IFN-γ treatment protocol for MHC-I<sup>lo</sup>/PD-L1<sup>+</sup> pancreatic tumor cells selectively restores their TAP-mediated presentation competence and CD8 T-cell priming potential

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ABSTRACT

Background Many cancer cells express a major histocompatibility complex class I low/programmed cell death 1 ligand 1 positive (MHC-I<sup>lo</sup>/PD-L1<sup>+</sup>) cell surface profile. For immunotherapy, there is, thus, an urgent need to restore presentation competence of cancer cells with defects in MHC-I processing/presentation combined with immune interventions that tackle the tumor-initiated PD-L1/PD-1 signaling axis. Using pancreatic ductal adenocarcinoma cells (PDACCs) as a model, we here explored if (and how) expression/processing of tumor antigens via transporters associated with antigen processing (TAP) affects priming of CD8 T cells in PD-1/-PD-L1-competent/-deficient mice.

Methods We generated tumor antigen-expressing vectors, immunized TAP-competent/-deficient mice and determined de novo primed CD8 T-cell frequencies by flow cytometry. Similarly, we explored the antigenicity and PD-L1/PD-1 sensitivity of PDACCs versus interferon-γ (IFN-γ)-treated PDACCs in PD-1/PD-L1-competent/-deficient mice. The IFN-γ-induced effects on gene and cell surface expression profiles were determined by microarrays and flow cytometry.

Results We identified two antigens (Cripto-1 and an endogenous leukemia virus-derived gp70) that were expressed in the Endoplasmic Reticulum (ER) of PDACCs and induced CD8 T-cell responses either independent (Cripto-1-K<sub>Δ30</sub> or dependent (gp70-K<sub>Δ15E</sub>) on TAP by DNA immunization. IFN-γ-treatment of PDACCs in vitro upregulated MHC-I- and TAP- but also PD-L1-expression. Mechanistically, PD-L1/PD-1 signaling was superior to the reconstitution of MHC-I presentation competence, as subcutaneously transplanted IFN-γ-treated PDACCs developed tumors in C57BL/6J and PD-L1<sup>-/-</sup> but not in PD-1<sup>-/-</sup> mice. Using PDACCs, irradiated at day 3 post-IFN-γ-treatment or PD-L1 knockout PDACCs as vaccines, we could selectively bypass upregulation of PD-L1, preferentially induce TAP-dependent gp70-K<sub>Δ15E</sub>-specific CD8 T cells associated with a weakened PD-1<sup>-</sup> exhaustion phenotype and reject consecutively injected tumor transplants in C57BL/6J mice.

Conclusions The IFN-γ-treatment protocol is attractive for cell-based immunotherapies, because it restores TAP-dependent antigen processing in cancer cells, facilitates priming of TAP-dependent effector CD8 T-cell responses without additional check point inhibitors and could be combined with genetic vaccines that complement priming of TAP-independent CD8 T cells.

BACKGROUND

CD8 T cells play a crucial role for the control of many human and murine tumors. In an optimal scenario, the immune system specifically detects and eliminates aberrant tumor progenitor cells before they develop into clinically manifested tumors. This implied that tumor cells per se are immunogenic and can present tumor-associated antigens (TAAs) to the immune system in a manner that allows priming of effector CD8 T-cell responses. However, many tumors developed mechanisms to escape from recognition and elimination by CD8 T cells. Downregulation or loss of molecules involved in MHC-I processing/presentation was evident in many primary human and murine cancer cells, thereby limiting their presentation competence to CD8 T cells. Furthermore, PD-L1 expression on tumor cells could function as a molecular shield and directly silence effector T-cell responses through coinhibitory PD-L1/PD-1 interactions. On the other hand, there was evidence that interferon-γ (IFN-γ) producing CD8 T cells could upregulate PD-L1 on tumors, indicating that a gradual increase in immune-suppressive PD-L1 signaling and/or the efficacy of anti PD-L1/PD-1 therapies may depend on initial CD8 T cell/tumor cell interactions. However, tumors like pancreatic ductal adenocarcinoma (PDAC) also...
generate among others of PD-L1 expressing myeloid-derived suppressor cells and/or regulatory CD4+Foxp3+ Tregs that may enhance the downregulation of tumor-specific CD8 T-cell responses and/or affect the limited responsiveness of murine and human PDAC to antibody-mediated PD-L1/PD-1 check point inhibition.8–10

The lack of presentation-competent MHC-I molecules on the surface of cancer cells depends on multiple factors, like alterations in MHC-I or beta-2 microglobulin expression and/or in the endogenous antigen processing/presentation machinery, that primarily affect the generation of antigenic peptides in the cytosol by the proteasome complex, or the import of these peptides from the cytosol into the Endoplasmic reticulum (ER) and their loading to nascent MHC-I molecules by transporters associated with antigen processing (TAP).11 In particular, TAP could be a central player for priming tumourspecific CD8 T cells, as some antigens/epitopes (ie, T-cell epitopes associated with impaired peptide processing; TEIPPs) were even exclusively presented under MHC-I presentation-prohibiting conditions in TAP-deficient tumors.11,12 A number of TAP-independent pathways have been described for secretory or cell surface associated antigens that are initially targeted to the ER, for example, antigens that contain NH2-terminal ER-targeting signal peptide (SP) sequences.13–14 SP and transmembrane helices contain a high density of MHC-I epitopes that are generated in the ER and/or in the trans Golgi network by various processing pathways, for example, involving ER-resident signal peptidase (SPase), SP peptidase, furin or ER aminopeptidases.15,16 In particular, expression and processing of self-antigens in the ER could improve their MHC-I presentation efficacy and antigenicity to prime CD8 T cells, at least, when the antigen processing machinery in the ER generates epitopes with free carboxyl (COOH)-termini that do not require further COOH-terminal trimming before loading to MHC-I molecules.14–18 Generation/presentation of these epitopes often was independent of proteasomes and/or TAP.15,16–18 However, antigens expressed in the ER and translocated into the cytosol, for example, by cross-presentation could also be processed/presented in a TAP-dependent manner.16,19

Little is known if (and how) processing of tumor antigens in the ER and/or in TAP-dependent/-independent pathways affects epitope presentation by MHC-I cancer cells and/or priming of effector CD8 T cells.

Here, we analyzed two central points for CD8 T cell-mediated immunotherapies: (1) processing/presentation requirements of TAAs that allow epitope presentation and CD8 T-cell priming, and (2) immune-suppressive PD-L1-signaling requirements of tumor cells that allow expansion and/or maintenance of functional effector CD8 T cells. To determine if TAP-dependent/-independent antigen processing of two tumor antigens/epitopes expressed in the ER of pancreatic ductal adenocarcinoma cells (PDACs) affects priming of CD8 T cells, that is, the Cr-1:Kb/cr16-24 epitope from the embryonic antigen Cripto-1 (Cr-1)21,22 and the gp70:Kb/p15E epitope from an endogenous leukemia virus-derived gp70 glycoprotein,23 we immunized TAP-competent/-deficient mice with antigen-expressing vector DNAs.24 Furthermore, we restored MHC-I presentation competence in PDACCs in vitro by IFN-γ-treatment and primed tumor-specific effector CD8 T-cell responses in PD-L1/PD-L1-competent/-deficient mice by cell-based immunization. Overall, we established a novel IFN-γ-treatment protocol for PDACCs that restored their MHC-I presentation competence, curtailing the coinhibitory PD-L1/PD-1 signaling axis and allowed priming of TAP-dependent effector CD8 T cells.

MATERIALS AND METHODS

Mice

C57BL/6J (B6) mice were obtained from Janvier (Le Genets-St-Ise; France). RAG1−/− (B6.129S7-Rag1tm1Mon/J; Jackson #002216), TAP1−/− (B6.129S2-Tap1tm1Sj/J; Jackson #002944), PD-L1−/− and PD-1−/− mice26 were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University. KPC mice were generated by breeding B6.129S2-Il2rgtm1Jgr/J (Jackson #002109), LSL-Kras(G12D)27 and p48Cre+/+ mice.28

Cell lines

HEK-293 (CRL-1573) and MC38 (CRL-2639) cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, Maryland, USA). The murine KPC tumor cell lines were obtained from PDAC developed in KPC mice.29 Briefly, PDAC were digested with collagenase D, trypsinized and passed through a 40 µm cell strainer (passage 0). Five cell lines were generated from individual mice/tumors, expanded in vitro for 3–4 passages, tested for Kras(G12D/+), p53 and Cre expression by PCR and frozen in liquid nitrogen. Cells from frozen bulk stocks were expanded in vitro for about 4–6 weeks and used in the experiments. All cell lines were tested ‘free of mycoplasma’ (PCR Mycoplasma Test Kit; cat. no. A3744, AppliChem, Darmstadt, Germany).

Construction of expression plasmids and characterization of antigen expression

The antigenic sequences of Cr-1 and gp70 were synthesized (codon optimized) by GeneArt (Regensburg, Germany) and cloned into the pCI vector (cat. no. E1731, Promega, Mannheim, Germany) using the NheI and NotI restriction sites. All antigen modifications were carried out using the Q5 Site-Directed Mutagenesis Kit (cat. no. E0554, NEB, Ipswich, USA). Batches of DNA were produced in Escherichia coli using the Qiagen Plasmid Mega Kit (cat. no. #002944), PD-L1-/-25 and PD-1-/- mice26 were tested for MHC-I presentation competence.

Immunization of mice and tumor models

Mice were immunized intramuscularly into both tibialis anterior muscles with 100 µg/mouse DNA, or transferred...
subcutaneously into the left/right flank with 2.5x10^5 tumor cells (in 100 µL PBS). For cell-based immunizations, tumor cells were pretreated with recombinant mouse IFN-γ (20 ng/mL, cat. no. 554587, BD Biosciences, Heidelberg, Germany) for 16–20 hours, washed and cultured for indicated times before gamma irradiation (30 Gy). Where indicated, anti-PD-1 (cat. no. BP0146; Bio X Cell), anti-CD8 (cat. no. BE0117; Bio X Cell), anti-CD4 (cat. no. BE0119; Bio X Cell) and rat IgG2a or IgG2b isotype control antibodies (cat. no. BP0089, BE0090; Bio X Cell) were injected intraperitoneally (100 µg/mouse). Tumor growth was monitored by regular palpation with calipers. Mice were sacrificed when the tumor diameter reached 1 cm. Determination of antigen-specific CD8+ T-cell frequencies by flow cytometry (FCM) was carried out as described previously.30

**RESULTS**

PDACCs established from highly aggressive tumors in Kras^{G12D/+}/Trp53^{−/−}/p48^{Cre/+} (KPC) mice29 barely express MHC-I molecules on the cell surface (online supplementary figure S1a). We here asked whether tumor antigens expressed in the ER of these PDACCs and processed in TAP-independent/-dependent pathways are preferentially presented to CD8 T cells under these conditions.

**TAP-dependent/-independent priming of CD8 T cells by TAAs**

We initially analyzed the Cr-1 antigen (Cr-1 or TDGF-1; www.uniprot.org/uniprot/P51865) that is expressed during embryonic development in men and mice and re-expressed in KPC-derived PDACCs (online supplementary figure S1b,c) and in about 44% of human PDAC.21 22 This antigen contains a single Cr-1:Kb/cr16-24 epitope located at the extreme COOH-terminus of its ER-targeting SP (SP<sub>Cr-1</sub>),22 which is released during Cr-1 maturation by ER-resident SPase (figure 1A). To determine TAP-dependent/-independent presentation of the Cr-1:Kb/cr16-24 epitope, we used a DNA immunization approach. For this, we generated a Cr-1-encoding vector (pCl/Cr-1; figure 1B) and determined Cr-1 expression in transiently transfected HEK-293 cells. Endoglycosidase H treatment of cell lysates showed that Cr-1 was mainly expressed in an about 20 kDa glycosylated Cr-1 form (figure 1C), confirming an almost quantitative SP<sub>Cr-1</sub>-mediated translocation of Cr-1 into ER/Golgi compartments. A single injection of pCl/Cr-1 efficiently primed Cr-1:Kb/cr16-24-specific CD8 T cells in B6 and TAP-deficient (TAP1-/-) mice, detectable in liver and spleen by ex vivo staining with Cr-1:Kb/cr16-24 dimers or after peptide-specific stimulation and determination of IFN-γ. Only Cr-1:Kb/cr16-24-specific CD8 T-cell frequencies by FCM (figure 1D; groups 2). Priming of Cr-1:Kb/cr16-24-specific CD8 T cells thus did not require TAP-mediated antigen processing.

It has been reported that introduction of a proline (P) immediately following the SP cleavage sites (position +1) generated non-cleavable antigen variants.34 The SP identification program SignalP V. 5.0 (http://www.cbs.dtu.dk/services/SignalP/data.php) predicted a cut at position 26 of the murine SP<sub>Cr-1</sub>, generating an epitope-containing SP<sub>26</sub> fragment (figure 1A). To confirm the crucial role of ER-resident SPase for priming Cr-1:Kb/cr16-24-specific CD8 T cells, we generated a pCl/Cr-1<sub>R26P</sub> vector that contained an amino acid exchange at Cr-1<sub>26</sub> from arginine (R) to proline (P) (figure 1A,B). This vector expressed an about 22 kDa gpCr-1<sub>R26P</sub> glycoprotein that migrated above the gpCr-1 protein band in SDS-PAGE, demonstrating that the SP<sub>Cr-1</sub> SP was not cleaved off in this

Alternatively, differences between two groups were evaluated using the unpaired student’s t-test. The p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****) values were considered significant.

**Gene expression analyses**

Total RNA was isolated from five individual cell lines. The quality was analyzed with a bioanalyzer (Agilent Technologies, Santa Clara, USA). Gene expression analysis was carried out using the SurePrint G3 Mouse Gene Expression 8×60K Microarray (Design ID 028005; Agilent Technologies). Samples were labeled with the Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer’s guidelines. Slides were scanned using a G2565CA microarray scanner (Agilent Technologies). Raw data files were extracted using the Feature Extraction software (v.10.7.3.1, Agilent Technologies). Raw microarray data were background corrected and quantile normalized using the R and Bioconductor package limma (https://www.R-project.org).31 32 After filtering control probes and low expressed probes, differential expression analysis was performed using limma.

**Determination of MHC-I and PD-L1 expression on cancer cells**

Expression of MHC-I (H2-Kb) and PD-L1 on established KPC cell lines and KPC cells isolated ex vivo from subcutaneous tumors was characterized by FCM using the following antibodies: PE Rat anti-mouse H2-Kb (cat. no. 116508, BioLegend), PE Rat anti-mouse CD274 (cat. no. 558091, BD Biosciences) and PE Rat anti-mouse CD11c (cat. no. 12-4321-83, eBioscience). To exclude contaminations with hematopoietic cells in ex vivo isolated tumor cell populations, CD45.2<sup>+</sup> and CD11c<sup>+</sup> cells were electronically gated and excluded. This ensured that histograms reflect H2-Kb<sup>+</sup> and PD-L1<sup>+</sup> levels on tumor cells, satisfying the respective forward/sideward (FSC/SSC) features.33

**Statistics**

Data were analyzed using PRISM software (GraphPad, San Diego, USA). Unless mentioned otherwise, all column scatter plots show respective frequencies±SD. Where indicated, the statistical significance of differences in the mean cell frequencies between multiple groups was determined by analysis of variance (ANOVA) (with 95% CI) followed by Tukey’s multiple comparisons test.
Figure 1  TAP-independent induction of Cr-1:Kb/cr16-24 specific CD8 T cells by DNA immunization. (A) Map of the Cripto-1 (Cr-1) protein. The NH2-terminal signal peptide (SP_{Cr-1}), the epidermal growth factor (EGF)-like domain, the Cripto_Frl-1_Cryptic domain (CFC) and the glycosylphosphatidylinositol anchor (GPI) are indicated. The amino acid sequences of SP_{Cr-1}, SP_{Cr-1/R26P}, the Kb/cr16-24 epitope and the predicted SPase cleavage site are shown. (B) Schematic presentation of pCI/Cr-1, pCI/Cr-1_{R26P} (Cr-1 containing an arginine to proline exchange at position 26) and of pCI/stT77Cr-1 (Cr-1 containing a strep-tag (ST) and Hsp73-capturing SV40 T1-77 (T77) domain). (C) lysates of non-transfected HEK-293 cells and HEK-293 cells transiently transfected with pCI/Cr-1 (lanes 1), pCI/Cr-1_{R26P} (Lane 2) or pCI/stT77Cr-1 (lanes 3) were subjected to SDS-PAGE followed by beta-actin-, Cr-1- or st- specific Western blotting. Where indicated, cell lysates were treated with endoglycosidase H (E-Hf) or remained untreated (E-Hf-) prior to SDS-PAGE. The positions of glycosylated (gp) and non-glycosylated forms of Cr-1 are indicated. (D) B6 mice were immunized with pCI (group 1, n=6, in two independent experiments), pCI/Cr-1 (group 2, n=6, in two independent experiments), pCI/Cr-1_{R26P} (group 3, n=3, one experiment) or pCI/stT77-Cr-1 (group 4, n=3, one experiment) and TAP1-/- mice (n=6, in two independent experiments) were injected with PCI (group 1) or pCI/Cr-1 (group 2). Frequencies of dimer+ Kb/cr16-24 specific CD8 T cells in liver and spleen of immunized mice were determined ex vivo by FCM at day 12 postinjection. Additionally, antigen-specific IFN-γ CD8 T cells were determined by ex vivo stimulation of spleen cells with K0/cr16-24 (closed circles) and control K0/ova257-264 peptides (open circles). Graphs show the actual proportions (%) of K0/cr16-24-dimer or IFN-γ’ cells of total CD8+ T cells±SD for statistical evaluation, indicated groups of mice were compared using one-way ANOVA (with 95% CI) followed by Tukey’s multiple comparisons test (B6) or by the unpaired Student’s t-test (TAP-/-). Only statistically significant differences are indicated (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). ANOVA, analysis of variance; Cr-1, Cripto-1; FCM, flow cytometry; IFN-γ, interferon-γ; SPase, signal peptidase; SP, signal peptide; TAP, transporter associated with antigen processing.
protein (figure 1C, lane 2). As a consequence, we could not induce K\(^{b}/cr_{16-24}\)-specific CD8 T cells in B6 mice by pCI/Cr-1\(\text{R26P}\) immunization (figure 1D, group 3). Expression/process of Cr-1 in the ER of antigen-presenting cells (APCs) targeted by DNA immunization was, thus, a prerequisite to induce Cr-1K\(^{b}/cr_{16-24}\)-specific CD8 T cells. Confirmatory, targeting Cr-1 to the cytosol, using a NH\(_{2}\)-terminal, DnaJ homologous SV40/T\(_{a}\) domain that stably binds cytosolic Hsp73 in transfected cells,\(^{36,37}\) (pCI/stT77Cr-1) (figure 1B,C; online supplementary figure S2) did not induce Cr-1K\(^{b}/cr_{16-24}\)-specific CD8 T cells in vaccinated B6 mice (figure 1D; group 4).

Next, we searched for an epitope-containing tumor antigen that, in contrast to Cr-1, is expressed in the ER of PDACCs but processed for MHC-I presentation and CD8 T cell priming in a TAP-dependent manner. We identified the glycoprotein 70 (gp70), originating from an endogenous murine leukemia virus integrated as a provirus in the mouse germline DNA (www.uniprot.org/uniprot/P03386), which fulfilled these criteria. Gp70 was stably expressed in PDACCs (online supplementary figure S1b,c) but not in somatic cells.\(^{23,30,38}\) In contrast to Cr-1K\(^{b}/cr_{16-24}\), the gp70K\(^{b}/p15E\) epitope is not located in the ER-targeting SP (SP\(_{gp70}\)) (figure 2A) and its processing/presentation apparently depends on proteasomal processing\(^{30}\) and TAP.\(^{38}\) Therefore, this antigen/epitope needs retrotranslocation from the ER to the cytosol and/or ‘cross-presentation’ for efficient MHC-I processing/presentation.\(^{30,38}\) Neither full-length gp70 nor epitope-containing gp70 fragments induced CD8 T cells in H-2\(^{b}\) BALB/c or H-2\(^{b}\) B6 mice.\(^{30,41,42}\) Confirmatory, we here show that an ER-targeted SP\(_{stgp70}\) antigen, composed of a gp70\(_{27-515}\) fragment and a NH\(_{2}\)-terminal heterologous Igx SP sequence (figure 2A,B), did not induce gp70K\(^{b}/p15E\)-specific CD8 T cells in B6 mice (figure 2C; groups 2). In contrast, a cytosolic Hsp73-bound st\(_{27}^{gp70}\) fusion antigen (pCI/stT77gp70) (figure 2A,B; online supplementary figure S2), associated with a high cross-priming capacity,\(^{30,43}\) induced high frequencies of gp70K\(^{b}/p15E\)-specific CD8 T cells in B6 but not in TAP\(^{-/-}\) mice (figure 2C; groups 3). Priming of gp70K\(^{b}/p15E\) and Cr-1K\(^{b}/cr_{16-24}\) specific CD8 T cells thus differed substantially, depending on expression of tumor antigens in the ER versus the cytosol (figures 1 and 2; online supplementary table 1). Furthermore, Cr-1K\(^{b}/cr_{16-24}\) and gp70K\(^{b}/p15E\)-specific CD8 T cells were functional in B6 mice and almost quantitatively and specifically eliminated CFSE-labeled APCs pulsed with Cr-1K\(^{b}/cr_{16-24}\) (online supplementary figure S3) or gp70K\(^{b}/p15E\) peptides\(^{30}\) in vivo cytotoxicity assays. This suggested that priming of these TAA-specific CD8 T cells in B6 mice was not restricted by central and peripheral tolerance mechanisms.

**Manipulation(s) of KPC cells for their usage as cell-based vaccines**

A major aim was to explore whether tumor cell vaccines could be used to prime anti-tumor-specific CD8 T-cell responses. We, thus, asked if MHC-I expression can be restored on KPC tumor cells and affect priming of TAP-independent (Cr-1K\(^{b}/cr_{16-24}\)) or TAP-dependent (gp70K\(^{b}/p15E\)) CD8 T-cell responses, and if tumor cell-initiated immune-suppressive (eg, PD-L1-mediated) mechanisms interfere with these immune responses.

In line with established data, we could upregulate MHC-I expression on different KPC cells by an in vitro treatment with IFN-\(\gamma\)\(^{41}\) (figure 3A). Microarrays performed on untreated and IFN-\(\gamma\)-treated KPC cells identified 291 significant differentially expressed probesets (Benjamini-Hochberg adjusted \(p<0.05\)). Among those probesets, 289 probesets (corresponding to 238 genes) were upregulated and only one gene (two probesets) was downregulated in IFN-\(\gamma\)-treated cells (figure 3B; online supplementary table 2). Unsupervised hierarchical clustering-based heatmap of the differentially expressed genes showed clear segregation of untreated and IFN-\(\gamma\)-treated cells (figure 3B). Furthermore, among 91 genes present in the KEGG pathway ‘Antigen processing and presentation’ (mmu04612) 27 genes were significantly upregulated; for example, TAP1 and TAP2 (figure 3C). In line with these findings, Gene Set Enrichment Analysis based on the REACTOME database showed significant upregulation of 105 gene sets in IFN-\(\gamma\)-treated cells (FDR<0.05) including ‘Antigen processing and presentation’ and ‘IFN-\(\gamma\) signaling’ (FDR q value<0.001; \(p<0.001\)) (online supplementary figure S4a). In particular, the upregulation of TAP1 was found within the top three leading-edge genes based on the immunological signatures database (online supplementary figure S4b). However, these analyzes also revealed a significant upregulation of coinhibitory PD-L1 (CD274) in IFN-\(\gamma\)-treated KPC cells (online supplementary table 2), correlating with a prominent upregulation of PD-L1 on the cell surface (figure 3A).

KPC7735 cells and IFN-\(\gamma\)-pretreated KPC7735 cells efficiently grafted as solid tumors in transplanted B6 mice (figure 4A). This suggested that upregulation of co-inhibitory PD-L1 on IFN-\(\gamma\)-treated KPC7735 cells was sufficient to prevent CD8 T cell-mediated rejection of transplanted tumors through direct activation of the PD-L1-signaling axis with PD-1+ CD8 T cells. To confirm this assumption, we transplanted KPC7735 cells into B6 mice and treated them with anti-PD-1 antibody. Anti-PD-1 but not isotype control antibodies inhibited the outgrowth of KPC7735 tumors in three out of six animals (figure 4B), indicating that transplanted KPC7735 cells per se induced CD8 T cells that unleashed their effector functions only after blocking PD-L1/PD-1 interactions.\(^{45}\) Confirmatory, untreated KPC7735 cells and, with a higher robustness, IFN-\(\gamma\)-treated KPC7735 cells induced an antitumor immunity that rejected tumor transplants in PD-1\(^{-/-}\) mice (figure 4C). Comparable to B6 mice, neither non-treated nor IFN-\(\gamma\)-treated KPC7735 cells were rejected in PD-L1\(^{-/-}\) mice (figure 4A–D). We, thus, concluded that PD-L1 molecules on the surface of KPCs function as a dominant gatekeepers that suppressed effector CD8 T-cell responses in B6 mice via the PD-L1/PD-1 signaling axis.
KPC7735 cells transplanted subcutaneously into B6 mice showed a prominent upregulation of both, MHC-I and PD-L1 molecules on the cell surface, resembling the effects of the in vitro IFN-γ-treatment figure 4E). The upregulation of MHC-I and PD-L1 cell surface expression was weakened in subcutaneous KPC7735 tumors developing in T- and B cell-deficient syngeneic RAG1−/− mice (figure 4E). This indicated that transplanted KPC7735 cells stimulated the adaptive immune system in immune-competent mice, which in turn induced both, MHC-I and PD-L1 expression on these tumor cells, and thereby established tumor-eradicating effector
CD8 T-cell responses when the PD-L1/PD-1 signaling axis was silenced/blockedin anti-PD-1-treated B6 or in PD-1−/−mice.

To proof that tumor cells induce PD-L1/PD-1-sensitive effector CD8 T cells, we generated PD-L1-deficient KPC7735PD1KO cells using the CRISPR/Cas9 system. KPC7735PD1KO cells upregulated expression of MHC-I but not PD-L1, Cr-1 or gp70 on IFN-γ-treatment (online supplementary figure S3a–c) and were quantitatively rejected in transplanted B6 mice (figure 5A). An additional challenge of these KPC7735PD1KO-immune, tumor-free B6 mice at day 28 with KPC7735 cells resulted in a quantitative rejection of these transplanted

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**Figure 3** IFN-γ-induced gene expression in KPC cell lines. (A) Different KPC cell lines were either untreated or treated with 20 ng/mL recombinant mouse IFN-γ for 20 hours, followed by FCM analysis of H2-Kb and PD-L1 surface expression. One representative histogram per cell line out of three independent experiments is shown. (B) Unsupervised hierarchical clustering based on differentially expressed probesets by comparing five IFN-γ-treated (for 20 hours) KPC cell lines (+IFN-γ) to the respective untreated KPC cell lines (ctrl). Red represents upregulated probesets while blue shows downregulated ones in the IFN-γ-treated (+IFN-γ) group. The samples clearly clustered based on the experimental arms (pink=ctrl; light blue=+IFN-γ). (C) KEGG ‘Antigen processing and presentation’ (mmu04612) gene expression profile in IFN-γ-treated KPCs (+IFN-γ) to untreated KPCs (ctrl). We identified 27 genes that showed significant differential expression (BH adjusted p<0.05) and are listed to the right of the heatmap. FCM, flow cytometry; IFN-γ, interferon-γ.
cells (figure 5A) along with a prominent CD8 T-cell response against the TAP-dependent gp70:Kb/p15E but not the TAP-independent Cr-1:Kb/cr16-24 epitope (figure 5B). Similarly, a single immunization of B6 mice with KPC7735PD-L1KO cells induced high frequencies of gp70:Kb/p15E-specific CD8 T cells and low frequencies of Cr-1:Kb/cr16-24-specific CD8 T cells (figure 5C). Interestingly, also KPC7735 cells primed gp70:Kb/p15E- and Cr-1:Kb/cr16-24-specific CD8 T cells in B6 mice, but the frequencies were lower than in KPC7735PD-L1KO-immune mice (figure 5C). It was striking that a significant higher proportion of gp70:Kb/p15E-specific CD8 T cells primed in KPC7735-immune B6 mice expressed the PD-1 exhaustion marker (figure 5D), which could limit their effector phenotype and explain the rejection of KPC7735PD-L1KO but not of KPC7735 tumor cells in these mice.

**Development of a novel IFN-γ-treatment protocol to selectively restore MHC-I presentation-competence on PDACCs**

Considering, that the deletion of PD-L1 in tumor cells with the CRISPR/Cas9 system is an attractive but
laborious and time-consuming way to generate effective cell-based tumor vaccines for PD-1-competent B6 mice, we explored conditions to manipulate KPC tumor cells for selectively restoring their MHC-I presentation competence. To determine whether the kinetics of IFN-γ-inducible MHC-I and PD-L1 surface expression differ, and could be exploited to manipulate the upregulation of PD-L1, tumor cells were treated for 20 hours with IFN-γ, washed and further cultured in vitro (figure 6A,B). This showed that upregulation of PD-L1 in different KPC cells was less sustained as compared with MHC-I and declined toward basal levels around day 3 (d3) post IFN-γ treatment start, whereas MHC-I expression still was prominent (figure 6B). In contrast, the expression of gp70 and Cr-1 tumor antigens was not (or rarely) affected by the IFN-γ treatment protocol (online supplementary figure S6).

For immunization studies, we additionally irradiated tumor cells and transplanted either non-treated irradiated KPC7735 cells (KPC7735-ir), KPC7735 cells irradiated immediately after IFN-γ treatment (KPC7735-I-ir) and KPC7735 cells irradiated at d3 post IFN-γ treatment (KPC7735-Id3-ir) into the left flank of B6 mice (figure 6A–C). We did not observe outgrowth of tumors in these mice, confirming the efficacy of our irradiation conditions (figure 6C). At d19 postvaccination, mice were challenged with 2.5×10⁵ naïve KPC7735 cells into the opposite right flank (figure 6A–C). KPC7735-ir and KPC7735-I-ir cells did not induce protective immune responses and consecutively transplanted KPC7735 cells formed solid tumors (figure 6C). In contrast, KPC7735-Id3-ir cells induced a tumor-specific immunity that efficiently suppressed tumor development of transplanted KPC7735 cells (figure 6C). Suppression of tumor growth in this group was reverted by the injection of anti-CD8 but not anti-CD4 mAbs (figure 6C), demonstrating that CD8 T cells played a crucial role in the rejection of KPC7735 tumor transplants. Similarly, KPC7735-Id3-ir cells primed a tumor-protective immunity in PD-1⁻/⁻ mice (figure 6D).

In contrast to B6 mice, vaccination of PD-1⁻/⁻ mice with KPC7735-ir cells was also sufficient to suppress
the outgrowth of consecutively transplanted KPC7735 cells (figure 6C,D). This suggested that the immune-mediated upregulation of MHC-I and PD-L1 (figure 4E) was, at least transiently active in transplanted KPC7735-ir cells and allowed priming of an anti-tumor response in PD-1−/− (lacking the PD-L1/PD-1 signaling axis) but not in B6 mice. Confirmatory, irradiation of KPC cells resulted in a prominent cell death (>90%) during a subsequent in vitro culture for 48 hours, but did not significantly affect the IFN-γ-induced upregulation of MHC-I and PD-L1
molecules on the small population of live cells (online supplementary figure S7).

KPC7735-Id3-ir cells primed a prominent gp70:Kb\textsuperscript{p15E}-specific CD8 T-cell response and a weak Cr-1:Kb\textsuperscript{cr162-24}-specific CD8 T-cell response in B6 mice (figure 6E; online supplementary table 1). Interestingly, gp70:Kb\textsuperscript{p15E}- and Cr-1:Kb\textsuperscript{cr162-24}-specific CD8 T-cell frequencies were comparable in KPC7735-Id3-ir-immune B6 and PD-L\textsuperscript{1/-} mice (figure 6E), indicating that a missing PD-1 expression did not affect de novo priming of CD8 T cells. However, as compared with KPC7735-ir cells that did not elicit a tumor-protective immunity in B6 mice (figure 4C), KPC7735-Id3-ir cells induced higher frequencies of gp70:Kb\textsuperscript{p15E}-specific CD8 T cells associated with a decreased single positive (PD-1\textsuperscript{+} or LAG-3\textsuperscript{+}) and double positive PD-1\textsuperscript{+}/LAG-3\textsuperscript{+} exhaustion marker profile (online supplementary figure S8) which could preserve their expansion and/or functional effector phenotype.\textsuperscript{46} The IFN-\(\gamma\)-treatment protocol could also be used in B6 mice to prime a tumor-specific immunity against different PD-L\textsuperscript{1/-}/MHC\textsuperscript{co} PDACCs (KPC7612-Id3-ir) (online supplementary figure S9). Interestingly, upregulation of PD-L1 in IFN-\(\gamma\)-treated PD-L\textsuperscript{1/-}/MHC\textsuperscript{co} MC38 colon adenocarcinoma cells was less sustained as compared with the inducible MHC-I expression and MC38-Id3-ir but not MC38 or MC38-ir cell-based vaccination induced a protective immunity in B6 mice that rejected a subsequent transplant of MC38 cells (online supplementary figure S10). Above analyses revealed clear differences in the priming of Cr-1:Kb\textsuperscript{cr162-24} and gp70:Kb\textsuperscript{p15E}-specific CD8 T-cell responses by DNA-based and cell-based immunization (online supplementary table 1). We, thus, tested whether DNA immunization could be used to complement the TAP-dependent gp70:Kb\textsuperscript{p15E}-specific CD8 T-cell immunity induced by KPC tumor cell-vaccines. Indeed, coinfection of B6 mice with pCI-Gp70 and KPC7735\textsuperscript{PD-L1KO} cells efficiently primed both, Cr-1:Kb\textsuperscript{cr162-24} and gp70:Kb\textsuperscript{p15E}-specific CD8 T-cell responses and the respective CD8 T-cell frequencies were even comparable to those primed by individual vaccines (online supplementary figure S11). This strongly suggested that de novo primed Cr-1:Kb\textsuperscript{cr162-24} and gp70:Kb\textsuperscript{p15E}-specific CD8 T cells did not interfere.

**DISCUSSION**

Here, we used PDACCs as a model to explore if (and how) expression and processing of tumor antigens in the ER and/or in TAP-dependent/independent pathways influences the MHC-I epitope presentation and priming of effector CD8 T cells by cell-based immunization.\textsuperscript{47 48} We showed that subcutaneous transplantation of KPC cells into immune-competent B6 but not into T- and B-cell deficient Rag1\textsuperscript{-/-} mice upregulated MHC-I and PD-L1 expression on the cell surface. This indicated that cells of the adaptive immune system in immune-competent B6 mice directly interfered with transplanted KPC tumor cells and triggered a balanced MHC-I presentation-competent, immune-suppressive cell surface expression profile.\textsuperscript{5} We could largely mimic this in vivo upregulation of MHC-I and PD-L1 molecules by treating in vitro cultured KPC cells with IFN-\(\gamma\). Microarrays performed on untreated and IFN-\(\gamma\)-treated KPC cells confirmed the upregulation of genes involved in MHC-I expression (eg, beta-2 microglobulin), the conventional MHC-I antigen processing/presentation pathway (eg, TAP1)\textsuperscript{49} but also of PD-L1 molecules on the small population of live cells (online supplementary figure S7).

The IFN-\(\gamma\)-treatment protocol could also be used in B6 mice to prime a tumor-specific immunity against different PD-L\textsuperscript{1/-}/MHC\textsuperscript{co} PDACCs (KPC7612-Id3-ir) (online supplementary figure S9). Interestingly, IFN-\(\gamma\)-initiated upregulation of PD-L1 were superior to the reconstitution of MHC-I presentation-competence, as IFN-\(\gamma\)-treated KPC7735 cells grew out to tumors in B6 and PD-L\textsuperscript{1/-} mice, but not in PD-L\textsuperscript{1/-} mice. In PD-L\textsuperscript{1/-} mice, PD-L1 molecules were exclusively expressed on transplanted tumor cells, demonstrating that the engagement of PD-1 by PD-L1 on tumor cells was sufficient to suppress the anti-tumor responses to KPC7735 cells. When PD-L1/ PD-1 signaling was blocked in PD-L\textsuperscript{1/-} mice or anti-PD-1 treated B6 mice, subcutaneously transplanted KPC7735 cells induced an antitumor immunity against themselves, pointing to a dominant negative PD-L1-mediated mechanism. Similarly, the depletion of PD-L1 in KPC7735 PD-L1\textsuperscript{-/-} tumor cells was sufficient to prevent the PD-L1/ PD-1 signaling axis and preferentially induce TAP-dependent gp70:Kb\textsuperscript{p15E}-specific CD8 T cells. Overall, the KPC7735 PD-L1\textsuperscript{-/-} induced immunity led to their own rejection and protected these B6 mice from consecutively transplanted KPC7735 tumors. Based on these observations, we established an in vitro IFN-\(\gamma\) treatment protocol for KPC tumor cells that enhanced their antigenic potential to prime CD8 T cells in B6 mice in an antigen specific manner. This method reconstituted TAP-dependent MHC-I presentation-competence and concomitantly bypassed the IFN-\(\gamma\)-mediated upregulation of PD-L1 in KPC cells. As a consequence, cell-based immunization with KPC7735-Id3-ir cells, but neither KPC7735-ir nor KPC7735-Id3-ir cells, induced a tumor-protective immunity in B6 mice that rejected a subsequent inoculum of naïve KPC7735 cells. An interesting aspect of this method was that codelivery of anti-PD-1 or anti-PD-L1 antibodies that provided potent tools for immune checkpoint control but also induced serious side effects, like the induction of autoimmune diabetes,\textsuperscript{45 50} were not necessary to induce this protective CD8 T-cell immunity by cell-based immunization.

Cr-1 and gp70 antigens expressed in their natural compartment (the ER) or as Hsp73-bound chimeric antigens in the cytosol elicited a complete opposite pattern of CD8 T-cell responses by DNA immunization (online supplementary table 1). Expression and processing of Cr-1 in the ER was a prerequisite to generate the Cr-1:Kb\textsuperscript{cr162-24} epitope and induce Cr-1:Kb\textsuperscript{cr162-24}-specific CD8 T cells in a TAP-independent manner. Confirmatory, a cytosolic Hsp73-complexed stT77Cr-1 antigen did not induce CD8 T cells, though Hsp73 facilitated cross-priming of CD8 T cells by DNA immunization.\textsuperscript{20 40} It is unknown if and which cross-presentation/-priming mechanism(s)
(ie, antigen transfer from antigen-expressing myocytes targeted by vector DNA injection to professional B7.1+ APCs, like dendritic cells) were involved in the priming of Cr-1:\(K^{b}/\alpha_{1221}\)-specific CD8 T cells by SPase-sensitive Cr-1 but not SPase-resistant Cr-1\_ex alpha antigens.Alternatively, Cr-1 could be directly expressed and processed in the ER of professional APCs targeted by intramuscular DNA injection. The expression/process requirements of Cr-1 for priming effector CD8 T cells by DNA immunization strongly resembled the preproinsulin (ppins) self-antigen. Ppins is targeted to the ER by a SP\_ppins domain and contains a \(K^{b}/\alpha_{1221}\)-specific CD8 T cells and autoimmune diabetes were induced in PD-1\_− and PD-L1\_− by injection of ppins DNA, as well as in ppins-immune B6 mice that replenished their diabetogenic potential after anti PD-L1 antibody treatment. Comparably to Cr-1, the expression of ppins in the ER was a prerequisite to generate a low-affine \(K^{b}/\alpha_{1221}\)-epitope and induce autoreactive CD8 T cells by DNA immunization, most likely because this epitope must not compete for MHC-I binding with the bulk of antigenic peptides generated in the conventional endogenous antigen presentation pathway for TAP-dependent transport into the ER. A primary effect of ER-expression/processing of self-antigens thus could be that epitopes with a relative weak avidity for MHC-I can directly bind to a substantial number of MHC-I molecules in the ER, necessary to prime/activate autoreactive CD8 T cells. In contrast, expression of gp70 antigens in the ER was not sufficient to directly stimulate gp70:K^{b}/p15E-specific CD8 T cells in B6 and TAP-deficient mice by DNA immunization. This showed that the gp70 antigen does not belong to the group of TEIPPs, which are exclusively immunogenic under TAP-deficient conditions. In line with previous reports, indicating that presentation of the gp70:K^{b}/p15E epitope depends on proteasomal processing and TAP, we showed that cytosolic Hsp73-bound stT77gp70 complexes induced gp70:K^{b}/p15E-specific CD8 T cells in B6 but not TAP-deficient mice. A stgp70 antigen lacking the Hsp73-binding domain did not induce gp70:K^{b}/p15E-specific CD8 T cells. This suggested that priming of gp70:K^{b}/p15E-specific CD8 T cells by cytosolic gp70 antigens critically depend on ‘help’ from Hsp73. Confirmatory, RNA-binding stgp70 designer antigens (eg, stgp70tat, a fusion antigen composed of stgp70 and a cationic HIV-tat domain) induced TLR-7-dependent innate ‘help’ and primed gp70:K^{b}/p15E-specific CD8 T cells by DNA immunization. Furthermore, considering that a proline (P) at position 3 of the K^{b}/p15E epitope (KSPWFTTL) greatly hindered its TAP-dependent transport and efficient presentation by K^{b} molecules, we think that these adjuvant-like immune-stimulatory immune responses might improve epitope presentation.

It is generally assumed that cross-presentation of tumor cells or tumor cell debris by professional APCs is a prerequisite to prime functional effector CD8 T-cell responses. In our transplanation model, KPC7735-Idd3-ir cells induced high frequencies of gp70:K^{b}/p15E-specific CD8 T cells in both, B6 and PD-1\_− mice. A comparable effector phenotype was induced by KPC7735\_PD-L1\_KO cells. Therefore, at least for the two antigens tested, the IFN-γ-treatment protocol selectively enhanced priming/expansion of TAP-dependent gp70:K^{b}/p15E-specific CD8 T cells that correlated with the rejection of consecutively transplanted KPC7735 cells. However, it is obvious that additional multi-specific CD8 T-cell responses against yet unknown antigens/epitopes were coprimed by KPC7735-Idd3-ir cells and facilitated the almost quantitative rejection of transplanted KPC7735 tumor cells.

In conclusion, we here showed a promising strategy to overcome two initial problems in the development of CD8 T-cell-mediated immunotherapies against cancer: (1) the identification of expression/process conditions for tumor-associated self-antigens that stimulate effector CD8 T-cell responses by cell-based vaccines and (2) the combination of CD8 T-cell priming with immune interventions that limit CD8 T-cell exhaustion by silencing the tumor-mediated PD-L1/PD-1 signaling axis.

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Contributors KS, JK, LR, JG, MW and AL designed and performed the experiments, researched and interpreted data. AK, MW, TS and RS conceived the experiments, secured funding, discussed and interpreted the data. KS and RS wrote manuscript. All authors edited and approved the final manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Except of the established human cell line HEK-293 used for detection of vector-expressed antigens, this study does not contain human materials. All mouse immunization studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German Federal Animal Protection Law. The protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Ulm (Tierforschungszentrum Ulm, Obererhofg) and the Regierungsspräsidium Tübingen (Permit Numbers: 1199 and 1273 to AK).

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Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The datasets generated in this study are now publicly available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140397.

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