AWARD NUMBER: W81XWH-19-1-0166

TITLE: miRNA-Mediated Rescue of NK Cell Cytotoxicity Against Drug-Resistant Quiescent Leukemia Stem Cells

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CONTRACTING ORGANIZATION: University of Maryland, Baltimore, MD

REPORT DATE: July 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2021	Annual	01Jul2020-30Jun2021

4. TITLE AND SUBTITLE
miRNA-Mediated Rescue of NK Cell Cytotoxicity Against Drug-Resistant Quiescent Leukemia Stem Cells

5a. CONTRACT NUMBER
W81XWH-19-1-0166

5b. GRANT NUMBER
CA180744

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the period covered by this report, we found that sustained pre-miR-155 expression partially rescued the inhibitory effect of the bone marrow mesenchymal stroma cell (MSC) conditioned medium (CM) on human NK cell cytotoxic activity against $CD34^+$ blast crisis CML (CML-BC) stem and progenitor cells. Similar results were obtained using NK cells from wt and miR-155 transgenic (tg) mice and BCR-ABL1-expressing mouse 32Dcl3 myeloid precursors. Mechanistically, we found that FAS and DR5, but not NK-G2D, ligands are overexpressed in primary BCR-ABL1 transgenic mouse stem/progenitor cells, and that tg miR-155 NK cells express TRIAL and FAS Ligand at high levels. Moreover, we gathered evidence showing that miR-155 tg NK-mediated killing of BCR-$ABL1^+$ cells is FAS dependent. Interestingly, we also found that overexpression of pre-MiR-155 leads to downregulation of pre-miR-300 levels, suggesting the existence of a negative feedback loop. We will continue investigating the pre-miR-155-dependent mechanism leading to restored NK cell cytotoxic activity toward leukemic stem cells and determine whether sustained pre-miR-155 combined with miR-300 inhibition will enhance killing of CML quiescent stem cells. Thus, the project goals and SOW remain the same for the approved no-cost extension period.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE		11	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		

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1. INTRODUCTION:

Qualitative and quantitative defects in natural killer (NK) cells is a feature contribute to survival of BCR-ABL1 tyrosine kinase inhibitor (TKI)-resistant quiescent leukemia-initiating stem cells (qLSCs) in TKI-treated chronic myelogenous leukemia (CML) patients. Our preliminary data indicate that the decrease in NK cell numbers and their inability to kill CML qLSC may result from unbalanced levels of the bone marrow microenvironment (BMM)-regulated miR-155 and miR-300 in NK cells. Thus, we proposed to determine whether altering the miR-155/miR-300 ratio by increasing miR-155 expression confers to NK cells the ability to overcome the BM mesenchymal stem cell (MSC) and hypoxia-induced inhibitory effects on their proliferation and anti-cancer cytotoxicity, and efficiently kill TKI-resistant CML qLSCs *in vitro* and *in vivo*. This study might serve as proof-of-concept for developing miRNA-based NK cell immunotherapies for the several stem cell-derived malignancies with dysfunctional NK cells.

2. KEYWORDS:

Leukemia, CML, BCR-ABL1, NK cells, miRNAs, miR-155, miR-300, quiescent LSC, MSC, hypoxia, immunotherapy.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

As stated in the approved SOW, the major goals of this funded project are:

Aim#1 To assess whether sustained miR-155 expression rescues NK cell killing of TKI-resistant qLSCs by determining whether 1A) constitutive miR-155 expression enhances NK cell killing of quiescent CML LSCs and restores selective cytotoxicity against leukemic but not normal HSCs in primary NK cells from leukemic animals *in vitro*; and 1B) constitutive miR-155 expression alone or associated with miR-300 downregulation override the hypoxia- and MSC-induced NK cells growth arrest and impaired cytotoxicity toward patient-derived qLSCs.

Aim#2 To determine the *in vivo* therapeutic potential of miR-155-activated NK cell anti-LSC cytotoxic activity by 2A) generation and *in vitro* functional characterization of BMM-resistant NK-92-155/anti-300 cells.; and 2B) assessing NK-92-155/300 cytotoxicity against CML qLSCs in CML mouse PDXs.

What was accomplished under these goals?

This is the outcome of the limited number of experiments we could perform during the pandemic period (06/30/2020 to 07/01/2021). Note that during 2020 the research activity at the awarded institution and PI laboratory has been first fully suspended, restarted at 25% in summer 2020 because of COVID-19 pandemic and it is currently not a full pace. As per School of Medicine Dean's letter of April 14th, 2021, research activities at UMB School of Medicine are planned to be fully restored by fall 2021.

Our research during this period was primarily focused on the *in vitro* experiments (Aim 1) since *in vivo* experiments (Aim 2) were precluded by the COVID-19 pandemic. We are now expanding the mouse colonies and generate the NK-92 cell line that co-express human pre-miR155 and anti-miR300-miR-Zip to perform experiments of Aim 2.

Project goals and SOW remain the same with a very slight modification of the proposed experiments as suggested by the Reviewers' panel.

Aim#1 To assess whether sustained miR-155 expression rescues NK cell killing of TKI-resistant qLSCs

1A) To determine whether NK cells constitutively expressing miR-155 show enhanced and selective killing of quiescent LSCs

Because miR-155 expression markedly and significantly increases absolute numbers of BM $NK1.1^+$(or $DX5^+$)$CD3^-$ cells and their cytotoxic activity against leukemic but not normal quiescent $Lin^-Sca^+Kit^+$ (LSK) stem cells (see last year report), we investigated whether altered FAS, DR5 and NKG2D ligands expression on BCR-$ABL1^+$ LSK cells and FAS ligand and TRIAL levels on NK cells may account for the selective and enhanced killing of leukemic stem cells (LSC) by miR-155-overexpressing NK cells.

Our data indicate that FAS and DR5 levels are significantly upregulated whereas NKG2D ligands' expression is barely detectable in leukemic LSK cells, suggesting that FAS and DR5 may mediate NK cell cytotoxic activity against CML quiescent LSCs. Similar FAS, DR5 and NK-G2D ligands' expression patterns were found in 32D-BCR-ABL mouse myeloid precursors. Analysis of FAS Ligand and TRIAL expression in primary $NK1.1^+CD3^-$ NK cells revealed that TRIAL levels are higher in miR-155 tg compared to wt NK cells maintained in resting condition. Conversely, FAS Ligand and TRIAL expression was not significantly different in IL-2-cultured wt and miR155 tg $NK1.1^+CD3^-$ NK cells cultured in the absence or presence of mouse MSC CM. Consistent with the expression studies, wt and miR-155-tg NK cell-mediated cytotoxic activity against 32D-BCR-ABL cells was FAS dependent but NKG2D independent.

Because we found that SHIP1 is downmodulated by MSC CM and hypoxia in a miR-155-independent manner, it is unlikely that miR-155-dependent SHIP1 inhibition regulates NK cell activity in CML patients. Thus, we will not perform experiments in mouse $NK1^+CD3^-$ miR-155 Tg NK cells aimed at assessing whether miR-155-induced NK cell cytotoxicity depends on reduced SHIP1 expression/function.

1B) To determine whether miR-155 overexpression overcomes BMM (MSC and/or hypoxia)-induced suppression of human and mouse NK cell proliferation and cytotoxic activity

Our data (*see previous report*) showing that MSC-induced miR-155-5p inhibition and, therefore, impaired miR-155-5p-dependent negative regulation of SHIP1 and CEBPB does not represent the mechanism leading to suppression of NK cell proliferation/activity, suggest that re-expression of pre-miR-155-5p is likely required to overcome the BMM inhibitory effects on NK cells.

To determine whether overexpression of pre-miR-155 overcomes the BMM inhibitory effect on NK cells, we ectopically express human pre-miR-155 in NK-92 cells using Lenti-miR-155 (PMIRH155PA1; SBI System Bioscience). As expected GFP-sorted pre-miR-155-overexpressing NK-92 cells showed reduced SHIP1 expression. Interestingly, pre-miR-155 also reduced pre-miR-300 levels, suggesting the existence of negative feedback loop between miR-155 and miR-300. Moreover, pre-miR-155 overexpression rescued NK-92 cell spontaneous cytotoxicity toward qLSCs and $CD34^+$ progenitors from CML patients (n=3) samples from the inhibitory effects of BM MSCs.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

The project goals and SOW are not changed. Thus, based on the data we obtained during the funded periods and on the ability of pre-miR-155 to selectively and efficiently enhance NK cell killing of self-renewing BCR-ABL$^+$ LSCs, we will a) assess whether overexpression of pre-miR-155 in combination with miR-300 inhibition has the ability to enhance NK cell proliferation and cytotoxic activity against CML qLSCs exposed to the inhibitory effects of BM MSCs and hypoxia.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No significant changes in objectives and scope were found. However, we found that the ability of miR-155 to enhance NK cell proliferation and cytotoxicity of $NK1.1^{+}CD3^{-}$ NK cells was not dependent on the activity of mature miR-155-5p only (as expected) but it depends on pre-miR-155 expression. Thus, expression of a pre-miR-155 (lenti-pre-miR-155 is available in the PI's lab) instead of mature miR-155-5p is now be used in the experiments proposed in the approved SOW.

Actual or anticipated problems or delays and actions or plans to resolve them

COVID-19 pandemic state significantly delayed and still delays the execution of in vivo experiments within the time frame anticipated in the approved SOW. We plan to execute the proposed Aim 2 experiments during the approved *No Cost Extension* period.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

The article below, which containing data relative to miR-300 and the effect of BM MSCs on pre-miR-155 expression in NK cells, came out in July 2020; however, a copy of this article has been submitted with the previous annual report <u>therefore it will not be included as an appendix in this annual report</u>.

Silvestri G.*, Trotta R.* Stramucci L., Ellis J., Harb J., Neviani P., Wang S., Eisfeld A.K., Walker C.J., Zhang B., Srutova K., Gambacorti-Passerini C., Pineda G., Jamieson CHM., Calabretta B., Stagno F., Vigneri P., Nteliopoulos G., May P., Reid A., Garzon R., Roy D.C., Moutuou M.M., Guimond M., Hokland P., Deininger M., Fitzgerald G., Harman C., Dazzi F., Milojkovic D., Apperley J.F., Marcucci G., Qi J., Machova-Polakova K., Zou Y., Fan X., Baer M. R., Calabretta B. and Perrotti D. *Persistence of drug-resistant leukemic stem cells and impaired NK cell immunity in CML patients depend on MIR300 anti-proliferative and PP2A-activating functions. **<u>Blood Cancer Discovery</u>**. 1(1):48-67, 2020.* (*) Co-first Authors

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

- **Website(s) or other Internet site(s)**

- https://bloodcancerdiscov.aacrjournals.org
- https://twitter.com (AACR Blood Cancer Discovery Twitter)

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Danilo Perrotti
Project Role: PI
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Nearest person month worked: 12
Contribution to Project: Conceptualization, formal data analysis, supervision, paper writing/editing.
Funding Support: N/A

Name: Rossana Trotta
Project Role: Co-I
Researcher Identifier: ORCID ID 345462615; Scopus ID: 7004543994
Nearest person month worked: 12
Contribution to Project: investigation, methodology, paper review and editing

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Nothing to Report.