Functional Significance of Zinc-Related Signaling Pathways in Immune Cells

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Abstract

Recent years have brought a paradigm shift for the role of the essential trace element zinc in immunity. Although its function as a structural component of many enzymes has been known for decades, current experimental evidence points to an additional function of the concentration of free or loosely bound zinc ions as an intracellular signal. The activity of virtually all immune cells is modulated by zinc in vitro and in vivo. In this review, we discuss the interactions of zinc with major signaling pathways that regulate immune cell activity, and the implications of zinc deficiency or supplementation on zinc signaling as the molecular basis for an effect of zinc on immune cell function.

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INTRODUCTION

Zinc Status, Human Health, and Immunity

Since its discovery in the 1960s (105), zinc deficiency has been known to compromise immune function, which is well illustrated by three examples. First, zinc deficiency is currently the fifth leading cause of mortality and morbidity in developing countries. Zinc deficiency leads to increased susceptibility to infection and parasitic disease (137), emphasizing the essentiality of zinc for immunity. Second, the disease acrodermatitis enteropathica results from zinc deficiency, in most cases based on an autosomal recessive mutation of the intestinal zinc uptake protein Zrt/Irt-like protein (Zip) 4. Acrodermatitis enteropathica leads to severe immunological consequences, including thymic atrophy, decreased lymphocyte counts and function, and death from infections (75, 133). Third, parallels exist between the decline of immune function with age and zinc deficiency. Restoring zinc levels by supplementation can significantly improve immune function in marginally zinc-deficient elderly, a finding that further supports a causal relationship between zinc status and immunity (47, 60).

Zip: Zrt/Irt-like protein

Cytokine: soluble protein that is released or recognized by immune cells and acts via specific receptors on the cell membrane as an intercellular mediator for the regulation of the immune response

MTF-1: metal-response element-binding transcription factor

Zinc and Immune Function

The effect of zinc on immune function is not based on a single mechanism; rather, zinc affects the expression of hundreds of genes in immune cells (7, 20, 46). Neither is the effect of zinc limited to one part of the immune system. Functional consequences of zinc deficiency affect lymphopoiesis (36), virtually all types of mature immune cells (56, 63), cytokine production (108), and the polarization of T helper subsets (13, 103).

The functional consequences of zinc deficiency are as multifaceted as the effects of zinc on the immune system. Zinc deficiency is associated with allergic, infectious, and autoimmune diseases (108), and experiments in mice indicate that its epigenetic consequences could even persist for several generations (6).

The Molecular Basis for the Role of Zinc

Zinc binding motifs are found in up to 10% of the proteins encoded by the human genome (2). These structural and/or catalytic roles of zinc have been summarized in detail elsewhere (3). On the other hand, zinc-related signaling pathways involve transient structural stabilization of signaling proteins [e.g., zinc fingers in metal-response element-binding transcription factor (MTF-1)], inhibition of enzymes (e.g., different types of phosphatases), and assembly of multiprotein complexes (e.g., between Lck and the T cell receptor). These main molecular interactions by which zinc exerts its function in signal transduction are discussed in detail below.

REGULATION OF FREE ZINC CONCENTRATIONS

Free intracellular zinc, which has also been called labile, mobile, or available zinc, can

reversibly bind to regulatory sites in signaling proteins. Consequently, changes of the free zinc concentration can affect signaling and might even act as zinc signals.

Cellular Zinc Homeostasis

Physiologically, free zinc is mainly regulated at the single cell level, and cells employ an intricate homeostasis that involves several dozen proteins. Zinc can be controlled by uptake from the extracellular space, redistribution between different intracellular compartments, or the reversible oxidative release of zinc ions from storage proteins. Taken together, these mechanisms regulate free zinc homeostasis and thereby zinc signaling (22, 109).

Mammalian zinc transporters belong to two major protein families. The first are 14 Zip (Zrt/Irt-like) proteins, designated SLC39A1–A14, which transport zinc into the cytosol either from the extracellular space or cellular organelles. The second are 10 ZnT transporters, designated SLC30A1–A10. In general, they transport zinc out of the cytosol, leading to export or intracellular sequestration, e.g., into zincosomes, which are vesicles that can accumulate high levels of free zinc (22, 109). It is known that there is considerable cell-specific expression of some of the transporters, which is dynamically regulated in response to zinc status (4, 95) and endocrine and cytokine signaling (22). On the other hand, expression of these transporters can regulate signal transduction via the level of intracellular zinc (125).

Metallothionein (MT) is a cysteine-rich 6–7 kD protein that binds metal ions, in particular zinc (86). Although all zinc ions are bound in similar tetrathiolate coordination environments, the affinities of the sites for zinc vary, with a logK between 7.7 and 11.8 (73), providing picomolar to nanomolar concentrations of free zinc ions to the cytosol. Also, the number of zinc binding sites of MT can be regulated by reversible oxidation (87), creating additional species of MT that are partially oxidized or even polymerized by intermolecular disulfide bonds (43, 45), linking free zinc ion homeostasis to cellular redox metabolism (87). A physiological role for MT is suggested by experiments with MT knockout mice, which show reduced proliferation of thymocytes in response to the T cell mitogen concanavalin A, and reduced cytokine production, antigen presentation, and phagocytic activity by macrophages (118).

The metal-response element-binding transcription factor (MTF)-1 is a cellular zinc ion sensor (76). It regulates transcription of genes whose promoters contain metal-response elements (106) because at least two of its six zinc fingers are not constitutively binding zinc, but sense free zinc ion availability by reversible binding, resulting in stabilization of the zinc-finger domains and subsequent binding to DNA (76). In addition, transcription is activated by phosphorylation of MTF-1 at serine and tyrosine residues by various signaling pathways that can be activated by zinc ions (79, 112). MTF-1 controls the expression of proteins that are essential for zinc homeostasis. It induces transcription of MT-1 and -2 and the transporter ZnT-1 (50, 78), and has been suggested to repress transcription of ZIP4 and 10 (22).

Nitric oxide synthase (NOS) catalyzes the synthesis of the second messenger nitric monoxide (NO) from arginine. NO can oxidize cysteine thiols, and S-nitrosylation and subsequent disulfide formation lead to the release of protein bound zinc, e.g., from MT (74). By this mechanism, the proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, which induce expression of the inducible NOS, can cause a NO-mediated release of zinc from nuclear MT (114). In turn, it was shown that zinc released from MT by NO can activate MTF-1-dependent gene expression (117).

Zinc Homeostasis and Inflammation

Remarkable changes in zinc homeostasis occur during an inflammatory immune response. Zinc plasma levels decrease in response to endotoxins such as lipopolysaccharide (LPS, a membrane component of gram-negative bacteria)

Free zinc: although the name is chemically incorrect, "free zinc" is generally used as an operative term describing zinc that is only loosely bound to its ligand environment. Free zinc is only a very small fraction of the total cellular zinc content. These metal ions can bind to regulatory binding sites of enzymes involved in signal transduction, whereby changes in the free zinc concentration can act as signals

Zinc signals: a change of the intracellular concentration of free zinc, leading to a change in the saturation of regulatory zinc binding sites in one or more signaling proteins, thereby affecting signal transduction

ZnT: zinc transporter

MT: metallothionein

Innate immunity: first barrier of the immune response, based on the recognition of conserved molecular patterns of pathogens. Due to the expression of receptors for antibodies, the innate immune system can also support the adaptive immune system

MAPKs: mitogen-activated protein kinases

or proinflammatory cytokines. This is due to translocation of zinc into organs, predominantly the liver (21). The proinflammatory cytokine IL-6 leads to upregulation of the zinc-import protein Zip14 in mouse liver (84). In rat hepatocytes, the zinc-binding protein MT and cellular zinc content are upregulated by IL-6 (23, 113), and experiments in MT knockout mice showed that this protein is required for hepatic accumulation of zinc (101), indicating that zinc is stored as an MT complex. Physiological importance for the upregulation of MT is suggested by the observation that survival of MT knockout mice (lacking MT-I and -II) in polymicrobial sepsis is reduced compared to the respective wild types (136).

Recent experiments indicate additional effects of inflammation on the zinc homeostasis of single immune cells. Zip8 is upregulated in monocytes in response to treatment with TNF-α and/or endotoxin (8, 22), and dendritic cell maturation in response to LPS leads to changes in intracellular zinc and an altered expression of zinc transport proteins (67). In addition, treatment of monocytes with LPS leads to increased expression of MT (81), and antisense experiments demonstrated an involvement of MT in LPS-induced adherence and production of H_2O_2 (80). These results show that inflammation alters zinc homeostasis on the level of the entire organism as well as in single cells, and that these events are involved in the immune response to the pathogen.

SIGNIFICANCE OF ZINC FOR SIGNALING PATHWAYS IN IMMUNE CELLS

Compared to the complexity and number of processes that are regulated on the cellular level, only a very limited number of signaling pathways exist. Depending on the type of cell, these pathways are utilized by different receptors and lead to expression of a specialized set of genes. Immune cells, but also a myriad of other cell types, share these identical signaling pathways. In this review, we cite data obtained in nonimmune cells if these signaling pathways are relevant and respective experiments have not been done in the immune cell type that is discussed. In addition, although the influence of zinc on certain pathways is discussed for one cell type, owing to redundancies, these pathways can be relevant for several other types of immune cells, which are only briefly indicated in the respective section.

Zinc and Innate Immunity

Innate immunity constitutes a fast but unspecific defense against pathogens. Its cells are activated by pathogen-associated molecular patterns (PAMPs), conserved structures in pathogens that are recognized by a set of conserved receptors, triggering phagocytosis, cytokine secretion, killing of target cells, or antigen presentation to cells of the specific immune system.

Monocytes/macrophages and dendritic cells. Monocytes/macrophages and dendritic cells (DCs) take up and present antigens to T cells, and they can release cytokines in order to coordinate the immune response. A potent activator for both cell types is LPS, which is sensed by Toll-like receptor (TLR)-4. TLR-4 signaling leads to secretion of proinflammatory cytokines by monocytes and maturation and antigen presentation in dendritic cells and macrophages.

Zinc has a dual effect on TLR-4-induced cytokine secretion in monocytes (summarized in **Figure 1**). Although a moderate increase of free zinc is a physiological signal involved in TLR-4 signal transduction, higher concentrations are inhibitory. Intracellular zinc signals can be observed within less than two minutes after stimulation with LPS (48). They are involved in the activation of mitogen-activated protein kinases (MAPKs) by TLR-4, and chelation of zinc completely abrogates MAPK stimulation in response to LPS (48). MAPK activation by zinc is a common event in many cell types. The MAPK extracellular regulated kinase (ERK) was previously shown to be activated by zinc treatment of fibroblasts (49), neurons, and neuroblastoma

cells (1, 98), as well as in oligodendrocytes as a result of zinc release by peroxynitrite (144). This effect is not limited to ERK; several examples exist that describe activation of other MAPKs like p38 and Jun N-terminal kinase (JNK) (1, 79).

No direct effect of zinc on MAPK activity has been shown so far. Hence, MAPKs are most likely not a direct molecular target of zinc but rather are activated indirectly. In human monocytes, inhibition of ERK and p38 dephosphorylation was shown (48). This suggests an effect of zinc on MAP-kinase phosphatases (MKPs), which are dual-specificity phosphatases that dephosphorylate both tyrosine and threonine residues in activated MAPKs. Zinc-inhibition of MKPs is involved in ERK activation during oxidative stress in neurons (52) and is one mechanism by which zinc stimulates expression of IL-8 in epithelial cells (66). However, not all of the zinc effects on MAPK phosphorylation and activation can be explained simply by zinc inhibition of MKP because zinc also affects targets upstream of the MAPKs. For example, MAPK activation in airway epithelial cells also depends on an effect of zinc on tyrosine phosphorylation upstream of ERK (138).

The effect of zinc on MAPKs is not always agonistic. In rat glioma cells, only low concentrations of zinc activate ERK, whereas higher concentrations were shown to reduce the activating phosphorylation (44). A potential molecular mechanism for such a negative regulation of Erk was identified in *Caenorhabditis elegans*. Here, the ZnT-1 homolog CDF-1 mediates zinc efflux, which leads to activation of the Ras/Raf/MEK/ERK pathway (11). It was suggested that zinc promotes the inhibitory phosphorylation of KSR (kinase suppressor of Ras) by a yet unidentified mechanism. KSR is a scaffolding protein that stabilizes the interaction of Ras, Raf, and MEK (141). Another hypothesis states that CDF/ZnT-1 binding to Raf-1 promotes its biological activity and that this interaction is inhibited by Zn^{2+} (58).

The inhibitory effect of elevated free zinc on TLR-4 signaling is based on a different mechanism, the modulation of cyclic nucleotide signals. The second messengers, cyclic adenosine monophosphate (cAMP) and its guanosine analogue (cGMP), are synthesized by adenylate cyclases (ACs) or guanylate cyclases (GCs), respectively. Their degradation is mediated by cyclic nucleotide phosphodiesterases (PDEs).

Several isoforms of PDEs are activated at low zinc concentrations because they require a catalytic zinc ion, bound to two tandem histidine-containing amino acid sequences in the catalytic domain (18). Conversely, slightly higher concentrations of zinc than those necessary for activation inhibit PDEs in vitro (29, 37, 99). There is no indication that binding of the catalytic zinc ion is influenced by the labile zinc concentration, whereas augmented concentrations of free zinc can inhibit PDE activity and reduce the rate of cyclic nucleotide degradation.

In human monocytes, degradation of cyclic nucleotides is mediated by PDEs 1, 3, and 4, all of which are inhibited by zinc (131). In addition to its effect on catalytic activity, zinc also reduces gene expression of most PDE isoforms and blocks the LPS-induced increase of PDE-4B transcription and PDE activity (131). The anti-inflammatory effect of zinc is due to cross-activation of protein kinase A (PKA) by elevated cGMP, resulting from zinc-mediated PDE inhibition. This leads to inhibitory phosphorylation of Raf-1 at serine 259 with subsequent Raf-1 inactivation. Because active Raf-1 is required for TLR-4-mediated NF-κB activation, the zinc/cGMP/PKA/Raf pathway blocks the activation of this transcription factor and thereby transcription of genes for proinflammatory cytokines (130).

Zinc inhibits the PDE-mediated cleavage of cAMP and cGMP in vitro, but only an increase of cGMP was found after zinc treatment of intact monocytes resulting from reduced formation of cAMP (69, 131). In addition to PDEs, the AC is also inhibited by zinc, whereas the synthesis of cGMP by the soluble GC is not affected. So far, two mechanisms for zinc inhibition of AC have been presented: Zinc may influence the enzymatic activity by altering the conformation of AC (68) or, alternatively, by inhibiting the activation of AC

by the heterotrimeric G-protein alpha subunit (38).

An alternative mechanism for the inhibition of proinflammatory cytokine formation in response to LPS has been demonstrated. In the promyelocytic cell line HL-60, zinc leads to upregulation of the mRNA of the cytoplasmic zinc finger protein A20 (104). In TLR signaling, tumor necrosis factor receptor-associated factor (TRAF)-6 ubiquitination leads to proteasome-independent activation of the transforming growth factor beta-activated kinase (TAK)1, which in turn phosphorylates IκB kinases (IKKs) and MAPK kinases (MKKs) (28, 132). A20 is induced by proinflammatory stimuli and is involved in the termination of TLR-induced activity of NF-κB and proinflammatory gene expression in macrophages (9). A20 deubiquitinates TRAF-6, inactivating this protein. Therefore, upregulation of A20 by zinc would lead to diminished NF-κB and MAPK activation in response to TLR stimulation.

Several proteins in this signaling pathway contain structural zinc sites, and reversible binding could mediate a zinc effect in a manner similar to that of the regulatory zinc fingers in MTF-1. A20 contains seven zinc fingers (94), and TRAF-6 contains one RING (really interesting new gene) and four zinc finger structures (77). The vast majority of zinc fingers bind the metal too tightly to respond to changes in free zinc, and there is no evidence that zinc may affect TRAF-6. Furthermore, A20 deubiquitinase activity is unaffected by the presence of millimolar concentrations of the zinc chelator 1,10-phenanthroline in vitro (9). Although the RING finger and first zinc finger of TRAF-6 are essential for activation of IKK and p38 MAPK, in the case of A20 the N-terminal domain is responsible for the deubiquitinase activity of A20, and the C-terminal zinc finger–rich region is a ubiquitin ligase not relevant for TRAF-6 deubiquitination (135). Taken together, there is no indication that inhibition of LPS-induced signaling by zinc involves its reversible binding to TRAF-6 or A20.

On a longer time scale, zinc also affects the response of DCs to LPS. It was shown that treatment of murine DCs with LPS led to a reduction of free intracellular zinc, based on a TIR-domain-containing adaptor protein inducing IFN beta (TRIF)-dependent change in the expression pattern of several zinc transporters. This involved a reduction of Zip 6 and 10 gene expression and upregulated expression of ZnT 1, 4, and 6. The reduction in free zinc was important for maturation of DCs (67).

Zinc is involved in the regulation of LPS signaling in cells of the innate immune system, but the effect of zinc is not limited to the response to LPS. In the human monocytic cell line THP-1, a role for free zinc in regulation of protein kinase C (PKC) activity during monocyte chemoattractant protein (MCP)-1-induced adhesion was postulated (71), and it remains to be investigated which other receptors on these cells depend on zinc for their signaling. In vivo, zinc deficiency will impair the function of monocytes, in particular their ability to mount an adequate cytokine response during infection, but because zinc is equally involved in negative regulation of the same pathways, a disturbance of zinc homeostasis could also affect the limitation of proinflammatory cytokine production, leading to an overproduction. Although this seems contradictory, it is supported by experimental evidence. In an elderly population, zinc supplementation reduced spontaneous cytokine production but at the same time improved the cytokine response to PAMPs (60).

Granulocytes and mast cells. Fc immunoglobulin receptors allow different immune cells to utilize antibodies for pathogen sensing. In granulocytes, this mechanism is involved in phagocytosis, a process that is known to be impaired under conditions of zinc deficiency (108). In mast cells, triggering of FcεR induces degranulation. Notably, the secretory mast cell granules are rich in zinc, which is released into the cellular environment together with a variety of immunological mediators (41, 51). The relationship between activation of FcRs and zinc status has been investigated in mast cells, but so far the results are ambiguous.

Measurements with fluorescent probes showed a reduction of free zinc in response to treatments that induce degranulation, including the triggering of FCεRs (51). On the other hand, another study reported a so-called zinc-wave, i.e., an increase in intracellular zinc by crosslinking of FcεRI, which depends on Ca^{2+} and MAPK signaling (140). One explanation could be the use of different fluorophores in the two studies, detecting different pools of cellular zinc. Whereas the decrease of zinc (measured with Zinquin) has been attributed to a loss of granule zinc, the increase (measured with Newport Green DCF) seemed to be cytosolic, originating from the perinuclear region (51, 140). However, the differences do not end there. Although treatment with the membrane-permeable zinc-chelator TPEN [N,N,N′,N′-tetrakis-(2-pyridyl-methyl)ethylenediamine] was originally shown to activate NF-κB, and TNF-α-induced NF-κB activation was blocked by treatment with zinc/pyrithione (51), in a later study, NF-κB activation in response to FcεR crosslinking was blocked by TPEN administration, and this effect was reversed by zinc treatment (59).

It remains to be analyzed under which circumstances zinc inhibits NF-κB and caspase-3 activity (51) or is involved in the activation of NF-κB, ERK, JNK, and PKCβI and thereby is required for degranulation and cytokine production in mast cells in vitro as well as allergic reactions in vivo (59, 140). No notable differences exist in the concentrations of TPEN, zinc, and pyrithione, or treatment of the cells with these substances, in the different studies. It also remains to be seen if the differential outcome of these experiments was based on the use of mast cells from different species or, in the case of primary cells, on the method by which these were obtained.

NK cells. Natural killer (NK) cells are a subset of lymphocytes that mediate the killing of either infected or transformed cells. The recognition of these cells is based either on "missing self" [the absence of major histocompatibility complex (MHC) class I molecules] or by antibodies bound to the cellular surface. So far, experimental evidence exists only for an influence of extracellular zinc on NK cells. Zinc has been shown to induce multimerization of killer Ig-related receptors, altering their binding to MHC class I molecules on the surface of target cells (128). Furthermore, zinc supplementation promotes the development of NK cells from $CD34^+$ hematopoietic stem cells (92) and increases the quantity of interferon-γ-producing NK cells (89).

Intracellular zinc signals have not been investigated in NK cells; however, lines of evidence connect major signaling pathways in NK cells to zinc. A multitude of different receptors are required for fine-tuning of NK cell activity (142). A common feature of many of these receptors is the presence of characteristic tyrosine phosphorylation sites that either activate (immunoreceptor tyrosine-based activation motif; ITAM) or inactivate (immunoreceptor tyrosine-based inhibitory motif; ITIM) NK cell function. In this respect, one feature of zinc ions becomes relevant: their ability to inhibit protein tyrosine phosphatases (PTPs) (10). This inhibition seems to be a common feature of PTPs, in which zinc interacts with the highly conserved catalytic domain, possibly by binding to the active site cysteine (44). Notably, a nucleophilic attack by the catalytically active cysteine residue is a shared feature of PTPs and MKPs, another target of zinc (5). On the other hand, serine/threonine phosphatases employ a dinuclear metal center in which water acts as the nucleophil (5), and no reports exist about a specific inhibition of these enzymes by zinc. Several PTPs are inhibited by low concentrations of zinc, including T cell PTP, with an IC_{50} of 200 nM (88), and PTP1B and SHP-1, with IC_{50} values of 17 nM and 93 nM, respectively (42). These values are close to the concentration range of physiological levels of free intracellular zinc and suggest that a partial inhibition of PTPs by free zinc could even occur at basal levels of free zinc.

Among the lymphocytes, CD16 (FcγRIII) is a characteristic marker for NK cells. It enables NK cells to recognize immune

Major histocompatibility complex (MHC): these protein complexes are required to present antigens to T cells and define the molecular basis of self-recognition

PTPs: protein tyrosine phosphatases

complexes of IgG on target cells, leading to their destruction by a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC) (17). In light of the involvement of zinc in the signal transduction of another Fc receptor, as discussed above for mast cells, it should be a worthwhile target to investigate the role of zinc in CD16-mediated ADCC of NK cells.

Many of the functions that zinc may have on signal transduction in NK cells have been hypothesized from the effects of zinc on comparable signaling pathways in other cell types. Clearly, experimental confirmation is required. In vivo observations during zinc deficiency indicate a reduced lytic activity of NK cells (34) that is restored by zinc supplementation (102), which corresponds to a requirement of zinc for NK cell activity.

Zinc and the Adaptive Immune System

In the adaptive immune system, T and B lymphocytes carry specific receptors to recognize a certain target structure. Altered zinc homeostasis in vivo affects lymphopoiesis and function of these cells, and the multiple interactions of zinc with relevant signaling pathways suggest its effect on the cellular signal transduction as the underlying molecular cause.

T cells. Signaling by the T cell receptor (TCR) is affected by zinc at several different levels, as summarized in **Figure 2**. The TCR has no intrinsic kinase activity and depends on an Src-family tyrosine kinase, the lymphocyte protein tyrosine kinase (Lck), for signal transduction. Signaling from the TCR is initiated by the specific interaction with an antigen-loaded MHC molecule on the surface of a neighboring antigen-presenting cell followed by the assembly of a multiprotein TCR-signaling complex. Lck is one of the first kinases to be activated and is critical for phosphorylation of the 10 ITAM motifs of the T cell antigen receptor-signaling complex and the kinase ZAP-70 (96). Lck expression is upregulated in T lymphocytes of zinc-deficient mice (82, 90), and several additional lines of evidence also point to a direct regulation of Lck activity by zinc.

Zinc ions support recruitment of Lck to the TCR signaling complex and its activation by linking two distinct protein interface sites. The N-terminal region of Lck is bound to the intracellular domains of the membrane proteins CD4 or CD8 (55, 83). In a "zinc clasp" structure, this interaction is stabilized by a zinc ion bound to two cysteine residues from each protein at the interface site between Lck and CD4/CD8 (64). Because CD4 and CD8 also bind to MHC with their extracellular domains and are thereby recruited to the vicinity of the TCR signaling complex, this brings Lck in close proximity to its substrates.

The second zinc-dependent interface site is required for homodimerization of Lck. Here, two zinc ions at the dimer interface of the SH3 domains stabilize the complex (110). With an estimated K_d value for zinc of $\leq$100 nM, this interaction is in the physiologically relevant concentration range of free zinc. The activation of Lck is a complex event that depends on two tyrosine phosphorylation sites. One is tyrosine 394 in the so-called activation loop. It is transphosphorylated between Lck molecules upon activation, increasing kinase activity (10, 96). Zinc-induced homodimerization brings two Lck molecules into close proximity, thereby facilitating its full activation. The other phosphorylation occurs in the C terminal negative regulatory site at tyrosine 505. Upon phosphorylation of this tyrosine residue, the kinase assumes a closed, inactive conformation. The transmembrane PTP CD45 selectively dephosphorylates tyrosine 505, keeping Lck in a primed state. TCR signaling and Lck phosphorylation are regulated by a number of additional PTPs (91), all of these being potential targets for zinc-inhibition. Consequently, it cannot be predicted if this would result in preferential dephosphorylation of an activating or inactivating tyrosine by zinc in vivo. Activation of Lck by micromolar zinc concentrations has been observed in cell free experiments, but although inhibition of tyrosine 394 dephosphorylation

Adaptive immune system: specific response against an antigen, based on the selective activation of a small subset of T and B cells that carry specific receptors for this particular antigen. The formation of an immunological memory allows a more efficient reaction when the same antigen is encountered again. This memory function is unique to the adaptive immune system

TCR: T cell receptor

could be an explanation for a direct activation of Lck in cells, increased kinase activity was found for the immunoprecipitated kinase, i.e., in the absence of PTP (100). Although this does not exclude a participation of PTP inhibition in intact cells, it shows that zinc can activate Lck directly in the absence of phosphatases, most likely as a result of dimerization. Both zinc-dependent homodimerization and heterodimerization are specific for Lck because the cysteine residues that form both intermolecular zinc-binding sites are unique for this kinase and are not present in other Src kinase family members (64, 110).

The c-src tyrosine kinase COOH-terminal Srk kinase (Csk) phosphorylates the inactivating tyrosine 505 of Lck, thereby inhibiting TCR signaling and T cell activation (16). Zinc can interfere with these events in several ways. First, Csk is inhibited by reversible binding of zinc ions (69, 119). This inhibition is mediated by substituting for Mg^{2+} at one of its binding sites, and zinc has a submicromolar IC_{50} value even in the presence of 6 mM Mg^{2+} (69, 119).

In addition, phosphorylation by PKA activates Csk in T cells (129). As discussed above, zinc has no direct influence on PKA activity (130), but it can inhibit AC and thereby the formation of the PKA activator cAMP (69). Also, zinc inhibition of PDE can block degradation of cyclic nucleotides, leading to activation of PKA (130). While inhibition of CSK and AC promote TCR signaling, PDE inhibition could antagonize it. Which outcome on TCR signaling results from the modulation of this pathway by zinc in vivo remains to be investigated.

The transcription factor nuclear factor of activated T cells (NFAT) mediates the expression of many genes in response to TCR stimulation, including IL-2. In resting cells, NFAT proteins are constitutively phosphorylated and reside in the cytoplasm. In response to TCR/CD28-mediated calcium signaling, NFAT is dephosphorylated by calcineurin (CN), a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, and translocates into the nucleus (85).

Although zinc and iron are essential cofactors for the catalytic domain of CN, which contains a Fe^{2+}-Zn^{2+} binuclear center, several reports describe an inhibition of CN by zinc but not by iron (53, 97, 121). In vitro experiments with CN from bovine brain even showed an inhibition at physiologically relevant concentrations of zinc (10 nM–10 μM). However, in these experiments CN was activated by the addition of nickel, and the inhibition was based on competition with Ni^{2+}, whereas no effect was found on the interaction of CN with the physiological activator calmodulin (121).

Phosphatidyl-inositol-3-kinase (PI3K) is a negative regulator of GSK-3β, one of the kinases that keeps CN in its phosphorylated, inactive state in resting cells. Hence, augmented PI3K activity can abrogate NFAT activity (85). Increased GSK-3β phosphorylation via a pathway involving PI3K has been shown in neuroblastoma cells (1), and zinc acts via the PI3K pathway in various cell types (31, 65, 79, 124). A mechanistic explanation could be an increased degradation of phosphatase and tensin homolog (139), which dephosphorylates the product of PI3K, phosphatidylinositol 3,4,5-trisphosphate, that mediates the activation of PDK-1/Akt/GSK-3β. These results suggest that a comparable activation of PI3K by zinc could also occur in T cells. Inhibition of CN by zinc would result in NFAT inactivation and a concomitant reduction of TCR-mediated transcription, whereas activation of PI3K signaling would act agonistic. An indication of which of these mechanisms may be more relevant in the cellular context is given by experiments in Jurkat cells, a human T cell line, in which zinc blocks NFAT-induced IL-2 mRNA expression and protein production (123).

In addition to TCR signaling, zinc also affects signals originating from the interleukin (IL)-1 receptor. High zinc concentrations (0.1 mM) inhibit IL-1β-stimulated IFN-γ production in primary human T cells and IL-1 dependent proliferation of a murine T cell line. It has been shown by an in vitro activity assay of IL-1 receptor-associated kinase (IRAK), a central kinase in the signaling pathways

downstream of the IL-1 receptor, that IRAK-1 activity is reduced by incubation with zinc (134).

The IRAK family of serine/threonine kinases is also involved in other pathways. IRAK4 has been found to participate in TCR-mediated NF-κB activation (120), although this observation is disputed (61). Hence, zinc can inhibit two central signaling pathways in the activation of T-cells, TCR and IL-1 receptor, by its effect on IRAK, making this a major target for negative regulation of T cells by zinc.

The role of IRAK is not limited to T cells. The high degree of similarity between TLR-4 and IL-1 receptor signal transduction, including the involvement of IRAK family members, suggests that IRAK inhibition could be another mechanism by which zinc negatively influences TLR-4 signaling.

A function of zinc as a mediator of T lymphocyte signal transduction was among the first reports of a potential role of this ion in signaling. Two decades ago, its interaction with PKC was identified as the biochemical basis of these observations (25).

The PKC family of serine/threonine kinases consists of several different isoforms. They are distinguished into classical PKCs, which are activated by cofactors such as Ca^{2+} and diacylglycerol; novel PKCs, which bind diacyglycerol but no Ca^{2+}; and atypical PKCs, which do not interact with either cofactor.

Each isoform has specific roles in the regulation of cellular functions. In T cells, PKC θ is involved in the activation of several transcription factors in response to TCR/CD28 stimulation. In addition, a role for PKC α in proliferation and IL-2 production of T cells was shown. In B cells, several PKC isoforms are involved in survival, pre-B cell development, and induction of tolerance toward self antigens. In innate immunity, primary targets for PKC are macrophage activation and mast cell degranulation (122).

The PKCs have four conserved domains (C1–C4). Structural analysis of PKC β1 has shown that the diacylglycerol binding C1 domain in the N-terminal regulatory part of the enzyme contains two homologous regions with six cysteines and two histidines, forming a total of four Cys_3His zinc binding motifs (54). No information about a differential effect of zinc on the different isoforms is available, and varying forms of C1 domains are present in conventional, novel, and atypical PKCs, indicating zinc binding to all known PKC isoforms (122).

Zinc treatment stimulates multiple steps during PKC activation, including augmented PKC kinase activity, increased affinity to phorbol esters, and enhanced binding to the plasma membrane and cytoskeleton; the induction of these events by physiological activators of PKC can be inhibited by membrane-permeable zinc chelators (26, 27, 35, 143).

The interaction between PKC and zinc is not limited to an effect of zinc on the activation of PKC. PKC itself can be a source for zinc release, as it has been shown that activation by lipid second messengers or thiol oxidation can lead to measurable zinc release from the regulatory domain (70, 72). In addition, PKC also regulates the intracellular free zinc concentration and distribution. In T cells, phorbol ester treatment results in the redistribution of zinc from the nucleus and mitochondria to the cytosol and microsomes (24), and treatment with phorbol esters leads to an increase of free zinc in the Jurkat human T cell line (48). Furthermore, the phorbol ester-induced differentiation of HL-60 cells into macrophages comprises a transient increase of nuclear zinc that depends on PKCβ (39).

A prediction of the effects of zinc on T cells is impossible because of the number of zinc-related signaling pathways. In vivo, there are several observations regarding zinc status and T cell function, such as an increase of the delayed-type hypersensitivity reaction upon correction of zinc deficiency (47), but it remains unclear if this is a direct effect on T cells or is mediated via the cytokine environment provided by other immune cells. On the other hand, zinc supplementation inhibits the allogeneic reaction in the mixed lymphocyte culture (33). This indicates, once again, that zinc may have multiple, opposing functions, depending on its concentration and certainly also on the interaction

with multiple other environmental factors that remain to be identified.

B cells. Many of the zinc-regulated signaling pathways discussed above are also important in B cells, e.g., tyrosine phosphorylation, PKC, MAPK, and activation of the transcription factors NFAT and NF-κB. However, in contrast to many of the cell types discussed above, mature B cell function is not so dependent on the organisms' zinc status. Rather, lymphopoiesis and pre-B cell development are mainly affected by zinc deprivation in vivo (36). During zinc deficiency, the loss of lymphoid tissue exceeds that of other tissues, and in addition to B cells, T cell development is also impaired (63). At several points in the development of lymphocytes, a rigorous selection ensures functionality and avoids autoreactivity by eliminating the majority of newly formed cells by apoptosis. Zinc deficiency increases the rate of apoptosis and leads to a depletion of B cells. In addition to an effect on glucocorticoid metabolism, which has been connected to the loss of pre-B cells (36), and an effect on the gene expression of several regulatory proteins, such as the ones from the Bcl/Bax family (127), zinc has been shown to influence several aspects of apoptotic signal transduction.

Zinc directly regulates the activity of enzymes in the apoptotic cascade. First, the calcium-dependent endonuclease that mediates DNA fragmentation is inhibited by zinc (30). However, this target is beyond the point of no return for programmed cell death, and an inhibition could explain a suppression of DNA fragmentation during apoptosis, but not the effect on cellular survival.

Another important group of enzymes in apoptosis are cysteine-aspartic acid proteases (caspases), which form a cascade to transduce initial apoptotic signals to the effector enzymes that mediate the organized destruction of cells characteristic for programmed cell death. In this process, inactive procaspases are activated by proteolytic cleavage. An additional regulatory mechanism is indicated by inhibition of caspases-3, -6, -7, and -8 by low micromolar zinc concentrations (116). Caspase-6 was most sensitive, being inhibited at an apparent binding constant of 0.3 μM (116). Still, these measurements were performed in the presence of β-mercaptoethanol, a thiol-based reducing agent that binds zinc and leads to an overestimation of the metal ion concentration required for inhibition. Accordingly, an effect of the β-mercaptoethanol concentration on the IC_{50} of zinc was shown for caspase-3 (116). When measurements were performed in the absence of reducing agents and chelators, and using HEPES, a buffer substance with minimal interaction with zinc, an IC_{50} below 10 nM was found for caspase-3 (88). This value is clearly in the physiological range of free intracellular zinc, suggesting that endogenous zinc can inhibit caspase-3. As in the case of PTP and MKP, a cysteine residue is involved in the catalytic mechanism of caspases, and it has been suggested that caspase-3 inhibition is based on reversible binding of zinc to cysteine 163 in the catalytic site (127).

It is interesting to speculate why the function of mature B cells, which employ the same signaling pathways as other immune cells, seems to be affected by zinc to a lesser extent. Even the reduced antibody production during zinc deficiency is based on reduced B cell numbers whereas it is unaffected on a per-cell basis (19), pointing to an effect on cellular development rather than on function. This may be caused by differences in zinc homeostasis, making mature B cells less susceptible to conditions of limited zinc availability. Although B cells are highly susceptible to apoptosis during development, and zinc is one factor that influences these signals, mature B cells can tolerate comparable conditions due to changes in zinc-regulating proteins, but certainly also by changing the expression patterns of several other factors that regulate the cellular responsiveness to apoptotic signals.

FUTURE RESEARCH

Despite the impressive progress zinc research has made since the discovery of the essentiality of zinc for humans in the early 1960s (105),

much remains to be done. Many molecular targets for zinc were identified, especially in signal transduction. Still, the majority of these effects were characterized looking at isolated signaling pathways, and in most cases, not even in cells of the immune system. Zinc affects multiple signaling pathways with a potentially opposite outcome for cellular activity, as shown for the effects on TCR signaling in **Figure 2**. Future efforts will have to elucidate the in vivo situation, i.e., which of these effects are actually physiologically relevant.

Much information is also lacking on the functional consequences of zinc in the context of whole organs. Although a role for zinc as an endocrine mediator is rather unlikely, it may be a paracrine mediator under certain circumstances. Occasionally, parallels in the organization of the brain and the immune system are pointed out. In the brain, zinc modulates neural transmission of information, and it may be a neurotransmitter itself (62). It is well established that lymph nodes and the spleen are neurologically innervated, and that nerve activation results in a response such as contraction of lymph nodes (111, 126). However, it is less clear how the nervous system and the immune system communicate. Zinc, which is a signal or modulator in both systems, may be a common language (48, 62).

In order to mediate a signal, the zinc concentration has to be intermediate. There have to be sufficient reserves for release during signaling, but levels must still be low enough to show an amplitude during signaling and avoid permanent inhibition of zinc-sensitive enzymes. Interestingly, the zinc content in immunologically or neurologically highly active tissues is low or intermediate, whereas organs with low immunological activity or immune-privileged organs show extremely high zinc content. For example, blood (5–6 μg/g zinc content) and skin (32 μg/g) as immunologically highly active organs have a low zinc content. In comparison, liver (58 μg/g) and kidney (55 μg/g), which are immunologically active but not in direct contact to pathogens, have an intermediate zinc content, whereas immune-privileged organs such as the retina (290 μg/g) and the prostate (700 μg/g) have a very high zinc content (12, 32, 57, 115). It is plausible that these high zinc concentrations suppress immune responses and inflammatory signals, as described above. Furthermore, in both tissues, reduced zinc concentrations are found in diseases such as age-related macular degeneration (AMD), prostate cancer, and prostatitis (32, 40). This hypothesis is supported by the fact that AMD can be treated by oral zinc application, and prostatitis can be treated by direct application of zinc into the prostate in an animal model (15, 93). Therefore, the high zinc concentration may be the reason for the absence of immune reactivity. However, high zinc concentrations may also have some advantages, since they could prevent undesired inflammatory processes that destroy tissues, such as those observed in AMD.

As mentioned above, the prostate is the organ with the highest zinc content in the body. Therefore, it should be questioned whether there could be another reason for the high content of the prostate and why it differs from that of the testis (170 μg/g), which has a high, but not extremely high, zinc content (115). One explanation is that effective defense against pathogens is essential for the reproductive system to ensure reproduction of the species. This is only possible if the innate immunity is active, which depends on the activation by pattern recognition receptors such as the TLRs (107). If TLR signaling would be inhibited by high zinc concentrations, the reproductive system would no longer be protected. Therefore, the intermediate concentration is the best choice for the testis.

In the light of the anti-inflammatory effect described in this review, the prostatic fluid is an anti-inflammatory agent that may have another function. A less-common but long-known cause of female infertility is the production of antisemen antibodies (14). However, semen is a foreign antigen to every woman, and therefore it is remarkable that this pathology is not more common. The high zinc concentration of the prostatic fluid may suppress the first

inflammatory response and, later on, antibody production.

Taken together, the modulation of signaling events by tissue levels of free zinc may be a general phenomenon, especially in the immune system, which could be easily mediated by different zinc concentrations. The local effect of this different zinc concentration on tissue-specific physiology and immune responses should be investigated in the future.

CONCLUDING REMARKS

Several signaling proteins have been shown to be zinc regulated. At times, it seems that the number of zinc-regulated pathways exceeds the pathways that are not affected. Although certainly not all of these interactions are physiologically relevant, it becomes clear that nanomolar concentrations of free zinc regulate the activity of enzymes such as PTP, MKP, and caspases by reversible binding. The use of chelators has demonstrated that zinc not only can trigger signals when added artificially, but also is an essential physiological component of signaling pathways. Therefore, the importance of zinc for immunity is not only based on its role as a static component of a plethora of different proteins, but also on the regulation of signal transduction by free zinc.

Based on the observation that free zinc concentrations can change upon stimulation of cells, it has been speculated that zinc ions may even be a second messenger, comparable to calcium. Because zinc appears to act mainly by inhibiting negative regulators such as phosphatases, we suggest a slightly different approach, in which zinc acts as a permissive signal. In this case, a certain intracellular threshold of free zinc would be required, partially inhibiting dephosphorylating enzymes and thereby protecting phosphorylation signals. In cells, phosphatase activity generally exceeds kinase activity by far, and mechanisms such as intracellular sequestration, oxidative inhibition, or the fast, reversible inhibition of phosphatases by zinc ions are necessary to permit or preserve phosphorylation signals. Therefore, the free intracellular zinc level may regulate the cells' ability to respond to extracellular stimulation.

Zinc deficiency impairs the immune response, and many diseases are accompanied by secondary zinc deficiency that may aggravate disease, similar to the effects described for the inflammatory response. In these cases, just supplementing zinc and expecting that a rise in plasma zinc will have the same effect as an intracellular zinc signal may not be an appropriate therapeutic treatment. Cellular homeostasis tightly controls zinc levels; otherwise, each meal that contained enough zinc to raise plasma levels would modulate the immune response. However, when intracellular zinc stores are depleted during zinc deficiency, supplementation could replenish zinc required for intracellular signaling and restore immune function.

In conclusion, we now know several distinct zinc-dependent signaling pathways and molecular targets of zinc. However, the overall influence of zinc homeostasis on immune cell reactivity and the resulting immune response remain to be uncovered.

SUMMARY POINTS

1. In several immune cells, zinc signals were observed after stimulation with lipopolysaccharide (monocytes, dendritic cells), phorbol esters (T cells), or crosslinking of receptors for antibodies (mast cells).
2. In addition to the effects on the cellular level, systemic changes of zinc homeostasis occur during the acute phase of an infection, especially an IL-6-induced sequestration into the liver via Zip 14, where the zinc remains stored in a complex with metallothionein.
3. Free zinc is involved in the regulation of cytokine production (monocytes), maturation (dendritic cells), degranulation (mast cells), and apoptosis (lymphocytes).

4. Many signaling pathways have been shown to be affected by free zinc, including MAPKs, PKC, NF-κB, cyclic nucleotides, IRAK, caspases, tyrosine phosphorylation, and PTPs, and transcription factors such as NFAT and MTF-1.
5. Zinc could be a permissive signal, regulating the cellular response to stimulation by inhibition of different kinds of phosphatases that depend on an active site cysteine.

FUTURE ISSUES

1. In many instances, the in vivo relevance of the molecular observations of zinc signaling still needs to be demonstrated.
2. In the case of several zinc-regulated signaling proteins in a single pathway, it has to be elucidated which ones have the predominant influence or if the effects occur at different concentrations, leading to a fine tuning by multiple zinc-sensitive steps.
3. It remains to be investigated whether whole-organ and systemic zinc homeostasis affect the immune response and, in particular, if there is a role of the high-zinc content of immune privileged organs in the regulation of immune function.
4. Finally, the knowledge that has been obtained on the molecular and cellular level has to be translated into therapeutic approaches to modulate the immune response.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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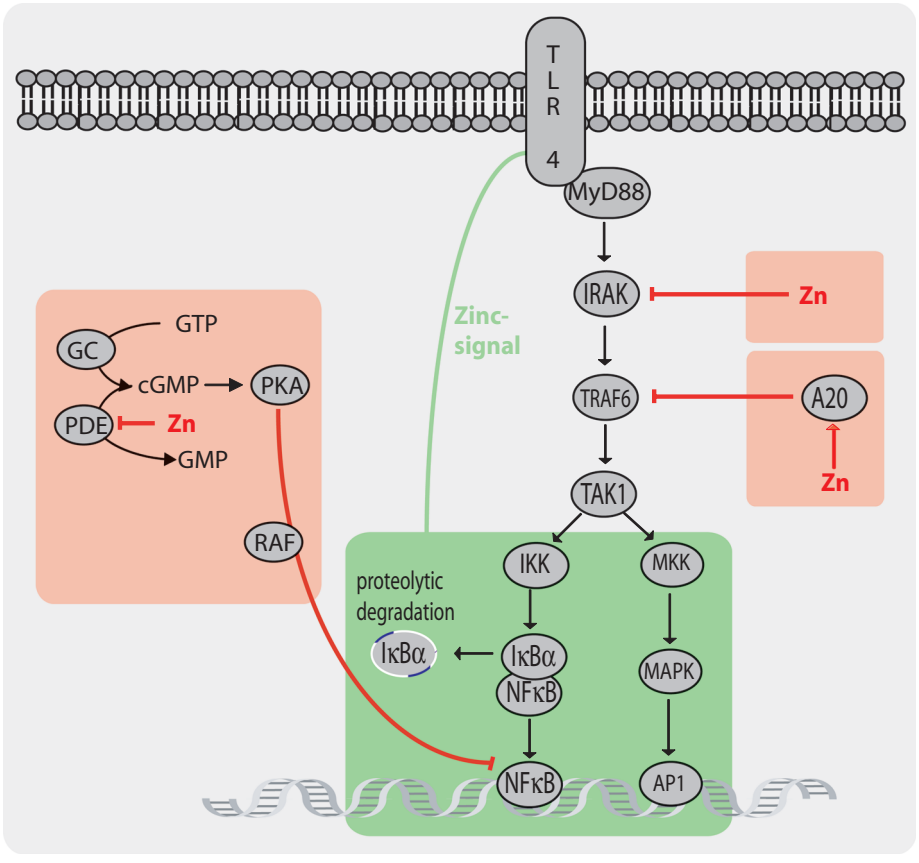

Figure 1

Zinc and Toll-like receptor 4 (TLR4) signaling. Zinc signals are required for activation of nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) signaling in response to TLR4(*green area*). Higher concentrations of zinc inhibit TLR4 signals (*red areas*). Suggested mechanisms are a direct inhibition of interleukin-1 receptor-associated kinase (IRAK), an upregulation of A20 that removes the activating ubiquitination of tumor necrosis factor receptor-associated factor 6 (TRAF6), or an inhibition of cyclic nucleotide phosphodiesterases (PDE). The latter leads to a rise in cGMP and cross-activation of protein kinase A (PKA), which then inhibits Raf and thereby TLR-mediated NFκB activation. GC, guanylate cyclase; IKK, I kappa B kinase; MKK, MAPK kinase; PDE, phosphodiesterase; PKA, protein kinase A; cGMP, cyclic guanosine monophosphate; GTP, guanosine trisphosphate.

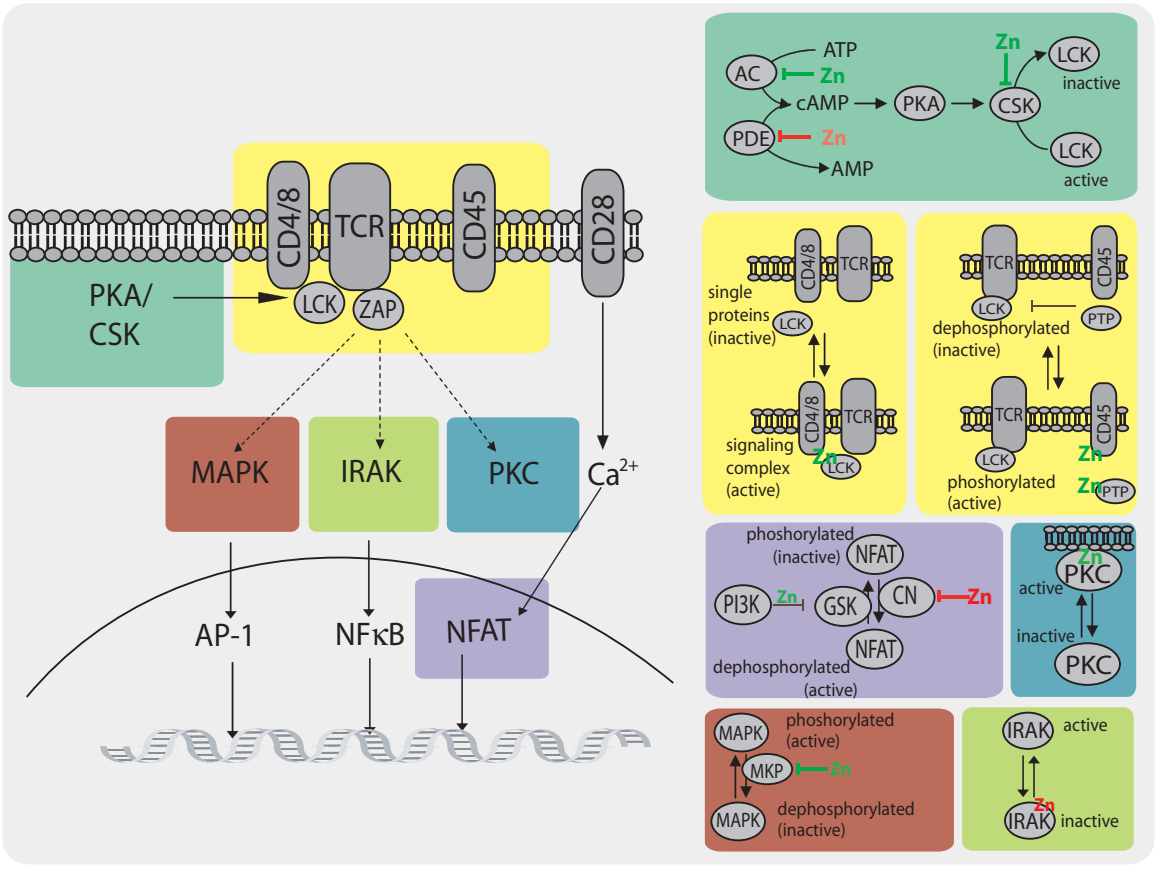

Figure 2

Zinc and TCR signaling. (*Left*) Overview of TCR signaling. Zinc-regulated components are highlighted by boxes in different colors. (*Right*) Detailed molecular interactions between zinc and components of TCR signaling. Green Zn indicates an overall activating influence of zinc on TCR signaling; red/orange indicates inhibition. AC, adenylate cyclase; AP-1, activator protein 1; ATP, adenosine trisphosphate; cAMP, cyclic adenosine monophosphate; CSK, c-src tyrosine kinase; IRAK, interleukin-1 receptor-associated kinase; LCK, lymphocyte-specific protein tyrosine kinase; MAPK, mitogen-activated protein kinase; MKP, MAP-kinase phosphatase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; PDE, cyclic nucleotide phosphodiesterase PI3K, phosphatidyl-inositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PTP, protein tyrosine phosphatase; TCR, T cell receptor; ZAP, zeta-chain (TCR)-associated protein kinase.

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Errata

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