SUPPLEMENTARY MATERIAL

FULL TITLE

Cytokine-armed vaccinia virus promotes cytotoxicity towards pancreatic carcinoma cells via activation of human intermediary CD56^{dim}CD16^{dim} natural killer cells.

AUTHOR INFORMATION

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TABLE OF CONTENTS

SUPPLEMENTARY METHODS (Detailed):

Culture of cell lines and primary cells: Human PDAC cell lines PANC-1 (RRID:CVCL_0480), BxPC3 (RRID:CVCL_0186), Capan-2 (RRID:CVCL_0026), MiaPACA-2 (RRID:CVCL_0428), AsPC-1 (RRID:CVCL_0152) and other carcinoma cells HeLa (RRID:CVCL_0030), CV-1 (RRID:CVCL_0229) were cultured with full growth culture media following the ATCC suggested formula (RPMI/DMEM based media). Carcinoma cell lines were a kind gift from Dieter Saur and Bartletts Laboratories. Chronic myelogenous leukemia cell line K562 cells (RRID:CVCL_0004) was cultured as suspension cells with RPMI1640 (Gibco Fischer Scientific) with 10% FBS. The NK cell line NK-92 (RRID:CVCL_2142) was cultured as suspension cells in Alpha minimum essential medium (Lonza) supplemented with 150 U/mL human recombinant IL-2 (Abcam), 0.2 mM myo-inositol, 0.1 mM 2-mercaptoethanol, 1.5 g/L sodium bicarbonate, 0.02 mM folic acid, 20% fetal bovine serum. Co-culture with PBMCs or NK cell line was realized with RPMI1640 with 10% human AB serum (Merck). Passage numbers from cell lines used did not exceed P30 and have been authenticated using STR profiling within the last three years. All experiments were performed with mycoplasma-free cells.
Freshly dissected pancreatic carcinoma tissue was minced and incubated with 5ml 2.5mg/ml collagenase P in complete RPMI media with 10μM Y-27632 dihydrochloride for 1h at 37°C. The tissue was mechanically disrupted by thin pipette to generate small cell clusters. After centrifugation, filtered cell pellet was diluted in complete RPMI medium and plated in a culture dish pre-coated with Collagen I from rat tail (Fisher scientific). Cells were growing under this condition until further use or discarded in cases of fibroblasts overgrowth (> 50% of total area).

Virus generation, titration and replication assay: Parental virus of vvDD, vvDD-IL2 and vvDD-IL15 were provided as kind gifts from Bartlett laboratories (Dr. Zhong Sheng Guo, UPMC Hillman Cancer Center, Pittsburgh). For expansion, HeLa cells were plated and passaged on 150 mm^2 cell culture dishes with full growth media. Cells were infected with parental virus at MOI 0.5. Following incubation, infected cells were scraped from the plate, spun down and resuspended in 10mM Tris-HCL. The cells were homogenized with a dounce homogenizer and supernatant was collected following short centrifugation. The supernatant was carefully layered on top of concentrated sucrose solution (36% sucrose with 10mM Tris-HCl) and centrifuged at 16,000rpm and 4°C for 1h. The pellet was subsequently resuspended in 10mM Tris-HCl with Benzonase-Nuclease solution to remove any contaminating cellular DNA before ultracentrifuging for 1h. The supernatant was removed and the pellet resuspended in 10mM Tris-HCl with subsequent storage at -80°C.
CV-1 cells were plated onto 6-well culture plates at 3.0×10^5 cells/well and incubated overnight. The following day, the stock virus was thawed at RT, serial diluted (1:10) in full growth media and added to CV-1 cells in duplicates. Following 48h incubation at 37°C, visible plaques were formed. Cells with plaques were fixed and stained with crystal violet in 20% ethanol. The plaques were counted in two wells with numbers ranging 10-100. The titer of the stock virus was estimated in consideration of average plaque number and dilution factor.
For viral replication assessment, tumor cells were plated on 6-well culture plates at 1.0×10^5 cells/well and incubated overnight. The next day, tumor cells were mock infected or infected with virus constructs at MOI of 0.1 and 1. At specified time points post infection (24h, 48h, 72h), supernatant was collected and 1mL distilled water was added into the wells with cells. 15mins later, cells at bottom were scraped and collected in a 15 mL Falcon tube. Subsequently, cells were spun down at 3000×g for 15 mins, supernatant collected and

combined with the previously collected supernatant from the same well. After serial dilution, supernatant with virus at different dilution factors (10^x) were added into 6-well plates with CV-1 cells. CV-1 cells were cultured for 48h at 37°C until visible plaques were formed, subsequently fixed and stained with crystal violet in 20% ethanol.

PBMC generation and co-culture: Human whole blood samples from healthy donors were collected in heparin coated collection tube and diluted with PBS at 1:1. The diluted blood was aliquoted on Ficoll (2:1) and spun down for 25 mins, 550×g at RT. Next, the interphase ring (the PBMC layer) was removed and mixed with PBS, spun down twice with PBS at 400×g to wash out residual Ficoll. The PBMC pellet was resuspended in RPMI (+10% human serum; +50 U/mL IL-2) and cultured at 37°C.
For co-culture, carcinoma cells were seeded in 12-well culture plates in their respective full growth medium at 1.0×10^5 cells/well. The next day, human derived PBMC, or NK-92 cells were isolated/sub-cultured and resuspended in RPMI (+10% human serum, +50 U/mL IL-2). PBMCs for positive and negative controls (activation assays) were cultured with 50 U/mL or 400 U/mL IL-2. The number of plated immune cells was counted with hematocytometer to obtain PBMC suspensions at E/T ratios of 1:1/10:1 or NK-92 cell suspensions at E/T ratio of 0.5:1. Calculated volume of counted PBMC-/NK cell suspension was transferred into wells and cultured for 1h, 24h and 48h. For co-culture of immune cells with virus infected tumor cells, tumor cells were first infected with virus constructs at MOI 0.1. After 12h, the immune cells were isolated (PBMC) or sub-cultured (NK-92 cells) and added to the tumor cells.

Flow cytometry: The immune cell suspensions (PBMC, NK-92) in flow cytometry buffer was spun down and incubated with anti-CD45 BV785 (Biolegend/clone: HI30), anti-CD3 BV510 (Biolegend/clone:UCHT1), anti-CD19 BV605 (Biolegend/clone:HIB19), anti-CD56 APC (Biolegend/clone:5.1H11), anti-CD16 FITC (Biolegend/clone:3G8), anti-CD69 PE (Biolegend/clone: FN50) antibodies (1:200) for 20 min at 4°C. After a final wash with flow cytometry buffer, cells were resuspended again in 200µL of flow cytometry staining buffer and analysed on flow cytometer (BD LSRFortessa™; BD, Becton, Dickinson and Company, Heidelberg, Germany) after adding 5µL 7-AAD for gating the viable cells. For immune cells co-cultured with virus infected carcinoma cells, immune cells were stained first with Zombie Aqua at RT for 20mins for gating the viable cells, and subsequently stained with anti-human CD45 BV785, anti-human CD3 Pacific Blue (Biolegend/clone:UCHT1), anti-human CD19 BV605, anti-human CD56 APC, anti-human CD16 FITC, anti-human CD69 PE antibodies (1:200) or anti-human CD56 APC, anti-human FasL PE (Biolegend/clone: NOK-1) antibodies, anti-human TRAIL PE Cyanine7 antibodies (1:200) for 20 min at 4°C. After a final wash with flow cytometry staining buffer, cells were fixed with 4% PFA for 20min to inactivate any residual virus. Cells were spun down and resuspended in 200µL of flow cytometry staining buffer.
For surface receptors and intracellular protein expression in immune cells co-cultured with virus infected carcinoma cells, immune cells were stained first with Zombie Aqua (1:500) at RT for 20min, and stained with anti-human CD56 APC, anti-human DNAM1 PE-Cyanine7 (Biolegend/clone:11A8), anti-human NKG2D BV785 (Biolegend/clone:1D11) (1:200) for 20min. Next, cells were fixed with 4% PFA for 20mins before being resuspended in 1X Intracellular Staining Perm Wash Buffer for 10mins. After centrifugation, cells were resuspended in 1X Intracellular Staining Perm Wash Buffer with anti-human IFN-gamma FITC (Biolegend/clone:4S.B3) and anti-human perforin APC-Cyanine7 (Biolegend/clone:dG9)

(1:100) for 20mins and analyzed on flow cytometer (BD LSRFortessa™; BD, Becton, Dickinson and Company, Heidelberg, Germany) and FlowJo software.
The expression of membrane-bound IL-2, PD-L1, Calreticulin and YFP from virus infected carcinoma cells were assayed via flow cytometry. Tumor cells were seeded in culture dishes and mock infected or infected with virus constructs at MOIs of 0.1, 1.0 and 5.0. 24h after virus infection, cells were detached with TrypLE express (Fisher Scientific), then stained with Zombie Aqua dye (1:500) for 20mins at RT. After centrifugation, cells were resuspended in 50µL flow cytometry staining buffer and stained with 50ul antibody mastermix for 20mins at 4℃, including anti-mouse IL-2 APC (1:200; clone JES6-5H4); and anti-human CD274 PE (1:200; clone MIH2); or only anti-human Calreticulin APC (1:200; clone EPR3924). Cells were washed with 1mL flow cytometry staining buffer to remove residual antibodies, and subsequently fixed in 300µL 4% PFA for 20mins. Cells were spun down and resuspended in 200µL of flow cytometry staining buffer and analysed on flow cytometer (BD LSRFortessa™; BD, Becton, Dickinson and Company, Heidelberg, Germany) and FlowJo software.

Cytotoxicity assay: Visual cytotoxicity assessment and screening was realized following plating of PDAC cell lines in 6-well culture plates and culture with full growth media. Upon confluency (90-100%) NK-92 cell suspension was co-cultured at E/T ratios of 0.5:1 for 48h. Cells were fixed and stained with crystal violet in 20% ethanol and compared with control (no co-culture).
To analyze tumor apoptosis after co-cultured with immune cells, K562 cells were pre-labelled with CSFE or Cell Trace Blue for identification from interfering suspension immune cells. After the pre-treatment, PDAC cells were detached using TrypLE while Cell Trace Blue-labelled K562 cells were collected directly. PDAC cells were stained with anti-human EpCAM Alexa Flour700 antibody (1:200) for 20mins at 4℃ for differentiation from parallel harvested immune cells. After single wash with Annexin V binding buffer, tumor cells were incubated with Annexin V PE (1:21) for 15mins. Another wash with Annexin V binding buffer followed. Cells were resuspended in 200µL annexin-V binding buffer and 5µL 7-AAD was added prior analysis. Of note, for analyses of virus infected tumor cells, Zombie Aqua was used instead to gate for viable cells together with EpCAM for tumor cell identification. For inactivation of residual virus 4% PFA was added, prior analysis on flow cytometer.

Immunofluorescence and TUNEL assay: YFP expression was detected using a ZEISS Axio Observer Z1 microscope. PDAC cells were plated on 12-well plates at the density of 1.0×10^5 cells and infected with vvDD,vvDD-IL2 or vvDD-IL15 at MOI 1. 24h later, cells were fixed with 4% PFA for 15min and washed with PBS, and analyzed under excitation at 524nm for YFP. Surface Calreticulin staining PANC-1(1.0×10^5 cells) and BxPC-3(1.5×10^5 cells) were seeded in 12-well plates and infected with vvDD at 0.1, 1 or 5 MOI the next day. 48h post infection, cells were incubated in 100% methanol at room temperature for 5mins. Cells were incubated with 1% BSA, 22.5 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 min to block unspecific binding of the antibody, and incubated in diluted calreticulin antibody(1:400) in 1%BSA in PBST overnight at 4°C. After cells washed with PBS, images were excited and obtained at 614nm using ZEISS Axio Observer Z1 microscope.
Terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) was performed with PANC-1 and BxPC-3 cells. Cells were cultured on glass 8-chamber slides and infected with viruses at MOI 0.1 or 1.0 the next day. At specified time points (24h, 48h, 72h) post infection, cells were then fixed with 4% formaldehyde for 15mins, permeabilized with

0.05% Triton-X100 for 2mins on ice and stained with 100µL/well In-Situ Cell Death Detection Kit for 1h at 37℃, protected from light. After being washed once with PBS, cell nuclei were labeled with DAPI (1:1000) for 10mins at RT. Images were obtained using a ZEISS Axio Observer Z1 microscope with an objective lens (20x magnification). YFP expressed by virus constructs, TUNEL staining and DAPI were excited at 524nm, 614nm and 465nm respectively. For illustration, channels were merged with the ImageJ software.

Real-time quantative PCR: Taqman based qPCR detection method was used. Tumor cells were seeded in culture dishes and treated with virus constructs at MOI 0.1,1.0, 5.0 or control medium (mock) the next day. Total RNA was extracted 24h after infection, and RNA (2µg) was reverse transcribed for cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), which contained 10X RT Buffer (2×1mL), 10X RT Random Primers (2×1mL), 25X dNTP Mix (100mM, 1mL), MultiScribe Reverse Transcriptase (1mL at 50U/µL), RNase Inhibitor (10 × 0.1mL at 20U/µL). The reaction was performed at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and cooling down at 4°C. Subsequently Master mixes for target and housekeeping genes were generated by adding 0.5µl primer, 5µl TaqMan Gene Expression Master-Mix, and 3.5µl DEPC water per sample, 1µl template DNA was added to each reaction mix aliquot (10µl per well). A run consisted of a 10min heating step and 40 cycles with each 10s for denaturation at 95 °C and 30s at the appropriate annealing temperature for annealing and elongation. For all samples, a detection probe for HPRT-1 was used to normalize the obtained data. 9µL master mix of corresponding primer pair and DNase water was served as a negative control. The cycle threshold (Ct) values of the genes of interest were normalized to the cells' corresponding Ct value for HPRT-1. Thus, the results were expressed in fold change (FC) using the formula: 2^(−ΔCt), where ΔCt = Ct (gene of interest) − Ct (HPRT-1). Pre-designed primers from Fisher Scientific were used for IL-15 (ID Mm04336046_m1), IL-2 (ID Mm00434256_m1), A34R (ID Mm02344630_s1).

Immunoblotting: Carcinoma cells were cultured in 6-well plates and infected with virus constructs at MOIs of 0.1, 1.0, and 5. At 24h post-infection, cells were scraped using RIPA buffer on ice. Cell lysates were spun in a microcentrifuge at 14,000rpm for 20min to remove cell debris. Protein concentration for each cell lysate was determined via BCA assay following standard protocols. 20µg of protein from each cell lysate was combined with lysis buffer and loading buffer, and immediately boiled at 100°C for 5 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10% polyacrylamide at 77V for the stacking gel and at 100V for the running gel, and transferred to nitrocellulose membrane for 2h at 238mA. The membrane was blocked with 5% defatted dried milk for 1h at room temperature and incubated overnight at 4°C with anti-Bad monoclonal antibody (Cell Signaling Technology/clone: D24A9), anti-P53 monoclonal antibody, Anti-Bcl-xl monoclonal antibody (Cell Signaling Technology/clone: 54H6) and Anti-GAPDH monoclonal antibody (Abcam/clone: 6C5) in TBS-T. The membranes were washed carefully with TBST, and incubated with HRP-Linked Anti-mouse IgG from sheep, HRP-Linked Anti-rabbit IgG from sheep diluted 1:1000. The bound antibodies were detected by chemoluminiscence reaction using Biorad Chemidoc imaging device.

ATP assay: Tumor cells were cultured in 12-well plates at the density of 1.0×10^5 cells/well and infected with mock or vvDD at MOIs of 0.1, 1.0, and 5. At 48h p.i, supernatant was collected

and 10μL supernatant from each sample or standard was added into a 96-well luminescence plate as triplicates. 10mL standard reaction solution, which contained 8.9 mL dH_2O, 0.5 mL 20X Reaction Buffer, 0.1 mL 0.1 M DTT, 0.5 mL of 10 mM D-luciferin, 2.5 μL of firefly luciferase 5 mg/mL stock solution was gently mixed. Subsequently 90μL standard reaction solution was added using a multichannel pipette before bioluminescence were detected by SpectraMax i3x (Molecular Devices).

Proteomics: For proteomic profiling by mass spectrometry, PANC-1 cells were plated in 6-well plates at 5.0×105cell/well and infected with virus constructs at 0.1MOI the next day. After 24h, the supernatant was discarded, and cells were scraped in cold PBS. Cells were subsequently centrifuged at 1000rpm, and were lysed by SDS based lysis buffer (2% SDS, 100 mM Tris pH8, 150mM NaCl, 1mM EDTA, phosphatase inhibitor cocktail 2 and 3 (Sigma, 1:100), 10mM NaF). Samples were heated for 10mins at 95℃ before being stored at -80℃. Lysates were reduced with 10 mM DTT (dithiothreitol, Sigma) for 45min followed by alkylation with 40mM CAA (2-chloroacetamide, Sigma) for 30min. After treatment with Benzonase® (Merck, 50 units) for 30 min at 37°C, samples were centrifuged for 10 minutes at high speed. The supernatant was collected and protein extracts were cleaned up using paramagnetic bead-based protocol (PMID: 30464214). For each sample 200 ug protein was digested with endopeptidase LysC (Wako) and sequence-grade trypsin (Promega) in 50 mM ammonium bicarbonate overnight at 37°C with an enzyme-to-protein ratio of 1:50. The resulting peptides were harvested, dried, resolved in 50 mM HEPES and labeled with 16-plex tandem mass tag (Fisher Scientific) reagents following the vendors instructions. Samples were combined, desalted on C18 SepPak columns (Waters, 200mg/1cc) and fractionated by high-pH reversed phase off-line chromatography (1290 Infinity, Agilent) into 30 fractions. Of each fraction, 10 % material was used for global proteome analyses. The remaining 90% were further pooled into 15 fractions and used for phosphopeptide enrichment, which was performed on an Agilent Bravo automated liquid handling platform using Fe(III)-NTA cartridges. For LC-MS/MS measurements, peptides were reconstituted in 3% acetonitrile with 0.1% formic acid and separated on a reversed-phase column (20 cm fritless silica microcolumns with an inner diameter of 75 μm, packed with ReproSil-Pur C18-AQ 1.9 μm resin (Dr. Maisch GmbH)) using a 98 min gradient with a 250 nl/min flow rate of increasing Buffer B (90% ACN, 0.1% FA) concentration (from 2% to 60%) on a High Performance Liquid Chromatography (HPLC) system (Thermo Fisher Scientific) and analyzed on a Q Exactive HF-X instrument (Thermo Fisher Scientific). The mass spectrometer was operated in data-dependent acquisition mode using the following settings: full-scan automatic gain control (AGC) target 3 x 106 at 60K resolution; scan range 350–1500 m/z; Orbitrap full-scan maximum injection time 10 ms; MS/MS scan AGC target of 1 x 105 at 45K resolution; maximum injection time 86 ms (for non-enriched peptides) or 120 ms (for phosphopeptides); normalized collision energy of 30 and dynamic exclusion time of 30 s; precursor charge state 2-6, 20 MS2 scans per full scan.
RAW data were analyzed with MaxQuant software package (v 1.6.10.43) using the Uniprot databases for human (UP000005640_2019_07) and the vaccinia (UP000000344). The search included variable modifications of methionine oxidation and N-terminal acetylation, deamidation (N and Q) and fixed modification of carbamidomethyl cysteine. For measurements of phosphopeptide enriched samples phosphorylation (STY) was added in addition as a variable modification. Reporter ion MS2 for TMT16 was selected (internal and N-terminal) and TMT batch specific corrections factors were specified. The FDR (false discovery rate) was set to 1% for peptide and protein identifications. Unique and razor peptides

were included for quantification. The resulting text files were filtered to exclude reverse database hits and potential contaminants.

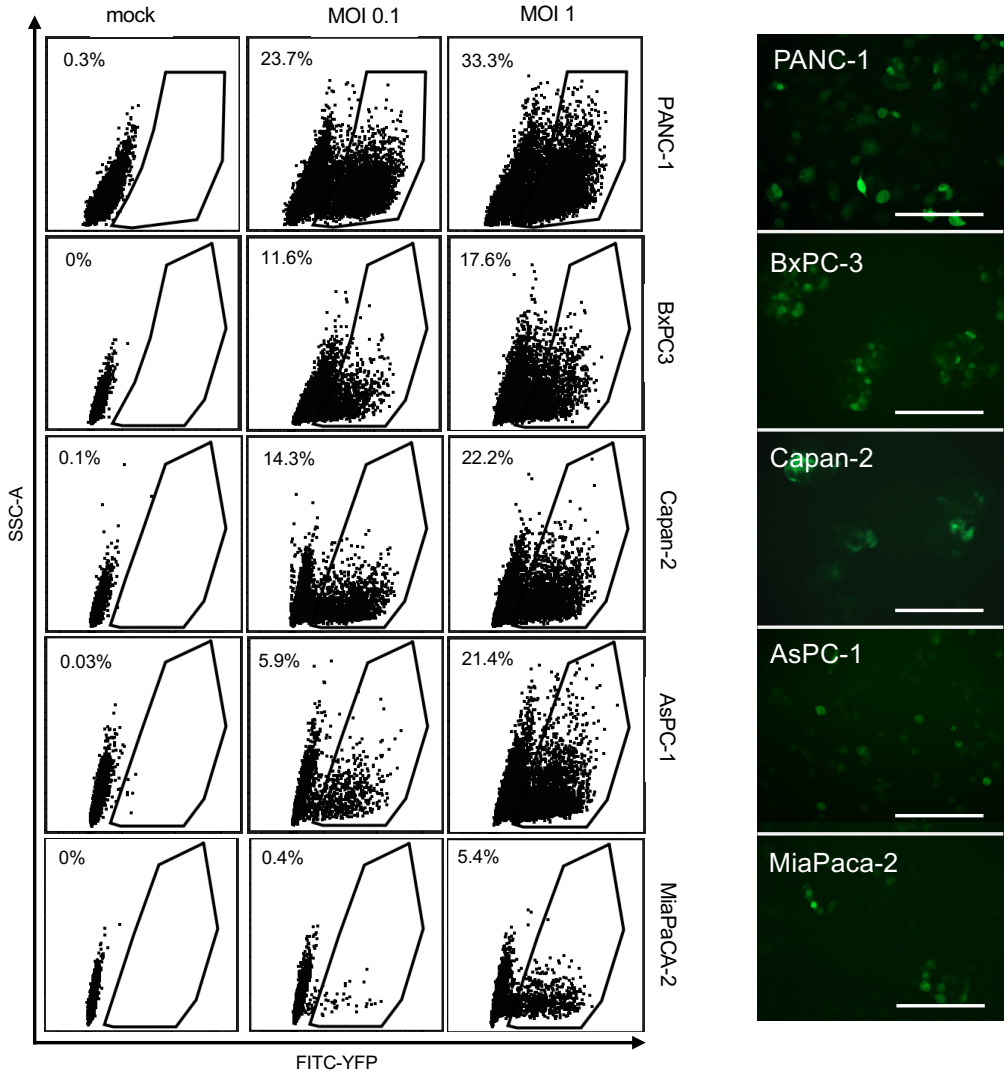
Statistics: GraphPad Prism version 9 (GraphPad Software, San Diego, CA) was used for creating graphs and analyzing data from *in vitro* experiments. The data analysis was performed using non-parametric Student's t test or two-way ANOVA. P values of < 0.05 were considered statistically significant. The symbols used in figures were standard ones: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; and ns, not significant. For proteomic data analysis the log2 transformed and normalized reporter ion intensities were used with a valid value filter of 100%. Differences in protein and phosphopeptide abundance between experimental groups were calculated using Student's test. Signals passing the significance cut-off of FDR 1 or 5% were considered differentially expressed.

Supplementary table 1: Virus proteins detected in infected PDAC cell line.

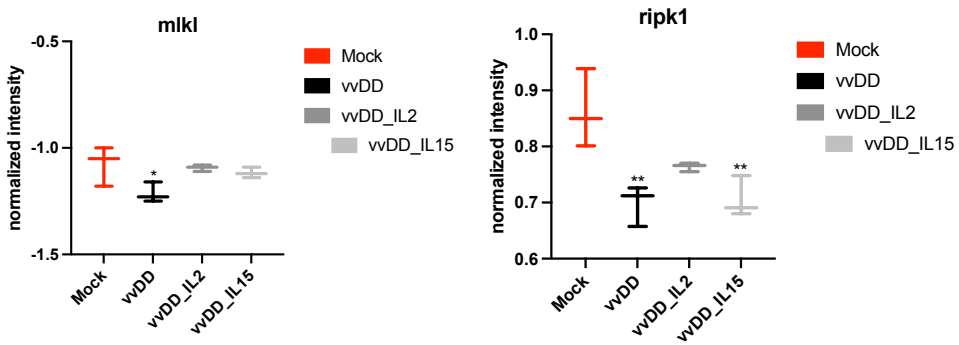
			vvDD vs mock		vvDD-IL2 vs mock		vvDD-IL15 vs mock	
Gene name	*Protein ID*	*Peptides*	*log2 ratios**	*q-value***	*log2 ratios**	*q-value***	*log2 ratios**	*q-value***
A11_VACCW	Q80HV8	31	0,6860	<0,0001	0,9163	0,0011	0,9341	0,0022
A13_VACCW	Q76ZQ4	7	1,0227	<0,0001	0,9579	0,0009	1,1661	0,0021
A21_VACCW	P68712	8	0,7293	<0,0001	0,8248	0,0007	0,9049	0,0011
A28_VACCW	P68633	6	0,7347	<0,0001	0,897	0,0006	0,9763	0,0012
A32_VACCW	P68615	7	0,8737	<0,0001	1,1738	0,0030	1,0592	<0,0001
B1_VACCW	P16913	18	0,9735	<0,0001	1,1025	0,0018	1,1432	<0,0001
B4_VACCW	P24769	1	0,4382	0,0007	0,5383	0,0006	0,5484	0,0006
C4_VACCW	P17370	7	0,7376	<0,0001	1,2952	0,0056	0,8880	0,0011
H2_VACCW	P04312	5	0,7477	<0,0001	1,0820	0,0013	0,9841	0,0023
I6_VACCW	P68462	6	0,8944	<0,0001	1,1305	0,0026	1,0658	<0,0001

*log2 fold changes

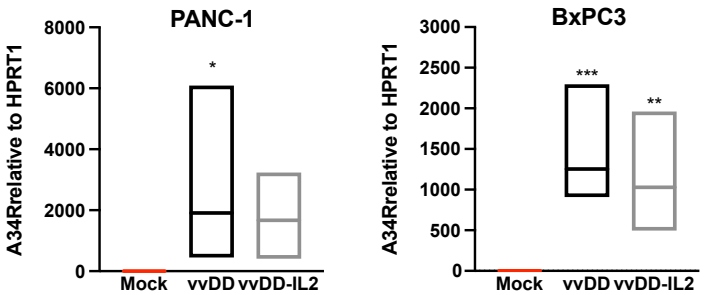
**TMT-based corrected p-value



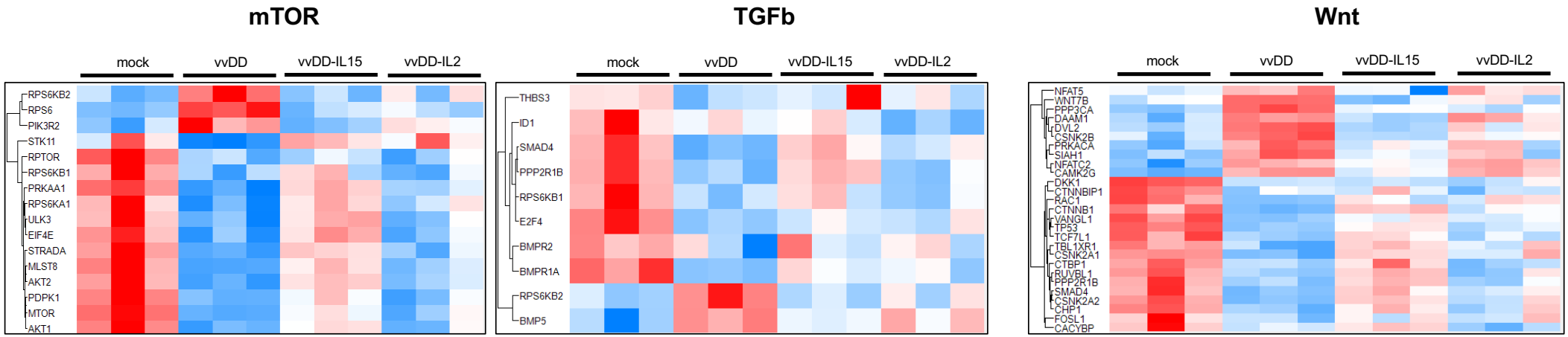
Suppl. Figure 1. Infection efficiencies of vvDD in screened PDAC cell lines.



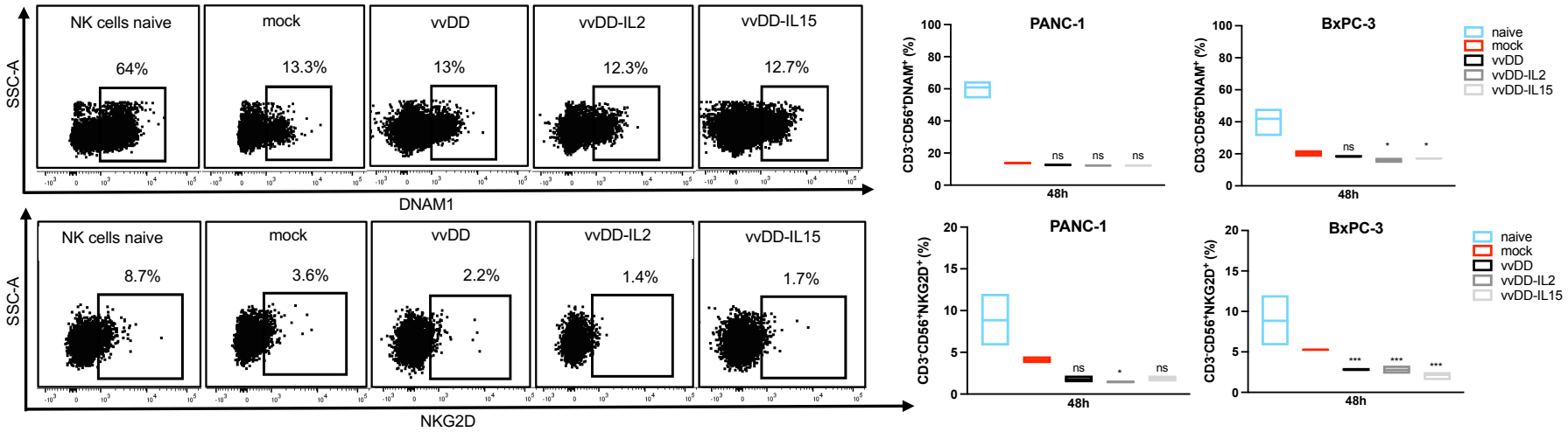
Suppl. Figure 2. Proteomic regulation of anti-apopototic mlkl and ripk1 following vaccinia virus infection.



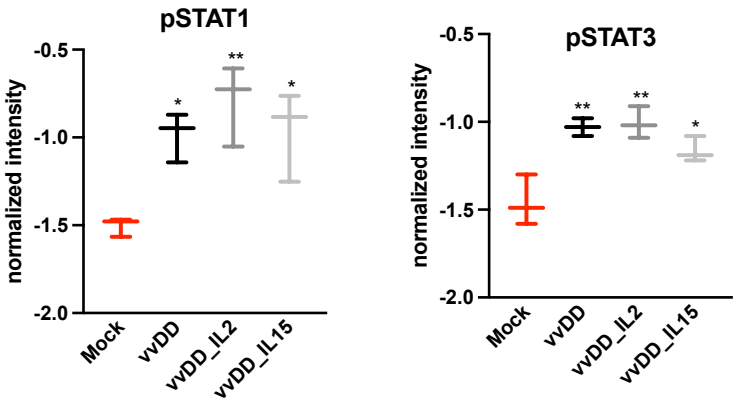
Suppl. Figure 3. Regulation of A34 expression following vaccinia virus infection.



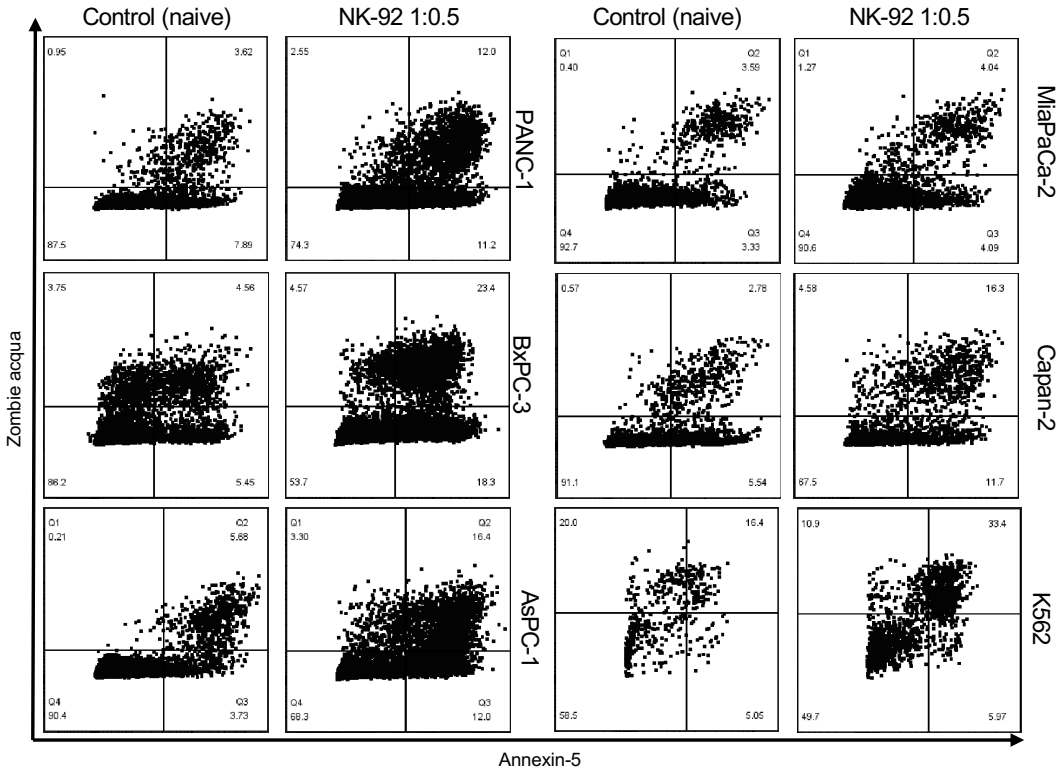
Suppl. Figure 4. Proteomic regulation of pancreatic carcinoma pathways following vaccinia virus infection.



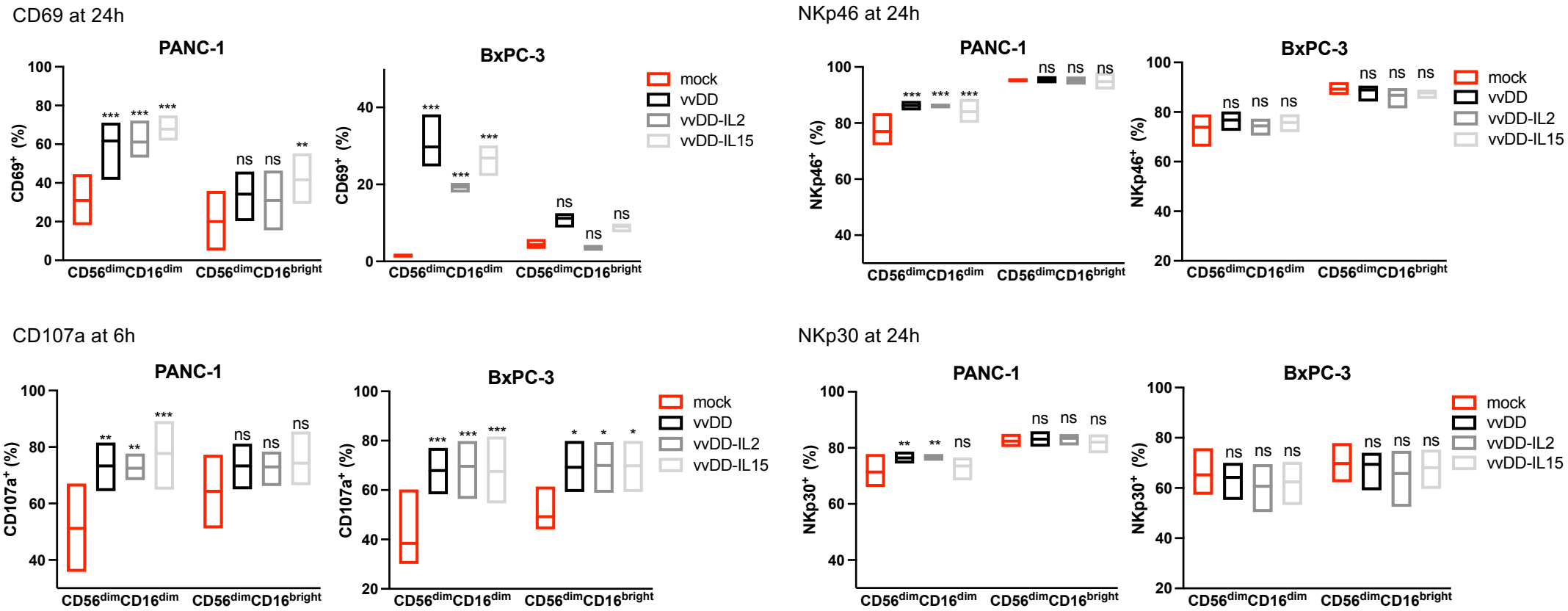
Suppl. Figure 5. Surface expression of DNAM1 and NKG2D on NK cells following vaccinia virus infection.



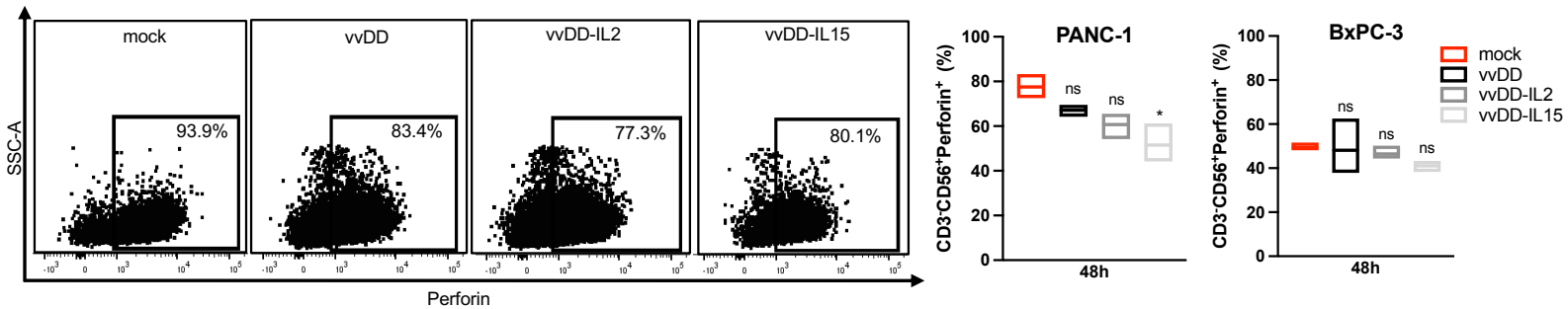
Suppl. Figure 6. STAT1 and STAT3 phosphorylation following vaccinia virus infection.



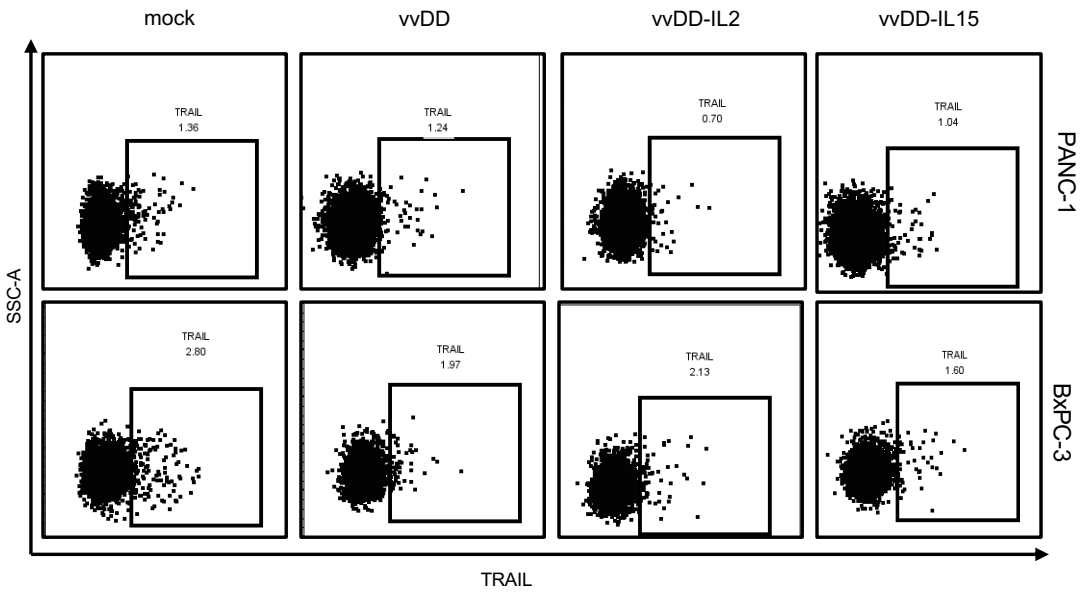
Suppl. Figure 7. NK cell mediated cytotoxicity via NK-92 cell line.



Suppl. Figure 8. CD69 and CD107a expression of respective effector NK cell sub-populations following vaccinia virus infection.



Suppl. Figure 9. Exhaustion of cytolytic granules following vaccinia virus infection.



Suppl. Figure 10. Regulation of TRAIL after vaccinia virus infection.