The Journal *of* Immunology

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Zinc Signals Are Essential for Lipopolysaccharide-Induced Signal Transduction in Monocytes[1]

Hajo Haase,* Julia L. Ober-Blöbaum,[†] Gabriela Engelhardt,* Silke Hebel,* Antje Heit,[‡] Holger Heine,[§] and Lothar Rink[2]*

Cytosolic alterations of calcium ion concentrations are an integral part of signal transduction. Similar functions have been hypothesized for other metal ions, in particular zinc (Zn^{2+}), but this still awaits experimental verification. Zn^{2+} is important for multiple cellular functions, especially in the immune system. Among other effects, it influences formation and secretion of proinflammatory cytokines, including TNF-α. Here we demonstrate that these effects are due to a physiological signaling system involving intracellular Zn^{2+} signals. An increase of the intracellular zinc ion concentration occurs upon stimulation of human leukocytes with _Escherichia coli_, LPS, Pam$_3$CSK$_4$, TNF-α, or insulin, predominantly in monocytes. Chelating this zinc signal with the membrane permeable zinc-specific chelator TPEN (_N,N,N′,N′_-tetrakis-(2-pyridyl-methyl)ethylenediamine) completely blocks activation of LPS-induced signaling pathways involving p38 MAPK, ERK1/2, and NF-κB, and abrogates the release of proinflammatory cytokines, including TNF-α. This function of Zn^{2+} is not limited to monocytes or even the immune system, but seems to be another generalized signaling system based on intracellular fluctuations of metal ion concentrations, acting parallel to Ca^{2+}. _The Journal of Immunology,_ **2008, 181: 6491–6502.**

Zinc is an essential trace element for immune function and is indispensable for an adequate immunological response to all pathogens (1). Recent reports indicate that zinc is required for the production of C-C chemokines from airway epithelium cells (2) and the activation of mast cells via FcεRIs (3–5). In contrast, stimulation of dendritic cells with LPS (3), a component of the cell wall of Gram-negative bacteria, affects expression of the zinc transporters ZIP-6 and ZIP-10 and ZnT-1, ZnT-4, and ZnT-6; an event that is required for the LPS-induced increase in MHC class II surface expression (6). So far, zinc ions have been primarily regarded as a static component of zinc enzymes and transcription factors (7). However, another role for this metal ion has been suggested, with the nonbound or loosely protein bound, so-called free zinc being a component of cellular signal transduction. Although an effect of in vitro zinc treatment on several signaling pathways has already been described in mammalian cells (8), and in particular in monocytes (9), a physiological function for zinc ions in signal transduction remains to be shown.

Several cells of the immune system sense certain components of pathogens, so-called pathogen associated molecular patterns, via specific receptors. One of them is TLR4, the receptor for LPS. TLR4 is expressed on monocytes/macrophages and to a lesser extent also on neutrophil granulocytes and B cells. Upon extracellular assembly of a complex of LPS with LPS binding protein, CD14, MD-2 and the extracellular domain of TLR4, intracellular signaling cascades are triggered via the Toll/IL-1R domain of TLR4, activating the MyD88- and Toll/IL-1R domain–containing adaptor-inducing IFN-β (TRIF)[3]-dependent pathways (10). By this mechanism, LPS stimulates several signaling pathways, including MAPK, protein kinase C (PKC), PI3K, Src family tyrosine kinases, and NF-κB (10–14). A major reaction of monocytes to LPS stimulation is the release of proinflammatory cytokines like TNF-α, IL-1β, and IL-6. It was shown that zinc influences the production of these proinflammatory cytokines, but those effects were somewhat contradictory depending on the concentration of zinc that was used. Some observations indicated that zinc treatment inhibits the production of these cytokines (15, 16). This reduction is mediated by inhibition of cyclic nucleotide phosphodiesterases by relatively high zinc concentrations (17, 18). Other reports describe zinc as an activator of proinflammatory cytokine production and that it acts synergistically with LPS if applied simultaneously at substimulatory concentrations (19, 20). The underlying molecular mechanism for this effect remains unknown.

This study investigated whether LPS-induced signaling involves physiological alterations of intracellular free zinc ions that participate in signal transduction, comparable to what has been shown for calcium many years ago. We found that incubation with LPS, as well as several other stimuli, triggers an increase in intracellular available zinc. This zinc signal is necessary for LPS-induced MAPK and NF-κB activation and transcription and release of proinflammatory cytokines.

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[3] Abbreviations used in this paper: TRIF, Toll/IL-1R domain–containing adaptor-inducing IFN-β; PKC, protein kinase C; pNPP, _p_-nitrophenol phosphate; PTP, protein tyrosine phosphatase; eGFP, enhanced GFP; IKK, IκB kinase.

FIGURE 1. Free zinc levels in leukocytes after stimulation of different receptors. Whole blood was loaded with the zinc-specific fluorescent probe FluoZin-3 and the zinc-dependent fluorescence recorded by flow cytometry for monocytes (■), lymphocytes (▨), and granulocytes (dark gray) after stimulation with *E. coli* (*A*), LPS (*B*), Pam_3CSK_4 (*C*), TNF-α (*D*), or insulin (*E*). Data are shown as free zinc compared with untreated control cells. Results represent mean of at least *n* = 6 different donors. *, *p* < 0.05 for data significantly different from controls determined by ANOVA/Tukey's honestly significant difference (HSD). *F*, Mono Mac1 cells were loaded with Zinquin or FluoZin-3, respectively. Intracellular distribution of fluorescence in control cells and after stimulation with LPS (500 ng/ml) for 30 min was documented by fluorescence microscopy. *G*, RAW 264.7 murine macrophages were grown in a 96-well plate and loaded with FluoZin-3. The fluorescence was recorded in 2-min intervals. After 10 min of recording of the baseline, cells were treated with buffer, 250 ng/ml, or 1 μg/ml LPS, and the resulting fluorescence was recorded for an additional 50 min. One representative experiment is shown as the mean of triplicates ± SD of *n* = 3 experiments conducted.
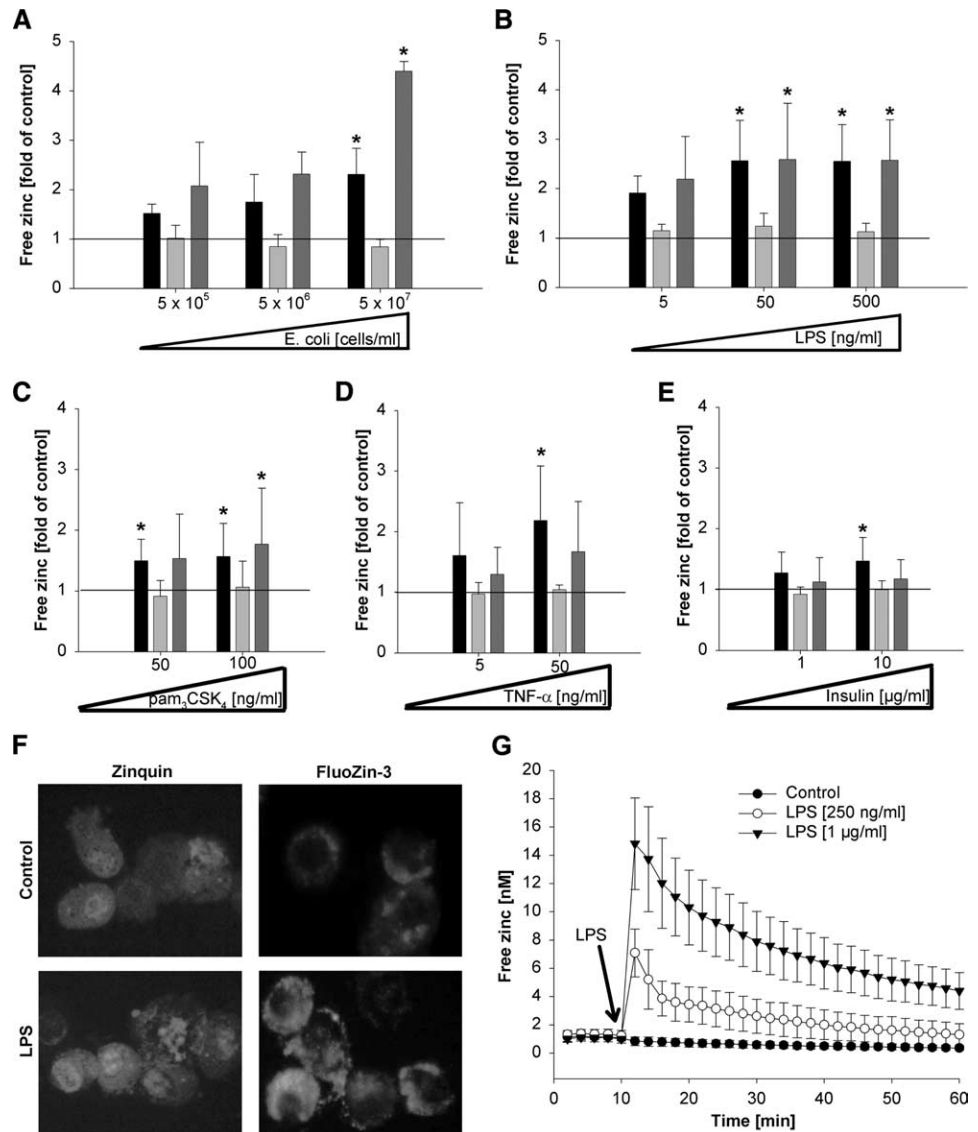
Materials and Methods

Isolation and culture of primary human cells

PBMC were isolated from heparinized peripheral venous blood from healthy donors by centrifugation over Ficoll-Hypaque, washed three times with PBS, and resuspended in RPMI 1640 medium containing 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). For enrichment of monocytes, PBMC were seeded on 100-mm-diameter plastic culture dishes at a density of 2×10^6 cells/ml and incubated for 3 h at 37°C and 5% CO_2. Nonadherent cells were removed by washing with medium at 37°C.

Leukocytes, consisting of granulocytes, monocytes, and lymphocytes (T cells, B cells, and NK cells), were isolated from heparinized whole blood by addition of 1 part of a 6% hydroxyethyl starch solution to 2 parts of blood and sedimentation for 30 min at room temperature, followed by two washes of the leukocyte fraction with PBS before transfer into culture medium.

Isolation of primary murine splenocytes

The 10-wk-old mice with knockouts for TLR4 signaling adaptor proteins MyD88 or TRIF on a C57BL/6 background were used. All experiments were performed in parallel with age- and sex-matched wild-type animals. The animals were sacrificed by cervical dislocation and the spleens were removed and placed in 100 mm diameter petri dishes containing 5-ml RPMI 1640 with 10% FCS. To obtain the splenocytes, the spleens were repeatedly pressed against the bottom of the dish with the upper end of the plunger of a single-use syringe. The suspension was filtered through

sterile gauze, followed by hypotonic lysis of erythrocytes. Splenocytes were washed twice with culture medium and immediately used for the experiments.

Ion measurements with fluorescent probes

Cultured cells were loaded in loading buffer (25 mM HEPES (pH 7.35), 120 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 0.3% BSA) for 30 min, either with 1 μM FluoZin-3-AM or 1 μM Fluo-4-AM (both from Invitrogen) at 37°C. Subsequently, cells were washed twice with measurement buffer (incubation buffer without albumin) and transferred into a 96-well plate at a density of 2×10^6 cells per ml. The resulting fluorescence was recorded on a Tecan Ultra 384 fluorescence well plate reader using an excitation wavelength of 485 nm and measuring the emission at 535 nm. Intracellular free zinc concentrations were calculated as previously described (21), using 50 μM TPEN to determine minimal and 100 μM $ZnSO_4$/50 μM pyrithione to determine maximal fluorescence, respectively.

For measuring extracellular zinc, cells were transferred into measurement buffer without dye loading. FluoZin-3 free acid, the zinc-sensitive membrane-impermeable form of this dye, which is normally formed after intracellular cleavage of the acetoxymethylester by unspecific esterases, was directly added to the measurement buffer to a final concentration of 1 μM.

For fluorescence microscopy, cells were loaded with FluoZin-3-AM ester (1 μM), or Zinquin ethyl ester (25 μM) in RPMI 1640 for 30 min at 37°C. Cells were stimulated for 30 min with LPS and fluorescence was
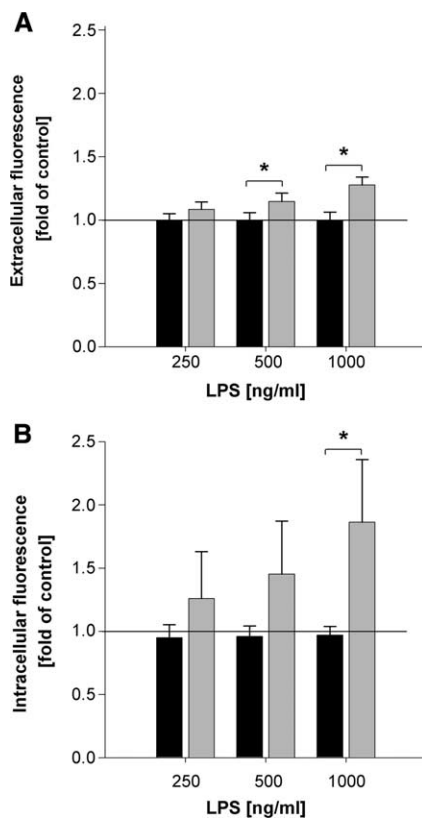
FIGURE 2. Intracellular and extracellular zinc levels after treatment with LPS. The human monocytic cell line Mono Mac1 was supplemented with the nonmembrane permeable FluoZin-3 free acid in the measurement buffer (*A*) or loaded with FluoZin-3-AM (*B*), and the zinc-dependent fluorescence was recorded before (■) and 30 min after addition of LPS (▨). No baseline drift was observed during these 30 min. Results were normalized to fold of untreated controls. Data are presented as mean ± SD of $n = 3$ independent experiments, performed in triplicates. ∗, $p < 0.05$ for results significantly different from controls determined by Student's t test.

FIGURE 3. Distinction between zinc and calcium signals. Cells were loaded with the zinc probe FluoZin-3 (*A*) or the calcium probe Fluo-4 (*B*) to monitor the levels of intracellular free ions. Results shown are of an additional untreated control (●) and of cells treated with either PMA (25 ng/ml) (○) or PHA (1 μg/ml) (▼) 10 min after the start of the experiment. One representative experiment of mean of triplicates ± SD of $n = 3$ experiments is shown.

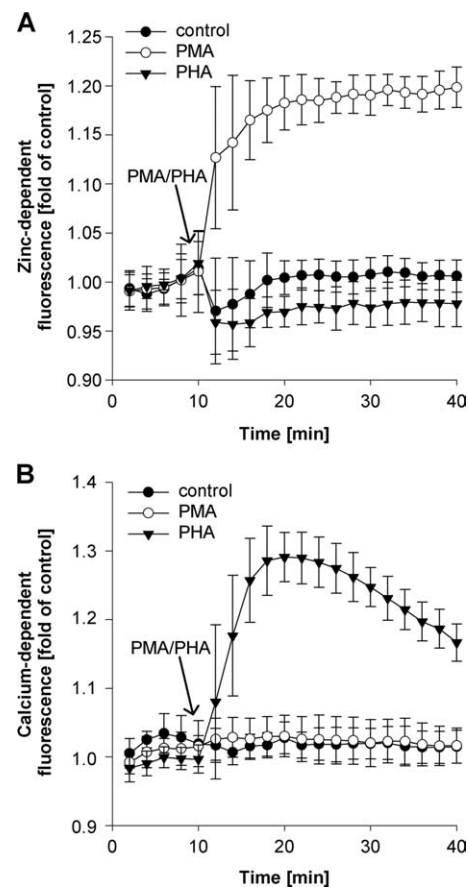monitored with a Zeiss Axioskop and photographed at ×40 magnification using a Nikon Coolpix 4500 digital camera.

For zinc measurements in primary leukocytes, heparinized whole blood from healthy, consenting donors was loaded with FluoZin-3-AM ester (10 μM) for 30 min at 37°C, and incubated for another 30 min as indicated in each experiment. After erythrocyte lysis with BD FACS lysing solution (BD Biosciences), cells were analyzed by flow cytometry using a BD FACSCalibur flow cytometer as previously described (21). Forward and side light scatter were used to distinguish monocyte, lymphocyte, and granulocyte populations. The minimum fluorescence was determined after addition of TPEN (200 μM), maximum fluorescence after addition of zinc (1 mM) and the ionophore pyrithione (50 μM).

Cytokine quantification

Before the incubations specified in the experiments, cells were seeded at a density of 1×10^6 cells/ml in 24-well tissue culture dishes overnight. Whole blood was used within one hour after the samples were taken. To measure cytokine release, samples were spun at 15,000 g for 1 min; supernatants were harvested and stored at −80°C. For measurement of cellular cytokine content, pellets were lysed in 50 mM Tris-HCl (pH 7.4), containing 1% (w/v) Triton X-100, 300 mM NaCl, 5 mM EDTA, and 0.02% (w/v) sodium azide. For quantification of TNF-α, IL-1β, and IL-6, ELISAs from BD Biosciences were used.

Measurement of phagocytosis and oxidative burst

Heparinized whole blood (100 μl) was incubated for 20 min at 37°C with *Escherichia coli* (strain BL-21) that had been transformed with the vector pGEX-4T-1 containing the GFP gene. Dihydroethidine (final concentration, 2.5 μM) was added and the incubation continued for another 10 min. BD FACS lysis solution was added and cells were washed with PBS and analyzed by flow cytometry. Forward and side light scatter were used to distinguish between monocyte and granulocyte populations.

Measurement of NF-κB activity

Cells were stimulated for 1 h as indicated in each experiment and NF-κB activity was measured with the Trans NF-κB p50 transcription factor assay kit (Active Motive). The ELISA-based method uses 96-well plates with immobilized NF-κB consensus site DNA. Detection of bound NF-κB is mediated by an HRP-coupled Ab against the NF-κB p50 subunit. The assay was performed according to the manufacturer's instructions.

Western blotting

After the incubation, cells were collected by centrifugation and lysed in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.1%(v/v) 2-ME, and 0.01% (w/v) bromophenol blue. An equivalent of 2×10^5 cells per lane was separated on 10% polyacrylamide gels at 150 V and blotted to nitrocellulose membrane. Uniform loading of gels was confirmed by staining with Ponceau S. After destaining, membranes were blocked overnight with TBS-T (20 mM Tris-HCl (pH 7.6), 136 mM NaCl, 0.1% (v/v) Tween 20) containing 5% fat-free dry milk, and incubated with the primary Abs (all from New England Biolabs) at 1/1000 dilution in TBS-T containing 5% BSA for 3 h. Subsequently, membranes were washed three times with 25 ml TBS-T and incubated for 1 h with goat anti-rabbit-HRP or goat anti-mouse-HRP followed by detection with LumiGlo Reagent (New England Biolabs). Density of the 120 kD band (see Fig. 9E) was determined with Quantity One Software (version 4.3.0; Bio-Rad).

FIGURE 4. Role of zinc in cytokine expression and release. *A*, Mono Mac1 cells were incubated for 4 h with the ionophore pyrithione (10 μM) and zinc sulfate as indicated, and TNF-α mRNA expression was analyzed by RT-PCR using β-actin as a housekeeping gene. *B*, TNF-α mRNA expression was monitored as in *A* after LPS stimulation (250 ng/ml) of untreated Mono Mac1 cells and after preincubation with the zinc chelator TPEN (2.5 μM, 30 min before the addition of LPS). *C*, TNF-α secretion measured by ELISA in the culture supernatant of Mono Mac1 cells after 4 h of incubation. Data shown are mean $\pm$ SD ($n = 3$ donors). $*$, $p < 0.05$ for relevant statistically significant changes determined by ANOVA/Tukey's honestly significant difference. *D*, The amount of TNF-α, IL-1β, and IL-6 was measured by ELISA in the culture supernatants of PBMC stimulated for 4 h with LPS (250 ng/ml) alone or after preincubation with TPEN (2.5 μM, 30 min before LPS). Results shown are mean $\pm$ SD ($n = 3$ donors). *E*, Intracellular available zinc was measured in Mono Mac1 cells loaded with FluoZin-3. Fluorescence was monitored in 10-min intervals for 60 min either in untreated control cells (●), after addition of zinc sulfate (1 μM) (○), or 1 μM zinc sulfate in the presence of pyrithione (10 μM) (▼). Results shown are mean $\pm$ SD ($n = 6$ donors). *F*, Cytokine release from Mono Mac1 cells after stimulation with LPS (250 ng/ml) alone, in the presence of zinc sulfate (1 μM), or in the presence of zinc sulfate (1 μM) and pyrithione (10 μM). Results shown are mean $\pm$ SD ($n = 3$ donors). Significant differences at $p < 0.05$, determined by ANOVA/Tukey's honestly significant difference, are indicated by different letters.
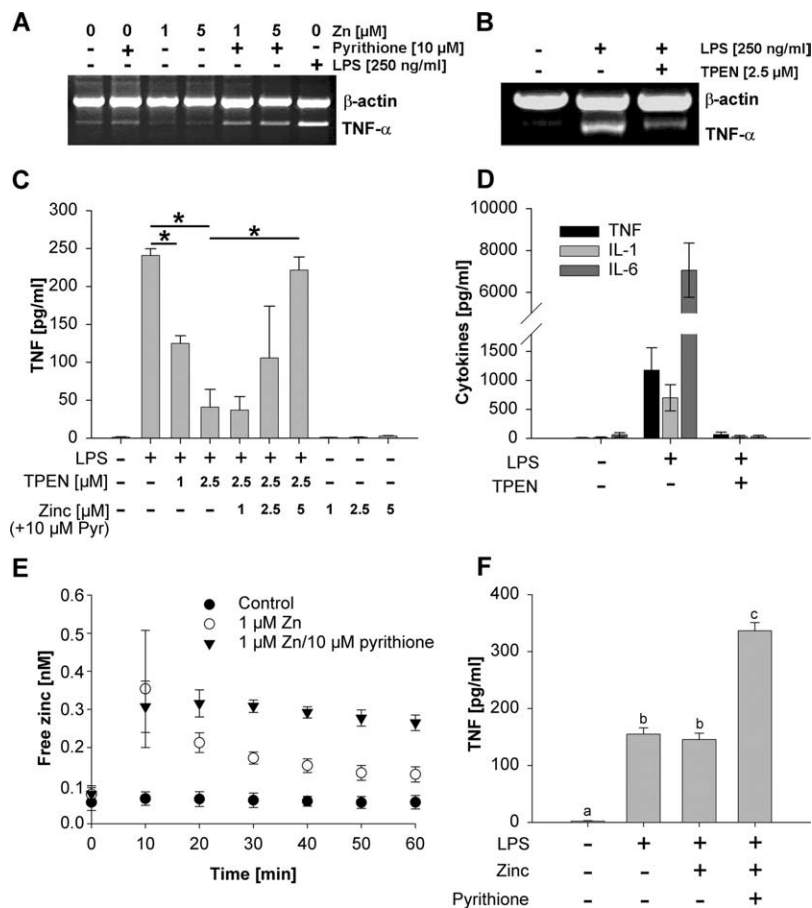
The influence of zinc on tyrosine kinase activity was investigated as previously described (22). Briefly, 2×10^7 cells were lysed by sonication with a Vibra Cell sonicator (Sonics & Materials) in 4 ml of ice-cold buffer (20 mM HEPES/NaOH, 20 mM MgCl$_2$ (pH 7.5)). After the times indicated in each experiment, aliquots were taken, mixed with 5X electrophoresis sample buffer and immediately heated to 95°C for 3 min. Tyrosine phosphorylation in these samples was investigated by Western blotting as described, using pTyr 100 anti-phosphotyrosine Ab (New England Biolabs).

Measurement of phosphatase activity with p-nitrophenol phosphate (pNPP)

A total of 2×10^6 cells/ml were lysed by sonication in buffer (20 mM HEPES/NaOH, 20 mM MgCl$_2$ (pH 7.5)). Cell lysate was given on a 96 multiwell plate (100 μl/well) and preincubated with different concentrations of zinc sulfate for 5 min. The reaction was started by addition of pNPP (1 mM) and performed at room temperature under gentle shaking. After 1 h, the reaction was stopped by addition of 100 μl NaOH (1 M) per well. The formation of p-nitrophenolate was quantified by its absorption at 405 nm.

RT-PCR

After incubation as indicated in each experiment, total RNA was isolated using the Qiagen RNeasy kit and cDNA was prepared using the Promega Reverse Transcription System according to the manufacturer's instructions. PCR was performed using HotStarTaq DNA polymerase (Qiagen) in a final volume of 50 μl using 50 ng of cDNA with the following parameters: 95°C for 15 min, 30 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 1 min, extension at 72°C for 3 min with a 5-min extension at the last cycle. Primer for β-actin (23) and TNF-α (24) were added at final concentrations of 0.8 μM (TNF-α) and 0.1 μM (β-actin). After PCR amplification, 10 μl of each sample were separated by electrophoresis on a 2% agarose gel containing 4 ng/ml ethidium bromide.

Cell viability assay

To measure cellular viability, cells were treated as indicated in each experiment and subsequently incubated with propidium iodide (10 μg/ml in

PBS) for 10 min at 4°C in the dark. The dye is membrane impermeable in intact cells, and stains dead cells as a result of the loss of plasma membrane integrity. Staining was detected by flow cytometry, using a FACSCalibur flow cytometer.

TNF promoter reporter assay

Murine RAW 264.7 macrophages stably transfected with the human TNF promoter in front of the gene for enhanced GFP (eGFP) were grown and incubated in DMEM containing 1 mg/ml glucose, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 400 μg/ml G418, at 37°C, 5% CO$_2$ and 100% humidity. Cells were plated at a density of 2×10^5/ml 24 h before the incubations. After the incubation, cells were cultured for another 24 h, dead cells labeled by staining with propidium iodide, and the eGFP fluorescence was measured by flow cytometry using a FACSCalibur flow cytometer. The fluorescence of untransfected RAW cells was subtracted.

Statistical analysis

Statistical significance of experimental results was calculated by Student's *t* test using Sigma Plot software or in case of multiple comparisons by one-way ANOVA followed by Tukey's honestly significant difference post hoc test using SPSS 15.0. All experiments presented have been performed at least three times independently with comparable results.

Results

Alterations of intracellular free zinc

If zinc ions have a function in signal transduction, zinc signals, i.e., alterations of the concentration of intracellular free zinc, would have to occur in response to stimulation. In leukocytes from whole blood that had been loaded with FluoZin-3, a fluorescent probe for zinc, a rise in free zinc was measured in response to treatment with *E. coli* (Fig. 1*A*). The increase was dependent on the amount of bacteria, and was observed in
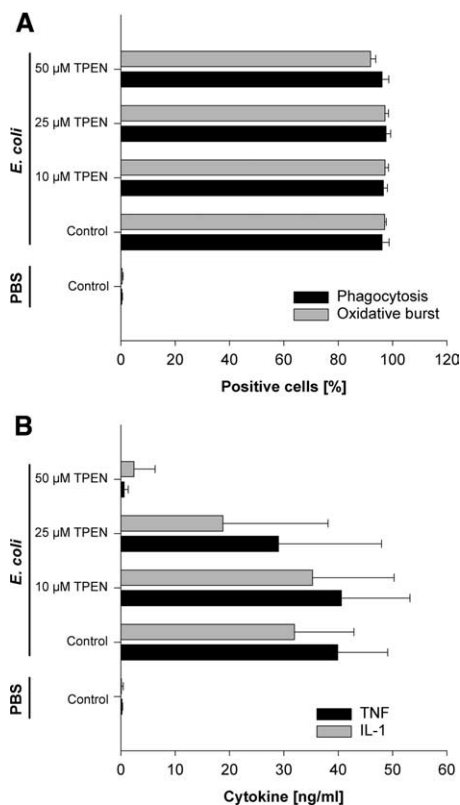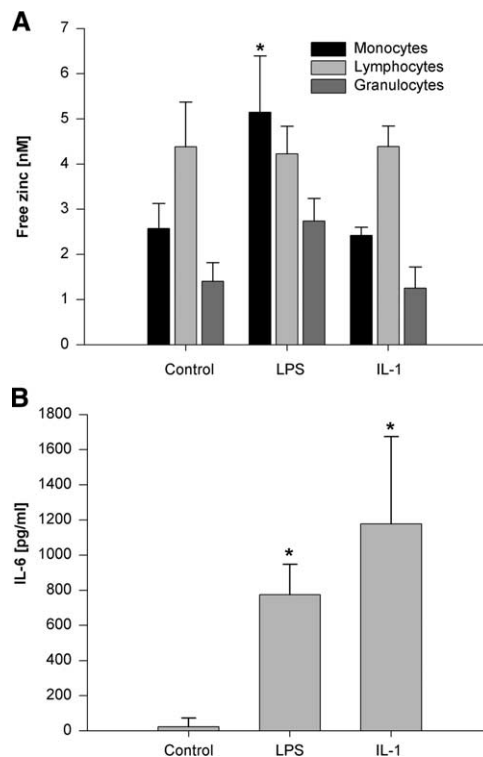
FIGURE 5. Effects of TPEN on phagocytosis and cytokine secretion in monocytes from whole blood. Heparinized whole blood was incubated with TPEN (10–50 μM) for 30 min before addition of GFP-expressing *E. coli*. *A*, Samples were loaded with the oxidation sensitive pro-fluorophore dihydroethidine, and phagocytosis and oxidative burst were measured by flow cytometry. Results are shown for monocytes only. *B*, After 4 h, cells were removed by centrifugation and the concentrations of TNF-α and IL-1β in the plasma were determined by ELISA. Data are mean $\pm$ SD from $n = 4$ healthy donors.

FIGURE 6. Effect of IL-1 on free zinc. *A*, Whole blood was loaded with the zinc-specific fluorescent probe FluoZin-3 and the resulting fluorescence recorded by flow cytometry. Available zinc in monocytes (■), lymphocytes (▥), and granulocytes (dark gray) is shown after stimulation with LPS (10 ng/ml) and recombinant human IL-1β (50 ng/ml). Results represent mean of $n = 4$ different donors. ∗, $p < 0.05$ for results significantly different from controls determined by ANOVA/Tukey's honestly significant difference. *B*, Leukocytes were incubated with LPS (10 ng/ml) and recombinant human IL-1β (50 ng/ml) for 24 h, and IL-6 in the supernatants was quantified by ELISA. Results represent mean of $n = 3$ different donors, each measured in duplicates. ∗, $p < 0.05$ for results significantly different from controls determined by ANOVA/Tukey's honestly significant difference.

monocytes and granulocytes, but not lymphocytes (Fig. 1*A*). This observation indicates that the zinc signals might be triggered by pattern recognition receptors, which are predominantly expressed on cells of the innate immune system.

One prominent group of such receptors is the family of TLRs. When leukocytes were stimulated with LPS or Pam$_3$CSK$_4$, which activate TLR4 or the heterodimer of TLR1/2, respectively, both substances induced a concentration dependent increase in free zinc, which was more prominent with LPS (Fig. 1, *B* and *C*). Triggering zinc signals is not limited to TLRs, stimulation of the receptors for TNF and insulin also resulted in higher levels of this metal ion, an effect that was only significant in monocytes (Fig. 1, *D* and *E*). The intracellular distribution after treatment with LPS was monitored in the monocytic cell line Mono Mac1 either with FluoZin-3 or Zinquin, another fluorescent probe for zinc (Fig. 1*F*). Both dyes yield different distribution patterns of zinc-dependent fluorescence, probably resembling differential cellular compartmentation of the probes. Treatment with LPS does not change the patterns of intracellular zinc distribution detected with both dyes, but rather increases free zinc in the entire cell.

When LPS was used to stimulate the murine macrophage cell line RAW 264.7 (Fig. 1*G*) or the human monocytic cell line Mono Mac1 (data not shown), the maximal zinc signal occurred within less than 2 min, but might be even faster because earlier time

points could not be resolved with the experimental setup, demonstrating a rapid zinc release in response to LPS.

Next, we analyzed whether the increase of free zinc is a result of uptake from the extracellular environment. Measurements with intracellular and extracellular probes both showed an increase after stimulation with LPS, demonstrating that zinc is not taken up from the outside, but is released from intracellular stores, with a small amount even exported into the surroundings (Fig. 2).

A contamination of the stimuli with zinc cannot account for the intracellular increases observed after stimulation. All substances were tested with FluoZin-3 in cell-free experiments (data not shown) and none of the stock solutions contained detectable amounts of zinc with the exception of insulin, but in this experiment the concentration was significantly below 10 μM. This concentration did not cause a significant change in available zinc when added directly to whole blood (data not shown), most likely due to the high zinc-buffering capacity of serum proteins.

Fluorescent probes are not absolutely specific for one metal ion, but detect multiple ions with different sensitivity. Hence, it was crucial to exclude that the signals observed in this study were caused by calcium. We and others have shown that FluoZin-3 does not detect calcium (21, 25). To confirm this specificity in intact cells, Jurkat cells were loaded either with FluoZin-3 to measure Zn^{2+} or with Fluo-4 to detect Ca^{2+} and stimulated with PMA or PHA (Fig. 3). The Zn^{2+} concentration increased in these cells after

FIGURE 7. Role of MyD88 and TRIF in zinc signaling. Freshly isolated murine splenocytes were loaded with FluoZin-3 and the resulting fluorescence was measured in a well plate reader. After 10 min of recording of the baseline, LPS (1 μg/ml) was added. One representative experiment of $n = 3$ for each knockout is shown as mean $\pm$ SD of three replicates. A and B, Comparison of wild-type littermates and MyD88$^{-/-}$ mice. C and D, Comparison of wild-type littermates and TRIF$^{-/-}$ mice. E, Primary human leukocytes were loaded with FluoZin-3 and free intracellular zinc was measured after 30 min of incubation with either 20 μg/ml HTA-125 anti-TLR4 Ab or IgG2a isotype control (both Abs were azide-free and contained low endotoxin). Data shown are mean of $n = 3$ donors. *, $p < 0.05$ for results significantly different from controls determined by Student's t test. F, Primary human leukocytes were incubated with 0.5 ng/ml LPS and 10 μg/ml HTA-125 (added 15 min before LPS), and the release of TNF-α or IL-1β was measured by ELISA after 4 or 24 h, respectively. Data shown are mean from $n = 3$ donors, presented as a percentage of inhibition of the LPS-induced effect. *, $p < 0.05$ for results significantly different from controls determined by Student's t test.
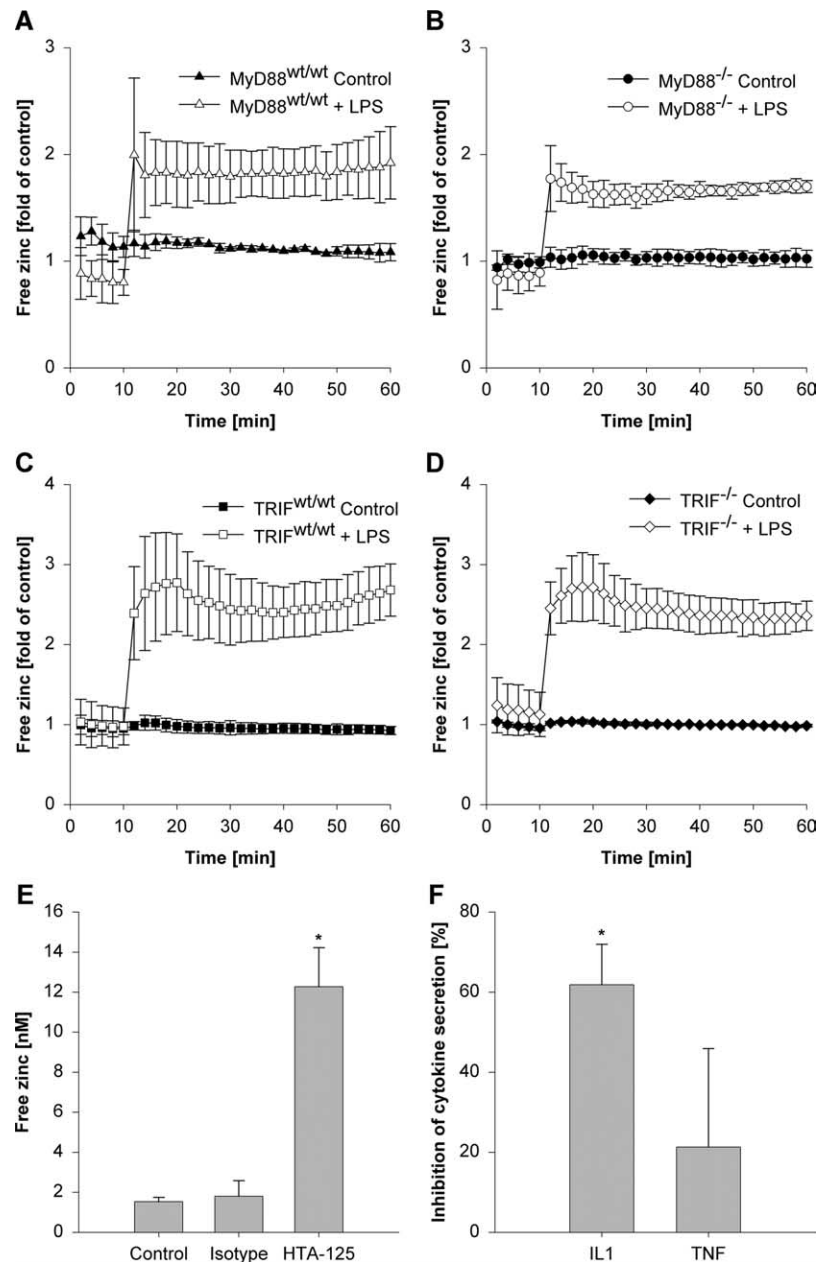
stimulation with PMA, but no effect on the calcium ion concentration was observed. Conversely, PHA did not change free zinc, but caused a rise in intracellular Ca^{2+} concentration, confirming that zinc and calcium are two independent signals, triggered by different stimuli.

Role of zinc in LPS-induced cytokine release

To clarify whether the fluctuations of free zinc after stimulation with LPS are part of the LPS-induced signal transduction we investigated its role in the expression and secretion of the proinflammatory cytokine TNF-α. Raising the intracellular free zinc by adding zinc in combination with the ionophore pyrithione to the culture medium increased TNF-α mRNA in Mono Mac1 cells, whereas addition of extracellular zinc was without effect in the absence of the ionophore. Pyrithione had only a small effect when applied alone, most likely due to the zinc background that was already present in the cell culture medium (Fig. 4A). Chelation of zinc with TPEN (N,N,N',N'-tetrakis-(2-pyridyl-methyl)ethylenediamine), a membrane-permeant zinc-specific chelator, blocked the LPS-

induced increase of TNF-α mRNA (Fig. 4B), and the release of TNF-α from Mono Mac1 cells (Fig. 4C). The latter effect was reversed by addition of zinc and pyrithione, indicating that TPEN acts via its function as a chelator for zinc and not by any other mechanism.

Although incubation with zinc and pyrithione did induce TNF-α mRNA in Mono Mac1 cells, it was not sufficient to cause secretion of TNF-α protein into the supernatant when applied in the absence of LPS (Fig. 4C). TNF-α is not the only cytokine depending on free zinc for its expression. When PBMC were pretreated with TPEN, LPS-triggered TNF-α, IL-1β, and IL-6 secretion were completely blocked (Fig. 4D).

Fig. 4A indicated that only intracellular changes of free zinc affect LPS-induced TNF-α mRNA transcription. As shown in Fig. 4E, zinc added to the culture medium was quickly taken up, but due to the homeostatic equilibrium of uptake and export, nearly normal levels were restored in less than 1 h. In the presence of the ionophore pyrithione, available intracellular zinc remains on a higher level. LPS-induced TNF-α secretion was not altered by simultaneous addition of 1 μM zinc to the culture

medium alone. When LPS was added together with zinc and pyrithione, TNF-α release was doubled (Fig. 4*F*), confirming that zinc has to be present within the cells to interact with LPS-induced signaling.

Two other functions of monocytes, phagocytosis and oxidative burst, were measured in whole human blood by flow cytometry with GFP-expressing *E. coli* and the oxidation sensitive pro-fluorophore dihydroethidine, respectively. No significant effect on either function was observed in monocytes (Fig. 5*A*) or granulocytes (data not shown) after preincubation with up to 50 μM TPEN. When the supernatant of these samples was analyzed for TNF-α and IL-1β, TPEN inhibited the cytokine secretion induced by the addition of *E. coli* (Fig. 5*B*). Compared with Mono Mac1 and PBMC, higher concentrations of TPEN were necessary to suppress cytokine secretion in whole blood. This effect is probably due to the higher amount of zinc present in the samples, resulting from serum and erythrocyte zinc as well as higher cell numbers.

*Characterization of signals that regulate free zinc*

Even though the IL-1R and TLR4 both trigger the MyD88-dependent signaling pathway (26), IL-1 stimulation had no effect on free zinc in leukocytes (Fig. 6*A*), although in both cases secretion of IL-6 was observed (Fig. 6*B*). Therefore, we tested the involvement of the two main adaptor proteins of TLR4 in triggering the zinc signal, by measuring these signals in splenocytes isolated from MyD88$^{-/-}$ or TRIF$^{-/-}$ mice in comparison to wild-type animals (Fig. 7, *A–D*). There was a clear signal in splenocytes from both knockouts in response to LPS stimulation, suggesting that alterations in free zinc are independent of MyD88 and TRIF.

To assess whether the LPS-induced signal involves TLR4, despite a lack of involvement of the two major signaling pathways, we used a TLR4-specific Ab (HTA-125). It triggered a strong increase of free zinc in primary human leukocytes, whereas the isotype (mouse IgG2a) control was ineffective (Fig. 7*E*). HTA-125 is regarded as a blocking Ab for TLR4 and it did block LPS-induced IL-1β production and, to a lesser extent, TNF-α (Fig. 7*F*).

To elucidate whether any LPS-activated downstream signaling pathways are involved in the increase of intracellular free zinc, we incubated the cells with different inhibitors for these pathways 30 min before treatment with LPS. U0126, SB202190, and genistein, which inhibit MEK1/2, p38 MAPK, and tyrosine kinases, respectively, all inhibited the production of TNF-α significantly, but U0126 and SB202190 were without a significant effect on the zinc signal (Fig. 8*A*). When genistein was used, the LPS-induced zinc signal was blocked. This response indicates a function for tyrosine phosphorylation in the increase of intracellular zinc.

PKC activation by PMA has been shown to cause zinc signals (27), and in monocytes these can be inhibited by the PKC inhibitor bisindolylmaleimide II (21). Preincubation with this inhibitor did not affect the LPS-induced zinc signal in RAW cells (Fig. 8*B*) and blocked LPS-induced TNF-α by ~40%, although this effect was not statistically significant. Conversely, TNF-α production in response to PMA was effectively blocked by bisindolylmaleimide II or TPEN (Fig. 8*C*), confirming that the inhibitor did inhibit PKC, and showing that PMA-induced cytokine secretion can also be inhibited by zinc chelation.

All inhibitors had no effect on cell viability at the tested concentrations (data not shown). The inhibitors could potentially interfere with the detection of zinc by the fluorophore, rather than affect cellular zinc levels. Therefore, cell-free controls have been performed with FluoZin-3 free acid, the form of FluoZin-3 that is
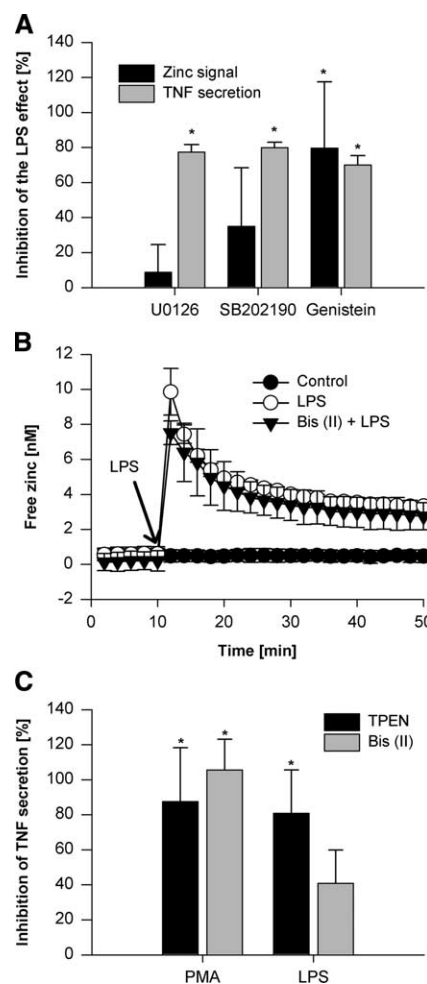
FIGURE 8. Role of signaling pathways for zinc signals. *A*, Mono Mac1 cells were treated with LPS (500 ng/ml) alone, or after 30 min of preincubation with inhibitors for MEK1/2 (U0126, 20 μM), p38 MAPK (SB202190, 1 μM), or tyrosine kinases (genistein, 50 μM). Treatment was followed by measurement of free zinc with FluoZin-3 after 30 min, or TNF-α secretion into the supernatant after 4 h by ELISA. Results are shown as a percentage of inhibition of the effect of LPS, and are mean $\pm$ SD of $n = 6$ (zinc) or $n = 3$ (TNF-α) experiments. *, $p < 0.05$ for significant different from cells treated with LPS alone using ANOVA/Tukey's honestly significant difference. *B*, RAW 264.7 cells were loaded with FluoZin-3 and the resulting fluorescence was measured in a well plate reader. After 10 min of recording of the baseline, LPS (100 ng/ml) was added either to untreated control cells, or cells to which 1 μM bisindolylmaleimide had been added 30 min before the experiment. One representative experiment as mean of triplicates $\pm$ SD of $n = 4$ experiments is shown. *C*, Stimulation of RAW 264.7 with PMA (50 ng/ml) or LPS (100 ng/ml), respectively, for 24 h, either as untreated controls or preincubated with 2 μM TPEN or 1 μM bisindolylmaleimide II. The formation of TNF-α was measured by ELISA. Results are shown as mean $\pm$ SD ($n = 3$ donors) of the percentage of inhibition compared with the effect of LPS or PMA alone, respectively. *, $p < 0.05$ significant difference of cells treated with PMA or LPS alone determined by ANOVA/Tukey's honestly significant difference.

present within cells after enzymatic cleavage of the AM ester. Zinc-dependent fluorescence is not directly affected by LPS, the inhibitors that were used, or calcium and magnesium ions (data not shown).

Genistein inhibited the rise in intracellular zinc (Fig. 8*A*). However, genistein-mediated inhibition of LPS-induced TNF-α
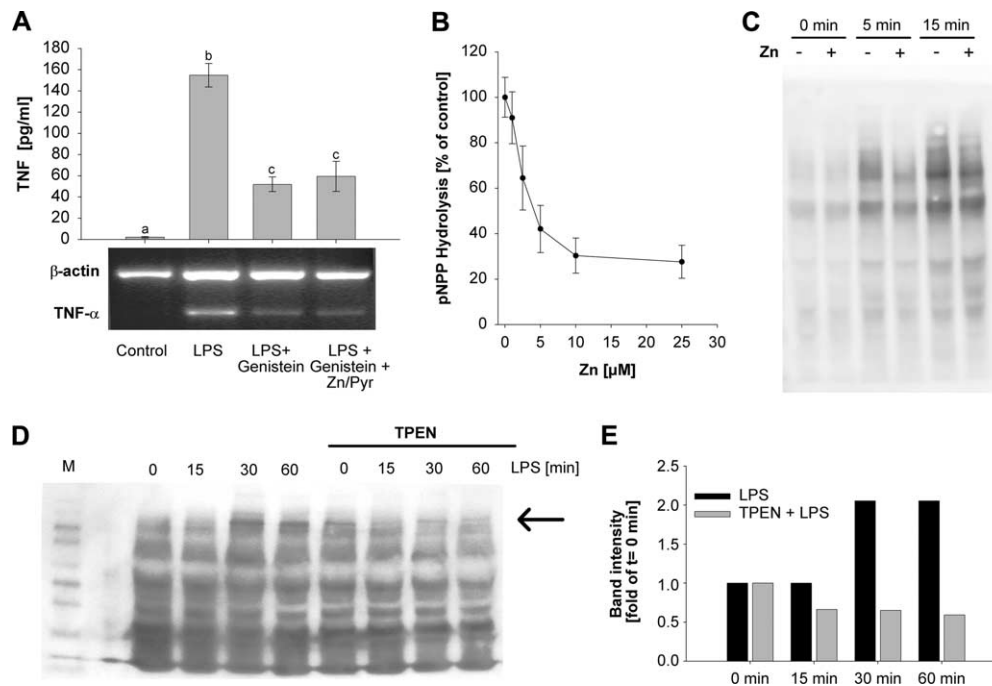
FIGURE 9. Tyrosine phosphorylation and zinc signaling. *A*, The influence of preincubation with genistein (50 μM, 30 min) on TNF-α secretion and mRNA expression was measured by ELISA and RT-PCR in Mono Mac1 cells after stimulation with LPS (250 ng/ml) alone, and after addition of zinc (1 μM) and pyrithione (10 μM) together with LPS. Data are presented as mean $\pm$ SD ($n = 3$ donors). Significant differences determined by ANOVA/Tukey's honestly significant difference are indicated by different letters. *B*, The hydrolysis of pNPP in lysate from Mono Mac1 cells was measured at 405 nm after 1 h in the presence of the indicated zinc concentrations. Data are shown as mean $\pm$ SD from $n = 3$ independent experiments, performed in triplicates. *C*, Mono Mac1 cells were lysed by sonication and phosphatase activity was inhibited by addition of sodium orthovanadate (100 μM). The time-dependent phosphorylation in response to 1 mM ATP was monitored in untreated controls and in the presence of 50 μM Zn by Western blotting with an Ab directed against phosphorylated tyrosine (using pTyr 100). One representative experiment of $n = 3$ experiments is shown. *D*, Western blot analysis of tyrosine phosphorylation in primary monocytes after stimulation with LPS (250 ng/ml) alone or after preincubation with TPEN (2.5 μM, 30 min). Arrow indicates a protein of ~120 kDa that is phosphorylated after LPS stimulation. The peak intensity of this band was quantified by densitometry (*E*).

mRNA synthesis and secretion from Mono Mac1 was not reversed by addition of zinc and pyrithione, indicating that tyrosine phosphorylation must have other functions in TLR4 signaling besides regulating intracellular zinc (Fig. 9*A*). Thus, not only is tyrosine phosphorylation essential for increased intracellular zinc, zinc is also ineffective in the absence of tyrosine kinase activity. Zinc is known to inhibit protein tyrosine phosphatases (PTP), so although early phosphorylation events are required for zinc signals, one role of zinc may be conserving tyrosine phosphorylation by inhibition of PTP. Concurrently, zinc ions inhibited intrinsic phosphatase activity in Mono Mac1 lysate, monitored by hydrolysis of pNPP (Fig. 9*B*), but did not modulate kinase activity in the lysate (Fig. 9*C*). A role of zinc in LPS-induced tyrosine phosphorylation was further investigated by Western blot analysis using an Ab against phosphotyrosine. The band of an unidentified protein of 120 kDa was found after LPS stimulation, but only in the absence of TPEN (Fig. 9, *D* and *E*). Notably, basal tyrosine phosphorylation was largely unaffected by the incubation with TPEN, indicating a specificity for the actions of zinc.

Zinc and MAPK signaling

Several signal transduction pathways are involved in LPS-induced synthesis and release of TNF-α. Inhibitors like U0126 for MEK (upstream of ERK) and SB202190 for p38 MAPK have been shown to block TNF-α release from monocytes (28, 29). *E. coli*-induced phosphorylations of p38 MAPK, MEK1/2, and ERK1/2 were completely abrogated when leukocytes were loaded with the zinc chelator TPEN before treatment with the bacteria (Fig. 10*A*).

Comparable observations were also made when LPS, TNF-α, or insulin were used for stimulation (data not shown). In contrast, incubation of leukocytes with zinc in the presence of the ionophore pyrithione induced MAPK phosphorylation (Fig. 10*B*). To exclude that the effect of TPEN in Fig. 10*A* was due to cytotoxicity, rather than chelation of a zinc signal, we tested the effect of the treatments on cellular viability, which was unaffected (Fig. 10*C*). Instead, zinc preserves phosphorylation of p38 and ERK1/2 in the lysate of RAW 264.7 cells, indicating an effect on MAPK dephosphorylation (Fig. 10*D*).

When the effects of TPEN, U0126, and SB202190 on TNF-α mRNA and TNF-α protein in the cellular lysate and the supernatant were compared, the LPS-induced increase of TNF-α mRNA in Mono Mac1 cells was only inhibited by TPEN, whereas U0126 and SB202190 were without effect (Fig. 10*E*). Nevertheless, all three substances inhibited the release of TNF-α into the supernatant (Fig. 10*E*). TPEN and U0126 blocked the synthesis of TNF-α. Inhibition of p38 did not affect the synthesis, but the release of TNF-α because the cytokine was present in the cellular fraction but not in the supernatant after cells had been pretreated with SB202190 (Fig. 10*E*). Taken together, zinc acts on the expression of TNF-α mRNA, upstream from p38 and ERK and, although zinc is important for MAPK activation, this is not sufficient to explain its effect on TNF-α transcription.

Zinc and transcriptional activation of TNF-α

The transcription factor NF-κB plays a major role in LPS-induced TNF-α transcription, some reports even mention a requirement for zinc in DNA binding of NF-κB. When Mono
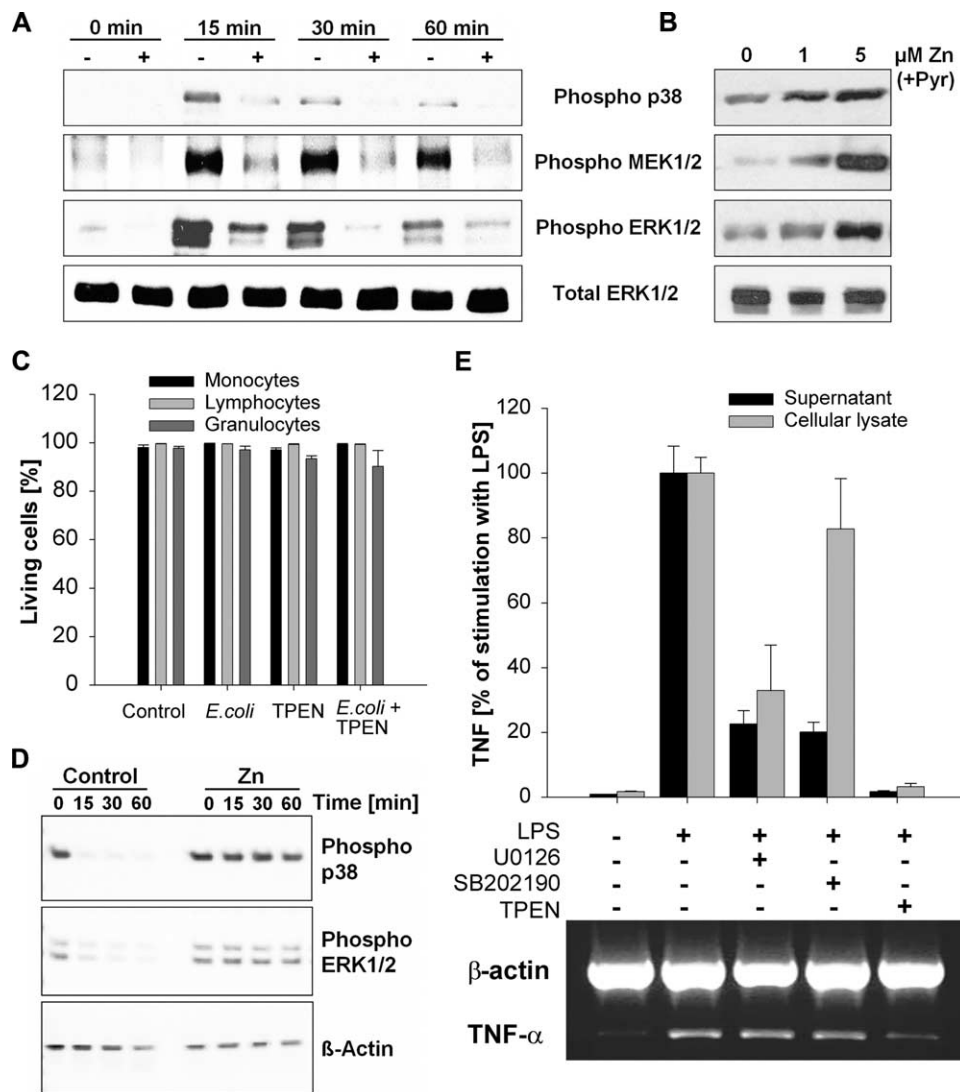
FIGURE 10. Role of free zinc in MAPK activation. *A*, Effect of chelation of zinc signals by TPEN on MAPK phosphorylation in human leukocytes stimulated with *E. coli* for up to 60 min, analyzed by Western blotting with Abs against phosphorylation of Thr^{180}/Tyr^{182} on p38 MAPK, phosphorylation of Ser^{217}/Ser^{221} on MEK1/2, phosphorylation of Thr^{202}/Tyr^{204} of ERK1/2, and total ERK1/2. All gels shown are representative of at least three independent experiments. *B*, Human leukocytes were stimulated with the indicated concentrations of zinc sulfate in the presence of 10 μM pyrithione for 30 min. MAPK phosphorylation was analyzed as shown in *A*. Representative results for $n = 3$ donors are shown. *C*, Human leukocytes were incubated for 60 min as described in the conditions used for *A*, followed by 24 h of incubation in normal culture medium to allow manifestation of toxic effects. Subsequently, membrane integrity was measured by addition of propidium iodide and analysis by flow cytometry as described in *Materials and Methods*. Results are shown as mean $\pm$ SD from $n = 4$ different donors. *D*, RAW 264.7 were stimulated by 30 min of incubation with LPS (500 ng/ml). After lysis by sonication on ice, cells were brought to 37°C and incubated for the times indicated either as untreated control or in the presence of 50 μM ZnSO$_4$. In these samples, dephosphorylation by intrinsic phosphatase activity was monitored by Western blot. One representative result of $n = 3$ independent experiments is shown. *E*, Mono Mac1 cells were stimulated with LPS (250 ng/ml) after 30 min of preincubation with MAPK inhibitors U0126 (25 μM; MEK1/2) and SB202190 (1 μM; p38 MAPK), or the zinc chelator TPEN (2.5 μM). After 4 h with LPS, the amount of cytokines was determined by ELISA in the supernatant and in the cellular lysate. The amount of TNF-α detected after stimulation with LPS was taken as 100%. Values were 174 $\pm$ 15 pg/ml (supernatant) and 594 $\pm$ 29 pg/10^5 cells (lysate). Data shown are mean $\pm$ SD for $n = 3$ donors. The gel shows TNF-α mRNA expression after incubation with LPS for 3 h, analyzed by RT-PCR using β-actin as a housekeeping gene. One representative experiment of $n = 3$ experiments is shown.

Mac1 cells were stimulated with LPS, NF-κB DNA binding activity increased 4-fold. This effect was suppressed by preincubation of the cells with TPEN, but not U0126 (Fig. 11*A*), indicating an effect of zinc on transcription, whereas ERK affects posttranscriptional events. However, this effect is not directly from zinc on the DNA binding ability because only treatment of intact cells with TPEN was effective, whereas the addition of TPEN to the lysate did not reduce the LPS-induced binding activity. Likewise, zinc treatment of the lysate from cells incubated with LPS in the presence of TPEN did not reverse the effects of the chelator (Fig. 11*A*). Hence, zinc affects

the pathway leading to NF-κB activation but not NF-κB directly. This affect was confirmed by Western blotting with phosphorylation-specific Abs against the kinases upstream of NF-κB, showing that the LPS-induced increase in IκB kinase (IKK)β phosphorylation was inhibited by TPEN in Mono Mac1 (data not shown) and in primary human monocytes (Fig. 11*B*).

When zinc was added to RAW 264.7 cells stably transfected with eGFP under the control of the human TNF promoter, we found that addition of either zinc or the ionophore pyrithione alone had no effect, but in combination these were sufficient to induce TNF promoter activation (Fig. 11*C*). When zinc and pyrithione
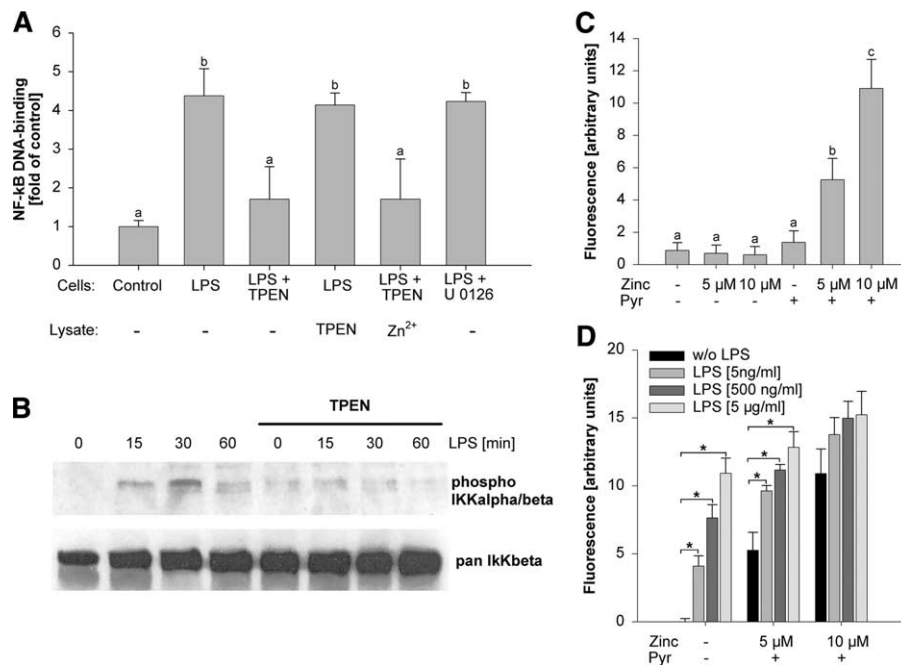
FIGURE 11. Effect of zinc on LPS-induced transcription of TNF-α. *A*, Mono Mac1 cells were treated for 1 h with LPS (250 ng/ml) alone, or after 30 min of preincubation with TPEN (2.5 μM) or the MEK1/2 inhibitor U0126 (20 μM). To investigate a possible involvement of zinc in the DNA binding of NF-κB, some lysates were additionally incubated with TPEN (2.5 μM) or zinc sulfate (1 mM) for 1 h in vitro as indicated. Results are expressed as mean $\pm$ SD for $n = 3$ donors. Significant difference determined by ANOVA/Tukey's honestly significant difference is indicated by different letters. *B*, IKK activation in primary monocytes after stimulation with LPS (250 ng/ml) with or without TPEN (2.5 μM, 30 min preincubation) was investigated by Western blotting using Abs against double phosphorylation of Ser^{176}/Ser^{180} (IKKα) or Ser^{177}/Ser^{181} (IKKβ) (*top*). The m.w. of the detected band corresponds to IKKβ (87 kDa), or total IKKβ (*bottom*). *C* and *D*, RAW 264.7 murine macrophages stably transfected with the human TNF promoter coupled to an eGFP reporter gene were incubated for 24 h as indicated and the resulting eGFP expression was measured by flow cytometry. Results represent mean $\pm$ SD of $n = 5$ independent experiments. *C*, Incubation in the presence of the indicated concentrations of ZnSO$_4$ and pyrithione (10 μM). Significant difference determined by ANOVA/Tukey's honestly significant difference is indicated by different letters. *D*, Incubation for 24 h with ZnSO$_4$, pyrithione (10 μM), and different concentrations of LPS as indicated. *, $p < 0.05$ for significant difference from the respective controls without LPS detemined by ANOVA/Tukey's honestly significant difference.

were administered together with LPS, the effects were not additive. Rather, zinc contributes to the effects of LPS and increases the response to suboptimal doses, whereas combined treatment with high LPS and zinc concentrations leads to saturation (Fig. 11*D*).

Discussion

Proinflammatory cytokine secretion from monocytes is affected by zinc. Although low concentrations act synergistically with LPS (19, 20), higher ones inhibit the production of these cytokines (15–18). Zinc cannot only reduce mRNA expression of proinflammatory cytokines in cultured cells (15), it also inhibits LPS-mediated toxicity in mice (16, 30). Although the molecular mechanism that leads to the inhibition by high zinc concentrations was found to be based on a modulation of cGMP signaling (17, 18), only few data exist regarding the mechanism furthering proinflammatory events. An effect of zinc on the fluidity state of LPS, and hereby the interaction with its receptor, was discussed (31). However, this effect cannot be the basis for those described in this study because the requirement for the ionophore pyrithione shows that zinc acts inside the cells. The data demonstrate that the intracellular zinc concentration rises in response to stimulation, especially in LPS-treated monocytes. This signal is required for the production of proinflammatory cytokines because it is necessary for the activation of MAPKs and the TNF-α promoter.

An increase of the intracellular free zinc ion concentration was induced by LPS, which triggers TLR4. In general, TLR4 is known to signal through at least two different mechanisms: a MyD88-dependent and a TRIF-dependent pathway (26). Our experiments

show that neither MyD88 nor TRIF are required to induce a rise of free zinc because the signal was still present in splenocytes from the respective knockout mice. However, the signal seems to originate from TLR4 because the Ab HTA-125 does not only block LPS-induced cytokine production, but it also triggers a zinc signal. It remains to be investigated what molecular mechanisms are involved in the formation of this zinc signal. So far, we were able to exclude MAPKs, which seem to be located downstream of zinc in the signaling pathways, and demonstrated an involvement of tyrosine phosphorylation. Although PKC can trigger zinc signals (21, 27) and this kinase is activated by LPS/TLR4 (10), inhibition of PKC had no effect on the zinc signal, and only partially inhibited LPS-induced cytokine production. In contrast, zinc is required for PKC-induced cytokine production because this was inhibited by the chelator TPEN.

Cellular zinc homeostasis is controlled by transport proteins (32); the expression of several of them is modulated by LPS (6, 33, 34). LPS also induces the zinc binding protein metallothionein (35), and inducible NO synthase, which can release metallothionein-bound zinc (36). Although these effects may induce long-term changes of free zinc, which might also affect signal transduction, the alterations described in our study occur within less than 2 min and can therefore not be mediated by changes in protein expression. They rather indicate a rapid translocation of zinc ions from an intracellular compartment that is not accessible for the fluorescent zinc probes that were used. Both dyes used in this study differ considerably in their properties. FluoZin-3 is a modification of the BAPTA based Fluo-3, forms a 1:1 complex with zinc and has a

dissociation constant of 8.9 nM (37). Conversely, Zinquin is a modification of the quinoline-based dye TSQ, and forms both a 1:1 (K_D = 370 nM) and a 2:1 (K_D = 85 nM) complex with zinc (38).

Stimulation of TLR4 with LPS activates a complex intracellular signaling network (10, 26). This activation includes IRAK1/4 and TRAF-6, and leads to the activation of several kinases like the MAPKs p38, JNK, and ERK, but also PKC, PI3K, and several Src family tyrosine kinases (11–14). The literature regarding the function of the MAPKs ERK and p38 in LPS-induced TNF-α secretion is contradictory. Different reports show an effect of ERK either on TNF-α mRNA synthesis or its stability and posttranscriptional processing (29, 39, 40). We found that in Mono Mac1 cells ERK influences mRNA stability or translation because U0126 inhibited formation of the TNF-α protein, but not NF-κB activation or TNF-α mRNA synthesis. In this experimental system the p38 MAPK inhibitor SB202910 did not affect synthesis of TNF-α protein, but posttranslational events, presumably processing or secretion of the precursor protein. Other groups have found that p38 MAPK is either involved in TNF-α transcription through regulation of NF-κB, or translational control of TNF-α expression, or mRNA stability (41–43). Because chelation of zinc signals with TPEN leads to a decrease in NF-κB activity and mRNA synthesis, this result is a sign. Because although zinc is required for LPS-mediated MAPK activation, this requirement does not wholly explain the need for zinc in proinflammatory signaling, and other pathways that lead to the activation of TNF-α transcription must also depend on zinc signals.

It has been reported that DNA binding of NF-κB is zinc dependent (44), but in previous studies only one metal ion chelator (1,10-orthophenanthroline) inhibited DNA binding of the transcription factor. However, most assays for NF-κB activity are routinely performed in the presence of millimolar concentrations of EDTA, which also chelates zinc with high affinity. Besides, NF-κB is not a zinc finger protein and the crystal structures of the p50 homodimer and the p50/p65 heterodimer bound to DNA do not contain zinc (45, 46). These results make it seem likely that zinc acts upstream, affecting the signals that regulate NF-κB activation, but not NF-κB directly. This hypothesis is supported by the data presented in Fig. 11, A and B, showing an influence of TPEN on IKKβ phosphorylation, but no effect of zinc on DNA binding of NF-κB.

We found an LPS-induced rise of free zinc in cells from human and murine origin suggesting the existence of zinc signaling in several species of mammals. In Jurkat cells, a human T cell line, free zinc increased upon activation by PMA, confirming that zinc signals are not restricted to cells of the myeloid lineage. They are not even limited to cells of the immune system, as we found a strong increase in intracellular free zinc after stimulation of murine L929 fibroblasts with TNF-α (data not shown), indicating that zinc ions could be a common element of signal transduction in multiple cell types and signaling pathways.

Zinc has previously been shown to influence cellular signal transduction by inhibition of several dephosphorylating enzymes like PTPs (47, 48), cyclic nucleotide phosphodiesterases (17), and dual specificity phosphatases (49). We propose a mechanism in which zinc acts as a permissive signal. The level of intracellular free zinc regulates the rate of dephosphorylation, and hereby the signal intensity of phosphorylation dependent signaling. Protein tyrosine kinase activity is required for LPS-induced signals (50), and tyrosine phosphorylation of TLR4 and MyD88 has been detected after stimulation with LPS (51, 52). Evidence is accumulating that tyrosine phosphorylation of TLR2 (53) and TLR4 (54) is required for signal transduction originating from these receptors. Once these early phosphorylation events are understood, it will be

possible to define the role of zinc in more detail. Additionally, dual specificity phosphatases, which dephosphorylate MAPKs, are important for negative regulation of TLR signaling and are involved in LPS-induced cytokine production (55–58). The results in Fig. 10 point toward an inhibition of these phosphatases by zinc.

Zinc and calcium are two redox-inactive cations involved in signal transduction, but seem to function by different principles, the former by preserving existing signals, the latter as a classical second messenger. Although calcium mediates most of its effects via a specific adaptor protein, calmodulin, zinc ions interact directly with a range of different target proteins. In addition, Fig. 3 shows that intracellular fluctuations of both ion concentrations are controlled by independent mechanisms. Our data indicate that zinc ions contribute to a second signaling system based on intracellular fluctuations of metal ion concentration, acting parallel to calcium.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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