AWARD NUMBER: W81XWH-16-1-0380

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

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CONTRACTING ORGANIZATION: Regents of the University of Minnesota

REPORT DATE: January 2020

TYPE OF REPORT: Final

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
Jan 2020	Final	09/30/2016 - 09/29/2019

4. TITLE AND SUBTITLE

Enhancing Natural Killer Cell Mediated Targeting and Responses to Myeloid Leukemias

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0380

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Regents of the University of
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Office of Sponsored Projects
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Minneapolis, MN 55455-2009

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

Dr. Felices, the PI in this proposal, is a trained immunologist with expertise on signal transduction in his first year as an assistant professor at the University of Minnesota. He obtained his training in academia (UMASS and UMN) and industry (Novartis) focusing in signal transduction in the immune system and tumorogenesis. As Jr. Faculty, Dr. Felices' research is aimed at maximizing the immunotherapeutic value of natural killer (NK) cells against myeloid leukemias, like CML, AML and MDS, covered by Myeloproliferative disorders FY15 topic area. He proposes to do this by incorporating his signaling knowledge into a novel platform of molecules, termed bi- or Tri-specific killer engagers (BiKEs and TriKEs). Our group has shown that BiKEs target NK cells to myeloid leukemic cells through generation of an immune synapse between CD33, on the myeloid leukemic cell, and CD16, on the NK cell. Dr. Felices' proposal aims to carry these molecules into the next generation and given the outstanding translational environment surrounding him at the University of Minnesota, he is uniquely positioned to move these molecules into the clinic and advance the current immunotherapeutic efforts against myeloid leukemias.

His mentor, Dr. Jeffrey S. Miller, is a world-renowned NK cell immunotherapy expert with vast experience in clinical approaches targeting myeloid leukemias using NK cells. He is well published and funded in this area and has a proven track record in mentoring. Besides his leadership role at the University of Minnesota and access to a well-populated bank of myeloid leukemia clinical samples, generated from several clinical trials led by him and his colleagues, Dr. Miller's group pioneered the NK BiKE and TriKE work making him the ideal mentor for this proposal.

15. SUBJECT TERMS

NK – Natural Killer, CML – Chronic Myeloid Leukemia, MDS – Myelodysplastic Syndromes, AML – Acute Myeloid Leukemia, BiKE – Bi-specific Killer Engager, TriKE – Tri-specific Killer

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	Unclassified		19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

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1. Intro (Year-3-Final):

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. Our research will first optimize the platform to better induce killing of myeloid malignancies. This will be achieved in several ways: 1) strengthening the main activating signal through incorporation of a strong single domain anti-CD16 arm into the platform in the place of the previous CD16 scFv; 2) swapping the main CD16 activating signal for a more NK cell specific NKG2C activating signal; 3) swapping the myeloid tumor targeting domain, targeting CLEC12A instead of CD33, in order to reduce toxicities and enhance myeloid cancer stem cell control; 4) incorporating different cytokine, IL-12, in the place of the IL-15; or 5) blocking inhibitory signals (PD-L1) provided by the tumor cells that interact with the PD-1 on NK cells. Our original first generation TriKE has just entered a Phase I clinical trial for treatment of high risk MDS and refractory AML (NCT03214666), where patients are being recruited currently. We believe these next generation TriKE molecules can substantially improve on the first generation TriKE currently being tested by further inducing tumor killing and driving 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.

2. Key words (Year-3-Final):

NK – Natural Killer
CML – Chronic Myeloid Leukemia
MDS – Myelodysplastic Syndromes
AML – Acute Myeloid Leukemia
TriKE – Tri-specific Killer Engager
scFv – Single-chain variable fragment
ADCC – Antibody-Dependent Cell-mediated Cytotoxicity
IL- – Interleukin
rhIL-15 – recombinant human IL-15
KIR – Killer-cell Immunoglobulin-like Receptor
161533 or scFv16-m15-33 – both refer to anti-CD16 x IL-15 x anti-CD33 TriKE. The m15 stands for mutant rhIL-15
cam16 – camelid anti-CD16 single domain antibody
cam161533 or cam16-wt15-33 – both refer to the camelid anti-CD16 x IL-15 x anti-CD33 TriKE. The wt15 stands for wild type rhIL-15
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
NKG2C – Natural Killer Group 2 C
CLEC12A - C-Type Lectin Domain Family 12 Member A
V_HH – Heavy domain antibody (also called single domain antibody or nanobody)

3. Accomplishments (Year-3-Final):

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.

As outlined in the reports from previous years, and this year, we had to alter the strategy due to the fact that some of the molecules proposed did not work as well. Thus our final products, for the end of the grant period, are as follows: 1) creation of second generation TriKEs including a much more potent single domain humanized antibody against CD16 in the place of the anti-CD16 scFv; 2) swapping the main CD16 activating signal for a more NK cell specific NKG2C activating signal; 3) swapping the myeloid tumor targeting domain, targeting CLEC12A instead of CD33, in order to reduce toxicities and enhance myeloid cancer stem cell control; 4) incorporating different cytokine, IL-12, in the place of the IL-15; or 5) blocking inhibitory signals (PD-L1) provided by the tumor cells that interact with the PD-1 on NK cells. Taken together, these family of molecules greatly alter and improve the potential of NK cell immunotherapy in the Myeloid Malignancies setting

A first generation TriKE, composed of single chain variable fragment (scFv) arms against CD16, on NK cells, and CD33, on myeloid malignancies, has just entered the clinic for a first in human trial for treatment of patients with high-risk MDS and refractory AML. We are now in the process of recruiting the first patient for this exciting trial. However, we have clear data showing that this original TriKE platform demonstrated reduced functionality and limited the number of other scFvs present in the molecule. We have optimized the platform by swapping in a humanized anti-CD16 arm derived from a llama single domain antibody (V_HH), which we call cam16, in the place of the anti-CD16 scFv.

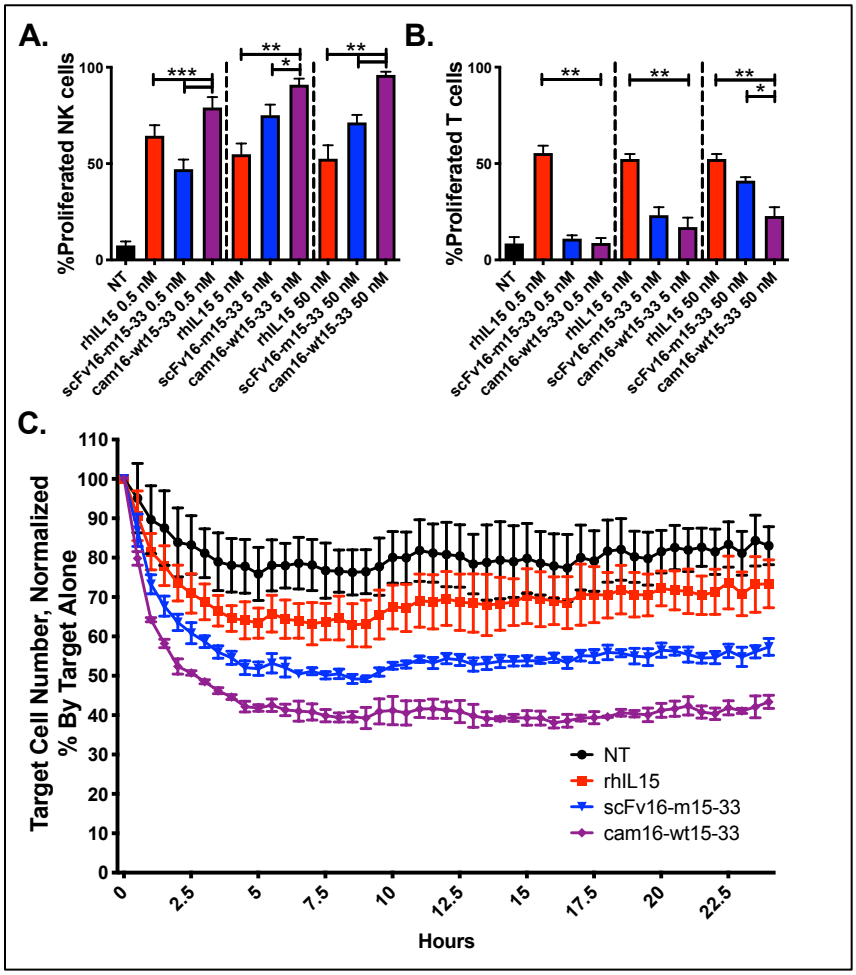

Figure 1: PBMCs were CellTrace labeled and incubated for 7 days with noted concentrations of rhIL-15 (red), scFv16-m15-33 TriKE (blue), and cam16-wt15-33 TriKE (purple). At the end of the 7 days cells were harvested, stained and evaluated. (a) The proportion of NK cells that proliferated or (b) the proportion of T Cells that proliferated. (c) An IncuCyte imaging assay was used to evaluate NK-mediated killing of CellTrace Far Red labeled targets over a 24-hour period with noted treatments (30 nM). Representative of 6 separate experiments.

The second generation cam16-wt15-33 TriKE not only outperforms the first generation scFv16-m15-33 TriKE and rhIL-15 in terms of NK cell proliferation (**Figure 1A**), but it also specifically delivers the IL-15 moiety to

NK cells, strongly reducing the amount of IL-15 activity on T cells when compared to rhIL-15 when evaluating proliferation (**Figure 1B**). Across the board, the cam161533 TriKE induces more NK cell activation, measured by surface CD69 surface expression and degranulation as measure by CD107a (data not shown**)** on NK cells, more HL-60 myeloid tumor killing in an IncuCyte imaging assay (**Figure 1C**). In vivo studies in HL-60 cell line xenogeneic mouse models show clearly enhanced activity of the second generation cam16-wt15-33 TriKE in comparison to the first generation scFv16-m15-33 in terms of tumor control and NK cell expansion **(Figure 2A-C)**. A PDX model of AML was also used to compare the activity of the cam16-wt15-33 TriKE to rhIL-15, being tested clinically, and results indicate that tumor control is enhanced while NK cell proliferation is slightly lower with the second generation TriKE **(Figure 2D-F)**. Taken together we believe this second generation cam16-wt15-33 TriKE molecule is far superior to the first generation scFv16-m15-33 TriKE molecule, which has just entered the clinic. The manuscript is in the second phase of review and we are currently in the process of generating a GMP batch of this drug as a follow up clinical trial to the first generation TriKE.

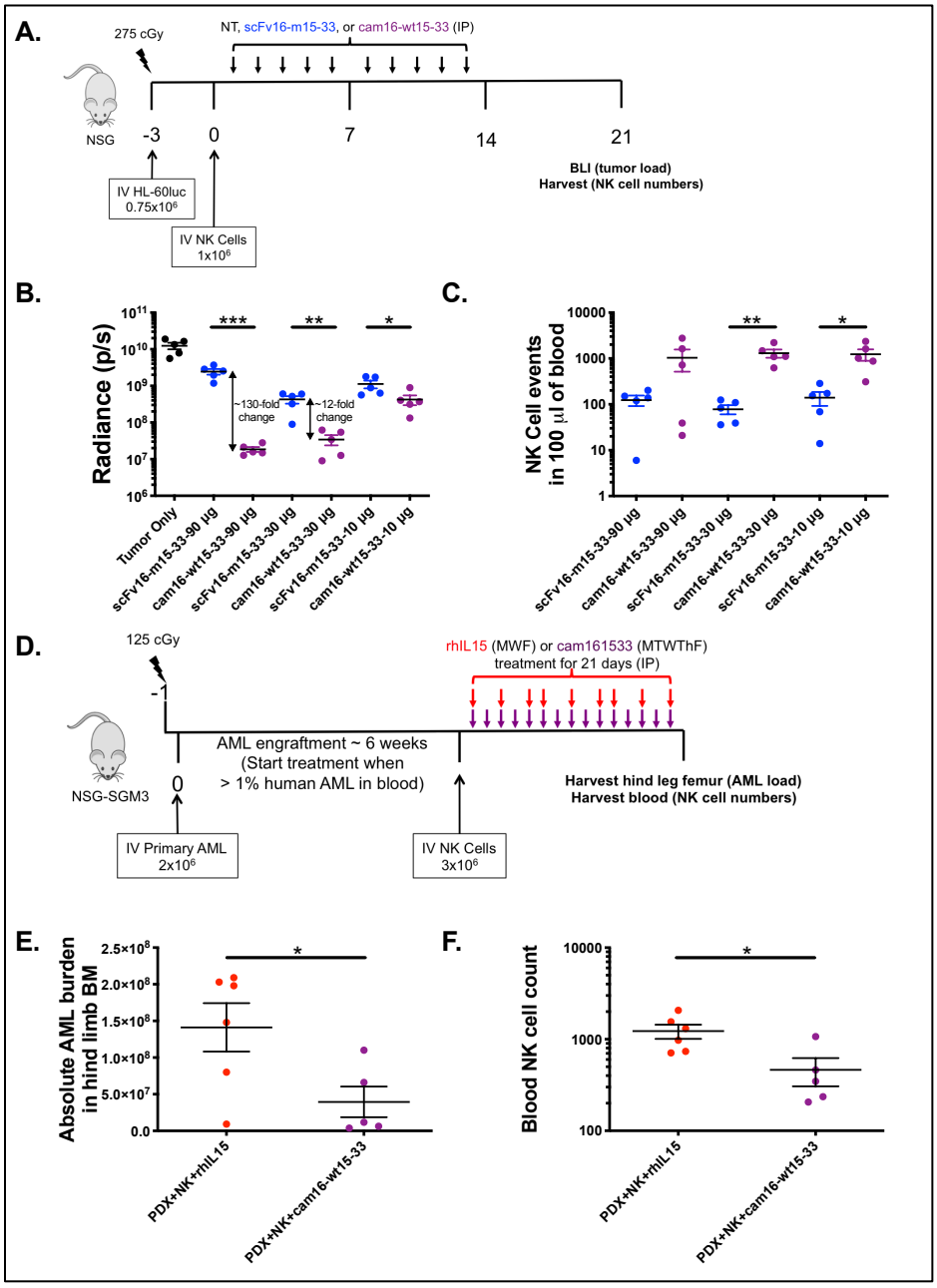

Figure 2: a) Diagram of HL-60/human NK cell xenogneic NSG model for evaluation of TriKE activity. Mice were then treated IP with noted concentrations of scFv16-m15-33 (blue) or cam16-wt15-33 (purple) 5 times weekly for 2 weeks. At day 21 tumor load was assessed by bioluminescent imaging and mice were bled to evaluate NK cell expansion. (b) Summary of tumor radiance and (c) NK cell numbers at day 21. (d) Diagram of AML patient derived xenograft (PDX) model used to compare the second-generation cam16-wt15-33 TriKE to rhIL-15. Upon AML engraftment mice were injected with NK cells and treatments and harvested 3 weeks later for evaluation of (e) AML blast (CD45+ CD33+) numbers in the bone marrow compartment and (f) NK cell numbers in a 100 µl of blood. Unpaired T test was used to calculate statistical differences.

The reason we selected CD16 as the receptor to trigger via TriKEs on the NK cells is that it is one of the most potent NK cell receptors. However, it is expressed in several other immune subsets, including monocytes and neutrophils, creating the possibility that the TriKEs will be 'sponged' by other cells. To bypass this issue we decided to create a TriKE swapping the anti-CD16 arm with an scFv targeting NKG2C on NK cells. NKG2C is an activating receptor within the NKG2A family. It is expressed in a population of more differentiated NK cells that arises in people that have been previously exposed to CMV. The interesting thing about NKG2C is that we can use it as an alternative to targeting activation of NK cells through CD16. Unlike CD16, NKG2C is not clipped upon activation and is not expressed on neutrophils and macrophages, making it a much more specific NK cell targeted agent. We generated an anti-NKG2C(scFv)xIL-15xanti-CD33(scFv) targeted TriKE and tested it in vitro on NKG2C low and NKG2C high NK cells. Results indicate that the TriKE selectively induces NK cell

degranulation on the NK cells that express NKG2C (High NKG2C+) when incubated with myeloid tumor targets, THP-1s, but does not induce degranulation of NK cells that don't express NKG2C (Low NKG2C+) when tested with THP-1 cells. Interestingly, in the post-transplant setting

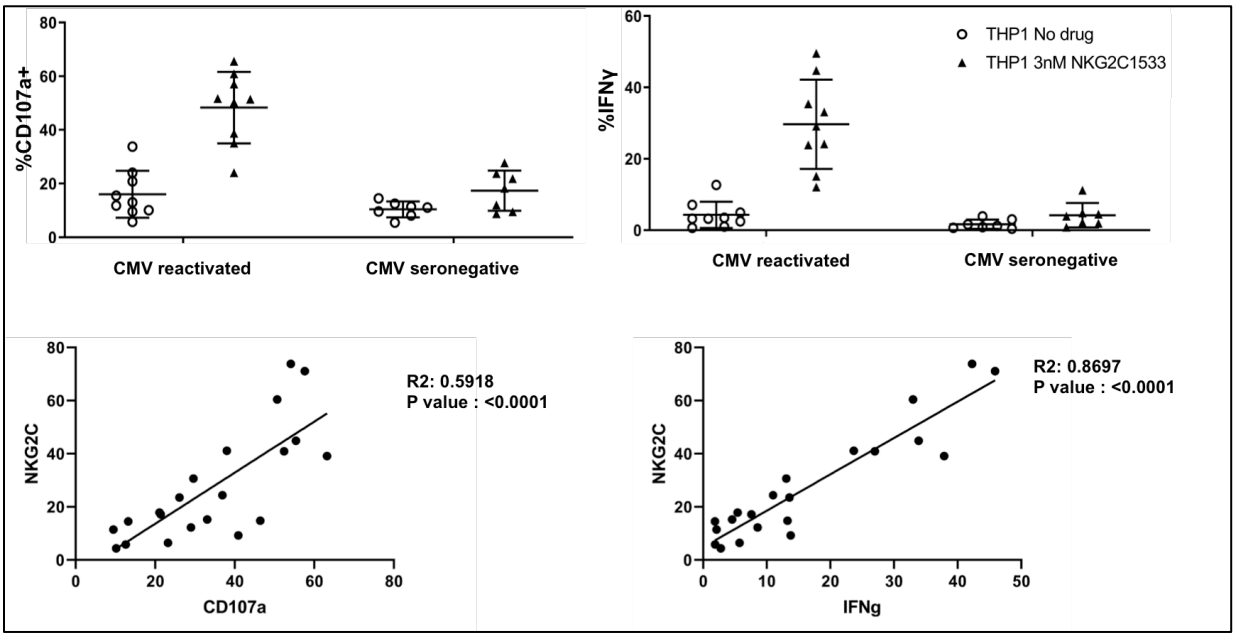

Figure 3: Thawed post transplant patient samples were evaluated 6 months post transplant and segregated into CMV reactivated or CMV seronegative. Cells were then incubated with THP-1 targets at a 2:1 ratio for 5 hours and degranulation (left) or IFNγ production (right) was determined. Data was graphed out as a function of CMV status (top) or NKG2C expression (bottom).

cytomegalovirus (CMV) reactivation has been shown to give rise to a population of NKG2C+ adaptive NK cells. We tested our TriKE in these samples to evaluate degranulation (CD107a) and inflammatory cytokine (IFNγ) activity against THP-1 cells as well (**Figure 3**). NKG2C expression, mediated by CMV reactivation also, correlated to specific proliferation, measured by CellTrace dilution, of the NKG2C expressing cells when treated with the NKG2C TriKE vs. when treated with rhIL-15 **(Figure 4)**. Finally, using induced pluripotent stem cells we generated a population of NK cells that uniformly expressed NKG2C or NKG2C and DAP12, an adaptor of NKG2C meant to enhance function. We tested the NKG2C TriKE in this population and show increased dynamic killing of tumor cells versus not TriKE treatment (data not shown). Taken together this data shows that we have created a functional NKG2C activating TriKE that can deliver specific activating signals to NK cells. This paper is being written up right now and will be submitted to Blood Advances shortly.

As the previous two projects demonstrate, the IL-15 moiety, located in the center of the TriKE molecules, has a prominent role in expansion of the NK cells. However, we wanted to evaluate if other cytokines can be leveraged in this platform as well. To this end we set out to explore IL-21 and IL-12. The intitial thought of using IL-21 as the cytokine within the TriKE had to do with the observation that feeder cells (K562s)

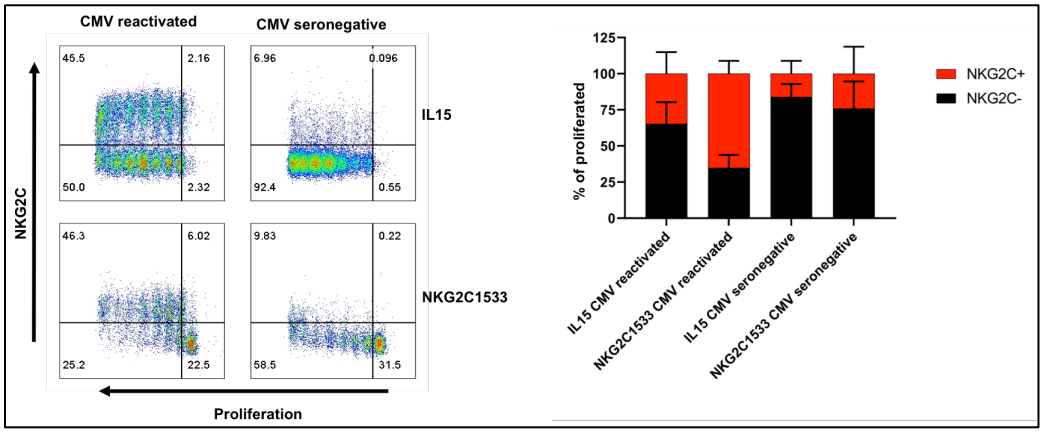

Figure 4: CMV reactivated or seronegative patient samples were incubated with CellTrace dye to evaluate proliferation and put in culture with rhIL-15 or the NKG2C TriKE for 7-days. Samples were then harvested and CellTrace dilution (i.e.: proliferation) was determined in the NKG2C+ and NKG2C- populations.

expressing membrane bound IL-21 and 41BB-ligand induce potent NK cell expansion that can be sustained long term without a resulting exhaustion of the NK cells, seen if IL-15 is expressed membrane bound in this system. However, our results clearly indicated that expression of IL-21 within the TriKE platform led to very robust NK cell death. Thus this cytokine has been abandoned for the time being, but we are planning to create

TetraKE molecules containing both IL-21 and 41BB-L in the near future to evaluate if this can bypass the issue. The goal of IL-12 expression in the TriKE platform had different objectives: 1) better induction of inflammatory signals; 2) escape from exhaustion mediated by repetitive antigen exposure at the tumor site; and 3) more potent bridging of the innate and adaptive immune responses through better priming of dendritic cells (DCs). The issues with the inhibitory TriKEs have also allowed us to push up the research on activating TriKEs. Besides progress described in the previous progress report on the IL-21 activating TriKE, we have worked on the IL-12 activating TriKE on the past year. This TriKE was initially geared to improve the inflammatory component of the NK cell response and it does just that, as shown on **Figure 5A**. However, using an assay in which NK cells are forced to exhaustion due to repeated exposure to tumor cells, we have shown that the IL-12 TriKE can also rescue NK cells from exhaustion (**Figure 5B**). We believe that NK cell exhaustion might very well be one of the major barriers to effective NK cell immunotherapy against myeloid malignancies, so these findings bare a lot of relevance. We have also tested how NK cell activation against myeloid tumors via an IL-12 expressing TriKEs modulates DC maturation, contrast to activation via an IL-15 expressing TriKE, and our data indicates that IL-12 TriKE supernatants better mature DCs (data not shown). We are in the process of determining whether this increased maturation also results in better T cell activation, and thus better T cell responses. Results from this project will be presented in the American Association of Immunology meeting this year and we are in the process of carrying out the remaining experiments needed to publish this work.

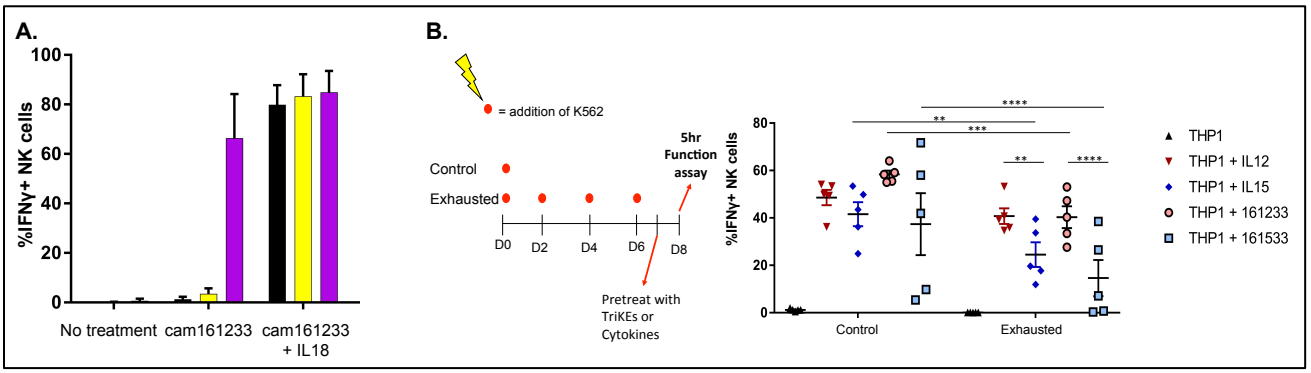

Figure 5: A) Interferon gamma production was evaluated against no targets (black), CD33- Raji cells (yellow) or CD33+ THP1 cells (purple), in the presence of no treatment (negative control), the cam161233 TriKE, or the TriKE plus IL-18 (as a positive control). PBMCs were incubated with noted targets for 5 hours and then the percentage of gated NK cells expressing intracellular IFNγ was determined by flow cytometry. B) Cells were exposed to K562 targets once (control) or 4 times (exhaustion condition) to determine the impact of restimulation on exhaustion and demonstrate if IL-12 expressing TriKEs can bypass this process.

For all of the projects noted above we have used CD33 as the target antigen. While this antigen offers broad detection of myeloid malignancies, it is also highly expressed in normal myeloid cells thus collateral damage is likely when using these TriKEs. To this end we have been exploring targeting of other myeloid tumor antigens. CLEC12A, also known as CLL1, is an interesting alternative CD33 as it is not expressed normally on myeloid cells, and is expressed at lower levels in myeloid progenitors, but is expressed in AML and MDS as well as myeloid tumor cancer stem cells. Also, 70% of CD33$^-$ AMLs express CLEC12A, making it a valuable target for CD33 antigenic escape strategies. Our data against primary patient AML blasts shows that the CLEC12A TriKE

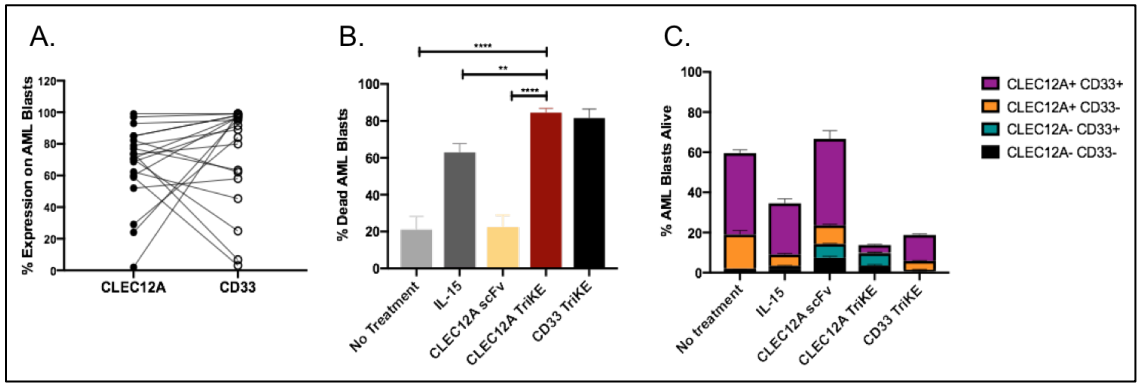

Figure 6: A) Primary AML blasts (SSCh low, CD45int, CD117+, CD14-, CD34+) were assessed for expression of CD33 and CLEC12A using flow cytometry (n=10). B) Enriched NK cells (CD56+, CD3-) from healthy donors (n=10) were incubated with primary AML blasts with the noted treatments (30nM) to evaluate target cell killing using flow cytometry and a live/dead marker over 48 hours. C) The proportion of different groups of AML blasts (based on CD33 and CLEC12A expression) were tracked over 48 hours to assess specificity of the 1615CLEC12A TriKE compared to the 1615CD33 TriKE. Enriched NK cells (CD56+, CD3-) from healthy donors (n=5) were incubated with bone marrow samples from AML patients with the noted treatments (30nM) to evaluate killing of cancer stem cells (SSCh low, CD45int, CD34+, CD38-) in a 4-hour assay at a 2:1 effector/target ratio.

induces robust killing of blasts, but also demonstrates that this TriKE specifically kills the CLEC12A subset of the blasts **(Figure 6)**. Furthermore, CLEC12A can be expressed in leukemic stem cells (LSCs), which should better protect against relapse. Flow cytometric assays evaluating LSC killing shows that CLEC12A TriKE induced the best killing of this population of cells **(Figure 7).** We have tested the CLEC12A TriKE in an in vivo xenogeneic model using a CD33+ and CLEC12A+ cell line, HL-60s, to evaluate dynamic tumor control over 21 days of treatment. Our results indicate that the CLEC12A TriKE performs similarly or outperforms the CD33 TriKE in this preclinical model **(Figure 8)**. A PDX mouse model using primary AML blasts shows similar results indicating that this approach will be suitable clinically. We are extremely excited about the potential of targeting CLEC12A as an alternative to CD33 and are planning to submit this manuscript to Leukemia within the next couple of months. Furthermore, we are currently exploring GMP generation of this TriKE molecule for follow up clinical trials targeting CLEC12A alone or in conjunction with the CD33 TriKE. Though our proposed studies have changed slightly due to issues with some of the inhibitory TriKEs, we have been extremely productive with alternative projects (cam161533 TriKE, NKG2C1533 TriKE, and cam1615CLEC12A TriKE) and have moved the IL-12 TriKE forward in the last year of the grant.

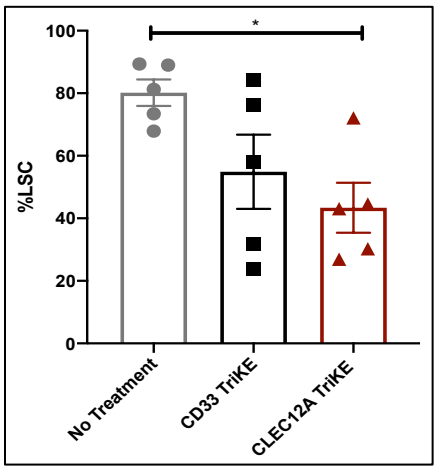

Figure 7: Enriched NK cells (CD56+, CD3-) from healthy donors (n=5) were incubated with bone marrow samples from AML patients with the noted treatments (30nM) to evaluate killing of cancer stem cells in a 4-hour assay at a 2:1 effector/target ratio. Combined data from cancer stem cell killing assay showing percentage of cancer stem cells present at the end of the assay. Error bars indicate +/- standard error of the mean. Statistical significance are determined as *P , .05.

In terms of training activities and professional development I used project funds to attend the AACR Tumor Immunology and Immunotherapy meeting in Miami on November 2018 and attend the American association of Immunologists meeting in San Diego on May 2019. I presented at both of those meetings and was also invited to present my TriKE related (cam161533 and CLEC12A TriKE) work later on in the year to Cord Blood Connect (in Miami) and Targeting Immunity (in Boston). I also attended and presented in several seminars at the University of Minnesota, ranging from the Cancer Center Seminar, to Garibaldi lecture and NK MIG meeting. The protected time also allowed me to recently author a blood advances paper and allowed to work on and submit the cam161533 Paper and work on the CLEC12A paper.

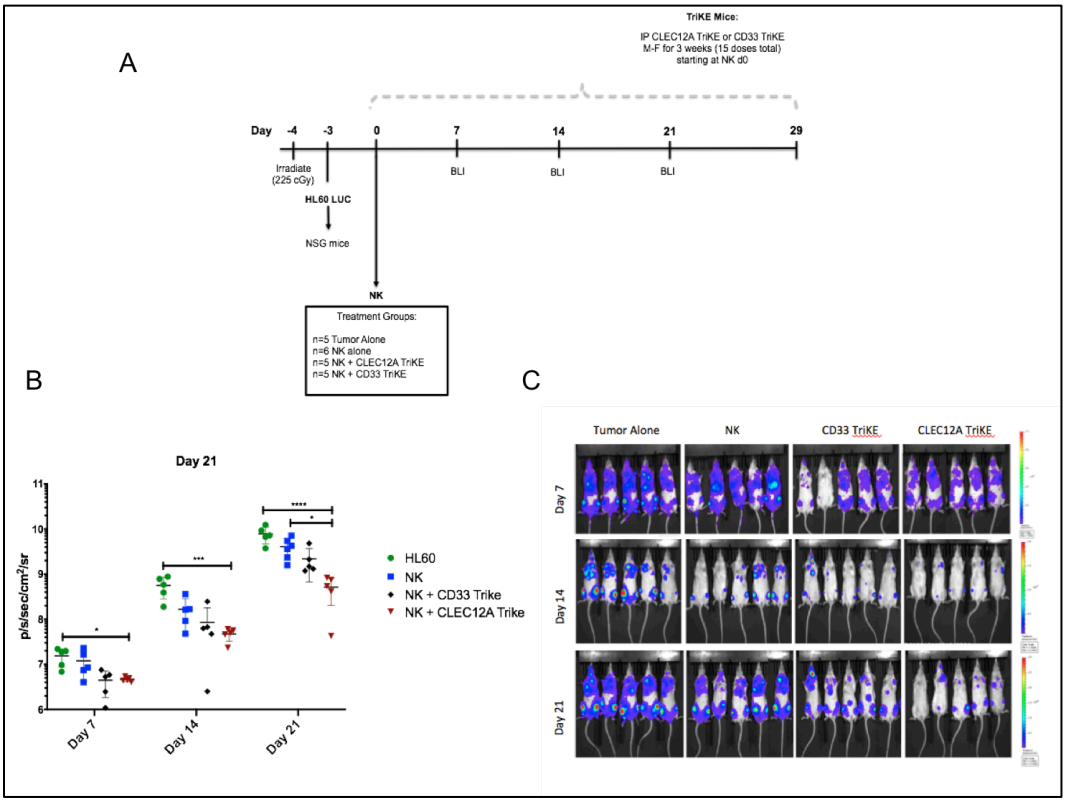

Figure 8: (A) Schematic of HL-60luc mouse experiment. The model was established by conditioning NSG mice (225cGy) and then injecting HL-60luc cells intravenously (7.5 × 105 cells/mouse). Three days later, 1 × 106 normal human donor NK cells (calculated from a magnetically depleted CD3/CD19 product) activated overnight with 10ng/ml IL-15 were infused. The 1615CLEC12A TriKE or 161533 TriKE (20ug) was administered MTWThF through the next 3 weeks of the study (15 doses total), and a control group received NK cells but no treatment. (B) Quantification of luminescence from the four treatment groups at day 7, day 14 and day 21 after NK infusion. Each dot represents a different mouse. (C) Individual mouse photoluminescence after 2-minute exposures on day 7, 14 and 21.

Overall, the work from this grant resulted in 6 publications (listed below), one manuscript in revision and two to be submitted within the next three months, one study in the works to be published within the next year. It also provided funding for attendance of 6 scientific meetings and resulted in 4 poster presentations, 5 National invited talks, and 5 locally invited oral presentations (listed below). Finally, the work from this grant aided in generating the data for filing of a patent for CLEC12A TriKEs (see full patent in products section) and laid the groundwork for several upcoming second-generation TriKE clinical trials.

Total Publications:

1. 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.
2. **Felices M**, Lenvik AJ, McElmurry R, Chu S, Hinderlie P, Bendzick L, Geller MA, Tolar J, Blazar BR, Miller JS. Continuous treatment with IL-15 exhausts human NK cells via a metabolic defect. JCI Insight. 2018 Feb 8;3(3). pii: 96219. doi: 10.1172/jci.insight.96219. [Epub ahead of print] PubMed PMID: 29415897; PubMed Central PMCID: PMC5821201.
3. Uppendahl LD, Dahl CM, Miller JS, **Felices M**, Geller MA. Natural Killer Cell-Based Immunotherapy in Gynecologic Malignancy: A Review. Front Immunol. 2018 Jan 5;8:1825. doi: 10.3389/fimmu.2017.01825. eCollection 2017. Review. PubMed PMID: 29354116; PubMed Central PMCID: PMC5760535.
4. Sarhan D, Brandt L, **Felices M**, Guldevall K, Lenvik T, Hinderlie P, Curtsinger J, Warlick E, Spellman SR, Blazar BR, Weisdorf DJ, Cooley S, Vallera DA, Önfelt B, Miller JS. 161533 TriKE stimulates NK-cell function to overcome myeloid-derived suppressor cells in MDS. Blood Adv. 2018 Jun 26;2(12):1459-1469. doi: 10.1182/bloodadvances.2017012369. PubMed PMID: 29941459; PubMed Central PMCID: PMC6020813.
5. Don Yun H, **Felices M**, Vallera DA, Hinderlie P, Cooley S, Arock M, Gotlib J, Ustun C, Miller JS. Trispecific killer engager CD16xIL15xCD33 potently induces NK cell activation and cytotoxicity against neoplastic mast cells. Blood Adv. 2018 Jul 10;2(13):1580-1584. doi: 10.1182/bloodadvances.2018018176. PubMed PMID: 29980573; PubMed Central PMCID: PMC6039654.
6. **Felices M**, Kodal B, Hinderlie P, Kaminski MF, Cooley S, Weisdorf DJ, Vallera DA, Miller JS, Bachanova V. Novel CD19-targeted TriKE restores NK cell function and proliferative capacity in CLL. Blood Adv. 2019 Mar 26;3(6):897-907. doi: 10.1182/bloodadvances.2018029371. PubMed PMID: 30890546; PubMed Central PMCID: PMC6436008.
7. **Felices M**, Lenvik TR, Kodal B, Lenvik AJ, Hinderlie P, Schirm DK, McElmurry R, Bendzick L, Geller MA, Eckfeldt CE, Vallera DA, Miller JS. A Novel Humanized Single Domain Camelid Sequence Engaging CD16 on NK Cells Enables Potent and Specific IL-15 Activity in a 2nd Generation Trispecific Killer Engager. Cancer Immunology Research. 2020: *In secondary review stage*.
8. *CLEC12A and NKG2C TriKE studies are being written currently for Leukemia and Blood Advances (respectively).* **Felices M** is the last author in both studies. IL-12 TriKE study in the works for a publication within the next year.

Poster Presentations:

1. Arvindam US, Van Hauten P, Hallstrom C, Vallera DA, Dolstra H, Miller JS, **Felices M**. "CD16-IL15-CLEC12A Trispecific Killer Engager (TriKE) drives NK cell expansion, activation, and antigen specific killing of Cancer Stem Cells in Acute Myeloid Leukemia." 60th annual ASH meeting in San Diego, CA, 2018.
2. **Felices M**, Kodal B, Lenvik AJ, Ettestad B, Bendzick L, Ryan C, Hinderlie P, Hallstrom C, Schirm DK, McElmurry R, Eckfeldt CE, Geller MA, Lenvik TR, Vallera DA, Miller JS. "Antigen-specific targeting of cancer by NK cells via next generation TriKE molecules." 17th meeting of the Society for Natural Immunity (SNI) in San Antonio, TX, 2018.

3. **Felices M**, Eckfeldt CE, Lenvik TR, Kodal B, Lenvik AJ, Bendzick L, Ryan C, Geller MA, Cooley S, Weisdorf DJ, Vallera DA, Miller JS. "Second-generation camelid TriKE induces improved NK cell mediated targeting of AML in pre-clinical models." 59th annual ASH meeting in San Diego, CA, 2018.
4. **Felices M**, Sarhan D, Brandt L, Guldevall K, McElmurry R, Lenvik AJ, Chu S, Tolar J, Taras EP, Spellman S, Warlick ED, Verneris MR, Cooley S, Weisdorf DJ, Blazar BR, Önfelt B, Vallera DA, Miller JS. "CD16-IL15-CD33 Trispecific Killer Engager (TriKE) Overcomes Cancer-Induced Immune Suppression and Induces Natural Killer Cell-Mediated Control of MDS and AML via Enhanced Killing Kinetics." 58th annual ASH meeting in San Diego, CA, 2016.

National Invited Talks:

1. Advances in Tri-specific Killer Engager (TriKE) Molecules for NK Cell Antigen-Specific Recognition of Tumor Cells in High Risk MDS and Refractory AML Settings. Invited Speaker: Targeting Innate Immunity Congress. September 2019, Cambridge, MA.
2. Leveraging Natural Killer Cell Immunotherapy through Cytokine-Bearing Antigen-Specific TriKE Molecules. Invited Speaker: Cord Blood Connect International Congress. September 2019, Miami, FL.
3. Improving NK cell immunotherapy for cancer. Invited speaker: Prostate Cancer Foundation (PCF) 25th retreat. October 2018, Carlsbad, CA.
4. Maximizing the potential of Natural Killer cell immunotherapy via cytokine signaling, checkpoint blockade and TriKE molecules. Invited speaker: UMASS medical school. June 2018, Worcester, MA.
5. Driving natural killer cell antigen-specific targeting of cancer via next generation Trivalent molecules. 2018 AACR annual meeting in Chicago, IL.

Local Invited Talks (at the University of Minnesota):

1. Driving NK cell immunotherapy with TriKE molecules (Flash Talk). 9th Annual Cancer Research Symposium; University of Minnesota; April 2019.
2. Driving NK cell immunotherapy against liquid and solid tumors via next generation TriKEs. Division of Hematology, Oncology and Transplantation Garibaldi Lecture; University of Minnesota, April 2018.
3. NK cell targeting of lung malignancies with TriKEs. Thoracic TWG Presentation, University of Minnesota; November 2017; Minneapolis, MN.
4. Novel approaches to enhance NK cell immunotherapy. Garibaldi Research Conference, HOT Division, University of Minnesota; April 2017; Minneapolis, MN.
5. Driving TriKEs from the bench to the clinic: an NK cell immunotherapy story. Masonic Cancer Center Seminar Series, University of Minnesota; February 2017; Minneapolis, MN.

4. Impact (Year-3-Final):

The goal of the proposed work is to significantly improve cellular therapies against chronic myeloid leukemia (CML), covered in the FY15 Myeloproliferative disorders topic area, and related myeloid disorders acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). CML has an incidence of 1-2 cases per 100,000 adults and accounts for about 15% of newly diagnosed cases of leukemia in adults. Prognosis for CML has significantly improved due to the advent of first, second, and third-generation tyrosine kinase inhibitors (TKIs), yet there is still worse survival in the elderly and alternative approaches remain necessary for patients who fail TKI treatment (due to TKI resistant mutation) or patients that present with advanced CML. CML and MDS can progress into AML, which predicts a worse prognosis and a requirement for transplantation as the only curative therapy. Even with transplantation, which is a high-risk procedure and is not well tolerated by elderly patients, the risk for relapse still remains high with current therapeutic options. As the population ages the incidence of AML, MDS, and CML cases increases. Cellular therapy that specifically targets the myeloid tumor cells by the immune system would be of great value independently at earlier stages of disease and as a complementary approach to current therapies at later stages. This type of therapy has the potential to be utilized on its own, depending the stage of intervention, reducing the need for toxic chemotherapeutic and radiation therapy intervention.

The immunotherapeutic value of NK cells is currently being exploited against myeloid malignancies in a variety of ways. NK cells can drive tumor killing in the allogeneic hematopoietic cell transplant (HCT) or NK cell infusion setting, where the graft versus leukemia (GVL) effect is driven through KIR-HLA ligand mismatch, preventing attenuation of NK cell activation through inhibitory KIR. KIR blocking monoclonal antibodies (mAbs) are currently being tested in the clinic to drive this effect in the autologous setting. To drive NK cell antibody-dependent cell-mediated cytotoxicity (ADCC) against myeloid tumors anti-CD33 mAbs have been tested, but results haven't been promising with use of the antibody on its own. Another approach to enhance NK cell immunotherapy is through cytokine treatment, both in the pre- and post-transplant settings. Although this approach is promising, off target effects are a major concern. One final approach generating excitement in the cellular immunotherapy field, also shown to enhance NK cell function, is disruption of the PD-1/PD-L1 checkpoint blockade axis through use of blocking mAbs.

The molecules created during the course of this will likely shape NK-cell based targeted immunotherapy in the myeloid malignancy field for years to come. Our original version of the TriKE targeting myeloid malignancies (through anti-CD33 and an anti-CD16 scFv), just entered the clinic for a first-in-human clinical trial. Through the course of this grant we have greatly improved the CD16 binding arm through generation of a humanized single domain antibody against CD16. Resulted in a much stronger second-generation TriKE platform that will be used in all future TriKE molecules as well as the next clinical test targeting myeloid malignancies. We have also generated anti-CD33 Targeting TriKE molecules that bind the activating NKG2C receptor instead of CD16. This allows for better targeting of NK cells, as CD16 is expressed in neutrophils and macrophages, as well as direct targeting of *adaptive* NK cells, which have more mature characteristics and can better kill tumor. We have also generated an IL-12 TriKE that is meant to drive NK cell mediated inflammation through induction of dendritic cell activation/maturation, which in terms results in better T cell responses. This TriKE will not only better bridge the innate and adaptive immune responses, but also better protect NK cells against exhaustion. Finally, we have generated and pre-clinically validated a CLEC12A targeting TriKE. Unlike CD33, CLEC12A can be used to target myeloid malignancies with minimal collateral damage on normal myeloid cells. Of importance, CLEC12A has also been shown to be a good marker for cancer stem cells in AML and MDS, hopefully allowing for better control of resurgence of disease. CLEC12A TriKEs will likely follow the anti-CD33 second generation TriKE in the clinical trials cue. Given the way the TriKE molecules integrate several signals at the NK/tumor cell synapse, they have the potential to achieve similar immunotherapeutic success to that seen by chimeric antigen receptor (CAR) approaches. The big difference is that they would do this in a more economical "off-the shelf" manner that does not require individualized gene therapy and can be applied to a large number of people. This would represent a major advance in NK cell based immunotherapies against tumors, significantly driving the field forward.

5. Changes/Problems (Year-3-Final):

As discussed briefly last year, as we gain insight into the biology modulated by the TriKE molecules we have been shifting our aims. The majority of the efforts over the past year have focused on: 1) improving the CD16 engaging portion of the platform which should have important effects on clinical implementation of the TriKE (Paper in secondary revision stage at Cancer Immunology Research); 2) Activation through the NKG2C activating receptor (all experiments done, paper being written); 3) Targeting CLEC12A instead of CD33 to improve myeloid tumor targeting (paper mostly written and will be submitted to Leukemia in the next month); 4) a PD-L1 targeting TriKE (we have good biology for this one but are in the process of screening (with non-DoD funds) for a different PD-L1 engager to allow for clinical translation; and 5) an IL-12 TriKE (about 60% of the way through experiments for this project). We have deeply explored biology of the IL-21 TriKE (SA2.2), and have found that delivery of IL-21 via the TriKE platform is very toxic to NK cells. Thus, due to this unexpected result, we are abandoning this TriKE.

6. Products (Year-3-Final):

Publications:

- **Felices M**, Kodal B, Hinderlie P, Kaminski MF, Cooley S, Weisdorf DJ, Vallera DA, Miller JS, Bachanova V. Novel CD19-targeted TriKE restores NK cell function and proliferative capacity in CLL. Blood Adv. 2019 Mar 26;3(6):897-907. doi: 10.1182/bloodadvances.2018029371. PubMed PMID: 30890546; PubMed Central PMCID: PMC6436008.
- **Felices M**, Lenvik TR, Kodal B, Lenvik AJ, Hinderlie P, Schirm DK, McElmurry R, Bendzick L, Geller MA, Eckfeldt CE, Vallera DA, Miller JS. A Novel Humanized Single Domain Camelid Sequence Engaging CD16 on NK Cells Enables Potent and Specific IL-15 Activity in a 2nd Generation Trispecific Killer Engager. Cancer Immunology Research. 2020: *In secondary review stage*.
- *CLEC12A and NKG2C TriKE studies are being written currently for Leukemia and Blood Advances (respectively).* **Felices M** is the last author in both studies.

Patents:

- A patent application was converted for the CLEC12A TriKE molecule on 10/17/2019. This is a conversion from provisional application no. US 62/747,983. As expected, this DoD award is listed as part of the government funding for said patent. An image of the first page of the patent application is copied here. The full patent conversion application can be found in Appendix C.

PATENT APPLICATION
Attorney Docket No. GTBIO2090-1WO

NK ENGAGER MOLECULES AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims benefit of priority under 35 U.S.C. §119(e) of U.S. Serial 62/747,983, filed October 19, 2018, the entire contents of which is incorporated herein by reference in its entirety.

GOVERNMENT FUNDING

[002] This invention was made with government support under CA111412 and CA65493, awarded by the National Institutes of Health, under CA36725, CA72669, CA077598 and CA197292, awarded by the National Cancer Institute and under CA150085, awarded by the U.S. Department of Defense. The government has certain rights in the invention.

SEQUENCE LISTING

[003] The material in the accompanying sequence listing is hereby incorporated by reference into this application. The accompanying sequence listing text file, name GTBIO2090_1WO_Sequence_Listing.txt, was created on October 15, 2019, and is 23 kb. The file can be accessed using Microsoft Word on a computer that uses Windows OS.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[004] The invention relates generally to immunotherapy and more specifically to compositions useful for engaging natural killer (NK) cells in an immune response.

BACKGROUND INFORMATION

[005] Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system capable of immune surveillance. Like cytotoxic T cells, NK cells deliver a store of membrane penetrating and apoptosis-inducing granzyme and perforin granules. Unlike T cells, NK cells do not require antigen priming and recognize targets by engaging activating receptors in the absence of MHC recognition. NK cells express CD16, an activation receptor that binds to the Fc portion of IgG antibodies and is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells are regulated by IL-15, which can induce increased antigen-dependent cytotoxicity, lymphokine-activated killer activity, and/or mediate interferon (IFN), tumor-necrosis factor (TNF) and/or granulocyte-macrophage colony-stimulating factor (GM-CSF) responses. All of these IL-15- activated functions contribute to improved cancer defense.

WEST:288081148.1
367038-000104

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7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS (Year-3-Final)

Name	Martin Felices
Project Role	PD/PI
Researcher Identifier (ORCID)	0000-0002-5945-0634
Nearest person month worked	4 calendar months
Contribution to Project	Experimental design, data analysis, drug design, project direction
Funding Support	See attached support page.

Name	Behiye Kodal
Project Role	Researcher 3 (Scientist)
Researcher Identifier (ORCID)	N/A
Nearest person month worked	6 calendar months
Contribution to Project	Experimental execution, data analysis
Funding Support	N/A

Name	Alexander Lenvik
Project Role	Researcher 3 (Scientist)
Researcher Identifier (ORCID)	N/A
Nearest person month worked	6 calendar months
Contribution to Project	Experimental execution, data analysis, drug design
Funding Support	N/A

PREVIOUS/CURRENT/PENDING SUPPORT:

MARTIN FELICES

1. Previous

W81XWH-16-1-0380/0010866661-0001(PI: Felices)

Title: CA150085 - Enhancing Natural Killer Cell-Mediated Targeting and Responses to Myeloid Leukemias

Effort/Salary: 30%

Agency: U.S. Department of Defense

Duration: 09/30/2016-09/29/2019

Funding: $359,979

Grants Officer: Elayne Seiler, Grants Specialist
elayne.k.seiler.civ@mail.mil
301.619.7358

Goal: Generate TriKEs targeting pathways involved in suppression of NK cell function and generate TriKEs targeting pathways involved in amplifying NK cell function.

Specific aims: 1. Generation of TriKes targeting pathways involved in suppression of NK cell function
2. Generation of TriKes targeting pathways involved in amplification of NK cell function

Overlap: None

R01HL122216 (PI: Jeff Miller)

Title: Inducing NK cells to remember and fight cancer

Effort/Salary: 5%

Agency: NIH/NHLBI

Duration: 12/01/2014-11/30/2018

Funding: $258,745

Grants Officer: Nahed El Kassar, nahed.elkassar@nih.gov

Goal: This program will improve transplant outcome by taking account of NK cell receptor genes to select donors that will reduce relapse of leukemia and increase survival of the transplant recipient.

Specific aims: Aim 1: Investigate the unique microRNA profile of adaptive NK cells and requirements for their expansion.
Aim 2: Determine which signaling and transcription factor networks promote the Development and survival of HCMV-induced adaptive NK cells.
Aim 3: In vivo analysis of adaptive NK cell survival, function and homing in a xenogeneic model of adoptive transfer of human NK cells and HCMV reactivation.

Overlap: None

T32HL007062 (PI: Vercellotti)
Title: Hematology Research Training Program

Effort/Salary: 100%

Agency: NIH

Duration: 06/01/2011-05/31/2013 (supported)

Funding: $437,036 (current annual direct)

Grants Officer: Manda C. Richards, Grants Management Specialist
301.827.7977
manda.richards@nih.gov

Goals: This training program is part of University of Minnesota Department of Medicine, Division of Hematology, Oncology, and Transplantation. It is focused on training MDs and PhDs for academic careers that emphasize Hematology-related Research.

Specific aims: N/A

Overlap: None

2. Current

P30CA77598 (Yee)
Title: *Cancer Center Support Grant*
Effort: 10%
Agency: NIH/NCI
Duration: 02/01/2019 – 01/31/2024
Funding: $230,000
Program Official: Sonya Roberson, robersos@mail.nih.gov
Goal: To provide an infrastructure for cancer research, education and patient care for the citizens of the Minnesota and the surrounding region. Our mission is to create a collaborative environment that advances knowledge about the causes, prevention, detection and treatment of cancer.
Aims: N/A
Role: Investigator
Overlap: None

Fate Therapeutics
Title: *Development of NK Cell Therapeutics*
Effort: 10%
Duration: 11/01/2019 – 10/31/2022
Funding: $820,776
Goal: The main goal of this project is to optimize production of natural killer cells from human pluripotent stem cells.
Role: Investigator
Overlap: None

Obsidian Therapeutics

Title: Preclinical Testing of Tunable Cytokine Receptors
Effort/Salary: 5%
Duration: 2/1/19 – 1/31/21
Funding: $219,000
Goal: Selection of a potential NK cell therapeutic that has shown great persistence and efficacy of cells with pulsed DD-IL-15 either in WT background or in the context of CRISPR PD1 knockout.
Overlap: None

R21CA216652 (PI: Moriarity)

Title: Genetically Modified Natural Killer Cells for Cancer Immunotherapy
Effort/Salary: 4%
Agency: NIH
Duration: 09/06/2018-08/31/2020
Funding: $159.420
Grants Officer: Anthony Welch
awelch@ncifcrf.gov
Goal: We will utilize a specific immune cell, termed Natural Killer cells, and modify their genetic code to enhance their ability to treat a pre-existing cancer.
Specific aims: Specific Aim 1: Optimize enrichment and expansion of genetically modified primary human NK cells
Specific Aim 2: Functionally test knockout and/or gene edited primary human NK cells for enhanced expansion, survival, and/or cancer cell killing in vitro and in vivo
Overlap: None

P01CA065493 (PI: John Wagner)

Title: Biology and Transplantation of the Human Stem Cell
Effort/Salary: 15%
Agency: NIH/NCI
Duration: 07/01/2015-06/30/2020
Funding: $192,000
Grants Officer: William Merritt, PhD
merrittw@mail.nih.gov
Goal: The main goal of this project is to understand how to exploit NK cell therapy along with umbilical cord blood transplantation.
Specific aims: Cancer Center Project 3 (Jeff Miller), Project Title: Adaptive NK Cell Therapy to Improve UCB Transplant Outcomes

1. Evaluate the clinical impact of IL-15 signaling on adaptive NK cells. CMV reactivation, which occurs in 50% of CMV seropositive patients undergoing UCBT, induces a unique population of adaptive NK cells and is correlated with protection from relapse.
2. Activation and antigen targeting of NK cells for prevention of relapse.

Overlap: None

P01CA111412 (PI: Jeff Miller)

Title: NK cells, their receptors, transplantation and cancer therapy
Effort/Salary: 24% (
Agency: NIH/NCI
Duration: 04/05/2016-03/31/2021
Funding: $1,416,372
Grants Officer: William Merritt, merrittw@mail.nih.gov
Goal: This program will improve transplant outcome by taking account of NK cell receptor genes to select donors that will reduce relapse of leukemia and increase survival of the transplant recipient.

Specific aims: Aim 1: Understand how KIRs interact with class I HLA to determine NK cell function
Aim 2: Test the hypothesis that trans-presentation of IL-15 is needed to optimally expand NK cells in vivo
Aim 3: Determine now hCMV induces the development of adaptive NK cells with enhanced ADCC capacity and memory-like properties.
Aim 4: Perform clinical trials to exploit enhanced NK cell activation with specific antigen targeting with bi-specific killer engagers (BiKE) to enhance anti-tumor potency of NK cells

Overlap: None

R35CA197292-02 (PI: Jeff Miller)

Title: Viral Priming and Targeting NK Cells Against Solid Tumor Malignancies
Effort/Salary: 5%
Agency: NIH/NCI
Duration: 08/05/2015-07/31/2022
Funding: $600,000
Grants Officer: Anthony R. Welch, awelch@ncifcrf.gov
Goal: The overarching goal is to develop strategies to enhance the anti-tumor activity of endogenous NK cells in patients with solid tumor malignancies. The objective is to develop "off the shelf" reagents to activate NK cells, overcome inhibitory receptor signaling, and target them to specific tumor antigens.
Specific aims: Aim 1: Use genetic epidemiologic studies to determine whether NK cells “adapted” by viral exposure (cytomegalovirus [CMV] ± human papilloma virus [HPV]) influence the risk of cancer development and response to therapy.
Aim 2: Characterize RHAMM as a promising target to eliminate tumor cells and Immunosuppressive non-malignant cells from the tumor microenvironment,
Aim 3: Test whether newly discovered “adaptive” NK cells mediate enhanced anti-tumor activity via CD16 signaling.
Aim 4: Develop and test novel agents to target activated NK cells against solid tumors.
Overlap: None

RSG-14-151-01-CCE (PI: Geller)

Title: Natural Killer Cell Immunotherapy for Ovarian Cancer
Effort/Salary: 10%
Agency: American Cancer Society
Duration: 01/01/2015-12/31/2018 (no cost extension to 12/31/2019)
Funding: $798,851
Grants Officer: The American Cancer Society
Extramural Grants Department
250 Williams Street NW, 6th Floor
Atlanta, GA 30303

Goal: We are developing an anti-mesothelin chimeric antigen receptor (CAR) in iPSCs to produce a targeted NK cell population effective against ovarian cancer, where 70% of tumors express mesothelin. Our goal is for CAR-expressing NK cells to be used as a readily available, "off-the-shelf" product for anti-tumor immunotherapy.
Specific aims: 1. Express an anti-mesothelin chimeric antigen receptor (CAR) in human induced pluripotent stem cells (iPSCs) to create targeted NK cells with increased ability to kill human ovarian cancer cells.
2. Evaluate in vivo anti-ovarian cancer activity of NK cells derived from human iPSCs expressing anti-mesothelin chimeric receptors.
Overlap: None

Cancer Center Clinical Research (PI Miller)

Title:	Development of NK Cell Therapeutics
Effort/Salary:	13%
Agency:	UMN internal
Duration:	02/01/2017-01/31/2020
Funding:	$200,000
Grants Officer:	
Overlap:	None

3. Pending

None

Appendix A: Publications

REGULAR ARTICLE

blood advances

Novel CD19-targeted TriKE restores NK cell function and proliferative capacity in CLL

Martin Felices,[1] Behiye Kodal,[1] Peter Hinderlie,[1] Michael F. Kaminski,[1] Sarah Cooley,[1] Daniel J. Weisdorf,[1] Daniel A. Vallera,[2] Jeffrey S. Miller,[1] and Veronika Bachanova[1]

[1]Division of Hematology, Oncology, and Transplantation, Department of Medicine, and [2]Department of Radiation Oncology, University of Minnesota, Minneapolis, MN

Key Points

- 161519 TriKE induces NK cell–mediated killing of primary CD19$^+$ CLL tumor cells while also inducing NK cell proliferation.
- 161519 TriKE enhances interferon γ production on CLL patient NK cells.

Chronic lymphocytic leukemia (CLL) is characterized by chronic clonal expansion of mature CD19-expressing B lymphocytes and global dysfunction of immune effectors, including natural killer (NK) cells. CLL remains incurable, and novel approaches to refractory CLL are needed. Our group has previously described trispecific killer engager (TriKE) molecules that redirect NK cell function against tumor cells. TriKE reagents simultaneously bind an activating receptor on NK cells, CD16, and a tumor antigen while also providing an NK cell expansion signal via an interleukin-15 moiety. Here we developed the novel CD19-targeting 161519 TriKE. We demonstrate that 161519 TriKE induced killing of a CD19-expressing Burkitt's lymphoma cell line and examined the impact on primary CLL targets using healthy donor and patient NK cells. 161519 TriKE induced potent healthy donor NK cell activation, proliferation, and directed killing. Furthermore, 161519 TriKE rescued the inflammatory function of NK cells obtained from CLL patient peripheral blood samples. Finally, we show that 161519 TriKE induced better directed killing of CLL in vitro when compared with rituximab. In conclusion, 161519 TriKE drives a potent activating and proliferative signal on NK cells, resulting in enhanced NK cell expansion and CLL target killing. Our findings indicate the potential immunotherapeutic value of 161519 TriKE in CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in Western countries.[1] The biology, genetics, and clinical behavior of this malignancy are highly variable.[2] Although recent novel targeted therapies, such as Bruton tyrosine kinase inhibitor ibrutinib, PI3-kinase inhibitor idelalisib, BCL-2 inhibitor venetoclax, and monoclonal antibodies obinutuzumab and ofatumumab, have demonstrated potent antitumor activity and some remarkably prolonged remissions, safer and more effective therapies for refractory CLL are still needed.[3] Allogeneic donor transplantation (alloHCT) is the only known therapy with curative potential.[3] The graft-versus-leukemia effect facilitated by donor T cells and NK cell effectors often leads to permanent eradication of CLL clones.[4] However, alloHCT is often not feasible for CLL patients because of their older age or declining overall fitness.[5] Novel therapies with capacity to revert immune dysfunction in CLL patients and harness immune effector–mediated CLL targeting are particularly attractive. CAR T-cell therapies are being explored in this setting, but they are associated with toxicities, and CAR T exhaustion has proven to be a major obstacle in this approach.[6,7] Natural killer (NK) cell–based immunotherapies represent an alternative approach to this problem.[8] Most CLL patients exhibit low numbers of NK cells compared with healthy individuals, indicating that an NK cell immunotherapeutic approach would have to involve

Submitted 4 December 2018; accepted 10 February 2019. DOI 10.1182/bloodadvances.2018029371.

The full-text version of this article contains a data supplement.

methodologies to drive expansion of a patient's NK cell population or to add allogeneic NK cells, as well as methodologies to improve NK cell–specific targeting of the tumor.[9,10]

NK cells are innate immune effectors comprising 5% to 15% of blood lymphocytes that are characterized by expression of CD56 and absence of surface CD3 and B-cell receptors. In their ontogeny, NK cells acquire inhibitory (killer immunoglobulin-like receptors [KIRs] and NKG2A) and activating receptors, which regulate their function.[11] NK cells mediate tumor control by secreting inflammatory cytokines that bridge the innate and adaptive immune responses and trigger Fas- or Trail-mediated tumor cell death. NK cells can also directly lyse the tumor via recognition of activating stress ligands on the surface of the tumor that trigger natural cytotoxicity receptors on NK cells or via CD16-mediated recognition of antibody-coated tumors through a process called antibody-dependent cell-mediated cytotoxicity.[12,13] CD16, 1 of the most powerful NK-activating receptors, binds the Fc portion of monoclonal antibodies and mediates cytotoxicity by inducing the release of cytotoxic granules containing perforin and granzyme (degranulation) and by inducing production of proapoptotic cytokines like interferon γ (IFNγ) and tumor necrosis factor α.[14,15] NK cell function, survival, and proliferation are physiologically regulated and can be therapeutically enhanced by cytokines, particularly interleukin-2 (IL-2) and IL-15.[16] Because IL-2 can potently induce regulatory T-cell expansion, recent clinical approaches leveraging NK cell immunotherapy have focused on treatment with different modalities of IL-15.[17-20] NK cells in CLL are reported to be hypofunctional, with impaired direct cellular cytotoxicity and cytokine production, a defect that can be partially bypassed by cytokine signaling.[21] CLL cells express several pan–B-cell proteins, including CD19, CD22, and CD20, which can be therapeutically targeted with antibodies or cellular therapies such as CAR19 T cells.

Our group has previously designed and described novel trispecific killer engager (TriKE) molecules that induce specific NK cell–mediated killing of tumor targets while also providing a cytokine signal to drive NK cell expansion.[22-27] These molecules are composed of 2 single-chain variable fragments (scFvs), 1 engaging the CD16 activating receptor on NK cells and 1 engaging a tumor associated antigen, connected by small linkers and the cytokine IL-15. Here, we describe a novel TriKE targeting the CD19 tumor antigen (termed 161519). We present data on preclinical efficacy of 161519 TriKE in vitro including testing against primary CLL samples using autologous NK cells from patients with CLL and healthy donor NK cells. Our findings indicate that this molecule potently leverages the immunotherapeutic value of NK cells for CLL.

Methods

161519 TriKE construct

A hybrid gene encoding 161519 TriKE was synthesized using DNA shuffling and ligation techniques. The fully assembled gene (from 5′ end to 3′ end) consisted of *Nco*I restriction site, ATG start codon, anti-human CD16 scFv,[28] 20–amino acid segment (PSGQAGAAASESLFVSNHAY), N72D-mutated human IL-15,[29] 7–amino acid linker (EASGGPE), anti-CD19 scFv,[30] and *Xho*I restriction site. The resulting 1914–base pair *Nco*I/*Xho*I fragment gene was spliced into the pET28c expression vector under the control of an isopropyl-β-D-thiogalactopyranoside–inducible T7 promoter. DNA sequence has been validated to confirm sequence and location of gene insertion (Biomedical Genomics Center, University of Minnesota, Minneapolis, MN).

TriKE production and isolation

Plasmid was transfected into *Escherichia coli* strain BL21 (DE3). Culturing and harvest conditions have been previously described.[22] The pellet was extracted using a solution of 0.3% deoxycholate, 5% Triton X-100, 10% glycerin, 50 mmol/L of Tris and sodium chloride (NaCl), and 5 mmol/L of EDTA at pH 8.0 and then washed. Refolding procedure was done by first dissolving inclusion bodies in solubilization buffer at a 20:1 ratio and incubating at 37°C. Solubilization buffer consisted of 7 M of guanidine hydrochloride, 50 mM of Tris, 50 mM of NaCl, 5 mM of EDTA, and 50 mM of dithiothreitol at pH 8.0. After incubation, supernatant was harvested and diluted at a 20:1 ratio in refolding buffer (50 mM of Tris and hydrogen chloride [HCl], 50 mM of NaCl, 0.8 mM of L-arginine, 20% glycerin, 5 mM of EDTA, and 1 mM of glutathione disulfide at pH 8.0). This buffer was later removed with 10-fold dialysis using 20 mM of Tris and HCl at pH 9.0 in 20 mM of Tris and HCl at pH 9.0 at 4-column volume. Purification was carried out using a Fast Flow Q ion exchange column in a 3-step elution process as previously described.[22-25] Purity was determined running sodium dodecyl sulfate polyacrylamide gel electrophoresis using Simply Blue Life Stain (Invitrogen, Carlsbad, CA). The size of 161519 TriKE was 67.62 kDa.

Cell culture, isolation of NK cells, and patient samples

The cancer cell lines Raji (Burkitt's lymphoma) and K562 (chronic myelogenous leukemia) were obtained from the American Type Culture Collection. They were maintained in suspension using RPMI 1640 supplemented with 10% fetal bovine serum in 5% carbon dioxide at 37°C.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors obtained by Memorial Blood Bank (Minneapolis, MN) after written consent was provided. PBMCs were separated from blood using density gradient Ficoll-Paque (GE Healthcare), underwent red blood lysis, and then were either used directly or controlled-rate frozen and maintained in liquid nitrogen. NK cell enrichment was carried out by magnetic bead isolation using kits from STEMCELL Technologies (catalog #19055) as suggested by the company's protocol to enrich NK cell products from fresh buffy coat products.

Viably cryopreserved CLL patient blood samples were obtained through the Translational Therapy Shared Resource of Masonic Cancer Center, University of Minnesota. Use of PBMCs from patients was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota in accordance with the Declaration of Helsinki.

Real-time tumor cell killing assay

Raji cells were stably transduced using red fluorescent protein NucLight Red lentiviral reagent (Essen Bioscience). NucLight Red Raji target cells were plated into 96-well plates, with caspase 3/7 added at 5 μM per well. Enriched NK cells were added at a 2:1 effector/target ratio, along with the appropriate treatments (at 30 nM for 161519 and rituximab and 0.3 nM for equifunctional

concentration of IL-15) and cocultured for 44 hours in RPMI media supplemented with 10% fetal bovine serum. The normalized percentage of live Raji cells was calculated from the number of caspase 3/7$^-$ NucLight Red$^+$ target cells acquired using IncuCyte Zoom software, at noted time points (every 15 minutes), normalized against targets alone and against 0 hours in coculture groups. A normalized killing count graph was generated using GraphPad Prism software.

Phenotypic flow cytometry

CLL patient samples and PBMC products from healthy donors were phenotyped for NK cell characteristics with the Live/Dead Fixable Aqua Staining Kit (catalog #L-34966; Thermo Fisher Scientific), to gate on live cells, and the following fluorochrome-conjugated antibodies: phycoerythrin (PE)-Cy7–conjugated CD56 (clone HCD56; BioLegend); PE-CF594–conjugated CD3 (clone UCHT1; BD Biosciences); APC-Cy7–conjugated CD16 (clone 3G8; BioLegend); Pacific Blue–conjugated CD57 (clone HCD57; BioLegend); PE-conjugated KIRs CD158, CD158b, and CD158e1 (clones HP-MA4, DX27, and DX9, respectively; BioLegend); and APC-conjugated CD159a (NKG2a; clone Z199; Beckman Coulter). NK cells were identified as CD56$^+$/CD3$^-$/live cells and gated into CD56 brights and CD56 dims. Cells were run on LSRII (BD Biosciences) for 60 seconds per sample, analyzed via FlowJo software (Tree Star Inc.), and graphed on GraphPad Prism software.

Proliferation assay

Enriched NK cells were labeled using CellTrace Violet Cell Proliferation Kit (catalog #C34557; Thermo Fisher Scientific) according to the company's protocol. The cells were incubated for 7 days with noted treatments (0.5 nM for IL-15 and 50 nM for the rest); 50 nM was used instead of 30 nM because of the length of the assay to preserve stability in vitro. Cells were stained with CD56, CD3, and Live/Dead Near IR (catalog #L34976; Thermo Fisher Scientific). The CD56$^+$/CD3$^-$/live NK cells were then measured for the amount of CellTrace diluted.

CD107a degranulation and IFNγ cytokine production assay

PBMCs and CLL patient cells were plated overnight and then cocultured for 4 hours with target cells (Rajis, K562s, or CLL patient cells) at an effector/target ratio of 2:1 with the noted treatments (30 nM for everything but IL-15 [used at equifunctional 0.3 nM]). Alternatively, enriched NK cells were plated and incubated overnight with treatments (30 nM for everything but IL-15 [used at 0.3 nM]) and washed in the morning before incubation with target cells at a 2:1 ratio, followed by replenishment of treatments. FITC-conjugated anti-CD107a (clone H4A3; BioLegend), used to evaluate NK cell degranulation, was added at the start of the 4-hour incubation period. After a 1-hour incubation, Golgi Stop and Golgi Plug (BD Biosciences) were added for the last 3 hours. At the end of the 4 hours, the cells were stained using the Live/Dead Fixable Aqua Staining Kit (Thermo Fisher Scientific), surface stained with anti-CD56 and anti-CD3, fixed with 2% paraformaldehyde, and permeabilized with intracellular staining buffer (BioLegend). The cells were then stained for BV650-conjugated IFNγ (clone 4S.B3; BioLegend).

Flow-based killing assay

CLL patient cells were labeled using CellTrace Violet dye (Thermo Fisher Scientific). Enriched NK cells were incubated with noted treatments (30 nM for everything but IL-15 [used at 0.3 nM]) in a 96-well plate for 18 hours. After the incubation period, NK cells were washed and then cocultured with CellTrace-labeled target cells at a 2:1 effector/target ratio. Immediately after the addition of target cells, the wells were replenished with the noted treatments. After 4 hours of incubation, cells were harvested and stained with BV785-conjugated CD19 (clone HIB19; BioLegend), APC-conjugated CD5 (clone UCHT2; BioLegend), and Live/Dead Near IR. The killing percentage was obtained by calculating the percentage of CellTrace$^+$/CD19$^+$/CD5$^+$/dead cells in treatment groups vs effectors plus CLL targets alone (baseline/no treatment).

Statistical analysis

Statistical tests were conducted using GraphPad Prism (GraphPad Prism Software, Inc., La Jolla, CA).

Results

NK cells from CLL patients exhibit diminished maturation and cytokine production

To explore the level of NK cell dysfunction in CLL patients, we compared NK cells from 9 patients with CLL with NK cells from 9 healthy donors. The frequency of circulating NK cells was 2.3-fold lower in CLL patients compared with healthy participants (Figure 1A). Expression of maturation markers such as CD56 bright, CD56 dim, CD16, and NKG2A was similar between CLL patient NK cells and healthy donors NK cells (Figure 1B-E). In contrast, expression of receptors associated with later stages of NK cell maturation, such as KIR and CD57, was decreased in CLL patient cells compared with healthy donor cells (Figure 1F-G). NK cell cytotoxicity against K562 targets measured by NK cell degranulation was similar in CLL patient NK cells and healthy donor NK cells (Figure 1H); however, unlike healthy donor NK cells, those from CLL patients showed no increase in IFNγ production upon encountering K562 targets (Figure 1I). This defect was accompanied by a higher baseline IFNγ production on NK cells from CLL patients. In summary, these data indicate that NK cells from CLL patients are less abundant and exhibit developmental and functional defects, including impaired cytokine production; however, the normal expression of CD16 suggests preserved capacity to degranulate in response to CD16 ligation.

161519 TriKE efficiently drives NK cell activation against a B-cell lymphoma cell line

To target CLL, we generated a novel TriKE, termed 161519 (Figure 2A), containing an scFv against CD16, IL-15 cytokine, and an scFv against CD19 as described.[25,31] Before functionally testing the TriKE, we evaluated the activity of the IL-15 moiety in 161519 TriKE using a 48-hour bioassay and discovered that IL-15 activity in 161519 TriKE was 100-fold lower than in monomeric IL-15 (supplemental Figure 1A). Therefore, all assays used equifunctional IL-15. When healthy donor PBMCs were incubated for 4 hours with 161519 TriKE in the absence of CD19$^+$ targets, low levels of NK cell CD107a degranulation and little to no intracellular IFNγ were induced (Figure 2B-C). The low

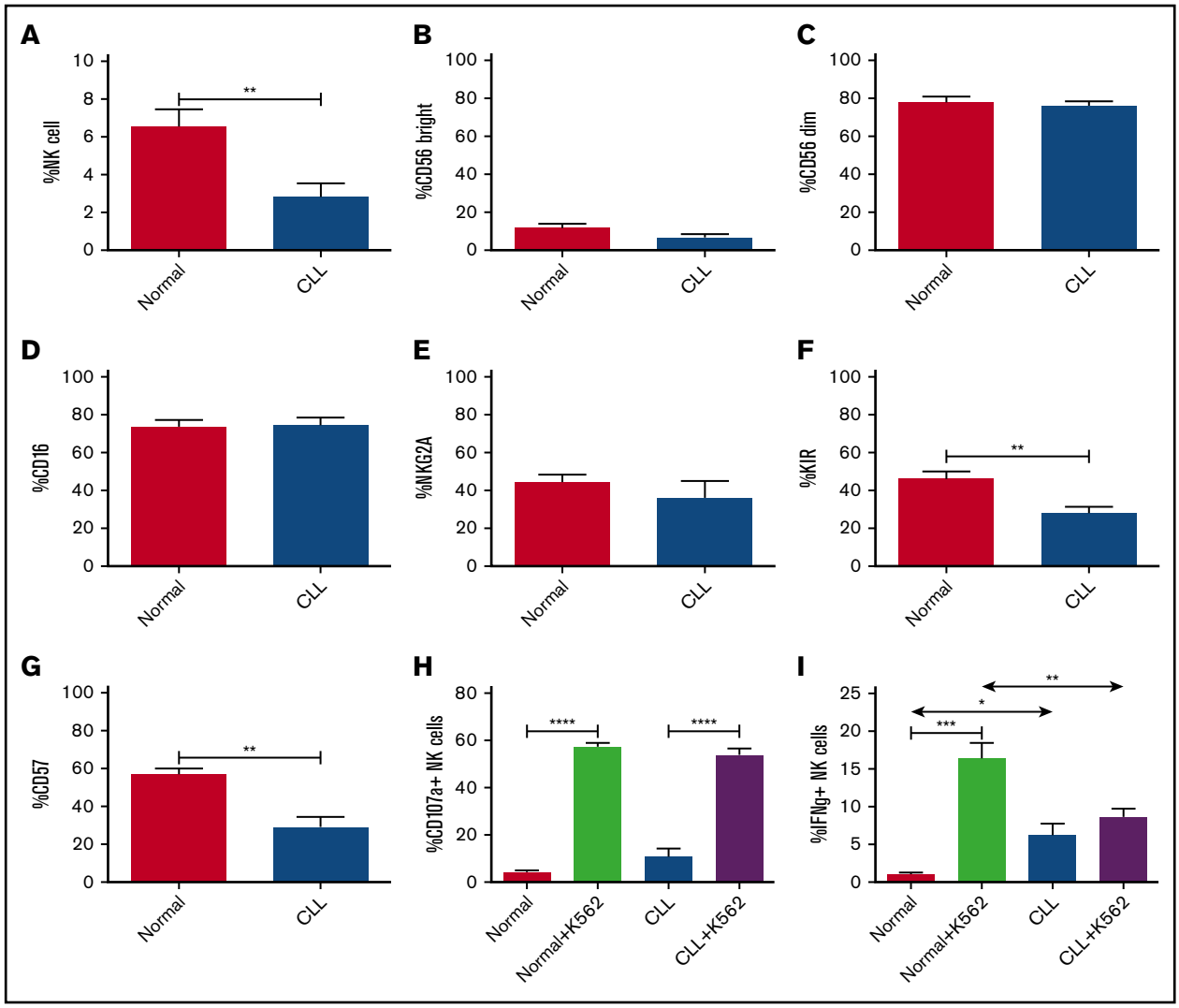

Figure 1. Phenotypic and functional characteristics of CLL patient vs healthy donor NK cells. Frozen PBMCs obtained from CLL patients (n = 9) and healthy donors (n = 9) were thawed and rested overnight and then used for flow cytometric analysis. (A) Pooled data showing proportion of $CD56^{+}/CD3^{-}$ NK cells within the lymphocyte gate in CLL patients vs healthy donors. While gating on NK cells, further subgating was carried out to evaluate the proportions of $CD56^{bright}$ (B) vs $CD56^{dim}$ (C) NK cells, as well as the proportions of NK cells expressing CD16 (D), NKG2A (E), KIR (F), and CD57 (G). NK cell degranulation via CD107a expression (H) and IFNγ production (I) were evaluated in CLL patient samples and healthy donors upon triggering natural cytotoxicity by incubation with K562 targets (n = 9 for CLL and for healthy donors, respectively). (A-G,I) Unpaired Student *t* test used for comparison between CLL samples and normal donor samples. (H-I) Paired Student *t* test used for internal group comparisons. Error bars indicate the mean ± standard error of the mean. Statistical significance are determined as $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

basal induction of degranulation was similar to that seen by incubation with equimolar amounts of the anti-CD20 monoclonal antibody rituximab or anti-CD16/CD19 bispecific killer engager (BiKE),[31] lacking the IL-15 moiety. To model the effect of these different treatments on $CD19/CD20^{+}$-expressing B-cell malignancies, we used the Raji (Burkitt's lymphoma) cell line. Incubation of PBMCs for 4 hours with Raji cells in the presence of 161519 TriKE, rituximab, or 1619 BiKE induced significantly higher NK cell degranulation compared with no drug or equifunctional amounts of monomeric IL-15 (Figure 2D). NK degranulation in the presence of Raji targets was similar among 161519 TriKE, 1619 BiKE, and rituximab; however, IFNγ production was significantly higher with TriKE treatment (Figure 2E). Addition of equifunctional amounts of IL-15 to rituximab or 1619 treatments mitigated the differential in IFNγ production (supplemental Figure 1B).

To evaluate the priming effect of the IL-15 moiety in 161519 TriKE on NK cell activation, NK cells were enriched with magnetic beads and incubated overnight alone or with IL-15, 1619 BiKE, rituximab, or 161519 TriKE. The next day, fresh reagents were added, and NK cells were cocultured for 4 hours with Raji targets. 161519 TriKE induced a significant increase in NK cell degranulation, measured by a higher proportion of CD107a-expressing cells with 161519 TriKE (84% ± 2.2%) compared with rituximab (60% ± 5.7%), 1619 BiKE (49% ± 5.7%), or IL-15 alone and at least a 3.5-fold increase in IFNγ-producing NK cells when compared with rituximab, 1619 BiKE, or controls (Figure 2F-G). As with the 4-hour incubation, addition of

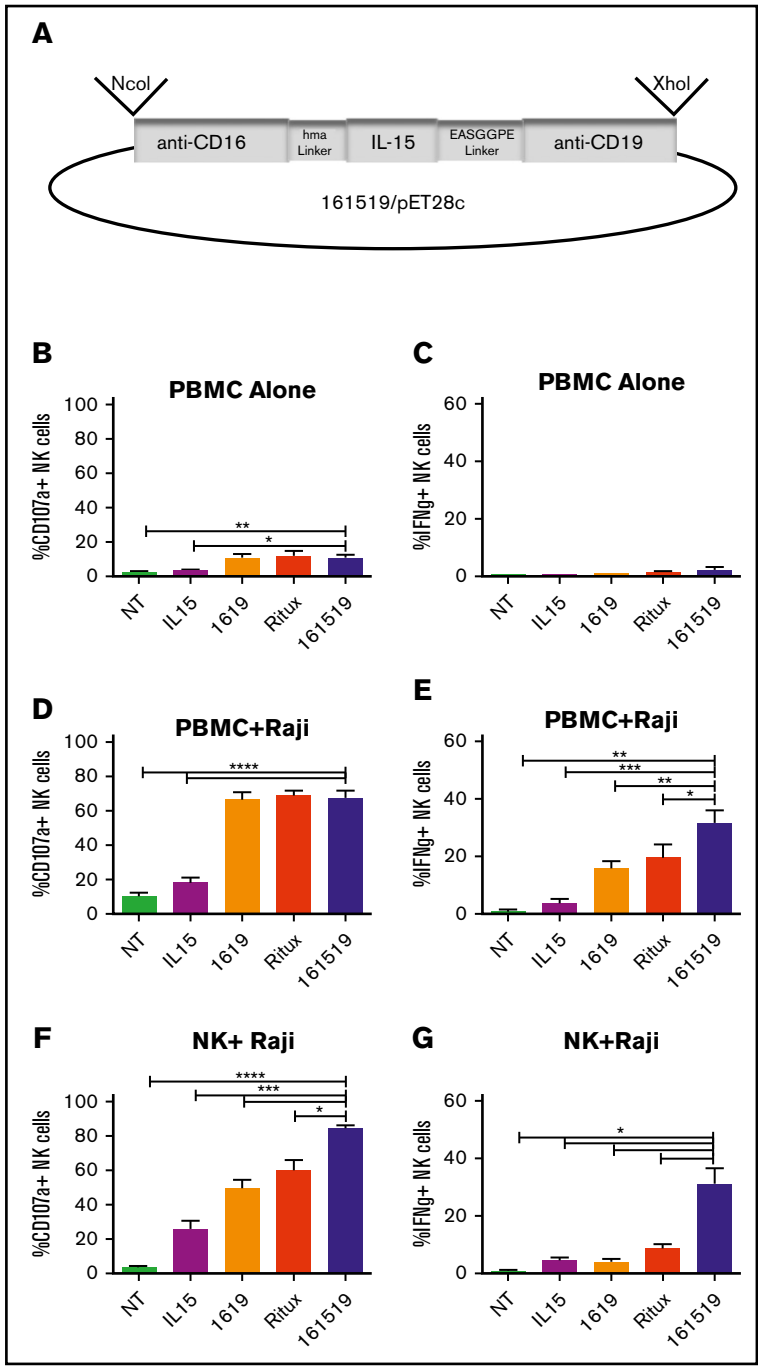

Figure 2. Functional validation of 161519 TriKE. (A) Simplified schema of 161519 TriKE construct, cloned into the pET28c vector via *Nco*I and *Xho*I restriction sites. The construct consists of 3 arms: an anti-CD16 scFv arm, IL-15 arm, and anti-CD19 scFv arm joined by 2 linkers (HMA and EASGGPE). Frozen PBMCs from healthy donors (n = 6) were incubated with noted treatments (30 nM for everything but IL-15 [used at 0.3 nM]) to evaluate background CD107a expression (degranulation) (B) or intracellular IFNγ production (C) on $CD56^{+}/CD3^{-}$ NK cells in a 4-hour assay. CD107a expression (D) and intracellular IFNγ (E) were also evaluated under the same conditions as stated before on NK cells within the PBMCs but in the presence of Raji targets at a 2:1 effector/target ratio. To evaluate the priming effects of the molecules on NK cells before target encounter, noted treatments (30 nM for everything but IL-15 [used at 0.3 nM]) were incubated overnight with enriched NK cells, and then CD107a (F) or IFNγ (G) was assessed after a 4-hour coculture with Raji targets on the next day (n = 5). One-way analysis of variance (ANOVA) with repeated measures was used to calculate differences against the 161519 group. Error bars indicate the mean ± standard error of the mean. Statistical significance are determined as $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$. NT, no treatment; Ritux, rituximab.

equifunctional amounts of IL-15 to rituximab or 1619 treatments overnight mitigated the differential in IFNγ production (supplemental Figure 1C). These data indicate that 161519 TriKE can induce substantial healthy donor NK cell activation against CD19-expressing tumor targets.

161519 TriKE induces NK cell proliferation

TriKE molecules containing the IL-15 moiety are endowed with the critical ability to drive NK cell expansion and maintenance. To examine the capacity of 161519 TriKE to induce NK cell proliferation, enriched CellTrace-labeled NK cells were exposed to monomeric IL-15 (equifunctional), 1619 BiKE, rituximab, or 161519 TriKE for a week, and CellTrace dilution was evaluated by flow cytometry. 161519 TriKE induced robust proliferation, which was similar to equifunctional monomeric IL-15, indicating the functional capacity of the IL-15 moiety within TriKE (Figure 3A-B). In contrast, 1619 BiKE and rituximab did not induce proliferation. Because IL-15 also sustains NK cell survival, NK cell viability was evaluated after a week of treatment. 161519 TriKE treatment resulted in better survival than treatment with 1619 BiKE or rituximab or no treatment and similar viability to that induced by equifunctional monomeric IL-15 (Figure 3C-D).

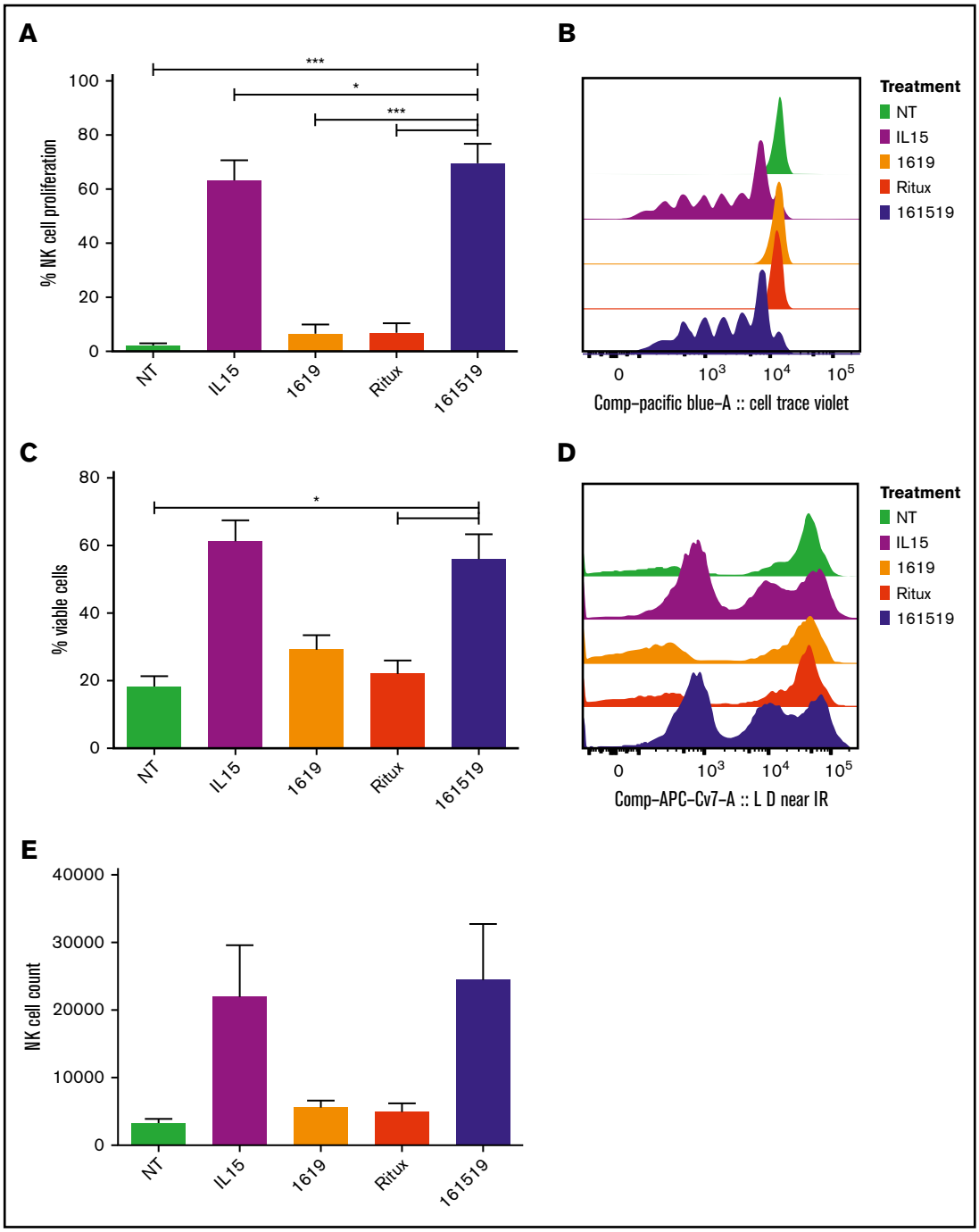

Figure 3. Unlike rituximab, 161519 TriKE induces potent NK cell proliferation and survival. NK cells were enriched from fresh healthy donor samples (n = 6), CellTrace Violet labeled, and incubated for 7 days with 161519 TriKE or control treatments at 50 nM (for everything but IL-15 [used at 0.5 nM]) concentration. After the incubation period, wells were harvested, and NK cells were evaluated by flow cytometry. Pooled data (A) and representative histograms (B) showing NK cell proliferation (by CellTrace dilution) on the different treatment groups. Pooled NK cell viability (C) and representative histograms (D) showing cell death (by incorporation of Live/Dead Near IR dye) on the different treatment groups. (E) Pooled NK cell count (60 seconds at constant speed) at the time of harvest. One-way ANOVA with repeated measures was used to calculate differences against the 161519 group. Error bars indicate the mean ± standard error of the mean. Statistical significance are determined as $*P < .05$ and $***P < .001$.

The 161519 TriKE and IL-15 groups had greater than fourfold higher numbers than the no treatment, 1619 BiKE, and rituximab groups after a week of treatment (Figure 3E). To evaluate the specificity of 161519 TriKE, PBMCs were CellTrace labeled to determine proliferation of NK cells and T cells treated with 161519 or IL-15 with or without 1619 or rituximab (supplemental Figure 1D). The data clearly indicate that all treatments induce NK cell proliferation, but 161519 treatment induces reduced proliferation of T cells compared with uncoupled IL-15.

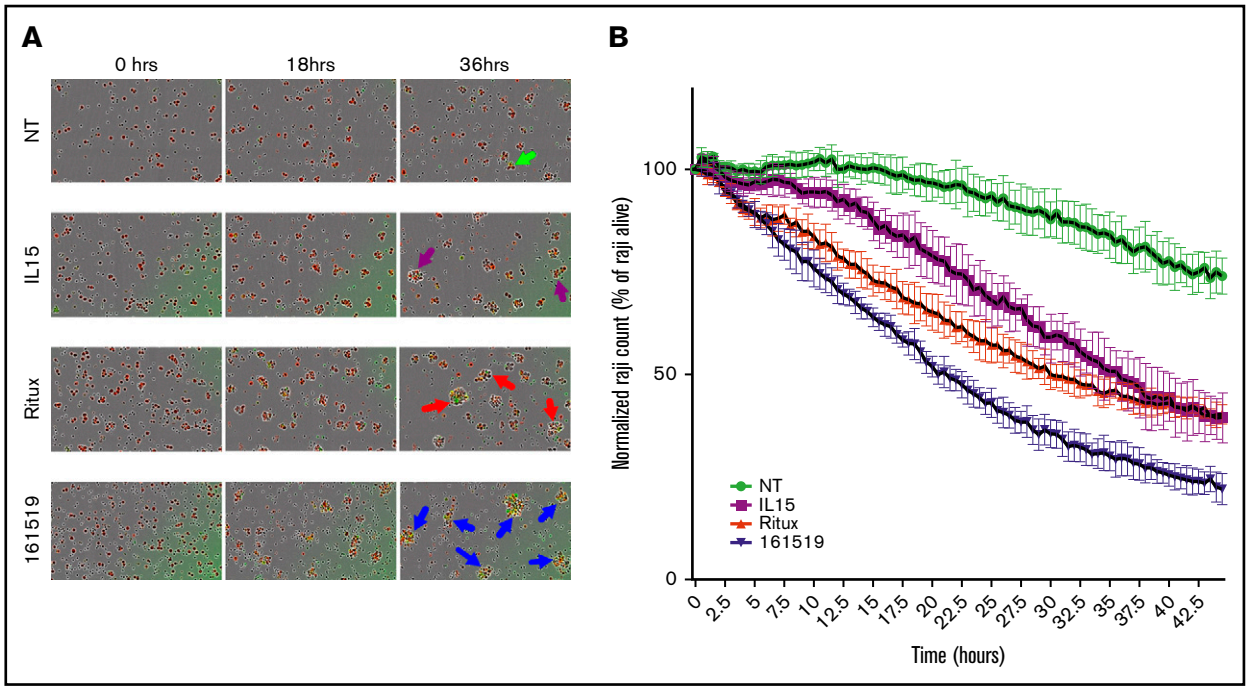

Figure 4. 161519 TriKE induces more tumor killing than rituximab in real-time 2-day imaging assay. Enriched NK cells were incubated with NucLight Red–transduced Raji cells at a 2:1 effector-to-target ratio with the noted treatments (30 nM for everything but IL-15 [used at 0.3 nM]) for 44 hours within an IncuCyte Zoom imager. Dead Raji cells were measured as caspase 3/7^{+} (green)/NucLight Red^{+} cells. (A) Representative images (original magnification ×4: 2.82 μm/pixel) at 0, 18, and 36 hours showing Raji cells (larger red cells) and NK cells (smaller black cells). Arrows point to killing clusters where dying Raji cells (yellow) are apparent. (B) Quantification of the percentage of live Raji tumor targets (Nuclight Red^{+}/caspase 3/7^{-}) normalized to targets alone and the 0-hour time point. Readings were taken every 15 minutes over a 44-hour period. Representative of 4 separate experiments.

161519 TriKE exhibits better tumor killing than rituximab in a longitudinal killing assay

Although previous assays showed the effect of 161519 TriKE on NK cell degranulation (Figure 2), we employed a longitudinal imaging killing assay to evaluate direct tumor cell killing. Compared with rituximab and other groups, 161519 TriKE induced more killing clusters, composed of NK cells, live Raji cells, and dying Raji cells at later time points (Figure 4A). The compiled data indicate that both 161519 TriKE and rituximab treatments induced similar tumor cell killing early on (6-hour time point), but 161519 TriKE induced the highest level of Raji killing after 12 hours compared with rituximab, suggesting augmented function that can be attributed to the IL-15 moiety (Figure 4B). Although it could be argued that the differences in function against the targets are mediated by increased expansion driven by TriKE, it is important to note that NK cells have a doubling time of >24 hours,[32] indicating that differences in tumor killing driven by antibody-dependent cell-mediated cytotoxicity within the first 24 hours are likely driven by differences in IL-15 priming between 161519 TriKE compared with rituximab.

161519 TriKE potentiates in vitro autologous CLL NK cell function and allogeneic NK cell function against CLL tumors

To evaluate the effect of 161519 TriKE on NK cells from CLL patients, CLL patient PBMCs were cultured with Raji targets for 4 hours, and NK function with different treatments was evaluated by flow cytometry. Although all treatments exhibited increased NK cell degranulation when compared with controls (Figure 5A), 161519 TriKE was the only treatment that significantly elevated IFNγ production of NK cells from CLL patients against Raji targets compared with lower IFNγ levels with 1619 BiKE or rituximab treatments (Figure 5B). Thus, the 161519 TriKE treatment reverted some of the cytokine response defects noted in CLL patient NK cells (Figure 1I). To evaluate if 161519 TriKE could be applied with an allogeneic infusion or in a transplantation setting, healthy donor NK cells were enriched, incubated with noted treatments overnight, and then washed and placed in culture with CellTrace-labeled CLL targets in the presence of noted treatments for 4 hours, and NK cell function was evaluated on the CellTrace^{-} healthy donor NK cells. In this allogeneic setting, 161519 TriKE induced better degranulation and IFNγ production than rituximab, 1619 BiKE, equifunctional IL-15, or no treatment (Figure 5C-D). These data indicate that 161519 TriKE displays a functional advantage in the autologous and allogeneic settings.

161519 TriKE amplifies NK cell–mediated killing of CLL patient tumor cells

It is important to measure primary CLL killing; therefore, a novel in vitro assay was developed to address the question. Because CLL patient blood displays a heterogeneous B-cell population, comprising both CLL cells and normal B cells, the IncuCyte Zoom killing assay could not be used in this setting. To that end, we designed a flow cytometry–based assay where CLL cells were CellTrace labeled and coincubated for 4 hours with indicated treatments and

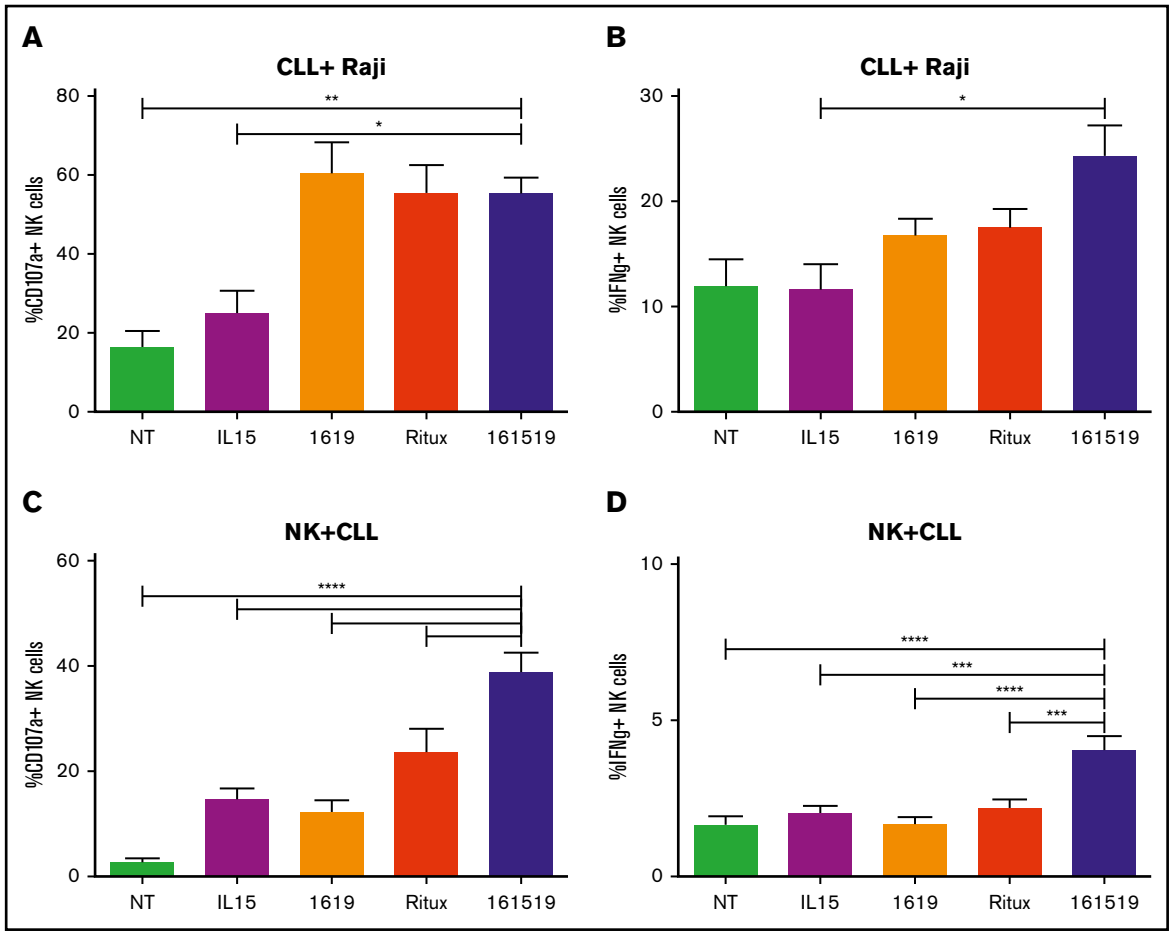

Figure 5. 161519 TriKE amplifies NK cell function in autologous and allogeneic in vitro settings. PBMCs obtained from CLL patients (n = 5) were cultured with Raji target cell line (2:1 effector-to-target ratio) for a 4-hour period in the presence of treatment conditions at 30 nM (for everything but IL-15 [used at 0.3 nM]). NK cells were determined by $CD56^{+}/CD3^{-}$ expression and degranulation, and IFNγ production was assessed. (A) Pooled data showing CD107a expression on NK cells from CLL patient samples incubated in the presence of Raji targets. (B) Pooled data showing intracellular IFNγ expression on NK cells from CLL patient samples incubated in the presence of Raji targets. To evaluate 161519 TriKE in an allogeneic setting, enriched allogeneic NK cells from healthy donors were incubated with noted molecules (30 nM for everything but IL-15 [used at 0.3 nM]) for 18 hours, washed, and then placed in culture alone or cocultured with CellTrace-labeled CLL patient cells (2:1 effector-to-target ratio) for a 4-hour period with outlined treatment conditions added again at a 30 nM (for everything but IL-15 [used at 0.3 nM]) concentration (n = 17; 2 experiments with 2-3 NK donors against 3-4 CLL targets). CellTrace dye was used to be able to distinguish healthy donor–enriched NK cells from CLL patient NK cells. (C) CD107a expression on $CellTrace^{-}$ NK cells cultured with CLL patient cells. (D) Intracellular IFNγ production on $CellTrace^{-}$ NK cells cultured with CLL patient cells. One-way ANOVA with repeated measures was used to calculate differences against the 161519 group. Error bars indicate the mean ± standard error of the mean. Statistical significance are determined as $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

healthy donor allogeneic NK cells, which had been preprimed overnight with said treatments. The 4-hour time point was selected because it is commonly used for NK cell chromium release killing assays, and it matches the degranulation and inflammatory cytokine production experiments using CLL targets (Figures 5C-D). Cells were then harvested, and CLL tumor target killing with each treatment was evaluated by gating on $CellTrace^{+}/CD19^{+}/CD5^{+}$ cells, evaluating the proportion of remaining live cells in comparison with baseline killing of NK cells with tumor targets alone (Figure 6). Our data indicate that in this NK cell–driven allogeneic assay, 161519 TriKE was superior to rituximab-mediated killing, inducing greater than sixfold more powerful killing. Taken together, these findings show that 161519 TriKE can improve on rituximab-based NK cell–mediated tumor killing approaches by costimulation through the IL-15 moiety.

Discussion

Novel immunotherapies in CLL will need to overcome the exhausted immune system and be target specific to be highly effective. Here, we report the first preclinical study using a novel CD19-targeted TriKE reagent that restores function of NK cells in CLL. We demonstrated the capacity of 161519 TriKE to revert inflammatory dysfunction in CLL and harness NK immune effector–mediated CLL killing.

Our group has previously reported on TriKE molecules targeting a number of tumor antigens, including a TriKE targeting CD33 (161533), which will be shortly tested in a phase 1 clinic trial (NCT03214666).[33] Using primary CLL targets, our data confirm effective elimination of CD19-expressing targets and reversal of CLL patient NK cell IFNγ dysfunction, important in inducing

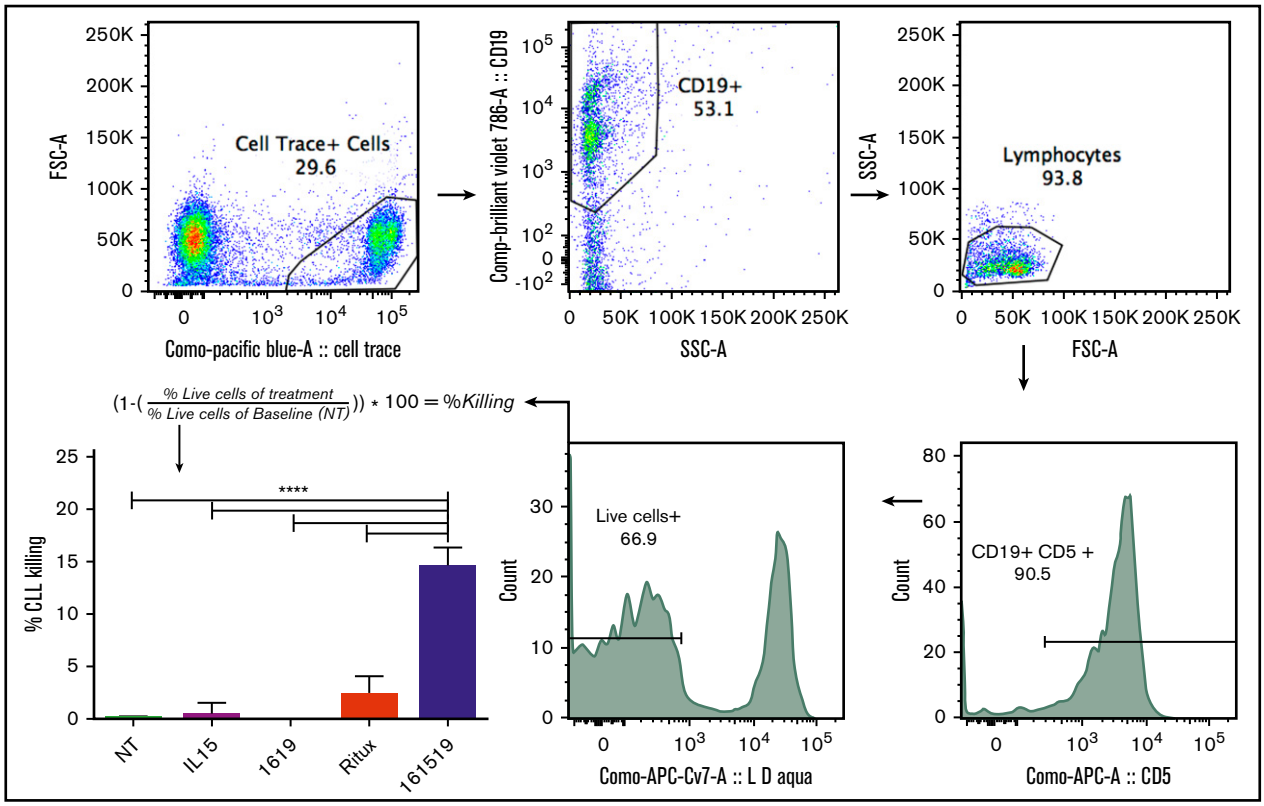

Figure 6. 161519 TriKE induces stronger NK cell–mediated killing of CLL patient tumor cells. Enriched allogeneic NK cells from healthy donors were incubated with noted molecules (30 nM for everything but IL-15 [used at 0.3 nM]) for 18 hours, washed, and then cocultured with CellTrace-labeled CLL targets in the presence of fresh noted treatments for a 4-hour period (n = 17; 2 experiments with 2-3 NK donors against 3-4 CLL targets). After the incubation period, CLL tumor cells were identified as CellTrace$^+$/CD19$^+$/CD5$^+$ cells. Flow chart outlines the gating schema used to measure live target CLL cell percentage. Killing percentage was then calculated from live target cell percentage. NT denotes NK cells incubated with CLL targets without any molecules added and is used as a baseline of killing. One-way ANOVA with repeated measures was used to calculate differences against the 161519 group. Error bars indicate the mean ± standard error of the mean. Statistical significance are determined as ****$P < .0001$.

adaptive immune responses, with a novel 161519 TriKE. Although target specificity is important for NK therapeutics, the absolute NK cell numbers may be critical for clinical efficacy. We demonstrate that the IL-15 moiety of 161519 TriKE delivers a powerful proliferative signal to NK cells, driving NK cell expansion. Our experimental testing provides the basis to explore efficacy of 161519 TriKE in clinical trials.

The data presented here support a prior report of NK cell hyporesponsiveness and defective NK cell maturation observed in patients with CLL, including comparisons between monozygotic twins.[9] Both studies showed NK cell defects in CLL patients. Of importance, we evaluated CD16 expression and found no difference between healthy donor NK cells and CLL patient NK cells. This is important, because 161519 TriKE activity is dependent on ligation of the CD16 receptor. Unlike the previous study, we noted an important defect in inflammatory cytokine secretion on NK cells from CLL patients, which was abrogated by 161519 TriKE, likely through costimulation induced by the IL-15 moiety.[23,34] The IL-15 moiety in 161519 TriKE may also overcome the survival defect noted in CLL patient NK cells described in the previous study.[9] Overall our experiments suggest that the 161519 TriKE reagent can restore defects present in NK cells from CLL patients.

In terms of clinical application, the reagent we developed differs significantly from rituximab not only in type of target (CD19 vs CD20) but also in mechanism of action. The most significant difference is the IL-15 moiety within TriKE, which enhances specific NK cell expansion and activation. Given the quantitative and qualitative defects in NK cells from CLL patients, this quality may be critical for clinical efficacy. Augmented IL-15 function in the context of the anti-CD16 and anti-CD19 scFv arms within the 161519 TriKE molecule is notable in comparison with exogenous IL-15 alone, which does not mediate the same effect.[17-20] As our data show, simple priming with IL-15 alone does not induce nearly as much tumor killing (Raji or primary CLL). Combination approaches using an IL-15 superagonist (ALT-803) and rituximab were recently presented at the American Association of Cancer Research annual meeting, showing exciting responses against relapsed or refractory indolent non-Hodgkin's lymphoma (NCT02384954). However, tumor-specific localization of IL-15 may be relevant in clinical applications, and TriKE will likely limit the systemic immune off-target toxicities of IL-15 in patients by homing it specifically to NK cells via the anti-CD16 arm.[20,25]

CLL patients with refractory disease have limited therapeutic options. AlloHCT and CAR19 T-cell approaches have both shown promise in CLL and conceptually demonstrated curative effects of

cellular therapy; however, both are highly complex individualized procedures that can be associated with substantial morbidity and mortality.[35] In addition, their application is restricted to patients without major comorbidities and is limited by autologous CAR T-cell exhaustion and toxicities.[7,36] By triggering an endogenous NK cell response, which displays a less toxic profile than T-cell responses, 161519 TriKE offers a potentially valuable off-the-shelf alternative to CAR T-cell therapies that could be used alone or in combination with adoptive NK cell therapy. Therefore, we propose that 161519 TriKE should be further evaluated as a treatment of refractory CLL patients in settings where there are no satisfactory therapeutic alternatives.

Acknowledgments

The authors acknowledge the Flow Cytometry Core and the Translational Therapy Laboratory at the University of Minnesota for excellent service and technical support.

This work was supported by the US Department of Defense (CA150085) (M.F.), the National Cancer Institute, National Institutes of Health (P01 CA111412 and P01 CA65493) (M.F. and J.S.M.), and the University of Minnesota Foundation Nelson/Bachanova CLL Fund.

GT Biopharma has licensed the TriKE platform from the University of Minnesota, but no funds from GT Biopharma were used for any aspect of this study.

Authorship

Contribution: Scientific concepts and design of research studies were conceived by M.F., D.A.V., J.S.M., and V.B.; experiments and data acquisition were conducted by B.K., P.H., and M.F.K.; and the manuscript was written and edited by M.F., B.K., S.C., D.J.W., D.A.V., J.S.M., and V.B.

Conflict-of-interest disclosure: M.F., J.S.M., and D.A.V. receive consulting honoraria from GT Biopharma. The remaining authors declare no competing financial interests.

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References

1. Li Y, Wang Y, Wang Z, Yi D, Ma S. Racial differences in three major NHL subtypes: descriptive epidemiology. *Cancer Epidemiol*. 2015;39(1):8-13.
2. Ferrer G, Montserrat E. Critical molecular pathways in CLL therapy. *Mol Med*. 2018;24(1):9.
3. Brown JR, Kim HT, Armand P, et al. Long-term follow-up of reduced-intensity allogeneic stem cell transplantation for chronic lymphocytic leukemia: prognostic model to predict outcome. *Leukemia*. 2013;27(2):362-369.
4. Ben-Bassat I, Raanani P, Gale RP. Graft-versus-leukemia in chronic lymphocytic leukemia. *Bone Marrow Transplant*. 2007;39(8):441-446.
5. Dreger P, Schetelig J, Andersen N, et al; European Research Initiative on CLL (ERIC) and the European Society for Blood and Marrow Transplantation (EBMT). Managing high-risk CLL during transition to a new treatment era: stem cell transplantation or novel agents? *Blood*. 2014;124(26):3841-3849.
6. Porter DL, Hwang WT, Frey NV, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med*. 2015;7(303):303ra139.
7. Kasakovski D, Xu L, Li Y. T cell senescence and CAR-T cell exhaustion in hematological malignancies. *J Hematol Oncol*. 2018;11(1):91.
8. Bachanova V, Cooley S, Defor TE, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood*. 2014;123(25):3855-3863.
9. MacFarlane AW 4th, Jillab M, Smith MR, et al. NK cell dysfunction in chronic lymphocytic leukemia is associated with loss of the mature cells expressing inhibitory killer cell Ig-like receptors. *OncoImmunology*. 2017;6(7):e1330235.
10. Bachanova V, Burns LJ, McKenna DH, et al. Allogeneic natural killer cells for refractory lymphoma. *Cancer Immunol Immunother*. 2010;59(11):1739-1744.
11. Foley B, Felices M, Cichocki F, Cooley S, Verneris MR, Miller JS. The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). *Immunol Rev*. 2014;258(1):45-63.
12. Davis ZB, Felices M, Verneris MR, Miller JS. Natural killer cell adoptive transfer therapy: exploiting the first line of defense against cancer. *Cancer J*. 2015;21(6):486-491.
13. Murphy WJ, Parham P, Miller JS. NK cells--from bench to clinic. *Biol Blood Marrow Transplant*. 2012;18(1 suppl):S2-S7.
14. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med*. 2005;202(7):1001-1012.
15. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*. 2006;107(1):159-166.
16. Meazza R, Azzarone B, Orengo AM, Ferrini S. Role of common-gamma chain cytokines in NK cell development and function: perspectives for immunotherapy. *J Biomed Biotechnol*. 2011;2011:861920.
17. Conlon KC, Lugli E, Welles HC, et al. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol*. 2015;33(1):74-82.
18. Dubois S, Conlon KC, Müller JR, et al. IL15 infusion of cancer patients expands the subpopulation of cytotoxic CD56^{bright} NK cells and increases NK-cell cytokine release capabilities. *Cancer Immunol Res*. 2017;5(10):929-938.

19. Wrangle JM, Velcheti V, Patel MR, et al. ALT-803, an IL-15 superagonist, in combination with nivolumab in patients with metastatic non-small cell lung cancer: a non-randomised, open-label, phase 1b trial. *Lancet Oncol*. 2018;19(5):694-704.

20. Romee R, Cooley S, Berrien-Elliott MM, et al. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood*. 2018;131(23):2515-2527.

21. Burger JA, Gribben JG. The microenvironment in chronic lymphocytic leukemia (CLL) and other B cell malignancies: insight into disease biology and new targeted therapies. *Semin Cancer Biol*. 2014;24:71-81.

22. Schmohl JU, Felices M, Oh F, et al. Engineering of anti-CD133 trispecific molecule capable of inducing NK expansion and driving antibody-dependent cell-mediated cytotoxicity. *Cancer Res Treat*. 2017;49(4):1140-1152.

23. Schmohl JU, Felices M, Taras E, Miller JS, Vallera DA. Enhanced ADCC and NK cell activation of an anticarcinoma bispecific antibody by genetic insertion of a modified IL-15 cross-linker. *Mol Ther*. 2016;24(7):1312-1322.

24. Schmohl JU, Felices M, Todhunter D, Taras E, Miller JS, Vallera DA. Tetraspecific scFv construct provides NK cell mediated ADCC and self-sustaining stimuli via insertion of IL-15 as a cross-linker. *Oncotarget*. 2016;7(45):73830-73844.

25. Vallera DA, Felices M, McElmurry R, et al. IL15 trispecific killer engagers (TriKE) make natural killer cells specific to CD33+ targets while also inducing persistence, in vivo expansion, and enhanced function. *Clin Cancer Res*. 2016;22(14):3440-3450.

26. Don Yun H, Felices M, Vallera DA, et al. Trispecific killer engager CD16xIL15xCD33 potently induces NK cell activation and cytotoxicity against neoplastic mast cells. *Blood Adv*. 2018;2(13):1580-1584.

27. Sarhan D, Brandt L, Felices M, et al. 161533 TriKE stimulates NK-cell function to overcome myeloid-derived suppressor cells in MDS. *Blood Adv*. 2018; 2(12):1459-1469.

28. McCall AM, Adams GP, Amoroso AR, et al. Isolation and characterization of an anti-CD16 single-chain Fv fragment and construction of an anti-HER2/neu/anti-CD16 bispecific scFv that triggers CD16-dependent tumor cytolysis. *Mol Immunol*. 1999;36(7):433-445.

29. Zhu X, Marcus WD, Xu W, et al. Novel human interleukin-15 agonists. *J Immunol*. 2009;183(6):3598-3607.

30. Kipriyanov SM, Kupriyanova OA, Little M, Moldenhauer G. Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. *J Immunol Methods*. 1996;196(1):51-62.

31. Gleason MK, Verneris MR, Todhunter DA, et al. Bispecific and trispecific killer cell engagers directly activate human NK cells through CD16 signaling and induce cytotoxicity and cytokine production. *Mol Cancer Ther*. 2012;11(12):2674-2684.

32. Liu Y, Wu HW, Sheard MA, et al. Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. *Clin Cancer Res*. 2013;19(8):2132-2143.

33. 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;31:64-75.

34. Rautela J, Huntington ND. IL-15 signaling in NK cell cancer immunotherapy. *Curr Opin Immunol*. 2017;44:1-6.

35. Schwarzbich MA, Witzens-Harig M. Cellular immunotherapy in B-cell malignancy. *Oncol Res Treat*. 2017;40(11):674-681.

36. Mahmoudjafari Z, Hawks KG, Hsieh AA, Plesca D, Gatwood KS, Culos KA. American Society for Blood and Marrow Transplantation Pharmacy Special Interest Group survey on chimeric antigen receptor T cell therapy administrative, logistic, and toxicity management practices in the United States. *Biol Blood Marrow Transplant*. 2019;25(1):26-33.

Appendix B: Manuscripts in Review

A Novel Humanized Single Domain Camelid Sequence Engaging CD16 on NK Cells Enables Potent and Specific IL-15 Activity in a Second Generation Trispecific Killer Engager

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Running Title: Second generation TriKE potentiates NK cell activity

Key Words: Natural Killer, TriKE, Tri-specific Antibodies, IL-15, Immunotherapy

Financial Support: This work was supported in part by DoD CA150085 (MF), NIH U01HL127479 (DAV), NCI P01 CA111412 (MF and JSM), P01 CA65493 (MF and JSM), R35 CA197292 (MF and JSM), and the Randy Shaver Cancer Research and Community Foundation (DAV). The TriKE™ work was also supported in part by funding provided by GT Biopharma, Inc (MF, DAV and JSM).

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Conflict of Interest Statement: MF, JSM and DAV receive consulting honorary from GT Biopharma, which has licensed the TriKE platform from the University of Minnesota.

Word Count: 4,937
Abstract: 202
Figures: 6

Abstract

Natural Killer (NK) cells are potent immune modulators that can quickly lyse tumor cells and elicit potent inflammatory responses. These characteristics make them ideal candidates for immunotherapy. However, unlike T cells NK cells do not possess clonotypic receptors capable of specific antigen recognition and cannot expand via activating receptor signals alone. To imbue NK cells with these capabilities we created and have previously described a Tri-specific Killer Engager (TriKE^{TM}) platform capable of inducing antigen specificity and cytokine mediated NK cell expansion. TriKE molecules have three arms: a single chain variable fragment (scFv) against the activating receptor CD16 on NK cells, to trigger NK cell activation, an scFv against a tumor associated antigen (CD33 in this study), to induce specific tumor target recognition, and an IL-15 moiety, to trigger NK cell expansion and priming. Here we demonstrate that by swapping the anti-CD16 scFv with a novel humanized single domain antibody against CD16 we greatly improve the functionality the TriKE molecules. A CD33-targeting second-generation TriKE induces stronger and more specific proliferation, enhanced in vitro NK cell activation and killing of CD33 expressing targets, and vastly improved tumor control in pre-clinical mouse models. Given these improved functional characteristics we propose quick translation of second-generation TriKEs.

Introduction

Natural Killer (NK) cell-based immunotherapies are quickly gaining traction pre-clinically and in early phase clinical trials (1). This is due to the ability of NK cells to lyse tumor with minimal priming, their lack of MHC restriction, their capacity to exert antibody-dependent cellular cytotoxicity (ADCC) and to robustly and rapidly produce inflammatory cytokines (2). A number of NK cell immunotherapy modalities are being actively explored, including cellular, cytokine, checkpoint inhibition, and ADCC-driven therapies (3). Developing affordable NK cell-based immunotherapeutic off-the-shelf products will significantly strengthen clinical practice.

Both cytokine- and ADCC-mediated immunotherapies are ideal candidates for off-the-shelf approaches. NK cells express cytokine receptors that modulate NK cell effector function, development, proliferation and homeostasis. IL-2 and IL-15 activate a host of downstream signaling molecules that result in enhanced function, proliferation and survival (4, 5). Because of reduced toxicities and its NK cell stimulating effects without the augmentation of Tregs, IL-15 has become the cytokine of interest in NK immunotherapy (6, 7). Recombinant human IL-15 and an IL-15 superagonist complex (N-803, formerly ALT-803) are under investigation in several tumor settings alone and in combination with adoptive transfer in patients with leukemia (8-11). Early clinical data indicate that IL-15 can induce NK cell expansion necessary to enhance immunotherapy (12, 13). IL-15 also has the ability to prime CD16 signaling on NK cells (14). Amongst these approaches, NK cell driven function through monoclonal antibodies and through bi- and tri-specific engagers is of significant clinical interest (15-19). Our group described a platform, termed Tri-specific Killer Engager (TriKE), which merges the concepts of cytokine signaling and bi-specifics into a single molecule to maximize NK cell immunotherapeutic potential (3).

TriKE molecules are composed of three arms: a single chain variable fragment (scFv) targeting the activating receptor CD16 on NK cells, a scFv targeting a tumor-associated antigen (TAA), and an IL-15 moiety to drive NK cell expansion, survival and priming (3, 16). We previously described TriKE molecules against a host of TAAs, including a molecule targeting CD33 meant to enhance NK cell immunotherapy against myeloid malignancies including acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), and other CD33-expressing malignancies such as systemic mastocytosis (20-22). This first-generation TriKE molecule, termed 161533 in previous studies, is called scFv16-m15-33 in this context for clarity and contains a mutant IL-15 with an N72D substitution (abbreviated m15) (23) and a Phase I/II clinical trial is underway for treatment of refractory AML, high-risk MDS and systemic mastocytosis (NCT03214666). The present study describes the effect of substituting a “humanized” anti-CD16 single domain camelid antibody to replace the anti-CD16 scFv, which enables presentation of wild type (wt) IL-15 without use of m15. The resulting molecule, cam16-wt15-33 TriKE, displays stronger IL-15 signaling capabilities, better NK cell activation, and increased NK cell mediated tumor control both *in vitro* and *in vivo.* Thus, we postulate that the second-generation TriKE molecule may prove more efficacious for clinical development.

Materials and Methods

Isolation of immune cells, transplant patient samples, and cell lines.

Healthy donor blood was obtained from Memorial Blood Bank (Minneapolis, MN), and processed to isolate peripheral blood mononuclear cells (PBMCs) using density gradient Ficoll-Paque (GE Healthcare). PBMCs were either cryopreserved in liquid nitrogen, were processed fresh to enrich NK Cells using EasySep Human NK Cell Enrichment Kit (Cat. No:19055, STEMCELL Technologies), or were CD3/CD19 depleted using Selection Kits (Cat. No:17851&17854, STEMCELL Technologies).

All human samples, including AML patient blasts, were obtained following informed consent, received in compliance with guidelines by the Committee on the Use of Human Subjects in Research and in accordance with the Declaration of Helsinki. PBMCs collected 28 days after double cord (N = 9) or matched sibling transplant (4 bone marrow and 5 peripheral blood stem cell grafts) were cryopreserved and stored by Translational Therapy Shared Resource (Masonic Cancer Center, University of Minnesota).

CD33+ Acute promyelocytic leukemia cell line HL-60 was received from American Type Culture Collection (ATCC), was authenticated using flow cytometry as previously described (20), and cultured in RPMI 1640 supplemented with 10 % Fetal Bovine Serum in 37^0C, at 5% CO2.

ELISA for *In Vitro* and *In Vivo* Detection of IL15

Equimolar concentrations of scFv16-m15-33 and rhIL-15 were serially diluted in RPMI media or heat-inactivated Human AB serum (Valley Biomedical, Inc.). The Quantikine ELISA kit (R&D Systems) was used per the manufacturer's protocol to detect IL-15. For *in vivo* detection of IL-15, NOD *scid* gamma (NSG) (Jackson Laboratories) mice were tested with no treatment or intravenous with 20 μg of scFv16-m15-33, or 5 μg of rhIL-15. Serum was collected 30 minutes post-injection for IL-15 determination.

Measuring NADP Activity of IL15

CTLL-2 cells (Sigma-Aldrich) were incubated for 48 hours with several concentrations of scFv16-m15-33 and rhIL-15. Cells were then incubated for 1 hour at 37^0C and 5% CO2 with Resazurin (Cat. No:AR002, R&D Systems), a blue reagent that is reduced by metabolically active viable cells to form pink resorufin, identifiable at 570 nm absorbance using a Tecan Infinite M200Pro plate reader (25).

cam16-wt15-33 TriKE Construct

Production steps of the second-generation TriKE cam16-wt15-33, including construction, verification, isolation, and purification, were performed as previously described for the first-generation TriKE (20, 24). The final construct of the cam16-wt15-

33 *Nco*I/*Xho*I gene fragment was encoded from a start codon, a humanized camelid anti-CD16 VHH (cam16), a 20 amino acid flanking sequence (PSGQAGAAASESLFVSNHAY), wild type human IL-15, a 7 amino acid flanking sequence (EASGGPE), and anti-CD33 scFv.

OCTET

An Octet based label-free binding assay (OctetRED96, ForteBio, Molecular Devices) was performed to measure binding affinity of the analytes scFv16-m15-33 and cam16-wt15-33 to the ligands CD16a and CD16b. The ligands were captured on biosensor tips using tag - specific dips (Anti-HIS tags for CD16a and CD16b). The biosensor coated with a ligand was dipped into buffer containing an analyte for association and then dipped into assay buffer (PBS). The binding and dissociation was measured using Bio-Layer Interferometry technology.

Proliferation Assay

The CellTrace Violet Proliferation Kit (Cat. No:C34557, Thermo Fisher) was used to evaluation proliferation (dye dilution) by flow cytometry. Cells were labeled, treated as indicated, harvested 7 days later and analyzed on an LSRII (BD Biosciences). For the characterization of NK and T cells, LIVE/DEAD NEAR IR (Cat. No: L34976, Thermo Fisher), PE-CY7 conjugated anti-CD56 (HCD56, BioLegend), and PE-CF594 conjugated anti-CD3 (UCHT1, BD BioSciences) were used. NK cells and T cells were identified as LiveDead-/CD56+/CD3- and LiveDead-/CD56-/CD3+ cells (respectively). Analysis of flow cytometric data was performed using FlowJo software (Tree Star Inc.).

Activation Assay

PBMCs were incubated overnight with noted treatments, harvested, and stained with Live/Dead Fixable Aqua Staining Kit (Cat. No: L-34966, Thermo Fisher), anti-CD56, anti-CD3, PE conjugated anti-CD69 (FN50, BioLegend), APC conjugated anti-CD25 (M-A251, BioLegend) to evaluate NK Cell activation marker expression with flow cytometry.

Function Assay Measuring CD107a Degranulation and IFNγ Cytokine Production

Flow cytometric assessment of NK cell function was carried out as previously described (24). Briefly, following addition of the treatments and targets, cells were stained with FITC conjugated anti-CD107a (H4A3, BioLegend) at the beginning of 4-hr incubation. One hour after the addition of anti-CD107a, cells were given Golgi Stop and Golgi Plug (BD Biosciences), and incubated for 3hrs. At the end of 4hrs, cells were stained with Live/Dead Fixable Aqua Staining Kit (Cat. No: L-34966, Thermo Fisher), anti-CD56 and anti-CD3, fixed and permeabilized. Permeabilized cells were stained with BV650 conjugated IFNγ (4S.B3, BioLegend), and were evaluated with flow cytometry. Effectors (normal donor or d28 post-transplant patient PBMCs) were co-cultured with targets in each of the experiments.

Real Time Tumor Killing

HL-60 cells were labeled using CellTrace Far Red Proliferation Kit (Cat. No:C34564, Thermo Fisher). Enriched NK cells were incubated at a 2:1 effector:target ratio with CellTrace Far Red labeled HL-60 targets with noted treatments and with IncuCyte Caspase-3/7 Green Apoptosis Assay reagent (Cat. No:4440, Essen BioScience). Plates were placed in an IncuCyte Zoom (Sartorius Inc.) for 24 hours. Readings were taken every 15 minutes. A graph was created representing percentage live HL-60 targets (CellTrace Far $Red^{+}Caspase3/7^{-}$), normalized to targets alone at the starting (0 hr) time point.

In Vivo Models

Mouse experiments were performed following the guidelines of Institutional Animal Care and Use Committee at the University of Minnesota.

The previously described HL-60luc/Human NK cell Xenogeneic NOD scid gamma (NSG) mouse model was implemented (20). Briefly, mice were irradiated (275 cGy), intravenously (IV) injected with $0.75x10^6$ HL-60luc cells, injected IV three days later with $1x10^6$ CD3/CD19 - depleted human NK Cells and treated for two weeks intraperitoneally (IP). At Day 21 tumor load was assessed after luciferin injection using bioluminescent imaging. Mice were bled on Day 21 (100 μl blood per mouse), RBCs lysed and cells stained with Brilliant Violet 605 conjugated anti-humanCD45 (HI30, BioLegend), anti-CD56, and anti-CD3, and acquired on LSRII for 60 secs/sample.

An AML Patient-Derived Xenograft (PDX) model was used to compare the activity of cam16-wt15-33 to rhIL-15. 6-8 week old NSG-SGM3 mice were irradiated (125 cGy) and injected IV the next day with $2x10^6$ primary AML. Progress of AML engraftment was monitored with weekly bleeds staining for anti-CD45 and APC-conjugated anti-CD33 (WM53, BioLegend) to determine proportion of CD45+/CD33+ blast cells until detection of at least 1% human AML in blood was achieved (in about 6 weeks). Mice then received $3x10^6$ CD3/CD19-depleted NK Cells and subsequently received IP treatments of either 5 μg rhIL-15 3 times weekly or 30 μg cam16-wt15-33 5 times weekly for a total of 3 weeks. Mice were harvested on Day 21 and blood was stained with anti-humanCD45, anti-CD56, and anti-CD3 to determine CD45+/CD56+/ CD3- NK cell expansion. Bone marrow was stained with anti-humanCD45, FITC conjugated anti-CD34 (561, BioLegend), and anti-CD33 to determine CD45+/CD33+ AML blast.

Statistical Analysis

GraphPad Prism (GraphPad Prism Software, Inc, La Jolla, CA) was used to generate graphs with error bars showing mean ± SEM, and to calculate statistical significance as *P<0.05, **P< 0.01, ***P<0.001, and ****P<0.0001.

Results

IL-15 potency is significantly diminished within the backbone of first-generation scFv16-m15-33 Trispecific Killer Engager (TriKE). We previously reported a TriKE composed of a single chain variable fragment (scFv) against CD16 (scFv16), an N72D mutant IL-15 (m15) shown to signal better than wild type IL-15, and an scFv against CD33 (33), termed scFv16-m15-33 (20, 23). Because versions of this molecule containing a wild type IL-15 moiety were not functional, the mutant IL-15 version of this first-generation TriKE, scFv-m15-33, was selected. While this molecule clearly displays cytotoxic activity mediated by NK cell engagement and proliferative activity by the IL-15 domain, we hypothesized that the activity of the IL-15 moiety might be compromised in the first-generation TriKE backbone. To test this, IL-15 protein detection in the scFv16-m15-33 TriKE was compared to detection of equimolar amounts of recombinant human IL-15 (rhIL-15) using an ELISA assay **(Figure 1A)**. Data indicate a significant reduction in IL-15 detection when comparing the scFv16-m15-33 TriKE to rhIL-15. Results are identical when comparing detection in media (RPMI with 10% FBS) or human serum. When NSG mice were injected with rhIL-15 or scFv16-m15-33 TriKE at similar doses and serum for analysis obtained 30 minutes later, IL-15 detected by ELISA was significantly greater after rhIL-15 injection than with scFv16-m15-33 TriKE despite the rhIL-15 being a smaller molecule (12.8 kDa vs. 66.05 kDa) with potential for more rapid clearance **(Figure 1B).** To determine whether functional activity was altered, the scFv16-mIL15-33 TriKE was compared to rhIL-15 over a concentration range using an assay that detects NADP activity on CTLL-2 cells that signal through IL-15 but do not contain CD16 or CD33 receptors **(Figure 1C)**. Together, results indicate that mIL-15 in the TriKE signals with about 25-fold less than rhIL-15.

Creation of a second-generation cam16-wt15-33 TriKE. IL-15 detection and signaling data indicate that incorporation of IL-15 into the TriKE resulted in reduced activity. Since the scFv16-mIL15-33 TriKE contains multiple scFvs, each of which contains a variable heavy (V_H) and variable light (V_L) chain, the impact on diminished IL-15 activity may be mediated by inefficient folding of the scFvs **(Figure 1D)**. Previous studies show that molecules containing multiple scFvs have potential for aggregation and nonspecific folding (26-29). The V_H of one scFv aggregates to the V_L of the other scFv and vice-versa. To overcome this challenge, a single domain antibody (sdAb or V_{HH}) sequence was substituted for the scFv anti-CD16 binding domain. Because the sdAb contains only one variable heavy domain that cannot aggregate with a V_L from an scFv, the TriKE molecule is forced to fold precisely without mispairing **(Figure 1E).** To accomplish this, the CDRs of a camelid (llama) anti-CD16 sdAb were cloned into a sdAb backbone (30, 31). A number of amino acids were altered to humanize the molecule to form the new cam16 NK cell engager **(Figure 1F).** cam16 was then cloned into a TriKE backbone containing wild type IL-15 (wt15) and an scFv anti-CD33 (cam16-wt15-33 TriKE) or a mutant IL-15 and anti-CD33 (cam16-m15-33). Because no difference has been detected in proliferative activity between the cam16-wt15-33 TriKE vs. the cam16-m15-33 TriKE, the wild type version was selected for development **(data not shown)**.

To compare the binding capabilities of the cam16-wt15-33 and scFv16-m15-33 TriKEs, Octet analysis was performed to measure the affinity of each TriKE against the CD16A and CD16B proteins **(Figure 1G)**. Addition of the cam16 arm in the cam16-wt-15-33 TriKE improved binding affinity 28-fold against CD16A when compared to the scFv16-m15-33 TriKE (KD = 2.32E-8 vs. 6.56E-7 M respectively). Cam16-wt15-33 TriKE had much stronger binding against CD16B than scFv16-m15-33 TriKE (KD = 3.40E-8 vs. 1.44E-6 M respectively). Both molecules bound CD33 with relatively similar affinity (KD = 2.56E-8 M for cam16-wt15-33 vs. 2.21E-8 M for scFv16-m15-33). Data indicate that the second-generation cam16-wt15-33 TriKE binds CD16 with greater affinity than the first-generation scFv16-m15-33 TriKE.

IL-15 activity in cam16-wt15-33 TriKE is significantly greater than the scFv-m15-33 Trike. To investigate the ability of this new construct to drive IL-15 signaling, NK cell proliferation was evaluated on CellTrace-labeled peripheral blood mononuclear cells (PBMCs) incubated with different concentrations of rhIL-15, scFv16-m15-33, or cam16-wt15-33. Total NK cell proliferation was highest on the cam16-wt15-33 at all concentrations **(Figure 2A)** and NK cell proliferation beyond three divisions **(Figure 2B)** was highest on the cam16-wt15-33 group at concentrations of 5 nM or greater. The IL-15 specificity was tested by evaluating proliferation on T cells **(Figure 2C and 2D)**. Despite driving the strongest NK cell proliferation, compared to rhIL-15 or scFv-m15-33, the cam16-wt15-33 TriKE drove T cell proliferation significantly less than rhIL-15 and the scFv-m15-33 TriKE at the highest concentration tested. Results indicate that the cam16 engager induces potent delivery of the wild type IL-15 within the TriKE backbone specifically to NK cells without cross stimulation to T cells. Since IL-15 signaling can also induce expression of activation markers CD69 and CD25, NK cells were incubated with rhIL-15, scFv16-m15-33, or cam16-wt15-33 overnight and expression of activation markers was evaluated. With the exception of the lowest dose CD69 proportion on NK cells was similar, but the median fluorescence intensity (MFI) for CD69 was higher at all concentrations tested on the cam16-wt15-33 TriKE when compared to the scFv16-m15-33 **(Figure 2E and 2F)**. The proportion and median florescence of CD25 expressing NK cells was substantially higher with cam16-wt15-33 treatment when compared to scFv16-m15-33 **(Figure 2G and 2H)**. When compared to rhIL-15 at the 30 nM concentration, the cam16-wt15-33 TriKE median CD25 expression was much higher. These data indicate that IL-15 in the cam16-wt15-33 TriKE backbone is substantially stronger than that on the scFv16-m15-33 TriKE and similar to rhIL-15.

The cam16-wt15-33 TriKE induces more potent NK cell anti-tumor activity. We next evaluated the ability of the cam16-wt15-33 TriKE to activate NK cell effector functions against CD33 expressing targets. To determine the differential in function between the second-generation cam16-wt15-33 TriKE and the first-generation scFv16-m15-33 TriKE, NK cells were incubated with tumor targets. A gradient of TriKE concentrations (61.7 pM to 15 nM) and degranulation and IFNγ expression was determined after 4 hours **(Figure 3A and 3B)**. The second-generation cam16-wt15-33 TriKE was clearly functionally superior to the first-generation scFv16-m15-33 TriKE throughout the spectrum of concentrations. Increased functionality was evident at the 30

nM concentration and when compared to rhIL-15 **(Figure 3C and 3D)**. To evaluate this function in a dynamic killing assay over time, we tested the TriKEs in an IncuCyte tumor killing assay **(Figure 3E)**. Enriched NK cells were plated with CellTrace Far Red labeled HL-60 tumor targets, the indicated drugs and Caspase 3/7 green reagent. The number of live tumor cells (Red^+Green-) was evaluated every 15 minutes for 24 hours. Data showed that the cam16-wt15-33 TriKE induces more rapid and complete killing kinetics than the scFv16-m15-33 TriKE or controls.

Increased binding stability of cam16-wt15-33 TriKE results in enhanced NK cell function. To determine whether the cam16-wt15-33 TriKE displays enhanced function through increased binding stability, PBMCs were incubated with TriKE molecules or rhIL-15 for 15 minutes, washed, and then cultured with HL-60 tumor targets for 4 hours to assess NK cell degranulation and cytokine production **(Figure 4A)**. The only activity detected was from TriKE or rhIL-15 stably bound to NK cells prior to washing. While the scFv16-m15-33 barely differed in activity from the rhIL15 control, the cam16-wt15-33 TriKE displayed greater functional activity, suggesting better binding stability. For CD33 binding stability, HL-60 cells were incubated for 15 minutes with TriKEs, washed, and then incubated with PBMCs to evaluate NK cell function. Similar results were seen **(Figure 4B)**. To evaluate the impact on proliferation, CellTrace-labeled PBMCs were incubated with TriKEs or rhIL-15 for 15 minutes, washed, and then incubated for 7 days **(Figure 4C)**. The cam16-wt15-33 TriKE induced the greatest stable binding and proliferation, followed closely by scFv16-m15-33 TriKE. rhIL-15 alone did not induce proliferation.

Second-generation TriKE induces enhanced activation of early post-transplant NK cells. To evaluate the impact of TriKE molecules post-transplant, patient NK cells were harvested at an early stage (day 28), where NK cells are thought to mediate relapse protection. Nine double-umbilical cord blood (dUCB) and 9 matched sibling stem cell transplant (Matched Sib) recipient samples were studied. Significant defects have been shown previously in IFNγ production despite high early engrafting NK cell numbers at this time point. (32).To determine the longer priming effect of the IL-15, cells were incubated with drugs and targets for 4 hours (standard) or were pre-incubated overnight with drugs. With dUCB, the cam16-wt15-33 TriKE induced higher degranulation than all other groups in the 4-hour incubation but little inflammatory cytokine production **(Figure 5A)**. With longer overnight incubation the cam16-wt15-33 TriKE still displayed degranulation but also induced potent IFNγ production similar to that seen with the rhIL-15 control but far greater than the scFv16-m15-33 TriKE **(Figure 5B)**. A similar pattern of activation occurred in the matched sibling samples incubated with treatments and targets for 4 hours. The cam16-wt15-33 TriKE induced better degranulation but no IFNγ in the short incubation **(Figure 5C)**. However, with overnight incubation of matched sibling samples, all treatment groups yielded similar levels of activation, indicating a differential effect on reconstituting NK cells from dUCB and matched sibling graft sources with TriKE molecule stimulation **(Figure 5D)**.

In vivo activity of the cam16-wt15-33 second-generation TriKE. Two different models were used to evaluate the efficacy of the cam16-wt15-33 TriKE in vivo. One employed intravenous (IV) injection of luciferase-labeled AML cell line (HL-60luc) into NSG mice to track tumor using live imaging. Three days after tumor inoculation, NK cells were injected IV and mice were treated 5 days a week (MTWThF) for two weeks with different concentrations (90, 30, or 10 μg/injection) of the second-generation cam16-wt15-33 TriKE or the first-generation scFv16-m15-33 TriKE **(Figure 6A)**. The mice were evaluated after three weeks for tumor burden using bioluminescence. Data show that at 90 and 30 μg doses, the cam16-wt15-33 TriKE treatment induced significantly better tumor control (130-fold and 12-fold respectively) than the scFv16-m15-33 TriKE **(Figure 6B)**. The differential in proliferative activity of the cam16-wt15-33 TriKE with the scFv16-m15-33 TriKE is illustrated by higher NK cell counts in the blood at day 21 at all doses tested **(Figure 6C)**.

The second in vivo system used a Patient-Derived Xenograft (PDX) tumor model in which primary acute myeloid leukemia (AML) blasts from cancer patients are injected into NSG-SGM3 mice. Tumor is allowed to grow for about 6 weeks. NK cells (IV) and drugs (IP) are then injected to evaluate efficacy **(Figure 6D)**. The efficacy of the cam16-wt15-33 TriKE was compared to rhIL-15 to evaluate the second-generation TriKE compared to an IL-15 product that has been tested clinically (11-13, 33). In this model, mice were treated for three weeks with the drugs and then harvested to assess absolute leukemia burden in the bone marrow and NK cell numbers in the blood. cam16-wt15-33 TriKE treatment resulted in less tumor in the bone marrow than rhIL-15 treatment **(Figure 6E)**, despite lower NK cell numbers found in the blood **(Figure 6F)**. In vivo data indicate that the cam16-wt15-33 TriKE induces better AML tumor control.

Discussion

The TriKE platform is unique among NK engagers because it incorporates cytokine signaling, specific tumor targeting, and activation of ADCC within a single molecule (16-19). TriKEs not only drive specific tumor killing but also induce NK cell proliferation and survival via the IL-15 moiety. Unlike T cells, which can be induced to expand through crosslinking of activating receptors, NK cells require an initial cytokine signal to induce proliferation. Such proliferation can be propagated further, but not substituted by, an activating receptor signal (34). The first-generation TriKE, scFv16-m15-33 (161533 in previous studies), provides both cytokine and activating signals (20). Our findings indicate that IL-15 functionality within this molecule is limiting. The IL-15 moiety within the first-generation TriKE shows in a 25-fold decrease in function when compared to rhIL-15. This decrease may be influenced by steric hindrance mediated by mispairing of the two scFvs (35). We therefore substituted a novel humanized single domain antibody (sdAb) against CD16 incapable of pairing with the light chain of the anti-CD33 scFv, resulting in improved functionality of the IL-15 moiety within the second-generation cam16-wt15-33 TriKE. Although we infer that this is due to decreased mispairing, the smaller size of the sdAb may also reduce steric hindrance. Incorporation of the cam16 sdAb within the TriKE allowed for use of wild type IL-15 within this platform (23). In all aspects, including proliferation, degranulation, inflammatory cytokine secretion, tumor killing and in vivo function, the second-generation cam16-wt15-33 is more potent than first-generation scFv16-m15-33. This differential in functionality is particularly evident in the HL-60luc xenogeneic mouse. cam16-wt15-33 TriKE induced striking increases in tumor control and NK cell numbers when compared to scFv16-m15-33.

Besides delivering a targeted ADCC signal, TriKEs specifically deliver IL-15 to NK cells mediated by the anti-CD16 arm (20, 24). The second-generation cam16-wt15-33 TriKE binds to CD16 on NK cells 28-fold better than scFv16-m15-33. This differential in binding is also reflected in the delivery of IL-15 to NK cells vs. T cells. Despite delivering a stronger IL-15 signal to NK cells, the cam16-wt15-33 TriKE, when compared to rhIL-15 and scFv16-m15-33, induces the weakest expansion signal on T cells, thereby minimizing the leaching of IL-15 by other immune cells and ensuring delivery to CD16-expressing cells. IL-15 can participate in the development of leukemias and solid tumors. Specific delivery would minimize IL-15 access to other tumors (36-39). IL-15 can also intensify autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematous, inflammatory bowel disease and multiple sclerosis (40). Thus, targeted delivery of IL-15 via the cam16-wt15-33 TriKE may limit NK-independent autoimmune effects.

In graft versus leukemia (GVL) transplant settings, specifically targeting the IL-15 to NK cells via the cam16-wt15-33 may enhance graft retention (1, 2, 41-44). NK cells are thought to play an important GVL role. IL-15 treatment can amplify NK cell expansion, although retention may be limited by expansion of host CD8 T cells and other subsets. Restricting CD8 T cell access to IL-15 may reduce GVHD in some clinical situations (47). The elegantly targeted delivery of IL-15 to NK cells, coupled with the ability to potently drive ADCC in vitro and in vivo, makes cam16-wt15-33 TriKE an exciting immunotherapeutic focus for our ongoing research.

Acknowledgements

We would like to acknowledge the Translational Therapy, Flow Cytometry, and Imaging cores at the University of Minnesota for their excellent services. This work was supported in part by NCI P30 CA077598, Minnesota Masonic Charities and the Killebrew-Thompson Memorial Fund. We would also like to thank Xianzheng Zhou, at New York Medical College, for use of his HL-60luc cells.

FIGURE LEGENDS

Figure 1. Design of second-generation cam16-wt15-33 TriKE. (a) ELISA-based detection of IL-15 in equimolar concentrations of scFv16-m15-33 TriKE (in blue) and rhIL-15 (in red). (b) NSG mice were injected with scFv16-m15-33 TriKE, rhIL-15, or nothing (control) and IL-15 levels in serum were detected by ELISA after 30 minutes (N=2). (c) Activity of IL-15 in the scFv16-m15-33 TriKE or rhIL-15 was evaluated after a 48-hours in culture with CTLL-2 cells. (d) Schematic of theoretic folding inefficiencies, between two scFvs, in the first-generation scFv16-m15-33 TriKE, causing steric hindrance of IL-15. (e) Schematic of second-generation cam16-wt15-33 TriKE, containing a humanized camelid single domain antibody (sdAb/VHH) anti-CD16 arm (in blue), leading to precise folding and reduced steric hindrance of IL-15. (f) anti-CD16 sdAb (cam16) map containing CDRs from camelid anti-CD16 antibody (red) cloned into a single domain backbone (in blue) with a number of amino acid modifications (in green) for humanization. (g) Octet analysis of binding affinity of scFv16-m15-33 TriKE binding to CD16a (green trace) and CD16b (orange trace) versus cam16-wt15-33 TriKE binding to CD16a (blue trace) and CD16b (red trace). Purple and light blue traces represent control unspecific binding of scFv16-m15-33 and cam16-wt15-33 TriKEs (respectively). Numbers represent 1) Biosensor probe preparation, 2) capture of ligand, 3) baseline in assay buffer, 4) association with antigen, and 5) disassociation in assay buffer.

Figure 2. Second-generation cam16-wt15-33 TriKE demonstrates potent IL-15 signaling with enhanced NK cell specificity. PBMCs were CellTrace labeled and incubated for 7 days with noted concentrations of rhIL-15 (red), scFv16-m15-33 TriKE (blue), and cam16-wt15-33 TriKE (purple). At the end of the 7 days cells were harvested, stained and evaluated. (a) The proportion of NK cells that proliferated or (b) that highly proliferated (greater than three divisions) with indicated treatments (N=5). (c) The proportion of T cells that proliferated or (d) that highly proliferated with indicated treatments (N=5). One-way ANOVA with repeated measures was used to calculate differences against the cam16-wt15-33 group within each of the concentrations. NK cells were activated overnight with noted stimuli or nothing (NT) and activation marker expression was assessed. (e) The CD69+ proportion and (f) median fluorescence intensity (MFI) as well as (g) CD25+ proportion and (h) MFI was assessed at the time of harvest on NK cells (N=7). Paired T tests between the scFv16-m15-33 and cam16-wt15-33 TriKEs were carried out within each of the concentrations.

Figure 3. Second-generation cam16-wt15-33 TriKE induces stronger NK cell activation against CD33+ targets. (a) NK cell degranulation (% CD107a+) and (b) IFNγ production evaluated within PBMCs incubated with CD33+ HL-60 tumor targets at a 2:1 ratio for 4 hours with indicated concentrations of TriKEs (N=4). (c) Proportion of NK cells expressing CD107a+ and (d) IFNγ after a 4-hour incubation with 30 nM TriKEs and HL-60 targets (N=8). One-way ANOVA with repeated measures was used to calculate differences against the rhIL-15 control group (brackets) while TriKEs were compared with paired T test (lines). (e) An IncuCyte imaging assay was used to

evaluate NK-mediated killing of CellTrace Far Red labeled targets over a 24-hour period with noted treatments (30 nM). Representative of 6 separate experiments.

Figure 4. Second-generation cam16-wt15-33 TriKE displays stable binding after short-term exposure. (a) PBMCs were incubated with the indicated treatments (30 nM) for 15 minutes, washed twice, and then incubated with HL-60 targets for 4 hours prior to evaluation of NK cell degranulation and IFNγ production (N=8). (b) HL-60 targets were incubated with the indicated treatments (30 nM) for 15 minutes, washed twice, and then incubated with PBMCs for 4 hours prior to evaluation of NK cell function (N=8). (c) PBMCs were CellTrace labeled, incubated with indicated treatments for 15 minutes, washed twice, and incubated for 7 days in culture with media (no cytokines) to evaluate total NK cell proliferation or those that highly proliferated (N=4). One-way ANOVA with repeated measures was used to calculate differences against the rhIL-15 control (brackets) while TriKEs were compared with paired T test (lines).

Figure 5. Second-generation cam16-wt15-33 TriKE enhances function in post-transplant patient samples. Samples from double cord (a-b) and matched sibling stem cell (c-d) transplant patients obtained day 28 after transplant were used to evaluate NK cell function with the indicated treatments at a 30 nM concentration (N=9 for both groups). Function was assessed with a standard 4-hour incubation assay against HL-60 targets and drug (a and c) or after overnight incubation with drug and then followed by a 4-hour incubation with HL-60 targets and fresh drug (b and d). NK cells were assessed for C107a (top) and IFNγ (bottom). One-way ANOVA with repeated measures was used to calculate statistical differences.

Figure 6. Enhanced function of second-generation cam16-wt15-33 TriKE against AML targets in vivo. (a) Diagram of HL-60/human NK cell xenogneic NSG model for evaluation of TriKE activity. Mice were then treated IP with noted concentrations of scFv16-m15-33 (blue) or cam16-wt15-33 (purple) 5 times weekly for 2 weeks. At day 21 tumor load was assessed by bioluminescent imaging and mice were bled to evaluate NK cell expansion. (b) Summary of tumor radiance and (c) NK cell numbers at day 21. (d) Diagram of AML patient derived xenograft (PDX) model used to compare the second-generation cam16-wt15-33 TriKE to rhIL-15. Upon AML engraftment mice were injected with NK cells and treatments and harvested 3 weeks later for evaluation of (e) AML blast (CD45+ CD33+) numbers in the bone marrow compartment and (f) NK cell numbers in a 100 μl of blood. Unpaired T test was used to calculate statistical differences.

References

1. Miller JS, Lanier LL. Natural Killer Cells in Cancer Immunotherapy. Annu Rev Canc Biol. 2019;3:77-103. doi: 10.1146/annurev-cancerbio-030518-055653. PubMed PMID: WOS:000462707500005.
2. Foley B, Felices M, Cichocki F, Cooley S, Verneris MR, Miller JS. The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). Immunol Rev. 2014;258(1):45-63. Epub 2014/02/13. doi: 10.1111/imr.12157. PubMed PMID: 24517425; PMCID: 3927144.
3. 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;31:64-75. doi: 10.1016/j.smim.2017.07.011. PubMed PMID: 28882429; PMCID: PMC5632228.
4. Fehniger TA, Cooper MA, Caligiuri MA. Interleukin-2 and interleukin-15: immunotherapy for cancer. Cytokine Growth Factor Rev. 2002;13(2):169-83. PubMed PMID: 11900992.
5. Waldmann TA. The biology of IL-15: implications for cancer therapy and the treatment of autoimmune disorders. J Investig Dermatol Symp Proc. 2013;16(1):S28-30. doi: 10.1038/jidsymp.2013.8. PubMed PMID: 24326545.
6. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol. 2006;6(8):595-601. Epub 2006/07/27. doi: 10.1038/nri1901. PubMed PMID: 16868550.
7. Kennedy-Nasser AA, Ku S, Castillo-Caro P, Hazrat Y, Wu MF, Liu H, Melenhorst J, Barrett AJ, Ito S, Foster A, Savoldo B, Yvon E, Carrum G, Ramos CA, Krance RA, Leung K, Heslop HE, Brenner MK, Bollard CM. Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity. Clin Cancer Res. 2014;20(8):2215-25. doi: 10.1158/1078-0432.CCR-13-3205. PubMed PMID: 24573552; PMCID: PMC3989436.
8. Cooley S, He F, Bachanova V, Vercellotti GM, DeFor TE, Curtsinger JM, Robertson P, Grzywacz B, Conlon KC, Waldmann TA, McKenna DH, Blazar BR, Weisdorf DJ, Miller JS. First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. Blood Adv. 2019;3(13):1970-80. doi: 10.1182/bloodadvances.2018028332. PubMed PMID: 31266741; PMCID: PMC6616260.
9. Wrangle JM, Velcheti V, Patel MR, Garrett-Mayer E, Hill EG, Ravenel JG, Miller JS, Farhad M, Anderton K, Lindsey K, Taffaro-Neskey M, Sherman C, Suriano S, Swiderska-Syn M, Sion A, Harris J, Edwards AR, Rytlewski JA, Sanders CM, Yusko EC, Robinson MD, Krieg C, Redmond WL, Egan JO, Rhode PR, Jeng EK, Rock AD, Wong HC, Rubinstein MP. ALT-803, an IL-15 superagonist, in combination with nivolumab in patients with metastatic non-small cell lung cancer: a non-randomised, open-label, phase 1b trial. Lancet Oncol. 2018;19(5):694-704. doi: 10.1016/S1470-2045(18)30148-7. PubMed PMID: 29628312; PMCID: PMC6089612.
10. Romee R, Cooley S, Berrien-Elliott MM, Westervelt P, Verneris MR, Wagner JE, Weisdorf DJ, Blazar BR, Ustun C, DeFor TE, Vivek S, Peck L, DiPersio JF, Cashen AF, Kyllo R, Musiek A, Schaffer A, Anadkat MJ, Rosman I, Miller D, Egan JO, Jeng EK, Rock A, Wong HC, Fehniger TA, Miller JS. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. Blood. 2018;131(23):2515-27. doi: 10.1182/blood-2017-12-823757. PubMed PMID: 29463563; PMCID: PMC5992862.

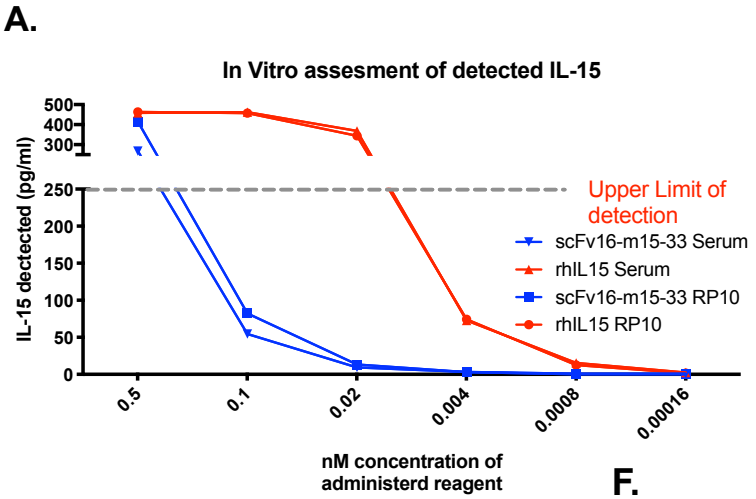
11. Miller JS, Morishima C, McNeel DG, Patel MR, Kohrt HE, Thompson JA, Sondel PM, Wakelee HA, Disis ML, Kaiser JC, Cheever MA, Streicher H, Creekmore SP, Waldmann TA, Conlon KC. A First-in-Human Phase 1 Study of Subcutaneous Outpatient Recombinant Human IL-15 (rhIL-15) in Adults with Advanced Solid Tumors. Clin Cancer Res. 2017. doi: 10.1158/1078-0432.CCR-17-2451. PubMed PMID: 29203590.
12. Dubois S, Conlon KC, Muller JR, Hsu-Albert J, Beltran N, Bryant BR, Waldmann TA. IL15 Infusion of Cancer Patients Expands the Subpopulation of Cytotoxic CD56(bright) NK Cells and Increases NK-Cell Cytokine Release Capabilities. Cancer Immunol Res. 2017;5(10):929-38. doi: 10.1158/2326-6066.CIR-17-0279. PubMed PMID: 28842470.
13. Conlon KC, Potter EL, Pittaluga S, Lee CR, Miljkovic MD, Fleisher TA, Dubois S, Bryant BR, Petrus M, Perera LP, Hsu J, Figg WD, Peer CJ, Shih JH, Yovandich JL, Creekmore SP, Roederer M, Waldmann TA. IL15 by Continuous Intravenous Infusion to Adult Patients with Solid Tumors in a Phase I Trial Induced Dramatic NK-Cell Subset Expansion. Clin Cancer Res. 2019. doi: 10.1158/1078-0432.CCR-18-3468. PubMed PMID: 31142503.
14. Nguyen QH, Roberts RL, Ank BJ, Lin SJ, Thomas EK, Stiehm ER. Interleukin (IL)-15 enhances antibody-dependent cellular cytotoxicity and natural killer activity in neonatal cells. Cell Immunol. 1998;185(2):83-92. doi: 10.1006/cimm.1998.1286. PubMed PMID: 9636686.
15. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. Br J Pharmacol. 2009;157(2):220-33. doi: 10.1111/j.1476-5381.2009.00190.x. PubMed PMID: 19459844; PMCID: PMC2697811.
16. Felices M, Lenvik TR, Davis ZB, Miller JS, Vallera DA. Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells. Methods Mol Biol. 2016;1441:333-46. doi: 10.1007/978-1-4939-3684-7_28. PubMed PMID: 27177679.
17. Rothe A, Sasse S, Topp MS, Eichenauer DA, Hummel H, Reiners KS, Dietlein M, Kuhnert G, Kessler J, Buerkle C, Ravic M, Knackmuss S, Marschner JP, Pogge von Strandmann E, Borchmann P, Engert A. A phase 1 study of the bispecific anti-CD30/CD16A antibody construct AFM13 in patients with relapsed or refractory Hodgkin lymphoma. Blood. 2015;125(26):4024-31. doi: 10.1182/blood-2014-12-614636. PubMed PMID: 25887777; PMCID: PMC4528081.
18. Gauthier L, Morel A, Anceriz N, Rossi B, Blanchard-Alvarez A, Grondin G, Trichard S, Cesari C, Sapet M, Bosco F, Rispaud-Blanc H, Guillot F, Cornen S, Roussel A, Amigues B, Habif G, Caraguel F, Arrufat S, Remark R, Romagne F, Morel Y, Narni-Mancinelli E, Vivier E. Multifunctional Natural Killer Cell Engagers Targeting NKp46 Trigger Protective Tumor Immunity. Cell. 2019;177(7):1701-13 e16. doi: 10.1016/j.cell.2019.04.041. PubMed PMID: 31155232.
19. Ellwanger K, Reusch U, Fucek I, Wingert S, Ross T, Muller T, Schniegler-Mattox U, Haneke T, Rajkovic E, Koch J, Treder M, Tesar M. Redirected optimized cell killing (ROCK(R)): A highly versatile multispecific fit-for-purpose antibody platform for engaging innate immunity. MAbs. 2019;11(5):899-918. doi: 10.1080/19420862.2019.1616506. PubMed PMID: 31172847; PMCID: PMC6601565.
20. Vallera DA, Felices M, McElmurry R, McCullar V, Zhou X, Schmohl JU, Zhang B, Lenvik AJ, Panoskaltsis-Mortari A, Verneris MR, Tolar J, Cooley S, Weisdorf DJ, Blazar BR, Miller JS. IL15 Trispecific Killer Engagers (TriKE) Make Natural Killer Cells Specific to CD33+ Targets While Also Inducing Persistence, In Vivo Expansion, and Enhanced Function. Clin Cancer Res. 2016;22(14):3440-50. doi: 10.1158/1078-0432.CCR-15-2710. PubMed PMID: 26847056; PMCID: PMC4947440.

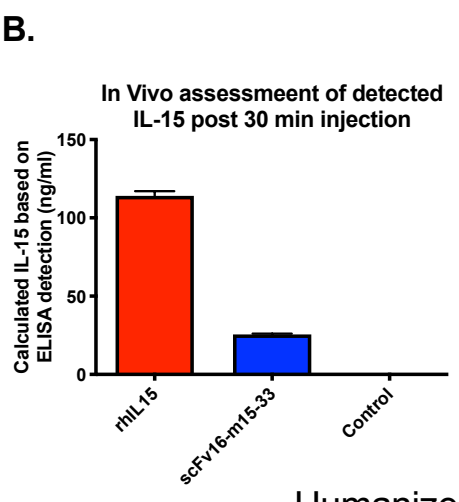
21. Sarhan D, Brandt L, Felices M, Guldevall K, Lenvik T, Hinderlie P, Curtsinger J, Warlick E, Spellman SR, Blazar BR, Weisdorf DJ, Cooley S, Vallera DA, Onfelt B, Miller JS. 161533 TriKE stimulates NK-cell function to overcome myeloid-derived suppressor cells in MDS. Blood Adv. 2018;2(12):1459-69. doi: 10.1182/bloodadvances.2017012369. PubMed PMID: 29941459; PMCID: PMC6020813.
22. Don Yun H, Felices M, Vallera DA, Hinderlie P, Cooley S, Arock M, Gotlib J, Ustun C, Miller JS. Trispecific killer engager CD16xIL15xCD33 potently induces NK cell activation and cytotoxicity against neoplastic mast cells. Blood Adv. 2018;2(13):1580-4. doi: 10.1182/bloodadvances.2018018176. PubMed PMID: 29980573; PMCID: PMC6039654.
23. Zhu X, Marcus WD, Xu W, Lee HI, Han K, Egan JO, Yovandich JL, Rhode PR, Wong HC. Novel human interleukin-15 agonists. J Immunol. 2009;183(6):3598-607. doi: 10.4049/jimmunol.0901244. PubMed PMID: 19710453; PMCID: PMC2814526.
24. Felices M, Kodal, B., Hinderlie, P., Kaminski, M. F., Cooley, S., Weisdorf, D. J., Vallera, D. A., Miller, J. S., & Bachanova, V. Novel CD19-targeted TriKE restores NK cell function and proliferative capacity in CLL. Blood Adv. 2019;3(6):897-907. doi: https://doi.org/10.1182/bloodadvances.2018029371.
25. Prabst K, Engelhardt H, Ringgeler S, Hubner H. Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. Methods Mol Biol. 2017;1601:1-17. doi: 10.1007/978-1-4939-6960-9_1. PubMed PMID: 28470513.
26. Glaven RH, Anderson GP, Zabetakis D, Liu JL, Long NC, Goldman ER. Linking Single Domain Antibodies that Recognize Different Epitopes on the Same Target. Biosensors (Basel). 2012;2(1):43-56. doi: 10.3390/bios2010043. PubMed PMID: 25585631; PMCID: PMC4263546.
27. Iezzi ME, Policastro L, Werbajh S, Podhajcer O, Canziani GA. Single-Domain Antibodies and the Promise of Modular Targeting in Cancer Imaging and Treatment. Front Immunol. 2018;9:273. doi: 10.3389/fimmu.2018.00273. PubMed PMID: 29520274; PMCID: PMC5827546.
28. Hassanzadeh-Ghassabeh G, Devoogdt N, De Pauw P, Vincke C, Muyldermans S. Nanobodies and their potential applications. Nanomedicine (Lond). 2013;8(6):1013-26. doi: 10.2217/nnm.13.86. PubMed PMID: 23730699.
29. Rozan C, Cornillon A, Petiard C, Chartier M, Behar G, Boix C, Kerfelec B, Robert B, Pelegrin A, Chames P, Teillaud JL, Baty D. Single-domain antibody-based and linker-free bispecific antibodies targeting FcgammaRIII induce potent antitumor activity without recruiting regulatory T cells. Mol Cancer Ther. 2013;12(8):1481-91. doi: 10.1158/1535-7163.MCT-12-1012. PubMed PMID: 23757164.
30. Behar G, Siberil S, Groulet A, Chames P, Pugniere M, Boix C, Sautes-Fridman C, Teillaud JL, Baty D. Isolation and characterization of anti-FcgammaRIII (CD16) llama single-domain antibodies that activate natural killer cells. Protein Eng Des Sel. 2008;21(1):1-10. doi: 10.1093/protein/gzm064. PubMed PMID: 18073223.
31. Vincke C, Loris R, Saerens D, Martinez-Rodriguez S, Muyldermans S, Conrath K. General strategy to humanize a camelid single-domain antibody and identification of a universal humanized nanobody scaffold. J Biol Chem. 2009;284(5):3273-84. doi: 10.1074/jbc.M806889200. PubMed PMID: 19010777.
32. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, Weisdorf DJ, Miller JS. NK cell education after allogeneic transplantation: dissociation between recovery of cytokine-producing and cytotoxic functions. Blood. 2011;118(10):2784-92. Epub 2011/07/16. doi: 10.1182/blood-2011-04-347070. PubMed PMID: 21757615; PMCID: 3172795.

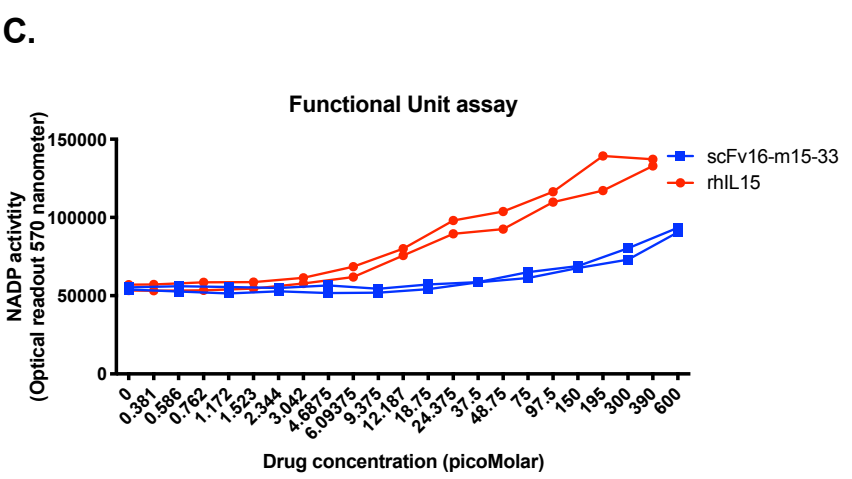
33. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, Fleisher TA, Dubois SP, Perera LP, Stewart DM, Goldman CK, Bryant BR, Decker JM, Chen J, Worthy TA, Figg WD, Sr., Peer CJ, Sneller MC, Lane HC, Yovandich JL, Creekmore SP, Roederer M, Waldmann TA. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. J Clin Oncol. 2015;33(1):74-82. doi: 10.1200/JCO.2014.57.3329. PubMed PMID: 25403209; PMCID: PMC4268254.
34. Ali AK, Nandagopal N, Lee SH. IL-15-PI3K-AKT-mTOR: A Critical Pathway in the Life Journey of Natural Killer Cells. Front Immunol. 2015;6:355. doi: 10.3389/fimmu.2015.00355. PubMed PMID: 26257729; PMCID: PMC4507451.
35. Dietrich S, Gross AW, Becker S, Hock B, Stadlmayr G, Ruker F, Wozniak-Knopp G. Constant domain-exchanged Fab enables specific light chain pairing in heterodimeric bispecific SEED-antibodies. Biochim Biophys Acta Proteins Proteom. 2019. doi: 10.1016/j.bbapap.2019.07.003. PubMed PMID: 31295556.
36. Hodge DL, Yang J, Buschman MD, Schaughency PM, Dang H, Bere W, Yang Y, Savan R, Subleski JJ, Yin XM, Loughran TP, Jr., Young HA. Interleukin-15 enhances proteasomal degradation of bid in normal lymphocytes: implications for large granular lymphocyte leukemias. Cancer Res. 2009;69(9):3986-94. doi: 10.1158/0008-5472.CAN-08-3735. PubMed PMID: 19366803; PMCID: PMC2786937.
37. Wu S, Fischer L, Gokbuget N, Schwartz S, Burmeister T, Notter M, Hoelzer D, Fuchs H, Blau IW, Hofmann WK, Thiel E. Expression of interleukin 15 in primary adult acute lymphoblastic leukemia. Cancer-Am Cancer Soc. 2010;116(2):387-92. doi: 10.1002/cncr.24729. PubMed PMID: 19924795.
38. Kuniyasu H, Ohmori H, Sasaki T, Sasahira T, Yoshida K, Kitadai Y, Fidler IJ. Production of interleukin 15 by human colon cancer cells is associated with induction of mucosal hyperplasia, angiogenesis, and metastasis. Clin Cancer Res. 2003;9(13):4802-10. PubMed PMID: 14581351.
39. Khawam K, Giron-Michel J, Gu Y, Perier A, Giuliani M, Caignard A, Devocelle A, Ferrini S, Fabbi M, Charpentier B, Ludwig A, Chouaib S, Azzarone B, Eid P. Human renal cancer cells express a novel membrane-bound interleukin-15 that induces, in response to the soluble interleukin-15 receptor alpha chain, epithelial-to-mesenchymal transition. Cancer Res. 2009;69(4):1561-9. doi: 10.1158/0008-5472.CAN-08-3198. PubMed PMID: 19190330.
40. Di Sabatino A, Calarota SA, Vidali F, Macdonald TT, Corazza GR. Role of IL-15 in immune-mediated and infectious diseases. Cytokine Growth Factor Rev. 2011;22(1):19-33. doi: 10.1016/j.cytogfr.2010.09.003. PubMed PMID: 21074481.
41. Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, Marsh SG, Guethlein LA, Parham P, Miller JS, Weisdorf DJ. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. Blood. 2009;113(3):726-32. Epub 2008/10/24. doi: 10.1182/blood-2008-07-171926. PubMed PMID: 18945962; PMCID: 2628378.
42. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, Marsh SG, Geraghty D, Spellman S, Haagenson MD, Ladner M, Trachtenberg E, Parham P, Miller JS. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. Blood. 2010;116(14):2411-9. Epub 2010/06/29. doi: 10.1182/blood-2010-05-283051. PubMed PMID: 20581313; PMCID: 2953880.

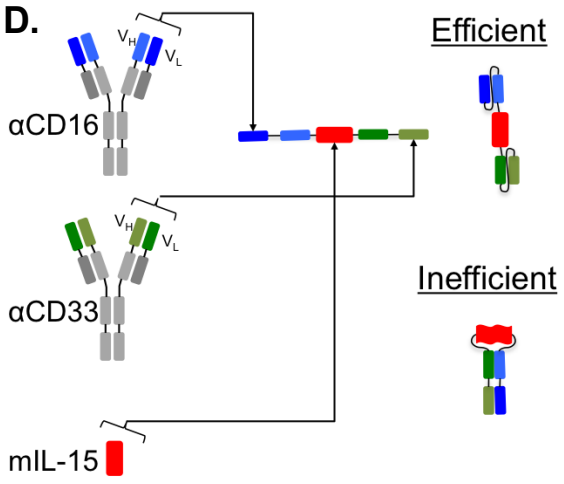
43. Davis ZB, Felices M, Verneris MR, Miller JS. Natural Killer Cell Adoptive Transfer Therapy: Exploiting the First Line of Defense Against Cancer. Cancer J. 2015;21(6):486-91. doi: 10.1097/PPO.0000000000000156. PubMed PMID: 26588681; PMCID: PMC4763946.
44. Grzywacz B, Moench L, McKenna D, Jr., Tessier KM, Bachanova V, Cooley S, Miller JS, Courville EL. Natural Killer Cell Homing and Persistence in the Bone Marrow After Adoptive Immunotherapy Correlates With Better Leukemia Control. J Immunother. 2019;42(2):65-72. doi: 10.1097/CJI.0000000000000250. PubMed PMID: 30489431; PMCID: PMC6365204.
45. Miller JS, Morishima C, McNeel DG, Patel MR, Kohrt HEK, Thompson JA, Sondel PM, Wakelee HA, Disis ML, Kaiser JC, Cheever MA, Streicher H, Creekmore SP, Waldmann TA, Conlon KC. A First-in-Human Phase I Study of Subcutaneous Outpatient Recombinant Human IL15 (rhIL15) in Adults with Advanced Solid Tumors. Clin Cancer Res. 2018;24(7):1525-35. doi: 10.1158/1078-0432.CCR-17-2451. PubMed PMID: 29203590.
46. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, McKenna D, Le C, Defor TE, Burns LJ, Orchard PJ, Blazar BR, Wagner JE, Slungaard A, Weisdorf DJ, Okazaki IJ, McGlave PB. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105(8):3051-7. Epub 2005/01/06. doi: 10.1182/blood-2004-07-2974. PubMed PMID: 15632206.
47. Perkey E, Maillard I. New Insights into Graft-Versus-Host Disease and Graft Rejection. Annu Rev Pathol. 2018;13:219-45. doi: 10.1146/annurev-pathol-020117-043720. PubMed PMID: 29099650.
48. Ssemaganda A, Kindinger L, Bergin P, Nielsen L, Mpendo J, Ssetaala A, Kiwanuka N, Munder M, Teoh TG, Kropf P, Muller I. Characterization of neutrophil subsets in healthy human pregnancies. PLoS One. 2014;9(2):e85696. doi: 10.1371/journal.pone.0085696. PubMed PMID: 24551035; PMCID: PMC3923728.
49. Felices M, Lenvik AJ, McElmurry R, Chu S, Hinderlie P, Bendzick L, Geller MA, Tolar J, Blazar BR, Miller JS. Continuous treatment with IL-15 exhausts human NK cells via a metabolic defect. JCI Insight. 2018;3(3). doi: 10.1172/jci.insight.96219. PubMed PMID: 29415897; PMCID: PMC5821201.

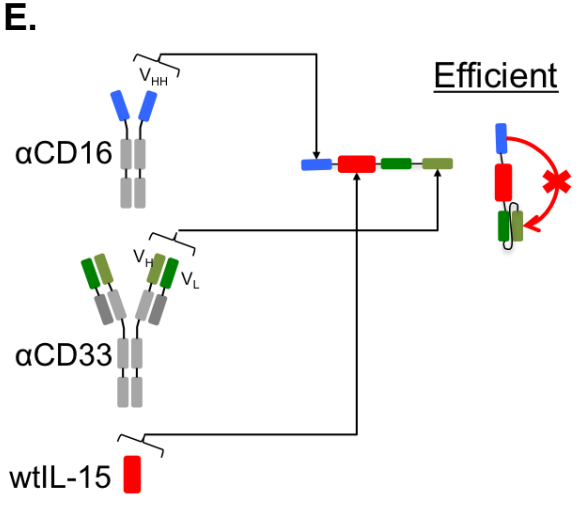
Figure 1:

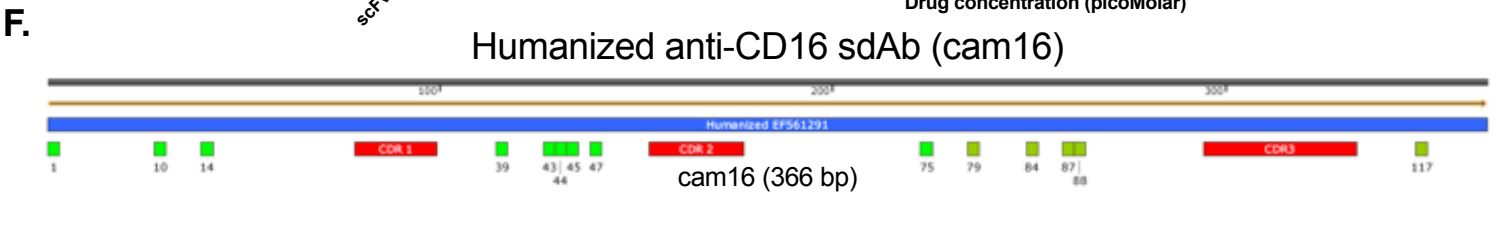

A.
In Vitro assesment of detected IL-15
IL-15 dectected (pg/ml)
500
400
300
250
200
150
100
50
0
Upper Limit of detection
scFv16-m15-33 Serum
rhIL15 Serum
scFv16-m15-33 RP10
rhIL15 RP10
0.5
0.1
0.02
0.004
0.0008
0.00016
nM concentration of administerd reagent


B.
In Vivo assessmeent of detected IL-15 post 30 min injection
Calculated IL-15 based on ELISA detection (ng/ml)
150
100
50
0
rhIL15
scFv16-m15-33
Control


C.
Functional Unit assay
NADP activity (Optical readout 570 nanometer)
150000
100000
50000
0
scFv16-m15-33
rhIL15
0
0.381
0.586
0.762
1.172
1.523
2.344
3.042
4.6875
6.09375
9.375
12.187
18.75
24.375
37.5
48.75
75
97.5
150
195
300
390
600
Drug concentration (picoMolar)


D.
Efficient
αCD16
Inefficient
αCD33
mIL-15


E.
Efficient
αCD16
αCD33
wtIL-15


F.
Humanized anti-CD16 sdAb (cam16)
Humanized EF561291
CDR 1
CDR 2
CDR3
cam16 (366 bp)

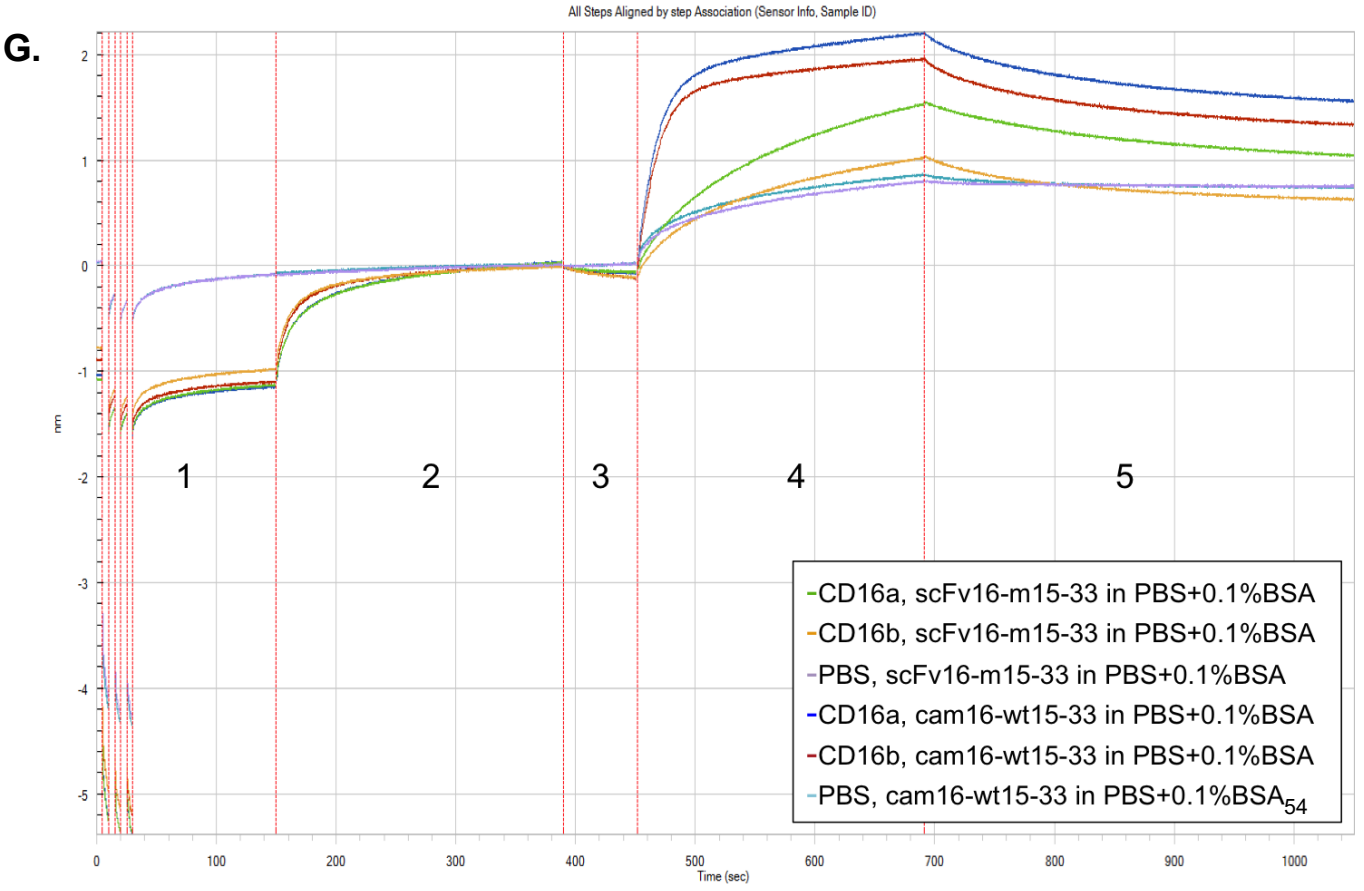

G.
All Steps Aligned by step Association (Sensor Info, Sample ID)
nm
1
2
3
4
5
-CD16a, scFv16-m15-33 in PBS+0.1%BSA
-CD16b, scFv16-m15-33 in PBS+0.1%BSA
-PBS, scFv16-m15-33 in PBS+0.1%BSA
-CD16a, cam16-wt15-33 in PBS+0.1%BSA
-CD16b, cam16-wt15-33 in PBS+0.1%BSA
-PBS, cam16-wt15-33 in PBS+0.1%BSA
Time (sec)

Figure 2:

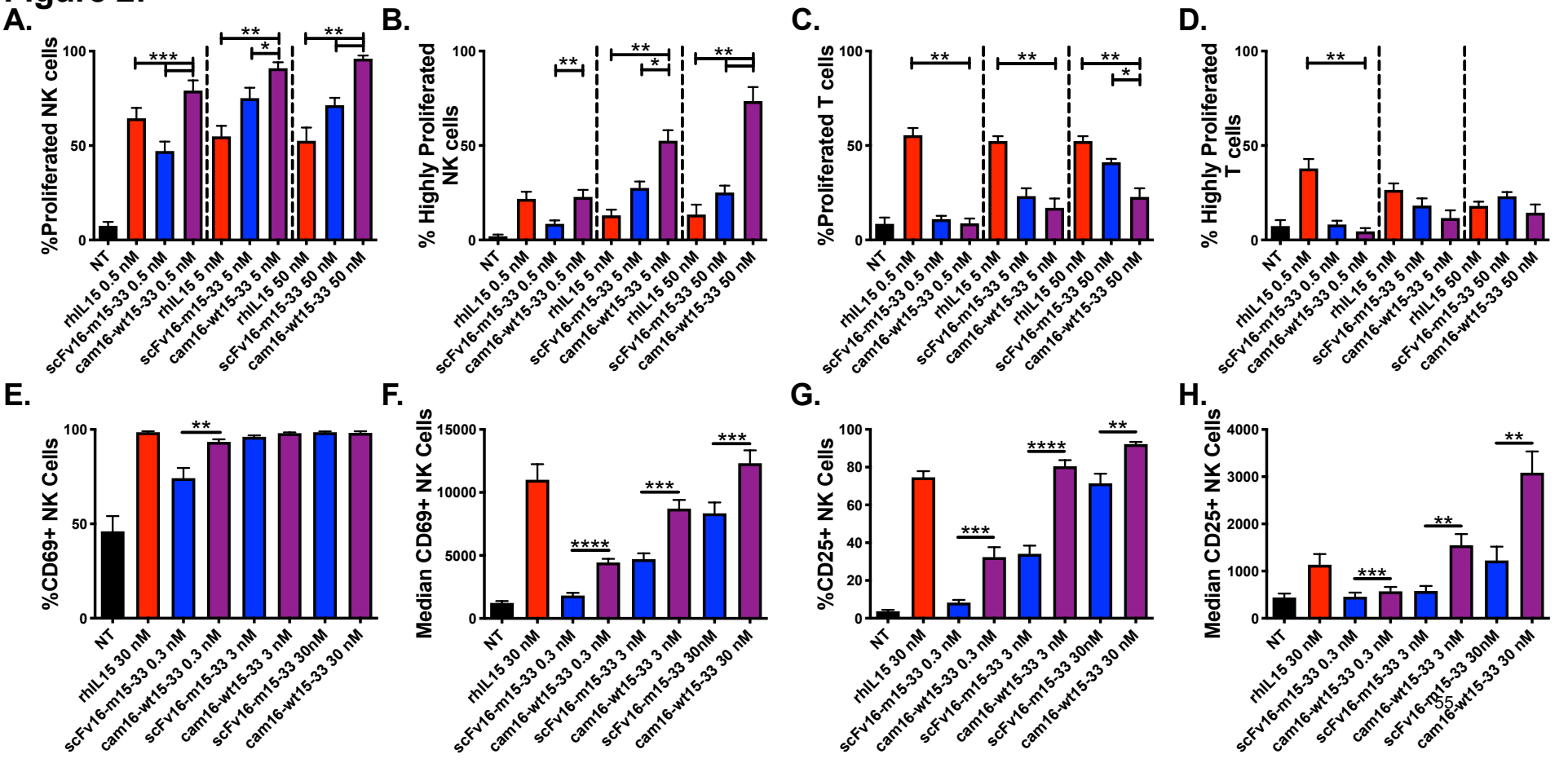

A.
%Proliferated NK cells
B.
% Highly Proliferated NK cells
C.
%Proliferated T cells
D.
% Highly Proliferated T cells
E.
%CD69+ NK Cells
F.
Median CD69+ NK Cells
G.
%CD25+ NK Cells
H.
Median CD25+ NK Cells
NT
rhIL15 0.5 nM
scFv16-m15-33 0.5 nM
cam16-wt15-33 0.5 nM
rhIL15 5 nM
scFv16-m15-33 5 nM
cam16-wt15-33 5 nM
rhIL15 50 nM
scFv16-m15-33 50 nM
cam16-wt15-33 50 nM
rhIL15 30 nM
scFv16-m15-33 0.3 nM
cam16-wt15-33 0.3 nM
scFv16-m15-33 3 nM
cam16-wt15-33 3 nM
scFv16-m15-33 30nM
cam16-wt15-33 30 nM

Figure 3:

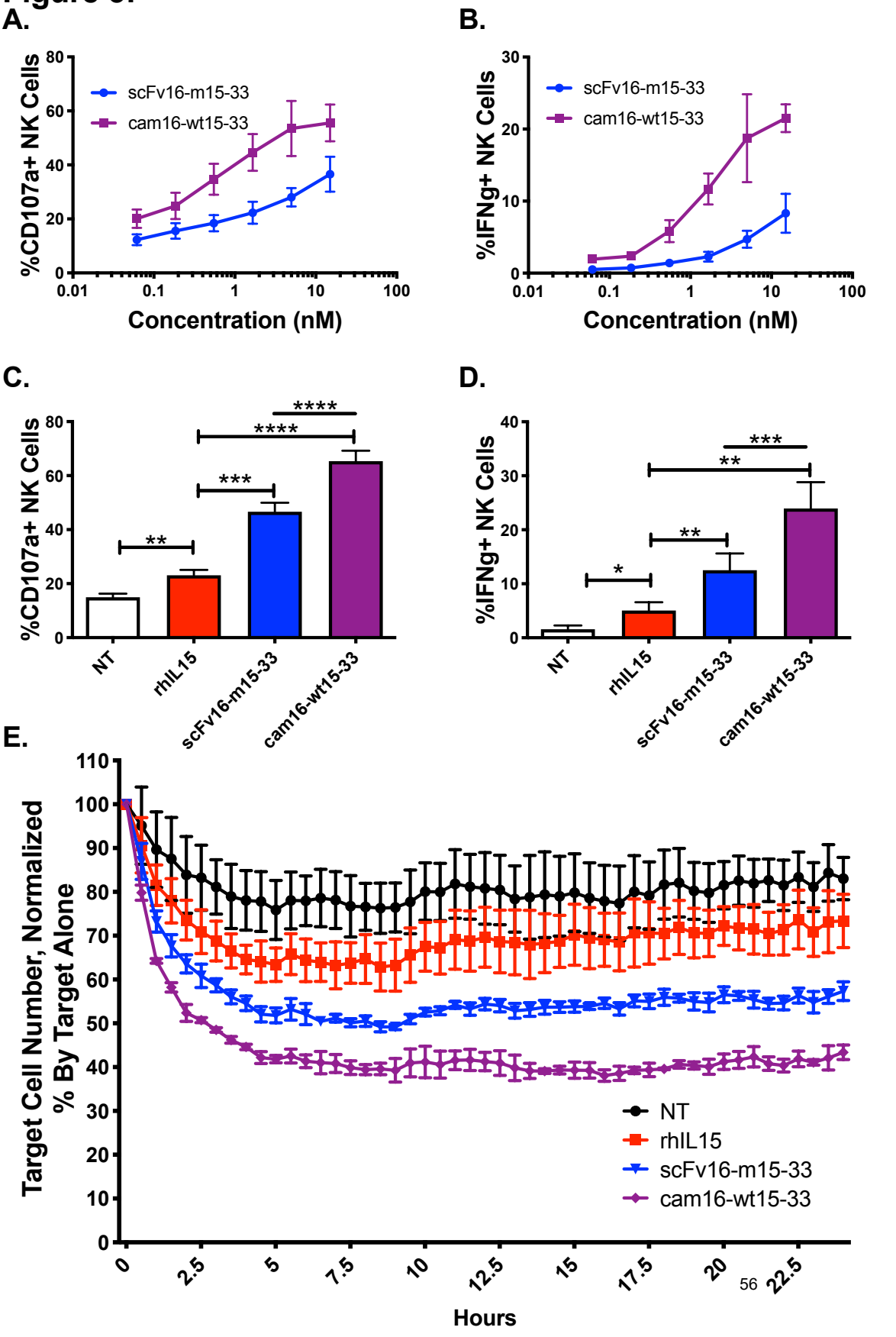

A.
%CD107a+ NK Cells
scFv16-m15-33
cam16-wt15-33
Concentration (nM)
B.
%IFNg+ NK Cells
scFv16-m15-33
cam16-wt15-33
Concentration (nM)
C.
%CD107a+ NK Cells
NT
rhIL15
scFv16-m15-33
cam16-wt15-33
D.
%IFNg+ NK Cells
NT
rhIL15
scFv16-m15-33
cam16-wt15-33
E.
Target Cell Number, Normalized % By Target Alone
NT
rhIL15
scFv16-m15-33
cam16-wt15-33
Hours

Figure 4:

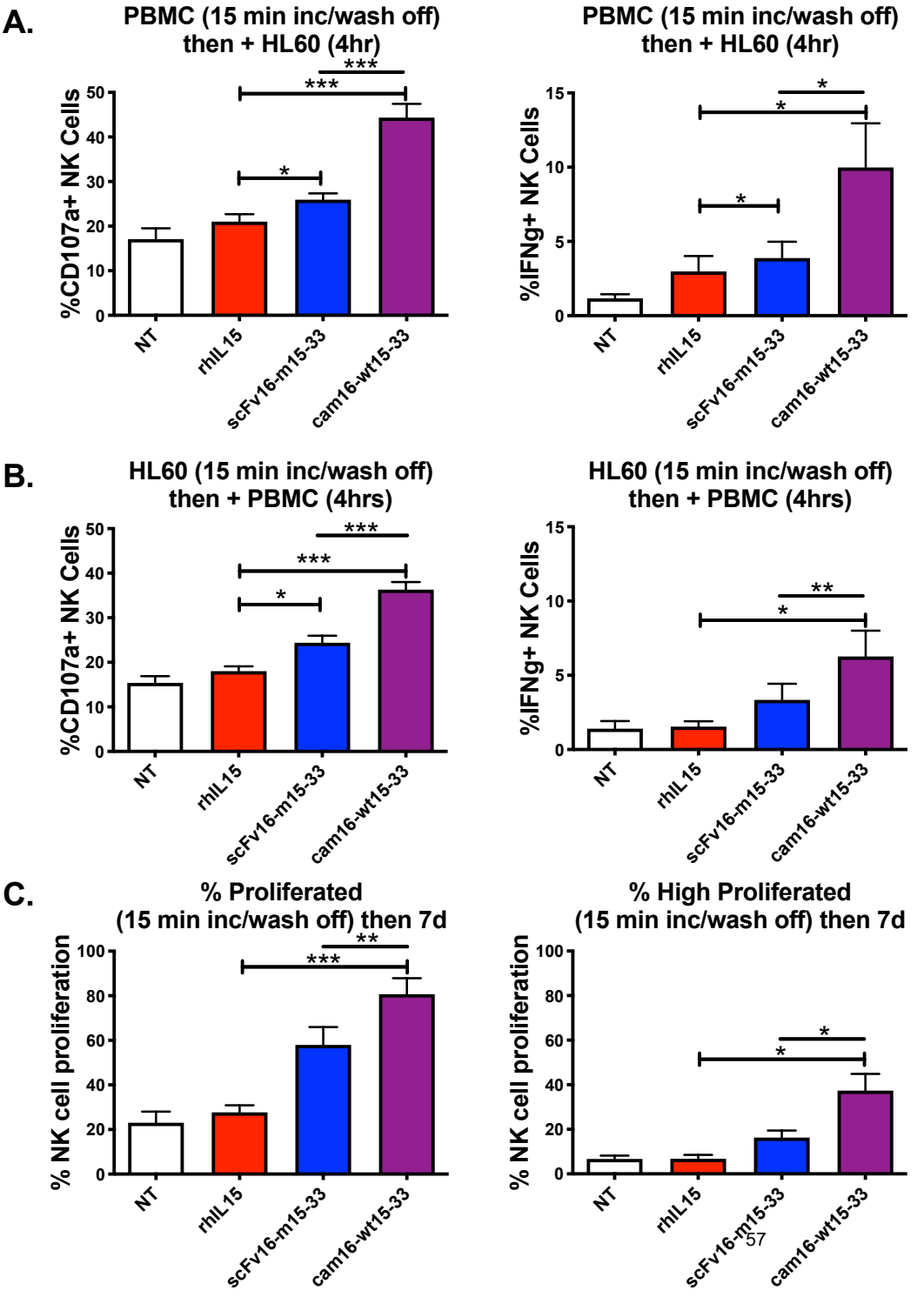

A.
PBMC (15 min inc/wash off) then + HL60 (4hr)
%CD107a+ NK Cells
PBMC (15 min inc/wash off) then + HL60 (4hr)
%IFNg+ NK Cells
B.
HL60 (15 min inc/wash off) then + PBMC (4hrs)
%CD107a+ NK Cells
HL60 (15 min inc/wash off) then + PBMC (4hrs)
%IFNg+ NK Cells
C.
% Proliferated (15 min inc/wash off) then 7d
% NK cell proliferation
% High Proliferated (15 min inc/wash off) then 7d
% NK cell proliferation
NT
rhIL15
scFv16-m15-33
cam16-wt15-33

Figure 5:

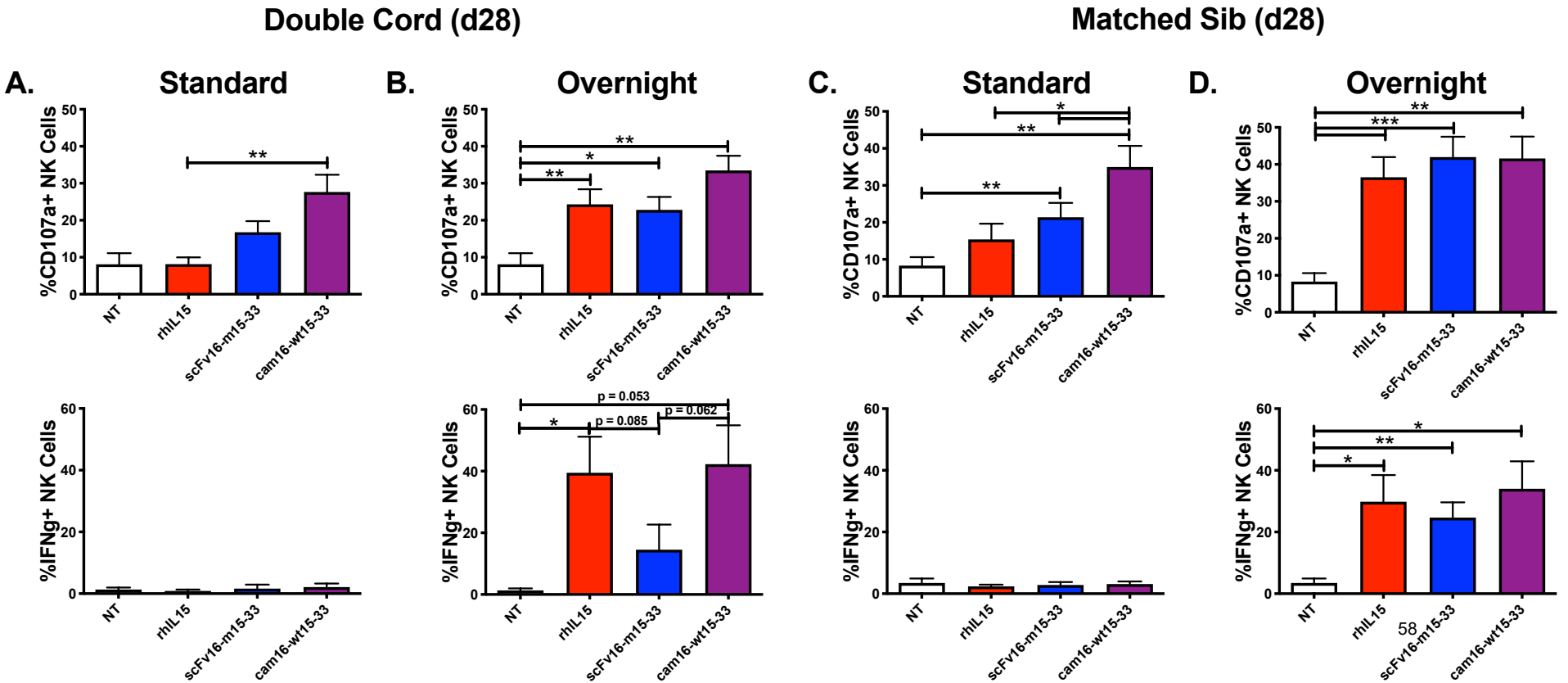
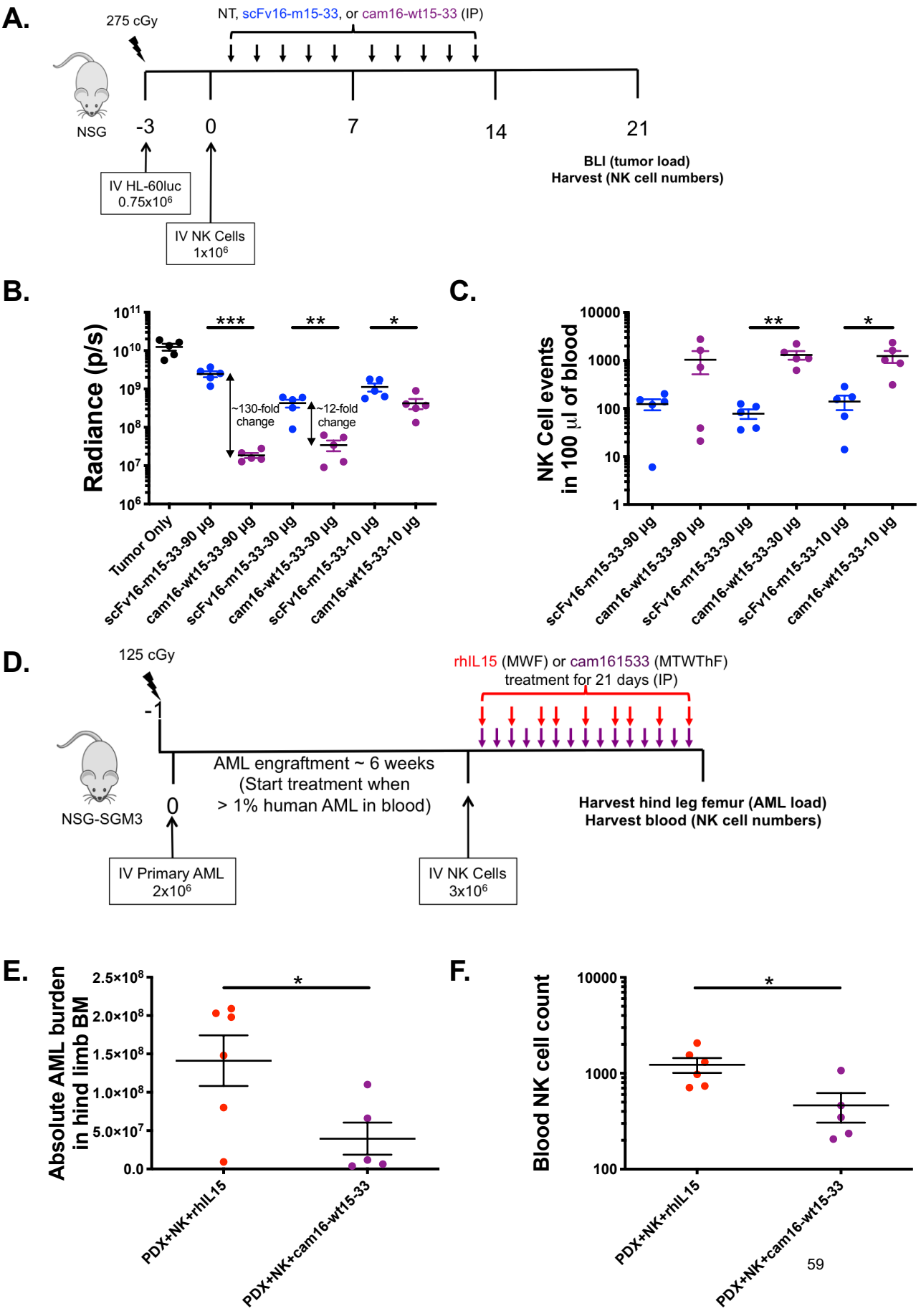

Double Cord (d28)
Matched Sib (d28)
A.
Standard
B.
Overnight
C.
Standard
D.
Overnight
%CD107a+ NK Cells
%IFNg+ NK Cells
NT
rhIL15
scFv16-m15-33
cam16-wt15-33
p = 0.053
p = 0.085
p = 0.062

Figure 6:


A.
275 cGy
NT, scFv16-m15-33, or cam16-wt15-33 (IP)
NSG
-3
0
7
14
21
BLI (tumor load)
Harvest (NK cell numbers)
IV HL-60luc
0.75x10^6
IV NK Cells
1x10^6
B.
Radiance (p/s)
***
**
*
~130-fold change
~12-fold change
Tumor Only
scFv16-m15-33-90 µg
cam16-wt15-33-90 µg
scFv16-m15-33-30 µg
cam16-wt15-33-30 µg
scFv16-m15-33-10 µg
cam16-wt15-33-10 µg
C.
NK Cell events in 100 µl of blood
**
*
scFv16-m15-33-90 µg
cam16-wt15-33-90 µg
scFv16-m15-33-30 µg
cam16-wt15-33-30 µg
scFv16-m15-33-10 µg
cam16-wt15-33-10 µg
D.
125 cGy
-1
rhIL15 (MWF) or cam161533 (MTWThF)
treatment for 21 days (IP)
AML engraftment ~ 6 weeks
(Start treatment when
> 1% human AML in blood)
NSG-SGM3
0
Harvest hind leg femur (AML load)
Harvest blood (NK cell numbers)
IV Primary AML
2x10^6
IV NK Cells
3x10^6
E.
Absolute AML burden in hind limb BM
*
PDX+NK+rhIL15
PDX+NK+cam16-wt15-33
F.
Blood NK cell count
*
PDX+NK+rhIL15
PDX+NK+cam16-wt15-33

Appendix C: Patent Conversion Application

NK ENGAGER MOLECULES AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims benefit of priority under 35 U.S.C. §119(e) of U.S. Serial 62/747,983, filed October 19, 2018, the entire contents of which is incorporated herein by reference in its entirety.

GOVERNMENT FUNDING

[002] This invention was made with government support under CA111412 and CA65493, awarded by the National Institutes of Health, under CA36725, CA72669, CA077598 and CA197292, awarded by the National Cancer Institute and under CA150085, awarded by the U.S. Department of Defense. The government has certain rights in the invention.

SEQUENCE LISTING

[003] The material in the accompanying sequence listing is hereby incorporated by reference into this application. The accompanying sequence listing text file, name GTBIO2090_1WO_Sequence_Listing.txt, was created on October 15, 2019, and is 23 kb. The file can be accessed using Microsoft Word on a computer that uses Windows OS.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[004] The invention relates generally to immunotherapy and more specifically to compositions useful for engaging natural killer (NK) cells in an immune response.

BACKGROUND INFORMATION

[005] Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system capable of immune surveillance. Like cytotoxic T cells, NK cells deliver a store of membrane penetrating and apoptosis-inducing granzyme and perforin granules. Unlike T cells, NK cells do not require antigen priming and recognize targets by engaging activating receptors in the absence of MHC recognition. NK cells express CD16, an activation receptor that binds to the Fc portion of IgG antibodies and is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells are regulated by IL-15, which can induce increased antigen-dependent cytotoxicity, lymphokine-activated killer activity, and/or mediate interferon (IFN), tumor-necrosis factor (TNF) and/or granulocyte-macrophage colony-stimulating factor (GM-CSF) responses. All of these IL-15- activated functions contribute to improved cancer defense.

[006] Therapeutically, adoptive transfer of NK cells can, for example, induce remission in patients with refractory acute myeloid leukemia (AML) when combined with lymphodepleting chemotherapy and IL-2 to stimulate survival and in vivo expansion of NK cells. This therapy can be limited by lack of antigen specificity and IL-2-mediated induction of regulatory T (Treg) cells that suppress NK cell proliferation and function. Generating a reagent that drives NK cell antigen specificity, expansion, and/or persistence, while bypassing the negative effects of Treg inhibition, can enhance NK-cell-based immunotherapies.

SUMMARY OF THE INVENTION

[007] The present invention relates to compounds and compositions for activating NK cells to stimulate an immune response for treating cancer and other disorders. In one embodiment, the invention provides a compound including an NK engaging domain; an NK activating domain operably linked to the NK engaging domain; and a targeting domain that selectively binds to a target cell and is operably linked to the NK activating domain and the NK engaging domain, wherein the targeting domain selectively binds to CLEC12A.

[008] In some embodiments, the NK engaging domain includes a moiety that selectively binds to CD16. In some embodiments, the NK engaging domain moiety includes an antibody or a binding fragment thereof or a nanobody, also known as single domain antibody (sdAb or VHH). In some embodiments, the antibody binding fragment includes an scFv, a F(ab)2, or a Fab. In some embodiments, the antibody or a binding fragment thereof or the nanobody is human or humanized. In some embodiments, the antibody or a binding fragment thereof or the nanobody is camelid.

[009] In some embodiments, the NK activating domain includes a cytokine or functional fragment thereof. In some embodiments, the NK activating domain includes IL-15 or a functional fragment thereof. In some embodiments, the IL-15 includes the amino acid sequence of SEQ ID NO:9 or a functional variant thereof. In one aspect, the functional variant of IL-15 includes an N72D or N72A amino acid substitution compared to SEQ ID NO:9.

[0010] In some embodiments, the targeting domain moiety includes an antibody or a binding fragment thereof or a nanobody. In some embodiments, the antibody binding fragment includes an scFv, a F(ab)2, or a Fab.

[0011] In some embodiments, the NK engaging domain includes a moiety that selectively binds to CD16, the NK activating domain includes IL-15, and the targeting domain selectively binds to CLEC12A.

[0012] In some embodiments, the compounds and compositions described herein include at least one flanking sequence linking two of the domains. In some embodiments, the

compounds and compositions described herein further include a second flanking sequence linking the two linked domains with a third domain. In some embodiments, the flanking sequences flank the NK activating domain. In some embodiments, a first flanking sequence is C-terminal to the NK engaging domain and a second flanking sequence is N-terminal to the anti-CLEC12A targeting domain.

[0013] In some embodiments, provided herein is an isolated amino acid sequence including SEQ ID NO.: 1. In some embodiments, provided herein is an isolated DNA sequence encoding the amino acid sequence of SEQ ID NO.: 1.

[0014] In some embodiments, provided herein is an isolated amino acid sequence including SEQ ID NO.: 2. In some embodiments, provided herein is an isolated DNA sequence encoding the amino acid sequence of SEQ ID NO.: 2.

[0015] In some embodiments, provided herein is an isolated amino acid sequence including SEQ ID NO.: 4. In some embodiments, provided herein is an isolated DNA sequence encoding the amino acid sequence of SEQ ID NO.: 4.

[0016] In some embodiments, provided herein are compositions including the compounds described herein and a pharmaceutically acceptable carrier.

[0017] In some embodiments, provided herein are methods including: administering to a subject a compound described herein in an amount effective to induce NK-mediated killing of a target cell. In some embodiments, the target cell is a cancer cell.

[0018] In some embodiments, provided herein are methods for stimulating expansion of NK cells in vivo, the methods including: administering to a subject an amount of a compound described herein effective to stimulate expansion of NK cells in the subject.

[0019] In some embodiments, provided herein are methods of treating cancer in a subject, the methods including: administering to the subject an amount of a compound described herein effective for treating the cancer. In some embodiments, the cancer includes prostate cancer, lung cancer, colon cancer, rectum cancer, urinary bladder cancer, melanoma, kidney cancer, renal cancer, oral cavity cancer, pharynx cancer, pancreas cancer, uterine cancer, thyroid cancer, skin cancer, head and neck cancer, cervical cancer, ovarian cancer, or hematopoietic cancer. In some embodiments, the methods provided herein further include administering the compound prior to, simultaneously with, or following chemotherapy, surgical resection of a tumor, or radiation therapy. In some embodiments, the chemotherapy includes altretamine, amsacrine, L-asparaginase, colaspase, bleomycin, busulfan, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytophosphane, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel,

doxorubicin, epirubicin, etoposide, fluorouracil, fludarabine, fotemustine, ganciclovir, gemcitabine, hydroxyurea, idarubicin, ifosfamaide, irinotecan, lomustine, melphalan, mercaptopurine, methotrexate, mitoxantrone, mitomycin C, nimustine, oxaliplatin, paclitaxel, pemetrexed, procarbazine, raltitrexed, temozolomide, teniposide, tioguanine, thiotepa, topotecan, vinblastine, vincristine, vindesine, and vinorelbine. In some embodiments, the hematopoietic cancer is AML.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] **FIGURES 1A-1C** illustrate NK cell proliferation (FIGURE 1A), NK cell killing (FIGURE 1B), and functional assays (FIGURE 1C) upon treatment with CLEC12A TriKE.

[0021] **FIGURE 2** illustrates the percentage of CD33 and CLEC12A surface expression on primary AML samples from 10 patients.

[0022] **FIGURES 3A-3B** illustrate the CD16-IL15-CLEC12A TriKE (FIGURE 3A) and mechanisms of action (FIGURE 3B).

[0023] **FIGURE 4** illustrates binding of the CD16-IL15-CLEC12A TriKE to targets that express CLEC12A.

[0024] **FIGURES 5A-5B** illustrate CD16-IL15-CLEC12A TriKE promotion of NK cell proliferation.

[0025] **FIGURES 6A-6C** illustrate CD16-IL15-CLEC12A TriKE induction of degranulation (FIGURE 6A) and cytokine production (FIGURES 6B-C) against AML target cells.

[0026] **FIGURES 7A-7B** illustrate CD16-IL15-CLEC12A TriKE induction of AML target cell killing.

[0027] **FIGURES 8A-8D** illustrate CD16-IL15-CLEC12A TriKE induced killing of primary AML targets in vitro.

[0028] **FIGURES 9A-9C** illustrate CD16-IL15-CLEC12A TriKE induction of NK cell proliferation.

[0029] **FIGURES 10A-10D** illustrate functional validation of the CD16-IL15-CLEC12A TriKE.

[0030] **FIGURES 11A-11C** illustrate CD16-IL15-CLEC12A TriKE induction of target cell killing in a real-time imaging assay. THP-1 tumor targets are shown.

[0031] **FIGURES 12A-12G** illustrate CD16-IL15-CLEC12A TriKE-induced killing of primary AML blasts.

[0032] **FIGURES 13A-13G** illustrate that CD16-IL15-CLEC12A TriKE limits tumor growth in vivo.

[0033] **FIGURES 14A-14C** illustrate binding validation of the CD16-IL15-CLEC12A TriKE.

[0034] **FIGURES 15A-15B** illustrate CD16-IL15-CLEC12A TriKE induction of target cell killing in a real-time imaging assay. HL-60 tumor targets are shown.

[0035] **FIGURES 16A-16B** illustrate CD16-IL15-CLEC12A TriKE-mediated target killing. Target gating strategy (FIGURE 16A) and target cell killing (FIGURE 16B) are shown. AML blast targets are shown.

[0036] **FIGURE 17** illustrates a gating strategy to identify cancer stem cells in bone marrow samples from AML patients.

[0037] **FIGURES 18A-18B** illustrate CLEC12A and CD33 expression within the CD34pos progenitor compartment in bone marrow. Cell populations from two representative donors (FIGURE 18A) and cell colonies after treatment with the indicated TriKE (FIGURE 18B) are shown.

[0038] **FIGURE 19** illustrates a gating strategy to determine different CD34pos progenitor subpopulations in healthy bone marrow samples.

DETAILED DESCRIPTION OF THE INVENTION

[0039] Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system capable of immune surveillance. Like cytotoxic T cells, NK cells deliver a store of membrane penetrating and apoptosis-inducing granzyme and perforin granules. Unlike T cells, NK cells do not require antigen priming and recognize targets by engaging activating receptors in the absence of MHC recognition. NK cells express CD16, an activation receptor that binds to the Fc portion of IgG antibodies and is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells are regulated by IL-15, which can induce increased antigen-dependent cytotoxicity, lymphokine-activated killer activity, and/or mediate interferon (IFN), tumor-necrosis factor (TNF) and/or granulocyte-macrophage colony-stimulating factor (GM-CSF) responses. All of these IL-15- activated functions contribute to improved cancer defense.

[0040] Therapeutically, adoptive transfer of NK cells can, for example, induce remission in patients with refractory acute myeloid leukemia (AML) when combined with lymphodepleting chemotherapy and IL-2 to stimulate survival and in vivo expansion of NK cells. This therapy can be limited by lack of antigen specificity and IL-2-mediated induction of regulatory T (Treg) cells that suppress NK cell proliferation and function. Generating a

reagent that drives NK cell antigen specificity, expansion, and/or persistence, while bypassing the negative effects of Treg inhibition, can enhance NK-cell-based immunotherapies.

[0041] This disclosure describes generating a tri-specific molecule that includes two domains capable of driving NK-cell-mediated killing of tumor cells (e.g., CD33+ and/or CD33- tumor cells) and an intramolecular NK activating domain capable of generating an NK cell self-sustaining signal. The tri-specific molecule can drive NK cell proliferation and/or enhance NK-cell-driven cytotoxicity against, for example, HL-60 targets, cancer cells, or cancer cell-derived cell lines.

[0042] The invention is based on the development of a CD16/IL-15/CD33 trispecific killer engager (TriKE) molecule to target acute myeloid leukemia (AML) cells using Natural Killer (NK) cells. This molecule contains an anti-CD16 camelid nanobody to activate NK cells, an anti-CD33 single chain variable fragment (scFv) to engage cancer targets, and an IL-15 molecule that drives NK cell priming, expansion and survival. Using an earlier version of this molecule, the CD33 TriKE was shown to be effective at activating NK cells against AML targets in vitro and in vivo. This preclinical data has led to the establishment of a clinical trial in refractory AML patients at the University of Minnesota, set to open Q3 2018. While these previous studies have validated the use of TriKEs as an effective strategy of harnessing NK cells in cancer immunotherapy, CD33 has limitations as a target antigen.

[0043] The high mortality and poor five-year survival rates (26%) for AML patients can be attributed to chemotherapy resistance and disease relapse. A majority of chemotherapy resistant leukemia stem cells (LSCs) that are hypothesized to facilitate relapse do not express CD33. In addition, all hematopoietic stem cells and normal myeloid cells express CD33, thus targeting this antigen can lead to severe defects in hematopoiesis and on-target/off-tumor toxicity. To address these limitations, described herein is the development of a TriKE that targets CLEC12A or C-type lectin-like molecule 1 (CLL-1). CLEC12A is highly expressed on AML cells and over 70% of CD33 negative cells express CLEC12A. It has been attributed as a stem cell marker in AML, being selectively overexpressed in LSCs. CLEC12A is expressed by CD34+/CD38- LSCs but not normal CD34+/CD38- hematopoietic stem cells in regenerating bone marrow, thus minimizing off-target effects. C-type lectin domain family 12 member A is a protein that in humans is encoded by the CLEC12A gene. This gene encodes a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell-cell signaling, glycoprotein turnover, and roles in inflammation and immune response. The protein encoded by this gene is a negative regulator of granulocyte and

monocyte function. Several alternatively spliced transcript variants of this gene have been described, but the full-length nature of some of these variants has not been determined. This gene is closely linked to other CTL/CTLD superfamily members in the natural killer gene complex region on chromosome 12p13.

[0044] BiKE and TriKE Compounds

[0045] Bi-specific fusions have been made that incorporate an anti-human anti-CD16 scFv derived from a human phage display library technology (McCall et al., 1999. Mol Immunol. 36:433-445). NK cells mediate antibody-dependent cell-mediated cytotoxicity (ADCC) through the CD16 (FcγRIII) receptor. Signaling through the CD16 receptor induces calcium fluxes and phosphorylation of ITAMs, triggering the release of lytic granules and cytokines such as interferon (IFNγ) and tumor necrosis factor (TNFα). A bi-specific molecule has been designed to trigger the CD16 receptor in conjunction with other targeting molecules (Gleason et al. Blood. 2014 (19):3016-26), a so-called bispecific killer engager (BiKE). With one scFv recognizing NK cells and a second scFv recognizing a tumor antigen, BiKEs can markedly enhance cytotoxic killing in various human cancers. One exemplary BiKE targeted CD33 and enhanced NK cell responses against acute myeloid leukemia (AML) and myelodyplastic syndrome (MDS). MDS is a clonal heterogeneous stem cell disorder characterized by normal or hypercellular bone marrow (BM) with peripheral blood (PB) cytopenias and an increased risk of progressing to AML.

[0046] NK cells are responsive to a variety of cytokines including, for example, IL-15, which is involved in NK cell homeostasis, proliferation, survival, activation, and/or development. For example, IL-15 can activate NK cells, and can restore functional defects in engrafting NK cells after hematopoietic stem cell transplantation (HSCT). IL-15 and IL-2 share several signaling components, including the IL-2/IL-15Rβ (CD122) and the common gamma chain (CD132). Unlike IL-2, IL-15 does not stimulate Tregs, allowing for NK cell activation while bypassing Treg inhibition of the immune response. Besides promoting NK cell homeostasis and proliferation, IL-15 can rescue NK cell functional defects that can occur in the post-transplant setting. IL-15 also can stimulate CD8+ T cell function, further enhancing its immunotherapeutic potential. In addition, based on pre-clinical studies, toxicity profiles of IL-15 may be more favorable than IL-2 at low doses. In accordance with some embodiments, the compositions described herein can be used to activate NK cells and drive NK cell priming, expansion and survival.

[0047] This disclosure describes, in one aspect, tri-specific killer engager (TriKE) molecules that generally include one or, one or more targeting domains (that target, e.g., a

tumor cell or virally-infected cell), and one or more cytokine NK activating domains (e.g., IL-15, IL-12, IL-18, IL-21, or other NK cell enhancing cytokine, chemokine, and/or activating molecule), with each domain operably linked to the other domains. As used herein, the term "operably linked" refers to direct or indirect covalent linking. Thus, two domains that are operably linked may be directly covalently coupled to one another. Conversely, the two operably linked domains may be connected by mutual covalent linking to an intervening moiety (e.g., and flanking sequence). Two domains may be considered operably linked if, for example, they are separated by the third domain, with or without one or more intervening flanking sequences.

[0048] Exemplary BiKE and TriKE molecules or compounds are described in WO2017062604, the disclosure of which is incorporated herein by reference in its entirety.

[0049] This disclosure describes, in some embodiments, compounds that include an NK engaging domain; an NK activating domain operably linked to the NK engaging domain; and a targeting domain that selectively binds to a target cell and is operably linked to the NK activating domain and the NK engaging domain, wherein the targeting domain selectively binds to a target molecule. The target molecule can be expressed on the surface of a target cell, for example. The target cell can be a tumor cell, for example. In some embodiments, the targeting domain selectively binds to CLEC12A.

[0050] As used herein, the terms "selectively binding" or "selectively binds" in reference to the interaction of a binding molecule or a domain described herein, e.g., an antibody or an engaging domain, an activating domain, or a targeting domain, and its binding partner, e.g., an antigen or a receptor, means that the interaction is dependent upon the presence of a particular structure, e.g., an antigenic determinant or epitope or amino acid sequence, on the binding partner. In other words, the binding molecule or domain preferentially binds or recognizes the binding partner even when the binding partner is present in a mixture of other molecules. The binding may be mediated by covalent or non-covalent interactions or a combination of both. The terms "selectively binding" or "selectively binds" and "specifically binding" or "specifically binds" may be used interchangeably.

[0051] The compounds described herein can possess or lack a His tag. A His tag allows for purification of a protein and can be useful in research applications, for example. The His tag may be placed at the C-terminus or at the N-terminus of the compounds or molecules described herein and may include a spacer N-terminal or C-terminal to the His tag. As an example, a His tag that is placed at the C-terminus of a compound or molecule described herein can include a spacer N-terminal to the His tag. As another example, a His tag that is

placed at the N-terminus of a compound or molecule described herein can include a spacer C-terminal to the His tag. An exemplary His tag with spacer is SEQ ID NO:3. SEQ ID NO:3 can be placed at the C-terminus of the compounds described herein. A person skilled in the art will appreciate that any number of His repeats can constitute a His tag and that any spacer sequence of any length or no spacer can be used.

[0052] In some embodiments, the TriKE compound or molecule that selectively binds to CLEC12A includes the isolated amino acid sequence of SEQ ID NO:1. In some embodiments, the TriKE compound or molecule that selectively binds to CLEC12A includes the isolated amino acid sequence of SEQ ID NO:2. In some embodiments, the targeting domain of the compounds described herein that selectively binds to CLEC12A includes the isolated amino acid sequence of SEQ ID NO:4.

[0053] Also described herein are nucleic acid sequences that encode the sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4. For example, SEQ ID NO:1 can be encoded by SEQ ID NO:5, SEQ ID NO:2 can be encoded by SEQ ID NO:6, and SEQ ID NO:4 can be encoded by SEQ ID NO:7. A person of skill in the art will appreciate that any functional variants of the nucleic acid molecules provided herein are contemplated in the present disclosure. Functional variants are nucleic acid sequences that can be translated to provide an amino acid sequence homologous or identical to that translated from a parent molecule.

[0054] NK Engaging Domain

[0055] The NK engaging domain can include any moiety that binds to and/or activates an NK cell and/or any moiety that blocks inhibition of an NK cell. Exemplary NK cell engaging domains include a moiety that binds to, e.g., CD16, CD16+CD2, CD16+DNAM, or CD16+NKp46. In some embodiments, the engaging domain includes a moiety that selectively binds to CD16. In some embodiments, the NK engaging domain activates an NK cell. In some embodiments, the NK engaging domain blocks inhibition of an NK cell.

[0056] In some embodiments, the NK engaging domain can include an antibody that selectively binds to a component of the surface of an NK cell. In other embodiments, the NK engaging domain can include a ligand or small molecule that selectively binds to a component of the surface of an NK cell. As used herein, the term “selectively binds” refers to the ability to differentiate between two or more alternatives such as, for example, having differential affinity, to any degree, for a particular target. As used herein, “antibody” refers generally an immunoglobulin or a fragment thereof and thus encompasses a monoclonal antibody, a fragment thereof (e.g., scFv, Fab, F(ab’)2, Fv or other modified forms), a combination of monoclonal antibodies and/or fragments thereof, and/or a combination of polyclonal

antibodies. Thus, for brevity, reference to an antibody that selectively binds to a component of the surface of an NK cell includes any antibody fragment that exhibits the described binding character. Similarly, reference to a ligand that selectively binds to a component of the surface of an NK cell includes any fragment of the ligand that exhibits the described binding character.

[0057] In some embodiments, the NK engaging domain can selectively bind to a receptor at least partially located at the surface of an NK cell. In certain embodiments, the NK engaging domain can serve a function of binding an NK cell and thereby bring the NK into spatial proximity with a target to which the targeting domain - described in more detail below - selectively binds. In certain embodiments, however, the NK engaging domain can selectively bind to a receptor that activates the NK cell and, therefore, also possess an activating function. As described above, activation of the CD16 receptor can elicit antibody-dependent cell-mediated cytotoxicity. Thus, in certain embodiments, the NK engaging domain can include at least a portion of an anti-CD16 receptor antibody effective to selectively bind to the CD16 receptor. In other embodiments, the NK engager cell domain may interrupt mechanisms that inhibit NK cells. In such embodiments, the NK engager domain can include, for example, anti-PD1/PDL1, anti-NKG2A, anti-TIGIT, anti-killer-immunoglobulin receptor (KIR), and/or any other inhibition blocking domain.

[0058] One can design the NK engaging domain to possess a desired degree of NK selectivity and, therefore, a desired immune engaging character. For example, CD16 has been identified as Fc receptors FcγRIIIa (CD16a) and FcγRIIIb (CD16b). These receptors bind to the Fc portion of IgG antibodies that then activates the NK cell for antibody-dependent cell-mediated cytotoxicity. Anti-CD16 antibodies selectively bind to NK cells, but also can bind to neutrophils. Anti-CD16a antibodies selectively bind to NK cells, but do not bind to neutrophils. A TriKE embodiment that includes an NK engaging domain that includes an anti-CD16a antibody can bind to NK cells but not bind to neutrophils. Thus, in circumstances where one may want to engage NK cells but not engage neutrophils, one can design the NK engaging domain of the TriKE to include an anti-CD16a antibody.

[0059] While described herein in the context of various embodiments in which the NK engaging domain includes an anti-CD16 receptor scFv, the NK engaging domain can include any antibody or other ligand that selectively binds to the CD16 receptor. Moreover, the NK engaging domain can include an antibody or ligand that selectively binds to any NK cell receptor such as, for example, the cell cytotoxicity receptor 2B4, low affinity Fc receptor CD16, killer immunoglobulin like receptors (KIR), CD2, NKG2A, TIGIT, NKG2C, LIR-1,

and/or DNAM-1. In one embodiment, the invention composition is a construct in operable linkage NKG2C/IL-15/CD33. It should be understood that the positioning of the moieties may be changed based on activity assays (e.g., CD33/IL-15/NKG2C).

[0060] In some embodiments, the NK engaging domain includes an antibody or a binding fragment thereof, or a nanobody. The antibody binding fragment can be an scFv, a F(ab)2, or a Fab. In some embodiments, the NK engaging domain includes a nanobody. In some embodiments, the NK cell engager can involve the use of a humanized CD16 engager derived from an animal nanobody. While an scFv has a heavy variable chain component and a light variable chain component joined by a linker, a nanobody consists of a single monomeric variable chain - i.e., a variable heavy chain or a variable light chain - that is capable of specifically engaging a target. A nanobody may be derived from an antibody of any suitable animal such as, for example, a camelid (e.g., a llama or camel) or a cartilaginous fish. A nanobody can provide superior physical stability, an ability to bind deep grooves, and increased production yields compared to larger antibody fragments.

[0061] In one exemplary embodiment, a nanobody-based NK engager molecule can involve a humanized CD16 nanobody derived from a published llama nanobody (GeneBank sequence EF561291; Behar et al., 2008. Protein Eng Des Sel. 21(1):1-10), termed EF91. Llama EF91 was initially constructed into a BiKE containing CD19 to test the ability of this CD16 engager to drive NK cell activation. It showed functionality similar to rituximab-mediated killing in a chromium release assay with Raji targets. Upon confirming functionality of the molecule, the CDRs were cloned into a humanized camelid scaffold (Vincke et al., 2009. J Biol Chem. 284(5):3273-3284) to humanize the CD16 engager, now termed HuEF91. The binding of HuEF91 is equivalent to binding observed using a standard CD16 scFv, indicating that incorporating the llama nanobody variable heavy chain into the humanized backbone has not hindered the specificity of the molecule. The use HuEF91 as an NK engager in the TriKE molecules described herein can increase drug yield, increase stability, and/or increase NK-cell-mediated ADCC efficacy.

[0062] Thus, in accordance with some embodiments, the antibody or a binding fragment thereof or the nanobody is human or humanized. In some embodiments, the antibody or a binding fragment thereof or the nanobody is camelid.

[0063] NK Activating Domain

[0064] The NK activating domain can include an amino acid sequence that activates NK cells, promotes sustaining NK cells, or otherwise promotes NK cell activity. The NK activating domain can be, or can be derived from, one or more cytokines that can activate

and/or sustain NK cells. As used herein, the term "derived from" refers to an amino acid fragment of a cytokine (e.g., IL-15) that is sufficient to provide NK cell activating and/or sustaining activity. In embodiments that include more than one NK activating domain, the NK activating domains may be provided in series or in any other combination. Additionally, each cytokine-based NK activating domain can include either the full amino acid sequence of the cytokine or may be an amino acid fragment, independent of the nature of other NK activating domains included in the TriKE molecule. Exemplary cytokines on which an NK activating domain may be based include, for example, IL-15, IL-18, IL-12, and IL-21. Thus, while described in detail herein in the context of an exemplary model embodiment in which the NK activating domain is derived from IL-15, a TriKE may be designed using an NK activating domain that is, or is derived from, any suitable cytokine.

[0065] For brevity in this description, reference to an NK activating domain by identifying the cytokine on which it is based includes both the full amino acid sequence of the cytokine, any suitable amino acid fragment of the cytokine, and or a modified version of the cytokine that includes one or more amino acid substitutions.. Thus, reference to an "IL-15" NK activating domain includes an NK activating domain that includes the full amino acid sequence of IL-15, an NK activating domain that includes a fragment of IL-15, or an NK activating domain such as, for example, IL-15N72D or IL-15N72A, that includes an amino acid substitution compared to the wild-type IL-15 amino acid sequence.

[0066] The use of an IL-15 NK activating domain in a TriKE can provide sustained NK cell activity - as evidenced in a mouse model showing human NK cells are dramatically elevated and cancer reduced - even after three weeks. NK cells are activated in mice to produce an array of anti-cancer factors and cytokines. Moreover, an IL-15 NK activating domain can alter the chemistry of these molecules so that they refold more easily and/or are recoverable in greater yield, thus rendering the TriKE molecules more suitable for clinical scale-up.

[0067] Thus, in some embodiments, the NK activating domain includes a cytokine or functional fragment thereof. In some embodiments, the activating domain includes IL-15 or a functional fragment thereof. In some embodiments, the IL-15 is wild-type IL-15. In some embodiments, the IL-15 is human. In some embodiments, the IL-15 is wild-type human IL-15. In some embodiments, the IL-15 comprises an amino acid sequence of SEQ ID NO:9 or a functional variant thereof. In some embodiments, the functional variant of IL-15 comprises an N72D or N72A amino acid substitution as compared to SEQ ID NO:9.

[0068] As used herein, the term "functional variant" refers to a molecule, including a binding molecule, for example, comprises a nucleotide and/or amino acid sequence that is altered by one or more nucleotides and/or amino acids compared to the nucleotide and/or amino acid sequences of the parent molecule. For a binding molecule, a functional variant is still capable of competing for binding to the binding partner with the parent binding molecule. In other words, the modifications in the amino acid and/or nucleotide sequence of the parent binding molecule do not significantly affect or alter the binding characteristics of the binding molecule encoded by the nucleotide sequence or containing the amino acid sequence, i.e., the binding molecule is still able to recognize and bind its target. The functional variant may have conservative sequence modifications including nucleotide and amino acid substitutions, additions and deletions. These modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and random PCR-mediated mutagenesis.

[0069] Functional variants can also include, but are not limited to, derivatives that are substantially similar in primary structural sequence, but which contain e.g., in vitro or in vivo modifications, chemical and/or biochemical, that are not found in the parent binding molecule. uch modifications include inter alia acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI-anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA-mediated addition of amino acids to proteins such as arginylation, ubiquitination, and the like.

[0070] Targeting Domain and Targets

[0071] The targeting domain can include any moiety that selectively binds to an intended target such as, for example, a tumor cell, a target in the cancer stroma, a target on an inhibitory cell such as myeloid derived suppressor cells that are CD33+, or a target on a virally-infected cell. Thus, a targeting domain can include, for example, an anti-tumor antibody such as rituximab (anti-CD20), afutuzumab (anti-CD20), trastuzumab (anti-HER2/neu), pertuzumab (anti-HER2/neu), labetuzumab (anti-CEA), adecatumumab (anti-EpCAM), citatuzumab bogatox (anti-EpCAM), edrecolomab (anti-EpCAM), arcitumomab (anti-CEA), bevacizumab (anti-VEGF-A), cetuximab (anti-EGFR), nimotuzumab (anti-EGFR),

panitumumab (anti-EGFR), zalutumumab (anti-EGFR), gemtuzumab ozogamicin (anti-CD33), lintuzumab (anti-CD33), etaracizumab (anti-integrin $\alpha_v\beta_3$), intetumumab (anti-CD51), ipilimumab (anti-CD152), oregovomab (anti-CA-125), votumumab (anti-tumor antigen CTAA16.88), or pemtumumab (anti-MUC1), anti-CD19, anti-CD22, anti-CD133, anti-CD38 anti-mesothelin, anti-ROR1, CSPG4, SS1, or IGFR1. Any tumor marker can be targeted. In some embodiments, the targeting domain, or tumor-associated antigen targeted, can include CD133, CD20, HER2, CEA, EpCAM, VEGF-A, EGFR, CD33, integrin αVβ3, CD51, CD152, CD125, CTAA16.88, MUC1, CD19, CD22, CD38, mesothelin, ROR1, CSPG4, SS1, or IGFR1, NKG2 family members, including but not limited to 2A, 2B, 2C, and the like, BCMA, APRIL, B7H3, and PSMA, by way of example.

[0072] In some embodiments, the target cell is a tumor cell. In some embodiments, the tumor cell is CD33+. In some embodiments, the tumor cell is CD33-. In some embodiments, the tumor cell is a hematopoietic cancer cell. In some embodiments, the tumor cell is a leukemic cell. In some embodiments, the leukemic cell is an acute myeloid leukemia (AML) cell. In other embodiments, the targeting domain can selectively bind to a target on a cell infected by a virus such as, for example, EBV, HBV, HCV, and/or HPV. In some embodiments, a viral target is a tumor marker or a tumor antigen. Any viral tumor marker or viral or non-viral tumor antigen can be targeted.

[0073] The targeting domain moiety can include an antibody or a binding fragment of an antibody, or a nanobody, as described above. The antibody binding fragment can comprise an scFv, a F(ab)2, or a Fab.

[0074] In certain particular embodiments, the targeting domain can include an anti-CLEC12A antibody. In other particular embodiments, a second targeting domain can be included. The second targeting domain can include a moiety that can bind to any of the targets described above. In some embodiments, the second targeting domain can selectively bind to CD33.

[0075] In some embodiments, the compounds described herein include an NK engaging domain having a moiety that selectively binds to CD16, an activating domain having IL-15, and a targeting domain that selectively binds to CLEC12A. The terms “CLEC12A Trike,” “1615CLEC12A TriKe,” and “CD16-IL15-CLEC12A TriKE” can be used interchangeably to refer to a TriKE that targets CLEC12A, unless the context clearly indicates otherwise.

[0076] Flanking Sequences

[0077] In some embodiments, the compounds described herein can further include a flanking sequence or linker sequence that can link two of the above-described domains. The

terms "flanking sequence" and "linker sequence" can be used interchangeably, unless context clearly indicates otherwise. In some embodiments, the presence of the flanking sequence can further increase NK cell activation. Any amino acid sequence can be a flanking sequence or a linker sequence. One exemplary flanking sequence includes the 20 amino acids of SEQ ID NO:13. Another exemplary flanking sequence includes the seven amino acids of SEQ ID NO:14. Yet other exemplary flanking sequences include SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:15. As yet another example, any number of repeats of an amino acid sequence can be a flanking sequence or a linker sequence. For example, any number of repeats of the sequence of SEQ ID NO:15 can be a flanking sequence or a linker sequence. Sequence repeats can be complete or partial, and complete or partial repeats can be at the beginning, i.e., at the N-terminus, or the end, i.e., at the C-terminus, of a flanking sequence or linker sequence. Flanking sequences can be in any orientation.

[0078] Certain embodiments (e.g., the 1615CLEC12A TriKE without a His tag, SEQ ID NO:1 or 1615CLEC12A TriKE with a His tag, SEQ ID NO:2) can include more than one flanking sequence. As one example, SEQ ID NO:1 and/or SEQ ID NO:2 include the flanking sequence of SEQ ID NO:11 to link the NK engaging domain (e.g., anti-CD16 receptor scFv) with the NK activating domain (e.g., IL-15). SEQ ID NO:1 and/or SEQ ID NO:2 also include the flanking sequence of SEQ ID NO:12 to link the NK activating domain with the targeting domain (e.g., anti-CLEC12A scFv). The flanking sequences that link the domains of the molecule can be the same or can be different. As an example, the same or different flanking sequences can link the NK engaging domain (e.g., anti-CD16 receptor scFv) with the NK activating domain (e.g., IL-15) and the NK activating domain with the targeting domain (e.g., anti-CLEC12A scFv). In some embodiments, constructs that lack a flanking sequence exhibit reduced activity compared to constructs that possess the flanking sequence.

[0079] In some embodiments, the compounds described herein include at least one flanking sequence linking two of the domains. In some embodiments, the compounds described herein further include a second flanking sequence linking the two linked domains with a third domain. In some embodiments, the flanking sequences are the same. In some embodiments, the flanking sequences are different.

[0080] In some embodiments, the flanking sequences flank the NK activating domain. In some embodiments, a first flanking sequence is C-terminal to the NK engaging domain. In some embodiments, a second flanking sequence is N-terminal to the anti-CLEC12A targeting domain. In some embodiments, a first flanking sequence is C-terminal to the NK engaging domain and a second flanking sequence is N-terminal to the anti-CLEC12A targeting domain.

[0081] Formulations

[0082] The compounds described herein may be formulated with a pharmaceutically acceptable carrier. As used herein, "carrier" includes any solvent, dispersion medium, vehicle, coating, diluent, antibacterial, and/or antifungal agent, isotonic agent, absorption delaying agent, buffer, carrier solution, suspension, colloid, and the like. The use of such media and/or agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions. As used herein, "pharmaceutically acceptable" refers to a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with a TriKE molecule without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

[0083] A TriKE molecule may therefore be formulated into a pharmaceutical composition. The pharmaceutical composition may be formulated in a variety of forms adapted to a preferred route of administration. Thus, a composition can be administered via known routes including, for example, oral, parenteral (e.g., intradermal, transcutaneous, subcutaneous, intramuscular, intravenous, intraperitoneal, etc.), or topical (e.g., intranasal, intrapulmonary, intramammary, intravaginal, intrauterine, intradermal, transcutaneous, rectally, etc.). A pharmaceutical composition can be administered to a mucosal surface, such as by administration to, for example, the nasal or respiratory mucosa (e.g., by spray or aerosol). A composition also can be administered via a sustained or delayed release.

[0084] Thus, a TriKE molecule may be provided in any suitable form including but not limited to a solution, a suspension, an emulsion, a spray, an aerosol, or any form of mixture. The composition may be delivered in formulation with any pharmaceutically acceptable excipient, carrier, or vehicle. For example, the formulation may be delivered in a conventional topical dosage form such as, for example, a cream, an ointment, an aerosol formulation, a non-aerosol spray, a gel, a lotion, and the like. The formulation may further include one or more additives including such as, for example, an adjuvant, a skin penetration enhancer, a colorant, a fragrance, a flavoring, a moisturizer, a thickener, and the like.

[0085] A formulation may be conveniently presented in unit dosage form and may be prepared by methods well known in the art of pharmacy. Methods of preparing a composition with a pharmaceutically acceptable carrier include the step of bringing a TriKE molecule into association with a carrier that constitutes one or more accessory ingredients. In general, a

formulation may be prepared by uniformly and/or intimately bringing the active molecule into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

[0086] Methods of Treatment

[0087] In some embodiments, provided herein, are methods that include administering to a subject a compound or molecule described herein in an amount effective to induce NK-mediated killing of a target cell. Any cell can be a target cell. In some embodiments, the target cell is a cancer cell. The methods described herein can include administering to the subject a TriKE molecule in an amount effective to induce NK-mediated killing of the target cells in the subject. In some embodiments, TriKE molecules are administered to treat a disease or condition of the subject.

[0088] As used herein, "treat" or variations thereof refer to reducing, limiting progression, ameliorating, or resolving, to any extent, the symptoms or signs related to a condition. As used herein, "ameliorate" refers to any reduction in the extent, severity, frequency, and/or likelihood of a symptom or clinical sign characteristic of a particular condition; "symptom" refers to any subjective evidence of disease or of a patient's condition; and "sign" or "clinical sign" refers to an objective physical finding relating to a particular condition capable of being found by one other than the subject or patient.

[0089] As used herein, the term "subject" refers to any individual or patient on which the methods disclosed herein are performed. The term "subject" can be used interchangeably with the term "individual" or "patient." A "subject" can be any animal such as, for example, a mammal (e.g., dog, cat, horse, cow, sheep, goat, monkey, etc.). In certain embodiments, the subject can be a human.

[0090] A "treatment" may be therapeutic or prophylactic. "Therapeutic" and variations thereof refer to a treatment that ameliorates one or more existing symptoms or clinical signs associated with a condition. "Prophylactic" and variations thereof refer to a treatment that limits, to any extent, the development and/or appearance of a symptom or clinical sign of a condition. Generally, a "therapeutic" treatment is initiated after the condition manifests in a subject, while "prophylactic" treatment is initiated before a condition manifests in a subject. Thus, in certain embodiments, the method can involve prophylactic treatment of a subject at risk of developing a condition. "At risk" refers to a subject that may or may not actually possess the described risk. Thus, for example, a subject "at risk" for developing a specified condition is a subject that possesses one or more indicia of increased risk of having, or developing, the specified condition compared to individuals who lack the one or more indicia,

regardless of the whether the subject manifests any symptom or clinical sign of having or developing the condition. Exemplary indicia of a condition can include, for example, genetic predisposition, ancestry, age, sex, geographical location, lifestyle, or medical history. Treatment may also be continued after symptoms have resolved, for example to prevent or delay their recurrence.

[0091] In other embodiments, methods for stimulating expansion of NK cells *in vivo* are provided herein that include administering to a subject a compound or molecule described herein in an amount effective to stimulate expansion of NK cells in the subject. In some embodiments, TriKE molecules are administered to treat a disease or condition of the subject. Using a TriKE molecule as a part of an *in vivo* treatment can make NK cells antigen specific with simultaneous co-stimulation, enhancement of survival, and expansion, which may be antigen specific. In other cases, the TriKE can be used *in vitro* as an adjuvant to NK cell adoptive transfer therapy.

[0092] In still other embodiments, methods of treating cancer are provided herein that include administering to s subject a compound or molecule described herein effective for treating the cancer. In some embodiments, the cancer is prostate cancer, lung cancer, colon cancer, rectum cancer, urinary bladder cancer, melanoma, kidney cancer, renal cancer, oral cavity cancer, pharynx cancer, pancreas cancer, uterine cancer, thyroid cancer, skin cancer, head and neck cancer, cervical cancer, ovarian cancer, or hematopoietic cancer. In some embodiments, the hematopoietic cancer is a myelodysplastic syndrome (MDS). In some embodiments, the hematopoietic cancer is a lymphoma. In some embodiments, the hematopoietic cancer is a leukemia. In some embodiments, the hematopoietic cancer is acute myeloid leukemia (AML).

[0093] As used herein, the term “myeloid leukemia” refers to leukemia characterized by proliferation of myeloid tissue and an abnormal increase in the number of granulocytes, myelocytes and myeloblasts in the circulating blood. This term is synonymous with the terms myelocytic leukemia, myelogenic leukemia, myelogenous leukemia and granulocytic leukemia. The term “myeloid leukemia” can represent inter alia acute and chronic myeloid leukemias (AML and CML), acute promyelocytic leukemia (APL), chronic myelomonocytic leukemia (“CMML”), myelodysplastic syndrome and juvenile myelomonocytic leukemia which involve the myeloid elements of the bone marrow (e.g., white cells, red cells and megakaryocytes) and includes all subtypes which are defined by morphological, histochemical and immunological techniques that are well known by those skilled in the art.

Subtypes of AML include according to the FAB classification FAB-M0, FAB-M1, FAB-M2, FAB-M3, FAB-M4, FAB-M5, FAB-M6 and FAB-M7.

[0094] As used herein, the term "myelodysplastic syndrome" encompasses a heterogeneous group of closely related clonal hematopoietic disorders that originate in an early blood-forming cell in the marrow. All disorders are characterized by a cellular marrow with impaired morphology and maturation (dysmyelopoiesis) and peripheral blood cytopenias, resulting from ineffective blood cell production. In other words, the maturing blood cells often die in the marrow before they reach full maturity and enter the blood, accounting for the low blood cell concentrations. In patients suffering from myelodysplastic syndrome there may also be an accumulation of very immature marrow cells, called leukemic blast cells.

[0095] The amount of TriKE molecule administered can vary depending on various factors including, but not limited to, the specific TriKE molecule being used, the weight, physical condition, and/or age of the subject, and/or the route of administration. Thus, the absolute weight of TriKE molecule included in a given unit dosage form can vary widely, and depends upon factors such as the species, age, weight and physical condition of the subject, and/or the method of administration. Accordingly, it is not practical to set forth generally the amount that constitutes an amount of TriKE molecule effective for all possible applications. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors.

[0096] In some embodiments, the method can include administering sufficient amounts of a TriKE molecule to provide a dose of, for example, from about 100 ng/kg to about 50 mg/kg to the subject, although in some embodiments the methods may be performed by administering a TriKE molecule in a dose outside this range. In some of these embodiments, the method includes administering sufficient amounts of a TriKE molecule to provide a dose of from about 10 μg/kg to about 5 mg/kg to the subject, for example, a dose of from about 100 μg/kg to about 1 mg/kg.

[0097] Alternatively, the dose may be calculated using actual body weight obtained just prior to the beginning of a treatment course. For the dosages calculated in this way, body surface area (m2) is calculated prior to the beginning of the treatment course using the Dubois method: m2 = (wt kg0.425 × height cm0.725) × 0.007184.

[0098] In some embodiments, the method can include administering sufficient amounts of a TriKE molecule to provide a dose of, for example, from about 0.01 mg/m2 to about 10 mg/m2.

[0099] In some embodiments, a TriKE molecule may be administered, for example, from a single dose to multiple doses per week, although in some embodiments the method can be performed by administering a TriKE molecule at a frequency outside this range. In certain embodiments, a TriKE molecule may be administered from about once per month to about five times per week.

[00100] In some embodiments, the method further includes administering one or more additional therapeutic agents. The one or more additional therapeutic agents may be administered before, after, and/or coincident to the administration of a TriKE molecule. A TriKE molecule and the additional therapeutic agents may be co-administered. As used herein, "co-administered" refers to two or more components of a combination administered so that the therapeutic or prophylactic effects of the combination can be greater than the therapeutic or prophylactic effects of either component administered alone. Two components may be co-administered simultaneously or sequentially. Simultaneously co-administered components may be provided in one or more pharmaceutical compositions. Sequential co-administration of two or more components includes cases in which the components are administered so that each component can be present at the treatment site at the same time. Alternatively, sequential co-administration of two components can include cases in which at least one component has been cleared from a treatment site, but at least one cellular effect of administering the component (e.g., cytokine production, activation of a certain cell population, etc.) persists at the treatment site until one or more additional components are administered to the treatment site. Thus, a co-administered combination can, in certain circumstances, include components that never exist in a chemical mixture with one another. In other embodiments, the TriKE molecule and the additional therapeutic agent may be administered as part of a mixture or cocktail. In some aspects, the administration of TriKE molecule may allow for the effectiveness of a lower dosage of other therapeutic modalities when compared to the administration of the other therapeutic agent or agents alone, thereby decreasing the likelihood, severity, and/or extent of the toxicity observed when a higher dose of the other therapeutic agent or agents is administered.

[00101] Exemplary additional therapeutic agents include altretamine, amsacrine, L-asparaginase, colaspase, bleomycin, busulfan, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytophosphane, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, fluorouracil, fludarabine, fotemustine, ganciclovir, gemcitabine, hydroxyurea, idarubicin, ifosfamaide, irinotecan, lomustine, melphalan, mercaptopurine, methotrexate, mitoxantrone,

mitomycin C, nimustine, oxaliplatin, paclitaxel, pemetrexed, procarbazine, raltitrexed, temozolomide, teniposide, tioguanine, thiotepa, topotecan, vinblastine, vincristine, vindesine, and vinorelbine.

[00102] Accordingly, in some embodiments, methods of treating cancer provided herein further include administering a compound, molecule, composition, or formulation described herein prior to, simultaneously with, or following chemotherapy, surgical resection of a tumor, or radiation therapy. The chemotherapy can include altretamine, amsacrine, L-asparaginase, colaspase, bleomycin, busulfan, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytophosphane, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, fluorouracil, fludarabine, fotemustine, ganciclovir, gemcitabine, hydroxyurea, idarubicin, ifosfamaide, irinotecan, lomustine, melphalan, mercaptopurine, methotrexate, mitoxantrone, mitomycin C, nimustine, oxaliplatin, paclitaxel, pemetrexed, procarbazine, raltitrexed, temozolomide, teniposide, tioguanine, thiotepa, topotecan, vinblastine, vincristine, vindesine, and vinorelbine, for example.

[00103] In some embodiments, the methods provided herein can include administering sufficient TriKE molecules as described herein and administering at least one additional therapeutic agent, with administration of TriKE molecules and at least one additional therapeutic agent demonstrating therapeutic synergy. In some aspects of the methods of the present invention, a measurement of response to treatment observed after administering both a TriKE molecule as described herein and the additional therapeutic agent is improved over the same measurement of response to treatment observed after administering either the TriKE molecule or the additional therapeutic agent alone. In some embodiments, an additional therapeutic agent can include an additional agent that targets EpCAM including, for example, an EpCAM specific monoclonal antibody, such as, for example, Catumaxomab, a monoclonal hybrid antibody targeting EpCAM and CD3.

[00104] As used herein, the term “and/or” means one or all of the listed elements or a combination of any two or more of the listed elements; the terms “comprises,” “comprising,” and variations thereof are to be construed as open ended - i.e., additional elements or steps are optional and may or may not be present.

[00105] As used herein, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Unless otherwise specified, “a,” “an,” “the,” and “at least one” can be used interchangeably and can mean one or more than one. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type

described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[00106] As used herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

[00107] "About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20%, or ±10%, or ±5%, or even ±1% from the specified value, as such variations are appropriate for the disclosed methods or to perform the disclosed methods.

[00108] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs.

[00109] As used herein, the term "protein" refers to any polymeric chain of amino acids. The terms "peptide" and "polypeptide" are used interchangeably with the term “protein” and also refer to a polymeric chain of amino acids. The term "protein" encompasses native or artificial proteins, protein fragments and polypeptide analogs of a protein sequence. A protein may be monomeric or polymeric. The term "protein" encompasses fragments and variants (including fragments of variants) thereof, unless otherwise contradicted by context.

[00110] As used herein, the term “nucleic acid” refers to any deoxyribonucleic acid (DNA) molecule, ribonucleic acid (RNA) molecule, or nucleic acid analogues. A DNA or RNA molecule can be double-stranded or single-stranded and can be of any size. Exemplary nucleic acids include, but are not limited to, chromosomal DNA, plasmid DNA, cDNA, cell-free DNA (cfDNA), mRNA, tRNA, rRNA, siRNA, micro RNA (miRNA or miR), hnRNA. Exemplary nucleic analogues include peptide nucleic acid, morpholino- and locked nucleic acid, glycol nucleic acid, and threose nucleic acid.

[00111] In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more embodiments.

[00112] For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. Further, as appropriate, any combination of two or more steps may be conducted simultaneously.

[00113] The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

EXAMPLE 1

[00114] This example describes development of the CD16-IL15-CLEC12A TriKE.

[00115] The 1615CLEC12A TriKE was developed in a mammalian cell system to ensure that appropriate post-translational modifications are present. Specific binding of the TriKE to HL-60 and THP-1 target cells that express CLEC12A compared to Raji cells that do not express CLEC12A was confirmed. Treatment of peripheral blood mononuclear cells (PBMCs) with the 1615CLEC12A TriKE drove a significant increase in NK cell specific proliferation over 7 days as measured by CellTrace dilution compared to treatment with a CLEC12A scFv or IL-15 alone (69.7 ± 6.7% vs 11.9 ± 2.5% vs 38.4 ± 7.3%) (**Figure 1A**). To measure NK cell killing, an IncuCyte Zoom assay was conducted. Here, HL-60 target cells were labeled with a caspase 3/7 reagent where a color change indicates target cell death. The 1615CLEC12A TriKE was able to induce more target cell killing than CLEC12A scFv or IL-15 as measured by number of live target cells at the end of the 48 hour assay (53.9 ± 1.9% vs 103.3 ± 3.4% vs 71.1 ±1.4%). The 1615CLEC12A TriKE induced an increase in NK cell degranulation, measured by CD107a expression against HL-60 AML tumor targets in a 4 hour functional assay compared to treatment with CLEC12A scFv or IL-15 alone (62.3 ± 1.1% vs 19.4 ± 3.8% vs 27.5 ± 4.9%). In this assay, there was also an increase in cytokine production, measured by IFNg and TNFa respectively (16.7 ± 4.2% vs 2.3 ± 1.5% vs 4.7 ± 1.9% and 18.0 ± 5.1% vs 2.5 ± 1.7% vs 4.6 ± 2.5%) (**Figure 1B**). A similar enhanced functional response with THP-1 AML tumor targets was observed. In these functional assays, treatment with the 1615CLEC12A TriKE produced less background activation compared to the CD33 TriKE, indicating less off-target effects on PBMCs. To confirm the clinical relevance of this molecule, the efficacy of the 1615CLEC12A TriKE against primary AML targets was tested. AML blasts were identified as SSC low, CD45 intermediate and CD34 high cells. Out of the 9 AML samples tested, 7 expressed high levels of CD33 (70.4 ± 6.3%) and CLEC12A (78.1 ± 5.2%). In functional assays with these samples, the 1615CLEC12A TriKE was able to induce greater CD107a and IFNg expression, and enhanced killing of tumor targets as measured by a live/dead stain compared to CLEC12A scFv or IL-15 (**Figure 1C**). In these assays, the efficacy of the 1615CLEC12A TriKE was comparable to the CD33 TriKE. These data demonstrate that the 1615CLEC12A TriKE drives NK cell specific

proliferation, degranulation, cytokine secretion, and killing of tumor targets in vitro. Apart from AML, CLEC12A is expressed on cancer cells and LSCs in patients with myelodysplastic syndromes (MDS). These findings highlight the clinical potential of the 1615CLEC12A TriKE individually or in combination with the CD33 TriKE for the treatment of MDS and AML.

EXAMPLE 2

[00116] This example describes expression of CD33 and CLEC12A on AML cells.

[00117] A majority of all deaths from hematopoietic malignancies are caused by acute myeloid leukemia (AML) which has a poor five-year survival rate of 26%, highlighting the need for new therapies. The most common antigen used to target AML cells is CD33. However, there are many limitations of developing therapies against CD33. For example, not all cancer cells express CD33, including cancer cells in patients with refractory AML. In addition, all cells of the myeloid lineage and some cells of the lymphoid lineage like activated NK cells and T cells express CD33, leading to off-target toxicity. Further, cancer stem cells, which are thought to facilitate relapse, do not express CD33.

[00118] A novel antigen called C-type Lectin-like molecule 1 (CLL-1) or CLEC12A was targeted to address the above limitations.

[00119] Figure 2 shows the percentage of CD33 and CLEC12A surface expression measured by flow cytometry analysis of primary AML samples from 10 patients. CLEC12A was highly expressed on AML cells. About 70% of CD33 negative cells expressed CLEC12A. The expression of CLEC12A was restricted to a subset of myeloid cells, limiting off-target toxicity. CLEC12A was present on leukemic stem cells but not hematopoietic stem cells.

[00120] These data establish CLEC12A as a surface marker on both primary AML cells that express CD33 and that lack CD33 expression. Thus, in accordance with some embodiments, CLEC12A can be targeted by a tri-specific killer engager (TriKE) molecule on AML and other cells that express or lack CD33.

EXAMPLE 3

[00121] This example describes the tri-specific killer engager (TriKE) molecule targeting CLEC12A.

[00122] To target cancer cells using Natural Killer (NK) cells, a tri-specific killer engager (TriKE) molecule was developed containing an anti-CD16 heavy chain antibody that activates NK cells, an IL-15 molecule that drives NK cell priming, expansion and survival, and an anti-CLEC12A single chain variable fragment (scFv) that engages cancer targets. A

schematic of the CD16-IL15-CLEC12A TriKE and mechanism of action is shown in Figures 3A-B.

[00123] The TriKE contains an anti-CD16 heavy-chain antibody constructed by incorporating the CDRs of a llama anti-CD16 V_{HH} into a humanized V_{HH} backbone (Figure 3A). This is linked to a wild type IL-15 molecule which is linked to the scFv from an anti-CLEC12A antibody. The TriKE (SEQ ID NO.:1) was produced in a mammalian system with Expi-293 cells and contains a His tag which was used to purify the molecule. In accordance with some embodiments, the TriKE molecule may lack a His tag. A TriKE molecule that lacks a His tag can be suitable for use in clinical applications, although aTriKE containing a His tag can be used as well. The TriKE creates an immunological synapse between a CLEC12A+ tumor cell and NK cell promoting release of cytotoxic granules and secretion of cytokines that kills the target cell (Figure 3B).

[00124] In addition to the scFv from an anti-CLEC12A antibody described above as an illustrative example (SEQ ID NO.:4; corresponding to SC02-357 of U.S. Patent No. 7,741,443), the TriKE targeting domain can comprise any sequence capable of targeting or binding to CLEC12A, such as scFvs SC02-378 and SC02-161 and any derivatives of scFvs SC02-357, SC02-378, and SC02-161. scFvs SC02-357, SC02-378, and SC02-161 are described in U.S. Patent No. 7,741,443, the disclosure of which is incorporated herein in its entirety, specifically with respect to scFvs SC02-357, SC02-378, and SC02-161 sequences.

EXAMPLE 4

[00125] This example describes binding validation of the CD16-IL15-CLEC12A TriKE to target cells.

[00126] CLEC12A+ HL-60 and CLEC12A- Raji targets were incubated with the 1615CLEC12A TriKE or scFv at equimolar concentrations. Binding was assessed by an anti-His antibody that binds to the His tag on the TriKE or scFv. A secondary Streptavidin antibody was used that was detected by flow cytometry. Data in Figure 4 shows that 1615CLEC12A TriKE bound to HL-60 targets, but not Raji targets.

[00127] Binding of the different components of the 1615CLEC12A TriKE was tested using an ELISA against CD16 (Figure 14A), IL15 receptor alpha (Figure 14B), and CLEC12A extracellular domain (ED; Figure 14C). The TriKE was tested in 3 fold serial dilutions from 900nM to 0.4nM and had the highest binding at 30nM, which was used in subsequent experiments, unless otherwise noted.

[00128] These data show that each component of the 1615CLEC12A TriKE bound to its respective target molecule and that CD16-IL15-CLEC12A TriKE specifically bound to targets that express CLEC12A.

EXAMPLE 5

[00129] This example describes NK cell proliferation induced by CD16-IL15-CLEC12A TriKE.

[00130] PBMCs were labeled with Cell Trace and incubated with equimolar concentrations of IL-15, CLEC12A scFv or CLEC12A TriKE for 7 days (Figure 5A). The NK cell population was assessed by evaluating dilution of Cell Trace dye in the CD56+CD3- population using flow cytometry (Figure 5B). The percentage of proliferated NK cells was calculated using FlowJo Analyzer. Statistics reflect significant differences between the groups as calculated with a One Way ANOVA, * $P < 0.05$, ** $P < 0.005$, $N = 10$. A greater percentage of proliferated NK cells was seen in the presence of CLEC12A TriKE as compared to IL-15 or CLEC12A scFv.

[00131] In other experiments, PBMCs were isolated from fresh healthy donor samples (n=6), CellTrace Violet labeled, and incubated for 7 days with 1615CLEC12A TriKE or control treatments at 30nM, as described above. After the incubation period, cells were harvested and NK cell (CD3-, CD56+) proliferation was evaluated by flow cytometry.

[00132] Pooled data (Figure 9A) and representative histograms (Figure 9B) show NK cell proliferation (by CellTrace dilution) for the different treatment groups. Figure 9C shows pooled NK cell count (45 seconds at constant speed) at the time of harvest. One-way analysis of variance (ANOVA) with repeated measures was used to calculate differences compared to the 1615CLEC12A group. Error bars indicate +/- standard error of the mean. Statistical significance are determined as **P<0.005, ****P<0.0001. Significantly greater percentages of proliferated NK cells were seen upon treatment with CLEC12A TriKE as compared to no treatment, treatment with IL-15, or treatment with CLEC12A scFv.

[00133] These data show that the 1615CLEC12A TriKE induced potent NK cell proliferation.

EXAMPLE 6

[00134] This example describes functional validation of the CD16-IL15-CLEC12A TriKE.

[00135] PBMCs were incubated with CLEC12A+ HL-60 and THP1 cells at a 2:1 effector to target ratio in the presence of IL-15, CLEC12A scFv or CLEC12A TriKE at equimolar concentrations. Surface CD107a, to evaluate degranulation (Figure 6A), intracellular IFNg (Figure 6B), and TNFa to evaluate inflammatory cytokine production (Figure 6C), were

assessed on $CD56^{+}CD3^{-}$ NK cells by flow cytometry. Statistics comparing treatment with CLEC12A TriKE to treatment with IL-15 or CLEC12A scFv controls reflect significant differences between the groups as calculated with a One Way ANOVA, ** $P < 0.005$, $N = 6$. A greater percentage of CD107a surface staining and greater percentages of IFNg and TNFa intracellular staining on NK cells were seen in the presence of CLEC12A TriKE as compared to no treatment, treatment with IL-15, or treatment with CLEC12A scFv for both HL60 and THP1 target cells.

[00136] In other experiments, frozen PBMCs from healthy donors (n=6) were incubated with the indicated treatments (30nM) to evaluate CD107a expression as a marker of degranulation (Figure 10A), intracellular IFNg production (Figure 10B), or intracellular TNFa expression in NK cells (CD3-, CD56+; Figure 10C) in a 4-hour assay. The cells were evaluated with PBMCs alone or in the presence of THP1 and HL-60 targets at a 2:1 effector/target ratio. Activation of NK cells (CD3-, CD56+) in PBMCs were evaluated using CD69 expression in a 4-hour assay with PBMCs alone or in the presence of THP1 and HL-60 targets at a 2:1 effector/target ratio (Figure 10D). One-way analysis of variance (ANOVA) with repeated measures was used to calculate differences against the 1615CLEC12A group. Error bars indicate +/- standard error of the mean. Statistical significance are determined as *P , .05, **P , .01, ***P , .001, and ****P , .0001.

[00137] Greater NK cell activation was seen, demonstrated by increased staining for CD107a, IFNg, TNFa, and CD69 upon incubation with CLEC12A TriKE as compared to no treatment, treatment with IL-15, or treatment with scFv for both THP1 target cells and HL60 target cells (Figures 10A-D).

[00138] These data show that the 1615CLEC12A TriKE induced degranulation and cytokine production against AML target cells, including THP1 and HL-60 targets.

EXAMPLE 7

[00139] This example describes CD16-IL15-CLEC12A TriKE-induced killing of AML targets.

[00140] Enriched NK cells were incubated with CLEC12A+ HL-60 (Figure 7A) and THP1 (Figure 7B) targets at a 2:1 effector to target ratio in the presence of IL-15, CLEC12A scFv or CLEC12A TriKE at equimolar concentrations. The target cells were labeled with a Cell Trace Far Red dye and a Caspase 3/7 green apoptosis assay reagent (Essen Biosciences). Killing was assessed using an Incucyte Zoom machine and analyzed by normalizing cell numbers to initial number of target cells. Graphs in Figures 7A-B depict the following (from the top): (i) no treatment (first from top); (ii) treatment with CLEC12A scFv (second from

top); (iii) treatment with IL-15 (third from top/second from bottom); (iv) treatment with CLEC12A TriKE (fourth from top/bottom). The percentage of live target cells was lowest upon treatment with CLEC12A TriKE.

[00141] 1615CLEC12A TriKE-mediated induction of target cell killing was evaluated in a real-time imaging assay. Enriched NK cells (CD3-, CD56+) were incubated with CellTrace Far Red labeled THP-1 cells at a 2:1 effector to target ratio with the noted treatments (30nM) for 48 hours within an IncuCyte S3 imager. Dead THP-1 cells were measured using a Caspase 3/7 reagent. Figure 11A shows quantification of the percentage of live THP-1 tumor targets (CellTrace Far Red/Caspase 3/7) normalized to targets alone at the 0-hour time point. Readings were taken every 30 minutes over a 48-hour period. Representative of 3 separate experiments. First from top corresponds to no treatment, second from top corresponds to treatment with CLEC12A scFv, third from top corresponds to treatment with IL-15, fourth from top corresponds to treatment with CLEC12A TriKE. The lowest percentage of live THP-1 cells was seen in the presence of CLEC12A TriKE.

[00142] Figure 11B are representative images (original magnification 34: 2.82 mm/pixel) at 0, 18, and 36 hours showing THP-1 cells (larger cells) and NK cells (smaller cells). At 0 hours, few dead cells were present for all indicated treatment conditions. At 18 and 36 hours, few clusters of dead THP-1 cells were apparent throughout the no treatment and CLEC12A scFv conditions, with some clusters of dying THP-1 cells present for treatment with IL-15 and many more clusters of dying cells present with treatment with CLEC12A TriKE.

[00143] Figure 11C shows quantification of the percentage of live THP-1 tumor targets at different effector to target rations (1:1, 2:1 and 5:1). First, second and third from top correspond to NK Alone, fourth from top corresponds to 1:1 NK + CLEC12A TriKE, fifth from top corresponds to 2:1 NK + CLEC12A TriKE, sixth from top corresponds to 5:1 NK + CLEC12A TriKE. Lower percentages of live THP-1 cells were seen for all effector to target ratios as compared to NK cells alone, with an effector to target ratio of 5:1 resulting in the lowest percentage of live THP-1 cells, but this decrease in viability of THP-1 cells was maximal when CLEC12A TriKE treatment was present.

[00144] In other experiments, enriched NK cells were incubated with CellTrace Far Red labeled HL-60 cells at a 2:1 effector to target ratio with the indicated treatments (30nM for each) for 48 hours within an IncuCyte s3 imager. Dead HL-60 cells were measured using a Caspase 3/7 reagent.

[00145] Figure 15A shows quantification of the percentage of live HL-60 tumor targets (CellTrace Far Red/Caspase 3/7) normalized to targets alone and the 0-hour time point.

Readings were taken every 30 minutes over a 48-hour period. Representative of 3 separate experiments. First from top corresponds to no treatment, second from top corresponds to treatment with CLEC12A scFv, third from top corresponds to treatment with IL-15, fourth from top corresponds to treatment with CLEC12A TriKE. The lowest percentage of live HL-60 cells was seen in the presence of CLEC12A TriKE.

[00146] Figure 15B are representative images at 0, 18, and 36 hours showing HL-60 target cells (larger cells) and NK cells (smaller cells). At 0 hours, few dead cells were present for all indicated treatment conditions. At 18 and 36 hours, few clusters of dead HL-60 cells were apparent throughout the no treatment and CLEC12A scFv conditions, with some clusters of dying HL-60 cells present for treatment with IL-15 and many more clusters of dying cells present with treatment with CLEC12A TriKE.

[00147] These data show that the CD16-IL15-CLEC12A TriKE induced killing of AML targets, including THP-1 and HL-60 target cells.

EXAMPLE 8

[00148] This example illustrates CD16-IL15-CLEC12A TriKE-induced killing of primary AML blast targets *in vitro*.

[00149] Enriched NK cells were incubated with primary AML blasts at a 2:1 effector to target ratio in the presence of IL-15, CLEC12A scFv, CLEC12A TriKE or CD33 TriKE at equimolar concentrations. Figure 8A shows the gating scheme to identify AML blasts using FlowJo Analyzer. Figure 8B shows the percentage of killing of AML blasts as assessed by the live/dead marker after gating on blasts cells after 48 hours. Surface CD107a expression to evaluate degranulation (Figure 8C) and intracellular IFNg to evaluate inflammatory cytokine production (Figure 8D) were assessed on $CD56^{+}CD3^{-}$ NK cells by flow cytometry after 4 hours. Statistics reflect significant differences between the groups as calculated with a One Way ANOVA, *P<0.05 ** P < 0.005, N = 10. The percentage of dead AML blast cells and percentages of staining for CD107a and IFNg were significantly greater upon incubation with CLEC12A TriKE as compared to incubation with IL-15 or incubation with CLEC12A scFv (Figures 8B-D.

[00150] In other experiments, primary AML blasts (SSCh low, CD45int, CD117+, CD14-, CD34+) were assessed for expression of CD33 and CLEC12A using flow cytometry (Figure 12A). Cells expressed CD33, CLEC12A, or both CD33 and CLEC12A as shown.

[00151] Enriched NK cells (CD56+, CD3-) from healthy donors (n=10) were incubated with primary AML blasts with the indicated treatments (30nM) to evaluate CD107a expression as a marker of degranulation (Figure 12B) and intracellular IFNg production

(Figure 12C) in a 4-hour assay at a 2:1 effector/target ratio. CLEC12A TriKE and CD33 TriKE induced degranulation and IFNg production as shown. Target cell killing was also evaluated using flow cytometry and a live/dead marker over 48 hours (Figure 12D). Treatment with IL-15, CLEC12A TriKE, and CD33 TriKE resulted in killing of AML blast cells as shown, with greater CLEC12A TriKE-mediated killing.

[00152] The proportion of different groups of AML blasts (based on CD33 and CLEC12A expression) were tracked over 48 hours to assess specificity of the 1615CLEC12A TriKE compared to the 1615CD33 TriKE (Figure 12E). Bars showing % AML blasts alive correspond to the following (from the top of each bar): (i) for no treatment, CLEC12A+CD33+ and CLEC12A+CD33-; (ii) for IL-15, CLEC12A+CD33+, CLEC12A+CD33-, and CLEC12A-CD33-; (iii) for CLEC12A scFv, CLEC12A+CD33+, CLEC12A+CD33-, CLEC12A-CD33+, and CLEC12A-CD33-; (iv) for CLEC12A TriKE, CLEC12A+CD33+, CLEC12A-CD33+, and CLEC12A-CD33-; (v) for CD33 TriKE, CLEC12A+CD33+ and CLEC12A+CD33-. These data confirm specificity of the 1615CLEC12A TriKE compared to the 1615CD33 TriKE.

[00153] In other experiments, enriched NK cells (CD56+, CD3-) from healthy donors (n=5) were incubated with bone marrow samples from AML patients with the indicated treatments (30nM) to evaluate killing of cancer stem cells (SSCh low, CD45int, CD34+, CD38-) in a 4-hour assay at a 2:1 effector/target ratio (Figure 12F). Representative flow plots show killing of CLEC12A and CD33 positive cancer stem cells.

[00154] Figure 12G shows combined data from the cancer stem cell killing assay showing percentage of cancer stem cells present at the end of the assay. One-way analysis of variance (ANOVA) with repeated measures was used to calculate differences against the 1615CLEC12A group. Error bars indicate +/- standard error of the mean. Statistical significance are determined as *P , .05, **P , .01, ***P , .001, and ****P , .0001. Treatment with CLEC12A TriKE resulted in significantly lower percentages of LSC compared to no treatment.

[00155] To examine primary AML blasts as target cells for killing mediated by the CLEC12A TriKE, a gating strategy was employed. The gating strategy to identify primary AML blasts is shown in Figure 16A. Primary AML blasts (n=5) were then incubated with the indicted treatments (30nM) to evaluate target cell killing using flow cytometry and a live/dead marker over 48 hours (Figure 16B). The percentage of dead AMP blasts at 48 hours is shown.

[00156] These data show that the 1615CLEC12A TriKE induced a slight increase in killing of primary AML blasts in patient samples at blast crisis, where NK cells are limiting.

EXAMPLE 9

[00157] This example describes 1615CLEC12A TriKE-mediated limitation of tumor growth *in vivo*.

[00158] Figure 13A shows a schematic of HL-60luc mouse experiments. The model was established by conditioning NSG mice (225cGy) and then injecting HL-60luc cells intravenously (7.5 × 105 cells/mouse). Three days later, 1×10^6 normal human donor NK cells (calculated from a magnetically depleted CD3/CD19 product) activated overnight with 10ng/ml IL-15 were infused. The 1615CLEC12A TriKE or 161533 TriKE (20ug) was administered MTWThF through the next 3 weeks of the study (15 doses total), and a control group only received HL-60luc cells.

[00159] Quantification of luminescence from the four treatment groups at day 7, day 14 and day 21 after NK infusion are shown in Figure 13B. Each dot represents a different mouse and bars denote mean +/- Standard Deviation. One-way analysis of variance (ANOVA) without matched comparisons was used to calculate differences against the 1615CLEC12A group. Statistical significance was determined as *P , .05, ***P , .001, and ****P , .0001. Figure 13C shows individual mouse photoluminescence (dark regions) after 2-minute exposures on day 7, 14 and 21. CLEC12A TriKE treatment resulted in decreased tumor burden at all time points examined compared to HL60 control. In addition, a significant reduction in tumor burden upon CLEC12A TriKE treatment relative to NK cell control treatment was seen at day 21.

[00160] Figure 13D shows a schematic of pdx mouse experiments. The model was established by conditioning NSG SGM3 mice (125cGy) and then injecting HL-60luc cells intravenously (7.5×10^5 cells/mouse). Tumors were allowed to grow until there were at least 1% AML Blasts in the blood. Then 1×10^6 normal human donor NK cells (calculated from a magnetically depleted CD3/CD19 product) activated overnight with 10ng/ml IL-15 were infused. The 1615CLEC12A TriKE or 161533 TriKE (20ug) was administered MTWThF for the next 3 weeks of the study (15 doses total) and a control group received NK cells but no treatment. The mice were sacrificed on day 21 and the percentage of AML blasts (CD45int, CD33+) in the bone marrow from the femur was calculated by flow cytometry (Figure 13E). Each dot represents a different mouse. The percentage of NK cells (CD3-, CD56+) was calculated in the bone marrow samples (Figure 13F) and peripheral blood (Figure 13G) by flow cytometry. Events were collected over 60 seconds and the number of human NK cell events was calculated. Representative dot plots are shown denoting the number of NK (CD56+CD3−) cell events within the CD45+ gate). One-way analysis of variance (ANOVA)

without matched comparisons was used to calculate differences against the 1615CLEC12A group. Error bars denote mean +/- Standard Deviation. Statistical significance was determined as *P , .05, ***P , .001, and ****P , .0001. Results show that NK + CLEC12A TriKE treatment significantly decreased the percentage of primary AML blast cells compared to tumor alone (Figure 13E). In addition, NK + CLEC12A TriKE treatment resulted in significantly increased percentages of NK cells in bone marrow (Figure 13F) and peripheral blood (Figure 13G) compared to tumor alone or tumor + NK treatment.

[00161] Taken together, these results show that 1615CLEC12A TriKE limited tumor growth *in vivo*.

EXAMPLE 10

[00162] This example describes analysis of stem and progenitor cells.

[00163] Cancer stem cells were identified in bone marrow samples using the gating strategy shown in Figure 17. A gating strategy to determine different $CD34^{pos}$ progenitor subpopulations in healthy bone marrow samples is shown in Figure 19. The gating strategy shown in Figure 19 was used to analyze cell populations shown in Figure 18A.

[00164] Figure 18A shows CLEC12A and CD33 expression within the $CD34^{pos}$ progenitor compartment in bone marrow from two representative healthy donors. HSC: hematopoietic stem cell, MPP: multipotent progenitor, LMPP: lymphoid-primed multipotent progenitor, CLP: common lymphoid progenitor, CMP: common myeloid progenitor, GMP: granulocyte-macrophage progenitor, MEP: megakaryocyte-erythroid progenitor. CLEC12A expression was seen in CMP, GMP, and MEP populations, but was relatively lower in HSC, MPP, CLP and LMPP populations. In addition, with the exception of the GMP population, CLEC12A was found at lower levels than CD33. Burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) colonies were counted after treatment with the 1615CLEC12A TriKE or the 161533 TriKE (Figure 18B). Greater BFU-E and CFU-GM colony numbers were seen upon treatment with 1615CLEC12A TriKE as compared to treatment with 161533 TriKE.

[00165] The data show that CLEC12A was differentially expressed in normal donor stem and progenitor cell populations and that treatment with 161533 TriKE decreased stem cell formation and/or differentiation compared to treatment with CLEC12A TriKE. Without being limited by theory, this indicates that while CLEC12A can be used to target leukemic stem cells, it should allow for normal hematopoietic reconstitution, while CD33 targeting is more likely to impact reconstitution. In other words, CLEC12A targeting should have less off target effects in terms of normal myeloid reconstitution.

[00166] In summary, taken together, the above data show that the CD16-IL15-CLEC12A TriKE bound specifically to target cells expressing CLEC12A, promoted proliferation of NK cells, enhanced the function of NK cells, promoted killing of AML cell lines in Incucyte zoom assays, and induced killing of primary AML and MDS blasts.

EXAMPLE 11

[00167] This example illustrates the generation of a TetraKE targeting CLEC12A and a second target or tumor antigen.

[00168] A TetraKE (tetramer) molecule can be designed that includes more than one targeting domain. As an example, a TetraKE can include a NK engaging domain, an NK activating domain and two targeting domains. Any of the NK engaging domains and NK activating domains described herein can be used. The targeting domains can target different targets or tumor antigens, for example. Any combination of targets or tumor antigens can be included in the TetraKE. For example, a first targeting domain can bind to CLEC12A, while a second targeting domain can bind to another target or tumor antigen.

[00169] Any of the targets or tumor antigens described herein can be included in a TetraKE with a first targeting domain that binds to CLEC12A, including, for example, a second targeting domain that binds to CD133, CD20, HER2, CEA, EpCAM, VEGF-A, EGFR, CD33, integrin αVβ3, CD51, CD152, CD125, CTAA16.88, MUC1, CD19, CD22, CD38, mesothelin, ROR1, CSPG4, SS1, or IGFR1, NKG2C, BCMA, APRIL, B7H3, and PSMA, or a viral antigen derived from EBV, HBV, HCV, and/or HPV. Further, TetraKE domains can be operably linked to each other using flanking or linker sequences as described herein. An exemplary TetraKE includes a compound that has a moiety that selectively binds to CD16, an NK activating domain that comprises IL-15, a first targeting domain selectively binds to CLEC12A, and a second targeting domain that selectively binds to CD33.

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SEQ ID NO.: 2

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SEQ ID NO.: 3

VDEHHHHHHHHHH

SEQ ID NO.: 4

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SEQ ID NO.: 5

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SEQ ID NO.:17

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VDE

SEQ ID NO.	Description
SEQ ID NO.:1	CLEC12A TriKE
SEQ ID NO.:2	CLEC12A TriKE with His tag and spacer
SEQ ID NO.:3	His tag and spacer
SEQ ID NO.:4	CLEC12A targeting domain
SEQ ID NO.:5	DNA encoding CLEC12A TriKE with His tag and spacer
SEQ ID NO.:6	DNA encoding CLEC12A TriKE
SEQ ID NO.:7	DNA encoding CLEC12A targeting domain
SEQ ID NO.:8	Humanized Cam16
SEQ ID NO.:9	Wild-type IL-15
SEQ ID NO.:10	Signal peptide
SEQ ID NO.:11	Linker
SEQ ID NO.:12	Linker
SEQ ID NO.:13	Linker
SEQ ID NO.:14	Linker
SEQ ID NO.:15	Linker
SEQ ID NO.:16	Signal peptide
SEQ ID NO.:17	Signal peptide
SEQ ID NO.:18	Non-humanized Cam16

[00170] Any and all references and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, that have been made throughout this disclosure are hereby incorporated herein by reference in their entirety for all purposes.

[00171] Although the present invention has been described with reference to specific details of certain embodiments thereof in the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A compound comprising:

an NK engaging domain;

an NK activating domain operably linked to the NK engaging domain; and

a targeting domain that selectively binds to a target cell and is operably linked to the NK activating domain and the NK engaging domain, wherein the targeting domain selectively binds to CLEC12A.

2. The compound of claim 1, wherein the NK engaging domain comprises a moiety that selectively binds to CD16.

3. The compound of claim 1, wherein the NK engaging domain moiety comprises an antibody or a binding fragment thereof or a nanobody.

4. The compound of claim 3, wherein the antibody binding fragment comprises an scFv, a F(ab)2, or a Fab.

5. The compound of claim 3, wherein the antibody or a binding fragment thereof or the nanobody is human or humanized.

6. The compound of claim 3, wherein the antibody or a binding fragment thereof or the nanobody is camelid.

7. The compound of claim 1, wherein the NK activating domain comprises IL-15 or a functional fragment thereof.

8. The compound of claim 7, wherein the IL-15 comprises an amino acid sequence of SEQ ID NO:9 or a functional variant thereof.

9. The compound of claim 1, wherein the targeting domain moiety comprises an antibody or a binding fragment thereof or a nanobody.

10. The compound of claim 9, wherein the antibody binding fragment comprises an scFv, a F(ab)2, or a Fab.

11. The compound of claim 1, wherein the NK engaging domain comprises a moiety that selectively binds to CD16, the NK activating domain comprises IL-15, and the targeting domain selectively binds to CLEC12A.

12. The compound of claim 1, comprising at least one flanking sequence linking two of the domains.

13. The compound of claim 12, further comprising a second flanking sequence linking the two linked domains with a third domain.

14. The compound of claim 13, wherein the flanking sequences flank the NK activating domain.

15. The compound of claim 14, wherein a first flanking sequence is C-terminal to the NK engaging domain and wherein a second flanking sequence is N-terminal to the anti-CLEC12A targeting domain.

16. An isolated amino acid sequence comprising SEQ ID NO.:1

17. An isolated DNA sequence encoding the amino acid sequence of SEQ ID NO.:1.

18. An isolated amino acid sequence comprising SEQ ID NO.:2.

19. An isolated DNA sequence encoding the amino acid sequence of SEQ ID NO.:2.

20. The compound of claim 1, wherein the CLEC12A domain is set forth in SEQ ID NO:4.

21. A composition comprising:
the compound of claim 1; and
a pharmaceutically acceptable carrier.

22. A method comprising:
administering to a subject the compound of claim 1 in an amount effective to induce NK-mediated killing of a target cell.

23. The method of claim 22, wherein the target cell is a cancer cell.

24. A method for stimulating expansion of NK cells in vivo, the method comprising:
administering to a subject an amount of the compound of claim 1 effective to stimulate expansion of NK cells in the subject.

25. A method of treating cancer in a subject, the method comprising:
administering to the subject an amount of the compound of claim 1 effective for treating the cancer.

26. The method of claim 25, wherein the cancer comprises prostate cancer, lung cancer, colon cancer, rectum cancer, urinary bladder cancer, melanoma, kidney cancer, renal cancer, oral cavity cancer, pharynx cancer, pancreas cancer, uterine cancer, thyroid cancer, skin cancer, head and neck cancer, cervical cancer, ovarian cancer, or hematopoietic cancer.

27. The method of claim 26, further comprising administering the compound prior to, simultaneously with, or following chemotherapy, surgical resection of a tumor, or radiation therapy.

28. The method of claim 27, wherein the chemotherapy comprises altretamine, amsacrine, L-asparaginase, colaspase, bleomycin, busulfan, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytophosphane, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, fluorouracil, fludarabine, fotemustine, ganciclovir, gemcitabine, hydroxyurea, idarubicin,

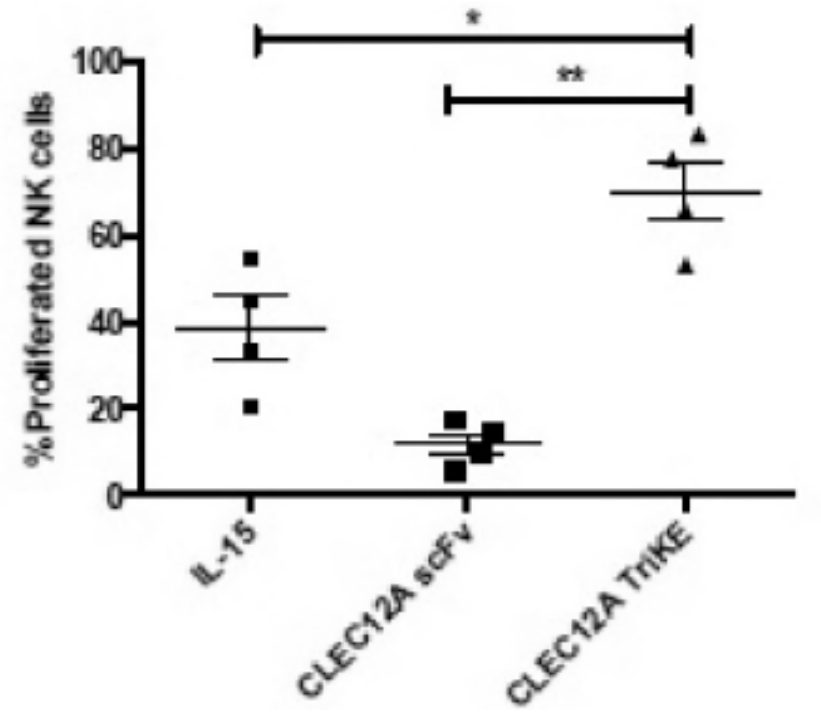
ifosfamaide, irinotecan, lomustine, melphalan, mercaptopurine, methotrexate, mitoxantrone, mitomycin C, nimustine, oxaliplatin, paclitaxel, pemetrexed, procarbazine, raltitrexed, temozolomide, teniposide, tioguanine, thiotepa, topotecan, vinblastine, vincristine, vindesine, and vinorelbine.

29. The method of claim 26, wherein the hematopoietic cancer is AML.

NK ENGAGER MOLECULES AND METHODS OF USE THEREOF

ABSTRACT

Provided are compositions for activating NK cells to stimulate an immune response for treating cancer and other disorders. In one embodiment, the invention provides a compound comprising an NK engaging domain that binds to CD16; an NK activating domain operably linked to the NK engaging domain; and a targeting domain that selectively binds to a target cell and is operably linked to the NK activating domain and the NK engaging domain, wherein the targeting domain binds to CLEC12A.


*
**
100
80
60
40
20
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% Proliferated NK cells
IL-15
CLEC12A scFv
CLEC12A TriKE

FIGURE 1A

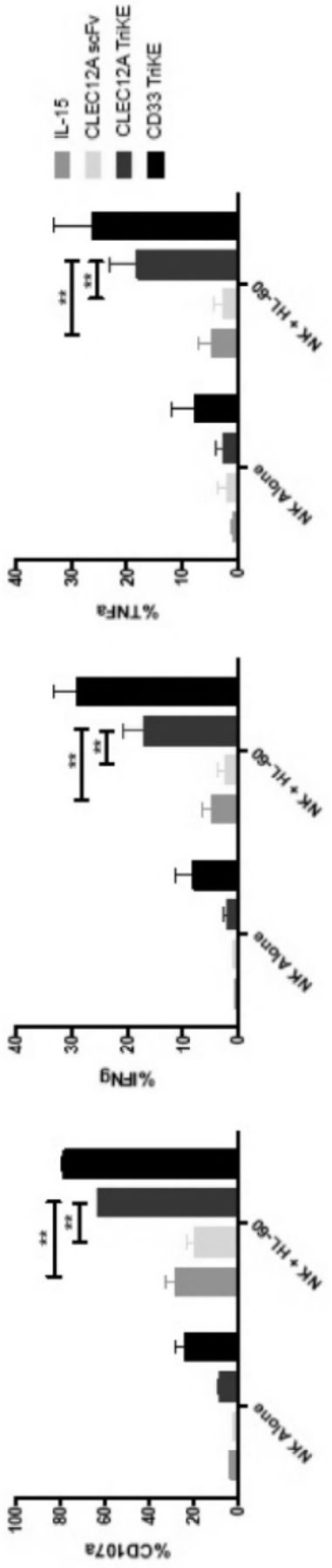
IL-15
CLEC12A scFv
CLEC12A TriKE
CD33 TriKE
%TNFa
%IFNg
%CD107a
NK Alone
NK + HL-60
**
**

FIGURE 1B

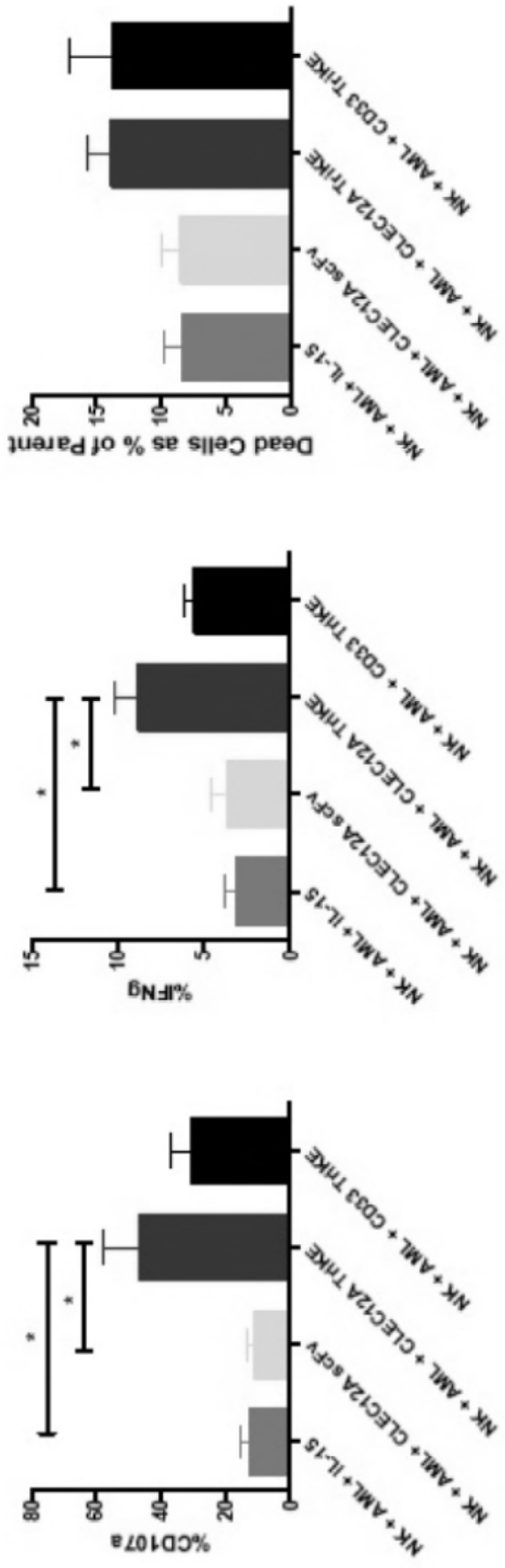
Dead Cells as % of Parent
20
15
10
5
0
NK + AML + IL-15
NK + AML + CLEC12A scFv
NK + AML + CLEC12A TriKE
NK + AML + CD33 TriKE
%IFNg
15
10
5
0
*
*
%CD107a
80
60
40
20
0
*
*

FIGURE 1C

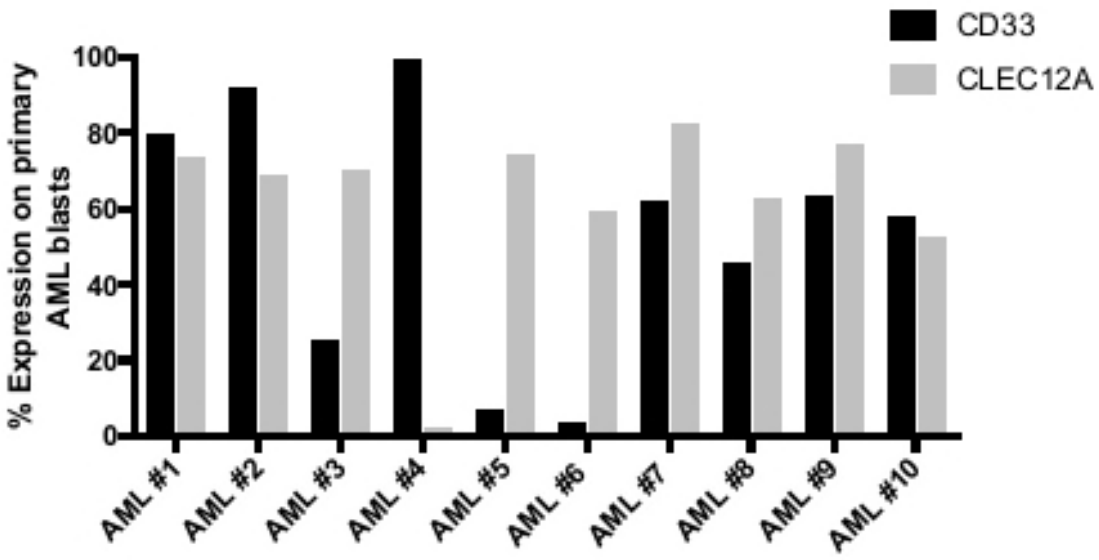
CD33
CLEC12A
% Expression on primary AML blasts
100
80
60
40
20
0
AML #1
AML #2
AML #3
AML #4
AML #5
AML #6
AML #7
AML #8
AML #9
AML #10

FIGURE 2

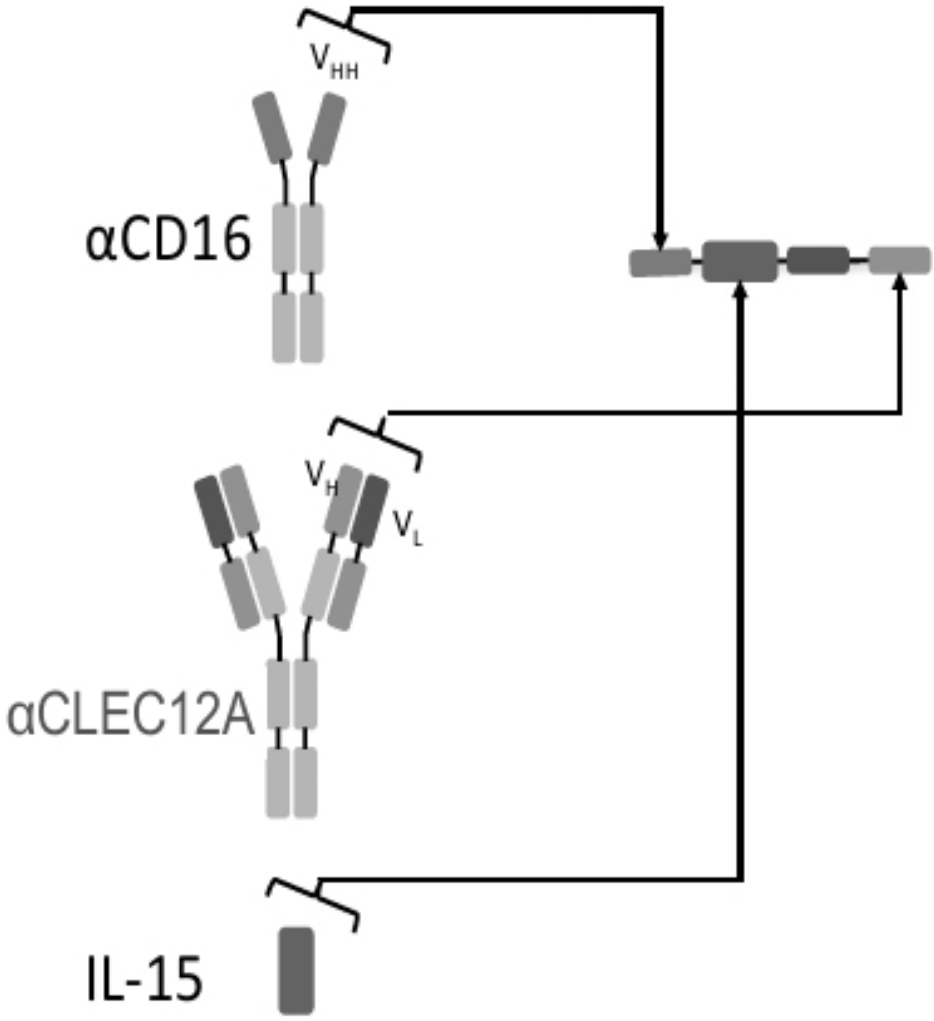

FIGURE 3A

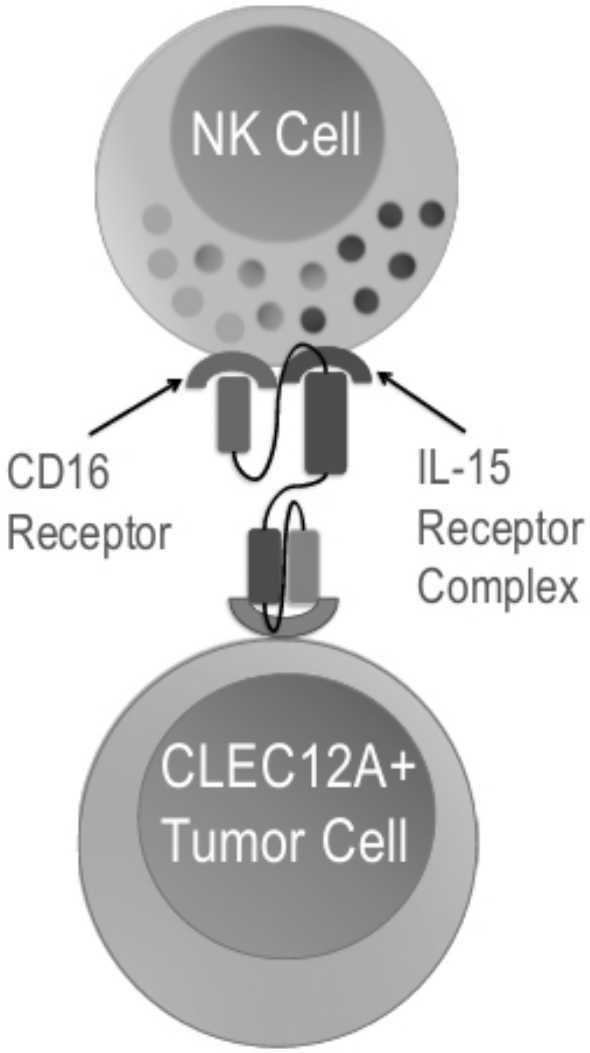
NK Cell
CD16
Receptor
IL-15
Receptor
Complex
CLEC12A+
Tumor Cell

FIGURE 3B

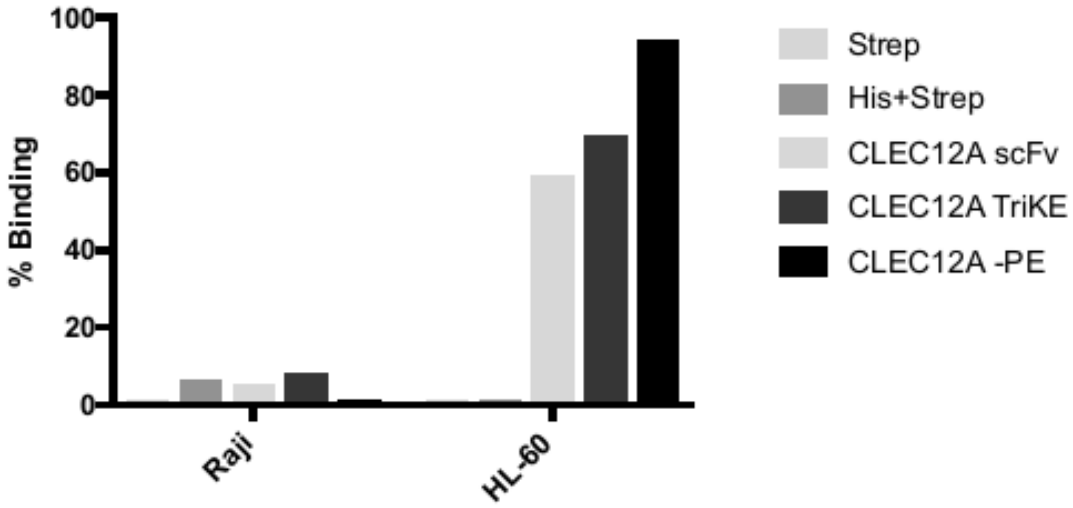
100
80
60
40
20
0
% Binding
Raji
HL-60
Strep
His+Strep
CLEC12A scFv
CLEC12A TriKE
CLEC12A -PE

FIGURE 4

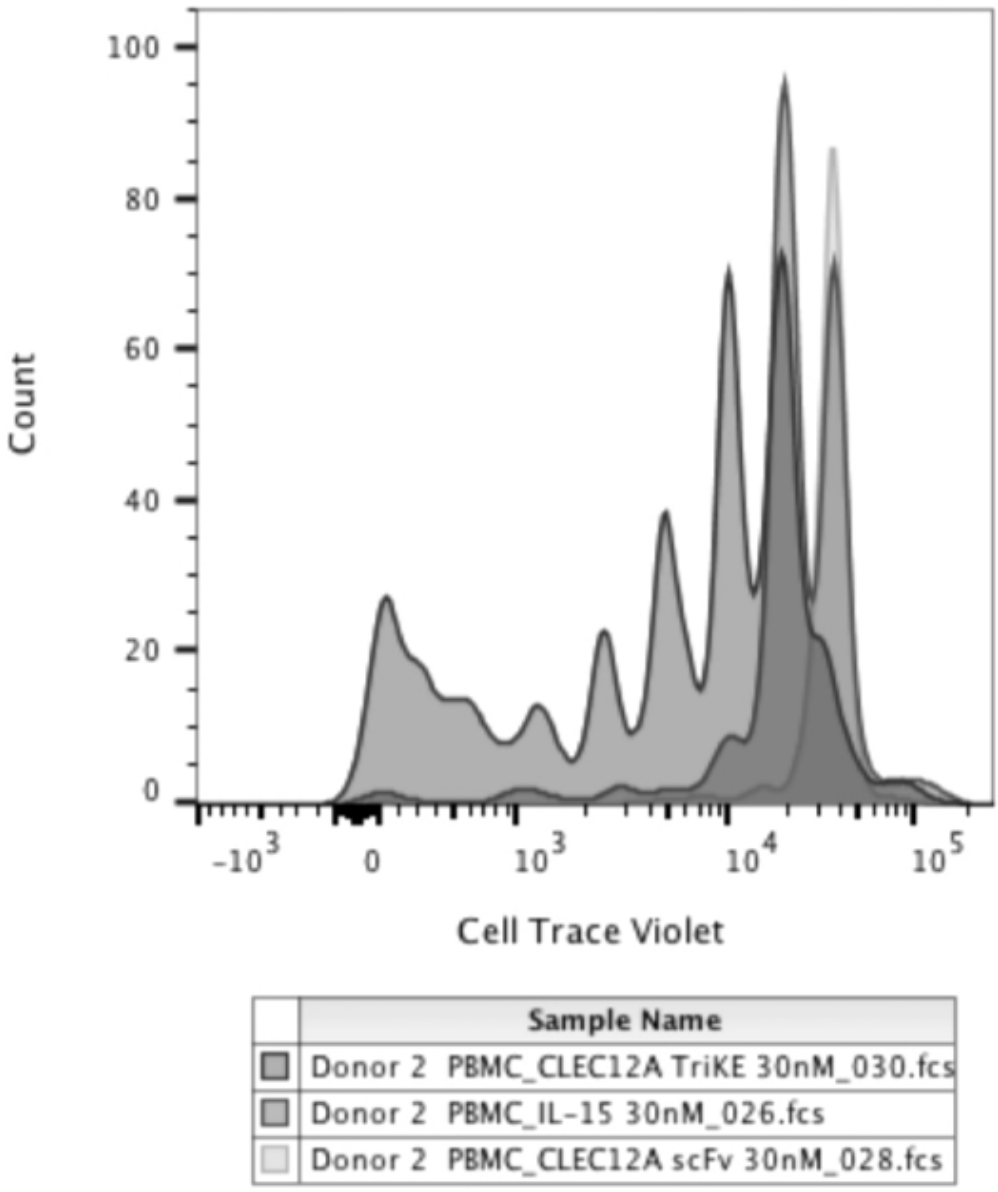
100
80
60
40
20
0
Count
-10^3
0
10^3
10^4
10^5
Cell Trace Violet
Sample Name
Donor 2 PBMC_CLEC12A TriKE 30nM_030.fcs
Donor 2 PBMC_IL-15 30nM_026.fcs
Donor 2 PBMC_CLEC12A scFv 30nM_028.fcs

FIGURE 5A

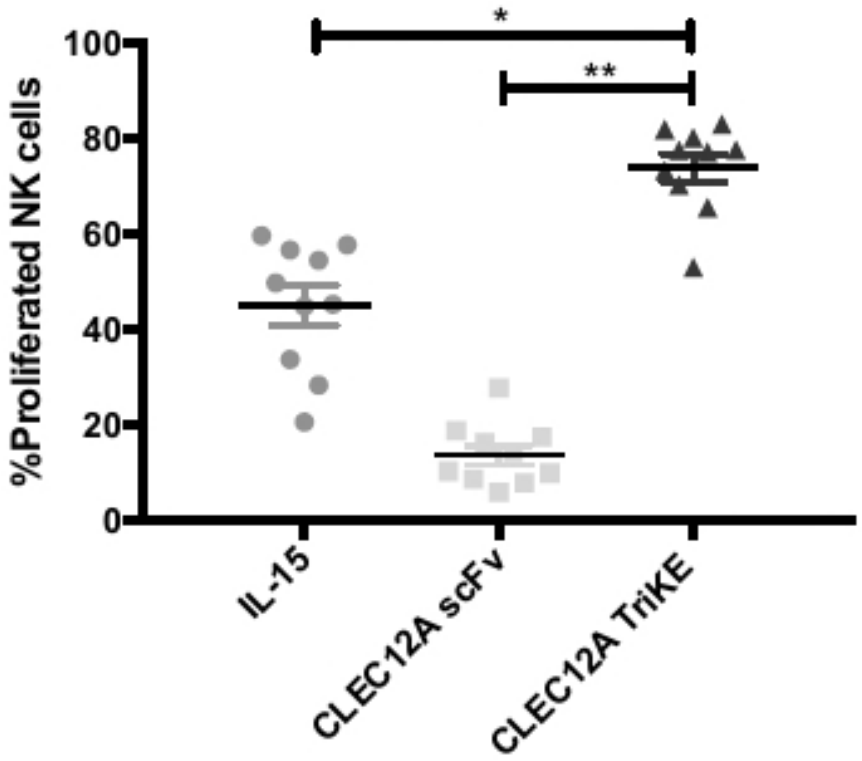

FIGURE 5B

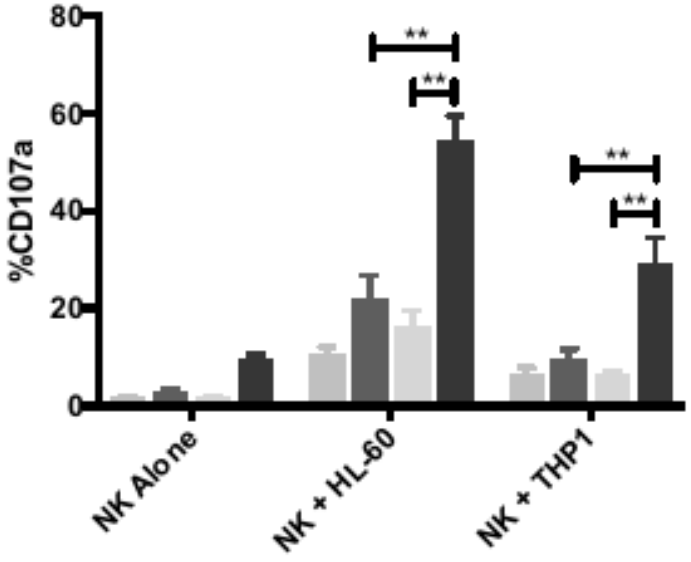

FIGURE 6A

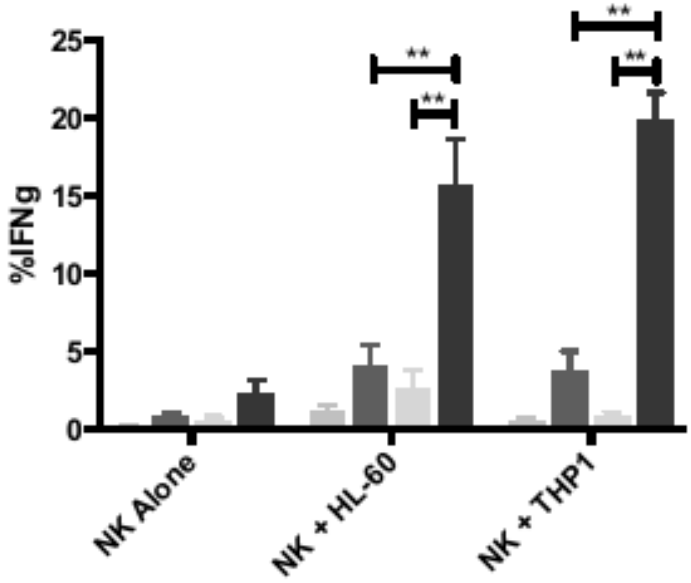

FIGURE 6B

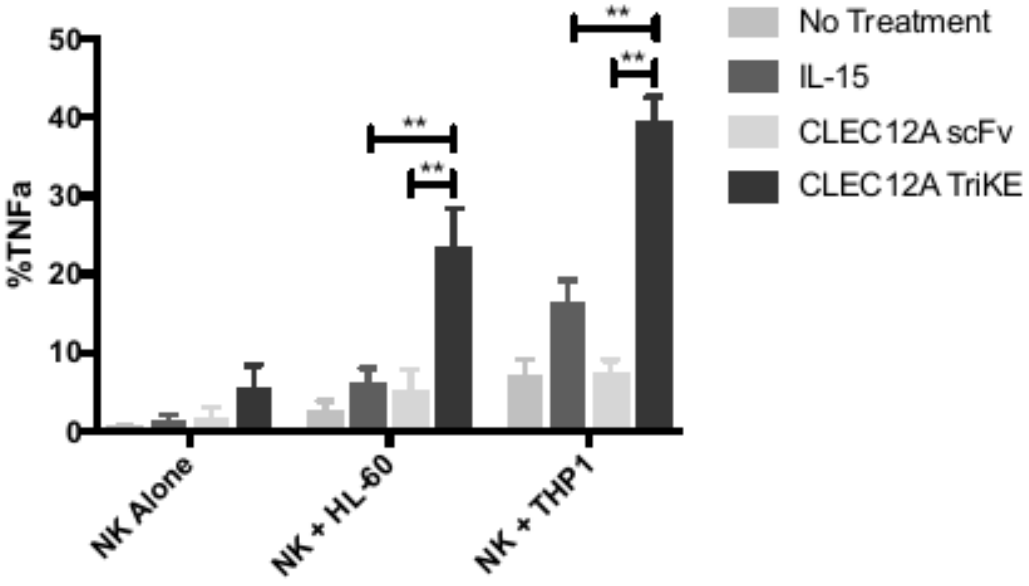

FIGURE 6C

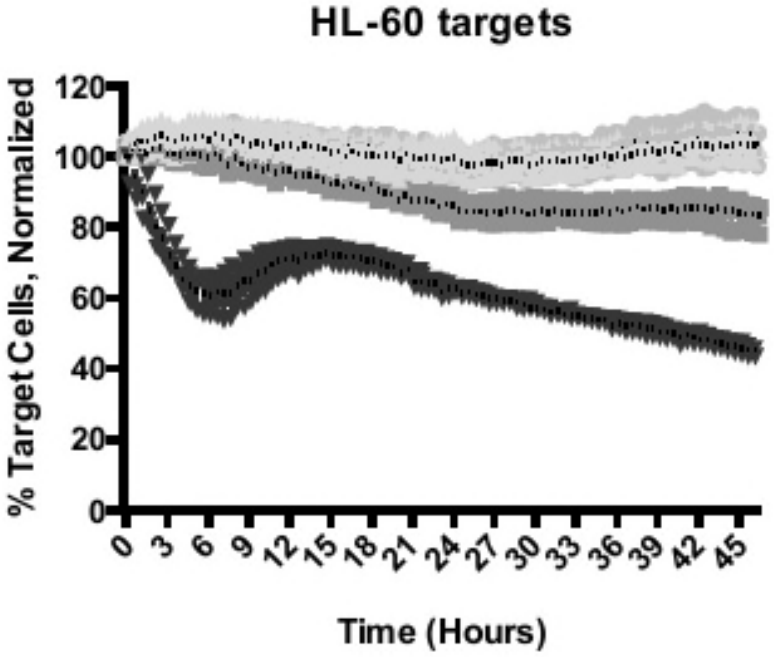

FIGURE 7A

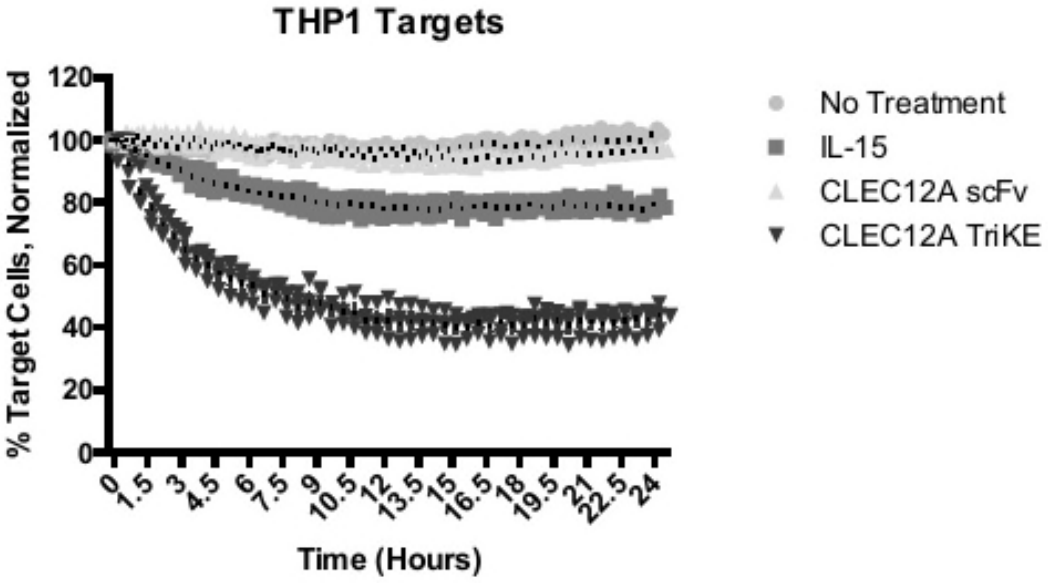

FIGURE 7B

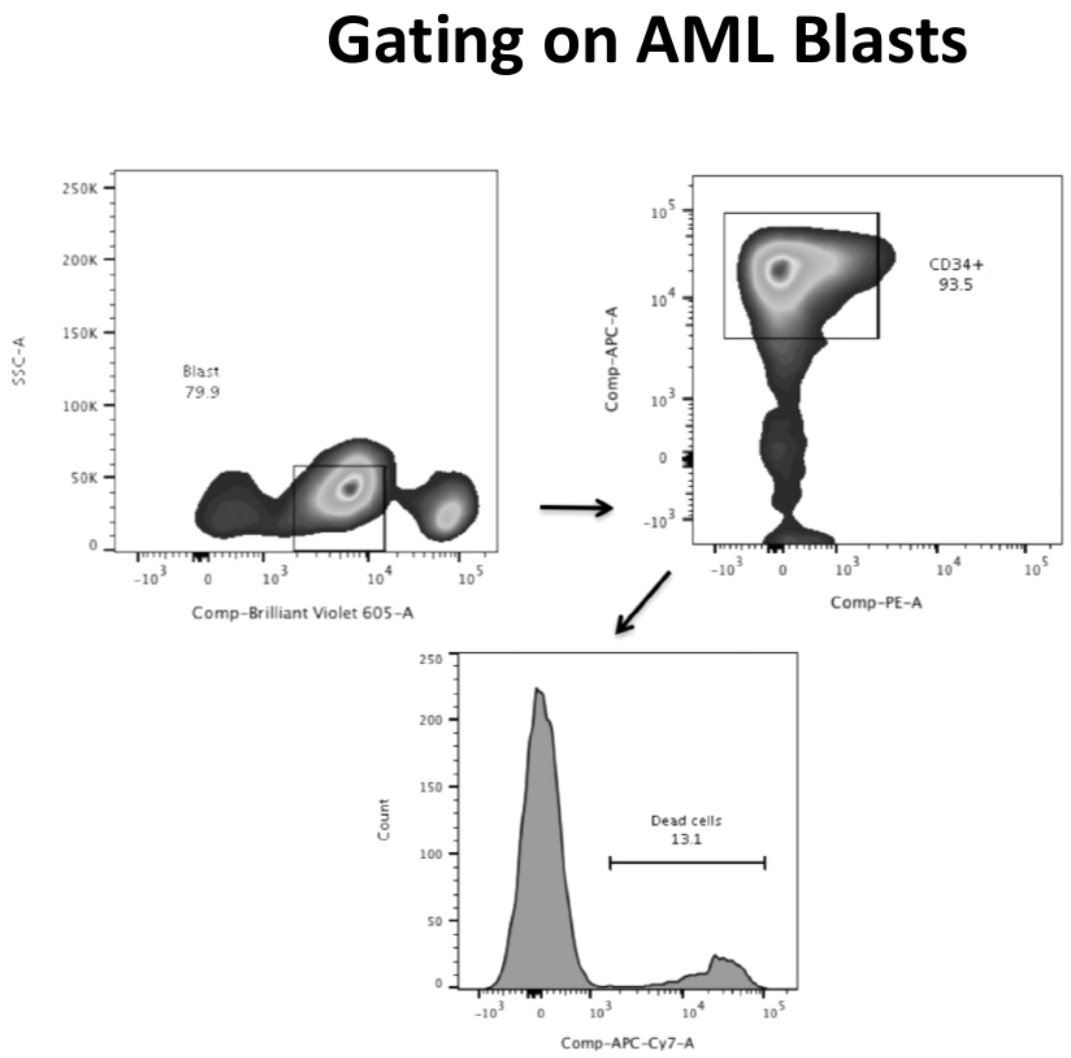
Gating on AML Blasts
SSC-A
250K
200K
150K
100K
50K
0
Blast
79.9
Comp-Brilliant Violet 605-A
Comp-APC-A
CD34+
93.5
Comp-PE-A
Count
Dead cells
13.1
Comp-APC-Cy7-A

FIGURE 8A

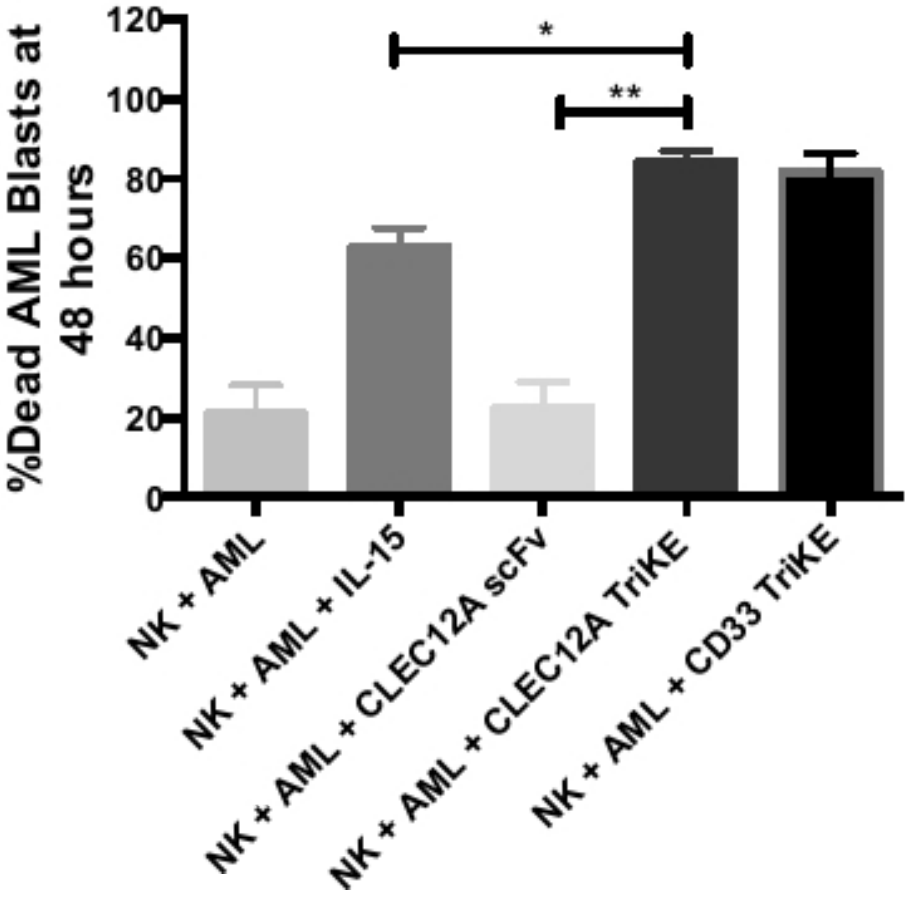
%Dead AML Blasts at 48 hours
120
100
80
60
40
20
0
*
**
NK + AML
NK + AML + IL-15
NK + AML + CLEC12A scFv
NK + AML + CLEC12A TriKE
NK + AML + CD33 TriKE

FIGURE 8B

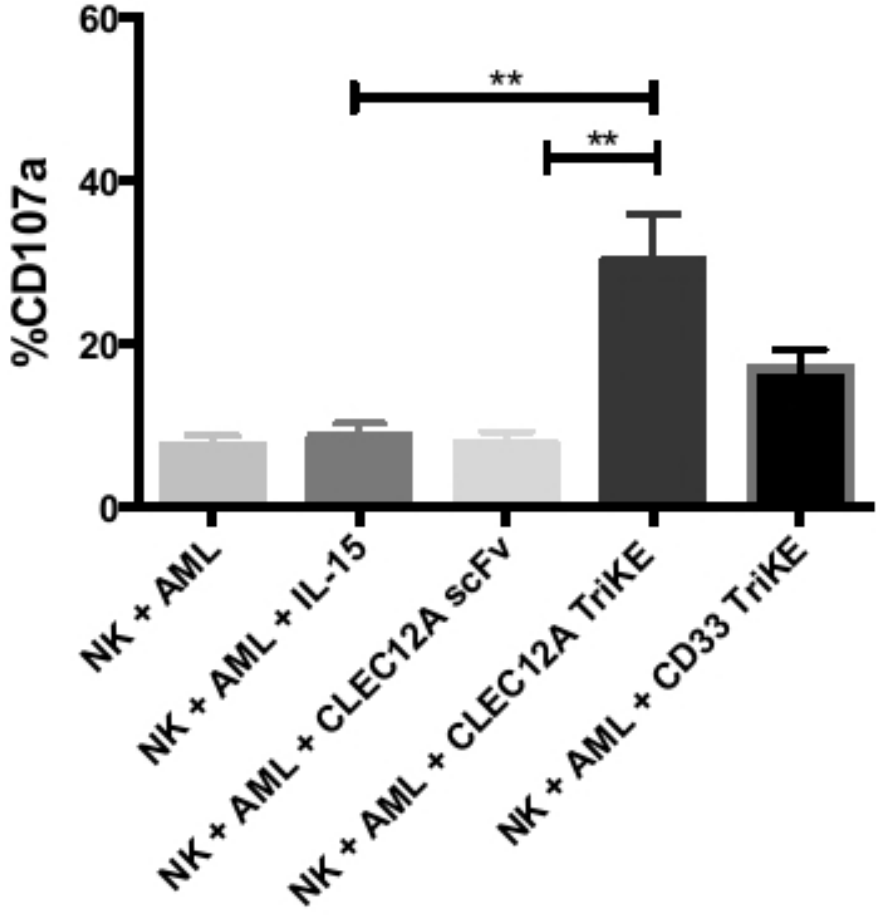
60
40
20
0
%CD107a
**
**
NK + AML
NK + AML + IL-15
NK + AML + CLEC12A scFv
NK + AML + CLEC12A TriKE
NK + AML + CD33 TriKE

FIGURE 8C

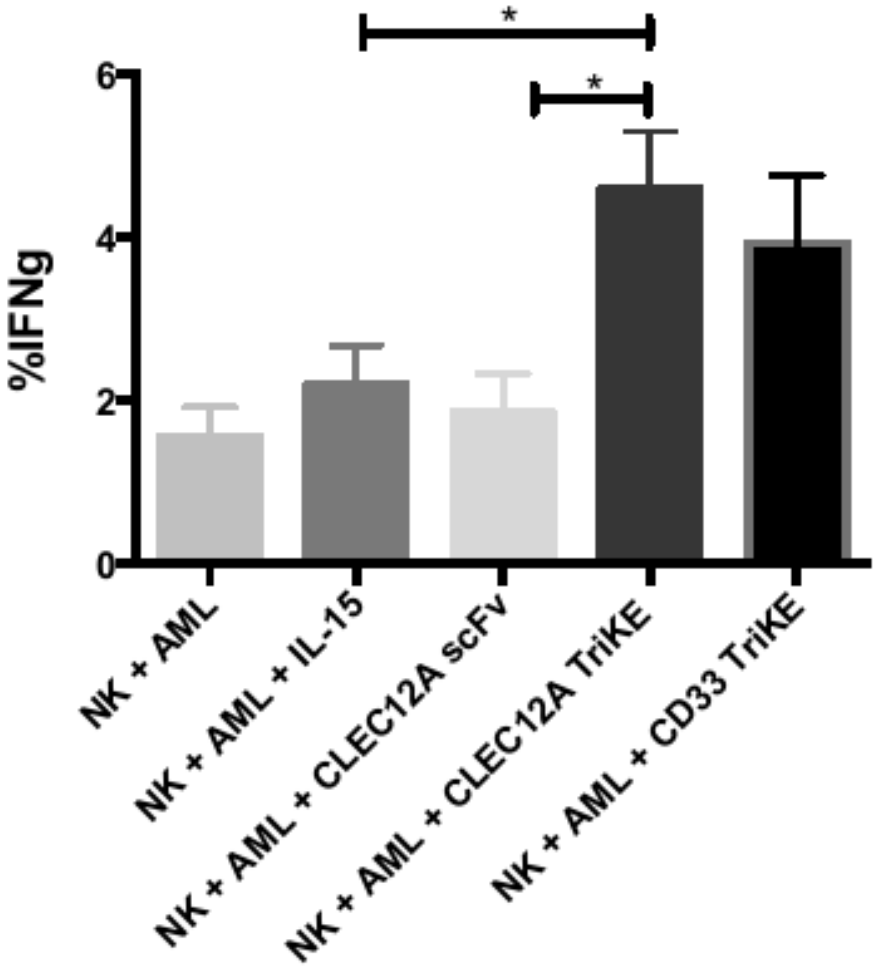
*
*
6
4
2
0
%IFNg
NK + AML
NK + AML + IL-15
NK + AML + CLEC12A scFv
NK + AML + CLEC12A TriKE
NK + AML + CD33 TriKE

FIGURE 8D

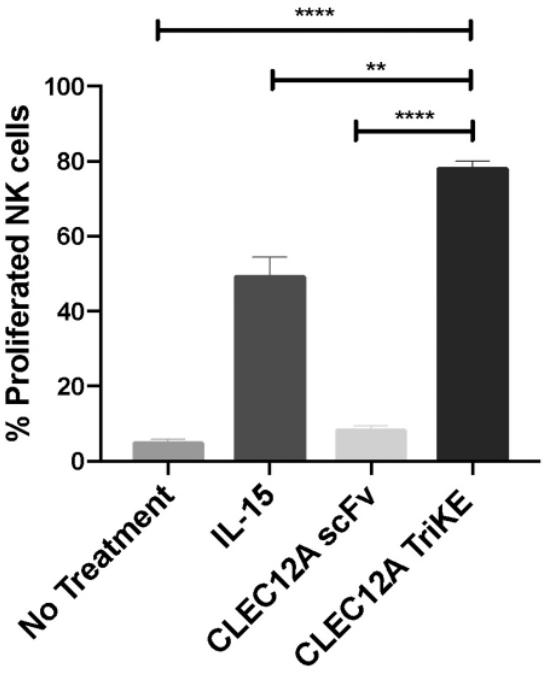

FIGURE 9A

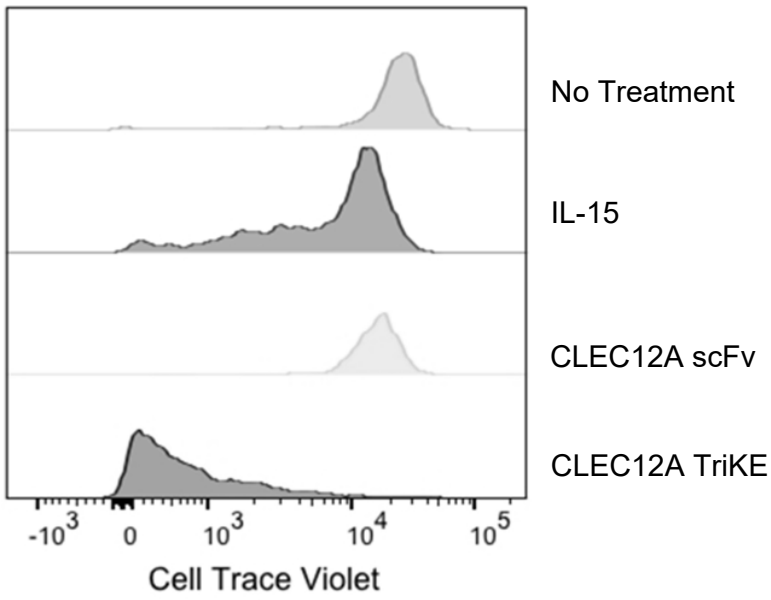

FIGURE 9B

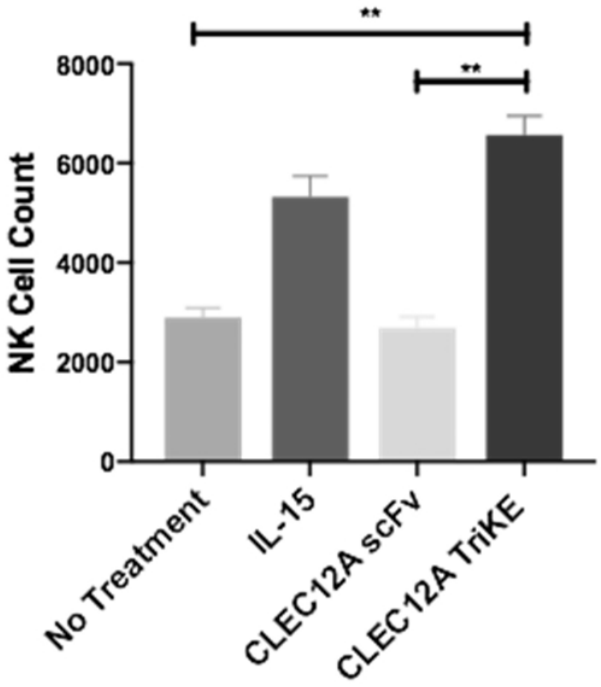

FIGURE 9C

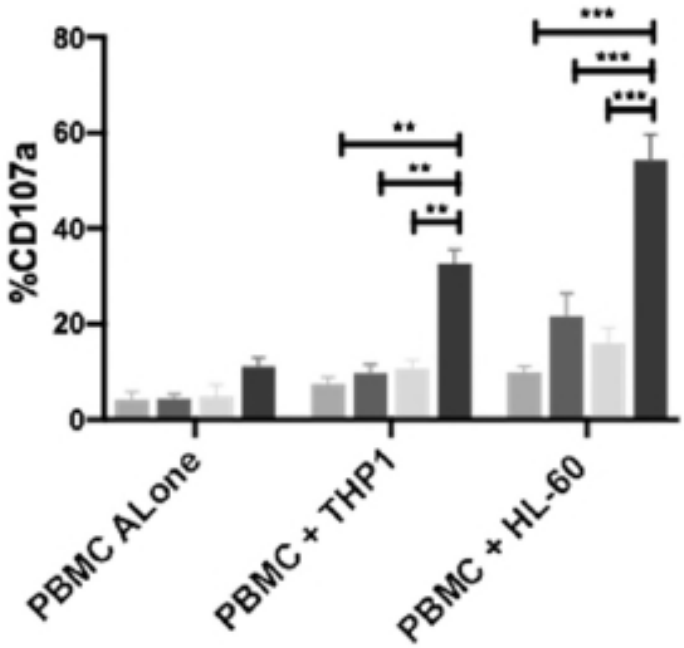

FIGURE 10A

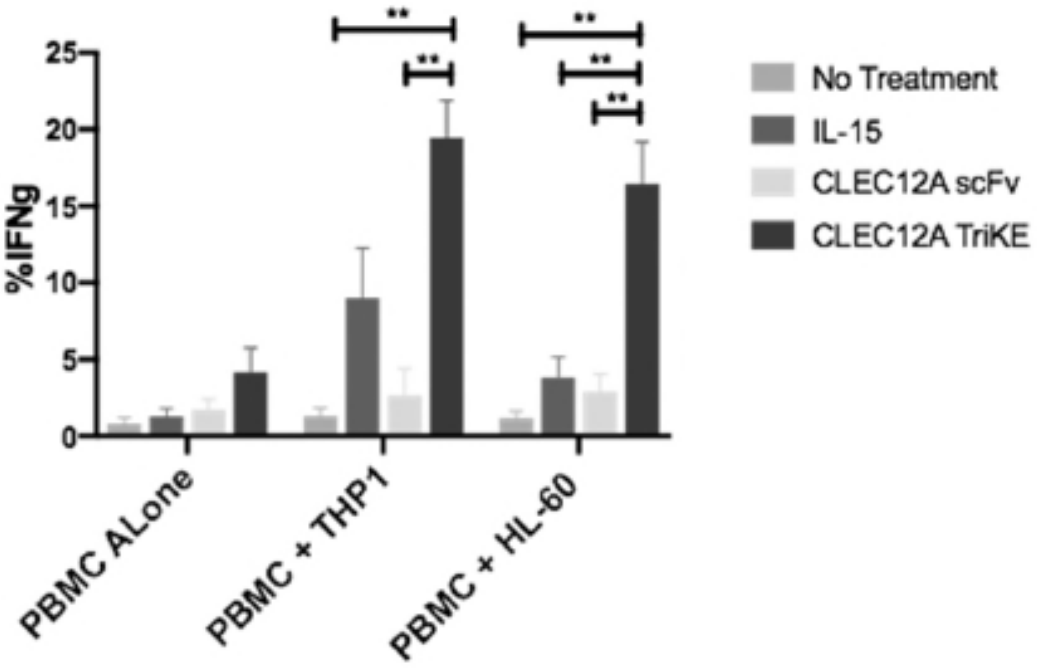

FIGURE 10B

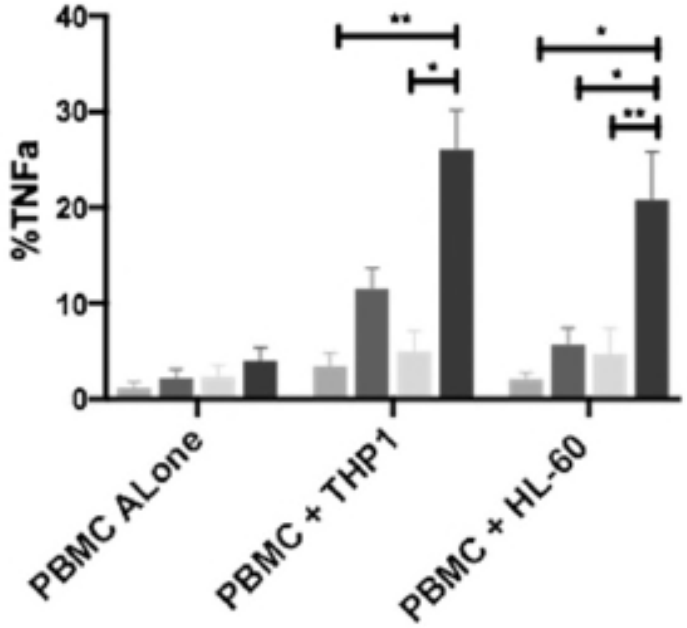

FIGURE 10C

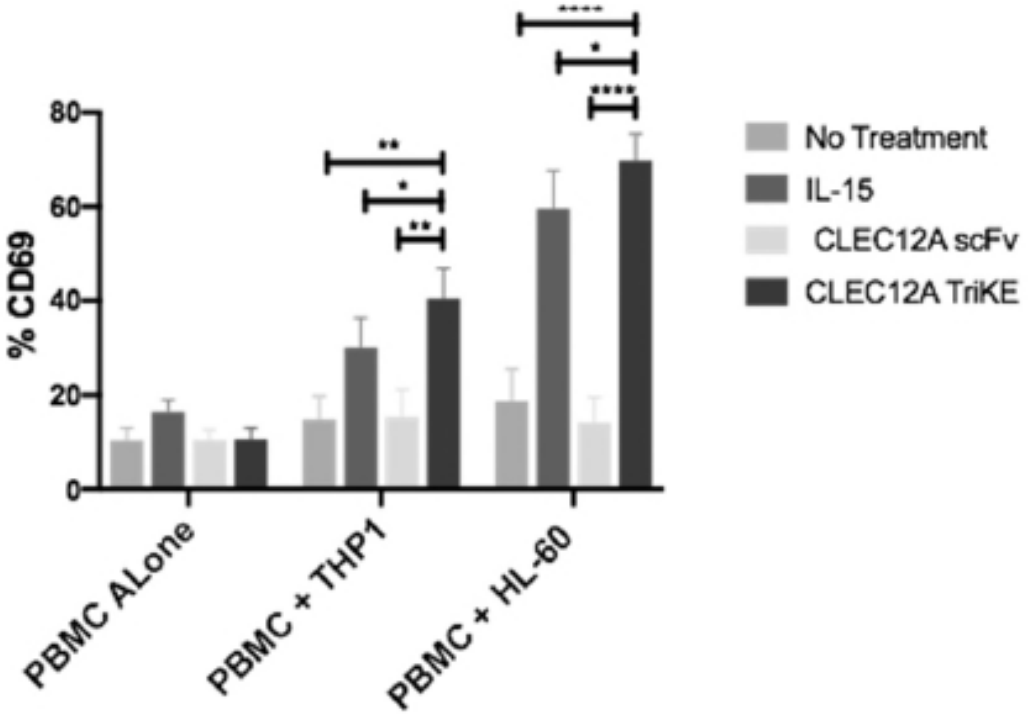

FIGURE 10D

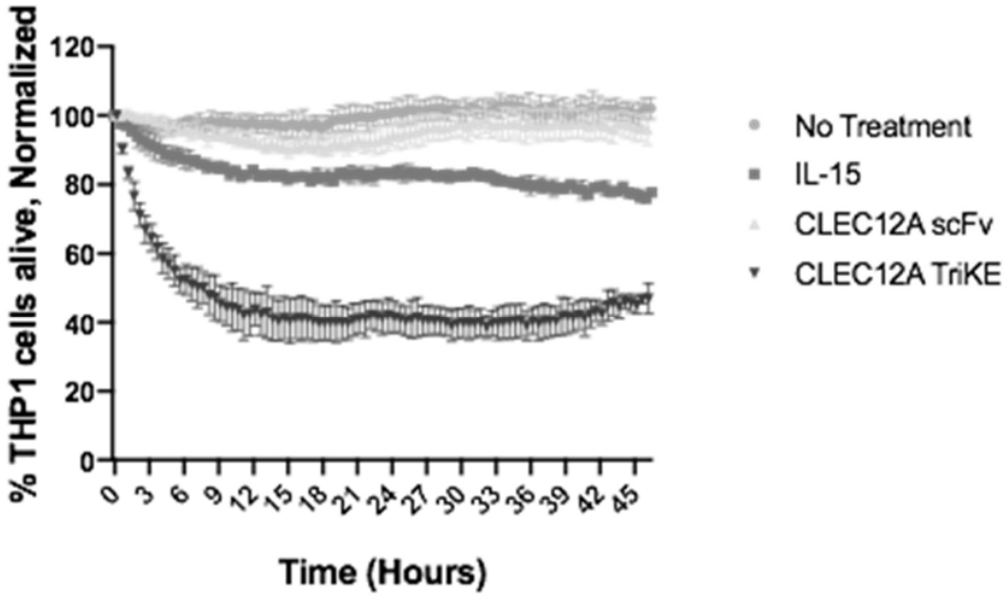

FIGURE 11A

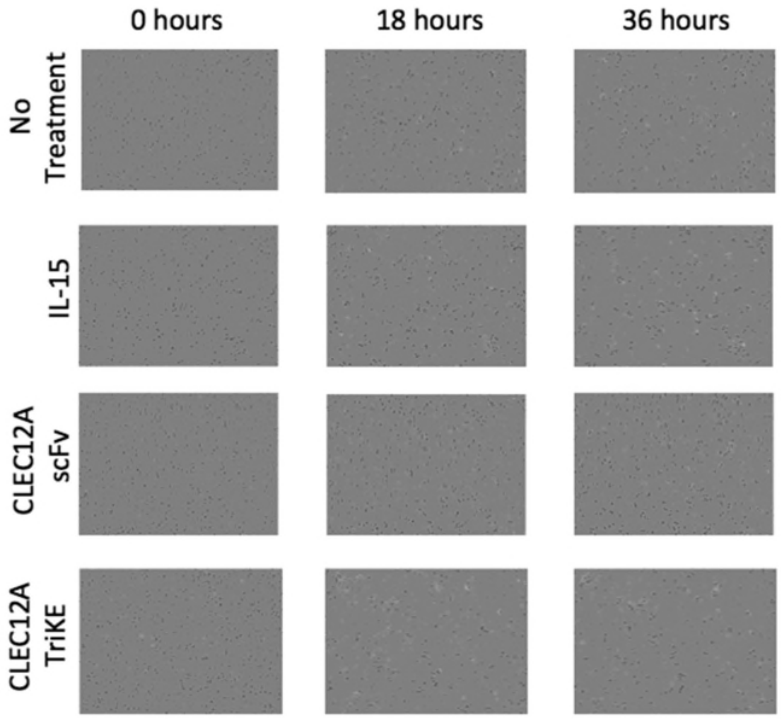

FIGURE 11B

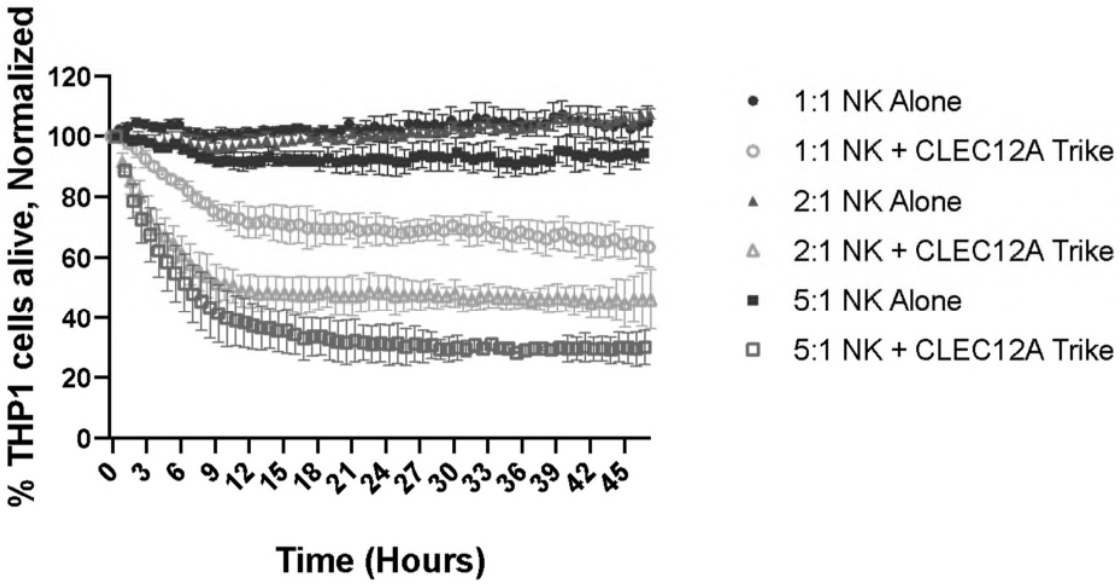

FIGURE 11C

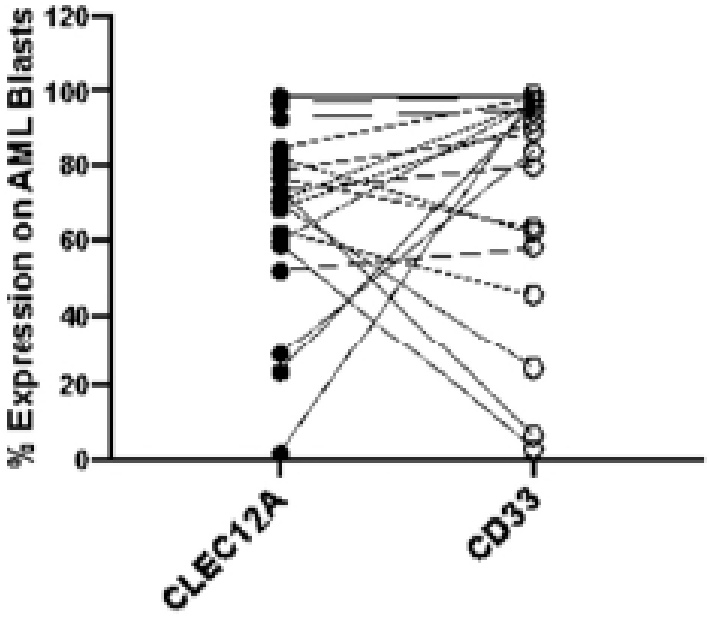

FIGURE 12A

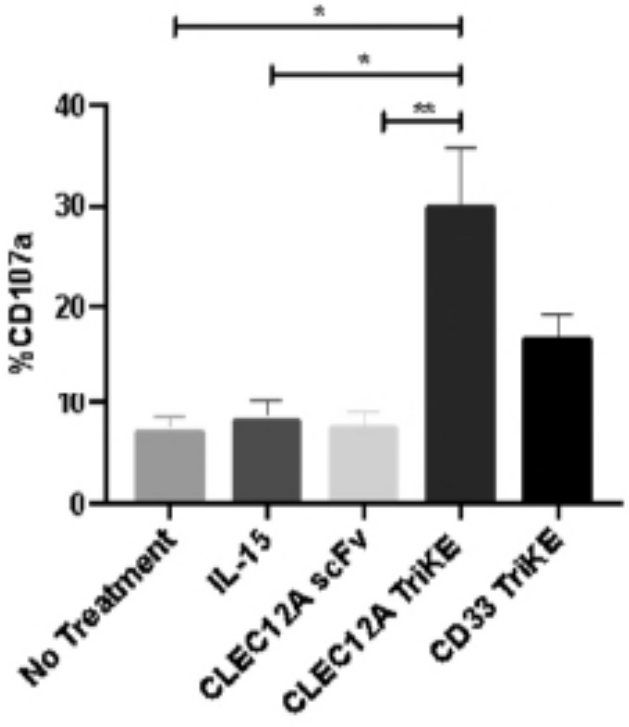

FIGURE 12B

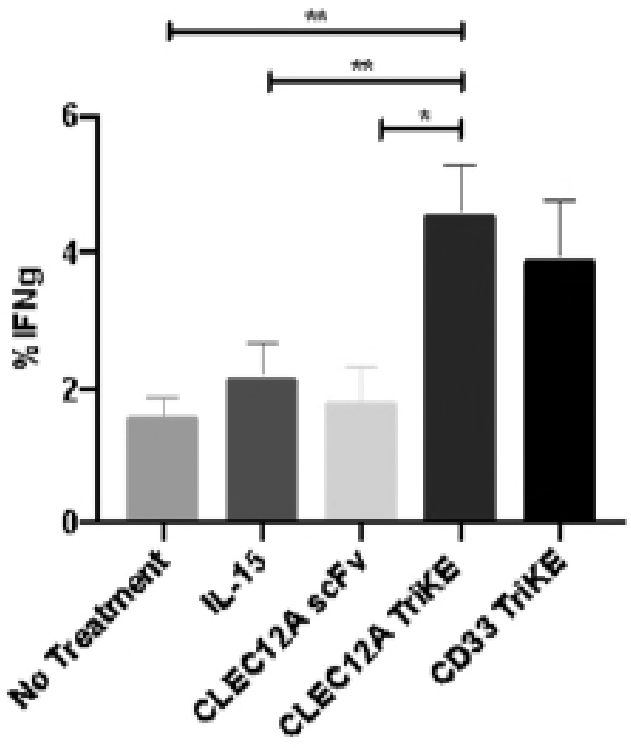

FIGURE 12C

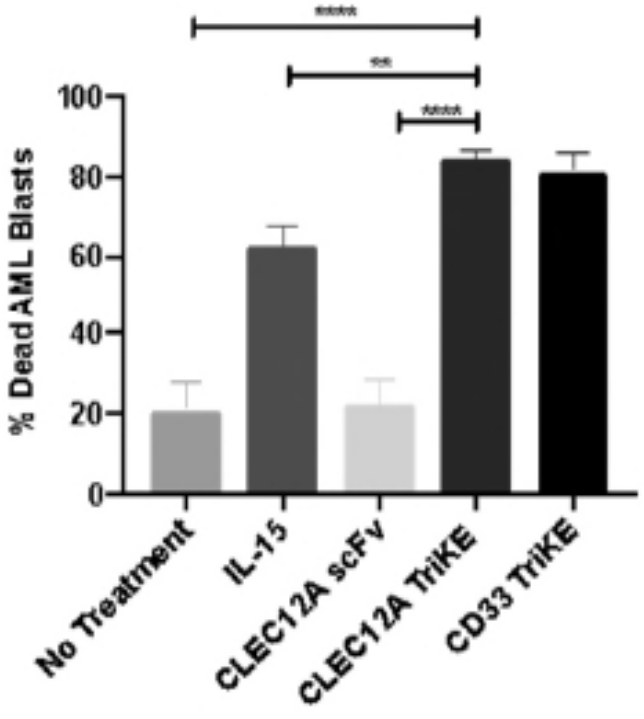

FIGURE 12D

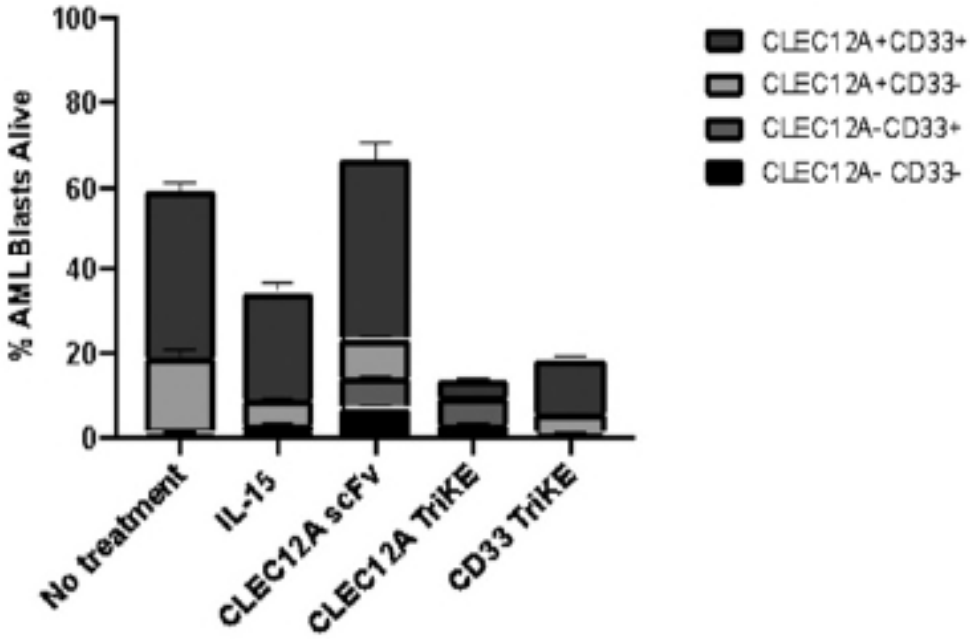
100
80
60
40
20
0
% AML Blasts Alive
No treatment
IL-15
CLEC12A scFv
CLEC12A TriKE
CD33 TriKE
CLEC12A+CD33+
CLEC12A+CD33-
CLEC12A-CD33+
CLEC12A- CD33-

FIGURE 12E

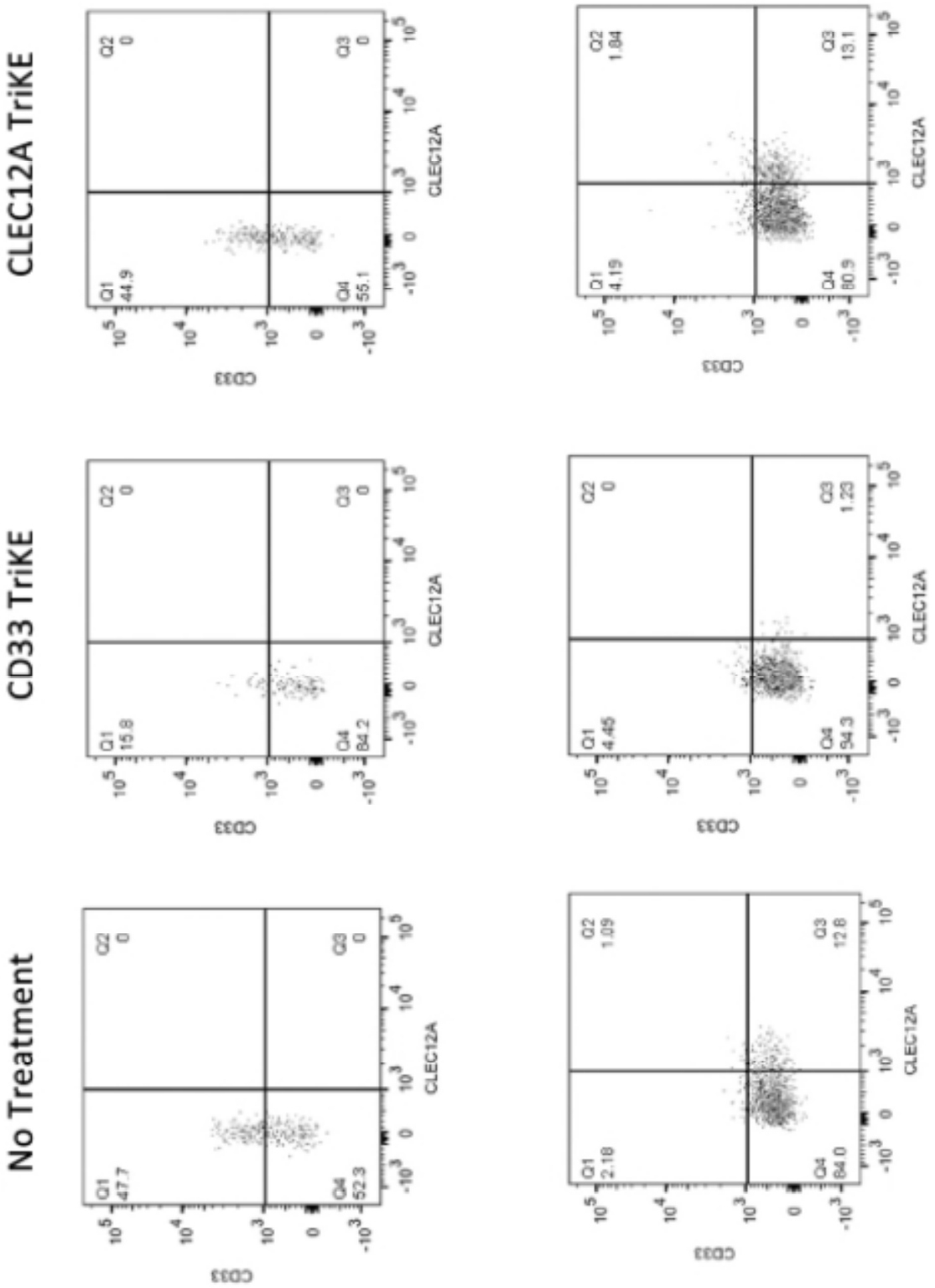
No Treatment
CD33 TriKE
CLEC12A TriKE
CD33
CLEC12A
Q1
47.7
Q2
0
Q3
0
Q4
52.3
Q1
15.8
Q2
0
Q3
0
Q4
84.2
Q1
44.9
Q2
0
Q3
0
Q4
55.1
Q1
2.18
Q2
1.09
Q3
12.8
Q4
84.0
Q1
4.45
Q2
0
Q3
1.23
Q4
94.3
Q1
4.19
Q2
1.84
Q3
13.1
Q4
80.9

FIGURE 12F

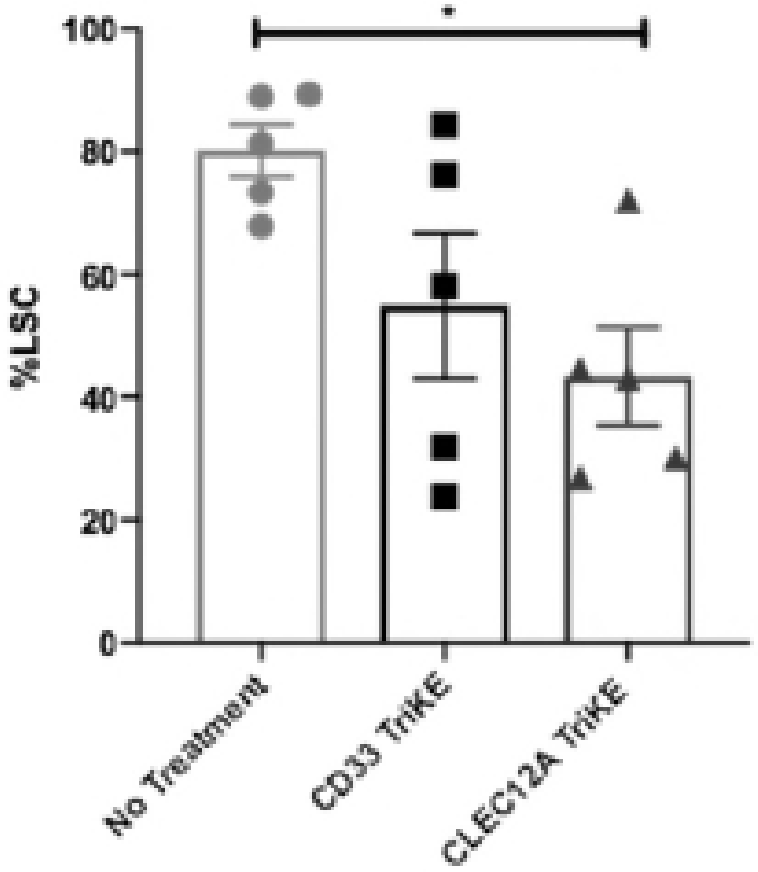

FIGURE 12G

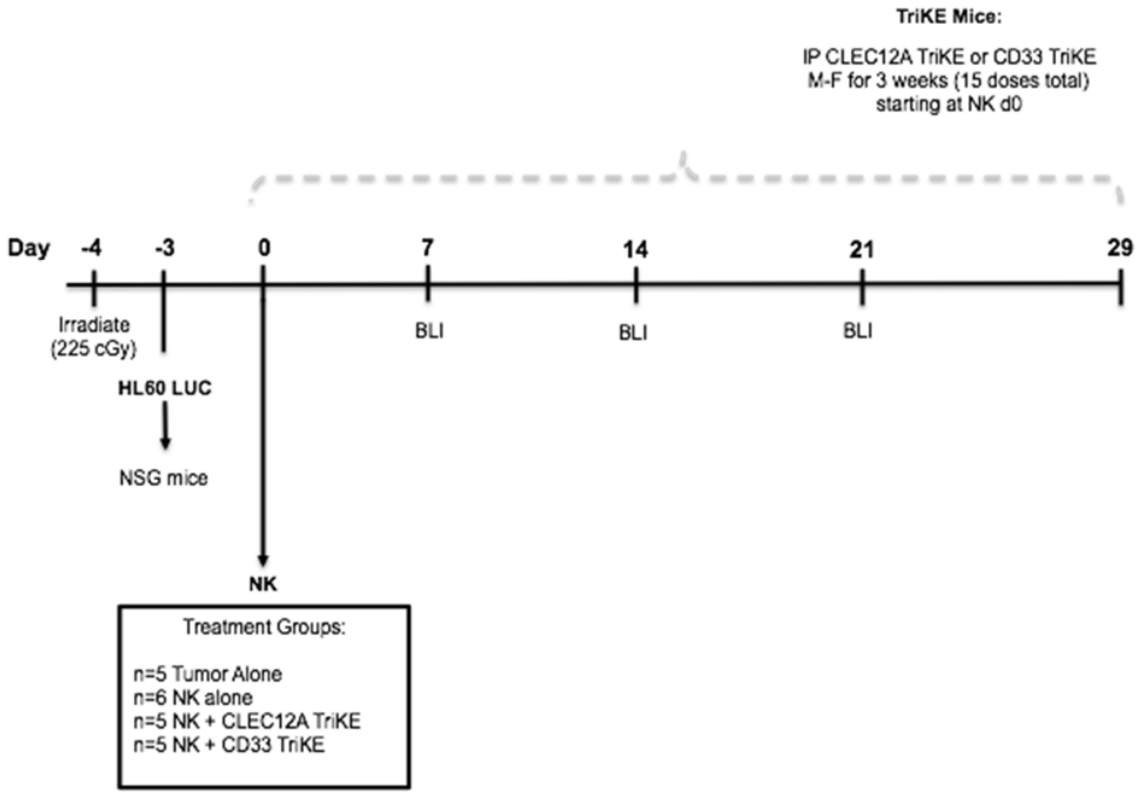

FIGURE 13A

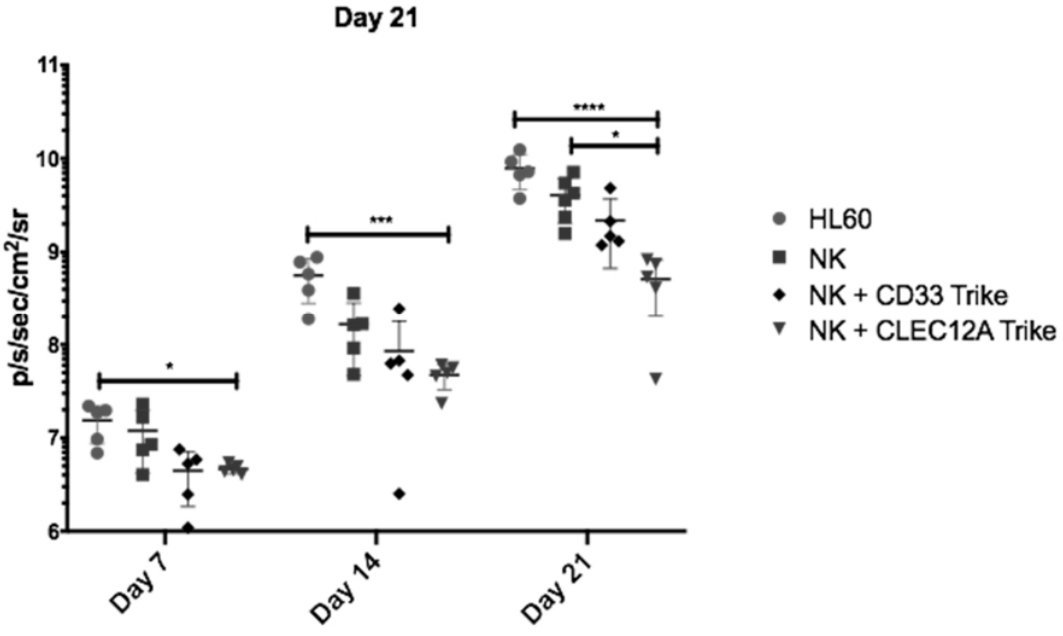

FIGURE 13B

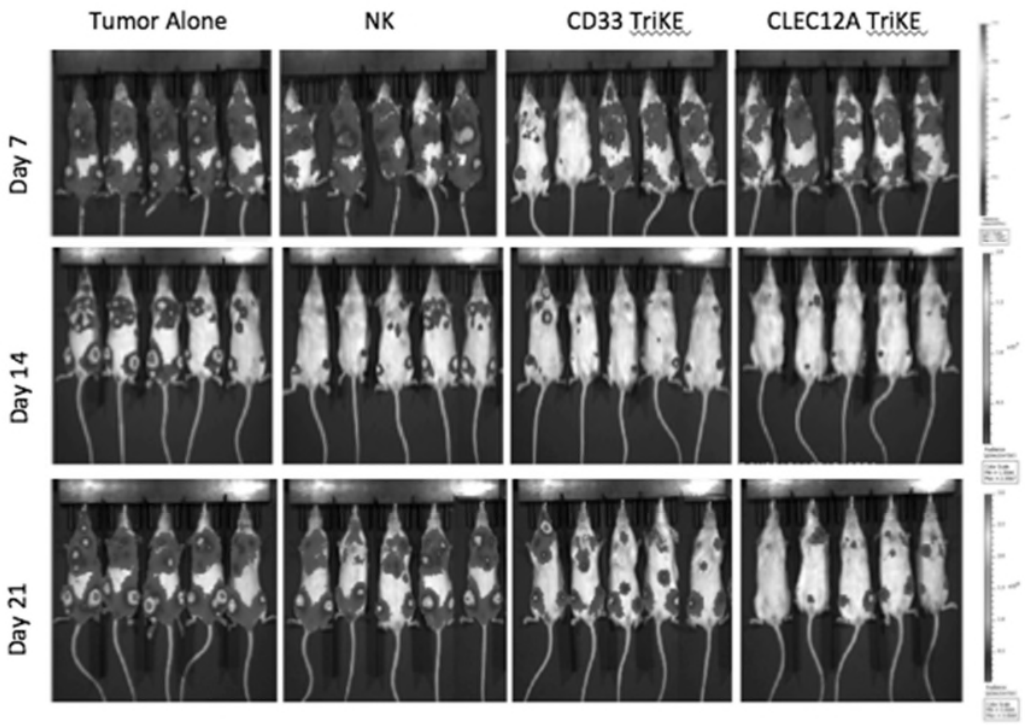

FIGURE 13C

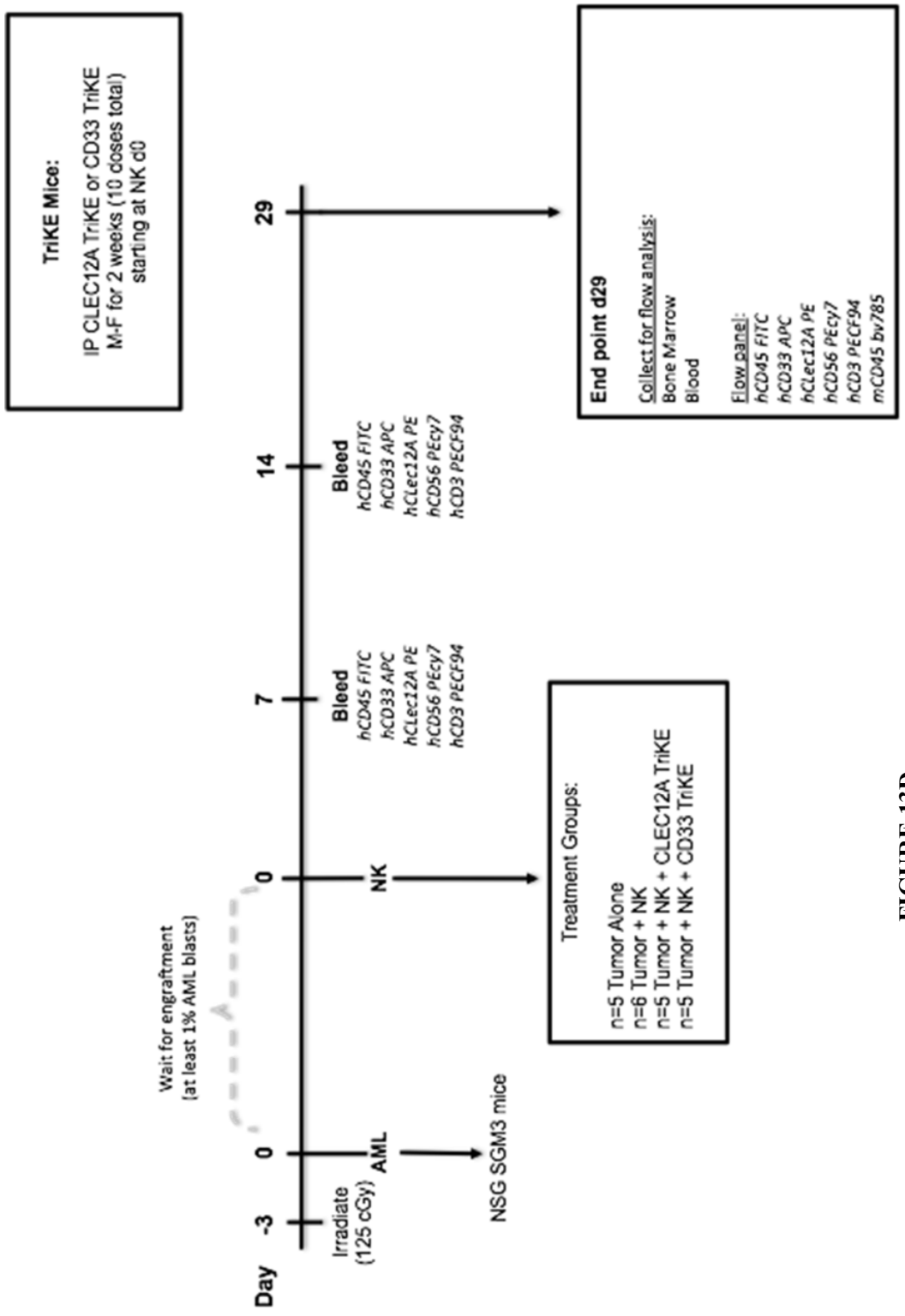
TriKE Mice:
IP CLEC12A TriKE or CD33 TriKE
M-F for 2 weeks (10 doses total)
starting at NK d0
Day
-3
0
7
14
29
Irradiate
(125 cGy)
AML
Wait for engraftment
(at least 1% AML blasts)
NSG SGM3 mice
NK
Bleed
hCD45 FITC
hCD33 APC
hCLec12A PE
hCD56 PEcy7
hCD3 PECF94
Bleed
hCD45 FITC
hCD33 APC
hCLec12A PE
hCD56 PEcy7
hCD3 PECF94
Treatment Groups:
n=5 Tumor Alone
n=6 Tumor + NK
n=5 Tumor + NK + CLEC12A TriKE
n=5 Tumor + NK + CD33 TriKE
End point d29
Collect for flow analysis:
Bone Marrow
Blood
Flow panel:
hCD45 FITC
hCD33 APC
hCLec12A PE
hCD56 PEcy7
hCD3 PECF94
mCD45 bv785

FIGURE 13D

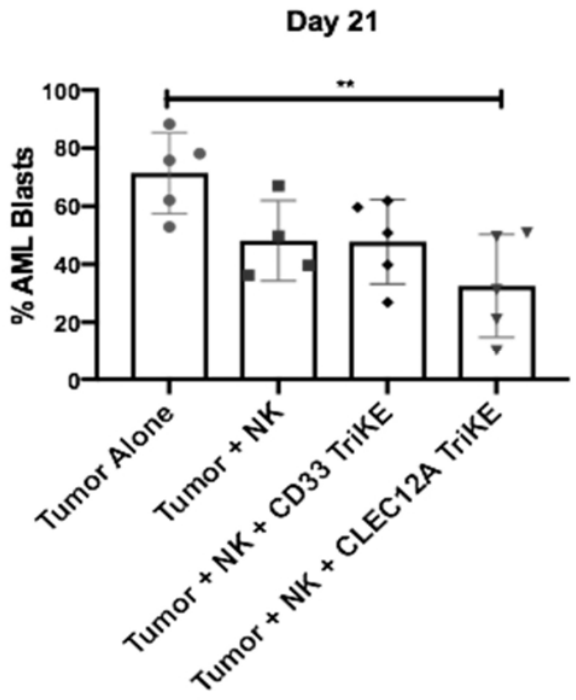

FIGURE 13E

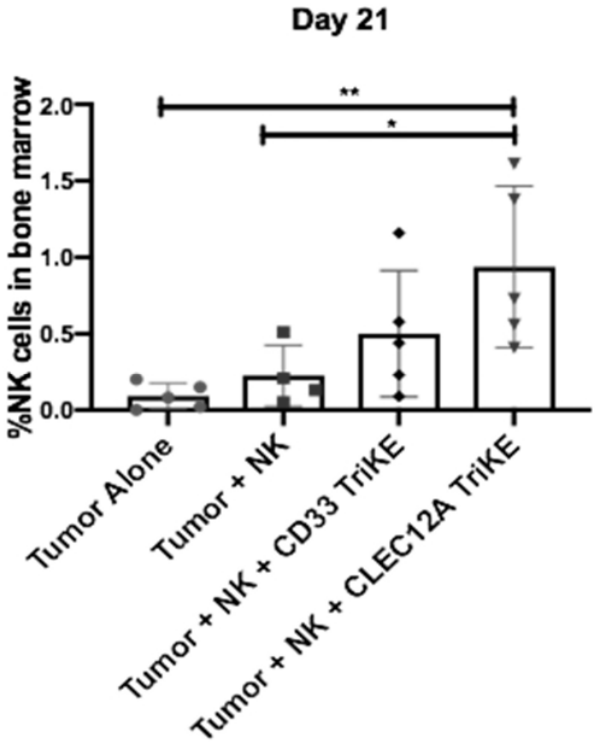

FIGURE 13F

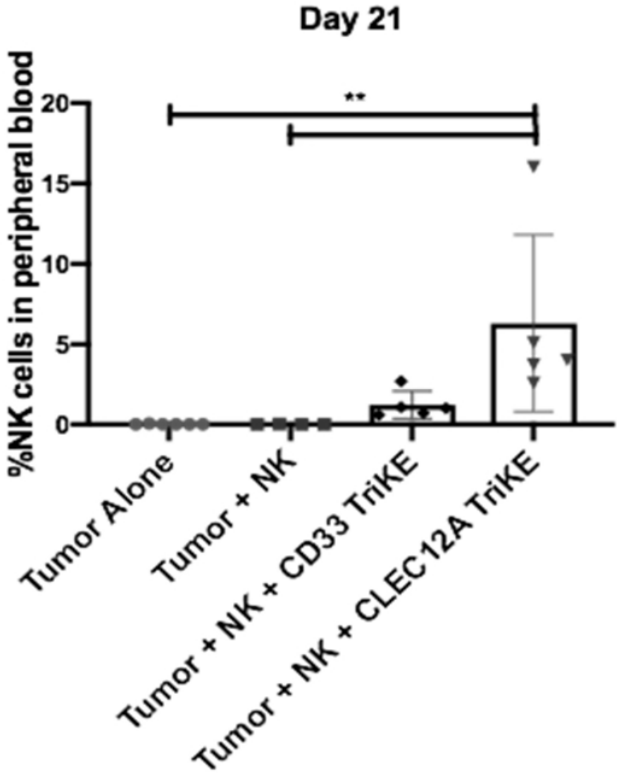

FIGURE 13G

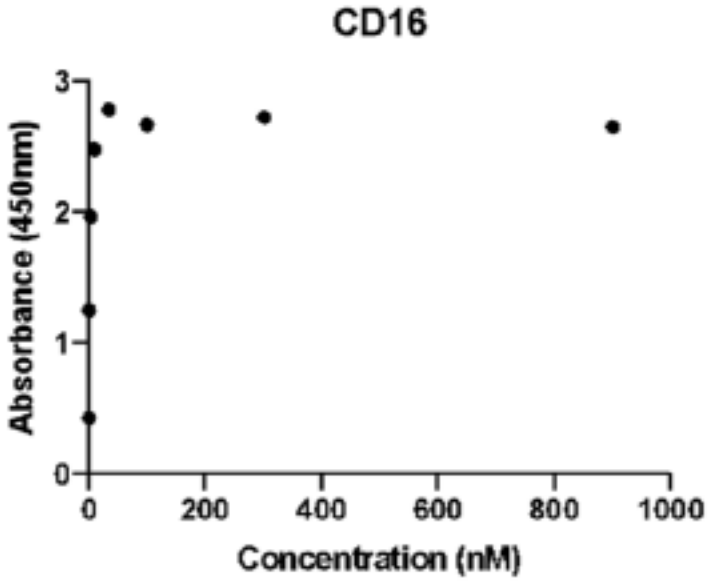

FIGURE14A

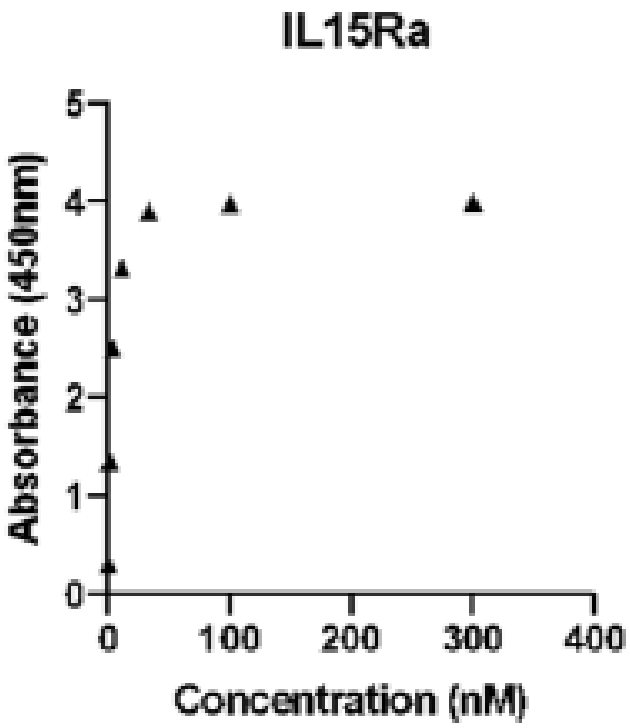

FIGURE 14B

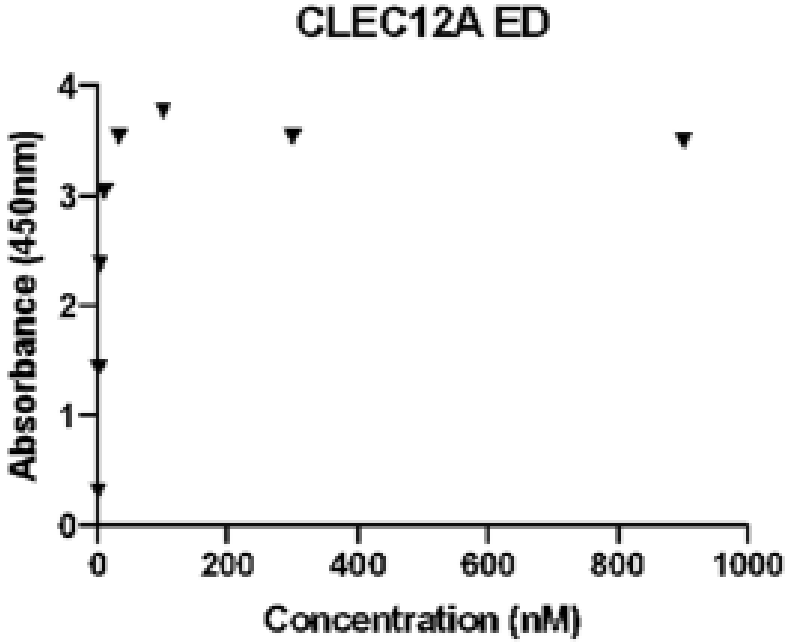

FIGURE 14C

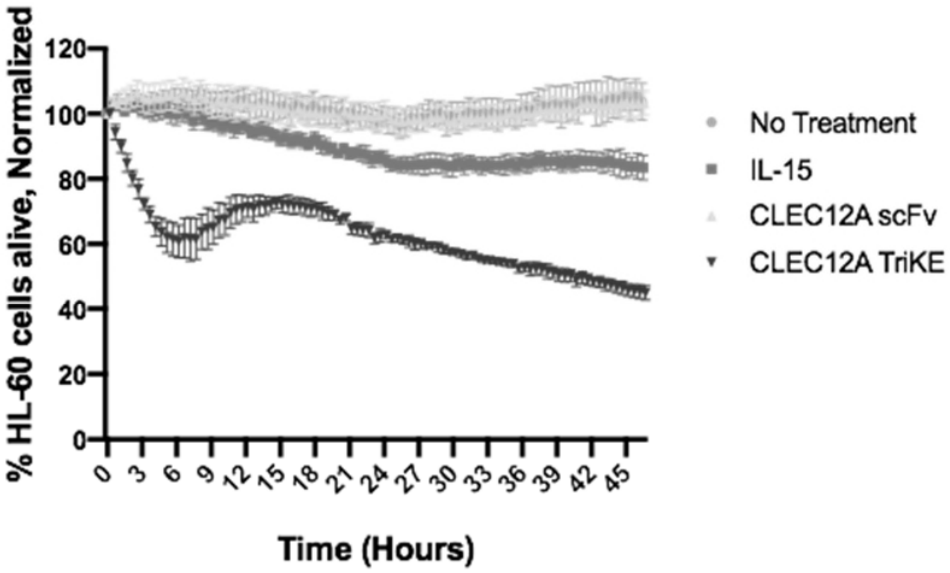

FIGURE 15A

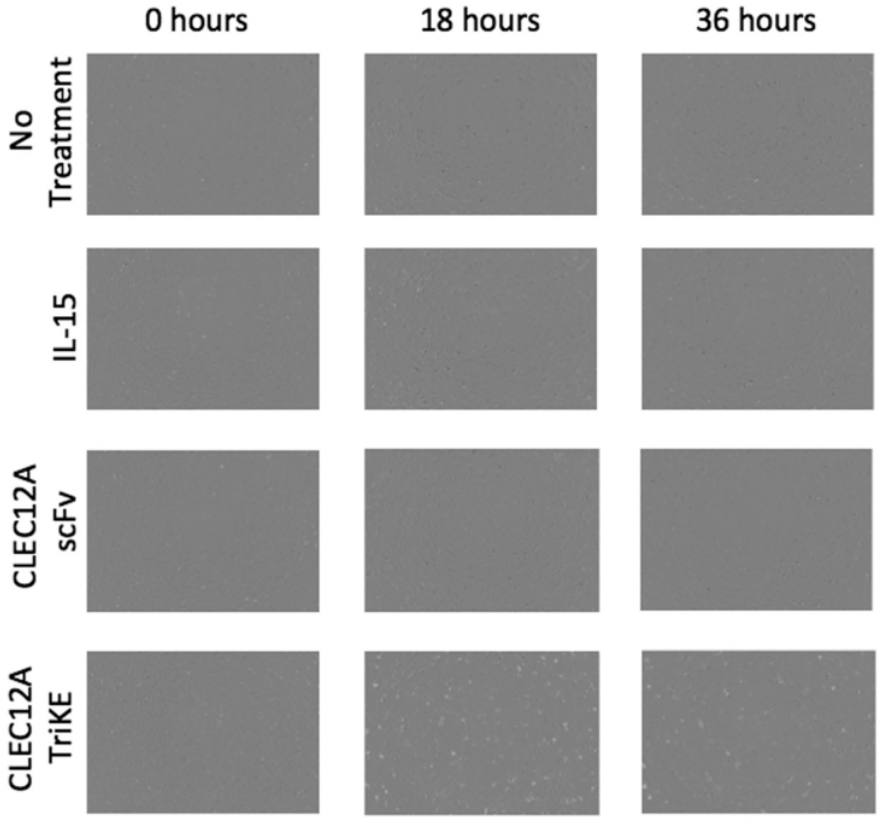

FIGURE 15B

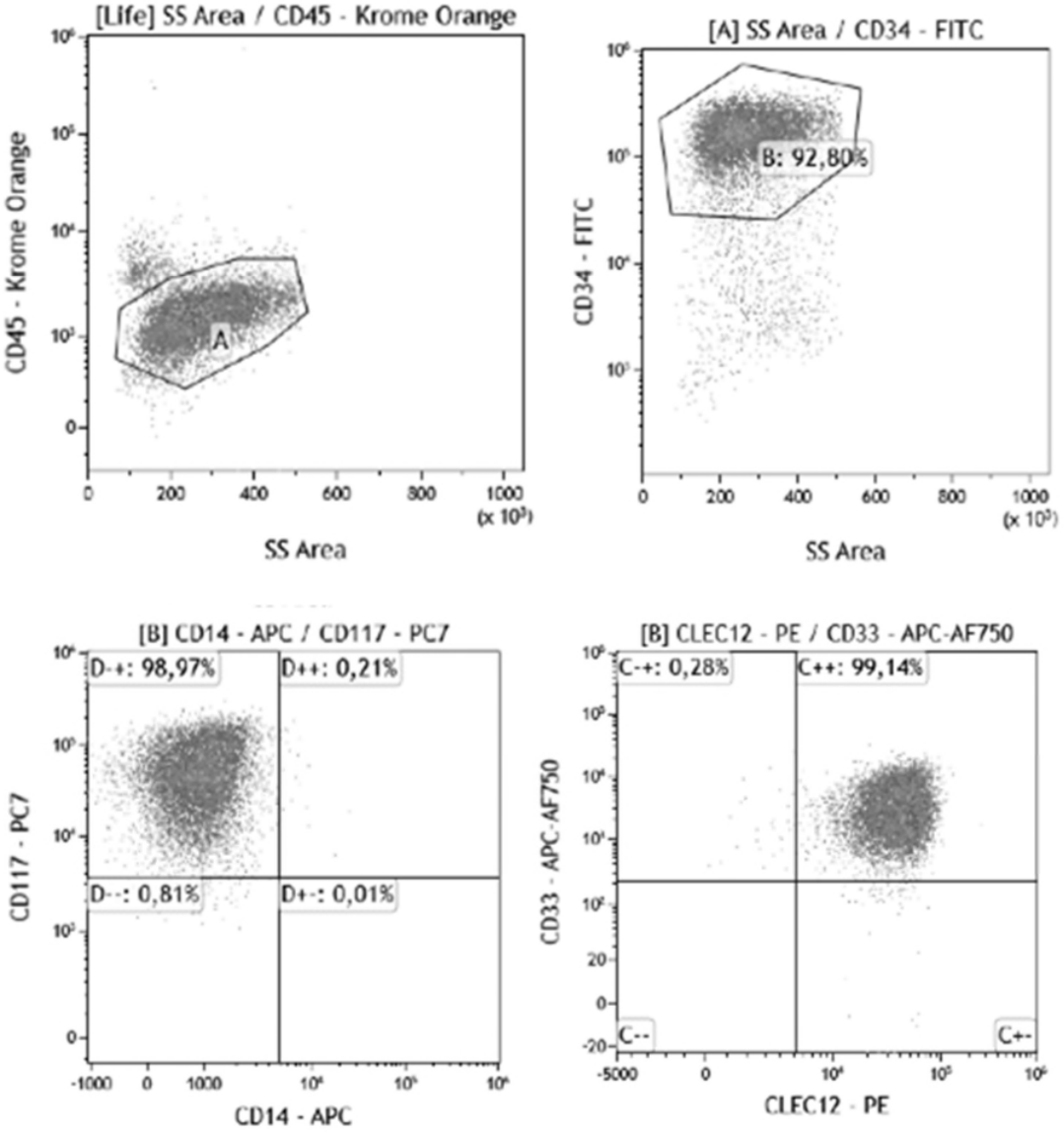
[Life] SS Area / CD45 - Krome Orange
CD45 - Krome Orange
SS Area
A
[A] SS Area / CD34 - FITC
CD34 - FITC
SS Area
B: 92,80%
[B] CD14 - APC / CD117 - PC7
D-+: 98,97%
D++: 0,21%
D--: 0,81%
D+-: 0,01%
CD117 - PC7
CD14 - APC
[B] CLEC12 - PE / CD33 - APC-AF750
C-+: 0,28%
C++: 99,14%
C--
C+-
CD33 - APC-AF750
CLEC12 - PE

FIGURE 16A

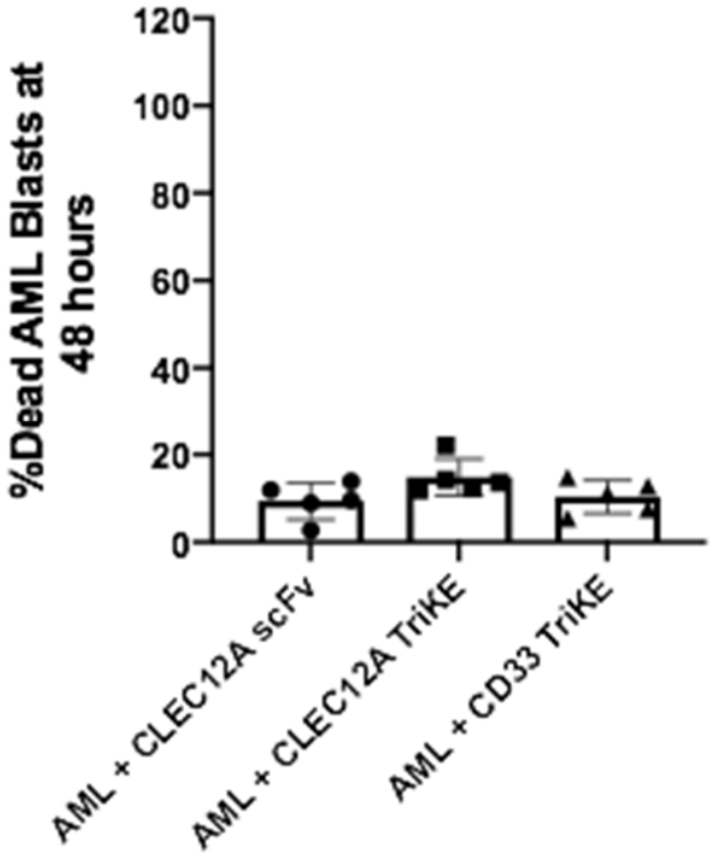
%Dead AML Blasts at 48 hours
120
100
80
60
40
20
0
AML + CLEC12A scFv
AML + CLEC12A TriKE
AML + CD33 TriKE

FIGURE 16B

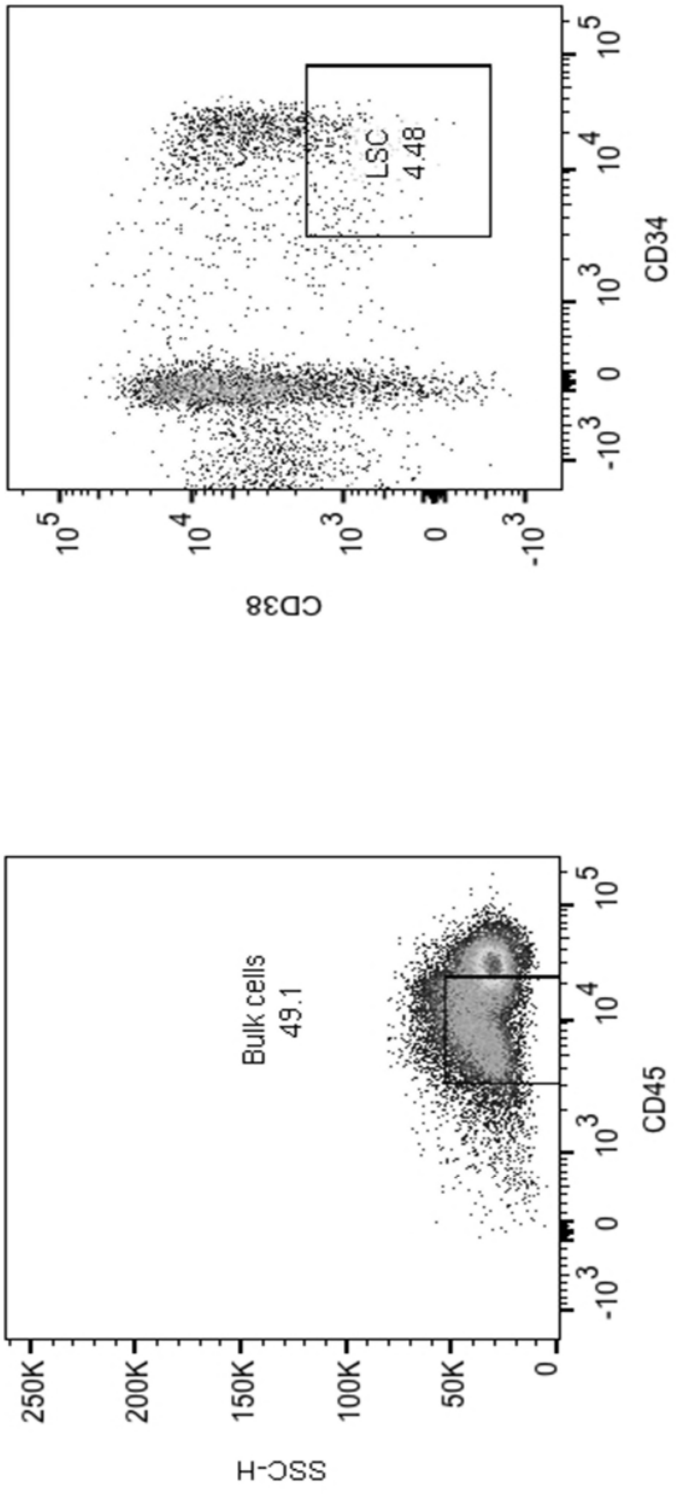
SSC-H
250K
200K
150K
100K
50K
0
Bulk cells
49.1
CD45
-10^3
0
10^3
10^4
10^5
CD38
10^5
10^4
10^3
0
-10^3
LSC
4.48
CD34

FIGURE 17

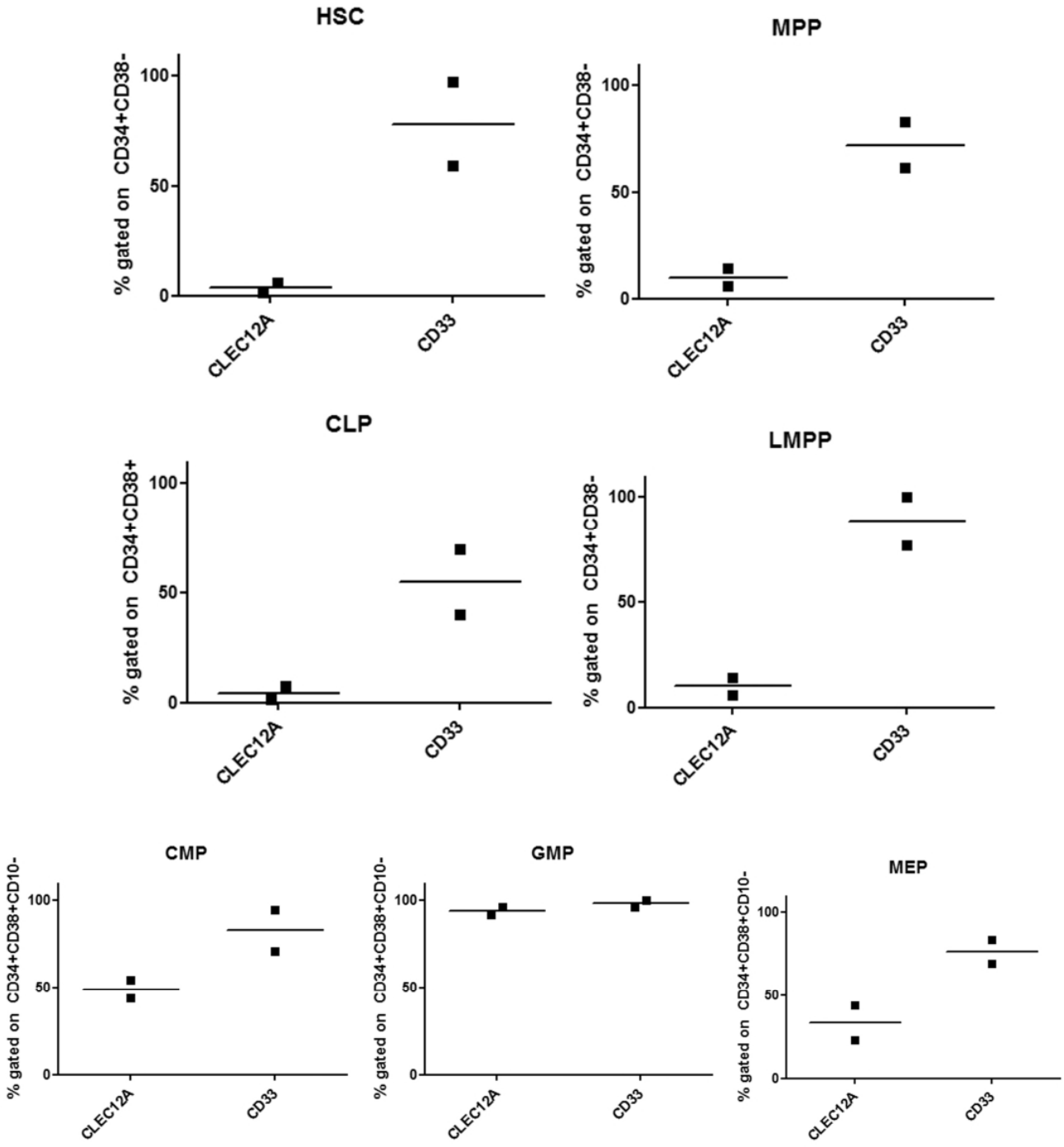
HSC
% gated on CD34+CD38-
100
50
0
CLEC12A
CD33
MPP
% gated on CD34+CD38-
100
50
0
CLEC12A
CD33
CLP
% gated on CD34+CD38+
100
50
0
CLEC12A
CD33
LMPP
% gated on CD34+CD38-
100
50
0
CLEC12A
CD33
CMP
% gated on CD34+CD38+CD10-
100
50
0
CLEC12A
CD33
GMP
% gated on CD34+CD38+CD10-
100
50
0
CLEC12A
CD33
MEP
% gated on CD34+CD38+CD10-
100
50
0
CLEC12A
CD33

FIGURE 18A

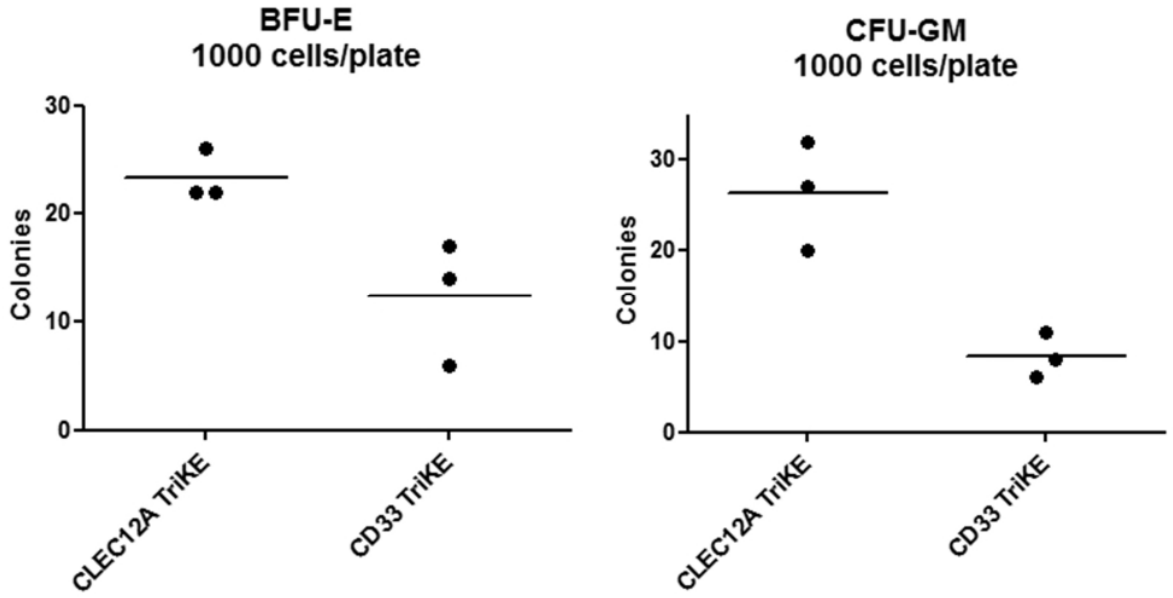
BFU-E
1000 cells/plate
Colonies
30
20
10
0
CLEC12A TriKE
CD33 TriKE
CFU-GM
1000 cells/plate
Colonies
30
20
10
0
CLEC12A TriKE
CD33 TriKE

FIGURE 18B

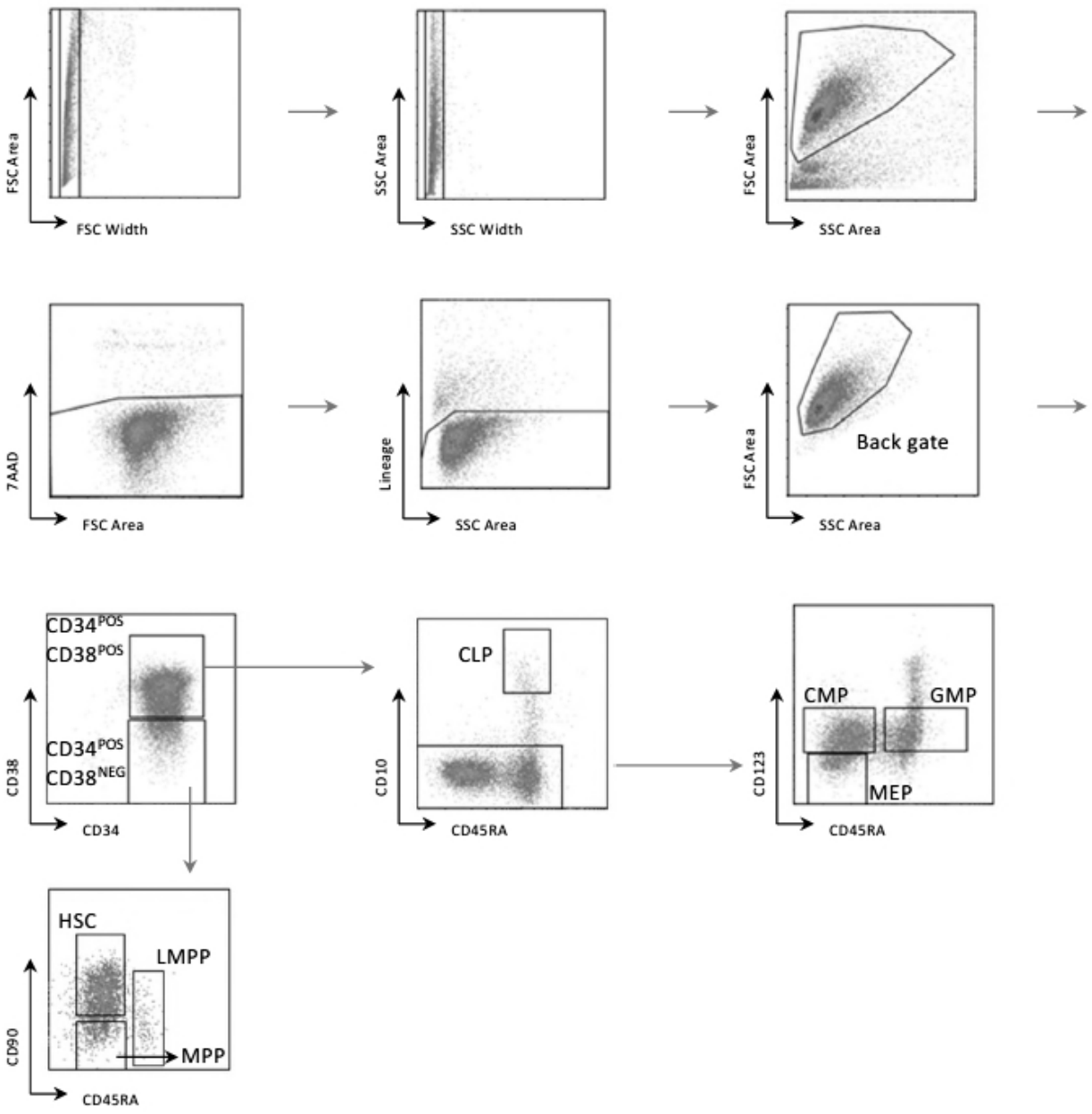
FSC Area
FSC Width
SSC Area
SSC Width
FSC Area
SSC Area
7AAD
FSC Area
Lineage
SSC Area
FSC Area
Back gate
SSC Area
CD34POS
CD38POS
CD34POS
CD38NEG
CD38
CD34
CLP
CD10
CD45RA
CMP
GMP
MEP
CD123
CD45RA
HSC
LMPP
MPP
CD90
CD45RA

FIGURE 19