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# Human melanoma cell secreting human leukocyte antigen–G5 inhibit natural killer cell cytotoxicity by impairing lytic granules polarization toward target cell

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ABSTRACT

Human leukocyte antigen–G (HLA-G) is a nonclassical tolerogenic molecule that can be expressed either as membrane bound (HLA-G1) or secreted (HLA-G5) isoforms. Upregulation of HLA-G1 or HLA-G5 expression by tumor cells constitutes an efficient way to escape from antitumoral immune responses. The inhibitory role of HLA-G1 on NK cell cytotoxicity is well characterized; however, that of the HLA-G5 isoform secreted by tumor is poorly understood. Our results indicate that the HLA-G5 isoform secreted by M8 melanoma cells is able to protect them from natural killer leukemia cell line (NKL) cytotoxicity. Analysis of NKL/M8-HLA-G5 conjugates by confocal microscopy demonstrates that the inhibition of NKL cytotoxic activity resulted from an impairment of NKL actin reorganization and perforin granules polarization toward M8-HLA-G5 target cell. This study also indicates that HLA-G5 soluble isoform remains evenly distributed in the cytoplasm of M8-HLA-G5 conjugated to NKL cells, suggesting that HLA-G5 does not require to polarize toward effector cell to induce efficient inhibition. These results highlight the inhibitory mechanisms mediated through HLA-G5 leading to tumor escape from NK cell cytotoxicity.



## 1. Introduction

Natural killer (NK) cells play a key role in the immune responses against tumors and viruses [1,2]. Their activation depends on the balance of activating versus inhibitory signals occurring through receptor–ligand interaction at the NK target cell interface [3]. Once activated, NK cells are able to kill target cells as well as to produce cytokines involved in the development of the adaptive immune response.

The interaction of NK cells with target cells has been shown to induce a specific reorganization of cell surface receptors and intracellular components at the area of contact that has been called immunological synapse [4–9]. The activating immunological synapse of NK cells is characterized by the accumulation of NK cell activatory receptors such as CD2 and by the reorganization of cytoskeleton at the area of contact. This molecular reorganization ultimately leads to NK cell lytic granules polarization toward target cell followed by their release in the interstice. The role of the activating NK cell synapse would be to properly direct the delivery of lytic granules toward target cell [10]. Such mechanism of directed secretion in a close environment would help to reach high concentration of perforin/granzyme but also to avoid hazardous diffusion that could affect healthy neighboring cells [10]. An inhibitory NK cell synapse has also been described [4]. It is induced by the target cell's HLA class I molecules and NK cell's inhibitory receptors interaction and is characterized by their accumulation at the center of the NK/target cell interface. The role of the inhibitory NK cell synapse would be to induce a strong and localized inhibitory signal to impair cytoskeletal reorganization and lytic granule polarization toward the target cell, thus preventing their killing [10].

The nonclassical HLA class I molecule HLA-G differs from classical HLA class I molecules by a low allelic polymorphism, seven alternative-spliced isoforms (four membrane-bound and three soluble) and a highly tissue-restricted expression [11–13]. The best characterized isoforms of HLA-G are membrane-bound HLA-G1 and its secreted counterpart HLA-G5. The HLA-G1 and HLA-G5 isoforms derive from the same transcript but differ because of alternative splicing. Both of them are composed of three globular domains associated noncovalently with $\beta_2$-microglobulin and a 9–11 amino acids–long peptide. The immunomodulatory function of HLA-G1 and HLA-G5 occurs through their interaction with inhibitory receptors selectively expressed by cells of the immune system. To date, three receptors, immunoglobulin-like transcript 2 (ILT2/CD85j/LILRB1), ILT4 (CD85d/LILRB2), and KIR2DL4 (CD158d) have been identified. The ILT2 inhibitory receptor that is expressed at the surface of some NK cells contains cytoplasmic immuno-tyrosine–based inhibition motifs (ITIM)

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that, upon phosphorylation, become docking sites for SHP-1 and SHP-2 phosphatases [11–13].

Even though in healthy conditions soluble HLA-G5 isoform expression is restricted to trophoblast, thymus, erythroblasts and human mesenchymal stem cells, its expression can be induced in various pathologic conditions [11–15]. In particular, it has been reported that HLA-G5 expression was induced in some heart-, kidney- and liver–kidney-transplanted patients [14–17]. In each case, expression of HLA-G5 in plasma from patients with transplants was correlated with a better graft acceptance. Additional studies showed that HLA-G5 extracted from liver–kidney-transplanted patient plasma was able to inhibit CD4+ T cell alloproliferation *in vitro* [18]. These studies strongly suggest that the interaction of HLA-G5 with inhibitory receptors expressed at the surface of immune effector cells may induce tolerance to allograft in transplanted patients.

In cancer, HLA-G5 and HLA-G1 have been found to be expressed by ovarian carcinoma, melanoma, and some hemopathic malignancies [19–21]. In breast and ovarian carcinoma, high levels of soluble HLA-G5 detected in plasma were associated with elevated tumor grade and development of metastasis [20,21].

Even though the expression of HLA-G1 membrane-bound isoform at the surface of tumor cell lines has been shown to confer protection against NK cell–mediated cytolysis, the impact of HLA-G5 expressed by tumor target cells on NK cell cytotoxicity remains unknown.

In this study, we investigated whether melanoma tumor target cells expressing exclusively the HLA-G5 secreted isoform can inhibit NK cell cytotoxic activity.

## 2. Subjects and methods

### 2.1. Cell lines and culture

Natural killer leukemia cell line was maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mmol/l L-glutamine, 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA) and 50 IU/ml of IL-2 (Chiron, Siena, Italy). Activated NKL cells were obtained by adding 200 IU/ml of IL-2 (Chiron, Siena, Italy) 24 hours before the experiments. The M8 melanoma cell lines transfected with the pcDNA3.1 vector (Invitrogen, Carlsbad, CA), either empty (M8-pcDNA) or containing the HLA-G5 cDNA (M8-HLA-G5), were obtained as previously described [22]. The M8-pcDNA and M8-HLA-G5 melanoma cell lines were maintained in culture with RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mmol/l L-glutamine, 10% heat-inactivated FCS (Invitrogen, Carlsbad, CA), and 50 $\mu$g/ml Hygromycin B (Invitrogen, Carlsbad, CA).

### 2.2. Flow-cytometric analysis

M8-pcDNA and M8-HLA-G5 cells were fixed for 10 minutes with 3% paraformaldehyde followed by permeabilization with PBS1X, 3% bovine serum albumin (BSA), and 0.3% saponin. Staining were performed for 30 minutes on ice using an anti–HLA-G monoclonal antibody (mAb; Clone 4H84, Exbio, Prague, Czech Republic) followed by a FITC-conjugated goat anti-mouse antibody in PBS 1X, 3% BSA, and 0.3% saponin. Isotype control antibody was systematically used.

Flow-cytometric analyses were performed on an Epics XL cytometer (Beckman Coulter, Villepinte, France) using EXPO32 software (Beckman Coulter, Villepinte, France).

### 2.3. Enzyme-linked immunoabsorbent assay analysis

HLA-G5 protein released in the supernatant of transfected M8-HLA-G5 melanoma cells were quantified by enzyme-linked immunosorbent assay (ELISA) as previously described [18].

### 2.4. Cytotoxicity assays

Cytolytic activity of NKL was assessed against $^{51}$Cr-labeled M8 target cells in a standard 4-hour $^{51}$Cr-release assay [22]. For each experiment, triplicate samples were used.

### 2.5. Confocal microscopy

NK cells were conjugated with M8-pcDNA or M8-HLA-G5 cells. After 15 minutes of incubation at 37°C, conjugates were gently resuspended and laid on poly-L-lysine–coated slides for 5 minutes at 37°C. Cells were then fixed for 10 minutes with 3% paraformaldehyde and permeabilized with PBS1X, 3% BSA, and 0.3% saponin. Staining was performed with Alexa-488 Phalloidin (Invitrogen, Carlsbad, USA), anti-perforin mAb (Clone δG9, BD Pharmingen, Le-Pont-de-Claix, France), anti–HLA-G5 mAb (clone 5A6, Exbio, Prague, Czech Republic), and goat anti-mouse isotype-specific Alexa 555 or 647 antibodies (Invitrogen, Carlsbad, USA). The samples were mounted in Vectashield medium with DAPI (Vector Laboratories, Montrouge, France) and examined on a Carl Zeiss LSM 510 confocal microscope using a plan apochromat 63×/1.4 oil immersion objective. Images were acquired at ×63 magnifications. Three-dimensional reconstructions were performed with the Amira 4.0 software (Visual Concept GmbH, TGS Inc., Berlin, Germany).

### 2.6. Statistical analyses

Data are presented as means ± standard deviation (SD). Student's $t$ test was used, and a $p$ value of $<0.05$ was taken to be significant. For figures showing representative experiments, error bars represent the SD of triplicates.

## 3. Results

### 3.1. Characterization of M8 melanoma cells expressing soluble HLA-G5

To characterize M8-HLA-G5 cells, HLA-G5 level expression and localization were analyzed. As shown in Fig. 1A, intracellular expression of HLA-G5 analyzed by flow cytometry indicated that whereas M8-HLA-G5 cells expressed soluble HLA-G5, M8-pcDNA cells did not. Moreover, visualization of intracellular HLA-G5 localization by confocal microscopy analysis showed that it was evenly distributed into the cytoplasm of M8-HLA-G5 cells (Figs. 1B and 1C).

Because the efficiency of HLA-G5 inhibitory function depends on its secretion in the environment, the level of HLA-G5 secreted by M8-HLA-G5 cells was measured by ELISA. As shown in Fig. 1D, after an overnight culture, the amount of soluble HLA-G5 present in culture supernatant of M8-HLA-G5 cells reached a 150 ng/ml concentration, which is in agreement with a previous value in plasma from melanoma patients [23]. These results indicate that HLA-G5 produced by M8-HLA-G5 cells is not localized to specific intracellular area and is secreted to physiologic levels.

### 3.2. M8-HLA-G5 melanoma target cells inhibit NKL cell cytotoxic function

It has been reported that the membrane bound HLA-G1 isoform inhibits the NK cell cytolytic function [22,24]. To investigate the ability of the secreted HLA-G5 isoform to inhibit NK cell cytolytic function, cytotoxic assays using the NKL cell line and M8 target cells expressing HLA-G5 (M8-HLA-G5) or not (M8-pcDNA) were performed. As shown in Fig. 2A, M8-HLA-G5 and M8-HLA-G1 cells were protected with similar extent from NKL cells cytotoxic function. By contrast, M8-HLA-G5 cells supernatant co-incubated with NKL cells was not sufficient to protect M8-pcDNA cells from NKL cytotoxicity (Fig. 2B). These results indicate that NK cell cytotoxicity is inhibited by M8 cells secreting HLA-G5 but not by M8-HLA-G5 cell supernatant alone.