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Inhibition of Interleukin-10 in the tumor microenvironment can restore mesothelin chimeric antigen receptor T cell activity in pancreatic cancer in vitro

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ABSTRACT

Background. Pancreatic cancer cells are known to shield themselves from immunosurveillance by secreting immune inhibitory cytokines such as Interleukin-10. Using mesothelin, a differentiating antigen that is overexpressed in pancreatic cancer, we assessed the negative effect of the tumor microenvironment on chimeric antigen receptor T cell–based immunotherapy and its reversal via depletion of Interleukin-10.

Methods. T cells cultured in pancreatic cancer–cell-conditioned medium were transduced with lentiviruses encoding mesothelin–chimeric antigen receptor in the presence or absence of anti-Interleukin-10–blocking antibody.

Results. Coculture supernatants of conditioned medium displayed significant inhibition of interferon γ and granzyme B secretion, both of which are crucial for induction of target cell cytotoxicity. In contrast, this inhibition was restored toward baseline when conditioned medium was Interleukin-10– depleted ($p < .05$ for both interferon γ and granzyme B). In addition, we observed a significant decrease in mesothelin–chimeric antigen receptor T cell–induced cytotoxicity of BxPC-3 target cells in the presence of conditioned medium. Furthermore, we observed a partial blunting of this inhibition when Interleukin-10 was depleted from the conditioned medium.

Conclusion. Substantial reversal of tumor-derived immunosuppression may be achieved by blocking Interleukin-10 in the local microenvironment, allowing for more effective cytotoxicity of mesothelin-engrafted chimeric antigen receptor T cells and enhancing the potential for clinical application.

The vast majority of patients with pancreatic cancer (PC) present with distant metastases at the time of diagnosis, thereby precluding attempts at curative resection.[1,2] Chemotherapy has had some effects on increasing overall survival, but lack of dramatic results underscores the need for novel therapies.[2] The lack of effective conventional therapeutic options has spurred research into immunotherapeutic approaches such as adoptive transfer of gene-modified T cells that can cause tumor regression.[3] One such promising T-cell approach is the utilization of chimeric antigen receptor (CAR) technology, which has ushered in a new era in immunotherapy.[4]

T cells engineered to express CARs are comprised of an extracellular, single-chain fragment variable (scFv) derived by joining the heavy- and light-chain variable regions of a monoclonal antibody against tumor-specific antigen (TSA). This scFv is connected to one or more intracellular, costimulatory domains via a transmembrane domain,[5] which in turn is linked to the cytoplasmic portion of the T-cell receptor ζ (TCR ζ), also known as cluster of differentiation (CD)3 ζ.[6] The costimulatory domains improve T-cell trafficking to tumor cells, while CD3 ζ induces activation of T cells. CAR T cells are superior to prior T-cell therapies in that a greater-affinity, antibody-like recognition of the tumor cell is achieved in a human leukocyte antigen (HLA)-independent fashion.[7] The latter is important, because both HLA and TSA processing and presentation are often dysfunctional in tumor cells and are involved in immune escape.[8] Mesothelin, a differentiating membrane glycoprotein, is

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overexpressed on the surface of several tumors including PC cells.[9] Mesothelin expression is associated with worsening tumor progression and chemoresistance, making it an attractive TSA for scFv-mediated immunotherapy.[10,11]

Although impressive tumor regression rates have been observed in clinical trials with CAR T cell therapy in several hematologic malignancies,[12] there has been limited success with solid tumors such as PC. One of the negative factors contributing to these results is the immunosuppressive tumor microenvironment, a biologic feature often observed in PC patients.[13,14] Both T regulatory and PC cells secrete Interleukin (IL)-10, and increased levels of IL-10, both in plasma and the tumor microenvironment, have been shown to inhibit mesothelin-specific, T-cell responses in PC patients.[9] With a focus on IL-10 as the main immunosuppressant, we explored the effect of the PC microenvironment and the presence of IL-10 on mesothelin-CAR T-cell activity in cell culture.

Methods

Cell lines and conditioned medium

We tested 4 human PC cell lines for mesothelin expression (BxPC-3, Capan-1, PANC-1, and MIA PaCa-2), all of which were positive. Although all were positive, BxPC-3 had the greatest expression and was the only nonmetastatic cell line. Because cancer cell lines derived from earlier-stage and lower-grade disease are better models in which to study the effects of novel therapies and establish proof of principle,[15] we chose not to include metastatic tumor cell lines in this initial study and could not identify any mesothelin-negative PC cell lines, in line with earlier studies.[16] BxPC-3 (American Type Culture Collection, Manassas, VA) was cultured in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum. The culture medium was replaced with serum-free medium at approximately 60% confluency, and the supernatant was harvested after 48 hours. This conditioned medium was used to simulate the tumor microenvironment. In some experiments, BxPC-3-conditioned medium was pretreated with 5 μg/mL anti-IL-10 blocking antibody for 30 minutes.

Human T-cell isolation and propagation

Blood was drawn by venipuncture from healthy individuals into heparin-lined BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and subjected to Ficoll gradient centrifugation.[17] The interface containing peripheral blood mononuclear cells (PBMCs) was harvested and incubated for 1 hour in the serum-free, human T-cell medium, ImmunoCult-XF T (STEMCELL Technologies Inc, Cambridge, MA). This step allows adherent monocytes to be separated from nonadherent lymphocytes that remain in the supernatant suspension. The supernatant was centrifuged at 500 g for 5 minutes, and the T-cell pellet was suspended in ImmunoCult-XF T. T cells were maintained at 1×10^6 cell/ml in 25 μl/ml ImmunoCult Human CD3/CD28 T Cell Activator (STEMCELL Technologies Inc) and 500 IU/ml of IL-2.

Mesothelin-CAR vector construction and lentiviral production

The mesothelin scFv sequence was derived from the University of Pennsylvania patent,[18] and the sequences of various components of CAR were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Construction of the third generation, mesothelin-CAR lentiviral vector with vesicular stomatitis virus-glycoprotein (VSV-G) pseudotyped vector generation was outsourced to VectorBuilder (Cyagen Biosciences Inc, Santa Clara, CA). Correct assembly of the third generation mesothelin-CAR construct was validated by analyses of the deoxyribonucleic acid (DNA) sequence. Viral transductions were performed for 3 consecutive days in 6-well plates using 2×10^6 cells in a total volume of 2 mL of lentiviral supernatant containing 8 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO) and 500 IU/mL human IL-2 at a multiplicity of infection of 50. Cells were centrifuged at 1,800 rpm at 37°C for 5 minutes, plated, and then placed in a CO_2 incubator at 37°C for 2 hours.

Electrophoresis and western blotting

Cells were lysed on ice with radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Sigma-Aldrich), and protein estimation was conducted using the Bradford Protein Assay kit (Bio-Rad, Hercules, CA). Approximately 50 μg of total protein was resolved on 4%–12% polyacrylamide gel and transferred to polyvinylidene fluoride membrane by Western blotting. Blots were developed with IRDye 680/800 secondary immunoglobulin G (IgG) antibodies followed by visualization of the protein bands on OdysseyCLx Imaging System (LI-COR Biotechnology, Lincoln, NE).

Fluorescence microcopy

Expression of enhanced green fluorescent protein (EGFP) was visualized under the Zeiss Observer.A1 fluorescence microscope with images captured using the cooled Axio CAM CCD camera. The same microscopic fields were viewed and photographed by phase-contrast microscopy.

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

2×10^5 mesothelin-positive BxPC-3 target cells (T) were cocultured with varying concentrations of mesothelin-CAR–transduced effector human T cells (E) in 96-well plates for 24 hours. Cell-free supernatants were stored in a –80°C freezer until assayed. IFN-γ and granzyme B secretion were measured by an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Data represent mean values of triplicate studies.

Cytotoxicity assays

Cells were seeded into 96-well plates at a density of 3×10^3 cells/well. At 24 hours, cell viability was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc, Rockville, MD) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± standard deviation from at least 3 independent experiments. Student *t* test was employed to evaluate differences between groups. All *P* values are 2 sided.

Results

Efficiency of CAR engraftment of T cells and lentiviral transduction

Third-generation, mesothelin-CAR vector was constructed using cell-surface scFv linked to the CD8α hinge and transmembrane region. This was connected intracellularly with the cytoplasmic portion of two costimulatory domains, CD28 and 4-1BB (CD137), as well as an activation domain derived from the cytoplasmic part of CD3ζ (Fig 1, *A*). The CAR construct was incorporated into lentiviral vectors driven by the promoter for elongation factor 1 alpha (EF1α), which is preferable for expression in T cells. The costimulatory

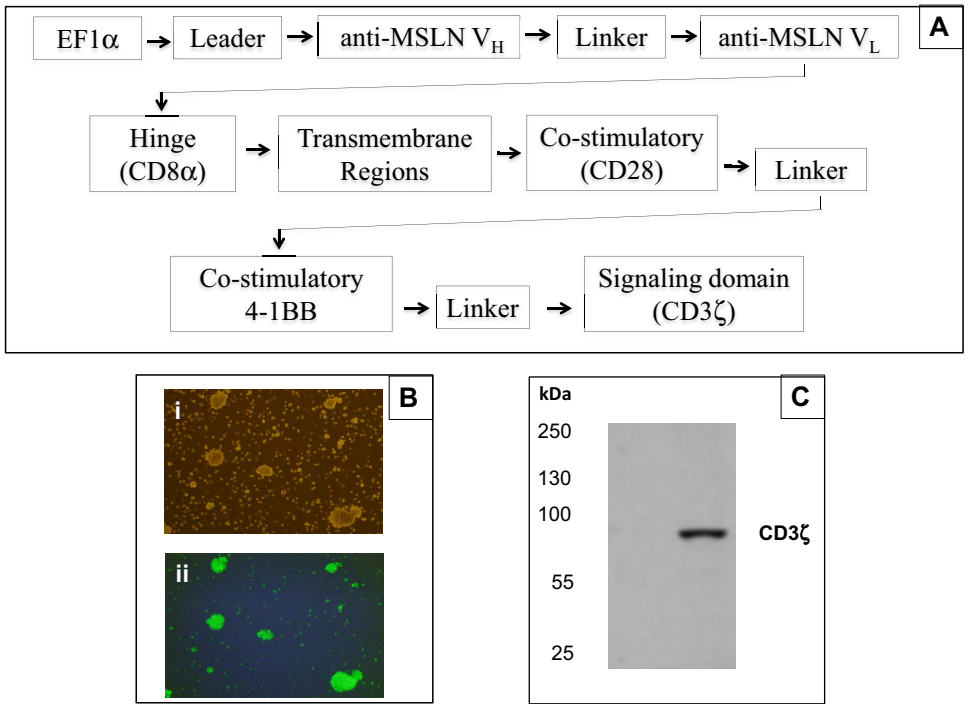

Fig. 1. Mesothelin CAR design, engraftment of T cells, and transduction efficiency. (*A*) Schematic representation of the single-chain fragment variable (scFv) of mesothelin (MSLN) fragments encoding variable regions of heavy (V_H) and light (V_L) chains connected by a linker. We designed the CAR sequence as follows: Toward the extracellular end, the MSLN scFv is connected to CD28, spanning the transmembrane region. The cytoplasmic portion consists of CD28 and 4-1BB costimulatory and CD3ζ activation domains. The mesothelin-CAR scFv expression cassette is driven by the EF1α promoter. The CAR construct was ligated into a lentiviral backbone with EGFP (*not presented*). (*B*) T cells were transduced with lentiviruses encoding mesothelin-CAR at a multiplicity of infection of 50. The lentiviral transduction efficiency reached > 70% as determined by EGFP expression. Presented are representative images of phase contrast microscopy (i) along with fluorescent expression (ii). (*C*) Western blot analysis confirmed the presence of exogenous CD3ζ in the transduced T cells (*right panel*) versus control (*left panel*).

molecules act by transmitting information within the immune effector cell by generating second messenger signaling that promotes activities to kill cancer cells. Furthermore, we incorporated the puromycin resistance gene for selection and EGFP for visualization of transfected cells. T cells were transduced with lentiviruses encoding mesothelin-CAR at a multiplicity of infection of 50. Examination of EGFP expression showed transduction efficiency > 70% (Fig 1, *B*). CD3ζ couples cell surface antigen recognition with several intracellular signaling pathways, an important aspect of T-cell activation. Low expression of CD3ζ results in an impaired immune response. Western blot analysis confirmed the intracellular expression of exogenous CD3ζ in the transduced T cells (Fig 1, *C*).

Mesothelin-engrafted CAR T cells generate proinflammatory signals and kill PC cells

To ascertain the functional ability of mesothelin-engrafted CAR T cells to induce proinflammatory signals, such as IFN-γ and granzyme B crucial for rapid induction of target cell lysis, we conducted coculture experiments with mesothelin-CAR T cells and BxPC-3 cells. ELISA assays of 24-hour coculture supernatants displayed robust secretion of IFN-γ (Fig 2, *A*; $P < .001$) and granzyme B (Fig 2, *B*; $P < .001$) when compared with controls. Cytotoxicity assays demonstrated lysis exceeding 50% and 65% at E:T ratios of 10:1 ($P < .05$) and 20:1 ($P < .01$), respectively, with no evidence of specific lysis of mock-transduced cells (Fig 2, *C*). These experiments indicated that mesothelin-engrafted CAR T cells can generate proinflammatory signals on contact with PC cells to induce cell death.

Tumor-mediated suppression of CAR T-cell activity and its reversal by blocking IL-10

Although in vitro interaction of T cells bearing antimesothelin-CAR with BxPC-3 cells enabled cytolysis, tumors are known to shield themselves from immunosurveillance by secreting immune inhibitory factors. Using BxPC-3–conditioned medium to simulate the tumor microenvironment, we assessed the secretion of proinflammatory signals in the presence and absence of the known immune inhibitor IL-10. When compared with coculture supernatant alone, ELISA assays of conditioned medium displayed inhibition of the secretion IFN-γ (Fig 3, *A*; $P < .05$) and granzyme B (Fig 3, *B*; $P < .05$). In contrast, this inhibition was significantly but incompletely restored to coculture baseline when conditioned medium, which contained IL-10 at approximately 100 pg/ml (data not shown), was IL-10–depleted ($P < .05$ for both IFN-γ and granzyme B). In addition, we observed a decrease in mesothelin-CAR T cell–induced cytotoxicity of BxPC-3 target cells in the presence of conditioned medium versus coculture alone at an E:T ratio of 10:1 (29% decrease in target cell lysis; $P < .05$) and a more pronounced effect at an E:T ratio of 20:1 (35% decrease; $P < .05$) (Fig 3, *C*). Furthermore, in line with our ELISA assays, we observed blunting of this inhibition in IL-10–depleted conditioned medium (14% and 15% decrease in cytotoxicity, respectively; P = NS).

Discussion

The PC microenvironment represents an immunosuppressed state, particularly in the more advanced stages of the disease, because

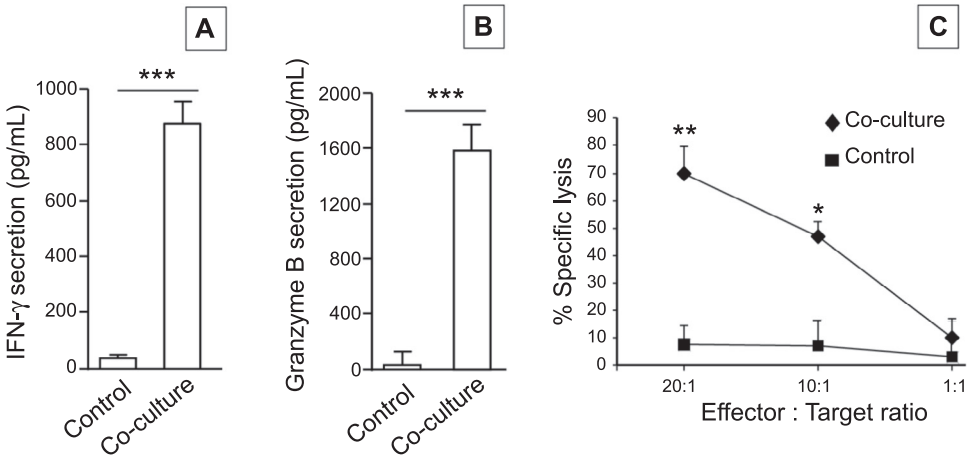

Fig. 2. CAR T-cell–induced secretion of proinflammatory signals and death of BxPC-3 cells. ELISA and cell viability assays of cocultures of mesothelin-CAR T cells and BxPC-3 cell line. Controls are BxPC-3 cells incubated with nontransduced human T cells. (Data are representative of at least 3 separate experiments.) (*A*) IFN-γ secretion and (*B*) Granzyme B secretion; $^{***}P < 0.001$. (*C*) Cell viability assays with Cell Counting Kit-8; $^{**}P < .01$; $^{*}P < .05$.

tumor cells in vivo secrete spontaneously protective anti-inflammatory cytokines, such as TGF-β and IL-10, that can specifically defend against immune attack.[13] Use of mesothelin-engrafted CAR T cells for PC immunotherapy is an active area of research,[12,19,20] but immune inhibitory mechanisms often observed in solid tumors, which may compromise the efficacy of mesothelin-engrafted CAR T cells have not been fully elucidated.[21]

The novel studies presented herein shed further light on the PC microenvironment by demonstrating inhibition of mesothelin-CAR T cell activity via IL-10 and its reversal with neutralizing antibody. Our study, however, has several limitations, including use of only one PC cell line, exclusion of mesothelin-negative cell lines, and confining our investigations to an in vitro setting with a focus on only IL-10. We also did not characterize the conditioned media with regard to the presence of other inhibitory cytokines.

IL-10 is a well-known suppressive cytokine implicated in blocking proliferation and cytokine production of T cells and plays a role in inducing T-cell anergy,[22] thus shielding tumor cells from immunosurveillance. Although we observed a significant negative influence of IL-10 on mesothelin-CAR T-cell activity, we have not been able to completely restore the activity by blocking IL-10. This lack of total effect may be due to the fact that in addition to IL-10, cytokines, such as TGF-β, IL-6, IL-8, and, to some extent, vascular endothelial growth factor, fibroblast growth factor, and epidermal growth factor, are all known to induce T-cell anergy in PC.[13] Further studies are required to understand the other factors present in the PC microenvironment that may also be negatively affecting mesothelin-CAR T-cell activity apart from IL-10.

IFN-γ not only coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes, but also integrates signaling with other cytokines to induce cancer cell death.[23] One of the mechanisms by which cytotoxic T cells induce apoptosis of cancer cells is by exocytosis of granzyme B, a serine proteinase that activates members of the caspase family.[24] We explored whether IL-10 in the PC microenvironment affected the secretion of these 2 proinflammatory signals by assessing coculture supernatants. Our results in these cell-culture experiments suggest that the secretion of both IFN-γ and granzyme B can be considered important endpoints by which to assess the influence of the PC microenvironment on mesothelin-CAR T-cell activity. Our study suggests that blocking IL-10 activity in the PC microenvironment may amplify the response to current mesothelin-CAR T-cell–based immunotherapeutic strategies and may further improve outcomes beyond those being obtained in ongoing clinical trials.[12] Along these lines, we are not aware of any prior clinical trial or application of anti-IL–10 antibody relating to PC but are currently

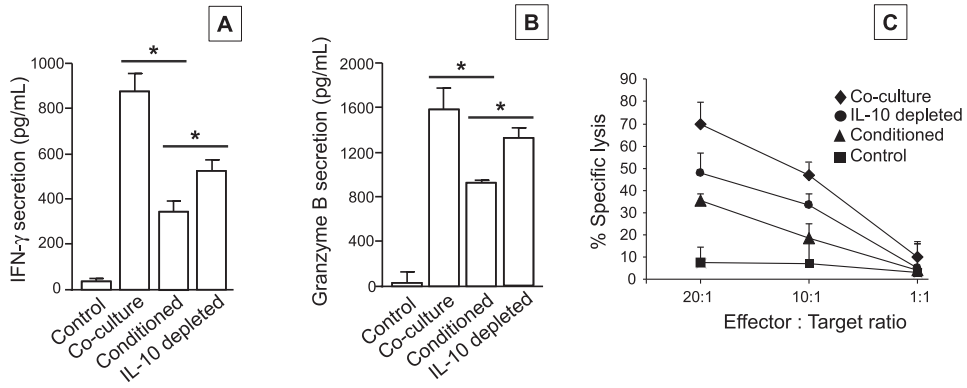

Fig. 3. Inhibition and reversal of proinflammatory signals and cytotoxicity by IL-10 and its blocking antibody. ELISA and cell viability assays of cocultures of mesothelin-CAR T cells and BxPC-3 cell line with tumor-conditioned medium in the presence and absence of IL-10 neutralizing antibody. Data are representative of at least 3 separate experiments. (*A*) IFN-γ secretion. (*B*) Granzyme B secretion. (*C*) Cell viability assays with Cell Counting Kit-8. $^{*}P < .05$.

evaluating the strengths and weaknesses of utilizing both cell line–derived and patient-derived xenograft murine cancer models for conducting animal trials.[25]

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Discussion

Dr Joshua Mammen (Kansas City, KS): I would like to commend the authors for a thought-provoking and excellent presentation and particularly explaining in such a clear fashion this new technology, which most of us are not particularly familiar with. The authors use a technology that's recently been demonstrated to be enormously promising in hematologic malignancies, which is a CAR T-cell therapy. However, as the authors note, little is known about its effect on overcoming immune resistance, particularly in an immune-resistant cancer like pancreatic cancer.

The presentation suggests a strategy that's both novel and promising. I have a few questions to clarify some aspects of the presentation. First, the authors offer that IL-10 is well known to cause immunosuppression in the microenvironment. I would like the authors to explain why they chose to concentrate exclusively on this one cytokine, rather than others that might be involved. Were other cytokines evaluated and excluded? And what methodology was used to make that determination?

Second, conditioned media has numerous stimulatory and suppressive factors within it. Do the authors characterize the conditioned media used in the experiments? In particular, is the concentration of IL-10 within conditioned media well known, and did the authors attempt to use IL-10 alone at the known conditioned media concentration in order to attempt to simulate and stimulate the tumor microenvironment?

In addition to demonstrating the role of IL-10 by using the blocking antibody in the setting of conditioned media, I think this experiment would be valuable to highlight the role IL-10 may play by itself, even though the authors acknowledge it may only have a partial effect.

And, finally, the authors demonstrate exciting results in the in vitro setting. I'm interested in your thoughts on how to proceed to an in vivo environment, particularly since the mouse model may likely have a very different immune response compared with a human model.

Ms Oksana Gruzdyn: I am going to answer your questions one by one, starting with the first one. We chose to concentrate on IL-10 instead of other cytokines in order to avoid difficulties with interpretation of results. We also studied TGF-beta, which is another inhibitor cytokine, and we obtained similar results but not to the same extent as to what we have seen with IL-10.

In addition, we have yet to evaluate various factors that are prevalent in the pancreatic tumor-cell microenvironment. Some pancreatic cancer patients may have different inhibitor pathways activated, so for that reason, CAR-T therapy becomes extremely patient specific.

To address your second question, conditioned media for the pancreatic cancer microenvironment for the cell line that we used has been characterized by previous investigators, and IL-10 concentrations have been well documented. For that reason, we did not choose to characterize IL-10 again. We did not attempt to use IL-10 alone to simulate the tumor microenvironment. I think this is an excellent suggestion, and it will help us better understand the role that it plays on CAR T-cell therapy.

To address your final question, How do we propose to validate the strategy in an in vivo model? CAR-T therapy is a synthetic approach that redirects T-cell specificity against tumor antigen. This is an adoptive immunotherapy where T cells are isolated from a patient. Chimeric antigen receptors are introduced ex vivo through

viral therapy expression, using antivirus or retrovirus in a nutrient-rich environment and then reintroduced back into the patient.

Now, a tumor microenvironment not only creates a physical barrier by decreasing T-cell ability to penetrate the tumor mass, but it also plays a significant role by secreting inhibitory cytokines. Individual strategies have been developed to address these challenges, such as coadministration of CAR with various drugs and therapies, co-administration with checkpoint blockade, as well as targeting specific inhibitory cytokines. So, what we propose to do is to directly inhibit IL-10 by cotransducing CAR with IL-10–dominant negative receptor. In addition, we propose to target or simulate the release of activating cytokines. So, for example, IL-12 and IL-18 are promising candidates because they have an ability to directly inhibit IL-10, as well as regulatory T cells that are found in the tumor microenvironment. We propose to design fourth-generation CAR by combining expression of CAR, mesothelin CAR with IL-12 secretion, as well as basically selecting an IL-10–double negative receptor to target IL-10 that way.

Dr Nicholas Zyromski (Indianapolis, IN): I think it is interesting work, and I have a couple quick questions for you. First is, I think that the point about doing this in vivo is right on target and you really have to prove this in vivo. Before you do that, have you been able to show this effect in any other pancreatic cancer cell lines?

Ms Oksana Gruzdyn: We have studied the BxPC-3 cell line and one other cell line.

Dr Nicholas Zyromski: Did you observe similar effects in the other cell lines?

Ms Oksana Gruzdyn: Yes. Also, we repeated the same experiments for ovarian cancer and obtained similar results.

Dr Nicholas Zyromski: And I understand you're using Interferon gamma and granzyme as surrogates for apoptosis. Did you actually measure apoptosis? That's the presumed mechanism by which this is working.

Ms Oksana Gruzdyn: We have not yet measured apoptosis in these experiments.