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The Expression of Functional Toll-Like Receptor 4 Is Associated With Proliferation and Maintenance of Stem Cell Phenotype in Endothelial Progenitor Cells (EPCs)

Jin He,[1,2] Zhilin Xiao,[1] Xiaobin Chen,[1] Meifang Chen,[1] Li Fang,[1] Mei Yang,[1] Qingshan Lv,[1] Yuehui Li,[3] Guancheng Li,[3] Jinyue Hu,[4**] and Xiumei Xie[1*]

[1]*Department of Geriatric Cardiology, Xiangya Hospital, Central South University, Changsha 410008, China*
[2]*Department of Cardiology, Renmin Hospital of Hunan Province, Changsha 410005, China*
[3]*Cancer Research Institute, Central South University, Changsha 410078, China*
[4]*Central Laboratory, Renmin Hospital, Wuhan University, Wuhan 430060, China*

ABSTRACT

Endothelial dysfunction is involved in various cardiovascular diseases such as atherosclerosis. Endothelial progenitor cells (EPCs) contribute to re-endothelialization and neo-vascularization, and the increase of EPCs in peripherial circulation benefits the prognosis of cardiovascular disease. However, little is known about the biological stimuli that initiate the proliferation and the maintenance of stem cell phenotype of EPCs. Here we reported that human umbilical vein blood derived EPCs expressed gene transcripts coding for Toll-like receptor (TLR) 1–6, TLR8–10, TLR4 co-receptor CD14, and myeloid differentiation factor 88 (MyD88), a TLR adaptor molecule. Protein expression of TLR2, 4, CD14, and MyD88 was also detected by FACS or Western blot. The activation of TLR4 by LPS modulated the expression of TLRs, induced the phosphorylation of NF-κB, P38, and ERK42/44, and up-regulated the gene expression of cytokines IL-8, IFN-α, IFN-β, and TNF-α, suggesting EPCs expressed functional TLR4. Unexpectedly LPS treatment failed to induce apoptosis in EPCs, but instead promoted cell proliferation of EPCs. Furthermore, the treatment of EPCs with LPS up-regulated stem cell markers AC133 and CD34 in both mRNA and protein levels, and down-regulated the protein expression of differential marker eNOS. These results suggested that TLR4 functions to maintain the stem cell phenotype of EPCs and enlarge its population, which reveals a novel aspect of the multiple-faced TLR biology, and may open new prospects for using TLR4 agonists to promote the production of EPCs for clinical use. J. Cell. Biochem. 111: 179–186, 2010.

KEY WORDS: TOLL-LIKE RECEPTOR 4; PROGENITOR ENDOTHELIAL CELLS; PROLIFERATION; LIPOPOLYSACCHARIDE; CELL SURFACE MARKERS

Despite changes in lifestyle and the use of new pharmacologic therapies, cardiovascular disease remains the leading cause of death in the United States, Europe, and much area of Asia [Braunwald, 1997; Breslow, 1997]. The development of atherosclerosis may results in myocardial infarction, heart failure, stroke, and peripheral artery disease, finally leading to irreversible organ damage [Werner and Nickenig, 2006]. It has been proposed that endothelial dysfunction is the initial step in the development of atherosclerotic plaques. According to the response-to-injury hypothesis, cardiovascular risk factors induce a chemical or mechanical injury of the endothelium that triggers and enables the concomitant invasion of macrophages and lipid deposition [Libby et al., 1997]. Various injuries to the endothelium may increase the local recruitment of monocytes, leukocytes, and platelets and lead to the loss of the anti-thrombotic properties of the vessel wall [Nakashima et al., 1998; Ross, 1999]. Endothelial progenitor cells (EPCs) are special sub-type progenitor cells with ability to differentiate into mature endothelial cells, and contribute to re-endothelialization and neo-vascularization which are highly associated with cardiovascular disease [Hristov and Weber, 2004]. In general, EPCs can be derived from bone marrow, peripheral blood, and umbilical cord blood, characterized by three surface markers: AC133, CD34, and KDR [Dzau et al., 2005]. An amount of experiments in animals proved that systemic transfusion or intrinsic

Jin He and Zhilin Xiao contributed equally to this work.

*Correspondence to: Prof. Xiumei Xie, Department of Geriatric Cardiology, Xiangya Hospital, Central South University, Changsha 410008, China. E-mail: xyxiexm@sina.com

**Correspondence to: Dr. Jinyue Hu, Central Laboratory, Renmin Hospital, Wuhan University, Wuhan 430060, China. E-mail: jinyuehu@whu.edu.cn

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mobilization of EPCs enhances the restoration of the endothelium after focal endothelial denudation [Griese et al., 2003; Kong et al., 2004]. However, in human, circulating CD34/KDR positive progenitor cells in patients with CAD are reduced to about 50% of that in control groups [Vasa et al., 2001]. Cardiovascular risk factors impair the number and function of EPCs, restricting the therapeutic potential of progenitor cells [Werner and Nickenig, 2006]. Therefore, how to improve the level of circulation EPCs has become the research focus for clinical scientists.

Toll-like receptors (TLRs), a kind of germline-encoded pattern-recognition receptors (PRRs), are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on non-immune cells such as fibroblasts and epithelial cells. TLRs can recognize distinct microbial components and directly activate immune cells [Akira et al., 2006]. Exposure of immune cells to the ligands of these receptors activates intracellular signaling cascades that rapidly induce the expression of a variety of overlapping and unique genes involved in the inflammatory and immune responses. It has been elucidated that TLRs can regulate both innate immunity and acquired immunity and play a key role in anti-microbial and anti-endogenous ligands host defense. To date, 12 members of the TLR family have been identified in mammals. Among of them, TLR4 is expressed in endothelial cells, macrophages and recognizes a very divergent collection of ligands such as lipopolysaccharide (LPS), heat-shock protein 60 and 70, fibrinogen, and the fusion protein of respiratory syncytial virus (RSV) [Akira et al., 2006]. Some studies found that there was a significant high expression of TLR4 in atherosclerotic plaques, and proved that the synthesis and release of a series of cytokines associated with atherosclerosis depends on the recognition function of TLR4 [Vasa et al., 2001], suggesting that the process of TLR4-mediated inflammatory response of immune cells acts as a bridge in connecting immune responses with chronic inflammation in atherogenesis. However, little is known about the effect of TLR activation on EPC's proliferation and function.

Recently, it had been reported that TLRs were expressed in various kind of stem cells, and functioned to induce cytokine expression, to promote proliferation and differentiation [Chen et al., 2009; Covacu et al., 2009; De Luca et al., 2009; Lee et al., 2009; Liotta et al., 2008]. In the present study, we analyzed the expression and the function of TLR4 in EPCs from human umbilical cord blood, and found that activation of TLR4 by LPS induced proliferation but not apoptosis in EPCs. Unexpectedly, TLR4 also functioned to maintain the stem cells phenotype of EPCs. These results suggested that TLR4 ligands may promote the production of EPCs for clinical use.

MATERIALS AND METHODS

CELL CULTURE

Human umbilical cord blood samples were collected in sterile blood packs containing heparin solution as an anticoagulant, as described previously [Murohara et al., 2000; Aoki et al., 2004]. The samples were stored at room temperature and were processed within 24 h after blood collection. Informed consent was obtained from all mothers before labor and delivery. Protocols for sampling cord blood were approved by the Hospital Ethical Review Board of Clinical Studies.

Mononuclear cells were isolated by density gradient centrifugation with Histopaque 1077 (Sigma–Aldrich) from 50 ml of human umbilical cord blood [Vasa et al., 2001], and $2–5 \times 10^6$ mononuclear cells were plated on six-well culture dishes coated with human fibronectin (Chemicon) in RPMI1640 medium supplemented with 10% fetal bovine serum, 10 ng/ml VEGF (R&D systems, Minneapolis, MN), 2 ng/ml bFGF (Peprotech). After 4 days in culture, non-adherent cells were removed by washing with PBS and adherent cells were cultured continually by the addition of fresh media for another three days, and adherent cells underwent cytochemical analysis. To detect the up-take of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL (DilLDL), cells were incubated with 2.4 μg/ml DiLDL at 37°C for 12 h, and then were fixed with 2% paraformaldehyde for 30 min at room temperature, and incubated with FITC-labeled lectin from Ulex europaeus (FITC-UEA-I, 10 μg/ml) at 37°C for 1 h. Dual-staining cells positive for both lectin and DiLDL were judged as EPCs.

REVERSE TRANSCRIPTION–PCR

Total RNA was extracted from $1–5 \times 10^6$ cells using Trizol (Life Technologies, Gaithersburg, MD) as described by the manufacturer. mRNA was reverse transcribed with RevertAid (MBI Fermemtas, Burlington Ontario, Canada) at 42°C for 60 min, and the resulted cDNA was subjected to PCR (95°C for 1 min followed by 25–35 cycles of 95°C for 30 s, 60°C for 30 s, 68°C for 60 s, and an extension for 10 min at 68°C). PCR products were separated on 1.0% agarose gels and visualized with ethidium bromide. Forward and reverse primer pairs are listed as follows:

AC133-F (5′-GTCTGACCAGCGTGAAAACTA-3′)
AC133-R (5′-CAACTCCAACCATGAGGAAGA-3′)
CD14-F (5′-TTGTGAGCTGGACGATGAAGA-3′)
CD14-R (5′-AGGCATGGTGCCGGTTAT-3′)
CD31-F (5′-CGGATCTATGACTCAGGGACA-3′)
CD31-R (5′-GCCATGACTGAGTACACAGCC-3′)
CD34-F (5′-ATAGCTCAGGACCTGGGACCT-3′)
CD34-R (5′-TTCCTGGGAGAAAATTGTAGC-3′)
eNOS-F (5′-TTCCGGGGATTCTGGCAGGAG-3′)
eNOS-R (5′-GCCATGGTAACATCGCCGCAG-3′)
GAPDH-F (5′-AATCCCATCACCATCTTCCA-3′)
GAPDH-R (5′-CCTGCTTCACCACCTTCTTG-3′)
IFN-α-F (5′-AGCTGCAAGTCAAGCTGCTCT-3′)
IFN-α-R (5′-TTCTTCACAGCCAAGATGGA-3′)
IFN-β-F (5′-TCTCCTCCAAATTGCTCTCCT-3′)
IFN-β-R (5′-TACTCCTTGGCCTTCAGGTAA-3′)
IFN-γ-F (5′-TGCAGGTCATTCAGATGTAGC-3′)
IFN-γ-R (5′-ATGCTCTTCGACCTCGAAACA-3′)
IL-1α-F (5′-TTCAAGGAGAGCATGGTGGT-3′)
IL-1α-R (5′-TGGCTTTTGGGTATCTCAGGCA-3′)
IL-1β-F (5′-TTGAAGCTGATGGCCCTAAAC-3′)
IL-1β-R (5′-CACCAAGCTTTTTTGCTGTG-3′)
IL-6-F (5′-CCAGTACCCCCAGGAGAAGAT-3′)
IL-6-R (5′-TTGCCTTTTTCTGCAGGAAC-3′)
IL-8-F (5′-TTGGCAGCCTTCCTGATTT-3′)

IL-8-R (5′-TCAAAAACTTCTCCACAACCC-3′)
IL-12P40-F (5′-CCCCTGAAGAAGATGGTATCA-3′)
IL-12P40-R (5′-TGATGAAGAAGCTGCTGGTGT-3)′
KDR-F (5′-GATTGTACACCTGTGCAGCAT-3′)
KDR-R (5′-TATTTCCTCCCTGGAAGTCCT-3′)
MyD88-F (5′-TTCTTGAACGTGCGGACACA-3′)
MyD88-R (5′-TTTCGATGAGCTCACTAGCAA-3′)
TLR1-F (5′-TAATGCATTTGATGCCCTGC-3′)
TLR1-R (5′-CCTTCAAGGAAGTGCCAGAAT-3′)
TLR2-F (5′-TTCTTTTTCTTCCCTGGGCA-3′)
TLR2-R (5′-TGAATTTGTTTCACCAGTGGA-3′)
TLR3-F (5′-AAAACCTTTGCCTTCTGCAC-3′)
TLR3-R (5′-GGAATCGTTACCAACCACATT-3′)
TLR4-F (5′-TGTGGCTCACAATCTTATCCA-3′)
TLR4-R (5′-CTAAATGTTGCCATCCGAAA-3′)
TLR5-F (5′-GCTTTTCAGGGACTGTTCCAT-3′)
TLR5-R (5′-CAAACCCATGTGAAAGATCCA-3′)
TLR6-F (5′-TCTTGGGATTGAGTGCTATGA-3′)
TLR6-R (5′-CGGTGTACAAAGCTGTCTGTG-3′)
TLR7-F (5′-GCTGACAAATTTGGAGTTGCT-3′)
TLR7-R (5′-GCCAACTTCACTTGAATCTCC-3′)
TLR8-F (5′-GCTGACAAATTTGGAGTTGCT-3′)
TLR8-R (5′-AAATGCAATGCCCGTAGAGA-3′)
TLR9-F (5′-TGAGGACCTGGCCAATCTGA-3′)
TLR9-R (5′-AAGGCCCTGAAGATGCCGA-3′)
TLR10-F (5′-CAAATGCACAAATGCCACAC-3′)
TLR10-R (5′-AAAATCCAGAGATGGGCTGA-3′)
TNF-α-F (5′-TGACAAGCCTGTAGCCCATGTT-3′)
TNF-α-R (5′-AGGGCAATGATCCCAAAGTAGA-3′)
VE-Cadherin-F (5′-TGCCATCGATAATTCTGGAC-3′)
VE-Cadherin-R (5′-AGAAAGGCTGCTGGAAAATG-3′)
vWF-F (5′-GAGGCTGAGTTTGAAGTGC-3′)
vWF-R (5′-CTGCTCCAGCTCATCCAC-3′).

For semi-quantitation, gels were scanned and the pixel intensity for each band was determined using the ImageMaster VDS (Pharmacia Biotech) and normalized with the level of GAPDH.

CELLULAR APOPTOSIS ASSAY

The apoptosis of cells were detected by propidium iodide (PI)/Annexin V staining. In general, 10^6 stimulated or unstimulated cells were washed twice with phosphate-buffered saline (PBS), then labeled by Annexin V–fluorescein isothiocyanate (FITC) and PI in binding buffer according to the instructions in the PI/Annexin V–FITC Apoptosis Detection Kit provided by Jingmei (Shenzhen, China). Fluorescence signals of FITC and PI were detected on a FACScan (BD Bioscience, San Jose, CA). The log of Annexin V–FITC fluorescence was displayed on the x-axis, and the log of PI fluorescence was displayed on the y-axis. For each analysis, 10,000 events were recorded.

FLOW CYTOMETRY

Human EPCs were grown to sub-confluency, and washed with fluorescence-activated cell sorting buffer (5 mM EDTA, 0.1% NaN_3, and 1% FCS, in Dulbecco's PBS). After incubation with a monoclonal antibody against human CD14, TLR2, and TLR4 (R&D systems) for 40 min on ice, the cells were stained with a FITC-labeled secondary antibody and examined for protein expression by flow cytometry (BD Bioscience). For one step staining, EPCs were incubated with PE-conjugated anti-AC133 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), or PE-conjugated anti-KDR mAb (Miltenyi Biotec), or FITC-conjugated anti-CD34 mAb (BD Pharmingen, San Diego, CA), respectively, for 40 min on ice, and washed with cold PBS for three times. The protein expression was measured by flow cytometry.

WESTERN BLOTTING ANALYSIS

EPCs were lysed in 150 μl of 1× SDS sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mM DTT], sonicated for 3 s, and then boiled for 5 min. The cell lysate was then centrifuged at 10,000*g* at 4°C for 10 min. Total protein was electrophoresed on 10% Tris–glycine precast gels (Invitrogen) and transferred onto Immunoblotin P membranes (Millipore). The membranes were blocked by incubation in 3% non-fat dry milk for 1 h at room temperature and then incubated with primary antibodies (CD14, TLR4 antibodies from R&D Systems, MyD88, eNOS antibodies from Santa Cruz, phospho-NF-κB P65, NF-κB P65, phospho-p38, p38, phospho-Erk, Erk antibodies from Cell Signaling, anti-GAPDH antibody from Beyotime, Hangzhou, China) in PBS containing 0.1% Tween-20 overnight at 4°C. After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected with Super Signal Chemoluminescent Substrate Stable Peroxide Solution (Pierce) and BIOMAX-MR film (Eastman Kodak). When necessary, the membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and reprobed with antibodies against various cellular proteins.

PROLIFERATIVE MTT ASSAY

The adherent cells were trypsinized and suspended in 100 μl medium at day 14. About 4,000 cells/well were placed on fibronectin-coated 96-well plates and cultured for 24 h. Twenty microliters of MTT (15 mg/ml, Oxiod) was added to each well. After incubation for 4 h at 37°C, the supernatant was removed and 150 μl of dimethyl sulfoxide (DMSO, Sigma) was replaced for 10 min, whereupon light absorbance at 490 nm was detected using an enzyme-linked immunosorbent assay (ELISA) plate reader.

STATISTICS ANALYSIS

All experiments were performed at least three times, and the representative results were shown. Results are expressed as the mean plus or minus the standard deviation (SD). Differences between groups were examined for statistical significance using Student's *t*-test, and *P* values equal to, or less than, 0.05 were considered statistically significant.

RESULTS

CHARACTERIZATION OF EPCs

It has been reported that in patients with bacterial pneumonia, the circulating EPCs is increased [Yamada et al., 2005], but the mechanism is not fully understood. To determine the effect of TLR4 activation on the survival, apoptosis and proliferation of EPCs,

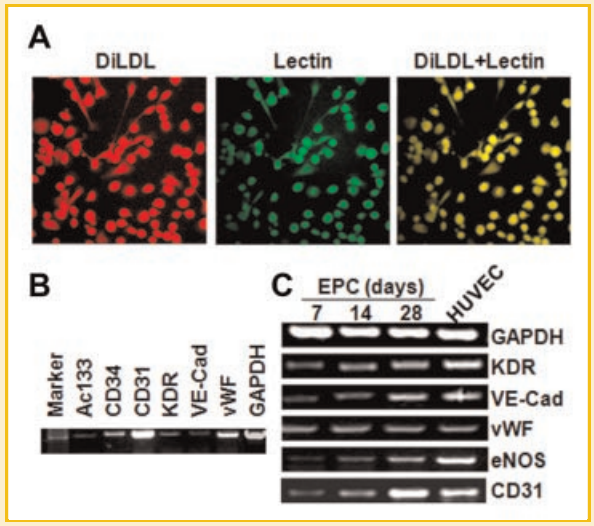

Fig. 1. Characterization of EPCs. A: DiLDL uptake (left panel) and lectin binding (middle panel) of isolated EPCs was determined by fluorescence microscopy. Overlay images are shown in right panel. B: The expression of stem cell markers in EPCs. Cells cultured at day 7 were harvested, and the total RNA were isolated and reverse transcripted for the detection of gene expression of AC133, CD34, CD31, KDR, VE-Cad, vWF, and eNOS by RT-PCR, GAPDH was detected as loading control. C: Gene expression of endothelial markers in EPCs. EPCs culture at days 7, 14, and 28 were harvested, total RNA were isolated and reverse transcripted for the detection of gene expression of KDR, VE-Cad, vWF, eNOS, and CD31. Genes expressed in HUVEC were detected as control.

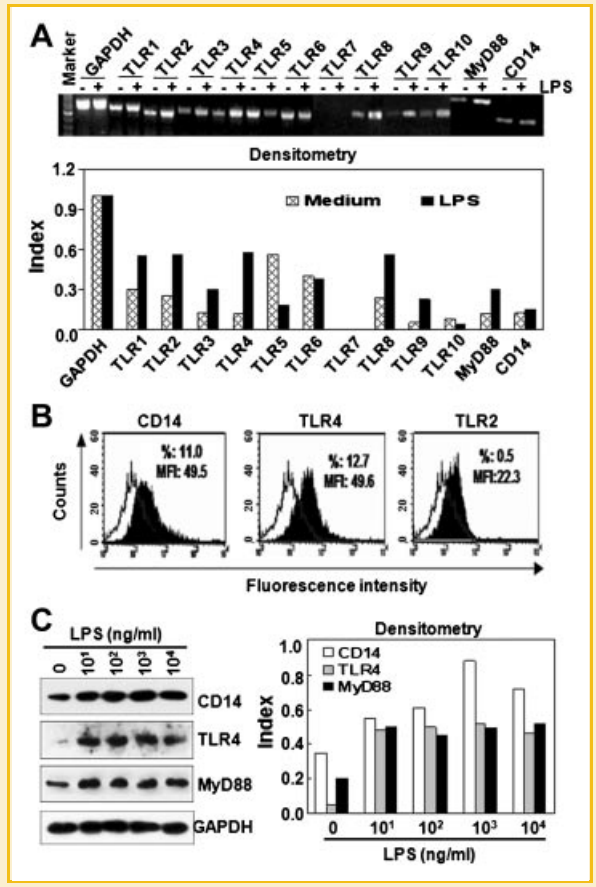

Fig. 2. TLRs expression in EPCs. A: Gene expression of TLRs in EPCs. EPCs treated with or without 100 ng/ml LPS for 24 h were harvested, total RNA were isolated, and reverse transcripted for the detection of TLR1–10, MyD88, and CD14 by RT-PCR. The levels of gene expression were semi-quantitated by scanning the pixel intensity of the bands using the ImageMaster VDS (Pharmacia Biotech) and normalized with the levels of GAPDH. B: Protein expression of CD14, TLR4, and TLR2. EPCs cultured on six-well plates were harvested, fixed, and stained with mouse anti-CD14, TLR4, and TLR2 antibodies, followed by incubation with FITC-labeled rabbit anti-mouse IgG. Protein expression was detected by FACS. C: Up-regulation of CD14, TLR4, and MyD88 induced by LPS. EPCs treated with indicated LPS at 37°C for 24 h were lysed. Equal amounts of total proteins were electrophoresed and blotted for the detection of CD14, TLR4, and MyD88 by Western blot, respectively. GAPDH protein was detected as loading control. The levels of protein expression were semi-quantitated by scanning the pixel intensity of the bands and normalized with the levels of GAPDH.

mononuclear cells were isolated from human umbilical cord blood. After the culture with 10 ng/ml VEGF and 2 ng/ml bFGF for 7 days, adherent EPCs were characterized by DiLDL uptake and concomitant lectin binding (Fig. 1A) [Vasa et al., 2001], and by expressing of AC133, CD34, and CD31, which are markers of stem cells. The endothelial origin was further documented by demonstrating the expression of KDR, vWF, and vascular endothelium cadherin by RT-PCR (Fig. 1B). Moreover, late stage EPCs expressed higher level of eNOS than early stage EPCs, indicating that late stage EPCs began to differentiate (Fig. 1C).

THE EXPRESSION OF TLRS IN EPCs

Pretreatment with LPS or IFN-γ significantly induces the expression of TLR2 and TLR4 in freshly isolated human umbilical vein endothelial cells (HUVEC) [Faure et al., 2001]. Also IFN-α sensitizes freshly isolated HUVEC to double-stranded RNA (dsRNA) induced apoptosis [Kaiser et al., 2004]. In this study, we detected the expression of TLRs in rest EPCs and LPS treated EPCs. The RT-PCR results indicated that rest EPCs expressed TLR1-6, TLR8-10, TLR4 co-receptor CD14 and MyD88, an adaptor molecule in signaling pathway downstream several TLRs (Fig. 2A). The protein expression of CD14, TLR4, and TLR2 was also detected in EPCs by FACS (Fig. 2B). LPS treatment of EPCs up-regulated the gene expression of TLR1, 2, 3, 4, 8, 9, 10, MyD88, and CD14, down-regulated gene expression of TLR5 and 6 (Fig. 2A), and LPS dose dependently up-regulated the protein expression of CD14, MyD88, and TLR4 (Fig. 2C). These results suggested that EPCs expressed functional TLR4.

THE TREATMENT OF EPCs WITH LPS UP-REGULATED THE EXPRESSION OF CYTOKINES AND INDUCED SIGNAL TRANSDUCTION

Toll-like receptors function to induce innate immune response by the induction of pro-inflammatory cytokines. We found that the treatment of HUVECs with LPS induced the up-regulation of cytokine IL-1β, TNF-α, IL-6, and IL-8 (data not shown). In this study, we found that the treatment of EPCs with 100 ng/ml LPS for 24 h up-regulated the gene expression of IL-8, IFN-α, IFN-β, and TNF-α

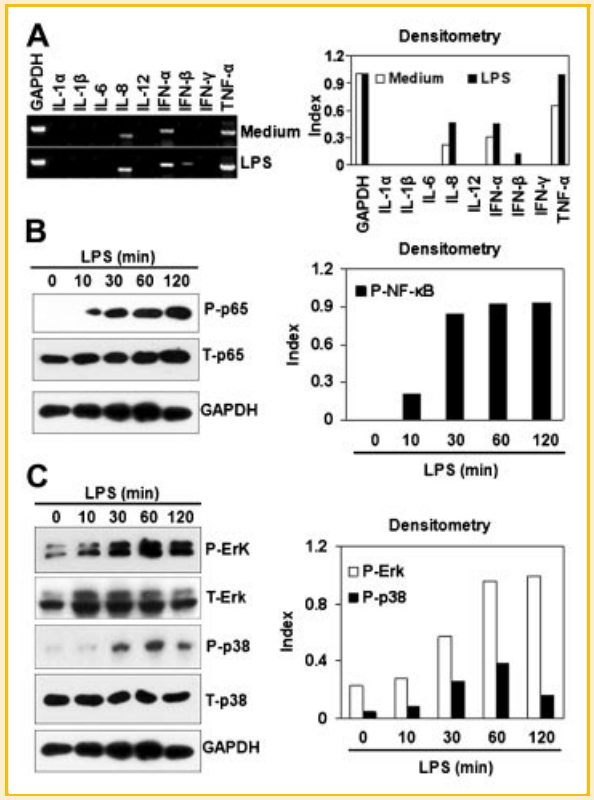

Fig. 3. EPCs express functional TLR4. A: LPS up-regulated gene expression of cytokines. EPCs untreated or treated with 100 ng/ml LPS at 37°C for 24 h were harvested. Total RNA was extracted, and RT-PCR was done to detect gene expression of cytokines indicated. The levels of gene expression were semi-quantitated by scanning the pixel intensity of the bands using the ImageMaster VDS (Pharmacia Biotech) and normalized with the levels of GAPDH. B: LPS induced the phosphorylation of NF-κB in EPCs. Cells starved with serum-free medium overnight were cultured in the presence of 100 ng/ml LPS at 37°C and lysed at the indicated time points. Equal amounts of total proteins were electrophoresed and blotted for the detecting of phosphorylated NF-κB P65 and total P65, respectively. GAPDH protein was detected as loading control. C: LPS induced the phosphorylation of Erk and P38 in EPCs. Cells were treated as (B). Phosphorylated p38, Erk and total p38, Erk were detected by Western blot. GAPDH protein was detected as loading control. The levels of protein phosphorylation were semi-quantitated by scanning the pixel intensity of the bands and normalized with the levels of total proteins in both panels (B) and (C).

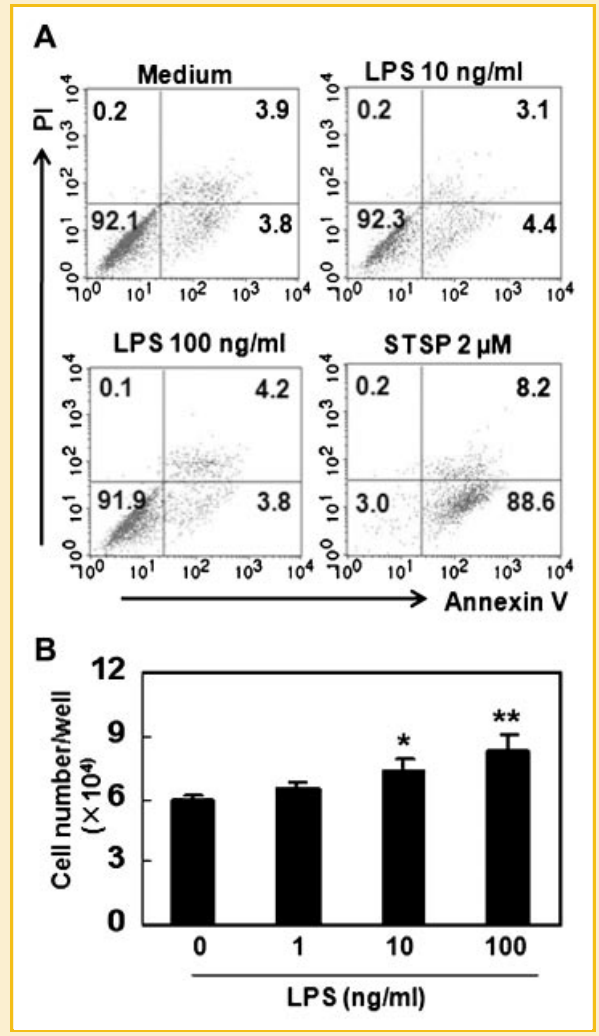

Fig. 4. The effect of TLR4 activation on proliferation and cell apoptosis in EPCs. A: LPS did not induced apoptosis in EPCs. Cells untreated or treated with 100 ng/ml LPS at 37°C for 24 h were harvested and stained by Annexin/PI staining. FACS was done to detect the cell apoptosis. Apoptosis were detected in cells treated with 2 μg/ml staurosporine (STSP) as positive control. B: LPS promoted proliferation of EPCs. 2×10^4 cells were incubated in 96-well plates with indicated LPS for 3 days. Cell number was measured by MTT assay. * $P < 0.05$ and ** $P < 0.01$ compared with medium alone group respectively.

(Fig. 3A). Meanwhile, LPS treatment induced the activation of signal molecules including NF-κB, P38, and Erk, which are important down-stream molecules for TLR signaling [Akira and Takeda, 2004]. Western blot results showed that NF-κB activated with the peak at 120 min, P38 activated with the peak at 60 min and Erk activated with the peak at 120 min (Fig. 3B,C). These results further suggested that EPCs expressed functional TLR4.

THE EFFECT OF TLR4 ACTIVATION ON APOPTOSIS AND PROLIFERATION OF EPCs

Endotoxin LPS is a deleterious factor for many cells and tissue such as endothelial cell and lung tissue by inducing cell apoptosis [Brass et al., 2008; Damico et al., 2008]. As EPCs expressed TLR4 and its co-receptor CD14, we detected the effect of LPS on EPC apoptosis. Unexpectedly, the PI/Annexin V staining results showed that the treatment of EPCs with 10 or 100 ng/ml LPS did not induce apoptosis (Fig. 4A). Then we detected the effect of TLR4 on the proliferation of EPCs. The results from MTT assay indicated that the treatment of EPCs with 10 or 100 ng/ml LPS for 72 h significantly promoted EPC growth ($P < 0.05$ and < 0.01, respectively, Fig. 4B).

THE EFFECT OF TLR4 ACTIVATION ON THE EXPRESSION OF STEM CELL MARKERS IN PECs

TLR agonists selectively promote B cell subsets to differentiate to terminal plasma cells [Genestier et al., 2007]. In response to a recombinant heat shock fusion protein, TLR4 activates DCs and induces the differentiation of cytolytic CD8+ T cells [Palliser et al.,

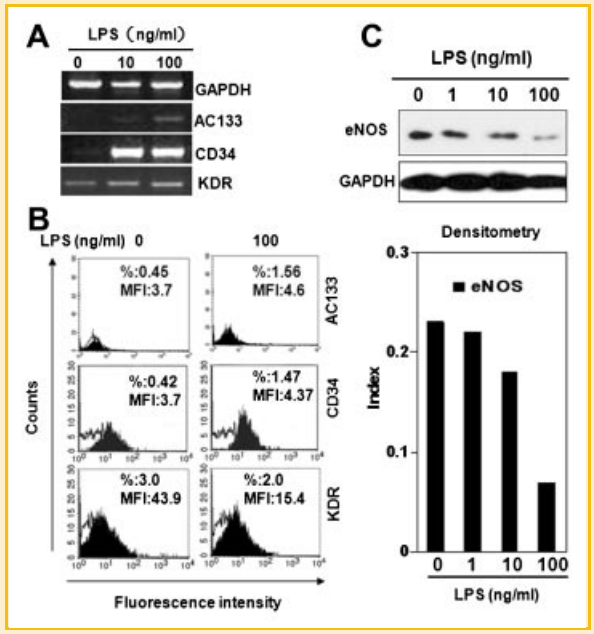

Fig. 5. The effect of TLR4 activation on the expression of stem cell markers in EPCs. A: TLR4 activation up-regulated gene expression of AC133, CD34. EPCs treated with indicated LPS for 24 h were harvested, and total RNA were isolated and reverse transcripted for detection of AC133, CD34, and KDR by RT-PCR. GAPDH was detected for loading control. B: TLR4 activation up-regulated protein expression of AC133 and CD34. EPCs treated with indicated LPS for 24 h were harvested, fixed, and stained with mouse anti-human AC133, CD34 antibodies, followed by incubation with FITC-labeled rabbit anti-mouse IgG. Protein expression was detected by FACS. C: TLR4 activation down-regulated the protein expression of eNOS. EPCs treated with indicated LPS at 37°C for 24 h were lysed. Equal amounts of total proteins were electrophoresed and blotted for the detecting of eNOS by Western blot. GAPDH protein was detected as loading control. The levels of protein expression were semi-quantitated by scanning the pixel intensity of the bands and normalized with the levels of GAPDH.

2004]. Here, we detected the stem cell markers in EPCs to test the effect of TLR4 activation on EPCs differentiation. Unexpectedly, RT-PCR results showed that the treatment of EPCs with 10 or 100 ng/ml LPS for 24 h up-regulated the expression of stem cell markers AC133 and CD34 (Fig. 4A). The treatment of EPCs with 100 ng/ml LPS also up-regulated the protein expression of AC133 and CD34 (Fig. 4B). Western blot results showed that LPS dose dependently down-regulated the expression of eNOS, a differential marker of endothelial cells (Fig. 5C). Taking together, these results indicated that TLR4 functions not to induce EPC differentiation, but instead, to maintain the stem cell phenotype of EPCs.

DISCUSSION

In this study, we have shown that human umbilical vein blood derived EPCs expressed gene transcripts of TLR1-6, TLR8-10, the signaling molecular MyD88 down stream of several TLRs such as TLR4 and TLR2, and the TLR4 co-receptor CD14. Furthermore we also demonstrated that EPCs expressed the proteins of TLR4 and CD14. TLR4 ligand LPS induced TLR4 signaling, resulted in the phosphorylation of NF-κB, P38, and ERK42/44, which all are TLR4 downstream signaling molecules [Akira and Takeda, 2004]. Up-regulation of the genes coding for cytokines including IL-8, IFN-α, IFN-β, and TNF-α was induced by LPS treatment, suggesting that EPCs expressed functional TLR4. Unexpectedly LPS treatment failed to induce apoptosis of EPCs, but instead promoted cell proliferation. In addition, the treatment of EPCs with LPS up-regulated stem cell markers AC133 and CD34 in both mRNA and protein levels, and down-regulated the differential maker eNOS, suggesting that TLR4 functions to maintain the stem cell phenotype of EPCs.

TLRs have reported to be expressed in various kind of cells, including monocyte, macrophages, DCs, B cells, specific types of T cells and even on non-immune cells such as vascular endothelial cells, fat cells, cardiac cells, and intestinal epithelial cells [Hornung et al., 2002]. Recently, TLRs had been reported to be expressed in stem cells. The viral double-strain RNA receptor TLR3 was expressed in human mesenchymal stem cells and function to induce a cytokine expression profile distinct from that in immune cells [Chen et al., 2009]. Human hematopoietic stem cells and hematopoietic progenitor cells have a comparable pattern of expression of TLR transcripts characterized by the predominance of TLR1, 2, 3, 4, and 6, and the TLR1/2 agonist PAM(3)CSK(4) instructs commitment of human hematopoietic stem cells to a myeloid cell fate [De Luca et al., 2009]. Functional TLRs were reported to be expressed on mouse embryonic stem cells and the TLR ligands stimulate stem cells to proliferate [Lee et al., 2009]. Adult neural stem cells also constitutively express TLR2 and 4. The expression of TLRs is increased by the treatment of proinflammatory cytokines, and TLR ligands induce the production of proinflammatory cytokines, suggesting that neural stem cells may be primed to participate in cytokine production during neuroinflammatory or traumatic conditions [Covacu et al., 2009]. Bone marrow derived mesenchymal stem cells express high levels of TLR3 and 4. The treatment of cells with ligands induces activation of NF-κB as well as the production of cytokines IL-6, 8, and CXCL10 [Liotta et al., 2008]. In our study, we detected the gene expression of all TLRs except TLR7 in EPCs, and TLR4 ligand LPS induced activation of the downstream signal molecules of TLR4, suggesting the TLR4 expressed on EPCs is functional.

LPS, known to be an endotoxin of Gram-negative bacteria, is generally the most potent immunostimulant among cell-wall components. After binding to the membrane receptor TLR4, LPS activates intracellular signaling cascades that rapidly induce the expression of genes involved in the inflammatory and immune responses, followed by the synthesis and release of pro-inflammatory cytokines, chemokines, growth factors, and others. It has been proposed that LPS up-regulates the expression of TLR2 and TLR4 on endothelial cells through NF-κB [Faure et al., 2001]. Our studies also showed that LPS could up-regulate the expression of TLRs in EPCs including TLR4 and TLR2 as well as MyD88 and CD14, the assisted molecules in TLR4 signal transduction, and the LPS treatment resulted in the expression of inflammatory factors including IL-8, IFN-α, IFN-β, and TNF-α. However, we found that LPS did not impact the expression of TLR7.

Many studies have proved that LPS causes morphology change and dysfunction of endothelial cell through both direct and indirect ways. The former refers to that LPS activates TLR and MAPK, NF-κB

signaling pathway which mediates the damage to endothelial cell. The latter refers to that activated TLR induced by LPS mediates signal transduction and activates target gene to produce inflammatory cytokines which further aggravate the damage [Dauphinee and Karsan, 2006]. The levels of circulating EPCs have been investigated in different inflammatory disease states. For example, rheumatoid arthritis (RA) is associated with significantly lower levels of circulating EPCs compared to healthy controls [Grisar et al., 2005]. Mayr also proposed that a low dose of LPS injected into healthy adults minimize the amount of EPCs, which may attribute to the abundant accumulation of TNF [Mayr et al., 2007]. In contrast, circulating EPCs had also been reported to increase in patients with acute lung injury [Burnham et al., 2005], where they seem to have a prognostic value, and in patients with bacterial pneumonia [Yamada et al., 2005], where they contribute to lung repair. Moreover, the circulating hematopoietic and EPCs had increased in the early phase of the acute myocardial infarction [Massa et al., 2005]. As microbial components such as LPS from infected bacteria and factors such as HSP and HMGB1 released from necrotic tissues are TLR4 ligands, we supposed that functional TLR may be contributed to the increase of EPCs. The data in our study that the treatment of EPCs with LPS increased the number of EPCs in in vitro culture, represent a link between TLR4 and the increase of EPCs in some cases of diseases.

The importance of TLRs in recognizing exogenous microbial pathogens and endogenous ligands by the innate defense system is well established [Akira and Takeda, 2004; Akira et al., 2006]. TLRs also functions to promote cell differentiation for various kinds of cells including T, B lymphocytes, plasma cells, bone marrow $CD34^+$ progenitors, and even osteoclast [Bi et al., 2001; Palliser et al., 2004; Rui et al., 2006; Genestier et al., 2007; Sioud and Floisand, 2007; Skokos and Nussenzweig, 2007; Capolunghi et al., 2008; Ha et al., 2008]. However, several studies had reported that TLR4 activation led to the block of cell differentiation. In lethal sepsis, TLR4 activation caused the expansion of the pool of hematopoietic stem cells in the bone marrow, and the block of hematopoietic stem cell (HSC) differentiation, resulting in the onset of neutropenia [Rodriguez et al., 2009]. Hyaluronan (HA), an extracellular matrix (ECM) component present in bone marrow and soft connective tissues, inhibits osteoclast differentiation through TLR4 by interfering with M-CSF signaling, indicating that the interaction between ECM components and innate immune receptors can play an important role in the regulation of bone metabolism [Chang et al., 2007]. TLR9 ligand had also been reported to inhibit osterclast differentiation [Amcheslavsky and Bar-Shavit, 2007]. In our studies, we found that human umbilical vein blood derived EPCs expresses several TLRs. The activation of TLR4 by LPS promoted cell proliferation, up-regulated the expression of CD133 and CD34, two important stem cell markers, and down-regulated the expression of eNOS, a important differential marker of endothelial cells, suggesting that TLR4 functions in EPCs to maintain the stem cell phenotype, but not to promote cell differentiation.

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