
**The Effect of Intracellular Zinc Concentration on
Immune Activation and Proinflammatory Response in
THP-1 Monocytes**

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The Effect of Intracellular Zinc Concentration on Immune Activation and Proinflammatory Response in THP-1 Monocytes

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Abstract

Zinc is an essential micronutrient required for many cellular functions, including the development and function of the immune system. The biological consequences of zinc deficiency illustrate the significance of zinc in human health. Zinc deficiency has been shown to cause the dysregulation of both innate and adaptive immune responses, as well as promote the development of chronic low-grade inflammation. Interestingly, the effects of aging and zinc deficiency on the immune system are remarkably alike, as aging is also strongly associated with impaired immune function and chronic low-grade inflammation. In fact, the older population is particularly susceptible to zinc deficiency, and zinc status declines with age. It is therefore thought that age-related zinc deficiency may contribute to the chronic inflammation commonly observed in the elderly. In contrast to severe zinc deficiency, the effect of marginal zinc deficiency on chronic inflammation is not well characterized. Additionally, the effect of zinc supplementation on immune function is unclear, since clinical trials of zinc supplementation exhibit conflicting results. The central questions of this study are whether marginal zinc

deficiency contributes to immune dysregulation, resulting in an enhancement of immune cell activation and increased inflammatory response, and whether zinc supplementation can reduce immune activation and decrease inflammation. Using cultured THP-1 human leukemic monocytic cells, we studied the impact of marginal zinc deficiency and zinc supplementation on the expression of well-known mediators of immune activation and proinflammatory response. We found zinc deficient cells consistently demonstrated signs of increased immune activation and proinflammatory response compared to zinc adequate cells. Marginal zinc deficient cells repeatedly produced increased markers of immune activation and proinflammatory response as well, but to a lesser degree than more severely zinc deficient cells. The effect of zinc supplementation on immune function was not as clear. Zinc supplemented cells produced reduced levels of some of these markers and increased levels of other markers. These findings suggest that age-related zinc deficiency, which is similar to marginal zinc deficiency, may also have negative impacts on the immune system. Furthermore, our findings provide preliminary data for further cell culture studies or human trials of marginal zinc deficiency and zinc supplementation to further clarify their effects on the immune system.

Keywords: Aging; Immunity; Inflammation; Zinc

1. Introduction

1.1. Age-related zinc deficiency may contribute to chronic inflammation in the elderly

Aging results in gradual changes in the body's life processes. One particular process that undergoes such changes is the immune system, which becomes progressively dysregulated with age. Age-related dysregulation of immune function results in an

increased frequency of infection, fatigue, and systemic low-grade inflammation (1). This chronic inflammation is of concern because it promotes the development of degenerative diseases, such as heart disease, cancer, diabetes, and autoimmune disease (2-4). The consequences of aging on the immune system bear fascinating similarities to those of zinc deficiency, as zinc deficiency is also implicated with dysregulated immune function and chronic low-grade inflammation (5-7). Intriguingly, zinc status has been found to decline with age, which occurs as a result of both decreased consumption and absorption of zinc in the elderly (8-10). National surveys have revealed that while 12% of the total US population does not consume adequate zinc (11-14), 40% of the elderly US population does not consume adequate zinc (9, 10). Therefore, age-related zinc deficiency is a potential contributing factor to the chronic inflammation and related diseases frequently experienced by the elderly (9-14).

1.2. The Importance of Zinc

Zinc is an essential micronutrient that plays crucial roles in a number of biological processes. On the cellular level, zinc functions in catalytic, structural, and regulatory roles. Close to 300 different enzymes require zinc for the catalysis of their critical chemical reactions (15, 16). For example, the enzyme methionine synthase relies on its zinc atom to form L-methionine, an amino acid essential for initiating the synthesis of all polypeptides for eukaryotes (17-19). Furthermore, zinc is necessary for the functional structure of many proteins and cell membranes. Certain polypeptides cannot fold into the proper form without one or more zinc atoms, such as proteins that fold into the zinc finger motif- a common pattern for DNA-binding proteins (20, 21). The regulatory roles of zinc include regulation of gene expression through zinc finger proteins, cell signaling

cascades, hormone secretion, and nerve impulse propagation (22). On the organismal level, zinc is important for a wide variety of physiological functions including proper growth and development, neurological function, and immune response (8, 23).

1.3. Zinc's role in immune function and inflammation

Zinc deficiency leads to the dysregulation of both innate and adaptive immune responses (24, 25). The innate immune response is a non-specific but rapid onset attack against infectious agents that is mediated by mast cells, natural killer cells, basophils, eosinophils, and phagocytic cells, including neutrophils and macrophages (9). Adaptive immunity involves defense strategies against specific pathogens and is mainly executed by T and B cells (9, 26, 27). Numerous studies reveal that zinc deficiency decreases the activity of these cells and decreases the production of precursor cells for some of these cell types (6, 28-31). Zinc deficiency is also associated with increased proinflammatory response. Both cell culture and animal studies demonstrate an increased production of proinflammatory cytokines and other markers of proinflammatory response as a result of zinc deficiency (32-34). Long-term overexposure to proinflammatory factors, such as proinflammatory cytokines and reactive oxygen species, assaults a variety of physiological processes, including cell replication, angiogenesis, apoptosis, and DNA repair (35, 36).

In contrast, zinc supplementation is a highly effective anti-inflammatory agent. Extensive research demonstrates that zinc supplementation decreases the production of inflammatory response factors in allergic inflammation and acute inflammation in mice (34). In addition, brief zinc repletion of zinc deficient mice corrects dysregulated proinflammatory responses and mitigates related tissue injury. Zinc supplementation

trials in humans provide less consistent results, likely because of differences in the dose and scheduling of supplementation (6, 37).

The precise mechanisms by which zinc deficiency affects the immune system remain under investigation. One well-supported method by which zinc deficiency causes immune dysregulation occurs through the increased nuclear translocation of proinflammatory response-mediating transcription factor, NF κ B (38). Another potential mechanism is through an alteration in the expression of zinc transporters, which regulate intracellular zinc homeostasis. Recent findings demonstrate that changes in intracellular zinc balance, as mediated by zinc transporter expression, disrupt signaling events in immune cells, resulting in dysregulation of the proinflammatory response (9, 39, 40).

1.4. Monocytes are a key participant in the proinflammatory response

Monocytes are a type of white blood cell involved in the innate immune response. They play crucial roles in the initiation and resolution of proinflammatory responses. For a proinflammatory response to be induced, monocytes must first undergo a series of changes called activation (41). A classic sign of monocyte activation is the increased expression of cell activation markers, such as intercellular adhesion molecule 1 (ICAM1). ICAM1 is a glycoprotein expressed on the extracellular surface of monocyte cells that functions primarily in monocyte migration and adhesion to sites of infection (42). Monocytes also perform vital functions in the progression of proinflammatory responses through the increased production of reactive oxygen species (ROS) and proinflammatory cytokines (43). ROS are involved in the signaling cascades leading to the increased expression of adhesion molecules like ICAM1 and proinflammatory cytokines (44, 45). Proinflammatory cytokines facilitate host defense against pathogens and promote

inflammation and fever. Short-term expression of proinflammatory cytokines provides beneficial protective effects, but aberrant expression of these proteins results in tissue damage, shock, and in some cases, death (46). The use of monocytes in a cell culture system provides the ability to characterize the effects of manipulating zinc status during different stages in the induction of an inflammatory response.

1.5. Research goals and hypotheses

Though the consequences of severe zinc deficiency on the immune system are well characterized, the consequences of marginal zinc deficiency on immune function are not fully elucidated. Marginal zinc deficiency a less severe form of zinc deficiency that is more prevalent than severe zinc deficiency in the US and more comparable to age-related zinc deficiency. However, most studies have focused on the effects of severe zinc deficiency to date (12). At the same time, the benefits of zinc supplementation in the elderly are controversial, as clinical trials of zinc supplementation to improve immune function in the elderly provided inconsistent results (6, 37). Previous work in vitro using cell culture indicates severe zinc deficiency causes inappropriately increased immune cell activation and amplified proinflammatory responses. The purpose of this research project is to determine the effect of marginal zinc deficiency and zinc supplementation on immune cell activation and intensity of the proinflammatory response in an in vitro cell culture model. We hypothesize marginal zinc deficiency will increase immune cell activation and proinflammatory response intensity, but to a lesser degree than that caused by zinc deficiency. Conversely, we expect zinc supplementation to reduce immune cell activation and proinflammatory response intensity. The outcomes of this study will help reveal whether marginal zinc deficiency, such as age-related zinc deficiency, causes

exaggerated proinflammatory responses and contributes to chronic inflammation and related diseases. The results of this research will also further our understanding of the potential of zinc supplementation to mitigate chronic inflammation. Taken together, the findings of this study will help identify nutritional therapies that can prevent and alleviate chronic inflammation and associated degenerative illnesses in the elderly.

2. Materials and methods

2.1. Cell culture, zinc depletion and augmentation, and lipopolysaccharide (LPS) stimulation

Human leukemic monocyte cell line THP-1 was attained from American Type Tissue Collection (Manassas, VA, USA). RPMI 1640 culture medium with 10% fetal bovine serum (FBS) was used to culture cells in humidified incubators at 37°C with 5% CO₂. Zinc was removed from FBS using a previous published chelation technique that entails incubating FBS with 10% Chelex 100 (wt/vol) (Sigma, St. Louis, MO, USA) overnight at 4°C with constant stirring (47). Cells were cultured in media that was either zinc deficient (RPMI with 10% Chelex-treated FBS and 0µM ZnCl₂), marginally zinc deficient (RPMI with 10% Chelex-treated FBS and 1µM ZnCl₂), zinc adequate (RPMI with 10% Chelex-treated FBS and 4µM ZnCl₂), or zinc supplemented (RPMI with 10% Chelex-treated FBS and 40µM ZnCl₂). Cells were cultured for 21 days, changing media every three to four days to establish respective zinc status. Monocyte activation and induction of proinflammatory response was accomplished through lipopolysaccharide (LPS) (Sigma) treatment. LPS is a potent and well-characterized activator of monocytes and induces a potent inflammatory response. Monocyte activation and proinflammatory cytokine assays involved stimulating cells with 100ng/mL LPS for 24 hours at 37°C. ROS assays involved stimulating cells with 10 µg/mL LPS for 3 hours at 37°C. After

LPS stimulation, cells were harvested for analysis of cell adhesion marker expression- a marker of cell activation. Supernatant was collected for determination of proinflammatory cytokine production and ROS generation.

2.2. Intracellular zinc quantification

Intracellular zinc concentration was assessed using FluoZin-3 acetoxymethyl ester (FluoZin-3), a fluorescent indicator that binds free intracellular zinc. Cells were seeded at 5×10^5 cells per well in a 24-well tissue culture plate and incubated with 1 μ M FluoZin-3 for 30 minutes at 37°C. Relative fluorescence was then measured with flow cytometry analysis.

2.3. Determination of cell proliferation rate

Cell proliferation rate was studied as a measure of cell viability. Cell counts were performed once or twice a week for four weeks. Prior to counting, cells were scraped from the culture flask, dyed with trypan blue, loaded onto a hemacytometer, and visualized under a light microscope. Total cell number was then calculated and plotted as a bar graph to compare cell proliferation.

2.4. Monocyte activation assay

After stimulation with LPS, cells were harvested, washed, and stained for cell activation marker, ICAM1. Staining involved incubating cells with a phycoerythrin-conjugated antibody against ICAM1 for 20 minutes in dark conditions. Relative fluorescence was subsequently measured using flow cytometry.

2.5. Proinflammatory response assays

Proinflammatory response was studied by measuring the production of proinflammatory cytokines and generation of ROS.

2.5a. Proinflammatory cytokine measurement

Cells were seeded equally at 5×10^5 cells per well in 24-well tissue culture plates. After LPS treatment, resulting supernatant was collected and analyzed for Interleukin-1 β (IL1 β), Interleukin-6 (IL6), and monocyte chemotactic protein-1 (MCP1) with human IL1 β , IL6, and MCP1 Ready-SET-Go ELISA kits from eBioscience (San Diego, CA, USA). Proinflammatory cytokine concentration in the supernatant was quantified using spectrophotometry at 450 nm, subtracting the value at 570 nm.

2.5b. Reactive oxygen species measurement

In 96-well flat-bottom tissue culture plates, cells were seeded equally at 1×10^5 cells/well and treated with LPS. Carboxy-H₂DCFDA (DCF), a ROS detection reagent, was added to the LPS-treated cells at 20 μ M for 30 minutes at 37°C. Fluorescence was measured using spectrophotometry at Ex 488 nm, Em 535 nm with auto-cutoff.

2.6. Statistical analyses

Statistical analyses were accomplished with GraphPad Prism Version 5.02 software (GraphPad, La Jolla, CA, USA). All analyses are stated as mean \pm S.E.M. Unpaired *t* tests and one-way analysis of variance (ANOVA) were used where applicable. Bonferroni post hoc tests were additionally applied to examine differences among the means when a significant main effect was found in one-way ANOVA. Statistical significance was defined as $P \leq .05$.

3. Results

3.1. Zinc supplementation results in increased intracellular zinc

The impact of zinc status on the induction and intensity of proinflammatory response was studied in an in vitro THP-1 cell culture model in which cells were grown

in either zinc deficient, marginally zinc deficient, zinc adequate, or zinc supplemented media. To assess whether culturing THP-1 cells in media with defined concentrations of zinc resulted in corresponding intracellular levels of zinc, free intracellular zinc ions were labeled with a fluorescent indicator called FluoZin3[®] after 21 days of subjecting the cells to their respective media. Flow cytometry analysis of the relative fluorescence revealed zinc supplementation significantly increases intracellular zinc levels compared to those of cells grown in zinc adequate conditions. No significant difference in the abundance of intracellular zinc was detected between cells grown in zinc deficient, marginally zinc deficient, and zinc adequate media since these changes in intracellular zinc were below the sensitivity of the assay (Fig. 1). Secondary confirmation of these trends is needed, but the timing of this thesis project did not allow. We intend to validate these results using inductively-coupled plasma mineral analysis in the near future.

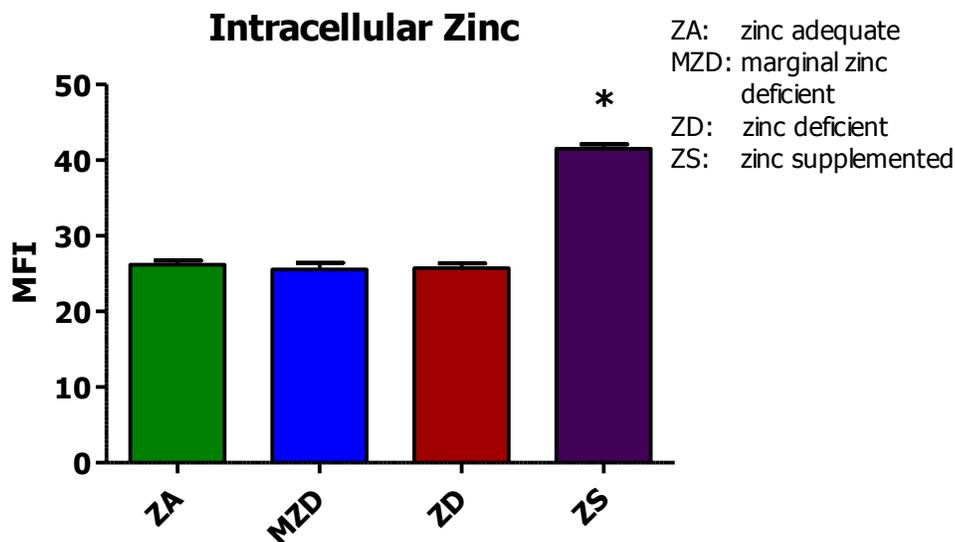


Fig. 1. Zinc supplementation resulted in increased intracellular zinc. THP-1 cells (n=6 per treatment) were cultured in ZA, MZD, ZD, or ZS media. Intracellular zinc was measured by FluoZin-3 flow cytometry. Data represent mean \pm S.E.M. MFI, mean fluorescence intensity. *P<.05 vs. ZA. Results are representative of 2 independent experiments.

3.2. Zinc deficiency decreases growth rate

Alterations in zinc status also caused significant differences in cell proliferation rate. Zinc deficiency consistently produced dramatic decreases in growth rate, but marginal zinc deficiency and zinc supplementation did not cause appreciable changes in growth rate (Fig. 2).

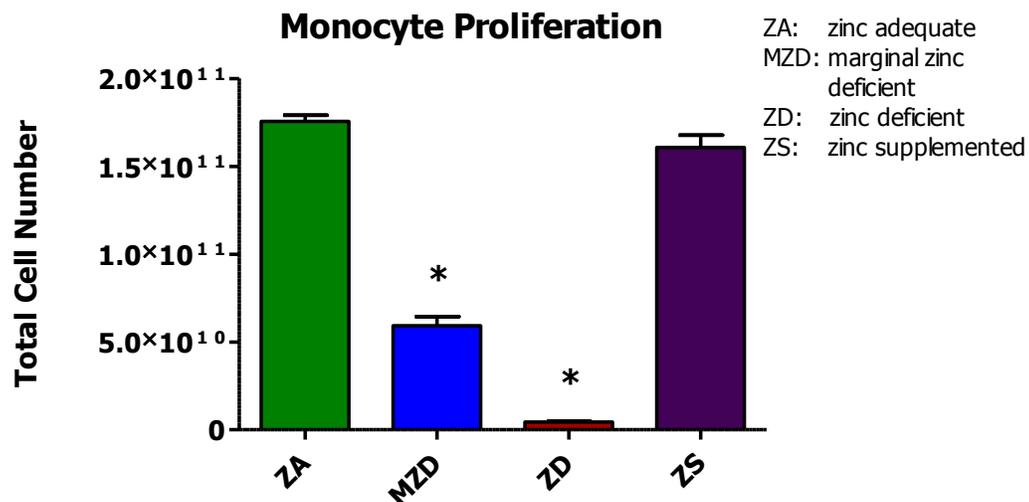


Fig. 2. Zinc deficiency decreased growth rate. THP-1 cells (n=3 per treatment) were cultured in ZA, MZD, ZD, or ZS media. Cumulative total cell number at the end of cell culture period was determined by performing cell counts every three or four days for 28 days. Data represent mean \pm S.E.M. *P<.05 vs. ZA. Results are representative of 2 independent experiments.

3.3. Zinc deficiency, marginal zinc deficiency, and zinc supplementation increase expression of monocyte activation marker ICAM1

3.3a. Zinc deficiency causes spontaneous expression of ICAM1

Along with noticeable changes in cell proliferation rates, alterations in zinc status resulted in noticeable morphological changes. Zinc deficient cells in particular became smaller, irregularly shaped, and adhered to the culture flask in comparison with zinc adequate cells, which were consistently round, larger, and not adherent. In response to proinflammatory stimuli, monocytes become activated and primed to elicit a

proinflammation response. A hallmark of monocyte activation involves the upregulation of cell activation markers, including cell adhesion molecules such as ICAM1, that aid in monocyte migration and adherence to sites of infection. Interestingly, ICAM1 expression was upregulated with zinc deficiency, even in the absence of LPS stimulation. This spontaneous upregulation of ICAM1 expression signifies severe zinc deficiency induces cell activation in THP-1 cells (figure not shown).

3.3b. Zinc deficiency, marginal zinc deficiency, and zinc supplementation increase expression of ICAM1 following induction of proinflammatory response with LPS

Stimulation of the THP-1 cultures with LPS resulted in greater expression of ICAM1 than untreated cells in all of the zinc groups. Zinc deficiency further increased ICAM1 expression, and LPS-induced zinc deficient cells expressed almost twice as much ICAM1 as LPS-induced zinc adequate cells. Cells grown in marginally zinc deficient and zinc supplemented media also exhibited increased expression of ICAM1 compared to zinc adequate cells, but to a lesser degree than cells grown in zinc deficient media (Fig. 3C).

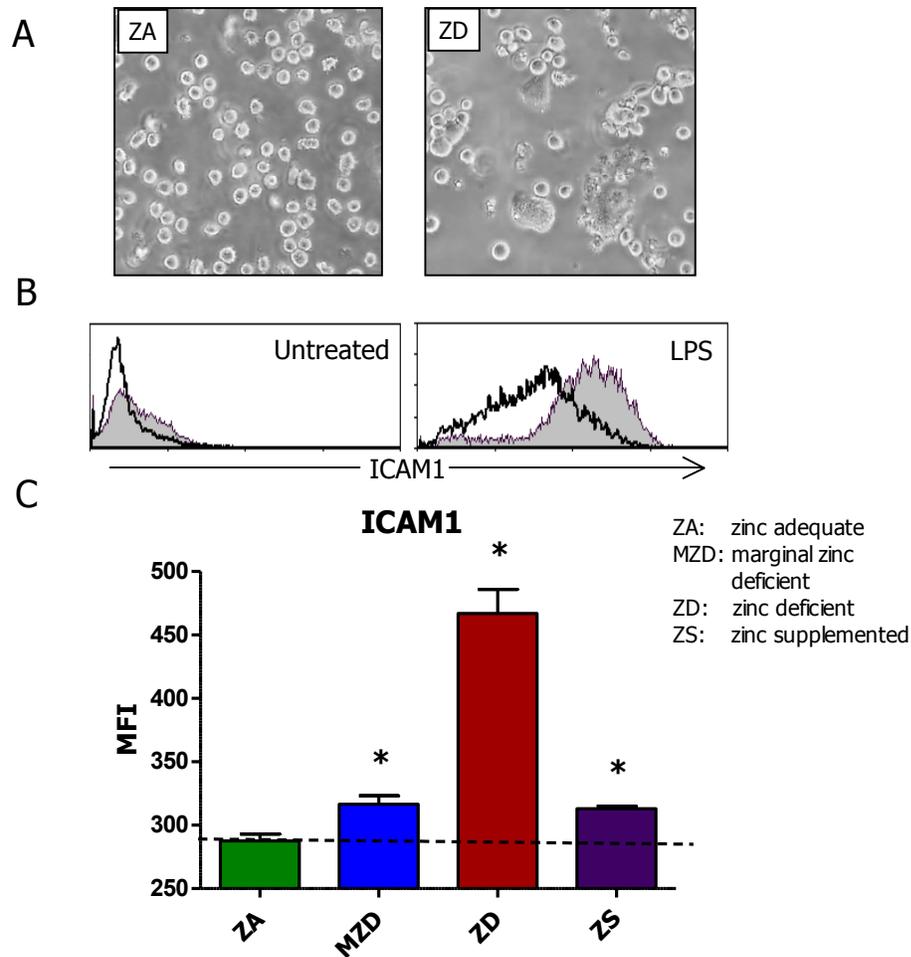


Fig. 3. Zinc deficiency increased cell adherence that paralleled with upregulated ICAM1 expression. Marginally zinc deficient and zinc supplemented cells demonstrated increased ICAM1 expression as well. THP-1 monocytes (n=3 per treatment) were cultured in ZA, MZD, ZD, or ZS media for 21 days. Cell adherence patterns were visualized under a light microscope (A). Cells were stimulated with 100 ng/mL LPS for 24h and subsequently stained for ICAM1 expression. ICAM1 expression was quantified using flow cytometry (B & C). Data represent mean \pm S.E.M. MFI, mean fluorescence intensity. *P<.05 vs. ZA. Results are representative of 2 independent experiments.

3.4. Zinc deficiency and marginal zinc deficiency increase production of proinflammatory cytokines

Having observed the effects of zinc deficiency in cell proliferation rate, cell morphology, and monocyte cell activation, we proceeded to study how zinc status affects the induction of proinflammatory response. One such hallmark is the increased production of proinflammatory cytokines, which are proteins secreted by monocytes to further the progression of the proinflammatory response through signal cascades. The

relative production of proinflammatory cytokines, Interleukin-1 β (IL1 β), Interleukin-6 (IL6), and monocyte chemoattractant protein-1 (MCP1), was measured with capture antibodies against these cytokines. Zinc deficient cells produced a significantly increased amount of IL1 β , IL6, and MCP1 than did zinc adequate cells. There was a dose dependent increase in proinflammatory cytokine level with increasing level of zinc deficiency. The production of proinflammatory cytokines by the zinc supplemented cells was less consistent. Compared to zinc adequate cells, zinc supplemented cells produced a slightly lesser but not significantly lesser amount of IL1 β , a significantly greater amount of IL6, and a slightly greater but not significantly greater amount of MCP1 (Fig. 4).

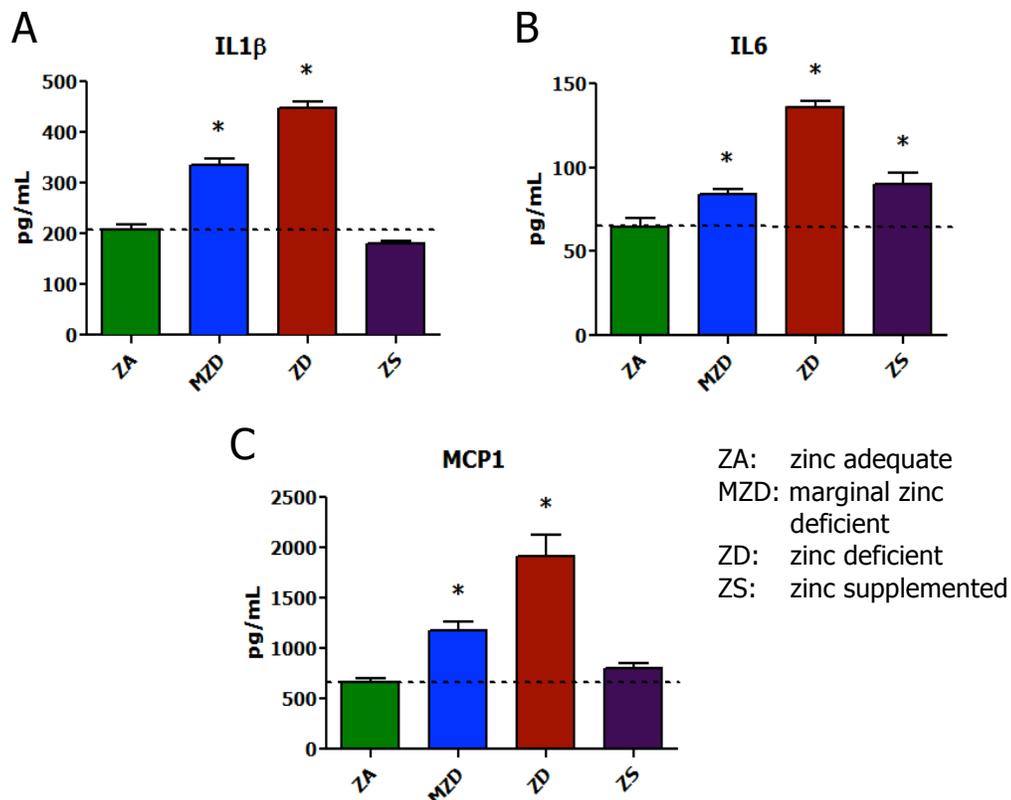


Fig. 4. Zinc deficiency and marginal zinc deficiency increased the production of proinflammatory cytokines. THP-1 cells (n=3 per treatment) were cultured in ZA, MZD, ZD, or ZS media for 3 weeks. Cells were then subjected to 100 ng/mL LPS for 24 h. Production of proinflammatory cytokines IL1 β (A), IL6 (B), and MCP1 (C) was quantified with ELISAs. Data represent mean \pm S.E.M. *P<.05 vs. ZA. IL1 β and IL6 results are representative of 3 independent experiments, and MCP1 results are representative of 2 independent experiments.

3.5. Zinc deficiency and marginal zinc deficiency increase generation of ROS

3.5a. Zinc deficiency causes spontaneous ROS generation

Another well-characterized hallmark of the proinflammatory response is the increased generation of ROS. ROS function in the proinflammatory response to kill bacteria within cells and induce the production of proinflammatory cytokines and cell activation markers (44, 45). DCF, a ROS detection reagent, was added to the cultures to measure the generation of ROS without stimulation of a proinflammatory response with LPS. Zinc deficient cells produced an increased amount of ROS compared to the baseline amount produced by zinc adequate cells, suggesting the increased proinflammatory response observed with zinc deficiency may in part be caused by the increased production of ROS. However, no significant difference in ROS generation was detected between zinc adequate, marginally zinc deficient, and zinc supplemented cells (Fig. 5).

3.5b. Zinc deficiency and marginal zinc deficiency increase ROS generation following induction of proinflammatory response with LPS

THP-1 monocytes were treated with LPS to stimulate a proinflammatory response, and the subsequent production of ROS was again measured by fluorescent labeling with DCF. LPS treatment caused an increased production of ROS in all of the zinc groups relative to that produced by untreated cells. LPS-treated zinc deficient cells produced a further increased level of ROS that was significantly greater than the level produced by LPS-treated zinc adequate cells. The LPS-induced increase in ROS response was also dose dependent with increasing level of zinc deficiency. No change was observed in the level of ROS produced by LPS-treated zinc supplemented cell and LPS-treated zinc adequate cells (Fig. 5).

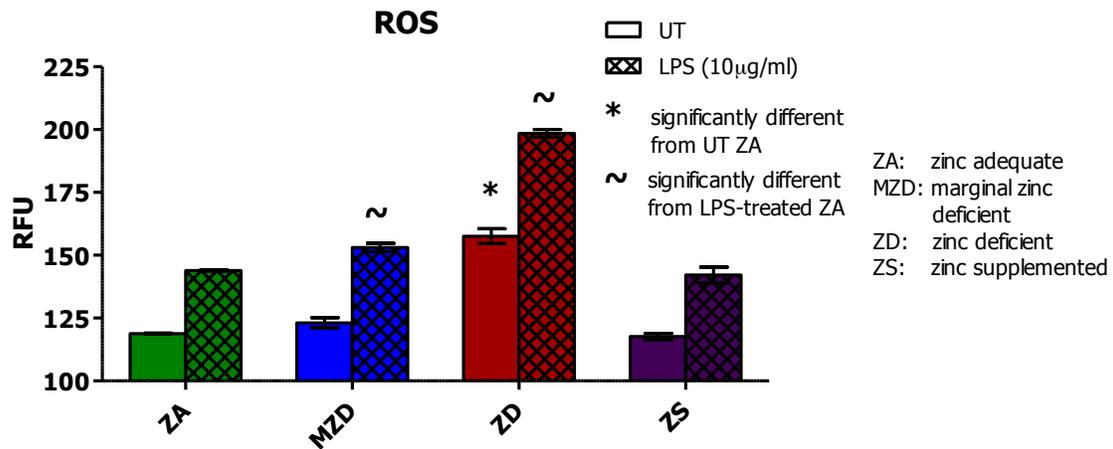


Fig. 5. Zinc deficiency and marginal zinc deficiency increased the production of ROS. THP-1 cells (n=3 per treatment) were cultured in ZA, MZD, ZD, or ZS media for 3 weeks. Cells then remained untreated or were incubated with 10 µg/mL LPS for 3h. ROS were labeled with DCF and detected using spectrophotometry. Data represent mean ± S.E.M. RFU, relative fluorescence units. *P<.05 vs. ZA. Results are representative of 2 independent experiments.

4. Discussion

Aging is commonly accompanied by declining immune function, resulting in less effective defense against infection, chronic low-grade inflammation, and related diseases (1-4, 8-10). The effects of zinc deficiency on the immune system mirror those of aging, and a decline in zinc status is also associated with age (5-10). Age-related zinc deficiency is thus a probable contributing factor to the chronic inflammation common to elderly populations (9-14). In developed nations, the zinc deficiency associated with aging is not as acute as severe zinc deficiency and is more akin to marginal zinc deficiency. While the immune-disrupting effects of severe zinc deficiency are well known, much less is known about the consequences of marginal zinc deficiency on immune function (12).

We anticipated cells grown in marginally zinc deficient media to demonstrate increased immune activation and proinflammatory response intensity. The findings in

this study generally support our hypothesis. After stimulating the cells to initiate a proinflammatory response, the marginally zinc deficient group demonstrated increased immune activation through increased expression of cell activation marker ICAM1 compared to the level expressed by the zinc adequate group (Fig. 3C). Furthermore, marginally zinc deficient cells produced increased proinflammatory cytokines and increased ROS compared to zinc adequate cells, suggesting marginal zinc deficiency, similar to severe zinc deficiency, can result in increased proinflammatory response (Fig. 4 & 5). The increased immune activation and proinflammatory response intensity demonstrated by the marginal zinc deficiency in this study has potential clinical relevance, as the elderly are highly susceptible to marginal zinc deficiency. This data suggests sub-acute zinc deficiency, such as the form of zinc deficiency associated with aging, contributes to chronic inflammation and increases susceptibility to related degenerative diseases.

Zinc supplementation has been explored as a treatment for the immune dysregulation and chronic inflammation associated with aging. Zinc has anti-inflammatory properties, and zinc supplementation regulates immune function during allergic and acute inflammation (34). In addition, zinc repletion of zinc deficient mice effectively restored proinflammatory response to normal levels, and zinc supplementation reduces markers of inflammation in populations prone to zinc deficiency, like children and sickle cell patients (48, 49). A number of clinical trials of zinc supplementation to improve immune function and reduce chronic inflammation in the elderly have been performed, but the results are inconclusive (6, 37). Some of the inconsistent data may be attributed to a lack of sensitive biomarker for zinc deficiency in humans.

In this study, we also explored the effect of zinc supplementation on immune function using the cell culture model described earlier. We expected zinc supplementation to decrease immune activation and proinflammatory response intensity, however zinc supplementation did not cause a reduction in any of the markers of immune activation and proinflammatory response intensity we measured. Instead, the cells grown in zinc supplemented media demonstrated an increase in cell activation marker ICAM1 and proinflammatory cytokine IL6 production (Fig. 3C & 4B). One potential explanation for these increases in ICAM1 and IL6 expression may be that the concentration of zinc in the zinc supplemented media was too high, causing toxicity in the cells. Repeating this trial of zinc supplementation with a lower concentration of zinc in the zinc supplemented media is needed to confirm.

The findings of this study indicate marginal zinc deficiency causes greater immune activation and exaggerated proinflammatory response. Elevated proinflammatory responses and resulting prolonged exposure to proinflammatory response mediators increase the likelihood of developing chronic inflammation and associated degenerative diseases (35, 36). Ultimately, these findings about marginal zinc deficiency suggest age-related zinc deficiency, though not as serious as severe zinc deficiency, has negative impacts on the immune system and deserves additional investigation. The effect of zinc supplementation on the proinflammatory response is less clear at this time. However, these trends provide preliminary data for further cell culture studies or human trials of marginal zinc deficiency and zinc supplementation to clarify their effects on the immune system. In combination, these research efforts may

help determine dietary practices to prevent chronic inflammation in the elderly and sustain quality of life through late stages of age.

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