HLA-G is a component of the CLL escape repertoire to generate immune suppression: impact of HLA-G 14 bp (rs66554220) polymorphism

by Roberta Rizzo, Valentina Audrito, Paola Vacca, Davide Rossi, Davide Brusa, Marina Stignani, Daria Bortolotti, Giovanni D’Arena, Marta Coscia, Luca Laurenti, Francesco Forconi, Gianluca Gaidano, Maria Cristina Mingari, Lorenzo Moretta, Fabio Malavasi, and Silvia Deaglio

Haematologica 2013 [Epub ahead of print]

doi:10.3324/haematol.2013.095281

Publisher’s Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors’ final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
HLA-G is a component of the chronic lymphocytic leukemia escape repertoire to generate immune suppression: impact of HLA-G 14 bp (rs66554220) polymorphism

Running title: HLA-G polymorphism in CLL

Roberta Rizzo¹, Valentina Audrito²,⁵, Paola Vaccá³, Davide Rossi⁴, Davide Brusa⁶, Marina Stignani¹, Daria Bortolotti¹, Giovanni D’Arena⁶, Marta Coscia⁷, Luca Laurenti⁸, Francesco Forconi⁹, Gianluca Gaidano⁴, Maria Cristina Mingari¹⁰, Lorenzo Moretta¹¹, Fabio Malavasi²,¹² and Silvia Deaglio²,⁵

¹Department of Medical Sciences, Sections of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy; ²Department of Medical Sciences, University of Turin, Turin, Italy; ³Department of Experimental Medicine, University of Genoa, Genoa Italy; ⁴Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy; ⁵Human Genetics Foundation (HuGeF), Turin, Italy; ⁶Department of Onco-Hematology, IRCCS “Centro di Riferimento Oncologico della Basilicata,” Rionero in Vulture, Italy; ⁷Division of Hematology, University of Torino, Azienda Ospedaliera Città della Salute e della Scienza di Torino, Torino, Italy; ⁸Institute of Hematology, Catholic University of the Sacred Heart, Rome; ⁹Cancer Sciences Unit, CRUK Centre, University of Southampton & Haematology Department, SUHT, Southampton, United Kingdom; ¹⁰AOU San Martino–Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy; ¹¹Giannina Gaslini Institute, Genoa, Italy; and ¹²Research Center for Experimental Medicine, Città della Salute e della Scienza Hospital, Turin, Italy

Correspondence
Silvia Deaglio, MD, PhD, Department of Medical Sciences, University of Turin School of Medicine & Human Genetics Foundation (HuGeF), via Nizza, 52, 10126 Torino, Italy.
E-mail: silvia.deaglio@unito.it

Roberta Rizzo, PhD, Department of Medical Sciences, Section of Microbiology, University of Ferrara, via Borsari 46, 44121 Ferrara, Italy. Email: roberta.rizzo@unife.it.

Funding
Work supported by grants from the Italian Ministries of Education, University and Research (Futuro in Ricerca 2008 # RBFR08ATLH, PRIN 2009 # 2009LMEEEH_002 e # NANLST_001, FIRB 2010 # RBAP11FXBC-005), Italian Ministry of Health (Bando Giovani Ricercatori 2008 # GR-2008-1138053), Associazione Italiana per la Ricerca sul Cancro Foundation (Special Program Molecular Clinical Oncology 5 x 1000 No. 10007; My First AIRC Grant No. 13470 and IG 12754), Fondazione Cariplo, Compagnia di San Paolo (No. PMN_call_2012_0071) and local funds of the University of Turin (ex-60%).
The Fondazione Ricerca Molinette provided valuable assistance and support.
Abstract

This work investigates the possibility that HLA-G, a molecule modulating innate and adaptive immunity, is part of an immune escape strategy of chronic lymphocytic leukemia cells. A 14 base pair insertion/deletion polymorphism (rs66554220) in the 3’-untranslated region of HLA-G influences mRNA stability and protein expression. The analysis of a cohort of chronic lymphocytic leukemia patients confirms that del/del individuals are characterized by higher levels of surface and soluble HLA-G than those of the other two genotypes. In line with its role in immunomodulation, the percentage of regulatory T lymphocytes is higher in del/del patients than in patients with the other genotypes and correlates with the amounts of surface or soluble HLA-G. Furthermore, addition of sHLA-G-rich plasma from chronic lymphocytic leukemia patients induces natural killer cell apoptosis and impairs natural killer cell lysis, with effects proportional to the amount of soluble HLA-G added. Lastly, the presence of HLA-G 14 base pair polymorphism is of prognostic value, with del/del patients showing reduced overall survival, as compared to the other genotypes. These results suggest that i) the HLA-G 14 bp polymorphism influences the levels of surface and soluble HLA-G expression, and that ii) the over-expression of HLA-G molecules contributes to create tolerogenic conditions.
Introduction

Chronic lymphocytic leukemia (CLL), the most common type of adult leukemia in Caucasian populations, is characterized by the progressive accumulation of mature CD5+/CD23+B cells in the peripheral blood and lymphoid organs (1). Several observations point to immune escape as a relevant mechanism of tumor promotion. First, CLL cells express high levels of immunomodulatory factors [e.g., TGF (2) and IL-10 (3)], which suppress response to antigens and influence activation, expansion, and effector functions of T lymphocytes (4). Second, an increase in the numbers of circulating regulatory T cells (Treg) parallels disease progression (5, 6). Lastly, adenosine production in the extracellular milieu by selected leukemic subpopulations shields the CLL clone from the actions of the immune system (7).

The aim of this work is to investigate the role of human leukocyte antigen G (HLA-G) as a further strategy adopted by CLL cells to evade immune defenses and to create protected niches where to grow and expand. HLA-G is a non-classical major histocompatibility complex (MHC) Class I product with limited sequence variability. It is exclusively expressed in tissues where the immune system needs to be constantly suppressed, including cytotrophoblast from early gestation placentas, amniotic cells, endothelial cells of chorionic blood vessels, thymic epithelial cells and corneas (8). HLA-G is a tolerogenic molecule, which inhibits cytolysis mediated by natural killer (NK) cells or T lymphocytes, induces T cell apoptosis and blocks transendothelial migration of NK cells (9). These functions are exerted upon binding the killer cell immunoglobulin-like receptor (KIR)2DL4 and the immunoglobulin-like transcript (ILT)2 and (ILT)4 ligands (10, 11). Hence, the immunosuppressive features of HLA-G are functional in pregnancy, organ transplantation, autoimmune diseases, and cancer immune escape (12).

The *HLA-G* gene encodes seven isoforms generated through alternative splicing: four are membrane-bound (namely, HLA-G1, -G2, -G3, and -G4), while three (HLA-G5, -G6, and -G7) are soluble and represent the counterparts of HLA-G1, -G2 and -G3, respectively. An alternative
mechanism to generate soluble HLA-G (sHLA-G) forms is represented by proteolytic cleavage of the membrane molecules (13).

*HLA-G* is characterized by different polymorphisms at the 5’ upstream regulatory and the 3’ untranslated regions (UTR) (14). One of these, characterized by the deletion/insertion (del/ins) of 14 base pairs (14 bp) (rs66554220), is responsible for mRNA stability and consequently protein production (15). The presence of the 14bp sequence is associated with unstable mRNA and reduced sHLA-G protein production (16). This polymorphism is implicated in autoimmune and chronic inflammatory conditions (8), while its role in cancer growth and progression is still controversial.

The role of HLA-G products in CLL patients has been evaluated in previous studies, which however focused exclusively on the expression either of the membrane or of the secreted isoform of the molecule. The results obtained indicate that i) HLA-G expression at transcription and protein levels is increased in CLL cells as compared to normal B lymphocytes (17-19); and ii) HLA-G expression correlates with worse clinical outcome in CLL (20, 21).

This work was undertaken with the aim of assessing the impact of the *HLA-G* 14 bp polymorphism on expression of the membrane and soluble forms of the HLA-G protein, and its role in promoting immune escape in a wide and well-characterized cohort of CLL patients.
Methods

Patients and controls

Five hundred and six individuals with a confirmed diagnosis of CLL were enrolled at diagnosis into a retrospective study and typed for *HLA-G* rs66554220 polymorphism. Patient characteristics are reported in Supplementary Table 1. Blood samples from patients or non-leukemic individuals were obtained after written informed consent in accordance with local institutional guidelines and the Declaration of Helsinki. The study has been approved by the Human Genetics Foundation Ethical Committee.

Peripheral blood mononuclear cells (PBMCs) and purified B lymphocytes were obtained as described (22).

Flow cytometric analyses

Antibodies used for flow cytometry are detailed in Supplementary Materials and Methods. Data were acquired using a FACSCanto II (BD Biosciences, Buccinasco, Italy) or Gallios (Beckman Coulter) flow cytofluorimeters, processed with DIVA v6.1.3 (BD Biosciences), and analyzed with FlowJo Version 9.01 softwares (TreeStar, Ashland, OR). At least 10,000 events were analyzed for each sample.

*HLA-G 14 bp polymorphism typing*

Genomic DNA was extracted from PBMCs using a DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy). The *HLA-G 14 bp polymorphism was genotyped by PCR (23, 24).

Soluble HLA-G detection by ELISA

Measurements of sHLA-G1 and HLA-G5 levels were performed as reported (25). After both ELISA measurements, the amount of sHLA-G1 was expressed as the difference between sHLA-G1/HLA-G5 and HLA-G5 concentrations (26).

Isolation and culture of NK cells
NK cells were isolated from PBMCs or from decidua were obtained as described (27) (28). Purified NK cells were cultured on allogeneic irradiated feeder cells in the presence of IL-2 (100 U/ml) and PHA (1.5 ng/ml, Gibco, Life Technologies) (27).

**NK cell apoptosis**
Freshly isolated or IL-2 activated NK cells were incubated with different serum samples from CLL patients representative of the different 14 bp HLA-G polymorphism. After overnight incubation, NK cell apoptosis was measured using annexin V and propidium iodide (Invitrogen, Eugene, OR, USA).

**NK cytolytic activity**
IL-2-activated NK cells were tested for cytolytic activity in a 4 h $^{51}$Cr-release assay against the K562 cell line (27).

**Statistical analyses**
Overall survival (OS) was measured from date of sampling to date of death (event) or last follow-up (censoring). Survival analysis was performed by the Kaplan-Meier method. The crude association between time-fixed exposure variables at diagnosis and survival was estimated by log-rank. Statistical significance was defined as $p$ value $<$0.05. Statistical tests were performed using the GraphPad Prism 6.0 software (Graphpad Software, San Diego, CA, USA) and the SPSS software v20.0 (Chicago, IL).
Results

Effects of HLA-G 14 bp polymorphism on membrane and soluble protein expression in CLL patients

The hypothesis behind this work is that patients with a del/del genotype of the 14 bp polymorphism are characterized by a more stable HLA-G mRNA, resulting in higher levels of the molecule on the cell surface and in biological fluids (16).

The hypothesis was tested by determining the effects of the 14 bp polymorphism on the expression of HLA-G protein at the surface of CLL B cells obtained from 126 patients. The observed levels of HLA-G expression on CD19+/CD5+ CLL B lymphocytes were highly variable (mean ± SEM 7.35 ± 1.13%, Figure 1A, B). Patients with a del/del genotype had a trend towards increased levels (n=51, mean 8.97 ± 1.85) of surface HLA-G, even if a comparison with ins/del (n=48, mean 6.38 ± 2) or ins/ins (n=27, mean 6.03 ± 1.77) patients was not statistically significant (Figure 1C). However, when divided into quartiles, 25.4% of the 126 patients presented a surface HLA-G expression by leukemic cells above the third quartile (third quartile value: 9.5%). Of these, 58% had a del/del, 23% an ins/del and 19% an ins/ins genotype (p<0.0001, Chi-Square test, Figure 1D).

Attention was next focused on HLA-G plasma levels assayed by ELISA assay in a cohort of 60 patients and 60 sex and age matched controls. Results indicate a marked variability in concentration in both CLL patients (mean ± SEM, 19.71 ± 2.83 ng/ml) and controls (mean ± SEM, 17.28 ± 23.64 ng/ml), without statistical differences between the two groups (Figure 2A). When CLL patients and controls were divided according to the genotype, del/del patients (n=27) displayed significantly higher levels of sHLA-G (mean 30.82 ± 4.8 ng/ml), than ins/del (n=18, mean 13.68 ± 3.67 ng/ml, p=0.015, Mann Whitney test) or ins/ins patients (n=15, mean 6.95 ± 2.74 ng/ml, p=0.003, Mann Whitney test, Figure 2B). Del/del controls showed a tendency towards a higher production of sHLA-G (n=22, mean 22.06 ± 27.32ng/ml) when compared to ins/del (n=25, mean 16.93 ± 24.87 ng/ml) or ins/ins (n=13, mean 9.88 ± 11.90 ng/ml) individuals, without reaching a statistically significant difference (Supplementary Figure 1A). After dividing sHLA-G levels into quartiles,
25% of CLL patients and of controls presented sHLA-G expression above the third quartile (third quartile value CLL patients: 31.75 ng/ml; third quartile value controls: 24.40 ng/ml). Of these, in the CLL cohort 80% presented a del/del genotype, while ins/del and ins/ins genotypes were 13% and 7%, respectively (p<0.0001, Fisher exact test, Figure 2C). When considering controls, 46% presented a del/del, 46% an ins/del and 8% an ins/ins genotype (p<0.0001, Fisher exact test, Supplementary Figure 1B).

We then asked whether sHLA-G levels correlate with HLA-G expressed on the membrane of CLL cells. As expected, the levels of expression of sHLA-G and membrane HLA-G presented a positive correlation (n=60, rho=0.4, p=0.003, Spearman Correlation test), in line with a relationship between surface HLA-G expression and release (Figure 2D). The analysis of covariance reported an independent effect of 14 bp polymorphism (p=0.003) and surface HLA-G expression (p=0.02) on sHLA-G plasma levels. Using an isoform-sensitive ELISA, sHLA-G1 was predominant in the plasma of CLL patients, indicating a derivation from shedding of the membrane form rather than from alternative splicing (sHLA-G1 vs HLA-G5 isoforms, p=0.001; Mann Whitney test, Figure 2E).

The conclusion is that patients with a del/del genotype present significantly higher sHLA-G levels and tend to express more membrane HLA-G on leukemic cells than the other genotypes.

**Effects of HLA-G 14 bp polymorphism on the number of circulating T lymphocytes in CLL patients**

CLL development and progression is paralleled by a progressive impairment of the host immune defenses, with clinically manifest immune defects of the T cell compartment. The next step of the work was to determine whether high levels of surface and sHLA-G would reflect the immune defects characterizing CLL. To this aim, the composition of T cell subsets was assessed in 52 CLL patients divided according to HLA-G 14 bp polymorphism.

CD4+ and CD8+ and T cell percentages were not significantly different in the three HLA-G 14 bp genotypes (Figure 3A, B). On the contrary, the number of Tregs, defined as
CD4+/CD25^{high}/CD127^{low}), was significantly higher in the del/del genotype (mean 6.97% ± 0.88), as compared to the ins/ins group (mean 3.23% ± 0.69, p=0.006, Mann Whitney test, Figure 3C). Heterozygous patients displayed intermediate values, not significantly different from both ins/ins and del/del homozygous patients (mean 5.77% ± 0.78). The percentage of Treg positively correlate with the levels of expression of surface HLA-G on CLL cells (n=33, rho=0.4, p=0.04, Spearman Correlation test), suggesting that the amount of HLA-G expressed by leukemic cells could impact on the frequency of Tregs (Figure 3D). In line with this observation was the finding that the percentage of circulating Tregs is higher in CLL with surface HLA-G expression >9.5% (third quartile) compared to CLL in which surface HLA-G expression was <9.5% (mean 6.95% ± 0.98 vs mean 4.31% ± 0.52, p=0.03, Mann Whitney U test; Figure 3E).

No statistically significant correlation could be detected between sHLA-G levels and the frequency of Tregs, likely due to the limited sample analysed (n=13, not shown). However, CLL patients in which sHLA-G levels were >31.75 ng/ml (third quartile), presented a trend towards a higher percentage of Tregs than patients in which sHLA-G were <31.75 ng/ml (mean 5.1% ± 1.44 vs mean 3.37% ± 0.52, p=0.30, Mann Whitney test, Figure 3F). Together, these data suggest that HLA-G expression is linked to an expansion of Tregs, as partly observed in other models(29).

**Effects of HLA-G polymorphism on NK cell function**

An alternative mechanism through which HLA-G molecules suppress the immune response is their inhibitory effect on NK cell activation and cytotoxic functions, mediated through the KIR2DL4 ligand (11). Therefore, we analyzed the possible implication of sHLA-G molecules in plasma samples on composition, activation and functional activities of NK cells in CLL samples. The reference control was represented by healthy individuals matched for gender and age.

The number of circulating NK cells (gated as CD56+/CD3−) was sharply decreased in CLL patients (n=9, mean 2.53% ± 0.88) as compared to controls (n=11, mean 11.52% ± 1.22, p<0.0001, Mann Whitney test, Figure 4A).
The cytolytic activity of NK cells isolated from CLL patients (n=9) was tested after culture for 2-4 weeks in the presence of IL-2. Cells were then assessed for their ability to lyse the K562 target cell line in a \(^{51}\)Cr-release cytolytic assay at different Effector:Target (E:T) ratios (from 40:1 to 0.25:1). Controls were IL-2-activated NK cells from healthy donors. As shown in Figure 4B, the NK-mediated cytolytic activity in CLL patients was lower than that in healthy donors. Thus, for example, 40% target cell lysis was obtained at 2.5:1 E:T ratio from healthy donors and at 20:1 for NK cells from CLL patients (Figure 4B).

We then asked whether KIR2DL4, the main HLA-G ligand (30), was expressed by NK cells. While resting NK cells from normal donors and from CLL patients lacked KIR2DL4, the ligand was induced upon IL-2-mediated NK cell activation, in agreement with published data (30) (Figure 4C). Representative expression plots are reported in Supplementary Figure 2, where decidual NK cells were used as positive control. No differential expression in KIR2DL4 was noted in HLA-G typed patients (not shown). The next step was to test whether sHLA-G present in plasma could induce apoptosis or inhibit the cytolytic function of NK cells obtained from normal donors. To answer this question, we used plasma from CLL patients containing different levels of sHLA-G to interfere with the viability and cytotoxic activity of NK cells. Plasma from CLL patients significantly compromised viability of NK cells from normal donors, by inducing apoptosis. The effect was directly correlated to sHLA-G levels in plasma, with significantly lower survival in the presence of plasma from patients with sHLA-G>31.75 ng/ml as compared to patients with undetectable sHLA-G (Figure 4D).

A significant inhibition of NK cell function was detected in the presence of CLL plasma samples with detectable sHLA-G (p<0.0001, Mann Whitney test, Figure 4E). At an E:T ratio of 10:1 donor NK cells in the absence of CLL plasma efficiently killed target cells (mean 85% ± 1.63), while in the presence of CLL plasma samples with detectable sHLA-G, the cytolytic activity was sharply reduced (mean 27.38% ± 4.72). CLL plasma samples with no sHLA-G were used as control. The presence of NK cell inhibition also in the presence of CLL plasma samples with undetectable
sHLA-G suggests the presence of other factors implicated in NK cell activation control. However, the degree of inhibition correlates with the concentrations of sHLA-G (Figure 4F). An inverse correlation between sHLA-G levels in CLL plasma samples (n=22) and the cytolytic activity of NK cells expressed as % cell lysis could be measured (rho=-0.49, p=0.02, Spearman Correlation test, Figure 4G). Consistent with the notion that CLL patients with del/del polymorphism display higher levels of sHLA-G than the other genotypes, plasma from del/del patients presented a higher inhibition than from the other genotypes. Thus, at 10:1 E:T ratio, del/del plasma inhibited NK cell lysis by 57.9%, while the effects exerted by ins/del and ins/ins plasma were 35.8% and 5.3%, respectively (p<0.0001, Fisher exact test, Figure 4H).

**Proof of principle: the HLA-G 14 bp polymorphism influences survival of CLL patients**

The results obtained so far indicate that the HLA-G 14 bp polymorphism dictates the amount of HLA-G protein present on the cell surface and in the plasma of CLL patients and that the molecule modulates quantitatively and qualitatively T and NK immunocompetence. In consideration of the immunosuppressive features of HLA-G, patients characterized by a del/del genotype would have higher levels of HLA-G levels, would be more immunosuppressed and ultimately present a worse clinical outcome. This issue was approached by testing the frequency of the HLA-G 14 bp polymorphism in 506 CLL patients (Supplementary Table 1). The genotype frequencies were in Hardy-Weinberg equilibrium: 176/506 patients (34.8%) were del/del homozygous, 81/506 (16%) were ins/ins homozygous and the remaining 249/506 (49.2%) were ins/del heterozygous. None of the demographic (age and sex), clinical (disease stage, splenomegaly, lymph node size), laboratory (LDH or β2m levels), or molecular variables (CD38, ZAP-70, IgHV mutational status, chromosomal aberrations) showed a preferential association with the HLA-G polymorphism (Supplementary Table 1). By survival analysis, patients harboring the del/del genotype were characterized by shorter survival than patients harboring the ins/del and/or ins/ins genotype (p=0.027, Log-rank test, Figure 5A). Consistently, CLL patients with sHLA-G levels above the third quartile presented lower survival (median, 63.9 months) compared to patients with sHLA-G
below the third quartile (median, 71.5 months, p=0.0215, Log-rank test; Figure 5B), and CLL patients with plasma samples showing inhibitory effects on NK cells showed a trend towards a lower survival than patients whose plasma lacked inhibitory effects (p=0.147, Log-rank test, not shown).
Discussion

HLA-G is a non-classical HLA protein that works by modulating main functions of NK cells and of Tregs. HLA-G-mediated signals are critical in mediating tolerance during specific ontogenetic moments (i.e., pregnancy). HLA-G may be expressed by tumor cells as part of a strategy to evade the action of the innate and adaptive immune system.

To test whether this may happen also in CLL cells, HLA-G expression was tested in a large cohort of CLL patients with well-defined molecular and clinical characteristics and with survival data available. The originality of the work is that the analyses of surface or sHLA-G expression were flanked by the characterization of the 14 bp polymorphism in the 3’ UTR untrascribed region. This point was dictated by evidence derived from different models, which allows to conclude that this polymorphism accurately predicts the amount of transcribed protein, either bound to the membrane or released in biological fluids.

The results indicate an association between the del/del genotype and increased levels of plasma HLA-G molecules. This association is apparent only in CLL patients, as plasma from age- and sex-matched controls failed to reach a statistically significant association between the del/del genotype and the amount of plasma HLA-G.

The situation on the cell membrane is less defined: del/del patients are more frequent in the group expressing higher levels of the molecule, even if a statistical significance is not reached. A potential explanation for this finding lies in the relative instability of HLA-G molecules on the CLL cell membrane. Results showing a correlation between membrane and sHLA-G levels favor the view that the main mechanism for the generation of sHLA-G is shedding rather than alternative splicing. This was confirmed by the analysis of HLA-G isoforms, which highlight a dominance of sHLA-G1, generated by proteolytic cleavage of membrane HLA-G1. No preference of isoform according to the 14 bp polymorphism was noted, in agreement with published data (31, 32).

A reasonable hypothesis to explain this result is that the membrane form represents a transitory step. A consequence is that quantification of soluble rather than surface HLA-G may be more accurate.
This is also in line with previous studies, which found that sHLA-G levels in plasma were higher in CLL patients than in healthy controls (17, 18). A second issue favoring the view that quantification of soluble rather than membrane HLA-G is an informative and dependable assay derives from old and new facts linked to the unique lipid structure of the CLL surface (33, 34), potentially increasing the instability of the membrane form. In agreement with this in myeloma cells mHLA-G is released from the cell membrane also in microparticles (35). These findings suggest that a quantification of sHLA-G may be clinically useful and more informative than surface analysis on tumor cells.

The second set of results obtained in this work may be considered as tiles composing a tolerogenic mosaic, where HLA-G molecules represent a link between innate and adaptive immunity. Accordingly, the presence of a del/del genotype (i.e., with high sHLA-G levels) is paralleled by an expansion of Tregs in the circulation. Supportive data come from other models, where HLA-G is reported to induce Tregs. As an example, PBMC exposed to sHLA-G5 acquire regulatory features, inhibiting allo-proliferative responses exerted by other T lymphocytes. It is also known that patients receiving combined liver/kidney transplants show high levels of sHLA-G5, which correlate with increased percentages of suppressor T cells (36). Similarly, stem-cell transplanted patients present high sHLA-G5 in the peripheral blood, with a simultaneous expansion of CD4+/CD25+/CD152+ T lymphocytes with suppressive activity. Indirect confirmation in the CLL model may be deduced from data showing that an increase in Tregs positively correlates with the presence of clinical and biological features of aggressive disease (37).

NK cells obtained from CLL patients present a lower cytotoxic activity when compared to NK cell populations obtained from healthy donors of a comparable age. This issue suggests that leukemic cells directly affect NK cell viability and/or activity. This would be achieved through binding of HLA-G to the KIR2DL4 ligand, which becomes expressed once NK cells are activated in the presence of IL-2. Our working hypothesis is that ectopic expression of HLA-G contributes to block NK cell functions. With the aim of reproducing physiological conditions the experiments were performed using whole plasma instead of purified HLA-G. The assumption was confirmed by
incubating NK cells obtained from normal donors with plasma from CLL patients containing variable amounts of sHLA-G. This was followed by a marked induction of NK cell apoptosis, which was proportional to the amount of sHLA-G present in plasma. Furthermore, also lysis inhibition was proportional to the amount of sHLA-G. Noteworthy, CLL plasma samples with undetectable sHLA-G were able to moderately induce NK cell apoptosis and to reduce NK cell cytotoxicity. These results suggest the presence of other factors than sHLA-G that are able to control NK cell activation in CLL condition. As expected, NK cell function of del/del patients was more impaired than that of ins/del or ins/ins patients.

In conclusion, data obtained in this work provide two different sets of information. The first one indicates that the 14 bp polymorphism influences quantitative analyses of sHLA-G. The amount of sHLA-G in plasma has a prognostic value, suggesting that this specific assay may be relevant in the management of CLL patients, rather than the mere measurement of membrane HLA-G. The second set of results indicates that the presence of HLA-G molecules in the neoplastic environment, either soluble or bound to the membrane, creates a favorable setting for CLL expansion. As a proof, the evaluation of the impact of 14 bp polymorphism on the clinical outcome of the disease shows that del/del patients display a lower overall survival than ins/del or ins/ins patients.

Taken together, our results sustain the view that HLA-G molecules are part of the escape strategies designed by CLL cells and indicates that a quantitative analysis of sHLA-G levels may be of clinical relevance in the management of CLL patients.

**Authorship and Disclosures:** RR, VA, PV, DR, DBr, MS, DBo: performed research; GDA, MC, LL, FF: contributed patient samples; GG, MCM, LM: designed research and interpreted data; FM, SD: designed research, interpreted data and together with RR wrote the paper. The authors declare no competing financial interests.
References


Figure Legends

Figure 1. Distribution of membrane HLA-G in a cohort of 126 CLL patients typed for the 14 bp polymorphism. (A) Density plots represent the gating strategy. Left panel indicates the morphological gate, right panel shows the staining for CD19 and CD5. (B) Histograms represent surface HLA-G expression in CD19+/CD5+ CLL B lymphocytes of three representative patients. (C) Patients were then divided according to the 14 bp polymorphism into del/del, ins/del and ins/ins categories. Box plots represent the distribution of mHLA-G in the different categories. (D) Graph representing the percentage of patients expressing mHLA-G above (black bars) or below (open bars) the third quartile (9.5%).

Figure 2. Quantification of soluble HLA-G levels in a cohort of 60 CLL patients typed for the 14 bp polymorphism. (A) Box plot analysis summarizing data obtained with a quantitative ELISA assay performed on 60 plasma samples from CLL patients and 60 plasma samples from control subjects (controls). The lower and upper limit of the box define the first and third quartiles, respectively, while the line inside the box represents the median. Whiskers identify minimum and maximum values. (B) Graph showing soluble HLA-G levels in CLL patients divided according to the 14 bp polymorphism into del/del, ins/del and ins/ins categories. (C) Graph representing the percentage of CLL patients expressing soluble HLA-G above (black bars) or below (open bars) the third quartile (31.75 ng/ml) in the three genotypes (D) Regression lines showing a positive correlation between the percentage of CLL cells expressing HLA-G on the cell surface and the amount of soluble HLA-G in the plasma. Spearman’s coefficient (rho) and the corresponding p value is listed. (E) Box plot showing the results of an ELISA assay using isoform specific antibodies to discriminate between soluble HLA-G1 (derived from shedding of the membrane form) and soluble HLA-G5 (derived from alternative splicing).

Figure 3. Evaluation of the T cell compartment in CLL patients typed for the 14 bp polymorphism. Percentage of total CD4+ (A) and CD8+ (B) circulating T lymphocytes and Tregs
(C) in CLL patients divided according to the 14 bp polymorphism. Tregs were defined as CD4+/CD25^{high}/CD127^{low}. (D) Regression line showing a positive correlation between membrane HLA-G and the percentage of circulating Tregs. Spearman’s coefficient (rho) and the corresponding p values are listed. (E) Percentage of Tregs in CLL patients expressing membrane HLA-G above or below the third (9.5%) quartile. (F) Percentage of Tregs in CLL patients expressing soluble HLA-G above or below the third (31.75 mg/ml) quartile.

**Figure 4. Evaluation of the NK cell compartment in CLL patients typed for the 14 bp polymorphism.** (A) Percentage of circulating CD56+/CD3^- NK cells in 7 CLL patients and in 11 healthy donors (controls) with a comparable age and male:female ratio. (B) ^{51}Cr cytotoxicity assay comparing the lytic potential of *in vitro* IL-2-activated NK cells from controls (circles) or CLL patients (squares) against the K562 target cell line. (C) Expression of KIR2DL4 in resting and IL-2-activated NK cells from CLL patients. Data are expressed as MFI ratio. (D) Percentage of NK cell viability in the presence of sHLA-G-high and sHLA-G-low plasma from CLL patients. (E) Inhibition of cytolytic activity of IL-2-activated HD-NK cells against K562 target cells in the presence or absence of plasma obtained from CLL patients. (F) The inhibitory effect of CLL plasma on NK cell lysis was studied in patients with high levels of soluble HLA-G and compared to patients with undetectable soluble HLA-G. (G) Regression line showing a negative correlation between the amount of soluble HLA-G and the percentage of cell lysis. Spearman’s coefficient (rho) and the corresponding p value is listed. (H) Graph representing the percentage of NK cell lysis inhibition obtained using plasma derived from patients categorized on the basis of HLA-G genotype.

**Figure 5. Kaplan-Meyer curves showing overall survival in 506 CLL patients.** (A) Kaplan-Meier estimates of overall survival according to according to *HLA-G* 14 bp genotype. (B) Kaplan-Meier estimates of overall survival according to according to soluble HLA-G levels.
FIGURE 1
FIGURE 2
**FIGURE 3**

(A) Box plots showing the percentage of CD4+ T cells for different HLA-G genotypes: del/del, ins/del, and ins/ins. The sample sizes are n=24, n=16, and n=12, respectively.

(B) Box plots showing the percentage of CD8+ T cells for different HLA-G genotypes: del/del, ins/del, and ins/ins. The sample sizes are n=24, n=16, and n=12, respectively.

(C) Box plots comparing the percentage of Treg cells for different HLA-G genotypes: del/del, ins/del, and ins/ins. The sample sizes are n=13, n=9, and n=6, respectively. The p-values are P=0.006 and P=0.45 for the comparisons del/del vs. ins/del and del/del vs. ins/ins, respectively.

(D) Scatter plot showing the relationship between membrane HLA-G (%) and Treg cell percentage. The correlation coefficient (rho) is 0.4, and the p-value is 0.04.

(E) Box plots comparing Treg cell percentage for different membrane HLA-G levels: >9.5% and <9.5%. The sample sizes are n=8 and n=21, respectively. The p-value is 0.032.

(F) Box plots comparing Treg cell percentage for different soluble HLA-G levels: >31.75 ng/ml and <31.75 ng/ml. The sample sizes are n=4 and n=9, respectively. The p-value is 0.30.
FIGURE 4
FIGURE 5
**Supplementary Table 1.** Demographic, clinical, laboratory and molecular variables of the CLL population subdivided according to rs 66554220 polymorphism.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HLA-G ins/ins n(%)</th>
<th>HLA-G ins/del n(%)</th>
<th>HLA-G del/del n(%)</th>
<th>Total</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD38</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤30%</td>
<td>40 (49)</td>
<td>158 (63)</td>
<td>108 (61)</td>
<td>306</td>
<td>0.076</td>
</tr>
<tr>
<td>&gt;30%</td>
<td>41 (51)</td>
<td>91 (37)</td>
<td>68 (39)</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>ZAP70</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20%</td>
<td>42 (52)</td>
<td>132 (53)</td>
<td>96 (55)</td>
<td>270</td>
<td>0.19</td>
</tr>
<tr>
<td>&gt;20%</td>
<td>39 (48)</td>
<td>117 (47)</td>
<td>80 (45)</td>
<td>236</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>FISH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13q-/-normal/+12</td>
<td>54 (67)</td>
<td>157 (63)</td>
<td>118 (67)</td>
<td>368</td>
<td>0.66</td>
</tr>
<tr>
<td>17p-/11q-</td>
<td>27 (33)</td>
<td>92 (37)</td>
<td>58 (33)</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>IGHV homology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤98%</td>
<td>40 (49)</td>
<td>157 (63)</td>
<td>104 (59)</td>
<td>301</td>
<td>0.09</td>
</tr>
<tr>
<td>&gt;98%</td>
<td>41 (51)</td>
<td>92 (37)</td>
<td>72 (41)</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>31 (38)</td>
<td>100 (40)</td>
<td>64 (36)</td>
<td>195</td>
<td>0.73</td>
</tr>
<tr>
<td>≥65</td>
<td>50 (62)</td>
<td>149 (60)</td>
<td>112 (64)</td>
<td>311</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>Binet Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>51 (63)</td>
<td>190 (76)</td>
<td>125 (71)</td>
<td>366</td>
<td>0.06</td>
</tr>
<tr>
<td>B - C</td>
<td>30 (37)</td>
<td>59 (24)</td>
<td>51 (29)</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>41 (51)</td>
<td>100 (40)</td>
<td>73 (41)</td>
<td>214</td>
<td>0.25</td>
</tr>
<tr>
<td>M</td>
<td>40 (49)</td>
<td>149 (60)</td>
<td>103 (59)</td>
<td>292</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15x10^9/l</td>
<td>50 (62)</td>
<td>132 (53)</td>
<td>97 (55)</td>
<td>279</td>
<td>0.39</td>
</tr>
<tr>
<td>&gt;15x10^9/l</td>
<td>31 (38)</td>
<td>117 (47)</td>
<td>79 (45)</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph Node Size</strong></td>
<td>≤3cm</td>
<td>71 (88)</td>
<td>198 (79)</td>
<td>143 (81)</td>
<td>412</td>
</tr>
<tr>
<td>&gt;3cm</td>
<td>10 (12)</td>
<td>51 (21)</td>
<td>33 (19)</td>
<td>94</td>
<td>0.26</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>Splenomegaly</strong></td>
<td>No</td>
<td>52 (64)</td>
<td>190 (76)</td>
<td>134 (76)</td>
<td>386</td>
</tr>
<tr>
<td>Yes</td>
<td>29 (36)</td>
<td>59 (24)</td>
<td>42 (24)</td>
<td>120</td>
<td>0.075</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>B2M</strong></td>
<td>&lt;2.5 mg/l</td>
<td>44 (54)</td>
<td>100 (40)</td>
<td>82 (47)</td>
<td>226</td>
</tr>
<tr>
<td>&gt;2.5 mg/l</td>
<td>37 (46)</td>
<td>149 (60)</td>
<td>94 (53)</td>
<td>280</td>
<td>0.07</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td><strong>LDH</strong></td>
<td>&lt;500 U/l</td>
<td>50 (62)</td>
<td>210 (84)</td>
<td>145 (82)</td>
<td>405</td>
</tr>
<tr>
<td>&gt;500 U/l</td>
<td>31 (38)</td>
<td>39 (16)</td>
<td>31 (18)</td>
<td>101</td>
<td>0.11</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>249</td>
<td>176</td>
<td>506</td>
<td></td>
</tr>
</tbody>
</table>
The genotypes ins/ins and ins/del were considered together as low and medium HLA-G producers and compared with the del/del high HLA-G producer genotype.

FISH, fluorescence in situ hybridization; *IGHV*, immunoglobulin heavy variable gene; B2M, beta-2-microglobulin.

p values were obtained by chi square test or Fisher’s exact test, when appropriate.
Supplementary Figure 1. Quantification of soluble HLA-G levels in a cohort of 60 controls typed for the 14 bp polymorphism. (A) Graph showing soluble HLA-G levels in control subjects divided according to the 14 bp polymorphism into del/del, ins/del and ins/ins categories. (B) Graph representing the percentage of control subjects expressing soluble HLA-G above (black bars) or below (open bars) the third quartile (24.4 ng/ml) in the three genotypes.
Supplementary Figure 2. Phenotypic analysis of KIR2DL4 on NK cells. (A) NK cells from decidual tissue (dNK), healthy donor (HD-NK) and 2 representative CLL patients were analyzed for surface expression of KIR2DL4 at day 0 and after 7 days of culture in IL-2. Staining with secondary antibody alone is shown (gray profile). One representative experiment out of 10 performed.
Supplementary Materials and Methods

Antibodies for flow cytometric analyses

Antibodies used were anti-HLA-G-PE (clone 87G), -CD19-FITC, -CD5-FITC, -CD4-PE-Cy5 (all from eBioscience, Milan, Italy), -CD38-PE (EXBIO Praha, Vestec, Czech Republic), -ZAP-70-AlexaFluor488 (Life Technologies, Invitrogen, Monza, Italy), -CD8-FITC (Biolegend, Milan, Italy), -CD3-PerCP, -CD56-APC, -CD19-APC (Miltenyi Biotech, Calderara di Reno, Italy). KIR2DL4 (R&D Systems, Abingdon, UK) was highlighted using a PE-conjugated rabbit anti-mouse secondary antibody (Southern Biotechnologies, Birmingham, AL). Tregs were detected using anti-CD4-PerCP/-CD25-PE/-CD127-AlexaFluor647 mix (Biolegend).