

Lithium Chloride Induces TNF α in Mouse Macrophages Via MEK–ERK–Dependent Pathway

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ABSTRACT

Lithium (Li) is one of the currently prescribed drugs for bipolar disorders (BPDs) and has many neuro-regulatory and immune-modulating properties. Because many neuro-pathological diseases including BPDs have been associated with some level of inflammation, Li's effect on inflammation may have some crucial consequences. Even though Li has been shown to have pro- and anti-inflammatory activities in different cell models, mechanisms involved in these effects are not well understood. Moreover, Li's effect on inflammation in the presence of activators of Toll-like receptors (TLRs), especially TLR-2 (that activates MyD88-dependent pathway) and TLR-3 (that activates TRIF-dependent pathway) is not known. Here we tested the role of Li in the presence and absence of TLR2, and TLR3 on MAPK and NF κ B pathways and the consequent production of tumor necrosis factor- α (TNF α) in Raw264.7 macrophages. Our results indicate that Li enhances TNF α production both in the absence and presence of TLR stimulation. Interestingly, Li differentially modulates MAPK and NF κ B pathways in the absence and presence of TLR2/3 ligands. Our results further indicate that the effect of Li on TNF α occurs at the post-transcriptional level. Together, these studies demonstrate that Li induces TNF α production in macrophages and that it modulates signaling at different levels depending on the presence or absence of TLR2/3 stimulation. *J. Cell. Biochem.* 115: 71–80, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: TNF α ; TLR2; TLR3; MACROPHAGE

Macrophages are innate immune cells with a tremendous ability to be plastic and heterogeneous depending on the tissue environment [Murray and Wynn, 2011]. These cells act as the first line of defense against invading microorganisms and have the capability to modulate a number of different immune functions including innate and adaptive immunity. By virtue of these abilities macrophages are thought to be central players in a number of inflammatory diseases [Sica and Mantovani, 2012]. Macrophages as well as other immune cells express pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns from the microbes as well as certain endogenous protein molecules [Moresco et al., 2011]. Activation of PRRs have been linked to the pathogenesis of a number of diseases caused by microbial as well as non-microbial sources. Among the PRRs, toll-like receptors (TLRs) play a critical role in inflammatory process in a diverse range of metabolic and non-metabolic diseases. TLRs are type-I membrane glycoprotein PRRs present on the cell membrane as well as the

intracellular membrane compartments. At present, 13 TLRs have been identified in humans and mice combined [Moresco et al., 2011].

Activation of TLRs and inflammatory responses have now been recognized to be important not only for microbial infections but also for many different disease processes including neuro-pathological diseases [Dantzer et al., 2008]. The nervous system has long been shown to influence inflammatory responses via neurotransmitter release and activation of the respective receptors on the surface of immune cells. Alternatively, the immune system has also been shown to influence psychological status including mood and behavior [Dantzer et al., 2008]. Several studies have examined the relationship between inflammatory markers, including cytokines and psychiatric disorders such as major depression and schizophrenia. While studies suggest an association between major depression and enhanced pro-inflammatory cytokines such as tumor necrosis factor- α (TNF α), whether cytokines play causative role remains somewhat controversial [Kenis and Maes, 2002]. In addition, effects of anti-depressants on

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cytokine levels have not been consistent between different studies [Kenis and Maes, 2002]. Lithium (Li) is one such pharmacological agent that has been shown to possess both pro- and anti-inflammatory properties [Shenkman et al., 1978; Kleinerman et al., 1989; Beyaert et al., 1991b; Martin et al., 2005; De Sarno et al., 2008; Chiu and Chuang, 2010].

Li is a monovalent cation and is a psychotropic anti-depressant drug that has been the standard pharmacological treatment for bipolar disorder (BPD) for more than five decades [Chiu and Chuang, 2010]. Yet, the precise molecular mechanisms of its therapeutic actions remain elusive. Several *in vivo* and *in vitro* studies have demonstrated that Li mediates a number of effects including regulation of receptors, ion transport, intracellular signaling, hormonal regulation as well as gene expression [Chiu and Chuang, 2010]. Because of its therapeutic effects, Li has been examined for its effects on various cytokines including TNF α in human monocytes as well as other immune cell models [Kleinerman et al., 1989; Wu and Yang, 1991; Kucharz et al., 1993; Maes et al., 1999; Szuster-Ciesielska et al., 2003]. How Li stimulates TNF α in macrophages and whether this could be modulated by TLR ligands other than lipopolysaccharide (LPS) is still not clear. We demonstrate here that Li induces TNF α production post-transcriptionally and selectively via the ERK pathway. We further show that in the presence of TLR2 and TLR3 ligands, Li differentially affects MAPK and NF κ B pathways and augments TNF α production via multiple MAPK and NF κ B pathways.

MATERIALS AND METHODS

MATERIALS

Ultra pure LPS, PolyI:C, and Pam3CSK4 were from Invivogen. Protease inhibitor cocktail tablet was from Roche. Monoclonal anti- α -tubulin antibody (mouse IgG1 isotype) was from Sigma. pERK1/2, pJNK, pP38, I κ B α , pP105 and pMEK antibodies were from Cell Signaling. ERK2 antibody was from Santa Cruz Biotechnology. Li chloride and sodium chloride were from Sigma Aldrich. TNF α ELISA kit was from eBiosciences. All other reagents were of the highest quality available.

CELL CULTURE

Raw264.7 macrophages were from ATCC and were cultured in DMEM with 10% FBS and penicillin-streptomycin at 37°C and 5% CO₂. For experiments cells were plated in 12-well plates. Twenty-four hours after plating, cells were treated with NaCl or LiCl at the indicated concentrations and time points followed by TLR2 and TLR3 ligands at the indicated concentrations and time points. Samples for ELISA, Western blotting and RNA were obtained accordingly.

WESTERN BLOTTING

After the various treatment protocols as indicated in the figure legends, cells were quickly washed with cold PBS followed by lysis with buffer containing 1% Triton X-100, protease and phosphatase inhibitors. The lysates were then clarified and protein concentration determined using Bradford assay. Equivalent amounts of protein were loaded on the gels for Western blot analysis. Immunoblotting was performed as described previously [Patil et al., 2010]. Secondary antibodies were IR dye conjugated and analyzed by Licor's Odyssey. The bands were quantified using Licor's Odyssey program.

ELISA

TNF α levels in the culture supernatants were analyzed using ELISA kit from eBiosciences following manufacturer's recommendations and as described previously [Porter et al., 2010].

RNA AND Q-PCR

RNA was isolated from the cells using Qiagen RNeasy mini kit using manufacturer's protocol and as described before [Packiriswamy et al., 2013]. Reverse transcription was performed using 1 μ g of RNA with the Promega cDNA synthesis kit. Q-RT-PCR was performed with ABI fast 7500 (Applied Biosystems) and genes were normalized to HPRT. Following primers were used: TNF α -forward: TCTCAT-CAGTTCTATGGCCC-3; reverse: GGGAGTAGACAAGCTACAAC; HPRT-forward: AAG CCT AAG ATG AGC GCA AG; reverse: TTA CTA GGC AGA TGG CCA CA.

ANALYSIS OF TLR EXPRESSION BY FLOW CYTOMETRY

After the respective treatments, the cells were rinsed with staining buffer (1% FBS and 0.09% sodium azide in PBS) and stained with the following antibodies: anti-TLR4 (MTS510, BD Biosciences, San Jose, CA); anti-TLR3 (11F8, Biolegend) and anti-TLR2 (6C2, eBiosciences). Data were acquired using a LSR II (BD Biosciences) and analyzed using Flowjo software (Tree Star, Inc., Ashland, OR) as described before [Patil et al., 2011b].

DATA ANALYSIS

All data are presented as the mean \pm SEM. Experiments were done in multiple passages. Two group comparisons were performed using Student's *t*-test and comparisons of more than two groups were done by ANOVA with post-Bonferroni multiple comparison test. All statistical analysis were performed using GRAPHPAD PRISM Software (San Diego, CA) and *P* < 0.05 were considered statistically significant.

RESULTS

LITHIUM INCREASES TNF α PRODUCTION IN MOUSE MACROPHAGES

To determine the effect of LiCl on TNF α production in mouse macrophage, Raw264.7 cells were treated with control (NaCl) or LiCl at various concentrations ranging from 0.1 to 30 mM for 3 and 6 h. LiCl but not NaCl caused a concentration- and time-dependent increase in TNF α release from Raw264.7 cells at both time points tested (Fig. 1A). To rule out the presence of endotoxin in LiCl, we treated the cells with LiCl in the presence or absence of polymixin B, an endotoxin inhibitor [Duff and Atkins, 1982]. As shown in Figure 1B, Polymixin B did not affect LiCl's effect on TNF α production either at 3 or 6 h. Polymixin-B however completely abrogated LPS-induced TNF α production, confirming Polymixin-B's effect in our system (Fig. 1C).

LITHIUM ENHANCES TLR-INDUCED TNF α PRODUCTION IN MOUSE MACROPHAGES

We then tested the effect of LiCl in the presence of TLR2 and TLR3 ligands (Pam3CSK4 and Poly I:C, respectively). Activation of both of

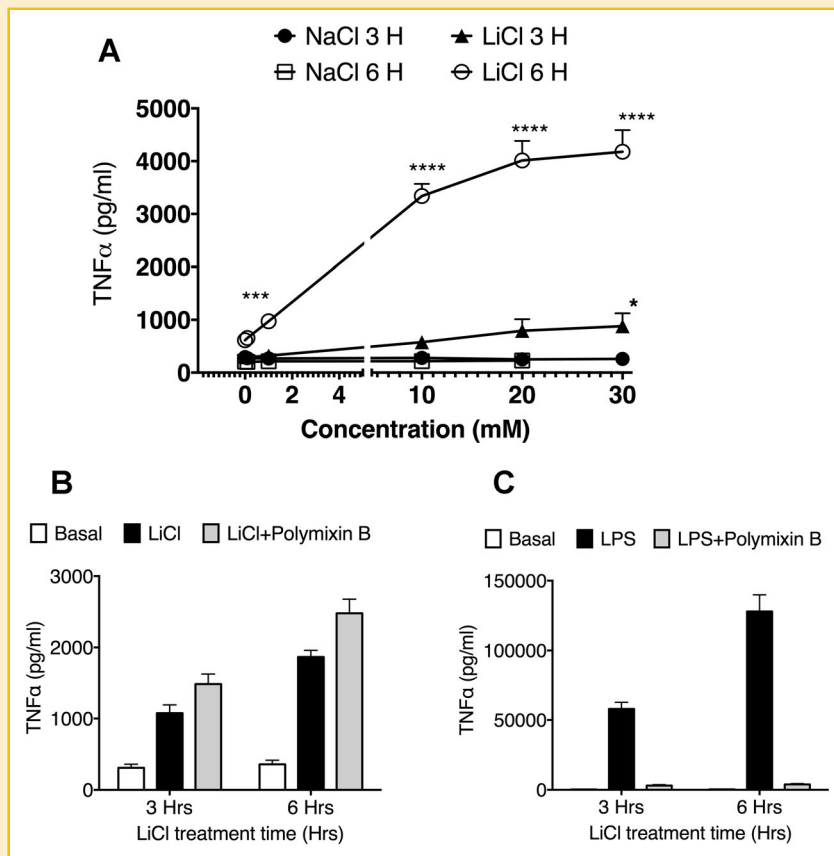


Fig. 1. A. Lithium induces a concentration-dependent increase in TNF α production in Raw264.7 macrophages. Cells were plated in 12-well plates at 1 million cells/well. Twenty-four hours after plating, cells were treated with LiCl or NaCl with the indicated concentrations and time. At the end of the treatment, the supernatants were collected and assayed for TNF α using ELISA kit from eBiosciences. In B and C, cells were treated with polymixin B (50 μ g/ml) along with NaCl, LiCl or LPS. N = 6. ** P < 0.01; *** P < 0.001; **** P < 0.0001; compared between the respective concentrations of LiCl and NaCl.

these receptors normally stimulates signaling via Myd88- and TRIF-dependent pathways, respectively [Kenny and O'Neill, 2008]. We pretreated Raw264.7 macrophages with NaCl/LiCl for 6 h and then stimulated the cells with the two TLR ligands for the various times as shown. Pretreatment with LiCl significantly enhanced Pam3CSK4- and Poly I:C-induced TNF α production (Fig. 2A). We also pretreated cells with various concentrations of NaCl/LiCl for 6 h and then stimulated with the two TLR ligands for another 6 h. As shown in Figure 2B, LiCl enhanced Pam3CSK4- and Poly I:C-induced TNF α production significantly in a dose-dependent manner. Importantly, increasing concentrations of NaCl did not influence TLR2 or TLR3-induced TNF α production. To further determine if the effect of LiCl (without or with TLR stimulation) on TNF α production is mediated at the level of transcription, we examined the mRNA expression of TNF α subsequent to LiCl (\pm TLR2 or TLR3) treatment. As shown in Figure 3A, TNF α expression was not significantly modulated by LiCl either in the absence or presence of TLR stimulation.

LiCl has been shown to mediate its effects via a number of different biochemical mechanisms, the most prominent one being inhibition of GSK3 β [Chiu and Chuang, 2010]. To determine if the effect of LiCl could be mediated via inhibition of GSK3 β , we pretreated macro-

phages with SB216763 (another potent and a more selective GSK3 β inhibitor [Coghlan et al., 2000]). SB216763 neither affected basal TNF α production nor TLR-induced TNF α production suggesting that the effect of LiCl on TNF α production is likely independent of GSK3 β inhibition (data not shown).

SIGNALING MECHANISMS REGULATING LITHIUM-INDUCED TNF α PRODUCTION

To begin to understand the mechanisms by which LiCl affects TNF α production, we first tested whether LiCl pretreatment enhances the expression levels of TLRs. As shown in Figure 3B, LiCl treatment did not affect the expression levels of any of the TLRs tested including TLR2, TLR3 and TLR4, as determined by flow cytometry. Because the levels of these receptors did not change with LiCl treatment, we focused on the cell signaling pathways stimulated by these TLRs, in the context of TNF α production. Previous studies have shown that TLR-induced ERK pathway plays an important role in the regulation of TNF α production [Coghlan et al., 2000]. Treatment of Raw264.7 macrophages with LiCl significantly induced ERK and MEK phosphorylation (Fig. 4). LiCl treatment did not affect either p105

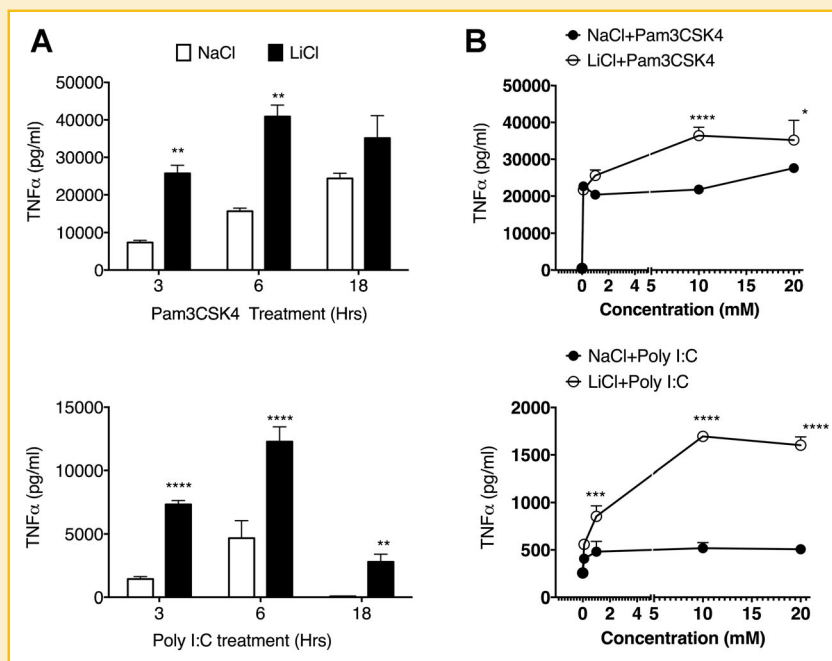


Fig. 2. Lithium pretreatment enhances TLR-2 and TLR3-induced TNF α production in mouse macrophages. Raw264.7 cells were plated in 12-well plate as indicated in Figure 1. In (A), cells were pretreated with NaCl or LiCl (30 mM) for 6 h and then treated with Pam3CSK4 (1 μ g/ml) or Poly I:C (1 μ g/ml) for the indicated time points. Culture supernatants were then assayed for TNF α using ELISA. In (B), cells were treated with NaCl/LiCl for 6 h with the various concentrations as shown and then treated with Pam3CSK4 (1 μ g/ml) or Poly I:C (1 μ g/ml). Supernatants were then assayed for TNF α using ELSIA. N = 3–8 for A and N = 6 for B. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; compared between the respective concentrations of LiCl and NaCl.

phosphorylation or p105 degradation (one of the upstream regulators of MEK–ERK pathway, especially critical in TLR-induced ERK activation in macrophages) [Patial et al., 2011a; Gantke et al., 2012]. Also, as shown in Figure 4, LiCl did not affect the levels of pP38, pJNK, or I κ B α .

To further confirm that the effect of LiCl on TNF α production is mediated via ERK pathway, we pretreated Raw264.7 macrophages with LiCl in the presence or absence of ERK inhibitor, PD98059 [Davies et al., 2000]. As shown in Figure 5, PD98059 significantly blocked LiCl-induced TNF α production demonstrating that LiCl-induced TNF α is at least in part mediated via ERK activation. Together these data suggest that the effect of Li on TNF α production in the absence of TLR ligands occurs in part via regulation of MEK–ERK pathway.

To understand the mechanisms by which LiCl regulates TLR-induced TNF α production, we examined p105–MEK–ERK pathway as well as JNK, p38 and I κ B α pathways. LiCl pretreatment significantly enhanced the levels of Pam3CSK4-induced phospho-P105–MEK–ERK pathway compared to the controls (Fig. 6). To confirm that p105 phosphorylation is upstream of ERK pathway, we pretreated cells with IKK β inhibitor prior to Pam3CSK4 \pm LiCl treatment. P105 is phosphorylated by IKK β and thus inhibition of IKK β has been shown to abrogate TLR-induced ERK activation [Patial et al., 2011a; Gantke et al., 2012]. As shown in Figure 6C, IKK β inhibitor (IKK-2 inhibitor IV) significantly blocked TLR2-induced ERK activation both in the absence and presence of LiCl. In addition to the ERK pathway, LiCl pretreatment also enhanced pJNK levels induced by Pam3CSK4.

Although I κ B α and pP38 levels did not markedly change with Pam3CSK4 alone at the time points tested, LiCl pretreatment significantly enhanced I κ B α degradation and pP38 levels.

Unlike LiCl's effect on Pam3CSK4-induced signaling, Poly I:C-induced signaling was differentially affected by LiCl. In the absence of LiCl, PolyI:C increased MEK–ERK phosphorylation but did not markedly increase pP105, pP38, pJNK, or I κ B α degradation at the time points tested (Fig. 7). Compared to these effects, LiCl pretreatment significantly enhanced PolyI:C-induced MEK–ERK phosphorylation, JNK phosphorylation as well as I κ B α degradation (Fig. 7) but did not affect either p38 or p105 phosphorylation. Together these results suggest that LiCl differentially affects TLR2 and TLR3 induced signaling pathways. Thus effects of Li by itself and in the presence of TLR ligands are likely to be different in terms of the signaling mechanisms and the consequent TNF α production that it regulates. To further test the role of these signaling molecules in TNF α production induced by TLR2 or TLR3 in the absence or presence of LiCl, we used selective inhibitors of ERK (PD98059), JNK (SP600125), p38 (SB203580), and IKK β (IKK-2 inhibitor IV). Interestingly, in the absence of LiCl, JNK and IKK β inhibitors significantly blocked Pam3CSK4-induced TNF α production, whereas in the presence of LiCl, MEK, and IKK β inhibitors markedly blocked Pam3CSK4-induced TNF α production (Fig. 8). Poly I:C-induced TNF α production was inhibited only by the IKK β inhibitor both in the absence or presence of LiCl (Fig. 8). These results suggest that TNF α production is differentially regulated by Pam3CSK4 and Poly I:C in the presence and absence of LiCl.

DISCUSSION

Li is a widely used pharmaceutical in the treatment of neurological diseases such as BPD. While neurological disorders themselves have been linked to higher levels of inflammatory cytokines, there have been a number of studies examining the role of anti-depressants as well as other neuro-regulatory agents in modulating inflammatory cytokines. In particular role of LiCl in modulating inflammatory cytokines has been examined with variable results depending on the cells used as well as the co-stimulating agents used. For example, using human monocytes, Kleinerman et al. [1989] showed that LiCl stimulates TNF α but not IL-1 secretion. Even though other studies have shown no change in TNF α secretion in the presence of LiCl alone [Maes et al., 1999; Nahman et al., 2012] our studies confirm the basic premise that LiCl indeed is capable of stimulating TNF α secretion from mouse macrophages. Our data also indicate that this occurs at the reported therapeutic concentrations of LiCl [Chiu and Chuang, 2010], suggesting that patients on LiCl, at least acutely are likely able to stimulate TNF α in macrophages. There is clinical evidence that LiCl is able to modulate psoriasis in patients since, in Li treated patients, psoriasis is triggered or aggravated as a side effect and that Li and TNF are able to synergistically induce IL6 that could play a key role in psoriasis [Beyaert et al., 1991a, 1992]. Even though how Li's effect on TNF α could directly influence bipolar treatment outcome is not known, studies have shown that cytokines from the systemic circulation can cross the blood brain barrier to influence the brain [McCusker and Kelley, 2013]. Additionally, systemic cytokines have also been shown to affect the integrity of the blood brain barrier [McCusker and Kelley, 2013]. Li could also immune-modulate glial

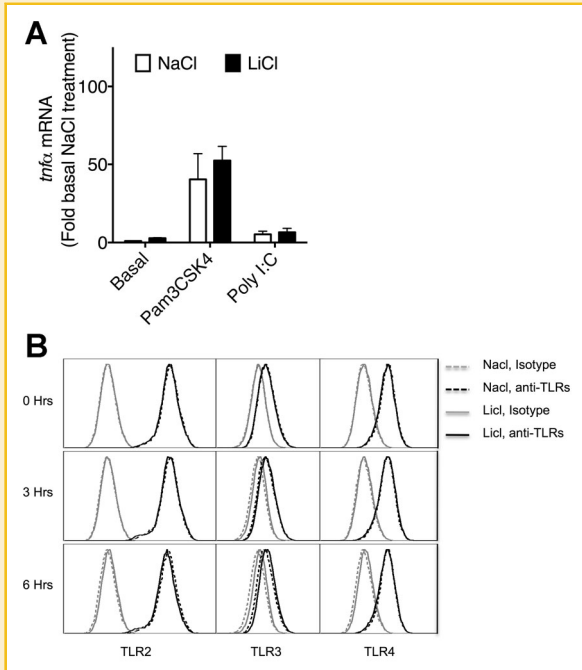


Fig. 3. Lithium's effect on TNF α in mouse macrophages is at the post-transcriptional level and downstream of TLR expression. **A:** Raw264.7 cells were pretreated with NaCl or LiCl (10 mM) for 6 h followed by treatment TLR ligands as indicated for additional 6 h. Cells were then processed for RNA extraction and TNF α mRNA expression determined by real time QPCR as described before [Loniewski et al., 2007]. **B:** Raw264.7 macrophages were treated with NaCl or LiCl (30 mM) for 3 or 6 h and the expression levels of the different TLRs examined by flow cytometry. A representative experiment from two such experiments is shown.

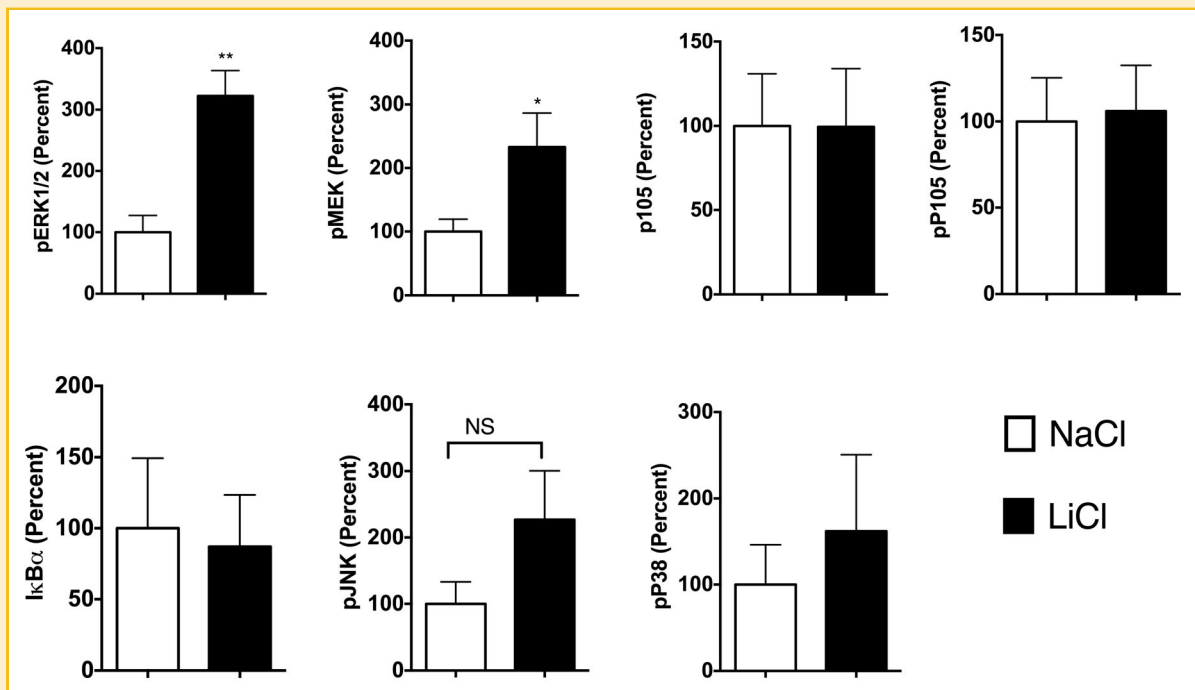


Fig. 4. Lithium induces pERK and pMEK levels in Raw264.7 macrophages. Raw264.7 macrophages were treated with NaCl or LiCl (30 mM) for 6 h and the cells were collected and Western blotting performed for the various proteins as shown. N = 5. * $P < 0.05$ and ** $P < 0.01$ compared to NaCl.

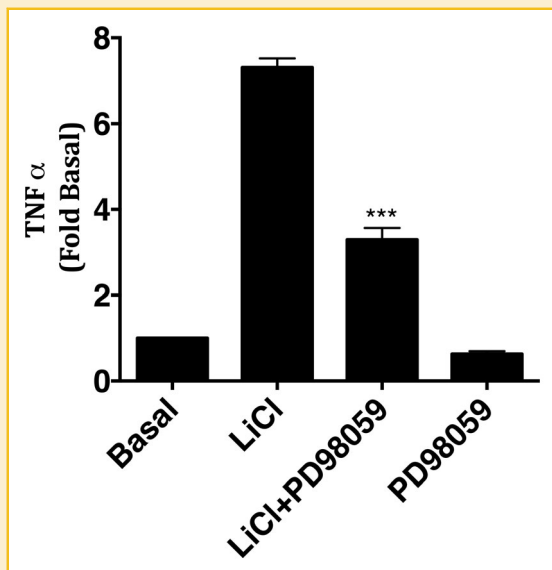


Fig. 5. Lithium-induced TNF α production is inhibited by PD98059 in Raw264.7 macrophages. Raw264.7 cells were treated with LiCl (30 mM) in the presence or absence of MEK-ERK inhibitor, PD98059 (10 μ M), for 6 h and then culture supernatants assayed for TNF α by ELISA. N = 3. *** P < 0.001 compared to LiCl treatment.

cells in the nervous system [Nahman et al., 2012]. Together these effects could affect the treatment outcomes.

Our studies suggest that Li induces TNF α production in part via the ERK pathway. Our data also provides evidence that the increase in TNF α is mediated at the post-transcriptional level. TNF α production has previously been shown to be regulated at transcriptional and post-transcriptional levels involving mechanisms such as mRNA stability [Shapira et al., 1994; Clark, 2000; Dumitru et al., 2000; Rousseau et al., 2008; Kim et al., 2011]. Data presented here also confirms that LiCl activates MEK-ERK pathway at or above the level of MEK phosphorylation. Although the mechanisms involved in how LiCl activates MEK is not clear, we have ruled out the role of GSK3 since inhibition of GSK3 using a more selective inhibitor of GSK3 did not induce TNF α production. Further confirming that our results are independent of GSK3 β inhibition, are studies showing that TLR-induced TNF α production is *diminished* in macrophages from GSK3 β knockout mice [Martin et al., 2005]. Even though the precise mechanism by which Li modulates ERK pathway is not clear, other studies have shown that Li can influence the ERK pathway in different cell types [Ghribi et al., 2003; Yan et al., 2007; Kim et al., 2012; Tsui et al., 2012]. For example, Li has been shown to regulate hippocampal neurogenesis via the ERK pathway [Yan et al., 2007]. Interestingly, in SHSY5Y cells, while Li was shown to increase ERK phosphorylation with wild type DUSP (dual specificity phosphatase), transfection with SNP variants of DUSP6 (dual specificity phosphatase) significantly decreased Li's effect on ERK phosphorylation [Kim et al., 2012]. In our studies we have ruled out the role of p105, one of the upstream regulators of the ERK pathway [Zhang et al., 2009]. Since Li appears to regulate the ERK at the level of MEK, it is possible that Li might influence phosphatases that

regulate this pathway [Zhen et al., 2002; Chiu and Chuang, 2010; Kim et al., 2012].

Li has been shown to have a number of different cellular and biochemical effects that are both dependent and independent of GSK3 β inhibition [Chiu and Chuang, 2010]. Even though Li is being widely used as an inhibitor of GSK3 β , recent studies have demonstrated that Li has other biochemical effects including inhibition of NMDA receptor-mediated calcium influx, inhibition of various phosphoinositol phosphatases including inositol polyphosphate-1-phosphatase and inositol monophosphatase [Chiu and Chuang, 2010]. In addition to direct inhibition of GSK3 β activity, Li has also been shown to indirectly inhibit GSK3 β activity via mechanisms such as disruption of β -arrestin-2 signaling complex with PP2A that consequently activates Akt phosphorylation and inhibition of GSK3 [Beaulieu et al., 2008]. One study [Yanagita et al., 2007] found that Li selectively inhibits Na $^+$ influx via Na $^+$ channels and subsequent Ca $^{2+}$ influx and catecholamine secretion independent of GSK3 β inhibition, in adrenal chromaffin cells. While many of these effects have been shown in neuronal cell types, mechanisms that regulate inflammatory cytokines such as TNF α in immune cells are not well understood.

One study recently found that treatment of bone-marrow-derived macrophages with Li induces degradation of p105 that subsequently leads to inhibition of LPS-induced ERK pathway and therefore LPS-induced TNF α transcription [Zhang et al., 2009]. Our studies in Raw264.7 macrophages however, suggest that p105 is not regulated by Li treatment even after 18 h of treatment (data not shown). While the reasons for the differences in mechanisms are not clear, macrophages are highly heterogeneous and therefore, different sources of macrophages will likely exhibit different mechanisms of p105 regulation. Interestingly, our studies suggest that LiCl not only regulates TNF α production by itself, it also modulates TNF α production stimulated by two different TLR ligands. The mechanism by which this occurs however is different between the two ligands. This could be because TLR2 activates MyD88-dependent pathway whereas TLR3 activates TRIF-dependent pathway. Whereas TLR2-induced ERK activation is regulated by IKK β -p105-dependent pathway, TLR3-induced ERK pathway appears to be independent of p105 pathway. Other signaling pathways such as the Ras-Raf pathway has also been shown to be important regulators of the MEK-ERK pathway [Geppert et al., 1994] and thus TLR3-induced ERK activation could be mediated via these mechanisms. Surprisingly, pretreatment of LiCl enhanced all of the major MAPK and NF κ B pathways induced by Pam3CSK4 and PolyI:C in these macrophages with particular differences between TLR2 and TLR3 on p105 and p38 phosphorylation. Even though I κ B α degradation itself was not evident at the time points tested with TLR2 or TLR3 ligands in the absence of LiCl, IKK β inhibitor blocked TNF α production in the absence of LiCl with both ligands. IKK β mediates NF κ B via several other I κ Bs including p105 and thus TLR2 and TLR3-induced TNF α production may be mediated via I κ Bs independent of I κ B α . Furthermore, it is possible that I κ B α degradation by TLR2/3 in the absence of LiCl occurred at the time points we did not test. Interestingly function of MEK-ERK and JNK pathways seem to be different in their role in mediating TNF α production in the absence and presence of LiCl especially with TLR2 stimulation. Taken together

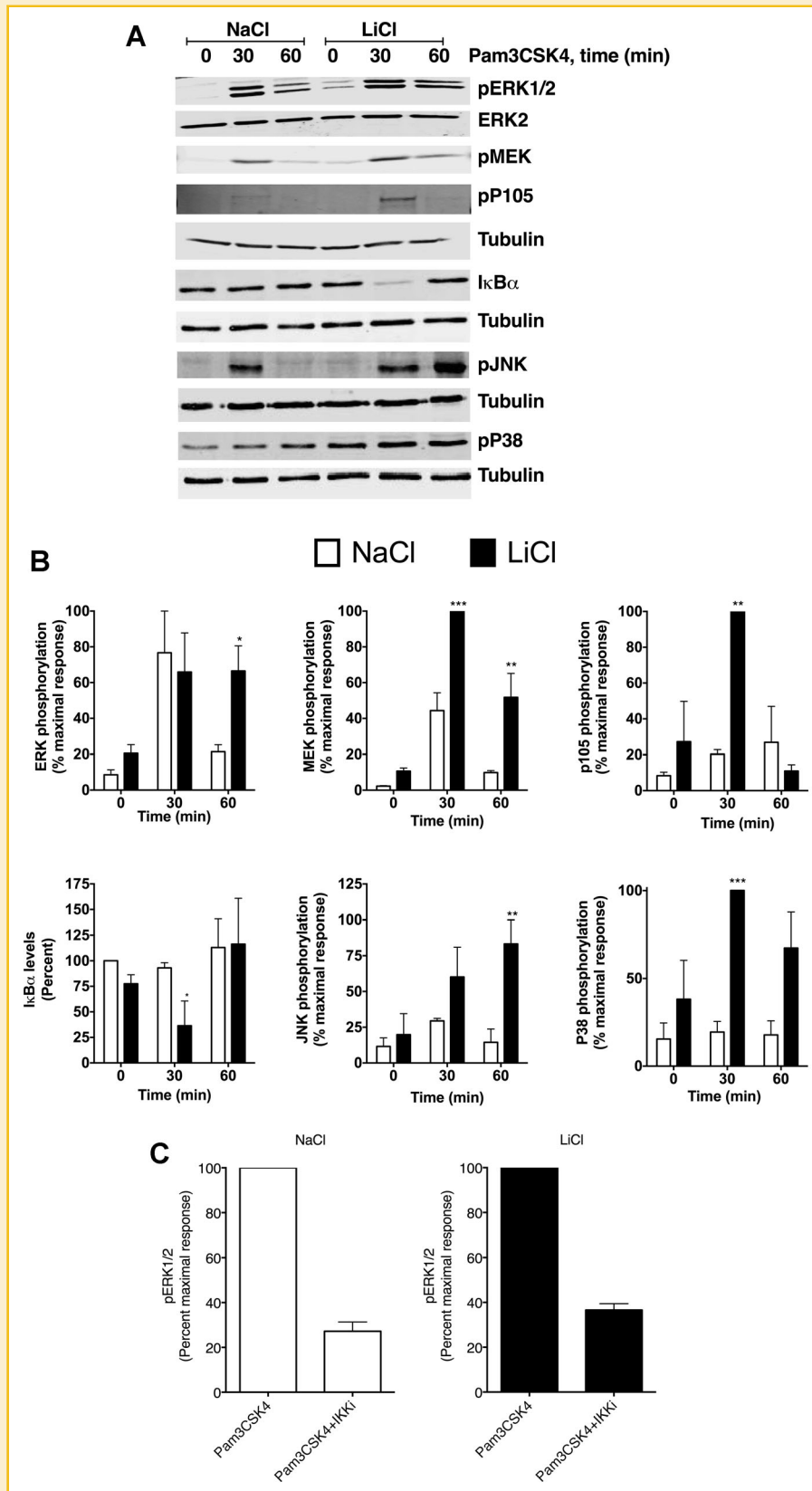


Fig. 6. Lithium pretreatment enhances TLR2-stimulated signaling pathways in mouse macrophages. Raw264.7 macrophages were plated as described in Figure 1 and then treated with NaCl or LiCl (30 mM) for 6 h followed by treatment with Pam3CSK4 (1 μ g/ml) for the indicated time points. Cells were then processed and Western blotting performed for the various proteins as shown (A,B); N = 3. * P < 0.05; ** P < 0.01; *** P < 0.001; compared between NaCl and LiCl at the respective time points. In (C), effect of IKK β inhibitor (IKK-2 inhibitor IV, Calbiochem) on Pam3CSK4-induced pERK levels is shown. Cells were treated with NaCl or LiCl followed by IKK β inhibitor prior to stimulation with Pam3CSK4. PhosphoERK levels were analyzed by Licor Odyssey as described in Materials and Methods Section, N = 3.

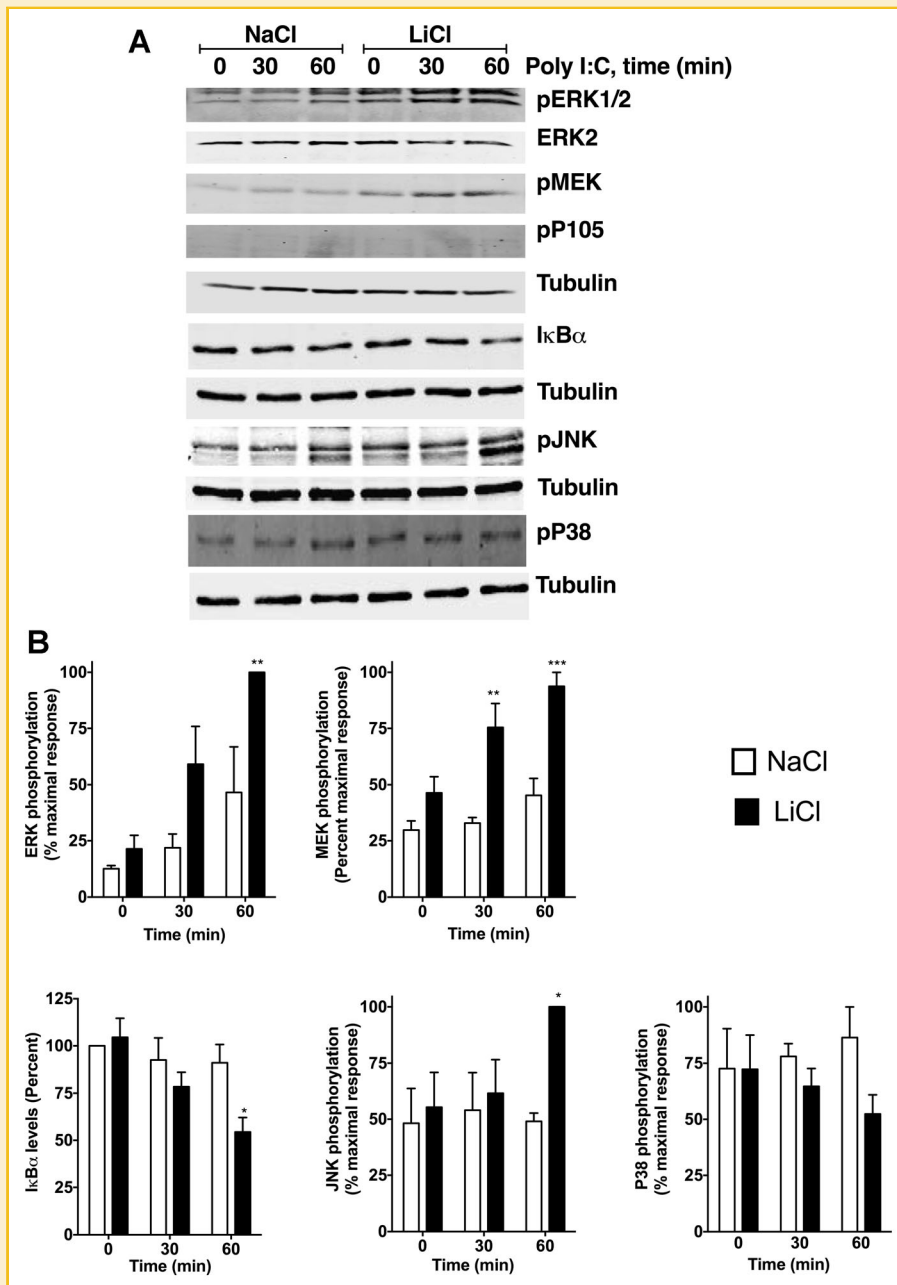


Fig. 7. Lithium pretreatment enhances TLR3-stimulated signaling pathways in mouse macrophages. Cells were plated and treated with NaCl or LiCl for 6 h as indicated in Figure 6 and then treated with Poly I:C (1 μ g/ml) for the indicated time points. Cells were then processed and Western blotting performed for the various proteins as shown, N=3. * P <0.05; ** P <0.01; *** P <0.001; compared between NaCl and LiCl at the respective time points. Representative blots are shown in A, and quantitation in B.

our studies suggest that LiCl may selectively modulate certain signalosomes of the TLR2 and TLR3 signaling pathways and mediate TNF α production in macrophages.

Li by virtue of also being a GSK3 inhibitor has been used in other settings or diseases where GSK3 β has been shown to play an important role. For example, Li has been shown to be effective in preventing and suppressing experimental autoimmune encephalomyelitis [De Sarno et al., 2008]. In addition it has been shown to reduce disease severity in other models of diseases including sepsis

[Wang et al., 2009], arthritis [Cuzzocrea et al., 2006], colitis [Whittle et al., 2006] and atherosclerosis [Choi et al., 2010]. In addition, Li has been used as a therapeutic target for BPD in humans [Chiu and Chuang, 2010]. Thus while Li may have other effects in addition to GSK3 β , its effects on GSK3 β has been therapeutically useful. However, understanding how these additional effects of Li occurs will help further understand some of the side effects of Li in patients. While a number of studies have examined the role of LiCl in modulating LPS-induced signaling in macrophages as well as other

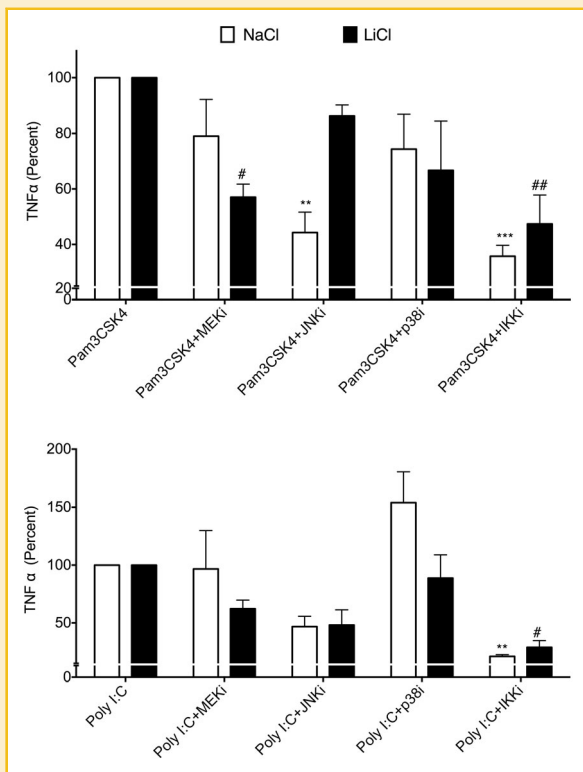


Fig. 8. Differential effects of MAPK and IKK β inhibitors on TLR2- and TLR3-induced TNF α production in the absence and presence of LiCl: Raw cells were plated and treated with NaCl or LiCl (10 mM) for 6 h followed by the indicated inhibitors 30 min prior to stimulation with Pam3CSK4 (A) or Poly I:C (B) for an additional 6 h. TNF α levels were determined in the culture supernatants using ELISA as described in Materials and Methods Section. TNF α levels in pg/ml were as follows: Pam3CSK4 + NaCl = 21962 \pm 4035; Pam3CSK4 + LiCl = 41994 \pm 3121 and Poly I:C + NaCl = 3397 \pm 1102; Poly I:C + LiCl = 21226 \pm 6476; N = 3. Data were normalized to percent TLR stimulation with or without LiCl and analyzed by ANOVA with post-Bonferroni. ** P < 0.001; *** P < 0.0001 compared to TLR ligand treatment with NaCl. # P < 0.05; ## P < 0.01 compared to TLR ligand treatment with LiCl.

cell types [Maes et al., 1999; Zhang et al., 2009; Kaufmann et al., 2011; Nahman et al., 2012], our studies here in macrophages are the first to examine the role of LiCl in modulating TLR2 and TLR3 signaling. Although the end result on TNF α production is similar between TLR2 and TLR3 activation, the signaling mechanisms modulated by LiCl in these two signal transduction pathways are slightly different. Further elucidation and understanding of these biochemical mechanisms will help us better target neurological diseases without the side effects.

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