**Articles** Chronic Lymphocytic Leukemia

HLA-G is a component of the chronic lymphocytic leukemia escape repertoire to generate immune suppression: impact of the *HLA-G* 14 base pair (rs66554220) polymorphism

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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 patients with chronic lymphocytic leukemia confirmed that del/del individuals are characterized by higher levels of surface and soluble HLA-G than subjects with 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 patients with chronic lymphocytic leukemia induces natural killer cell apoptosis and impairs nat ural killer cell lysis, with effects proportional to the amount of soluble HLA-G added. Lastly, the presence of an *HLA G* 14 base pair polymorphism is of prognostic value, with del/del patients showing reduced overall survival, as com pared to those with other genotypes. These results suggest that: (i) the *HLA-G* 14 base pair polymorphism influences the levels of surface and soluble HLA-G expression, and (ii) the over-expression of HLA-G molecules contributes to creating tolerogenic conditions.

Introduction

Chronic lymphocytic leukemia (CLL), the most common type of adult leukemia in Caucasian populations, is charac terized by the progressive accumulation of mature CD5+/CD23+ B cells in the peripheral blood and lymphoid organs.1 Several observations point to immune escape being a relevant mechanism of tumor promotion. First, CLL cells express high levels of immunomodulatory factors (*e.g.*, transforming growth factor2 and interleukin-103), which sup press responses to antigens and influence activation, expan sion, and effector functions of T lymphocytes.4 Secondly, an increase in the numbers of circulating regulatory T cells (Treg) parallels disease progression.5,6 Lastly, adenosine pro duction in the extracellular milieu by selected leukemic sub populations shields the CLL clone from the actions of the immune system.7

The aim of this work was 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 in which 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 tis sues where the immune system needs to be constantly sup pressed, including cytotrophoblast from early gestation pla centas, amniotic cells, endothelial cells of chorionic blood ves sels, thymic epithelial cells and corneas.8 HLA-G is a tolero genic 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-2 and -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 mecha nism to generate soluble HLA-G (sHLA-G) forms is represent ed by proteolytic cleavage of the membrane molecules.13

*HLA-G* is characterized by different polymorphisms at the

*©2014 Ferrata Storti Foundation.This is an open-access paper.doi:10.3324/haematol.2013.095281 The online version of this article has a Supplementary Appendix. Manuscript received on July 23, 2013. Manuscript accepted on December 17, 2013. Correspondence: silvia.deaglio@unito.it or roberta.rizzo@unife.it.*

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5’ upstream regulatory and the 3’ untranslated regions.14 One of these, characterized by the deletion/insertion (del/ins) of 14 base pairs (14 bp) (rs66554220), is responsi ble for mRNA stability and consequently protein produc tion.15 The presence of the 14 bp sequence is associated with unstable mRNA and reduced sHLA-G protein pro duction.16 This polymorphism is implicated in autoim mune 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, although these 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 transcrip tion and protein levels is increased in CLL cells as com pared to normal B lymphocytes;17-19 and (ii) HLA-G expres sion 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 large, 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. The patients’ char acteristics are reported in *Online Supplementary Table S1*. Blood samples from patients or non-leukemic individuals were obtained after written informed consent in accordance with local institu tional guidelines and the Declaration of Helsinki. The study was approved by the Human Genetics Foundation Ethical Committee.

Peripheral blood mononuclear cells and purified B lymphocytes were obtained as described elsewhere.22

*Flow cytometric analyses*

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

*Typing the* HLA-G *14 base pair polymorphism* Genomic DNA was extracted from peripheral blood mononu clear cells using a DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy). The *HLA-G* 14 bp polymorphism was genotyped by poly merase chain reaction.23,24

*Soluble HLA-G detection by enzyme-linked immunosorbent assay*

sHLA-G1 and HLA-G5 levels were measured as reported previ ously.25 After both enzyme-linked immunosorbent assay (ELISA) measurements, the amount of sHLA-G1 was expressed as the dif ference between sHLA-G1/HLA-G5 and HLA-G5 concentra tions.26

*Isolation and culture of natural killer cells* NK cells were isolated from peripheral blood mononuclear cells or from decidua as described elsewhere.27,28 Purified NK cells were cultured on allogeneic irradiated feeder cells in the presence of

HLA-G polymorphism in CLL

interleukin-2 (100 U/mL) and phytohemagglutinin (1.5 ng/mL, Gibco, Life Technologies).27

*Natural killer cell apoptosis*

Freshly isolated or interleukin-2 activated NK cells were incu bated with different serum samples from CLL patients representa tive 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).

*Natural killer cell cytolytic activity*

Interleukin-2-activated NK cells were tested for cytolytic activi ty in a 4 h 51Cr-release assay against the K562 cell line.27

*Statistical analyses*

Overall survival 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 analysis. Statistical significance was defined as a *P* value <0.05. Statistical tests were performed using GraphPad Prism 6.0 software (Graphpad Software, San Diego, CA, USA) and SPSS software v20.0 (Chicago, IL, USA).

Results

*Effects of the* HLA-G *14 base pair polymorphism on membrane and soluble protein expression in patients with chronic lymphocytic leukemia*

The hypothesis underlying this study is that patients with a del/del genotype of the 14 bp polymorphism have more stable *HLA-G* mRNA, resulting in higher levels of the molecule on the cell surface and in biological fluids.16

This 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 ± standard error of 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 sur face HLA-G, even if a comparison with levels in 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 had 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, χ2 test, Figure 1D).

Attention was next focused on HLA-G plasma levels assayed by ELISA in a cohort of 60 patients and 60 sex and age-matched controls. Results indicate a marked vari ability 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 statistically significant dif ferences between the two groups (Figure 2A). When CLL patients and controls were divided according to genotype, del/del patients (n=27) had significantly higher levels of sHLA-G (mean 30.82±4.8 ng/mL) than had either 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

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G (n=22, mean 22.06±27.32 ng/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 the differ ence reaching statistical significance (*Online Supplementary Figure S1A*). After dividing sHLA-G levels into quartiles, 25% of CLL patients and of controls had sHLA-G expres sion above the third quartile (third quartile value in CLL patients: 31.75 ng/mL; third quartile value in controls: 24.40 ng/mL). Of these, in the CLL cohort 80% had a del/del genotype, while 13% had an ins/del genotype and the remaining 7% had an ins/ins genotype (*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, *Online Supplementary Figure S1B*).

**A**

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 mem brane HLA-G were positively correlated (n=60, rho=0.4, *P*=0.003, Spearman correlation test), in line with a rela tionship between surface HLA-G expression and release (Figure 2D). The analysis of covariance showed an inde pendent effect of 14 bp polymorphism (*P*=0.003) and sur face HLA-G expression (*P*=0.02) on sHLA-G plasma levels. Using an isoform-sensitive ELISA, sHLA-G1 was predom inant in the plasma of CLL patients, indicating a derivation from shedding of the membrane form rather than from alternative splicing (sHLA-G1 *versus* HLA-G5 isoforms, *P*=0.001; Mann-Whitney test, Figure 2E).

The conclusion is that patients with a del/del genotype

C SS

250k

200k

150k

100k

50k

0

0 50k 100k 150k 200k 250k

5

DC

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104

103

102

101

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FSC CD19

**B**

# 1 low # 2 intermediate # 3 high

100 80

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1 % 19% 80

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100 101 102 103 104 105 100 101 102 103 104 105 100 101 102 103 104 105 HLA-G

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P<0.0001 >9.5% <9.5%

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

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morphological gate, right panel shows the staining for CD19 and CD5. (B) Histograms represent sur face HLA-G expression in CD19+/CD5+ CLL B lymphocytes from three representative patients. (C) Patients were then divided according to the 14 bp polymorphism into groups with del/del, ins/del or ins/ins genotype. Box plots represent the distribution of mHLA-G in the dif ferent categories. (D) Graph repre senting the percentages of patients

del/del ins/del ins/ins

del/del ins/del ins/ins

expressing mHLA-G above (black

HLA-G genotype HLA-G genotype

bars) or below (open bars) the third quartile (9.5%).

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have significantly higher sHLA-G levels and tend to express more membrane HLA-G on leukemic cells than do patients with the other genotypes.

*Effects of the* HLA-G *14 base pair polymorphism on the number of circulating T lymphocytes in patients with chronic lymphocytic leukemia*

CLL development and progression are paralleled by a progressive impairment of host immune defenses, with clinically manifest immune defects of the T-cell compart ment. The next step of the study 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 polymor phism.

CD4+ and CD8+ and T-cell percentages were not signifi cantly different among the groups with the three *HLA-G* 14 bp genotypes (Figure 3A,B). In contrast, the number of Treg, defined as CD4+/CD25high/CD127low), was significant ly higher in patients with the del/del genotype (mean 6.97% ± 0.88) than in the group with the ins/ins genotype (mean 3.23% ± 0.69, *P*=0.006, Mann-Whitney test, Figure 3C). Heterozygous patients displayed intermediate values, not significantly different from those in either the ins/ins or del/del homozygous patients (mean 5.77% ± 0.78). The percentage of Treg correlated positively 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 influ ence the frequency of Treg (Figure 3D). In line with this observation was the finding that the percentage of circu lating Treg was higher in cases of CLL with surface HLA G expression >9.5% (third quartile) than in CLL in which

**A B C**

HLA-G polymorphism in CLL

surface HLA-G expression was <9.5% (mean 6.95% ± 0.98 *versus* 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 Treg, likely due to the limited sample analyzed (n=13, *data not shown*). However, CLL patients in whom sHLA-G levels were >31.75 ng/mL (third quartile) tended to have a higher per centage of Treg than patients in whom sHLA-G levels were <31.75 ng/mL (mean 5.1% ± 1.44 *versus* 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 Treg, as partly observed in other models.29

*Effects of the* HLA-G *polymorphism on natural killer cell function*

An alternative mechanism through which HLA-G mole cules suppress the immune response is their inhibitory effect on NK cell activation and cytotoxic functions, medi ated through the KIR2DL4 ligand.11 We, therefore, ana lyzed the possible implication of sHLA-G molecules in plasma samples on composition, activation and functional activities of NK cells in CLL samples. The reference con trol 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 interleukin-2. Cells were then assessed for their ability to lyse the K562 target cell line in a 51Cr-release cytolytic assay at different effector:target (E:T) ratios

Figure 2. Quantification of soluble HLA-G

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n=60

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*P*=0.003

*P*=0.015 *P*<0.0001

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n=27

75

<31.75 ng/mL >31.75 ng/mL

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 per formed on 60 plasma samples from CLL

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patients and 60 plasma samples from control subjects (controls). The lower and upper limits 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 sol uble HLA-G levels in CLL patients divided

0

CLL controls

0

0 del/del ins/del ins/ins HLA-G genotype

del/del ins/del ins/ins HLA-G genotype

according to the 14 bp polymorphism into del/del, ins/del and ins/ins cate gories. (C) Graph representing the per centages of CLL patients expressing sol uble HLA-G above (black bars) or below

**D E**

100

80

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*P*=0.001

n=60

(open bars) the third quartile (31.75 ng/mL) in the three genotypes. (D) Regression lines showing a positive cor relation between the percentage of CLL

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n=60 rho=0.4

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cells expressing HLA-G on the cell sur face and the amount of soluble HLA-G in the plasma. Spearman coefficient (rho) and the corresponding *P* value are pro vided. (E) Box plot showing the results of an ELISA using isoform-specific antibod ies to discriminate between soluble HLA G1 (derived from shedding of the mem brane form) and soluble HLA-G5 (derived

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HLA-G (%)sHLA-G1 sHLA-G5 0 20 40 60 80

from alternative splicing).

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**A B C** 10

*P*=0.006

*P*=0.45

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del/del ins/del ins/ins

HLA-G genotype

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HLA-G genotype

del/del ins/del ins/ins HLA-G genotype

**E F D**

*P*=0.30

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15 10 5

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n=33

rho=0.4 *P*=0.04

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15 10 5

0

*P*=0.032

n=8

n=21

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8 6 4 2 0

n=4

n=9

0 5 10 15 20 25 >9.5 <9.5 >31.75 <31.75 membrane HLA-G (%) membrane HLA-G (%) soluble HLA-G (ng/mL)

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 Treg (C) in CLL patients divided according to the 14 bp polymorphism. Treg were defined as CD4+/CD25high/CD127low. (D) Regression line showing a positive correlation between membrane HLA-G and the percentage of circulating Treg. Spearman coefficient (rho) and the corresponding *P* values are provided. (E) Percentage of Treg in CLL patients expressing membrane HLA-G above or below the third quartile (9.5%). (F) Percentage of Treg in CLL patients expressing soluble HLA-G above or below the third quartile (31.75 mg/mL).

(from 40:1 to 0.25:1). Controls were interleukin-2-activat ed NK cells from healthy donors. As shown in Figure 4B, the NK-cell-mediated cytolytic activity in CLL patients was lower than that in healthy donors. Thus, for example, 40% target cell lysis was obtained at an E:T ratio of 2.5:1 from healthy donors and at 20:1 for NK cells from CLL patients (Figure 4B).

We next asked whether KIR2DL4, the main HLA-G lig and,30 was expressed by NK cells. While resting NK cells from normal donors and from CLL patients lacked KIR2DL4, the ligand was induced upon interleukin-2- mediated NK cell activation, in agreement with published data30 (Figure 4C). Representative expression plots are reported in *Online Supplementary Figure S2*, showing results from an experiment in which decidual NK cells were used as a positive control. No differential expression in KIR2DL4 was noted in HLA-G typed patients (*data 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 the 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 of the NK cells when exposed to plasma from patients with sHLA-G >31.75 ng/mL as compared to plasma from patients with unde tectable sHLA-G (Figure 4D).

Similarly, NK cell function was significantly impaired 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 the 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 correlated with the concentrations of sHLA-G (Figure 4F). There was an inverse correlation

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*P*=0.01

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**E F**

*P*<0.0001*P*<0.0001*P*=0.001

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*P*<0.0001

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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 seven CLL patients and in 11 healthy donors (controls) with a comparable age and male:female ratio. (B) 51Cr cytotoxicity assay comparing the lytic potential of *in vitro* interleukin-2-activated NK cells from controls (circles) or CLL patients (squares) against the K562 target cell line. (C) Expression of KIR2DL4 in resting and interleukin-2-activated NK cells from CLL patients. Data are expressed as mean fluorescence intensity (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 interleukin-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 that in 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 coefficient (rho) and the corresponding *P* value are provided. (H) Graph representing the percentage of NK cell lysis inhibition obtained using plasma derived from patients categorized on the basis of HLA-G genotype.

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between sHLA-G levels in CLL plasma samples (n=22) and

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the cytolytic activity of NK cells expressed as percentage cell lysis (rho= –0.49, *P*=0.02, Spearman correlation test, Figure 4G). Consistent with the notion that CLL patients

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with del/del polymorphism have higher levels of sHLA-G )

compared to the levels in patients with the other geno

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types, plasma from del/del patients showed greater inhibi

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Thus, at an E:T ratio of 10:1, del/del plasma inhibited NK

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cell lysis by 57.9%, while the effects exerted by ins/del

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and ins/ins plasma were 35.8% and 5.3%, respectively

o

p

o

(*P*<0.0001, Fisher exact test, Figure 4H).

20

r

P

*Proof-of-principle: the* HLA-G *14 bp polymorphism* 0

*influences survival of patients with chronic*

*lymphocytic leukemia*

The results obtained so far indicate that the *HLA-G* 14

HLA-G ins/del ins/ins

HLA-G del/del

n=506

*P*=0.027

0 24 48 72 96 120 144 168 192 216 240 264 288 312 Months

bp polymorphism dictates the amount of HLA-G protein **B**

present on the cell surface and in the plasma of CLL 100

patients and that the molecule quantitatively and qualita tively modulates T and NK immunocompetence. In con sideration of the immunosuppressive features of HLA-G,

80

patients characterized by a del/del genotype would have

)

%

(

higher levels of HLA-G, would be more immunosup

g

n

pressed and ultimately have a worse clinical outcome. 60

i

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v

This issue was approached by testing the frequency of the

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HLA-G 14 bp polymorphism in 506 CLL patients (*Online*

n

40

o

*Supplementary Table S1*). The genotype frequencies were in

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t

r

o

Hardy-Weinberg equilibrium: 176/506 patients (34.8%)

p

o

r

were del/del homozygous, 81/506 (16%) were ins/ins 20

P

homozygous and the remaining 249/506 (49.2%) were ins/del heterozygous. None of the demographic (age and 0

sHLA-G >31.75 ng/mL

sHLA-G >31.75 ng/mL

n=109

*P*=0.021

sex), clinical (disease stage, splenomegaly, lymph node size), laboratory (lactate dehydrogenase or β2-microglobu lin levels), or molecular variables (CD38, ZAP-70, *IGHV* mutational status, chromosomal aberrations) showed a preferential association with the *HLA-G* polymorphism (*Online Supplementary Table S1*). According to the survival analysis, patients harboring the del/del genotype had a shorter survival than patients harboring the ins/del or ins/ins genotype (*P*=0.027, log-rank test, Figure 5A). Consistently, CLL patients with sHLA-G levels above the third quartile had a shorter survival (median, 63.9 months) than patients with sHLA-G levels 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 having a shorter survival than patients whose plasma lacked inhibitory effects (*P*=0.147, log-rank test, *data not shown*).

Discussion

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

To test whether this may happen in CLL cells, HLA-G expression was assessed in a large cohort of CLL patients with well-defined molecular and clinical characteristics for whom survival data available. The originality of this work is that the analyses of surface or sHLA-G expression were

0 24 48 72 96 120144 168 192 216 240 264 288 312 336 Months

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

accompanied by characterization of the 14 bp polymor phism in the 3’ untranslated region. This choice was dic tated by evidence derived from different models indicat ing that this polymorphism accurately predicts the amount of transcribed protein, either bound to the mem brane or released into biological fluids.

Our results indicate an association between the del/del genotype and increased levels of plasma HLA-G mole cules. This association is apparent only in CLL patients, as plasma from age- and sex-matched controls failed to show 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 more frequently expressed higher levels of the molecule, although statistical significance was 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 mem brane and sHLA-G levels support the view that the main mechanism of generation of sHLA-G is shedding rather than alternative splicing. This was confirmed by an analy sis of HLA-G isoforms, which demonstrated a dominance of sHLA-G1, generated by proteolytic cleavage of mem

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brane HLA-G1. No preference of isoform according to the 14 bp polymorphism was noted, in agreement with previ ously published data.31,32

A reasonable hypothesis to explain this result is that the membrane form represents a transitory step. A conse quence 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 plas ma were higher in CLL patients than in healthy con trols.17,18 A second issue favoring the view that quantifica tion of soluble rather than membrane HLA-G is an inform ative and dependable assay derives from old and new facts linked to the unique lipid structure of the CLL cell sur face,33,34 potentially increasing the instability of the mem brane form. In agreement with this, in myeloma cells mHLA-G is also released from the cell membrane in microparticles.35 These findings suggest that quantification of sHLA-G may be clinically useful and more informative than analysis of the surface of tumor cells.

The second set of results obtained in this study may be considered as tiles creating a tolerogenic mosaic, in which 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 Treg in the circulation. Supportive data come from other models, in which HLA-G is reported to induce Treg. As an example, peripheral blood mononuclear cells exposed to sHLA-G5 acquire regulatory features, inhibit ing allo-proliferative responses exerted by other T lym phocytes. It is also known that patients receiving com bined liver/kidney transplants show high levels of sHLA G5, which correlate with increased percentages of sup pressor T cells.36 Similarly, stem-cell transplanted patients have high levels of sHLA-G5 in the peripheral blood, with a simultaneous expansion of CD4+/CD25+/CD152+ T lym phocytes with suppressive activity. Indirect confirmation in the CLL model may be deduced from data showing that an increase in Treg positively correlates with the presence of clinical and biological features of aggressive disease.37

NK cells obtained from CLL patients have less cytotoxic activity than NK cell populations obtained from healthy donors of a comparable age. This suggests that leukemic cells directly affect NK cell viability and/or activity. This would be achieved through binding HLA-G to the KIR2DL4 ligand, which becomes expressed once NK cells are activated in the presence of interleukin-2. Our working hypothesis is that ectopic expression of HLA-G con tributes to block NK cell functions. With the aim of repro ducing physiological conditions the experiments were per formed using whole plasma instead of purified HLA-G. The assumption was confirmed by incubating NK cells obtained from normal donors with plasma from CLL

HLA-G polymorphism in 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, lysis inhibition was also propor tional to the amount of sHLA-G. It is worth noting that CLL plasma samples with undetectable sHLA-G were able to induce moderate NK cell apoptosis and to reduce NK cell cytotoxicity. These results suggest the presence of fac tors other than sHLA-G that are able to control NK cell activation in CLL. As expected, NK cell function of del/del patients was more impaired than that of ins/del or ins/ins patients.

In conclusion, the data obtained in this study 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, in either a soluble form or bound to the membrane, creates a favor able setting for CLL expansion. As proof of this, evalua tion of the impact of the 14 bp polymorphism on the clin ical outcome of the disease showed that del/del patients have a poorer overall survival than do either ins/del or ins/ins patients.

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

***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 N. 10007; My First AIRC Grant N. 13470 and IG 12754), Fondazione Cariplo, Compagnia di San Paolo (N. PMN\_call\_2012\_0071) and local funds of the University of Turin (ex-60%).*

*The Fondazione Ricerca Molinette provided valuable assistance and support.*

***Authorship and Disclosures***

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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