ERK negatively regulates TLR2 induced transcription of TNFα, IL-6, iNOS, Cox2, and IL1β in Bone Marrow Derived Macrophages.

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Chulhan Kim

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This certifies that the master's thesis of Chulhan Kim is approved.

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The Graduate School

Yonsei University

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Abbreviations

ERK: Extracellular signal-regulated kinase

JNK: c-Jun NH(2)-terminal kinase

TLR: Toll-like Receptor

LPS: Lipopolysaccharide

Poly (I:C) : Polyinosinic-polycytidylic acid

LTA: Lipoteichoic acid

BMDM: Bone marrow derived macrophages

iBMDM: Immortalized bone marrow derived macrophage

PAMP: pathogen-associated molecular pattern

JAK: Janus kinase

STAT: Signal Transducers and Activators of Transcription

NO: nitric oxide

iNOS: inducible nitric oxide synthase

ROS: reactive oxygen species

TRIF: TIR-domain-containing adapter-inducing interferon-β

TRAM: TRIF-related adaptor molecule

Myd88: Myeloid differentiation factor 88

IRAK: IL-1 receptor associated kinase

TAK: TGF-b - activated kinase

TIR: Toll/interleukin 1 receptor

MAL: MyD88 adaptor-like

SARM: Sterile alpha motif and Armadillo motif domain-containing protein

TRAF6: TNF receptor-associated factor 6

TpL2: Tumor progression locus 2

IRF3: interferon regulatory factor3

CCL5: Chemokine (C-C motif) ligand 5

TNFα: Tumor necrosis factor alpha

IL-6: Interleukin 6

Cox2: Cyclo-oxygenase 2

IL1β: Interleukin 1 beta

CREB: cAMP response element-binding protein

IκBα: Inhibitor of kappa b alpha

IKK: IkB kinases

MEK: Mitogen-activated protein kinase kinase

MAPK: Mitogen-activated protein kinase

MKK: MAPK kinase

qRT-PCR: quantitative Real-time Reverse Transcription Polymerase Chain Reaction

CRISPR-CAS9: Clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9

ABSTRACT

ERK negatively regulates TLR2 induced transcription of TNFα, IL-6, iNOS, Cox2, and IL1β in Bone Marrow Derived Macrophages.

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Extracellular signal-regulated kinase (ERK) was recently identified as a type of mitogen-activated protein kinase involved in Toll-like Receptor (TLR) downstream signaling and its corresponding cytokine production in bone marrow derived macrophages. Previous papers focused on the correlation between TLR4 induced transcription and ERK when introduced to Lipopolysaccharide (LPS). Here, I evaluate the relationship between ERK and TLR2 induced transcription when Pam3CSK4 is applied. Specifically, I assessed ERK dependent regulation of pro-inflammatory cytokines such as TNFα, IL-6, iNOS, Cox2, and IL1β in order to illustrate the pathway in which ERK negatively regulates TLR2 induced transcription. Since no direct inhibitor of ERK exists, I used a well-defined inhibitor (U0126) of Mitogen-activated protein kinase kinase (MEK1/2), an upstream factor known to directly regulate ERK, to test ERK-dependent negative regulation. In order to detect protein and mRNA level of the cytokines, I used Western Blotting and quantitative Real-time Reverse Transcription Polymerase Chain Reaction(qRT-PCR), respectively. Furthermore, to locate the point in which

negative regulation of TLR2 downstream transcription by ERK occurs, I evaluated potential pathways suggested by previous studies involving MEK1/2 and phosphorylated Signal Transducers and Activators of Transcription (p-STAT) using Western Blotting. Results showed that activation by Pam3CSK4 took a distinct pathway from LPS, leading to diagnosis of other potential pathways involving phosphorylation of p38, JNK, IκBα, and CREB. In addition, I tested macrophage-like cell lines: J774A.1, Immortalized bone marrow derived macrophage (iBMDM), and Raw264.7 to create a MEK1/2 knockout cell line using the CRISPR-CAS9 system for substitution of in vivo experimentation with knockout mice. In final, to confirm that the negative regulation effect is TLR2 specific, TLR2 specific ligands: Pam3CSK4 and Lipoteichoic Acid (LTA) are contrasted with ligands of TLR3, TLR4, and TLR9. Collectively, these data suggest that ERK negatively regulates TLR2-induced transcription of pro-inflammatory cytokines: TNFα, IL-6, iNOS, Cox2, and IL1β. These findings may contribute in the development of drugs that modulate TLR activity to treat inflammatory diseases and cancer.

Key words : Pam3CSK4, LPS, TLR2, TLR4, ERK, cytokine

Introduction

There are ten known types of human TLRs. These ten types can be categorized into two main groups. (Doyle, O'Neil et al. 2006) The first group: TLR1, TLR2, TLR4, TLR5, and TLR6 triggers anti-bacterial responses (Kumar et al. 2009). TLR4, the dominantly analyzed TLR, senses LPS, a component of the gram-negative bacteria cell wall (Akira, Uematsu et al. 2006). TLR2 works synergistically with TLR1, TLR5, and TLR6 to verify distinct pathogen-associated molecular patterns (PAMPs) (Kumar et al. 2009). To be more specific, TLR2 cooperates with TLR1, TLR5, and TLR6 to recognize triacylated lipop eptides, bacterial flagellin, and diacylated lipopeptides, respectively (Akira, Uematsu et al. 2006). Moreover, TLR2 also senses numerous fungal and protozoal products (Akira, Uematsu et al. 2006). The second group: TLR3, TLR7, TLR8, and TLR9 initiates anti-viral reactions (Kumar et al. 2009). TLR3 acknowledges double stranded RNA, TLR7 and TLR8 recognize single stranded RNA, and TLR9 distinguishes CpG motifs, which exist in both virus and bacteria (Kumar et al. 2009). In short, here I analyze the downstream signaling of two TLRs: TLR4 and TLR2 from the anti-bacterial group, that is divergent from the remaining eight types of TLRs.

TLR4 stimulation is known to activate numerous signaling pathways including two pathways involving mitogen-activated protein kinases (MAPKs) and Janus kinase(JAK)-STAT (Xiaoyu Hu et al. 2007; Kaisho and Akira 2006). MAPKs, include p38 kinase, c-Jun NH(2)-terminal kinase (JNK), and ERK (Kaisho and Akira 2006). MAPK activation by phosphorylation is regulated by MAPK kinase (MKK). A direct known MKK for p38, JNK, and ERK is MKK3/6, MKK4/7, and MEK1/2, respectively (Jeffrey, Rommel and Mackay et al. 2007). In short, TLR signaling is transferred through MKK kinase to MKKs and finally to p38, JNK, and ERK. Phosphorylation of these three MAPKs leads to the release of

inflammatory cytokines such as TNF-α and IL-6 (Kawai and Akira 2006). Inflammatory cytokines then trigger adjacent cells to produce chemokines or adhesion molecules, thereby recruiting inflammatory cells such as macrophages to the sites of infection. Sequentially, the macrophages engulf the invasive pathogens and digest them by nitric oxide (NO) produced from inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), or defensins (Flannagan, Cosio and Grinstein 2009).

JAKs have four isoforms: JAK1, JAK2, JAK3, and Tyk2, while STATs have seven isofroms: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Specific combinations of JAK and STAT isoforms allow distinct cytokine receptor signaling (Kisseleva et al. 2002). STAT1 and STAT3 have been shown to be important in IL-10 production (Bouhamdan et al. 2015). Furthermore, IL-10, fuctions as an anti-inflammatory cytokine, thus, inhibits the production of proinflammatory cytokines such as TNFα and IL-6 in macrophages (Couper, Blount and Riley et al. 2008).

In contrast to TLR4 stimulation, TLR2 stimulation is achieved by Pam3CSK4, a synthetically made triacylated lipopeptide that imitates the acylated amino terminus of bacterial lipopeptides, and LTA (Takeuchi, Akira et al. 1999). Compared to the well-defined TLR4 downstream pathway, an overall view of TLR2 downstream signaling requires further clarification. However, the converging point of TLR2 and TLR4 downstream pathways: myeloid differentiation factor 88 (Myd88) has been identified (Janssens and Beyaert 2002), shedding light to potential TLR2 downstream pathways as well as regulation mechanisms between TLR induced transcription.

MyD88, a universal adapter protein for all TLR except TLR3, consists of a TIR and death domain at its C- and N-terminal region, respectively (O'Neil and Bowie 2007). The TIR domain interacts with TLRs, while the death domain recruits IL-1

receptor - associated kinase (IRAK) family members: IRAK-1, IRAK-2, IRAK-4, and IRAK-M. IRAK-1 and IRAK-4 are known to play a critical role in the MyD88-dependent pathway, signaling TRAF6 to trigger TGF-b - activated kinase (TAK) 1 activation through ubiquitination and the canonical IkB kinases (IKK): IKKα and IKKβ (Kaisho and Akira 2006). Sequentially, IKKs phosphorylate IkB and initiate degradation leading to NF-kB downstream signaling (Kaisho and Akira 2006). Moreover, TAK 1 activation can also lead to the activation of MAPKs: p38, JNK, and ERK (Ninomiya-Tsuji1, Cao and Matsumoto et al. 1999). Specifically, though the complete mechanism is unknown, TAK 1 activation signals through Tumor progression locus 2 (TpL2), facilitating phosphorylation of MEK1/2 and subsequently, ERK (Arthur and Ley 2013). In brief, Myd88 dependent signaling of TLR2 and TLR4 leads to MAPK or NF-kB activation, causing transcription of inflammatory cytokine genes.

Furthermore, out of the five defined adaptors: MyD88, TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), MyD88 adaptor-like (MAL), and sterile alpha motif and Armadillo motif domain-containing protein (SARM) (Medzhitov et al. 1998; Kawai et al. 1999; Fitzgerald et al. 2001; Horng et al. 2001, 2002; Kaisho et al. 2001; Yamamoto et al. 2003; O'Neil and Bowie 2007), the ones that bind to the intracellular domain of TLR2 and TLR4 besides Myd88 are TRAM and TRIF (Nilsen et al. 2015). TLR4 is well known for utilizing the adaptors TRAM and TRIF, to activate the interferon regulatory factor3 (IRF3) (Fitzgerald, Pitha and Golenbok 2003). In comparison, recent studies confirm TRAM and TRIF involvement in TLR2 signaling regarding induction of CCL5, a chemokine that recruits leukocytes to inflammation sites (Nilsen et al. 2015). Overall, much is yet to be discovered concerning TLR2 downstream signaling.

Nonetheless, recent studies have provided the backbone for potential pathways in

which TLR2 associates with MyD88, TRIF, or TRAM to trigger MAPK or NF-kB activation, which in turn regulates the transcription of pro-inflammatory cytokine genes, such as TNFα, IL-6, iNOS, Cox2, and IL1β (Nilsen et al. 2015; Arthur and Ley 2013; Rayamajhi and Miao 2013). All in all, here I contribute to understanding this overall structure by providing a novel contrasting transcription pattern of pro-inflammatory cytokines in relation to LPS activated TLR4 and Pam3CSK4 activated TLR2.

2. Materials and Methods

2.1. Chemicals and antibodies

Pam3CSK4, LPS, LTA, Poly(I:C), and CpG DNA (ODN2006) were purchased from Invivogen (San Diego, CA). p38 inhibitor (SB203580), JNK inhibitor (AEG3482), and ERK inhibitor (U0126) were purchased from Calbiochem (San Diego, CA). Phospho-specific antibodies against ERK1/2, p38, JNK, CREB, STAT1, STAT3, as well as total CREB, iNOS, STAT1, and STAT3 are from Cell Signaling Technology (Beverly, MA). Anti-rabbit IgG secondary antibody was also purchased from Cell Signaling Technology. Anti-goat IgG secondary antibody, phospho-specific antibody against IκBα, as well as total ERK1/2, Cox2, p38, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IL1β antibody was purchased from BioVision (San Francisco, CA).

2.2 Mice and isolation of bone marrow derived macrophages

6 to 12 week-old mice were dissected, and the bone marrow was flushed out. Macrophages were cultured with DMEM media supplemented with 30% L929 supernatant containing macrophage-stimulating factor, glutamine, sodium pyruvate, 10% heat-inactivated Fetal Bovine Serum, and antibiotics for 7 days. BMDMs were re-plated at a density of 1×10^6 cells/well in 12well plates the day before the experiment.

2.3 Protein extraction and immunoblotting

After the appropriate treatments, cells were washed with cold Phosphate Buffered Saline (PBS), and harvested in Radio-immunoprecipitation assay buffer (RIPA) containing protease inhibitor and phosphatase inhibitor cocktails. Equal amounts of proteins (20 μg) were mixed with the proper volume of 5X sample buffer, separated on 10% or 12% SDS-polyacrylamide gel electrophoresis and transferred to Nitrocellulose membrane at 50 V for 150-180 min. The NC membrane was blocked with 5% dry milk in TBST (Tris-buffered saline with 0.1% Tween-20), rinsed, and incubated with primary antibody overnight. The blots were washed and incubated with secondary anti-IgG antibody. Membranes were washed and immuno-reactive bands were visualized using a chemiluminescent substrate (ECL-Plus, GE Healthcare, Pittsburgh, PA).

2.4 Nitrite determination by Griess reaction

The nitrite concentration in the culture media was used as a measure of NO production. After stimulation/incubation, the generation of NO in the cell culture supernatant was determined by measuring nitrite accumulation in the medium using Griess reagent(Sigma). 100μl of culture supernatant and 100μl of Griess reagent were mixed and incubated for 5 min. The absorption was measured in an automated plate reader at 540 nm. Sodium nitrite (NaNO2; Sigma) was used to generate a standard curve for quantification. Background nitrite was subtracted from the experimental values.

2.5 RNA extraction and quantitative reverse transcriptase/real-time PCR

Rinse cell monolayer with ice cold PBS once. Lyse cells directly in a culture dish by adding 500μl of TRIZOL Reagent per well of 12well plate and pass the cell lysate several times through a pipette. Add 100μl of chloroform and centrifuge the samples at 13000rpm for 20 minutes at 4℃. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing RNA. Precipitate the RNA from the aqueous phase by mixing with equal amount of isopropyl alcohol and centrifuge(13000rpm, 20min, 4℃). Wash the RNA pellet once with 75% ethanol, air-dry, then dissolve RNA in DEPC-treated water to create cDNA for qRT-PCR analysis. Using Gapdh as a house-keeping gene control, CFX Connect Real-time system (BioRad) was used to analyze mRNA level.

3. Results

3.1 ERK-dependent negative regulation of TLR2 induced response under Pam3CSK4 challenge

3.1.1 ERK negatively regulates TLR2 induced secretion of iNOS and NO in BMDMs.

Previous studies focused on LPS-induced TLR4 downstream signaling and its relationship to ERK in BMDMs (Bouhamdan et al. 2015). In order to investigate the credibility of previous observations and contrast it with the acquired results regarding Pam3CSK4-induced TLR2 downstream signaling, I constructed the following experiment. Since one of the main ways macrophages use to attack pathogens is by nitric oxide production (Christian Bogdan et al. 2000), I first focused on the production of NO in relation to iNOS (Fugen Akten 2004). I cultured wild type BMDMs for a week and analyzed iNOS production in response to LPS and Pam3CSK4 along with the presence and absence of U0126. BMDMs were treated beforehand with U0126 (10 mM) for 30 min, and then LPS or Pam3CSK4 was applied for 24 hours. iNOS was assessed using Western blotting and nitric oxide in the conditioned media was measured using Griess assay. The western blot (Figure 1B) showed drastic increase in Pam3CSK4-induced expression of iNOS in the presence of U0126 compared to sole treatment of Pam3CSK4, certifying negative regulation via ERK. Moreover, NO production level was comparable in LPS-challenged BMDMs with or without U0126 (Figure 1A), matching the results of previous studies (Bouhamdan et al. 2015) and illustrated significant increase of NO release in Pam3CSK4+U0126 treated BMDMs in comparison to BMDMs treated with only Pam3CSK4 (Figure 1A). In brief, the

Western blot and Griess assay results validated that ERK negatively regulates secretion of iNOS and NO by Pam3CSK4-triggered TLR2.

3.1.2 ERK negatively regulates TLR2 induced transcription and positively regulates TLR4 induced transcription of TNFα, IL-6, Cox2, and IL1β.

Previous studies showed decrease of TNFα and IL-6 protein level using ELISA when LPS challenged TLR4 downstream pathway was inhibited by U0126 (Bouhamdan et al. 2015). RT-PCR results displayed mRNA expression levels relative to Gapdh (Figure 2) that were in agreement with the ELISA results of previous studies (Bouhamdan et al. 2015). In other words, TNFα, IL-6, Cox2, and IL1β exhibited decreased levels of mRNA expression when U0126 pre-treatment inhibited the LPS-triggered TLR4 downstream pathway, proving ERK's positive regulation of TLR4 induced transcription. Furthermore, Pam3CSK4 activated TLR2 induced transcription of pro-inflammatory cytokines was upregulated when ERK was blocked (Figure 2). Therefore, ERK negatively controls TLR2 induced transcription under Pam3CSK4 challenge. Overall, ERK negatively regulates TLR2 induced transcription and positively regulates TLR4 induced transcription of pro-inflammatory cytokines: TNFα, IL-6, Cox2, and IL1β.

3.2 Analysis of ERK-dependent regulation upon TLR downstream pathways

3.2.1 ERK-dependent negative regulation of Pam3CSK4-triggered TLR2 pathway does not signal via MEK1/2 and p-STAT.

In order to confirm the IL-10 regulation pathway through ERK and subsequent STAT phosphorylation (Bouhamdan et al. 2015), I assessed phosphorylation of STAT1, STAT3, and ERK in response to LPS. BMDMs were treated with LPS

for different time points up to 8 hours to assess STAT1 (Tyr701) and STAT3 (Tyr705) phosphorylation. As in previous studies, BMDMs treated with or without U0126 showed similar total STAT1 levels, and comparable STAT1 phosphorylation in the case of U0126 treatment (Figure 3A). Likewise, BMDMs treated with U0126 responded to LPS challenge with similar STAT3 phosphorylation and comparable total STAT3 levels (Figure 3A). It was possible that contrasting phosphorylation patterns of STAT led to the contrasting transcription results by LPS and Pam3CSK4. However, the assumption that Pam3CSK4 triggered TLR2 took the same pathway of LPS was incorrect. The Western blot results exhibiting barely induced p-STAT1 and p-STAT3 expression (Figure 3A) proved that Pam3CSK4 triggered TLR2 downstream signaling took a divergent path from LPS triggered TLR4 downstream signaling.

3.2.2 ERK-dependent negative regulation of Pam3CSK4-triggered TLR2 pathway does not signal through phosphorylation of p38, JNK, IκBα, and CREB.

Since Pam3CSK4-triggered TLR2 took a differing path from LPS-triggered TLR4 downstream pathway, I tested several potential pathways regarding ERK signaling such as: JNK, p38, CREB, and IκBα (Yoav D. Shaul, Rony Seger 2007). In the Western blot results, p38, JNK, IκBα, and CREB showed similar levels of phosphorylation up to 8 hours when applied with or without U0126 under Pam3CSK4 challenge (Figure 3B). Moreover, phosphorylation patterns of p38, JNK, IκBα, and CREB were also comparable under LPS challenge. Thus, ERK and p-STAT are the dominant factors regulating the LPS-triggered TLR4 downstream pathway. In brief, activation patterns of p38, JNK, IκBα, and CREB did not match the contrasting patterns of transcription, leading to the need for further research of potential factors that may be responsible for the two divergent pathways regarding

Pam3CSK4 and LPS.

3.3 TLR2-specific regulation by ERK

3.3.1 Nitric oxide secretion and mRNA expression levels of TNFα, IL-6, Cox2, and IL1β are negatively regulated by ERK in TLR2 specific manner under Pam3CSK4 challenge.

Finally, I verified that the negative regulation mediated by ERK is TLR2 specific. I treated BMDMs with TLR2 specific ligands: Pam3CSK4 and LTA, and contrasted NO level and mRNA expression level with ligands of TLR3 (Poly (I:C)), TLR4 (LPS), and TLR9 (ODN2006) (Figure 4). Ligands of TLR3 and TLR9 resulted in minimal expression of NO (Figure 4A) and basal mRNA expression of TNFα, IL-6, Cox2, and IL1β (Figure 4B). Therefore, these results collectively indicate that the hypothesis: ERK specifically targets and negatively regulates TLR2-induced transcription of TNFα, IL-6, iNOS, Cox2, and IL1β under Pam3CSK4 challenge is most likely true. However, additional experimentation of various ligands of the TLR ligand family is needed to strengthen the hypothesis. Moreover, further research is required to clarify the exact reason for LTA resulting in comparably low levels of nitric oxide and mRNA expression of pro-inflammatory cytokines. Overall, ERK dependent negative regulation of NO secretion and mRNA expression of pro-inflammatory cytokines: TNFα, IL-6, Cox2, and IL1β is a unique phenomenon restricted to TLR2 triggered by Pam3CSK4.

4. Discussion

TLR has been acknowledged as a key factor linked to the prevention and treatment of cancer and inflammatory diseases: rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus (Stefan K. Drexler, Brian M. Foxwell 2010). Moreover, inhibitors of TLR2, TLR4, TLR7, and TLR9 are being selected for preclinical and clinical trials (Elizabeth J. Hennessy, Andrew E. Parker and Luke A. J. O'Neill 2010). Thus, here, I provide additional insight into the TLR downstream signaling pathway which is crucial in the capability of regulating TLR activity without triggering unwanted side effects.

LPS triggers TLR4 to pass on signaling to Myd88 and TRIF (Akira S., Takeda K. 2004). Moreover, it has been shown that LPS-induced expression of iNOS in macrophages is positively regulated by Src-family tyrosine kinases via the TRIF-dependent signaling pathways of TLR4 (Hwang DH et al. 2005). Thus, ERK dependent regulation of iNOS can be determined only when TRIF knockout mice is available. Nevertheless, it is clear that Pam3CSK4-induced expression of iNOS is negatively regulated via ERK since, the western blot (Figure 1B) shows drastic increase in iNOS when comparing the Pam3CSK4 and Pam3CSK4+ERK inhibitor (U0126) lanes. iNOS mRNA RT results showed minimum reading at the 24hr time point (data not shown). It is likely that iNOS mRNA fluctuated between the 24hr time point and requires additional time-frame based collection of Pam3CSK4+U0126 mRNA samples to visualize the increase of iNOS mRNA expression leading to the confirmed increase in the secretion of iNOS. Furthermore, NO level does not seem to have significant difference in LPS and LPS+U0126 samples (Figure 1A). This is most likely due to LPS signaling via TRIF affecting iNOS signaling, therefore, also needs TRIF knockout mice for

confirmation.

In comparison with iNOS, the mRNA levels of TNFα, IL-6, Cox2, and IL1β show obvious contrasting results between LPS and Pam3CSK4 (Figure 2). In brief, LPS+U0126 mRNA levels of the four pro-inflammatory cytokines: TNFα, IL-6, Cox2, and IL1β indicate decreased expression compared to mRNA levels of samples applied with only LPS, which are in agreement with results provided in previous studies (Bouhamdan et al. 2015). While, Pam3CSK4+U0126 mRNA levels of the cytokines reveal increased expression as to mRNA levels of samples applied solely with Pam3CSK4.

The way that Pam3CSK4 shows contrasting cytokine level patterns to LPS is likely by signaling through different transcription factors rather than STAT. Considering previous studies that validate phosphorylation variations of STAT is the signaling mechanism downstream of LPS triggered TLR4 and ERK (Bouhamdan et al. 2015), and the fact that Pam3CSK4 barely induced any p-STAT expression (Figure 3A), I tested several potential pathways regarding ERK signaling such as: JNK, p38, CREB, and IκBα (Yoav D. Shaul, Rony Seger 2007) to confirm the hypothesis above. None of them showed significant difference in the level of phosphorylation when applied with U0126 (Figure 3B). Further investigation is required to find the factor responsible for the differing transcription patterns of pro-inflammatory cytokines induced by LPS and Pam3CSK4 under ERK-dependent regulation.

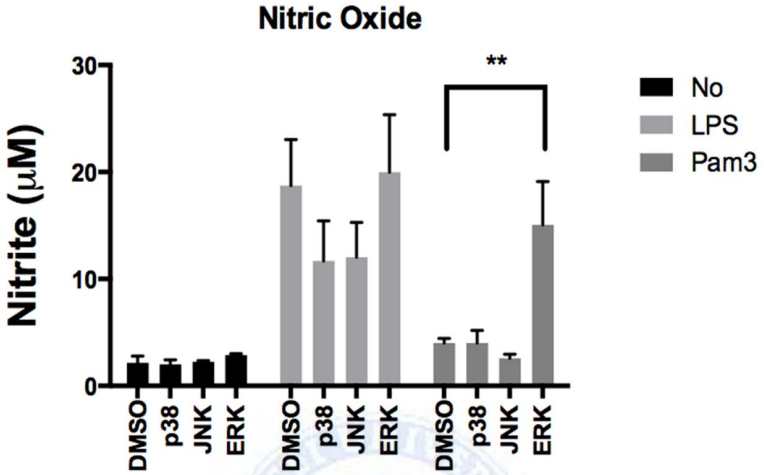
Due to the lack of Mek1(d/d) Sox2 (Cre/+) mice (Bouhamdan et al. 2015) for in vivo experimentation, I attempted to create a MEK1/2 deficient cell line using the CRISPR-CAS9 system on macrophage-like cell lines: J774A.1, Raw264.7, and iBMDM. However, none of the cell lines showed increased mRNA expression level or nitric oxide secretion when comparing treatment with both Pam3CSK4 and U0126 to sole treatment of Pam3CSK4 (data not shown). Therefore, it is necessary

to acquire Mek1/2 deficient mice for in vivo experimentation.

In final, I attempted to certify that the ERK-dependent negative regulation of cytokine mRNA level expression and NO secretion is TLR2 specific. Griess assay and RT-PCR results verified that Pam3CSK4 induced TLR2 specific responses via ERK (Figure 4). However, LTA showed basal level of expression. The fact that LTA-induced expression of genes are substantially delayed compared to other TLR2 ligands (Elizabeth M. Long et al. 2009) and that a murine model of acute inflammation showed Pam3CSK4's capability to stimulate robust leukocyte recruitment in vivo, while LTA remained functionally inert (Elizabeth M. Long et al. 2009), explains the basal mRNA and NO readings when BMDMs were treated with LTA and collected at the 24hr time point. In short, the underlying reasons for basal readings regarding LTA may be due to the prolonged period required for LTA stimulation or an unknown mechanism in which LTA fails to initiate inflammatory responses.

Overall, I acquired a partial structure of the pathway (Figure 5) resulting in the contrasting transcription patterns of pro-inflammatory cytokines due to LPS-induced TLR4 signaling and Pam3CSK4-induced TLR2 signaling. This structure provides insight into the regulation mechanisms of TLR downstream signaling. All in all, this paper provides further comprehension in the designing of drugs regulating TLR activity, which is connected to inflammatory diseases and cancer.

A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Nitrite (μM)
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20
10
0
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No
LPS
Pam3
DMSO
p38
JNK
ERK
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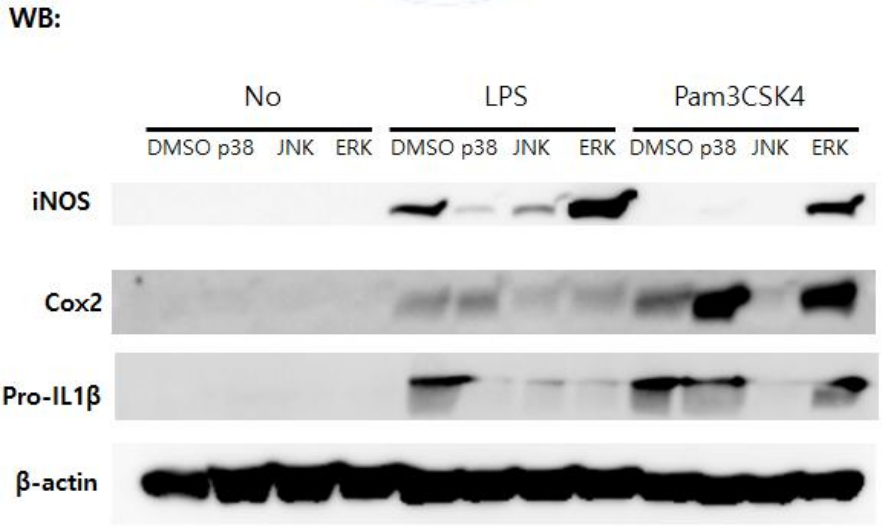
p38
JNK
ERK
DMSO
p38
JNK
ERK

B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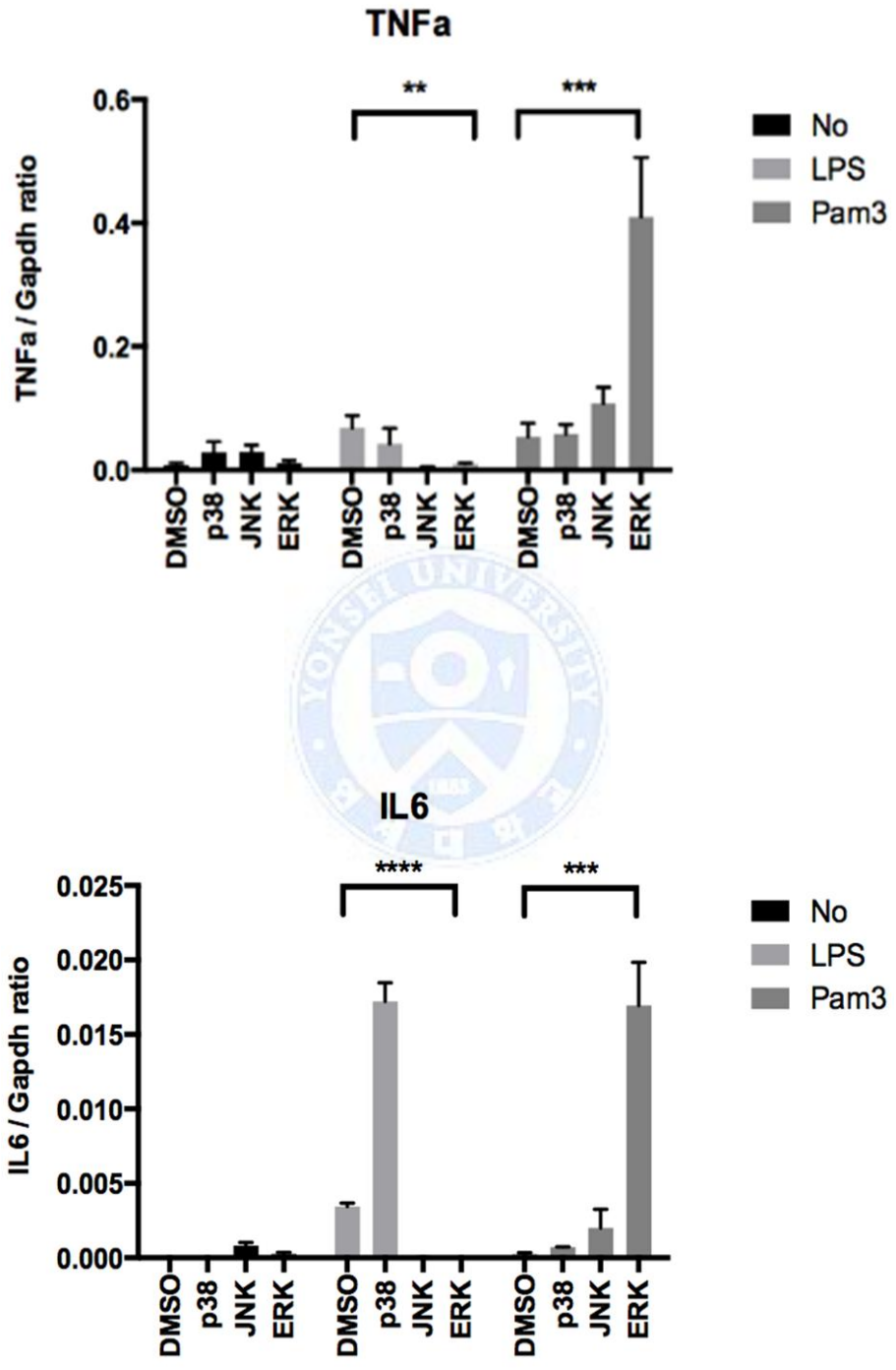
No
LPS
Pam3CSK4
DMSO p38 JNK ERK DMSO p38 JNK ERK DMSO p38 JNK ERK
iNOS
Cox2
Pro-IL1β
β-actin

Fig.1 Pam3CSK4 activated ERK negatively regulates iNOS and nitric oxide production. BMDMs derived from WT mice were treated with LPS(100ug/mL) and Pam3CSK4(1mg/mL) for 24 h, along with p38 inhibitor(SB203580), JNK inhibitor(AEG3482), or ERK inhibitor(U0126) at a concentration of 10mM. (A) The nitrite concentration in the culture medium was used as a measure of NO production. After stimulation/incubation, the generation of NO in the cell culture supernatants was determined by measuring nitrite accumulation in the medium using Griess reaction. Data are presented as mean of three independent experiments. Using unpaired t-test, a p value<0.05 was considered significant and error bars indicate SD. (B) Whole cell extracts were prepared and 20 μg total protein was subjected to SDS-PAGE and Western blot analysis using antibodies for iNOS, Cox2, and IL1β. Equal loading was confirmed by total β-actin antibody.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TNFa / Gapdh ratio
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0.2
0.0
**
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JNK
ERK
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IL6
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0.020
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0.010
0.005
0.000
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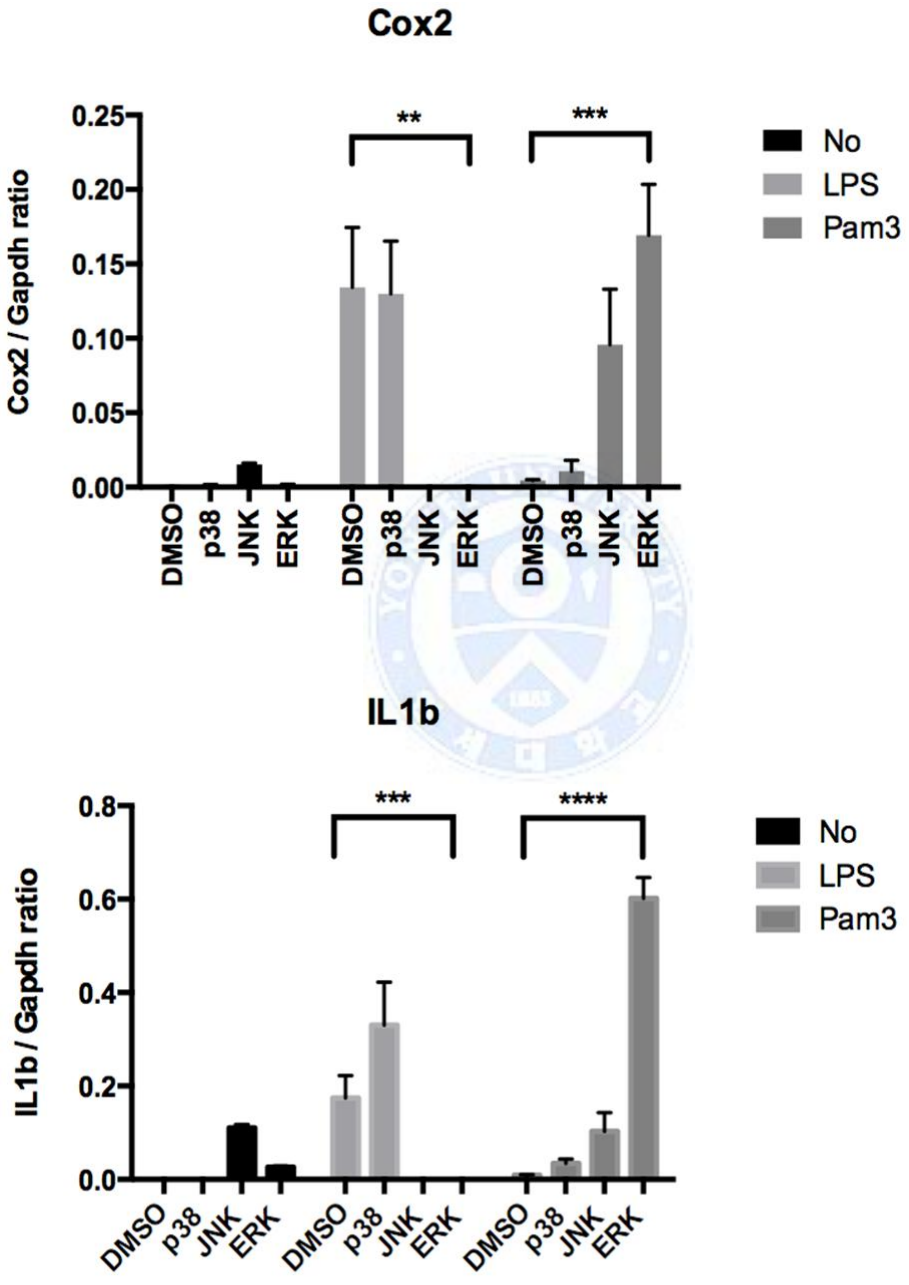
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Cox2 / Gapdh ratio
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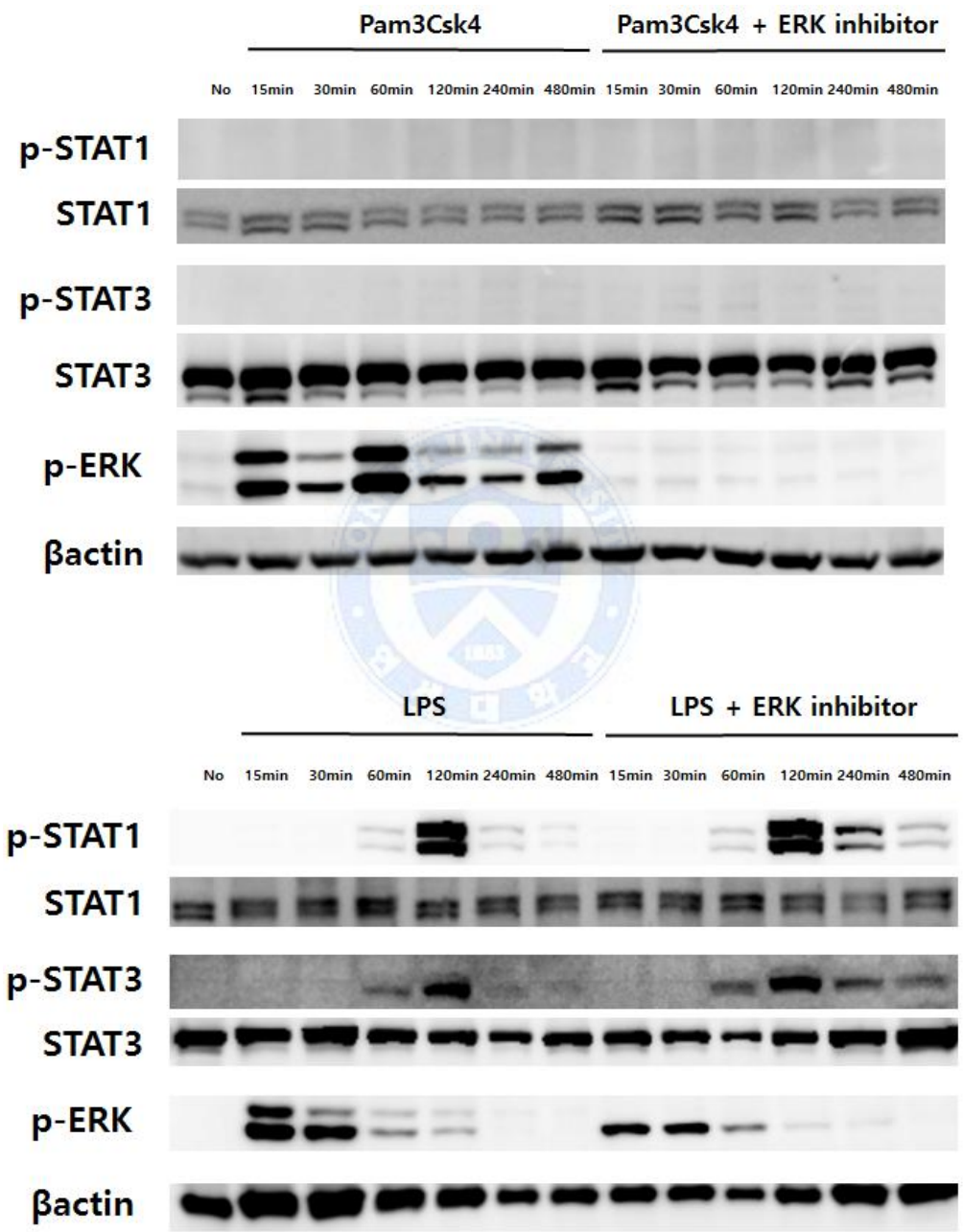
0.20
0.15
0.10
0.05
0.00
**
***
DMSO
p38
JNK
ERK
No
LPS
Pam3
IL1b
IL1b / Gapdh ratio
0.8
0.6
0.4
0.2
0.0
***
****

Fig.2 ERK-dependent negative regulation of pro-inflammatory cytokines in response to Pam3CSK4. BMDMs derived from WT mice were treated with LPS(100ug/mL) and Pam3CSK4(1mg/mL) for 24 h, along with p38 inhibitor(SB203580), JNK inhibitor(AEG3482), or ERK inhibitor(U0126) at a concentration of 10mM. Using Gapdh as a house-keeping gene control, CFX Connect Real-time system (BioRad) was used to analyze mRNA level. Data are presented as mean of three independent experiments. Using unpaired t-test, a p value<0.05 was considered significant and error bars indicate SD.

A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Pam3Csk4 + ERK inhibitor
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p-STAT1
STAT1
p-STAT3
STAT3
p-ERK
βactin
LPS
LPS + ERK inhibitor
No 15min 30min 60min 120min 240min 480min 15min 30min 60min 120min 240min 480min
p-STAT1
STAT1
p-STAT3
STAT3
p-ERK
βactin

B

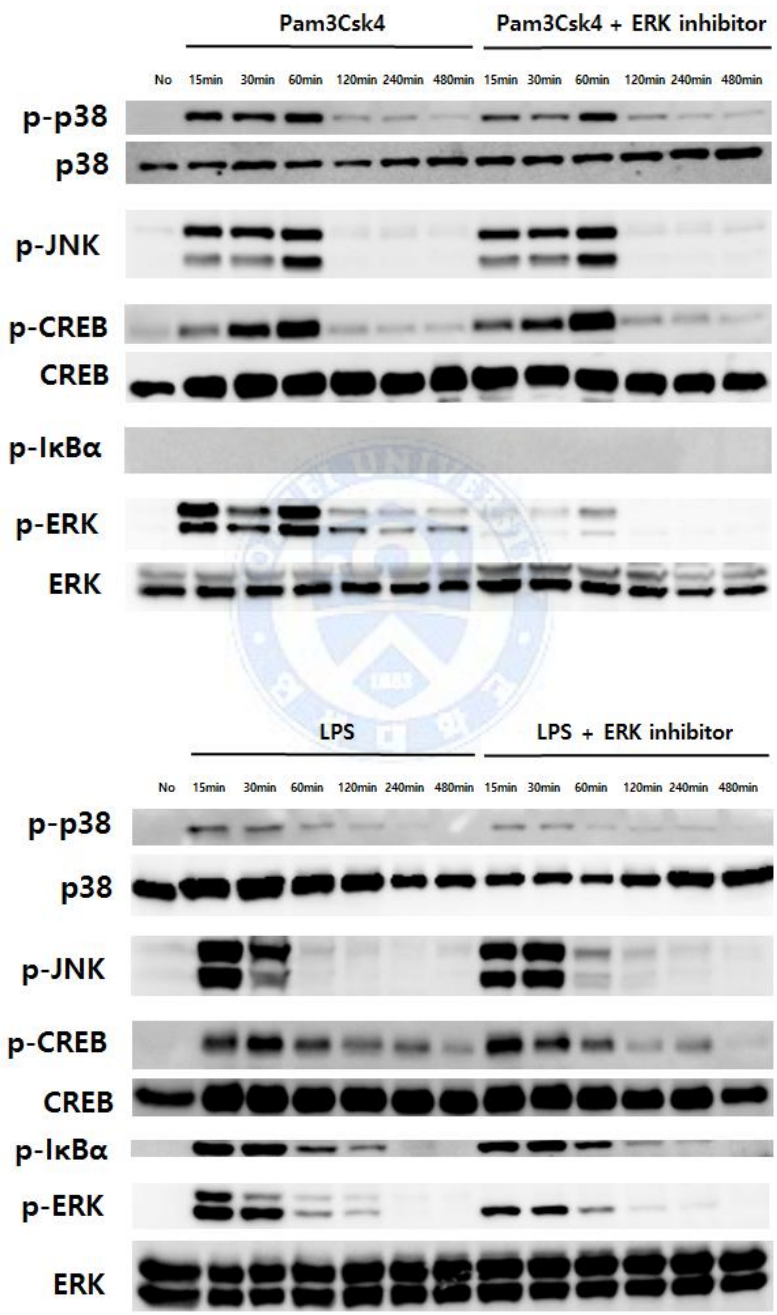
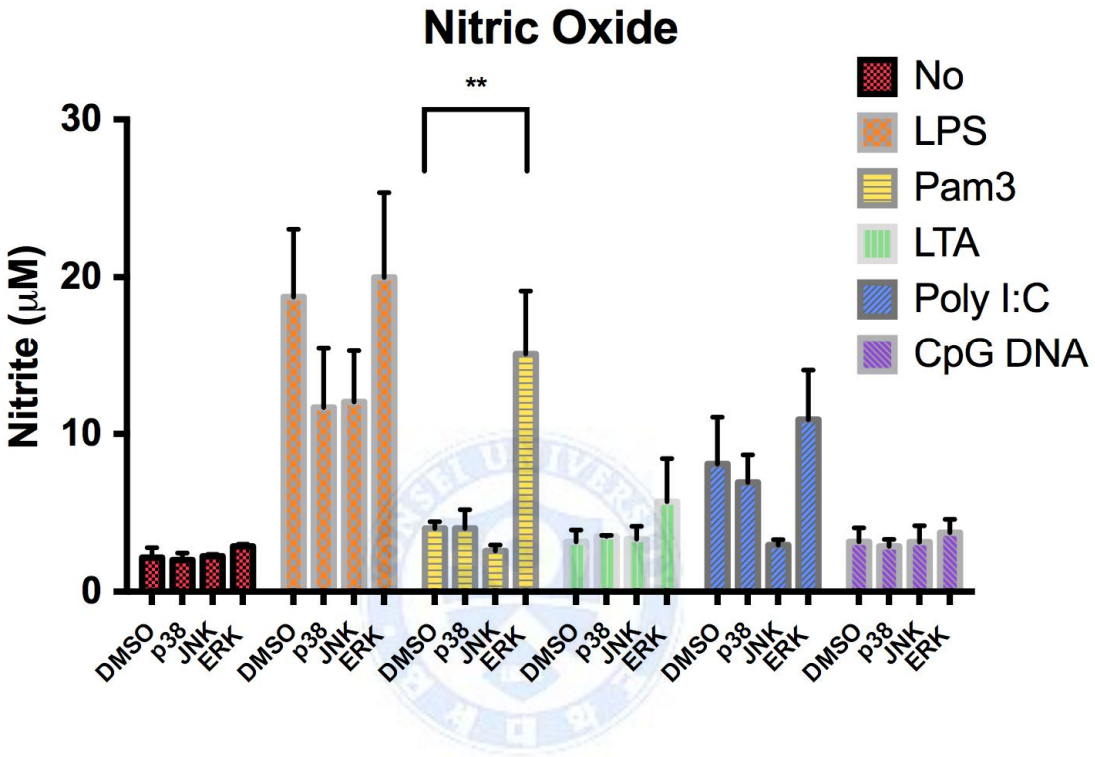
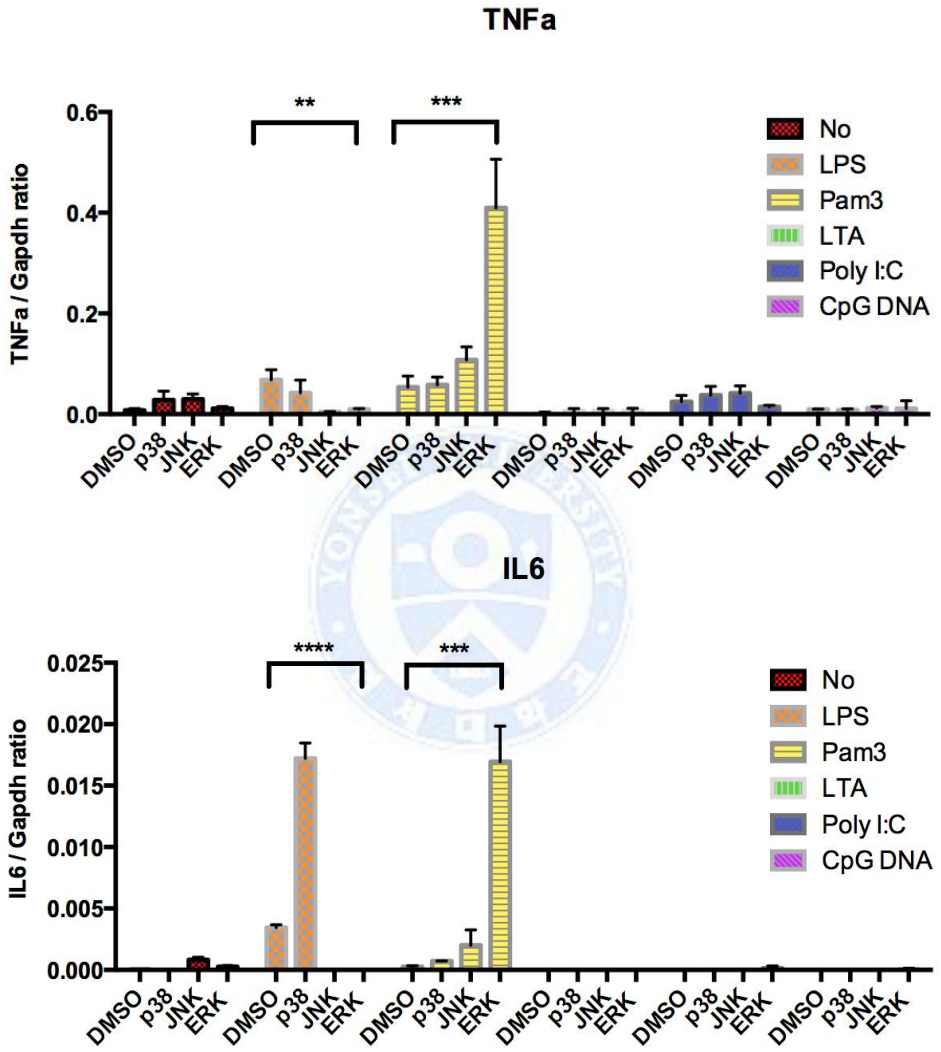

Pam3Csk4
Pam3Csk4 + ERK inhibitor
No 15min 30min 60min 120min 240min 480min 15min 30min 60min 120min 240min 480min
p-p38
p38
p-JNK
p-CREB
CREB
p-IκBα
p-ERK
ERK
LPS
LPS + ERK inhibitor
No 15min 30min 60min 120min 240min 480min 15min 30min 60min 120min 240min 480min
p-p38
p38
p-JNK
p-CREB
CREB
p-IκBα
p-ERK
ERK

Fig.3 Pam3CSK4 via ERK pathway does not involve p-STAT, p-p38, p-JNK, p-CREB, and p-IκBα. Whole cell extracts were prepared and 20 μg total protein was subjected to Western blot analysis. (A) Phospho-specific antibodies for STAT1 (Tyr701), STAT3 (Tyr705), and ERK (Thr202/Tyr204) were used. Equal loading was confirmed by total STAT1, STAT3, and β-actin antibody. (B) Detection of phospho-specific antibodies for p38 (Thr180/Tyr182), JNK (Thr183/Tyr185), CREB (Ser133), IκBα (Ser32), and ERK (Thr202/Tyr204) in response to Pam3CSK4 and LPS treatment for different time points are shown. Equal loading was determined using antibodies against total p38, CREB, and ERK.

A


Nitric Oxide
**
Nitrite (μM)
30
20
10
0
No
LPS
Pam3
LTA
Poly I:C
CpG DNA
DMSO
p38
JNK
ERK

B


TNFa
TNFa / Gapdh ratio
0.6
0.4
0.2
0.0
**
***
No
LPS
Pam3
LTA
Poly I:C
CpG DNA
DMSO
p38
JNK
ERK
IL6
IL6 / Gapdh ratio
0.025
0.020
0.015
0.010
0.005
0.000
****
***

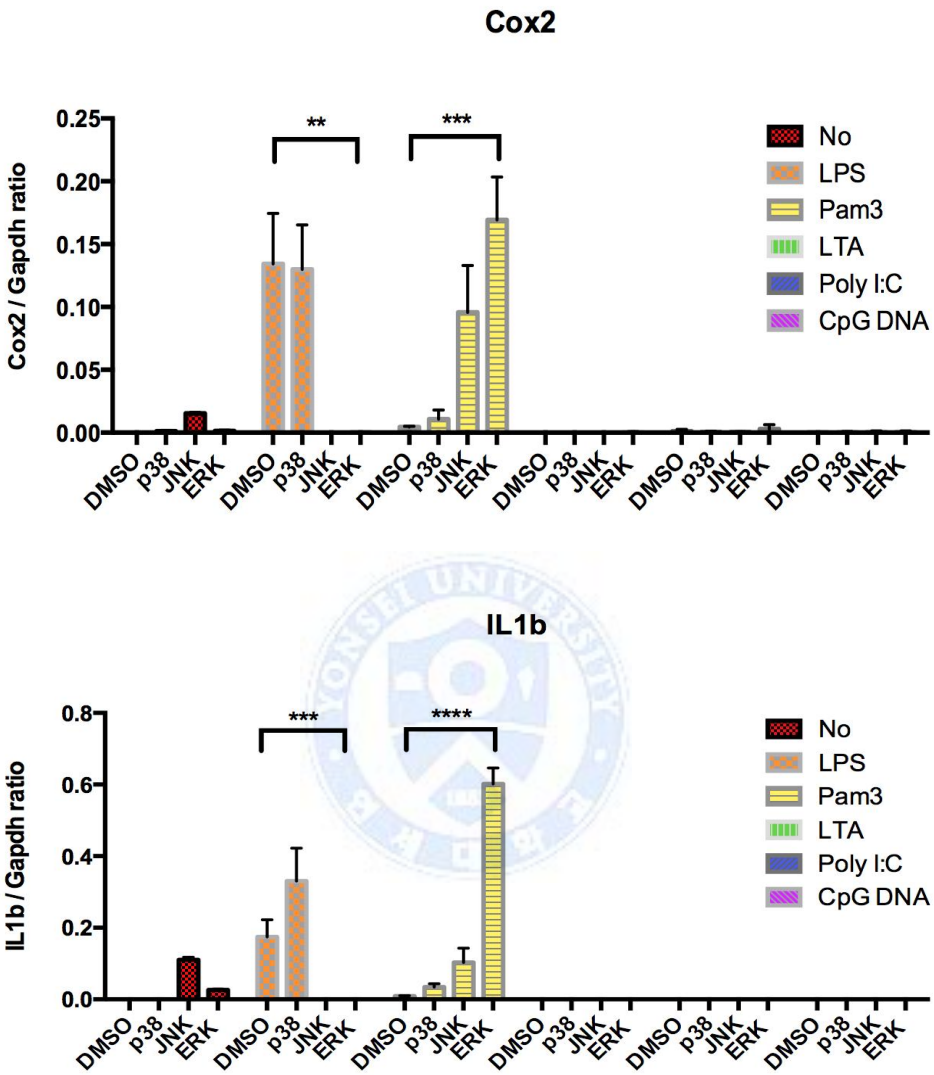

Cox2
Cox2 / Gapdh ratio
0.25
0.20
0.15
0.10
0.05
0.00
**
***
No
LPS
Pam3
LTA
Poly I:C
CpG DNA
DMSO
p38
JNK
ERK
IL1b
IL1b / Gapdh ratio
0.8
0.6
0.4
0.2
0.0
***
****

Fig. 4 ERK-dependent negative regulation of nitric oxide and pro-inflammatory cytokines are TLR2 specific under Pam3CSK4 challenge. BMDMs derived from WT mice were treated with LPS(100ug/mL) and Pam3CSK4(1mg/mL) for 24 h, along with p38 inhibitor(SB203580), JNK inhibitor(AEG3482), or ERK inhibitor(U0126) at a concentration of 10mM. (A) The nitrite concentration in the culture medium was used as a measure of NO production. After stimulation/incubation, the generation of NO in the cell culture supernatants was determined by measuring nitrite accumulation in the medium using Griess reaction. Data are presented as mean of three independent experiments. Using unpaired t-test, a p value<0.05 was considered significant and error bars indicate SD. (B) Using Gapdh as a house-keeping gene control, CFX Connect Real-time system (BioRad) was used to analyze mRNA level. Data are presented as mean of three independent experiments. Using unpaired t-test, a p value<0.05 was considered significant and error bars indicate SD.

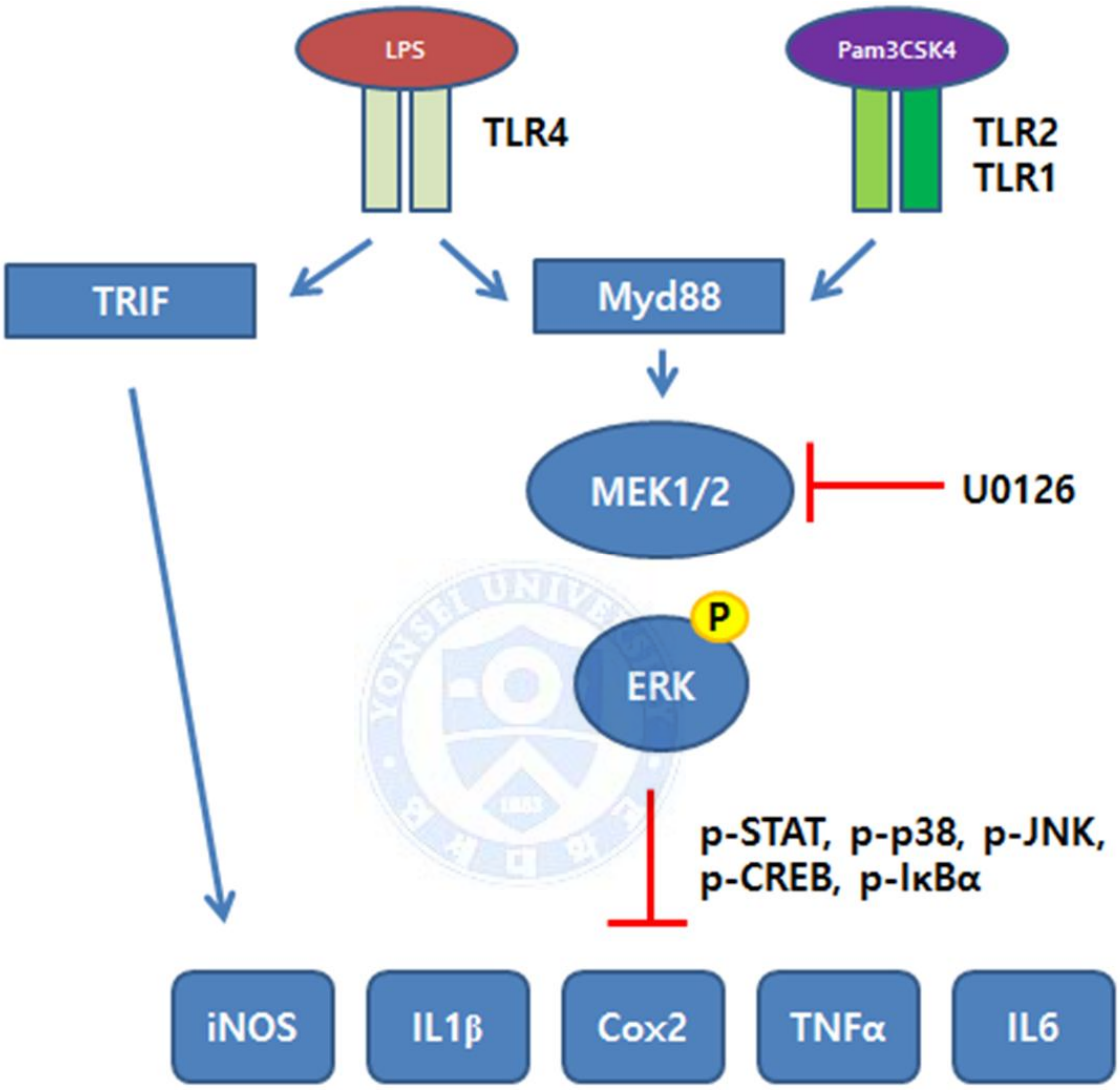

LPS
TLR4
Pam3CSK4
TLR2
TLR1
TRIF
Myd88
MEK1/2
U0126
P
ERK
p-STAT, p-p38, p-JNK,
p-CREB, p-IκBα
iNOS
IL1β
Cox2
TNFα
IL6

Fig. 5 Schematic of LPS and Pam3CSK4 stimulated pathways leading to the contrasting patterns of ERK-dependent regulation.

Gene	Primer sequence
TNF-alpha q1F	ATGTCCATTCCTGAGTTCTG
TNF-alpha q1R	AATCTGGAAAGGTCTGAAGG
IL-1 beta q1F	TCAACCAACAAGTGATATTCTC
IL-1 beta q1R	ACACAGGACAGGTATAGATTC
IL-6 q1F	CCAAGACCATCCAATTCATC
IL-6 q1R	CCACAAACTGATATGCTTAGG
Ptgs2(Cox2) q1F	GCCCGTGCTGCTCTGTCTTAAC
Ptgs2(Cox2) q1R	GTTGCTCTAGGCTTTGCTGGCTAC
Gapdh_F	GGCAAATTCAACGGCACAGTCAAG
Gapdh_R	TCGCTCCTGGAAGATGGTGATGG
iNOS_F	AATCTTGGAGCGAGTTGTGG
iNOS_R	CAGGAAGTAGGTGAGGGCTTG

Table 1. Forward and reverse primers used for RT-PCR

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Abstract in Korean

쥐 골수세포에서 ERK의 TLR2 관련 cytokine인 TNFa, IL-6, iNOS, Cox2, IL1β에 대한 음성조절 기작

ERK는 mitogen-activated protein kinase의 한 종류로 TLR activation과 이와 관련된 cytokine production에 중요 역할을 하는 것으로 밝혀졌다. 위와 같은 사실을 기재한 논문은 LPS에 의한 TLR4 induced transcription과 ERK사이의 상관관계에 초점을 맞췄으나, 이 논문은 ERK와 Pam3CSK4로 인한 TLR2 induced transcription 사이의 관계를 규명하고자 한다. 더 나아가, 기존 논문에 기록된 결과들을 재확인하고, TNFa, IL-6, iNOS, Cox2, IL1β와 같은 pro-inflammatory cytokine들의 변화가 ERK dependent한지 여부와 TLR2 downstream을 ERK가 negatively regulate하는 pathway를 확인하는 것이 최종 목적이다. ERK dependency를 확인하기 위해서는 ERK에 대한 검증된 inhibitor가 없는 관계로, ERK를 직접적으로 regulate하는 것으로 알려진 upstream factor인 MEK에 대한 inhibitor를 이용하여 실험을 setting한 후, 각각의 cytokine에 대해 Western Blotting을 통한 protein level과 RT-PCR을 통한 mRNA level 변화 경향성에 대한 자료를 얻었다. 더 나아가 ERK가 TLR2 downstream을 조절하는 지점을 찾기 위해 기존 논문에서 명시한 MEK1/2와 p-STAT 관련 기작은 물론, 가능성 있는 p38, JNK, IκBα, CREB의 인산화 변화 정도를 Western Blotting을 이용하여 확인하였다. 추가적으로 in vivo 실험을 위하여 macrophage-like cell line들을 testing하여 MEK1/2에 대한 KO cell line을 얻으려고 시도하였다. 마지막으로 TLR2 specific한 ligand인 Pam3CSK4를 통해 얻은 결과가 TLR3, TLR4, TLR9에서는 일어나는지 않을 것이라는 가설을 검증하였다. 종합하자면, 위의 자료들은 ERK의 TLR2 관련 pro-inflammatory cytokine들에 대한 음성조절 기작을 규명함으로써 TLR 관련 기작을 조절하여 염증성 질환과 암을 치료하는 약을 제조하는데 도움을 줄 수

있을 것이다.

핵심 단어: Pam3CSK4, LPS, TLR2, TLR4, ERK, cytokine