Immunogenomic determinants of tumor microenvironment correlate with superior survival in high-risk neuroblastoma

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ABSTRACT

Background Tumor-infiltrating CD8+ T cells and neoantigens are predictors of a favorable prognosis and response to immunotherapy with checkpoint inhibitors in many types of adult cancer, but little is known about their role in pediatric malignancies. Here, we analyzed the prognostic strength of T cell-inflamed gene expression and neoantigen load in high-risk neuroblastoma. We also compared transcriptional programs in T cell-inflamed and non-T cell-inflamed high-risk neuroblastomas to investigate possible mechanisms of immune exclusion.

Methods A defined T cell-inflamed gene expression signature was used to categorize high-risk neuroblastomas in the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program (n=123), and the Gabriella Miller Kids First (GMKF) program (n=48) into T cell-inflamed, non-T cell-inflamed, and intermediate groups. Associations between the T cell-inflamed and non-T cell-inflamed group, MYCN amplification, and survival were analyzed by Cox proportional hazards models. Additional survival analysis was conducted after integrating neoantigen load predicted from somatic mutations. Pathways activated in non-T cell-inflamed relative to T cell-inflamed tumors were analyzed using causal network analysis.

Results Patients with T cell-inflamed high-risk tumors showed improved overall survival compared with those with non-T cell-inflamed tumors (p<0.05), independent of MYCN amplification status, in both TARGET and GMKF cohorts. Higher neoantigen load was also associated with better event-free and overall survival (p<0.005) and was independent of the T cell-inflamed signature. Activation of MYCN, ASCL1, SOX11, and KMT2A transcriptional programs was inversely correlated with the T cell-inflamed signature in both cohorts.

Conclusions Our results indicate that tumors from children with high-risk neuroblastoma harboring a strong T cell-inflamed signature have a more favorable clinical outcome, and neoantigen load is a prognosis predictor, independent of T cell inflammation. Strategies to target SOX11 and other signaling pathways associated with non-T cell-inflamed tumors should be pursued as potential immune-potentiating interventions.

BACKGROUND

The presence of effector T cells in the tumor microenvironment has been associated with improved survival in adults with many types of cancer.1-3 Several studies of melanoma and other solid tumors have demonstrated that expression of dendritic cell (DC) and CD8+ T cell-associated genes, or a T cell-inflamed gene signature, is correlated with favorable prognosis and response to immunotherapy with checkpoint blockade therapy or tumor vaccines.4-8 T cell-inflamed tumors are characterized by type I interferon (IFN) activation, immune potentiating chemokines, antigen presentation, cytotoxic effector molecules, and activated CD8+ T cells.9 The inflamed tumor microenvironment is additionally characterized by IFN-induced inhibitory pathways such as programmed death-ligand 1 (PD-L1) and indoleamine-2, 3 dioxygenase, and higher proportions of FOXP3+ regulatory T cells.9 Other known predictors of response to immunotherapy include, but are not limited to, a high tumor mutational burden (TMB)10 and a high neoantigen load.11 While TMB and neoantigen load often highly correlate with each other,12 previous studies have demonstrated both markers have low correlation with the presence of T cell inflammation,10 12 13 and TMB (or neoantigen load) and T cell-inflamed gene expression may represent non-redundant predictive biomarkers of immune checkpoint inhibitors efficacy.14

In contrast, resistance to immunotherapy has been correlated with tumors that lack the T cell-inflamed signature. There is increasing evidence that signaling pathways intrinsic to the neoplastic cells may impair the local immune response in tumors. Tumor cell-intrinsic activation of the WNT/β-catenin pathway has been associated with a lack of T
cell infiltration in melanoma, bladder cancer, and more broadly across cancer. Activation of the phosphoinositide 3-kinase (PI3K) signaling pathway through loss-of-function mutations in phosphatase and tensin homolog (PTEN) can likewise mediate a non-T cell-inflamed tumor microenvironment in melanoma, and inactivation of LKB1 can have a similar effect in lung adenocarcinoma. Further, in lymphoma, diminished activation and recruitment of T cells have been reported with MYC activation, largely through inhibition of macrophage activation. MYC and several other activated transcriptional pathways have more broadly been associated with non-T cell-inflamed tumors across cancer types.

In contrast to adult cancers, pediatric neoplasms have low mutational burden and most are non-T cell-inflamed, with scarce tumor-infiltrating lymphocytes (TILs) among anti-inflammatory M2 tumor-associated macrophages (TAMs). Although the response to immune checkpoint inhibition is poor for many pediatric cancers, post-consolidation immunotherapy with monoclonal antibodies targeting the GD2 ganglioside combined with cytokines significantly improves survival for children with high-risk neuroblastoma. Further, high response rates were also reported in newly diagnosed patients in a single institutional study with induction chemotherapy combined with anti-GD2 antibody, and significant anti-tumor immunity was observed in a Children’s Oncology Group (COG) clinical trial testing irinotecan and temozolomide combined with anti-GD2 antibody and GM-CSF in patients with relapsed/refractory neuroblastoma. The immunobiology of the neuroblastoma microenvironment is an emerging field. To increase our understanding about how immunogenic determinants influence neuroblastoma phenotype, we analyzed the correlation between patient survival and T cell-inflamed gene expression and neoantigen load in tumor. We demonstrate that both biomarkers are prognostic in children with high-risk neuroblastoma and identify tumor-intrinsic oncogenic signaling pathways activated in neuroblastomas with a non-T cell-inflamed phenotype. These findings enhance a framework, whereby T cell-inflamed expression and neoantigen load can provide new prognostic information to inform treatment decisions, and may also lead to the development of future immune therapeutic interventions.

METHODS

Study cohorts and datasets

Two neuroblastoma cohorts were analyzed. The discovery cohort included patients from Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program (n=149; 123 high-risk) (dbGAP accession ID phs000218.v22.p8) (online supplemental table 1). RNAseq paired-end (PE) FastQ files, whole exome sequencing (WES) alignment BAM files, somatic mutation MAF (Mutation Annotation Format) files, and clinical data were downloaded from Genomic Data Commons (GDC) (https://portal.gdc.cancer.gov) (accessed 07/2017). The validation cohort included patients with clinical information in the International Neuroblastoma Risk Group (INRG) Data Commons and tumor RNAseq data in the Gabriella Miller Kids First (GMKF) program (n=198; 48 high-risk) (online supplemental table 2). Universal system identification (USI) numbers were used to link the datasets. Access to RNAseq PE FastQ files in GMKF could not be obtained at the time of study, and therefore, preprocessed gene expression TSV files from the GMKF data portal (https://kidsfirstdrc.org/) (accessed 08/2020) were used for analysis. Of the 209 patients identified, 11 were determined by USI number to also be included in the discovery cohort, hence were excluded from the validation cohort; 198 were kept for validation (online supplemental table 2).

RNAseq gene expression quantification

The quality of raw sequencing reads was assessed by FastQC (V.0.11.5) for the tumor samples in the discovery cohort. Read counts were quantified at transcript level using Kallisto (V.0.44.0) with human reference assembly GRCh38 and Gencode gene annotation (V.28), summarized into gene level using tximport (V.1.4.0), normalized by trimmed mean of M-values (TMM) method, and log-transformed.

Identification of T cell-inflamed and non-T cell-inflamed tumor groups

Using a defined T cell-inflamed gene expression signature, the tumors in the discovery cohort were categorized into three groups (T cell-inflamed, non-T cell-inflamed, and intermediate) using consensus clustering methods following previous protocols. In brief, an expression matrix consisting of the 160 genes from the T cell-inflamed signature was subset from the TMM-normalized and log-transformed RNAseq gene expression quantification matrix and was used to cluster tumors into 12 clusters by ConsensusClusterPlus (V.1.42.0) using hierarchical clustering with Euclidean distance and Ward.D2 linkage (2000 bootstraps and 80% usage of gene features). Tumors were then assigned with each of the three immune groups based on high, low, or intermediate expression of the T cell-inflamed signature. The number of clusters was determined using the elbow method.

Mapping of T cell-inflamed and non-T cell-inflamed tumor groups between discovery and validation cohorts

The assignment of tumor groups in the discovery cohort cannot be migrated directly to that of tumors in the validation cohort due to the relative nature of gene expression data without spike-in controls. To address this issue, we projected T cell-inflamed gene expression of the validation cohort to the space of the discovery cohort using 11 patients that overlap between the two cohorts. First, we normalized and log-transformed gene expression within each cohort. We calculated a T cell-inflamed score for each tumor, defined as the mean expression of all
genes from the signature. Then, we fit a linear regression model on the T cell-inflamed scores of the discovery cohort and validation cohort using tumors from the 11 overlapping patients. \( \text{Score}_T = -0.7798 + 1.2418 \times \text{Score}_n \)
(adjusted \( R^2 = 0.967 \)), where \( \text{Score}_T \) represents T cell-inflamed score of the discovery cohort (T as TARGET), and \( \text{Score}_n \) represents T cell-inflamed score of the validation cohort (G as GMKF). We used this model to convert all T cell-inflamed scores of the validation cohort (\( \text{Score}_n \)) to values comparable to that of the discovery cohort (\( \text{Score}_T \)), then sorted all tumors by T cell-inflamed scores lower to higher. Lastly, we assigned new tumor groups to the validation cohort based on existing tumor groups from the discovery cohort, employing the rules as follows: for all tumors harboring a score less than or equal to that of the last non-T cell-inflamed tumor on the sorted list, assign as non-T cell-inflamed; for all tumors harboring a score greater than or equal to that of the first T cell-inflamed tumor on the sorted list, assign as T cell-inflamed; otherwise, assign as intermediate.

**Differential gene expression detection and pathway activation prediction**

For the discovery cohort analysis, we focused on 19,883 protein-coding genes defined in Genecode annotation (\( V.28 \)) and followed the protocol established in our previous work. In brief, after removing genes with low expression (defined as CPM (counts per million of mapped reads) \( \leq 3 \)), 15,580 genes with CPM>3 in at least 30 tumors were TMM-normalized and log-transformed. Differentially expressed genes (DEGs) comparing non-T cell-inflamed with T cell-inflamed groups were identified using Linear Models for Microarray Data (limma) \(^{27} \) with precision weights (\( V.3.36.2 \)) and filtered by false discovery rate (FDR)-adjusted \( p \leq 0.05 \), and fold change \( \geq 1.5 \) or \( \leq -1.5 \). Upstream transcriptional regulators and change of direction (activation or inhibition) as a result of target molecules (encoded by DEGs) were predicted using Ingenuity Pathway Analysis (IPA) (QIAGEN, Germany) causal network analysis\(^{26} \) with the curated Ingenuity Knowledge Base (accessed 12/2017). Transcriptional programs activated in non-T cell-inflamed relative to T cell-inflamed tumors were filtered at overlap \( p \leq 0.05 \) (measuring the enrichment of target molecules in the dataset) and z-score \( \geq 2.0 \) (measuring the predicted activation level of the pathways). For the validation cohort analyses, preprocessed RNAseq expression data downloaded from the GMKF data portal was quantified using Kallisto\(^{33} \) (\( V.0.44.0 \)) and the per-tumor gene expression files were downloaded and aggregated into cohort level, TMM-normalized, and log\(_2\) transformed for further analysis.

**Somatic mutation detection, HLA genotyping, and neoantigen prediction**

For the discovery cohort, the somatic mutations were harmonized using four somatic variant callers (MuTect2, VarScan2, SomaticSniper, and MuSE)\(^{30} \). After rigorous filtering following GDC’s guidelines (https://docs.gdc.cancer.gov/Data/File_Formats/MAF_Format), somatic variants that were detected by at least two callers and passed all the filters were selected for further analysis. Total TMB was defined as the total number of non-synonymous somatic mutations (NSSMs), those that were predicted to alter protein sequence in tumor (insertions/deletions, missense/nonsense/stopgain mutations, and those that modify splicing sites). Putative neoantigens were predicted from NSSMs using netMHCpan\(^{17} \) (\( V.4.0 \)), filtered by gene expression from the RNAseq data described as follows. Patients’ major histocompatibility complex (MHC) class I haplotypes were predicted from WES of germline DNA using Optitype (\( V.1.3.1 \)). Nine-mer peptides were generated from the mutated site through a sliding window approach using in-house python scripts. Our previous work had suggested that peptides of SYFPEITHI\(^{38} \) mutant score \( >25 \) or delta score (mutant – wildtype)>5 bind to MHC class I molecules.\(^{13} \) In this study, we used netMHCpan that covers more human leukocyte antigen (HLA) genotypes than SYFPEITHI. To select neoantigens that are likely to have strong binding affinity to HLA-A molecules and expressed in tumor, we filtered for 9-mer peptides of netMHCpan mutant score \( >0.638 \) (equivalent to IC 50 nmol, strong binding) or delta score \( >0.070 \) (correlated with SYFPEITHI delta score 5) and derived from genes upregulated compared with the median of its expression across all tumors.

**T cell-inflamed gene expression and pathway score calculation**

For each tumor, a T cell-inflamed score was computed as the mean expression of the 160 genes involved in the signature after scaling and centering across all tumor samples.\(^{21} \) A pathway activation score was calculated to each tumor following our published protocol\(^{17} \) requiring at least 50% of the pathway-specific target molecules to be upregulated in a tumor sample (relative to its median expression across all tumor samples) in non-T cell-inflamed relative to T cell-inflamed group. For pathways in which less than 10 target molecules were present, 5 or more molecules were required to be upregulated to classify the pathway as activated. For pathways in which less than 5 target molecules were present, only pathways with all molecules upregulated were classified as activated. In addition, for each pathway identified in this study (MYCN, ASCL1, SOX11, and KMT2A), the expression level of a pathway was defined by the mean expression of all target molecules from this pathway, which was then used to correlate with the T cell-inflamed gene expression across all tumors by Spearman’s correlation.

**Survival analysis**

Cox proportional hazards (PH) models were used to test the association between the tumor group (T cell-inflamed, non-T cell-inflamed, and intermediate) and the survival outcome (event-free survival (EFS); overall survival (OS)) in the discovery (\( n=118 \) high-risk patients diagnosed between 2000 and 2010) and validation cohorts (\( n=17 \) high-risk patients with survival data available) using R.
package survival (function `coxph`) (V.2.41.3). Univariable
and multivariable Cox PH models were used to assess the
significance of tumor group as a single predictor or after
adjusting for covariates including age, MYCN status, and
ploidy. In addition, Kaplan-Meier (KM) estimator with
log-rank test was performed using R package survminer
(V.0.4.2).

Immunohistochemistry immunofluorescence staining
Immunofluorescence (IF) staining on human neuroblasto-
toma tumors was performed by the Human Immunologic
Monitoring Core Facility at The University of Chicago
using tissue from 17 intermediate or high-risk neuroblas-tomas (5 MYCN-amplified and 12 MYCN-non-amplified).
Briefly, slides were baked, cleared, and rehydrated. After
heat-induced epitope retrieval, the slides were placed in
a humidity chamber, blocked by 10% donkey serum for
1 hour, incubated with anti-CD8 Ab (Dako, M7103)
at 1:100 dilution for 1 hour, followed by Cy3 donkey
anti-Mouse IgG (Jackson Immunological Research Lab,
715-165-150) at 1:500 dilution for 1 hour. The slides then
incubated with anti-Batf3 Ab (Novus, AF7437) at 1:40
dilution for 1 hour, followed by Cy5 donkey anti-Rabbit
IgG (Jackson Immunological Research Lab, 711-175-152)
at 1:200 dilution for 1 hour. After thorough wash, slides
were incubated in DAPI and mounted with Fluoromount
(Sigma, F4680). Images of the slides were taken using
a Leica SP8 laser scanning confocal microscope at Inte-
grated Light Microscopy Core Facility. A pathologist (PP)
scored tumors for intensity and distribution of CD8+ cells
and Batf3+ cells in a blinded fashion.

Statistical analysis
For analysis of contingency tables including compar-
tion of tumor sample frequency between groups, Fish-
er’s exact test was used. Differential gene expression
analysis between groups were performed using empirical
Bayes regression models in limma voom with preci-
sion. Spearman’s correlation ρ was used for measuring statistical dependence between normalized and
log2-transformed expression level of different genes
and between gene expression of the T cell-inflamed
signature and pathways, p<0.05 was considered statistically
significant. Statistical analysis was performed using R
(V.3.5.2) and Bioconductor (release 3.8).

RESULTS
A T cell-inflamed gene expression signature defines three
distinct groups in neuroblastoma
Using a defined T cell-inflamed gene expression signature,
we categorized the 149 primary neuroblastoma tumors
from the discovery cohort (TARGET) into three subsets
(figure 1A). High expression of T cell signature genes (T
cell-inflamed) was detected in 57 (38.3%) tumors, low or
no expression (non-T cell-inflamed) was identified in 45
(30.2%) tumors, and 47 (31.5%) had intermediate levels of
expression (intermediate) (table 1). In the validation
cohort (n=198, GMKF), 89 (44.9%) were categorized as
T cell-inflamed; 55 (27.8%) were non-T cell-inflamed; 54
(27.3%) were intermediate (figure 1B, table 2). In the
discovery cohort, 123 of 149 patients were classified as
high-risk, whereas 48 of 198 patients in validation cohort
have high-risk neuroblastoma. In analyses restricted to
high-risk patients, 53 (43.1%) and 23 (47.9%) were catego-
rized as T cell-inflamed in the discovery and validation
cohorts, respectively, and 33 (26.8%) and 18 (37.5%) were
categorized as non-T cell-inflamed.

In the discovery cohort, MYCN amplification was significa-
cantly more prevalent in the non-T cell-inflamed tumors
(17/45, 37.8%) compared with the T cell-inflamed tumors (3/56, 5.4%) (p=0.000080, odds ratio [OR]=10.5,
two-sided Fisher’s exact test). Additionally, patients
diagnosed at age <18 months had tumors that were
enriched in the non-T cell-inflamed tumor group (12/45,
26.7% non-inflamed vs 6/57, 10.5% inflamed; p=0.04, OR=3.06). The enrichment of MYCN amplification in
non-T cell-inflamed tumors was also observed in the vali-
dation cohort (14/54, 25.9% non-inflamed vs 4/89, 4.5%
inflamed; p=0.00038, OR=7.33). In addition, patients <18
months of age in the validation cohort had tumors that were
enriched in the non-T cell-inflamed tumor group
(40/54, 74.1% non-inflamed vs 44/89, 49.4% inflamed;
p=0.049, OR=2.90).

T cell-inflamed gene expression is prognostic of survival in
high-risk neuroblastoma
We analyzed EFS and OS according to the level of expres-
sion of the T cell-inflamed signature in 118 high-risk
patients diagnosed between 2000 and 2010 from the
discovery cohort, which consists of 51 T cell-inflamed,
33 non-T cell-inflamed, and 34 intermediate tumors. In
Cox PH univariable models, patients with T cell-inflamed
tumors had significantly better OS compared with those
with non-T cell-inflamed tumors (p=0.043, hazard ratio
[HR]=0.56) (table 3). A similar trend was observed in
EFS but the results did not reach statistical significanc
(p=0.17, HR=0.69) (table 3). Similar to other high-risk
cohorts, MYCN status was not statistically significantly
associated with OS or EFS (OS: p=0.38; EFS: p=0.58)
(table 3). However, OS, but not EFS, was better for high-
risk patients with hyperdiploid neuroblastoma compared
with those with diploid tumors (OS: p=0.05; EFS: p=0.10)
(table 3). In Cox PH multivariable models adjusting for
age, MYCN status, and ploidy, the T cell-inflamed signa-
ture maintained independent statistical significance for
OS (p=0.035, HR=0.48). Stage and histology were not
included in the multivariable analysis because the high-
risk patients had predominantly stage 4 disease and
unfavorable histology tumors (117/118, 99.1% as stage
4; 107/110, 97.3% with unfavorable histology tumors, 8
unknown).

In the discovery cohort, the T cell-inflamed and inter-
mediate groups showed similar probabilities of survival.
Therefore, we combined these two groups and compared probability of survival to patients with non-T cell-inflamed tumors (OS: p=0.0076, EFS: p=0.10, log-rank test) (KM estimator shown in figure 2A,B). Similar associations between T cell-inflamed/intermediate tumors and improved survival were observed in the 17 high-risk patients with available survival data from the validation cohort (OS: p=0.016, EFS: p=0.0098) (figure 2C,D). No significant association with survival outcome was detected for age, MYCN status, or ploidy. Patients were not selected by diagnosis year 2010 or earlier due to small sample size. However, EFS and OS were not significantly different

Figure 1  Immunogenomic landscape of neuroblastoma tumor microenvironment. (A) Heatmap of the T cell-inflamed gene expression signature in the discovery cohort (TARGET). Genes are on the row and tumor samples are on the column. The non-T cell-inflamed (blue), intermediate (gray), and T cell-inflamed (red) tumor groups, consensus clusters C1 to C12, MYCN amplification status, clinical and demographic factors are shown above the heatmap. n=149 primary tumors shown, including 123 high-risk. (B) Heatmap of the T cell-inflamed gene expression signature in the validation cohort (GMKF). Same annotation as in (A). n=198 primary tumors shown, including 48 high-risk. COG, Children’s Oncology Group; GMKF, Gabriella Miller Kids First; TARGET, Therapeutically Applicable Research to Generate Effective Treatments.
between the 13 patients diagnosed between 2008 and 2010 and the four patients diagnosed between 2011 and 2012 (OS: p=0.98, EFS: p=0.66), and hence these are unlikely to contribute to the significantly better survival outcome observed in T cell-inflamed group relative to non-T cell-inflamed group in the validation cohort.

### Table 1: Characteristics of patients from T cell-inflamed, intermediate, and non-T cell-inflamed tumor groups in the discovery cohort (TARGET)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T cell-inflamed N=57, no (%)</th>
<th>Intermediate N=47, no (%)</th>
<th>Non-T cell-inflamed N=45, no (%)</th>
<th>P-value</th>
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<td>Low risk</td>
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<td>4 (8)</td>
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*P values were calculated using one-way analysis of variance, χ² test, Fisher’s exact test.

* p<0.05, **p<0.01, ***p<0.001.

COG, Children’s Oncology Group; INSS, International Neuroblastoma Staging System; TARGET, Therapeutically Applicable Research to Generate Effective Treatments.
### Table 2  Characteristics of patients from T cell-inflamed, intermediate, and non-T cell-inflamed tumor groups in the validate cohort (GMKF)

<table>
<thead>
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<th>Characteristic</th>
<th>T cell-inflamed N=89, no (%)</th>
<th>Intermediate N=54, no (%)</th>
<th>Non-T cell-inflamed N=55, no (%)</th>
<th>P-value</th>
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<td>36 (67)</td>
<td>22 (40)</td>
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</table>

P values were calculated using one-way analysis of variance, χ² test, Fisher's exact test.

*p<0.05, **p<0.01, ***p<0.001.

COG, Children's Oncology Group; GMKF, Gabriella Miller Kids First; INSS, International Neuroblastoma Staging System; NE, Not Evaluable.
Neoantigen load is a prognostic marker independent of the T cell-inflamed expression signature

Neoantigens are mutant antigens that are only expressed on tumor cells and not normal cells. Neoantigen-derived epitopes (neoepitopes) are recognized by antigen-specific CD8+ T cells.43 To evaluate the neoantigen load in neuroblastoma tumors, WES data from 198 matched tumor/normal pairs of the discovery cohort carrying one

Table 3  OS and EFS of high-risk neuroblastoma patients according to T cell inflammation group and other established prognostic markers

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<th>Characteristic</th>
<th>Comparison</th>
<th>Overall survival</th>
<th>Event-free survival</th>
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<td>HR</td>
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<td>T cell inflammation group</td>
<td>T cell-inflamed vs non-T cell-inflamed</td>
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<td>0.317</td>
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<tr>
<td></td>
<td>Intermediate vs non-T cell-inflamed</td>
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<td>0.218</td>
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<td>Age (months)</td>
<td>Continuous variable</td>
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<td>0.991</td>
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</table>

HR and p-values were calculated using Cox proportional hazards models.
*p<0.05, **p<0.01, ***p<0.001.
CI, confidence interval; EFS, event-free survival; HR, hazard ratio; OS, overall survival.

Figure 2  T cell-inflamed gene expression signature predicts overall survival in high-risk neuroblastoma patients. (A,B) Kaplan-Meier estimator of the T cell-inflamed (red), intermediate (gray), and non-T cell-inflamed (blue) tumor groups from the discovery cohort (TARGET), with overall survival (OS) shown in (A) and event-free survival (EFS) shown in (B). Of 123 high-risk patients, n=118 diagnosed between year 2000 and 2010 are shown; all have survival data available. (C,D) Kaplan-Meier estimator of the T cell-inflamed (red), intermediate (gray), and non-T cell-inflamed (blue) tumor groups from the validation cohort (GMKF), with OS shown in (C) and EFS shown in (D). Of 48 high-risk patients, n=17 with survival data available shown. P-values were calculated by log-rank test in (A) to (D), comparing T cell-inflamed/intermediate tumors as one combined group to non-T cell-inflamed tumors. GMKF, Gabriella Miller Kids First; TARGET, Therapeutically Applicable Research to Generate Effective Treatments.

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or more somatic single nucleotide variants (SNVs) were analyzed. After combining calls of four somatic callers and rigorous quality filtering, 4235 somatic SNVs were identified in 3369 genes. Each tumor harbors a median of 17 somatic SNVs (range, 1–168 SNVs), with 15 somatic SNVs predicted to alter protein sequences (range, 1–162), which is consistent with the somatic mutation profile previously reported in high-risk neuroblastomas. To investigate if neoantigen load was associated with outcome in high-risk patients, of 118 high-risk patients diagnosed between 2000 and 2010 in the discovery cohort, we analyzed tumors from 89 patients with both WES and RNAseq data available. The total number of neoantigens in tumor was determined by filtering for those predicted to bind to MHC class I molecule HLA-A. We focused on HLA-A molecule because the prediction algorithm for this allele is the most reliable.

A median of 4 (range 1–30) candidate neoantigens were identified in 78 of 89 tumors. Seventy-four patients diagnosed between year 2000 and 2010 were included in survival analysis. We found that the neoantigen load was significantly associated with OS (ρ = 0.00022, log-rank test) (figure 3A) and EFS (p = 0.0044) (figure 3B), although there was no significant difference in neoantigen load between non-T cell-inflamed and T cell-inflamed groups (p = 0.22, two-sided Wilcoxon rank-sum test) (figure 3C).

We defined four patient groups (hereafter referred as, quadrants (Q)) split by the threshold of T cell-inflamed (Tinfl) gene expression in non-T cell-inflamed tumors and median of neoantigen load (Neo) (Spearman’s correlation coefficient p = 0.053, p = 0.65) (figure 3D): Q1 (n = 8), Tinfl<sub>low</sub>Neo<sub>high</sub>; Q2 (n = 19), Tinfl<sub>low</sub>Neo<sub>low</sub>; Q3 (n = 20), Tinfl<sub>high</sub>Neo<sub>low</sub>; Q4 (n = 27), Tinfl<sub>high</sub>Neo<sub>high</sub>. OS and EFS were significantly different according to quadrant assignment (OS: p = 0.00083; EFS: p = 0.0061, log-rank test) (figure 3E,F). Patients in Q1 and Q4, who had tumors harboring high level of neoantigens, had superior outcome compared with those in Q2 and Q3 (figure 3E,F).

### Tumor-intrinsic oncogenic transcriptional programs associated with a non-T cell-inflamed phenotype

To investigate if tumor-intrinsic transcriptional programs may play a role in inhibiting T cell infiltration in non-T cell-inflamed neuroblastomas, we first analyzed tumors from the discovery cohort for signaling pathways intrinsic to the neoplastic cells that were previously reported to impair the local immune response in other tumor types. This includes somatic activation mutations in CTNNB1 or damaging mutations in repressors of the pathway (APC/AXIN2/β-catenin), somatic copy number loss in AXIN2 (p.A113T), and two tumors had PIK3CA missense (p.K111N) or nonsense mutations (p.E888X), but none occurred at the known PIK3CA activation mutation positions (AA 345, 542, 545, 546, 1043, 1044, 1047).

We next took an unbiased approach to identify transcriptional programs that are activated in non-T cell-inflamed tumors by comparing the whole transcriptome RNAseq expression of 33 non-T cell-inflamed to 53 T cell-inflamed tumors from the high-risk patients in the discovery cohort. A total of 1730 genes were identified that were significantly differentially expressed between the two tumor groups, with 230 upregulated in non-T cell-inflamed group and 1500 upregulated in the T cell-inflamed group (FDR-corrected p < 0.05, fold change ≥1.5 or ≤–1.5). Causal network analysis with Ingenuity Knowledge Base (Qiagen) identified activation of MYCN signaling in non-T cell-inflamed tumors (activation z-score ≥2.0, p < 0.05), consistent with our findings showing enrichment of MYCN amplification in non-T cell-inflamed tumors (figure 4A). Immunohistochemistry (IHC) staining of a limited number of available intermediate or high-risk neuroblastoma tumors (n = 17, 5 MYCN-amplified and 12 MYCN-non-amplified; images of all IHC slides are provided at https://github.com/riuyuebao/NBL-TME-Immunogenomics) demonstrated lower infiltration with CD8<sup>+</sup> T cell and Batf3<sup>+</sup> DCs in MYCN-amplified tumors compared with MYCN-non-amplified neuroblastomas (figure 4B), although the difference did not reach statistical significance in this small cohort (p = 0.22, two-sided Fisher’s exact test). In both the discovery and validation cohorts, the DC genes are highly correlated with the T cell-inflamed gene expression (p < 0.05, Spearman’s correlation) (online supplemental figure 1).

To determine if activation of transcriptional programs other than MYCN signaling is associated with the non-T cell-inflamed phenotype, we repeated the differential gene expression and causal network analyses using only MYCN non-amplified tumors (n = 91). Genes significantly upregulated in 16 non-T cell-inflamed neuroblastomas compared with 49 T cell-inflamed tumors were used to predict upstream regulators. Three pathways (ASCL1, SOX11, and KMT2A) were identified to be activated in non-T cell-inflamed tumors without MYCN amplification (activation z-score ≥2.0, p < 0.05) (figure 4C). We next calculated an activation score for each pathway using previously described methods. The results showed that the three pathways operate in a partially exclusive manner (online supplemental figure 2), with activation of SOX11, KMT2A, and ASCL1 signaling detected in 66%, 30%, and 30% of non-T cell-inflamed tumors, respectively, compared with less than 5% of the T cell-inflamed tumors (figure 4D,E). Taking together, the activation of one or more pathways regulated by MYCN, ASCL1, SOX11, or KMT2A was found in 85% of the non-T cell-inflamed tumors. The inverse correlation between the expression of T cell-inflamed gene signature and the four pathways (MYCN, ASCL1, SOX11, KMT2A) was confirmed in the validation cohort (online supplemental figure 3A,B), providing strong evidence that the activation of the four
Figure 3  Neoantigen load predicts overall and event-free survival in high-risk neuroblastoma patients. (A,B) Kaplan-Meier estimator of the neoantigen load high and low tumor groups in association with overall survival (OS) shown in (A) and event-free survival (EFS) shown in (B). (C) Neoantigen load between non-T cell-inflamed, intermediate, and T cell-inflamed groups. (D) Quadrants (Q1 to Q4) according to expression of T cell-inflamed gene signature (x-axis) and the neoantigen load (y-axis). Vertical line labels the separation of non-T cell-inflamed group versus intermediate or T cell-inflamed group, and horizontal line labels the median of neoantigen load across samples. (E,F) Kaplan-Meier estimator of the four groups from (D), with OS shown in (E) and EFS shown in (F). n=78 patients from the discovery cohort (TARGET) with both WES and RNAseq data available and having at least one candidate neoepitope detected are shown in (C). n=74 patients diagnosed between year 2000 and 2010 are shown in (A, B, D, E, and F). P-values were calculated by two-sided Wilcoxon rank-sum test in (C), Spearman’s correlation in (D), and log-rank test in (A, B, E, and F). TARGET, Therapeutically Applicable Research to Generate Effective Treatments; WES, whole-exome sequencing.
transcriptional programs was significantly associated with a non-T cell-inflamed phenotype.

**DISCUSSION**

Although improved survival and response to immunotherapy have been observed in adults with cancers showing T cell infiltration, the immunobiology of neuroblastoma tumors and its association with outcome had been poorly understood. In this study, we categorized 149 clinically annotated primary neuroblastoma tumors in the TARGET program as T cell-inflamed, non-T cell-inflamed, and intermediate using a defined T cell-inflamed gene expression signature. The gene signature also identified the same three tumor groups in an independent cohort comprised of publicly available tumor genomic data housed in the GMKF program linked to clinical information in the INRG Data Commons. In both cohorts, MYCN amplification was significantly more prevalent in the non-T cell-inflamed tumors compared with the T cell-inflamed tumors. Interestingly, we also found that patients in both cohorts diagnosed at age <18 months had tumors that were enriched in the non-T cell-inflamed tumor group.

In analyses restricted to high-risk patients in the TARGET cohort, OS was significantly better for those with T cell-inflamed tumors compared with those with non-T cell-inflamed tumors. A similar trend was observed for EFS, although statistical significance was not reached. Further, the T cell-inflamed signature maintained independent statistical significance for OS in multivariable analysis adjusted for age, MYCN status, and ploidy. This association between T cell-inflamed tumors and superior outcome was validated in the clinically annotated GMKF cohort of tumors. Because neoantigens are recognized by the immune system and can be targeted to increase anti-tumor immunity, we also analyzed neoantigen load in the neuroblastoma tumors. Although no significant difference in neoantigen load was detected among T cell-inflamed, non-T cell-inflamed, or intermediate groups, superior OS was seen in the cohort of patients with tumors harboring a high neoantigen load. Taken together, these results suggest that T cell-inflamed gene expression and high neoantigen load may independently

![Figure 4](image-url)
impact the clinical behavior of neuroblastoma tumors, resulting in improved survival.

The lack of correlation between the T cell-inflamed expression signature (also known as an IFN-γ-associated expression signature) and TMB, which is highly correlated with neoantigen load, 31 has been reported in many adult cancers, including melanoma, 13 head and neck, 7 and pancreas. 50 It is well established in the literature that TMB (or neoantigen load) and T cell-inflamed expression are both prognostic but seemly have little correlation. 10–12 In particular, the pan-cancer study reports four groups of patients determined by high/low IFN-γ-associated expression signature and high/low TMB. 52 Only patients possessing high levels of both signatures had the best response rate, and a significant number of patients only showed high levels of one of the signatures. 52 The mechanism underlying the decoupling of T cell-inflamed expression signature and TMB and neoantigen load remains to be explored.

Others have evaluated inflammatory cell infiltrates in neuroblastoma tumors using different methodologies and markers. Asgharzadeh and colleagues 53 assessed the neuroblastoma tumors using different methodologies. The inverse correlation between MYCN amplification and T cell-inflamed tumors seen in our study and others 53,54 suggests that MYCN signaling inhibits T cell infiltration in neuroblastoma tumors. In support of MYCN's role in mediating exclusion of T cells from the microenvironment of neuroblastoma tumors, we identified activation of MYCN signaling in non-T cell-inflamed tumors (activation z-score ≥2.0, p<0.05) comparing expression profiles between non-T cell-inflamed and T cell-inflamed tumors. In addition, we identified three transcriptional programs, ASCL1, SOX11, and KMT2A, that were active in non-T cell-inflamed tumors without MYCN amplification.

ASCL1 (alias hASH1 in human) is a known proneural transcription factor essential for neurogenesis. However, in neuroblastoma ASCL1 represses genes involved in neuron differentiation. 58 Recent studies have demonstrated that ASCL1 is a MYCN-dependent and LMO1-dependent member of the adrenergic neuroblastoma core regulatory circuitry (CRC), an interconnected autoregulatory loop of transcription factors whose expression is driven by themselves and other members of the CRC. 59 Interestingly, LMO1 and the CRC members bind to enhancer elements and directly upregulate the ASCL1 gene, resulting in promotion of cell growth and repression of neuronal differentiation. 59 Activation of ASCL1 signaling is also predictive of poor prognosis in neuroendocrine lung cancers. 60 In glioblastoma, ASCL1 is critical to the maintenance of stem cells through activation of WNT signaling. 33 SOX11 is a transcription factor essential for neuron survival and neurite outgrowth. 61 In our study, the expression of MYCN and SOX11 pathways is positively correlated (Spearman’s r=0.81 in TARGET and 0.83 in GMK, respectively, p<0.0001), suggesting the two mechanisms may interact. Indeed, recent studies reported that SOX11 was a direct target of MYCN. 63 However, 30% of MYCN-non-amplified tumors showed SOX11 pathway activation, which may indicate other signaling routes independent of MYCN. KMT2A (alias MLL1 in human) is an epigenetic regulator of neuronal function. In pancreatic cancer where anti-PD/L1 immunotherapy is ineffective, MLL1 directly binds to the promoter of the checkpoint inhibitor PD-L1 and activates its transcription, and combinatorial therapy of anti-MLL1 and anti-PD/L1 was proven to suppress tumor growth in mouse models. 64 Taken together, these transcriptional programs support a stem cell-like phenotype in neural tissues, which is a consistent theme with what has been observed in adult tumors for a state of epithelial–mesenchymal transition being associated with immuno-oncology resistance. 65

In conclusion, the association of improved survival with T cell-inflamed neuroblastoma and high neoantigen load indicate that crosstalk between tumor cells and components of the microenvironment influence neuroblastoma
phenotype. Our studies also suggest that tumor-intrinsic MYCN, ASCL1, SOX11, or KMT2A signaling may mediate immune exclusion in neuroblastoma. Understanding the molecular mechanisms that drive the presence or absence of T cell infiltration and neoantigen load should enable more personalized treatment approaches and provide insight for the development of new therapies that may enhance response to immunotherapy and improve outcome. Clinical trials testing the efficacy of anti-GD2 antibodies and other modalities of immunotherapy in patients with neuroblastoma tumors that are T cell-inflamed or harbor high neoantigen load are warranted.

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Contributors
RB, SLC, and AVD conceived the study. AVD supervised the project. RB acquired the data, developed the methodology, performed the computations, and analyzed the data. KH performed the neoantigen prediction and filtering. YZ performed the IHC staining and scanned the slides. PP examined the pathology slides. RS, SS, JLL, TFG, SLC, and AVD interpreted the results. RB, SS, SLC, and AVD wrote the manuscript. All authors contributed to the final manuscript.

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Competing interests
RB declares patents: (all provisional) PCT/US15/612657 (Cancer Immunotherapy), PCT/US15/63052 (Microbiome Biomarkers for Anti-PD-1/PD-L1 Responsiveness: Diagnostic, Prognostic and Therapeutic Uses Thereof), PCT/US18/36052 (Microbiome Biomarkers for Anti-PD-1/PD-L1 Responsiveness: Diagnostic, Prognostic and Therapeutic Uses Thereof). SLC declares a patent on WNT3a/catenin targeting to enhance anti-tumor immune responses (PCT/15/155,099), serves on the SAB on Venn Therapeutics, Tango Therapeutics, Arcus Bisciences and consults for TAKEDA, Replimune, Ribon, Dragonfly and Merck. TFG has received consultancy fees from Merck, Roche-Genentech, Abbvie, Bayer, Jounce, Aduro, Fog Pharma, Adaptimmune, FivePrime, and Sanofi. TFG has received research support from Roche-Genentech, BMS, Merck, Incyte, Seattle Genetics, Celldex, Ono, Evelo, Bayer, and Adur. TFG has intellectual property/licensing agreements with Aduro, Evelo, and BMS. TFG is a co-founder/shareholder of Jouvis and Poxis Oncology. AVD declares Research funding (all to institution for clinical trials): Merck, Roche, Julubiat Druadmage, YMrbs. GlaixoSmithKline, Actihe Therapeutics, Lilly; Scientific Advisory Board: Merck; Consultancy: Ology Medical Education; Travel/Accommodations: GlaixoSmithKline; Stock: Pfizer (all outside the submitted work). SLC declares Research funding (to the institution for clinical trials): Merck and United Therapeutics; Stock (personal or immediate family member): United Therapeutics, Merck, Stryker, Amgen, Pfizer, Abbvie; Jazz Pharmaceuticals, Lilly, Sanofi, Varex Imaging, Accelerated Medical Diagnostics, Anthem, Cardinal Health, Novo Nordisk, Regeneron, Zimmer BioMet (all outside the submitted work). The remaining authors declare no competing interests.

Patient consent for publication
Not required.

Ethics approval
Each country or cooperative group submitting data from clinical trials to the INRG Data Commons obtained institutional review board approval and informed patient consent for their respective studies. The INRG Data Commons has approval from The University of Chicago Institutional Review Board.

Provenance and peer review
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Data availability statement
All data relevant to the study are included in the article or uploaded as supplementary information. The gene expression, somatic mutations, and clinical data were downloaded from GDC data portal (https://portal.gdc.cancer.gov), and GMKF data portal (https://portal-kidsfirstdc.org/dashboard). Patient-level clinical data of the GMKF cohort are under controlled access at INRG Data Commons and can be requested by contacting the INRG Review Committee (https://inrgdb.org). HIC image data files were deposited on a publicly accessible GitHub Repository (https://github.com/ryuehas/NBL-TME-immunogenomics). Other data will be provided upon request from the corresponding author.

Supplemental material
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REFERENCES


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Supplementary Materials

for

Immunogenomic determinants of tumor microenvironment correlate with superior survival in high-risk neuroblastoma

Running title: Immunogenomic determinants correlate with superior survival in high-risk neuroblastoma

Feature: Immunotherapy Biomarkers

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This document contains **Supplementary Figures 1 to 3. Supplementary Tables 1 and 2** are provided separately as an Excel Spreadsheet file.
Supplementary Figure 1: Correlation of DC gene expression with CD8A gene expression in T cell-inflamed, intermediate, and non-T cell-inflamed tumors. Spearman’s correlation of expression levels of BATF3, IRF8, THBD (CD141), and CD1C are shown for the TARGET (n=123 high-risk patients) and GMKF (n=48 high-risk patients) cohorts. \( \rho \) = Spearman’s correlation coefficient. \( P \) = p-value.
Supplementary Figure 2. Partially exclusive activation of the four pathways in non-T cell-inflamed tumors. Numbers represents the number of tumors that harbor activation of each pathway or multiple pathways.
Supplementary Figure 3. Neuroblastoma-intrinsic oncogenic pathway activation correlates with non-T cell-inflamed tumor microenvironment in the discovery (TARGET) and validation (GMKF) cohorts. (A) T cell-inflamed gene expression is significantly higher in MYCN non-amplified tumors compared to MYCN amplified tumors. n=123 and 48 tumors from high-risk patients in TARGET and GMKF are shown, respectively. (B) Correlation between T cell-inflamed gene expression and pathway activation score of ASCL1, KMT2A and SOX11. n=91 and 29 MYCN non-amplified tumors from high-risk patients in TARGET and GMKF are shown, respectively. Two-sided Welch Two Sample t-test was used in A. Spearman’s correlation was used in B. \( \rho \) = Spearman’s correlation coefficient. \( P \) = p-value.