

## RESEARCH ARTICLE

## Tumor Immunology and Microenvironment

# Cytokine-armed vaccinia virus promotes cytotoxicity toward pancreatic carcinoma cells via activation of human intermediary CD56<sup>dim</sup>CD16<sup>dim</sup> natural killer cells

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## Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains a particularly aggressive disease with few effective treatments. The PDAC tumor immune microenvironment (TIME) is known to be immune suppressive. Oncolytic viruses can increase tumor immunogenicity via immunogenic cell death (ICD). We focused on tumor-selective (vvDD) and cytokine-armed Western-reserve vaccinia viruses (vvDD-IL2 and vvDD-IL15) and infected carcinoma cell lines as well as patient-derived primary PDAC cells. In co-culture experiments, we investigated the cytotoxic response and the activation of human natural killer (NK). Infection and virus replication were assessed by measuring virus encoded YFP. We then analyzed intracellular signaling processes and oncolysis via in-depth proteomic analysis, immunoblotting and TUNEL assay. Following the co-culture of mock or virus infected carcinoma cell lines with allogenic PBMCs or NK cell lines, CD56<sup>+</sup> NK cells were analyzed with respect to their activation, cytotoxicity and effector function. Both, dose- and time-dependent release of danger signals following infection were measured. Viruses effectively entered PDAC cells, emitted YFP signals and resulted in concomitant oncolysis. The proteome showed reprogramming of normally active core signaling pathways in PDAC (e.g., MAPK-ERK signaling). Danger-associated molecular patterns were released upon infection and stimulated co-cultured NK cells for enhanced effector cytotoxicity. NK cell subtyping revealed enhanced numbers and activation of a rare CD56<sup>dim</sup>CD16<sup>dim</sup> population. Tumor cell killing was primarily triggered via Fas ligands rather than granule release, resulting in

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marked apoptosis. Overall, the cytokine-armed vaccinia viruses induced NK cell activation and enhanced cytotoxicity toward human PDAC cells *in vitro*. We could show that cytokine-armed virus targets the carcinoma cells and thus has great potential to modulate the TIME in PDAC.

#### KEYWORDS

immune modulation, immunogenic cell death, modified vaccinia virus, natural killer cells, proteome analysis

#### What's New?

Pancreatic adenocarcinoma is characterized by a high stroma content and an immunosuppressive tissue environment. Here, the authors investigated the capacity of vaccinia viruses to stimulate the human innate immune system upon exposure to pancreatic carcinoma cells. Infection of pancreatic adenocarcinoma cells with modified and novel cytokine-armed vaccinia viruses led to cellular re-programming of core signaling pathways and initiation of immunogenic cell death that stimulated natural killer cells and resulted in enhanced cytotoxicity. Natural killer cell activation by cytokine-armed vaccinia viruses shows great potential for immune modulation in the pancreatic duct adenocarcinoma microenvironment.

## 1 | INTRODUCTION

Pancreatic carcinoma remains a deadly disease with few curative treatment strategies. Albeit promising pre-clinical effects, immunotherapies have largely been ineffective in PDAC patients. Reasons are thought to lie in the suppressive immunomodulatory set-point and overall cold tumor immune microenvironment (TIME) with few effector T-lymphocytes and natural killer (NK) cells.<sup>1</sup> The stroma-rich environment likely impedes the immunological cross-talk and chemoattraction, further isolating the neoplastic carcinoma cells, that are already disguised by relatively low mutational burden and poor neoantigen expression.<sup>2,3</sup> Main concepts to induce anti-tumor immune response in PDAC have been experimentally explored by improving T-cell responses toward the tumor via adoptive T-cell transfer<sup>4,5</sup> or alternatively priming T-cells by cancer vaccines<sup>6</sup> sometimes in combination with immune checkpoint inhibitors.<sup>7</sup> Clinical studies demonstrated therapeutic effects of pembrolizumab in selected PDAC patients with extensive mutational burden (MSI high, <1% of patients).<sup>8</sup> However, this single-agent targeted concept proves ineffective in classic, immune-deprived PDAC.<sup>9</sup> Active T-cell priming via molecular cancer vaccines may affect the TIME but likely requires additional immunomodulatory strategies.

Oncolytic viruses (OV) can be used for specific tumor cell targeting resulting in direct oncolysis and immune stimulation. They commonly harness tumor cell targeting entry mechanisms for replication with downstream induction of immunogenic cell death (ICD).<sup>10</sup> Many viral derivatives have been investigated, of which thymidine kinase- and vaccinia growth factor modified vaccinia viruses (vvDD) showed high tropism for TP53 mutated carcinoma cells.<sup>11</sup> Vaccinia viruses contain large dsDNA (>190 kb) enabling the insertion of immunostimulatory elements. For instance, GM-CSF-armed OV (Pexa-Vec; T-Vec) have been tested with good response rates in HCC and

melanoma patients.<sup>12,13</sup> Similar constructs carrying other transgenes were used to design “tumor-selective” vvDD.<sup>14,15</sup> Indeed, pre-clinical studies using IL-2- and IL-15 transgenes have shown augmented anti-tumor immunity resulting in tumor regression in combination with immune checkpoint blockade.<sup>16,17</sup>

Chemokines and cytokines typically have pleiotropic effects and thereby induce complex immune responses *in vivo*. IL-2 predominantly acts by supporting T-cell responses after engaging with the IL2-R (CD25) on the cell surface.<sup>18</sup> It induces expansion of effector CD8<sup>+</sup> cells, maintenance of NK cells as well as influencing the homeostasis of Treg cells.<sup>19,20</sup> IL-15 has related functions with stronger and more sustained effects on NK- and memory T cells.<sup>21</sup> The innate immune system plays a critical role in inducing both, cytotoxic T cells and NK cells, and is important for potent anti-tumor immune responses, especially in the setting of low immunogenicity.<sup>22</sup> NK cells are highly specialized effector cells that can induce direct cytotoxicity via cytolytic granules (perforin and granzyme B) and death cell receptors (TRAIL and FasL) or indirectly via immunoreactive cytokines (such as IFN- $\gamma$  and TNF- $\alpha$ ).<sup>23</sup> Their cytotoxicity can be unleashed by cells with reduced MHC-I expression (missing-self-concept),<sup>24</sup> cell surface Fc-receptor bound antibodies mediating antibody-dependent cellular cytotoxicity<sup>22</sup> as well as triggered by released damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs).<sup>25</sup> DAMPs and PAMPs can be released upon tumor cell killing following virally induced oncolysis and may thus elicit an immediate response of NK cells during surveillance.<sup>26</sup> Indeed, Nelson et al. recently demonstrated the activation and increased cytotoxicity of NK and CD8<sup>+</sup> T-cells with subsequent tumor regression in subcutaneous and orthotopic pancreatic carcinoma models following oncolytic VSV-IL15 infection.<sup>27</sup> We hypothesized that cytokine-armed oncolytic vaccinia virus strains (vvDD-IL2 and vvDD-IL15) can enhance

stimulation of NK cells compared to mock infected or wildtype vvDD, resulting in increased cytotoxicity toward pancreatic carcinoma cells via molecular effectors for ICD.

In the present study, we characterized NK cell activation following exposure to pancreatic carcinoma cells *in vitro*. Likewise, we explored susceptibility of PDAC cells from different patients toward infection with modified (vvDD) and cytokine-armed vaccinia virus strains (vvDD-IL2 and vvDD-IL15) informing on their clinical potential to initiate an anti-tumor response. We identify distinctive features of NK cell effectors with a phenotypic shift toward an intermediary activation state (CD56<sup>dim</sup>CD16<sup>dim</sup>), that correlated with enhanced cytotoxic effects. Intracellular proteomic as well as further molecular analysis of infected tumor cells elucidated ICD as one mechanism by which effective T-cell priming and immunomodulation of the tumor microenvironment could be established.

## 2 | MATERIALS AND METHODS

### 2.1 | Culture of cell lines and primary cells

Human PDAC and other carcinoma 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), HeLa (RRID:CVCL\_0030) and CV-1 (RRID:CVCL\_0229) were cultured with full growth culture media following the ATCC suggested formula. Cell lines NK-92 (RRID:CVCL\_2142) and K-562 (RRID:CVCL\_0004) were cultured as suspension cells with media specified in supplementary methods. The cell lines were provided as kind gifts from Dieter Saur- (carcinoma cell lines), Thomas Kammertöns- (suspension cell lines) and Bartlett's laboratories (CV-1 cells). Passage numbers from cell lines did not exceed P30 and have been authenticated using STR profiling within the last 3 years. All experiments were performed with mycoplasma-free cells. Primary tissue cultures were realized with enzymatic and mechanic dissociation procedures detailed in supplementary methods.

### 2.2 | Virus generation, titration, and replication assay

Parental viruses of vvDD, vvDD-IL2, and vvDD-IL15 were provided as kind gifts from Bartlett laboratories (Dr. Zhongsheng Guo, UPMC Hillman Cancer Center, Pittsburgh). For expansion, larger quantities of HeLa cells were infected at MOI 0.5. After 48 h incubation, purified viral pellets were collected in 10 mM Tris-HCl following multistep centrifugation procedures (see supplementary methods) and stored at  $-80^{\circ}\text{C}$ . For titration, CV-1 cells were cultured in 6-well culture plates overnight. Stock virus was thawed at RT, serially diluted (1:10) in full growth media, and added to CV-1 cells. The titer of the stock virus was calculated based on average plaque number and dilution factor (detailed in supplementary methods). For assessment of viral replication, carcinoma cells were cultured and infected with viral constructs

at MOI 0.1 or 1. At specified time points post-infection (24, 48, and 72 h), supernatant was collected, centrifuged, and serially diluted onto CV-1 cells. The virus count was estimated in consideration of average plaque number and dilution factor (detailed in supplementary methods).

### 2.3 | PBMC generation and co-culture

Human whole blood samples from healthy donors were collected in heparin-coated collection tube and diluted with PBS. PBMCs were extracted using standard Ficoll protocols described in supplementary methods. PBMC pellet was resuspended in RPMI (+10% human serum; +50 U/mL IL-2) and cultured at  $37^{\circ}\text{C}$ . Co-cultures were realized by generating suspensions at E/T ratios of 1:1/10:1 or NK-92 cell suspensions at E/T ratio of 0.5:1. For co-culture of immune cells with virus-infected tumor cells, tumor cells were first infected with virus constructs at MOI 0.1 and co-cultures established at 12 h post-infection. Co-cultures were analyzed 1, 24, and 48 h post-infection. More detailed information under supplementary methods.

### 2.4 | Flow cytometry

At specified time points, the immune cell suspensions (PBMC and NK-92) in flow cytometry buffer were 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  $4^{\circ}\text{C}$ , 5  $\mu\text{L}$  7-AAD added and viable cells analyzed on flow cytometer (BD LSRFortessa™; BD, Becton, Dickinson, Heidelberg/Germany). In the setting of infected carcinoma cells, immune cells were stained first with Zombie Aqua 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 or anti-human CD56 APC, anti-human FasL PE (Biolegend/clone: NOK-1), anti-human TRAIL PE Cyanine7, anti-human DNAM1 PE-Cyanine7 (Biolegend/clone: 11A8), anti-human NKG2D BV785 (Biolegend/clone: 1D11), anti-human IFN- $\gamma$  FITC (Biolegend/clone: 4S.B3) or anti-human perforin APC-Cyanine7 (Biolegend/clone: dG9) (all 1:100–200) for 20 min at  $4^{\circ}\text{C}$ . Cells were fixed with 4% PFA for 20 min to inactivate any residual virus and subsequently analyzed on flow cytometer with FlowJo software. The expression of membrane-bound IL-2, PD-L1, Calreticulin, and YFP from virus-infected carcinoma cells followed after infection and dissociation. Cells were stained with antibody reaction mix for 20 min at  $4^{\circ}\text{C}$ , using 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). Fixed cells were analyzed on flow cytometer with FlowJo software. The antibody combination

varied in the individual experimental settings specified under supplementary methods.

## 2.5 | Cytotoxicity assay

For visual cytotoxicity assessment and screening, PDAC cell lines were cultured in wells. Upon confluency (90%–100%) NK-92 cell suspension was co-cultured at E/T ratios of 0.5:1 for 48 h. Cells were fixed and stained with crystal violet in 20% ethanol and compared with control. To analyze tumor apoptosis following co-culture with immune cells, K-562 cells were pre-labeled with CFSE or Cell Trace Blue. After the pre-treatment, PDAC cells and K562 cells were collected. PDAC cells were identified with anti-human EpCAM Alexa Fluor700 antibody (1:200) and incubated with Annexin V PE (1:21) for 15 mins while 5  $\mu$ L 7-AAD was added prior to analysis. Modification in virus-infected tumor cells is specified under supplementary methods.

## 2.6 | Immunofluorescence and TUNEL assay

PDAC cells were infected vvDD, vvDD-IL2, or vvDD-IL15 at MOI 1, and fixed carcinoma cells analyzed under excitation at 524 nm for YFP using ZEISS Axio Observer Z1 microscope. For surface calreticulin expression, PANC-1 and BxPC-3 cells were infected for 48 h with vvDD at MOI 0.1, 1, or 5. Fixed cells were incubated in diluted calreticulin antibody (1:400; clone EPR3924) and images obtained at 614 nm excitation. Terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) was performed with infected PANC-1 and BxPC-3 cells (MOI 0.1; MOI 1.0). At specified time points (24, 48, and 72 h), fixed cells were stained with protocol from In-Situ Cell Death Detection Kit (Sigma). Images were obtained for YFP, TUNEL, and DAPI at 524, 614, and 465 nm excitation. For illustration, merged channels were generated by the ImageJ software.

## 2.7 | Real-time quantitative PCR

Taqman-based qPCR detection methodology is specified in more detail under supplementary methods. In short, carcinoma cells were infected with MOI 0.1, 1.0, 5.0, or control medium (mock). Total RNA was extracted 24 h after infection (2  $\mu$ g) and reverse transcribed (High-Capacity cDNA Reverse Transcription Kit; Fisher Scientific). Pre-defined reaction mix for target and housekeeping genes were incubated in reaction cycler which considered a 10 min heating step and 40 cycles for 10 s denaturation at 95°C and 30 s for annealing and elongation. HPRT-1 was used to normalize the obtained data and negative controls were included. The cycle threshold (Ct) values of the genes of interest were normalized to the cells' corresponding Ct value for HPRT-1. Established, pre-designed primers (Fisher Scientific) were used for IL-15 (ID Mm04336046\_m1), IL-2 (ID Mm00434256\_m1), A34R (ID Mm02344630\_s1). Results are presented in fold change using the formula:  $2^{(-\Delta Ct)}$ , where  $\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{HPRT-1})$ .

## 2.8 | Immunoblotting

Following 24 h infection of carcinoma cells (MOIs 0.1, 1.0, 5), RIPA-generated cell lysates were collected and centrifuged. Calculated amount of protein (20  $\mu$ g) was denatured (100°C for 5 min) and separated by electrophoresis (SDS-PAGE) and subsequently transferred onto nitrocellulose membrane. Membrane was blocked in 5% defatted dried milk and stained with anti-Bad monoclonal antibody (Cell Signaling Technology/clone: D24A9), anti-P53 monoclonal antibody, Anti-Bcl-2 monoclonal antibody (Cell Signaling Technology/clone: 54H6) and Anti-GAPDH monoclonal antibody (Abcam/clone: 6C5) overnight at 4°C in TBS-T. Following the secondary HRP reaction, antibodies were detected by chemoluminescence using Biorad Chemidoc imaging device.

## 2.9 | ATP assay

Carcinoma cells were infected with mock or vvDD (MOIs 0.1, 1.0, 5). Supernatant was collected at 48 h added into a 96-well luminescence plate. Standard reaction solution (specified in supplementary methods) was added to each well and bioluminescence detected by SpectraMax i3x (Molecular Devices).

## 2.10 | Multiplex immunoassay

Mock and infected (vvDD, vvDD-IL2, and vvDD-IL15) tumor cells (PANC-1; BxPC3) were co-cultured with NK-92 cells at E/T ratios of 0.5:1 for 24 and 48 h. Supernatant was collected after centrifugation and sterile filtration using a 0.2  $\mu$ m filter. Perforin, granzyme B, and TRAIL were measured in cell supernatants using the human ProcartaPlex Mix&Match (PPX-05-MX47X7V) following the manufacturer's recommendations. Data were acquired with the Luminex100 machine and BioPlex Manager 6.1 software. Standard curves were fitted using the logistic-5PL regression. The minimal detectable concentrations were: Hu granzyme B (13 pg./mL), hu Perforin (86 pg./mL), and hu TRAIL (1.5 pg./mL).

## 2.11 | Proteomics

For proteomic profiling by mass spectrometry, PANC-1 cells were infected with virus constructs at MOI 0.1 for 24 h. Cell lysates were treated with phosphatase inhibitor cocktail (Sigma Aldrich, 1:100), reduced with 10 mM DTT (dithiothreitol, Sigma), and alkylated with 40 mM CAA (2-chloroacetamide, Sigma). After treatment with Benzonase® (Merck, 50 units) for 30 min at 37°C, the supernatant was collected and cleaned. Processed peptides were labeled with 16-plex tandem mass tag (Fisher Scientific) reagents. Samples were desalted and fractionated by high-pH reversed-phase off-line chromatography (1290 Infinity, Agilent). Peptides were reconstituted in 3% acetonitrile with 0.1% formic acid and separated on a High-Performance Liquid



Chromatography system (Fisher Scientific). 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). Reporter ion MS2 for TMT16 was selected (internal and N-terminal) and TMT batch-specific correction factors were specified. The false discovery rate (FDR) was set to 5%. Unique and razor peptides were included for quantification. More detailed information is provided under supplementary methods.

## 2.12 | Statistics

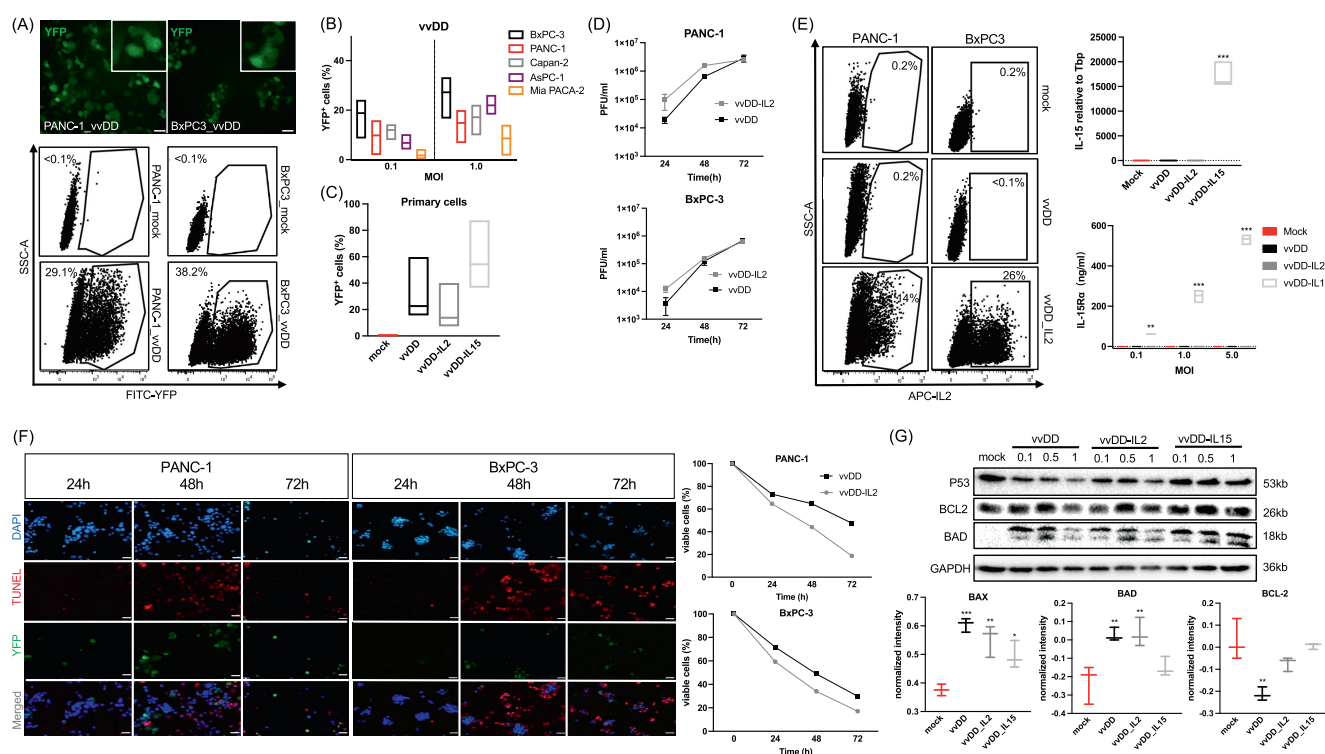
GraphPad Prism version 9 (GraphPad Software, San Diego, CA) was used for creating graphs and analyzing data from in vitro experiments. The data analysis was performed using non-parametric Student's *t*-test or two-way ANOVA. *p*-values of <0.05 were considered statistically significant. The symbols used in figures were standard ones: \**p* < .05; \*\**p* < .01; \*\*\**p* < .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 by Student's test. Signals passing the significance cut-off of FDR 5% were considered differentially expressed.

## 3 | RESULTS

### 3.1 | Oncolysis of human PDAC cells via modified vaccinia virus with enhanced tumor selectivity

Upon exposure with the OV vvDD, neoplastic cells with enhanced thymidine kinase activity are selectively targeted via altered endogenous signaling pathways (e.g., EGFR/Ras/MAPK; type-I IFN).<sup>28</sup> We sought to explore infection efficiency and replication of modified (vvDD) and cytokine-armed (vvDD-IL2 and vvDD-IL15) vaccinia virus strains both, in human PDAC cell lines and primary patient tissue cultures. Infection at low MOIs demonstrated virus entry with transcription indicated by emitted intracellular fluorophore signals (YFP<sup>+</sup>) (Figure 1A). Screening of PDAC cell lines revealed moderate efficiencies of tumor cell infection across samples (26.2%, STD ±8.7 p.p.),



**FIGURE 1** Oncolytic vvDD viruses infect PDAC cells and induce expression of YFP, cytokine transgenes and apoptosis. (A) Fluorescent YFP signals in PANC-1 and BxPC-3 cells of intracellular vaccinia virus following 24 h infection at MOI1. Scale bar = 10  $\mu$ m. (B) YFP signals (flow cytometry) of PDAC cell lines following vvDD infection. Replicate data presented as mean, min/max. (C) At same conditions, patient-derived PDAC cell cultures (*n* = 6) are efficiently infected with modified Western-reserve (vvDD) and cytokine-armed vaccinia virus (vvDD-IL2 and vvDD-IL15). Replicate data presented as mean, min/max. (D) Plaque titration assay shows a time-dependent increase in virus titer following infection of PANC-1 and BxPC-3 at 24, 48, and 72 h. (E) The expression of GPI (rigid peptide linker) anchored IL2 was measured via flow cytometry, while IL15 was detected in the supernatant by ELISA. Replicate data presented as mean, min/max. (F) TUNEL assay indicated apoptosis of infected PANC-1 and BxPC-3 cells (MOI1) that peaked at 48 h post-infection. In parallel the viability of carcinoma cells decreased time dependent as shown by MTS-assay. Scale bars = 50  $\mu$ m. (G) Western blot analyses shows increased expression of pro-apoptotic BCL-2 and BAD and down-regulation of cell cycle checkpoint P53 (top). Proteomic analyses confirmed these observations (below).

with higher rates in BxPC-3 and PANC-1 cells (Figure 1B, Suppl. Figure 1). Moreover, primary PDAC patient samples showed higher infection rates (36.7%, STD  $\pm 20.6$  p.p.), suggesting clinical applicability of these constructs (Figure 1C, Table 1). The vaccinia virus strains used retained their viability and reproducibility as deduced from virus quantification post-infection. We detected increasing viral loads over time (Figure 1D). Larger cytokine-armed constructs did not impede the infection efficiency and were able to express their respective transgenes as surface proteins (IL2) or as soluble cytokine (IL15) (Figure 1E). Vaccinia virus infection resulted in increased cell death, known to be triggered via oncolysis-related pathways. In fact, we observed apoptosis in vaccinia virus-infected cells peaking at 48 h post-infection (Figure 1F). When investigating the molecular mechanism of virally induced cell death, we identified apoptosis-related proteins to be highly regulated. For instance, protein expression levels of BAX and BAD were up-regulated, while BCL-2, MLKL, and RIPK1 were down-regulated following virus infection (Figure 1G, Suppl. Figure 2). Overall, PDAC cells appear amenable to vaccinia virus infection and oncolysis by inducing apoptosis in the cells.

### 3.2 | Cellular re-programming of virus-infected PDAC cells in core signaling pathways

To explore the intracellular changes induced by vaccinia virus infection in PDAC cells, we characterized the (phospho-)proteome of mock, vvDD, vvDD-IL2, and vvDD-IL15 infected PANC-1 cells, covering 8669 proteins and 29,428 high confident (class 1) phosphosites. In addition to human metabolites, viral proteins required for entry, protein transcription, cytoplasmatic replication, and encapsidation (e.g., A28, A21, H2, A20, D4, B1, A13, A32, and I6) were detected in the human host cells 24 h post-infection (Figure 2A, Suppl. Table 1).

Likewise, important molecules responsible for effective infection and oncolysis, such as A34R were up-regulated (Suppl. Figure 3). PCA analyses show differential expression in the global protein and phospho-proteome for vvDD, vvDD-IL2, and vvDD-IL15 virus-infected PANC-1 cells when compared to mock (Figure 2B). Differentially expressed proteins are involved in proliferation/apoptosis (DNM1L and IGF2BP1), immunogenicity (HLA-G), and transcriptional regulation (MEF2C, KIF2C, and CNBP) (Figure 2C). We next explored default core

signaling pathways regularly affected in PDAC cells in response to their genomic driver gene alterations (Cell cycle, MAPK-ERK, mTOR, TGF- $\beta$ , and Wnt/Notch signaling). Altogether 12 proteins involved in the cell cycle/apoptosis, 20 proteins in MAPK-ERK, 16 proteins in mTOR, 10 proteins in TGF- $\beta$ , and 27 proteins in Wnt-related pathways (KEGG annotated) were selected from whole proteome supervised clustering. In proteins related to cell cycle regulation, 5 (41.6%) proteins were found up-regulated, including BAX and BAD, key regulators of apoptosis, while 7 (58.4%) proteins were found downregulated in vvDD and vvDD-IL2 treated cells (Figure 2D). Interestingly, relevant gene products of genes involved in cell cycle regulation (*TP53*, *CDK2*, and *CDK4*) were downregulated. This observation is in line with previous studies that vaccinia virus controls the level of P53 through MDM2 activation.<sup>29</sup> While 7 (35%) proteins related to MAPK-ERK signaling (*HRAS*, *KRAS*, and *MAP2K1*) showed increased expression in wildtype vaccinia virus-treated cells, the majority of components (65%) showed reduced expression after virus treatment, including key regulators (*NFKB1*, *SOS1*, *MAPK1*, and *EGFR*) (Figure 2E). Still, specific members of the Ras family (*EGFR*, *KRAS*, *HRAS*, and *NRAS*) showed increased expression after vvDD or vvDD-IL2 treatment but not with vvDD-IL15, underscoring possible cytokine-mediated downstream effects. Signaling pathways, involved in metabolism (mTOR), invasion (TGF- $\beta$ ), and stromal activation (Wnt/Notch) were all downregulated following vvDD infection pointing to an anti-proliferative and pro-apoptotic effect of vaccinia virus treatment (Suppl. Figure 4).

In summary, vaccinia virus infection induces cellular re-programming and changes major signaling pathways in PDAC cells in a way that can have a relevant therapeutic, anti-tumorigenic effect on PDAC cells.

### 3.3 | Induction of immunogenicity via vaccinia virus treatment

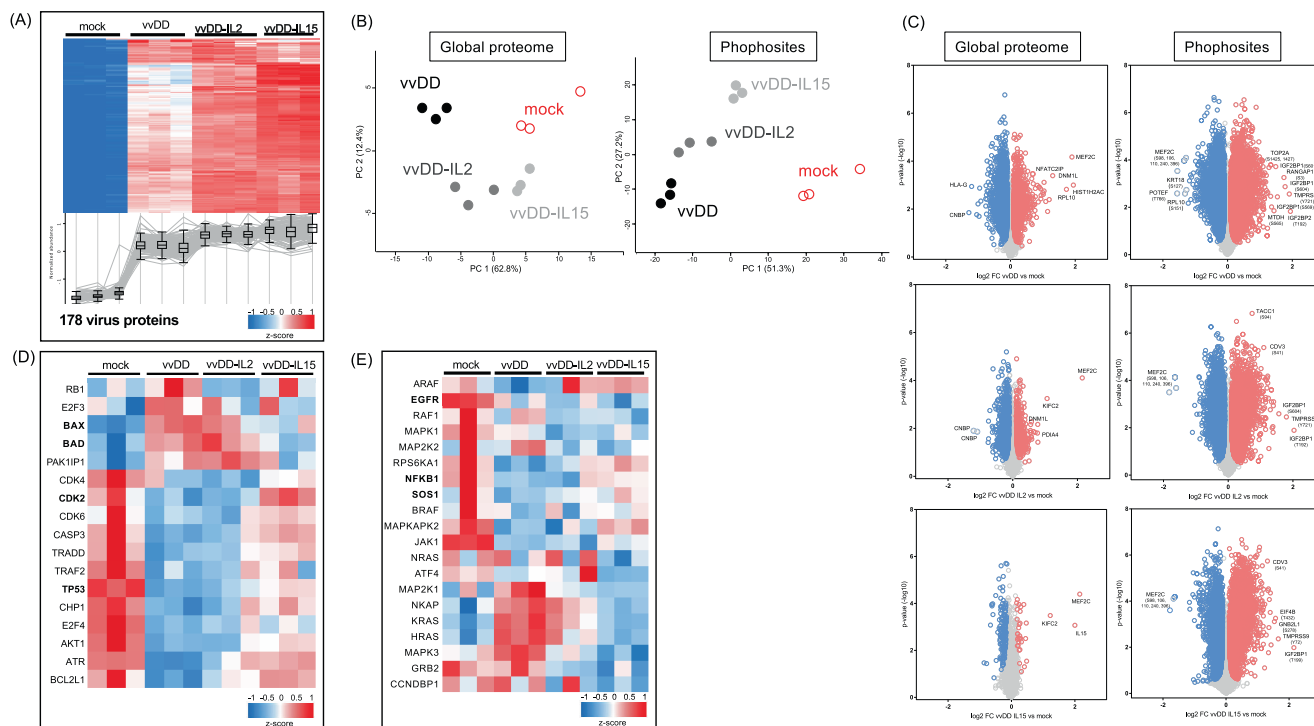
Induction of immunogenicity has been suggested to be a major therapeutic effect of OV. This property is of particular importance in PDAC, where the TIME has been described as “cold” and dominated by suppressive signals from resident immunomodulatory myeloid and Treg cells.<sup>30</sup> We examined molecules on carcinoma cells involved in the cross-talk with the TIME. First, we investigated the expression of MHC-I and MIC-A/B molecules on the surface of infected PDAC cell

**TABLE 1** Patient demographics of infected primary cells from pancreatic duct adenocarcinoma.

Identifier	Histopathology	TNM <sup>a</sup>	Neoadjuvant therapy	Passage number	Infection efficiency <sup>b</sup>
P123	Ductal adenocarcinoma	T3N0L0V0Pn1G2	No	2	33.3 ( $\pm$ 23.9)
P130	Ductal adenocarcinoma	T2N1L0V0Pn1G3	No	4	24.4 ( $\pm$ 18.1)
P167	Ductal adenocarcinoma	T2N2L0V0Pn1G3	No	2	53.0 ( $\pm$ 23.8)
P170	Ductal adenocarcinoma	T3N1L1V1Pn1G3	No	3	62.5 ( $\pm$ 23.9)
P182	Ductal adenocarcinoma	T3N1L0V0Pn1G3	No	2	19.8 ( $\pm$ 15.1)
P185	Ductal adenocarcinoma	T1N1L0V0Pn1G2	No	2	26.9 ( $\pm$ 19.0)

<sup>a</sup>8th edition AJCC classification.

<sup>b</sup>Average infection efficiency (% YFP+ cells) of vvDD at MOI1 in three replicate experiments.



**FIGURE 2** Proteomic configuration of pancreatic carcinoma cells following modified and cytokine-armed oncolytic virus infection (A) Proteomic analyses of 178 viral proteins in infected Panc-1 cells presented as cluster blot with triplicate z-score values, demonstrating the intracellular active viral machinery for protein transcription, cytoplasmatic replication and encapsidation. (B) Whole proteome (left) and phosphosite PCA analyses on infected Panc-1 cells (triplicate) with vvDD, vvDD-IL2, vvDD-IL15 compared to mock. (C) Volcano blot of regulated proteins (left) and phosphosites (right) in vvDD, vvDD-IL2 and vvDD-IL15 infected PANC-1 cells. (D) Cluster blot with triplicate z-score values of proteins involved in cell cycle regulation/apoptosis. (E) Cluster blot with triplicate z-score values on proteins involved in MAPK-ERK signaling.

lines as compared to mock (Figure 3A). We observed down-regulation of MHC-I and MIC-A/B in BxPC-3 cells, but not in other PDAC cell lines. Next, we assessed cell surface expression following the infection of primary PDAC cells from six patients but found stable MHC-I and MIC-A/B expression across samples (Figure 3B). When the antigen-presenting/processing machinery (APM) was analyzed in more detail, we found up-regulation of major regulators including TAP1, TAP2, Calnexin, and Calreticulin on proteomic levels in vvDD and vvDD-IL2 infected PANC-1 cells (Figure 3C). Still, proteins for antigen presentation (HLA-B/-G/-E) were downregulated in infected cells which may reflect an autophagy-dependent mechanism of MHC-I regulation in PDAC.<sup>31</sup> Interestingly, PDAC cells infected with vvDD-IL15 were configured differently and may predict adverse downstream effects with enhanced sensitivity toward NK cells.

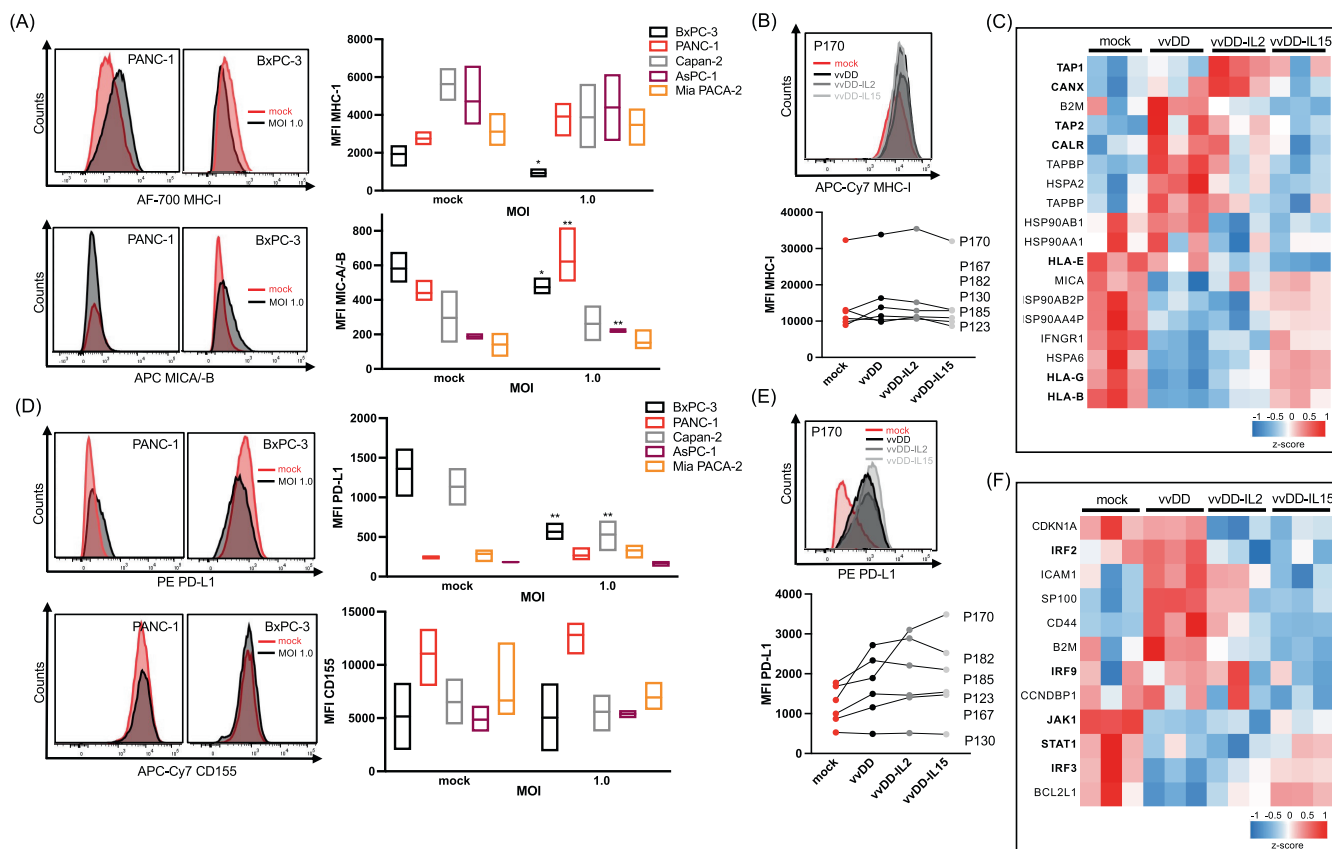
Another important mechanism for immune evasion of carcinoma cells is the involvement of CD155 and an active PD-1/PD-L1 axis. While the latter represents an attractive target for therapeutic intervention, CD155 is thought to be more important for NK cell-mediated regulation via engagement of activating (DNAM-1) or inhibitory (TIGIT) signals.<sup>32</sup> We explored the role of PD-L1 expression following vaccinia virus infection and characterized its expression on the surface of PDAC cell lines. Only BxPC-3 and Capan-2 showed detectable PD-L1 expression which was downregulated after vaccinia virus infection (Figure 3D). By contrast, PD-L1 expression was more readily observed in primary tissue samples of PDAC patients. Infection with

vvDD, vvDD-IL2, and vvDD-IL15 up-regulated the surface expression of PD-L1 in 5 of 6 patients, suggesting their promising role as adjuvant in individual patients for more efficient immune checkpoint blockade (Figure 3E). The expression of CD155, on the other hand, was not affected by vvDD infection which may explain why DNAM-1 and NKG2D were both downregulated, likely not being engaged in this process (Suppl. Figure 5). Additionally, we assessed the overall effect on pathways related to the immunogenicity that are induced by interferon-gamma (IFN- $\gamma$ ) signaling. We identified 12 crucial genes related to the IFN- $\gamma$  pathway with significant changes in expression compared to mock (Figure 3F). Key gene products (JAK1, STAT1, and IRF3) were downregulated post-infection, while phosphorylation of downstream effectors (pSTAT1 and pSTAT3) was increased in vvDD-treated cells (Suppl. Figure 6).

Together, these findings suggest that wildtype- and cytokine-armed vaccinia viruses have a direct impact on the immune reactivity of tumor cells through changes in antigen presentation, checkpoint regulation, and pathways regulating immune responsiveness.

### 3.4 | NK cell activation and cytotoxicity are induced upon vaccinia virus infection

In line with previous observations, we identified MHC-I depletion as a putative mechanism by which the modified vaccinia virus with

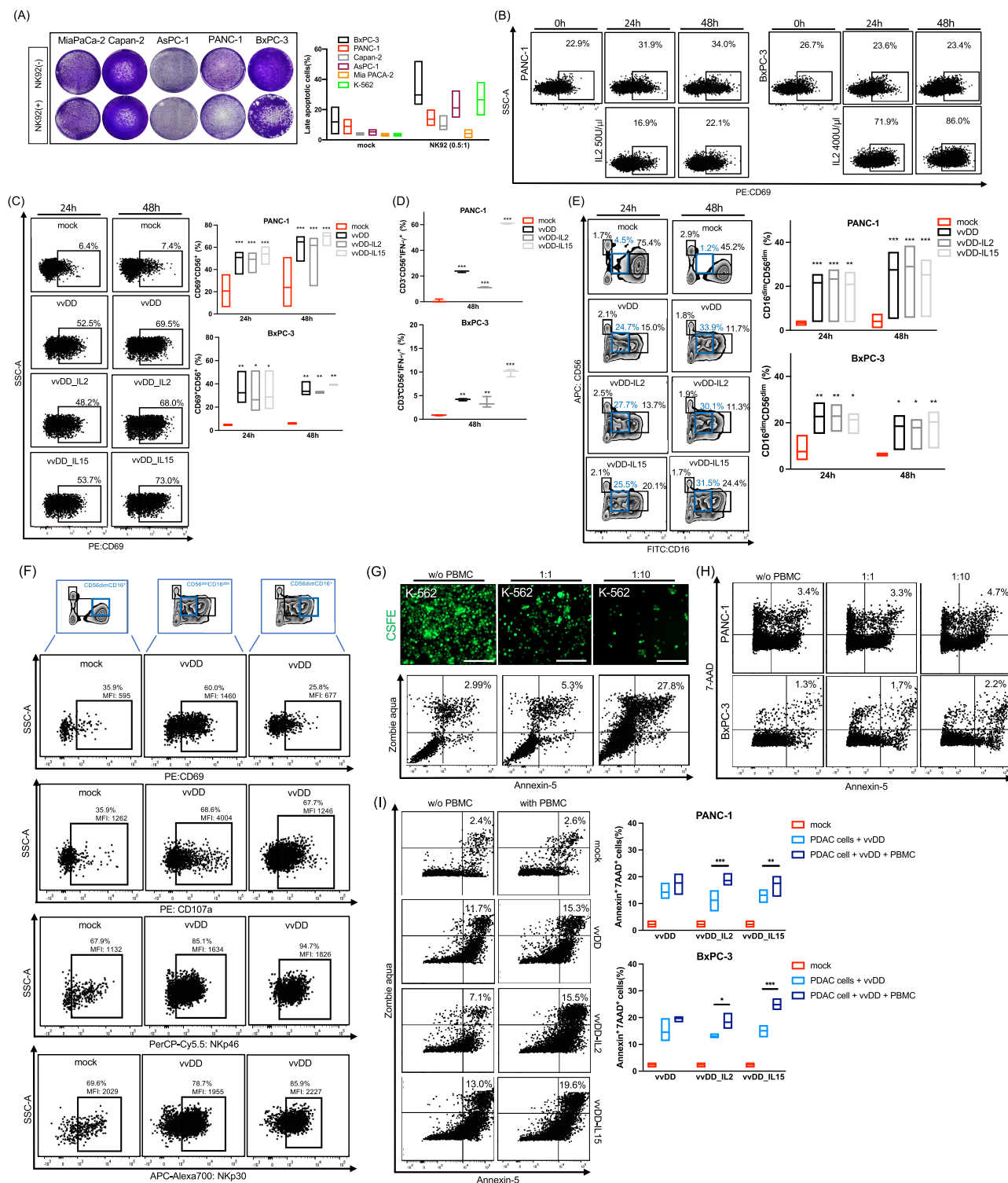


**FIGURE 3** Virus infection changes MHC-I expression and other proteins involved in immune regulation and immunogenicity. (A) Surface expression of MHC-I and MICA/B in mock and vDD infected cells was negatively regulated solely in BxPC-3 cells. (B) Surface expression in primary PDAC cells show stable MHC-I expression in primary, patient derived PDAC cells. (C) Cluster blot with triplicate z-score values of proteins involved in the antigen presenting machinery (APM). (D) Surface expression of PD-L1 and CD155 across PDAC cell lines. (E) Up-regulation of PD-L1 in selected, vDD infected primary PDAC cells. (F) Cluster blot with triplicate z-score values of proteins involved in IFN- $\gamma$  signaling.

enhanced tumor selectivity can evade immune cell recognition.<sup>33</sup> Subsequently we explored whether the missing self-concept might contribute to NK cell activation by infected PDAC cells. Screening of available PDAC cell lines in co-culture with the NK92 cell line identified PANC-1 and BxPC3 cells to be sensitized toward NK cell-mediated cytotoxicity inducing a high degree of dead or late apoptotic cells (Figure 4A, Suppl. Figure 7). In the co-culture setting of tumor cells with human donor PBMCs, we examined the activation of CD56<sup>+</sup> NK cells by analyzing their profile of relevant activation markers (CD16; CD69). NK cells cultured with non-infected tumor cells (native) showed negligible induction of cytotoxic effectors (CD56<sup>dim</sup>CD16<sup>+</sup>) and little upregulation of the activation marker CD69 when compared to positive controls (Figure 4B). However, the infection with OV (vDD, vDD-IL2, and vDD-IL15) resulted in increased activation upon co-culture (Figure 4C). This also resulted in an increased IFN- $\gamma$  secreting NK cell population likely mediating other downstream cytotoxic effector functions, particularly pronounced after vDD-IL15 infection (Figure 4D). During co-culture, a shift toward a population with intermediary CD56<sup>dim</sup>CD16<sup>dim</sup> expression profile appeared while the fraction of classic CD56<sup>dim</sup>CD16<sup>+</sup> cytotoxic NK cell effectors was reduced (Figure 4E). This specific NK

cell sub-population showed a high activation with an enhanced cytolytic potential as indicated by the increased frequency of CD69<sup>+</sup> cells and enhanced CD107a expression (Figure 4F, Suppl. Figure 8). Previous data has shown that poxviral hemagglutinins (HA) can be responsible for increased activation and cytotoxicity of NK cells after exposure to OV.<sup>34</sup> Indeed, we observed increased expression of the HA binding receptors Nkp46 and Nkp30 on CD56<sup>dim</sup>CD16<sup>dim</sup> NK cell subset when exposed to vaccinia virus-infected cells.

In order to assess functionality of our healthy donor-derived PBMCs, they were co-cultured with CFSE labeled myelogenous K-562 cell line. We confirmed increased NK cell-mediated killing of K-562 cells, which are prototypic NK cell targets due to their low to negative expression of MHC class I (Figure 4G). Next, we examined the cytotoxic effect of human donor NK cells from PBMCs on PDAC cells. In contrast to NK cell lines (Figure 4A), we could not observe any induction of early- (7-AAD<sup>-</sup>/Annexin<sup>+</sup>) or late apoptosis (7-AAD<sup>+</sup>/Annexin<sup>+</sup>) in the absence of virus infection (Figure 4H). However, after infection with vaccinia virus constructs, the cytotoxic effect during co-culture with PBMCs/NK cell line exceeded that of virus exposure alone, indicating increased NK cell reactivity (Figure 4I).



**FIGURE 4** Induction of NK cell mediated cytotoxicity following cytokine-armed vaccinia virus. (A) Screening of PDAC cell lines stained with crystal violet following 48 h co-culture with NK92 revealed BxPC3 and PANC-1 cells as sensitive candidates for NK cell mediated cytotoxicity. (B) Co-culture with human PBMC indicated high tolerance with little if any activation of NK cells (CD69<sup>+</sup> among gated CD3<sup>+</sup>CD56<sup>+</sup> human NK cells). (C) vvDD infected cells induced NK cell activation as evidenced by shift toward CD69<sup>+</sup> expression. (D) Co-culture of infected tumor cells with human PBMCs revealed induced expression of INF- $\gamma$  in CD3<sup>+</sup>CD56<sup>+</sup> NK cells. (E) A previously unappreciated immature NK cell population with presence of CD56<sup>dim</sup>CD16<sup>dim</sup> marker expression develops after 48 h co-culture with infected tumor cells. (F) The CD56<sup>dim</sup>CD16<sup>dim</sup> subset was characterized by high fraction of CD69<sup>+</sup>, CD107a<sup>+</sup>, NKp46<sup>+</sup> and NKp30<sup>+</sup> cells when compared to CD56<sup>dim</sup>CD16<sup>+</sup>. (G) Dose dependent CFSE labeled K-562 suspension cells with a large fraction of late-apoptotic cells after 48 h. Scale bar = 100  $\mu$ m. (H) Cytotoxicity assay using untreated carcinoma cells (BxPC-3 and PANC-1) co-cultured with or without PBMC after 48 h. (I) Cytotoxicity assay using infected carcinoma cells (BxPC-3 and PANC-1) in co-culture with PBMC, compared to carcinoma cells without PBMC and mock.



Overall, this data informed on the effect of vaccinia virus treatment which enhanced the cytotoxicity of NK cells toward PDAC cells.

### 3.5 | Mediators of NK cell-dependent cytotoxicity following vaccinia virus infection

The cytolytic function of NK cells upon recognition of foreign or diseased cells is typically mediated through degranulation releasing effector proteins such as perforin and granzyme B. We detected perforin in CD3<sup>+</sup>CD56<sup>+</sup> NK cells with moderate reduction after co-culture with canonical NK-sensitive tumor cell line K-562 over time. However, the reduction in perforin was less when exposed to pancreatic carcinoma cells indicating a tolerance of PDAC cells toward lytic granule-mediated NK cell killing (Figure 5A). While observing enhanced cytotoxicity following vaccinia virus infection (vvDD, vvDD-IL2, and vvDD-IL15), this was not accompanied by increased release of granzyme B or perforin into the supernatant or exhaustion of cytotoxic granules in NK cells (Figure 5B, Suppl. Figure 9). Besides the release of cytotoxic molecules, NK cells may also kill through engagement of their surface markers FasL or TRAIL with cognate ligands on the tumor cells. Indeed, FasL but not TRAIL was up-regulated on NK cells following co-culture with vaccinia

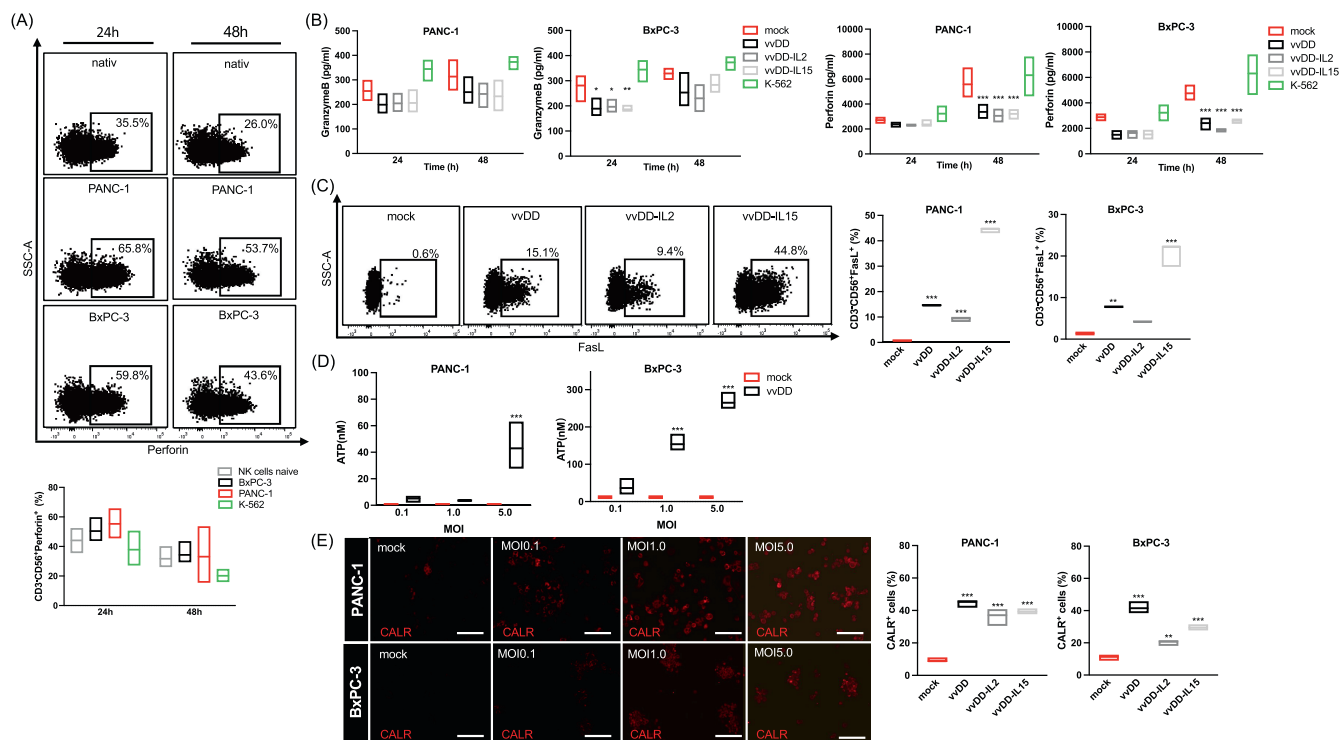
virus-infected PANC-1 or BxPC-3 cells (Figure 5C, Suppl. Figure 10). This effect was particularly prominent in vvDD-IL15 infected cells and may explain increased cytotoxicity in this setting.

To assess the degree to which vaccinia virus strains induced ICD in PDAC cells, we measured the membrane-exposed calreticulin on tumor cells as well as ATP in the supernatant of vaccinia virus-infected PANC-1 and BxPC3 cells. We observed that ATP was released in a dose-dependent manner after vvDD infection but not after mock treatment (Figure 5D). Similarly, the endoplasmic reticulum chaperone calreticulin was increasingly identified on the tumor cell surface with ascending viral dosage (Figure 5E). Together, the results reveal the capacity of vaccinia virus treatment to induce ICD and death ligands may contribute to enhanced cytotoxicity toward infected cells.

We were able to characterize potential mechanisms on how human NK cells can be attracted and activated toward carcinoma cells that were infected with cytokine-armed OV.

## 4 | DISCUSSION

NK cells together with effector T-cells can become protagonists in the TIME and may then, at least under refined experimental conditions,



**FIGURE 5** FasL but not perforin up-regulation correlates with NK cell mediated cytotoxicity following oncolysis. (A) Intracellular perforin granules from PBMC derived NK cells following 24 and 48 h co-culture with PANC-1, BxPC-3, and K-562 cells. (B) Granzyme B and perforin release from NK cells measured from supernatant via Luminex assay following co-culture with mock or vvDD treated carcinoma cell lines. (C) Co-culture of NK cells with vvDD infected tumor cells induces FasL expression on NK cells. (D) Infected carcinoma cells (vvDD infected) release ATP into supernatant in dose-dependent manner. (E) Infected carcinoma cells (vvDD, vvDD-IL2, and vvDD-IL15 infected) transport calreticulin to the cell surface with dose-dependence. 20 $\times$  magnification, scale bar = 100  $\mu$ m. On the right shown the fraction of calreticulin surface expression of infected carcinoma cells (vvDD, vvDD-IL2, and vvDD-IL15 infected) by FACS analysis.

effectively contain the local tumor spread in PDAC.<sup>35</sup> They can act directly on carcinoma cells through cytolytic or apoptosis-inducing mechanisms and orchestrate immunogenicity via the release of cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) and chemokines (CCL3 and CCL5).<sup>23,36</sup> PDAC is characterized by low infiltration of effector T-cells and NK cells, likely due to lack of signals for chemoattraction, and reduced MHC-I restricted antigen recognition on cancer cells, which renders it a poor target for T-effector cells.<sup>31,37</sup> In our co-culture experiments, we observed low activity of human NK cells toward PDAC cell lines, when compared to positive controls (IL-2-induced NK cells, NK cells in co-culture with K-562). Only the potent NK92 cell line was able to induce moderate apoptosis upon co-culture with BxPC3 and AsPC-1 cell lines, indicating susceptibility of PDAC cells toward NK cell-mediated killing. In contrast to previous findings, we observed that, with exception of BxPC-3, PDAC cell lines show overall high MHC-I surface expression and negligible PD-L1 expression.<sup>31,38</sup> In primary cells, a distinct picture occurred where heterogeneous PD-L1 expression was observed and up-regulation following infection in selected patient samples was induced (e.g., P123 and P182). MHC-I expression may only be one mechanism accounting for the tolerance of pancreatic carcinoma cells toward the innate immune cell machinery. Distant homologs of MHC-I (stress-inducible antigens), MICA/-B are potent regulators for NK cell-mediated antigen recognition of cells via interaction with NKG2D as ligand.<sup>39</sup> Similarly, CD155 on the tumor cell surface acts as an activating immunomodulator primarily via DNAM-1 receptor interaction or induces NK cell inhibition via interaction with TIGIT and CD96.<sup>40</sup> However, only a minor effect was observed following vaccinia virus infection which may explain the negative feedback loop of activating NKG2D and DNAM-1 receptors that likely responded with downregulation due to lack of engagement upon co-culture with PDAC cells. In any case, the regulation of activating and inhibitory receptors on NK cells may further contribute to the evasion of pancreatic carcinoma from NK cell-mediated killing. Considering the complex interplay of activating and inhibitory receptors as well as antigen recognition in the tumor microenvironment, a greater scale molecular analysis in vivo (in whole tumor tissue) is required for better understanding of the NK cell tolerance in PDAC.

OVs were designed to overcome the innate immune tolerance of solid tumors.<sup>10</sup> We used modified WR vaccinia virus strain (vvDD) that incorporated and expressed immune-activating cytokines IL-2 or IL-15.<sup>16,17</sup> We demonstrated infection of human PDAC cells with moderate efficiency (26.2%, STD  $\pm$ 8.7 p.p.) resulting in intracellular replication and immediate oncolysis. We have observed a tendency of increased incorporation of infectious particles (YFP signals) for the vvDD-IL15 compared to other constructs (vvDD and vvDD-IL2). Since all constructs are based on identical viral backbones, we assume this to be due to increased replication, and cross-infection capacities, while blocking apoptosis mechanisms in response to interaction with carcinoma-specific receptors (e.g., presence of IL15R $\alpha$ ), as has been previously described.<sup>41</sup> The viral infection was repeatedly followed by changes in intracellular signaling pathways of the host cells interfering with important pathways responsible for carcinogenic hallmarks in PDAC but also corrupting cell cycle and inducing apoptosis. On the

other hand, regulatory proteins for viral replication, transcription, and encapsidation were all produced, reprogramming the infected cells toward viral reproduction. Indeed, within 72 h the viral load in the supernatant increased by 10<sup>2</sup>-fold while more than two-thirds of the carcinoma cells were killed during the same time interval. Other studies using the same virus reported similar destructive cascades on cell lines of colon (MC38), ovarian carcinoma (A2780, OVCAR-3, and IGROV-1), or even in primary carcinoma cells from patients.<sup>17,42,43</sup> Together, our data indicate a dual role of vvDD on pancreatic carcinoma cells leading to direct tumor cell killing, but also re-programming of infected cells via pro-apoptotic, anti-proliferative, and anti-invasive molecular signaling in favor of viral replication and encapsidation.

Clonal TP53 alterations and abrogated signaling pathways (MEK-ERK and Wnt) can be expected in >70% across PDAC samples and thus modified vaccinia virus represents a particularly attractive vehicle.<sup>44</sup> After 24 h virus incubation, we observed targeting efficiencies of 26.2% ( $\pm$ 8.7 p.p.) across carcinoma cell lines and 36.7% ( $\pm$ 20.6 p.p.) across primary cells, providing evidence that PDAC cells can be a therapeutic target of cytokine-armed vvDD constructs. Previous studies have addressed potential synergistic effects of oncolysis with chemo- or radiotherapy targeting PDAC.<sup>45,46</sup> Clinical trials with oncolytic vaccinia virus in HCC and malignant melanoma demonstrated direct and indirect (distant from virus-infiltrated tissue) tumor cell killing, suggesting immunological priming may be responsible for systemic treatment effects in those patients.<sup>12,43</sup>

Immune-edited, cytokine-armed vaccinia viruses may deliver additive benefits by providing cytokine-mediated activation of various immune cell subtypes in the local microenvironment of carcinoma cells. We showed effective transgene expression for either construct, vvDD-IL2 and vvDD-IL15. Both cytokines are relevant for activation of NK cells and have been shown to guide and support tumor regression in vivo with few or no cytotoxic side effects.<sup>16,17</sup> Following infection of PDAC cells, and subsequent co-culture, NK cell activation occurred in vitro. Classically, NK cells are subdivided on the basis of their relative expression of CD56 and CD16; blood circulating CD56<sup>bright</sup>CD16<sup>dim</sup> NK cells are viewed as cytokine producers, while the larger fraction of the CD56<sup>dim</sup>CD16<sup>+</sup> subset are considered cytotoxic effectors.<sup>22</sup> Interestingly, we observed a shift of NK cells toward an "immature" intermediary state that is CD56<sup>dim</sup>CD16<sup>dim</sup>; a yet less appreciated NK cell sub-population that is poorly characterized but recently gained attention in a study with blood from immune deficient (HIV) and healthy donors.<sup>47,48</sup> Our data suggest that this population may be an important effector sub-population responsible for enhanced tumor cell killing following vvDD infection. It displayed a particularly large proportion of activated CD69<sup>+</sup> NK cells and showed enhanced capacity for degranulation as indicated by induced CD107a surface expression. Previous studies demonstrated that the interaction of viral hemagglutinins with neutral cytotoxicity receptors (e.g., NKp46 and NKp30) in the setting of vaccinia virus infection may increase NK cell reactivity and susceptibility for tumor cell lysis.<sup>34</sup> In line with this study, we observed a relevant up-regulation of the NKp46 and NKp30 receptors especially in the CD56<sup>dim</sup>CD16<sup>dim</sup> cells following vvDD infection, providing support to the assumption that this specific subtype for NK cell mediates

tumor cell killing. The downstream effects of this cytolytic activity were observed following 24 h co-culture with infected pancreatic carcinoma cells. On average 17.9%/20.9% of the infected PDAC cells (PANC-1/BxPC-3 cells) were apoptotic when co-cultured with PBMCs versus 12.8%/14.2% without ( $p < .001$ ). This effect was more pronounced when tumor cells were infected with cytokine-armed vvDD suggesting stronger activation of NK cells via exposure toward membrane-bound IL2 and soluble IL15.

NK cells are a critical factor in the innate immune response. Via surveillance of the tissue microenvironment, they react to danger signals released from apoptotic or dying cells (danger-associated molecular patterns).<sup>26</sup> Upon recognition of infected cells, they release cytolytic granules (perforin and granzyme B) or engage death receptors that initiate apoptotic signals.<sup>23</sup> We propose that cellular infection with OV5 causes release of intracellular components such as HMGB1 and ATP that can elicit intratumoral immunogenicity via ICD.<sup>10,26,44</sup> This has been demonstrated to occur following infection with modified vaccinia virus strains in other tumor entities.<sup>11,49</sup> With increasing MOI, we measured increasing concentrations of ATP in the supernatant. In addition, surface expression of calreticulin on carcinoma cell lines was induced upon infection. Thus, modified VV constructs likely provoke intracellular changes inducing ICD which in turn can attract NK cells via the release of danger signals. We propose that this pathway can be therapeutically used for effective immune modulation in pancreatic carcinoma that is characterized by an immune excluded TIME. We observed that perforin in co-cultured PBMC was reduced over time indicating granule release associated with cell killing, while the death receptor FasL was up-regulated. This may imply a switch toward death-receptor mediating killing pathways as has been proposed in previous studies.<sup>50</sup>

Our study was designed specifically to address and investigate NK cell reactivity toward human pancreatic carcinoma cells in the treatment naïve setting and after virus infection with modified (vvDD) and cytokine-armed vaccinia viruses. The data must be interpreted with caution. First, all experimental settings were conducted in vitro while immunological responses in vivo normally include the complex cellular and paracrine interplay of the entire tumor microenvironment (tumor cells, immune cells, stromal cells, and vessels). Especially, the pleiotropic effects of cytokine-armed vaccinia viruses cannot be well assayed in foreseeable manner. Still, in contrast to adaptive immune responses which require the knowledge of cognate T cells, the innate immunoreaction regularly follows an antigen-independent, immediate molecular program that can be closely reconditioned in the experimental, standardized in vitro setting. We found it informative, to expose allogenic human donor PBMCs with native and infected human PDAC cell lines and primary cells, in order to deduce information on the immediate response of co-cultivated NK cells. Future in vivo assessment (animal and human) of NK cell activation via single-cell or spatial analyses may further elucidate the effects of virotherapy on NK cell-mediated cytotoxicity toward carcinoma cells. Second, albeit we observed a less well-documented NK cell population (CD56<sup>dim</sup>CD16<sup>dim</sup>) and suggested its involvement in augmented cytotoxicity, we could not directly trace or identify mechanisms by which this novel NK cell subtype occurred.

Although we have demonstrated the release of components associated with ICD-related molecular pathways which included danger signals normally prompting NK cell reactivity, we lack high-throughput molecular information on NK cell phenotype (activating vs. inhibitory) following the oncolysis of PDAC cells. Cell numbers were overall too small restricting detailed molecular analyses of this subtype. Lastly, we were able to analyze effects of virotherapy in only limited number of pancreatic carcinoma patient samples. Additional data with other tumor entities or specific molecular subtypes of PDAC may provide a broader mechanistic pattern of how modified vaccinia virus shapes the innate immune cell responses.

In summary, we investigated the capacity of wild-type and cytokine-armed vaccinia viruses to stimulate and enhance the human innate immune system upon exposure to pancreatic carcinoma cells. In this study, we collected in vitro data that demonstrated targeting of human PDAC cells by cytokine-armed vaccinia viruses with local release (IL-15) or surface expression (IL-2). The modified WR vaccinia virus strain led to tumor cell killing via oncolysis and modulated the carcinogenic signaling pathways known to be responsible for the PDACs aggressive phenotype. The release of danger signals (ATP and calreticulin) activated neighboring NK cells and permitted more effective tumor cell killing, in addition to the direct oncolysis. Further studies will focus on the analysis of cytotoxic T-cells (CD8<sup>+</sup>) and T-cell homeostasis that can collaborate with NK cells via direct cytotoxic effects on PDAC cells. Immune-editing effects of vvDD will be analyzed in more complex, in vivo immune microenvironments. Our data provides further evidence, that cytokine-armed vaccinia virus can effectively modulate the immune tolerant and regulatory tumor microenvironment toward more effective anti-tumor immune responses in PDAC.

## AUTHOR CONTRIBUTIONS

**Ruonan Wang:** Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft. **Mengwen Hu:** Formal analysis; investigation; methodology; writing – review and editing. **Isis Lozzi:** Formal analysis; investigation; methodology; writing – review and editing. **Cao Zhong Jing Jin:** Formal analysis; investigation; methodology; writing – review and editing. **Dou Ma:** Formal analysis; investigation; methodology; writing – review and editing. **Katrin Splith:** Formal analysis; investigation; methodology; software; writing – review and editing. **Jörg Mengwasser:** Formal analysis; investigation; methodology; writing – review and editing. **Vincent Wolf:** Formal analysis; investigation; methodology; writing – review and editing. **Linda Feldbrügge:** Formal analysis; investigation; methodology; writing – review and editing. **Peter Tang:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Lea Timmermann:** Investigation; writing – review and editing. **Karl Herbert Hillebrandt:** Investigation; writing – review and editing. **Marieluise Kirchner:** Formal analysis; investigation; methodology; resources; software; visualization; writing – review and editing. **Philipp Mertins:** Resources; software. **Georg Hilfenhaus:** Conceptualization; formal analysis; investigation; methodology; writing – review and editing. **Christopher Claudius Maximilian Neumann:** Formal analysis; investigation; methodology; writing – review and editing.

**Thomas Kammertoens:** Conceptualization; data curation; formal analysis; investigation; methodology; supervision; writing – original draft; writing – review and editing. **Johann Pratschke:** Resources; supervision. **Thomas Malinka:** Resources; supervision. **Igor Maximilian Sauer:** Project administration; resources; supervision; writing – review and editing. **Elfriede Noessner:** Conceptualization; data curation; formal analysis; investigation; methodology; resources; supervision; validation; visualization; writing – original draft. **Zong Sheng Guo:** Data curation; formal analysis; investigation; methodology; resources; supervision; writing – original draft. **Matthäus Felsenstein:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing – original draft.

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## CONFLICT OF INTEREST STATEMENT

All listed authors confirmed that there are no competing interests to declare.

## DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article or uploaded as online supplemental information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD048276. Other data that support the findings of this study are available from the corresponding author upon request.

## ETHICS STATEMENT

The study was approved by the institutional ethics committee (Charité—Universitätsmedizin Berlin, EA4/020/21).

## INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

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