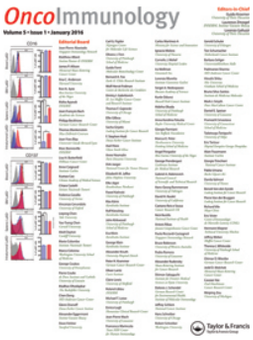

OncoImmunology

ISSN: (Print) 2162-402X (Online) Journal homepage: https://www.tandfonline.com/loi/koni20

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To cite this article: Alessandra Dondero, Fabio Pastorino, Mariella Della Chiesa, Maria Valeria Corrias, Fabio Morandi, Vito Pistoia, Daniel Olive, Francesca Bellora, Franco Locatelli, Aurora Castellano, Lorenzo Moretta, Alessandro Moretta, Cristina Bottino & Roberta Castriconi (2016) PD-L1 expression in metastatic neuroblastoma as an additional mechanism for limiting immune surveillance, OncoImmunology, 5:1, e1064578, DOI: 10.1080/2162402X.2015.1064578

To link to this article: https://doi.org/10.1080/2162402X.2015.1064578

ONCOIMMUNOLOGY
2016, VOL. 5, NO. 1, e1064578 (9 pages)
http://dx.doi.org/10.1080/2162402X.2015.1064578

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ORIGINAL RESEARCH

PD-L1 expression in metastatic neuroblastoma as an additional mechanism for limiting immune surveillance

Alessandra Dondero[a], Fabio Pastorino[b], Mariella Della Chiesa[a], Maria Valeria Corrias[b], Fabio Morandi[b], Vito Pistoia[b], Daniel Olive[c], Francesca Bellora[a], Franco Locatelli[d], Aurora Castellano[d], Lorenzo Moretta[b], Alessandro Moretta[a,e], Cristina Bottino[a,b,*], and Roberta Castriconi[a,e,*]

[a]Dipartimento di Medicina Sperimentale, Università degli Studi di Genova, Italy; [b]Istituto Giannina Gaslini, Laboratorio di Oncologia, Genova, Italy; [c]CRCM, Team Immunity and Cancer, Inserm, Institut Paoli-Calmettes, Aix-Marseille Université, CNRS, UM 105, U1068, UMR7258, Marseille, France; [d]Università di Pavia and Dipartimento di Onco-Ematologia Pediatrica, Ospedale Bambino Gesù, Roma, Italy; [e]Centro di Eccellenza per le Ricerche Biomediche, Università degli Studi di Genova, Viale Benedetto XV, Genova, Italy

ABSTRACT

The prognosis of high-risk neuroblastoma (NB) remains poor, although immunotherapies with anti-GD2 antibodies have been reported to provide some benefit. Immunotherapies can be associated with an IFNγ storm that induces in tumor cells the "adaptive immune resistance" characterized by the *de-novo* expression of Programmed Death Ligands (PD-Ls). Tumor cells can also constitutively express PD-Ls in response to oncogenic signaling. Here, we analyze the constitutive and the inducible surface expression of PD-Ls in NB cells. We show that virtually all HLA class I^{pos} NB cell lines constitutively express PD-L1, whereas PD-L2 is rarely detected. IFNγ upregulates or induces PD-L1 both in NB cell lines *in vitro* and in NB engrafted nude/nude mice. Importantly, after IFNγ stimulation PD-L1 can be acquired by NB cell lines, as well as by metastatic neuroblasts isolated from bone marrow aspirates of high-risk NB patients, characterized by different *MYCN* amplification status. Interestingly, in one patient NB cells were poorly responsive to IFNγ stimulation, pointing out that responsiveness to IFNγ might represent a further element of heterogeneity in metastatic neuroblasts. Finally, we document the presence of lymphocytes expressing the PD-1 receptor in NB-infiltrated bone marrow of patients. PD-1^{pos} cells are mainly represented by $\alpha\beta$ T cells, but also include small populations of $\gamma\delta$ T cells and NK cells. Moreover, PD-1^{pos} T cells have a higher expression of activation markers. Overall, our data show that a PD-L1-mediated immune resistance mechanism occurs in metastatic neuroblasts and provide a biological rationale for blocking the PD-1/PD-Ls axis in future combined immunotherapeutic approaches.

ARTICLE HISTORY
Received 20 April 2015
Revised 15 June 2015
Accepted 16 June 2015

KEYWORDS
Anti-tumor immunity; immune checkpoints; neuroblastoma; natural killer cells; T cells; PD-1; PD-L1; PD-L2; INF-γ; TNF-α

Introduction

Neuroblastomas (NB) are extra-cranial neuroectodermal tumors that account for 15% of all childhood cancer deaths. Different prognostic factors are critical for identifying high-risk NB and guiding therapeutic choices. These factors include age, stage and amplification of *MYCN* (*MYCN*^{ampl}), the major oncogenic driver.[1] High-risk patients present with metastatic disease (stage 4 or M) at diagnosis and have a grim prognosis due to resistance to conventional therapies and early relapse, which not only occur at the primary tumor site but frequently arises in the bone marrow.[2,3]

Natural Killer (NK) cells, when appropriately activated, are capable of killing NB cells. This has been demonstrated, *in vitro* and in animal models, using as targets long-term cultured NB cell lines as well as bone marrow-infiltrating neuroblasts isolated from stage M patients,[4–6] although the latter appear to be more resistant to NK-mediated killing as compared to cell lines.[4] The degree of susceptibility to the NK-mediated cytolytic activity relies on both the repertoire and the surface density of ligands expressed on NB cell surface. In particular, neuroblasts lack HLA class I molecules or show a level of their expression insufficient to generate signals turning off NK-cell function via the inhibitory killer-cell immunoglobulin-like receptors (KIRs).[7] Vice versa, tumor cells can express different ligands engaging receptors that trigger the NK cytolytic machinery and the release of immunostimulatory cytokines, such as IFNγ. These include UL16-binding proteins (ULBP)-2 and ULBP-3, ligands of NKG2D, and Poliovirus Receptor (PVR, CD155) and Nectin-2 (CD112) that are recognized by DNAM-1.[8,9]

Clinical evidences show that the immune system is unable to guarantee a long-lasting control of the disease and, in particular, an efficient NK-mediated destruction of NB fail to occur *in vivo*, suggesting the existence of mechanisms allowing tumor evasion of host immunity. For example, in some patients, metastatic neuroblasts lack the expression of PVR, and its absence

CONTACT Alessandro Moretta alemoret@unige.it
*C. Bottino and R. Castriconi share co-seniorship of this paper.
Supplemental material for this article can be accessed on the publisher's website.

correlates with poor susceptibility to NK-mediated killing.[10] Moreover, in all patients, tumor cells stably express B7-H3,[11] a transmembrane surface glycoprotein endowed with pro-tumoral properties[12,13] that, interacting with an (still unknown) inhibitory receptor, is capable of limiting both NK and T cell-mediated cytolytic activity.[11,14] B7-H3 is considered an unfavorable prognostic factor in both hematological malignancies[15] and solid tumors, including NB,[16-22] and clinical trials with a fully human antibody[23] are ongoing.

An additional mechanism of escape could be the exploitation by tumors of the immune checkpoints, inhibitory pathways that physiologically maintain self-tolerance and limit the duration and amplitude of immune responses, thus minimizing tissue damage.[24,25] One possible pathway is represented by the PD-1/PD-Ls axis. Programmed cell death 1 (PD-1, CD279) is an inhibitory receptor, mainly expressed by $\alpha\beta$ and $\gamma\delta$ T cells. Interestingly however, some reports demonstrated the expression of PD-1 also in activated NK cells.[26,27] Most data on PD-1 functions are referred to $\alpha\beta$ T cells, where PD-1 has been demonstrated to switch off the T cell function mostly in peripheral tissues. Indeed, unlike CTLA-4, PD-1 is expressed during the late phase of T cell activation and, upon engagement with its ligands, it inhibits kinases involved in T cell activation.[28] In $\gamma\delta$ T cells, TCR triggering might partially overcome the inhibitory effect mediated by PD-1. In particular, while proliferation rate might be affected by PD-1 engagement, slight differences in either cytokine production or cytotoxicity were observed in $\gamma\delta$ T cells interacting with PD-L1^{pos} or PD-L1^{neg} tumors.[29] PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are members of the B7 family and represent ligands of PD-1.[30] PD-L2 expression is mainly restricted to antigen presenting cells (APC), whereas PD-L1 is expressed in several normal tissues.[24,25] Interestingly, certain tumors have been shown to express PD-L1 and its interaction with the receptor has been suggested to play a crucial role in immune evasion.[24,31]

In full agreement with preclinical experimental data, combined therapies that include the blockade of the PD-1/PD-L1 pathway resulted in long-term responses in patients with advanced melanoma and, very recently, two anti-PD-1 antibodies obtained FDA approval.[25] Interestingly, clinical responses were observed also in patients affected, at the time of therapeutic decision, by PD-L1^{neg} melanoma and carcinomas. In this context, it has been shown that tumor cells can upregulate PD-L1 surface density upon stimulation with IFNγ and TNF-α,[24,32] cytokines that are released by T lymphocytes, NK cells and macrophages during effective Th1-polarized antitumor responses.

In the present study, we analyzed the constitutive and the inducible expression of PD-Ls in human NB cell lines and *ex vivo* isolated neuroblasts, and evaluated PD-1 expression on lymphocytes in tumor-infiltrated bone marrow aspirates.

Results

Analysis of the constitutive expression of PD-L1 and PD-L2 in NB cell lines

HLA class I and PD-Ls might be key regulators of both NK- and T cell-mediated immune surveillance. We analyzed their constitutive surface expression in twelve human NB cell lines characterized by either the presence or the absence of *MYCN* amplification (*MYCN*^{ampl}) (Fig. 1 and Fig. S1). Although, HLA-I was mainly detected on non-*MYCN*^{ampl} cell lines, exceptions to the rule existed. Indeed, MYCN^{ampl} LAN-1 cells expressed significant levels of HLA-I molecules and, conversely, non-*MYCN*^{ampl} SK-N-SH cells consistently lacked their expression. Similarly, when analyzing PD-L1 expression, it appeared mostly restricted to non-*MYCN*^{ampl} cells. However, it was detectable in *MYCN*^{ampl} LAN-1 cells and absent in non-*MYCN*^{ampl} SK-N-SH cells (Fig. 1 and Fig. S1). Thus, as for HLA-I, in the NB cell lines analyzed, the constitutive expression of PD-L1

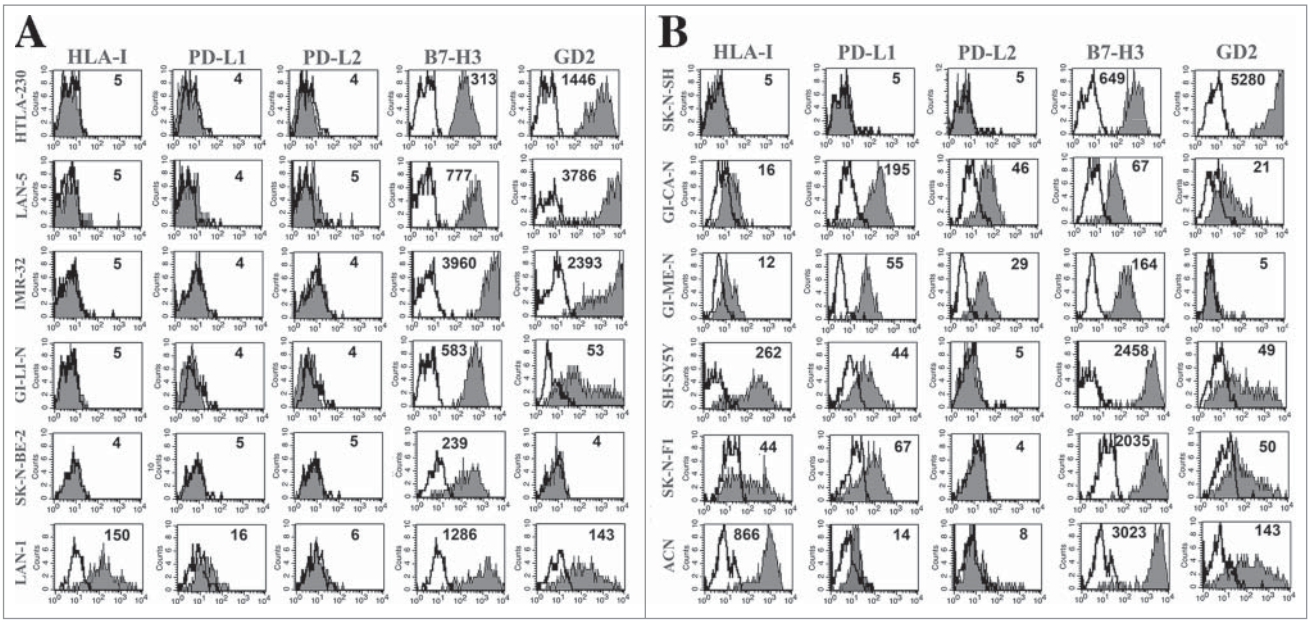

Figure 1. Analysis of the constitutive expression of PD-L1 and PD-L2 in NB cell lines. Representative cytofluorimetric analysis of the expression of PD-L1, PD-L2 and HLA-I in *MYCN*^{ampl} (panel A) and non-*MYCN*^{ampl} (panel B) NB cell lines. B7-H3 and GD2 are shown for comparison. White profiles refer to cells incubated with isotype-matched controls. Values inside each histogram indicate the Median Fluorescence Intensity (MFI).

did not appear to strictly correlate with *MYCN* status. However, HLA-I positive cell lines co-expressed in all instances PD-L1, thus suggesting a possible link in the capability to express these molecules. The constitutive expression of PD-L2 was rarely detected and it was restricted to GICAN and GIMEN, non-*MYCN*^{ampl} HLA-I^{pos} PD-L1^{pos} cell lines. It is of note that HLA-I^{pos} PD-L1^{pos} NB cell lines, although expressing one or more ligand for the DNAM-1 and NKG2D activating NK (and T) cell receptors[4,33] (Fig. S2) shared the expression of B7-H3 (Fig. 1), a ligand involved in modulation of NK and T cell-mediated cytotoxicity whose expression has been shown to correlate with a worse prognosis in different tumor histotypes, including NB.[4]

Cytokines-mediated induction or upregulation of PD-Ls in NB cell lines

Representative *MYCN*^{ampl} or non-*MYCN*^{ampl} cell lines were cultured in the presence of IFNγ or TNF-α. As shown in Fig. 2, in *MYCN*^{ampl} (HTLA-230, IMR-32) cells, IFNγ induced the *de novo* surface expression of HLA-I and PD-L1 molecules, while it did not promote that of PD-L2. On the other hand, in non-*MYCN*^{ampl} cell lines (SH-SY5Y and SK-N-F1), it induced both expression of PD-L2 and upregulation of HLA-I and PD-L1. The capability of TNF-α to modulate ligands expression was reduced as compared to that of IFNγ. Indeed, the two representative *MYCN*^{ampl} cell lines were totally unresponsive to TNF-α, preserving their PD-L1^{neg} PD-L2^{neg} HLA-I^{neg} phenotype. Moreover, in non-*MYCN*^{ampl} cell lines, TNF-α conditioning resulted in a smaller increase of HLA-I and PD-L1, as compared to those observed with IFNγ (Fig. 2).

Interestingly, in *MYCN*^{ampl} cells the *de novo* expression of PD-L1 showed a kinetics even more rapid than that of HLA-I (Fig. S3). Indeed, the maximal PD-L1 expression was observed at 24 h after IFNγ stimulation (mean of fold increase = 6.4), with no significant increase (or decrease) at later time, whereas HLA-I expression was significantly increased after 24 h (mean of fold increase = 96.6) but reached the highest level of expression at 48 h (mean of fold increase = 329.7). On the other hand, in non-*MYCN*^{ampl} cells the kinetics of upregulation of these molecules was comparable, with a maximal expression at 24 h. Regarding PD-L2, it was undetectable at any time in *MYCN*^{ampl}, whereas it was acquired by non-*MYCN*^{ampl} cells, and progressively increased until 48 h (Fig. S3).

IFNγ induces PD-L1 expression in a human NB mouse model

In order to verify whether NB cells could acquire PD-Ls in an *in vivo* setting, we used an animal model based on the subcutaneous injection of the human (HLA-I^{pos} PD-L1^{pos} PD-L2^{neg}) SH-SY5Y NB cell line in immunodeficient mice (see Materials and Methods for more details). This model allowed a rapid quantification of the tumor burden and an easy access for the intra-tumor injection of IFNγ, with minimal suffering of the animal, when compared with the invasive surgery required to treat orthotopic tumor models. At the end of the treatments, tumors were removed and single cell suspensions of NB cells from untreated or IFNγ-treated xenografts were analyzed for ligands expression, gating on cells expressing the GD2^{pos} B7-H3^{pos} phenotype (Fig. 3 and Fig. S4A). Remarkably, the engraftment resulted in NB cells lacking PD-L1 and HLA-I expression, while preserving their original GD2^{pos} B7-H3^{pos} phenotype. Importantly, in line with data obtained *in vitro*

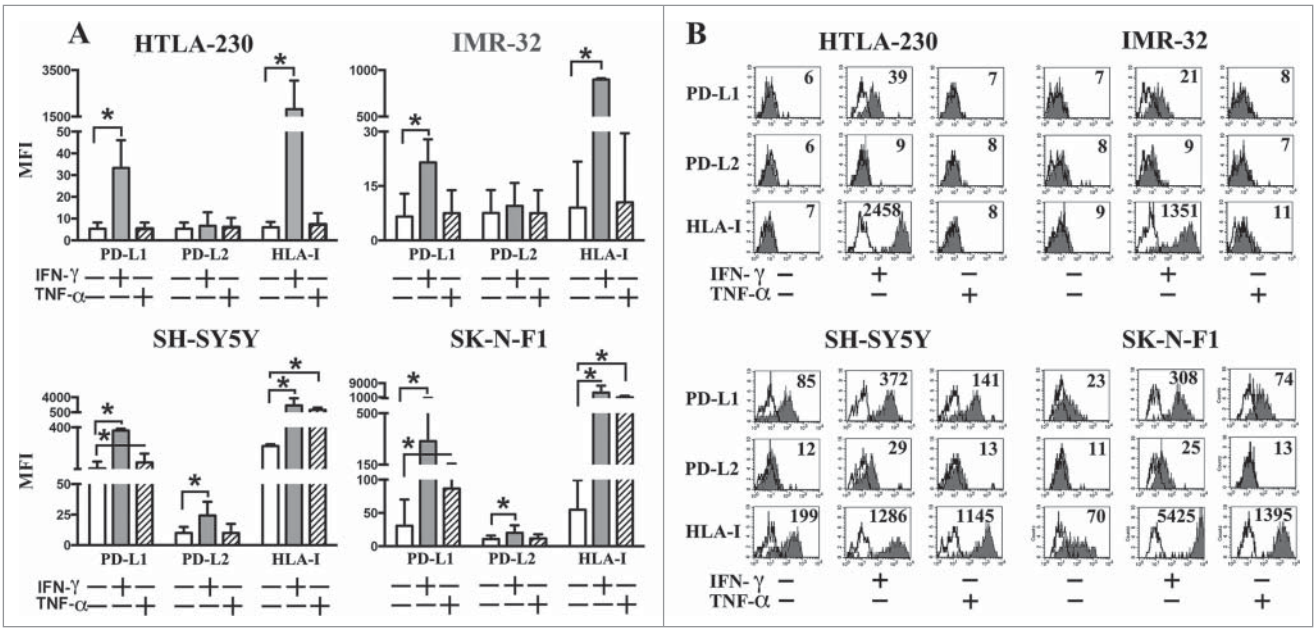

Figure 2. PD-L1 and PD-L2 expression in INFγ− or TNF-α−treated NB cell lines. Panel A: cytofluorimetric analysis of the expression of PD-L1, PD-L2 and HLA-I in representative *MYCN*^{ampl} (HTLA-230, IMR-32) and non-*MYCN*^{ampl} (SH-SY5Y and SK-N-F1) cell lines cultured for 48 h either in the absence (white bars) or in the presence of IFNγ (gray bars) or TNF-α (striped bars). Mean of MFI and 95% confidence intervals are indicated. $^{*}p < 0.05$. Panel B: Representative cytofluorimetric analysis of PD-L1, PD-L2 and HLA-I expression in untreated or cytokine-treated NB cell lines. White profiles refer to cells incubated with isotype-matched controls. Values inside each histogram indicate the MFI.

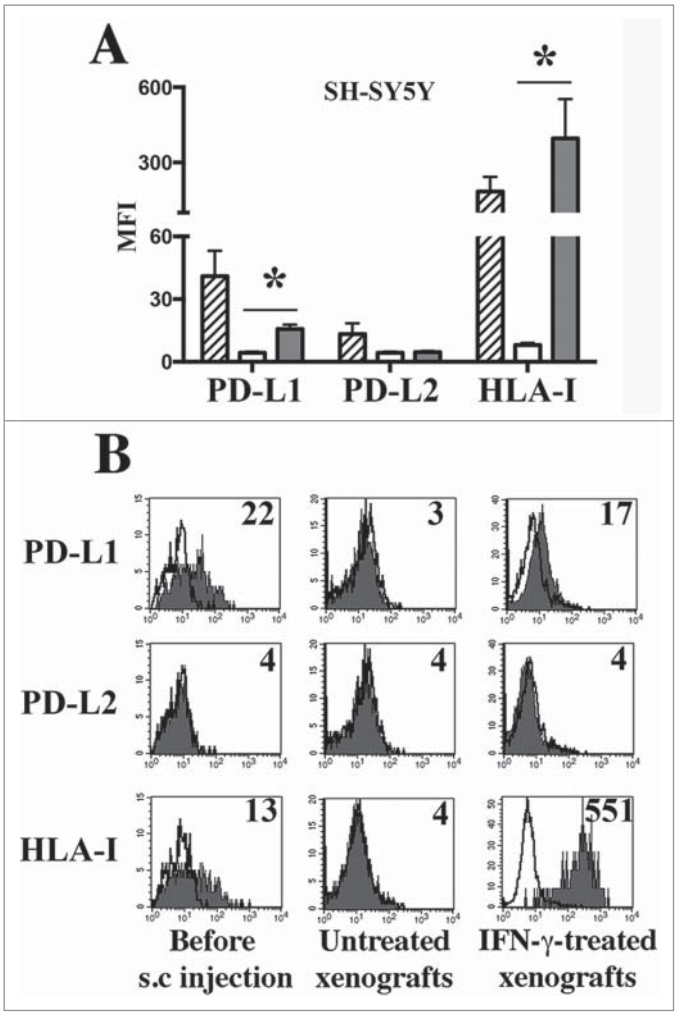

Figure 3. PD-L1 expression in human NB xenografts. Panel A: Cytofluorimetric analysis of PD-L1, PD-L2 and HLA-I expression in the SH-SY5Y cell line just before subcutaneous (s.c.) injection in animals (stripped bars), and in xenografts derived from untreated (white bars) or IFNγ-treated mice (gray bars). Mean of MFI, 95% confidence intervals and significance are indicated. $^{*}p < 0$ .05. Panel B: Representative cytofluorimetric analysis of PD-L1, PD-L2 and HLA-I in the SH-SY5Y cells before s.c. injection, in untreated or IFNγ-treated xenografts. White profiles refer to cells incubated with isotype-matched controls. Values inside each histogram indicate the MFI.

(Fig. 2), intra-tumor injection of IFNγ restored the surface expression of both PD-L1 and HLA-I (Fig. 3). On the other hand, no significant changes were observed in terms of PD-L2 expression (Fig. 3).

Analysis of the PD-Ls and PD-1 expression in high-risk NB patients

We analyzed the constitutive and inducible expression of PD-Ls in neuroblasts purified from bone marrow aspirates of patients with stage M NB (Fig. 4 and Fig. S4B). GD2^{pos} B7-H3^{pos} neuroblasts did not constitutively express PD-L1 and PD-L2. In all patients analyzed, neuroblasts maintained their PD-L2-negative phenotype even in the presence of IFNγ. On the contrary, IFNγ stimulation induced the expression of PD-L1 in three out four patients analyzed, whose *MYCN* status were amplified (PT#1), gain (PT#2) or non-amplified (PT#4) (Fig. 4). Responsiveness to the cytokine was confirmed by the *de novo* expression or upregulation of HLA-I expression, which reached the highest median intensity in neuroblasts from the non-*MYCN*^{ampl} PT#4. Interestingly, PT#3, who was characterized as PT#2 by a *MYNC*^{gain} status, was poorly responsive to IFNγ conditioning. Indeed, upon cytokine stimulation, neuroblasts did not show any induction of PD-L1 expression, and that of HLA-I molecules was restricted to a very small percentage of cells (Fig. 4).

We analyzed PD-1 expression on NK and T lymphocytes in NB-infiltrated bone marrow aspirates. T cells represented the large majority (70%) of lymphocytes at this site. Among NK lymphocytes, a small percentage (up to 2%) of PD-1^{pos} cells were detected. On the other hand, about 40% of CD3^{pos} cells expressed PD-1 (Fig. 5A). PD-1^{pos} CD3^{pos} lymphocytes showed a significant higher percentage of CD25^{pos} and CD69^{pos} cells, as compared to the PD-1 negative counterpart (Fig. 5B and C). Interestingly, PD-1^{pos} T cells also included a subset of γ/δ T cells (Fig. 5B and C).

Discussion

An important challenge for pediatric oncologists is represented by stage M NBs, a disease often refractory to standard therapies, that frequently shows recurrence or progression.[3] Current therapeutic strategies are based on risk factors that do not take into consideration the expression, in tumor cells, of surface molecules crucial for the recognition by the immune system, probably underestimating a further element of heterogeneity existing among patients. Phenotypic and functional analysis showed that infiltrating neuroblasts can either lack or express low levels of HLA-I,[11] thus representing non-optimal targets for HLA-restricted T cell-based immunotherapies. In these cases, tumor cells can display susceptibility to NK-mediated killing, which, however, in some patient, is limited by the absence on tumor cells of key ligands for activating NK receptors.[10] Moreover, in all patients, NB cells constitutively and stably express at the cell surface B7-H3, a molecule endowed, not only with an immune-regulatory activity that limits T and NK cell-mediated killing, but also with direct tumor promoting properties.[4] The characterization of the biological features of B7-H3, together with its poor expression in most normal tissues, is recently driving novel immunotherapeutic approaches targeting this tumor-associated marker.[23,25,34]

Here we show that, together with the constitutive expression of B7-H3, NB cells can exploit inducible members of the B7 family to regulate key effectors of the immune system. Indeed, the surface expression of PD-Ls, and in particular of PD-L1 can be induced in NB cells by inflammatory cytokines such as INFγ and TNF-α. As demonstrated in other tumors,[35] in NB cells, INFγ stimulation is more potent than TNF-α. INFγ induced the expression of PD-L1 in various *MYCN*^{ampl} and *non-MYCN*^{ampl} NB cell lines *in vitro* as well as in an animal tumor model based on the use of SH-SY5Y cells. Different from most NB cell lines (including the prototypic *MYCN*^{ampl} HTLA-230), which require orthotopic models for *in vivo* growth, the SH-SY5Y cell line has the ability to both respond to IFNγ stimulation and grow subcutaneously. The use of an

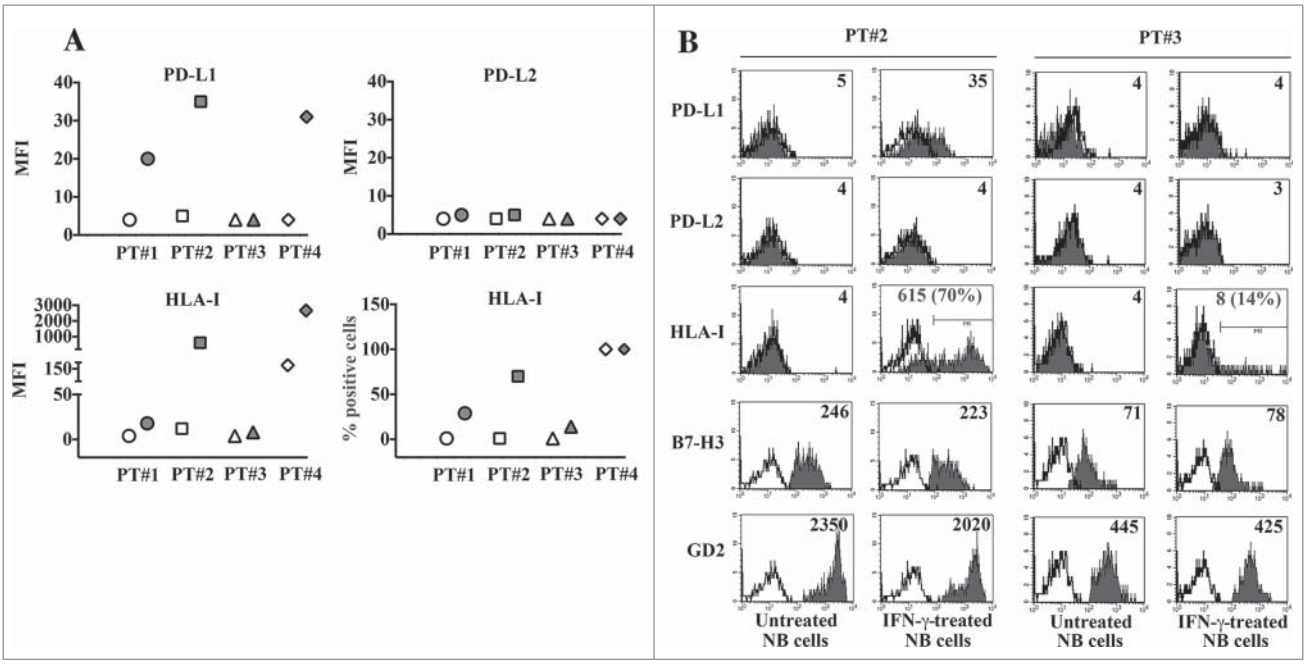

Figure 4. Analysis of the constitutive and inducible PD-L1 and PD-L2 expression in neuroblasts from NB patients. Panel A: Neuroblasts from (CD45-depleted) bone marrow aspirates of stage M patients ($GD2^{pos}$ $B7\text{-}H3^{pos}$), untreated (white symbol) or treated with IFNγ over 5 d (gray symbol) were analyzed by flow cytometry for the expression of the indicated molecules. Raw values are plotted. Patient 1 (PT#1) (*MYCN*^{ampl}), Patient 2 (PT#2) (*MYCN*^{gain}), Patient 3 (PT#3) (*MYCN*^{gain}), Patient 4 (PT#4) (non-*MYCN*^{ampl}). MFI or percentage of positive cells are indicated. Panel B: Representative cytofluorimetric analysis of PD-L1, PD-L2 and HLA-I expression in IFNγ-responsive (PT#2) and IFNγ-unresponsive (PT#3) neuroblasts. White profiles refer to cells incubated with isotype-matched controls. Values inside each histogram indicate the MFI and, in brackets, the percentage of positive cells.

orthotopic mouse model as a new experimental approach could be considered in future investigations.

Importantly, INFγ also induced the expression of PD-L1 in freshly-isolated metastatic neuroblasts from patients, which did not present detectable levels of this molecule at the cell surface. PD-L1 expression occurs independently of the *MYCN* amplification status, whereas it is apparently coordinated with that of HLA-class I molecules. Moreover, the INFγ-mediated *de novo* induction of PD-L1 shows a more rapid kinetics as compared to HLA-I molecules, thus suggesting that in an inflammatory microenvironment PD-L1 could prematurely limit the activity of T lymphocytes and precede the acquisition of HLA-I levels optimal for the KIR-mediated inhibition of NK cell functions. This observation is in line with data recently published by Boes M. et al., who shows that in NB cell lines stimulated by poly(I:C), an agonist of Toll-Like Receptor 3 (TLR3), the kinetic of PD-L1 expression is more rapid than that of HLA-I.[36] Importantly, they also demonstrate that the antibody-mediated blocking of PD-L1 increases the T-cell stimulatory properties of poly (I:C)-stimulated NB cell lines.[36]

B7-H3 is considered a negative prognostic factor in different solid tumors, whereas the analysis of PD-L1 expression gave conflicting results.[37–42] No strict correlation has been demonstrated between PD-L1 mRNA levels in carcinomas and survival.[35] Accordingly, in human NB primary tumors the levels of *PD-L1* expression did not show correlation with overall and relapse-free survival (Fig. S5). In this context, further analysis of NB specimens will allow to validate the relevance of epigenetic mechanisms regulating PD-Ls expression at post-transcriptional levels.[43,44] Other studies reported a better survival in patients carrying $PD\text{-}L1^{pos}$ tumors that were characterized by elevated lymphocyte infiltration, and the presence of infiltrating $PD\text{-}1^{pos}$ T cells has been considered a favorable prognostic marker.[45] Both conditions reflect the existence of effective anti-tumor responses, but also highlight the opposite, beneficial versus detrimental, effects of an inflammatory/Th1-polarized microenvironment. Indeed, the release of large amounts of INFγ by activated T and NK cells amplifies the immune responses, but also forces the expression of PD-L1, which could result in premature inhibition of lymphocyte proliferation and effector function.[46] Overall, our present data emphasize the complexity of the PD-1/PD-L1 axis and suggest that, in NB, PD-L1 might be considered a *MYCN*-independent "tumor-inflammatory" prognostic factor rather than simply a tumor-associated marker.

The susceptibility of NB cells to INFγ stimulation supports the usefulness of the blockade of the PD-1/PD-L1 pathway in combined therapeutic approaches aimed at achieving long-lasting remission in patients with high-risk NB. Indeed, both conventional and innovative therapeutic protocols generate an inflammatory microenvironment capable of influencing PD-L1 expression in tumor cells. It is of note that INFγ is highly represented in the serum of tumor patients suffering of the Cytokine Release Syndrome (CRS), which frequently occurs with targeted immunotherapeutic approaches.[47] These include the use of antibodies directed against tumor-associated antigens and adoptive therapies with chimeric antigen receptor (CAR)-modified T cells. The Ab-mediated targeting of the oncofetal antigen GD2 has been already included in the standard cure of high-risk NB[3] and approaches based on the infusion of CAR-T cells specific for GD2 are tested in preclinical[48,49] and clinical[50] studies.

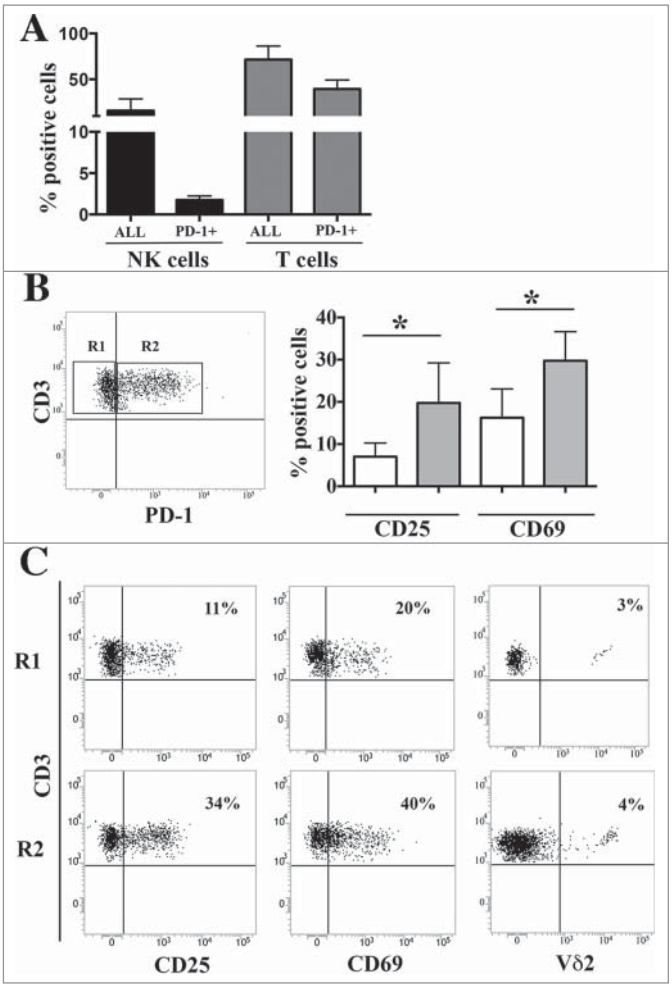

Figure 5. Surface expression of PD-1 in lymphocytes from NB-infiltrated bone marrow aspirates of patients with stage M. Panel A: Bone marrow infiltrating NK and T cells were analyzed by multicolor flow cytometry. The percentage of the two lymphocyte populations and of the PD-1^{pos} subsets is shown. Mean and 95% confidence intervals are indicated. Panel B: Gating strategy (left) and CD25 and CD69 expression (right) in CD3^{pos} PD-1^{neg} (R1, white bars) and CD3^{pos} PD-1^{pos} (R2, gray bars) T cells. Mean of percentage of positive cells, MFI and 95% confidence intervals are indicated. * $p < 0$ .05. Panel C: Representative cytofluorimetric analysis of CD25, CD69 and Vδ2 expression in CD3^{pos} PD-1^{neg} (R1) and CD3^{pos} PD-1^{pos} (R2) T cells. Percentage of positive cells and MFI are indicated.

Notably, metastatic neuroblasts appear to display variability in the response to INFγ stimulation. In particular, in one patient analyzed in our study (PT#3), tumor cells were very poorly responsive to the cytokine and did not express detectable levels of PD-L1 upon cytokine stimulation. More information about the frequency of IFNγ resistant NB will derive from the analysis of cohort of patients larger than that used in the present study. In this context, it is of note that NB is a rare pediatric disease and both the number and the volume of samples are scarce. Since the beginning of our study, we received 19 bone marrow aspirates from NB patients (Table S1). However, 12 samples were NB free and in 3 cases, due to the poor tumor infiltration (<1 % of total cells), purified NB cells were insufficient to perform *in vitro* IFNγ stimulation. Thus, although NB resistance to IFNγ needs to be supported by the analysis of a wider number of patients, susceptibility or resistance to INFγ stimulation by tumor cells might be taken into consideration when selecting patients for PD-1/PD-L1 blocking approach. On the other hand, it is conceivable that also NB patients characterized by PD-L1 negative tumors might benefit from the block of the PD-1/PD-L1 axis. Indeed, both PD-L1 and PD-L2 expression is induced in APC including macrophages and dendritic cells and, together with chronic antigen exposure, play a role in exhaustion of the immune response.[25,51] Thus, interfering with the PD-1/PD-Ls immune checkpoint might also reactivate the crosstalk between APC and lymphocytes. According to this hypothesis, clinical responses with anti-PD-1 antibodies have been observed also in patients whose tumors were considered negative for PD-L1 expression.[52] In this context, the analysis of bone marrow aspirates from stage M patients showed the presence of infiltrating NK and T lymphocytes and, although T cells were clearly more represented, a PD-1^{pos} cell subset was detectable in both lymphocyte populations. Interestingly, PD-1^{pos} T cells also included $\gamma\delta$ T cells, which, lacking HLA-I restriction, might be involved in the early phase of endogenous or adoptive immune responses against NB.[53,54]

In conclusion, our study provides a biological rationale for considering blocking the PD-1/PD-L1 axis as an additional immunotherapeutic approach in combined therapies of high-risk NB patients. Such therapies might include also targeting of B7-H3, an immunomodulatory pro-tumoral molecule that shows a striking stability at the NB cell surface.

Materials and methods

Neuroblastoma cell lines

GI-LI-N, GI-ME-N and GI-CA-N NB cell lines were established at the Laboratory of Oncology, Giannina Gaslini Institute, Genova, Italy; SH-SY5Y, SK-N-F1, IMR-32, LAN-1, LAN-5, SK-N-BE-(2) and SK-N-SH cell lines were purchased from Banca Biologica and Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino-IST, Genova, Italy). NB cell lines are periodically checked for *MYCN* amplification by fluorescence in situ hybridization analysis. HTLA-230 and ACN were kindly provided by Dr. E. Bogenmann (Children's Hospital Los Angeles, Los Angeles, CA) and by the late Dr S Carrel, respectively.[55]

NB cell lines were cultured in the presence of RPMI 1640 medium supplemented with 10% heat inactivated FCS (Sigma-Aldrich), 50 mg/mL streptomycin, 50 mg/mL penicillin and 2 mM glutamine (henceforth referred to complete medium). The NB cell lines used in this study were checked for morphology, proliferation rate and mycoplasma contamination, after thawing and within four passages in culture.

Neuroblastoma patients

After informed consent, bone marrow was aspirated from iliac crests of children diagnosed with stage M NB and admitted at the Oncology Unit of the Giannina Gaslini Institute (Table S1). Diagnosis and staging were performed according to the INRG-SS.[1] The study was approved by the Istituto Giannina Gaslini Ethics Committee and the procedures were in accordance with the Helsinki Declaration of 1975. NB were purified from bone marrow aspirates as previously described.[11]

Mouse tumor model

All animals were purchased from Harlan Laboratories (Harlan Italy, S.Pietro al Natisone, Italy) and housed under specific pathogen-free conditions. Experiments involving animals were reviewed and approved by the Licensing and Ethical Committee of IRCCS Azienda Ospedaliera Universitaria San Martino − IST (Genova, Italy), and by the Italian Ministry of Health. *In vivo* experiments were performed with three mice for group. 2×10^7 SH-SY5Y cells were subcutaneously injected in the mid-dorsal region of five-week-old female nude/nude mice, as previously described.[56] Tumors were allowed to grow for 3 weeks, and then intratumorally treated with 300 ng/mL of IFNγ in complete medium, every day for 2 d. Control mice received complete medium alone. 24 h after the end of treatments, mice were sacrificed by cervical dislocation after being anesthetized with xilezine (Xilor 2%, Bio98 Srl, Milan, Italy), NB tumors removed, immersed in complete medium, and minced by an homogenizer at 4°C. The single cell suspensions were subjected to erythrocytes lysis (1.54 M NH4Cl; 99.8 mM KHCO3; 0.988 mM EDTA), and washed in PBS before the cytofluorimetric analysis performed gating $GD2^{pos}$, $B7\text{-}H3^{pos}$ cells.

Cytofluorimetric analysis, IFNγ and TNF-α treatment

For one-color cytofluorimetric analysis (FACSCalibur Becton Dickinson & Co, Mountain View, CA) cells were stained with the appropriate mAbs followed by Phycoerythrin (PE)-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL).[11] On every experimental session, the flow cytometer performances were monitored, the reproducibility of the fluorescence intensity was aligned by calibrated microsfere (Becton Dickinson & Co, Mountain View, CA) and isotype matched antibodies were used as controls. For multicolor cytofluorimetric analysis of bone marrow samples, NK or T cells were gated by physical parameters and the combined use of anti-CD56, anti-CD3, anti-CD19, anti-CD45 mAbs. The analyses were performed on FACSVerse (Becton Dickinson & Co) and data were analyzed by FacsSuite software 1.0.5 version.

For cytokine stimulation NB cell lines or freshly-isolated neuroblasts were seeded at 200.000 cells/well in round flat bottom plates and cultured (for 2 or 5 days, respectively) in the presence of TNF-α or INFγ (PeproTech, Rock Hill, NJ) at the final concentration of 100 ng/mL. Cytofluorimetric analysis was performed by gating $GD2^{pos}$, $B7\text{-}H3^{pos}$ cells.

Monoclonal antibodies

A6136 (IgM, anti-HLA class-I), M5B14 (IgM) and NE97 (IgG2b) (anti-B7-H3), M5A10 (IgG1, anti-PVR), U191 (IgM, anti-Nectin-2), KRA236 (IgG1, anti-CD226), c227 (IgG1, anti-CD69), MAR93 (IgG1, anti-CD25) and BAB281 (IgG1, anti-NKp46) mAbs were produced in our lab. Anti-PD-L1.3.1 (IgG1, anti-PD-L1), anti-PD-L2 (IgG1, anti-PD-L2) and anti-PD-1 mAbs were produced in D. Olive's lab, Anti-ULPB2 (165903, IgG2a) and anti-ULBP3 (166510, IgG2a) mAbs (SantaCruz biotechnology, inc). Anti-GD_2 mAb (14.G2a, IgG2A), anti-CD16-PerCPCy5.5 and anti-CD45-V500 (BD Bioscience PharMingen, San Diego, CA). Anti-CD56-PC7 (C218 clone) (Beckman Coulter, Immunotech, Marseille, France); anti-CD45-FITC anti-CD3-VioGreen, anti-CD19-VioBlue, anti-Vdelta1-VioBlue, anti-Vdelta2-APC and anti-TCRab-APC-Vio770 mAbs (Miltenyi Biotec, Bergisch Gladbach, Germany); Goat anti-mouse isotype specific secondary reagents (anti-IgG1-APC-Cy7 and anti-IgG2B-PE) (Southern Biotec). The KL247 (IgM, anti-NKp46), DF200 (IgG1 anti-KIRs) and AZ158 (IgG2a, anti-KIRs) mAbs produced in our lab were used as isotype-matched controls.

PD-L1 gene expression analysis in NB patients

Correlation of overall and relapse-free survival of NB patients and levels of *PD-L1* expression were obtained from the Versteeg database containing whole-genome sequence data from 88 human NB primary tumors,[57] evaluated using R2 Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi).

Statistical analysis

Wilcoxon−Mann−Whiteny p value test (non-parametric significance test) was employed. The statistical level of significance (p) is indicated. Graphic representation and statistical analysis were performed using the PASW Statistic version 20.0 software (formerly SPSS Statistics) (IBM, Milan Italy) and GraphPad Prism 6 (GraphPad Software La Jolla, CA).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. G. Reggiardo (Medi Service, Genova, Italy) for help in statistical analysis, Dr A. Garaventa (Istituto G. Gaslini, Genova) for providing *neuroblastoma* BM aspirates. We apologize to the colleagues whose work we could not cite because of space constraints.

Funding

This work was supported by Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.) (Investigator Grant 15704 and special project 5×1000 9962), Ministero dell'Istruzione, dell'Università e della Ricerca (M.I.U.R) (PRIN 20103FMJEN) and Ministero della Salute (5 × 1000 e Ricerca Corrente) and Equipe FRM DEQ20140329534. F. Bellora is recipient of a fellowship awarded by A.I.R.C. (special project 5×1000 9962).

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