Differential Gene Expression after Zinc Supplementation and Deprivation in Human Leukocyte Subsets

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An individual's zinc status has a significant impact on the immune system, and zinc deficiency, as well as supplementation, modulates immune function. To investigate the effects of zinc on different leukocyte subsets, we used microarray technology to analyze and compare the changes in mRNA expression in cell culture models of monocytes (THP-1), T cells (Jurkat), and B cells (Raji), in response to supplementation for 40 h with 50 μM zinc or 2.5 μM of the membrane-permeant zinc chelator TPEN (N,N,N′,N′- tetrakis-(2-pyridyl-methyl)ethylenediamine), respectively. In each cell type, several hundred genes were identified to be zinc sensitive, but only a total of seven genes were commonly regulated in all three cell lines. The majority of those genes were involved in zinc homeostasis, and none in immune function. Nevertheless, further analysis revealed that zinc affects entire functional networks of genes that are related to proinflammatory cytokines and cellular survival. Although the zinc-regulated activities are similar throughout the gene networks, the specific genes that are affected vary significantly between different cell types, a situation that helps to elucidate the disparity of the effects that zinc has on different leukocyte populations.

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INTRODUCTION

Zinc ions were found to affect multiple cellular signaling pathways on the molecular level (1), suggesting zinc may play a role in the regulation of a considerable number of genes. This zinc function has been confirmed by microarray experiments investigating the effects of zinc supplementation and/or deprivation. Such experiments have revealed that zinc influences the expression of hundreds of genes (2–4). Consequently, zinc affects virtually all aspects of the immune system. Zinc deficiency occurs as a result of malnutrition, aging, and inborn or acquired impairment of zinc uptake, and can lead to thymic atrophy, malfunction of immune cells, and subsequently, high incidence of infections (5). In addition, several diseases with immunological etiologies, including inflammation, rheumatoid arthritis, diabetes, and asthma, are accompanied by zinc deficiency (6–8). Many studies indicate that pharmacological zinc supplementation can be beneficial. In nonobese diabetic mice, an animal model for type I diabetes, the incidence of spontaneously occurring diabetes was reduced by zinc supplementation (9). Zinc supplementation has been shown to have beneficial effects in rheumatoid arthritis (10), and the negative correlation between serum zinc and pro-inflammatory cytokine levels in rheumatoid arthritis indicates a link between zinc and the severity of the disease (6). In elderly people, who have a particularly high risk of zinc deficiency, zinc supplementation improves responsiveness to immunization, serum thymulin activity, delayed type hypersensitivity reactions, and T-cell proliferation in response to mitogens (11).

These studies show the potential benefits of zinc supplementation as a means to modulate immune function. However, zinc supplementation also entails the possibility of unwanted side effects. The production of proinflammatory cytokines by monocytes is antagonized by an inhibition of phosphodiesterases by zinc (12). Accordingly, zinc can suppress proinflammatory cytokine release during sepsis in in vivo models, but on the other hand, it can also worsen the symptoms (13,14). While low doses of zinc reduce the risk for prostate cancer, higher dosage enhances the incidence (15). Zinc supplementation also suppresses certain aspects of immune function, such as the allogeneic reaction (16). Thus, despite the many beneficial effects that have been observed, lack of knowledge about the molecular effects of zinc on different cell types in the immune system makes the actions of zinc unpredictable, thus restricting the use of zinc as an immunomodulator.

The aim of this study was to analyze and compare the effects of zinc supplementation and deprivation in cell culture models for monocytes, T and B cells. We used microarray technology to identify genes that are affected by a change of the zinc status in each cell type or are commonly regulated in all of these cells, to contribute to a better understanding of the alterations that occur during zinc-mediated immunomodulation.

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MATERIALS AND METHODS

Cell Culture

Cells were plated at a density of 2 ×10^5 per mL in RPMI 1640 (Cambrex, Taufkirchen, Germany) supplemented with 10% FCS (PAA, Cölbe, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured for 40 h at 37°C, 100% humidity, and 5% CO_2 as untreated controls or in the presence of either 50 µM $ZnSO_4$ (zinc supplementation) or 2.5 µM of the membrane permeant zinc chelator TPEN [N,N,N′,N′-tetrakis-(2-pyridyl-methyl)ethylenediamine] (zinc deprivation). At the end of the treatment, fluorescent staining with acridin orange and ethidium bromide was performed to determine the viability of the cells. No effect of zinc supplementation or deprivation on viability was observed.

Measurement of Labile Zinc

Cells were incubated as specified above, and labile zinc was measured at the end of the incubations as reported previously (17). Briefly, FluoZin-3 AM ester (Invitrogen, Karlsruhe, Germany) was added directly to the culture medium (final concentration 1 µM), and cells were loaded at 37°C for 30 min, washed with phosphate buffered saline (PBS), and resuspended in PBS containing 10% FCS at a density of 2 ×10^6 cells per mL. The labile zinc concentration was estimated according to the formula of Grynkiewicz et al. (18), using 50 µM TPEN or 100 µM zinc in the presence of 50 µM pyrithione to determine minimal and maximal fluorescence, respectively.

Target RNA Preparation, Hybridization, and Microarray Analysis

Total RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany), and RNA from control cells was used as the reference channel against which RNA from treated cells was compared in the arrays. All samples were prepared in triplicate, and each individual sample was run on two arrays, the duplicate array being a dye swap. In this design, for each treatment condition, a total of six arrays, including three dye swaps, were analyzed.

The human oligonucleotide library was purchased from Sigma-Compugen (Oligo library, release 1.0, Jamesburg, NJ, USA) with a further additional set of 700 oligonucleotides custom synthesized according to the manufacturer's standard design guidelines. Oligonucleotides were resuspended in phosphate buffer and printed onto Codelink slides (GE Healthcare, Amersham, St.Giles, UK) using a Biorobotics TAS microarray system. Probe labeling was performed using the Genisphere 350HS labeling system (Hatfield, PA, USA) according to the manufacturer's standard protocol using 5.5 µg total RNA per sample. Reverse transcription was performed using Powerscript RT (BD Biosciences, Oxford, UK) with final dNTP concentrations of 0.5 mM each and using the 5 pmol/µL modified oligo dT primer provided (Genisphere). Hybridization was performed using a Lucidea SlidePro (GE Healthcare, Amersham) for 17 h, at 42°C. Dendrimer hybridization was performed essentially under the same conditions with the following changes: dextran concentration was reduced to 2.5% final (w/v), hybridization time was reduced to 4 h at 50°C, and wash conditions were as previously described with the exception that the initial wash was performed at 50°C. Scanning was performed using a Perkin Elmer Scanarray 4000XL scanner (Buckinghamshire, UK) for both Cy3 and Cy5. Image analysis and data extraction were performed using Bluefuse V3.2 (Bluegnome Ltd, Cambridge, UK). Outliers due to contamination were removed by manual exclusion.

Bioinformatics Analysis

Data sets were analyzed using GeneSpring v7.2 (Silicon Genetics, Redwood City, CA, USA). Loess and dye-swap normalizations were applied to all data sets. A subset of genes for data interrogation was generated that excluded controls, genes that did not dye swap, spots of poor quality, and gene probes that were present in less than 50% of samples. One-way, parametric ANOVA tests were performed followed by Benjamini and Hochberg multiple test correction with a false discovery rate of 0.10. From these selected genes, relative expression compared with untreated cells was determined (1.5-fold up or down), and gene changes common to two of three of the triplicates for each condition were considered significant. Microsoft Excel templates were prepared for each of the cell lines containing genes that were over- and underexpressed after zinc and TPEN treatment. Ingenuity pathway analysis 3.0 was used to assemble functional networks altered by zinc status (Ingenuity Systems, Redwood City, CA, USA) and to identify the biological functions and/or diseases that were most significant to the data set. Fischer's exact test was used to calculate a *P*-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

Real Time Quantitative Reverse-Transcription PCR

The genes and corresponding Taqman (Applied Biosystems, Foster City, CA, USA) probes and primer sequences used for RT-PCR are listed in Table 1. RNA was prepared from cells as described. hZIP-1 mRNA was transcribed with the Reverse Transcription System (Promega, Mannheim, Germany), and analyzed on an AbiPrism 7000 (Applied Biosystems) using porphobilinogen deaminase (PBGD) as housekeeping gene. The sequences of the PBDG primers and the probe have been published elsewhere (19). Cycler conditions were 95 °C for 15 min and 50 cycles of 95 °C for 15 s and 60 °C for 60 s. For all other genes, in vitro transcription was performed with the Superscript III First-Strand Synthesis System with random hexamer primers (Invitrogen, Paisley, Scotland, UK) and the Bio-Rad I-Cycler (Bio-Rad, Hercules, CA, USA) was used for real-time RT-PCR. Reactions were prepared using Platinum qPCR supermix with Taqman probes (FAM-490) or Platinum SYBR green

Table 1. Taqman probes and primer sequences used for real–time RT–PCR.

Gene	Probe/Primer
HAX1	Hs01573086_g1
KIAA1109	Hs01567746_m1
PTPRN	Hs01090888_g1
SLC30A1	Hs00253602_m1
MT1H	Hs00823168_g1
SLCO1C1	Hs00908742_m1
STK4	Hs01070055_m1
GAPDH	Hs99999905_m1
MT1L	5′-TCGCCTCTCCCGTCATTT-3′
	5′-AGCAGGGCTGTCCCCA-3′
β-globin	5′-GCTTCTGACACAACTGTGTTCACTAGC-3′
	5′-CACCAACTTCCACGTTCACC-3′
hZIP-1	5′-GCCTGACTACCTGGCTGCCATAGA-3′
	5′-CGGCCCTGACTGCTCGTAA-3′
	5′-CCTTGCACGTGACGCTCCAGTTCCCACT-3′ (FAM/TAMRA probe)

qPCR supermix (Invitrogen, UK). Taqman PCR thermocycler conditions were 50 °C for 2 min, 90 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. SYBR green PCR conditions were 40 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. All samples were run in triplicate with both test probes and the control gene human GAPDH or β-globin to control for differences in amount of starting material. A standard curve was created for each PCR reaction. Fold-changes were calculated by normalizing the test-crossing threshold (Ct) with the housekeeping control Ct.

RESULTS

Three human leukocyte cell lines, THP-1 (monocytes), Jurkat (T cells), and Raji (B cells), were cultured in the presence of 50 µM $ZnSO_4$ or 2.5 µM of the membrane permeant zinc chelator TPEN for 40 h to compare the effect of zinc supplementation or deprivation on different types of leukocytes. The zinc status at the end of the incubation was investigated by staining of labile zinc using FluoZin-3 (Figure 1), confirming that the levels of intracellular zinc were altered by this treatment.

The effect of these incubations was analyzed by mRNA array expression analysis. A list with the MIAME (Minimum Information About a Microarray Experiment) compliant data sets can be found in supplementary Table 1. As shown in Table 2, the incubations resulted in an altered expression of several hundred genes in each cell line. Although the effect of zinc treatment on labile zinc in THP-1 cells was much more pronounced than that of TPEN (Figure 1), 10-fold more genes in THP-1 cells were affected by TPEN than by zinc, and five-fold more genes in THP-1 cells were affected than in the other two cell lines, indicating that even moderate zinc deprivation can have a significant impact on gene expression in monocytes. In all cases treatment with zinc or TPEN resulted in a higher number of up- than downregulated genes, indicating increased transcriptional activity as a result of a disturbance of zinc homeostasis (Table 2).

Next, we performed a detailed analysis to determine if the expression of the regulated genes correlated with the cellular zinc status in general, or responded specifically to either zinc deprivation or supplementation (Table 3). Only a few genes were identified to be regulated both by zinc deprivation and supplementation, while the majority responded

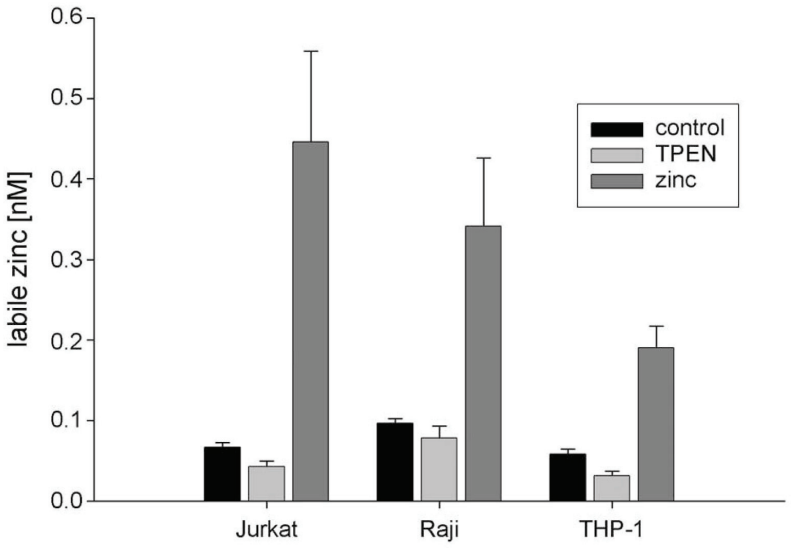

Figure 1. Intracellular labile zinc following incubation with zinc or TPEN. Cells were cultured in the presence of 50 µM $ZnSO_4$ or 2.5 µM TPEN for 40 h. For the last 30 min, cells were loaded with the zinc-specific fluorescent dye FluoZin-3, and the resulting zinc-specific fluorescence was measured by flow cytometry and transformed into labile zinc concentrations as described in the methods section. Data shown are means from n = 4 independent experiments ± SE.

Table 2. Total number of genes considered in the analysis and number of genes that were regulated by zinc or TPEN incubation.

	Total	Regulated by zinc		Regulated by TPEN	
Raji	11440	287	↑ 262	256	↑ 228
			↓ 25		↓ 28
Jurkat	11944	205	↑ 171	359	↑ 272
			↓ 34		↓ 87
THP-1	11913	122	↑ 110	1357	↑ 805
			↓ 12		↓ 552

↑, Upregulated; ↓, downregulated.

Table 3. Genes regulated by zinc deprivation and supplementation

	Zn↑ TPEN↑	Zn↑ TPEN↔	Zn↑ TPEN↓	Zn↓ TPEN↑	Zn↓ TPEN↔	Zn↓ TPEN↓	Zn↔ TPEN↑	Zn↔ TPEN↓
Raji	16	243	3	0	25	0	210	25
Jurkat	7	164	0	1	33	0	264	87
THP	8	93	9	5	6	1	792	542

↑, Upregulated; ↓, downregulated; ↔, not changed.

to only one treatment. This result shows that the expression of only a small percentage of genes is under direct control of zinc, and that zinc supplementation and deprivation affect different subsets of genes and thereby different physiological processes.

The array results from the three cell lines were compared to identify common genes that were affected in all cell types. Despite the high total numbers of genes that were influenced by the cellular zinc status, only a very small fraction was commonly regulated (Figure 2). Among the seven genes that were changed either by zinc or TPEN treatment in each of the cell lines (Table 4) were three proteins with direct involvement in zinc homeostasis: two isoforms of the zinc-storage protein metallothionein and SLC30A1, which is also known as ZnT-1, a zinc export protein on the plasma membrane.

It is likely that several other MT isoforms were also upregulated by zinc, but those were not investigated on the array. Additional zinc transporters, namely ZnT-3, -4, -5, and -7 and hZIP-3, -4, -8, -9, -10, -11, and -14, were investigated, but showed no significant regulation by any treatment in any cell type. Zinc deprivation can be expected to induce an altered expression of at least one zinc importer in an attempt to restore normal zinc levels. Therefore, the expression of the plasma membrane zinc import protein hZIP-1, which was not investigated on the array, was measured by quantitative PCR, and the results confirmed significant upregulation in all three cell lines after TPEN treatment (Figure 3).

To assess the reliability of the array experiments, the seven genes in Table 4 were also investigated by quantitative PCR (Table 5). Although the absolute fold expression values showed considerable differences between both methods, their regulation was confirmed for most cases, showing a high degree of comparability between the array experiments and quantitative PCR.

Although all three cell lines are derived from human leukocytes, the effects of zinc are specific for each cell line and thus cannot be generalized, because only a few genes are commonly regulated and most of these are involved in zinc homeostasis. To investigate whether zinc acts on similar networks of functionally related genes, we analyzed the data with the Ingenuity Pathway™ visualization tool. This tool systematically encodes findings from peer-reviewed scientific publications into ontologies, or groups of genes or proteins related by common function. Molecular networks of direct physical, transcriptional, and enzymatic interactions were computed from this knowledge base. Genes identified through microarray analysis (focus genes) and network score (probability of a network being assembled by chance alone) were determined. A score higher than two corresponds with a probability of 99.9% that the networks were not assembled by chance alone. The total numbers of genes regulated in more than one cell line after Zn treatment were too small to yield significant functional networks, but in the case of TPEN treatment, functional rela-

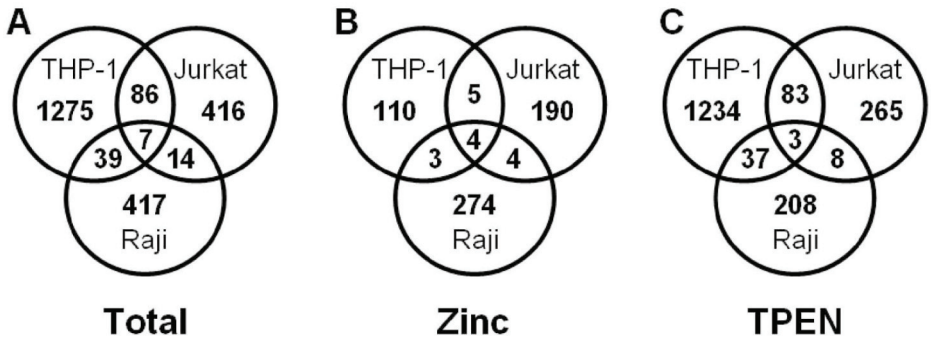

Figure 2. Commonly regulated genes. The datasets of genes whose expressions were changed > 1.5 fold in at least one cell line were compared between THP-1, Raji, and Jurkat. Displayed are the numbers of genes that are regulated in one cell line alone, or in two or all three cell lines by zinc and/or TPEN (A), or zinc alone (B), or TPEN alone (C).

Table 4. List of genes regulated in all three cell lines (array data)

		THP-1		Jurkat		Raji	
Genbank	Name	Zinc	TPEN	Zinc	TPEN	Zinc	TPEN
NM_006118	HAX1	1.2	1.7	-1.0	1.6	1.1	1.9
AL137384	KIAA1109	1.1	1.8	1.3	1.5	ND	2.1
NM_002846	PTPRN	1.6	2.3	1.5	2.6	1.3	1.8
U68494	SLC30A1	3.7	-1.3	2.2	-1.2	1.5	ND
NM_002450	MT 1L	5.0	-3.8	2.1	-1.3	2.1	-1.8
X64177	MT 1H	7.8	-7.6	2.8	-1.3	2.1	-1.4
NM_017435	SLCO1C1	8.1	-2.5	4.7	-1.1	3.6	-1.4

Two different sequences for another gene, STK4 (NM_006282), were used on the arrays and only one sequence indicated zinc-regulation in all three cell lines. Since quantitative RT PCR did not confirm a regulation in all cell types, this gene was not included in the table. ND, not detected.

tionships were found in genes commonly regulated in THP-1 and Jurkat as well as in THP-1 and Raji. The 86 genes regulated by TPEN in THP-1 and Jurkat cells gave three networks, two of which had high significance (scores of 29 and 20, respectively). These networks were merged and are displayed in Figure 4. Notably, zinc does not necessarily have the same effect on the expression of a particular gene in different cells; for example, the α subunit of the IL-10 receptor is three-fold upregulated by TPEN in THP-1, but downregulated in Jurkat.

When the expression of the genes in this network was shown for Raji (see Figure 4C), most genes were found not to be regulated. However, when another network was created out of the genes commonly regulated by TPEN in THP-1 and Raji (Figure 5), this network also involved TNF and a set of related genes as well as caspase 3. TNF was not significantly regulated in any cell line, and the dataset did not even contain caspase 3. Both genes were automatically identified because of their interaction with other genes in these networks. The independent inclusion of these genes in both networks suggests that in all three investigated cell types, the effects of zinc deprivation involve many genes related to proinflammatory cytokines and cellular survival. In THP-1 cells, TNF-related inflammatory genes were affected by zinc, and in addition, after TPEN treatment several genes related to the proinflammatory cytokine IL-1β were upregulated. These were IL-1β itself (3.6-fold upregulated), the IL-1β processing caspase 1 (2.4-fold upregulated), and Myeloid differentiation primary response gene 88 (2.6-fold upregulated), a protein that is involved in IL-1β–induced signaling.

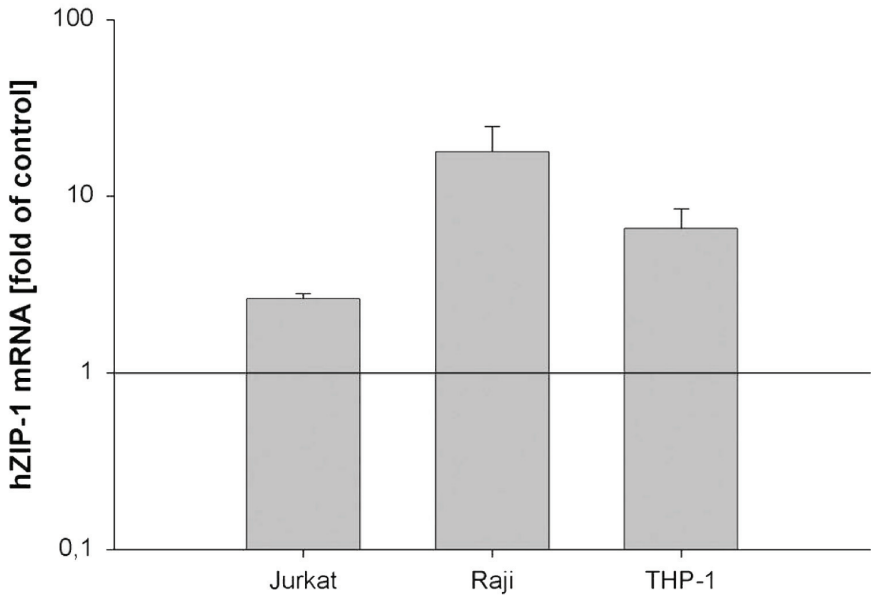

Figure 3. Quantitative hZIP-1 PCR. The expression of the zinc import protein hZIP-1 mRNA after 40 h incubation with 2.5 μM TPEN was measured by quantitative PCR. Data shown are expression values relative to untreated control cells presented as means from n = 3 (Jurkat), n = 6 (Raji), or n = 7 (THP-1) independent experiments ± SE.

DISCUSSION

Previous microarray experiments have shown that zinc can affect hundreds of different target genes (3), and zinc also affected the three leukocyte models investigated in this study. In array experiments focusing on liver tissue, zinc was found to mainly affect energy and lipid metabolism (20,21). In the leukocyte models investigated here, the functional networks contained genes that were mainly involved in inflammation and cellular survival. These findings show that the effects of zinc are organ specific. Although the three cell lines investigated in this study were immune cells derived from human leukocytes, even among these functionally related cells only a small number of genes were commonly affected. Some recent studies have looked at the effects of zinc on gene expression of leukocytes (22) and lymphocytes (23), but our results suggest that it may be necessary to investigate the effect of zinc for each cell type, because of significant differences.

Three of the common genes were not unexpected, because when zinc homeostasis is disturbed, the cell acts to restore optimal zinc levels. This process explains the upregulation of metallothioneins 1H and 1L and the plasma membrane zinc exporter ZnT-1 as a result of incubation with zinc. These three genes are under

Table 5. List of genes regulated in all three cell lines (PCR data)

		THP-1		Jurkat		Raji	
Genbank	Name	Zinc	TPEN	Zinc	TPEN	Zinc	TPEN
NM_006118	HAX1	-1.1	1.4	-1.4	1.8	1.1	1.2
AL137384	KIAA1109	1.1	2.3	1.5	2.8	1	1.8
NM_002846	PTPRN	1.8	3.4	1.2	2.9	1.1	2.2
U68494	SLC30A1	30.5	ND	1.8	-1.7	1.1	1.2
NM_002450	MT 1L	5	-3.3	2.1	-1.4	2.1	-2.0
X64177	MT 1H	17.9	1.7	ND	ND	3.5	ND
NM_017435	SLCO1C1	3.2	-1.7	4.2	-1.3	1.8	1.1

ND, not detected.

the control of MTF-1 (metal responsive element binding transcription factor-1), which directly senses intracellular zinc levels and regulates the transcription of genes by binding to a metal response element in their promoter region (24).

The relationship between the remaining 4 genes and zinc is not so clear. SLCO1C1, a member of the solute carrier for organic anions family, is generally considered to be a transporter for thyroid hormone (25). However, other reports indicate a role in glutathione (GSH) transport (26). Because SLCO1C1 is strongly upregulated by zinc, GSH could serve to complex zinc and be involved in zinc buffering. On the other hand, there is an interaction between zinc and the cellular redox system (27), of which GSH is an important part. Hence, the change could also be a response to a disturbance of the cellular redox balance by zinc. The receptor type protein tyrosine phosphatase (PTP) RN, also known as IA-2, is an autoantigen in diabetes that is found in pancreatic islets and the brain (28). For KIAA1109 and HAX1, no functions have thus far been published. Also, there are no reports indicating a particular role of any of these 4 genes in the immune response. Therefore, a central role of the corresponding proteins in zinc-mediated immunomodulation seems unlikely.

The array analysis showed that zinc has an extensive impact on leukocyte gene expression, but, despite some homeostatic proteins, there is no common protein, or group of proteins, for example a transcription factor or signaling protein, that mediates the immunological effects of zinc. In particular, no remarkable effects on gene expression were found for several different kinase or phosphatase families, or transcription factors. It is likely that zinc interacts with the gene expression by regulating signaling pathways through posttranslational events. Recent characterizations of such interactions show that many proteins that are involved in signal transduction are regulated by zinc (1). Specific effects that were found in the different cell lines may occur because the active signaling pathways are dependent on the cell type.

Also, the expression of most genes identified by our experiments did not seem to depend directly on zinc, because the genes are regulated by either zinc or TPEN treatment rather than by both conditions. This finding is in contrast to another study that investigated the short-term effects of zinc supplementation and deprivation in THP-1 cells and identified 283 genes that were responsive to zinc depletion as well as excess (2). These different findings for the same cell line are probably attributable to the different experimental approaches employed. We used only 2.5 μM TPEN instead of 10 μM, and an incubation time of 40 h instead of four hours. The conditions from (2) cause a short term, massive depletion of labile zinc, and are suitable to identify genes whose expression is most likely under the direct control of zinc. We were focusing on the effect of moderate long-term zinc deficiency, as occurs in most zinc-deficient individuals. The different results in both studies show that the validity of the data is strongly limited to the conditions under which they were obtained, and that the acute and long-term effects of zinc have to be distinguished.

In our experiments the decrease in labile zinc after TPEN treatment seems to be only minor, given that micromolar amounts of the chelator were added. In this respect it has to be noted that a significant part of the TPEN will interact with extracellular zinc and not even enter the cells. Also, the zinc status was measured at the end of the incubation period, after the cells had been adjusting to the altered availability of zinc by increased expression of hZIP-1, and probably also by mobilization of protein bound cellular zinc. It is important that labile zinc, measured at a subnanomolar level (see Figure 1), is in equilibrium with the other cellular zinc pools, and represents only a minor fraction of total cellular zinc, which is in the order of several hundred micromolar (29). The high number of genes affected by TPEN treatment clearly shows a profound impact on gene expression, even in response to a seemingly minor reduction of intracellular labile zinc.

When functional networks were used to analyze common genes in two of three cell types, it was found that while zinc may not act on the same proteins, it does affect comparable functional networks. First, in both networks (see Figures 4 and 5) the proinflammatory cytokine TNF holds a central position. An effect of zinc on TNF and other cytokines, especially in combination with an additional stimulation, is well known (5). What is notable is the number of other genes with a functional relationship to TNF that are also affected by the altered zinc status, showing that the effect of zinc status on proinflammatory signaling is not an isolated event in a few cytokines, but involves many more genes than previously expected.

Another remarkable protein is caspase 3. This enzyme is not only directly inhibited by low nanomolar zinc concentrations (30); it is also present in both networks, although the gene was not included in the array data. Recent research shows a relationship between zinc and apoptosis (31) and between zinc deprivation and cell death (32). Our data

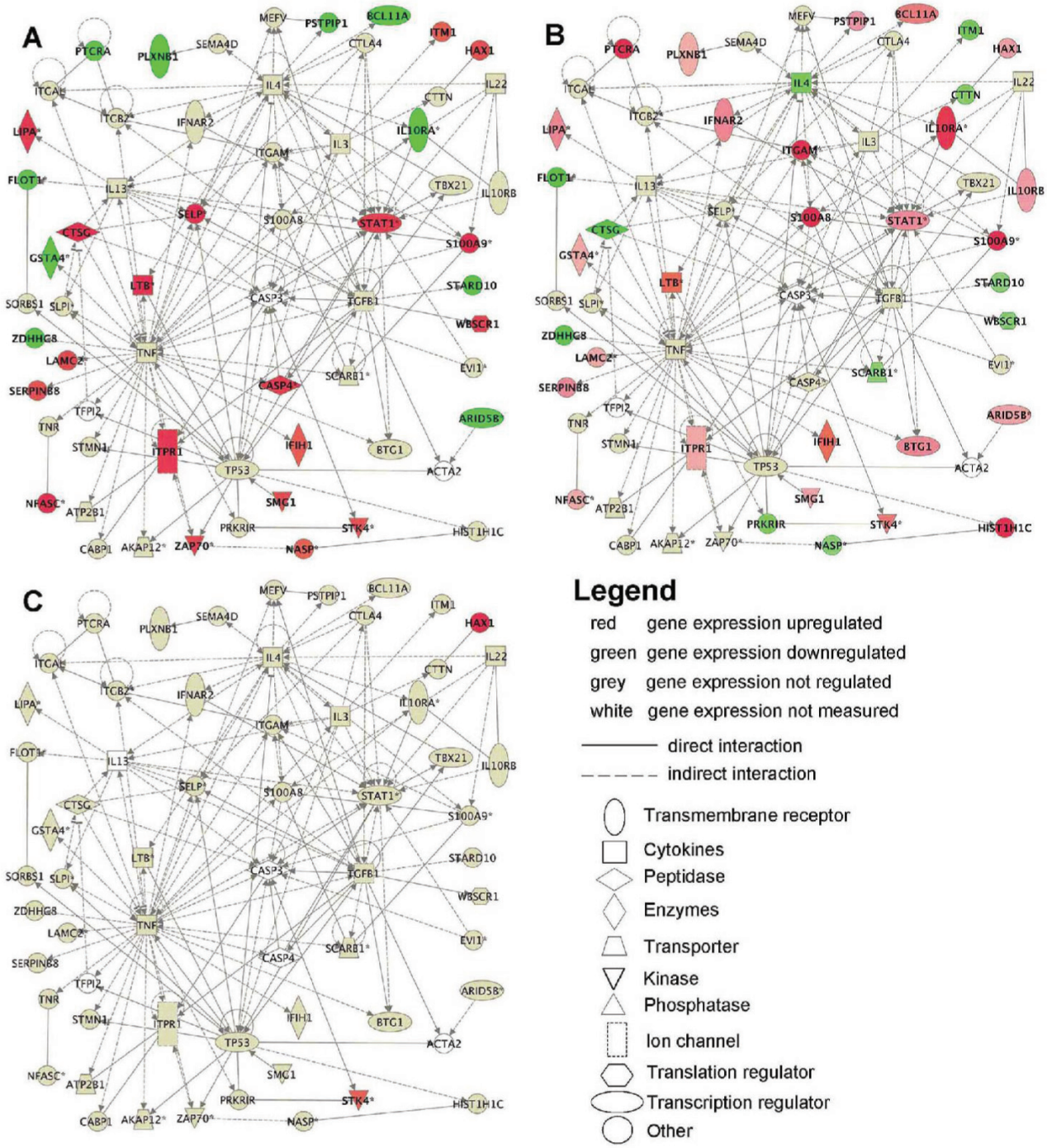

Figure 4. Functional networks of genes regulated by TPEN in THP-1 and Jurkat cells. The 86 genes whose expression was significantly altered by TPEN treatment in both THP-1 and Jurkat were analyzed with Ingenuity pathway analysis. Two networks with high significance were identified and merged. The resulting network is displayed, and the regulation of the genes is shown for Jurkat (A), THP-1 (B), and Raji (C). A network is a graphical representation of the molecular relationships between genes and gene products. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product as indicated in the figure legend.

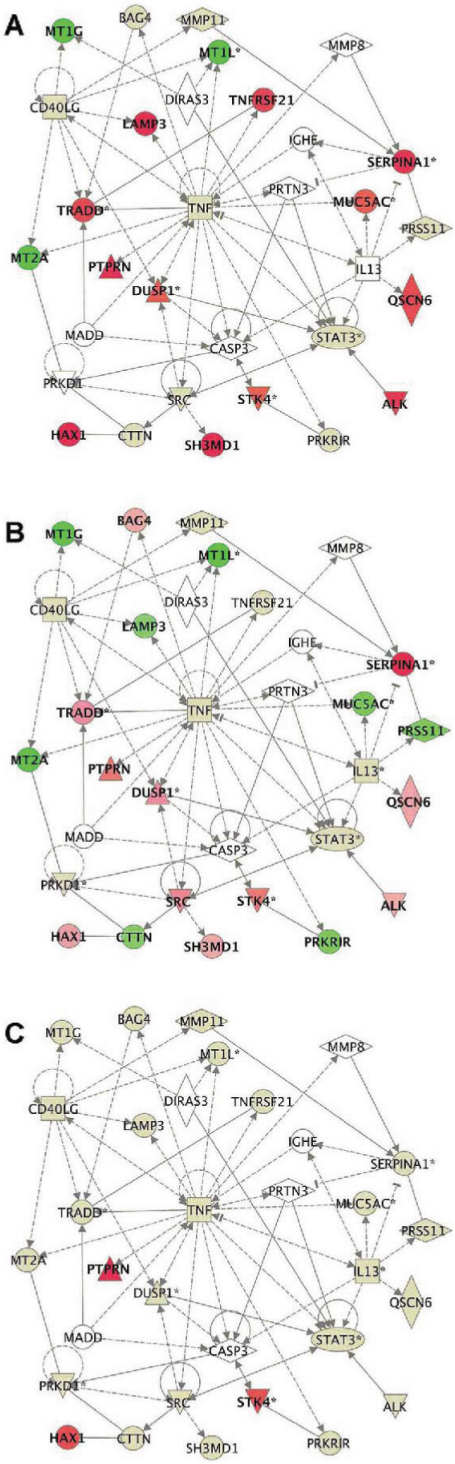

Figure 5. Functional networks of genes regulated by TPEN in THP-1 and Raji cells. The 40 genes whose expression was significantly altered by TPEN treatment in both THP-1 and Raji were analyzed with Ingenuity pathway analysis. The resulting network is displayed, and the regulation of the genes is shown for Raji (A), THP-1 (B), and Jurkat (C). For an explanation of the networks see Figure 4.

suggest that these relationships are not isolated events limited to the regulation of caspases by zinc, but involve an entire group of interacting genes. Future work should focus on a detailed analysis of the contribution of the genes in those networks to the proinflammatory and apoptotic effects of zinc on immune cells.

The significance of the functional networks is two-fold; on the one hand they demonstrate that, despite the lack of commonly regulated genes, certain cellular processes are a target for zinc regulation in more than one leukocytes subset, even though the effects on single genes may be opposite in different cells, as shown for the IL-10 receptor α subunit. On the other hand, the functional networks aid in the identification of additional targets related to the known cellular functions of zinc.

Zinc supplementation is a way to modulate the immune system that can profoundly affect human health, and is a treatment with low acute toxicity and generally only mild side effects. One major group of genes affected by zinc status is related to inflammation, and zinc can directly affect several kinds of inflammatory diseases, including rheumatoid arthritis, diabetes, asthma, and sepsis (6-10,12-14). Another interesting aspect of zinc status is found in elderly individuals, who in general show a significant reduction of their plasma or serum zinc levels. Immunosenescence, the age-related decline of immune function and subsequent higher susceptibility to infections, seems to correlate with this marginal zinc deficiency, and zinc supplementation has been shown to counteract many symptoms of immunosenescence (11). The data obtained here may contribute to understanding of these events on the molecular level. These data also show, however, that the serum zinc level acts on leukocytes, a group of functionally diverse cells, which may lead to an unexpected and sometimes unfavorable outcomes, especially in the context of an immune response. This finding indicates that a more detailed understanding of the impact of zinc on immune function is needed to optimize the use of its im-

munomodulatory potential. The data presented here are a starting point for this analysis. Of course, mRNA microarray experiments have several limitations. First, they give information only about transcriptional effects and not about translational and posttranslational events. Results will have to be verified on the protein level. It is promising, however, that the published observations about the effects of zinc on proinflammatory cytokines correspond well to the microarray data. A limitation of microarrays is the number of samples that can be analyzed with this method, allowing the study of only a restricted number of treatment conditions. The genes identified by this approach will have to be investigated at different time points and zinc concentrations to get a more complete picture of the influence of zinc on immune cells.

This study identified zinc-dependent genes and functional networks, which are shared by different types of leukocytes. Separate further analyses of the data for each cell line are needed to identify the specific effects of zinc on the different cell types. Respective work is under way in our laboratory and has already identified several groups of genes that are affected by zinc status in particular leukocytes. Although cell lines were used as models in the present study, once the most promising genes for each cell type are identified, further investigations will seek to confirm the effect of zinc on those genes in vivo and to elucidate the precise role of the products of these genes within the immunomodulatory action of zinc.

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