의학석사 학위논문

사람 유래 수용성 CD14 재조합 단백질에 의해 증가된 LPS 유도 단핵구 활성화

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2014 년 8 월

서울대학교 대학원

의과학과 의과학전공

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A thesis of the Master's degree

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2014 년 7 월

위 원 장 (인)

부위원장 (인)

위 원 (인)

Enhanced Lipopolysaccharide Induced Monocyte Activation by Recombinant Human Soluble Cluster of Differentiation 14

by
Seong Jun Kanng

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

July 2014

Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

ABSTRACT

Introduction: CD14 is the first described pattern recognition receptors that detect LPS. It has two form, one is membrane-bound form and the other is soluble form. Over the past few decades, a considerable number of studies have been conducted on soluble CD14. But the exact physiological role of soluble CD14 is still controversial. In this thesis, I would like to investigate the precise physiological role of soluble CD14 on human blood monocytes activation.

Methods: To investigate the physiological role of soluble CD14, Recombinant human soluble CD14 was cloning, expression, and purified. Then, pro-inflammatory cytokine IL-6 and TNF-a secretion level from human PBMC was checked by CBA assay. After that, Phosphorylation level of NF-kB was measured by western blot from THP-1 human monocytic cell line.

Results: The following results were obtained. First, soluble CD14 increased pro-inflammatory cytokine IL-6 and TNF-a secretion in human PBMC at steady state (10 pg/ml) and septic state (1ng/ml) concentration of LPS. Second, soluble CD14 augmented LPS-induced phosphorylation of NF-kappa B in THP-1 cell line.

Conclusions: Taken together, these results lead to the conclusion that soluble CD14 can increase LPS-induced immune responses like pro-inflammatory cytokine secretion, and phosphorylation of NF-kappa B. Therefore, it seems to be reasonable to conclude that soluble CD14 can augment LPS-induced immune responses.

Keywords: CD14, soluble CD14, LPS, Phospho NF-kB, PBMC, Proinflammatory cytokines, IL-6, TNF-a

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LIST OF ABBREVIATIONS

CD: cluster of differentiation
LPS: Lipopolysaccharide
LBP: Lipopolysaccharide binding protein
PRR: Pattern recognition receptor
TLR: Toll like receptor
CHO: Chinese hamster ovary
DMEM: Dulbecco’ s modified Eagles medium
FBS: Fetal bovine serum
CDS: Coding DNA sequence
DNA: Deoxyribonucleic acid
RNA: Ribonucleic acid
PCR: Polymerase chain reaction
RT-PCR: Reverse transcriptase- polymerase chain reaction
SDS: Sodium dodecyl sulfate
PAGE: Polyacrylamide Gel Electrophoresis
kDa: Kilo Dalton
CBA: Cytometric bead array
IL: Interleukin
TNF: Tumor necrosis factor
HRP: Horse radish peroxidase
Ab: Antibody
Ag: Antigen
pNF-kB: phospho nuclear factor kappa B
NF-kB: Nuclear factor kappa B
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

INTRODUCTION

Monocyte Antigen CD14 (Cluster of differentiation 14), also known as myeloid cell-specific leucine-rich glycoprotein was first discovered in 1985 (1). After that, CD14 was identified as a receptor for the endotoxin lipopolysaccharide (LPS) (2). Also, CD14 is the first described well-known pathogen pattern recognition receptors (PRRs) (3). The representative ligand is LPS. CD14 has an important role as a co-receptor for TLR4 (4). CD14 transfer LPS to TLR4, and through this process the TLR4 signaling pathway is turned on (5). CD14 plays important roles in the immune system. It makes up a complex with TLR4 and MD2, which detects LPS (6, 7).

CD14 is a 53kDa glycosylphosphatidylinositol (GPI)-anchored protein, and it is also known to have a 56kDa soluble form (8). Membrane-bound CD14 is anchored to the exoplasmic surface of mature monocytes, macrophages, granulocytes and dendritic reticulum cells of renal nonglomerular endothelium by a cleavable phosphoinositol tail (9-11). Meanwhile, the soluble form of CD14 has no GPI-anchoring, and it is present in normal

sera and in the urine of nephrotic patients. The concentration of CD14 in plasma from healthy adults is 2 to 4 ug/ml (12).

Lipopolysaccharide (LPS) is also known as an endotoxin (13). It is a large molecule that comprises the outer membrane of gram-negative bacteria. It is detected by TLR4-CD14 complex (14, 15). Lipopolysaccharide binding protein (LBP) also helps LPS binding, but LPS can be detected by CD14 independently (16). LPS in the blood stream induces severe inflammatory response throughout the whole body called sepsis, yet healthy person also has LPS in the blood stream, because intestine is permeable to endotoxin in several conditions (17, 18). Endotoxin concentration of healthy individual is about 5.1±7.3 pg/ml. However, it increased to 110-726pg/ml (the mean value is 581±49 pg/ml) in septic patients (19).

Over the past few decades, a considerable number of studies have been conducted on soluble CD14. Although numerous attempts have been made to study the role of soluble CD14, the exact physiological role is still controversial. There is a conflict of opinions about the physiological role of soluble CD14. One group proposed that soluble CD14 can neutralize LPS-induced

responses (20), and another proposed that the soluble CD14 mediates the activation of cells by LPS (9, 21) In this thesis, I would like to investigate the precise physiological role of soluble CD14 on human blood monocyte activation.

MATERIALS AND METHODS

1. Cell culture

Chinese hamster ovary (CHO) and Human embryonic kidney 293(HEK293) cell lines were cultured and maintained with high glucose Dulbecco's modified Eagles medium (DMEM, HyClone, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% of heat inactivated fetal bovine serum (FBS, Gibco/BRL, Grand islands, NY, USA) supplemented with 10mM HEPES (Sigma-aldrich, St. Louis, MO, USA), 100 μM non-essential amino acids, 55 μM beta-mercaptoethanol (β-ME) and 5ml of 100x PenStrep (Thermo Fisher Scientific Inc., Waltham, MA, USA). THP-1, MKN45, and mouse Kupffer cells were cultured by hy glucose RPMI 1640 (HyClone, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% FBS and 5ml of 100x PenStrep. The two-compartment bioreactor for the adhesion cells, CELLine AD 1000(INTEGRA Bioscience, Zizers, Switzerland) was used for the mass production of CD14 overexpressed cell culture supernatant according to the

manufacturer's instructions. In order to obtain optimal performance of the bioreactor, 50ml of growth medium (10% FBS DMEM) was poured into the medium compartment. Next, pre-cultured viable target cells (2.5×10^7 cells) were prepared in 15ml of fresh growth medium. Then, the cell suspension was inoculated into the cell compartment through black silicon cap. It was important to minimize the introduction of air-bubbles in the cell compartment during inoculation. After that, 950ml of nutrient medium was poured into the media compartment. The procedure was repeated every 7 days.

2. Preparation of total RNAs

Human fore skin fibroblasts (6×10^6) were homogenized in 1ml of TRIzol® (Invitrogen, Carlsbad, CA, USA). The samples were incubated for 5 minutes at 20° C. Then, 0.2ml of Chloroform (per 1ml of TRIzol® reagent) was added to each sample, and the samples were vigorously shaken. Then the samples were incubated for 3 minutes at 20° C. After that, the samples were centrifuged with 12000g for 10 minutes at 4° C. The aqueous phases were transferred to fresh tubes and mixed with 0.5ml of

isopropanol per 1ml of TRIzol® reagent. The solution was incubated for 10 minutes at 20° C, and then centrifuged with 12000g for 10 minutes at 4° C. After centrifugation, the samples were washed with ice-cold 75% Ethanol. Then the samples were air-dried for 5 minutes, and were finally dissolved in RNase-free water.

3. Synthesis of 1st strand cDNA

Total RNA from Human fibroblast was used as the template RNA for RT-PCR. Takara PrimeScript™ RT-PCR kit (TaKaRa, Japan) was used for this experiment. At first, the mixture for reaction was prepared. dNTP mixture(10mM) 1㎕, Random 6-mer(20㎕M) 1㎕, Template RNA 5㎕g, RNase-free water up to 10㎕l were placed in a tube. The tube was placed in a thermal cycler the temperature was set at 65° C for 5 min for denaturation and annealing of the random 6-mer and then stored at 4° C. After the reaction, elongation step was performed. 5X PrimeScript™ buffer 4㎕, RNase inhibitor 0.5㎕, PrimeScript™ RTase 0.5㎕, and RNase free water 5㎕ were added to the tube that contains 10㎕ of reaction mixture from

denaturing and annealing step. Then the tube was placed in a thermal cycler and reverse transcription was performed using parameters according to the following conditions: at 30° C for 10 minutes, 42° C for 30 minutes, 95° C for 5 minutes. The resultant was stored at 4° C.

4. Primer design for human CD14 cloning

Forward and reverse primer for mouse and human CD14 cloning were designed as following. Human CD14 (for pcDNA3.1 myc/his "B" type) Forward primer sequence is 5' – ggtaccatggagcgcg cgtcctg –3', which contains *Kpn* I restriction enzyme site, and Reverse primer sequence is 5' – gaattccaggcaaagcccc gggcc –3', which contains *Eco* RI restriction enzyme site.

5. PCR

Mixture for PCR was prepared by combining the following reagents. 10x PCR buffer 10 μl, dNTP mixture (10mM),

Forward primer 0.5 μl, Reverse primer 0.5 μl, TaKaRa ExTaq™ 0.5 μl (2.5U), cDNA 5 μl, RNase-free water up to 50 μl. After mixing the reagents, the tubes were put in the PCR machine and PCR was conducted under the following program: at 94° C for 30 seconds, then 33 cycles of 94° C for 30 seconds, 60° C for 30 seconds, 72° C for 2 minute 30 seconds. After that, the final elongation step was performed at 72° C for 15 minutes, and the resultant was stored at 4° C.

6. DNA agarose electrophoresis

1% (w/v) Agarose (Promega, Madison, WI, USA) was dissolved in 1x TAE buffer. Then it was heated in a microwave. EtBr-treated dissolved gel was poured into the gel cast. After the gel had hardened, PCR samples were loaded on the gel with 6x DNA sample buffer. Electrophoresis was performed at 100 V for about 30 minutes.

7. DNA gel extraction

This experiment was performed by DNA gel extraction kit (Qiagen, USA) according to the manufacturer's instructions.

8. T vector cloning

pGEM-T-Easy vector systems(Promega, Madison, WI, USA) was used for this experiment. The human and mouse CD14 gene that had been amplified by RT-PCR was cloned into pGEM-T- vector.

9. pcDNA3.1 myc/his vector

pcDNA3.1 myc/his vector systems (Invitrogen, Carlsbad, CA, USA) was used to produce recombinant protein in this experiment.

10. Western blot and Coomassie blue staining

Different amount samples were harvested and lysed by RIPA buffer and resolved by SDS-PAGE and then transferred to NC membranes. Next, the NC membranes were incubated in 5% BSA in PBST(0.05% tween-20) and proved with specific primary antibody. Mouse anti-human CD14 (Santacruz biotech, USA), mouse anti-His antibody (Cell signalling, USA), rabbit anti-human phosphor NF-kB (p65), rabbit anti-human NF-kB(p65) antibody (Cell signalling, USA) and mouse anti-human GAPDH antibody(Santacruz, USA) were used as primary antibodies, and were detected by goat anti-mouse IgG: HRP (Santacruz, USA) and goat anti-rabbit IgG: HRP (Santacruz, USA). Primary antibodies were usually incubated for overnight in a cold room (4° C). Secondary antibodies were usually incubated for 45 minutes in the cold room (4° C). Between primary and secondary antibody incubation and after the secondary antibody incubation, 5 times of washing with PBST was performed. The positive signals were developed by adding 1ml of Enhanced Chemoluminescent reagent (ECL, Pierce,

USA). For coomassie blue staining, the SDS PAGE gel was soaked in 0.25% coomassie blue staining solution (w/v 0.25% Coomassie blue R in 10% glacial acetic acid, 40% methanol, 50% DW) and gently shaken for overnight at room temperature. The next day, stained SDS PAGE gel was destained by destaining solution (10% glacial acetic acid, 40% methanol, 50% DW).

11. Protein purification using Ni-NTA agarose bead.

At first, culture supernatant from CD14 stable cell line mass culture system (Bioreactor CL1000, INTEGRA Bioscience, Zizers, Switzerland) was centrifuged to remove cell debris. The Ni-NTA agarose resin (GE healthcare, USA) was equilibrated with more than 10X volume of resin with binding buffer (more than 10ml), and was gently shaken for 1 hour. The equilibrated resin left upright for 30 minutes and the buffer was discarded. The cell supernatant was added to the resin and mixed gently for 1 hour. The mixture was poured to the column and packing, and the supernatant flew through the column 5 times. Next, the resin was washed with more than 10X volume with the binding buffer for 5 times. At last, the protein was eluted 20 times with

500 ul of elution buffer. Next, the concentration was checked by using the Nanodrop device.

12. PBMC isolation

Whole blood cells were obtained from healthy human volunteers. The donors were free of prescribed and over-the-counter medications. This study was approved by the Seoul National University Hospital Institutional Review Board (IRB). The IRB approval number is 1403-036-563. Peripheral blood mononuclear cell (PBMC) were isolated using Ficoll-paque plus(GE healthcare, AB, Sweden). Human blood was mixed with PBS about 1:2 ratio. 8 ml of blood+PBS was layered over 3 ml of Ficoll reagent in 15 ml conical tube. Then, the 15 ml tubes were centrifuged at 1200 x *g* for 30 minutes at room temperature with slow acceleration and without brake in the centrifuge. After centrifugation, buffy coat containing PBMCs was obtained and the remaining Ficoll reagent was washed out. After washing and cell counting, PBMCs were cultured at 4 x 10^5 cells / 96 well in several experimental conditions, in 5% CO_2 incubator at 37 °C

13. Cytokine profile analysis using a cytometric bead array(CBA) assay

The concentration of cytokines secreted into the culture supernatant of soluble CD14 and LPS-treated PBMC with various conditions were measured using the CBA assay kit (BD Bioscience, San Diego, CA, USA) according to manufacturer's protocol. 50㎕ of culture supernatants were incubated with capture beads and detection antibody for 3 hours at room temperature without light exposure, and the data were acquired by FACScanto II and analyzed by FACSDiva software.

14. Statistical analysis

Statistical analyses were performed by student's t-test with P value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

1. Information on human CD14 gene

Total RNA was obtained from human foreskin fibroblast, and it was used as a source of human CD14 cDNA. cDNA encoding Met 1 to Ala 375 of human CD14(Figure 1. A, B) was generated by polymerase chain reaction (PCR). The primers used for this reaction were as follows: Forward primer sequence of 5' – *GGTACC* ATG GAG CGC GCG TCC TG–3' containing a *Kpn* I restriction site (*Italic*) and Reverse primer sequence of 5' – *GAATTC* CAG GCA AAG CCC CGG GCC–3' containing an *Eco* RI restriction site (*Italic*). The amplified PCR products were ligated to pGEM T easy vector. pGEM T easy vector with human CD14 was transfected to XL–10 bacteria. The white colonies were picked and cultured for overnight. The T vector isolated from the colonies was double digested by *Kpn* I and *Eco* RI according to the enzyme corporation's protocol.

The digested DNA was gel extracted and ligated to pcDNA3.1 myc/his mammalian cell expression vector, and the vector was transfected into DH5α bacteria.

(A)

```
Homo sapiens CD14 molecule (CD14), transcript variant 1, mRNA
LOCUS          NM_000591 1648 bp    mRNA    linear
PRI 15-JUN-2013
DEFINITION     Homo sapiens CD14 molecule (CD14), transcript
variant 1, mRNA.
ACCESSION      NM_000591
VERSION        NM_000591.3  GI:291575160
SOURCE         Homo sapiens (human)
ORGANISM       Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Euarchontoglires; Primates;
Haplorrhini;Catarrhini; Hominidae; Homo.

translation="MERASCLLLLLLPLVHVSATTPEPCELDDEDFRCVCNFSEPQPDWSEAFQC
VSAVEVEIHAGGLNLEPFLKRVDADADPRQYADTVKALRVRRLTVGAAQVPAQLLVGALRVLAY
SRLKELTLEDLKITGTMPPLPLEATGLALSSLRLRNVSWATGRSWLAELQQWLKPGLKVLSIAQ
AHSPAFSCEQVRAFPALTSLDLSDNPGLGERGLMAALCPHKFPAIQNLALRNTGMETPTGVCAA
LAAAGVQPHSLDLSHNSLRATVNPSAPRCMWSSALNSLNLSFAGLEQVPKGLPAKLRVLDLSCN
RLNRAPQPDELPEVDNLTLDGNPFLVPGTALPHEGSMNSGVVPACARSTLSVGVSGTLVLLQGA
RGFA"
```

(B)

```
   1 cagagaaggc ttaggctccc gagtcaacag ggcattcacc gcctggggcg cctgagtcat
  61 caggacactg ccaggagaca cagaacccta gatgccctgc agaatccttc ctgttacggt
 121 ccccctccct gaaacatcct tcattgcaat atttccagga aaggaagggg gctggctcgg
 181 aggaagagag gtggggaggt gatcagggtt cacagaggag ggaactgaat gacatcccag
 241 gattacataa actgtcagag gcagccgaag agttcacaag tgtgaagcct ggaagccggc
 301 gggtgccgct gtgtaggaaa gaagctaaag cacttccaga gcctgtccgg agctcagagg
 361 ttcggaagac ttatcgacca tggagcgcgc gtcctgcttg ttgctgctgc tgctgccgct
 421 ggtgcacgtc tctgcgacca cgccagaacc ttgtgagctg gacgatgaag atttccgctg
 481 cgtctgcaac ttctccgaac ctcagcccga ctggtccgaa gccttccagt gtgtgtctgc
 541 agtagaggtg gagatccatg ccggcggtct caacctagag ccgtttctaa agcgcgtcga
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 721 gtactcccgc ctcaaggaac tgacgctcga ggacctaaag ataaccggca ccatgcctcc
 781 gctgcctctg gaagccacag gacttgcact ttccagcttg cgcctacgca acgtgtcgtg
 841 ggcgacaggg cgttcttggc tcgccgagct gcagcagtgg ctcaagccag gcctcaaggt
 901 actgagcatt gcccaagcac actcgcctgc cttttcctgc gaacaggttc gcgccttccc
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1441 ttcgaccctg tcggtggggg tgtcgggaac cctggtgctg ctccaagggg cccggggctt
1501 tgcctaagat ccaagacaga ataatgaatg gactcaaact gccttggctt caggggagtc
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1621 cttaaacaac gggtcaaaaa aaaaaaaa
```
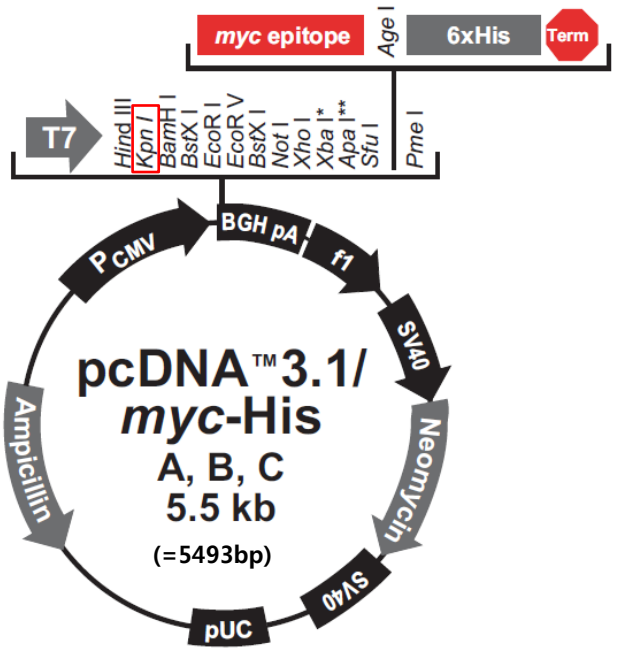

(C)



(D)

Forward, Name: HCD14_pcDNA3.1b_F, AT 10℃ + GC 48℃ = 58℃, GC content: 70%

5′- *Kpn* I GGTACC atg gag cgc gcg tcc tg -3′

Reverse, Name: HCD14_pcDNA3.1b_R, AT 6℃ + GC 52℃ = 58℃, GC content: 81%

5′- *Eco* RI GAATTC **CA** ggc aaa gcc ccg ggc c -3′

Figure 1. Information on human CD14 gene

(A) Genbank database information about human CD14 mRNA variant 1.

(B) Coding DNA sequence (CDS) of human CD14. (379~1507, 1128bp)

(C) Vector map of pcDNA3.1 myc/his

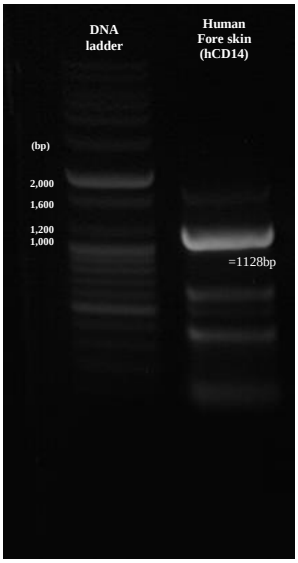
(D) Forward and reverse primer design for human CD14 cloning.

2. Construction of human CD14 expression vector pcDNA3.1 myc/his

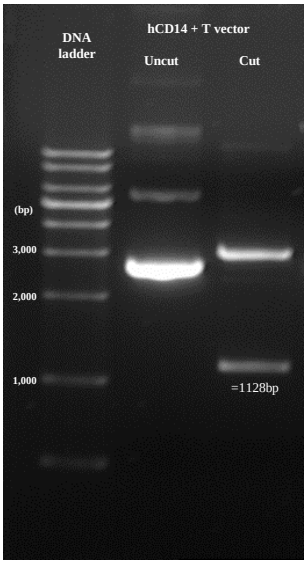
PCR was conducted by using the designed primers in Figure 1D. Total RNA from human foreskin fibroblast was used to generate 1st strand cDNA. It was used as the source for human CD14. At an appropriate annealing temperature (Human: 60.5), the precise DNA size (human CD14: 1128bp) was obtained (Figure 2A). After cloning, gel extraction was performed, and the precise DNA size was checked by agarose gel electrophoresis. As a result, T vector cloning and pcDNA3.1 myc/his vector cloning were successfully done, and they have precise DNA size of human CD14 (Figure 2B, C).

DNA sequencing were conducted, and the results matched with original sequence 100% (Figure 2D).

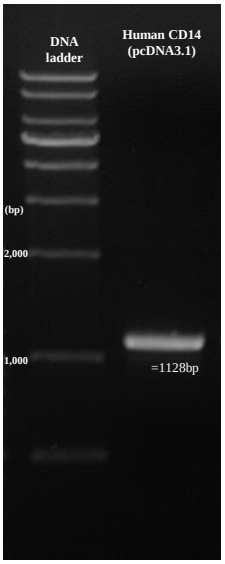
(A)


DNA ladder
Human Fore skin (hCD14)
(bp)
2,000
1,600
1,200
1,000
=1128bp

(B)


DNA ladder
hCD14 + T vector
Uncut
Cut
(bp)
3,000
2,000
1,000
=1128bp

(C)


DNA ladder
Human CD14 (pcDNA3.1)
(bp)
2,000
1,000
=1128bp

(D)

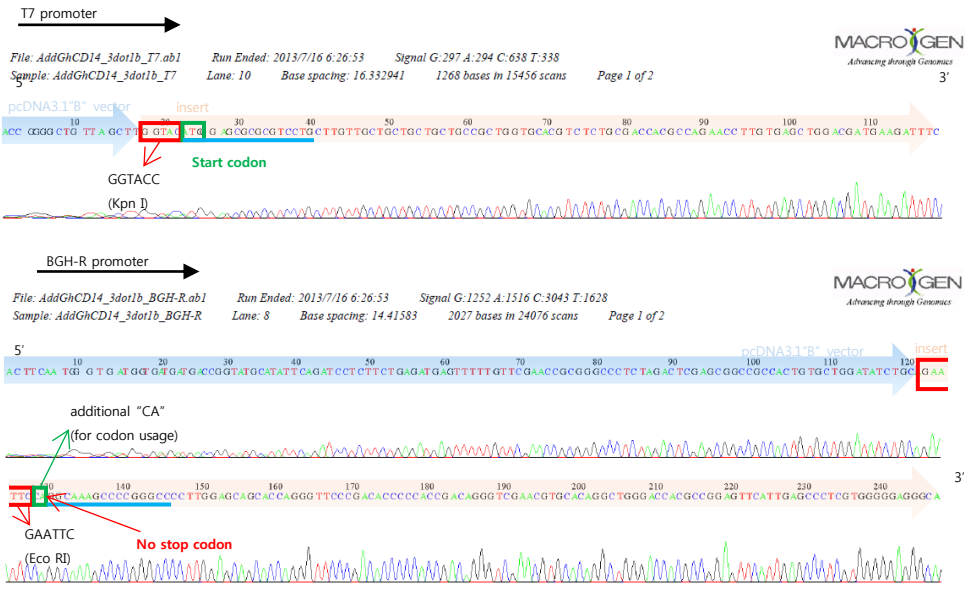

T7 promoter
File: AddGhCD14_3dot1b_T7.ab1 Run Ended: 2013/7/16 6:26:53 Signal G:297 A:294 C:638 T:338
Sample: AddGhCD14_3dot1b_T7 Lane: 10 Base spacing: 16.332941 1268 bases in 15456 scans Page 1 of 2
MACROGEN
Advancing through Genomics
5'
3'
pcDNA3.1"B" vector
insert
Start codon
GGTACC
(Kpn I)
BGH-R promoter
File: AddGhCD14_3dot1b_BGH-R.ab1 Run Ended: 2013/7/16 6:26:53 Signal G:1252 A:1516 C:3043 T:1628
Sample: AddGhCD14_3dot1b_BGH-R Lane: 8 Base spacing: 14.41583 2027 bases in 24076 scans Page 1 of 2
additional "CA"
(for codon usage)
GAATTC
(Eco RI)
No stop codon

Figure 2. Construction of human CD14 expression vector pcDNA3.1 myc/his

(A) Human CD14 DNA band. PCR product by using appropriate primer for human CD14. DNA template was 1st strand cDNA obtained from human foreskin fibroblast total RNA.

(B) After T vector cloning, Insert and vector size were confirmed by electrophoresis.

(C) After pcDNA3.1 myc/his vector cloning. Insert size was confirmed by electrophoresis.

*0.8% Agarose gel was used for electrophoresis.

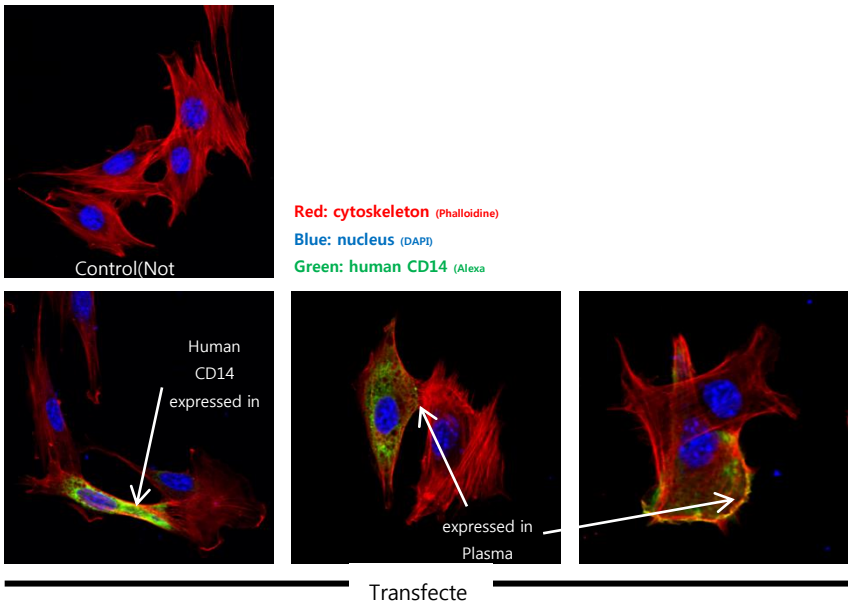
(D) DNA sequencing results from MACROGEN.

3. Establishment of stable cell lines that overexpress human CD14

NIH-3T3 cells and Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. To confirm the expression of CD14, NIH-3T3 cells were transfected with pcDNA3.1_hCD14. Then using confocal microscopy, the expression of human CD14 in cytosol and plasma membrane was checked. The results shows that pcDNA3.1_hCD14 vector can over-express human CD14 successfully. (Figure 3A)

Next, to establish human CD14 over-expressing stable cell line, CHO cells were transfected with pcDNA3.1_hCD14 vector, and stable cell line was generated using Neomycin (G418) treatment. Finally, 10 clones of hCD14 overexpressed CHO cell lines were obtained. To check the over-expression of hCD14, western blot analysis was conducted. Among the 10 clones, hA11, hA22, hA42, hB31, hC41 over-expressed human CD14. (Figure 3B)

(A)


Control(Not
Red: cytoskeleton (Phalloidine)
Blue: nucleus (DAPI)
Green: human CD14 (Alexa
Human
CD14
expressed in
expressed in
Plasma
Transfecte

(B)

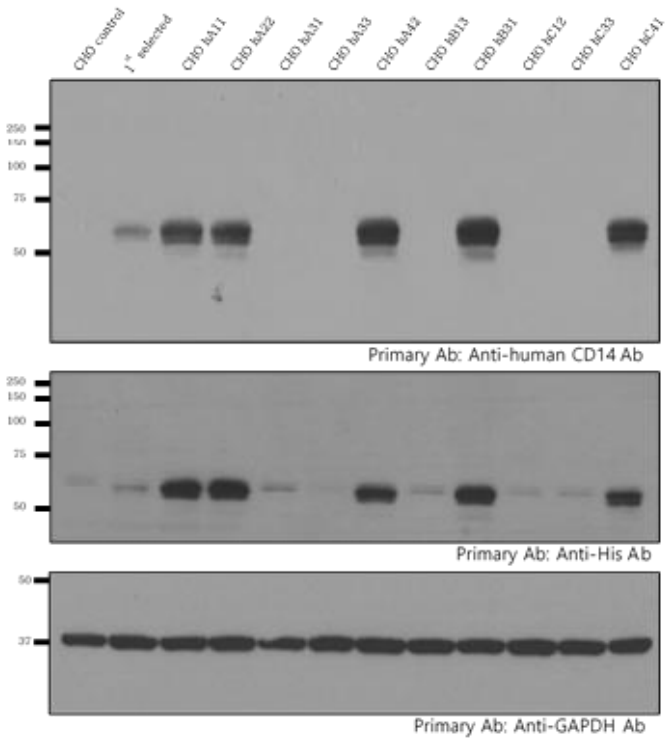

CHO control
1st selected
CHO bA11
CHO bA22
CHO bA31
CHO bA33
CHO bA42
CHO bB13
CHO bB31
CHO bC12
CHO bC33
CHO bC41
250
150
100
75
50
Primary Ab: Anti-human CD14 Ab
Primary Ab: Anti-His Ab
50
37
Primary Ab: Anti-GAPDH Ab

Figure 3. Expression of human CD14

(A) Confocal microscopy images of human CD14 transfected NIH-3T3 fibroblasts stained with TRITC phalloidin for actin filaments (red), nuclei counterstained with DAPI (blue), and human CD14 stained with anti-CD14 antibody (primary antibody) and anti-mouse Alexa 488(secondary antibody) (Green). Upper image is the negative control (Not transfected). Bottom images are transfected cells

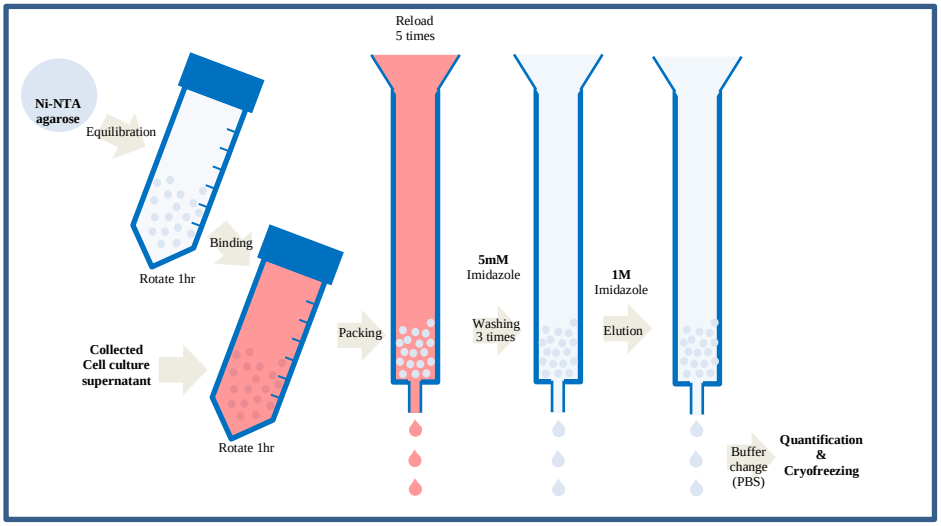
(B) Western blot analysis for human CD14 over-expressed cell line

4. Purification of recombinant human soluble CD14

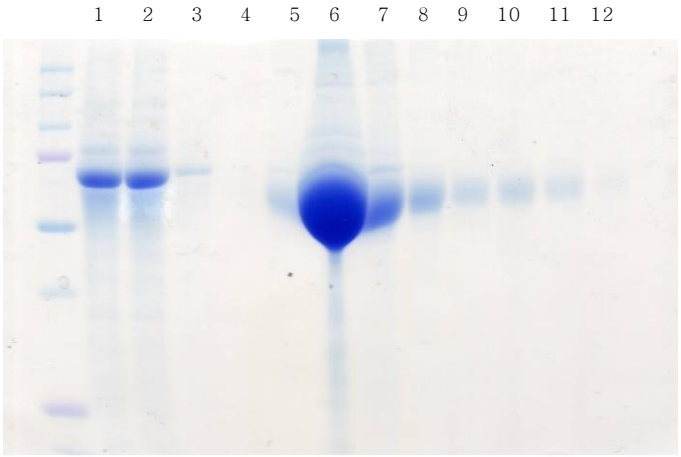
Human CD14 stable cell lines also produce the soluble form of CD14 into the culture supernatant. To obtain recombinant soluble human CD14 protein, human CD14 stable cell line culture supernatant was gathered using bio-reactor system, and purified by Ni-NTA agarose bead. (Figure 4A)

To confirm the purity of recombinant human soluble CD14, Coomassie blue staining of eluted samples were conducted. (Figure 4B) And the concentration was measured by Nanodrop. From the results, I found that recombinant human soluble CD14 can be secreted to culture supernatant and successfully purified by Ni-NTA agarose bead. (Figure 4C)

(A)


Reload
5 times
Ni-NTA
agarose
Equilibration
Binding
Rotate 1hr
Collected
Cell culture
supernatant
Rotate 1hr
Packing
5mM
Imidazole
Washing
3 times
1M
Imidazole
Elution
Buffer
change
(PBS)
Quantification
&
Cryofreezing

(B)


1 2 3 4 5 6 7 8 9 10 11 12

(C)

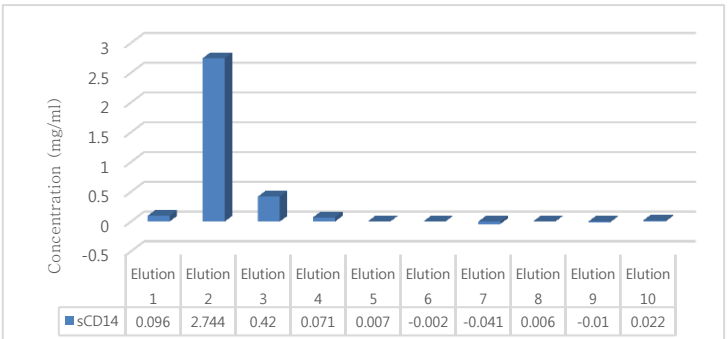

Concentration (mg/ml)
3
2.5
2
1.5
1
0.5
0
-0.5
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8
Elution 9
Elution 10
sCD14
0.096
2.744
0.42
0.071
0.007
-0.002
-0.041
0.006
-0.01
0.022

Figure 4. Recombinant human soluble CD14 purification

(A) Diagram of the experimental layout

(B) Coomassie blue staining of SDS PAGE gel. Lane 1; Culture supernatant, Lane 2; Flow through, Lane 3; wash 1, Lane 4; wash 2, Lane 5−12; eluted sample 1−8.

(C) Nanodrop result of each eluted sample.

5. The increase of pro-inflammatory cytokine IL-6 and TNF-alpha production

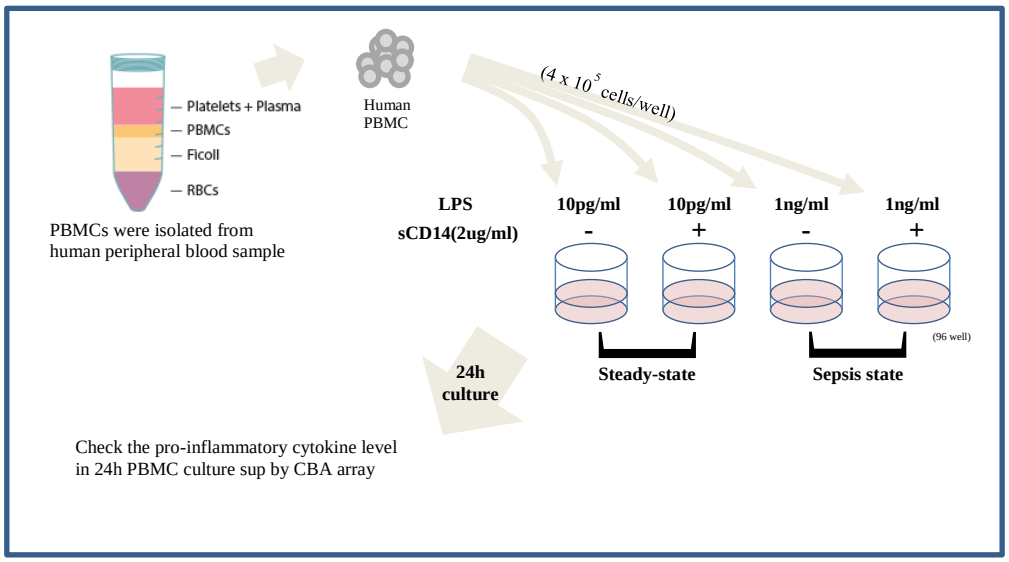
Isolated 4 x 10^5 of human PBMC was cultured for 24 hours in 96-well plate with steady-state concentration of LPS (10pg/ml) or higher sepsis LPS level (1ng/ml). (Figure 5A)

The culture supernatant was obtained and cytokine production was checked by CBA assay.

It was found from the result that IL-6 and TNF-alpha concentration was increased when recombinant soluble CD14 was co-treated with LPS.

In low-concentration of LPS, sCD14 treatment seemed to increase IL-6 (*P*-value = 0.0009) and TNF-alpha (*P*-value = 0.0017) concentration significantly. However, in sepsis concentration, 1 ng/ml of LPS also increased the concentration of these pro-inflammatory cytokines, but it was not significant (IL-6; *P*-value = 0.1504, TNF-alpha; *P*-value = 0.2704). (Figure 5B)

(A)


Platelets + Plasma
PBMCs
Ficoll
RBCs
PBMCs were isolated from human peripheral blood sample
Human PBMC
(4 x 10^5 cells/well)
LPS
sCD14(2ug/ml)
10pg/ml
10pg/ml
1ng/ml
1ng/ml
-
+
-
+
(96 well)
24h culture
Steady-state
Sepsis state
Check the pro-inflammatory cytokine level in 24h PBMC culture sup by CBA array

(B)

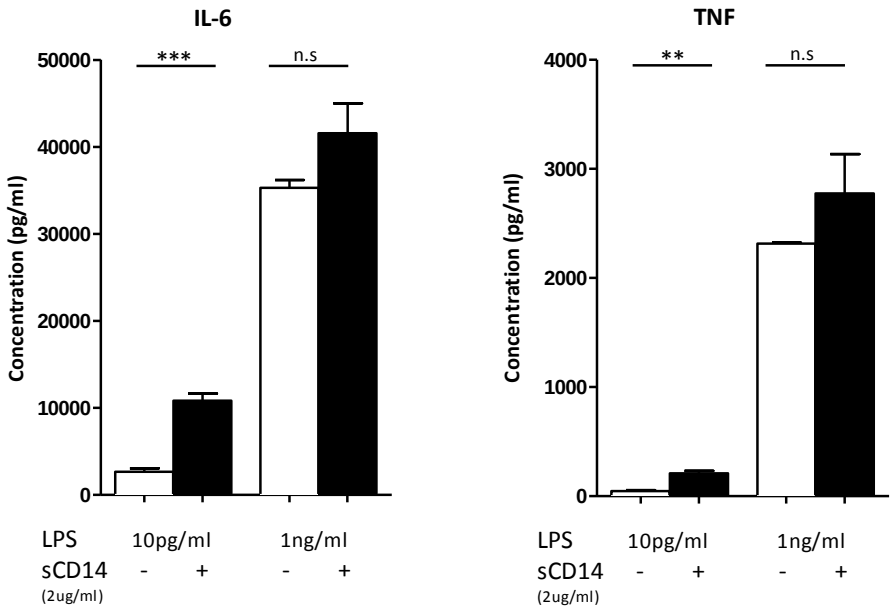

IL-6
***
n.s
50000
40000
30000
20000
10000
0
Concentration (pg/ml)
LPS
10pg/ml
1ng/ml
sCD14
(2ug/ml)
-
+
-
+
TNF
**
n.s
4000
3000
2000
1000
0
Concentration (pg/ml)
LPS
10pg/ml
1ng/ml
sCD14
(2ug/ml)
-
+
-
+

Figure 5. Cytokine secretion analysis from human PBMC

(A) Diagram of the experimental layout

(B) Cytometric bead array result. The data were acquired by FACScanto II and analyzed by FACSDiva software.

*The statistical significance was judged by *P*-values (***; *P*-value<0.001, **; *P*-value<0.01, n.s; not significant, *P*-value>0.05)

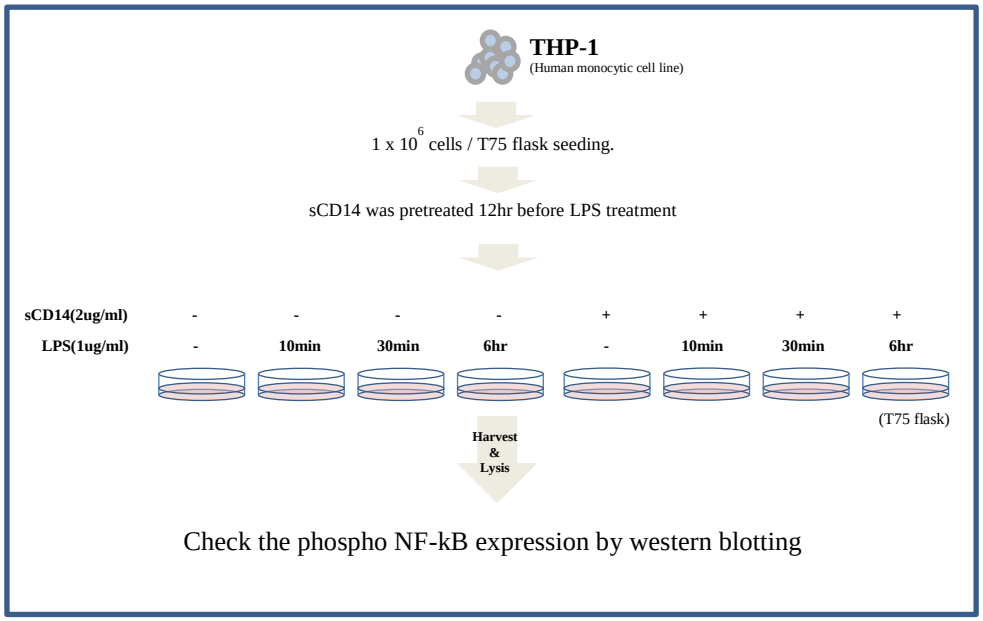
6. Phosphorylation of NF-kB is increased in human monocytic cell line THP-1

The major source of IL-6 and TNF-alpha in PBMC were monocytes. So, this figure, I want to check phosphorylation level of NF-kB in human monocytic cell line.

1 x 10^6 cells of human monocytic cell line THP-1 cells were seeded in T75 flask. After 24 hours of culturing in 5% CO2 37C incubator, sCD14 and LPS were treated by several different condition. (Figure 6A)

As a results, comparing LPS treated group with LPS and sCD14 co-treated group, the phosphorylation of NF-kB level was increased in LPS and sCD14 co-treated group(in 10min, 30min, 6hr). (Figure 6B)

(A)


THP-1
(Human monocytic cell line)
1 x 10^6 cells / T75 flask seeding.
sCD14 was pretreated 12hr before LPS treatment
sCD14(2ug/ml) - - - - + + + +
LPS(1ug/ml) - 10min 30min 6hr - 10min 30min 6hr
(T75 flask)
Harvest & Lysis
Check the phospho NF-kB expression by western blotting

(B)

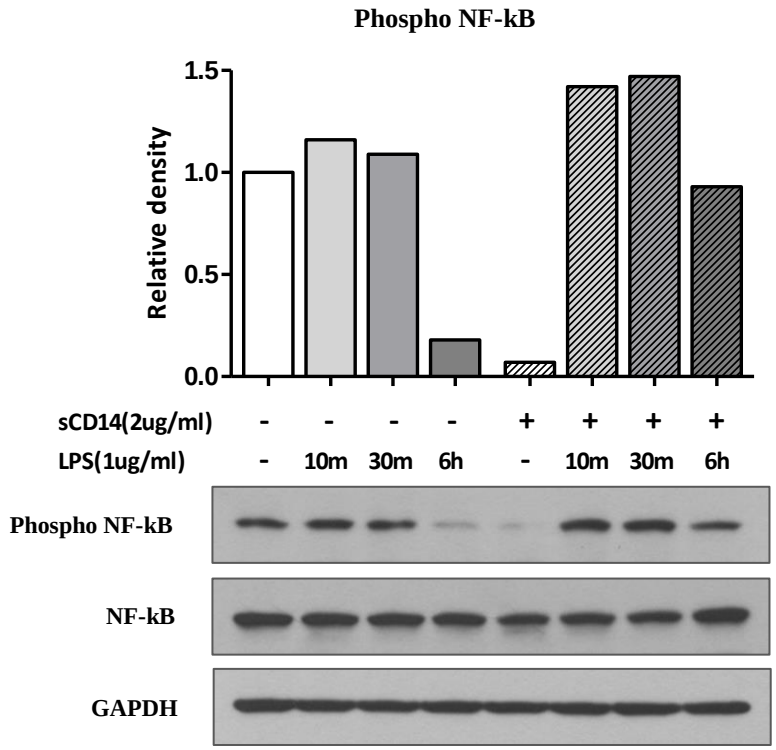

Phospho NF-kB
Relative density
1.5
1.0
0.5
0.0
sCD14(2ug/ml) - - - - + + + +
LPS(1ug/ml) - 10m 30m 6h - 10m 30m 6h
Phospho NF-kB
NF-kB
GAPDH

Figure 6. Phosphorylation of NF-kB in THP-1 human monocytic cell line

(A) Diagram of the experimental layout

(B) Western blot analysis. Each lane 25ug of cell lysate was loaded. Y-axis of the graph represented relative density of western blot band. And all bands were normalized by GAPDH.

DISCUSSION

The purpose of this study is to investigate the physiological roles of soluble CD14 on monocyte activation. My study revealed that recombinant human soluble CD14 increased LPS-induced pro-inflammatory cytokine (IL-6 and TNF-alpha) secretion on PBMC culture, and increased phosphorylation of NF-kB in human monocytic cell line, THP-1. In conclusion, the results of this thesis suggest that human soluble CD14 can augment LPS-induced immune responses, especially monocyte activation. Compared to previous conflict about the role of soluble CD14, this study lends weight to the idea that sCD14 can activate the cells with LPS, This is contrast to the idea that sCD14 can neutralize LPS-induced responses. Therefore, my result suggest that neutralizing soluble CD14 could be used for therapeutic treatment of excessive LPS-induced immune responses like sepsis or septic shock.

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국문 초록

서론: CD14 는 최초로 발견된 패턴 인식 수용체로서 패혈증의 원인이 되는 그람 음성 세균의 LPS 와 결합하는 특성이 있다. CD14 는 두 가지 형태를 가지고 있는데 하나는 세포막에 발현되는 세포막 CD14 이고 나머지 하나는 수용성 CD14 이다. CD14 가 발견된 이후 이 수용성 CD14 의 생리학적 기능을 밝히기 위한 많은 연구가 이루어 졌지만 정확한 수용성 CD14 의 역할은 여전히 논란이 많다. 여기서는 수용성 CD14 가 사람 혈액 내 단핵구의 LPS 에 의한 활성화에 어떤 영향을 미칠 것 인지를 연구하고자 하였다

방법: 사람 수용성 CD14 의 정확한 역할을 연구하기 위해 사람 수용성 CD14 재조합 단백질을 클로닝하여 발현시키고 단백질을 정제하였다. 그리고 사람 말초 혈액 세포를 분리하여 그 세포에서 인터루킨 6 와 TNF-a 와 같은 염증성 사이토카인의 분비를 CBA 를 이용해 확인하였다. 그 후 사람 단핵구 세포주인 THP-1 을 이용해 NF-kappa B 의 인산화 정도를 Western blot 으로 확인하였다.

결과: 사람 수용성 CD14 는 평상시(LPS 농도 10pg/ml)와 패혈증시(LPS 농도 1ng/ml) 사람 말초 혈액 세포 배양액에 분비되는 인터루킨-6 와 TNF-a 농도를 증가시켰다. 또한 사람 수용성 CD14 는 THP-1 cell line 에서 LPS 에 의한 NF-kappa B 의 인산화를 증가 시키는 것으로 나타났다.

결론: 결론적으로 사람 수용성 CD14 는 LPS 에 의해 유도되는 면역반응을 증가시키는 역할을 하는 것으로 보인다.

주요어 : CD14, 수용성 CD14, LPS, 인산화 NF-kB, 말초 혈액 세포, 염증성 사이토카인, 인터루킨-6, TNF-a

학 번 : 2012-21781