Tumor-wide RNA splicing aberrations generate immunogenic public neoantigens

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ABSTRACT

Tumor heterogeneity and low somatic mutational burden are challenges facing immunotherapy in gliomas. Recent studies detected alternative-splicing (AS) events in various cancer types that could potentially translate into tumor-specific proteins. Our study investigates tumor-specific AS to identify novel MHC-I-presented neoantigen candidates through an integrative transcriptomic and proteomic computational pipeline, which is complemented by an extensive spatial analysis of AS candidates in glioma samples. **With this unique *in silico* pipeline, we reveal immunogenic neoantigen candidates generated by cancer-specific, tumor-wide, public AS events.** Bulk pan-cancer RNA-sequencing data from TCGA was analyzed against GTEx normal tissue data to identify tumor-specific AS events (neojunctions). Proteosome processing and MHC-binding predictions were used subsequently to identify neojunction-derived neoantigen candidates that were likely presented by MHC Class I. Expression of neojunctions and their peptide derivatives was validated by RNA-seq and mass spectrometry data sets. Moreover, our unique spatially-mapped database allowed for the identification of tumor-wide neojunctions. 2 HLA-A*0201-binding candidates were presented and detected by HLA-IP MS, elicited a CD8+ T-cell-specific immune response and initiated a tumor-killing response in GBM tumor lines. These findings unveils a new class of epitopes for T-cell-based immunotherapies against public AS-derived targets in tumors.

IDENTIFICATION OF NEOJUNCTIONS IN TUMORS

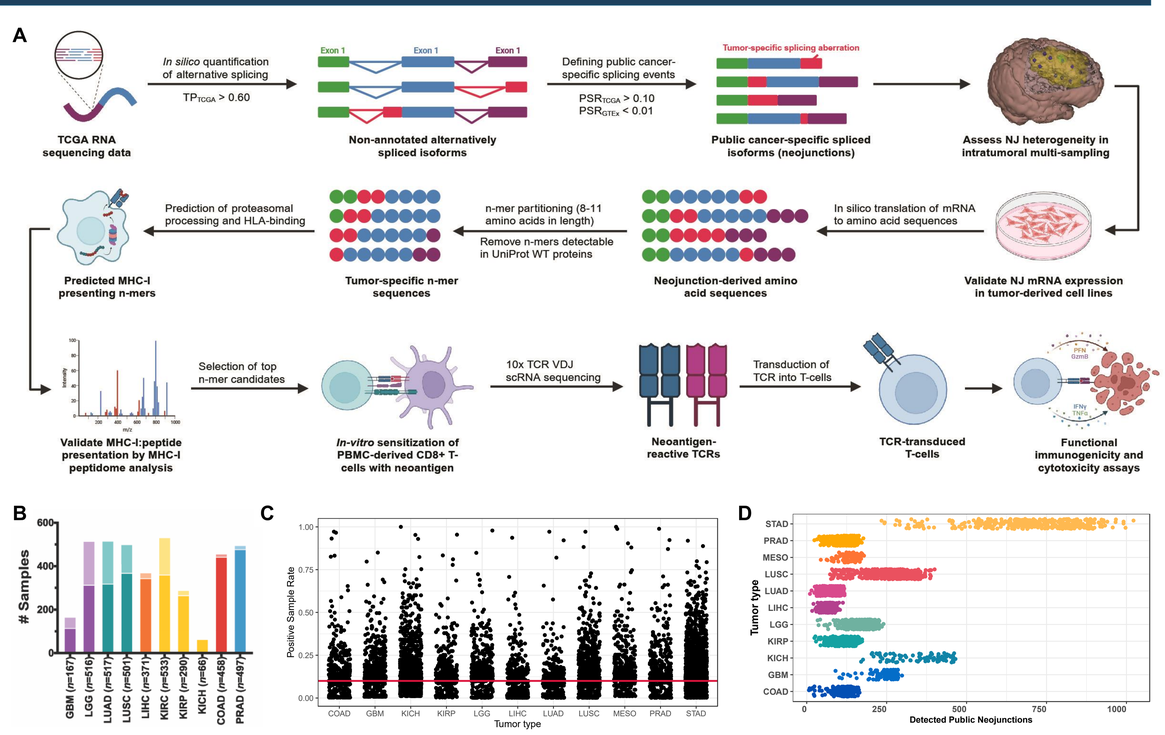

Figure 1: Identification of neojunctions (NJs) in TCGA samples. A. Pipeline for identifying putative, tumor-wide, cancer-specific alternative splicing events from RNA-sequencing data from The Cancer Genome Atlas (TCGA) **B.** Number of TCGA samples by disease subgroups. Samples that retained for downstream analysis (upper bars) have a tumor purity of at least 60%. **C.** Interpatient frequency (positive sample rate; PSR) of putative neojunctions identified in each tumor type. Public neojunctions are defined as those with a PSR ≥ 10% (red line). **D.** Total public neojunctions detected per sample across tumor types.

NEOANTIGEN:HLA-BINDING BINDING PREDICTION

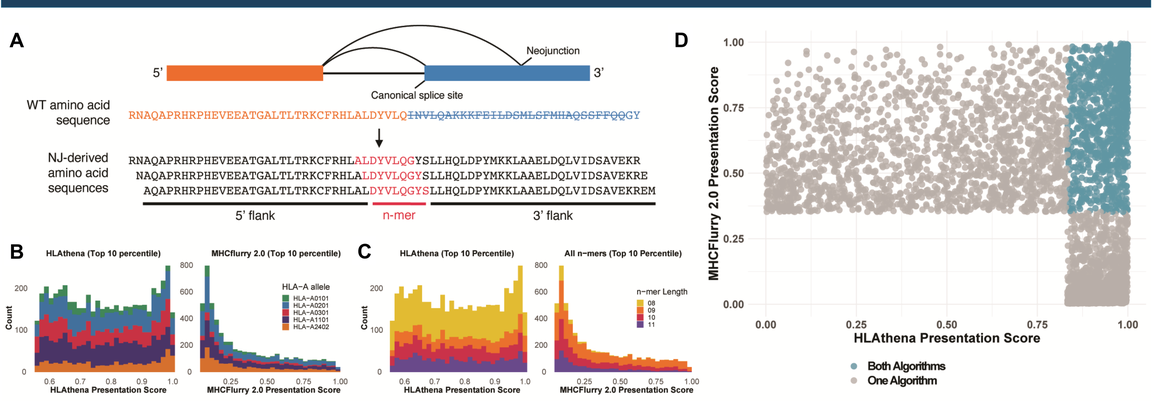

Figure 2: *in silico* prediction of neojunction-derived peptide presentation by HLA. A. Diagram illustrating the mechanism of neoantigen production by the introduction of a neojunction. Multimer partitioning was employed subsequently to generate a peptide bank for prediction analysis. **B-C.** Histogram illustrating the scores pertaining to the likelihood of peptide presentation calculated from each algorithm for the top scoring 10-percentile of n-mers categorized by HLA-allele (**B**) or n-mer length (**C**). **D.** Dot plot showing the overlay of the top scoring 10-percentile of both algorithms. Top-scoring final candidates are indicated in blue as the candidates that scored in the top 10 percentile in both algorithms.

NJ-SPECIFIC TCRS ARE IDENTIFIED FROM DONOR-DERIVED PBMCS

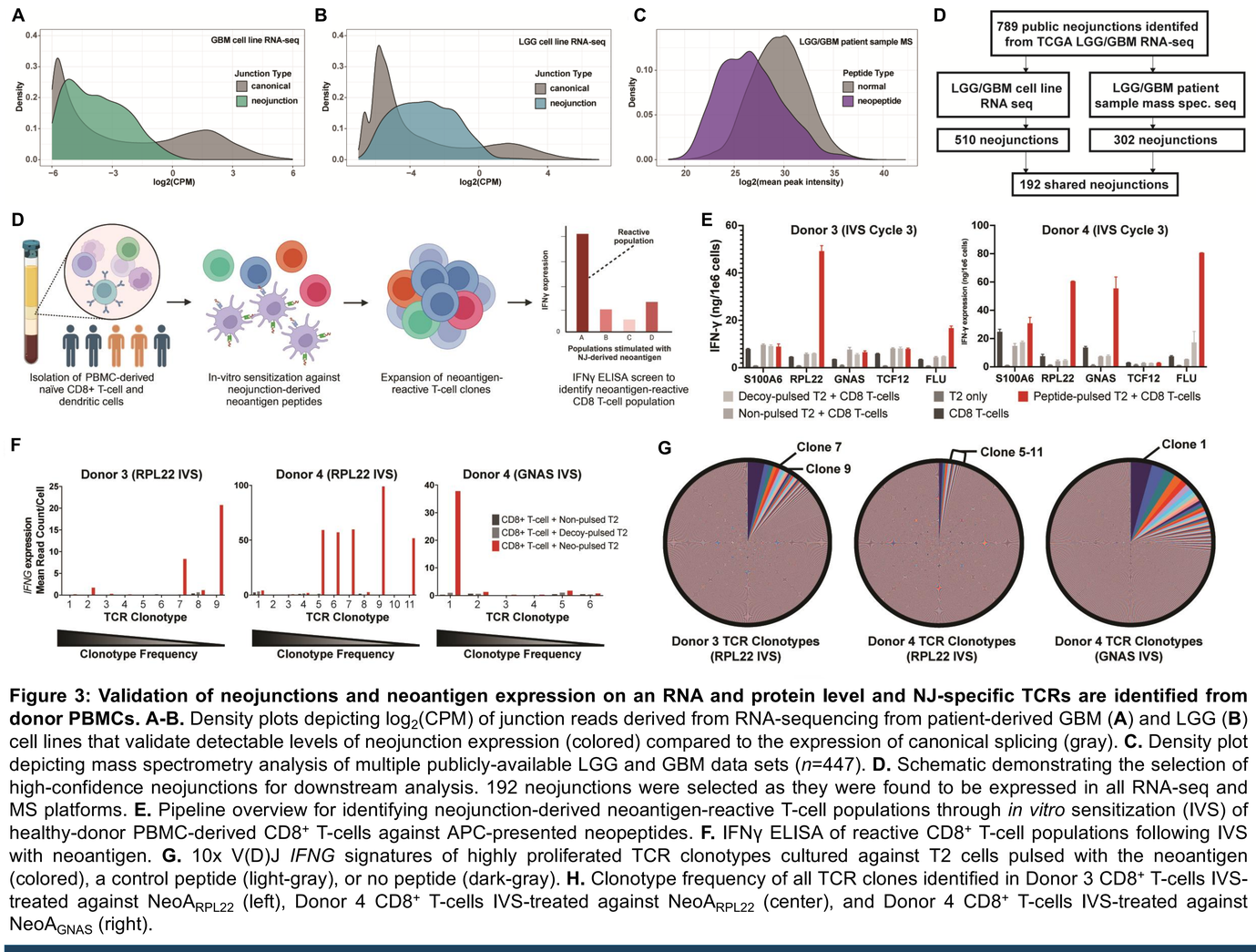

Figure 3: Validation of neojunctions and neoantigen expression on an RNA and protein level and NJ-specific TCRs are identified from donor PBMCs. A-B. Density plots depicting $\log_2$(CPM) of junction reads derived from RNA-sequencing from patient-derived GBM (**A**) and LGG (**B**) cell lines that validate detectable levels of neojunction expression (colored) compared to the expression of canonical splicing (gray). **C.** Density plot depicting mass spectrometry analysis of multiple publicly-available LGG and GBM data sets (*n*=447). **D.** Schematic demonstrating the selection of high-confidence neojunctions for downstream analysis. 192 neojunctions were selected as they were found to be expressed in all RNA-seq and MS platforms. **E.** Pipeline overview for identifying neojunction-derived neoantigen-reactive T-cell populations through *in vitro* sensitization (IVS) of healthy-donor PBMC-derived CD8⁺ T-cells against APC-presented neopeptides. **F.** IFNγ ELISA of reactive CD8⁺ T-cell populations following IVS with neoantigen. **G.** 10x V(D)J *IFNG* signatures of highly proliferated TCR clonotypes cultured against T2 cells pulsed with the neoantigen (colored), a control peptide (light-gray), or no peptide (dark-gray). **H.** Clonotype frequency of all TCR clones identified in Donor 3 CD8⁺ T-cells IVS-treated against $NeoA_{RPL22}$ (left), Donor 4 CD8⁺ T-cells IVS-treated against $NeoA_{RPL22}$ (center), and Donor 4 CD8⁺ T-cells IVS-treated against $NeoA_{GNAS}$ (right).

SPATIAL VALIDATION IN GBM/LGG AND PAN-CANCER

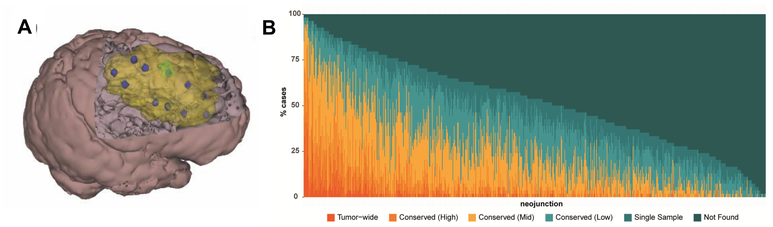

Figure 4: Spatial validation of neojunctions and neoantigen expression. A. 3-D models of 10 mapped and maximally distanced biopsies (blue) were taken within each tumor. **B.** Distribution of glioma-specific neojunctions (*n*=789, columns) ordered by intratumoral heterogeneity in patients. Neojunctions are considered tumor-wide if they are found across 100% of intratumor samples within the same patient, highly conserved if found in > 70% of samples but not 100% of samples, moderately conserved if found in > 30% of samples but ≤ 70% of samples, or lowly conserved if found in at least two samples but ≤ 30% of samples.

NJ-DERIVED PEPTIDES ELICIT CD8+ T-CELL RESPONSE

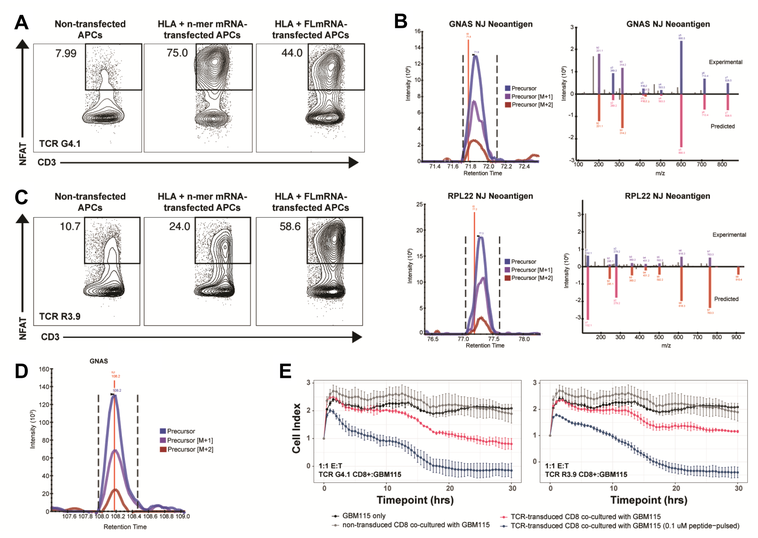

Figure 5: Neojunction-derived peptides elicit CD8+ T-cell driven responses. B-C. NJ_{GNAS}-derived (**B**) and NJ_{RPL22}-derived (**C**) neoantigen-specific TCR-transduced triple-reporter Jurkat76 cells were co-cultured against transfected COS7. TCR activation of TCR-transduced triple-reporter Jurkat76/CD8 was measured by flow cytometry analysis of NFAT-GFP. **D.** Mass spectrometry spectra of NJ_{GNAS}-derived (bottom) and NJ_{RPL22}-derived (top) neoantigen n-mers detected through IP-MS/MS following HLA-A*02:01 pulldown of HLA-A*02:01 and full-length neojunction-encoding mRNA-transduced COS7 cells and **E.** NJ_{GNAS}-derived (left; colored), NJ_{RPL22}-derived (right; colored) neoantigen-specific TCR-transduced, or non-transduced (gray) CD8⁺ T-cells cultured against GBM115.

CONCLUSION

- Novel integrative *in silico* pipeline identified public, tumor-wide neojunctions.
- 3-D spatial sample analysis revealed neojunctions expressed tumor-wide.
- Neojunction-derived neoepitopes elicited a robust CD8⁺ T-cell response.

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