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Engraftment of mesothelin chimeric antigen receptor using a hybrid Sleeping Beauty/minicircle vector into NK-92MI cells for treatment of pancreatic cancer

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ABSTRACT

Background: We have previously demonstrated in vitro cytotoxicity of mesothelin-chimeric antigen receptor autologous T cells against pancreatic cancer cells using lentiviral vectors, but these vectors pose safety concerns. Here, we incorporated Sleeping Beauty and minicircle design enhancements into interleukin-2–secreting natural NK-92MI cells to eliminate both bacterial and viral components and address inhibition by the tumor microenvironment.

Methods: Parental (conventional deoxyribonucleic acid)-mesothelin-chimeric antigen receptor and minicircle-mesothelin-chimeric antigen receptor vectors were electroporated into NK-92MI cells and engraftment was visualized by immunofluorescence analysis with protein-L staining. Interferon-γ and granzyme B secretion were measured by enzyme-linked immunosorbent assay from cocultures of parental-mesothelin-chimeric antigen receptors and minicircle-mesothelin-chimeric antigen receptors with human pancreatic cancer cells, and cytotoxicity of chimeric antigen receptor NK-92MI cells was tested against three pancreatic cancer cell lines.

Results: Cloning of mesothelin-chimeric antigen receptor Sleeping Beauty into a minicircle vector removed its bacterial backbone and reduced its size with improved electroporation efficiency. Chimeric antigen receptor engraftment, Interferon-γ and granzyme B secretion, and specific lysis against all three pancreatic cancer lines were significantly increased with minicircle-mesothelin-chimeric antigen receptor versus parental-mesothelin-chimeric antigen receptor NK-92MI cells.

Conclusion: We provide proof of concept that allogeneic mesothelin-chimeric antigen receptor NK-92MI cells with hybrid Sleeping Beauty and minicircle technologies provide increased engraftment and cytotoxicity in vitro with potential safety benefits when translated to the clinical arena.

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Introduction

Pancreatic cancer (PC) is relatively resistant to chemotherapy and typically diagnosed at an advanced stage, thereby precluding surgical resection and underscoring the need for novel therapies.[1] One such promising T cell–based approach is the utilization of chimeric antigen receptor (CAR) technology, which has ushered in a new era in cancer immunotherapy because of its ability to overcome the need for antigen processing and presentation coupled with human leukocyte antigen–independent recognition of the tumor target.[2]

However, despite achieving high remission rates in selected hematologic malignancies, CAR T cell therapy has several important limitations:

- Autologous T cell preparation is time consuming and labor intensive.

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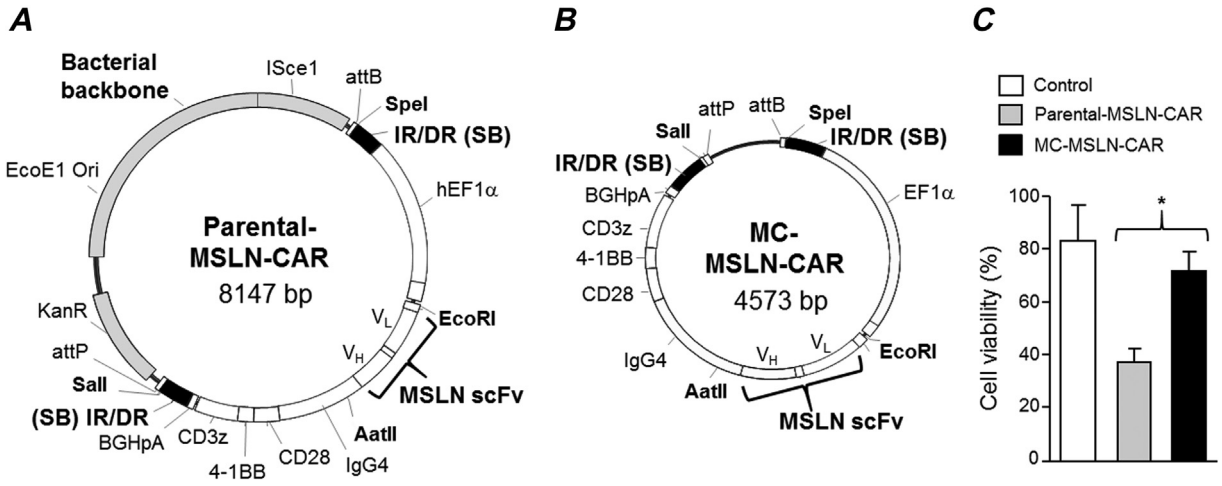

Fig 1. Construction of parental-MSLN-CAR and MC-MSLN-CAR vectors, electroporation, and cell viability. Schematic representations of (*A*) parental-MSLN-CAR and (*B*) MC-MSLN-CAR T cell SB transposon vectors that possess an hEF1α promoter with MSLN-CAR consisting of CD28, 4-1BB, and CD3ζ. The entire MSLN-CAR cassette is positioned within recombination attB/attP sites that assist in MC generation while enzymatically degrading the bacterial backbone. (*C*) Bar diagram depicting cell viability via trypan blue staining of mock (control), parental-MSLN-CAR, and MC-MSLN-CAR electroporation of NK-92MI cells. *IR/DR*, SB inverted repeat/direct repeat; *BGHpA*, bovine growth hormone polyadenylation sequence; *EcoE1 Ori*, *E. coli* origin of replication; and *KanR*, Kanamycin resistance gene. $^{*}P < .05$.

- The functional ability of CAR T cells is inhibited by the tumor microenvironment.[3]
- Viral vectors[4] and bacterial elements[5] often possess safety concerns.

Bone marrow–derived primary natural killer (NK) cells are considered a viable alternative to autologous CAR T cell generation because they can be easily expanded for clinical application and provide a potential source of allogeneic "off-the-shelf" cellular therapy.[6] Interleukin (IL)-2 contributes to the ability of CAR T cells to overcome the negative effects of the tumor microenvironment, but its systemic infusion in clinical trials produced severe side effects.[7] The NK-92MI cell line, which is derived from NK cells but modified to secrete IL-2, can be utilized as part of a CAR T cell treatment strategy to localize IL-2 intratumorally to reduce systemic toxicity.[7]

Sleeping Beauty (SB) transposons are mobile genetic elements that contain genes of interest flanked by inverted terminal repeats enabling chromosomal integration and therefore represent a safer alternative to viral vectors that may activate oncogenes.[8] Minicircle (MC) plasmid vectors are devoid of redundant bacterial elements, such as antibiotic-resistant genes, and are not only safe, but also highly efficient vector systems because of their smaller size.[9]

Mesothelin (MSLN), a differentiating membrane glycoprotein, is overexpressed on the surface of several tumors, including PC cells.[10] MSLN expression is associated with worsening tumor progression and chemoresistance, making it an attractive tumor-specific antigen for CAR T cell–mediated therapy.[11]

To address the limitations of CAR T cell therapy by incorporating the possible solutions discussed, we have generated functional NK-92MI MSLN-CAR T cells with a hybrid, SB/MC vector that we demonstrate herein to increase engraftment, enhance production of interferon (IFN)-γ and granzyme B, and more effectively kill PC cells in vitro.

Methods

Design and production of the MSLN-SB-MC-CAR vector

Restriction enzymes and other reagents were obtained from Sigma-Aldrich Corporation (St. Louis, MO). The MSLN single chain Fv fragment (scFv) sequence was derived from a published University of Pennsylvania patent,[12] and the sequences of various components of CAR were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The pSB11 transposase vector and pDonR-SB transposon to clone MSLN scFv were both obtained from Creative Biolabs (Shirley, NY). Subcloning of MSLN scFv using two-step polymerase chain reaction (PCR) was performed in the pGEM-T vector (Promega, Madison, WI). Removal of bacterial backbone from parental-MSLN-CAR to generate MC-MSLN-CAR was performed either using MC-easy Kit (System Biosciences, Palo Alto, CA) or outsourcing to Aldevron (Madison, WI) (Fig 1, *A* and *B*). Briefly, MC producer-specific ZYCY10P3S2T *E. coli* competent cells were transformed with parental-MSLN-CAR and induced with arabinose induction medium followed by MC-MSLN-CAR isolation using PureLink HiPure Kit (Invitrogen, Carlsbad, CA).

Cell lines and medium

The MSLN-positive PC cell lines BxPC-3, MiaPaCa-2, and PANC-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 with 10% fetal bovine serum. NK-92MI cells, derived from the NK-92 cell line by stable transfection of human IL-2 cDNA, were also obtained from ATCC and maintained in the following culture medium: αMEM without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum, and 12.5% fetal bovine serum.

Electroporation of parental- and MC-MSLN-CAR into NK-92MI cells

Co-electroporation of either parental-MSLN-CAR or MC-MSLN-CAR along with pSB11 SB transposase at a 1:1 ratio into NK-92MI cells was performed using a Nucleofector Kit and 4DNucleofector electroporation unit (Lonza Inc, Allendale, NJ). Trypan blue staining was conducted to assess the viability of cells after electroporation.

Protein-L staining and fluorescence microcopy

Biotinylated protein-L FITC-streptavidin binding to κ light chains was used to assess CAR engraftment of NK-92MI cells by confocal immunofluorescence. CAR engraftment was visualized

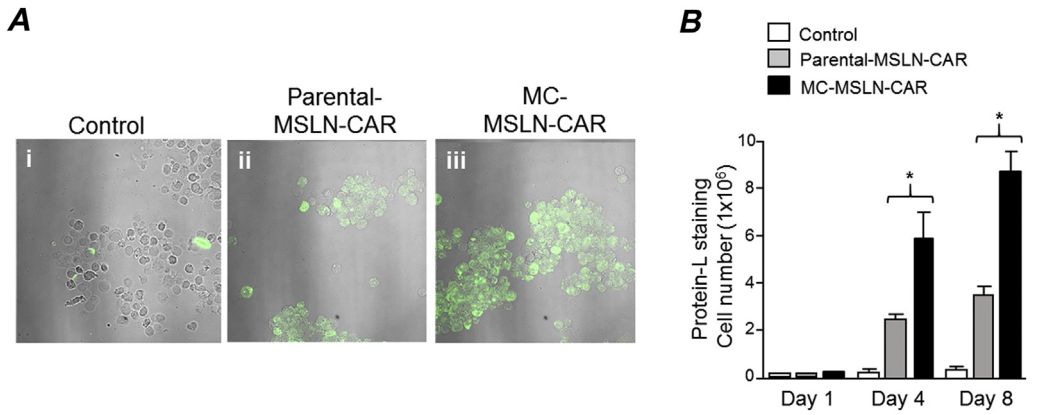

Fig 2. Protein-L staining of NK-92MI cells to determine CAR engraftment. (*A*) Representative fluorescent images of protein-L staining 8 days after electroporation of NK-92MI cells: (i) control, (ii) parental-MSLN-CAR, and (iii) MC-MSLN-CAR. (*B*) Number of Protein-L stained cells on days 1, 4, and 8 for mock (control), parental-MSLN-CAR, and MC-MSLN-CAR electroporation of NK-92MI cells. $^{*}P < .05$.

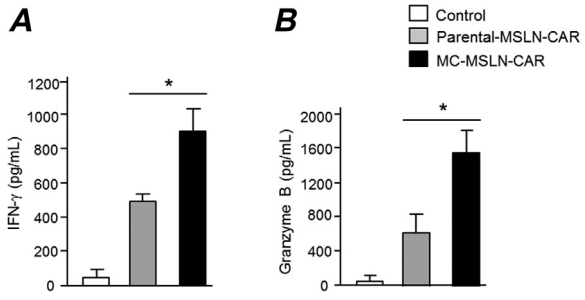

Fig 3. CAR T cell-induced secretion of proinflammatory signals. (*A*) IFN-γ and (*B*) Granzyme B secretion measured by ELISA assays of 48 h cocultures of mock (control), parental-MSLN-CAR, and MC-MSLN-CAR with BxPC-3 cells. $^{*}P < .05$.

under the Zeiss Observer.A1 fluorescence microscope, and the images were captured using the cooled Axio CAM CCD camera.

IFN-γ and granzyme B enzyme-linked immunosorbent assay (ELISA)

The 2×10^5 MSLN-positive BxPC-3 target cells were cocultured with varying concentrations of MSLN-CAR-transduced NK-92MI effector cells in 96-well plates for 48 hours. Cell-free supernatants were stored at −80°C until assayed. IFN-γ and granzyme B secretions were measured by an ELISA kit (R&D Systems, Minneapolis, MN).

Cytotoxicity assays

Control and NK-92MI CAR T cells and PC cells were seeded into 96-well plates at indicated effector-to-target (E:T) ratios. At 48 hours, cell viability was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc, Rockville, MD).

Statistical analysis

Data are expressed as mean ± standard deviation from a minimum of 3 independent experiments. Student's *t* test was employed to evaluate differences between groups. All *P* values are 2-sided, with ≤ 0.05 being statistically significant.

Results

Generation of parental-MSLN-CAR and MC-MSLN-CAR vectors and viability of NK-92MI cells after electroporation

MSLN-CAR was synthesized and cloned into EcoRI and XbaI sites of pDonR-SB vector to generate pDonR-MSLN-SB-CAR. An overlapping 2-step PCR was conducted to amplify the MSLN-SB-CAR cassette, using pDonR-MSLN-SB-CAR as a template to generate (1) SpeI and SalI restriction sites at the 5' and 3' ends of the cassette and (2) a G-to-C mutation creating the AatII restriction site (Fig 1, *A* and *B*). These modifications allowed creation of a master vector with greatly enhanced utility for easy manipulation and switching of various scFv sequences. The resultant PCR product was cloned into pGEM-T vector with SpeI and SalI restriction digestions (data not presented). The entire MSLN-CAR-SB cassette was clipped with SpeI and SalI restriction enzymes and ligated into pMC cloning vector generating parental-MSLN-CAR that contains the required bacterial elements for plasmid amplification and antibiotic selection (Fig 1, *A*). Production of MC-MSLN-CAR resulted not only in a vector size reduction of 2.5 kb, thereby increasing electroporation efficiency, but also removed the unwanted bacterial backbone (Fig 1, *B*). We found superior viability of NK-92MI cells after MC-MSLN-CAR versus parental-MSLN-CAR electroporation (Fig 1, *C*).

CAR engraftment of NK-92MI cells

When compared with mock-electroporated cells (control) (Fig 2, *A* [i]), we observed 35% CAR engraftment of NK-92MI cells on day 8 with parental-MSLN-CAR (Fig 2, *A* [ii]), which was further increased to 80% with MC-MSLN-CAR (Fig 2, *A* [iii]). Along these lines, CAR engraftment, as measured by protein-L staining, was significantly increased on both days 4 ($P < .05$) and 8 ($P < .05$) post-electroporation for MC-MSLN-CAR versus parental-MSLN-CAR (Fig 2, *B*).

MSLN-CAR T cells generate proinflammatory signals

To ascertain the functional ability of MSLN-CAR NK-92MI cells to induce proinflammatory signals, such as IFN-γ and granzyme B, which are crucial for rapid induction of target cell lysis, we conducted coculture experiments with B×PC-3 cells. ELISAs of 48-hour supernatants of parental-MSLN-CAR displayed robust secretion of IFN-γ and granzyme B when compared with controls, and both were significantly increased with MC-MSLN-CAR (Fig 3, *A* and *B*; $P < .05$).

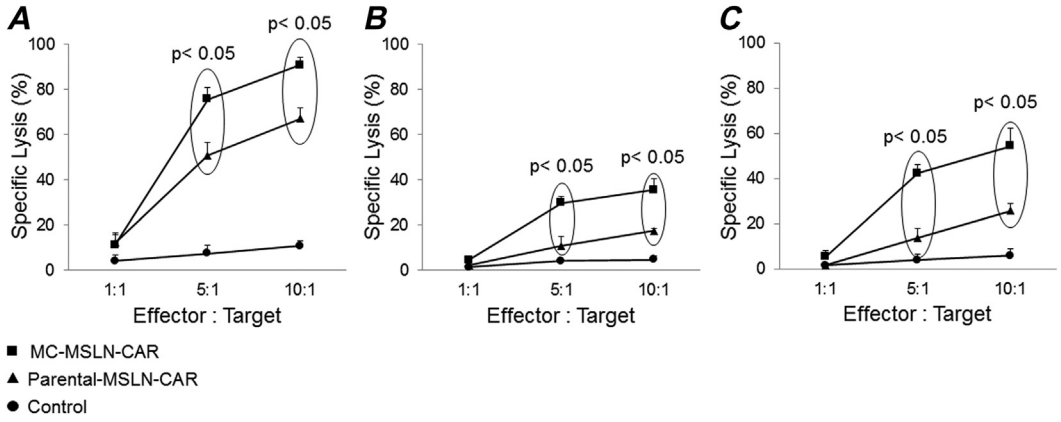

Fig 4. CAR T cell-induced death of human PC cells. Cell viability assays of 48 h cocultures of mock (control), parental-MSLN-CAR, and MC-MSLN-CAR with (*A*) BxPC-3, (*B*) MiaPaCa-2, and (*C*) PANC-1 cells. $^{*}P < .05$.

MC-MSLN-CAR kills PC cells significantly more efficiently than parental-MSLN-CAR

When compared with control (lysis < 5%), parental-MSLN-CAR NK-92MI cells displayed a specific lysis of 48% at an E:T ratio of 5:1 and 64% at an E:T ratio of 10:1 against B×PC-3 cells (Fig 4, *A*). We observed a further significant increase in specific lysis with MC-MSLN-CAR to 69% and 94% at E:T ratios of 5:1 and 10:1, respectively ($P < .05$ for both), corroborating CAR engraftment immunofluorescence results. We also found significant increases in cytotoxicity between MC-MSLN-CAR and parental-MSLN-CAR at both E:T ratios when Mia-PaCa-2 cells and PANC-1 cells were substituted for B× PC-3 cells; however, the specific lysis achieved was somewhat lower (Fig 4, *B* and *C*).

Discussion

We have demonstrated in vitro cytotoxicity of MSLN-CAR autologous T cells against PC cells using lentiviral vectors elsewhere,[13] but these vectors pose safety concerns. In the present report, we re-engineered the MSLN CAR vector into NK-92MI cells without viral or bacterial components but with the addition of both SB and MC design enhancements. As a result, we demonstrated improved CAR engraftment and enhanced IFN-γ and granzyme B secretion with increased cytotoxicity when compared with a conventional parental DNA vector. Given that the B×PC-3 cell line demonstrates the highest levels of MSLN expression among the three human PC cell lines tested,[14] it was not surprising to observe higher absolute levels of specific lysis with these cells.

MSLN expression in normal cells is limited to mesothelial cells lining the pericardium and peritoneum. Ongoing clinical trials using MSLN-CAR T cell therapy for PC have not demonstrated any adverse effects related to these cells.[15] Nevertheless, we do need to confirm the expression of MSLN on biopsied PC cells from patients before considering therapy. If the expression levels are too low, the patient will not be suitable for this therapy. We tested four human PC cell lines, B×PC-3, Capan-1, PANC-1, and MIA PaCa-2, for MSLN expression, and all were found to be positive, in line with earlier biopsy studies by other investigators.[14]

In contrast to most lentiviral vectors, SB integrates into the human genome without any preference for active transcriptional units, thus decreasing the risk of insertional mutagenesis.[8] MC vectors, without bacterial plasmid sequences containing unmethylated CpG, are superior to their plasmid DNA analogues in that they do not trigger inflammatory responses.[16] Further, given the smaller size of MC-MSLN-CAR when compared with their analogous parental-MSLN-CAR plasmids, we observed enhanced engraftment of CAR on the surface of NK-92MI cells, thus increasing their ability to kill PC cells. In addition, given the lack of viral vector preparation, MC CAR NK-92MI cells may reduce manufacturing costs and regulatory hurdles, which thus far have limited the range of application of CAR-based immunotherapy. Finally, fluorescence activated cell sorting of MSLN-CAR positive NK-92MI cells before their clinical use could boost their efficacy in patients, but two potential drawbacks of this approach are that the cells could suffer shear- or flow-induced damage, and given the large number of cells involved, it adds an additional challenging step and safety concern to the overall process. We expect that our ongoing optimization experiments will further improve the electroporation efficiency of MSLN-CAR positive NK-92MI cells, thus obviating the need for cell sorting.

Unlike CAR therapy with autologous T cells, NK-92MI cells can be maintained as a continuously available and expandable off-the-shelf therapy. NK-92MI cells are not human leukocyte antigen–restricted and would not be expected to elicit an immune response when administered therapeutically to patients because NK-92 cells do not.[17] However, because they are derived from a lymphoma patient, they must be irradiated before clinical use as a safety measure to inhibit proliferation. Despite irradiation, CAR NK-92 cells have been shown to have impressive in vivo cytotoxicity and are clinically well tolerated.[18]

Although using MSLN-engrafted CAR T cells for PC immunotherapy is an active area of research,[19] the tumor microenvironment may compromise its efficacy. However, because IL-2 is known to activate and expand NK cells to overcome the negative effects of the tumor microenvironment,[7] we theorize that IL-2 enhances the cytotoxic effects of CAR T cells in vivo. Along these lines, the use of modified recombinant NK-92MI effector CAR cells secreting IL-2 in our present system instead of autologous T cells might be more beneficial in the clinical setting. We are conducting experiments comparing NK-92 versus NK-92MI MSLN-CAR activity in the presence of tumor-conditioned medium, which we expect to provide more direct evidence of the importance of IL-2 in overcoming negative effects of the tumor microenvironment.

In conclusion, we provide proof of the concept that allogeneic MSLN-CAR NK-92MI cells with hybrid SB and MC technologies provide increased engraftment and cytotoxicity in vitro with potential safety benefits when translated to the clinical arena.

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Conflict of interest/Disclosure

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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Discussion

Dr Carlos Chan (Iowa City, IA): I would like to first congratulate the authors for taking on this important mission to treat this deadly cancer. I would also like to thank the program committee for giving me an opportunity to discuss their exciting work.

As you can see, CAR T cell therapy is a type of immunotherapy that can potentially make a big impact on cancer treatment, but it's often viewed as a very expansive therapy with a lengthy processing time, as the authors have discussed. Here the authors utilized modified bone marrow derived NK cells engrafted with MSLN-CAR in a very compacted format without any bacterial components. These MSLN-CAR NK cells were shown to lyse pancreatic cancer cells in vitro. This is very exciting.

Here are my questions:

1. To improve the cytotoxic effect, can you sort out the MSLN-CAR positive NK cells by FACS prior to coculture with cancer cells? I would assume this will be an essential step in the clinical setting.
2. From the practical standpoint in the clinical setting, what will be the sequence of events? It was not clear to me in the manuscript. I assume you will need to use autologous cells from patients. So, will patients get their bone marrow biopsy at diagnosis and generate the MSLN-CAR NK cells? How will this be different in comparing to other CAR T cell therapy?
3. What is the percentage of MSLN positive pancreatic cancers? Should MSLN expression be tested for an individual patient first? Will MSLN-CAR NK cells target normal cells with MSLN expression?

Dr Oksana Gruzdyn: Thank you for your questions, Dr. Chan. In the first question, you refer to the NK-92 cells prior to clinical trials. The first step is to increase the efficiency of the NK-92 cells to 80% MSLN-CAR expression. If we achieve an efficiency greater than 80%, then we will not need to use FACS analysis to sort the NK-92 cells.

With regard to the second question, conventional CAR T cell therapy is autologous T cell vaccination followed by infusion back into the patient. MSLN-CAR NK-92 MI cells are a natural killer cell line which can be prepared to be presented 'off the shelf' to patients right away without the need for leukapheresis of autologous T cells. NK-92 MI cells are not immunogenic and they do not elicit an immune response like autologous T cells, so they may be prepared for the patient that is expressing MSLN on pancreatic cancer cells.

With regard to the third question, we have not found a cell line that is negative for MSLN thus far. However, the expression of MSLN varies between different cell lines. We have yet to test the percentage of expression of MSLN in pancreatic cancer patients.

Dr Sarkis Meterissian (Montreal, QC): Your presentation is very good. I work on melanoma and breast cancer and work on PD-1 and PD-L1. I have two questions for you.

I am just curious, in the literature, there's a lot of work being done on combining vaccines with PD-L1 therapy, and in melanoma, of course, it's much more successful than other tumors.

Could you comment on combining your approach to that type of upregulation of the immune system?

My second question, in breast cancer I do a lot of work looking at the immune environment of the tumor, particularly the triple negative breast cancers, where we know that immunostimulation or immunomodulation is important. Can you comment on the role of tumor infiltrating lymphocytes? In other words, the more infiltration of lymphocytes and neutrophils that you have, do you find that you could predict a different type of response?

Dr Oksana Gruzdyn: With regard to the first question, there's clinical work going on right now that combines the expression of tumor-specific antigen with IL-12 or IL-15 that is known to enhance efficiency of CAR T cells. There are different ways to make the CAR T cell more specific to a particular tumor by combining it with PD-1 therapy. To address your second question, in our research, we are using NK-92MI cells that secrete IL-2, which is known to inhibit the tumor microenvironment. So we plan to compare NK cells versus NK-92MI cells with regard to the effect on the tumor microenvironment.