ONCOIMMUNOLOGY
2024, VOL. 13, NO. 1, 2289738
https://doi.org/10.1080/2162402X.2023.2289738

ORIGINAL RESEARCH

OPEN ACCESS

PD-L1-expressing natural killer cells predict favorable prognosis and response to PD-1/PD-L1 blockade in neuroblastoma

Mengjia Song[a,b*], Yue Huang[a,d*], Ye Hong[a,b*], Juan Liu[a,b,e], Jia Zhu[a,b], Suying Lu[a,b], Juan Wang[a,b], Feifei Sun[a,b], Junting Huang[a,b], Jiaqian Xu[a,b,e], Yan Tang[a,c], Jian-Chuan Xia[a,c], and Yizhuo Zhang[a,b]

[a]Collaborative Innovation Center for Cancer Medicine, State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou, China; [b]Department of Pediatric Oncology, Sun Yat-sen University Cancer Center, Guangzhou, China; [c]Department of Biotherapy, Sun Yat-sen University Cancer Center, Guangzhou, China; [d]Department of Medical Oncology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China; [e]Department of Pediatric Oncology, The Fifth Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China

ABSTRACT

T/NK cell-based immunotherapy has achieved remarkable success in adult cancers but has limited efficacy in pediatric malignancies including high-risk neuroblastoma (NB). Immune defects of NB tumor microenvironment are poorly understood compared with adults. Here, we described the unique characteristics of NB immune contexture and determined the phenotype signatures of PD-L1-expressing CD8$^+$ T and NK cells in NB tumors by systemically analyzing the spatial distribution of T and NK cells and the distinct expression of programmed death 1 (PD-1) and its ligand (PD-L1) in patients with NB. We found that PD-L1-expressing CD8$^+$ T and NK cells in NB tumors were highly activated and functionally competent and associated with better clinical outcomes. Intratumoral NK cells were a favorable prognostic biomarker independent of CD8$^+$ T cells, PD-1/PD-L1 expression, tumor stage, *MYCN* amplification, and risk classification. NK cells combined with anti-PD-1/PD-L1 antibodies showed potent antitumor activity against both *MYCN*-amplified and non-amplified NBs *in vitro* and *in vivo*, and PD-L1-expressing NK cells associated with improved antitumor efficacy. Collectively, we raise novel insights into the role of PD-L1 expression on CD8$^+$ T-cell and NK-cell activation. We highlight the great potential of intratumoral NK cells in better defining risk stratification, and predicting survival and response to anti-PD-1/PD-L1 therapy in NB. These findings explain why single anti-PD-1/PD-L1 therapy may not be successful in NB, suggesting its combination with NK cell-adoptive cellular therapy as a promising strategy for relapsing/refractory NB. This study provides a potential prospect that patients with PD-L1-expressing NK cells may respond to anti-PD-1/PD-L1 therapy.

ARTICLE HISTORY
Received 10 August 2023
Revised 30 October 2023
Accepted 27 November 2023

KEYWORDS
Immunotherapy; natural killer cells; neuroblastoma; programmed death ligand 1

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood, and it occurs mostly in children <5 years of age, accounting for 15% of childhood cancer-related deaths. It arises from neural crest progenitor cells in the sympathetic nervous system and presents with tumor masses in the adrenal glands and/or sympathetic ganglia.[1] Despite recent advances in surgical tumor resection, chemotherapy, radiotherapy, and autologous stem cell transplantation, the 5-year survival rate of high-risk patients is still <40%.[2] Antibody-based immunotherapy targeting disialoganglioside and B7-H3 has achieved encouraging success in some patients with NB over the past decade, and anti-disialoganglioside antibody dinutuximab was incorporated into the standard treatment regimen, which substantially improved patient survival.[3,4] However, the current prognosis of <50% survival in high-risk patients remains dismal, suggesting an urgent need for developing novel and effective immunotherapeutic approaches.

T/natrual killer (NK) cell-engineering therapies such as immune checkpoint inhibitors (ICIs) and adoptive cell therapy, particularly anti-programmed death-1 (PD-1) and anti-programmed death ligand 1 (PD-L1) antibodies, have recently achieved remarkable success in adult cancers.[5,6] However, these therapies have not shown similar efficacy in high-risk NB or other pediatric malignancies because of the nature of the tumor immune microenvironment (TIME).[7,8] Specifically, low-risk NB has the highest spontaneous regression rate among human cancers, which may be attributed to the induction of host immune responses against tumor cells.[9] This underscores the importance of an active TIME for favorable prognosis of NB patients. Nevertheless, high-risk NB, especially those accompanied by *MYCN* gene amplification, is generally recognized as a poor immunogenic tumor with low

CONTACT Yan Tang tangyan@sysucc.org.cn; Jian-Chuan Xia xiajch@mail.sysu.edu.cn; Yizhuo Zhang zhangyzh@sysucc.org.cn Collaborative Innovation Center for Cancer Medicine, State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, 651 Dongfeng Road East, Guangzhou 510060, China

*These authors contributed equally to this work.

This article has been republished with minor changes. These changes do not impact the academic content of the article.
Supplemental data for this article can be accessed online at https://doi.org/10.1080/2162402X.2023.2289738

© 2023 The Author(s). Published with license by Taylor & Francis Group, LLC.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

tumor mutation burdens (TMBs), a paucity of neoantigens, low major histocompatibility complex (MHC) antigens, immature immune system, lack of tumor-infiltrating lymphocytes, and immunosuppressive tumor microenvironment.[10] In such a contexture, classic T-cell immunity, which relies on tumor-derived peptides presented by MHC, is no longer available, complicating the hurdles for ICIs. To date, the complexity and diversity of the immune contexture and its influence on the response to ICIs are largely unidentified in NB.

Tumor-infiltrating lymphocytes (TILs) and PD-L1 expression were reportedly key biomarkers correlated with favorable prognosis and the response to anti-PD therapy.[11] The novel concept of 'TIME spatial heterogeneity' suggests that apart from the density of TILs, their localization, distribution, and relationship to PD-L1 expression status have prognostic implications.[12] PD-L1 on tumor cells is conventionally considered an oncogenic driver and a co-opted “shield” that contributes to immune escape.[13] Notably, PD-L1 is also expressed in immune cells, such as tumor-associated macrophages (TAMs), dendritic cells, myeloid-derived suppressor cells, T cells, and NK cells.[12,14–16] Recent insights on the role of PD-L1 in T and NK cells remain controversial. PD-L1 in NK cells was identified as an activation marker in a PD-1-independent manner, resulting in enhanced function and preventing cell exhaustion.[17] PD-L1$^+$ T cells also reportedly promoted self-tolerance and suppression of neighboring macrophages and effector T cells in cancer.[18] However, to our knowledge, the function and clinical significance of PD-L1 expression in T and NK cells in NB tumors and their role in ICB immunotherapy are still unknown.

To address these issues, we comprehensively analyzed the spatial distribution of CD8$^+$ T and NK cells, the distinct expression of PD-1/PD-L1 signaling, and their prognostic value in two cohorts of human NB. We identified different subclasses of TIME, performed functional phenotype analysis for PD-L1-expressing CD8$^+$ T and NK cells, and explored potential combination therapy of CD8$^+$ T or NK cells with anti-PD-1/PD-L1 antibodies in different NB models, aiming to advance the strategies in T cell and NK cell-based immunotherapies in NB patients.

Materials and methods

Patients and samples

Cohort_1 included 96 NB tissue samples collected at Sun Yat-sen University Cancer Center (Guangzhou, China) and the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) between 2004 and 2016. Cohort_2 was an NB tissue microarray obtained from Outdo Biotechnology Company (Outdo Biotechnology, Shanghai, China) that included 89 NB tissue samples collected between 2010 and 2015. For isolating tumor-infiltrating lymphocytes (TILs), fresh NB tissues were obtained from nine patients with primary NB. All samples in the study were confirmed by pathologic analysis. None of the patients had received prior anticancer treatment. The use of human samples was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center (Approval No. SL-B2022-273-01), and the requirement for informed consent was waived by the institutional review committee. All experiments involving humans were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Diagnosis and histology were determined using the International Neuroblastoma Staging System (INSS) and the International Neuroblastoma Pathology Classification, respectively. Risk stratification was performed according to the Children's Oncology Group (COG) Risk Group System.

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 or Prism 8 (Graph Pad Software Inc.). Data are expressed as the mean ± SD according to the distribution level. The chi-square test was used to analyze the correlation between intratumoral CD8, intratumoral NCR1, and IC-PD-L1 expressions and the clinicopathological features of NB patients. Differences between groups with normally distributed continuous variables were analyzed using the paired t-test. The association between the expression levels of two markers was analyzed using Pearson's correlation coefficient. The Kaplan-Meier method and log-rank test were used to plot survival curves and analyze differences in survival time between patient subgroups. Cox's proportional hazards regression model was used to evaluate the prognostic value of the risk factors. In all analyses, a two-tailed $P < 0.05$ was considered statistically significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Results

The spatial distribution pattern of CD8$^+$ T, NK, and PD-L1$^+$ cells in NB tumor

Tumor tissues of patients with NB were collected from two cohorts, including one cohort from Sun Yat-Sen University (cohort_1, n = 96) and one from a tissue microarray (cohort_2, n = 89). The clinicopathological features of NB patients in the two cohorts are shown in Supplemental Table S1. To evaluate the distribution pattern of CD8$^+$ T, NK, and PD-L1$^+$ cells in NB tissues, we performed immunohistochemistry (IHC) staining to detect CD8$^+$ T and NCR1 (also named NKp46)$^+$ NK cells infiltrated into the tumor nest (intratumoral lymphocytes) and those infiltrated into the surrounding stroma (peritumoral lymphocytes), as well as PD-L1$^+$ tumor and immune cells. A spatial discordance of CD8 and NCR1 expressions in peritumoral and intratumoral regions was observed in NB tissues from both cohort_1 (Figure 1a) and cohort_2 (Figure 1b), indicating a mixed immune infiltration pattern in the tumor nest and surrounding stroma in NB. PD-L1 showed distinct expression patterns on both tumor cells and immune cells, as well as marked heterogeneity with intense membrane staining and weak cytoplasmic staining in NB tissues (Figure 1a,b).

Density of CD8$^+$ T and NCR1$^+$ NK, and PD-L1$^+$ cells are positively correlated in NB tumor

A negative or weak expression of CD8 (intratumoral, peritumoral, and total), NCR1 (intratumoral, peritumoral, and total), and PD-L1 (tumor cells, immune cells, and total) was observed in

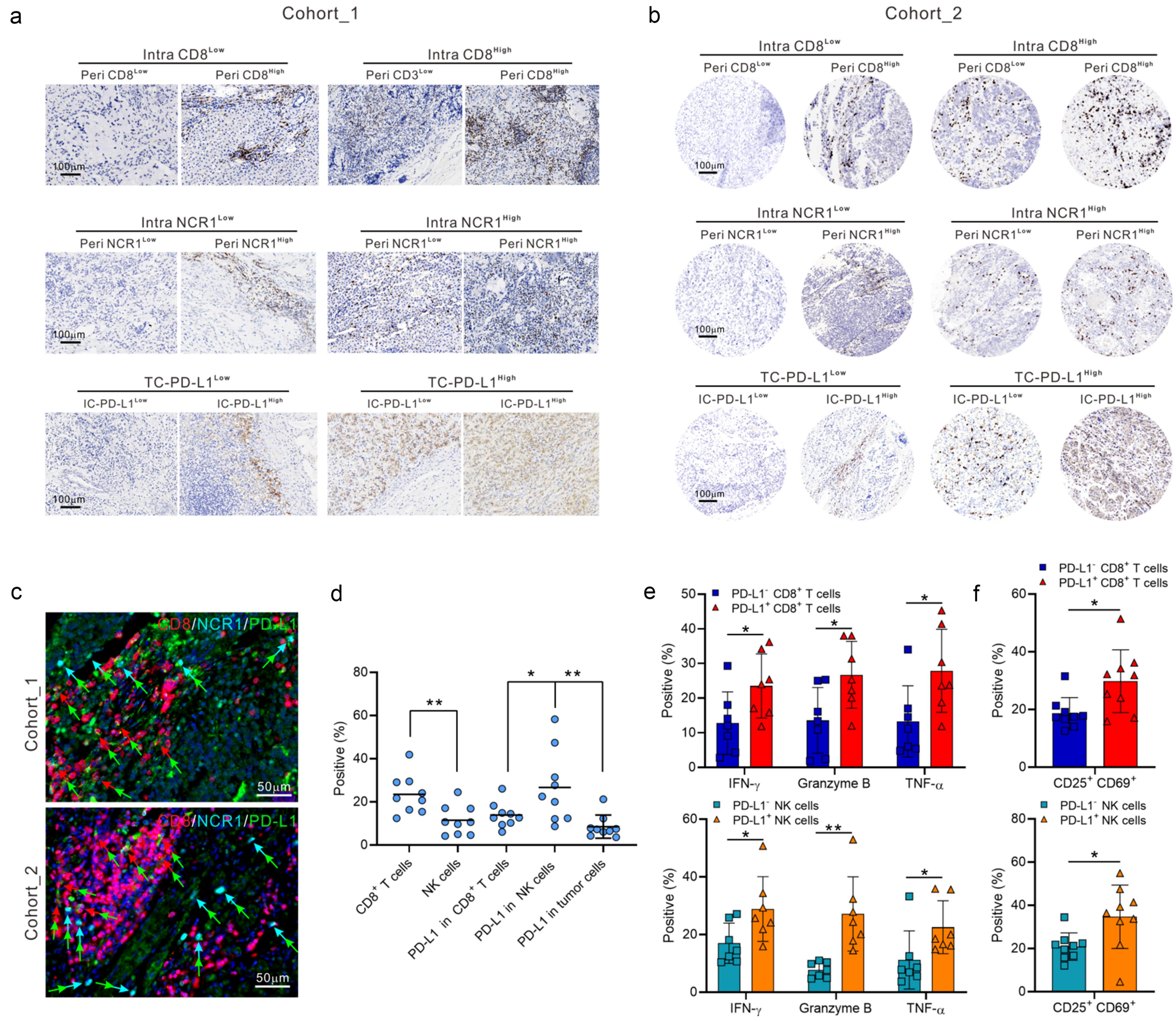

Figure 1. Spatial distribution of $CD8^+$ T cells, $NCR1^+$ NK cells, and PD-$L1^+$ cells in NB patients. a-b. Representative photomicrographs of IHC staining for intratumoral and peritumoral CD8, intratumoral and peritumoral NCR1, and tumor and immune cell PD-L1 protein in NB tissues from cohort_1 (a) and cohort_2 (b); Scale bars, 100μm. c. Representative images of immunofluorescent staining for the colocalization of CD8 (red), NCR1 (cyan), and PD-L1 (green) in NB tissues from cohort_1 (top) and cohort_2 (bottom). DAPI, blue. The figure panel pairs the representative images taken with different zooming options. Scale bars, 50μm. d. Flow cytometry analyses for the percentage of $CD8^+$ T and NK cells in tumor-infiltrating leukocytes and the proportion of PD-$L1^+$ cells in $CD8^+$ T, $CD56^+$ NK, and tumor cells isolated from human NB tumors ($n = 9$). $CD8^+$ T cells were identified as $CD45^+$ $CD3^+$ $CD8^+$ cells, NK cells were identified as $CD45^+$ $CD3^-$ $CD56^+$ cells, tumor-infiltrating leukocytes were identified as $CD45^+$ cells, and tumor cells were identified as $CD45^-$ $NCAM^+$ cells. E-F. Flow cytometry analyses for the expression of cytotoxic markers IFN-γ, granzyme B, and TNF-α ($n = 7$) (e) and activation markers CD25 and CD69 ($n = 9$) (f) by PD-$L1^+$ and PD-$L1^-$ subsets in $CD8^+$ T and NK cells isolated from human NB tumors. Intra, intratumoral; peri, peritumoral; TC, tumor cell; IC, immune cell; ns, no significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

the majority of samples from the two cohorts (Supplemental Figure S1A), and a generally positive correlation between the three was also observed (Supplemental Figure S1B). By stratifying patients, we next analyzed the association of CD8, NCR1, and PD-L1 with INSS stage, *MYCN* amplification, and COG risk classification. As shown in Supplemental Figure S1C, D, significant differences in the distribution of intratumoral NCR1 and immune cell (IC)-PD-L1 in cohort_1 and that of intratumoral NCR1, total PD-L1, and IC-PD-L1 in cohort_2 were observed between different INSS stages, with lower expression levels in stage 4 than in others; the expression of intratumoral CD8, intratumoral NCR1, total PD-L1, and tumor cell (TC)-PD-L1 were lower in tissues with *MYCN* amplification than in those without in both cohorts; in high-risk patients, low expression of intratumoral NCR1 and IC-PD-L1 was observed in cohort_1, and low expression of peritumoral NCR1, intratumoral NCR1, total PD-L1, TC-PD-L1, and IC-PD-L1 was observed in cohort_2.

PD-L1 expression marks highly activated and functionally competent $CD8^+$ T and NK cells in NB tumor

Above IHC staining data showed PD-L1 expression for all immune cells, and we wondered whether PD-L1 was preferentially expressed on $CD8^+$ T and NK cells. Multiplex

immunofluorescence (mIF) staining revealed that PD-L1 was co-localized with some $CD8^+$ T and $NCR1^+$ NK cells in human NB tumors from both cohorts (Figure 1c. We then performed the phenotypic analysis of immune cells isolated from fresh tumor tissues of patients with NB by flow cytometry. As shown in Figure 1d and Supplementary Figure S1E, the percentage of $CD8^+$ T and NK cells in tumor-infiltrating leukocytes were approximately 25% and 15%, respectively. Consistent with mIF staining data, PD-L1-expressing $CD8^+$ T-cell and PD-L1-expressing NK-cell populations existed in the majority of NB tumors. Interestingly, a substantial proportion of PD-$L1^+$ subset in NK cells (median, 26.7%; range, 8.7% to 58.5%) was detected, which was higher than that in $CD8^+$ T cells (median, 13.9%; range, 6.1% to 26.1%), indicating that PD-L1 was associated preferentially with NK cells as compared with $CD8^+$ T cells. We also examined the expression of PD-L1 on tumor cells and found a relatively low proportion (median, 8.5%; range, 3.6% to 21.1%) of PD-$L1^+$ tumor cells.

Next, we performed functional phenotype analysis on the PD-$L1^+$ subsets in $CD8^+$ T and NK cells. The expression of cytotoxic markers IFN-γ, granzyme B, and TNF-α (Figure 1e) and activation markers CD69 and CD25 (Figure 1f) was significantly increased in PD-$L1^+$ subsets compared with PD-$L1^-$ subsets in both $CD8^+$ T and NK cells, suggesting that PD-L1 expression on T and NK cells appears to define highly activated and functionally competent cells. No difference was observed in proliferation between PD-$L1^+$ and PD-$L1^-$ subsets in $CD8^+$ T and NK cells (Supplemental Figure S1F).

ScRNA-seq analysis reveals that PD-L1-positive NKT and NK cells in NB tumor have an activated status

To decipher the status of PD-L1-positive T and NK cells in human NB samples, we next performed scRNA-seq analysis based on GSE154037 dataset of NB focused on NKT cells (Figure 2a). Differential expression analysis and GO functional analysis were conducted on two groups of PD-L1-negative and positive NKT cells. Intriguingly, the differentially expressed genes in PD-L1-positive NKT cells were primarily enriched in GO terms involving in activated T cells, such as T cell activation, immune response-activating signaling pathways, T cell costimulation, tumor necrosis factor production, and cell killing (Figure 2b). To gain a more comprehensive understanding, Gene Set Variation Analysis was executed on gene sets deeply intertwined with these terms. Consequently, PD-L1 -positive NKT cells had higher gene set scores associated with cytotoxic signature, T cell activation, and NK cell activation (Figure 2c).

We also performed scRNA seq analysis on another GSE14766 dataset of NB available for NK cells (Figure 2d) and obtained similar results. The top GO terms enriched in PD-L1-positive NK cells were implicated with activated NK cells, such as immune response-activating signaling pathways, activation of immune response, regulation of immune effector process, tumor necrosis factor production, positive regulation of lymphocyte activation, lymphocyte costimulation, cell killing, and NK cell-mediated cytotoxicity (Figure 2e). Gene Set Variation Analysis revealed higher gene set scores associated with cytotoxic signature, NK cell activation, and chemokines (CCL2, CCL5, and CCL19) related to effector cells, in PD-L1-positive NK cells (Figure 2f). Together, such observations indicate that NKT and NK cells expressing PD-L1 in NB tumors trend to show a functional activation status.

Higher density of intratumoral $CD8^+$ T, intratumoral $NCR1^+$ NK, and PD-$L1^+$ immune cells are associated with a favorable prognosis in patients with NB

Survival analysis was performed to determine the prognostic value of $CD8^+$ T cells (intratumoral, peritumoral, and total), $NCR1^+$ NK cells (intratumoral, peritumoral, and total), and PD-$L1^+$ cells (tumor cells, immune cells, and total) in NB patients. Patients with intratumoral $CD8^{High}$ expression had better over survival (OS) and/or event-free survival (EFS) than those with intratumoral $CD8^{Low}$ expression (Supplemental Figure S2A), whereas peritumoral and total CD8 expressions were not statistically significant prognostic factors (Supplemental Figure S2B, C). A similar pattern was observed in the survival analysis based on NCR1 (intratumoral, peritumoral, and total) (Supplemental Figure S3). The favorable prognostic value of intratumoral CD8 and NCR1 was supported by univariate Cox regression analysis, which showed that high expression of intratumoral CD8 and NCR1 was associated with a lower risk of death and incidence of events in the two cohorts (Table 1).

Kaplan–Meier analysis and univariate Cox regression analysis supported the favorable prognostic value of high IC-PD-L1 expression in NB (Supplemental Figure S4A, Table 1). A positive association between total PD-L1 expression and superior OS and EFS was observed in cohort_1, whereas the association was not statistically significant in cohort_2 (Supplemental Figure S4B, Table 1). There was no significant correlation between TC-PD-L1 expression and patient survival in the two cohorts (Supplemental Figure S4C, Table 1).

Intratumoral $CD8^+$ T and $NCR1^+$NK cells correlate with increased survival in NB regardless of PD-$L1^+$ immune-cell density, INSS stage, MYCN *amplification, or COG risk classification*

To predict the response to anti-PD therapy, four types of TIME have been defined according to T cell infiltration and PD-L1 expression levels from tumor biopsies:[19] $CD8^{low}$PD-$L1^{low}$ (type I), $CD8^{high}$PD-$L1^{high}$ (type II), $CD8^{high}$PD-$L1^{low}$ (type III), and $CD8^{low}$PD-$L1^{high}$ (type IV). Using the same method, four groups were defined based on NK cell infiltration and PD-L1 expression levels: $NCR1^{low}$PD-$L1^{low}$ (type I), $NCR1^{high}$PD-$L1^{high}$ (type II), $NCR1^{high}$PD-$L1^{low}$ (type III), and $NCR1^{low}$PD-$L1^{high}$ (type IV). However, based on total expression levels, there was no significant difference in survival between the four types in both cohorts except the superior EFS of type II groups ($CD8^{high}$PD-$L1^{high}$ and $NCR1^{high}$PD-$L1^{high}$, respectively) in cohort_1 (Supplemental Figure S5).

Survival analysis was further performed based on the combination of prognostic factors (intratumoral CD8, intratumoral NCR1, and IC-PD-L1). The clinical outcome was better in patients with $CD8^{low}$PD-$L1^{high}$ (type IV) than in those with $CD8^{low}$PD-$L1^{low}$ (type I), and survival was longer in

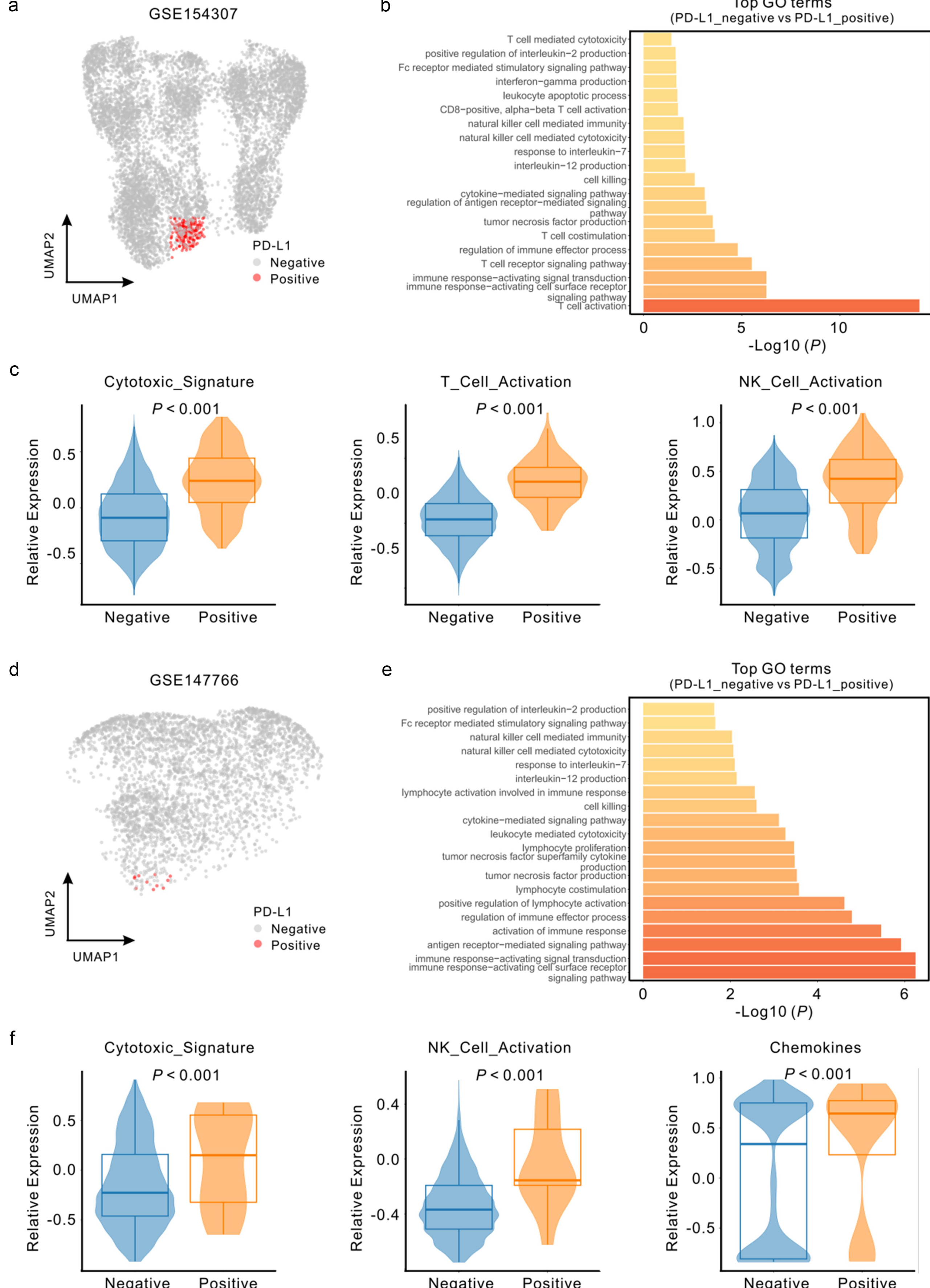

Figure 2. ScRNA-seq analysis reveals that PD-L1-positive NKT and NK cells have an activated status in NB tumor. a. PD-L1-negative or -positive NKT cell type annotation by the expression of marker gene signatures in human NB tumors from GSE154037 dataset based on t-Uniform Manifold Approximation and Projection (UMAP). Color

CD8^{high}PD-L1^{high} (type II) than in CD8^{high}PD-L1^{low} (type III) patients (Figure 3a). Consistently, high IC-PD-L1 expression was associated with better EFS in cohort_1 and better OS in cohort_2 among patients with intratumoral NCR1^{High} expression (Figure 3b). These results indicated that PD-L1^{+} immune cells were a favorable prognostic factor in patients with intratumor-infiltrating CD8^{+} T and NCR1^{+} NK cells. Indeed, intratumoral PD-L1^{+} CD8^{+} T-cell and PD-L1^{+} NK-cell populations showed a tight correlation with better clinical outcomes based on the results of mIF staining (Figure 3c,d). Together with the phenotype analysis in Figure 1e,f, this finding suggests that the function activated CD8^{+} T and NK cells may be identified by their expression of PD-L1. Notably, high expression of intratumoral CD8 and NCR1 was associated with better outcome in patients with either high or low IC-PD-L1 expression in both selected cohorts (Figure 3a,b), suggesting intratumoral CD8^{+} T and NCR1^{+} NK cells correlate with increased survival in NB regardless of PD-L1^{+} immune-cell density.

Next, we wondered whether combining intratumoral CD8^{+} T and NCR1^{+} NK cell status with variables that affect patient survival, such as INSS stage, *MYCN* amplification, and COG risk classification, could provide a more accurate prediction of clinical outcome. Patients were grouped based on the combination of INSS stage with intratumoral CD8^{+} T and NCR1^{+} NK cells. In both cohorts, high expression of intratumoral CD8 and NCR1 was associated with superior survival in patients with both favorable (stage 1–3/4S) and unfavorable (stage 4) prognosis (Supplemental Figure S6A, B). Patients were also stratified according to *MYCN* status combined with intratumoral CD8^{+} T and NCR1^{+} NK cells. High expression of intratumoral CD8 and NCR1 was correlated with increased survival in both patients with and without *MYCN* amplification (Supplemental Figure S6C, D). Similar results were obtained in the survival analysis according to COG risk classification in combination with intratumoral CD8 and NCR1 expression, which showed that high expression of intratumoral CD8 and NCR1 was associated with better prognosis in patients with any risk (Supplemental Figure S6E, F).

Intratumoral NCR1^{+} NK cells correlate with increased survival in NB regardless of PD-1 expression or spatial distribution of CD8^{+} T cells

For IHC staining on PD-1 (Figure 4a), a negative or weak PD-1 expression was observed in the majority of NB tissues (Supplemental Figure S7A). PD-1 expression was correlated with CD8 (total, intratumoral, and peritumoral), NCR1 (total and intratumoral), and PD-L1 (total) in both cohorts and with TC-PD-L1 and IC-PD-L1 in cohort_1 (Figure 4b). PD-1 expression differed significantly according to INSS stage in both cohorts and COG risk classification in cohort_2, with lower PD-1 expression in stage 4 and high-risk patients (Supplemental Figure S7B, D). There were no significant differences in PD-1 expression between patients with and without *MYCN* amplification (Supplemental Figure S7C). The mIF staining (Figure 4c) demonstrated that the total number of CD8^{+} T cells was much higher than NCR1^{+} NK cells in the TIME of NB, consistent with the results of flow cytometry analysis in Figure 1d; the number of CD8^{+} PD-1^{+} T cell infiltration was also higher than NCR1^{+} PD-1^{+} NK cells (total, intratumoral, and peritumoral); compared with peritumoral TILs, PD-1 was preferentially co-expressed with intratumoral TILs. However, there was no significant difference in the proportion of PD-1^{+} subsets in CD8^{+} T and NK cells isolated from human NB tumors, both of which were more than 20% (Figure 4d).

PD-1 expression alone was not significantly correlated with patient outcome in the survival analysis (Figure 4e, Supplemental Figure S7E). Further analysis was performed by combining PD-1 expression with intratumoral CD8^{+} T and NCR1^{+} NK cells. Partial results from cohort_1 showed that high intratumoral CD8 expression was correlated with better OS in patients with PD-1 high or low expression (Figure 4f), whereas inconsistent results were obtained in the analysis of EFS in cohort_1 and OS in cohort_2 (Supplemental Figure S7F). Conversely, consistent results from both cohorts demonstrated that high intratumoral NCR1 expression was associated with better survival regardless of PD-1 expression (Figure 4g, Supplemental Figure S7G).

Moreover, a high density of intratumoral NCR1^{+} NK cells was associated with superior survival regardless of the density (high or low) and location (total, intratumoral, or peritumoral) of CD8^{+} T cells (Supplemental Figure S8). Multivariate Cox regression analysis showed that only intratumoral NCR1 expression was a favorable prognostic factor for OS and/or EFS independent of peritumoral and total NCR1 expression, intratumoral, peritumoral, and total CD8 expression, PD-1 and PD-L1 expression, tumor stage, *MYCN* amplification, and risk classification (Table 1). Taken together, these results suggest that intratumoral NCR1^{+}NK cells are a potential prognostic biomarker for better predicting survival in NB patients.

PD-1/PD-L1 blocked NK cells exhibit robust cytotoxicity to NB cells in vitro

MYCN amplification often occurs in high-risk NBs and correlates with rapid tumor progression and a poor prognosis overall.[20] In contrast to low-risk NBs without *MYCN* amplification, high-risk NBs with *MYCN* amplification have the

key from gray to red indicates relative expression levels from low to high. b. This functional enrichment analysis of differentially expressed genes between PD-L1-negative and PD-L1-positive NKT cells. Bar plot ranking of the top GO terms. c. Gene Set Variation Analysis on gene sets related to Cytotoxic_Signature (left), T_Cell_Activation (middle), and NK_Cell_Activation (right) to obtain the corresponding gene set scores in PD-L1-positive and PD-L1-negative NKT cells. The difference of the gene sets between the two groups were analyzed. d. PD-L1-negative or -positive NK cell type annotation by the expression of marker gene signatures in human NB tumors from GSE147766 dataset based on UMAP. Color key from gray to red indicates relative expression levels from low to high. e. This functional enrichment analysis of differentially expressed genes between PD-L1-negative and PD-L1-positive NK cells. Bar plot ranking of the top GO terms. f. Gene Set Variation Analysis on gene sets related to Cytotoxic_Signature (left), NK_Cell_Activation (middle), and Chemokines (right) to obtain the corresponding gene set scores in PD-L1-positive and PD-L1-negative NK cells. The difference of the gene sets between the two groups were analyzed. ***$P < 0.001$.

Table 1. Univariate and multivariate analysis of overall survival and event-free survival in neuroblastoma patients.

	Cohort_1 (n = 96)								Cohort_2 (n = 89)			
	Overall survival				Event-free survival				Overall survival			
	Univariate cox		Multivariate cox		Univariate cox		Multivariate cox		Univariate cox		Multivariate cox	
Variable	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)
Age ≥18 months or <18 months	0.836	1.10 (0.45–2.71)			0.053	2.11 (0.99–4.51)			0.324	1.63 (0.62–4.28)		
Gender Male or female	0.258	1.63 (0.701–3.79)			0.938	0.98 (0.55–1.74)			0.160	1.96 (0.77–4.98)		
INSS stage 4 or 1/2/3/4S	0.030	2.43 (1.09–5.41)	0.168	1.90 (0.76–4.73)	0.001	2.77 (1.49–5.16)	0.345	1.49 (0.65–3.40)	0.041	2.64 (1.04–6.72)	0.152	0.30 (0.06–1.56)
MYCN amplified Yes or no	0.010	3.44 (1.34–8.82)	0.433	1.51 (0.54–4.19)	0.014	0.42 (0.21–0.84)	0.769	1.13 (0.50–2.54)	0.005	4.37 (1.58–12.1)	0.198	1.97 (0.70–5.52)
Risk group High or low/intermediate	0.053	2.40 (0.99–5.85)			0.001	3.68 (1.78–7.58)	0.021	3.26 (1.20–8.90)	0.006	3.52 (1.43–8.67)	0.139	3.45 (0.67–17.8)
Total CD8 expression High or low	0.219	0.65 (0.32–1.29)			0.043	0.57 (0.33–0.98)	0.758	0.90 (0.45–1.80)	0.186	0.53 (0.21–1.36)		
Peritumoral CD8 expression High or low	0.796	0.91 (0.46–1.83)			0.735	0.91 (0.53–1.56)			0.643	0.81 (0.33–1.99)		
Intratumoral CD8 expression High or low	0.003	0.30 (0.14–0.67)	0.410	0.67 (0.253–1.75)	0.009	0.47 (0.27–0.83)	0.677	0.85 (0.38–1.87)	0.011	0.24 (0.08–0.72)	0.642	0.76 (0.24–2.42)
Total NCR1 expression High or low	0.236	0.66 (0.33–1.32)			0.100	0.633 (0.37–1.09)			0.068	0.41 (0.15–1.07)		
Peritumoral NCR1 expression High or low	0.601	1.20 (0.60–2.40)			0.705	1.11 (0.65–1.90)			0.541	1.33 (0.53–3.30)		
Intratumoral NCR1 expression High or low	<0.001	0.153 (0.06–0.40)	0.019	0.25 (0.08–0.80)	<0.001	0.26 (0.14–0.47)	0.003	0.29 (0.12–0.66)	0.003	0.05 (0.01–0.36)	0.007	0.05 (0.06–0.45)
Total PD-L1 expression High or low	0.032	0.43 (0.20–0.93)	0.641	1.30 (0.43–3.91)	0.015	0.51 (0.29–0.88)	0.997	1.00 (0.47–2.16)	0.213	0.55 (0.22–1.41)		
Tumor cell-PD-L1 expression High or low	0.394	0.73 (0.35–1.51)			0.056	0.59 (0.34–1.02)			0.517	0.74 (0.30–1.84)		
Immune cell-PD-L1 expression High or low	0.003	0.28 (0.12–0.65)	0.301	0.56 (0.19–1.68)	0.002	0.406 (0.23–0.72)	0.920	1.04 (0.50–2.19)	0.080	0.42 (0.16–1.11)		

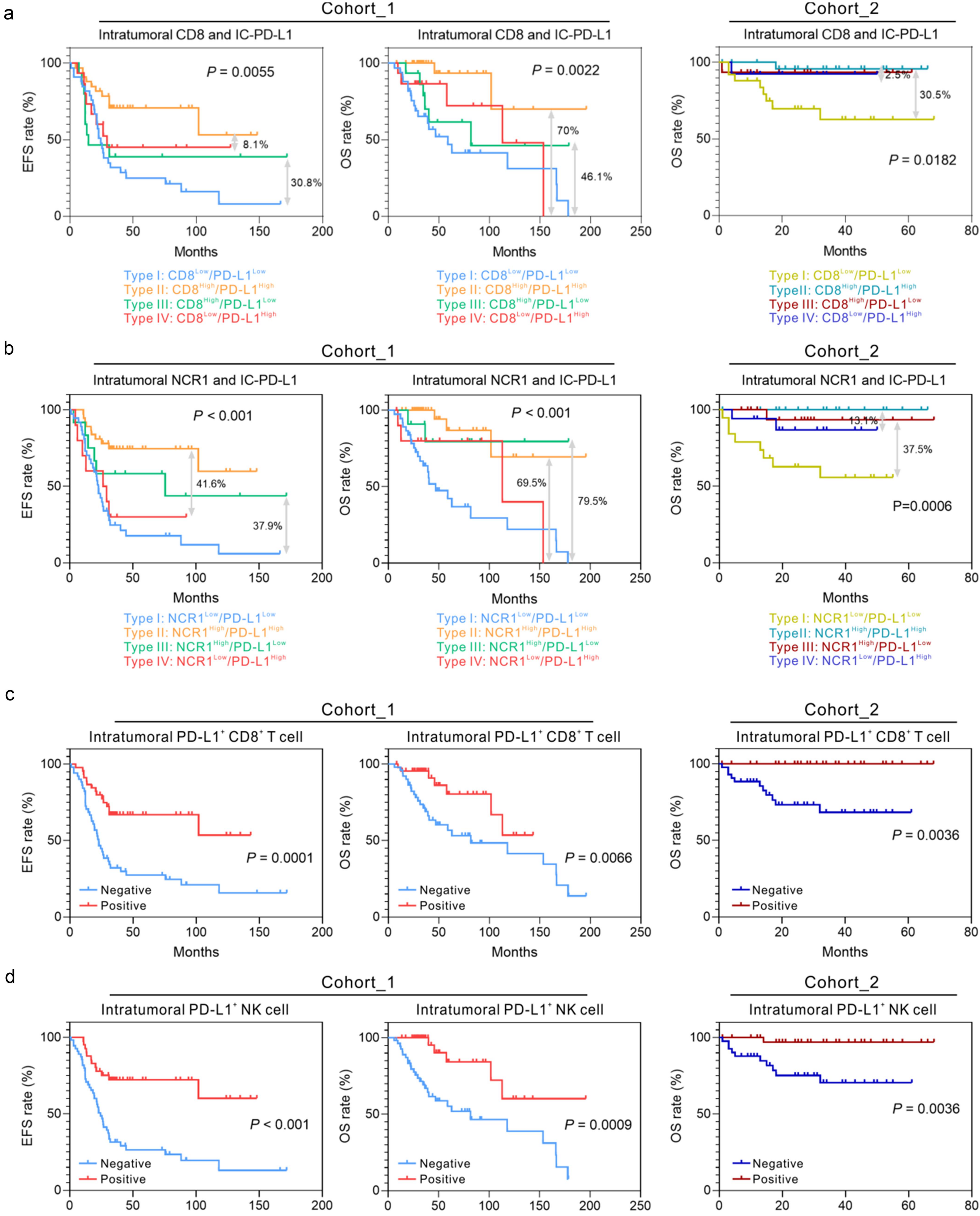

Figure 3. Expression levels of intratumoral CD8 and intratumoral NCR1 correlate with increased survival in NB patients regardless of immune cell-PD-L1 expression. a-b. Kaplan–Meier curves of EFS and OS in patients from cohort_1 (left) and OS in those from cohort_2 (right) based on intratumoral CD8 (a) or intratumoral NCR1 (b) combined with immune-cell PD-L1 expression in IHC staining. IC, immune cell. C-D. Kaplan–Meier curves of EFS and OS in patients from cohort_1 (left) and OS in those from cohort_2 (right) based on the levels of intratumoral PD-L1^{+} CD8^{+} T cell (c) or intratumoral PD-L1^{+} NCR1^{+} NK cell (d) in mIF staining.

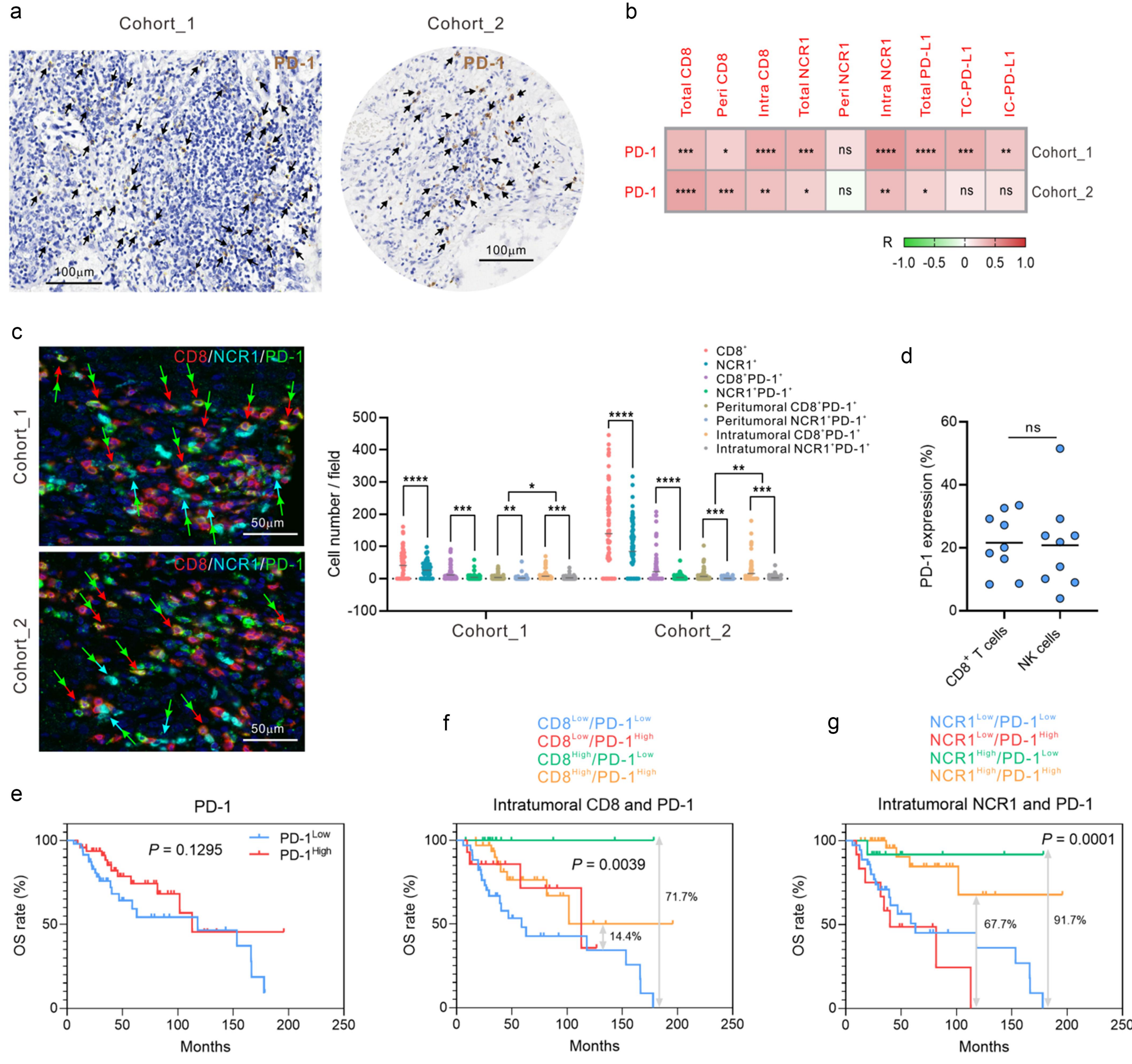

Figure 4. Intratumoral NCR1 expression levels correlate with increased survival in NB patients regardless of PD-1 expression and the density and spatial distribution of $CD8^+$ T cells. a. Representative photomicrographs of IHC staining for PD-1 in NB tissues from cohort_1 (left) and cohort_2 (right); Scale bars, 100μm. b. Correlation analyses of PD-1 with total CD8, intratumoral CD8, peritumoral CD8, total NCR1, intratumoral NCR1, peritumoral NCR1, total PD-L1, tumor cell-PD-L1, and immune cell-PD-L1 based on IHC staining score. c. Representative images of immunofluorescent staining for the colocalization of CD8 (red), NCR1 (cyan), and PD-1 (green) in NB tissues from cohort_1 (top) and cohort_2 (bottom) cohorts. DAPI, blue. The figure panel pairs the representative images taken with different zooming options. Scale bars, 50μm. Number of $CD8^+$, $NCR1^+$, $CD8^+$ $PD\text{-}1^+$, $NCR1^+$ $PD\text{-}1^+$, peritumoral $CD8^+$ $PD\text{-}1^+$, peritumoral $NCR1^+$ $PD\text{-}1^+$, intratumoral $CD8^+$ $PD\text{-}1^+$, and intratumoral $NCR1^+$ $PD\text{-}1^+$ cells was quantified. d. Flow cytometry analyses for the expression of PD-L1 in $CD8^+$ T and NK cells isolated from human NB tumors (n = 9). e-g. Kaplan–Meier curves of OS according to PD-1 alone (e), PD-1 combined with intratumoral CD8 (f), or intratumoral NCR1 (g) expression in patients from cohort_1. Intra, intratumoral; peri, peritumoral; TC, tumor cell; IC, immune cell; ns, no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

characteristics of cold tumors, endowed with immune evasion mechanisms, such as infiltrated by $CD163^+$ tumor-associated macrophages, downregulating human leukocyte class I antigens and NKG2D ligands, expressing high levels of immunosuppressive gangliosides and sialic acid-containing sugars and proteins, and upregulating PD-1/PD-L1 axis.[8] It seems coherent that we found lower $CD8^+$ T cells, NK cells, and PD-L1 in NB tissues with *MYCN* amplification (Supplemental Figure S1C). We next assessed the surface expression of immune-related markers by flow cytometry in a panel of five NB cell lines, including two *MYCN* amplified and three *MYCN* non-amplified (Figure 5a). High levels of PD-L1 was observed in *MYCN* non-amplified cells compared to those in *MYCN* amplified cells (Figure 5b), and similar results were obtained in MHC-I, MHC-II, as well as the NK-cell-activating receptor ligands, MICA and MICB (Supplemental Figure S9 A, B).

To investigate the cytotoxicity of $CD8^+$ T and NK cells to NB cells, we first induced tumor-associated $CD8^+$ T and NK cells by co-culturing $CD8^+$ T cells and NK cells from the

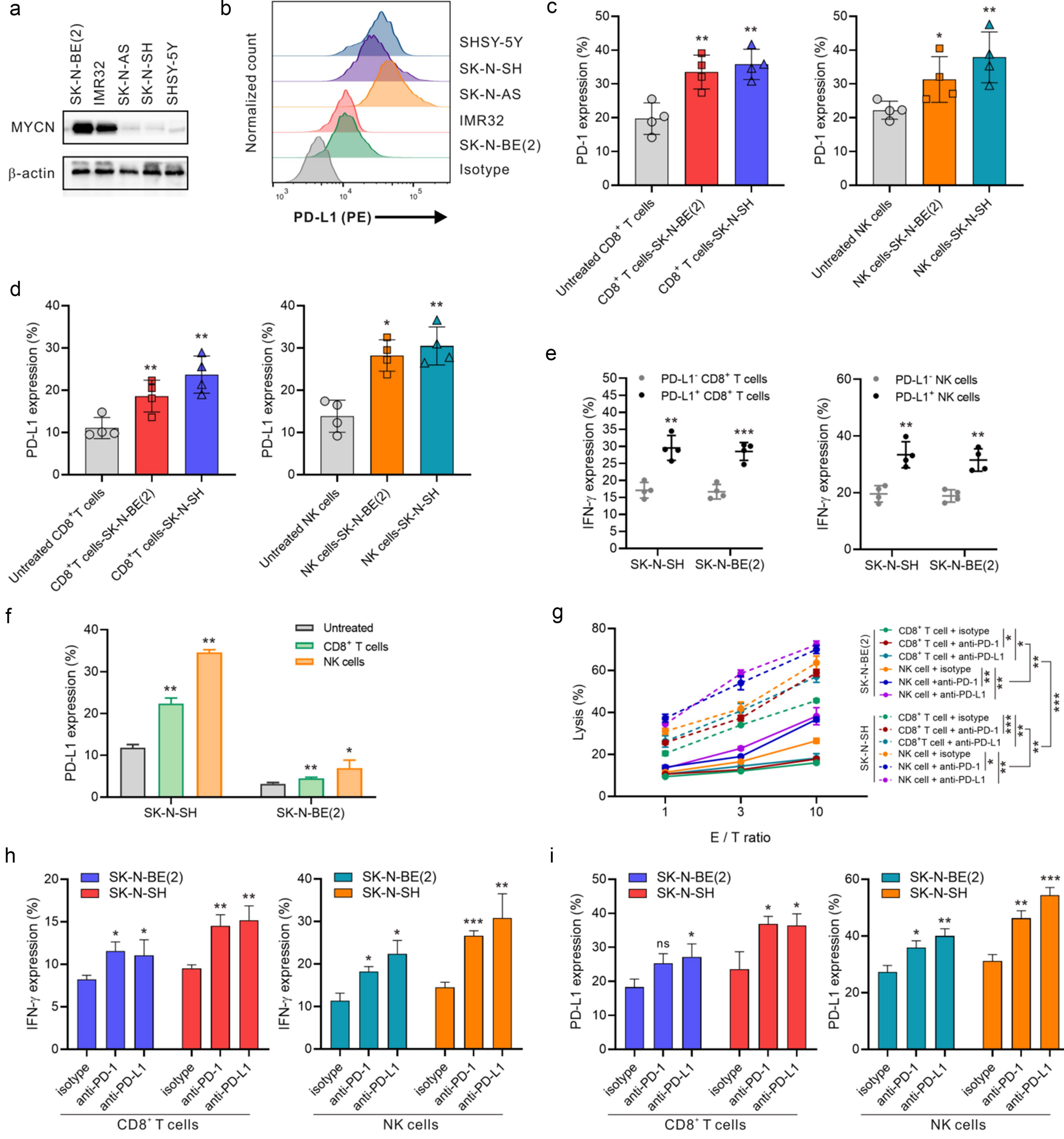

Figure 5. Cytotoxicity of CD8$^+$ T or NK cell combined with anti-PD-1/PD-L1 antibodies to NB cells *in vitro*. a. The expression of MYCN protein in five NB cell lines was detected by western blot. b. The surface expression of PD-L1 by five NB cell lines was assessed by flow cytometry. c-f. CD8$^+$ T and NK cells from the PBMCs of healthy donors were co-cultured with or without *MYCN* amplified (SK-N-BE.[2]) and non-amplified (SK-N-SH) NB cells in Transwell system for 6 h. The expression of PD-1 (c) and PD-L1 (d) by CD8$^+$ T and NK cells were examined by flow cytometry. The expression of IFN-γ in PD-1$^-$ or PD-1$^+$ subsets in CD8$^+$ T and NK cells was assessed by flow cytometry (e). The expression of PD-L1 by SK-N-BE.[2] and SK-N-SH cells was examined by flow cytometry (f). $n = 4$. g-i. *In vitro* induced CD8$^+$ T and NK cells blocked with or without anti-PD-1 or anti-PD-L1 antibodies were co-cultured SK-N-BE.[2] and SK-N-SH cells for 6 h with an effector: target ratio of 1:1. The lysis of SK-N-BE.[2] and SK-N-SH cells was assessed by cytotoxicity assay (g). The percentage of IFN-γ (h) or PD-L1 (i) positive cells in CD8$^+$ T and NK cells activated with or without anti-PD-1 or anti-PD-L1 antibodies was assessed by flow cytometry. $n = 3$. ns, no significance. Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

PBMCs of healthy donors with NB cell lines with (SN-N-BE.[2]) or without (SK-N-SH) *MYCN* amplification *in vitro*. Compared with the controls, such *in vitro* induced CD8$^+$ T and NK cells showed concordant higher expression of PD-1 (Figure 5c) and PD-L1 (Figure 5d). A higher level of IFN-γ expression was found in the PD-L1$^+$ subsets compared with the PD-L1$^-$ subsets (Figure 5e), indicating PD-L1 expressing CD8$^+$ T and NK cells are more functionally active. These results demonstrate that the phenotype of *in vitro* induced CD8$^+$ T and NK cells generally resembles that of CD8$^+$ T and NK

cells in the tumor (Figure 1d, E; Figure 4d). Meanwhile, we also observed enhanced PD-L1 expression on tumor cells co-cultured with $CD8^+$ T and NK cells (Figure 5f). These data show the rationale for using anti-PD-1/PD-L1 therapies.

Then, *in vitro* induced $CD8^+$ T and NK cells were used for cytotoxicity assay. As shown in Figure 5g, *MYCN* non-amplified SN-N-SH cells were significantly more susceptible to both $CD8^+$ T-cell- and NK-cell-mediated lysis than *MYCN* amplified SN-N-BE.[2] cells, indicating that *MYCN* non-amplified NB cells are more immune-reactive. Intriguingly, the NK cells exhibited higher cytotoxicity than $CD8^+$ T cells in both *MYCN* amplified and non-amplified cells. We hypothesize that the upregulation of PD-1 on $CD8^+$ T and NK cells limits their cytotoxicity through ligation with PD-L1 on NB cells.[5,19] After adding anti-PD-1 antibodies, we observed a significantly increased cytotoxicity of NK cells to both *MYCN* amplified and non-amplified cells, whereas the cytotoxicity of $CD8^+$ T cells was only significantly increased in *MYCN* non-amplified SN-N-SH cells, with a slight increase in *MYCN* amplified SN-N-BE.[2] cells. This indicates that NK cells may contribute more than $CD8^+$ T cells in the antitumor immunity of NB. Accordingly, after blockade of PD-1/PD-L1 axis with anti-PD-1 antibodies, we found a slight and significant increase of IFN-γ secretion levels in effector cells co-cultured with *MYCN* amplified and non-amplified cells, respectively; moreover, PD-1 blocked NK cells demonstrated a more activated status compared with PD-1 blocked $CD8^+$ T cells (Figure 5h).

The above data have demonstrated that PD-L1 expression in both patient-derived and *in vitro* induced $CD8^+$ T and NK cells marks functional competent cells. To further evaluate the function of PD-L1 on effector cells, anti-PD-L1 antibodies were used for the treatment of *in vitro* induced $CD8^+$ T and NK cells. Indeed, we observed an increased cytotoxicity (Figure 5g) and IFN-γ secretion levels (Figure 5h) of $CD8^+$ T cells and NK cells after adding anti-PD-L1 antibodies, with more significant levels in NK cells. In addition, PD-L1 expression on effector cells co-cultured with tumor cells was further elevated at both the mRNA (Supplemental Figure S9C) and protein (Figure 5i) levels after treatment with anti-PD-1 or anti-PD-L1 antibodies, supporting the view that PD-L1 expression on $CD8^+$ T cells and NK cells may represent a functional activation status, which can be further upregulated by PD-1/PD-L1 blockade. Collectively, these results indicate that compared with $CD8^+$ T cells, NK cells exhibit strong cytotoxicity to NB cells *in vitro* and PD-1/PD-L1 blockade can further enhance their effector function.

Combination therapy of NK cell infusion and anti-PD-1/PD-L1 antibodies demonstrates potent anti-tumor activity in xenograft models of NB

We next sought to test the influence of PD-1/PD-L1 blockade on the antitumor activity of $CD8^+$ T and NK cells *in vivo*. A subcutaneous xenograft model of NB was designed in NCG mice (Figure 6a). Mice were intravenously injected with *in vitro* induced human $CD8^+$ T and NK cells pretreated with or without anti-PD-1 or anti-PD-L1 antibodies. As shown in Figure 6b–p, the tumor growth of *MYCN* amplified NB was more rapid than that of *MYCN* non-amplified NB; *MYCN* non-amplified tumors were more susceptible to immune-killing mediated by both $CD8^+$ T and NK cells than *MYCN* non-amplified tumors; NK cells exhibited stronger anti-tumor activity than $CD8^+$ T cells in both *MYCN* amplified and non-amplified tumors; the addition administration of anti-PD-1 or anti-PD-L1 antibodies to NK cells led to a more significant delay in tumor growth both *MYCN* amplified and non-amplified tumors than $CD8^+$ T cells. Moreover, a prolonged survival was found in mice treated with NK cells blocked by anti-PD-1 or anti-PD-L1 antibodies as compared with $CD8^+$ T cells blocked by anti-PD-1 or anti-PD-L1 antibodies in both *MYCN* amplified and non-amplified tumors, with a more prolonged survival time in mice bearing non-amplified tumors (Figure 6q).

We next examined the intratumoral infiltration of NK and $CD8^+$ T cells in mouse tumors. Compared with isotype groups, infiltrated NK and $CD8^+$ T cells blocked with anti-PD-1 or anti-PD-L1 anti-bodies had increased IFN-γ secretion levels, with higher levels in infiltrated NK cells than $CD8^+$ T cells, especially those in *MYCN* non-amplified tumors (Supplemental Figure S9D). Additionally, both infiltrated NK and $CD8^+$ T cells constitutively expressed PD-L1, which responded to anti-PD-1 or anti-PD-L1 antibodies and showed a concordant higher expression pattern with IFN-γ (Supplemental Figure S9E). Together, these data further support that PD-L1 expression on effector cells might be a functionally active biomarker. Our *in vivo* study indicates that NK cells may be more tumor-reactive than $CD8^+$ T cells in both *MYCN* amplified and non-amplified NBs, and that combination therapy with NK-cell infusion and anti-PD-1/PD-L1 antibodies may be a potential immunotherapeutic strategy for NB patients.

T-cell and NK-cell gene signatures and CD274 predict a good clinical outcome in a large-scale database of human NB

An external cohort of a GEO database consisting of 493 NB samples was used for validation. Gene expression profile in total tumor was analyzed at mRNA level. The T cell score, NK cell score, *CD274* expression, and *PDCD1* expression were significantly positively correlated (Supplemental Figure S10A) and were all lower in patients with stage 4, *MYCN* amplification, and high-risk classification, with no significant differences in NK cell score and *PDCD1* between INSS stages and in *PDCD1* between the COG risk groups (Supplemental Figure S10B-D). High T cell score, NK cell score, and *CD274* expression alone, but not *PDCD1* expression, showed a strong association with a good clinical outcome (Supplemental Figure S10E).

In the survival analysis based on the combination of T cell score and NK cell score with others, *CD274* (Supplemental Figure S11A, B) and *PDCD1* (Supplemental Figure S11C, D) did not improve patient stratification when combined with either T cell score or NK cell score. However, the results were consistent with those of the above two cohorts to some extent. For instance, patients with T-cell-score^{low}*CD274*^{Low}, NK-cell-score^{low}*CD274*^{Low}, T-cell-score^{low}*PDCD1*^{Low}, and NK-cell-score^{low}*PDCD1*^{Low} had the poorest survival in different subgroups.

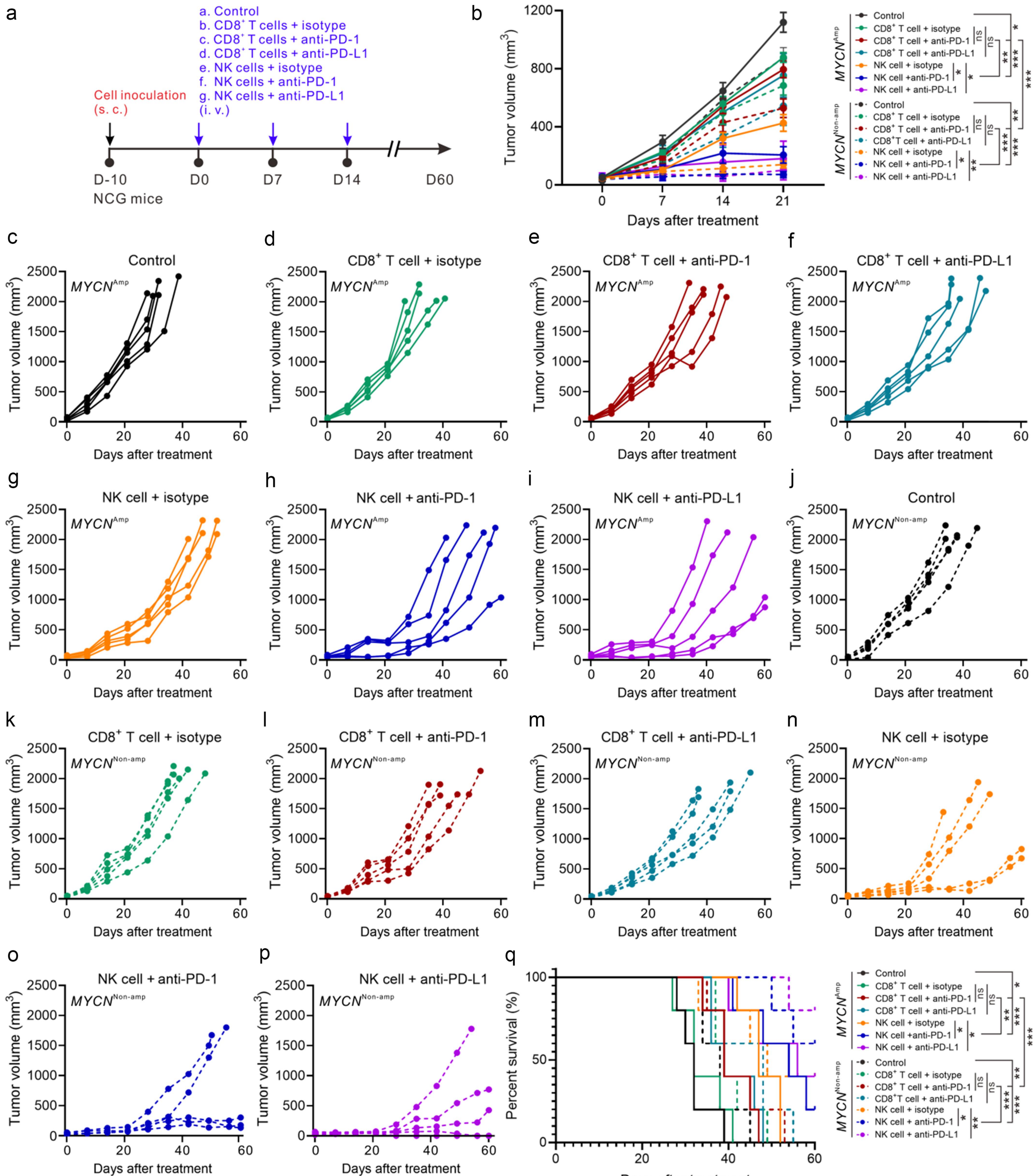

Figure 6. Antitumor activity of CD8+ T or NK cell combined with PD-1/PD-L1 blockade in xenograft models of NB. a. Experimental scheme for the subcutaneous xenograft models of NB in NCG mice. Mice are inoculated with *MYCN* amplified (SK-N-BE.[2]) and non-amplified (SK-N-SH) NB cells subcutaneously. Then, mice were intravenously injected with *in vitro* induced human CD8+ T and NK cells that were blocked with or without anti-PD-1 or anti-PD-L1 antibodies; $n = 5$ for each group. b. Tumor volumes for mice in different treatment groups in A were recorded on the indicated days; $n = 5$ for each group. c-p. Tumor volume of mice in each group as indicated were recorded on the indicated days and plotted individually. q. Kaplan–Meier survival curves for the mice in different treatment groups in A. Survival curves were compared using the log-rank test (two-tailed). $n = 5$ for each group. ns, no significance. Data are representative of three independent experiments. Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Discussion

Distinct temporal expression patterns of PD-L1 were identified on tumor cells and host immune cells, and the spatial discordance of PD-L1 between primary and metastatic tumor lesions was also confirmed.[12,14–16,21,22] In this study, we showed that PD-L1 expression on intratumoral CD8$^+$ T and NK cells marks a functionally activated status and associates with favorable survival and improved anti-PD-1/PD-L1 efficacy by *ex-vivo* and *in-vivo* analyses. Consistently, it has been revealed that NK cells expressing PD-L1 was a cytolytic effector and blocking PD-L1 activates PD-L1$^+$ NK cells via a p38 pathway independent of PD-1 to control growth,[17] which provides a possible explanation for the activation role of PD-L1 on CD8$^+$ T and NK cells. These findings offer novel insights into the activation of T and NK cells via PD-L1, as well as a possible explanation for why some patients whose tumor cells lack PD-L1 expression still respond to ICIs. Consistent with our findings, Dondero et al. reported that PD-L1 expression appeared mostly restricted to *MYCN* non-amplified cells, but not *MYCN* amplified cells.[23] After IFN-γ stimulation, PD-L1 was found to be induced in both *MYCN* amplified and non-amplified NB cell lines, as well as in metastatic neuroblasts derived from bone marrow of NB patients characterized by different *MYCN* status.[23] However, neuroblasts from one patient with a *MYCN* gain status were poorly responsive to IFN-γ stimulation,[23] indicating the heterogeneity of PD-L1 induction in neuroblasts.

Actually, emerging evidence has mentioned the roles of IC-PD-L1. *Schreiber et al.* suggested that IC-PD-L1 is a more accurate biomarker for predicting the response to ICIs or prognosis in sarcoma.[14] Differences in tumor immunogenicity determined the relative contribution of IC-PD-L1 and TC-PD-L1 to anti-PD therapy because IC-PD-L1 is critical for inhibiting antitumor immunity in poorly immunogenic tumors, whereas TC-PD-L1 is sufficient to suppress antitumor immunity in highly immunogenic tumors.[15] As a result, IC-PD-L1 has a higher prognostic value in poorly immunogenic tumors such as NB, particularly *MYCN* amplified NB. By contrast, TC-PD-L1 might be more predictive in highly immunogenic tumors. In the present study, we confirmed the PD-L1 expression by infiltrated CD8$^+$ T and NK cells and its association with improved therapeutic efficacy in the immune system reconstituted NCG mouse, and these results warrant validation in immunocompetent mouse with a syngeneic NB model. The role of PD-L1 in other immune cells has also been extensively investigated. For instance, PD-L1 expression by activated myeloid cells fosters immune privilege and tumor progression and predicts response to anti-PD-1 antibodies.[24] *Fu et al.* demonstrated that PD-L1 expressed in antigen-presenting cells plays an essential role in checkpoint blockade therapy in different tumor models.[16] Nevertheless, PD-L1$^+$ T cells were also demonstrated to have diverse tolerogenic effects on tumor immunity through restraining effector T cells via the canonical PD-L1-PD-1 axis, inducing PD-1$^+$ macrophages M2-like polarization, and reducing CD4$^+$ T cell TH1-polarization via STAT3-dependent signal.[18] Further studies will be needed to dissect the comprehensive effects of PD-L1 engagement on T cells and other immune cells in the TME.

In the present study, NK cells have great prognostic value and therapeutic potential in NB patients. Interestingly, we showed that the spatial location of NK cells is important since only intratumoral NK cells are a favorable prognostic marker independent of CD8$^+$ T cells and PD-1/PD-L1 expression, as well as known factors affecting patient outcome. This important finding may help improve patient stratification and provide proof of principle that intratumoral NK cells serve as a potential marker for predicting survival and response to ICIs in NB, although this remains to be confirmed in a prospective study with a larger number of samples. Despite the greater number of CD8$^+$ T cells, our study also implies that NK cells may play a predominant role in antitumor immunity in NB, which was further validated by our functional and therapeutic data. This notion is also supported by recent studies showing that NB tumors are often infiltrated by NK cells.[25] The phenomenon may be attributed to the following factors: first, NB is characterized by a low mutational load and low MHC-I expression[10] and thus could not trigger an efficient T cell response, whereas the immune killing of NK cells is not restricted by MHC expression and antigen stimulation. Second, the ligand of the DNAM1 activating receptor which triggers the cytolytic activity of NK cells is expressed in NB and upregulated in the tumor vasculature,[26] and the NK-cell-activating receptor ligands such as MICA and MICB were also expressed by NB cells in this study. Additionally, we found a higher proportion of functional PD-L1-expressing NK cells than CD8$^+$ T cells in the TME of NB. Therefore, it has been proposed to further illustrate the molecular mechanism underlying the interaction between NB and NK cells, thus establishing a convincing biological rationale for novel NK cell-based immunotherapies.

In line with other studies,[27,28] we found that intratumoral CD8$^+$ T cells were associated with favorable clinical outcomes in patients with NB. However, most of NB cells, particularly *MYCN* amplified cells, are human leukocyte antigen class I negative.[29] It has been revealed that in addition to alfabeta T cells, there are small populations of gammadelta T cells and NK cells in the tumor microenvironment of NB.[23] Therefore, it's quite possible that gammadelta T cell and/or NK cell subsets that are not restricted by MHC expression[30] play a major role in CD8-expressing cells in NB. In cytotoxicity assay, we derived tumor associated CD8$^+$ T cells by using a similar method described elsewhere,[31] and the killing of such tumor associated CD8$^+$ T cells is not MHC-restricted. It is probably that NKG2D and DNAM1 expressed by NK cells are responsible for this killing. Meanwhile, this killing may also be mediated by gammadelta T cells that are activated in an MHC-independent manner.[30] We cannot exclude the presence of gammadelta T cell and NK cell subsets in our system. Overall, NB is a cold tumor with poor infiltration of immune cells, especially PD-1$^+$ T and NK cells,[7] which is further confirmed by the present study. For PD-1/PD-L1 blockade immunotherapy, a key issue is to ensure the sufficient infiltration of PD-1$^+$ T and NK cells in the tumor site. Therefore, PD-1/PD-L1 blockade immunotherapy should be used only following a precise personalized approach, such as adoptive T and/or NK cell transfer therapy, that may improve the immune infiltration of NB tumor to some extent.

Our study has several limitations. We examined only a small fraction of total tumors in cohort_2 because of the use of tissue microarray, and we only performed survival analyses related to OS due to lack of EFS follow-up data. Some results in the GEO database that were inconsistent with the two selected cohorts may be explained by the differences in detection levels, analysis dimensions, people, and sample size. A larger prospective study is needed to validate the present study findings. In addition, GSE154037 used in scRNA seq analysis is a dataset that includes only engineered NKT cells, rather than NK or conventional T cells.[32] NKT cells are conventionally recognized as an important effector cell type that expresses both NK and T cell receptors on their surface. NKT cells can produce a large number of cytokines and play a cytotoxic role similar to NK and T cells. Therefore, in terms of functional status, NKT cells are considered to be representative of NK and conventional T cells to some extent.[33]

In summary, our study offers new insights into the role of PD-L1 expression in $CD8^+$ T-cell and NK-cell activation. We also highlight the potential of intratumoral NK cells for independently predicting prognosis and response to anti-PD-1/PD-L1 therapy, providing a rationale for combination therapy with NK cells and anti-PD-1/PD-L1 therapy that will be helpful to advance the immunotherapeutic strategies in NB. This work provides an explanation for why single anti-PD-1/PD-L1 therapy may not be successful in NB and provides a potential prospect that patients with PD-L1 expression in TILs, especially NK cells, may respond to anti-PD-1/PD-L1 therapy. Additionally, our findings help better define risk stratification and pave the way for an in-depth study of immune effects in the establishment of T/NK cell-based antitumor immunity in NB patients at a spatial level.

Acknowledgments

We thank the Gene Expression Omnibus database for providing expression and clinical data on human neuroblastoma.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the National Key Research and Development Program of China under Grant No. 2022YFC2705005, the National Scientific Foundation of China under Grant No. 82203754, China Postdoctoral Science Foundation under Grant No. 2022M713606 and BX20220364, Basic and Applied Basic Research Project of Guangzhou, Guangdong province, China under Grant No. 2023A04J1770, and Basic and Applied Basic Research Project of Guangdong province, China under Grant No. 2021A1515110234.

ORCID

Mengjia Song http://orcid.org/0000-0002-6399-3412
Jian-Chuan Xia http://orcid.org/0000-0002-1268-3967

Authors' contributions

Conception and design: Y. Zhang, J. Xia, M. Song; Development of methodology: Y. Huang, Y. Hong, H. Chen, J. Xu, J. Liu; Acquisition of data: J. Zhu, S. Lu, J. Wang, F. Sun, J. Huang; Analysis and interpretation of data: M. Song, Y. Hong, J. Liu; Writing, review, and/or revision of the manuscript: M. Song, Y. Hong, Y. Zhang; Administrative, technical, or material support: Y. Zhang, J. Xia, M. Song.

Ethics approval and consent to participate

The use of human samples was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center (Approval No. SL-B2022-273-01), and the requirement for informed consent was waived by the institutional review committee. All experiments involving humans were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All experiments involving animals were approved by the Animal Care and Use Committee of the Sun Yat-Sen University Cancer Center (Approval No. 2022080E).

List of abbreviations

COG	Children's Oncology Group
EFS	event-free survival
GEO	Gene Expression Omnibus
IC	immune cell
ICIs	immune checkpoint inhibitors
IFN-γ	interferon-gamma
IHC	immunohistochemistry
INSS	International Neuroblastoma Staging System
MHC	major histocompatibility complex
mIF	multiplex immunofluorescence
NB	neuroblastoma
NK cell	natural killer cell
OS	over survival
PBMCs	peripheral blood mononuclear cells
PD-1	programmed death 1
PD-L1	programmed death ligand 1
TAMs	tumor-associated macrophages
TC	tumor cell
TILs	tumor-infiltrating lymphocytes
TIME	tumor immune microenvironment.

References

1. Maskalenko NA, Zhigarev D, Campbell KS. Harnessing natural killer cells for cancer immunotherapy: dispatching the first responders. Nat Rev Drug Discov. 2022;21(8):559–577. doi:10.1038/s41573-022-00413-7.
2. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. Lancet. 2007;369(9579):2106–2120. doi:10.1016/S0140-6736(07)60983-0.
3. Theruvath J, Menard M, Smith BAH, Linde MH, Coles GL, Dalton GN, Wu W, Kiru L, Delaidelli A, Sotillo E, et al. Anti-GD2 synergizes with CD47 blockade to mediate tumor eradication. Nat Med. 2022;28(2):333–344. doi:10.1038/s41591-021-01625-x.
4. Ladenstein R, Potschger U, Valteau-Couanet D, Luksch R, Castel V, Yaniv I, Laureys G, Brock P, Michon JM, Owens C, et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. Lancet Oncol. 2018;19(12):1617–1629. doi:10.1016/S1470-2045(18)30578-3.
5. Sanmamed MF, Chen L. A paradigm shift in cancer immunotherapy: from enhancement to normalization. Cell. 2018;175(2):313–326. doi:10.1016/j.cell.2018.09.035.

6. Kim JM, Chen DS. Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). Ann Oncol. 2016;27(8):1492–1504. doi:10.1093/annonc/mdw217.
7. Wienke J, Dierselhuis MP, Tytgat GAM, Kunkele A, Nierkens S, Molenaar JJ. The immune landscape of neuroblastoma: challenges and opportunities for novel therapeutic strategies in pediatric oncology. Eur J Cancer. 2021;144:123–150. doi:10.1016/j.ejca.2020.11.014.
8. Park JA, Cheung NV. Targets and antibody formats for immunotherapy of neuroblastoma. J Clin Oncol. 2020;38(16):1836–48. doi:10.1200/JCO.19.01410.
9. Bolande RP. A natural immune system in pregnancy serum lethal to human neuroblastoma cells: a possible mechanism of spontaneous regression. Perspect Pediatr Pathol. 1992;16:120–133.
10. McNerney KO, Karageorgos SA, Hogarty MD, Bassiri H. Enhancing neuroblastoma immunotherapies by engaging iNKT and NK cells. Front Immunol. 2020;11:873. doi:10.3389/fimmu.2020.00873.
11. Litchfield K, Reading JL, Puttick C, Thakkar K, Abbosh C, Bentham R, Watkins TBK, Rosenthal R, Biswas D, Rowan A, et al. Meta-analysis of tumor- and T cell-intrinsic mechanisms of sensitization to checkpoint inhibition. Cell. 2021;184(3):596–614.e14. doi:10.1016/j.cell.2021.01.002.
12. Toki MI, Merritt CR, Wong PF, Smithy JW, Kluger HM, Syrigos KN, Ong GT, Warren SE, Beechem JM, Rimm DL, et al. High-plex predictive marker discovery for melanoma immunotherapy–treated patients using digital spatial profiling. Clin Cancer Res. 2019;25(18):5503–12. doi:10.1158/1078-0432.CCR-19-0104.
13. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252–264. doi:10.1038/nrc3239.
14. Noguchi T, Ward JP, Gubin MM, Arthur CD, Lee SH, Hundal J, Selby MJ, Graziano RF, Mardis ER, Korman AJ, et al. Temporally distinct PD-L1 expression by tumor and host cells contributes to immune escape. Cancer Immunol Res. 2017;5(2):106–17. doi:10.1158/2326-6066.CIR-16-0391.
15. Juneja VR, McGuire KA, Manguso RT, LaFleur MW, Collins N, Haining WN, Freeman GJ, Sharpe AH. PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. J Exp Med. 2017;214(4):895–904. doi:10.1084/jem.20160801.
16. Tang H, Liang Y, Anders RA, Taube JM, Qiu X, Mulgaonkar A, Liu X, Harrington SM, Guo J, Xin Y, et al. PD-L1 on host cells is essential for PD-L1 blockade–mediated tumor regression. J Clin Invest. 2018;128(2):580–8. doi:10.1172/JCI96061.
17. Dong W, Wu X, Ma S, Wang Y, Nalin AP, Zhu Z, Zhang J, Benson DM, He K, Caligiuri MA, et al. The mechanism of anti–PD-L1 antibody efficacy against PD-L1–negative tumors identifies NK cells expressing PD-L1 as a cytolytic effector. Cancer Discov. 2019;9(10):1422–37. doi:10.1158/2159-8290.CD-18-1259.
18. Diskin B, Adam S, Cassini MF, Sanchez G, Liria M, Aykut B, Buttar C, Li E, Sundberg B, Salas RD, et al. PD-L1 engagement on T cells promotes self-tolerance and suppression of neighboring macrophages and effector T cells in cancer. Nat Immunol. 2020;21(4):442–454. doi:10.1038/s41590-020-0620-x.
19. Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer–response. Clin Cancer Res. 2013;19(19):5542. doi:10.1158/1078-0432.CCR-13-2234.
20. Ambros PF, Ambros IM, Brodeur GM, Haber M, Khan J, Nakagawara A, Schleiermacher G, Speleman F, Spitz R, London WB, et al. International consensus for neuroblastoma molecular diagnostics: report from the International neuroblastoma risk group (INRG) biology committee. Br J Cancer. 2009;100(9):1471–1482. doi:10.1038/sj.bjc.6605014.
21. Cabrita R, Lauss M, Sanna A, Donia M, Skaarup Larsen M, Mitra S, Johansson I, Phung B, Harbst K, Vallon-Christersson J, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. Nature. 2020;577(7791):561–565. doi:10.1038/s41586-019-1914-8.
22. Mansfield AS, Aubry MC, Moser JC, Harrington SM, Dronca RS, Park SS, Dong H. Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor infiltration between paired primary lesions and brain metastases in lung cancer. Ann Oncol. 2016;27(10):1953–8. doi:10.1093/annonc/mdw289.
23. Dondero A, Pastorino F, Della Chiesa M, Corrias MV, Morandi F, Pistoia V, Olive D, Bellora F, Locatelli F, Castellano A, et al. PD-L1 expression in metastatic neuroblastoma as an additional mechanism for limiting immune surveillance. Oncoimmunol. 2016;5(1):e1064578. doi:10.1080/2162402X.2015.1064578.
24. Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, Zheng L. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. J Exp Med. 2009;206(6):1327–37. doi:10.1084/jem.20082173.
25. Wei JS, Kuznetsov IB, Zhang S, Song YK, Asgharzadeh S, Sindiri S, Wen X, Patidar R, Najaraj S, Walton A, et al. Clinically relevant cytotoxic immune cell signatures and clonal expansion of T-cell receptors in high-risk MYCN-not-amplified human neuroblastoma. Clin Cancer Res. 2018;24(22):5673–5684. doi:10.1158/1078-0432.CCR-18-0599.
26. Bottino C, Dondero A, Bellora F, Moretta L, Locatelli F, Pistoia V, Moretta A, Castriconi R. Natural killer cells and neuroblastoma: tumor recognition, escape mechanisms, and possible novel immunotherapeutic approaches. Front Immunol. 2014;5:56. doi:10.3389/fimmu.2014.00056.
27. Mina M, Boldrini R, Citti A, Romania P, D'Alicandro V, De Ioris M, Castellano A, Furlanello C, Locatelli F, Fruci D, et al. Tumor-infiltrating T lymphocytes improve clinical outcome of therapy-resistant neuroblastoma. Oncoimmunol. 2015;4(9):e1019981. doi:10.1080/2162402X.2015.1019981.
28. Melaiu O, Chierici M, Lucarini V, Jurman G, Conti LA, De Vito R, Boldrini R, Cifaldi L, Castellano A, Furlanello C, et al. Cellular and gene signatures of tumor-infiltrating dendritic cells and natural-killer cells predict prognosis of neuroblastoma. Nat Commun. 2020;11(1):5992. doi:10.1038/s41467-020-19781-y.
29. Forloni M, Albini S, Limongi MZ, Cifaldi L, Boldrini R, Nicotra MR, Giannini G, Natali PG, Giacomini P, Fruci D, et al. NF-κB, and not MYCN, regulates MHC class I and endoplasmic reticulum aminopeptidases in human neuroblastoma cells. Cancer Res. 2010;70(3):916–24. doi:10.1158/0008-5472.CAN-09-2582.
30. Silva-Santos B, Mensurado S, Coffelt SB. Gammadelta T cells: pleiotropic immune effectors with therapeutic potential in cancer. Nat Rev Cancer. 2019;19(7):392–404. doi:10.1038/s41568-019-0153-5.
31. Xue R, Zhang Q, Cao Q, Kong R, Xiang X, Liu H, Feng M, Wang F, Cheng J, Li Z, et al. Liver tumour immune microenvironment subtypes and neutrophil heterogeneity. Nature. 2022;612(7938):141–147. doi:10.1038/s41586-022-05400-x.
32. Heczey A, Courtney AN, Montalbano A, Robinson S, Liu K, Li M, Ghatwai N, Dakhova O, Liu B, Raveh-Sadka T, et al. Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. Nat Med. 2020;26(11):1686–1690. doi:10.1038/s41591-020-1074-2.
33. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? Nat Rev Immunol. 2004;4(3):231–237. doi:10.1038/nri1309.