Journal of Immunology Research

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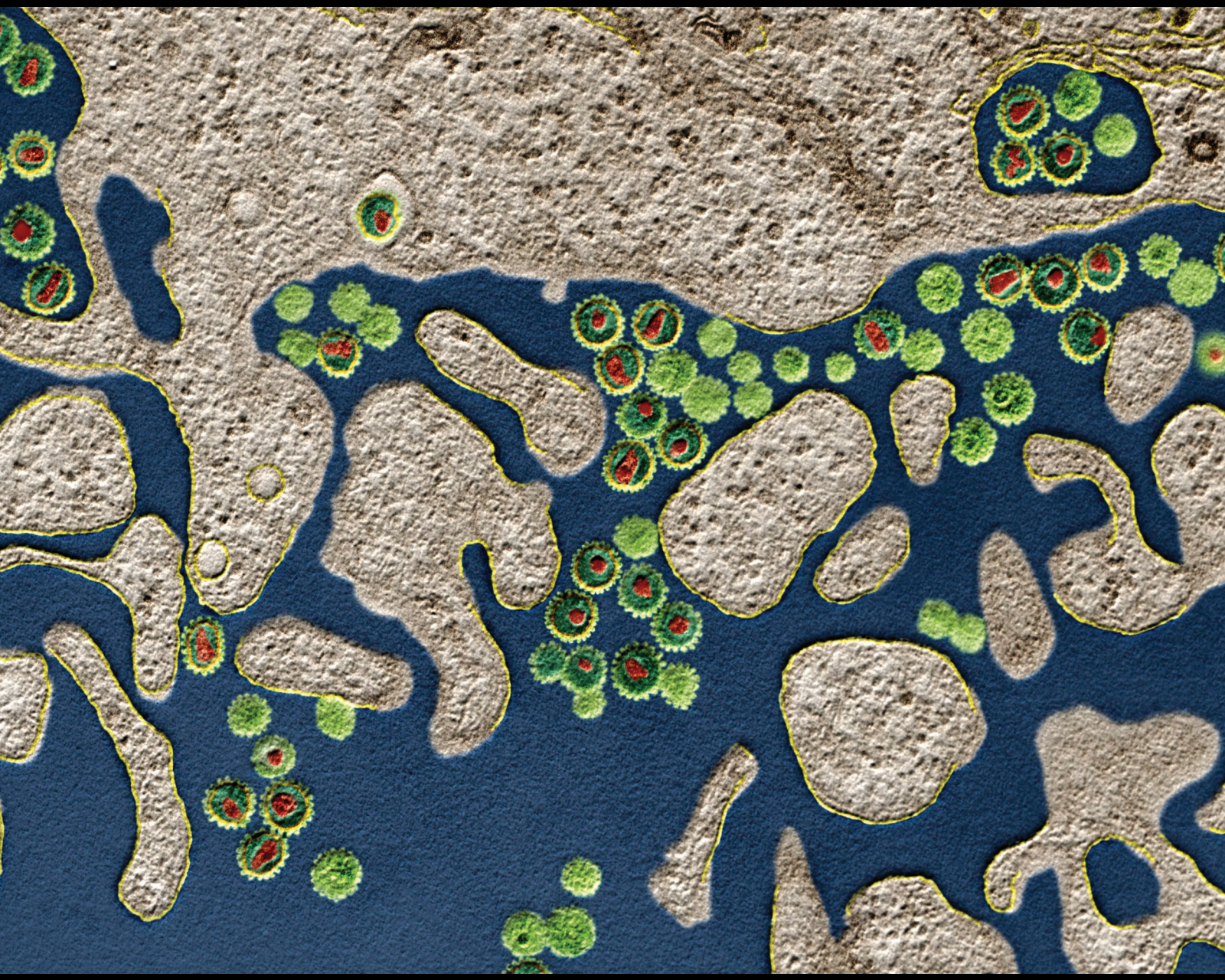
Guest Editors: Fabio Morandi, Enrico Fainardi, Roberta Rizzo, and Nathalie Rouas-Freiss



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Editorial

The Role of HLA-Class Ib Molecules in Immune-Related Diseases, Tumors, and Infections

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HLA-class Ib family include HLA-E, -F, -G, and -H molecules that, in contrast with high polymorphic HLA-class Ia molecules (HLA-A, -B, and -C), display a limited polymorphism, with a small number of alleles encoding limited functional proteins. Similar to HLA-class Ia molecules, HLA-class Ib molecules bind peptides generated from cytosolic antigens and present them to $CD8^+$ T lymphocytes, but their main function is the regulation of immune responses, both in physiological and pathological conditions.

HLA-G, the best characterized HLA-Ib molecule, is expressed on fetal cytotrophoblast cells during pregnancy and abrogates maternal NK cell cytotoxicity against fetal tissues. However, HLA-G is also expressed (or released as soluble molecules) by different cells and tissues in pathological contexts such as tumors, grafted organs, pathogen-infected cells, and inflammatory tissues. HLA-G interacts with specific receptors on T and B lymphocytes, NK cells, neutrophils, and antigen presenting cells, inhibiting their function. HLA-E is virtually expressed by all nucleated cells and binds peptides derived from HLA-class I molecules leader sequence. In physiological conditions, it interacts with CD94/NKG2A inhibitory receptor on NK cells, inhibiting their cytotoxicity against cells displaying normal HLA-class I molecules expression. When such molecules are downregulated (i.e., transformed or infected cells), HLA-class I-derived peptides are lower and subsequently HLA-E expression is dampened, allowing NK cells lo lyse these cells. However, different transformed cells upregulate HLA-E expression to avoid NK-cell mediated lysis. Limited information is available regarding HLA-F function. This molecule acts as chaperone for the β2-microglobulin-free heavy chain of other HLA-class I molecules, and it is expressed on the surface of activated lymphocytes. So far, no functional HLA-H molecules encoded by HLA-H genes have been characterized.

In the present issue dedicated to these nonclassical HLA-class Ib molecules, two papers have analyzed some basic aspects of HLA-G. First, E. C. Castelli et al. have reviewed some interesting data regarding the regulation of HLA-G gene expression. They analyzed different mechanisms that operate at transcriptional and posttranscriptional level, with emphasis on polymorphisms that are present both in the untranslated and coding region. Second, E. Alegre et al. have described the factors involved in the generation of different HLA-G isoforms. Moreover, the authors analyzed the data present in the literature regarding posttranslational modifications of HLA-G molecules and the expression and function of different HLA-G specific receptors.

The murine homologue of HLA-G and –E and Qa2 and Qa1 has been analyzed by B. L. Melo-Lima et al., who have provided some interesting data regarding the expression of these molecules during fetal and postnatal development of thymus and other tissues.

Three papers have explored the role of HLA-G in physiological conditions. The review by M. Dahl et al. has analyzed factors that regulate HLA-G expression during pregnancy, with emphasis on differential spatiotemporal expression of HLA-G. Moreover, the authors analyzed functional differences between membrane-bound and soluble HLA-G. F. Montespan et al. have demonstrated that human mesenchymal stem cells derived from bone marrow or adipose tissue maintain HLA-G expression and consequently retain their immunoregulatory properties after osteodifferentiation, thus suggesting that these cells may be used for bone repair. Finally, E. Alegre et al. have analyzed HLA-G biochemistry with special emphasis to the mechanisms that regulate its expression and how the protein modifications affect the quantification.

The role of HLA-G in pathological conditions is the key topic of this special issue. M. Ezeakile et al. have demonstrated that the presence of HLA-G dimers in kidney transplant patients prolonged kidney allograft survival. L. Amiot et al. have analyzed data regarding HLA-G expression in patients with bacterial, viral, or parasitic infections, with particular attention on factors released by parasites that may affect HLA-G expression. Novel interesting data regarding HLA-G expression and function in human tumors have been described in two papers. G. Locafaro et al. have demonstrated that HLA-G expressing DC-10 and $CD4^+$ T cells are highly represented in AML patients with HLA-G^+ blasts and may contribute to immune escape of tumor cells. In contrast, M. J. Rutten et al. have demonstrated that HLA-G expression on tumor cells in ovarian carcinoma patients is an independent prognostic factor that predicts a better overall and event-free survival and response to chemotherapy.

Finally, the expression and function of HLA-E have been investigated by F. Morandi et al., whohave demonstrated that IL-27 treatment upregulated HLA-E expression on human monocytes, rendering the latter cells immunosuppressive, through the inhibition of IFN-γ secretion by NK cells.

In conclusion, the papers in this issue will help to enrich the current knowledge regarding HLA-Ib molecules and their role in physiological and pathological conditions.

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Research Article

HLA-G Expression Is an Independent Predictor for Improved Survival in High Grade Ovarian Carcinomas

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Aberrant expression of human leukocyte antigens (HLA) class I has prognostic importance in various cancers. Here, we evaluated the prognostic value of classical (A/B/C) and nonclassical (G/E) HLA expression in 169 high grade epithelial ovarian cancer samples and linked that to clinicopathological characteristics and survival. Expression of HLA-A, -B/C, or -E was not correlated with survival. Survival was prolonged when tumours expressed HLA-G ($P = 0.008$) and HLA-G was an independent predictor for better survival ($P = 0.011$). In addition, HLA-G expression was associated with longer progression-free survival ($P = 0.036$) and response to chemotherapy ($P = 0.014$). Accordingly, high expression of HLA-G mRNA was associated with prolonged disease-free survival ($P = 0.037$) in 65 corresponding samples. Elevated serum-soluble HLA-G levels as measured by enzyme-linked immunosorbent assay in 50 matched patients were not correlated to HLA-G protein expression or gene expression nor with survival. During treatment, sHLA-G levels declined ($P = 0.038$). In conclusion, expression of HLA-G is an independent prognostic factor for improved survival in high grade epithelial ovarian cancer and a predictor for platinum sensitivity.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynaecological cancer and the second cause of cancer related death among women [1]. Stage at diagnosis, result of debulking surgery, histological type, and response to chemotherapy all influence the prognosis [2–4]. All patients are treated with cytoreductive surgery and adjuvant combination chemotherapy, usually consisting of paclitaxel and carboplatin. Although response to treatment is high, approximately 70% of patients with advanced disease develop recurrence, suggesting that effectiveness of treatment protocols is low.

Morphologically and histologically, there are large differences within epithelial ovarian cancer, suggesting different patterns of development. Therefore, a classification based on a dualistic model of carcinogenesis is developed [5]. Type I tumours are low grade, are slowly growing, and develop from premalignant stage towards malignant lesion, while type II tumours are high grade, more aggressively growing, and often present at an advanced stage [6–8]. However, even within type II tumours, large differences exist regarding response to treatment and patient survival.

Molecular variations or interference of the immune system with the disease process may be accountable for this [9, 10]. As in other solid tumours like breast, colon, and cervical cancer, in epithelial ovarian cancer expression of human leukocyte antigens (HLA) is also associated with prognosis [9, 11–14]. The role of these molecules is to present intracellular peptides to cytotoxic T cells and herewith trigger an adequate immune response against the aberrant cells [15]. In contrast, nonclassical HLA expression is thought to play a role in immune tolerance by inhibiting natural killer (NK) cell-mediated lysis [16–21]. Studies have also indicated that soluble HLA-G (sHLA-G) could play a role in suppressing the functions of various immune competent cells [22–24].

Downregulation of classical HLA is associated with an unfavourable prognosis in ovarian cancer [25, 26]; however, the association of HLA-G with prognosis is controversial

[12, 27–32]. Upregulation of HLA-G by IL6, IL8, or IL10 was suggested to help cancer cells evade the immune response by inhibition of NK cell- and CTL-mediated lysis [33–35]. However, the opposite was seen in melanoma cells [30]. The same controversies regarding prognosis are seen between studies analysing HLA-G expression in ovarian cancer. These controversies could be due to the fact that heterogeneous groups of patients were analysed [7, 36]. As HLA-G is frequently expressed in high grade ovarian tumours and almost never in low grade tumours, the role of this molecule could be different within these tumour types [37]. To optimize treatment regimens it is important to search for prognostic markers in homogenous cohorts of patients.

The purpose of this study was to evaluate whether expression of classical and nonclassical HLA influences survival in a cohort of 169 clinically well-characterized high grade epithelial ovarian cancer patients. In addition, we analysed HLA-G gene expression and serum sHLA-G concentration in all available matched frozen biopsies and serum samples, respectively.

2. Material and Methods

2.1. Patients and Material. This study includes patients treated for primary epithelial ovarian cancer in the Gynaecologic Oncologic Centre of the Academic Medical Centre Amsterdam and one of its referral hospitals, the Deventer Hospital, between 1993 and 2010. Haematoxylin/eosin (H&E) stained slides of the tumours were retrieved from the pathology department archives and were reviewed. Histological features, including histological grade, were assessed by an experienced pathologist (MV) blinded to the clinical data. In total, 169 patients with type II, high grade serous and undifferentiated tumours, were selected. Representative tumour areas were marked on the H&E slides to be cored for the array blocks. Furthermore, corresponding frozen tissue of 65 type II tumours was collected for RNA isolation. From 50 patients matched serum samples were available.

Clinical data of these patients were obtained from a prospectively maintained database at the Department of Gynaecologic Oncology at the Academic Medical Centre Amsterdam and missing data were abstracted from patient charts. Staging of the disease was done according to the criteria of International Federation of Gynaecologists and Obstetricians (FIGO). All patients were treated with primary debulking surgery (PDS) or interval debulking surgery (IDS) and a platinum based combination chemotherapy if indicated by disease stage. Patients who had more than one centimetre of residual tumour after primary debulking surgery underwent an interval debulking, solely when there was no progression during chemotherapy treatment. Progression-free survival (PFS) and disease specific survival (DSS) were calculated from the date of first surgery or start of chemotherapy to the date of progression, death, or last follow-up, respectively. Result of surgery was scored as no macroscopic disease or any residual tumour. Furthermore, sensitivity to platinum containing chemotherapy was classified according to moment of recurrence. When recurrence occurred within 6 months after the last cycle of chemotherapy was given, tumours were classified as platinum resistant. If recurrence occurred more than one year after the last cycle of chemotherapy was given, tumours were classified as platinum sensitive [38, 39]. The tissue and serum as well as the clinical data were used according to the guidelines of the Medical Ethical Committee of the Academic Medical Centre Amsterdam.

2.2. Tissue Microarray. To construct the tissue microarrays (TMA), formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected from the pathology archive of both hospitals. We retrieved FFPE samples of primary tumour or metastasis at surgical intervention, which was either before treatment with chemotherapy or after 2 cycles of chemotherapy. Of each tumour, 3 representative 0.6 mm diameter cores were taken, resulting in 20 to 24 tumours on each TMA block (Beecher, Silverspring, MD, USA, TMA instrument). Sections of 4 μm were obtained from each TMA block and placed on coated glass slides to allow for immunohistochemical staining.

2.3. Immunohistochemistry and Evaluation of Staining. For HLA class I staining, antibodies against HLA-A (HCA2) and HLA-B/C (HC10) heavy chains were used (a kind gift from Professor Dr. Jaques Neefjes, NKI, Amsterdam, The Netherlands). Normal epithelium of benign ovarian cysts and liver and renal tissue served as control. In addition, 4 μm sections of the TMAs were stained with the mouse monoclonal antibodies clone MEM-E/02 (MCA2193, AbD Serotec, Kidlington, UK) and clone 4H84 (LifeSpan Biosciences, WA, USA) against HLA-E and HLA-G, respectively.

First, the tissue sections were deparaffinised and rehydrated using graded concentrations of ethanol to distilled water; endogenous peroxidise activity was blocked with 0.03% H_2O_2/MeOH for 20 minutes. Antigen retrieval was performed in boiling 0.01 M citrate buffer (pH 6.0) for 12 minutes. After 2 hours of cooling in citrate buffer, slides were washed twice in distilled water and twice in phosphate-buffered saline (PBS). Subsequently, incubation was performed overnight at room temperature with the primary antibodies diluted in PBS containing 1% bovine serum albumin. Second, sections were incubated with BrightVision polyhorseradish peroxidase anti-mouse/rabbit/rat IgG (Immunologic BV, Duiven, The Netherlands) for 30 minutes at room temperature. Washing between incubations was performed 3 times for 5 minutes in PBS. Immune complexes were visualized by applying a 0.05 M *tris*-HCl buffer (pH 7.6) containing 0.05% of 3,3′-diamino-benzidine-tetrahydrochloride and 0.0018% of H_2O_2. After 10 minutes, the reaction was stopped by rinsing with demineralised water. Finally, the tissue sections were counterstained with Mayer's haematoxylin before addition of a coverslip.

Immunohistochemical staining was scored without prior knowledge of clinical parameters and based on the intensity and percentage of positively stained tumour cells. The percentage of positively stained tumour cells was scored from 0 to 5: absent (<1%, 0), sporadic (1–5%, 1), local (6–25%, 2), occasional (26–50%, 3), majority (51–75%, 4), or large

majority (>75%, 5). The staining intensity was scored from 0 to 3 to reflect negative (0), weak (1), moderate (2), or strong (3) staining intensity. Samples stained for classical HLA class I were categorized in one of three categories of expression based on the sum of the scores: normal expression, total score 7-8; weak, 3–6; and total loss, 0–2. Any variation in expression was considered as downregulation (score ≤ 6).

For HLA-G, membrane or combined membrane and cytoplasmic expression were considered positive. Scoring of HLA-G and -E was similar to classical HLA class I scoring, except that labelling of categories was different (strong expression 7-8; low expression 3–6; and no expression 0–2). Samples with any expression (>3) were considered unregulated. HLA-E positivity was also analysed using 5 or higher as cut-off as described earlier for ovarian cancer [12]. To obtain high concordance rate with whole tissue slides, a minimum of 2 cores with representative tumours tissue had to be present on the TMA to be used in statistical analyses.

2.4. Total RNA Isolation and Microarray Hybridization. RNA was isolated from frozen sections that contained at least 50% of tumour collected at surgery for ovarian cancer. Of these, 65 samples matched with tissue used to construct the TMAs. For this study, we used HLA-G gene expression data of these 65 matched cases.

For each specimen, 30 sections of 20 μm were cut for RNA isolation. Total RNA was isolated and extracted using RNA Bee (Amsbio, UK) and the RNeasy Mini kit (DNAse-treated; Qiagen, The Netherlands) and amplified using the Illumina TotalPrep RNA Amplification kit (Ambion, Life Technologies). Samples with RIN values ≥7, as evaluated using the Bioanalyzer (Agilent, California, USA), were used for genetic analyses. RNA of the 65 samples was hybridized to Illumina Human HT-12 v4 Expression BeadChip 47 K Arrays (Illumina, CA, USA). The arrays provide intensity data for each probe or probe set, indicating a relative level of hybridization with the labelled target. Genes that were not detected above background level in at least one sample were excluded from the analysis. The data were then normalized by applying between array simple scaling (i.e., mean centring between arrays) and a subsequent log2 transformation.

2.5. sHLA-G Enzyme-Linked Immunosorbent Assay. Serum samples were collected from patients visiting the department of gynaecologic oncology and stored at −80°C. Samples were collected before starting the treatment, after 2 courses of chemotherapy, at the end of treatment, and at time of recurrence. Of those patients whose tissue was used to construct TMAs, we could retrieve serum of 50 patients.

sHLA-G was quantified using a commercially available ELISA kit (Exbio, Prague, Czech Republic) according to the manufacturer's instructions. Briefly, calibrators and samples were incubated in microtitration wells precoated with monoclonal antibody, mouse anti-HLA-G monoclonal antibody (MEMG/9), which recognized the most abundant soluble isoforms (shedded sHLA-G1 and intron4 containing secreted HLA-G5). After 18 hours of incubation and washing, monoclonal anti-human beta2-microglobulin antibody labelled with horseradish peroxidase (HRP), which recognized the immobilized antibody sHLA-G complex, was added to the wells and incubated for 60 minutes. Following rinsing, the substrate solution (H_2O_2 with tetramethylbenzidine) was added to react with the remaining HRP-conjugated antibody. After the addition of acidic stop solution, the absorbance of the resulting yellow product was measured at 450 nm using a microplate reader (BIO-RAD Model 550, CA, USA). According to absorbance values proportional to sHLA-G concentrations of calibrators, a calibration curve was constituted and used to determine the sHLA-G concentrations of serum samples. The interassay coefficient variation of the human sHLA-G ELISA kit was 1.3–16.9% and the limit of detection was 2 U/mL. The ELISA was performed in a blinded manner.

2.6. Statistical Analysis. Association of clinicopathologic parameters with HLA expression was analysed with two-sided Chi-square tests. To correlate loss of expression or upregulation of classical and nonclassical HLA with clinicopathologic parameters or survival, expression of classical HLA class I was analysed as normal expression versus downregulation. Nonclassical HLA was analysed as no expression versus upregulation.

Survival was expressed as progression-free survival (PFS) and disease specific survival (DSS). For DSS and PFS, the Kaplan-Meier method and log-rank test were used to calculate difference between groups with and without expression of classical and nonclassical HLA. To determine whether clinicopathologic characteristics as well as HLA class I expression were related to survival, univariable and multivariable Cox regression analyses were performed.

Correlation of sHLA-G and HLA-G expression in tissue was analysed with Spearman's rho. Differences in concentration of sHLA-G before, during, or after treatment and at recurrence were compared between matched cases using the Wilcoxon-rank test for the median. In all cases P values < 0.05 were considered statistically significant. Statistical analysis was performed in IBM SPSS (Version 19.0, IBM SPSS, Chicago, IL).

Gene expression levels of HLA-G were analysed using R2: microarray analysis and visualization platform (http://r2.amc.nl/). Differential gene expression analysis of HLA-G of tissue with and without HLA-G protein expression and sHLA-G concentration was performed with ANOVA. For survival analyses, Kaplan-Meier method and log-rank test were used to calculate differences between groups with expression levels above and below the median.

3. Results

3.1. Patient Characteristics. Patients with high grade epithelial ovarian carcinoma treated with primary or interval debulking surgery were included ($n = 169$). From 141 patients, ovarian tissue was available of which 108 samples were collected before chemotherapy started.

Median age at diagnosis was 60 years (range 36–88 years). The majority of cases had advanced stage disease ($n = 154$)

Table 1: Patient characteristics.

Baseline characteristics	All patients (n = 169)
Mean age, years (SD)	61 (11.7)
FIGO stage	
I	8 (5%)
II	7 (4%)
III	125 (74%)
IV	29 (17%)
Histologic classification	
High grade serous	141 (83%)
Undifferentiated	28 (17%)
Residual disease after debulking surgery	
No macroscopic tumour	37 (22%)
Less than 1 cm residual disease	46 (27%)
1 cm or more residual disease	86 (51%)
Kind of debulking surgery	
PDS	134 (79%)
IDS	35 (21%)
Tissue origin	
Ovary	141
Metastases	28
Kind of chemotherapy given	
None	9 (5%)
Single drug platinum	5 (3%)
Multidrug carboplatin/paclitaxel	123 (19%)
Multidrug with platinum	32 (73%)
Amount of cycles	6 (5–7)
Platinum sensitivity	
Refractory disease	34 (20%)
Platinum resistant disease	36 (21%)
Partial chemosensitive	25 (15%)
Platinum sensitive disease	65 (38%)

Refractory disease: recurrence occurred during chemotherapy treatment; platinum resistant disease: recurrence occurred within 6 months after the last cycle of chemotherapy; partial platinum sensitive disease: recurrence occurred after 6 months or within 1 year after last cycle of platinum based chemotherapy; platinum sensitive disease: recurrence occurred more than 1 year after the last cycle of chemotherapy.

and residual disease after debulking surgery (n = 132). Median follow-up was 31 months (0.7–186); 125 patients had recurrence or progression. Median DSS was 38 months (95% CI 29–47) and PFS was 15 months (95% CI 12–18). Clinicopathologic characteristics are summarized in Table 1.

3.2. Correlation of HLA Class I Protein Expression and Clinicopathologic Characteristics. Expression of all classical and nonclassical HLA class I molecules could be evaluated in 137 tumours (81%) (Figure 1). Downregulation of HLA-A occurred in 74.6% of the cases; in 37.2%, there was no expression at all. Downregulation of HLA-B/C was observed in 82.7%; in 46%, tumours expression was totally absent. HLA-E and HLA-G upregulation was observed in 73.4% and 47.9%, respectively. Clinical factors related to HLA protein expression were residual disease after surgery and sensitivity to chemotherapy (Table 2). In univariable analysis, residual disease after surgery (OR: 2.42; 95% CI 1.06–5.52) and sensitivity to platinum based chemotherapy (OR: 2.10; 95% CI 1.05–4.19) were significantly related to upregulation of HLA-G. In multivariable analysis sensitivity to platinum based chemotherapy (OR: 2.46; 95% CI 1.05–5.75) was independently associated with HLA-G upregulation. Furthermore, residual disease after surgery was independently associated with HLA-A downregulation (OR 2.86; 95% CI 1.17–7.03).

Besides, combined absence of HLA-A and HLA-G expression, which was correlated to residual disease after surgery (P = 0.038), combination of expression levels of HLA-E and -G or classical and nonclassical HLAs, did not correlate with clinicopathologic characteristics.

When correlating HLA expression with survival, expression of HLA-G was significantly correlated with a favourable prognosis. Both PFS and DSS were significantly better when tumours expressed HLA-G. PFS was 19 months and decreased to 6 months, if there was no expression (P = 0.038). DSS of patients with HLA-G expression was 56 months versus 30 months, when there was no expression of HLA-G (P = 0.008). Five-year survival of patients with tumours expressing HLA-G was significantly better (P = 0.001) (Figure 2(a)). In the group of patients of whom ovarian tissue was collected before chemotherapy treatment started (n = 108), PFS for HLA-G positive cases was 28 months versus 16 months for those lacking expression (P = 0.027). DSS advantage in this group was 40 months (P = 0.011).

In univariate analysis, advanced stage disease (HR 4.7; 95% CI: 1.16–19.17), IDS (HR 1.8; 95% CI: 1.15–2.91), macroscopic residual tumour (HR 2.6; 95% CI: 1.57–4.43), and lack of expression of HLA-G (HR 1.69; 95% CI: 1.14–2.51) were related to worse survival. In multi-variate analysis, HLA-G expression remained an independent prognostic factor for improved survival (P = 0.020), as well as no residual disease after surgery and PDS. However FIGO stage did not retain prognostic significance (Table 3).

Downregulation of HLA-A resulted in a shorter PFS, 14 months (95% CI 11.1–16.9) instead of 25 months (95% CI 15.5-34.5) (P = 0.047), when there was normal expression. Disease specific survival at five-year follow-up was not different for patients with tumours lacking HLA-A expression (Figure 2(b)). Within the whole follow-up period, DSS was worse, when there was downregulation (36 versus 58 months); however, this was not significant (P = 0.066). Expression or absence of expression of HLA-B/C and -E (any upregulation or with a cut-off of 5) or combination of HLA-E/G did not influence prognosis. Also when stratified for classical HLA class I expression no difference in survival was observed according to HLA-E and/or -G expression (Figure 2(c)).

3.3. HLA-G Gene Expression in Frozen Biopsies and sHLA-G Concentration in Serum of High Grade EOC Patients. The median level of HLA-G gene expression in tumour tissue was 230.38 fluorescence units. Patients with high HLA-G gene

Table 2: Correlation of HLA-A, HLA-B/C, HLA-G, and HLA-E expression with clinical parameters.

	HLA-A			HLA-B/C			HLA-E			HLA-G		
	Normal expression	Downregulation	*P*	Normal expression	Downregulation	*P*	Upregulation	Normal expression	*P*	Upregulation	Normal expression	*P*
N (%)	51 (36)	91 (64)		29 (17)	124 (83)		124 (83)	28 (17)		81 (53)	71 (47)	
Variables												
Age												
<60	30	42	0.15	14	63	0.78	65	10	0.26	40	38	0.61
>60	21	49		12	61		59	15		33	41	
FIGO stage												
Stages I-IIA	4	4	0.39	1	6	0.83	5	2	0.39	3	3	0.87
Stage >IIB	47	87		25	118		119	23		78	68	
Residual tumor												
No residual	18	14	**0.006**	11	22	**0.006**	27	4	0.51	23	10	**0.033**
Any residual	33	77		15	102		97	21		58	61	
Platinum sensitivity												
Resistant	15	40	0.08	9	48	0.66	52	9	0.66	25	35	**0.014**
Sensitive	33	46		15	65		66	14		53	32	

Table 3: Cox regression analysis on disease specific survival including clinicopathological factors and HLA expression.

Disease specific survival	Univariable analysis			Multivariable analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (years)	1.01	0.99–1.03	0.185			
FIGO stage						
Low (I-IIA)	1			1		
High (IIB–IV)	4.70	1.16–19.17	0.030	3.74	0.48–28.99	0.207
Kind of surgery						
PDS	1			1		
IDS	1.80	1.15–2.91	0.011	2.48	1.49–4.15	0.001
Residual disease						
Microscopic	1			1		
Macroscopic	2.60	1.57–4.43	<0.000	2.21	1.21–4.02	0.009
HLA-A expression						
Normal	1					
Downregulation	1.30	0.85–1.98	0.231			
HLA-B/C expression						
Normal	1					
Downregulation	1.13	0.68–1.88	0.647			
HLA-E expression						
Normal	1					
Upregulation	1.31	0.75–2.27	0.341			
HLA-G Expression						
Normal	1.69	1.14–2.51	0.009	1.62	1.08–2.42	0.020
Upregulation	1					

HR: hazards ratio, CI: confidence interval, Ref.: referent, PDS: primary debulking surgery, IDS: interval debulking surgery.

expression had a significant better prognosis than those with low expression for 5-year disease specific survival ($P = 0.027$) (Figure 2(d)). Protein expression of HLA-G in tumour tissue was not correlated with HLA-G gene expression ($P = 0.43$) and also no correlation was observed between serum sHLA-G levels and protein expression ($R = 0.26$, $P = 0.066$) or sHLA-G levels and gene expression.

The concentration of sHLA-G in serum at start of treatment had a median of 10.3 U/mL (interquartile range (IQR) 3.0–23.5). Median concentration at the start of the treatment was higher than after 2 cycles of chemotherapy 5.7 U/mL (IQR 3.0–7.3) ($P = 0.038$). Concentration of sHLA-G decreased during the treatment and increased at recurrence (Figure 3). At recurrence, levels of sHLA-G were equally high as at the start of the treatment (median 7.4 U/mL (IQR 2.8–27.5)). Finally, no difference was seen in disease specific survival of patients with sHLA-G levels in serum above the median ($n = 19$) and below the median ($n = 17$) ($P = 0.70$).

4. Discussion

In this study, we analysed expression of classical and non-classical HLA class I in high grade ovarian carcinomas. Upregulation of HLA-G expression was an independent predictor for improved survival. Furthermore, our analyses showed that HLA-G expression in these tumours is correlated with residual disease after surgery and sensitivity to platinum chemotherapy. In addition, loss of expression of HLA-A resulted in shorter progression-free survival. The expression or loss of expression of other classical and nonclassical HLA molecules in combination or alone was not associated with survival.

In concordance with protein expression, elevated gene expression of HLA-G was associated with good prognosis. However, besides an elevated concentration of sHLA-G at the start of the treatment, the concentration of serum sHLA-G did not show any prognostic relevance.

Prognostic significance of HLA class I molecules has been described in various solid as well as heamatopoetic tumours, including ovarian cancer. Several groups showed that intact HLA phenotype confers better prognosis in terms of overall survival [9, 25, 26]. Only one study, by Vitale et al. [40], did not show a survival advantage for HLA class I in ovarian cancer. Mostly, the lack of expression of classical HLA class I is associated with poor survival, whereas expression of HLA-E or -G results in a worse prognosis [12, 13, 17, 25, 35, 41, 42]. Recently, however, it was shown that not only the absence of HLA-G and -E expression but also the absence of HLA class I expression was associated with worse survival in colon cancer patients [43]. This examples the controversies which exists regarding HLA and survival, which could be due to differences in staining techniques and scoring or definition of expression.

Although expression of HLA-G is often correlated with poor survival, which is explained by inhibition of NK cell and

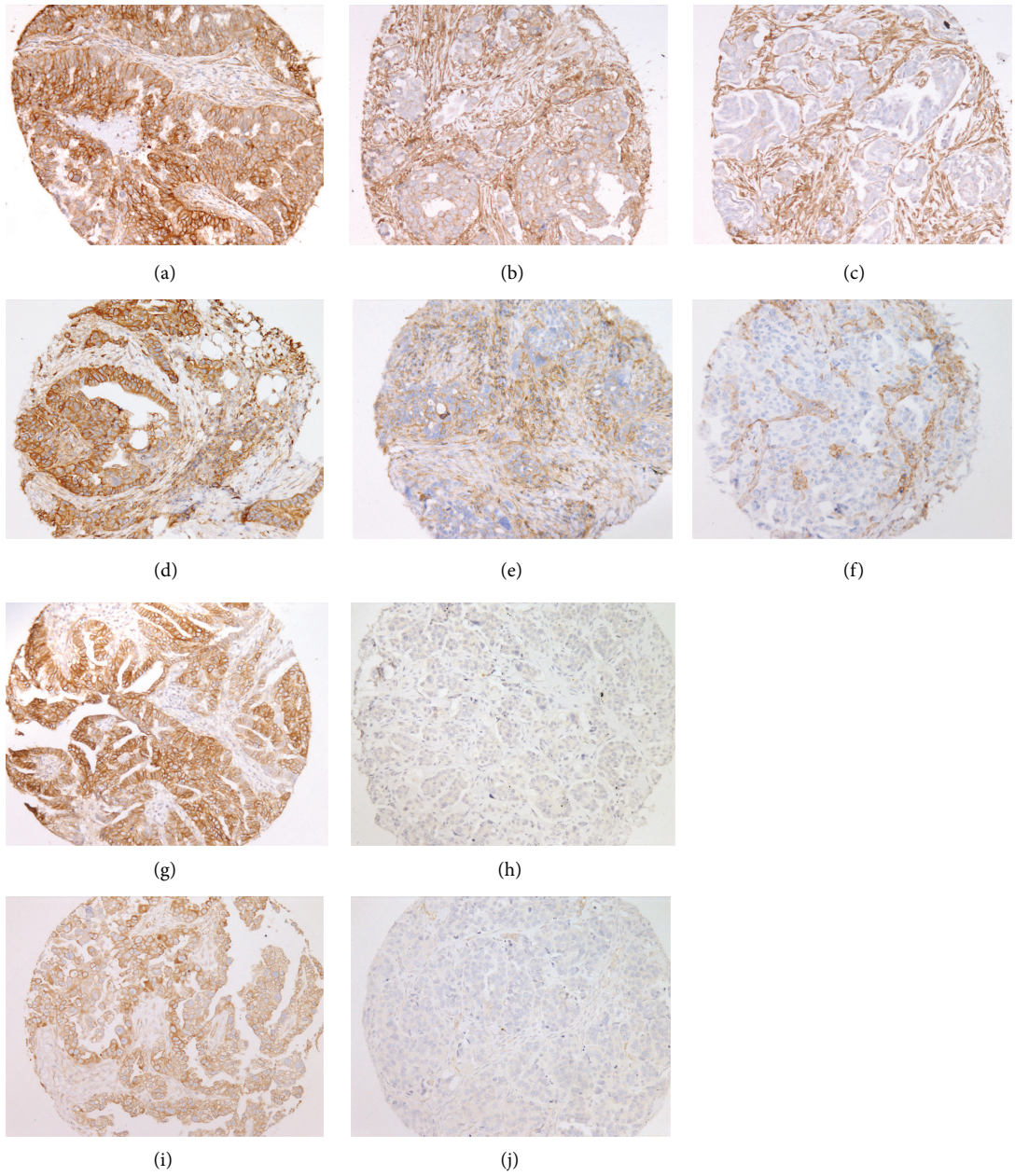

Figure 1: Representative examples of HLA immunohistochemical staining, HLA-A ((a)–(c)), HLA-B/C ((d)–(f)), HLA-E ((h)–(j)), and HLA-G ((i)-(j)). Strong expression ((a), (d), (g), and (i)), weak expression ((b) and (e)), and loss of expression ((c), (f), (h), and (j)).

CTL-mediated lyses, our data show that, in high grade EOC, expression of HLA-G is associated with a good prognosis. It is assumed that upregulation helps the tumour to evade the immune response of the host by blocking NK cell activity whilst others dispute this [27, 31]. Improved survival of patients with epithelial ovarian cancer expressing HLA-G in tumour cells in ascites has been described [31]. Yet, the biological significance of HLA-G remains uncertain at present.

HLA-G is more often expressed in high grade tumours [37, 44, 45]; therefore, it is thought that HLA-G plays a role in tumour growth and aggressive behaviour of high grade EOC. HLA-G is also thought to influence chemosensitivity of high grade EOC [31].

Furthermore, as described in melanoma cell lines, expression of HLA-G can account for susceptibility to NK-mediated lysis due to a switch in alternative splicing [30]. The primary transcript of HLA-G is alternatively spliced, producing seven different mRNA molecules encoding four membrane-bound (HLA-G1 to HLA-G4) and 3 soluble (HLA-G5-G7) protein isoforms. The susceptibility to NK-mediated lysis could be explained by a switch of alternative splicing leading to the loss of cell surface HLA-G1 and its replacement by intracellular HLA-G2, through which tumour cells become

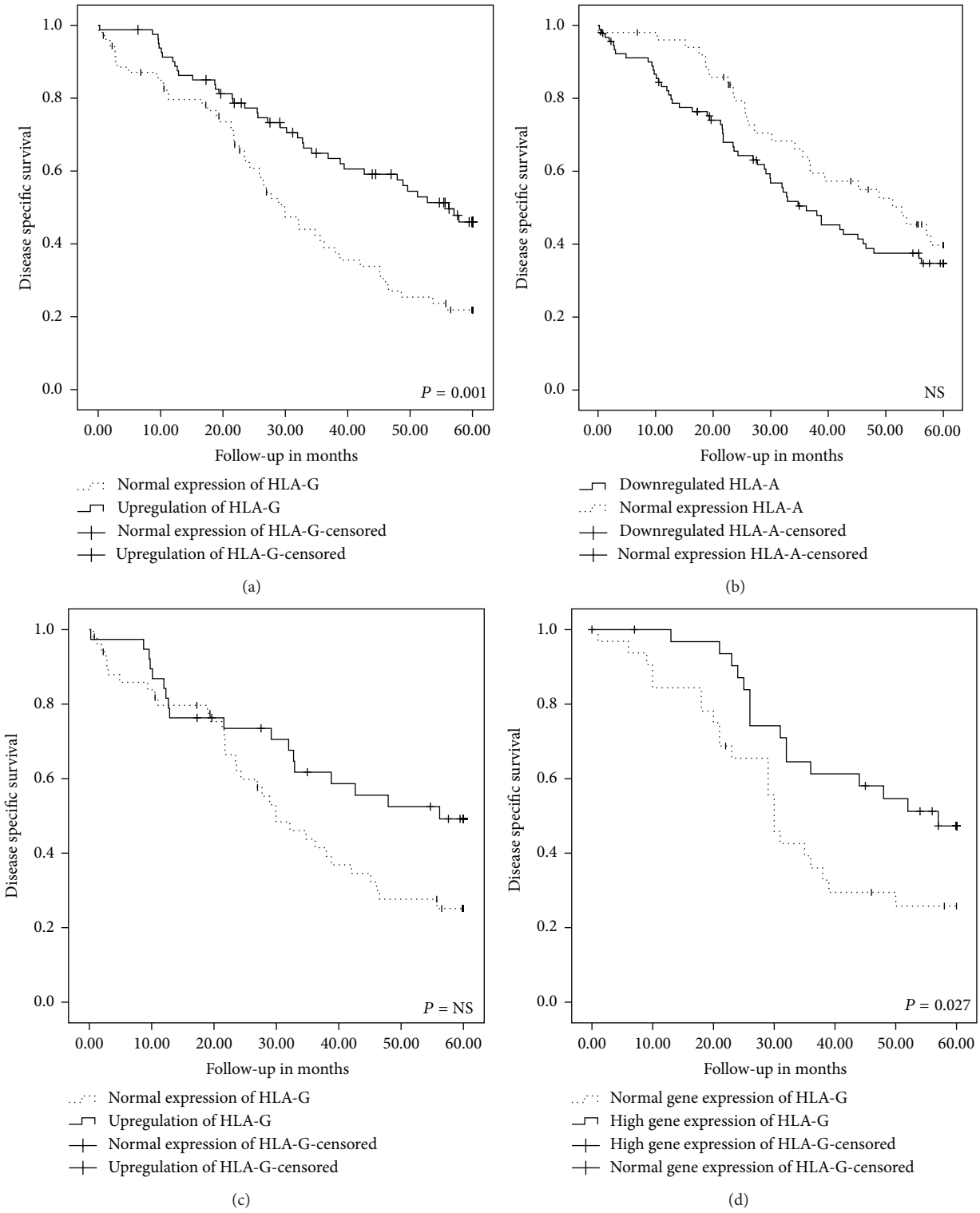

Figure 2: Survival analyses. Kaplan-Meier 5-year survival curves and log-rank test for HLA-G expression (a), HLA-A expression (b), HLA-G expression in tumours with downregulation of HLA-A (c), and HLA-G gene expression at the median expression cut-off of 230.4 fluorescence units (d) (NS = not significant).

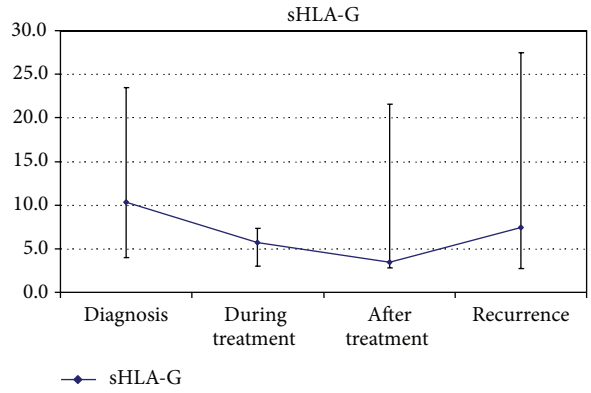

Figure 3: Median concentration of sHLA-G (u/mL) at diagnosis, after 2 cycles of chemotherapy, after primary treatment, and at recurrence. $\perp$ is the lowest interquartile and $\top$ is the highest interquartile.

more susceptible to NK cell-mediated lysis [30, 35]. Recent findings show that HLA-G interacts with KIR2DL4 on NK cells to regulate the production of cytokines and chemokines [46].

This binding leads to proangiogenic factors and inflammatory cytokines production activating the immune response. To further examine how NK and T cells respond to HLA-G, we are currently analysing the expression of KIR2DL4 on CTLs and NK cells isolated from high grade EOC patients. On the contrary, recently Lin et al. described that tumour invasiveness or metastasis correlated with HLA-G expression may rely on the induction of MMP-15 expression by HLA-G in ovarian cancer [47].

Expression of nonclassical HLAs in tumours lacking expression of classical HLA has been described in several solid tumours to influence prognosis in a negative way [28, 42, 48]. This is explained by the mechanism of downregulation of classical HLA class I resulting in escape of tumours from cytotoxic T cell immune recognition. By upregulation of nonclassical HLA, they may further escape immune recognition [35]. We could not demonstrate this in the current high grade ovarian carcinoma cohort. Although we could not indisputably demonstrate a significant correlation between HLA-G gene expression and expression at the protein level, high gene expression was found to be associated with a good prognosis. There is evidence of upregulation of HLA-G mRNA in response to transformation, neovascularisation, inflammation, and infection [49]. Increased vascularity may suggest improved tumour oxygenation and drug delivery and thereby improved response to chemotherapy [50]. High gene expression of HLA-G could reflect highly vascularised tumours and susceptibility to chemotherapy.

In contrast, detection of soluble HLA-G in ascites was described to be higher in malignant ascites of ovarian and breast cancer than in ascites of benign disease [44], but detection of sHLA-G in serum of ovarian cancer patients has not been described. Although the concentration of sHLA-G at diagnosis was higher than after the treatment, in the present study, no prognostic value of sHLA-G could be demonstrated. Schütt et al. described in a subgroup of lung cancer patients with squamous cell carcinoma a better prognosis for patients with low concentrations of sHLA-G [51]. Similar to the findings in our study, no prognostic significance for sHLA-G concentration was observed in breast, renal cell, and esophageal carcinoma [52–54].

Expression of HLA-G on tumour cells in malignant effusions has been demonstrated to be related to survival and proposed as a possible marker of tumour susceptibility to chemotherapy [31]. In concordance with the findings of Davidson et al., who found reduced expression of HLA-G in effusions obtained after the start of chemotherapy, we found a reduced concentration of sHLA-G in serum in samples obtained during and after treatment. This could indicate a role in susceptibility to chemotherapy.

In order to validate the use of HLA-G expression as a predictive marker in selecting patients for treatment, independent, large, and homogeneous cohorts should be evaluated to make definite conclusions on the prognostic influence of HLA expression in EOC.

In conclusion, this is the first study to describe the correlation of classical and nonclassical HLA class I expression levels with survival in high grade epithelial ovarian cancer and to correlate both membrane-bound and soluble HLA-G protein and HLA-G gene expression. The here presented data show that, in high grade epithelial ovarian carcinomas, HLA-G expression is an independent parameter for improved survival.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Osteodifferentiated Mesenchymal Stem Cells from Bone Marrow and Adipose Tissue Express HLA-G and Display Immunomodulatory Properties in HLA-Mismatched Settings: Implications in Bone Repair Therapy

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Mesenchymal stem cells (MSCs) are multipotent cells that can be obtained from several sources such as bone marrow and adipose tissue. Depending on the culture conditions, they can differentiate into osteoblasts, chondroblasts, adipocytes, or neurons. In this regard, they constitute promising candidates for cell-based therapy aimed at repairing damaged tissues. In addition, MSCs display immunomodulatory properties through the expression of soluble factors including HLA-G. We here analyse both immunogenicity and immunosuppressive capacity of MSCs derived from bone marrow and adipose tissue before and after osteodifferentiation. Results show that HLA-G expression is maintained after osteodifferentiation and can be boosted in inflammatory conditions mimicked by the addition of IFN-γ and TNF-α. Both MSCs and osteodifferentiated MSCs are hypoimmunogenic and exert immunomodulatory properties in HLA-mismatched settings as they suppress T cell alloproliferation in mixed lymphocyte reactions. Finally, addition of biomaterials that stimulate bone tissue formation did not modify MSC immune properties. As MSCs combine both abilities of osteoregeneration and immunomodulation, they may be considered as allogenic sources for the treatment of bone defects.

1. Introduction

Bone is among the most frequently transplanted tissues with about 1 million procedures annually in Europe. Despite their considerable disadvantages, including the risk of disease transfer and immunologic rejection, limited supply of bone, costs, and complications, allografts and autografts account for more than 80% of total graft volume. Significant growth opportunities exist for synthetic bone grafts in association with mesenchymal stem cells (MSCs) from autologous or allogenic sources as alternatives to biological bone grafts in orthopaedic and maxillofacial surgery [1, 2]. In a classical approach, bone tissue engineering consists of harvesting bone marrow from a patient, isolating MSCs by their adherence to tissue culture plastic, expanding and differentiating those cells in culture to a sufficient number, and then seeding them onto a suitable synthetic scaffold prior to implantation into the same patient [3].

MSCs can be isolated from different tissues including bone marrow (BM), adipose tissue (AT), and perinatal sources [4]. Many reports highlighted the immunomodulatory properties of MSCs relying on three

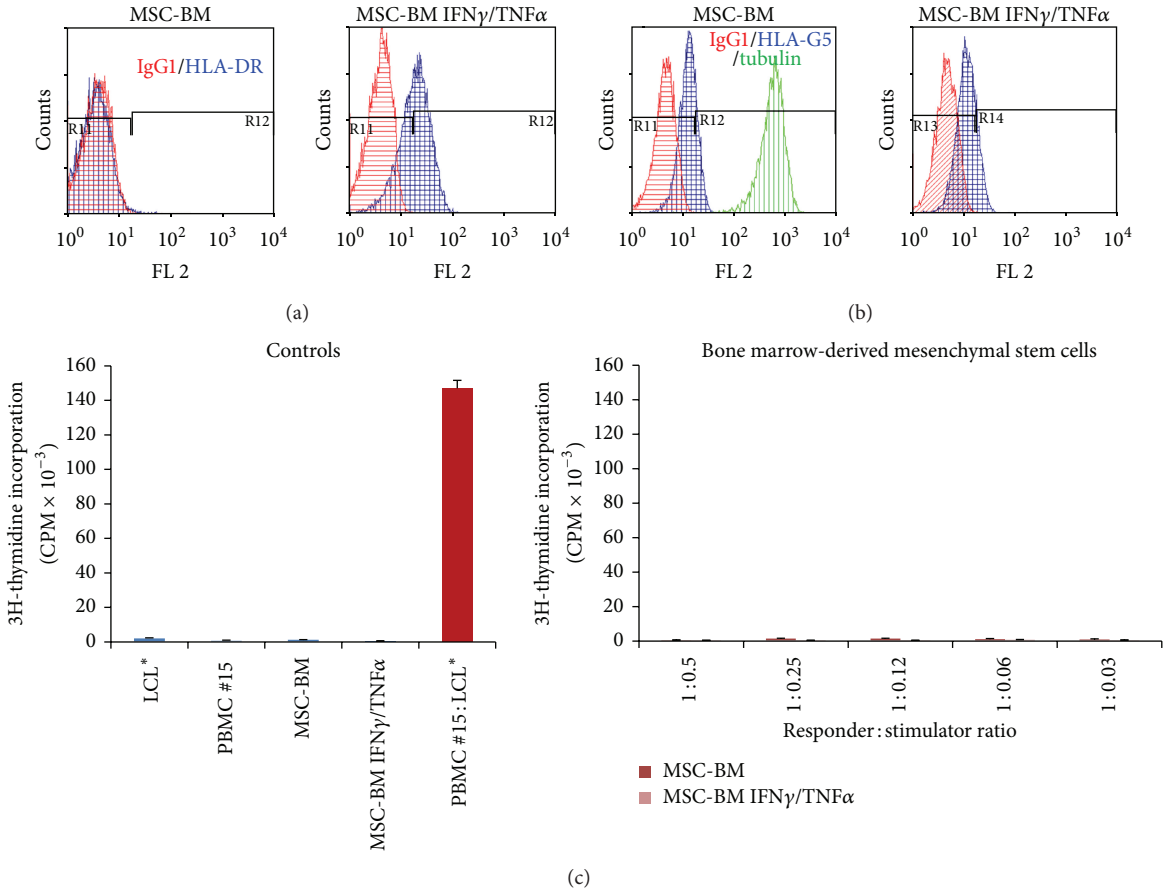


Figure 1: BM-derived MSCs express HLA-G and are hypoimmunogenic. (a) Expression of HLA-DR molecules was evaluated by flow cytometry analysis on BM-derived MSCs pretreated with IFNγ and TNFα (MSC-BM IFNγ/TNFα) or not (MSC-BM). IgG1 was used as isotype control Ab. (b) Expression of HLA-G5 was evaluated by intracellular flow cytometry analysis on BM-derived MSCs pretreated with IFNγ and TNFα (MSC-BM IFNγ/TNFα) or not (MSC-BM). Tubulin was used as positive control of cell permeabilization. (c) PBMC from healthy individual (#15) were used as responder cells towards BM-derived MSCs pretreated with IFNγ and TNFα (MSC-BM IFNγ/TNFα) or not (MSC-BM) as stimulating cells at various responder : stimulator ratios. Irradiated LCL* were used as positive control of T cell alloproliferation. Results are given as mean cpm ± s.e.m.; one representative experiment is shown.

main mechanisms: (1) cell cycle arrest of immune cells at the G1 phase, (2) direct interaction with immune cells, and (3) paracrine effect through secretion of various factors including HLA-G, prostaglandin E2, cytokines (TGFβ, IL6, IL10, HGF, VEGF, etc.), and enzymes (indoleamine 2,3-dioxygenase and inducible nitric oxide synthase) [5–8]. Based on these tolerogenic properties, allogenic MSCs are currently tested in various clinical trials [9, 10].

HLA-G molecules expressed by mesenchymal stem cells fulfill an important function since blockade of HLA-G using HLA-G neutralizing antibodies could reverse MSC ability to (i) generate *in vitro* the expansion of $CD4^+CD25^+$ $FoxP3^+$ regulatory T cells, (ii) inhibit the alloproliferative T cell response, and (iii) suppress the cytotoxic function of NK cells. These results show that HLA-G molecules, mainly soluble HLA-G5, actively contribute to the immunosuppressive properties exerted by MSCs [11, 12].

In clinical trials aimed at repairing bone defects, the main objective is to develop new biomaterials that simulate bone issue formation in combination with MSCs. In this context, our work entailed assessing, from an immunological perspective, whether allogenic MSCs could be used without a risk of rejection instead of autologous MSCs. The results obtained *in vitro* validate this hypothesis since the MSCs proved to be hypoimmunogenic and immunosuppressive in allogenic conditions. Moreover, following infusion in bone, MSCs may undergo osteodifferentiation process under the influence of *in vivo* osteogenic factors. We thus evaluated whether (1) allogenic MSCs committed to osteodifferentiation process can be rejected or not due to histoincompatibility and (2) combination with biomaterials modifies MSCs immune properties.

2. Materials and Methods

2.1. Isolation of PBMC. PBMC were isolated from blood of healthy volunteer donors (after informed consent) from the French Blood Establishment (EFS, Saint-Louis Hospital,

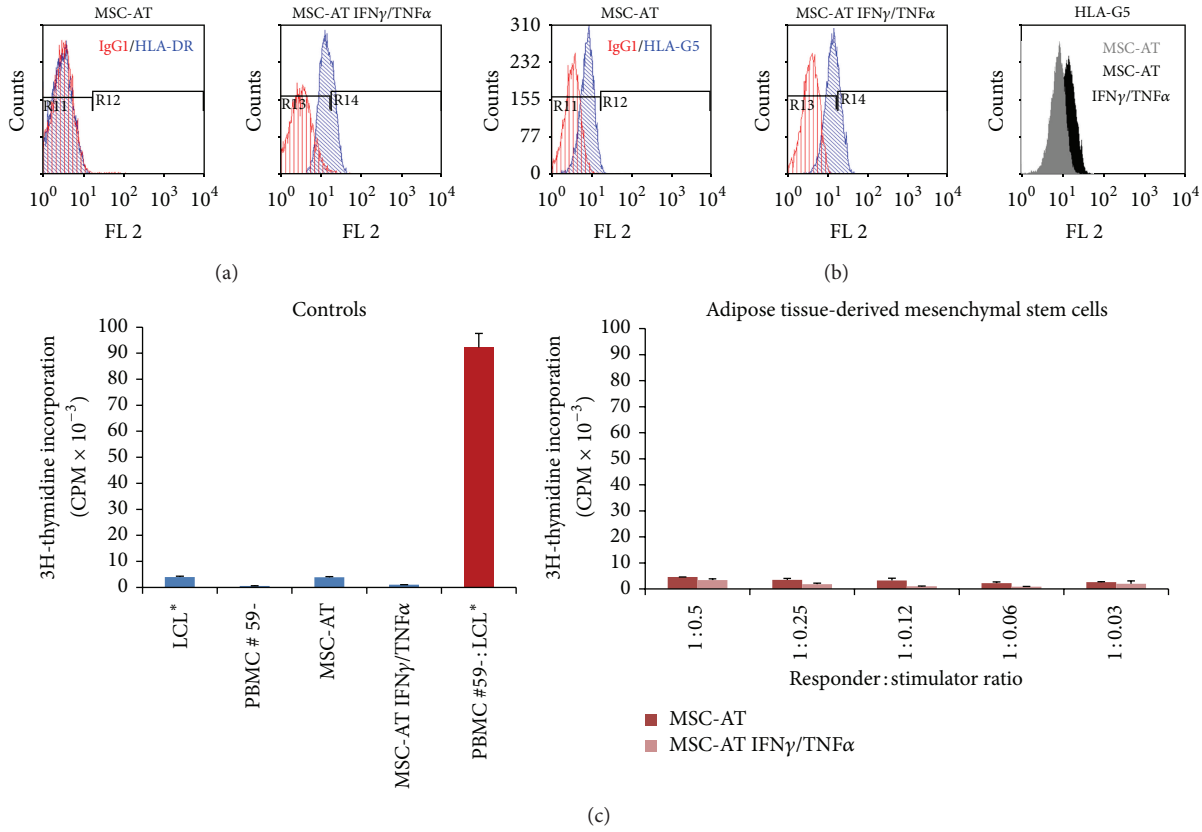


Figure 2: AT-derived MSCs express HLA-G and are hypoimmunogenic. (a) Expression of HLA-DR molecules was evaluated by flow cytometry analysis on AT-derived MSCs pretreated with IFNγ and TNFα (MSC-AT IFNγ/TNFα) or not (MSC-AT). IgG1 was used as isotype control Ab. (b) Expression of HLA-G5 was evaluated by intracellular flow cytometry analysis on AT-derived MSCs pretreated with IFNγ and TNFα (MSC-AT IFNγ/TNFα) or not (MSC-AT). (c) PBMC from healthy individual (#59-) were used as responder cells towards AT-derived MSCs pretreated with IFNγ and TNFα (MSC-AT IFNγ/TNFα) or not (MSC-AT) as stimulating cells at various responder : stimulator ratios. Irradiated LCL* were used as positive control of T cell alloproliferation. Results are given as mean cpm ± s.e.m.; one representative experiment is shown.

Paris, France) by density-gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare). These cells were used as HLA-mismatched responding cells in MLR.

2.2. In Vitro Osteodifferentiation. MSCs from BM or AT were obtained from Reborne consortium center (http://www.reborne.org/). Before experiments, MSCs were thawed and expanded through seeding 1000 cells/cm^2 in T75 flasks. When cultures reach 60–70% confluence cells were harvested and seeded for immunological assays. The osteodifferentiated MSCs used as stimulating cells in MLR were obtained as previously described [13]. Briefly, MSCs were cultured in osteoblastic differentiation medium consisting of αMEM, FBS, ascorbic acid, NaH_2PO_4, and BMP-4 during 14 to 23 days.

2.3. Preparation of MSC-Biomaterial Complex For Immunological Tests. 24-well (ultralow attachment) plates containing disks of MBCP+ (macroporous biphasic calcium phosphate) granules with the same diameter of each well bottom were prepared in collaboration with Biomatlante (France) to achieve a complete adherence of seeded MSCs to the biomaterial and then a complete induction of osteoblastic differentiation of MSCs. The disks of MBCP+ were washed for 48 hours before use to avoid any toxic effect on MSCs. Then MSCs were seeded on MBCP discs during 5 days in order to colonize homogenously the particles. Then, MSC-biomaterial complex was tested in mixed lymphocyte reactions.

2.4. Mixed Lymphocyte Reaction (MLR) Using MSC as Either Stimulating Cells Or Third-Party Cells Facing HLA-Mismatched PBMC as Responder Cells. PBMC from different donors were used as responder cells and MSCs or osteodifferentiated MSCs from BM or AT were used as either allogenic stimulating cells or third-party cells at various ratios. The HLA class II$^+$ human B-lymphoblastoid cell line LCL 721.221 (ATCC) was used as positive control to stimulate HLA-mismatched PBMC. When LCL cells were used as stimulator cells, a 75 Gy dose irradiation was given and responder: stimulator ratio was of 1 : 0.5 with a final concentration of PBMC responder cells of 10^5 cells/well in a 96-well flat bottomed plate.

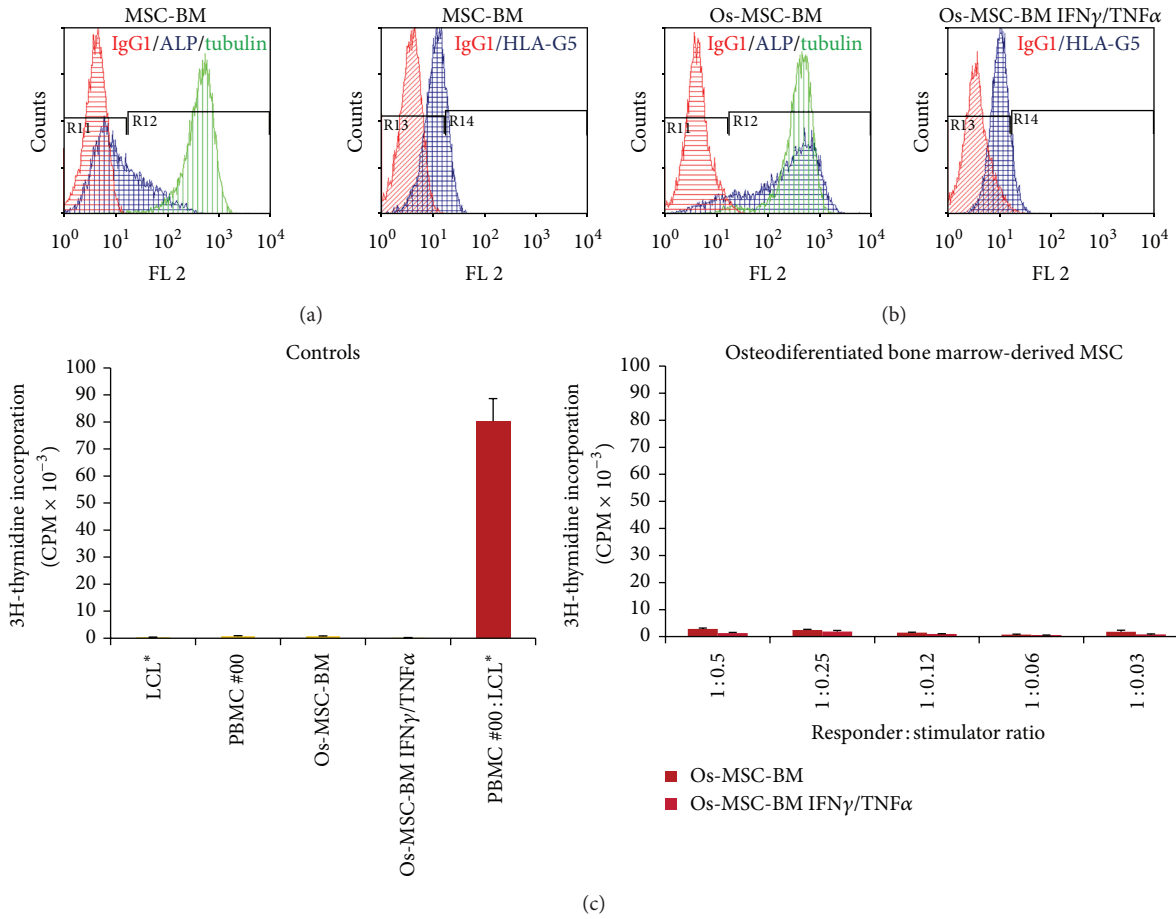


Figure 3: BM-derived MSCs committed to osteodifferentiation process express HLA-G and are hypoimmunogenic. (a and b) Expression of ALP and HLA-G5 was evaluated by intracellular flow cytometry analysis on BM-derived MSCs committed to 14-day osteodifferentiation process and pretreated with IFNγ and TNFα (Os-MSC-BM IFNγ/TNFα) or not (Os-MSC-BM). IgG1 was used as isotype control Ab. Tubulin was used as positive control of cell permeabilization. (c) PBMC from healthy individual (#00) were used as responder cells towards BM-derived MSCs committed to osteodifferentiation process and pretreated with IFNγ and TNFα (Os-MSC-BM IFNγ/TNFα) or not (Os-MSC-BM) as stimulating cells at various responder: stimulator ratios. Irradiated LCL* were used as positive control of T cell alloproliferation. Results are given as mean cpm ± s.e.m.; one representative experiment is shown.

Particularly, in MBCP experiments, MLR were performed in 24-well bottomed plates with a final concentration of PBMC responder cells of 10^6 cells/well. Experiments comparing the effects of standard culture condition (no MBCP) and three-dimensional culture setting using MBCP discs were both performed in 24-well bottomed plates.

Cultures were incubated at 37°C in a humidified 5% CO_2 air atmosphere. PBMC proliferation was measured at day 6 by [^{3}H]-thymidine incorporation (1 μCi/well, Perkin Elmer) during the last 18 hours of culture. Cells were then harvested on filtermats A and thymidine incorporation into DNA was quantified, using a *beta* counter (Wallac 1450, Pharmacia). All samples were run in triplicate. The influence of MSC licensing with inflammatory cytokines such as IFN-γ at 10 ng/mL (Peprotech) and plus TNF-α at 15 ng/mL (R&D systems) was analyzed by adding these cytokines in cultures 48 hours before MLR [14].

2.5. Flow Cytometry. The cytokine (IFN-γ and TNF-α) treatment efficiency was checked by cytofluorometry analysis through the upregulation of HLA-DR expression on MSC. Briefly, cells were washed in PBS and stained with the anti-HLA-DR conjugated with PE (Beckman Coulter) in PBS 2% heat-inactivated fetal calf serum for 30 minutes at 4°C. Control aliquots were stained with an isotype-matched antibody to evaluate nonspecific binding to target cells. Fluorescence was detected by using the Epics XL4 flow cytometer (Beckman Coulter, Brea, CA, USA).

The expression of the HLA-G5 soluble isoform by MSCs was assessed by using the 2A12 mAb (Exbio) after cell permeabilization. The osteodifferentiation process was verified by cytofluorometry analysis through the induction of alkaline phosphatase (ALP) expression in osteodifferentiated MSC after cell permeabilization. Briefly, cells were first permeabilized by using saponin (Sigma) and then stained with 2A12 or anti-ALP (R&D systems) for 30 minutes at 4°C. After washing, cells were subsequently stained with an F(ab′)2 goat anti-mouse IgG antibody conjugated with PE (Beckman Coulter) for 30 minutes at 4°C. Control aliquots were stained with an isotype-matched antibody to evaluate nonspecific

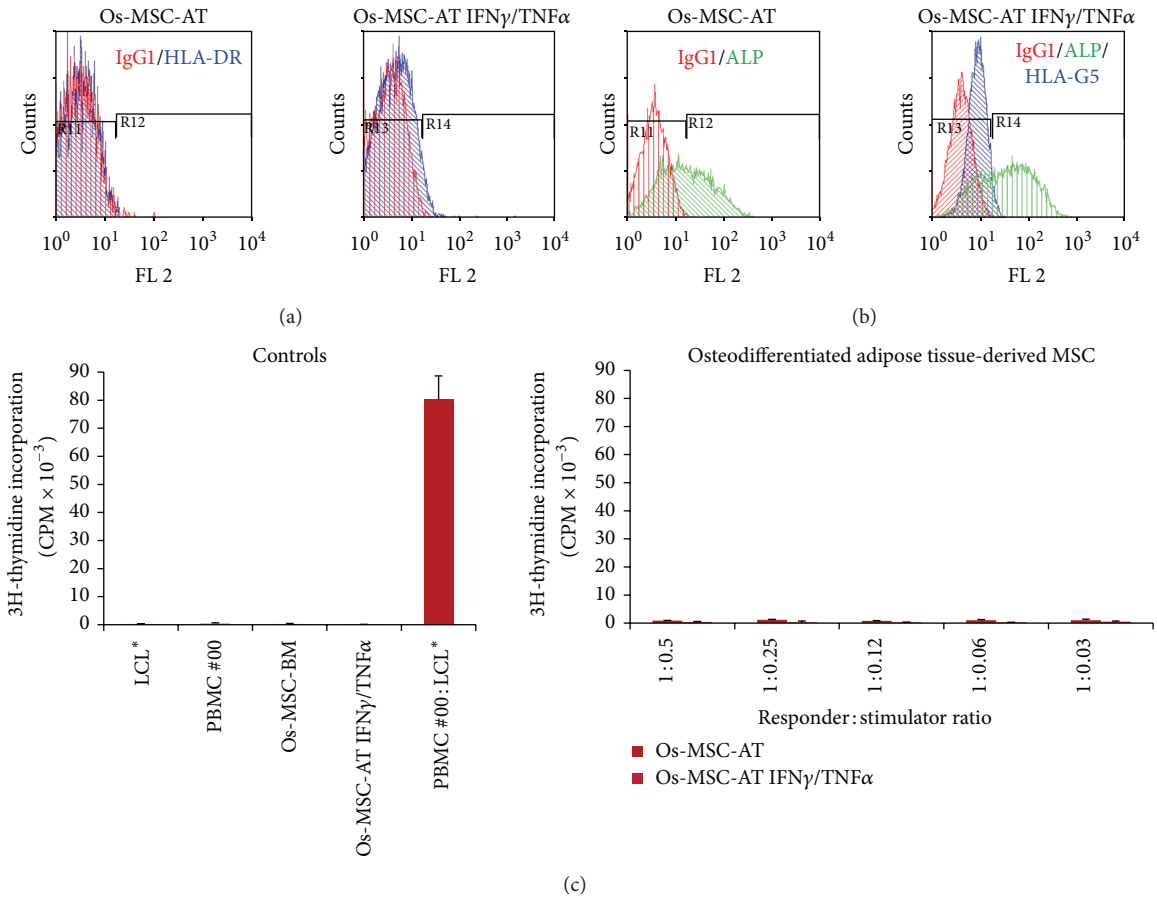


Figure 4: AT-derived MSCs committed to osteodifferentiation process express HLA-G and are hypoimmunogenic. (a) Expression of HLA-DR molecules was evaluated by flow cytometry analysis on AT-derived MSCs committed to 14-day osteodifferentiation process and pretreated with IFNγ and TNFα (Os-MSC-AT IFNγ/TNFα) or not (Os-MSC-AT). (b) Expression of ALP and HLA-G5 was evaluated by intracellular flow cytometry analysis on AT-derived MSCs committed to 14-day osteodifferentiation process and pretreated with IFNγ and TNFα (Os-MSC-AT IFNγ/TNFα) or not (Os-MSC-AT). IgG1 was used as isotype control Ab. (c) PBMC from healthy individual (#00) were used as responder cells towards AT-derived MSCs committed to osteodifferentiation process and pretreated with IFNγ and TNFα (Os-MSC-AT IFNγ/TNFα) or not (Os-MSC-AT) as stimulating cells at various responder : stimulator ratios. Irradiated LCL* were used as positive control of T cell alloproliferation. Results are given as mean cpm ± s.e.m.; one representative experiment is shown.

binding to target cells. Fluorescence was detected by using the Epics XL4 flow cytometer.

2.6. Statistical Analysis. Significance was assessed by using a nonparametric Mann-Whitney test, assuming a P value < 0.05 as significant, and was marked with $*$ in the figures.

3. Results and Discussion

Two main questions were addressed in the present study relying on whether immune regulatory properties of MSCs are modified by (1) MSC differentiation towards the osteoblastic cell lineage or (2) the addition of synthetic biomaterial (i.e., MBCP+ granules). In this regard, we analyzed both the immunogenic and immunosuppressive properties of MSCs from BM or AT in allogenic conditions, that is, facing HLA-mismatched PBMC. To identify low immunogenic MSC types, we studied their ability to be recognized as allogenic cells by HLA-mismatched PBMC in MLR using MSCs as stimulating cells and PBMC from various healthy donors as responder cells. To examine their immunosuppressive properties, we studied their ability to modulate T cell alloproliferation as third-party cells in a classical MLR. All the functional experiments were performed by considering the differentiation status of MSC, either immature or osteodifferentiated, and seeded onto biomaterial or not.

No PBMC alloproliferation was observed in response to various doses of allogenic MSCs derived from bone marrow (Figure 1(c)) or adipose tissue (Figure 2(c)) even after licensing with IFN-γ and TNF-α (Figures 1(c) and 2(c)). Tables 1 and 2 summarize the results obtained with PBMC from distinct healthy donors. The efficiency of cytokine treatment was attested by the induction of HLA-DR expression on MSCs (Figures 1(a) and 2(a)). In order to evaluate the influence of osteodifferentiation process on the immunogenicity of MSCs, similar functional assays were performed using BM-derived and AT-derived MSCs committed to preosteoblastic

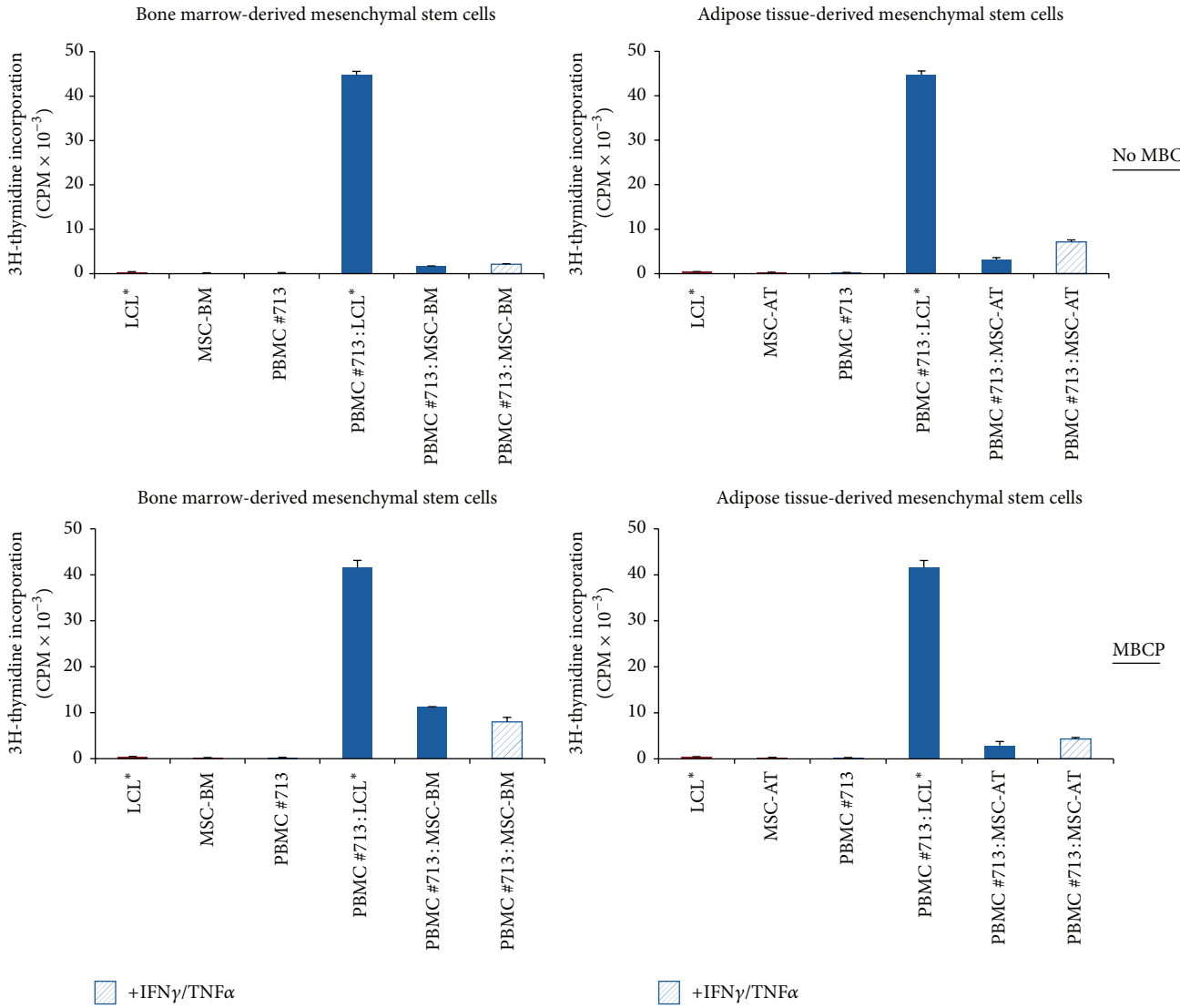
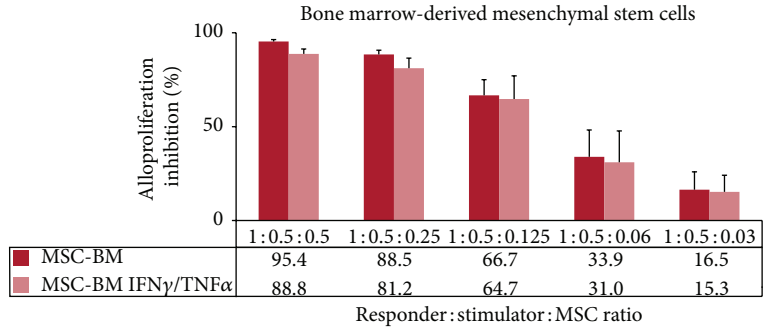


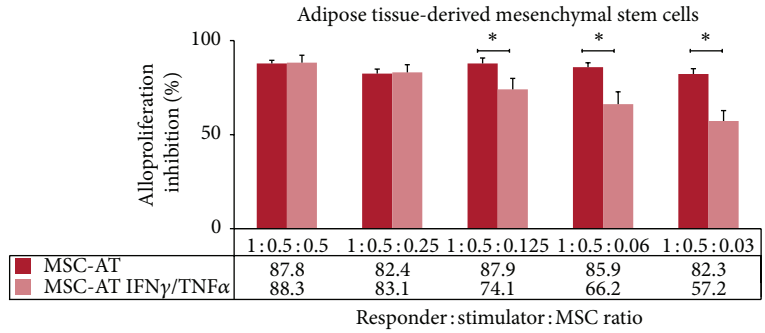
Figure 5: Both BM- andAT-derived MSCs when combined to MBCP biomaterial keep their hypoimmunogenicity. PBMC from healthy individual (#713) were used as responder cells towards either BM-derived MSCs (MSC-BM) or AT-derived MSCs (MSC-AT) that were pretreated with IFNγ and TNFα (IFNγ/TNFα) or not and used as stimulating cells at the responder : stimulator ratio of 1 : 0.2. MSCs were combined to MBCP biomaterial (MBCP) or not (No MBCP). Irradiated LCL* were used as positive control of T cell alloproliferation. Results are given as mean cpm ± s.e.m.; one representative experiment is shown.

MSCs as stimulating cells. The osteodifferentiation process was validated through the upregulation of ALP expression in osteodifferentiated MSCs (Figures 3(a), 3(b), 4(a), and 4(b)). Results show that both BM-derived and AT-derived MSCs committed to osteodifferentiation are still hypoimmunogenic whether they are pretreated or not with IFN-γ and TNF-α (Figures 3(c) and 4(c) and Tables 1 and 2). Then, we looked at whether combination of biomaterial (i.e., MBCP) with MSCs alters their immunogenicity. No differences were found between standard 2D-coculture conditions (MSC + PBMC) and 3D-coculture conditions (MSC + MBCP + PBMC). One representative allogenic combination is shown (Figure 5) for which the mean percentage of T cell alloproliferation is presented in Table 3 ($n = 3$ healthy donors). It is of note that the addition of MBCP to BM-derived MSCs treated or not with cytokines modifies slightly their immunogenicity, although no statistical difference was observed between both conditions ($P > 0.1$) (Table 3). Consequently, we can conclude that both the osteodifferentiation process and the presence of biomaterial (MBCP) did not abrogate the hypoimmunogenicity of MSCs.

Notably, expression of the tolerogenic soluble HLA-G5 protein was observed in MSCs derived from BM and AT and could be enhanced following treatment with IFN-γ and TNF-α (Figures 1(b) and 2(b)). Such enhanced expression of HLA-G by IFN-γ treatment was previously reported for other cell types such as monocytes [15], bronchial epithelial cells [16], thymic epithelial cells [17], and various tumor cells [18]. Nevertheless, HLA-G expression levels varied among the various batches of MSCs tested (data not shown). In



(a)



(b)

Figure 6: Both BM- andAT-derived MSCs display immunosuppressive properties in a dose-dependent manner. PBMC from healthy individuals were used as responder cells towards irradiated LCL* used as stimulating cells in presence of either (a) BM-derived MSCs (MSC-BM) or (b) AT-derived MSCs (MSC-AT) that were pretreated with IFNγ and TNFα (IFNγ/TNFα) or not and used as third-party cells at various responder : stimulator : MSC ratios. Results are given as mean percentage of alloproliferation inhibition ± s.e.m. according to the maximal alloproliferation observed with PBMC+LCL* using PBMC from 4 and 6 distinct healthy donors in BM- and AT-derived MSCs experiments, respectively.

Table 1: MSC from BM after osteodifferentiation and licensing by IFN-γ and TNF-α do not induce PBMC proliferation in allogenic conditions.

R : S[a] ratio	MSC-BM	MSC-BM IFN-γ/TNF-α	Os-MSC-BM	Os-MSC-BM IFN-γ/TNF-α
1 : 0.5	4.3 ± 2.1[b]	1.1 ± 0.3	3.3 ± 0.3	1.7 ± 0.2
1 : 0.25	4.5 ± 1.4	1.0 ± 0.3	3.1 ± 0.1	1.8 ± 0.5
1 : 0.12	5.3 ± 1.9	1.1 ± 0.4	3.2 ± 1.0	2.3 ± 1.2
1 : 0.06	5.4 ± 2.4	1.4 ± 0.5	2.6 ± 1.1	3.9 ± 3.1
1 : 0.03	4.3 ± 1.7	1.4 ± 0.5	2.2 ± 0.2	2.1 ± 1.2

[a]PBMC from healthy individuals were used as responder cells towards BM-derived MSCs pretreated with IFN-γ and TNF-α (MSC-BM IFN-γ/TNF-α) or not (MSC-BM) as stimulating cells at various responder : stimulator (R : S) ratios. Similar experiments were performed with BM-derived MSCs committed to osteodifferentiation process and pretreated with IFN-γ and TNF-α (Os-MSC-BM IFN-γ/TNF-α) or not (Os-MSC-BM) as stimulating cells.

[b]Data are mean ± s.e.m. of alloproliferation percentage obtained with 5 and 3 distinct healthy donors for MSC-BM and Os-MSC-BM experiments, respectively. This percentage is calculated considering PBMC stimulated with LCL* as 100% alloproliferation.

Table 2: MSC from AT after osteodifferentiation and licensing by IFN-γ and TNF-α do not induce PBMC proliferation in allogenic conditions.

R : S[a] ratio	MSC-AT	MSC-AT IFN-γ/TNF-α	Os-MSC-AT	Os-MSC-AT IFN-γ/TNF-α
1 : 0.5	5.6 ± 1.0[b]	7.2 ± 3.1	1.1 ± 0.2	0.9 ± 0.6
1 : 0.25	5.5 ± 1.5	5.2 ± 2.6	1.3 ± 0.3	0.8 ± 0.5
1 : 0.12	5.3 ± 1.5	4.7 ± 2.9	1.0 ± 0.3	1.1 ± 0.8
1 : 0.06	5.3 ± 2.0	5.5 ± 3.0	1.0 ± 0.2	1.3 ± 1.0
1 : 0.03	4.6 ± 1.1	3.9 ± 2.0	1.4 ± 0.3	1.8 ± 1.3

[a]PBMC from healthy individuals were used as responder cells towards AT-derived MSCs pretreated with IFN-γ and TNF-α (MSC-AT IFN-γ/TNF-α) or not (MSC-AT) as stimulating cells at various responder : stimulator (R : S) ratios. Similar experiments were performed with AT-derived MSCs committed to osteodifferentiation process and pretreated with IFN-γ and TNF-α (Os-MSC-AT IFN-γ/TNF-α) or not (Os-MSC-AT) as stimulating cells.

[b]Data are mean ± s.e.m. of alloproliferation percentage obtained with 4 and 3 distinct healthy donors for MSC-AT and Os-MSC-AT experiments, respectively. This percentage is calculated considering PBMC stimulated with LCL* as 100% alloproliferation.

agreement with recent findings, we observed enhanced HLA-G expression by MSCs following osteodifferentiation (Figures 3(a), 3(b), and 4(b)) [13].

Then, we investigated the immunomodulatory properties of MSCs as third-party cells in MLR. Results showed that both BM-derived and AT-derived MSCs display immunosuppressive properties in a dose-response manner (Figure 6). It is

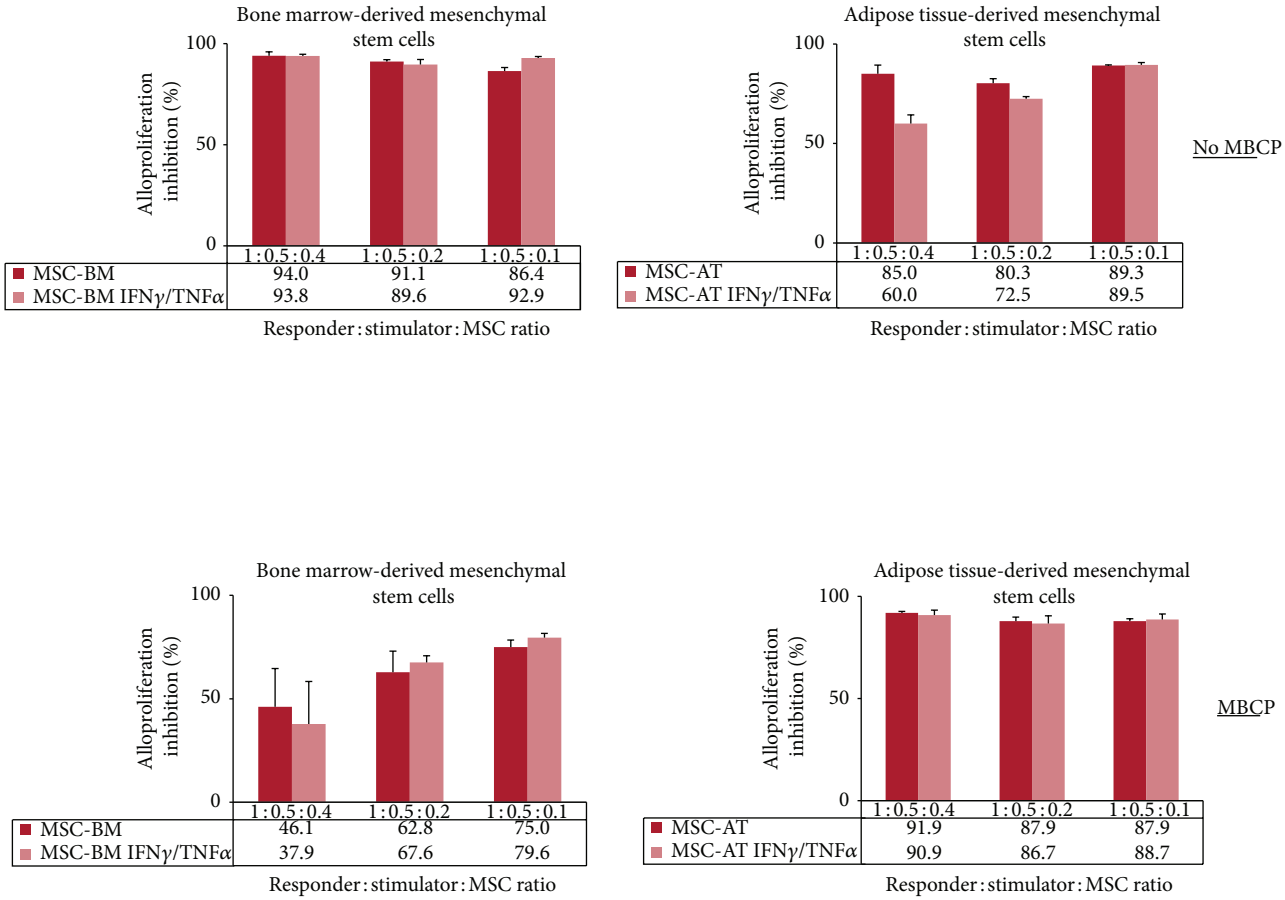


Figure 7: Both BM- andAT-derived MSCs when combined to MBCP biomaterial keep their immunosuppressive properties. PBMC from healthy individuals were used as responder cells towards irradiated LCL* used as stimulating cells in presence of either BM-derived MSCs (MSC-BM) or AT-derived MSCs (MSC-AT) that were pretreated with IFNγ and TNFα (IFNγ/TNFα) or not and used as third-party cells at various responder : stimulator : MSC ratios. MSCs were combined to MBCP biomaterial (MBCP) or not (No MBCP). Results are given as mean percentage of alloproliferation inhibition ± s.e.m. when compared to PBMC+LCL* using PBMC from 3 distinct healthy donors.

Table 3: Immunogenicity of MSC from bone marrow and adipose tissue combined with MBCP biomaterial and licensing by IFN-γ and TNF-α.

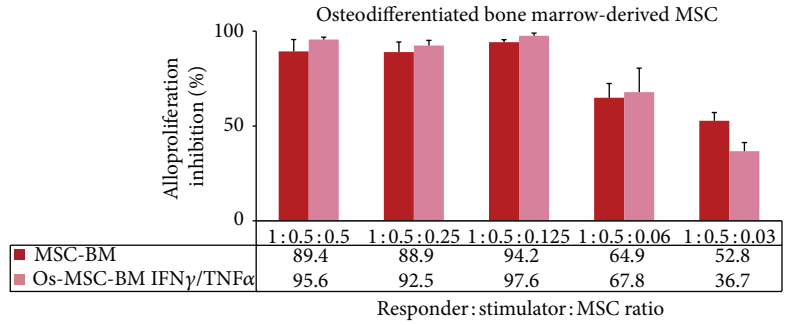
	MSC-BM	MSC-BM IFN-γ/TNF-α	MSC-AT	MSC-AT IFN-γ/TNF-α
No MBCP[a]	7.5 ± 2.2[b]	9.8 ± 4.0	13.9 ± 3.9	23.0 ± 5.2
MBCP	51.9 ± 14.1	41.1 ± 11.6	8.1 ± 0.8	11.0 ± 0.8

[a]PBMC from healthy individuals were used as responder cells towards BM- or AT-derived MSCs pretreated with IFN-γ and TNF-α or not as stimulating cells at 1 : 0.2 responder : stimulator ratio.

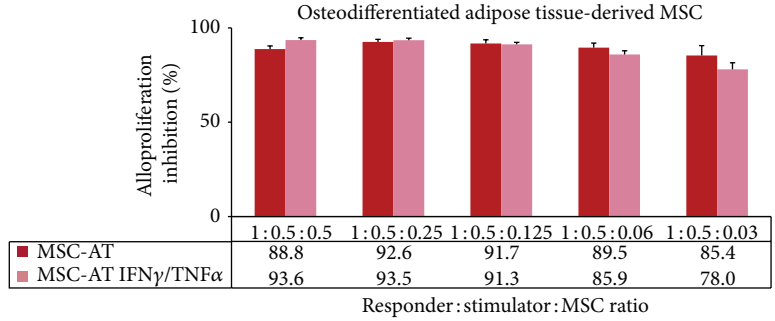
[b]Data are mean ± s.e.m. of alloproliferation percentage obtained with 3 distinct healthy donors. This percentage is calculated considering PBMC stimulated with LCL* as 100% alloproliferation.

of note that (i) AT-derived MSCs are more potent at low doses compared to BM-derived MSCs ($P < 0.05$) (Figures 6(a) and 6(b)) and (ii) licensing with cytokines reduces significantly the immunosuppressive properties of AT-derived MSCs ($P < 0.05$) (Figure 6(b)). When combined to biomaterial (MBCP), both BM-derived and AT-derived MSCs still exert immunosuppressive properties as they greatly inhibit T cell alloproliferation with or without being seeded with MBCP (Figure 7). Although not statistically significant, addition of MBCP reduces the immunomodulatory properties of BM-derived MSCs at high responder: stimulator: MSC ratios. This could be due to steric hindrance when high numbers of cells are used. Indeed, such MBCP effect is no longer observed at low ratios (Figure 7). Also, both BM-derived and AT-derived MSCs when committed to preosteoblastic MSCs inhibit T cell alloproliferation and remain thus able to induce a tolerogenic microenvironment (Figure 8). Licensing with IFN-γ and TNF-α did not modify such MSC-derived immunosuppression (Figures 7 and 8). It is of note that once osteodifferentiated AT-derived MSCs are more potent at low doses compared to BM-derived MSCs as they display higher immunosuppressive effects ($P < 0.05$) (Figure 8). Such higher immunomodulatory capacity of adipose tissue-derived multipotent stromal cells compared to their bone marrow-derived counterparts has been previously reported [19].

Our present results are in agreement with previous reports showing that differentiation of stem cells does not



(a)



(b)

Figure 8: Both BM- andAT-derived MSCs when committed to osteodifferentiation process keep their immunosuppressive properties. PBMC from healthy individuals were used as responder cells towards irradiated LCL* used as stimulating cells in presence of either osteodifferentiated BM-derived MSCs (Os-MSC-BM) or AT-derived MSCs (Os-MSC-AT) that were pretreated with IFNγ and TNFα (IFNγ/TNFα) or not and used as third-party cells at various responder : stimulator : MSC ratios. Results are given as mean percentage of alloproliferation inhibition ± s.e.m. when compared to PBMC+LCL* using PBMC from 3 and 5 distinct healthy donors in BM- and AT-derived MSCs experiments, respectively.

alter their low immunogenicity and immunomodulatory properties. For instance, human amniotic epithelial cells, which have stem cell-like properties, retain their immunosuppressive functions after differentiation into hepatocyte-like cells [20]. Also, human Wharton's jelly-derived MSCs maintain the expression of immunomodulatory molecules, such as HLA-G, when subjected to osteogenic differentiation *in vitro* [21].

In conclusion, MSCs from BM or AT display tolerogenic properties which are maintained following osteodifferentiation process or addition of biomaterial and may thus be considered as allogenic sources for regenerating bone defects in orthopaedic and maxillofacial surgery [22].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Differential Transcript Profiles of MHC Class Ib (Qa-1, Qa-2, and Qa-10) and *Aire* Genes during the Ontogeny of Thymus and Other Tissues

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Qa-2 and Qa-1 are murine nonclassical MHC class I molecules involved in the modulation of immune responses by interacting with T $CD8^+$ and NK cell inhibitory receptors. During thymic education, the *Aire* gene imposes the expression of thousands of tissue-related antigens in the thymic medulla, permitting the negative selection events. Aiming to characterize the transcriptional profiles of nonclassical MHC class I genes in spatial-temporal association with the *Aire* expression, we evaluated the gene expression of *H2-Q7*(Qa-2), *H2-T23*(Qa-1), *H2-Q10*(Qa-10), and *Aire* during fetal and postnatal development of thymus and other tissues. In the thymus, *H2-Q7*(Qa-2) transcripts were detected at high levels throughout development and were positively correlated with *Aire* expression during fetal ages. *H2-Q7*(Qa-2) and *H2-T23*(Qa-1) showed distinct expression patterns with gradual increasing levels according to age in most tissues analyzed. *H2-Q10*(Qa-10) was preferentially expressed by the liver. The *Aire* transcriptional profile showed increased levels during the fetal period and was detectable in postnatal ages in the thymus. Overall, nonclassical MHC class I genes started to be expressed early during the ontogeny. Their levels varied according to age, tissue, and mouse strain analyzed. This differential expression may contribute to the distinct patterns of mouse susceptibility/resistance to infectious and noninfectious disorders.

1. Introduction

Immune tolerance has been assigned to two broad categories according to the places where it occurs, that is, central and peripheral tolerance [1]. It is well known that nonclassical Major Histocompatibility Complex class I molecules (MHC Ib), like HLA-G and HLA-E, are associated with the regulation of immune responses in the periphery; however, little is known regarding the effect of these molecules at the central level. Human MHC Ib molecules exhibit restricted tissue distribution and do not have an important role in antigen presentation, and the coding regions of the respective genes have relatively low variability, particularly at exons that code major functional regions of the molecules [2].

In mice, two molecules have been described to be functional homologues of HLA-G and HLA-E: Qa-2 and

Qa-1, respectively. Like humans, Qa-2 and Qa-1 molecules are involved in the regulation of immune responses and are encoded by the *H2-Q7/H2-Q9* (also called *PED gene—preimplantation embryo development gene*) and *H2-T23* genes, respectively, both located in Histocompatibility Complex-2 (H-2) [3, 4].

The H-2 spans approximately 4 Mb of chromosome 17 (23.0 cM, cytoband B-C) and contains 3 major classes of highly polymorphic gene sets: class I (*H-2*-K, *H-2*-D, Q, and *H-2*-T18 genes), class II (*H-2*-I genes), and class III (*H-2*-S genes). These genes are involved in many immunological processes, including graft rejection, immune response, antigen presentation, and complement component [5]. The number of class I genes, their organization, structural characteristics, and their patterns and levels of expression differ from species to species [3]. In general, C57BL/6 and BALB/c mice are considered in two different haplotypes: $H\text{-}2^{b}$ and $H\text{-}2^{d}$, respectively [4] (http://www.informatics.jax.org/).

The murine Qa-2 genes map to the H-2Q region, between the classical class I H-2D locus and the H-2TL cluster of class lb sequences. This locus encodes a variable number of class Ib genes in different strains [3]. Strains expressing high ($Qa\text{-}2^{high}$), medium ($Qa\text{-}2^{med}$), low ($Qa\text{-}2^{low}$), and no Qa-2 ($Qa\text{-}2^{nul}$) were identified [3]. In general, BALB/c strain is considered to be $Qa\text{-}2^{med}$ having two Qa-2 genes ($Q6^{d}$ and $Q7^{d}$). In C57BL/10, considered to be $Qa\text{-}2^{high}$, four genes with the Qa-2-coding properties are located within the Q regions: $Q6^{b}$, $Q7^{b}$, and, additionally, $Q8^{b}$ and $Q9^{b}$ [3].

Qa-2 is a 40-kDa glycoprotein found as membrane-bound and soluble isoforms generated by alternative splicing [3, 6, 7]. Qa-2 is expressed mostly by lymphoid-derived cells and plays an important role in controlling growth and murine fetal development and, like HLA-G, the molecule is related to the protection of the fetus by inhibiting maternal NK cell-mediated lysis [8–10].

Qa-1 is a 48 kDa cell surface glycoprotein currently found in association with β2-microglobulin [4, 11]. Its surface expression is found in lower levels virtually in all tissues and is increased in activated hematopoietic, T, B, and antigens presenting cells [4]. Qa-1 is involved in suppression of $CD4^{+}$ T cell and NK cell responses through a preferential interaction with inhibitory CD94/NKG2A receptors [4, 12]. The suppression and modulation of autoreactive T $CD4^{+}$ and B clones is mediated by T $CD8^{+}$ regulatory cells that recognize autoantigens presented by Qa-1 molecules [4, 12, 13]. These regulatory cells perform perforin-mediated lysis and production of immunomodulatory cytokines such as TGF-β and IL-10 [4, 12, 13].

Another murine MHC class Ib molecule involved in the modulation of the immune system is Qa-10, encoded by the *H2-Q10* gene. Qa-10 is synthesized at high levels by the liver parenchymal cells and is easily detectable in the serum as a high multivalent complex [14]. It has been suggested that Qa-10 liver cell expression may be responsible for the relative lack of immunogenicity of liver transplants and better acceptance of liver allografts [15, 16].

It has been postulated that the regulatory role played by MHC class Ib molecules is an additional mechanism that controls autoimmune reactions in peripheral autoreactive lymphocytes that escaped from central tolerance during the ontogeny of the thymus [13]. Indeed, the mechanisms of central tolerance that occur during thymus development are critical processes for the prevention of autoimmunity during the fetal and neonatal periods [17, 18]. This process characterizes the negative selection that purges the T cell repertoire of self-reactive clones through clonal deletion, inactivation, or deviation [18, 19]. The medullary thymic epithelial cells (mTECs) are primarily associated with negative selection through the expression of a wide array of tissue-restricted antigens (TRAs), a process also termed promiscuous gene expression (PGE) [20–22].

PGE is greatly dependent on the *Aire* gene *(autoimmune regulator)* [18, 21]. A mutation in this gene leads to a severe multiorgan autoimmune polyglandular syndrome type I (APS 1, also called APECED), in both mice and humans [17, 18]. It has currently been reported that most genes encoding promiscuously expressed TRAs in the thymus are regulated by a single *Aire* gene product, which is involved in a multiprotein complex transcriptional process responsible for transcription initiation, modifications of chromatin, transcriptional regulation of mRNA during the productive elongation phase, and regulation of alternative splicing events of the pre-mRNA [23, 24].

Interestingly, the expression of several MHC class Ib molecules has been reported on certain thymic cell subpopulations in mice and humans. Qa-2 expression has been used to identify functionally competent medullary thymocytes [25–27]. HLA-G is highly expressed on mTECs and stromal cells at the corticomedullary junction, and high levels of soluble HLA-G are observed in the thymus medullary compartment [28]. Qa-1 is expressed on the surface of hematopoietic cells responsible for the positive selection of Qa1-restricted $CD8^{+}$ T cells, allowing the maturation and selection of potentially self-reactive T $CD8^{+}$ regulatory cells [4, 11]. In addition to expressing immunoregulatory MHC Ib molecules, the thymus is the primary site of *Aire* expression, which is characteristically synthesized by mTEC subsets, intrathymic dendritic cells, and thymic macrophages [18].

During thymus ontogeny in the fetal stage, the expression of MHC class I and class II molecules is crucial for the education and selection of the repertoire of lymphocytes [29]. The early fetal thymus from E13.5 to E17.5 day *p.c.* is primarily composed of a homogeneous population of double-negative (DN) $CD4^{-}/CD8^{-}$ precursor T cells. The gradual acquisition of the CD4 antigen occurs around E18.5 day, with the positive selection process starting thereafter [30]. This period (E16.5–E18.5) coincides with the onset of V(D)J recombination of T cell receptor in DN thymocytes and with the beginning of promiscuous gene expression, in which the *Aire* gene has a well-recognized role [31]. From the E18.5 day p.c., thymocytes gradually gain the phenotypic markers resembling the T $CD4^{low}$ lymphocyte precursors in adults. These events allow the recognition and interaction of the T cell receptor with the MHC-peptide complex, allowing the occurrence of positive and negative selection in these cells [30]. The negative selection process occurs during perinatal

ages and extends during the first 15 days after birth [17, 32]. This period is a critical stage in thymus ontogeny that is related to the mechanisms operating in the prevention of autoimmune processes [17, 32].

Considering that (i) many immunomodulatory MHC class Ib molecules are expressed in the thymus, (ii) Aire is a paradigmatic thymic immunomodulatory molecule that may have the ability to induce the expression of other molecules involved in the regulation of immune responses; (iii) many immunomodulatory molecules have differential temporal expression during thymus ontogeny, and (iv) different experimental strains may show distinct profiles of immunoregulatory molecules, we hypothesized that nonclassical MHC class I gene expression, such as *H2-Q7*(Qa-2), *H2-T23*(Qa-1), and *H2-Q10*(Qa-10), could be temporally related to *Aire* expression, participating in the occurrence of the central tolerance process and extending through the postnatal thymus development. To achieve this goal, we assessed the pattern of *Aire* expression and also the expression of nonclassical MHC class I genes by evaluating the temporal transcript profiles of *Qa1, Qa2, Qa10,* and *Aire* in the thymus of C57BL/6 and BALB/c mice, starting from the embrionary (E14.5 days) period and continuing till adulthood (60 days). The temporal transcript profile of these genes was also evaluated in some lymphoid and nonlymphoid tissues (spleen, liver, and gut) and in immunologically privileged sites (brain and placenta) to correlate with thymus findings.

2. Material and Methods

2.1. Animals. C57BL/6 and BALB/c mice were bred in an isolated cage provided with 0.45 μm pore size air filter. To obtain an accurate day of gestation, the presence of a post coitum vaginal plug observed at 7:00 am was considered to be day zero. Fetuses were surgically collected from the uterus, and p.c. age was confirmed according to the morphological characteristics of each developmental phase [32]. Tissue samples were obtained in triplicate from (i) fetuses aged E14.5, E15.5, E16.5, E17.5, E18.5, E19.5, and E20.5 days of gestation, (ii) newborns aged 1, 5, 10, and 15 days, and (iii) adults aged 45 and 60 days. For each age were used at least 3 different animals. For tissue harvesting all tissues were washed in saline solution and then processed. For thymus both lobes were processed. For spleen, liver, gut, placenta, and brain only the same portions of tissues were analyzed. There was no separation between hematopoietic cells and parenchyma. Experimental procedures followed ethical guidelines under strict guidance and approval from the University of São Paulo Ethics Committee for Animal Experimental Research (Protocol number 043/2009).

2.2. RNA Extraction. After tissue isolation, total RNA samples were obtained by maceration of each tissue in TRizol reagent using a Potter homogenizer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and treated with DNAse (deoxyribonuclease I amplification grade, Invitrogen). RNA integrity was checked by the presence of the 28S and 18S bands in 1.5% agarose gel, and only protein-free, phenol-free, and undegraded RNA species were used, as determined by UV spectrophotometry.

2.3. Analysis of Expression by Real-Time PCR. Total RNA isolated from samples was reverse-transcribed to cDNA using the High Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. cDNA amplification was initially carried out in a total volume of 25 μL, corresponding to 500 ng of the initial RNA.

We assessed the expression of the *H2-Q7*(Qa-2), *H2-T23*(Qa-1), *H2-Q10*(Qa-10), and *Aire* genes by quantitative real-time PCR using TaqMan Probe-Based Gene Expression Analysis (Applied Biosystems) in a total volume of 10 μL containing 75 ng total RNA, 5 μL TaqMan PCR Universal Master Mix (Applied Biosystems), and 0.5 μL TaqMan Gene Expression Assays. An ABI System Sequence Detector 7500 (Applied Biosystems) was used with the following regimen of thermal cycling: stage 1—1 cycle for 2 minutes at 50°C; stage 2—1 cycle for 10 minutes at 95°C; stage 3—40 cycles for 15 seconds at 95°C, followed by the last cycle for 1 minute at 60° and 25 seconds at 72°C. Gene expression was normalized relatively to the TaqMan endogenous controls (Applied Biosystems), using *glyceraldehyde-3-phosphate dehydrogenase* and *β-actin* genes. The relative quantification of transcript levels at the different ages and tissues was performed by the comparative $2^{-\Delta\Delta Ct}$ method for each different analysis using the ΔCt minimum as control sample. Each sample was tested in triplicate. The TaqMan Inventoried Assays and TaqMan Gene Expression Assay reference are listed as follows: *H2-Q7*: Mm00843895_m1; *H2-Q10*: Mm01275264_m1; *H2-T23*: Mm00439246_m1; *Aire*: Mm00477461_m1; *GAPDH*: 4352339E; *ACTB*: 4352341E.

Statistical analysis was performed using one-way ANOVA followed by the Bonferroni multiple comparison test for analysis of gene expression profiles in different tissue samples. To compare age-related tissue samples between lineages, we used the Student's *t*-test. The analyses involving several variables (type of tissue, age, and genes) were performed using the two-way ANOVA Bonferroni's multiple comparison test with the aid of the Graphpad Prism V.5 software (San Diego, CA, http://www.graphpad.com/prism/). The Pearson product-moment correlation coefficient was calculated using R software version 2.14.0 (http://www.r-project.org/). $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant.

3. Results

3.1. Analysis of MHC Class Ib and Aire Transcripts in Different Tissues of C57BL/6 Mice. Aiming to discriminate the primary organ of gene expression, we first compared each individual's gene transcript levels among the different tissues analyzed. We found that the *H2-Q7* transcripts were primarily observed in thymus, followed by spleen and liver at all ages, being higher in adult thymus compared with other tissues along at different ages ($P < 0.01$) (Figure 1(a)). The transcript levels of *H2-T23* were homogeneously expressed among all tissues, showing higher levels only in liver at adult ages ($P < 0.01$)

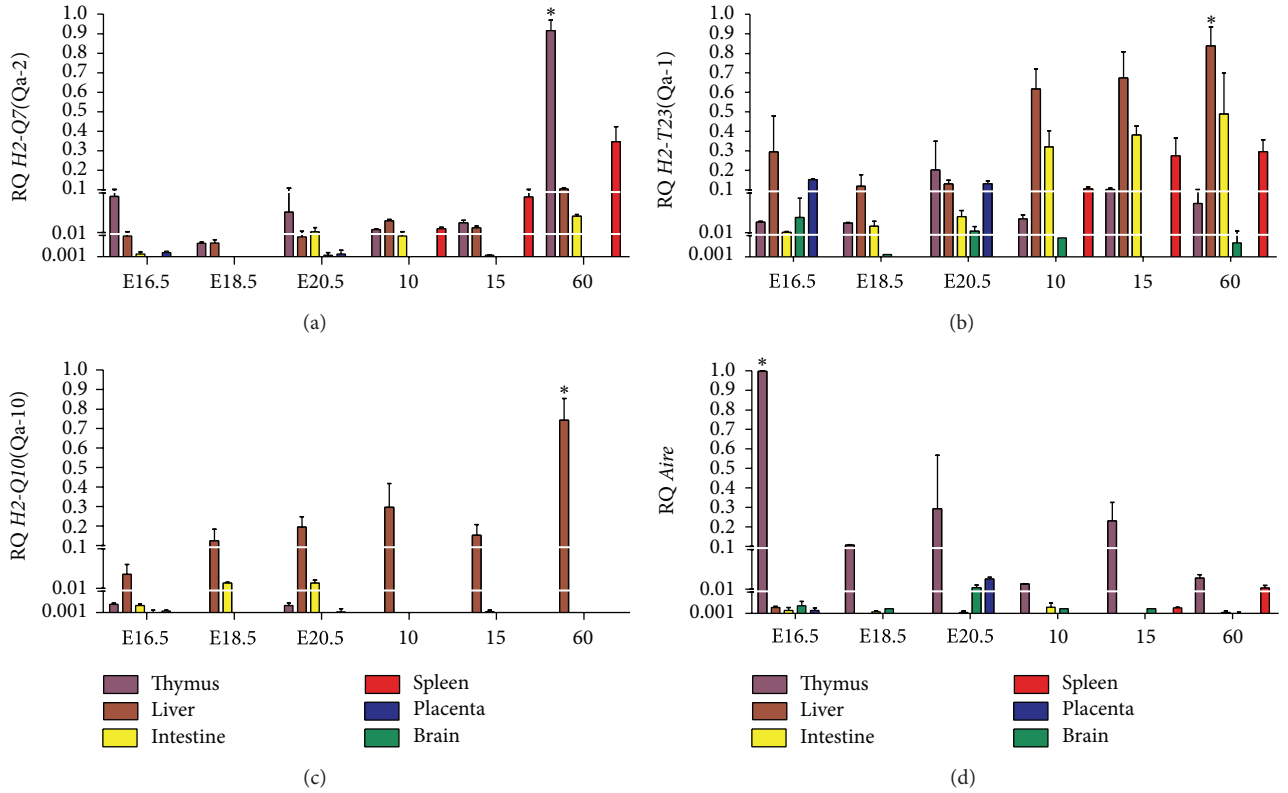

Figure 1: Analysis of MHC class Ib and *Aire* transcripts in different tissues of C57BL/6 mice. Relative quantification (RQ) of messenger RNA for (a) *H2-Q7*(Qa-2), (b) *H2-T23*(Qa-1), (c) *H2-Q10*(Qa-10), and (d) *Aire* is representative of the mean values of $2^{-\Delta\Delta Ct}$. Tissue samples were obtained in triplicate from different animals for each age analyzed. Each experiment was independently performed at least three times. Data were analyzed statistically by two-way ANOVA followed by the Bonferroni multiple comparison test. Values close to the level of significance are marked with (∗).

(Figure 1(b)). *H2-Q10* proved to be liver-specific, gradually increasing with age ($P < 0.001$). *H2-Q10* expression was also detected in the fetal thymus and intestine (Figure 1(c)). Abundant levels of *Aire* transcripts were observed in the thymus during fetal ages, with a peak occurring at day E16.5 ($P < 0.001$), although, at reduced levels, *Aire* transcripts were observed at all postnatal ages. In addition, restricted levels of this gene were observed in brain and placenta (during fetal periods) and in spleen of adult mice (Figure 1(d)). In this analysis the gene transcript levels of each gene singly were compared between all tissues and ages.

3.2. Comparisons of Gene Expression Profiles of MHC Class Ib and Aire during Development of Thymus, Lymphoid, and Nonlymphoid Tissues in C57BL/6 Mice. To compare the transcription pattern during organ ontogeny, we analyzed each transcript in isolated tissues according to age. During fetal thymus development, both *H2-Q7* and *Aire* transcripts were significantly increased at E16.5 days in comparison to *H2-Q10* and *H2-T23* at all ages ($P < 0.001$). In addition, *H2-Q7* was the most widely expressed gene throughout the period of thymus development, reaching significance at E16.5, 45, and 60 days ($P < 0.001$). In adult thymus, *Aire* transcripts were observed at levels similar to *H2-Q10* and *H2-T23* (Figure 2(a)). In liver samples, *H2-Q10* expression was high and increased with aging (Figure 2(b)); however, in gut samples, these transcripts were elevated during fetal and perinatal ages ($P < 0.05$). During postnatal periods, the *H2-T23* gene was the most expressed ($P < 0.001$), followed by *H2-Q7* ($P < 0.01$) (Figure 2(c)). In the spleen, a gradual transcript increase was observed for *H2-Q7* and *H2-T23*. *H2-Q7* was significantly higher in spleen samples from 60-day-old animals ($P < 0.001$). A diminished expression of *Aire* was observed in spleen samples at postnatal ages (Figure 2(d)). Interestingly, during the evolution of pregnancy, the *H2-T23* transcripts levels were higher than *H2-Q7* at day E15.5 ($P < 0.001$) and day E16.5 ($P < 0.001$), and at day E20.5 ($P < 0.05$) in placenta samples (Figure 2(e)). Overall, MHC class Ib transcripts were observed at very restricted levels in brain at all ages analyzed, except for a faint expression of *H2-Q7* transcripts during the perinatal period (Figure 2(f)).

3.3. Individual Transcript Patterns of H2-Q7(Qa-2), H2-T23(Qa-1), H2-Q10(Qa-10), and Aire throughout Development in C57BL/6 Mice. The individual values of the transcripts for *H2-Q7*, *H2-Q10*, *H2-T23*, and *Aire* throughout the development are shown in Table 1 and are representative of the mean values of $2^{-\Delta\Delta Ct}$.

Table 1: Relative Quantification of gene expression profiles of *H2-Q7, H2-Q10, H2-T23* and *Aire* throughout the development of C57BL/6 mice. The lines are representative of the mean values of $2^{-\Delta\Delta Ct}$. Tissue samples were obtained in triplicate from different animals for each age analyzed. Each experiment was independently performed at least three times. Data were analyzed statistically by One-way ANOVA followed by the Bonferroni multiple comparison test. The comparison was performed between the transcript levels of one target gene in the same tissue but at different ages. Values close to the level of significance are marked with (∗). Values near the significance level ($P < 0.05$) are marked with (†), ($P < 0.01$) with (•) and ($P < 0.001$) with (∗). was utilized.

	Thymus				Liver				Intestine			
	H2-Q7 (Qa2)	*H2-T23 (Qa1)*	*H2Q10 (Qa10)*	*Aire*	*H2-Q7 (Qa2)*	*H2-T23 (Qa1)*	*H2Q10 (Qa10)*	*Aire*	*H2-Q7 (Qa2)*	*H2-T23 (Qa1)*	*H2Q10 (Qa10)*	*Aire*
E14.5	0.0102	0.3810	0.0344	0.1617	0.1355	0.0735	0.0187	0.0000	0.0515	0.0080	0.0365	0.1550
E15.5	0.0966	0.1100	**0.1967**†	0.2500	0.1465	0.0725	0.0286	0.0000	0.0340	0.0045	0.0365	0.2893
E16.5	0.0920	0.2220	**0.6565***	**0.9500***	0.088	0.1447	0.0765	0.0000	0.0333	0.0203	0.1503	0.2180
E17.5	0.0785	0.3020	0.0200	**0.9850***	0.09	0.1160	0.0330	0.0000	0.0426	0.0156	0.1273	0.7205
E18.5	0.0066	0.2060	0.0172	0.1075	0.1008	0.1535	0.1156	0.0000	0.0246	0.0296	0.2140	0.3527
E19.5	0.1070	0.2300	**0.3683**$^{\dagger *}$	0.1367	0.0625	0.1330	0.0956	0.0000	0.0185	0.0355	**0.7255**$^{\dagger \bullet *}$	0.3760
E20.5	0.0560	0.3540	**0.5505***	0.0200	0.112	0.1308	**0.1775**†	0.0000	0.0280	0.0660	**0.949**$^{\dagger *}$	0.3000
1	**0.1645**†	**0.4323**$^{\dagger \bullet}$	**0.9955***	0.0733	0.0625	0.2020	0.0370	0.0000	0.0267	0.1137	0.2960	0.2175
5	0.0243	**0.6658**$^{\dagger \bullet *}$	**0.5250***	0.1700	**0.2225**†	**0.6850**$^{\bullet *}$	0.0917	0.0000	0.0695	0.1997	0.2400	**0.7715**†
10	0.0166	0.3185	0.0172	0.0200	**0.332**$^{\bullet *}$	**0.6167**$^{\dagger \bullet}$	0.1347	0.0000	0.1900	0.4580	0.0080	0.3810
15	0.0320	**0.6668**$^{\bullet *}$	0.0423	0.1850	0.185	**0.6743**$^{\dagger \bullet *}$	0.1513	0.0000	0.1905	**0.5445**†	0.0230	0.2750
45	**0.2190**†	**0.4633**$^{\bullet}$	0.0563	0.0866	**0.9995***	**0.6380**$^{\dagger \bullet}$	**0.7717***	0.0000	**0.6305**$^{\bullet *}$	0.3805	0.0530	0.3080
60	**0.9158***	0.2993	0.0425	0.0360	**0.91***	**0.8385***	**0.5723***	0.0000	**0.9175***	**0.699**$^{\dagger \bullet *}$	0.0360	0.1310
	Spleen				Placenta				Brain			
	H2-Q7 (Qa2)	*H2-T23 (Qa1)*	*H2Q10 (Qa10)*	*Aire*	*H2-Q7 (Qa2)*	*H2-T23 (Qa1)*	*H2Q10 (Qa10)*	*Aire*	*H2-Q7 (Qa2)*	*H2-T23 (Qa1)*	*H2Q10 (Qa10)*	*Aire*
E14.5	—	—	—	—	0.0326	0.2435	**0.7957**†	0.0210	0.0580	0.0350	0.0170	0.6820
E15.5	—	—	—	—	0.0450	0.6507	0.2425	0.0596	0.1823	0.0230	0.0330	0.7630
E16.5	—	—	—	—	0.0413	0.3227	0.2490	0.0693	0.1673	0.0060	0.0370	0.3703
E17.5	—	—	—	—	—	—	—	—	0.1453	0.0185	0.0170	0.2370
E18.5	—	—	—	—	—	—	—	—	0.1570	0.0140	0.0150	0.2220
E19.5	—	—	—	—	—	—	—	—	0.1375	0.0120	0.0535	0.3280
E20.5	—	—	—	—	0.0233	0.2803	0.0430	**0.8767***	0.3205	0.0140	0.6965	0.6355
1	0.0165	0.1047	**0.2103***	0.0295	—	—	—	—	0.3030	0.0000	0.0855	0.4105
5	0.0085	0.0965	0.0190	0.0836	—	—	—	—	0.0000	0.0880	0.0140	0.1940
10	0.0343	0.2217	0.0126	0.0126	—	—	—	—	0.6560	0.0510	0.0270	0.1980
15					—	—	—	—	0.0440	0.0050	0.0330	0.2750
45	0.1667	0.5817	0.0533	0.0806	—	—	—	—	0.1110	0.0250	0.0530	0.1820
60	**0.7665***	0.5973	0.0553	**0.2073**$^{\bullet *}$	—	—	—	—	0.3530	0.0400	0.1165	0.1355

†($P < 0.05$); $^{\bullet}$($P < 0.01$); *($P < 0.0001$).

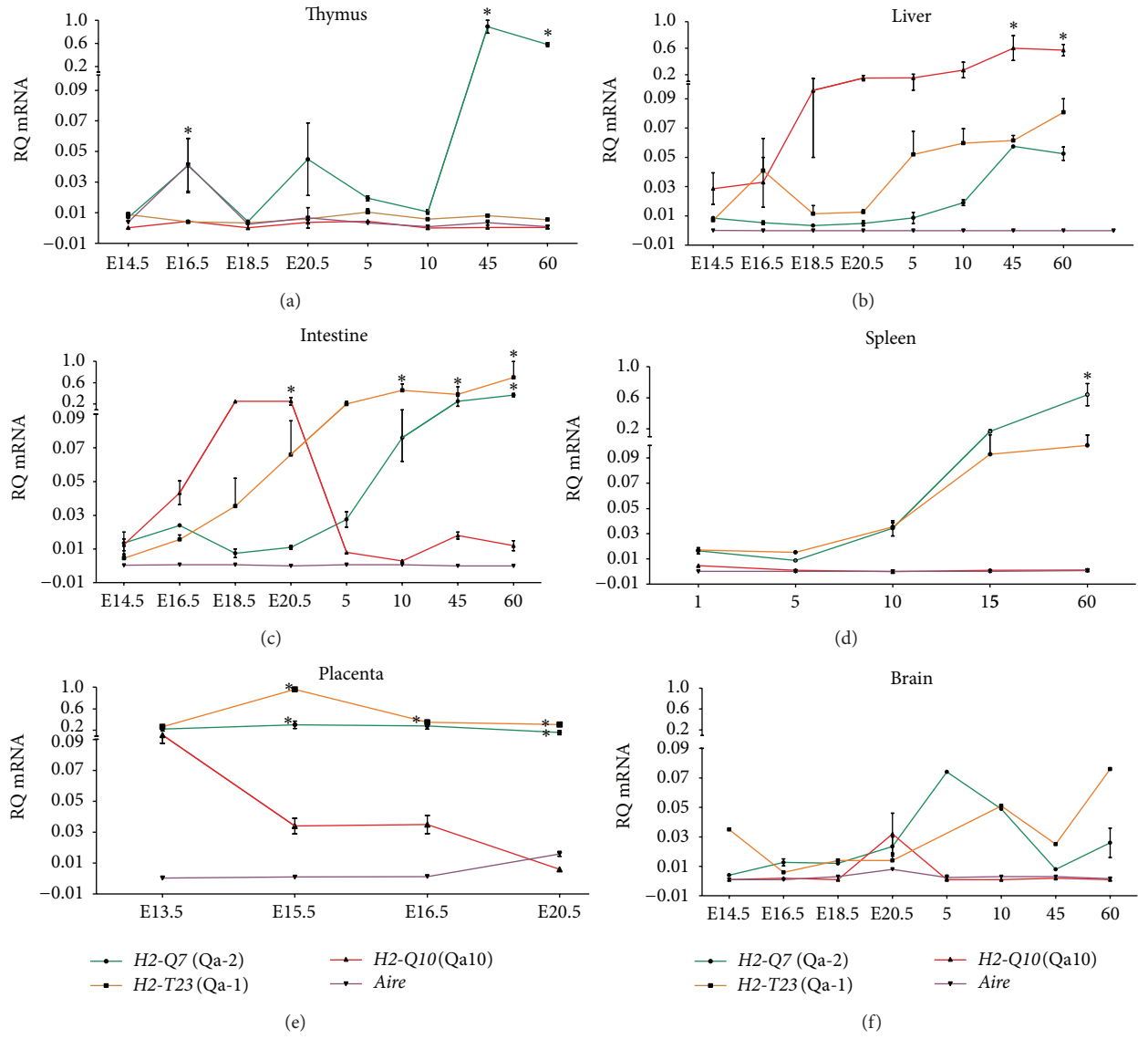

Figure 2: Comparisons of gene expression profiles of MHC class Ib and *Aire* during development of thymus, lymphoid, and nonlymphoid tissues in C57BL/6 mice. Relative quantification (RQ) obtained from (a) thymus, (b) liver, (c) intestine, (d) spleen, (e) placenta, and (f) brain is representative of the mean values of $2^{-\Delta\Delta Ct}$. obtained from comparisons of the four genes in each tissue independently. Tissue samples were obtained in triplicate from different animals for each age analyzed. Each experiment was independently performed at least three times. Data were analyzed statistically by two-way ANOVA followed by the Bonferroni multiple comparison test. Values close to the level of significance are marked with (∗).

In the thymus, transcript levels of *H2-Q7* gradually increased with age, reaching significance in mice aged 1, 45, and 60 days compared to other ages. *H2-T23* expression was increased in animals aged 1, 5, 15, and 45 days compared to fetal and adult ages. Although it is considered to be liver-specific, *H2-Q10* expression was surprisingly increased during the fetal period of thymus development at days E15.5, E16.5, E19.5, and E20.5 and during the perinatal period at days 1 and 5. *Aire* expression was significantly increased during fetal thymus development, primarily at E16.5 and E17.5. *Aire* expression was maintained at reduced levels during postnatal periods in the thymus of newborn and adult animals.

In the liver, gene expression profiles of *H2-Q7*, *H2-T23*, and *H2-Q10* were detected at low levels at fetal and perinatal ages, increasing after day 5. Postnatal expression of *H2-Q7* and *H2-Q10* peaked at days 45 and 60, whereas *H2-T23* expression continued to be elevated from day 5 to 60. *Aire* gene transcription was not detected during liver development.

In the gut, the expression of *H2-Q7* and *H2-T23* was increased in the postnatal period, primarily from day 15 to day 60. In contrast, *H2-Q10* transcripts peaked at late fetal periods on days E19.5 and E20.5. *Aire* expression was observed at all ages, exhibiting

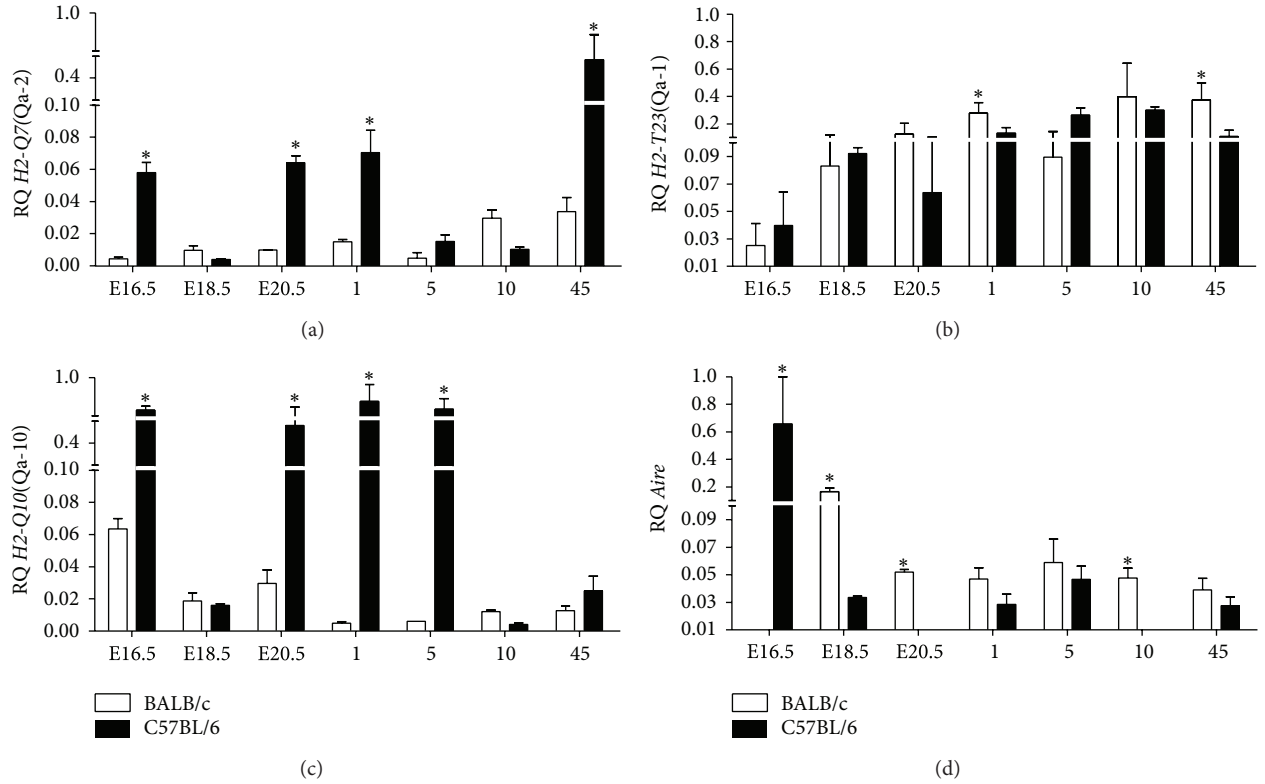

Figure 3: Comparison of gene expression profiles during thymus development between C57BL/6 and BALB/c mice. Relative quantification (RQ) of messenger RNA for (a) *H2-Q7* (Qa-2), (b) *H2-T23* (Qa-1), (c) *H2-Q10* (Qa-10), and (d) *Aire* is representative of the mean values of $2^{-\Delta\Delta Ct}$. Tissue samples were obtained in triplicate from different animals and lineages for each age analyzed. Each experiment was independently performed at least three times. Data were analyzed statistically by the Student's *t*-test. Values close to the level of significance are marked with (∗).

significant peak expression at day E14.5 and at perinatal day 5.

Due to the late formation of the spleen, the tissue samples were obtained only during postnatal periods. *H2-Q7* and *Aire* showed a closely similar expression pattern, exhibiting peak levels at day 60. In contrast, *H2-Q10* transcripts peaked at day 1. No significant differences in *H2-T23* expression were observed.

In placenta samples, the gene expression profiles of *H2-Q7* and *H2-T23* showed no significant differences from day E14.5 to day E20.5. On the other hand, the expression of *H2-Q10* peaked at day E14.5 and the expression of *Aire* peaked at day E20.5.

In the brain, analysis of the expression of *H2-Q7, H2-T23*, *H2-Q10*, and *Aire* in fetal and adult mice showed no significant differences.

3.4. Comparison of Gene Expression Profiles between C57BL/6 and BALB/c Mice. Compared with BALB/c mice, the C57BL/6 mouse thymus showed (i) higher transcript levels for *H2-Q7* and *H2-Q10* during fetal and postnatal development; (ii) increased levels of *H2-Q7* on the following days: E16.5 ($P = 0.0142$), E20.5 ($P = 0.0063$), 1 ($P = 0.0037$), and 45 ($P < 0.0001$) (Figure 3(a)); (iii) closely similar *H2-T23* transcript levels, except at days 1 and 45, when *H2-T23* expression levels were significantly increased in BALB/c mice ($P = 0.01$ and $P = 0.0058$, resp.) (Figure 3(b)); (iv) increased levels of *H2-Q10* mRNA in mice aged E16.5 ($P < 0.01$), E20.5 ($P < 0.01$), 1 ($P < 0.001$), and 5 postnatal days ($P < 0.001$) (Figure 3(c)); (v) a peak of *Aire* transcripts at day E16.5 ($P < 0.001$), while in BALB/c mice this peak occurred at day E18.5 of development ($P = 0.0001$). Overall, BALB/c mice expressed higher levels of *Aire* in the thymus at all other ages evaluated in this study, reaching significance at fetal E18.5 ($P = 0.0001$) and E20.5 days ($P = 0.0318$) and at 10 days after birth ($P = 0.0004$) (Figure 3(d)).

Regarding other organs (liver, gut, spleen, placenta, and brain), C57BL/6 mice expressed considerably higher levels of *H2-Q7* than BALB/c mice. In addition, *H2-Q7* transcripts in fetal E17.5 liver ($P = 0.0125$), in E16.5 and E18.5 placentas ($P = 0.00137$), in 10 day spleen ($P = 0.0146$), and in 15-day gut ($P = 0.0002$) were higher in C57BL/6 mice (data not shown). *H2-T23* transcripts were more abundant in BALB/c mice at most ages and in most organs compared to C57BL/6 mice, being significantly increased at E17.5, E18.5, and E20.5 day in the fetal liver ($P = 0.0057$, $P = 0.0196$, and $P = 0.0174$, resp.) and at day 10 in the gut ($P < 0.001$). No significant differences in *H2-T23* levels were observed in the spleen of these mice (data not shown). *H2-Q10* was significantly increased in fetal liver and fetal gut of C57BL/6 mice aged E20.5 ($P = 0.0020$) and E18.5 ($P = 0.0210$),

respectively. C57BL/6 mouse placentas with E16.5 and E20.5 days of pregnancy ($P = 0.0094$ and $P = 0.018$, resp.) showed augmented *H2-Q10* levels. Overall, *Aire* transcripts were higher in BALB/c mice compared with C57BL/6 mice, being significantly increased in 10-day spleen ($P = 0.0242$), in E18.5, E20.5, and 15-day gut ($P = 0.0002$, $P = 0.0067$, and $P < 0.01$, resp.), and in E20.5 day placenta ($P = 0.0009$) (data not shown).

3.5. Correlation of Gene Expression Profiles during Thymus Ontogeny. Considering the thymus tissue samples obtained at fetal ages, we found a positive correlation between *Aire* and *H2-Q7* in both strains analyzed ($R = 0.0378/P = 0.4025$ for C57BL/6, and $R = 0.03618174/P = 0.9638$ for BALB/c). Negative correlations between *Aire* and *H2-T23* were found for C57BL/6 ($R = -0.0408/P = 0.9306$) and for BALB/c mice ($R = -0.1852341/P = 0.8148$). In C57BL/6 mice, the expression of the *H2-Q10* gene showed a positive correlation with *Aire* ($R = 0.1311/P = 0.7793$); however, a negative correlation was observed in BALB/c mice ($R = -0.5067555/0.4932$). After birth, negative correlations between *Aire* and *H2-Q7* were observed for both strains ($R = -0.424/P = 0.4016$ for C57BL/6, and $R = -0.1140859/P = 0.8076$ for BALB/c). A positive correlation between *Aire* and *H2-Q10* and *Aire* and *H2-T23* was observed for C57BL/6 and BALB/c mice ($R = 0.3409015/P = 0.5085$ and $R = 0.8148293/P = 0.04826$, resp.). However, in BALB/c mice this correlation was negative ($R = -0.4232537/P = 0.3441$ for *H2-Q10*, and $R = -0.1459941/P = 0.7548$ for *H2-T23*).

4. Discussion

4.1. MHC Class Ib Genes and the Thymic Selection. Although the *Aire* gene has a well-recognized role in central tolerance, the role of nonclassical MHC molecules, which also have tolerogenic properties, is not fully understood. To evaluate the relationship between *Aire* and *nonclassical MHC class I* genes, we studied the simultaneous expression of these genes during the ontogeny of lymphoid and nonlymphoid organs, from embryonic ages to adulthood.

In the present study, the *H2-Q7* gene (Qa-2 molecule) was abundantly expressed in the thymus compared with other genes and in other tissues at any age analyzed. Several lines of evidence indicate that Qa-2 may be involved in migration, maturation, and effector mechanisms of cells that emigrate from the thymus. Cells exhibiting a high expression of Qa-2 do migrate to the periphery and perform their effector mechanisms [26, 27], corroborating previous results showing that Qa-2 is a marker of medullary thymocytes in the final stages of development [25–27]. In addition, Qa-2 may be involved in the generation of T $CD8^+$ lymphocytes specific for antigens that are presented at the periphery in the context of these molecules [12, 26] and in the selection and regulation of $CD8\alpha\alpha$/$TCR\alpha\beta$ intraepithelial T cells [34].

In humans, the expression of HLA-G in the fetal thymus may be related to the inhibition of thymus NK cells, potentially capable of destroying thymocytes expressing classical HLA class I molecules at low density [35] or even inducing apoptosis of $CD8^+$ T cells via Fas expression [8, 28]. Also, the expression of HLA-G by mTECs may induce immune tolerance driven by antigen-specific T cells through the expansion of natural regulatory $CD4^+$ $Foxp3^+$ T cells [8, 36]. Taken together, these findings indicate that the high expression of *H2-Q7* during perinatal and adult ages may be related to the formation of subtypes of functionally mature thymocytes residing in thymic medulla. The increased expression of *H2-Q7* in adulthood may further indicate that, instead of thymic involution, the presence of mature and functional thymocytes may maintain the functionality of the thymus in thymocyte generation.

It is interesting to observe that the expression of the *H2-Q7* and *Aire* genes exhibited closely similar profiles and showed positive significant correlations in C57BL/6 and in BALB/c mice only during fetal ages. Negative selection is crucial to maintain the homeostasis of the immune system, a process in which the *Aire* gene plays a central role, since it is directly implicated in the control of the expression of thousands of TRAs [21, 22]. Based on the positive correlations between the transcript profiles of *Aire*, *H2-Q7*, and *H2-Q10* in the fetal thymus, we may hypothesize that nonclassical MHC class I genes are also under the transcriptional control of *Aire*. Additionally, it has been proposed that *Aire* may activate genes that are usually silenced or expressed at low levels due to methylation marks (H3K4me0) in their promoter regions. The low expression and the presence of methylation marks may be a feature of *Aire*-dependent activation of genes which are not normally expressed, as in mTECs [23, 24]. In humans, the expression of the *HLA-G* gene is regulated at epigenetic levels due to the presence of methylation of CpG motifs in the promoter region [37, 38] and could also be under the transcriptional regulation of *Aire*.

In contrast to *H2-Q7*, we found a negative correlation between *Aire* and *H2-T23*(Qa-1) expression during fetal thymic development in both mouse strains. Indeed, *H2-T23* transcription levels were reduced during the fetal period of thymus development, increasing during postnatal ages. These results corroborate previous studies reporting that Qa-1 is primarily expressed by antigen-presenting cells and activated lymphocytes during the effector phase of the immune response [4, 11, 12]. The expression of Qa-1 (*H2-T23*) in the fetal thymus, as observed in the present study, may be related to the generation of regulatory T cells, since Qa-1 is involved in the regulation of autoimmunity by suppressive $CD8^+$ T regulatory cells. The positive selection of potentially Qa-1-dependent $CD8^+$ Tregs may allow the expansion of these regulatory cells in the peripheral lymphoid organs after encountering the cognate antigen [4, 11, 12].

There are no previous studies evaluating the expression of *H2-Q10* in the thymus, and there are few studies reporting the immunomodulatory role of the Qa-10 molecule. In this context, it has been reported that Qa-10 is liver-specific and may be related to better acceptance of hepatic allografts [14, 15]. At least at a transcriptional level, we demonstrated the expression of *H2-Q10* outside the liver. Although a reduced expression of *H2-Q10* during thymus ontogeny was observed in this study, the expression levels were higher during the fetal

period. Additionally, we found a significant positive correlation between *Aire* and *H2-Q10* in C57BL/6 mice both for embrionary and for postnatal ages. In contrast, we observed a significant negative correlation between *Aire* and *H2-Q10* in BALB/c mice for embrionary and postnatal ages. As proposed for *H2-Q7*, *Aire* may also be involved in the transcriptional regulation of *H2-Q10*(Qa-10), which in turn may be involved in tolerance in the thymus. Since the expression patterns of *Aire* and *H2-Q10* are distinct in different strains, one may hypothesize that *H2-Q10* expression may be associated with differential immune response patterns, yielding differential susceptibility to foreign or modified self antigens.

4.2. MHC Class Ib Genes during Ontogeny of Lymphoid and Nonlymphoid Tissues. Overall, in peripheral organs such as the spleen, liver, and gut, the transcription profiles of *H2-Q7*, *H2-Q10*, and *H2-T23* were characterized by a gradual increase of transcript levels with aging. In the spleen, the increased expression of *H2-Q7* in C57BL/6 mice supports previous studies reporting that the expression of Qa-2 by spleen cells is sufficiently high to prime $CD8^+$ T cells [39]. Additionally, *H2-T23* transcripts found in this organ may be related to a higher state of activation of T cells, since the expression of Qa-1 preferentially occurs in activated immunocompetent cells [12].

In the liver, both during the fetal period and in adulthood, we observed an increased level of nonclassical MHC transcripts, particularly those encoding the Qa-10 molecule. The increased *H2-Q10* liver transcription rates could serve to modulate immune responses maintaining the "immunosuppressed" state of the liver. This idea is corroborated by studies reporting that hepatocytes expressing Qa-10 are apparently free of autoimmune processes and show little evidence of cell damage [14]. The early expression of MHC class Ib genes may be involved in the maintenance of this "immunosuppressed" state from embryonic stage to adulthood. This maintained state of tolerance in liver cell subpopulations possibly contributes to the high rates of acceptance of liver transplants; however, it may also contribute to liver vulnerability to chronic pathogens, such as hepatitis viruses and *Plasmodium* spp. [40]. We agree with the idea raised by Stroynowski and Tabaczewski in which since Qa-10 appears in the circulation as a soluble molecule, the high expression of Qa-10 would be an additional mechanism by which the liver could impose systemic immunological tolerance, influencing the immune responses at other body sites, particularly in autoimmune manifestations or in allografts [3].

The transcription of the *H2-Q7*, *H2-Q10*, and *H2-T23* genes was detected from E13.5 day during the embryonic development of liver. It is well established that during embryogenesis the fetal liver acts as an important hematopoietic organ, producing diverse cell types as the progenitors of T, B, NK, and dendritic cells and monocytes [41, 42]. Indeed, under normal conditions, these cells and hematopoietic stem cells are able to express Qa-2 and Qa-1 [3, 4]. It has also been demonstrated that fetal liver mesenchymal stem cells express HLA-G molecules [43], and some subtypes of $CD8^+$ T cells have been identified as natural $CD8^+$ T cells, exhibiting the $CD8^+$ $HLA\text{-}G2^+$ and $CD8^+$ $CD122^+$ phenotypes [40]. Apparently, the expression of HLA-G in liver cells may contribute to immunosuppression events observed in the liver, favoring the chronification of infections [44, 45].

H2-Q7 expression in the gut is of potential interest, since several lines of evidence show that Qa-2 is involved in the selection and maintenance of mucosal $CD8\alpha\alpha$/$TCR\alpha\beta$ intraepithelial lymphocytes and therefore in the regulation of immune responses [39]. It is known that regulatory T cells are abundantly found in the lamina propria of the gut, which can be generated at these sites or may migrate through homing receptors to the gut [46]. Thus, the increased levels of *H2-T23* transcripts observed in the gut may be related to the involvement of Qa-1 in the suppression of NK cell responses and the maintenance and generation of $CD8^+$ regulatory T cells, contributing to oral tolerance in the gut [12, 13].

The importance of Qa-1 and Qa-2 in the placenta was demonstrated by studies showing that $\gamma\delta$ TCR lymphocytes present in the decidua are oligoclonal and restricted to antigens presented by class Ib molecules. Interestingly, these populations of $\gamma\delta$ T cells are selected in the thymus during the fetal period [47]. The expression profiles of *H2-Q7* and *H2-T23* observed in our study are consistent with human studies showing that the HLA-G molecule has well-marked temporal regulation during pregnancy, with high expression in the first months and decreased expression in the third trimester of pregnancy [47]. Our observation that *H2-T23* transcripts are more expressed *H2-Q7* transcripts is surprising, considering that the placenta and embryonic tissues are described as the relevant sites of expression of Qa-2 and a major component of the *H2-Q6/Q7/Q8/Q9* genes (*Ped* gene) involved in preimplantation and embryonic development [6, 9].

Overall, we did not observe expression of MHC class Ib genes in the brain, except for a faint expression of *H2-Q7* and *Aire* transcripts in both mouse strains (data not shown). Under physiological conditions, brain Qa-2 expression has been associated with the development and plasticity of the organ [48]. On the other hand, in humans, induced brain expression of HLA-G has been reported during the course of inflammatory diseases such as multiple sclerosis and has been associated with inhibition of responses mediated by cytotoxic T cells, NK cells, and inhibition of T cell proliferation [46].

The expression of *Aire* transcripts outside the thymus, found here to be reduced in the brain and to occur in considerable levels in peripheral organs such as the spleen and gut, is quite interesting, since in recent years several studies have attempted to identify the expression of *Aire* and the occurrence of PGE in tissues other than the thymus [17, 18, 32]. The relevance and functionality of the expression of *Aire* in peripheral lymphoid organs are still very controversial. Recently, it has been reported that the stromal cells of lymph nodes, spleen, and Peyer's patches express reduced levels of Aire. The expression of Aire by these organs occurs in certain eTACS *(extra thymic Aire-expressing cells)*. In a similar way to mTECs, eTACS can perform promiscuous gene expression and are able to mediate deletion of autoreactive T cells [17, 18].

4.3. Differential Transcript Profiles between Mice Strains. The comparisons of *Aire* gene expression profiles between strains

corroborated our previous studies, showing a peak of *Aire* expression in C57BL/6 thymus at day E16.5 and in BALB/c thymus at day E18.5. *Aire* expression anticipated the PGE phenomenon in both strains and occurred after the beginning of TCR V(D)J recombination. This process starts on the E14.5 day and E16.5 day in C57BL/6 and BALB/c mice, respectively [49, 50]. Therefore, the timing of T cell maturation during thymus development apparently differs between these strains, suggesting an important role of the genetic background in the modulation of these important thymus events [51, 52]. In addition, differences in imunomodulatory MHC class Ib gene expression profiles between strains, as observed in this study, may provide further evidence of the patterns of susceptibility and resistance to infections, autoimmune diseases, and cancers of these strains. In most age groups analyzed in this study, the expression of *H2-Q7* in the thymus and other peripheral organs was significantly higher in C57BL/6 mice than in BALB/c mice. This may reflect the fact that BALB/c mice usually express lower levels of Qa-2 as they present a deletion of both *H2-Q6* and *H2-Q9 loci*, and therefore the BALB/c strain is characterized as medium producers of Qa-2 (Qa-2^{med}) [3].

Considering that *Q7* and *Q9* genes synergistically contribute to the expression of Qa-2, the genetic differences between BALB/c and C57BL/6 mice may contribute to differential expression of Qa-2, which is approximately 4 to 5 times higher in C57BL/6 mice [3]. In contrast, *H2-T23* and *AIRE* transcript levels were significantly higher in BALB/c mice compared to C57BL/6 mice. Compared to C57BL/6 mice, BALB/c mice are more vulnerable to infections triggered by *Staphylococcus aureus, Mycoplasma pulmonis*, and *Leishmania major* [51–53]. Considering that Qa-1 is clearly involved in the generation of regulatory T cells [4, 12, 13] and that *Aire* may shape the repertoire of regulatory T cells [18], increased expression of these transcripts may account for increased central regulatory function and increased susceptibility to infections [54].

5. Conclusions

The present study raised the idea of a potential transcriptional link between *Aire* and nonclassical MHC class I genes acting at a central level during thymic education and potentially influencing and modulating the immune responses at the periphery. Although using a generalist approach, this study aimed to characterize the transcription patterns of relevant immunomodulatory genes and may be useful for further studies regarding the involvement of nonclassical MHC class I molecules in immune tolerance events. The evaluation of the protein product encoded by these genes is crucial to understand the relationship and possible transcriptional regulation of nonclassical MHC class I molecules by Aire. Further analysis will be performed in this area in order to verify the potential influence of Aire on the promoter regions of *H2-Q7*, *H2-Q10*, and *H2-T23*. Studies involving appropriate animal models for MHC class Ib molecules can contribute to the current knowledge about HLA-G and HLA-E in humans and are a prerequisite for the development of therapeutic strategies such as the production of nonclassical MHC recombinants molecules associated with immunosuppressive therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Immunomodulatory Properties of HLA-G in Infectious Diseases

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HLA-G is a nonclassical major histocompatibility complex molecule first described at the maternal-fetal interface, on extravillous cytotrophoblasts. Its expression is restricted to some tissues in normal conditions but increases strongly in pathological conditions. The expression of this molecule has been studied in detail in cancers and is now also beginning to be described in infectious diseases. The relevance of studies on HLA-G expression lies in the well known inhibitory effect of this molecule on all cell types involved in innate and adaptive immunity, favoring escape from immune control. In this review, we summarize the features of HLA-G expression by type of infections (i.e, bacterial, viral, or parasitic) detailing the state of knowledge for each pathogenic agent. The polymorphism, the interference of viral proteins with HLA-G intracellular trafficking, and various cytokines have been described to modulate HLA-G expression during infections. We also discuss the cellular source of HLA-G, according to the type of infection and the potential role of HLA-G. New therapeutic approaches based on synthetic HLA-G-derived proteins or antibodies are emerging in mouse models of cancer or transplantation, and these new therapeutic tools may eventually prove useful for the treatment of infectious diseases.

1. Introduction

HLA-G was first described by Geraghty et al. in 1987 [1] as a member of the nonclassical human leukocyte antigen (HLA) family, which also includes HLA-E and F [2, 3]. The HLA-G gene is located within the major histocompatibility complex on the p21.31 region of chromosome 6. It has eight exons and seven introns, and its sequence is about 86% identical to the consensus sequence of the HLA-A, -B, and -C genes. Unlike classical class I molecules, HLA-G has a short cytoplasmic tail of six amino acids, due to premature stop codon in exon 6 [1]. Alternative splicing of the primary transcript generates four membrane-bound isoforms and three soluble forms. HLA-G1 has a structure similar to that of classical HLA class I molecules: a heavy chain consisting of three extracellular globular domains (α1, α2, α3) noncovalently associated with the β-2 microglobulin and a monomer peptide. The membrane-bound isoforms, HLA-G2, -G3, and -G4, are truncated isoforms lacking the α2 and/or α3 domains of the heavy chain [4, 5] and they should not, therefore, bind β-2 microglobulin [6]. Soluble HLA-G isoforms are generated either by alternative splicing of the HLA-G primary transcript (HLA-G5, -G6, and -G7) or by proteolysis of the HLA-G1 isoform (HLA-G1s) [7–9]. Indeed, the HLA-G5, -G6, and -G7 isoforms are highly unusual, as they are spliced variants of the HLA-G mRNA retaining introns 4 and 2 [7, 9, 10].

HLA-G is structurally diverse, with (i) different isoforms resulting from alternative splicing, (ii) some β2 M-free molecules [11], and (iii) all isoforms other than HLA-G3 being able to form homomultimers [12]. Indeed, HLA-G isoforms can also form homotrimers and homodimers, through the establishment of disulfide bridges between cysteine residues located in positions 42 and 147 [13]. Truncated isoforms of HLA-G can also carry out the biological functions of this molecule. Indeed, multimeric structures of HLA-G isoforms

function by differential binding to LILRB receptors [12]. Thus, HLA-G has specific features not found in other HLA class I molecules, such as (i) limited polymorphism [14, 15], (ii) restricted expression in physiological conditions [16], (iii) a shorter cytoplasmic tail region due to a stop codon in exon 6, (iv) unusual regulatory mechanisms due to the use of a promoter unique among HLA class I genes [17–20], and (v) numerous immunomodulatory properties, as described below.

HLA-G expression was initially described as restricted to the maternal-fetal interface, on extravillous cytotrophoblasts [21]. In healthy, nonfetal subjects, the HLA-G protein is found only on the cornea [22], thymic medulla [23], nail matrix [24], beta cells of the islets of Langerhans [25], mesenchymal stem cells [26], and endothelial precursors [27].

Levels of this protein are upregulated in many diseases and this upregulation may modulate the immune response.

The immunosuppressive properties of HLA-G have been thoroughly described. Indeed, the role of this molecule in immunotolerance was first described following its detection at the maternal-fetal interface, in *in vitro* studies, and has recently been confirmed by *in vivo* studies in mice. HLA-G can inhibit all types of immune competent cells (Figure 1). This effect is mediated by the direct binding of both completely soluble and membrane-bound isoforms to inhibitory receptors via the α3 domain. Indeed, B and T lymphocytes, NK cells, and monocytes of the myeloid lineage express the immunoglobulin-like transcript ILT2 (CD85j, ILIRB1) [28]; monocytes, macrophages, and dendritic cells express ILT-4 (CD85d, LILRB2) [29]. The killer cell immunoglobulin-like receptor (KIR2DL4/p49) is specific for HLA-G and is expressed by decidual NK cells. Unlike other inhibitory receptors, it may also mediate activation [30, 31]. In addition, soluble HLA-G triggers the apoptosis of T and NK cells via CD8-like classical class I soluble molecules [32]. HLA-G modulates adaptive and innate immunity by interacting with T or B lymphocytes and NK cells or polymorphonuclear cells (Figure 1).

HLA-G can inhibit all steps in the immune response: differentiation, proliferation, cytolysis, cytokine secretion, and immunoglobulin production. It can also alter antigen presentation to T lymphocytes, by inhibiting dendritic cell function and maturation [33–36] and by specific effects on T and B lymphocytes during effector activities. Indeed, this molecule inhibits the cytolytic activity of T and NK cells [37, 38] and the proliferation of B lymphocytes, together with the differentiation of these cells and their immunoglobulin secretion [39]. It also affects cooperation between B and T lymphocytes, by inhibiting T4 alloproliferation [36, 40] and inducing different types of regulatory T cells [41, 42]. Trogocytosis can generate different types of temporary regulatory cells *in situ*, accounting for the immunosuppressive effect of HLA-G-positive cells, despite their small numbers [43]. In addition, HLA-G inhibits the function of neutrophils, key cells in host immune defense against pathogens. Indeed, its interaction with its receptor, ILT4, on neutrophils impairs phagocytosis and the respiratory burst of neutrophils responsible for reactive oxygen species production [44].

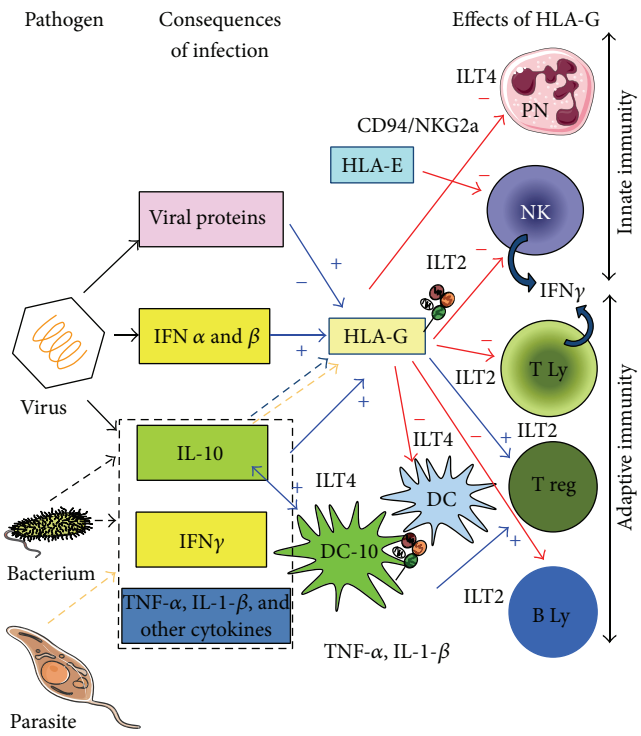

Figure 1: Causes and consequences of HLA-G modulation in infectious diseases. Positive and negative effects of HLA-G are shown in blue and red, respectively. Parasites, bacteria, or viruses induce the secretion of various cytokines, including IL-10 and interferon (-γ for bacterium and IFN-α and -β for virus). These cytokines upregulate the expression or secretion of HLA-G. In addition, IL-10 induces IL-10-producing human dendritic cells (DCs), termed DC-10, expressing HLA-G and ILT4. HLA-G induces tolerogenic DC in addition to DC-10 and regulatory cells via direct interaction with ILT2 and/or ILT4. HLA-G, through direct interaction with ILT2, inhibits the function of T and NK cells and B cells, whereas it inhibits the function of granulocytes and myeloid DC via direct interaction with ILT4. Indirect effects of HLA-G are mediated by the induction of HLA-E cell surface expression, which inhibits CD94/NKG2a on NK and T cells. The consequence of HLA-G action is a downregulation of innate and adaptive immunity.

Many studies have focused on HLA-G in tumoral processes, highlighting its role in tumor escape from the immune response [45]. The expression of this molecule is also beginning to be reported in other diseases, including infectious diseases. As host immune defense mechanisms efficiently eliminate most infections, studies on HLA-G in infections are based on the rationale that this molecule decreases the efficacy of the immune response through wide-ranging effects on all cell types involved in the immune response.

2. Features of HLA-G Expression by Infection Type

The main studies on HLA-G and infectious diseases are summarized in Table 1.

Table 1: Summary of the main studies on HLA-G and infectious diseases.

	HLA-G level changes			Model or patients	Characteristics	References
	Cell surface Increase	Cell surface Decrease	Blood sHLA-G Increase			
Infectious nonviral diseases						
Septic shock			↗	$n = 64$	Marked early and persistent increase: predictor of survival	[47]
Parasitic infections due to *Plasmodium falciparum*		↘		Malaria-infected placenta ($n = 15$)	Extravillous trophoblast cells (42% versus 90% in controls) Association with an increase in NK cells	[118]
Leishmania infantum visceral leishmania (VL)			↗	$n = 94$ 31 HIV+ 24 VL (7 HIV+ and 17 HIV−) 39 healthy subjects	HIV and VL: 57% HIV alone: 81% VL-HIV seronegative: 35%	[50]
Toxoplasma gondii	↗			*In vitro* infection of human trophoblast and BeWo cells	At mRNA and protein levels. Treatment with IL-10 decreases HLA-G expression.	[53]
			↗	Amniotic fluid: 58 women infected 24 noninfected	Significantly higher levels when the fetus is congenitally infected.	[52]
Viral infections						
		↘		Cotransfection experiments on glioma cell line and macrophages	Nef-independent, Vpu-dependent	[119]
HIV			↗		Before HAART, correlated with viral clearance and increase in $CD4^+$ T-cell levels. Decrease after treatment (36 months) Role of interferons and cytokines Increase in shedding	[54]
	↗			Infection treated (20) or not treated (3)	Indirect induction by viral products and/or cytokines (IL-10) T lymphocytes and monocytes Monocytes in treated patients	[57]
	↗			Treated by HAART ($n = 12$)	Expression on monocytes decreases after treatment to block cytokines (IL-10)	[56]
	↗			Patients treated ($n = 7$) with HAART or with a protease inhibitor regime or after HAART stopped	Monocytes (50%) on HAART Increase with nucleoside reverse transcriptase inhibitors but not with protease inhibitors Decrease when HAART removed	[58]
			↗	Longitudinal study in 24 infected patients	In early phases, restored to normal level in chronic phases of untreated normal progressors and long-term nonprogressors Secretion by monocytes, dendritic cells Role of IL-10	[120]

Table 1: Continued.

	HLA-G level changes			Model or patients	Characteristics	References
	Cell surface Increase	Cell surface Decrease	Blood sHLA-G Increase			
hCMV	↗			hCMV reactivation in *in vitro* activated macrophages ($n = 10$) Patients with HCMV pneumonitis	Day 20 poststimulation: expression in 45% of macrophages Bronchoalveolar macrophages Cooperative action of pp72 and pp86	[61]
	↗		↗	Patients ($n = 75$)	Increase on peripheral monocytes (6.3% versus 1.6%) Association with increase in plasma IL-10 concentration and no significant increase in IFN-γ concentration	[62]
Neurotropic virus (HSV-1 and RABV)	↗			*In vitro* infection of human neuron cell line (NT2-N)	Activation of HLA-G transcription Cell surface expression during RABV infection but not during HSV-1 infection	[63, 64]
Influenza virus (IAV) H1N1	↗			*In vitro* IAV infection of human alveolar epithelial cell line A549	Upregulation of HLA-G m RNA and proteins	[65]
	↗			(101) HIN1 patients (58 pandemic and 43 seasonal H1N1)	Monocytes and T lymphocytes (T reg CD4CD25FOXP3)	[66]
HPV		↘		Biopsies of invasive cervical carcinoma ($n = 79$)	Low HLA-G5 expression in all HPV-related cases	[67]
Hepatitis B virus	↗		↗	90 acute, 131 chronic, and 152 resolved cases of hepatitis B	Chronic > acute Resolved = normal Expression on monocytes and Treg	[68]
	↗			Chronic hepatitis B ($n = 74$)	Hepatocytes and biliary epithelial cells	[70]
Hepatitis C virus			↗	Chronic hepatitis C ($n = 67$)	sHLA-G = sHLA-G1 and-G5 Increase in plasma IL-10 and IFN-γ concentrations	[69]
	↗			Liver biopsies of patients with chronic hepatitis C ($n = 89$)	Hepatocytes More frequent in milder stages Significant correlation with the area of liver fibrosis	[121]
	↗			Liver biopsies of patients with chronic hepatitis C ($n = 20$)	HLA-G-positive cells are mast cells Soluble HLA-G secretion by human mast cells regulated by class I interferons	[71]

(a) Bacterial Infections. Septic shock is characterized by high mortality (40–50%) despite adequate initial treatment. Indeed, during septic shock, the initial huge systemic inflammatory response is immediately followed by an anti-inflammatory process, acting as negative feedback. However, this compensatory inhibitory response may subsequently become deleterious, as nearly all immune functions are compromised [46].

Monneret et al. [47] reported that marked, persistent HLA-G5 expression in septic shock was predictive of survival. The exocytosis-mediated upregulation of ILT4 expression on neutrophils is inhibited in conditions of sepsis, so the large amounts of HLA-G5 found in the plasma samples of patients surviving sepsis may have allowed them to control neutrophil inflammatory activity [44]. However, soluble HLA-G concentration was not found to be predictive of the detection of bacteremia and sepsis in pediatric oncology patients with chemotherapy-induced febrile neutropenia [48].

(b) Parasitic Infections. Few clinical data for parasitic infections are available, and those published relate mostly to plasma concentrations of sHLA-G, with the exception of one study of the protective role of HLA-G polymorphism in malaria [49]. We previously reported an increase in soluble HLA-G levels in 35% of cases of visceral leishmaniasis (*Leishmania infantum*) (VL) in HIV-seronegative patients and 57% of patients coinfected with HIV and *Leishmania infantum* [50]. However, the percentage of HLA-G-positive patients and the mean sHLA-G value were significantly lower in patients with both HIV infection and VL than in the patients with HIV infection alone. These results suggest that the increase in sHLA-G levels in HIV-infected patients with VL may contribute to a general tolerogenic environment, favoring the persistence of *Leishmania* and shortening the life expectancy of HIV-infected patients. sHLA-G may also be an immune biomarker of successful treatment. Thus, levels of sHLA-G with indoleamine 2,3 dioxygenase (IDO) activity may thus constitute, together with Th1/Th2 cytokine levels, surrogate markers for the resolution of VL, at least in immunocompetent patients [51]. High levels of sHLA-G are found in the amniotic fluid in women acquiring toxoplasmosis during pregnancy. The levels of this protein are the highest when the fetus is congenitally infected. However, all fetuses were born alive in our small series of patients, consistent with adequate downregulation of the inflammatory response. HLA-G may, therefore, play an immunomodulatory role that is necessary to avoid fetal loss but that may lead to the maternal-fetal transmission of *Toxoplasma gondii* [52]. HLA-G expression increases upon the *in vitro* infection of primary human trophoblasts and BeWo cells with *Toxoplasma gondii*, probably due to the secretion of proinflammatory cytokines in response to the parasite [53].

(c) Viral Infections. Many extensive studies have been carried out on cancers, but HLA-G expression has also been studied in many viral infections, with HIV infections being the most extensively studied (at least 30 published studies).

2.1. HIV Infection. Levels of sHLA-G are significantly higher in HIV-infected patients before treatment than in healthy controls [54]. The increase in plasma sHLA-G concentration in these patients has been attributed to an increase in HLA-G secretion from intracellular stores in monocytes and dendritic cells [55]. Indeed, a longitudinal study of plasma sHLA-G concentration in HIV-infected individuals with different rates of clinical progression showed that sHLA-G expression was associated with HIV disease progression [56]. HLA-G levels are high early in infection and remain high in rapid progressors. However, these concentrations return to normal levels in the chronic phase of infection, in both untreated normal progressors and long-term nonprogressors, when the infection is controlled. Cell surface expression of HLA-G is also detected on 93% of monocytes and 34% of T lymphocytes in patients [57]. Serum concentrations of HLA-G, like those of the other classical class I molecules (sHLA-A, -B, -C), also increase in HIV-infected patients and are significantly decreased by antiretroviral therapy (highly active antiretroviral therapy, or HAART), in cases in which HIV-1 replication is strongly inhibited.

Moreover, HAART significantly decreases the concentration of circulating soluble HLA-G molecules, this decrease being correlated with viral clearance and an increase in $CD4^+$ T cells, as reported for classical class I molecules. The decrease in sHLA-G levels after HAART reported by Cabello et al. is not consistent with the finding of an increase in HLA-G expression on monocytes following HAART in another study [56]. The agents responsible for this increase are nucleoside reverse transcriptase inhibitors rather than protease inhibitors [58]. Murdaca et al. explain these conflicting findings in terms of the membrane expression of HLA-G inducing an increase in soluble HLA-G molecule shedding [59]. However, high levels of HLA-G in peripheral monocytes were also observed in two of the 12 untreated patients, suggesting other causes unrelated to HAART [56]. High levels of HLA-G molecules are also found in the monocytes of untreated HIV-positive patients [57], possibly due to the pathogenesis of infection.

HLA-G expression may allow these cells to evade the immune system, because the protective function of HLA-G occurs after the induction of this molecule in HAART-treated HIV-1 patients, accounting for both the consistently defective function of monocytes in HIV-1-infected patients and the role of the viral reservoir present in monocytes during infection [56]. It inhibits myeloid dendritic antigen-presenting capacity via ILT4 and enhances the secretion of inflammatory cytokines [55]. $HLA\text{-}G^+$ regulatory T cells decrease in both absolute numbers and relative proportions during progressive HIV-1 infection. Their levels are thus inversely correlated to those of phenotypic markers of immune activation. $HLA\text{-}G^+$ T regulatory cells can decrease harmful bystander activation and may protect against HIV-1-associated immune activation and HIV-1 disease progression [60].

2.2. Human CMV (hCMV). Both membrane-bound and soluble plasma HLA-G concentrations increase during hCMV

infection. The induction of HLA-G protein in macrophages has been observed after the generation of these cells *ex vivo* from latently infected monocytes and after the reactivation of hCMV infection [61]. HLA-G protein has also been detected *ex vivo* on bronchoalveolar macrophages from patients suffering from acute hCMV pneumonitis, on peripheral monocytes and in plasma [62]. Blood sHLA-G concentration has been shown to be correlated with blood IL-10 and IFN-γ concentrations.

2.3. Neurotropic Virus. HLA-G protein has been reported to be expressed in human neurons after infection with rabies virus or herpes simplex type I, following the activation of gene transcription [63, 64].

2.4. Influenza A Virus (IAV). HLA-G expression was first demonstrated *in vitro* in an alveolar epithelial cell line, at the mRNA and protein levels, after treatment with various IAV strains [65]. HLA-G expression has been detected *in vivo* in patients infected with the pandemic H1N1 or seasonal H1N1 [66] viruses. It has been detected on monocytes and T lymphocytes, including T4 regulatory cells in particular. This cellular HLA-G expression contrasts with the absence of an increase in the plasma concentration of this protein.

2.5. Human Papilloma Virus (HPV). Low levels of HLA-G5 expression are observed in all HPV-related cases of invasive cervical cancer [67]. Indeed, HPV E5 may be involved in the decrease in HLA-G expression at the cell surface, because high-risk HPV oncoproteins may inhibit the promoters of HLA class I heavy chain genes and may modulate the levels of the transporter associated with antigen processing (TAP1) protein.

2.6. Hepatitis B and C Viruses (HBV and HCV). Plasma HLA-G concentration is higher during hepatitis infection than in healthy subjects without HBV infection. It is higher in cases of chronic hepatitis B than in acute hepatitis B and it returns to normal after resolution of the infection. In addition, an increase in HLA-G cell surface expression is observed on peripheral monocytes and regulatory T cells [68]. Similarly, an increase in blood sHLA-G concentration has been reported in patients with chronic hepatitis infection [69], associated with an increase in blood IL-10 and IFN-γ concentrations.

HLA-G expression in the liver has been detected by immunohistochemical methods, in hepatocytes and biliary epithelial cells from patients with chronic hepatitis B, by Souto et al. [70]. We [71] found that the number of HLA-G^+ cells was significantly correlated with the area of tissue affected by fibrosis. This led to the first demonstration that HLA-G^+ cells were mast cells. HLA-G secretion was significantly induced in human mast cells stimulated with IL-10 or class I interferons.

3. Mechanisms of HLA-G Modulation during Infection

These mechanisms (polymorphism, interference of infectious proteins with HLA-G intracellular trafficking and shedding, and cytokines) are summarized in Figure 1 and Tables 2 and 3.

(a) Polymorphism, Alleles, and Single-Nucleotide Polymorphisms. Firstly, HLA-G polymorphism, although limited with 40 alleles identified [15], is involved in susceptibility to viral infections, particularly those caused by HIV and HCV (Table 1). Indeed, the G*010108 allele has been reported to be associated with an increase in the risk of HIV-1 infection, whereas the G*0105N allele (null allele) has been shown to be associated with protection from infection in African women [72, 73] but a greater risk of infection in a population from north-eastern Italy [74]. Da Silva et al. have shown that HLA-G variants influence the horizontal transmission of HIV horizontal in African-derived HIV-infected patients, with a higher frequency of alleles and genotypes associated with low levels of HLA-G expression (i.e., a higher frequency of the 14 bp insertion allele) in African-derived HIV-infected individuals and a higher frequency of the 14 bp insertion +3142G (insG) haplotype and the insG/insG diplotype. In addition, a higher frequency of the ins/ins genotype is found among African-derived HIV-infected patients also infected with HCV [75].

Thus, the transmission of HIV-1 from infected mothers to their infants may be influenced by dissimilarities in their HLA-G sequences [76]. HLA-G*01:03+ mothers have recently been shown to be less likely to transmit HIV-1 to their children during the perinatal period [77]. The polymorphic sites may affect miRNA binding to the HLA-G mRNA, thereby influencing HLA-G translation [19, 78].

HLA-G polymorphism may also affect susceptibility to HCV infection in patients with sickle cell disease, because the C allele seems to confer protection against HCV, by a mechanism associated with an increase in HLA-G expression [79]. Homozygosity for the 14 bp deletion and the allele containing this deletion (010401) seems to be a risk factor for the vertical transmission of HCV, whereas the 0105N allele confers protection [80]. The HLA-G 14 bp insertion/deletion polymorphism is also a putative susceptibility factor for active hCMV infection in children [81]. Two polymorphisms in the 3′ untranslated region of the HLA-G gene (3′UTR) (14 bp ins/del, +3142C>G) are involved in susceptibility to HPV infection; indeed, the 14 bp del allele is associated with a high risk of HPV infection, and the del/C haplotype is associated with the development of invasive cervical cancer [82].

An association of HLA-G 3′UTR polymorphisms with the antibody response to *Plasmodium falciparum* has also recently been reported [49, 83].

(b) Interference of Viral Proteins with the Intracellular Trafficking of HLA-G. Viral proteins have generally been reported to decrease HLA class I expression, but their effect on HLA-G expression at the cell surface is more ambiguous (Table 3). Indeed, they may have no effect [84–86] or an inhibitory

Table 2: Influence of HLA-G polymorphism on susceptibility to infectious diseases.

Pathogens	Protection	Susceptibility	Vertical transmission (mother-to-child)	References
HIV	HLA-G*0105N (null allele)			[72, 73]
		G*010108 allele		[72]
		G*010108/010401		
		G*010101/010108		
		G*0105N		[74]
		14 bp (ins) allele		[75]
		+3142G (insG) haplotype		[75]
	G*01:01:01 genotype	G*01:04:04 genotype		[122]
			Differences in the HLA-G gene DNA sequence between mother and child	[76]
		14 bp insertion allele 14 bp + 3142G (insG) haplotype		[75]
		insG/insG diplotype in HCV coinfected		[75]
HCV		insG/insG diplotype in HIV coinfected		[75]
	+3142C allele in sickle cell disease patients			[79]
		−14 bp/−14 bp genotype		[81]
	HLA-G*0105N		G*010401 homozygosity for HLA-G 14 bp deletion	[80]
HPV	14 bp ins allele	14 bp del allele del/C haplotype with ICC development		[82]
Plasmodium falciparum	+3187G allele and haplotype UTR1	Haplotype UTR3		[83]
	+3010G and +3142C +3010G and +3196G			[49]

effect [87–90], and one study, carried out by Onno et al. [61], even reported HLA-G induction after viral reactivation in activated macrophages, through the cooperative action of the early HCMV proteins pp72 and pp86. By contrast, another team showed that HLA-G1 levels at the cell surface were downregulated and that this downregulation was dependent on hCMV short viral US glycoproteins [89]. Some US proteins have differential effects on the expression of classical HLA class I and HLA-G molecules at the cell surface, due to the shorter cytoplasmic tail of HLA-G [91] and other structural characteristics [91].

These conflicting results for hCMV may be accounted for by differences between the cell types studied (monocytes, trophoblasts, or the U373-MG astrocytoma cell line). The effects of viral proteins differ with the infected cell target, the type (classical or otherwise) of HLA class I molecules, and the membrane-bound or soluble nature of the HLA-G protein. These conclusions are illustrated by the following examples. US10 downregulates the cell surface expression of HLA-G but not that of classical class I MHC molecules [88], because the short cytoplasmic tail of HLA-G (RKKSSD) acts as a US10 substrate. On the other hand, the US2 protein decreases levels of HLA class I molecules by supporting proteasome-mediated degradation, unlike HLA-G1, which lacks the residues essential for interaction with US2 [84]. Moreover, HLA-G1 has also been reported to be targeted for degradation, independently of the cytoplasmic tail [84].

For HIV infections, the short cytoplasmic tail of HLA-G confers resistance to Nef-induced downregulation [85], whereas Nef downregulates MHC class I molecules [92].

(c) Cytokines. Many viruses have also developed other strategies for escaping host immune surveillance, such as a deregulation of the host cytokine network through the secretion of cytokines. Cytokines are also important in bacterial infections.

Table 3: Interference of viral proteins with HLA-G intracellular trafficking; comparison with classical HLA class I molecules.

Virus	Viral protein	Classical HLA class I		HLA-G			References
		Mechanism	Downregulation	Downregulation	No change	Upregulation	
HIV	Nef	Interacts directly with class I domain Redirects to endolysosomal pathway	↘		→ truncated cytoplasmic domain		[85]
	Vpu	Redirects to degradation pathway Affects early step in biosynthesis	↘	↘			[119]
HCMV	US2 US11	Exports for cytosolic degradation	↘		→ truncated cytoplasmic domain		[84, 86]
	US3 US6	Retention in endoplasmic reticulum	↘	↘			[87]
	US10			↘ cytoplasmic tail			[88]
	pp72 and pp86					↗	[99]
Herpes Virus	ICP47	Inhibits TAP (transporter associated with antigen processing)	↘	↘			[90]

The interleukin- (IL-) 10 family of cytokines and the related interferon (IFN) family form the larger class II cytokine family [93]. The IL-10 family consists of three subgroups, defined on the basis of biological functions: IL-10, the IL-20 subfamily cytokines (including IL-19, IL-20, IL-22, IL-24, and IL-26), and the type III IFN group (IFNλs).

Several viruses have been shown to upregulate the expression of cellular IL-10, which is produced by monocytes and, to a letter extent, by lymphocytes and, possibly, mast cells. Other viruses, such as the Epstein-Barr virus and HCMV, have functional orthologs of IL-10. Indeed, blood IL-10 and IFN-γ concentrations are high in hCMV infection [62] and in chronic hCMV infection [69]. In bacterial infections, IL-10 is also produced during sepsis [94]. High IL-10 levels are associated with bacteremia and sepsis in febrile pediatric cancer patients with neutropenia [95].

IL-10 is a pleiotropic cytokine with both immunostimulatory and immunosuppressive properties [96]. HLA-G expression is induced following IL-10 stimulation in experiments *in vitro* and is associated with IL-10 expression *in vivo* in a context of cancer. IL-10 selectively induces HLA-G expression, at both the mRNA and protein levels, in human trophoblasts and monocytes [97]. By contrast, Zhao et al. [53] have reported that IL-10 downregulates HLA-G expression in an *in vitro* model based on the infection of human trophoblasts with *Toxoplasma gondii*.

Interferons trigger important antiviral effects during viral infections. They can be classified into three classes: (i) class I (IFN-α, -β), produced by NK cells, lymphocytes, macrophages and fibroblasts, and other molecules, such as IFN-ω and -ζ, produced by leukocytes, (ii) class II, consisting solely in IFN-γ produced by NK and T cells, and (iii) class III, recently described and including IFN-λ1 (IL-29), -λ2 (IL-28A), and -λ3 (IL-28B), produced by numerous cell types, including plasmacytoid dendritic cells. Types I and III interferons are produced by virus-infected cells. In these cells, double-stranded RNA activates the signaling cascades leading to the transcription of the IFN-α and -β genes. Following their secretion, these interferons interact with a specific IFNα/β receptor on neighboring uninfected cells and on the initial infected cells, activating a signaling cascade that produces antiviral proteins that act on viruses and upregulate HLA class I expression. IFN-γ is involved in both innate and adaptive immunity. Type III IFNs signal through a receptor complex consisting of IL10R2 and IFNL-R1 (IL-28RA). HLA-G induction by interferons has been reported in numerous studies. Indeed, different types of IFN (α, β, and γ) can induce HLA-G expression in different cell types. Yang et al. [98] reported the induction of HLA-G on Jeg 3 cells by different interferons. The induction of HLA-G on monocytes has also been reported [99]. IFN-β and -γ have recently been shown to activate HLA-G expression in a human neuron cell line infected with rabies virus [63]. HLA-G expression after IFN treatment has also been demonstrated in several tumor models, including a melanoma cell line. Thus, treatment with IFN-β or -γ increases the dimer/monomer ratio and, subsequently, affinity for the ILT2 receptor [100]. An increase in HLA-G expression, in monocytes and serum, is also

observed in patients treated systemically with IFN-α [101]. Similar effects have also been reported after treatment with IFN-β1 [102]. Interferons are known to induce HLA class I expression by binding to the interferon-stimulated response element (IRSE) motif in the proximal promoter region of class I genes. This motif is absent from the HLA-G promoter [15], so the upregulation of HLA-G expression by interferons was unexpected. This upregulation was accounted for by the identification of another specific functional IRSE in the distal promoter, at a position −744 bp upstream from the ATG [103].

The early phase of septic shock is characterized by a massive release of inflammatory mediators, causing organ dysfunction and hypoperfusion. These cytokines include tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta), and IFN-γ. Like IFN-γ, TNF-α and IL-1 can also induce HLA-G. Indeed, TNF-α has been shown to induce a moderate increase in steady-state levels of HLA-G mRNA in human trophoblast cell lines [98]. IL-1β increases the expression of HLA-G and Toll-like receptor 4 (TLR4) in an HIF-1α-dependent manner [104].

Protease levels generally increase during bacterial and viral infections and this may lead to the proteolytic shedding of membrane-bound HLA-G in a soluble form, resulting in an increase in blood HLA-G concentration.

4. General Discussion

An upregulation of HLA-G expression has been reported in most studies of viral infection. Reported discrepancies in the results concerning HLA-G expression in hCMV or HIV infections may reflect differences in the models used or in infection status or stage between studies. This upregulation of HLA-G expression results principally from an increase in the secretion of cytokines, such as IL-10 and class I interferons. HLA-G levels increase, either at the cell surface or in the blood (sHLA-G). Indeed, concentrations of soluble HLA-G in the blood increase in some viral infections caused by HIV, hCMV, HCV, and HBV viruses, similar to classical soluble class I antigens. The increase in the secretion of cytokines, including interferons in particular, during the course of viral infection, and the use of interferons as therapeutic agents may account for the increase in HLA-G levels. Shedding, due to metalloprotease digestion, is favored by interferons and also contributes to the increase in soluble HLA-G concentration in the blood. The peripheral cells expressing HLA-G during viral infections are monocytes and T lymphocytes (HIV, influenza). Neurons and bronchoalveolar macrophages have been shown to express HLA-G in infected tissues. In HCV hepatitis, Souto et al. [70] found that hepatocytes and biliary epithelial cells expressed HLA-G, whereas we identified HLA-G-positive cells as mast cells [71]. This discrepancy can also be accounted for a difference in the definition of positivity, because we also observed a weak staining of hepatocytes but took only strong staining into account. These findings were confirmed by our findings for a human mast cell line showing that this cell line expressed HLA-G and secreted class I interferons. Moreover, mast cells may promote liver fibrosis [105] by stimulating collagen synthesis and fibroblast chemotaxis. Cytokines involved in liver fibrosis, such as IL-4 or IL-33 [106], act as chemoattractants, driving the activation of mast cells [107, 108]. In addition, mast cells secrete tryptase and many cytokines involved in fibroblast proliferation [109] and fibrogenesis [110], including IL-10 [111]. However, the role of HLA-G in viral infections remains unclear, because two hypotheses are possible. It may promote virus immune escape, as in cancers. This hypothesis is supported by the immunosuppressive properties of HLA-G, which act on all the cells involved in the immune response. In addition, sHLA-G downregulates CXCR3 levels on peripheral blood and tonsil CD56 cells [112]. This dysregulation of CXCR3 signaling due to CXCL10 deficiency impairs antiviral responses *in vivo,* including the antiviral response to herpes simplex virus 1 infection [113].

Alternatively, HLA-G expression or secretion may reflect an appropriate and efficient response to the inflammatory process occurring during viral infection or septic shock. Indeed, HLA-G may be beneficial during viral infection, because an increase in HLA-G concentration occurs following the secretion or therapeutic administration of interferons, classes I and III IFNs are secreted as physiologic antiviral responses, and IFN-α is an effective treatment for chronic HCV infection. We can hypothesize that the antiviral effect of classes I and III IFNs may be mediated by the properties of HLA-G, which is induced by IFN, as described above.

The immunosuppressive properties of HLA-G have been clearly demonstrated *in vitro*, and the role of this protein has now been elucidated *in vivo*. Indeed, two studies have demonstrated the involvement of this protein in tumor progression in a mouse model *in vivo*. In a xenograft model, the HLA-G1 isoform promotes tumor progression in immunocompetent Balb/c mice, affecting both innate and adaptive immunity. By contrast, no tumor development is observed when HLA-G is blocked by a specific antibody, demonstrating the specificity of the effect [114]. HLA-G plays a role in tumor escape, through expansion of the population of myeloid-derived suppressor cells and an alteration of the cytokine balance in favor of a Th2 response rather than a Th1/Th17 response. HLA-G expression is associated with tumor metastasis and poor survival in the Balb/c nu/nu mouse model of ovarian cancer [115]. In another model used to assess the efficacy of synthetic HLA-G proteins for therapeutic purposes in a context of transplantation, it was shown that a single treatment of skin allograft recipient mice with these proteins was sufficient to prolong graft survival significantly and that four weekly treatments were sufficient to ensure graft survival [116].

The feasibility of synthesizing effective HLA-G-derived molecules opens up new possibilities in the fields of tumor diseases and infection. For example, HCV infections are a worldwide public health problem and may be suitable for treatment with such molecules, because HLA-G expression is correlated with the area of fibrosis.

In the future, it may be possible to modulate HLA-G transcription with a miRNA, such as the hsa mir-148a and mir-152, which bind to the 3′ untranslated region of the HLA-G gene (3′UTR) [19], downregulating its mRNA levels. Indeed a polymorphism of the binding site for this miRNA

(the 263del/ins SNP) has been associated with poor control of HIV infection [117].

5. Conclusions

As in cancers, HLA-G expression is upregulated in infectious diseases, in response to changes in the cytokine microenvironment, relating principally to increases in the levels of IL-10 and interferons. HLA-G expression may occur in infected tissues and/or, more frequently, in peripheral blood, in the form of sHLA-G or a membrane-bound form on monocytes or different types of T cells (CD4, T reg). This molecule may have deleterious effects, promoting pathogen escape from immune control, as reported in cancers, or it may be beneficial, as in septic shock [47], reflecting appropriate and effective feedback control of inflammatory process. The role of this protein in parasitic and viral infections remains to be elucidated. Thus, HLA-G may be a single marker of infectious diseases, related to pathogens and/or to the immune response, or it may constitute a therapeutic target, once its function has been clarified in particular types of infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Some Basic Aspects of HLA-G Biology

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Human leukocyte antigen-G (HLA-G) is a low polymorphic nonclassical HLA-I molecule restrictively expressed and with suppressive functions. HLA-G gene products are quite complex, with seven HLA-G isoforms, four membrane bound, and other three soluble isoforms that can suffer different posttranslational modifications or even complex formations. In addition, HLA-G has been described included in exosomes. In this review we will focus on HLA-G biochemistry with special emphasis to the mechanisms that regulate its expression and how the protein modifications affect the quantification in biological fluids.

1. Introduction

Human leukocyte antigen-G (HLA-G) is a major histocompatibility complex class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules by its restricted tissue distribution and limited polymorphism in the coding region. HLA-G role in immune tolerance was uncovered studying its expression in trophoblast cells at fetus-maternal interface [1]. Several studies have found an aberrant or reduced expression of both HLA-G mRNA and protein in pathological conditions such as preeclampsia [2] or recurrent spontaneous abortion [3] in comparison with normal placentas. HLA-G expression has been documented in few tissues during physiological conditions, such as cornea, thymus, erythroid, and endothelial precursors [4–6], and in a variable percentage of serum/plasma samples from healthy subjects [7] where the main producers seem to be activated CD14+ monocytes [8]. An ectopic expression of HLA-G molecules has been observed during "no-physiological" conditions, such as viral infection [9–12], cancer [13], transplantation [14–18], and in inflammatory and autoimmune diseases [19–21]. Thus, a growing body of evidence has indicated HLA-G as a suitable key actor in different pathologies. In fact, HLA-G may exhibit two distinct effects in pathological conditions: it could be protective in inflammatory and autoimmune diseases [22] or it could be dangerous, for example, in tumors or infectious diseases.

2. HLA-G Expression and Regulation

The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region (3′ UTR) that modify the affinity of gene targeted sequences for transcriptional or posttranscriptional factors, respectively [24]. Twenty-nine single nucleotide polymorphisms (SNPs) have been identified in the HLA-G promoter region, which may be involved in the regulation of HLA-G expression, considering that many of these polymorphisms are within or close to known or putative regulatory elements (Figure 1). The *HLA-G* 5′ upstream regulatory region (URR) is unique among the *HLA* genes [25] and is unresponsive to NF-κB [25] and IFN-γ [26], due to the presence of a modified enhancer A (enhA) and a deleted interferon-stimulated response element (ISRE). A locus control region (LCR) located −1.2 kb from exon 1 exhibits a binding site for CREB1 factor, which also binds to two additional cAMP response elements at −934 and −770 positions from the ATG start codon. In addition, an ISRE for IFN response factor-1 (IRF-1) is located at

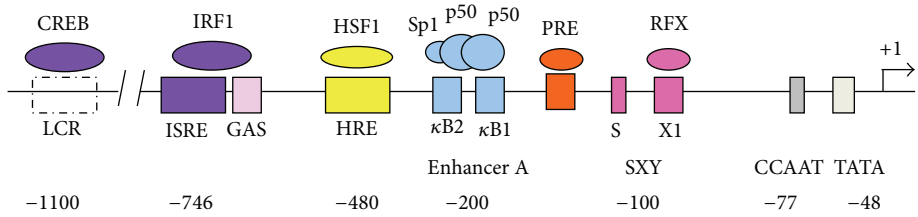

Figure 1: HLA-G unique promoter region. Enhancer A element (KB1, KB2, Sp1): NF-KB; interferon-stimulated regulatory element (ISRE); interferon regulatory factor (IRF); interferon-gamma activated site (GAS); SXY region; progesterone response element (PRE); hypoxia response element (HRE).

the −744 bp position [24] and is involved in *HLA-G* transactivation following IFN-β treatment [27]. The *HLA-G* promoter also contains a heat shock element at the −459/−454 position that binds heat shock factor-1 (HSF-1) [28] and a progesterone receptor binding site at −37 bp from ATG start codon [29]. Several promoter region polymorphisms coincide with or are close to known or putative regulatory elements and thus may affect the binding of *HLA*-G regulatory factors [30]. The −725 C>G/T SNP is very close to ISRE, and the −725 G allele is associated with a significantly higher expression level compared with the other alleles [31]. The polymorphic sites at the 5′ URR are frequently in linkage disequilibrium (LD) with the polymorphic sites identified at the 3′ UTR, some of them influencing alternative splicing and mRNA stability [25].

A 14 base pair (14 bp) insertion/deletion (INS/DEL) polymorphism (rs66554220) in exon 8 involves mRNA stability and expression [32]. In particular, the DEL allele stabilizes the mRNA with a consequent higher HLA-G expression [33, 34]. The presence of an adenine at position +3187, modifying an AU-rich motif in the *HLA-G* mRNA, decreases its stability [35]. One single nucleotide polymorphism (SNP) C>G at the +3142 bp position (rs1063320) has been explored by Tan and coauthors [36]. The presence of a guanine at the +3142 position may influence the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs miR-148a, miR-148b, and miR-152, therefore decreasing the mRNA availability for translation by mRNA degradation and translation suppression. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The contrasting results obtained by Manaster and coauthors [37], who have reported the absence of +3142 C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression. Other SNPs have been identified as implicated in miRNA interaction. In particular, +3003, +3010, +3027, and +3035 SNPs are influenced by miR-513a-5p, miR-518c*, miR-1262 and miR-92a-1*, miR-92a-2*, miR-661, miR-1224-5p, and miR-433 miRNAs [35]. The miR-2110, miR-93, miR-508-5p, miR-331-5p, miR-616, miR-513b, and miR-589* miRNAs target the 14 bp INS/DEL fragment region, and miR-148a, miR-19a*, miR-152, mir-148b, and miR-218-2 also influence the +3142 C/G polymorphism.

HLA-G is a stress-inducible gene: heat shock, hypoxia, and arsenite increase different HLA-G alternative transcripts [28, 38]. The indolamine 2,3-dioxygenase (IDO), an enzyme which metabolizes tryptophan, induces HLA-G expression during monocyte differentiation into dendritic cells [39]. Interestingly, HLA-G exerts its immune tolerogenic function towards T cell alloproliferation following an independent pathway from IDO [40]. Nitric oxide-dependent nitration of both cellular and soluble HLA-G protein decreases total HLA-G cellular protein content and expression on the cell surface, while it increases HLA-G shedding into the culture medium. This effect was posttranscriptional and the result of metalloprotease activity [41–43]. Several evidences indicate that the soluble HLA-G1 (sHLA-G1) form is generated through the shedding of the membrane bound HLA-G1 by metalloproteinase (MP) [44–47]. In particular, matrix metalloproteinase-2 (MMP-2), a zinc-containing and calcium-requiring endopeptidase known for the ability to cleave several extracellular matrix constituents, as well as nonmatrix proteins, is responsible for HLA-G1 membrane-shedding via three possible highly specific cleavage sites [48].

The anti-inflammatory and immunosuppressive interleukin- (IL-) 10 has been correlated with concomitant HLA-G expression [33]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor (LIF) [49] and methotrexate cell exposure [50]. Furthermore, interferon (IFN)-α, -β, and -γ enhance HLA-G cell-surface expression by tumors or monocytes [51, 52]. HLA-G expression could be acquired by trogocytosis, where a "donor" cell that expresses membrane HLA-G exchanges membrane parts containing HLA-G with a "recipient" cell that is not expressing HLA-G molecules. In this particular situation, "recipient" cells will acquire and make use of membrane HLA-G molecules from a "donor" HLA-G positive cell without the activation of HLA-G gene transduction into protein. Trogocytosis of HLA-G from antigen presenting cell (APC) by T cells in humans makes these T cells unresponsive [53]. It has been shown that NK cells can acquire HLA-G1 from tumor cells, which provokes an arrest of NK cells proliferation and cytotoxic activity,

behaving like suppressor cells capable of inhibiting other NK cell functions [54].

3. HLA-G Transcription Products

To date, 50 alleles (IMGT HLA database, December 2013) and 16 proteins are known. Seven HLA-G isoforms exist due to mRNA alternative splicing and differential association with β2-microglobulin (β2-m). Four of them are found on the cell surface (HLA-G1, -G2, -G3, and -G4), while the other three are soluble forms released from the cell (HLA-G5, -G6, and -G7), due to the lack of the transmembrane and intracellular domains of membrane-bound HLA-G (Figure 2). The HLA-G 14 bp INS/DEL polymorphism is involved in the expression of both HLA-G1 and HLA-G5 isoforms, with decreased HLA-G1 and HLA-G5 concentrations in 14 bp INS samples in comparison with 14 bp DEL samples [32, 34].

The overall structure of HLA-G resembles other class I MHC molecules, in which a heavy chain comprised of three extracellular domains is noncovalently associated with β2-m (Figure 2). A nine-residue self-peptide is bound within a cleft formed by two alpha-helices and a beta-sheet floor. An extensive network of contacts is formed between the peptide and the binding cleft, leading to a constrained mode of binding reminiscent of that observed in HLA-E [65].

4. HLA-G Receptors

HLA-G exerts its immunomodulatory functions through the interaction with multiple receptors such as LILRB1 (ILT2/CD85j), LILRB2 (ILT4/CD85d), and KIR2DL4 (CD158d), which are differentially expressed by immune cells. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated CD8+ T cells [66], modulates the activity of NK cells [67] and dendritic cells (DC) [68], blocks allocytotoxic T lymphocyte response, induces expansion of T cell populations such as $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells [69] and $CD3^+CD4^{low}Foxp3^-$ and $CD3^+CD8^{low}Foxp3^-$ [70], and inhibits Vγ9Vδ2 T-cell proliferation and cytotoxicity without inducing apoptosis [71]. Moreover, HLA-G is expressed at high levels on DC-10 cells, human DCs with tolerogenic activity and an outstanding ability to produce IL-10 [72]. Interestingly, the expression of membrane-bound HLA-G1 and its receptors is upregulated by IL-10 on DC-10 and the expression of high levels of membrane-bound HLA-G1, ILT4, and IL-10 by DC-10 is critical to the generation of allergen-specific Tr1 cells by DC-10.

Whereas LILRB1 is expressed by NK cells, T cells, DCs, and decidual macrophages, LILRB2 expression is restricted to monocytes, macrophages, and DCs. These receptors can bind both classical and nonclassical HLA-I molecules [73, 74]. However, they present more affinity for HLA-G than for classical HLA-I molecules [75]. Also, HLA-G interaction with LILRB1 on NK cells and the resultant inhibitory function do not require tumor cell lipid raft integrity [76]. This differs from classical HLA-I, which are recruited in lipid rafts upon receptor engagement [77].

LILRB1 and LILRB2 possess 4 extracellular domains (D1–D4) and four and three immunoreceptor tyrosine-based inhibitory motifs (ITIMs), respectively, in their long cytoplasmic tails. These ITIM motifs confer them inhibitory characteristics, contrary to other LILR family receptors with activating properties that lack these ITIM motifs and possess an Arg residue in the transmembrane domain [74]. Interaction of LILRB1 and LILRB2 with their ligands causes phosphorylation of these ITIMS and recruitment of SHP phosphatases that initiate the inhibitory cascade. The D1 and D2 domains mediate the interaction of these receptors with HLA-I molecules and in the case of LILRB1 that occurs with the α3-domain and β2-m [74]. In fact, β2-m free HLA-G molecules are not recognized by LILRB1 [78]. However, in the case of LILRB2, it seems that interactions of these receptors with HLA-I molecules implicate the conservative residues of α3-domain but not of β2-m [73, 74]. HLA-G can form dimers that bind to LILR receptors with even a higher affinity than HLA-G monomers [79], being able to bind two receptors simultaneously [80].

Another HLA-G receptor is KIR2DL4 or CD158d, the only receptor of the killer cell immunoglobulin-like receptors (KIR) family that is expressed in all NK cell types [67]. KIR family includes receptors with activating properties and receptors with inhibitory properties. KIR2DL4 has unique structural properties among the rest of KIR receptors: it possesses a long cytoplasmic tail characteristic of inhibitory receptors, a charged amino acid in the transmembrane domain similarly to activating KIR receptors (reviewed [81]), and a mixed structure in the extracellular part with D0 and D2 domains. Contrary to other KIR receptors, KIR2DL4 expression is transitory on NK cell surface, with a main expression in endosomes, reached by an endocytic process. KIR2DL4 seems to participate to HLA-G endocytosis when it is transient expressed on NK cell surface, as both HLA-G and KIR2DL4 can be simultaneously colocalized in endosomes [82]. This could explain why soluble HLA-G or anti-KIR2DL4 antibodies, but not solid-phase bound antibodies, can induce cytokine secretion by NK resting cells. However, KIR2DL4 expression can be induced by IL-2 and its activation upon antibodies engagement provokes a weak cytotoxic activity with a strong IFN-γ production [83].

In vitro studies have shown that KIR2DL4 is able to interact with β2-m free HLA-G molecules, inducing IFN-γ production [84] and increasing NK cell cytotoxicity [19]. Contrary to LILR receptors, KIR does not bind HLA-I molecules through its α3 domain but through α1 and α2 domains which are much more polymorphic than α3 domain [85, 86]. This could account for the broader specificity of LILR receptors in comparison with KIR2DL4 that binds specifically HLA-G and no other HLA-I molecules. Also, structural studies suggest that KIR2DL4 cannot bind HLA-G dimers due to steric reasons [22].

The expression of LILRB1, LILRB2, and KIR2DL4 can be induced by HLA-G without any costimulatory requirement, which indicates that it can occur independently from any immune response [87].

Besides these receptors, HLA-G can also bind to CD8 without TCR interaction, provoking NK cells and activated

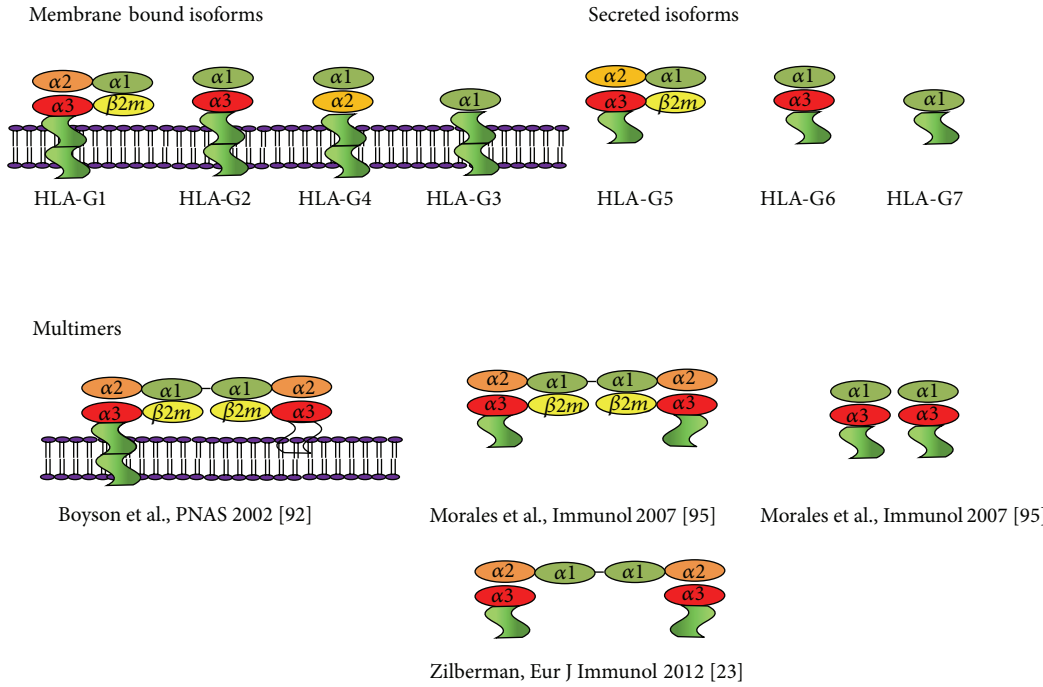

Figure 2: HLA-G isoforms and conformations. Membrane and soluble HLA-G isoforms are reported as monomeric and dimeric conformation Zilberman, Eur J Immunol 2012 [23].

CD8+ T cells apoptosis, and FasL upregulation and secretion [88]. Another putative HLA-G receptor is CD160. Interaction of HLA-G with CD160 expressed by endothelial cells induces the apoptosis of these cells [89] and inhibits cell proliferation, migration, and tubule formation [90], inhibiting the angiogenic process.

5. Posttranslational Modifications of HLA-G Molecule

Although most studies are related to β2-m bound HLA-G molecules that correspond to the originally described structure, several results have demonstrated the existence of modified variants of this structure. For example, expression of β2-m free HLA-G, which can be originated by dissociation of HLA-G complete isoforms [45], has been demonstrated in different tissues such as placenta [78] or pancreatic endocrine cells [91].

Besides Cys residues in α2 and α3 domains that allow intramolecular disulphide bonds, HLA-G molecule presents other important Cys residues. Cys42 in α1 domain and Cys147 in α2 domain can form intermolecular disulphide bonds giving rise to HLA-G dimers that can be observed by SDS/PAGE under nonreducing conditions [92]. These structures have been observed for all HLA-G isoforms except HLA-G3 [93]. It has been estimated that about 40% of HLA-G molecules at trophoblastic cells surface are in a dimeric form; meanwhile, only a small fraction of soluble HLA-G would be constituted by HLA-G dimers [94]. Even more, villous cytotrophoblast cells can produce dimers of β2-m free HLA-G5 molecules [95].

Immunoblot analysis with 4H84 antibody rends bands of diverse molecular weights (35–50 kDa) due to a glycosylation of HLA-G through an Asn residue (Asn86). This modification has been observed for both soluble and membrane bound HLA-G [96]. Another posttranslational modification observed in HLA-G is nitration in Tyr residues. Presence of 3-nitrotryrosine in HLA-G has been demonstrated in vivo in biological fluids both in monomeric and multimeric form [42] and in vitro after treatment with NO donors, which also increase HLA-G shedding by metalloproteases [43]. The detection of this modified HLA-G may characterize HLA-G synthesized at sites of inflammation where there is an important peroxide production.

Recently, HLA-G of molecular weights (70–76 kDa) higher than those expected were observed in biological fluids even when SDS/PAGE prior to western blot was performed under reducing conditions [64]. These molecules were associated with β2-m and could form dimers through disulphide bonds. The importance of these structures resides in the fact that they are not equivalently recognized by anti-HLA-G antibodies and can originate discrepancies in HLA-G quantification results. These molecules were later identified as ubiquitinated HLA-G molecules [97] with an intracellular origin demonstrated by their presence in exosomes, which are microvesicles of 50–100 nm originated from the endolysosomal pathway and secreted by many different cell types [98].

These particles carries mRNA, miRNA, and proteins, such as classical HLA-I molecules [98], and can exert distant immune functions [98]. Exosomes could act as a mechanism

to spread HLA-G tolerogenic functions because HLA-G presence has been demonstrated in exosomes produced by melanoma cells [99] and by early and term placenta [100]. Furthermore, in serum from pregnant women HLA-G can be detected incorporated into exosomes [101].

6. Analytical Challenging in Soluble HLA-G Analysis

Searching in PubMed with the words HLA-G and ELISA there are 175 papers published until November, 2013, measuring soluble HLA-G in different biological fluids, including serum, plasma, and exudates. From these papers, it is clear that the measurement of soluble HLA-G is a potential biomarker for diagnostic and/or prognostic in some physiopathological situations, such as obstetric complications or cancer [102]. In addition detectable levels of soluble HLA-G in medium from embryo culture are associated with success in vitro fertilization. For this reason, the disposal of a good and widely accepted method to measure the soluble HLA-G levels is of crucial importance to achieve a good translation of results between different laboratories. Most are in-house ELISA assays (Table 1) using as capture antibody the mAb MEM-G/9, which has been raised against recombinant human HLA-G refolded with β2-m and peptide [56]. Other ELISAs are designed to measure exclusively HLA-G5 and/or -G6 using anti-pan HLA-I antibody W6/32 as detection antibody and the antibody 5A6G7 as capture antibody [103], that reacts with the intron 4, which is exclusive of these two isoforms [104]. As detection antibody most assays use an anti-β2-m antibody or W6/32. These assays perform very well in vitro using cell cultures, but the procedure for HLA-G measurement is far from being resolved in vivo, and it has been a source of conflicting results and interesting discussions [105–108]. More than 15 years have passed since first reports of a method for measuring HLA-G [55] and meanwhile some important efforts have been carried out by several authors to validate a method and a standard to measure soluble HLA-G [58]. However, four main problems remain to be solved: the identification of the main circulating HLA-G molecules in vivo, the obtaining of a purified standard widely available, the selection of the antibodies used in the procedure, and the sensitivity of the methodology.

Probably the most important issue is related to the types of HLA-G molecules present in biological fluids, as we do not know yet the predominant isoform and whether it circulates free or included in microvesicles, that is, exosomes [64], or if they are mainly free molecules or associated with β2-m, or even the influence of modifications such as dimerization [92], nitration [42], or ubiquitination [97]. The presence of these altered structures could be more relevant in cancer where there is a deeply altered microenvironment. Probably, the predominant structures in biological fluids are the dimeric or multimeric forms, considering that the extracellular redox status is more oxidized than the redox status and that there is a low proportion of free SH groups from the Cys in circulation [41]. It is not known if these proteins react equally with different antibodies employed to measure HLA-G in ELISA. Assuming the statement that only shed HLA-G1 and HLA-G5 are released to circulation, we and others have calculated the amount of sHLA-G1 by the difference between the concentrations of sHLA-G1/HLA-G5 (using MEM-G/9 as capture mAb) and HLA-G5 (using 5A6G7 as capture mAb) [109]. However, under the new vision of circulating HLA-G molecules we cannot be sure now that this always occurs in vivo. Unexpected results probably due to anomalous structures were already documented in the Wet-Workshop for Quantification of Soluble HLA-G held in 2004 [58]. In this workshop it was observed in some samples that there were HLA-G 5A6G7-immunoreactive molecules that were not recognized by MEM-G/9. These different structures were later elucidated to be new high molecular weight HLA-G complexes [64].

A second important problem is the lack of a widely available purified HLA-G molecule that could serve as a standard. The only commercial soluble HLA-G available kit nowadays for quantitative measurement (EXBIO Praha, Czech Republic) uses a sHLA-G standard calibrator in terms of arbitrary units/mL, but its equivalence to a protein concentration or biological activity is unknown. A high useful method to produce a protein is by plasmid transfection in bacteria, and both HLA-G1 and a fusion protein have been produced by this methodology [110]. As synthetized in a prokaryotic model, there are not the posttranslational modifications produced in eukaryotes, mainly glycosylation [96], and probably their conformation is not equivalent to the native protein. For example, the fusion proteins produce inhibition in NK cells only at levels much higher than the native protein. HLA-G5 molecules purified from detergent lysates of SF9 cells transfected with HLA-G5 and human β2-m have been used as a standard [58], while others have purified the protein from HLA-G transfected cell culture supernatants by affinity chromatography [111]. Also, other studies use dilutions of tested cell supernatants as standard, but the concentrations obtained cannot be extrapolated to other studies [109]. A standard widely available that could serve for data comparison between different laboratories could be of interest, so data could be transferred between papers. Until this standard becomes available, HLA-G level comparisons between different laboratories should be taken with caution. Same precautions should be taken when transferring the reference values that depend on not only both the standard and methodology used, but also on the population studied.

The third issue is related to the capability of the antibodies to recognize all forms of HLA-G. Most of the anti-HLA-G antibodies used in the ELISA recognize the native protein, are very specific, and do not react with other HLA-I molecules (Table 1) as it has been discussed elsewhere [112]. We do not know yet if the reaction is equimolar with all HLA-G molecules, and probably some proteins could be underrecognized. For example, it was recently shown by flow cytometry that MEM-G/9 can also react with HLA-G3, but the intensity of the signal is weaker than with HLA-G1 [113]. Some HLA-G complexes are underrecognized by MEM-G/9 and react better with the anti-HLA-G antibody G-233 [64]. In addition, although HLA-G polymorphism is quite low with only 16

Table 1: Examples of methods developed for measuring soluble HLA-G.

Type	Standard	Capture antibody	Detection antibody	Detection	Detection limit	Reference
ELISA-sandwich	HLA-G1/LCL 722.221 transfected cells	W6.32 after depletion with TP25.99	anti-β2-m	Colorimetric	2.1 ng/mL	[55]
ELISA-sandwich	None	87G, BFL.1 or MEM-G/9	W6/32	Colorimetric	O.D.	[56]
ELISA-sandwich	HLA-G transfected CHO cells	G233	56B	Colorimetric	1 ng/mL	[57]
ELISA-sandwich	HLA-G5 protein derived from insect SF9 cells	MEM-G/9	anti-β2-m	Colorimetric	5 ng/mL	[58]
ELISA-sandwich	HLA-G5 protein derived from insect SF9 cells	5A6G7	W6/32	Colorimetric	5 ng/mL	[58]
Luminex	HLA-G5 and beta2 m transfected SF9 cells	MEM-G9	anti-β2-m	Fluorescence	0.3 ng/mL	[59]
ELISA-sandwich	HLA-G transfected LCL 721.221 cells	MEM-G/9	W6/32	Fluorescence	1 ng/mL	[60]
Bio-Plex	HLA-G5 transfected HeLa cells	MEM-G/9	W6/32	Fluorescence	0.3 ng/mL	[61]
ELISA-sandwich	HLA-G transfected LCL 721.221 cells	MEM-G/9	W6/32	Chemiluminescence	2 ng/mL	[62]
ELISA-sandwich	Purified HLA-G	HGY (noncommercial)	Polyclonal anti-HLA-G	Colorimetric	1 U/mL	[63]
ELISA-sandwich	HLA-G1 transfected LCL-721.221 cells	G233	anti-β2-m	Colorimetric	4 ng/mL	[64]

proteins described to date, we do not know yet how they affect the binding to the antibodies. Of particular interest is that although the capture antibody in ELISA is HLA-G specific, only a few authors have used a specific antibody for HLA-G as detection antibody [63]. Instead, as we mentioned before, the detection antibody used in most of the ELISAs is an anti-β2-m antibody. The fact that HLA-G1 and -G5 from cell cultures are complexed with β2-m does not imply that the same occurs always in vivo in all clinical situations. Some of HLA-G released by the embryo is not bound to β2-m [95], so these molecules would not be detected in this type of assays. Although some authors have used the anti-HLA-G mAb 4H84 in ELISA [114], its use is not recommended as it can produce some nonspecific reactions with classical HLA-I molecules, under certain methodological conditions [113].

Finally, another problem to be solved is the sensitivity of the method. An important issue is that neither the functional sensitivity nor the analytical sensitivity is usually reported. Most methods are sandwich-ELISAs with colorimetric detection, whose reported detection limit is in the order of 1–10 ng/mL and soluble HLA-G levels are below this detection limit in many occasions. Thus, it is not known if there is no circulating HLA-G or if the procedure is not sensitive enough for quantification of low HLA-G levels. Some authors have improved the methodology, using fluorescence detection or with procedures based on microspheres technology. The detection limit decreased one order of magnitude compared to the colorimetric based ELISA methodology [59, 61]. This last methodology seems more appropriate for measuring HLA-G in media from embryo culture during in vitro fertilization [59].

7. Conclusions

HLA-G is a molecule that has been deeply studied during the last two decades where the almost exclusive expression in placenta has been well documented. When the HLA-G gene is expressed, it can produce seven isoforms that exert immune-suppressive functions by binding to its receptors. However, there are some important basic concepts in its biochemistry that remain not well explained yet. Among them, the knowledge of the regulation of the protein expression is a corner stone to understand how it can be expressed ectopically in different pathological situations. This could help to induce HLA-G in a tissue when a suppressive action is convenient (e.g., organ transplantation) or to suppress it when its expression is harmful (e.g., tumor). Also, along recent years multiple HLA-G protein modifications have been described, such as HLA-G dimers that bind LILRB receptors with an affinity even higher than monomers, or nitration.

Moreover, high molecular weight molecules of HLA-G have been described as HLA-G complexed with ubiquitin. Furthermore, circulating HLA-G has also been observed as included in exosomes. The complete identification of these circulating HLA-G structures would improve not only the knowledge of this molecule but also the design of better methods for analysis. These are important questions that should be elucidated in order to understand the biology of HLA-G and to clarify some discrepant results.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

HLA-G Dimers in the Prolongation of Kidney Allograft Survival

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Human leukocyte antigen-G (HLA-G) contributes to acceptance of allografts in solid organ/tissue transplantation. Most studies have determined that soluble HLA-G isoforms are systematically detected in serum/plasma of transplanted patients with significantly fewer episodes of acute and/or chronic rejection of allogeneic tissue/organ. Current models of the interactions of HLA-G and its specific receptors explain it as functioning in a monomeric form. However, in recent years, new data has revealed the ability of HLA-G to form disulfide-linked dimeric complexes with high preferential binding and functional activities. Limited data are available on the role of soluble HLA-G dimers in clinical pathological conditions. We describe here the presence of soluble HLA-G dimers in kidney transplant patients. Our study showed that a high level of HLA-G dimers in plasma and increased expression of the membrane-bound form of HLA-G on monocytes are associated with prolongation of kidney allograft survival. We also determined that the presence of soluble HLA-G dimers links to the lower levels of proinflammatory cytokines, suggesting a potential role of HLA-G dimers in controlling the accompanying inflammatory state.

1. Introduction

Human leukocyte antigen-G (HLA-G) is a natural molecule involved in the establishment and maintenance of maternal tolerance to semiallogeneic fetal tissues [1–6]. Decreased expression of HLA-G during pregnancy has been noted as a contributing factor to preeclampsia and multiple miscarriages [7–11]. In addition, HLA-G expression has been identified in pancreas, adult thymic cells, and stem cells, as well as in pathological conditions including cancer, transplantation, HIV infection, and inflammatory diseases [12–27]. HLA-G binds to several receptors, including ILT2, ILT4, and KIR2DL4 receptors, to inhibit immune responses of myelomonocytic cells, dendritic cells, T cells, B cells, and NK cells [5, 28–35]. In addition to membrane-bound forms (HLA-G1, -G2, -G3, and -G4), HLA-G is also presented by several soluble isoforms (sHLA-G1, -G5, -G6, and -G7) generated through two mechanisms: alternative splicing and proteolytic release, which is known to be mediated by metalloproteases [36, 37]. Significantly high levels of sHLA-G were determined in several physiological and pathological conditions, including an association with higher pregnancy and implantation rates [8]. It has been determined that a high level of sHLA-G is correlated with clinical manifestation of several diseases, rheumatoid arthritis, systemic lupus erythematosus, asthma, and HIV infection. Increased levels of sHLA-G were correlated with disease progression in patients with hematological malignancies and solid tumors, including patients with acute leukemia, lymphoma, chronic lymphatic leukemia, melanoma, breast cancer, glioma, and renal and lung carcinomas [25, 38]. Recent studies showed that sHLA-G molecules are involved in prolongation of allograft survival in patients with organ/tissue transplantation [39–41]. In most studies plasma/serum levels of HLA-G was determined by ELISA. However, HLA-G-specific ELISA has limitations and does not discriminate the presence of monomer or dimer isoforms of HLA-G. New data has revealed that disulfide-linked dimeric complexes of HLA-G have high preferential

binding to immune inhibitory receptors, induce efficient immune inhibitory receptor signaling, and have strong functional activities [32, 42–45]. Limited data are available on the role of HLA-G dimers in clinical pathological conditions. The outstanding questions are whether these HLA-G dimers are formed in patients with organ transplantation and what their function might be in the prolongation of allograft survival. Here we report the analysis of sHLA-G dimers in kidney transplant patients. First, we determined that the levels of sHLA-G dimers were significantly higher in patients with no rejection episode compared with patients that have a chronic rejection of a kidney transplant. The high levels of sHLA-G dimers were associated also with increased expression of the membrane-bound form of HLA-G on monocytes from patients that have no rejection episode of kidney transplant.

HLA-G also has the ability to reduce inflammatory responses through the inhibition of immune cells to produce proinflammatory molecules. One of the potential candidates of such molecules includes matrix metalloproteinase (MMP). Increased expression of MMPs was observed in several human diseases, including cancer and autoimmune diseases, suggesting an involvement of these enzymes in immunity, inflammatory responses, and repair mechanisms. MMP-9 and MMP-2 are especially able to modulate inflammatory responses via cytokine/chemokine actions. Here we demonstrated that the high levels of sHLA-G dimers in kidney transplant patients that have no episodes of rejection were associated with decreased plasma levels of MMP-9. We also determined that the increased levels of sHLA-G dimers linked to the lower levels of proinflammatory cytokines, suggesting the potential role of sHLA-G dimers in controlling the accompanying inflammatory state. From these findings, sHLA-G dimers might be useful as a potential marker to control rejection and the inflammatory status of human kidney allotransplants.

2. Materials and Methods

2.1. Patients. We enrolled kidney transplant recipients in the study, of which 50 had no evidence of rejection (NR) and 17 had chronic rejection (CR). Kidney function and rejection was evaluated by creatinine level and verified by biopsy. Table 1 describes the clinical and demographic characteristics of the patient population. Kidney recipients in both categories had similar distribution with respect to gender, age, and race. The distribution of donor source (living or deceased), cold ischemia time, primary cause of renal failure, and immunosuppressive treatment between each group of recipients was not statistically different. Average creatinine levels of CR patients were significantly higher than NR patients ($P < 0.05$). The protocol was approved by the Human Assurance Committee of Georgia Regents University, and written informed consent was obtained from all subjects in the study.

2.2. Separation of Human Plasma, PBMCs, and Red Blood Cells from Whole Blood. Blood samples were obtained from patients and collected in EDTA tubes. Aliquots of plasma were stored at −80°C. PBMCs and red blood cells were isolated from buffy coats using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation. Aliquots of PBMCs were stored in liquid nitrogen. Aliquots of red blood cells were stored at −80°C.

2.3. Zymography. Total protein of plasma samples was measured using the Bradford method (Bio-Rad, Richmond, CA, USA). Diluted (1 : 50) plasma samples were loaded onto 10% gelatin gels, and electrophoresis was performed at 100 constant voltages. Gels were washed twice with 2.5% Triton-X for 20 min and then incubated at 37°C overnight in zymography Development Buffer (Bio-Rad). The following day, to visualize the bands, gels were stained with Coomassie Brilliant Blue Dye (Bio-Rad) for 3 hrs and destained with Destaining Solution for 45 min. The gels were rehydrated in water overnight, then scanned and analyzed using ImageJ program developed at the National Institutes of Health (USA).

2.4. Depletion of Albumin and IgG from Plasma before Immunoprecipitation. Depletion of unwanted proteins that could interfere with the immunoprecipitation of HLA-G from plasma was achieved using ProteoPrep Immunoaffinity Albumin and IgG Depletion kit (Sigma-Aldrich) following the manufacturer's protocol.

2.5. Immunoprecipitation of Depleted Plasma and Western Blot Analysis for Detection of HLA-G Monomer and Dimer. 100 μL of depleted plasma were mixed with 100 μL of cold RIPA buffer and incubated on ice for 15 min. 20 μL of protein G bead slurry was added to the plasma lysate, then incubated at 4°C for 60 min and centrifuged at 10,000 g for 10 min. 2 μL of MEM-G/9 mAb (Santa Cruz Biotechnology, Dallas, TX, USA) was added to the supernatant and the mixture was incubated at 4°C overnight. After incubation, 50 μL of Protein G bead slurry was added to the plasma lysate, incubated at 4°C for 1 hr, and centrifuged at 10,000 g for 30 sec. 50 μL of Laemmli sample buffer was added to the bead pellet. Samples were run under both reduced and nonreduced conditions. The mixture was denatured at 95°C for 5 min and centrifuged at 10,000 g for 5 min. The supernatant was loaded onto gels for electrophoresis. 30 μL of immunoprecipitated plasma was separated on 10% running gel and 5% stacking gel and transferred to PVDF membrane. The membrane was blocked with 5% BSA and incubated with MEM-G/9 primary mAb, followed by goat anti-mouse IgG-HRP secondary Ab (Santa Cruz Biotechnology). Chemiluminescent HRP-conjugated detection reagent was used for detection. Quantification of blotted proteins was determined by densitometry analysis of scanned films using ImageJ software.

2.6. Cytokine and Chemokine Analysis. Cytokine and chemokine plasma levels were measured using the Multi-Analyte ELISArray Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The Array Kits are designed for the simultaneous detection of up to 12 pro- and anti-inflammatory cytokines and chemokines

Table 1: Demographic, clinical, therapeutic, and transplant-related parameters of the patients.

	NR	CR
Total, *n*	50	17
Gender, *n*		
Male	14	4
Female	36	13
Recipient age, yr, mean (range)	51.28 (24–75)	47.94 (27–75)
Race, % (*n*)		
Caucasian	26% (13)	29.4% (5)
African American	70% (35)	64.7% (11)
Hispanic	4% (2)	5.9% (1)
Primary cause of renal failure, *n*		
Diabetic nephropathy	15	3
Lupus nephritis	—	3
Renal cystic disease	6	1
Glomerulosclerosis	5	1
Hypertension	11	3
Hypertensive kidney disease	3	2
Other	14	4
Donor type, % (*n*)		
Deceased	80% (40)	70.6% (12)
Living	20% (10)	29.4% (5)
Cold ischemia time, hr, mean ± SD	16.00 ± 10.01 ($n = 45$)	14.57 ± 0.39 ($n = 13$)
Creatinine level, mg/dL, mean (range)	1.48 (0.79–2.56)	2.71 (1.04–6.46)
(HLA-A, B, DRB1) matches, mean ± SD	1.83 ± 0.71 ($n = 36$)	2.08 ± 0.66 ($n = 13$)
(HLA-A, B, DRB1) mismatches, mean ± SD	1.42 ± 0.71 ($n = 36$)	3.62 ± 0.70 ($n = 13$)
Immunosuppressive treatment, *n*		
Azathioprine	2	—
Cyclosporine	5	4
Mycophenolate	30	14
Prednisone	34	13
Rapamycin (Sirolimus)	5	3
Tacrolimus	36	9

NR: no rejection; CR: chronic rejection.

(IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12, IL17A, IFNγ, TNFα, and GM-CSF).

2.7. Flow Cytometry. PBMCs from both groups of patients (NR and CR) were treated with human TruStain FcX (Fc receptor blocking solution; BioLegend, San Diego, CA, USA) and stained using fluorochrome-conjugated human-specific mAbs against CD3, CD4, CD8, CD14, CD19, and HLA-G. All mAbs were purchased from BD Biosciences (San Jose, CA, USA) or from BioLegend. Cytometry was performed on a cytometer FACSCanto (BD, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA) or Cell Quest software (BD Biosciences). Some results are expressed as percentage of positive cells obtained with specific Ab compared to irrelevant isotype-matched Ab.

2.8. Statistical Analysis. Statistical analysis was performed using NCSS (NCSS LLC, Kaysville, Utah, USA) and GraphPad (GraphPad Inc., La Jolla, CA, USA) packages. Normality and continuous numeric data was checked using the Kolmogorov-Smirnoff one-sample test, and comparisons were performed by Student's t test or by Mann-Whitney U test when appropriate. The P value of ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. The Levels of sHLA-G1/HLA-G5 Monomer and Dimer Forms Are Increased in Plasma of Nonrejected Kidney Transplant Patients. In this study we evaluated the levels of monomer and dimer forms of sHLA-G1 and HLA-G5 isoforms in patients with nonrejected (NR) and chronic rejected (CR) kidney allografts. Immunoprecipitation and Western blot analysis of plasma patients resulted in the discovery of two bands; one corresponding to the expected molecular mass of 39 kDa, which represents the sHLA-G monomer, and one approximately twice that (sHLA-G dimer) (Figure 1(a)).

The supernatant from HLA-G5-transfected 721.221 human lymphoblastoid cells was used as a positive control. Both forms of sHLA-G had been determined in the plasma of NR and CR patients, and there was considerable variation in sHLA-G levels within each group. However, the level of total sHLA-G was significantly higher ($P = 0.03$) in NR compared with CR kidney transplant patients (Figure 1(b)). In addition, the level of the monomer form of sHLA-G was slightly higher in the NR group compared with the CR group (Figure 1(c)). We determined that the dimer form of sHLA-G was dominant in the plasma of both groups of patients. Moreover, the level of the dimer form of sHLA-G was significantly elevated ($P = 0.03$) in the NR group of patients (Figure 1(d)). These data indicate that the dimer form of sHLA-G is present and dominates in the plasma of kidney transplant patients.

3.2. Increased Expression of the Membrane-Bound Form of HLA-G1 in Monocytes from Nonrejected Kidney Transplant Patients. It is known that sHLA-G proteins can be generated by two mechanisms: alternative splicing and proteolytic release, which is mediated by metalloproteases. To determine the potential contribution of the membrane-bound form of HLA-G1 shedding into the pool of sHLA-G molecules in plasma patients, the expression of HLA-G1 on the cell surface of peripheral monocytes and T and B cells has been analyzed in both groups of patients. There was no significant difference in the number of HLA-G1-positive T cells and B cells between NR and CR patients (Figures 2(a) and 2(d) and data not shown). Overall, in both groups of patients, HLA-G1-positive cells represent a small fraction (2–6% of the total) of T and B cells. As expected, the majority of HLA-G1-positive cells in the peripheral blood of both NR and CR patients were determined in the population of monocytes (Figures 2(a), 2(b), and 2(c)). However, the number of HLA-G1-positive monocytes was significantly elevated ($P = 0.002$) in the NR patients, but not in the CR patients (Figure 2(e)). These data revealed that the increased expression of HLA-G1 on monocytes from NR patients might have a substantial contribution to the elevated plasma levels of sHLA-G monomer and dimer forms in those patients.

3.3. Analysis of MMP-2 and MMP-9 in Plasma of NR and CR Kidney Transplant Patients. Since one of the mechanisms of the production of sHLA-G involves shedding of the membrane-bound form of HLA-G1 by metalloproteases and since MMPs play a crucial role this class of enzymes, we investigated the levels of MMP-2 and MMP-9 in the plasma of kidney transplant patients. Zymography analysis of transplant patients showed the presence of two bands at 68 kDa and 90 kDa, which correspond to MMP-2 and MMP-9, respectively (Figure 3(a)). As shown in Figure 3(a), plasma from both groups of patients contains substantial amounts of MMP-2 and MMP-9, with no significant difference in the plasma levels of MMP-2 and MMP-9 between NR and CR patients (Figures 3(c) and 3(d)). However, a tendency toward elevation of MMP-9 levels was observed in the CR kidney transplant patients (Figure 3(d)). Since plasma from both the NR and CR groups of patients contains sHLA-G, this data additionally support the possibility that MMPs might play a role in contributing to the pool of total sHLA-G by shedding the membrane-bound HLA-G1 molecules.

Since HLA-G, MMP-2, and MMP-9 are all involved in regulation of the inflammatory response by modulation of cytokines and chemokines and the inflammatory response represents a critical stage in rejection or survival of allogeneic transplants, we next determined the levels of proinflammatory cytokines in kidney transplant patients.

3.4. Increased Levels of Proinflammatory Cytokines IL-1β, IL-2, and IL-6 in Plasma from Patients with Chronic Rejection of Kidney Transplant. We thus investigated whether the plasma level of proinflammatory cytokines differs between NR and CR kidney transplant patients. For this purpose, we have used a Multi-Analyte ELISArray Kits designed to simultaneously assess the levels of 12 pro- and anti-inflammatory cytokines and chemokines (IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12, IL17A, IFNγ, TNFα, and GM-CSF). There was considerable variation in cytokines levels within both the NR and CR patients, especially for IL-1α, IL-4, IL-12, and IFN-γ (Figure 4). However, the CR kidney transplant patients had significantly elevated levels of proinflammatory cytokines IL-2 ($P = 0.005$), IL-1β ($P = 0.05$), and IL-6 ($P = 0.05$) (Figure 4). In addition, the level of IL-17A was elevated in CR patients. These data support our observation that the dimer form of sHLA-G associates with control of inflammatory responses in kidney transplants patients.

4. Discussion

HLA-G is natural molecule involved in the establishment and maintenance of maternal tolerance to fetal semiallogeneic tissues. HLA-G binds to several immune cell inhibitory receptors, for example, ILT2, ILT3, and ILT4, to downmodulate immune responses of myelomonocytic cells, T cells, B cells, and NK cells. Limited polymorphisms, restricted tissue expression, and a relatively restricted peptide presentation make HLA-G a unique molecule, unlike the classical HLA class I molecules. Recently, another unusual characteristic of HLA-G has been discovered: its ability to form a disulfide-linked dimer form both in solution and at the cell surface. HLA-G, unlike most other MHC class I molecules, has two free cysteine residues located in positions 42 and 147 in extracellular domains α1 and α2, respectively. HLA-G molecules refolded *in vitro* form a disulfide-linked dimer with an intermolecular Cys42-Cys42 disulfide bond [46]. Soluble HLA-G5 molecules expressed by human 293 T cells also form disulfide-linked dimeric and additional oligomeric forms, which can reduce the level of CD8 expression on cytotoxic T lymphocytes (CTLs) [47]. The efficiency of inhibitory signaling is dependent upon several factors, including the stability and avidity of the ligand and its proper structural orientation, which significantly affects the affinity and signaling to targeted inhibitory receptors. Mutagenesis studies of the free cysteines suggested that the HLA-G dimer more efficiently inhibits NK killing than the monomer and increases the

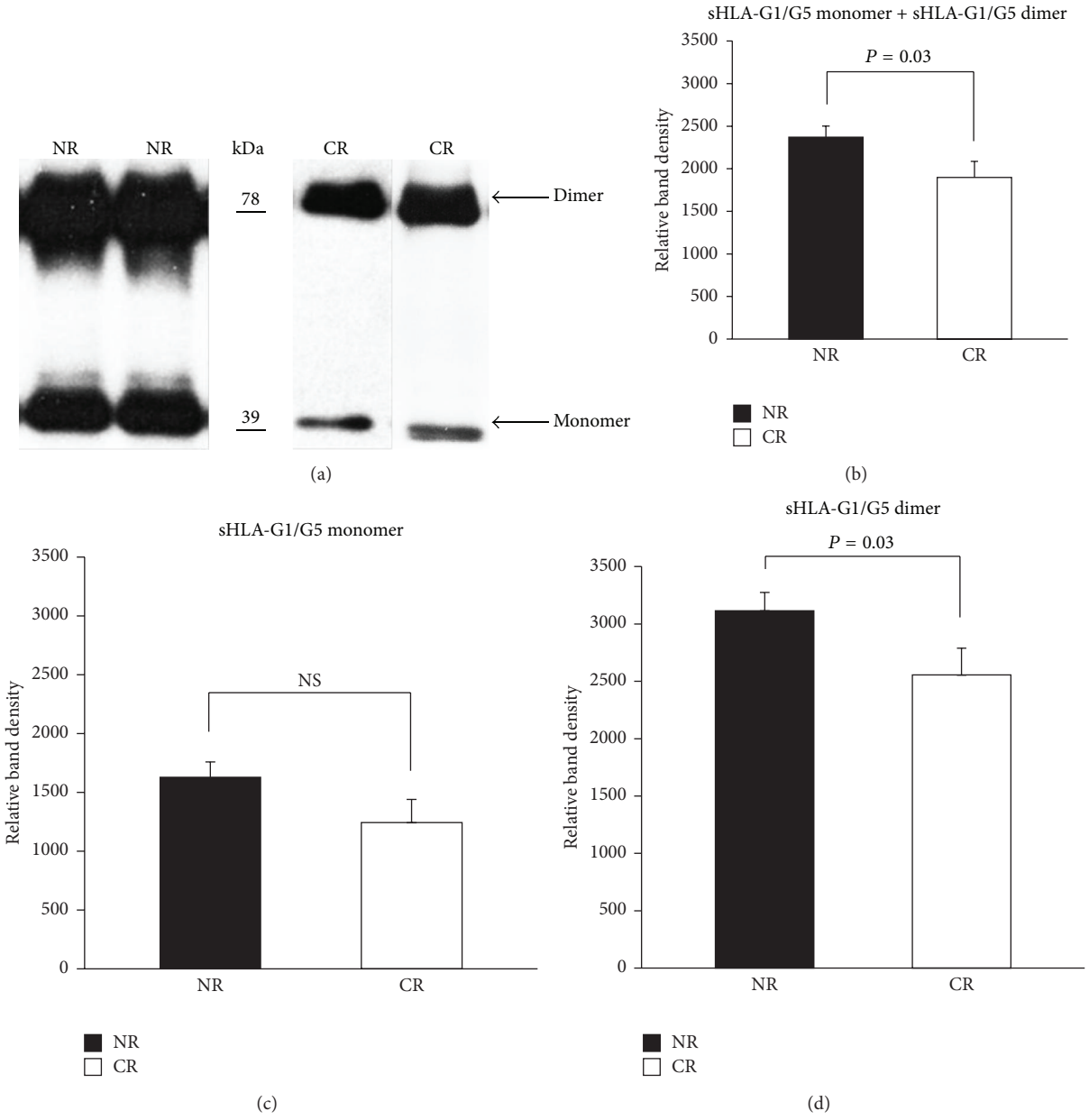


Figure 1: Increased levels of monomer and dimer isoforms of sHLA-G1/G5 in plasma of nonrejecting kidney transplant patients. (a) Plasma from kidney transplant patients was immunoprecipitated with MEM-G/9 mAb. Immunoprecipitates were electrophoresed under nonreducing conditions and Western blot analysis was performed to determine the levels of monomer and dimer of sHLA-G. Representative data from patients with no rejection (NR) and patients with chronic rejection (CR) are demonstrated. Mean relative band density of total sHLA-G (b), sHLA-G monomer (c), and sHLA-G dimer (d) in NR ($n = 42$) and CR ($n = 17$) patients was determined by densitometry. Data are shown as mean ± SEM and analyzed by Student's t-test. NS: not significant.

efficiency of ILT2 signaling [32, 42, 43, 48]. Experimental data from several groups, including our laboratory, suggest that HLA-G dimer has increased avidity and proper structural orientation to induce efficient inhibitory signaling during ligation with human ILT2 and ILT4, and murine PIR-B-inhibitory receptors [32, 44, 45]. This makes HLA-G dimer as the most powerful ligand form for modulation of inflammatory and alloimmune responses in several pathological conditions, including the prolongation of kidney allograft survival or graft acceptance. Many studies have been designed to determine sHLA-G in the plasma or serum of patients suffering from various diseases. sHLA-G levels were determined in spontaneous miscarriages, in autoimmune diseases, in solid organ transplantation, and in various malignancies. In almost all the studies, the quantification of sHLA-G was analyzed using ELISA. Unfortunately, the available sHLA-G ELISA determines the total amount of sHLA-G protein only, which includes both monomer and dimer forms. Since the dimer represents the most powerful form of sHLA-G, it is very critical to analyze the levels of sHLA-G dimer in healthy

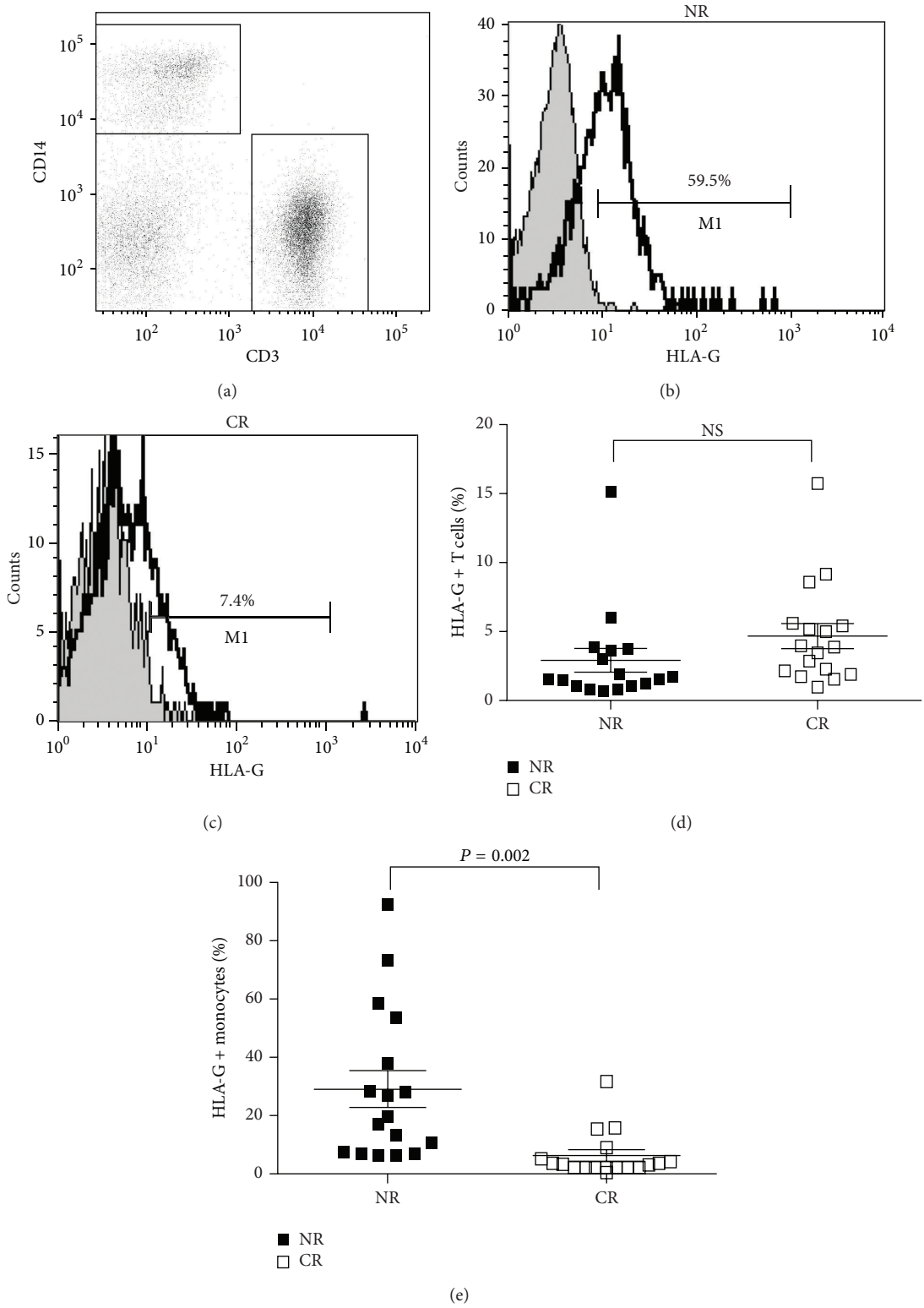
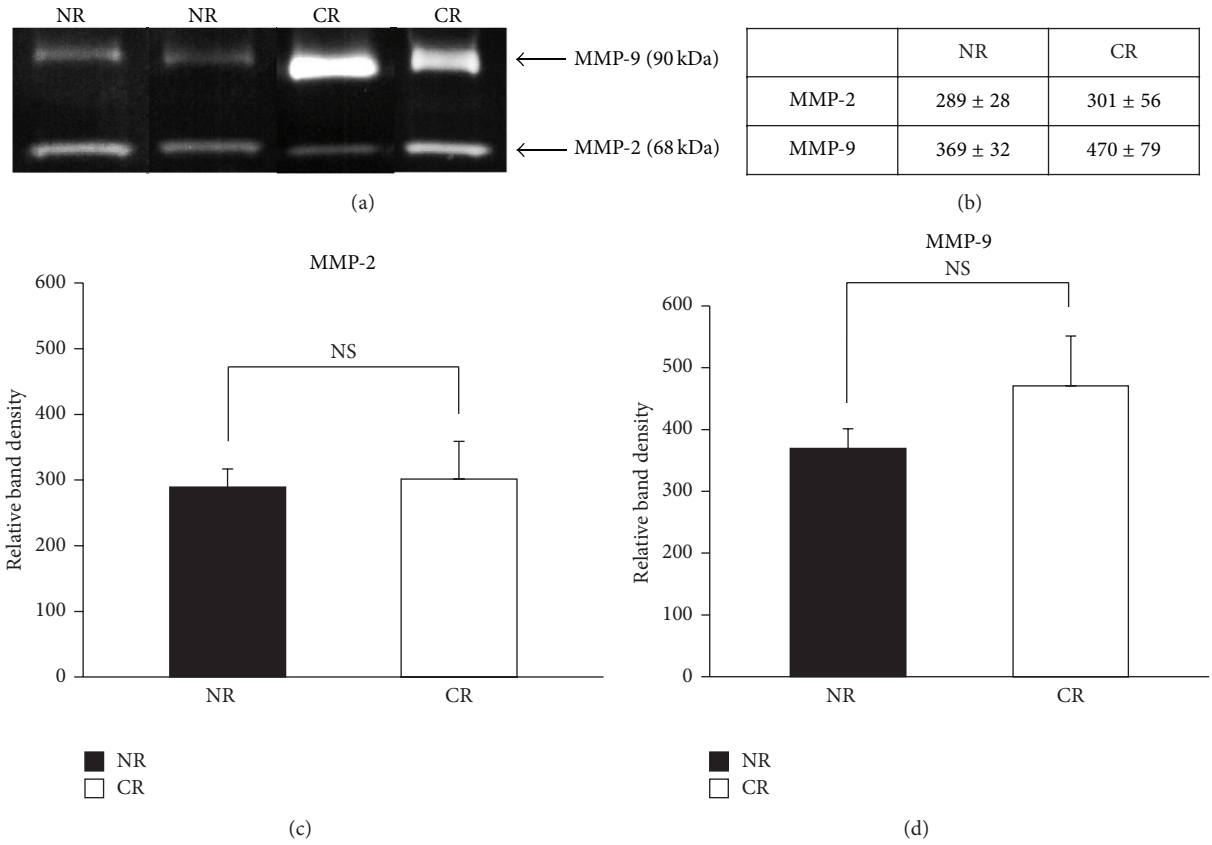


Figure 2: Increased percentages of HLA-G-positive monocytes in nonrejecting kidney transplant patients. (a, b, c) PBMCs from renal allograft patients in both the nonrejected group (NR) and chronic rejected (CR) groups were stained with anti-CD3, anti-CD14, and anti-HLA-G mAbs, and FACS analysis was performed. Representative flow cytometric analysis of PBMCs from indicated groups of renal allograft recipients is shown. (b and c) Histograms shown here were gated on a CD14-positive population. Filled histograms represent the isotype control. Numbers indicate the percentage of HLA-G-positive monocytes. (d) Numbers indicate the percentage of HLA-G-positive T cells in both groups of patients (gated on CD3-positive population). Filled boxes represent data from NR ($n = 17$) and open boxes represent data from CR ($n = 17$) patients. (e) Numbers indicate the percentage of HLA-G-positive monocytes in both groups of patients (gated on CD14-positive population). Data are shown as mean ± SD and analyzed by Student's t-test. NS: not significant.



	NR	CR
MMP-2	289 ± 28	301 ± 56
MMP-9	369 ± 32	470 ± 79

Figure 3: Gelatin zymography of human plasma from kidney transplant patients. Bradford protein assay was used to quantify the amount of plasma needed for zymography that was then loaded onto gelatin gels. The gels were scanned and analyzed using imageJ. (a) Representative gels are of MMP-2 and MMP-9 from patients with no rejection (NR) and patients with chronic rejection (CR). (b) Mean relative band density of MMP-2 and MMP-9 in NR ($n = 50$) and CR ($n = 17$) patients was measured by densitometry. Statistics are shown as mean ± SEM. (c, d) Graphical representation of Figure 3(b). The P value was calculated using Student's t-test. NS: not significant.

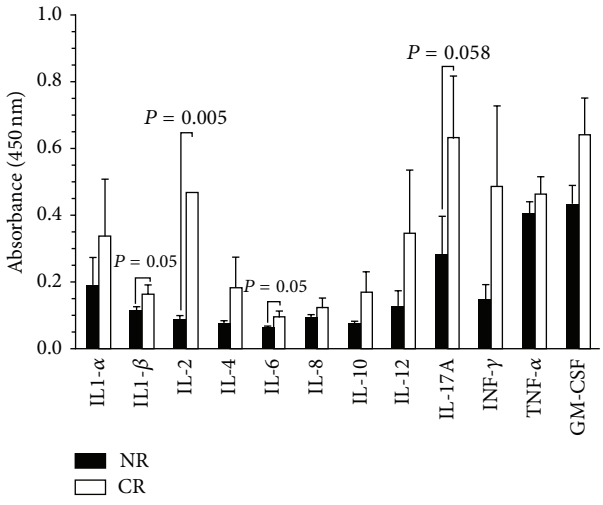


Figure 4: Elevated level of proinflammatory cytokines in plasma of rejected kidney transplant patients. Cytokine and chemokine levels were analyzed using Multi-Analyte ELISArray kits. Cytokine levels from kidney transplant patients with no evidence of rejection ($n = 9$) and with chronic rejection ($n = 15$) are presented as the mean of absorbance values ± SEM. The P value was calculated using Mann-Whitney U test.

and disease conditions. To date, no study has investigated whether HLA-G disulfide-linked homodimers are present in plasma from kidney transplant patients. Here we show that sHLA-G dimers are present in plasma from kidney transplant patients. The levels of sHLA-G dimer were significantly elevated in patients with no rejection episodes compared with patients with chronic rejection, indicating the association of sHLA-G dimers with the prolongation of kidney allograft survival. In support of that, similar levels of expression and percent of immune inhibitory receptor ILT2- and ILT4-positive cells has been determined on monocytes and T and B cells in both groups of patients (data not shown). This clinical finding is in agreement with our previous study using animal models demonstrating that HLA-G dimers prolong the survival of allogeneic skin transplants in ILT transgenic mice ([32, 49, 50] and unpublished data). In the future, it will be important to determine the percentages of sHLA-G1 and sHLA-G5 dimers in total sHLA-G. However, to date, there is no data available demonstrating that sHLA-G1 and sHLA-G5 dimers have different binding and/or different efficiency to induce inhibitory receptor signaling. Our results show that the number of HLA-G1-positive monocytes is significantly increased in NR patients, indicating that the percent of sHLA-G1 dimer might have been elevated within the total level of

sHLA-G dimers in plasma of the patients with no episodes of rejection. Recently, Rizzo et al. [37] demonstrated an effective link between MMP-2 and HLA-G1 shedding in 721.221-G transfected cell line, JEG3 cell line, and IL-10-treated PBMCs from five healthy donors using an *in vitro* experiments. However, the process of shedding and especially dimerization of sHLA-G1 *in vivo* and in pathological conditions, including kidney transplant patients, could be affected by several factors and requires additional investigation.

The anti-inflammatory effects of sHLA-G dimers represent a new finding for this form of HLA-G. Recently published data by Kuroki et al. [45] demonstrating that in an animal model of collagen-induced arthritis, HLA-G dimer interacting through a murine ILT homologue, the PIR-B receptor exhibited significantly more anti-inflammatory effects compared to monomer. To date, no data is available on studies of the anti-inflammatory effect of sHLA-G dimers in clinical applications. We demonstrate here that an increased level of sHLA-G dimers in kidney transplant patients with no rejection episodes is linked to significantly lower levels of proinflammatory cytokines IL-2, IL-1β, and IL-6. It will be important to dissect the mechanisms of sHLA-G dimers controlling inflammatory responses. The anti-inflammatory effect of sHLA-G dimers opens a new strategy to generate useful agents to control inflammatory responses with minimal side effects.

In conclusion, our study shows that sHLA-G dimers are associated with better survival of kidney allografts and control of the accompanying inflammatory response in kidney transplant patients. Thus sHLA-G dimers can be a potential biomarker to control human alloimmune and inflammatory responses.

Conflict of Interests

The authors declare that there is no potential conflict of interests.

Acknowledgments

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Research Article

HLA-G Expression on Blasts and Tolerogenic Cells in Patients Affected by Acute Myeloid Leukemia

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Human Leukocyte Antigen-G (HLA-G) contributes to cancer cell immune escape from host antitumor responses. The clinical relevance of HLA-G in several malignancies has been reported. However, the role of HLA-G expression and functions in Acute Myeloid Leukemia (AML) is still controversial. Our group identified a subset of tolerogenic dendritic cells, DC-10 that express HLA-G and secrete IL-10. DC-10 are present in the peripheral blood and are essential in promoting and maintaining tolerance via the induction of adaptive T regulatory (Treg) cells. We investigated HLA-G expression on blasts and the presence of HLA-G-expressing DC-10 and $CD4^+$ T cells in the peripheral blood of AML patients at diagnosis. Moreover, we explored the possible influence of the 3′ untranslated region (3′UTR) of *HLA-G*, which has been associated with HLA-G expression, on AML susceptibility. Results showed that HLA-G-expressing DC-10 and $CD4^+$ T cells are highly represented in AML patients with HLA-G positive blasts. None of the HLA-G variation sites evaluated was associated with AML susceptibility. This is the first report describing HLA-G-expressing DC-10 and $CD4^+$ T cells in AML patients, suggesting that they may represent a strategy by which leukemic cells escape the host's immune system. Further studies on larger populations are required to verify our findings.

1. Introduction

Human Leukocyte Antigen (HLA)-G is a nonclassical HLA class I molecule, originally described essential for promoting fetus-maternal tolerance [1, 2]. It is now clear that HLA-G is involved in promoting beneficial tolerance in several settings, such as autoimmunity and organ transplantation, and in contributing to detrimental tolerance in viral infections and cancer [3]. HLA-G is expressed in seven different isoforms, four of which are membrane-bound (HLA-G1, G2, G3, and G4) and three are soluble (HLA-G5, G6, and G7) [4, 5]. Among these isoforms, the best characterized are HLA-G1, the most stable membrane-bound isoform, soluble HLA-G5, and shed HLA-G1. HLA-G modulates immune responses through several nonexclusive mechanisms: it inhibits cytolytic activities of NK and $CD8^+$ cytotoxic T cells [6] and proliferation of T cells [7, 8], and it modulates antigen presenting cell (APC) differentiation and function [9]. APCs overexpressing HLA-G are poor stimulators and are able to induce anergic/suppressor $CD4^+$ T cells [9–12]. Our group described a subset of tolerogenic IL-10-producing DC (DC-10) that is present in the peripheral blood [13, 14]. DC-10 are characterized by the expression of membrane-bound HLA-G and by their ability to induce adaptive IL-10-producing T regulatory (Treg) cells [13, 14]. We demonstrated that DC-10 accumulate in human decidua during pregnancy where they

Table 1: Clinical patients' characteristics.

Variable	All patients	Blasts in PB Mean % (range)	HLA-G$^+$ blasts *n* (mean %)	HLA-G$^-$ blasts *n* (mean %)	*P* value$^\$$
Number of patients (%)	22	65.6 (13.3–96.4)	15 (68.2)	7 (31.8)	
Age at diagnosis (year)	59 (22–83)				
Male (%)	8 (36)	73.9 (13.3–94.5)	8 (100)	0 (0)	0.022
Female (%)	14 (64)	60.8 (23.9–96.4)	7 (50)	7 (50)	
AML	3	58.1 (42.2–85.2)	2 (66.7)	1 (33.3)	
AML-M0	2	35.6 (25.4–45.7)	1 (50)	1 (50)	
AML-M1	1	68	/	1 (100)	
AML-M2	7	62.4 (23.9–94.5)	5 (71.5)	2 (28.5)	
AML-M3	1	94.4	1 (100)	/	
AML-M4	6	81.5 (40–95.3)	4 (66.6)	2 (33.4)	
AML-M5a	1	96.4	1 (100)	/	
AML-M6	1	13.3	1 (100)	/	

$^\$$Comparison between HLA-G$^+$ and HLA-G$^-$ blasts using Fisher's exact test.

contribute in generating a tolerogenic microenvironment limiting immune responses *in vivo* [15].

Studies on solid tumors revealed that HLA-G could be potentially expressed by all tumors, either as membrane-bound or as soluble isoform [16, 17]. In these contexts, HLA-G acts as a negative regulator of the immune response through different mechanisms, including inhibition of angiogenesis, prevention of antigen recognition and T cell migration, and suppression of T and NK cytotoxicity [16, 18, 19]. HLA-G expression by leukemic cells is still controversial. Analysis performed on blasts from patients with different leukemia, including Acute Myeloid Leukemia (AML), Acute Lymphoid Leukemia (ALL), and Chronic Myeloid Leukemia (CML), demonstrated that neither mRNA for any HLA-G isoforms nor HLA-G antigen was detected [20, 21]. However, more recently it has been shown that the expression of HLA-G by circulating blasts from AML, CML, but not B-ALL, and by B-CLL cells was strongly associated with an unfavorable outcome of the disease [22–24]. In addition, a correlation between soluble HLA-G plasma levels and AML, ALL, and B-CLL was proposed [25, 26].

Despite low degree of genetic variability in the coding region of *HLA-G*, several polymorphisms are present in the noncoding region of the gene both at 5′ upstream regulatory region (URR) and 3′ untranslated region (UTR), which may influence the HLA-G expression [27]. The most studied polymorphism at the 3′ UTR is the presence (Ins) or absence (Del) of a fragment of 14 base pairs (14 bp Ins/Del) that has been associated with *HLA-G* mRNA stability [28–30]. In addition, the +3142 C/G single nucleotide polymorphism (SNP) controls the magnitude of mRNA production, since the presence of the G may increase the affinity of this region for miR-148a, miR-148b, and miR-152 [31, 32]. The +3187 A/G SNP has been reported to affect mRNA stability due to its proximity to an AU-rich motif, which mediates the *HLA-G* mRNA degradation [33]. Beside these polymorphic sites, other less studied SNPs of the 3′UTR are located at positions +3001 T/C, +3003 T/C, +3010 C/G, +3027 C/A, +3035 C/T, and +3196 C/G [34, 35].

The 14 bp Ins/Del has been associated with tolerance in different clinical conditions including autoimmunity [36–38], pathological pregnancy [39–42], recurrent spontaneous abortions [39, 40, 42, 43], and preeclampsia [29, 41, 43–45], although the results on the two latter conditions are contradictory. The presence of the 14 bp Del has been found to be predictive of the incidence of graft versus host disease after unrelated [46] and HLA-identical sibling [47] hematopoietic stem cell transplantation (HSCT) for beta-Thalassemia, suggesting a role for this polymorphism in the establishment of immunological tolerance also in the context of HSCT. The association of HLA-G polymorphisms with malignancies has been studied in a wide range of solid tumors, including breast and cervical cancers [48–52], but, thus far, it has not been evaluated in leukemia.

We investigated the expression of HLA-G on leukemic blasts and tolerogenic immune cells, DC-10 and CD4$^+$ T cells, in the peripheral blood of AML patients at diagnosis. We also determined whether polymorphisms at 3′ UTR of *HLA-G* locus correlate with AML susceptibility.

2. Materials and Methods

2.1. Patients. All protocols were approved by the institutional review board and samples were collected under written informed consent according to the Declaration of Helsinki. 22 patients affected by AML were included in this retrospective study and analyzed for biological and clinical characteristics. AML diagnosis was based on standard cytological criteria according to the French-American-British (FAB) classification. Patients' diagnosis was subclassified by morphological and immune phenotyping. None of the patients received medical interventions before the study. Patients' characteristics are listed in Tables 1 and 2.

Table 2: HLA-G expression and cytogenetic karyotype.

Patient number	FAB classification	Sex	Age	HLA-G expression	Karyotype
1	AML-M2	F	83	Positive	46XX +8[4];[16]
3	sAML	F	69	Negative	46XX
5	AML-M4	F	54	Positive	46XX
9	AML-M4	F	44	Negative	46XX
10	AML-M2	M	60	Positive	46XY t(8; 21)(q22;q22), del(9)(q?22)
11	AML-M4	M	70	Positive	46XY[19]; +21[2]; iso p21[1]
18	AML-M2	F	68	Negative	46XX iso 8p
19	AML-M4	F	76	Positive	46XX
20	AML-M0	F	83	Positive	46XX −7/t(1;7;4;12)[12]
21	AML-M2	F	66	Positive	46XX +8[4];[16]
23	AML-M2	M	74	Positive	46XY
34	AML-M0	F	72	Negative	46XX
36	AML-M6	M	53	Positive	46XY complex(del5q,-10 14; 19, 7p-)[20]
37	AML-M2	F	59	Negative	46XX
38	AML	M	59	Positive	46XY
39	AML-M1	F	47	Negative	46XX
41	AML-M4	F	41	Negative	46XY
44	AML	M	36	Positive	46XY
45	AML-M4	F	44	Positive	46XX
49	AML-M2	M	77	Positive	46XY
54	AML-M3	M	22	Positive	46XY[6], t(15;17)[5]
55	AML-M5a	F	58	Positive	46XX

2.2. Cells Isolation and Serum Collection. Peripheral blood mononuclear cells (PBMCs) from AML patients were isolated by Ficoll density gradient centrifugation and cryopreserved in gas phase of liquid nitrogen to the time of analysis. Serum was obtained from the blood samples of AML patients by centrifugation and cryopreserved in gas phase of liquid nitrogen for ELISA test.

2.3. Cytogenetic Analysis. Cytological analysis was performed using standard G-band karyotyping technique. Results were described according to the International System for Human Cytogenetic Nomenclature [53].

2.4. Flow Cytometry Analysis. Frozen PBMCs were thawed in X-VIVO 15 medium (Lonza, Italy), supplemented with 5% pooled AB human serum (Lonza, Italy) and 100 U/mL penicillin/streptomycin (Lonza, Italy), and washed twice in Phosphate Buffered Saline (PBS) (Sigma, CA, USA) with 2% Fetal Bovine Serum (FBS) (Lonza, Italy). PBMCs were initially incubated for 10 min at room temperature with FcR blocking reagent (Miltenyi Biotech, Germany) and stained for additional 20 min at room temperature in the dark with monoclonal antibodies (mAbs) specific for the following human antigens: CD45 (BioLegend, USA), CD16 (BD Pharmigen, CA, USA), CD4 (Becton Dickinson, CA, USA), CD14 (Becton Dickinson, CA, USA), and HLA-G (MEM-G9, Exbio, Czech Republic). Cells were identified using a multiparametric approach based on the combination of mAbs. Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, CA, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). Quadrant markers were set accordingly to unstained controls. Leukemic blasts were identified as $CD45^{dim}$ according to Lacombe et al. [54].

2.5. Detection of Soluble HLA-Gs. Levels of shed HLA-G1 and soluble HLA-G5 were determined by enzyme-linked immunosorbent assay (ELISA), as previously described [55, 56]. To detect sHLA-G (shed HLA-G1 and HLA-G5) plates (Nunc-Immuno Plate PolySorp, Thermoscientific, Denmark) were coated with the mAb G233 (Exbio, Czech Republic), whereas to detect HLA-G5 plates were coated with the mAb 5A6G7 (Exbio, Czech Republic). sHLA-G or HLA-G5 was detected with biotinylated β_2-microglobulin or W6/32 mAbs (Exbio, Czech Republic), respectively. Supernatants from HLA-G transfected LCL721.221 [57] and HeLa HLA-G5 transfected cells (kindly provided by Dr. R. Rizzo, Università di Ferrara) purified by affinity chromatography by using the W6/32 mAb were used for the generation of standard calibration curves for sHLA-G and HLA-G5, respectively. The limit of sensitivity was 0.5 ng/mL.

2.6. Amplification and Sequencing of 3′UTR of the HLA-G Gene. Genomic DNA was extracted from PBMCs using a commercial kit (QIAamp, QIAGEN, Italy) according to the manufacturer's instructions. Briefly, 100 ng of genomic DNA was amplified in a 25 μL reaction containing 1X polymerase chain reaction (PCR) buffer (Roche, USA), 0.2 mM dNTP

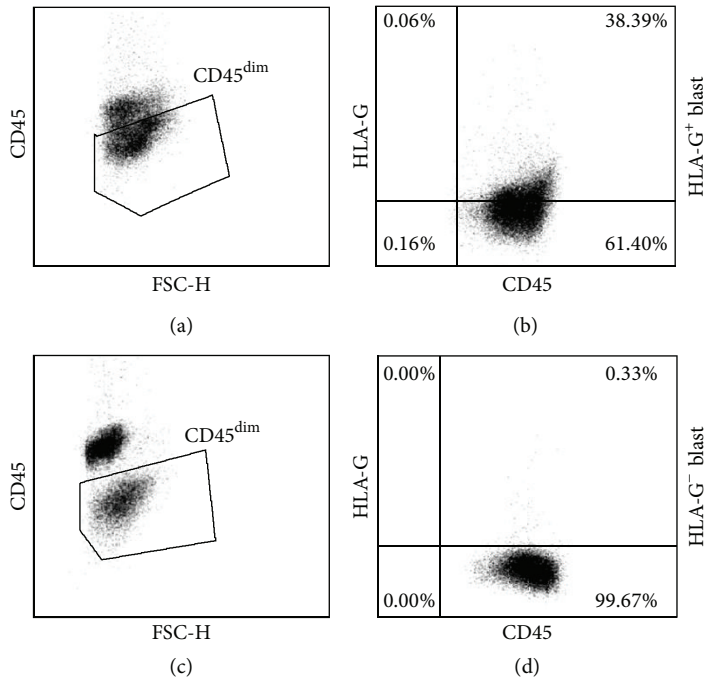

Figure 1: HLA-G expression on leukemic blasts. PBMCs of AML patients were analyzed by flow cytometry to determine the expression of HLA-G on blasts. An anti-human CD45 mAb was used to discriminate leukemic cells from normal mononuclear cells, being blasts $CD45^{dim}$. Representative plots from a patient with HLA-G^+ blast ((a) and (b)) or with HLA-G^- blast ((c) and (d)) are shown.

mix (Roche, USA), 1.5 mM $MgCl_2$ (Roche, USA), 0.8 U *Taq* Polymerase (Roche, USA), and 1 μM of each primer (For.: 5′ TCACCCCTCACTGTGACTGA 3′; Rev.: 5′ TTCTCATGTCTTCCATTTATTTTGTC 3′). The initial denaturation step was carried out at 95°C for 3 min, followed by 30 cycles at 93°C for 60 s, 58°C for 60 s, and 72°C for 60 s and by a final extension step at 72°C for 10 min. The amplification product was evaluated using a 2.5% agarose gel, purified using a commercial kit (Wizard SV Gel and PCR Clean-Up System, Promega, WI, USA) according to the manufacturer's instructions, and subjected to direct sequencing on both strands. All polymorphic sites observed at the 3′UTR were individually annotated and named according to previous reports [35].

2.7. Statistical Analysis. All results are presented as mean values ± SD. Comparison of parameters between subgroups of patients was performed using the nonparametric Mann-Whitney *U* test for continuous variable and Fisher's exact test for categorical data. Differences were regarded as significant at $P < 0.05$. The results were analyzed using GraphPad Prism 3.0 (GraphPad Software, USA).

3. Results and Discussion

We investigated the expression of HLA-G on leukemic blasts from 22 patients affected by Acute Myeloid Leukemia (AML), referring to the San Raffaele Hematology and Bone Marrow Transplantation Program (Table 1). The cohort of patients analyzed was characteristic of AML, with a median age of 59 years, both male and female (36% and 64%, resp.), with AML-M2 and AML-M4 subtype predominance. The mean percentage of blasts in the peripheral blood of patients was 65.6 ± 27.97% (mean ± SD, $n = 22$; range 13.3–96.4%). Patients' PBMCs were analyzed by flow cytometry and the expression of HLA-G was determined on blasts identified as $CD45^{dim}$ cells with MEM-G/9 mAb, which detects the full-length HLA-G1 isoform. HLA-G expression on AML blasts less than 1% was considered negative. Results showed that HLA-G was expressed in 15 out of 22 (68.2%) AML patients, and percentage of HLA-G^+ blasts varied from 1.5% to 59% (Figure 1). Although our study has been performed in a small cohort of Caucasian AML patients, it confirmed previous analyses in Chinese AML patients [58, 59], and indicate that membrane-bound HLA-G can be expressed on AML blasts. The analysis of the association between the expression of HLA-G on blasts and clinical parameters, including patient age, gender, subtype of AML, and percentage of blasts at diagnosis, revealed that HLA-G expression is independent of all the abovementioned variables except for gender. In our cohort of AML patients, all males showed HLA-G^+ blasts (Table 1). Nevertheless, these results are partially in accordance with previous analyses demonstrating that the HLA-G expression on leukemic blasts is not associated with specific patients' characteristics [58].

We next investigated whether HLA-G expression on blasts can be associated with cytogenetic karyotype abnormalities. Results indicated that abnormalities were present in 7 out of 15 AML patients with HLA-G^+ blasts and only

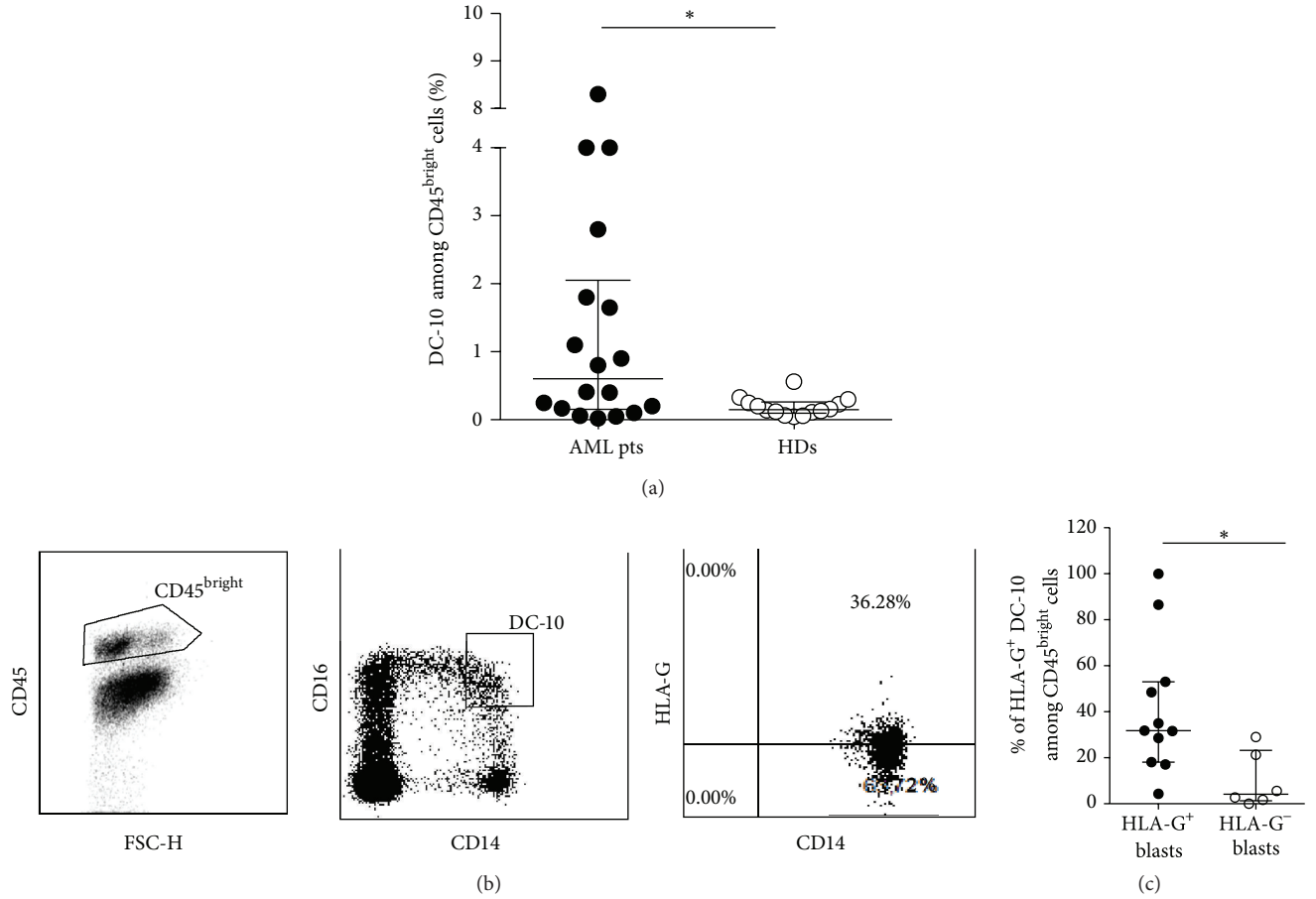

Figure 2: Flow cytometric analysis of cells containing DC-10 in the peripheral blood of AML patients. (a) Percentages of DC-10, identified among the $CD45^{bright}$ cells according to the co-expression of CD14 and CD16, in the peripheral blood of AML patients (AML pts) and healthy donors (HDs) are shown. Each dot represents single AML patient or HD. Lines indicate median and interquartile range of positive cells detected in all patients and donors analyzed. $^{*}P < 0.05$. (b) Representative dot plots of HLA-G^{+} DC-10 are depicted. (c) Percentages of HLA-G^{+} DC-10 in patients with HLA-G^{+} or HLA-G^{-} blasts are shown. Each dot represents single AML patient. Lines indicate the median and interquartile range of HLA-G^{+} DC-10. $^{*}P < 0.05$.

in 1 patient with HLA-G^{-} blasts (Table 2). These results are in contrast to previous data in which a marked difference in the frequency of cytogenetic abnormalities between HLA-G^{+} and HLA-G^{-} AML blasts was observed [24]. It cannot be excluded that this discrepancy can be due to the different ethnic population analyzed. Nevertheless, it remains to define whether a correlation between cytogenetic abnormalities and HLA-G expression exists by enlarging the cohort of patients.

One of the strategies by which tumors escape the host's immune surveillance is the upregulation of HLA-G expression on both cancer and non-tumor cells, such as mononuclear cells [17]. Our group described DC-10, which constitutively expressed HLA-G and are involved in promoting tolerance [13–15]. We postulated that DC-10 might play a role in favoring tumor escape in AML patients. The population of cells containing DC-10 was identified by the concomitant expression of CD14 and CD16 on $CD45^{bright}$ cells. Results showed a significantly higher frequency of DC-10 in the peripheral blood of AML patients compared to that observed in healthy donors (1.5 ± 2.13%, $n = 18$, mean ± SD versus 0.19 ± 0.13%, $n = 14$, mean ± SD; $P = 0.027$; Figure 2(a)). The percentage of human DC-10 in AML patients with HLA-G^{+} and HLA-G^{-} blasts was similar (1.4±2.5, $n = 12$, mean ± SD versus 1.6 ± 1.26%, $n = 6$, mean ± SD, resp.; data not shown). Previous reports indicated that HLA-G itself promotes the expression of HLA-G on T and myeloid cells [10]; thus, we compared the frequency of HLA-G-expressing DC-10 in patients with HLA-G^{+} or HLA-G^{-} blasts. Results showed a significantly higher frequency of HLA-G^{+} DC-10 in patients with HLA-G^{+} blasts as compared to those with HLA-G^{-} blasts (41.3 ± 29.25%, mean ± SD, $n = 12$ versus 10.05 ± 12.08%, mean ± SD, $n = 6$; $P = 0.013$, Figures 2(b) and 2(c)). In parallel, the presence of naturally occurring $CD4^{+}$ Treg cells constitutively expressing HLA-G (HLA-G^{+} $CD4^{+}$ Treg cells), which have been identified in the peripheral blood of healthy donors and patients [15, 60, 61], was analyzed. The proportion of $CD4^{+}$ T cells in the peripheral blood of leukemic patients and of healthy donors was similar (29.6 ± 18.59%, mean ± SD, $n = 21$ versus 31.43 ± 8.57%, mean ± SD, $n = 16$; Figure 3(a)).

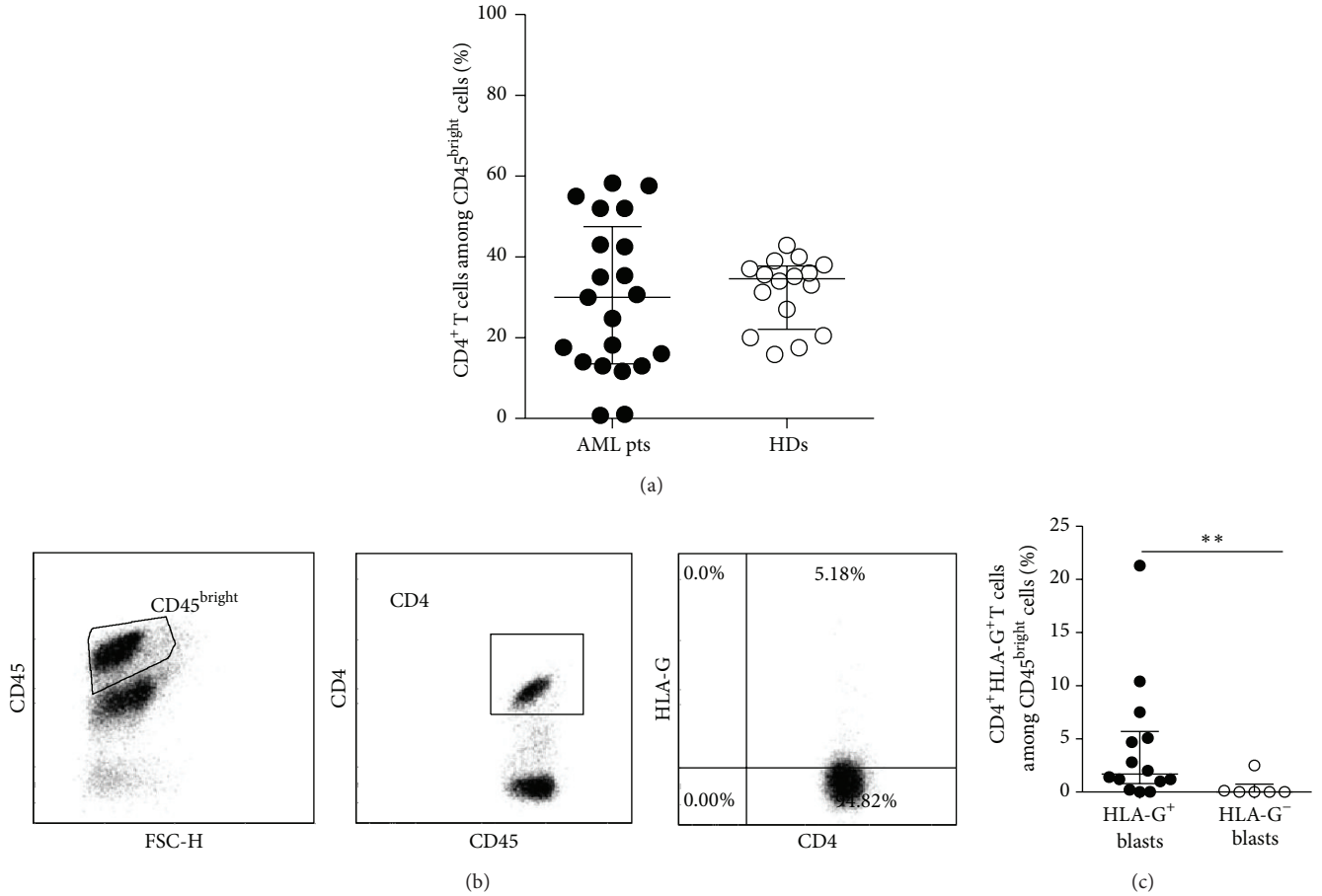

Figure 3: Flow cytometric analysis of $CD4^+$ T cells in the peripheral blood of AML patients. (a) Percentages of $CD4^+$ T cells among the $CD45^{bright}$ cells in the peripheral blood of AML patients (AML pts) and healthy donors (HDs) are shown. Each dot represents single AML patient or HD. Lines indicate the median and interquartile range of positive cells detected in all patients and donors analyzed. (b) Representative dot plots of HLA-G^+ $CD4^+$ T cells are depicted. (c) Percentages of HLA-G^+ $CD4^+$ T cells in patients with HLA-G^+ or HLA-G^- blasts are shown. Each dot represents single AML patient. Lines indicate the median and interquartile range of HLA-G^+ $CD4^+$ T cells. $^{**}P < 0.01$.

Interestingly, as for DC-10, the percentage of HLA-G^+ $CD4^+$ Treg cells was significantly higher in patients with HLA-G^+ blasts than in those with HLA-G^- blasts (4.2 ± 5.79%, mean ± SD, $n = 14$ versus 0.44 ± 1.01%, mean ± SD, $n = 6$; $P = 0.0072$ Figures 3(b) and 3(c)). This is the first report demonstrating the presence of HLA-G-expressing DC-10 and $CD4^+$ T cells in the peripheral blood of leukemic patients. Our findings indicate that the frequency of regulatory cells, DC-10 and HLA-G^+ $CD4^+$ T cells, is increased in patients with HLA-G-expressing blasts, supporting the hypothesis that the expression of HLA-G on blasts may be a strategy by which leukemia promotes a tolerogenic microenvironment limiting anti-tumor responses. This mechanism of immune escape has been previously proposed for solid tumor where both infiltrating cells and tumor cells can express HLA-G [16]. It remains to be defined whether HLA-G-expressing tolerogenic cells are present in the bone marrow of AML patients where leukemic blasts reside and proliferate before emerging in the periphery. Moreover, correlation studies between HLA-G expression on blasts and the frequency of DC-10 and HLA-G^+ $CD4^+$ T cells will elucidate whether the microenvironment enriched in immunomodulatory factors allows the recruitment or the induction of tolerogenic cells inhibiting the antileukemic effects.

Thus far, the presence of increased levels of soluble HLA-G has been associated with malignancies, including AML [25, 26]. We therefore sought to evaluate the amounts of soluble HLA-G in the serum of AML patients that were previously analyzed for HLA-G expression. sHLA-G (shed HLA-G1 and HLA-G5) was detected in 15 out of 18 patients with a mean value of positive samples of 1.78 ± 1.28 ng/mL (mean ± SD) and no differences were obtained between sera from patients with HLA-G^+ and HLA-G^- blasts (1.79 ± 1.29 ng/mL, $n = 10$ versus 1.75 ± 1.42 ng/mL, $n = 5$, mean ± SD, Figure 4). In line with previous findings demonstrating that sHLA-G plasma levels were significantly higher in AML-M4 and AML-M5 acute leukemia subtypes [25], we detected higher amounts of sHLA-G in sera of AML-M4 patients as compared to those of other AML subtypes (2.8 ± 1.28 ng/mL $n = 5$ versus 1.2 ± 0.93 ng/mL, $n = 10$ mean ± SD; data not shown). In parallel,

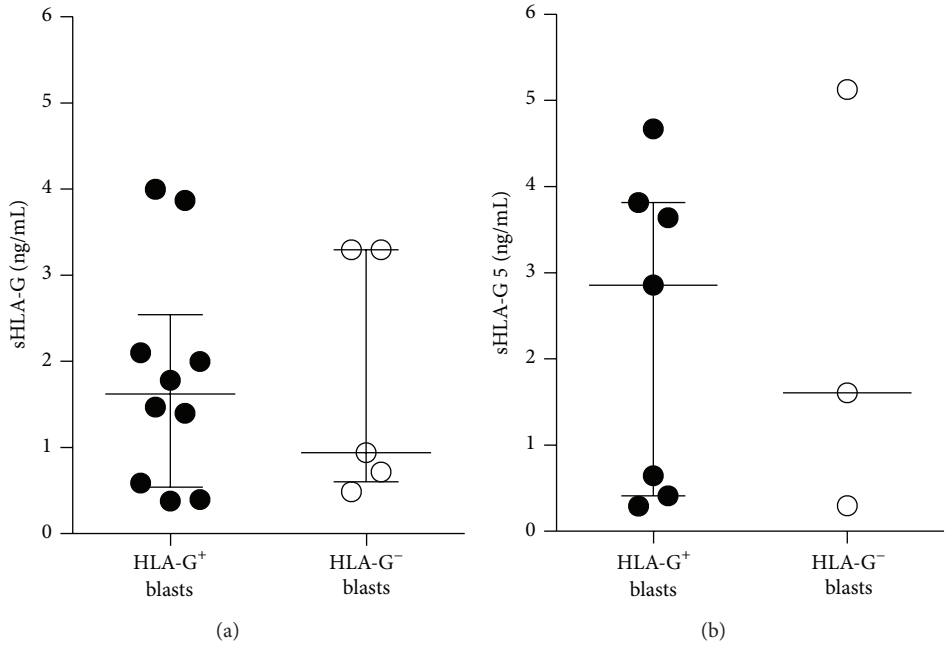

Figure 4: Soluble HLA-G levels in the sera of AML patients. The concentration of (a) sHLA-G (shed HLA-G1 and HLA-G5) and (b) HLA-G5 in the sera of AML patients was evaluated using specific sandwich ELISA. Each dot represents single AML patient. Lines indicate the median of serum levels (ng/mL) of positive samples in patients with HLA-G$^+$ or HLA-G$^-$ blasts.

Table 3: Frequency of 3′UTR haplotypes and genotypes.

3′UTR polymorphic sites	AML patients (n = 19)	HDs (n = 141)
Ins/Ins	0.26	0.22
Del/Del	0.37	0.30
Ins/Del	0.37	0.47
InsG/InsG	0.26	0.23
DelC/DelC	0.15	0.22
InsG/DelC	0.26	0.32
DelG/X	0.32	0.23
UTR-1	0.21	0.32
UTR-2	0.29	0.3
UTR-3	0.16	0.09
UTR-4	0.13	0.11
UTR-5	0.03	0.05
UTR-6	0.05	0.02
UTR-7	0.08	0.08
UTR-8	0.05	0.032

we detected HLA-G5 in 10 out of 18 patients with a mean value of positive samples of 2.33 ± 1.84 ng/mL (mean ± SD), and similar to sHLA-G, no differences were found between sera from patients with HLA-G$^+$ and HLA-G$^-$ blasts (2.33 ± 1.84 ng/mL, n = 7 versus 2.35 ± 2.5 ng/mL, n = 3; mean ± SD; Figure 4). Overall the levels of sHLA-G observed in our cohort of AML patients were lower compared to a previous work [25]. This discrepancy can be due to the fact that in our study we measured sHLA-G serum levels whereas Gros et al. [25] reported results from plasma samples. It has been indeed demonstrated that the levels of sHLA-G (shed HLA-G1 and HLA-G5) are significantly higher in plasma treated with EDTA as compared to those in plasma treated with heparin or in serum [62].

To define whether variations in the 3′UTR of HLA-G are associated with HLA-G expression, we analyzed 8 polymorphic sites at the *HLA-G* 3′UTR segment, including the 14 bp Ins/Del (rs1704), +3003 C/T (rs1707), +3010 C/G (rs1710), +3027 A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142), and +3196 C/G (rs1610696), previously described [34, 35]. The frequency of the 14 bp genotypes was similar in AML patients and healthy donors (Table 3). Since the 14 bp Ins is in strong linkage disequilibrium with the G in position +3142, we classified patients and controls according to the presence of the 14 bp Ins/Del and the +3142 C/G polymorphisms as InsG/InsG, DelC/DelC, DelC/InsG, and DelG/X. The relative frequencies of these genotypes in healthy donors were for InsG/InsG 23%, for DelC/DelC 22%, for DelC/InsG 32%, and for DelG/X 23% (Table 3). Interestingly, in AML patients we found a higher frequency of DelG/X genotype as compared to that observed in healthy donors (32% and 23%, resp.; Table 3). In line with these results, the frequency of UTR-3 haplotype (14 bp Del, +3003T, +3010C, +3027C, +3035C, +3142G, +3187A, and +3196C) was highly represented in AML patients than in healthy donors (16% and 9%, resp.; Table 3). Polymorphisms at the 3′UTR of *HLA-G* locus, and particularly the 14 bp Ins/Del, have been associated with

different clinical conditions including autoimmunity and pathological pregnancy. So far, limited information has been published on the association of HLA-G polymorphisms in tumor cells with the levels of HLA-G expression and/or clinical outcome of patients [17]. Recently, studies in small cohort of patients investigated the association of 14 bp Ins/Del with the susceptibility to cervicovaginal and breast cancer, with controversial results [52, 63, 64]. Although in the present study the variation in the 3′UTR of HLA-G was evaluated in a limited number of AML patients, results showed no specific association, with the exception of UTR-3 haplotype. A more extensive study is warranted in a large cohort of patients in order to define whether specific UTRs of HLA-G might be associated with AML or can be used as genetic risk factor for the disease susceptibility.

4. Conclusions

Results from this study further improve the knowledge on the role of HLA-G in promoting tolerance. Moreover, they opem new clinical perspectives: HLA-G expression can be used as prognostic tumor biomarker to monitor disease state or as therapeutic target for improving immune responses against leukemia. The expression of HLA-G on blasts and the analysis of DC-10 and HLA-G$^+$ CD4$^+$ Tregs can be used to evaluate the effectiveness of anti-tumor therapies. Moreover, the analysis of HLA-G polymorphisms will allow the identification of specific HLA-G genotypes that could be associated with AML susceptibility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Grazia Locafaro and Giada Amodio contributed equally to this work. Grazia Locafaro and Giada Amodio performed the experiments and the analysis of the data and contributed to the preparation of the paper; Daniela Tomasoni performed some of the experiments; Cristina Tresoldi and Fabio Ciceri provided the clinical samples; Silvia Gregori conceived the scientific idea, supervised the project, and wrote the paper.

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Review Article

Transcriptional and Posttranscriptional Regulations of the *HLA-G* Gene

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HLA-G has a relevant role in immune response regulation. The overall structure of the *HLA-G* coding region has been maintained during the evolution process, in which most of its variable sites are synonymous mutations or coincide with introns, preserving major functional *HLA-G* properties. The *HLA-G* promoter region is different from the classical class I promoters, mainly because (i) it lacks regulatory responsive elements for IFN-γ and NF-κB, (ii) the proximal promoter region (within 200 bases from the first translated ATG) does not mediate transactivation by the principal HLA class I transactivation mechanisms, and (iii) the presence of identified alternative regulatory elements (heat shock, progesterone and hypoxia-responsive elements) and unidentified responsive elements for IL-10, glucocorticoids, and other transcription factors is evident. At least three variable sites in the 3′ untranslated region have been studied that may influence *HLA-G* expression by modifying mRNA stability or microRNA binding sites, including the 14-base pair insertion/deletion, +3142C/G and +3187A/G polymorphisms. Other polymorphic sites have been described, but there are no functional studies on them. The *HLA-G* coding region polymorphisms might influence isoform production and at least two null alleles with premature stop codons have been described. We reviewed the structure of the *HLA-G* promoter region and its implication in transcriptional gene control, the structure of the *HLA-G* 3′UTR and the major actors of the posttranscriptional gene control, and, finally, the presence of regulatory elements in the coding region.

1. Introduction

The nonclassical HLA-G molecule presents several properties that differ from other classical class I HLA (-A, -B, and -C) molecules, including restricted tissue distribution; limited protein variability; presence of several membrane-bound and soluble isoforms; unique molecular structure, presenting a particular peptide-binding groove that impairs peptide presentation to T cells; ability to form dimers and polymers and a reduced cytoplasmic tail that impairs molecule turnover; and, most importantly, the molecule that modulates several functions of immune system cells (reviewed by [1]). The interaction of HLA-G with leukocyte receptors, particularly ILT-2 and ILT-4, downregulates the cytotoxic activity of T CD8 and Natural Killer cells and inhibits antigen presentation and lymphocyte proliferation [1, 2]. Dendritic cells expressing IL-10 and HLA-G can induce regulatory T cells [3]. Due to all of these properties, HLA-G has been recognized as a tolerogenic molecule, and the tissue expression of HLA-G may protect or harm; that is, it may protect allografts against attack by the recipient immune system and may impair the cytotoxic immune response against tumor cells.

The *HLA-G* gene also presents unique features. The coding region exhibits few polymorphic sites randomly

distributed along exons and introns, contrasting with the high rate of exonic polymorphic sites observed in classical HLA class I genes. The exonic nucleotide sequences encoding residues that are important for molecule dimerization and molecule interaction with leucocyte receptors are usually conserved, indicating that the overall structure of the molecule was maintained throughout human evolution [1, 2, 4, 5]. Considering that HLA-G is expressed on the surface of placenta trophoblast cells, allowing the fetus to properly develop despite the maternal immune response, some sort of functional conservation was expected. On the other hand, gene regulatory regions present several polymorphic sites close to nucleotide sequences that serve as gene regulatory elements [6–9]. Nucleotide variability in the promoter region may influence HLA-G levels by modifying binding affinity for transcription factors. In contrast to classical HLA class I genes, the promoter region of *HLA-G* does not have responsive elements for IFN-γ or NF-κB. Similarly, nucleotide variability at the 3′ untranslated region (3′UTR) may influence *HLA-G* mRNA stability, microRNA targeting, or both, affecting the posttranscriptional gene regulation.

Considering that the structure of HLA-G molecules has been maintained throughout evolution, the quantity of produced molecules may primarily depend on factors that modulate gene expression by transcriptional and posttranscriptional mechanisms. Firstly, we will review the structure of the *HLA-G* promoter region and its implication in transcriptional gene control; secondly, the structure of the *HLA-G* 3′UTR and the major actors of the posttranscriptional gene control; and, finally, the presence of elements in the coding region that may regulate gene expression and differential mRNA splicing.

There is no consensus regarding the positions of the nucleotide variation in the *HLA-G* promoter and 3′UTR, mainly because (i) the IMGT/HLA database only presents sequences within 300 bases upstream to the first translated ATG, (ii) the complete 3′UTR gene segment is not considered in the IMGT database, and (iii) several HLA alleles were described presenting only some exon sequences. Therefore, the nucleotide positions used in the present study follow the one presented in the NG_029039 sequence (http://www.ncbi.nlm.nih.gov/nuccore/NG_029039). The nucleotide named as +1 is the Adenine of the first translated ATG (position 5867 at NG_029039). Variations within regulatory elements in the upstream 5′ untranslated region and 5′ promoter were denoted as negative values, considering position 5866 at NG_029039 as nucleotide –1.

2. HLA-G Transcriptional Regulation

The HLA class I genes are usually very similar in nucleotide sequence and structure because most of these genes have been generated in a series of imperfect duplications [10]. Therefore, in general, the same regulatory elements are acting in HLA class I genes, with some differences for each HLA class I locus. The HLA class I promoters are usually conserved, presenting cis-acting regulatory elements mainly within 220 bases upstream to the first translated ATG. However, the *HLA-G* promoter is atypical compared to other HLA class I genes since most of these regulatory elements are not functional.

The *HLA-G* locus presents a tissue-restricted expression pattern, being expressed in physiological conditions only in certain tissues such as trophoblast at the maternal-fetal interface, thymus, cornea, pancreas, proximal nail matrix, erythroblast, and mesenchymal stem cells [1, 11–18]. In view of the immunomodulatory properties of the HLA-G molecule, its expression must be under a tight tissue-specific regulation.

Overall, HLA class I genes present two main regulatory modules in the proximal promoter region (200 bases upstream to the translation start point), including (i) the Enhancer A (EnhA) combined with an interferon-stimulated response element (ISRE) and (ii) the SXY module, in which the transcription apparatus is mounted (Figure 1) [19–24]. However, these regulatory elements present locus-specific differences leading to different levels of HLA class I constitutive- and induced-expression (reviewed at [24, 25]).

The EnhA element includes two adjacent palindromic NF-κB binding sites (κB1 and κB2) that interact with the NF-κB family of transcription factors, both important to the constitutive and/or induced expression of HLA class I genes. This family includes several members, such as p50, p65 (also known as RelA), p52, c-Rel, and RelB, all usually acting by forming homo- or heterodimers [19]. Theoretically, the interaction of these factors with the EnhA element may transactivate (acting on any κB binding site) any HLA class I gene [20]. Thus, the HLA locus-specific transcription rate would be determined by (i) the levels of NF-κB/Rel family proteins in different tissues, (ii) modifications in the regulatory sequences, and (iii) potential activation of different NF-κB/Rel dimers [20]. In addition, EnhA may be a target sequence for other DNA-binding proteins, such as proteins of the leucine zipper transcription factor family [20]. For instance, p65 has a potent transactivation domain and might operate as a p65/p50 heterodimer or p65/p65 homodimer, while p50 lacks this transactivation domain and may not transactivate as a p50/p50 homodimer [20]. EnhA also mediates the TNF-induced transcription of HLA class I molecules [20, 29].

Due to variations in the EnhA nucleotide sequences among different HLA class I genes, NF-κB/Rel factors may interact as homo- or heterodimers resulting in different transcription levels [20]. The *HLA-G* EnhA element (including κB1 and κB2 sites) encompasses nucleotides –198 and –172 (regarding NG_029039) and, compared to other HLA class I genes, it is the most divergent one [19, 20]. In fact, the κB-sites in the *HLA-G* promoter (EnhA) are reported to bind only p50/p50 homodimers [25] (Figure 1). As presented earlier, p50 homodimers are not potent HLA class I gene transactivators [20]. Thus, although *HLA-G* possesses an NF-κB responsive element, it is not as efficient as the HLA class I classical genes [25].

ISRE is a target site for the interferon regulatory factor family, including the interferon regulatory factor-1 (IRF-1, activator), IRF-2, and IRF-8 (inhibitors) [19]. Interferon-γ (IFN-γ) is the most potent cytokine inducing HLA class I gene expression. IFN-γ induces the expression of IRF-1 by the activation of the Janus kinases (jak) 1 and 2 and

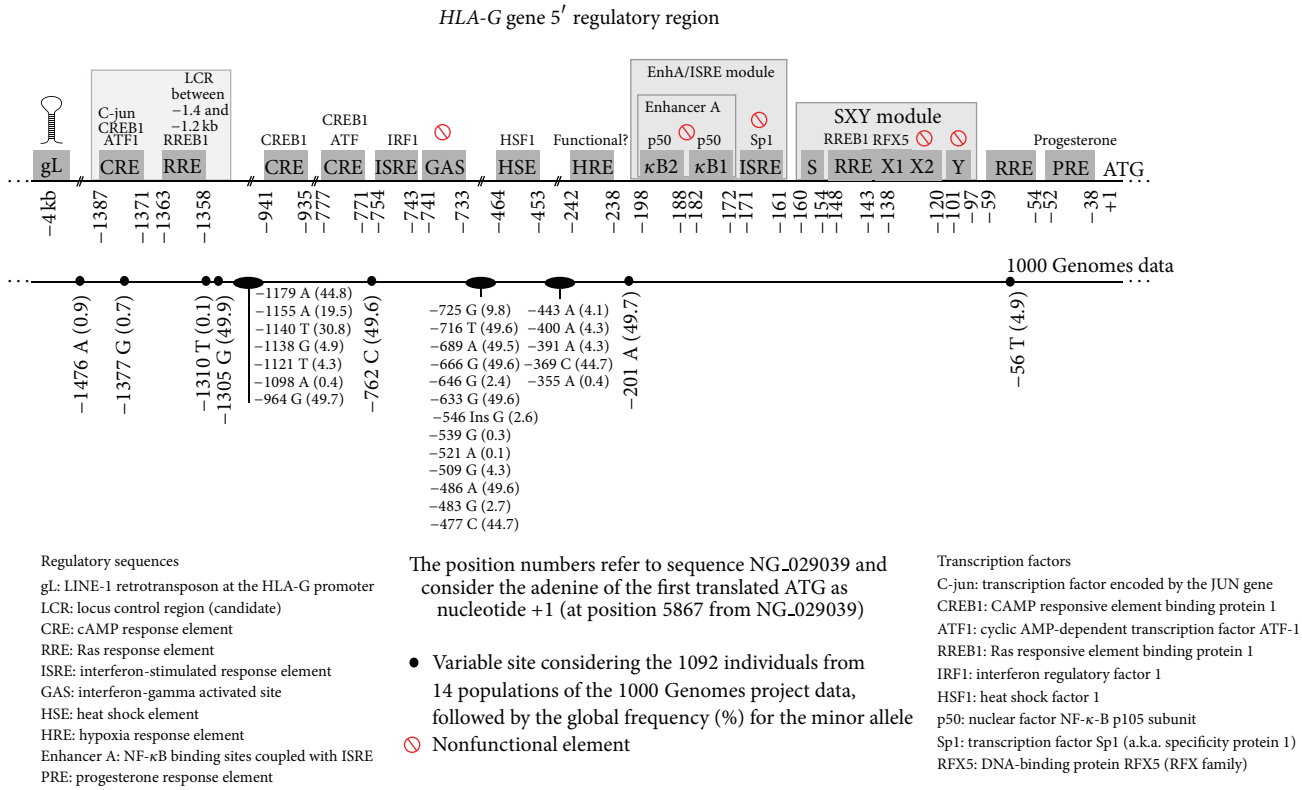

Figure 1: The *HLA-G* 5′ regulatory region with its known regulatory elements and variable sites according to the 1000 Genomes data.

phosphorylation of Stat1 (JAK/STAT pathway) [19, 21]. ISRE is adjacent to the EnhA element (constituting the module EnhA/ISRE presented earlier) and, thus, ISRE and EnhA regulate HLA class I expression cooperatively (Figure 1). ISRE also participates in the transactivation of β2-microglobulin, which is associated with the heavy α chain of the HLA class I molecule [21], and this information is important because an unbalanced production of these chains may impair correct HLA molecule assembly.

The nucleotide sequence of ISRE also varies among HLA class I loci. In this respect, locus-specific differences were observed in the IFN-induced expression levels of HLA class I genes [21, 29–31]. The *HLA-A* locus, for instance, does not respond to IFN at the same level as *HLA-B* and *HLA-C*, probably because of differences in the ISRE structure [19, 21, 29–31]. However, when comparing ISRE of the *HLA-G* locus with other class I genes, encompassing nucleotides −171 to −161, the *HLA-G* gene presents the most divergent ISRE compared to the ISRE consensus sequence, followed by *HLA-E* [19, 21], raising the issue of whether or not this element is fully functional for *HLA-G* and *HLA-E*. In fact, neither *HLA-G* nor *HLA-E* ISREs mediate IFN-γ-induced transactivation, and the binding of IRF-1 is not detected for *HLA-G* [21]. However, in the same way, probably because of the defective nature of the *HLA-G* ISRE, the binding of IRF-2 (transcription repression) was also not detected for *HLA-G* [21].

ISRE is also a target for other protein complexes that may mediate HLA class I transactivation. However, *HLA-G* κB2 (EnhA) and ISRE seem to bind only the constitutively expressed factor Sp1 (also known as Specificity Protein 1) [21, 25]. Nevertheless, the binding of Sp1 does not modulate the constitutive or IFN-induced transactivation of HLA class I genes, including *HLA-G* [21]. On the other hand, a candidate interferon-gamma activated site (GAS) was described between the −741 and −733 positions, presenting a sequence that would be compatible with a GAS consensus sequence (Figure 1). However, besides this new candidate, IFN-α, IFN-β, and IFN-γ treatments failed to increase HLA-G expression, a fact that was accredited to the defective nature of both the new candidate and the EnhA/ISRE region [32–34]. Nonetheless, another study showed that IFN-β enhances HLA-G expression by another ISRE present next to the nonfunctional GAS element at positions −754 to −743 [34].

The SXY module comprises the S, X1, and X2 (also known as site α) boxes and the Y box (also known as the Enhancer B or CCAAT box). The X1 box is a target for the multiprotein complex regulatory factor X (RFX), including the RFX5, the regulatory factor X-associated protein (RFXAP), and RFXANK [20, 25, 35, 36]. These RFX members have been shown to interact with the class II transactivator (CIITA) [37, 38], which is also an important element for HLA class I gene transactivation [25]. The X2 box is a binding target for the ATF/CREB (Activating Transcription Factor/cAMP Response Element Binding protein) transcription factor family [39]. Box Y is a binding target for Nuclear Factor Y (NFY), including subunits alpha (NFYA), beta (NFYB), and gamma (NFYC) [25, 40]. The function of box S has not yet

been elucidated. The binding of these factors to the SXY module allows the further binding of the coactivator CIITA and the NOD-like receptor family CARD domain containing 5 (NLRC5) factors [25, 41, 42]. The CIITA is constitutively expressed by antigen presenting cells and is induced by IFN-γ, and it transactivates HLA class I genes [41, 43]. NLRC5 transactivates HLA class I genes (but not HLA class II) and is constitutively expressed in a series of different tissues, mainly hematopoietic cells, or is induced by INF-γ [44–46].

For *HLA-G*, the SXY module presents sequences compatible only with the S and X1 elements, but divergent from X2 and Y elements (Figure 1) [25]. Therefore, CIITA, which is dependent on a functional SXY module, does not transactivate *HLA-G*, mainly because of the missing X2 and Y elements [25, 41, 42, 47, 48].

The *HLA-G* promoter region is unique among the HLA genes. Considering all the elements discussed above, it became clear that the *HLA-G* proximal promoter (within 200 bases) did not mediate transactivation by the principal HLA class I transactivation mechanisms [25]. In addition, studies evaluating the *HLA-G* promoter region within 1438 bp from ATG did not detect differences in the basal level of transactivation for different *HLA-G* promoters in different cell types [49, 50].

Some alternative regulatory elements within the *HLA-G* gene promoter have been described. A heat shock element (HSE) which would respond to the presence of heat shock proteins (HSP), especially the heat shock factor 1 (HSF1), was described in the *HLA-G* promoter region [51] (Figure 1). Stress-induced HSP are potent components that modulate immune responses. In general, *HLA-G* transcription is induced by heat shock (physical stress) or arsenate treatment (chemical stress) in human melanoma and glioblastoma cell lines, in which stress-induced HSF1 binds to an HSE lying between the −464 and −453 positions. This HSE response was detected for *HLA-G* but not for other HLA class I genes [51].

HLA-G expression may also be induced by progesterone [52], which is an immunomodulatory steroid hormone secreted both by the corpus luteum and placenta, allowing endometrium maintenance and embryo implantation. The mechanism underlying this induction is primarily mediated by the activation of the progesterone receptor (PR) and its subsequent binding to an alternative progesterone response element (PRE) found in the *HLA-G* promoter between positions −52 and −38, overlapping the *HLA-G* TATA box [53] (Figure 1).

Experiments with transgenic mice allowed the identification of a Locus Control Region (LCR) candidate located at least 1.2 kb upstream to the first translated ATG. This region is critical for the HLA-G expression regarding when and where it should be expressed. It is possible that this region acts by maintaining the chromatin in an open state or active configuration, enhancing gene expression [54, 55]. In addition, it may bind protein complexes associated with activation and inhibition of *HLA-G* transcription [56, 57].

At least three CRE/TRE candidate sites (cAMP Response Element/TPA Response Element) have been already considered, the first one being situated between the −1387 and −1371 positions (inside the putative LCR region discussed earlier), the second between the −941 and −935 positions, and the third between the −777 and −771 positions (Figure 1). The first CRE site (at LCR) was described to be an *in vitro* target site for c-jun by using electrophoretic mobility shift assay (EMSA). C-Jun, together with c-Fos, forms the AP-1 early response transcription factor. In addition, this same site was reported to bind ATF1/CREB1 *in vitro* and *in situ* by using chromatin immunoprecipitation (ChIP) [39]. The second CRE/TRE site binds *in vitro* to CREB1 and the third site binds *in vitro* to ATF1/CREB1 [39]. Mutations in all three CRE/TRE sites have been reported to reduce the *HLA-G* CREB1 transactivation, but a stronger inhibition was observed when the first CRE/TRE site (at the LCR) was mutated [39].

The repressor factor RREB1 (Ras Responsive Element Binding 1) may also be implicated in *HLA-G* expression regulation. At least three binding sites for RREB1, known as Ras Response Elements (RRE), in the *HLA-G* promoter region have been described. The consensus sequence GGTCCT, corresponding to one of the binding sites for RREB1, was found in the proximal promoter between the −59 and −54 positions (one direct site) and between the −148 and −143 positions and the −139 and −134 positions (a direct site and an inverted site). A target site related to the other consensus-binding site for RREB1, CCCCACCATCCCC, was found within the LCR between the −1363 and −1358 positions (Figure 1). The mechanism underlying RREB1 repression is probably associated with the recruitment of the corepressor C-terminal binding protein 1 or 2 (CtBP-1 or CtBP-2), or both, and the deacetylase 1 (HDAC1), which is involved in chromatin remodeling probably increasing chromatin condensation and hampering transcription factor accessibility [58, 59].

The GLI-3 repressor, a signal transducer of the Hedgehog pathway (HH), has also been reported to regulate *HLA-G* during the maturation of osteoblasts [60], especially in the production of the HLA-G5 isoform. It acts by a direct interaction of the HH signaling transducer factor GLI-3 with the *HLA-G* promoter. However, it is not clear whether the HH signaling pathway, a highly conserved molecular pathway involved in the development of several tissues, directly regulates HLA-G5 expression in other cell types.

A negative regulator of gene expression is observed in a sequence about −4 Kb upstream to the *HLA-G* translation starting point, overlapping with a LINE-1 sequence [61] (Figure 1). LINEs (Long Interspersed Elements) are a group of retrotransposons, which are highly repetitive elements from the eukaryotic genome that contribute to genome variability. The LINE-1 element described for *HLA-G* (named gL) is an AT-rich sequence (about 60%) that presents more sites with a high probability of forming hairpin loops than the general LINE sequence. These hairpin loops might directly or indirectly interact with the *HLA-G* promoter and interfere with the binding of transcriptional factors and enhancers [61]. LINE elements are frequently found lying in the 5′ upstream regulatory region of other HLA class I genes, including *HLA-A*. However, the LINE sequence found in the *HLA-A* promoter (named aL) is not transcriptionally active and is shorter than the one found in *HLA-G* (gL). Therefore,

the presence of this gL element in the *HLA-G* promoter would explain its limited expression compared with other class I genes. However, it should be noticed that this gL element is also present in HLA-G-expressing cells; thus, other regulatory elements might inhibit or overcome this negative regulation [61].

Hypoxia is an important physiological microenvironment for placentation and for the formation of the maternal-fetal interface [62]. The microenvironment is also crucial for the function of T and B cells. In this scenario, hypoxia is also associated with increased HLA-G expression. The Hypoxia-Inducible Factor (HIF) is involved in the control of cellular responses to oxygen depletion [62]. The HLA-G expression (membrane and soluble) is 2 times increased when extravillous cytotrophoblasts are cultivated under only 2% oxygen [63]. Likewise, hypoxia is associated with increased *HLA-G* transcription in a series of HLA-G-negative tumor lineages, such as 1074mel [64, 65] and M8 [66]. A consensus Hypoxia Response Element (HRE) [67] is located between the −242 and −238 positions (Figure 1). However, the functionality of this element has not been explored [64].

Some inducers of HLA-G expression have been described; but the underlying induction mechanisms are unknown. Interleukin 10 (IL-10), which is produced by lymphocytes, monocytes, macrophages, placenta, and some tumors, may induce HLA-G expression and the down-regulation of other HLA class I and II genes [68–70]. Cortisol, a glucocorticoid produced by the adrenal gland, is a potent immunomodulatory hormone at high doses. HLA-G expression in trophoblastic cells was increased following treatment with dexamethasone or hydrocortisone [71], but no complete Glucocorticoid Response Element (GRE) has been identified in the *HLA-G* promoter.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a protein secreted by macrophages, T cells, mast cells, NK cells, endothelial cells, fibroblasts, and uterine epithelium. GM-CSF increases HLA-G expression when combined with INF-γ treatment, but no effect is observed for GM-CSF alone [72, 73].

The Leukemia Inhibitory Factor (LIF) is a cytokine expressed at the maternal-fetal interface in the cytotrophoblast that plays an important role in implantation. LIF is mainly expressed in the implantation window. By using the choriocarcinoma cell line JEG3, *HLA-G* transcription was increased by about 3.6 times after LIF treatment. It was demonstrated that LIF induces full-length membrane HLA-G (HLA-G1) expression on the JEG3 cell surface [74]. In addition, LIF may induce HLA-G1 expression in the presence of ERAP1 (Endoplasmic Reticulum Aminopeptidase-1) expressed in the endoplasmic reticulum. Repression of ERAP1 in JEG3 cells treated with LIF diminishes HLA-G expression, suggesting a role for ERAP in *HLA-G* regulation [75].

Some drugs may also induce HLA-G production, such as methotrexate (MTX), one of the most used antirheumatic drugs for the treatment of rheumatoid arthritis (RA). MTX can induce apoptosis of mitogen-stimulated peripheral blood mononuclear cells (PBMCs) resembling the mechanisms underlying the inhibition of cytotoxic T CD8+ cell activity by soluble HLA-G molecules. MTX can induce the production of sHLA-G in unstimulated RA or healthy individual PBMCs and may have a role in the clinical outcome of RA patients. The mechanisms underlying sHLA-G production after MTX treatment are unknown, but it was reported that MTX therapy mediates an increase of interleukin-10-producing cells, which in turn may stimulate HLA-G production [76].

The *HLA-G* promoter exhibits numerous polymorphic sites (Figure 1). Data from the 1000 Genomes project, including 1092 individuals from 14 different populations, showed 32 variable sites within 1500 nucleotides upstream to the first translated ATG. Most of these variable sites have been already described in other populations or samples different from those evaluated by the 1000 Genomes consortium [6–9, 77–81]. Of those, 24 variable sites present frequencies higher than 1% and 14 present frequencies higher than 10% in the global 1000 Genomes data (all 1092 individuals). These variable sites may be important for the regulation of HLA-G expression and may act in different ways. Polymorphisms in the proximal promoter of *Paan-AG*, the functional homologue of *HLA-G* in the Olive Baboon, have been shown to influence NF-κB binding and transcription activity [82, 83]. However, the human variable sites may act by mechanisms differing from those described above because, generally, these variable sites do not coincide with known regulatory elements (Figure 1).

Variation in regulatory elements may affect the binding of the corresponding regulatory factors. In this respect, only four variable sites coincide with known regulatory elements: (i) position −1377 in the first CRE site of the LCR, (ii) positions −1310 and −1305 of the LCR, and (iii) position −56 of the Ras Response Element (RRE) in the proximal promoter. Of these, only the ones at positions −1305 and −56 are frequently found worldwide (Figure 1). Other variable sites are close to known regulatory elements and may somehow influence the binding of transcription factors. In this group we may observe variable sites at positions −762 (between a CRE and ISRE), −725 (next to a nonfunctional GAS element), −477 and −433 (around the HSE), and −201 (next to Enhancer A) (Figure 1).

Few studies have associated promoter polymorphisms and differential HLA-G expression. The variable site at position −725, in which the minor allele (G) is present in 9.8% of the chromosomes evaluated in the 1000 Genomes project, was associated with differential HLA-G expression. *HLA-G* promoter haplotypes (between −1389 and −55 and not considering primer sequences) were cloned into luciferase expression vectors and transfected to the HLA-G expressing cell JEG-3, resulting in a significantly higher expression level of the promoters presenting Guanine at position −725 [84]. In addition, another study described the same influence of position −725 on HLA-G expression levels [85]. This same polymorphism (−725 G) has been reported to be associated with sporadic miscarriage [7] and end-stage renal disease [86], while the most frequent allele (−725 C) has been reported to protect against multiple sclerosis [87]. Nevertheless, despite the lack of studies regarding *HLA-G* promoter polymorphisms and HLA-G expression, some polymorphic sites have already been associated with several conditions. The polymorphism at position −964, which is very frequent

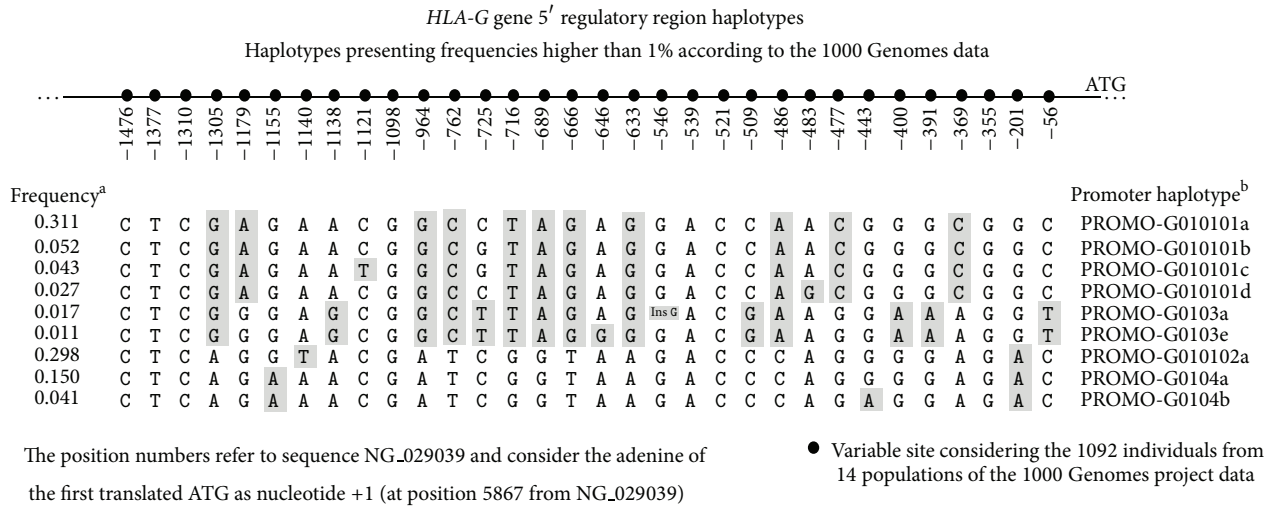

Figure 2: The most frequent haplotypes of the *HLA-G* 5′ regulatory region according to the 1000 Genomes data. [a]Haplotype frequencies considering the 1092 individuals from the 1000 Genomes project [26]. [b]Haplotypes were named according to [6].

among the populations evaluated by the 1000 Genomes consortium, was associated with asthma. The −964 G/G genotype was associated with asthmatic children of affected mothers, whereas the A/A genotype was associated with asthmatic children of unaffected mothers [88]. The −964 A and −486 C alleles, together with the −725 G allele, were also associated with protection against end-stage renal disease [86]. The polymorphism at position −1305, also very frequent among the 1000 Genomes populations, was associated with nonsegmental vitiligo [89].

The methylation status of the *HLA-G* promoter is also very important to the *HLA-G* transcriptional activity [90]. It has been reported that the CpG islands in the *HLA-G* promoter region of JAR (choriocarcinoma) cells, which does not express HLA-G, were fully methylated, whereas for an HLA-G expressing cell such as JEG-3, the CpG islands were only partially methylated [91, 92]. In addition, HLA-G expression was induced in several tumor cell lines by using demethylation agents, such as 5-aza-2′deoxycytidine [93–97]. Moreover, the levels of histone acetylation in the *HLA-G* promoter chromatin have been reported to be significantly enhanced in FON+ (melanoma) and JEG-3 (Human placental choriocarcinoma cell line) cell lines, both expressing HLA-G, while in non-HLA-G expressing cell lines, such as M8 (melanoma) and JAR, histones seem to be hypomethylated [94, 96, 98]. Histone acetylation is usually associated with a relaxed chromatin structure, therefore, with greater levels of gene expression [99, 100]. In this respect, polymorphisms in the *HLA-G* promoter, especially in the CpG islands, might also be associated with different methylation profiles [84].

Although most of the *HLA-G* promoter variable sites do not occur inside known regulatory elements (Figure 1), balancing selection has been reported to maintain divergent haplotypes in the 5′ promoter [6, 8, 9, 78, 79] and 3′UTR regulatory regions [6, 27, 28, 78, 101, 102]. In fact, at least 14 variable sites in the promoter region do present frequencies higher than 10%, and 11 variable sites present frequencies higher than 44% (Figure 1). However, considering the haplotypes described for the *HLA-G* promoter, which seem to be the same worldwide [6, 8, 9, 77–79], most of these frequent variable sites are in complete Linkage Disequilibrium (LD), and just four main *HLA-G* promoter lineages are associated with these variable sites. These promoter lineages were first proposed by Ober's group [8] and subsequently confirmed and named in a Brazilian study as PROMO-G010101, PROMO-G010102, PROMO-G0103, and PROMO-G0104 [6]. In addition, considering data from the 1000 Genomes Project, only nine promoter haplotypes present frequencies higher than 1% in worldwide populations (Figure 2), but two of them, PROMO-G010101a and PROMO-G010102a, which are the most divergent ones, account for more than 60% of all haplotypes. Nevertheless, despite the fact that most of these frequent *HLA-G* variable sites are not within known regulatory elements, several lines of evidence indicate balancing selection acting on the *HLA-G* promoter found in several populations [6, 8, 9, 78, 79], suggesting that divergent promoters have been maintained with high heterozygosis. This observation is probably related to a possible better fitness of individuals carrying both high- and low-expressing promoters. Therefore, these divergent *HLA-G* promoter haplotypes are probably associated with differential HLA-G expression, but the mechanisms are unknown. However, as discussed later, the pattern of LD observed for the promoter region extends up to *HLA-G* 3′UTR [6, 8, 9, 27, 77–79] and at least 20 kb beyond the *HLA-G* 3′UTR [102]. Thus, selective pressures acting on other *HLA-G* regions as well as adjacent sequences might also influence *HLA-G* promoter variability and heterozygosis. Figure 2 illustrates major *HLA-G* promoter region haplotypes observed in worldwide populations.

3. Posttranscriptional Regulation of *HLA-G*

As previously stated, there is no consensus regarding the positions of the nucleotide variation in the *HLA-G* 3′UTR,

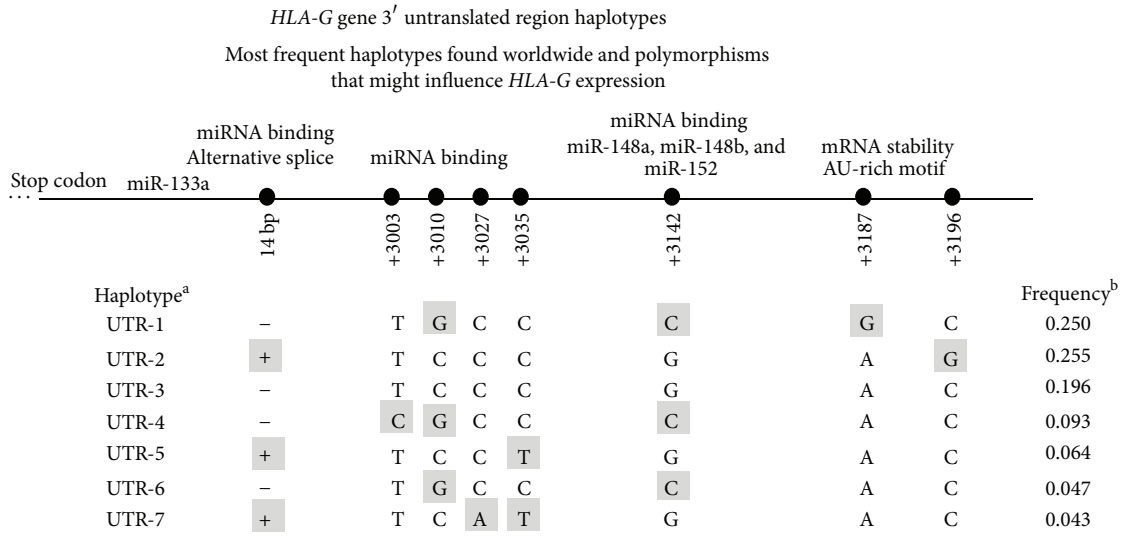

Figure 3: The most frequent haplotypes of the *HLA-G* 3′ untranslated region according to the 1000 Genomes data. [a]Haplotypes were named according to [27]. [b]Haplotype frequencies considering 21 worldwide populations [28].

considered to be located mostly in exon 8. Since there is no official information regarding the *HLA-G* 3′UTR sequences, the nucleotide positions used in the present study follow those previously reported by our group [1, 6, 27], that is, inferring polymorphic sites in 3′UTR using the original *HLA-G* sequence described by Geraghty and colleagues [103] and considering nucleotide +1 as the Adenine of the first translated ATG (similar to the IMGT/HLA description). In the *HLA-G* 3′UTR, there is a well-studied polymorphism that consists of a 14-nucleotide deletion (rs371194629 or rs66554220), also known as the 14-bp indel (insertion/deletion) polymorphism. The sequence used as a model for the *HLA-G* promoter structure (NG_029039) does not present this 14-nucleotide sequence (that would be inserted between nucleotides +2960 and +2961). Given that the presence of these 14 nucleotides is also found in gorillas and chimpanzees, it should represent the ancestor allele, and the 14 bp sequence should be included in the 3′UTR reference sequence. Therefore, any position after nucleotide +2960 is taken considering the original NG_029039 sequence plus 14 bases. For instance, the polymorphism at the +3142 position discussed later in this review refers to the +3128 nucleotide in the NG_029039 reference sequence.

Due to a premature stop codon (positions +2536 to +2538 in NG_029039), the *HLA-G* gene presents a relatively large 3′UTR genomic sequence that extends up to the +3292 nucleotide, encompassing approximately 754 nucleotides. Inside the 3′UTR genomic region, there is an intron that is spliced out, giving rise to the mature *HLA-G* mRNA with a 3′UTR sequence of approximately 397 nucleotides (considering the presence of the 14 bases discussed earlier). This 3′UTR is a key feature for transcriptional *HLA-G* regulation, which is important for (i) *HLA-G* mRNA stability, (ii) targeting specific microRNAs [104], and (iii) polyadenylation signal in the AU-rich regulatory mRNA element [105]. The mRNA availability for translation, as well as consequent protein production and maturation, is constantly balanced by the opposing forces of transcription levels and mRNA decay. The transcription level is mainly driven by the 5′ regulatory region and the presence of specific transcription factors, while mRNA decay is mainly driven by its intrinsic stability (which is dependent on the nucleotide sequence) and the action of microRNAs. MicroRNAs may negatively regulate gene expression by translation suppression, RNA degradation, or both [104, 106–108]. The first miRNA was reported in 1993 [109], and more than 2000 human microRNAs have been reported to date [110, 111].

The *HLA-G* 3′UTR presents several polymorphic sites, some of which have been associated with differential HLA-G expression profiles. Although the *HLA-G* 3′UTR segment is quite short compared to the same region in other genes, it presents at least eight polymorphic sites that are frequently found in worldwide populations (Figure 3). The *HLA-G* 3′UTR variability and haplotypes were systematically explored in a Southeastern Brazilian population, in which seven frequent haplotypes were described, encompassing these eight polymorphic sites, designated UTR-1 to UTR-7, and a rare one named UTR-8 [27]. The relationship between *HLA-G* 3′UTR polymorphisms (especially for the 14 bp polymorphism) and other variable sites in the *HLA-G* coding and promoter region was also previously explored [77, 78, 105, 112, 113]. Furthermore, several populations were evaluated regarding these polymorphic sites, including additional samples from other Brazilian regions and other worldwide populations, and the same pattern of 3′UTR variability has been observed [6, 27, 28, 85, 102, 114–121]. Recently, the variability at the *HLA-G* locus was explored by using the 1000 Genomes data [28, 102] and, taking together all of these studies in the last decade, it became clear that the same 3′UTR pattern observed in Brazilians [27] is found worldwide, with just some new low frequency haplotypes.

Most of the polymorphisms present in the *HLA-G* 3′UTR may influence the HLA-G expression profile by different mechanisms. Since they are present in a short mRNA sequence with just some nucleotides apart, and since the pattern of haplotypes is quite conserved [28, 102], the influence of each polymorphic site on the HLA-G expression profile may not be independent of other polymorphic sites; that is, extended haplotypes should be considered due to the cumulative effects of different polymorphisms. For example, the +3003, +3010, +3027, and +3035 polymorphic sites encompass only 32 nucleotides that are also in linkage disequilibrium with each other and in linkage disequilibrium with variable sites in the coding and promoter segments [6] (Figure 3).

The first *HLA-G* 3′UTR polymorphic site associated with HLA-G expression levels was an indel (insertion/deletion) variant known as the 14 bp polymorphism. This polymorphism is characterized by the removal of a 14-nucleotide segment [122] between positions +2961 and +2974, and it presents high frequency in all populations studied so far. The ancestor allele (the 14 bp presence or insertion) is also found in gorillas and chimpanzees [1]. The 14 bp polymorphism has been associated with the magnitude of HLA-G production [77, 123–125], modulating *HLA-G* mRNA stability [113, 126–128] and also as a target for microRNAs [106]. In general, the presence of the 14-nucleotide sequence (5′-ATTTGTTCATGCCT-3′) has been associated with lower HLA-G production for most membrane-bound and soluble isoforms in trophoblast samples [77, 78, 123, 125, 128]. However, Svendsen and colleagues observed the opposite when K562 cells were transduced with ins-14 bp HLA-G1 or with del-14 bp HLA-G1, in which the expression of HLA-G1 was found to be higher for ins-14 bp cells compared to del-14 bp cells [124]. Moreover, this 14-base sequence was also associated with an alternative splicing of the *HLA-G* transcript, in which 92 bases from the mature 3′UTR *HLA-G* mRNA were removed (including the 14-base sequence itself) [113, 128], and these smaller transcripts were reported to be more stable than the complete transcript [126]. Though influencing mRNA stability, only a fraction of the mRNA bearing these 14 nucleotides is further processed with the removal of 92 bases, and the greater stability apparently does not compensate for the lower HLA-G levels associated with the 14-base sequence. Nevertheless, there are controversial results regarding the influence of this polymorphism in HLA-G expression and alternative splicing.

The following four polymorphic sites, frequently found in the *HLA-G* 3′UTR in worldwide populations, are present at positions +3003, +3010, +3027, and +3035 [6, 27]. Although no specific regulation mechanism has been described regarding these polymorphic sites, they might influence microRNA binding [106]. Additional polymorphic sites around this small *HLA-G* 3′UTR segment are infrequently observed in worldwide populations, including the +3001 C/T polymorphism observed in Senegalese and Northeastern Brazilian populations [115, 116] and the +3033 C/G polymorphism observed among Northeastern Brazilians [115]. Although there are no studies evaluating the functional properties of these polymorphic sites, an *in silico* study reported that several microRNAs might target this small segment [106].

The nucleotide variation at position +3142 has been associated with the magnitude of HLA-G expression by posttranscription mechanisms, such as the interaction with microRNAs. It was functionally and computationally demonstrated that this variation site would influence the binding of specific microRNAs, including miR-148a, miR-148b, and miR-152 [129]. The presence of a Guanine at the +3142 position increases the affinity of this region for these microRNAs, hence decreasing *HLA-G* expression by mRNA degradation and translation suppression [106, 129, 130]. This polymorphism, together with the 14-bp polymorphism, has been considered to be the most important one regarding *HLA-G* posttranscription regulation, and methodologies have been proposed to quickly type these polymorphic sites [131, 132]. At least two studies have demonstrated that the +3142 C/G polymorphic site may influence HLA-G expression by modulating the mRNA interaction with miR-152, particularly in bronchial asthma [129, 133]. However, there is no consensus regarding the influence of this polymorphic site on the binding of such microRNAs, since another functional study did not detect this influence [134]. Instead, it was reported that both miR-148a and miR-152 downregulate HLA-G expression, irrespective of the +3142 C or G alleles [134]. These microRNAs have already been reported to modulate the expression of another classical HLA class I gene, *HLA-C* [135]. Interestingly, only HLA-C and HLA-G are usually found at the maternal-fetal interface, indicating the presence of some sort of coordinated regulation. Similarly to miR-148a, miR-148b, and miR-152, other microRNAs have the potential to bind to the *HLA-G* mRNA 3′UTR and to influence HLA-G expression. The binding ability of these microRNAs may be potentially influenced by polymorphisms observed in the *HLA-G* 3′UTR [106].

Another polymorphic site that has been associated with the magnitude of HLA-G expression is located at position +3187 A/G. This polymorphism was associated with preeclampsia in a Canadian population [136]. The mechanism underlying such association has been attributed to the proximity of this polymorphic site to an AU-rich motif that mediates mRNA degradation. Then, the presence of an Adenine instead of a Guanine at position +3187 would lead to a decreased HLA-G expression due to the increased number of Adenines in this AU-rich motif [136].

In addition to the microRNAs that might target polymorphic sequences in the *HLA-G* 3′UTR, some microRNAs would bind to nonpolymorphic sequences and modulate HLA-G expression irrespectively of the individual genetic background. However, such approach has not yet been used and only microRNAs targeting polymorphic sequences have been evaluated. Nevertheless, the microRNA miR-133a was found to target a nonpolymorphic sequence upstream to the 14-b sequence fragment, between nucleotides +2945 and +2952, downregulating HLA-G expression (Figure 3). This phenomenon was associated with the pathogenesis of recurrent spontaneous abortion [137].

Taken together, the conserved patterns of *HLA-G* 3′UTR haplotypes and the few frequent haplotypes found worldwide

[102, 116] show that only one haplotype does carry all alleles that have been associated with high HLA-G expression. This haplotype, known as *HLA-G* UTR-1 [27] (14 bp Deletion/+3003 T/+3010 G/+3027 C/+3035 C/+3142 C/+3187 G/+3186 C), does not present the 14 bp sequence; that is, it presents a 14 bp deletion, which was associated with highly soluble HLA-G expression; it presents a Cytosine at position +3142 (less sensitive to specific microRNAs targeting this region), and it exhibits a Guanine at position +3187 (increased mRNA stability). Besides possessing these three polymorphic alleles associated with high HLA-G production, UTR-1 presents some other interesting features: (i) it is one of the most frequent 3′UTR haplotypes found worldwide [116], (ii) it has been described as one of the most recent *HLA-G* 3′UTR haplotypes among the frequent ones due to its exclusive association with the presence of an Alu element that is close to *HLA-G* (20 Kb downstream the 3′UTR) [102], and (iii) UTR-1 was recently associated with higher HLA-G expression [138].

Several studies have reported that the *HLA-G* 3′UTR segment is also under selective pressures, whereby balancing selection is maintaining high levels of heterozygosis in this region [6, 27, 28, 101, 139, 140]. As observed worldwide [27, 28, 102], the two most frequent *HLA-G* 3′UTR haplotypes (UTR-1 and UTR-2) are also the most divergent ones (Figure 3). They differ in all known variable sites that might influence HLA-G expression. Therefore, the same phenomenon observed for the promoter region is also seen in the 3′UTR, in which high heterozygosis is observed between high- and low-expression haplotypes. Moreover, the rate of recombination in the *HLA-G* locus is quite low, and the pattern of linkage disequilibrium found in the *HLA-G* locus encompasses the promoter region, the coding region, the 3′UTR, and at least 20 kB downstream of the 3′UTR [102]. Thus, in general, only few frequent extended haplotypes do exist and a specific promoter haplotype is usually accompanied by the same *HLA-G* coding sequence and the same 3′UTR haplotype [6–8, 28, 77, 78, 102]. The UTR-1 haplotype, for example, is usually associated with the coding sequence for the HLA-G*01:01:01:01 allele and the PROMO-G010101a promoter haplotype [6–8, 28, 102]. Therefore, the influence of each variable site at the HLA-G transcriptional level must be considered.

4. *HLA-G* Coding Region Polymorphisms Influencing HLA-G Expression

The HLA-G genetic structure resembles the class I structure, in which the first translated exon encodes the peptide signal, the second, third, and fourth ones encode the extracellular α1, α2, and α3 domains, respectively, and the fifth and sixth ones encode the transmembrane and the cytoplasmic domain of the heavy chain. Considering the *HLA-G* coding region (from the first translated ATG to the stop codon), at least 75 single nucleotide polymorphisms (SNP) have been observed, defining the 50 currently described *HLA-G* alleles, encoding only 16 distinct proteins (IMGT, database 3.14.0, November 2013). Similarly to what has been described for other genes such as *IRF4, MYC, IFNG,* and others [141–146], it is possible that intronic or exonic nucleotide sequences may exhibit affinity for transcription factors, thereby regulating the expression of the gene; however, this subject has not yet been studied in the context of the *HLA-G* gene.

The presence of certain polymorphic sites in the coding region may also regulate the expression of the seven described HLA-G isoforms generated by alternative splicing of the primary transcript. Four of the HLA-G isoforms are membrane-bound (HLA-G1, G2, G3, and G4) and 3 are soluble (G5, G6, and G7) ones. HLA-G1 is the complete isoform exhibiting a structure similar to that of the membrane-bound classical HLA molecule, associated with β2-microglobulin, HLA-G2 has no α2 domain, HLA-G3 presents no α2 and α3 domains, and HLA-G4 has no α3 domain. The soluble HLA-G5 and HLA-G6 isoforms present the same extracellular domains of HLA-G1 and HLA-G2, respectively, and the HLA-G7 isoform has only the α1 domain [147–149]. In contrast to most of the currently described *HLA-G* alleles that may produce all membrane-bound and soluble isoforms, the presence of stop codons in the coding region may yield truncated or missing HLA-G isoforms. The *HLA-G*01:05N null allele presents a Cytosine deletion in the last nucleotide of codon 129 or in the first nucleotide of codon 130 (exon 3), leading to a TGA stop signal in codon 189, yielding incomplete formation of the HLA-G1, -G4, and -G5 isoforms and normal expression of HLA-G2, -G3, and -G7 [1, 150, 151]. Similarly, the *HLA-G*01:13N allele presents a C → T transition in the first base of codon 54 (α1 domain), yielding the formation of a premature TAG stop codon, preventing the production of all membrane-bound and soluble isoforms, and therefore it is probably not expressed [1, 152, 153].

Humans bearing allele G*01:05N in homozygosis have been reported [154–157], a fact that may indicate that soluble HLA-G molecules or molecules lacking the α3 domain are sufficient for HLA-G function. The frequency of the G*01:05N allele varies among different populations [1], ranging from complete absence in Amerindian populations from the Amazon, Mayans from Guatemala, and Uros from Peru [139, 151, 158], to intermediate frequencies in Africa [155] and higher than 15% in some populations of India [159], while allele G*01:13N is quite rare [152, 153]. It has been proposed that high G*01:05N frequencies are associated with high pathogen load regions, and intrauterine pathogens would act as selective agents, with increased survival of G*01:05N heterozygous fetuses. In this case, the reduced HLA-G1 expression may result in an improved intrauterine defense against infections [139, 151, 154, 160]. To the best of our knowledge, no homozygous G*01:13N has been described.

5. Concluding Remarks

Due to the important role of HLA-G in the regulation of the immune response and its relevant function during the course of pregnancy, the overall structure of the molecule has been maintained during the evolution process, preserving major HLA-G binding sites to leukocyte receptors and HLA-G dimer formation. On the other hand, several variable sites

have been observed along the *HLA-G* regulatory regions. Although a perfunctory analysis of the many variable sites observed in the promoter region of several worldwide populations indicates that some known transcription factor target regions have also been conserved, one cannot rule out the influence of the differential action of distinct transcription factors according to promoter region variability. In contrast, most of the variable sites found in the *HLA-G* 3′UTR might influence HLA-G expression by facilitating or hindering microRNA binding and/or influencing mRNA stability.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

IL-27 Driven Upregulation of Surface HLA-E Expression on Monocytes Inhibits IFN-γ Release by Autologous NK Cells

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HLA-G and HLA-E are HLA-Ib molecules with several immunoregulatory properties. Their cell surface expression can be modulated by different cytokines. Since IL-27 and IL-30 may either stimulate or regulate immune responses, we have here tested whether these cytokines may modulate HLA-G and -E expression and function on human monocytes. Monocytes expressed gp130 and WSX-1, the two chains of IL27 receptor (R), and IL6Rα (that serves as IL-30R, in combination with gp130). However, only IL27R appeared to be functional, as witnessed by IL-27 driven STAT1/ STAT3 phosphorylation. IL-27, but not IL-30, significantly upregulated HLA-E (but not HLA-G) expression on monocytes. IFN-γ secretion by activated NK cells was dampened when the latter cells were cocultured with IL-27 pretreated autologous monocytes. Such effect was not achieved using untreated or IL-30 pretreated monocytes, thus indicating that IL-27 driven HLA-E upregulation might be involved, possibly through the interaction of this molecule with CD94/NKG2A inhibitory receptor on NK cells. In contrast, cytotoxic granules release by NK cell in response to K562 cells was unaffected in the presence of IL-27 pretreated monocytes. In conclusion, we delineated a novel immunoregulatory function of IL-27 involving HLA-E upregulation on monocytes that might in turn indirectly impair some NK cell functions.

1. Introduction

HLA-class Ib family represents a small group of HLA-class I molecules that include HLA-G, HLA-E, HLA-F, and HLA-H [1]. In contrast with highly polymorphic classical HLA-class Ia molecules that are mainly involved in the presentation of peptides recognized by the T cell receptor on T lymphocytes, as well as in the interaction with killer Ig-like receptors on NK cells, HLA-Ib molecules are characterized by a very low degree of polymorphism and display several immunoregulatory properties [2].

HLA-G and -E are the best characterized among HLA-Ib molecules. HLA-G can interact with four inhibitory receptors, namely, immunoglobulin-like transcript (ILT)2, ILT4, KIR2DL4, and CD160, and this interaction leads to the inhibition of immune effector cell functions. The physiological role of HLA-G is to abrogate the maternal NK cell response against fetal tissue at maternal/fetal interface [3].

HLA-E presents peptides derived from the leader sequence of other HLA-class I molecules to NK cells, by interacting with the CD94/NKG2A receptor. When target cells express normal levels of HLA and consequently HLA-derived peptides are generated, this interaction takes place, leading to the inhibition of NK cell lysis. In contrast, cells with low to absent HLA-class I expression (i.e., tumor cells and virus-infected cells) generate low amounts of HLA-derived peptides, and consequently their surface expression of HLA-E is low. The loss of interaction between surface HLA-E and CD94/NKG2A provide a "kill" signal to NK cells [4].

HLA-G expression can be modulated by different cytokines, such as IL-10 [5, 6], IFN-γ [7], IFN-α [8], IFN-β [9], and TGF-β [10]. Similarly, modulation of HLA-E expression has been reported by different authors, in response to IFN-γ [11–16], TNF-α, and IL-1β [12]. In this context, a possible modulation of HLA-G and -E expression

by IL-27 may be interesting, since both immunostimulatory and immunoregulatory properties have been ascribed to this cytokine [17].

IL-27 belongs to the IL-12 family and is formed by EBI3 (also shared by IL35) and p28 (homologous to p35 and p40 subunits of IL-12) [18]. The IL-27 receptor (R) is composed by WSX-1 (also known as IL-27Rα/TCCR) and gp130 chains [19]. Signal transduction initiated by IL-27 involves the phosphorylation of STAT molecules, especially STAT-1 and -3, in different immune effector cells [18, 20–22]. Very recently, the IL-27 p28 subunit has been described as an independent cytokine, also known as IL-30, that may function in the absence of the EBI3 subunit [23, 24]. p28 exerts anti-inflammatory effects by acting as antagonist of gp-130 mediated signaling initiated by IL-6 or IL-27 [24]. In addition, IL-30 may signal independently through IL-6Rα, by recruiting gp130 homodimers [25].

In this paper, we report for the first time the modulation of a member of the HLA-class Ib molecule family by IL-27 in human monocytes, and we provide the first evidence of a differential effect of IL-27 and IL-30 on a specific human immune cell population.

2. Materials and Methods

2.1. Cell Isolation and Flow Cytometry. Peripheral blood samples were obtained from six different normal donors afferent to the blood bank of Istituto Giannina Gaslini, after written informed consent. Monocytes and NK cells were isolated using RosetteSep Human Monocyte Enrichment Cocktail and RosetteSep Human NK cells Enrichment Cocktail, respectively (StemCell Technologies), following manufacturer's protocol.

The expression of gp130, WSX-1, and IL-6Rα was evaluated on freshly isolated monocytes using the following monoclonal antibodies (mAbs): FITC-conjugated anti-gp130, PE-conjugated anti-WSX-1, and PE-conjugated anti-IL-6Rα (R&D Systems). Fluorochrome and isotype-matched irrelevant antibodies (Beckman Coulter) were used as negative control. Cells were stained for 20 min in the dark at 4°C and then washed in PBS (Sigma) supplemented with 1% FBS (Euroclone).

IL-27 and IL-30 driven signal transduction was analyzed on monocytes cultured for 30′ at 37°C and 5% CO_2 in the presence or absence of human recombinant (hr)IL-27 (R&D System, 100 ng/mL) or hrIL-30 (Abnova, 100 ng/mL), using FITC-conjugated anti-phospho (p)STAT1, anti-pSTAT3, and anti-pERK1/2 mAbs (Cell Signaling) following manufacturer's protocol.

HLA-G and -E expression was evaluated on monocytes cultured for 24 hours at 37°C and 5% CO_2 in the presence or absence of hrIL-27 (R&D System, 100 ng/mL) or hrIL-30 (Abnova, 100 ng/mL), using purified MEM-G9 (Exbio) and 3D12 (Biolegend) mAbs, respectively. Isotype-matched irrelevant antibody (Beckman Coulter) was used as negative control. Cells were stained for 20 min in the dark at 4°C and washed in PBS (Sigma) supplemented with 1% FBS (Euroclone). Cells were then incubated with PE-conjugated goat anti-mouse IgG1 (Beckman Coulter) as secondary reagent.

Cells were run on Gallios cytometer (Beckman Coulter). 10^4 events were collected. FACS analysis was performed using Kaluza software (Beckman Coulter). Data were expressed as mean relative of fluorescence intensity (MRFI = mean of fluorescence intensity obtained with specific antibody/mean of fluorescence intensity obtained with irrelevant isotype-matched antibody).

2.2. Degranulation Assay. Freshly isolated NK cells (10^5 cells) were cultured in RPMI-1640 medium (Euroclone) supplemented with 10% FBS (Euroclone) using round-bottom 96-well plates (Corning), in the presence or absence of target cells (K562 cell line) at 1 : 4 effector/target ratio. In some experiments, autologous monocytes (2.5×10^4 cells, pretreated or not with IL-27 or IL-30, as described above) were added as third-party cells. PE-conjugated anti-CD107a mAb (Miltenyi Biotec) was added to each well. Cells were incubated for 5 hours at 37°C and 5% CO_2. Cells were then washed and run on Gallios cytometer (Beckman Coulter). 10^4 events were collected. FACS analysis was performed using Kaluza software (Beckman Coulter). Data were expressed as percentage of $CD107a^+$ cells, gating on NK cells.

2.3. IFN-γ Release by NK Cells. Flat-bottom 96-well plates (Corning) were coated overnight at 4°C with 100 μL of anti-NKp46 activating receptor mAb (2.5 μg/mL, Miltenyi Biotec) or PBS. Plates were then washed 3 times in PBS. NK cells were plated at 10^5 cells/well in RPMI-1640 10% FBS supplemented with IL12p70 (R&D System, 0.1 ng/mL), in the presence or absence of autologous monocytes (2.5×10^4 cells, pretreated or not with IL-27 or IL-30, as described above). Cells were incubated at 37°C and 5% CO_2 for 24 hours. Supernatants were then collected and centrifuged at 3000 g for 10 minutes. IFN-γ was measured using IFN-γ ELISA set (Immunotools), following the manufacturer's protocol. Absorbance at 450 nm was measured using Infinite 200 PRO spectrometer (Tecan Group Ltd.). Results are expressed as ng/mL IFN-γ.

2.4. Statistical Analysis. Statistical analysis was performed using Prism Software (GraphPad Software Inc.). The normality of each variable was checked by using the Kolmogorov-Smirnov test. When normality of data distribution was found in all variables, statistical analysis was performed by a parametric approach. Conversely, when normality of data distribution was rejected in several variables, a nonparametric analysis was applied. Accordingly, t-test or Mann-Whitney test was used.

3. Results

3.1. Human Monocytes Expressed Complete and Functional IL-27R. The expression of gp130 and WSX-1 (the two subunits of IL-27R) and IL-6Rα (that serves as receptor for IL-30 in combination with gp130) was tested on freshly isolated human monocytes. As shown in Figure 1(a), monocytes expressed very high levels of gp130 (MRFI ± SD: 44.35 ± 8.69)

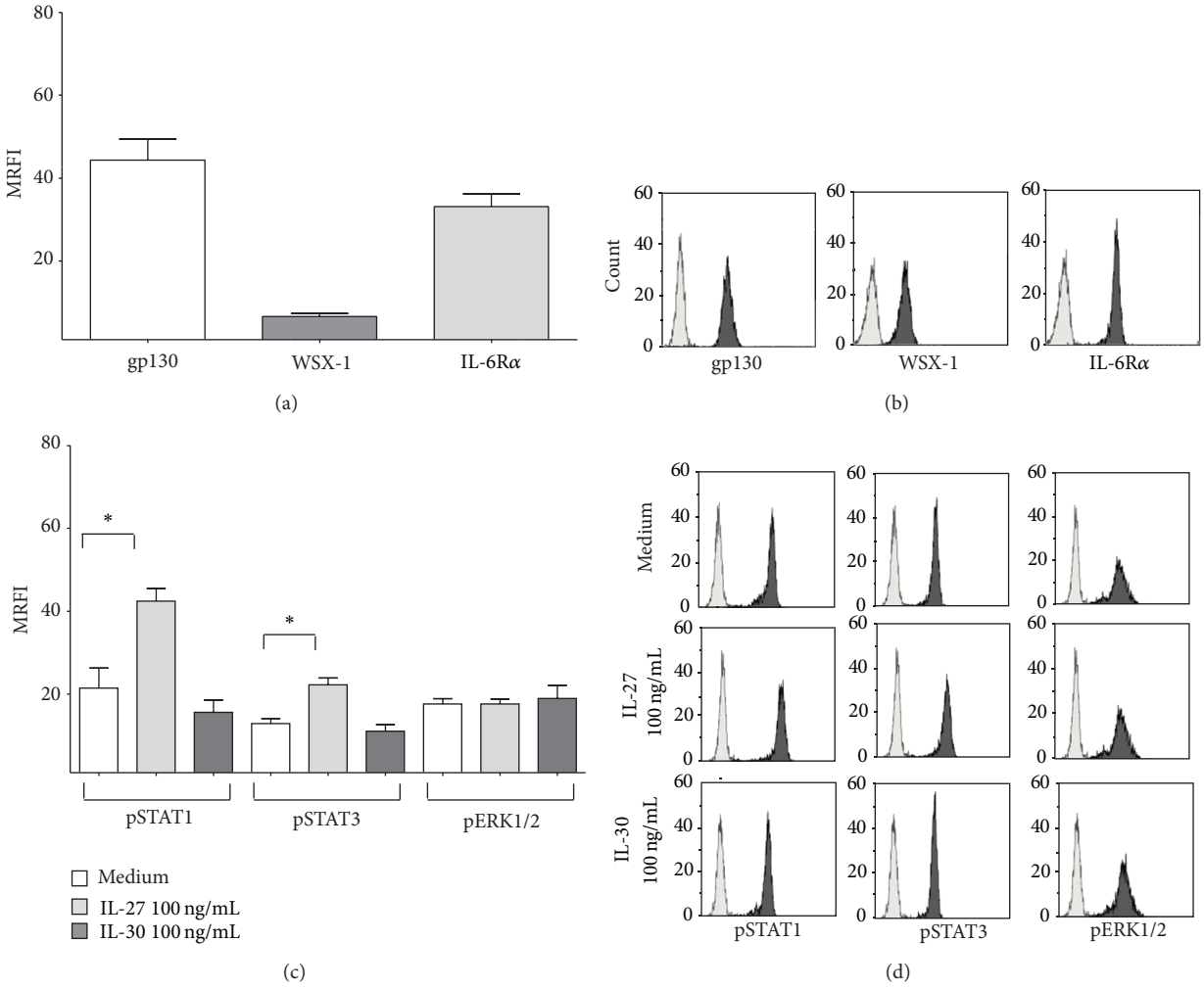

Figure 1: Expression and function of IL27R and IL30R in human monocytes. (a) Expression of gp130 (white bar), WSX-1 (grey bar), and IL6Rα (light grey bar) was evaluated by flow cytometry in freshly isolated monocytes. Results are represented as MRFI. Histograms represent mean of six different experiments ± SD. One representative FACS analysis is reported in (b). Grey profiles show staining with irrelevant fluorochrome and isotype matched mAb. Black profiles show staining with specific mAb. (c) STAT1, STAT3, and ERK1/2 phosphorylation was investigated in monocytes cultured with medium alone (white bars), 100 ng/mL IL-27 (light grey bars), or 100 ng/mL IL-30 (grey bars). Results are represented as MRFI. Histograms represent mean of six different experiments ± SD. Asterisks indicate statistical significance. One representative FACS analysis is reported in (d). Grey profiles show staining with irrelevant fluorochrome and isotype matched mAb. Black profiles show staining with specific mAb.

and IL6Rα (MRFI ± SD: 33.11 ± 5.37), whereas WSX-1 expression (MRFI ± SD: 6.56 ± 1.33) was lower. A representative FACS analysis is shown in Figure 1(b).

Next, we asked whether IL-27R and IL-30R expressed by human monocytes were functional. As shown in Figure 1(c), IL-27 treatment increased the phosphorylation of STAT1 (MRFI ± SD: medium 21.44 ± 8.32; IL-27 42.45 ± 5.26; $P = 0.05$) and STAT3 (MRFI ± SD: medium 12.85 ± 2.1; IL-27 22.2 ± 2.86; $P = 0.05$), but not of ERK1/2. Conversely, no significant upregulation of pSTAT1, pSTAT3, or pERK1/2 was observed after treatment of monocytes with IL-30. A representative FACS analysis is shown in Figure 1(d).

Thus, taken together, these data suggested that IL27R, but not IL30R, was functional in human monocytes. Another possible explanation is that IL-30 driven signaling may be mediated by the activation of additional pathways that do not include STAT or ERK molecules, as reported in other human immune cells.

3.2. IL-27 Treatment Induced Surface HLA-E Upregulation on Monocytes. We have next investigated whether IL-27 or IL-30 may modulate the surface expression of two HLA-class Ib molecules on human monocytes. As shown in Figure 2(a), IL-27 treatment significantly upregulated HLA-E (MRFI ± SD: medium 4.33 ± 1.95; IL-27 9.75 ± 3.31; $P = 0.01$) but not HLA-G expression on monocytes. In contrast, no modulation of HLA-G and -E expression was observed after treatment of monocytes with IL-30. A representative FACS analysis is shown in Figure 2(b).

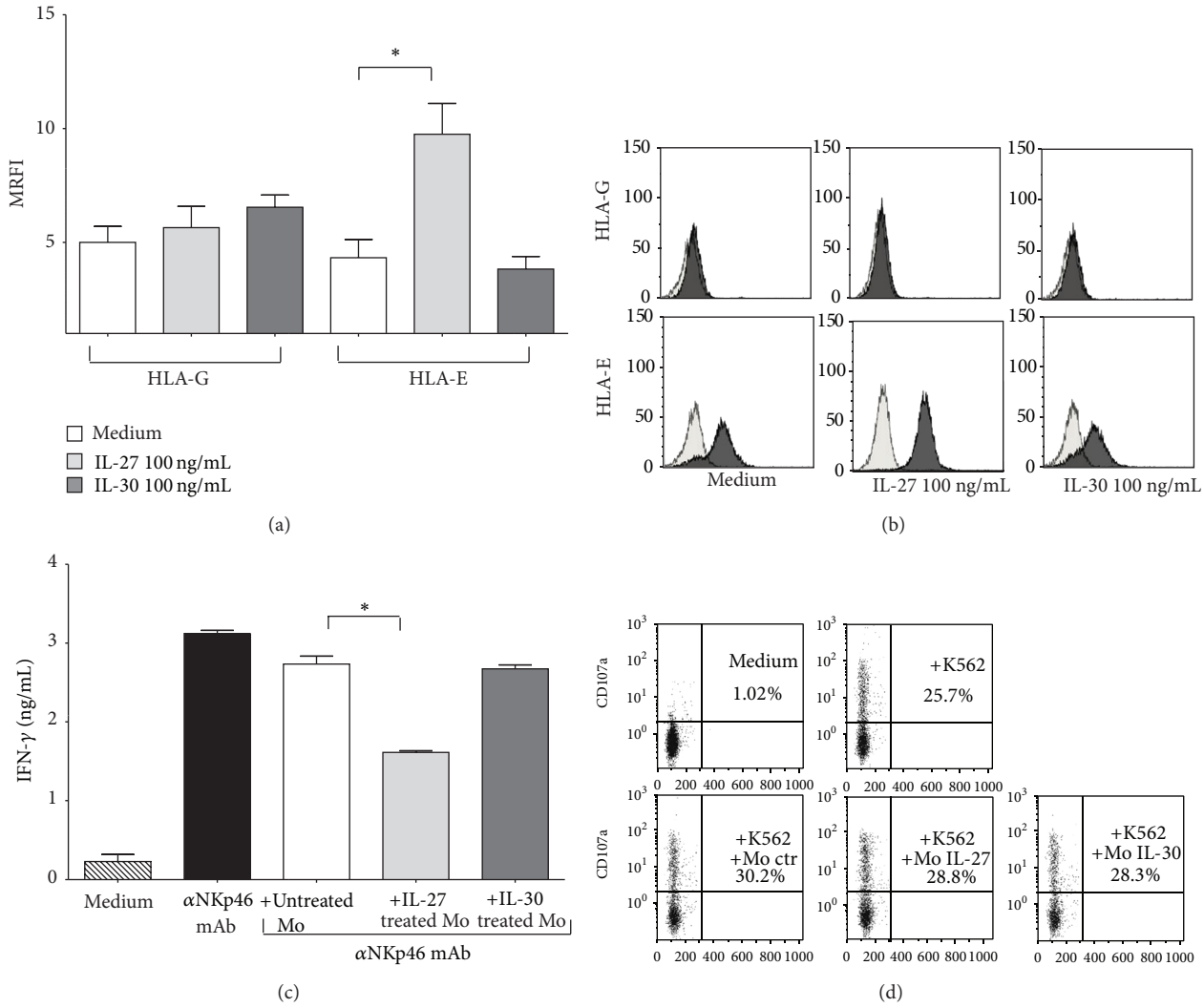

Figure 2: Modulation of HLA-G and HLA-E expression in human monocytes and functional assays on NK cells. (a) HLA-G and -E expression was investigated in monocytes cultured with medium alone (white bars), 100 ng/mL IL-27 (light grey bars), or 100 ng/mL IL-30 (grey bars). Results are represented as MRFI. Histograms represent mean of six different experiments ± SD. Asterisks indicate statistical significance. One representative FACS analysis is reported in (b). Grey profiles show staining with irrelevant fluorochrome and isotype matched mAb. Black profiles show staining with specific mAb. (c) IFN-γ was analyzed in supernatants from NK cells cultured with (i) medium alone (stripped bar), (ii) in the presence of coated anti-NKp46 mAb (black bar) or in the presence of coated anti-NKp46 mAb and autologous monocytes, (iii) untreated (white bar), (iv) pretreated with IL-27 (light grey bar), or (v) pretreated with IL-30 (grey bar). Results are represented as ng/mL IFN-γ. Histograms represent mean of six different experiments ± SD. Asterisks indicate statistical significance. (d) Expression of CD107a was evaluated on NK cells cultured with (i) medium alone, (ii) target cells (K562) at 4 : 1 effector : target ratio, and target cells (K562) in the presence of autologous monocytes, (iii) untreated, (iv) pretreated with IL-27, or (v) pretreated with IL-30. One representative experiment out of three performed is shown. The percentage of CD107a$^+$ cells (gating on NK cells using physical parameters) is indicated.

3.3. IL-27 Treated Monocytes Inhibited IFN-γ Release by NK Cells but Not Cytotoxicity. Finally, we have investigated whether the IL-27 driven upregulation of HLA-E on monocytes may functionally impair autologous NK cell function. To this end, we have investigated IFN-γ secretion and cytotoxic activity of NK cells cultured in the presence or absence of autologous monocytes, previously treated with medium alone, IL-27, or IL-30.

IFN-γ secretion was investigated on supernatants from NK cells cultured in different experimental conditions. As shown in Figure 2(c), IFN-γ secretion was low to absent in NK cells cultured with medium alone (mean ng/mL ± SD: 0.22 ± 0.18), whereas NK cells cultured in the presence of coated anti-NKp46 agonist mAb secreted high amounts of IFN-γ (mean ng/mL ± SD: 3.12 ± 0.08). Such secretion was significantly reduced when NK cells were cocoltured with autologous monocytes pretreated with IL-27 (mean ng/mL ± SD: 1.61 ± 0.04, $P = 0.01$), but not with untreated (mean ng/mL ± SD: 2.73 ± 0.19) or IL-30 pretreated (mean ng/mL ± SD: 2.67 ± 0.1) monocytes.

Cytotoxic activity of NK cells was next assessed by investigating the secretion of lytic granules in response to target cells, witnessed by CD107a expression on the cell surface. NK cells cultured with medium alone displayed a low to absent degranulation (% $CD107a^+$ cells ± SD: 0.81 ± 0.29) that was dramatically increased in the presence of K562 cell line (% $CD107a^+$ cells ± SD: 29.21 ± 4.95). NK cells preserved the ability to secrete lytic granules in response to K562 cell line when cultured in the presence of autologous monocytes pretreated with IL-27 (% $CD107a^+$ cells ± SD: 34.06 ± 7.43), IL-30 (% $CD107a^+$ cells ± SD: 30.35 ± 2.77), or medium alone (% $CD107a^+$ cells ± SD: 31.47 ± 1.73). A representative FACS analysis is shown in Figure 2(d).

Our results indicated that IL-27 treated monocytes that upregulated surface HLA-E expression were able to dampen IFN-γ secretion by activated autologous NK cells, probably through the interaction of HLA-E with the inhibitory receptor CD94/NKG2A expressed on NK cells. Conversely, such interaction was not sufficient to inhibit the release of cytotoxic granules by NK cells in the presence of HLA-class I deficient target cells.

4. Discussion

The role of HLA-class Ib molecules in the control of the immune system has been clearly described in the last years [26, 27]. Surface and soluble HLA-G molecules abrogate the function of different immune effector cells, such as NK cells, T cells, and B cells, through different mechanisms [3]. A high expression of HLA-E on the surface of target cells may protect them from NK cell mediated lysis [28], and this feature is commonly used as immune escape mechanism by virus-infected cells [15] or tumor cells [29]. In addition, several authors have demonstrated that cells with high surface HLA-E expression may modulate other NK cell functions through the interaction with CD94/NKG2A on NK cells, for example during trophoblast recognition by decidual NK cells [30].

No information is available regarding a possible modulation of HLA-G and -E expression by IL-27. It has been previously reported that IL-27 exerts a proinflammatory activity on human monocytes, inducing an augmented response to TLR signals [31, 32] and the release of proinflammatory cytokines and chemokines [33] mainly through STAT1, STAT3, and NF-κB activation.

In line with these data, we have here demonstrated that in monocytes IL-27 signals through STAT1 and STAT3 phosphorylation, whereas ERK pathway was unaffected by IL-27 treatment. In addition, we have demonstrated for the first time that IL-30 displays a different behavior, since molecules involved in IL-27 driven signaling pathway were unaffected by IL-30. It is conceivable that additional pathway(s) other than that of STAT and ERK might be involved in IL-30 signaling in monocytes. Another possible explanation may be related to a defective function of IL-30R on monocytes.

We have here provided the first demonstration that IL-27 treatment upregulated HLA-E (but not HLA-G) expression on the cell surface of human monocytes. The upregulation of this immunoregulatory molecule on the latter cells is apparently in contrast with the literature, since different studies have demonstrated a proinflammatory activity of IL-27 on human monocytes [31–33]. However, in the last years, several evidences support the concept that this cytokine may function either as proinflammatory or immunoregulatory factor [34]. In addition, we have very recently demonstrated that IL-27 may act as homeostatic cytokine, limiting the duration and the intensity of adaptive immune response by inhibiting the function of immature dendritic cells [35]. In this view, we may hypothesize that the upregulation of HLA-E on monocytes may function as a negative feedback signal to NK cells, leading to decreased IFN-γ secretion by the latter cells that may in turn limit their helper function and consequently the activation of other immune effector cells. In support of this hypothesis, we have previously demonstrated that HLA-E is upregulated in peripheral blood and synovial monocytes from patients affected by juvenile idiopathic arthritis [36], thus suggesting that HLA-E upregulation takes place during autoimmune and inflammatory conditions.

5. Conclusions

In conclusion, we delineated a novel potential mechanism of action for IL-27 that may regulate some NK cell functions indirectly through the upregulation of surface HLA-E on human monocytes. Future studies on patients affected by different autoimmune and inflammatory diseases will help to clarify whether this feature may be relevant in physiological and pathological settings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Irma Airoldi and Vito Pistoia equally contributed as last authors.

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Review Article

The Many Faces of Human Leukocyte Antigen-G: Relevance to the Fate of Pregnancy

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Pregnancy is an immunological paradox, where fetal antigens encoded by polymorphic genes inherited from the father do not provoke a maternal immune response. The fetus is not rejected as it would be theorized according to principles of tissue transplantation. A major contribution to fetal tolerance is the human leukocyte antigen (HLA)-G, a nonclassical HLA protein displaying limited polymorphism, restricted tissue distribution, and a unique alternative splice pattern. HLA-G is primarily expressed in placenta and plays multifaceted roles during pregnancy, both as a soluble and a membrane-bound molecule. Its immunomodulatory functions involve interactions with different immune cells and possibly regulation of cell migration during placental development. Recent findings include HLA-G contributions from the father and the fetus itself. Much effort has been put into clarifying the role of HLA-G during pregnancy and pregnancy complications, such as preeclampsia, recurrent spontaneous abortions, and subfertility or infertility. This review aims to clarify the multifunctional role of HLA-G in pregnancy-related disorders by focusing on genetic variation, differences in mRNA stability between *HLA-G* alleles, differences in HLA-G isoform expression, and possible differences in functional activity. Furthermore, we highlight important observations regarding *HLA-G* genetics and expression in preeclampsia that future research should address.

1. Introduction

The human leukocyte antigen (HLA)-G is a HLA class Ib protein, which in contrast to the highly polymorphic classical HLA molecules shows limited polymorphism and restricted tissue distribution and has a unique alternative splice pattern [1–3].

HLA-G is expressed as several different splice variants including four membrane-bound (HLA-G1 to -G4) and three soluble isoforms (HLA-G5 to -G7). In addition, both membrane-bound β2-microglobulin (β2m)-linked and free dimers, membrane bound β2m-free heavy chains, and possibly soluble β2m-free dimers have been reported [1, 4–6]. Approximately 50 *HLA-G* alleles corresponding to 16 HLA-G proteins have been reported (The IMGT database; Nov. 2013). Investigations of *HLA-G* genetics in relation to risk of certain pregnancy complications have increased during recent years [7–9].

The expression of HLA-G was first described in placenta during pregnancy as the conventional β2m-linked membrane-bound form, and subsequently several other isoforms have been reported in this compartment [1, 10]. To date, there is a good amount of evidence to support that the extra-villous trophoblast (EVT) cells express membrane-bound HLA-G1, soluble HLAG-5/-G6, and possibly other isoforms, whereas the HLA-G5 and HLA-G2/-G6 expression in villous trophoblast (VT) and syncytiotrophoblast (ST) cells have been proposed but are still a matter of controversy [6, 11, 12]. Furthermore, membrane-bound HLA-G1 can be shed from the membrane and released as soluble HLA-G1 [13]. In addition to placental expression, soluble HLA-G (sHLA-G) has been detected in peripheral blood from men, pregnant and nonpregnant women, follicular fluid, fertilized oocytes, and in male reproductive tissues including semen [14–18]. It is still not clear, however, exactly which of the isoforms of HLA-G that are responsible for the immunomodulatory functions during pregnancy.

HLA-G has been shown to be a ligand for the immune receptors immunoglobulin-like transcript 2 (ILT-2), ILT-4,

and the killer immunoglobulin-like receptor 2 (KIR2DL4) [19–22]. The immunomodulatory effects that result from these interactions include inhibition of T cells, inhibition of NK cell proliferation and cytotoxic functions, enhancement of the generation of regulatory T cells, inhibition of the differentiation of antigen-presenting cells (APC), and alterations in cytokine secretion [23, 24].

During pregnancy, immunoregulatory functions must be initiated to secure acceptance of the semiallogenic fetus. The fetus displays antigens inherited from the father on the cell surfaces. Originally, the maternal acceptance of the fetus was explained partly by the fact that trophoblast cells lack expression of the highly polymorphic classical HLA-A and -B molecules and partly because the immune system shifted from a T helper 1 (Th1) cytokine profile towards a Th2 cytokine profile [33, 34]. However, this would generate a problem in relation to natural killer (NK) cell-mediated lysis of cells lacking HLA expression; uterine NK cells constitute a large part of the immune cells in the uterine compartment [35]. This dilemma was overcome, when the expression of nonpolymorphic HLA class Ib molecules, HLA-E, -F, and -G, on EVT cells, was discovered and explored. EVT cells also express HLA-C, apparently in low amount [36]. The restricted tissue distribution of HLA-G and its immunomodulatory functions have generated much effort into clarifying the function of HLA-G during pregnancy, and which role HLA-G might have in pregnancy complications, such as preeclampsia, recurrent spontaneous abortions, and subfertility or infertility.

Preeclampsia is a pregnancy disorder that can be roughly divided into two stages [37]. The first is characterized by the trophoblast migration into decidua creating the first materno-fetal interface, and the second is when the syncytiotrophoblast comes into direct contact with maternal blood. The disorder affects 2–7% of all pregnancies in varying degree from mild hypertension, proteinuria, and oedema to kidney and liver dysfunction, impairs the blood coagulation system, and in worst cases cerebral haemorrhage [8]. The pathology of this disorder is still unknown, but it has been proposed that preeclampsia evolves from dysfunctional immunoregulation. As a first step, during first trimester, it has been hypothesised that lack of an adequate immunological response may lead to failure of trophoblast invasion and failure of spiral arteries remodelling, resulting in poor blood supply and possibly hypoxia in the placenta. At the second step during second trimester, where signs of preeclampsia are recognizable in the maternal organ systems, it has been difficult to identify the triggering factors. Several studies have linked *HLA-G* genotypes and aberrant HLA-G protein expression to preeclampsia; however, other studies have not obsered any significant associations [38–41]. In addition, several studies have linked certain *HLA-G* genotypes and aberrant HLA-G protein expression to the risk of recurrent spontaneous abortions (RSA) [9].

This review aims to clarify the role of HLA-G in pregnancy-related disorders such as preeclampsia by focusing on genetic variation, differences in mRNA stability between different *HLA-G* alleles, differences in HLA-G isoform protein expression, and possible differences in receptor interactions and functional activity. The review will also highlight important and partly conflicting observations regarding *HLA-G* genetics and HLA-G expression in preeclampsia that further research needs to address.

Table 1: A comparison of differences in *HLA-G* gene and protein expression and functional characteristics between the *14 bp deletion* and *14 bp insertion* alleles. The observed differences may be influenced by other DNA and mRNA polymorphisms in the 3′-untranslated region (3′UTR), especially the *+3142* and *+3187* SNPs, and/or the 5′-upstream regulatory region of HLA-G that are in linkage disequilibrium with the *14 bp ins/del* polymorphism (see the text and Figure 1 for details). (Based on a large number of references listed in the text).

	14 bp deletion HLA-G allele	*14 bp insertion HLA-G* allele
Alternative splicing of *HLA-G* mRNA that includes a deletion of 92 bp of the 3′UTR	No	Yes
Levels of *HLA-G* mRNA (not including the 92 bp splice variants)	+++	++
Levels of soluble HLA-G1	?	?
Levels of HLA-G5 during pregnancy*	+	++
Levels of soluble HLA-G in blood plasma from healthy nonpregnant donors**	+++	++
Membrane-bound expression of HLA-G1*	++	+++
HLA-G mRNA stability	+	+++
Inhibition of NK cytotoxicity*	+	++

*Only one or very few studies; needs further verification.
**β2-microglobulin-associated soluble HLA-G1 and HLA-G5.

2. *HLA-G* Polymorphisms in relation to Alternative Splicing and Protein Expression

Given the immunoregulatory functions of HLA-G, studies have focused on possible gene variations influencing HLA-G expression. Harrison et al. reported a *14 bp insertion/deletion* (*14 ins/14 del*) polymorphism in the 3′-untranslated region (3′UTR) of the *HLA-G* gene at position +2961 (Figure 1) [42]. A summary of the most important known differences in posttranscriptional processing, protein expression, and functional activity of the *14 del HLA-G* allele and *14 ins HLA-G* allele is depicted in Table 1 and Figure 2.

In the current review, the positions of polymorphisms in the 3′UTR of the *HLA-G* gene are listed as in Castelli et al. [27]. These positions differ by 15 nucleotides after the *14 bp ins/del* polymorphism when compared to the original published *HLA-G* gene sequence by Geraghty et al. [26]. Nucleotide +1 is the adenine (A) of the first translated ATG. Furthermore, *HLA-G* allele nomenclature is in the current review listed according to the WHO nomenclature versions (The IMGT database) used in the specific original publications reviewed, because conversions to the current nomenclature can be difficult and inaccurate.

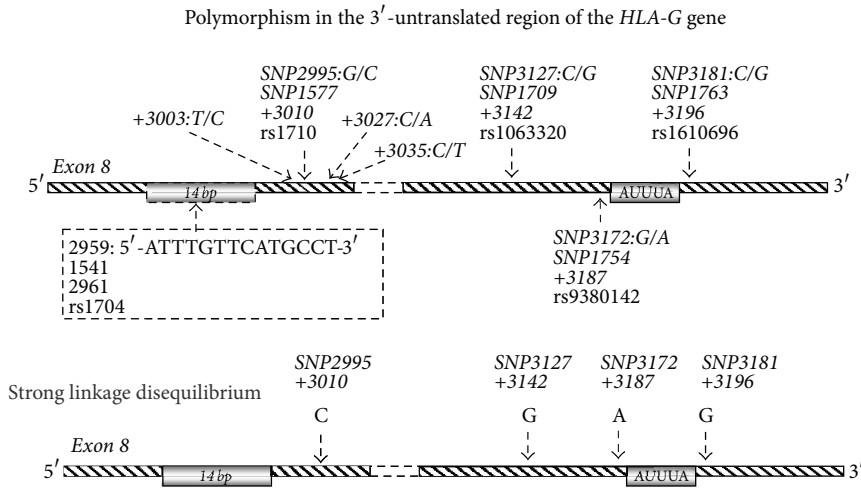

Figure 1: Polymorphisms in the 3′-untranslated region (3′UTR) of the *HLA-G* gene. The 3′UTR haplotype shown below has been associated with the risk of developing severe preeclampsia [25]. (Nomenclature used by different authors is shown for clarification. "*SNPxxxx*," for example, *SNP3127*, is based on the original publication of the HLA-G gene sequence by Geraghty et al. [26] and the study of HLA-G 3′UTR haplotypes in cases of severe preeclampsia by Larsen et al. [25]; "*+xxxx*," for example, *+3142*, by the publication by Castelli et al. [27]. (Based on [25, 27, 28])).

The *14 ins* allele has been associated with significantly lower *HLA-G* mRNA levels in first trimester trophoblast cells compared to the *14 del* allele [30, 41]. Furthermore, studies have shown unique alternative splice patterns in relation to the *14 ins* allele, where 92 bp are spliced out due to an introduction of a cryptic branchpoint not found in the *14 del* allele [1, 30]. This alternative splicing was more prominent in the *G∗010103* (*G∗01:01:03:0x*) allele compared to the *G∗010102* (*G∗01:01:02:0x*) allele, and interestingly, homozygous individuals carrying the *G∗010103* allele showed the same HLA-G1 expression as the high secretor *G∗010101* (*G∗01:01:01:0x*) allele, and even higher expression of HLA-G2/-G4. Another study found that, when the 92 bp is deleted, a more stable *HLA-G* transcript is obtained [31]. This has led to a hypothesis that the deletion of 92 bp might be a compensatory mechanism in individuals carrying the *14 ins* allele in an attempt to increase sHLA-G protein expression.

In placental tissue, a reduced expression of HLA-G has been linked to preeclampsia, but these studies did not differentiate between membrane-bound HLA-G and sHLA-G; and most of these studies did not examine a possible association with the *14 bp* polymorphism [40, 41, 43, 44]. A number of studies have reported an association between fetuses carrying the *14 ins* allele and/or the *14 ins/14 ins* HLA-G genotype and risk of preeclampsia [25, 38, 41, 45, 46]. On the other hand, some studies have not observed any association [39].

Several studies have found that the *14 ins* allele is associated with decreased levels of soluble HLA-G in blood plasma [47–50], while a few studies found no association [51, 52]. In most of the studies, sHLA-G has been determined with a commercially available sHLA-G enzyme-linked immunosorbent assay (ELISA) kit (Exbio, Praha), based on the capture antibody MEMG/9, capturing sHLA-G1/-G5 in association with β2m and a detecting antibody against β2m. Interestingly, the study by Wu et al., who failed to report an association, used a different ELISA assay with a higher limit of detection, compared to the Exbio kit. This could account for the differences in results, whereas there is no obvious explanation to the reported lack of association in the study by Zhang et al. examining children with atopic asthma and positive controls. In a study by Rizzo et al., sHLA-G1 and HLA-G5 were determined by performing two different ELISA assays: one capturing both sHLA-G1 and HLA-G5 and one capturing only HLA-G5 by the use of the monoclonal antibody (mAb) 5A6G7, which is specific for HLA-G5/-G6 [53]. Surprisingly, this showed that women with severe preeclampsia had significantly higher levels of soluble HLA-G5 than women with uncomplicated pregnancies. In addition, there was a trend towards lower sHLA-G1 expression in women with severe preeclampsia [53]. This is in accordance with a study of *HLA-G* mRNA expression in preeclampsia, in which a high expression of *HLA-G5* mRNA was detected in preeclampsia compared to controls [54]. Also, it is partly in accordance with another study measuring HLA-G5 levels in blood plasma of pregnant women in relation to the *14 bp* polymorphism. This study showed no difference in HLA-G5 levels between pregnant women and control women, and levels of HLA-G5 did not seem to change during pregnancy. However, HLA-G5 levels were higher among women carrying the *14 ins* polymorphism either displaying heterozygosity or homozygosity than women without the *14 ins* allele [55].

In addition to the *HLA-G 14 bp* polymorphism, other regions of the *HLA-G* gene have been studied for possible association with HLA-G protein expression. The null allele *G∗01:05N* is defined by a single nucleotide deletion polymorphism in codon 130 (also known as *1597C*) in exon 3 leading to a frameshift mutation resulting in abnormal full-length HLA-G1 and -G5 isoforms. However, other alternatively

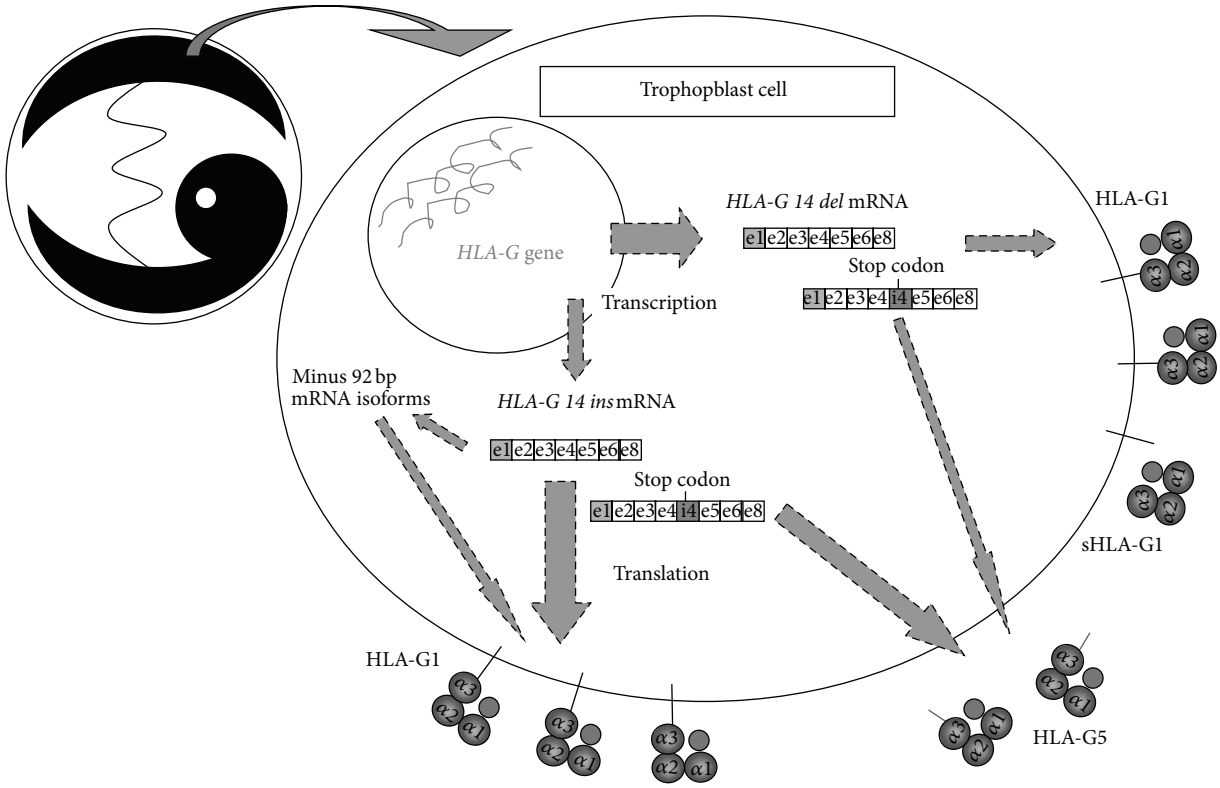

Figure 2: A simplified illustration of current status regarding the dynamic differences in transcription, mRNA stability, and translation in (extravillous) trophoblast cells between the *14 bp del* and *14 bp ins HLA-G* alleles. Only the full-length mRNA isoforms are shown for clarity. The relative thicknesses of the specific arrows are putative and should be interpreted with caution. The effect of the *HLA-G 14 bp ins/del* polymorphism in the 3′-untranslated region (3′UTR) on HLA-G expression may be influenced by linkage disequilibrium with single nucleotide polymorphisms (SNPs) in the 3′UTR, especially the *+3142* and *+3187* SNPs according to one study [29], and in the 5′-upstream regulatory region. (Based primarily on [1, 30–32]).

spliced HLA-G isoforms lacking exon 3 are generated, and some studies support that these isoforms might also possess functional capacity resembling the function of the full length proteins [56]. This is further supported by recent experiments with synthetic HLA-G protein variants [57]. One study found that sHLA-G levels in healthy pregnant women were significantly lower in women carrying this *1597C* deletion mutation [58]. In addition, it was shown that the deletion mutation was more frequent in a group of preeclamptic women compared to normal controls.

Recently, increasing interest has been drawn towards examining single nucleotide polymorphisms (SNPs) in the 3′UTR region of the *HLA-G* gene. One study examined a C/G SNP at position +3142 in relation to microRNAs (miRNAs) [59]. MicroRNAs are noncoding single-stranded RNAs modulating gene expression by targeting mRNA. Based on thermodynamic calculations, the authors hypothesized that the binding of miRNA would be more stable in genotypes carrying a G at position +3142 compared to genotypes carrying a C. The sHLA-G level in JEG-3 cells expressing the *+3142G* SNP was markedly decreased after transfection with miRNA-148a. Although no comparison with the *3142C* genotype was made, this study indicates that *+3142G* might be associated with decreased expression of HLA-G [59]. Yie et al. found that a C/A SNP at position +3187 of exon 8 resulted in reduced half-life of mRNA-transcripts [60]. Interestingly, the *14 ins* allele is in linkage disequilibrium with both the *+3142G* and the *+3187A* SNPs. This illustrates that linkage disequilibrium in the *HLA-G* gene region plays a pivotal role. Other studies have identified more SNPs in the 3′UTR as well [27, 28, 61]. Larsen et al. studied *HLA-G* 3′UTR polymorphism in severe preeclamptic cases and controls in a North European population [25]. Castelli et al. showed that SNPs in the 3′UTR are target sites for different miRNAs [61]. In the Brazilian population, Castelli et al. tried to characterize polymorphisms and examine the linkage disequilibrium between them. Interestingly, they were able to group the gene variations into eight different UTR haplotypes [62]. *UTR-1* and *UTR-2* were the most frequently distributed, accounting for 52% of the haplotypes. On this basis, Di Cristofaro et al. examined the correlation of the UTR haplotypes with sHLA-G expression and found that *UTR-1* homozygous individuals displayed high secretion, whereas individuals homozygotic for the *UTR-2* haplotype were low secretors [63]. Furthermore, this study found that the highest secretors were the *UTR-5*, regardless of whether individuals were homozygous or heterozygous. In a recent study, however, *UTR-5* was found to be a low

secretor [29]. A possible explanation to these contradictory results is that 5′URR polymorphisms affecting expression that are associated with the *UTR-5* haplotype may vary among populations. Furthermore, although the ELISA in the two studies was both based upon capture of sHLA-G1/HLA-G5 with the MEM-G/9 mAb, the study by Martelli-Palomino et al. used an in-house ELISA with a HLA-G5 protein as standard and tested blood *plasma* samples, while the study by Di Cristofaro et al. used the commercial Exbio sHLA-G kit with no well-defined standard and tested blood *serum* samples [29, 63]. Serum samples have been shown to be less reproducible in relation to sHLA-G measurements [64]; however, differences in sHLA-G concentrations may turn out more pronounced when analysing serum samples. These technical discrepancies may also partly explain differences in results between the two studies. Future clarifying studies should investigate blood plasma and a large number of samples. The study by Martelli-Palomino et al. showed that *UTR-1 (14 del/+3142C /+3187G)* is a high secretor, whereas *UTR-5* and *UTR-7 (14 ins/+3142G/+3187A)* are low secretors, while the rest of the haplotypes show intermediate sHLA-G levels [29]. *UTR-1* includes the *14 del* allele. This is in accordance with previous studies showing that the *14 ins* sequence and *+3142G* are associated with lower HLA-G expression [50, 65, 66]. The *14 del* allele is also found in *UTR-3*, *UTR-4*, and *UTR-6*, but all of these have the *+3187A* allele, whereas *UTR-1* displays the *+3187G* allele, which might also contribute to *UTR-1* showing higher sHLA-G expression than the other haplotypes. Of interest is also the fact that the *G∗01:05N* allele and the *G∗01:06* are derived from the *UTR-2* lineage, which was shown to be a low or intermediate secretor. This is in accordance with the study by Loisel et al. examining the *G∗01:05N* allele, as discussed above, and a study by Moreau et al. linking the *G∗01:06* fetal genotype to preeclampsia [45, 58]. In addition, the *UTR-2* is the only haplotype displaying a G at position +3196, whereas the other haplotypes display a C. From this it can be speculated that the *+3196G* allele might be a binding site for miRNA or the target of other regulatory mechanisms affecting HLA-G expression. No studies have linked this SNP to sHLA-G expression yet, and curiously no miRNA binding sites were found at positions +3187 and +3196 when testing with an array of miRNAs identified by affinity calculations, whereas binding sites were localized at positions +3003, +3010, +3027, +3035, and +3142 and at the 14 bp ins/del polymorphic site [61]. However, these two SNPs are located in close proximity to an AUUUA-pentamer sequence (Figure 1). Such AU-rich elements (AREs) have been described in the 3′UTR of labile mRNAs encoding, for example, cytokines. Therefore, sequence variation close to AREs may influence mRNA stability [67, 68].

At present, it seems that the *14 ins* polymorphism and the *+3142* SNP are the most important gene variations independently correlated with HLA-G protein expression. Also, a consensus can be made in the direction that classification based only on the *14 bp HLA-G* polymorphism will result in low-to-medium sHLA-G secretors in healthy donors with *14 ins/14 ins* genotypes and medium-to-high secretors for *14 del/14 del* genotypes.

3. Are There Any Functional Differences between Membrane-Bound and Soluble HLA-G?

Accumulating evidence suggests that the membrane-bound HLA-G1, the shedded sHLA-G1, the soluble HLA-G5, and possibly other isoforms might exhibit different functions during pregnancy. Early studies have not been able to support this because no antibodies have been available for distinguishing HLA-G isoforms. Given that shedded sHLA-G1 levels seem to be lower in blood plasma of pregnant women with severe preeclampsia in late pregnancy, as discussed earlier, it can be speculated that HLA-G1 might be the most important source of HLA-G in the pathogenesis of preeclampsia. However, as this effect is observed in late pregnancy, where the interface involving the VT and ST cells seems to play the most important role, the results are puzzling. HLA-G5 might be the most important isoform in the uterine compartment, although this is controversial. It might also be that the sHLA-G1 released during second trimester has important interactions with maternal peripheral immune cells, thereby inducing tolerance to the fetus. The role of circulating HLA-G5, though, still remains to be elucidated. A recent study based on HLA-G sequences transduced into K562 cells examining differences between the *14 ins* and the *14 del* alleles actually found that membrane-expression of HLA-G1 was higher in the *14 ins* transfectants than in the *14 del* transfectants. On the contrary, the 14 del allele showed higher secretion rates of the shedded HLA-G1 than the *14 ins* allele. Furthermore, it was shown that the *14 ins* transfectants were more efficient in inhibiting NK cytotoxicity than the *14 del* transfectants in accordance with a high HLA-G1 expression [32].

In the male reproductive system, HLA-G5 seems to be the central molecule. Our group and Langat et al. have detected HLA-G5 in tissues such as the testis, the epididymis and the prostate gland, and sHLA-G expression in seminal plasma [16, 17]. Levels of sHLA-G in blastocyst media from *in vitro* fertilization (IVF) have also been examined, and it has been shown that high sHLA-G levels correlate well with fertility success [69, 70]. However, these studies did not differentiate between HLA-G isoforms and no studies have tried to correlate HLA-G expression in blastocysts and in IVF media to genetic variations. One study did, however, use an antibody stated to capture β2m-free HLA-G molecules (4H84) [71], but cross reactivity with other class I molecules has been reported using this mAb, questioning these results [72].

As described above, HLA-G has been found to interact with the immune receptors ILT-2, ILT-4, and KIR2DL4. ILT-2 is expressed on the surface of a wide variety of immune cells including NK cells, $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and monocytes, whereas ILT-4 is predominantly expressed on the surface of APCs such as macrophages, monocytes, and dendritic cells [22]. Another receptor for HLA-G is KIR2DL4, and up until now HLA-G has been thought to be the only known ligand for this receptor; however, this was recently challenged [73]. KIR2DL4

is mainly expressed on $CD56^{bright}$ NK cells, the major proportion of NK cells in the uterus, whereas this cell type is almost exclusively absent in the pool of NK cells circulating in peripheral blood [35]. It was shown that membrane-bound HLA-G induced inhibition of uterine NK cell-mediated cytolysis through KIR2DL4, whereas peripheral NK cells were almost devoid of this receptor and conversely did not show inhibition of cytolysis [74, 75]. However, inhibition of peripheral NK cell cytotoxicity by HLA-G1 in an EVT cell line has been demonstrated. Additionally, KIR2DL4 surface expression was upregulated when cocultured with the HLA-G positive TEV1-cell line [76, 77]. Interestingly, KIR2DL4 has not yet been shown to interact with soluble HLA-G although a concept of endosomal signalling between these two has been suggested [78]. Intriguingly, a woman with several successful pregnancies has been identified, homozygous for a genotype not encoding KIR, stating that the interaction is not fundamental for successful pregnancy [79]. On the contrary, it has been shown that the expression of KIR2DL4 on the surface of uterine NK cells was higher in fertile women than among RSA women, indicating that the interaction between membrane-bound HLA-G and KIR2DL4 may favour induction of tolerance at the materno-fetal interface [80]. Moreover, shedded HLA-G has also been found to have the capacity to prevent NK-mediated cell lysis in a sHLA-G transfected HLA-negative cell line [81]. This study did not characterize any receptor interactions, making it difficult to determine whether the inhibitory effect is due to interaction with KIR2DL4, or alternatively another immune receptor such as ILT-2. One study indicated that HLA-G5 is more potent than HLA-G1 in inhibiting NK cell-mediated lysis, when HLA-G1 and -G5 transfectants were studied in K562 cells. However, the combination of HLA-G1 and -G5 had a significantly additive effect on the inhibition of NK cytotoxicity [82]. A recent study shows that the KIR2DL4 receptor also has the potential of acting through its activating motif. In a transfection study, it was shown that cytotoxicity of NK cells towards a cell line could actually be induced by the receptor interaction of KIR2DL4 and the unconventional β2m-free HLA-G isoforms. In addition proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ were expressed [83]. Since some studies point towards that β2m-free HLA-G isoforms are expressed at the fetomaternal interface, one could speculate that these proinflammatory cytokines participate in angiogenesis leading to vascular remodelling and migration of the trophoblast. This is in accordance with another study showing that sHLA-G does not affect the cytolytic activity of uterine mononuclear lymphocytes but induces IFN-γ secretion in both uterine and peripheral NK cells [84]. However, these findings are in direct contrast to the traditional view of pregnancy as an immunological shift from a Th1 to a Th2 response. Based on what we know today, this hypothesis seems to be too simplistic, and it is possible that membrane-bound HLA-G interacts with inhibitory immune receptors to induce tolerance of the fetus, and at the same time sHLA-G is serving as an activating molecule promoting proinflammatory cytokine secretion allowing trophoblast migration and vascular remodelling. It is hypothesized that the interaction between early trophoblast cells and endothelial cells of the spiral arteries is crucial for trophoblast invasion. This interaction has been shown to increase in a proinflammatory environment characterized by cytokines such as TNF-α and IL1β [85]. This study showed, by using blocking antibodies, that the adhesion molecules VCAM-1 and $\alpha 4\beta 1$ were crucial for the interaction, but whether there is any interaction between these adhesion molecules and HLA-G on trophoblast cells remains to be elucidated. A recent study on JAR and JEG-3 cell lines found that HLA-G5 was able to stimulate trophoblast invasion through KIR2DL4 and ILT-2 probably through the ERK pathway [86]. This is in contrast to a previous study showing that sHLA-G actually inhibited trophoblast invasion [87]. Interestingly, these studies varied in concentrations of sHLA-G added in the invasion assay. In the study that suggested inhibition of trophoblast invasion, recombinant sHLA-G protein was added to the trophoblast cells at a concentration one hundred times higher in comparison to the study reporting a stimulation of trophoblast invasion. Taken together these studies could indicate that the effect of sHLA-G on trophoblast invasion was concentration-dependent.

In addition to the HLA-G receptor interactions described above several other immune cells might contribute to the induction of tolerance at the materno-fetal interface. Amodio et al. identified a specific dendritic cell population by flow cytometric analysis on first trimester decidual samples from healthy pregnancies undergoing elective abortions [88]. The DC-10 can either be recruited from peripheral blood, by induction of resident decidual dendritic cells, or by de novo induction promoted by the decidual microenvironment and have been shown to express high amounts of HLA-G and ILT-4 and promote IL-10 secretion. The IL-10 secretion is proposed to induce expression of HLA-G, ILT-2, and ILT-4 on immature decidual cells converting them to DC-10. The DC-10 cells can be important in inducing tolerance as they have been shown to be potent inducers of a specific subset of $CD4^+CD25^+FOXP3$ regulatory T cells called Tr1 cells *in vitro* [89]. Another specific subset of regulatory $CD4^+$ T cells constitutively expressing HLA-G has been shown to accumulate at inflammatory sites [90]. The study by Amodio et al. also showed that levels of $CD4^+HLA\text{-}G^+$ T cells were significantly higher in the peripheral blood of pregnant women compared to healthy controls [88]. From this it can be speculated that these cells are recruited to the fetomaternal interface during early implantation, where inflammatory responses might be involved in trophoblast invasion. In addition, these HLA-G positive cells have been shown to suppress T-cell proliferation through a reversible regulation of inflammation dependent on IL-10 and HLA-G [91].

4. Tissue Specific HLA-G Expression in relation to *HLA-G* Genetics

At present, not many published studies have addressed possible differences in *HLA-G* genotype-associated expression between different types of cells and tissues. This could be accomplished by cell- or organ-specific differences in

stimulatory or inhibitory substances, for example, hormones, or for independent types of cells, transcription factors or miRNAs. A range of studies have been published that show significant associations between sHLA-G concentrations in blood plasma and serum and the *HLA-G 14 bp ins/del* genotype, alternatively *HLA-G* 3′UTR haplotypes, as described previously [29, 50, 63, 65]. In these studies, homozygous *14 ins/14 ins* individuals show general lower sHLA-G1/HLA-G5 protein levels than *14 del/14 del* individuals, as discussed above. However, recently, we have shown that the *14 ins* allele has the highest membrane-bound expression of HLA-G1 in transduced K562 cells [32]. Altogether, the relationship between *HLA-G* genetics and HLA-G expression levels may turn out to be more complicated than previous thought, and it may even be tissue-specific.

5. Clarification of *HLA-G* Allele Associations in Preeclampsia and Related HLA-G Expression Is Needed

An increasing number of studies have indicated a role for HLA-G in the pathogenesis of preeclampsia. Several studies have reported reduced sHLA-G concentrations in maternal blood in preeclamptic cases compared to controls in all three trimesters of pregnancy [53, 71, 92–94]. HLA-G protein and mRNA expression in the placenta seem to be reduced in preeclampsia [40, 43, 44]. Furthermore, several studies have observed significant associations between certain *HLA-G* alleles, genotypes, and haplotypes [25, 38, 41, 45, 46]. Special attention has been drawn to the *14 ins* allele and an increased risk of severe preeclampsia in pregnancies, where the fetus is homozygous for an *HLA-G* 3′UTR haplotype that includes the *14 ins*, *+3010C*, *+3142G*, *+3187A*, and *+3196G* polymorphisms [25]. Whether this 3′UTR haplotype is a low or intermediate sHLA-G secretor in healthy donors is currently controversial [29, 63]. Furthermore, it is not known if these findings can be extrapolated to HLA-G expression in trophoblast cells, and thereby maternal blood sHLA-G levels during pregnancy, which is higher than in nonpregnant women. The soluble HLA-G concentration during pregnancy must be a mix of contributions from the mother, most probably from maternal immune cells, and from the fetal trophoblast cells in the placenta. Two studies suggest that the relationship between *HLA-G* polymorphism and HLA-G expression during pregnancy might be complex. A small study of HLA-G expression in term placenta in relation to *HLA-G* genotypes and polymorphisms using immunohistochemical staining of HLA-G indicates that *14 ins/14 ins* trophoblast cells do not show a clearly reduced expression of HLA-G [95]. Finally, a recent study of *14 ins* and *14 del* transfectants in the K562 cell line revealed that the *14 ins* transfectants had a higher cell surface expression of HLA-G1 than the *14 del* transfectants [32]. These controversies need to be clarified in future studies. In relation to a possible importance of HLA-G expression in the pathogenesis of preeclampsia, it is important to study the influence of the *HLA-G* polymorphisms in the 5′URR and the 3′UTR on transcription, mRNA stability, and alternative splicing. This is also important in the context that there might be differences between HLA-G expression linked to these polymorphisms in trophoblast cells and in immune cells. It can be speculated that this might be due to different profiles of miRNAs, other regulatory factors, and methylation status. Some of these interactions might be abnormal in preeclampsia and associated with specific *HLA-G* haplotypes, for example, the *14 ins/+3142G/+3187A* haplotype as one study indicates [25].

Maybe the predominating soluble HLA-G isoform in nonpregnant female donors and in male donors is HLA-G5, and during pregnancy the rise in sHLA-G in the maternal blood might primarily be a result of shedded HLA-G1 from trophoblast cell membranes in the placenta. Therefore, HLA-G protein expression in trophoblast cells in relation to *HLA-G* genetics needs to be investigated in more detail.

That the reduced sHLA-G blood levels observed in preeclampsia by a range of studies should merely be a result of a specific fetomaternal *HLA-G* genotype combination is probably not the case. It can be hypothesized that it might be a combination of predisposing *HLA-G* polymorphism in interaction with one or several other possible pathogenic factors, for example, an aberrant miRNA profile, defects in metalloproteinase activity that have been reported in preeclampsia, or the presence of certain viruses in the placenta that contribute to development of preeclampsia [96, 97]. Several studies have elucidated how human cytomegalovirus (HCMV) interferes with and downregulates HLA-G expression [98, 99]. Interestingly, a small pilot study has linked the presence of HCMV sequences and certain *HLA-G* alleles with increased risk of preeclampsia, and there might be some evidence for an association between CMV infection and preeclampsia [100, 101].

In conclusion, in future studies for clarification of the role of HLA-G in the development of preeclampsia, ideally *HLA-G* genetics, maternal blood sHLA-G levels, metalloproteinase activity, and the presence of specific viruses should be studied in the same cohort of pregnant women including a substantial number of pregnancies complicated with preeclampsia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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