

ARTICLE

Myeloid-targeting immunotherapies overcome inhibitory barriers in immune-evasive neuroblastoma

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Neuroblastomas are highly heterogeneous tumors originating from neural crest-derived cells destined to form the sympathetic nervous system. Nearly half of high-risk tumors present with amplification of the *MYCN* proto-oncogene. Here, we describe a *Mycn*-driven, transplantable, non-germline, genetically engineered mouse model (*Mycn*-nGEMM). *Mycn*-nGEMM tumors recapitulate the immune-evasive, macrophage-rich tumor microenvironment of high-risk, *MYCN*-amplified human neuroblastoma. Treatment of tumor-bearing mice with anti-PD-L1, but not anti-PD-1 or anti-CTLA-4, inhibited tumor growth, profoundly remodeling the tumor microenvironment by depleting anti-inflammatory macrophages and increasing T cell infiltration. Surprisingly, while tumor cells showed low expression of PD-L1, anti-inflammatory macrophages from both murine and human neuroblastoma expressed PD-L1. We identified cytokines, including macrophage migration inhibitory factor, secreted by the *Mycn*-nGEMM cancer cells that drive expression of PD-L1 on macrophages. Combining anti-PD-L1 with CD40 agonist antibodies further improved survival in *Mycn*-nGEMM mice, demonstrating the potential for myeloid-targeting immunotherapies to overcome inhibitory barriers in immune-evasive neuroblastoma.

Introduction

Neuroblastoma (NB), the most common extracranial solid tumor of childhood, is generally diagnosed in very young children and accounts for 12% of childhood cancer-related deaths (Qiu and Matthay, 2022). NB arises from neural crest cells (NCC), a population of pluripotent cells delaminating from the neural tube during embryogenesis. NCC notably form the sympathetic ganglia and the adrenal medulla, the most frequent sites from which NBs arise. While low- and intermediate-risk NB show survival rates of 95% with little or no cytotoxic therapy, survival for high-risk NB remains <50% despite intensive multimodal therapies (Qiu and Matthay, 2022).

NB is highly heterogeneous, displaying genomic instability, which results in large chromosome deletions or gains (Ma et al., 2018). Amplification of the *MYCN* transcription factor and proto-oncogene occurs in 45% of high-risk tumors (Pugh et al., 2013). Interestingly, NB tumors develop a complex immunosuppressive

tumor microenvironment (TME). Tumors notably evade T cells and natural killer (NK) cells by downregulating human leukocyte antigens (Raffaghello et al., 2005; Bernards et al., 1986; Lenardo et al., 1989). High-risk NB tumors are devoid of T, B, or NK cells, and the immune compartment is instead dominated by immunosuppressive tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (Pistoia et al., 2013). Understanding mechanisms of immune evasion in NB has uncovered new therapeutic opportunities.

Immunotherapy using a tumor-directed anti-disialoganglioside (anti-GD2) antibody is standard of care, making NB a model for immunotherapy in pediatrics (Yu et al., 2010). In contrast, other immunotherapy approaches, notably immune checkpoint inhibitors (CPIs), have shown a striking lack of activity in the context of pediatric solid tumors, including NB (Qiu and Matthay, 2022). The paucity of mutations and the lack of neoantigens present major

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challenges to immunotherapies that target T cells. Furthermore, levels of PD-L1 and their association with T cell infiltration and outcome have led to conflicting results in several preclinical studies (Majzner et al., 2017; Saletta et al., 2017; Wei et al., 2018), making it difficult to identify biomarkers predictive of response to immune checkpoint inhibition in NB. Progress depends critically on better understanding the unique immunobiology of pediatric cancers and developing novel immunotherapeutic strategies.

Immunocompetent *in vivo* models are therefore central to test new therapeutic strategies and promising combinations of targeted therapies, chemotherapy, and immunotherapy. However, currently available *in vivo* models for NB are often inadequate to test therapeutic combinations. The first genetically engineered mouse model (GEMM) of NB, the tyrosine hydroxylase (TH)-MYCN model, targeted expression of the human MYCN transgene to mouse sympathoadrenal precursors using the rat TH promoter (Weiss et al., 1997). This model is highly penetrant only in strain 129X1/SvJ (Hackett et al., 2014), whereas most immunological mutants exist in strain C57BL/6J (B6). Since 1997, three additional immunocompetent GEMMs of NB have been published, two driven by activating mutations in *ALK* (Schulte et al., 2013; Berry et al., 2012) and a third indirectly activating MYCN through mis-expression of the small RNA-binding protein LIN28B (Molenaar et al., 2012). Others directed expression of *Mycn* in a mouse NCC-derived system with low penetrance, requiring loss of p53, which occurs rarely in NB, for complete penetrance (Olsen et al., 2017).

In this study, we describe an immunocompetent *Mycn*-driven, transplantable, non-germline, GEMM (*Mycn*-nGEMM) for NB. This highly manipulable model is ideal for immunoncology discovery and preclinical therapeutic testing. Immune profiling by mass cytometry recapitulated a TME characteristic of high-risk human NB. *Mycn*-nGEMM tumors did not respond to checkpoint inhibition using anti-PD-1 and anti-CTLA-4 but did respond to anti-PD-L1, despite poor expression of PD-L1 in tumor cells. We demonstrate that tumors arising in the *Mycn*-nGEMM showed profound immunosuppression mediated by PD-L1-expressing, immunosuppressive TAMs. In this context, anti-PD-L1 immunotherapy depleted immunosuppressive TAMs and robustly inhibited tumor growth, especially when combined with a CD40 agonist, demonstrating the potential for myeloid-targeted immunotherapies to overcome the immuno-inhibitory barriers in a “cold” TME.

Results

Expression of *Mycn* in migrating NCC drives NB in immunocompetent, syngeneic hosts

We developed an immunocompetent, easily manipulable *in vivo* model for NB transplantable into B6 hosts to enable immune characterization using the toolbox of immunological mutants available in B6. NCC were isolated from B6 embryos by dissecting out the trunk neural tube at stage mouse embryonic day (E) 9.5 and transferring to a fibronectin-coated plate to allow for NCC migration (Fig. 1 A). These trunk NCC expressed migration markers Sox10, Foxd3, and *Ascl1* (Fig. S1 A) with low levels of sympathoadrenal markers *Hand2*, *Phox2a*, *Phox2b*, and *TH* (Fig.

S1 B). Migrating NCC were then transduced *in vitro* with a retrovirus carrying cDNA for mouse *Mycn* and sorted for expression of GFP (Fig. 1 A). Expression of *Mycn* in NCC, confirmed by quantitative RT-PCR (qRT-PCR) and western blot (Fig. S1, C and D), increased proliferation *in vitro* compared with empty vector transduced (Fig. S1 E). *Mycn*-transduced NCC were isografted subcutaneously into immunocompetent B6 syngeneic hosts and monitored for tumor growth and survival. Out of 13 allografted mice, four developed tumors that reached endpoint (tumor volume 2,000 mm³) between 115 and 161 days after injection (Fig. 1 B). H&E staining shows a representative small round blue cell tumor (Fig. 1 C). Tumors stained positively for markers typically found in human NB, including *Phox2b*, *TH*, synaptophysin, NCAM, and N-Myc (Fig. 1 C).

Four cell lines were derived from two primary tumors. These cell lines differed in levels of *Mycn*, assessed by qRT-PCR and western blot (Fig. 1, D and E; and Fig. S1 F). The cell line expressing the highest level of *Mycn* (*Mycn*-nGEMM-high) showed accelerated proliferation *in vitro*, compared with *Mycn*-nGEMM-low, expressing the lowest levels of *Mycn* (Fig. S1 G). Both cell lines were transduced with a firefly luciferase gene and implanted into the renal capsule of B6 hosts. This allowed monitoring of tumor growth by bioluminescent imaging (BLI) (Fig. 1 F and Fig. S1 H). As expected, latency was shorter for cell line-derived tumors than for *de novo* primary tumors, and faster growth kinetics were observed for tumors derived from the *Mycn*-nGEMM-high cell line. Time-to-endpoint ranged from 30 days for *Mycn*-nGEMM-high to 60 days for *Mycn*-nGEMM-low (Fig. 1 G). These results suggest a correlation between levels of *Mycn* expression and aggressiveness (Fig. S1 I).

We therefore show that mis-expression of *Mycn* in mouse embryo-derived NCC is sufficient to initiate tumors in immunocompetent, syngeneic hosts. The resulting tumors express markers of NB and can be secondarily transplanted orthotopically to form tumors with short latency and full penetrance. In contrast to existing germline GEMM models for NB, cell lines can be easily derived from these *Mycn*-driven non-germline (nGEMM) tumors (*Mycn*-nGEMM) and subsequently modified genetically, facilitating easy manipulation of the model *in vitro* and *in vivo*.

Bulk RNA sequencing (RNA-seq) was performed on: (1) naïve NCC isolated from embryonic day (E) 9.5 mouse embryos, (2) NCC expressing the *Mycn* construct, (3) the *Mycn*-nGEMM-high and *Mycn*-nGEMM-low cell lines derived from primary tumors, and (4) cells isolated from TH-MYCN tumors. Expression data were compared with human cancer datasets, including acute myeloid leukemia (AML), kidney sarcoma, osteosarcoma, rhabdoid tumor, Wilms tumor, and two NB datasets: TARGET and Gabby Miller Kids First (GMKF). Uniform manifold approximation and projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE) plots showed that NCC, *Mycn*-nGEMM, and TH-MYCN samples clustered closely with human NB samples with MYCN amplification from the GMKF dataset (Fig. 1, H and I). Murine samples did not clearly cluster with the TARGET NB dataset, which might in part derive from lower tumor purity levels in this dataset. Further analysis showed that both models clustered with MYCN-amplified tumors, tumors classified as high-risk (Fig. S1 J).

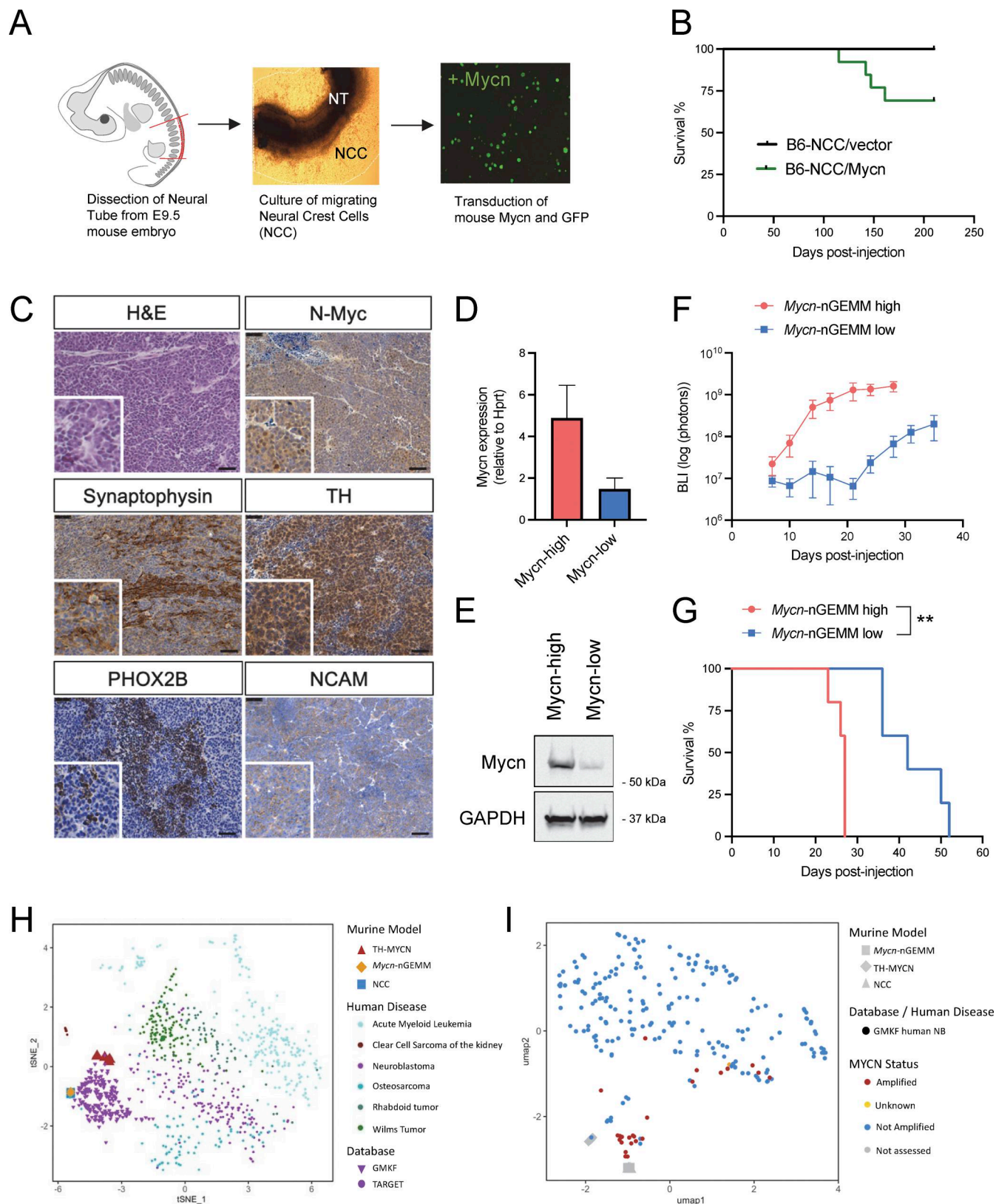


Figure 1. Expression of Mycn in migrating mouse NCC leads to tumor formation in immunocompetent, syngeneic C57BL/6J hosts. (A) Neural tubes (NT) from E9.5 C57BL/6J embryos were dissected between somite 16 and 24 (indicated by red lines) and cultivated in vitro on laminin-coated plates for 24 h. The migrating NCC were transduced with a *Mycn*-IRES-GFP retroviral construct. GFP-positive cells were sorted and formed spheres. (B) Mycn-expressing NCC (green, $n = 13$) and NCC transduced with an empty vector (black, $n = 10$) were injected in the flank of C57BL/6J mice. Tumor formation was monitored over time until endpoint was reached. (C) H&E and IHC staining for N-Myc, Phox2B, TH, synaptophysin, and NCAM on *Mycn*-nGEMM tumor shows high expression of all

markers. Scale bar = 50 μ m. **(D)** Mycn expression relative to HPRT as assessed by qRT-PCR in *Mycn*-nGEMM-high and *Mycn*-nGEMM-low cell lines. Data represent mean values $\pm$ SD ($n = 2$ independent experiments). **(E)** N-Myc expression assessed by western blot in *Mycn*-nGEMM-high and *Mycn*-nGEMM-low tumors. **(F)** Growth curves for *Mycn*-nGEMM-high ($n = 6$ mice) and *Mycn*-nGEMM-low ($n = 7$ mice) tumors as assessed by BLI. Data represent mean values $\pm$ SEM. **(G)** Kaplan–Meier curves for C57BL/6J mice injected with *Mycn*-nGEMM-high or *Mycn*-nGEMM-low cell lines in the renal capsule ($n = 5$ mice per group). Kaplan–Meier survival analyses were performed by log-rank test. $^{**}P < 0.01$ **(H)** t-SNE clustering including TH-MYCN, *Mycn*-nGEMM, NCC, and *Mycn*-NCC samples and indicated human cancer datasets. **(I)** UMAP plot using GSKF dataset depending on MYCN-amplification. Source data are available for this figure: SourceData F1.

We also carried out single-cell RNA sequencing (scRNA-seq) on *Mycn*-nGEMM-low tumors and evaluated chromosomal copy number changes in tumor cells. We identified several clusters of tumor cells characterized by different chromosomal abnormalities, including chromosomes 7, 9, and 14 loss and chromosomes 8 and 10q gain. These chromosomal copy number abnormalities reveal significant intra-tumoral heterogeneity and chromosomal instability, well-known features of neuroblastoma (Fig. S1 K).

Immune profiling reveals a cold TME dominated by immunosuppressive TAMs

Using a 40-parameter mouse-specific cytometry by time-of-flight (CyTOF) mass cytometry panel (Table S1), we initiated a detailed characterization of the TME of *Mycn*-nGEMM tumors. Medium-sized tumors (400 mm³) were harvested from both *Mycn*-nGEMM-high and *Mycn*-nGEMM-low models, dissociated into single cells, and stained with the mass cytometry panel. Single-cell data for a total of 1.8 million cells were clustered initially with the FlowSOM algorithm (Van Gassen et al., 2015), then meta-clustered with the PhenoGraph algorithm (Levine et al., 2015). Data were visualized using the tSNE algorithm (Amir et al., 2013). The first analysis showed that only one meta-cluster out of 20 (cluster 15) expressed the lymphocyte common antigen CD45 (Fig. 2 A and Fig. S2 A). The CD45-positive cluster represented ~3% of total cells in the tumor. Tumor cells, identified by expression of GFP, represent up to 90% of total cells (Fig. 2 B). The remainder included endothelial cells (GFP⁺, CD45⁺, and CD31⁺) and fibroblasts (GFP⁺, CD45⁺, and CD90⁺).

A second clustering, focusing only on the CD45⁺ cells (Fig. 2, C and D), showed the immune infiltrate to be dominated by immunosuppressive (M2-like) macrophages (CD11b⁺, F4/80⁺, MHCII⁺, and CD206⁺), representing up to 40% of total immune cells in both *Mycn*-nGEMM-high and *Mycn*-nGEMM-low tumors. The second most represented immune type were MHCII⁺ macrophages (CD11b⁺, F4/80⁺, and MHCII⁺), ~20% of total immune cells, while inflammatory, CD11b⁺, F4/80⁺, MHCII⁺, and CD206⁺ macrophages represented 10% of immune cells. 80% of the immune infiltrate consisted of myeloid cells, and <10% comprised T cells or B cells (Fig. 2 E). NK cells and dendritic cells (DCs) were very rare (<1%). No regulatory T cells were detected. Interestingly, proportions of the different immune subsets were very similar between *Mycn*-nGEMM-high and *Mycn*-nGEMM-low tumors (Fig. 2 E). Thus, despite differences in tumor growth kinetics, both models developed a highly immunosuppressive TME, recapitulating human MYCN amplified NB (Costa et al., 2022).

Expression of cytokines, chemokines and growth factors by *Mycn*-nGEMM-high and *Mycn*-nGEMM-low cells was assessed by RNA-seq (Fig. 2 F). Both cell lines expressed and secreted high

levels of macrophage migration inhibitory factor (MIF; Fig. 2 G), a pleiotropic inflammatory cytokine upregulated in NB, contributing to tumor aggressiveness (Bin et al., 2002; Cavalli et al., 2019) and inhibiting T cell-mediated anti-tumor immunity (Zhou et al., 2008). CSF1, a well-characterized ligand of CSF1-R that promotes monocytes and macrophages recruitment to the TME, was also highly expressed in both cell lines (Metelitsa et al., 2004; Hashimoto et al., 2016; Hadjidaniel et al., 2017). Blockade of CSF1-R improved response to chemotherapy in NB preclinically (Webb et al., 2018). A cytokine array performed on the supernatant of *Mycn*-nGEMM-high cultures confirmed these findings, with CSF1, C-C Motif chemokine ligand 2 (CCL2), and C-X-C motif ligand 1 (CXCL1) all detected (Fig. S2 B). Secretion of tissue inhibitor of metalloproteinases-1 was also observed, associated with poor survival in patients with NB (Paul et al., 2017).

Overall, *Mycn*-nGEMM tumors show poor immune infiltration, characteristic of immunologically cold, high-risk human NB tumors. *Mycn*-nGEMM tumors were almost devoid of T, B, and NK cells. The immune compartment was dominated by myeloid cells and immunosuppressive TAMs, likely attracted to the tumor by cancer cell-secreted cytokines.

Anti-PD-L1 treatment of *Mycn*-nGEMM tumors depletes immunosuppressive TAMs, remodeling the intra-tumoral immune landscape

While outcomes for high-risk disease have improved modestly through escalation of chemotherapy and a cocktail of immune-activating cytokines plus anti-GD2 monoclonal antibody, the success of immune checkpoint blockade has notably been limited for patients with NB.

We first tested the response of the *Mycn*-nGEMM model to CPI. B6 mice with established *Mycn*-nGEMM tumors by BLI were treated with five doses of CPI (anti-PD-1 and anti-CTLA-4), delivered every 3 days, starting 7 days after orthotopic injection. Consistent with results of clinical trials in NB (Qiu and Matthay, 2022), neither tumor growth nor survival were impacted significantly in response to CPI (Fig. S3, A and B). While mass cytometry analyses performed on tumors at endpoint showed an increase of CD4⁺ T and CD8⁺ T cells in the spleen of CPI-treated animals, no changes in the TME were observed (not shown).

In contrast, mice-bearing orthotopic *Mycn*-nGEMM-low tumors showed significant inhibition of tumor growth when treated with anti-PD-L1 therapy alone (Fig. 3, A and B). Anti-PD-L1 treatment also resulted in significantly improved survival for *Mycn*-nGEMM-low tumor-bearing mice compared with IgG-treated mice (Fig. 3 C). After five doses of anti-PD-L1, tumor volume was significantly decreased for *Mycn*-nGEMM-high

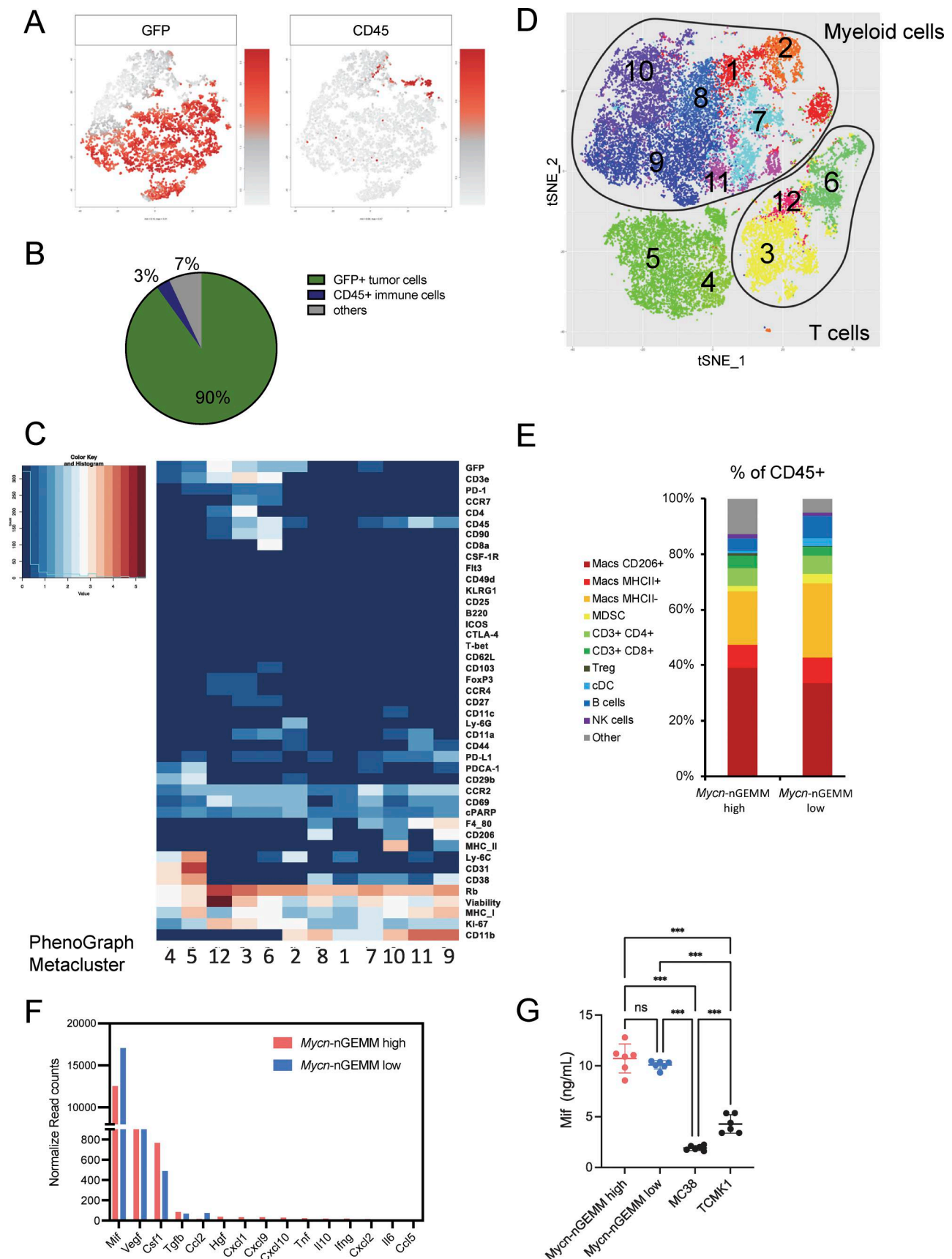


Figure 2. **Immune profiling of *Mycn*-nGEMM tumors reveals a highly immunosuppressive TME dominated by TAMs.** (A) t-SNE representation of GFP and CD45 signals in *Mycn*-high and *Mycn*-low nGEMM tumors ($n = 3$ tumors per cell line). (B) Average proportion of tumor cells (GFP⁺), immune cells (CD45⁺),

and other cells in *Mycn*-nGEMM tumors ($n = 3$ tumors per cell line). **(C)** Heatmap indicating levels of expression for each marker for all CD45⁺ meta clusters identified with the mouse mass cytometry panel. **(D)** t-SNE representation of clustering for CD45⁺ cells in *Mycn*-nGEMM tumors ($n = 3$ tumors per cell line). **(E)** Percentage of the different immune subsets in *Mycn*-nGEMM tumors ($n = 3$ tumors per cell line). **(F)** Expression of cytokines assessed by RNA-seq analysis on *Mycn*-nGEMM-high and *Mycn*-nGEMM-low cells, represented as normalized read counts. **(G)** Secreted MIF expression from conditioned cell media of *Mycn*-nGEMM, MC38 murine colon adenocarcinoma and TCMK1, mouse kidney epithelial cells determined by ELISA assay. Data represent mean $\pm$ SD from three independent experiments. Statistical significance was determined by one-way ANOVA multiple comparisons test (G). *** $P < 0.001$, ns = no significance.

tumors as well, although survival was not improved significantly in this more aggressive model (Fig. S3, C and D).

To understand effects on the immune landscape, we treated *Mycn*-nGEMM tumors with three doses of anti-PD-L1, then analyzed tumors by mass cytometry at day 14 after implantation. Significantly more inflammatory macrophages (CD11b⁺, F4/80⁺, MHCII⁺, and CD206⁺) and fewer immunosuppressive TAMs (CD11b⁺, F4/80⁺, MHCII⁻, CD206⁺, and PD-L1⁺) were detected in the TME of anti-PD-L1-treated tumors (Fig. 3 D and Fig. S3 E). While PD-L1⁺, CD206⁺ TAMs represented 40% of the immune cells in the IgG-treated tumors, almost no PD-L1⁺ immunosuppressive TAMs were detected in anti-PD-L1-treated tumors. Conversely, the proportion of inflammatory TAMs increased from 10 to 40% of the total immune cells in response to anti-PD-L1 treatment (Fig. 3, E and F), reducing the ratio of immunosuppressive to inflammatory TAMs from 2.5 in the IgG-treated tumors to 0.2 in the anti-PD-L1-treated tumors (Fig. S3 F). A significant increase in CD4⁺ T cells and CD8⁺ T cells infiltration, up to 6% and 17% of total immune cells, respectively, was also observed for tumors treated with anti-PD-L1 (Fig. 3, G and H).

To clarify how treatment with anti-PD-L1 impacted the TME in these models, we treated *Mycn*-nGEMM cells, bone marrow-derived macrophages (BMDM), and MC38 colorectal carcinoma cells with IgG or anti-PD-L1 in vitro. Treatment with anti-PD-L1 did not affect cell viability regardless of expression levels of PD-L1 (Fig. S3, G and H). We assessed potential antibody-dependent cell-mediated cytotoxicity (ADCC) through in vitro ADCC assays using *Mycn*-nGEMM and MC38 as target cells. This assay did not reveal mFCγRIV activity in the presence of the anti-PD-L1 antibody (Fig. S3 I). Importantly, in vivo depletion of CD4⁺ T and CD8⁺ T cells impaired tumor growth suppression induced by anti-PD-L1 treatment in the *Mycn*-nGEMM-low model (Fig. 3 I; and Fig. S3, J and K). These results suggest that the anti-tumor effects of anti-PD-L1 in this model are dependent on T cell activity.

To determine if rejection of tumors in response to treatment with anti-PD-L1-induced immunological memory, we re-challenged seven complete responders from the initial cohort by injecting *Mycn*-nGEMM-low cells subcutaneously. While five out of the seven mice did develop tumors, the latency was increased compared with naïve mice (Fig. 3 J). Additionally, two of the re-challenged mice remained tumor free after injection, suggesting that the initial anti-PD-L1 treatment promoted an immune response that led to immunological memory.

Our results highlight a previously underappreciated mechanistic function of PD-L1 blockade, with anti-PD-L1 treatment decreasing PD-L1⁺ immunosuppressive TAMs in the TME, subsequently remodeling the immune landscape of the tumors toward a more immunostimulatory environment, promoting

infiltration of T cells, decreasing tumor burden, improving survival, and resulting in immunological memory.

Expression of PD-L1 is restricted to TAMs in MYCN-amplified human NB

While PD-L1 expression in tumors is a commonly used biomarker of response to CPIs, PD-L1 expression on immune cells, including the myeloid compartment, is also a prognostic factor for the efficacy of anti-PD-L1 therapy in lung and breast cancers (Kowanetz et al., 2018; Emens et al., 2021). We therefore sought to determine the contribution of tumor and myeloid cells to overall expression of PD-L1 in MYCN-driven NB.

Studies in NB are conflicting regarding whether levels of expression of PD-L1 associate with outcome. Notably, while positive PD-L1 staining was observed in most low- and intermediate-risk cases and associated with longer overall survival in these categories, high level expression of PD-L1 was associated with increased risk of relapse and decreased survival in high-risk tumors (Majzner et al., 2017; Saletta et al., 2017). Interestingly, MYCN non-amplified tumors showed higher levels of PD-L1 expression than MYCN-amplified tumors (Saletta et al., 2017). Our analysis of a human NB cohort confirmed an inverse correlation between expression of MYCN and PD-L1. A positive correlation was observed between expression of MYC (c-myc) and PD-L1 in NB (Fig. S4 A), in line with reports showing that Myc can drive immunosuppression through upregulation of PD-L1 expression on tumor cells (Casey et al., 2016).

Mass cytometric data from the *Mycn*-nGEMM tumors confirmed that only a very limited population of GFP⁺ tumor cells expressed PD-L1 (Fig. 4 A and Fig. S4 B). However, a majority of the CD11b⁺ myeloid cells expressed high levels of PD-L1. Interestingly, a positive correlation between PD-L1 and CD163, a macrophage marker, was also observed in the human NB cohort independently of MYCN expression status (Fig. S4 A), suggesting that macrophages contribute to a significant proportion of PD-L1 expression in the TME of NB tumors.

To confirm relevance to human NB, we analyzed MYCN-amplified human NB samples to determine the proportion of tumor cells and macrophages expressing PD-L1. Serial immunohistochemical analyses for PD-L1, PHOX2B, and CD163 proteins were performed and analyzed using an image processing pipeline (Fig. 4, B and C). Quantification showed that PD-L1 was expressed mainly on CD163⁺ cells (Fig. 4 D and Fig. S4 C). Expression of PD-L1 on PHOX2B⁺ tumor cells was detectable only at very low frequency (<1%). These data, along with previous reports (Shirinbak et al., 2021) confirm that TAMs are the main source of PD-L1 expressed in human MYCN-amplified NB, and suggest the translatability of our preclinical results with anti-PD-L1 in *Mycn*-nGEMM tumors to human NB.

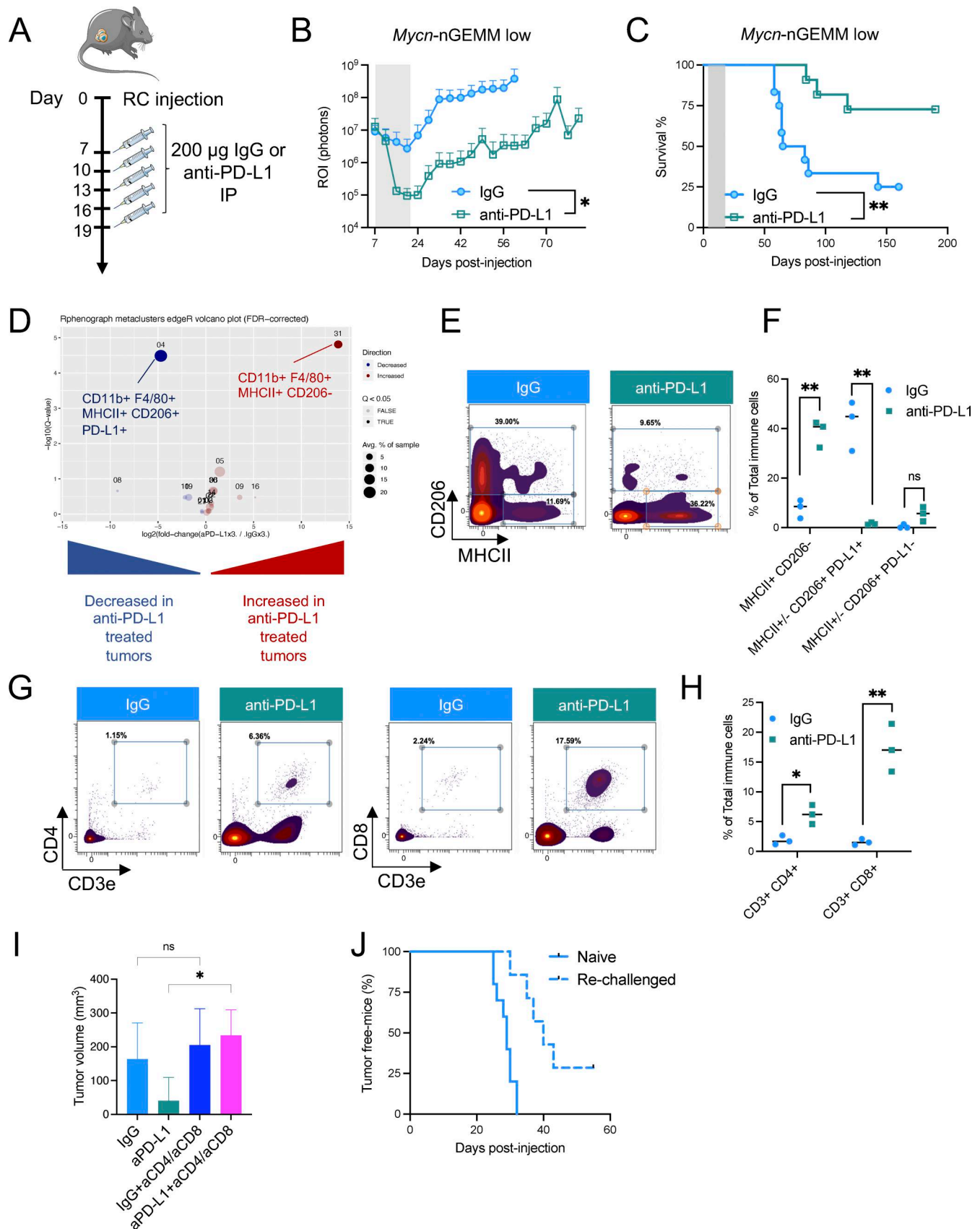


Figure 3. **Anti-PD-L1 treatment of *Mycn*-nGEMM tumors depletes CD206⁺ TAMs and remodels the intra-tumoral immune landscape.** (A) *Mycn*-nGEMM-low cells were transplanted in the renal capsule of C57BL/6J hosts on day 0. After BLI imaging and randomization, treatments started on day 7 with 200

μg of anti-PD-L1 (or corresponding IgG) injected i.p. every 3 days for a total of 5 doses. **(B)** Growth curves of *Mycn*-nGEMM-low tumors treated with IgG or anti-PD-L1 antibodies, as assessed by BLI. Grey area indicates treatment window. Data represent mean values ± SEM ($n = 5$ mice per group). **(C)** Kaplan–Meier curves for C57BL/6J mice injected with *Mycn*-low cell line in the renal capsule and treated with 5 doses of IgG or anti-PD-L1 antibodies ($n = 10$ mice per group). **(D)** Volcano plot comparing abundance of tumor-infiltrating leukocyte (TIL) subpopulations in anti-PD-L1-treated tumors (red) and IgG-treated tumors (blue) using the mouse mass cytometry immune cell panel ($n = 3$ tumors per group). Statistically significant clusters in volcano plots are highlighted in opaque color and indicated with a cell type label. **(E)** Mass cytometry data from *Mycn*-nGEMM tumors were filtered on CD45⁺CD11b⁺F4/80⁺ TAMs, then gated manually to identify cells positive or negative for CD206 or MHC-II. Frequencies of TAMs expressing CD206 and MHCII was quantified for IgG-treated and anti-PD-L1-treated tumors ($n = 3$ tumors per group). **(F)** Percentage of CD11b⁺F4/80⁺ TAMs expressing MHC-II, CD206, or PD-L1 markers in *Mycn*-nGEMM tumors after treatment with IgG or anti-PD-L1 antibodies ($n = 3$ tumors per group). **(G)** Mass cytometry data from *Mycn*-nGEMM tumors were filtered on CD45⁺ cells, then gated manually to identify cells positive or negative for CD3e, CD4, or CD8. Frequencies of T cells expressing CD4 and CD8 was quantified for IgG-treated and anti-PD-L1-treated tumors ($n = 3$ tumors per group). **(H)** Percentage of CD4⁺ T cells and CD8⁺ T cells in *Mycn*-nGEMM tumors after treatment with IgG or anti-PD-L1 antibodies ($n = 3$ tumors per group). **(I)** After CD4⁺ T and CD8⁺ T cell depletion by neutralizing antibodies and treatment of IgG and anti-PD-L1, tumor volume was measured by ultrasound on day 21 after injection ($n = 5$ mice per group). **(J)** Kaplan–Meier curves representing percentage of tumor-free mice for naïve ($n = 10$ mice) and anti-PD-L1-cured mice ($n = 7$ mice) rechallenged with *Mycn*-nGEMM-low cells in the right flank. Statistical significance was determined by two-way ANOVA (B), unpaired *t* test (F and H), and one-way ANOVA multiple comparisons test (I). Kaplan–Meier survival analyses were performed by log-rank test (C and J). * $P < 0.05$, ** $P < 0.01$ and ns = no significance.

Cytokines secreted from *Mycn*-nGEMM cells induce expression of PD-L1 on TAMs

To determine whether factors secreted by tumor cells could induce expression of PD-L1 on TAMs, we polarized BMDM cells into immunosuppressive macrophages and co-cultured in a transwell system with cell lines from four different murine cancers. After 3 days, expression of PD-L1 on the cell surface of the macrophages was measured by flow cytometry. Interestingly, macrophages co-cultured with the *Mycn*-nGEMM cells showed significantly increased expression of PD-L1 (Fig. 4 E). In contrast, expression of PD-L1 remained low on the macrophages co-cultured with SB28 glioma, MC38 colorectal carcinoma, or B16F10 melanoma cells, suggesting that cytokines specifically secreted by the *Mycn*-nGEMM cells play a significant role in the upregulation of PD-L1 on the immunosuppressive TAMs.

We observed that MIF was highly secreted by *Mycn*-nGEMM (Fig. 2, F and G). MIF is upregulated in a variety of tumor types. The interaction of MIF with CD74 has been reported to upregulate expression of PD-L1 on melanoma cells, helping tumor cells to escape anti-tumor immune response (Imaoka et al., 2019). To test whether MIF secreted by the *Mycn*-nGEMM cells could induce PD-L1 on TAMs, BMDM-derived M2-like macrophages were co-cultured with *Mycn*-nGEMM-high cells in a transwell system in the presence of a MIF inhibitor (4-Iodo-6-phenylpyrimidine [4-IPP]) or vehicle control. Treatment with 4-IPP partially reversed the expression of PD-L1 at the surface of the macrophages (Fig. S4 D). These data suggest secretion of MIF as a mechanism contributing to the increased expression of PD-L1 on TAMs. Bin and colleagues previously reported potential roles for MIF in both aggressiveness and escape from anti-neuroblastoma immune response (Bin et al., 2002). Our results suggest that these escape mechanisms could include upregulation of PD-L1 on TAMs in the TME.

scRNA-seq analysis of the TME in *Mycn*-nGEMM tumors

To further clarify how anti-PD-L1 influences the immune landscape, we carried out scRNA-seq on *Mycn*-nGEMM-low tumors. UMAP of 11,943 tumor and CD45⁺ cells identified seven clustered subsets of cells (tumor, T cells, B cells, NK cells, DCs, TAMs, and granulocytes—Fig. 5 A), all of which showed *Mif* expression, with highest levels in the tumor cell cluster (Fig. 5 B). These data

were compared with published scRNA-seq datasets of human neuroblastoma and TH-MYCN tumors, showing high expression of MIF similar to *Mycn*-nGEMM tumors (Fig. S5, A–D) (Dong et al., 2020; Costa et al., 2022).

By scRNA-seq, we characterized *Mycn*-nGEMM TAMs into three subtypes—Arg1^{hi}, Cxcl9^{hi} T, and Ace^{hi} (Fig. 5, C and D). Consistently, Arg1^{hi} TAMs displayed an M2-like signature, whereas Cxcl9^{hi} TAMs exhibited a more M1-like signature (Fig. 5 E). Ace^{hi} TAMs scored negatively for both M1 and M2 signatures, suggesting a more undifferentiated M0 cell state. We next compared *Mycn*-nGEMM TAMs to M2-like human TAMs defined by Wienke et al. (2024). Arg1^{hi} and Cxcl9^{hi} murine TAMs resembled MAF^{hi} M2-like human TAMs, whereas Ace^{hi} murine TAMs resembled S100^{hi} M2-like human TAMs (Fig. 5 F). Extending this analysis to murine TH-MYCN, we showed that TH-MYCN TAMs were clustered into three subpopulations. Both were M2-like, one population was Ace^{hi} and one was Cxcl9^{hi} (Fig. S5, E and F). These results indicate that (1) *Mycn*-nGEMM tumors expressed high levels of *Mif*, (2) *Mycn*-nGEMM TAMs were heterogeneous and displayed predominantly M2-like signatures, and (3) *Mycn*-nGEMM TAMs exhibit greater heterogeneity than TH-MYCN TAMs. Additionally, we examined levels of N-Myc protein. *Mycn*-nGEMM tumors exhibited higher levels of N-Myc protein compared with TH-MYCN tumors, supporting our hypothesis that N-Myc also contributes to an immunosuppressive TME in *Mycn*-nGEMM (Fig. S5 G).

Anti-PD-L1 cooperates with CD40 agonist to remodel the TME and suppress *Mycn*-nGEMM NB

While we observed regression and significantly longer overall survival in *Mycn*-nGEMM-low tumor-bearing mice following treatment with anti-PD-L1, no significant survival benefit was observed in mice-bearing fast-growing *Mycn*-nGEMM-high tumors, despite a significant decrease in tumor size after treatment. These observations suggest that increased infiltration of proinflammatory TAMs and T cells following the depletion of PD-L1⁺ immunosuppressive TAMs was insufficient to trigger a strong anti-tumor immune response in the more aggressive *Mycn*-nGEMM-high model.

CD40 agonists promote antigen presentation and enhance anti-tumor activity of macrophages. We hypothesized that

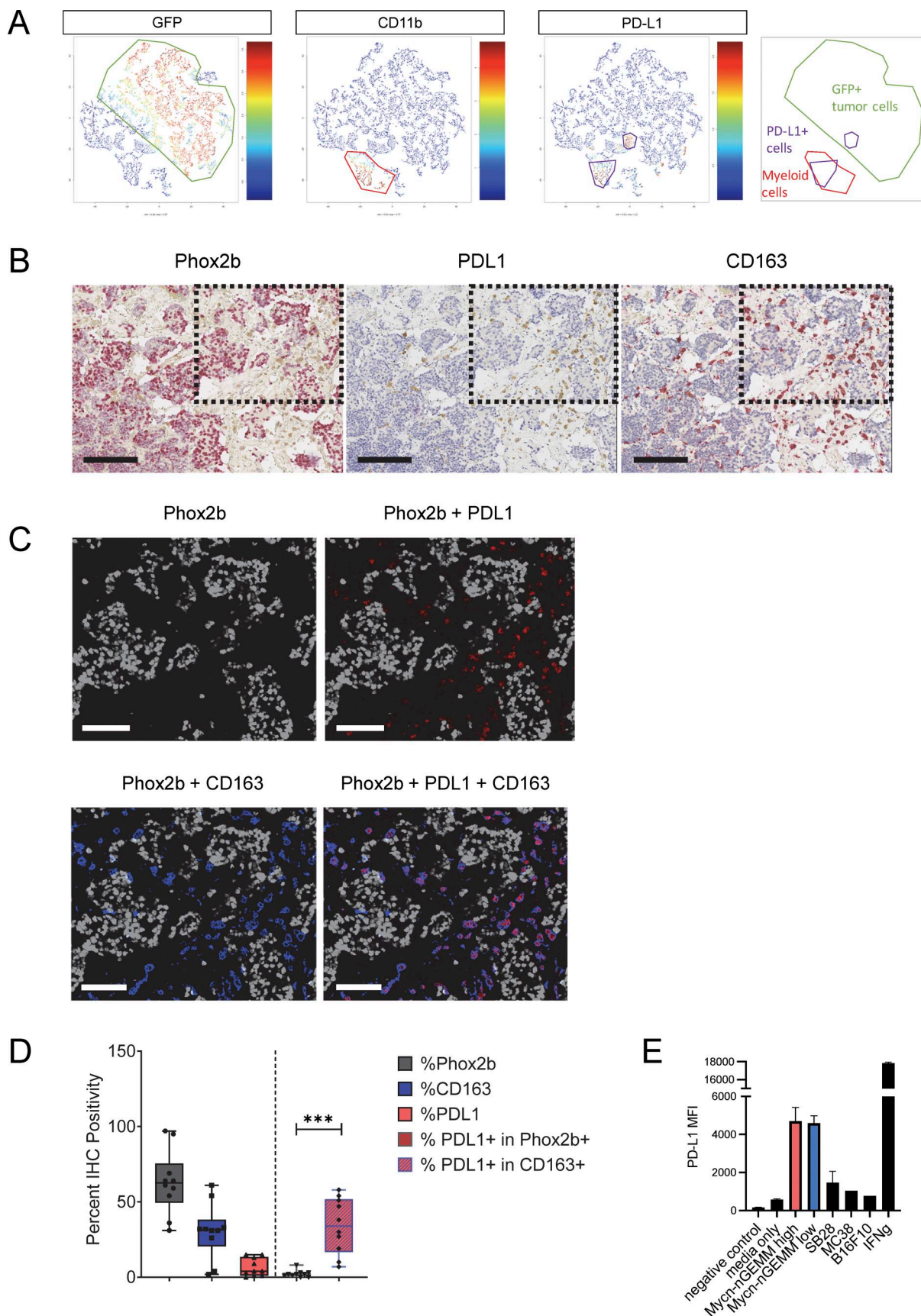


Figure 4. **PD-L1 is expressed on TAMs in both *Mycn*-nGEMM and human NB tumors.** (A) t-SNE representation of GFP, CD11b, and PD-L1 signals in *Mycn*-nGEMM-high and *Mycn*-nGEMM-low tumors ($n = 3$ tumors per cell line) shows that PD-L1 is expressed mostly on myeloid cells in *Mycn*-nGEMM tumors.

(B) Representative serial IHC of human *MYCN*-amplified NB tumor and pseudo-fluorescence visualization. Scanned images (10×) of serial IHC of Phox2b (left, nuclear staining, red), PD-L1 (middle, membrane staining, brown), and CD163 (right, membrane staining, red) of a representative formalin-fixed paraffin-embedded section of a human *MYCN* amplified NB obtained after chemotherapy. Scale bar = 200 μ m. **(C)** Magnified region from B (dotted black lines) of deconvoluted images stacked to demonstrate IHC of Phox2b, Phox2b with PD-L1, Phox2b with CD163, and Phox2b with CD163 and PD-L1. Scale bar = 100 μ m. **(D)** Quantitative image analysis of *MYCN*-amplified tumors for CD163, Phox2b, and PD-L1. Boxplot of the percent positivity of Phox2b, CD163, and PD-L1 in *MYCN*-amplified NB tumors. Percent positivity is also shown for Phox2b⁺PD-L1⁺ cells (mean 2.2%) and CD163⁺PD-L1⁺ cells (mean 34.1%). **(E)** PD-L1 expression assessed by flow cytometry on BMDM co-cultured with indicated cell lines (*Mycn*-high, *Mycn*-low, SB28, MC38, or B16F10) or treated with IFN γ for 72 h. Mean fluorescent intensity (MFI) for the PD-L1 signal is indicated for each condition. Data represent mean $\pm$ SD from two independent experiments. Statistical significance was determined by one-way ANOVA multiple comparisons test (D); ***P < 0.001.

combining CD40 agonist antibody and anti-PD-L1 blockade could therefore enhance anti-tumor immunity in the *Mycn*-nGEMM-high model. Mice injected orthotopically with *Mycn*-nGEMM-high cells were treated with five doses of anti-PD-L1, CD40 agonist, or a combination (Fig. 6 A). Treatment with CD40 agonist led to increased overall survival and significantly decreased tumor volume after five doses and synergized with anti-PD-L1 (Fig. 6, B–D; and Fig. S5, H and I). However, CD40 agonist did not sensitize *Mycn*-nGEMM tumors to anti-PD-1 (Fig. S5 J).

Analysis of the TME at day 21 after implantation revealed a significant increase in CD45⁺ cells in tumors, up to 10% for the combination of anti-PD-L1 and CD40 agonist (Fig. 6 E and Fig. S5 K). Increased expression of MHC-I on tumor cells was also observed, suggesting enhanced antigen presentation and tumor immunogenicity (Fig. 6 F). The anti-PD-L1 and CD40 agonist combination also led to increased expression of CD38 on TAMs (Fig. 6 G), a marker of activated proinflammatory, anti-tumor macrophages. Finally, proportions of CD11c⁺ DCs (Fig. 6 H), CD4⁺ T and CD8⁺ T cells (Fig. 6, I and J), and B220⁺ B cells (Fig. 6 K) were also higher in tumors treated with the combination compared with each single agent or IgG control.

Overall, the anti-PD-L1-mediated depletion of immunosuppressive TAMs, combined with increased antigen presentation and activation of proinflammatory macrophages by CD40 agonist led to remodeling of the TME, increasing tumor immunogenicity, enhancing tumor growth inhibition, and extending survival in the aggressive *Mycn*-nGEMM-high model.

Discussion

Here, we describe a novel *Mycn*-driven, transplantable, immunocompetent mouse model for NB, a new system that accurately mirrors the TME of human *MYCN*-amplified NB tumors. In contrast to classical autochthonous GEMM that recapitulates a limited number of drivers, demonstrates long latencies, and is difficult to monitor and further manipulate genetically, this mouse NCC-based non-germline *Mycn*-nGEMM model shows short latency and is readily amenable to BLI monitoring in vivo. Tumor cells can be readily modified genetically to incorporate additional mutations relevant to primary or relapsed NB. Tumor cells can also be implanted into immunological mutants in the B6 background to explore interactions between tumor cells and the TME.

While amplification of *MYCN* is the most prominent genetic lesion in high-risk NB, human NB presents considerable intra- and inter-tumoral heterogeneity, in part due to recurrent copy number aberrations. Analysis of copy number has shown intra-

tumoral heterogeneity in the *Mycn*-nGEMM models as well, with multiple chromosomal aberrations.

Results from early stage clinical trials with CPI in pediatric cancers have been disappointing. The lack of strong tumor-associated antigens in NB, a copy number-driven cancer, and the low immune infiltration in the TME likely contribute to the failure of T cell-targeted CPI in the clinic. Therefore, elucidating mechanisms of immunosuppression is essential to improve clinical responses to CPI in NB. Here, we showed that, similar to human *MYCN*-amplified NB, the TME of *Mycn*-nGEMM tumors is almost devoid of immune cells. *Mycn*-nGEMM TAMs constitute the dominant immune subset and the main source of PD-L1 expression. While tumoral PD-L1 is often used as a predictive biomarker for response to checkpoint inhibition, PD-L1 expression on tumor-infiltrating myeloid cells is also an independent predictor of response (Kowanetz et al., 2018; Emens et al., 2021; Herbst et al., 2014; Powles et al., 2014). PD-L1-expressing myeloid cells serve an essential role in restricting T cell responses through the PD-1/PD-L1 axis (Tang et al., 2018; Oh et al., 2020), suggesting blockade of PD-L1 signaling in myeloid cells as an important mechanism for successful application of CPI.

While the *Mycn*-nGEMM models were resistant to treatment with anti-PD-1 and anti-CTLA-4 in combination, *Mycn*-nGEMM-low tumors responded to anti-PD-L1. PD-L1-expressing immunosuppressive macrophages were depleted following treatment with anti-PD-L1, leading to profound remodeling of the TME, with increased proportions of anti-tumoral and inflammatory macrophages and increased infiltration of CD4⁺ T and CD8⁺ T cells. These observations confirm previous reports of a differential immunological impact of PD-L1 blockade compared with anti-PD-1 treatment. Indeed, anti-PD-L1 treatment was shown to directly affect the myeloid compartment in tumors by promoting repolarization of M2-like TAMs into anti-tumoral inflammatory M1-like macrophages and triggering macrophage-mediated anti-tumor activity (Hartley et al., 2018; Xiong et al., 2019). Together, these results suggest that anti-PD-L1 therapy can promote anti-tumor immunity, even in highly immune evasive tumors like *Mycn*-driven NB, where anti-PD-1 therapy had a limited effect.

Tumor-infiltrating macrophages express much higher levels of PD-L1 compared with monocytes in culture (Hartley et al., 2017). Cytokines produced locally in the tumor are drivers of PD-L1 expression on TAMs and include TNF α (Hartley et al., 2017), IL-10 (Bloch et al., 2013), the IL-27/STAT3 axis (Horlad et al., 2016), IL-4, IL-6, IL-13, and CXCL8 (Zhang et al., 2018; Lin et al., 2019). Analysis of cytokines produced in the TME of the *Mycn*-nGEMM tumors revealed high expression of MIF, CSF1,

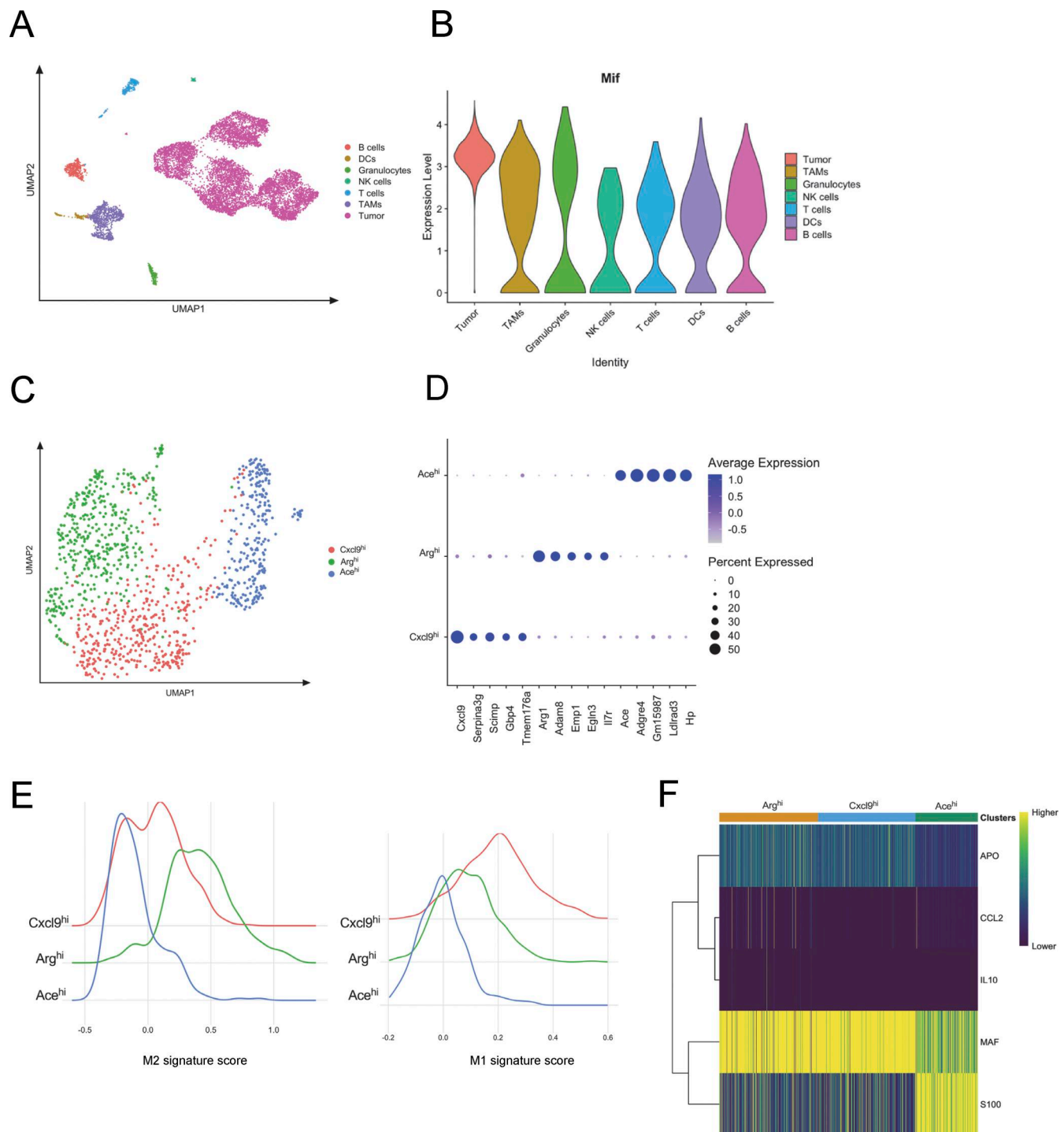


Figure 5. scRNA-seq identifies heterogeneity among TAMs in *Mycn*-nGEMM tumors, which correlates with TAMs in human NB. (A) UMAP of 11,943 CD45⁺ cells and neuronal cells obtained from three replicates of *Mycn*-nGEMM tumors after integration. **(B)** Violin plot of *Mif* mRNA expression by cell type of the *Mycn*-nGEMM tumors. **(C)** UMAP of 1,780 *Mycn*-nGEMM TAMs. Three major clusters were identified and annotated according to marker genes and pathway analysis (see text for details). **(D)** Dotplot presenting the marker genes for each cluster of C. **(E)** Distributions of M2 signature (left) and M1 signature (right) scores for each TAM subtypes in C. **(F)** Heatmap of correlation scores between *Mycn*-nGEMM TAMs and human TAM subtypes defined by Wienke et al. (2024). Columns represent *Mycn*-nGEMM TAM cells and rows represent human TAM subtypes. The colored bar at the top represents the *Mycn*-nGEMM macrophage clusters (subtypes).

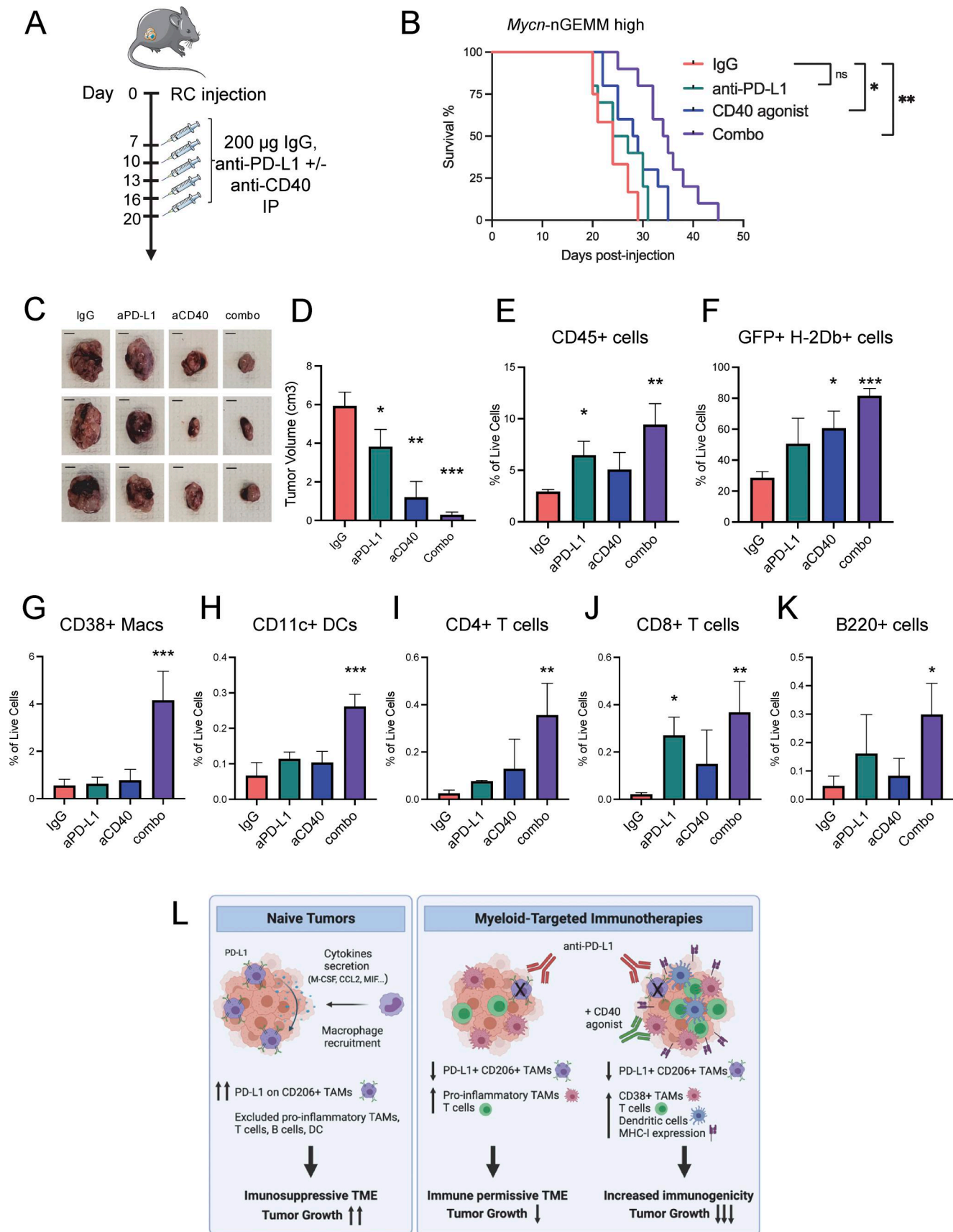


Figure 6. **Combining anti-PD-L1 with anti-CD40 significantly impairs tumor growth and increases survival in the *Mycn*-high nGEMM model.** (A) *Mycn*-nGEMM-high cells were transplanted in the renal capsule (RC) of C57BL/6J hosts on day 0. After BLI imaging and randomization, treatments started on

day 7 with 200 μ g of anti-PD-L1 (or corresponding IgG), CD40 agonist, or a combination of both injected i.p. (IP) every 3 days for a total of 5 doses. **(B)** Kaplan–Meier curves for C57BL/6J mice, transplanted with *Mycn*-nGEMM-high cells in the renal capsule on day 0, and treated with indicated antibodies ($n = 10$ –12 mice per group). **(C)** Pictures of three tumors harvested at day 21 (5 treatment doses) for indicated treatment group. **(D)** Tumor volumes were measured for tumors harvested at day 21 for each treatment group. Indicated values are mean of $n = 3$ tumors per group $\pm$ SD. **(E)** Proportion of CD45⁺ cells in tumors from each treatment group as a percentage of total cells in the tumors (mean values of $n = 3$ tumors) $\pm$ SD. **(F)** Proportion of GFP⁺H-2Kd/H2Dd⁺ cells in tumors from each treatment group as a percentage of total cells in the tumors (mean values of $n = 3$ tumors) $\pm$ SD. **(G)** Proportion of CD38⁺ macrophages as a percentage of total cells in the tumors (mean values for $n = 3$ tumors) $\pm$ SD. **(H)** Proportion of CD11c⁺ DCs as a percentage of total cells in the tumors (mean values for $n = 3$ tumors) $\pm$ SD. **(I)** Proportion of CD4⁺ T cells as a percentage of total cells in the tumors (mean values for $n = 3$ tumors) $\pm$ SD. **(J)** Proportion of CD8⁺ T cells as a percentage of total cells in the tumors (mean values for $n = 3$ tumors) $\pm$ SD. **(K)** Proportion of B220⁺ B cells as a percentage of total cells in the tumors (mean values for $n = 3$ tumors) $\pm$ SD. **(L)** Cartoon summarizing effect of anti-PD-L1 and CD40 agonist therapies on the TME and growth of *Mycn*-nGEMM tumors. Statistical significance was determined by ordinary one-way ANOVA test (D–K), and Kaplan–Meier survival analyses were performed by log-rank test (B). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and ns = no significance.

CCL2, and CXCL1, all implicated in recruitment of myeloid cells to the tumor. TAMs showed no detectable levels of the other cytokines described as drivers of PD-L1 expression. Nevertheless, co-culture experiments confirmed that factors secreted by *Mycn*-nGEMM cells led to significant upregulation of PD-L1 on M2-like TAMs in vitro. Interestingly, MIF, the most highly expressed cytokine in the *Mycn*-nGEMM system and a known contributor to tumorigenesis and aggressiveness in NB (Cavalli et al., 2019; Garcia-Gerique et al., 2022), has been described as a driver of PD-L1 expression on melanoma cells through its interaction with CD74 (Imaoka et al., 2019). Here, we showed that inhibition of MIF decreased PD-L1 expression on immunosuppressive TAMs co-cultured with *Mycn*-nGEMM cells, suggesting that MIF could participate in the regulation of PD-L1 expression on TAMs as well.

No tumor regression has been observed to date in pre-treated NB patients who received the anti-PD-L1 antibody atezolizumab, with at best stable disease for 4 out of 11 patients (Geoerger et al., 2020). While *MYCN*-amplification status of the 11 treated patients was not detailed in this manuscript, anti-PD-L1 therapy alone did not appear sufficient to drive immune reactivation against the tumor. CD40 agonists are being actively investigated in a variety of tumor types, notably in the context of tumors with insufficient baseline T cell activation like pancreatic adenocarcinoma (Beatty et al., 2011; Byrne and Vonderheide, 2016; Jiang et al., 2022; Li and Wang, 2020). CD40 activation on antigen-presenting cells leads to immune activation, notably potentiating inflammatory macrophage function (Beatty et al., 2011). CD40 agonists were also shown to trigger a T cell-independent, anti-tumor immune response in the non-*Mycn*-driven, syngeneic NXS2 NB models by stimulating macrophage function (Lum et al., 2006). The increase in M1-like macrophages observed after anti-PD-L1 therapy in *Mycn*-nGEMM tumors led us to investigate combination of anti-PD-L1 with CD40 agonist in the more aggressive *Mycn*-nGEMM-high model. Combining both agents induced significant tumor growth inhibition, TME remodeling, and extended survival in these poorly immunogenic tumors. CD40 agonists are currently being evaluated in pediatric patients with tumors of the central nervous system (Lindsay et al., 2020) but have not yet been evaluated in other pediatric cancers to date.

Overall, our work demonstrates that the novel *Mycn*-driven, immunocompetent *Mycn*-nGEMM model for NB represents a promising new system to accurately model the highly immune

evasive TME of human *MYCN*-amplified NB tumors and evaluate the impact of different immunotherapy strategies. While *Mycn*-nGEMM tumors were resistant to anti-PD-1/anti-CTLA-4 immunotherapies, our study highlights the potential of harnessing the tumor-infiltrating myeloid compartment to drive TME remodeling through depletion of immunosuppressive TAMs via anti-PD-L1 therapy and to enhance anti-tumor immune response by targeting the CD40/CD40L axis in previously untreated tumors. A future challenge will be to model therapy-induced changes in the TME—including radiotherapy, chemotherapy, and targeted therapy—to determine the best combination strategy with immune-based therapy in pre-treated tumors.

Materials and methods

Trunk neural tube dissection and NCC culture

Neural tubes were dissected from E9.5 B6 embryos to allow outgrowth of trunk NCC, as previously described (Pfaltzgraff et al., 2012). Briefly, trunk neural tubes were isolated and cultured in vitro on fibronectin-coated plates and maintained in a 50% DMEM low glucose (11885084; Gibco) + 30% neurobasal media (21103049; Gibco), supplemented with 15% Chick Embryo Extract (100-163P; GeminiBio), 1% penicillin-streptomycin (15140122; Gibco), 1X N2 (LS17502048; Gibco), 1X B27 (LS12587010; Gibco), 117 nM retinoic acid (R2625; Sigma-Aldrich), 50 μ M 2-mercaptoethanol (21985023; Gibco), 20 ng/ml basic fibroblast growth factor (bFGF) (300-305P; GeminiBio), and 20 ng/ml IGF-1 (300-310P; GeminiBio). After 24 h in culture, neural tubes were removed to retain only the migrating trunk NCC. Migrating NCC were transduced with retroviruses expressing N-Myc and GFP and sorted 48 h after transduction for GFP expression. 1e6 cells were resuspended in a 1:1 solution of Geltrex (A1569601; Gibco) and DMEM media and injected subcutaneously in the flank of 6-wk-old B6 mice.

Virus production and cell transduction

HEK293T cells were plated in 10-cm petri dishes. For retroviruses, 80% confluent HEK293T cells were transfected 24 h after plating with a pMSCV plasmid expressing N-Myc and GFP (a gift from Jeff Bluestone lab at University of California, San Francisco [UCSF], San Francisco, CA, USA), plasmids pUMVC (gift from Bob Weinberg, #8449; Addgene) and pCMV-VSV-G (gift from Bob Weinberg, #8454; Addgene), using TransIT-LT1

reagent following manufacturer's instructions (MIR2300; Mirus). For lentiviruses, 80% confluent HEK293T cells were transfected 24 h after plating with a pCMV plasmid expressing luciferase, psPAX2 (a gift from Didier Trono #12260; Addgene) and pCMV-VSV-G plasmids using TransIT-Lenti reagent following the manufacturer's instructions (MIR6600; Mirus). Supernatants were harvested 48 h after transfection. Viruses were concentrated overnight using Lenti-X concentrator (631232; Takara), centrifuged at $2,000 \times g$ for 45 min at 4°C, and re-suspended in appropriate media before being added to the cells. Media was changed after overnight incubation of the cells with viruses.

Tumor dissociation and *Mycn*-nGEMM cell lines

Subcutaneous tumors obtained after injection of N-Myc expressing NCC into B6 mice were dissociated into single cells using TrypLE (12604013; Gibco). Single-cell suspensions were filtered through a nylon 70- μ m filter, and erythrocytes were lysed by incubation in ammonium-chloride-potassium lysing buffer (A10492-01; Gibco) for 5 min. Cells were resuspended in PBS and counted, and 5×10^5 living cells were plated per 150-cm² cell culture flasks in neurobasal media (21103049; Gibco) supplemented with 1X B27 (LS12587010; Gibco), 1X L-glutamine (25030149; Gibco), 20 ng/ml of FGF (450-33; Peprotech), 20 ng/ml of EGF (315-09; Peprotech), and 1% penicillin-streptomycin (15140122; Gibco). Spheres were passaged by dissociation using TrypLE. *Mycn*-nGEMM cell lines were transduced with a lentiviral construct expressing luciferase and blasticidin and selected with 10 μ g/ml of blasticidin (A1113903; Thermo Fisher Scientific) for 10 days.

Cell culture

Cell lines (SB28, MC38, B16F10, and TCMK1) were grown in DMEM or RPMI with 10% FBS. All cells were tested and authenticated. Mycoplasma status was checked monthly using Plasmotest Mycoplasma Detection Kit (InvivoGen). Cells were passaged every 3–5 days with TrypLE Express Enzyme (Life Technologies) when confluent and replated in T75 flasks at 1×10^5 – 5×10^5 cells in 15 ml media.

Renal capsule injections in mice and treatments

Animal experiments were performed in accordance with national guidelines and regulations and UCSF Institutional Animal Care and Use Committee (IACUC) protocol numbers AN169783-01C or AN152965-02A. 6–8-wk-old C57BL/6J female mice were acquired from Jackson Laboratory (Stock number 000664) and acclimated for 7 days prior to injection date. Mice were injected in the left renal capsule with 4×10^5 cells resuspended in 5 μ l of a 1:1 solution of Geltrex and Neurobasal media. 7 days after injection, luminescence was measured, and mice with a signal above 10^6 were enrolled for treatment. Mice were treated i.p. with 200 μ g of antibody or the corresponding isotype control diluted in saline to a volume of 100 μ l per mouse, every 3 days for a total of five doses. Endpoint criteria by which mice were euthanized were defined per our approved IACUC protocol as exhibiting >15% weight loss from baseline weight taken on the day of injection and/or exhibiting signs of pain (i.e., hunch and grimace),

Table 1. Antibodies used for in vivo studies

Antibody	Corresponding isotype control
Anti-mouse PD-1 BioXCell BE0146	Rat IgG2a isotype BioXCell BE0089
Anti-mouse CTLA-4 BioXCell BE0131	Polyclonal Syrian Hamster BioXCell BE0087
Anti-mouse PD-L1 BioXCell BE0101	Rat IgG2b isotype BioXCell BE0090
Anti-mouse CD40 BioXCell BE0016	Rat IgG2a isotype BioXCell BE0089
Anti-mouse CD4 BioXCell BE0003	Rat IgG2a isotype BioXCell BE0090
Anti-mouse CD8a BioXCell BE0061	Rat IgG2a isotype BioXCell BE0090

ulceration of the skin overlying tumors without improvement over a period of 7 days, tumor width exceeding 2 cm, abnormal neurological signs (such as seizures), or decreased motility (Table 1).

T cell depletion in mice

Seven days after orthotopic tumor injection, mice were treated with 200 μ g anti-PD-L1 and/or 200 μ g anti-CD4/anti-CD8 or corresponding isotype control (Table 1) i.p. every 3 days. On day 21, tumor volumes were measured by ultrasound, and peripheral blood was collected from the submandibular vein and analyzed for T cell populations by flow cytometry (Table 4).

Bioluminescent imaging of tumor-bearing mice

Bioluminescent signals from tumors were measured twice a week, starting 7 days after injection. Mice received i.p. injection of potassium-salt D-luciferin (GoldBio) diluted in sterile saline at 20 mg/ml for a dose of 64 mg/kg 20 min prior to measurement of radiance. Mice were anesthetized with isoflurane and imaged using an IVIS Spectrum Imaging System (PerkinElmer). Photon intensity was measured for each mouse using the Living Image software (PerkinElmer).

Immunohistochemical staining

Tumors were fixed in 4% PFA for 24 h, then 70% EtOH for 48 h. After embedding in paraffin, 5- μ m paraffin sections were cut. Sections were deparaffinized using xylene and rehydrated. Antigen retrieval was performed using 1X, pH 6.0, citrate buffer (C9999-100 ML; Sigma-Aldrich) in a pressure cooker (2 min at 125°C then 10 min at 90°C). Endogenous peroxidase activity was blocked by incubating sections in 0.3% hydrogen peroxide solution (H1009; Sigma-Aldrich) for 15 min. Sections were permeabilized with 0.4% Triton (X-100; Sigma-Aldrich) in PBS. Blocking was performed with 10% normal goat serum (50062Z; Thermo Fisher Scientific). Sections were incubated overnight with primary antibodies at dilutions indicated. After washing three times in PBS, sections were incubated with the biotinylated secondary antibody (immunohistochemistry [IHC] selected goat anti-rabbit IgG biotinylated, 21537; Millipore Sigma) for 1 h at room temperature (RT). Sections were incubated in Vectastain ABC-HRP solution (PK4000; Vector

Laboratories) for 30 min at RT, then in DAB substrate solution (SK4105; Vector Laboratories) for 10 min following the manufacturer's instructions. Sections were washed in water then dehydrated and mounted for imaging on a Leica microscope given in Table 2.

IHC and image analysis on human samples

IHC for PD-L1 (clone M3653; DAKO), Phox2b (clone ab183741; abcam), and CD163 (clone NCL-L-CD163; Leica Novocastra) was performed on 10 MYCN amplified tumors. The human tissue collection was approved by the Children's Hospital Los Angeles Institutional Review Board. In brief, tumor sections were serially stained using Leica BOND RX and scanned at 40× magnification after each staining using Aperio Image Scanner.

Serial images for PD-L1, CD163, and Phox2b markers were processed in the following manner using custom automation macros in FIJI ImageJ software (Schindelin et al., 2012). Tiles of 16,384 × 16,384 pixels were imported from each large Aperio image in a gridded fashion using the Bio-Formats plugin (Linkert et al., 2010) and saved in TIFF format. For each tile position, the marker images were aligned twice for better accuracy using the "Linear Stack Alignment with SIFT" plugin with default parameters and affine transformation (Lowe, 2004). The aligned marker images were color deconvolved (Landini et al., 2021; Ruifrok and Johnston, 2001) with vectors for FastRed-FastBlue-DAB, inverted, and color channels corresponding to each marker plus nuclei were saved as one pseudo-fluorescence multichannel TIFF per tile (nuclei, blue from CD163; Phox2b-red; PD-L1-DAB; CD163-red). Tumor tissue regions with abundant Phox2b positivity were selected for detection and classification of cells using QuPath Imaging Software (version 0.3.0). Positive cells for each stain (PD-L1, CD163, and Phox2b) were identified by setting threshold values for each pseudo-fluorescence marker. The classifiers then enumerated single-, double-, and triple-positive cells for the markers.

RNA extraction and qRT-PCR

RNA was extracted from cells or tumors using the Quick-RNA miniprep kit from Zymo Research (R1054). RNA was quantified by NanoDrop (Thermo Fisher Scientific). For qRT-PCR, 800 ng of RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (4368814). Detection was performed using the SYBR green PCR master mix from Applied Biosystems (4309155) following the manufacturer's instruction on a Quantstudio 5 Real-time PCR system

Table 2. Antibodies used for IHC

Antibody	Reference	Dilution
Phox2B	Abcam ab183741	1:200
TH	Abcam ab137869	1:200
Synaptophysin	Abcam ab14692	1:500
NCAM	Abcam ab95153	1:100
MYCN	Abcam ab16898	1:100
MYCN	Cell signaling 51705	1:400

(Applied Biosystems). The following primers were designed using Primer-BLAST (Ye et al., 2012) (Table 3).

RNA-seq and analysis

RNA extracted from *Mycn*-nGEMM tumors was sequenced. 180 µg of total RNA from quality-controlled samples was processed for mRNA sequencing and sequenced on an Illumina HiSeq 2000, with a 150-bp paired-end read length.

Raw fastq files from RNA-seq data were aligned to the mouse mm10 genome using the STAR aligner v 2.7.3a. Gene expression was quantified using RSEM v1.3.3 and Ensembl v92 gene annotation to get transcript per million expression values. Mouse gene IDs were mapped to human gene IDs using biomaRt v2.48.3.

Western blot

1e6 NCC were lysed in 200 µl of radioimmunoprecipitation assay lysis buffer (#89900; Thermo Fisher Scientific) supplemented with protease inhibitors (#87785; Thermo Fisher Scientific) for 15 min on ice. Lysates were sonicated and then centrifuged at 4°C for 10 min at 13,000 rpm. 4X Laemmli buffer (Bio-Rad) was added to the supernatants to a final concentration of 1X, and samples were incubated at 95°C for 5 min. 1 µg of each sample was loaded on a 10% acrylamide gel (Bio-Rad) and run at 150 V for 30 min. Gels were transferred on nitrocellulose membrane using the TransBlot Turbo machine (Bio-Rad) following the manufacturer's instructions. Membranes were blocked in 5% milk in TBS with 0.1% Tween 20 detergent (TBS-T) for 1 h at RT, then incubated with MYCN antibody (ab24193; Abcam) or GAPDH antibody (AM4300; Ambion) diluted at 1:500 and 1:1,000 respectively, in 5% milk in TBS-T overnight. Membranes were washed three times in TBS-T and incubated with secondary antibody (W4018 goat anti-rabbit Promega or W4028; goat anti-mouse Promega) diluted in 5% milk for 1 h at RT. Membranes were washed three times in TBS-T. Signal was detected

Table 3. Primers used for qRT-PCR

Gene	Forward primer	Reverse primer
mHPRT	5'-TGCTGACCTGCTGGATTA-CA-3'	5'-TTATGTCCCCCGTTGACTGA-3'
mMYCN	5'-AGCACCTCCGAGAGGAT-AC-3'	5'-CGGTGACCACATCGATTTC-3'
mSox10	5'-CTAGCCGACCAGTACCCT-CA-3'	5'-GGCGCTTGTCACCTTCGTTCC-3'
mFoxD3	5'-GTCCGAGGACATGTTTCA-CAA-3'	5'-GCTCCGAAGCTCTGCATC-ATC-3'
mASCL1	5'-GTCAGAGCTGTCTTAGCC-CC-3'	5'-GTCCGAGAACTGACGTTGCT-3'
mHand2	5'-CAAGGACGACCAGAACGG-AG-3'	5'-GTGCTTTTCAAGATCTCATTC-AGC-3'
mPhox2A	5'-TCCTCCAAGTGTGCGCTT-3'	5'-CTCGTGAACGTTGTGCGGAT-3'
mPhox2B	5'-TATGGCCGGGATGGATAC-CT-3'	5'-GGCCCCAAAAGTGGTCCTTA-3'
mTH	5'-CAAGCAGGGTGAGCCAAT-TC-3'	5'-TGGGTAGCATAGAGGCCCTT-3'

using Pierce ECL western blotting substrate (32209; Thermo Fisher Scientific) following the manufacturer's instructions.

Cell proliferation assay

Cells were plated in 96-well plates at 1,000 cells per well on day 0. Cells were quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions.

ADCC reporter bioassay

The assay was performed using mFC γ RIV ADCC Reporter Bioassay Kit following the manufacturer's protocol (Promega). Briefly, *Mycn*-nGEMM, MC38, and mFC γ RIV effector cells were mixed at a ratio of 1:5, and the mixture was seeded into white 96-well plates and treated with IgG and anti-PD-L1 antibodies. After 6 h of incubation at 37°C with 5% CO₂, luciferase activity was quantified by a micro plate reader.

Dissociation of tumors for mass cytometry

Tumors were harvested after treatment, and 200–400 mg were dissociated into single cells. Tumors were cut into 5-mm pieces using a scalpel. Tumor fragments were washed in PBS, then resuspended in 1.5 ml of a solution of collagenase IV at 3.2 mg/ml (LS004209; Worthington), deoxyribonuclease I at 1 mg/ml (LS002007; Worthington), and soybean trypsin inhibitor (LS003587; Worthington) at 2 mg/ml diluted in PBS and incubated at 37°C under agitation for 30 min. Cell suspensions were strained through a 70- μ m mesh cell strainer and washed with 10 ml of PBS. Cells were centrifuged at 300 $\times$ *g* for 5 min at 4°C. Pellets were resuspended in 500 μ l of PBS. 5 ml of ammonium-chloride-potassium lysing buffer (#A10492-01; Gibco) was added to lyse erythrocytes for 5 min at RT. Reactions were quenched with 5 ml of PBS, and cells were centrifuged at 300 $\times$ *g* for 5 min at 4°C. Pellets were resuspended in PBS and counted. 30e7 cells were resuspended in 5 ml of PBS, and 1 μ l of 5 mM Cisplatin-Pt198 was added (#201198; Fluidigm) for 5 min at RT. Reaction was quenched with 5 ml of Cell Staining Media (CSM) buffer (Miltenyi AutoMACS running buffer). Cells were centrifuged at 300 $\times$ *g* for 5 min at 4°C. Pellets were resuspended in 5 ml of CSM, and 500 μ l of 16% PFA were added to fix the cells for 10 min at RT. Reactions were quenched with 5 ml of CSM, and cells were centrifuged at 600 $\times$ *g* for 5 min at 4°C. Pellets were resuspended in 10 ml of CSM to wash, and cells were centrifuged at 600 $\times$ *g* for 5 min at 4°C. Finally, pellets were resuspended at 1e7 cells per mL in CSM supplemented with 10% DMSO, and three tubes were frozen at –80°C (1e7 cells per tube) at –80°C for long-term storage.

Mass cytometry antibody conjugation and validation

Metal-tagged antibodies were purchased pre-conjugated (Fluidigm Corporation) or conjugated in-house using the MAXPAR X8 chelating polymer kit (Fluidigm Corporation). Some metal isotopes were obtained from Trace Sciences International and were prepared by the UCSF Single Cell Analysis Center (SCAC). The concentration of final conjugated antibody stocks was measured by 280-nm absorbance on a NanoDrop and diluted to a maximum concentration of 0.8 mg/ml for storage at 4°C. Metal-

tagged antibody stocks were validated and titrated using positive control and negative control cells on the Fluidigm Helios mass cytometer at UCSF SCAC as described previously (Simonds et al., 2021).

Staining of mouse samples with antibodies for CYTOF mass cytometry

Mouse tumor, spleen, and lymph node samples were thawed, counted, and stained 24–72 h prior to data acquisition on the Helios mass cytometer. Samples were barcoded with Fluidigm Cell-ID 20-Plex Pd Barcoding Kit according to the manufacturer's protocol. Barcoded samples were pooled and then washed by centrifuging at 600 $\times$ *g* 5 min and resuspending in AutoMACS running buffer. For mouse samples, Fc blocking was performed with anti-mouse CD16/CD32 antibody (clone 2.4G2) (UCSF SCAC) at 1 μ g per 1e6 cells for 10 min at RT. For human samples, Fc blocking was performed with TruStain FcX (Bio-Legend) at 8 μ l per 100 μ l of cell suspension for 5 min at RT. Mouse samples were stained with FITC-conjugated anti-CCR2 and biotin-conjugated anti-CCR4 for 30 min, then washed twice with AutoMACS running buffer. Cells were then stained with metal-tagged antibodies against surface-expressed targets for 30 min at RT, then washed twice with AutoMACS running buffer. Surface-stained cells were resuspended in 3 ml of Perm-S Buffer (Fluidigm) and incubated for 30 min at 4°C (mouse samples) for permeabilization. Permeabilized cells were centrifuged and resuspended in Perm-S Buffer with a cocktail of antibodies against intracellular markers, then incubated 30 min at RT. Cells were washed twice with Perm-S Buffer and twice with AutoMACS running buffer. Mouse samples were intercalated and postfixed at 4°C for up to 7 days in PBS with 7% Perm-S buffer (vol/vol), 0.1 μ M Ir intercalator (Fluidigm), and 4% PFA. Cells were centrifuged and resuspended in 18 M Ω water with EQ Four Element Calibration Beads (Fluidigm) prior to running on the Fluidigm Helios mass cytometer at UCSF SCAC using a Super Sampler (Victorian Airship and Scientific Apparatus, LLC) as the sample input device.

Clustering method for mass cytometry data

Data were acquired on the Helios cytometer and then pre-processed as described previously (Simonds et al., 2021) by normalizing, debarcoding, and gating on viable cell events. Clustering was performed in two steps, first by identifying 900 self-organizing map (SOM) nodes with FlowSOM (Van Gassen et al., 2015) and then clustering the SOM nodes with PhenoGraph (Levine et al., 2015).

Differentiation and co-culture of BMDM

Bone marrow was harvested from the femur of C57BL/6J mice, and monocytes were grown in DMEM media, supplemented with 10% FBS and 20% of 3T3-mCSF conditioned media for 7 days for differentiation into macrophages. BMDM were treated with a combination of IL4 (10 ng/ml; PeproTech) and IL10 (10 ng/ml; PeproTech) or IFN γ (10 ng/ml; PeproTech) for 3 days. Polarization into M2-like macrophages was confirmed by staining with CD206 antibody by flow cytometry. M2-like BMDM were then co-cultured in Transwell system (3412; Corning) with

indicated tumor cell lines for 3 days in DMEM media. Where indicated, 4-IPP, a selective MIF inhibitor (3429; Tocris), was added to the culture (1 μ M). PD-L1 levels were assessed by flow cytometry.

scRNA-seq data processing and analysis

Raw sequencing data from a single tumor sample were aligned to the GRCm39-2024-A mouse reference genome using Cell Ranger (v8.0.0). The resulting output was processed using Seurat (v5). Quality control measures were applied to filter out cells with <200 or >6,500 detected genes, <500 total aligned reads, or >10% mitochondrial RNA content. This filtration yielded 11,943 high-quality cells for subsequent analysis.

Gene expression counts were normalized using the “Log-Normalize” method in Seurat, wherein counts for each cell were divided by the total counts for that cell, multiplied by a scale factor of 10,000, and natural log-transformed. The top 2,000 variable features were identified using the “FindVariableFeatures” function. Principal component analysis was performed on these variable features, and the first 15 principal components were used to compute 20 nearest neighbors for each cell using the “Find-Neighbors” function. Cells were then clustered using the “FindClusters” function. Dimensionality reduction was achieved through UMAP using the first 15 principal components.

Initial cell type annotation was performed using the SingleR tool with the MouseRNAseqData function from the celldex library as a reference. Macrophages were identified based on the expression of key marker genes: *Ptpcr*, *Adgre1*, *Cd68*, *Cd14*, and *Itgam*. This resulted in the selection of 1,175 macrophage cells for further analysis.

Visualization of gene expression patterns was accomplished using the “VlnPlot,” “DimPlot,” and “DotPlot” functions from the Seurat package. Cluster-specific marker genes were identified using the “FindAllMarkers” function with the following parameters: *only.pos* = TRUE, *logfc.threshold* = 0.25, and *min.pct* = 0.3. Genes exhibiting significant expression in multiple clusters were largely excluded from the final marker gene list.

M1/M2 macrophage signatures were derived from Wienke et al. (2024), and module scores for these signatures were calculated for each cell using Seurat’s “AddModuleScore” function.

Cell type annotation and correlation analysis between *Mycn*-nGEMM TAMs and human TAM subtypes were performed using the SingleR algorithm (Aran et al., 2019). The reference dataset consisted of labeled scRNA-seq data of TAMs obtained from Wienke et al. (2024). SingleR utilized this reference to compute correlations and assign labels to the nGEMM TAM populations based on transcriptional similarities to the human TAM subtypes.

Flow cytometry

BMDMs were harvested using TrypLE, filtered through a 70- μ M filter, and then stained with indicated antibodies for 30 min on ice. Cells were fixed in 4% PFA for 10 min at RT and analyzed on a SH800 (Sony Biotechnology).

List of antibodies given in Table 4.

Cytokine array

Mycn-nGEMM-high cells were cultured for 3 days, then 500 μ l of supernatant was assessed on a Cytokine Array following the

Table 4. Antibodies used for flow cytometry

Brilliant Violet 421 anti-mouse CD274	BioLegend 124315
PE anti-mouse CD45	BioLegend 147711
Alexa Fluor 647 anti-mouse F4/80	BioLegend 127313
PerCP/Cyanine5.5 anti-mouse I-A/I-E	BioLegend 107625
PE/Cyanine7 anti-mouse CD206	BioLegend 141719
Brilliant Violet 421 anti-mouse CD45	BioLegend 103134
PE anti-mouse CD4	BioLegend 116006
Alexa Fluor 594 anti-mouse CD3 ϵ	BioLegend 152318
PE/Cyanine 7 anti-mouse CD8a	BioLegend 100722

manufacturer’s instructions (ARY006; R&D Systems). Signal was analyzed using Image J Software.

ELISA

Mycn-nGEMM, MC38, and TCMK1 cells were seeded on 12-well plate. After 24 h, conditioned cell media was collected and used to determine secreted MIF expression. The assay was performed following the protocol of mouse MIF ELISA kit (Abcam).

Statistical analyses

All statistical analyses were performed using GraphPad Prism9. Differences in means were determined with Student’s *t* test or ANOVA, not assuming equal variances. Differences in survival were determined using the log-rank test.

Online supplemental material

Fig. S1 shows expression of *Mycn* in migrating mouse NCC leads to tumor formation in immunocompetent, syngeneic C57BL/6J hosts. Fig. S2 shows immune profiling of *Mycn*-nGEMM tumors. Fig. S3 shows response of *Mycn*-nGEMM tumors to checkpoint inhibitors. Fig. S4 shows PD-L1 expression in human MYCN-amplified NB and murine *Mycn*-nGEMM model. Fig S5 shows analysis of Mif expression by scRNA-seq. Table S1 shows the mouse CyTOF antibody panel.

Data availability

Sequencing data are available at the Gene Expression Omnibus accession number: GSE299403.

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Supplemental material

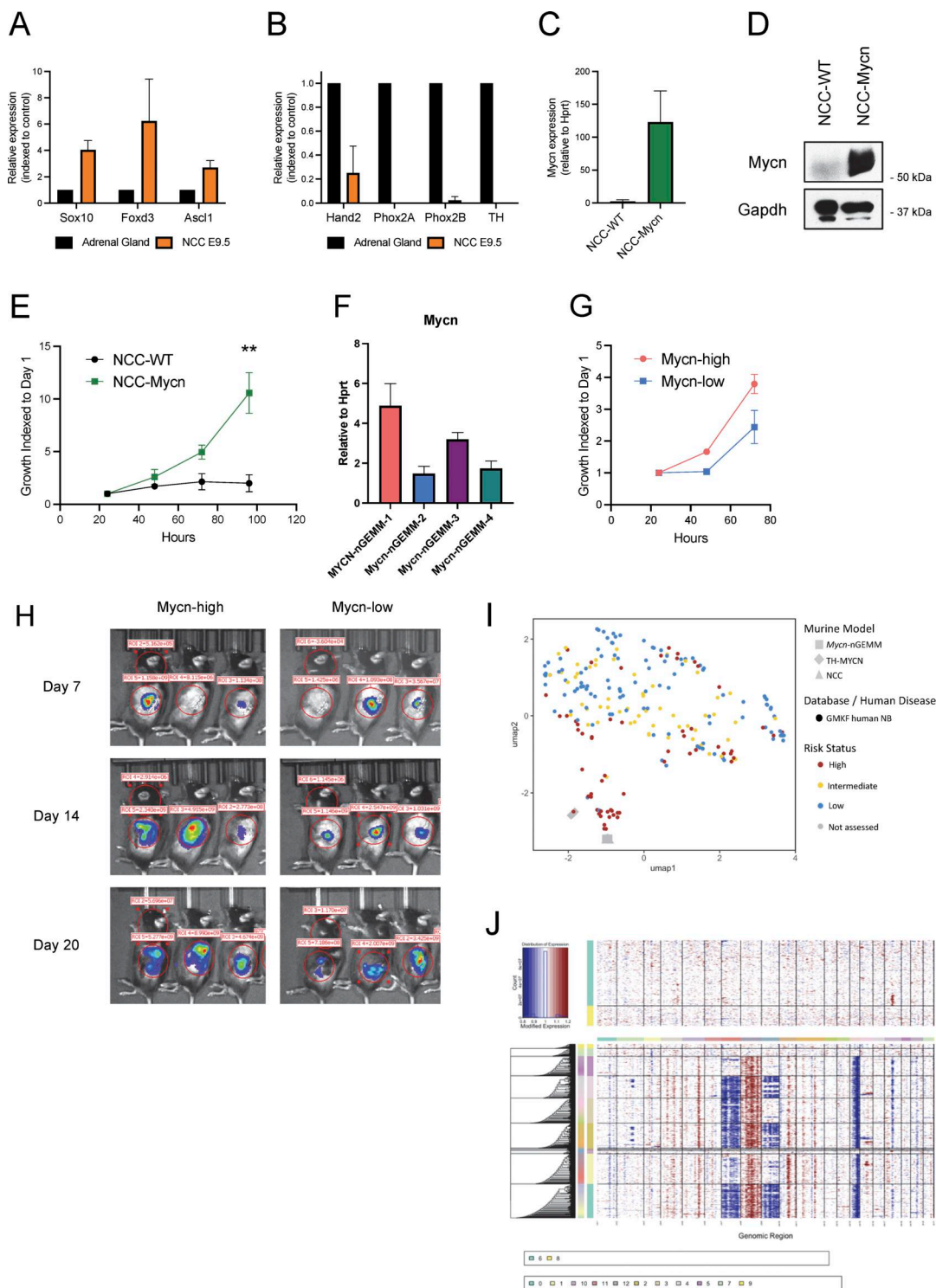


Figure S1. Expression of Mycn in migrating mouse NCC leads to tumor formation in immunocompetent, syngeneic C57BL/6J hosts. (A) Sox10, Foxd3, and Ascl1 expression relative to HPRT as assessed by qRT-PCR in adrenal gland and NCC from E9.5 embryos. Data represent mean values $\pm$ SD from $n = 3$ independent experiments. (B) Hand2, Phox2a, Phox2b, and TH expression relative to HPRT as assessed by qRT-PCR in adrenal gland and NCC from E9.5 embryos. Data represent mean values $\pm$ SD from $n = 2$ independent experiments. (C) Mycn expression relative to HPRT as assessed by qRT-PCR in NCC-WT (non-transduced) and NCC-Mycn (transduced with the Mycn-GFP construct). Data represent mean values $\pm$ SD from $n = 2$ independent experiments. (D) N-Myc expression as assessed by western blot in NCC-WT (non-transduced) and NCC-Mycn (transduced with the Mycn-GFP construct). (E) Growth kinetics of NCC-WT (non-transduced) and NCC-Mycn (transduced with the Mycn-GFP construct) cells assessed by CTG assay. Data represent mean values $\pm$ SD of $n = 3$ independent experiments. (F) Mycn expression relative to HPRT as assessed by qRT-PCR in four cell lines derived from Mycn-nGEMM primary tumors. Data represent mean values $\pm$ SD from $n = 2$ independent experiments. (G) Growth kinetics of Mycn-nGEMM-high and Mycn-nGEMM-low cells assessed by CTG assay. Data represent mean values $\pm$ SD of $n = 2$ independent experiments. (H) Representative BLI images of Fig. 1 F. (I) Umap using GMKF dataset depending on risk. (J) Heatmap on copy number variation from Mycn-nGEMM tumors. Source data are available for this figure: SourceData FS1.

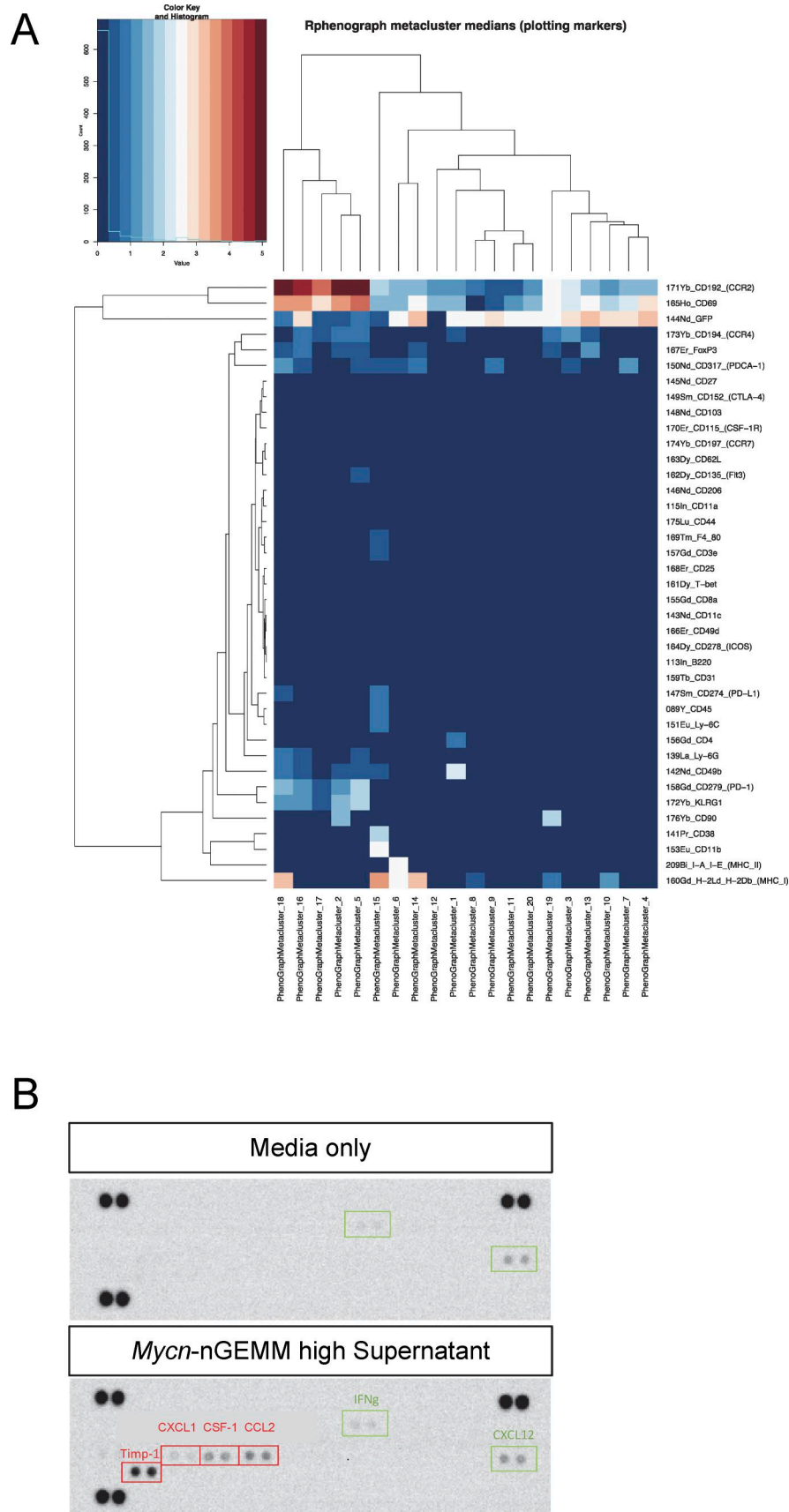


Figure S2. **Immune profiling of *Mycn*-nGEMM tumors.** (A) Heatmap indicating levels of expression for each marker for all meta clusters identified with the mouse mass cytometry panel. (B) Cytokine array performed on supernatant from media only (no cells) and *Mycn*-nGEMM-high-conditioned media.

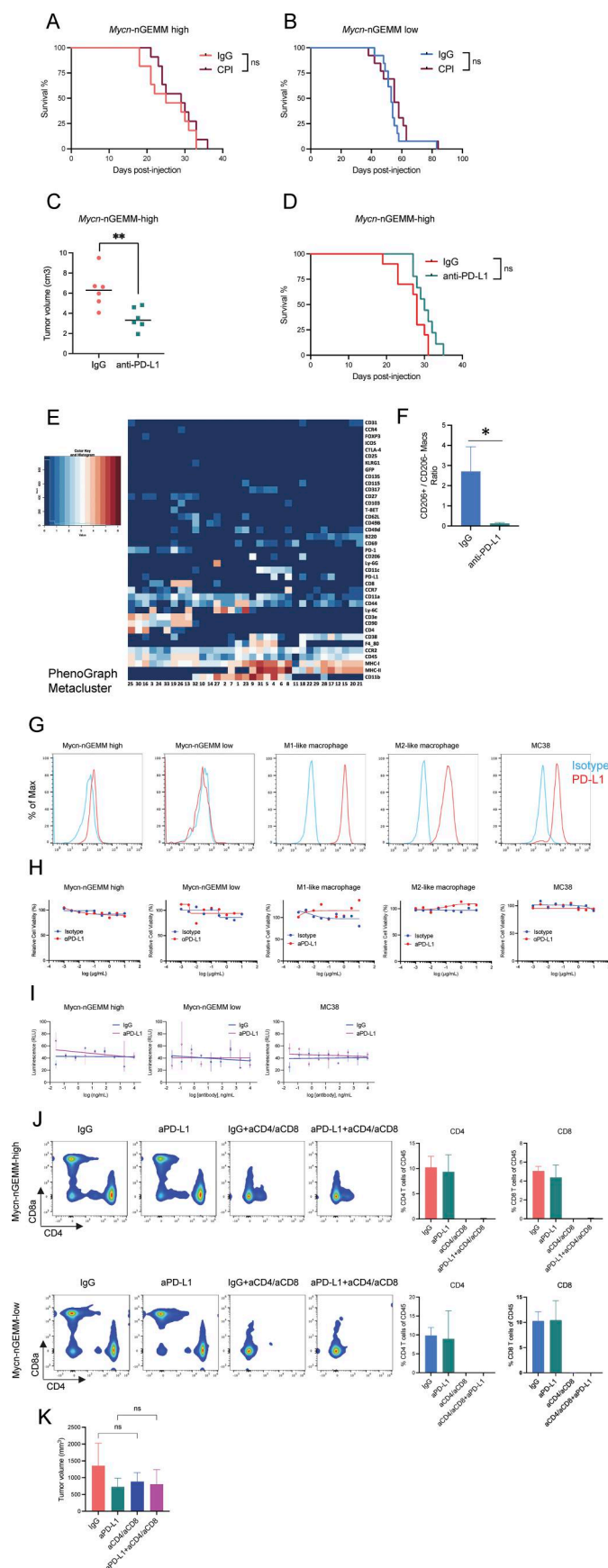
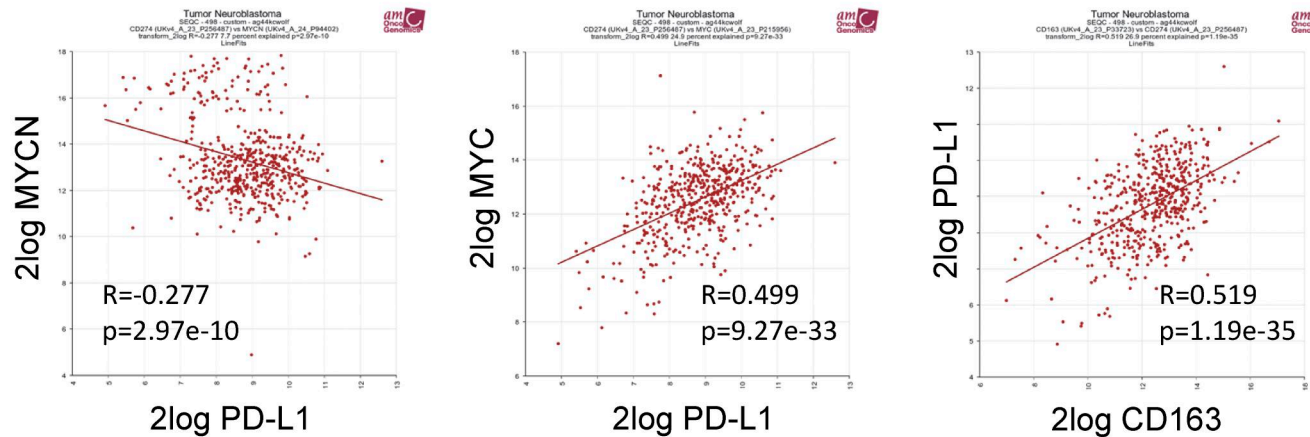
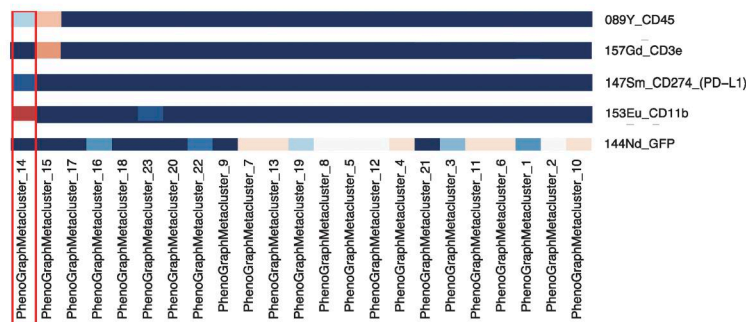


Figure S3. **Response of *Mycn*-nGEMM tumors to checkpoint inhibitors.** (A) Kaplan–Meier curves for C57BL/6J mice injected with *Mycn*-nGEMM-high cell line in the renal capsule and treated with five doses of IgG or anti-PD-1+anti-CTLA-4 antibodies ($n = 11$ mice per group). (B) Kaplan–Meier curves for C57BL/6J mice injected with *Mycn*-nGEMM-low cell line in the renal capsule and treated with 5 doses of IgG or anti-PD-1+anti-CTLA-4 antibodies ($n = 13$ mice per group). (C) Tumor volume of *Mycn*-nGEMM-high tumors implanted in the renal capsule of C57BL/6J mice and treated with five doses of anti-PD-L1 antibody or corresponding IgG antibody ($n = 6$ mice per group). (D) Kaplan–Meier curves for C57BL/6J mice injected with *Mycn*-nGEMM-high cell line in the renal capsule and treated with five doses of IgG or anti-PD-L1 antibody ($n = 9$ – 10 mice per group). (E) Heatmap indicating levels of expression for each marker for all CD45⁺ PhenoSOM meta clusters identified with the mouse mass cytometry panel for *Mycn*-nGEMM tumors treated with IgG or anti-PD-L1. (F) CD206⁺/CD206[−] macrophage ratio from mass cytometry analysis of renal capsule tumors from *Mycn*-nGEMM-low mice treated with three doses of anti-PD-L1 antibody or corresponding IgG. (G) Flow cytometry data from *Mycn*-nGEMM, BMDM-derived M1 or M2-like macrophages, and MC38 cells for isotype control and PD-L1. (H) Cell proliferation of *Mycn*-nGEMM, BMDM-derived M1 or M2-like macrophages and MC38 cells after treatment with IgG control and anti-PD-L1 for 72 h. Data represent mean $\pm$ SD from three independent experiments. (I) *Mycn*-nGEMM and MC38 cells (T; target) and mFCyRIV cells (E; effector) were cultured at E: T ratio is 5:1 under IgG control or anti-PD-L1 conditions. After 6 h, ADCC activity was determined. Data represent mean $\pm$ SD from three independent experiments. (J) Flow cytometry data from *Mycn*-nGEMM-high (top) and low (bottom) tumors after treatment with IgG, anti-PD-L1, and anti-CD4 and anti-CD8 were filtered for CD45⁺ and CD3e cells and showed cell populations for CD4 and CD8a ($n = 5$ mice per group). (K) Tumor volume was measured by ultrasound on day 21 after injection ($n = 5$ mice per group). Statistical significance was determined by unpaired t test (C and F), one-way ANOVA multiple comparisons test (K), and Kaplan–Meier survival analyses were performed by log-rank test (A, B, and D). * $P < 0.05$, ** $P < 0.01$, and ns = no significance.

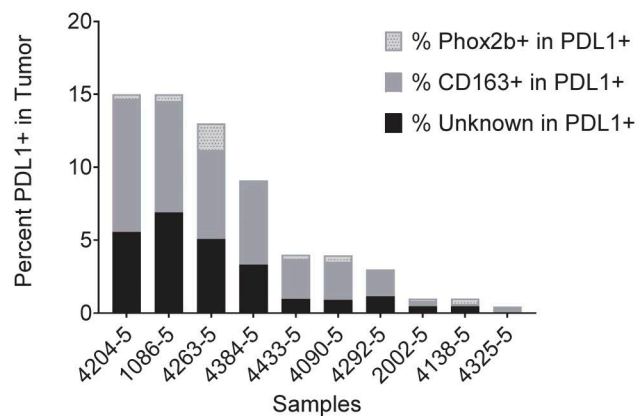
A



B



C



D

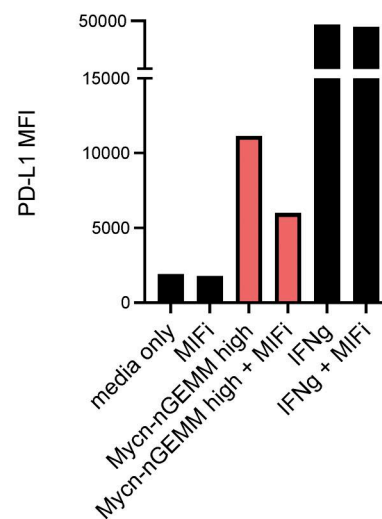


Figure S4. PD-L1 expression in human MYCN-amplified NB and murine Mycn-nGEMM model. (A) Analysis of MYCN, PD-L1, MYC, and CD163 expression in a cohort of human neuroblastoma samples. (B) Heatmap representing expression of CD45, CD3e, PD-L1, GFP, and CD11b in Mycn-nGEMM tumors from Fig. 2. (C) Percentage of PD-L1⁺ cells in Phox2B⁺ and CD163⁺ population of individual samples included in Fig. 4 D. (D) PD-L1 expression assessed by flow cytometry on BMDM co-cultured with indicated conditioned media (Mycn-high, Mycn-low), treated or not with a MIF inhibitor (MIFi) or IFN γ for 72 h. Mean fluorescent intensity (MFI) for the PD-L1 signal is indicated for each condition.

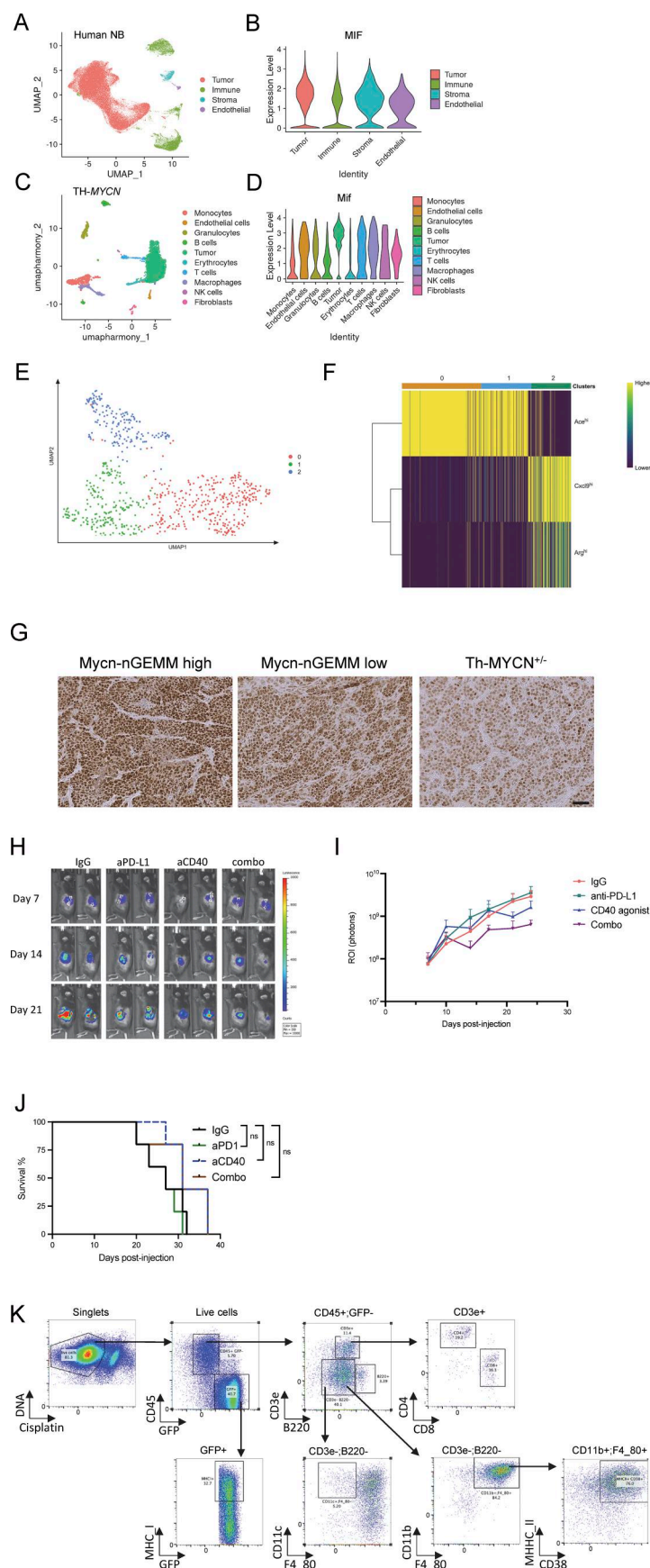


Figure S5. **Analysis of Mif expression by scRNA-seq.** (A) UMAP of human neuroblastoma cells, clustered and colored by cell type. (B) Violin plot presenting MIF expression in the different clusters of A. (C) UMAP of TH-MYCN mouse NB cells, clustered and colored by cell type. (D) Violin plot presenting Mif expression in the different clusters of C. (E) UMAP of selected macrophage cells reclustered from C. (F) Heatmap of correlation scores between TH-MYCN TAMs and *Mycn*-nGEMM TAMs subtypes. Columns represent *Mycn*-nGEMM TAMs and rows represent TH-MYCN TAMs subtypes. The colored bar at the top represents the *Mycn*-nGEMM macrophage clusters (subtypes). (G) Levels of N-Myc protein in *Mycn*-nGEMM-high, *Mycn*-nGEMM-low, and TH-MYCN^{+/−} tumors, assessed by immunostaining with Cell Signaling Technologies antibody against N-Myc. Scale bar = 50 μ m. (H) Representative BLI images from experiment described in Fig. 5 A. (I) Growth curves of *Mycn*-nGEMM-high tumors treated with IgG, anti-PD-L1, and CD40 agonist of the combination, as assessed by BLI. Grey area indicates treatment window. Data represent mean values $\pm$ SEM (n = 5 mice per group). (J) Kaplan–Meier curves for C57BL/6J mice injected with *Mycn*-nGEMM-high cell line in the renal capsule and treated with IgG, anti-PD-L1, and CD40 agonist of the combination (n = 5 mice per group). (K) Gating strategy for data represented in Fig. 6, E–K. Kaplan–Meier survival analyses were performed by log-rank test (I). ns = no significance.

Provided online is Table S1. Table S1 shows the mouse CyTOF antibody panel.