohmoto, Y., Mizutani, T., Murata, M., Shimizu, M. Role of increased production of monocytes TNF-alpha, IL-1beta and IL-6 in psoriasis: relation to focal infection, disease activity and responses to treatments. *Journal of dermatological science.* **14** (2), 145-153 (1997).

51. Pietrzak, A., Lecewicz-Torun, B., Chodorowska, G., Rolinski, J. Interleukin-18 levels in the plasma of psoriatic patients correlate with the extent of skin lesions and the PASI score. *Acta Dermato-Venereologica.* **83** (4), 262-265, (2003).
52. Cai, Y. et al. A critical role of the IL-1β-IL-1R signaling pathway in skin inflammation and psoriasis pathogenesis. *Journal of investigative Dermatology*. **139** (1), 146–156 (2018).
53. Arican, O., Aral, M., Sasmaz, S., Ciragil, P. Serum levels of TNF-α, IFN-γ, IL-6, IL-8,IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators of Inflammation*. **2005** (5), 273–279 (2005).
54. Grossman, R. M. et al. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proceedings of the National Academy of Sciences of the United States of America*. **86** (16), 6367–6371 (1989).
55. Nair, R. P. et al. Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. *Nature Genetics.* **41** (2), 199-204 (2009).
56. Boukamp, P. et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *The Journal of Cell Biology.* **106** (3), 761-771 (1988).
57. Deyrieux, A. F., Wilson, V. G. In vitro culture conditions to study keratinocyte differentiation using the HaCaT cell line. *Cytotechnology.* **54** (2), 77-83 (2007).
58. Micallef, L. et al. Effects of extracellular calcium on the growth-differentiation switch in immortalized keratinocyte HaCaT cells compared with normal human keratinocytes. *Experimental Dermatology.* **18** (2), 143-151 (2009).
59. Wilson, V. G. in *Epidermal Cells*. Springer. 33-41 (2013).
60. Ding, J. et al. Gene expression in skin and lymphoblastoid cells: Refined statistical method reveals extensive overlap in cis-eQTL signals. *American Journal of Human Genetics.* **87** (6), 779-789 (2010).
61. Res, P. C. et al. Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis. *PLoS One.* **5** (11), (2010).
62. Groves, R. W., Mizutani, H., Kieffer, J. D., Kupper, T. S. Inflammatory skin disease in transgenic mice that express high levels of interleukin 1 alpha in basal epidermis. *Proceedings of the National Academy of Sciences.* **92** (25), 11874-11878 (1995).
63. Chan, J. R. et al. IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2–dependent mechanisms with implications for psoriasis pathogenesis. *The Journal of Experimental Medicine.* **203** (12), 2577-2587 (2006).
64. Zheng, Y. et al. Interleukin-22, a TH 17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature.* **445** (7128), 648-651 (2007).
65. Ma, H.-L. et al. IL-22 is required for Th17 cell–mediated pathology in a mouse model of psoriasis-like skin inflammation. *The Journal of Clinical Investigation.* **118** (2), 597-607 (2008).
66. Lowes, M. A., Russell, C. B., Martin, D. A., Towne, J. E., Krueger, J. G. The IL-23/T17 pathogenic axis in psoriasis is amplified by keratinocyte responses. *Trends Immunol.* **34** (4), 174-181 (2013).

67. Banno, T., Gazel, A., Blumenberg, M. Effects of tumor necrosis factor-α (TNFα) in epidermal keratinocytes revealed using global transcriptional profiling. *Journal of Biological Chemistry.* **279** (31), 32633-32642 (2004).
68. Boniface, K. et al. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *The Journal of Immunology.* **174** (6), 3695-3702 (2005).
69. Boniface, K. et al. Oncostatin M secreted by skin infiltrating T lymphocytes is a potent keratinocyte activator involved in skin inflammation. *The Journal of Immunology.* **178** (7), 4615-4622 (2007).
70. Terui, T. Role of neutrophils in induction of acute inflammation in T-cell-mediated immune dermatosis, psoriasis: A neutrophil-associated inflammation-boosting loop. *Experimental Dermatology.* **9**, (2000).
71. Chiang, C.-C., Cheng, W.-J., Korinek, M., Lin, C.-Y., Hwang, T.-L. Neutrophils in psoriasis. *Frontiers in Immunology.* **10**, 2376 (2019).
72. Kennedy-Crispin, M. et al. Human keratinocytes' response to injury upregulates CCL20 and other genes linking innate and adaptive immunity. *The Journal of Investigative Dermatology.* **132** (1), 105-113 (2012).
73. Morizane, S., Gallo, R. L. Antimicrobial peptides in the pathogenesis of psoriasis. *The Journal of Dermatology.* **39** (3), 225-230 (2012).
74. Vandal, K. et al. Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. *The Journal of Immunology.* **171** (5), 2602-2609 (2003).
75. Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., Tessier, P. A. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *The Journal of Immunology.* **170** (6), 3233-3242 (2003).
76. Nukui, T. et al. S100A8/A9, a key mediator for positive feedback growth stimulation of normal human keratinocytes. *Journal of Cellular Biochemistry.* **104** (2), 453-464 (2008).
77. Yang, D. et al. β-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science.* **286** (5439), 525-528 (1999).
78. Hsu, K. et al. Anti-infective protective properties of S100 calgranulins. *Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Inflammatory and Anti-Allergy Agents).* **8** (4), 290-305 (2009).
79. Nestle, F. O., Kaplan, D. H., Barker, J. Psoriasis. *The New England journal of medicine.* **361** (5), 496-509 (2009).
80. Tsoi, L. C. et al. Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nature Genetics.* **44** (12), 1341 (2012).
81. Swindell, W. R. et al. RNA-seq identifies a diminished differentiation gene signature in primary monolayer keratinocytes grown from lesional and uninvolved psoriatic skin. *Scientific Reports.* **7** (1), 1-13 (2017).
82. Melino, G. et al. The cornified envelope: A model of cell death in the skin. **24** (4), 175 (1998).

83. Frost, P., Weinstein, G. D., Van Scott, E. J. The ichthyosiform dermatoses II. *Journal of Investigative Dermatology.* **47**, 561-567 (1966).
84. Reichelt, J., Furstenberger, G., Magin, T. M. Loss of keratin 10 leads to mitogen-activated protein kinase (MAPK) activation, increased keratinocyte turnover, and decreased tumor formation in mice. *Journal of Investigative Dermatology.* **123** (5), 973-981 (2004).
85. Hu, Z. et al. Loss-of-function mutations in filaggrin gene associate with psoriasis vulgaris in Chinese population. *Human Genetics.* **131** (7), 1269-1274 (2012).
86. Giardina, E. et al. Characterization of the loricrin (LOR) gene as a positional candidate for the PSORS4 psoriasis susceptibility locus. *Annals of Human Genetics.* **68** (6), 639-645 (2004).
87. Suga, H. et al. Skin barrier dysfunction and low antimicrobial peptide expression in cutaneous T-cell lymphoma. *Clinical Cancer Research.* **20** (16), 4339-4348 (2014).
88. Iotzova-Weiss, G. et al. S100A8/A9 stimulates keratinocyte proliferation in the development of squamous cell carcinoma of the skin via the receptor for advanced glycation-end products. *PLoS One.* **10** (3), (2015).
89. Seo, M.-D., Kang, T. J., Lee, C. H., Lee, A.-Y., Noh, M. HaCaT keratinocytes and primary epidermal keratinocytes have different transcriptional profiles of cornified envelope-associated genes to T helper cell cytokines. *Biomolecules & Therapeutics.* **20** (2), 171-176 (2012).
90. Soboleva, A. G. et al. Genetically predetermined limitation in the use of HaCaT cells that affects their ability to serve as an experimental model of psoriasis. *Genetika.* **50** (10), 1222-1231 (2014).
91. Wang, Z. et al. Systematic screening and identification of novel psoriasis‐specific genes from the transcriptome of psoriasis‐like keratinocytes. *Molecular Medicine Reports.* **19** (3), 1529-1542 (2019).
92. Inoue, K. et al. Mechanism underlying ATP release in human epidermal keratinocytes. *The Journal of Investigative Dermatology.* **134** (5), 1465-1468 (2014).
93. Harden, J. L*. et al.* CARD14 expression in dermal endothelial cells in psoriasis. *PLoS One.* **9** (11), e111255 (2014).
94. Krueger, J. G. et al. IL-17A is essential for cell activation and inflammatory gene circuits in subjects with psoriasis. *The Journal of Allergy and Clinical Immunology.* **130** (1), (2012).
95. Hegyi, Z. et al. Vitamin D analog calcipotriol suppresses the Th17 cytokine-induced proinflammatory S100 "alarmins" psoriasin (S100A7) and koebnerisin (S100A15) in psoriasis. *The Journal of Investigative Dermatology.* **132** (5), 1416-1424 (2012).
96. Schonthaler, H. B. et al. S100A8-S100A9 protein complex mediates psoriasis by regulating the expression of complement factor C3. *Immunity.* **39** (6), 1171-1181 (2013).
97. Wilsmann-Theis, D. et al. Among the S100 proteins, S100A12 is the most significant marker for psoriasis disease activity. *Journal of the European Academy of Dermatology and Venereology : JEADV.* **30** (7), 1165-1170 (2016).
98. Leuner, K. et al. Reduced TRPC channel expression in psoriatic keratinocytes is associated with impaired differentiation and enhanced proliferation. *PLoS One.* **6** (2), e14716 (2011).

99. Gao, L. et al. Ozone therapy promotes the differentiation of basal keratinocytes via increasing Tp63-mediated transcription of KRT10 to improve psoriasis. *Journal of Cellular and Molecular Medicine.* **24** (8), 4819-4829 (2020).
100. Kim, B. E. et al. TNF-α downregulates filaggrin and loricrin through c-Jun N-terminal kinase: role for TNF-α antagonists to improve skin barrier. *The Journal of Investigative Dermatology.* **131** (6), 1272-1279 (2011).

Figure 1

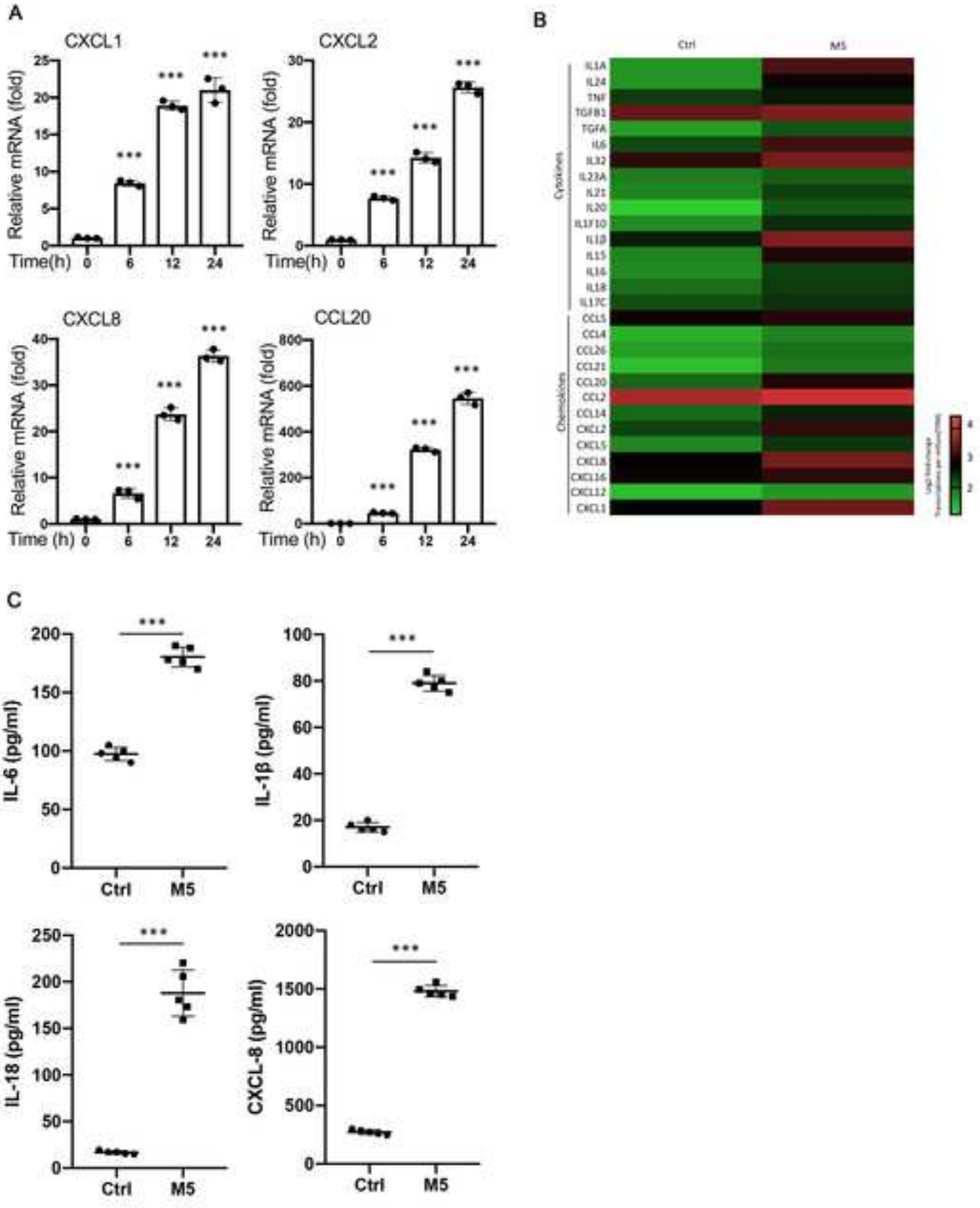


Figure 2

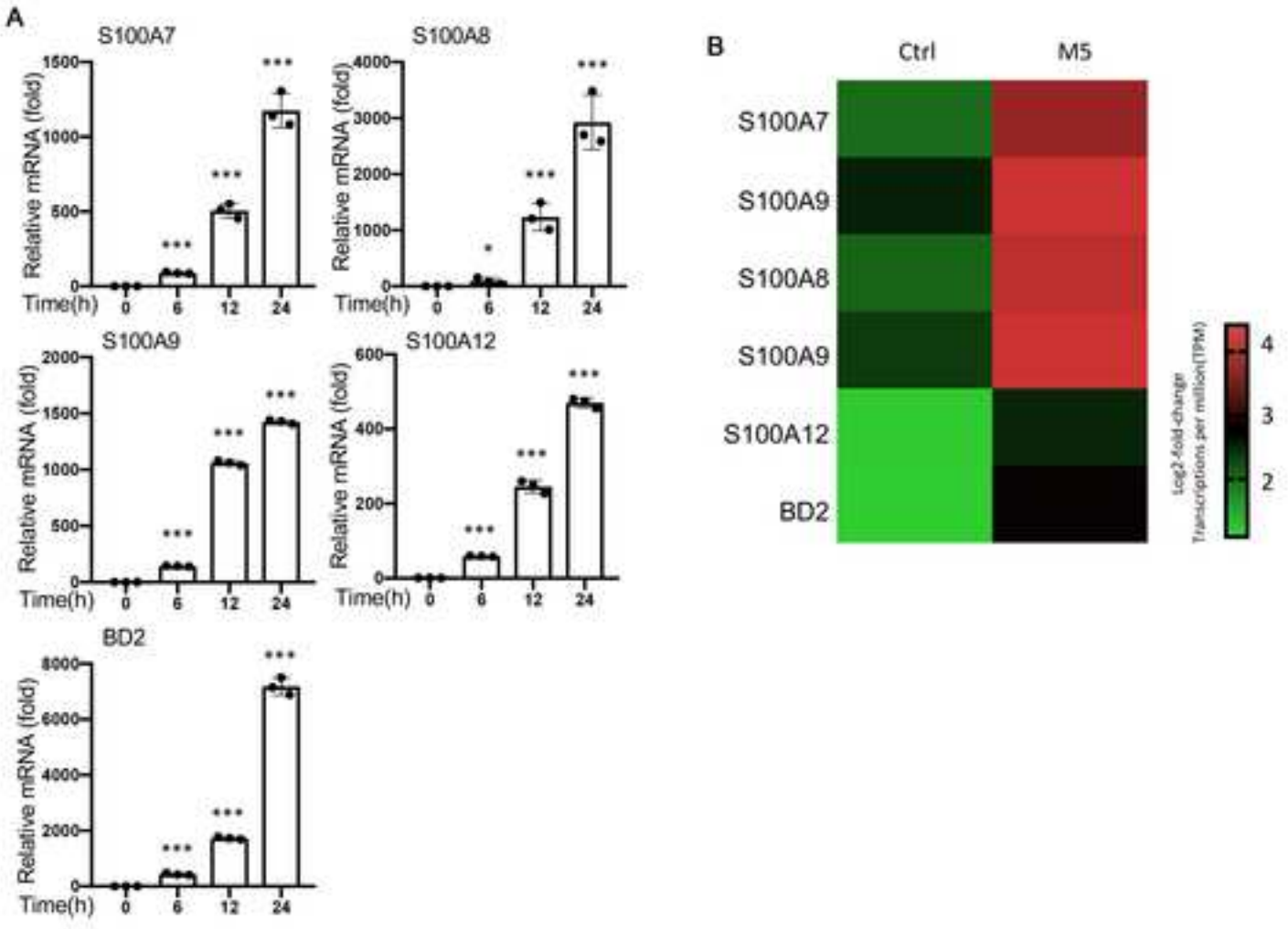
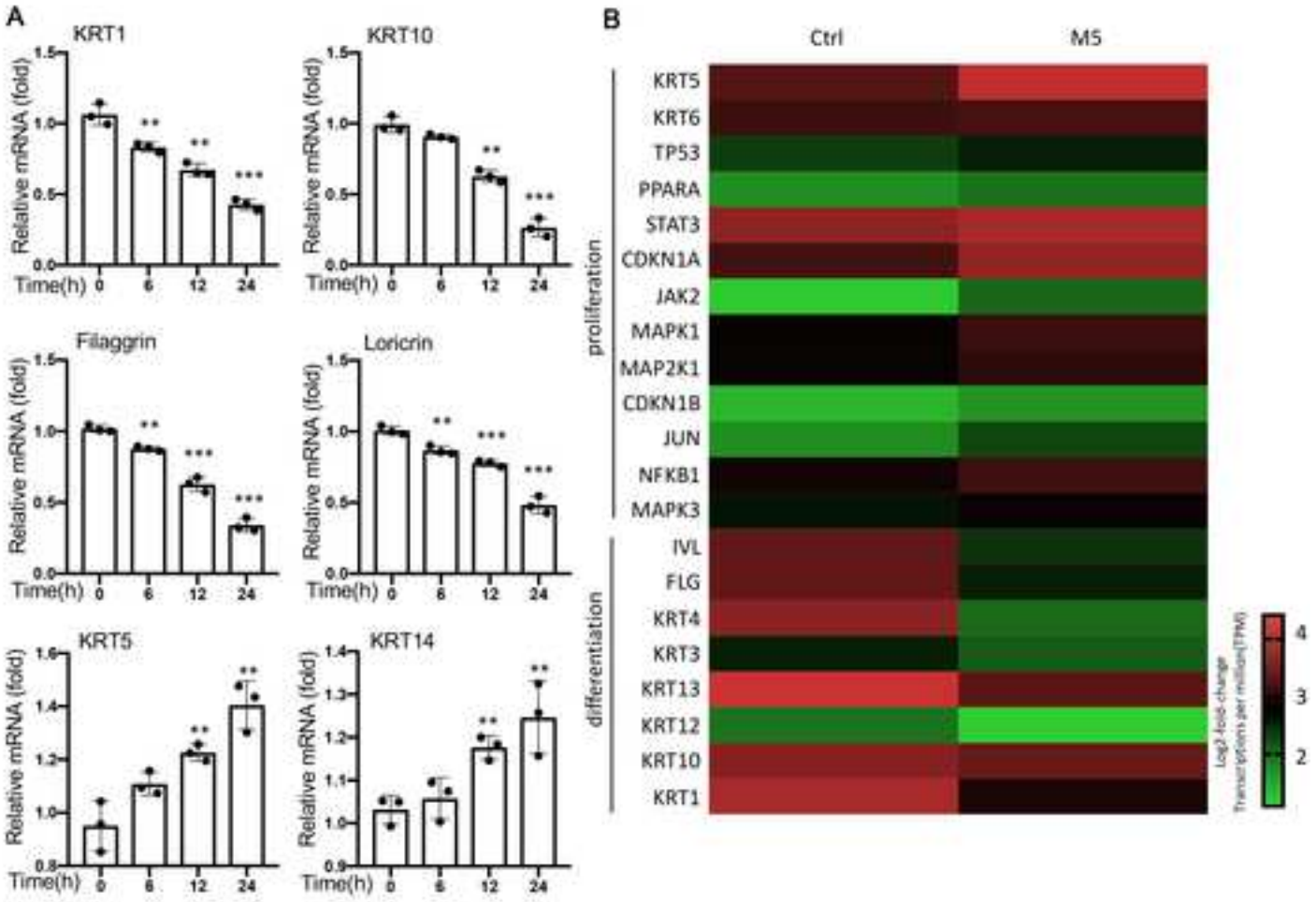


Figure 3



Primer Sequ

Gene	Forward (5' to 3')
β-actin	CCACGAAACTACCTTCAACTCC
S100A7	CCTTAGTGCCTGTGACAA
S100A8	AGTGTCCTCAGTATATCA
S100A9	CAACACCTTCCACCAATAC
S100A12	CAATACTCAGTTCGGAAGG
CXCL8	GTCCTTGTTCCACTGTGCCT
CCL20	TGACTGCTGTCTTGGATACACAGA
CXCL1	GCCAGTGCTTGCAGACCCT
CXCL2	CAAACCGAAGTCATAGCCAC
BD2	TTCTCGTTCCTCTTCATA
Filaggrin	TGAGGGCACTGAAAGGCAAA
Loricrin	GGGCACCGATGGGCTTAG
Krt14	CCAGTTCTCCTCTGGATCGC
Krt10	CCTGCTTCAGATCGACAATGCC
Krt5	TGTCAAGAAACAGTGCGCCA
Krt1	CCCTCCTGGTGGCATACAAG

ence
Reverse (5’ to 3’)
GTGATCTCCTTCTGCATCCTGT
CTGCTTGTGGTAGTCTGT
CATCTTTATCACCAGAATG
TCATTCTTATTCTCCTTCTTGAG
CTTTGATATTCTTGATGGTGTTT
GCTTCCACATGTCCTCACAA
TGATAGCATTGATGTCACAGCCT
GGCTATGACTTCGGTTTGGG
TCTGGTCAGTTGGATTTGCC
ATATGGCTCCACTCTTAA
TGGCCACATAAACCTGGGTC
GGTAGGTTAAGACATGAAGGATTTGC
TCCAGTGGGATCTGTGTCCA
ATCTCCAGGTCAGCCTTGGTCA
GCTGCTGGAGTAGTAGCTTCC
GTTGGTCCACTCTCCTTCGG

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
DMEM—Dulbecco's Modified Eagle Medium	Gibco	11965092	
Fetal Bovine Serum	Gibco	10100139C	
HaCaT cells	China Center for Type CultureCollection	GDC0106	Less than 15 generations
Human IL-1β ELISA Kit	Beyotime	PI305	
Human IL-6 ELISA Kit	Beyotime	PI330	
Human IL-8 ELISA Kit	Beyotime	PI640	
IL-1 alpha Human	Prospec	CYT-253	Recombinant protein
IL-17 Human	Prospec	CYT-250	Recombinant protein
IL-22 Human	Prospec	CYT-328	Recombinant protein
OSM Human	Prospec	CYT-231	Recombinant protein
PBS	Gibco	10010049	pH 7.4
Penicillin-Streptomycin	Gibco	15140163	
PrimeScrip RT reagent Kit	TAKARA	RR047A	
TB Green Premix Ex Taq	TAKARA	RR420A	
TNF alpha Human	Prospec	CYT-223	Recombinant protein
TRIzo Reagent	Invitrogen	15596018	
Trypsin-EDTA (0.25%), phenol red	Gibco	25200072	

Response to the Editor

Dear Dr. Bajaj,

The comments have been carefully taking into account and new revised submission have been uploaded. We highlighted all the altered passages in light yellow. The responses are as follows,

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

2. Please address all the specific comments marked in the manuscript.

Reply: We have addressed comments using the attached file.

3. Once done please highlight 3 pages of the protocol section including headings and spacings, to be used for filming purposes.

Reply: We have highlighted protocol section. However, I am a little concerned about the need for RT-PCR and Elisa procedures to be taken.

Reviewer #8:

Manuscript Summary:
This paper describes a method of producing inflammatory keratinocytes from HaCaT cells. The biggest advantage associated with this method is that M5 stimulated HaCaT cells were found to express chemokines, cytokines and keratins which are crucial in the pathophysiological process of psoriasis in a reproducible and consistent way.

Major Concerns:
Nil

Minor Concerns:
In line 82: as you know the stratum lucidum is found in the epidermal layer of thick skin of the palms and soles.

Reply: Thank you for your comments, we have annotated the stratum lucidum in the manuscript.

In line 275: keratins 5 and 14 are described as associated with proliferating

keratinocytes The keratin pair is usually expressed in basal keratinocytes regardless. Keratin 6 was upregulated, what about keratin 16?

Reply: Thank you for your comments, KRT16 associates with KRT6 isomers and co-expressed with KRT14 in number of epithelial tissues. We analysis microarray expression profiling, showed KRT16 slightly increased in M5 stimulated HaCaT compared to control, so we didn't show it in the manuscript.

Supplementary Table

Gene	Ratio Psoriasis/Control	p Value
chemokine		
CXCL1	5.34	<0.001
CXCL2	10.14	<0.001
CXCL8	18.59	<0.001
CCL20	7.07	<0.001
Antimicrobial peptide		
BD2	193.55	<0.001
S100A7	15.31	<0.001
S100A8	10.14	<0.001
S100A9	52.46	<0.001
S100A12	52.48	<0.001
Keratinocyte differentiation		
Filaggrin	0.58	<0.001
Loricrin	0.47	<0.001
KRT10	0.92	<0.001