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Selective impact of zinc on human circulating CD56^{dim} NK cells

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ABSTRACT

Purpose: Natural Killer (NK) cells are essential for recognizing aberrant and virus-infected cells. Adequate levels of the trace element zinc are known to be important for proper immune functionality against infections and tumor growth. So far, not many studies have analyzed the effects of zinc on NK cells. We previously observed higher levels of activated CD69^{+} NK cells in zinc-adequate compared to zinc-deficient donors. We therefore aimed to analyze the influence of zinc on the activation and functionality of NK cell *in vitro*.

Methods: Peripheral blood mononuclear cells were freshly isolated from young healthy donors. Serum zinc levels and Adjusted Zinc Diet Score (AZDS) were determined. NK cells, CD56^{dim} NK cells, and CD56^{bright} NK cells were analyzed *ex vivo* or cultivated in zinc-deficient, zinc-adequate, and zinc-supplemented media for 1 or 20 h ± specific inhibitors. CD56^{dim} NK cell frequencies, phenotype, and functionality were analyzed via flow cytometry, qPCR, and Western blot.

Results: The frequencies of cytotoxic CD56^{dim} NK cells positively correlated with serum zinc as well as AZDS. CD69 expression was significantly increased after 20 h of zinc supplementation on CD56^{dim} NK cells. CD69^{+} CD56^{dim} NK cells showed significant higher degranulation capacity and IFNγ production. Furthermore, NFκB inhibition upregulated CD69 expression and *NFKB* mRNA was significantly decreased in zinc-supplemented conditions. In addition, perforin levels were sensitive to zinc deprivation.

Conclusion: Extracellular zinc selectively activates CD56^{dim} NK cells enhancing degranulation and IFNγ production. Zinc supplementation might be a useful tool for *in vitro* NK cell expansion protocols for future cellular therapies.

1. Introduction

Natural Killer (NK) cells are an essential part for the first line response of the innate immune system. After T and B cells, NK cells are the most frequent lymphocytes within the human blood (10–20 %) [1]. Here, two NK cell subsets with different functionality can be identified based on their CD56 expression: CD56^{bright} and CD56^{dim} NK cells. CD56^{bright} NK cells are predominantly immunomodulatory and secrete high levels of IFNγ [2]. CD56^{dim} NK cells, on the other hand, are highly cytotoxic and can effectively kill infected or aberrant cells. To execute this functionality, CD56^{dim} NK cells have cytotoxic granules containing perforin and granzymes, in particular granzyme B [3], which are released upon activation.

NK cells express specific receptors, which can either activate or inhibit NK cell functionality [3]. Most receptors, which belong to the NKG2 or Killer cell Immunoglobulin-like Receptor (KIR) family, recognize human leukocyte antigen (HLA) class I molecules and thus inhibit NK cell functionality to protect against elimination. The expression patterns of NKG2A and KIR on NK cells have been shown to correspond to their maturation state. During their development NK cells first express NKG2A, then co-express KIR as well as NKG2A, finally NK cells downregulate NKG2A expression to become mature KIR^{+}NKG2A^{-} NK cells [4]. Of note, KIR^{+}NKG2A^{-} CD56^{dim} NK cells exhibit the most mature phenotype [4]. HLA class I molecules are expressed on all nucleated body cells and are downregulated by viral proteins to escape T cell recognition or during neoplasm development [5,6]. Hence, NK cells effectively kill cells with decreased HLA class I expression due to a lack of inhibition [7]. However, inhibitory signals can be overruled if NK cells receive stronger activation than inhibitory signals [8].

These activation signals can be induced by antibody recognition via antibody dependent cell cytotoxicity, natural cytotoxicity receptor engagement, and/ or soluble factors, such as cytokines and interleukins [8], but potentially also other not yet identified stimuli. One cell surface receptor, which is upregulated early after NK cell activation, is CD69

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[9]. In general, understanding the process of NK cell activation is highly desirable, as NK cells are promising tools for cellular therapeutic applications. Particularly, since NK cells, in contrast to T cells, do not contribute to graft-versus-host disease (GvHD), but instead contribute to the beneficial graft-versus-leukemia effect [10]. Simultaneously, NK cells are important for promoting anti-tumor surveillance and several clinical trials use CAR-NK cells against B cell acute lymphatic leukemia (B-ALL) [11] Therefore, getting a better understanding on different pathways for NK cell activation, is highly desirable.

Zinc is an essential trace element, which enhances immunological functions in physiological concentrations [12]. It is known that zinc deficiency disrupts immune functionality. Since zinc deficiency is a common problem worldwide [12], studying the influence of zinc on the human immune system and to determine if and when zinc supplementation should be administered is of great importance. Studying the effects of zinc can either be done *in vivo* with clinical studies or *in vitro*. To enable the study on zinc, two techniques have been developed for zinc level quantification. Serum zinc levels are widely measured by atomic absorption spectroscopy (AAS) [13]. In addition, our institute developed an 18-item food questionnaire to calculate the uptake of zinc by a phytate corrected Adjusted Zinc Diet Score (AZDS) [14]. For *in vitro* studies, culture media are either deprived of zinc or zinc is added.

So far, not many studies have analyzed the effects of zinc on human NK cells. We previously observed a significant increase in NK cytotoxicity after a one-hour *in vitro* zinc supplementation of the NK cell line YTS as well as peripheral blood NK cells [15]. In line with this, we observed a significant increase in NK cell cytotoxicity after a 2-week *in*

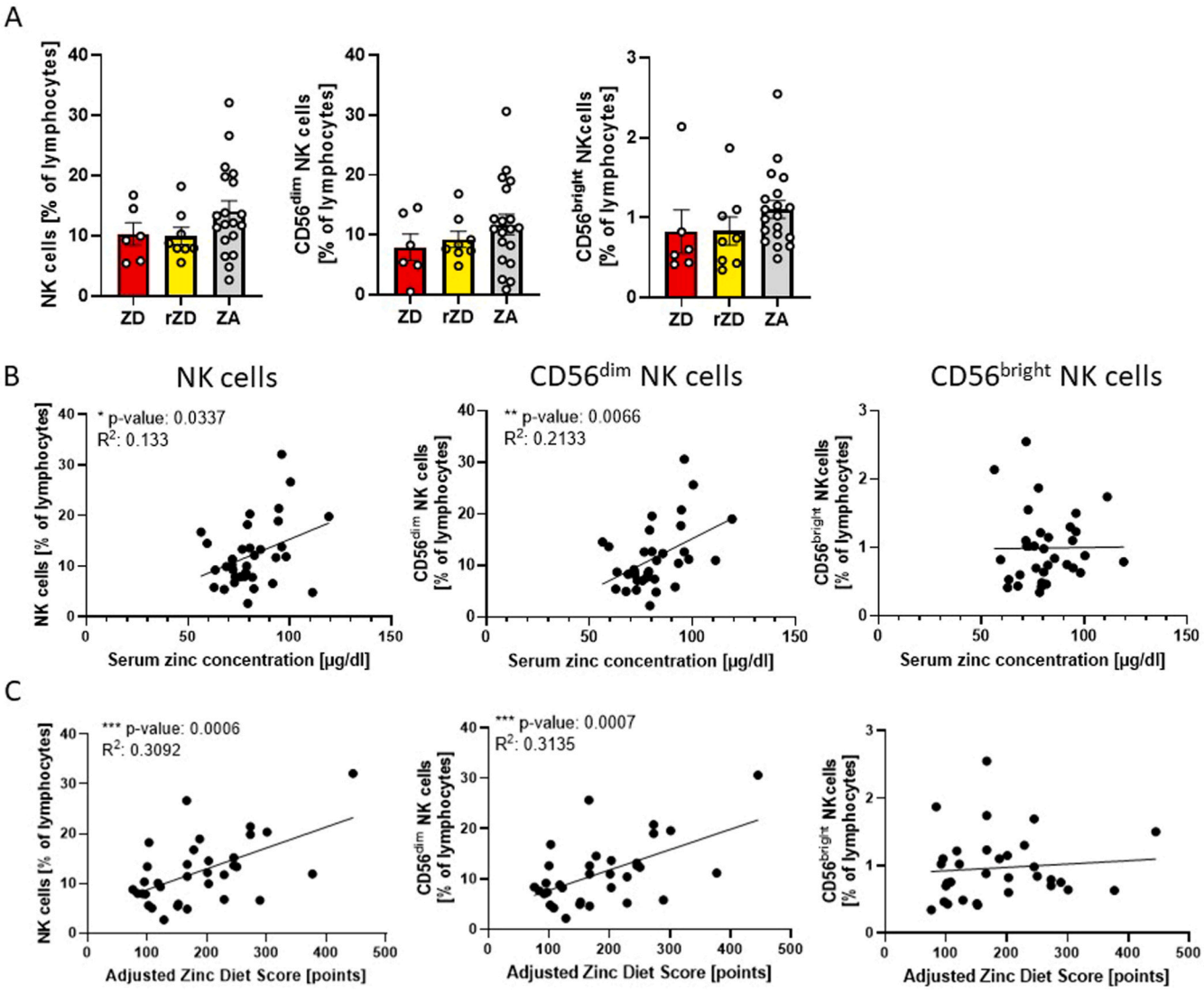

Fig. 1. Total NK cell and CD56^{dim} NK cell frequencies correlate with the adjusted zinc diet score and serum zinc levels. Young healthy volunteers donated venous blood in the morning. Each donor completed the FFQ and one serum tube as well as one heparin blood sample was taken. Of note, for two participants one value of either the serum zinc level or AZDS score was missing, therefore matched values were available for n = 33. PBMCs were isolated and flow cytometrically analyzed for total NK cells, CD56^{dim} NK cells, and CD56^{bright} NK cells. Donors were classified as zinc-deficient (ZD, red bars), if the serum zinc level were < 70 mg/dL, at risk of ZD (rZD, yellow bars), if the serum zinc level > 70 mg/dL but the FFQ was < 113 points or zinc-adequate (ZA, grey bars) if both values were above > 70 mg/dL and > 113 points. Bar graphs showing total NK cell frequencies (left hand side), CD56^{dim} NK cells frequencies (middle), and CD56^{bright} NK cell frequencies (right hand side) for ZD (n = 6), rZD (n = 8), and ZA donors (n = 19) ex vivo (A). Correlations of total NK cell frequencies (left hand side), CD56^{dim} NK cell frequencies (middle), and CD56^{bright} NK cell frequencies with serum zinc concentrations (n = 33, B) or Adjusted Zinc Diet Score (AZDS) (n = 33, C). Each dot represents an individual donor. The data is representative of > 3 independent experiments. The height of the bars represents the mean ± SEM. Levels of significance were calculated using an Ordinary one-way ANOVA (total NK cells and CD56^{dim} NK cells) or non-parametric ANOVA (Kruskal-Wallis test, CD56^{bright} NK cells) (A) or a simple linear regression (B/C), * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, p-values as indicated.

vivo zinc supplementation in young healthy adults [16]. However, we observed no correlation between the initial zinc status and NK cell frequencies, which might have been due to the observed low serum zinc levels [16], since 72 % of our cohort was zinc-deficient [16]. However, we observed significant higher frequencies of activated NK cells, based on CD69 expression, in individuals with adequate serum zinc levels compared to individuals with zinc-deficient serum levels *ex vivo* [16]. This suggests that adequate extracellular zinc levels might activate NK cells.

In order to investigate the effects of zinc on the frequencies and the activation of human peripheral blood NK cells in more detail, we recruited a second cohort of young healthy adults with higher initial serum zinc levels. We co-cultured isolated peripheral blood mononuclear cells (PBMCs) from young healthy donors in different zinc media conditions at two different time points (1 h and 20 h). We studied the effects of zinc on peripheral blood NK cell frequencies *ex vivo* and *in vitro* as well as the functionality and activation state *in vitro*.

2. Results

2.1. $CD56^{dim}$ NK cell frequencies significantly correlate with donor's initial zinc status

In our first study on young healthy adults, we observed no correlation between NK cell frequencies and serum zinc or AZDS *ex vivo* [16]. Since 72 % of our analyzed cohort were classified as zinc-deficient (ZD), we were wondering if a correlation between NK cells and zinc might require a cohort with a broader distribution of initial zinc levels. We therefore recruited a second cohort of young healthy adults (n = 35, of n = 33 participants we had matched serum zinc levels as well as AZDS scores) and divided our cohort in ZD (n = 6), at risk of zinc deficiency (rZD, n = 8), and zinc-adequate (ZA, n = 19) according to a previously published study [17]. Here, donors classified as ZD have serum zinc levels < 70 mg/dl, donors were classified as at risk of ZD if they show adequate serum zinc levels, but an Adjusted Zinc Diet Score < 113 points, and the rest of the donors were classified as zinc-adequate (ZA) (Fig. 1A) [17]. We isolated PBMCs of our cohort and analyzed NK cells, $CD56^{dim}$ NK cells, and $CD56^{bright}$ NK cells via flow cytometry.

As expected, ZA donors showed significantly higher serum zinc levels as well as AZDS compared to ZD donors (Supplementary Figure 1A). As previously reported [14], we observed a significant correlation between serum zinc levels and AZDS (Supplementary Figure 1B). A non-significant tendency of higher total NK cell as well as $CD56^{dim}$ NK cell mean frequencies was observed in ZA donors compared to ZD and rZD donors (total NK cells: ZD vs. ZA p-value: 0.4283, ZA vs rZD p-value: 0.3021; $CD56^{dim}$ NK cells: ZD vs ZA p-value: 0.4222, ZA vs rZD p-value: 0.6336), while hardly any differences between the means in $CD56^{bright}$ NK cells were determined between the three groups (Fig. 1A). We next correlated total NK cell, $CD56^{dim}$ NK cell, and $CD56^{bright}$ NK cell frequencies with the serum zinc levels (Fig. 1B) and AZDS (Fig. 1C). A significant positive correlation was detectable between serum zinc concentration as well as AZDS with total NK cells frequencies (serum zinc: R^2: 0.133, * p-value= 0.0377, Fig. 1B, AZDS: R^2: 0.3092, *** p-value: 0.0006, Fig. 1C). This correlation could be attributed to a significant positive correlation between $CD56^{dim}$ NK cells with serum zinc concentrations as well as AZDS (serum zinc: R^2: 0.2133, ** p-value= 0.0066, Fig. 1B, AZDS: R^2: 0.3135, *** p-value: 0.0007, Fig. 1 C). We observed no correlation between $CD56^{bright}$ NK cell frequencies with serum zinc concentration or the AZDS (Fig. 1B/ C).

Our data demonstrates that the donor`s initial zinc status positively correlated with total NK cell frequencies, in particular $CD56^{dim}$ NK cell frequencies, within a dominantly zinc-adequate cohort. Our observations suggest that changes in extracellular zinc levels have a greater influence on human peripheral blood $CD56^{dim}$ NK cell frequencies compared to $CD56^{bright}$ NK cell frequencies.

2.2. 20 h of zinc supplementation selectively upregulates CD69 expression on $CD56^{dim}$ NK cells

Since we observed positive correlations of initial zinc status to NK cell frequencies, in particular $CD56^{dim}$ NK cell frequencies (Fig. 1), we next wanted to investigate the influence of zinc on peripheral blood NK cells *in vitro*. Hence, we isolated PBMCs from healthy young donors and cultured the freshly isolated PBMCs for 1 h or 20 h in ZD, ZA or zinc-supplemented (ZS) culture media. After 1 h or 20 h, we determined the frequencies of total NK cells, $CD56^{dim}$ NK cells or $CD56^{bright}$ NK cells and their activation status via flow cytometry.

We observed no significant differences between the three zinc media conditions (ZD, ZA, ZS) for the frequencies of total NK cells, $CD56^{dim}$ NK cells or $CD56^{bright}$ NK cells after 1 h of culture (Fig. 2A). We also observed no difference in their activation status (Fig. 2B), which was assessed via CD69 expression.

After 20 h in the three conditions, we observed a significant increase between the ZD and the ZS media conditions for total NK cell frequencies (p-value: 0.0181) (Fig. 2C). However, no change was seen for $CD56^{dim}$ NK cell or $CD56^{bright}$ NK cell frequencies (Fig. 2C). Of note, we observed a significant increase in frequencies of lymphocytes after both time points independent of the zinc condition compared to *ex vivo* (t = 0) (Supplementary Figure 2), which might be explained by the attachment of monocytes during the culture. No significant differences in lymphocyte frequencies were detectable between the three zinc conditions (Supplementary Figure 2).

However, after 20 h of zinc supplementation, a significant upregulation of CD69 expression was seen on total NK cells compared to the ZD or ZA media conditions (p-values: ZS vs. ZD 0.0121, vs. ZA 0.0122) (Fig. 2D). This increase was due to a significant increase in CD69 expression on $CD56^{dim}$ NK cells after zinc supplementation compared to ZD or ZA media conditions (p-values: ZS vs. ZD 0.0101, vs. ZA 0.0125) (Fig. 2D). On the other hand, $CD56^{bright}$ NK cells showed no change in CD69 expression independent of the culture medium.

It is known that CD69 expression on NK cells can be induced by IL-2 [18]. We therefore tested our culture supernatant for IL-2 to determine if CD69 expression was induced by elevated extracellular zinc levels or due to IL-2 production from by-stander populations within the PBMCs. In 2 of 4 donors, we observed a slight increase of IL-2 within the supernatant after zinc supplementation (mean: 12.3 pg/ml IL-2, not detectable < 7.8 pg/ml, data not shown). As expected, CD69 expression on zinc-supplemented $CD56^{dim}$ NK cells cultured for 20 h was significantly higher after an additional stimulation for 3 h with IL-2 (p-value: 0.0312) (Fig. 3E).

Our data demonstrates that neither zinc deprivation nor supplementation for either 1 h or 20 h have an immediate impact on NK cell frequencies. However, in our hands a 20 h zinc supplementation induces the upregulation of CD69 expression on $CD56^{dim}$ NK cells, which suggests a selective activation of $CD56^{dim}$ NK cells by extracellular zinc, which can be further elevated by IL-2 addition.

2.3. $CD69^{+}$ $CD56^{dim}$ NK cells show significantly higher degranulation capacity and higher IFNγ secretion

Our data indicates a selective activation of $CD56^{dim}$ NK cells, a subset with high cytotoxic potential, when supplemented with extracellular zinc (Fig. 2). CD69 expression is known to be induced early after NK cells activation and $CD69^{+}$ NK cells have been described to be highly cytotoxic [9]. Hence, we first aimed to investigate NK cytotoxicity after 20 h of cultivation in the three different zinc media conditions (ZD, ZA, ZS). Therefore, we co-cultured PBMCs after 20 h culture in ZD, ZA, or ZS medium with the HLA class I-deficient cell line K562 in the presence or absence of IL-2 for 4 h. As previously described [16,19], the killing rate was determined by carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and propidium iodide (PI) staining via flow cytometry. We observed no significant differences in the killing rate between the three

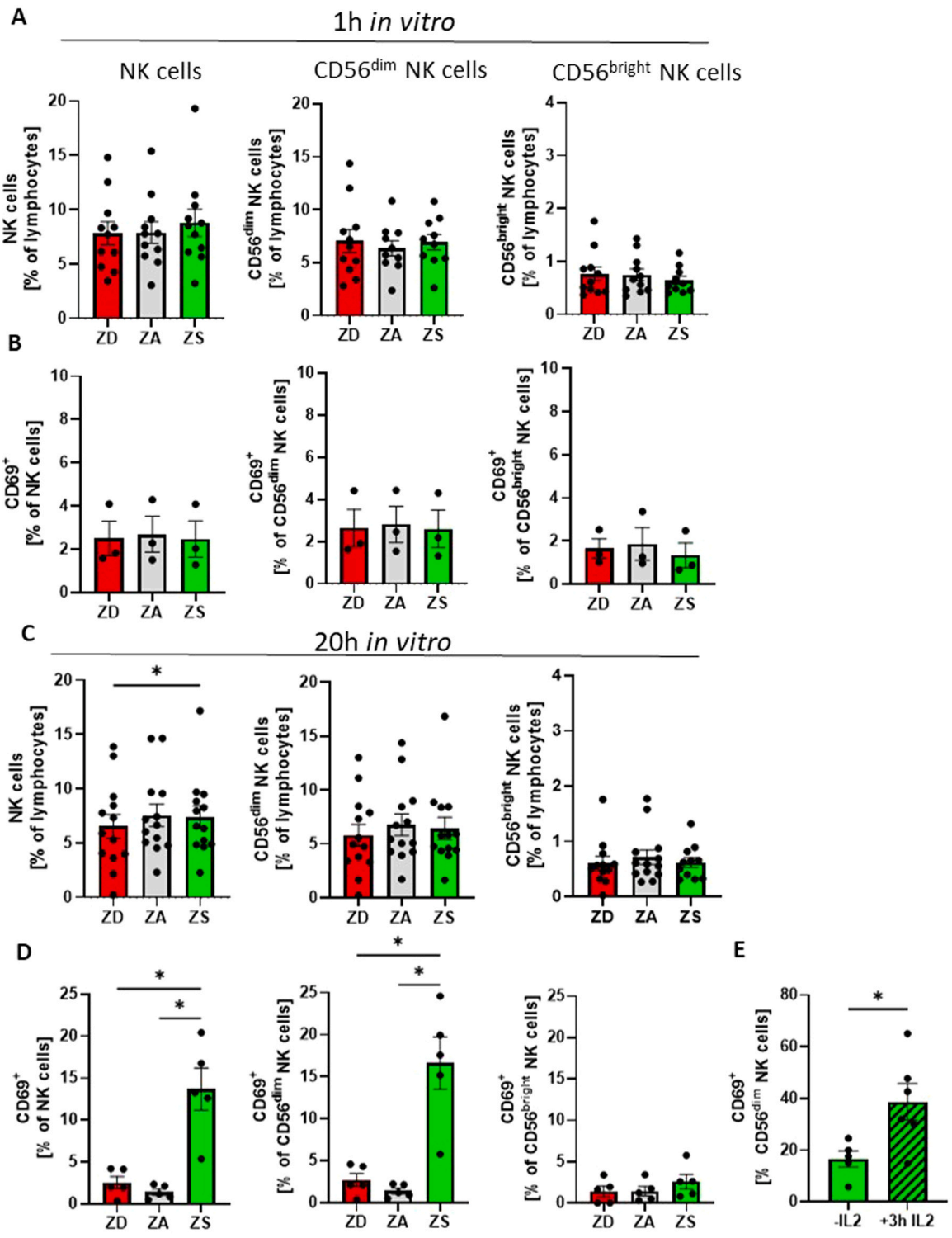

Fig. 2. *In vitro* zinc media conditions do not impact NK cell frequencies. Fresh PBMCs were isolated and cultivated for 1 h (A/B) or 20 h (C/D) in zinc-deficient (ZD; red bars), zinc-adequate (ZA, grey bars), and zinc-supplemented (ZS, green bars) media followed by flow cytometric analyses of the NK cell, $CD56^{dim}$ NK cell, and $CD56^{bright}$ NK cell frequencies (A/C) and corresponding CD69 expression (B/D). Bar graphs showing the frequencies (n = 10, A) and CD69 expression (n = 3, B) of total NK cell, $CD56^{dim}$ NK cell, and $CD56^{bright}$ NK cell frequencies after 1 h incubation in ZD, ZA, or ZS media. Bar graphs showing the frequencies (n = 13, C) and CD69 expression (n = 5, D) of total NK cell, $CD56^{dim}$ NK cell, and $CD56^{bright}$ NK cell frequencies after 20 h incubation in ZD, ZA, or ZS media. Bar graphs showing CD69 expression after 20 h of ZS (green bar) and after 20 h with 3 h 75 Units IL-2 (green/ black bar) (n = 5–6). Each dot represents an individual donor. The experiments are representative of > 3 independent experiments (A-D) or two individual experiments (E). The height of the bars represents the mean ± SEM. Statistical outliers were removed using GraphPad prism. Levels of significance were calculated using an Ordinary one-way ANOVA ((A)-$CD56^{dim}$ NK cells, (D)-all), non-parametric ANOVA (Friedmann test) ((C)-total NK cells, $CD56^{dim}$ NK cells), non-parametric ANOVA (Kruskal-Wallis test) ((A/C) $CD56^{bright}$ NK cells), a repeated measurement one-way ANOVA ((A)-total NK cells, (B)-all) or an unpaired *t*-test (Welch`s test, E), * p-value < 0.05.

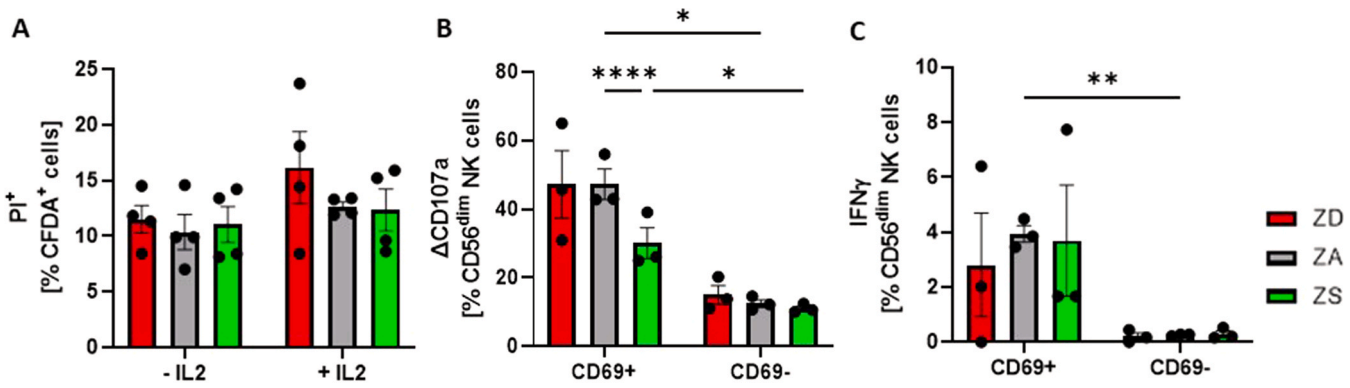

Fig. 3. $CD69^+$ $CD56^{dim}$ NK cells show higher degranulation capacity and IFNγsecretion. Fresh PBMCs were isolated and cultivated for 20 h in zinc-deficient (ZD; red bars), zinc-adequate (ZA, grey bars), and zinc-supplemented (ZS, green bars) media. The HLA-class I deficient cell line K562 was added in an effector target ratio 12.5:1 with (A) or without 75 Unit IL-2 addition (B/D). After 4 h, cytotoxicity (A), degranulation capacity (B), and IFNγ (C) secretion was analysed via flow cytometry. Bar graphs showing the PI^+ $CFDA^+$ K562 cells without or with IL-2 in ZD, ZA, and ZS medium (n = 4, A). Bar graphs showing the degranulation capacity (n = 3, B) and IFNγ (n = 3, C) of $CD69^+$ and $CD69^-$ $CD56^{dim}$ NK cells. Each dot represents an individual donor. The data is representative of one experiment. The height of the bars represents the mean ± SEM. Levels of significances were calculated using a two-way ANOVA (A-C), * p-value < 0.05, ** p-value < 0.01, and **** p-value < 0.0001.

zinc conditions independent of IL-2 addition (Fig. 3A). Of note, with IL-2 addition NK cells cultivated in ZD medium showed a slightly higher mean cytotoxic activity compared to ZA or ZS media conditions (Fig. 3A).

Since we observed no significant differences in cytotoxicity, we compared the degranulation capacity and IFNγ secretion between $CD69^+$ and $CD69^-$ $CD56^{dim}$ NK cells. PBMCs were co-cultured with the HLA class I-deficient cell line K562 as described above, except that we here determined degranulation capacity and intracellular IFNγ levels without IL-2 addition. Degranulation capacity (based on CD107a expression) and intracellular IFNγ were analyzed via flow cytometry. $CD69^+$ $CD56^{dim}$ NK cells showed significantly higher degranulation capacity after cultivation in ZA as well as ZS media conditions compared to $CD69^-$ NK cells cultured in the same media conditions (Fig. 3B). A non-significant higher degranulation capacity was also seen in $CD69^+$ $CD56^{dim}$ NK compared to $CD69^-$ $CD56^{dim}$ NK cells in ZD media conditions (p-value: 0.0736) (Fig. 3B). This suggests an overall higher degranulation capacity of $CD69^+$ compared to $CD69^-$ $CD56^{dim}$ NK cells. When comparing between the different zinc conditions, the degranulation capacity was significantly higher in $CD69^+$ $CD56^{dim}$ NK cells in ZA compared to ZS media condition. No differences in degranulation capacity between the zinc conditions were seen for $CD69^-$ $CD56^{dim}$ NK cells (Fig. 3B).

As IFNγ levels within the supernatant were below the detection limit of our ELISA (data not shown), we analyzed intracellular IFNγ contents via flow cytometry. Detected intracellular IFNγ levels varied between the individual donors (Fig. 3C). Nevertheless, independent of the zinc condition, $CD69^+$ $CD56^{dim}$ NK cells showed higher intracellular IFNγ levels compared to $CD69^-$ $CD56^{dim}$ NK cells (Fig. 3C), which was significant when comparing the ZA media conditions (Fig. 3C). Our data confirms that $CD69^+$ $CD56^{dim}$ NK cells are indeed activated seen by significantly higher degranulation capacities and elevated IFNγ secretion compared to $CD69^-$ $CD56^{dim}$ NK cells.

2.4. *Perforin is sensitive to zinc deprivation in vitro independent of the activation status*

$CD69^+$ $CD56^{dim}$ NK cells showed a significant higher degranulation capacity and IFNγ secretion compared to $CD69^-$ $CD56^{dim}$ NK cells (Fig. 3). The cytotoxic granules granzyme B and perforin are hallmarks for $CD56^{dim}$ NK cells cytotoxicity [3]. We therefore analyzed granzyme B and perforin levels between $CD69^+$ and $CD69^-$ $CD56^{dim}$ NK cells *ex vivo*, after 20 h incubation in different zinc media, and with an additional three-hour IL-2 incubation via flow cytometry.

Ex vivo $CD69^+$ $CD56^{dim}$ NK cells expressed significantly lower granzyme B as well as perforin levels compared to $CD69^-$ $CD56^{dim}$ NK cells (Fig. 4A). After 20 h in the different zinc conditions, no significant differences in granzyme B or perforin levels were observed due to the activation status. Instead, we observed significant differences due to the zinc media conditions. Granzyme B levels showed a slight non-significant increased trend after zinc supplementation in $CD69^+$ $CD56^{dim}$ NK cells (p-value: 0.05333) (Fig. 4B). On the other hand, perforin levels were significantly decreased in ZD media conditions from both $CD56^{dim}$ NK cell activation states compared to ZS media conditions. $CD69^+$ $CD56^{dim}$ NK cells cultured in ZD medium also showed significantly decreased perforin content compared to ZA conditions (Fig. 4C).

We next added IL-2 for additional three-hours to our 20 h conditions and analyzed granzyme and perforin content as well as spontaneous intracellular IFNγ via flow cytometry. In line with our previous observation, we observed a non-significant increase of granzyme B levels after ZS condition in $CD69^+$ $CD56^{dim}$ NK cells (p-value: 0.0743) (Fig. 4D). In line with our observation above, granzyme B levels showed no significant differences between the three zinc conditions (Fig. 4D). Perforin levels were significantly decreased in ZD conditions compared to ZA as well as ZS conditions for $CD69^+$ $CD56^{dim}$ NK cells and to ZA condition in $CD69^-$ $CD56^{dim}$ NK cells (Fig. 4E).

We next compared the spontaneous intracellular IFNγ levels between $CD69^+$ $CD56^{dim}$ NK cells and $CD69^-$ $CD56^{dim}$ NK cells after zinc supplementation and IL-2 addition. $CD69^+$ $CD56^{dim}$ NK cells showed significant higher contents of intracellular IFNγ compared to $CD69^-$ $CD56^{dim}$ NK cells (Fig. 4 F). All in all, our data show perforin is sensitive to extracellular zinc deprivation after 20 h of *in vitro* incubation and $CD69^+$ $CD56^{dim}$ NK cells produce more intracellular IFNγ after zinc supplementation.

2.5. *Zinc media conditions influence KIR and NKG2A expression on $CD56^{dim}$ NK cells*

KIR and NKG2A expression on $CD56^{dim}$ NK cells depend on the age and the underlining genomics of the individual donor [20]. Zinc has been shown to stabilize the binding of KIR with HLA class I molecules and is necessary for their inhibitory function [21–24]. Therefore, we wanted to determine the impact of different zinc media on KIR/ NKG2A expression on $CD56^{dim}$ NK cells and if the activation might impact their expression patterns. PBMCs were isolated and cultured for 20 h in ZD, ZA or ZS media conditions. $CD56^{dim}$ NK cells were analyzed for their CD69, KIR (via a KIR-MIX containing KIR2DL3/L2/S1, KIR3DL1/S1, and KIR2DL1/S1), and NKG2A expression via flow cytometry. The

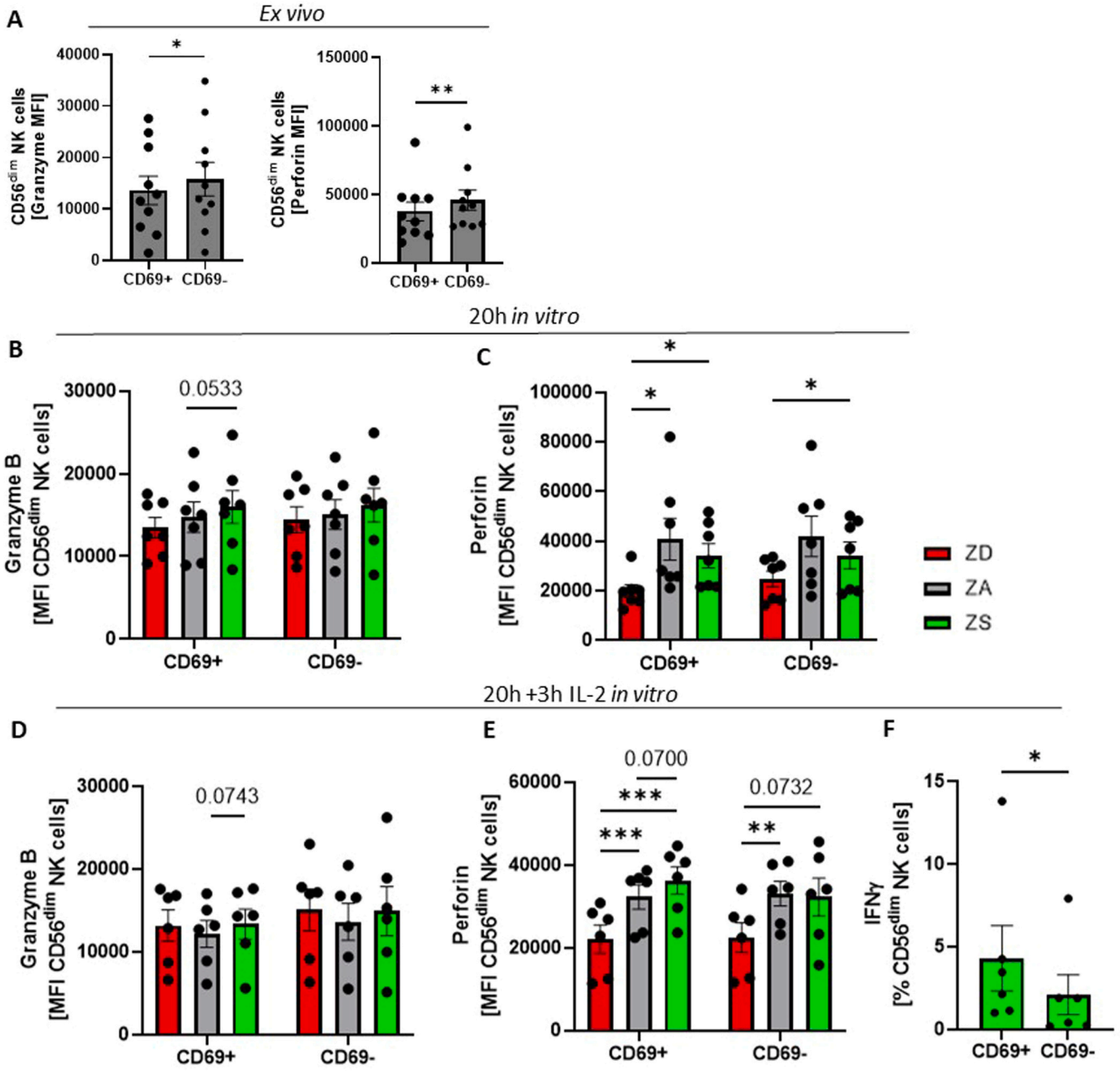

Fig. 4. Perforin levels are sensitive to zinc deficiency. PBMCs were freshly isolated and CD69^{+} and CD69^{-} CD56^{dim} NK cells were compared for their granzyme B and perforin content (by mean fluorescence intensity, MFI) *ex vivo* (A), after 20 h in zinc-deficient (ZD; red bars), zinc-adequate (ZA, grey bars), and zinc-supplemented (ZS, green bars) media (B/C), or 20 h in ZD, ZA, or ZS media plus 3 h of 75 Units IL-2 (D/E) or their spontaneous IFNγsecretion (F). Bar graphs showing granzyme B and perforin levels of CD69^{+} and CD69^{-} CD56^{dim} NK cells *ex vivo* (n = 10, A). Bar graphs showing granzyme B (B) and perforin levels (C) after 20 h of ZD, ZA, or ZS media between CD69^{+} and CD69^{-} CD56^{dim} NK cells (n = 7). Bar graphs showing granzyme B (D) and perforin (E) after 20 h of ZD, ZA, or ZS media with 3 h 75 Units IL-2 between CD69^{+} and CD69^{-} CD56^{dim} NK cells (n = 6). Bar graphs showing spontaneous IFNγ secretion of CD69^{+} and CD69^{-} CD56^{dim} NK cells after 20 h of ZS media with 3 h 75 Units IL-2 culture. The height of the bars represents the mean ± SEM. Each dots represents an individual donor. The data is representative of two individual experiments. Levels of significances were calculated using a paired *t*-test (A, granzyme B), paired Wilcoxon test (A, perforin, F), and two-way repeated measurements ANOVA (B-E), * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

following CD56^{dim} NK cell subsets were identified: KIR^{+}NKG2A^{-}, here called KIR^{only} CD56^{dim} NK cells, KIR^{+}NKG2A^{+} CD56^{dim} NK cells, KIR^{-}NKG2A^{+}, here called NKG2A^{only} CD56^{dim} NK cells, and KIR^{-}NKG2A^{-} CD56^{dim} NK cells.

Overall, no significant differences in KIR or NKG2A expression pattern between CD69^{+}and CD69^{-} CD56^{dim} NK cells cultivated in the same zinc media conditions were observed. KIR^{only} CD69^{+}CD56^{dim} NK cells showed a slight increase in ZS medium compared to ZD medium (p-value 0.2760) (Fig. 5A). While KIR^{only} CD69^{-}CD56^{dim} NK cells decreased significantly in ZS medium compared to ZA and ZD medium (Fig. 5A). An inverse trend was seen for NKG2A^{only} CD56^{dim} NK cells. NKG2A^{only} CD69^{+} CD56^{dim} NK cells decreased in ZS media compared to ZD medium (Fig. 5B). In contrast, NKG2A^{only} CD69^{-} CD56^{dim} NK cells showed a significant increase in ZS medium compared to ZA and ZD medium (Fig. 5B).

KIR^{-}NKG2A^{-} CD56^{dim} NK cells showed a significant increase after zinc supplementation compared to ZD media condition in CD69^{+} CD56^{dim} NK cells or compared to ZA as well as ZD media conditions in

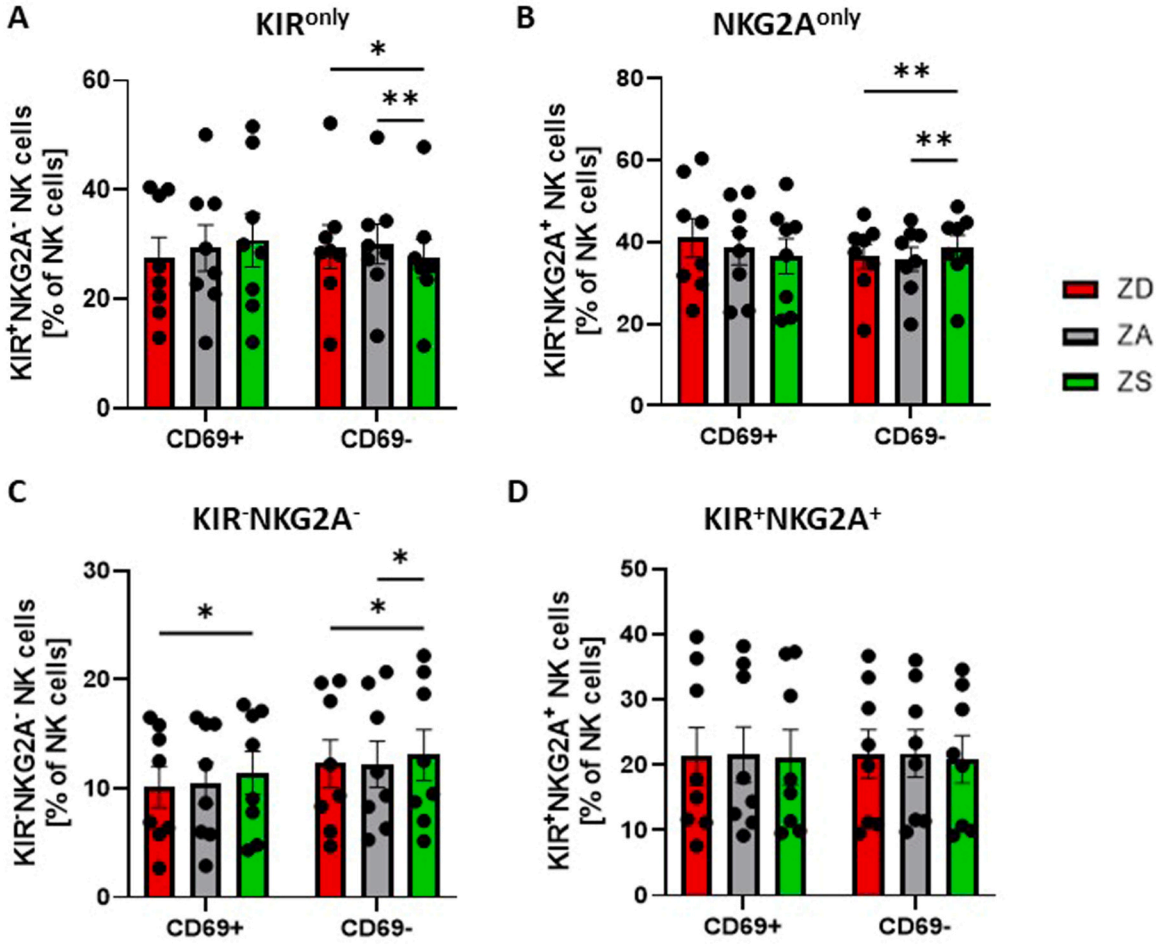

Fig. 5. Zinc media conditions influence KIR/ NKG2A expression on CD56^{dim} NK cells. PBMCs were freshly isolated and incubated for 20 h in zinc-deficient (ZD; red bars), zinc-adequate (ZA, grey bars), and zinc-supplemented (ZS, green bars) media conditions. KIR (comprising a KIR- MIX of anti-human KIR2DL3/L2/S1, KIR3DL1/S1, and KIR2DL1/S1 antibodies) and NKG2A were analyzed on CD69^{+} and CD69^{-} CD56^{dim} NK cells via flow cytometry. Bar graphs showing KIR^{only} (KIR^{+}NKG2A^{-}) (A), NKG2A^{only} (KIR^{-}NKG2A^{+}) (B), KIR^{-}NKG2A^{-} (C), and KIR^{+}NKG2A^{+} (D) CD69^{+} (left hand side) and CD69^{-} (right hand side) CD56^{dim} NK cells (n = 8). The height of the bars represents the mean ± SEM. Each dots represents an individual donor. The data is representative of two individual experiments. Levels of significances were calculated using a two-way repeated measurements ANOVA (A-D), * p-value < 0.05, ** p-value < 0.01.

CD69^{-} CD56^{dim} NK cells (Fig. 5C). No differences between the zinc conditions nor the activation status was seen for KIR^{+}NKG2A^{+} CD56^{dim} NK cells (Fig. 5D).

Our data indicates that extracellular zinc levels might influence KIR and NKG2A expression on CD56^{dim} NK cells, while their expression was independent of the activation status.

2.6. *NFκB/p65 inhibition significantly upregulates CD69 expression and pre-activates NK cells*

Our results indicate that extracellular zinc promotes the selective activation of CD56^{dim} NK cells with higher degranulation capacity and IFNγ secretion (Figs. 2,3). Since we detected hardly any IL-2 in our zinc-supplemented culture supernatants (not shown), we aimed to determine the mechanism of the CD69 up-regulation. Therefore, we added four different inhibitors to our 20 h ZS culture media: JAK inhibitor, MEK inhibitor, NFκB/p65 inhibitor and a p38 MAPK inhibitor and analyzed CD69 expression.

As expected, JAK inhibition prevented the upregulation of CD69 expression on CD56^{dim} NK cells. This was also seen after MEK inhibitor as well as p38 MAPK inhibitor treatment (Fig. 6A). Unexpectedly, inhibition of NFκB/p65 significantly increased CD69 expression on CD56^{dim} NK cells (Fig. 6A/B). An increase in CD69 expression was also seen in CD56^{dim} NK cells cultivated overnight in ZA media as well as the NFκB/p65 inhibitor (Fig. 6C). This suggests NFκB/p65 upregulates CD69 expression independently of additional extracellular zinc levels. However, the CD69^{+} CD56^{dim} NK cells after NFκB/p65 inhibitor treatment showed increased unspecific degranulation and therefore reduced target specific degranulation capacity (Fig. 6D). This suggests CD56^{dim} NK cells might be in a pre-activated or exhausted state after prolonged NFκB/p65 inhibition.

Zinc has been described to activate [25] as well as inhibit NFκB [26]. Hence, we conducted quantitative Real-Time Polymerase Chain Reaction (qPCR) for NFκB mRNA and normalized the expression to GAPDH [27] as housekeeping gene from PBMCs cultured in the three different zinc conditions. We observed a significant reduction of *NFKB* in ZS media compared to ZD media conditions and a reduced expression between ZA to ZD conditions (p-value: 0.0591) (Fig. 6E). This suggests extracellular zinc might decrease NFκB expression in CD56^{dim} NK cells to promote CD69 expression.

We further observed a decrease in CD69 expression after MEK as well as p38 inhibition. We therefore conducted Western blot analyses for pan as well as phospho CREB, a downstream target of MEK [28], and p38. We observed no clear indication that CREB is increased in our cultures after zinc supplementation (Supplementary Figure 3). However, we observed a significant increase in p38 phosphorylation after zinc supplementation compared to ZA conditions (Fig. 6F). This might indicate a regulation of CD69 expression by phosphorylation of p38 in CD56^{dim} NK

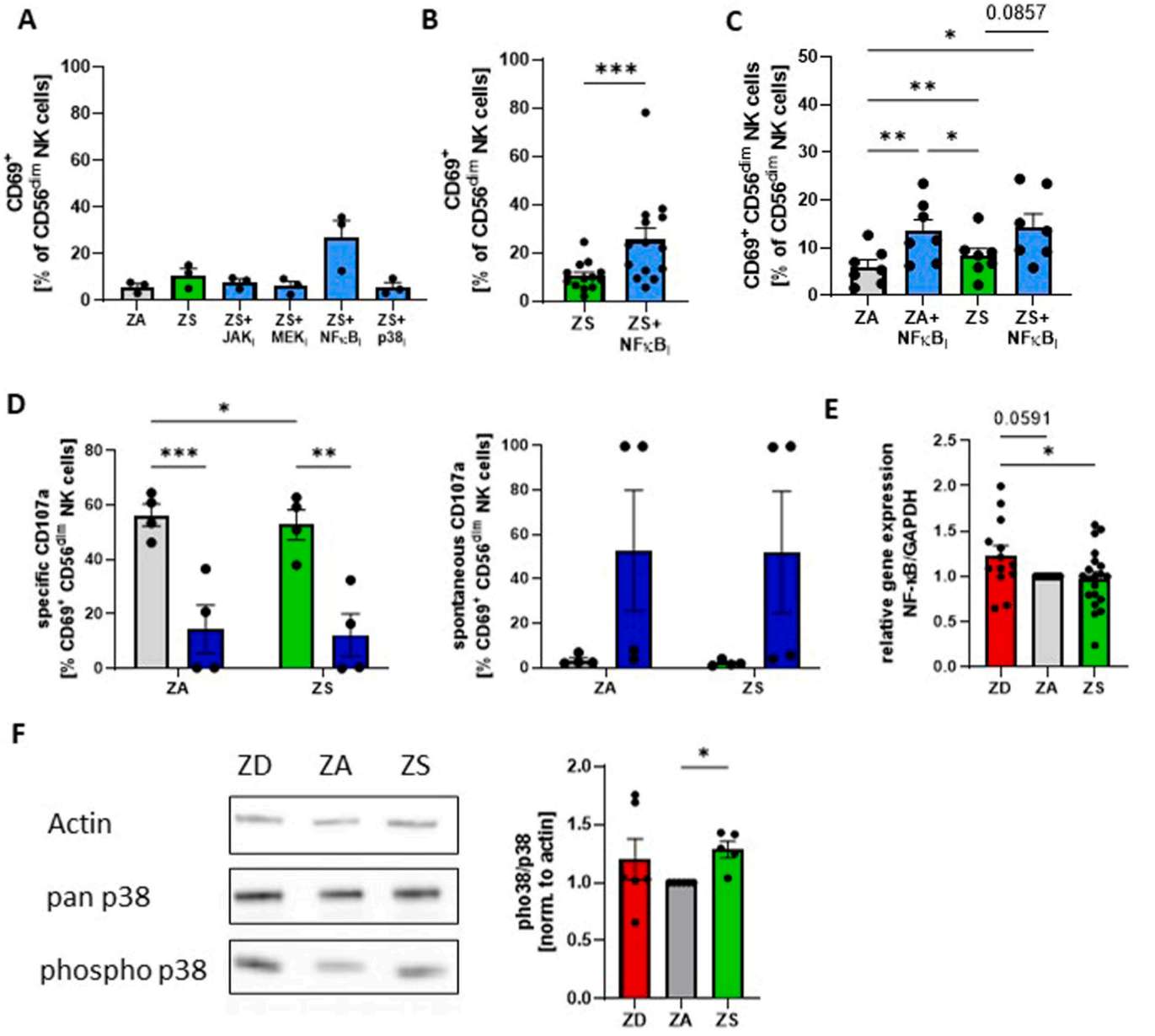

Fig. 6. NFκB inhibition increases CD69 expression on CD56^{dim} NK cells. PBMCs were isolated and cultivated for 20 h in zinc-adequate (ZA, grey bars), zinc-supplemented (ZS, green bars) media conditions or ZS plus JAK (300 nM), MEK (5μM), p38 MAPK (10μM), or NFκB inhibitor (1μM, all blue bars). CD69 expression was determined via flow cytometry. Bar graphs showing CD69 expression on CD56^{dim} NK cells from ZA, ZS or ZS plus inhibitor condition (n = 3, A). Bar graph showing CD69 expressing on CD56^{dim} NK cells after ZS media conditions and ZS + NFκB inhibitor treatment (n = 12–14, B) as well as ZA media conditions and ZA + NFκB inhibitor treatment (n = 7, C). After 20 h of ZA, ZS ± NFκB inhibitor treatment, the HLA class I-deficient cell line was added in an effector target ratio of 12.5:1. Bar graphs showing target specific (right hand side) or spontaneous (left hand side) CD107a expression after 4 h of co-cultivation (addition of monensin after 1 h) on CD56^{dim} NK cells (n = 4, D). After 20 h of ZS, ZA or ZS media conditions, RNA (E) or proteins (F) were isolated. Bar graphs showing the relative NFκB expression GAPDH after ZD, ZA or ZS media conditions (n = 13–15, E). Exemplary Western Blot bands and their quantification of Actin, pan p38, and phospho p38 after ZD, ZA or ZS media conditions (n = 5–6, F). The height of the bars represents the mean ± SEM. Each dots represents an individual donor. The data is representative of > 3 individual experiments. Levels of significance were calculated with repeated measurements one-way ANOVA (A, C, D), an unpaired *t*-test (Wilcoxon test) (B), Ordinary one-way ANOVA (E), and Kruskall-Wallis test (F), * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

cells after zinc supplementation. Our data indicate that CD69 expression is increased in CD56^{dim} NK cells after NFκB inhibition and that extracellular zinc might promote NFκB inhibition.

3. Discussion

The trace element zinc has been previously described to be vital for immune cell functionality [12], however so far, studies investigating the effect of zinc on NK cell function are scarce. Our study demonstrates for the first time a selective influence of extracellular zinc on peripheral blood CD56^{dim} NK cells, as extracellular zinc levels did not influence CD56^{bright} NK cells. We observed a significant correlation between serum zinc levels and CD56^{dim} NK cell frequencies within our zinc-adequate cohort. In line with the *ex vivo* data, in our hands *in vitro* zinc supplementation selectively activated CD56^{dim} NK cells by up-regulating CD69 expression. CD69^{+} CD56^{dim} NK cells showed significantly higher degranulation capacity and IFNγ production compared to CD69^{-} CD56^{dim} NK cells of the same donors validating their activated state. We also observed that perforin is very sensitive to zinc deprivation. Our study further suggests that NFκB inhibition and

phosphorylation of p38 MAPK might be involved in CD69 expression. Our study provides interesting insights into the interaction between extracellular zinc and $CD56^{dim}$ NK cells. Since cytotoxic $CD56^{dim}$ NK cells are very important for anti-tumor and anti-viral immunity, adequate zinc levels might be an inexpensive, but effective tool to decrease the risk of these burdens. This knowledge might be further applicable for *in vitro* NK cell expansion protocols for adoptive transfers.

CD69 expression is upregulated in most immune cells after activation [29]. In line with this, CD69 expression is also induced early after NK cell activation [18]. We here observed that the addition of extracellular zinc leads to a selective upregulation of CD69 expression on $CD56^{dim}$ NK cells promoting the generation of functional activated $CD56^{dim}$ NK cells. The induction of CD69 expression has been previously described due to different activation stimuli on NK cells [18], such as IL-2 [30]. Indeed, CD69 expression significantly increased further on $CD56^{dim}$ NK cells when we stimulated our 20 h zinc-supplemented cultures for additional 3 h with IL-2. Since we did not culture enriched $CD56^{dim}$ NK cells, but supplemented PBMCs with zinc in our cultures, there might have been the chance that a small fraction of pre-activated immune cells, such as T cells, secrete IL-2 to the supernatant. However, we hardly detected any IL-2 within our zinc-supplemented culture supernatants. Since peripheral blood $CD56^{dim}$ NK cells hardly express the high affinity IL-2 receptor CD25 [31], it is very unlikely that NK cells are stimulated by these very low IL-2 amounts to upregulate CD69 expression. Therefore, we might exclude CD69 expression due to IL-2 within our experiments. Nevertheless, blocking IL-2 or CD25 could be a future experimental approach to determine the role of IL-2. As mentioned above, PBMCs were used instead of purified NK cells. Using purified NK cells in future studies could provide deeper insights into the selective activation of extracellular zinc on $CD56^{dim}$ NK cells. Nevertheless, our data suggests that extracellular zinc might activate $CD56^{dim}$ NK cells directly.

Our study provided evidence that changes in extracellular zinc can change the activation status of $CD56^{dim}$ NK cells selectively. However, the question remains, what the mechanisms of CD69 up-regulation might be. To the authors knowledge the exact mechanism behind CD69 up-regulation on $CD56^{dim}$ NK cells in general is so far unknown. We previously observed intracellular zinc and calcium ion influx to be vital for T cell activation [32]. Therefore, high extracellular zinc levels might also promote an increase of intracellular zinc levels and/ or calcium levels within $CD56^{dim}$ NK cells. In line with this, an increase of intracellular zinc was observed in the NK cell line YTS after a three day *in vitro* zinc supplementation [15]. This suggests that NK cells also accumulate intracellular zinc if high extracellular zinc levels are available. Indeed, intracellular zinc accumulation has been described in several immune cell subsets [33]. To study this further, in the future it will be interesting to determine the expression of zinc transporter known to induce zinc influx [34] on NK cells.

$CD69^{+}$ $CD56^{dim}$ NK cells showed higher degranulation capacity and IFNγ secretion. This is in line with previous published studies showing that $CD69^{+}$ $CD56^{dim}$ NK cells secrete higher IFNγ levels [18,30,35,36]. However, unlike current literature [15,16], we observed no significant differences in NK cytotoxicity. As we did not specifically determine cytotoxicity for $CD69^{+}$ compared to $CD69^{-}$ $CD56^{dim}$ NK cells, a higher cytotoxicity of CD69-expressing NK cells cannot be ruled out. If $CD69^{+}$ $CD56^{dim}$ NK cells are more cytotoxic, it might not directly contribute to the measured cytotoxicity, since $CD69^{+}$ $CD56^{dim}$ NK cells are with a mean frequency of 15 % the minority of all NK cells. Nevertheless, we observe the activation of $CD56^{dim}$ NK cells simultaneously with a functional switch after zinc supplementation towards immune modulatory functions. This has been previously observed in other lymphocytes, as zinc supplementation can induce a switch from B cells [37] as well as T cells [38,39] towards regulatory B cells [37] and T cells [38,39]. Of note, zinc influx in T cells promotes the differentiation into T_{helper} 1 subsets, too [38]. One explanation of this observation might be the experimental set-up. We here supplemented PBMCs of dominantly zinc-adequate donors *in vitro* for 20 h with zinc, while an *in vivo* zinc supplementation study with zinc-deficient donors could show a significant increase in NK cell cytotoxicity after 2-weeks [16]. Hence, the duration and the donor`s initial zinc status might play an important role, even though the activation of NK cell might be fast, an intracellular change in cytotoxicity might be a slower process or is dependent on the timing of the different zinc influxes [40].

Zinc ions have been described to be important signaling ions [33], antioxidants [41,42] as well as influencers of transcriptional activity [43], epigenetic regulation [44], and protein phosphorylation [43]. So far, the influence of zinc has been described to inhibit as well as activate NFκB [25,26]. Our data suggests that inhibition of NFκB/p65 of the canonical NFκB pathway increases CD69 expression on $CD56^{dim}$ NK cells. We further observed a significant decrease of *NFKB* mRNA after zinc supplementation compared to zinc-deficient medium. Our observations suggest that elevated extracellular zinc levels might inhibit *NFKB* mRNA transcription or increase *NFKB* mRNA degradation by so far unknown mechanisms. Once NFκB/p65 expression is decreased, CD69 expression is upregulated. The canonical pathway has been suggested to be important for early induction of CD69 expression [45], while the non-canonical pathway sustains CD69 expression [45]. This suggests that our observed CD69 expression might indeed be induced early on $CD56^{dim}$ NK cells. In contrast to the NFκB/p65 inhibitor, p38 inhibition during 20 h of zinc supplementation did not upregulate CD69 expression. This indicates p38 is important for the upregulation of CD69. In line with this, we observed significantly higher p38 phosphorylation after zinc supplementation compared to zinc-adequate conditions. This suggests that zinc supplementation enhances p38 phosphorylation, which we previously observed in activated T cells [32]. The confirmation of p38 has been described to be redox dependent [46], however if zinc ions play a role in this change needs to be further investigated. Prior studies have described p38 to activate NFκB [47] rather than to inhibit NFκB. However, here, we observe low NFκB/p65 expression with increased p38 phosphorylation and an up-regulation of CD69 expression. So far, no study has described that p38 is regulated by NFκB/p65. Therefore, currently we favor a model where zinc supplementation decreases/inhibits NFκB mRNA transcription and simultaneously increases p38 phosphorylation, which in combination leads to CD69 expression on $CD56^{dim}$ NK cells (overview in Fig. 7). It needs to be mentioned that these observations were made by analyzing PBMCs, instead of isolated NK cells. Since we observed selective activation on $CD56^{dim}$ NK cells after zinc supplementation, it is very likely that the seen effect was dominantly contributed by NK cells. However, we cannot rule out the involvement of other immune cells within the PBMC contributing to our observations.

Zinc has been described to be vital to enhance and restore NK cell functionality in zinc-deficient individuals [16,48,49]. Previously, zinc deficiency was thought to be dominantly a problem of malnutrition, elderly, or individuals with low bioavailable zinc uptake, such as vegetarians or vegans [16,48,49]. However, in our last study, we observed 72 % of young healthy adults were zinc-deficient [16]. This might have serious consequences, especially if zinc uptake is low during fetal development or childhood. Zinc homeostasis is crucial for the development of human T cells in the thymus [50,51], as zinc deficiency directly leads to T cell deficiency. The importance of zinc within the thymus is also visible, as apoptotic T cells release their intracellular zinc to replenish extracellular zinc within the human thymus [50]. The role of zinc during NK cell development is understudied and more studies are needed to determine the influence of zinc. In this study, we describe the selective activation of $CD56^{dim}$ NK cells via extracellular zinc supplementation. We further observed changes for KIR/ NKG2A expression on $CD56^{dim}$ NK cells. This is in line with current literature, as zinc ions have been described to be vital for KIR functionality and their formation [21–24]. We previously identified a unique human NK cell progenitor within the fraction of circulating innate lymphoid cells 1 (cILC1), which generates high levels of KIR^{+} NK cells [19]. We speculated that this NK precursor might be developmentally linked with T cells due to their

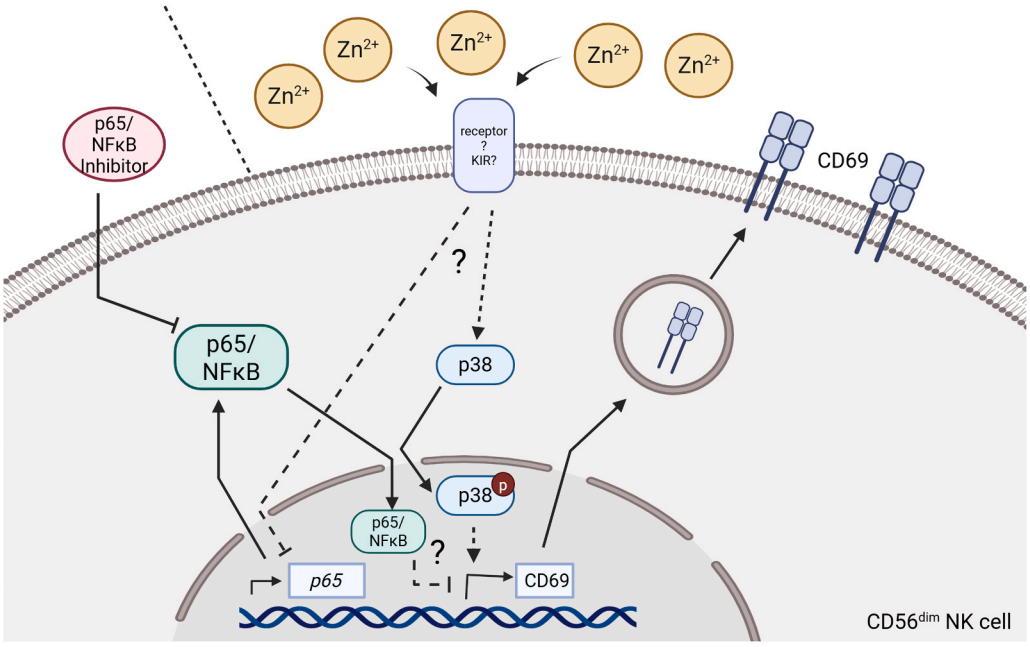

Fig. 7. Proposed scheme of intracellular events for CD69 upregulation. Schematic overview of proposed intracellular events leading to CD69 upregulation in $CD56^{dim}$ NK cells. Legend: Arrows (→) indicate a chain of events, blunt arrows (–|) symbolize an inhibition, solid lines represent known mechanisms, dotted lines represent proposed interaction. Question mark (?) indicates down-stream events are unknown. Abbreviations: KIR (Killer cell Immunoglobulin-like Receptor), Zn^{2+} (Zinc ion), red p (phosphorylated).

shared T cell associated characteristics [19,52]. Indeed, we could now identify an ILC1 subset within the human thymus, which also generates high levels of KIR^+ NK cells [53]. Further, thymic ILC1s might be precursors of cILC1s. *FOXN1*-heterogygous patients as well as DiGeorge patients with athymia, who both have defects in the thymus architecture, show significant decreased cILC1 frequencies and total cell counts in peripheral blood compared to healthy age-matched controls [53]. Hence, the thymus might play an important role in the generation and potential priming of KIR^+ NK cells. Due to the unique role of zinc within the thymus and based on our current data, we speculate that zinc might be one factor during NK cell development to promote KIR/ NKG2A expression.

Our *in vitro* observation is in line with our initial observation that zinc-adequate donors showed significantly higher CD69 expression compared to zinc-deficient donors [16]. However, we observed no significant difference in CD69 expression on $CD56^{dim}$ NK cells before and after a 2-week zinc supplementation of young healthy adults [16]. As the life span of NK cells from young donors is quite short (10–14 days) [54], one explanation could be a potential migration of $CD69^+$ NK cells into the tissues, since CD69 has been described to be important for cell retention into tissues [29]. In line with this, tissue resident NK cells are characterized by CD69 expression [55]. In this scenario, $CD69^+$ NK cells migrate into the tissues and are replaced by newly developed $CD69^-$ NK cells within the periphery. Supporting this hypothesis, we did not observe changes in peripheral blood $CD56^{dim}$ NK cell frequencies in our previous *in vivo* supplementation study [16].

The knowledge from our study could be a promising first step for future clinical therapeutic interventions against tumor cells with NK cells. Clinical usage of NK cells have been particularly focused to fight CD20-expressing B cell acute lymphoblastic leukemia [11]. Studies have identified no direct adverse effects after zinc supplementation in leukemic patients or patients with solid tumors [56–58]. Especially zinc supplementation has been suggested to reduce oral toxicities during radiation for leukemic as well as solid tumors [56–58]. Furthermore, zinc supplementation has been suggested as potential therapeutic strategy for hepatocellular carcinoma [59]. Increasing serum zinc levels in leukemic patients might be one strategy to enhance anti-tumor surveillance by NK cells. However, leukemic cells accumulate intracellular zinc for oncofusion protein stabilization [60]. Furthermore, intracellular zinc protected tumor cells from T cell cytotoxicity in an experimental mouse model [61]. Suggesting that zinc supplementation might also be beneficial for tumor growth or escape of immunosurveillance. Nevertheless, the induction of $CD69^+$ $CD56^{dim}$ NK cells via zinc supplementation might be one strategy to promote anti-tumor surveillance. However, CD69 expression has also been linked to an exhausted phenotype in T cells [62] as well as NK cells [36]. Therefore, anti-CD69 treatment has been suggested to promote anti-tumor surveillance [62]. In line with this, an experimental tumor model showed significantly higher cytotoxicity of murine NK cells after anti-CD69 treatment [63]. More research regarding the effects of zinc on NK cells is needed as well as more insights into NK cell functionality in cancer patients with regard to their zinc status before routine zinc supplementation can be recommended.

The data from this study could be applicable for future therapeutic strategies, since "of the shelf" NK cell therapies are currently actively investigated [64]. Zinc supplementation has been suggested to increase $CD56^+$ frequencies in NK cell differentiation cultures from $CD34^+$ hematopoietic stem and precursor cells [65,66]. However, so far it is not known if this increase might be due to NK cell or Innate Lymphoid Cell 3 (ILC3) differentiation, as both populations can be generated within the same cultures [67–69]. Therefore, zinc supplementation to *in vitro* NK cell cultures either by expanding *ex vivo* NK cells [70] or differentiating NK cells from $CD34^+$ hematopoietic stem and precursor cells [71,72] might enhance the generation and functionality of the generated NK cells. Once the NK cells have been expanded and zinc supplemented, the NK cells could be given via adoptive transfer to the cancer patients. This would decrease the risk of fueling tumor cells with zinc *in vivo* but have the beneficial aspect of pre-activating high functional NK cells. If this is suitable for NK cell differentiation protocols and transferring these NK cells into patients' needs to be further investigated.

4. Materials and methods

4.1. Human samples and ethics statement

Healthy young blood donors have given their informed consent to donate venous blood. All donors provided blood (serum monovettes, Li-heparin/ heparin tubes) in the morning and peripheral blood mononuclear cells (PBMCs) were isolated immediately. The study was approved by the Institutional Ethics Committee of the Medical Faculty of RWTH Aachen University (EK 23–148). As previously published, a 18-item Food Frequency questionnaire (FFQ) via Zinc-App [73] was used, which asseses the net zinc score based on frequency, portion size, and quantity of daily food intake. The FFQ calculates a phytate corrected adjusted zinc diet score [14]. Serum zinc values were determined as previously described [13,16]. Individuals were classified as zinc-deficient (ZD) with a zinc serum score < 70 mg/dl, as at risk of zinc deficiency (rZD) with a zinc serum score > 70 mg/dl but < 113 points in the FFQ, or zinc-adequate (ZA) with a serum zinc > 70 mg/dl as well as > 113 points in the FFQ.

4.2. Cell lines

The HLA class I-deficient erythroleukemic cell line K562 was cultivated in RPMI 1640 (Sigma-Aldrich, Darmstadt) with 10 % heat-inactivated fetal calf serum FCS (Lot #: CP-21–4515, Capricorn Scientific, Ebsdorfergrund) and 1 % (v/v) L-Glutamine (Sigma-Aldrich, Darstadt) at 37°C and 5 % CO_2. Twice a week the cells were splitted 1:10 or 1:15.

4.3. Serum zinc measurement

Blood was collected in serum monovettes (Sarstedt) and was centrifuged for 10 min at 1841xg. Serum was diluted 1:1 with deionized water and serum zinc concentrations were determined by flame atomic adsorption spectrometry (ASS) using an AAnalyst 800 (Perkin-Elmer).

4.4. Isolation of PBMCs

Venous blood from young healthy donors were collected in the morning either in lithium-heparin tubes (16 IU heparin/ml blood, Sarstedt) or 50 U heparin (Braun) per ml blood. Donor demographics are displayed in Table 1. The blood was diluted 1:2 with 1x PBS (Sigma-Aldrich). The cells were layered on using a lymphocyte separation medium (density: 1.077 g/ml, Capricorn Scientific) and PBMCs were separated using a 20 min 800xg centrifugation with minimal acceleration and deceleration. After one washing step, erythrocytes were lysed with 5 ml of erythrocytes-lysingbuffer containing 1.5 M ammonium chloride (AppliChem), 100 mM sodium hydrogen carbonate (AppliChem), and 10 mM triplex-111 (EDTA, Merck). Followed by three additional washing steps with 1x PBS. PBMCs were counted using a Bürker Counting Chamber and adjusted to the needed concentration. PBMCs were either analysed *ex vivo* or cultured for 1 h (h) or 20 h in different zinc media as described below.

Table 1
Donor demographics.

Parameters	Cohort	Sex, n (%)
General	n = 35, for n = 33 both values (serum zinc level & AZDS score) were available	13 (37.1 %) Male 22 (62.9 %) Female
Age	< 40, > 18 years	n/d
Zinc status ZD rZD ZA	n = 6 n = 8 n = 19	5 (83.3 %) Female 7 (87.5 %) Female 10 (52.6 %) Female

4.5. Calculations of NK cells frequencies

Frequencies of lymphocytes were calculated, as following

$$: \frac{\#\ of\ cell\ population\ (FlowJo)}{\#\ of\ lymphocytes\ (FlowJo)}\ x\ 100$$

4.6. PBMC incubation in different zinc media

1–2.5 × 10^6 PBMCs were cultured in medium containing RPMI 1640 (Sigma-Aldrich, Darstadt) with 10 % heat inactivated fetal calf serum (FCS) (Capricorn Scientific, Ebsdorfergrund, Lot #: CP-21–4515), 1 % (v/v) L-Glutamine (200 mM, Sigma-Aldrich), 1 % (v/v) Penicillin (Stock: 10000 U/ml, Sigma Aldrich), and 1 % (v/v) Streptomycin (10000 mg/ml, Sigman-Aldrich). This medium is the zinc-adequate (ZA) medium. For the zinc-supplemented (ZS) medium, 50 mM Zinc sulfate ($ZnSO_4$ x 7 H_2O, Sigma-Aldrich, Darmstadt) was added. When specifically described, ZS medium also contained 15 mM or 30 mM Zinc sulfate. To create a zinc-deficient (ZD) medium, the ZA medium was treated with CHELEX beads (Chelex 100, sodium form, Sigma-Aldrich) for 1 h at room temperature. As previously described [74], CHELEX beads also capture other cations, such as Magnesium Mg^{2+} and Calcium Ca^{2+}, from the medium. Therefore, both cations were added after CHELEX treatment to the medium (500 mM $CaCl_2$ (Merck, Darmstadt) and 400 mM $MgCl_2$ (Sigma-Aldrich, Darmstadt)). Zinc content of the individual media were verified by ASS measurement.

To determine the intracellular pathway of CD69 up-regulation, the PBMCs were cultured in the different zinc media conditions, as well as the following inhibitors: NF-κB activation inhibitor (1 μM, Cat # 481406, Calbiochem, Hyderabad India), p38 MAPK inhibitor SB202190 (10 μM, Cat.#S7067–5MG, Sigma-Aldrich, St. Louis, USA), MEK inhibitor U0126 (5 μM, Cat. # 1144, Tocris Bioscience, Wiesbaden-Nordenstadt) or JAK inhibitor (300 nM, Cat. # 420099, Calbiochem, Hyderabad; India).

PBMCs were cultivated as indicated either for 1 h or 20 h at 37°C with 5 % CO_2. Where indicated the cells were incubated for 20 h with an additional 3 h stimulation of 75 U IL-2 (Proleukin, Novartis). After cultivation, the cells were further processed for flow cytometric analyses, the CD107a assay, perforin/ granzyme content determination or for Western Blot and qPCR. Due to handling reasons, some samples were frozen after the 20 h zinc media conditions, defrosted and stained extracellularly.

4.7. Flow cytometric analyses

Isolated PBMCs were stained in 1x PBS (Sigma-Aldrich, Darmstadt) with 1 % BSA (Fluka, Buch, Switzerland) with the following antibodies extracellularly: anti human -CD3 (UCTH1)-FITC, -CD56 (HCD56)-PE or -BV510™, -KIR2DL3/L2/S2 (CD158b/j, DX27) -FITC, -KIR3DL1/NKB1 (CD158e1, DX9)-FITC, -KIR2DL1/S1/S3/S5 (CD158, HP-MA4)-FITC all from Biolegend. Additionally, CD69 (FN50)-APC or -BV421™ from BD Bioscience, Heidelberg and NKG2A (CD159a, Z199)-PE/Cy7 from Beckman Coulter. Intracellular staining was performed according to the manufactures protocol. In brief, PBMCs were extracellularly stained with the antibodies described above. The cells were centrifuged for 10 min at 300x g, the supernatant completely removed, and fixated for 20 min at 4°C using 1x Fixation buffer (Biolegend). The cells were washed with 1x Permwash buffer (10x concentrate, diluted in aqua dest, Biolegend) and centrifuged for 10 min at 300xg. Anti-human Perforin-PE (δG9, eBioscience), anti-human Granzyme B-PE (GB11, BD Bioscience) or anti-human IFNγ-APC (4S.B3, Invitrogen) were added for 30 min 4°C. Permwash buffer was added to the cells before a 10 min 4°C centrifugation step. The cells were washed additional two times (first with 1x Perm wash, second with 1xPBS) with 10 min 4°C centrifugation steps in between. Read out was the mean fluorescence intensity (MFI) of perforin and granzyme B for each cell population or frequencies of IFNγ

secretion. All samples were acquired on a flow cytometer Canto II (BD Bioscience) and analysed using FlowJo (Version 10.10.0).

4.8. CD107a assay

Freshly isolated PBMCs were counted and cultured for 20 h in ZD, ZA or ZS medium with and without inhibitors. After 20 h, 1 ml CD107a-BV510 (H4A3, Biolegend) as well as K562 cells were added in an effector target ratio of 12.5:1. After 1 h of co-culture at 37°C and 5 % CO_2, Monensin (1x, Biolegend) was added, and co-cultured for additional 3 h. After washing with 1x PBS, the cells were only extracellularly stained and analysed with the Canto II (BD). Alternatively, the cells were also stained intracellularly with anti-human IFNγ - PE/Cy7 (4S.B3, Biolegend) for 30 min at 4°C. Of note, for samples were the IFNγ secretion was analysed, Golgi Plug (BD Bioscience) was added simultaneously to Monensin. Permwash buffer was added to the cells before a 10 min 4°C centrifugation step. The cells were washed additional two times (first with 1x Perm wash, second with 1xPBS) with 10 min 4°C centrifugation steps in between. Spontaneous degranulation based on CD107a expression and spontaneous IFNγ secretion was determined from PBMCs without added K562. Specific degranulation based on CD107a expression as well as specific IFNγ secretion was calculated by subtracting the spontaneous CD107a expression/ IFNγ secretion (PBMC -K562) from the total CD107a expression/ IFNγ secretion (PBMC +K562). All samples were acquired on a flow cytometer Canto II (BD Bioscience) and analysed using FlowJo (Version 10.10.0).

4.9. Cytotoxicity assay

Freshly isolated PBMCs were counted and cultured for 20 h in ZD, ZA or ZS medium. CFDA-SE labelled K562 cells were added in an effector target ratio of 12.5:1 a with or without IL-2 (75 Units) and the samples were centrifuged for 3 min at 120xg. After 4 h at 37°C and 5 % CO_2 of co-culture, the cells were stained with 2.5 % propidium iodide (PI, Sigma Aldrich) for 10 min and measured by flow cytometry using a FACS Calibur (BD Bioscience) to determine the dead K562 cells. CFDA-SE labelled K562 without PBMCs were used as negative control for spontaneous dead cells. The data was analyzed by gating on $PI^{+}CFDA^{+}$ K562 cells. To determine the target-specific killing rate, the frequencies of K562 without PBMCs (spontaneous dead) were subtracted from the K562 with PBMCs (total dead). The data was analysed with FlowJo (Version 10.10.0).

4.10. Quantitative PCR (qPCR)

RNA was isolated with a phenol-chloroform method using TRIzol. RNA concentration was determined via Nanodrop (Nanodrop 1000, Thermo Fisher Scientific, Schwerte). In brief, all samples were centrifuged, resuspended in TRIzol reagent (Ambion, Life Technologies, Darmstadt) and incubated for 5 min before freezing at −80°C. Within one month, the samples were defrosted and handled at RT during processing. Chloroform was added, the samples shaken, and incubated for 15 min. After a 15 min centrifugation step at 12000x g at 4°C, the aqueous phase was taken, mixed with isopropanol, and vortexed shortly. The samples were then incubated for 10 min with an additional centrifugation step (10 min 12,000 xg). After removing the supernatant, 75 % ethanol was added followed by a short vortexing step. The samples were centrifuged, the pellet was dried with a vacuum pump and resuspended in DEPC water. Samples were stored at −80°C before cDNA synthesis. 1 mg of RNA was transcribed with the qScript cDNA Synthesis Kit (Quantabio, Gaithersberg, MD, USA) according to manufacturer's instructions. qPCR was performed for GAPDH (60°C annealing temperature, forward primer: 5´-TGC TGA TGC CCC CAT GTT C-3´, reverse primer: 5´-GGC AGT GAT GGC ATG GAC TG-3´), CREB1 (60°C annealing temperature, forward primer: 5´-ACT GTA ACD DTG CCA ACT CC-3´, reverse primer: 5´-GAA TGG TAG TAC CCG GCT GA-3´), and NF-kB (63°C annealing temperature, 5´-CCA GAC CAA CAA CAA CCC CT-3´, reverse primer: 5´-TCA CTC GGC AGA TCT TGA GC-3´) on the QuantStudio™ Design & Analysis (Thermo Fisher Scientific, Waltham, USA).

4.11. Western blot analyses

After 20 h of culturing in different zinc media with or without inhibitor, PBMCs were centrifuged and resuspended in sampling buffer (65 mM Tris-HCl (pH 6.8) (Roth, Karlsruhe, Germany), 2 % [w/v] SDS (Merck, Darmstadt, Germany), 26 % [v/v] glycerol Roth, Karlsruhe, Germany) with 1 μM sodium orthovanadate (Sigma-Adrich, St. Louis, MO, USA), 1 % β-mercaptoethanol (Merck, Darmstadt, Germany), and 2 % protease inhibitor cocktail (Boster Biological Technology, Pleasanton, CA, USA), as previously described [75]. Cell lyses was performed with a Vibra Cell sonicator (Sonics & Materials, Newtown, PA, USA) and heated at 95 °C for 3 min. Cell lysates were stored at −20°C until further usage within 3 months after storage. Cell lysates were defrosted, 2 % bromophenol blue (Merck, Darmstadt, Germany) was added, and the lysates were heated at 95 °C for 3 min. A 10 % polyacrylamide gel was used for protein separation with a 4 % collection gel. Protein concentrations of each condition was determined by Pierce 660 nm Protein Assay (Sigma-Aldrich) according to manufacturer's instructions. The same amount of protein was loaded for each sample per lane with a colored prestained standard (New England BioLabs, Frankfurt am Main, Germany) for molecular weight determination. The proteins were separated at 170 V.

Samples were blotted on nitrocellulose membranes with a pore size of 0.45 μM at 100 V for 60 min. To determine equal protein loading per lane, the membrane was stained with Ponceau S (PanReac, AppliChem, Darmstadt, Germany). Afterwards, the membranes were washed in TBS-T (20 mM Tris (pH 7.6) 137 mM NaCl, and 0.1 % [v/v] Tween 20) for 5 min. Subsequently, the membranes were blocked for 1 h in TBS-T plus 5 % fat-free dry milk (Saliter J.M. Gabler-Saliter Milchwerk GmbH & Co. KG, Obergünzburg) and washed in TBS-T 3 times.

The following primary antibodies were incubated over night at 4°C: phospho-CREB (SER133) (Clone: 87G3, Rabbit, Cat.No.: 9198S) (diluted 1:1000 in TBS-T + 5 % BSA), CREB (Clone: 48H2, Rabbit, Cell Signaling Technology, Danvers, MA, USA, Cat. No.: 9197S) (diluted 1:500 in TBS-T + 5 % BSA, both 43 kDa), phopho-p38 MAPK (Thr180/Tyr182, Rabbit, clone: 3D7, Cat. 9215 S), p38 MAPK (both 40 kDa, Cat. 9212 S), and β-Actin (45 kDa, Rabbit, Cat. 4967 L) all from Cell Signaling Technology, Danvers, MA, USA. The following day the membranes were washed three times with TBS-T. The membrane was incubated with the following secondary antibodies for 3 h at room temperature: anti-mouse HRP-linked antibody for phospho-CREB and anti-rabbit HRP-linked antibody for CREB, p38, and phopho-p38 (both Cell Signaling Technology, Danvers, MA, USA) were both diluted 1:2000 in TBS-T + 5 % fat-free dry milk. Before detection with Westar Antares (Cyanagen, Bologna, Italy) using LAS-3000 (Fujifilm Lifescience, Tokyo, Japan), the membranes were washed three more times. Band intensity was determined by ImageJ (Version 1.54 g, NIH, Bethesda, MD, USA).

Statistical analyses

All data was tested for normal distribution using the Shapiro-Wilk and Kolmogorov-Smirnov tests. All data was further tested for outliers using the GraphPad Prism software Version 8.0.1 and 10.04.2 (GraphPad Software, La Jolla, USA). The tests used for statistical analyses are indicated in each figure legend. Significances are indicated as following: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

CRediT authorship contribution statement

Lothar Rink: Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Kim Ohl:** Investigation, Formal analysis. **Jana Jakobs:** Writing – review & editing,

Investigation, Formal analysis. **Laura Heenen:** Visualization, Validation, Investigation, Formal analysis. **Ronja Stöcker:** Visualization, Investigation, Formal analysis, Data curation. **Sabrina B. Bennstein:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtemb.2025.127788.

Data Availability

Data of this study can be provided upon reasonable request.

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