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14. ABSTRACT

Multiple studies show that leukocytes infiltrating solid tumors have a hyporesponsive phenotype, yet the various molecular pathways remain unclear. We found one novel mechanism by TGFb, abundant in the tumor microenvironment, which induces miR-183 in human NK cells to downregulate the stimulatory signaling protein, DAP12 shutting down NK tumoricidal function. The goal of this project is to target miR-183 as a new therapeutic strategy to restore DAP12 in NK cells and recover immune function against cancer. Using immunodeficient NSG mice, the protocol is to optimize conditions to deliver anti-sense miR183 to NSG mice bearing human A549 lung tumors in combination with intravenous administration of human NK cells. The design of the anti-sense miR183 vector and its testing in vitro for efficacy is then followed by its use in vivo in tumor bearers to reconstitute NK function for regression of the established tumor.

15. SUBJECT TERMS

None provided

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1. INTRODUCTION:

Natural killer (NK) cells play an essential role in innate immunity against lung cancer but they can be compromised in the tumor microenvironment due to immunosuppressive factors produced by tumor cells. One such factor is transforming growth factor-beta (TGFb). We recently discovered that TGFb can turn on microRNA (miR)-183 in infiltrating NK cells which then degrades DAP12 that is critical for anchoring activating NK receptors on the cell surface. This event effectively blinds NK cells to the tumor cells. This project introduces a new concept to target miR183 for restoration of NK cells to combat lung cancer. It employs a human xenograft *Nod-scid-IL2Rg*$^{-/-}$ (NSG) mouse model using A549 human lung tumor cells with engraftment of human NK cells in vivo.

2. KEYWORDS:

Natural Killer Cell, Transforming Growth Factor-beta, lung cancer, microRNA-183, DAP12, NK receptor, NKp44, NKp46, Immunotherapy, Nanoparticle

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of this project is to test whether removing Natural Killer (NK) cell suppression by blocking microRNA-183 (miR-183) function helps to control early stage lung cancer in a fully humanized xenograft mouse model. These findings help to develop miR-based therapeutics that restores NK cells, which are critical for early immune surveillance against lung cancer.

What was accomplished under these goals?

Month 1-6: Established a robust human xenograft mouse model of lung carcinoma. During the first two months, we established luciferase-expressing human lung tumors in NSG mice. We generated stable luciferase- expressing A549 (A549-Luc) and optimized the tumor implantation in NSG hosts via subcutaneously injection. A549-Luc developed tumors successfully in NSG hosts and mice bearing A549-Luc tumors were the subject of all subsequent studies. An orthotopic model of SCLC was also tested at this time. However, a caveat of this model was the uneven distribution and growth of the cell line inside the mouse lungs making it an inadequate model for the study of NK delivery of anti-miR183 making the study of the orthotopic SCLC model unfeasible at this time. Because of this, from that point on we focused our efforts on the subcutaneous model of NSCLC A549-injection with consistent results.

Because healthy human NK cells are critical for the project, we developed and optimized cell isolation, culture and activation of NK cells from whole blood. Next, we evaluated NK cells and host interaction by transferring NK cells into A549-tumor bearing NSG host via tail vein injection. Using bioluminescence imaging, we observed that tumors in mice injected with NK cells grew significantly slower than in mice that did not receive NK cells.

At day 28, we harvested tumors, blood and tissues from tumor-bearing mice to analyze for NK presence in the different organs. We found that NK cells pre-cultured in IL2 and IL15 persisted in mouse blood, liver, and spleen as long as one week after being injected. By staining for NK activation markers, such as CD56, NKp46, NKp44, and NKG2D, we found that NK cells were retained all the phenotypes in blood and other organs but a in tumors, suggesting that the tumor microenvironment can suppress NK cell activity (Fig. 1). These results indicate that additional treatments are needed together with the NK therapy against lung cancer.

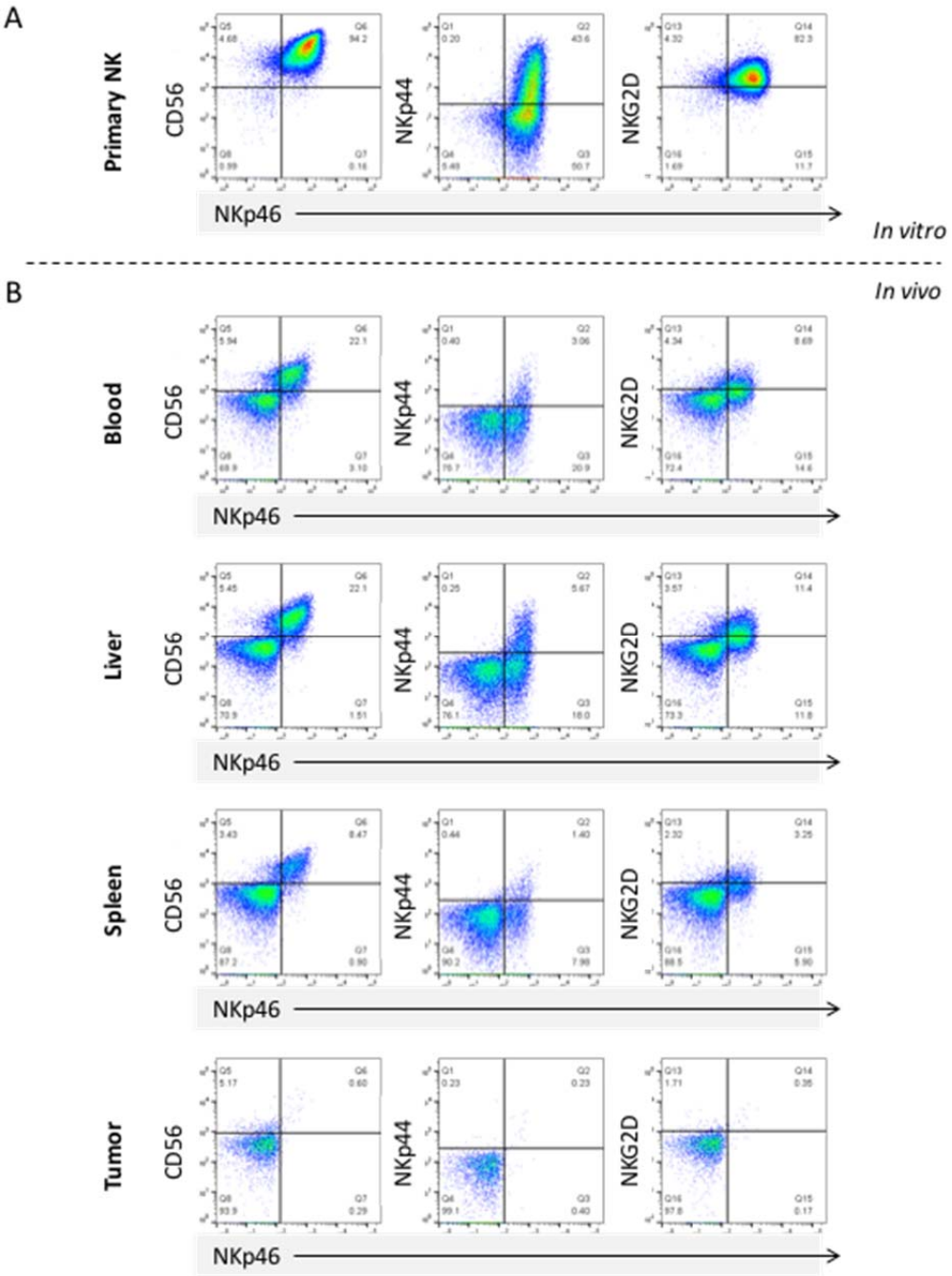

Figure 1 Flow cytometry analysis of surface CD56, NKp44 and NKG2D on A) Primary NK cells cultured in vitro; and B) Primary NK cells in vivo isolated from blood, liver, spleen, and tumor of A549 lung tumor-bearing mice. Mice were subcutaneously inoculated with $5x10^6$ A549-luciferase on the back followed 7 days later by intravenous injection with $1x10^7$ NK cells in phosphate-buffered saline supplemented with IL2- and IL-15. Mice were sacrificed 3 days later and lymphocytes were isolated from tumors and different tissues using Ficoll density centrifugation.

Month 6-12: Evaluate miR-183 blockade by anti-miR-183 as a new immunotherapeutic strategy.

We hypothesized that TGFb, abundantly produced by tumor cells in the tumor environment, can induce miR-183 up-regulation in NK cells to cause the down-regulation of NK function. Indeed, NK cells treated with TGFb displayed fewer activation markers, quantitated by FLOW staining and western blot, as well as impaired killing function determined by cytotoxic assays. In addition, miR-183 was also at least five times higher in NK cells treated with TGFb compared to untreated controls.

Because establishing an effective way to block miR-183 function of NK cells is crucial for the study, we have created an anti-miR-183 lentiviral construct and developed different techniques for maximum virus uptake. We noted that actively dividing NK cells stimulated with IL2 and IL15, and high titer lentivirus expressing anti- miR183 were both critical for optimal anti-miR-183 uptake into NK cells, hence maximizing miR-183 blockade. We have spent six months to create anti-miR-183 constructs, producing high titer virus and optimizing the technique of virus uptake into large numbers of NK cells for in vivo delivery into NSG mice bearing A549 human lung tumors.

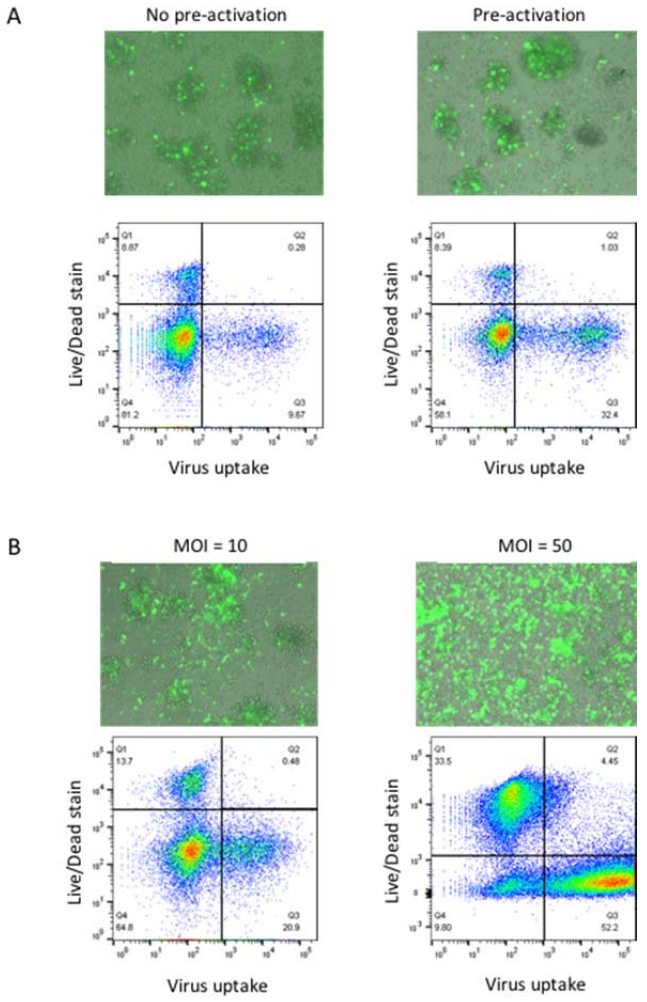

Figure 2 Infectivity of anti-miR183 lentivirus in NK cells showing different levels of viral uptake. A) With or without pre-activation by cytokine for 24 hours before being transduced with lentivirus. B) with low titer virus (MOI=10) and high titer virus (MOI=50). Viral uptake was measured by fluorescence microscopy or FLOW cytometry staining for GFP co-expression. Dead NK cells were gated out by Live/Dead staining.

Month 12-24: Evaluate miR-183 blockade by anti-miR-183 as a new immunotherapeutic strategy.

We have optimized conditions to grow and maintain human NK cells before and after uptaking of anti-miR-183 lentiviral construct. To maintain NK cells in vivo, we notice that the culture of NK cells with IL15 prior to in vivo tail vein injection into mice and the addition of IL15 to the injection medium significantly improved the detection of NK cells in tissues of mice bearing human A549 lung tumors. However, we faced the problem of recovering few NK cells in tumors of mice (0.6%), comparing to significant numbers in the blood, liver or spleen (22.1%, 22.1%, 8.47%, respectively). We surmised that the reason of recovering few NK cells in the tumor bed was due to harsh conditions to isolate human NK cells. We found that the commercial digestion buffer to dissolve the tumor causes cell death of both tumor cells and infiltrating NK cells. Viability of tumor cells ranges from 50% to 60%, and because the injected NK cells form a very small percentage of the cells within the tumor tissue, they could easily be lost. We attempted to use other commercial digestion buffers and finally found one from BD Life Sciences that provided us with 94-97% viability (Fig. 3A). With this product, we have resumed to examine the trafficking of human NK cells into organs of A549-tumor bearing NSG mice.

Using either CD56/NKp46 or NKp44/NKp46 as specific NK markers and the BD Digestion Buffer, we found that NK cells still did not appear to enter the tumor but can be detected in the blood, liver, and spleen **(**Fig. 3**).** To determine if this is a phenomenon related to the tumor cell line used, we next tested NK cell infiltration into either A549 tumor-bearing mice or H1299 lung tumor-bearing mice **(**Fig. 4A, B**).** Gating on CD45+ human lymphocytes, the same results were obtained with both cell lines in vivo, using NKp46/NKp44 or NKp46/NKG2D markers to identify NK cells.

The lack of significant detection of NK cells in tumor tissue could be due to loss of chemokine receptors in NK cells caused by the tumor microenvironment or due to lack of chemokines produced by the tumor cells to attract NK cells. The chemokine and chemokine receptor family is extensive but NK cells are known to express certain chemokine receptors, including CCR2. We have reported earlier that A549 tumor cells lack CCL2 which bind CCR2, and thus may not attract NK cells. We are attempting to define if CCL2/CCR2 or other chemokine/receptors are involved.

One current caveat of lentivirus introduction of anti-sense miR183 on primary NK cells is the delicate state of these cells making them highly susceptible to cell death induction by lentiviral stimulation. We observe that this effect decreases the efficacy of injected NK cells in our in vivo tumor model making it hard to reach optimal effects by these NK cells. Taking advantage of our current collaboration with Dr. Blanka Sharma from the University of Florida, we successfully encapsulated miRNA183 into PLGA nanoparticles (NP)s while maintaining miRNA integrity, with a loading efficiency of 25% to improve the viability of NK cells for in vivo injection, ensuring the success of the goals in task 2 of the SOW. These NPs also have no untoward cytotoxic effects on NK cells in vitro. PLGA nanoparticles (NP) appear to be easily taken up by human NK cells in vitro and they are not targeted into lysosomes, thus indicating that the anti-sense miR183 cargo within PLGA NPs will not be degraded inside the NK cell.

In order to test the product in vitro prior to in vivo therapeutics in NSG mice, we first developed a 3D polyethylene glycol-based hydrogel system for the study of NK cell-lung cancer cell interactions. In this system, NK cells migrated into hydrogels containing primary A549 cells but only at 24h after tumor implantation. The tumor cells were incubated in the hydrogels for 24 h (early stage) or 7 days (mature stage) prior to introduction of NK cells to mimic various stages of the tumor microenvironment. With increasing culture time, the A549 cancer cells remain viable and form cell aggregates/clusters in the gel. Interestingly, after culturing A549 cells for 7 days, NK cells show significantly lower migration into the A549 gels (Fig 5A). These finding recapitulate in vivo results showing that when NK cells are co-injected with cancer cells in mice, tumors do not form; however, when the cancer cells are injected first and NK cells 7 days later, the NK cells do not impact tumor development, and tumors do form. Therefore, we believe our in vitro model may

recapitulate important features of the in vivo tumor microenvironment. Cytokine analysis for the 3D models revealed that NK cells are indeed less activated in the “mature” tumors than in “early stage” tumors, as determined by decreased levels of RANTES, a chemokine secreted by activated NK cells to recruit other immune cells (Fig 5B).

We plan to complete our characterization of this model system in vitro and compare the results with tumor xenografts in vivo, in order to validate that our in vitro system models in vivo mechanisms and is an effective system for evaluating immunotherapies in vitro.

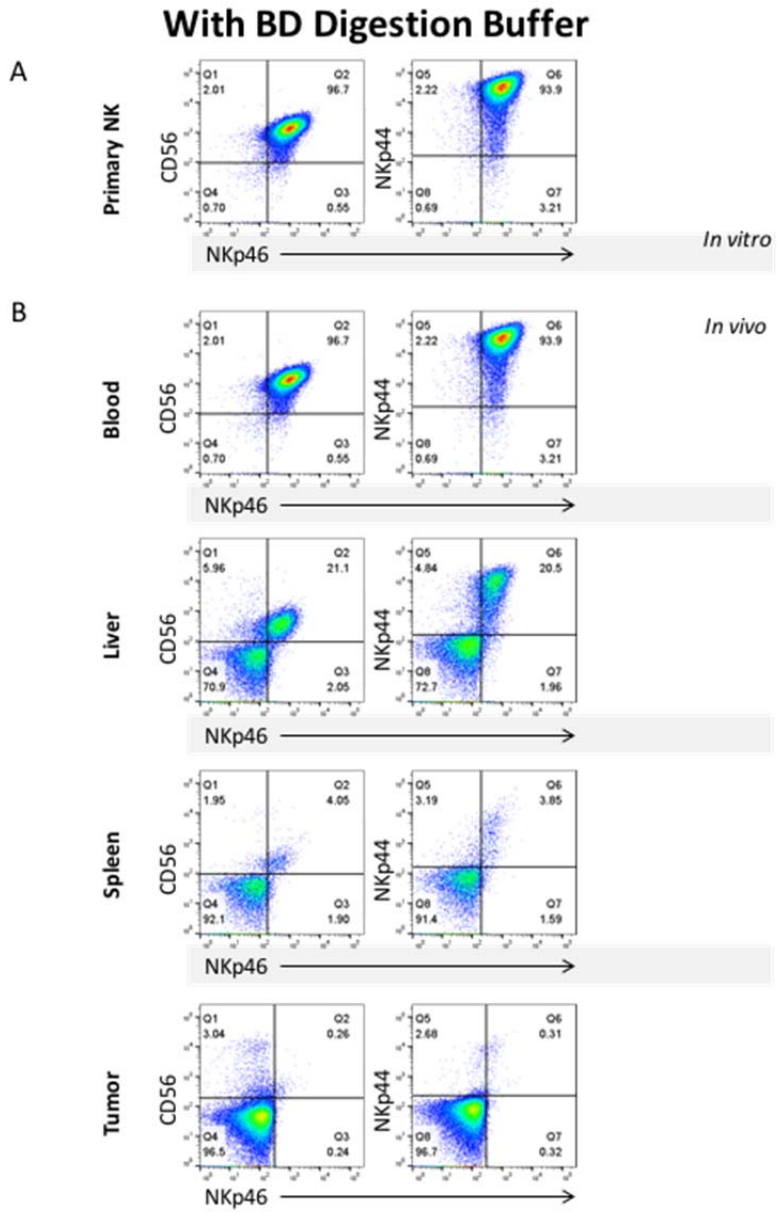

Figure 3 Flow cytometric analysis of primary human NK cells cultured in vitro (A) and recovered in vivo in different organs after intravenous injection of 1 x107 NK cells into NSG mice bearing human A549 lung tumors (B). Mice were subcutaneously inoculated with $5x10^5$ A549-Luciferase on the back. When the tumors reached a detectable size, mice were injected via tail vein with total of 200ul of 10^7 NK cells, supplemented with 500U/ml IL-2 and 50ng/ml IL-15. Mice then were sacrificed at day 3 and lymphocytes were isolated from tumors and different tissues using Ficoll density gradient centrifugation. The isolated lymphocytes were analyzed for CD56+NKp46+ or NKp44+NKp46+ NK cells. These NK cells are readily detected in blood, liver, and spleen but not in the tumor bed.

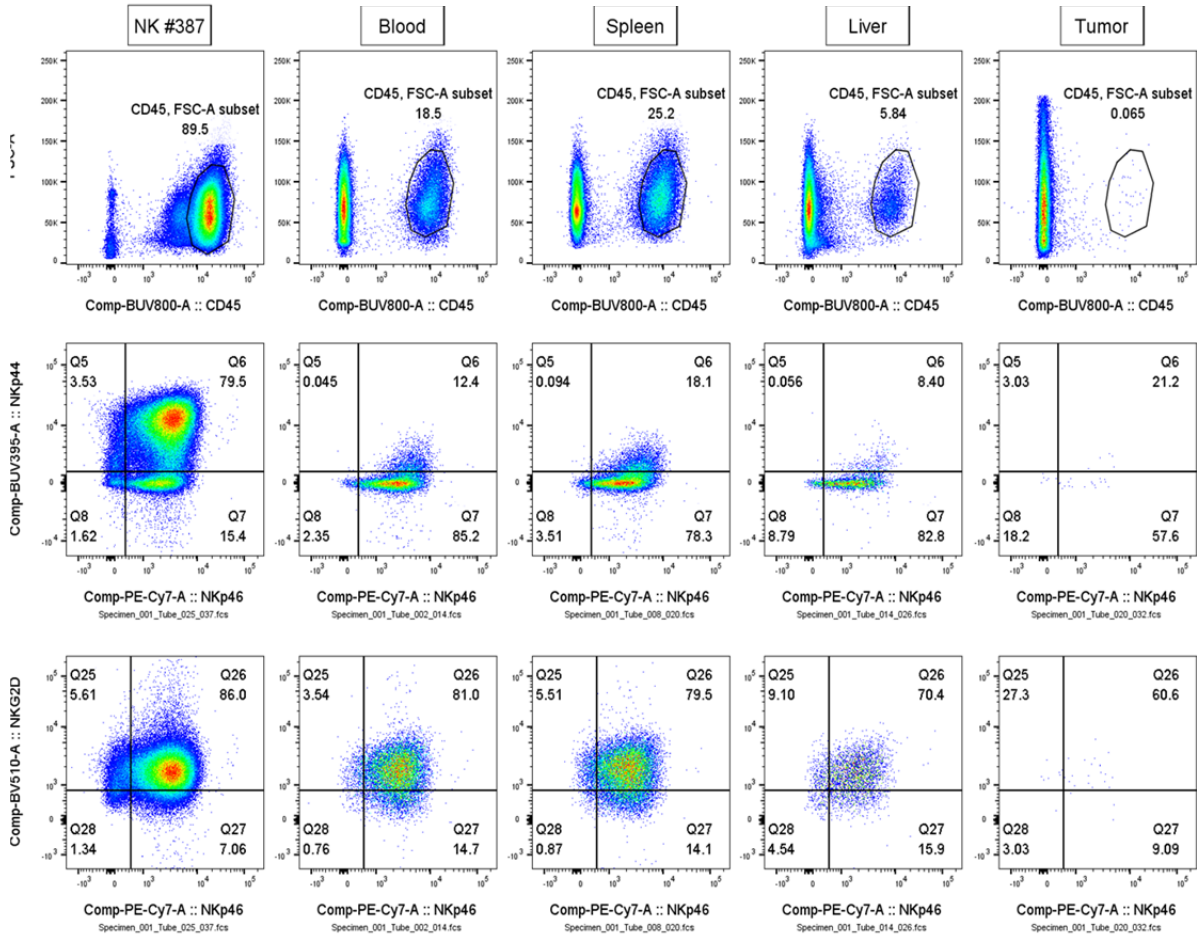

Fig. 4A. Percentages of CD45+ human lymphocytes in different organs of A549 lung tumor bearing NSG mice.
Gating on CD45+ human lymphocytes, the percentages of NKp46+NKp44+ cells, or

NK cells in NSG mice bearing H1299

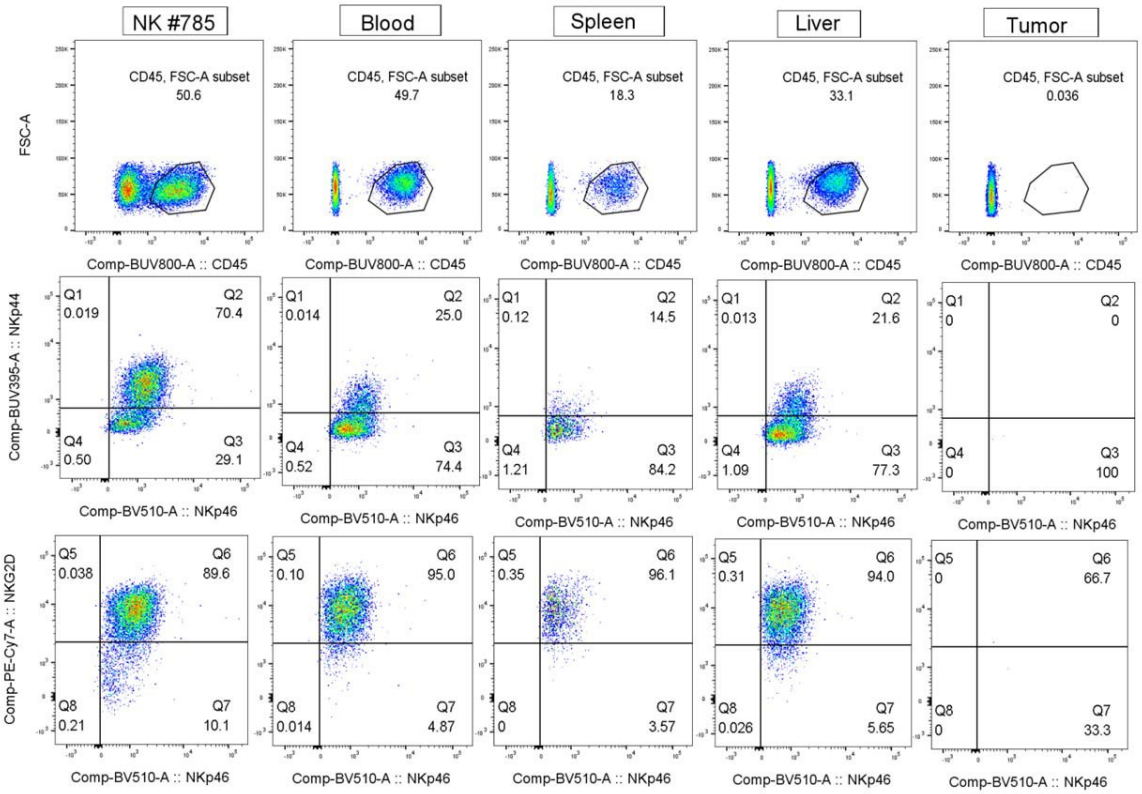

Fig. 4B. Percentages of CD45+ human lymphocytes in different organs of H1299 lung tumor bearing NSG mice.
Gating on CD45+ human lymphocytes, the percentages of NKp46+NKp44+ cells or

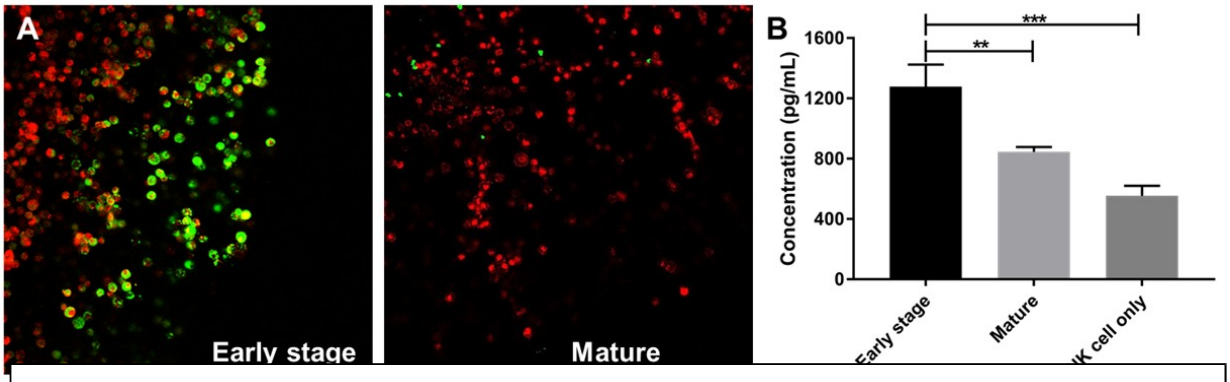

Figure 5. (A) Migration of NK cells into a 3D tumor model is impacted by the incubation period of the A549 lung cancer cells in the hydrogel. Red = cancer cells; yellow = NK cells. "Early stage" tumors refer to hydrogels in which A549 cells were cultures for 1 day, and "mature" tumors refer to hydrogels in which A549 cells were cultured for 7 days prior to the introduction of NK cells. There is decreased NK cell migration into mature tumors. (B) Secretion of RANTES by NK cells in

What opportunities for training and professional development has the project provided?
I learnt a great deal from this award, first in gaining the complex molecular technical training to clone and construct viral vectors and second in becoming proficient in all the immune assays required to conduct a human NK lung immunotherapy project. What I learnt most was how to plan an experiment and solve each problem as it develops, gaining confidence to think independently. Attendance at the annual meeting of the American Association of Cancer Research was eye-opening to the myriads of approaches that can be considered to combat cancer, offering me new ideas for my career development.

How were the results disseminated to communities of interest? Nothing to report

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

In the remaining no-cost extension period, I plan to carry out the final steps to evaluate miR-183 blockade strategy in the human xenograft mouse model. We are in the process to transduce sufficient numbers of NK cells with control lentiviral vector or anti-miR183 construct and inject them intravenously into NSG mice bearing A549 subcutaneous tumors. We will also inject unmanipulated NK cells into mice as additional controls. We expect that we will need to generate sufficient NK cells (for untreated controls, empty vector-containing NK cells, and anti- miR183-containing NK cells) for at least 5 mice per group for each experiment. We intend to repeat it again to obtain statistically-valid data.

4. **IMPACT:**

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

This project is of high impact because it will establish a new strategy based on microRNA targeting for immunotherapy of cancer.

What was the impact on other disciplines? *Nothing to report*

What was the impact on technology transfer? *Nothing to report*

What was the impact on society beyond science and technology? Nothing to report

5. **CHANGES/PROBLEMS:** *We found some issues with lentiviral toxicities in primary NK cells. We also found that the viability of NK decreased in tumor bearing mice and that the intra-tumoral infiltration of NK cells was not efficient leading us to focus our efforts on the study of NK intratumoral phenotype.*

Changes in approach and reasons for change: In order to improve the survival of NK cells pre-injection we took advantage of a collaboration to encapsulate anti-miR183 into nanoparticles. This procedure increased the viability of these cells which translated into higher efficacy in vivo. The viability of NK cells in vivo was also an issue which was correlated to the systemic presence of tumor in the mice, as we saw no decrease in viability in control mice injected with primary NK cells only. We hypothesized that the availability of TGFB was a main culprit of this effect and to overcome it will be beyond of the scope of the current proposal but will be addressed in future separate studies. Lastly, intratumoral infiltration of NK cells was low which we hypothesize has to do with modulation of chemokine and chemokine receptors in our system. For that reason we expanded our studies into understanding the changes in expression of chemokine receptors including CX3CR1 and CCR2. The expression of these receptors was low to no expression of CXC3R1 in NK cells inside the tumor compared to adjacent and normal nearby tissues. This is an important discovery that will be critical moving forward to enhance the efficacy of anti-miR183 therapeutic delivery which we are currently trying to confirm by overexpression of CCL2 and CX3CL1 (ongoing) on the tumor cells to attract NK cells. We are also attempting to overexpress CCR2 or CX3CR1 (ongoing) on NK cells which is at the moment challenging based on the description provided earlier on why we moved into nanoparticle use for primary NK cell delivery. If this overcomes the issue it will be important to address the expression of this receptor on NK cells in future studies.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. *No changes*

Significant changes in use or care of human subjects. *No changes*

Significant changes in use or care of vertebrate animals. *No changes*

Significant changes in use of biohazards and/or select agents. *No changes*

6. **PRODUCTS:** *Nothing to report*

Website(s) or other Internet site(s): *Nothing to report*

Technologies or techniques: *Nothing to report*

Inventions, patent applications, and/or licenses: *Nothing to report*

Other Products: *Nothing to report*

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	*Thu Le Trinh*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*No change*
Funding Support:	*No change*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? *No change*

What other organizations were involved as partners? *Nothing to report*

8. **SPECIAL REPORTING REQUIREMENT** *Nothing to report*

APPENDICES *Nothing to report*