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The Human Lysyl-tRNA Synthetase Secreted by Shiga Toxins Triggers Proinflammatory Response to the Toxin-sensitive Cells

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석사학위논문

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Abstract

Shiga toxin producing *Escherichia coli* (STEC) strains are foodborne pathogens that cause fatal systemic complications such as hemolytic uremic syndrome and central nervous system (CNS). In patients infected with STEC, inflammatory responses are involved in reducing or exacerbating disease severity upon infections with the pathogen and require the participation of cytokine/chemokine in concert of leukocytes on Stxs-induced intestinal, renal, and CNS vascular lesions. To date, although numerous studies have been reported defining the proinflammatory response to Shiga toxin type 1 (Stx1) or Shiga toxin type 2 (Stx2) in *in vivo* and *in vitro*, no coherent mechanism has been proposed to explain the dynamic immune regulation involving cytokines or unknown mediators of inflammation during intoxication.

A complex of aminoacyl-tRNA synthetases, known as Multi-aminoacyl-tRNA synthetase complex (MSC), is only founded in higher organisms such as yeast, zebra-fish, mouse, and human. In human, MSC is composed of eight aminoacyl-tRNA synthetases and three axillary proteins. It is well known that each aminoacyl-tRNA synthetases has noncanonical functions such as immune response, tumorigenesis, angiogenesis, and inflammatory response.

Classical role of human lysyl-tRNA synthetase (KRS) within MSC is to support translational regulation. Otherwise, KRS also has been known that cytosolic KRS can regulate transcriptional activity of transcription factor MITF and USF2.

Furthermore, the form of secreted KRS engages in secondary cellular function triggering inflammatory response. However, the function of KRS against infection of human pathogenic bacteria was poorly understood.

This study identified the release of KRS to the extracellular space from the Stx1 or Stx2-treated human cervical cancer cells (HeLa) or macrophage-like THP-1 cells (D-THP-1). As I predicted, incubations of the D-THP-1 cells with enzymatic deficient Stxs were not capable of secreting KRS into the media. Furthermore, neither Stx1 nor Stx2 induced transcription of KRS mRNA and nucleus translocation. Unlike KRS, aminoacyl-tRNA synthetase complex-interacting multifunctional protein 2 (AIMP2), known as cytosolic binding partner of KRS, remained in cytosol without extracellular secretion when it is exposed to Stx2. Furthermore, addition of the exogenous KRS or co-treatment of KRS with Stxs activated secretion of inflammatory cytokines including IL-1β or TNF-α and chemokines including IL-8 or MIP1α in autocrine and paracrine manner. To support the pathogenesis mechanisms of Stxs associated with KRS, mouse model following the administration with Stx2 LD50 (10 ng/kg) demonstrated that the survival rate from the intoxicated KRS-hetero knockdown mice was significantly higher than that of the wild-type mice. In conclusion, this study implicated that KRS may be a key player to mediate proinflammatory signal transductions in susceptible host infected with STEC.

Keywords: Shiga toxins, Lysyl-tRNA synthetase, Proinflammatory response

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I. INTRODUCTION

Shiga toxins (Stxs) are major virulence factors generated by food-borne pathogenic bacteria, Shiga toxin-producing *Escherichia coli* (STEC) and *Shigella dysenteriae* serotype 1. In particular, infections with STEC as pathogen of significant public health concern have been strongly implicated in attribution of cause for the hemolytic uremic syndrome (HUS) which is characterized by acute renal failure, thrombocytopenia, and endothelial damage of glomeruli and kidney arterioles (Tarr *et al*., 2005). STEC produces Shiga toxins that may be mainly divided into two classes Stx1 and Stx2, based on their antigenic similarity to the prototypical Shiga toxin expressed by *Shigella dysenteriae* serotype 1 (Strockbine *et al*., 1986).

All Stxs have AB_5 molecular structure composed of enzymatic A-subunit in non-covalent association with homopentameric B subunits (Fraser *et al*., 1994). The pentameric B subunits are responsible for toxin binding to neutral glycolipid receptor globotriaosylceramide (Gb3, or CD77) on the surface of host cells mediating cytoplasmic delivery of the A-subunit (Lingwood *et al*., 2010; O'Brien, *et al*., 1992). The monomeric A-subunit of the toxin is responsible for eliciting highly specific N-glycosidase activity that mediates selective depurination of 28S ribosomal RNA within the large 60S of eukaryotic ribosomes (Endo Y. *et al*., 1988). Following endocytosis, the A-subunit intracellularly transports via the Golgi complex to the endoplasmic reticulum and then to the cytoplasm; a process termed retrograde transportation (Sandvig *et al*., 2001). During retro translocation, the

disulfide bond is reduced, then the A-subunit is cleaved into 2 fragments, A_1 (amino terminus, 1–251 amino acid) and A_2 (carboxyl terminus, 252–293 amino acid) leading to the release of A_1-fragment from the holotoxin (Tam P. J. *et al*., 2012). Subsequently, proteolytically processed A_1 - fragment results in ribosomal inactivation and protein synthesis inhibition by blocking eEF-1 and eEF -2 dependent peptidyl elongation process (Endo Y. *et al*., 1988). The translocation into ER-lumen to reach cytosol of the Stxs as multi-functional proteins is required for inducing ribotoxic stress responses, apoptosis through ER stress, autophagy, and proinflammatory response (Lee *et al*., 2008).

Production of cytokines and chemokines such as interleukin-1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), macrophage chemoattractant monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α/β), and Gro-β may induce injury in specific illness such as neurological abnormalities and Hemolytic Uremic Syndrome in development of life threatening conditions such as acute renal failure (Hemolytic Uremic Syndrome) and neurological abnormalities. (Lee M. S. *et al*., 2013)

A number of studies have been reported the Stxs play not only multifunctional role but also ribotoxic stress response. Stxs trigger apoptosis through various mechanisms in different cell types (Cherla R. P. *et al*., 2003). Human monocytes or macrophage-like cell lines respond to the toxins by stimulating signaling pathways via cell stress- activated protein kinases. These include p38 MAPK/ERK and JNK who lead upregulating expression of cytokines and chemokines (Harrison L. M. *et*

al., 2004). Stxs stimulate cytokine production through activation of transcription factors, nuclear factor κB (NF-κB) and activator protein 1 (AP-1) at transcriptional and posttranscriptional levels (Sakiri R. *et al*., 1998). Moreover, Stxs may act in communication with bacterial lipopolysaccharide (LPS) and induce the production of TNF-α, IL-1 and IL-6 by macrophages, thus rendering the vascular endothelial cells are more sensitive to the toxin (Tesh V. L. *et al*., 1994).

Aminoacyl-tRNA synthetases (AARSs) are proteins that are involved in protein translation. They catalyse the ligate of amino acids to their cognate tRNAs with a high fidelity. Mammalian AARSs carry out highly conversed catalytic domains acting same function as their prokaryotic counterparts but they also contain other domains in addition to their catalytic domains, such as GST-like domains, WHEP domains, leucine zipper domains and α-helical appendices (Guo M. *et al*., 2010). These domains enable themselves to interact with each other or other various proteins, which are defined as noncanonical functions.

Interestingly, in human, AARSs are organized into a MSC, where eight AARSs (IRS, LRS, QRS, KRS, RRS, DRS, MRS and EPRS) and three AARS-interacting multifunctional proteins (AIMP1, AIMP3, AIMP3) (Mirande M. *et al*., 1982). This complex may promote protein synthesis and is also serve as a reservoir of regulation molecules for functions beyond aminoacylation (Kyriacou S. V. *et al*., 2008). Several AARSs have extracellular signaling functions that are stimulated in response to inflammatory cytokines, and these AARSs play some roles in angiogenesis, tumorigenesis, inflammatory response and immune response. Human

biofunctional enzyme EPRS has two catalytic domains linked by three tandem repeat WHEP domains (Rho S. B. *et al*., 1998), which are critical for targeting and regulating GAIT complex by binding with RNA (Jia J. *et al*., 2008). EPRS has been shown to recognize and bind target mRNAs (Sampath P. *et al*., 2004). The interaction between individual components of GAIT complex requires their phosphorylation which is induced by IFN- gamma (Arif A. *et al*., 2012; Vyas K. *et al*., 2009). This complex regulates gene expression during inflammation by binding at 3'untranslated region elements within a family of mRNAs encoding inflammatory proteins and suppresses their translation (Turpaev K. T. *et al*., 1996). Several AARSs have been reported as secreted cytokines that regulate immune responses and angiogenesis. Under IFN-gamma stimulation, N-terminal 47 amino acids of WRS is truncated and generate alternative splicing form (mini-WRS) (Tolstrup A. B. *et al*., 1995). Both of the full-length WRS and mini-WRS have enzymatic activity but mini-WRS only has a function of angiogenetic cytokines (Tzima E. *et al*., 2005). The amino acids sequence DTIEEHRQ is located in tRNA anticodon binding domain corresponding for angiostatic activity (Wakasugi K. *et al*., 2002). The mini-YRS (N terminal 364 amino acid N-terminal of YRS) is generated by cleavaging from YRS into two separate domains by leukocyte elastase (Wakasugi K. *et al*., 1999; Quevillon S. *et al*., 1999). Inside MSC, AIPM2 is the scaffolding protein, where KRS interacts tightly with AIMP2 (Choi J. W. *et al*., 2009). AIMP2 is also found in TNF signaling pathway as a pro-apoptotic mediator through TRAF2. AIMP2 binds to TRAF2 after a TNF signal and induces the ubiquitylation of TRAF2 through the E3 ligase BIRC2 (Han J. M. *et al*., 2008). In

response to DNA damage, AIMP2 is translocated to the nucleus and binds the tumor suppressor p53 and prevents p53 from the ubiquitylation by MDM2 (Wang Z. *et al*., 2010; Labirua A. *et al*., 2010). The study showed the connection between TRS and autoimmune diseases such as polymyositis and dermatomyositis through its identification as the target for the myositis autoantibody PL-7 (Williams T. F. *et al*., 2013). TRS is also secreted in response to inflammatory or angiogenic signaling of endothelial cell migration through VEGF and/or TNF-α receptors (Levy C. & Fisher F. E. *et al*., 2011).

Canonical function of KRS is translocated to the nucleus and binds transcription factor MITF (microphthalmia-associated transcription factor) which is an oncogenic transcriptional activator (Razin E. *et al*., 1999). MITF is sequestered by its interaction with HINT1 (histidine triad nucleotide-binding protein 1) (Lee Y. N. & Razin E. *et al*., 2005). Upon binding, KRS can introduce Ap4A molecule that binds HINT1, leading to the release of HINT1 from the complex that allows the transcription of MITF responsive genes (Lee Y. N. *et al*., 2004). The extracellular signaling activity and functional versatility of human KRS have been identified with pathophysiological significance in various cancer cell types (Park *et al*., 2005). KRS is also secreted from cancer cells in response to TNF. The secreted KRS bound to macrophages and peripheral blood mononuclear cells to enhance their migration and TNF-α production (Kim D. G. *et al*., 2012). The MAPK, ERK and p38 were determined to be involved in the signal transduction triggered by KRS (Park *et al*., 2005).

The function of KRS has been revealed in many studies. However, the host response mechanism of KRS by bacterial toxins was poorly understood. Therefore, I characterized the secretion of KRS within the relation with Shiga toxin intoxication and the following effect of the process.

In this study, I observed secretion of KRS from immune sensitive cells using western blotting and ELISA. Furthermore, I found out that secreted KRS was derived from MSC, not from its overexpression. Moreover, the function of released KRS was identified as proinflammatory mediator in immune sensitive cells. In addition, as *in vivo* data, toxicity of Stx2 was attenuated in KRS heterozygous knockout mice compared to wild-type mice. These results might be the influence of the reduction of KRS production in KRS heterozygous knockout mice.

Thus, This study suggested that human KRS secreted by Stxs induced proinflammatory response in autocrine and paracrine manners.

II. MATERIALS AND METHODS

Antibodies and reagents. The following antibodies from Cell Signaling Technology (CST; Danvers, MA) were used: anti-Myc with HRP (Horseradish peroxidase) conjugated anti-human-LysRS (9990), anti-β-Actin (12620). The following antibodies were purchased from Neomics (Gyeonggi province, South Korea): anti-aspartyl-tRNA synthetase (NMS-01-0013), anti-human-AIMP1 (NMS-01-0019), and anti-human-AIMP2 (NMS-02-0011). The primary antibodies were bound by monoclonal anti-mouse or anti-rabbit HRP conjugate secondary antibodies (7074; CST). In order to immunoprecipitate myc-tagged proteins, Anti-c-Myc Agarose Conjugate (A7470; Sigma Aldrich, St. Louis, MO) beads were used. To purify KRS from mammalian cells, a Strep-Tactin column system (IBA, Goettingen, Germany) was used. G-418 (A1720; G-418 disulfate salt) antibiotic reagent was purchased from Sigma Aldrich for the selection of KRS-expressing stable cell line. Transfection reagent, X-tremeGENE HP DNA, from Roche Diagnostics (06365809001; Mannheim, Germany) was used.

Expression vector. Expression plasmid pEXPR-IBA5 (IBA) containing a twin-strep tag on the N-terminus was constructed for the expression of KRS (LysRS) and AIMP2 genes. The expression plasmid pcDNA3-myc with myc-tag on N-terminus was constructed for the expression of KRS gene. All of these constructs were provided by Professor Sunghoon Kim from Seoul National University.

Toxins. Stx1 was prepared as previously described (Tesh *et al*., 1993). Briefly, Stx1 was purified from cell lysates prepared from *E. coli* DH5α (pCKS112), a recombinant strain containing a plasmid encoding the *stx1* operon, by sequential ion exchange and immuno-affinity chromatography. The purity of toxin preparations was assessed by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) with silver staining and Western blotting analysis. Toxin preparation were passed through ActiClean Etox columns (Sterogene Bioseperations, Calsbad, CA) to remove trace endotoxin contaminants and were determined to contain < 0.1 ng of endotoxin per ml as determined by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA). Purified Stx1 holotoxin containing a double mutation (E167Q and R170L) in the A subunit which dramatically reduces enzymatic activity. Recombinant purified Stx2, Stx2 A^{-} toxoid (Y77S/E167Q/R170L), and Stx2 B-subunits were obtained from the NIAID, NIH Biodefense and Emerging Infections Research Repository (BEI Resources, Manassas, VA).

Cell culture. The FreeStyle™ 293-F Cell line (Life Technologies, Grand Island, NY) which is adapted to the suspension culture system was cultured in FreeStyle™ 293 Expression Medium (Gibco, Grand Island, NY) in 50 ml Erlenmeyer flask at 37℃ in 8% CO_2 inside a humidified incubator with suspension at 120 rpm. Cells were subcultured every three days at a density of 2×10^6 cells/ml. The human myelogenous leukemia cell line THP-1 was purchased from the American Type

Culture Collection (ATCC; Manassas, VA). Cells were maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% Antibiotic-Antimycotic (Gibco) at 37℃ in 5% CO_2 in a humidified incubator. These cells were classified as undifferentiated monocytic cells. THP-1 cells (2.5×10^6 cells/well) were treated with 50 ng/ml of Phorbol-12-myristate-13-acetate (PMA; Sigma Aldrich) for 48 h prior to the experiments to differentiate THP-1 cells to the adherent macrophage-like state. Cells were incubated on 6-well plate (Nunc, Waltham, MA) at 37℃ in 5% CO_2 in a humidified incubator. After 48h, non-attached cells were washed with cold Dulbecco's phosphate-buffered saline (Gibco) twice, and adhered cells were incubated in a fresh complete RPMI media to give a final concentration, approximately 2.5×10^6 differentiated THP-1 cells/well for 3 days. The cell culture media was changed to fresh complete RPMI without PMA every 24 h for the next 3 days. Experiments were performed on the fourth day after PMA removal. The cervix epithelial cell line HeLa from ATCC was maintained in MEM (Gibco) supplemented with 10% FBS and 1% Antibiotic-Antimycotic at 37℃ in 5% CO_2 in a humidified incubator. The human kidney cortex/proximal tubule epithelial cell line HK-2 was purchased from the ATCC. HK-2 cells were maintained in Keratinocyte-Serum Free Medium (K-SFM) (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract, human recombinant epidermal growth factor, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Preparation of cellular lysates and western blotting. Undifferentiated and

differentiated THP-1 cells were treated with Stx1, Stx2, holotoxins with mutations in the A-subunit (Stx1 A^{-}, Stx2 A^{-}) or B-subunit alone without A-subunit (Stx1 B-sub, Stx2 B-sub) for 0 to 24 h. Following stimulation, cells were harvested with CHAPS [50 mM Pipes/HCl (pH 6.5), 2 mM EDTA, 0.1% Chaps, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride] (CST) buffer supplemented with protease inhibitor [50 μM PMSF, 10 μM Pepstatin A, 20 μM Leupeptin, 100 μM Bebzamidine, 50 μM Bestatin] (Gendepot, Barker, TX) and phosphatase inhibitor [Sodium flroride, Sodium orthovanadate, Sodium pyrophosphate, Sodium glycerophosphate] (Gendepot) cocktails and centrifuged at 16,000 × *g* for 20 min at 4℃. Protein quantification was determined using the DC Protein Assay (Bio-rad, Hercules, CA). Equal amounts of protein samples were separated by Bolt 4-12% Bis-Tris plus gels (Life Technologies) via SDS-PAGE in MES running buffer (Life Technologies) and transferred to polyvinylidene difluoride (PVDF) membranes using a iBlot-2 dry blotting system (Life Technologies). Membranes were blocked with Casein blocking buffer (Sigma Aldrich) [200 mM Tris pH 7.6, 1.38 mM NaCl, 0.1% Tween 20] and washed three times for 5 min with TBST [20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20]. Membranes were then incubated with primary antibodies with TBST + 4% FBS at 4℃ overnight. Membranes were then washed three times and incubated with HRP-labeled secondary antibodies at 1 h at room temperature in Casein blocking buffer (Sigma Aldrich). Membranes were developed using Image Quant LAS 4000 imaging system (GE Healthcare, Amersham, UK).

KRS secretion test in HeLa cells. pcDNA-LysRS-myc transfected HeLa cells (10 × 10^6 cells/well) were both stimulated and unstimulated with Stx2 (1 ng/ml) for 24 h. Cells and cell-free supernatants were collected after toxin treatment. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer [150 mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA] supplemented with protease inhibitor and phosphatase inhibitor cocktails. Cell free-supernatants were cleared by centrifugation at 16000 × *g* for 20 min at 4℃ and concentrated using the Amicon® Ultra centrifugal filter system. Concentrated samples were separated by 4-12% Bis-Tris plus gels via SDS-PAGE in MES SDS Running buffer and transferred to PVDF membranes.

Purification of Multi-aminoacyl-tRNA synthetase complex in HeLa cells. Human cervix epithelial cell line HeLa was cultured in 50 plates of 150 mm (10 × 10^6 cells/plate) cell culture dish in MEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic at 37℃ in 5% CO_2 in a humidified incubator. Upon 80% confluences of cells, cells were transfected with AIMP2-expressing vector (pEXPR-IBA5-AIMP2) using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. Following transfection for 48 h, 25/50 plates of cells were washed with DPBS twice, and stimulated with Stx1 (10 ng/ml) for 12 h in a 10% serum containing MEM. After 12 h, cells were washed out with DPBS twice, and harvested with MSC lysis buffer [250 mM Tris/HCl pH 7.4, 37.5% glycerol, 750 mM NaCl, 5 mM EDTA] supplemented with protease inhibitor and phosphatase

inhibitor cocktail. The remaining 25 plates were harvested using a lysis buffer without any stimulus. Cell lysates were centrifuged at 16000 × *g* for 90 min at 4℃. The supernatant was filtered using Steritop-GP 0.2 μm polyethersulfone, 1000 ml 45 mm, radio-sterilized (Merck Millipore, Darmstadt, Germany). The filtered sample was then loaded into the Strep-tag-Strep Tactin (IBA) 1 ml column. The column was washed with Strep-tag washing buffer [100 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA] (IBA) and eluted with Strep-tag elution buffer [100 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin] (IBA). Eluted protein samples were concentrated and injected to a Superdex 75 10/300 GL gel filtration column (GE Healthcare), installed on an AKTA FPLC system (GE Healthcare) which was equilibrated with the sample buffer [50 mM Tris-HCl, pH 8.0, 250 mM NaCl]. Protein was eluted at a flow rate of 0.4 ml/min at room temperature. Purified MSC complex was visualized using Coomassie Blue staining in a 4-12% gradient SDS-PAGE gel (Life Technologies).

KRS-expressing stable cell line and purification. 293F cells were cultured in 50 ml (1×10^6 cells/ml) Free-style 293 Expression Medium (Gibco) in an Erlenmeyer flask at 37℃ in 5% CO_2 in a humidified incubator with suspension at 120 rpm. Upon accumulating 2×10^6 cells/ml, cells were transfected with KRS-expressing vector (pEXPR-IBA5-KRS) using X-tremeGENE HP DNA reagent according to the manufacturer's protocol. Following transfection, cell culture media was utilizing 200 μg to 800 μg of G 418 sulfate (Sigma Aldrich) incrementally every three days

until the cells were expressing KRS-stably. 500 ml (2×10^6 cells/ml) of KRS-expressing 293F cells were centrifuged at 500 × *g* for 30 min at 4℃. The cell pellet was lysed using MSC lysis buffer supplemented with the protease and phosphatase inhibitor. Cell lysates were centrifuged at 16000 × *g* for 90 min at 4℃. Supernatant was filtered using Steritop-GP 0.2 μm polyethersulfone, 1000 ml 45 mm, radio-sterilized. The filtered sample was loaded into the Strep-tag-Strep Tactin column. The column was washed with Strep-tag washing buffer and eluted with Strep-tag elution buffer. Purified KRS was confirmed by SDS-PAGE coomassie blue staining and western blotting.

Isolation of nuclear and cytoplasmic extracts. In order to fractionate the nucleus and cytoplasm of KRS-transfected cells, D-THP-1 cells were treated by Stxs for 0 to 240 min. Treated cells were fractionized using NE-PER nuclear and cytoplasmic extraction kit (Thermo scientific, Waltham, MA) following the manufacture's protocol. Protease inhibitor and phosphatase inhibitor were added to cytosolic (Cyto) and nucleic (NE) extracts.

Immunoprecipitation. HeLa cells were transfected with KRS-expressing vector (pcDNA3-KRS-myc). Cells were treated with Stx1 (10 ng/ml) or Stx2 (100 pg/ml) for 30 to 360 min and harvested with RIPA lysis buffer supplemented with protease inhibitor and phosphatase inhibitor. Equal amounts of cell lysates were incubated with anti-c-myc agarose beads for 3 h at 4℃. Following incubation, beads were

washed out with ice-colded DPBS and centrifuged at 500 × *g* for 3 min for three times. Immunoprecipitated samples were acquired by boiling off the beads with 5 × SDS loading buffer for 10 min at 95℃.

Enzyme-linked immunosorbent assay (ELISA). Quantification of human KRS protein was performed using Lysyl-tRNA synthetase ELISA kit from Neomics (Gyeonggi province, South Korea). D-THP-1 cells in 6-well plate (2.5×10^6 cells/well) were treated with Stx1 (400 ng/ml) or Stx2 (10 ng/ml) for 0 to 24 h to stimulate KRS secretion. Cell cultured media was collected and concentrated using an Amicon® Ultra centrifugal filter system. Specified volumes of the samples were added into 96-well polystyrene microtiter plates coated with KRS. Two independent experiments were performed by following the manufacturer's instructions.

To examine the quantity of inflammatory cytokine production in cell-free supernatants, D-THP-1 cells (2.5×10^6 cells) and HK-2 (2.0×10^6 cells) cells were treated by KRS (0.1 μM), Stx1 (400 ng/ml) or Stx2 (10 ng/ml) for 0 to 24 h in serum free RPMI medium. Cell-free supernatants were collected and quantification of cytokine production by IL-8 or IL-1β or TNF-α or MIP1α ELISA kit (KOMA biotech, Seoul, South Korea) was performed.

RNA Isolation. 2.5×10^6 D-THP-1 cells were plated in 6-well plates. Cells were treated with either Stx1 (400 ng/ml) or Stx2 (10 ng/ml) for 4 h or 12 h in serum free RPMI medium. Following treatment, total RNA was isolated from the cells using

the column based purification system, PureLink® RNA Mini Kit (Ambion, Austin, TX). Isolation was performed according to the manufacturer's procedures. For the elimination of genomic DNA contamination, the purified RNA samples were treated with TURBO DNase^{Tm} (Ambion) for 30 min. The purity and concentration of RNA were measured using spectrophotometry (Nanodrop Technologies, Wilmington, DE).

Real-time PCR. Real-time (RT)-PCR was performed with isolated RNA using the RealHelix^{TM} qRT-PCR system (Nanohelix, Daejeon, South Korea) according to the manufacturer's instructions. The following real-time primers were purchased from Bioneer (Daejeon, South Korea):

KRS, 5′-GCCTCAAAGACAAGGAAACAAG-3′ (forward),
5′-TGTCCAGCTCGTTGTGATAAG-3′ (reverse);

β-Actin, 5′-CCTGGCACCCAGCACAAT-3′ (forward),
5′-GCCGATCCACACGGAGTACT-3′ (reverse).

All PCR primers for target genes were designed through the Roche Assay design Center (http://www.universalprovelibrary.com). Quantitative RT-PCR reactions were carried out in a LightCycler 96 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. A total of 20 ng of RNA were utilized in the reverse transcription reactions. The following run protocol was used: cDNA synthesis (50˚C, 40 min); denaturation (95 ˚C, 12 min); amplification for 40 cycles (95˚C, 20 sec; 60˚C, 1 min). The raw fluorescence data was normalized by β-Actin RNA expression level. Relative expression values were calculated using LightCycler 96 software version 1.01.

MTS-based cell cytotoxicity assay. D-THP-1 cells (2.5×10^6 cells/well) in 6-well plates were trypsinized and washed three times with DPBS. Prior to treatment, cells (1.0×10^4 cell/well) were seeded in 96-well microtiter plates (Nunc) in RPMI medium supplemented with 10% FBS. Cells were treated with KRS (0.1 μM), Stx1 (400 ng/ml) or Stx2 (10 ng/ml) for 24 h. Cytotoxicity was determined by colorimetric assay using the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenylsulfophenyl)-2-(4-H-tetrazolium] inert salt; (MTS; Promega, Madison, WI). Twenty microliter of MTS reagents was added to each well and incubated for 1 h at 37˚C in a 5% CO2 humidified incubator. Optical density was recorded with an automated microtiter plate reader at an absorbance of 490 nm (Biotek, Winooski, VT). Cell death percentages were calculated using the following equation: percentage of cell death = [(average OD_{490} of treated cells-average OD_{490} of control cells) ÷ average OD_{490} of control cells] × 100. Untreated cell measurements with the background absorbance at 630 nm were subtracted from each sample reading. The reference wavelength of 630 nm was used to subtract background absorbance contributed by excess cell debris and other nonspecific factors.

Animal studies. Female 8-week-old KRS knock down (KD) mice with a C57BL/6 wild-type (WT) control mice were provided from professor Sunghoon Kim (Seoul National University, Seoul, South Korea). Prior to experiments, mice were acclimatized with unlimited water and food in a specific pathogen-free facility.

Appropriate amount of Stx2 (10 ng/kg) was dissolved in ice-colded PBS before it was intraperitoneally administration to mice. Survival rate and body weight were observed following injection until death. All animal experiments were approved by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of health.

Statistical analysis. Data are reported as means ± standard errors of the mean (SEM) for at least three independent experiments. Data were analyzed for statistical significance by Excel (Microsoft, Redmond, WA). The student's *t* test was performed to measure differences in samples. A *P* value of <0.05 and <0.01 were considered statistically significant.

III. RESULTS

Secretion of KRS in Stxs intoxicated macrophage-like human monocytic (D-THP-1) cells.

To test whether Stxs induce KRS secretion, human monocytic leukemia cell line (THP-1) and macrophage-like monocytic (D-THP-1) cells were either untreated or treated with Stx1, Stx1 A^{-}, Stx1 B-subunit, Stx2, Stx2 A^{-} or Stx2 B-subunit for 12 h. Cell lysates and cell-free supernatants were harvested for KRS measurement by western blotting and ELISA. Western blotting data indicated that secretion of KRS was not observed from non-differentiated human monocytic cells but differentiated THP-1 cells (Fig. 1A). Moreover, enzymatic deficient Stxs and B-subunit deficient Stxs treated D-THP-1 cells were not capable of releasing KRS into the media. Other MSC component such as EPRS and AIMP2 were not detected in toxin treated cell culture supernatants. These data suggest macrophage-like THP-1 cells released KRS specifically via Stxs intoxication. Furthermore, KRS secretion level quantities were determined using ELISA (Fig. 1B). KRS secretion was significantly increased by Stx1 or Stx2 in D-THP-1 cells. Overall, these data suggests that Stxs had an impact on inducing KRS secretion in macrophage-like monocytic cells.

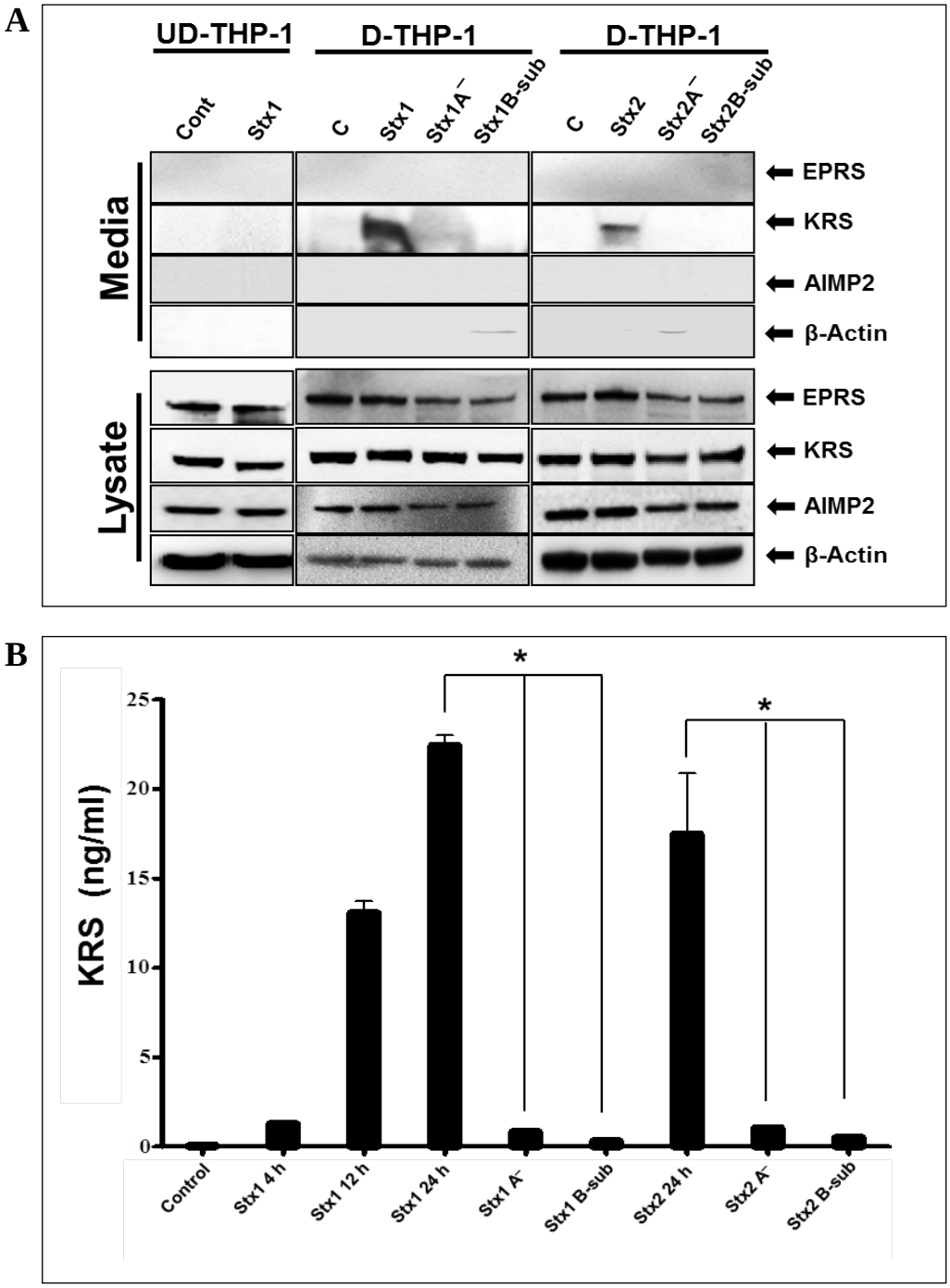


Fig. 1. Induction of KRS secretion in D-THP-1 cells by Stxs. (A)

Undifferentiated THP-1 (UD-THP-1) or differentiated THP-1 (D-THP-1) cells were either untreated or treated with Stx1 (400 ng/ml), Stx2 (10 ng/ml), holotoxins with mutations in the A-subunit (Stx1 A^{-}, 400 ng/ml; Stx2 A^{-}, 10 ng/ml) or B-subunits alone without enzymatic A^{-} subunit (Stx1 B-sub, 400 ng/ml; Stx2 B-sub, 10 ng/ml) at the indicated time points. The upper panels show immunoblots of KRS, AIMP2, EPRS, and actin in culture supernatants as determined with each of the specific antibodies. The lower panels show immunoblots of KRS, AIMP2, EPRS, and β-Actin in the lysates of the same samples. this data is representative of three independent experiments. (B) ELISA of KRS released from D-THP-1 cells left unstimulated (Cont) or stimulated with Stx1, Stx2, Stx1 A^{-}, Stx2 A^{-}, Stx1 B-subunit, and Stx2 B-subunit for 12 h. Standards were provided with the kits were used to calculate soluble cytokine/chemokine protein amounts. Values are given as pg/ml and expressed as the mean ± standard error of three independent experiments. Statistical significance was calculated using one-way ANOVA.

Identification of the origin of secreted KRS by Stxs treatment.

It was reported that KRS from cytosolic MSC can translocate to the nucleus to promote producing proinflammatory cytokines (Park S. G. *et al*., 2005).

To identify whether secreted KRS was derived from KRS expression by Stxs treatment, RT-PCR with KRS primers was performed in D-THP-1 cells. Neither Stx1 nor Stx2 induced an increase of KRS mRNA, suggesting that the KRS, presence in the media, was not due to an increase in KRS expression (Fig. 1A). Furthermore, The translocation of KRS into the nucleus by Stx2 treatment was further investigated. Stx2 was treated to the D-THP-1 cells for 0 to 240 min and separated to cytosolic fraction (Cyto) and nucleus fraction (NE). Western blotting data revealed that the translocation level of KRS was maintained constantly in cytosolic extracts and nuclear extracts by Stx2 intoxication (Fig. 2B).

These data suggest that KRS is neither overexpressed nor translocated into the nucleus by Stxs.

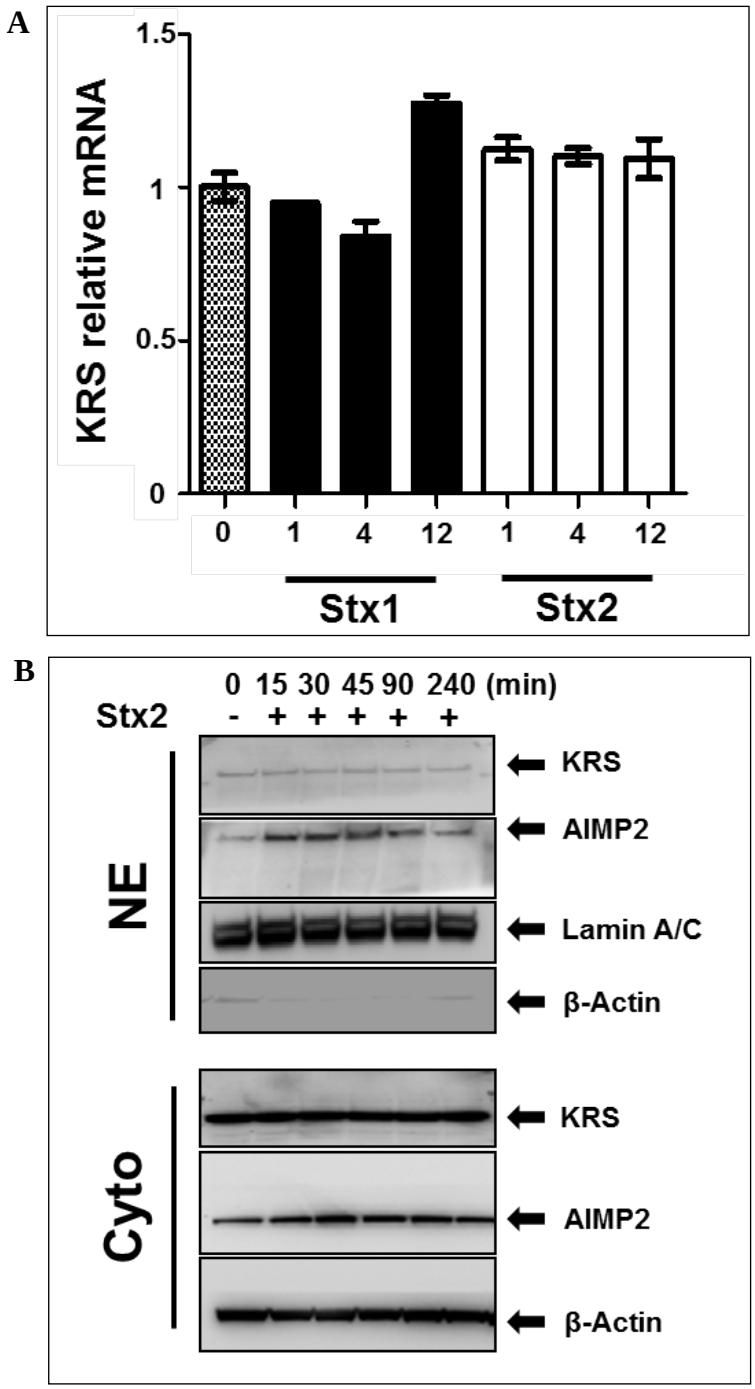

A
KRS relative mRNA
1.5
1
0.5
0
0
1
4
12
1
4
12
Stx1
Stx2
B
0 15 30 45 90 240 (min)
Stx2
- + + + + +
NE
KRS
AIMP2
Lamin A/C
β-Actin
Cyto
KRS
AIMP2
β-Actin

Fig. 2. Real-time PCR and nuclear fractionation for the identification of a source of secreted KRS by Stxs. (A) D-THP-1 cells (2.5×10^6) were treated with Stx1 (400 ng/ml) or Stx2 (10 ng/ml) at indicated time points. RNA extracts of each sample were isolated for real-time PCR analysis. RT-PCR was performed with primers specified for human KRS and β-actin to measure relative expression levels of the genes in the unstimulated or stimulated D-THP-1 cells with Stx1 or Stx2. Values from the results of RT-PCR were normalized to the expression of β-actin. The data shown are the mean fold induction ± SEM of three independent experiments. (B) D-THP-1 cells (2.5×10^6) were treated with Stx2 (10 ng/ml) for indicated time points and fractionated to cytosol and nucleus. Equivalent nucleus and cytosolic fractions were subjected to SDS-PAGE followed by immunoblotting of KRS, AIMP2 and β-actin.

Secretion of KRS in HeLa cells by Stx2.

It has been reported that secretion of KRS is induced by TNF-α in cancer cells and secreted KRS triggers proinflammatory response (Park S. G. *et al*. 2005). It is plausible to believe that KRS secretion in HeLa cells may be induced by Stxs treatment.

To verify KRS secretion in HeLa cells by bacterial toxins, HeLa cells were transfected with N-terminus myc-tagged KRS expression vector and challenged with Stx2. The release of KRS in cultured supernatant was observed in western blotting analysis (Fig. 3).

These data indicate that Stxs stimulate the secretion of KRS not only in macrophage-like monocytic cells but also in cervical cancer cells.

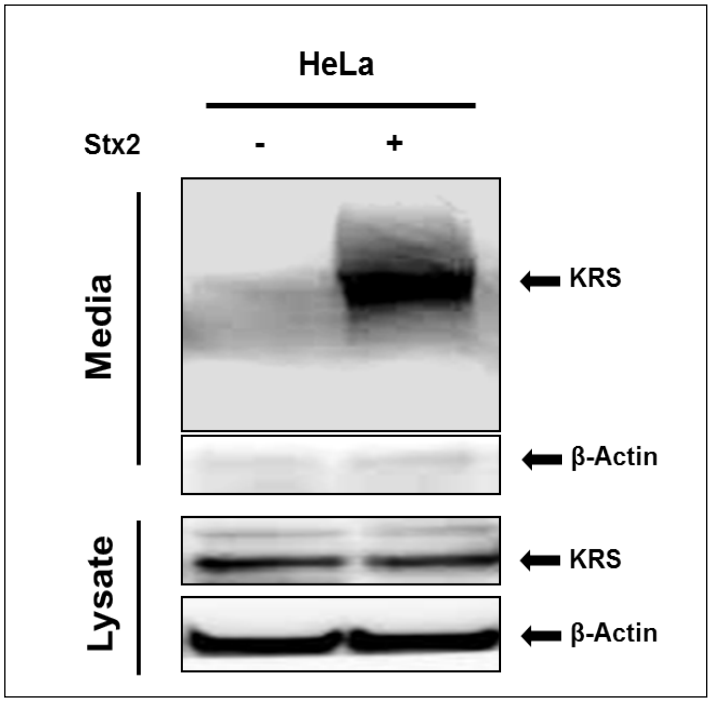


Fig. 3. KRS secretion in Stx2 treated HeLa cells. Prior to the experiments, HeLa cells (2×10^8) were transfected with myc-tagged KRS-expressing construct for 36 h. Cells were treated with Stx2 (100 pg/ml) for 12 h, and the whole cell lysates and cell-free supernatant were collected for western blotting. Equivalent media and lysates were subjected to SDS-PAGE followed by immunoblotting of KRS, AIMP2 and β-actin

Dissociation of Multi-aminoacyl-tRNA synthetase complex in Stx1 intoxicated HeLa cells.

Previous data demonstrated that secreted KRS from D-THP-1 cells was not originated from overexpression. Besides, it is known that 90% of AARSs are associated with MSC. Thus, it is hypothesized that secreted KRS by Stxs intoxication may be derived from MSC. To demonstrate this hypothesis, it is necessary to make a construct which can gather MSC components. Because, AIMP2 has a critical role for maintaining structural stability of the MSC (Kim *et al*., 2002), pEXPR-IBA-3-AIMP2 construct was used due to the Strep-tag on the C-terminus. Coomassie blue staining data indicated that MSC was successfully purified in HeLa cells (Fig. 3A). Moreover, there was an observed dissociation of MSC using western blotting analysis. Although the binding affinity of KRS with MSC was significantly decreased, one of the MSC component, DRS still bound with MSC (Fig. 3B).

These data indicate that Stxs induce disintegration of KRS from MSC, and trigger KRS secretion to extracellular medium in D-THP-1 and HeLa cells.

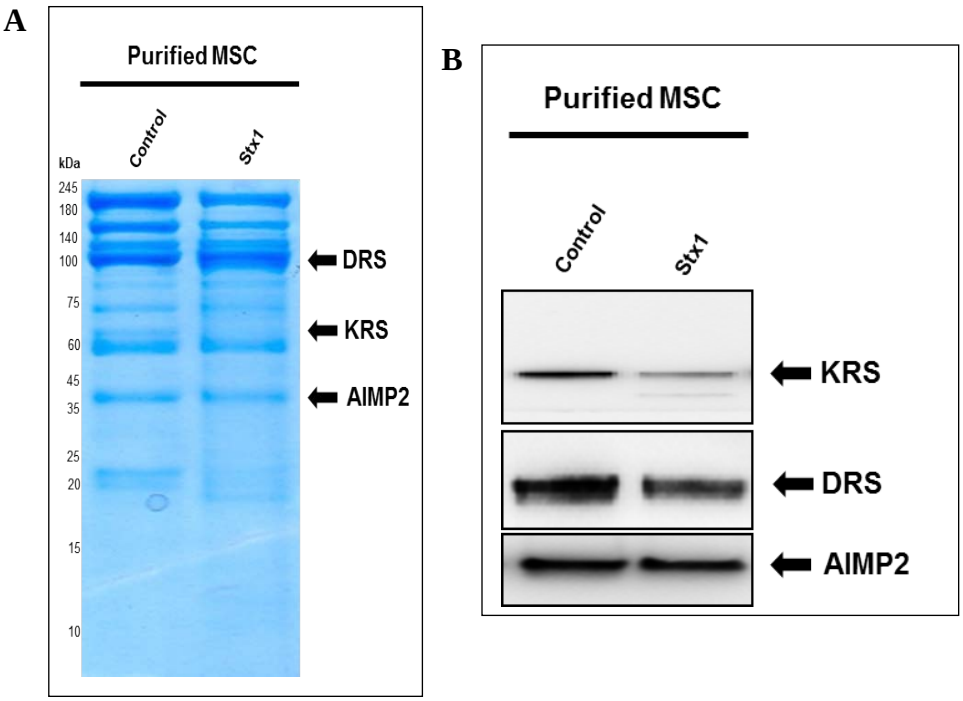


Fig. 4. Dissociation of KRS from MSC in Stx1 challenged HeLa cells. Prior to the experiments, HeLa cells (1×10^7) were transfected with C-terminus strep-tagged AIMP2 expression construct. Cells were treated with Stx1 (10 ng/ml) for 12 h and purified using a Strep-Tactin column. (A) The purity of the human MSC preparations was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie blue staining. (B) Components of the endogenous MSC in the toxin treated or untreated cell were detected by KRS, DRS, and AIMP2-specific antibodies.

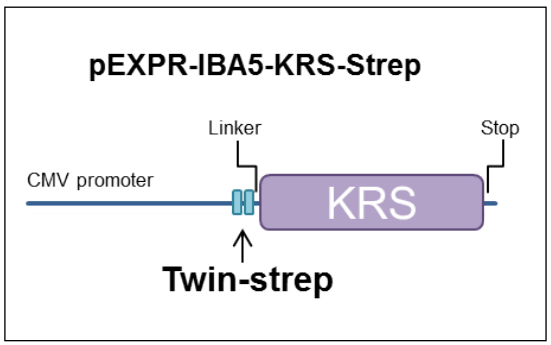
Scheme of generating KRS-expressing stable cell line and purification.

To examine the functions of secreted KRS, N-terminus strep-tagged KRS expression construct was designed (Fig. 5A). Mammalian cell suspension culture systems were selected to purify the large amounts of KRS. Designed construct was transfected in 293 F cells with transfection reagent. Overexpression of KRS was confirmed by western blotting (Fig. 5B). Within the cell culture media being changed every 3 days, the concentration of antibiotic reagent, G 418, was increased from 200 μg/ml to 800 μg/ml utilizing steady incremental amounts. As a result, stable KRS-expressing free-style 293 cell line was generated.

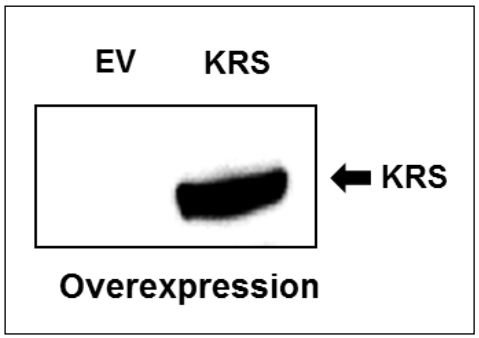
To confirm KRS expression within a stable cell line, Real-time PCR (Fig. 5C) and genomic DNA PCR (Fig. 5D) experiments were performed. Prior to the functional study of KRS, KRS was purified with affinity chromatography using Strep-Tactin system and fast protein liquid chromatography (FPLC) from stable cell lysates (Fig. 5E). Purified KRS was identified by western blotting analysis (Data not shown).

Generation of KRS-expression stable cell line and purification of human KRS was successfully performed.

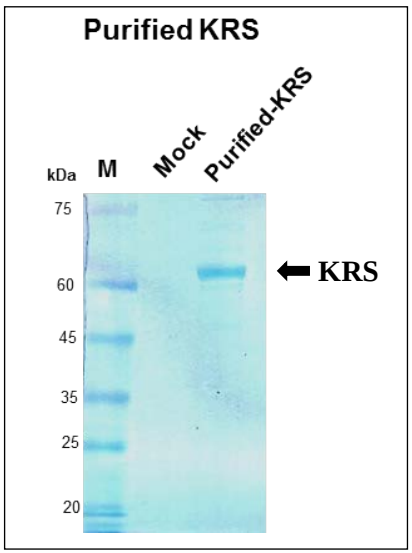
A


pEXPR-IBA5-KRS-Strep
Linker
Stop
CMV promoter
KRS
Twin-strep

C


EV
KRS
KRS
Overexpression

E


Purified KRS
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Mock
Purified-KRS
75
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45
35
25
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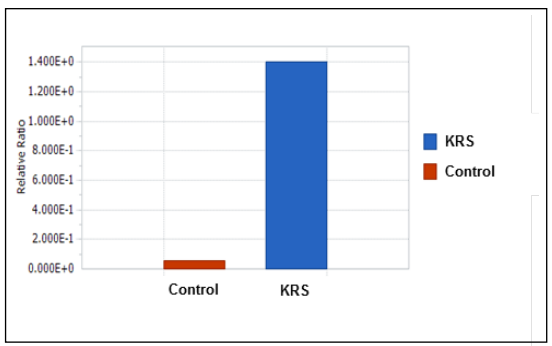
KRS

B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Relative Ratio
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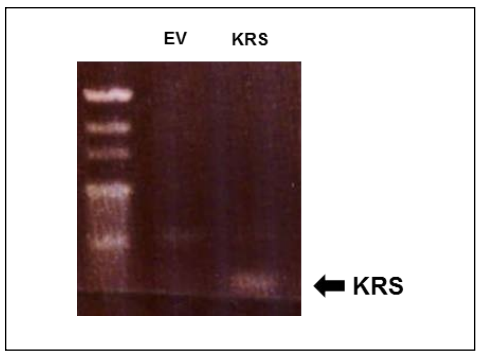
EV
KRS
KRS

Fig. 5. Purification of human KRS in free-style 293 cells. (A) Cloning was performed to express human KRS in mammalian cell. KRS gene was integrated in pEXPR-IBA5 expression vector which have twin-strep-tag in N-terminus. Constructed vector was transfected in free-style 293 cells. (B) Expression of KRS was observed by strep specific antibody. (C) KRS-expressing stable cell line was identified using RT-PCR with KRS-targeted primer. Values from the results of RT-PCR were normalized to the expression of β-actin. The data shown are the mean fold induction ± SEM of two independent experiments. (D) Genomic DNA from KRS-expressing stable cell line was isolated to examine integration of KRS genome. Insertion of KRS genome was identified by genomic DNA PCR. (E) KRS was purified in KRS-expression free-style 293 stable cell line by Strep-Tactin affinity chromatography and fluid protein liquid chromatography. Purified KRS was denatured and separated by SDS-PAGE using 4-12% gradient acrylamide gel coomassie blue stained.

Cytokines and chemokines production in D-THP-1 cells by KRS.

To identify the function of KRS in mammalian cells, purified KRS was treated to D-THP-1 cells. Cell-free supernatants were collected and analyzed with ELISA. The amount of cytokines and chemokines production was increased based on a dose dependent manner presented by KRS. Secretion of Macrophage inflammatory protein 1α (MIP1α) and IL-8 were significantly increased (Fig. 6A and 6B). Inflammatory cytokine IL-1β and inflammation signaling protein Tumor necrosis factor-α (TNF- α) were released by KRS (Fig. 6C and 6D). Increment of inflammatory cytokines and chemokines in KRS treated D-THP-1 cells demonstrated that KRS might be mediator of proinflammatory response against Stxs intoxication.

Together, immune-sensitive cells stimulate KRS secretion against Stxs to activate proinflammatory cytokines and chemokines within their proximal environment. Thus, it is possible to conclude the role of KRS is involved in cytokine production regulation and autocrine signaling molecule.

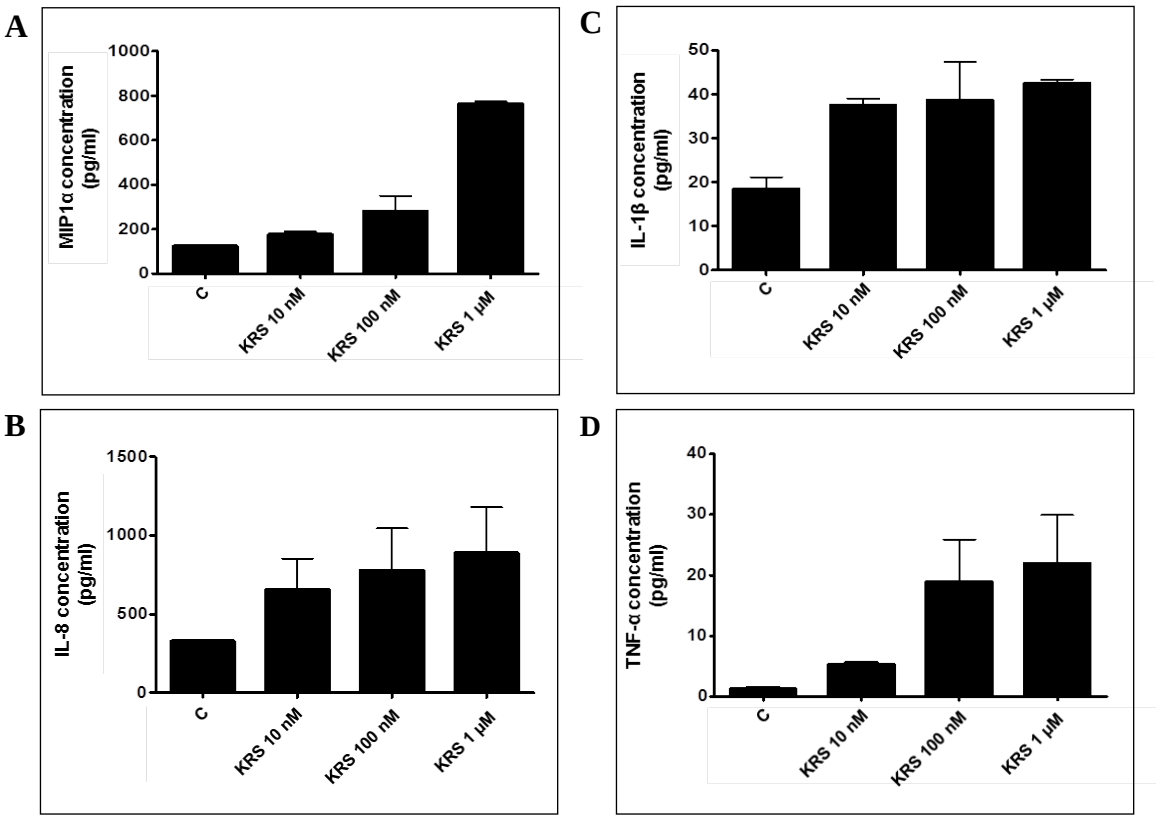

Fig. 6. Production of proinflammatory cytokines in D-THP-1 cells by human KRS. D-THP-1 (2.5×10^6) cells were challenged with KRS (0.1 μM) for 12 h. Cell-free supernatants were collected and multiplexing immunoassay was performed using ELISA kit from KOMA Biotech. (A) Secretion of MIP1α was significantly increase by KRS dose dependent manner. (B) Secreted chemokine, IL-8 was increased by KRS treatment. (C) IL-1β secretion in D-THP-1 cells was increased compared with control cells. (D) Secretion of TNF-α was increased by KRS in dose dependent manner. Values are given as pg/ml and expressed as the mean ± standard error of three independent experiments. Statistical significance was calculated using

one-way ANOVA (P value versus control, #, <0.01; P value versus Stx1 or Stx2, * < 0.001).

Enhancement of proinflammatory cytokines production via co-treatment of KRS with Stxs.

In order to demonstrate further effects of KRS in Shiga toxin intoxicated condition, D-THP-1 cells were co-treated with KRS and Stxs. Cell free supernatants were collected and analyzed by ELISA. Data showed that KRS treatment with Stxs enhanced production of chemokine MIP1α and IL-8 rather than Stxs alone after treatment for 4 h (Fig. 7A and B). Moreover, co-treatment of KRS and Stxs induced proinflammatory cytokine IL-1β and TNF-α more significantly rather than Stxs alone after treatment for 12 h (Fig. 7C and D).

Taken together, production of proinflammatory cytokines against Stxs intoxication are synergistically increased by presence of KRS. Thus, these data reveal that KRS is proinflammatory mediator that enhances cytokines and chemokines production by Shiga toxin intoxication in D-THP-1 cells.

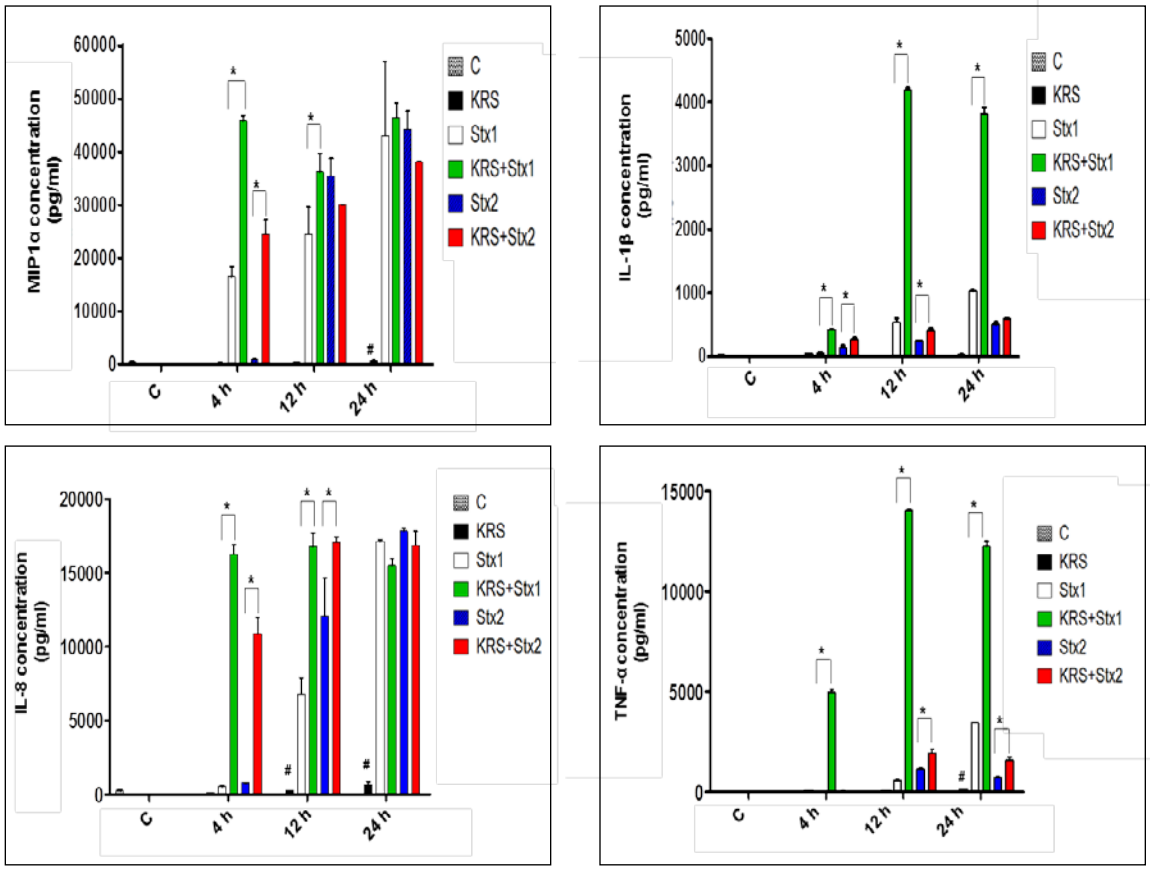

Fig. 7. Synergistic effects of the production of proinflammatory cytokines by KRS in Stxs treated D-THP-1 cells. D-THP-1 cells (2.5×10^6) were incubated with Stx1 (400 ng/ml) or Stx2 (10 ng/ml) in presence or absence of purified KRS (0.1 μM) for indicated time points. Cell-free supernatants were collected, and soluble (A) IL-1β, (B) TNF- α, (C) MIP1α, and (D) IL-8 were measured using a sandwich ELISA. Standards provided with the kits were used to calculate soluble cytokine/chemokine protein amounts. Values are given as pg/ml and expressed as the mean ± standard error of three independent experiments. Statistical significance

was calculated using one-way ANOVA (*P* value versus control, #, <0.01; *P* value versus Stx1 or Stx2, * < 0.001).

Paracrine effects of KRS in human kidney cells.

To identify further role of KRS, correlated cell line with Stxs was selected for further experiments. It was reported that infections with STEC of humans cause pathogenic damage of kidney arterioles and glomeruli (Tarr *et al.*, 2005). This raises the possibility that the secreted KRS via Stxs within the macrophages associated with kidney cells may be responsible for inducing proinflammatory responses within the kidney cells. Thus, human kidney (HK-2) cell line was selected and treated with KRS. Cell-free supernatant was collected and ELISA was performed. The results suggest that HK-2 cells are induced IL-8 secretion by KRS treatment (Fig. 7A). Furthermore, The effect of KRS with Stxs also investigated. Unlike D-THP-1 cells, co-treatment of KRS with Stxs in HK-2 cells did not trigger enhancement of cytokine or chemokine production (Fig. 7B)

These results indicate that KRS may have impacts on triggering inflammatory chemokine production through a paracrine-like manner within human kidney cells.

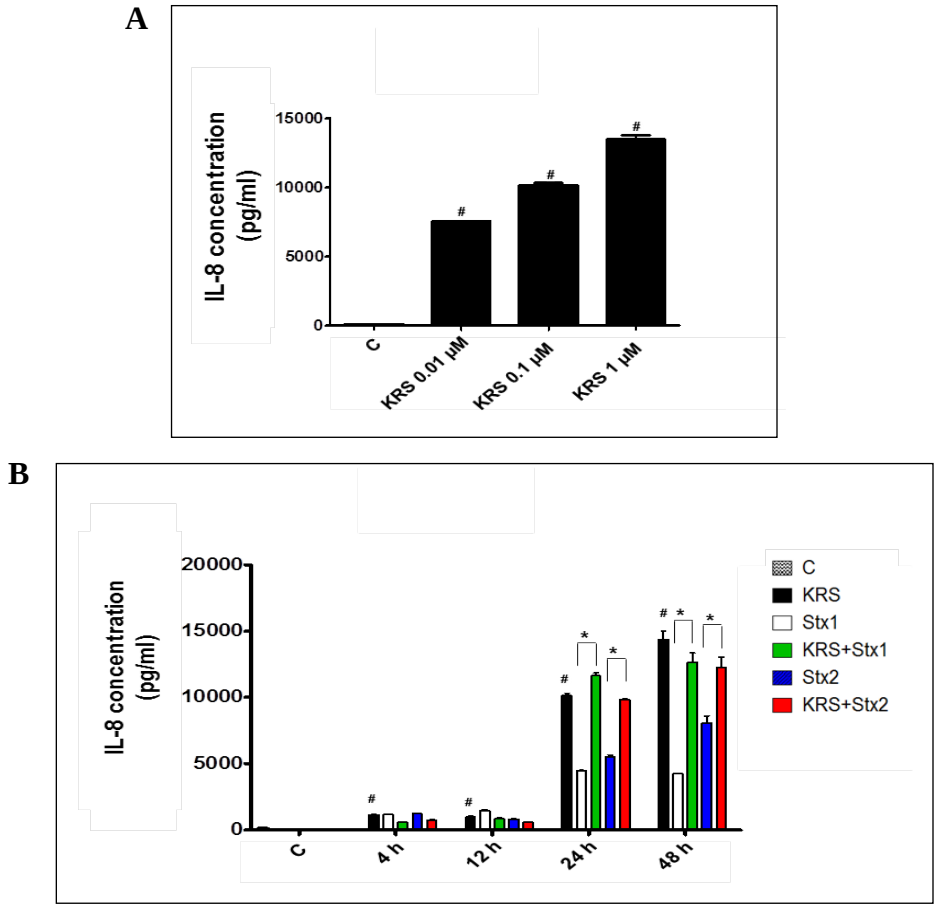

Fig. 8. Proinflammatory chemokine production in human kidney cells by KRS. HK-2 cells (1.5×10^6) were incubated with KRS (0.1 µM) or Stx1 (400 ng/ml) or Stx2 (10 ng/ml) or KRS (0.1 µM) with Stxs at indicated time points. Cell-free supernatants were obtained and secreted IL-8 was quantified by ELISA. (A) KRS treatment in HK-2 cells induced production of inflammatory cytokine IL-8 and was increased in a KRS does dependent manner. (B) Co-treatment of KRS and Stxs triggered generation of IL-8 chemokine production. Standards provided with the kits were used to calculate soluble cytokine/chemokine protein amounts. Values are

given as pg/ml and expressed as the mean ± standard error of three independent experiments.

Statistical significance was calculated using one-way ANOVA (P value versus control, #, <0.01; P value versus Stx1 or Stx2, * < 0.001)

Cytotoxicity of KRS in D-THP-1 cells.

It is possible to think that the proinflammatory response of KRS to the D-THP-1 cells are responsible for the cytotoxic effects of KRS. Toxicity of KRS was observed using a MTS assay in D-THP-1 cells. In the KRS administrated D-THP-1 cells, the viability of cells was not decreased like as control cells (Fig. 8). However, 60% of Stx1 or Stx2 challenged cells underwent a decrease of mitochondrial dehydrogenase. Thus, these data revealed that non-cytotoxic events of KRS when treated with D-THP-1 cells, are not significantly different than the control cells.

The proinflammatory response are therefore not attributed to the cytotoxic effects of KRS.

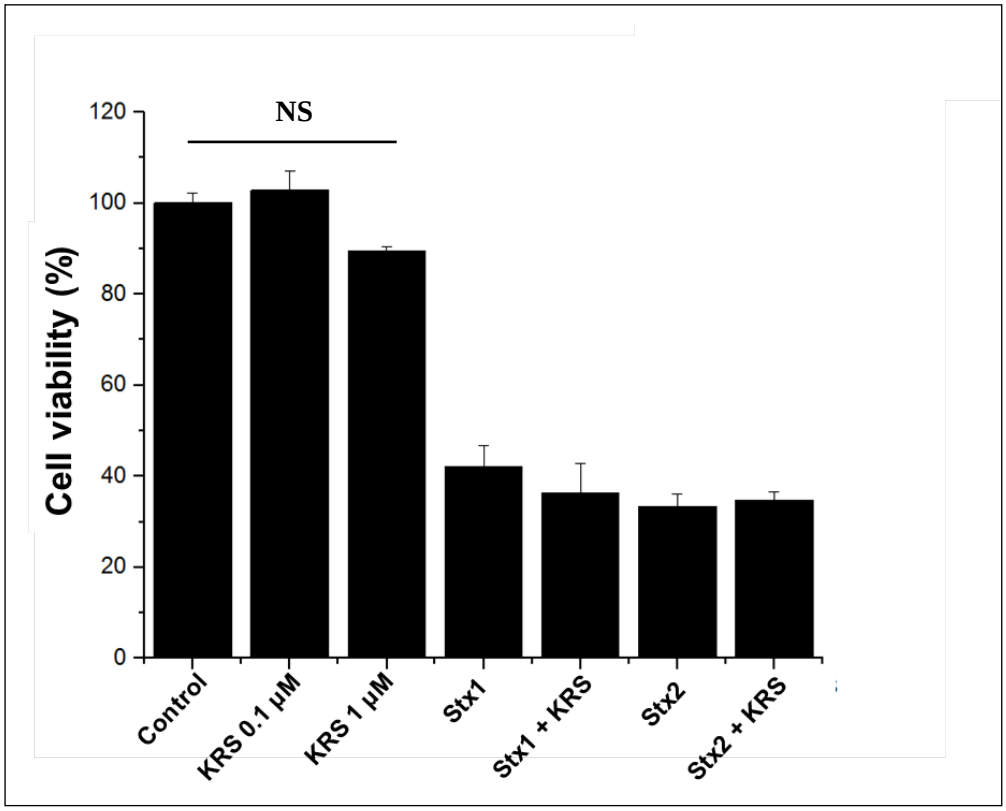

Fig. 9. Cell cytotoxicity assay of KRS treated D-THP-1 cells. D-THP-1 (1×10^4) cells were seeded to 96-well white plate. KRS (0.1 µM, 1 µM) Stx1 (400 ng/ml) or Stx2 (10 ng/ml) or KRS (0.1 µM) with Stxs were treated to plated cells for 24 h. Cell viability was measured by MTS assay. The values represented are average data from three independent experiments. Statistical significance was calculated using an one-way ANOVA.

***In vivo* experiments of Stx2 administrated KRS heterozygous knockout mice.**

Previous data suggested that KRS triggered proinflammatory response in autocrine and paracrine manners. To evaluate if KRS production between KRS heterozygous knockout (KD) mice and wild-type (WT) mice has an effect on survival rate and body weight, *in vivo* experiments were performed.

Control mice were injected with 200 μl of PBS and all of these mice were showed 100% survival rate after intraperitoneal injection (Data not shown).

As shown in Figure 9A, two days following toxin injection, 30% of WT mice were died. By the third day, 100% of the WT mice were completely died. Otherwise, only 60% of KRS KD mice were died in that time point. Four days after injection, KRS KD mice were completely died. Survival rate of WT and KRS KD mice showed that KRS KD mouse can survive more than a day compared to WT mouse. Evaluation of the body weight of the WT compared to the KRS KD mice showed more rapid decrease within the WT mice following toxin injection (Fig. 9B)

These results demonstrated that the toxin injection had a significant effect on WT mice compared to KRS KD mice, with the WT mice showing higher mortality immediately following the injection time. These findings may be attributed to the effect of the KRS secretion within the KRS KD mice, reducing the proinflammatory response leading to the prolonged death compared the WT mice.

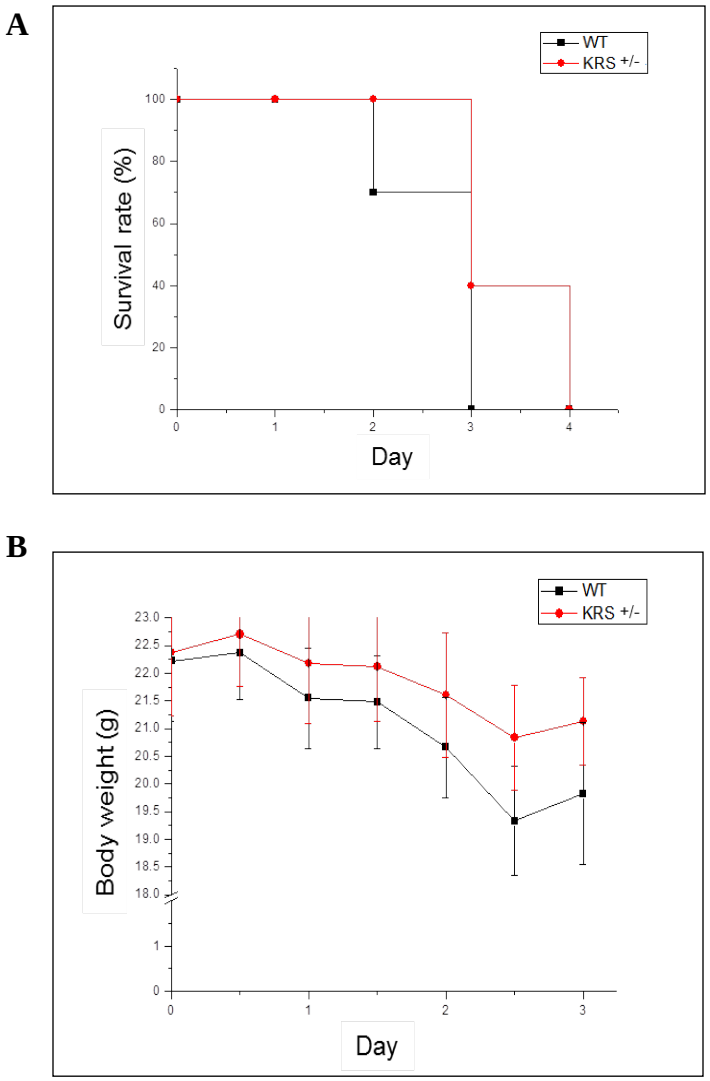

Fig. 10. Survival time increments of KRS heterozygous knockout mice versus wild-type mice against Stx2 administration. WT and KRS KD mice were challenged with Stx2 10 ng/kg by intraperitoneal injection. (A) Mouse survival rate was observed every day following the Stx2 injection. Significant difference ($P < 0.01$) between WT mice treated with Stx2 versus KRS KD mice treated with Stx2

was observed. (B) The body weight of each type of mice was measured every 12 h. Mouse mortality was examined using the log rank test software (IBM SPSS Statistics). A *P* value of < 0.01 was considered statistically significant.

IV. DISCUSSION

Infection with Shiga toxin-producing *Escherichia coli* (STEC) leads to the development of HUS and acute renal failure associated with elevated risk of patient morbidity and mortality. Although the exotoxin Stxs, major causative agent of the pathogen, are well characterized as multifunctional virulence factors inducing protein synthesis inhibition and apoptosis followed by ribotoxic stress, many studies have reported that contribution of the innate immune response to the potentially fatal complications by the toxin is clinically important to define the pro-inflammatory response *in vivo*. One key challenge in understanding pro-inflammatory host signaling mechanism behind the Stxs-mediated disease progression lies in exploring specific regulation and the redundant role of pro- and anti-inflammatory response activated by the toxins. Cytokines and chemokines secreted from macrophages by Stxs may contribute to damage in the colon and development of systemic complications such as acute kidney injury and neurological abnormalities, implying inflammatory cytokines toward adverse effects in primary target organs of the toxins. In a murine model of Stx-mediated renal damage, many studies have reported that challenge with the toxins elicits macrophages chemotactic responses in kidney that may lead to pathophysiology (Keepers *et al.*, 2007; Erin Lenzt *et al.*, 2011). These results highlight the need of substantial clarification for the Stxs-induced immune signaling mediators such as cytokines or cytokine-like molecules involving inflammation and activation of

innate immune responses.

Beyond the primary role of human AARSs are translational regulation by aminoacylating their specific tRNA, it has been reported that unlike prokaryotic AARSs, the human AARSs incorporated additional domains and have critical role in regulation of important biological processes including intracellular signaling transduction, metabolic process, tissue differentiation, angiogenesis and inflammation (Park *et al*., 2005; Rho S. B. *et al*., 1998; Tamara F. Williams *et al*., 2013). Among these AARSs, interestingly, it has been known that non-canonical functions of human KRS are involved in immune system by triggering pro-inflammatory responses, implying human KRS as cytokine-like molecules in signal dependent manner (Park *et al*., 2005).

In this study, I investigated the signaling properties of Stxs-induced KRS secretion as inflammatory mediator to stimulate immune response in a autocrine and paracrine manner. My primary observations are that, in the toxin-sensitive cells, KRS is dissociated from cytosolic binding partner AIMP2 within MSC in response to Stx1 or Stx2 and that the dissociated KRS is secreted in order to induce enhanced proinflammatory response in macrophage-like monocytic cells and human renal proximal tubular epithelial cell lines during intoxication.

Park *et al.* have demonstrated that human KRS is secreted to trigger proinflammatory response with TNF-α production through the activation of monocyte/macrophage as a signaling mediator. This suggested that human KRS works as the active cytokine (Park *et al*., 2005). In recent study, human KRS has been demonstrated as a regulator of the immune response in mast cells with

activation of MAPK cascade by regulating target gene expression (Lee Y. N. *et al*., 2004).

I show here that KRS is secreted by the cell in response to the Stxs in the HeLa and human macrophage-like THP-1 cells. However, the secretion was failed when the cells were incubated with N-glycosidase activity deficient Stx1 A^{-}, Stx2 A^{-}, Stx1 B-subunit and Stx2 B-subunit alone without the enzymatic A subunit of the toxin (Fig. 1). These data suggest that endogenous KRS secretion is strongly increased in the enzymatic ability-dependent manner of the Stxs. AIMP2 has been shown to dissociate from MSC complex following oncogenic stimuli and translocated to the nucleus (Park *et al*., 2005, PNAS). Human KRS is secreted from the various cancer cell lines in the presence of TNF-α but not from the mouse macrophage RAW264.7. Thus, human KRS may be secreted in the stimulus signal-dependent manner, because I detect the increased KRS secretion following the Stxs-treatments in the human macrophage-like THP-1 cells. However, I did not detect any effect of Stxs on the cytosolic expression of KRS at transcriptional or translational levels (Fig. 2). Based on these data I have hypothesized that Stxs may induce dissociation of KRS from AIMP2 in the cytosol following the toxin-mediated ribotoxic stress. Data of Stx1 treatment in AIMP2 overexpressed HeLa cells, suggesting that the toxin-induced ribotoxic stress appears to be involved in signal transduction to promote modification to release KRS from the MSC complex (Fig. 4).

The data collected from the human macrophage-like THP-1 cells and human kidney cells with KRS treatment indicated secreted KRS from MSC dissociation mediates proinflammatory responses (Fig. 6 and Fig. 8). *In vivo* data of Stx2 injected KRS

WT and KD mice verified the effect that secreted KRS lead to acute body weight loss and rapid death due to the promotion of proinflammatory response (Fig. 10).

It is then propose that secreted human KRS may be directly or indirectly correlated with Shiga toxin-mediated inflammatory responses in various types of host cells. Investigation of the regulation of Stxs-activated proinflammatory cytokines and their mediators is warranted as serious infectious disease can be caused by toxin producing bacteria. Besides, demonstrating the regulatory effects of Stxs-induced secretion of KRS may provide valuable insights to the underlying immunological mechanism that mediates systemic inflammation on various target organs during intoxication. Further study whether the secreted multifunctional human KRS is related to coordinate various signaling pathways with pathophysiological implications will be necessary to evaluate precise physiological significance of the KRS. Moreover, further studies are needed to develop botanical drugs to regulate infection caused by food-borne bacteria, such as STEC or *Shigella dysenteriae*.

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VI. 국문초록

Shiga toxins (Stxs)은 식품매개 병원균인 Shiga toxin producing *Escherichia coli* (STEC) 또는 *shigella dysenteriae* serotype 1 으로부터 생성되는 toxin 이다. 하나의 A 소 단위체와 다섯 개의 B 소 단위체로 구성된 Stxs 는 세포 외막의 Gb3 수용체를 통하여 세포 내부로 침입하게 된다. 세포 내로 들어온 Stxs 는 엔도좀을 형성하며 퇴행적 수송 (retrograde transportation)을 통해 골지체를 거쳐 소포체로 이동하며, A 소단위체가 분리되어 세포질 로 이동한 후, 분리된 A 소단위체 N-말단의 A_1 조각이 세포질 내부에 존재하는 숙주 세포의 28S-rRNA 와 결합하여 단백질 합성을 막게 된다. 이를 통해 세포에 ribotoxic 스트레스를 주게 되고, 이러한 스트레스로 인해 세포 내에서 Nuclear factor kappa B, inflammation, mTOR 등과 같은 기작이 활성 되지만, 이러한 기작과 연관된 중간 매개체에 대한 자료는 현재까지 잘 알려진 바가 없다.

효모균 이상의 고등생물에서만 발견되는 Multi aminoacyl-tRNA synthetase complex (MSC) 는 사람의 경우 8 개의 아미노아실 tRNA 합성효소와 3 개의 보조 단백질로 구성된 단백질 복합체로서, 외부로부터 자극이 주어졌을 때, 각각의 아미노아실 tRNA 합성효소와 보조단백질 들이 기존의 알려진 기능 외에 면역반응, 혈관신생합성 등과

같은 다른 기능을 하게 된다고 알려져 있다. 이 중 Lysyl-tRNA synthetase (KRS)의 경우 면역 전 반응 (proinflammatory response)에 중요한 역할을 한다고 알려져 있으나, 박테리아가 인체 내에 감염되었을 때의 반응 기작은 밝혀진 바가 없다.

따라서, 본 논문에서는 Stxs 에 대한 세포의 면역 반응 중, KRS 가 면역 전 반응에 대한 매개체로 작용하여, 세포 밖으로 분비되는 것과, 분비된 KRS 의 타깃 세포에 대한 염증반응 유도에 대하여 알아보고자 한다.

우선 대식세포의 특성을 띄는 대식구 유사세포에서 Stxs 가 처리되었을 때 세포 내 KRS 의 변화에 대해 분석하였다. 그 결과 Stx1 과 Stx2 에 의해 세포 밖으로 분비된 KRS 를 단백질 흡입법과 고상효소 면역 검정법을 통해 확인하였다. 반면, 세포독성이 없다고 알려진 A 소 단위체가 변종 된 Stxs 와 B 소 단위체만이 존재하는 Stxs 가 처리된 세포 에서는 세포 밖으로의 KRS 가 검출되지 않았다.

분비된 KRS 가 세포의 과 발현에 의한 것인지 확인하기 위하여 Stx1 과 Stx2 가 처리된 대식구 유사세포에서 RT-PCR 을 통하여 전사상 변화가 없는 것을 확인하였고, 세포의 핵과 세포질을 분리하여 단백질 흡입법으로 확인해본 결과, KRS 의 발현양의 변화가 없는 것을 확인하였다.

세포 내 KRS 의 발현양 자체의 변화가 없으나, 세포 밖으로 분비되는 KRS 가 존재하는 이유를 확인하기 위해 MSC 로부터의 KRS 변화를 확인하였다. MSC 를 형성하는데 중심이 되는 단백질인 AIMP2 를 과발현시킨 HeLa 세포에서 Stx1 을 처리 하였을 때, KRS 가 AIMP2 로부터 분리되는 것을 확인하였고, 이를 통해 KRS 가 MSC 로부터 세포 밖으로 분비된다는 것을 확인하였다.

분비된 KRS 의 기능을 확인하기 위하여, 다량의 KRS 의 확보가 필요하였다. 따라서 KRS 의 정제를 위해 KRS 를 안정적으로 발현하는 부유 배양 세포인, 293F stable cell line 을 제작하였다. 제작된 세포로부터 KRS 를 정제한 후, KRS 가 세포에 대한 염증 전 반응에 어떠한 영향을 미치는지에 대한 실험을 수행하였다. KRS 가 분비된 대식구 유사 세포에서 자가분비 효과에 따른 염증 전 반응 활성화와 근거리 분비를 통한 사람 신장 세포에 염증 전 반응을 일으킨다는 것을 고상효소 면역 검정법과 단백질 흡입법을 통해 확인하였다.

또한, 이러한 반응들이 KRS 의 독성에 의한 세포 사멸과의 연관성을 알아보기 위하여, 세포독성 실험을 통해 확인한 결과, KRS 에 의한 대식구 유사세포의 세포사멸이 발견되지 않았다. 이를 통해 KRS 에 의한 염증 전 반응은 KRS 가 갖는 고유한 효과임을 알아내었다.

마지막으로, *in vivo* 상에서 Stxs 에 대한 KRS 의 효과를 확인하기 위하여, KRS 유전자가 억제된 쥐와 야생형의 쥐에 Stx2 를 복강 내

주사 하였을 때, 생존율 변화와 몸무게 변화를 확인하였고, 이를 통해 KRS 유전자가 억제된 쥐가 Stx2 에 대한 사망 효과가 줄어드는 것을 확인하였다. 이를 통해 KRS 야생형의 쥐가 KRS 유전자가 억제된 쥐보다 KRS 에 의한 더 많은 수의 사이토카인 분비가 일어나, 위와 같은 결과가 얻어진 것이라 추측할 수 있다.

모든 결과를 종합해보면, Stxs 에 의해 대식구 유사세포 에서 KRS 가 MSC 로 부터 KRS 가 분리되게 되고, 분리된 KRS 는 자가분비 또는 근거리분비를 통하여 염증 전 반응을 일으키게 되는 것을 확인하였다. 더욱이 동물 실험을 통하여 KRS 가 염증 전 반응을 매개하는데 있어 중요한 역할을 하는 것 또한 확인하였다.

본 연구에서 밝혀진 KRS 의 생물학적 기능을 이용하여, 염증 전 반응을 조절 한다면, 식품매개 병원균인 Shiga toxin producing *Escherichia coli* (STEC) 또는 *shigella dysenteriae* serotype 1 에 오염된 식품을 섭취한 사람에서의 급성 패혈증 등에 의한 사망을 막을 수 있는 약물 개발에 기여할 수 있을 것이다.