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Differential impact of zinc deficiency on phagocytosis, oxidative burst, and production of pro-inflammatory cytokines by human monocytes

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Zinc deficiency has a fundamental influence on the immune defense, with multiple effects on different immune cells, resulting in a major impairment of human health. Monocytes and macrophages are among the immune cells that are most fundamentally affected by zinc, but the impact of zinc on these cells is still far from being completely understood. Therefore, this study investigates the influence of zinc deficiency on monocytes of healthy human donors. Peripheral blood mononuclear cells, which include monocytes, were cultured under zinc deficient conditions for 3 days. This was achieved by two different methods: by application of the membrane permeable chelator *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) or by removal of zinc from the culture medium using a CHELEX 100 resin. Subsequently, monocyte functions were analyzed in response to *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Zinc depletion had differential effects. On the one hand, elimination of bacterial pathogens by phagocytosis and oxidative burst was elevated. On the other hand, the production of the inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 was reduced. This suggests that monocytes shift from intercellular communication to basic innate defensive functions in response to zinc deficiency. These results were obtained regardless of the method by which zinc deficiency was achieved. However, CHELEX-treated medium strongly augmented cytokine production, independently from its capability for zinc removal. This side-effect severely limits the use of CHELEX for investigating the effects of zinc deficiency on innate immunity.

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Introduction

Since the discovery of zinc being an essential micronutrient in 1963,[1,2] its impact on human health has been the subject of numerous investigations. Zinc influences all cellular subsets of the immune system, affecting both proliferation and function. Not only severe, but also marginal zinc deficiency caused by malnutrition, malabsorption, disease or aging increases the risk for infections.[3] The innate immune system, which acts as a first line of the immune defense, has been shown to be weakened by zinc deficiency. The effects include decreased natural killer cell activity and impaired chemotaxis and oxidative burst of neutrophil granulocytes.[4] For monocytes, which are also part of the first line immune defense, zinc can have both an activating and an deactivating influence. On the one hand, severe zinc depletion negatively affects monocyte functions and survival,[4] but on the other hand an environment of high zinc concentration can also inhibit their activation.[5]

One major monocyte function is cytokine production, and zinc influences the release of inflammatory cytokines, even though the results so far have been highly contradictory.[6] Lipopolysaccharide (LPS), part of the cell wall of gram-negative bacteria, is one of a myriad of agents that leads to secretion of monokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. Their release in response to stimulation using LPS can be enhanced in peripheral mononuclear blood cells (PBMC) by zinc supplementation,[4,7] and intracellular zinc signals are required for the production of pro-inflammatory cytokines in response to LPS.[8] Moreover, secretion of pro-inflammatory cytokines can also be directly induced by incubation with high extracellular Zn^{2+} concentrations.[4] In contrast, high extracellular zinc concentrations have also been shown to inhibit LPS-induced monokine production.[8]

During zinc deficiency the immune system undergoes a reprogramming, leading to a shift from adaptive to innate immune defense.[9] Lymphocytes, and in particular T cells, show

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increased occurrence of apoptosis. In contrast myeloid cells, such as neutrophils and monocytes, seem to be protected. Their number increases in the bone marrow as well as in the periphery. Because the production of immune cells is costly in times of limited availability of essential nutrients, the body seems to set priorities and to channel its resources toward the first line of immune defense.[9]

The present study examines the influence of relatively mild zinc deficiency on monocyte functions such as phagocytosis, oxidative burst, and secretion of the pro-inflammatory cytokines TNF-α and IL-6 in response to gram-negative *E. coli* and gram-positive *S. aureus* or *S. pneumoniae*. Two distinct approaches for zinc depletion were compared: culturing cells in the presence of the chelator *N*,*N*,*N*′,*N*′-tetrakis-2(pyridyl-methyl)ethylenediamine (TPEN) or in medium depleted of zinc by treatment with CHELEX resin.

Materials and methods

Materials

RPMI 1640, L-glutamine, penicillin, streptomycin and PBS were from Lonza (Verviers, Belgium) and FCS was from PAA (Coelbe, Germany). Biocoll separating solution was purchased from Biochrom AG (Berlin, Germany). TPEN, CHELEX 100 (sodium form), propidium iodide, LPS (*E. coli* serotype 0111:B4), sodium pyrithione and dihydroethidium (DHE) were obtained from Sigma Aldrich (Taufkirchen, Germany). FluoZin-3 AM and dihydrorhodamine123 (DHR123) were from Invitrogen (Karlsruhe, Germany). $ZnSO_4 \times 7\ H_2O$ was purchased from Merck (Darmstadt, Germany). OptEIA ELISA kits for human TNF-α and IL-6 were from BD (Heidelberg, Germany). All other chemicals were from standard sources and of analytical quality.

Isolation and cultivation of human PBMC

Heparinized peripheral venous blood from 24 healthy consenting donors between the ages of 21 and 29 was used to isolate PBMC by density gradient centrifugation using Ficoll as described.[10] Plasma samples of each donor were produced by centrifugation (3000 × *g*, 10 minutes) of heparinized blood and stored at −20 °C. Depending on the experiment, the number *n* varies between 20 and 24, either because of an insufficient number of cells isolated, or because of unsuccessful analysis. The cells (2×10^6 ml^{-1}) were cultured in RPMI 1640 containing 10% heat inactivated, low-endotoxin FCS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. To modulate the zinc status, PBMC were incubated for 3 days at 37 °C in a humidified 5% CO_2 atmosphere under four different conditions: (1) culture medium without additional treatment ($RPMI^{Control}$), (2) culture medium supplemented with 1.5 μM TPEN ($RPMI^{TPEN}$), (3) culture medium in which Zn^{2+} was reduced to 170 nM (10.9 μg L^{-1}, Table 2) by treatment with CHELEX as previously described[11] ($RPMI^{CHE}$), and (4) $RPMI^{CHE}$ supplemented with Zn^{2+} to yield a final concentration of 6.77 μM ($RPMI^{CHE+Zn}$). Samples for flow cytometric measurements were kept in sterile 5 ml PPN-tubes (Greiner, Frickenhausen, Germany), whereas the cells for ELISAs were cultivated in 48-well-plates (BD, Heidelberg, Germany).

Inductively coupled plasma mass spectrometry (ICP-MS)

The concentrations of Mg, Ca, Mn, Fe, Ni, Co, Cu, Zn, As, Se and Mo were quantified by ICP-MS (Agilent 8800 ICP-QQQ, Agilent Technologies Deutschland GmbH, Böblingen, Germany) simultaneously in the single quad-mode using helium as a collision gas. Further ICP-MS conditions are listed in Table 1. The nebulizer gas flow and parameters of lenses, Q1, collision cell and Q2 were tuned daily for maximum sensitivity (oxide ratio <1.0% ($^{140}Ce^{16}O^+/^{140}Ce^+$), double charged ratio <1.5% ($^{140}Ce^{++}/^{140}Ce^+$), background counts <0.1 cps). For quantification an element mix (100 mg L^{-1}, SPETEC (Erding, Germany)) was diluted in 2% HNO_3. Medium samples were diluted (1 : 2–1 : 100) with 2% HNO_3 for determination.

Flow cytometric measurement of cell viability

After 3 days of incubation in different media, cells (2×10^6 ml^{-1}) were transferred into PBS and incubated in the dark with 10 μg ml^{-1} propidium iodide for 10 minutes. Subsequently, the cells were washed using PBS and dye uptake was measured by flow cytometry on a FACScan (BD Biosciences).

Flow cytometric measurement of intracellular free zinc ions

The cells were loaded with FluoZin-3 AM (1 μM, 30 min, 37 °C) and free Zn^{2+} was measured by flow cytometry as described,[12] using a dissociation constant for the Zn^{2+}/FluoZin-3 complex of 8.9 nM.[13] The supernatants of the samples for the phagocytosis/burst assays were retained and used for washing- and incubation steps in the FluoZin-3 assay, to avoid contact with media or buffer with normal Zn^{2+} content.

Flow cytometric measurement of phagocytosis and oxidative burst

The cells were transferred into 100 μl of the donors' own plasma and incubated for 30 minutes at 37 °C (positive controls) or 4 °C (negative controls), respectively, in polypropylene tubes. This was followed by addition of 20 μl of PBS containing 2×10^7 bacteria transformed to express fluorescent proteins; either DSred-*E. coli* (strain BL21),[11] GFP-*S. aureus* (strain ATCC29213)[14] or GFP-*S. pneumoniae* (strain R6).[15] Additionally, redox sensitive pro-fluorophores were added, either DHR123 (1 μM) for DsRed-*E. coli* or DHE (2.5 μM) for bacteria expressing GFP, respectively. Subsequently, samples were

Table 1 Experimental conditions for ICP-MS

Forward power	1550 W
Cool gas flow	15 L min^{-1}
Auxiliary gas flow	0.9 L min^{-1}
Nebulizer gas flow	1 L min^{-1}
Nebulizer type	MicroMist
Mode	Single quad
Collision gas flow	He: 5 ml min^{-1} (purity 99.9999%)
Quadrupole	*m*/*z* 24 (Mg), 43, 44 (Ca), 55 (Mn), 56 (Fe), 58, 60 (Ni), 59 (Co), 63 (Cu), 64, 66 (Zn), 75 (As), 76, 77, 78 (Se), 95, 98 (Mo)
Integration time	0.3 s
Replicates	3
Limit of quantitation (μg L^{-1})	Mg: 0.73, Ca: 13.58, Mn: 0.037, Fe: 0.88, Ni: 0.20, Co: 0.003, Cu: 0.40, Zn: 0.43, As: 0.02, Se: 1.22, Mo: 0.007

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incubated at the same temperatures as before for further 30 minutes, washed using ice-cold PBS, followed by measurement of phagocytosis and oxidative burst by flow cytometry (FACScan, BD Biosciences). Data are expressed as the difference between median fluorescence intensity of the samples incubated at 37 °C and the respective 4 °C negative controls.

Cytokine quantification by enzyme-linked immunosorbent assay (ELISA)

Cells were stimulated using DSred-*E. coli*, GFP-*S. aureus*, and GFP-*S. pneumoniae* (in each case 20 μl of PBS containing 2×10^7 bacteria per ml) or using LPS (250 ng ml^{-1}) for 4 hours and supernatants were stored at −80 °C until further use. TNF-α- and IL-6 concentrations were measured by OptEIA ELISAs according to the manufacturer's instructions.

Statistical analysis

Statistical significance of the results was analyzed by a paired *t*-test using GraphPad Prism software version 5.01 (Graph pad software, La Jolla, CA, USA).

Results

Free intracellular zinc levels and viability

To measure the effects of culture in the presence of the chelator TPEN or after removal of zinc from the medium using CHELEX on intracellular free zinc levels, these were measured by flow cytometry using the zinc-selective fluorescent probe FluoZin-3 (Fig. 1A). In comparison to monocytes grown in normal culture medium, intracellular free zinc levels were significantly lowered using TPEN. A similar effect was also observed when free zinc was measured in lymphocytes, the other population of cells present in PBMC. In contrast, culture in $RPMI^{CHE}$ showed no difference to $RPMI^{CHE+Zn}$ in either cell type. Remarkably, culture in CHELEX-treated media elevated the level of free zinc in monocytes, but reduced it in lymphocytes, independently from the presence of added zinc.

To exclude that cell survival had been affected by zinc deprivation, the membrane integrity was measured using propidium iodide (Fig. 1B). There was no impact on the survival rate of monocytes cultured using TPEN or in CHELEX-treated medium. Lymphocytes were also unaffected by TPEN, but there was a negligible (although statistically significant) elevation of viability from 99.6% to 99.7% in $RPMI^{CHE}$.

Phagocytosis and oxidative burst

Next, the impact of zinc deficiency on the phagocytosis of DSred-*E. coli*, GFP-*S. aureus*, and GFP-*S. pneumoniae* was analyzed (Fig. 2). Three day culture in the presence of TPEN augmented phagocytosis of all three bacterial species, although this was only statistically significant for DSred-*E. coli* and GFP-*S. aureus*. In contrast, zinc removal by CHELEX had no statistically significant impact on phagocytosis of either bacterium, although there were trends toward increased phagocytosis of GFP-*S. aureus*, and GFP-*S. pneumoniae*.

Simultaneously to phagocytosis, the oxidative burst was measured in the same samples (Fig. 3). There were no differences between zinc adequate and zinc deficient controls for DSred-*E. coli* and GFP-*S. pneumoniae*. In contrast, GFP-*S. aureus*-induced oxidative burst was significantly elevated by zinc-deficient culture in

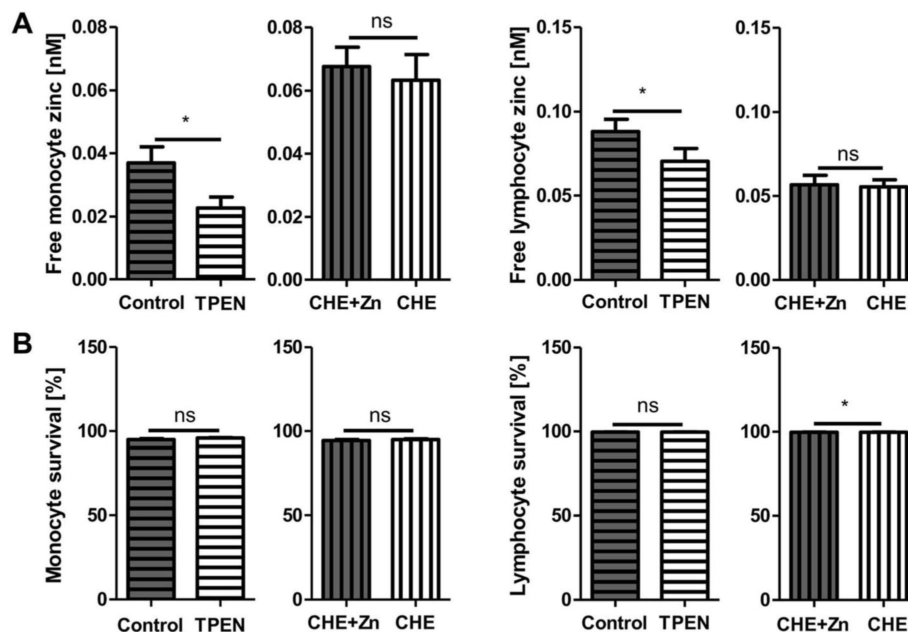

Fig. 1 Impact of zinc deficiency on intracellular free zinc and viability of PBMC. PBMC were cultured in two experimental models for zinc-deficiency, comparing $RPMI^{Control}$ with $RPMI^{TPEN}$ and $RPMI^{CHE+Zn}$ with $RPMI^{CHE}$, respectively. After three days, flow cytometry was used for measuring intracellular free zinc levels using FluoZin-3 (A) and membrane integrity using propidium iodide (B) in monocytes and lymphocytes. Data are shown as means + SEM from at least $n = 22$ donors. Statistical significance was analyzed by Student's *t*-test (ns, not significant; *, $p < 0.05$).

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Fig. 2 Influence of zinc deficiency on monocyte phagocytosis. The effects of three days of zinc deficiency induced by either TPEN (RPMI^{Control} *vs.* RPMI^{TPEN}) or CHELEX (RPMI^{CHE+Zn} *vs.* RPMI^{CHE}) on phagocytosis of three different species of fluorescently labeled bacteria were measured: DSred-*E. coli*, GFP-*S. aureus* and GFP-*S. pneumoniae*. Data are shown as means + SEM from at least n = 20 donors. Statistical significance was analyzed by Student's t-test (ns, not significant; **, $p < 0.001$).

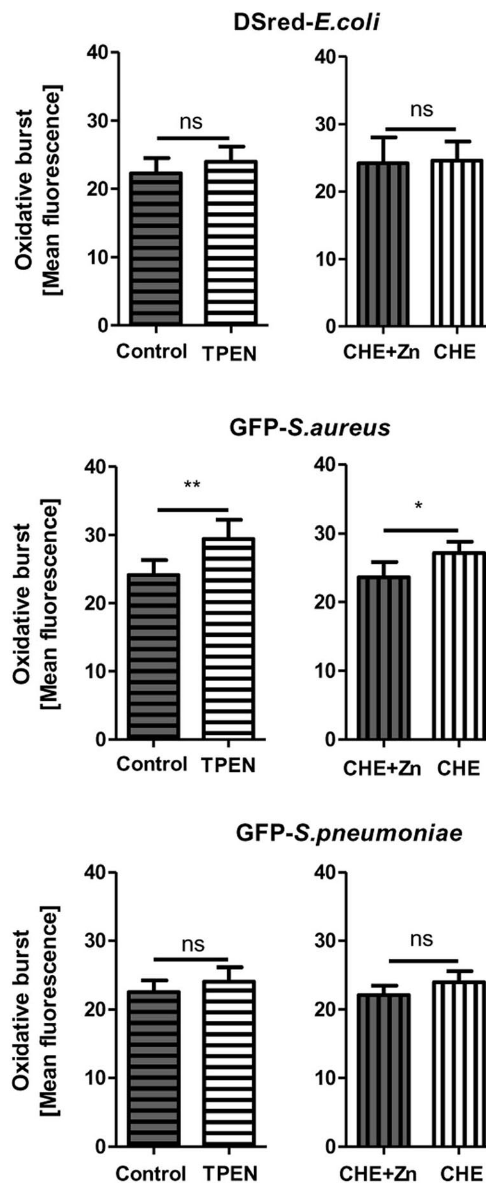

Fig. 3 Influence of zinc deficiency on monocyte oxidative burst. In parallel with the measurement of phagocytosis shown in Fig. 2, the oxidative burst in monocytes was quantified using the redox-sensitive pro-fluorophores dihydrorhodamine 123 (for DSred-*E. coli*) or dihydroethidium (for GFP-*S. aureus* and GFP-*S. pneumoniae*). Data are shown as means + SEM from at least n = 20 donors. Statistical significance was analyzed by Student's t-test (ns, not significant; *, $p < 0.05$; **, $p < 0.001$).

RPMI^{TPEN} and RPMI^{CHE}. These data indicate a general trend toward increased phagocytosis and oxidative burst in response to zinc deficiency.

Production of pro-inflammatory cytokines

For examination of the effect of zinc deficiency on cytokine production, TNF-α (Fig. 4) and IL-6 (Fig. 5) were measured by ELISA. PBMC were stimulated using the three bacterial species used for analysis of phagocytosis and oxidative burst, as well as LPS. Zinc depletion by TPEN reduced the production of TNF-α in all experiments, and the effect was statistically significant in response to DSred-*E. coli*, GFP-*S. aureus* and LPS. For IL-6, trends similar to TNF-α were observed. These were only statistically significant for LPS, due to the high inter-individual variation that is typical for experiments with human subjects.

Corresponding to the data obtained using TPEN, RPMI^{CHE} compared to RPMI^{CHE+Zn} showed lower TNF-α secretion in response to GFP-*S. aureus* and LPS. Notably, cytokine values were elevated by roughly one order of magnitude in all media that had been in contact with CHELEX, regardless of if they had been reconstituted with zinc or not. CHELEX-treated medium

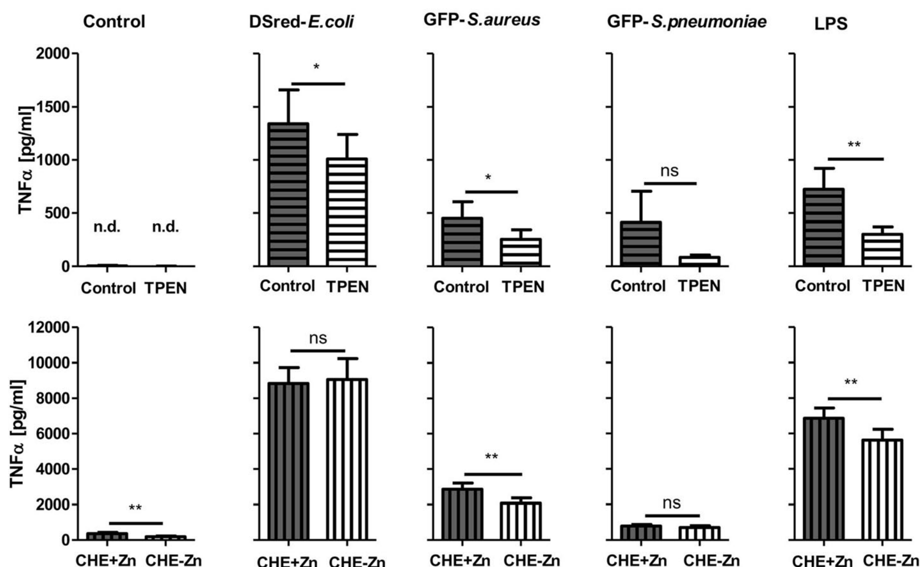

Fig. 4 Influence of zinc deficiency on the production of TNF-α. After 3 days of incubation in $RPMI^{Control}$ or $RPMI^{TPEN}$ and $RPMI^{CHE+Zn}$ or $RPMI^{CHE}$, PBMC were either left untreated or stimulated using DSred-*E. coli*, GFP-*S. aureus*, GFP-*S. pneumoniae* or LPS (250 ng ml^{-1}) for further 4 hours. Subsequently, TNF-α-production was measured by ELISA, investigating the effects of zinc deficiency induced by TPEN ($RPMI^{Control}$ *vs.* $RPMI^{TPEN}$) or CHELEX ($RPMI^{CHE+Zn}$ *vs.* $RPMI^{CHE}$). Data are shown as means + SEM from n = 24 donors. Statistical significance was analyzed by Student's t-test (ns, not significant; *, $p < 0.05$; **, $p < 0.001$). N.d. = not detected, values were below the detection limit of 7.8 pg ml^{-1}.

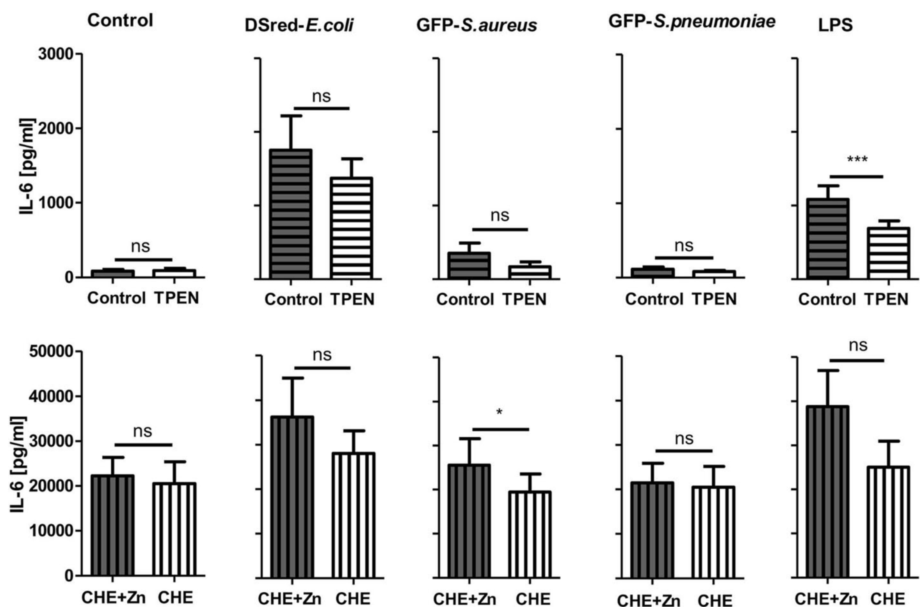

Fig. 5 Influence of zinc deficiency on the production of IL-6. Analogous to the measurement of TNF-α in Fig. 4, IL-6 production was measured by ELISA, comparing the effects of zinc deficiency induced by TPEN ($RPMI^{Control}$ *vs.* $RPMI^{TPEN}$) or CHELEX ($RPMI^{CHE+Zn}$ *vs.* $RPMI^{CHE}$). Data are shown as means + SEM from n = 24 donors. Statistical significance was analyzed by Student's t-test (ns, not significant; *, $p < 0.05$; ***, $p < 0.001$).

induced a basal production of IL-6 that even superseded the levels found after stimulation using LPS or bacteria in normal medium. Most likely, the influence of CHELEX contributes the major part of IL-6 production in the corresponding samples stimulated using bacteria or LPS. Notably, $RPMI^{CHE}$ alone did not cause any signals in the cytokine ELISAs. Furthermore, the augmented cytokine production was not observed when PBMC were treated with LPS immediately after transfer into $RPMI^{CHE}$, without the three day culture period (data not shown). This excludes an artifact by direct interaction of $RPMI^{CHE}$ with cytokine detection. Taken together, zinc depletion reduces the production of TNF-α and IL-6. Additionally, there is a major impact of CHELEX on cytokine production that is unrelated to zinc.

To examine a possible impact of CHELEX on elements other than zinc, we analyzed the contents of eleven biologically

Table 2 Element contents of culture media

	$RPMI^{control}$	$RPMI^{TPEN}$	$RPMI^{CHE}$	$RPMI^{CHE+Zn}$
Ca [mg L^{-1}]	23.82 ± 2.01	26.83 ± 0.58	28.65 ± 1.75	28.86 ± 2.07
Mg [mg L^{-1}]	11.84 ± 0.36	12.17 ± 0.02	9.62 ± 0.57	9.32 ± 0.19
Zn [μg L^{-1}]	169.72 ± 8.11	170.76 ± 7.89	10.90 ± 0.32	439.25 ± 19.71
Fe [μg L^{-1}]	208.57 ± 4.98	213.34 ± 9.32	207.88 ± 6.61	208.43 ± 7.71
Cu [μg L^{-1}]	11.99 ± 0.35	12.18 ± 0.44	7.43 ± 0.18	7.17 ± 0.11
Mn [μg L^{-1}]	3.45 ± 0.05	3.73 ± 0.32	3.38 ± 0.26	3.31 ± 0.15
Se [μg L^{-1}]	n.d.	n.d.	n.d.	n.d.
Mo [μg L^{-1}]	2.61 ± 0.16	2.56 ± 0.14	9.99 ± 0.40	10.05 ± 0.37
Ni [μg L^{-1}]	n.d.	n.d.	n.d.	n.d.
Co [μg L^{-1}]	0.35 ± 0.02	0.34 ± 0.00	0.35 ± 0.01	0.33 ± 0.01
As [μg L^{-1}]	0.22 ± 0.06	0.22 ± 0.05	0.24 ± 0.06	0.22 ± 0.04

n.d. = not detected; values were below the limit of quantitation for Se (1.22 μg L^{-1}) or Ni (0.2 μg L^{-1}), respectively.

relevant elements in the cell culture media by ICP-MS (Table 2). Zinc was most prominently depleted (>90%), whereas lesser reductions were observed for copper and magnesium. Most other elements remained unaffected, with the exception of molybdenum. Approximately a fourfold increase in molybdenum content was observed in $RPMI^{CHE}$ and $RPMI^{CHE+Zn}$, indicating a release of molybdenum from CHELEX during treatment of the media.

Discussion

In the present study, two approaches for depleting cells of zinc are compared. TPEN is a membrane-permeable chelator with high affinity for zinc. Other physiologically important metals that are bound by TPEN with lower affinity, such as calcium, are unaffected under the conditions used in our experiments.[16] In contrast, CHELEX removes metal ions from the culture medium and calcium and magnesium have to be reconstituted subsequently.[11] Specificity is achieved by adding zinc back to CHELEX-treated medium, making the zinc concentration the only difference between the two kinds of media.[17,18]

In Fig. 1, the chelator TPEN reduces free intracellular zinc, whereas no significant difference is found between $RPMI^{CHE+Zn}$ and $RPMI^{CHE}$. However, free zinc is only a small fraction of the total cellular zinc.[19] In zinc deficient cells, Zip-transporters responsible for the movement of zinc into the cytosol are upregulated, while transporters of the ZnT-type needed for the reverse transport are downregulated.[8,20–24] In our hands zinc is removed from the culture medium by CHELEX with over 90% efficiency. The remainder might still be sufficient for the cells to maintain free zinc, despite a state of general zinc deficiency. In contrast, TPEN passes through the cell membrane and chelates free zinc directly in the cytosol. Nevertheless, both methods for zinc depletion yield similar impacts on phagocytosis, oxidative burst, and cytokine production, as discussed below. In some cases, not all effects did reach a statistical significance, but this is most likely due to a high degree of variation between different human donors, compared to cell cultures or genetically identical inbred strains of experimental animals.

For DSred-*E. coli* and GFP-*S. aureus* phagocytosis is significantly augmented in the presence of TPEN, while there are no statistically significant differences between cells kept in $RPMI^{CHE+Zn}$ and $RPMI^{CHE}$, although a respective trend is visible for GFP-*S. aureus*. It has been reported that CHELEX causes a weaker form of zinc deficiency than TPEN, which might be reflected by a lesser impact on phagocytosis.[25] Both kinds of zinc deprivation elevate phagocytosis of GFP-*S. pneumoniae*, even though the differences do not reach a statistical significance. The fluorescence of GFP-*S. pneumoniae* is lower than that of DSred-*E. coli* and GFP-*S. aureus* (data not shown), most likely due to lower expression of GFP. Nevertheless, this should only affect the fluorescence intensity of the samples, but not the outcome in general. Previous studies have shown that staphylococci and streptococci differ in their sensitivity to phagocytosis, depending on the expression of neutrophil surface receptors.[26] Hence, the impact of zinc deficiency on phagocytosis by monocytes may also vary with the bacterial species.

A general trend toward increased phagocytosis in zinc-deficient monocytes supports the theory of reprogramming of the immune system during zinc deficiency, because a switch to myeloid cells is only useful if these are reliable in function. In addition, there are other conditions under which zinc homeostasis is altered. A lower plasma zinc level is usually found in septic patients, combined with a higher degree of inflammation.[27] Moreover, in monocytes a decrease of the intracellular zinc level can be caused by the induction of intracellular zinc binding proteins through LPS or inflammatory cytokines.[28] Finally, free intracellular zinc is reduced during vitamin D_3-induced differentiation of HL60 cells into macrophages, which is accompanied by acquisition of macrophage functions such as phagocytosis. Additional zinc deprivation during differentiation further increases phagocytosis and oxidative burst.[11] Taken together, these data could indicate that during an immune response systemic and cellular zinc levels are downregulated and reduced zinc promotes monocyte differentiation and activation, increasing their ability for phagocytosis.

Once ingested into a monocyte, pathogens are killed by reactive oxygen species (ROS) after fusion of phagosomes with lysosomes. Among other antimicrobial proteins, lysosomes contain the key enzyme of ROS production, NADPH oxidase.[29] NADPH oxidase activity is connected to the intracellular zinc status of the cell: it is inhibited both by zinc excess and zinc deficiency.[8] Furthermore, zinc deprivation of *Histoplasma capsulatum* has been

shown to lead to increased susceptibility of the pathogen against ROS, whereas at the same time zinc accumulation improves ROS-tolerance of the host cell.[30,31]

Oxidative burst is increased by zinc deficiency during macrophage differentiation.[11] Accordingly, in the present study zinc deficiency promotes the oxidative burst of human monocytes, but seemingly only in response to GFP-*S. aureus*, thereby excluding a general effect. Monocytes can discriminate between gram-positive and gram-negative bacteria, *e.g.*, when it comes to cytokine secretion,[32] but the differences in ROS production in response to different bacteria cannot be attributed to the composition of their cell wall. The response toward GFP-*S. aureus* (gram-positive) is augmented under zinc deficiency, whereas this is not observed for gram-negative DSred-*E. coli* and gram-positive GFP-*S. pneumoniae*.

The impact of zinc on the production of pro-inflammatory cytokines, such as TNF-α and IL-6, is still controversial. In monocytes zinc has both activating and inhibiting roles in pro-inflammatory cytokine production, depending, amongst other factors, on its concentration and the duration of the altered zinc status.[3] A study with the monocyte cell line HL-60 showed an augmented expression of mRNA for TNF-α, IL-1β, and IL-8 in response to stimulation using phorbol-12-myristate-13-acetate in cells cultured in zinc-deficient culture medium depleted by CHELEX.[33] It has further been shown that levels of pro-inflammatory cytokines in elderly are increased during zinc deficiency and that this can be reversed through zinc supplementation.[34–36] In contrast, the production of TNF-α and IL-6 can be increased through zinc supplementation in PBMC.[4,7]

This study indicates that TNF-α and IL-6 production is decreased after zinc deprivation for three days. In the context of a reprogramming of the immune system during zinc deficiency,[9] a lower release of pro-inflammatory cytokines seems adequate: TNF-α and IL-6, among other cytokines, have many functions connecting the innate with the adaptive immune system. As the reprogramming shifts immunity toward a solitary defense by the innate immune system, the communication between both sides can be economized.

Notably, there is one major difference between the results obtained using TPEN and CHELEX. CHELEX-treated medium activates monocytes, independently of zinc. This effect is even stronger than the one of LPS, which is a very potent inducer of inflammatory cytokines in monocytes. This effect could be independent from metal ion binding. The CHELEX beads are removed and do not get in direct contact with the cells. Nevertheless, the material could come off and act on macrophages directly, possibly *via* unspecific activation of cell surface receptors. Alternatively, the effect of CHELEX-treatment might result from chelation of another metal in addition to zinc. Calcium and magnesium were routinely added back to CHELEX-treated medium.[11] Still, magnesium levels were slightly below those of the control, but this seems to be only a minor difference. Moreover, the findings presented in Table 2 differ from the literature with regard to the removal of several other elements, indicating that different protocols can lead to differential depletion of trace elements from cell culture media. Copper was reported not to be depleted from culture medium by treatment with CHELEX,[25] whereas ICP-MS showed a reduction of copper by roughly 40% in the CHELEX-treated media compared to $RPMI^{control}$. On the other hand, manganese was previously found to be removed by CHELEX,[25] but its levels were unaffected by CHELEX treatment in the present study. Remarkably, there was a release of molybdenum from the CHELEX, quadrupling the content of this element in $RPMI^{CHE+Zn}$ and $RPMI^{CHE}$. Molybdenum is known to elevate the expression of pro-inflammatory cytokines, including TNF-α and IL-6, in human monocytes/macrophages.[37] Although molybdenum concentrations higher than in the present study were used, and the effects did not reach the levels induced by treatment with LPS, this may be due to differences in experimental conditions, such as the shorter incubation times of 24 to 48 h.[37] Hence, a release of molybdenum might be the explanation for the non zinc-related impact of CHELEX on cytokine production, although this needs to be confirmed by further investigations.

Conclusion

Zinc depletion differentially affects monocyte functions. Direct elimination of bacterial pathogens by phagocytosis and oxidative burst is elevated, whereas production of inflammatory messengers is reduced. These results were obtained independently of the method by which zinc deficiency was achieved. Still, CHELEX has side-effects on monocytes that are unrelated to its capability for zinc removal. Some cellular functions, such as phagocytosis, can be investigated without interference. In contrast, cytokine production is directly affected by CHELEX *via* an unknown mechanism, limiting the use of CHELEX for studying innate immunity.

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