"Grey Genome" derived MHC-presented peptides are a novel source of tumor specific targets for cancer immunotherapies

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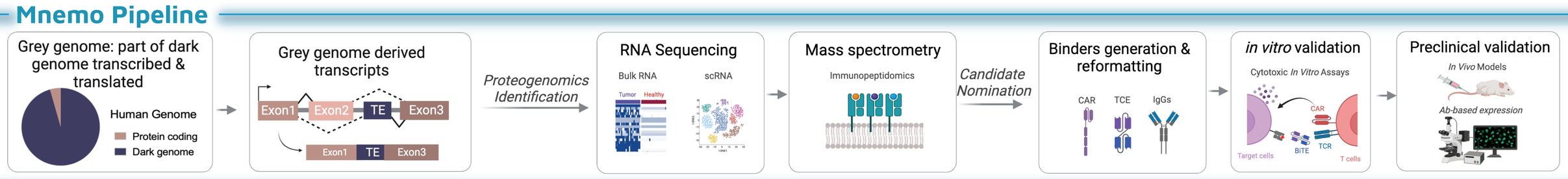
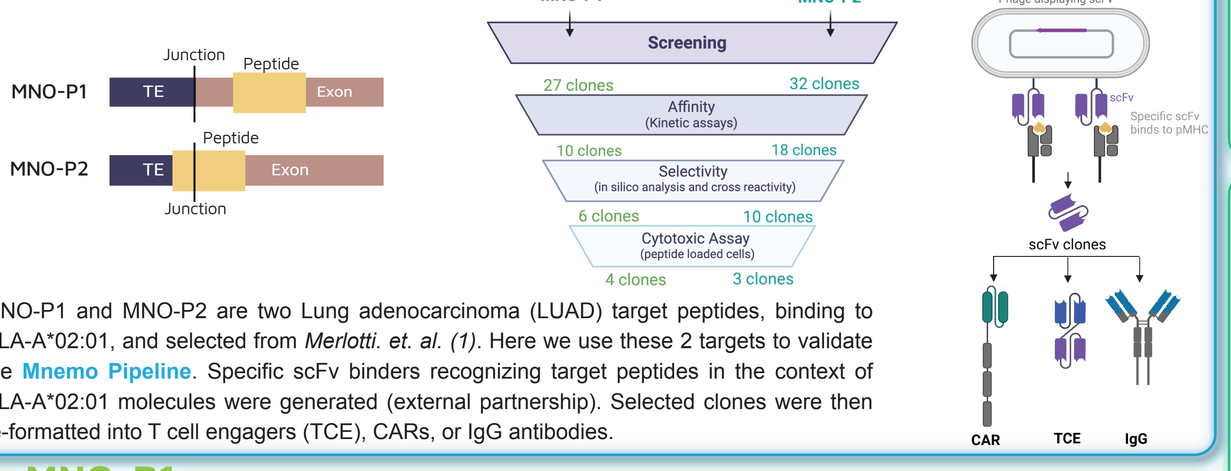
The 'dark genome' represents >90% of human genome and is composed mainly of unannotated regions and Transposable Elements (TEs). We define as **'grey genome'** the part of the dark genome which is transcribed and translated. A major challenge for cancer-specific therapeutics resides around specificity and recurrence of cancer targets. **We developed a discovery engine that enables the identification of recurrent tumor-specific targets for immunotherapies, exploiting a new family of antigens derived from the grey genome. These antigens arise from junctions between exons and TEs as the result of non-canonical splicing events.** First discovered in non-small cell lung cancer (NSCLC), these junctions are shared across patients, tumor specific and generate peptides bound to HLA-I molecules. Moreover, junction-derived peptides are immunogenic *in vitro* and specific cytotoxic T cells are found in primary NSCLC tumor and draining lymph nodes samples (1). After generating de novo TCR-like restricted antibodies against specific junction-derived peptides, we demonstrate their expression on target tumor cell lines using high resolution microscopy. The TCR-like antibodies display antigen-specific cytotoxicity against target cells, both as bispecific T cell engagers (TCEs) and as chimeric antigen receptors (CARs). In summary, we present end-to-end validation of our in silico identified peptide antigens, from expression characterization to in vitro preclinical validation in multiple immunotherapeutic modalities.

Mnemo Pipeline



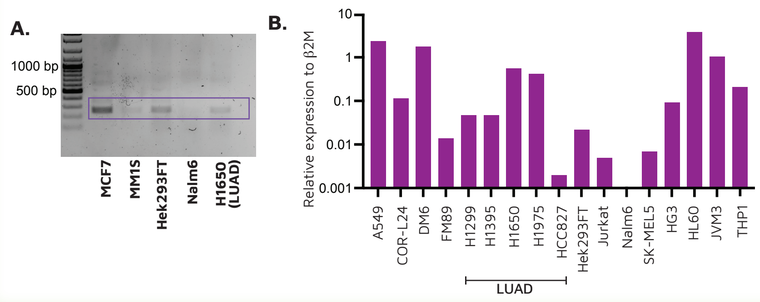
Mnemo POC targets and binder generation



MNO-P1 and MNO-P2 are two Lung adenocarcinoma (LUAD) target peptides, binding to HLA-A*02:01, and selected from *Merlotti. et. al. (1)*. Here we use these 2 targets to validate the **Mnemo Pipeline**. Specific scFv binders recognizing target peptides in the context of HLA-A*02:01 molecules were generated (external partnership). Selected clones were then re-formatted into T cell engagers (TCE), CARs, or IgG antibodies.

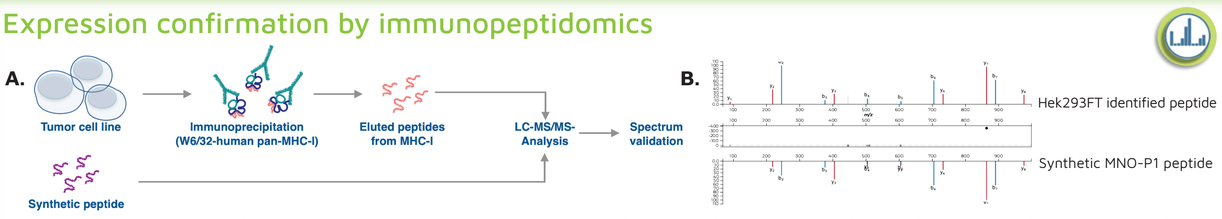
MNO-P1

Transcript validation and quantification



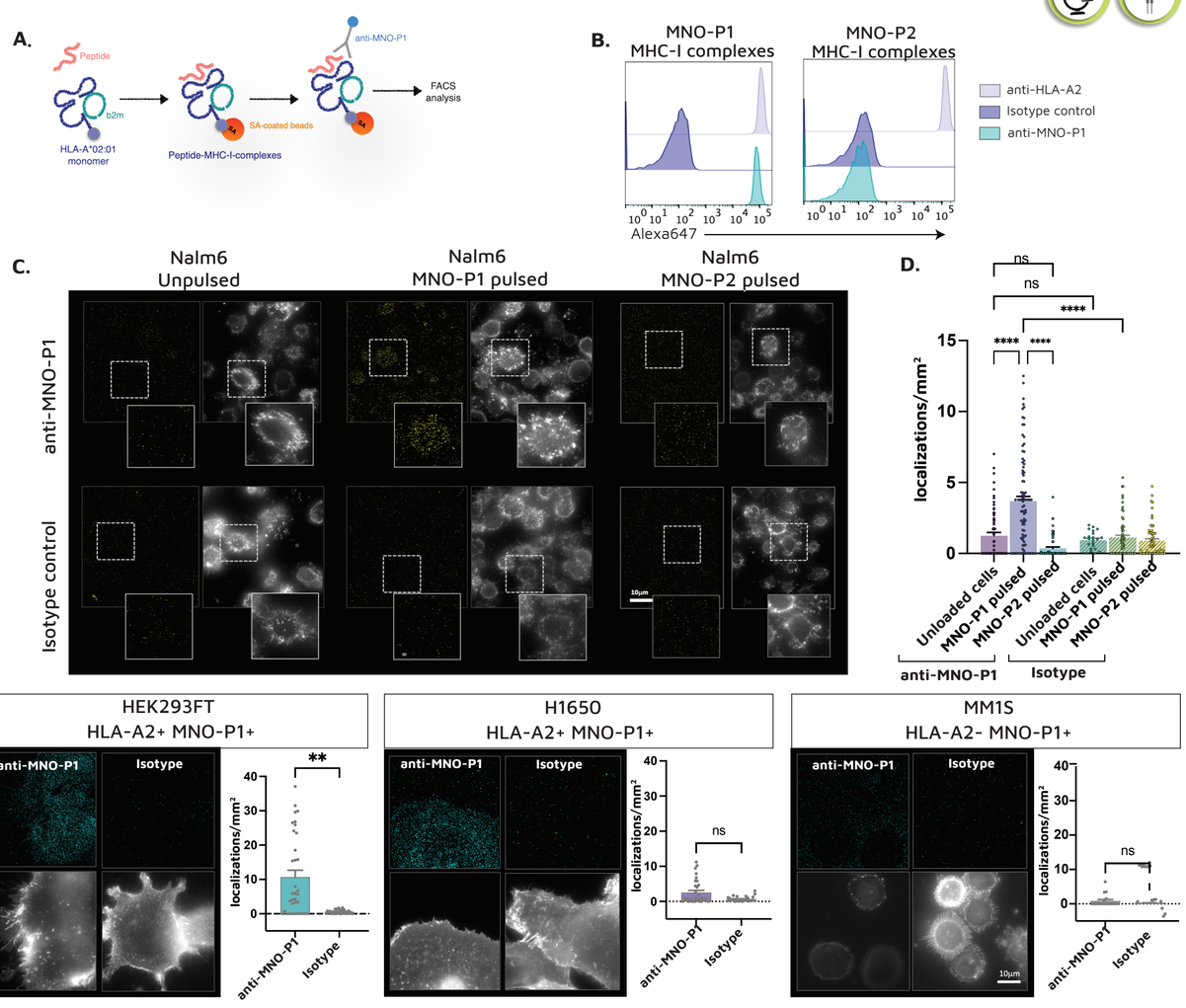
MNO-P1 RNA expression is validated and quantified using PCR-based assays. A, RT-PCR for MNO-P1 transcripts in different cell lines. PCR products were run in 2% agarose gel obtaining the expected amplification product size and sequence after purification. **B**, qRT-PCR using TaqMan probes for MNO-P1 transcript in different tumor cell lines. Relative expression to housekeeping gene is shown.

Expression confirmation by immunopeptidomics



MNO-P1 peptide is identified in immunopeptidomic samples. A, Schematic of immunopeptidomics detection and spectrum validation. MNO-P1 was identified in different tumor cell lines including HEK293FT and Nalm6. **B**, MS2 spectrum comparison between endogenous detected MNO-P1 peptide in HEK293FT and synthetic peptide using Universal Spectrum Explorer. Top spectrum was generated by searching HEK293FT immunopeptidome against a proprietary library using HTSequestsearch engine. Bottom spectrum results from the LCMS analysis of synthetic MNO-P1 peptide.

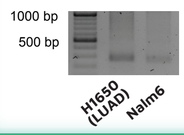
Expression characterization using high resolution microscopy



MNO-P1 is presented on MHC-I molecules at the cell surface. A. Schematic of MNO-P1 antibodies FACS test. **B.** MNO-P1 antibodies or isotype control were used to stain specific peptide-MHC-I-complexes by FACS. Anti-HLA-A2 antibodies were used as positive control of peptide-MHC-I complexes folding. **C-D**. High resolution microscopy of MNO-P1 expression on Nalm6 using dSTORM. Cells were loaded or not with MNO-P1 peptide or with MNO-P2 as irrelevant peptide control. After fixation, cells were stained with MNO-P1 antibodies or isotype control Alexa647 coupled IgGs (left images) and WGACF488 was used as membrane counterstain (right images). (B). MNO-P1 localizations per surface area was quantified (D). **E**. High resolution microscopy of MNO-P1 expression on HLA-A2+ or HLA-A2- cells, expressing MNO-P1 at the transcript level (HEK293FT, H1650 and MM1S), and their localizations per surface area (E). WGACF488 was used as membrane counterstain (lower images). All images were acquired in a Nanoimager microscope. Reconstruction of dSTORM images was done using CODI alto. Representative images are shown and n>22 cells per condition were quantified. Kruskal-Wallis test was applied (****p<0.0001)(**p<0.001).

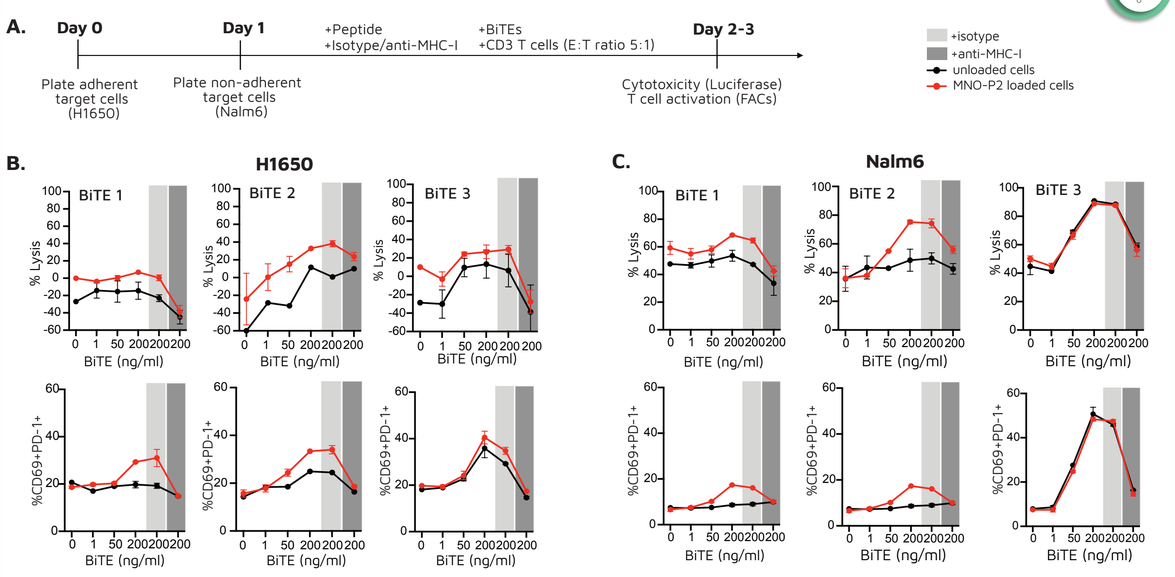
MNO-P2

Transcript validation on target cells



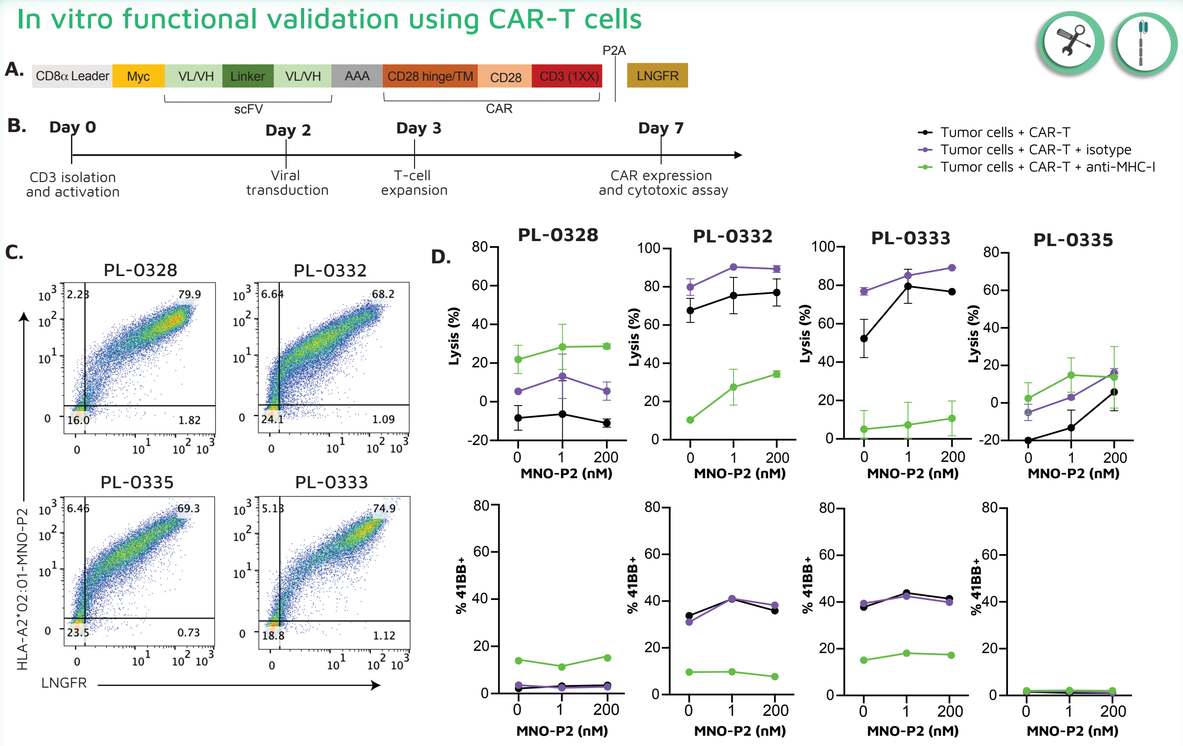
MNO-P2 RNA expression is validated by RT-PCR. A, RT-PCR for MNO-P2 transcripts in 2 target cell lines used for target validation. PCR products were run in 2% agarose gel obtaining the expected amplification product size and MNO-P2 sequence.

In vitro functional validation using Bispecific T cell Engagers (BiTEs)



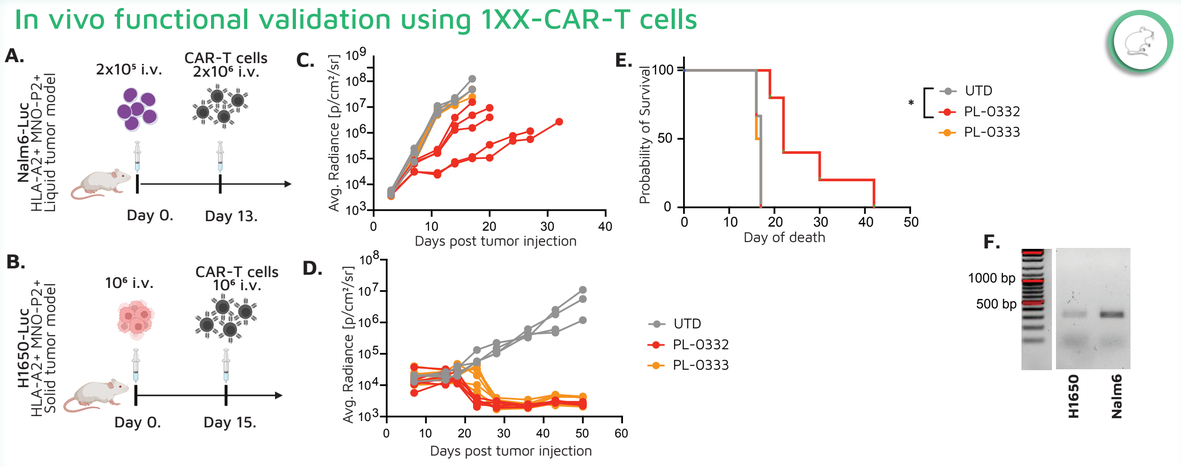
MNO-P2 targeting BiTEs redirect in vitro T-cell killing in the presence of MNO-P2-expressing cells. A, Schematic for cytotoxic assay. **B**, Dose–response curves of anti-MNO-P2 BiTEs molecules against luciferase-labeled, MNO-P2-expressing H1650 (B) and Nalm6 (C). Target cells loaded or not with MNO-P2 peptide at 1µM, in the presence of blocking anti-MHC-I antibody or isotype control, were co-cultured with human T cells at an E:T cell ratio of 5:1, using a dose range of anti-MNO-P2 targeting BiTEs. 24 or 48 hours later, specific cytotoxicity was assessed with a luminescence readout, and effector T cell expression of CD69 and PD-1 was measured by flow cytometry. 3 different clones for BiTEs were used and are showed in left, middle and right panel, respectively,for each cell line.

In vitro functional validation using CAR-T cells



MNO-P2 CAR-T cells mediate potent cytotoxic activity in vitro. A, Schematic diagram of MNO-P2-CAR constructs. **B**, Schematic of CAR-T cell production. **C**, FACS analysis showing CAR expression for MNO-P2 CAR constructs screened after CAR optimization. Expression was measured 5 days after CAR retrovirus transduction (based on anti-LNGFR and HLA-A*02:01-MNO-P2 tetramer staining) **D**, Top: cytotoxicity of MNO-P2 CAR-T cells determined by luciferase-based killing assay following a co-culture with MNO-P2-expressing **Cell line 5**, for 48h at a 1:1 E:T ratio. Bottom: cell surface expression of activation marker 41BB on CAR-T cells following cytotoxic assay

In vivo functional validation using 1XX-CAR-T cells



MNO-P2 CAR-T cells mediate in vivo antitumor response. **A-B**, Schematic of target and effector cells injections using a liquid tumor model (Nalm6) (A), and a solid tumor model (H1650) (B). **C, D**, In vivo tumor growth was followed by measuring luciferase intensity in an IVIS imaging system, after MNO-P2 CAR-T cells injections (PL-0332 or PL-0333) or untransduced (UTD) T cells, for both liquid (C) and solid (D) tumor model. **E**, Survival curves for Nalm6 liquid tumor model (*p=0.0177). **F**, Expression of MNO-P2 detected by RT-PCR in target cells. H1650 cells were isolated from mouse lungs after 29 days post injection. Nalm6 cells were isolated from mouse bone marrows after 13 days post injection.

Conclusions

End-to-end validation of Mnemo's pipeline, from in silico detection to preclinical functional assays:

- MNO-P1 and MNO-P2 target expression was validated in multiple tumor cell lines at the transcript level by PCR-based assays
- MNO-P1 expression was confirmed and characterized the protein level by mass spectrometry and imaging using IgG reformatted binders
- MNO-P2 binders were reformatted in different immunotherapy formats (BiTE and CAR) and tested in different screening assays to select optimal format
- MNO-P2 targeting binders promote specific cytotoxicity against MNO-P2 expressing cell lines in both BiTE and CAR format, and MNO-P2 targeting CAR-T cells show in vivo antitumour response

Grey Genome Antigens can be used to specifically target tumor cells in vitro and in vivo

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References

1. Noncanonical splicing junctions between exons and transposable elements represent a source of immunogenic recurrent neo-antigens in patients with lung cancer. ***Sci Immunol**, 2023. Feb 3;8 (80):eabm6359.*