

# Elevated levels of soluble non-classical major histocompatibility class I molecule human leucocyte antigen (HLA)-G in the blood of HIV-infected patients with or without visceral leishmaniasis

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## Introduction

Human leucocyte antigen-G (HLA)-G are non-classical class I histocompatibility complex molecules. The soluble forms human leucocyte antigen (HLA)-G5 and sHLA-G1 are, respectively, generated by alternative splicing and by metalloproteinase cleavage of the full-length membrane-bound isoform HLA-G1. HLA-G molecules display tissue-restricted expression: they are expressed in the cytotrophoblast, where they play a major role in maternal-fetal tolerance. Membrane-bound and soluble HLA-G molecules inhibit natural killer (NK) cell-mediated cytotoxicity [1] and allogeneic T cell proliferation [2,3]. HLA-G genotype has been reported to be associated with interleukin (IL)-10 expression in activated peripheral blood mononuclear cells (PBMC) [4]. The immunosuppressive properties of HLA-G may be involved in the persistence and progression of HIV infection. Recent studies have shown that several HLA-G polymorphisms influence susceptibility to HIV-1 infection [5] and that monocyte and T cell HLA-G surface expression is elevated in HIV-1-seropositive patients [6]. No data are available on the expression of the soluble forms HLA-G5 and sHLA-G1 in HIV-infected patients with and without opportunistic infections.

## Summary

The non-classical class I major histocompatibility complex molecules human leucocyte antigen (HLA)-G have been shown to play a role in HIV persistence, but no data are available on the expression of the soluble forms HLA-G5 and sHLA-G1 in HIV-infected patients with and without opportunistic infections. The soluble HLA-G isoform was measured with an enzyme-linked immunosorbent assay (ELISA) method in plasma from 94 subjects: 31 HIV-1-seropositive, 17 with visceral leishmaniasis (VL), seven with both VL and HIV-1 infection and 39 healthy HIV-seronegative subjects. Between groups, the frequency of sHLA-G positivity was statistically different: 81% of HIV-infected patients were positive, as were 57% of HIV-*Leishmania infantum* co-infected patients, 35% of HIV-seronegative patients with VL and 3% of healthy controls. Levels of the soluble forms of the immunomodulatory molecules HLA-G are elevated during HIV infection. In HIV-*Leishmania* co-infected patients, sHLA-G secretion could contribute to the tolerogenic environment and to *Leishmania* immune evasion.

**Keywords:** HIV, HLA-G, *Leishmania donovani*, soluble HLA-G, visceral leishmaniasis

Visceral leishmaniasis (VL) is caused by protozoan parasites of the genus *Leishmania*, which are obligate intracellular parasites of the macrophage-dendritic cell lineage. The main causative species are *L. donovani* in India and Africa, *L. infantum* in all countries of the Mediterranean littoral, in central Asia and China, and *L. chagasi* (considered as synonymous with *L. infantum*) in the New World. Patients with acute VL have a T helper 2 (Th2) serum cytokine profile with high IL-4 and IL-10 levels and their peripheral blood mononuclear cells (PBMC) exhibit defective antigen-specific proliferation. This defect seems to be due mainly to immunosuppressive factors produced by PBMC themselves. Indeed, antigen-specific proliferation of PBMC from patients cured of VL is inhibited by co-culture with autologous PBMC obtained during the acute phase of the disease. IL-10 was the first immunosuppressive factor to be implicated in this setting, but IL-6, IL-4 and IL-13 are now also thought to play a part [7].

Acute VL is associated with immunosuppressive processes and may be favoured by severe immunodeficiency, as occurs in AIDS. The World Health Organization has classified *Leishmania*/HIV co-infection as a 'new and frightful disease' which is becoming increasingly frequent. Cases of opportunistic *Leishmania* infection have been reported in 35

countries, but mainly in south-western Europe (1911 cases in Spain, France, Italy and Portugal) [8]. In the recent past 70% of adult cases of acute VL have been associated with HIV infection, and up to 9% of AIDS patients develop opportunistic VL (<http://www.who.int>). Since the introduction of highly active anti-retroviral therapy (HAART) during AIDS, a reduction in the annual incidence of VL relapses among the patients has been observed [8].

The objectives of this study were to compare circulating levels of soluble HLA-G isoforms in HIV-seropositive and -seronegative patients with or without *L. infantum* VL.

## Patients and methods

### Blood samples

sHLA-G can be assayed in plasma or serum. Blood samples from 94 subjects were obtained from three French teaching hospitals (71 from Rennes, 21 from Marseille and two from Créteil, with thanks to Professor M. Deniau). Thirty-one patients were HIV-1-seropositive (group 1) and 24 patients had VL: seven HIV-1-seropositive (group 2) and 17 HIV-seronegative (group 3); the remaining 39 subjects were healthy blood donors (group 4). Samples from HIV-infected and/or *Leishmania*-infected patients were obtained either for HIV plasma load assay or for *Leishmania* serology. All samples were anonymized according to the French legislation for biomedical research and stored at  $-20^{\circ}\text{C}$  until use.

### Soluble HLA-G (sHLA-G1 and HLA-G5) assay

Soluble HLA-G concentrations were measured with a specific sandwich enzyme-linked immunosorbent assay (ELISA) method validated at the 'Wet Workshop for Quantification of Soluble HLA-G' (2004, Essen, Germany). Microtitre plates (Corning Costar, Issy-les-Moulineaux, France) were coated with 10  $\mu\text{g/ml}$  MEM-G/9 (Exbio, Prague, Czech Republic) in 0.01 M phosphate-buffered saline (PBS), pH 7.4. After three washes in PBS containing 0.05% Tween-20, the plates were saturated with 2% bovine serum albumin-PBS for 30 min at room temperature. Serum or plasma (100  $\mu\text{l}$ ) was added to each well and tested in triplicate. After 1 h incubation at room temperature the plates were washed three times in PBS containing 0.05% Tween-20. Anti- $\beta$ 2-microglobulin horseradish peroxidase complex ( $\beta$ 2m-HRP; Dako, Trappes, France; 100  $\mu\text{l}$ ) was added to each well, and the plates were incubated further for 1 h at room temperature. After three washes the plates were incubated for 30 min with the substrate (ortho-phenylenediamine dihydrochloride; Dako), and optical density (OD) was measured at 490 nm after adding 1 N  $\text{H}_2\text{SO}_4$ . Standard OD curves were prepared with serial dilutions of HLA-G5 purified from LCL-721-221-G5 cell-line culture supernatant. Results are expressed in ng/ml (mean of three wells).

## Statistical analysis

SPSS version 12.0 for Windows software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Analysis of variance with the non-parametric Kruskal-Wallis test was used to compare sHLA-G concentrations among the groups of subjects. When a significant difference was observed, the groups were compared  $2 \times 2$  using the Student-Newman-Keuls test. The frequency of sHLA-G positivity in each group was compared using the  $\chi^2$  test. *P*-values  $< 0.05$  were considered statistically significant.

## Results

Patient characteristics, plasma soluble HLA-G concentration and percentage of positive patients are shown in Table 1. The sHLA-G positivity cut-off was determined as the mean of the healthy control values plus two standard deviations (31 ng/ml). The frequency of sHLA-G positivity in each group was statistically different between groups using the  $\chi^2$  test ( $P < 0.01$ ). sHLA-G concentrations are shown using box-plots in Fig. 1 and were statistically different among the groups of subjects using the Kruskal-Wallis test ( $P < 0.01$ ).

The sHLA-G concentrations in the 39 healthy subjects ranged from 3.27 to 32.92 ng/ml, with a median of 16.71 ng/ml and mean value of  $17.02 \pm 6.93$  ng/ml. Twenty-five of the 31 HIV-infected patients without VL were positive for sHLA-G, with concentrations ranging from 19.07 to 150.28 ng/ml (median 52.02; mean  $60.77 \pm 34.81$  ng/ml). Ten patients had sHLA-G levels above 62 ng/ml, i.e. more than twice the cut-off. Using the Student-Newman-Keuls test to compare groups  $2 \times 2$ , the sHLA-G levels were significantly higher in this group than in the other three groups ( $P < 0.05$  compared to the control, HIV/VL and VL groups).

Among the patients with VL, sHLA-G concentrations in HIV-seronegative patients ranged from 6.62 to 86.37 ng/ml, with two very high concentrations (83.55 and 86.37 ng/ml). The median sHLA-G concentration was of 24.57 ng/ml (mean  $31.31 \pm 22.62$  ng/ml). sHLA-G ELISA was positive ( $> 31$  ng/ml) in 35% of these subjects (not significantly different in the Student-Newman-Keuls test from the control group or from HIV-infected patients with VL). In HIV-infected patients with VL, sHLA-G levels ranged from 17.82 to 60.03 ng/ml, with a median of 40.71 ng/ml and a mean of  $39.25 \pm 15.71$  ng/ml ( $P < 0.05$  versus controls and HIV-infected patients). Fifty-seven per cent of these subjects had positive results.

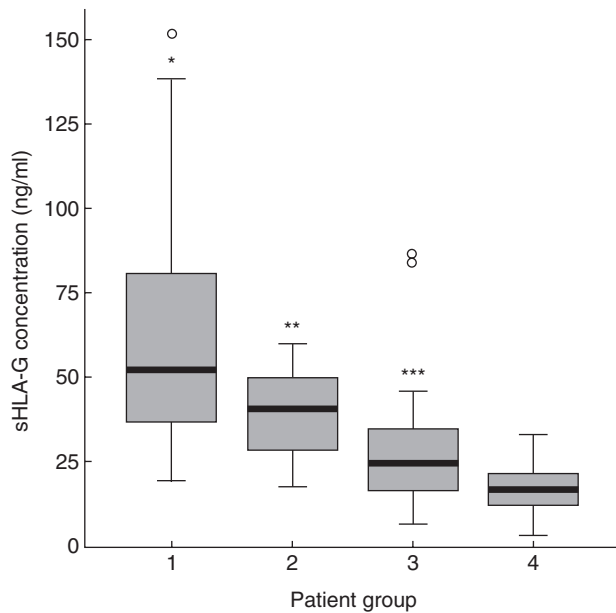
## Discussion

The non-classical class I major histocompatibility complex molecules HLA-G were implicated initially in maternal-fetal tolerance. Among others, it has since been shown to play a role in HIV persistence. Cell surface expression of the

**Table 1.** Plasma soluble human leucocyte antigen (HLA)-G concentration (ng/ml) and percentage of positive patients according to clinical status.

Group	Patient	Adult (A) or children (C)	HIV status	HAART	Visceral leishmaniasis status	sHLA-G concentration (ng/ml)	% of positive patients*
Group 1 ( <i>n</i> = 31): HIV-infected	1	A	+	+	-	73.03	81% (25/31)
	2	A	+	+	-	55.35	
	3	A	+	+	-	94.12	
	4	A	+	+	-	84.47	
	5	A	+	+	-	150.28	
	6	A	+	+	-	57.87	
	7	A	+	+	-	37.97	
	8	A	+	+	-	49.26	
	9	A	+	+	-	78.08	
	10	A	+	+	-	89.82	
	11	A	+	+	-	138.70	
	12	A	+	+	-	39.18	
	13	A	+	+	-	19.07	
	14	A	+	+	-	59.29	
	15	A	+	+	-	53.04	
	16	A	+	+	-	28.16	
	17	A	+	+	-	35.66	
	18	A	+	+	-	32.37	
	19	A	+	+	-	43.73	
	20	A	+	+	-	87.58	
	21	A	+	+	-	39.86	
	22	A	+	+	-	48.16	
	23	A	+	+	-	43.61	
	24	A	+	-	-	27.37	
	25	A	+	-	-	52.02	
	26	A	+	-	-	26.80	
	27	A	+	-	-	24.98	
	28	A	+	-	-	22.03	
	29	A	+	-	-	54.91	
	30	A	+	-	-	125.94	
	31	A	+	-	-	111.21	
Group 2 ( <i>n</i> = 7): HIV-infected with VL	32	A	+	+	Primary VL	42.60	57% (4/7)
	33	A	+	+	Primary VL	17.82	
	34	A	+	+	Primary VL	40.71	
	35	A	+	+	Primary VL	25.92	
	36	A	+	+	Primary VL	57.19	
	37	A	+	+	Relapse	30.42	
	38	A	+	+	Relapse	60.03	
Group 3 ( <i>n</i> = 17): VL only	39	A	-	-	Primary VL	20.71	35% (6/17)
	40	A	-	-	Primary VL	24.48	
	41	A	-	-	Primary VL	27.38	
	42	A	-	-	Primary VL	83.55	
	43	A	-	-	Primary VL**	46.04	
	44	A	-	-	Primary VL	38.53	
	45	A	-	-	Primary VL	16.21	
	46	A	-	-	Primary VL	6.62	
	47	A	-	-	Primary VL	16.44	
	48	A	-	-	Relapse**	8.56	
	49	A	-	-	Primary VL	24.57	
	50	C	-	-	Primary VL	33.36	
	51	C	-	-	Primary VL	24.15	
	52	C	-	-	Primary VL	34.48	
	53	C	-	-	Primary VL	25.61	
	54	C	-	-	Primary VL	15.19	
	55	C	-	-	Primary VL	86.37	
Group 4 ( <i>n</i> = 39): healthy control	56-94	A		Healthy blood donors		Mean ± s.d.: 17.02 ± 6.93	3% (1/39)

HAART: highly active anti-retroviral therapy; s.d.: standard deviation. \*sHLA-G positivity cut-off >31 ng/ml; \*\*patients undergoing solid organ transplantation.



**Fig. 1.** Plasma soluble human leucocyte antigen (HLA)-G concentration in ng/ml (minimum value, 25th percentile, median, 75th percentile and maximum value) according to clinical status (group 1: HIV-infected patients; group 2: HIV-infected patients with visceral leishmaniasis; group 3: visceral leishmaniasis only; group 4: healthy control patients). \*Group 1 statistically different from groups 2, 3 and 4 ( $P < 0.05$ ); \*\*Group 2 statistically different from groups 1 and 4 ( $P < 0.05$ ). \*\*\*Group 3 statistically different from group 1 ( $P < 0.05$ ).

membrane-bound isoform HLA-G1 has been reported to be both up-regulated and down-regulated in HIV-1-infected patients [6,9]. Like membrane-bound forms, sHLA-G molecules have tolerogenic effects, such as inhibition of allogeneic T cell proliferation and natural killer cell-mediated cytotoxicity [1–3]. It is frequently observed, especially in haematological tumoral malignancies, that high soluble HLA-G levels can be determined, whereas no surface expression is observed. It is possible that strong shedding activity during some diseases decreases HLA-G surface expression and in consequence increases plasma levels [10]. Here we measured the soluble isoforms HLA-G5 and sHLA-G1 with a standardized ELISA method [10].

We found that circulating sHLA-G levels were markedly higher in HIV-infected subjects than in healthy controls. Indeed, 87% of HIV-infected patients exhibited elevated sHLA-G serum levels. Increased membrane-bound HLA-G1 expression has been reported on monocytes and some T lymphocyte subsets in HIV-infected individuals [6]. Cabello *et al.* hypothesized that HAART is able to induce the membrane expression of HLA-G on peripheral monocytes from HIV-1 seropositive individuals [11]. In our series, we found that four of eight HIV-infected patients without HAART (50%) were positive for sHLA-G, in contrast with 25 of 40 treated patients (83%) who were on HAART, including those

co-infected with *Leishmania*. HIV proteins themselves can modulate cell surface expression of HLA class I molecules. In particular, Vpu-dependent HLA-G1 down-regulation has been implicated in decreased surface expression [9]. The high circulating levels observed in this study could be due in part to shedding of the membrane-bound isoform, and could participate in the pathogenesis of HIV infection by inducing tolerance [12].

sHLA-G levels were positive in one-third of our HIV-seronegative patients with VL. A low number of patients could explain the absence of statistical difference between the mean concentration in this group with that observed in the control group. In some patients, high sHLA-G levels might represent an additional evasion strategy developed by *Leishmania* which, like HIV, can establish latent infection in macrophages [13]. Patients with VL have a Th2 cytokine profile that could be related to sHLA-G secretion. IL-10 is a potent inducer of HLA-G1 expression on monocytes/macrophages and the resulting suppression of NK cells, whose activity is essential for innate immunity to VL, could undermine host resistance to *Leishmania* [14]. sHLA-G assay in patients with subclinical or asymptomatic VL and high levels of interferon (IFN)- $\gamma$ , IL-2 and IL-12 could be of interest.

More than half of our HIV-infected patients with VL were positive for sHLA-G. However, the percentage of positive patients and the mean sHLA-G value were significantly lower in patients with both HIV infection and VL than in the patients with HIV infection alone. *Leishmania* and HIV have cumulative immunosuppressive effects. Macrophage infection by *L. donovani*, followed by *in vitro* infection by HIV, increases intracellular proliferation of the parasite [13]. HIV-1 alters IL-12 and IL-18 secretion by PBMC during *L. donovani* infection [15] and inhibits *Leishmania*-induced lymphocyte proliferation without affecting IL-6 production. IL-6, which is detectable in the serum of patients with acute VL, modulates HIV replication in T cells and macrophages, suggesting that *Leishmania* might transactivate HIV-1 [13]. Our results suggest that elevated sHLA-G levels in HIV-infected patients with VL might participate in the general tolerogenic environment, favouring the persistence of *Leishmania* and shortening the life expectancy of HIV-infected individuals.

In conclusion, the ELISA method used here is a simple and sensitive tool for measuring circulating soluble HLA-G isoforms in patients infected by HIV and/or *L. infantum*. Elevated circulating levels of HLA-G molecules might help *Leishmania* parasites to evade cell-mediated immune responses in some patients, even in patients non-infected with HIV. More experiments are warranted to understand the contribution of HLA-G to immunosuppression during VL.

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## References

- 1 Lopez-Botet M, Llano M, Navarro F, Bellon T. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol* 2000; **12**:109–19.
- 2 Le Fric G, Laupeze B, Fardel O *et al.* Soluble HLA-G inhibits human dendritic cell-triggered allogeneic T-cell proliferation without altering dendritic differentiation and maturation processes. *Hum Immunol* 2003; **64**:752–61.
- 3 Lila N, Rouas-Freiss N, Dausset J, Carpentier A, Carosella ED. Soluble HLA-G protein secreted by allo-specific CD4+ T cells suppresses the allo-proliferative response: a CD4+ T cell regulatory mechanism. *Proc Natl Acad Sci USA* 2001; **98**:12150–5.
- 4 Rizzo R, Hviid TV, Stignani M *et al.* The HLA-G genotype is associated with IL-10 levels in activated PBMCs. *Immunogenetics* 2005; **57**:172–81.
- 5 Matte C, Lajoie J, Lacaillle J, Zijenah LS, Ward BJ, Roger M. Functionally active HLA-G polymorphisms are associated with the risk of heterosexual HIV-1 infection in African women. *Aids* 2004; **18**:427–31.
- 6 Lozano JM, Gonzalez R, Kindelan JM *et al.* Monocytes and T lymphocytes in HIV-1-positive patients express HLA-G molecule. *Aids* 2002; **16**:347–51.
- 7 Wilson ME, Jeronimo SM, Pearson RD. Immunopathogenesis of infection with the visceralizing *Leishmania* species. *Microb Pathog* 2005; **38**:147–60.
- 8 Desjeux P, Alvar J. *Leishmania*/HIV co-infections: epidemiology in Europe. *Ann Trop Med Parasitol* 2003; **97** (Suppl. 1):3–15.
- 9 Derrien M, Pizzato N, Dolcini G *et al.* Human immunodeficiency virus 1 downregulates cell surface expression of the non-classical major histocompatibility class I molecule HLA-G1. *J Gen Virol* 2004; **85**:1945–54.
- 10 Sebt Y, Le Fric G, Pangault C *et al.* Soluble HLA-G molecules are increased in lymphoproliferative disorders. *Hum Immunol* 2003; **64**:1093–101.
- 11 Cabello A, Rivero A, Garcia MJ *et al.* HAART induces the expression of HLA-G on peripheral monocytes in HIV-1 infected individuals. *Hum Immunol* 2003; **64**:1045–9.
- 12 Carosella ED, Moreau P, Aractingi S, Rouas-Freiss N. HLA-G: a shield against inflammatory aggression. *Trends Immunol* 2001; **22**:553–5.
- 13 Wolday D, Berhe N, Akuffo H, Britton S. *Leishmania*–HIV interaction: immunopathogenic mechanisms. *Parasitol Today* 1999; **15**:182–7.
- 14 Korbel DS, Finney OC, Riley EM. Natural killer cells and innate immunity to protozoan pathogens. *Int J Parasitol* 2004; **34**:1517–28.
- 15 Wolday D, Berhe N, Britton S, Akuffo H. HIV-1 alters T helper cytokines, interleukin-12 and interleukin-18 responses to the protozoan parasite *Leishmania donovani*. *Aids* 2000; **14**:921–9.