HLA-G and lymphoproliferative disorders

Laurence Amiot [a,*], Gaëlle Le Friec [a], Yasmine Sebti [a], Bernard Drénou [a],
Céline Pangault [a], Valérie Guilloux [a], Xavier Leleu [b],
Marc Bernard [c], Thierry Facon [b], Renée Fauchet [a]

[a] *Laboratoire Universitaire d'Hématologie et de la Biologie des Cellules Sanguines,
UPRES EA 22-33, Faculté de Médecine, Université de Rennes 1, Rennes, France*
[b] *Service des maladies du sang, CHRU Lille, France*
[c] *Service d'Hématologie clinique, CHU Pontchaillou, Rennes, France*

Abstract

The immunomodulatory properties of the HLA-G molecule explain its relevance in malignancies. Our investigations in lymphoproliferative disorders show (i) a frequent and variable distribution of alternatively spliced HLA-G mRNA isoforms, (ii) a rare cell surface expression in diffuse large cell lymphomas with HLA class I loss in half of cases, and (iii) an increased serum level of sHLA-G in half of cases. The potential role of the microenvironment and/or tumoral process in HLA-G expression is discussed in the light of these data. HLA-G rather through its soluble isoform might provide a new way of immune evasion for lymphoid proliferations.
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1. Introduction

The nonclassical MHC class I molecule HLA-G, in contrast to classical class I molecules, is characterised by specific features such as tissue-restricted distribution [1–4] and low polymorphism [5]. Furthermore, several HLA-G isoforms have been described, resulting from alternative splicing of its primary transcript: four membrane-bound proteins including the full-length isoform HLA-G1 and the truncated isoforms HLA-G2, -G3, and -G4 [6,7] as well as three soluble isoforms HLA-G5, -G6, and -G7 [8,9]. The role of this molecule initially described on cytotrophoblast cells [4], would not be restricted to materno-fetal immune tolerance but would be involved more generally during immune responses. This immunomodulating role is partially explained by its capacity to protect target cells from NK and T cytolysis through direct [10–14] or indirect [15,16] interaction with several inhibitory receptors. This function seems to be shared by soluble isoform HLA-G5 corresponding to HLA-G1 with its three extracellular domains α1, α2, and α3 lacking the transmembrane and cytoplasmic domains because of the presence of a stop codon in retained intron 4. Furthermore, HLA-G5 would be able to trigger apoptosis in activated T CD8+ cells similarly to soluble classical HLA I [17,18], however this property remains discussed in other studies [19–21]. The determination of HLA-G expression in tumoral process is of particular interest because it would constitute a mechanism of tumoral escape from immune surveillance. HLA-G expression has been yet demonstrated on certain types of tumours such as melanomas [22], renal [23], breast [24] and lung carcinomas [25], gliomas [20] or cutaneous lymphomas [26]. Soluble HLA-G (sHLA-G) was also reported to be elevated during melanomas [27]. We here present our updated data on HLA-G expression in malignancies developed from effective cells of immune response, the lymphoproliferative disorders. These malignancies defined as neoplastic proliferations of B or T lymphocytes constitute a heterogeneous group of disorders. Chronic lymphoid leukaemia (CLL) is defined as malignant proliferation of mature lymphocytes involving the blood and bone marrow. Non-Hodgkin lymphomas (NHL) are due to the uncontrolled proliferation of lymphoid cells of B or T type affecting primarily spleen or lymph nodes and are separated into several broad categories according to the WHO classification [28]. Multiple myelomas (MM) are defined as malignant plasma cells proliferations. We have previously shown a high frequency of HLA-G transcripts [29,30], our updated data confirmed the rare cell surface protein expression in these pathologies [31]. The dissociation between the transcription and the protein expression

* Corresponding author. Tel.: +33-2-99-28-42-72;
fax: +33-2-99-28-41-52.
E-mail address: laurence.amiot@chu-rennes.fr (L. Amiot).

led us to research HLA-G through its soluble isoform in serum of patients suffering from lymphoid proliferations. Thus, we report here for the first time an increased serum level of sHLA-G in malignant lymphoid proliferations.

2. HLA-G mRNA expression in lymphoproliferative disorders

Before the availability of specific anti-HLA-G antibodies, different studies have only investigated HLA-G mRNA expression in normal and tumoral cells or tissues. Using RT-PCR with exon-specific HLA-G primers followed by hybridisation with exon-specific probes, these studies have described alternatively spliced mRNAs in normal mononuclear cells both in lymphocytes and monocytes [7,31,32]. HLA-G transcription in B or T lymphocytes is not a permanent feature. In transcriptionnally active lymphocytes, the HLA-G primary transcript or HLA-G1 is the major form and is differentially spliced in B and T lymphocytes [30]. Similarly to normal lymphocytes, our previous studies have evidenced a variable distribution of alternative mRNAs with no specific pattern according to the pathology in the majority of lymphoid disorders (Fig. 1). Numerous mRNA isoforms (HLA-G1, -G2, -G3, -G4, -G5, and -G6) could be observed in these malignancies, however HLA-G1 and HLA-G5 appeared to be the main transcribed isoforms. These results have been confirmed by a recent work of Urosevic et al. [26] in cutaneous lymphomas using RT-PCR showing the presence of the full-length HLA-G1 transcript in all cases of lymphomatous skin lesions. However, no statistically difference was observed between lymphoma samples and control group. Nevertheless, real-time quantitative RT-PCR will be useful to precise the level of HLA-G transcripts in lymphomas according to their subtype, in comparison with the basal level observed in normal lymphocytes. Furthermore, these data compared to those of protein expression would be helpful to clarify the factors regulating its expression.

3. HLA-G protein cell surface expression in lymphoproliferative disorders

HLA-G cell surface expression was studied in 50 cases of B-NHL using flow cytometry [33] with the 87G antibody (kindly provided from D. Geraghty) or MEM-G/9 (Exbio, Prague, Czech rep.), recognising both HLA-G1 and HLA-G5 isoforms. The NHL included 20 cases of B-NHL selected on the basis of a low HLA class I expression using flow cytometry analysis with W6/32, a pan HLA class I monoclonal antibody (mAb). The 30 remaining B-NHL cases presented normal class I expression. The characteristics of lymphomas are specified in Table 1. HLA-G cell surface expression was not detected in the majority of lymphomas (47/50) except in three cases of lymphomas with variable level of positive cells (20, 64, and 90%). These three cases were characterised by partial classical HLA class I defect. The common clinico-biological characteristics of HLA-G expressing lymphomas were diffuse large B cell lymphoma (DLCBL) histology from germinal origin, a frequent extra-nodal localisation and a poor outcome. In a study currently in progress, we found four new cases expressing HLA-G protein out of 60 cases of lymph-nodes NHL (Table 1). The histological diagnosis of these HLA-G positive cases were four DLCBL with normal class I expression including two transformations of follicular NHL (Fig. 2, bottom graphs). In summary, HLA-G positive expression was found in seven lymphomas out of 110 NHL cases with a HLA class I partial loss in three out of seven cases. None of the 52 low grade NHL expressed HLA-G protein in contrast to DLCBL or high grade NHL (Table 1). Among the seven DLCBL expressing HLA-G; two appeared to be de novo cases with one Epstein–Barr virus (EBV)-induced
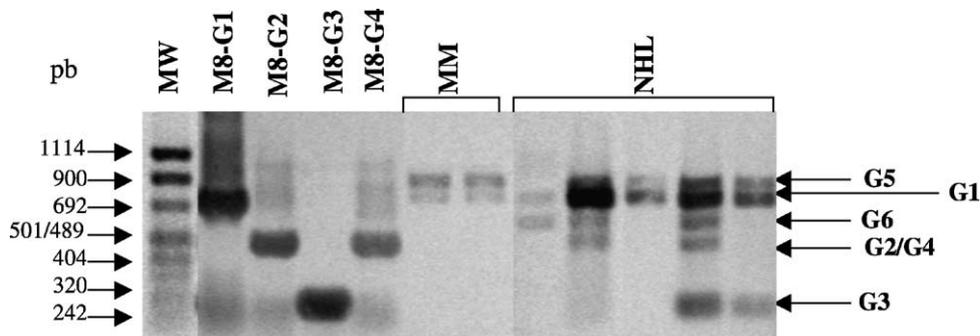

Fig. 1. Variable distribution of HLA-G mRNA spliced isoforms in NHL. After amplification using pan-HLA-G primers set (G257/G1004), PCR products were hybridized with exon 2-specific GR probe recognising all isoforms as previously described [52]. The molecular weight markers (MW) are on the left. The transfected M8-G1, -G2, -G3, and -G4 cell lines provided from E.D. Carosella (Preworkshop HLA-E, -F, -G) are used, respectively as controls of HLA-G1, -G2, -G3, and -G4. Seven cases of lymphoproliferative disorders including two cases of MM and five cases of NHL are represented. Arrows indicate the bands corresponding to G5, G1, G2/G4, G6, and G3 mRNA isoforms. The splicing of the primary transcript is variable according to the case. All mRNA isoforms except HLA-G7 are found in the fourth NHL case whereas HLA-G1 and -G5 mRNA are, respectively present in seven and six out of seven cases.

Table 1
HLA-G cell surface expression compared to HLA class I expression in lymphoproliferative disorders using flow cytometric analysis with 87G or MEM-G/9

		HLA-G negative		HLA-G positive	
		Normal class I	Partial class I loss	Normal class I	Partial class I loss
NHL ($n = 110$)	$(34 + 20^*)_1 + (39 + 17^*)_2$	69	34	4	3
B-NHL ($n = 106$)	$(30 + 20^*)_1 + (39 + 17^*)_2$	65	34	4	3
FL ($n = 34$)	$(5 + 4^*)_1 + (19 + 6^*)_2$	24	10	–	–
MCL ($n = 11$)	$(2 + 1^*)_1 + (8)_2$	10	1	–	–
MZL ($n = 3$)	$(3)_2$	3	–	–	–
LL ($n = 3$)	$(3)_1$	3	–	–	–
LPL ($n = 1$)	$(1)_1$	1	–	–	–
DLCBL					
De novo ($n = 28$)	$(10 + 7^*)_1 + (6 + 5^*)_2$	14	12	2	–
Transformation ($n = 14$)	$(6^*)_1 + (2 + 6^*)_2$	–	9	2	3
Burkitt ($n = 7$)	$(5 + 2^*)_1$	5	2	–	–
Lymphoblastic ($n = 5$)	$(5)_1$	5	–	–	–
T-NHL ($n = 4$)	$(4)_1$	4	–	–	–
CLL ($n = 76$)	$(30)_{1'} + (46)_2$	75	–	–	1

Different subtypes of lymphoproliferative disorders are represented: B- or T-NHL, B- or T-non-Hodgkin lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal/malt lymphoma; LL, lymphocytic lymphoma; LPL, lymphoplasmacytic lymphoma; DLCBL, diffuse large B cell lymphoma; and CLL, chronic lymphoid leukaemia. An asterisk (∗) indicates cases with partial HLA class I loss. 1 and 1′ correspond to previous studies: Ref. [33] for NHL and Ref. [31] for CLL; 2 indicates the new studies.

NHL occurring in one case of acquired immunodeficiency syndrome (AIDS). In the five remaining cases, HLA-G expression appeared related to transformation of quiescent lymphoma in DLCBL whereas no detection is observed in lymphomas with high level of proliferation (Burkitt or lymphoblastic lymphomas). Furthermore, in transformed follicular lymphoma (FL), HLA-G expression appears associated with HLA class I decrease. The mechanism underlying HLA class Ia defect combined with HLA-G expression may be a selection induced by the immune cells. Firstly, a partial loss of HLA class I molecules could occur in some tumoral cells which would become potential targets for NK lysis and secondly HLA-G would consequently protect them against NK and T cytotoxicity.

Seventy-six cases of CLL obtained from two successive studies [31] were similarly investigated (Table 1). HLA-G expression was only observed in cerebral fluid of one case of unusual CLL with meningeal involvement (40%) associated
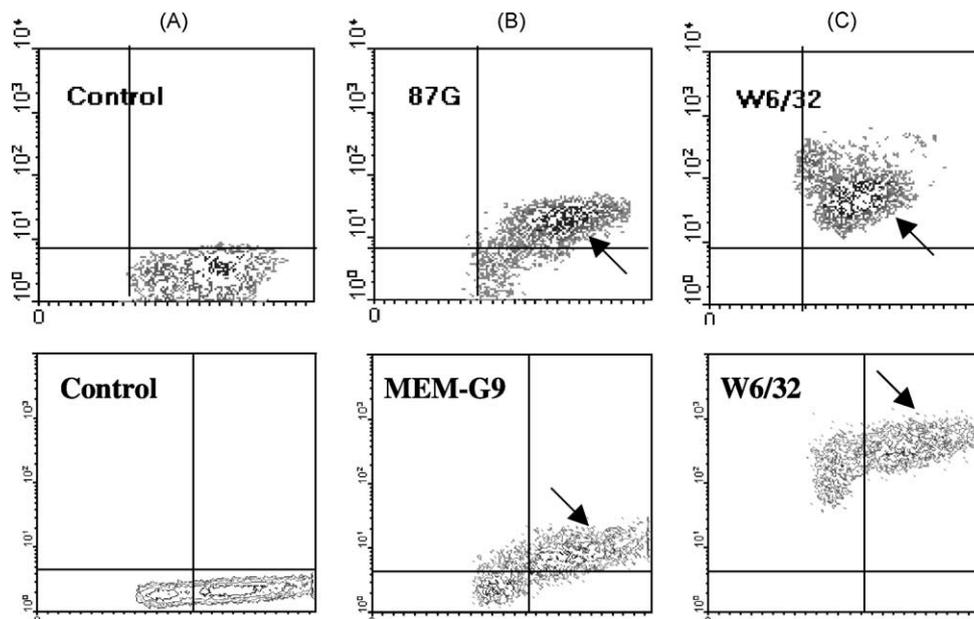

Fig. 2. HLA-G expression on lymphomatous cells. Representative cases with class I defective expression or normal class I positive expression are represented, respectively at the top and bottom of the figure. The size parameter is represented on the horizontal axis and the fluorescence intensity on the vertical axis. The two spots correspond, respectively to the small reactive cells and the large tumoral cell population (arrow). (A) Staining with the isotypic control; (B) staining with an anti-HLA-G 87G or MEM-G/9 mAb; and (C) staining with W6/32 mAb.

with class I partial loss (Table 1) but not on tumoral cells from blood or bone marrow ($n = 75$). HLA-G expression was also investigated by Urosevic et al. [26] in primary T and B cutaneous lymphomas, defined by a clonal accumulation of lymphocytes initially restricted to skin. Using immunohistochemistry with 4H84, HLA-G protein expression was detected in 51% of 45 cases. HLA-G positivity was found in indolent types of cutaneous B cell lymphomas while HLA-G expression was rather associated with high grade histology and advanced stage of disease in cutaneous T lymphomas. The discrepancy in the frequency of HLA-G expressing lymphomas between the different reported studies could be mainly explained by (i) the difference of lymphomatous localisation (blood, bone marrow or lymph-node in the former one and cutaneous in the latter), (ii) a higher number of T cases in cutaneous lymphomas, (iii) the difference of sample type (isolated cells in suspension or paraffin-embedded tissue sections), (iv) the difference of techniques, flow cytometry versus immunohistochemistry.

4. Increased serum level of sHLA-G in lymphoproliferative disorders

Since lymphoid malignancies exhibit frequently HLA-G transcripts but no HLA-G protein cell surface expression [31], we have investigated the level of sHLA-G in sera of these patients. sHLA-G molecules have been detected in plasma of healthy subjects [34] with no difference between males (24.9 ± 3.0 ng/ml) and females (20.1 ± 2.1 ng/ml). Since different ELISA methods allowing to detect sHLA-G isoforms have been reported, we developed another specific method of sandwich ELISA using new available antibodies. MEM-G/9 and peroxydase anti-beta2 microglobuline (β2-m) were used, respectively as the capturing and the detecting antibodies. This method reveals the complete soluble isoform associated to β2-m, but do not allow to differentiate HLA-G5 defined as the intron 4-retaining form and the shed membrane-bound HLA-G1. sHLA-G level was investigated in sera of 9 CLL, 53 B-NHL and 4 T-NHL compared to 30 healthy subjects (Fig. 3). In healthy subjects, the basal sHLA-G level was 18.57 ± 9.29 ng/ml ($n = 30$). Increased sHLA-G level was defined as the mean of basal sHLA-G + 2 standard error of the mean (>37.17 ng/ml). The mean of sHLA-G was significantly increased in CLL (49.03 ± 24.39 ng/ml, $P < 0.0001$), in B-NHL (46.51 ± 23.03 ng/ml, $P < 0.0001$) compared to normal controls. The secretion of sHLA-G appears also increased in two out of four T-NHL cases (41.13 ± 20.5 ng/ml), however these results have to be confirmed on a larger number of patients.
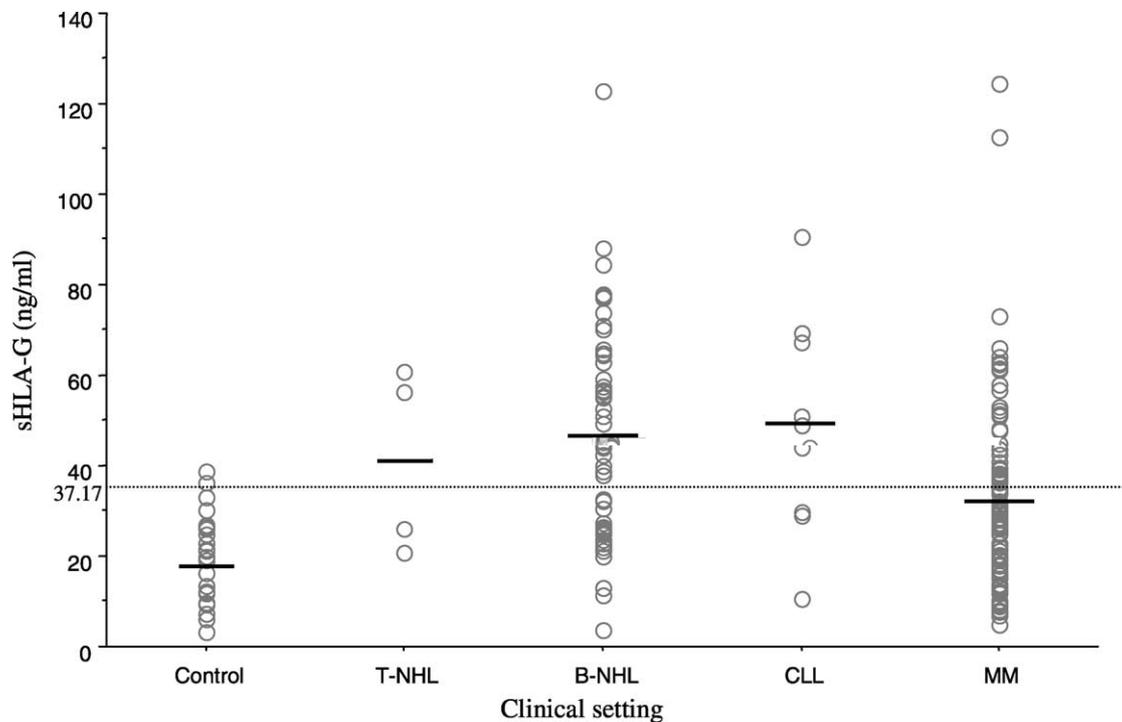

Fig. 3. Quantitative determination of sHLA-G levels (ng/ml) using ELISA in different clinical settings (T-NHL, B-NHL, CLL, MM) compared to controls. Each sHLA-G sample is represented by a circle and average of each setting is represented by a black horizontal bar. Scale bar (37.17 ng/ml) corresponds to the sHLA-G mean in controls + 2 standard errors. When compared to control (18.57 ng/ml), sHLA-G is significantly increased in T-NHL (41.13 ng/ml), in B-NHL (46.51 ng/ml, $P < 0.0001$), in MM (31.45 ng/ml, $P < 0.001$), and in CLL (49 ng/ml, $P < 0.0001$). A wide range of values is observed in all lymphoproliferative disorders.

5. Increased sHLA-G in multiple myeloma

Serum β2-m, the light chain of HLA class I molecules, is still one of the most powerful survival prognostic factor in MM [35,36]. We therefore assessed sHLA-G associated with β2-m in a retrospective and collaborative study with the "Intergroupe francophone du myélome" (IFM group) including 101 cases of MM. Main patient characteristics were as follows: median age = 63 years; stage I, II, and III: 36, 19, and 45%, respectively; Ig-G, Ig-A, light chain M component 64, 27, and 6%, respectively; 51% with at least one bone lesion; 31% chromosome 13 deletion ($n = 70$); median level of β2-m = 2.8 mg/l. Sixteen patients were untreated. Among the 85 treated patients, 37 received an intensive treatment. In MM, the sHLA-G median value was 31.45 ± 20.09 ng/ml (range 4.8–124.5) and was significantly increased ($P < 0.001$) compared to the controls (Fig. 3). Statistical studies did not reveal a significant correlation with neither β2m nor global survival. However, the functional consequences of this increase in 30% of patients remain to be investigated.

6. Concluding remarks

HLA-G expression could be observed at different levels during lymphoproliferative disorders: despite a common transcription of its gene, HLA-G protein expression is rarely observed at cell surface on tumoral cells [31] in our experience contrasting with the frequent sera detection of the soluble isoform. The factors inducing this expression are probably dependent on microenvironment factors such as cytokines. Indeed the capacity of some cytokines to induce HLA-G expression has been previously demonstrated in vitro in monocytic lineage [31,32]. The HLA-G modulation by IFNγ was also shown in other models such as myoblasts [37], glioma [20] and ovarian carcinoma cells [38]. Further evidence of this HLA-G-inducing role of cytokines was suggested by the in vivo association between IL10 expression and HLA-G protein in cutaneous lymphomas [26] and lung carcinomas [25]. The regulatory mechanisms controlling IFN-γ [39] or IL10 [32] induced HLA-G gene activation are both transcriptional or post-transcriptional depending on the cell type and involve regulatory pathways not shared by other classical class I genes [40,41]. Additional arguments are in favour for a general role of the microenvironment such as those of lung or skin. In addition to malignancies, HLA-G protein was often evidenced in several pulmonary diseases [42] or in chronic skin diseases [43,44]. The frequency of HLA-G protein in cutaneous NHL in contrast to that of lymph node or leukemic disorders could be partially explained by the difference of lymphomatous localisation.

In addition, an HLA-G positive case is observed in an EBV-induced NHL in AIDS suggesting the potential role of other factors such as viral infection. The induction of HLA-G molecules have been yet shown in activated macrophages harbouring human CMV (HCMV) infection [45] or in peripheral monocytes and lymphocytes during HIV-1 infection [46]. Thus the production in vivo of IL10 by tumoral cells in AIDS lymphomas has previously been reported [47] and could subsequently favour the appearance of HLA-G expression. HLA-G protein should be researched in EBV-induced lymphoproliferative disorders such as HIV-related lymphomas, post-transplant lymphoproliferative disorders and nasal NK/T lymphomas. These factors do not allow to exclude the intrinsic role of tumoral cells since HLA-G expression have been frequently found in transformation of low grade lymphomas in association with HLA class I loss [33]. These data suggest that HLA class I abnormalities may occur as secondary event during lymphomagenesis. Environmental factors or intrinsic abnormalities of tumoral cell could be involved in HLA-G protein expression.

The decreased HLA class I expression could be observed in lymphomas similarly to other solid tumours [48]. This inverse balance of HLA class I and HLA-G expression observed in half of NHL cases have also been reported in lung carcinomas [25] and melanoma metastasis unresponsive to IFNα2β treatment [49]. This differential expression of classical HLA class I versus HLA-G genes is in accordance with their tight transcriptional control as suggested by divergence in their regulatory modules [40,41]. However, the partial decrease of HLA class Ia molecules is not a sufficient nor necessary condition to induce HLA-G expression in lymphoproliferative disorders (Table 1) or in solid tumours as previously reported [22–24]. The dissociation between transcriptional activity and protein expression in lymphoid malignancies has prompted us to investigate the presence of soluble isoforms in sera. sHLA-G detected in healthy subjects [34] sera was poorly investigated in tumoral pathologies except in melanomas [27] for which an elevated sHLA-G serum level was detected. Using specific ELISA, we found increased sHLA-G level in 60% of B and T lymphoid malignancies and in 30% of myeloma cases. These results suggest that sHLA-G secretion is more frequent than its cell surface expression in our experience. Even if no correlation with clinical and biological data has been yet evidenced, the variations in sHLA-G serum level such as its increase in lymphoid malignancies or its decrease in septic shocks (personal data) suggest a functional relevance. The functional property of HLA-G5 similarly to HLA-G1 concerns the modulation of primary and secondary immune responses suggesting an important role during tumoral process as well as transplantation [50,51]. Gene transfer experiments of HLA-G5 as well HLA-G1 in glioma [20] or muscle rhabomyosarcoma [21] cell lines rendered these cells resistant to alloreactive lysis by direct inhibition of natural killer and CD4, CD8 T cells. Further HLA-G5 reduced alloproliferation [19], interfered with effective priming of antigen-specific cytotoxic T cells and reduced antigen-specific alloreactive lysis [20,21]. We can speculate that the negative effects of HLA-G5

on T function described in allogeneic situations could be applied in autologous reactions such as anti-tumoral response. An increased secretion of HLA-G soluble isoform, may have similar negative immunomodulatory effects than membrane-bound isoforms in favouring escape of tumoral cells from immune response. Thereby, HLA-G through its soluble isoform might constitute a new way of immune escape for lymphoid proliferations. Further clinical and biological data are currently collected to perform statistical studies on a larger series of lymphoid malignancies in order to assess a correlation between sHLA-G and prognosis. These studies may provide a clinical basis for using membrane or sHLA-G modulation as a relevant goal in lymphoproliferative disorders.

Acknowledgements

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