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ORIGINAL CONTRIBUTION

Zinc supplementation induces CD4$^+$CD25$^+$Foxp3$^+$ antigen-specific regulatory T cells and suppresses IFN-γ production by upregulation of Foxp3 and KLF-10 and downregulation of IRF-1

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Abstract

Purpose The essential trace element zinc plays a fundamental role in immune function and regulation since its deficiency is associated with autoimmunity, allergies, and transplant rejection. Thus, we investigated the influence of zinc supplementation on the Th1-driven alloreaction in mixed lymphocyte cultures (MLC), on generation of antigen-specific T cells, and analyzed underlying molecular mechanisms.

Methods Cell proliferation and pro-inflammatory cytokine production were monitored by [^{3}H]-thymidine proliferation assay and ELISA, respectively. Analysis of surface and intracellular T cell marker was performed by flow cytometry. Western blotting and mRNA analysis were used for Foxp3, KLF-10, and IRF-1 expression.

Results Zinc supplementation on antigen-specific T cells in physiological doses (50 µM) provokes a significant amelioration of cell proliferation and pro-inflammatory cytokine production after reactivation compared to untreated controls. Zinc administration on MLC results in an increased induction and stabilization of CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$CD25$^+$CTLA-4$^+$ T cells ($p < 0.05$). The effect is based on zinc-induced upregulation of Foxp3 and KLF-10 and downregulation of IRF-1. However, in resting lymphocytes zinc increases IRF-1.

Conclusion In summary, zinc is capable of ameliorating the allogeneic immune reaction by enhancement of antigen-specific iTreg cells due to modulation of essential molecular targets: Foxp3, KLF-10, and IRF-1. Thus, zinc can be seen as an auspicious tool for inducing tolerance in adverse immune reactions.

Keywords Zinc · Regulatory T cells · Mixed lymphocyte culture (MLC) · Foxp3 · IRF-1 · KLF-10

Introduction

Induction of tolerance is critical for prevention of autoimmunity and maintenance of immune homeostasis by active suppression of inappropriate immune responses. In this regard, CD4$^+$CD25$^+$ regulatory T cells (Treg) play a major role in the maintenance of self-tolerance and immune suppression, although the mechanisms controlling Treg cell development and suppressor function remain incompletely understood. Treg cells are characterized by constitutive expression of CD25, the interleukin (IL)-2 receptor α-chain that is a component of the high-affinity IL-2R and cytotoxic T lymphocyte antigen 4 (CTLA-4). Additionally, they specifically express the forkhead/wingedhelix family transcriptional repressor Foxp3 that is essential for Treg cell development and function [1].

Treg cells actually comprise several subsets, including naturally occurring Treg cells (nTreg) arising during thymic development. nTreg cells are exported from the thymus to peripheral tissues and comprise about 5–10 % of CD4$^+$ T helper (Th) cells [2]. Furthermore, Treg cells can also be induced in vivo or in vitro after T cell receptor (TCR) stimulation, in mice [3, 4] and men [5, 6], referred to as iTreg cells. In these in vitro models, mostly antigen-specific Treg cells are generated. Studies demonstrated that autoantigen-specific Treg cells are much more potent suppressors of the induction of autoimmune disease than

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polyclonal Treg cells [7, 8]. Thus, active antigen-specific Treg cells are generated for potent suppression of the induction of organ-specific and systemic autoimmune disease [8, 9].

Despite extensive studies on the role of Foxp3 in inducing and maintaining tolerance, little information on regulation of its expression is available. Some studies show a correlation between expression of Foxp3 and other transcription factors, including the interferon regulatory factor (IRF)-1 [10], and Krüppel-like factor (KLF)-10 [11]. IRF-1 is referred to as a negative regulator of Foxp3 expression, as in vivo IRF-1 deficiency results in a selective and marked increase in highly differentiated and activated Treg cells [10]. Thus, IRF-1 seems to play a direct role in the generation and expansion of Treg cells by specifically repressing Foxp3 transcriptional activity. In contrast to IRF-1, KLF-10 is mentioned as an essential transcription factor for proper Treg cell function, because animals carrying a disruption in KLF-10 are impaired in Foxp3 activation. Furthermore, KLF-10-deficient Treg cells have impaired cell differentiation, skewed cytokine profiles with enhanced Th1, Th2, and Th17 cytokines, and a reduced capacity for suppression of effector cells [11].

Besides influences of transcription factors on Foxp3 expression and activation, we lately showed that zinc is involved in iTreg cell induction and stability by inhibition of sirtuin 1 [12]. Consistent with that, we and others noted a beneficial effect of moderate zinc supplementation on the outcome of unwanted T cell-mediated immune reactions [13–18]. Zinc inhibited the allogeneic reaction in the mixed lymphocyte culture (MLC), a model for the Th1-driven graft-versus-host disease (GVHD) [14], but also ameliorated experimental autoimmune encephalomyelitis (EAE), a commonly used animal model for the inflammatory autoimmune disorder multiple sclerosis (MS) [16, 18]. Zinc, as an essential trace element, is indispensable for proper immune function, as zinc deficiency compromises immune function and increases the risk of infectious diseases, allergies, and autoimmunity [19–21]. In particular, T cell development and T cell activation are zinc-dependent processes [22, 23] and a variety of studies have already shown a strong impact of zinc deficiency on cell-mediated immunity, causing various T cell defects [23, 24]. In this context, we investigated the capacity of zinc supplementation to modulate the allogeneic immune response of antigen-specific T cells and analyzed the molecular mechanisms responsible for the ameliorated alloreaction.

Materials and methods

Reagents

Zinc sulfate (Sigma-Aldrich, Steinheim, Germany) was dissolved in sterile water to obtain a stock solution of 100 mM and further diluted in nonsupplemented protein-free medium (Ultradoma P.F., Lonza, Switzerland) to a final concentration of 2 mM, which was used for experiments. TPEN (Sigma-Aldrich) was dissolved in sterile water to obtain a stock solution of 2 mM. Phytohemagglutinin (PHA; Becton–Dickinson, Heidelberg, Germany) was dissolved in RPMI 1640 medium supplemented with 10 % FCS to obtain a stock solution of 1 mg/ml. hIL-2 (Novartis, Neuss, Germany) was dissolved in RPMI 1640 medium supplemented with 10 % FCS to obtain a concentration of 100 U/µl.

Antibodies

Anti-human CD4 (FITC), anti-human CD25 (APC), anti-human Foxp3 (PE), anti-human CD152 (PE-Cy5), were obtained from BD Biosciences (Heidelberg, Germany).

Human PBMC isolation and generation of mixed lymphocyte culture

PBMC (peripheral blood mononuclear cells) were isolated from whole blood samples of young healthy donors by using Biocoll (Biochrom, Berlin, Germany) density centrifugation. PBMC were collected at the interface, washed twice with PBS, and resuspended in RPMI 1640 containing 10 % FCS (heat inactivated for 30 min at 56 °C), 2 mM L-glutamine, 100 U/ml potassium penicillin, and 100 U/ml streptomycin sulfate (all from Sigma-Aldrich). For experimental setups, cells were adjusted to a final concentration of 2×10^6 cells/ml. For generation of two-way mixed lymphocyte cultures, 2×10^6 PBMC/ml of two genetically diverse donors were pre-incubated with medium or supplemented with 50 µM zinc sulfate 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 % CO_2 atmosphere.

Generation of alloantigen-specific T cells

For T cell priming (first MLC), PBMC were adjusted to a concentration of 2×10^6 PBMC/ml. Priming B cells (BJAB cells) were fixed using the same volume of a 3 % paraformaldehyde solution (Sigma-Aldrich) for 3 min. After washing the B cells

three times with PBS (Sigma-Aldrich), 2×10^6 PBMC/ml and fixed BJAB were pre-incubated with or without 50 μM zinc sulfate for 15 min followed by mixing at a 5:1 ratio in pyrogen-free 24-well dishes (VWR) for 5 days. Afterward, cell culture medium was removed and cells were adjusted to 5×10^5/ml in fresh culture medium. T cell expansion was initiated by addition of 2.5 μg/ml PHA (Sigma-Aldrich) for 2 days followed by the addition of 100 U/ml hIL-2 (Novartis) for 3 days. 2×10^6/ml antigen-specific expanded T cells were used for the restimulation experiments by mixing at a 5:1 ratio with priming antigen (fixed BJAB cells), or foreign antigen (fixed Raji cells), or at a 1:1 ratio with autologous or allogeneic PBMC for 5 days. All incubation steps were carried out at 37 °C in a humidified 5 % CO_2 atmosphere.

Flow cytometry

For cell surface staining, 1×10^6 cells were incubated with respective antibodies 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 manufacturer's instructions and incubated with Foxp3 or CTLA-4 antibodies. Fluorescence was detected by flow cytometry, using a FACS Calibur.

Western blotting

A total of 2×10^6 cells were collected by centrifugation, lysed by sonication in 100 μl sample buffer (62.5 mM Tris–HCl [pH 6.8], 2 % [w/v] SDS, 27 % [v/v] glycerol, 0.1 % [v/v] 2-ME, 0.01 % [w/v] bromo-phenol blue, 1 mM Na_3VO_4), and heated for 3 min at 95 °C. An equivalent of 4×10^5 cells/lane were separated on 10 % (H3) polyacrylamide gels at 160 V and blotted to nitrocellulose membranes. Uniform loading of gels was confirmed by staining with Ponceau S. After destaining, membranes were blocked for 1 h with TBST (20 mM Tris–HCl [pH 7.6], 136 mM NaCl, 0.1 % [v/v] Tween 20) containing 5 % fat-free dry milk. Subsequently, primary antibodies were incubated overnight (dilution 1/1000 in TBST containing 5 % BSA). Membranes were washed and incubated for 1 h with goat anti-rabbit HRP or horse anti-mouse HRP followed by detection with LumiGlo reagent on a LAS-3000 (Fujifilm Lifescience, Düsseldorf, Germany). IRF-1 and β-actin antibodies and reagents were purchased from Cell Signaling Technology except of anti-Foxp3 (Abcam, Cambridge, UK). Densitometric quantification was performed with Image J.

Real-time PCR

The mRNA of 4×10^6 cells was isolated after lysis in 1 ml Tri Reagent (Ambion, Life Technologies, Carlsbad, CA) and transcribed into cDNA with the qScript cDNA Synthesis Kit (Quanta Biosciences, Darmstadt, Germany) according to the manufacturers' instructions. Quantitative real-time PCR was performed on a Step-OnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with the following oligonucleotide sequences.

PBGD: 5′-ACGATCCCGAGACTCTGCTTC-3′ (forward) and 5′-GCACGGCTACTGGCACACT-3′ (reverse), T-bet: 5′-AGGGACGGCGGATGTTCCCA-3′ (forward) and 5′-GCTGCCCTCGGCCTTTCCAC-3′ (reverse), GATA-3: 5′-GCCCGGTCCAGCACAGAAGG-3′ (forward) and 5′-TGAGGGGCCGGTTCTGTCCG-3′ (reverse), RORC2: 5′-CAGTCATGAGAACACAAATTGAAGTG-3′ (forward) and 5′-CAGGTGATAACCCCGTAGTGGAT-3′, Foxp3: 5′-CACCTGGCTGGGAAAATGG-3′ (forward) and 5′-GGAGCCCTTGTCGGATGAT-3′, IRF-1: 5′-GTACCGGATGCTTCCACCTC-3′ (forward) and 5′-GGAATCCCCACATGACTTCCT-3′ (reverse), KLF-10: 5′-AAGGAGTCACATCTGTAGCC-3′ (forward) and 5′-TCCAGCTACAGCTGAAAGGC-3′.

The components of each PCR sample (final 20 μl) were 6 μl dH_2O, 10 μl SYBR Select PCR Master Mix (Applied Biosystems), 1 μl forward and reverse primer each (4 μM), and 2 μl of the respective cDNA (50 ng/ml) or dH_2O as a negative control. The real-time PCR was performed in duplicate with the following parameters for all oligonucleotides: 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 56 °C for 30 s. For quantification, the comparative cycle threshold method ($\Delta\Delta CT$) was used, normalizing the results to the housekeeping gene PBGD.

IFN-γ quantification

Supernatants were harvested, stored at −20 °C until measurement, and only thawed once for cytokine detection. hIFN-γ protein concentration was quantified using OptEIA assays from BD PharMingen (Heidelberg, Germany), according to the manufacturer's instructions.

## [³H]-thymidine proliferation assay

Cells were incubated at a concentration of 2×10^6/ml in 200 μl/well in a pyrogen-free 96-well dish (VWR). The proliferation assay was performed in triplicate. 18.5 kBq/well [³H]-thymidine (GE Healthcare, Germany) was added for the last 16 h of incubation before harvesting. The amount of incorporated [³H]-thymidine was assessed by a liquid scintillation β-counter (LBK Wallace).

Statistical analysis

Statistical significance was calculated by Student's *t* test (Figs. 1, 2, 3, 4) and Wilcoxon signed-rank test (Fig. 5) using GraphPad Prism software (version 5.01).

Fig. 1 Induction of Treg cells via zinc supplementation in MLC. PBMC were adjusted to 2×10^6 cells/ml and remained untreated (*white bars*) or were pre-incubated with 50 μM zinc (*black bars*) for 15 min prior to MLC generation. After 5 days of MLC incubation, the percentage of Treg cells was analyzed by flow cytometry. Gating was performed on activated CD4+SSC^{hi} blasts. The percentage of **a** CD4+CD25^{high} ($n = 8$), **b** CD4+Foxp3+ ($n = 7$), **c** CD4+sCTLA-4+ (surface) ($n = 8$), **d** CD4+iCTLA-4+ (intracellular) ($n = 7$) cells is shown. *Significance of $p < 0.05$, **significance of $p < 0.01$ (Student's *t* test). Mean values + SEM are shown
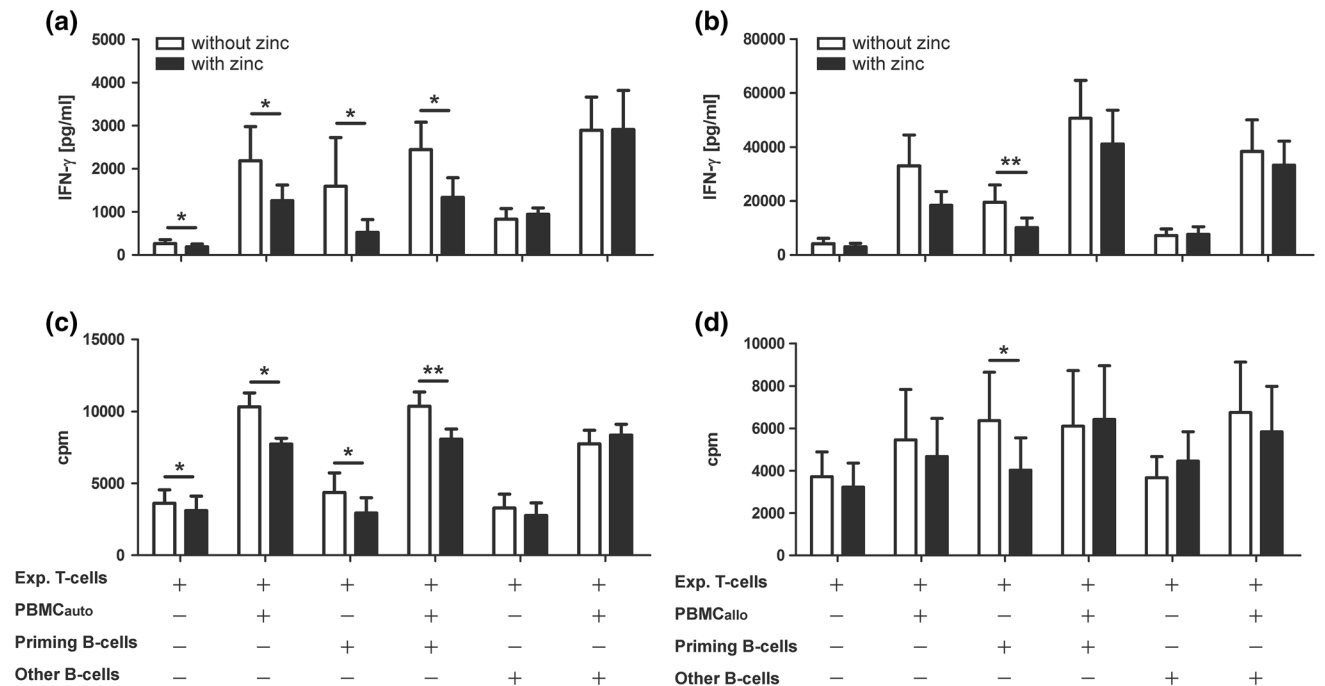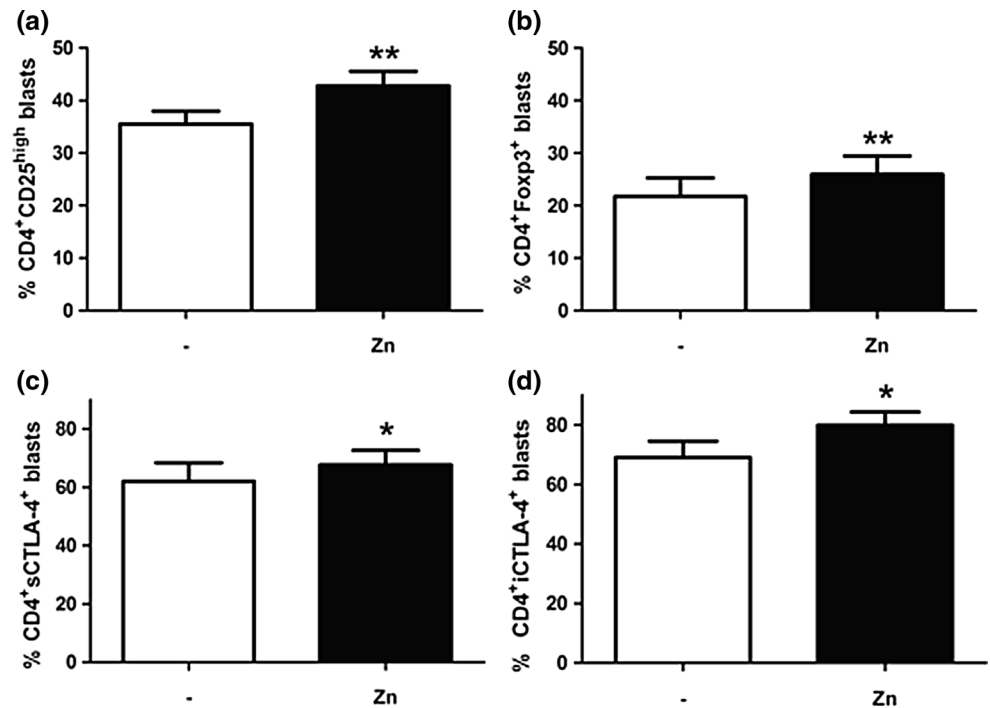

Fig. 2 Zinc supply provokes downregulated reaction of antigen-specific T cells after reactivation. Priming and expansion of antigen-specific T cells as well as T cell restimulation was performed as described in "Materials and methods" section. Untreated controls are shown as *white bars*; zinc supplementation (50 μM) is shown as *black bars*. **a**, **b** IFN-γ cytokine production was measured by ELISA. **c**, **d** Cell proliferation was determined by [³H]-thymidine assay. **a**, **c** Restimulation experiments with autologous PBMC (PBMC$_{auto}$) were performed. **b**, **d** Cross-reactivity experiments were performed by using allogeneic PBMC (PBMC$_{allo}$). Results are presented as mean values + SEM of $n = 6$ (**a**, **c**), $n = 8$ (**b**, **d**) independent experiments. *Significance of $p < 0.05$, **significance of $p < 0.01$ (Student's *t* test)
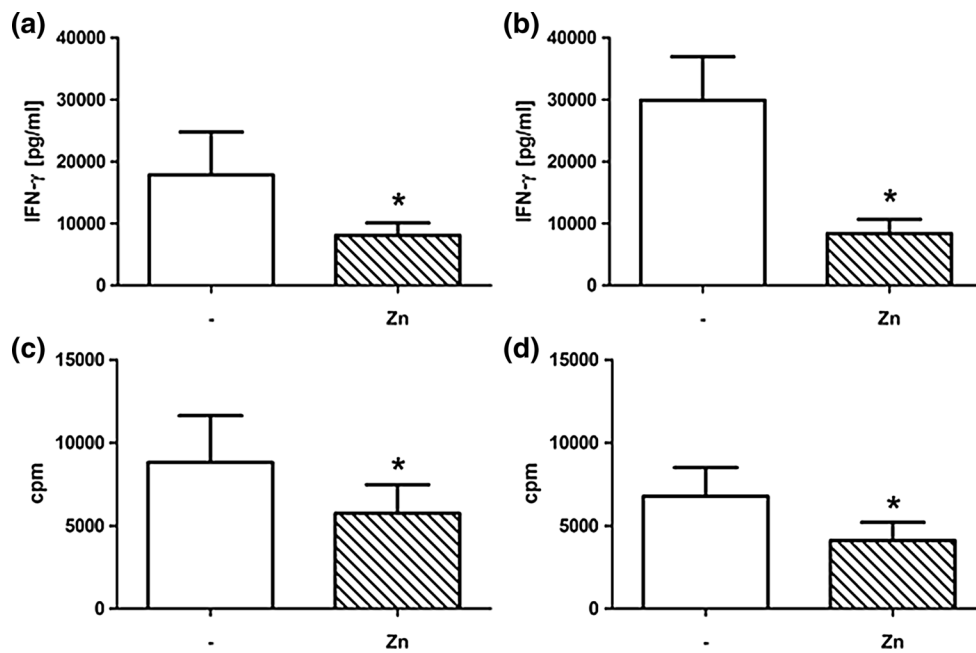
Fig. 3 Diminished allogeneic reaction due to zinc supply after T cell activation. Priming and expansion of antigen-specific T cells as well as T cell restimulation was performed as described in "Materials and methods" section. Zinc supplementation (50 μM, *striped bars*) was not performed until restimulation (second MLC). **a, b** IFN-γ cytokine production was measured by ELISA. **c, d** Cell proliferation was determined by [3H]-thymidine assay. **a, c** Restimulation of expanded T cells with priming antigen. **b, d** Restimulation of expanded T cells with priming antigen and autologous PBMC. Results are represented as mean values + SEM of at least $n = 6$ independent experiments. *Significance of $p < 0.05$ (Student's *t* test)
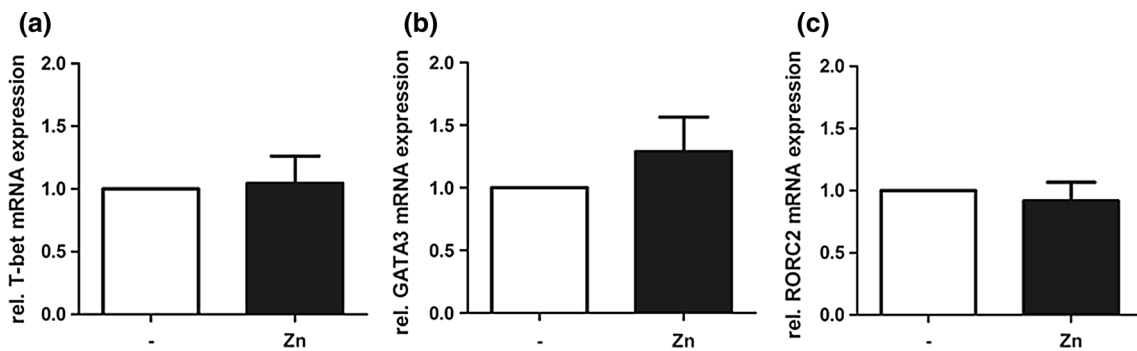
Fig. 4 Th1-, Th2-, and Th17-specific transcription factor expression is not influenced by zinc supplementation. 2×10^6 PBMC/ml remained untreated (*white bars*) or were pre-incubated with 50 μM zinc (*black bars*) for 15 min prior to MLC generation. After 5 days of MLC incubation, the mRNA expression of **a** T-bet for Th1, **b** GATA-3 for Th2, and **c** RORC2 for Th17 was measured. Results are normalized to the untreated control and represent mean values + SEM of at least $n = 6$ independent experiments

Results

Zinc alleviates the allogeneic immune reaction by induction of regulatory T cells

Co-cultivation of peripheral blood mononuclear cells (PBMC) of genetically diverse individuals is a well-known model to assess allogeneic immune reactions, also known as mixed lymphocyte culture (MLC). Alloreactive T cells, with Th1 cytokine production [interferon (IFN)-γ] or cell proliferation, are used to reflect its severity.

We confirmed the results of a previous study [12] and showed that zinc supplementation reduced the severity of MLC reaction compared to nonsupplemented controls, via stabilization of regulatory T cells (Treg) in MLC after 5 days of incubation (Fig. 1). Zinc supplementation significantly increases surface marker expression of Treg cells (Fig. 1a, c), and the intracellular Treg-specific transcription
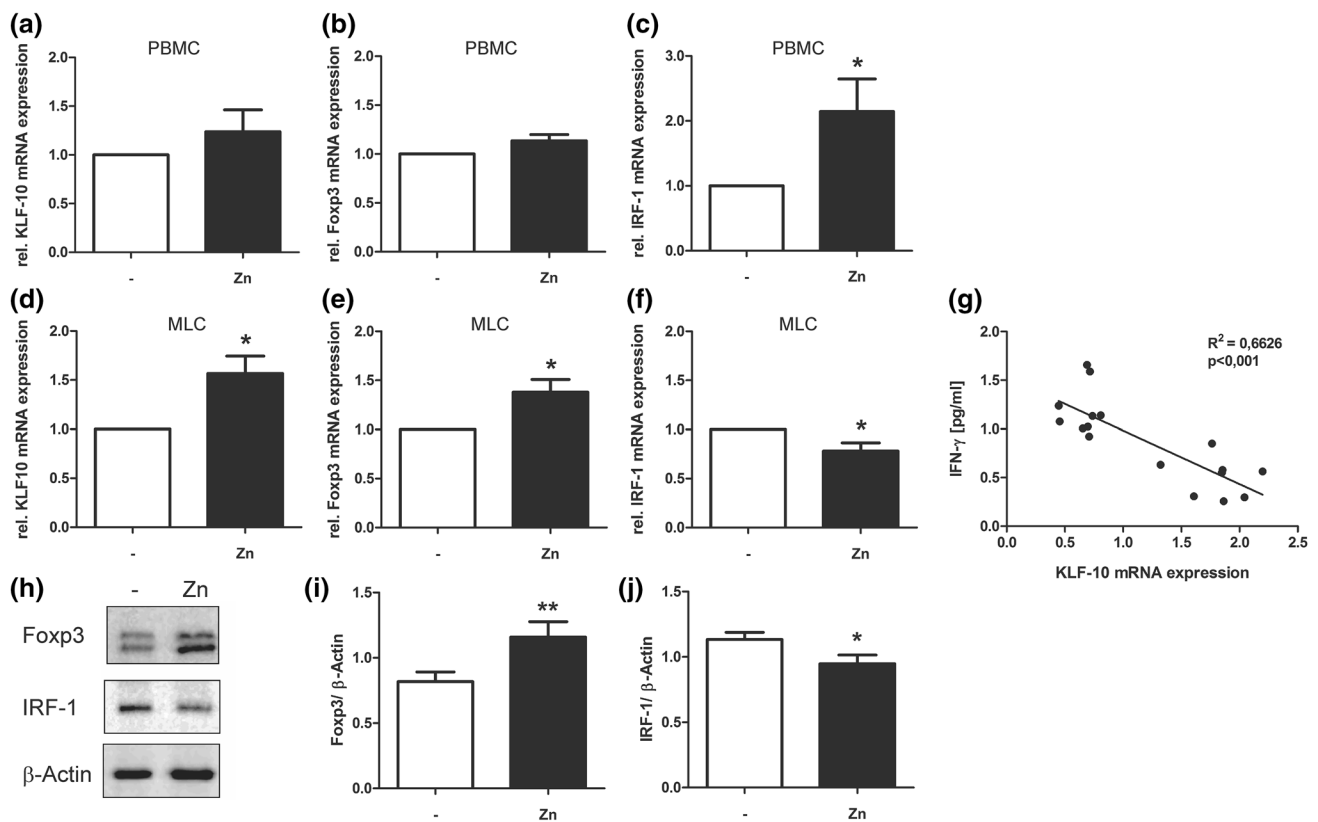
Fig. 5 Mechanism of iTreg cell induction and stabilization by zinc supplementation in MLC. 2×10^6 PBMC/ml were pre-incubated with 50 μM zinc (*black bars*) or remained untreated (*white bars*) for 15 min prior to MLC generation for 5 days. **a–f** mRNA expression of KLF-10, Foxp3, and IRF-1 was measured in **a–c** PBMC and **d–f** MLC. Results are normalized to the untreated control and represented as mean values + SEM of at least $n = 5$ independent experiments. **g** Correlation of KLF-10 mRNA expression and IFN-γ cytokine secretion was performed after 5 days of MLC ($n = 17$). **h–j** Foxp3 and IRF-1 protein was measured by Western blotting after 5 days of un-supplemented (*white bars*) or zinc-supplemented (50 μM) (*black bars*) MLC. Results show mean values + SEM of **i, j** densitometric quantifications and **h** one representative experiment out of $n = 13$ independent experiments. *Significance of $p < 0.05$, **significance of $p < 0.01$ (Wilcoxon signed-rank test)

factor Foxp3 (Fig. 1b) and CTLA-4 (Fig. 1d). These results confirm former findings showing a zinc-induced enrichment of iTreg cells during the allogeneic immune response.

Zinc-supplemented antigen-specific T cells show diminished reaction during reactivation

Since zinc supplementation provoked a beneficial effect on the severity of MLC alloreaction due to induction of Treg cells, we now investigated the potential zinc effect on generating antigen-specific T cells. Therefore, we analyzed and compared IFN-γ cytokine production (Fig. 2a, b) as well as cell proliferation (Fig. 2c, d) of primed and expanded T cells with or without zinc treatment.

For priming, T cells were cultivated with or without zinc supplementation over a period of 5 days in one-way MLC using fixed BJAB cells as priming antigen. Afterward, T cells were expanded over 5 days and restimulated in a second one-way MLC by using priming B cells or genetically different B cells (Raji cells). To test potential cross-reactivity of expanded T cells, we used both autologous and allogeneic PBMC during restimulation (Fig. 2b, d).

Antigen-specific T cells subjected to zinc treatment showed less IFN-γ cytokine production and less cell proliferation compared to untreated T cells. In addition, reactivation of zinc-supplemented T cells by priming B cells showed a far lower responsiveness compared to expanded and restimulated T cells from control expansions. This effect was seen either if restimulation was performed solely with priming antigen or in combination with autologous PBMC. However, T cells derived either from zinc-supplemented or control expansion showed no difference in cytokine production and proliferation when restimulated with genetically different antigen, as well as in combination with autologous PBMC (Fig. 2a, c). Subsequently, we elucidated whether this observed zinc effect can be reproduced by foreign PBMC suggesting cross-reactivity of expanded T cells. Therefore, we expanded T cells from

zinc-treated and control MLC and performed re-incubation as shown in Fig. 2a, c. Restimulation of expanded T cells with the priming antigen again showed a significantly reduced proliferation and IFN-γ secretion when comparing zinc-supplemented and control samples. Instead, no difference between zinc-treated and control samples could be observed when experiments were done with allogeneic PBMC (Fig. 2b, d).

These results indicate an ameliorating zinc effect on reactivated antigen-specific T cells, whereas no effect was detectable in cross-reactivity experiments. Upregulation of iTreg cell populations upon zinc supply as seen in 5-day MLC (see Fig. 1) could constitute a possible reason for these effects, as we saw similar outcomes for FACS analysis of antigen-specific T cells while reactivation (data not shown). This leads to the presumption that zinc supplementation increases the stability of antigen-specific iTreg cells, developed from activated and expanded T effector cells, but allows immune responses against neoantigens.

Zinc supplementation diminishes allogeneic reaction even after T cell activation

Next, we investigated whether it is possible to modulate the allogeneic T cell response after a first reaction due to exposure to a priming antigen had occurred already. Accordingly, we performed the experimental setup as used in "Materials and methods" section, but without zinc administration during T cell priming or expansion. Instead, zinc supplementation was performed for the first time 15 min before restimulation (second MLC) with the same antigen used for priming. Figure 3a, b shows reduced IFN-γ production due to zinc treatment after reactivation of expanded T cells with priming antigen or a combination of priming antigen and autologous PBMC. Furthermore, cell proliferation in both setups was decreased (Fig. 3c, d).

These results indicate a positive modulation of the allogeneic T cell reaction by zinc supplementation, namely reduction of pro-inflammatory cytokine production, and cell proliferation, even if the allogeneic immune response to an antigen had occurred already.

Zinc does not modulate Th1, Th2, and Th17 cells in MLC

As we saw in the previous experiments, zinc administration reduces cell proliferation, IFN-γ cytokine expression and induces iTreg cells in the MLC, resulting in an amelioration of the alloreaction. Zinc supplementation is known to influence the Th1-mediated immune reaction, whereas the Th2-mediated immune reaction remains unaffected [25–27]. Whether zinc administration in the MLC specifically modulates Treg cells or also acts on other T cell populations, like Th1, Th2 or Th17 cells, remains to be elucidated. Therefore, we analyzed Th1 cell-specific (T-bet) and Th2 cell-specific (GATA3) transcription factors (Fig. 4a, b). Inasmuch as Th17 cells are described as the direct counterpart of Treg cells, we furthermore analyzed RORC2 expression (Fig. 4c). As shown in Fig. 4, we observed no significant difference in any T cell-specific transcription factor mRNA expression, leading to the conclusion that zinc supplementation during MLC selectively affects Treg cell induction and stability.

Zinc supplementation induces iTreg cell stabilization in MLC by modulation of KLF-10 and IRF-1

To uncover the molecular mechanism behind induction and stabilization of Treg cells in MLC by zinc administration, we analyzed Foxp3, KLF-10, and IRF-1 mRNA expression in zinc-supplemented samples and untreated controls after 5 days of MLC (Fig. 5b, d–g). The same treatment was applied to the respective PBMC (Fig. 5a–c). Additionally, protein expression of Foxp3 and IRF-1 was determined in MLC after 5 days of incubation (Fig. 5h–j).

Zinc supplementation did not influence mRNA expression of KLF-10 and Foxp3 in PBMC after 5 days of incubation compared to nonsupplemented controls, whereas a significant upregulation due to zinc supply was observed in MLC. In contrast to that, IRF-1 mRNA expression was significantly downregulated in zinc-supplemented MLC, whereas a significant increase was found in PBMC. Moreover, a correlation of Treg cell induction and pro-inflammatory cytokine production was done (Fig. 5g). We found an existing negative correlation between KLF-10 mRNA expression and IFN-γ secretion: The higher the KLF-10 mRNA expression was, the lower was the IFN-γ secretion in MLC. Besides the rising Foxp3 mRNA expression levels in zinc-supplemented MLC, also the protein amount was significantly increased in zinc-treated MLC compared to controls (Fig. 3i). In addition, IRF-1 protein expression was similarly reduced like IRF-1 mRNA expression (Fig. 3j). Moreover, zinc deficiency had no impact on IRF-1 or Foxp3 expression (data not shown).

Hence, we showed that zinc administration acts beneficial on iTreg cell modulation via upregulation of KLF-10 and Foxp3, and inhibition of IRF-1.

Discussion

The essentiality of zinc for appropriate immune function is well known since a variety of cellular functions are regulated by zinc [24, 28, 29]. Depending on cell type and applied zinc dose, either immune suppression or activation can be observed [24, 30, 31]. Particularly, T cell

development and T cell activation are zinc-dependent processes [22, 23], as zinc deficiency results in various T cell defects manifested in thymus atrophy and lymphopenia [32]. Since zinc is essential for T cell functionality, one aim of our study was to investigate the influence of zinc supplementation on the Th1-driven alloreaction in MLC. Zinc supplementation in physiological doses lead to an ameliorating effect by stabilization of iTreg cells in MLC after 5 days of incubation. This confirmed our previous data [12] and is in line with our former finding showing a suppressed IFN-γ production in MLC due to zinc supply [14, 33]. Cytokine concentrations, like IFN-γ, are sensitive parameters for possible graft rejection in MLC [34, 35], thus correlating with the severity of the acute GVHD [36]. Therefore, it can be assumed that zinc has the ability to downregulate allogeneic immune reactions due to stabilization of induced Treg cells.

Secondly, we thought to analyze whether there is an influence of zinc supplementation on the generation of antigen-specific T cells. The comparison of zinc-supplemented versus untreated antigen-specific T cells originated from T cell expansion showed an ameliorated pro-inflammatory cytokine production as well as cell proliferation. These parameters were significantly reduced after challenging antigen-specific T cells for a second time with the same antigen used during priming, whereas equal proliferation and IFN-γ production were observed by challenging with another antigen. In line with that, Faber et al. showed a zinc-induced reduction of IFN-γ production in allogeneic activated conditions without affecting tetanus toxoid-triggered immune responses [14]. Other studies found beneficial effects of zinc supplementation in allogeneic transplantation models. In a rodent cardiac transplantation model, allograft rejection was reduced by zinc in a dose-dependent manner [13, 37], and the maintenances of functional grafts in intraportal-islet-transplanted rat recipients were significantly increased due to zinc supply [38]. The authors either suggested an inhibited allograft-cell apoptosis or reduced pro-inflammatory cytokine production as possible reason. Thus, zinc seems to positively affect adverse reactions, supporting our findings of diminished IFN-γ production due to zinc supplementation in antigen-specific expanded T cells. As we could see in MLC experiments, zinc administration results in a stabilization of iTreg cells. In antigen-specific expanded T cells, the same mechanism is involved in ameliorating the immune response. Zinc signals are known to upregulate phosphorylation of MAP kinase Akt and inhibit PTEN. A modulation of this pathway occurs upstream of Akt but downstream of Jak1, because Stat5 signaling is not influenced by zinc [39]. Furthermore, zinc induces p38 MAP kinase phosphorylation [40], which is also highly increased in Treg cells [41]. IL-2 is essential for the generation of Treg cells and for stable Foxp3 expression [42, 43].

Zinc signals are shown to promote IL-2-induced proliferation, indicating that zinc is indispensable for IL-2-dependent proliferation of T cells [31]. These observations lead to the assumption that besides Treg cell survival also MAP kinase signaling might be essential for the peripheral generation of iTreg cells, wherein zinc-dependent mechanisms play an important role.

It is known that iTreg cells can prolong the survival of heart allografts without any additional immunosuppression in a mice model using completely MHC-mismatched animals [44]. Moreover, antigen-specific or rather organ-specific Treg cells from TCR transgenic mice seem to be more efficient at preventing autoimmunity than a polyclonal population of Treg cells with unknown antigen specificities [7, 45]. In this respect, zinc alone seems to attenuate reactivity of antigen-specific T cell due to antigen challenge conditioned by iTreg cell stabilization.

Due to this positive zinc effect, we investigated whether it is possible to modulate the allogeneic T cell response via zinc supplementation after first cell activation by stimulation with a foreign antigen had occurred already. Surprisingly, the ameliorating zinc effect on expanded T cells remained, even if T cell priming and expansion occurred without zinc treatment. Zinc supplementation occurred for the first time 11 days after priming: during reactivation of expanded T cells. This still diminished cell proliferation as well as pro-inflammatory cytokine production, but enabled unchanged reaction to foreign antigens, thus maintaining the antigenic potency of the host. This is of main importance as many studies only see beneficial effects of zinc supplementation if administered pre- or simultaneously to disease induction [12, 16–18] or allogeneic transplantation [13, 37, 38]. In contrast, we showed a beneficial zinc effect in the allogeneic immune reaction when zinc treatment started about 2 weeks after induction, disclosing novel treatment options in already triggered adverse immune reactions. As zinc is able to stabilize iTreg cells in MLC, zinc supplementation also results in tolerance induction in antigen-specific expanded T cells, indicated by blunted pro-inflammatory cytokine production and cell proliferation. Thus, zinc represents a low-cost stimulant for Treg cell expansion in vitro, which plays a fundamental role in today's transplantation models.

Of major interest was furthermore the identification of molecular mechanisms responsible for induction and stabilization of iTreg cells in MLC in regard to zinc supplementation. Zinc is known to be indispensable for proper polarization of mature T cells as zinc deficiency results in a disturbed Th1/Th2 ratio leading to unbalanced cell-mediated immune responses [25, 26]. Hence, we analyzed Th1 cell-specific (T-bet), Th2 cell-specific (GATA3), Th17 cell-specific (RORC2), and Treg cell-specific (Foxp3) transcription factor expression in allogeneic MLC reaction. We

found no significant difference in Th1, Th2, or Th17 cell-specific transcription factor mRNA expression, leading to the conclusion that zinc supplementation during MLC selectively affects the population of Treg cells.

Besides Foxp3 expression, other transcription factors are described to influence Treg cell development and functionality. Some studies show a correlation between Foxp3 expression and IRF-1 [10], and KLF-10 [11]. IRF-1 is expressed at low basal levels by most types of resting cells, including T cells [46], but accumulates in response to several stimuli and cytokines including IFN-γ, the strongest IRF-1 inducer. We found a significant increase in IRF-1 mRNA expression in PBMC stimulated with zinc. This is in line with former investigation of showing a significant increase of IFN-γ due to zinc supplementation in vitro [47] and in vivo [48]. In contrast to that, we observed a significant reduction of IRF-1 mRNA in MLC due to zinc supplementation. This perfectly matches with the significantly decreased IFN-γ production as well as significantly increased Foxp3 mRNA and protein expression in MLC, as in vivo IRF-1 deficiency resulted in a selective and marked increase in highly differentiated and activated Foxp3+ Treg cells [10].

On the molecular level, IRF-1 plays a direct role in the generation and expansion of Treg cells specifically repressing Foxp3 transcriptional activity. Thus, we could show for the first time that IRF-1 mRNA and protein expression can be reduced by zinc application in MLC. In contrast to IRF-1, KLF-10 is mentioned as an essential transcription factor for proper Treg cell function, because animals carrying a disruption in KLF-10 no longer show Foxp3 activation. Furthermore, KLF-10-deficient Treg cells have impaired cell differentiation, skewed cytokine profiles with enhanced Th1, Th2, and Th17 cytokines, and a reduced capacity for suppression by wild-type co-cultured T cell effector cells, and accelerated atherosclerosis in immunodeficient, atherosclerotic mice [11]. In our MLC studies, we found an increased expression of KLF-10 due to zinc administration that furthermore negatively correlated with the IFN-γ cytokine production. Hence, we uncovered novel molecular targets influenced by zinc supplementation, as induction of KLF-10, stabilization of Foxp3, and suppression of IRF-1 expression. Thus, zinc stabilizes iTreg cells in MLC, leading to a diminished IFN-γ cytokine production and consequently to amelioration of the allogeneic immune reaction.

Zinc supplementation turns out to be a promising treatment strategy for antigen-specific Treg cell stabilization, by influencing molecular target gene expression of Foxp3, IRF-1, and KLF-10. Moreover, zinc ameliorates cell proliferation and pro-inflammatory cytokine expression in Th1-driven allogeneic immune reaction. Treg cell induction and stabilization play a fundamental role in adverse immune reactions and today's transplantation models. Our findings support zinc as an easy and cheap therapeutic agent showing great promise for clinical treatment of T cell-induced disorders, such as allergies, autoimmune diseases, as well as organ transplantations, which has to be further validated an analyzed in in vivo studies.

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