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HLA-G1 and HLA-G5 active dimers are present in malignant cells and effusions: The influence of the tumor microenvironment

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Dimers of the nonclassical HLA-G class I molecule have recently been shown to be active structures that mediate inhibition of NK-cell cytotoxic activity through interaction with the immunoglobulin-like transcript (ILT)-2 inhibitory receptor. However, this has only been proven in trophoblasts and HLA-G transfectants. Here, we document for the first time the existence of HLA-G dimers in cancer. Indeed, we identified both surface and soluble HLA-G dimers in tumor cells and malignant ascites respectively. Interestingly, factors from the tumor microenvironment, such as interferons, enhanced the formation of HLA-G dimers and increased the protection of tumors from NK cell-mediated lysis. These data emphasize the impact of HLA-G conformation on its efficiency at inhibiting the antitumor response and thus favoring tumor progression. In view of these results, the effect of the tumor microenvironment on upregulation of HLA-G function deserves particular attention when designing cancer immunotherapy protocols.

Keywords: Cancer • Dimers • HLA-G • Malignant effusions • Tumor microenvironment

Supporting Information available online

Introduction

HLA-G is a human MHC class I molecule that has been described as nonclassical due to differences in its tissue distribution and properties compared to the classical MHC class I molecules, HLA-A, -B, and -C. The major differences lie in its expression and in its immunosuppressive properties [1,2]. HLA-G expression is restricted to a few healthy tissues [3,4] but can be induced during the course of numerous pathologies [5]. Microenvironmental factors account for this selective expression [1,6]. Another characteristic of HLA-G is that its primary transcript is alternatively spliced, producing seven mRNA encoding four membrane-bound (HLA-G1 to HLA-G4) and three soluble (HLA-G5 to HLA-G7) protein isoforms. Among these isoforms, the HLA-G1 and HLA-G5 are the most frequently observed. HLA-G1 was the first of the isoforms to be discovered in healthy tissue, namely in invasive trophoblasts at the maternofetal interface [7], and has been implicated in maternofetal tolerance [8]. HLA-G-mediated protection of the fetus occurs through its binding to inhibitory receptors

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expressed by immune cells. The resulting inhibitory signal has been demonstrated to be the major signal involved in immunosuppression compared with those generated by other activating and inhibitory ligands [5]. The immunoglobulin-like transcripts ILT-2 and ILT-4 (also known as LIR-1 and LIR-2, LILRB-1 and LILRB-2, and CD85j and CD85d, respectively) [9, 10] are two receptors that have been clearly shown to interact with HLA-G to inhibit lymphoid and myeloid cell responses [11–13]. Importantly, the inhibitory function of ILT-2 is enhanced upon binding to HLA-G1 oligomers [14–17]. In vitro studies have shown that HLA-G1 is able to form disulfide-linked homodimers on the cell surface [14, 15, 18–20] that bind to ILT-2 with increased avidity compared to HLA-G1 monomers [21]. The physiological relevance of this phenomenon was defined by the observation that HLA-G dimers are present on the surface of trophoblast cells [16, 22]. A trophoblast is defined as a pseudomalignant tissue because it shares common features with neoplastic tissues, with the greatest similarity to melanomas. In this regard, we described in 1998, the expression of HLA-G in tumors such as melanomas [23]. This expression was characterized as heterogeneous and associated with a malignant phenotype [23, 24]. Also, HLA-G expression has been suggested to serve as a means by which tumors escape elimination by the immune system [23], and HLA-G expression in melanoma cell lines is indeed able to inhibit NK cell- and CTL-mediated cytolysis [25, 26]. To follow-up our previous investigations in tumor immunology, the present study was designed to further our understanding of the relationship between HLA-G structure and function (i.e., β2m association, oligomerization, NK-cell function) and how factors from the tumor microenvironment may modulate this.

Results

β2m-associated HLA-G1-ILT-2 interaction protects melanoma cells from NK cytolysis

To determine whether HLA-G1 heavy chain activity requires association with the β2m light chain, we studied β2m-deficient melanoma cells into which the HLA-G1 gene was introduced. For this purpose, we first conducted experiments using the human FO-1 melanoma cell line [27] and showed that in the absence of β2m, HLA-G1 does not reach the cell surface and cannot protect melanoma cells from NK cell-mediated lysis (See Supporting information Fig. 1).

To confirm that β2m deficiency is the causative factor in the lack of HLA-G1 cell surface expression, we introduced the β2m gene into HLA-G1-positive β2m-negative melanoma cells. Because FO-1-HLA-G1 cells have reduced HLA-G1 protein levels and cannot be efficiently transfected or transduced to express the β2m gene, we used a previously described β2m-deficient Fon melanoma cell line (referred to as Fon^-) [26]. In the presence of β2m, HLA-G1 is correctly expressed at the cell surface in the Fon^+ melanoma cell line [26], which is a melanoma cell line derived from a surgically removed HLA-G1-positive melanoma lesion. We have shown that, upon long-term in vitro culture, β2m and HLA-G1 gene expression is lost, and concomitantly, the sensitivity of the resultant Fon^- cells to NK cell-mediated lysis is restored [26].

Here, Fon^- cells were transduced with an HLA-G1 lentiviral construct alone or together with a β2m lentiviral construct (the resulting cell lines are referred to as Fon^- HLA-$G1^+$ and Fon^- HLA-$G1^+$ $\beta2m^+$, respectively). Fon^- HLA-$G1^+$ cells did not express the classical HLA class I, HLA-E, and HLA-G proteins on the cell surface similar to what was observed in the Fon^- cells used as a control (Fig. 1A and B). Conversely, HLA class I membrane expression was restored in Fon^- HLA-$G1^+$ $\beta2m^+$ cells. Notably, membrane HLA-G1 proteins were associated with β2m in Fon^- HLA-$G1^+$ $\beta2m^+$ cells as shown by flow cytometry analysis using the MEM-G/09 mAb. Importantly, β2m-free HLA-G1 proteins were not present in Fon^- HLA-$G1^+$cells, as shown by the absence of staining with the 4H84 mAb [28] (Fig. 1B). Finally, the lack of HLA-G1 cell surface expression in Fon^- HLA-$G1^+$ cells is not due to a reduction in HLA-G1 protein levels but rather due to the absence of β2m because coexpression of β2m and HLA-G1 restores cell surface expression of HLA-G1 in Fon^- HLA-$G1^+$ $\beta2m^+$ cells. In addition, intracellular HLA-G1 protein levels are similar in Fon^- HLA-$G1^+$ and Fon^- HLA-$G1^+$ $\beta2m^+$ cells (Fig. 1C). Thus, HLA-G1 cell surface expression is not dependent on its intracellular protein levels but on its association with β2m.

In agreement with these results, Fon^- HLA-$G1^+$cells were efficiently lysed by ILT-2-positive NKL cells to a level similar to that of Fon^- cells (Fig. 1D). Conversely, Fon^- HLA-$G1^+$ $\beta2m^+$ cells were protected from NKL cell-mediated lysis (Fig. 1D and E).

We have previously demonstrated that the expression of HLA-G1 in Fon^+ cells leads to the inhibition of NKL cell-mediated lysis through the interaction of HLA-G1 with ILT-2 [26]. In accordance, we show here that blocking interaction with ILT-2 reverses HLA-G1-mediated inhibition of NKL cell-mediated lysis of Fon^- HLA-$G1^+$ $\beta2m^+$ cells (Fig. 1E). Taken together, these results demonstrate that β2m is required for cell surface expression of HLA-G1 and its interaction with ILT-2.

IFN-β and IFN-γ boost HLA-G1 expression to protect melanoma cells from NK cytolysis

Neither HLA-G1 expression nor function could be restored in Fon^- cells by cytokines such as IFNs [26]. In contrast, IFN-β and IFN-γ enhanced HLA-G1 expression on the surface of Fon^+ cells compared with untreated Fon^+ cells (Fig. 2A). Indeed, IFN-β and IFN-γ induced a relative enhancement of HLA-G1 expression, as disclosed by a 1.9 ± 0.3 (median MFI ± SD, $n = 6$) and 2.1 ± 0.7 fold increase, respectively, compared with untreated cells, as shown in Fig. 2B.

HLA-G1 expression protected Fon^+ cells from NKL cell-mediated cytolysis, while Fon^- cells, which do not express HLA-G1 (Fig. 2A), were sensitive to NKL cell-mediated cytolysis, as shown here (Fig. 2C) and previously [26]. Using an E:T ratio as high as 200 fold more NK cells than tumor cells, we showed that both IFN-β and IFN-γ treatments still protected Fon^+ melanoma

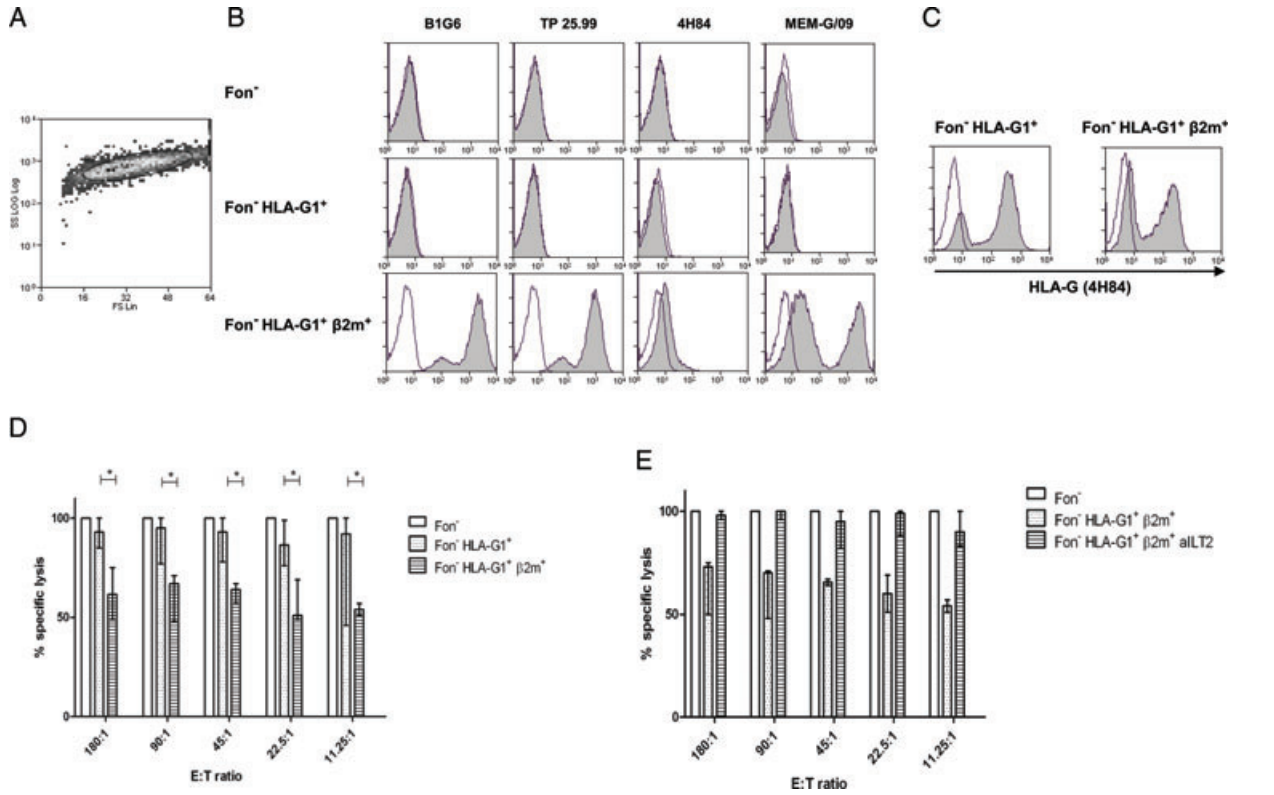


Figure 1. β2m-associated HLA-G1–ILT-2 interaction protects melanoma cells from NK cytolysis. (A, B, and C) Flow cytometry analysis of Fon$^-$ cells. (A) A representative dot plot of viable Fon$^-$ gated cells is shown. (B) Analysis of cell surface expression of β2m (B1G6 mAb), HLA class I, and HLA-E (TP25.99 mAb) and HLA-G (4H84 and MEM-G09 mAbs) in Fon$^-$, Fon$^-$HLA-G1$^+$, and Fon$^-$ HLA-G1$^+$ β2m$^+$ cells. Grey-filled histograms correspond to relevant mAb staining, and open histograms correspond to the isotype control. The results are representative of at least three different experiments. (C) Analysis of intracellular HLA-G expression in Fon$^-$ HLA-G1$^+$ and Fon$^-$ HLA-G1$^+$ β2m$^+$ cells using the 4H84 pan HLA-G mAb. (D, E) Fon$^-$, Fon$^-$ HLA-G1$^+$, and Fon$^-$ HLA-G1$^+$ β2m$^+$ cells were used as targets (T) and NKL cells used as effectors (E) at different E: T ratios in 4 h ^{51}Cr release assays. The percentage of lysis of Fon$^-$ cells was considered as 100%. (D) The percent of specific lysis of Fon$^-$, Fon$^-$ HLA-G1$^+$, and Fon$^-$ HLA-G1$^+$ β2m$^+$ cells mediated by NKL effector cells was evaluated in four independent cytotoxicity assays; each assay was performed in triplicate. Mean specific lysis of triplicates was calculated and the data are shown as the median ± range of these means. $^*p < 0.05$, Mann–Whitney test. (E) The percent of specific lysis of Fon$^-$ and Fon$^-$ HLA-G1$^+$ β2m$^+$ cells mediated by NKL effector cells with or without blocking of ILT-2 receptors was evaluated in three independent cytotoxicity assays; each assay was performed in triplicate. The data are presented as in (D).

cells from NKL cell-mediated lysis (Fig. 2C). Concomitantly, these cytokines enhanced HLA-G1 cell surface expression in Fon$^+$ cells (Fig. 2A and B). In conclusion, both IFN-β and IFN-γ significantly upregulate HLA-G1 expression and function.

We also analyzed whether IL-10, which is a cytokine that has been previously shown to upregulate HLA-G1 expression [29], could modulate HLA-G1 expression. No significant increase in HLA-G1 expression was observed (Supporting information Fig. 2A), which is in accordance with our previous study [26]. In agreement with this result, IL-10-treated Fon$^+$ cells were protected from NKL cell-mediated lysis to a similar extent as untreated Fon$^+$ cells (Supporting information Fig. 2B).

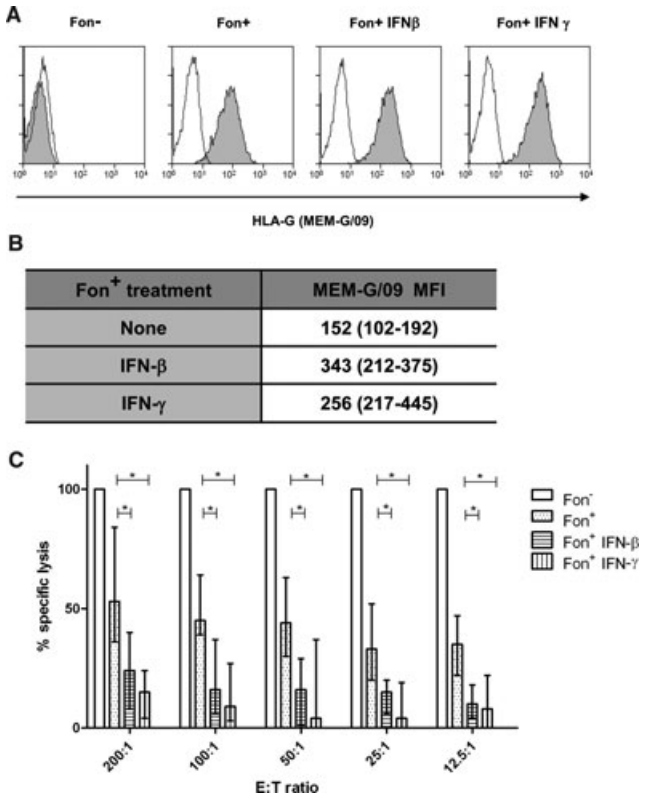
Several cytokines and growth factors from the tumor microenvironment are important in melanoma development and progression [30]. Among these, binding of vascular endothelial growth factor, SCF, and FGF to their cognate receptors activates the MAPK signal transduction pathway that plays a key role in the proliferation of most tumors, including melanomas [31]. However, treatment with these ligands did not alter HLA-G1 expression levels in Fon$^+$ melanoma cells (Supporting information Fig. 2C).

IFN-β and IFN-γ enhance HLA-G1 dimers formation on the surface of melanoma cells

To determine whether the inhibition of NK-cell cytolytic activity by HLA-G1 was related to an increase in the number of HLA-G1 dimers, we performed Western blot analysis of cell surface HLA-G1 proteins from Fon$^+$ cells treated with or without IFN-β or IFN-γ under nonreducing conditions. Only the results in which HLA-G1 membrane-bound proteins were detected i.e. no tubulin detected in the gels, are presented.

With the aim of developing this technique, we first used the M8-HLA-G1 cells, which express high levels of HLA-G1, and conducted Western blot analysis under both nonreducing and reducing conditions. As expected, HLA-G1 could be detected as a monomer of 39 KDa, but high molecular weight multimers, including dimers of about 74 KDa, were also detected (Fig. 3A).

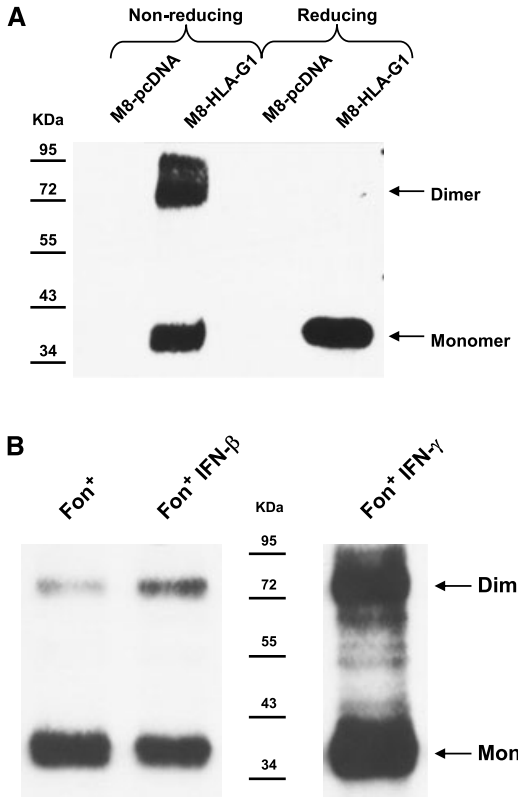
Using the same methodology, we observed that Fon$^+$ melanoma cells express HLA-G1 dimers on their cell surface (Fig. 3B). Interestingly, untreated Fon$^+$ cells had a higher ratio



B

Fon+ treatment	MEM-G/09 MFI
None	152 (102-192)
IFN-β	343 (212-375)
IFN-γ	256 (217-445)

Figure 2. IFN-β and IFN-γ increase HLA-G1 expression and protection from NKL-mediated lysis. (A, B) Flow cytometry analysis of HLA-G1 cell surface expression in Fon$^-$ and Fon$^+$ cells treated with or without IFN-β or IFN-γ using the MEM-G/09 mAb. (A) Histogram plots from one representative experiment are shown. (B) HLA-G1 cell surface expression under the indicated conditions is presented as the median (range) MFI of four different experiments. (C) Fon$^+$ cells treated with or without IFN-β or IFN-γ were used as targets of NKL effector cells at the indicated E:T ratio in 4 h ^{51}Cr release assays. The percent of specific lysis of these target cells was evaluated in four independent cytotoxicity assays; each assay was performed in triplicate. The percentage of lysis of Fon$^-$ cells was considered as 100%. Mean specific lysis of triplicates was calculated and the data are shown as the median ± range of these means.* $p < 0.05$, Wilcoxon's test.



C

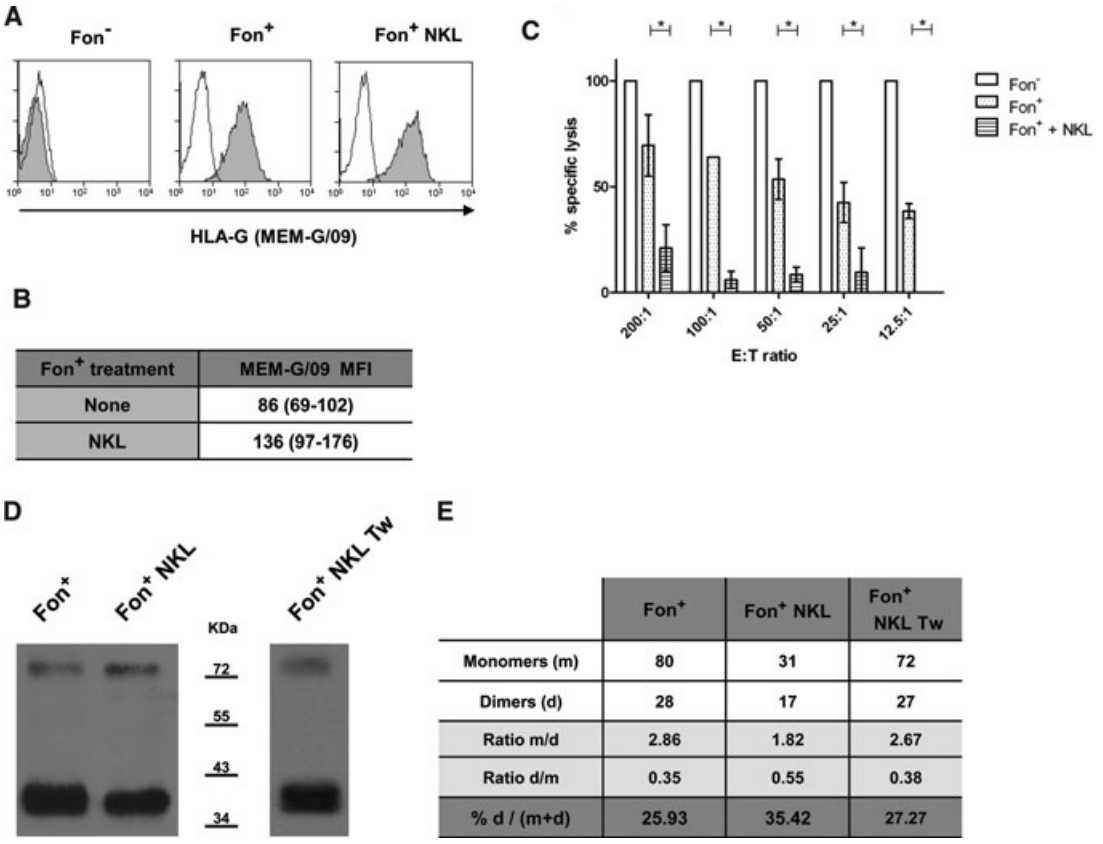
	Fon$^+$	Fon$^+$ IFN-β	Fon$^+$ IFN-γ
Monomers (m)	50	44	88
Dimers (d)	15	26	80
Ratio d/m	0.30	0.59	0.91
% d / (m+d)	23.08	37.14	47.62

Figure 3. IFN-β and IFN-γ enhance HLA-G1 dimer formation on the surface of melanoma cells (A) Cell surface proteins from M8-pcDNA or M8-HLA-G1 cells were biotinylated, immunoprecipitated, and then resolved on 10% SDS-PAGE gels under nonreducing or reducing conditions and subjected to immunoblotting using the 4H84 mAb. The results from one experiment representative of three are shown. (B, C) Cell surface proteins from Fon$^+$ cells treated with or without IFN-β or IFN-γ were biotinylated and analyzed as in (A). (B) The results shown are representative of four independent experiments. (C) Corresponding densitometry analysis of the chemiluminescent signals. The ratio d/m represents the proportion of HLA-G1 dimers (d) compared with HLA-G1 monomers (m), and % d/(m + d) correspond to the percentage of HLA-G1 dimers relative to total HLA-G1 protein. Both were calculated using the mean densitometry values from data pooled from four independent experiments.

of HLA-G1 monomers to dimers compared to cells treated with IFN-β or IFN-γ (Fig. 3B). Densitometry analysis of chemoluminescent signals allowed a more precise quantification of the HLA-G1 dimer/monomer ratios (Fig. 3C). The percentages of HLA-G1 dimers relative to total HLA-G1 were 24.5 ± 7.5, 38.3 ± 7.1, and 39.8 ± 11.3 ($n = 4$) for untreated, IFN-β-treated, and IFN-γ-treated Fon$^+$ cells, respectively. These results show that both IFN-β and IFN-γ increase the formation of active structures, that is, HLA-G dimers.

The interaction between NK cells and melanoma cells favors HLA-G1 dimer formation

Next, we investigated whether the interaction between NK cells and tumor cells may influence the formation of HLA-G1 dimers in tumor cells. For this purpose, NKL cells and Fon$^+$ melanoma cells were cocultured. These NKL-sensitized Fon$^+$ cells were then analyzed by flow cytometry and also used as targets in NK-cell cytotoxic assays. Our results show that NKL-sensitized Fon$^+$ cells express more membrane-bound HLA-G1 protein (Fig. 4A), as revealed by the 1.6 ± 0.2 ($n = 3$) fold increase in



Fon+ treatment	MEM-G/09 MFI
None	86 (69-102)
NKL	136 (97-176)

	Fon+	Fon+ NKL	Fon+ NKL Tw
Monomers (m)	80	31	72
Dimers (d)	28	17	27
Ratio m/d	2.86	1.82	2.67
Ratio d/m	0.35	0.55	0.38
% d / (m+d)	25.93	35.42	27.27

Figure 4. The interaction between NKL cells and melanoma cells favors HLA-G1 dimer formation. (A, B) Flow cytometry analysis of HLA-G1 cell surface expression in Fon$^-$, untreated (Fon$^+$), and NKL-sensitized (Fon$^+$ NKL) Fon$^+$ cells using the MEM-G/09 mAb. (A) The histogram plots shown are representative of three independent experiments. (B) HLA-G1 surface expression is presented as the median (range) MFI of three independent experiments. (C) Untreated and NKL-sensitized Fon$^+$ cells were used as targets for NKL effectors at the indicated E:T ratios in 4 h ^{51}Cr release assays. The results from four independent experiments, which were performed in triplicate, are presented as the median with range with the percentage of lysis of Fon$^-$ cells being 100%. $^*p < 0.05$, Wilcoxon's test. (D, E) Cell surface proteins from untreated Fon$^+$ cells and NKL-sensitized Fon$^+$ cells that were (Fon$^+$ NKL Tw) or were not (Fon$^+$ NKL) cultured in Transwell inserts for 48 h were biotinylated and analyzed as described in Figure 3A. (D) The western blot shown is representative of three independent experiments. (E) Corresponding densitometry analysis of the chemiluminescent signals was performed as described in Figure 3C and is presented as the mean values pooled from three experiments.

MEM-G/09 MFI values, compared with untreated Fon$^+$ cells (Fig. 4B). In agreement with this finding, NKL-sensitized Fon$^+$ cells became almost completely resistant to NKL cell-mediated lysis (Fig. 4C).

In agreement with the above results, we found that NKL-sensitized Fon$^+$ cells display a higher ratio of dimers/total HLA-G1 (32.2 ± 4.5%) than untreated Fon$^+$ cells (24.9 ± 1.4%; $n = 3$) (Fig. 4D and E). As IL-2-stimulated NKL cells secrete IFN-γ, the effects observed in NKL-sensitized Fon$^+$ cells may be due to IFN-γ rather than the interaction of HLA-G with ILT-2. To address this question, we conducted similar experiments using semipermeable membranes (Transwell) to separate the Fon$^+$ and NKL cells. Our results show that the percentages of dimers/total HLA-G1 did not vary with or without the Transwells (25.7 ± 2.3% and 24.9 ± 1.4%, respectively; $n = 3$) (Fig. 4D and E). Altogether, these results show that close contact between NKL and Fon$^+$ cells rather than the involvement of soluble factors such as IFN-γ, which are secreted by NKL cells, favors HLA-G1 dimer formation.

Malignant effusions from ovarian carcinoma patients contain HLA-G5 dimers

To provide clinical relevance for our findings, we examined whether soluble HLA-G5 oligomers could be detected in malignant effusions as described above for membrane-bound HLA-G1. For this purpose, we measured soluble HLA-G levels in ascitic fluid collected from ovarian carcinoma patients by ELISA. Here, we describe the results obtained from four effusions displaying various HLA-G5 protein concentrations. These effusions, called no. 1, no. 2, no. 3, and no. 4, contained 38, 62.5, 26.5, and 32.5 ng/mL of soluble HLA-G respectively, when measured using a MEM-G/09 ELISA [32] and 181.7, 59, and 19.6 ng/mL, respectively, when measured using a 5A6G7 ELISA [4]. Notably, the HLA-G isoform present in malignant ascite no. 1 is likely HLA-G5 and not shed HLA-G1. Immunoprecipitation of soluble HLA-G proteins from these ascitic fluids followed by Western blot analysis revealed that the malignant ascites contained HLA-G5 dimers (≈ 74 kDa) and potentially higher molecular weight multimers

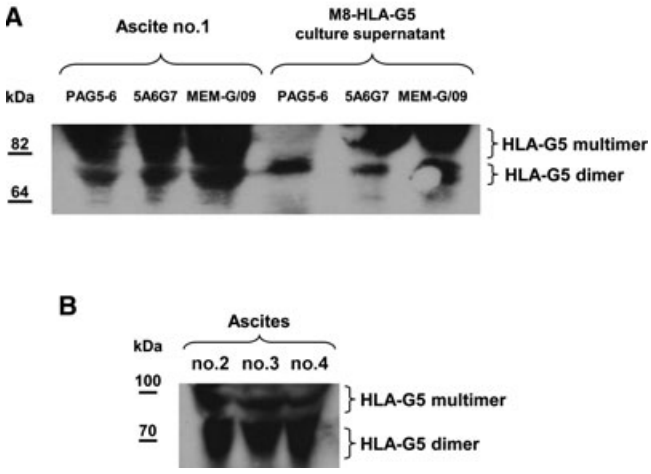


Figure 5. HLA-G5 dimers are contained in malignant effusions from ovarian carcinoma patients. (A) Soluble HLA-G5 proteins from the ovarian carcinoma ascite of patient no. 1 were immunoprecipitated using the PAG5-6, MEM-G/09, and 5A6G7 Abs and subjected to Western blot analysis. Immunoblotting was performed using the 4H84 mAb. (B) A similar analysis was performed with ascites from patients no. 2, no. 3, and no. 4 using the MEM-G/09 mAb for immunoprecipitation and the PAG5-6 Ab for immunoblotting.

(Fig. 5). The supernatant from confluent M8-HLA-G5 cells was used as a positive control.

Discussion

Tumors can escape from immune surveillance by several means. Among these, some tumors downregulate HLA class I antigens, which allows the tumors to avoid recognition and destruction by $CD8^+$ T cells but renders them susceptible to NK cell-mediated lysis. Mutations in components of the antigen processing machinery [33] or in the β2m gene underlie this loss of HLA class I antigens [34]. One strategy used by tumor cells to resist NK cell-mediated cytotoxicity is the neoexpression of immunosuppressive molecules, such as the nonclassical HLA-G class I molecule. To date, HLA-G expression has been observed in malignant lesions in about 3000 patients. The HLA-G protein has been found in almost all types of cancer regardless of their origin, that is, ectodermic, mesodermic, or endodermic. The proportion of tumors expressing HLA-G ranges from 10 to 95%, with the exception of trophoblastic tumors (100% expression) that are excluded due to the nature of this tissue [35]. Upregulation of HLA-G in melanocytes has been shown to be a better predictor of malignancy than defects in classical HLA class I antigens [24]. Here, we studied two β2m-deficient human melanoma cell lines, FO-1 and Fon^-. In both cell lines, introduction of the HLA-G1 gene did not result in localization of the protein to the cell membrane. Consequently, HLA class I-negative FO-1-HLA-G1 and Fon^- $HLA\text{-}G1^+$ cells remained sensitive to NK cell-mediated lysis. Transduction of β2m into Fon^- $HLA\text{-}G1^+$ cells led to cell surface expression of the HLA-G1 protein and conferred protection from NK cell-mediated lysis. Antibody-blocking cytotoxicity assays demonstrated that this protection occurred through the interaction of HLA-G1 with ILT-2. One could hypothesize that classical HLA class I and HLA-E proteins, which are also expressed following β2m transduction into Fon^- $HLA\text{-}G1^+$ cells, are also involved in resistance of these cells to NK cell-mediated lysis. However, previous studies have shown that ILT-2 binds to HLA-G with a three- to four-fold higher affinity than to classical HLA class I proteins [12] and that expression of classical HLA class I and HLA-E proteins in Fon cell lines weakly affects NKL cell-mediated lysis through interactions with the CD94/NKG2A receptor [26]. These results demonstrate that HLA-G1 free heavy chains need to associate with β2m to reach the cell surface and mediate inhibition of NK cell-mediated lysis, which is in accordance with a previous study [15].

β2m-associated HLA-G molecules are able to form oligomers on the cell surface through formation of disulfide bonds between two cysteine residues located in positions 42 [18] and 147 [14] of the monomers. However, HLA-G homodimers cannot interact with the KIR2DL4 receptor [18], which is selectively expressed by NK cells [36]. In contrast, these oligomers enhance the inhibitory signal mediated by ILT-2 due to increased avidity toward this receptor compared with monomers [14, 21], thereby favoring inhibition of NK cell-mediated cytotoxicity [14]. Notably, ILT-2 cannot interact with free HLA-G heavy chain complexes that are present in small amounts on the cell surface of transfectants and of the JEG-3 choriocarcinoma cell line [15]. The crystal structure of a disulfide-linked HLA-G homodimer has been determined [19, 21] and is consistent with cell surface expression of these oligomers. HLA-G is the only MHC class I molecule that displays the potential to form such disulfide-linked complexes physiologically. Indeed, β2m-associated HLA-G1 [16] and β2m-free HLA-G5 [22] homodimers have been observed in trophoblasts. To date, no study has investigated whether HLA-G disulfide-linked homodimers are present in malignant cells derived from cancer patients. Here, we show that a surgically removed $HLA\text{-}G1^+$ melanoma lesion-derived cell line, Fon^+ [26], exhibits such HLA-G homodimers. Although HLA-G monomers were predominant on the cell surface, Fon^+ cells have sufficient amounts of HLA-G dimers to mediate efficient inhibition of NK cell-mediated killing. Indeed, in agreement with our previous results [26], Fon^+ cells were almost entirely resistant to NKL cell-mediated lysis in contrast to Fon^- cells.

Interferons are cytokines that are frequently present in the tumor microenvironment and are known to upregulate HLA-G expression. Indeed, IFN-β and, to a greater extent, IFN-γ increase cell surface expression of HLA-G1 in Fon^+ cells and ensure better protection from NK cell-mediated lysis. Remarkably, enhanced resistance to NK cell-mediated killing is also associated with an increase in HLA-G dimerization. These results are in agreement with a previous study demonstrating the importance of HLA-G1 dimers on the cell surface of transfected cells for efficient inhibition of NK cell-mediated killing by ILT-2 [14]. NK cells are among the effectors present in the tumor microenvironment. Here, we also demonstrate that NK cells favor the formation of HLA-G1 dimers,

which increases inhibition of their own function. In addition, the interaction of HLA-G1 with ILT-2 decreases IFN-γ production by NK cells [37, 38], thus acting as a negative feedback that limits effector function.

The examination of biological fluids from cancer patients showed that high levels of the soluble forms of HLA-G were associated with advanced stages of disease, high-grade histology, and poor prognosis [35]. In particular, the soluble HLA-G5 protein isoform was found in malignant effusions from ovarian cancer patients [39, 40]. Interestingly, we describe here that HLA-G5 proteins are present as homodimers in the ascitic fluid from four ovarian carcinoma patients containing various levels of HLA-G5. Remarkably, HLA-G5 dimers from these malignant ascites and from the supernatant of M8-HLA-G5 cells displayed a slightly altered molecular weight. This may be due to differences in glycosylation of the protein [41]. Indeed, it is well known that cancer cells exhibit high levels of glycosylation. In addition, a recent study showed changes in the glycosylation of serum proteins in ovarian cancer patients [42]. The identification of soluble HLA-G dimers, which represent the biologically active form of this molecule, strongly suggests that their interaction with ILT-2 and/or ILT-4 receptors expressed by tumor-associated leucocytes leads to inhibit tumor antigen-specific immune responses. Indeed, serum HLA-G5 has been shown to be secreted by monocytes activated by tumor cells in neuroblastoma patients and to inhibit cytotoxic effector cells [43]. Moreover, the HLA-G5 dimer, but not monomer, induces strong ILT-4-mediated signaling in vitro, and inhibits the maturation of ILT-4-expressing dendritic cells in vivo [44]. Finally, the tumor antigen-specific immune response can be dampened by induction of immunosuppressive T cells. This could be mediated by the soluble HLA-G5 isoform as previously demonstrated in the context of transplantation [45, 46].

In conclusion, we here provide evidence for the first time that HLA-G1 and its soluble counterpart HLA-G5 are present as dimers in cancer. Moreover, we highlight the influence of the tumor environment on upregulation of such active structures. Given that HLA-G exhibits immunosuppressive properties, our findings support a role for HLA-G in escape of solid tumors from the immune system, which should be taken into consideration in therapeutic protocols that use interferons.

Materials and methods

Cell cultures

M8 and Fon are HLA class I-positive melanoma cell lines [23, 47]. Fon$^-$ HLA class I-negative cells and M8 transfectants were obtained as previously described [26, 28]. The NKL cell line was established from a patient with aggressive NK cell leukemia and expresses ILT-2 and KIR2DL4 [48]. This cell line was maintained and used as previously described [11].

Fon$^+$ and NKL cell coculture experiments were performed as described below. NKL cells were first activated overnight with 30 U/mL of IL-2 (kindly provided by Chiron, Suresnes, France). Then, activated NKL cells were seeded on a Fon$^+$ cell monolayer (at a ratio of 5:1, respectively) in a 75 cm^2 flask or in six wells plates for experiments with Transwells (Dustcher D, Brumath, France). Both cell types were cocultured for 48 h. Then, NKL cells were discarded, and the NKL-sensitized Fon$^+$ cells were subjected to cell surface biotinylation.

Lentiviral constructs and Fon$^-$ cell transduction

Production of lentiviral particles and Fon$^-$ cell transduction were performed as previously described [49]. Briefly, Fon$^-$ cells were cotransduced with β2m and HLA-G1 lentiviral particles, and cell surface expression of HLA-G1 was checked by flow cytometry analysis 48 h after lentiviral transduction.

Fon$^+$ cell treatments

Fon$^+$ cells were treated for 48 h with 500 U/mL of IFN-γ and 1000 U/mL of IFN-β (Peprotech, Neuilly-sur-Seine, France).

Antibodies and flow cytometry

The following antibodies were used: B5-1-2, a mouse IgG1 anti-human tubulin antibody (Sigma, St Quentin Fallavier, France); B1G6, a mouse IgG2a anti-human β2m antibody (Beckman Coulter, Marseille, France); TP25.99, a mouse IgG1 anti-human HLA-A, -B, -C, and -E antibody (kindly provided by Soldano Ferrone, University of Pittsburgh, PA, USA); 87G, a mouse IgG2a antibody that reacts with HLA-G1 and HLA-G5 isoforms in their β2m associated form (Exbio Praha, Vestec, Czech Republic); MEM-G/09, a mouse IgG1 antibody exhibiting the same specificity as the 87G mAb (Exbio Praha); 5A6G7, a mouse monoclonal IgG1 antibody; PAG5-6, a rabbit polyclonal antibody that specifically recognizes the soluble isoforms of both HLA-G5 and HLA-G6 [4, 50]; and 4H84, a mouse IgG1 antibody that recognizes the free heavy chain of all the HLA-G isoforms (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA). The MEM-G/09 and 5A6G7 mAbs have been previously validated by our laboratory [51], and the 4H84, 87G, and PAG5-6 Abs were validated during the HLA-G, -E, -F international preworkshop [50].

Flow cytometry analysis was performed as previously described [51]. EPICS XL4 flow cytometer and Expo32 software (Beckman Coulter, Brea, CA, USA) were used for data acquisition and Summit 4.3 (Dako) for analysis.

Cytotoxicity assays

Cytotoxicity assays were performed as previously described [11].

Immunoprecipitation of biotinylated cell surface proteins

Cell surface proteins in viable Fon$^+$ cells were biotinylated as previously described [28]. Cells were then lysed in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, protease inhibitors, 1% NP-40, and 50 mM iodoacetamide (Sigma) for 1 h on ice. The insoluble material was removed, and biotinylated proteins were then precipitated with 120 μL of 50% streptavidin-agarose slurry (Sigma) at 4°C. After three washes in lysis buffer containing 0.1% SDS, precipitates were boiled in 30 μL of 2× Laemmli buffer with or without 100 mM DTT and used in Western blotting assays under reducing or nonreducing conditions.

Immunoprecipitation of soluble HLA-G from malignant effusions

Malignant effusions were recovered during surgery of ovarian carcinoma patients after their informed consent (Tenon hospital, France) and were processed within 6 h. These ascitic fluids were centrifuged at 1500 rpm for 10 min. Supernatants were collected, and 1 mL was incubated with either the PAG5-6, MEM-G/09, or 5A6G7 antibody overnight at 4°C with stirring. In parallel, 1 mL of supernatant from confluent M8-HLA-G5 cells was subjected to the same treatments as a positive control. Immune complexes were precipitated using protein A/G plus-agarose (Santa Cruz Biotechnology; Tebu-Bio, Le Perray en Yvelines, France) for 3 h at 4°C with stirring. Immunoprecipitates were then treated as described above.

Western blot analysis

Total protein aliquots from M8 or FO-1 transfectant cell lysates or precipitated proteins from Fon$^+$ cells were separated on 10% SDS-PAGE gels as previously described [52]. The 4H84 mAb or the PAG5-6 Ab were used for immunoblotting at a 1:1000 dilution, and peroxidase-conjugated sheep anti-mouse IgG Ab (Sigma) or goat anti-rabbit IgG Ab (Cell Signaling Technology, Danvers, MA, USA) at 1:10,000 and 1:2000 dilutions respectively. For biotinylation experiments, membranes were subsequently probed with an anti-tubulin mAb (Clone B 5-1-2; Sigma) diluted 1:5000, and immune complexes were detected as described above. Quantification of blotted proteins was determined by densitometry analysis of scanned films using the Fluorchem™ imaging system and AlphaEaseFC™ software (Alpha-Innotech, CA, USA).

Statistical analysis

Assays were conducted in triplicate for each experiment. All results are shown as median ± range (Prism 5.0) for cytotoxic assays or median (Microsoft Office Excel) (range) for other experiments. The nonparametric Wilcoxon and Mann–Whitney tests were performed using Prism 5.0 software (Graph Pad, San Diego, CA, USA) with p values < 0.05 considered significant.

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Abbreviations: **ILT**: immunoglobulin-like transcript

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