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PRINCIPAL INVESTIGATOR: Sheng Wei

CONTRACTING ORGANIZATION: H. Lee Moffitt Cancer Center & Research Institute
Tampa, FL 33612

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12902 Magnolia Drive
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14. ABSTRACT

The goal of this application is to investigate how a microRNA, namely miR183, can disrupt the expression of a critical molecule, DAP12, that controls tumoricidal function in human Natural Killer (NK) Cells and to understand how nicotine, contained in tobacco smoke, utilizes this mechanism to abort immunity against lung cancer. In addition, we seek to explore the viability of targeting miR183 to restore NK cells as a new form of immunotherapy for early stage lung cancer. We have encountered problems with nicotine instability and work is still in progress to identify conditions to probe the effect of nicotine on NK cells. However, nicotine instability is not an issue in vivo and we have collected blood samples from tobacco smokers, e-cigarette users and past smokers to compare with never smokers for NK function. In terms of anti-miR183 therapeutics, we found that PLGA nanoparticles are readily taken up by NK cells but do not enter the lysosome, thus are not degraded within the NK cell. Nanoparticles will next be loaded with anti-sense miR183 to confirm that NK cell preloaded with anti-miR183 will resist TGFb suppression.

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1. INTRODUCTION:

Immune escape has emerged as one of the hallmarks of cancer and conquering this barrier is critical to early resistance against cancer. However, its mechanism remains obscure, especially related to natural killer (NK) cells. The goal of this application is to uncover how a microRNA, namely miR183, can disrupt the expression of a critical molecule, DAP12, that controls tumoricidal function in human Natural Killer (NK) Cells and to understand how nicotine, contained in tobacco smoke, utilizes this mechanism to abort immunity against lung cancer. In addition, we seek to explore the viability of targeting miR183 to restore NK cells as a new form of immunotherapy for early stage lung cancer. The specific aims are 1) to decipher the role of miR183 in nicotine-directed inhibition of human NK cell function, 2) to examine if the use of tobacco or nicotine-containing e-cigarettes induces miR183 expression and disrupt NK function,
3) to determine if targeting miR183 can serve as an immunotherapeutic modality for treating early stage lung cancer. These aims will be achieved through (i) analysis of molecular basis for nicotine control of the miR183 promoter, (ii) analysis of heavy tobacco smokers and e-cigarette smokers as well as former heavy smokers for loss of NK function, and (iii) analysis of a human xenograft *Nod-scid-IL2Rg*$^{-/-}$ (NSG) mouse model for the ability of human NK cells to respond to NK-directed nanoparticles bearing antagomiRs against miR183 to provoke human lung tumor regression in vivo. Such approaches will yield new insight into the pathogenic role of miR183 in nicotine-derived NK cell suppression and define a new miR-based immunotherapeutic strategy to treat lung cancer.

KEYWORDS: Natural Killer Cell, NK receptors, tumor microenvironment, Transforming Growth Factor-beta, nicotine, tobacco smokers, e-cigarette-users, lung cancer, microRNA-183, DAP12, NKp44, NKp46, nanoparticle-based immunotherapy

2. ACCOMPLISHMENTS:

What were the major goals of the project?

Lung cancer mortality remains a leading therapeutic issue because of the heterogeneity of cell types and the diversity of genetic changes involved. New drugs offer an improved overall survival to only a small subset of patients and the majority of lung cancer patients can only be treated with palliative chemotherapy. Immune surveillance, on the other hand, can be effective against any form of lung cancer, if mobilized in the early stages of disease. Natural killer (NK) cells are particularly effective as the first line of innate immunity against cancer. However, we recently made the seminal finding that NK cells in the lung tumor microenvironment are significantly at a disadvantage because of loss of DAP12, a critical adaptor protein that anchors activating NK receptors on the cell surface to recognize tumor cells. This loss is caused by transforming growth factor beta (TGFb) produced by tumor cells that can induce microRNA (miR)-183 in infiltrating NK cells to downregulate DAP12 expression. In addition to TGFb, tobacco smoking has long been established to cause lung cancer, and nicotine contained in tobacco smoke is reported to be immunosuppressive. With the discovery of miR183 as a critical NK cell regulator, the goals of the project are to investigate (i) if nicotine associated with tobacco smoking and lung cancer induces the miR183/DAP12 circuit to suppress NK function, (ii) if use of tobacco or e-cigarettes induce miR183 an disrupt NK function, and (iii) if miR183 blockade can be targeted to treat early stage lung cancer by reactivating NK cell function.

What was accomplished under these goals?

Due to the retirement of Dr. Julie Djeu, the original PI of this grant, Dr. Sheng Wei, MD, Professor in the Department of Immunology, has taken over this project as a new PI on March 2018. By the time of this

change was approved by DOD, this grant was in a non-cost extension period already. Dr. Wei has used the remaining funds and resources to continue the focus of the original proposed goals on which his group made significant progress.

This is the current progress on the proposed three aims:

Aim 1: To examine the role of miR183 in nicotine inhibition of human NK cell function.

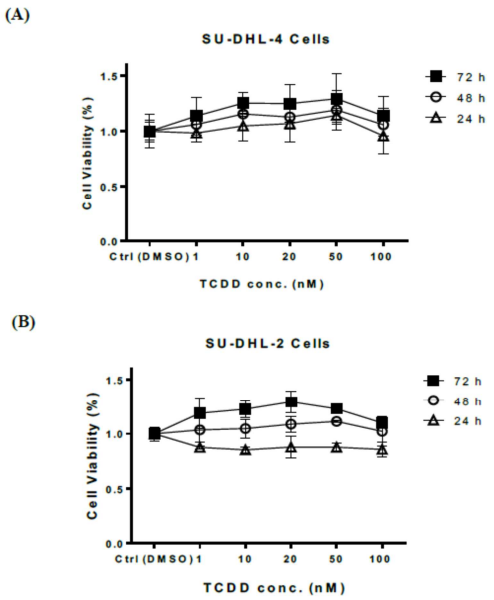

Figure 1.- TCDD and DMSO(control) treated cells at various concentrations and times (as shown). The viability was measured with a live/dead dye by flow cytometry.

During the last progress report we have reported that we are unable to reach the goals as proposed in the original application. For example we have conducted all the proposed experiments with no significant results. We have tested a number of new lots of nicotine and each lot was tested on different normal donor NK cells. In particular, this reagent has no effect in downregulation of NK activating receptors expression. By then we made the conclusion that we were unable to demonstrate that nicotine had any effect on NK function. Based on these facts we decided to continue to look for other sources of nicotine or equivalent environmental toxic chemicals to investigate their potentially negative role on immune competent cells. One of the chemical is 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) which also share the AhR receptor with nicotine. TCDD is one of the most toxic dioxin complexes and also a well-known ligand of aryl hydrocarbon receptor (AhR). We compare TCDD effects between on two cell lines including SUDHL-2 & SUDHL-4 derived from lymphoma. In both cell lines, cell viabilities are not significantly different in various concentrations of TCDD, but there are trends of increasing cell viabilities following exposure from 1 nM to 50 nM TCDD at each time point. However, the treatment of 100 nM TCDD does not correspond to the dose-dependent increasing trends of cell viabilities, which might be relevant to the cytotoxicity of TCDD (Figure 1A, B). These data did not show significant impact on cell biology as measured by cell death as we have shown using nicotine. It is well established that exposure of TCDD is associated with immune abnormality and cancer and its effect depend on its interaction with AhR receptor. The AhR is a key transcriptional regulatory protein involved in the altered gene expression and toxicity. Upon binning of TCDD, AhR signals through the translocation from cytoplam into the nucleus. In order to further compare if TCDD, Nicotine and cigarette smoke extract could have a role in regulating AhR receptor mediated signaling, an immunostaining experiment was performed using PBMC isolated from healthy donors. Surprisingly, all these reagents have a significant effect on AhR translocation upon treatment. In particular, Nicotine- and TCDD high dose-treated cells show a higher increase in AhR translocation compared to other reagents. These preliminary observations suggest that the bioassays we performed and reported in the last progress report to study NK function may not be the appropriate

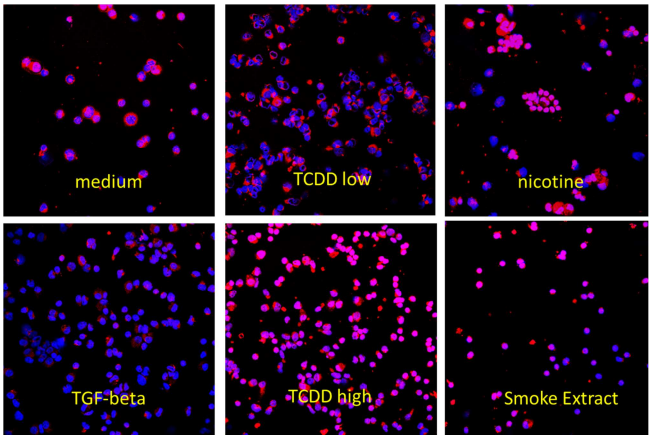

Figure 2.- Immunostaining of AhR (red) translocation into the nucleus upon treatment with various treatments as indicated. Blue is DAPI for nuclear staining.

biomarker. It may explain why we failed to see any positive results. Nonetheless, we believe the immuno-staining shown in Figure 2, is very encouraging, because these results are very meaningful and novel observation. We plan to further study if there are any changes of the downstream signaling of AhR, especially at the transcription level to modulate cytokine production.

Aim 2. To verify that use of tobacco or e-cigarettes can induce miR183 and disrupt NK function.

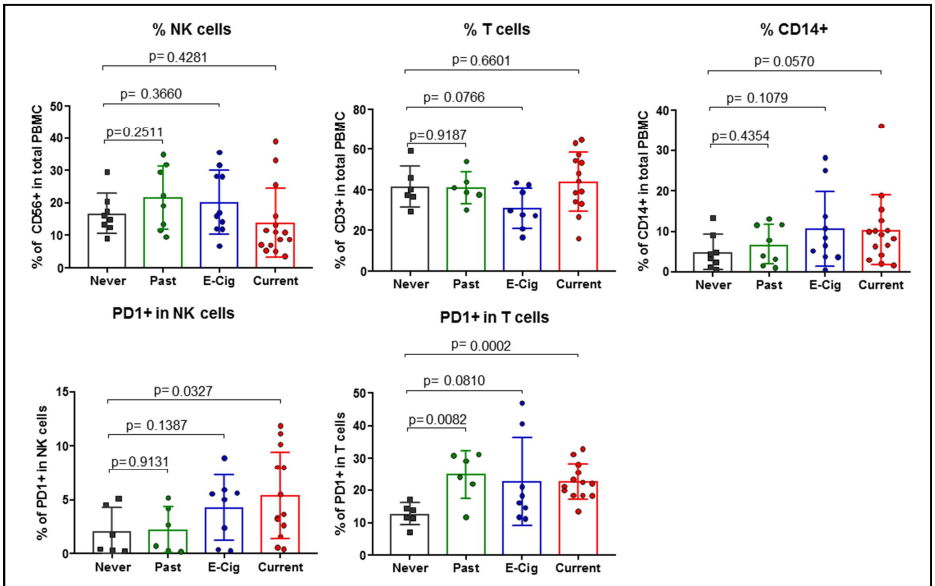

Figure 3.- NK, T cells and myeloid cells are changed in current and E-cigarette smokers by monitoring their numbers and the level of PD1 expression.

Within this aim, we have reported in last progress report that there is a trend of reduction of NK cells, in particular reduction of $CD16^+$ NK cells, where CD16 FcR is critical for antibody-dependent cell cytotoxicity against microbial-infected cells. This observation is more meaningful in current smokers. However these data need to be further validated in more detail with more donors, therefore we performed detailed analysis on PBMCs isolated from non-smoker, past-smoker, E-smoker and current smoker. We firstly analyzed the immune check point protein, PD-1, expression level in both NK and T cells in these groups by flow cytometric analysis. As shown in Figure 3, 1) there is a significant reduction of NK cells in current smoker and it seems no effect on T cells, 2) The expression of PD-1 are significantly increased in NK cells in both E-smoker and current smokers, 3) The expression of PD-1 are significantly increased in past, E-smoker and current smokers, and 4) CD14 positive macrophages are significantly increased in both E-smoker and current smokers. These results are quite striking because it indicated that exposure to cigarette, even in E-smoker, there is increased immune suppression in both NK and T cells. Increased $CD14^+$ cells after exposure to smoker demonstrated that smoking contribute to tumor suppressive microenvironment because $CD14^+$ monocytes is a major component and source of myeloid derived suppressor cells (MDSC). MDSCs are key players in the induction of suppressive cytokines and inflammatory mediators that contribute to immune suppression linked to cancer. To further prove this, we next analyzed if there is increased production of the suppressive cytokine, IL-10, and suppressive mediator, S100A9. Both are key mediators produced by MDSC and are responsible for failure of immunotherapy. As shown in Figure 4. IL-10 are significantly increased in both E-smoker and current smoker, while S100A9 is dramatically increased in current smokers. These novel observations demonstrate that smoking, especially in both E-smoker and current smokers, can induce immune suppression that is a potential marker for future tumor escape from immune surveillance. We provide first evidence that E-smoking can significantly play a role in the induction of immune-suppression.

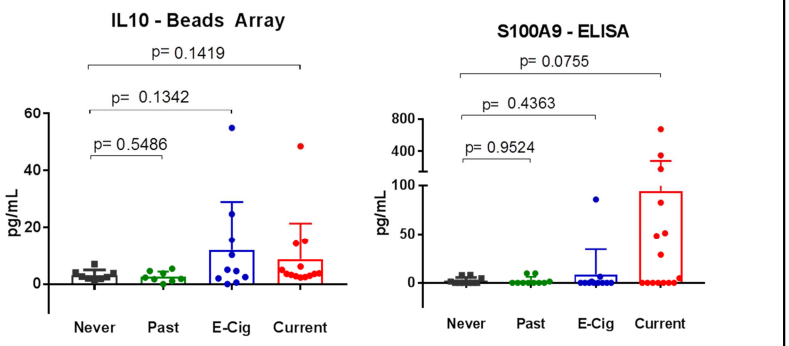

Figure 4.- Increased suppressive mediators circulating in the blood of E-smokers and current smokers.

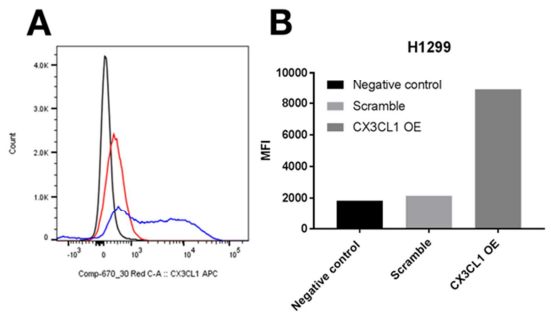

Figure 5.- Flow cytometric analysis of H1299 cell lines infected with either scramble or overexpressing CX3CL1 viral particles. (A) Histogram representation of CX3CL1 expression in negative control (black), scramble (red), or over expressing CX3CL1 (blue). (B) Mean fluorescent intensity (MFI) of intracellular CX3CL1 protein levels.

This information will need to be further validated.

<u>Aim 3. To construct stable anti-sense miR and nanoparticles targeted to human NK cells.</u>

The overreaching goal of this aim is to provide proof-of-concept that targeting TGFβ or miR183 can restore NK function in vivo against lung cancer. For the most part, we have developed novel strategies by using nanoparticle as a delivery vehicle to introduce siRNA against mir183 and TGF signaling pathway to NK cells. We have been able to observe thus far the proof of our initial hypothesis in vitro in primary NK cells. We saw that while the NK cells are not as resilient to nanoparticles treatment as NK92, demonstrated by a shift in their scatter properties, they are alive and able to reduce the expression of TGFbR2 after nanoparticle uptake. Another main issue this year was the change of PI from Dr. Djeu, who retired this year from research, to Dr. Sheng Wei a close collaborator. The transition of the project to the Wei lab was smooth as some of the scientists involved in the project migrated to his lab and the loss of time was minimal considering the change. However, it was still a reduction of our working time for this project since among the changes was not only the lab closure and transfer of assets and personnel to Dr. Wei but also the transfer of IACUC protocols which were frozen until the transition was completed to the new PI. Hence, in some degree the work progress has been delayed.

Nonetheless, we still manage to make significant research progress as summarized below:

1) Successful establishment of a murine model using IL-15 pre-activated NK cells 24h prior to in vivo injection, supplemented with IL-15 during injection into NSG mice bearing CX3CL1-A549 tumor cells. This allows the intra-tumoral infiltration of primary NK cells.

2) Successful in vitro uptake of labeled particles and selection of optimal dose for NK cell survival and uptake efficiency.

3) Successful reduction of TGFbR2 in the surface of primary NK cells.

4) Continue to test the in vitro functionality of siRNA loaded nanoparticle in primary NK cells in cytotoxic assays.

5) In the process of obtaining designed anti-miR183 for nanoparticle–loading and testing in vitro and in vivo in primary NK cells.

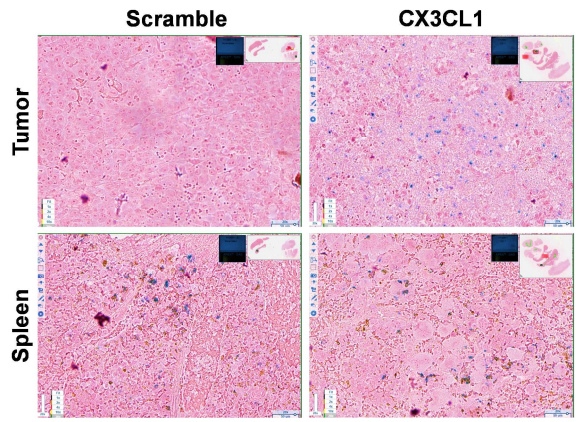

Figure 6.- Immunohistochemical analysis of infiltration of human CD45+ NK cells in CX3CL1 overexpressing A549 tumor-bearing mice. NK cells isolated from healthy donors and pre-cultured in the presence of 10ng/mL IL15 for 3 days. They were then injected, in IL15 supplemented vehicle (10ug/1x106 cells), into NSG tumor-bearing mice. The tumors were A549 cells transfected with a scrambled lentivirus or lentivirus for the overexpression of CX3CL1 and injected subcutaneously into the back of NSG immunodeficient mice. Spleens and tumors were harvested at the end of the experiment and fixed in formalin, embedded into paraffin from where slides were cut. The image represents cells stained for human CD45 (NK cells, blue cells) and counterstained pink. Representative images from one mouse each.

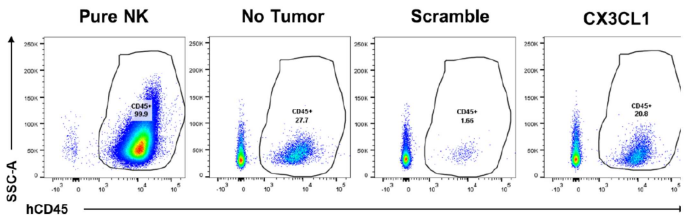

Figure 7. Flow cytometric analysis of circulating primary human CD45+ NK cells in the blood of CX3CL1 overexpressing A549 tumor-bearing mice. A549 cells were transfected with CX3CL1 or scrambled control lentivirus NK cells isolated from healthy donors and incubated with IL-15. Cells were injected into tumor bearing mice and blood collected after euthanasia, stained with Live/Dead stain and with anti-human CD45 and analyzed on a flow cytometer.

Experimental strategy 1 – Analysis of miR183 and DAP12 in adoptive-transferred human NK cells in NSG mice.

This past year was set with resolving the main issue of providing a fully working murine model of an established tumor for the testing of the nanotherapeutic delivery of antagomirs for miR183. We showed in the past that there is no delivery of NK cells to the tumor bed. After solving the survival of NK cells through the use of co-culture of primary NK cells with IL-15 followed by co-injection of IL-15 with NK cells, to aid in their survival, precluding our studies of NK cell effectiveness after treatment with anti-sense loaded nanoparticles. However, while this solved the survival of circulation of primary NK cells in the NSG mice, we still had issues in the recovery of NK cells from the tumor bed. The answer came from some of our data demonstrating that CX3CL1 expression is minimal in the two human lung cancer cell lines, A549 and H1299 while its receptor in NK cells CX3CR1 remains in these cells. Therefore, we hypothesized that CX3CL1 acts as a chemoattractant to drive NK cells towards the tumor site and that its reduction in the tumor prevents the immune-surveillance activity of NK cells. To test this we generated A549 and H1299 cell lines infected with lentiviral particles to overexpress CX3CL1, or a scramble control (**Figure 5**), and injected them subcutanously into the backs of NSG mice for our murine tumor-bearing development. We then injected primary healthy NK cells via tail vein into the mice and examine infiltration of NK cells into the circulation and the tumors. As shown in **Figure 6**, there was a strong mobilization of injected NK cells inside the tumor as observed in the immunohistochemistry of tumor tissues from A549 (see blue cells which are human $CD45^+$ cells). Moreover, when analyzing the total percentage of live human $CD45^+$ NK cells in the blood of these animals we observed an increase in the total circulating numbers of these cells comparable to non-tumor bearing mice demonstrating the capacity of CX3CL1 expression in the tumor of restoring immune-surveillance (**Figure 7**). However, while in the past we have observed a reduction of the numbers of NK cells in the circulation of the model animals, compared to non-tumor mice, blood in the presence of tumor in this model, regardless of the supplementation with IL15 demonstrating the a tumor-factors are involved in the reduced mobilization of NK cells in the periphery. To our surprise, overexpression of CX3CL1 also increased the circulating levels of human $CD45^+$ NK cells suggesting that this allows increased mobilization and survival of these cells in the murine model. It will be interesting to see the role of either TGF□ and/or miR-183 in this chemokine (either directly or indirectly) and how does it correlate with primary specimens. To our surprise this suggests that just the presence of CX3CL1 is able to recruit NK cells tumor infiltration in a murine model not only giving us a full model to test the nanoparticles as expected in aim3 but also suggests the importance of this chemokine as a potential target for future therapeutic development to augment NK-mediated anti-tumor immunity.

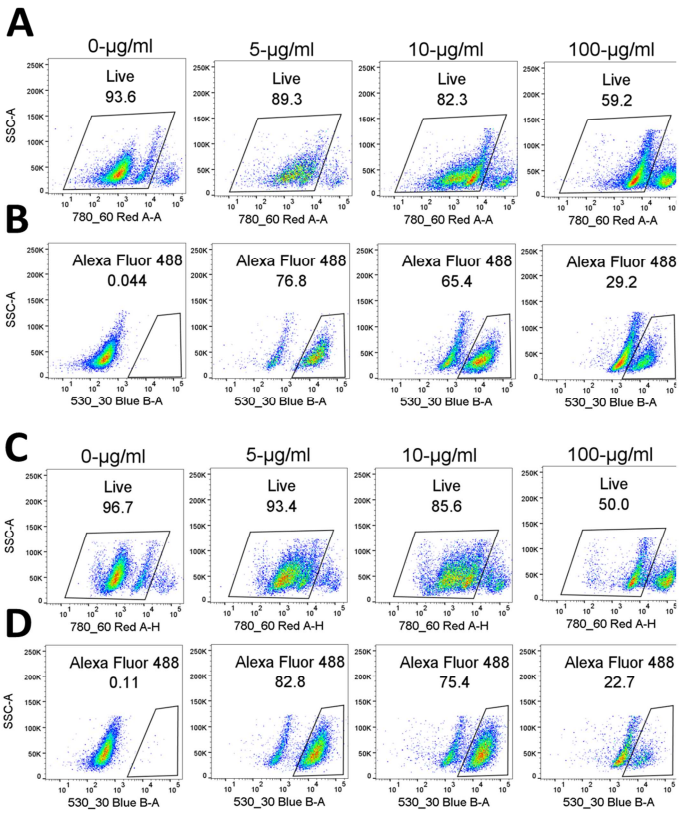

Figure 8. Flow cytometric analysis of the uptake of MnO_2 nanoparticles labeled with Alexa Fluor 488 by primary NK cells. NK cells isolated by two different donors: #357 (A & B) or #562 (C & D) were incubated with 5-µg/ml, 10-µg/ml, or 100-µg/ml MnO_2 nanoparticles for 24-hours. Cells were collected following incubation, stained with Live/Dead stain, fixed, and analyzed for Alexa Fluor 488 on a flow cytometer.

Experimental strategy 2 – Analysis of TGFβ involvement in miR183 induction and DAP12 suppression in vivo.

In order to improve NK functional activation, we took a novel strategy by using the MnO_2 nanoparticles that can be readily internalized by NK-92 cells. The goal of this approach is that we wanted to test the uptake and effect of nanoparticles on the more clinically relevant primary NK cells derived from healthy donors in preparation for testing in the CX3CL1 tumor model. It is evident, by previous attempts in our lab, that primary NK cells are less susceptible to internalization of extracellular materials, rendering it difficult to genetically manipulate gene expression in primary NK cells. It is clear that MnO^2 nanoparticles provide a promising alternative for effective delivery of genetic components such as antisense RNA into primary NK cells due to their relatively small size. We wanted to determine whether primary NK cells can internalize the MnO^2 nanoparticles as effectively as NK-92 cells. For this purpose, we isolated primary NK cells from several healthy donors and incubated 2×10^5 cells with 5, 10 or 100 µg/ml nanoparticles overnight at 37°C, 5% CO_2. Our flow cytometric results demonstrated that primary NK cells isolated from both donors #357 and #562 (**Figure 8 & 9**) are capable of efficient internalization of the Alexa 488 loaded MnO_2 nanoparticles at various concentrations although we did noticed that the increasing concentration of nanoparticles had an adverse effect on cell survival in both donors (**Figure 8A & C**). While concentrations of 5 and 10ug/mL were well tolerated, a dose of 100ug/ml had an adverse effect on viability, reducing it to ~50% on both donors. Interestingly most of the surviving cells at that point were not Alexa 488 positive potentially indicating that at that dose the cells that took nanoparticles died. These results were confirmed through confocal microscopy where we observed the localization of the nanoparticles in discreet structures in the cytoplasm (**Figure 9**) although a minute amount of nanoparticles were able to make it into the nucleus. At 5-µg/ml, we observed a higher number of cells with intact nuclei and there were a higher percentage of cells that have internalized the nanoparticles compared to the two higher concentrations. With these methodology is also patently clear to see the damage to the cells that had taken the nanoparticles with a diffuse and burst phenotype where green is localized although this phenotype is seen at the 100ug/ml dose and none at the 5ug/ml dose which is the reason why that dose is selected for our continued studies with the MnO_2 nanoparticles.

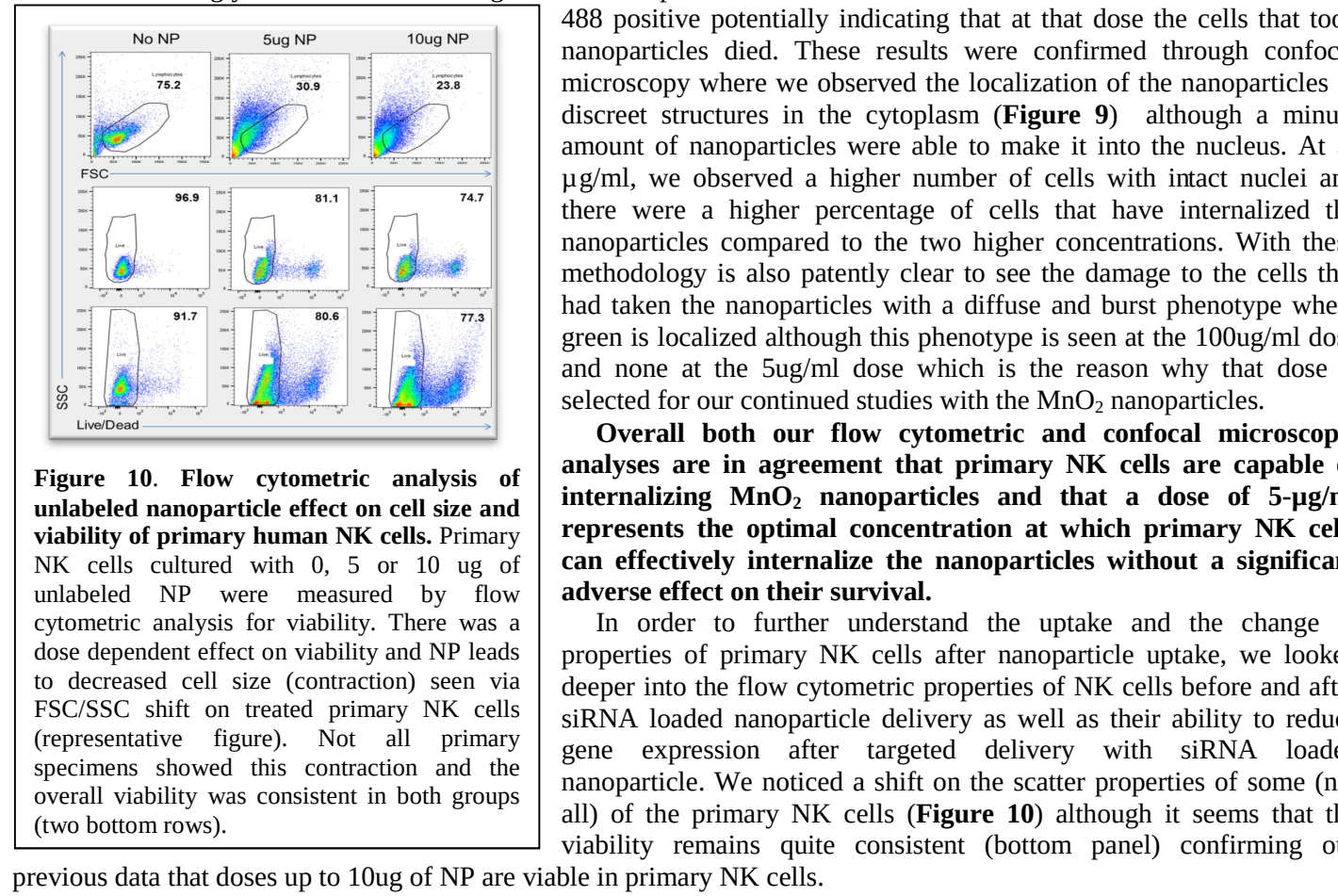

Figure 9. Confocal microscopic analysis of the uptake of MnO_2 nanoparticles labeled with Alexa Fluor 488 by primary NK cells. NK cells isolated by two different donors: #357 (A) or #562 (C) were incubated with 5-µg/ml, 10-µg/ml, or 100-µg/ml MnO_2 nanoparticles for 24-hours. Cells were collected following incubation, fixed with paraformaldehyde, and analyzed for Alexa Fluor 488 (green) on a confocal microscope. Cell nuclei were stained with DAPI (blue).

Figure 10. Flow cytometric analysis of unlabeled nanoparticle effect on cell size and viability of primary human NK cells. Primary NK cells cultured with 0, 5 or 10 ug of unlabeled NP were measured by flow cytometric analysis for viability. There was a dose dependent effect on viability and NP leads to decreased cell size (contraction) seen via FSC/SSC shift on treated primary NK cells (representative figure). Not all primary specimens showed this contraction and the overall viability was consistent in both groups (two bottom rows).

Overall both our flow cytometric and confocal microscopic analyses are in agreement that primary NK cells are capable of internalizing MnO_2 nanoparticles and that a dose of 5-µg/ml represents the optimal concentration at which primary NK cells can effectively internalize the nanoparticles without a significant adverse effect on their survival.

In order to further understand the uptake and the change in properties of primary NK cells after nanoparticle uptake, we looked deeper into the flow cytometric properties of NK cells before and after siRNA loaded nanoparticle delivery as well as their ability to reduce gene expression after targeted delivery with siRNA loaded nanoparticle. We noticed a shift on the scatter properties of some (not all) of the primary NK cells (**Figure 10**) although it seems that the viability remains quite consistent (bottom panel) confirming our previous data that doses up to 10ug of NP are viable in primary NK cells.

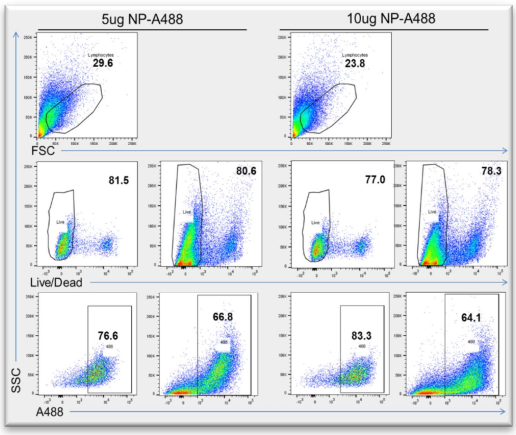

Figure 11. **Flow cytometric analysis of Alexa488-loaded nanoparticle effect on viability, shifting and uptake in primary human NK cells.** Primary NK cells cultured with 5 or 10 ug of A488-loaded NP were measured by flow cytometric analysis for viability (Live/Dead, either from side scatter gate -second row- or from ungated cells –bottom row-) and uptake (A488). We observed a dose dependent increase in SSC/FSC pre-gated cells (unshifted) versus the ungated population which showed equal A488 expression regardless of the dose of NP treatment (representative figure).

Next we repeated the experiments with the dye conjugated nanoparticless in primary NK cells. We previously shown robust uptake of these conjugated nanoparticles but first, we needed to confirm these results with more donors as well as observe the shift characteristics we just described in these cells to see if there is any correlation between the shift and the uptake of pre-loaded nanoparticles. Our first foray was using Alexa 488 (A488) loaded nanoparticles at the same two doses we have tested previously: 5ug and 10ug (**Figure 11,** representative figure of three separate experiments). We measured the uptake by flow cytometry following the two gating strategies shown in Figure 1 for the purpose of understanding the uptake capabilities of the shifted cells. While overall viability is similar for both groups, the un-shifted cells had an apparently higher uptake of the A488 loaded nanoparticles and displayed a dose dependency while shifted cells did not. Similarly, the use of Alexa 594 beads produce equivalent results which suggests that the fluorescence observed is specific to the uptake of the nanoparticles and not due to background fluorescence when carrying out this experiment (data not shown). Therefore, it will be good to understand whether un-shifted primary NK cells are the right population to enhance the anti-tumor efficiency of these cells in the future. Once we focus more on the animal experiments we will perform sorting experiments of both populations to assess which one has better anti-tumor immune-surveillance capabilities.

In order to confirm that the observation of NK shifting in primary cells is not due to a different experimental variability issues we also performed these experiments with NK-92 cells to repeat the observations. This will be critical for the development of primary NK cells that are targeted with MnO_2 nanoparticle system and siRNA for TGF-beta receptor 2 (TGF-bRII) downregulation which has already been carried out with NK-92 cells shown previously. In our hands, delivery of nanoparticles conjugated with A488 at 5ug and 10ug, as we did with the primary NK cells, did not induce the size contraction that we saw in primary NK cells and viability remained high on these cells (**Figure 12**). We did observe high uptake of A488-loaded nanoparticles, even higher than we observed for primary NK cells. Combined, the data suggests that loaded nanoparticles uptake is feasible and efficient in primary NK cells which can overtake the sensitivity induced by MnO2 nanoparticle delivery. The nanoparticles uptake by primary NK cells is specific and discrepancies observed in the past between primary NK cells and NK92 are due to cell differences and not to differences in protocol or NP batches. Moving forward we will pay attention to viability of NK cells after delivery and if there is a correlation between the contraction and their ability to go into the tumor.

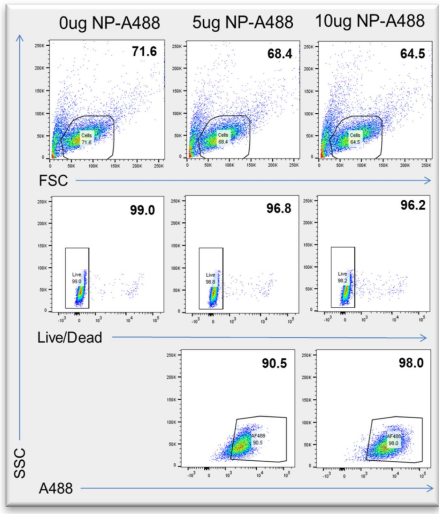

Figure 12.- **Flow cytometric analysis of Alexa488-loaded nanoparticle effect on viability, shifting and uptake in NK92 cells.** NK92 cells cultured with 5 or 10 ug of A488-loaded NP were measured by flow cytometric analysis for viability (Live/Dead, from side scatter gate) and uptake (A488). We observed a slight dose dependent decrease in SSC/FSC unshifted cells although viability did not show a change. However we did see an increased dose-dependent uptake of labeled NP (representative figure).

Once we confirmed particle uptake, we moved forward with the conjugated siRNA against TGFBR2 that we have shown in NK92 previously. We focused our work on the uptake by primary NK cells and importantly if there is reduction of receptor expression in the surface of NK cells, which will be critical when we move to the in vivo work. Just as we saw with the contraction, some of the attempts with siRNA loaded NK cells attempts were not successful and seemed to affect viability although this seem to be due to donor to donor variability. We also had some issues as TGFBR2 expression in unstimulated cells is null or highly reduced (data not shown) so although we did see some level of reduction it is not

ideal for our purposes. Our latest attempt saw expression of TGFBR2 in the surface of primary NK cells and a strong reduction in the receptor expression in these cells specific to the siRNA loaded nanoparticles (**Figure 13**). This suggests that the siRNA nanoparticles is efficiently taken by primary NK cells and will be modifying receptor expression which will be useful for our in vivo studies which are currently underway. While we are in the process of confirming this data and the changes in cytotoxicity of these NK cells in killing assays, these data confirms our earlier findings that the MnO_2 nanoparticles facilitate RNA delivery, and hope that in the next quarterly report we can confirm the changes in cytotoxic activity both in vitro and in vivo in our animal established tumor model. We will also start considering the use of anti-miR183 in our assays once we are fully confident of the TGFBR2 expression results.

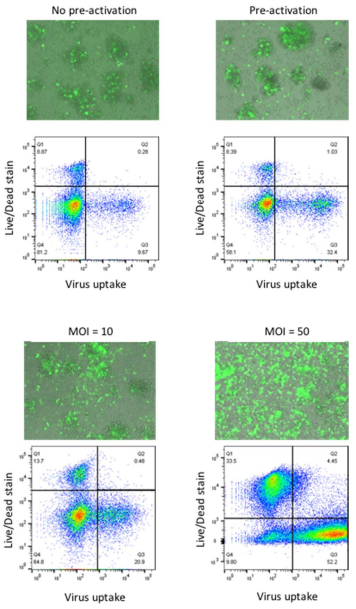

Figure 14 Infectivity of anti-miR183 lentivirus in NK cells showing different levels of viral uptake. A) With or without pre-activation by cytokine for 24 hours before being transduced with lentivirus. B) with low titer virus (MOI=10) and high titer virus (MOI=50). Viral uptake was measured by fluorescence microscopy or FLOW cytometry staining for GFP co-expression. Dead NK cells were gated out by Live/Dead staining.

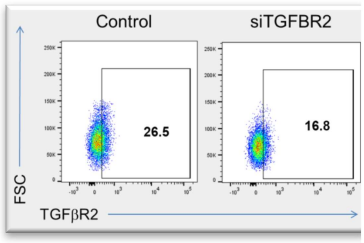

Figure 13. Flow cytometric analysis of TGFBR2 reduction after treated with siRNA-loaded nanoparticle. Primary NK cells cultured for 48 hours with 10 ug of TGFBR2-loaded NP were measured by flow cytometric analysis for TGFBR2 reduction.

Experimental strategy 3 **– Genetic deletion of miR183 in NK cells prior to adoptive transfer.**

Based on the results just shown, it is important to emphasize that we have already established the miR-183 knock-down lentivirus. 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 IL-2 and IL-15, 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 created 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 the CX3CL1-overexpressing A549 human lung tumors (**Figure 14**). We had also successfully encapsulated miRNA183 into the old PLGA nanoparticles while maintaining miRNA integrity, with a loading efficiency of 25%. These NPs also had no untoward cytotoxic effects on NK cells in vitro. PLGA nanoparticles appeared 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 the next round of experimentations, before the testing of anti-miR183 primary NK cells in our new murine model, we will test the uptake of this anti-miR183 in the MnO_2 nanoparticles, their effect on the efficiency of miR183 expression and their functional activity in vitro. We will also test if there are any changes in the scatter properties of primary NK cells treated with anti-miR183.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

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

The current proposal ended after a one year non-cost extension. We will continue this work and publish our findings in the near future.

3. **IMPACT:**

TGFb has long been known to be immunosuppressive but clinical trials with anti-TGFb or TGFb inhibitor in cancer patients have not produced notable successes, likely due to numerous off- target effects related to the pleiotrophic nature of TGFb with various physiological effects. Direct targeting of miR183 could avoid such issues to constitute a new molecular and precise strategy for lung cancer therapy. Most importantly, we have discovered a new immune checkpoint inhibitor, miR183, that disrupts NK function against cancer.

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

To date, no attempts have been made to address microRNA therapeutics to correct tumor immunity against cancer and our project is of high impact in introducing precision medicine to control a new immune checkpoint inhibitor.

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

4. **CHANGES/PROBLEMS:**
5. *Nothing to report*

Changes in approach and reasons for change:
Nothing to report

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:	*Sheng Wei*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
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 REQUIREMENTS** *Nothing to report*

9. **APPENDICES:** *Nothing to report*