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Arsenic treatment of acute promyelocytic leukemia affects neutrophil function in a compensatory manner

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Abstract

Background Arsenic (ATO) and retinoic acid (ATRA) are successfully used as chemotherapy-free regimens to treat acute APL. Compared to traditional chemotherapy approaches, this therapy evokes fewer haematological side effects, such as severe neutropenia and thrombocytopenia, but little is known about the impact of the treatment on neutrophil function.

Methods We included three patients undergoing consolidation treatment for APL. To evaluate the functionality of neutrophils, we assessed chemotaxis, ROS production, and neutrophil extracellular trap (NET) release during different time points of the treatment and compared them with neutrophils from healthy donors.

Results We revealed that the chemotactic ability of neutrophils isolated from APL patients was decreased before starting each cycle of treatment. However, there was an increase in chemotactic ability in the first week of treatment compared to other time points. Additionally, we observed increased ROS production at the start of the treatment cycle. In vitro exposure of isolated neutrophils from healthy donors to ATO led to decreased chemotaxis at high ATO concentrations exceeding those achieved in vivo, while ROS production was not affected. Chemotaxis and ROS production were not altered by exposure to ATRA in vitro and neither ATO nor ATRA had an effect on neutrophils' ability to release NETs.

Conclusions Our study suggests that ATO and ATRA therapy alter neutrophil function by increasing chemotaxis and reducing ROS production. The effect on neutrophil function does not, however, seem to impact infection susceptibility in our patients, indicating that the enhanced functionality might compensate for the lowered neutrophil count.

Keywords Acute promyelocytic leukemia, Neutrophils, Chemotaxis, Arsenic, Infection risk assessment

Background

Acute promyelocytic leukemia (APL) is a subgroup of acute myeloid (AML) and a rare disease. Only 5-8% of all newly recorded AML cases are classified as APL [1]. APL is specified by the unique translocation t(15:17), which creates the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) oncogene [2, 3]. Although APL is fatal when untreated, it has become a success story of precision therapy in oncology due to the use of inorganic arsenic (ATO), with the formula As_2O_3, in combination with retinoic acid (ATRA) [4]. ATRA

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affects the RARα part of the oncogene and elevates its transcription, which reverts the differentiation blockage of the cells. ATO binds to the PML part of the oncogene and induces differentiation and apoptosis [2]. Chemotherapy-free treatment appears to be at least as effective for APL patients as chemotherapy-based regimens [5, 6] and is now widely used. The chemotherapy-free regimen has benefits such as less severe hematological toxicity, i.e., neutropenia and thrombocytopenia [6]. However, even though most patients with APL are cured, early death (death within the first 30 days) is still a clinical problem, and infection is the second most common cause of early death among patients over 50 years of age [7].

Neutrophils are part of innate immunity and are the most frequent circulating leukocytes [8]. Neutrophils exercise their antimicrobial functions by leaving circulation and migrating towards the site of infection following a gradient of chemokines, so-called chemotaxis [9]. Once at the site of infection, they fight microbes by engulfing them into phagosomes, degranulating and releasing neutrophil extracellular traps (NETs) [10–12]. The oxidative burst, namely, the production of reactive oxygen species (ROS), constitutes a central activation mechanism in neutrophils with both antimicrobial and signalling properties [8]. Neutropenia is common in patients with hematological diseases and is a consequence of the disease itself and of the applied therapeutic interventions [13]. Neutropenia results in an increased risk of severe infections with microbes such as bacteria and fungi [13–15] and infections are a common complication in this patient population [16, 17]. In the absence of a clinically-established tools for evaluation of neutrophil function, clinicians can only rely on the absolute neutrophil count (ANC) to assess the individual risk level of susceptibility to infection.

Although the effect of ATO on neutrophils is not fully known, it has been shown that neutrophils exposed to ATO in vitro have a decreased ability to perform phagocytosis, phorbol 12-myristate 13-acetate (PMA)-induced ROS production and NET formation [18]. In vitro exposure to ATO also induces apoptosis and increases the release of cytokines, including IL-8 [19]. There are also contradicting results showing increased phagocytosis presiding cell death in neutrophils exposed to ATO [20]. However, in these studies neutrophils were exposed to ATO in vitro and there is a lack of understanding how neutrophil isolated from patients undergoing ATO treatment exercise their functions. Oral ATRA supplementation has been shown to induce an increase in spontaneous migration in neutrophils and increase the expression of β2 integrin CD11b [21]. However, little is known about the functionality of neutrophils isolated from APL patients receiving combination treatment with ATO and ATRA.

In our study, we evaluated the functionality of neutrophils isolated from patients with APL during consolidation treatment with ATO and ATRA to supply a more detailed risk assessment regarding susceptibility to infections for patients undergoing this therapy approach. We found altered neutrophil function before treatment with decreased chemotactic ability and increased ROS production compared to healthy donors. Both functions subsequently changed during consolidation treatment to increased chemotaxis and decreased ROS production. These findings, taken together with the absence of severe neutropenia and no infection-related hospitalization within our patient group, suggest that ATO and ATRA consolidation treatment affects neutrophil functions in a manner compensating for decreased neutrophil counts.

Methods

Ethical statement

Ethical approval (register number 09-210 M, project number CFU-12/09) was carried out by the Regional Ethical Review Board, Umeå, Sweden. The study was performed in agreement with the Declaration of Helsinki. The patients were informed both orally and in writing and were included after providing written consent.

Subjects

Study subjects were patients diagnosed with APL (patient characteristics shown in Table 1). Patients were included between 2018 and 2021, and blood samples were collected between 2018 and 2022. For patient inclusion, written informed consent was obtained. All subjects were diagnosed and treated at the Haematology Unit, Umeå University Hospital.

Blood samples were collected at four different timepoints during cycles of consolidation treatment with ATO and ATRA (Table 2). The time points for sampling were selected to reflect the different exposures during the established treatment regimen. Sampling at day 1 was taken before start of consolidation i.e. before treatment, sampling at day 5 after 4 days of treatment with ATO in combination with ATRA, sampling at day 22-25 after two weeks of treatment with ATO only and the last sampling

Table 1 Descriptive data of the patients included in the study

	Gender	Age at diagnosis	Risk group
Subject No 1	Male	72	Intermediate
Subject No 2	Female	71	Low
Subject No 3	Male	31	Low

The risk group is stratified according to the Swedish national AML guidelines

Table 2 Numbers of samples from each patient

Day of treatment	Day 1	Day 5	Day 22-25	The 6th week during ATRA
Subject No 1	2 samples	2 samples	2 samples	2 samples
Subject No 2	4 samples	4 samples	4 samples	1 sample
Subject No 3	2 samples	2 samples	2 samples	1 sample

Day 1, 5, 22-25 and week 6 refer to when the sample was taken during the 8 weeks of treatment

during the sixth week when the patient was treated with ATRA only.

The consolidation treatment was administered according to the national recommendations by the Swedish AML group (Tables 3 & 4). Each consolidation cycle consisted of eight weeks of treatment either with ATO, ATRA or both treatments in combination [5]. Weeks seven and eight in each cycle are without treatment and thus represent two weeks of recovery. The patients were in remission at the time of blood sampling. All samples were harvested during consolidation cycles one to three except for subject 2, for which one sampling cycle was taken during consolidation cycle four.

Blood from healthy donors was collected at Blood Central, Umeå, in conjunction with blood donation. The donors were informed orally by the staff and gave oral and/or written consent. No information concerning the healthy donors was obtained, and the donation was anonymous to the person performing the neutrophil isolation.

Neutrophil isolation

Blood samples were collected in tubes containing EDTA. For isolation of neutrophils, a two-step gradient procedure was used. The blood was initially carefully placed on a layer of Histopaque (Histopaque 1119, Sigma Life Science) and centrifuged at 800 g for 40 minutes. The interphase between the plasma and the red blood cells were collected subsequently, washed with PBS and placed on a Percoll (Cytiva) gradient extending from a 65% to an 85% isotonic Percoll solution. Isotonicity was adjusted using ten-fold-concentrated PBS. The gradient was centrifuged at 800 g for 20 minutes and the layer between 70 and 75% containing neutrophils was collected. After washing with PBS and RBC lysis (Biolegend), cell viability was determined in a cell counter (Bio-Rad TC20 automated cell counter) using trypan blue. The isolation process usually yields >95% mature neutrophils [22].

Table 3 Consolidation numbers 1-3

Consolidation 1-3	Treatment
Day 1-14	ATRA 45 mg/m^2
Day 29-42	ATRA 45 mg/m^2
Day 1-5	ATO 0,30 mg/kg
Day 8, 11, 15, 18, 22 and 25	ATO 0,25 mg/kg

Table 4 Consolidation number 4

Consolidation 4	Treatment
Day 1-14	ATRA 45 mg/m^2
Day 1-5	ATO 0,30 mg/kg
Day 8, 11, 15, 18, 22 and 25	ATO 0,25 mg/kg

Chemotaxis

Neutrophil chemotaxis was examined using a transwell system as previously described [22]. In brief, neutrophils isolated from the three subjects were resuspended in Rowell Park Memorial Institute) medium (RPMI 1640, Lonza) with 0.05% human serum albumin (HSA, alburex CSL Behring) and stained with 3.3 µM bis-2-carboxyethyl-5-[and-6]-carboxy-fluorescein-AM (Sigma–Aldrich) in a 24-well fluorescence-blocking transwell system (BD Falcon, HTS FluoroBlok, 3 µm pore size, PET membrane) for 20 minutes at room temperature. After washing and resuspending in RPMI with 0.05% HSA, 5×10^5 neutrophils were seeded in fluorescence-blocking transwell inserts. The inserts were placed in a 24-well plate loaded with 600 µl of RPMI and 10 nM N-formyl-methionyl-leucyl-phenylalanine (fMLF). A plate reader (FLUOstar OMEGA; BMG Labtech) was used with 5% CO_2 and 37 °C. Fluorescence intensity (FI) with excitation 485 nm and emission 520 nm was detected from below every minute over the course of 30 min. Neutrophils inserted directly into the well were used as a 100% FI control, and medium only was used as a blank. When using neutrophils isolated from healthy donors, the assay was performed in a similar manner, but neutrophils were resuspended in As_2O_3 (ATO, Sigma Aldrich) diluted in RPMI with 0.05% HSA to final concentrations of 5 µM, 50 µM, 100 µM and 250 µM or ATRA 1 µM. For evaluation, we calculated migration as percentage of labeled cells in absence of a transwell insert. The migration of unstimulated cells was subtracted from the fMLF-induced migration. We calculated the time required for 50% of the maximum migration to determine the half maximum migration time. The percentage difference per minute at half maximum migration time was used as a measure of velocity according to previous work [22].

Reactive oxygen species production

A luminol-based chemiluminescence assay was used to quantify reactive oxygen species production. Neutrophils (5×10^4), resuspended in RPMI, were seeded into 96-well

plates together with 50 µM luminol and 1.2 U of horseradish peroxidase. After 15 minutes of preincubation at 37 °C with 5% CO_2, 20 nM phorbol 12-myristate 13-acetate (PMA) was added to trigger the oxidative burst. Chemiluminescence was measured at 37 °C every other minute for 3 hours (Varioskan Flash, Thermo Scientific). The result was presented as area under the curve (AUC). AUC for the 90 values measured during the experiment was calculated using Graph Pad Prism 6 (formula: ΔX*([(Y1+Y2)/2]-Baseline). When using blood from healthy donors, isolated neutrophils were resuspended in As_2O_3 diluted in RPMI to final concentrations of 5 µM, 50 µM, 100 µM and 250 µM. Neutrophils unexposed to PMA served as an unstimulated control. ROS production was calculated as the area under the curve (AUC) and presented as the AUC of PMA-stimulated neutrophils minus the AUC of unstimulated neutrophils as described previously [22].

DNA fluorescence assay

To quantify NET formation upon phosphokinase activation using PMA, we seeded 5×10^4 neutrophils per well in a 96-well plate together with 0.5x Sytox green nucleic acid stain (Thermo Scientific). Triton X-100 was added to wells serving as a 100% lysis control. After 15 minutes of incubation at 37 °C and 5% CO_2, 100 nM PMA was added. FI was measured using a plate reader (FLUOstar OMEGA; BMG Labtech) with 5% CO_2 and 37 °C, and excitation at 485 nm and emission at 520 nm were detected every 10 minutes for 10 hours. Unstimulated neutrophils served as a negative control. Fluorescence was calculated as a percentage of lysis control after subtraction of background signal obtained from unstimulated cells at the respective time points. Data are presented as a percentage of extracellular DNA.

Flow cytometry for cell viability quantification

After isolation was performed, 1×10^6 neutrophils per sample were resuspended in RPMI and 5 µM, 50 µM, 100 µM or 250 µM ATO. Fixation with 2% paraformaldehyde (PFA) served as a control. Cells were incubated at 37 °C with 5% CO_2 for 3 hours, centrifuged and subsequently resuspended in PBS. Propidium iodide (2.5 µg, Immunochemistry Technology) was added to each sample. The samples were incubated on ice for 20 minutes followed by flow cytometry measurement performed in a ZE5 Cell Analyser (Bio-Rad). A total of 1×10^5 cells were examined per condition. Cells positive for propidium iodide were considered nonviable.

Measurement of ATO concentrations

When isolating neutrophils, plasma was collected and frozen at -80 °C. Coded plasma samples were sent to ALS Scandinavia, Luleå, Sweden for analysis. The ATO concentration was determined using ICP-SFMS according to SS-EN ISO 17294-2:2016 and US EPA Method 200.8:1994.

Statistics

We used Graph Pad Prism 6 for our statistical analysis. When evaluating data, we tested for normal distribution of data points using Shapiro-Wilk test and the Kolmogorov–Smirnov test. To be able to make multiple comparisons, one-way ANOVA was used for normally distributed data. For data without normal distribution, the Kruskal–Wallis test was used. A p value below 0.05 was considered statistically significant. Pearson's or Spearman´s test of correlation was used to determine any relation between samples. Statistical power was not calculated due to the study being explorative in nature.

Results

The leukocyte count and absolute neutrophil count (ANC) decreased in patients undergoing treatment with ATO (Fig. 1A, B). This finding was expected since ATO is known to induce neutrophil apoptosis and decrease the ANC (Binet, et al 2011). However, from all patient samples, only two values were below 1×10^9 cells/L, and none of them fell below 0.5×10^9 cells/L. Thus, the treatment did not lead to severe neutropenia defined as ANC<0.5×10^9 cells/L [23]. Notably, the reduction was already noticeable after four days of treatment. Treatment with ATRA exclusively did not affect the leukocyte count or the ANC.

In addition to neutrophil count, the functionality of neutrophils from patients undergoing consolidation treatment with ATO and ATRA was impacted. We observed that neutrophil chemotaxis towards fMLF was decreased before starting consolidation (i.e., after two weeks without treatment) compared to healthy controls as determined by percentage migration (Fig. 1C), as was the calculated velocity at half-maximum migration (Fig. 1D). After four days of treatment with ATO and ATRA, the migration percentage increased compared to the migration of healthy donor neutrophilss. After four weeks of consolidation treatment (day 22-25, during treatment with ATO only), the migration was again reduced slightly compared to the migration of healthy donors, whereas the calculated velocity remained at earlier levels (Fig. 1C).

Next, we investigated whether the leukocyte and neutrophil counts correlated with chemotactic ability. We found a negative correlation indicating an association of lower leukocyte counts and ANCs with increased migration (Spearman's test: p:0.01, r: -0.50 and p:0.01 and r:-0.55, respectively) (Fig. 1E, F).). To gain insight into

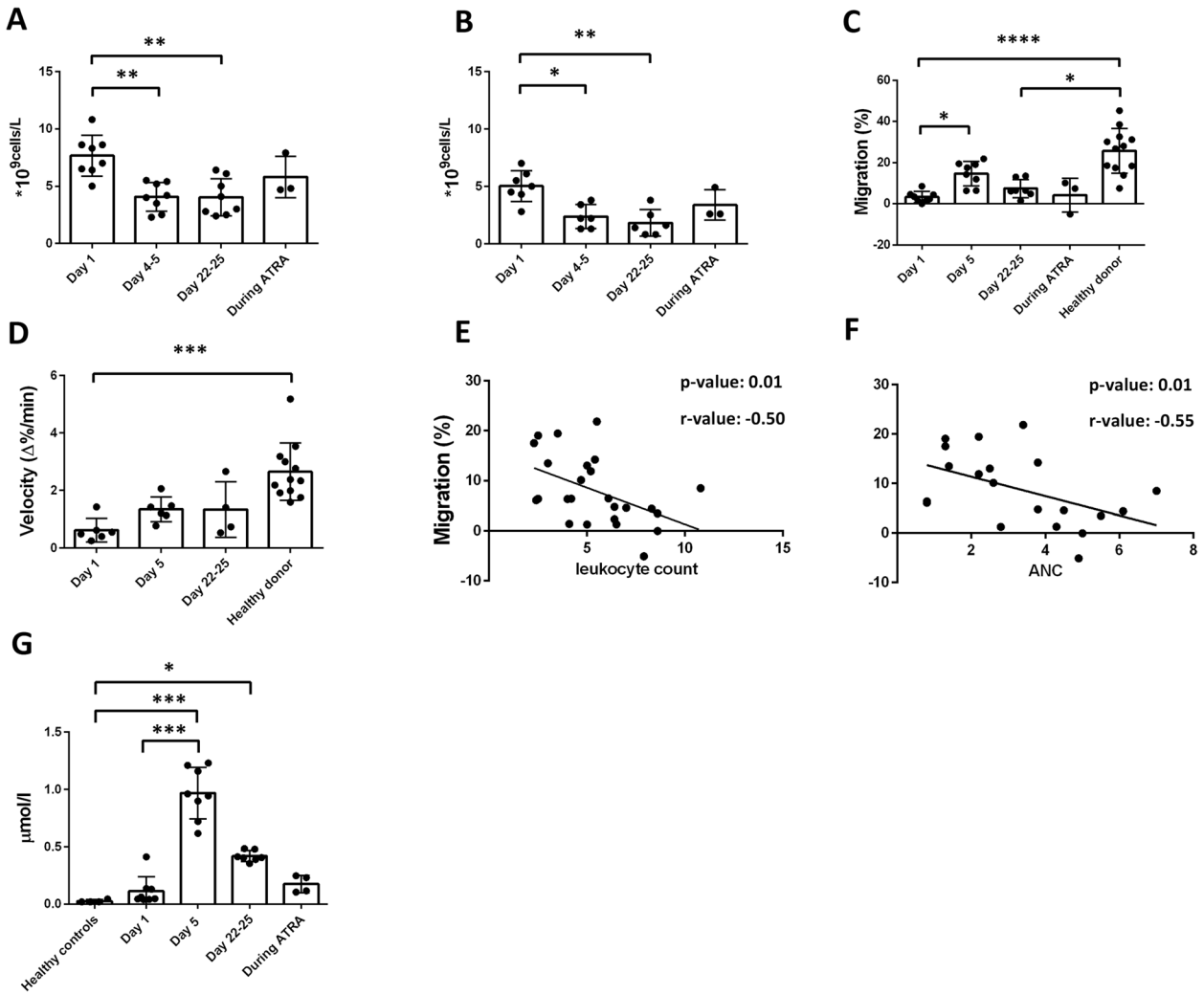

Fig. 1 Low neutrophil count and increased neutrophil chemotaxis correlate in APL patients treated with ATO. The leukocyte count (**A**) and absolute neutrophil count (ANC) (**B**) were reduced during ATO treatment (at days 4-5 and days 22-25) compared to before treatment (day 1). When only on treatment with ATRA (week 6), there was no decrease in leukocyte and absolute neutrophil counts. Chemotaxis of neutrophils isolated from subjects at different time points during treatment with ATO and ATRA and from healthy donors are presented as percent migrated cells (**C**) and velocity at half-maximum migration time (**D**). Migration and velocity are decreased before treatment compared to healthy donors, indicating a reduced chemotactic ability. The correlation between leukocyte count and chemotaxis (**E**) and ANC and chemotaxis (**F**) was examined using Spearman´s test of correlation. There was a negative correlation, indicating that a lower leukocyte count or ANC was correlated with increased chemotaxis. Levels of ATO measured in plasma collected from study subjects and healthy donors presented as µM/L (**G**). There was an increase in ATO levels in study subjects during ATO treatment (days 5 and 22-25) compared to before treatment and in healthy donors. Since the migration of unstimulated cells was subtracted from the fMLF-induced migration to calculate percentage migration, some of the values reported fell below 0, due to the unstimulated value reaching a higher total migration than the examined condition (C, E, F)

actual concentrations in patients, ATO levels were measured in plasma collected from study subjects when samples for neutrophil isolation were taken. To determine baseline, we also included plasma from healthy controls. We observed higher levels of ATO in patients during ATO treatment (day 5) compared to before the start of a treatment cycle (day 1) and to untreated, healthy controls (Fig. 1G). The highest levels of ATO close to 1 µmol × l^{-1} were measured on day 5 of treatment.

To test the direct effect of ATO on neutrophils isolated from healthy donors, we exposed neutrophils in vitro to ATO and ATRA separately and examined the chemotactic ability. Chemotaxis towards fMLf decreased with increasing concentrations of ATO, whereas ATRA did not have an effect on chemotaxis (Fig. 2).

In addition to chemotaxis, oxidative stress constitutes a crucial function of neutrophils. We evaluated ROS production in samples taken from subjects 2 and 3. We

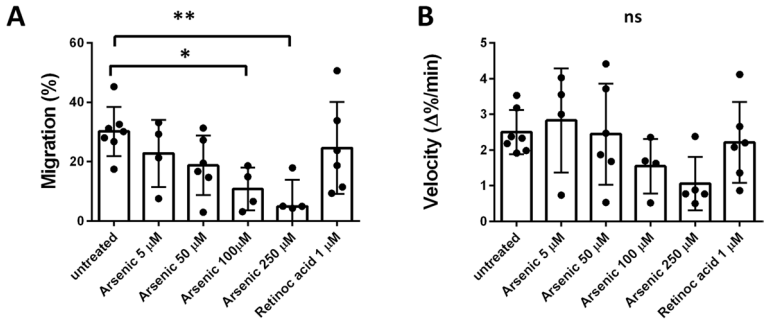

Fig. 2 In vitro, ATO but not ATRA decreases neutrophil chemotaxis. Neutrophils isolated from healthy donors were exposed to ATO or ATRA at different concentrations. Chemotaxis is shown as the percentage of migrated cells (**A**) and velocity at half-maximum migration time (**B**). Chemotaxis was reduced at higher concentrations of ATO but was not affected by ATRA

found more ROS production on day one compared to the levels produced by healthy donors (Fig. 3A). On day 25 of treatment (after 4 weeks of ATO therapy), ROS production decreased back to the levels of healthy donors. Comparison of leukocyte counts, ANC and ROS production showed that there was a significant correlation of higher leukocyte counts and ANC with increased ROS production (Pearson´s test: p=0.03, r=0.49 and p=0.03, r=0.49, respectively) (Fig. 3B, C).

To test the direct effect of ATO on neutrophils, we determined ROS production in isolated neutrophils from healthy donors in vitro. When neutrophils from healthy donors were exposed to ATO, we found a tendency of decreased ROS production with increased ATO levels, but the findings were not significantly different from each other (Fig. 4A). Neutrophils exposed to ATRA in vitro showed a similar trend of decreased ROS production at higher concentrations of ATRA, but the findings were not significantly different (Fig. 4B).

Next, we determined the impact of ATO on NET release, since NET formation is an important antimicrobial defence mechanism of neutrophils. To quantify the release of NETs, we used a fluorescence-based assay measuring PMA-induced NET formation over a period of six hours as previously described [24–26]. When evaluating neutrophils isolated from patients during different time points of treatment and comparing them to healthy donors, there were no differences in NET release at three (Fig. 5A) or six hours post stimulation (Fig. 5B). We performed similar experiments with neutrophils isolated from healthy donors and exposed the cells to ATO in vitro. Neither in these experiments could we observe effects on NET release upon ATO exposure (Fig. 5C & D). According to previous experience, the fluorescence-based assays correlate well with microscopic, image-based assays for NET quantification [24–26]. As the fluorescence-based assay did not reveal differences in ATO-exposed and unexposed neutrophils, we concluded that corroboration with microscopy-based assays was not required.

To evaluate whether our overall findings were influenced by an increase in neutrophil death during our assays, we determined neutrophil viability. We performed flow cytometry on neutrophils isolated from healthy donors after three hours of incubation with different concentrations of ATO (Fig. 5E). Propidium iodide (PI) was used to label nonviable cells. There was no difference in the number of PI-positive cells, confirming our previous finding that ATO exposure at the concentrations used in our assays does not increase cell death within the time limit of our experiments.

Discussion

Chemotherapy-based treatment of APL negatively impacts neutrophil function [27]. Our exploratory study is based on the hypothesis that ATO and ATRA may also affect the functions of neutrophils in patients treated for APL. The aim was to improve the possibility of performing risk assessments for patients' susceptibility to infections based on neutrophil function data. The sampling from day one of the treatment regimen is taken before administration of ATO and ATRA. It takes 10 days for neutrophils to reach full maturity [28]. This means that the majority of neutrophils present in circulation at the start of each treatment regimen matured in the absence of ATO and ATRA. Notably, the last 14 days of each regimen did not include any treatment. Previous treatment with chemotherapy can cause a defect in neutrophil functions.

The chemotactic ability, i.e., the total migration of neutrophils, was decreased at the beginning of the treatment compared to the total migration performed by neutrophils isolated from healthy controls (Fig. 1C & D). In children undergoing chemotherapy, it has been reported that neutrophils have a decreased phagocytic function before the start of treatment [27]. This is in line

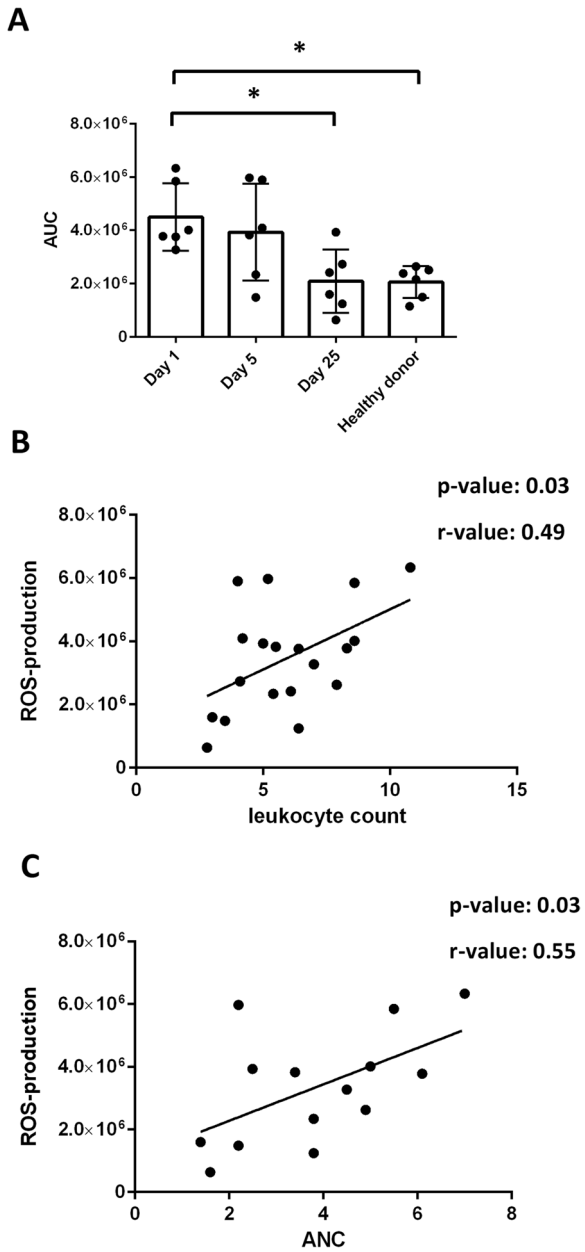

Fig. 3 ROS production by neutrophils decreases during ATO treatment cycles but is increased compared to that in healthy donors. ROS production was measured as the area under the curve (AUC) over a time period of three hours (**A**). Neutrophils isolated from subjects undergoing treatment with ATO showed higher ROS production before treatment than those from healthy donors. There was also a decrease after four weeks of ATO treatment compared to before treatment. Correlation between ROS production and leukocyte count (**B**) and ANC (**C**) was tested using Pearson´s test of correlation showing an association between higher leukocyte counts, ANC and increased ROS production

with our findings suggesting that a suppressive effect of a myelotoxic treatment might linger for a longer time and affect neutrophils that are being matured in the chemotherapy-free interval. However, in our study, chemotaxis was improved after four days of treatment with ATO and ATRA (Fig. 1C & D). The findings were consistent among different consolidation rounds, and the increased total migration correlated with a decrease in leukocyte count and ANC. Since the patients were treated with both ATO and ATRA during the second sampling, it is not possible to attribute the findings to one of them specifically. It is known that chemotactic ability declines in older patients [29]. However, we could not see a difference in the findings when comparing the two older patients to the younger patient, the trend was the same for all subjects. Previous studies together with our findings (Fig. 2) related to exposing neutrophils from healthy donors to ATO or ATRA in vitro indicate that ATO and ATRA decrease neutrophil chemotaxis [30]. Due to the explorative nature of our study we tested different concentrations of ATO to evaluate the effects on chemotaxis. We only used one concentration of ATRA since higher concentrations (125μM) have been proven to induce apoptosis in neutrophils [31]. The concentration of 1μM ATRA was selected, since this concentration is well established to induction of differentiation of HL-60 cells into mature neutrophils [32]. A study on children with APL showed that ATRA-concentrations in plasma peaked at mean 429,7 ng/ml, which is lower than our chosen concentration but they were treated with a lower ATRA-dose ($30mg/m^2$) compared to our study subjects ($45mg/m^2$) [33]. The levels of exposure to the drugs in our in vitro experiments were higher than the estimated levels in the blood of patients undergoing APL treatment, which could in part explain the deviating results. In addition, the exposure in blood during APL treatment occurs at lower concentrations but over a prolonged period of time compared to in vitro experiments, both of which may affect outcome.

The correlation between a decrease in leukocyte count and ANC and an increase in total migration was significant but not pronounced, with an r-value of approximately -0.5 (Fig. 1E & F). This implies that the decreased amounts of neutrophils could at least partially be compensated for with an increased chemotactic ability, while other, unknown factors may be involved. It has been shown in other studies that subjects with a lower ANC have higher plasma levels of IL-8 [34, 35]. ATO exposure to neutrophils isolated from healthy donors increases the neutrophil release of IL-8 [19, 29]. This may be the case in our study subjects and could contribute to an increased chemotactic ability of neutrophils. In accordance, high levels of IL-8 in the blood of children with febrile

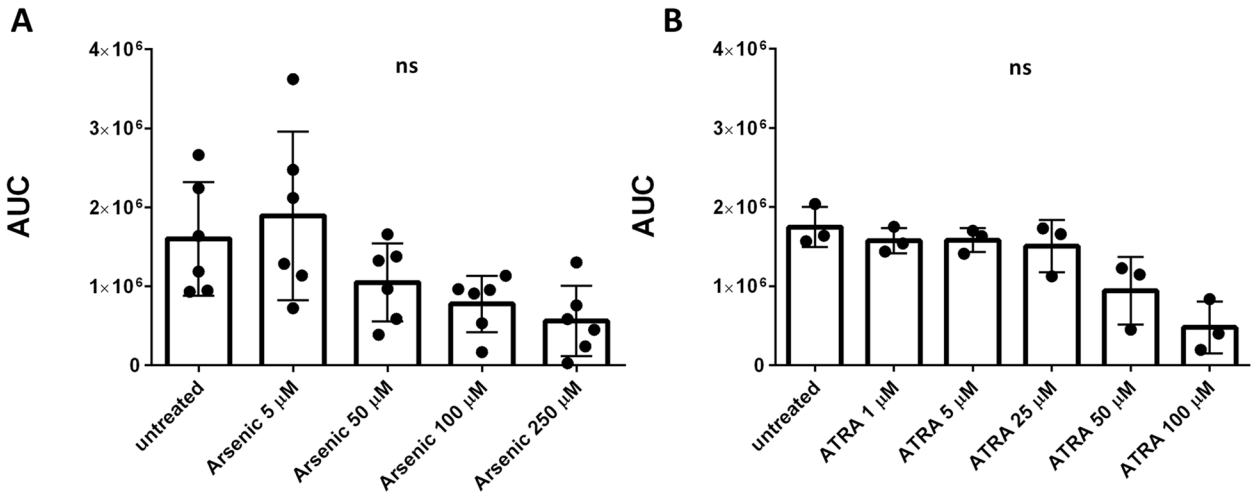

Fig. 4 Direct effect of ATO and ATRA on neutrophil ROS production in vitro is not statistically significant. Neutrophils isolated from healthy donors were exposed in vitro to ATO (**A**) and ATRA (**B**). They were then activated by PMA, and ROS production was measured for three hours and calculated as the AUC. There was a tendency of decreased ROS production with increased levels of ATO and ATRA, but the values were not significantly different as determined using a one-way ANOVA test

neutropenia failed to predict infection severity [36]. An increased neutrophil function due to higher levels of IL-8 could at least in part act as a compensatory mechanism for decreased neutrophil counts. However, such a connection has not been studied yet and, therefore, we may only speculate at this point, but this issue could be an interesting topic for future research. An increased chemotactic function of neutrophils in the neutropenic phase could very well be part of the explanation for why our patients did not suffer from severe infection during the time of sampling.

In previous studies, ATO concentration was measured in APL patients before and four hours after intravenous ATO treatment, showing levels of ATO varying between 0.05 µmol/l before treatment and 0.54 µmol/l four hours after treatment [37]. The dosage of ATO in that study was lower than the ATO dose given to the subjects in our study (0,16 mg/kg compared to 0,25 and 0,30 mg, respectively). This correlates to the concentrations of ATO being higher in the plasma samples from our study subjects with an increase on day 5 (mean value 0,97 µM/L) compared to day 1 (mean value 0,11 µM/L) and an increase on day 25 (mean value 0,42 µM/L) compared to healthy donors (mean value 0,03 µM/L). ATO is eliminated through renal excretion and has a half-life in plasma of approximately 10-14 hours [38]. The concentrations are considerably lower than the concentrations used for in vitro ATO exposure, but this corroborates that the levels of ATO in plasma in our study subjects are higher throughout each consolidation cycle during ATO treatment (four weeks in total per cycle) compared to the levels seen in healthy donors (Fig. 1G).

ROS production after stimulation with PMA was increased in neutrophils isolated from our study subjects before a treatment cycle, compared to neutrophils from healthy donors, indicating increased functionality (Fig. 3A). It has been shown that neutrophils from patients with chronic lymphocytic leukemia are more prone to produce ROS than neutrophils from healthy donors [39], but for APL patients, this remained unknown. Four weeks into ATO therapy, ROS production returned to levels comparable to those of neutrophils from healthy donors. This is in line with an earlier report describing lowered neutrophil ROS production upon ATO exposure in vitro [18] and is corroborated by our findings of slightly decreased ROS production upon in vitro exposure of neutrophils from healthy donors to high ATO concentrations (Fig. 4). In our setting, the in vitro exposure of neutrophils from healthy donors to ATO was relatively short (3 hours), which may explain why observed differences were not more pronounced. More importantly, we found a positive correlation between high leukocyte count and ANC in our study subjects and increased neutrophil ROS production (Fig. 3B & C), which is contrary to the correlation of cell count and chemotactic ability (Fig. 1E & F). The r-values comparing ROS-production with leukocyte count and ANC indicate a moderate correlation which may be due to direct or indirect effects of ATO and ATRA treatment on neutrophils.

Moreover, we demonstrated that the formation of NETs upon PMA stimulation was unaltered in neutrophils isolated from APL patients (Fig. 5). This is in contrast to a previous study finding that neutrophils of

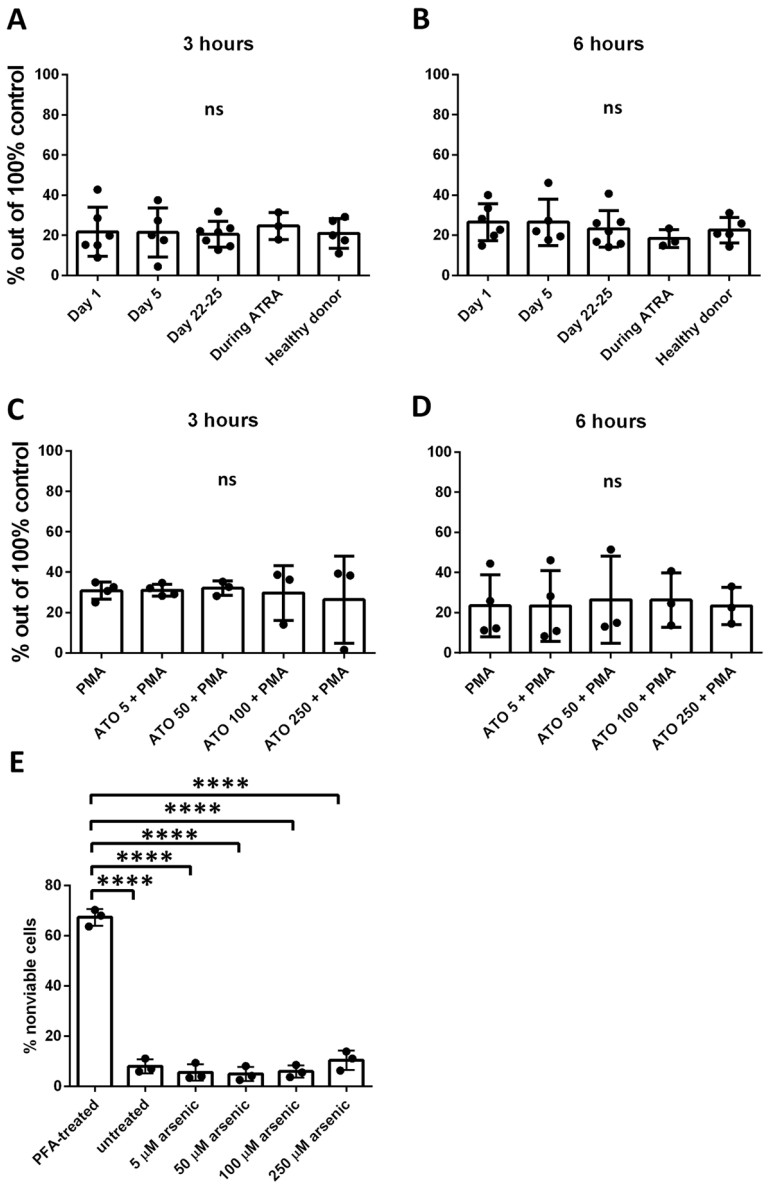

Fig. 5 Neither ATO treatment of APL patients nor direct exposure to ATO in vitro affects NET release of neutrophils. Neutrophils isolated from patients undergoing APL treatment and from healthy donors. The figure shows the quantification of PMA-induced NET production by neutrophils calculated as percentage of 100% control after three hours (**A**) and six hours (**B**). The figure also shows the quantification of PMA-induced NET production by neutrophils isolated from healthy donors treated with ATO in vitro at three hours (**C**) and six hours (**D**). There was no significant difference between neutrophils from different times of treatment compared to neutrophils from healthy controls measured with one-way ANOVA. Neutrophils stained with propidium iodide (PI) as a marker of nonviable cells measured by flow cytometry after three hours of exposure to ATO at different concentrations (**E**). Cell viability was considerably decreased after PFA treatment, which served as a control, whereas cell viability was not affected in the range of ATO concentrations used throughout our experiments

APL patients undergoing induction treatment showed increased NET formation [40]. The discrepancy might stem from the fact that our study subjects were in remission at the time of sampling and hence, the neutrophils examined were more likely to be mature displaying less responsivity to ATO and ATRA exposure as the neutrophils from APL patients undergoing induction treatment. Consistently, in vitro ATO treatment of isolated neutrophils from healthy donors did not result in altered NET production (Fig. 5C & D), despite being in contrast to a previous study showing that healthy donor neutrophils exposed to ATO in vitro release fewer NETs than their untreated counterparts [18]. Hence, our study demonstrates that NET production is virtually unaffected

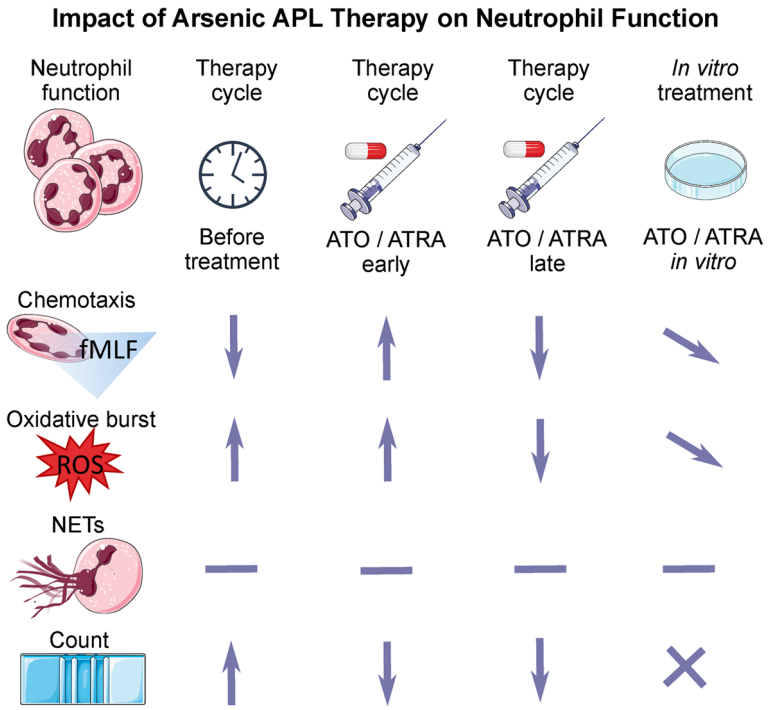

Fig. 6 Summary image to illustrate main findings of the study. Indicated are the time points before and under consolidation treatment cycles when patient neutrophils were isolated from peripheral blood and functionally tested, as well as direct in vitro treatment of isolated neutrophils. The effect on the different functions, chemotaxis, reactive oxygen burst, NET formation, and ANC (count) are depicted by arrows in relation to healthy donor neutrophils. An upward-oriented arrow represents an increased, a downward-oriented arrow represents a decreased functionality. A dash represents unchanged functionality compared to control. An "X" means that the function is not applicable for the respective condition

by ATO treatment, both in exposed neutrophils from healthy donors and in neutrophils from APL patients under remission. A limitation of our study remains the low number of included subjects undergoing treatment for APL which is mainly due to rarity of APL.

Conclusions

We observed decreased chemotaxis but increased ROS production in neutrophils isolated from our study subjects undergoing treatment for APL compared to healthy controls (Fig. 6). During the course of ATO/ATRA treatment, the chemotactic ability of neutrophils recovers, and ROS production returns to the levels of healthy donor neutrophils. As low neutrophil counts correlate with an elevated chemotactic ability, while high neutrophil counts correlate with increased ROS production, we conclude that the amount and functionality of neutrophils is likely to be compensated in APL patients undergoing ATO/ATRA therapy. Hence, the therapy is unlikely to increase the patients' susceptibility to severe microbial infection, which is corroborated by the absence of severe infection incidences in our study subjects. Moreover, in vitro exposure of neutrophils to ATO and ATRA only poorly reflects the effects of ATO/ATRA therapy on neutrophil function. In vitro experiments should, therefore, only be used in combination with experiments using neutrophils isolated from APL patients. Our study indicates that alterations in neutrophil function are compensatory and strongly support the advantageous clinical impact of chemotherapy-free leukemia treatment. Further studies will be helpful to better understand the cellular processes that give rise to our observations.

Abbreviations

AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APL	Acute promyelocytic leukemia
ATO	Inorganic arsenic
ATRA	Retinoic acid
CD	Cluster of differentiation
fMLF	N-formyl-methionyl-leucyl-phenylalanine
HL-60 cells	Human leukemia cell line 60
NET	Neutrophil extracellular trap
PBS	Phosphate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species

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Authors' contributions
Conceptualization, ATS and CFU; methodology, CFU and ATS; formal analysis, ATS; investigation, ATS; writing—original draft preparation, ATS; writing—review and editing, CFU; funding acquisition, ATS and CFU. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethical Review Board, Umeå, Sweden (register number 09-210 M, project number CFU-12/09, date 2010-01-12) with patients' consent.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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