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Stromal-like Wilms tumor cells induce human Natural Killer cell degranulation and display immunomodulatory properties towards NK cells

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ABSTRACT

The similarity of stromal-like Wilms tumor (str-WT) cells with mesenchymal stem cells (MSC), suggests their relevant role in the interplay with immune cells in the tumor microenvironment. We investigated the interaction between str-WT cells and NK cells. We observed that str-WT cells expressed some major ligands for activating and inhibitory NK cell receptors. Moreover, they expressed inhibitory checkpoint molecules involved in the negative regulation of anti-tumor immune response. The analysis of the interaction between str-WT cells and NK lymphocytes revealed that activated NK cells could efficiently degranulate upon interaction with str-WT cells. On the other hand, str-WT cells could exert potent inhibitory effects on cytokine-induced activation of NK cell proliferation and phenotype, which were mediated by the production of IDO and PGE2 inhibitory factors. Our data provide insight into the molecular interactions between str-WT cells and NK lymphocytes that may result in different outcomes possibly occurring in the WT microenvironment.

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Introduction

Wilms Tumor (WT) or nephroblastoma is the most common pediatric renal tumor, with an estimated annual incidence of 1 in 10.000 children.[1] It is a malignant embryonal neoplasm derived from nephrogenic blastemal cells. At the molecular level, in some patients, WT is the result of aberrations in the *WT1* gene, located on chromosome 11p13[2,3] and coding for a protein necessary for the development of the kidney and gonads before birth.[4] Other low frequency recurrent genetic mutations have been identified within CTNNB1, TP53, and IGF-II genes.[5,6] Histologically, many nephroblastomas replicate the histology of the developing kidney.[7] A variety of cell types (which include blastema, epithelium, and stroma) are present in most lesions. The relative proportion of each cell or tissue type varies from case to case and the diverse cell types may express variable degree of differentiation. Most tumors have triphasic pattern, containing blastemal, stromal, and epithelial cell types, but biphasic and monophasic lesions are often observed. Wilms tumors with WT1 mutations have predominant stromal-type histology (str-WT) and have been shown to display morphological, phenotypic, and biological features similar to mesenchymal stem cells (MSC).[8] Indeed, str-WT cells expressed typical MSC surface markers, including CD105, CD73, and CD90. Moreover, they showed stem cell-like properties being able to differentiate toward adipogenic, chondrogenic, and osteogenic lineages.

The importance of inflammation in tumor development is well known, and it is now well established that an inflammatory microenvironment is a key component of many tumors.[9] Indeed, within the tumor microenvironment, a delicate balance between antitumor and tumor-promoting activities exists, which involves tumor cells, tumor-associated fibroblasts, endothelial cells, innate and adaptive immune cells. There is a plethora of publications describing the interplay between inflammation and the development of tumors in adults. On the contrary, only few studies investigated the molecular links between WT and inflammation. Recent studies showed that, compared with normal kidney, WT exhibited infiltration of inflammatory immune cells and overexpression of several inflammatory transcription factors and other inflammatory markers.[10–12] Notably, the leucocyte infiltrate was predominantly localized in the stromal component of WT tissue, thus suggesting a preferential attraction of immune cells toward tumor cells displaying an MSC-like phenotype .[12]

Different effector cells are involved in immune responses against tumors, including α/β and γ/δ T lymphocytes, and Natural Killer (NK) cells. The anti-tumor effect of NK cells is primarily related to their ability to kill cancer cells and to

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secrete soluble factors that act, either directly or indirectly, by recruiting/activating other effectors in tumor tissues. In particular, NK cells release large amounts of immunostimulatory cytokines, such as IFN-γ, and are capable of recognizing surface ligands that are primarily expressed/up-regulated by tumor cells. These molecules are recognized by an array of activating NK receptors that include NKp46, NKp30, and NKp44 (named Natural Cytotoxicity Receptors, NCR), NKG2D, and DNAM-1.[13–16] In addition, NK cells express KIR (Killer-cell Immunoglobulin-like Receptors) and CD94/NKG2A, inhibitory receptors specific for classical and non-classical HLA-class I molecules, respectively, which regulate their function .[17,18]

The relevance of NK cells in tumor control is well established in mouse models and human hematologic malignancies; however, their contribution to controlling human solid tumors is still debated. The tumor microenvironment and the associated abnormal inflammatory response may negatively influence differentiation, recruitment, and NK cell efficiency at the tumor site.[19–22] In cancer, immune cells may display phenotypic and/or functional alterations responsible for a reduced ability to display optimal anti-tumor responses. One of the main mechanisms involves the activation of the immune checkpoint pathways. It has been shown that the effector functions of T cells can be negatively regulated by the expression of inhibitory immune checkpoint receptors (including PD-1 and CTLA-4) that recognize their cognate ligands (PD-L1/L2 and B7.1/.2 molecules, respectively), expressed by tumor cells or by cells in the tumor microenvironment, including macrophages.[23] Interestingly, the expression of PD-1 has been also demonstrated in NK cells.[24,25] The blockade of these inhibitory immune checkpoint pathways could result in an increased anti-tumoral immune response. Indeed, therapeutic strategies masking PD-1 or CTLA-4 with chimeric or humanized monoclonal antibodies have shown remarkable clinical responses in different tumor types, including melanoma and lung adenocarcinoma .[24,26,27]

More recently, other inhibitory checkpoint molecules have been proposed as new targets for cancer immunotherapies. These include B7-H3 (CD276), an immune checkpoint ligand expressed at high levels in many adult and pediatric solid tumors, such as neuroblastoma, glioma, sarcoma, and Wilms tumor.[20,28,29] Currently, clinical trials are exploring the efficacy of modulating or targeting the B7-H3R/B7-H3 axis by employing anti-B7-H3 mAbs[30] or B7-H3 CAR-T cells .[20,29,31]

The interaction between tumor cells and the immune system is essential for the control of tumor growth. Thus, a better knowledge of the mechanisms involved would critically contribute to the development of novel therapeutic strategies. In Wilms tumor, very few data are available about the function of immune cells in the response against WT cells. In particular, a relevant role could be played by str-WT cells in view of their similarity with MSC. In this context, a large body of literature supports the evidence for a strong immunosuppressive effect mediated by MSC on many cells of the immune system, [32–34] including NK cells .[35,36]

In the present study, we provide a detailed characterization of the immune phenotype of patient-derived str-WT cells, in particular analyzing the expression of surface ligands relevant to the interaction with human NK cells. We show that activated NK cells degranulate and produce inflammatory cytokines in the presence of tumor cells. In addition, similar to MSC, str-WT cells exert an immunomodulatory effect on NK cell function. This property could be taken into consideration for alternative therapeutic approaches in the treatment of WT.

Materials and methods

Antibodies

For cytofluorimetric analysis, cells were stained with the following fluorochrome-conjugated monoclonal antibodies (mAbs): phycoerythrin (PE)-anti-CD105 (clone MEM-226, IgG2a; Immunotools, 21271054); fluorescein isothiocyanate (FITC)-anti-CD3 (clone UCHT1, IgG1; 300406), FITC-anti-CD47 (clone CC2C6, IgG1; 323106), PE-anti-CD73 (clone AD2, IgG1; 344004), PE-anti-NKp30 (clone P30-15, IgG1; 325208) purchased from Biolegend; PE-anti-CD107a (clone H4A3, IgG1; 121079942), PE-anti-CD146 (clone P1H12, IgG1; 12146942) from eBioscience; allophycocyanin (APC)-anti-CD56 (clone NCAM16.2, IgG2b; 341027), PE-anti-PD-L1 (clone MIH1, IgG1; 557924), PE-anti-PD-L2 (clone MIH18, IgG1; 558066) from Becton Dickinson; PE-anti-NKp46 (clone BAB281, IgG; IM3711), PE-anti-NKp44 (clone Z231, IgG1; IM3710), PE-anti-NKG2A (clone Z199, IgG2b; IM329IU), PE-anti-NKG2D (clone ON72, IgG1; A08934), PE-anti-2B4 (clone C1.7, IgG1; IM1608) from Beckman Coulter; APC-anti-CD90 (clone DG3, IgG1; 130095402); PE-anti-HLA class I (clone Tu149, IgG2a; MHBC04) from Invitrogen.

For indirect staining, the following primary monoclonal antibodies (mAbs) were used: anti-ULBP1 (clone 170818, IgG2a; MAB 1380100), anti-ULBP2 (clone 165903, IgG2a; MAB 1298100), anti-ULBP3 (clone 166510, IgG2a; MAB 1517100), anti-ULBP4 (clone 709116, IgG2b; MAB 6285), anti-B7-H6 mAb (clone 875001, IgG1; MAB7144), anti-Nidogen-1 (clone 302117, IgG1; MAB2570) all from R&D Systems; The following mAbs were produced in our laboratory: F22 (IgG1, anti-DNAM-1); 5B14 (IgM, anti-B7-H3), L14 (anti-Nectin-2, IgG2a), L95 (anti-PVR, IgG1), BAM195 (anti-MICA, IgG1), A6136 (anti-HLA class I, IgM).

The goat anti-mouse antibodies PE-anti-IgG1 (Life Technologies; P21129), PE-anti-IgG2a (Southern Biotech; 108009), PE-anti-IgG2b (Southern Biotech; 109009), PE-anti-IgM (Beckman Coulter; IM0555), and PE AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG (Jackson ImmunoResearch, 109–116-170) were used as secondary reagents.

The following commercial antibodies were utilized in this study for Western blot analysis: mouse anti-vimentin (clone V9, IgG1; Santa Cruz Biotechnology, sc-6260); mouse anti-pan Cytokeratin mAb (clone PCK-6; IgG1; Abcam, ab6401); rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAb (clone 14 C10; Cell Signaling, 2118); rabbit anti-cyclooxygenase-2 (COX-2) mAb (clone D5H5; Cell Signaling, 12282); rabbit anti indoleamine 2,3-dioxygenase (IDO) Ab (ThermoFisher, 711778); goat anti-mouse Ig HRP-conjugated mAb (Southern Biotech, 1031–05); goat anti-rabbit HRP-conjugated mAb (Southern Biotech, 4050–05).

Isolation and expansion of str-WT cells from WT tissue specimens

Str-WT cell cultures were obtained from primary WT specimens derived from patients undergoing nephrectomy for tumor removal (see Table 1 for information about WT patients' clinical data and histology of tumor samples). Residual material from histological analysis was used for tumor cell isolation and expansion. To this aim, tumor samples were dissociated into single-cell suspensions by combining mechanical dissociation with enzymatic degradation of the extracellular matrix using the gentleMACS™ Dissociator (Miltenyi Biotec, 130–093-235) and the Tumor Dissociation Kit (Miltenyi Biotec, 130–095-929), according to the manufacturer's instructions. After dissociation, the sample was applied to a 70 μm filter to remove any remaining larger particles. Then, the single-cell suspension was plated at a density of 4×10^5 cells/ml in T75 tissue-culture flasks in the MesenPRO RS™ Medium (Gibco, 12746012), supplemented with 2 mM L-Glutamine (Gibco, A29168-01) in order to promote the preferential expansion of tumor cells of stromal type. Half the medium volume was replaced twice a week. After reaching ≥80% confluence, tumor cells were harvested by trypsin/EDTA (Euroclone, ECB3052), counted, and re-plated for expansion at 6000 cells/cm^2. Culture expanded str-WT cells were then phenotypically and functionally characterized.

As normal counterpart of renal epithelial cells, the gW4K cell population was isolated from the healthy renal tissue specimen of patient #4, and cultured in MesenPRO RS™ Medium.

For cytokine stimulation experiments, str-WT cells were cultured for 72 h in the presence of 20 ng/ml IFN-γ (Miltenyi Biotec, 130–096-482) or for 48 h with 20 ng/ml IL-1β (Miltenyi Biotec, 130–093-897) and 10 ng/ml TNF-α (Miltenyi Biotec, 130–094-014).

NK cell isolation and culture

NK cells were isolated from the PBMC of healthy donors using the Human NK Cell Isolation Kit (Miltenyi Biotec, 130–092-657), according to the manufacturer's instructions. Purity of NK cells, evaluated by cytofluorimetric analysis of CD56+CD3-CD19- lymphocytes, ranged from 95% to 98%. Purified NK cells were cultured for up to 7 days in RPMI 1640 (Lonza, BE12-167 F) 10% FBS (Gibco, 10270–106), supplemented with 2 mM L-Glutamine, Penicillin/Streptomycin (Euroclone, ECB3001D), and 100 U/ml IL-2 Proleukin (Novartis, 18A08294) in order to obtain short-term activated polyclonal NK cells.

Table 1. Information on WT patients. Clinical data and histology of the tumor from which str-WT cells were derived.

Patient	Sex	Age at surgery	Nephroblastoma histologic pattern	Pre-surgery chemotherapy
gW1	F	17 months	Biphasic (stromal+epithelial)	Yes
gW3	F	10 months	Triphasic	No
gW5	M	30 months	Biphasic (stromal+epithelial)	Yes
gW6	F	44 months	Biphasic (stromal+blastema)	Yes

Other cells

The immortalized MSC-like WT1, WT6, and WT10 cells lines were generated and characterized in the laboratory of Prof. B. Royer-Pokora from WT patients displaying WT1 gene mutations.[8] Tumor cells were maintained in culture with MesenPRO RS™ Medium supplemented with 2 mM L-Glutamine.

MSCs were derived from the spongy tissue of discarded bone fragments of seven pediatric patients undergoing surgery to correct major scoliosis at the Orthopedic Department of G. Gaslini Institute, as previously described.[36] Cells were cultured with MesenPRO RS™ Medium supplemented with 2 mM L-Glutamine and used in the experiments after at least four expansion passages to ensure depletion of hematopoietic cells.

The erythroleukemia cell line K562 was maintained in culture in RPMI 1640 supplemented with 10% FBS, 2 mM L-Glutamine, and Penicillin/Streptomycin.

Flow cytometric analysis

For surface staining, 50.000 to 150.000 cells were firstly incubated with marker-specific fluorochrome-labeled mAbs for 30 minutes at 4°C. Then, cells were washed and resuspended in RPMI 1640 5% FBS before the analysis.

For indirect staining, cells were firstly incubated with unconjugated marker-specific mAbs or sNCRs for 30 min at 4°C. Then, after washing, PE-conjugated goat anti-mouse IgG or IgM or Goat PE-anti-human IgG secondary antibodies were added to the samples, respectively, for an additional 30 min incubation. sNCRs were prepared as previously described .[37]

Cytofluorimetric analysis of NK cells was performed by triple color staining. Briefly, PBMC or purified NK cells were stained with anti-CD56-APC, FITCanti-CD3-, and marker-specific mAbs and incubated for 30 minutes. Expression of informative markers was assessed by gating on CD56+CD3- lymphocytes.

All samples were run on the MACSQuant Analyzer (Miltenyi Biotec) and BD FACS Calibur (Becton-Dickinson) flow cytometers and analyzed using Flow Jo 10.0.8 software (TreeStar Inc., http://www.flowjo.com/). Data are represented as Mean Fluorescence Intensity (MFI) or as Stain Index (S.I.): $(MFI_{pos} - MFI_{neg})/2\sigma_{neg}$.

SDS-PAGE and Western blot analysis

Cells were lysed either in 1% Nonidet *P*-40 (NP-40, Sigma) in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, or in RIPA buffer (ThermoFisher, 89900) in the presence of Halt protease Inhibitor Single-Use Cocktail (ThermoFisher, 78430).

Samples were run on 10% polyacrylamide gel and transferred to Immobilon-P PVDF membranes (Millipore IPVH00010). Membranes were blocked with 5% BSA in Tris-buffered-saline containing 0.05% Tween-20 (TBS-T) and probed with primary antibodies (see Antibodies section) followed by the appropriate HRP-conjugated secondary reagent. The SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher 34080) was used for detection. Images were

acquired with ChemiDoc Touch Imaging System (Bio-Rad) and analyzed with Image Lab software (Bio-Rad).

RT-PCR analysis

Total RNA was extracted from different cell lines using RNAeasy Mini Kit (Qiagen, 74104). Oligo(dT)-primed cDNA was prepared starting from 1 μg of RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, 04379012001) following manufacturer's instructions. RT reaction was conducted for 10′ at 42°C followed by 50′ at 55°C. PCR reactions were carried out for 30 (or 35) cycles utilizing Platinum TAQ DNA Polymerase (ThermoFisher, 10966034) at an annealing temperature of 58°C (β-actin and IDO) or 60°C (COX-2). Primers used were: β-actin for 5′ ACTCCATCATGAAGTGTGACG and β-actin rev 5′ CATACTCCTGCTTGCTGATCC; IDO ORF up 5′ GACTACAAGAATGGCACACG and IDO ORF dw 5′ AATGTGCTCTTGTTGGGTTAC; COX-2 for2 5′ ATGATTGCCCGACTCCCTTG and COX-2 rev 5′ CCCCACAGCAAACCGTAGAT. PCR products (249 bp β-actin; 1244 bp IDO; 395 bp COX-2) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

NK cell degranulation assay

NK cell activation following interaction with tumor cell targets was evaluated as the frequency of degranulating NK cells, measured by the expression of CD107a (lysosome-associated membrane protein-1). Short-term IL-2-activated NK cells were incubated for 4 h with str-WT cell populations or WT cell lines at a 2:1 effector-to-target ratio. The K562 erythroleukemia cell line was used as NK-sensitive control target in parallel degranulation assays. For masking of HLA-class I molecules, saturating amounts of the anti-HLA class I A6136 mAb (IgM isotype) were added to wells. PE-anti-CD107a was added directly to the wells. After 1 h incubation, the monensin containing Golgi Stop reagent (BD Biosciences, 554724) was added and incubated for another 3 h. Cells were analyzed on a MACSQuant cytofluorimeter.

NK cell proliferation assay

Freshly isolated NK cells were plated at 2.5×10^5 cells/well in U-bottom 96-well plates either alone or in the presence of irradiated tumor cells or MSC at an NK-to-tumor cell/MSC ratio of 20:1, 10:1, and 5:1. After culture for 4 days in RPMI supplemented with 10% FBS and 100 U/mL IL-2 to induce NK-cell proliferation, 5 μM 5-ethynyl-2′-deoxyuridine (EdU) was added for the last 18 h, and uptake was evaluated by flow cytofluorimetric analysis after cell staining with CD56-APC mAb and the Click-iT® Plus EdU Flow Cytometry Assay Kit (Molecular Probes, C10633). In some experiments, co-culture of NK cells and tumor cells was set also in the presence of the COX-2 inhibitor NS-398 (5 μM; Sigma, N194) and the IDO inhibitor 1-methyl-D-Tryptophan (1-M-Trp, 0.5 mM; Cayman Chemicals, CAY-16456), used singularly or in combination.

Co-culture experiments for the evaluation of NK cell proliferation were also performed in the absence of cell contact, by plating 2.5×10^5 NK cells in the upper chamber and 2.5×10^4 str-WT cells in the lower chamber of a transwell culture system (0.4 μm pore size; Costar, 3413).

ELISAs

PGE2 production by str-WT populations was evaluated: (i) on culture supernatants derived from cells cultured alone (basal level), (ii) stimulated for 48 h with 20 ng/ml IL-1β and 10 ng/ml TNF-α, and (iii) on supernatants collected after 72 h from NK–str-WT cell co-cultures. PGE2 quantification was performed by competitive ELISA technique using the Prostaglandin E2 Parameter Assay Kit (R&D Systems, KGE004B), according to the manufacturer's instructions. PGE2 concentrations were calculated by comparison with known standards with a lowest detection limit 39 pg/mL. All determinations were made in duplicate.

IFN-γ and TNF-α production was evaluated on 48 h supernatants collected from cultures of short-term IL-2-activated NK cells and from co-cultures of the same NK cell populations and str-WT cells. Cytokine concentrations were measured employing the quantitative sandwich enzyme immunoassay technique using the Human IFN-γ ELISA Kit (Invitrogen, EHIFNG) and the Human TNF-alpha Quantikine ELISA Kit (R&D Systems, DTA00D). All determinations were made in duplicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA). To determine statistical significance, matched paired Students' t-test was used for all experiments. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$.

Ethics statement

All biological samples were obtained after approval by the ethic committees of the institutional review boards of Giannina Gaslini Institute and of Regione Liguria, Genova, Italy (protocols 339–256REG2014 and 737-WILMS). Informed consent was obtained from patients or patients' legal guardians in accordance with the Declaration of Helsinki.

Results

Isolation and in vitro expansion of stromal-type WT cell populations

Renal tumor samples were derived from nine patients hospitalized at the IRCCS Giannina Gaslini Institute. Eight were diagnosed as Wilms tumors by clinical observation and histopathological examination. In four cases, we were able to isolate and culture str-WT cell populations, namely, gW1, gW3, gW5, and gW6 (Table 1). Patients #1 and #5 presented with biphasic WT characterized by stromal and epithelial components; patient #3 had WT with classical triphasic histology with

a predominant blastemal component; patient #6 had biphasic WT with stromal and blastemal cells. All patients presented unilateral tumors and, except for patient #3, they had received chemotherapy before nephrectomy.

Enrichment in stromal-type component was obtained by culturing cells in an MSC-specific medium, that allowed the preferential expansion of tumor cells of stromal type. Initially, cultures showed morphological heterogeneity, as deriving from a starting cell population composed by different tumor cellular elements. Following culture passages, a preferential expansion of stromal-type tumor cells was observed. *In vitro* expanded cells displayed the typical spindle-shaped MSC morphology (Figure 1a) and phenotype (Figure 1b), as documented by the expression of CD105, CD90, CD73, and CD146 surface markers. Western blot analysis further supported the MSC-like nature of these cells, with absence or low expression of cytokeratin, and high levels of vimentin (Figure 1c).

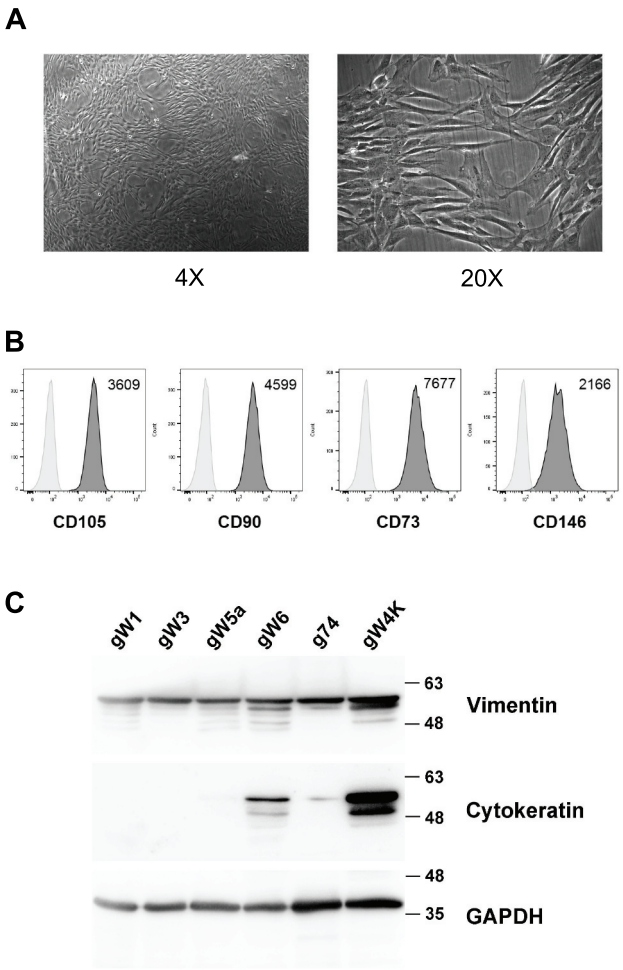


Figure 1. Str-WT cells display morphological and phenotypic features of MSC. (a) Spindle-shaped, MSC-like morphology of *in vitro* expanded str-WT cells. The representative gW3 population is shown. Left: 4x magnification; right: 20x magnification. (b) Flow cytometric analysis of the expression of the indicated MSC surface markers in gW3 cells. Light gray and dark gray histograms represent negative control and marker expression, respectively. Numbers indicate Mean Fluorescence Intensity (MFI). (c) Western blot analysis of vimentin and cytokeratin expression in the four str-WT cells, in MSC (g74), and in epithelial renal cells (gW4K). GAPDH expression was analyzed as positive control. Molecular weight (MW) markers (kDa) are indicated on the right. A representative experiment of three is shown.

The phenotype of str-WT cells was similar to that of the three immortalized WT cell lines, WT1, WT6, and WT10, previously generated and characterized in the laboratory of Prof. Pokora[8] (Figure S1).

Stromal-type WT cells express ligands for immune cell receptors

To investigate a possible interaction between str-WT cells and NK lymphocytes, we first analyzed the expression on tumor cells of the ligands for the receptors that regulate NK cell activation and function .[15,16,18,38,39]

We found that str-WT cells expressed various ligands of inhibitory NK receptors, including HLA-class I molecules, and the immune checkpoint ligands PD-L1 and PD-L2, and B7-H3 (Table 2 and Figure 2a). The surface density of HLA-class I molecules varied consistently among different WT populations, with the lowest expression in gW3 (S.I. = 10.0) and the highest in gW5 (S.I. = 87.0). PD-L1 expression was very weak in all str-WT populations, and always inferior to that of PD-L2.

Table 2. Surface expression of the ligands of activating and inhibitory receptors in str-WT cells and WT cell lines. Data are represented as Stain Index (S.I.). For NKG2D ligands, only the S.I. of the molecule expressed at highest level among MICA and ULBP1,2,3,4 is indicated.

WT	HLA-I	PD-L1	PD-L2	B7-H3	PVR	Nectin-2	ULBPs/MICA
gW1	32.1	0.4	1.8	10.0	19.5	19.4	2.0 (MICA)
gW3	10.0	0.5	1.5	16.3	18.3	18.9	3.0 (ULBP2)
gW5	87.0	1.1	5.4	13.1	24.7	15.5	2.2 (ULBP2)
gW6	45.1	0.6	0.5	22.0	23.6	22.6	1.0 (MICA)
WT1	33.4	3.7	5.9	5.3	10.3	7.3	1.5 (ULBP2)
WT6	49.7	0.7	7.1	4.4	11.8	15.2	3.4 (ULBP2)
WT10	28.9	1.4	9.6	5.1	5.7	14.3	3.8 (ULBP2)

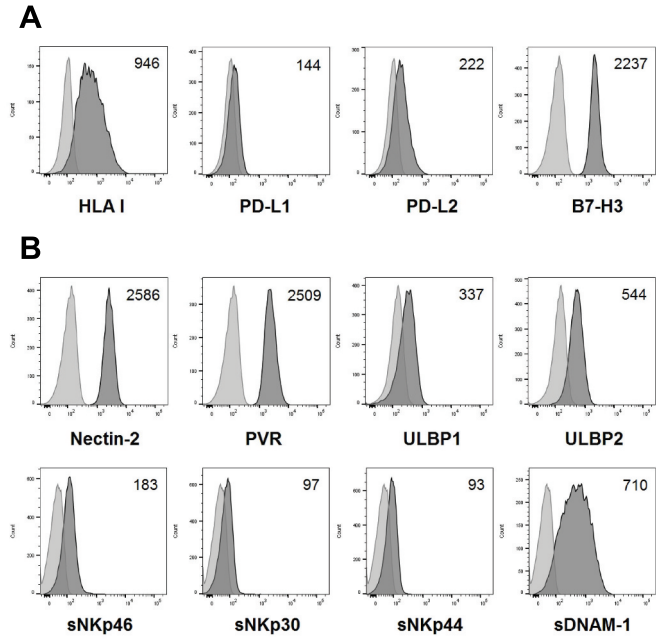


Figure 2. Str-WT cells express ligands for immune cell receptors. Flow cytometric analysis of the expression of ligands for inhibitory (a) or activating (b) NK cell receptors in str-WT cells. The representative gW3 str-WT cell population is shown. Light gray and dark gray histograms represent negative control and marker expression, respectively. Numbers indicate MFI. (See Table 2 for the phenotypic data of all WT cell populations).

This is in line with the data obtained by analyzing in parallel the WT cell lines (Table 2) and MSC populations from healthy children (Figure S2), which also showed PD-L2 expression levels higher than those of PD-L1. Moreover, str-WT cell populations, as well as WT cell lines and MSC, displayed a very high expression of B7-H3 molecule (Table 2, Figure 2a and S2).

We then tested str-WT cells for the expression of ligands of activating NK receptors. Similar to MSC, str-WT populations, as well as WT cell lines, expressed the DNAM-1 ligands, namely, PVR and Nectin-2. However, different from MSC, which were reported to express higher levels of PVR, [36] tumor cells displayed similar surface densities of PVR and Nectin-2. Tumor cells were also positive for NKG2D ligands, with predominant expression of ULBP2 or MICA (Table 2 and Figure 2b).

The availability in our laboratory of soluble activating receptors, namely, sNKp46, sNKp30, sNKp44, and sDNAM-1, allowed us to analyze the expression of their ligands on str-WT cells (Figure 2b and S3A). The soluble receptors variably reacted with tumor cells, depending on the tumor population analyzed. Interestingly, although reacting with sNKp30, the str-WT populations were all negative for B7-H6 (Figure S3B), the known ligand of this receptor, [40] thus suggesting that str-WT cells may express an alternative, still unidentified, NKp30 ligand. Regarding sNKp44, all str-WT cell populations and the WT1 cell line positively stained with the soluble receptor, but the surface expression of the NKp44 ligand Nidogen-1[41] was detected only in gW6 (Figure S3C), indicating that other ligands, such as PCNA[42] and MLL-5, [43] may be expressed by tumor cells. sNKp46 reacted, although at variable extent, with the str-WT populations and WT1. Finally, sDNAM-1 strongly reacted with both the str-WT cells and the WT cell lines, in line with the expression of high levels of PVR and Nectin-2.

It is of note that the surface phenotype of all WT cell populations analyzed was stable, being not modified by expansion passages.

Str-WT cells induce NK cell degranulation and cytokine production

The activation of effector functions in NK cells is the result of an equilibrium between activating and inhibitory signals, which are generated during the interaction between NK and target cells. In this regard, most of the str-WT cell populations expressed low or intermediate levels of HLA-class I molecules and were positive for various ligands of activating NK receptors. Therefore, we hypothesized that tumor cells could be susceptible to NK cell-mediated lysis. Indeed, in degranulation assays, short-term IL-2-activated NK cells acquired the surface expression of CD107a degranulation marker upon 4 h-incubation with str-WT cells (Figures 3a and 3b). The HLA-class I negative K562 leukemia cell line was used as control given its high susceptibility to NK cell-mediated killing.

Different degranulation levels were observed, depending on the tumor population used as target. In particular, the percentages of degranulating lymphocytes were higher in str-WT populations, compared to the WT cell lines. Moreover, unlike the WT cell lines, in str-WT cell populations masking of HLA-class I molecules could not significantly increase degranulation.

In order to further analyze the interaction between activated NK lymphocytes and str-WT cells, we performed co-culture experiments to evaluate cytokine production. Collected 48 h culture supernatants, tested in ELISA assays, revealed that NK cells could produce IFN-γ and TNF-α upon interaction with tumor cell populations (Figure 3c).

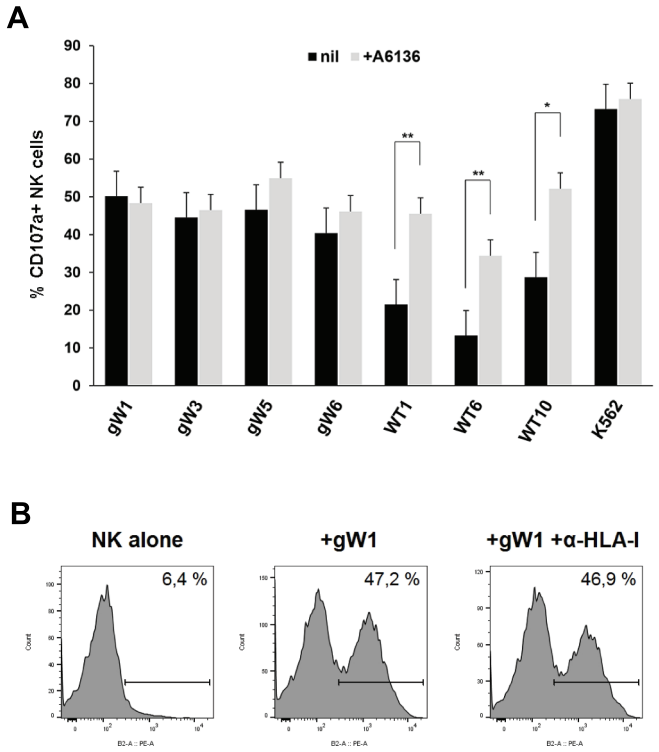
Str-WT cells display immunosuppressive properties toward NK cells

The previous demonstration by Prof. Pokora's group of the similarity between str-WT and MSC was based on the analysis of cell phenotype and gene expression profiles. An additional important characteristic of MSC is their strong immunosuppressive activity toward different cells of the immune system, including NK cells.[35,36] Therefore, we tested the inhibitory potential of str-WT cell populations, in co-culture experiments with NK lymphocytes. As control, co-cultures of NK cells and MSC were set in parallel. As shown in Figure 4a, str-WT cell populations and WT cell lines exerted a significant inhibitory effect on the cytokine-induced proliferation of NK cells. Indeed, the percentage of EdU+ NK cells, induced to proliferate *in vitro* by the addition of IL-2, was significantly reduced by co-culture with tumor cells. The inhibitory effect was dependent on the NK-to-tumor cell ratio used. As shown in Figure 4b, the strongest inhibition was observed at 5:1 ratio, and it decreased progressively at higher ratios. Notably, inhibition was contact-dependent, as co-culture experiments performed using transwell chambers to physically separate NK lymphocytes and tumor cells did not result in str-WT cell-mediated inhibition of NK cell proliferation (Figures S4A and S4B).

The inhibitory effect exerted by str-WT cells affected not only proliferation but also the acquisition of the phenotype typical of cytokine-activated NK cells. Indeed, in NK cells stimulated with IL-2 and co-cultured with str-WT populations, the expected up-regulated expression of NKp30, NKp44, NKG2D, and 2B4 activating receptors was not observed (Figure 4c and 4d). On the other hand, the expression of other receptors, such as NKp46 and DNAM-1, that are not modulated by IL-2, was not affected by the presence of tumor cells (Figure 4c).

Str-WT cells produce inhibitory factors upon exposure to proinflammatory stimuli

We investigated the mechanisms possibly involved in the inhibitory effect exerted by str-WT cells on NK lymphocytes. Firstly, we analyzed in tumor cells the expression of COX-2, previously reported as a marker of an inflammatory tumor microenvironment in WT tissue specimens, that was primarily localized in the tumor stromal component.[11,12] COX-2 activity leads to the production of PGE2, which represents a major player in the immunosuppressive effect mediated by MSC.[11] As COX-2 is generally induced by inflammatory mediators, we analyzed its expression before and after exposure of str-WT cells to IL-1β + TNF-α.[44] We found that str-WT populations did not constitutively express COX-2. Notably, in three out of



IFN-γ

	-	+gW1	+gW3	+gW5	+gW6	g58
NK1	104	1634	1902	456	520	766
NK2	122	2910	1930	1720	804	660
NK3	43	260	851	223	208	194
NK4	104	1143	1877	3299	1690	2401

TNF-α

	-	+gW1	+gW3	+gW5	+gW6	g58
NK1	90	514	751	125	238	326
NK2	54	530	487	425	291	436
NK3	50	47	171	53	57	41
NK4	70	476	886	572	318	393

Figure 3. NK cells degranulate and produce cytokines upon interaction with str-WT cells. (a) Short-term IL-2-activated NK cells were used as effectors in degranulation assays in the presence of str-WT cells and WT cell lines. The E/T ratio used was 2:1. For each target, at least three effector cells from different donors were used. The prototypical NK-sensitive, HLA class I negative, K562 cell line was used in parallel as positive control. Experiments were performed either in the absence (black bars) or in the presence (gray bars) of mAb-mediated masking of HLA class I molecules (A6136 mAb, IgM isotype). Results are expressed as percentage of CD107a+ NK cells and represent mean ± SD of data obtained from at least three independent experiments performed. * $p < .05$, ** $p < .01$. (b) A representative degranulation experiment is shown, using gW1 as target cells. Numbers indicate percentages of CD107a+ NK cells. (c) Short-term IL-2-activated NK cells were cultured with str-WT cells (or g58 MSC) at an 8:1 NK/str-WT cell ratio. After 48 h, culture supernatants were collected and tested by ELISA assays for the evaluation of IFN-γ and TNF-α production. Data are expressed as cytokine concentration (pg/ml). Four NK cell populations derived from different donors were used.

four str-WT cells analyzed, IL-1β + TNF-α stimulation-induced COX-2 protein and mRNA expression (Figure 5a and S5A). We also tested PGE2 production by performing ELISA assays on the supernatants of str-WT cells cultured in the absence or in the presence of IL-1β + TNF-α. Although with a certain degree of variability, the str-WT populations constitutively secreted PGE2, whose levels were significantly increased by cytokine stimulation in three str-WT cells (Figure 5b, left). PGE2 production was also observed in the supernatants of NK–str-WT cell co-cultures (Figure 5b, right), suggesting that, similarly to MSC, str-WT cells can exert their inhibitory effect through the synthesis of PGE2.

Another important factor known to mediate MSC inhibitory effect is Indoleamine 2,3-dioxygenase (IDO), [35,45] an inducible enzyme that can be up-regulated by inflammatory cytokines, including IFN-γ. Regarding WT, IDO expression was previously demonstrated in tumor tissue specimens by PCR analysis.[11] As shown in Figure 5c and S5B, analysis of IDO expression in the str-WT populations cultured in the absence or in the presence of IFN-γ revealed that cytokine stimulation could significantly induce IDO protein and mRNA expression in all str-WT populations analyzed.

To demonstrate an effective role of PGE2 and IDO in the inhibition of cytokine-induced NK cell proliferation, we performed co-culture experiments with NK and str-WT cells in the presence or in the absence of inhibitors of these factors, namely, NS-398 (a selective COX-2 inhibitor) and 1-M-Trp (an inhibitor of IDO enzymatic activity), used singularly or in combination to assess their possible additive (or synergistic) effects. As shown in Figure 6, the addition of 1-M-Trp significantly counteracted, although not completely, the inhibitory effect exerted by str-WT cells, whereas NS-398 did not have any substantial effect. Remarkably, however, the simultaneous blocking of IDO and PGE2 could almost fully restore the NK cell proliferation, leading to a not statistically significant difference in comparison to standard condition (NK cells cultured alone).

In addition to soluble mediators, cell-to-cell contact may be responsible, at least in part, for the immunosuppressive effect mediated by str-WT cells on NK lymphocytes. In this regard, immune checkpoint ligands, known to be regulated by inflammatory cytokines, may be involved.[46] To investigate this aspect, str-WT cell populations were stimulated with IFN-γ and their phenotype was analyzed by flow cytometry. IFN-γ could significantly up-regulate HLA-class I, PD-L1, and, to a lesser extent, PD-L2 molecules, whereas it had no significant effect on B7-H3 expression. Interestingly, also CD47 molecule, known as one of the main "don't eat me" signals, [47] was up-regulated on str-WT cells following IFN-γ exposure (Figure 5d).

Discussion

Our study provides novel information on the expression in str-WT cells of markers relevant to the interaction with cells of the immune system, with a particular focus on NK cells. In addition, our results support the evidence for a significant immunosuppressive potential of str-WT cells, that may have relevant implications on tumor expansion and on the outcome of therapeutic interventions. In this context, the stromal component of WT may be primarily responsible for an immunoregulatory microenvironment that could influence the overall susceptibility of tumor cells to immune response.

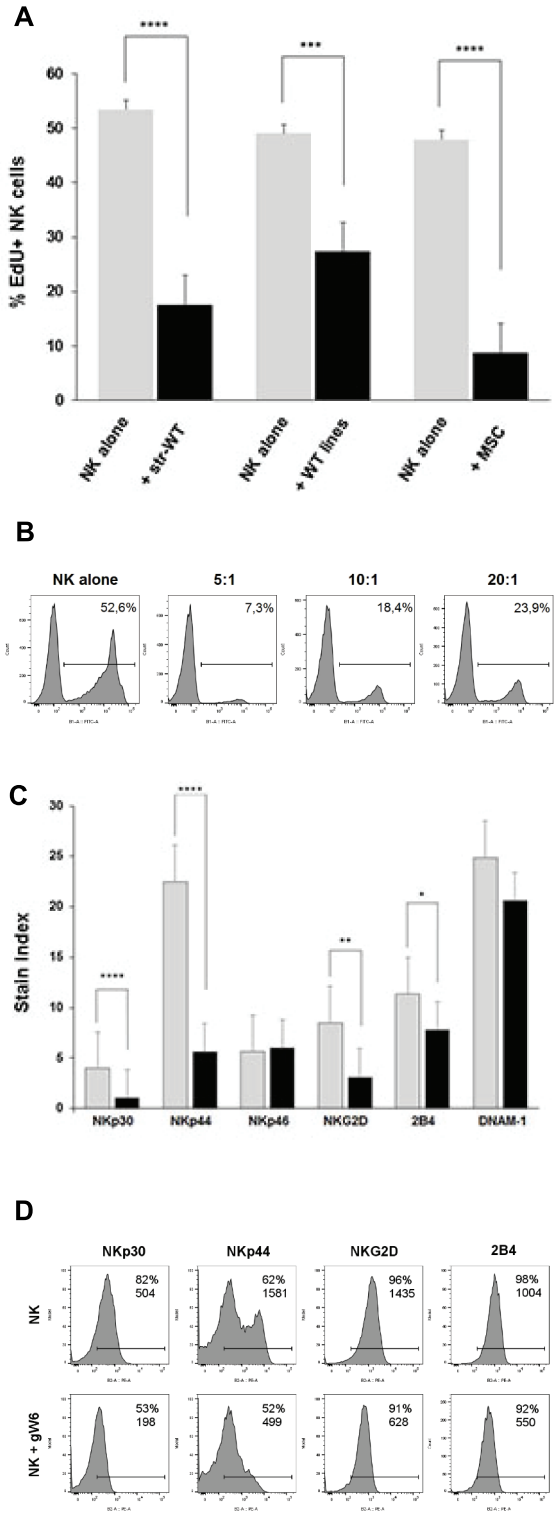


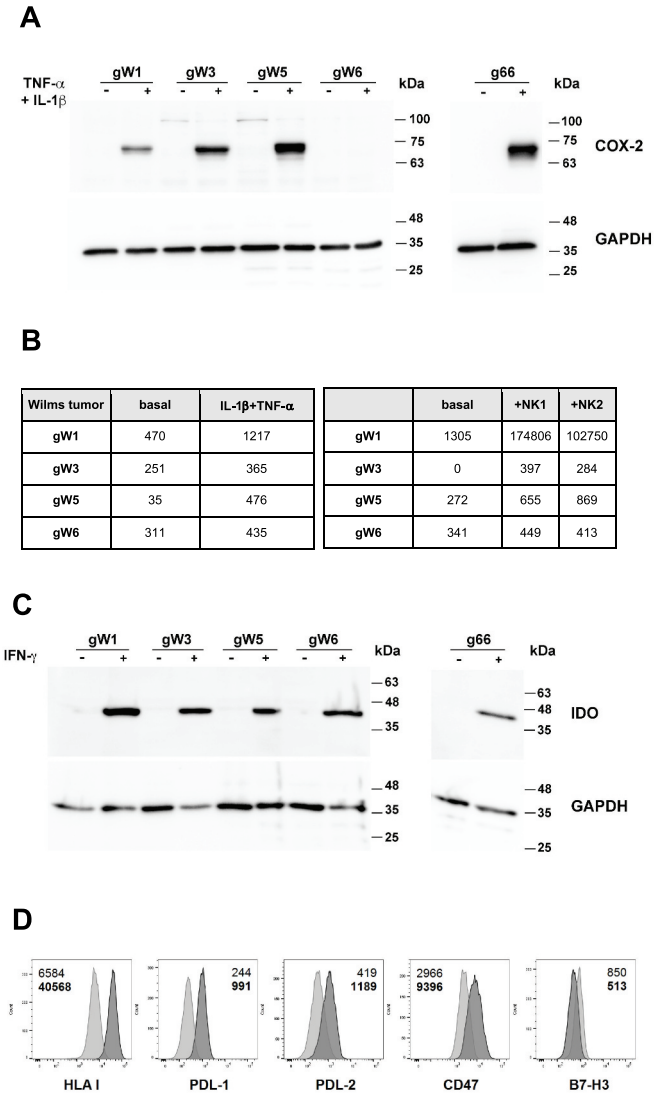
Figure 4. Str-WT cells inhibit IL-2-induced proliferation and phenotype of NK cells. (a) Freshly isolated NK cells were cultured with IL-2 in the absence or in the presence of str-WT, WT cell lines, or MSC at a 10:1 NK-to-WT ratio. NK cell proliferation was analyzed after 5-day culture by EdU incorporation method. Data are expressed as percentage of EdU+ NK cells. Bars represent mean ± SD of independent experiments performed analyzing the effect of the four str-WT cells, three WT cell lines, or three MSC on at least three different NK cell populations. (b) Representative experiment showing the inhibitory effect of gW3 on NK cell proliferation at different NK/WT ratios. (c) IL-2-activated NK cells were cultured for 6 days alone (gray bars) or in the presence of str-WT cells (black bars) and analyzed by flow cytometry for the expression of the various activating NK receptors. Results are represented as mean ± SD of S.I. data obtained from independent experiments analyzing the effect of the four str-WT cells on at least 2 NK cell populations. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. (d) A representative experiment showing the capability of gW6 str-WT cells to modulate receptor expression on NK cells. Histograms represent marker expression. Upper numbers represent the percentage of positive cells, while lower numbers indicate MFI.

We could obtain the in vitro selective expansion of WT cells of stromal type by the use of an MSC-specific medium that promoted the preferential proliferation of str-WT cells. As previously reported, [8] these tumor cells showed the surface and intracellular phenotype typical of the mesenchymal lineage (Figure 1).

A more detailed phenotypic analysis revealed that str-WT cells expressed different ligands for activating and inhibitory receptors known to regulate the effector responses of NK cells (Table 2 and Figure 2 and S3). In particular, tumor cells expressed the ligands of NKG2D, with predominant expression of MICA and ULBP2, and the ligands of DNAM-1, namely, PVR and Nectin-2. In addition, the availability in our laboratory of the soluble forms of NKp46, NKp30, and NKp44 receptors allowed the indirect detection of the NCR-specific ligands on the surface of str-WT cells, that showed a variable expression of these molecules. Tumor cells also expressed HLA-class I molecules, as well as PD-L1, PD-L2, and B7-H3, ligands of immune checkpoint inhibitory receptors.

The expression of different molecules known to be involved in the interaction with NK cells and regulation of NK effector functions was suggestive of a possible cytotoxic effect exerted by NK lymphocytes toward tumor cells. As expected, short-term IL-2-activated NK cells showed efficient degranulation and IFN-γ and TNF-α production following interaction with all str-WT cells, indicating that activating receptor-ligand interactions prevailed on inhibitory signals. MAb-mediated masking of HLA-class I molecules did not increase NK cell degranulation toward str-WT cells, whereas it had a significant effect in the case of the three long-term cultured WT cell lines (Figure 3). A possible explanation may be related to the concomitant expression in these cell lines of high levels of inhibitory HLA-class I molecules and low levels of ligands of activating NK receptors (DNAM-1 and NKG2D) (Table 2). Accordingly, the removal of the main inhibitory signals could unlock the activating pathways triggered by NK cell activating receptor engagement. On the other hand, it can be speculated that in str-WT cells the efficient triggering of activating receptors could prevail on the inhibitory HLA-class I-mediated inhibitory signals, thus leading to high percentages of degranulating NK cells. The demonstration that activated NK cells can degranulate upon interaction with str-WT cells adds novel information about the important role that these effectors may play in the immune response against WT. Notably, it has been recently shown that NK cells are present in WT tissues in a significantly high percentage, [10] suggesting that NK cells can be recruited toward WT sites where they can mediate anti-tumor response.

Another relevant finding of our study is the immunosuppressive effect that str-WT cells exert on NK cells. This capability represents an additional feature of WT cells of stromal type, which is highly reminiscent of MSC characteristics. The MSC-like immunomodulatory activity was observed for all tumor populations tested, although with some variability



Wilms tumor	basal	IL-1β+TNF-α
gW1	470	1217
gW3	251	365
gW5	35	476
gW6	311	435

	basal	+NK1	+NK2
gW1	1305	174806	102750
gW3	0	397	284
gW5	272	655	869
gW6	341	449	413

Figure 5. Str-WT cells up-regulate inhibitory factors on exposure to inflammatory cytokines. (a) Western blot analysis of COX-2 expression in str-WT cells, and in g66 MSC, either unstimulated or stimulated with IL-1β + TNF-α for 48 h. GAPDH expression was analyzed as positive control. Molecular weight (MW) markers (kDa) are indicated on the right. A representative experiment of three is shown. (b) Production of PGE2 by str-WT cells cultured alone (basal), stimulated by IL-1β + TNF-α, or co-cultured with NK cells derived from two different healthy donors (NK1 and NK2). Culture supernatants were collected after 48 h (for cytokine stimulation experiments) or after 72 h (for co-culture experiments) and analyzed by ELISA assay. Data refer to the levels of PGE2 expressed as pg/ml. (c) Western blot analysis of IDO expression in str-WT cells and in g66 MSC, either unstimulated or stimulated with IFN-γ for 48 h. GAPDH expression was analyzed as positive control. Molecular weight (MW) markers (kDa) are indicated on the right. A representative experiment of two is shown. (d) gW1 cells were stimulated with IFN-γ and analyzed by flow cytometry for the expression of HLA-class I and the indicated immune checkpoint molecules. Light and dark gray histograms represent unstimulated and IFN-γ-astimulated cells, respectively. Upper numbers represent MFI of unstimulated cells, while bold lower numbers refer to MFI of stimulated cells.

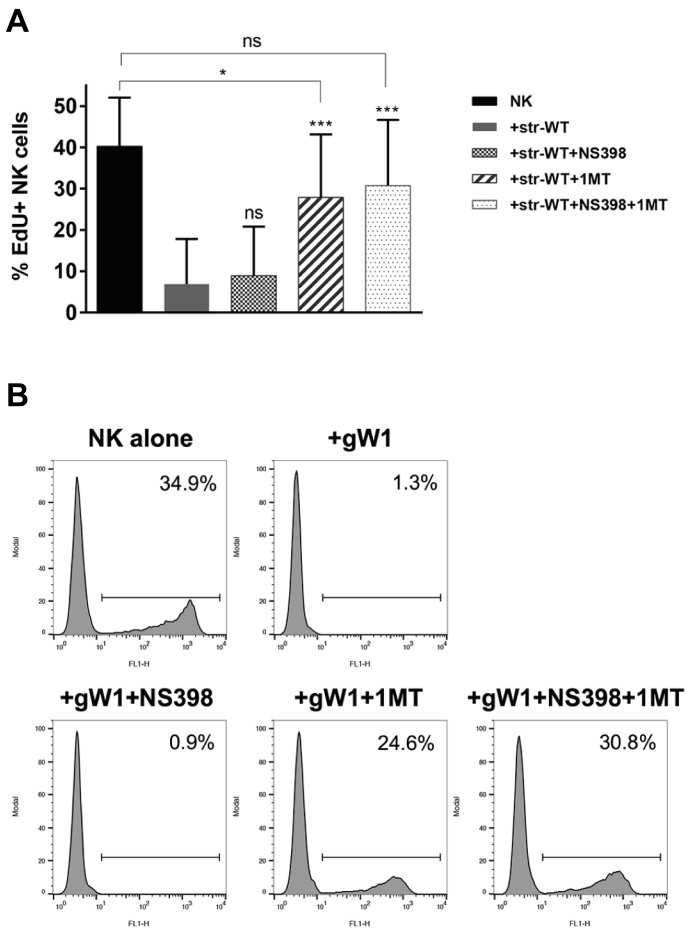


Figure 6. Role of PGE2 and IDO in the str-WT cell-mediated inhibition of NK-cell proliferation. (a) Freshly isolated NK cells were cultured with IL-2 in the absence or in the presence of str-WT cells, at a 10:1 NK-to-str-WT cell ratio, with or without the addition of the PGE2 synthesis inhibitor NS-398 or the IDO inhibitor 1-M-Trp, used singularly or in combination. NK cell proliferation was analyzed after 5-day culture by EdU incorporation method. Data are expressed as percentage of EdU+ NK cells. Bars represent mean ± SD of 13 independent experiments performed analyzing the effect of the four str-WT cells on at least three NK cell populations derived from different donors. * $p < .05$, *** $p < .001$ (NK+str-WT vs NK+str-WT +1MT or str-WT+NS-398 + 1MT). (b) Representative experiment showing the inhibitory effect of gW1 on NK cell proliferation under the different culture conditions.

(Figures 4a and 4b). The degree of inhibition was dependent on the NK-to-str-WT cell ratio and on the direct contact between the two cell types (Figure S4). Moreover, it affected not only NK cell proliferation but also the acquisition of the "activated" phenotype induced by immunostimulatory cytokines such as IL-2 (Figures 4c and 4d). Notably, an impaired up-regulation of activating NK receptors was also observed, and was restricted to those molecules whose expression is known to be increased or *de novo* induced by IL-2 stimulation. Overall, these data indicate that str-WT cells strongly affect the cytokine-induced activation of NK cells.

The mechanisms underlying the immunomodulatory effect of MSC have been demonstrated to be dependent on an inflammatory microenvironment.[34] Indeed, similar mechanisms could be involved in the inhibitory activity exerted by str-WT cells. The interaction between tumor and NK cells may induce the release by NK cells of inflammatory mediators, which in turn activate in tumor cells the production of inhibitory factors. These include PGE2, a soluble factor that is produced through the activity of the constitutive COX-1 and inducible COX-2 enzymes.[44] COX-2 expression has been reported in many tumor tissues, including WT, [11] as a marker of an inflammatory microenvironment. Thus, it represented a major candidate as a mediator of the str-WT cell-induced inhibitory effect on NK cells. Our results revealed that, except for gW6, COX-2 expression could be induced in str-WT cells

upon stimulation by IL-1β and TNF-α (Figure 5a and S5A). Accordingly, PGE2 levels in the supernatants of tumor cells cultured without cytokines were usually low, while they were significantly increased in the supernatants of tumor cells stimulated by IL-1β and TNF-α or cultured with NK cells (Figure 5b). It has also to be considered that PGE2 production in str-WT cells may be partially ascribed to COX-1 activity, especially under basal conditions.

We also focused on IDO enzyme as a possible major player in the inhibition of NK cell activation.[48] As for COX-2, IDO expression, which is generally inducible, was weakly positive in some str-WT cells but it was significantly induced in all WT populations by the stimulation with IFN-γ (Figure 5c and S5B).

The definitive proof of PGE2 and IDO involvement in the inhibition of NK cell proliferation was given by co-culture experiments in which COX-2 and IDO inhibitors were used to counteract the str-WT cell-mediated inhibitory effect, thus restoring NK cell proliferation (Figure 6). These data are in line with previous results reported by Spaggiari et al. for MSCs, that accounted for a major role of IDO in the MSC-mediated inhibition of NK cell proliferation and only a synergistic effect of PGE2.[35] Notably, the required initial physical interaction between NK lymphocytes and tumor cells would promote cytokine production by NK cells that in turn induce COX-2 and IDO expression. Altogether, these findings demonstrate that str-WT cells can mediate their immunosuppressive effect through the production of soluble factors that are induced by an inflammatory microenvironment. Moreover, it must be considered that these factors are involved in the inhibition not only of NK cells, but also of other important effectors of anti-tumor response, including T cells and macrophages, that have been detected in the WT microenvironment, mostly confined in the stromal component of the tumor.[10–12] Thus, the use of novel therapies interfering with COX-2 and IDO activity may reveal a good strategy to unlock the immune response against WT.

An innovative immunotherapeutic approach is the use of immune checkpoint inhibitors. The blockade of the PD-1/PD-L1 and -L2 interactions is currently applied in different tumors, and additional molecular targets are now being considered that include B7-H3 and CD47.[30,47] Our analysis has shown that str-WT cell populations express PD-L1, PD-L2, B7-H3, and CD47 molecules and that inflammatory stimuli, such as IFN-γ, can up-regulate the expression of most of these inhibitory ligands (Figure 5d). Thus, checkpoint blockade immunotherapy may represent a novel therapeutic approach, which may reveal effectiveness in unlocking the immune response also against WT.

In conclusion, our study reveals novel aspects of the phenotype and function of stromal-type WT cells and sheds new light on the cellular interactions which, in the tumor microenvironment, WT cells may establish with NK cells and, possibly, with other cells of the immune system. In this context, it must be considered that the outcome of the interaction between NK lymphocytes and str-WT cells is not *a priori* predictable, and it may depend on several conditions/factors, including the activation state of NK cells and the presence of an inflammatory microenvironment. Additional therapeutic strategies for WT are strongly needed, since some patients do not respond to conventional treatments or undergo disease relapse. Moreover, a proportion of patients experiences early and/or late treatment-related adverse events. Our results may contribute to the design of novel therapeutic strategies that could represent an alternative, effective therapeutic option for the successful treatment of WT.

Notes on contributions

C.C. and G.M.S. designed and performed the experiments and wrote the manuscript; M.S. performed most of the experiments; E.P. performed experiments and analyzed data; B.A., L.M., G.C., and G.M.G. critically revised the manuscript; A.R.S. examined the tumor samples and is responsible for the histological diagnoses of the cases; A.R.S. and L.A.N. provided tissue samples for the isolation of WT cells and MSC, respectively; C. B. analyzed data and critically revised the manuscript.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Declaration of interest statement

The Authors declare no competing interests.

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