

## Original Article

# Effects of *CD14* and *TLR4* on LPS-mediated normal human skin fibroblast proliferation

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Received November 16, 2014; Accepted January 8, 2015; Epub February 15, 2015; Published February 28, 2015

**Abstract:** Background: This study was designed to elucidate the effects of *CD14* and *TLR4* on the proliferation of LPS-mediated normal human skin fibroblasts and to gain new insights into the mechanisms underlying LPS-induced scar formation. Methods: Cell morphology and density were observed using an inverted microscope. Proliferation curves were described by MTT. The number of proliferating cells was counted by staining the cells with trypan blue. Cells were stained with propidium iodide (PI) and the phase of cell generation cycle was determined by flow cytometry. Results: The growth rate detected in groups B, C, D was slower compared to group A after 48 h. All proliferation curves were approximately S shaped and the peak was seen on days 6, followed by a sharp decline on days 7. The proliferation rate of groups B, C, D was significantly slower than group A. After the staining, we found a significantly smaller number of proliferating cells in groups B, C, D as compared to group A. Flow cytometry revealed that the ratio of proliferating cells at S phase was lower in the groups B, C, D as compared to group A. Conclusions: Both separate and combined use of anti-*CD14* and anti-*TLR4* enable a significant decline in the rate and capacity of proliferation, with the latter pretreatment resulting in the sharpest reduction. Therefore, it seems likely that anti-*CD14* along with anti-*TLR4* is significantly engaged in the signaling mechanisms of LPS-mediated fibroblast proliferation.

**Keywords:** Toll like receptor 4, *cd14*, lipopolysaccharide, fibroblast, proliferation

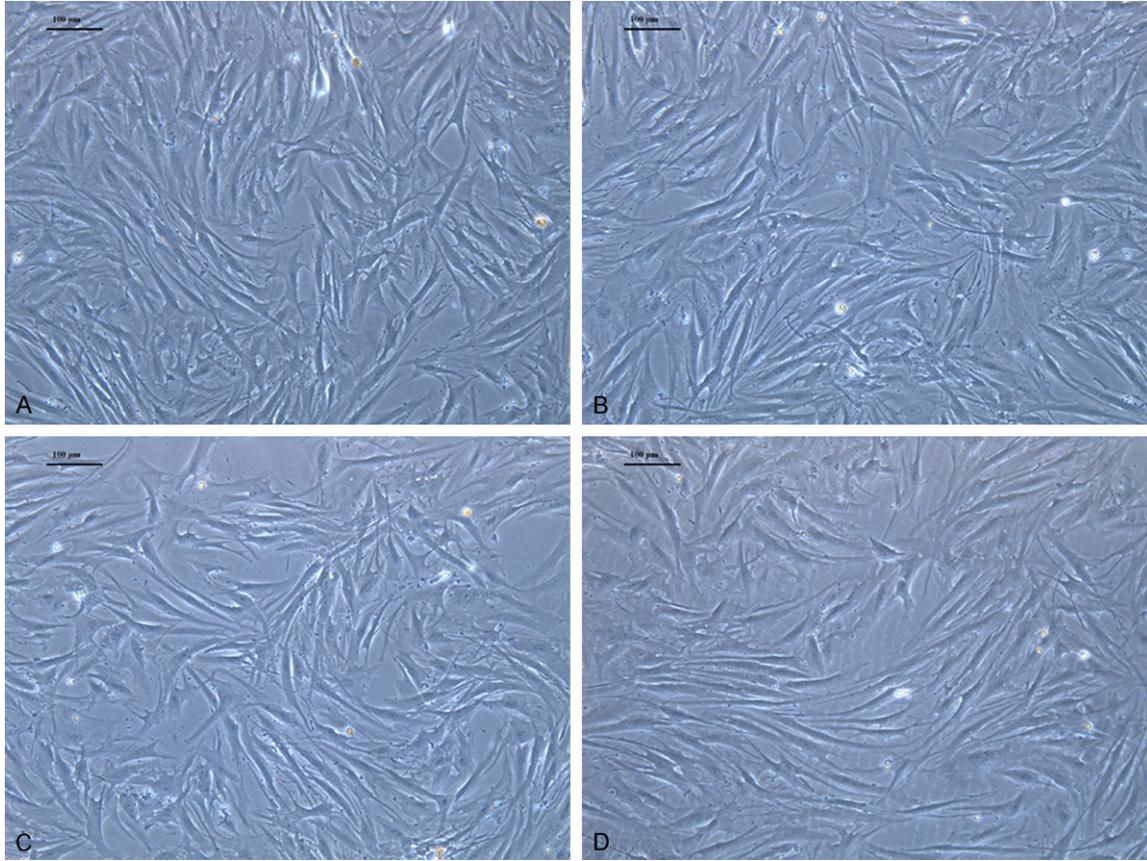
## Introduction

Prevention and treatment of hypertrophic scar have always been the focuses and difficulties in surgical trauma community. Hypertrophic scar is being thought of as the consequence of fibroblast hyperplasia, excessively aggregated extracellular matrix and unavailability of the signals responsible for degrading these matrixes during the repair process. Available data have documented that fibroblasts as the main effector cells are involved in the fibrosis of many target organs, such as liver, and lungs [1-3]. The pathogenic bacteria engaged in the late stage of trauma consist predominantly of gram negative bacteria [4-6]. Lipopolysaccharide (LPS) present in gram negative bacteria could alter the biological characteristics of normal skin fibroblasts, and has similar biology to hypertrophic scar fibroblasts [7-9].

Toll-like receptor 4 (named TLRs thereafter) is a class of transmembrane proteins latching onto

the cell membrane or transwell membrane, and could identify LPS, bacterial lipoprotein or double stranded RNA virus [10]. Existing data have shown that LPS activates the TGF-beta signaling pathway by cooperating with *TLR4*, leading to advanced liver fibrosis and more serious injuries consequently [11]. Results of earlier research demonstrated that LPS and *TLR4* interactions can activate the downstream signals unless the engagement of *CD14* [12]. Conversely, more recent data have shown that *CD14* is not a must in the activation [13]. In view of this, we pretreated normal human skin fibroblasts by using *CD14* and *TLR4* in isolation or in combination, then stimulated the fibroblasts with LPS, attempting to observe the changes in cell growth characteristics and cell cycle, to elucidate the role *CD14* and *TLR4* play in LPS-stimulated normal human skin fibroblast proliferation, and to gain new insights into the molecular mechanisms of LPS-induced scar formation.

## CD14 and TLR4 on skin fibroblast proliferation



**Figure 1.** Morphologic appearance of human normal skin fibroblasts in distinct groups (inverted microscope, 100 ×; A. Group A, B. Group B, C. Group C, D. Group D).

### Materials and methods

#### Reagents and instruments

Reagents were composed of RPMI 1640 basic medium, FBS, trypsin-EDTA (Gibco company, USA), antibiotics of penicillin and streptomycin (PAA company, USA), human normal skin fibroblast cell lines (provided by Burn Research Institute), LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), RNA enzyme, propidium iodide (PI) (Sigma, USA), and analytical reagents (China). Instruments consisted of CO<sub>2</sub> incubator (SANYO, Japan), inverted microscope (Leica company, Germany), microplate reader model 450 (Bio-Rad company, USA), and flow cytometry (Becton Dickinson, USA).

#### Grouping

The frozen normal human skin fibroblasts cell lines were resuscitated, and subsequently cultured in RPMI 1640 medium composed of 10% FBS at 37°C with 5% CO<sub>2</sub>. Cell digestion and

passage were done with 0.25% trypsin. We selected the third to the tenth generation cells for later experiments and categorized the cells into four groups, including 0.1 g/mL LPS reference group (group A), anti-CD14 pretreatment + LPS group (group B), anti-TLR4 pretreatment + LPS group (group C), anti-CD14 combined with anti-TLR4 pretreatment + LPS group (group D).

#### Morphologic observation of fibroblasts

The third generation fibroblasts were cultured in T-25 flasks with RPMI 1640 complete medium containing 10% FBS for 24 h, then with pretreatment liquid (5 ml) for 6 h, and with a final step of incubation with 0.1 µg/mL LPS for 48 h. An inverted microscope was utilized to determine the cell morphology and density.

#### Proliferation curve of fibroblasts

Normal human skin fibroblasts ( $1 \times 10^4$ /ml) were cultured in 96-well plates (0.2 ml/well) for 24 h, then cultured with pretreatment liquid

## CD14 and TLR4 on skin fibroblast proliferation

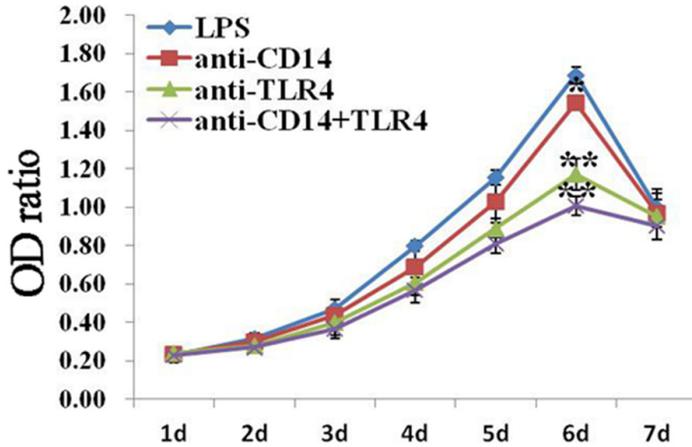


Figure 2. Proliferation curve of human normal skin fibroblasts in distinct groups.

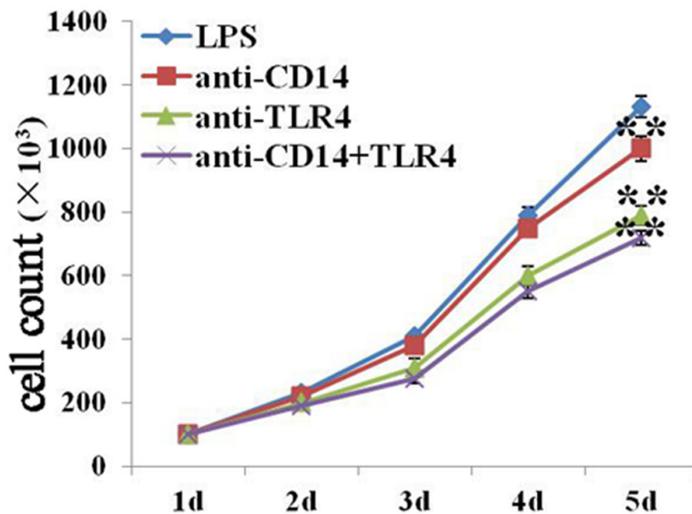


Figure 3. Proliferation quantities of human normal skin fibroblasts in distinct groups.

(0.2 ml), and continued to be cultured with 0.1  $\mu\text{g/ml}$  LPS. A single plate was removed out on days 0, 1, 2, 3, 4, 5, 6, 7, and 5 mg/ml MTT was supplemented (20  $\mu\text{l/well}$ ). After incubation at 37°C for 4 h, we discarded the supernatant, and added 200  $\mu\text{l}$  DMSO into each well, followed by oscillation for 5 min. Microplate reader was used to test the OD490 values based on the growth curve. 8 wells were prepared for each group at each time point.

### Numbering of proliferating fibroblasts

Normal human skin fibroblasts ( $0.5 \times 10^5/\text{ml}$ ) were cultured in 12-well plates (2 ml/well). After 24 h, the cells were cultured with pretreatment liquid (2 ml) for 6 h and with 0.1  $\mu\text{g/ml}$  LPS for

another 48 h. The cells were harvested, and counted by staining with trypan blue.

### Cell cycle analysis

Fibroblasts were cultured in T-25 flasks containing RPMI 1640 medium for 24 h. All cells were further cultured with pretreatment liquid (5 ml) for 6 h, followed by incubation with 0.1  $\mu\text{g/ml}$  LPS for 48 h. The cells digested with 0.25% trypsin were harvested, and fixed by 75% ice-cold ethanol, followed by washing with PBS, resuspension, filtering with 300 mesh nylon screen, addition of RNase to remove RNA-related interference, and addition of PI to label the cells. Cell cycle was determined by use of flow cytometry.

### Statistical methods

Statistical analyses were performed using software SPSS16.0. Data are shown as mean  $\pm$  SD. Univariate analyses were done using one-way ANOVA. Pairwise comparisons were undertaken using SNK-q. Kruskal-Wallis H test was used in case of heterogeneity of variance. Between-group comparisons were carried out by Nemenyi test. The significance level was set at  $P < 0.05$ .

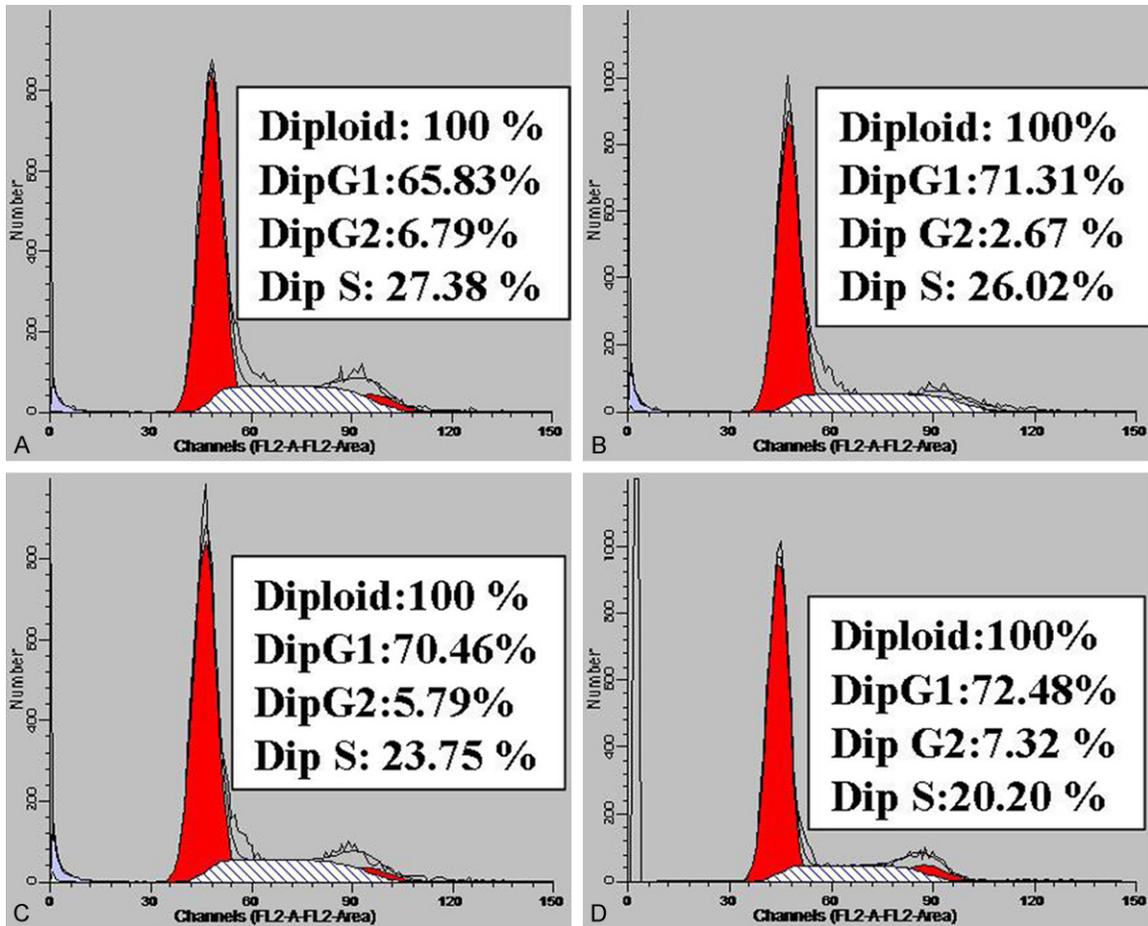
## Results

### Morphologic appearance

Inverted microscope showed that all cells were morphologically similar, elongated and spindle shaped. After culture for 48 h, cell density of groups A, B, C, D, was ( $99 \pm 1\%$ ), ( $94 \pm 2\%$ ), ( $83 \pm 3\%$ ) and ( $76 \pm 2\%$ ), respectively. The observed fusion speed of the latter three groups, compared with the former group, was statistically significantly slower, with group D showing the slowest speed ( $P < 0.01$ ), as illustrated in Figure 1.

### Proliferation curve

Approximately S shaped curves were observed for all investigated groups. The cells were in incubation period on days 1~2. The logarithmic growth started on days 3, and the peak was



**Figure 4.** Cell cycle of human normal skin fibroblasts in distinct groups (A. Group A, B. Group B, C. Group C, D. Group D).

observed on days 6, followed by a rapid decrease on days 7. The number of proliferating cells was significantly smaller in groups B, C, D, as compared to group A. The minimum number was seen in group D ( $P < 0.01$ ). The results are shown in **Figure 2**.

*Number of proliferation cells*

The cells were stained with trypan blue to count the number of proliferation cells. On days 5, the number in groups A, B, C, D was  $(1132 \pm 32) \times 10^3$ ,  $(1000 \pm 40) \times 10^3$ ,  $(790 \pm 31) \times 10^3$  and  $(720 \pm 23) \times 10^3$ , respectively. Groups, B, C, D, compared to group A, showed a significant decline after amplification, with the smallest number detected in group D ( $P < 0.01$ ), as illustrated in **Figure 3**.

*Cell cycle changes*

After having been cultured for 48 h, the rate of cells at S phase in groups A, B, C, D was (27.58

$\pm 0.32$ )%,  $(25.82 \pm 0.27)$ %,  $(23.91 \pm 0.44)$ % and  $(20.24 \pm 0.63)$ %, respectively. We observed significantly lower rate in groups B, C, D than in group A, and the minimum rate was observed in group D ( $P < 0.01$ ). The results are displayed in **Figure 4**.

**Discussion**

Hypertrophic scar occurs in case of wound healing after severe trauma. The pathogenesis remains as yet to be elucidated. We have previously found that LPS plays a significant role in promoting the proliferation of normal human skin fibroblasts, and its proliferation characteristics are almost the same as scar fibroblast [7, 8]. It is also reported that severe wound infection is the main promoter of hypertrophic scar. We therefore speculated that not only the severe wound infection but also the skin fibroblast are involved in the formation of hypertrophic scar.

## CD14 and TLR4 on skin fibroblast proliferation

TLRs are important pattern recognition receptors, and have an essential role in protecting hosts against a large repertoire of microorganisms. Previous data have shown that TLRs are expressed in an array of skin cells, such as keratinocytes, Langerhans cells, macrophages, T cells, B cells, columnar cells and endothelial cells [14, 15]. Several lines of evidence have indicated that *TLR4* as a member of the TLRs family is also expressed in skin fibroblasts [16, 17]. Due to the severe wound infection and wound bacteria composed predominantly of gram negative bacteria at the later stage of serious burns, there are a large number of LPSs [18]. The present work has provided evidence that both separate and combined use of *CD14* and *TLR4* to pretreat cells resulted in notably reduced capability in the proliferation of LPS-mediated human normal fibroblasts. We noted the most significant reduction when *CD14* and *TLR4* were used in combination. We thus believed that *CD14* along with *TLR4* was involved in LPS-mediated human normal fibroblast proliferation. This finding is consistent with an earlier observation that *TLR4* is the target of gram negative bacteria induced sepsis treatment [19].

In conclusion, this study shows that *CD14* together with *TLR4* is significantly engaged in the proliferation of LPS-mediated human normal fibroblasts. Our study may provide new insights for better understanding the molecular basis of LPS-induced scar formation. Additional research is needed to confirm the findings being presented in the current work.

### Disclosure of conflict of interest

None.

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