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약학 석사 학위논문

선천 면역을 유도하는 **Tryptophanyl-tRNA synthetase**에 작용하는 치료용 항체의 개발 연구

Development of Therapeutic Antibody Targeting Tryptophanyl-tRNA Synthetase Inducing Innate Immunity

2019 년 8 월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 의약생명과학전공

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이 논문을 약학 석사 학위논문으로 제출함

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서울대학교 융합과학기술대학원
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고 서 연

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2019 년 8 월

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ABSTRACT

Development of therapeutic antibody targeting Tryptophyl-tRNA synthetase inducing innate immunity

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Overactivation of the innate immune system upon bacterial infection leads to sepsis. Specific bacterial compounds potently stimulate immune cells via Toll-Like Receptor 4 (TLR4)-Myeloid Differentiation protein 2 (MD2). Released LPS from gram-negative bacteria induce a TLR4-MD2 signal, and production of inflammatory cytokines from macrophage cells. In current study, it is reported that Tryptophanyl-tRNA synthetase (WRS) is secreted by monocyte upon bacterial infection. WRS is one type of aminoacly-tRNA synthetases which are commonly known as ligation enzyme with amino acids and their cognate tRNAs. WRS has a crucial role of inflammatory reaction by directly binding to TLR4-MD2 complex expressed in macrophages surface to induce phagocytosis and inflammatory cytokine production. To neutralize TLR4-MD2 signaling in experimental models of sepsis, we develop monoclonal antibodies toward the WRS. In this study, we

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addressed the question of whether neutralization of WRS by systemic administration of a monoclonal antibody interfere with sepsis pathology induced by Gram-negative bacterial infection.

Most importantly, blockade of TLR4-MD2 pathway upon neutralizing of WRS was beneficial with respect to decrease in inflammatory cytokine, tumor necrosis factor-alpha (TNF-α). Thus, we propose WRS inhibition as a strategy for therapeutic prevention of sepsis.

Key words: Tryptophanyl-tRNA synthetase, innate immune, TLR4-MD2, monoclonal antibody, TNF-α

Student Number: 2017-224424

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ABBREVIATION LIST

ARS: Aminoacyl-tRNA synthetase

WRS: Tryptophanyl-tRNA synthetase

TLR4-MD2: Toll-Like Receptor 4 -Myeloid Differentiation factor 2

LPS: Lipopolysaccharide

TNF-α: Tumor necrosis factor-alpha

ELISA: Enzyme-linked immunosorbent assay

TnT: Transcription and Translation

SPR: Surface plasmon Resonance

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INTRODUCTION

Uncontrolled systemic innate immune response, such as sepsis, is predominantly a problem in intensive care units (ICUs). Sepsis patients with overactivated immune system have a mortality rate of about 40-60%. Currently, antibiotics and corticosteroid hormone are used in the treatment of sepsis. The antibiotics is principally used to remove pathogen in early stage of sepsis, but it cannot affect the overactivation of immune system. Moreover, the mechanism of corticosteroid hormone to infection and innate immunity is at present unclear. For the most part, it possibly relieves hyper inflammation, but the patient's long-term prognosis is poor. Therefore, a novel treatment for blocking of innate immunity is required to improve survival rate and prognosis of sepsis patients. There were several strategies of antibody therapy targeting tumor necrosis factor alpha (TNF-α) via toll-like receptor 4 (TLR4)-myeloid differentiation factor-2 (MD2) [1], which is related in hyper inflammation [2]. However, TNF-α and TLR4-MD2, targeting antibody therapy had failed to improve survival rate compared to placebo [3,4]. It was reported that Tryptophanly-tRNA synthetase (WRS) is immediately secreted from monocyte upon pathogen infection, and has a key role in stimulating inflammation directly bound to TLR4-MD2 in macrophages which is fundamental cause of sepsis by over-activating of innate immune system [5]. Here, we report that direct blockade of WRS decreases in inflammatory cytokines, followed by inhibition of dimerization of TLR4-MD2. We developed an antibody that specifically binds to human WRS by panning a library of phage-displayed human single chain variable fragments (scFv), and conversion to IgG formation. The treatment of anti-WRS antibody showed a

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significant decrease in TNF-α production from macrophage via TLR4-MD2 inhibition [6]. Taken together, our results suggest that WRS antibody can be used as a novel treatment for sepsis by inhibiting TLR4-MD2 pathway.

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MATERIALS AND METHODS

Cell culture and materials

Human monocyte THP-1 cells were provided from Biobank (Biocon). RPMI-1640 medium (HyClone, Cat # SH30255) containing 10% fetal bovine serum (FBS, HyClone, Cat # SH30084) and 1% penicillin and streptomycin (HyClone, Cat # SV30010) at 37℃ in a 5% CO2 incubator were used for THP-1 cultivation.

Macrophage differentiation

THP-1 cells were differentiated with 5 ng/ml phorbol-12-myristate 13-acetate (PMA, Sigma-Aldrich, Cat # P8139) in RPMI media without serum for 2 days. After 2 days, the cells were refeeded with fresh medium containing 10% FBS and without PMA for 1 day to allow cells recovery [7]. Cell differentiation was verified by evaluating cell adhesion and mass of cytosol under optical microscope [8].

Tryptophanyl-tRNA synthetase protein purification

The cDNA for human WRS were cloned into pET-28a (6X His-tag) vector. The WRS protein were overexpressed in *Escherichia coli*, BL21. The bacterial cells were lysed by sonication for five times (10 sec durations with 1 min interval). His-tagged proteins in supernatants were purified by using Ni-NTA resin. Protein eluted solution was dialyzed in 15% glycerol in PBS buffer and LPS removal filtration through Mustang E membrane (0.2 μm, Pall Corporation). Coomassie Blue staining were done to confirm the purity and expression of WRS.

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Biopanning

All biopanning procedure [9] and Human IgG conversion [10] was kindly done by Biocon antibody team.

Human TNF-α secretion ELISA

5x10^5 cells per well were seeded in 24 well plate. The plates were incubated for 3days for macrophage differentiation. Cultured media were removed from the wells, and serum free media were added for 2 hours. Then, we treated with lipopolysaccharide (LPS), and WRS for 3 hours. The levels of TNF-α in culture supernatants were determined using commercially available ELISA kit (BD bioscience, cat # 555212) according to the manufacturer's protocol.

TNF-α inhibition test

WRS antibodies and WRS protein were pre-incubated at 37℃ in a 5% CO2 incubator with WRS protein for 2 hours, and treated to differentiated THP-1 cells for 3 hours [11].

Immunoblotting

THP-1 cell were lysed with RIPA buffer for 30min at 4℃ with vigorous shaking. The cell lysate centrifuged 18000xg for 15 min at 4℃. The protein concentration in supernatant were measured by Pierce BCA protein assay kit (Thermo scientific, cat # 23225) according to the manufacturer's protocol. After measuring, diluted each samples at the same concentration (1 μg/μl) in 5X sample buffer, and boiled for 5 min at 99℃. 10 μg of each sample were loaded into SDS-PAGE gel, and detection

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with target antibodies in 5% skim milk.

Transcription and Translation (TnT)

TNT Quick Coupled Transcription/Translation system (Promega cat # L1170) was used for overexpression of WRS protein by using T7 promoter according to the manufacturer's protocol.

SPR

SPR experiments were performed using a Biacore T200 (GE Healthcare) with Series S sensor chip (GE Healthcare). PBS buffer was used as a running buffer for the immobilization step. Immobilization was performed using an amine coupling kit (GE Healthcare). Flow cells were activated with pulse of a 1:1 mixture of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) according to the manufacturer's protocol. WRS WHEP domain peptide were diluted with 10 mM sodium acetate and immobilized to a Series S sensor chip, CM5. Flow cells were then blocked with a 7-min pulse of 1 M ethanolamine-HCl (pH 8.5). Serial dilution of WRS antibodies or buffer were injected for 1– 10 min at 30 μl min–1 flow rate and washed out for 20 min according to the analyte.

Peptide preparation

WRS WHEP domain and its 2 fragment peptides were synthetized by GL biochem and Peptron.

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Alanine mutagenesis

pSG5-HA-WRS (1-150 amino acids) clone was provided from Biocon, and used for point-mutation of WHEP domain. Each amino acid (8-27, 33-47) were substituted to alanine.

Protein 3D structure analysis

3D structure of WRS WHEP domain was produced by PyMOL program [12].

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RESULTS

Anti-WRS antibody inhibits TNF-α secretion from human differentiated macrophage

Before we started to determine the antibody inhibition effect, we confirmed the activity of purified His-tagged WRS protein cloned in pET28a vector (Fig. 1A). And we confirmed the TNF-α induction effect in THP-1 cells by human TNF-α ELISA kit (Fig. 1B). Following the previous study, secreted WRS has shown the role of a stimulating factor to produce inflammatory cytokine such as TNF-α. Therefore, we tested time and dose condition with different concentration of WRS protein by measuring secreted TNF-α level. In addition, we treated boiled sample of WRS protein to check the gram-negative endotoxin, lipopolysaccharide (LPS), contamination. We choose the final condition as 100nM for further WRS treatment test.

From the screening of anti-WRS antibody (performed by Biocon antibody team, Korea), we identified 4 scFv clones which are targeting WRS (Fig. 2). After selecting scFv clones, we produced CDR regions of scFv into IgG plasmid and produced recombinant IgG WRS antibody. Finally, we identified 1 mouse IgG clone (1A10), and 3 human IgG clones (4H9, 3B6, 3C6).

We measured TNF-α in supernatant of THP-1 cells after treatment with anti-WRS (Fig. 3A). Macrophage cells differentiated from THP-1 cells were preincubated with 10 μg/ml of anti-WRS antibodies at 37 ℃ for 3 hours and then treated with human WRS protein. Antibody 4H9 and 3C6 showed inhibitory effect of TNF-α secretion in THP-1, but antibody 1A10 and 3B6 did not inhibit TNF-α secretion. Inhibition of

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Erk signaling by WRS neutralizing antibody was identified by western blotting using the same cell lysate (Fig. 3B). Western blotting data also shows that antibody 4H9 and 3C6 decreased in phosphor-Erk levels. According to this data, anti-WRS antibody successfully binds to WRS and inhibits stimulation of innate immune system, and down-regulates TNF-α production and Erk signaling pathway from human macrophage.

Anti-WRS antibody binds to WRS N-terminal, specifically WHEP domain

To identify epitope of WRS antibodies, we overexpressed HA-tagged WRS fragment clones (Full length, 1-150, 48-471, 94-471) cloned in pSG5-HA vector which has a T7 promoter by using transcription and translation (TnT) method (Fig. 4A). To confirm expression level of WRS fragments, we detected TnT products with HA antibody (Fig. 4B). Antibody 1A10 binds to 48-471 and 94-471 fragments (C-terminal of WRS). Antibody 4H9 and 3C6 binds to 1-150 fragment, not to 48-471 and 94-471. This data indicates that antibody 4H9 and 3C6 binds to N-terminal of WRS (WHEP domain). WRS WHEP domain is known as key domain to help TLR4-MD2 dimerization. Therefore, these two antibodies (4H9 and 3C6) had an inhibition effect with the same binding domain (WRS WHEP domain). In Figure 4, C presents binding result of 4 WRS antibodies. Above all, we decided to use antibody 3C6 in the following experiments because of its binding site and inhibition effect.

Titration of anti-WRS antibody to suppress TNF-α secretion from macrophage.

In previous inhibition test using anti-WRS antibodies, we used 10 μg/ml of each anti-WRS antibody. For titration of antibody 3C6, we reperformed TNF-α secretion test under same condition of screening test. Firstly, we diluted antibody

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3C6 in 10 folds from 10 μg/ml to 1 ng/ml (Fig. 5A). This data indicates 10 ng/ml of 3C6 antibody is sufficient to block WRS effect. Then, we diluted antibody 3C6 in 2 folds from 100 ng/ml to 1.06 ng/ml. EC50 of antibody 3C6 was 35 pM from Figure 5B data (Fig. 5B). Additionally, we tested human IgG preincubated with WRS protein, because antibody 3C6 has IgG construction. IgG has no effect onto TNF-α secretion (Fig. 5C). Furthermore, antibody 3C6 was also treated with LPS under same condition as above tests without WRS, antibody 3C6 has no effect on TNF-α secretion of macrophage by LPS (Fig. 5D).

Antibody 3C6 binds to helix-turn-helix form of WRS WHEP domain, not to single helix.

Following current study for novel function of WRS in innate immune system, N-terminal of WRS WHEP domain has a key function to TLR4-MD2 dimerization. And we checked the epitope of antibody 3C6 in figure 4. Therefore, we determined 3D structure of WRS WHEP domain (Fig. 6B), and ordered WRS WHEP domain and single helix peptides to identify binding affinity of antibody 3C6 to WRS WHEP domain (Fig. 6B). Binding affinity by SPR (Woojung bio, Korea) are shown in Figure 6C. This data indicates that antibody 3C6 binds to WRS WHEP domain, not to single helix of WHEP domain (Fig. 6C).

Furthermore, for the detailed recognition of binding site between WRS WHEP domain and antibody 3C6, we performed alanine scanning of WRS WHEP domain, and overexpressed mutated clones by TnT method (Fig. 7A and B).

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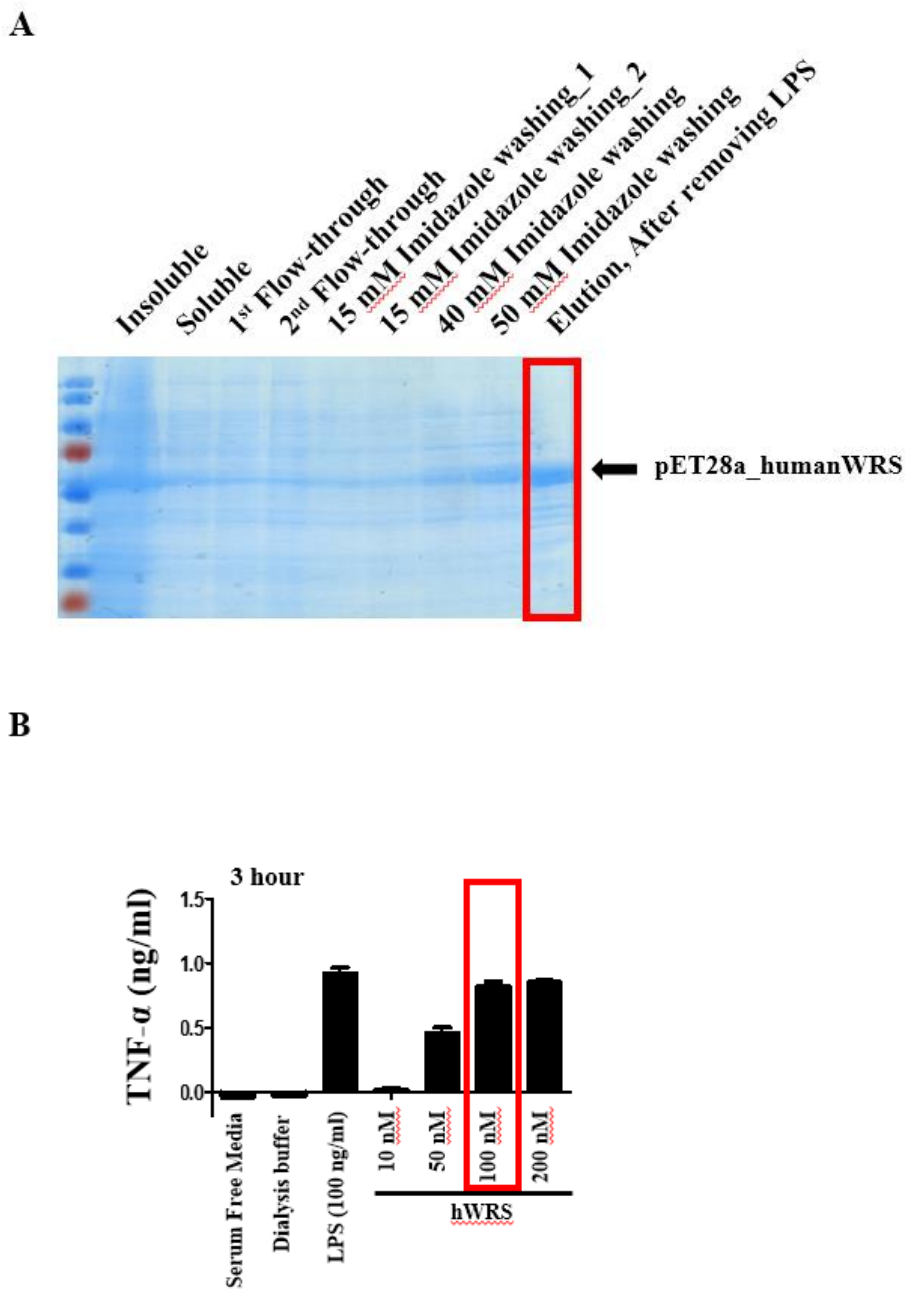

Figure 1. Purification and TNF-α secretion of human WRS

(A) WRS protein were purified using affinity chromatography. Ni-NTA lesin was used to purify His-tagged WRS protein.

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(B) Secreted TNF-α analysis after treatment of purified WRS. 10 to 1000 nM of purified WRS protein was administered to differentiated human macrophage (THP-1) cells for 3 and 18 hours. Serum free media and dialysis buffer were used as negative control and LPS was used as positive control.

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A

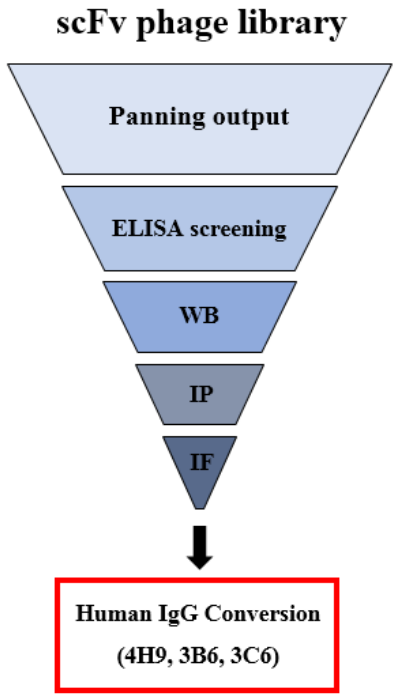

Figure 2. Selection of anti-human WRS antibody by biopanning

Overall schematic of screening of human WRS binding antibody.

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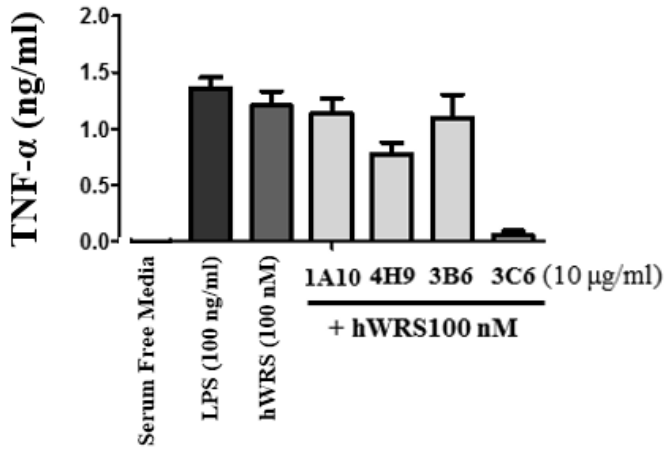
A



B

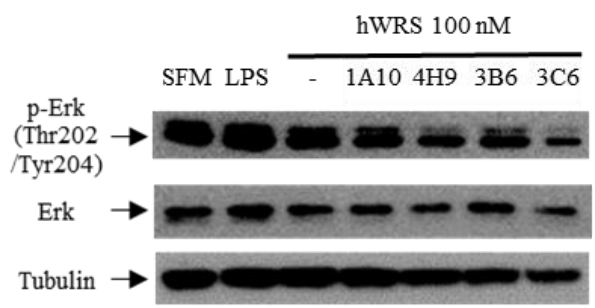


Figure 3. Effect of WRS neutralizing antibodies on TNF-α secretion from human macrophage cells

(A) Differentiated THP-1 cells were treated with human WRS protein and anti-WRS antibodies for 3 hours. Before treatment of WRS and antibodies, they were preincubated for at 37℃ for 2 hours. Monoclonal anti-WRS antibodies have shown an inhibitory effect on TNF-α secretion measured by ELISA

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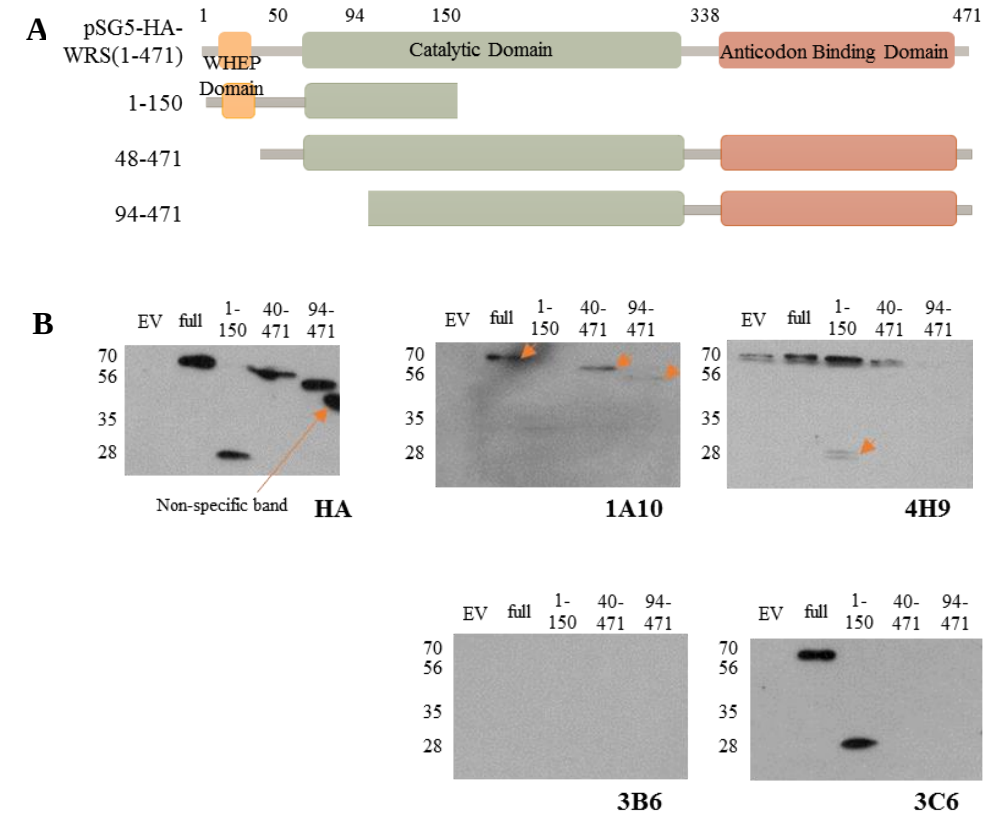
(B) Inhibition of Erk signaling by anti-WRS antibodies. The levels of Erk and phosphorylated-Erk activation were detected by western blot analysis.



C

	1A10	4H9	3B6	3C6
Full Length	O	O	-	O
1-150 AA	X	O	-	O
40-471 AA	O	X	-	X
94-471 AA	O	X	-	X

Figure 4. Epitope mapping of WRS antibodies by TnT and Western blotting

(A) Overexpressed WRS fragments were detected by anti-WRS antibodies

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(B) Expression of pSG5-HA-tagged clones was confirmed with HA antibody to quantify protein expression, and 4 types of anti-WRS antibodies bound to respectively each fragment.

(C) Table shows binding site of each antibody. Antibody 1A10 binds to C-terminal of WRS. Antibody 4H9 and 3C6 binds to N-terminal of WRS. Antibody 3B6 did not bind to WRS.

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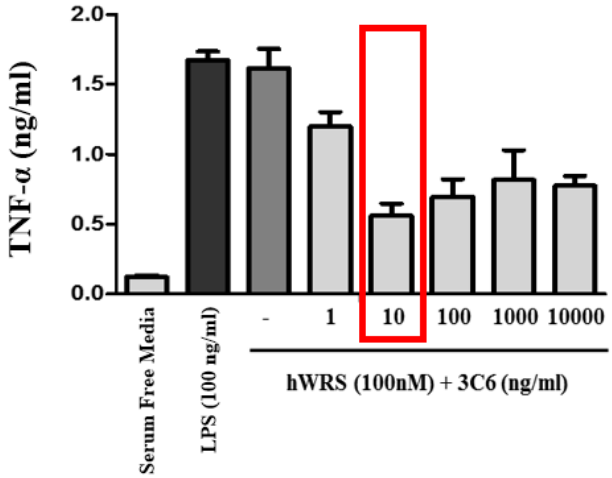
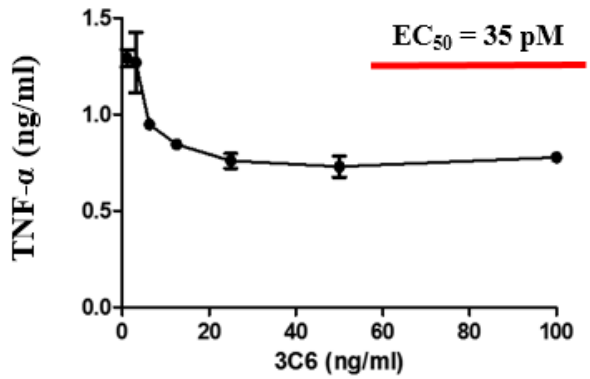
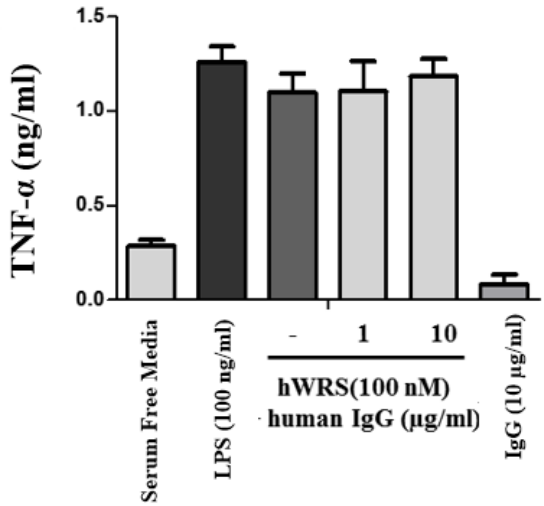
A


TNF-α (ng/ml)
2.0
1.5
1.0
0.5
0.0
Serum Free Media
LPS (100 ng/ml)
-
1
10
100
1000
10000
hWRS (100nM) + 3C6 (ng/ml)

B


TNF-α (ng/ml)
EC50 = 35 pM
1.5
1.0
0.5
0.0
0
20
40
60
80
100
3C6 (ng/ml)

C


TNF-α (ng/ml)
1.5
1.0
0.5
0.0
Serum Free Media
LPS (100 ng/ml)
-
1
10
hWRS(100 nM)
human IgG (µg/ml)
IgG (10 µg/ml)

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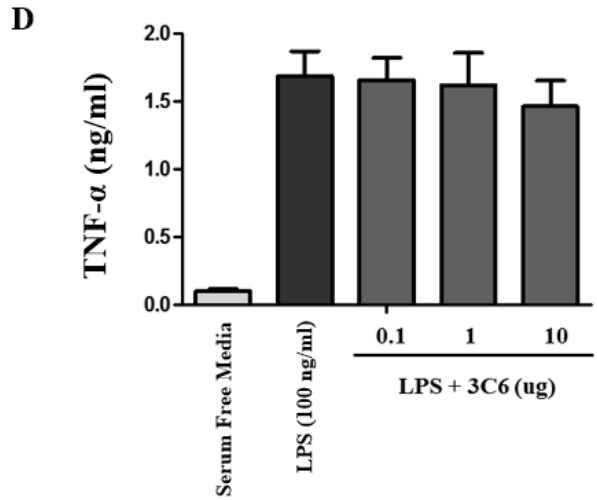

Figure 5. 3C6 antibody inhibits TNF-α secretion in human macrophage cells

(A, B) Levels of TNF-α in the cell culture supernatant were determined by ELISA. 3C6 antibody was diluted in 10 fold from 10 μg/ml (A), and 2 fold from 100 ng/ml (B)

(C) IC50 determination by using logarithm concentration.

(D, E) Human IgG were preincubated with WRS (D), 3C6 antibody were preincubated with LPS (E).

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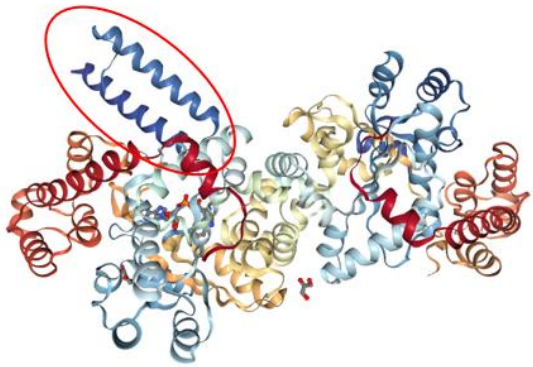
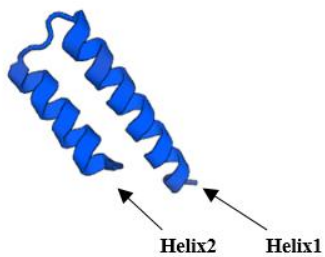
A Human WRS full length (1-471)



B Human WRS WHEP domain (1-47)



1. HumanWRS (1-47) : MPNSEPASLLELFNSIATQGELVRSLKAGNASKDEIDSAVKMLVSLK
2. Helix1 (7-30) : ASLLELFNSIATQGELVRSLKAGN
3. Helix2 (31-47) : ASKDEIDSAVKMLVSLK

C

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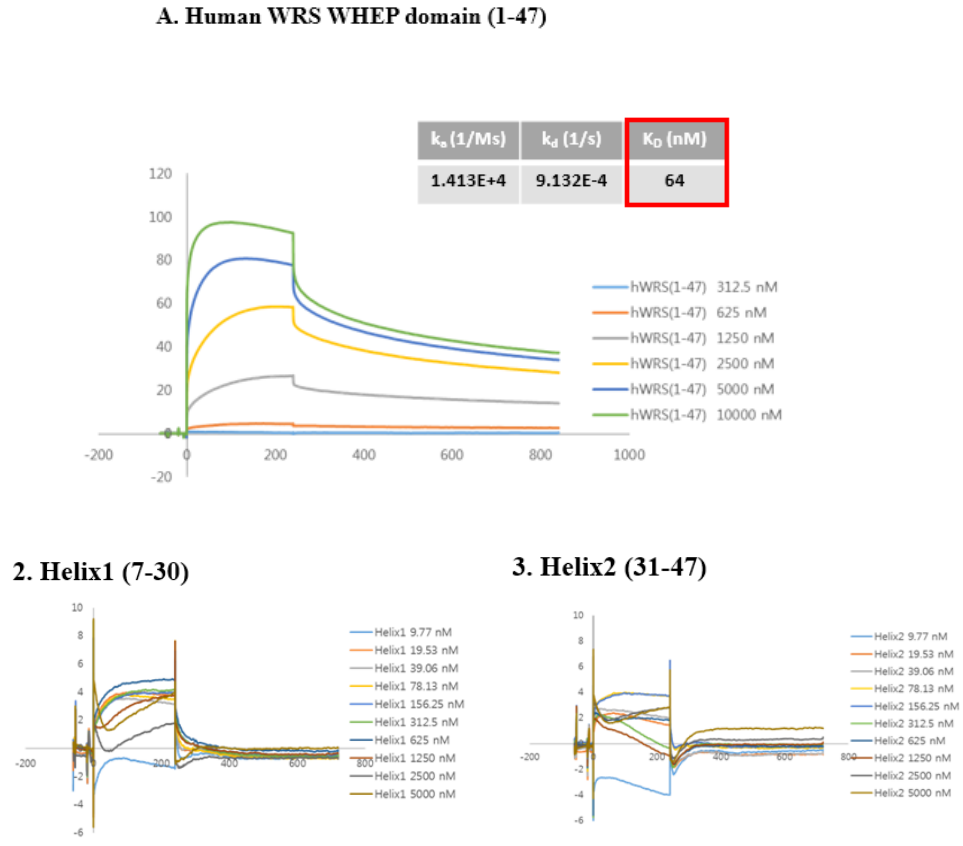

Figure 6. 3C6 antibody binding affinity test with WRS WHEP domain by SPR

(A) 3D structure of human WRS

(B) 3D structure of human WRS WHPE domain and amino acid sequence of WHEP domain and 2 helixes

(C) SPR sensogram graph between 3C6 antibody and WRS WHEP domain fragments (Human WRS (1-47), Helix1 (7-30), Helix2 (31-47)).

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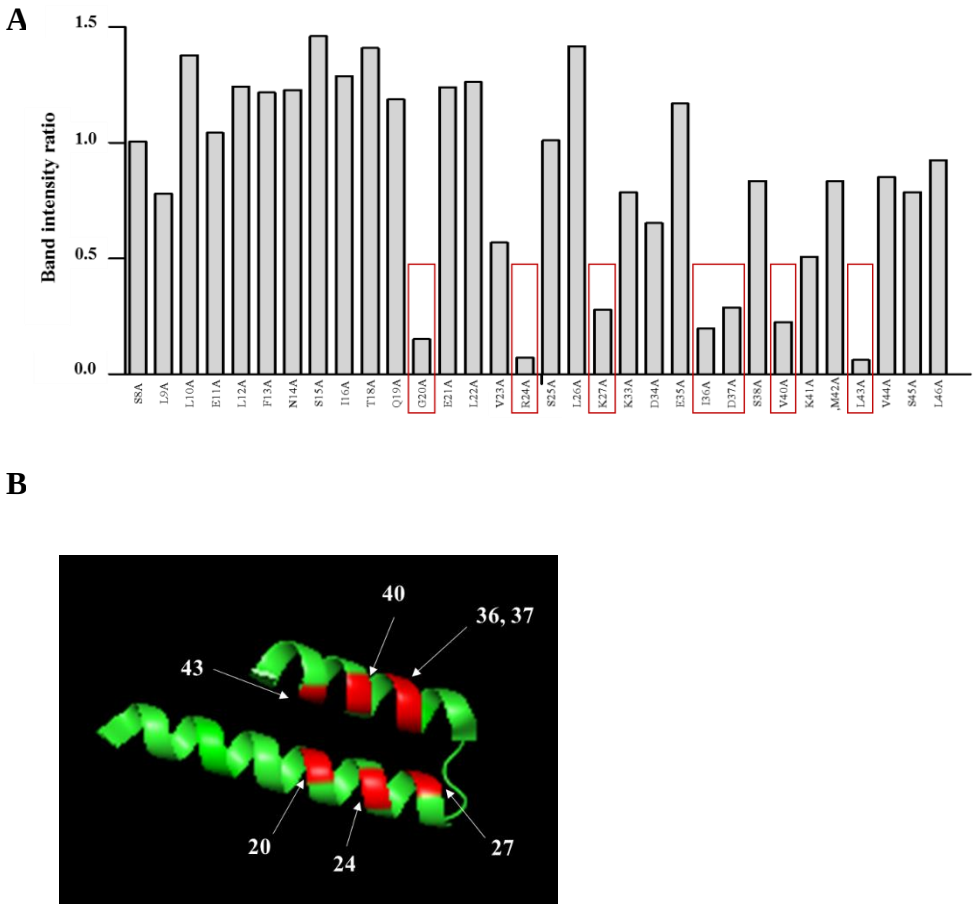

Figure 7. Identification of 3C6 binding site to WRS WHEP domain

(A) Alanine scanning of WRS WHEP domain were detected by using 3C6 antibody.

(B) Following the alanine scanning data, binding site of 3C6 were highlighted with red color and arrow.

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DISCUSSION

LPS is the major stimulator of innate immune responses contributing to the adverse effects of Gram-negative bacterial infection on host organism. LPS-induced signal transduction is known to be initiated by TLR4-MD2 complex [2]. Current study indicated that WRS has a key function of inflammatory cytokine production mechanism when host was under gram-negative bacterial infection [5]. WRS was secreted upon pathogen infection, and directly bound to TLR4-MD2 complex to induce phagocytosis and chemokine production [13]. This study provides possibility suggesting anti-WRS antibody as a novel therapeutic treatment of autoimmune disease by down-regulating of inflammatory cytokine levels. Using an anti-WRS antibody, 3C6, we showed here its cytokine reduction function in human macrophage cells. 3C6 binds to the N-terminal 47 amino-acid peptide (WHEP domain) of WRS and is specific and potent inhibitor of LPS-mediated cell activation *in vitro* [14]. Furthermore, decrease in cytokine level was not directly affected by 3C6 antibody alone, but preincubation with WRS protein. Most likely, 3C6 inhibited TLR4-MD2 signaling by blocking WRS-mediated dimerization of TLR4-MD2 complex [15]. In addition, we mapped the epitope of 3C6 to 47 amino acid region of the N-terminal domain of WRS. This region has previously been shown to be functionally important in determining the dimerization of TLR4-MD2 complex. Moreover, further studies are underway to increase binding affinity of 3C6 by antibody maturation process, and determine the inhibitory effect of antibody 3C6 in sepsis mouse model. There are several types of sepsis animal model, LPS injection, nose tip bacteria inoculum,

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CLP (Cecal ligation and puncutre), CASP (colon ascendens stent peritonitis), etc. Traditionally, LPS injected mouse model is used to induce hyper immune system, but CASP is known as the suitable model for sepsis study. Additionally, to figure out that antibody 3C6 also has an effect to other cytokines related to autoimmune disease such as interleukin 6(IL-6) and interleukin 10(IL-10), the level of these cytokines should be determined in human macrophage cells under hyper immune condition [16]. Finally, future studies should also address the effect of anti-WRS monoclonal antibody in combination with a single dose of antibiotic in sepsis mouse model. In conclusion, the result of this study demonstrated the existence of neutralizing effect of anti-WRS antibody in human macrophage cells. Such treatment of overexpressed innate immune system like sepsis, which was described in this paper, has a possible beneficial effect to downregulate of autoimmune disease.

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요약 (국문초록)

Tryptophanyl-tRNA Synthetase를 표적하는 항체를 통한 패혈증의 면역 사이토카인 억제 효능 연구

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고서연

신체에 병원균, 독소, 외래 물질 등과 같은 항원이 감염되면 이를 방어하기 위해서 선천 면역 반응이 일어난다. 그 중 대표적인 방어체계는 대식세포에서 외래 물질을 인식하는 TLR4(Toll-Like Receptor 4)-MD2(Lymphocyte antigen 96) 복합체가 dimer를 형성하여 면역 사이토카인을 생성함으로써 면역반응을 촉진하는 것이다. 그런데 감염된 외래 물질이 성공적으로 제거되었더라도 면역 사이토카인이 줄어들지 않고 체내에서 계속 높은 상태를 유지하여 끊임없는 면역반응이 일어나는 문제가 발생하는 것이 패혈증이다. 최근 연구에서 패혈증 환자의 혈액에 아미노아실 tRNA 합성효소 (Aminoacyl-tRNA synthetase)의 한 종류인 트립토파닐 tRNA 합성효소(Tryptophanyl-tRNA synthetase, WRS)가 높은 농도로 존재함이 알려졌다. WRS는 트립토판과 RNA를 연결하는 본래의 기능 이외에도 혈관신생 등의 다양한 기능들이 밝혀지고 있으며, 특히 이번 연구에서 집중할 것은 선천 면역 반응을 유도하는 기능이다. 선천 면역 반응에서 WRS의 역할은 TLR4-

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MD2 복합체에 직접적으로 결합하는 것이다. 그러므로 외부 물질에 감염 되었을 때, WRS가 선천면역반응을 자극해서 inflammatory cytokine의 생성에 관여한다는 것이 밝혀졌다. 본 연구에서는 WRS에 직접 결합하는 neutralizing antibody를 개발하여 선천 면역을 억제하고자 하였다. 인간 대식세포에 neutralizing antibody를 처리하여 면역 사이토카인 생성이 감소되는 것을 확인하였으며, TLR4-MD2 dimerization에 관여한다고 알려진 WRS의 WHEP domain에 binding 함으로써 면역 사이토카인이 감소한다는 것을 밝혔다. 이러한 결과를 토대로, 대식세포에서의 선천 면역 반응을 억제하는 항체를 사용하여 새로운 패혈증 치료제로서의 가능성을 제시하였다.

주요어 : Tryptophanyl-tRNA synthetase, innate immune, TLR4-MD2, monoclonal antibody, TNF-α

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