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Evelyn Fast , Jana Jakobs , Jens Bertram , Inga Weßels , Judith Sailer , Bernhard Michalke , Lothar Rink

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HIGHLIGHTS

- Zn and $Fe^{2+}$ lower IFN-· production in MLC, but not $Fe^{3+}$
- Iron de□ciency decreased IFN-· production, but not zinc deficiency
- Iron mediated effects are due to electron donation ($Fe^{2+}$) and electron acceptance ($Fe^{3+}$)
- Iron effects are equal in different lymphocyte stimulation models
- Zinc effects differ in MLC and PHA-stimulation of lymphocytes

Title:

Zinc, $Fe^{2+}$ and $Fe^{3+}$ differentially influence IFN-γ production in human peripheral blood mononuclear cells

Author names and affiliations:

Evelyn Fast[a], Jana Jakobs[a], Jens Bertram[b] Inga Weßels[c], Judith Sailer[d] , Bernhard Michalke[e] and Lothar Rink[a]*

[a] Institute of Immunology, Medical Faculty, RWTH Aachen, Pauwelsstraße 30, 52074 Aachen, Germany

[b] Institute of Occupational, Social and Environmental Medicine, RWTH Aachen, Pauwelsstraße 30, 52074 Aachen, Germany

[c] Center of Allergy & Environment (ZAUM), Technical University of Munich/Helmholtz Munich, German Research Center for Environmental Health, Munich, Germany

[d] Institute of Toxicology and Environmental Hygiene, TUM School of Medicine and Health, Technical University Munich (TUM), Munich, Germany

[e] Independent Researcher

* Corresponding author: Phone: +49 (0)241 8080208 Fax: +49 (0)241 8082613

E-mail: Lothar.Rink@RWTH-Aachen.de

Running Title: $Zn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ differentially influence IFN-γ

## ABSTRACT:

Background: Iron overload is a common phenomenon in patients undergoing transfusions or organ transplantation. Clinical studies indicate that iron overload interferes with immune function. Baseless supplementation of iron leads to higher morbidity and mortality. In iron overload T-cell differentiation is skewed towards a Th2 response, with lower levels of interferon (IFN)-γ. Zinc is known for its immune balancing abilities, e.g. by induction of regulatory T cells. This study aims to investigate the interaction of iron and zinc in mixed lymphocyte cultures (MLC).

Methods: MLC, peripheral blood mononuclear cells (PBMC) stimulation with phytohemagglutinin, ELISA, PCR, ICP-MS

Results: $Zn^{2+}$ supplementation leads to a significantly lower IFN-γ production in MLC compared with control ($p<0.03$). $Fe^{2+}$ supplementation lowers the IFN-γ production in MLC ($P<0.0017$), too. However, $Fe^{3+}$ has a slightly increasing effect on IFN-γ release which differs significantly from $Fe^{2+}$ ($p<0.03$). In 2,2-Bipyridyl-induced iron deficiency IFN-γ production is lowered ($p<0.0003$ ), whereas zinc deficiency does not significantly affect IFN-γ production. Examinations of Interleukin (IL)-2 and IL-6 show comparable tendencies. The $Fe^{2+}$ effect can be imitated by sodium sulfite. $Fe^{3+}$ treatment increases intracellular free iron in PBMC significantly compared to $Fe^{2+}$ treatment ($p<0.02$).

Conclusion: Iron (II) and zinc both suppress cytokine production in MLC. $Fe^{3+}$ shows a significantly different effect on IFN-γ production. The underlying mechanism is likely a donation of electrons by $Fe^{2+}$ or oxidative stress. These findings provide mechanistic insights on how the oxidation state of iron differentially modulates human immune cell function and highlights the importance of iron speciation in nutritional immunology.



## KEYWORDS:

zinc, iron, immunology, cytokine production, mixed lymphocyte culture

## 1.INTRODUCTION:

Iron is an essential transition metal in the human body, and it plays important roles for DNA-synthesis, respiratory chain and hematopoiesis(1).
The majority of plasma iron is bound to transferrin, forming a complex that is nontoxic and well soluble under physiological conditions. Since the iron-binding capacity of transferrin is limited, iron excess leads to non-transferrin-bound iron (NTBI) formation in the plasma. Iron not being bound to transferrin facilitates its cellular uptake and will subsequently lead to deposition of iron in parenchymal cells of various organs. Consequences of parenchymal iron accumulation are organ dysfunction and tissue damage (2).

Iron overload is a common phenomenon in patients that have received multiple transfusions but is also found in hereditary iron metabolism disorders and neoplastic diseases (2).
In patients undergoing organ transplantation iron overload often occurs due to multiple transfusions and underlying diseases. Especially in the case of liver transplantation, underlying diseases such as liver cirrhosis or hemochromatosis go along with iron overload. Therefore many patients already enter the transplantation process with disturbances in their iron metabolism (3).

Being at the intersection between the risk of an immunological transplant rejection and an increased risk of infection, organ transplantation means facing challenges in immune balancing.
Clinical studies indicate that iron overload interferes with the physiological function of the immune system (4). It has been seen that a baseless supplementation of iron leads to a higher morbidity and mortality due to infectious diseases (5). Therefore, it can be assumed that excess iron in the body leads to a dysbalance of the immune system. Clinical studies have shown that iron overloaded patients undergoing solid organ transplantation show an increased risk of developing *Staphylococcus aureus* bacteriemia (6) and a decrease in 5-year survival rate with sepsis identified as a leading cause of increased mortality (7). Not only solid organ transplantations but also in hematopoietic stem cell transplantation, iron overload is a common complication going along with adverse effects. Elevated pretransplant ferritin levels are associated with an increased risk of bloodstream infections and acute graft versus host disease (GVHD) (8).
With regard to organ and hematopoietic cell transplantation, examination of the immune function in iron overload could explain tendencies of developing infection and help to discover means of prevention.

Zinc is an essential trace element that is well known for its ability to act balancing on the immune system. The underlying mechanisms are various. Zinc induces regulatory T cells and therefore improves immune balance without suppression. (9) Production of proinflammatory T helper (Th) 17-cells, playing a role in the pathogenesis of autoimmune diseases, is suppressed by zinc (10). In mixed lymphocyte cultures zinc has been shown to dampen the allogenic proinflammatory reaction. On the

other hand, zinc acts differently on resting T cells, improving their reactive capacity by enhancement of interferon (IFN)-$\gamma$ and interleukin (IL)-2 production (11).

Especially in the aged and hospitalized population high prevalence of both, zinc and iron deficiency, occur. While zinc deficiency, showing mild and unspecific symptoms, is often overlooked, iron deficiency is frequently detected in routine blood examinations. Therefore, iron is one of the most popular nutritional supplements worldwide (12).
Clinical studies show that zinc supplementation in a variety of pathological conditions as well as on healthy subjects will lead to positive immunological effects (13) while it is shown that baseless supplementation of iron leads to unfavorable outcomes (5). Therefore, with regard to the literature, an antagonistic effect of zinc and iron treatment can be assumed, due to this opposite effects of zinc and iron. By now it is not fully explained which underlying effects cause the immunological dysregulation seen in iron overload. Still, zinc as an immune balancing agent could be potentially counteracting immune dysregulation in iron excess. Therefore, an examination of zinc and iron interaction on the immune function is necessary.
Previous studies have already shown that zinc and sodium show antagonistic effects on the immune function that are neutralized in a combined supplementation (14). Thus, the question arises if zinc and iron supplementation show interactions as well.
The interaction of zinc and iron in cell cultures has mostly been studied in the context of cellular uptake. Previous studies on intestinal cells, such as Caco-2-cells indicate that zinc can enhance iron influx by increasing the expression of DMT-1 (15). The interaction of iron and zinc in immune cell cultures has still to be investigated.

The aim of this study was to investigate the interaction of iron and zinc in mixed lymphocyte cultures and other T-cell stimulation models.



## 2. MATERIAL AND METHODS:

### 2.1 Cell donor characteristics:

All experiments were conducted using peripheral blood of healthy donors. Before blood samples were collected all participants have been asked about their recent medical history to assure that the immune function was not altered underlying medical conditions. Individuals with recent infections, autoimmune diseases, immune defects or conditions that predispose to malnutrition were excluded from the study. In addition, donors with a known diagnosis of iron or zinc deficiency were not included in the study.

2.2 Choice of cell models:

All experiments were performed with peripheral blood mononuclear cells (PBMC) as they provide a physiologically relevant source of human immune cells, without confounding effects of immortalization ore genetic modification . Moreover the diversity of included cells, including lymphocytes and monocytes allows to assess effects on both, innate and adaptive immunity.

A mixed lymphocyte culture was used as a functional assay to examine alloreactivity. Since this method allows assessment of proliferation and cytokine production, it is a suitable model for our aim to compare immune competence between differently stimulated cell populations, whereas PHA-stimulation is a strong activation overwriting some influences on immune cell activation.

2.3 Preliminary testing of cytotoxicity:

All used cell culture supplements except $Fe_2(SO_4)_3$ and $ZnSO_4$ were tested for their toxicity in titration experiments before performing the main experiments (see supplementary data). Viability was assessed by propidium iodide (PI) staining followed by flow cytometric analysis (FACS Calibur, Becton & Dickinson). For the main experiments we only used supplement concentrations that we identified to show no significant reduction of viability in PBMC compared to the control group. For $Fe_2(SO_4)_3$ no dose titration was performed, since it was necessary for our experimental setup to use the same concentration as in the $FeSO_4$ group. For $ZnSO_4$ we refer to a previously performed titration experiment (21).

2.4 Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Generation of Mixed Lymphocyte Cultures (MLC).

Peripheral venous blood of healthy donors was taken after informed consent and ethics committee approval (RWTH Aachen University Hospital, statement no. EK 23-234). PBMC were isolated as previously described (16). For the experimental setups, cells were cultured in RPMI 1640 medium containing 10% FCS (Capricorn Scientific, Ebsdorfergrund), 1% L-glutamine (Sigma-Aldrich, Steinheim, Germany) and 1% penicillin-streptomycin (Sigma-Aldrich, Steinheim, Germany). The cells were adjusted to a final concentration of $2 \times 10^6$ cells/mL. $2 \times 10^6$ PBMC/mL per donor were preincubated in medium alone or in medium supplemented with 50 µM $ZnSO_4$, 50 µM $FeSO_4$ , 2.5 µM TPEN (N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine), 200 µM 2,2-Bipyridyl (BIP), 50 µM $Fe_2(SO_4)_3$ , 0.5 mM ascorbic acid, 1 mM sodium sulfite, 250 µm nicotinamide, 100 µM potassium chlorate (all supplements from Sigma-Aldrich, Steinheim, Germany) or combinations of the above for 15 min. The cultures were continued in the same supplement-containing medium for the entire incubation period without washing the cells.

For the generation of MLC, PBMC of two genetically diverse donors were combined in a 1:1 ratio in a pyrogen-free 24-well dish (Becton Dickinson, Falcon, Heidelberg) for 96 h. All incubation steps were performed at 37°C in a humidified 5% $CO_2$ atmosphere.

2.5 Stimulation of PBMC with Phytohemagglutinin (PHA)

For the experimental setups, PBMC were adjusted to a final concentration of $1\times10^6$ cells/mL in culture medium. $1 \times 10^6$ PBMC per donor were preincubated in medium alone or in medium supplemented with 50 µM $ZnSO_4$, 50 µM $FeSO_4$, 2.5 µM TPEN, 200 µM BIP, 50 µM $Fe_2(SO_4)_3$ or combinations of the above for 15 min. In the following, 2.5 µg PHA (Becton Dickinson, Heidelberg ) were added to 1 ml of each of the preincubated cell solutions. After preincubation the cells stayed in the supplement-containing medium. Cells were incubated at 37 °C in a humidified 5% $CO_2$ atmosphere for 48 h.

2.6 Cell viability and proliferation controls

To ensure that the observed cytokine modulation was not due to cytotoxic or antiproliferative effects, PBMC and MLC cultures were analyzed for viability and proliferation under all treatment conditions (see supplementary data). Viability was assessed by propidium iodide (PI) staining followed by flow cytometric analysis (FACS Calibur, Becton & Dickinson). The proliferation was quantified by cell counting (Sysmex hemocytometer XN-330) after culture.

2.7 ELISA

Supernatants of MLC were harvested after 4 days or supernatants of stimulated PBMC were harvested after 2 days and stored at -20°C until measurements. For the quantification of IFN-γ, IL-2 and IL-6 protein expression OPTEia kits from Becton Dickinson (Heidelberg, Germany) were used according to the manufacturer's instructions. Supernatants were diluted as necessary to fall within the standard curve range, depending on the examined cytokine we used dilutions from 1:1 to 1:101 for the measurements.

2.8 mRNA Isolation and Real-Time PCR

PBMC were collected from MLC after 4 days of incubation. After lysis in 1 ml Trizol Reagent (Ambion, Life Technologies, Carlsbad, CA) the RNA was isolated. Transcription into cDNA using the qScript DNA Synthesis Kit (Quanta Biosciences, Darmstadt, Germany) according to the manufacturers' instructions. Quantitative real-time (RT) PCR was performed on a real-QuantStudio 3 System.
The following primers were used:
PBGD: 5'ACG ATC CCG AGA CTCTGC TTC 3' (forw.) 5'GCA CGG CTA CTG GCA CACT 3'(rev.)
IFN-γ: 5'AGAATTGGAAAGAGGAGAGTGACAG 3'(forw.) 5'GTCTTCCTTGATGGTCTCCACAC 3' (rev.)
Zip 8: 5' CCT CGG ATT GAT TTT GAC TCC ACT 3' (forw.) 5'AGC AGG ATT TGC ATA GCA TGT CAC 3'(rev.)
Expression of the target genes was normalized to the expression of the housekeeping gene PBGD. All samples were run in duplicates. $^{\Delta\Delta}$CT-method was used for gene quantification as described previously (17).

2.9 Inductively Coupled Plasma- Mass Spectrometry (ICP-MS)

The total intracellular iron content was assessed by ICP-MS (8900 ICP-MSMS Triple Quad, Agilent Technologies). PBMC were isolated as previously described and adjusted to a final concentration of $1\times10^6$ cells/mL. $1 \times 10^6$ PBMC per mL per donor were preincubated in medium or in medium supplemented with 50 µM $FeSO_4$, 50 µM $Fe_2(SO_4)_3$ or 200 µM BIP for 15 min. Cells were incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 4 days. The samples were frozen at -80°C for 15 min to assure cell lysis.

Residues of the cell pellets were resuspended with 2.25% $HNO_3$ and briefly vortexed. After that resuspension in 2.25% $HNO_3$ was performed thrice, resulting in a total volume of 3 mL. Rh103 was added as internal standard. A calibration curve consisting of 12 calibration points was recorded from 0.2 µg/L to 800 µg/L. To avoid interferences He was used as a collision gas in single quadrupole mode.

Further ICP-MS parameters were as follows: RF Power 1550 W, RF Matching 1.50 W, Sample Depth 8.0 mm

Nebulizer Gas 1.15 L/min, Integration time 0.1 s.

2.10 Speciation of Iron in PBMC

For redox speciation analysis of $Fe^{2+}$ and $Fe^{3+}$ in PBMCs, the method outlined in a work by Mandrioli et al. (18) was significantly modified to reduce the overall analysis time (including analysis and column cleaning) and to optimize detection for ICP-KED-MS.

PBMC were isolated and adjusted to a concentration of $1 \times 10^6$ cells/mL as previously described. $1 \times 10^6$ PMBC of healthy donors were incubated for 15 min with 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$, or left untreated as control. Cells were incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 4 days. Cell pellets were resuspended in 100 µL water and 5 mM EDTA and stored at -80°C until analysis.

The separation of the redox species $Fe^{2+}$ and $Fe^{3+}$ was conducted using a NexSAR® PEEK HPLC gradient system, which featured an autosampler programmed for a 50 µL sample volume, a column oven maintained at 30 °C, and a Dionex IonPac™ CS5A RFIC analytical cationic column (4 x 250 mm). For the detection of iron in the chromatograms, the column was directly connected to the NexIon® ICP mass spectrometer (PerkinElmer, Rodgau-Jügesheim, Germany), which operated in KED mode and was equipped with platinum cones, a glass concentric nebulizer, and a cyclone spray chamber (PerkinElmer, Shelton, CT, USA).

The HPLC conditions were as follows: Eluent A consisted of 50 mM ammonium citrate and 7.0 mM PDCA at pH 4.2, while Eluent B contained 30 mM EDTA and 250 mM $NH_4Ac$ at pH 6. Unlike the method used by Mandrioli et al. (18), a gradient elution was implemented to expedite column cleaning after each run and to ensure reproducibility. Gradient elution was programmed as follows:

Table 1: HPLC conditions

| Time (min) | B % | Flow rate (ml/min) |
|---|---|---|
| 0 | 0 | 0.5 |
| 2 | 0 | 0.8 |
| 3 | 0 | 0.8 |
| 6 | 50 | 0.8 |
| 7.5 | 100 | 0.8 |
| 13 | 100 | 0.8 |
| 13.5 | 0 | 0.8 |
| 18 | END | 0.8 |

The operating conditions for the NexIon® ICP mass spectrometer were: RF power was set to 1,200 W, with a plasma gas flow of 16 L/min, an auxiliary gas flow of 1.05 L/min, and a nebulizer gas flow of 0.98 L/min (optimized daily). Helium was utilized for KED mode at a flow rate of 1 mL/min. The isotopes 54 Fe, 56Fe and 57Fe were monitored during the analysis. Clarity software was used for comprehensive instrument control of both the NexSAR® PEEK HPLC gradient system and the NexIon® ICP mass spectrometer, as well as for the evaluation of iron chromatograms and peak area calculations. The $Fe^{2+}$ /$Fe^{3+}$ ratios were determined based on the respective peak areas.
Statistical significances were calculated by Friedman test with Dunn's post-hoc test ($n = 6$; * $p < 0.05$; ** $p < 0.01$).

2.11 Statistics

Calculation of statistical significances was performed by one-way ANOVA and mixed-effects analysis using GraphPad Prism software (version 9.4.1). ANOVA was used to compare balanced data sets, whereas a mixed-effects-model was used when data sets were imbalanced due to missing values, accounting for donor variability as a random effect. Tukey's test was used as post-hoc test. Statistically significant different means do not share any indicated letter ($p < 0.05$).

## 3. RESULTS:

### 3.1 Cell viability and proliferation controls

Both MLC (72 h; Supplementary Fig. 1A) and PBMC (48 h; Supplementary Fig. 2A) displayed viability consistently above 85%. There was no significant difference in viability between treatment groups and the control groups. Total cell numbers after culture were not significantly altered across treatments (Supplementary Fig. 1B, 2B). These data confirm that the shown effects cannot be explained by general cytotoxicity or growth inhibition and reflect immunomodulatory processes of the tested substances.

### 3.2 Zinc and Iron Supplementation Lead to Significantly Lowered Production of IFN-γ in MLC

Since clinical studies have indicated that zinc and iron have different effects on the immune system, the effects of both elements and their deficiencies were analyzed. We expected to see an antithetic effect of zinc and iron on the cytokine production.

The MLC served as an in vitro model for allogenic immune reaction, mediated by T cells. Examination of IFN-γ, as one of the leading cytokines produced by Th1-cells, serves to quantify and compare the allogenic inflammatory reaction (19).

Previous experiments have shown that treatment with zinc in physiological doses leads to a significant decrease of IFN-γ production in MLC, which served as a benchmark for our experimental setup (20). In order to examine the effects of iron incubation on MLC, a relevant concentration of iron was administered to the medium. Application of 50 µM iron equals an iron excess in the serum, while a range from 9-29 µmol/l is considered normal for an adult male. Zinc and iron were administered using zinc sulfate and iron sulfate, assuring that all observed effects are induced by the cations, respectively. In designing the experiments, the concentrations of zinc and iron were selected based on their physiological levels in the human body as well as on typical ranges observed under conditions of excess. In addition, a range of concentrations was tested to ensure that the treatments remained within non-toxic limits for the cultured cells. In designing the experiments, the concentrations of zinc and iron were selected based on their physiological levels in the human body as well as on typical ranges observed under conditions of excess. To ensure cellular tolerance we performed a preliminary concentration range, to identify non-toxic doses. Preliminary testing included concentrations ranging from 15 to 200 µM for iron. For zinc, dose-dependent effects on the IFN-γ and tolerable concentrations in MLC have been already examined in previous studies (21)

We decided to use equal concentrations of zinc and iron for all experiments since both showed a significant dose response and no significant toxicity in a concentration of 50µM. The zinc chelator TPEN was used to model serum hypozincemia (22).

Zinc treatment led to a significantly lowered production of IFN-γ in MLC in comparison to an untreated control (Fig. 1 A). Treatment with the zinc chelator TPEN showed a slight increase in IFN-γ production. Thus, a rather antagonistic effect of zinc supplementation and zinc deficiency can be assumed.

In contrast, treatment with iron induced a significant decrease in the production of IFN-γ, as well as iron deficiency by treatment with BIP did (Fig. 1 A). Therefore, an antagonistic effect of iron treatment and iron deficiency could not be observed.

Although the effects of zinc and iron are directed in the same way, a combined treatment with iron and zinc did not show a synergistic effect. There was no significant effect of the combined treatment compared to the single effects of iron and zinc treatment (Fig. 1 A).

### 3.3 Zinc and Iron Supplementation Lead to Significantly Lowered Production of IL-2 in MLC

Since the anticipated contrary effect of iron and zinc supplementation could not be observed in the measurement of IFN-γ production the question arose if effects could be seen on other T-cell-specific cytokines. IL-2 is a cytokine that is produced by activated T cells and therefore also represents the activation of cellular immunity in allogenic immune reactions (23).
In the following experiment the influence of iron and zinc and their chelators on the IL-2 production in MLC was determined.
Zinc treatment and iron treatment lead to a significantly lowered production of IL-2 compared to an untreated control, consistent with their effects on the IFN-γ production (Fig. 1 B). As seen in the IFN-γ production no synergistic effect of iron and zinc treatment can be seen. Single treatment with iron shows a significantly lower production of IL-2 than the combined treatment of iron and zinc (Fig. 1 B). Thus, it can be estimated that iron has a stronger influence on the IL-2 production or interferes with the cytokine production in a different way than zinc does.
Iron deficiency induced by BIP lowers the production of IL-2 significantly and shows no significant difference to treatment with iron. Again, both agents have a suppressive effect, as seen on the IFN-γ expression. In contrast to that, chelation of zinc using TPEN does not lead to a significant effect compared to the control, while zinc supplementation suppresses the IL-2 production significantly. Hence, the antithetic effect between treatment with zinc and zinc chelation remains consistent (Fig. 1 B).Thus, it can be observed that the effects of iron and zinc deficiency on cytokine production are consistent between the assessment of IFN-γ and IL-2 production.

### 3.4 Iron Supplementation Shows a Significant Decrease on IL-6 Production in MLC

For further assessment of systemic inflammatory processes the production of the proinflammatory and pyrogenic cytokine IL-6 was examined. IL-6 is holding a key role in the coordination of inflammatory processes. Since IL-6 can be produced not only by lymphocytes, but also by monocytes, examining the IL-6 production shifts the focus from lymphocytes to all PBMC. Treatment with iron led to a significantly lowered production of IL-6 in MLC compared to an untreated control. (Fig. 1 C) Contrary to the observations in IL-2 and IFN-γ measurement, treatment with zinc did not significantly affect the IL-6 production (Fig. 1 C).

### 3.5 $Fe^{2+}$ and $Fe^{3+}$ have Significantly Different Effects on the IFN-γ and IL-2 Production in MLC

Since iron in the human body is mainly bound to plasma proteins and non-transferrin-bound-iron (NTBI) only occurs in pathophysiological conditions, the oxidation state of NTBI is not well examined. Therefore, the question arose if treatment with $Fe^{3+}$ would lead to different effects than observed in the treatment with $Fe^{2+}$. Both $Fe^{2+}$ and $Fe^{3+}$ were administered as sulfate, using the same concentration, thus all observed effects can be ascribed to the oxidation state of iron.

Effects of $Fe^{2+}$ and $Fe^{3+}$ admission in MLC were examined by measurement of cytokine production. Treatment with $Fe^{2+}$ lead to a significantly lowered production of IFN-γ (Fig. 2 A) and IL-2 (Fig. 2 B) as shown in the previous experiments. On the contrary, treatment with $Fe^{3+}$ did not show significant effects compared to the untreated control. With regard to the production of IFN-γ, treatment with $Fe^{3+}$ even shows a slight increase in cytokine production (Fig. 2 B).
Contrary to the expectations, the effect of $Fe^{3+}$ is significantly different from the observed effect of $Fe^{2+}$ and requires a further examination.

### 3.6 $Fe^{2+}$ and $Fe^{3+}$ have Significantly Different Effects on the Expression of IFN-γ mRNA

To further elucidate the effects of $Fe^{2+}$ and $Fe^{3+}$ on the IFN-γ production a RT-PCR analysis of the expression of IFN-γ mRNA was performed. The aim of this experiment was to see if the effect on cytokine production is already indicated on transcriptional or post-transcriptional levels. Moreover, it was of interest to examine whether it might become visible that $Fe^{2+}$ chelation and supplementation suppress the IFN-γ production by different mechanisms.
The PCR analysis results are consistent with the ELISA results. Treatment with $Fe^{2+}$ significantly lowers the expression of IFN-γ mRNA (Fig. 3). BIP also decreased the IFN-γ mRNA expression. In contrast to that a slight increase in IFN-γ mRNA expression, but not significantly different to the control, is observed in treatment with $Fe^{3+}$.
Thus, the altered expression of IFN-γ in treatment with $Fe^{2+}$ and BIP measured in the ELISA experiments is consistent with alterations in the expression of IFN-γ mRNA. The consistency of $Fe^{2+}$ and $Fe^{3+}$ effects in ELISA and PCR delivers further evidence that the effect of iron on PBMC is dependent on the oxidation state.

### 3.7 Iron Uptake in PBMC is Increased in Supplementation with $Fe^{3+}$

With regard to the discrepancy of $Fe^{2+}$ and $Fe^{3+}$ effects it was necessary to clarify if iron uptake into PBMC was successful with both substances. $Fe^{3+}$ did not show an effect on the IFN-γ production that was significant to the control, therefore the question arose if $Fe^{3+}$ could be taken up into PBMC. If uptake of $Fe^{3+}$ into PMBC was not possible at all or at least impaired, it would provide a direct explanation for the missing effect on the IFN-γ production. For clarification an ICP-MS measurement of cellular iron after supplementation was performed.
The measurement of iron-uptake showed that treatment with $Fe^{2+}$ and $Fe^{3+}$ lead to a significant increase in cellular iron, respectively (Fig. 4). In opposition to the hypothesis, the cellular uptake of $Fe^{3+}$ is significantly higher than the uptake of $Fe^{2+}$.
In the scope of these results, it becomes evident that the different effects of $Fe^{2+}$ and $Fe^{3+}$ cannot be explained by a lack of iron uptake in $Fe^{3+}$ treated PBMC. Hence, further examinations were needed to elucidate the underlying mechanisms.

### 3.8 Stimulation with $Fe^{3+}$ increases the intracellular $Fe^{2+}$ and $Fe^{3+}$ ratio

It was necessary to clarify the impacts on the intracellular $Fe^{2+}$ and $Fe^{3+}$ ratio, to further investigate the different $Fe^{2+}$ and $Fe^{3+}$ effects on immune function in PBMC. Since our previous experiments have shown, that uptake into PBMC was successful with both substances, the question arose if the intracellular ratio $Fe^{2+}$ and $Fe^{3+}$ might be altered. For further clarification a speciation of intracellular iron by HPLC-ICP-MS was performed.

In untreated PBMC the intracellular iron species is predominantly $Fe^{2+}$, resulting in a $Fe^{3+}/Fe^{2+}$ ratio below 1. After supplementation with $Fe^{2+}$ , there is a significant increase of intracellular $Fe^{3+}$. The $Fe^{3+/}Fe^{2+}$ ratio is shifted towards a dominance of $Fe^{3+}$ (Fig 5 A). Stimulation with $Fe^{3+}$ also shows a reversal of the $Fe^{3+/}Fe^{2+}$ ratio in comparison to the control. The ratio of $Fe^{3+}/Fe^{2+}$ is significantly higher in $Fe^{3+}$ stimulation compared to stimulation with $Fe^{2+}$ (Fig 5 A).

Therefore alterations in the intracellular ratio of $Fe^{2+}$ and $Fe^{3+}$ might provide an explanation for the observation of different effects of $Fe^{2+}$ and $Fe^{3+}$.

### 3.9 $Fe^{2+}$ Effect can be simulated by Using Sodium Sulfite

Since the treatment with $Fe^{2+}$ and $Fe^{3+}$ was conducted under the same conditions, the only difference in both treatments is found in the oxidation status of the iron ion. The previous experiment also clarified that both iron species can be taken up well by PBMC, while $Fe^{3+}$ shows an even better uptake than $Fe^{2+}$.

Hence, we analyzed, if replacement of $Fe^{2+}$ and $Fe^{3+}$ by other electron donors and electron acceptors would still result in the same effects. We hypothesized that $Fe^{2+}$ might act as an oxidant and might disturb the cytokine production by applying oxidative stress to the cells. To verify whether this might be the case, a comparison to other oxidants was necessary. In the following experiment sodium sulfite and ascorbic acid were used as electron donor and electron acceptor, respectively.

Treatment with sodium sulfite led to a significantly decreased production of IFN-$\gamma$ compared to the control (Fig. 6 A). There was no significant difference to the treatment with $Fe^{2+}$. Going along with our hypothesis, the effect of $Fe^{2+}$ could be simulated by the use of another oxidant.

Contrary to expectation, treatment with ascorbic acid significantly decreased the production of IFN-$\gamma$ as well (Fig. 6 A). This is disaccording to the $Fe^{3+}$, which is not significantly affecting IFN-$\gamma$ levels.

The experiment was performed again using nicotinamide and potassium chlorate as alternative electron donators. Both, nicotinamide and potassium chlorate did not lower the IFN-$\gamma$ production significantly (Fig. 6 B). This is consistent with the results observed with $Fe^{3+}$. This might support our hypothesis, that ascorbic acid has a special influence on immunity and was not suitable as a random electron acceptor. Thus, it can be assumed that the effects seen with $Fe^{2+}$ and $Fe^{3+}$ are due to oxidative effects. $Fe^{2+}$, showing an effect comparable to sodium sulfite, is most likely acting as an electron donator. Since $Fe^{3+}$ is an electron acceptor, the effects on the cytokine production seen with $Fe^{2+}$ are not replicable with $Fe^{3+}$ as well as with other electron acceptors.

### 3.10 Treatment with $Fe^{3+}$ Significantly Enhances Expression of Zip8 mRNA

Since we demonstrated differences in iron uptake in $Fe^{2+}$ and $Fe^{3+}$ supplementation of PBMC, there might be changes in the expression of metal transporters. The Zip transporter family is a group of transmembrane transporters enabling the influx of zinc, iron, manganese, and cadmium. Previous studies have also indicated that Zip8 is likely involved in the regulation of IFN-γ expression (24). Thus, Zip8 being in the interface of cytokine and metal uptake regulation, was an important target for investigation. We hypothesized that a decrease in Zip8 expression might provide an explanation for lowered IFN-γ levels in the expression of Zip8 in $Fe^{2+}$ treatment or iron chelation. Not according to our hypothesis, both $Fe^{2+}$ and BIP do not significantly affect the expression of Zip8 mRNA.
Downregulation of IFN-γ in a Zip8 dependent manner can therefore be excluded as a possible explanation for altered cytokine production in dysregulation of the iron status.
In contrast, treatment with $Fe^{3+}$ was associated with increased Zip8 mRNA, which may contribute to the observed increase in iron uptake.(Fig. 7).

### 3.11 Zinc Effects Differ Between MLC and PHA-Stimulated PBMC While Iron Effects Remain Consistent

In the previous experiments all effects of zinc and iron treatments have been shown in MLC as a stimulation model. To further discriminate T-cell activation model dependent effects from independent effects, stimulation with PHA was consulted as an alternative model of T-cell activation. PHA is a plant lectin that binds and unspecifically activates lymphocytes.
Preincubation with zinc did not show a suppression of the IFN-γ production in PHA-stimulated PBMC, while preincubation with the zinc-chelator TPEN significantly lowered the production of IFN-γ (Fig. 8). The effects of zinc supplementation and chelation appear to be reversed in the PHA-stimulation. Thus, it can be assumed that the effect observed in MLC is depended on the T-cell activation model used. Preincubation with $Fe^{2+}$ leads to a significant decrease of IFN-γ production in PHA-stimulated PBMC, as previously seen in MLC as well. $Fe^{3+}$ does not induce a significant reduction in IFN-γ production (Fig. 8), consistent with the observations in MLC. Since a change in the inflammation model did not alter the effects of $Fe^{2+}$ and $Fe^{3+}$, a rather independent effect can be assumed.
Chelation of divalent iron by BIP did not significantly lower the production of IFN-γ in PHA stimulated PBMC, while it had a strong suppressive effect on the IFN-γ production in MLC. Nevertheless, a slight decrease can be seen in the PHA-stimulation as well. Thus, it can be assumed, that iron deficiency has a stronger impact on cells in the MLC than in PHA-stimulation. This provides the first difference in effects of $Fe^{2+}$ and BIP treatment we could observe in our experiments.

## 4. DISCUSSION:

In this study we investigated the interaction of iron and zinc in mixed lymphocyte cultures and stimulations with PHA.

Previous studies by Bryan et al. have shown a dose-related impairment of the MLC response after treatment with iron citrate (24). Until now, the alteration in cytokine production by iron in MLC has not been assessed. Furthermore, citrate itself has an immunosuppressive effect in MLC (25). We have shown that zinc and iron treatment significantly lowered the production of IFN-γ and IL-2 in MLC, respectively. IFN-γ is a typical cytokine produced by Th1 cells, which are responsible for the allogenic immune response. Hence, the observed decrease in IFN-γ expression indicates that the allogenic reaction in MLC is dampened or even partially suppressed in $Fe^{2+}$ and zinc treatment. Not according to our hypothesis that zinc and iron might show antagonistic effects, here it becomes evident that both effects on the IFN-γ production are rather suppressive. However, it can not be assumed that zinc and iron affect immunity in the same way.

Since we could demonstrate a drastic lowering of IL-2 production and a significant suppression of IL-6 production in MLC after treatment with $Fe^{2+}$ as well, it appears that $Fe^{2+}$ has a more general suppressive effect on the cytokine production. Nevertheless, a reduction in cell count or cell viability does not provide a possible explanation for the general suppression in cytokine production since we performed viability tests and counted the cells after the incubation (see supplementary data). Also, we tested for differences in the expression of different T-cell subpopulations by examination of subpopulation specific transcription factors (data not shown). A significant change in T helper cell subpopulations could not be observed and does therefore not provide an explanation for the observed effects of $Fe^{2+}$ treatment.

We also investigated the expression of IL-6 as a marker for monocytes and macrophages to include all cellular subtypes in PBMC into the study and also emphasize crosstalk between cell types. IL-6 is a cytokine that is particularly expressed in the early phase of infections and is responsible for the coordination of the systemic response to infections, including upregulation of the body temperature and acute phase proteins. Although IL-6 is primarily produced by monocytes and macrophages, its secretion is linked to cytokine crosstalk and T-cell activation (26).

The present data demonstrates that iron administration shows a significant decrease in IL-6, while zinc treatment does not. A possible explanation could be that zinc alters the expression of Th1-cytokines by induction of regulatory T cells (27). This would only alter the balance between Th-cell cytokines and therefore does not have a generalized effect on the cytokine production. Iron might inhibit cytokine production by a less specific mechanism and therefore shows a generalized suppressive effect on the expression of various cytokines.

Thus, the generalized suppression of cytokine production in iron overload that we observed in our experiments agrees well with the observations in clinical studies that identified severe infections as a major risk in iron overload (6, 28). Especially, disturbances in the IL-6 production may explain a tendency towards development of bloodstream infections and sepsis, because the coordination of the early infection stage is affected.

Another important difference between the $Fe^{2+}$ and zinc effect in MLC and PHA-stimulated PBMC can be seen in our chelation experiments, functioning as a comparative model for iron and zinc deficiency. In the MLC a chelation of zinc, using the zinc chelator TPEN leads to a slight, but not significant increase in the production of proinflammatory cytokines, while treatment with zinc significantly lowers the production of IL-2 and IFN-γ. Thus, an antithetic effect of zinc supplementation and zinc deficiency, as observed in previous studies (29), can be seen in our MLC experiments as well. In the PHA-stimulation these effects were reversed. PBMC stimulated with PHA show a significant suppression of IFN-γ production when treated with TPEN, while zinc supplementation does not induce a significant effect. This underlines on the one hand that the zinc effect differs between different stimulation models, but on the other hand that the zinc effect likely follows specific pathways and is not generalized. Also, a clear antagonism between zinc supplementation and zinc deficiency can be observed.

Considering $Fe^{2+}$ and the divalent iron chelator BIP no antithetic effect can be observed. Both, $Fe^{2+}$ supplementation and iron deficiency induce a significant lowering of the IFN-γ production in MLC. Moreover, the $Fe^{2+}$ effect remains consistent in PHA-stimulated PBMC, whereas BIP does not significantly affect the IFN-γ levels in PHA-stimulation. Still, a slight lowering of IFN-γ production in PHA-stimulated PBMC can be observed after incubation with BIP.

The divergence of the BIP effect between MLC and PHA models can be explained by the difference of the stimulation model itself. MLC represents a physiological cell response to antigens and therefore is more sensitive to metabolic disruptions such as a limitation in trace element availability. In contrast to that PHA stimulation represents a strong, non-specific activation that can override metabolic limitations. The pronounced suppression in the MLC model can therefore highlight the importance of iron-dependent reactions under physiological conditions, that might not be captured by strong activation models such as PHA stimulation. These observations underline that it is crucial to observe immunomodulatory effects under physiological conditions, since aggressive stimulation can wash out sensitive metabolic effects.

To observe effects under physiological conditions it was also important that all supplements remained present in the medium for the whole culture period, as trace metal excess or deficiency in the serum rather represents a persistent extracellular stimulus over time than a short term fluctuation. Moreover this supports the hypothesis that the observed effects can be explained by more complex redox reactions rather than transient uptake effects.

Previous studies indicate that T-lymphocytes have an increased iron demand after their activation and are functionally impaired by iron scarcity. Iron deficiency appeared to disturb several signaling pathways including those for histone demethylation. Thus, lymphocyte gene expression might be altered in iron deficiency (30). Studies that put lymphocytes in intracellular iron deficiency by blocking of transferrin receptors could show that T-cell proliferation and differentiation can be inhibited by iron deficiency (31). A possible underlying mechanism could be inhibition of DNA synthesis, as previously shown (32).

Since a variety of enzymes converting $Fe^{2+}$ to $Fe^{3+}$ and vice versa are known, it is widely accepted that serum iron is in a dynamic balance (33). Therefore, different effects of treatment with $Fe^{2+}$ and $Fe^{3+}$ would not be expected. Still the present data clearly shows significantly distinct outcomes depending on the oxidation state of iron.

Since $Fe^{2+}$ can function as an electron donator, we further examined whether the effects seen with $Fe^{2+}$ can be replicated with other electron donators. The consistency within the observations in MLC and PHA-stimulation support the hypothesis of a model independent effect of $Fe^{2+}$. Our experiments have shown that sodium sulfite, in its function as an alternative electron donator, can induce a decrease in IFN-$\gamma$ production, that resembles the effect seen with $Fe^{2+}$ administration. Thus, suppression of cytokine production by $Fe^{2+}$ donating electrons delivers a possible explanation.
Electron donation to oxygen can lead to formation of reactive oxygen species, that cause oxidative stress. Immune cells are prone to be affected by oxidative stress, leading to disturbances in protein and gene expression and eventually cell death. Liu et al. have already shown in an animal model that excessive nutritional iron intake will lead to oxidative damage on immunological organs such as the spleen and lymph nodes and might therefore impair the systemic immune function (34).
Flescher et. al have shown that oxidative stress leads to suppression of cytokine production in lymphocytes by impairment of DNA-binding of the transcription factors NF-κB, AP-1 and NFAT. These transcription factors are crucial in the regulation of IL-2 production, which was suppressed by $Fe^{2+}$ in our experiments as well (35).
In the synopsis of previous studies and our results, it becomes evident that a tight regulation of serum iron levels is crucial to the function of the immune system. Iron deficiency negatively affects the immune function as well as excess iron does.
To further evaluate the contribution of redox-balance to cytokine production, we examined antioxidant compounds.
Due to high percentages of polyunsaturated fatty acids in the cell membrane immune cells contain a higher amount of intracellular antioxidants than other cells (36). Ascorbic acid is well known to influence immune function in various ways, for instance by modulation of gene expression in lymphocytes (37). It is one of the most common intracellular antioxidants in the human body. Therefore, the observed effect might be caused by confounding factors.
Ascorbic acid can counteract the activation of NFκB through oxidative stress and therefore lead to anti-inflammatory effects and reduce the production of proinflammatory cytokines (38). This provides an explanation why ascorbic acid treatment led to a lowered cytokine production in our experiments while other antioxidants did not show significant effects on the cytokine production. Nevertheless, it might be of interest for further studies to examine whether supplementation with ascorbic acid as an intracellular acting antioxidant might alleviate the immunological impairment going along with iron overload.
In our study comparison to other electron acceptors, not known to be immunomodulating, was conducted in order to clarify that the suppression seen in ascorbic acid treatment was caused by

independent factors. The use of sodium sulfite also has its limitations, since the substance is known to exhibit cytotoxicity. Further investigations with other antioxidant substances could help clarify if the observed effects can be explained by antioxidative capacities only.

Even though zinc does not have antioxidative capacities on its own, zinc could still provide beneficial effects on oxidative stress in iron overload in vivo. Since zinc is an important co-factor of many enzymes, including various enzymes involved in the antioxidant system response (39). These effects might not have been visible in the MLC, since the time span of four days is rather short and only isolated PBMC were examined. Our results show that both zinc and $Fe^{2+}$ reduce the expression of IL-2 significantly, but still the IL-2 cytokine levels in $Fe^{2+}$ treated PBMC are significantly lower than in the zinc treatment. With regard to the literature, this might be explained by different mechanisms causing the same effect. $Fe^{2+}$ appears to be rather immune suppressive (40) while zinc only dampens overshooting cytokine production by strong immune activation (41). Therefore, patients in iron overload might still benefit from a moderately dosed zinc supplementation, since zinc is known to counteract immune overshoot, without acting immune suppressive.

Our experiments show significant differences in the IFN-$\gamma$ expression between PBMC treated with $Fe^{2+}$ and PBMC treated with $Fe^{3+}$ in the MLC as well as in stimulation with PHA. Set into context with the literature, it can be assumed that the different effects are linked to the oxidation state of iron. Still, we focused on finding alternative explanations for the observed phenomenon, such as differences in the uptake and alterations in Zip8 expression.
We have shown an increase of iron uptake into PBMC after incubation with $Fe^{3+}$ compared to $Fe^{2+}$ incubation. Previous studies have already shown that T-lymphocytes preferentially take up non-transferrin-bound iron in a manner that resembles hepatocytes (42). This underlines that the immune system can be counted as an organ being affected by situations of iron overload. Descriptions by Pinto et al. are going even further and describe T-lymphocytes as a “circulating NTBI storage compartment“ involved in the regulation of tissue iron levels in iron overload (43). The studies by Pinto and Arezes used Fe(III)citrate for their simulation of NTBI in iron overload and thus refer to the uptake of $Fe^{3+}$ as well (43). However, a potential citrate effect, as described previously (25), was ignored.

Previous studies by Aydemir et al. have shown that the zinc transporter Zip8 plays an important role in the regulation of IFN-$\gamma$ expression (44). Our experiments show that incubation with $Fe^{3+}$ leads to a significantly enhanced expression of Zip8 mRNA. This might indicate that the underlying mechanisms of the different $Fe^{2+}$ and $Fe^{3+}$ effects on the IFN-$\gamma$ production might be found on transcriptional or posttranscriptional levels and require further examination. Since the mRNA expression does not necessarily correlate with protein expression, function and activity, further analysis of protein-levels or functional assays would be needed to confirm this hypothesis. Moreover, an enhancement of Zip8 transporter expression might on the one hand explain, why PBMC show higher intracellular iron levels when treated with $Fe^{3+}$ compared to $Fe^{2+}$. On the other hand, a higher Zip8 expression also facilitates the uptake of zinc and other trace elements into PBMC. Thus, a variety of intracellular pathways may

be influenced. Since Zip8 is only one of various transmembrane transporters involved in intracellular zinc and iron uptake, a differential analysis of Zip transporters could further elucidate changes in metal influx.

With regards to the fact that all of our performed experiments were in vitro experiments, using isolated PBMC, it is important to know the limitations of our results. The mixed lymphocyte culture serves as a standard model for allogenic immune response and transplantation. Therefore, it is a well-fitting cellular model and may even be representative for stem cell or bone marrow transplantation. Since iron uptake and storage in the human body is regulated by various mechanisms, the effects seen on isolated PBMC may differ in whole blood or animal models. Particularly the small intestine and the liver are involved in the regulation of the iron homeostasis, for example by expression of the proteohormone hepcidin. Still the continuity of the $Fe^{2+}$ and $Fe^{3+}$ effects in MLC and PHA-stimulations indicate, that the effects are independent from the use of MLC as stimulation model. Thus, it can be assumed that once iron excess might affect immune function as shown in our experiments, as soon as iron accumulation exceeds the systemic regulation mechanisms. Nevertheless, further examination of the immunological impact of $Fe^{2+}$ and $Fe^{3+}$ in animal models might be of interest with regard to involving systemic regulation mechanisms.

The clinical studies by Soofi et al, that showed an increase in morbidity caused by baseless iron supplementation (5) refer to oral supplementation of iron, which is in most cases an application of $Fe^{2+}$. This is consistent with our findings, that show a decrease in cytokine production under $Fe^{2+}$ administration. With regards to the results of our experiments, it could be assumed that application of $Fe^{3+}$, would not lead to an increase in infections. Since $Fe^{3+}$ has a poor bioavailability, supplementation of $Fe^{3+}$ means intravenous iron supplementation. A systematic review and meta-analysis of clinical studies comparing intravenous to oral iron application, shows a significantly elevated risk of infection in treatment with intravenous iron (45).
This is not consistent with our findings, since cytokine production was not significantly impaired in $Fe^{3+}$ treated PBMC. Nevertheless, it must be taken into consideration, that intravenous iron replacement is primarily used in cases of severe iron deficiency, as seen in malignant and chronic inflammatory diseases or patients undergoing hemodialysis. Therefore, the patient collectives receiving intravenous iron therapy might already be more prone to infections than the patient collectives that received oral iron treatment.
Besides host immunity effects, alterations in the availability of iron can may as well benfit pathogens through mechanisms of nutritional immunology.
In some clinical studies, more frequent and systemic infections are explained by principles of nutritional immunity, underlining that increased serum iron may provide nutritional benefits for bacteria and enhance bacterial growth (46). In addition to that our results can provide evidence that the cellular immune response is impaired as well. Moreover, important acute phase proteins such as caeruloplasmin and hepcidin, being crucial to the nutritional (iron) deprivation of pathogens are

excreted in an IL-6 dependent manner. Since iron overload impairs the expression of IL-6, already elevated iron levels cannot be chelated in the early infection phase and enable bacterial growth. In the synopsis of an impairment of innate and adaptive immune function and an increased potential for bacterial growth, it becomes evident, that iron supplementation should always have an indication and requires observation that early infections are not overlooked.

In conclusion our results show that zinc and iron both have suppressive effects on the cytokine production in MLC, which might be due to different underlying mechanisms. We showed that the effects of incubation with iron on MLC is dependent on the oxidation status of iron. Oxidative effects might provide a possible explanation for this phenomenon, but still the underlying mechanisms are not fully elucidated and need further examination. Nevertheless, it becomes clear that iron overload and iron supplementation require tight observations because adverse immunological effects might occur. Zinc treatment does not act immunosuppressive, but enhances the antioxidative capacities of the immune system and might therefore be helpful in stabilizing immune function in iron overload. Future studies integrating in vivo models and patient data will be essential to confirm these mechanisms and to define safe therapeutic ranges for iron and zinc supplementation.

Declarations of interest: none

Author contributions: Conceptualization: E.F., J.J., and L.R.; methodology: E.F., J.J., J.B, I.W. J.S. and L.R.; validation: E.F., J.J. and L.R.; formal analysis: E.F., J.J. and L.R.; investigation: E.F., J.B., I.W. and J.S.; resources: L.R.; data curation: E.F., J.J. and L.R.; writing - original draft preparation: E.F.; writing - review and editing: E.F., J.J., J.B. I.W. J.S. and L.R. visualization: E.F., J.J.; supervision: L.R., J.J.; project administration: L.R.

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**Figure Legends**

Figure 1

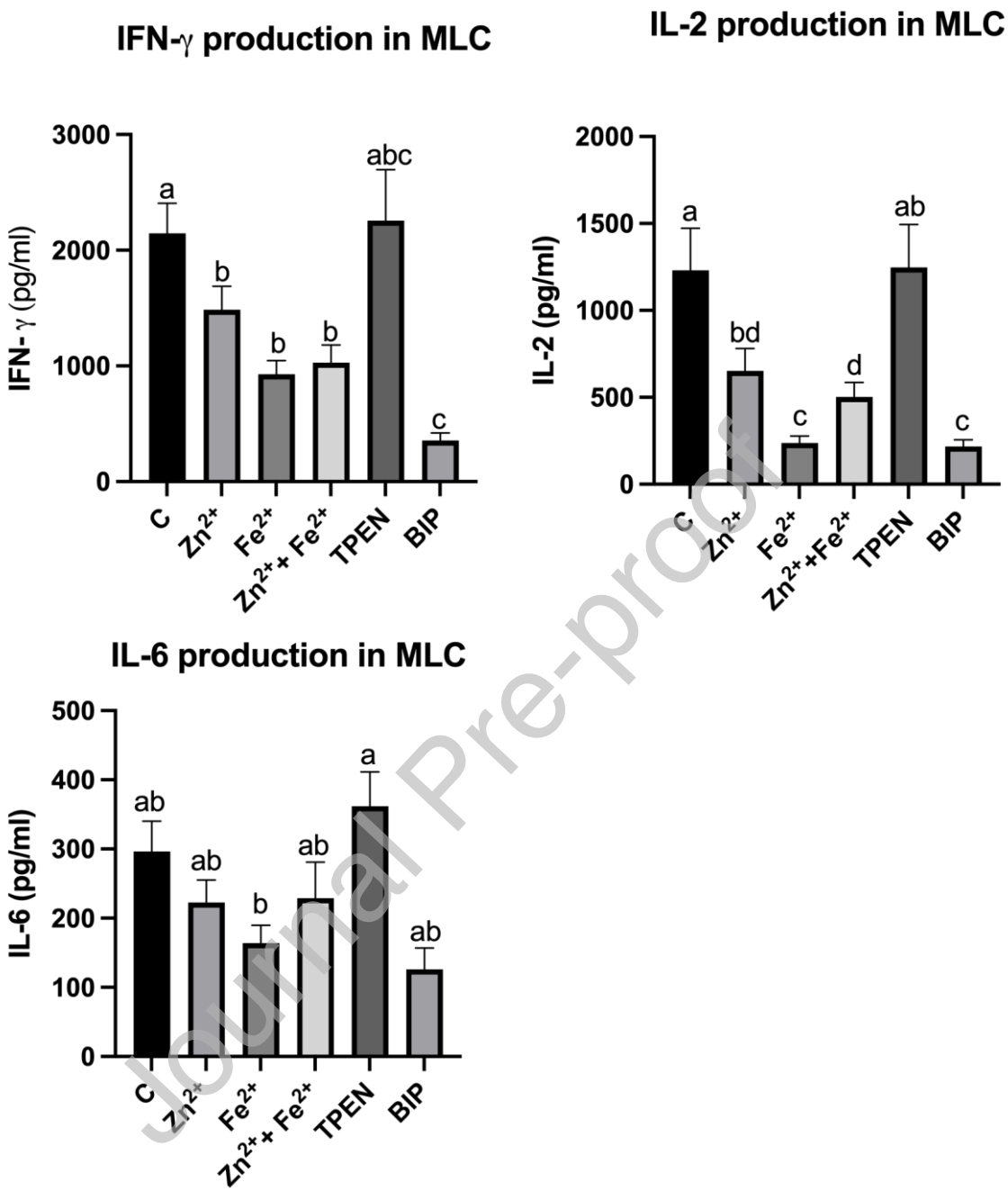



**Fig. 1. Zinc and iron treatment significantly lower the expression of proinflammatory cytokines in MLC.**

PBMC were adjusted to 2 × $10^6$ cells/mL. PBMC were pre-incubated for 15 min with 50 µM zinc, 50 µM iron ($Fe^{2+}$), a combination of 50 µM zinc and 50 µM iron, 2.5 µm TPEN or 200 µM BIP for 15 min or left untreated as a control. MLC were generated and incubated for 4 days with indicated concentrations. (A) The production of IFN-γ was measured by ELISA (n = 13). (B) The production of IL-2 was measured by ELISA (n = 20). (C) The production of IL-6 was measured by ELISA (n = 13). Results show mean values + SEM. Statistical significances were calculated by one-way ANOVA (A, B) or mixed effects analysis (C) using Tukey´s test as a post-hoc analysis for multiple pairwise comparisons among all treatment groups. Groups not sharing the same letter differ significantly ($p < 0.05$)

Figure 2

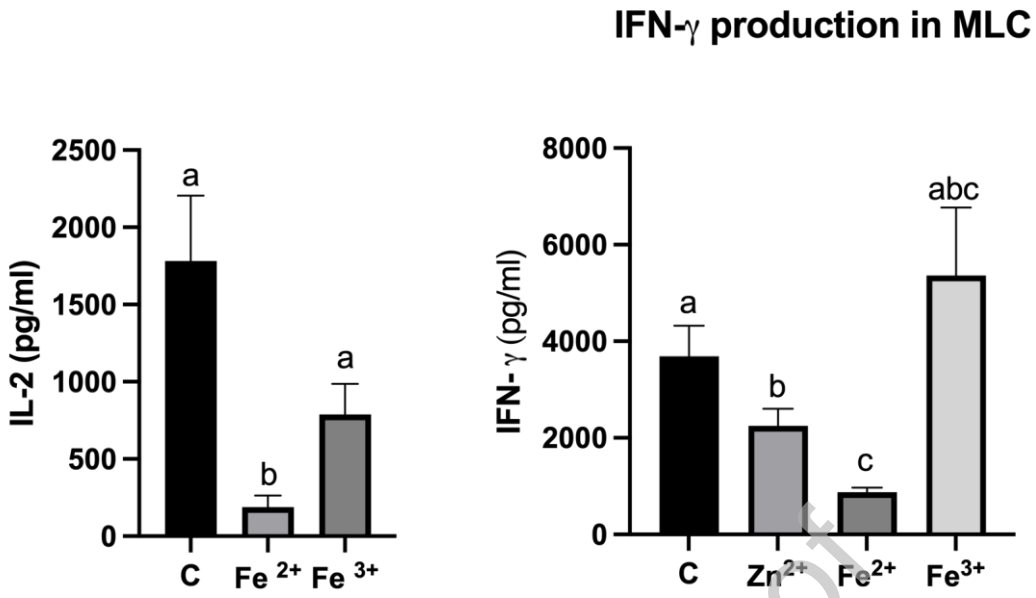



**Fig. 2. $Fe^{2+}$ and $Fe^{3+}$ treatment have significantly different effects on cytokine production in MLC.** PBMC were adjusted to 2 × $10^6$ cells/mL. PBMC were pre-incubated with 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$ for 15 min or left untreated as a control. MLC were generated and incubated for 4 days with indicated concentrations. (A) The production of IL-2 was measured by ELISA (n = 6). (B) The production of IFN-γ was measured by ELISA (n = 19). Results show mean values + SEM. Statistical significances were calculated by one-way ANOVA (A) or mixed-effects analysis (B) using Tukey´s test as a post-hoc test for multiple pairwise comparisons among all treatment groups. Groups not sharing the same letter differ significantly ($p < 0.05$)

Figure 3

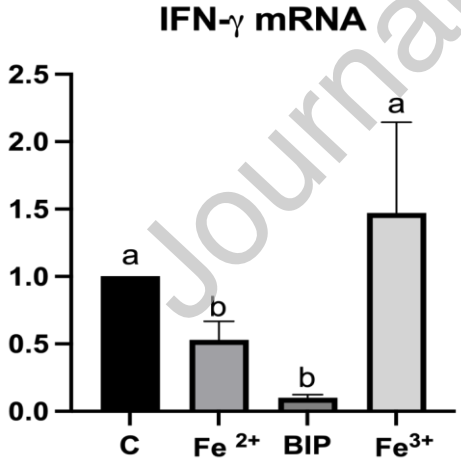



**Fig. 3. Iron deficiency and IFN-γ production in MLC.**
Supplementation with $Fe^{2+}$ and iron deficiency induced by BIP both have a suppressive effect on the expression of IFN-γ mRNA in MLC. Treatment with $Fe^{3+}$ does not significantly alter the expression of IFN-γ mRNA. PBMC were adjusted to 2 × $10^6$ cells/mL. PBMC were pre-incubated with 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$, 200 µM BIP for 15 min or left untreated as a control. MLC were generated and incubated for 4 days with indicated concentrations. The expression of IFN-γ mRNA (n = 9) was investigated by real-time PCR. Results show mean values + SEM. Statistical significances were calculated by mixed-effects analysis using Tukey´s test as a post hoc test for multiple pairwise comparisons among all treatment groups. Groups not sharing the same letter differ significantly ($p < 0.05$)

Figure 4

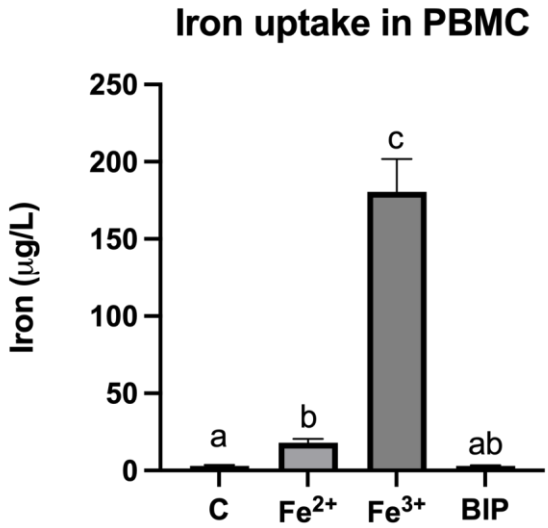
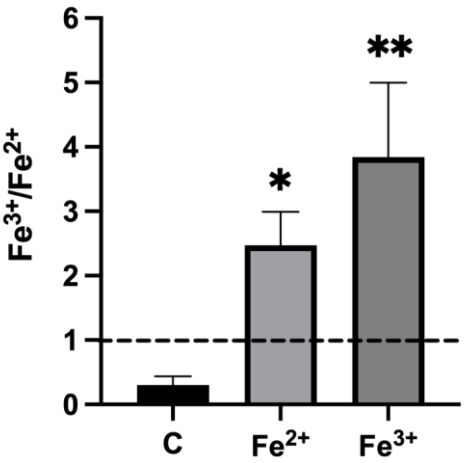



**Fig. 4. Impact of $Fe^{2+}$ and $Fe^{3+}$ treatment on the intracellular free iron concentration.**

$1 \times 10^6$ PBMC of healthy donors were incubated for 4 days with 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$, 200 µM BIP or left untreated as control. The total intracellular iron amount was analyzed by inductively coupled-plasma mass-spectrometry (ICP-MS) (n = 4). Results show mean values + SEM. Statistical significances calculated by one-way ANOVA using Tukey´s test as a post-hoc test for multiple pairwise comparisons among all treatment groups. Groups not sharing the same letter differ significantly ($p < 0.05$).

Figure 5



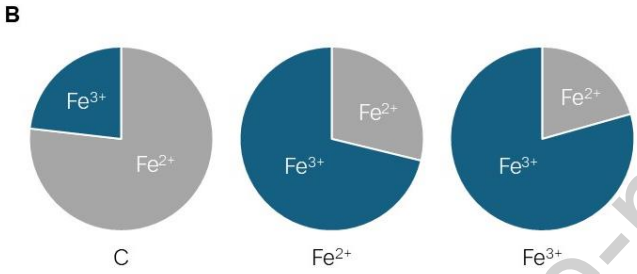




**Fig. 5. Impact of $Fe^{2+}$ and $Fe^{3+}$ treatment on the intracellular $Fe^{2+}$ and $Fe^{3+}$ ratio.**
$1 \times 10^6$ PMBC of healthy donors were incubated for 4 days with 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$, or left untreated as control (C). (A) The intracellular $Fe^{3+}$ /$Fe^{2+}$ ratio was quantified by ICP-MS (n = 6). (B) Mean relative proportions of $Fe^{2+}$ and $Fe^{3+}$ are shown. Data represent mean ± SEM. Statistical significance was assessed by Friedman´s test with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$).

Figure 6

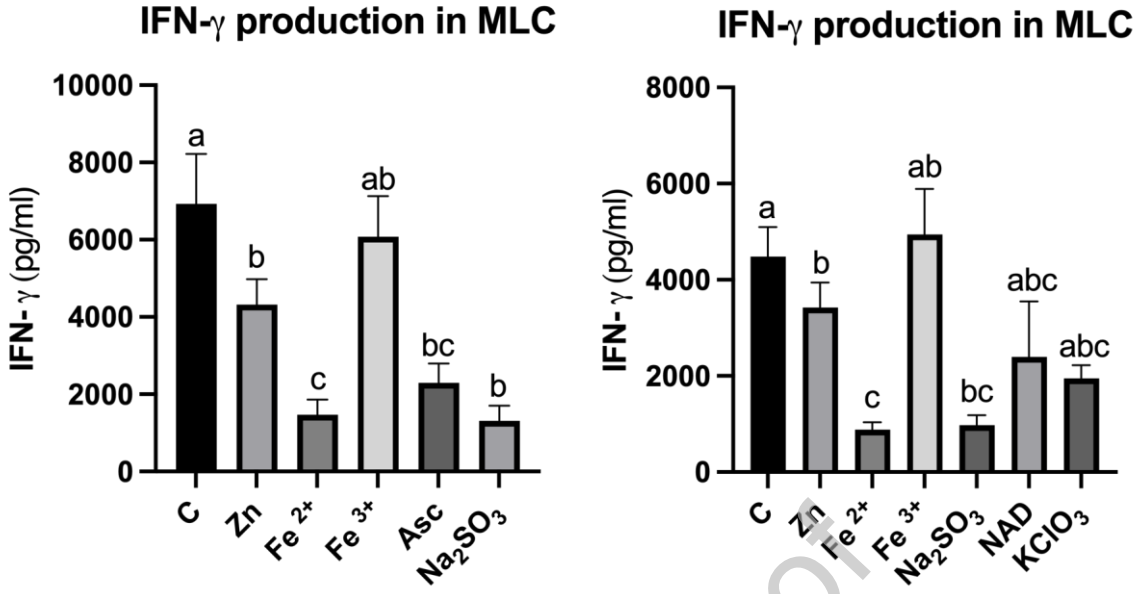



**Fig. 6. Electron donation and IFN-γ production in MLC.**
Influence of zinc, iron ($Fe^{2+}$), iron ($Fe^{3+}$), ascorbic acid and sodium sulfite incubation on IFN-γ production in MLC. (A) PBMCs were preincubated for 15 min with 50 µM $Zn^{2+}$ , 50 µM $Fe^{2+}$ , 50 µM $Fe^{3+}$ , 0.5 mM ascorbic acid (Asc), or 1 mM sodium sulfite ($Na_2$ $SO_3$ ), or left untreated (control). (B) Separate PBMC cultures were treated with 50 µM $Zn^{2+}$ , 50 µM $Fe^{2+}$ , 50 µM $Fe^{3+}$ , 250 µM nicotinamide (NAD), or 100 µM potassium chlorate ($KClO_3$ ), or left untreated (control). MLCs were generated and incubated for 4 days. IFN-γ production was measured by ELISA (n = 13). Data represent mean ± SEM. Statistical significance was determined by one-way ANOVA (A) or mixed-effects analysis (B) with Tukey´s post hoc test for multiple pairwise comparisons among all treatment groups.; groups not sharing the same letter differ significantly ($p < 0.05$).

Figure 7

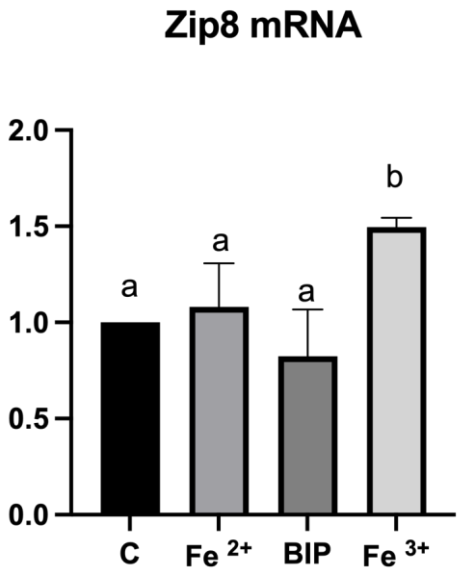



**Fig. 7. Treatment with $Fe^{3+}$ significantly enhanced the expression of Zip8 mRNA in MLC.** $Fe^{2+}$ treatment did not significantly affect the expression of Zip8 mRNA. PBMC were adjusted to 2 × $10^6$ cells/mL. PBMC were pre-incubated with 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$, 200 µM BIP for 15 min or left untreated as a control. MLC were generated and incubated for 4 days with indicated concentrations. The expression of Zip8 mRNA (n = 5) was investigated by real-time PCR. Results show mean values + SEM. Statistical significances calculated by one-way ANOVA using Tukey´s test as a post-hoc test for multiple pairwise comparisons among all treatment groups. Groups not sharing the same letter differ significantly ($p < 0.05$).

Figure 8

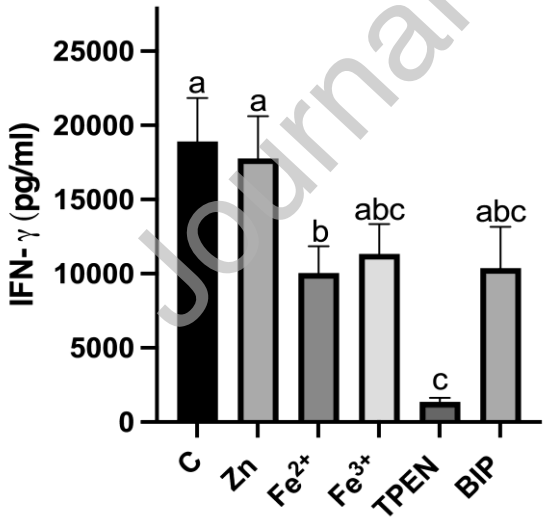



**Fig. 8. Treatment with $Fe^{2+}$ has a suppressive effect on the production of IFN-γ in PHA-stimulated PBMC.** PBMC were adjusted to 1 × $10^6$ cells/mL. PBMC were pre-incubated with 50 µM zinc, 50 µM $Fe^{2+}$, 50 µM $Fe^{3+}$, 2.5 µM TPEN or 200 µM BIP for 15 min or left untreated as a control. PBMC were incubated for 48 h with the indicated concentrations. The production of IFN-γ was measured by ELISA (n = 12). Results show mean values + SEM. Statistical significances calculated by one-way ANOVA using Tukey´s test as a post-hoc test for multiple pairwise comparisons among all treatment groups. Groups not sharing the same letter differ significantly ($p < 0.05$)

**Graphical abstract**