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The Role of Zinc in GM-CSF-Induced Signaling in Human Polymorphonuclear Leukocytes

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Scope: Zinc is suggested to be necessary for functional signaling induced by certain growth factors. The granulocyte-macrophage colony-stimulating factor (GM-CSF) is a key factor for differentiation and activation of myeloid cells. This report analyses the impact of different zinc concentrations on GM-CSF-induced signaling in mature polymorphonuclear leukocytes (PMN). Methods and results: As measured by flow cytometry, zinc increases surface GM-CSF receptor (GM-CSFR) in PMN, whereas monocytes respond with decreased GM-CSFR surface expression. Since total cellular GM-CSFR expression remains unaffected, the observed zinc-induced GM-CSFR surface dynamics may be explained by receptor redistribution. In PMN, zinc enhanced phosphorylation of mitogen-activated protein kinases (MAPK) in a dose-dependent manner as found in western blot. Zinc-induced MAPK phosphorylation is additionally augmented by moderate GM-CSF stimulation. Conclusion: The present study demonstrates the opposing influence of zinc on GM-CSFR surface expression in monocytes and PMN. Zinc and GM-CSF, use in optimized concentrations, augment MAPK signaling, and increase expression of MAPK-induced myeloid cell leukemia-1 (Mcl-1) in PMN. Thus, this study concludes that zinc strengthens growth factor-induced signaling. Hence, the study provides a basis for further in vivo studies, focusing on the therapeutic value of zinc in patients with a disturbed GM-CSF signaling.

1. Introduction

During granulopoiesis myelopoetic growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) are major players inducing proliferation and differentiation of hematopoietic progenitor cells. GM-CSF was found to affect mature cells as well. However, here activation rather than proliferation is achieved.[1] There is evidence from the literature that zinc is a major regulator of intracellular signaling in immune cells such as monocytes[2] and T-cells.[3] Recently, especially growth factor-induced signaling was observed to involve zinc-dependent mechanisms.[3,4]

GM-CSF transmits its effect via the GM-CSF receptor (GM-CSFR), which consists of four alpha (CD116)- and four beta (CD131) receptor subunits, forming a multimeric complex upon binding of four GM-CSF molecules.[4–6] The GM-CSFR does not have an intrinsic tyrosine kinase activity, hence signaling is transmitted via recruitment of the Janus kinase 2 (JAK2), a nonreceptor tyrosine kinase.[5,6] Activation of the mitogen-activated protein kinases (MAPK) pathway is initiated among others by extracellular-signal regulated kinases (ERK1/2) and p38 mitogen-activated protein kinases (p38) which phosphorylate a variety of different cytoplasmatic molecules and nuclear proteins, that in turn regulate gene expression in neutrophils.[5,6]

The importance of zinc for cellular signaling and function of myeloid cells was already emphasized in previous studies.[4,7,8] Aster et al.[4] found that zinc affects GM-CSF-induced signaling in the early myeloid cell line U937, probably explained by zinc's influence on membrane fluidity. As indicated above, effects of GM-CSF depend on the developmental stage of the myeloid cell and may also differ between leukemia cell lines and primary cells. Thus, we decided to take a closer look at zinc's influence on GM-CSFR-induced signaling in mature primary polymorphonuclear leukocytes (PMN) as well.

Increasing our understanding of the role of zinc in GM-CSF-induced signaling in primary cells, we hope to promote the clinical use of zinc and to uncover novel potential fields of zinc application. Our novel data presented here may provide a basis for subsequent clinical in vivo studies in patients with a disturbed GM-CSF signaling.

2. Experimental Section

2.1. Isolation and Culture of Primary Human Leukocytes and PMN

Leukocytes were isolated from heparinized blood of healthy, consenting donors. Ethical approval for the use of blood from

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human volunteers had been obtained from the institutional ethics review board of RWTH Aachen University Hospital under protocol number EK 023/05. One part of a 6% hydroxyethyl starch solution (Fresenius Kabi, Bad Homburg, Germany) was added to two parts of blood and sedimentation was allowed for 60 min at room temperature. Supernatants were centrifuged, cell pellets were washed with PBS (Dulbecco's PBS, St. Louis, MO, USA), and remaining erythrocytes were removed using hypotonic lysis.

Peripheral blood mononuclear cells (PBMC) and PMN were isolated using ficoll and percoll gradients, respectively, as described previously.[9,10] Monocytes and lymphocytes were enriched using adherence to culture dishes. Cells were cultured at 37 °C in a humidified, 5% carbon dioxide atmosphere in RPMI-1640 Medium (Sigma-Aldrich, Steinheim, Germany) containing 10% heat-inactivated FCS (Capricon Scientific, Ebsdorfergrund, Germany) and supplemented with 2 mM L-glutamine, 100 U mL^{-1} potassium penicillin, and 100 µg mL^{-1} streptomycin sulfate (all Sigma-Aldrich). Gating strategy for assessing the purities of the isolated populations were illustrated in Figure S1 (Supporting Information). The mean purity of nonactivated PMN was 98%, of enriched monocytes was 73.2% and of enriched lymphocytes was 68.2% (Figure S1, Supporting Information).

2.2. Dosage Information and Experimental Conditions

Following previous research, GM-CSF (ImmunoTools, Friesoythe, Germany) concentration from 20 to 200 U mL^{-1} and zinc sulfate (Sigma-Aldrich) concentrations between 5 and 500 µM were tested. To achieve a significant acute intracellular zinc increase within 30 min, zinc was combined with 1 µM pyrithione (sigma-Aldrich) for 30 min.[4,11] Optimal effects and low toxicity were found for total leukocytes, monocytes, and lymphocytes at 50 µM zinc, 1 µM pyrithione, and 100 U mL^{-1} GM-CSF. For neutrophil stimulation, 25 µM zinc, 1 µM pyrithione, and 50 U mL^{-1} were best. Those optimal concentrations were used for key experiments. Due to known toxicity of the combination of zinc and pyrithione when used for longer incubations, zinc alone was used to stimulate cells for 24 h.[12] Regarding GM-CSF incubation, 10–180 min of stimulation were tested. Optimal times depended on the experiment and could be found in the figure legends.

2.3. Western Blot

Western blot was performed as previously described by Schröder et al.[13] Membranes were incubated overnight with primary antibodies ((pan ERK1/2, phosphorylated ERK1/2 (T202/Y204), pan p38, phosphorylated p38 (T180/Y182) (all Cell Signaling Technologies, Beverly, USA)), gently shaking overnight. After three times of washing with TBS-T (Tris-buffered saline with Tween (20 mM Tris-HCl (Karl Roth, Karlsruhe, Germany), 150 mM NaCl (AppliChem, Darmstadt, Germany), 0.1% Tween 20 (Sigma-Aldrich)), membranes were incubated with a secondary antibody (HRP-coupled antirabbit IgG, Cell Signaling Technology, Frankfurt, Germany) for at least 4 h at room temperature. To visualize the protein bands, Westar Antares Luminol Enhancer solution (Cynanagen, Bologna, Italy) was used for the detection of total and phosphorylated proteins. Luminescence was analyzed by using a LAS 3000 (Fujifilm Lifescience, Düsseldorf, Germany) and quantified with ImageJ (ImageJ 1.46r, Wayne Rasband, National Institute of Health, USA).

2.4. Reverse Transcription and Real-Time PCR

The total messenger RNA (mRNA) was isolated after cell lysis in 1 mL Extrazol Reagent (7Bioscience GmBH, Neuenburg, Germany) and transcribed into complementary DNA (cDNA) using the qScript cDNA synthesis kit (Quanta Biosciences, Darmstadt, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed on a QuantStudio 3 PCR System.

PCRs for Mcl-1 (hMcl-1_FOR: ATG CTT CGG AAA CTG GAC AT; hMcl-1_REV: TCC TGA TGC CAC CTT CTT CTA GG) and the housekeeping gene glycerinaldehyde 3-phosphate dehydrogenase (GAPDH; hGAPDH_FOR: GAA GGT GAA GGT CGG AGT C; hGAPDH_REV: GAA GAT GGT GAT GGG ATT TC) (all Sigma Aldrich) were performed with 5 µL cDNA in 20 µL reaction volumes in duplicates using Power SYBR Green PCR Mastermix (Applied Biosystems by Thermo Fisher Scientific, Warrington, UK) with the following parameters: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for Mcl-1 or GAPDH. Expression was calculated as fold of control using the ΔΔCt method.

2.5. Flow Cytometry

2.5.1. Free Zn^{2+} Measurement

Free intracellular zinc measurement was performed as described previously.[14] In brief, free intracellular zinc was measured using the membrane-permeant fluorescent sensor ZinPyr-1 (Santa Cruz Biotechnology, St. Finell, Dallas, TX, USA) at final concentration of 10 µM. Flow cytometric data were recorded on a FACSCalibur (BD Biosciences, Heidelberg, Germany) using Cellquest software 3.0. The concentration of intracellular labile zinc was calculated from the mean fluorescence with the formula $[Zn] = K_D \times [(F-F\text{min})/(F\text{max}-F)]$ using a specific dissociation constant (Zn/ZinPyr-1 complex: 0.7 nM).

2.5.2. Cell Surface and Intracellular Staining of GM-CSF Receptor

Cells were harvested for surface staining, washed, and resuspended in 100 µL PBS+ 1 % BSA (AppliChem, Darmstadt, Germany). Surface staining of the cells was performed by incubating with FITC-conjugated antihuman CD116 antibody (BioLegend, Koblenz, Germany) and APC-labeled CD14 monoclonal antibody (BD Biosciences) or respective isotype controls (FITC Mouse IgG1κ isotype control, APC Mouse IgG2a isotype control, all BD Biosciences). This was followed by a washing step.

For intracellular staining, cells were harvested, washed with PBS, and resuspended in 100 µL PBS+ 1% BSA. Cells were fixed and permeabilized using BD Cytofix/Cytoperm buffer and washed twice with the BD Perm/Wash buffer (both solutions obtained from BD Biosciences). Intracellular staining was then performed by incubating with antihuman antibody against CD116

or respective isotype. The cells were washed two times with BD Perm/Wash buffer and resuspended in 300 µL PBS+ 1% BSA.

Samples were recorded on a FACSCalibur (BD Biosciences) and analyzed using Cellquest software 3.0. Gating strategies for analyzing cell type specific GM-CSFR expression were illustrated in Figure S2 (Supporting Information).

2.6. Quantification of Neutrophil Apoptosis Using Annexin V and Propidium Iodide Staining

For determination of cell viability concerning FACS assay conditions and longer GM-CSF incubation times (4 and 24 h), zinc preincubated and GM-CSF stimulated PMN were washed and taken up in PBS.

Isolated cells were washed once with PBS and resuspendend in PBS with calcium chloride (2.5 mM). 5 µL annexin V APC (ImmunoTools) were added according to the manufacturers' protocoll for 10 min. Subsequently, propidium iodide (PI, 10 µg mL^{-1}, Sigma-Aldrich) was added for another 5 min. In the case of only analyzing PI staining, PI was added after washing the cells for 5 min without previous Annexin V staining. Using flow cytometry, the percentage of annexin V and PI positive cells was determined.

2.7. Fluorescence Microscopy

Neutrophils were stained with ZinPyr-1 (5 µM, zinc), Hoechst (nucleus), and either ER tracker (endoplasmic reticulum), lysotracker (lysosomes), or golgi tracker (golgi apparatus), using commercially available kits, according to the manufacturer's instructions. LysoTracker Red (Molecular Probes, Eugene, OR, USA) was used to label the lysosomes, BODIPY TR Ceramide (Thermo Fisher, USA) was used to visualize the golgi apparatus and labeling of the endoplasmatic reticulum (ER) was performed with ER-ID Red assay kit (Enzo Life Sciences, Lörrach). Images were captured using a AXIO observer (Carl Zeiss, Jena, Germany) with 400× magnification.

2.8. Statistical Analysis

After testing for normal distribution by Shapiro–Wilk normality test and elimination of outliers by Grubb's test, statistical significances were calculated by the appropriate test indicated in the figure legend. Comparisons among different conditions were implemented using GraphPad prism software (V.8, GraphPad software, San Diego, CA, USA).

Statistical analysis was performed by a paired student's *t*-test, provided that the values were normally distributed. Statistically significant differences were represented by different asterisks (*t*-tests: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

For multiple comparisons one-way ANOVA was used together with Tukey's post hoc test if values were normally distributed. If values were not normally distributed, Friedman test was used together with Dunn's multiple comparison test. All titration series (Supporting Information) were analyzed using Dunnet's test as follow up test to ANOVA. Statistically significant differences were represented by different letters (ANOVA with respective post hoc test as indicated in the figure legend, $p < 0.05$).

3. Results

3.1. Zinc Incubation Increases GM-CSFR Surface Expression in PMN and Lymphocytes Whereas Monocytes Show an Internalization of Surface GM-CSFR

To assess the role of zinc in GM-CSF signaling of neutrophils, we investigated GM-CSFR expression in leukocyte subsets by FACS examination. According to our titration experiment with zinc and pyrithione (Figure S3, Supporting Information), only the combination of 50 µM zinc and 1 µM pyrithione had a significant effect regarding GM-CSFR expression (CD116) for all three leukocyte subsets (lymphocytes, monocytes, PMN) and was thus used for the first sets of zinc supplementation experiments. The effects of GM-CSF stimulation on GM-CSFR surface expression were tested after 10, 15, and 30 min of stimulation (Figure S4, Supporting Information). Although alterations in GM-CSFR expression induced by zinc were obtained for all time points, the 10 min stimulation achieved the most significant results (Figure S4, Supporting Information). We excluded any significant toxicity of the chosen stimulants and time frame, since the number of dead cells was always below 10% in PI staining of leukocytes, gated on PMN, monocytes as well as lymphocytes (Figure S5, Supporting Information).

Since zinc often has a direct influence on the substrates, as shown for LPS,[15] preincubation instead of a simultaneous incubation with zinc and GM-CSF was performed. Preincubation with zinc and pyrithione in this concentration induced a significant increase of GM-CSFR expression on the cell surface of gated PMN. When zinc treatment was immediately followed by GM-CSF stimulation no additional effect on the zinc-induced GM-CSFR upregulation was detected (**Figure 1**A). Similarly, GM-CSF incubation alone did not affect GM-CSFR surface expression (Figure 1A). Hence, GM-CSFR upregulation was primarily driven by zinc preincubation for 30 min. Equivalent results were obtained when purified neutrophils were examined (data not shown).

To see if the increase in GM-CSFR surface levels is specific to PMN or can be generalized to cells of the myeloid lineage, we took a closer look at monocytes and lymphocytes regarding their GM-CSFR expression. Interestingly, lymphocytes react to zinc preincubation with an increase in GM-CSFR expression (Figure 1C). In contrast to PMN and lymphocytes, monocytes show a decrease of GM-CSFR surface expression after zinc preincubation for 30 min (Figure 1B). Such as for PMN, GM-CSF stimulation alone or in combination with zinc treatment did not alter the GM-CSFR surface expression in lymphocytes and monocytes (Figure 1A–C).

Figure 1D illustrates the opposing reactions of PMN and monocytes with respect to their GM-CSFR surface expression after zinc administration: Unstimulated PMN (MFI = 15.83) shows a significantly lower basal GM-CSFR surface expression compared to unstimulated monocytes (MFI = 36.35) (Figure 1D). The low basal GM-CSFR expression in neutrophils increases after zinc administration (MFI = 21.91) (Figure 1D). Thus, zinc-increased GM-CSFR expression in PMN approach levels of the GM-CSFR expression in zinc-treated monocytes (MFI = 24.57) (Figure 1D). Hence, zinc treatment results in a comparable GM-CSFR expression in monocytes and PMN (Figure 1D). In

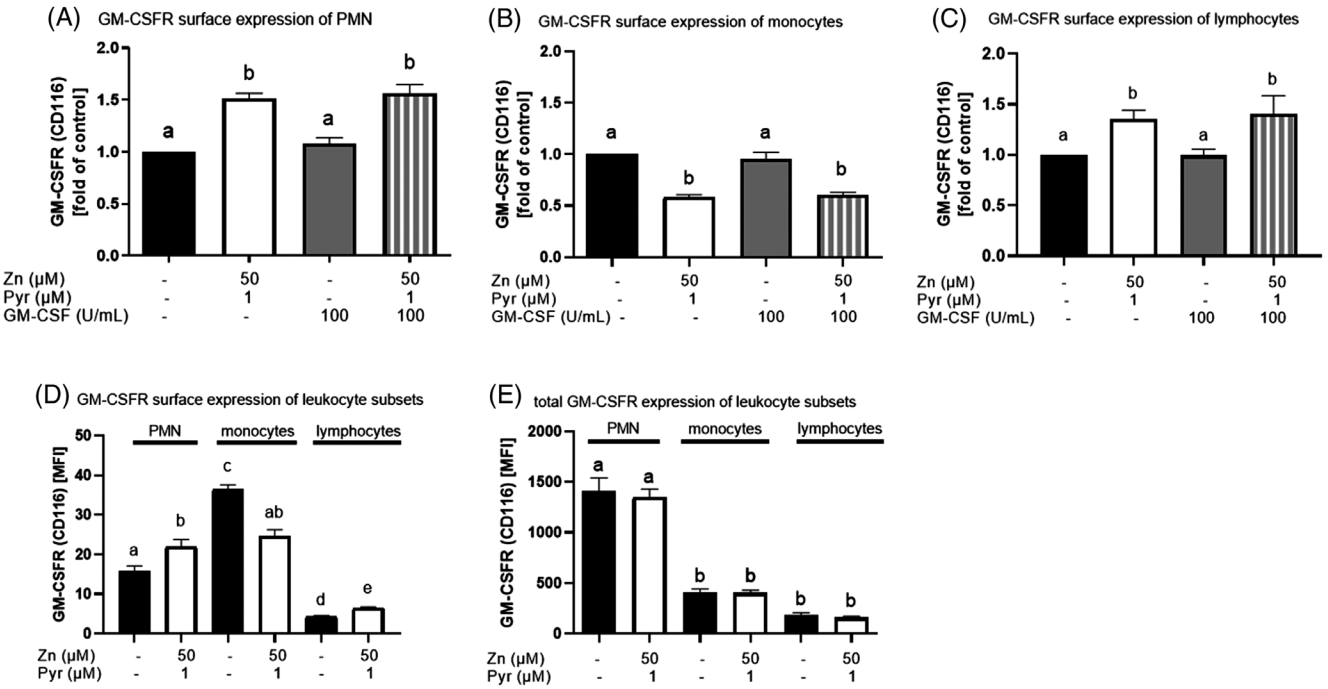

Figure 1. Influence of zinc on cell surface and total GM-CSFR expression in PMN, monocytes and lymphocytes. Expression of GM-CSFR on the surface of gated PMN A), monocytes B), and lymphocytes C) was detected using flow cytometry. Cells were incubated with or without zinc (50 µM) and pyrithione (1 µM) for 30 min. Subsequently, GM-CSF (100 U mL^{-1}) was added for 10 min. The results are normalized against the unstimulated control. Monocytes, PMN, and lymphocytes differ significantly in GM-CSFR surface expression D), whereas total GM-CSFR expression is highest in PMN E). Only GM-CSFR surface expression is altered by zinc D, E). Values are presented as mean + SEM from at least $n = 4$ experiments A, B, C) or $n = 7$ experiments D, E). Statistical significances, calculated by one-way ANOVA with Tukey test, are indicated. Bars that do not share the same letters are significantly different ($p < 0.05$) from each other.

contrast, lymphocytes show negligibly low GM-CSFR expression on the cell surface and in total compared to PMN and monocytes (Figure 1D,E).

3.2. Zinc Supplementation has No Influence on the Total GM-CSFR Expression in Each Leukocyte Subset

Figure 1A–D show cell surface expression of GM-CSFR. To explore a possible mechanism, explaining the quick alteration of GM-CSFR on the cell surface of PMN and monocytes after zinc treatment, we analyzed the total GM-CSFR content in leukocytes (Figure 1E): only GM-CSFR surface expression in leukocyte subgroups was altered by zinc whereas total GM-CSFR expression remains unaffected (Figure 1E). Accordingly, new receptor formation is unlikely as explanation for receptor dynamics at the cell surface of PMN, but receptor redistribution can be assumed. Compared to other leukocyte subgroups, PMN shows the highest total GM-CSFR expression (Figure 1E). Hence, a high intracellular receptor pool for PMN is proven.

3.3. GM-CSF Stimulation Causes a Short-Term Redistribution of Intracellular Zinc Using ZinPyr-1 Probe

For a variety of stimuli a zinc flux has been described,[16] thus, we investigated if stimulation with GM-CSF influences intracellular zinc homeostasis. Using the fluorescent probe ZinPyr-1,[17–19] intracellular free labile zinc of gated PMN (**Figure 2**A), monocytes (Figure 2B), and lymphocytes (Figure 2C) was measured 15 min after GM-CSF stimulation by flow cytometry. We saw a transient intracellular redistribution of zinc after GM-CSF stimulation in PMN (Figure 2A) whereas there was no change in the intracellular zinc content after GM-CSF stimulation in monocytes and lymphocytes (Figure 2B,C). Implying that ZinPyr-1 stains zinc in organelles,[17–19] a transient redistribution of zinc into the cytoplasm can be assumed, this redistribution may be involved in regulating GM-CSF-induced signaling which was subsequently further investigated. To verify this hypothesis, we carried out a microscopic examination of the intracellular distribution of ZinPyr-1 (Figure S6, Supporting Information). We did not observe cytosol-wide staining with ZinPyr-1 but rather local accumulation (Figure S6, Supporting Information). There was also only little clear costaining of ZinPyr-1 with dyes labeling the golgi apparatus (Figure S6B, Supporting Information). However, we could show a partial overlap of ZinPyr-1 with staining of the ER and lysosomes (Figure S6A,C, Supporting Information). This implies that ZinPyr-1 in PMN detects zinc rather in the organelles than in the cytosol, which is in line with the literature on ZinPyr-1 for other cell types.[17–19] Accordingly, we assume that the GM-CSF-induced increase in free intracellular zinc might explain the additive effect of zinc and GM-CSF on protein phosphorylation (**Figures 3** and 5A).

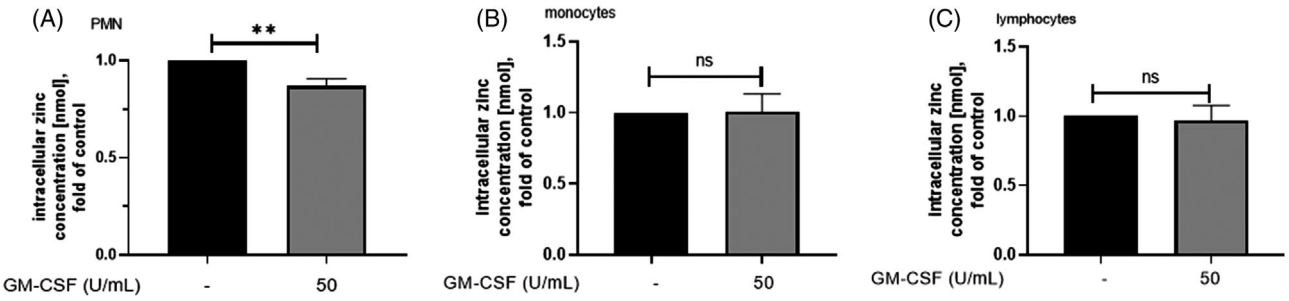

Figure 2. Stimulation with GM-CSF reduces intracellular free zinc level. Zinc flux was detected for PMN A), monocytes B), and lymphocytes C) using ZinPyr-1 levels in flow-cytometry after GM-CSF stimulation (50 U mL^{-1}) for 15 min. Values are presented as mean + SEM from at least n = 14 experiments. Statistical significances, analyzed by Student's *t*-test, is indicated (*$p < 0.05$).

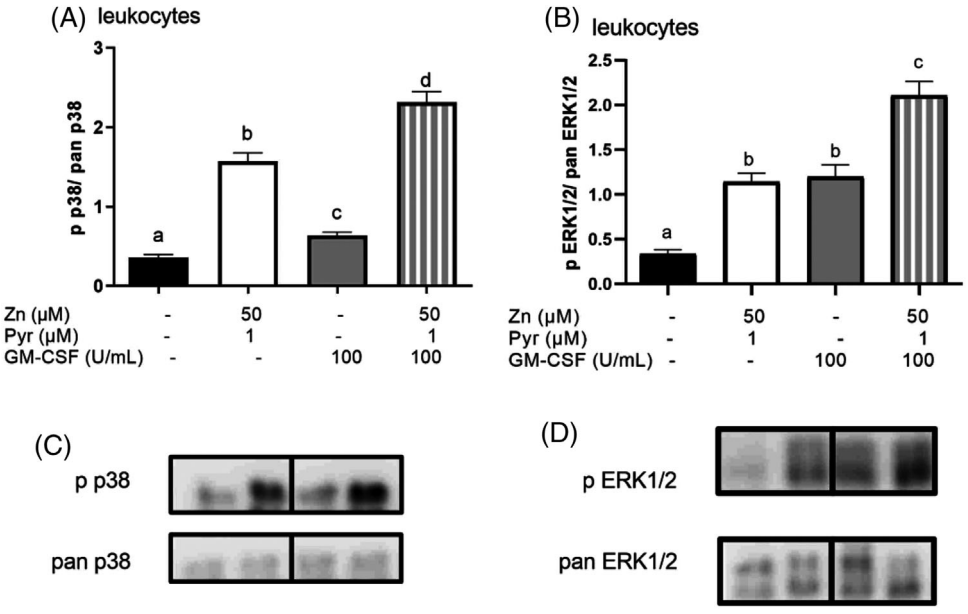

Figure 3. Zinc activates MAPK signaling and augments GM-CSF-induced signaling in human leukocytes. Total leukocytes show a significant increase in phosphorylation of p38 A) and ERK1/2 B) after GM-CSF stimulation, which was further increased by zinc and pyrithione preincubation. Leukocytes were preincubated with zinc (50 μM) and pyrithione (1 μM) for 30 min and subsequently stimulated with GM-CSF (100 U mL^{-1}) for another 15 min. Quantification was performed via optical density determination of $n = 23$ (p38) and $n = 24$ (ERK1/2) blots. Shown are representative western blot for p38 C) and ERK1/2 D). Since additional stimulants were run on the same gel, blots were spliced to only illustrate bands representing the stimulations quantified in A) and B). Results are presented as mean + SEM. Significant different data sets at $p < 0.05$, determined by one-way ANOVA with Tukey's test, do not share the same letters.

3.4. Zinc Activates MAPK Signaling and Augments GM-CSF-Induced Signaling in Total Leukocytes

Since an altered GM-CSFR surface expression was detected after zinc administration and a GM-CSF-induced zinc flux was observed, the effect of zinc homeostasis on signaling in human leukocytes was investigated. The key signaling molecules of the MAPK signaling pathway are ERK1/2 and p38.[5,6,20,21] First, total leukocytes were analyzed for changes in the activation levels of p38 and ERK1/2. Both kinases were activated in response to stimulation with GM-CSF (Figure 3A–D). Zinc strongly increased the phosphorylation of the kinases and GM-CSF application further enhanced the zinc-induced ERK1/2 and p38 phosphorylation in human leukocytes (Figure 3A–D).

3.5. Moderate Zinc and GM-CSF Concentrations Increase p38 and ERK1/2 Phosphorylation, but Avoid Hyper-Stimulation of PMN

Since we found market differences in the effects of zinc regarding GM-CSFR expression for the different leukocyte subtypes, we continued with assessing activation of p38 and ERK1/2 for the three main leukocyte subsets: The separately conducted experiments for monocytes and lymphocytes (Figure S7, Supporting Information) demonstrated that preincubation with zinc (50 μM) and pyrithione (1 μM) increased the phosphorylation of ERK1/2 and p38 in both monocytes and lymphocytes. A subsequent GM-CSF (100 U mL^{-1}) stimulation enhanced the phosphorylation of both kinases to the highest, similar to what was observed for

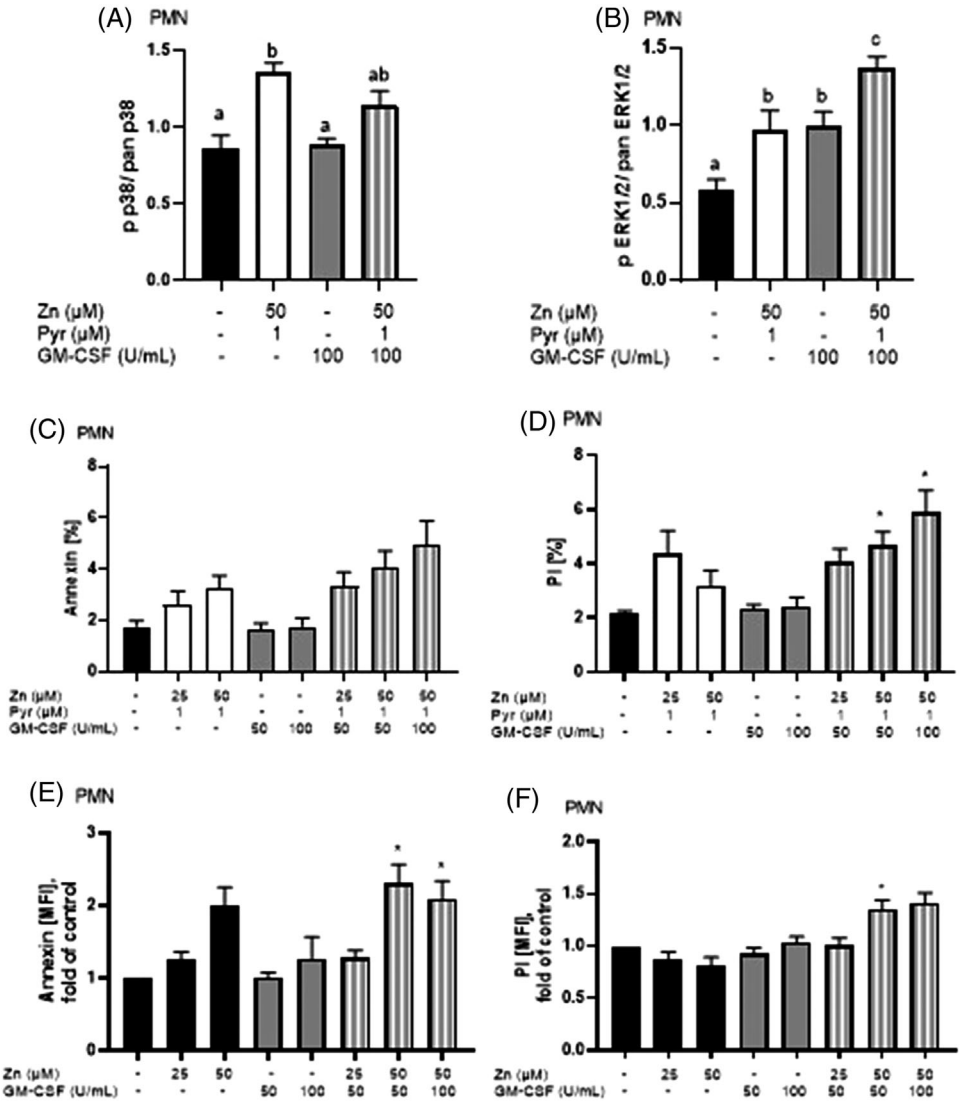

Figure 4. Dose-dependent effect of zinc and GM-CSF concentration on MAPK signaling and apoptosis rate of PMN. In PMN the combination of zinc (50 µM), pyrithione (1 µM), and GM-CSF (100 U mL^{-1}) caused a slightly lower increase in the phosphorylation of p38 compared to incubation with zinc/pyrithione exclusively A). GM-CSF (100 U mL^{-1}) did not increase p38 phosphorylation A). PMN shows a significant increase in ERK1/2 phosphorylation after GM-CSF (100 U mL^{-1}) stimulation, additionally increased by zinc (50 µM) and pyrithione (1 µM) phosphorylation B). Quantification was performed via optical density determination of at least $n = 6$ (p38) and $n = 16$ (ERK1/2) blots. Significant differences at $p < 0.05$, determined by one-way ANOVA with Tukey test, do not share the same letters. Apoptosis and necrosis rates of PMN were verified by annexin V staining after 4 h C) and 24 h E) as well as PI staining after 4 h D), and 24 h F). PMN was preincubated with zinc alone or combined with pyrithione (respective concentration as described in the figure) for 30 min and subsequently stimulated with or without GM-CSF (50 U mL^{-1} or 100 U mL^{-1}) as indicated. Means + SEM of at least $n = 5$ independent experiments are shown. Statistical significances, calculated by one-way ANOVA/Dunnett's multiple comparison test, are depicted (*$p < 0.05$).

total leukocytes (Figure 3). Lymphocytes show significant results, whereas only a trend can be seen in monocytes (Figure S7, Supporting Information).

For PMN, effects of zinc and GM-CSF on ERK1/2 phosphorylation (**Figure 4**B) were comparable to what we observed for total leukocytes. p38 phosphorylation also increased after incubation with zinc (50 µM) and pyrithione (1 µM), whereas GM-CSF (100 U mL^{-1}) stimulation had no significant effect on p38 phosphorylation (Figure 4A). Unexpectedly, the combination of zinc (50 µM), pyrithione (1 µM), and GM-CSF (100 U mL^{-1}) led to a lower increase in p38 phosphorylation compared to preincubation with zinc (50 µM) and pyrithione (1 µM) alone (Figure 4A). Accordingly, we suspected overstimulation of PMN after administration of zinc (50 µM), pyrithione (1 µM), and GM-CSF (100 U mL^{-1}) and determined apoptosis and necrosis rates of PMN. Although no significant increase in annexin V was detected within 4 h of stimulation (Figure 4C), combining 50 µM of zinc and 1 µM ofpyrithione with either 50 U mL^{-1} or 100 U mL^{-1} of GM-CSF resulted in significantly increased PI signals (Figure 4C,D).

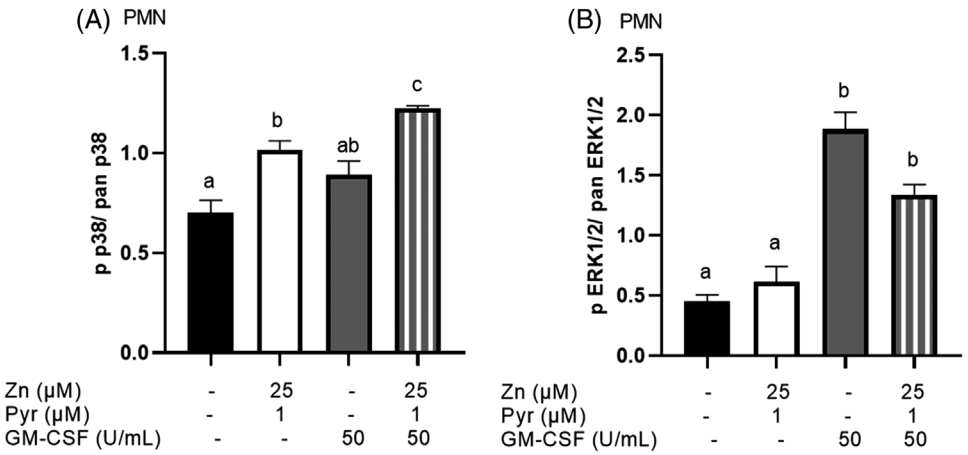

Figure 5. A moderate zinc and GM-CSF concentration activates MAPK signaling and augments GM-CSF-induced signaling in PMN. Working with lower concentrations of zinc (25 μM), pyrithione (1 μM), and GM-CSF (50 U mL $^{-1}$) causes a significant increase in the phosphorylation of both kinases, p38 A) and ERK1/2 B) in PMN. Quantification was performed via optical density determination of at least $n = 6$ (p38) and $n = 16$ (ERK1/2) blots. Results are presented as mean + SEM. Significant differences at $p < 0.05$, determined by one-way ANOVA with Tukey test, do not share the same letters.

Since the use of zinc together with pyrithione is only suitable for short-term experiments, e.g., analyses of intracellular signaling but is largely toxic when used for 24 h,[22] PMN were incubated for 24 h with 50 μM zinc but without pyrithione to assess long-term survival (Figure 4E,F). Combining 50 μM zinc with either 50 U mL^{-1} or 100 U mL^{-1} GM-CSF significantly increased annexin V (Figure 4E) as well as PI signals (Figure 4F) for PMN after 24 h, reinforcing our suggestion that PMN are hyperstimulated. Hence, we tested lower concentrations of zinc and GM-CSF regarding their impact on apoptosis. In annexin V and PI staining lower concentrations of zinc (25 μM) and GM-CSF (50 U mL^{-1}) did not alter the apoptosis and necrosis rates compared to the control after 4 and 24 h (Figure 4C–F). To test if those reduced concentrations are sufficient to activate p38 signaling in PMN, we conducted a titration series for Western Blotting (Figure S8, Supporting Information). We observed that especially low concentrations of zinc had a significant effect on p38 phosphorylation (Figure S8, Supporting Information). From the titration series we concluded that administration of zinc (25 μM), pyrithione (1 μM), and GM-CSF (50 U mL^{-1}) is optimal regarding p38 activation of neutrophils (Figure S8, Supporting Information, and **Figure 5**A). Moreover, the slightly lower concentrations of zinc (25 μM) combined with pyrithione (1 μM) were still sufficient to significantly increase GM-CSFR expression on the cell surface of PMN as measured by FACS (Figure S3, Supporting Information).

In contrast, a significant increase in ERK1/2 phosphorylation is only caused by more than 50 μM of zinc compared to the control (Figure S8, Supporting Information and Figures 4B and 5B). Consequently, the use of only 25 μM zinc has no significant effect on ERK1/2 phosphorylation (Figure 5B). Compared to p38 activation, GM-CSF (50 U mL^{-1}) has a significant effect on ERK1/2 phosphorylation, whereas subsequent zinc administration (25 μM) does not cause any further increase in ERK1/2 phosphorylation (Figure 5B). Thus, we suggest a higher zinc sensitivity for p38 in comparison with ERK1/2.

For PMN, we examined the phosphorylation of p38 and ERK1/2 after 15, 30, 60, and 180 min of GM-CSF stimulation (Figure S9, Supporting Information). The most significant results were found for 15 min of GM-CSF stimulation (Figure S9, Supporting Information), similar to the western blot in total leukocytes (Figure 3).

3.6. Zinc Pre-Incubation Increases mRNA Expression of MAPK-Dependent Gene Mcl-1

Having determined the optimal concentration of zinc and GM-CSF based on apoptosis measurements (Figure 4C–F) and the titration series (Figure S8, Supporting Information) we were interested in ERK1/2 and p38-specific gene expression of Mcl-1. Mcl-1 has already been described in literature as a downstream event in the MAPK signaling being characteristic for PMN activation, functioning, and survival.[23] Incubation with zinc (25 μM), pyrithione (1 μM), and GM-CSF (50 U mL^{-1}) led to a significant increase in Mcl-1 expression (**Figure 6**). We used concentrations of zinc, pyrithione, and GM-CSF as evaluated in the titration series (Figure S8, Supporting Information) to avoid a suggested overactivation in the case of higher concentrations.

4. Discussion

Mature neutrophils are the first line of defense against infection and any misreaction has severe consequences. The importance of zinc for cellular signaling and function of myeloid cells was suggested by previous studies,[4,7,8] but there are hardly any studies that have been conducted with primary cells on this topic. Hence, our report focuses on the effect of zinc supplementation on GM-CSFR expression and GM-CSF-induced signaling in mature PMN. Zinc concentrations chosen (25–50 μM) for our in vitro studies were proven to be physiological relevant and equivalent to effective doses chosen for zinc supplementation in humans in vivo.[11,24] Our study reports for the first time a different zinc-affected GM-CSFR expression within the myeloid lineage. Zinc administration increases the GM-CSFR surface expression in PMN while it diminishes expression in monocytes.

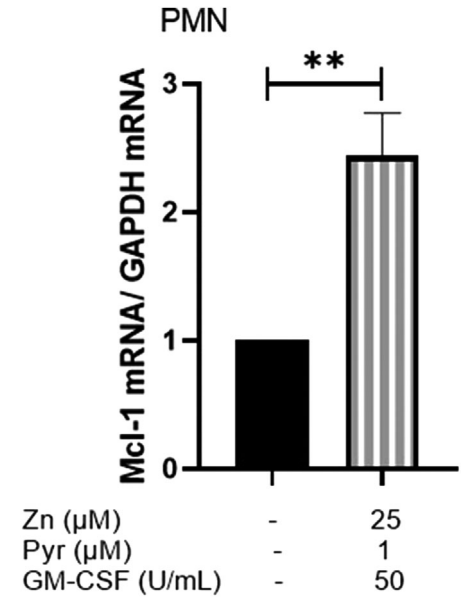

Figure 6. Impact of zinc on Mcl-1 expression. Effect of zinc (25 μM) and pyrithione (1 μM) preincubation for 30 min with subsequent GM-CSF stimulation (50 U mL^{-1}) for 3 h on Mcl-1 mRNA expression level in isolated PMN was quantified by RT-PCR and analysis was implemented using the ΔΔCt method for calculation. Bars indicate mean +SEM of $n = 7$ independent experiments. Statistical significances, analyzed by student's *t*-test, is indicated (**$p < 0.01$).

Moreover, mild zinc administration enhances the phosphorylation of the MAPK signaling pathway which is further increased after GM-CSF stimulation. Only moderate doses of zinc and GM-CSF should be used, otherwise PMN overactivation may occur. Ultimately, activation of the MAPK signaling pathway by combined treatment with zinc and GM-CSF is followed by increased MAPK-dependent Mcl-1 mRNA expression.

Previous studies by Aster et al. revealed an effect of zinc on GM-CSFR surface expression using the promyeloid cell line U937.[4] We were curious to see, if similar effects may be achieved in primary mature myeloid cells and to test, if there are differences depending on the leukocyte cell type.

Firstly, we found that in unstimulated cells, GM-CSFR surface expression is significantly higher in mature primary monocytes than in PMN. This corroborates already published data in unstimulated cells.[25–27] Secondly, we found striking differences between monocytes and PMN after zinc supplementation: the GM-CSFR surface expression of mature PMN was temporarily increased by zinc preincubation. In contrast, monocytes showed a decrease of GM-CSFR surface expression after zinc supplementation. The zinc-induced decrease of GM-CSFR on the cell surface in monocytes is not further affected by GM-CSF stimulation. A novel finding is the approximation of GM-CSFR surface levels between monocytes and PMN which occurs after short-term preincubation with zinc.

Referring to literature, our observation in mature monocytes fits well with data of Aster et al.[4,28] who detected a decrease in GM-CSFR surface expression for the early myeloid cell line U937 after zinc and GM-CSF administration. Since Aster et al.[4] suggested that zinc affects receptor endocytosis in GM-CSF-induced signaling in U937 cells, zinc might have an impressive impact on the alteration of membrane fluidity in mature granulocytes and monocytes. Thus, we propose that this could be a mechanism responsible for the downregulation of GM-CSFR expression in mature primary monocytes, as well. However, little is known about the influence of zinc on membrane composition in primary cells, which should be the focus of further studies to assess the clinical significance of this in vitro observation.

Thirdly, we were interested to explain the rapid increase in GM-CSFR on the cell surface of PMN. We determined the intracellular GM-CSFR content and found that zinc supplementation does not change the total content of GM-CSFR in monocytes, lymphocytes, and PMN. Thus, zinc probably influences the distribution of GM-CSFR between the cell surface and the intracellular reservoir. This largely rules out de novo GM-CSFR synthesis.

Differences in GM-CSFR surface expression between PMN and monocytes were already suggested by the literature.[25–27] We verified this finding and extended it regarding a difference in GM-CSFR surface expression of lymphocytes compared to PMN and monocytes. Since the mean fluorescence of GM-CSFR surface expression is rather independent from the number of cells analyzed, the difference in MFI of surface GM-CSFR expression is hardly explained by higher numbers of PMN, compared to lymphocytes or monocytes. According to our FACS data, monocytes and PMN have roughly the same diameter (Figures S1,S2, Supporting Information). Thus, the higher basal GM-CSFR surface expression detected for monocytes is probably not due to a difference in the cell size. A difference in cell size and thus surface area may however explain the low level of GM-CSFR, found for lymphocytes, which remains to be examined further.

We were the first describing that PMN show the highest total GM-CSFR expression compared to monocytes and lymphocytes. It is notable that the total GM-CSFR expression of PMN is several times higher in relation to the cell surface expression.

Walker et al.[29] discussed internalization and GM-CSF receptor recycling and delineated that an appreciable amount of internalized surface GM-CSFR is recycled intracellularly for renewed ligand binding. However, the exact mechanisms underlying the assumed transport of GM-CSFR to the cell surface in PMN remain to be clarified. Further in vivo studies should focus on the link between total and surface GM-CSFR expression in all leukocyte subsets. Moreover, it would be interesting to find out whether the zinc-induced increase in GM-CSFR surface expression by PMN and thus approximation of GM-CSFR levels expressed by monocytes, has clinical relevance. Since we had seen a modified GM-CSFR expression after zinc supplementation and a transient intracellular redistribution of zinc, we investigated the effect of zinc on two important signaling molecules of the MAPK cascade, ERK1/2 and p38. As was partly expected from literature, we observed a zinc-mediated increase in p38 and ERK1/2 phosphorylation in total leukocytes, isolated monocytes and lymphocytes in great detail.[3,20,21,30] The respective mechanisms by which zinc manifests its influence have been reported.[3,21,31,32] According to those reports zinc probably exerts the effect on ERK1/2 and p38, found here, via the inhibition of dual-specific MAPK phosphatases of MEK and ERK.[3] Normally, phosphatases terminate the MAPK signaling.[3] Since the MAPK phosphatase activity was inhibited, zinc supplementation results in a slower

dephosphorylation and consequently longer activity of MAPK.[3] This enables a prolonged transmission of the stimulation signal into the cell nucleus.[3]

Interestingly, in the case of PMN the administered doses needed to be optimized and lower concentrations of zinc (25 μM), pyrithione (1 μM), and GM-CSF (50 U mL^{-1}) resulted in synergistic effects on phosphorylation of p38. Those data underline the high sensitivity of PMN as regularly reported by others.[33,34] However, the difference in the optimal dosage for synergistically activating MAPK signaling in total leukocytes compared to PMN is probably due to the interaction between monocytes, lymphocytes, and PMN within the leukocyte samples. This point is quite interesting and it awaits further investigation how the interplay between the cell types may buffer zinc. Still, the synergistic effect of GM-CSF and zinc on the MAPK signaling pathway in total leukocytes and PMN has not been described before. Our data are in line with data from other studies, where zinc was administered together with other substances and synergistic effects were observed as well.[35–37] It has for example been observed that zinc can augment effects of other drugs, trace elements, and vitamins such as vitamin D3 as well as it may reduce the dosage necessary to reach optimal effects.[35–37] This was demonstrated here especially for the sensitive PMN, while higher concentrations may be necessary to affect GM-CSFR-induced signaling in monocytes and lymphocytes.

Furthermore, it has been reported that not every stimulus influences the activation of p38 and ERK1/2 to the same extent.[38] Consistent with the literature is our observation that GM-CSF is a less potent stimulus of p38 activity in PMN and leukocytes compared to ERK1/2 activity.[39]

Results from Sun et al.[40] support our observation that the quantity of GM-CSF determines whether monocytes or granulocytes and respective cellular processes for both leukocyte subtypes are favored. In short, Sun et al.[40] demonstrated that low quantities of GM-CSF promote the granulocytic lineage, most likely through prolonging survival, while high quantities reinforce the monocytic lineage, mainly through proliferation. This evidence promotes our observation that investigation of GM-CSF-induced signaling in PMN is best at mild, moderate GM-CSF concentrations.

A significant zinc-mediated increase in Mcl-1 mRNA expression points out the importance of an adequate zinc status for MAPK-dependent gene expression in PMN. This is not surprising, since Mcl-1 is reported to be ERK1/2 and p38 dependent[23] and an exclusive marker protein of PMN.[33,34] Dyugovskaya et al.[38] delineated that, depending on the stimulus, especially the p38-mediated Mcl-1 expression in PMN is crucial. This is in line with the data described here, suggesting that zinc's significant augmentation of Mcl-1 expression is probably largely due to activation of p38 signaling. Since increased Mcl-1 mRNA expression is suggested to lead to an increased Bcl-2/Bax ratio, this may explain the observed decrease in apoptosis as well.[41] Additional in vitro studies should explore if there is a synergistic or redundant relationship between zinc and GM-CSF with respect to their role in apoptosis, before carefully extrapolating to the in vivo situation.

Experiments performed within the scope of presented study were limited due to the short survival time of PMN.[33,34] Since the focus of this study is on very short-lived, extremely sensitive PMN, only short term effects were investigated here, whereas chronic changes in zinc homeostasis cannot be studied with this model due to the short life span.[33,34] Further studies on long-term effects of zinc on GM-CSF-induced signaling in PMN would require investigating cells from zinc adequate and zinc supplemented individuals.

The present study demonstrates that GM-CSF stimulation and zinc administration affect receptor expression, signaling, protein, and gene expression in a dose-dependent fashion and depending on the leukocyte subgroup. As a novel finding, we present the cell-type-dependent effects of zinc on GM-CSFR surface expression: PMN shows a zinc-induced upregulation of surface GM-CSFR, whereas monocytes respond with an internalization of GM-CSFR. Our results indicate a dose-dependent enhancement of MAPK phosphorylation in PMN after zinc preincubation which was additionally augmented after moderate GM-CSF stimulation. Hence, our study provides a basis for in vivo studies focusing on the therapeutic value of zinc in patients with a disturbed GM-CSF signaling. One possible therapeutical scenario would be to restore the response to GM-CSF in PMN of the elderly: Tortorella et al.[42] and Larbi et al.[43] have already shown that sensitivity to GM-CSF decreases with age. This contributes to a decreased rescue of PMN from apoptosis in elderly individuals.[42,43] According to the fact that the elderly are generally at risk of suffering from zinc deficiency,[44] we presume that mild zinc administration is beneficial to modulate and balance the activity of PMN. Further in vivo studies are strongly required to elucidate the underlying mechanisms in patients with an impaired response to GM-CSF. This would enable to estimate the preventive and therapeutic efficacy of zinc in GM-CSF-induced signaling. Zinc might be an optimal low-threshold and cost-effective therapeutic agent for patients with a compromised GM-CSF response. As mentioned, detailed in vivo research is needed to put this from bench to bedside.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: I.W., M.L., and L.R.; methodology: I.W. and M.L.; validation: M.L., I.W., A.B., J.W., and L.R.; formal analysis: M.L., A.B., J.W., and I.W.; investigation: M.L., A.B., J.W., and I.W.; resources: L.R., I.W.; data curation: M.L., J.W., A.B., and I.W.; writing — original draft preparation: M.L.; writing — review and editing: M.L., A.B., J.W., I.W., and L.R.; visualization: M.L.; supervision: I.W.; project administration: I.W. and L.R. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

GM-CSF receptor modulation, GM-CSF-induced signaling, human polymorphonuclear leukocytes, zinc homeostasis, zinc supplementation

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