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Research Article

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# The effects of different stimulated macrophages on HaCaT proliferation and secretion.

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## Abstract

***Background-****As an important cell of innate immunity, macrophages secrete a large number of inflammatory cytokines, including TNF-α, which may play crucial role in the initiation stage of psoriatic inflammation. Keratinocytes are the effector cells of psoriasis and may produce circulatory stimulation networks with other immune cells in the lesions. Understanding the effect of macrophages induce keratinocytes' biological function is necessary to understand the mechanisms of psoriasis.*

***Objectives-****To evaluate the effects of different polarized macrophages on the proliferation, differentiation, apoptosis and secretion of immortalized human keratinocytes (HaCaT).*

***Materials and Methods-****Establish co-culture system of macrophages and HaCaT in vitro to mimic macrophage infiltration microenvironment of psoriasis skin. The proliferation of HaCaT was detectd by CCK8, Cell cycle. Apoptosis of HaCaT was analysed by flow cytometry. The secretion of IL-1β, TNF and IL-6 by HaCaT was analysed by ELISA.*

***Results-****The results showed a decreased proliferation of HaCaT when co-cultured with LPS-induced macrophage and upregulated IL-6, IL-1β and TNF-α. While more HaCaT cells were blocked in the G0/G1 phase, the number of mitotic phase cells decreased and apoptotic cells increased. On the contrast, HaCaT showed increased proliferation when co-cultured with IL-4-induced macrophage, while no difference in apotosis of HaCaT. Moreover, proinflammatory cytokine TNF-α and IL-12 production by LPS-induced macrophage were significantly increased.*

***Conclusion-****Considering the changes in the biological function of HaCaT cells caused by the infiltration microenvironment of macrophages, these findings indicate that the polarization process of macrophage is a crucial mechanism in the development of psoriasis, and the underlying immunological mechanisms should be understood.*

**Key Words:** Macrophages; Hacat; Proliferation; Apoptosis; Cytokine

## Introduction

Excessive proliferation, differentiation and aptosis of keratinocytes and infiltration of inflammatory cells are important feature of psoriasis [1]. Parts of psoriasis patients and their families called psoriasis "deathless cancer". More recently, it has been gradually recognized that the innate immune system may be involved in the initiation of psoriasis inflammation. Macrophages are an important cell of innate immune system which have powerful functions of recognizing, phagocytosis, clearing bacteria and foreign bodies. Macrophages can be polarized into two different subsets named inflammatory M1 and anti-inflammatory M2 macrophages which play an important role in maintaining the stable state of the immune system and participate in the development of many diseases

[2]. As another major cellular component of innate immunity, keratinocytes are the main manifestation of psoriasis. They play an important role in skin inflammation and immune response by expressing cytokines and antimicrobial peptides [3]. While the crosstalk between macrophage and keratinocytes are still unknown. This paper mainly studies and discusses the effects of macrophages on proliferation, differentiation, secretion of keratinocyte to provide theoretical basis for the possible pathogenesis of psoriasis.

## Material

HaCaT human immortalized epidermal cells were purchased from KeyGen Biotech, and U937 human histiocytic lymphoma cells were purchased from Kunming cell bank, CNS. FITC Annexin V Apoptosis Detection Kit (556547) and PI/RNase Staining Buffer (550825) were obtained from Biosciences Pharmingen (San Diego, CA, US), and Cell Counting Kit-8 cell proliferation/toxicity test kit (FC101-03) were obtained from TransGen Biotech company (Pek, CH). ELISA kits targeting IL-12p70 (EK1121), IL-10 (EK1101), IL-1β (EK101B2), IL-6 (EK1062) and TNF-α (1822) were from LiankeBio company (Hangzhou, CH) and IL-4 from Sigma-Aldric (St Louis, MO). Phorbol ester (PMA; P1585), bacterial lipopolysaccharide (LPS; L2630) and DMSO (D2650) were purchased from Sigma-Aldric (St Louis, MO).

## Cell culture

The immortalized human epidermal line (HaCaT) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% PS; U937 cells, a line of human monocytic cells, were grown in Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 1% PS; All were cultured at 37 ℃ and a saturated humidity incubator of 5% CO2.

## Activation and Polarization of the Monocytic

U937 cells monocytes were activated into macrophages using 100ng/mL phorbol-12-myristate-13-acetate (PMA) for 48h, the U937 cells changed from suspension to adherent state and then washed with medium. Activated U937 cells monocytes into macrophages by 100 ng/mL phorbol-12-myristate-13-acetate (PMA) for 48h, when it changed from suspension to adherent state, washed with medium every other day twice. Then polarized U937 macrophages were with 100ng/mL LPS for the M1 macrophages and 20ng/mL IL-4 for the M2 macrophages.

## Co-culture of macrophages and HaCaT cells

Prepared HaCaT cells with trypsin digestion for single cell suspension of 1×105 cells/mL and convergence rate of 90%. Then inoculated the suspension into the lower chamber of 24-well Transwell culture plate (membrane diameter 0.4μm) at 500μL/well for 24h. After digestion, a single cell suspension of 5×105 cells/mL of macrophages which activited by different activation pathways, according to the set-up experimental groups, was inoculated into the upper chamber of 24-well Transwell culture plate at 200μL/well and cultured for 24h, 48h, 72h, and 96 h, respectively. Then removed the upper chamber and collected the media in the lower chamber for subsequent tests.

## HaHaT cells proliferation detection by CCK8

Added 10% CCK-8 solution into each lower chamber of 24-well transwell culture plate and incubated in the incubator. After 1h, moved the CCK-8 solution in the hole to the 96 hole plate, and detected OD value at 450nm by enzyme labeling instrument.

## Enzyme-Linked Immunosorbent Assay for Cytokine Production

Macrophage and HaCaT cells were treated with or without different macrophages as indicated for 24h, 48h, 72h and 96h. The cell culture media were collected for measurement of cytokine productions using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocol.

## Flow cytometric analysis for cell cycle

Trypsin digested HaCaT cells after 72 hours of co-culture, washed by 1×PBS, and resuspensed cells with 500μL of precooled PBS. Then added the cell suspension into 3.5ml of precooled 80% ethanol at 4℃for one night. Centrifuged at 2500 rpm for 5 minutes and precipitated the cells, removed the supernatant, washed with pre-cooled PBS twice and discarded the supernatant. Adding 50μL PI/RNase Staining Buffer resuspension cells over 200 mesh nylon screen to make a single cell suspension and then incubated at 4℃ for 30 minutes. Cells in different cell cycles were analyzed on a FACSCalibur flow cytometer (BD FACSAriaII) with CellQuest software (BD FACSDiva).

## Flow cytometric analysis for cell apotosis

After 72 hours of co-culture, the HaCaT medium in the lower chamber was sucked out into the centrifuge tube (containing cells that had been suspended for apoptosis or necrosis), washed adherent cells twice by PBS, collected medium with PBS into the centrifuge tube, trypsin digested, transferred the cells into the centrifuge tube, centrifuged at 1000rpm for 5 minutes, discarded the supernatant. Washed with pre-cooled PBS twice and discarded the clean supernatant. Added 500μL 1×Binding Buffer heavy suspension cells over 200 mesh screen to make a single cell suspension. Added 5μL Annexin V-PE and 10μL 7-AAD to each tube and mixed gently, stayed away from light at 4℃ for 5 minutes. The number of apoptotic cells was detected by FACSCalibur flow cytometry (BD FACSAriaII) within 30 minutes.

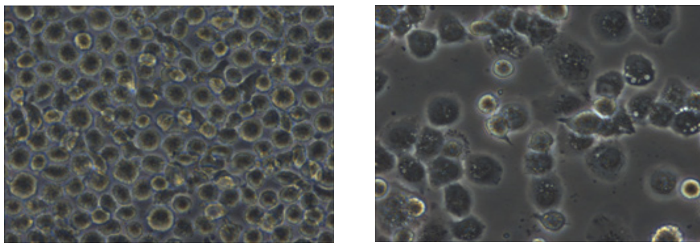
## Statistical processing

The results were expressed as the mean ± SD. One-way repeated-measures ANOVA was used for the analysis of the differences between the groups. All statistical analyses were carried out by SPSS 19.0 software, P-value $<0.05$ or P-value $<0.01$ were considered to be statistically significant.

## Results

### Macrophage induction and differentiation

After 48h induced by PMA, U937 cells' growth pattern changed from suspended state to adherent stat, protruding pseudopodia, and then monocytes differentiated into macrophages. After removed the unadherence cells by PBS, then added PMA, LPS and IL-4 respectively to further induce differentiation of macrophages. The induced macrophages were long spindle with much more pseudopodia. There was no obvious difference in morphology between the three groups under microscope (Figure 1).



A. Uninduced U937 (400×)  B. U937 induced by PMA (400×)

**Figure 1:** U937 were induced to macrophages by PMA.

In addition, compared with the PMA-induced group, IL-12 as well as TNF-α, which are signatures of M1 macrophages, increased in LPS-induced macrophages (*P* <0.05, *P* <0.01, respectively), and IL-10, which are signatures of M2 macrophages, decreased in LPS-induced macrophages (*P* <0.01). In contrast, IL-10 increased and TNF-α decreased in IL-4 induced macrophages than in only PMA-induced group (*P* <0.01). There was no significant difference in IL-12p levels (*P* >0.05). The results confirmed that Mφ was successfully induced and polarized to M1 and M2 macrophages by LPS and IL-4 respectively (Table 1 and Figure 2).

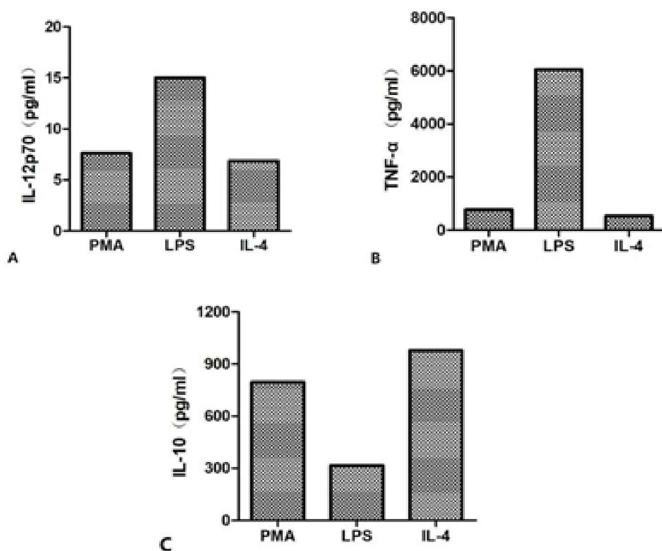



**Figure 2:** IL-10, IL-12p70, TNF-α of macrophage secretion in different activation pathways.

A.B. IL-12p70 and TNF-α showed a statistically significant higher in LPS-induced macrophages than IL4 and PMA-induced macrophages (*P* <0.05, *P* <0.01, respectively) .
C. IL-10 decreased in LPS-induced macrophages (*P* <0.01).

## Effects of different stimulated macrophages to HaCaT proliferation

After co-cultured with macrophages for 24h, 48h, 72h and 96h, HaCaT in different groups showed significantly different proliferation rates (*P* <0.01). Proliferation rate of HaCaT in LPS-induced M1 macrophages group was obvious lower than Mφ and M2 macrophages (Figure 3A).

## Effect of macrophages activated by different pathways on differentiation of HaCaT cells

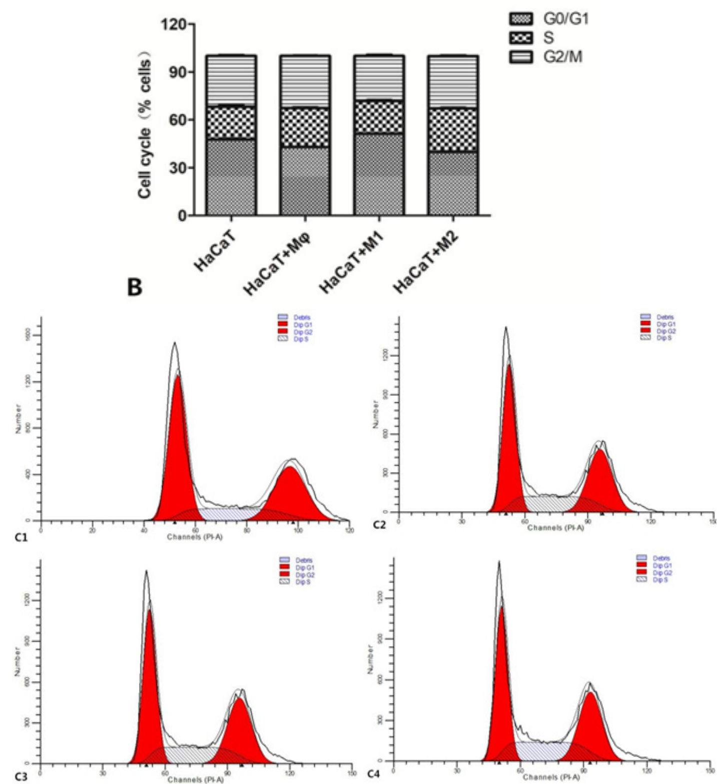
The cycle number of HaCaT cells was measured after 72h of co-culture with macrophages. Compared with the HaCaT culture group, more HaCaT co-cultured with M1 macrophages group were blocked in G0/G1 phase and less into the mitotic phase (M), while although the number of cells in G0/G1 phase was decreased when HaCaT cells co-cultured with M2 macrophages, the number of cells in S phase was relatively increased, so the number of cells in the mitotic phase was not significantly different (Figure 3B).

## Effect of macrophages activated by different pathways on apoptosis of HaCaT cells

After co-cultured with macrophages for 72h, HaCaT cells in M1 macrophage group showed significantly higher apoptosis rate than that in HaCaT group, Mφ and M2 group (P <0.01), but there was no significant difference between HaCaT group, Mφ and M2 group (Figure 3C and 3D).

## Detection of HaCaT cell secretory function

The secretion of IL-1β/IL-6 in HaCaT cells co-cultured with M1 were significantly higher than HaCaT, Mφ and M2 group. The Mφ group was higher than M2 group (all P <0.05) in 24h, 48h, 72h and 96h. And the secretion of TNF-α in HaCaT cells co-cultured both with M1 and M2 macrophage were higher than HaCaT and Mφ (P <0.05). There was no significant difference between M1 and M2 group (Figure 4).



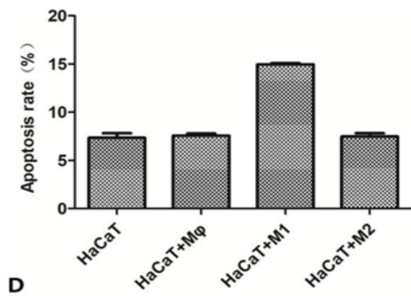



**Figure 3:** Effects of Macrophages in different activation pathways on Proliferation, differentiation and dapoptosis of HaCaT Cells.
A. Proliferation rate of HaCaT in LPS-induced M1 macrophages group was obvious lower than Mφ and M2 macrophages.
B. More HaCaT co-cultured with M1 macrophages group were blocked in G0/G1 phase and less into the mitotic phase (M).
C. Effect of macrophages activated by different pathways on apoptosis of HaCaT cells.
D. After co-cultured with macrophages for 72h, HaCaT cells in M1 macrophage group showed significantly higher apoptosis rate than that in HaCaT group, Mφ and M2 group (*P* <0.01).

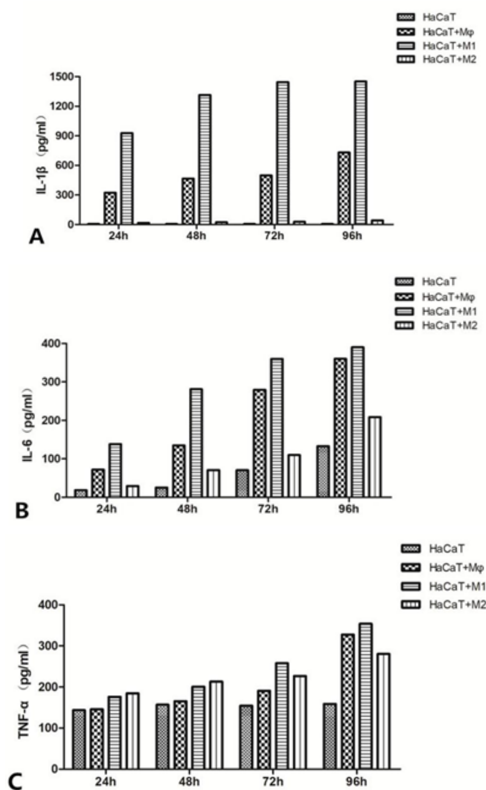



**Figure 4:** Effects of Macrophages in different activation pathways on secretion of HaCaT Cells.
A, B. IL-1β and IL-6 in HaCaT cells co-cultured with M1 were significantly higher than Mφ and M2 group.The Mφ group was higher than M2 group (all *P* <0.05) in 24h,48h,72h and 96h.
C.The secretion of TNF-α in HaCaT cells co-cultured both with M1 and M2 macrophage were higher than HaCaT and Mφ (*P* <0.05). There was no significant difference between M1 and M2 group.

## Discussion

Psoriasis is a chronic inflammatory skin disease involving both the innate and the adaptive immune compartments, crosstalking with skin tissue cells, while the pathogenesis of psoriasis has not been fully understood [4]. In the current model, a crosstalk between keratinocytes (KC), neutrophils, mast cells, T cells, and dendritic cells is thought to create inflammatory and pro-proliferative circuits mediated by chemokines and cytokines [5]. Various triggers, including recently identified autoantigens, Toll-like receptor agonists, chemerin, and thymic stromal lymphopoietin may activate the pathogenic cascade resulting in enhanced production of pro-inflammatory and proliferation-inducing mediators such as interleukin (IL)-17, tumor necrosis factor (TNF)-α, IL-23, IL-22, interferon (IFN)-α, and IFN-γ by immune cells. Macrophages and neutrophils are key components involved in the regulation of numerous chronic inflammatory diseases [6]. macrophages might play an important role in the initiation stage of many inflammatory disease through secreting a large number of inflammatory cytokines, such as TNF-α, IL-6. The role of macrophages in psoriasis is not yet fully characterised, however, they are speculated to contribute to the disease as there is a threefold increase in cell numbers in lesional skin, with evidence of normalisation after successful treatment [7]. Mouse models with skin phenotypes that resemble human psoriasis support involvement from skin macrophages in disease development and maintenance [8]. Macrophage markers and inflammatory cytokines significantly increased in psoriatic tissues over those in normal tissues. In animal studies, depletion of macrophages in mice ameliorated imiquimod, TLR7 agonist induced psoriatic response, in addition, treatment with this TLR7 agonist shifted macrophages in the psoriatic lesions to a higher M1/M2 ratio [9]. Psoriasis is characterized by abnormal proliferation of KCs and infiltration of inflammatory cells In the skin. Whether or not there is a crosstalk between macrophage and KCs and how does the crosstalk be performed to trigger psoriasis remains poorly articulated.

In the present study, we investigated the effects of different activation pathways on proliferation, apoptosis and secretory function of HaCaT cells to determine the crosstalk of macrohage and keratinocyte in psoriatic in flammation. As shown in the present studies, LPS-induced M1 macrophages could inhibit the proliferation of HaCaT cells, inhibited HaCaT cell differentiation through block more cells in G1 phase of cell differentiation, and promote apoptosis after co-culture. In contrast, the IL-10-Induced M2 macrophages promoted the proliferation of HaCaT cells, decreased the cell number in G1 phase and blocked more HaCaT cells in S phase, while had no significant effect on apoptosis.

KCs are believed to be crucial in both the early stages of psoriasis pathogenesis and later amplification of chronic inflammatory circuits [10]. KCs' hyperproliferation are a secondary phenomenon induced by immune activation and inflammation. KCs are the main constituent cell of the epidermis, differentiated from the basal layer cells of the epidermis, gradually differentiated and matured in the process of moving to the cuticle, and finally fell off the surface of the skin. In normal epidermis, the ratio of KCs increases and apoptosis is equal to maintain the dynamic balance of epidermal cells. This process is regulated by precision and complexity. The growth kinetics of KCs in psoriatic lesion area showed a spe-

cial disorder state, which showed that the proliferation of epidermal basal cells was accelerated, the mitotic cycle was shortened, while the kc differentiation process was delayed and the apoptosis process was delayed. Ar-turmerone suppressed cell proliferative ability and attenuated inflammatory cytokine expression by inactivating Hedgehog pathway in HaCaT cells, Some scholars believed that the occurrence and development of psoriasis is related to the runaway of KCs apoptosis, while the point of view is different. Scientists found that the pro-apoptosi genes fas, p53, c-myc hige expression in the lesions of psoriasis whereas the anti-apoptosis gene bcl-2 expression decreased [11, 12].

Positive correlation between the caspase-3 expression and the early onset psoriatic lesion confirmed that the apoptosis of KCs in psoriatic lesions was significantly increased, and there was positively correlated with the severity score of local lesions in skin lesions and PASI score. And Eding CB showed a general upregulation of anti-apoptotic genes and downregulation of pro-apoptotic genes in both involved and uninvolved psoriatic KCs compared with controls by microarray transcriptome analysis [13]. In vitro culture of KCs derived from both involved and uninvolved psoriatic skin, revealed higher viability and resistance to apoptosis following exposure to ultraviolet B compared with cells from healthy controls. This distinct apoptosis-resistant phenotype, unrelated to the inflammatory component of the disease, implies that intrinsic abnormalities in KCs may contribute to the pathogenesis of psoriasis.

Since KCs bear receptors for the majority of psoriasis-signature cytokines, they represent the “key responding” tissue cells to the psoriatic microenvironment. They respond to psoriatic cytokines by proliferating and amplifying inflammation through the production of other cytokines (i.e., IL-1, (IL-36γ), TNF α, IL-17C, IL-19, TSLP), chemokines, proliferation-stimulating factors and other pro-inflammatory products, such as AMPs [14-17]. Although KCs have a relevant role in mediating inflammation, this hypothesis considers keratinocyte response as secondary to immune cell activation. For instance, IL-17 and TNF-α strongly induce the synthesis of pro-inflammatory mediators with additive and synergistic effects on KCs gene expression [18]; similarly, IL-22 and other IL-20 cytokine family members (i.e., IL-19 and IL-20) stimulate keratinocyte hyperplasia, promoting epidermal thickness [19]. However, a recent study confirmed their immune relevance showing that keratinocyte genetic defects yield mice more susceptible to specific IL-17-mediated psoriasis-like inflammation [20]. On the contrary, KCs could produce a variety of cytokines by various endogenous or exogenous stimuli, such as proinflammatory cytokines include IL-1, IL-6, the IL-12, IL-20, IFN-γ, and TNF-α, while antiinflammatory cytokines include IL-10. These cytokines would affect the migration of inflammatory cells, the proliferation and differentiation of KCs, enhance or weaken the secretion of other cytokines, and even play an important role in systemic immune function.

As shown in the present and previous studies, macrophages can be polarized into two major differential subsets named inflammatory M1 and anti-inflammatory M2 macrophages. M1 macrophages produce proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, and IL-12, which are proved involvement in psoriasis pathology; M2 macrophages generate anti-inflammatory cytokines and are thought to improve tissue repair after inflammation or injury as pro-inflammatory and proliferation-inducing mediators [21, 22].

The results from our study showed HaCaT cell co-culture with M1 macrophages produce large amounts of IL-1β, IL-6 and TNF-α than HaCaT cell co-culture with M2 macrophages which demonstrate the ability of HaCaT produce proinflammation cytokines from stimulation of M1 macrophage and identify M1 macrophage as a critical promoter of HaCaT cell activation, independent of T cells. Though a network of cytokines is responsible for the inflammation of psoriasis, TNF-α has been implicated as a master proinflammatory cytokine of the innate immune response due to its widespread targets and sources. TNF-α does not affect the proliferation and differentiation of kc in normal people and psoriasis patients, but it can induce the expression of keratin k6, k16, k17 genes through the p38 MAP kinase pathway and indirectly promote the proliferation of KCs [23]. In psoriasis, TNF-α supports the expression of adhesion molecules (intercellular adhesion molecule 1 and P- and E-selectin), angiogenesis via vascular endothelial growth factor, the synthesis of proinflammatory molecules (IL-1, IL-6, IL-8, and nuclear factor κβ), and KCs hyperproliferation via vasoactive intestinal peptide [24].

In patients with psoriasis, IL-1β and IL-8 was significantly more expressed in the early phase of psoriasis lesional skin than in normal skin. While IL-12, IL-17, IL-23 and TNF-α and IFN-γ were slightly more expressed in psoriasis in normal skin without reaching statistical significance [25]. IL-1β is a pivotal cytokine in the innate immune response, and it is well known that its over-production plays a crucial role in the autoinflammation, which is a state of sterile inflammation not mediated by circulating autoantibodies and autoreactive T cells [26]. Activated IL-1β thus released has several paracrine effects including the production of TNFα by local KCs and upregulation of leucocyte chemotactic proteins e.g. selectins, which promote the skin infiltration and activation of T cells. IL-18 and IL-1β are further involved in the differentiation of Th1 cells and Th17 cells, respectively. Interleukin-6 is a multifunctional pro-inflammatory cytokine. Interleukin-6 is proved to be associated with many chronic inflammatory diseases and autoimmunity diseases such as psoriasis [27]. Serum IL-6 levels significantly correlated with PASI scores in patients with psoriasis vulgaris and significantly decreased by infliximab and adalimumab therapy [28]. γ-irradiation of KCs induces ATP release via activation of the TRPV4 channel, and then ATP activates P2Y11 receptor and p38 MAPK-NF-κB signaling, resulting in IL-6/IL-8 production [29].

## In conclusion

In summary, our study identified that LPS-treated monocyte/macrophages (M1) selectively produced IL-12p70, TNF-α in sharply contrast to IL-4-treated monocyte/macrophages (M2) subsets and further induced HaCaT cells secrete more proinflamatory cytokines such as IL-1β, IL-6 and TNF-α, which suggested that the previously identified that the blocking M1 macrophage polarization could be a strategy which enables inhibition of psoriatic inflammation activated proinflammatory molecules.

In addition, this study showed a distinct role of M1 macrophage inhibiting proliferation of HaCaT cells and promoting their differentiation and apoptosis. This effect of M1 macrophage to inhibit HaCaT proliferation was through inducing G0/G1/S phase arrest and inducing tumor cell apoptosis. On the contrary, M2 macrophages has no effect on apoptosis but makes HaCaT cells block the S phase of cell cycle and inhibits the proliferation of HaCaT cells. Of translational importance, our findings provide evidence that the distinct pro-apoptosis phenotype of KC unrelated to the inflammatory component secretion of M1 macrophage, implies that M1 macrophage polarization may contribute to the pathogenesis of psoriasis by induce keratinocyte apoptosis.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**Conflict of interest:** None.

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