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Original Research

Observation of TNF-α, IL-10 and HB-EGF gene expression by peripheral blood CD14+ mononuclear cells: a case of guttate psoriatic patient

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Abstract

Introduction: Guttate is a type of psoriasis in which patients are sensitive to *Streptococcus pneumoniae* throughout innate immune responses. During the inflammation, tumour necrosis factor alpha (TNF-α), a well-known pro-inflammatory cytokine, is expressed; meanwhile interleukin 10 (IL-10) and heparin-binding EGF-like growth factor (HB-EGF), which are capable of inhibiting transcription of the *TNF*-α gene, are also prominent. Furthermore, HB-EGF only impacts fibroblasts and keratinocytes which promote psoriatic lesions. In this study, we looked for differences of TNF-α, IL-10 and HB-EGF expression between a psoriatic patient and a non-psoriatic relative. **Methods**: To achieve our target, peripheral blood mononuclear cells (PBMCs) expressing LPS receptors or CD14 (CD14+ cells) derived from a guttate patient, and the donor's father (without psoriatic symptoms), were activated for 7 days by a lysate of *Streptococcus pneumoniae* for 24 hours before being harvested. **Result**s: Results showed detectable mRNAs of TNF-α, IL-10 and HB-EGF from isolated CD14+ cells of guttate patient were more intensive expression than the non-psoriatic one at 24 hours after engaging the bacterial components. In addition, transcription of *HB-EGF* gene from the guttate patient was maintained over 168 hours, while its mRNA level from the non-psoriatic volunteer was only expressed within 24 hours. **Conclusion**: Finally, in initial results of inflammatory effects between strains, the *Streptococcal* lysate was seen to have stronger immune responses than the *Staphylococcal* lysate on the immune cells of the guttate psoriasis.

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Keywords

CD14, guttate psoriasis, HB-EGF, IL-10, LPS, TNF-α

Introduction

Psoriasis is a chronic cutaneous inflammation in dermatology. To date, there have been a possibility of 40 susceptibility loci identified from various forms of psoriasis (Baurecht et al., 2015; Tsoi et al., 2012). The vast majority of these loci encode major histocompatibility complex (MHC), pro-inflammatory cytokines and nuclear transcriptional factors in both innate and adaptive immune responses (Jordan et al., 2012; Nair et al., 2006; Sato et al., 2015). Those elements are abnormally increased in gene expression in active peripheral blood mononuclear cells. Gervin et al. (2012) illustrated that there was a correlation between DNA methylation and expressions of *IL13* and *TNFSF11* genes in CD4 lymphocytes of monozygotic twins deferring psoriatic phenotype (Gervin et al., 2012). Interestingly, a streptococcal infection in the throat could lead to an exacerbation of almost all lesions on psoriasis patients during inflammation (Gudjonsson et al., 2003). According to these results, forming a psoriatic lesion might require environmental stimulators to activate cytokines involved in immune responses. Therefore, psoriasis might be a consequence of imbalance of inflammatory homeostasis.

Tumour necrosis factor alpha (TNF-α) is one of the first pro-inflammatory cytokines produced in innate immune response. Typical immune cells such as T helper 17 lymphocytes (Th17), dendritic cells (DCs) and macrophages are stimulated throughout trauma by pathogens, especially lipopolysaccharide (LPS) of bacteria (Evans et al., 2009; Haider et al., 2008). When LPS receptors or CD14 on these cells are activated, inflammation is triggered by the NF-κB pathway. As results of LPS induction, the activated immune cells immediately secrete TNF-α as well as other pro-inflammatory cytokine which probably lead to IL17, IL24, and IL33 production from keratinocytes (Balato et al., 2012; Chiricozzi et al., 2011; Kumari et al., 2013; Volpe et al., 2014). Furthermore, mutations in loci coding the transcriptional factors may initiate lesions. For example, the A20 mutant in plaque psoriasis could enhance TNF-α expression through the NF-κB pathway (Evans et al., 2004; Shembade et al., 2010). Characteristically, when a large number of psoriatic lesions derive from skin injuries, it is known as the Koebner phenomenon (Arias-Santiago et al., 2013; Thorarensen et al., 2015).

Moreover, the epidermal growth factors (*EGFs)* may play a key role in psoriasis. In fact, there are three predominant EGFs, including heparin-binding EGF-like growth factor (*HB-EGF)*, transforming growth factors (TGFs) and amphiregulin (AREG), which are involved in psoriasis. In post-inflammation, the presence of TGF-β and AREG prevent the transcription of inflammatory cytokine genes in

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immune cells at damaged skin. In addition, these two factors have similar anti-inflammatory function as IL-10, activating M2 macrophages and inflammatory suppression (Gratchev et al., 2008; Inoue et al., 2014; Maheshwari et al., 2011). Similarly, *HB-EGF* plays a potential key role for common psoriatic types via epidermal growth factor receptors (EGFRs). It induces EGFRs in response to the hyperproliferation, hyperdifferentiation, or hyperplasia of keratinocytes (Johnson et al., 1993). Moreover, expression of HB-EGF in human gastric carcinoma cells is also influenced by activation of the NF-κB pathway (Baek et al., 2008). Some mutations in the *HB-EGF* gene in animal models can lead to psoriasis-like phenotype (Poumay and De Rouvroit, 2012).

This study will observe the expression of TNF-α, IL-10 and HB-EGF in peripheral blood mononuclear cells expressing CD14+ (CD14+ cells) from a patient with guttate psoriasis, and his father without psoriasis, in a 7-day period. Additionally, the inflammatory effects of lysates of *Streptococcus pneumoniae* and *Coagulase-negative Staphylococci* on the CD14+ cells of guttate psoriasis were compared.

Materials-Methods

Study design

Two volunteers (a 34-year-old patient with guttate psoriasis, and his father with non-psoriasis as a control) agreed and signed the consent form to enroll the study, which was approved by the Ethic Medical Research Committee, University of Medicine Pham Ngoc Thach, Vietnam, according to the Declaration of Helsinki. Major criteria were: the psoriatic patient was diagnosed at the Ho Chi Minh City Hospital of Dermato–Venereology, Vietnam; PASI >10%; psoriatic symptoms for over 10 years; neither systemic therapy nor antiviral drugs were applied; no acute bacterial infections nor immune suppression, and no severe renal or deficient immune diseases or cancers (**Fig. 1**).

Peripheral blood isolation

Each volunteer had 6mL of peripheral venous blood taken once per two-weeks and the process was repeated in three times. All peripheral blood samples were collected into sterile lithium heparin tubes (Hong Thien My, Ho Chi Minh City, Vietnam), which were stored at approximately 4°C and transported to the laboratory within 2 hours.

Bacterial lysis

Streptococcus pneumoniae strain (ID: ATCC®49619™) and *Coagulase-negative Staphylococci* strain which were kindly provided by the Department of Microbiology, University of Medicine Pham Ngoc Thach. Generally, those strains were re-confirmed as *Streptococcus pneumoniae* and *Coagulase-negative*

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Staphylococci strains at the Department of Microbiology. To preserve functional proteins, the bacteria were lysated at extremely low temperature. In general, an individual colony of each strain was inoculated and dipped into the brain-heart infusion (BHI) medium (Merck, Darmstadt, Germany) at 37°C overnight. Subsequently, this overnight medium was centrifuged to obtain pellet (around 23 mg). The pellet was thawed by liquid nitrogen and rethawed at 37°C five times before being mixed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH of 7.2.) supplemented with 1% of penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), made up to a final volume of 500 mL and were stored at -20°C for further steps.

CD14+ cell preparation and inflammatory induction

Human peripheral blood mononuclear cells (PBMCs) from the heparinised venous blood of the participants were divided into two groups: guttate and control, respectively. In each group, PBMCs were sorted by Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). Then, they were washed and resuspended three times in cold RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 1% of penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Next, the 10^7 mononuclear cells in each group were incubated with 20 μL of CD14 MicroBeads, before the CD14 MicroBead-bound mononuclear cells (CD14+ cells) in the cell suspension were positively selected by magnetic activated cell sorting technology (Miltenyi Biotech, Bergisch Gladbach, Germany) in accordance with the manufacturer's instructions. Finally, 10^4 cells of isolated CD14+ cells were aliquoted into seven samples for each group and were cultured with RPMI 1640 medium at 37°C, 5% CO_2 overnight. For stimulation of inflammation, 10μL of the thawed *Streptococcal* or *Staphylococcal* lysate was added into six samples which were harvested at hours 1, 24, 48, 72, 120, 168, while the remaining sample was not. The RPMI 1640 medium was replaced every 24 hours.

RNA extraction and complementary DNA synthesis

Total RNA was extracted from activated CD14+ cells by TriPure (Roche, Mannheim, Germany) following the manufacturer's instructions. First strand complementary DNA (cDNA) was synthesised from total RNA with Random Primer Mix (New England BioLabs, MA) by reverse transcription-polymerase chain reaction (RT-PCR), according to a standard cDNA synthesis protocol of the manufacturer. The procedure of RT-PCR was conducted at 42°C for 60 min prior to inactivating at 80°C for 5 min. The cDNA products were stored at -20°C.

Analysis of mRNA gene expression

To observe mRNA level of TNF-α, IL-10, HB-EGF, and GAPDH, their primers were created using the Primer3 (version 0.4.0) and the published genetic sequences from the National Center for Biotechnology Information (NCBI). Reactions in PCR were performed with the One Taq® 2X Master mixed and

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Standard Buffer kit (New England BioLabs, MA, USA) in accordance with the manufacturer's directions. Each reaction was optimized with 950 ng of cDNA template using the following primers: TNF-α forward: 5'-ACAAGCCTGTAGCCCATGTT-3', TNF-α reverse: 5'-AAAGTAGACCTGCCCAGACT-3' (GenBank: NM_000594.3); IL-10 forward: 5'-TGCCTTCAGCAGAGTGAAGA-3', IL-10 reverse: 5'-GGTCTTGGTTCTCAGCTTGG-3' (GenBank: NM_000572.2); HB-EGF forward: 5'-GGTGGTGCTGAAGCTCTTTC-3', HB-EGF reverse: 5'-GCTTGTGGCTTGGAGGATAA-3' (GenBank: NM_001945.2); GAPDH forward: 5'-GAGTCAACGGATTTGGTCGT-3', GAPDH reverse: 5'-TTGATTTTGGAGGGATCTCG-3' (GenBank: NM_001289746.1). All primers were purchased from the Integrated DNA Technologies Pte Ltd (Singapore). Reactions were run on the Eppendorf® Mastercycler for 1 min at 95°C; and 30 cycles of 15 sec at 95°C followed by 60 sec at 50°C (TNF-α, IL-10 and HB-EGF) or 60°C (GAPDH). All PCR products were run on electrophoresis in 1.5% TBE agarose gel, and the results were analyzed with the UVP-GelDoc-It Imaging System to determine a relative amount of cDNA bands. Generally, this software compares the brightness of bands of interest with the 500 bp band of the Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb) (New England BioLabs, MA, USA), and then 12.4 ng/μL of DNA was multiplied with the ratios of brightness between each segment of interest and 500bp band. A 623bp band from IL-10 PCR product was sequenced by a commercial DNA sequencing service (First BASE Laboratories Sdn Bhd, Malaysia). Finally, the Unipro UGENE software (Version 1.26.0) was used for cDNA sequence analysis.

Date analysis

Statistical calculation for intensity of electrophoretic bands, which were compared different gene expression between guttate and control group in RT-PCR analysis, was conducted by two-way ANOVA with replication using Microsoft Excel software (Microsoft Inc, WA, USA). Standard error of the mean was utilized for error bars on three graphs.

Results

Streptococcal lysate and inflammation from activated CD14+ cells of guttate psoriasis

To monitor innate immune response of CD14+ cells from a patient with guttate psoriasis *in vitro*, the streptococcal lysate was used for this purpose. Further, the non-stimulated cell sample (0h) was used to verify all CD14+ cells which were not activated from previous steps. This ensures that those cells will naturally respond to the pathogen as well as exposing abnormal transcriptions between guttate and control groups. Besides, FBS was not supplemented into the RPMI 1640 medium because it contains several elements which could overlap with the transcriptional effects of HB-EGF.

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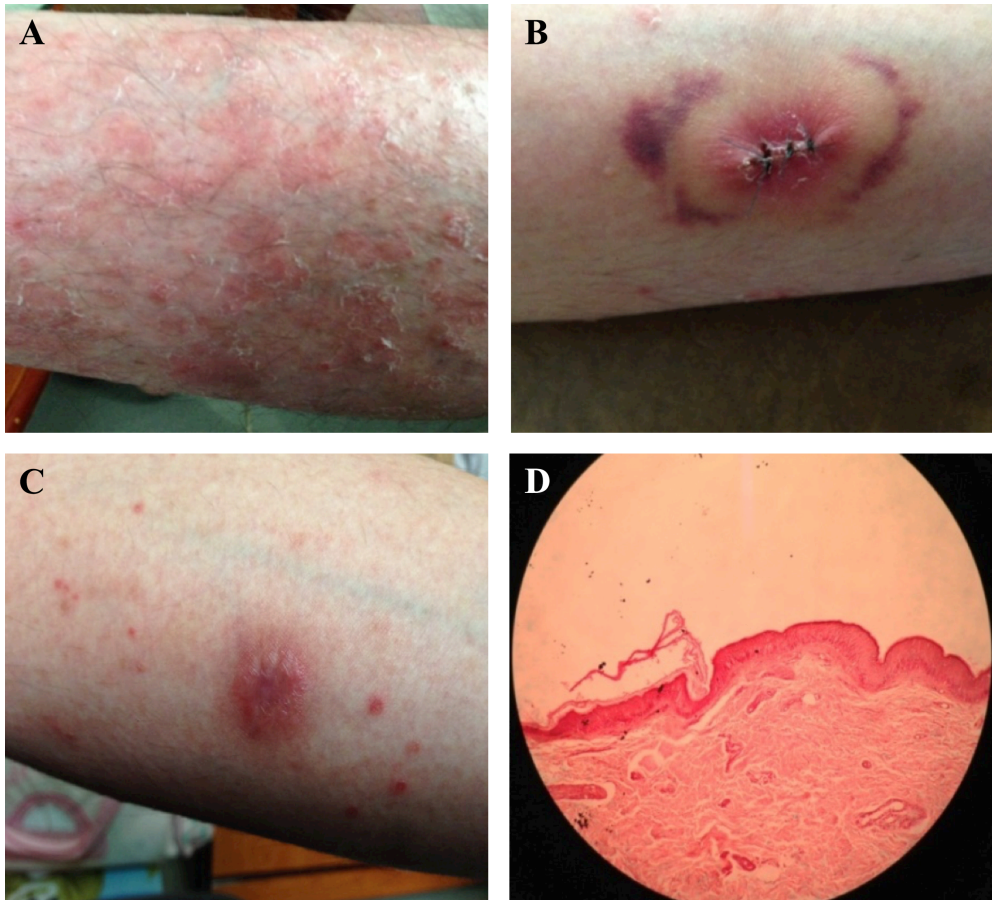

Figure 1. Psoriatic lesions on limbs and histology of guttate psoriatic patient: traumas on leg (**A**); position of biopsy on arm, where a psoriatic lesion was operated (**B**) and a new psoriatic lesion- Koebner phenomenon appeared within the next 7 days (**C**). The biopsy was stained with hematoxylin-eosin (**HE**) (**D**) at the Department of Histology, the Ho Chi Minh City Hospital of Dermato Venereology, Vietnam.

There were no cDNA signals of TNF-α, IL-10 and HB-EGF from the CD14+ cells in the non-stimulated samples (0) from both groups (psoriasis and control). After samples were stimulated by the lysate of *Streptococcus pneumoniae*, *TNF-α*, *IL-10* and *HB-EGF* gene transcription occurred in the first few hours until hours 168. Regarding the TNF-α signal, it occurred around 24 hours. However, the level of TNF-α mRNA in CD14+ cells of guttate was over 50-folds stronger than that of control approximately 153 ng and 3 ng of cDNA, respectively. In addition to the inflammatory response from the CD14+ cells, the signal of TNF-α from guttate continued to persist for the next 24 hours (48h) and there was no band at hour 72 (72h). On the other hand, a weak signal was detected from the 24-hour sample of control and no signal was seen in the remaining five samples (Fig. 2A,B).

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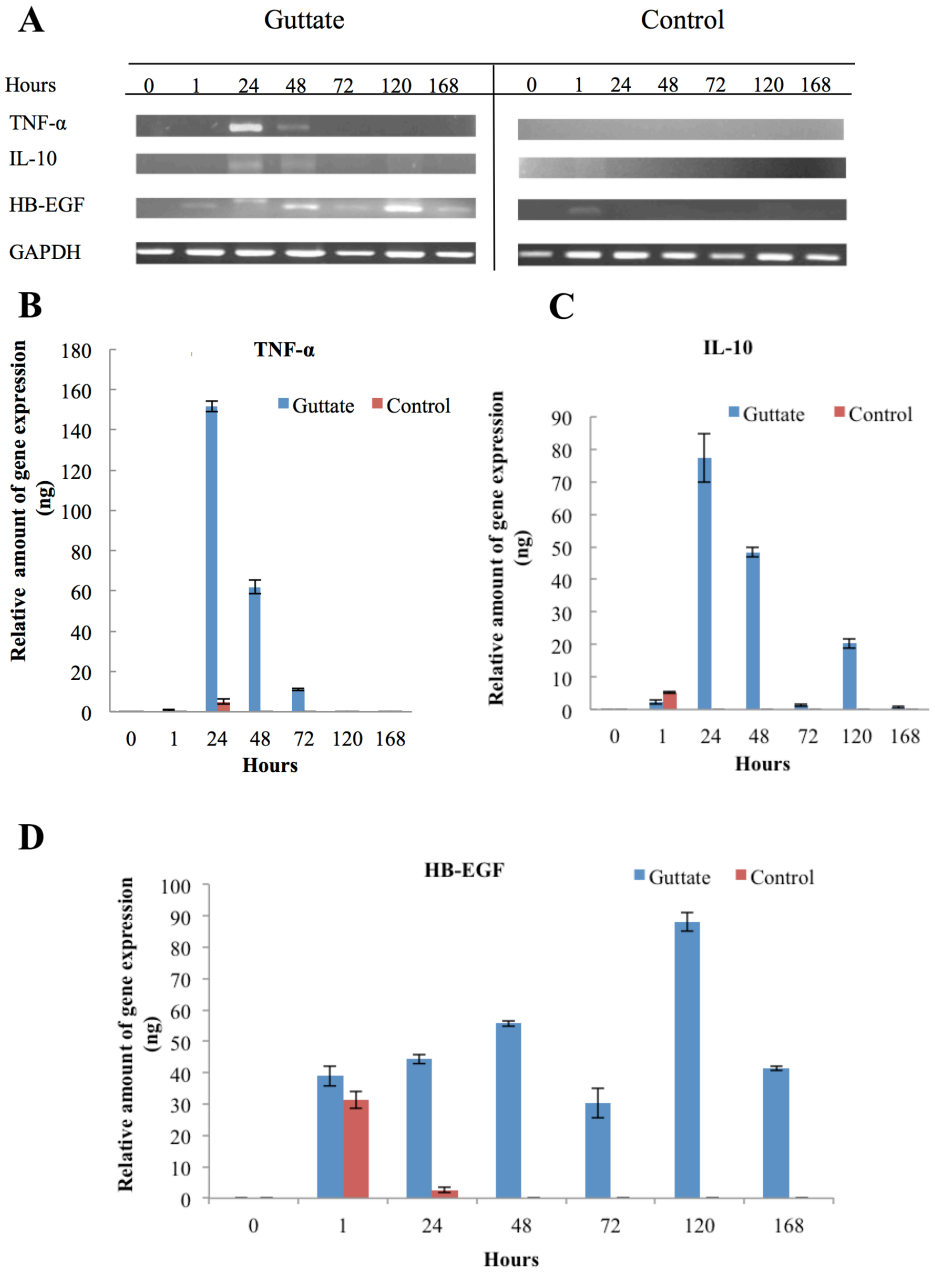

Figure 2. Innate immune response of psoriasis through expression of TNF-α, IL-10 and HB-EGF (**A**) Gene expression of the psoriatic patient (Guttate) and non-psoriasis person (Control) in hours 1, 24, 48, 72, 120, and 168; Relative amount of amplified cDNA of TNF-α (**B**), IL-10 **(C).** and HB-EGF **(D)** represent indirectly the original mRNA level in 10,000 activated CD14+ cells of each sample. Blank samples (0): CD14+ cells were not stimulated by the Streptococcal lysate. PCR products were run on 1.5% TBE agarose gel. Relative amount of amplified cDNA in each band was measured by the UPV software, according to intensity of the 500bp band, which contains 124ng of cDNA, is in the 100kb ladder. The error bars represent the mean ±SD within each experiment which was performed in three times with identical results. The cDNA intensities of TNF-α, IL-10 and HB-EGF of guttate were higher than results for the control group.

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Query  15          GATGT-CATGCAGA-GATACCCACCTCAGCATGG-AGGGGAGAATGATCTCTCTTCACCA  71
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Query  132         TCTATTAGTAGCACTTCTTACCTGGATACACTGATTCCAACTTTTGGTGATTCCCTGGGA  191
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  192         TCCttttttttCTCAGCTCTGACTTGGAAGCACAGAAGATTTCTCAGTTTCCAAATCATGT  251
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Query  252         GACAATACCTATTTGCATTTGTGAAAATTAAGCTTCTGAATGATTAGTAAATTCAGGCCA  311
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Query  612         GAACCA--AGACCA  623
                   ||  |||  ||||||
Sbjct  21869809    GA-CCATCAGACCA  21869821
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Figure 3. Sequencing the 623 bp band from the PCR product of IL-10 expression. The BLAST program was used to screen the sequence from the NCBI database. This sequence is homologous to the sequence of chromosome 2 as well as two ORFs. Reference number: NC 00002.12.

The IL-10 and HB-EGF gene expressions of more prolonged to 120 and 168 hours, respectively. Although IL-10 is a well-known anti-inflammatory cytokine, our results revealed that the mRNA of IL-10 and TNF-α from guttate and control groups were simultaneously expressed around the first 24 hours (24h). Nevertheless, the CD14+ cells of the control group expressed the *IL-10* gene in the first hour (1h), while expression of this anti-inflammatory cytokine from the guttate group was established around 24 hours to 120 hours (120h).

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Subsequently, downregulation of this cytokine proceeded to occur over the next 48 hours (Fig. 2A,C).

Similarly, transcription of the HB-EGF gene also progressed in the first hour for the two groups. The CD14+ cells of the control group showed weak expression at nearly 24h. Nonetheless, for the guttate group, this growth factor showed a gradual increasing expression before reaching a peak of gene expression at about 120 hours and persisting to over 168 hours (Fig. 2A,D). Interestingly, regarding the IL-10 PCR product, there was an unknown band of approximately 623bp. The date of cDNA sequencing were compared with the database from NCBI throughout the BLAST programme. It showed that the sequences were homologous to chromosome 2 and there are two open reading frames (ORFs) in the sequences (Fig. 3).

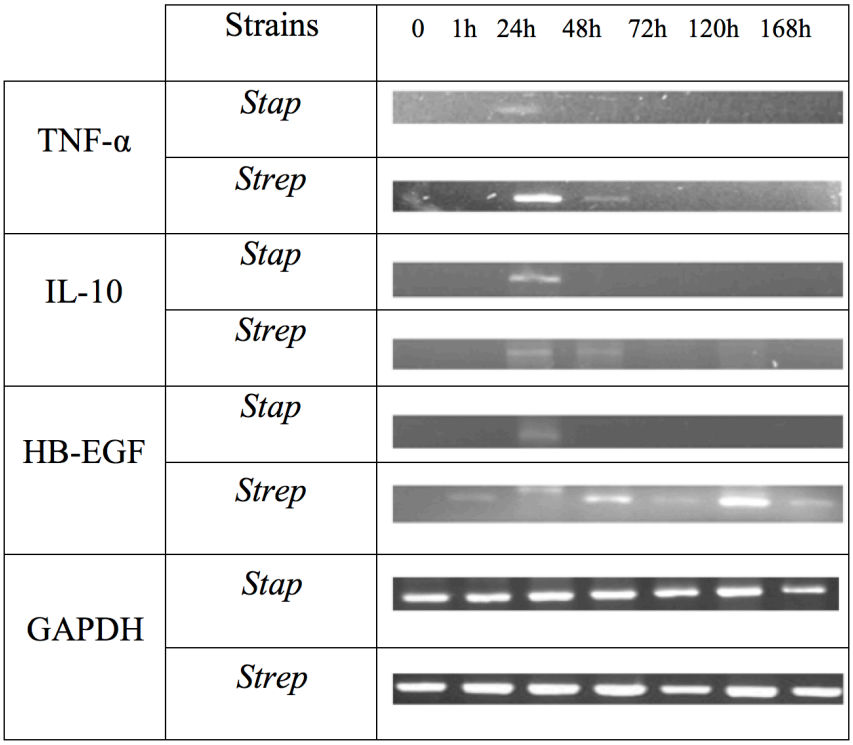

Figure 4. The innate immune response of the CD14+ cells stimulated by lysates of *Streptococcus pneumoniae (Strep)* and Coagulase-negative *Staphylococci (Stap).* Expression of TNF-α, IL-10, and HB-EGF were conducted for hours 0, 1, 24, 48, 72, 120, and 168. All PCR products were run on 1.5% TBE agarose gel. The mRNA levels of these three genes stimulation by staphylococcal lysate, is expressed just around 24 hours and is less intensive than the streptococcal lysate stimulated CD14+ cells.

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The inflammatory stimuli of Streptococcus pneumoniae and Coagulase-negative Staphylococci on CD14+ cells of guttate

To understand the transcriptional effects of topical bacteria on the innate immune response, *Coagulase-negative Staphylococci* (*Stap*) was chosen because it pertains to human microbiota on skin. Our results demonstrated that this strain partly affects the CD14+ cells of the guttate group. The expressions of TNF- α, IL-10 and HB-EGF only occurred in 24 hours, and the mRNA level was probably lower than the yields obtained from *Streptococcus pneumoniae* (*Strep*)-stimulated CD14+ cells (**Fig. 4**).

Regarding the induction of inflammation on CD14+ cells by *Coagulase-negative Staphylococci*, the staphylococcal lysate was insufficiently strong to cause strong gene expressions of TNF-α, IL-10, and HB-EGF, in comparison with the streptococcal lysate. Additionally, mRNA expression of IL-10 and HB-EGF in the guttate group did not persist as with *strep* stimulation, and they were similar to the control group stimulated by streptococcal lysate. Therefore, CD14+ cells in the guttate psoriatic patient are less sensitive to the staphylococcal components in react with a strong immune response. In fact, streptococcal infection leads to aggravation of the vast majority of lesions of patients with psoriasis, whilst skin damage only causes a topical inflammatory response.

Discussion

The guttate psoriasis patient was sensitive to the sore throat, especially *Streptococcus pneumoniae*. This strain is one of the local bacteria of the upper respiratory tract which plays an important role in the lesional exacerbation. Previous studies have shown that psoriatic lesions can trigger inflammation via LPS, and that Strep possibly enhance the release of pro-inflammatory cytokines from $CD4^+$ T cells and macrophages through the *Streptococcal* M6 protein (Johnston et al., 2004; Wang et al., 2006). However, in psoriasis, over-expression of mononuclear cells causes secretion of TNF-α, which significantly influences the large number of lesions. In this study, the signals of TNF-α, from the guttate psoriatic patient were more strongly expressed than the control (with non-psoriasis), although both participants were homologous in up to 50% of their genetic material. This means that suppressing gene expression of TNF-α in psoriatic patients less effective than normal people. Furthermore, approximately 160 genes involve inflammation such as S100A7, SerpinB4, and DEFB4 could be up-regulated by the TNF /IL-17 induction (Chiricozzi et al., 2011). This might impact on immune cells which regulate inflammation through TNF-α expression.

Additionally, the role of IL-10 is to suppress transcription of the *TNF*-α gene, but our results of these cytokines showed concomitant expression in the early 24 hours. . In fact, the majority of CD14+ cells as macrophages will release IL-10 to suppress transcript level of TNF-α through the LPS receptor induction (Wang et

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al., 2014). Saraiva et al. (2005) showed that there is a tight correlation between LPS-activated NF-κB complex and signal transduction of IL-10 through its promoter (Saraiva et al., 2005). Moreover, several studies have demonstrated that IL-10 production can be possibly up-regulated from 2 to 8 hours by apoptotic neutrophils, proopiomelanocortin peptides, or CD23 activation (Bhardwaj et al., 1996; Byrne and Reen, 2002; Dugas et al., 1996). Therefore, IL-10 production in CD14+ cells of guttate psoriasis corresponds to the signal transduction of TNF-α.

In this study, our hypothesis was that HB-EGF might play an important role in psoriatic pathway. This growth factor not only suppresses transcription of pro-inflammatory elements such as *TNF*-α gene (Rocourt et al., 2007). Moreover, HB-EGF also induces hyperdifferentiation or hyperproliferation of keratinocytes the during the recovery of cutaneous injuries in psoriasis (Shirakata et al., 2005). In our results of guttate, HB-EGF was produced in the first hour. In fact, we did not utilize FBS for cell culture so that other growth factors in FBS would not affect the LPS-induction of CD14+ cells. Despite differing signal transductions, it is probable that the streptococcal stimulus on the immune cells of the guttate group might activate a potential mediator which regulates the transcriptional systems of TNF-α as well as HB-EGF. Furthermore, the HB-EGF expression from guttate persisted to over 7 days (168 hours), while expression of the growth factor from the control group was just over 1 h after the inflammatory stimulation. The results of the control in this study correlates with research by Yoshizumi et al. (1992), which showed that TNF expression enhanced HB-EGF expression in 24 hours (Yoshizumi et al., 1992). Conversely, the HB-EGF mRNA level in guttate continues to persist to over 168 hours. It is possible to systematically activate the NF-κB and PKB pathways causing the over-expression of HB-EGF (Yotsumoto et al., 2010). In another clinical research of psoriasis, Anderson et al. (2010) demonstrated that serum HB-EGF and TNF-α concentration sustainably rise in patients with psoriatic plaques (Anderson et al., 2010). This is substantial evidence that there is a correlation between induction of the NF- κB pathway and HB-EGF gene expression in psoriasis.

There are several limitations in this study. It lacks date of TNF-α, IL-10 and HB-EGF concentrations in cell culture and lacks identification of some transcriptional factors which may respond to expression of these elements. Subsequently, the sample size is too small and the pattern is only guttate without plaque psoriasis (the most common type of psoriasis). However, our data in this study will become a preliminary database for next studies. Regarding HB-EGF expression and the NF-κB pathway, the identification of transcriptional mediators in activated macrophages (CD14+, CD163+) of guttate and plaque psoriasis is ongoing.

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Conclusion

In initial conclusion, the concomitant expression of TNF-α, IL-10 and HB-EGF, as well as abnormal transcription of the *HB-EGF* gene in a 168-hour period, may be indicators that CD14+ cells of guttate psoriasis are activated by LPS. In addition, streptococcal components are more pathogenic than staphylococcal ones. Control of *HB-EGF* gene transcription in immune cells is not only one of our targets improving the quality of life for psoriatic patients, but it is also probably applicable to immunotherapy and gene therapy for other autoimmune diseases and solid tumors in the future.

Abbreviations

AREG: amphiregulin
CD14: Cluster of differentiation 14
cDNA: complementary DNA
EGFR: Epidermal growth factor receptor
GADPH: Glyceraldehyde 3-phosphate dehydrogenase
HBEGF: Heparin-binding EGF-like growth factor
HE stain: Hematoxylin and eosin stain
IL: Interleukin
LPS: Lipopolysaccharide
MHC: Major histocompatibility complex
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
PASI: Psoriasis area severity index
PCR: Polymerase chain reaction
PKB: Protein kinase B
Th17: T helper 17 lymphocytes
TNF-α: Tumour necrosis factor alpha
TGFs: Transforming growth factors

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Author Contribution

All authors equally contributed on all experiments: designed the study; wrote the manuscript.

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