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Zinc differentially regulates mitogen-activated protein kinases in human T cells☆

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Abstract

Zinc is an essential nutrient with remarkable importance for immunity, in particular for T-cell function. This is, at least in part, based on an involvement of zinc ions in immune cell signal transduction; dynamic changes of the intracellular free zinc concentration have recently been recognized as signaling events. Because the molecular targets of zinc signals remain incompletely understood, we investigated the impact of elevated intracellular free zinc on mitogen-activated protein kinase (MAPK) activity and MAPK-dependent cytokine production in human T-cells. p38 was activated by treatment with zinc and the ionophore pyrithione, whereas ERK1/2 and c-Jun N-terminal kinases were unaffected. In contrast, after T-cell receptor stimulation with antibodies against CD3, ERK1/2-phosphorylation was selectively suppressed by intracellular zinc. Mechanisms that had been shown to mediate zinc-effects in other cells, such as activation of the Src kinase Lck, inhibition of the protein tyrosine phosphatase CD45 or MAPK phosphatases and cyclic nucleotide/protein kinase A signaling were not involved. This indicates that the differential impact of zinc on the MAPK families in T-cells is mediated by mechanisms that differ from the ones observed in other cell types. Further investigation of the activation of p38 by zinc demonstrated that this MAPK is responsible for the zinc-mediated activation of CREB and mRNA expression of the Th1 cytokines interferon-gamma and interleukin-2. In conclusion, regulation of MAPK activity contributes to the impact of zinc on T-cell function.

Keywords: Zinc; T-cell; Signal transduction; p38; ERK1/2; Zinc supplementation

1. Introduction

The trace element zinc is an essential nutrient. It is a cofactor of over 300 enzymes, is crucial for transcriptional and replication factors, e.g., in zinc finger motifs, and plays an important role in signal transduction [1,2]. Disturbance of the cellular zinc availability can be caused by inadequate intake, poor gastrointestinal absorption or enhanced excretion [3] and leads to a compromised function of the immune system, including enhanced susceptibility to infectious diseases. Hereby, zinc deprivation compromises predominantly the cell-mediated immune response, especially T-cells [4].

Among the different kinds of T-cells, $CD4^+$ T helper (Th)-cells are predominantly affected by zinc deficiency. Th-cells are further distinguished into several subtypes, including Th1 and Th2. Among these subtypes, mainly Th1-cells are affected by changes in the zinc availability. These cells produce cytokines such as interferon (IFN)-γ, activating a cellular immune response by macrophages. In contrast, Th2-derived cytokines promote the humoral response of antibody production by B-cells [5]. Zinc deprivation leads to reduced production of Th1 cytokines such as IFN-γ and interleukin (IL)-2. Accordingly, the cell-mediated immune response is impaired during zinc deficiency, while Th2-dependent functions remain largely intact [6–8].

The impact of zinc on T-cells could result from modulating several signaling pathways which finally lead to the activation of transcription factors such as CREB and nuclear factor kappa B (NFκB) [1]. First of all, zinc influences T-cell receptor (TcR) signaling. Here, it directly activates the Src kinase Lck, which is located upstream of all TcR-dependent signaling pathways [9,10]. By binding to two distinct protein interface sites, zinc induces recruitment of Lck to the TcR signaling complex and its activation by auto-phosphorylation [11–13]. Lck's target zeta-chain (TcR) associated protein kinase 70 kDa (ZAP70) can activate p38 without involvement of MAPK kinases (MAPKK), through phosphorylation of tyrosine 323. This triggers auto-phosphorylation of p38 at its Thr180/Tyr182 residues [14]. Protein tyrosine phosphatases (PTP) dephosphorylate multiple phosphotyrosine motifs in TcR signaling, e.g., of CD3 (the signal transduction component of the TcR) and Lck [15]. In addition to Lck activation zinc also inhibits PTPs [16,17], indicating a second mechanism by which this metal can regulate TcR signaling.

Some zinc-regulated downstream pathways of the TcR involve mitogen activated protein kinases (MAPKs) [1]. Mammalian cells express three main groups of MAPKs, extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPKs, and c-Jun N-terminal kinases (JNKs) [18]. Their activity is regulated by dual phosphorylation of a Thr-X-Tyr motif. Activation by phosphorylation is mediated by upstream MAPKK, and dephosphorylation by MAPK phosphatases (MKP) [18]. The latter are a subgroup of the PTP family, and several reports demonstrate their susceptibility to inhibition by zinc [19–22].

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Table 1
Primer sequences for RT-PCR

Gene	forward sequence (5′→3′)	reverse sequence (5′→3′)
β-Actin[a]	CGCCCCAGGCACCAGGGC	GCTGGGGTGTTGAAGGT
IL-2	TCCTGTCTTGCATTGCACTAAG	CATCCTGGTGAGTTTGGGATTC
IFN-γ	AGAATTGGAAAGAGGAGAGTGACAG	GTCTTCCTTGATGGTCTCCACAC
IL-4	TTGAACAGCCTCACAGAGCAGA	GTTGTGTTCTTGGAGGCAGCA
T-bet	AGGGACGGCGGATGTTCCCA	GCTGCCCTCGGCCTTTCCAC
GATA-3	GCCCGGTCCAGCACAGAAGG	TGAGGGGCCGGTTCTGTCCG
c-maf	TCTCCCGAGTTTTTCATAACTGAGCCC	ACACACTGGTAAGTACACGATGCTGG

[a] The β-Actin primer pair was from Ref [30].

Finally, zinc could affect TcR downstream signaling by inhibiting the degradation of cyclic nucleotides by phosphodiesterases (PDE) [23]. In monocytes, PDE inhibition leads to accumulation of the cyclic nucleotide 3′,5′-cyclic guanosine monophosphate (cGMP), with subsequent cross-activation of the protein kinase A (PKA) [24]. In T-cells, the PKA activator 3′,5′-cyclic adenosine monophosphate (cAMP) inhibits the expression of IL-2 and influences Th-cell polarization [25], indicating that PDE-inhibition with subsequent PKA activation could also be a mechanism by which zinc modulates T-cell activity. Furthermore, PKA-activation by zinc could also regulate MAPK activity by inhibiting the Raf/MEK/ERK pathway through phosphorylation of Raf on serine 259 [26].

An important downstream target of TcR signaling is the transcription factor CREB, which controls Th1 immune responses by inducing expression of IFN-γ. Not only is CREB activated by PKA [27], but several studies showed activation of CREB by p38, ERK1/2 or zinc [28,29]. Taken together, these observations suggest that zinc might act on cytokine expression *via* modulation of CREB, potentially by interfering with MAPK or PKA signaling.

The aim of this study was to analyze the impact of zinc on T-cell signaling, in particular on MAPKs. We found that only p38 was activated by zinc. In contrast, ERK1/2 phosphorylation in response to TcR-stimulation was inhibited, whereas JNK was entirely unaffected. Furthermore, different potential mechanisms for p38 activation were investigated and the contribution of zinc-induced PKA and MAPK activation in CREB activation and Th1/Th2 cytokine secretion was analyzed.

2. Methods and materials

2.1. Chemicals and reagents

Phosphate-buffered saline (PBS) and cell culture media and supplements were purchased from Lonza (Belgium). Fetal calf serum (FCS) was obtained from PAA (Germany) and heat-inactivated for 30 min at 56°C. Ficoll-Hypaque was from Biochrom (Germany). IL-2 and FluoZin-3 tetrapotassium salt were obtained from Peprotech (Germany) or Invitrogen (Germany), respectively. Forskolin, IBMX, SB202190, EDTA, propidium iodide, H-89, and pyrithione (sodium salt) were purchased from Sigma (Germany). Anti-CD3 antibody Okt3 was obtained from eBioscience (Germany) and $ZnSO_4 \times 7\ H_2O$ from Merck (Germany). The antibodies used for Western blot analysis were from New England Biolabs (Germany) and the antibodies used for FACS analysis from BD Pharmingen (Germany). db-cAMP and 8-Br-cGMP were from BioLog (Germany). All other reagents were purchased from standard sources.

2.2. Isolation and culture of primary human T-cells

Peripheral blood mononuclear 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% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). For enrichment of activated T-cells, PBMC were incubated for 2 days with 2.5 μg/ml PHA. Monocytes and B-cells were depleted by adherence to plastic and supernatants were transferred into fresh culture medium in which T-cells were expanded in the presence of 50 U/ml IL-2 for 4 days. The purity of the T-cell populations obtained by this method was controlled by flow cytometric measurement of CD3 expression (Suppl. Figure 1), with cells generally being >96% $CD3^+$.

2.3. Cell lines and culture

Cell lines were cultured at 37°C in a humidified 5% CO_2 atmosphere. Normal Jurkat cells, CD45 negative ($Jurkat^{CD45\ neg}$) and Lck negative Jurkat [$Jurkat^{Lck\ neg}$ (=JCAM1.6)] were grown in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and nonessential amino acids. ZAP70 negative Jurkat cells ($Jurkat^{ZAP70\ neg}$) were grown in IMDM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and non-essential amino acids. MOLT-4 cells were maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. To ensure that incubations did not affect cellular viability, cytotoxicity tests were routinely performed for all experimental conditions (Suppl. Figure 2).

2.4. Western blot

Western Blots and MAPK in vitro dephosphorylation were performed as previously described [19]. Phosphorylation site specific antibodies were directed against Thr202/Tyr204 (ERK1/2), Thr180/Tyr182 (p38 MAPK), Ser133 (CREB), Tyr416 (phospho-Src family) and serine or threonine phosphorylation of the PKA target sequence RRXS*/T*. Stimulation with UV light (312 nm) was carried out for 1 min with an UV-Transilluminator TI2 (Biometra, Germany).

2.5. Measurement of CD3 and CD45 cell surface expression

Cells were incubated with either FITC-labeled anti-CD3 antibody, or PE-labeled anti-CD45 antibody, or the corresponding isotype matched controls for 20 min in the dark at room temperature. Fluorescence was detected by flow cytometry, using a FACS Calibur (BD Pharmingen).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Isolation of mRNA and preparation of cDNA were carried out with the Macherey-Nagel Total RNA Isolation Kit (Germany) and the Quanta cDNA synthesis kit (USA) according to the manufacturers' instructions. PCR was performed with the Brilliant

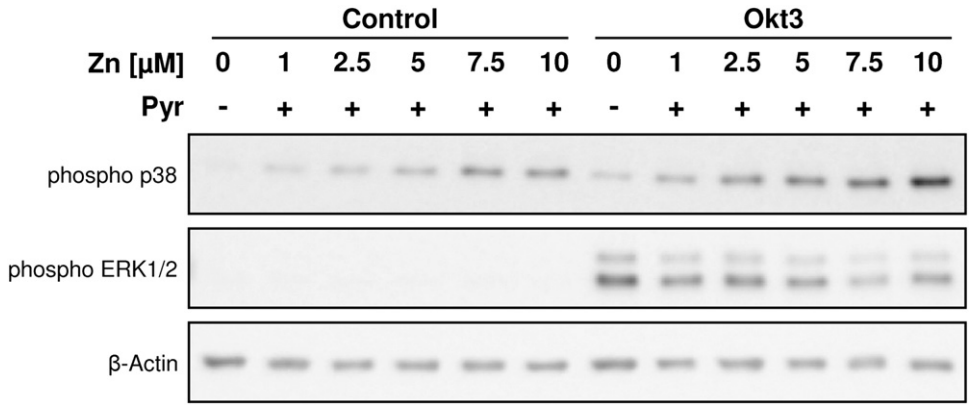

Fig. 1. Differential effect of zinc on p38 and ERK1/2 MAPK phosphorylation. Primary human T-cells were stimulated for 10 min with the indicated zinc concentrations and the ionophore pyrithione (Pyr, 10 μM). MAPK phosphorylation was detected by Western Blot analysis with phosphorylation state specific antibodies either directly, or after additional incubation with the anti-CD3 antibody Okt3 (100 ng/ml for 10 min). Representative blots for $n=3$ independent experiments are shown.

SYBR Green QPCR Master Mix (Stratagene, Germany), under the following conditions: an initial step of 95°C for 10 min was followed by 50 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 1 min. Primer (all from Sigma) was used at final concentrations of 0.1 μM. Sequences are shown in Table 1.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Cells were treated as indicated in Fig. 9 and supernatants were collected 24 h after incubation. IL-2 and IFN-γ were quantified by OptEIA ELISAs (BD) according to manufacturer's instructions.

2.8. Determination of cAMP and cGMP

The LANCE cAMP 384 kit (Perkin Elmer, Germany) was used to measure the cellular cAMP content as previously described [31]. To determine cGMP, the cyclic GMP EIA Kit (Cayman, Germany) was used. After incubation as indicated, Jurkat cells (2.5×10^6 cells) were harvested by centrifugation, resuspended in EDTA (30 mM in PBS) and heated to 95°C. After addition of absolute ethanol samples were homogenized, incubated on ice and harvested by centrifugation. Supernatants were dried with a concentrator 5301 (Eppendorf, Germany). Sample acetylation and measurement were carried out according to the manufacturer's instructions, using an Ultra 384 fluorescence well plate reader (Tecan, Germany).

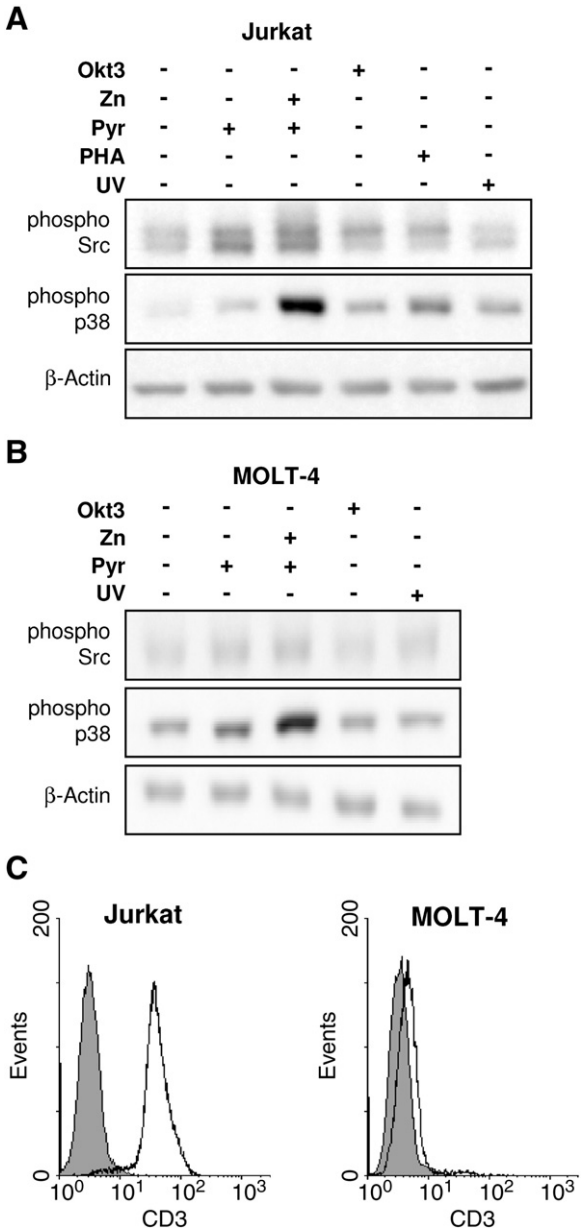

Fig. 2. Role of CD3 in zinc-induced p38 phosphorylation. Cells were incubated with Okt3 (100 ng/ml), zinc sulfate (Zn, 7.5 μM), pyrithione (Pyr, 10 μM) or PHA (10 μg/ml) for 10 min, or exposed to ultraviolet light (UV, 1 min) as indicated. Phosphorylation of Src kinases and p38 was determined by Western Blot in (A) Jurkat or (B) MOLT-4 cells. Representative blots for at least $n=3$ independent experiments are shown. (C) CD3 expression (black lines) compared to isotype-matched control (grey, filled histogram) was measured by flow cytometry for Jurkat and MOLT-4 cells.

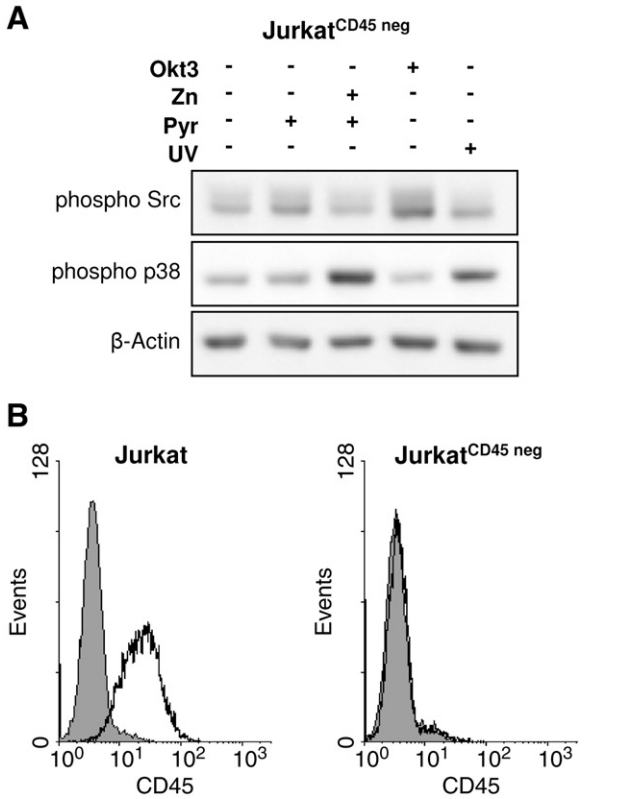

Fig. 3. Role of CD45 in zinc-induced p38 phosphorylation. (A) Jurkat$^{CD45\ neg}$ were incubated as described in the legend to Fig. 2 and phosphorylation of Src kinases and p38 was determined by Western Blot. Data are representative of $n=5$ independent experiments. For respective data in wild type Jurkat cells, see Fig. 2. (B) CD45 expression (black lines) compared to isotype-matched control (grey, filled histogram) was measured by flow cytometry for Jurkat and Jurkat$^{CD45\ neg}$ cells.

2.9. Quantification of free zinc

Free zinc in cell lysates was measured as described [21]. Briefly, cells were lysed by sonication in buffer [20 mM HEPES/NaOH, 20 mM $MgCl_2$, 250 μM Tris(2-carboxyethyl) phosphine, pH 7.5]. Lysates were incubated with 1 μM FluoZin-3 tetrapotassium salt for 15 min at 37°C, transferred to 96-well plates and incubated with different concentrations of $ZnSO_4$ for 5 min. Fluorescence was recorded on an Ultra 384 fluorescence well plate reader (Tecan, Germany) using excitation and emission wavelengths of 485 nm and 535 nm, respectively. Free zinc concentrations were calculated using 50 μM TPEN for determination of minimal and 100 μM $ZnSO_4$ for maximal fluorescence.

2.10. Statistical analysis

Statistical significance of experimental results was calculated by Student's t-test using Excel software or, in case of multiple comparisons, by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc test, using SPSS 15.0. All experiments have been performed at least three times independently with comparable results.

3. Results

3.1. Impact of Zinc on MAPK Phosphorylation

Primary human T-cells were treated with zinc sulfate and the ionophore pyrithione. Pyrithione was added together with zinc, because it forms a complex, shuttling the metal ion into the cells. This eliminates the involvement of cellular uptake mechanisms,

thereby allowing to induce a controlled increase of intracellular zinc that does not depend on individual zinc transporter expression of the different cells. This treatment caused a concentration-dependent increase of Thr180 and Tyr182 phosphorylation in the activation loop of p38 (Fig. 1; Suppl. Figure 3). Additionally, a relatively low amount of antibody against CD3 (clone Okt3), the signal transduction complex of the TcR, was used to induce sub-maximal stimulation of TcR signaling. Okt3 caused weak p38 phosphorylation, which was augmented by zinc/pyrithione.

In contrast to p38, zinc and pyrithione did not induce phosphorylation of ERK1/2. Furthermore, Okt3-induced phosphorylation of this kinase was reduced by addition of zinc and the ionophore (Fig. 1; Suppl. Figure 3). The third family of MAPK, JNK, was also investigated. Zinc had no impact on its phosphorylation, neither alone or after activation by antibodies against CD3 (Suppl. Figure 4).

3.2. Role of TcR-Signaling in Zinc-Induced p38 Phosphorylation

The effects of zinc on p38 and ERK1/2 phosphorylation seem to result from interaction with intracellular targets because no influence of zinc on MAPK phosphorylation, even at significantly higher zinc concentrations, was observed in the absence of an ionophore (data not shown). Because stimulation of the TcR leads to p38 activation, triggering of the TcR or components of its intracellular signaling pathways by zinc could lead to p38 phosphorylation. Upstream components of the TcR pathway are indeed activated by zinc. Jurkat cells incubated with zinc/pyrithione show not only phosphorylation of p38, but also of the activating tyrosine of Src family kinases (Fig. 2A; Suppl. Figure 5A).

To investigate the role of the TcR signaling complex CD3, comparable experiments were performed in MOLT-4 cells, which lack CD3 (Fig. 2B,C; Suppl. Figure 5B). The robust increase of p38 phosphorylation in these cells indicates that CD3 is not required for the effect of zinc on p38.

The PTP CD45 dephosphorylates several tyrosine residues of upstream components of TcR signaling and may therefore be involved in the impact of zinc on p38. However, in Jurkat$^{CD45\ neg}$ cells, zinc-induced phosphorylation of p38 was observed in the absence of this phosphatase (Fig. 3A,B; Suppl. Figure 6). On the other hand, there was no zinc-induced phosphorylation of Src kinases in Jurkat$^{CD45\ neg}$ cells, indicating that inhibition of CD45 by zinc is relevant for activation of Src, but not p38. From this it can be concluded that p38 activation by zinc does not require phosphorylation of Src kinases. This was supported by the observation that p38 was still phosphorylated after incubation of Jurkat$^{Lck\ neg}$ cells with zinc and pyrithione (Fig. 4A,B; Suppl. Figure 7A). Notably, no signal of phosphorylated Src kinase was found in these cells, indicating that Lck was the Src kinase that had caused this signal in Figs. 2 and 3.

One downstream target of Lck is the kinase ZAP70, which can activate p38 by phosphorylation on tyrosine 323 [32]. Because p38 phosphorylation after zinc/pyrithione treatment was also observed in Jurkat$^{ZAP70\ neg}$ cells (Fig. 4C and D; Suppl. Figure 7B) and incubation of wild-type Jurkat with zinc/pyrithione resulted only in phosphorylation of threonine 180 and tyrosine 182 but not tyrosine 323 of p38 (data not shown), ZAP70 is also not involved in zinc-mediated p38 phosphorylation.

Taken together, there are several steps that can be regulated by zinc in the signaling pathway leading from the TcR to p38, but none

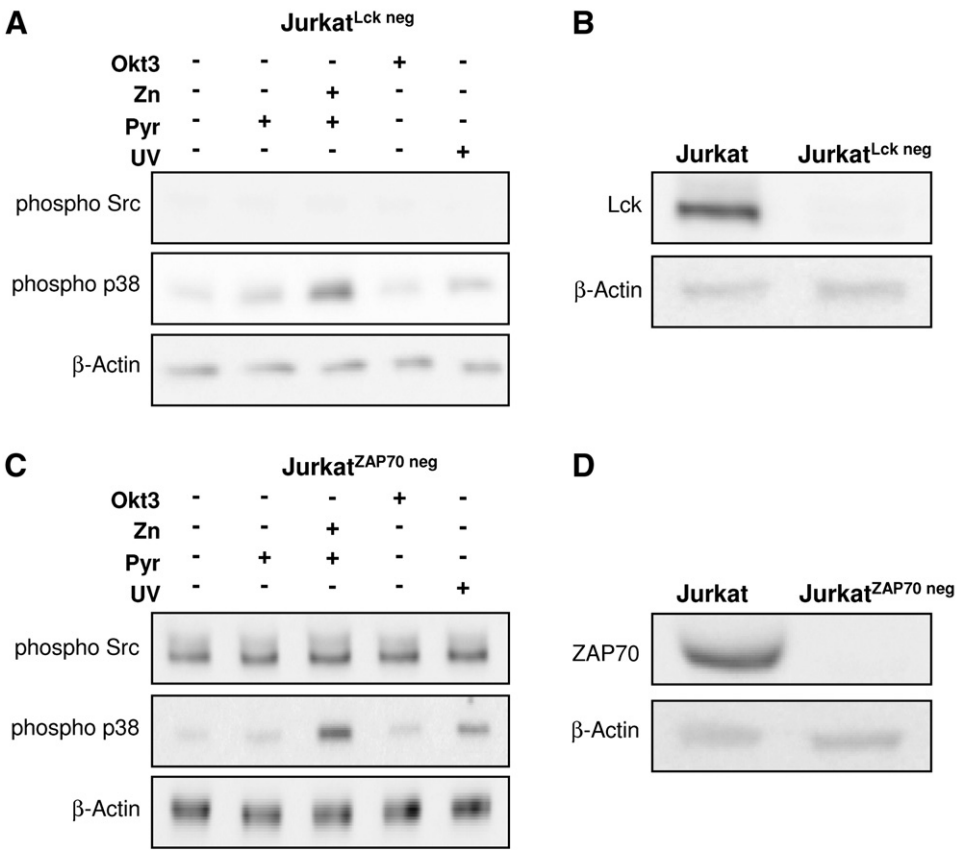

Fig. 4. Role of downstream kinases in zinc-induced p38 phosphorylation. Cells were incubated as described in the legend to Fig. 2 and phosphorylation of Src kinases and p38 was determined by Western Blot for (A) Jurkat$^{Lck\ neg}$ and (B) Jurkat$^{ZAP70\ neg}$ cells. Representative blots for $n=4$ independent experiments are shown. For respective data in wild type Jurkat cells, see Fig. 2. (C,D) The absence of Lck in Jurkat$^{Lck\ neg}$ (C) or ZAP70 in Jurkat$^{ZAP70\ neg}$ (D) respectively, was confirmed by Western blots.

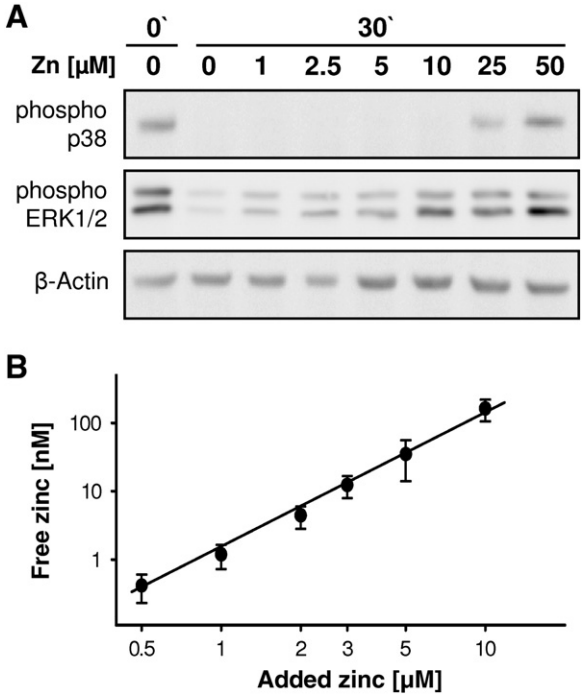

Fig. 5. Influence of zinc on MAPK dephosphorylation. The impact of zinc on MAPK dephosphorylation was monitored in the lysate of Jurkat cells supplemented with 0-50 μM zinc sulfate. (A) Prior to lysis, cells were stimulated with PHA (10 μg/ml for 30 min) to induce MAPK phosphorylation. After addition of zinc, lysates were incubated for further 30 min at 37°C to allow dephosphorylation by phosphatases. The remaining phosphorylation was documented by Western Blot. Representative blots for $n=3$ independent experiments are shown. (B) To determine the extent by which the lysates buffer the added zinc, free zinc was measured with the fluorescent probe FluoZin-3. Values are expressed as means of $n=3$ independent experiments±S.E.M.

of these is required for the elevated p38 phosphorylation in response to zinc.

3.3. Role of MAPK-Dephosphorylation in the Effect of Zinc on p38

Zinc could activate p38 independently from upstream signaling events by blocking its dephosphorylation by MKPs. To analyze the impact of zinc on these events, Jurkat cells were first incubated with PHA to induce ERK1/2 and p38 phosphorylation. This was followed by lysis, addition of different zinc concentrations and incubation to allow dephosphorylation by intrinsic phosphatase activity. ERK1/2 dephosphorylation was partially inhibited by addition of 1 μM $ZnSO_4$, whereas p38 was not affected unless much higher zinc concentrations were used (Fig. 5A; Suppl. Figure 8). However, even low micromolar concentrations significantly exceed the physiological concentrations of free intracellular zinc. To evaluate the physiological relevance, the amount of free zinc in our samples was quantified with the fluorescent probe FluoZin-3, showing that the added zinc had been buffered two to three orders of magnitude by the lysate. Hence, zinc inhibited ERK1/2 dephosphorylation in the physiological, nanomolar concentration range (Fig. 5B), but MKP-inhibition is not a likely cause for selective phosphorylation of p38.

3.4. Role of PKA in the Interaction between Zinc, MAPK and Cytokine Expression

Inhibition of PDEs by zinc can lead to activation of PKA, which inhibits the Raf/MEK/ERK pathway [23,24]. Consistently, activation of PKA by the PDE inhibitor IBMX and the adenylate cyclase activator

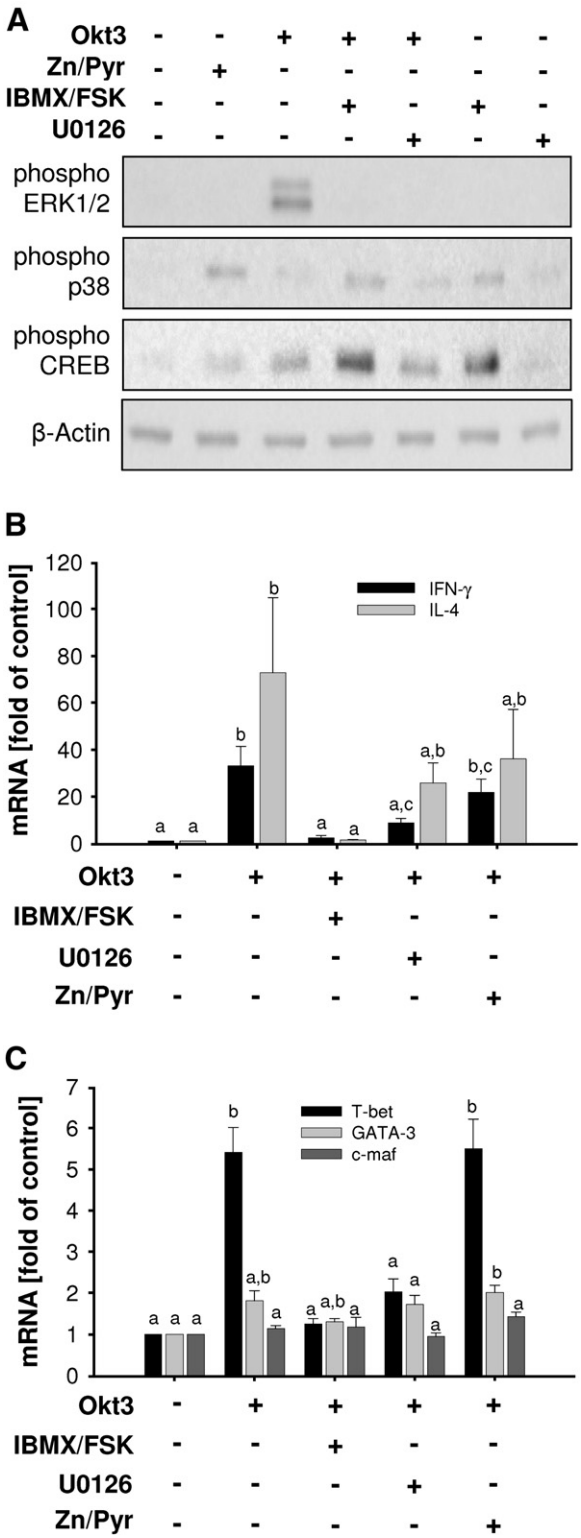

Fig. 6. Effect of PKA, ERK1/2, and zinc on the Th1/Th2 balance. Primary human T-cells were preincubated for 1 h with IBMX (1 mM) and forskolin (FSK, 10 μM), for 30 min with U0126 (5 μM), or for 10 min with zinc sulfate (Zn, 7.5 μM) and pyrithione (Pyr, 10 μM), followed by activation with Okt3 (100 ng/ml). (A) Ten minutes after the addition of Okt3, phosphorylation of ERK1/2, p38 and CREB was analyzed by Western Blot. Data are representative of $n=4$ independent experiments. (B,C) 4 h after the addition of Okt3, real-time PCR was used to determine the expression of IFN-γ and IL-4 (B) or the Th1-related transcription factor T-bet and the Th2-associated transcription factors GATA-3 and c-maf (C). Values are expressed as means+S.E.M. from $n=7$ different donors. Significantly different results ($P<.05$, One-way ANOVA with LSD post hoc test) do not share the same character.

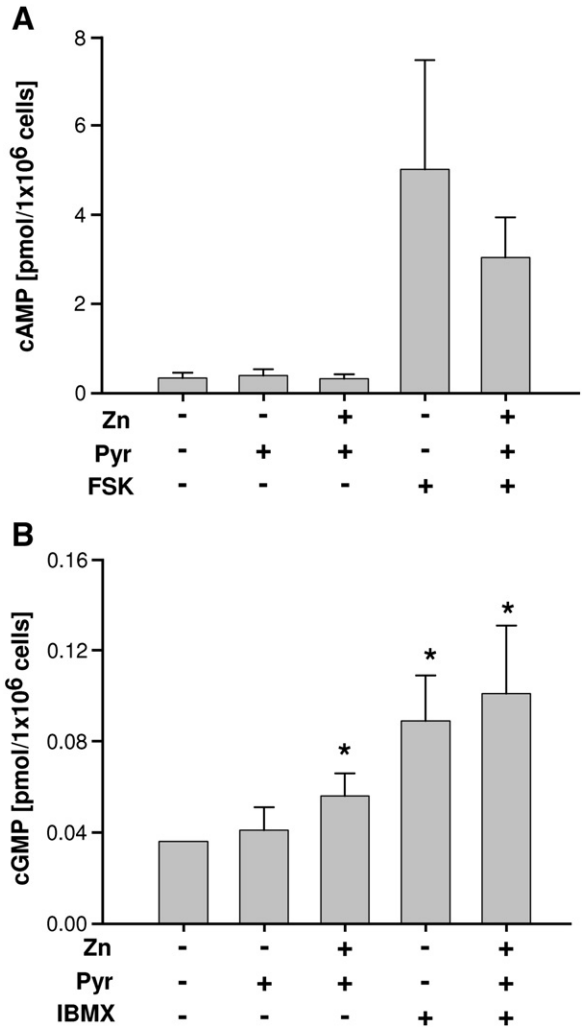

Fig. 7. Effect of zinc on cyclic nucleotide levels. Intracellular cyclic nucleotides were measured in Jurkat cells after 1h of stimulation with zinc sulfate (Zn, 7.5 μM), pyrithione (Pyr, 10 μM), IBMX (1 mM), and forskolin (FSK, 10 μM) as indicated. Samples were processed as described under Methods and materials and analyzed for cAMP (A) or cGMP (B). Data are representative of $n=3$ (A) or $n=8$ (B) independent experiments. Values are expressed as means+S.E.M. and values that differ significantly from the untreated controls are indicated (*$P<.05$, Student's t test).

forskolin reduced Okt3-induced ERK1/2 phosphorylation (Fig. 6; Suppl. Figure 9). This was also observed with the MEK inhibitor U0126 (Fig. 6A; Suppl. Figure 9). Furthermore, the transcription factor CREB is phosphorylated by PKA and has been shown to be regulated by zinc [28,27]. CREB was strongly phosphorylated in response to PKA activation (Fig. 6A; Suppl. Figure 9).

Next, events downstream of PKA, ERK and CREB, such as the activation of cytokines and Th-cell transcription factors, were investigated. Measurement of Okt3-induced mRNA expression of the cytokines IFN-γ and IL-4 showed complete reduction after PKA activation and partial decrease after inhibition of MEK (Fig. 6B). A similar suppression was observed by zinc/pyrithione, but it was incomplete, comparable to the zinc-effect on ERK1/2 phosphorylation in Fig. 1. The key transcription factor for Th1 polarization, T-bet, was strongly induced by Okt3. Notably, Th2 factors GATA-3 and *c*-maf were not activated under these conditions (Fig. 6C). T-bet expression was suppressed by IBMX/forskolin or U0126, but not by zinc/pyrithione. This shows that PKA and ERK control T-bet, whereas zinc acts only on cytokine expression, but not on the upstream transcription factors that control Th-cell polarization.

To investigate if the PKA pathway is activated by zinc/pyrithione, the impact on cyclic nucleotide levels was measured. There was no effect on cAMP (Fig. 7A), whereas cGMP was slightly but significantly elevated (Fig. 7B). As a consequence, there is no evidence for the classic activation of PKA by cAMP, but still cGMP could cross-activate PKA. However, only IBMX and forskolin, but not zinc/pyrithione-treatment caused phosphorylation of the RRXS*/T* PKA target motif (Fig. 8; Suppl. Figure 10), confirming that zinc does not activate PKA under these conditions.

3.5. Role of Zinc-Induced p38 Phosphorylation for CREB Activation

Whereas PDE-inhibition and subsequent activation of PKA would explain CREB phosphorylation triggered by zinc/pyrithione, the results in Figs. 7 and 8 indicate that this phosphorylation occurs independently of cyclic nucleotide signaling. Alternatively, CREB can also be phosphorylated by p38, and the p38 inhibitor SB202190 blocked zinc-induced CREB phosphorylation (Fig. 9A; Suppl. Figure11). The inhibitor had no effect on the phosphorylation of CREB in response to Okt3, demonstrating that only activation by zinc, but not by the TcR, depends on p38 (Fig. 9A; Suppl. Figure 11).

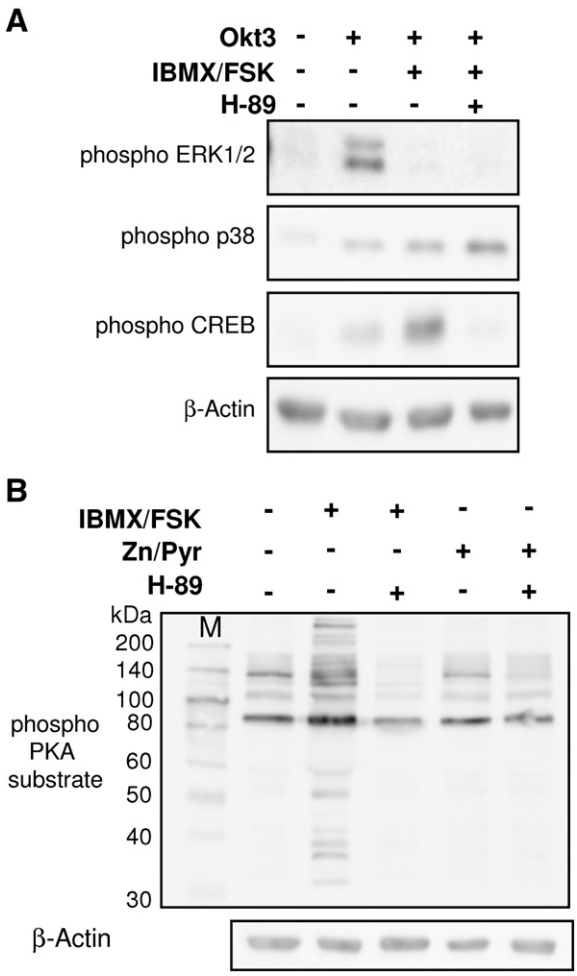

Fig. 8. Impact of zinc on PKA activity. Primary human T cells were incubated for 1 h with IBMX (1 mM) and forskolin (FSK, 10 mM), or zinc sulfate (Zn, 7.5 μM) and pyrithione (Pyr, 10 μM) for 10 min in the absence or presence of the PKA inhibitor H-89 as indicated. Western blot analysis was used to detect phosphorylation of (A) ERK1/2, p38, and CREB, or (B) a characteristic PKA substrate sequence (RRXS/T). In addition, a molecular weight standard has been run in one lane (M); the corresponding weights are denoted on the left hand side of the gel. All data are representative of $n=3$ independent experiments.

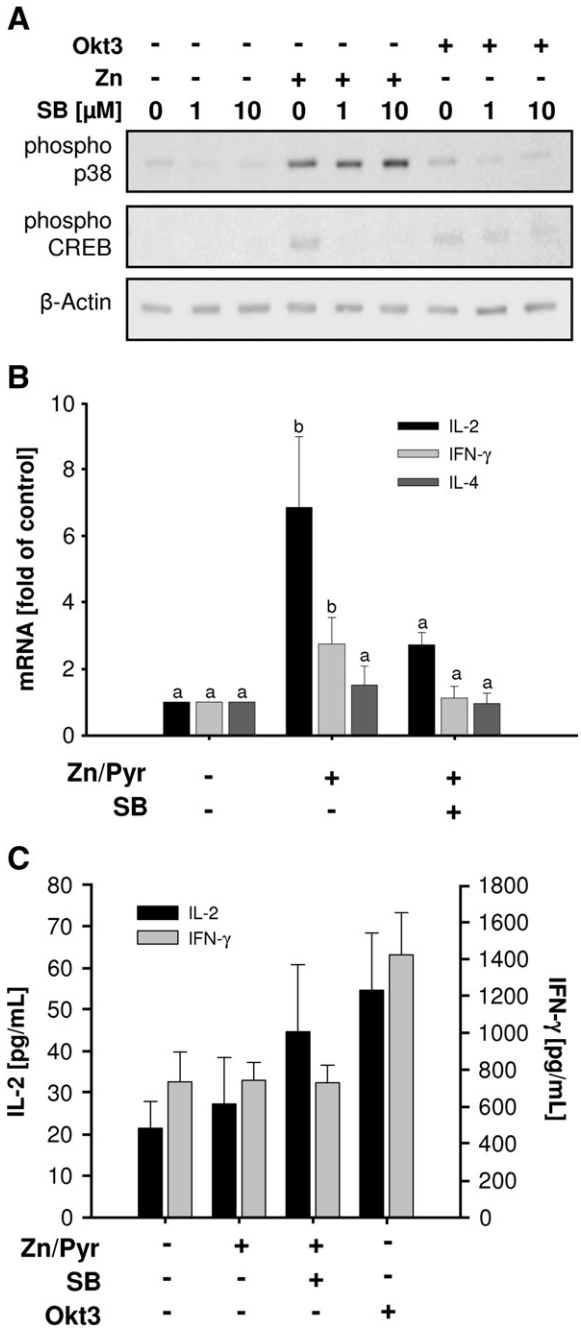

Fig. 9. Role of p38 in zinc-mediated CREB phosphorylation and cytokine expression. Primary human T-cells were preincubated for 30 min with the p38 specific inhibitor SB202190 (SB, concentrations as indicated or 10 µM), followed by zinc sulfate (7.5 µM) and pyrithione (10 µM), or Okt3 (100 ng/ml). (A) Ten minutes after the addition of zinc or Okt3, phosphorylation of p38 and CREB was analyzed by Western Blot. Data are representative of $n=3$ independent experiments. (B) Cytokine expression of IL-2, IFN-γ and IL-4 was examined with real time PCR after 4 h. (C) Primary human T-cells were incubated for 24 h as indicated and cytokine production was determined for IL-2 and IFN-γ with ELISA. Values for (B) and (C) are expressed as means+S.E.M. from $n=7$ different donors. Means that differ with statistical significance ($P<.05$, ANOVA, LSD post hoc test) do not share the same character.

Zinc promotes expression of IL-2 and IFN-γ mRNA, and inhibition of this effect by SB202190 shows that it involves p38 (Fig. 9B). No activation of cytokine secretion by zinc is observed at the protein level, indicating that zinc mediates p38-dependent mRNA expression of selected cytokines but not protein expression in the absence of additional stimulation (Fig. 9C).

4. Discussion

Zinc is an essential trace element, which is particularly important for immune responses involving T-cells. For example, during zinc deprivation the balance between the Th-cell subtypes Th1 and Th2 is disturbed, leading to reduced production of the Th1 cytokines IFN-γ and IL-2. In vivo zinc supplementation of zinc-deficient subjects reconstitutes Th1-mediated immune responses [8].

In recent years, it has been realized that zinc is a component of immune cell signal transduction [1]. Although this is now recognized as one basis for the requirement of zinc for the immune system, the molecular role of zinc signaling in different types of immune cells still needs to be investigated in more detail. The present study was done to explore the effect of zinc on MAPK signaling in human T-cells. Notably, the three MAPK families were differentially regulated. Zinc activated p38, but not ERK1/2 or JNK. On the other hand, ERK1/2 activity in response to stimulation of CD3 with antibodies was inhibited by zinc, whereas this was not observed for p38 or JNK.

In leukocytes, zinc and pyrithione stimulate the stress-induced p38 [19]. Furthermore, chelatable zinc is required for the inhibition of ERK1/2 dephosphorylation in IL-2 signaling [21]. In the present study, administration of zinc/pyrithione alone was not sufficient to induce phosphorylation of ERK1/2, but inhibited Okt3-induced ERK1/2 phosphorylation. This indicates that there is no linear relationship between zinc and ERK1/2, but several competing mechanisms occur at different zinc concentrations. This corresponds well to previous observations in C6 rat glioma cells, where ERK1/2 is activated by low zinc concentrations plus pyrithione, whereas higher concentrations do not affect ERK1/2 phosphorylation [33].

Several zinc-regulated events are involved in TcR signaling (Fig. 10), indicating its components as potential molecular targets for zinc-mediated activation of MAPK and MAPK-dependent cytokine production. Early events include tyrosine phosphorylation of the signaling complex CD3, recruitment and activation of Lck, and assembly of scaffolding proteins. This is followed by phosphorylation of ZAP-70 and activation of different signaling pathways, including MAPK.

Tyrosine phosphorylation occurs at CD3, Lck, and ZAP70. Among other PTPs, CD45 plays an important role for tyrosine phosphorylation in TcR signaling [15]. Strong p38 phosphorylation in zinc-treated Jurkat$^{CD45\ neg}$ cells showed that CD45 is not required for zinc-induced p38 phosphorylation. On the other hand, Src-kinase phosphorylation in response to zinc was not detected in Jurkat$^{CD45\ neg}$, indicating that the PTP participates in this effect of zinc.

The group of cysteine-based PTP that are sensitive to inhibition by zinc includes dual specificity MKPs that dephosphorylate MAPK. In human airway epithelial cells, the activation of ERK1/2 and p38 by zinc is explained by zinc-mediated inhibition of MKPs [22]. In T-cells, we have recently demonstrated that ERK1/2 dephosphorylation is sensitive to nanomolar concentrations of zinc [21]. This was confirmed in Fig. 5, but p38 dephosphorylation was only affected at significantly higher concentrations, indicating that zinc inhibition of MKPs is an unlikely explanation for selective activation of p38.

Another mechanism for zinc to interfere with T-cell signaling is direct activation of Lck. Zinc binds to two distinct protein interface sites. One is between Lck and CD4 or CD8, which induces recruitment of Lck to the TcR signaling complex. The other is located in the SH3 domain of Lck and promotes homodimerization and activating autophosphorylation [10–13]. Furthermore, the *c*-src tyrosine kinase COOH-terminal Srk kinase, which can phosphorylate the inhibitory tyrosine 505 of Lck, is reversibly inhibited by binding of zinc ions [34]. Via all three mechanisms, zinc could lead to activation of Lck and, subsequently, p38. Even though activating phosphorylation of Src

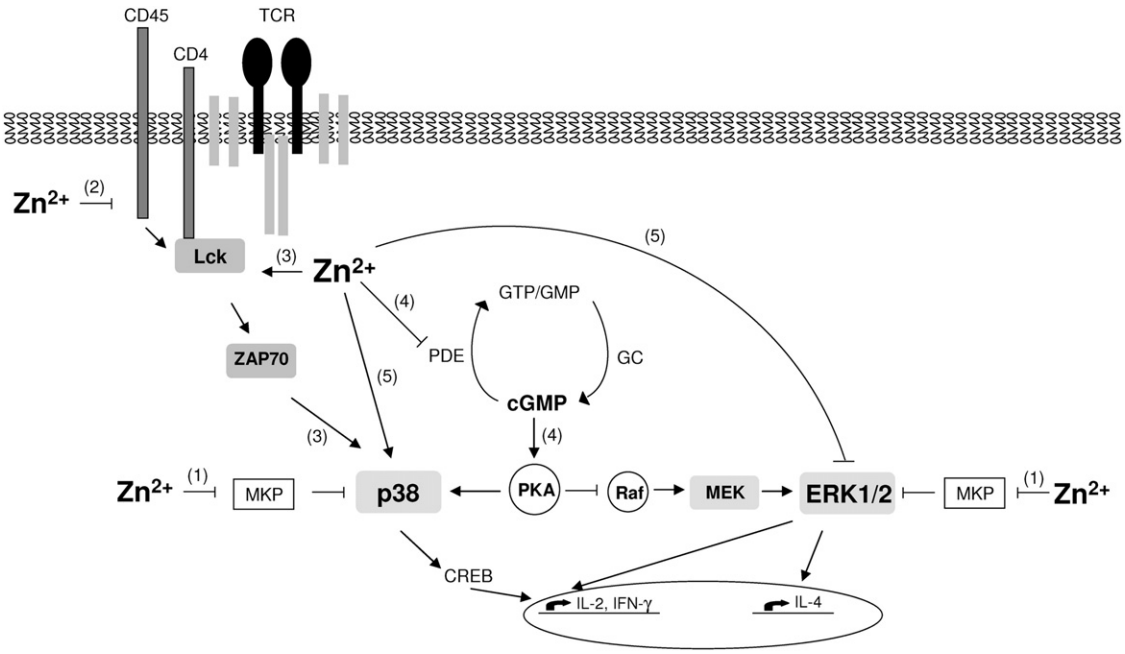

Fig. 10. Overview of the differential zinc effects on T-cell signaling. Other studies have identified several possible mechanisms for zinc to influence T-cell signal transduction. (1) Zinc inhibits MKPs that regulate p38 and ERK1/2 activity [19,21,22]. (2) Zinc inhibits PTP such as CD45 thereby influencing the activity of the Src kinase Lck [17]. (3) Zinc directly stimulates Lck activity [10], which would result in activation of p38 via the Syk kinase ZAP70 [14]. (4) Zinc inhibits PDEs and leads to cGMP accumulation with subsequent PKA activation [23,24]. This could cause inhibition of the Raf/MEK/ERK pathway and activate p38 and CREB [27]. (5) In this study, the existence of additional mechanisms for zinc to influence p38 and ERK1/2 was demonstrated.

kinases was observed in regular Jurkat cells in response to zinc treatment, experiments with Jurkat$^{Lck\ neg}$ showed that this kinase is not relevant for p38 activation.

The MAPK p38 is activated via Lck and ZAP70 after TcR engagement, which is independent of MAPKK and therefore known as alternative activation [14]. However, zinc-mediated p38 phosphorylation in Jurkat$^{Lck\ neg}$ and Jurkat$^{ZAP70\ neg}$ cells and the absence of tyrosine 323 phosphorylation in zinc-treated Jurkat exclude the possibility that zinc triggers this alternative pathway.

Another explanation for the effects of zinc on p38 and ERK1/2 could have been an impact on cyclic nucleotide signaling. PDE inhibition by zinc would lead to accumulation of cyclic nucleotides and PKA activation. PKA inhibits the Raf/MEK/ERK pathway by phosphorylation of Raf on serine 259 [26] and activates p38 and CREB [27]. Our results illustrate that only cGMP is significantly elevated in zinc-treated Jurkat cells, no PKA activation by zinc was observed, and neither p38 or CREB activation, nor ERK1/2 inhibition in response to zinc supplementation depended on PKA.

In addition to CREB, NFκB is another transcription factor important for T-cell activation that is modulated by zinc [1]. Furthermore, in macrophages NFκB is activated by PKA in a p38-dependent fashion [35]. However, conditions that activated p38 and CREB did not induce phosphorylation of the NFκB upstream kinases IKKalpha/beta or lead to DNA-binding of NFκB in an electrophoretic mobility shift assay (data not shown). This indicates that the zinc-dependent signaling pathways investigated here neither affect nor involve NFκB.

Notably, p38 has been shown to be involved in Th1 development, whereas ERK1/2 is required for Th2 [18]. This matches the effects of zinc, which is not only predominantly required for Th1 in vivo but also activates the Th1 MAPK p38 and inhibits the Th2 MAPK ERK1/2. Two recent studies show that zinc is a regulator of the transcription factor CREB, which controls the expression of the Th1 lead cytokine IFN-γ [28,36]. In T-cells, TcR-induced zinc release from lysosomes leads to inhibition of the phosphatase calcineurin, hereby preventing dephosphorylation of CREB and inducing CREB-dependent IFN-γ expression [28]. As shown in Fig. 9, the activating phosphorylation of CREB depends on p38, emphasizing that phosphorylation and dephosphorylation are both under the control of zinc. As a result, the induction of IFN-γ mRNA by zinc links the zinc status to the regulation of Th1/Th2 polarization. This matches the in vivo observation that zinc deficiency leads to reduced Th1-mediated immune functions [6,7].

The present study focused on supplementation of zinc/pyrithione to unstimulated cells or weak TcR stimulation by anti CD3 antibodies, in order analyze the effect of zinc with minimal interference by other signals. Normally, T-cells are activated by a complex interplay of multiple signals. The outcome of T-cell activation depends on many factors, leading either to tolerance or a precisely adjusted immune response, based on the formation of regulatory or effector T-cells. Stronger TcR stimulation, e.g., by cross-linking of Okt3 with secondary antibodies, in combination with CD28 costimulation, and recruitment of CD4 or CD8 will have to be investigated next. A stepwise approach will be required to further analyze the effect of zinc in all these different pathways, and to integrate them into the net effect on T-cell activation.

In conclusion, zinc differentially regulates ERK, p38, and JNK phosphorylation in T-cells, employing mechanisms that differ from those in other immune cells. Induction of Th1 cytokine mRNA expression after zinc/pyrithione stimulation depends on p38-mediated activation of the transcription factor CREB and gives a glimpse at the biochemistry behind the impact of zinc status on Th-cell polarization. Further experiments will now have to elucidate the contribution of zinc to different other components of this complex system. This will allow to finally understand the complex regulation of T-cell immunity by zinc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.10.007.

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