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# Expression of functional soluble human leucocyte antigen-G molecules in lymphoproliferative disorders

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## Summary

Membrane-bound and soluble human leucocyte antigen-G (sHLA-G) molecules display immunotolerant properties favouring tumour cell escape from immune surveillance. sHLA-G molecules have been detected in several tumour pathologies; this study aimed to evaluate sHLA-G expression in lymphoproliferative disorders. sHLA-G *plasma* level was significantly increased in 110 of 178 newly diagnosed lymphoid proliferations cases i.e. 59% of chronic lymphocytic leukaemia, 65% of B non-Hodgkin lymphomas (NHL) and 58% of T-NHL. To assess the mechanisms involved in this secretion, the differential effect of cytokines was tested in *in vitro* cultures of NHL cells. A significant induction of sHLA-G level was shown in T-NHL in contrast with B-NHL and normal equivalent cells, after cytokine stimulation with (i) interferon$\gamma$ (IFN$\gamma$), interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor, (ii) IL-10 and (iii) transforming growth factor $\beta$. An impact of microenvironment on sHLA-G expression was found in B-NHL as shown by the *in vitro* effect of addition of normal monocytes. Finally, a functional effect of sHLA-G molecules purified from pathologic *plasma* was demonstrated by their strong capacity to inhibit T-cell proliferation at concentrations currently observed during these disorders. These results suggest that functional sHLA-G molecules are upregulated in lymphoproliferative disorders which can support their potential immunomodulatory role during this pathology.

**Keywords:** soluble human leucocyte antigen-G, lymphoproliferative disorders, immunosuppression, chronic lymphocytic leukaemia, non-Hodgkin lymphoma.

Human leucocyte antigen-G (HLA-G) proteins are non-classical class I major histocompatibility complex-1 (MHC-I) molecules that were initially described in the foeto-maternal context, expressed by trophoblast cells (Kovats *et al*, 1990). Unlike classical MHC-I genes, the HLA-G gene (*HLA-G*) exhibits a limited polymorphism (Kirszenbaum *et al*, 1999) and is transcribed into seven isoforms generated by an alternative splicing encoding four membrane-bound proteins (HLA-G1 to HLA-G4) (Ishitani & Geraghty, 1992) and three soluble molecules (HLA-G5 to HLA-G7) (Fujii *et al*, 1994; Paul *et al*, 2000).

Membrane-bound HLA-G molecules, physiologically expressed on thymic and extravillous cytotrophoblast cells (Crisa *et al*, 1997), have also been detected in pathological situations. HLA-G membrane expression has been reported in melanomas (Paul *et al*, 1998), renal (Ibrahim *et al*, 2001), breast (Lefebvre *et al*, 2002) and lung carcinomas (Urosevic *et al*, 2001), gliomas (Wiendl *et al*, 2002) and cutaneous lymphomas (Urosevic *et al*, 2002) but also during inflammatory (Wiendl *et al*, 2000) and viral diseases (Lozano *et al*, 2002). HLA-G expression can be induced by different cytokines combinations as demonstrated in several models : (i) the combination of interferon-$\gamma$ (IFN$\gamma$) (IL)-2/granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to increase HLA-G expression on the myelomonocytic cell line U937 (Amiot *et al*, 1998) (ii) IFN$\gamma$ was able to

 
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modulate HLA-G expression in myoblasts (Wiendl *et al*, 2000), gliomas (Wiendl *et al*, 2002), ovarian carcinoma (Malmberg *et al*, 2002) and (iii) an association between IL–10 and HLA-G expression has been shown, *in vivo*, in lung cancers (Urosevic *et al*, 2001) and in cutaneous lymphomas (Urosevic *et al*, 2002). Soluble HLA-G (sHLA-G) molecules, consisting of HLA-G5, HLA-G6 and HLA-G7, are characterised by the persistence of intron 4 (HLA-G5 and G6) (Fujii *et al*, 1994) or intron 2 (HLA-G7) (Paul *et al*, 2000) in the main transcript leading to the generation of truncated molecules. Another kind of soluble molecule exists, which consists of shedding forms of membrane molecules, such as sHLA-G1 generated by proteolytic cleavage of HLA-G1 membrane-bound isoform using a similar mechanism to classical HLA class I molecules (Park *et al*, 2004). sHLA-G molecules have been detected in amniotic fluid and at basal level in the *plasma* of healthy subjects without difference between sexes (Rebmann *et al*, 1999). Moreover, sHLA-G molecules seem to be more frequently expressed in pathological conditions than membrane-bound isoforms. An increase of sHLA-G molecules has been described in *plasma* of melanoma patients (Ugurel *et al*, 2001), in ascites of breast and ovarian carcinomas (Singer *et al*, 2003) and also during multiple sclerosis (Fainardi *et al*, 2003).

HLA-G plays an important role during pregnancy but more generally in regulation of the immune response. Indeed, these molecules display immunotolerant properties, which could contribute to the immune escape of tumour or virus-infected cells. The immunomodulating properties, exhibited by both membrane-bound and soluble isoforms, are (i) natural killer (NK) and T-cell cytolytic inhibition (Wiendl *et al*, 2003), (ii) induction of CD95/CD95L-mediated apoptosis on NK and T8 cells (Fournel *et al*, 2000) and (iii) inhibition of T4-cell proliferation in response to allogeneic stimulation (Lila *et al*, 2001; Le Friec *et al*, 2003). These immunomodulating functions are mediated through interaction with inhibitory receptors: KIR2DL4, Ig-like transcript-2 (ILT-2) (CD85 j) and ILT-4 (CD85d) expressed by several immune cells (Shiroishi *et al*, 2003). Studies on the impact of HLA-G expression have concerned essentially solid tumours and few data are available on haematopoietic malignancies. We previously showed dissociation between the rarity of HLA-G cell surface expression and the frequency of transcript expression. In addition, we found a significant increase of sHLA-G *plasma* level in non-Hodgkin lymphomas (NHL) and chronic lymphocytic leukaemia (CLL) patients compared with healthy patients (Sebti *et al*, 2003). Lymphoproliferative disorders are characterised by malignant proliferations of B or T lymphocytes at different stages of differentiation, classified according to the World Health Organisation (WHO) classification using histological, immunophenotypical, molecular and cytogenetical criteria. This heterogeneous group of diseases consists of (i) CLL defined by a clonal proliferation of mature B-cells occurring in blood and bone marrow and (ii) NHL corresponding to B- or T-cells proliferations taking place in lymph nodes or spleen.

The present, broader prospective study investigated sHLA-G expression in lymphoproliferative disorders. We showed a significant increase of sHLA-G *plasma* levels in patients suffering from lymphoproliferative disorders, as well as an induction of sHLA-G expression by cytokines in *in vitro* cultures of T-tumour cells, in contrast to B-tumour cells, suggesting that distinct factors are implicated in sHLA-G secretion between B- and T-NHL. Furthermore, we demonstrated, for the first time, a functional effect of sHLA-G molecules on immune response in the *plasma* of patients suffering from lymphoproliferative disorders.

## Materials and methods

### *Patients*

Between January 2002 and September 2004, 178 patients with lymphoproliferative disorders were included in this prospective study and analysed for their sHLA-G *plasma* levels. These patients were treated in the Department of Clinical Haematology in Rennes Hospital and were classified according to the WHO classification. *Plasma* of 139 mature B-NHL, 12 mature T-NHL and 27 B-CLL were obtained after centrifugation at 1000 ***g***, 4°C of ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood. *Plasma* from healthy subjects ($n = 37$), provided by the Etablissement Francais du Sang Bretagne, were used as controls. The different histological subtypes of the tested pathologies are specified in Table I.

**Table I.** Different histological subtypes of B and T non-Hodgkin lymphoma (NHL) studied.

| |
|---|
| (**a**) B-NHL ($n = 139$) |
| High grade ($n = 93$) |
| Acute transformation of follicular lymphoma ($n = 7$) |
| Burkitt lymphoma ($n = 2$) |
| Diffuse large B-cell lymphoma ($n = 63$) |
| Mantle cell lymphoma ($n = 9$) |
| Other high-grade lymphomas ($n = 12$) |
| Low grade ($n = 46$) |
| Follicular lymphoma ($n = 30$) |
| MALT lymphoma ($n = 5$) |
| Other low-grade lymphomas ($n = 11$) |
| (**b**) T-NHL ($n = 12$) |
| T-lymphoblastic lymphoma ($n = 1$) |
| Sezary syndrome ($n = 4$) |
| Anaplastic large cell lymphoma ($n = 3$) |
| Intestinal T lymphoma ($n = 2$) |
| Peripheral T lymphoma ($n = 2$) |

(a) represents the different histological subtype and grade of B-NHL analysed during this study, (b) represents the different histological subtype of T-NHL analysed. MALT, mucosa-associated lymphoid tissue.

 

### *Cytokines*

Interleukin-2 (IL-2), IL-4, IL-6, IL-10 IFNγ_and transforming growth factor (TGF) β were supplied by Tebu Peprotech (Le Perray en Yvelines, France). Granulocyte–macrophage colony-stimulating factor (GM-CSF) was provided by Gentaur (Brussels, Belgium). Enhancer CD40-L was supplied by Alexis (Ilkirsch, France).

### *Antibodies*

MEM-G/9 specific for HLA-G1 and HLA-G5 molecules were purchased from Exbio (Praha, Czech Republic). Horseradish peroxidase (HRP)-antiβ2-microglobulin was supplied by DAKO (Trappes, France). 5A6G7 (CEA, Paris) recognises the intron 4-encoded epitope present in HLA-G5 and HLA-G6 molecules. W6/32-biotin antibody, specific for HLA class I heavy chain molecules, was purchased from Interchim (Montlucon, France).

### *Cell lines*

LCL721.221-G5, a B-lymphoblastoïd cell line transfected with HLA-G5, was kindly provided by D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). HK is a follicular dendritic cell (FDC)-like cell line, kindly provided by YS.Choi (Laboratory of Cellular immunology, New Orleans, USA).

## Methods

### *Specific soluble HLA-G enzyme-linked immunosorbent assay*

sHLA-G concentration was evaluated by a specific sandwich enzyme-linked immunosorbent assay (ELISA) using MEM-G/9 and HRP-antiβ2-microglobulin as capture and detection antibodies respectively.

Briefly, microtitre plates (Corning costar, Issy-les-Moulineaux, France) were coated overnight at 4°C with MEM-G/9 (10 μg/ml). Plates were then saturated with phosphate-buffered saline (PBS), 2% bovine serum albumin for 30 min. Triplicate cell culture supernatants or *plasma* were incubated for 1 h. After 1 h incubation with detection antibody (HRP-antiβ2-microglobulin), plates were incubated for 30 min in the dark with the substrate (ortho-phenyllenediaminedihydrochloride; DAKO). Between each step, plates were washed 3 times with PBS 0·05% Tween. All the incubations were performed at room temperature. After addition of $H_2SO_4$ (1 N), optical densities were measured at 490 nm. This ELISA detected both HLA-G5 molecules and sHLA-G1 molecules (HLA-G1 shedding form).

Another sandwich ELISA was performed by the Service de Recherches en Hémato-Immunologie of CEA in Paris, using 5A6G7 at 5 μg/ml as capture antibody and W6/32-biotin (Interchim) + streptavidin-HRP as detection antibody (Amersham, Pharmacia Biosciences, Orsay, France). For this ELISA, tetramethylbenzidine (Sigma-Aldrich, Saint-Quentin Fallavier, France) was used as substrate and optical densities were measured at 450 nm. This ELISA specifically detected soluble HLA-G5 (sHLA-G5) molecules. Recombinant HLA-G5 molecules were used as standard reagent for the determination of sHLA-G level in both cases. These two-specific ELISA were validated by the Wet Workshop for Quantification of sHLA-G (Rebmann *et al*, 2005).

### *Cytokine quantification*

Determination of IFNγ, tumour necrosis factor-α (TNFα), IL-2, IL-4, IL-6 and IL-10 *plasma* concentrations was performed in nine cases of T-NHL, 59 cases of B-NHL and 10 cases of controls using a CBA (Cytometric bead array, Pharmingen, Le Pont de Claix, France) kit according to the manufacturer's instructions. The results were expressed as pg/ml.

### *Cell cultures*

Lymph nodes cells from 14 cases of T-NHL and 12 cases of B-NHL (six follicular and six diffuse large B-cell lymphomas) were cultured for 48 h in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mmol/l glutamine, 1% sodium pyruvate and 1% penicillin–streptomycin in six-well plates ($1 \times 10^6$/ml). Cell samples constituted more than 80% tumour cells. The different conditions tested were as follows: (i) IL-2 (220 U/ml), IFNγ (100 U/ml) and GM-CSF (100 U/ml), previously tested on the myelomonocytic cell line U937 (Amiot *et al*, 1998) (ii) IL-10 (50 ng/ml); (iii) TGFβ 25 ng/ml) which are immunosuppressive cytokines; and (iv) cocultures with the enhancers CD40L (1 mg/ml), IL-2 (20 U/ml), IL-4 (50 ng/ml) and IL-6 (50 ng/ml), which mimic a T signal.

Similar stimulations were performed on five samples of peripheral blood mononuclear cells (PBMC), monocytes, total lymphocytes and three samples of purified B and T lymphocytes from healthy donors. PBMC were separated by Ficoll-Hypaque density gradient centrifugation (Invitrogen, Cergy-Pontoise, France). Monocytes, B and T lymphocytes were isolated from PBMC of healthy subjects, using CD14, CD19 and CD3 human microbeads respectively (Miltenyi Biotech, Paris, France) according to the manufacturer's instructions.

Cocultures were performed with monocytes from healthy subjects and PBMC from B-NHL patients at the different ratios indicated: 0:1, 1:0, 1:1; 1:2 and 1:4. Cocultures were also performed between the HK cell line and PBMC from B-NHL patients with or without different combinations of cytokines previously described.

### *sHLA-G molecules purification*

Soluble HLA-G molecules were purified from the plasma of patients with a lymphoproliferative disorder using Hi Trap

 

NHS 1 ml (*N*-hydroxysuccimide)-activated high performance columns (Amersham, Saclay, France) coated with MEM-G/9 mAb (5 mg/ml) as previously described for W6/32 (Le Friec *et al*, 2003). The columns were blocked with 100 mmol/l ethanolamine pH 9·0, overnight before use. After washing with PBS, pooled *plasma* were incubated in the columns overnight at 4°C. Bound antigens were eluted with 0·1 mol/l glycine buffer pH 2·6 neutralised with 1 mol/m Tris-buffer solution, pH 7·5. The presence of sHLA-G in the eluted fractions was determined by ELISA. Positive fractions obtained were pooled and sHLA-G concentration was then determinate by ELISA. Lastly, sHLA-G molecules were concentrated using a vacuum centrifuge before use in functional experiments. The absence of contamination by classical class I molecules was checked by ELISA.

### *Mixed leucocyte reactions*

Peripheral blood mononuclear cells were isolated from the heparinised blood of healthy donors by Ficoll-Hypaque density gradient centrifugation. For allogeneic stimulation experiments, $1 \times 10^5$ responder PBMC were incubated with $1 \times 10^5$ γ-irradiated stimulator cells in 200 μl of RPMI-1640 medium in round-bottomed 96-well microtitre plates. The coculture was set in the presence or absence of sHLA-G purified from patient plasma. Different concentrations of sHLA-G were tested: 10, 25, 50, 100, 150, and 200 ng/ml. After 5 d of culture, cells were pulsed with 37 kBq [$^3$H] thymidine per well for 18 h. Cells were then harvested onto filters (Perkin Elmer, Boston, USA) and tritium incorporation was measured on a beta counter (Perkin Elmer). All conditions were tested in triplicate.

### *Statistical analysis and clinical-biological correlations*

Student's *t*-test, Mann–Whitney *U*-test and the Wilcoxon matched-pair signed rank test were the different statistical tests used in this study. Clinical-biological criteria correlations with HLA-G levels were evaluated using a one-way analysis of variance (ANOVA). Tests were considered significant when $P < 0{\cdot}05$.

## Results

### *Increase of sHLA-G plasma level in lymphoproliferative disorders*

sHLA-G plasma level was assessed in 139 B-NHL, 12 T-NHL and 27 CLL patients and compared with 37 controls. The mean sHLA-G plasma level in healthy subjects was 16·23 ± 6·17 ng/ml. An increased sHLA-G level threshold was defined as the mean of the controls plasma levels +2SD (>28·6 ng/ml). A significant increase of sHLA-G level was observed in 91 of 139 cases of B-NHL (50·26 ± 34·66 ng/ml $n = 139$, $P < 0{\cdot}0001$), 7 of 12 cases of T-NHL (54·92 ± 52·16 ng/ml $n = 12$, $P < 0{\cdot}0005$) and 16 of 27 cases of CLL (45·65 ± 29·21 ng/ml $n = 27$, $P < 0{\cdot}0001$) according to Student's *t*-test (Fig 1). The range of sHLA-G levels was: 2·6–171·7 ng/ml for B-NHL, 6·7–181·4 ng/ml for T-NHL and 6·5–107·4 ng/ml for CLL.

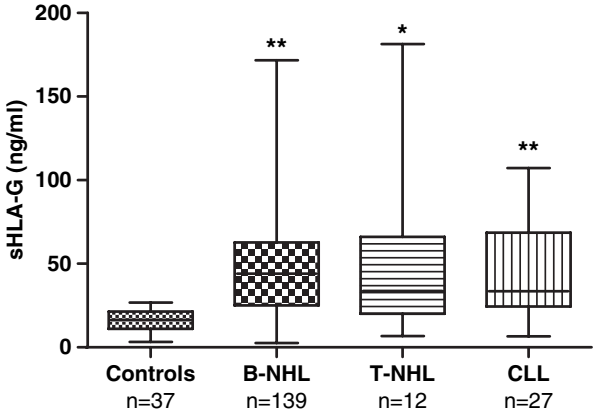


**Fig 1.** Soluble human leucocyte antigen-G (sHLA-G) plasma level in the different lymphoproliferative disorder subtypes compared with controls. sHLA-G level is shown for the different pathologies and compared with healthy subjects. The upper and lower limits of each box represent the interquartile range (25–75th percentile) and the horizontal line within each box shows the median value. sHLA-G level was significantly increased in 91 of 139 cases of B non-Hodgkin lymphomas (NHL), in 7 of 12 cases of T-NHL and in 16 of 27 cases of chronic lymphocytic leukaemia (CLL) according to Student's *t*-test. * and **indicates $P < 0{\cdot}0005$; **$P < 0{\cdot}0001$ respectively.

### *Comparison of sHLA-G5 and sHLA-G1 plasma level in lymphoproliferative disorders*

Soluble HLA-G5-specific plasma level was assessed by ELISA using 5A6G7 and W6/32-biotin on a smaller sample of lymphoproliferative disorders (27 B-NHL and 4 T-NHL). The mean HLA-G5 level was 54·59 ± 28·89 ng/ml in B-NHL and 43·42 ± 30·67 ng/ml in T-NHL. Using similar methodology to that previously described for sHLA-G, the positivity threshold of HLA-G5 was set at 37 ng/ml. Thus, an increase of HLA-G5 was observed in 19 of 27 cases of B-NHL and in 3 of 4 cases of T-NHL. The mean HLA-G5 plasma level was then compared with the plasma level of sHLA-G, composed of HLA-G5 and sHLA-G1, assessed by MEM-G/9/Anti-β2m-biotin sandwich ELISA. The mean level of HLA-G5 and sHLA-G were similar in B-NHL and T-NHL (Fig 2). In addition, 5 of 31 cases showed similar HLA-G5 and sHLA-G levels, 16 exhibited higher levels of HLA-G5 and 10 showed higher levels of sHLA-G.

### *Cytokine profile of B- and T-NHL*

Interferon-γ, TNFα, IL-10, IL-6, IL-4 and IL-2 plasma levels were determined in 9 of 12 cases of T-NHL and 59 of 139 cases of B-NHL and compared with 10 controls. The selected patients comprised half of the cases that exhibited a high-sHLA-G *plasma* level and the other half exhibited a

 

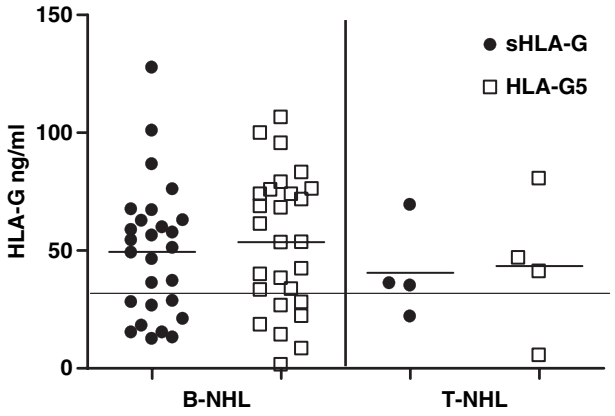


**Fig 2.** Comparison of human leucocyte antigen-G5 (HLA-G5) and sHLA-G plasma level in lymphoproliferative disorders sHLA-G (HLA-G5 + sHLA-G1) (●) and HLA-G5 (□) levels are shown for B non-Hodgkin lymphomas (NHL) and T-NHL. Each symbol represents the plasma level of one case measured by a specific sandwich ELISA, using MEM-G/9/β2 m and 5A6G7/biotinylated W6/32 for sHLA-G and HLA-G5 detection. Black horizontal bars indicate the mean sHLA-G plasma levels. The mean levels of HLA-G5 and sHLA-G appear comparable in B-NHL and T-NHL and are respectively: 54·59 ± 28·89 ng/ml vs. 49·44 ± 28·23 ng/ml in B-NHL and 43·42 ± 30·67 ng/ml vs. 40·56 ± 20·2 ng/ml in T-NHL

low-sHLA-G *plasma* level. Comparison of cytokine plasma levels between controls and patients was performed by Mann–Whitney *U*-test. Plasma levels of IL-6 and IL-10 were significantly increased ($P < 0{\cdot}0005$) in T-NHL (IL-6: 238·34 ± 147·11 pg/ml; IL-10: 190·38 ± 129·47 pg/ml) and B-NHL (IL-6: 39·11 ± 8·65 pg/ml; IL-10: 155·96 ± 70·81 pg/ml) when compared with controls (IL-6: 0 ± 0 pg/ml; IL-10: 3·28 ± 0·91 pg/ml) with higher cytokine plasma levels shown in T-NHL (Table II). Furthermore, a significant increase of IL-4 ($P < 0{\cdot}05$) was only found in T-NHL (8·98 ± 2·27 pg/ml) compared with controls. (3·19 ± 1·366 pg/ml). No difference was found in the different pathologies between the group with high- and low-sHLA-G *plasma* level (data not shown).

**Table II.** B-NHL and T-NHL cytokine profile.

| | Controls | B-NHL | T-NHL |
|---|---|---|---|
| Mean ± SE | | | |
| IFNγ | 8·19 ± 2·53 | 62·87 ± 40·17 | 147·46 ± 91·18 |
| TNFα | 3·46 ± 0·54 | 4·63 ± 0·36 | 3·56 ± 1·21 |
| IL-2 | 3·23 ± 0·74 | 20·84 ± 17·11 | 3·6 ± 0·70 |
| IL-4 | 3·19 ± 1·366 | 5·66 ± 0·977 | 8·98 ± 2·27 * |
| IL-6 | 0 ± 0 | 39·11 ± 8·65 ** | 238·34 ± 147·11 *** |
| IL-10 | 3·28 ± 0·91 | 155·96 ± 70·81 *** | 190·38 ± 129·47 *** |

This table represents the mean plasma levels of interferon γ (IFNγ), tumour necrosis factor α (TNFα), interleukin (IL)-2, IL–10, IL–6 and IL–4 in 10 controls, 59 cases of B-NHL and 9 cases of T-NHL. The results are expressed as mean ± Standard Error (SE) in pg/ml. Statistical comparison of cytokine plasma level was performed using Mann-Whitney U test. Tests were considered significant when $P < 0.05$. IL–4 was significantly increased in T-NHL (* $p < 0.05$) whereas IL–6 and IL–10 were increased in both types of NHL (** $p < 0.0005$ and *** $p < 0.0001$).

IFN, interferon; IL, interleukin; NHL, non-Hodgkin lymphomas; TNF, tumor necrosis factor.

### *Differential effects of cytokines on sHLA-G expression on B- and T-lymphomatous cells*

Lymph node cells from B- and T-NHL samples were cultured in the presence or absence of cytokines for 48 h as described above. sHLA-G level was then assayed in the culture supernatant by a specific sandwich ELISA. Increased sHLA-G expression was observed after cytokine stimulation in T-NHL *in vitro* cultures. In 14 T-NHL cases, the mean sHLA-G level was significantly increased, by 2·3- and 2·4-fold, after IFNγ + IL-2 + GM-CSF and IL-10 treatments respectively ($P < 0{\cdot}005$), and by 2·8-fold in all of the six tested cases stimulated with TGFβ ($P < 0{\cdot}05$; the Wilcoxon matched-pair signed rank test) (Fig 3A). Contrary to T-NHL, none of these treatments, IFNγ + IL-2 + GM-CSF or IL-10 ($n = 12$) or TGFβ $n = 5$) was able to modulate sHLA-G secretion in B-NHL cases (Fig 3B). Normal equivalent cells, such as PBMC ($n = 5$), monocytes ($P = 5$), total lymphocytes ($n = 5$) and purified B ($P = 3$) and T lymphocytes ($n = 3$), were also cultured in similar conditions and no effect of cytokine stimulation was observed on sHLA-G secretion in all cases (Fig 3C).

### *Effect of microenvironment on sHLA-G expression by B-lymphomatous cells*

As no effect of cytokine stimulation was observed on sHLA-G secretion in B-NHL *in vitro* cultures, additional experiments were performed to determine the impact of the tumour microenvironment. Impact of normal residual cells was firstly addressed. The addition of normal haematopoietic cells, such as monocytes, at different ratios (residual cell/B-NHL cell: 0:1, 1:0, 1:1, 1: 2 and 1: 4) significantly increased sHLA-G secretion ($n = 6$, $P < 0{\cdot}05$; the Wilcoxon matched-pair signed rank test) when compared with control conditions (B-NHL cells or monocytes alone) (Fig 4A). Similar experiments in the presence or in absence of cytokines (as previously used) showed no significant difference when compared with the control situation without cytokines (data not shown). Moreover, to evaluate the effect of T-residual cells, cytokines mimicking the T signal, i.e., IL-2, IL-4, IL-6 and CD40L, were added to B-NHL cells ($n = 6$); no effect on sHLA-G secretion was observed (Fig 4B).

In order to assess the effect of stromal cells, centrogerminative B-NHL cells were cocultured with HK cells (an FDC-like cell line), either alone or with B-NHL cells in the presence or absence of cytokines ($n = 3$). None of the culture conditions described influenced sHLA-G expression by B-NHL cells (Fig 4C).

 

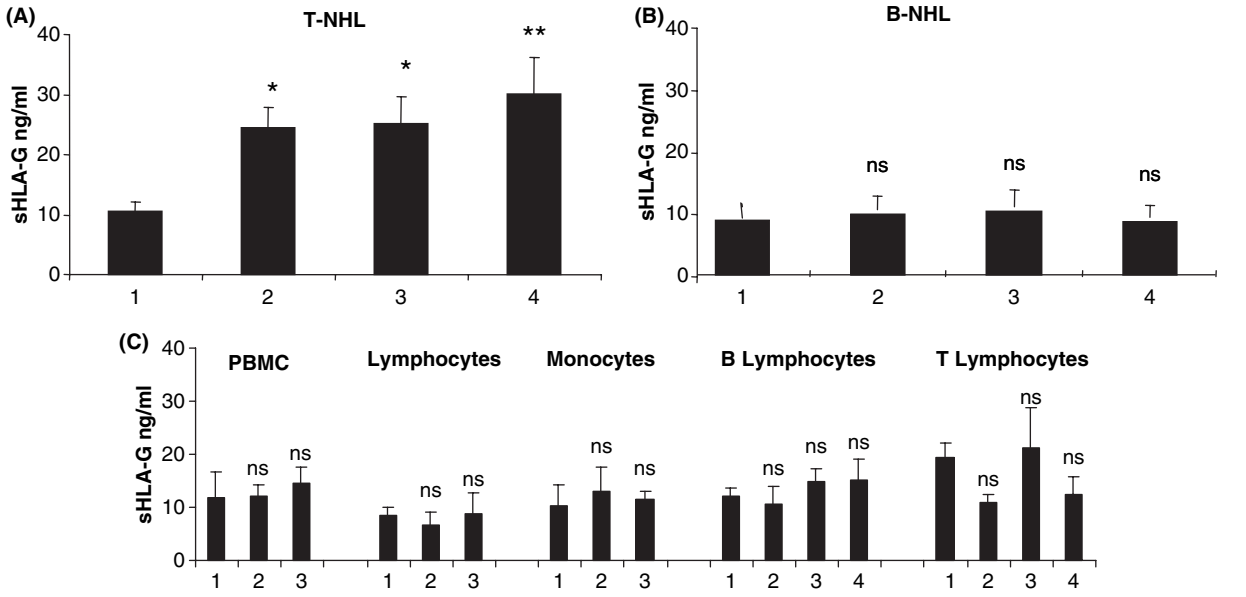


**Fig 3.** Effect of cytokines on soluble human leucocyte antigen-G5 (sHLA-G5) expression on tumour cells from B non-Hodgkin leucocyte (NHL) and T-NHL. Cells from T-NHL ($n = 14$), B-NHL ($n = 12$) patients, and normal equivalent cells i.e.: peripheral blood mononuclear cells, monocytes, total lymphocytes ($n = 5$) and B- and T-purified lymphocytes ($n = 3$) were cultured for 48 h in different conditions: (1) Control, (2) interferon γ, IL−2 + granulocyte–macrophage colony-stimulating factor (GM-CSF) (IFNγ + IL-2 + GM-CSF), (3) IL-10 (4) transforming growth factor β (TGFβ). Histograms represent sHLA-G levels (ng/ml) ±SE, measured by specific ELISA, for the different conditions. (A) Significant increase of sHLA-G level was observed in T-NHL cultures in presence of the different cytokine combinations. * and ** indicates $P < 0{\cdot}05$ and $P < 0{\cdot}005$ respectively. (B) No effect of cytokines was observed on sHLA-G secretion in B-NHL cultures. (C) No effect of cytokines was observed on sHLA-G secretion in any of the normal equivalent cell cultures.

### *Functional effect of sHLA-G molecules purified from plasma of patient*

Soluble HLA-G molecules were purified from the plasma of patients with lymphoproliferative disorders using a specific affinity column coupled with MEM-G/9. A range of sHLA-G concentrations (10–200 ng/ml) was tested in mixed leucocyte reactions (MLR) and a gradual inhibition of T-cell alloproliferation was observed ($n = 3$) (Fig 5). A control was performed with the negative fractions of the purification process to check for the absence of toxicity in the purified eluate (data not shown). We observed a range of T-cell proliferation inhibition, from 17% (10 ng/ml) to 95% (200 ng/ml), reaching a plateau at 100 ng/ml with a strong inhibition (80%). Thus, a notable functional effect of patient *plasma* sHLA-G was shown at concentrations frequently observed in lymphoproliferative disorders (from 50 ng/ml).

### *Clinico-biological correlations*

ANOVA analysis between sHLA-G levels of patient's *plasma* and relevant clinico-biological criteria in lymphoproliferative disorders were performed. No correlation was found with histological classification, grade, IPI score, lymphocytosis, remission delay, relapse occurring or death in NHL. In CLL, no correlation was found with Binet classification, CD38 expression, lymphocytosis, remission delay, relapse occurring or death. However hypogammaglobulemia, which is representative of immunosuppressive status, was correlated with high-sHLA-G level (Fig 6).

## Discussion

Human leucocyte antigen-G molecules are involved in immune suppression and could play a role in tumour cell escape from immune surveillance. Membrane-bound isoforms have been described in several cancers (Paul *et al*, 1998; Ibrahim *et al*, 2001; Urosevic *et al*, 2001; Wiendl *et al*, 2002) and up to now, soluble isoforms have been less well-studied in tumour pathologies. We have previously described a rare cell surface HLA-G expression in haematopoietic malignancies (Amiot *et al*, 1998, 2003) contrasting with a frequent transcription in cells from monocytic and lymphoid lineages (Amiot *et al*, 1996a,b). Moreover, recently, we have found a significantly increased sHLA-G *plasma* level in acute leukaemia (Gros *et al*, 2006). Consequently, this study investigated the role of sHLA-G expression in lymphoproliferative disorders. An increased sHLA-G *plasma* level was found in a broad cohort of patients suffering from lymphoproliferative disorders, i.e. 59% of CLL, 65% of B-NHL and 58% of T-NHL. To analyse the mechanisms involved in sHLA-G secretion, we studied *in vitro* and *in vivo* implication of cytokines on sHLA-G secretion in lymphoid proliferations. Finally, to assess the functional relevance of this secretion, we determined the effect of purified patient plasma sHLA-G on T-cell proliferation.

 

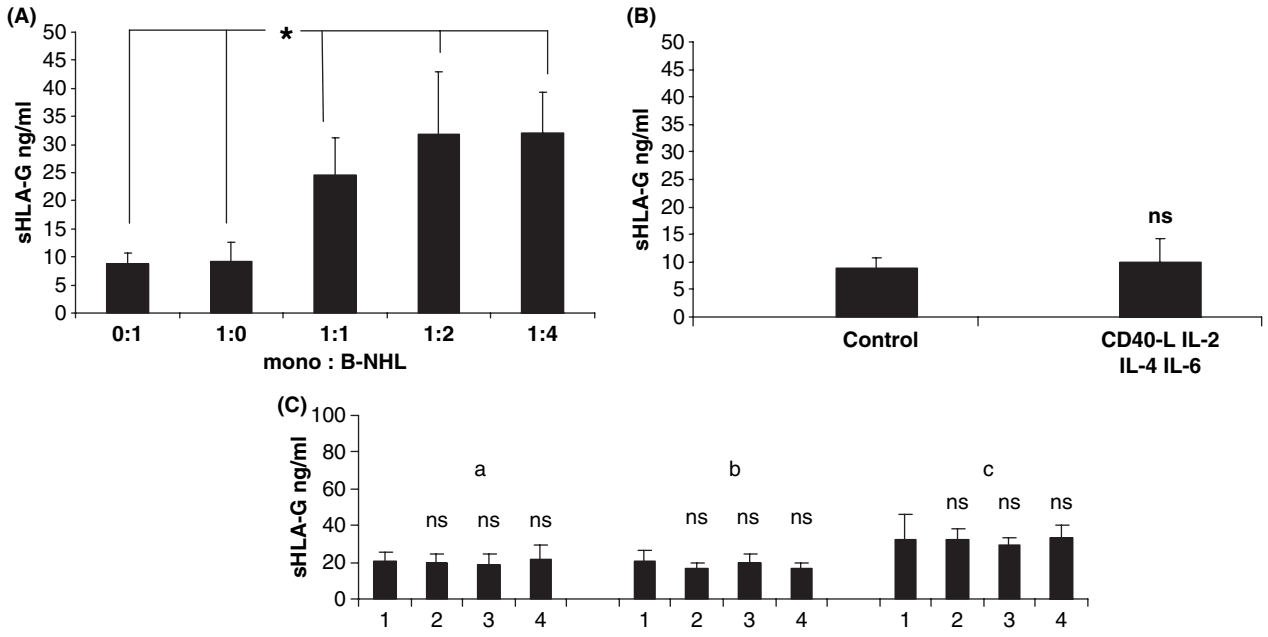


**Fig 4.** Effect of microenvironment cells on soluble human leucocyte antigen-G (sHLA-G) expression by B non-Hodgkin leucocyte (NHL) cells. Cocultures of monocytes and mononuclear cells from B-NHL were performed at different ratios: 0:1, 1:0, 1:1, 1:2, and 1:4 for 48 h. Histograms represent sHLA-G levels (ng/ml) ±SE measured by specific ELISA. (A) Addition of monocytes to B-NHL cells significantly increased sHLA-G expression compared with isolated monocytes or B-NHL. ($n = 6$). *$P < 0{\cdot}05$. (B) No effect of stimulation by a T-signal (IL-2, IL-4, IL-6 and CD40-L) was observed on sHLA-G secretion on B-NHL cultures ($n = 6$). (C) (a) and (b) represent B-NHL and HK cell lines cultured in the presence or in absence of cytokines respectively: (1) Control, (2) (interferon (IFN)γ, IL−2 + granulocyte–macrophage colony-stimulating factor (GM-CSF) (IFNγ + IL-2 + GM-CSF), (3) IL-10 (4) transforming growth factor β (TGFβ) (c) coculture of B-NHL cells with HK in presence or in absence of the same cytokines displays no variation of sHLA-G expression ($n = 3$).

The sHLA-G level observed in lymphoproliferative disorders was approximately 3 times more elevated when compared with controls, with a strong variation according to the pathology.

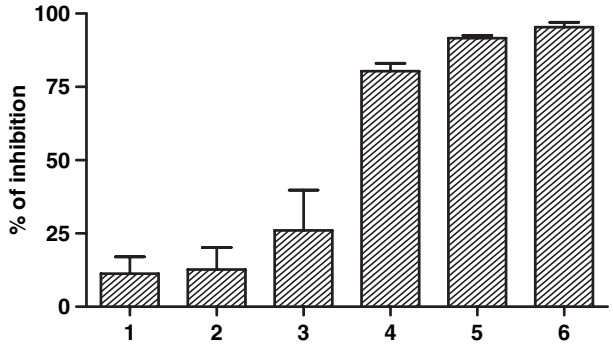


**Fig 5.** Evaluation of the functional effect of soluble human leucocyte antigen-G (sHLA-G) purified from patient plasma. Several concentrations of soluble HLA-G, purified with specific affinity columns coated with MEM-G/9, from patients plasma were tested in mixed leucocyte reactions: 10 ng/ml (1), 25 ng/ml (2), 50 ng/ml (3), 100 ng/ml (4), 150 ng/ml (5) and 200 ng/ml (6). The inhibition of T-cell alloproliferation in each condition is shown as histograms ±SE ($n = 3$). A progressive inhibition of T-cell alloproliferation was increased from, 11% to 95%, according to tested sHLA-G concentrations (10–200 ng/ml).

Moreover, a large individual variation was observed within a given pathology type. These findings are consistent with our previous study on a smaller cohort of patients (Sebti *et al*, 2003). The mean levels appeared similar in B and T pathologies and all histological subtypes. The frequency of sHLA-G increase, observed in 60% of cases, contrasted with the rarity of HLA-G membrane-bound expression previously reported (Amiot *et al*, 2003). These data are in accordance with a previous report describing HLA-G expression in 50% of cutaneous T-lymphomas by immunohistochemistry using the anti-HLA-G antibody 4H84, which recognises all isoforms including soluble isoforms (Urosevic *et al*, 2002). More recently, it has been shown that HLA-G cell surface expression is significantly associated with unfavourable outcome and immunodeficiency in CLL (Nuckel *et al*, 2005). However, this present work (data not shown) and our previous study (Amiot *et al*, 1998) did not find any HLA-G membrane-bound expression in CLL, although a frequent secretion of sHLA-G was detected in this pathology.

To complete this analysis, a sub-group of patients (B and T) were also studied using a HLA-G5-specific ELISA. Although the mean sHLA-G (HLA-G5 + sHLA-G1) and HLA-G5 levels appear similar, approximately 33% of cases exhibited higher levels of sHLA-G. These latter cases strongly suggest the presence of shedding HLA-G1 form in lymphoproliferative

 

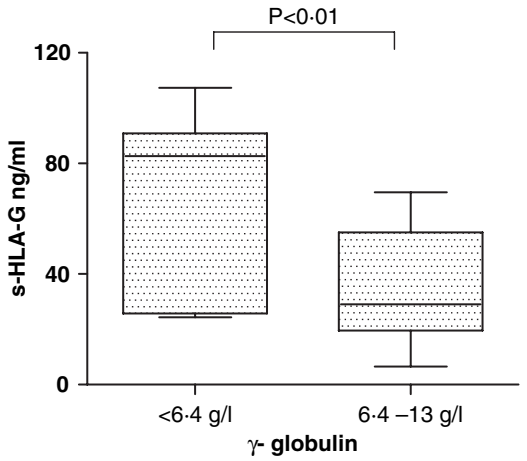


**Fig 6.** Correlation of plasma soluble human leucocyte antigen-G (sHLA-G) levels with γ-globulin level in chronic lymphocytic leukaemia. Plasma sHLA-G levels are shown in patients with hypogammaglobulemia (<6·4 g/l) and patients presenting a normal level of γ-globulin (6·4–13 g/l). Upper and lower limits represent the interquartile range (25–75th percentile) and the horizontal line indicates median value. ANOVA showed a significant difference in sHLA-G levels between patients with and without hypogammaglobulemia ($P < 0·01$).

disorders. Indeed, it has been shown that sHLA-G molecules, such as HLA class I molecules, could be generated by a metalloproteinase-dependent shedding mechanism (Park *et al*, 2004). Moreover, it has been shown that matrix metalloproteinases (MMPs), particularly MMP-9, are increased in NHL and that this expression is linked to aggressive pathology, characterised by poor overall survival (Sakata *et al*, 2004). However, 50% of cases showed a higher level of HLA-G5, which could be explained by the specificity of antibodies. Indeed, 5A6G7 recognises intron 4-encoded epitope whereas MEM-G/9 reacts with native form of human HLA-G1 on the cell surface, as well as with the sHLA-G5 isoform combined with β2-microglobulin, suggesting that 5A6G7 has a stronger affinity for HLA-G5. Altogether, these results indicate that sHLA-G resulting from alternative splicing is the predominant secreted form in lymphoproliferative disorders. Therefore, this data confirms the existence of HLA-G5 expression which is in conflict with recent publications (Blaschitz *et al*, 2005; Sargent, 2005).

In addition, the increased levels of IL-6, IL-10 and IL-4 shown in T-NHL, and of IL-6 and IL-10 in B-NHL, give rise to a T-helper cell type 2 (Th2) cytokine pattern in NHL. This is in accordance with the literature, which reported an elevation of IL-10 and IL-6 *plasma* levels in NHL (el-Far *et al*, 2004) and also a secretion of IL-4, IL-6 and IL-10 by T-lymphomatous cells in cutaneous lymphomas (Echchakir *et al*, 2000). However, no relationship was observed *in vivo* between these Th2 cytokine profile, notably IL-10, and sHLA-G *plasma* level. This discrepancy could be explained by the fact that the cytokine quantification was performed in peripheral blood whereas previous studies, which showed a relationship between HLA-G and IL-10 were performed *in situ* by immunohistochemistry (Urosevic *et al*, 2002).

The role of cytokines on sHLA-G expression has been reported in different studies. Our previous work has shown an induction of HLA-G expression on the U937 myelomonocytic cell line after stimulation with a combination of IFNγ, IL2, GM-CSF (Amiot *et al*, 1998). In the present study, IL-10 and TGFβ, two immunosuppressive cytokines, were also investigated with regard to sHLA-G expression *in vitro* on B- and T-NHL cells. Our results revealed a significant increase of sHLA-G expression in, *in vitro,* models of T-NHL cultured with all the different cytokine combinations. TGFβ seems to induce the strongest sHLA-G expression increase on T-NHL cells. This role of TGFβ has not been previously reported but could be explained by the immunosuppressive properties of that cytokine, which is known to induce tolerance in peripheral lymphocytes (Nakamura *et al*, 2004). This cytokine is also secreted in some cancers, allowing immune escape (de Visser & Kast, 1999). These findings, together with a previous study demonstrating an increase of TGF-β1 production in U937 myelomonocytic cell line stimulated by recombinant sHLA-G molecules (McIntire *et al*, 2004), are in favour of a potential-regulation loop between TGFβ and sHLA-G molecules expression. In addition, IL-10 elevated sHLA-G secretion but did not appear to be more significant than the effect of the other tested cytokines. IL-10 has been previously shown to induce HLA-G expression in human trophoblasts and monocytes in physiological situations (Moreau *et al*, 1999) and was associated with HLA-G expression in pathologies such as cutaneous lymphomas and lung cancers (Urosevic *et al*, 2001, 2002). However, in accordance with our data, no association between IL-10 and sHLA-G was found in U937 myelomonocytic cell lines (McIntire *et al*, 2004). Furthermore, no effect of cytokines has been shown on normal T-cells, suggesting a specific role in the tumour context.

Different results were obtained for B-NHL, the different combinations of cytokines displayed no effect on sHLA-G expression suggesting the implication of other factors in the microenvironment. B lymphomagenesis, similarly to normal B differentiation, is supported by the lymph node microenvironment, which is composed of haematopoietic cells associated with a stromal compartment. Moreover, tumour proliferation developed from B-lymphoid cells in germinal centre B lymphomas is organised *in vivo* into nodules around follicular dendritic cells. The coculture of germinal centre B-NHL cells with HK, a FDC-like cell line, did not show any variation of sHLA-G secretion. In addition, the haematopoietic residual compartment is mainly composed of T-lymphocytes and cells of monocytic lineage. Cocultures with monocytes triggered an increased sHLA-G expression compared with isolated B-NHL or monocytes, suggesting a role of the association of these two cell types on sHLA-G secretion. In contrast, stimulation of B-lymphomatous cells with a cytokine cocktail mimicking a T-signal displayed no effect *in vitro* on sHLA-G secretion. Consequently, monocytes, unlike

 

T lymphocytes, seem to participate in mechanisms involved in sHLA-G secretion by B-NHL. These results are in accordance with previous data showing an association between monocyte and HLA-G expression in both tumour and physiological contexts (Pangault *et al*, 2002; Le Friec *et al*, 2004). In addition, it has been shown that lymphoma-associated macrophage content in follicular lymphoma is an independent predictor of survival (Farinha *et al*, 2005) that perhaps could be linked to sHLA-G expression.

However, the present study failed to demonstrate any correlation between sHLA-G level and prognosis in NHL and CLL. It was suggested by V. Rebman during the last international conference on HLA-G molecules in July 2006 (unpublished observation) that the impact of HLA-G expression on prognosis during cancer pathology is linked to the functional binding between HLA-G molecules and ILT2 receptor. Nevertheless, in CLL high-sHLA-G level has been correlated to hypogammaglobulemia, which is related to immunodepressive status. These data are in accordance with the results reported by Nuckel *et al* (2005), which showed an association between HLA-G surface expression and immunodeficiency during CLL.

This study examined the functionality of sHLA-G molecules in the plasma of patient with lymphoproliferative disorders. Using a protocol purifying both sHLA-G1 and HLA-G5, we showed an inhibition of T-cell alloproliferation in MLR with sHLA-G concentrations (from 50 ng/ml) that are frequently observed in lymphoproliferative disorders. HLA-G inhibitory effect on T-cell alloproliferation was previously described in MLR with HLA-G cell line transfectant (Riteau *et al*, 1999; Wiendl *et al*, 2002). Similar properties were found for sHLA-G when secreted or added during MLR (Lila *et al*, 2001; Le Friec *et al*, 2003). This property, associated with the capacity to inhibit NK and T-cell cytotoxicity (Wiendl *et al*, 2003) and the induction of the apoptosis of CD8 cells (Fournel *et al*, 2000), favours immunosuppression. To date, functional experiments using sHLA-G molecules have mainly been achieved with proteins obtained from cell line transfectants (Bainbridge *et al*, 2000; Wiendl *et al*, 2002) or recombinant molecules (Marchal-Bras-Goncalves *et al*, 2001). This study is in accordance with a recent study by Le Rond *et al* (2006), which showed that HLA-G5 contained in the plasma from transplanted patients inhibited T-cell alloresponse.

In conclusion, this work demonstrated high levels of sHLA-G molecules in the plasma of patients with lymphoproliferative disease and its functional efficiency on immune response inhibition. We also confirmed the correlation between HLA-G expression and immunodeficiency in CLL. The immunosuppressive role of sHLA-G on NK cells, which play a major role in anti-tumour response, is well-known. The impact of NK cells in haematological malignancies is now emerging (Karadimitris *et al*, 2006). Together, these data support the potential immunomodulatory role of sHLA-G molecules in lymphoproliferative disorders, which could constitute an additional escape mechanism from immune surveillance. Further studies are necessary to identify functional inhibitory receptors and the signalling pathways involved in these pathologies. The negative modulation of sHLA-G secretion might contribute to improve the efficiency of classical therapeutic strategies in this type of tumour proliferation.

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