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Separation of zinc-dependent and zinc-independent events during early LPS-stimulated TLR4 signaling in macrophage cells

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ABSTRACT

Free zinc is required for proper lipopolysaccharide (LPS)-stimulated signaling, but potential sites of action in the pathway have not been defined. In this work, we provide *in vitro* and *ex vivo* evidence that zinc is not required for phosphorylation or ubiquitylation of IRAK1, a kinase functioning early in the TLR4 pathway. However, degradation of ubiquitylated IRAK1 occurred via a zinc-dependent, proteasome-independent pathway. These results provide evidence of a novel site of action for zinc during TLR4-mediated inflammatory responses.

1. Introduction

Zinc is a micronutrient that is essential for a wide variety of biochemical functions. In particular, zinc is essential for cell-mediated innate immunity, affecting the function of neutrophils, natural killer cells and macrophages. Many studies have documented the adverse effects of zinc deficiency on the ability to mount an immune response [1–6]. While certain requirements for zinc in immune function undoubtedly relate to its roles as an enzymatic cofactor [7,8] or a component of various structural motifs [9] (e.g., zinc fingers), more recent evidence points to additional roles for zinc in regulating immune cell signaling [10–13]. Although free ionic zinc in the cytoplasm is maintained at extremely low concentrations [14,15], the activation of certain immune cell types has been shown to trigger the release of free zinc within cytoplasm [10,11]. This transient pool of zinc, sometimes referred to as a "zinc wave", has been observed in response to a number of stimuli, including FcεRI activation in mast cells [10] and TLR1-, TLR2-, TLR4- and TNF-α activation in monocytes [11]. The levels of intracellular zinc transiently increases, usually reaching a peak a few minutes after induction by extracellular stimuli [11,16]. Although the effects of the transient zinc wave remain poorly understood, it is possible that transient fluxes in cytosolic zinc concentrations may serve as a type of a second messenger to modulate signaling pathways elicited during early phases of the innate immune response [12,16].

Evidence exists that zinc may be required for signal transduction events downstream of TLR recognition. TLR1, 2, and 4 recognize pathogen associated molecular patterns of bacterial components, such as triacyl lipoprotein, lipoprotein, and lipopolysaccharide (LPS) respectively [17]. Recognition of these extracellular ligands by their cognate receptors initiates an intracellular signaling cascade resulting in the elevated transcription of many genes that encode a range of proinflammatory mediators. Numerous studies have shown that zinc plays a critical role in these pathways; however, the underlying molecular mechanisms are unknown. For example, chelation of intracellular zinc in primary monocytes or cultured macrophage cell lines blocks the activation of MAPK and NF-κB DNA binding activity, as well as the expression and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [11]. Consistent with a requirement for zinc in these pathways, recent studies indicate that addition of physiological concentrations of zinc enhances MAPK(s) activation and cytokine

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production following LPS treatment [10,11,18]. However, the molecular targets affected by zinc remain poorly understood. To better understand how zinc availability regulates immune cell signaling, we investigated the effects of zinc deficiency and supplementation on TLR4 signaling in the RAW264.7 macrophage cell line as well as primary macrophages. Zinc depletion was found to block the LPS-induced phosphorylation of several kinases including IKK, MKK, and MAPKs. The phosphorylation and ubiquitylation of the early kinase, IRAK1, was unperturbed by zinc deficiency, thus demonstrating a degree of selectivity in the signaling events requiring zinc. However, the subsequent degradation of ubiquitylated IRAK1 occurred in a zinc-dependent manner that was independent of the proteasome. These zinc dependent steps involved in LPS signaling were confirmed in mouse primary peritoneal macrophages. Our findings identify discreet target proteins whose participation in the TLR4 signaling pathway is dependent on the availability of cytoplasmic zinc, thus providing new insights into the molecular mechanisms by which zinc deficiency suppresses the innate immune system.

2. Materials and methods

2.1. Animals and ethics

All animal husbandry and euthanasia procedures were performed in accordance with and under the approval of the Animal Care and Use Committee of the University of Missouri. Mice were euthanized by isofluorane anesthesia followed by cervical dislocation. Mice were maintained on 12 h light–dark cycle and food and water provided *ad libitum*.

2.2. Antibodies and reagents

Antibodies to phospho-ERK1/2 (T202/Y404), phospho-p38 (T180/Y182), phospho-IκB (S32), IκB, phospho-IKKα (S176)/IKKβ (S177), MKK6, phospho-MKK3/MKK6 (S189/207) were from Cell Signaling Technologies (Danvers, MA). Antibodies to ERK1/2, IRAK1, ubiquitin, IKKα/β were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to beta tubulin, mouse IgG, and rabbit IgG were from Sigma (St. Louis, MO). The antibody to actin was from EMD Chemicals (Billerica, MA), lambda phosphatase was from New England Biolabs (Ipswich, MA). MG132 and Epoxomicin were from BIOMOL (Farmingdale, NY). Lipopolysaccharide, N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), were from Sigma. $ZnCl_2$ was from Fisher Scientific (Pittsburgh, PA).

2.3. Cell culture

The mouse macrophage-like cell line RAW 264.7 was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 μM non-essential amino acids, 2 mM L-glutamine and 100 units/mL penicillin and streptomycin in 5% CO_2 at 37 °C. Cells were passaged at 1:8 dilution every 2 days.

Primary activated peritoneal macrophage cells isolated from C57 BL/6 mice as described previously [19].

2.4. Cell treatments

For TPEN treatments, cells were incubated with 20 μM of TPEN for 50 min. Subsequently, cells were treated with LPS with or without the addition of zinc for 20 min before harvesting cells. For testing for the role of the proteasome, RAW264.7 cells were pretreated with 20 μM of MG132 or 10 μM of epoxomicin for 50 min prior to treatment with LPS.

2.5. Immunoblot analysis

After treatments, RAW 264.7 cells were washed twice with ice cold 1X TBS (Tris-buffered saline) and collected with 2X Laemmli buffer (20 mM sodium phosphate buffer pH 7.0, 0.01% Bromophenol blue, 20% glycerol, 4% w/v SDS, add 40 mM DTT before use). After sonication to shear DNA, lysates were heated at 95 °C for 5 min and protein extracts (10 μg for p-ERK, p-p38, and ERK, 80 μg for p-IKKα/β, and 20 μg for other proteins) were separated by 8% (ubiquitin), 10% (IKK α/β) or 11% (all other proteins) SDS–PAGE gels, and transferred to PVDF membrane (Millipore) using 60 Volts for 2 h on ice. For proteins larger than 72 kD, 0.02% SDS was added to transfer buffer, and the time for transfer was 3.5 h. Membranes were incubated in blocking buffer (5% non-fat dry milk in TBS/T) for 1 h at room temperature (RT). Membranes were washed two times for 5 min each with 10 mL TBS/T and incubated with primary antibody (dilutions of 1:250 for IRAK1, 1:500 for ubiquitin; and 1:1000 for all others) with gentle agitation overnight at 4 °C. Membranes were washed four times for 5 min each with 10 mL of TBS/T before incubating with appropriate HRP-conjugated secondary antibody (1:5000 for p-IKKα/β and 1:10,000 for other proteins) in blocking buffer with gentle agitation for 1 h at RT. Membranes were washed at least five times for 5 min each with 10 ml of TBS/T and then incubated with SuperSignal West Pico chemiluminescence (Pierce) with or without the addition of 2–10% Femto chemiluminescence (Pierce) for 3 min prior to exposure. For stripping of blots, the membrane was incubated twice in stripping buffer (25 mM glycine–HCl, pH 2, 1% SDS) for 10–15 min at RT with shaking before washing with PBST for 5 min with shaking.

2.6. Phosphatase assays

After TPEN and LPS treatment, cells were washed twice with ice cold 1× TBS and collected in 60 μL of appropriate lysis buffer. For controls, Buffer 1 was used (100 mM HEPES-KOH pH7.5, 5% glycerol, 0.1% SDS, 0.5% deoxychiolic acid, 1% NP-40, 1 mM PMSF, 10 mM leupeptin, 10 nM calyculin A, 5 mM NEM, 5 μg/mL aproptinin and 50 μM MG132). To inhibit phosphatases, Buffer 2 was used (Buffer 1 with 15 mM EGTA, 5 mM EDTA, 5 mM NaPP, 1 mM NaMo, 50 mM NaF and 10 mM activated Na_3VO_4). For phosphatase treatments, Buffer 3 was used (Buffer 1 with 8 mM of $MnCl_2$ and 1 μL of lambda phosphatase). For phosphatase treatments in the presence of inhibitors, Buffer 4 was used (Buffer 2 with 8 mM of $MnCl_2$ and 1 μL of phosphatase). Cell lysates were incubated at 37 °C water for 20 min before terminating the reaction by adding 40 μL of 2.5 × loading buffer and heating to 95 °C for 5 min.

2.7. Immunoprecipitation

After treatments, cells were washed twice with ice-cold TBS and resuspended in 900 μL cold lysis buffer (100 mM HEPES-KOH, pH 7.5, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% of SDS, 0.5 mM EDTA, 2 mM N-ethylmaleimide, 50 μM MG132, 1 mM PMSF, 5 μg/mL aprotinin, 10 μM leupeptin, 50 mM NaPP, 25 mM NaF, 150 mM NaCl, 1 nM calyculin A, 2.5 mM NEM). Cell lysates were sonicated three times for 10 s on ice. Cell debris was removed by centrifugation for 20 min at 16,000 g at 4 °C. 30 μL of supernatant was saved as input. The remaining supernatant was precleared by incubating with 50 μL Protein G agarose beads with rotation at 4 °C for 2 h and then removing beads by centrifugation at 16,000 g for 5 min. The supernatant was incubated with appropriate antibody overnight with rotation at 4 °C prior to incubating with 50 μL Protein G agarose beads for 1 h at 4 °C with rotation. Collected beads were washed three times with 500 μL of Low Salt Buffer (25 mM Tris, pH 7.4, 5 mM EDTA, 100 mM NaCl, 0.1% Triton X-100), three times with 500 μL of High Salt Buffer (25 mM Tris, pH

7.4, 5 mM EDTA, 1 M NaCl, 0.1% Triton X-100), and one time in water. Finally, beads were resuspended in 50 μL of 2× Leammeli buffer and boiled at 95 °C for 5 min prior to loading samples on SDS–PAGE gels.

3. Results

3.1. Zinc enhances LPS-induced ERK activation in RAW264.7 macrophages

Recent studies demonstrated that activation of the TLR4 receptor in primary monocytes and granulocytes resulted in a rapid rise in free zinc levels in the cytoplasm [11]. In the mouse macrophage cell line, RAW264.7, this rise in free zinc was shown to occur within 2 min of LPS stimulation resulting in a 7–14-fold increase in free zinc [11]. Zinc depletion was found to block MAPK activation and subsequent pro-inflammatory cytokine production [11]. While these studies provided clear evidence of a requirement for zinc, the complement of molecular targets within the LPS signaling pathway affected zinc deficiency remains poorly understood.

Our initial studies were aimed at defining zinc-dependent steps involved in LPS-signaling. First, we demonstrated a positive effect of zinc by treating RAW264.7 cells for 20 min with a physiological concentration of $ZnCl_2$ (30 μM) together with increasing concentrations of LPS (5–50 ng/mL). The addition of zinc alone did not induce the phosphorylation of ERK (Fig. 1A). However, the addition of zinc increased ERK phosphorylation in response to LPS, particularly at low LPS concentrations (Fig. 1A). Cells without zinc co-treatment required approximately 10-fold more LPS to achieve the same level of ERK phosphorylation as in cells treated with zinc plus LPS, indicating that the presence of zinc potentiates LPS-induced signaling responses in RAW264.7 cells.

To further demonstrate the requirement for zinc in LPS-induced signaling, RAW264.7 cells were made zinc-deficient by pretreatment for 50 min with 20 μM of TPEN, a cell permeable zinc chelator with a zinc/chelate ratio of 1:1 [20]. Consistent with previous results [11], TPEN treatment was found to markedly inhibit phosphorylation of ERK in response to 50 ng/mL of LPS (Fig. 1B). This effect of TPEN was due to zinc chelation since the addition of 30 μM Zn^{2+} prevented TPEN-mediated suppression of LPS-induced activation of ERK (Fig. 1B). Moreover, this effect of TPEN was selective for zinc since the addition of other divalent metals (also added as chloride salts) could not prevent TPEN-induced block of LPS-induced ERK phosphorylation (Fig. 1C).

3.2. Zinc is broadly required for TLR4 signaling protein phosphorylation induced by LPS

The results above confirmed the specific requirement for zinc during LPS-induced MAPK phosphorylation. However, the extent to which zinc is more broadly required for LPS signaling has not been fully explored. Therefore, we investigated the requirements for zinc in LPS-induced phosphorylation of a broad range of proteins in the TLR4 pathway. TPEN treatment markedly suppressed LPS-induced phosphorylation of MKK3/6 and p38, the kinases which form part of the p38 pathway (Fig. 2A). We also tested the effect of zinc depletion on signaling via IκB, a protein which inhibits the NF-κB pathway by sequestering the NF-κB transcription factor in the cytoplasm and preventing its access to the nucleus [21]. In response to LPS stimulation, IκB is rapidly phosphorylated and ubiquitylated, which targets this protein for proteasomal degradation. LPS-induced IκB phosphorylation was inhibited by TPEN treatment, but reapplication of zinc restored phosphorylation (Fig. 2A). Similarly, the subsequent IκB degradation in response to LPS was also blocked in TPEN-treated cells, but the addition of zinc ameliorated these effects (Fig. 2A). We then focused on events upstream of IκB which might be dependent on zinc availability. The kinases, IKKα/β, required to phosphorylate IκB are also phosphorylated in response to LPS, and IKKα/β phosphorylation was similarly dependent on zinc availability (Fig. 2A). Collectively, these results demonstrate that zinc is required in multiple divergent pathways downstream of LPS stimulation (summarized in Fig. 2B, open circles).

3.3. Zinc is not required for IRAK1 phosphorylation

Because a broad spectrum of LPS-dependent phosphorylation responses were dependent on zinc, we hypothesized that zinc is required early in the TLR4 signaling pathway. One of the earliest events in the activation of the TLR4 pathway is phosphorylation of IRAK1, a kinase that forms part of a scaffold complex linked to the TLR4 protein. Normally, LPS treatment results in phosphorylation, poly-ubiquitylation, and degradation of IRAK1 [22–29]. Consistent with these previous studies, the levels of IRAK1 greatly

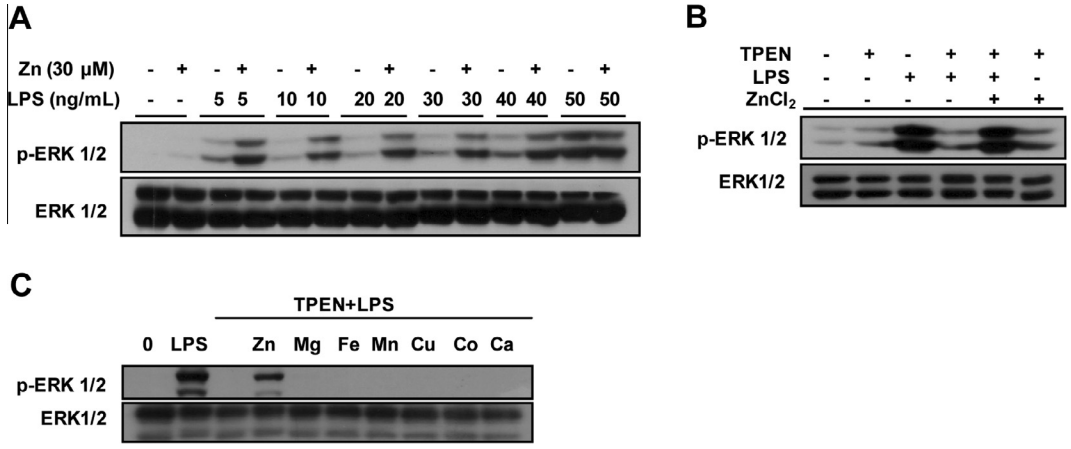

Fig. 1. Physiological concentrations of zinc synergistically amplify LPS-induced activation of ERK. (A) RAW264.7 cells were treated with (+) or without (−) 30 μM zinc and with increasing concentrations of LPS for 20 min. Immunoblot analysis was performed using antibodies against the activated form of ERK (p-ERK 1/2) or against the native ERK protein as a loading control (ERK 1/2). (B) Cells were incubated with (+) or without (−) 20 μM TPEN for 50 min. Subsequently, cells were treated with 50 ng/mL LPS, and/or 30 μM zinc for 20 min. Immunoblot analysis was performed as described in (A). Experiments were performed at least three times with similar results as shown. (C) Cells were treated as described for (B), but 30 μM of the divalent metal indicated (added as chloride salts) was included with the treatment. Immunoblot analysis was performed as described in (A). Experiments were performed at least three times with similar results as shown.

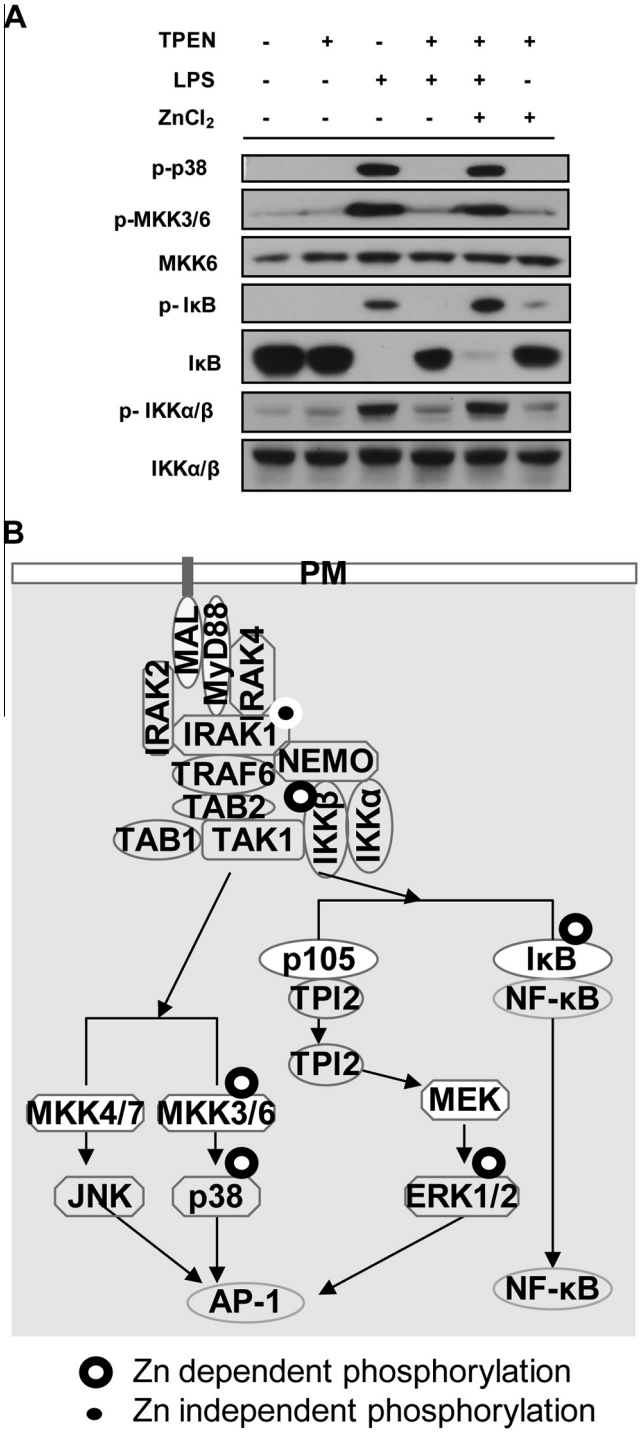

Fig. 2. Zinc is broadly required for LPS-induced phosphorylation of proteins in signaling pathways. (A) Cells were treated as described in Fig. 1B. Immunoblot analysis of protein extracts from treated cells were performed using antibodies against activated (p-p38) p38 kinase, activated (p-MKK3/6) or native (MKK6) MAPK kinases, phosphorylated (p-IκB) or native (IκB) IκB protein, or activated (p-IKKα/β) or native (IKKα/β) IκB Kinase. Experiments were performed at least three times with similar results as shown. (B) Model summarizing zinc-dependent (open circles on protein) and zinc-independent (closed circles on protein) phosphorylation of proteins involved in LPS-induced inflammatory signaling as described in this work.

decreased within 20 min in LPS-treated RAW 264.7 cells (Fig. 3A). The depletion of zinc using TPEN did not greatly affect the LPS-induced decrease of native IRAK1. However, this treatment resulted in the appearance of a slower migrating form of IRAK1

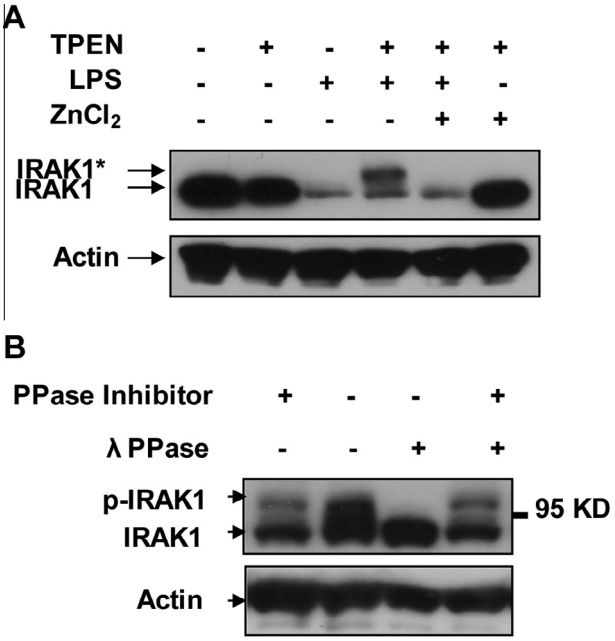

Fig. 3. Zinc is not required for LPS-induced phosphorylation of IRAK1 but is required for its degradation. (A) Cells were treated as described in Fig. 1B. Immunoblot analysis of protein extracts from treated cells were performed using antibodies against IRAK1 or actin proteins. Phosphorylated IRAK1 (IRAK1*) migrates as an apparently higher MW form. (B) The apparently higher MW form is confirmed as phosphorylated IRAK1 (p-IRAK1) by phosphatase assay. Protein extracts were treated in the presence (+) or absence (–) of phosphatase inhibitors or lambda (λ) phosphatase prior to immunoblot analysis as described in (A). Experiments were performed at least three times with similar results as shown.

(IRAK1* in Fig. 3A, lane 4). The accumulation of this slower migrating form of IRAK1 was generated specifically in response to zinc depletion as it was barely detectable in zinc replete cells that were stimulated with LPS (Fig. 3A, lane 3) or when zinc was added to the TPEN-treated LPS-stimulated cells (Fig. 3A, lane 5). In other cell types IRAK1 is known to become hyperphosphorylated in response to proinflammatory cytokines [22,24,25,27]. Therefore, we suspected that the slower migrating species of IRAK1 in the zinc depleted cells might represent the stabilization (or accumulation) of a hyperphosphorylated form. This hypothesis was supported by the finding that phosphatase treatment of protein lysates resulted in the disappearance of the higher molecular weight species of IRAK1, shifting it to the native form of IRAK (Fig. 3B, lane 3). A control sample that included a phosphatase inhibitor during the phosphatase treatment prevented this shift (Fig. 3B, lane 4). Taken together, these data indicate that zinc depletion stabilizes the accumulation of the phosphorylated form of IRAK1.

3.4. Zinc depletion does not block IRAK1 ubiquitylation

Previous studies have shown that IL-1/LPS stimulation of HEK293 cells resulted in phosphorylation of IRAK1 and subsequent K63-linked ubiquitylation and degradation [28]. Because IL-1R and TLR4 share a common cytoplasmic motif (the Toll/IL-1R homology domain) as well as MyD88 dependent signaling pathways [30], we considered the possibility that zinc may be essential for IRAK1 ubiquitylation and/or degradation of IRAK1. This hypothesis would account for (a) the disappearance of native IRAK1 in LPS-treated cells and (b) the stabilization of the phosphorylated form owing to the blockage of ubiquitylation and/or degradation by TPEN treatment.

Consistent with this possibility, addition of TPEN during LPS stimulation of cells resulted in the appearance of the high molecular

weight form of IRAK1 (Fig. 4A, lane 4), which failed to accumulate in the presence of added zinc (Fig. 4A, lane 5). Because longer exposures were required to visualize the higher molecular weight form IRAK1 in these immunoblot experiments, the decrease in levels of native IRAK1 in response to LPS alone was less obvious than in Fig. 4 due to near saturation of the signal.

The most likely explanation for the appearance of this high MW form is that IRAK1 becomes phosphorylated and poly-ubiquitylated after LPS treatment but that zinc is required for the degradation of IRAK1 once it is ubiquitylated. To address this possibility, we directly assessed whether the high MW form of IRAK1 is, in fact, ubiquitylated IRAK1. IRAK1 was immunoprecipitated from LPS-stimulated, zinc-depleted cells; and the presence of ubiquitin was determined by immunoblot analysis using anti-ubiquitin antibodies. As shown in Fig. 4B, ubiquitylated protein was readily detected in the higher MW species of immunoprecipitated IRAK1 in samples from zinc-deficient cells in response to LPS-stimulation, but not from untreated control cells (Fig. 4B, lanes 5 and 6). As a control, no ubiquitin was detected in cell lysates immunoprecipitated with equal amount of mouse IgG alone (i.e. no anti-IRAK1 antibody; Fig. 4B, lane 7–8). These results, together with those in Fig. 3, demonstrate that IRAK1 is both phosphorylated and ubiquitylated, and that these modified forms accumulate during zinc depletion.

3.5. IRAK1 degradation is independent of the proteasome

As poly-ubiquitylation is often associated with proteasomal degradation [31], we evaluated whether the zinc-dependent degradation of poly-ubiquitylated IRAK1 was also proteasome-dependent. RAW264.7 cells were pre-incubated with the proteasome inhibitor, MG132, or TPEN, then incubated with LPS Surprisingly, MG132 did not inhibit LPS-induced clearance of the native form of IRAK nor did it induce the appearance of the higher molecular weight forms in zinc replete cells (Fig. 5A, lane 7–9). The inability of MG132 to block IRAK1 degradation was not due to a lack of potency of the MG132 inhibitor because IκB degradation after LPS treatment was blocked by MG132 in the same experiments (Fig. 5A). Moreover, another proteasome inhibitor, epoxomycin, which blocks a broader range of protease activities in the proteasome [32] was found to inhibit LPS-induced degradation of IκB but not the degradation of IRAK1 (Fig. 5B, lane 7–9). These results indicate that although zinc is required for the degradation of poly-ubiquitylated IRAK1, this process is independent of the proteasome.

3.6. IRAK1 is degraded in a zinc dosage dependent manner

To further investigate the possible relation between the zinc requirement for IRAK1 degradation and the downstream effects on phosphorylation, we examined the minimal concentration of zinc required to reverse the stabilization of ubiquitylated IRAK1 by TPEN. The form of Ub-IRAK1 stabilized by 20 μM TPEN in LPS treated cells required between 20 and 30 μM zinc (Fig. 6A). Considering that TPEN chelates zinc at equimolar amounts [20], these findings are consistent with low levels of zinc in the physiological range required to elicit IRAK1 degradation. Interestingly, there was a strong correlation between the concentrations of zinc required to restore the degradation of IRAK1 and the restoration of phosphorylation of the downstream ERK, indicating that the stabilization of IRAK1 may be responsible for the inhibition of downstream signaling events. Furthermore, the addition of zinc (30 μM) potentiated LPS induced IRAK degradation similar to the additive effect found for ERK activation (Fig. 6B). These results further support our findings that the degradation of IRAK1 during early LPS responses is a novel site of action for zinc in TLR4 signaling.

3.7. Zinc is required for LPS signaling via IRAK1 in primary peritoneal macrophages

To verify whether our *in vitro* observations in RAW264.7 cells occur in primary macrophages, we assessed these responses in peritoneal macrophages isolated from mice. Consistent with the results using RAW264.7 cells, the LPS-induced phosphorylation of ERK1/2, p38, MKK3/6, IκB, and IKKs was inhibited by TPEN and restored by zinc supplementation in the cultured primary macrophages (Fig. 7). Moreover, zinc depletion of the primary macrophages using TPEN was found to stabilize both the phosphorylated and ubiquitylated forms of IRAK1 (Fig. 7). Taken together, these results from *in vitro* and *ex vivo* macrophage cultures demonstrate a requirement for zinc in LPS-induced IRAK degradation and subsequent downstream phosphorylation cascades.

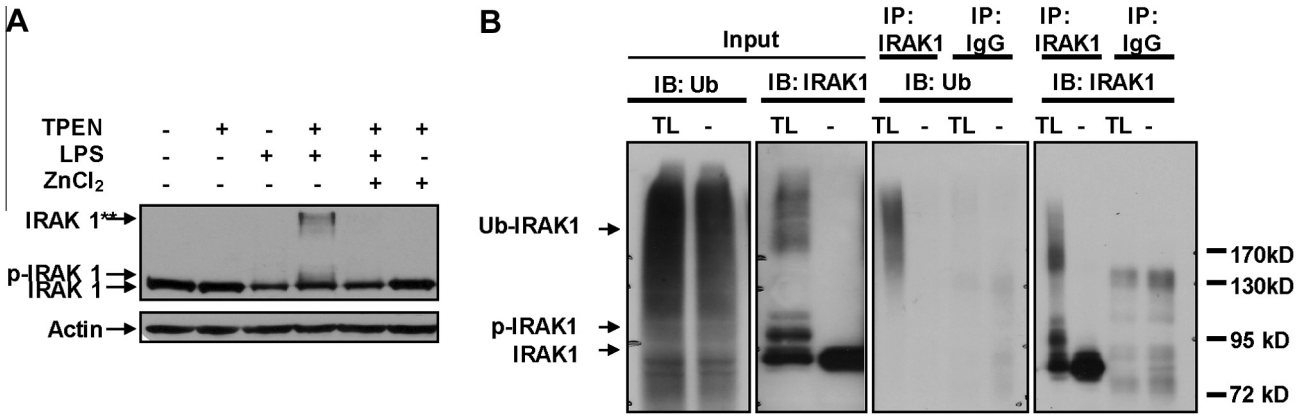

Fig. 4. Zinc is not required for LPS-induced ubiquitylation of IRAK1. (A) Cells were treated as described in Fig. 1B. Immunoblot analysis was performed as described in Fig. 3A except that images were exposed for longer to visualize the higher MW form of IRAK1 (IRAK1**). (B) Cells were incubated for with 20 μM TPEN 50 min, and then treated with 50 ng/mL LPS (TL) for 20 min or not treated (−). Lanes show samples from either total protein input (Input) or after immunoprecipitation (IP) with either IRAK1 or IgG as a control. Immunoblot (IB) analyses were performed using either anti-ubiquitin (Ub) or anti-IRAK1 (IRAK1) antibodies. The high MW form cross-reacting with both Ub and IRAK1 antibodies is indicated as ubiquitylated IRAK1 (Ub-IRAK1). Experiments were performed at least three times with similar results as shown.

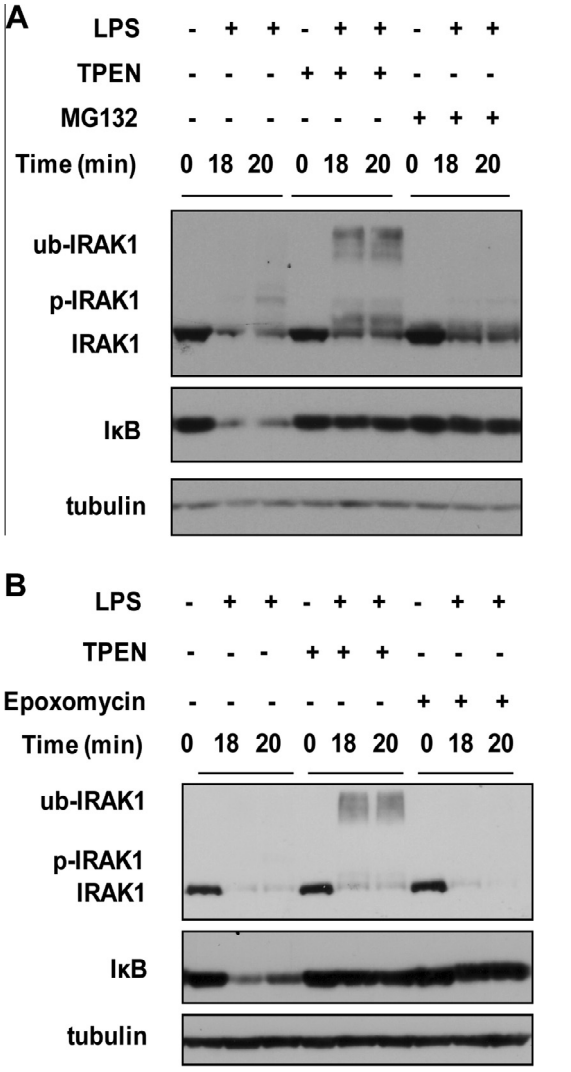

Fig. 5. LPS-induced degradation of IRAK1 does not require proteasome activity. Cells were incubated with (+) or without (−) 20 μM TPEN, or the proteasome inhibitors (A) 20 μM MG132 or (B) 10 μM epoxymcyin for 50 min. Then cells were treated with 50 ng/mL LPS for indicated time. Immunoblot analyses revealed the presence or absence of unmodified IRAK1, phosphorylated IRAK1 (p-IRAK1), or ubiquitylated IRAK1 (Ub-IRAK1). As a control for the effectiveness of proteasome inhibition, immunoblot analysis of IκB, which is degraded via the proteasome following LPS treatment, was used. Immunoblot analysis of tubulin was used as a loading control. Experiments were performed at least three times with similar results as shown.

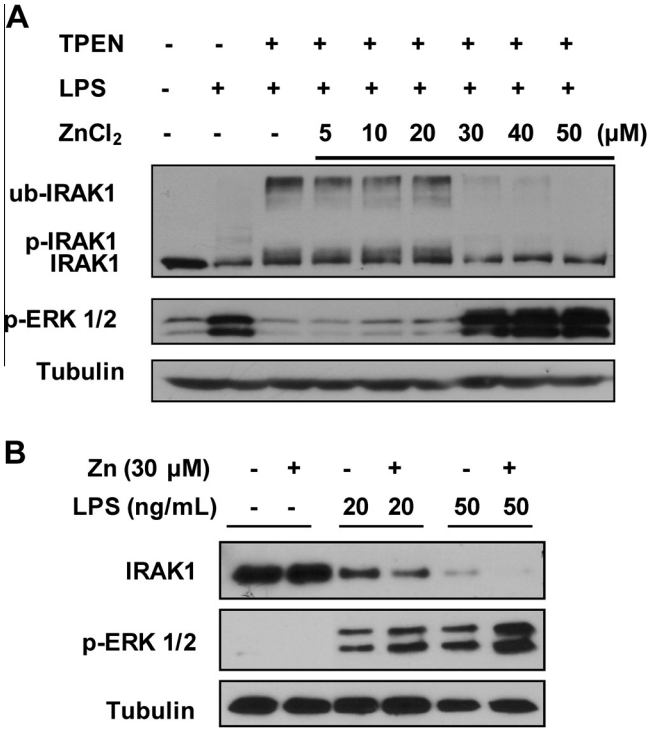

Fig. 6. LPS-induced degradation of IRAK1 is dependent on the concentration of zinc. (A) Cells were treated as described in Fig. 1B except with multiple different concentrations of zinc. (B) Cells were treated as described in Fig. 1A. Immunoblot was performed to detect the forms of IRAK1 described in Fig. 4, activated ERK (p-ERK 1/2) as described in Fig. 1, and tubulin as a loading control.

4. Discussion

To investigate potential roles for zinc in TLR4 signaling, 30 μM zinc chloride was used in these studies without the use of any zinc ionophores [33], which approximately mimics the physiological concentration of zinc in blood plasma [34,35]. This low-dose zinc treatment avoids potential complications of extended effects from high zinc concentrations [36–39].

Although zinc has previously been demonstrated to be required for proper TLR4 signaling [11,13], a potential site of action for zinc has not been elucidated. Our finding that zinc deficiency affected a broad range of protein phosphorylation in disparate pathways downstream of TLR4 (ERK1/2 MKK3/6, IKKβ, and IκB) suggested that zinc is required during early TLR4 signaling events. A requirement for zinc in LPS binding to the TLR4 receptor itself was ruled out because zinc depletion did not block phosphorylation and ubiquitylation of IRAK1. However, zinc depletion blocked IRAK1 degradation, resulting in the accumulation of phosphorylated and ubiquitylated forms of this protein.

IRAK1 is part of a membrane-associated protein complex that contains the regulatory kinases IKKα and IKKβ which become phosphorylated in response to LPS [28,29,40] (Fig. 2B). The ensuing phosphorylation and degradation of IRAK1 triggers the release of these p-IKKs to cytoplasm, which permits p-IKK-mediated phosphorylation and degradation of IκB and p105 [21]. The removal of IκB allows nuclear access of the NF-kB transcription factor, whereas the degradation of p105 releases active Tpl2, a MEK kinase, leading to the phosphorylation of ERK1/2 [21,41–43]. Thus, our finding that zinc depletion prevented IRAK1 degradation, resulting in the accumulation of the phosphorylated/ubiquitylated forms, provides a possible explanation for the inhibition of multiple pathways stemming from IRAK1.

Although our data clearly demonstrate a novel role for zinc in the degradation of IRAK1, it is important to note that zinc could have multiple sites of action in TLR4 signaling. For instance, many studies have described the ability of zinc to inhibit at least some protein tyrosine phosphatase activities, presumably by competing with the metal cofactors required for proper enzyme activity [44–47]. The general inhibition of phosphatase activity may promote the increase in protein phosphorylation upon activation of the upstream kinase(s); and in many immune signaling pathways, including the TLR4 pathway, zinc has been found to prolong tyrosine protein phosphorylation, probably due to the suppression of some phosphatases activity [10,11]. For instance, zinc has been shown to inhibit protein tyrosine phosphatase 1B (PTP1B) [47]; and PTP-1B can directly dephosphorylate the phosphor-tyrosine residue of activated MAPKs such as p38 in its activation loop [48]. Therefore, some or all of the phosphorylation events downstream

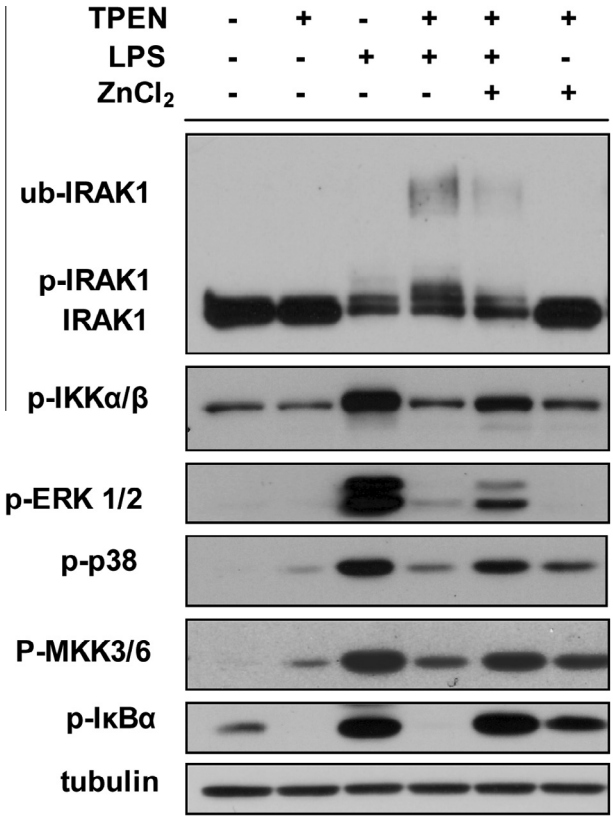

Fig. 7. Requirement for zinc in LPS-induced signaling events is also observed in primary mouse peritoneal macrophage cells. Primary mouse peritoneal macrophage cells were treated as described in Fig. 1B. Immunoblot analyses were performed as described for the RAW264.7 cells in Figs. 1, 2 and 4.

of IRAK1 may also be regulated at the level of transient phosphatase inhibition.

Our results clearly indicate that at the initial stage, the stabilization of modified IRAK1 forms during zinc deficiency occurred independently of the proteasome. Proteasome-dependent degradation typically targets Lys48-linked ubiquitylated proteins, such as IκB in response to LPS [43]. However, unlike IκB, IRAK1 has been reported to be Lys63-linked ubiquitylated, a modification essential in signal transduction but not classically associated with degradation [28,49–51]. Although the mechanisms of ubiquitylated IRAK1 degradation are poorly understood, our proteasome-independent model at the early stage of stimulation is consistent with numerous studies. For instance, using polyubiquitin linkage-specific antibodies, Newton et al. proposed that, IRAK1 is initially K63-linked polyubiquitylated upon IL-1β treatment; but after an hour of treatment, IRAK1 becomes Lys48-linked ubiquitylated [52]. Accordingly, in the first hour of stimulation, treatment with the proteasome inhibitor MG132 did not inhibit the degradation of native IRAK1 nor result in the accumulate of ub-IRAK1. Similarly, others also reported that inhibition of the proteasome was unable to inhibit IRAK1 degradation upon IL-1β treatment in IL-1R cells [29] or in 293 cells [24] in the first 30 min. Reports of proteasome-dependent IRAK1 degradation usually involve longer treatment times (1–4 h) [24,52,53]. Therefore, our results are consistent with a model where IRAK1 degradation occurs by two separate mechanisms, a proteasome-independent event in the first hour of response and a proteasome-dependent mechanism at later time points. Moreover, our results indicate the early response involves a previously unidentified zinc-dependent mechanism.

What might be the mechanism by which zinc depletion prevents degradation of ubiquitylated/phosphorylated IRAK1 during these early time points? The most likely possibility is that zinc deficiency prevents the activity of zinc-containing metalloproteases that might be required for IRAK1 degradation independently of the proteasome. It is also possible, however, that zinc may function at multiple steps in TLR4 signaling. For instance, zinc deficiency may limit the function of NEMO and TAB2, which are components of the TLR4 signaling complex that have zinc-containing ubiquitin binding domains (UBDs) essential for TLR4 signal transduction. As NEMO serves as a scaffolding protein to recruit the IKKs for phosphorylation [49,54,55], the failure of NEMO to bind through its UBD may prevent the proper recruitment of IKKβ for phosphorylation. Similarly, the zinc finger domain of TAB2 is critical for the recruitment of TAK1 for activation and subsequent IKKβ activation [56].

In summary, our findings identify a key step in TLR4 signaling that is sensitive to zinc deficiency; and in so doing, we have provided possible new directions for identifying proteasome-independent pathways for targeted protein degradation.

Author contributions

Conceived and designed the experiments: Y.W., M.J.P., S.C.P. Performed the experiments: Y.W. Analyzed the data: Y.W., M.J.P., S.C.P. Contributed reagents/materials/analysis tools: M.J.P., S.C.P. Wrote the paper: Y.W., M.J.P., S.C.P. Contributed to the interpretation of data and revisions of the drafts and final manuscript: Y.W., M.J.P., S.C.P.

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