Double-stranded RNA-mediated Toll-like receptor 3 activation is enhanced by ribosomal protein L19

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먼저 대학원 과정동안 맘껏 실험하고 공부할 수 있게 지도해주시고 한결 같은 모습으로 본을 보여주신 최인홍 교수님께 말로는 다 할 수 없는 깊은 감사를 드립니다. 면역학에 흥미를 갖게 자상하게 가르쳐주신 신전수 교수님께 진심으로 감사드립니다. 언제나 따뜻한 관심으로 조언해 주신 김종선 교수님, 바쁘신 가운데 논문지도를 해주신 김명희 교수님과 이영호 교수님께 감사드립니다. 조교생활을 하는 동안 많은 가르침을 주신 박전한 교수님께 감사드립니다. 인자한 모습으로 때론 날카로운 질문으로 깨닫게 해주신 김세종 교수님과 이원영 교수님, 자상하게 대해주신 이봉기 교수님, 묵묵히 지켜봐주신 조상래 교수님과 이재면 교수님께 감사드립니다.

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게 고맙다는 말을 하고 싶습니다.

늘 나의 말을 잘 들어주고 따라주며 힘이 되어준 진원이와 대학원 생활을 같이 하면서 삶의 활력을 더해준 정신이, 병주, 희열이, 지금은 실험실을 나갔지만 잘 챙겨줬던 정 많은 현규, 착하고 말 잘 들었던 자윤이, 여송이, 얼마 전 결혼을 한 현정이, 논문교정에 도움을 준 승환이에게 고마움을 전합니다. 또한 함께한 조교 선생님들과 2년 동안 의국장을 맡으면서 고생했던 김주영 선생님께도 감사의 말을 전합니다. 실험실 생활을 하면서 기쁨과 슬픔을 함께 나눈 깜찍한 미화, 의학전문 기자가 꿈인 영주, 밤 늦도록 항상 열심히 실험하는 상은이, 정화에게도 고마움을 전합니다.

저에게는 항상 곁에서 도움과 용기를 준 친구들이 있었습니다. 10년 이상을 한결같이 옆에 있어준, 미선이, 미소, 보경이에게 고마움을 전합니다. 대학생활을 하는 동안 기쁨을 함께 나누었던 경희, 경지, 지영, 상미에게 고마움을 전합니다.

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저자 씀

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ABBREVIATION

CBP	**CREB-binding protein**
cDNA	**complementary DNA**
CREB	**cAMP response element-binding protein**
DMEM	**Dulbecco's modified Eagle's medium**
DNA	**deoxyribose nucleic acid**
dsRNA	**double-stranded RNA**
EDTA	**ethylenediaminetetraacetic acid**
FBS	**fetal bovine serum**
FITC	**fluorescein isothiocyanate**
IFN	**Interferon**
IKK	**IkappaB-related kinases**
IRF	**Interferon regulatory factor**
ISGF	**IFN-stimulated gene factor**
ISRE	**interferon-sensitive response element**
LPS	**lipopolysaccharide**
LTA	**lipoteichoic acid**
MyD88	**myeloid differentiation protein 88**
NF-κB	**nuclear factor-kappaB**
PAMP	**pathogen-associated molecular pattern**
PBS	**phosphate buffered saline**
PBST	**PBS-0.1% Tween 20**
PKR	**double stranded RNA –activated protein kinase**
Poly(I:C)	**poly inosinic-cytidylic acid**

PRR	**pattern recognition receptors**
RNA	**ribonucleic acid**
RHIM	**RIP homotypic interaction motif**
RIG	**retinoid inducible gene**
RIP	**receptor interacting protein**
RPL19	**ribosomal protein L19**
SDS-PAGE	**sodium dodecyl sulfate polyacrylamide gel electrophoresis**
STAT	**signal transducer and activator of transcription**
TBK	**TANK (TRAF family member-associated NF-κB activator) binding kinase**
TIR	**Toll-IL-1 receptor homology**
TIRAP	**TIR-domain containing adaptor protein**
TLR	**Toll-like receptor**
TLR3-ECD	**TLR3 extracellular domain**
TRAF	**tumor necrosis factor receptor associated factor**
TRAM	**TRIF-related adaptor molecule**
TRIF	**Toll/IL-1 domain-containing adapter inducing IFN-β**

ABSTRACT

Double-stranded RNA-mediated TLR3 activation is enhanced by ribosomal protein L19

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TLR3 is activated by dsRNA, the genetic material of some viruses, and a viral replication intermediate in other viruses. Ligand recognition by TLR3 results in recruitment of the adapter molecule TRIF to the cytoplasmic TIR domain of TLR3. The recruitment of TRIF triggers a kinase cascade ultimately leading to IRF3 activation and the transcription of type I IFN genes. Here, we identified RPL19, which is found in the large ribosomal subunit (60S) of eukaryotes, as a protein that enhances TLR3 signaling. First, we have cloned TLR-ECD (1-23 LRR and 10-23 LRR) genes and RPL19 gene. Second, to identify whether TLR3 and RPL19 physically associate with each other, we

performed *in vitro* translation of TLR3-ECD genes and RPL19 gene and *in vitro* binding assay of them. To study this interaction in cells, RPL19 gene was transfected into TLR3 expressing cells. As results, TLR3 and RPL19 bound together *in vitro* and the presence of the RPL19 in the immunoprecipitate of TLR3 in cell lysate was detected by immunoblot analysis. This binding was not influenced by the absence or the presence of poly(I:C). These data clearly showed that TLR3 constitutively interacts with RPL19 independent of poly(I:C) involvement. Third, we detected interaction between poly(I:C) and RPL19 using poly(I:C)-agarose 'pull-down' assay, and showed that RPL19 bound poly(I:C). Fourth, the over-expression of RPL19 resulted in a significant induction of ISRE-Luc and a weak induction of NF-κB reporter activity after poly(I:C) stimulation. Fifth, we determined whether the activation of ISRE or NF-κB by RPL19 requires endosomal maturation. As a result, the enhancement of ISRE and NF-κB promoters by RPL19 requires endosomal maturation because the activation of these promoters was inhibited by the treatment of chloroquine. Sixth, we investigated the effect of RPL19 on the phosphorylation of IRF3. Over-expression of RPL19 induced phosphorylation of STAT1 in TLR3 expressing cells. Seventh, RPL19 appeared to co-localize with TLR3 in endosomes. Taken together, our results demonstrate that RPL19 augmented the activation of poly(I:C)-induced TLR3 signaling and type I IFN-mediated immune response. In conclusion, our results suggest that RPL19 may be an endogenous protein regulating TLR3 signaling *in vivo*.

Key words : Toll-like receptor 3, ribosomal protein L19, dsRNA, IRF3, STAT1, endosome

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I. INTRODUCTION

Toll-like receptors (TLRs) recognize different pathogen-associated molecular patterns (PAMPs), leading to the activation of an innate immune response and the shaping of the subsequent adaptive immune response. The ten known human TLRs recognize pathogen-associated molecules, such as lipoteichoic acid (LTA) by TLR2, lipopolysaccharide (LPS) by TLR4, flagellin by TLR5, and unmethylated CpG DNA motifs by TLR9. Binding of these ligands to TLRs initiates a series of signaling processes that stimulate and orchestrate the innate and adaptive immune responses.[1, 2] Human TLRs are

implicated in a number of diseases and, hence, constitute potential therapeutic targets. [3, 4]

Human TLR3 is activated by double-stranded RNA (dsRNA) associated with viral infection[5], endogeneous cellular mRNA[6], and sequence-independent small interfering RNAs.[7] TLRs utilize their common cytoplasmic Toll-interleukin-1 receptor (TIR) domain to transmit intracellular responses through the recruitment of TIR-containing adaptors.[1, 8] These adaptors include myeloid differentiation protein 88 (MyD88), Toll/IL-1 domain-containing adapter inducing IFN-β **(**TRIF), TRIF-related adaptor molecule (TRAM), and TIR-domain containing adaptor protein (TIRAP), which mediate cellular events that lead to the induction of antimicrobial and inflammatory genes.[9] TLR3 is distinct from other TLRs in that it is not dependent on MyD88 but rather on TRIF for signaling.[10] Other key features of TLR3 signaling include a requirement for phosphorylation of tyrosine residues in the TIR domain[11] and the involvement of phosphatidylinositol-3 kinase.[12] In turn, TLR3 activates genes for secreted antiviral cytokines, such as interferon ß (IFN-ß), and those that encode intracellular, viral, stress-inducible proteins.[13]

TRIF signaling leads to activation of interferon regulatory factor (IRF) 3 and IRF7 and induces type-I IFN production.[14, 15, 16] Two kinases have been demonstrated to directly phosphorylate and activate IRF3 and IRF7: TANK binding kinase (TBK) 1 and IKKε (IKKi). [17, 18, 19, 20] Although the contribution of IKKε in TLR3 signaling is under debate, TBK1 can be recruited to the N-terminal region of TRIF to activate IRF3. TRIF-induced NF-κB activation may proceed through two ways. First, TRIF recruits the tumor necrosis factor receptor associated factor (TRAF)-6 by means of its N-terminal region.[21, 22]

Second, TRIF contains a C-terminal RIP homotypic interaction motif (RHIM)[23] that interacts with the RHIM of both receptor interacting protein (RIP)-1 and RIP3 kinase.[24] Whereas RIP1 transmits TRIF-induced NF-κB activation, RIP3 appears to bear regulatory functions.[24, 25]

TRIF is not a TLR3-specific adaptor molecule but is also involved in IRF3 activation by TLR4. However, its recruitment to TLR4 is mediated by an additional adaptor molecule, TRAM.[26] Therefore, the TRIF pathway is differentially activated by TLR3 and TLR4. Consistent with this, IFN-stimulated response element induction by TLR4, but not by TLR3, requires the NF-κB subunit p65[27]. In addition, TLR3 engagement leads to a much stronger induction of type I IFNs than does TLR4 signaling.[28]

Although normally present at the cell membrane to detect extracellular PAMPs, a few TLRs, including TLR3, TLR7, TLR8, and TLR9, recognize their ligands in intracellular compartments such as endosomes. TLRs share the ability of nucleic acid recognition, detecting dsRNA (TLR3), ssRNA (TLR7 in mice, TLR8 in humans), and nonmethylated CpG DNA motifs (TLR9).[5, 29, 30, 31, 32] Even though these TLRs can recognize virally derived nucleic acids, they also have the propensity (at least TLR7/8 and TLR9) to interact with self nucleic acids.[29, 33] Importantly, their intracellular localization may prevent them from recognizing potentially dangerous self molecules and activating signals in the absence of infection.[33]

TLR3 localizes to an intracellular vesicular compartment in dendritic cells and cannot be detected on the cell surface.[34] This compartment has been assumed to be endosomes because inhibition of endosomal acidification

abrogates poly(I:C) signaling. TLR3 has to encounter dsRNA in these vesicles, which is probably achieved through phagocytosis of dsRNA released into the extracellular space by necrotic or lysed cells after viral infection. The other possibility would be the exposure to dsRNA during viral entry through receptor-mediated endocytosis. In contrast to TLR3, dsRNA activated protein kinase (PKR) and retinoid inducible gene (RIG)-I are located in the cytoplasm and, therefore, suited for the direct recognition of dsRNA produced during viral replication. For the establishment of an adaptive immune response against virus-infected cells, it is crucial to induce dendritic cell maturation. Therefore, the relative contribution of RIG-I versus TLR3 for the response to a particular virus might, among other factors, depend on the ability of the virus to infect dendritic cells. It is conceivable that the host response to a virus, such as the Sendai virus, which infects dendritic cells and enters the cell by plasma membrane fusion, which avoids the endosomes, would depend on RIG-I rather than TLR3. In fact, the host response to the Sendai virus is completely independent of TLR3, 7, 8 or 9[35] and is mediated by RIG-I.[36] During infections with the West Nile virus, a single-stranded (ss) RNA virus, a peripheral inflammatory response is initiated through TLR3 and disrupts the blood–brain barrier, which enables virus entry into the brain[37]. Therefore, *TLR3*$^{-/-}$ mice were more resistant to lethal West Nile virus infection, and as a result, the virus appears to benefit from its interaction with TLR3.[37] TLR3 also seems to be the TLR most strongly expressed in the brain, specifically in astrocytes and glioblastoma cell lines[26, 38] and there has been a description of a TLR3 isoform that seems to be exclusively expressed in the brain.[26] These findings might indicate that TLR3 has a specific role in the brain and/or in the response to encephalitogenic viruses.

The TLR family has a structurally conserved architecture consisting of an extracellular domain connected by a transmembrane region to an intracellular component. The extracellular domain is composed of between 19 and 25 repeats of a 24-residue leucine rich repeat (LRR) sequence that conforms to the typical LRR consensus sequence. The LRRs of TLR3 extracellular domain (TLR3-ECD) follow the typical consensus motif of a 24-residue repeat, consisting of $xL^{2}xxL^{5}xL^{7}xxN^{10}xL^{12}xxL^{15}xxxxF^{20}xxL^{23}x$, where L represents obligate hydrophobic residues, which for TLR3 include leucine (most prevalent), isoleucine, valine, methionine, and phenylalanine; F is a conserved phenylalanine; and N is a conserved asparagine.[39, 40] The seven conserved hydrophobic residues of the LRRs (at positions 2, 5, 7, 12, 15, 20, and 23) point inward into the solenoid and form a tightly packed hydrophobic core that provides lateral stability to the repeating LRR motifs. Seventeen of the 23 human TLR3 LRRs have the canonical 24-residue motif, and only LRR12 and LRR20 have insertions longer than 5 residues.[39]

The extracellular domain of TLR3 consists of a horseshoe-shaped array of LRRs with a characteristic curvature, as anticipated from amino acid sequence-based comparisons with the structures of homologues. However, several additional unpredicted structural features are evident. The crystal structures showed extensive glycosylation of one side of the convex surface and parts of the concave surface, a feature suggested to be common in other TLRs.[41] Additionally, a crystallographic dimer exsists with adjacent C-terminal regions close enough to bring the intracellular TIR domains together and allow signaling. This prompted speculation into the mode of dsRNA binding to the TLR3 extracellular domain and the biological significance of

the dimer.[42] It was postulated that the ligand-binding site for dsRNA was on the glycosylation-free faces of the TLR3 dimer in which dsRNA binds into a region along the top of the TLR3 dimer through a cluster of basic residues on the TLR3 surface.[39] Three alternative ligand-binding models have also been suggested. Docking of dsRNA to bound sulphate ions was suggested to bind to the phosphate backbone of dsRNA.[43] The second model suggested the binding to the glycosylated concave surface, whereas the third model utilized the unglycosylated surface placing dsRNA in a shallow groove between LRRs 12 and 20. Further mutational analysis has identified several residues particularly H^{539} and N^{541} that appear critical for TLR3 activity. In addition, two patches of residues form charged surfaces that appear to contribute cumulatively towards ligand binding.[44]

Not all TLRs function by themselves. In some instances, TLRs require co-receptors to generate a signaling complex or mediate an enhancement in signaling. Several non-TLR pattern recognition receptors (PRRs) appear to contribute towards the TLR response by presenting ligands to the TLRs: CD36 presents microbial diacylglycerides to TLR2–TLR6 heterodimers[45], TLR4 requires MD2, whereas TLR2 associates with TLR members, TLR1 or TLR6. CD14 was originally identified as a LPS receptor, which is attached to the cell surface by a glycosylphosphatidylinositol anchor.[46] Later, it was shown to function as an essential component of the TLR4/MD2 complex in LPS signaling.[3]

CD14 also plays an essential role in cellular uptake of ligands, including LPS and phosphatidylinositol[47], and interacts with ligands, including bacterial and nonbacterial products, to enhance ligand-mediated cell activation.[48] CD14

physically interacts with poly(I:C) and enables poly(I:C) internalization. This CD14-dependent poly(I:C) uptake enhances intracellular TLR3 activation, explaining why $CD14^{-/-}$ mice exhibited impaired responses to poly(I:C).[49]

In the process of searching for endogenous proteins which bind TLR3, we found that ribosomal protein L19 (RPL19) to be a candidate molecule. The ribosome consists of a small 40S subunit and a large 60S subunit. These subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. RPL19 is found in the large ribosomal subunit (60S) of eukaryotes and archaea. RPL19 consists of two small globular domains connected by an extended segment. RPL19 is located towards the surface of the large subunit, with one exposed end involved in forming the inter-subunit bridge with the small subunit.[50, 51] The other exposed end is involved in forming the translocon binding site, along with L22, L23, L24, L29, and L31e subunits.

The aim of this study was to identify whether RPL19 regulates TLR3 signaling. The starting point of this study was to clone the TLR3 gene and RPL19 gene. In TLR3 expressing cells we investigated whether RPL19 was physically associated with TLR3. We also performed poly(I:C)-agarose 'pull-down' assay to detect the interaction between poly(I:C) and RPL19. Over-expression of RPL19 was performed to investigate the activation of interferon-sensitive response element (ISRE) or nuclear factor-kappaB (NF-κB). Activation of IRF3 and STAT1 was assessed after over-expression of RPL19. Finally we analyzed the subcellular localization of TLR3 and RPL19. With all these experiments we aimed to investigate the role of RPL19 in TLR3 signaling.

II. MATERIALS AND METHODS

1. Cell culture

HEK293-TLR3 and HEK293-TLR2 cell lines were maintained and subcultured in 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, U.S.A.)-Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, U.S.A.) at 37ºC in 5% CO_2. The cell culture medium contained 10 μg/ml of blasticidin (InvivoGen, San Diego, CA, U.S.A.) or 50 μg/ml of HygroGold™ (InvivoGen, San Diego, CA, U.S.A.) as selection antibiotics. The cells were stained with anti-TLR3 antibody (eBioscience, San Diego, CA, U.S.A.) or anti-TLR3 antibody (eBioscience, San Diego, CA, U.S.A.) to confirm whether they expressed TLR2 or TLR3. The human glioblastoma cell line A172 was obtained from ATCC (CRL-1620™) and was maintained in minimal essential medium (MEM, Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% FBS (Gibco BRL, Grand Island, NY, U.S.A.) and penicillin and streptomycin Gibco BRL, Grand Island, NY, U.S.A.) at 37℃ in 5% CO_2. A549 cells originated from lung epithelial carcinoma (ATCC, CCL-185™), were cultured in MEM (Gibco BRL, Grand Island, NY, U.S.A.) containing 10% FBS (Gibco BRL, Grand Island, NY, U.S.A.) penicillin and streptomycin.

2. DNA construction

Deletion mutants of human TLR3-ECD (1-23 LRR and 10-23 LRR) were created into the *EcoRI/XhoI* site of the Gal4 DNA-binding domain fusion vector pGBKT7 (Clontech, Palo Alto, CA, U.S.A.) using pSG5-Flag-hTLR3 as a template and the upper: 5’-CCGGAATTCGCATCCTCCACCACCAAGT

GCAC-3’ and the lower: 5’-CCGCTCGAGTTCAAAGGGGGCACTGTCTT TGCA-3’for 1-23 LRR of TLR3, the upper: 5’-CCGGAATTCA ATCTCACT ATGCTCGATCTTTCC-3’ and the lower: 5’-CCGCTCGAGTTC AAAGGGGGCACTGTCTTTGCA-3’ for 10-23 LRR of TLR3 primers containing the underlined *EcoRI/XhoI* sites, respectively. Amplified PCR products were isolated from gel, digested with the appropriate enzymes, and ligated into the pGBKT7-*myc* (Clontech, Palo Alto, CA, U.S.A.) yeast expression vectors. Mammalian expression plasmids or *in vitro* translation plasmids for RPL19 were amplified by PCR and cloned into the *EcoRI/XhoI* –digested pcDNA3.1/V5 (Invitrogen, California, USA) or pGADT7 (Clontech, Palo Alto, CA, U.S.A.) vectors.

3. Yeast two-hybrid assay

pGBKT7-TLR3-ECD, which expresses mutants of TLR3 (1-23 LRR and 10-23 LRR) fused with Gal4-DNA binding domain in yeast, was used as bait. cDNA library expressing human fetal brain cDNA fused with Gal4 activation domain was purchased from BD Biosciences (Franklin Lakes, NJ, U.S.A.). Yeast strain, AH109 was used for the library screening. AH109 cells were transformed with pGKBKT7-TLR3-ECD and human placental cDNA library by the lithium method (Clontech). Transformants that could grow on the selective medium lacking leucine, tryptophan and histidine & containing 0.5 mM 3-aminotriazole (3-AT) were isolated. Only the colonies that turned into blue in the β-galactosidase assay were selected. Library-derived plasmids from the candidate clones were rescued into the *E. coli* and studied further.

4. β-galactosidase assay

Yeast transformants were grown on nylon filter in medium lacking leucine and tryptophan for 2 days. The colonies on the filter were frozen with liquid nitrogen and incubated for 30 min at 30℃ in Z buffer (38.6 mM β-mercaptoethanol, 1 mg/ml X-gal, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). When colonies turned blue, these gene products were scored as a positive interaction with each other.

5. *In vitro* co-immunoprecipitation of proteins

For immunoprecipitation, genes of TLR3 and RPL19 were clones and were *in vitro* transcribed by T7 RNA polymerase and translated with a T_NT ® T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI, U.S.A.) to prepare ^{35}S-Met-labeled bait and library proteins. *In vitro* translation reactions combined the reagents (10 μl *in vitro* translated ^{35}S-Met-labeled bait and prey protein) with each other for 1 hr at room temperature after resuspension in immunoprecipitation buffer [50 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM EDTA, 5 mM DTT, 0.1% Triton X-100 and 1x Complete Protease inhibitor (Roche, Indianapolis, IN, U.S.A.)] in a 1.5 ml microcentrifuge tube. The mixture was added with 10 μl (i. e., 1 μg) of HA-Tag polyclonal antibody (Sigma-Aldrich, St. Louis, MI, U.S.A.), mixed gently and incubated at room temperature for 1hr. The resulting immunocomplex was collected on prewashed protein A Sepharose beads (Bio-Rad, Hercules, CA, U.S.A.) by incubation for 1–2 hrs at 4°C and washed 5 times with PBS. The immunoprecipitates were resuspended with 20 μl of SDS-PAGE loading buffer and denatured at 80℃ for 5 min. Then the immunoprecipitates were

separated on 12% SDS-PAGE and transferred onto pre-wetted Whatman 3MM paper. It was then covered with Saran wrap, dried at 80℃ under constant vacuum. The gel was exposed to X-ray film overnight at room temperature.

6. Transient transfection and luciferase reporter gene assay

Luciferase reporter gene assay was performed with pIL6-κB-Luc (NF-κB promoter) (Stratagene, West Cedar Creek, TX) and pISRE-Luc plasmid (Stratagene, West Cedar Creek, TX). HEK293-TLR3 or HEK-TLR2 cells were grown in 24-well plates with 10% FBS (Gibco BRL)-DMEM at 37ºC in 5% CO_2. Cells were cotransfected with 0.05 mg/ml pIL6-κB-Luc, pISRE-Luc plasmids, and pCMV-β-galactosidase vectors (Promega, Madison, WI, U.S.A.) using FuGENE 6 Reagent (Roche, Indianapolis, IN, U.S.A.). After 24 hrs, cells were stimulated with poly(I:C) (Amersham Pharmacia Biotech, Milwaukee, WI. U.S.A.) for 6 hrs, and cell extracts were prepared. The luciferase activity was measured by a Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.) and β-galactosidase activity was measured with O-nitrophenyl-*β*-D-galactopyranoside (Sigma-Aldrich, St. Louis, MI, U.S.A.), as the substrate. Luciferase activity was normalized for transfection efficiency with the β-galactosidase activity.

7. Co-immunoprecipiation and western blot analysis

For transient transfection and co-immunoprecipitation experiments, HEK293-TLR3 or A172 cells (~1 x 10^6) were transfected with RPL19 plsmids (0.5 μg/ml each) for 24 hrs. The transfected cells were lysed in 300 μl of lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/ml aprotinin, 10 μg/ml

leupeptin, 1 mM phenylmethylsulfonyl fluoride]. For each immunoprecipitation, 0.2 ml aliquot of lysate was incubated with 2 μg of the anti-TLR3 antibody (R&D, Minneapolis, MN), anti-V5 antibody (Invitrogen, California, USA) or control IgG and 25 μl of a 1:1 slurry of protein G bead (Sigma-Aldrich, St. Louis, MI, U.S.A.), at 4℃ for 4 hrs. The beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl. The precipitates were analyzed by western blot using anti-V5 (marker protein of RPL19) or anti-TLR3 antibody was performed as followings.

Total protein from HEK293-TLR3 or A172 cells transfected with RPL19 or control plasmids were separated with 10-12% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked in PBS-0.1%Tween 20 (PBST) containing 5% non-fat milk and incubated with appropriate primary antibodies at a dilution of 1:1000. After three washes in PBST, peroxidase-conjugated secondary antibodies were incubated at a dilution of 1:5000, washed three times with PBST and developed with an ECL system (Amersham Pharmacia Biotech, Milwaukee, WI. U.S.A.). The anti-TLR3 antibody was polyclonal goat antibody (R&D, Minneapolis, MN) or monoclonal antibody (IMGENEX, San Diego, CA, U.S.A.). Anti-IκB-α, anti-phospho-IκB-α, anti-IRF3, anti-phospho-IRF3, anti-STAT1 or anti-phospho-STAT1 antibodies were all purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). For secondary antibodies, goat anti-mouse IgG, goat anti-rabbit IgG (Jackson Immuno Research, Baltimore, MD, U.S.A.) were used.

8. Poly(I:C) pull-down assay

To generate poly(I:C)-coated agarose beads, poly(C)-coated beads (Sigma-Aldrich, St. Louis, MO, U,S,A.) were resuspended in a double volume of 2 mg/ml poly(I:C) (Sigma-Aldrich) prepared in 50 mM Tris (pH7.0) -150 mM NaCl. The mixture was then rocked gently overnight at 4℃, collected by centrifugation at 1,000 x *g*, washed with 50 mM Tris (pH 7.0)-150 mM NaCl, resuspened in the same buffer as a 10% final slurry, and stored at 4℃ for use.

For poly(I:C) pull-down assay, poly(I:C)-coated beads were equilibrated in binding buffer [50 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40] as a 10% slurry and combined with an equal volume of extract from cells transiently transfected with RPL19 plasmids. The cell extracts were supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MI, U.S.A.), and 25 U of RNase inhibitor/ml (Promega, Madison, WI, U.S.A.). The mixtures were incubated with gentle agitation for 2 hrs at 4℃. Beads were then centrifuged at 1,000 x g, washed three times with lysis buffer and resuspended in 2x SDS-PAGE sample buffer. Samples were boiled at 100℃ for 5 min, centrifuged at 13,000 x g for 30 s, loaded immediately on to SDS-PAGE gels, and processed for immunoblot analysis with anti-V5 antibody.

9. Virus infection

A549 cells were transfected with V5-tagged RPL19 genes or mock vectors. Transfected A549 cells were grown to 80~90% confluence and were infected with 1 MOI of vesicular stomatitis virus (VSV) in serum free medium.

10. Analysis by confocal microscopy

HEK293-TLR3 cells or A172 cells were grown in 8-well Nunc Lab-Tec II chambered coverglasses (Nalge Nunc, Naperville, IL, U.S.A.) with 10% FBS (Gibco BRL, Grand Island, NY, U.S.A.)-DMEM containing 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MI, U.S.A.), penicillin and streptomycin (Gibco BRL, Grand Island, NY, U.S.A.) at 37℃ in 5% CO_2. Approximately 1×10^4 cells were transfected with RPL19 plsmids (0.5 μg/ml each) for 24 hrs and were fixed, permeabilized and stained with anti-V5 (marker protein of RPL19) (Invitrogen, California, USA), anti-EEA1 antibody (BD Biosciences), or anti-TLR3 polyclonal antibody (R&D, Minneapolis, MN) for 45 min at room temperature. Cells were washed three times and further incubated with 2.5 μg/ml of Alexa fluor-conjugated secondary antibody (Alexa-594, Invitrogen, California, USA) or FITC-conjugated goat anti-mouse IgG secondary antibody (Jackson Immuno Research, Baltimore, MD, U.S.A.) for 1 hr at room temperature. After incubation, cells were washed with PBS three times and analyzed by confocal microscopy. Confocal microscopic analysis was performed with a Zeiss Axiovert 100-M inverted microscope equipped with an LSM 510 (Carl Zeiss, Jena, Germany) laser scanning unit and a 1.4 NA × 63 Plan-Apochromat oil-immersion objective.

III. RESULTS

1. Structure and cloning of TLR3-ECD genes

Genomic structure of human TLR3 gene contains a unique intron that divides the coding sequences of the TIR domain. TLR3 transcripts are initiated in exon 1 and exon 2, which suggests the presence of two alternative promoters (Fig. 1A, *upper*). To identify human TLR3-interacting proteins, we generated TLR3-ECD constructs (Fig. 1A, *lower*). Two types of TLR3-ECD constructs (1-23 LRR, 10-23 LRR) were generated (Fig. 1B). Yeast cells were transfected with these two TLR3-ECD genes, and expressed 1-23 LRR or 10-23 LRR (Fig. 1B, *right*)

2. Screening of TLR3-ECD interacting proteins

To identify endogenous TLR3-ECD interacting proteins, we used the yeast two-hybrid system to screen human fetal brain cDNA library with TLR3-ECD (1-23 LRR, 10-23 LRR) as bait (Fig. 2A). We screened a total of ~ 1×10^6 independent clones and 112 clones were obtained. These clones were further screened by α-galactosidase activity and β-galactosidase activity (Fig. 2B). Finally 6 clones (8-1, 4-1, 4-111, 4-28, 4-78 and 4-93) were selected (Fig. 2B).

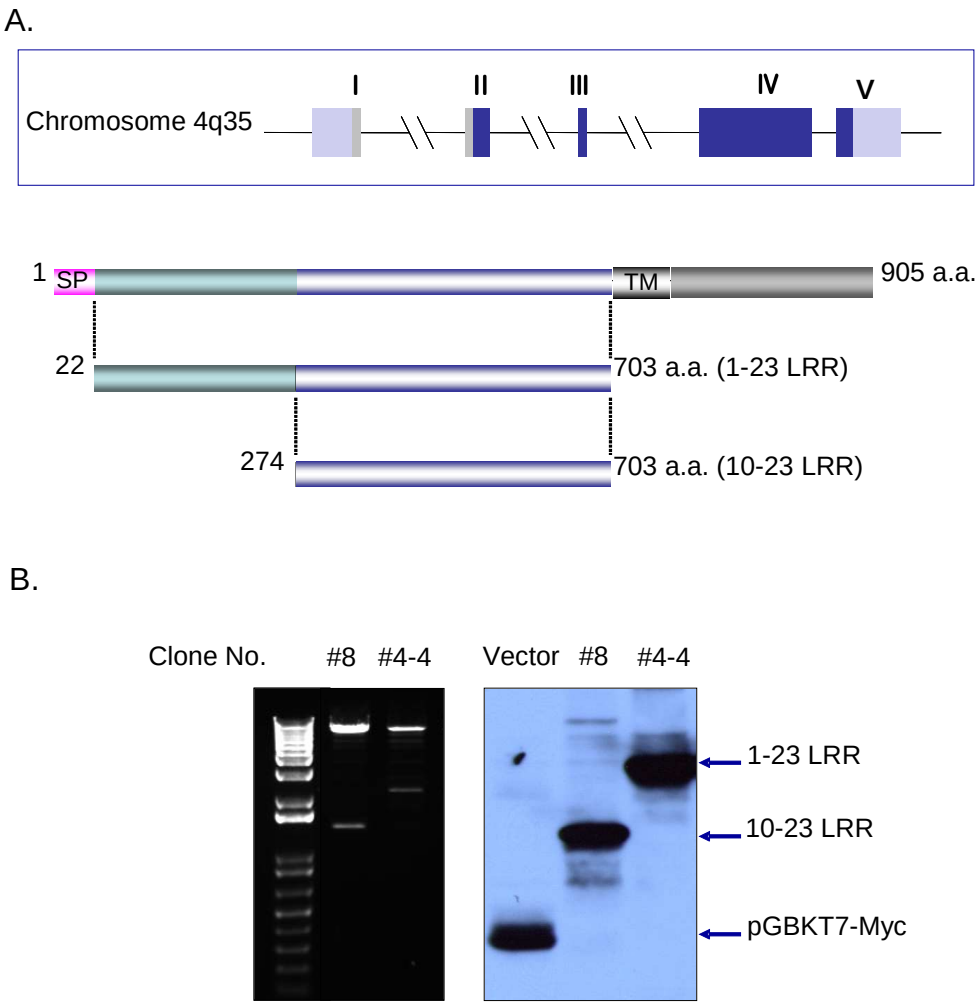


Fig. 1. Structure of TLR3 constructs and expression in yeast.
(A) Proximal promoter regions (light grey) and exons (non-coding sequences: dark grey, coding sequences:coloured) are shown as boxes. TLR3 transcripts are initiated in exon 1 and exon 2, suggesting the presence of two alternative promoters *(upper)*. The schematic diagram represents mutant plasmids containing 1-23 LRR or 10-23 LRR of TLR3 ectodomain *(lower)*. SP, signal peptide; TM, transmembrane. (B) TLR3 constructs were generated by PCR from cDNA derived from human brain astrocytoma cell line (LN215) and cloned into yeast expression vectors pGBKT7 *(left)*. Yeast AH109 was transformed with TLR3 constructs (1-23 LRR, 10-23LRR) or vector only (pGBKT7-Myc). Cell lysates were analyzed by SDS-PAGE immunoblotting using antibody specific for anti-myc antibody *(right)*.

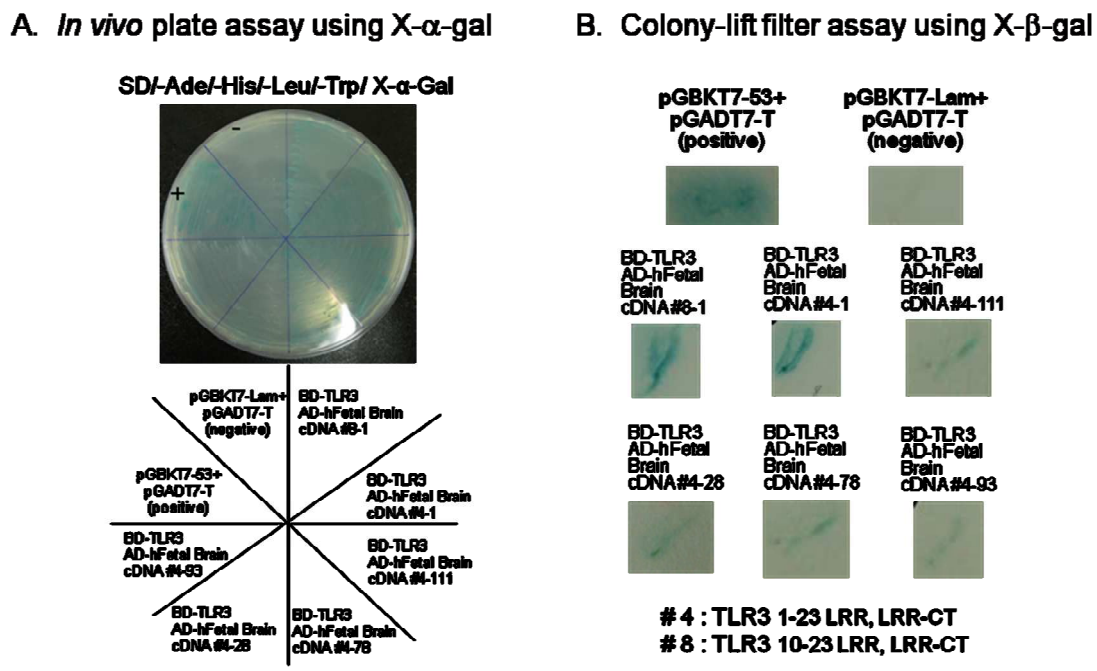


Fig. 2. Screening of proteins interacting with TLR3.

Yeast two-hybrid analysis was done to screen TLR3 interacting proteins. 1×10^6 independent clones of human fetal brain cDNA library were screened, and 112 clones were obtained. Cells with prey (AD-fetal brain cDNA library) and bait plasmids (BD-hTLR3 ECD) were streaked onto SD-AHLT plates. The cells grow on the plates when the prey and bait interact. Also, these clones were grown on the X-α-gal plates with blue color (A) or colored with blue using colony-lift filter assay (X-β-gal to assay β-galactosidase activity) (B).

3. Identification of RPL19 as a TLR3 binding protein and cloning of the RPL19 gene

Using TLR3-ECD (1-23 LRR or 10-23 LRR) as bait in a yeast two-hybrid screening we isolated one clone encoding 80-amino acid fragment that was identical to a portion of a 196-amino acid protein of RPL19 (Fig. 3A). The selected protein products corresponded exactly to amino acids from 1 to 80 of RPL19, which is identified using blast searches of the GenBank databases (Fig. 3A).

To clone the full-length gene of RPL19, we performed PCR with cDNA derived from human fetal brain cDNA library and the PCR product was cloned into mammalian expression vectors (Fig. 3B).

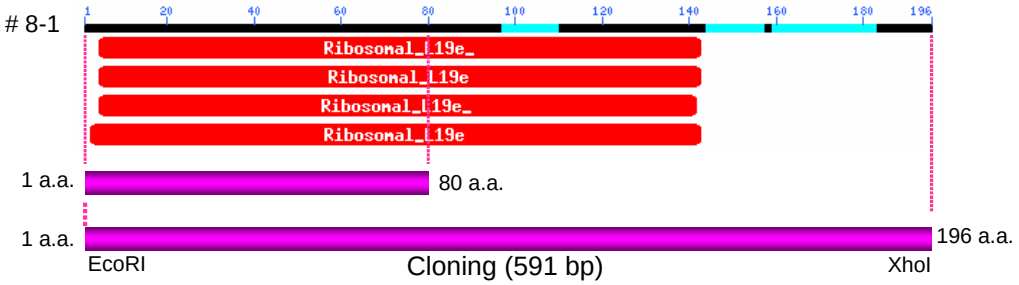
4. Interaction of TLR3 with RPL19 *in vitro* and *ex vivo*

To investigate whether RPL19 interacts with TLR3-ECD *in vitro*, RPL19 and TLR3-ECD proteins (1-23 LRR, 10-23 LRR) were translated *in vitro* and analyzed for binding. All the proteins were translated properly *in vitro* (Fig. 4A, *left*). These translated and [^{35}S]methionine-labeled TLR3-ECD (1-23 LRR, 10-23 LRR) and RPL19 were reacted and co-immunoprecipitated with anti-HA antibody, which is a marker protein for RPL19. As results, only 10-23 LRR proteins bound to RPL19 (Fig. 4A), *right*]. The whole TLR3-ECD (1-23 LRR) protein was not co-immunoprecipitated with RPL19 (Fig. 4A, *right*).

We further tested whether TLR3 and RPL19 physically associate with each other in HEK293-TLR3 cells. We generated V5-tagged RPL19 fusion constructs and transiently transfected them in HEK293-TLR3 cells. Total

protein from these cells was immunoprecipitated with anti-TLR3 antibody and the presence of RPL19 was analyzed by immunoblotting with anti-V5, which is a marker protein of RPL19 expression vector. As a result RPL19 was detected in the protein sample immunoprecipitated with anti-TLR3 antibody (Fig. 4B). The presence of RPL19 was not affected with stimulation of poly(I:C) (Fig. 4B). These data suggest that TLR3 interacts constitutively with RPL19 in HEK293-TLR3 cells and the binding is independent of poly(I:C) stimulation.

A. **Ribosomal Protein L19**



gi|4506609|ref|NP_000972.1| ribosomal protein L19 [Homo sapiens]
Length = 196a.a (591 bp)

Score = 161 bits (408), Expect = 5e-39
Identities = 80/80 (100%), Positives = 80/80 (100%)
Frame = +1

Query:	4	MSMLRLQKRLASSVLRCGKKKVWLDPNETNEIANANSRQQIRKLIKDGLIIRKPVTVHSR	183
		MSMLRLQKRLASSVLRCGKKKVWLDPNETNEIANANSRQQIRKLIKDGLIIRKPVTVHSR	
Sbjct:	1	MSMLRLQKRLASSVLRCGKKKVWLDPNETNEIANANSRQQIRKLIKDGLIIRKPVTVHSR	60

Query:	184	ARCRKNTLARRKGRHMGIGK	243
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Sbjct:	61	ARCRKNTLARRKGRHMGIGK	80

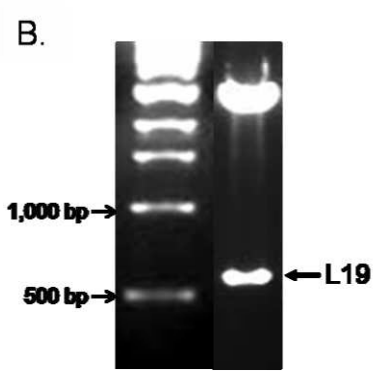

Fig. 3. Identification of RPL19 sequence.
(A) One clone, which was isolated using yeast two-hybrid screening, was characterized for its sequence. The blast searches and GenBank databases were used to identify the matched sequence as RPL19. (B) RPL19 cDNA was cloned.

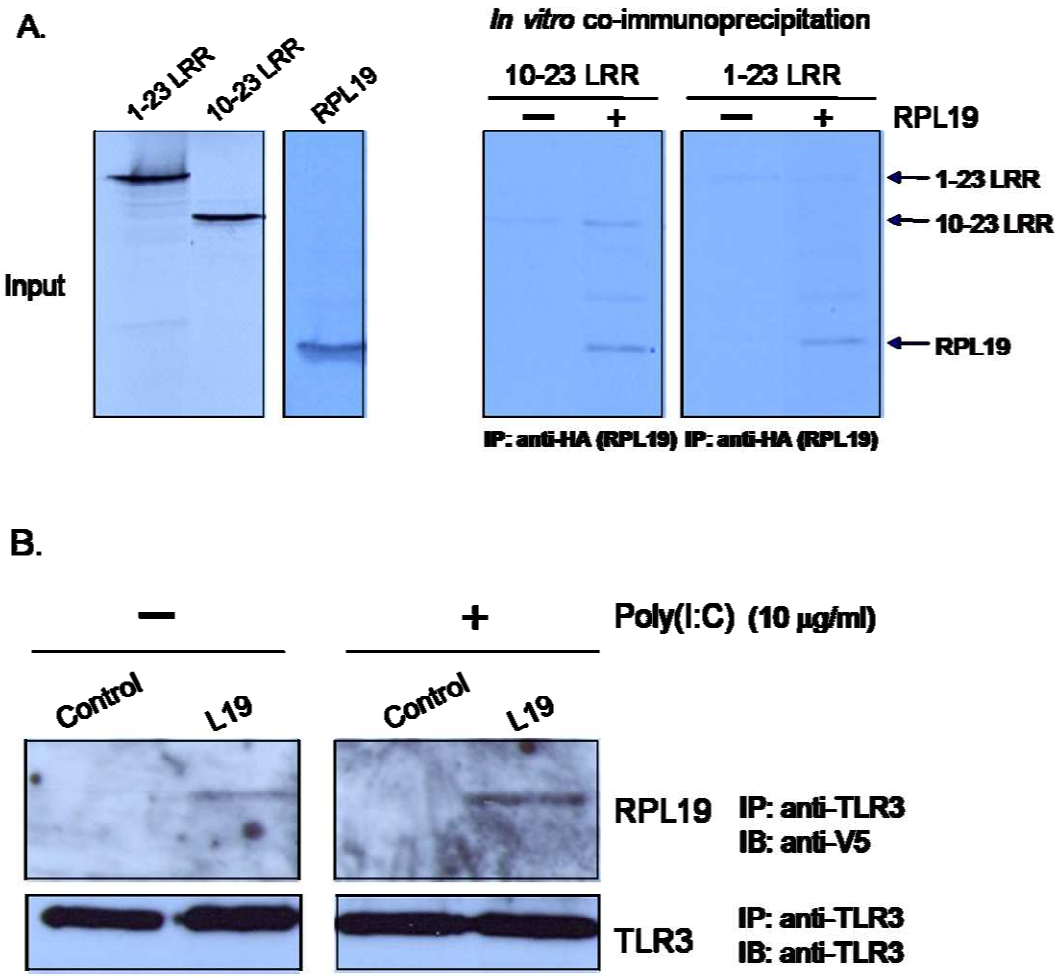

Fig. 4. Interaction of RPL19 with TLR3 *in vitro* and *ex vivo*.
(A) Indicated proteins were produced by *in vitro* translation, labeled with [^{35}S]methionine and analyzed by SDS-PAGE and fluorography (left). *In vitro* translated and [^{35}S]methionine-labeled TLR3-ECD (1-23 LRR and 10-23 LRR) or RPL19 were immunoprecipitated with anti-HA antibody. 10 μl of immunoprecipitate was loaded onto a SDS/12% polyacrylamide gel. (B) HEK293-TLR3 cells were transiently transfected with RPL19 plasmids (0.5 μg/ml each). After 48 hrs, cells were lysed and immunoprecipitated with anti-TLR3 antibody. Poly(I:C) (10 μg/ml) was added to the cells for 6 hrs. Western blotting was done with anti-V5 (RPL19) or anti-TLR3 antibody.
IP, immunoprecipitation; IB, immunoblotting.

5. Interaction of RPL19 with poly(I:C)

Next we investigated whether TLR3 interacts directly with poly(I:C). To examine the interaction with poly(I:C), we transfected the RPL19 gene into HEK293-TLR3 cells and performed a poly(I:C)-agarose 'pull-down' assay. As a result, direct interaction between poly(I:C) and RPL19 was detected (Fig. 5).

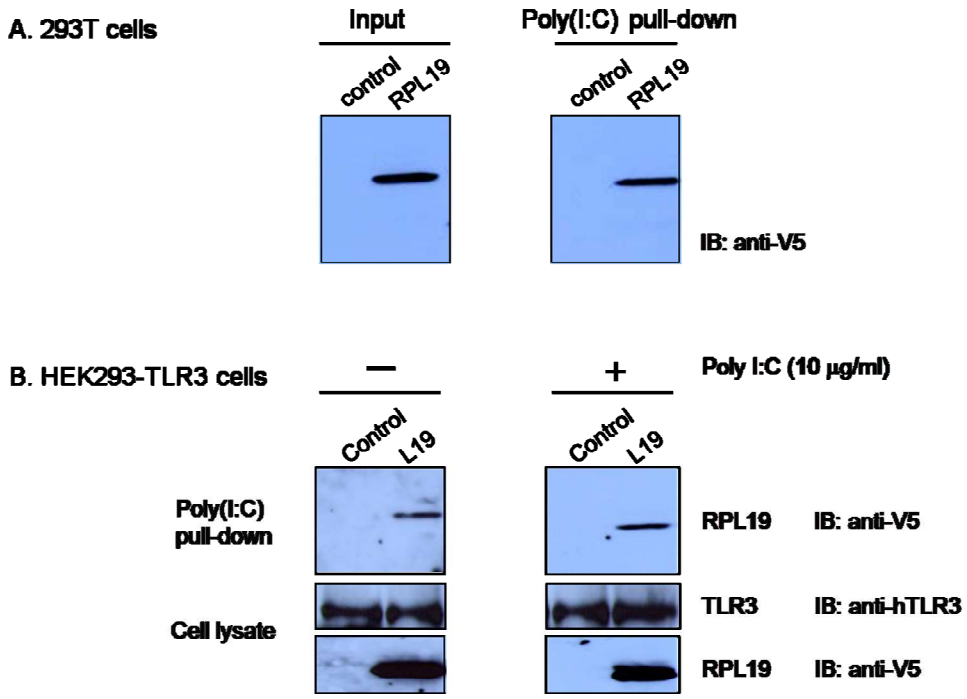

Fig. 5. Interaction of RPL19 with poly(I:C).
293T cells (A) or HEK293-TLR3 cells (B) were transiently transfected with V5-tagged RPL19. After 48 hrs, cell lysates were subjected to a poly(I:C) pull-down assay. SDS-PAGE was performed with poly(I:C)-agarose-associated complexes and immunoblotting was done with an anti-V5 antibody. The expression of each protein before pull-down is shown by immunoblot with anti-TLR3 or anti-V5 antibody in the input or in cell lysate. Poly(I:C) (10 μg/ml) was treated for 6 hrs prior to cell lysis.

6. Requirement of endosomal maturation for activation of ISRE or NF-κB by RPL19.

The involvement of RPL19 in poly(I:C)-induced activation of NF-κB and ISRE was measured by luciferase reporter gene assay in HEK293-TLR3 cells transiently transfected with RPL19. Over-expression of RPL19 resulted in a weak induction of ISRE-Luc activation (Fig. 6A) and NF-κB activation (Fig. 6B) after poly(I:C) stimulation.

Next we determined whether the activation of ISRE or NF-κB by RPL19 requires endosomal maturation. Chloroquine, which inhibits endosomal maturation, effectively blocked TLR3-mediated ISRE activation (Fig. 6A) or NF-κB activation (Fig. 6B) after poly(I:C) stimulation without RPL19 transfection. Over-expression of RPL19 increased ISRE activity to 1.5 fold (Fig. 6A, black column) and NF-κB activity to 1.7 fold (Fig. 6B, black column). Chloroquine, however, blocked the RPL19 effect completely (Fig. 6 A and B). These results suggest that RPL19 may interact with TLR3 in the endosomal compartment.

Next we investigated whether RPL19 is specifically worked on TLR3. LTA is a ligand for TLR2. We thus examined the impact of RPL19 on LTA -induced activation of the NF-κB promoter in TLR2 signaling. When RPL19 was expressed in TLR2 expressing cells, the activation of NF-κB was not increased with RPL19 when compared to LTA-treated control cells which were not transfected with RPL19 (Fig. 7A). As expected, the inhibitors of endosomal maturation, chloroquine (Fig. 7A) or bafilomycin (Fig. 7B) did not inhibit LTA-induced TLR2 signaling. These data demonstrate that RPL19 is

specific for TLR3 signaling.

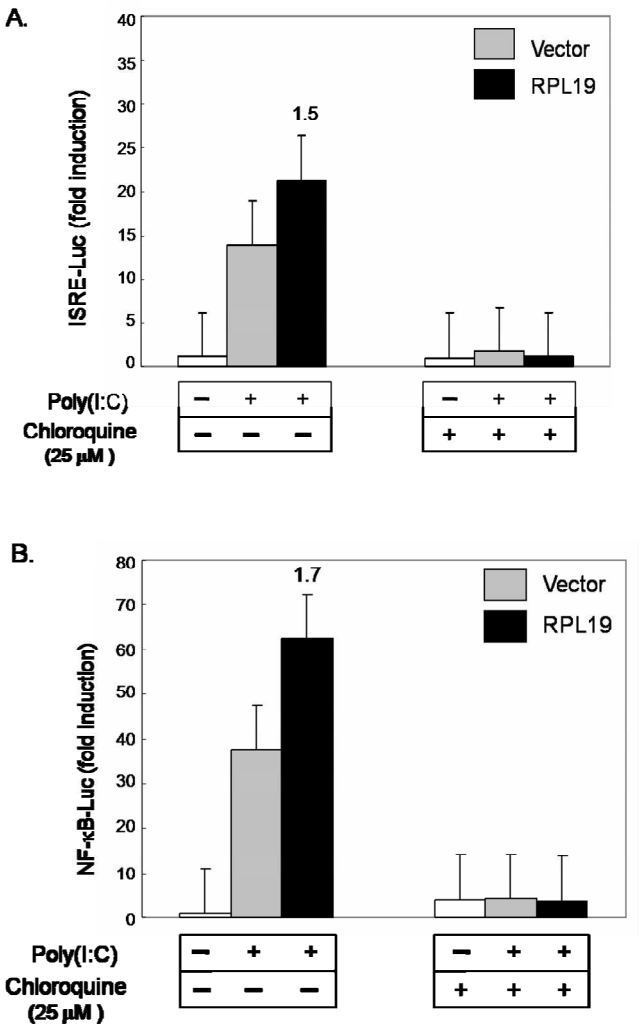

Fig. 6. Effect of RPL19 on poly(I:C)-induced activation of ISRE or NF-κB.
HEK293-TLR3 cells were transfected with empty vectors or expression vectors for RPL19 together with ISRE reporter plasmids (A) or NF-κB reporter plasmid (B). 24 hrs after transfection, cells were treated with poly(I:C) (10 μg/ml) for 6 hrs in the presence of 25 μM chloroquine or no inbibitor. 6 hrs after poly(I:C) treatment luciferase assays were performed. Luciferase activity is normalized to β-galactosidase activity; results are means ± s.d. from three separate transfections.

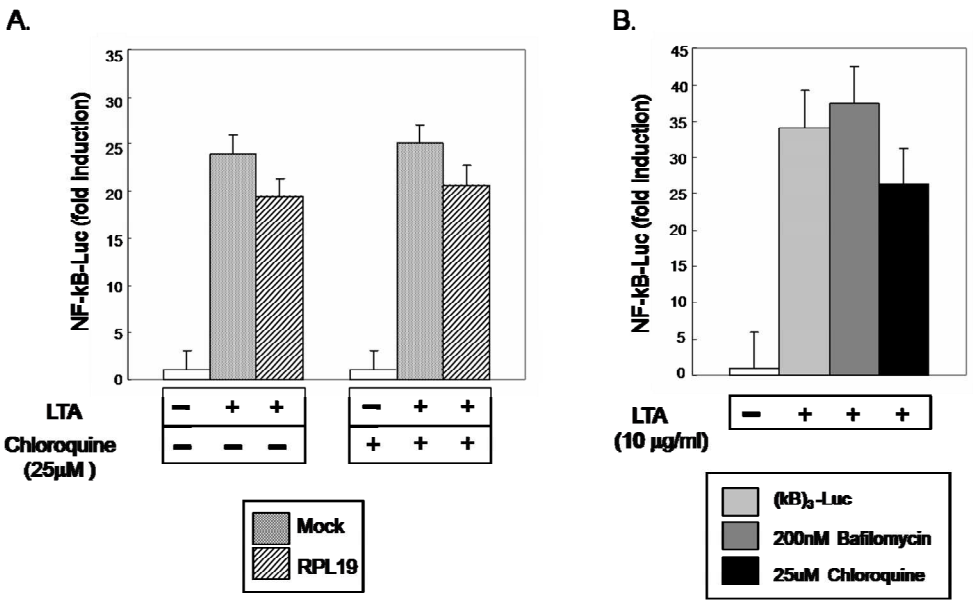

Fig. 7. Effect of RPL19 on LTA-induced activation of NF-κB.
HEK293-TLR2 cells were transfected with empty vectors or expression vectors for RPL19 together with NF-κB reporter plasmids. 24 hrs after transfection, cells were treated with LTA (10 μg/ml) for 6 hrs in the presence of 25 μM chloroquine, 200nM bafilomycin or no inhibitor. Luciferase assays were performed at 6 hrs after poly(I:C) treatment. Luciferase activity is normalized to β-galactosidase activity; results are means ± s.d. from three separate experiments.

7. Enhancement of RPL19 on activation of IRF3 and STAT1 in TLR3 signaling.

To investigate the phosphorylation of IRF3 and STAT1 after the over-expression of RPL19, we performed transfection of RPL19 in HEK293-TLR3 cells. With poly(I:C) stimulation, RPL19 induced relatively rapid phosphorylation of IRF3 (Fig. 8A and Fig 8 B) and STAT1 (Fig. 8A). Slight induction of STAT1 was also detected by RPL19 transfection itself (Fig. 8A). As shown in Fig 8B, transfection of RPL19 itself induced IRF3 phosphorylation without poly(I:C) treatment.

To further confirm whether RPL19 activates ISRE or NF-κB, we induced over-expression of RPL19 in A172 cells. A172 cells are glioblastoma cells which express TLR3 abundantly. In addition to the results with HEK293-TLR3 transfectant cells, we found that RPL19 induces rapid activation of IRF3 after poly(I:C) treatment in A172 cells (Fig. 9A). With over-expression of RPL19 in A172 cells, the phosphorylation of IRF3 occurred to 6.0 fold when compared to 3.5 fold in control cells 1 hr after poly(I:C) stimulation (Fig. 9B). But RPL19 did not augment the NF-κB signaling pathway in A172 cells. Phosphorylation of IκBα occurred at the same level in control cells and in cells over-expressed with RPL19 (Fig. 9C). These data suggest that RPL19 has an effect on the activation of IRF3-STAT1 signaling rather than the NF-κB pathway in A172 cells.

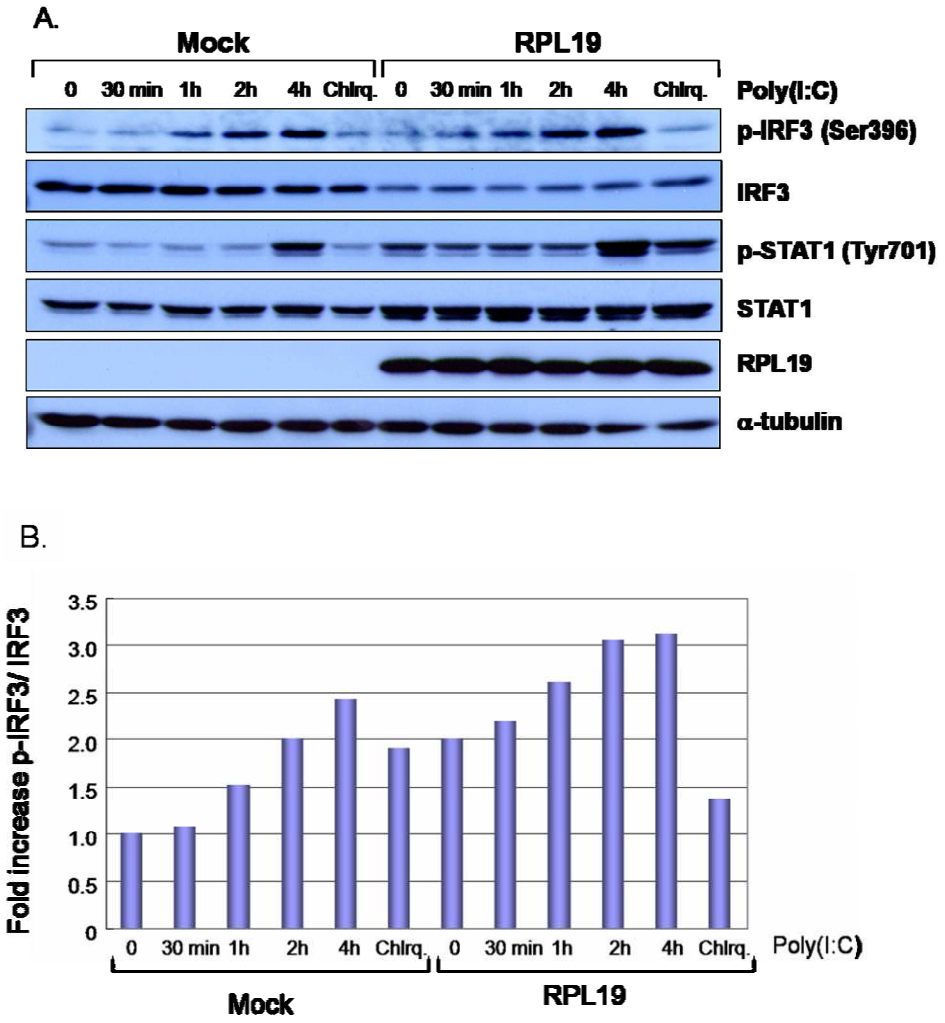

Fig. 8. Effect of RPL19 on activation of IRF3 and STAT1 during TLR3 signaling.
HEK293-TLR3 cells grown in 60mm plates were transfected with control (pcDNA3.1) or RPL19. 48 hrs after transfection, cells were stimulated with 10 μg/ml poly(I:C) in the presence of 25uM chloroquine or no inhibitor for the indicated time. Phosphorylated STAT1 (p-STAT1), phosphorylated IRF3 (p-IRF3), total STAT1 and total IRF3 were determined by western blotting using anti-p-STAT1, anti-p-IRF3, anti-STAT1 and anti-IRF3 antibodies. The expression level of RLP19 in the cell lysates was analyzed with anti-V5 antibody. To confirm equal loading, membranes were re-probed with anti-α-tubulin antibody. (B) The level of IRF3 phosphorylation was determined by densitometric analysis of phospho-IRF3 blots and quantification as a ratio to total IRF3 blots.

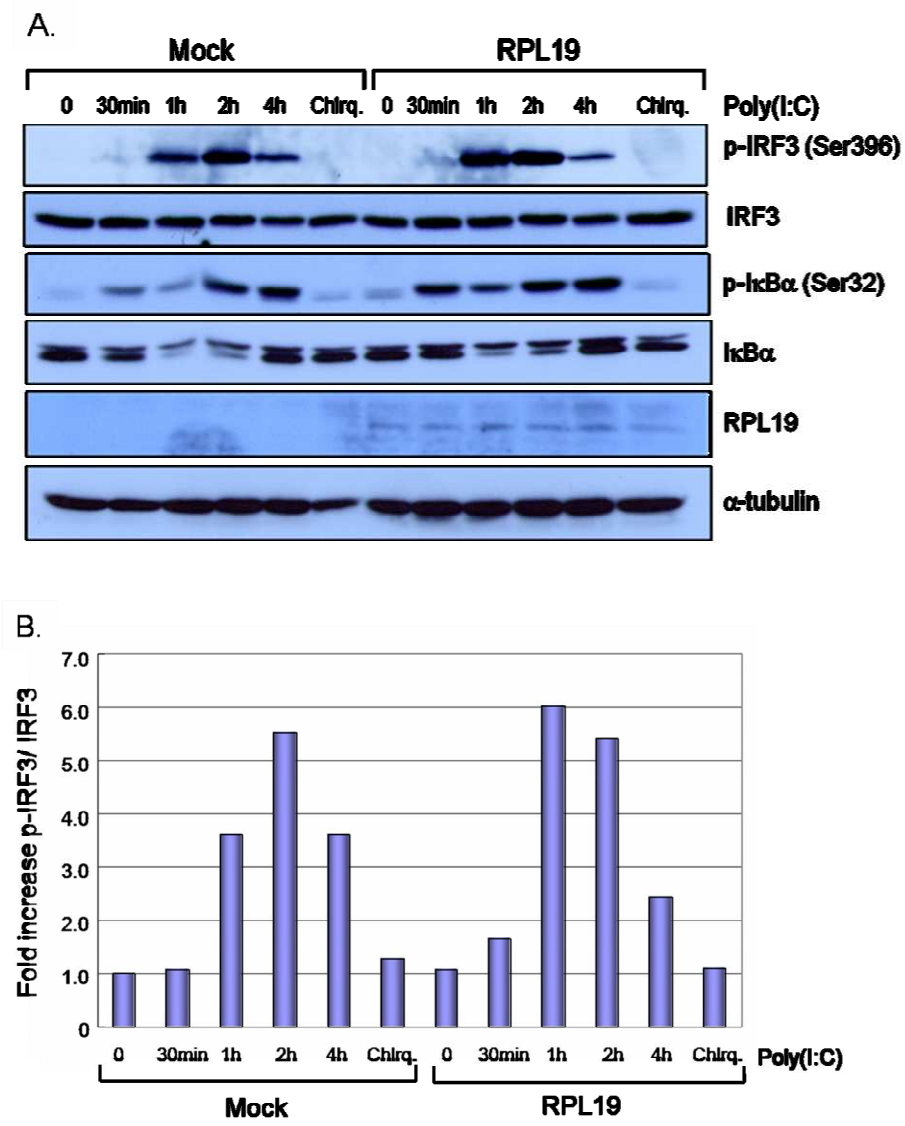

Fig. 9. Effect of RPL19 on activation of IRF3 or IκBα in TLR3 signaling. (Continued)

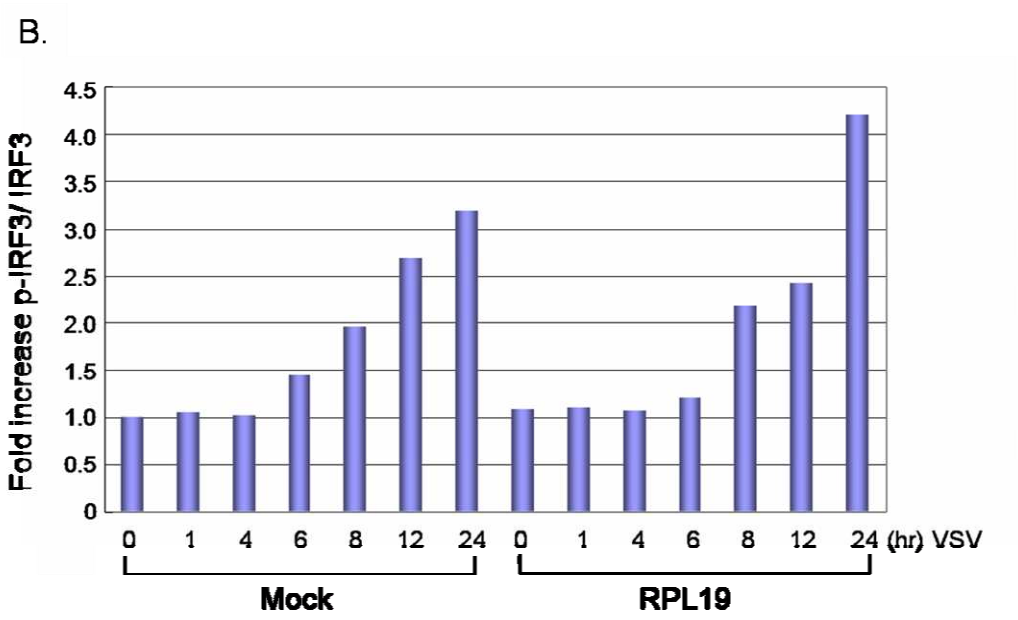

Fig. 9. Effect of RPL19 on activation of IRF3 or IκBα in TLR3 signaling. (A) A172 cells were transfected with control (pcDNA3.1) or RPL19. 48 hrs after transfection, cells were stimulated with 10 μg/ml poly(I:C) in the presence of 25μM chloroquine or no inhibitor for the indicated time. Phosphorylated IRF3 (p-IRF3), p-IκBα and residual IRF3, IκBα were determined by western blotting using anti-p-IRF3, anti-p-IκBα, anti-IRF3 and anti-IκBα antibodies. The expression level of transfected proteins in the lysates was analyzed by anti-V5 antibody (for RPL19). To confirm equal loading, membranes were reprobed with anti-α-tubulin antibody. (B) The level of IRF3 phosphorylation were determined by densitometric analysis of phospho-IRF3 specific western blots and were quantitated as a ratio to total IRF3 blots. (C) The level of IκBα phosphorylation was determined by densitometric analysis of phospho-IκBα blots and quantification as a ratio to total IκBα blots.

8. Effect of RPL19 on phosphorylation of IRF3 in VSV infected cells.

We analyzed the activation of endogenous IRF3 after infection with the vesicular stomatitis virus (VSV) in A549 cells with over-expression of RPL19. We performed transfection of RPL19 into A549 cells, which originated from lung epithelium and was sensitive to VSV infection. The phosphorylation of IRF3 was similar during 1-12 hrs after VSV infection in RPL19 over-expressed cells when compared to control cells (Fig. 10A and Fig. 10B). However, 24 hrs after VSV infection the phosphorylation of IRF3 was higher in RPL19 over-expressed A549 cells (4.2 fold) than in control cells (3.2 fold) (Fig. 10A and Fig. 10B). These results suggest that RPL19 controls antiviral immune responses, probably through the production of antiviral cytokines such as type I IFNs.

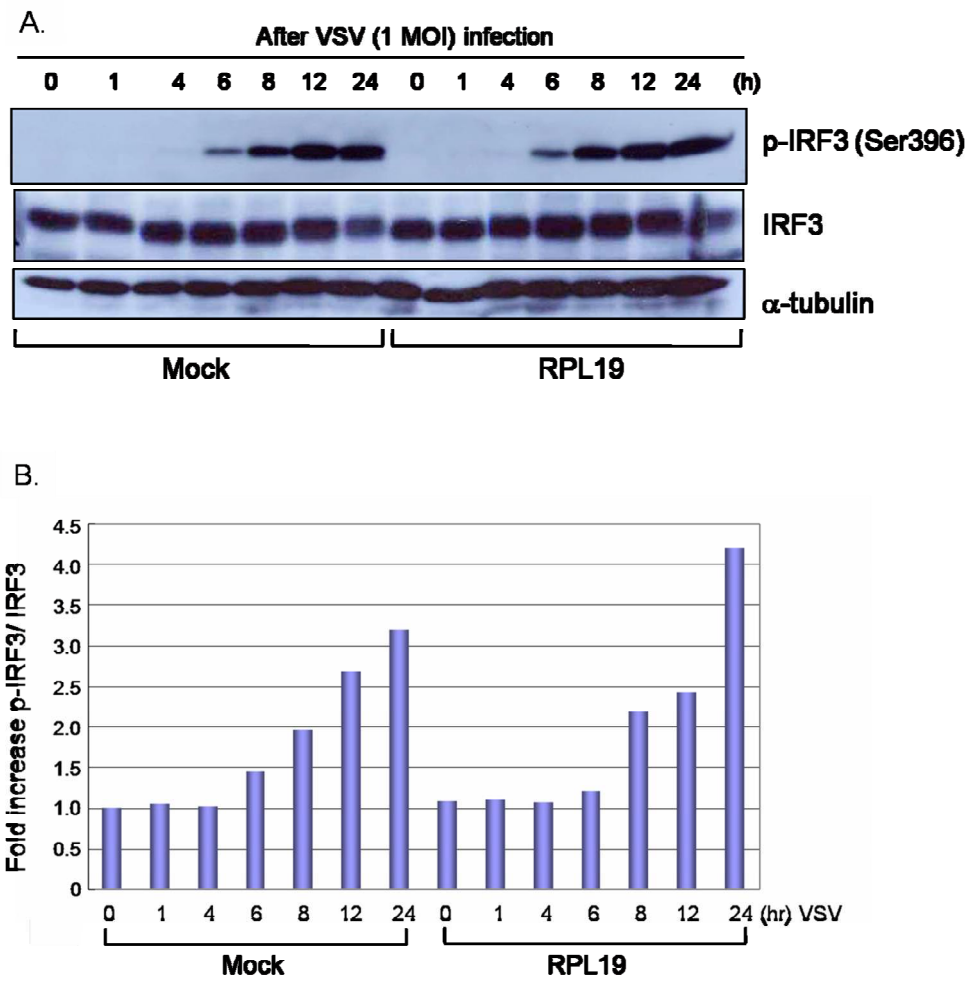

Fig. 10. Effect of RPL19 on IRF3 phosphorylation in VSV infected cells.
(A) A549 cells grown in 60mm plates were transfected with control (pcDNA3.1) or RPL19. 48 hrs after transfection, cells were infected with 1 MOI of VSV for the indicated time. Cell lysates were prepared and subjected to western blotting using anti-phospho-IRF3 (p-IRF3) antibody and anti-IRF3 antibody. To confirm equal loading, membranes were re-probed with an anti-α-tubulin antibody. (B) The level of IRF3 phosphorylation was determined by densitometric analysis of phospho-IRF3 blots and quantification as a ratio to total IRF3 blots.

9. Localization of RPL19 in the endosomes.

To examine the subcellular localization of RPL19, A172 cells transfected with RPL19 were stained with anti-V5 antibody (RPL19, red) and anti-EEA1 antibody (green) detecting early endosomes marker proteins. As a result, RPL19 was detected abundantly in the cytoplasm (Fig. 11). However, it is localized in the endosomes at relatively high levels in early endosomes, which was shown by merging with EEA-1 (Fig. 11). Therefore, RPL19 was found to co-localize with EEA1.

10. RPL19 co-localizes withTLR3.

We examined subcellular localization of TLR3 and RPL19 because TLR3 was known to be localized in the endosomes and, as shown in Fig. 11, RPL19 was also localized in the endosomes. RPL19 was transiently transfected into HEK293-TLR3 cells or into A172 cells. Cells were stained with anti-V5 antibody to detect RPL19 (red) or anti-TLR3 antibody (green). By confocal microscopic analysis RPL19 was found to co-localize with TLR3 in HEK293-TLR3 cells (Fig. 12A) or in A172 cells (Fig. 12B).

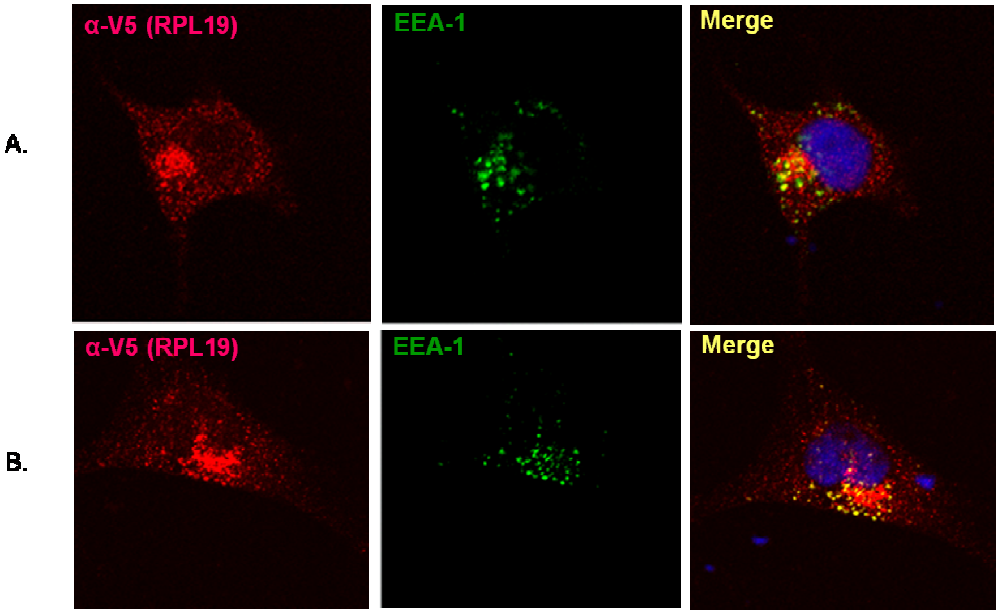

Fig. 11. Localization of RPL19 in endosomes.
(A and B) A172 cells were transiently transfected with 0.5 μg of V5-tagged RPL19. Cells were fixed and stained with anti-V5 (RPL19, red) antibody for 45 min at room temperature. Cells were washed three times and incubated with 2.5 μg/ml of Alexa Fluor-conjugated secondary antibody (Alexa-594) or anti-EEA1 antibody for 1 hr at room temperature. Then, cells were washed and nuclei were stained with DAPI (blue: 4,6-diamidino-2-phenylindole) and analyzed by confocal microscopy. Original magnification, ×40. A and B show two different cells.

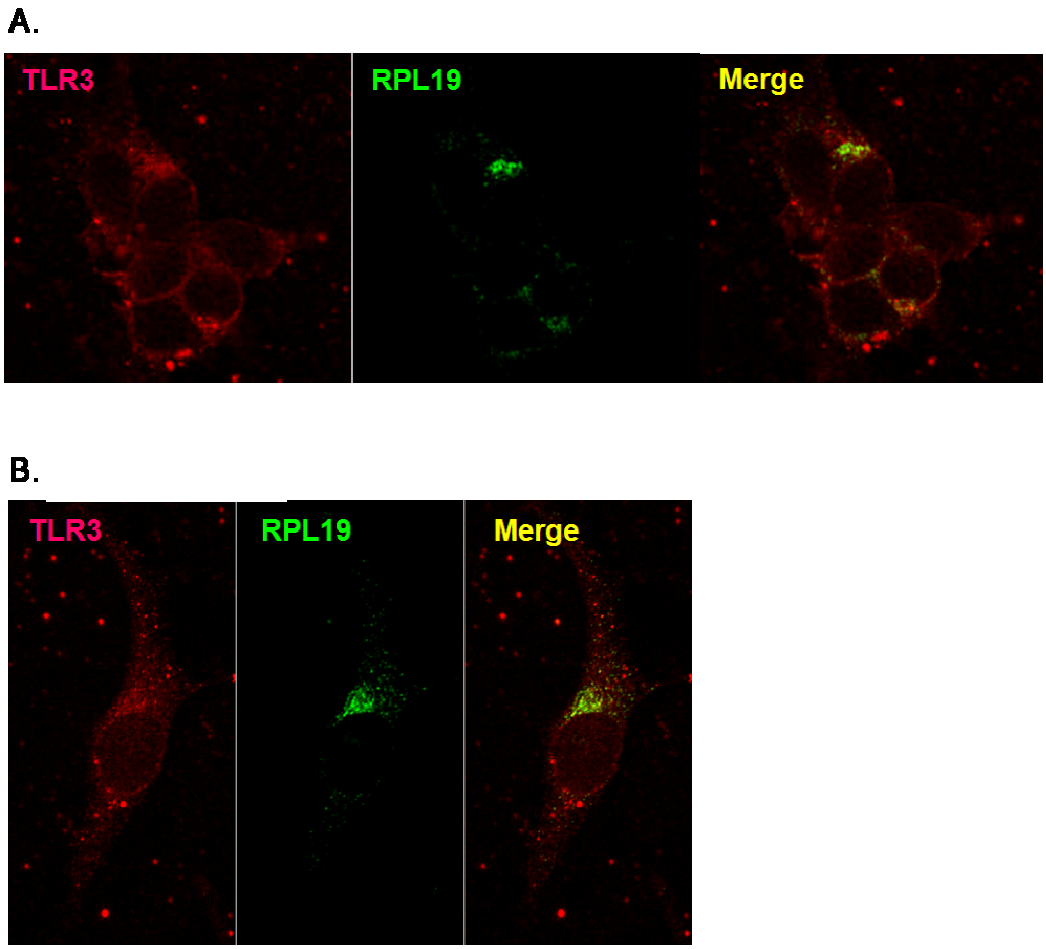

Fig. 12. Co-localization of RPL19 with TLR3.
HEK293-TLR3 cells (A) or A172 cells (B) were transfected transiently with 0.5 μg of V5-tagged RPL19. Cells were fixed and stained with anti-V5 (RPL19, red) antibody or anti-TLR3 antibody for 45 min at room temperature. Cells were washed three times and incubated with 2.5 μg/ml of Alexa Fluor-conjugated secondary antibody for anti-V5 antibody or goat anti-mIgG-FITC antibody for anti-TLR3 antibody for 1 hr at room temperature. After incubation, cells were washed and analyzed by confocal microscopy. Original magnification, ×40.

IV. DISCUSSION

In the process of studying the function and expression pattern of TLR3 in brain astrocytes[52], it has been the scientific question why brain astrocytes express higher levels of TLR3 than immune cells such as macrophages or even dendritic cells came up. When activated with poly(I:C), which mimics the acute phase of a viral infection[53, 54], brain astrocytes and microglial cells secrete type I IFNs, IFNα and IFNβ. Besides activated PKR, which phosphorylates the alpha subunit of eIF2[55], was associated with degenerating neurons in Alzheimer's disease.[56] These reports suggest that dsRNA or TLR3 signaling pathway might have a significant role in brain's immune system.

Therefore, we hypothesized that dsRNA, whether it is of viral origin or from an unknown source, might have the impact of causing brain pathology and that some endogenous molecules elicit dsRNA-mediated immune response to a certain level of overt pathological state. As already reported, not all TLRs function by themselves. TLR4 requires MD2 for signaling and CD14 for LPS transfer, whereas TLR2 associates with TLR1 or TLR6. CD14 is known as an essential component of the TLR4/MD2 complex in LPS signaling. But CD14 also physically interacts with poly(I:C) and enables poly(I:C) internalization.[49] CD14-dependent poly(I:C) uptake enhances intracellular TLR3 activation, explaining why $CD14^{-/-}$ mice exhibited impaired responses to poly(I:C). [49] So we investigated whether some endogenous molecules bind and control TLR3 signaling.

We screened endogenous molecules interacting with TLR3 in brain cells. This study started with cloning the TLR3-ECD gene and generating two types

of TLR3-ECD constructs (1-23 LRR, 10-23LRR). 1-23 LRR contains the whole extracellular portion of TLR3 and 10-23 contains the putative ligand binding site of TLR3. To identify TLR3-ECD interacting proteins in human cells, the yeast two-hybrid system was performed to screen human fetal brain cDNA library with 1-23 LRR or 10-23 LRR as bait. From the beginning ~1 x 10^6 independent clones, only one clone was found to interact with TLR3 *in vitro*. This clone was identified as RPL19.

RPL19 is found in the large ribosomal subunit (60S) of eukaryotes, which function as RNA chaperones during translation and coordinates interaction between the ribosome and RNA.[57, 58] Recently RPL19 is suggested to be a tumor marker. Among tumor cells, differential expression of RPL19 has been identified in epithelial malignancies. Increased levels of RPL19 are found at the core of malignant melanomas when compared with levels expressed at the tumor margin.[59] In addition to melanoma, RPL19 has been highlighted as a potential marker of breast cancer[60] or prostate cancer.[51] Other functions of RPL19 were not known until the present.

So we narrowed down the aim of this study to the functions of RPL19 on poly(I:C)-induced TLR3 signaling. First, to identify whether TLR3 and RPL19 physically associate the each other, we performed *in vitro* translation of RPL19 and TLR3-ECD (1-23 LRR, 10-23 LRR) and immunoprecipitation revealed that RPL19 is bound to TLR3. As shown in our results only 10-23 LRR was co-precipitated with RPL19 whereas 1-23 LRR did not. We suggest that *in vitro* the putative ligand binding site (10-23 LRR) interacts with RPL19 more strongly than the whole TLR3-ECD (1-23 LRR) does.

We further investigated the interaction between TLR3 and RPL19 in TLR3 expressing cells. HEK293 cells are cells transfected to over-express TLR3. In HEK293-TLR3 cells, RPL19 and TLR3 were immmunoprecipitated together. This interaction was detected in both conditions of absence or presence of poly(I:C). These data clearly showed that TLR3 constitutively interacts with RPL19 independent of poly(I:C) involvement. Next, we assessed interaction between poly(I:C) and RPL19, which was performed by using poly(I:C)-agarose ‘pull-down’ assay. From this study RPL19 was found to bind poly(I:C). These result shows that RPL19 may mediate the transfer of dsRNA to TLR3.

dsRNA signaling through TLR3 leads to the activation of at least three families of transcription factors: IRF-3, NF-κB and AP-1. Here we focused on the activation of IRF3 and NF-κB in response to poly(I:C). The involvement of RPL19 in poly(I:C)-induced activation of NF-κB and ISRE was measured by luciferase reporter genes assay. Over-expression of RPL19 in HEK293-TLR3 cells resulted in a weak induction of ISRE-Luc activation and NF-κB activation after poly(I:C) stimulation. These results suggest that RPL19 potentiate TLR3 signaling. As described previously CD14 is the only reported molecule potentiating TLR3 signaling by binding poly(I:C).

Then we determined whether the activation of ISRE and NF-κB by RPL19 requires endosomal maturation. Human TLR3 has been described as a cytosol-localized receptor and interaction with its ligand may occur in subcellular compartments within the endocytic pathway. These intracellular compartments display an acidic pH, a demonstrated requirement for

interaction between TLR3 and poly(I:C). Our study shows that the activation of ISRE and NF-κB by RPL19 requires endosomal maturation. In our study the activation of these promoters was inhibited by the treatment of chloroquine (inhibitor of endosomal maturation). This finding suggests clearly RPL19 is involved at the endosomal level, the major compartment, in which TLR3 signaling initiates. To know whether RPL19 is involved in other TLR members we examined the effect of RPL19 on TLR2 signaling. In TLR2 signaling LTA induces NF-κB activation. Transfection of RPL19 into HEK293-TLR2 cells had no effect on LTA-mediated activation. LTA induced NF-κB activation occurred at the same level in RPL19 transfected cells as in control cells. Inhibitors of endosomal maturation, chloroquine or bafilomycin, did not block LTA induced NF-κB activation in either of the cells. These data demonstrate that RPL19 has no effect on LTA-mediated TLR2 activation and acts specifically on TLR3.

To examine the effect of RPL19 on the production of type I IFNs, we investigated the phosphorylation of IRF3. With poly(I:C) stimulation, RPL19 induced relatively rapid phosphorylation of IRF3 and STAT1 in HEK293-TLR3 cells. Moreover, transfection of RPL19 itself induced IRF3 phosphorylation without poly(I:C) treatment. To further confirm whether RPL19 activates IRF3, we induced over-expression of RPL19 in A172 cells. A172 cells are glioblastoma cells which express TLR3 abundantly. In addition to the results with HEK293-TLR3 transfectant cells, we obtained more convincing data that RPL19 induces the activation of IRF3 after poly(I:C) treatment rapidly in A172 cells. The interesting finding was that RPL19 did not control the NF-κB signaling pathway. Phosphorylation of IκBα occurred

at the same level in cells over-expressed with RPL19 as control cells. These data suggest that RPL19 has an effect on the activation of IRF3-STAT1 signaling rather than the NF-κB pathway.

The C-terminal phosphorylation of IRF3 facilitates its association with the co-activators CBP [CREB (cAMP response element-binding protein)-binding protein] and p300.[61] IRF3 primarily activates the IFN-β promoter with the co-operation of transcription factors, especially NF-κB. Once IFN-β is produced, it subsequently activates a transcription factor complex, known as IFN-stimulated gene factor 3 (ISGF3), the comprising signal transducer and activator of transcription (STAT) 1, STAT2 and IRF9. ISG3 in turn induces the expression of IRF7 and this is subject to virus-induced phosphorylation in a manner similar to IRF3. This leads to activation of IRF7 and its stimulation of IFN-α/β promoters. The induction of type I IFNs establishes a positive feedback loop, ensuring high-level expression of type I IFN and IFN-inducible genes. According to our data, RPL19 augmented the activation of poly(I:C)-induced TLR3 signaling and, therefore, RPL19 may mediate type I IFN-mediated immune response.[62]

Next we analyzed the activation of IRF3 after infection with VSV in A549 cells with over-expression of RPL19. A549 cells originated from lung epithelium and was sensitive to VSV infection. During 1-12 hrs after VSV infection the phosphorylation of IRF3 was similar in RPL19 over-expressed cells when compared to control cells. However, 24 hrs after VSV infection the phosphorylation of IRF3 was higher in RPL19 over-expressed A549 cells than in control cells. These results suggest that RPL19 controls antiviral immune

responses, probably through the production of antiviral cytokines such as type I IFNs.

Our data shows that augmentation of TLR3 signaling by RPL19 was blocked by inhibitors of endosomal maturation and this finding leads us to presume that RPL19 may be localized in the endosome. To examine the localization of RPL19, we analyzed A172 cells transfected with RPL19 by immunostaining. Cells were stained with anti-V5 (RPL19) antibody or FITC-conjugated EEA1. As a result, RPL19 was expressed in the cytoplasm and also in early endosomes. Immunostaining of TLR3 and RPL19 showed that these molecules co-localized in the endosome. Therefore, we suggest that RPL19 interacts with TLR3 in the endosome.

To summarize, as illustrated in Fig 13, we suggest that the endogenous ribosomal protein RPL19 may interact with TLR3 in the endosomes and may augment TLR3 signaling.

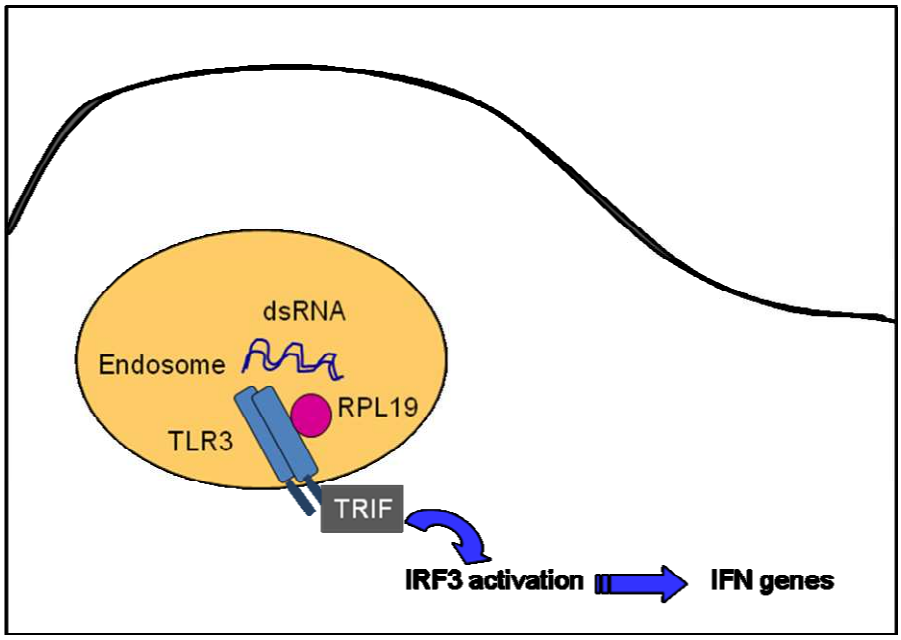

Fig. 13. Interaction between TLR3 and RPL19 in the endosomes to enhance TLR3 signaling. TLR3, Toll-like receptor 3; RPL19, ribosomal protein L19; TRIF, tumor necrosis factor receptor associated factor; IRF3, interferon regulatory factor

V. CONCLUSION

In this study, we investigated the interaction between RPL19 and TLR3 and the functional implication of the interaction.

1. Using TLR3-ECD (1-23 LRR or 10-23 LRR) as bait in a yeast two-hybrid screening we isolated one clone encoding ribosomal protein L19 (RPL19).

2. The putative ligand binding site of TLR3 (10-23 LRR) interacts with RPL19 more strongly than the whole TLR3-ECD (1-23 LRR) does *in vitro*. In addition TLR3 constitutively interacts with RPL19 independent of poly(I:C) in HEK293-TLR3 cells.

3. RPL19 was found to bind poly(I:C), which was detected by poly(I:C)-agarose 'pull-down' assay, suggesting that RPL19 may mediate the transfer of dsRNA to TLR3.

4. Over-expression of RPL19 in HEK293-TLR3 cells resulted in the induction of ISRE activation and NF-κB activation after poly(I:C) stimulation. Inhibitors of endosomal maturation, chloroquine and bafilomycin, blocked poly(I:C)-induced ISRE or NF-κB activation. Therefore, the activation of ISRE and NF-κB by RPL19 after poly(I:C) stimulation requires endosomal maturation as typical TLR3 signaling does.

5. Transfection of RPL19 into HEK293-TLR2 cells had no effect on LTA-mediated activation, which mediates TLR2 signaling. LTA induced NF-κB activation occurred at the same level in RPL19 transfected cells as in control cells. Chloroquine or bafilomycin did not block LTA induced NF-κB activation

in either of the cells. These data demonstrate that RPL19 has no effect on TLR2 activation and acts specifically on the TLR3 pathway.

6. With poly(I:C) stimulation, RPL19 induced relatively rapid phosphorylation of IRF3 or STAT1 in HEK293-TLR3 and also in A172 cells. Moreover, transfection of RPL19 itself induced IRF3 phosphorylation without poly(I:C) treatment.

7. Phosphorylation of IRF3 was induced at a similar level during 1-12 hrs after VSV infection in cells over-expressing RPL19 when compared to control cells. However, 24 hrs after VSV infection the phosphorylation of IRF3 was higher in A549 cells over-expressing RPL19 than in control cells. These results suggest that RPL19 controls antiviral immune responses, possibly through the production of antiviral cytokines such as type I IFNs.

8. RPL19 was detected abundantly in the cytoplasm. And it is localized in the endosome at relatively high levels, which is shown by merging with staining by early endosomal marker, EEA-1.

9. RPL19 was found to co-localize with TLR3 in HEK293-TLR3 cells or in A172 cells. Immunostaining of TLR3 and RPL19 showed that these molecules co-localize in endosomes. Therefore, we suggest that RPL19 interacts with TLR3 in endosome.

In summary, our results suggest that the endogenous ribosomal protein RPL19 may interact with TLR3 in the endosome and may augment TLR3 signaling.

REFERENCES

1. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499-511.

2. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987-95.

3. Ulevitch RJ. Therapeutics targeting the innate immune system. Nat Rev Immunol 2004;4:512-20.

4. Zuany-Amorim C, Hastewell J, Walker C. Toll-like receptors as potential therapeutic targets for multiple diseases. Nat Rev Drug Discov 2002;1:797-807.

5. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

6. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem 2004;279:12542-50.

7. Kariko K, Bhuyan P, Capodici J, Weissman D. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. J Immunol 2004;172:6545-9.

8. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev

Immunol 2002;20:197-216.

9. O'Neill LA, Fitzgerald KA, Bowie AG. The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol 2003;24:286-90.

10. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301:640-3.

11. Sarkar SN, Smith HL, Rowe TM, Sen GC. Double-stranded RNA signaling by Toll-like receptor 3 requires specific tyrosine residues in its cytoplasmic domain. J Biol Chem 2003;278:4393-6.

12. Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol 2004;11:1060-7.

13. Sen GC, Sarkar SN. Transcriptional signaling by double-stranded RNA: role of TLR3. Cytokine Growth Factor Rev 2005;16:1-14.

14. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med 2003;198:1043-55.

15. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 2003;4:161-7.

16. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al.

Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol 2002;169:6668-72.

17. Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, et al. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. Embo J 2000;19:4976-85.

18. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491-6.

19. Peters RT, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. Mol Cell 2000;5:513-22.

20. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;300:1148-51.

21. Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BR, Li X. Poly(I-C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFkappa B and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. J Biol Chem 2003;278:16713-9.

22. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)

associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol 2003;171:4304-10.

23. Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM. Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem 2002;277:9505-11.

24. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, et al. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 2004;5:503-7.

25. Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem 2005;280:36560-6.

26. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 2003;278:49751-62.

27. Wietek C, Miggin SM, Jefferies CA, O'Neill LA. Interferon regulatory factor-3-mediated activation of the interferon-sensitive response element by Toll-like receptor (TLR) 4 but not TLR3 requires the p65 subunit of NF-kappa. J Biol Chem 2003;278:50923-31.

28. Doyle SE, O'Connell R, Vaidya SA, Chow EK, Yee K, Cheng G. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. J Immunol 2003;170:3565-71.

29. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;303:1529-31.

30. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526-9.

31. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5.

32. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 2004;101:5598-603.

33. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol 2006;7:49-56.

34. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154-62.

35. Lopez CB, Moltedo B, Alexopoulou L, Bonifaz L, Flavell RA, Moran TM.

TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. J Immunol 2004;173:6882-9.

36. Foy E, Li K, Sumpter R, Jr., Loo YM, Johnson CL, Wang C, et al. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci USA 2005;102:2986-91.

37. Gohda J, Matsumura T, Inoue J. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. J Immunol 2004;173:2913-7.

38. Kaiser WJ, Offermann MK. Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. J Immunol 2005;174:4942-52.

39. Choe J, Kelker MS, Wilson IA. Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science 2005;309:581-5.

40. Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol 2003;24:528-33.

41. Weber AN, Morse MA, Gay NJ. Four N-linked glycosylation sites in human toll-like receptor 2 cooperate to direct efficient biosynthesis and secretion. J Biol Chem 2004;279:34589-94.

42. Kirk P, Bazan JF. Pathogen recognition: TLRs throw us a curve. Immunity

2005;23:347-50.

43. Bell JK, Botos I, Hall PR, Askins J, Shiloach J, Davies DR, et al. The molecular structure of the TLR3 extracellular domain. J Endotoxin Res 2006;12:375-8.

44. Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 3. Proc Natl Acad Sci USA 2006;103:8792-7.

45. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K, et al. CD36 is a sensor of diacylglycerides. Nature 2005;433:523-7.

46. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431-3.

47. Dunzendorfer S, Lee HK, Soldau K, Tobias PS. TLR4 is the signaling but not the lipopolysaccharide uptake receptor. J Immunol 2004;173:1166-70.

48. Dziarski R, Ulmer AJ, Gupta D. Interactions of CD14 with components of gram-positive bacteria. Chem Immunol 2000;74:83-107.

49. Lee HK, Dunzendorfer S, Soldau K, Tobias PS. Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. Immunity 2006;24:153-63.

50. Maisnier-Patin S, Paulander W, Pennhag A, Andersson DI. Compensatory evolution reveals functional interactions between ribosomal proteins S12,

L14 and L19. J Mol Biol 2007;366:207-15.

51. Bee A, Ke Y, Forootan S, Lin K, Beesley C, Forrest SE, et al. Ribosomal protein l19 is a prognostic marker for human prostate cancer. Clin Cancer Res 2006;12:2061-5.

52. Yang E, Shin JS, Kim H, Park HW, Kim MH, Kim SJ, et al. Cloning of TLR3 isoform. Yonsei Med J 2004;45:359-61.

53. Guha-Thakurta N, Majde JA. Early induction of proinflammatory cytokine and type I interferon mRNAs following Newcastle disease virus, poly [rI:rC], or low-dose LPS challenge of the mouse. J Interferon Cytokine Res 1997;17:197-204.

54. Traynor TR, Majde JA, Bohnet SG, Krueger JM. Sleep and body temperature responses in an acute viral infection model are altered in interferon type I receptor-deficient mice. Brain Behav Immun 2006;20:290-9.

55. Williams BR. PKR; a sentinel kinase for cellular stress. Oncogene 1999;18:6112-20.

56. Peel AL. PKR activation in neurodegenerative disease. J Neuropathol Exp Neurol 2004;63:97-105.

57. Maguire BA, Zimmermann RA. The ribosome in focus. Cell 2001;104:813-6.

58. Ramakrishnan V. Ribosome structure and the mechanism of translation.

Cell 2002;108:557-72.

59. Roesch A, Vogt T, Stolz W, Dugas M, Landthaler M, Becker B. Discrimination between gene expression patterns in the invasive margin and the tumour core of malignant melanomas. Melanoma Res 2003;13:503-9.

60. Henry JL, Coggin DL, King CR. High-level expression of the ribosomal protein L19 in human breast tumors that overexpress erbB-2. Cancer Res 1993;53:1403-8.

61. Masumi A, Yamakawa Y, Fukazawa H, Ozato K, Komuro K. Interferon regulatory
factor-2 regulates cell growth through its acetylation. J Biol Chem 2003;278(28):25401-7.

62. Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). Blood 2006;107(5):2112-22.

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ABSTRACT (in Korean)

Double-stranded RNA가 매개하는 Toll-like receptor 3의 신호전달에 대한 ribosomal protein L19의 영향

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양은정

Toll-like receptor 3 (TLR3)는 double stranded RNA, 바이러스 유전자 또는 바이러스 복제시 생성되는 중간산물에 의해서 활성화된다. TLR3의 extracellular domain이 ligand를 인지하면 adapter 분자인 Toll/IL-1 domain-containing adapter inducing IFN-β (TRIF)가 세포질 내에 존재하는 TLR3의 Toll-IL-1 receptor homology domain에 결합하게 된다. TLR3와 TRIF의 결합은 주로 IRF3를 인산화시키고 제1형 인터페론 유전자의 전사를 활성화시킴으로써 바이러스 면역에 중추적인 역할을 담당한다.

본 연구자는 TLR3와 결합하는 세포 내재 단백을 찾기 위하여 yeast two hybrid system을 사용하였다. 그 결과 진핵생물의 large ribosomal subunit (60S)에서 존재하는 ribosomal protein L19 (RPL19)이 TLR3와 결합함을 관찰하였다. 그리하여 RPL19 단백이 TLR3와 결합하고 TLR3 신호전달을 증가시킨다는 가설을 증명하기 위하여 다음 실험을 수행하였다.

1) TLR3와 RPL19의 유전자를 클로닝하였다. 2) 이들 단백의

결합여부를 관찰하기 위하여 시험관 내에서 단백을 합성하여 두 단백간의 결합을 측정한 결과 TLR3와 RPL19 단백이 결합함을 확인하였다. 그리고 TLR3를 과발현하는 HEK293 세포에 RPL19 유전자를 transfection 시키고 총단백을 얻어 항-TLR3 항체로 immunoprecipitation을 수행한 후 RPL19 단백에 대한 western blot을 통하여 세포 내에서도 TLR3와 RPL19이 결합함을 확인하였다. TLR3와 RPL19의 결합은 TLR3의 ligand인 poly(I:C) 처리에 상관없이 관찰되었다. 3) Poly(I:C)-agarose 'pull-down' 방법으로 시험관 내에서 RPL19가 poly(I:C)와 결합함을 확인하였다. 4) ISRE promoter reporter gene assay를 통하여 RPL19을 과발현시키면 poly(I:C)에 의해서 유도되는 ISRE의 활성화가 증가함을 관찰하였다. 5) Endosome 성숙을 억제하는 chloroquine을 처리하면 RPL19에 의하여 증가되는 NF-κB와 ISRE의 활성화가 억제되었다. 따라서 RPL19에 의한 NF-κB와 ISRE 활성도가 증가하기 의하여 endosome의 성숙이 필요함을 확인하였다. 6) RPL19가 제1형 인터페론 생성에 필요한 전사인자인 IRF3와 STAT1의 인산화에 미치는 영향을 측정하였다. TLR3를 발현하는 세포에 RPL19를 과발현시켰을 때 IRF3와 STAT1의 인산화가 증가되었다. 7) RPL19가 endosome 내에서 TLR3와 함께 존재함을 confocol microscopy로 확인하였다.

이러한 결과들은 RPL19가 poly(I:C)에 의해서 유도되는 TLR3 신호전달을 증가시키고, 제1형 인터페론이 매개하는 면역반응 또한 증가시킬 수 있음을 뒷받침한다. 따라서 본 연구자는 RPL19가 생체 내에서 TLR3 신호전달을 조절하는 내인성 단백으로 작용할 수 있음을 제시하고자한다.

핵심되는 말 : Toll-like receptor 3, ribosomal protein L19, dsRNA, IRF3, STAT1, endosome