nature portfolio

Peer Review File

AML-Modified BM EPCs Could be Remodeled to Facilitate Normal Hematopoiesis Recovery After Complete Remission

Corresponding Author: Professor Xiaojun Huang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript by Drs. Xing, Hu, and colleagues is very well-written and attempts to decipher the differences the bone marrow microenvironment of leukemic patients, those in remission, and healthy marrows from donors, with a focus on the features of bone marrow endothelial progenitor cells. They found marked morphologic differences among them, suggest that AML-modified BM endothelial cells could be partially reversed, that this can support hematopoietic recovery, and conduct a small trial to assess the role of NAC to reinvigorate endothelial cells, decrease reactive oxygen species, and promote hematopoiesis. I commend the authors on their work, and provide a few questions and suggestions below.

-We do have CR, leukemic, and HD samples here that have been assessed. But I think the group that is of most interest, perhaps are those with delayed count recovery, patients who remain cytopenic or hypoplastic/aplastic following count recovery - should one assess those samples for EPC function and ROS, etc. Do the authors feel that this may provide a better estimate of what is driving delayed count recovery following induction, rather than CR samples.

-I have some concerns regarding the clinical trial data - we don't have specifics regarding the right dose of NAC. How do we know that the NAC dose used was sufficient in achieving its pharmacodynamic effect. I realize that these patients received NAC, and and the authors are telling us they have better platelet recovery and are less cytopenic, but how can we know this is from NAC? A small cohort study like this cannot be powered sufficiently or well matched to eliminate any potential bias and establish a difference.

-The authors state that "Among the CR patients in NAC and control groups, the median periods of myeloid and platelet recovery were 14 days and 14 days(P=0.30), and 12 days and 15 days, respectively(P=0.0001)." This seems unusual. Our experience with induction regimens like IA is that time to count recovery is typically 28-35 days from time of treatment. The authors should explain this. They should also explain why count recovery was not improved for myeloid cells.

-The authors also state that the "... dose of G-CSF(P=0.002) and amount of platelet transfusion (P<0.0001) were markedly lower in NAC group than in control group". This is unclear to me. What is meant by "dose of GCSF". Is this the number of doses or daily dose? Did all patients receive GCSF? What is meant by "amount of platelet transfusion".

-These details are important because otherwise one cannot compare your data to real world experience. We do not see count recovery in 12 days following chemotherapy, we do not give GCSF to all patients, etc...

-The authors state that "Compared with CR, NAC improved the percentage of HSCs (... 0.67%±0.08% vs. 0.41%±0.06%; P=0.03)". It is also important to clarify when these measurements were made - at what point following CR were they checked?

Reviewer #2

(Remarks to the Author)
In this paper, the authors compared that Endothelial cells of patients with AML at diagnosis, post-remission comparted to healthy donor. They show as shown by others uisng mouse models or PDX, that EC are being affacted by AML. Nevertheless the novelty here is the fact that EC are still not completely normal at remission. They show that they

nevertheless are closer to normal HD EC than AML-EC and can promote HSC support. They show nevertheless that the support can be increase by reducing the ROS level via treatment via NAC.
Using AML-ETO mouse model, they further confirmed effect of NAC.
They further include a clinical trial data where they treated the pateinst at CR with or without NAC to help increase the regeneration of normal HSPCs.
Nevertheless there are major comments that can be raised and that will need to be addressed.
1- Using the mouse model, it is important to add a control group where they could dissect whether the effect on EC is due to AML or to the chemotherapy and how chemotherapy treatment has a long effect on EPC and whether this related to the EPC-CR phenotype observed. No where they discuss or incorporate mouse treated with chemo and not injected with AML-ETO. It will be also possible to use this normal control group to dissect what are the effect of chemo on normal HSPC from the mouse at different time point after chemotherapy and see whether 14 days post-chemo normal EPC will be similar or different to EC at CR.
2- The EC are composed a different sub-types which could be at least studied in mouse models: using for example Sca.1 to dissociate efefct on sinusoidals versus arterioles EC. The effect on human ECs: could they provide further info on what subtype of EC is mostly affected by AML??
3- The clinical data is of interest but do not provide any direct info on the potential effect fo NAC on EC. Could be that NAC acts on other BM niche components and or even on residual normal HSPCs.
4- In the co-culture provided of EPC with AML, or after CR it is unclear how many EPCs were used, from how many AML or HD?

Reviewer #3

(Remarks to the Author)
Abstract

The abstract is well written and summarizes the findings well.

Introduction

The introduction is well written overall. However, the authors should discuss other potential causes of failure to achieve normal hematopoiesis following successful anti leukemia therapy. These include the HSC depletion, inhibition of production of downstream hematopoietic cells by impeding differentiation at the HSC–progenitor transition, immune mediated hematopoietic failure, clonal hematopoiesis, and co existing myelodysplastic syndrome.

Methods

Patients and control individuals
Is the age given of patients the median? This median and range age of the patients should be moved to the results section.

Were the AML samples collected at time of diagnosis? What was the outcome of these patients? Were the CR samples taken at time of count recovery after induction? A table of patient characteristics of all groups should be provided including sex, age, wcc at diagnosis, molecular and cytogenetic abnormalities, and ELN classification.

Study design of a registered prospective single-arm study

What classification was used to designate the AML risk groups? Reference 7 is a review article of AML.

“NAC 400 mg tid D1-28 was added on the basis of routine supportive
therapy(blood transfusion)”. This statement is unclear. Did patients begin NAC when a blood transfusion was needed. D1 to D28 implies that NAC was given concurrently with chemotherapy induction but it is stated that it was started after chemo was finished. Please clarify. The primary endpoint was the time to hematopoietic
1 recovery white blood cell(WBC) (>109/L and platelet(PLT)(> 20 109/L). Usually, hematopoietic is defined as neutrophil count greater than 1 and platelets greater than 100. The endpoint defined here is problematic since the patients received GCSF which is not usually done in patients before CR, and platelets greater than 20 can be achieved with routine platelet transfusion.

Results
Overall the preclinical data is well presented, follows logically and presents a coherent argument for the impact of the leukemia microenvironment on normal hematopoeisis. However, The labels of the figures are very small in places making it difficult to read. The clinical trial data is weak due to poor definition of endpoints including count recovery and definition of infections. Since all patients in both groups were still alive no impact on mortality can be seen. A larger, randomized study is necessary including more frail patients is necessary to determine if indeed there is an impact of NAC on treatment.

Some specific comments:

Figure 1 h and J should be labeled with “crystal violet” or ” tube length” and the I and K should be placed adjacent to the representative images for clarity.

Figure 3. The labels of the figures are difficult to read.
Figure 3m – were the cd34 leukemia cells primary leukemia cells or cell lines?

Figure 4

Figure 4c. The difference in platelet count between AC and NAC would be unlikely to be clinically significant and NAC does not seem to increase the time to platelet count recovery in a clinically significant manner.

Figure 6 a and b. The median time to myeloid and platelet count recovery should be defined.
The comparison of infections in the intervention and control group should be shown in figure 6. How was severe infection defined. What was the incidence of febrile neutropenia in both groups? What was the rate of fungal infections in both groups?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for your kind revisions. I do not think adding a small pilot study to your preclinical translational experiments in this paper is a good idea. I would suggest separating these into two separate papers. Count recovery median period of 21 days seem very brief to me for a population of AML patients with various presentations.

Reviewer #2

(Remarks to the Author)
The authors provide a new revised version where provided new information related to the effect of AD treatment on BM EC as well as effect on sinusoidal/arteriolar EC in the AML mouse model.
Compare to the human data provided, the mouse model at diagnosis (when achieving >20%) they do not show angiogenesis. Only when the AML is > 80% they show increase in EC.Thus when treated with AD, they show that AML+AD is similar to normal HD. Nevertheless when compare to AD treatment, they show that the presence of AML might have protected EC from AD treatment as they show clear increase to EC in AML-AD group. When considering frequency of arteriole/sinusoid, they mentioned that they show similar data that was reported by others i.e: increase in arterioles and decrease in sinusoids with AML. The increase is nevertheless modest. What is clear is that after AD treatment the level of arterioles increase substantially both in normal AD group and AML+AD group, indicating that arterioles are clearly surviving better AD treatment. Thus, based on the new data and the differential effect of AD on arterioles/sinusoids, it question of overall conclusion merging both EC subsets.
Similarly,it will make sense to divide the arterioles/sinusoids when looking at the effect of ROS, transcriptomic as the ratio in the EC group comparing Normal HD versus AML, versus CR is different. They also mentioned that at day 10 after AD, the level of arterioles/sinusoids is similar , indicating that the effect of AD in EC is long-term. When looking at the effect of NAC, then it questioned whether the increase in EC observed is preferential on one subtype to the others.

- Why in Figure 4F, the percentage of EC in CR group is quite different to what is presented in Figure 2J, where in CR the percentage of EC is similar to normal.If Figure 2J is correct, then the effect of NAC will not be significant.
- Based on the long-term effect of AD on EC, I believe that the analysis of the transcriptome, the addition of the AD effect on normal mice should be added as a better control than HD.

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed my concerns. I have no further comments.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have provided a new revised version of their manuscript in which they deleted the preliminary data on the clinical trial which makes the paper much more concise and focused more on potential mechanistic aspects.
They also add a discussion on potential limitations of their study.
They have thus reliably improved the quality of the manuscript and provided answers to all my concerns.

Response to the Reviewer Comments (NCOMMS-24-09119)

Reviewer #1 (Remarks to the Author):

This manuscript by Drs. Xing, Hu, and colleagues is very well-written and attempts to decipher the differences the bone marrow microenvironment of leukemic patients, those in remission, and healthy marrows from donors, with a focus on the features of bone marrow endothelial progenitor cells. They found marked morphologic differences among them, suggest that AML-modified BM endothelial cells could be partially reversed, that this can support hematopoietic recovery, and conduct a small trial to assess the role of NAC to reinvigorate endothelial cells, decrease reactive oxygen species, and promote hematopoiesis. I commend the authors on their work, and provide a few questions and suggestions below.

Question 1: We do have CR, leukemic, and HD samples here that have been assessed. But I think the group that is of most interest, perhaps are, patients who remain cytopenic or hypoplastic/aplastic following count recovery - should one assess those samples for EPC function and ROS, etc. Do the authors feel that this may provide a better estimate of what is driving delayed count recovery following induction, rather than CR samples.

Answer 1: Many thanks for your good instruction. We do agree that evaluation of BM EPCs in those with delayed count recovery after chemotherapy may provide a better estimate of what is driving delayed count recovery following induction, rather than complete remission(CR) samples.

According to the ELN 2022 recommendations[1], CR with incomplete hematologic recovery(CRi) was defined as all CR criteria except for residual

neutropenia <1 × 10^9/L or platelet<100 × 10^9/L. Therefore, we performed additional experiments using BM samples from AML patients in CRi(N=12) as delayed count recovery control to analyze the percentage of BM EPCs and their ROS levels.

The related revision has been added in the **updated Figure 1 and *Results* section(pages 10,11, lines 235-238; 248-255).**

【Reference】

1.Hartmut Döhner, Andrew H Wei, Frederick R Appelbaum, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. Blood. 2022 Sep 22;140(12):1345-1377.

Question 2: I have some concerns regarding the clinical trial data - we don't have specifics regarding the right dose of NAC. How do we know that the NAC dose used was sufficient in achieving its pharmacodynamic effect. I realize that these patients received NAC, and the authors are telling us they have better platelet recovery and are less cytopenic, but how can we know this is from NAC? A small cohort study like this cannot be powered sufficiently or well matched to eliminate any potential bias and establish a difference.

Answer 2: We agree that dose-optimization study is important to investigate whether the NAC dose used here was sufficient in achieving its pharmacodynamic effect. In the current study, we used the routine dosage of oral NAC(400 mg 3 times a day) according to our previous studies[1-4].

The following evidences support that the clinical effect is from NAC.

(1) As a reactive oxygen species (ROS) scavenger, *N*-acety-L-cysteine (NAC) is widely used as an antioxidant and a mucolytic drug without significant side

effects. Recently, we reported that oral NAC(400 mg 3 times a day) was safe and effective in patients with corticosteroid-resistant immune thrombocytopenia(ITP) patients, with prolonged isolated thrombocytopenia(PT) after allo-HSCT, as well as in reducing the incidence of poor graft function(PGF) or PT in patients with acute leukemia after allo-HSCT, by improving the reduced and dysfunctional BM EPCs[1-6].

(2) As shown in **Figure 6,** the current pilot cohort study(NCT06024031) showed that NAC was safe and effective in promoting normal hematopoiesis recovery through improving the quantity and functions of BM EPCs from AML CR patients.

According to the kind instruction from you and **Reviewer 3,** "We are aware that the small sample size and single-arm study design are limitations of this study…., which provides a rationale for further prospective randomized clinical trials with larger numbers of patients and longer follow-up periods to validate our preliminary findings in the future." has been added as the limitation of the current study in the ***Discussion section*****(pages 21, 22, lines 507-514).**

【Reference】

1. **Yuan Kong**, Xie-Na Cao, Xiao-Hui Zhang, Min-Min Shi, Yue-Yun Lai, Yu Wang, Lan-Ping Xu, Ying-Jun Chang, **Xiao-Jun Huang***. Atorvastatin enhances bone marrow endothelial cell function in corticosteroid-resistant immune thrombocytopenia patients. ***Blood.***2018;131(11):1219-1233.
2. **Yuan Kong**, Min-Min Shi, Yuan-Yuan Zhang, Xie-Na Cao, Yu Wang, Xiao-Hui Zhang, Lan-Ping Xu, **Xiao-Jun Huang***. N-acetyl-L-cysteine improves bone marrow endothelial progenitor cells in prolonged isolated thrombocytopenia patients post allogeneic hematopoietic stem cell transplantation. ***American Journal of Hematology.*** 2018; 93(7):931-942.
3. **Yuan Kong**,Yu Wang, Yuan-Yuan Zhang, Min-Min Shi, Xiao-Dong Mo, Yu-Qian Sun, Ying-Jun Chang, Lan-Ping Xu, Xiao-Hui Zhang, Kai-Yan Liu, **Xiao-Jun Huang***. Prophylactic oral NAC reduced poor hematopoietic reconstitution by improving

endothelial cells after haploidentical transplantation. ***Blood Advances.*** 2019;3(8):1303-1317

4. Yu Wang , **Yuan Kong** , Hong-Yan Zhao, Yuan-Yuan Zhang, Ya-Zhe Wang, Lan-Ping Xu, Xiao-Hui Zhang, Kai-Yan Liu, **Xiao-Jun Huang*.** Prophylactic NAC promoted hematopoietic reconstitution by improving endothelial cells after haploidentical HSCT: a phase 3, open-label randomized trial. ***BMC Med***. 2022;20(1):140.
5. Shu-Qian Tang, Tong Xing, Zhong-Shi Lyu, Li-Ping Guo, Mi Liang, Chen-Yuan Li, Yuan-Yuan Zhang, Yu Wang, Lan-Ping Xu, Xiao-Hui Zhang, **Xiao-Jun Huang**, **Yuan Kong*.** Repair of Dysfunctional Bone Marrow Endothelial Cells Alleviates Aplastic Anemia. ***Sci China Life Sci***. 2023; 66(11): 2553-2570.
6. Min-Min Shi#,**Yuan Kong**#, Yang Song,Yu-Qian Sun,Yu Wang, Xiao-Hui Zhang,Lan-Ping Xu, Kai-Yan Liu, **Xiao-Jun Huang***. Atorvastatin enhances endothelial cell function in posttransplant poor graft function. ***Blood.*** 2016;128(25):2988-2999.

Question 3: The authors state that "Among the CR patients in NAC and control groups, the median periods of myeloid and platelet recovery were 14 days and 14 days(*P*=0.30), and 12 days and 15 days, respectively(*P*=0.0001)." This seems unusual. Our experience with induction regimens like IA is that time to count recovery is typically 28-35 days from time of treatment. The authors should explain this. They should also explain why count recovery was not improved for myeloid cells.

Answer 3: Many thanks for your kind comments. Our initial calculation began from the first day after the end of chemotherapy, until the first occurrence of a WBC count >1×10^9/L for three consecutive days, and a PLT count > 20×10^9/L sustained without transfusion for seven consecutive days. Consequently, among CR patients in the NAC and control groups, the median periods for myeloid and platelet recovery were 14 days and 14 days(*P*=0.30), and 12 days and 15 days(*P*=0.0001), respectively. Following your suggestion, we recalculated from the first day of chemotherapy to hematopoietic recovery, with the median recovery times for WBC and platelets being 21 days and 21

days(*P*=0.30), and 19 days and 22 days(*P*=0.0001) in the two groups, respectively. We apologize for unclear English expression and the related revisions could be found in ***Methods section*** **(page 8, lines 168-171)** and ***Results section*** **(page 17, lines 405-409)**.

The shorter WBC recovery time observed in this study may be related to the chemotherapy dosages employed (IDA 10mg/m^2 and cytarabine 100mg/m^2), which are potentially lower than the dosages recommended (IDA 10-12mg/m^2 and cytarabine 100-200mg/m^2). And another reason may be the use of G-CSF in our study.

Question 4: The authors also state that the "... dose of G-CSF(*P*=0.002) and amount of platelet transfusion (*P*<0.0001) were markedly lower in NAC group than in control group". This is unclear to me. What is meant by "dose of G-CSF". Is this the number of doses or daily dose? Did all patients receive G-CSF? What is meant by "amount of platelet transfusion". These details are important because otherwise one cannot compare your data to real world experience. We do not see count recovery in 12 days following chemotherapy, we do not give GCSF to all patients, etc.

Answers 4: Thanks for your comments and we apologize for the confusions owning to your English expression. The term 'dose of G-CSF' refers to the cumulative doses. G-CSF was used when WBC was <0.5×10^9/L(5 μg per kilogram of body weight per day) after the completion of chemotherapy. Additionally, 'amount of platelet transfusion' denotes the total volume of PLT transfusions received before PLT>20×10^9/L. In our clinical practice, we do use G-CSF after chemotherapy when the WBC

count fell below 0.5×10^9/L. Our initial calculations started from the day after the end of chemotherapy, focusing on the time until the first three consecutive days with a WBC count $>1\times10^9$/L, resulting in a median myeloid recovery time of 14 days.

According to your suggestion, we recalculated the median WBC recovery time from the first day of chemotherapy to myeloid recovery, which is 21 days. The shorter WBC recovery time in our study may be related to the lower chemotherapy dosage and the use of G-CSF.

According to your kind comments, the related details have been added in ***Methods section*****(pages 7-8, lines 164-176)**.

Question 5: The authors state that "Compared with CR, NAC improved the percentage of HSCs (... 0.67%±0.08% vs. 0.41%±0.06%; *P*=0.03)". It is also important to clarify when these measurements were made - at what point following CR were they checked?

Answer 5: CR patients were detected at 28 days(± 2 days) after chemotherapy.

Based on your kind suggestion, we added "The quantities and ROS level of BM EPC and BM HSCs were detected in patients with CR at 28 days(± 2 days) after chemotherapy." in ***Methods section*****(page 8, lines 186-187).**

Reviewer #2 (Remarks to the Author):

In this paper, the authors compared that Endothelial cells of patients with AML at diagnosis, post-remission comparted to healthy donor. They show as shown by others using mouse models or PDX, that EC are being affected by AML. Nevertheless, the novelty here is the fact that EC are still not completely normal at remission. They show that they nevertheless are closer to normal HD EC than AML-EC and can promote HSC support. They show nevertheless that the support can be increase by reducing the ROS level via treatment via NAC. Using AML-ETO mouse model, they further confirmed effect of NAC.

They further include a clinical trial data where they treated the patients at CR with or without NAC to help increase the regeneration of normal HSPCs. Nevertheless, there are major comments that can be raised and that will need to be addressed.

Question 1: Using the mouse model, it is important to add a control group where they could dissect whether the effect on EC is due to AML or to the chemotherapy and how chemotherapy treatment has a long effect on EPC and whether this related to the EPC-CR phenotype observed. No where they discuss or incorporate mouse treated with chemo and not injected with AML-ETO. It will be also possible to use this normal control group to dissect what are the effect of chemo on normal HSPC from the mouse at different time point after chemotherapy and see whether 14 days post-chemo normal EPC will be similar or different to EC at CR.

Answer 1: According to your good instruction, we performed additional experiments to incorporate mouse treated with chemo and not injected with

AML-ETO as normal control group(AD group). We found that there were significant differences in the number of BM ECs between normal mice after chemotherapy(AD group) and AML mice after chemotherapy(AML+AD group), and such differences still existed 10 days later. Based on the current data, it is still difficult to answer whether the remodeling of BM EC-CR after chemotherapy is due to AML remission or chemotherapy, and who plays a key role in the remodeling process. However, our in vitro and in vivo data demonstrated for the first time that AML-modified BM EPCs could be partially remodeled to support normal hematopoiesis after CR.

As instructed, we added "The quantity of post-chemo normal ECs in AD group was lower than those in AML+AD group(Figure 2j; 1.2±0.2% vs.2.2±0.2%; *P*=0.007)." in ***Results section*** **(page 13, lines 305-307)**.

Question 2: The EC are composed a different sub-types which could be at least studied in mouse models: using for example Sca.1 to dissociate effect on sinusoidals versus arterioles EC. The effect on human ECs: could they provide further info on what subtype of EC is mostly affected by AML?

Answer 2: As previously reported[1-2], AML has an impact on both arterial and venous endothelium, with a significant decrease in venous endothelium and a significant increase in arterial endothelium.

According to your kind instruction, we performed additional experiments to analyze sinusoidals ECs versus arterioles ECs in AML mice model. Although the AML mice models utilized were different, we found the similar results as previously reported[1-2]. The related results have been added in ***Results section*** **(pages 13,14, lines 309-313)**.

In addition, our data demonstrated that chemotherapy had an effect on both sinusoidals ECs and arterioles ECs. The manifestation is: the frequencies of sinusoidals ECs and arterioles ECs increased after chemotherapy in AML mice. Because both are significant, it is difficult to say which category has the greater effect, and the effect seems to exist simultaneously.

【References】

1. Passaro D, Di Tullio A, Abarrategi A, Rouault-Pierre K, Foster K, Ariza-McNaughton L, Montaner B, Chakravarty P, Bhaw L, Diana G, Lassailly F, Gribben J, Bonnet D. Increased Vascular Permeability in the Bone Marrow Microenvironment Contributes to Disease Progression and Drug Response in Acute Myeloid Leukemia. Cancer Cell. 2017 Sep 11;32(3):324-341.e6.
2. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, Kokkaliaris KD, Mercier F, Tabaka M, Hofree M, Dionne D, Papazian A, Lee D, Ashenberg O, Subramanian A, Vaishnav ED, Rozenblatt-Rosen O, Regev A, Scadden DT. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. Cell. 2019 Jun 13;177(7):1915-1932.e16.

Question 3: The clinical data is of interest but do not provide any direct info on the potential effect of NAC on EC. Could be that NAC acts on other BM niche components and or even on residual normal HSPCs.

Answer 3: Many thanks for your kind comments. We have provided the following information on the potential effect of NAC on EC in the clinical trial. As shown in **Figure 6**, NAC treatment improved the percentages, and reduced ROS level of BM EPCs from CR patients. NAC treatment improved the functions of BM EPCs from AML CR patients, as indicated by increased double-positive cells, elevated tube formation and migration abilities. Notably, the hematopoiesis-supporting ability of BM EPCs from AML CR patients was improved by NAC treatment, as indicated by decreased ROS levels, decreased

apoptotic ratios of HSCs and elevated CFU-GM efficiency. In contrast, the leukemia-supporting ability of BM EPCs in NAC group was not significantly different from that of control group. Therefore, we demonstrated that NAC treatment improved the quantity and functions of BM EPCs from AML CR patients.

We do agree that NAC may act on other BM niche components and or even on residual normal HSPCs. Therefore, "which may be through repairing BM EPCs, other BM niche components and/ or even on residual normal HSPCs" has been added in the ***Discussion section*****(page 22, lines 511-512).**

Question 4: In the co-culture provided of EPC with AML, or after CR it is unclear how many EPCs were used, from how many AML or HD?

Answer 4: According to your kind instruction, we have added "Primary leukemia cells were isolated from BMMNCs of newly diagnosed AML patients(N=6) using a CD34 MicroBead Kit(Miltenyi Biotec, Bergisch Gladbach, Germany). HSCs(1×10^5 per well) were cocultured with adherent BM EPCs (1×10^5 per well) for another 5 days" and "Primary leukemia cells(1×10^5 per well), KG-1 cells(5×10^4 per well) or HL-60 cells(5×10^4 per well) were cultured in a noncontact culture system with BM EPCs(1×10^5 per well) in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin for an additional 5 days." in ***Supplementary Methods section*****.**

Reviewer #3 (Remarks to the Author):

Question 1: Abstract

The abstract is well written and summarizes the findings well.

Answer 1: Thanks very much for your attention to our work. We are quite encouraged by your kind comments.

Question 2: Introduction

The introduction is well written overall. However, the authors should discuss other potential causes of failure to achieve normal hematopoiesis following successful anti leukemia therapy. These include the HSC depletion, inhibition of production of downstream hematopoietic cells by impeding differentiation at the HSC–progenitor transition, immune mediated hematopoietic failure, clonal hematopoiesis, and co existing myelodysplastic syndrome.

Answer 2: According to your good instruction, the above discussion has been added in ***Introduction section*** **(page 5, lines 96-101).**

Question 3: Methods

Patients and control individuals

Is the age given of patients the median? This median and range age of the patients should be moved to the results section. Were the AML samples collected at time of diagnosis? What was the outcome of these patients? Were the CR samples taken at time of count recovery after induction? A table of patient characteristics of all groups should be provided including sex, age, wcc at diagnosis, molecular and cytogenetic abnormalities, and ELN classification.

Answer 3: The age given here is median and range age of patients, and the

AML samples were collected at time of diagnosis.

As instructed, we have added the characteristics of all patients in the **updated Table S1**.

Question 4: Study design of a registered prospective single-arm study

What classification was used to designate the AML risk groups? Reference 7 is a review article of AML.

Answer 4: We are sorry for the inconvenience brought to you. We have conducted the risk stratification according to the ELN 2022 recommendations[1], and the reference has been changed.

【References】

1. Hartmut Döhner, Andrew H Wei, Frederick R Appelbaum, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. Blood. 2022 Sep 22;140(12):1345-1377.

Question 5: (1) "NAC 400 mg tid D1-28 was added on the basis of routine supportive therapy(blood transfusion)". This statement is unclear. Did patients begin NAC when a blood transfusion was needed. D1 to D28 implies that NAC was given concurrently with chemotherapy induction but it is stated that it was started after chemo was finished. Please clarify.

(2) The primary endpoint was the time to hematopoietic 1 recovery white blood cell(WBC) (>10^9/L and platelet(PLT)(> 20×10^9/L). Usually, hematopoietic is defined as neutrophil count greater than 1 and platelets greater than 100. The endpoint defined here is problematic since the patients received GCSF which is not usually done in patients before CR, and platelets greater than 20 can be achieved with routine platelet transfusion.

Answer 5: Thank you very much for your kind instruction.

(1) We are really sorry for the confusions brought to you owning to our poor English expression. As instructed, we have clarified "Patients who are willing to accept oral NAC treatment (400 mg 3 times per day) from +1 day to +28 day after the completion of induction chemotherapy continuously were enrolled in the cohort study." in ***Methods section*** **(page 7, lines 159-161).**

(2) The WBC recovery time was starting from the first day of chemotherapy to the first occurrence of a WBC count>1×10^9/L for three consecutive days. According to your suggestion, we analyzed the time when the neutrophil count ≥0.5×10^9/L and ≥1.0×10^9/L from the first day of chemotherapy in CR patients, and found no differences between the two groups (21 days vs. 21 days, and 22 days vs. 22 days).

We apologize again for our unclear English expressions. The PLT recovery time was starting from the first day of chemotherapy to the first occurrence of a platelet count >20×10^9/L sustained without transfusion for seven consecutive days. Consequently, among CR patients, the median PLT recovery time was 19 days in the NAC group, significantly shorter than the 22 days observed in the control group(P=0.0001). According to your suggestion, we also calculated the time when the platelet count≥50×10^9/L and ≥100×10^9/L, and there were no statistically significant differences between the two groups, which were 22 days vs. 23 days and 24 days vs. 24 days, respectively.

According to the good instruction from you and **Reviewer 2**, the related revision has been clarified in ***Methods section*****(pages 7,8, lines 168-178)**.

Question 6: Results

Overall the preclinical data is well presented, follows logically and presents a coherent argument for the impact of the leukemia microenvironment on normal hematopoeisis. However, the labels of the figures are very small in places making it difficult to read. The clinical trial data is weak due to poor definition of endpoints including count recovery and definition of infections. Since all patients in both groups were still alive no impact on mortality can be seen. A larger, randomized study is necessary including more frail patients is necessary to determine if indeed there is an impact of NAC on treatment.

Answer 6: According to the good instruction, we have revised the labels of the figures to making it easy to read. Moreover, we have clarified the definition of endpoints of count recovery, which could be found in ***Methods section*****(pages 7,8, lines 167-178).**

The definition of infections was added in the ***Supplementary Methods*** section.

Based on the kind instruction of you and **Reviewer 1,** "We are aware that the small sample size and single-arm study design are limitations of this study…., which provides a rationale for further prospective randomized clinical trials with larger numbers of patients and longer follow-up periods to investigate the dose-optimization of NAC and to validate our preliminary findings in the future." has been added as the limitation of the current study in the ***Discussion section*****(pages 21,22, lines 507-514).**

Some specific comments:

Question 1: Figure 1 h and J should be labeled with “crystal violet” or ” tube length” and the I and K should be placed adjacent to the representative images for clarity.

Answer 1: According to your kind suggestions, we have updated the labels of **Figure 1**.

Question 2: Figure 3. The labels of the figures are difficult to read.

Figure 3m – were the CD34 leukemia cells primary leukemia cells or cell lines?

Answer 2: Thank you for your good instruction, we have rewritten the labels of the **Figure 3**. We have clarified the source of the HSCs in ***Results section*** **(page 14, lines 333-336)** and in **Figure legend** of **Figure 3m(pages 30, 31, lines 768-770)**. "

Question 3: Figure 4c. The difference in platelet count between AD(changed to CR in the updated Figure 4c) and NAC(changed to CR+NAC in the updated Figure 4c) would be unlikely to be clinically significant and NAC does not seem to increase the time to platelet count recovery in a clinically significant manner.

Answer 3: Many thanks for your kind comments. As suggested by **Reviewer 2**, we performed additional mice experiments to evaluate quantities and functions of BM ECs from normal mice post chemotherapy treatment with(AD+NAC) or without NAC(AD).

As shown in mice study, the PLT count in NAC+CR group was increased than that in CR group(Figure 4c; 3172±168 vs. 2628±128; P=0.03). Consistently, in the clinical trial, the median period of PLT recovery was 19 days and 22 days,

respectively(*P*=0.0001) among the CR patients in NAC and control groups. Moreover, the total volume of PLT transfusions received before PLT≥ 20×10^9/L were markedly lower in NAC group than in control group(*P*<0.0001).

Question 4: Figure 6 a and b. The median time to myeloid and platelet count recovery should be defined.

Answer 4: Thank you for your suggestions. We have updated **Figure 6a** and **6b**.

Question 5: The comparison of infections in the intervention and control group should be shown in figure 6. How was severe infection defined. What was the incidence of febrile neutropenia in both groups? What was the rate of fungal infections in both groups?

Answer 5: In this study, severe infection was defined by the following criteria[1]: septicemia with a confirmed pathogen, radiological evidence of definite pulmonary infection, severe skin and soft tissue infection, and fever exceeding 38.5°C that is not controlled after 3 days of anti-infective treatment. Febrile neutropenia was defined as single temperature: ≥38.3°C orally or ≥38.0°C over 1 h, and neutropenia: <500 neutrophils/mcL or <1000 neutrophils/mcL and a predicted decline to ≤500 neutrophils/mcL over the next 48 h.

The incidence rates of febrile neutropenia in NAC and control group were 93.3% and 100%(*P*=0.043), respectively. In this study, antifungal prophylaxis was used in the two groups, but neither group showed definite fungal pathogen evidence of fungal infection.

According to your good instruction, the related revision could be found in ***Supplementary Methods section*****(page 2, lines 15-22)**, ***Results section***

(page 18, lines 414-418).

【References】

1. Ayman Saad, Alison Loren, Javier Bolaños-Meade, George Chen, Daniel Couriel, Antonio Di Stasi, Areej El-Jawahri, Hany Elmariah, Sherif Farag, Krishna Gundabolu, Jonathan Gutman, Vincent Ho, Rasmus Hoeg, Mitchell Horwitz, Joe Hsu, Adetola Kassim, Mohamed Kharfan Dabaja, John Magenau, Thomas Martin, Marco Mielcarek, Jonathan Moreira, Ryotaro Nakamura, Yago Nieto, Cameron Ninos, Caspian Oliai, Seema Patel, Brion Randolph, Mark Schroeder, Dimitrios Tzachanis, Asya Nina Varshavsky-Yanovsky, Madhuri Vusirikala, Frankie Algieri, Lenora A Pluchino. NCCN Guidelines® Insights: Hematopoietic Cell Transplantation, Version 3.2022. J Natl Compr Canc Netw. 2023 Feb;21(2):108-115.

Response to the Reviewer Comments (NCOMMS-24-09119B)

Reviewer #1 (Remarks to the Author):

Thank you for your kind revisions. I do not think adding a small pilot study to your preclinical translational experiments in this paper is a good idea. I would suggest separating these into two separate papers. Count recovery median period of 21 days seem very brief to me for a population of AML patients with various presentations.

Answer: Many thanks for your kind instruction. As instructed by you and locum chief editor, the clinical trial data have been removed from the current manuscript, which will be prepared for a separated paper in the future.

Reviewer #2 (Remarks to the Author):

The authors provide a new revised version where provided new information related to the effect of AD treatment on BM EC as well as effect on sinusoidal/arteriolar EC in the AML mouse model.

Question 1: Compare to the human data provided, the mouse model at diagnosis (when achieving >20%) they do not show angiogenesis. Only when the AML is > 80% they show increase in EC. Thus when treated with AD, they show that AML+AD is similar to normal HD. Nevertheless when compare to AD treatment, they show that the presence of AML might have protected EC from AD treatment as they show clear increase to EC in AML-AD group. When considering frequency of arteriole/sinusoid, they mentioned that they show similar data that was reported by others i.e: increase in arterioles and decrease in sinusoids with AML. The increase is nevertheless modest. What is clear is that after AD treatment the level of arterioles increase substantially both in normal AD group and AML+AD group, indicating that arterioles are clearly surviving better AD treatment. Thus, based on the new data and the differential effect of AD on arterioles/sinusoids, it questions of overall conclusion merging both EC subsets. Similarly, it will make sense to divide the arterioles/sinusoids when looking at the effect of ROS, transcriptomic as the ratio in the EC group comparing Normal HD versus AML, versus CR is different. They also mentioned that at day 10 after AD, the level of arterioles/sinusoids is similar, indicating that the effect of AD in EC is long-term. When looking at the effect of NAC, then it questioned whether the increase in EC observed is preferential on one subtype to the others.

Answer 1: We are very grateful for your thorough, detailed and constructive critiques of our manuscript. Moreover, thanks very much for instructing us to perform and analyze additional murine experiments to provide new information related to the effect of AD treatment on BM ECs as well as effect on sinusoidal/arteriolar ECs in the AML mouse model, which has helped us to greatly improve it. Although we couldn't provide the related data in the current study owning to the technical limitations, we do believe your great instruction is very helpful and shed light on valuable directions for our future study. We will try our best to elucidate the good questions step by step in the future.

According to your kind instruction, the related revision "BM ECs are heterogeneous, which can be further subdivided into arteriole/sinusoid ECs. Consistent with the previous reports[1-2], we verified that the increase in BM arteriole ECs whereas the decrease in sinusoid ECs in AML mice. After AD treatment, the level of arterioles increased substantially both in normal AD group and AML+AD group, indicating that chemotherapy may act on arteriole/sinusoid ECs differently. Consequently, the current study indicates that it will make sense to divide the arteriole/sinusoid EPC subsets to evaluate the effect of ROS, transcriptomic, the ratios of human arteriole/sinusoid EPC subsets among the HD, AML-CR and AML patient groups, and the murine transcriptome of BM EC subsets, which shed light on valuable directions for the future study." has been added as the limitation of the study in ***Discussion section*****(pages 21, 22, lines 503-513).**

【References】

1. Passaro D, Di Tullio A, Abarrategi A, Rouault-Pierre K, Foster K, Ariza-McNaughton L, Montaner B, Chakravarty P, Bhaw L, Diana G, Lassailly F, Gribben J, Bonnet D.

Increased Vascular Permeability in the Bone Marrow Microenvironment Contributes to Disease Progression and Drug Response in Acute Myeloid Leukemia. Cancer Cell. 2017 Sep 11;32(3):324-341.e6.

2. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, Kokkaliaris KD, Mercier F, Tabaka M, Hofree M, Dionne D, Papazian A, Lee D, Ashenberg O, Subramanian A, Vaishnav ED, Rozenblatt-Rosen O, Regev A, Scadden DT. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. Cell. 2019 Jun 13;177(7):1915-1932.e16.

Question 2: Why in Figure 4F, the percentage of EC in CR group is quite different to what is presented in Figure 2J, where in CR the percentage of EC is similar to normal. If Figure 2J is correct, then the effect of NAC will not be significant.

Answer 2: We are very sorry for the inconvenience brought to you owning to our incomplete expression in the manuscript. AML+AD group in **Figure 2J** and the CR group in **Figure 4F** were analyzed at different time points after chemotherapy. The BM ECs data of the AML+AD group in **Figure 2J** were detected on day 1 after the end of chemotherapy in AML mice, whereas the BM ECs data of the CR group in **Figure 4F** were analyzed on day 10 after the end of chemotherapy in AML CR mice, which is acted as the control group of the AML CR mice treated with NAC(CR+NAC group). Therefore, NAC still has a certain significant effect on CR mice.

According to your good instruction, we clarified the BM EC analysis time points "The quantity of post-chemo normal ECs in AD group was lower than those in AML+AD group on day 1 after the end of chemotherapy" and "NAC was administered to AML mice after CR and hematopoiesis and BM microenvironment were detected in mice that treated with NAC(CR+NAC group) or not(CR group) on day 10 after the end of chemotherapy(Figure S4)." in

***Result section*(pages 15,17, lines 355-356; 397-399).**

Question 3: Based on the long-term effect of AD on EC, I believe that the analysis of the transcriptome, the addition of the AD effect on normal mice should be added as a better control than HD.

Answer 3: We greatly admire your high-level instruction. Indeed, the additional murine data about the long-term effect of AD on ECs are consistent with your speculation. We agree that the addition of the AD effect on normal mice is a better control than HD. Although we couldn't provide the analysis data of the mouse transcriptome in the current study owning to limited cell number of BM ECs, we do believe your great instruction will be very helpful for your future study.

According to your good instruction, the related revision "BM ECs are heterogeneous, which can be further subdivided into arteriole/sinusoid ECs. Consistent with the previous reports, we verified that the increase in BM arteriole ECs whereas the decrease in sinusoid ECs in AML mice. After AD treatment, the level of arterioles increase substantially both in normal AD group and AML+AD group, indicating that chemotherapy may act on arteriole/sinusoid ECs differently. Consequently, the current study indicate that it will make sense to divide the arteriole/sinusoid EPC subsets to evaluate the effect of ROS, transcriptomic, the ratios of human arteriole/sinusoid EPC subsets among the HD, AML-CR and AML patient groups, and the murine transcriptome of BM EC subsets, which shed light on valuable directions for the future study." has been added as the limitation of the study in ***Discussion section*(pages 21, 22, lines 503-513).**

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my concerns. I have no further comments.

Answer: Thanks very much for your attention to our work. Moreover, we are very grateful for the thorough, detailed and constructive critiques of our manuscript, which has helped us to greatly improve it.

Response to Reviewer Comments (NCOMMS-24-09119C-Z)

Reviewer #2 (Remarks to the Author):

The authors have provided a new revised version of their manuscript in which they deleted the preliminary data on the clinical trial which makes the paper much more concise and focused more on potential mechanistic aspects.

They also add a discussion on potential limitations of their study.

They have thus reliably improved the quality of the manuscript and provided answers to all my concerns.

Answer: We are very grateful for the thorough, detailed and constructive critiques of our manuscript, which has helped us to greatly improve it.