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TITLE: Enhancing Natural Killer Cell Mediated Targeting and Responses to Myeloid Leukemias

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Myeloid Leukemias represent a growing problem in our aging population. Although treatment of these leukemias has advanced considerably over the past couple of decades, most of these myeloid leukemias still have poor prognosis, particularly in the elderly, and require hematopoietic cell transplants to fully kill the tumor, which is both costly and risky. A great deal of excitement has recently been placed on cellular therapies to treat tumors. Natural Killer (NK) cell immunotherapies could be used to treat myeloid tumors, but these cells require help in being targeted to the tumor and overcoming inhibitory signals that help the tumor escape recognition and killing. Our group has recently described small bivalent molecules (termed BiKEs) that target NK cells to myeloid tumors and induce NK cell mediated tumor killing. Though these molecules show promise, the work scope proposed here builds on them through incorporation of signals that will maximize NK cell function. This will be achieved by creating trivalent molecules (termed TriKEs) that target and activate NK cells but also either block inhibitory signals provided by the tumor cells, further enhance the activating signals provided by the BiKE, or drive expansion and maintenance of the NK cells killing the tumor cells.					
<b>15. SUBJECT TERMS</b> NK - Natural Killer, CML - Chronic Myeloid Leukemia, MDS - Myelodysplastic Syndromes, AML - Acute Myeloid Leukemia, BiKE - Bi-specific Killer Engager, TriKE - Tri-specific Killer					
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## 1. Introduction:

Myeloid leukemias represent a growing problem in our aging population. In military personnel the incidence of myeloid malignancies is increased due to exposure to ionizing radiation, chemicals, and other agents during deployment. Although treatment of these leukemias has advanced considerably over the past couple of decades, most of these myeloid leukemias still have poor prognosis, particularly in the elderly, and require hematopoietic stem cell transplants to fully kill the tumor. These transplants are costly, risky, and quite harsh on the patient, especially if the patient is older and frail. A great deal of excitement has recently been placed on cellular therapies to treat tumors. Rather than eradicating the tumors through chemicals and radiation, cellular therapies enhance immune function in the patients so the immune cells themselves can kill the tumors. One type of immune cells whose role is to find and kill tumors is the Natural Killer (NK) cell. Upon clinical diagnosis of myeloid leukemia the NK cells require help in being targeted to the tumor and overcoming inhibitory signals that help the tumor escape recognition and killing. Our group has described small bivalent molecules, termed BiKEs (Bi-specific Killer Engagers), which target NK cells to myeloid tumors and induce NK cell mediated tumor killing. The targeting in the BiKE is mediated by an anti-CD33 scFv, as CD33 is expressed in a number of these myeloid tumors, while the NK cell activation is mediated by an anti-CD16 scFv, as CD16 is a potent NK cell activating receptor. Recently we have published on a TriKE (Tri-specific Killer Engager) molecule that incorporates the cytokine IL-15 into the BiKE platform to enhance NK cell function, demonstrating our capability to expand on this platform. Though these molecules show promise, the work scope proposed here builds on them through incorporation of other signals that will maximize NK cell function. This will be achieved by creating two new families of TriKEs. The first target and activate NK cells but also block inhibitory signals provided by the tumor cells that interact with the PD-1, NKG2A, or KIR receptors on NK cells. The second family further enhances the activating signals provided by the BiKE by incorporating new cytokines (IL-21 or IL-12) or a new co-stimulatory component, via CD137 crosslinking. We believe these new families of TriKEs will further induce tumor killing and drive expansion and maintenance of the NK cells in order to maximize NK cell based immunotherapies. Attaining an immunotherapy that would bypass the need for costly and dangerous hematopoietic stem cell transplants but also diminish the rate of relapse in myeloid malignancies would greatly impact the way we treat these patients. Of note, the potential of these TriKE molecules is highlighted by the fact that our earlier version of the TriKE molecule incorporating IL-15 is headed into a Phase I clinical trial at the University of Minnesota early 2018 for treatment of refractory AML and high risk MDS.

## 2. Key words:

NK – Natural Killer

CML – Chronic Myeloid Leukemia

MDS – Myelodysplastic Syndromes

AML – Acute Myeloid Leukemia

BiKE – Bi-specific Killer Engager

TriKE – Tri-specific Killer Engager

scFv – Single-chain variable fragment

CDR – Complementarity-determining region

ADCC – Antibody-Dependent Cell-mediated Cytotoxicity

IL- – Interleukin

HLA – Human Leukocyte Antigen

KIR – Killer-cell Immunoglobulin-like Receptor

1633 – anti-CD16 x anti-CD33 BiKE

161533 – anti-CD16 x IL-15 x anti-CD33 TriKE

1633KIR – anti-CD16 x anti-CD33 x anti-KIR TriKE

1633NKG2A – anti-CD16 x anti-CD33 x anti-NKG2A TriKE

1633PDL1 – anti-CD16 x anti-CD33 x anti-PD-L1 TriKE

161233 – anti-CD16 x IL-12 x anti-CD33 TriKE

162133 – anti-CD16 x IL-21 x anti-CD33 TriKE

1633137 – anti-CD16 x anti-CD33 x anti-CD137 TriKE

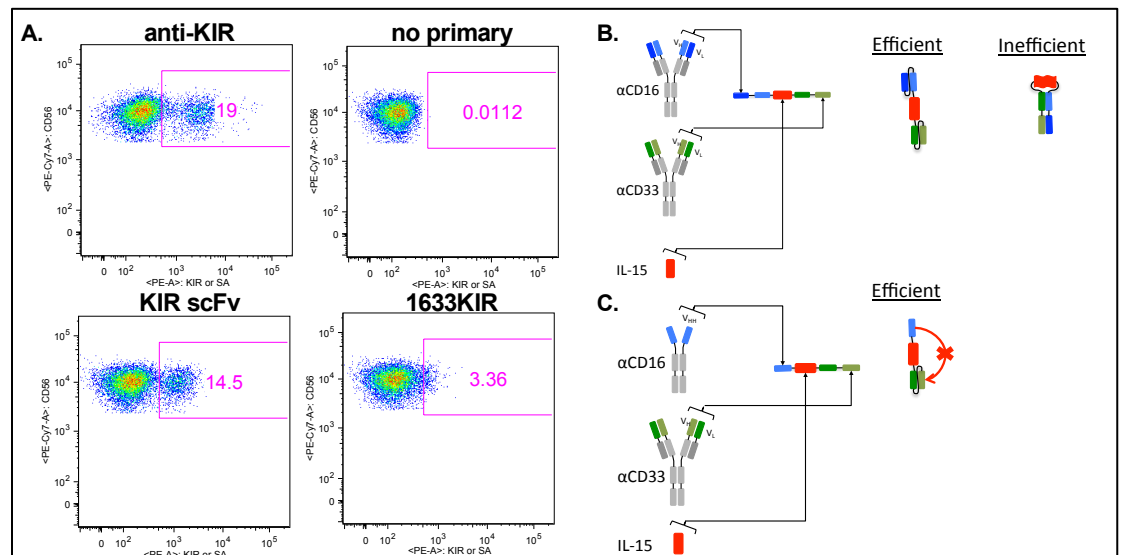
### 3. Accomplishments (Year-1):

The major goal of this proposal is to generate novel Tri-specific Killer Engager (TriKE) molecules in order to improve natural killer (NK) cell based immunotherapies against myeloid malignancies including AML, MDS, and CML. The molecules proposed build on a Bi-specific Killer Engager (BiKE) platform containing an anti-CD33 scFv, for myeloid tumor targeting, joined by a linker to an anti-CD16 (termed 1633 henceforth), to robustly induce NK cell activation. Six new TriKEs were proposed in this grant to enhance the NK cell activity mediated by the former 1633 BiKE; three targeting blockade of inhibitory pathways on NK cells (1633KIR, 1633NKG2A and 1633PDL1) and three enhancing NK cell activation (161233, 162133 and 1633137). To achieve this goal the proposal was split up into three major tasks, each occupying roughly one year of the proposal. The first major task, which takes place in year 1, involves construction of the TriKEs, as well as obtaining local IRB and DoD HRPO approval. The second major task, in year 2, involves testing of the three TriKEs targeting inhibitory pathways on NK cells. The third and last major task, in year 3, involves testing of the three TriKEs targeting enhancement of NK cell function via cytokines and co-stimulatory receptors.

Though slightly delayed both IRB and HRPO approval was achieved within the first six months of year 1. Subtasks 1 and 2, cloning and generation of anti-KIR, anti-NKG2A, anti-PD-L1, anti-CD137 scFvs and IL-21 and IL-12 cytokines, were mostly achieved within the first six months (see **Figure 1A** bottom left panel for scFv binding example). The anti-NKG2A has been problematic, folding seems to require secondary structure beyond the scFv, but we are trouble shooting by testing other clones. Instead of utilizing an anti-CD137 (41BB) scFv we have decided to clone the 41BBL protein instead hypothesizing that this would yield a more physiologic signal without differing much from the scFv strategy. With the exception of the anti-NKG2A scFv, we believe that all of the scFvs, cytokines, and ligands (in the case of 41BBL instead of anti-CD137) are functional thus mostly accomplishing the first subtasks (Major Task 1, subtasks 1 and 2, months 1-6).

While tackling subtask 3 in Major Task 1 (months 7-12), cloning of scFv, cytokine and ligand constructs into 1633 BiKE backbone, we ran into issues with protein output and binding. As can be seen in **Figure 1A**, although the anti-KIR scFv (bottom left) binds similarly to commercial anti-KIR antibodies (top left), incorporation of the anti-KIR scFv into the 1633KIR TriKE (bottom right) led to an ablation of KIR binding on NK92s treated with 5-AZA, which induces KIR expression but doesn't induce CD16 in these cells. After scouring the literature for answers we hypothesized that the decreased protein production output and binding capability was likely mediated by inefficient folding driven by unspecific binding of the light chain ( $V_L$ ) of one scFv to the heavy chain ( $V_H$ ) of another scFv. This of course could be amplified with the more scFvs that are present in a molecule.

To evaluate if this could be the case we resorted to a better characterized 161533 (anti-CD16 scFv x IL-15 cytokine x anti-CD33 scFv) TriKE recently published by our group (**Figure 1B** left). Efficient folding of this molecule (**Figure 1B** center) should yield proper binding and minimal disruption of IL-15 signaling. However the IL-15 moiety in this molecule is 13-fold less functional than monomeric IL-15, perhaps indicating that, at least in part, the  $V_H$  and



**Figure 1: Trouble shooting TriKE molecule structures**  
A) NK92 cells treated with 5-AZA, to induce KIR expression on these cells, were incubated with a commercial anti-KIR-PE antibody cocktail (top left), only anti-HIS secondary anti-body + SA-PE as a background staining control (top right), KIR scFv + anti-HIS + SA-PE (bottom left), and 1633KIR + anti-HIS + SA-PE (bottom right). B) In TriKE molecules, consisting of two scFvs, each with a  $V_H$  and  $V_L$  domain, and a cytokine (IL-15) resulting folding can lead to efficient folding, where the  $V_H$  and  $V_L$  domains bind to their adequate partners, or inefficient folding, where  $V_H$  and  $V_L$  domains bind the wrong partner. Inefficient folding results in decrease binding affinity and steric hinderance on components of the TriKE, likely reducing their function. C) To bypass inefficient binding a humanized camelid anti-CD16  $V_{HH}$  domain can be used to replace the anti-CD16 scFv. This  $V_{HH}$  domain cannot bind  $V_L$  domains thus maximizing efficient folding.

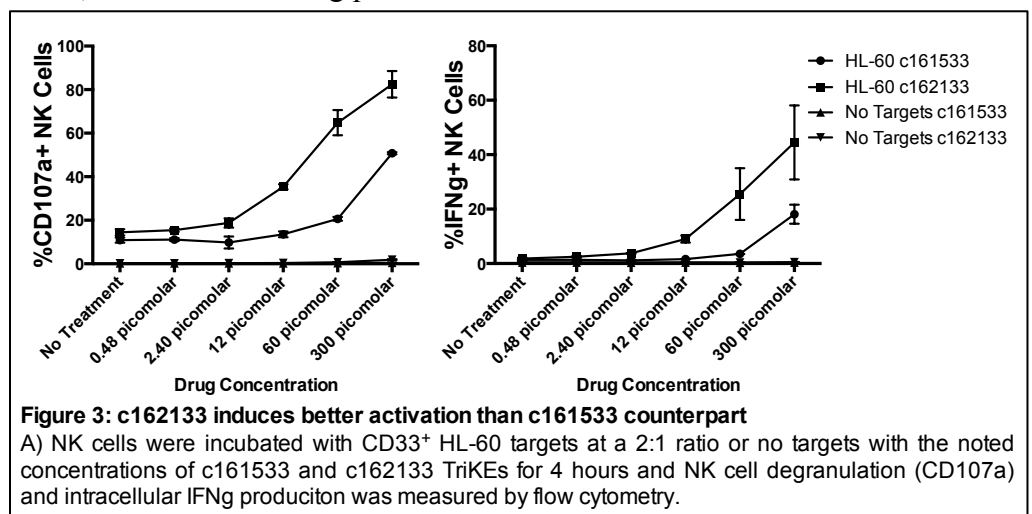
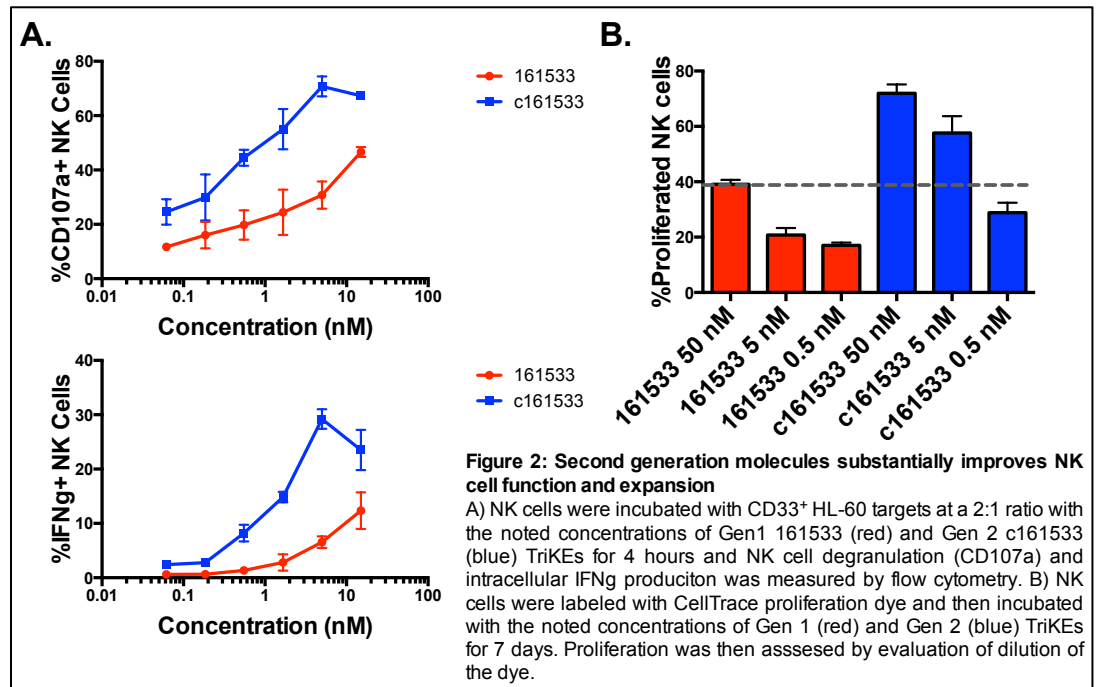
$V_L$  portions of the anti-CD16 and anti-CD33 scFvs are erroneously binding with each other (**Figure 1B** right). To resolve, or minimize, the issue of unspecific binding of scFv components to each other we hypothesized that we could use the  $V_{HH}$  portion of a camelid antibody, which rather than having two chains ( $V_H$  and  $V_L$ ) have only one chain ( $V_{HH}$ ) that cannot bind to  $V_L$  chains (**Figure 1C**).

We found a Llama camelid  $V_{HH}$  anti-CD16 antibody in the literature and cloned the CDR1, CDR2, and CDR3 regions from said  $V_{HH}$  antibody into a humanized camelid  $V_{HH}$  backbone to enhance future clinical applications. We swapped in the humanized camelid anti-CD16  $V_{HH}$  in place of the anti-CD16 scFv to create a second generation TriKE termed c161533. Of interest the c161533 TriKE yielded much better production characteristics, including increased output and purity, likely a

consequence of the higher stability found in  $V_{HH}$  engagers when compared to scFvs. Furthermore, when compared to the old 161533 molecule the c161533 vastly increased NK cell degranulation, via measurement of CD107a, and cytokine production against AML cell line tumor targets (**Figure 2A**). The IL-15 moiety in the c161533 also increased NK cell proliferation considerably when compared to the old 161533 molecule (**Figure 2B**). These findings indicated to us that incorporation of the humanized camelid anti-CD16  $V_{HH}$  into the TriKE platform went a long way to mitigate possible miss-folding outcomes incurred by the former molecule. Though time consuming, the findings from these troubleshooting efforts have great clinical implications for moving a next generation of these molecules into the clinic to treat myeloid malignancies and will be submitted for publication early next year. Furthermore, the troubleshooting puts our aims back on track.

We have now generated 4 of the 6 molecules (c1633KIR, c1633PDL1, c163341BBL and c162133) proposed of Major Task 1 subtask 3 with the humanized camelid anti-CD16  $V_{HH}$  and will starting testing on all of those. The first molecule to be tested, c162133 (Major Task 3, subtask 2.2 (proposed under year 3)), displays tantalizing functional characteristics at very low levels of molecule usage vs. its c161533 counterpart indicating

that usage of IL-21, vs. IL-15, might be beneficial for NK cell immunotherapy (**Figure 3**). We are currently setting up assays to compare real time killing of AML tumor cells (in an IncuCyte Zoom platform) when NK cells are treated with the c161533 vs. the c162133 TriKEs. Next step will be to evaluate the effect of the c162133 TriKE in long-term NK cell expansion assays. Given initial results we believe that we should be



preparing a manuscript on this TriKE Q2-Q3 of 2018. Though it is not guaranteed that the improvement in function noted with the c162133 TriKE will translate to all of the other manufactured TriKEs for this proposal, we should be able to answer that question within the next 3-4 months for most of them. To clarify, though this scFv issue presented a troubleshooting problem that delayed work slightly (while moving forward the TriKE technology), the experimental plan and SOW for this proposal remains the same.

In terms of training activities and professional development the project provided me the ability to attend two premier conferences. The first, the 2016 ASH (American Society of Hematology) meeting held in San Diego in December, focuses primarily on hematologic malignancies. I attended a large number of talks focusing on the state of AML, MDS, and CML clinical therapies as well as myeloid malignancy biology. At this meeting I gave a talk focusing on cytokine induced exhaustion of NK cells (relevant to dosing of the cytokine TriKEs) and presented a separate poster focusing on the mechanism of action of TriKEs. The second meeting I attended was the 2017 AAI (American Association of Immunologists) meeting held in Washington DC in May. This meeting focuses primarily on immunology and I attended a large number of talks focused on NK cell biology, cytokine signaling, and checkpoint blockade. Besides the meetings the project has provided protected time allowing me to attend several local seminars, including the cancer center seminar and the Garibaldi lecture at the University of Minnesota. The protected time also allowed me to recently author a review in *Seminars in Immunology* on possible utilization of BiKEs and TriKEs and checkpoint blockade to improve NK cell immunotherapy (reference listed below).

Davis ZB, Vallera DA, Miller JS, **Felices M**. Natural killer cells unleashed: Checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Semin Immunol.* 2017 Jun;31:64-75. doi: 10.1016/j.smim.2017.07.011. Epub 2017 Sep 5. Review. PubMed PMID: 28882429; PubMed Central PMCID: PMC5632228.

During the next reporting period we plan to validate the new camelid constructs (c1633KIR, c1633PDL1, c163341BBL), create and validate a c161233 construct, and try to troubleshoot the NKG2A construct. We will then finish testing the efficacy of the c162133 construct in the hopes of preparing a manuscript on Q2-Q3 of 2018. If functional, we will also fully test the efficacy of the c1633KIR and c1633PDL1 constructs. Finally we plan to prepare a manuscript describing the new c161533 construct (used to troubleshoot the folding issue) in Q1 2018.



**4. Impact:**

Nothing to Report

**5. Changes/Problems:**

Nothing to Report

**6. Products:**

Nothing to Report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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Funding Support	N/A