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Differential Regulation of TLR-Dependent MyD88 and TRIF Signaling Pathways by Free Zinc Ions

Anne Brieger, Lothar Rink, and Hajo Haase

Zinc signals are utilized by several immune cell receptors. One is TLR4, which causes an increase of free zinc ions (Zn\(^{2+}\)) that is required for the MyD88-dependent expression of inflammatory cytokines. This study investigates the role of Zn\(^{2+}\) on Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF)–dependent signals, the other major intracellular pathway activated by TLR4. Chelation of Zn\(^{2+}\) with the membrane-permeable chelator N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine augmented TLR4-mediated production of IFN-β and subsequent synthesis of inducible NO synthase and production of NO. The effect is based on Zn\(^{2+}\) acting as a negative regulator of the TRIF pathway via reducing IFN regulatory factor 3 activation. This was also observed with TLR3, the only TLR that signals exclusively via TRIF, but not MyD88, and does not trigger a zinc signal. In contrast, IFN-γ-induced NO production was unaffected by N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine. Taken together, Zn\(^{2+}\) is specifically involved in TLR signaling, where it differentially regulates MyD88 and TRIF signaling via a zinc signal or via basal Zn\(^{2+}\) levels, respectively. The Journal of Immunology, 2013, 191: 1808–1817.
20% sterile-filtered L929 cell culture supernatant for a total of 8 d. On
day 4, 10 ml fresh culture medium was added. On day 7, the medium was
completely replaced. On the next day, experiments were performed.

Production of IFN-β–containing cell-free culture supernatant

The supernatants of 3T3 and 293T cells stably transduced to produce mouse
IFN-β were collected 4 d after seeding and were centrifuged for 10 min at
300 × g. Supernatants were sterile-filtered with a 0.22-μm filter (Millex-
GV; Millipore, Billerica, MA), and aliquots were stored at −80˚C until
use. The activities of the IFN-β–containing cell supernatants were
determined by an IFN-β bioassay with the L929 cell line and the Encephalo-
myocarditis virus. For all experiments, 200 U/ml IFN-β was applied.

Purification of monoclonal anti–IFN-β Abs

7FD3 hybridoma cells produce rat IgG1 anti-mouse IFN-β Abs. The cells
were grown for 3–4 d under normal culture conditions until confluency and
cultured for 3 more days in serum-free medium (21). Supernatants were
collected, sterile-filtered, and concentrated (Minicon B15 commercial
sample concentrator; Millipore). The Ab was purified using an IgG Purification Kit
(Pierce, Thermo Scientific, Rockford, IL) following the manufacturer’s
instructions and quantified using an IgG-Standard with a Bradford assay
(Bio-Rad Laboratories). The purified Ab solution was sterile-filtered with
a 0.22-μm filter (Millex-GV; Millipore), and 0.01–1 μg/ml of the Abs
applied as depicted in Fig. 5C.

Zn2+ measurements with fluorescent probes

Cells were grown on a 96-well plate and incubated in loading buffer (25 mM
HEPES [pH 7.35], 120 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.3 mM
CaCl2, 1 mM MgCl2, 1 mM Na2PO4, 0.5% BSA) for 30 min with 1 μM
FluoZin-3-AM (Invitrogen, Eugene, OR) at 37˚C. Subsequently, cells were
washed twice with measurement buffer (incubation buffer without BSA).
FluoZin-3-AM is specific for free Zn2+ concentrations were calculated as previously described (6), using
50 μM N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; Sigma-
Aldrich) to determine minimal and 100 μM ZnSO4/50 μM pyritoline to
determine maximal fluorescence, respectively. TLR ligands were pur-
 chased from Invivogen (San Diego, CA).

Western blotting

A total of 5 × 105 cells was collected by centrifugation, lysed by soni-
cation in 100 μl sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v]
SDS, 27% [v/v] glycerol, 0.1% [v/v] 2-ME, 0.01% [w/v] bromphenol blue, 1 mM Na3VO4), and heated for 3 min at 95˚C. An equivalent of 1 × 105
cells/lane was separated on 10 or 14% (H3) polyacrylamide gels at 150
V, washed twice with measurement buffer (incubation buffer without BSA).
The resulting fluorescence was recorded on a Tecan Ultra 384 fluorescence
well plate reader (Tecan, Crailsheim, Germany) using an excitation
wavelength of 520 nm in a Sunrise well plate reader (Tecan) and
neutral red uptake was quantified by its absorption at 540 nm in a Sunrise well plate reader (Tecan) and
neutral red uptake was determined by measuring the absorption at 520 nm in a Sunrise well plate reader (Tecan) and
neutral red uptake was quantified with a standard curve from 0.78 to 50 μM nitrite.

Neutral red assay

Cellular viability was measured by neutral red uptake. RAW 264.7 cells
(5 × 105 cells/ml) were grown for 24 h and treated for further 24 h with
LPS and TEPEN. Cells were loaded for 3 h with neutral red (final concen-
tration, 55 μM), washed with PBS, lysed in a mixture of ethanol/H2O/Oraftacidic
acid (50:49:1), and neutral red uptake was quantified by its absorption at
540 nm in a Sunrise well plate reader (Tecan).

Flow cytometric measurement of endocytosis

TLR4 endocytosis was measured by flow cytometry as described previously
(10). After incubation, RAW 264.7 cells were treated with anti-CD16/CD32
blocking Ab for 5 min in ice-cold PBS. Anti–TLR4-PE (clone
MTS510) or PE-conjugated rat IgG2a,κ as isotype-matched control were
added (all Abs were from BD Pharmingen, Heidelberg, Germany). Abs
and cells were incubated for 20 min at 4°C in the dark and washed twice with ice-cold PBS. Fluorescence was measured with a FACScan (Becton Dickinson, Heidelberg, Germany).

**Statistical analysis**

Statistical significance was calculated by ANOVA using GraphPad Prism software (version 5.01). For the experiments with two variables (Fig. 1B–F), two-way ANOVA was used; the remaining data were analyzed by one-way ANOVA. A p value <0.05 was considered statistically significant.

**Results**

**Role of Zn²⁺ in NO release by macrophages**

In the mouse macrophage cell line RAW 264.7, the fluorescent probe FluoZin-3 detects an elevation of free intracellular Zn²⁺ in response to LPS (Fig. 1A). Accordingly, an impact of Zn²⁺ on the TLR4-induced release of NO is investigated. To this end, the concentration of nitrite, a degradation product of NO, is taken as a measure for production of the short-lived NO. Addition of the membrane-permeable Zn²⁺ chelator TPEN before stimulation of primary murine bone marrow–derived macrophages (BMMs) with LPS (Fig. 1B) and of RAW 264.7 cells with LPS or *E. coli* (Fig. 1C, 1D) leads to a concentration-dependent increase of NO in the cell culture supernatants. Incubation of RAW 264.7 cells with equimolar concentrations of Zn²⁺ in addition to TPEN abolishes the effect of the chelator (Fig. 1C), confirming that TPEN augments NO production by chelation of Zn²⁺. In contrast, increasing the intracellular Zn²⁺ concentration by addition of Zn²⁺ together with the ionophore pyrithione is without effect (Fig. 1D).

To exclude a contribution of the other NO synthases, eNOS and nNOS, the selective iNOS inhibitors 1400W and SMT are used. Both inhibitors completely abrogate LPS-induced NO production, regardless of the presence of TPEN (Fig. 1E, Supplemental Fig. 1A). This confirms that the entire NO production in response to LPS is mediated exclusively by iNOS. Next, the effect of zinc deficiency was investigated independently from TPEN by culturing RAW 264.7 cells for 3 d either in CHELEX-treated medium (zinc depleted) or CHELEX-treated medium reconstituted with 10 μM Zn²⁺ (zinc sufficient). The NO release of RAW 264.7 cells cultivated in zinc-depleted medium is markedly higher than in cells cultivated in zinc-sufficient medium. Furthermore, addition of Zn²⁺ and pyrithione simultaneous to LPS stimulation diminishes NO production in cells grown in zinc-depleted medium (Fig. 1F).

iNOS activity is mainly regulated on the transcriptional level. Accordingly, iNOS transcription in response to LPS stimulation of RAW 264.7 cells is elevated in the presence of TPEN (Fig. 2A). Notably, these conditions do not affect cellular viability (Supplemental Fig. 1B), and iNOS transcription is unaffected by TPEN alone (Supplemental Fig. 1C). Furthermore, iNOS protein expression after LPS stimulation is also increased by TPEN in a concentration-dependent manner (Fig. 2B). Altogether, the LPS-induced NO release is increased by Zn²⁺ chelation through elevated iNOS expression.

**Impact of Zn²⁺ chelation on TLR4 signaling**

TPEN has a differential impact on the mRNA expression of LPS-inducible genes. In contrast with iNOS, the LPS-induced transcription of IL-1β, IL-6, and IL-10 is significantly reduced by incubation of RAW 264.7 cells with the chelator (Fig. 3). On the other hand, transcription of type 1 IFNs is upregulated by Zn²⁺ chelation, in particular, IFN-β (Fig. 3). Rantes transcription is only reduced by 1.25 μM TPEN, the lowest applied TPEN concentration, and there is no effect of TPEN on MCP-1 and IP-10 (Fig. 3). The transcription of the cell-surface protein CD40 is slightly increased, but the effect is not statistically significant. CD80 and CD86 transcription is significantly elevated (Fig. 3). The gene transcription pattern suggests differential effects of Zn²⁺ chelation on MyD88 and TRIF-dependent TLR4 signaling, reducing MyD88 signaling whereas promoting the TRIF-dependent pathway. Activation of the two pathways is balanced through endocytosis of...
the TLR4R complex (10). However, the LPS-induced endocytosis of TLR4 is not affected by TPEN (Fig. 4A). TRAF3-ubiquitination is another mode of balancing MyD88 and TRIF-dependent signaling. TRAF3 is ubiquitinated on Lys$^{48}$ for MyD88 signaling (with subsequent degradation), whereas it is ubiquitinated on Lys$^{63}$ for TRIF signaling (23). No TRAF3 degradation is induced in BMMs treated from 15 min to 3 h with LPS, independently of TPEN preincubation (Fig. 4B).

A time-dependent comparison between the activation of the transcription factors NF-$\kappa$B and IRF3 was made in whole cells and nuclear extracts (Fig. 4C, 4D). The MyD88-dependent early nuclear translocation of NF-$\kappa$B 30 min and 1 h after LPS stimulation is reduced in the presence of TPEN. In contrast, the TRIF-dependent delayed NF-$\kappa$B activation after 2 and 3 h is not diminished by TPEN. IRF3 phosphorylation is detected in the whole cell lysate starting at 1 h (Fig. 4D). After 2 and 3 h, phospho-IRF3 is found in the nucleus and in whole cells, but only nuclear phospho-IRF3 is increased by TPEN (Fig. 4C, 4D). Hence, Zn$^{2+}$ chelation upregulates TRIF signaling upstream of the transcription factor IRF3 and leads to increased nuclear accumulation of phospho-IRF3.

To further evaluate the time dependence of Zn$^{2+}$ chelation for the enhancement of NO release, we added TPEN at different time points relative to LPS, ranging from 5 min prior (as in the previous experiments) to 8 h after addition of the TLR ligand. The addition of TPEN 5 min before LPS stimulation doubles NO release in comparison with LPS alone, but the effect is even more pronounced at 30 min to 1 h after LPS stimulation (Fig. 4E). Starting 2 h after LPS stimulation, the effect of TPEN addition decreases slightly, but up to 8 h after LPS stimulation still increases NO release ~2.5 times compared with LPS alone. By this time, the LPS-induced zinc signal has already returned to baseline levels (Fig. 4F). Hence, the effect of Zn$^{2+}$ chelation is most effective at the time when the TRIF pathway is activated, and basal Zn$^{2+}$ levels, not the initial zinc signal, alter NO release.

Involvement of IFN-$\beta$ in the impact of Zn$^{2+}$ chelation on iNOS

The main function of IRF3 in monocytes is the induction of IFN-$\beta$. Because IFN-$\beta$ transcription is elevated by Zn$^{2+}$ chelation, the
impact of supplementation with additional IFN-β on NO release was examined. Stimulation with a high amount of IFN-β (2000 U/ml) alone does not induce the release of NO but augments LPS-induced NO release in a manner similar to TPEN. Notably, TPEN does not cause a further increase of NO production (Fig. 5A, Supplemental Fig. 2A) and iNOS protein levels (Fig. 5B; Supplemental Fig. 2B) in the presence of IFN-β. Purified rat IgG1 Abs against murine IFN-β cause a concentration-dependent reduction of LPS-induced NO release by RAW 264.7 cells treated with LPS and IFN-β alone (Fig. 5C). Together, these results imply that Zn²⁺ chelation augments the NO release through increased IFN-β levels.

IFN-β activates a JAK-STAT pathway; hence the impact of Zn²⁺ chelation on STAT1 phosphorylation was investigated. RAW 264.7 cells were preincubated with TPEN and stimulated with IFN-β. Western blot analysis of STAT1 phosphorylation shows no effect of TPEN on Tyr701 and Ser727 phosphorylation. However, there is a slight decrease in total STAT1 protein in TPEN-preincubated and IFN-β–stimulated samples (Fig. 5D, Supplemental Fig. 2C). Consequently, transcription of STAT1 mRNA after preincubation with TPEN and stimulation with LPS or IFN-β was measured. Stimulation increases STAT1 expression, which is abrogated by preincubation with TPEN (Fig. 5E, 5F, Supplemental Fig. 2D).

Role of Zn²⁺ in NO release in response to other receptors
To elucidate whether zinc signals are relevant for signaling by other TLRs in addition to TLR4, we tested a panel of different ligands. Stimulation of RAW 264.7 cells with pam3CSK4 (TLR1/2 ligand), heat-killed Listeria monocytogenes (TLR2 ligand), flagellin (TLR5 ligand), FSL-1 (TLR6/2 ligand), imiquimod and ssRNA40 (TLR7 ligands), and ODN1826 (TLR9 ligand) induces zinc signals (Fig. 6). The only examined TLR ligand unable to induce a zinc signal is poly(I:C) (TLR3 ligand; Fig. 6). Comparable observations were made using human primary monocytes (Supplemental Fig. 3). The inability of poly(I:C) to induce a zinc signal was confirmed in RAW 264.7 cells with the fluorescent probe ZinPyr1 (Supplemental Fig. 4A), and biological activity of the poly(I:C) in RAW 264.7 cells was confirmed by measuring TNF-α secretion (Supplemental Fig. 4B).

FIGURE 4. Impact of Zn²⁺ chelation on TLR4 signaling. (A) Surface expression of TLR4 after stimulation with LPS (100 ng/ml) alone or in the presence of TPEN (1.75 μM, 5 min before LPS) was measured by flow cytometry. (B) Western blot of TRAF3 protein levels in BMMs after stimulation with LPS (100 ng/ml) alone or in the presence of TPEN (1.75 μM, 5 min before LPS). (C and D) RAW 264.7 cells were stimulated for the indicated times with LPS (100 ng/ml) and TPEN (5 min before LPS). Western blots for NF-κB, phosphorylated (Ser705) and total IRF3 were performed with nuclear extracts (C) and whole cells (D), using histone H3 and β-actin as respective housekeeping genes. (E) RAW 264.7 cells were incubated with LPS (100 ng/ml), and TPEN (1.75 μM) was added at different time points relative to LPS as indicated. A Griess assay was performed with the supernatant 24 h after addition of LPS. (F) Intracellular Zn²⁺ was measured as described for Fig. 1A. After the first hour, measurements were made in 30-min intervals. Results are shown as means ± SEM of n = 3 independent experiments (A, E, F) or one representative experiment of n = 3 (B–D).
Further investigations determined the effect of Zn2+ chelation on NO release induced by another TLR that triggered a zinc signal, TLR7, and by TLR3, which is inactive with respect to zinc signaling. Comparable with LPS, the TLR7 ligand imiquimod induces the release of NO, which can be further elevated by TPEN (Fig. 7A). iNOS protein expression is also increased by incubation of imiquimod-stimulated RAW 264.7 cells with TPEN (Fig. 7B). Similar to TLR4, Zn2+ chelation affected the transcription of several genes. Whereas the mRNA levels of IL-1β and IL-6 are unaltered by TPEN, there is a significant decrease of IL-10 mRNA (Fig. 7C). Comparable with the observations with TLR4, the levels of IFN-α, IFN-β, CD40, CD80, and CD86 mRNA are increased, although the effect is only statistically significant for IFN-β, CD80, and CD86 (Fig. 7C). The effect of TPEN on TLR3-induced NO release and IFN-β mRNA transcription was also examined. NO release in response to poly(I:C) increases in a concentration-dependent manner in TPEN-treated samples (Fig. 7D). In addition, IFN-β transcription is elevated after preincubation with TPEN and stimulation with poly(I:C) (Fig. 7E). Similar to the effect on TLR4 signaling, increasing the intracellular free Zn2+ concentra-
The administration of Zn<sup>2+</sup> and pyrithione does not alter TLR3-mediated NO release or IFN-β transcription (Fig. 7D, 7E).

IFN-γ induces iNOS expression independently from IFN-β (24). In contrast with LPS, IFN-γ does not trigger a zinc signal (Fig. 8A) and IFN-γ–mediated NO production is not augmented by TPEN (Fig. 8B). Notably, the absence of TPEN-induced elevation of iNOS activity is not due to toxicity of the chelator in combination with IFN-γ (Fig. 8C). Consequently, regulation by free Zn<sup>2+</sup> is not a general feature of iNOS expression but is specific for the TRIF/IRF/IFN-β pathway.

**Discussion**

Binding of LPS to TLR4 leads to recruitment of the adaptor proteins TIRAP and MyD88, resulting in the early activation of NF-κB, triggering mRNA expression of inflammatory cytokines (25). Subsequently, the receptor complex is internalized and binds TRAM and TRIF, inducing the delayed activation of NF-κB and phosphorylation of IRF3. Phosphorylated IRF3 translocates into the nucleus and induces the transcription of IFN-β (25). In turn, IFN-β production is essential for LPS-induced iNOS expression (17), because IFN-β activates the type I IFNR and the JAK-STAT...
pathway in an autocrine and paracrine fashion (16). Together, NF-κB and STAT1 induce iNOS transcription (15).

Activation of TLR4 also triggers a zinc signal. It supports MAPK signaling through inhibition of dephosphorylation, and is required for the phosphorylation of IKKα/β and for NF-κB–dependent production of inflammatory cytokines (7). This involvement of a zinc signal in MyD88 signaling is confirmed by this study. After TLR4 or TLR7 stimulation, the transcription of NF-κB–dependent genes such as IL-1β, IL-6, and IL-10 decreased, at least to some extent, when the zinc signal was chelated by TPEN. In addition, Zn²⁺ chelation reduced the MyD88-dependent nuclear translocation of NF-κB. Nevertheless, free intracellular Zn²⁺ is not an unspecific activator of all aspects of TLR signaling. The transcription of TRIF-dependent genes, such as IFN-β and the IFN-β–induced genes CD80 and CD86, is enhanced by Zn²⁺ chelation. Moreover, the Zn²⁺ chelator TPEN increases LPS-induced NO release through upregulation of iNOS expression. Simultaneously, there is no distinct effect of Zn²⁺ chelation on the TRIF-dependent genes Rantes, IP-10, and MCP-1. All three genes are thought to be mainly induced by TRIF signaling (26), but, possibly, other signaling pathways such as PI3K, NF-κB, and the MAPK ERK are also involved in TRIF-dependent induction of Rantes, MCP-1, and IP-10 (27, 28), explaining the differential outcome.

Signaling via several homodimers and heterodimers of TLR1–9 induces zinc signals, with TLR3 being the only exception. Notably, TRL3 is also the only TLR that does not activate the MyD88 pathway (29) but is capable of inducing IFN and iNOS expression via TRIF–dependent activation of NF-κB and IRF3 (30). Hence, not only does the zinc signal selectively support MyD88-dependent signaling, it only occurs in response to stimulation of TLRs triggering that particular pathway. Nevertheless, LPS-induced zinc signals occur in BMMs isolated from MyD88 knockout mice; therefore, the adaptor molecule itself is not required for the induction of zinc signals (7).

To identify the aspect(s) of TRIF signaling that are negatively regulated by Zn²⁺, we investigated several details of the pathway. Endocytosis of TLR4 was not altered by Zn²⁺ chelating, pointing toward an impact of Zn²⁺ that is located downstream of receptor internalization. Also, TRAF3 degradation was not involved. In contrast, an increase of nuclear, but not total cellular phospho-IκB, was measured in response to Zn²⁺ chelation. This suggests that Zn²⁺ affects nuclear phosphorylation of IκB, or nuclear translocation or retention of phospho-IκB. Furthermore, TLR7–induced IFN-β and iNOS transcription also increases by Zn²⁺ chelation, but TLR7 signals via MyD88 and IRF7. IFR3 activation depends on phosphorylation by TBK1 and IKKε (31). The kinases are activated by the recruitment of TRAF3 to TRIF (32) and Lys⁶³ ubiquinitination of TRAF3 (23). IRF7, in contrast, is phosphorylated by the kinases IL-1R–associated kinase 1 and IKK-α (33). Unphosphorylated IRF3 shuttles between cytoplasm and nucleus with nuclear export being the predominant effect (34, 35). Transport proteins for IRF3 import are a subset of importin-α receptors, namely, Qip1 and KPNAA3, which associate with importin-β receptors and the small GTPase Ran. Nuclear export is mediated by chromosome region maintenance/exportin 1 (34). Phosphorylated IRF3 and IRF7 bind to the histone acetylases CREB-binding protein and p300 (35, 36), and are localized in the nucleus through retention by CREB-binding protein/p300 (35). Therefore, activation and nuclear translocation of IRF3, as well as IRF7, offer many possibilities for regulation by Zn²⁺, which should be elucidated in more detail in future investigations.

Enhancement of iNOS induction by Zn²⁺ chelation also depends on the IRF3-dependent increase in IFN-β production. NO release was almost completely abrogated by an IFN-β–specific Ab, and more importantly, TPEN was ineffective in the presence of excess IFN-β. Further downstream, a potential influence of Zn²⁺ chelation on the IFN-β–dependent activation of the STAT pathway was examined. Treatment of cells with TPEN had no effect on IFN-β–mediated STAT1 phosphorylation. A comparable result has been observed for STAT5 phosphorylation in response to stimulation of the T cell line CTLL-2 with IL-2 (37), indicating that zinc signals might not be involved in JAK/STAT phosphorylation signaling by cytokine receptors in general. In contrast, TPEN reduced STAT1 transcription in response to stimulation with LPS or IFN-β. This finding will have no impact on the signaling events described in this article because the reported half-life for STAT1 is 16 h (38). It could, however, affect long-term signaling via the IFN-βR, representing a possible feedback mechanism to limit iNOS induction in response to prolonged exposure to LPS. This effect also could influence the subsequent activation of other signaling pathways involving STAT1 phosphorylation and signals depending on the level of unphosphorylated STAT1 protein for transcriptional activity (39).

Zn²⁺ frequently mediates its effects on signal transduction by inhibiting protein tyrosine phosphatases (PTPs) (40). PTPs known to be involved in TRIF signaling are SHP-2, SHIP1, and PTP1B (41–43). Nevertheless, all three PTPs are negative regulators of TRIF signaling, and activating them by reducing free Zn²⁺ could not explain augmentation of the TRIF pathway (41–43). Moreover, the reduction of basal intracellular Zn²⁺ concentrations modifies TRIF signaling, whereas most PTP inhibition constants measured so far are higher than basal intracellular Zn²⁺ level; for example, PTP1B is inhibited by Zn²⁺ with an IC₅₀ of 17 nM (40), a concentration that is well above the basal intracellular Zn²⁺ measured in monocytes (6). Yet, there is significant divergence in the inhibition constants of different PTP for Zn²⁺. For the RPTPβ, an inhibition constant of 21 μM was recently shown, indicating...

![Diagram](image-url)
that under normal conditions, RPTPβ could exist predominantly in its Zn2+-bound inactive state, being activated by a reduction of basal Zn2+ levels (44). Consequently, there may be one or more PTPs positively regulating TRIF signaling, which are controlled by intracellular free Zn2+ in a similar manner. It was suggested that Zn2+ inhibits PTPs by binding to a catalytically active thiol in the active site (45). Other enzymes, such as deubiquitinases, also have thiols in their catalytic center with a comparably low pKa, and hence similar chemical reactivity, which is illustrated by the fact that both classes of enzymes can be inactivated reversibly by thiol oxidation (46). It is conceivable that these enzymes also have a similar reactivity toward Zn2+, making them potential molecular targets for an effect of Zn2+ in addition to PTPs.

Dendritic cell maturation in response to LPS and monocytic differentiation of myeloid precursors stimulated by calcitriol both depend on alterations of cellular zinc homeostasis, ultimately leading to reduced free Zn2+ (20, 47). This shows that Zn2+ exerts regulatory functions not only through short-term spikes, but also via modulation of basal Zn2+ levels. Both effects are combined in TLR4 signaling, balancing MyD88 and TRIF signaling (Fig. 9).

After the initial increase in free Zn2+, facilitating MyD88 signaling, the concentration returns to basal values within a few hours, but these are still sufficiently high to limit TRIF signaling.

The reaction of macrophages toward TLR stimulation is multifaceted. An immune inflammatory response is triggered through MyD88-dependent production of cytokines, such as IL-1β and IL-6. In addition, TRIFs induce an antiviral immune response through TRIF-mediated release of IFN-β (48). TLR-induced NO release has various effects. NO is involved in antimicrobial, antiviral, and antimicrobial immune defense, but can also damage host tissue and suppress beneficial as well as harmful immune responses (49). This is of particular importance for the adequate activation of innate immunity. On the one hand, TRIFs are necessary for the induction of protectively immune against infection; on the other hand, an inappropriate TRIF response is associated with acute and chronic inflammation and systemic autoimmune diseases (48). This study identifies Zn2+ ions as a signal differentially affecting all of these functions at multiple levels, hereby fine-tuning TLR signaling.

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Disclosures

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