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Therapy-Induced MHC I Ligands Shape Neo-Antitumor CD8 T Cell Responses during Oncolytic Virus-Based Cancer Immunotherapy

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Abstract

Oncolytic viruses (OVs), known for their cancer-killing characteristics, also overturn tumor-associated defects in antigen presentation through the MHC class I pathway and induce protective neo-antitumor CD8 T cell responses. Nonetheless, whether OVs shape the tumor MHC-I ligandome remains unknown. Here, we investigated if an OV induces the presentation of novel MHC I-bound tumor antigens (termed tumor MHC-I ligands). Using comparative mass spectrometry (MS)-based MHC-I ligandomics, we determined differential tumor MHC-I ligand expression following treatment with oncolytic reovirus in a murine ovarian cancer model. In vitro, we found that reovirus changes the tumor ligandome of cancer cells. Concurrent multiplexed quantitative proteomics revealed that the reovirus-induced changes in tumor MHC-I ligand presentation were mostly independent of their source proteins. In an in vivo model, tumor MHC-I ligands induced by reovirus were detectable not only in tumor tissues but also the spleens (a

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Supporting Information

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source of antigen-presenting cells) of tumor-bearing mice. Most importantly, therapy-induced MHC-I ligands stimulated antigen-specific IFN γ responses in antitumor CD8 T cells from mice treated with reovirus. These data show that therapy-induced MHC-I ligands may shape underlying neo-antitumor CD8 T cell responses. As such, they should be considered in strategies promoting the efficacy of OV-based cancer immunotherapies.

Graphical Abstract

Keywords

oncolytic virus; reovirus; cancer immunotherapy; mass spectrometry; MHC ligandome; CD8 T cell epitopes; antitumor immunity

■ INTRODUCTION

Therapy-induced antitumor immune responses correlate with better cancer outcomes and thus are highly desired in clinics.^{1–3} Immunotherapies, administered either alone or in combination with conventional therapies, focus on promoting antitumor CD8 T cell responses as the presence of CD8 T cells predicts better clinical outcomes from many of the commonly occurring cancers.^{4,5} Furthermore, immunotherapies like immune checkpoint blockade with anti-PD-1 and anti-CTLA-4 antibodies promote neo-CD8 T cell expansion, which otherwise remain undetectable in nontreated cancer patients.^{1,6,7} These therapy-induced CD8 T cell responses can recognize and target cancer cells and also establish protection against relapse.^{6,8,9} As the activation of CD8 T cells is defined by the epitopes presented in MHC class I molecules (also known as MHC-I ligands), the tumor MHC-I ligandome shapes antitumor CD8 T cell responses. The potential role of therapy-driven MHC-I ligandome changes is poorly understood.

Reovirus, in its natural form, preferentially replicates in and kills cancer cells and is thus considered an oncolytic virus (OV) leading to several clinical trials for its use in cancer treatment. Reovirus also induces potent antitumor CD8 T cell immune responses, in part by

overturning several tumor-associated immune evasion mechanisms.^{10–19} This is manifested by a localized release of cytokines within the tumor microenvironment, leading to the activation and recruitment of various innate and adaptive immune cells.^{20,21} In addition, antigen-presenting cells that ingest cell debris released during oncolysis may activate tumor antigen-specific CD8 T cells.^{21,22} We have previously demonstrated that reovirus OV therapy enhances the expression of proteins involved in antigen processing (e.g., TAP1, TAP2) and presentation (e.g., MHC-I, β 2-microglobulin) in tumors of various cancer types.^{23,24} Furthermore, using cancer cells expressing ovalbumin as a surrogate tumor-associated antigen, we have demonstrated reovirus-mediated enhancement of tumor antigen-specific CD8 T cell immunity.²³ This robust oncolytic virus-mediated antitumor CD8 T cell immunity can be further invigorated by genetically engineering OVs to express immunostimulatory transgenes,²⁵ adding immune checkpoint inhibitors,^{26,27} or combining with chemotherapeutic drugs.²⁸

Our laboratory has recently reported MHC-I ligandome changes in response to the chemotherapeutic drug doxorubicin.²⁹ Although both preferential tumor replication and increased antigen presentation by OVs are well established, whether OVs also change the tumor MHC-I ligandome has not been explored. Here, we used MHC-I immunoprecipitation and LC-MS/MS with label-free quantitation to determine oncolytic reovirus-induced MHC-I ligands. We found that oncolytic reovirus induces new MHC-I ligands in tumor cells in vitro and in vivo, and from spleens (a source of antigen-presenting cells) of tumor-bearing animals. Quantitative proteomic analysis of virus-infected cancer cells revealed that reovirus-induced changes within the tumor MHC-I ligandome occur mostly independent of their source proteins. Most importantly, IFN γ assays showed that these therapy-induced tumor MHC-I ligands are immunogenic. Together, these findings highlight the importance of considering the effect of therapies such as OVs on the tumor MHC-I ligandome. The data provide rationale for exploiting the OV-induced tumor MHC-I ligands for cancer immunotherapies due to OV's preferential replication in tumors.

■ EXPERIMENTAL SECTION

Reovirus, Cell Lines, and Reagents

Reovirus (serotype 3, Dearing strain) was cultured, amplified, and isolated using a previously established protocol.¹⁰ A mouse ovarian surface epithelial cell line (MOSE, clone ID8) was obtained from Edith Lord (University of Rochester, Rochester, NY),³⁰ and grown at 37 °C, 5% CO₂ in DMEM containing 10% fetal bovine serum, 1× sodium pyruvate, 1× nonessential amino acids, and 1× Anti-Anti (all obtained from Invitrogen, Carlsbad, CA). Functional grade purified antimouse CD28 (37.51) was from BioLegend (San Diego, CA). Purified antimouse MHC-I antibodies were produced in-house from hybridoma clones B22.249 (H-2D^b specific) and Y3 (H-2K^b specific). For validation experiments, peptides were purchased from JPT Peptide Technologies (Berlin, Germany). An antimouse IFN γ DuoSet ELISA kit was purchased from R&D Systems (Minneapolis, MN).

MHC-I Peptide Isolation and Mass Spectrometry Analysis

MHC-I peptide immunoprecipitation was conducted for the mouse ID8 cell line as previously described.³¹ In brief, 1×10^8 cells for each treatment group (nontreated, IFN- γ treated, and reovirus-treated) were lysed in PBS containing 0.4% CHAPS and mini-complete protease inhibitor tablets (Roche, Indianapolis, IN). MHC-I proteins were precipitated from the cell lysates using 2 mg of anti-MHC-I antibody (both H-2D^b and H-2K^b for mouse) coupled to 80 mg of CNBr-activated Sepharose 4B resin (Uppsala, Sweden). Following overnight incubation in 10 mL glass tubes at 4 °C, bound MHC-I proteins and peptides were washed with 40 mL of PBS, then 30 mL of Milli-Q water, and peptides were eluted from the antibody-resin by acid treatment (eight times with 200 μ L of 0.2% trifluoroacetic acid [TFA]). Eluates were purified by ultrafiltration through 3 kDa molecular weight cutoff filters (Millipore, Cork, Ireland), and then the filtrates were lyophilized and desalted using Stage-tips.

Lyophilized peptides were solubilized in 12 μ L of 1% formic acid and analyzed by LC-MS/MS. For each antibody eluate, an aliquot of 1 μ L of peptides was injected onto a 75 μ m $\times$ 30 cm column (New Objective, Woburn, MA) self-packed with 4 μ m, 90 Å, Proteo C18 material (Phenomenex, Torrance, CA). Online chromatography was performed using a Dionex Ultimate 3000 UHPLC (Thermo Scientific, San Jose, CA) at a flow rate of 300 nL/min. Peptides were separated and eluted into the mass spectrometer using a gradient of 3 to 35% acetonitrile (0.1% formic acid) over 65 min, followed by 5 min at 95% acetonitrile (0.1% formic acid). MS was performed using an Orbitrap Velos Pro (Thermo Fisher Scientific, San Jose, CA) operated in data-dependent mode. Survey scans (MS1) were performed using the Orbitrap over a scan range of 350–650 m/z and resolution setting of 60 000. A lock mass of 445.12003 m/z was used to achieve internal mass calibration. On the basis of MS1 scans, MS2 scans were performed using the ion trap, selecting the top 10 most intense precursor (MS1) ions for fragmentation by collision-induced dissociation (CID) at 35% collision energy with a precursor isolation window of 2 m/z . MS2 scans were only collected on peptides with charge states of 2⁺ or 3⁺ with a minimum MS1 intensity of 50 counts. Advanced gain control (AGC) settings were 5×10^5 for Orbitrap scans and 2×10^5 for ion trap scans.

MHC-I Peptide Identification

MHC-I peptides were identified using a previously described targeted search strategy.³² Briefly, MHC-I peptides were predicted from all mouse proteins for the H-2D^b and H-2K^b alleles using NetMHC with a rank cutoff of 2% and these peptides were used to create a FASTA database. MS/MS data were searched using Sequest with “no cleavage” enzyme specificity with an MS1 tolerance of 5 ppm and MS2 tolerance of 0.5 Da. False discovery rates were controlled to 5% using Percolator. Searches were implemented in Proteome Discoverer (PD) version 2.2 (Thermo Fisher Scientific, San Jose, CA). Label-free quantitation (LFQ) was also implemented in PD version 2.2 using the Minora peak alignment algorithm. All peptides were normalized based on the summed peptide intensity for the entire sample.

Protein Extraction, Digest, and TMT Labeling of ID8 Cells

Cells were scraped into 2 mL of lysis buffer containing 2% SDS, 150 mM NaCl, 50 mM Tris (pH 8.5), 5 mM DTT, and 1 tablet of protease inhibitor (Sigma-Aldrich, St. Louis, MO) per 10 mL of buffer. Samples were homogenized with an Omni homogenizer (Omni International, Kennesaw, GA) with 3 cycles of 12 s, with cooling on ice between cycles, plus sonication with probe sonication. Samples were incubated at 56 °C for 30 min, cooled, then cysteines were alkylated using 14 mM iodoacetamide, and protein was purified by methanol-chloroform precipitation. Proteins were dried then resolubilized in 1.5 mL of 8 M urea, 50 mM Tris, pH 8.8 then protein content in the samples was measured using a BCA assay (Thermo Fisher Scientific, San Jose, CA). An aliquot of 100 μ g of protein was diluted to 1.5 M urea, digested for 4 h with trypsin (Promega, Madison, WI) then again overnight (both at a ratio of 1:100 trypsin:protein) at 37 °C. The pH of the digest was adjusted to <3 using formic acid and peptides were desalted using 60 mg solid phase Oasis HLB extraction cartridges (Waters, Milford, MA) then lyophilized. Peptides were resuspended in 100 μ L of 100 mM HEPES, 30% acetonitrile and 10 μ L of TMT10 reagents (Thermo Fisher Scientific, San Jose, CA) prealiquoted at a concentration of 20 μ g/mL in anhydrous acetonitrile. The reaction was quenched with 0.5% hydroxylamine (Sigma-Aldrich, St. Louis, MO), mixed equally, desalted using a 60 mg solid phase Oasis HLB extraction cartridge (Waters, Milford, MA), and lyophilized. Peptides were fractionated using an Onyx monolithic 100 $\times$ 4.6 mm C18 column (Phenomenex, Torrance, CA) using a flow rate of 800 μ L/min, with a gradient of 5–40% acetonitrile (10 mM ammonium formate, pH 8) applied over 60 min using an Agilent 1100 pump (Agilent Technologies, Santa Clara, CA). Twelve fractions were collected and desalted using homemade Stage-tips packed with Empore C18 extraction material (Sigma-Aldrich, St. Louis, MO) as previously described,³³ then lyophilized and subjected to LC-SPS-MS3.

LC-SPS-MS3, Protein Identification and Quantitation

Labeled peptides were separated by online nLC similarly to the MHC-I peptides. SPS-MS3 was performed on the Orbitrap Fusion (Thermo Fisher Scientific, San Jose, CA). MS1 scans were acquired over an m/z range of 350–1400, 120K resolution, AGC target of 5×10^5 , and maximum injection time of 100 ms. MS2 scans were acquired on the 10 most-abundant MS1 ions of charge state 2–8 using an isolation window of 0.7 Th, CID activation with a collision energy of 35%, rapid scan rate, AGC target of 20 000, dynamic exclusion for 120 s, and maximum injection time of 150 ms. MS3 scans were acquired using SPS of 10 isolation notches, m/z range of 100–1000, 50K resolution, AGC target of 2.5×10^5 , HCD activation at 65%, and maximum injection time of 200 ms.

Mass spectrometry data files were converted to mzXML using a modified version of ReadW.exe. MS2 spectra were searched against the mouse UniProt database (downloaded August, 2011) using Sequest (Ver28)³⁴ concatenated with a reovirus protein sequence database. TMT was set as a fixed modification (229.162932) on lysine residues and peptide N-termini, and carbamidomethylation (15.99492) as a fixed modification on cysteine. The allowable precursor mass tolerance was 10 ppm and product ion mass tolerance was 1 Da. False positive rates were controlled using the target-decoy approach³⁵ with a concatenated reversed database employing linear discriminant analysis to distinguish correct and incorrect

peptide identifications based on XCorr, CN, peptide length, and charge state. Peptides were grouped into proteins and their multiplied linear discriminant analysis probabilities were used to sort proteins and filter to a 1% maximum false discovery rate. The sum of all reporter ion summed signal-to-noise (S/N) values for peptides matching each protein was used for protein quantitation.

In Vivo Experimental Procedures

All in vivo experimental procedures were approved by the Ethics Committee at Dalhousie University, Halifax, Nova Scotia, Canada. Six to eight week old female wildtype C57BL/6 mice were obtained from Charles River Laboratories (Montreal, QC, Canada). Mice were injected as follows: reovirus (5×10^8 plaque forming unit [PFU], intraperitoneally [i.p.]), ID8 (3×10^6 cells, i.p.). ID8 tumor-bearing mice were injected with reovirus at 35 days post tumor implantation. Spleens and tumors were harvested at 7–10 days post reovirus injection.

T Cell IFN γ Assay

Spleens were harvested from mice and mechanically disrupted using the end of a syringe plunger. Cells were filtered through a 40 μ m cell strainer and treated with red blood cell-lysing ammonium-chloride-potassium (ACK) buffer. The resulting single-cell suspension of splenocytes was cultured in RPMI 1640 containing 1% (v/v) Glutamax, 10% fetal bovine serum, 1 $\times$ sodium pyruvate, 1 $\times$ nonessential amino acids, and 1 $\times$ Anti-Anti (all obtained from Invitrogen, Carlsbad, CA) for in vitro stimulation. Splenocytes (1×10^6 cells/well in a round-bottom 96-well plate) were cultured in the presence of peptide (10 μ g/mL) and purified antimouse CD28 antibody (1 μ g/mL) for 48 h. The concentration of IFN γ in the supernatant was assessed using the IFN γ ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Statistical Analysis

Statistical analysis of all MHC-I and proteomic data was performed in R. F-tests were performed on the quantitative MHC-I and proteome data using the *genefilter* package. Histogram ridge plots were generated using *ggridges*, position-specific frequency matrices (PSFM) were calculated using *peptools*, sequence logos were generated using *ggseqlogo*, and heatmaps were generated using *pheatmap* R packages. One-way ANOVA with Bonferroni post-test or a two-tailed Student's t-test with 95% confidence interval was used for statistical analyses where indicated. *p*-values were corrected for multiple hypothesis (adjusted *p*-values) using Benjamini-Hochberg and represented as follows: ns = *p* > 0.05, **p* 0.05, ***p* 0.01, and ****p* 0.001.

RESULTS AND DISCUSSION

Oncolytic Reovirus Induces the Expression of Otherwise Low-Abundant MHC-I Ligands in Mouse Ovarian Cancer Cells in Vitro

On the basis of our previous findings that reovirus (type 3 Dearing strain) initiates CD8 T cell antitumor immunity,^{23,24} we first sought to determine whether reovirus infection differentially modifies MHC-I ligandome constituents in cancer cells grown in vitro. Mouse ID8 ovarian cancer cells (C57BL/6 background; H-2D^b and H-2K^b MHC class I alleles)

Virus-Induced Alterations in the Tumor MHC-I Ligandome Are Largely Independent of Their Source Proteins

MHC-I peptide presentation is proposed to correspond to source protein abundance.⁴¹ We therefore next determined whether reovirus-induced alterations in the tumor MHC-I ligands are due to changes at the proteome level. To explore the MHC-I ligand and proteome relationship quantitatively, we performed 10-plex tandem mass tag (TMT)-based quantitative proteomics⁴² on virus-infected ID8 cells over a 48-h time course with two MOI of reovirus (1 and 10) (Figure 2A). In total, we quantified 6561 proteins and observed temporally distinct patterns of protein expression following k-means clustering (Figure 2A). GO annotation analysis using PANTHER of Cluster 1, which contained proteins that increased due to reovirus infection, showed enrichment of proteins involved in virus response, interferon response, and antigen processing (Figure 2B). Similar to the reovirus proteins that we identified (e.g., Lambda1, Mu1; Figure 2C), virus response proteins (e.g., APOL9, CCL5, GBP4, OAS1A; Figure 2D) and antigen presentation proteins (e.g., B2M, H2-D1, TAP1, TAP2; Figure 2E) all showed increased relative intensity over time in response to reovirus. These data confirm results from our previous work showing the effect of reovirus on antigen presentation in ID8 cells.²⁴ The remaining proteins (five of the six clusters, representing most of the data set) showed only minor changes, or decreases, in relative intensity over time (Figure 2A). Proteomics data have been submitted to the PRIDE repository, accession #PXD013066.

To match peptides to potential source proteins, we matched MHC-I peptides to all potential protein accessions from the UniProt database and then determined whether any of those proteins were in our proteomics data set (Figure 3A). This resulted in 1022 (66%) and 912 (68%) respective H-2D^b and H-2K^b peptides (Figure 3B) with successful matches to the proteome data set. These matches equated to 1070 and 1022 peptide-accession combinations for H-2D^b- and H-2K^b-specific peptides, respectively. Comparison of the log₂ fold change in MHC-I peptide abundance against the log₂ fold change in protein abundance at 48 h revealed poor correlation between MHC-I peptide presentation and protein abundance (Figure 3C). For example, AALDFKNV from the protein SMC4 was induced 29-fold but showed no difference at the source protein level (Figure 3D). Although most MHC-I peptides indeed showed no protein-level changes, a small fraction of the MHC-I peptides did increase both at the MHC-I peptide and source protein levels (Figure 3C). For example, AALTGTHVL from APOL9B was increased 9.2- and 290-fold at the MHC-I peptide and protein levels, respectively (Figure 3D).

The general lack of correlation between the MHC-I peptide and source protein levels in our data somewhat contrasts with previous studies on peptide presentation and protein abundance. However, our data concern differential measurements rather than overall abundance, which suggests that external effects on protein abundance, such as those affected by virus infection, are not represented by the MHC-I ligandome changes. These data suggest that virus-induced differences in protein cleavage and peptide trimming, as opposed to the synthesis of new proteins, may play a greater role in MHC-I peptide generation during viral infections. To investigate this, we analyzed source protein sequences of reovirus-induced MHC-I peptides compared to unchanged peptides. We observed only minor differences in

the frequency of amino acid residues ten positions upstream or downstream of these MHC-I peptides, between induced or all other MHC-I peptides in the data set (Figure S2A). There was also no observable difference in the distance to the source protein Nor C-terminus between the induced, unchanging, or repressed MHC-I peptides (Figure S2B). Altogether, our data suggest that MHC-I ligand differences from reovirus infection are not caused by differential protein abundance or peptide cleavage. MHC-I peptide presentation is complex and factors such as protein synthesis rates, turnover, and proteostasis should be investigated in further studies to determine how antigen presentation is governed.

In Vivo, Reovirus Induces Tumor MHC-I Ligands That Are Exclusive to Tumor-Bearing Mice

Although it has been observed that OV_s elicit antitumor immunity, whether induced antigens drive this immune response remains unexplored. Further, a major characteristic of OV_s is their preferential replication in tumors as a result of their ability to hijack the active replicative machinery of cancer cells.⁴³ Having established that reovirus alters the MHC-I ligandome in an in vitro setting, we next sought to understand whether the virus preferentially alters the MHC-I ligandome in tumors and whether this is reflected in MHC-I ligands of immune cells. To do this, spleens and/or tumors from nontreated or reovirus-treated, naïve or ID8 tumor-bearing (TB) mice were collected and analyzed by MHC-I immunoprecipitation and LC-MS/MS with LFQ (Figure 4A). In this experiment, we quantified 2447 and 1681 H-2D^b and H-2K^b peptides, respectively, matching to 6170 protein accessions in the search database (Figure 4B). Consistent with recently reported differences in tissue-specificity of mouse MHC-I ligands,⁴⁴ we observed only partial overlap with the in vitro experiment, whereby 1071 (20% of the 5199 total peptides between the two experiments) of the in vitro-identified MHC-I peptides were not quantified in vivo (Figure S3A). Importantly, preferential tumor replication of reovirus was indeed reflected in the MHC-I ligandome. We observed far greater differential MHC-I abundance in the TB mice (both in the spleen and the tumor) than in the naïve mice. For example, 2.9- and 7.8-fold more H-2D^b peptides were induced by reovirus in the spleen and tumor, respectively, of TB mice (normalized log₂[reovirus/NT]) 1.0, adjusted p-value 0.05) (Figure 4B). H-2K^b peptides showed this same effect whereby 6.4- and 14-fold more peptides were induced in the TB mice by reovirus at the spleen and tumor, respectively, than in the naïve mice (Figure 4B). To further illustrate the preferential reovirus infection in tumors, we directly compared reovirus-induced MHC-I ligands across the experiment. Of the MHC-I peptides that were significantly induced (normalized mean log₂[reovirus/NT]) 1.0, adjusted p-value 0.05) at the tumor, approximately 50% were also induced in the spleen of TB mice but very few of these were induced in the naïve mice (Figure S3B). Representative label-free quantitation data for several of these MHC-I peptides induced in both the spleen and tumor, tumor-only, or reovirus-derived are exemplified in Figure 4C. These data suggest that TB mice display a reovirus-induced tumor MHC-I ligandome that is not found in reovirus-treated naïve mice, PBS-treated naïve mice, or PBS-treated TB mice.

Reovirus-Induced Tumor MHC-I Ligands Contain Biologically Active Antitumor CD8 T Cell Epitopes

Having determined the preferential reovirus-induced MHC-I ligand changes in the ID8 tumor, we next investigated whether these therapy-induced MHC ligands elicit antitumor

CD8 T cell responses. To do this, we chose 16 *highly* virus-induced tumor MHC-I ligands (normalized $\log_2[\text{reovirus/NT}] - 3.0$, adjusted p -value 0.05) across a range of NetMHC % rank (but all $\geq 2\%$ rank) and peptide spectrum matches (PSMs), and excluding those that were also induced in the naive spleen (Figure 5A). These highly induced peptides were essentially absent in the untreated tumors and 13 of them were also induced in the spleen of TB mice (Figure 5B). These 16 peptides were synthesized and their capacity to elicit CD8 T cell responses in splenocytes of untreated and reovirus-treated TB mice was measured by IFN γ ELISA in the presence of costimulation with an anti-CD28 antibody. We observed no IFN γ response in splenocytes from reovirus-treated mice in the absence of any of the peptides or with anti-CD28 costimulation alone. In a validation screen, four of these peptides (GIIRFLIGF, INYVVAHV, STLSHVVL, and RSYRFMVM) produced strong responses (>150 pg/mL) in splenocytes of reovirus-infected TB mice, nearly equaling IFN γ production of the concanavalin A positive control (Figure 5C). Taken together, these data strongly suggest that reovirus-induced tumor MHC-I ligands can activate CD8 T cells. Although these MHC-I peptides are nonmutated self-peptides, they are absent without infection and thus may be immunogenic. These results support findings that the breadth of antitumor CD8 T cell immune response is majorly dictated by tumor-associated antigens (TAAs).^{45,46} Most importantly, our data show that OV-induced MHC-I ligands may shape the therapy-induced neo-antitumor CD8 T cell responses.

■ CONCLUSIONS

Similar to the already proposed implications for cancer neoepitopes,^{6,7} our data suggest that another promising strategy to enhance antitumor CD8 T cell immunity could be to exploit the OV therapy-induced tumor MHC-I ligandome. The exclusive identification of virus-induced MHC-I peptides in TB mice demonstrates the potential for therapy-induced human HLA-ligand-based vaccines to be developed and used in combination with OVs currently being tested in clinical trials. Finally, induced MHC-I ligands may be valuable not only for OV-based cancer therapies but other treatments that induce neo-antitumor CD8 T cell responses (e.g., doxorubicin, and checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 antibodies⁶), ultimately leading to next generation cancer immunotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Delineation of the reovirus-induced tumor MHC-I ligandome in vitro. (A) Schematic of the experimental setup. Mouse ID8 MOSE ovarian carcinoma cells (1×10^8) were treated for 24 h with IFN γ , reovirus (MOI 10) or left untreated. MHC-I peptides were immunoprecipitated with antibodies specific to the H-2K^b and H-2D^b C57BL/6 mouse alleles and analyzed by mass spectrometry. Spectra were then identified with SpectMHC and compared across samples by label-free quantitation. (B) Total number of unique peptides specific to the H-2D^b and H-2K^b mouse alleles that were quantified across the experiment. (C) Volcano plots of the IFN γ and reovirus-regulated MHC-I peptides. Shown are the $-\log(p\text{-value})$ based on F-tests (y-axis) versus the $\log_2$ fold change (reovirus or IFN γ -treated/control) (x-

axis). Induced (blue dots) or repressed (green dots) peptides are those with normalized $\log_2$ fold changes ≥ 1 or ≤ -1 and p -values ≤ 0.05 (adjusted using Benjamini–Hochberg multiple hypothesis correction). Peptides derived from reovirus source proteins are shown in red. (D) MHC-I peptides that are specifically induced by reovirus (mean $\log_2$ fold change ≥ 1 and p -values ≤ 0.05) and not by IFN γ including several examples and enriched GO terms (biological process) pertaining to the source proteins. (E) MHC-I peptides that are induced by both reovirus and IFN γ (mean $\log_2$ fold change ≥ 1 and p -values ≤ 0.05) including several examples and enriched GO terms (biological process) pertaining to the source proteins.

Figure 2.

Summary of temporal quantitative proteomics of reovirus-infected ID8 cells. (A) ID8 cells were harvested at five time points over 48 h of reovirus infection at MOI 1 or MOI 10 followed by multiplexed, quantitative proteomics using tandem mass tags (TMT). Shown is a heatmap with six k-means clusters (colored alongside) of the multiplexed relative temporal proteomics data set ($n = 6561$ proteins). (B) Enriched GO terms (derived using PANTHER) in k-means Cluster 1 (highly upregulated by reovirus) from A. (C) Temporal relative intensity from the proteomics data set of reovirus proteins Lambda 1 and Mu 1 during the 48 h course of infection (MOI 1 and 10, five time points each). (D) Temporal relative intensity from the proteomics data set of representative viral response proteins (APOL9B, CCL5, GBP4, and OAS1A) during the 48 h course of infection (MOI 1 and 10, five time points each). (E) Temporal relative intensity from the proteomics data set of representative antigen presentation proteins (B2M, H-2D1, TAP1, and TAP2) during the 48 h course of infection (MOI 1 and 10, five time points each).

Figure 3. Matching MHC-I peptide and source proteome quantitation during reovirus infection in ID8 cells. (A) MHC-I peptides were matched to all mouse protein accessions from the UniProt database and then these accessions were considered source proteins if they appeared in the proteomics data set shown in Figure 2. Some peptides match multiple source proteins. (B) Number of MHC-I peptides from the data set in Figure 1, for which protein matches were found (matched) or not found (unmatched) in the quantitative proteomics data set (both H-2D^b (Db) and H-2K^b (Kb) specific MHC-I peptides). (C) Correlation of log₂ ratios (Reovirus/Control) between MHC-I peptides from the MHC-I ligandome analysis from Figure 1 and their matching source proteins from the proteomics data set. Data are compared at 48 h of reovirus infection. (D) Representative examples of MHC-I peptides for which log₂ ratios (Reovirus/Control) are correlated (APOL9B) and uncorrelated (SMC4) at the MHC-I peptide and source protein levels.

Figure 4.

Discovery of tumor MHC-I ligands in tumor and immune cells *in vivo*. (A) Experimental setup for *in vivo* MHC-I ligandome monitoring. Naive mice or those harboring ID8 MOSE tumor cells were injected with either PBS or reovirus (5×10^8 PFU, *i.p.*). Spleens and tumors (spleens only from naïve mice) were harvested at 7–10 days post injection and MHC-I peptides were compared by immunoprecipitation and LC-MS/MS with label-free quantitation. (B) Volcano plots for the number of increased (blue dots) and decreased (green dots) MHC-I peptides ($\log_2$ fold changes ≥ 3 or ≤ -3 and p -values ≤ 0.05) in the naïve mice spleens, tumor-bearing mouse spleens, and tumors. The $-\log(p\text{-value})$ is based on F-tests implemented in the R package *genefilter* and adjusted using Benjamini–Hochberg multiple hypothesis correction. (C) Representative examples of the MHC-I peptide intensities increased by reovirus across tumors or spleen of the tumor-bearing mice, but not in naïve mice. Shown also are intensities of example MHC-I peptides derived from reovirus source proteins quantified in the experiment.

Figure 5.

Immunogenicity testing of reovirus-induced tumor MHC-I peptides. (A) Distribution of mean log₂ fold changes, number of PSMs identified (as an indication for MHC-I abundance), and NetMHC % rank for significantly induced peptides at the tumor from the data in Figure 4, showing the MHC-I peptides chosen (randomly) for validation by IFN γ assay. Any MHC-I peptides also induced by reovirus in the naive spleen (shown in purple) were excluded from being chosen for IFN γ assays. (B) Heatmap of relative intensity in the tumor and spleen of tumor-bearing mice of MHC-I peptides selected for IFN γ assays (from the data in Figure 4). * = oxidized methionine. (C) MHC-I peptides from B were incubated with splenocytes collected from reovirus-infected and nontreated mice (both tumor-bearing). All incubations were done in the presence of CD28 stimulation, and concanavalin A (ConA, 2 μ g/mL) was used as a positive control. IFN γ production (y -axis) was determined by ELISA.