The Interaction of Sodium and Zinc in the Priming of T Cell Subpopulations Regarding Th17 and Treg Cells

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Scope: Nutrition is a critical determinant of a functional immune system. The aim of this study is to investigate the molecular mechanisms by which immune cells are influenced by zinc and sodium.

Methods and Results: Mixed lymphocyte cultures and Jurkat cells are generated and incubated with zinc, sodium, or a combination of both for further tests. Zinc induces the number of regulatory T cells (Treg) and decreases T helper 17 cells (Th17), and sodium has the opposite effect. The transforming growth factor beta receptor signaling pathway is also enhanced by zinc and reduced by sodium as indicated by contrary phosphoSmad 2/3 induction. Antagonistic effects can also be seen on zinc transporter and metallothionein-1 (MT-1) mRNA expression: zinc declines Zip10 mRNA expression while sodium induces it, whereas MT-1 mRNA expression is induced by zinc while it is reduced by sodium.

Conclusion: This data indicate that zinc and sodium display opposite effects regarding Treg and Th17 induction in MLC, respectively, resulting in a contrary effect on the immune system. Additionally, it reveals a direct interaction of zinc and sodium in the priming of T cell subpopulations and shows that Zip10 and MT-1 play a significant role in those differentiation pathways.

1. Introduction

A well operating immune system is important to defend pathogenic organisms and to tolerate harmless organisms or food components, respectively. Nutrition is a critical determinant of a functional immune system and malnutrition is the most common cause of immunodeficiency worldwide. For instance, the micronutrients zinc, selenium, iron, copper, vitamins A, C, D, E, and B-6 as well as folic acid have an important influence on immune responses. Deficiency as well as over-nutrition of single nutrients can result in altered immune responses.

Salt, also known as sodium chloride (NaCl), is the most important mineral nutrient for humans. It adds flavor to food and is used as a preservative, binder, and stabilizer. It also prevents the growth of pathogenic and spoilage microorganisms in a variety of food systems. The human body needs a very small amount of sodium to conduct nerve impulses, contract and relax muscles, and maintain the proper balance of water and minerals. However, excessive sodium consumption, mainly seen in the developed countries, leads to pathologically increased blood pressure, heart disease, and stroke. Additionally, elevated sodium intake negatively influences the innate immune system, consequently mediating development of autoimmune diseases.

Although the exact pathologic mechanism still has to be investigated, it could be shown that increased sodium concentrations found locally in vivo dramatically increase the number of murine and human T helper (Th) 17 cells. Those interleukin (IL)-17-producing CD4-expressing T cells are interleukin (IL)-23-dependent and activate neutrophil granulocytes. On the one hand T helper 17 (Th17) cells are critical for clearing extracellular pathogens but on the other hand they also induce multiple autoimmune diseases. The sodium-induced Th17 polarization is implicated with the activation of the p38/mitogen-activated protein (MAP) kinase signaling pathway involving the toxicity-responsive enhancer binding protein (TonEBP/NFAT5) and the serum/glucocorticoid-regulated kinase 1 (SGK1). Studies already illustrated that physiologically elevated sodium concentrations alter the SGK1 signaling pathway leading to a marked impairment of regulatory T cell (Treg) function in vitro and in vivo.

Treg cells play an opposite role to Th17 cells in immune tolerance and autoimmune diseases. They are highly important for proper immune function, since they are the main repressor cell population in the human body. They maintain peripheral...
tolerance and thereby prevent autoimmune diseases and allergies. Treg cells can be distinguished from other Th cells by specific expression of the transcription factor Foxp3, which is responsible for their stability and function. Consumption of a sodium-rich diet favors the SGK1-dependent interferon (IFN)γ secretion in Treg cells, which as a consequence would lead to the induction of the pro-inflammatory Treg phenotype. Thus, excessive sodium consumption not only increases pro-inflammatory Th17 cells, but simultaneously inhibits the suppressive function of Treg cells.

Interestingly, studies show that the essential trace element zinc acts contrary to sodium. In contrast to sodium, zinc intoxication by excessive exposure is rare, whereas zinc deficiency is widespread and has a detrimental impact on the immune system. Hence, a proper zinc homeostasis is essential for adequate immune function. In the human body, zinc distribution differs in the extracellular space and intracellular compartments. For instance, high amounts of free zinc are located in specific lysosomes, called zincoinsomes, or are bound to proteins, for example, metallothionein (MT).

Zinc homeostasis is regulated by specific zinc transporter, which are grouped into two major protein families: 14 Zip (Zrt/Irt-like) and 10 zinc transporter (ZnT) proteins. Zips transport zinc out of the extracellular space or cellular organelles into the cytosol, whereas ZnTs carry zinc out of the cytosol.

Furthermore, the metal-binding protein MT has a specific redox-dependent function in cellular zinc metabolism. MT has two major isoforms MT-1 and MT-2 and is induced by inflammatory cytokines or lipopolysaccharide. It provides cellular zinc ions in a chemically available form and buffers zinc physiologically by cellular redistribution and compartmentalization of transiently elevated zinc-ion concentrations in the pre-steady state. Oxidative conditions lead to higher amount of available zinc, while the opposite effect is seen in reductive conditions. MT itself is induced by zinc which modulates various signaling pathways leading to phosphorylation of the zinc-binding transcription factor (MTF-1), a cellular zinc ion sensor. MTF-1 not only controls the expression of MT-1 and MT-2 but has also been suggested to repress transcription of Zip4 and Zip10.

Zinc supplementation is known to enhance the number of Treg cells and promote a shift to Th2 cytokine expression in adverse immune reactions, like mixed lymphocytes cultures (MLC) in vitro. The Th1 cytokine production, mainly IFNγ, is inhibited thereby decreasing the IFNγ/IL-10 ratio and lowering the proliferation index. Furthermore, a reduced number of Th17 cells has been noticed in vivo leading to an amelioration of the adverse immune reaction. On a molecular level, zinc is known to modulate signaling pathways causing altered immune reactions. These pathways are cell-specific and zinc-induced alterations in cellular responses or cellular differentiation are highly diverse. T cells are particularly affected by zinc as it influences the T cell receptor signaling by changing lymphocyte protein tyrosine kinase (Lck) or MAPK phosphatase activity leading to an altered MAPK signaling.

Since both sodium and zinc influence T cell differentiation and signaling cascades as MAPK signaling, there might be an interaction of sodium and zinc in the priming of T cell subpopulations regarding Th17 and Treg cells. Therefore, in this study, we investigated for the first time whether sodium and zinc have opposite effects on signaling cascades related to Treg or Th17 cell development, zinc transporter expression, and whether sodium alters the intracellular zinc level directly.

2. Materials and Methods

2.1. Reagents

Zinc sulfate (ZnSO₄) (Sigma-Aldrich, Steinheim, Germany) was dissolved in sterile water to obtain a stock solution of 100 mM, and further diluted in non-supplemented protein-free medium (Ultradoma P.F., Lonza, Switzerland) to a final concentration of 2 mM, which was used for experiments. Sodium chloride (NaCl) was purchased from (AppliChem, Darmstadt); transforming growth factor beta 1 (TGF-β1) from R&D Systems, Wiesbaden, Germany; IL-10 from Peprotech, Hamburg, Germany; N,N,N’,N’-tetraakis-(2-pyridylmethyl)ethane-1,2-diamine (TPEN) and Pyrithion from Sigma-Aldrich; nitric acid (69.0%) (HNO₃) from Sigma-Aldrich; and p-nitrophenyl phosphate from Thermo Fischer Scientific.

2.2. Human Peripheral Blood Mononuclear Cell Isolation and Generation of Mixed Lymphocyte Culture

Peripheral blood mononuclear cells (PBMC) were isolated as described before. For experimental setups, cells were adjusted to a final concentration of 2 × 10⁶ cells per mL⁻¹. For generation of two-way mixed lymphocyte cultures (MLC), 2 × 10⁶ PBMC per mL of two genetically diverse donors were pre-incubated with medium or supplemented with 50 μM zinc, 40 mM sodium, or both for 15 min followed by mixing at a 1:1 ratio in pyrogen-free 24-well dishes for indicated periods (VWR, Radnor, PA, USA). All incubation steps were carried out at 37 °C in a humidified 5% CO₂ atmosphere.

2.3. Cell Culture

Jurkat cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere and were grown in RPMI 1640, containing 10% FCS, 1% l-glutamine, 1% penicillin, and 100 U mL⁻¹ streptomycin sulfate (all from Sigma-Aldrich). RPMI is described by the company to contain 6000 mg L⁻¹ (≈ 102.67 mmol L⁻¹) NaCl (equivalent to 2360 mg L⁻¹ sodium). Finally, zinc and sodium was measured by inductively coupled plasma–mass spectrometry (ICP-MS) in the final medium of 117 ± 4 μg L⁻¹ (≈ 1.79 ± 0.06 μmol L⁻¹) zinc and 3200 ± 17 mg L⁻¹ (≈ 139.2 ± 0.7 mmol L⁻¹) sodium.

2.4. Flow Cytometry

2.4.1. Intracellular Zinc Measurement

1 × 10⁶ Jurkat cells per mL were incubated with 50 μM zinc, 40 mM sodium, or the combination of both for 48 h. Subsequently, they were loaded with 1 μM FluoZin3-AM in PBS at 37 °C for 30 min and were washed once with PBS. The maximum value was determined by using 100 mM zinc and 50 mM pyrithion and
the minimum by using 2 mM TPEN. Fluorescence was measured by flow cytometry using a FACS Calibur (BD Biosciences Heidelberg, Germany). Free zinc concentrations were calculated as described elsewhere.[25]

2.4.2. Foxp3 and RORC2 Measurement

For cell surface staining, 1 x 10^6 cells were incubated with anti-human CD4-FITC and anti-human CD25-APC antibodies (BD Biosciences) for 20 min in the dark at room temperature. For additional intracellular staining, cells were fixed and permeabilized using a fix/perm kit (BD Biosciences) according to the manufacturers’ instructions and incubated with anti-human Foxp3-PE (BD Biosciences) or anti-human/mouse related orphan receptor C2 (RORC2)/RORγT-PE (R&D Systems) antibodies, respectively. Fluorescence was detected by flow cytometry using a FACS Calibur (BD Biosciences).

2.5. Western Blotting

Western blotting was performed as described previously.[22] A total of 2 x 10^6 cells per lane was separated on 10% (H3) polyacrylamide gels. pSmad2/3, pSmad1/5/8, IRF-1, and β-Actin antibodies and reagents were purchased from Cell Signaling Technology except of Foxp3 antibody (Abcam, Cambridge, UK). Densitometric quantification was performed with Image J.

2.6. p-Nitrophenyl Phosphate Assay

A total of 2 x 10^6 cells per mL were lysed by sonication in buffer (20 mM HEPES/NaOH, 20 mM MgCl₂ [pH 7.5]). Cell lysate was preincubated with 50 µM zinc, 40 mM sodium, or both for 5 min. Samples were diluted (1:4 in dilution buffer) and distributed on 96 well-plates at 100 µL. The reaction was started by addition of p-nitrophenyl phosphate (pNPP) (1 mM) and performed at 37 °C under gentle agitation for 1 h. The reaction was stopped by addition of 100 µL NaOH (1 M). The formation of p-nitrophenolate was quantified by its absorption at 405 nm.[26]

2.7. Real-Time PCR

The total mRNA was isolated after lysis in 1 mL Trizol Reagent (Ambion, Life Technologies, Carlsbad, CA) and transcribed into cDNA using the qScript cDNA synthesis kit (Quanta Bio-science, Gaithersburg, MD, USA). Quantitative real-time PCR was performed on a Step-OnePlus real-time PCR System using Power SYBR Green (Applied Biosystems, Warrington, UK). Expression was calculated as fold of control using the ΔΔCt method.

For investigation of the expression of the zinc transporter and MT-1 (with all subtypes: MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, and MT-1X), Jurkat cells were incubated for 24 or 48 h with 50 µM zinc, 40 mM sodium, and the combination of zinc and sodium. For investigation of IFNγ and IL-17 MLC were used.

2.8. Inductively Coupled Plasma Mass Spectrometry

The total zinc and sodium content of the samples were quantified by ICP-MS (Agilent 8800 ICP-QQQ, Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Therefore, samples were ashed in 500 µL 69% HNO3 at 85 °C. The residue of the ashed pellets was re-suspended in 10% HNO3 containing 0.5 µg L⁻¹ Rh as internal standard and shaken for 3 h. For measurement the samples were diluted 1:5 and 1:500 for zinc and sodium, respectively. Helium was used as collision gas to avoid interferences. Further ICP-MS parameters are listed in Table 1. The nebulizer gas flow and parameters of lenses, Q1, Q2, and Q3 were tuned daily for maximum sensitivity (oxide ratio <1.0% (140Ce₁₆O⁺/140Ce⁺), double charged ratio <1.5% (140Ce²⁺/140Ce⁺), and background counts <0.1 cps).

2.9. Statistical Analysis

Statistical significances were calculated by one-way ANOVA with Tukey post hoc test using GraphPad Prism software (version 5.01). Statistical significances calculated by one-way ANOVA are represented by different letters.

3. Results

3.1. Antithetic Effect of Zinc and Sodium on the Number of Treg and Th17 Cells in MLC

Since zinc and sodium seem to differentially influence T cell subpopulation differentiation, Th17 as well as Treg were analyzed. Therefore, MLC were used serving as in vitro model for T cell mediated allogenic immune reactions, characterized by strong Th1 cytokine production.[27]

Zinc treatment in physiological doses led to a significant increase of CD4⁺Foxp3⁺ Treg cells (Figure 1a) and CD25⁺Foxp3⁺ Treg cells (Figure 1b), as shown before.[22,23] Administration of a physiologically relevant sodium concentration (40 mM, which induced a slight hypernatriemia with 179.2 mmol L⁻¹ sodium in the medium) reduced the number of human Tregs in comparison to the untreated control and zinc administration, as indicated for both CD4⁺Foxp3⁺ Treg cells (Figure 1a) and CD25⁺Foxp3⁺
Treg cells (Figure 1b). Moreover, the zinc-mediated induction and the sodium-mediated reduction of Tregs were attenuated when used as combined treatment. Here, the detected amount of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells was significantly higher (Figure 1a) and CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells (Figure 1b) were similar to the untreated control. Thus, the effects neutralize each other. Similar results were obtained by administering zinc and sodium using zinc chloride and sodium sulfate, respectively (Figure S1a, Supporting Information), showing that only the cations are relevant for this effect.

Additionally, the percentage of CD4<sup>+</sup>RORC2<sup>+</sup> and CD25<sup>+</sup>RORC2<sup>+</sup> T cells were analyzed. A significant increase of Th17 cells due to 40 mM sodium stimulation compared to the untreated control was measured in MLC (Figure 1c,d), which was so far observed in other models only.[9] In contrast, sole zinc supplementation slightly reduced CD4<sup>+</sup>RORC2<sup>+</sup> and CD25<sup>+</sup>RORC2<sup>+</sup> T cells (Figure 1c,d). Combined zinc and sodium treatment diminished the sodium effect on Th17 polarization, which shows for the first time that the negative effect of sodium could be antagonized by zinc. Comparable results were obtained by administering zinc and sodium using zinc chloride and sodium sulfate, respectively, (Figure S1b, Supporting Information).

Hence, sodium treatment triggers the pro-inflammatory Th17 immune reaction, whereas zinc promotes tolerance by Treg cell induction, and both counteract each other.

### 3.2. Influence of Zinc and Sodium Treatment on the Smad Signaling Pathway and Treg Induction

In literature, it is well-accepted that a direct connection between Treg cell induction and transforming growth factor (TGF)-β1 signaling exists.[28,29] Former investigations uncovered that simple TGF-β1 stimulation or zinc treatment are able to induce significantly higher amounts of Tregs in MLC compared to untreated controls. Moreover, combined zinc treatment and TGF-β1 stimulation resulted in a synergistic effect.[25] To elucidate molecular mechanisms for zinc- and sodium-mediated alterations of Th cell differentiation, Foxp3 protein expression was determined without TGF-β1 stimulation (Figure 2a,b) and with TGF-β1 stimulation (Figure 2a,c).
Figure 2. Influence of zinc and sodium on TGF-β1-induced Foxp3 expression and Smad signaling in MLC. PBMC were adjusted to 2 × 10^6 cells per mL and remained untreated (striped bars) or were pre-incubated with 50 µM zinc (white bars), 40 mM sodium (black bars), or both for 15 min (grey bars). MLC generation was performed, either left untreated (b,d–f) or followed by TGF-β1 stimulation for 5 days (c). Representative a) Western blots are shown for protein expression of the following densitometric analysis: b,c) Foxp3 (n = 12), d) pSmad 2/3 (n = 12), and e) IRF-1 (n = 8). f) Phosphatase activity was measured by pNPP Assay. Results show mean values + SEM of densitometric quantifications (b–e) or absorption of the converted phosphatase substrate at 405 nm. Statistical significances calculated by one-way ANOVA are represented by different letters.
Zinc pre-treatment of MLC resulted in a significant increase of Foxp3 expression compared to the untreated control as shown before,[25] whereas we report for the first time that sodium pre-treatment induced a decline (Figure 2a,b). However, the effect of sodium predominates when both sodium and zinc were used in combined treatment, as there is still a significant decrease of Foxp3 compared to the untreated control. These data are slightly different to the data shown in Figure 1a, since Figure 1a shows data gated on CD4+ Foxp3+ cells, whereas the Western blot in Figure 2b shows mixed PBMC.

With TGF-β1 stimulation, similar results were obtained. Expression of Foxp3 protein in MLC was significantly increased after zinc treatment and decreased after sodium treatment (Figure 2c). Combined zinc and sodium treatment reduced Foxp3 expression significantly compared to simple zinc treatment. Here, the Foxp3 expression was similar to the untreated control, leading to the assumption that zinc and sodium might interact with each other causing altered protein expression as other ions (potassium and calcium) tested increased the expression of Foxp3 protein in MLC (Figure S4, Supporting Information).

Foxp3 upregulation by zinc and a downregulation by sodium were shown; however, clear explanations for this phenomenon were not provided. Nevertheless, several mechanisms were uncovered in previous studies showing how zinc influences Foxp3 expression, for example, manipulation of the histone deacetylase Sirt-1, manipulation of the expression of several transcription factors including interferon regulatory factor 1 (IRF-1), or influencing signaling pathways like the TGF-β-dependent Smad signaling.[22,25,30] Therefore, the converse effect of sodium regarding activity and expression of the Smad signaling protein Smad 2/3 and the expression of the transcription factor IRF-1 were investigated in this study as a possible mechanism to inhibit Treg cells.

An upregulation of Smad 2/3 phosphorylation in zinc pre-treated MLC was uncovered. Moreover, phosphorylation of Smad 2/3 was significantly downregulated by sodium treatment (Figure 2d). Combined zinc and sodium treatment induced a decrease of Smad 2/3 phosphorylation compared to simple sodium treatment (Figure 2a,d).

Other molecular mechanisms described to be involved in Treg cell induction as zinc-induced down-regulation of IRF-1 (Figure 2e) or upregulation of pp38 (Figure S2, Supporting Information) were similarly regulated by sodium supplementation.

Thus, the Smad signaling pathway can be considered as one mechanism influenced by both zinc and sodium that can explain the induction or repression of Treg cells.

### 3.3. Zinc and Sodium Treatment Act Antagonistically on Phosphatase Activity

In previous studies, stimulation of T cells with zinc and the consequent increase of intracellular zinc concentrations lead to an activation of mitogen-activated protein kinase (MAPK),[31] such as higher phosphorylation of MAPK p38 and of the nuclear factor “kappa-light-chain-enhancer” of activated B cells (NF-κB) subunit p65.[32,33] Furthermore, an absence of active phosphatase leading to higher ERK1/2 phosphorylation has been described previously.[26,34] Moreover, zinc-related effects on several dephosphorylating enzymes have been described.[35,36]

In our study, a higher phosphorylation of Smad 2/3 by zinc administration and a lowered phosphorylation by sodium administration was found, leading to the presumption that phosphatases might also be involved in the alteration of the protein activity. Therefore, the phosphatase activity in human T cells was quantified by pNPP assay, as phosphatases dephosphorylate both tyrosine and threonine residues. Zinc administration led to a significant decrease of phosphatase activity, whereas only a slight decline by sodium administration compared to untreated control was found (Figure 2f). Comparing zinc and sodium treatment, zinc treatment induced a significantly lower phosphatase activity and this effect was still detectable for combined sodium and zinc administration (Figure 2f).

Hence, zinc is an effective phosphatase inhibitor leading to prolonged and increased phosphorylation of proteins involved in signaling cascades which eventually influence Treg cell development.

### 3.4. Sodium Affects the Intracellular Zinc Concentration in Human T Cells

Until now, only the divergent effects of zinc and sodium on Treg cells and Th17 cells were uncovered by investigating different molecular targets and signaling pathways. Hence, we analyzed whether zinc homeostasis is directly influenced by sodium administration.

Pre-incubation with zinc lead to a significantly higher intracellular free zinc concentration, whereas sole sodium incubation did not change the intracellular free zinc concentration (Figure 3a). Furthermore, a decrease of the free intracellular zinc concentration was shown for the first time when zinc and sodium were supplemented together compared to simple zinc supplementation. Thus, a direct interaction of zinc and sodium can be assumed. In addition, the total zinc amount was measured by ICP-MS. Here, a slightly lower zinc concentration after sodium incubation was observed (Figure 3b).

Furthermore, an increase of sodium after sole sodium treatment and a significant increase after combined zinc and sodium treatment was observed (Figure 3c).

### 3.5. Zinc and Sodium Act Contrary on the Induction of Metallothionein and the Zinc Transporter Zip10

Since we demonstrated antithetic effects of zinc and sodium administration in previous experiments, the role of sodium in zinc transporter and metallothionein (MT) mRNA expression was investigated as a possible mechanism for altered zinc homeostasis. Hereby, MT-1 protein comprises many subtypes encoded by a set of MT-1 genes (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, and MT-1X).[37]

An antagonistic effect of zinc and sodium supplementation on Zip10 mRNA expression compared to the untreated control was observed for the first time. This effect can be seen after 24 (Figure 4a) and 48 h (data not shown) incubation. The relative Zip10 mRNA expression decreased after zinc treatment versus...
Figure 3. Impact of zinc and sodium on the intracellular free zinc and sodium concentration. $1 \times 10^6$ Jurkat cells per mL were incubated for 48 h with 50 $\mu$M zinc (white bars), 40 mM sodium (black bars), both (grey bars) or left untreated as control (striped bars). a) The intracellular free zinc concentration was determined by using 1 $\mu$M FluoZin-3 AM as described in materials and methods ($n = 10$). b) The total zinc and c) the total sodium amount was analyzed by inductively coupled-plasma mass-spectrometry (ICP-MS) ($n = 13$). Results show mean values $\pm$ SEM. Statistical significances calculated by one-way ANOVA are represented by different letters.

Figure 4. Effect of zinc and sodium on the induction of metallothionein and the zinc transporter Zip10. $1 \times 10^6$ Jurkat cells per mL were incubated for 24 h with 50 $\mu$M zinc (white bars), 40 mM sodium (black bars), both (grey bars) or left untreated as control (striped bars). The mRNA expression of a) Zip10 ($n = 12$) and b) MT-1 ($n = 12$) was investigated by real-time PCR. Results show mean values $\pm$ SEM. Statistical significances calculated by one-way ANOVA are represented by different letters.

the untreated control, whereas a significant sodium-induced up-regulation was uncovered. Combined zinc and sodium treatment for 24 h (Figure 4a) alleviated the effect showing neither an up- nor downregulation of Zip10 mRNA expression compared to the untreated control (Figure 4a). The remaining zinc transporters were not contrarily regulated by zinc or sodium treatment (Figure S3, Supporting Information).

Zinc supplementation significantly increased MT-1 mRNA expression in comparison to the untreated control (Figure 4b). On the contrary, sodium treatment slightly decreased MT-1 mRNA expression (Figure 4b). The combined zinc and sodium treatment canceled each other out; thus, there was no effect compared to the untreated control (Figure 4b).

Hence, Zip10 and MT-1 mRNA expression is influenced by both zinc and sodium treatment which resulted in an altered zinc homeostasis.

4. Discussion

The balance of immune activation and suppression is well-known to be essential for a proper immune system. An
adequate immune response is necessary to defend against invading pathogens but an overactive immune system can lead to autoimmune diseases and allergies.\cite{38,39} As the incidence of autoimmune diseases in western countries has increased, the underlying patho-mechanisms and participating immune cells are the focus of interest. As Treg cells play an important role in establishing immune tolerance\cite{13} and Th17 cells activate the pro-inflammatory immune cascades,\cite{8} they are important antigens of a well-balanced immune system.

Former studies revealed the trace element zinc to enhance the number of Treg cells when applied in physiological doses and therefore inducing immune tolerance.\cite{38,40} In contrast, sodium supplementation induces the Th17-driven pro-inflammatory immune response.\cite{9,41} These findings align with our results showing a significant increase of CD4+CD25+Foxp3+ Treg cells by zinc administration and a decrease by sodium administration, whereas concerning the pro-inflammatory Th17 cells, zinc and sodium act the other way around (Figure 1). Furthermore, this study reveals a direct interaction of zinc and sodium in the priming of T cell subpopulations for the first time (Figure 3).

Our results are based on the assumption that Foxp3+ and RORC2+ T cells are two independent and mutually exclusive cell populations.

However, due to the lack of appropriate antibodies for human cells, we cannot rule out that the described cells are double positive for Foxp3 and RORC2. So far, Foxp3+ Th17 cells have been only described in rodents.\cite{39} Furthermore, these Foxp3 and RORC2 double positive cells seem to be an intermediate differentiation state, unlikely to be present in the MLC system, where zinc-induced Treg cells have a proven suppressing function.\cite{42} Lastly, we could not detect an increase in IL-17 production (data not shown) after zinc supplementation, which would be assumed if Th17 cells increase.

In literature, there are numerous described signaling pathways, transcription factors, and enzymes involved in the priming and differentiation of T cells, such as the Smad signaling pathway, MAPK signaling pathway, the transcription factors Foxp3, KLF-10, IRF-1, as well as the histone-deacetylase Sirt-1.\cite{43–46} Previous research on the Smad signaling pathway uncovered that treating PBMC and MLC with TGF-β1 induced the phosphorylation of Smad 2/3 leading to elevated Foxp3 expression. Additionally, zinc supplementation augmented TGF-β1-dependent Treg cell induction.\cite{25}

In this study, we showed for the first time that zinc and sodium antagonistically influenced the Smad signaling pathway during Treg cell and Th17 cell development. Zinc supplementation induced whereas sodium diminished Smad 2/3 phosphorylation in MLC (Figure 2d). This is in line with other findings, describing a Smad 2/3 phosphorylation via activation of certain receptors, leading to complex-formation translocating into the nucleus and modulating target gene expression such as those responsible for the induction of Treg cells.\cite{25,47} Moreover, signaling cascades might also be altered by changed phosphatase activity due to zinc and sodium supplementation (Figure 2f). Sodium application showed only minor effects on phosphatase activity, whereas zinc administration significantly reduced phosphatase activity contributing to prolonged signaling pathway activity. This is in agreement with other studies showing zinc-dependent effects on several dephosphorylating enzymes like protein tyrosine phosphatases\cite{35,48} cyclic nucleotide phosphodiesterases,\cite{49} and dual specific phosphatases.\cite{36}

An additional mechanism controlling Treg cell induction is the transcription factor IRF-1, which negatively regulates Treg cell differentiation by repressing Foxp3 expression.\cite{44} Hence, we assumed a contrary effect of sodium but our experiments showed a reduction of IRF-1 by both zinc and sodium treatment (Figure 2e). Thus, the different effects of zinc and sodium on Treg cell development seem to be IRF-1-independent.

Since zinc homeostasis and intracellular zinc distribution are highly important for cellular function and differentiation,\cite{15} the direct influence of zinc and sodium treatment on the intracellular free zinc level was investigated. In this study, a significant increase of the intracellular free zinc level in human T cells due to zinc treatment was found (Figure 3a). Interestingly, the combined sodium and zinc treatment significantly decreased the intracellular zinc level compared to sole zinc treatment. Therefore, a direct interaction of zinc and sodium on the zinc homeostasis can be assumed. On the molecular level, a proper zinc homeostasis is crucially dependent on the expression of zinc transporter importing extracellular located zinc into the cytoplasm and organelles or in the opposite direction. The zinc transporter Zip10 is located either on the outer membrane or intracellular membranes to transport zinc into the cytosol from extracellular space or from cellular compartments.\cite{16} Zip10 is expressed in T cells and in specific areas particularly in brain and liver.\cite{44} Furthermore, Zip10 plays a role in cell survival during early B cell development.\cite{50} Our data revealed a downregulation of Zip10 expression by zinc treatment (Figure 4a). This is in accordance with a previous study, showing an upregulation due to zinc deficiency, whereas zinc repletion decreases Zip10 expression.\cite{51} Therefore sodium may influence the zinc concentration by upregulating Zip10 expression as seen in our experiments for the first time (Figure 4a). Nevertheless, the role and importance of Zip10 in zinc homeostasis is not fully uncovered yet and needs further investigation.

Additionally, imported zinc is directly buffered by zinc-binding proteins as MT.\cite{52} In contrast to Zip10, the physiological role of MT in zinc homeostasis and immunity is better understood. MT is well-known to provide zinc ions by serving as a zinc buffer and is induced by zinc itself.\cite{24} Interestingly, this study uncovered a sodium-mediated reduction of MT-1 expression (Figure 4b). Hence, we revealed another possible explanation of the contrary impact of zinc and sodium on the intracellular zinc homeostasis and consequently Treg cell induction. By reducing the MT expression, sodium indirectly diminished the buffering of zinc and therefore prohibited the zinc-related modulation on the immune cells.

Our data indicate that zinc and sodium display opposite effects regarding Treg cell and Th17 cell induction in MLC resulting in a contrary effect on the immune system. They function antagonistically through the Smad signaling pathway but there are multiple mechanisms that still have to be investigated.

Furthermore, the intracellular zinc level influences cellular function and differentiation directly.\cite{15,25} In this study, we uncovered that sodium directly diminished the intracellular free zinc concentration in human T cells (Figure 3a). It is yet to be confirmed if this is relevant in a clinical setting. For instance, high sodium intake could disturb the zinc homeostasis and thereby
zinc’s subsequent protective effect on the immune system. This would be yet another negative effect of sodium in addition to inducing, for instance, the development of cardiovascular diseases, and autoimmunity diseases. Patients suffering from zinc deficiency should take this into consideration.\textsuperscript{[7,9]}

Nevertheless, these results reveal the huge impact of daily nutrition on the immune system. High salt consumption directly influences zinc homeostasis in a negative way and consequently impairs the tolerogenic immune response.\textsuperscript{[14]} In contrast, the pro-inflammatory immune response is favored which contributes to autoimmunity diseases. Thus, excessive dietary sodium intake and simultaneous low zinc intake, as commonly seen in fast food, might cause pathologically altered immune responses, which may result in the loss of healthy life years in western countries.\textsuperscript{[33]}

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

S.D. performed all experiments if not otherwise defined and wrote the manuscript; M.M. established the experimental setup and performed experiments for Figure S1, Supporting Information, and corrected the manuscript. T.S. and S.M. performed the ICP-MS measurements and corrected the manuscript. A.K.S. established the experimental setup and performed experiments for Figure S4, Supporting Information, measured IL-17, and corrected the manuscript. L.R. designed and supervised the study and corrected the manuscript.

Keywords

Foxp3, regulatory T cells, sodium, T helper 17 cells, zinc

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