

# Human Neuroblastoma Cells Trigger an Immunosuppressive Program in Monocytes by Stimulating Soluble HLA-G Release

Fabio Morandi,<sup>1</sup> Isabella Levreri,<sup>2</sup> Paola Bocca,<sup>1</sup> Barbara Galleni,<sup>3</sup> Lizzia Raffaghello,<sup>1</sup> Soldano Ferrone,<sup>4</sup> Ignazia Prigione,<sup>1</sup> and Vito Pistoia<sup>1</sup>

<sup>1</sup>Laboratory of Oncology, <sup>2</sup>Clinical Pathology, and <sup>3</sup>Data Handling, Neuroblastoma, G. Gaslini Children's Hospital, Genoa, Italy and <sup>4</sup>Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York

## Abstract

**HLA-G is overexpressed in different tumors and plays a role in immune escape. Because no information is available on HLA-G in relation to human neuroblastoma, we have investigated the expression of membrane-bound and secretion of soluble isoforms of HLA-G in neuroblastoma and functionally characterized their immunosuppressive activities. At diagnosis, serum soluble HLA-G (sHLA-G) levels were significantly higher in patients than in age-matched healthy subjects. In addition, patients who subsequently relapsed exhibited higher sHLA-G levels than those who remained in remission. Neuroblastoma patient sera selected according to high sHLA-G concentrations inhibited natural killer (NK) cell and CTL-mediated neuroblastoma cell lysis. Such lysis was partially restored by serum depletion of sHLA-G. In 6 of 12 human neuroblastoma cell lines, low HLA-G surface expression was not up-regulated by IFN- $\gamma$ . Only the ACN cell line secreted constitutively sHLA-G. IFN- $\gamma$  induced *de novo* sHLA-G secretion by LAN-5 and SHSY5Y cells and enhanced that by ACN cells. Primary tumor lesions from neuroblastoma patients tested negative for HLA-G. Neuroblastoma patients displayed a higher number of sHLA-G-secreting monocytes than healthy controls. Incubation of monocytes from normal donors with IFN- $\gamma$  or pooled neuroblastoma cell line supernatants significantly increased the proportion of sHLA-G-secreting cells. In addition, tumor cell supernatants up-regulated monocyte expression of CD68, HLA-DR, CD69, and CD71 and down-regulated IL-12 production. Our conclusions are the following: (a) sHLA-G serum levels are increased in neuroblastoma patients and correlate with relapse, (b) sHLA-G is secreted by monocytes activated by tumor cells rather than by tumor cells themselves, and (c) sHLA-G dampens anti-neuroblastoma immune responses. [Cancer Res 2007;67(13):6433–41]**

## Introduction

Neuroblastoma, a tumor originating from the sympathetic nervous system, is the most common extracranial malignancy in childhood. Whereas stage I or II tumors are localized and well differentiated and can usually be cured by surgical resection only, patients with stage III or IV tumors present regional or disseminated disease, respectively, often characterized by relapse after response to conventional treatments and poor prognosis (1).

The search for novel therapeutic strategies for neuroblastoma is warranted. Among them, immunotherapy has attracted interest,

but its success may be limited by several mechanism adopted by neuroblastoma cells to evade the control of the immune system (2–14).

HLA-G is a nonclassic MHC class I molecule (class Ib) with immunotolerant properties. It is structurally related to classic MHC class Ia (HLA-A, HLA-B, HLA-C), but at variance with the latter molecules, HLA-G shows a limited polymorphism, with only seven different isoforms encoded by alternative splicing of the same mRNA, that include membrane-bound HLA-G1, HLA-G2, HLA-G3, and HLA-G4 and soluble secreted HLA-G5, HLA-G6, and HLA-G7.

Membrane-bound HLA-G isoforms interact with the inhibitory receptors KIR2D4L (killer inhibitory receptor) and ILT2 [immunoglobulin (Ig)-like transcript 2] on natural killer (NK), B, and T cells, and ILT4 (Ig-like transcript 4) on DC and macrophages (15, 16), impairing host immune response.

The soluble HLA-G (sHLA-G) isoforms can also inhibit NK and CTL function by inducing apoptosis through CD8 ligation (17) or by enhancing the expression of Fas ligand on cytotoxic effectors and triggering Fas/Fas ligand mediated cell death (18).

The physiologic role of HLA-G is to establish immune tolerance at the maternal-fetal interface, abrogating the activity of maternal NK cells against fetal tissue (19). In normal tissues, HLA-G shows a limited distribution, being detected only on cytotrophoblast cells (20), thymic epithelial cells (21), cytokine-activated monocytes (22), mature myeloid and plasmacytoid dendritic cells (23), and inflamed muscle fibers (24).

It has been shown that HLA-G can be overexpressed in many human tumors, like glioma (25), melanoma (26), different lymphoproliferative disorders (B and T non-Hodgkin lymphoma, B-chronic lymphocytic leukemia, B and T acute lymphocytic leukemia; refs. 27, 28), ovarian, breast (29), renal (30), and lung (31) carcinoma, providing them with an immune-escape mechanism (32).

Moreover, patients with melanoma, breast cancer, ovarian cancer, and glioma show high levels of sHLA-G in serum (33) and in malignant ascites (34), pointing to possible correlations between the secretion of this molecule and disease progression.

Because elucidation of novel immune escape mechanisms may help design more efficacious immunotherapeutic protocols, we have investigated the expression of membrane-bound and secretion of soluble isoforms of HLA-G in neuroblastoma patients and functionally characterized their immunosuppressive activities.

## Materials and Methods

**Patients.** Sera were obtained at diagnosis from 53 patients with neuroblastoma at different stages, namely, 10 stage 1, 5 stage 2, 10 stage 3, 20 stage 4, and 8 stage 4s, according to the International Neuroblastoma Staging System (1). All patients were untreated at study. Controls were sera from 53 age-matched healthy individuals. All serum samples were obtained from the serum bank of the Clinical Pathology Laboratory, G. Gaslini Institute, Genoa.

**Requests for reprints:** Fabio Morandi, Laboratory of Oncology, G. Gaslini Institute, Largo G. Gaslini 5, 16148 Genoa, Italy. Phone: 39-10-5636342; Fax: 39-10-3779820; E-mail: fabiomorandi@ospedale-gaslini.ge.it.

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Monocytes were purified from heparinized peripheral blood (PB) samples of 10 neuroblastoma patients and 10 healthy age-matched controls upon informed consent of the legal guardians using a 50.6% PERCOLL (Pharmacia) density gradient as published (35). The purity of monocytes was evaluated by flow cytometry using a CD14 monoclonal antibody (mAb; see below) and ranged from 95% to 98%.

Dendritic cells were generated from PB monocytes cultured for 5 days in X-VIVO medium (Cambrex Bio Science Verviers) with 50 ng/mL rhIL-4 (Immunotools) and 200 units/mL rhGM-CSF (PeproTech).

Macrophages were generated from PB monocytes cultured for 6 days in RPMI (Euroclone) with 10% human serum.

**Cell lines.** The human neuroblastoma cell lines GI-CA-N, HTLA 230, ACN, GI-LI-N, GI-ME-N, IMR-32, SH-SY-5Y, LA-N-1, LA-N-5, and SK-N-SH were cultured in 75-cm<sup>3</sup> plastic flasks (Corning Incorporated) using DMEM (Euroclone) supplemented with 10% fetal bovine serum (FBS; GIBCO), 1% glutamine, 2% HEPES, and penicillin (100 IU/mL)/streptomycin (100 µg/mL; Cambrex Bio Science Verviers).

In some experiments, neuroblastoma cell lines were treated for 48 h with 1,000 units/mL of rhIFN-γ (Imuchin, Boehringer Ingelheim Italia) as described (11) before undergoing functional studies.

Supernatants from neuroblastoma cell lines grown at confluence were collected after 24 or 72 h culture.

The human lymphoblastoid cell line 721.221.G1 (kindly provided by Dr. Francesco Puppo, DIMI, Genoa) was obtained from the 721.221 parental cell line after transfection with human HLA-G1 cDNA and cultured in the same medium described above (36).

**ELISA.** Serum sHLA-G concentration was determined using a specific ELISA kit (Exbio). Briefly, a mouse mAb specific for human sHLA-G1 and sHLA-G5 (MEM-G/9) was used as capture reagent and a horseradish peroxidase (HRP)-conjugated rabbit polyclonal anti-human β<sub>2</sub> microglobulin antibody was used as detection reagent.

The lowest threshold of this assay was 1.6 ng/mL sHLA-G. Each sample was tested in duplicate.

Serum levels of intercellular adhesion molecule-1 (ICAM-1) and HLA class I and class II were detected using Maxisorp loose Nunc Immuno-module (Nunc A/S), coated with the following monoclonal antibodies: VF27-516 (capture) and CL203.4 (probe) for ICAM-1; TP25.99 (capture) and NAMB-1 (probe) for HLA-class I; and LGII-612.14 (capture) and Q5-13 (probe) for HLA-class II (37–40).

Probes were conjugated with HRP using HRP conjugation kit (Alpha Diagnostic). Calibration curves were done using total extract from peripheral blood mononuclear cells (PBMC) from healthy donors for ICAM-1 and HLA-class I, and total extract from RAJI cell line for HLA-class II, using arbitrary units.

Optical densities were quantified using a microtiter plate reader (SpectraFluor Plus).

**Flow cytometry.** A total of  $5 \times 10^5$  cells were incubated with 0.5 µg of the HLA-G specific mAb MEM-G/9 (Exbio) or with 0.5 µg of an irrelevant mouse IgG1 antibody (Serotec) as negative control for 30 min at 4°C.

After washing with staining buffer (PBS 1% FBS), cells were incubated with PE-conjugated goat anti-mouse IgG1 antibody (Serotec) for 30 min at 4°C.

Cytofluorimetric analysis of monocytes was done using the following antibodies: anti-CD14 PE, anti-CD68 FITC (Caltag), anti-CD69 PC5 (Beckman Coulter) anti-CD71 FITC, anti-HLA DR FITC, and anti-CD86 PE (Becton Dickinson). Cells were treated with specific antibodies or irrelevant isotype-matched controls (Serotec) for 30 min at 4°C. After final washing, cells were resuspended in PBS 1% FBS and analyzed using a FACScalibur flow cytometer (Becton Dickinson).

Intracellular staining was done as described (41) using anti-IL-12 FITC mAb (Diacclone) or anti-IL-10 PE mAb (Becton Dickinson) or irrelevant isotype-matched controls (Serotec).

Mean relative of fluorescence intensity (MRFI) was obtained by the following formula: (specific mean fluorescence intensity)/(isotype control mean fluorescence intensity).

**sHLA-G-specific ELISPOT.** ELISPOT assay for sHLA-G was done using MAIP54510 Multiscreen-IP Millipore plates (Millipore) coated overnight at

4°C with 70 µL per well of capture mAb MEM-G/9 (Exbio, 7 µg/mL in PBS) and then washed three times with PBS.

Plates were blocked with 150 µL of RPMI supplemented with 3% of bovine serum albumin (BSA; Sigma-Aldrich) for 2 h at 37°C.

Cells were resuspended in X-VIVO medium (Cambrex) and then seeded at the concentration of 5,000 cells per well for neuroblastoma cell lines and monocytes, and 2,500 cells per well for positive control (721.221.G1 cell line). In some experiments, monocytes were pretreated overnight with medium alone or pooled supernatants from four neuroblastoma cell lines (10–50% v/v) that were tested as such or following pretreatment for 2 h at room temperature (RT) with anti-IL-10 (19F1 mAb, 30 µg/mL), anti-transforming growth factor-β (TGF-β; 1D11 mAb, 10 µg/mL) or anti-GD2 (14G.2A, 20 µg/mL; ref. 42) neutralizing antibodies. Anti-IL-10 and anti-TGF-β mAbs were kindly donated by Dr. Franca Gerosa (University of Verona, Italy).

After 24 h incubation at 37°C in a moist atmosphere containing 5% CO<sub>2</sub>, cells were discarded. Plates were washed six times with PBS/Tween 0.05% (PBST) and then incubated with rabbit polyclonal anti-β<sub>2</sub> microglobulin antibodies (Sigma, 20 µg/mL in PBS 0.5% BSA) for 1 h at RT.

After another washing step (six times with PBST), plates were incubated with a dextran polymer (DakoCytomation) conjugated with goat-anti-mouse and anti-rabbit IgG, and alkaline phosphatase, to enhance sHLA-G detection. The polymer was diluted 1:5 in PBS 0.5% BSA and 10% mouse serum. After 30 min of incubation at RT, plates were washed six times with PBST, and then the assay was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Promega) for 3 min in the dark and then stopped under flowing tap water. sHLA-G spots were counted using an ELISPOT reader (BIO-READER 2000, Biosys).

**Cytotoxicity assay.** PBMC were isolated by Ficoll-Hypaque density gradient centrifugation from healthy donors following informed consent. Next, PBMC were depleted of T cells (by rosetting with neuraminidase-treated sheep RBC) and monocytes (by PERCOLL 50.6% density gradient centrifugation) and then were cultured in RPMI 10% FBS (GIBCO) with 500 units/mL of rhIL-2 (Proleukin, Chiron Corporation).

Neuroblastoma-specific CTL were generated from an HLA-A1<sup>+</sup> healthy donor using autologous mature monocyte-derived DC transfected with mRNA extracted from neuroblastoma cell lines, as previously described (43).

Sera from four neuroblastoma patients (with high concentration of sHLA-G) were depleted of sHLA-G by incubation with anti-mouse IgG Dynabeads (Invitrogen), coated with anti-HLA-G-specific antibody MEM-G/9 (Exbio) for 1 h at 4°C following magnetic separation.

Cytotoxic effectors were incubated for 30 min at 4°C with medium alone or recombinant sHLA-G or neuroblastoma patients sera before and after depletion of sHLA-G. Cells were then washed and used as effector cells in a standard 4 h <sup>51</sup>Cr release assay, using two different HLA-A1<sup>+</sup> neuroblastoma cell lines (ACN and SH-SH-5Y) as targets.

To test CTL lysis, neuroblastoma cell lines were pretreated with IFN-γ (1,000 units/mL) for 48 h to induce or enhance HLA-class I expression. Specific lysis was determined according to the formula: % specific lysis = cpm (sample – spontaneous)/cpm (total – spontaneous) × 100.

**Immunohistochemical staining of tissues.** Immunohistochemical staining of tissue sections was done using the Envision System HRP mouse (Dako). Briefly, 5-µm-thick sections were cut from formalin-fixed, paraffin-embedded blocks, deparaffinized with xylene, and rehydrated by passages through decreasing concentrations of ethanol (from 100% to 80%). Endogenous peroxidase activity was blocked by a 30-min incubation at RT with methanol containing 3% H<sub>2</sub>O<sub>2</sub>. Tissue sections were then incubated at 98°C for 40 min in citrate buffer (pH 6.0) for antigen retrieval (ChemMate, Dako). After rinsing in Optimax Wash Buffer (Menarini Diagnostics), tissue sections were incubated overnight at 4°C with optimal amounts of anti-HLA-G (MEM-G/9, Exbio) or isotype control (mouse IgG<sub>1</sub>, Caltag). Tissue sections were washed twice in Optimax Wash Buffer and incubated for 30 min at RT with Dako Envision System horseradish peroxidase mouse. After washing in Optimax Wash Buffer, peroxidase activity was detected by incubating tissue sections for 6 to 10 min at RT with Dako Liquid DAB Substrate Chromogen System (Dako). Tissue sections were counterstained with Mayer's hematoxylin (Sigma).

**Statistics.** Data were analyzed by PRISM 3.0 (Graphpad software) using two-tailed Wilcoxon ranked test. *P* values <0.01 or <0.05 were considered as significant.

## Results

**Serum levels of sHLA-G in neuroblastoma patients.** As shown in Fig. 1A, sHLA-G serum levels in neuroblastoma patients at diagnosis ( $n = 53$ ;  $26.1 \pm 6.97$  ng/mL) were significantly higher ( $P = 0.0003$ ) than those detected in sera from age-matched healthy subjects ( $n = 53$ ;  $4.409 \pm 0.808$  ng/mL).

Next, we analyzed serum sHLA-G concentrations according to the disease stage, but we did not find significant differences among these groups (stage 1,  $11.33 \pm 3.49$  ng/mL; stage 2,  $14.18 \pm 4.44$  ng/mL; stage 3,  $30.26 \pm 26.04$  ng/mL; stage 4,  $17.29 \pm 6.46$  ng/mL; stage 4s,  $71.95 \pm 29.38$  ng/mL). Stage 4s neuroblastoma patients showed a trend to higher serum sHLA-G levels than those detected in other groups, but the difference was not significant.

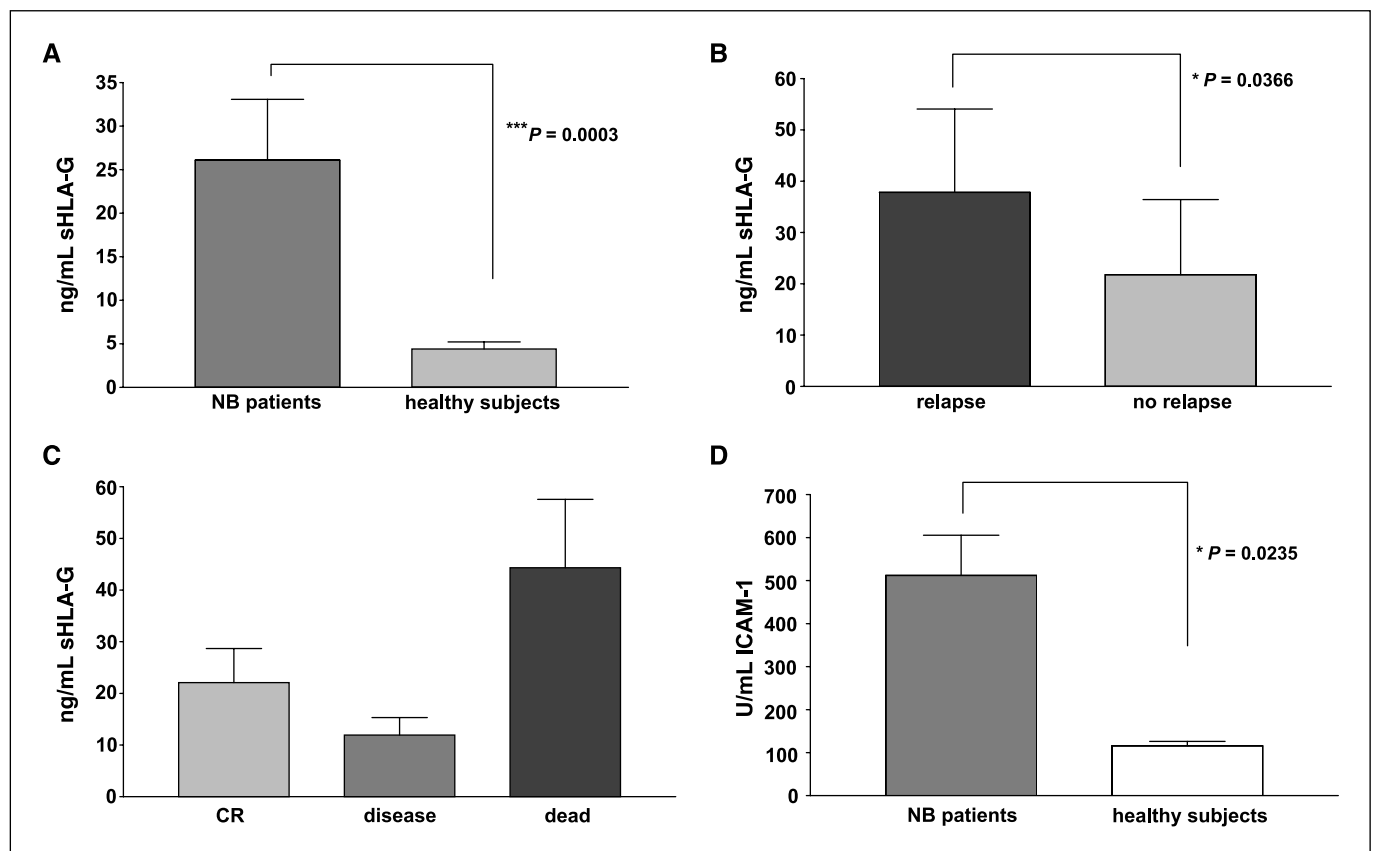
Correlations between serum sHLA-G concentrations and patient clinical features over a 3- to 6-year follow-up were subsequently analyzed. As shown in Fig. 1B, we found significantly higher levels of sHLA-G in sera from patients who developed local or disseminated relapse ( $36.51 \pm 10.84$  ng/mL) than in those who remained in remission ( $16.93 \pm 4.66$  ng/mL;  $P = 0.0366$ ).

No significant differences in sHLA-G serum levels was detected between patients with poor clinical outcome (dead,  $44.33 \pm 13.22$  ng/mL; alive with disease,  $11.92 \pm 3.4$  ng/mL) and patients in complete remission ( $22.10 \pm 6.59$  ng/mL; Fig. 1C). sHLA-G serum levels were higher in patients who died than in those alive with disease (Fig. 1C), but the difference was not significant.

Serum levels of sHLA class I and II, tested as controls, were similar in 15 neuroblastoma patients (sHLA-I,  $597.2 \pm 118.6$  units/mL; sHLA-II,  $45.20 \pm 2.03$  units/mL) and in 15 age-matched healthy controls (sHLA-I,  $498 \pm 145.2$  units/mL; sHLA-II,  $45.17 \pm 3.73$  units/mL).

No correlations were found between serum levels of sHLA-G and those of neuron-specific enolase or urinary concentrations of vanilmandelic acid and homovanillic acid. The latter three molecules are routinely tested as diagnostic markers in neuroblastoma (44, 45).

Serum levels of ICAM-1, which is involved in tumor immune escape via inhibition of cytotoxic effectors (46), were finally tested in the attempt of establishing correlations with serum concentrations of sHLA-G, which dampens antitumor immune responses using similar mechanisms (15, 16). Although ICAM-1 serum levels were significantly higher ( $P = 0.0235$ ) in neuroblastoma patients ( $512.1 \pm 93.24$  units/mL) than in age-matched healthy controls ( $116.1 \pm 10.12$  units/mL; Fig. 1D), no correlation was found with sHLA-G serum levels.



**Figure 1.** Serum sHLA-G levels in neuroblastoma (NB) patients and correlation with disease relapse. **A**, sHLA-G concentrations in sera from 53 neuroblastoma patients (mean,  $26.10 \pm 6.97$ ) were significantly higher ( $P = 0.0003$ ) than those detected in sera from 53 age-matched healthy controls (mean,  $4.409 \pm 0.808$ ). **B**, sHLA-G concentrations were tested in sera from 53 neuroblastoma patients, of whom 21 subsequently developed local/systemic relapse ( $36.51 \pm 10.84$  ng/mL), and 32 remained in remission ( $16.93 \pm 4.66$  ng/mL). A significant difference was found between these two groups, with a higher sHLA-G concentration in the former ( $P = 0.0366$ ). **C**, sHLA-G concentrations were detected in sera from 53 neuroblastoma patients, of whom 21 subsequently remained in complete remission (CR,  $22.10 \pm 6.59$  ng/mL), 13 survived with disease ( $11.92 \pm 3.4$  ng/mL), and 19 died ( $44.33 \pm 13.22$  ng/mL). No significant differences were found among these groups, but the last group displayed a trend to higher sHLA-G concentrations than the other groups. **D**, ICAM-1 serum levels, tested in 15 neuroblastoma patients ( $512.1 \pm 93.24$  units/mL) and 15 healthy age-matched controls ( $116.1 \pm 10.12$  units/mL), were significantly higher in the former than in the latter group ( $P = 0.0235$ ).

**Table 1.** Expression of surface HLA-G (HLA-G) and secretion of sHLA-G in 12 human neuroblastoma cell lines

Cell line	1p alteration		N-myc ampl.		N-myc s.c.	Chr 17	HLA-G			
	Del	Tr	HSR	DM			Surface		Soluble	
							Basal	IFN- $\gamma$ pretreated	Basal	IFN- $\gamma$ pretreated
IMR-32	+	-	+	+	-		+	+	-	-
SK-N-SH	-	-	-	-	-		-	-	-	-
SK-N-BE 2	+	+	+	-	+	t (3;17); (p21;q21)	+	+	-	-
HTLA 230			+	+			++	++	-	-
GI-ME-N	+		-		+		-	-	-	-
GI-LI-N	+						++	++	-	-
GI-CA-N	-		-		+		-	+	-	-
LA-N-1	+		+				+	+	-	-
LA-N-5	+		+				+	+	-	++
SHSY5Y+	-	-	-	-	+		-	-	-	++
SHSY5Y-	-	-	-	-	+		-	-	-	+++
ACN							-	-	+	++

NOTE: For surface HLA-G expression, a score was given on the basis of MRFI: - (MRFI, < 2), + (MRFI range, 2-2.5), ++ (MRFI, >2.5). For sHLA-G, data are expressed as number of spots/5,000 cells. A score was given on the basis of the number of sHLA-G spots counted: - (spots, <60); + (spots range, 60-120); ++ (spots range, 150-300); +++ (spots, >300). Cytogenetic and molecular features of these cell lines are also shown.

### Expression of HLA-G in human neuroblastoma cell lines.

Expression of surface membrane-bound HLA-G was next investigated by flow cytometry in 12 neuroblastoma cell lines with different cytogenetic and molecular features that are summarized in Table 1.

As shown in Fig. 2A, the baseline expression of HLA-G was variable, with five neuroblastoma cell lines (SK-N-SH, GI-ME-N, GI-CA-N, SH-SY-5Y, and ACN) virtually lacking HLA-G (MRFI < 2) and six neuroblastoma cell lines (IMR-32, SK-N-BE 2, HTLA 230, GI-LI-N, LA-N-1, and LA-N-5) showing low to moderate expression (MRFI, 2-3.5). Surface HLA-G tended to be more expressed in neuroblastoma cell lines with *MYCN* amplification (both HSR or DM) than in those with single-copy *MYCN* (Table 1), in the absence of statistical significance.

In contrast with classic HLA-class I molecules (9), 48 h treatment with IFN- $\gamma$  did not enhance HLA-G expression in neuroblastoma cell lines (47).

Next, we investigated by ELISPOT assay the secretion of sHLA-G in the same panel of neuroblastoma cell lines, and a score was given on the basis of the number of sHLA-G spots counted (Table 1). As shown in Fig. 2B, only the ACN cell line contained a low number of sHLA-G-secreting cells in baseline conditions (52 spots/5,000 cells), whereas the other cell lines were completely negative.

After overnight treatment with IFN- $\gamma$ , the LA-N-5, SH-SY-5Y, and ACN neuroblastoma cell lines were found to contain sHLA-G-secreting cells (150, 407, and 266.5 spots/5,000 cells, respectively). In contrast, under the same experimental conditions, the other cell lines were virtually devoid of sHLA-G-secreting cells (range, 0-40 spots; Fig. 2B).

**HLA-G expression in primary neuroblastoma lesions.** HLA-G expression was next evaluated by immunohistochemistry in formalin-fixed, paraffin-embedded tissue sections from 12 newly diagnosed primary neuroblastoma, 5 Schwannian stroma-poor and 7 stroma-rich, more differentiated tumors. The former were composed of groups or nests of neuroblastic cells separated by

delicate, often incomplete stromal septa without Schwannian proliferation. Schwannian stroma-rich tumors were characterized by the presence of mature or maturing ganglion cells scattered in a background of highly developed Schwannian stroma (48). Whereas Schwannian stroma-rich histology is usually favorable, Schwannian stroma-poor histology may range from unfavorable to favorable depending on other associated prognostic factors (49).

Neuroblastoma tissue sections from all cases tested negative for HLA-G expression. One representative experiment is shown in Fig. 3A, whereas HLA-G staining of a trophoblast tissue section tested as positive control is shown in Fig. 3B. Figure 3C shows some HLA-G<sup>+</sup> fully differentiated ganglionic cells that were detected only in one stroma-rich neuroblastoma sample.

**Expression of HLA-G in monocytes from neuroblastoma patients.** The above results pointed to a discrepancy between the high levels of sHLA-G in sera from neuroblastoma patients and the low to absent HLA-G expression in primary tumor cells and in neuroblastoma cell lines. Therefore, subsequent experiments were done to investigate whether monocytes, which are a major source of sHLA-G (50), were responsible for the production of sHLA-G in neuroblastoma patients.

To this end, monocytes were purified from PBMC of 10 stage 4 neuroblastoma patients and 10 healthy donors; the number of sHLA-G-secreting cells was assessed by ELISPOT assay, and HLA-G surface expression was evaluated by flow cytometry, both in baseline condition and after overnight stimulation with IFN- $\gamma$  (1,000 units/mL).

No differences were found in monocyte HLA-G surface expression between neuroblastoma patients and age-matched healthy controls (MRFI,  $3.78 \pm 1.19$  and  $4.37 \pm 2.45$ , respectively), and IFN- $\gamma$  treatment did not increase HLA-G expression in either group.

Conversely, a higher number of monocytes secreting constitutively sHLA-G was detected in neuroblastoma patients ( $189.8 \pm 27.6$  spots per 5,000 cells) than in age-matched healthy subjects ( $50.95 \pm 7.09$  spots per 5,000 cells), and this difference was

statistically significant ( $P < 0.0001$ ). Overnight treatment with IFN- $\gamma$  significantly increased the number of sHLA-G-secreting monocytes from healthy controls ( $186 \pm 28.57$  spots per 5,000 cells;  $P = 0.0029$ ), but not neuroblastoma patients ( $238.9 \pm 38.49$  spots per 5,000 cells; Fig. 2C).

To investigate if the elevated production of sHLA-G by monocytes from neuroblastoma patients was related to cell activation, surface expression of CD69, a previously reported early activation marker (51, 52), was assessed by flow cytometry on freshly isolated peripheral blood monocytes from four neuroblastoma patients and four healthy controls. As shown in Fig. 2D, the percentage of CD69<sup>+</sup> monocytes was significantly higher in neuroblastoma patients (% of positive cells,  $88.3 \pm 1.32$ ) than in healthy controls (% of positive cells,  $2.2 \pm 0.11$ ;  $P = 0.0286$ ), thus confirming the initial hypothesis.

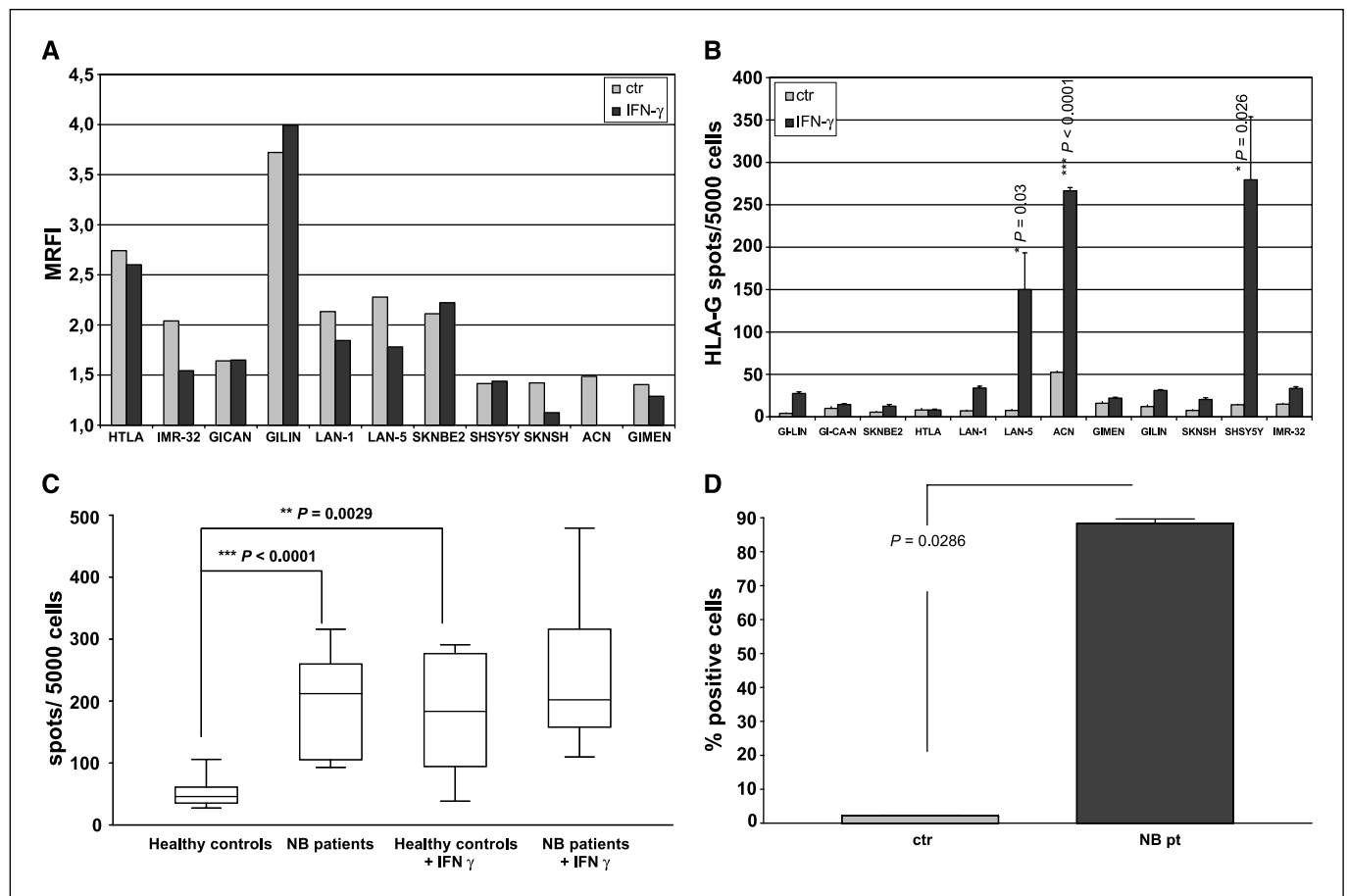
**Neuroblastoma cell line-derived soluble factors enhance sHLA-G secretion by monocytes from healthy donors.** To investigate the potential role of neuroblastoma cells in the induction

of sHLA-G secretion by monocytes from neuroblastoma patients, we tested the ability of supernatants from neuroblastoma cell lines to enhance HLA-G expression in monocytes from normal donors.

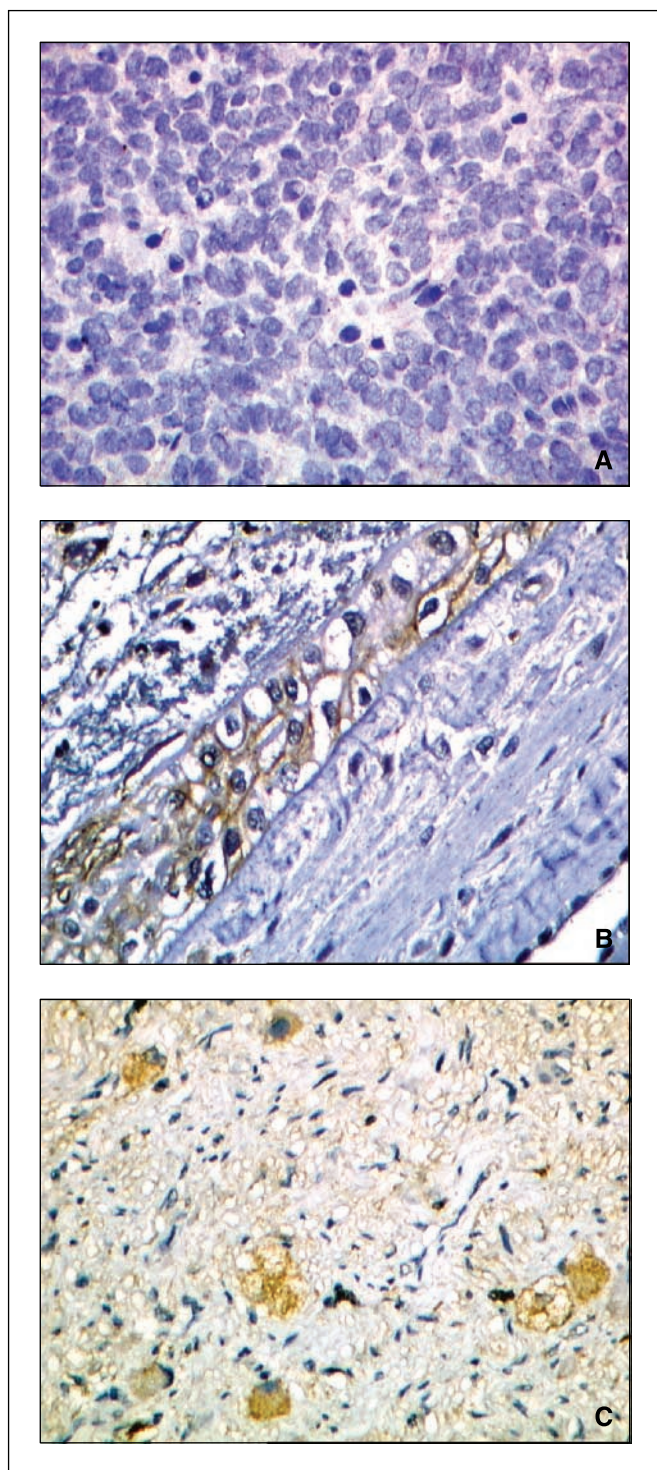
Monocytes from six healthy controls were incubated overnight with medium alone, IFN- $\gamma$  (1,000 units/mL), or 72 h pooled supernatants from four neuroblastoma cell lines. Cells were then analyzed for surface HLA-G expression by flow cytometry. The baseline surface expression of HLA-G (MRFI,  $1.88 \pm 0.07$ ) was not significantly increased by IFN- $\gamma$  ( $1.97 \pm 0.22$ ) or neuroblastoma supernatant ( $2.41 \pm 0.22$ ).

Next, we analyzed the number of sHLA-G-secreting normal monocytes by ELISPOT assay after overnight culture with medium or pooled supernatants from four neuroblastoma cell lines. In some experiments, supernatants were pretreated with anti-IL-10, anti-TGF- $\beta$ 1, or anti-GD2 neutralizing antibodies.

As shown in Fig. 4A, the baseline number of sHLA-G-secreting normal monocytes was significantly increased after stimulation with supernatants from neuroblastoma cell lines tested at the 10%



**Figure 2.** Expression of surface HLA-G and secretion of sHLA-G in neuroblastoma cell lines and monocytes. **A**, HLA-G surface expression was tested by flow cytometry on 12 human neuroblastoma cell lines, before (gray columns) and after (black columns) 48 h treatment with 1,000 units/mL IFN- $\gamma$ . Results are expressed as MRFI, obtained by the following formula: specific antibody fluorescence intensity/isotype-matched control intensity. One representative experiment out of three done is shown. **B**, sHLA-G secretion was analyzed by ELISPOT assay on 12 human neuroblastoma cell lines, before (gray columns) and after (black columns) 48 h treatment with 1,000 units/mL IFN- $\gamma$ . Results are expressed as specific HLA-G spots/5,000 cells. **C**, sHLA-G secretion was tested by ELISPOT assay in monocytes from nine neuroblastoma patients and nine age-matched healthy controls, either immediately after isolation (two left columns) or after overnight incubation with IFN- $\gamma$  (two right columns) or medium alone. The number of freshly isolated sHLA-G secreting monocytes in neuroblastoma patients ( $189.8 \pm 27.6$  spots/5,000 cells) was significantly higher ( $P < 0.0001$ ) than that detected in healthy controls ( $50.95 \pm 7.09$  spots/5,000 cells). The number of sHLA-G secreting monocytes was significantly increased by IFN- $\gamma$  in healthy controls ( $186 \pm 28.57$  spots/5,000 cells;  $P = 0.0029$ ), but not in neuroblastoma patients ( $238.9 \pm 38.49$  spots/5,000 cells). The numbers of sHLA-G spots detected in monocytes cultured overnight with medium alone were similar to those observed in the same freshly isolated cells (data not shown). **D**, PBMC from four neuroblastoma patients and four healthy controls were double stained with CD14 and CD69 monoclonal antibodies and analyzed by flow cytometry. Monocytes from healthy controls displayed lower expression of CD69 (% of positive cells,  $2.2 \pm 0.11$ ) than monocytes from neuroblastoma patients (% of positive cells,  $88.3 \pm 1.32$ ;  $P = 0.0286$ ).



**Figure 3.** Expression of HLA-G in primary neuroblastoma lesions. HLA-G expression was evaluated on formalin-fixed, paraffin-embedded tissue sections from primary neuroblastoma. Neuroblasts (A) were completely negative for HLA-G expression, as compared with tissue section from human trophoblast that tested positive (B). C, a tissue section from a stroma-rich, fully differentiated neuroblastoma tumor, containing occasional HLA-G<sup>+</sup> ganglionic cells.

final dilution ( $50.95 \pm 7.09$  versus  $97.13 \pm 7.06$  spots/5,000 monocytes;  $P = 0.0031$ ). Such effect was not inhibited by anti-IL-10, anti-TGF- $\beta$ 1, or anti-GD2 neutralizing antibodies, making an involvement of these factors unlikely (data not shown).

Neuroblastoma supernatants did not enhance secretion of sHLA-G by monocyte-derived dendritic cells or macrophages from normal donors; however, these cell fractions contained a higher number of cells secreting spontaneously sHLA-G than monocytes (data not shown).

These data showed that soluble factors secreted by neuroblastoma cell lines were able to activate monocytes and to induce sHLA-G secretion. Such conclusion was reinforced by the finding that monocytes cocultured with neuroblastoma cell lines in a transwell system showed a significantly increased expression of the activation markers CD69 and CD71 (Fig. 4B).

Moreover, monocytes cultured with pooled neuroblastoma cell line supernatants showed a significantly increased expression of CD86, CD68, and HLA-DR (Fig. 4C) and a significantly lower production of IL-12 (Fig. 4D). In contrast, IL-10 production was never detected irrespective of monocytes had been treated or not with neuroblastoma cell supernatants (data not shown).

Such findings suggest the occurrence of macrophage-like differentiation in normal monocytes incubated with neuroblastoma cell supernatants in conjunction with reduced ability of the latter cells to support IL-12-driven antitumor immune responses.

**Inhibition of cytotoxic effector cells by serum sHLA G.** To test the biological activity of sHLA-G in sera from neuroblastoma patients, we analyzed its ability to inhibit NK- and CTL-mediated neuroblastoma lysis (Fig. 5).

Activated NK cells from four healthy donors were tested against ACN and SH-SY-5Y neuroblastoma cell lines at the *E/T* ratio of 30:1. Neuroblastoma-specific HLA-A1<sup>+</sup> CTLs were generated as previously described (43) and challenged at the *E/T* ratio of 50:1 with HLA-A1<sup>+</sup> SH-SY-5Y neuroblastoma cell line that had been pretreated for 48 h with IFN- $\gamma$ .

Both NK cells and CTLs were pretreated for 1 h at 4°C with medium alone, or recombinant sHLA-G (100 ng/mL), or sera from four neuroblastoma patients (sHLA-G range, 184–212 ng/mL) either untreated or sHLA-G depleted, or sHLA-G<sup>-</sup> sera from four age-matched healthy donors. Cytotoxic effector cells were then challenged with target cells in a standard 4-h <sup>51</sup>Cr release assay.

Soluble recombinant HLA-G (100 ng/mL) inhibited both NK and CTL-specific lysis.

As shown in Fig. 5, sera from neuroblastoma patients were able to inhibit both NK activity (Fig. 5A, specific lysis  $47.75 \pm 6.17$  versus  $70.36 \pm 6.29$ ;  $P = 0.0041$ ) and CTL activity (Fig. 5B, specific lysis  $6.8 \pm 0.73$  versus  $21.64 \pm 0.73$ ;  $P = 0.0022$ ). No inhibition was detected using sera from healthy donors (data not shown).

Depletion of sHLA-G from selected neuroblastoma patient sera partially restored cytotoxic activity of both effector cell types (NK-specific lysis,  $56.32 \pm 5.8$ , and CTL-specific lysis,  $17 \pm 1.09$ ), and such effects were statistically significant ( $P = 0.0156$  and  $P = 0.0313$ , respectively).

## Discussion

Human neuroblastoma tumors have developed several mechanisms to elude the control of the immune system. These include (a) the down-regulation of HLA-A, HLA-B, and HLA-C molecule expression (that can be partially restored by IFN- $\gamma$  or retinoids; refs. 8, 9); (b) multiple defects of the antigen-processing machinery that could impair tumor-specific cytotoxic T-cell function (11); (c) production of soluble MIC-A, a ligand for NKG2D receptor, that can decrease NK and CTL function (10); (d) expression of 41g-B7-H3 that protects tumor cells from NK lysis (7); (e) expression of Fas-L

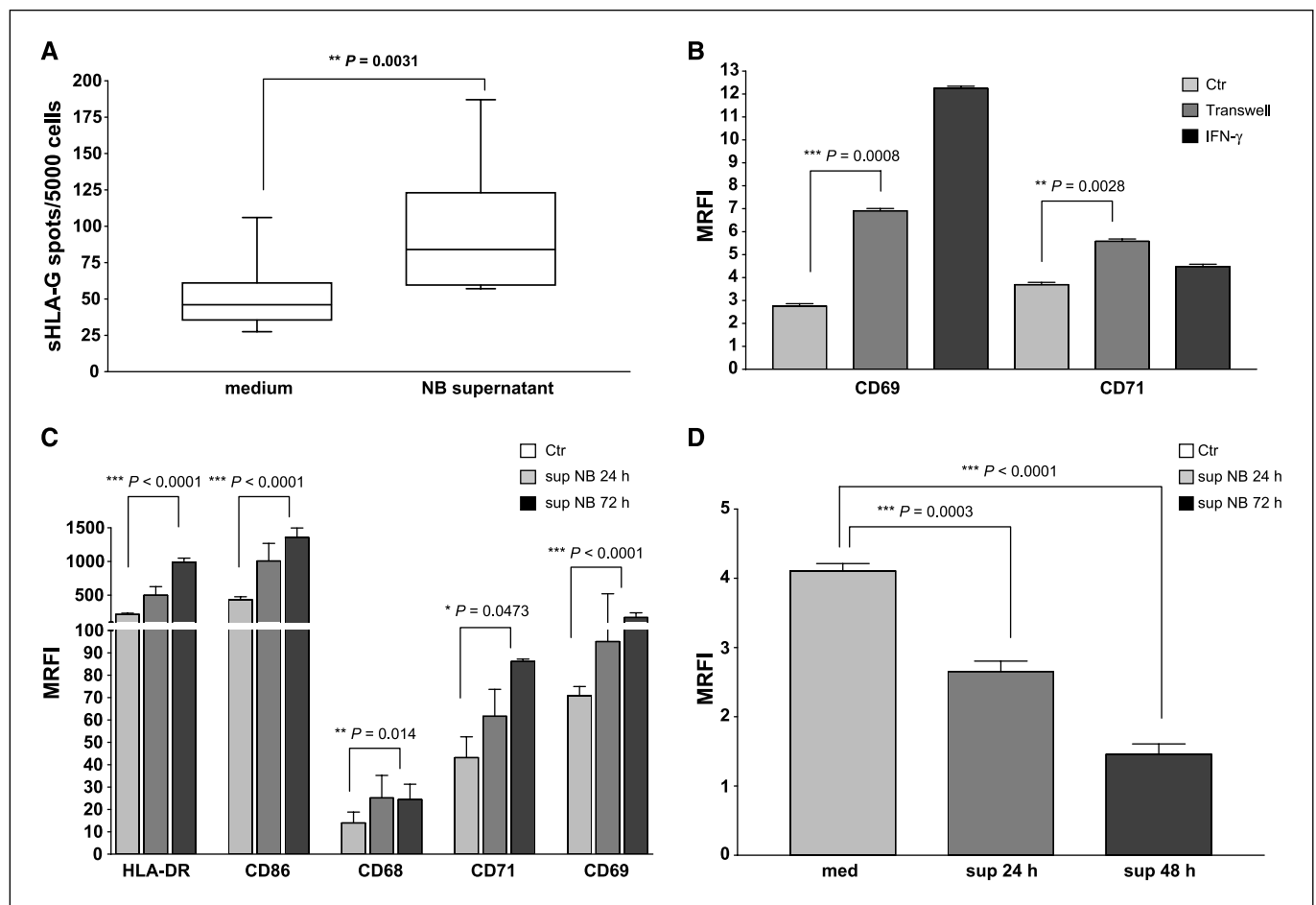
on neuroblastoma cells that interacts with Fas on T lymphocytes inducing their apoptosis (12); (f) and inhibition of dendritic cell generation and function by soluble gangliosides secreted by neuroblastoma cells (13, 14).

The involvement of HLA-G in the progression of several human tumors has been previously reported (25–32). Both soluble and membrane-bound isoforms were found to dampen the cytotoxic activity of NK cells and of tumor-specific CTL by interacting with inhibitory receptors expressed on these effector cells or inducing their apoptosis via CD8 ligation or Fas-FasL pathway (15–18).

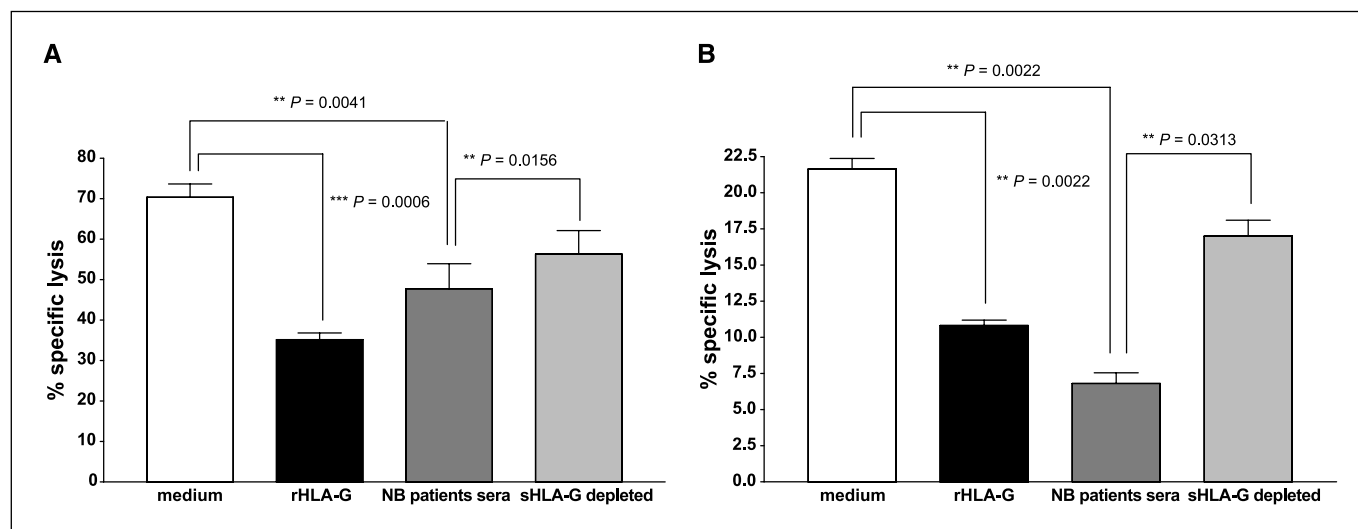
In the present study, we have investigated for the first time the expression of HLA-G in human neuroblastoma and its potential role in the immune escape of this tumor. Neuroblastoma patients showed higher serum levels of sHLA-G than age-matched healthy donors; in addition, sHLA-G, at the same concentrations detected in patient sera, inhibited the *in vitro* cytotoxic activities of activated NK cells and neuroblastoma-specific CTL against

neuroblastoma cell lines. The specificity of the observed sHLA-G increase in patient sera was confirmed by control experiments in which serum levels of sHLA class I and sHLA class II were comparable in patients and controls. sICAM-1 was found to be significantly overexpressed in patient sera, providing the first demonstration that this already reported mechanism of tumor immune evasion (46) operates also in neuroblastoma.

Rather unexpectedly, experiments done with neuroblastoma cell lines and primary tumor cells showed low to absent expression of surface HLA-G and secretion of the soluble molecule. Thus, of the 12 neuroblastoma cell lines analyzed, 6 displayed moderate surface expression of HLA-G (that was not up-regulated by IFN- $\gamma$ ), and only the ACN cell line secreted constitutively sHLA-G. After IFN- $\gamma$  treatment, two neuroblastoma cell lines (LA-N-5 and SH-SY-5Y) secreted *de novo* sHLA-G and ACN up-regulated such production. Although obtained from the analysis of a limited number of samples, immunohistochemical analysis of primary neuroblastoma



**Figure 4.** Effects of pooled supernatants from neuroblastoma cell lines on monocytes from normal donors. **A**, sHLA-G secretion was tested by ELISPOT assay on monocytes from 10 healthy controls, cultured overnight with medium alone or with 24 h pooled supernatants from four neuroblastoma cell lines tested at final dilution of 10%. The number of sHLA-G spots was significantly higher ( $P < 0.031$ ) in monocytes treated with neuroblastoma supernatant ( $84 \pm 16.06$  spots/5,000 cells) than in cells cultured with medium alone ( $46 \pm 7.09$  spots/5,000 cells). **B**, surface expression of the activation markers CD69 and CD71 was analyzed by flow cytometry on monocytes purified from normal donors and cultured overnight with medium alone (light-gray columns), or with 1,000 units/mL IFN- $\gamma$  (black columns), or in a transwell system with the GI-ME-N neuroblastoma cell line (gray columns). Results are expressed as MRFI. Columns, mean of three experiments. **C**, surface expression of HLA-DR, CD86, CD68, CD71, and CD69 was analyzed by flow cytometry on monocytes from normal donors cultured overnight with medium alone (light-gray columns), pooled 24 h supernatant (gray columns), or 72 h supernatant (black columns) from neuroblastoma cell lines. Results are expressed as MRFI. Columns, mean of three experiments. **D**, intracellular IL-12 was analyzed by flow cytometry on monocytes from normal donors cultured overnight with medium alone (light-gray column), pooled 24 h supernatant (gray column), or 72 h supernatant (black column) from neuroblastoma cell lines. Results are expressed as MRFI. Columns, mean of three experiments.



**Figure 5.** Inhibition of NK cell or CTL-mediated neuroblastoma cell lysis by sHLA-G-enriched sera from neuroblastoma patients. **A**, lysis of the SH-SY-5Y neuroblastoma cell line by activated NK cells following 1 h preincubation of the effector cells with medium (control), rHLA-G, or pooled sera from four neuroblastoma patients (sHLA-G range, 184–212 ng/mL), either unmanipulated or depleted of sHLA-G by immunomagnetic beads. Cytotoxicity was tested in a  $^{51}\text{Cr}$  release assay. **B**, lysis of the IFN- $\gamma$  stimulated SH-SY-5Y neuroblastoma cell line by CTLs following 1 h preincubation of the effector cells with medium (control), rHLA-G or pooled sera from four neuroblastoma patients (sHLA-G range, 184–212 ng/mL), either unmanipulated or depleted of sHLA-G by immunomagnetic beads. Cytotoxicity was tested in a  $^{51}\text{Cr}$  release assay.

lesions of different histology showed consistently that neither neuroblasts nor tumor stroma expressed HLA-G *in vivo*. The limited to absent expression of HLA-G in tumor cells prompted us to investigate if another cell type was mainly responsible for the secretion of sHLA-G in neuroblastoma patient sera. To this end, we focused on monocytes because these cells are known to be the major source of sHLA-G in peripheral blood (22, 50).

Monocytes from neuroblastoma patients and age-matched healthy donors did not differ in HLA-G surface expression either before or after IFN- $\gamma$  treatment. However, we detected higher numbers of freshly isolated monocytes secreting sHLA-G in neuroblastoma patients than in normal subjects. IFN- $\gamma$  increased the number of sHLA-G-secreting monocytes from normal individuals, but not neuroblastoma patients, possibly because the latter cells were already activated. Consistent with this hypothesis, monocytes freshly isolated from the peripheral blood of neuroblastoma patients showed significantly higher expression of the CD69 activation marker than monocytes from healthy donors.

We reasoned that soluble factors released by neuroblastoma cells could reach the systemic circulation and activate monocytes, thus enhancing their ability to release sHLA-G. Indeed, monocytes from normal donors incubated with supernatants from a pool of four neuroblastoma cell lines (*a*) contained higher numbers of sHLA-G secreting cells and (*b*) up-regulated surface expression of CD69 and CD71, indicative of an activated state, and of HLA-DR, CD68, and CD86, suggestive of macrophage-like differentiation. Furthermore, monocytes that had been treated with neuroblastoma cell line supernatants were found to down-regulate IL-12 production. Taken together, these findings suggest that tumor supernatant exposed monocytes undergo a program of “frustrated activation” because they express features of macrophage-like activated cells but release higher amounts of immunosuppressive sHLA-G and produce less IL-12, indicating a shift towards a more anergic phenotype.

Attempts at investigating the mechanism involved in neuroblastoma cell-mediated monocyte activation ruled out that TGF- $\beta$ 1,

IL-10, or soluble GD2 released by tumor cells were involved in the phenomenon.

An interesting translational implication of our study is the significant correlation between sHLA-G serum levels and the occurrence of disease relapse in neuroblastoma patients. Thus, when all neuroblastoma patients studied were divided in two groups, the first with local/systemic relapses and the second with complete remission over a 3- to 6-year follow-up, it was found that sHLA-G serum levels at diagnosis were significantly higher in the former than the latter patients. These results, which warrant confirmation in larger cohort of neuroblastoma patients, suggest that sHLA-G serum levels may represent a novel and reliable predictor of disease relapse in this pediatric tumor.

In conclusion, (*a*) sHLA-G serum levels are higher in neuroblastoma patients than healthy controls and, if confirmed in future studies, may help predict tumor relapse; (*b*) neuroblastoma cells show low expression of surface and sHLA-G; (*c*) a source of sHLA-G in neuroblastoma patients are tumor cell-instructed circulating monocytes that display the features of frustrated activation; and (*d*) the novel finding of a crosstalk between host cells and neuroblastoma cells through soluble factors likely released by the latter in the systemic circulation opens up new perspectives for the investigation of cancer-related immune evasion mechanisms.

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