CT26 Neoantigen Presentation and Immunogenicity

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Seeta Nyayapathy[1], Richard Jones[2], Michael Ford[2], Micah Doty[1], Julie M Rumble[1]

[1]Cayman Chemical Company, Ann Arbor, Michigan, United States, [2]MS Bioworks LLC, Ann Arbor, MI

MS Bioworks
Protein Mass Spectrometry Services

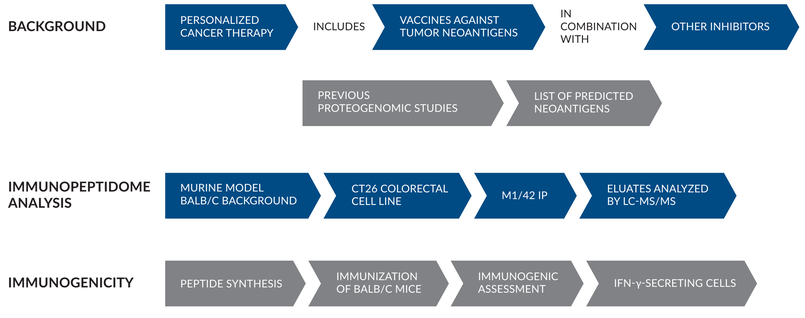
KEY FINDING

Immune responses elicited to neoantigens are identified through immunopeptidome profiling.

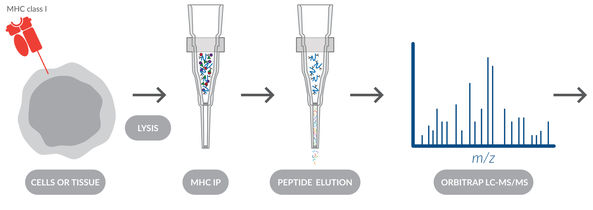
ABSTRACT

The foundation of personalized cancer therapy is teaching a patient's own immune system to directly kill the cancer cells. Vaccination against tumor antigens is one method being explored to generate immune responses against tumors and may be particularly effective in conjunction with therapies currently in the clinic, such as checkpoint inhibition. Identification of antigens for use in a vaccine is the pinch point of this method, and many algorithms have been developed in an attempt to predict what epitopes will be both presented and immunogenic. Importantly, both public (common to multiple individuals/cancer types) and private (single individual) epitopes may contribute to the development of effective vaccines. Experimental identification of presented and immunogenic epitopes is crucial to building effective anti-cancer vaccines. Using immunopeptidome profiling, we have narrowed a collection of published potential neoantigens to those that were presented by MHC class I in the CT26 mouse colorectal cancer cell line. These peptides were synthesized and used to immunize BALB/c mice. The immunogenicity of the peptide mix was then evaluated by ELISpot to assess IFN-γ responses in immunized mice. These experiments support a complete workflow for the selection of potential cancer neoantigens that experimentally exhibit both MHC presentation and immunogenicity.

EXPERIMENTAL APPROACH



CT26 MHC CLASS I IMMUNOPEPTIDOME PROFILING

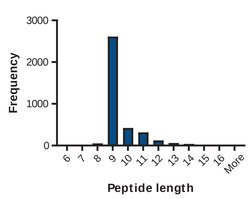


CT26 Neoantigens
GYQKMKALL
SPSYVYHQF
KYLSVQSQLF
MPHSLLPLVTF
QGPMALRIF

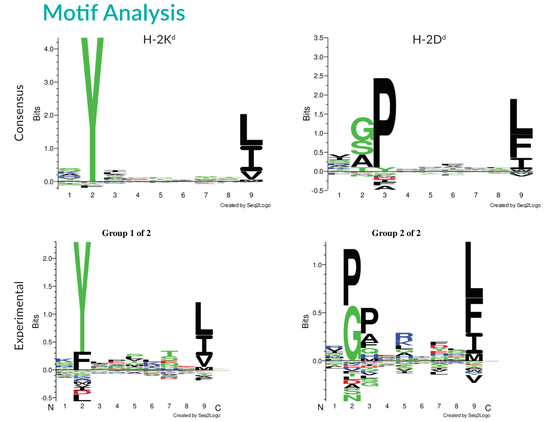
- CT26 cells were lysed, and the resulting preparations were subjected to immunoaffinity capture with M1/42 CnBr resin. Following multiple washes, MHC class I peptides were eluted from the MHC cleft with acid.
- Purified peptides were analyzed by LC-MS/MS using nano-scale chromatography combined with an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer using collision-induced dissociation (CID) or electron-transfer/higher-energy collision dissociation (EThcD) fragmentation.
- PEAKS was used for peptide identification. Peptides identified in Laumont *et al.* were appended to the mouse proteomic database. Five of these were experimentally confirmed in the CT26 immunopeptidome.

CT26 IMMUNOPEPTIDOME – PEPTIDE LIST

Peptide Distribution



Motif Analysis



- **(Left)** Histogram distribution of peptide length showed that majority of the peptides are 9-mers, consistent with MHC class I presentation.
- **(Right)** An unbiased motif analysis was performed with GibbsCluster to identify motifs of the peptide list. MHC class I peptide sequences clustered into two motifs, which resemble consensus.

PEPTIDE SYNTHESIS

Peptide Properties

Peptide	Mass (MW)	Length	Observed MW on MS Spectrum	Purity (%)
GYQKMKALL	1,050.59	9	1,050.2	88.29
SPSYVYHQF	1,126.51	9	1,126.2	78.51
KYLSVQSQLF	1,211.66	10	1,211.4	92.19
MPHSLLPLVTF	1,253.68	11	1,253.4	74.49
QGPMALRIF	1,031.56	9	1,031.2	93.03

Crude peptides were prepared by Fmoc-based SPPS (CEM, Liberty Blue™). Peptides were removed from their resins by cleavage in high trifluoroacetic acid. These crude peptides were purified on C18 semi-prep reversed phase chromatography by a methanol gradient (Phenomenex) by HPLC (Agilent). Rotary evaporation was used to remove methanol and lyophilized the aqueous fractions. MW of the HPLC-purified peptides was confirmed by mass spectrometry (FIA-MS).

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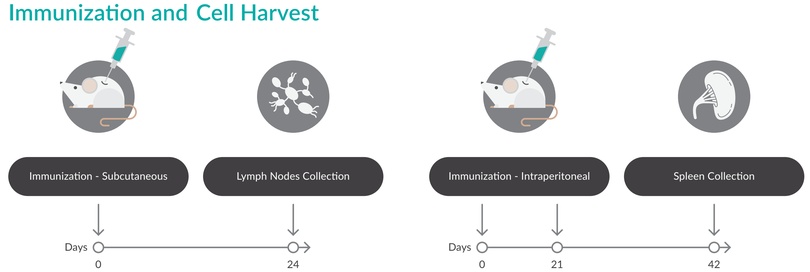
References and Resources

1. Laumont, C.M., Vincent, K., Hesnard, L., *et al.* Noncoding regions are the main source of targetable tumor-specific antigens. *Sci. Transl. Med.* **10(470)**, eaau5516 (2018).
2. Castle, J.C., Loewer, M., Boegel, S., *et al.* Immunomic, genomic and transcriptomic characterization of CT26 colorectal carcinoma. *BMC Genomics* **15(1)**, 190 (2014).
3. GibbsCluster: https://services.healthtech.dtu.dk/service.php?GibbsCluster-2.0
4. MotifViewer: https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1

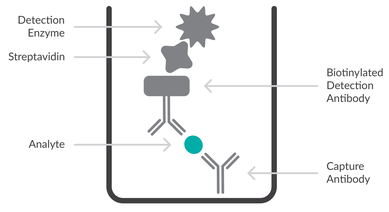
IMMUNOGENICITY BY ELISPOT

Immunization and Cell Harvest

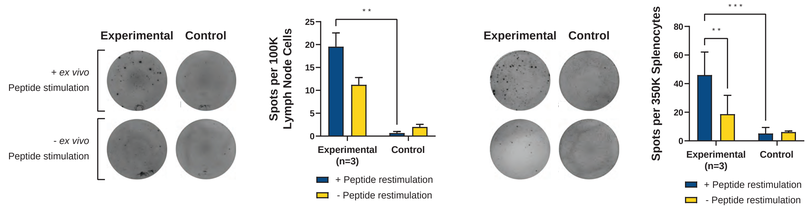


- **(Left)** 100 µg of peptide pool in 200 µl emulsion of IFA with 30 µg of CpG-1826 (mTLR9 stimulator) administered subcutaneously into BALB/c mice (n=3) and euthanized at 24 days.
- **(Right)** Supplemented with a booster at 21 days and euthanized at 42 days.
- Controls consisted of immunizations without peptide pool.
- Draining lymph nodes or spleens were dissected and a single cell suspension was made using a 70 µm cell strainer. Cells were seeded at multiple densities ± *ex vivo* peptide restimulation.

IFN-γ ELISpot



- Post-immunization immunoreactivity to the peptide pool was evaluated using IFN-γ ELISpot.
- Analysis was performed on a Mabtech IRIS™ FluoroSpot and ELISpot reader.
- The number of spots in each well corresponds to the number of IFN-γ-secreting cells.



ELISpot data shows a significant increase in number of IFN-γ-secreting cells in lymph node cells **(Left)** and splenocytes **(Right)** of mice administered the peptide pool and *ex vivo* peptide restimulation when compared to control mice and cells without *ex vivo* peptide restimulation, suggesting the immunogenicity of the CT26 neoantigen peptide pool. ** indicates p<0.005, *** indicates p<0.0005

SUMMARY

- We have demonstrated a complete workflow from experimentally identifying potential neoantigens to assessing immunogenicity of the peptide pool.
- Immunogenicity of murine epitopes can be assessed through multiple immunization strategies.
  - Route of administration can be tailored to the cells that need to be evaluated.
  - Prime/boost may be required to achieve optimal immunogenicity.
- Cayman Services has the capacity to work with different cell types and multiple readouts of immunogenicity, including ELISpot/FluoroSpot, flow cytometry, and functional assays.
- Cayman Services' experienced scientists can help to design your experimental approach to best answer your research questions about neoantigen identification.