Soluble HLA-G molecules impair natural killer/dendritic cell crosstalk via inhibition of dendritic cells

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HLA-G molecules are known to exert immunosuppressive action on DC maturation and on NK cells, and can in consequence inhibit respectively T cell responses and NK cytolysis. In this study, we show that monocyte-derived DC, differentiated in the presence of GM-CSF and IL-4, are sensitive to soluble (s) HLA-G molecules during LPS/IFN-γ maturation as demonstrated by the decrease of CD80 and HLA-DR expressions and IL-12 secretion. Moreover, DC pretreated with sHLA-G were found to activate NK/DC crosstalk less than non-treated DC. Early activation of NK cells co-cultured with autologous DC was diminished as assessed by CD69 expression. The IFN-γ production was impaired whereas a slight inhibition of the NK cell cytotoxicity against Daudi cell line was observed. Since sHLA-G is expressed in grafts or sites of tumour proliferation, its indirect action on NK cells via DC could constitute a pathway of early inhibition for both innate and specific immune responses.

Introduction

HLA-G is a non-classical MHC class I molecule, first described in the pregnancy context [1]. Its immunosuppressive properties explain its importance for tolerance of foetal semi-allogeneic cells by the maternal immune system [2]. Unlike classical MHC class I molecules, HLA-G exhibits, in physiological conditions, a very restricted tissue distribution [1, 3, 4]. In recent years, much work has focused on elevated HLA-G expression in various situations of graft tolerance and tumour development as well as on the possible consequences of this expression. To date, authors have concluded that HLA-G can favour graft tolerance, but also tumour escape.

HLA-G is transcribed into seven transcriptional isoforms by alternative splicing. The transcripts HLA-G1, HLA-G2, HLA-G3 and HLA-G4 lead to four different membrane-bound molecules [5, 6]. The transcripts HLA-G5, HLA-G6 and HLA-G7 lead to translation of three soluble molecules [7, 8]. It is noteworthy that another soluble (s) form of the molecule generated by shedding of the complete membrane-bound HLA-G1 isoform (sHLA-G1) coexists with the complete sHLA-G5 translated from the spliced transcript [9, 10]. Little is known about the functions of these truncated molecules (HLA-G2, HLA-G3, HLA-G4, HLA-G6 and HLA-G7). However, the complete isoforms HLA-G1, sHLA-G1 and HLA-G5 share the following immunosuppressive properties: (1) inhibition of allogeneic T cell proliferation [11, 12], (2) inhibition of allogeneic lysis in vitro glioma and myoblast models [13–15] and (3) interference with naive CD4+ T cell priming [16]. Moreover, HLA-G1 has

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been shown to inhibit cytolysis by CTL and by NK cells [2, 17, 18], whereas sHLA-G molecules could induce apoptosis of CD8+ T cells [19], NK cells [20] and endothelial cells playing a role in angiogenesis through engagement of CD160 [21].

The immunoregulatory properties of HLA-G result from interactions with diverse inhibitory receptors, i.e. directly via Ig-like transcript (ILT)-2 (LILR-B1, CD85j) expressed by several myeloid and lymphoid cells, or indirectly via CD94/NKG2A expressed on NK cells after induction of HLA-E-presenting HLA-G leader peptide [22]. It is also important to underline that HLA-G can interact with ILT-4 (LILR-B2, CD85d), a molecule specifically expressed on APC, including DC [23].

The action of HLA-G molecules through APC is of great interest. Previous work in our laboratory demonstrated the potential expression of HLA-G isoforms by myeloid and plasmacytoid DC in an in vitro model [24]. Other studies have focused on the influence of HLA-G molecules on T responses triggered by DC. This work investigates another aspect of APC-related immunity, the crosstalk between NK cells and DC.

Results

sHLA-G molecules inhibit maturation marker surface expression on monocyte-derived DC

Five-day-old monocyte-derived DC were matured for 48 h with LPS and IFN-γ in the presence or absence of purified sHLA-G. Flow cytometry (FCM) analysis indicated that HLA-DR and in greater extent CD80 expression were reduced in the presence of sHLA-G in a dose-dependent manner (Fig. 1, Table 1). Comparison of the means of the MFI ratio (specific mAb MFI/isotype control MFI) showed that, after treatment with 2 μg/mL sHLA-G, surface expression of CD80 and HLA-DR was significantly decreased (61% and 28%, respectively) (Table 1). No statistically significant differences could be observed in CD40, CD83, CD86 and HLA class I molecule expression. In contrast to sHLA-G, sHLA-B7 did not alter the expression of CD80 and HLA-DR on mature DC (Fig. 1).

Expression of the HLA-G receptors ILT-2 (CD85j) and ILT-4 (CD85d) was also investigated. At day 5, immature DC expressed ILT-2 and to a lesser extent ILT-4 (Fig. 2). The expression level of these markers was unchanged after 48 h of maturation with LPS and IFN-γ either in the presence or absence of sHLA-G. In addition, neither mature nor immature monocyte-derived DC expressed HLA-G in FCM analysis performed with MEM/G-9 mAb (data not shown).

Table 1. Influence of sHLA-G on mature DC markersa)

<table>
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<tr>
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<th>without sHLA-G</th>
<th>1 μg/mL sHLA-G</th>
<th>2 μg/mL sHLA-G</th>
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<tr>
<td>CD83</td>
<td>8.3 ± 4.1</td>
<td>8.2 ± 4.5</td>
<td>6.4 ± 4.2</td>
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<tr>
<td>CD40</td>
<td>38.7 ± 13.4</td>
<td>43.1 ± 18.8</td>
<td>37.8 ± 23</td>
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<td>CD80</td>
<td>18.2 ± 16.2</td>
<td>9.7 ± 4</td>
<td>7.4 ± 2.7c)</td>
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<tr>
<td>CD86</td>
<td>368.4 ± 328.4</td>
<td>375.1 ± 350.5</td>
<td>308.2 ± 279.4</td>
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<tr>
<td>HLA-DR</td>
<td>84.9 ± 23.3</td>
<td>77.5 ± 15.4</td>
<td>60.9 ± 12.6c)</td>
</tr>
<tr>
<td>W6/32</td>
<td>335 ± 55</td>
<td>Not determined</td>
<td>348 ± 56</td>
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a) Five-day-old monocyte-derived DC were matured for 48 h with 500 ng/mL LPS and 100 U/mL IFN-γ in the presence of 1 or 2 μg/mL purified sHLA-G.

b) Ratio fluorescence intensity = (MFI specific mAb/MFI isotype control) ± SD from five independent experiments.

c) *p<0.05 when compared to control mature DC.
IL-12 secretion by monocyte-derived DC is impaired by sHLA-G treatment

Cytokine secretion assay of DC during maturation with or without sHLA-G treatment was performed on culture supernatants. Though no effect of sHLA-G treatment on DC could be detected on IL-10 secretion (Fig. 3A), a dose-dependent effect was noted on IL-12 secretion (Fig. 3B). Compared with untreated cells, inhibition of IL-12 secretion after treatment reached 33% with 1 \( \mu \)g/mL sHLA-G and 43% with 2 \( \mu \)g/mL sHLA-G and was significant (Wilcoxon's paired test) for both concentrations. Four comparative experiments showed that sHLA-B7, as sHLA-G, induced a decrease of IL-12 secretion. Concentrations were 24.9/62.4 and 30/72.7 ng/mL in the presence of sHLA-B7 and sHLA-G, respectively, versus 36.4 ± 9 ng/mL with the untreated DC.

sHLA-G-treated DC are less efficient early NK activators than untreated DC

To evaluate the early activator potential of sHLA-G-treated DC, 24-h co-cultures were carried out at two NK/DC ratios (3:1 and 10:1) with purified autologous peripheral blood NK cells. Expression of the early activation marker CD69 by NK cells was then investigated. CD69 expression by NK cells increased in co-cultures with DC (Fig. 4). There was an inhibition of CD69 expression when NK cells were co-cultured with sHLA-G-treated DC compared to non treated DC. This decrease was not found when NK cells were co-cultured with sHLA-B7-pretreated DC (not shown).

Peripheral NK cells co-cultured in presence of sHLA-G-treated DC are lower IFN-\( \gamma \) secretors

Then, we measured the IFN-\( \gamma \) level in the supernatant of 48-h NK cell/DC co-cultures by ELISA. IFN-\( \gamma \) concentration in the supernatant of the co-culture with sHLA-G-pretreated DC was significantly reduced in comparison to control co-culture supernatant (Fig. 5). This decrease was not found when NK cells were co-cultured with sHLA-B7-pretreated DC (Fig. 5). Intracellular FCM analysis allowed to exclude the secretion of IFN-\( \gamma \) by the DC, defined as CD1a+ cells in the co-culture (data not shown).

sHLA-G DC reduced NK cell lysis of Daudi cell line

At last, we investigated cytotoxic activity of NK against Daudi cells. At an effector-to-target ratio of 50/1, Daudi cells were lysed at 43 ± 5% when NK cells were stimulated by untreated DC. The lysis was slightly
Discussion

This study aimed at evaluating the impact of immunomodulatory sHLA-G molecules on DC in the NK/DC crosstalk context. The choice of sHLA-G concentrations used in this study (1 and 2 μg/mL) was based on previous report [19] and on dose-response experiments performed in our laboratory [12]. These doses are consistent with physiopathological situations such as pregnancy [25], acute leukaemia or lymphoproliferative disorders [26, 27]. sHLA-G rates are lower in peripheral blood than soluble classical HLA class I antigens but exhibit higher affinity for ILT-2 and ILT-4 receptors than their classical counterparts [28].

First, the impact of sHLA-G molecules on DC was investigated in terms of cytokine secretion and phenotypic expression. sHLA-G inhibits IL-12 secretion and alters CD80 and HLA-DR surface expression. This result differs from that obtained with classical soluble HLA class I molecules such sHLA-B7 which do not trigger a diminution of cell surface presentation molecules. The decrease after sHLA-G treatment of CD80 and HLA-DR expressions is not surprising as an inhibition of T responses by sHLA-G-treated DC has been previously documented [29].

It is interesting to note that CD80 was shown to be a more potent inducer of Th1 cytokines, CD86 triggering Th2 responses, in particular IL-4 secretion [30]. Other work concluded that CD80 was a superior ligand for CD28 than CD86 and as a consequence committed a greater number of T cells to divide [31]. HLA-G is often considered as a Th2 response inducer molecule. This is consistent with the strong decrease of CD80 expression compared to CD86, observed in this study. Previous researches postulated that maintaining low costimulation and expression of presentation molecules could induce anergy of T cells and generation of regulatory T cells [32]. The decrease of IL-12 secretion by DC in the presence of sHLA-G molecules could also contribute to immunosuppressive properties observed on T cells.
Horuzsko et al. [32] showed that HLA-G molecules could impair maturation of murine and human DC (differentiated without IL-4) via ILT-4 interaction. These tolerized DC could in consequence reduce T cell allogenic activation and induce anergic and immunosuppressive CD4+ and CD8+ T cells. Another work highlighted the fact that a KG1 cell line transfected by HLA-G1 cDNA, was able (i) to inhibit the proliferation of CD4+ T cells, (ii) to shed HLA-G1, providing additional immunosuppressive signals, (iii) to induce CD4+ T cell anergy, and (iv) to allow the generation of suppressive CD4+ T cells [29]. Finally, it was proven that APC lines up-regulated their surface expression of inhibitory receptors such as ILT-2 and ILT-4 when stimulated by HLA-G1 or HLA-G5 [33].

The action of sHLA-G molecules on maturing DC is here confirmed but for the first time in a conventional GM-CSF/IL-4 differentiation protocol using human peripheral blood monocytes. These DC expressed ILT-2 and ILT-4 receptors at low levels, but blocking experiments with ILT-2 and anti-ILT-4 mAb did not reverse the inhibition of IL-12 secretion and on the contrary, led to an additive effect when associated with sHLA-G (data not shown). Further investigations are needed to fully understand the precise receptors that mediate inhibition of DC. Moreau et al. [34] recently evidenced that other receptors than ILT-2 and ILT-4 play a role in the transduction of signal from HLA-G and Fons et al. [21] described CD160 receptor expressed by endothelial cells as a new receptor of sHLA-G1.

sHLA-G expression is involved in immunosuppressive contexts such as graft tolerance and tumour proliferation. sHLA-G-pretreated DC are known to inhibit T cell alloproliferation. In this work, we demonstrate that sHLA-G-pretreated DC also affect NK cell activation. Indeed, our results show that co-culture of NK cells with DC pretreated with sHLA-G, but not DC pretreated with sHLA-B7, triggers a diminution of the expression of the NK early activation marker CD69 and a decrease of IFN-γ secretion by NK cells. The decrease of IL-12 and CD80 expressions observed in sHLA-G-pretreated DC alters NK/DC crosstalk and especially the IFN-γ secretion by the NK cells. This might consequently compromise T cell differentiation towards Th1 phenotype. In our experiments, we did not observe an association between IFN-γ secretion and cytotoxicity, which could be explained by distinct regulation pathways [35, 36].

A large body of evidence from work on this junction between innate and adaptive immunity allows the following statements. The NK/DC interaction is highly dependent on two factors: IL-12 secretion by DC and interaction via the NKp30 receptor, whose ligand is unknown for the moment [37, 38]. The secretion of IL-12 by maturing DC after immunologic synapse formation activates the lytic functions of NK cells as well as their secretion of cytokines, mainly IFN-γ and TNF-α [39]. Moreover, the CD56bright NK cell subset seems to proliferate in presence of such DC [40]. The engagement of NKp30 by DC gives an additional activation signal and allows the killing of the immature DC pool [37]. More recently it has been shown in infected mice that trans-presented IL-15 by DC through their IL-15Rα to NK cells in draining lymph nodes was a prerequisite for priming of NK functions [41]. It seems according to these statements that a contact between NK cells and DC is not dispensable. Importance of secreted molecules such as type I IFN, however, cannot be excluded. In response to NK activation, DC up-regulate their presentation and copresentation molecules and increase their IL-12 secretion, mainly through secretions of NK cell cytokines [38, 42].

All these results sustain the idea that innate mechanisms initiate or at least favour adaptive T cell responses by leading to fully mature DC and highly cytolytic and cytokine-secreting NK cells. It is possible that HLA-G molecules impact the NK/DC crosstalk in draining lymph nodes in some infectious or tumoral diseases either as soluble forms as suggested by these data or as membrane-bound forms. It would be of interest to evaluate HLA-G expression in lymph nodes, where cell-cell contact is suspected to take place, and put it in relation with possible variations of expression of crucial molecules such as IL-15 or IL-15Rα.

In conclusion, if NK/DC interaction is, as suspected, a major event in initiation of graft rejection or anti-tumour response, sHLA-G expression could play an important role in this context. The direct action of sHLA-G molecules on NK cells, that is to say inhibition of cytolyis [2, 18, 43] and apoptosis [20], was previously described. However, this novel mechanism of indirect action via DC could also be involved in a very early phase of the immune response by inhibiting cytokine secretions. Moreover, such a model sustains the idea of counteraction on T cell responses, as NK/DC crosstalk is tightly linked to adaptive immunity. These findings point the way to new fields of investigations, in particularly the signaling pathways of this sHLA-G interference in IL-12 secretion, a central cytokine for effective specific and innate immune responses.

**Materials and methods**

**Cytokines, antibodies and cell lines**

IL-4 and IFN-γ were provided by Tebu-Peprotech (Le Perray-en-Yvelines, France), GM-CSF by Gentaur (Brussels, Belgium) and *Escherichia coli* LPS by Sigma-Aldrich (Lyon, France). FITC-, PE- or PEC-conjugated mouse mAb were purchased from Immunotech/Beckman Coulter (Marseille, France; anti-CD1a, anti-CD3, anti-CD56, anti-CD69, anti-CD80, anti-HLA-DR),
from Becton Dickinson/Pharmingen (Le Pont de Claix, France; anti-CD40, anti-CD83, anti-CD86, anti-CD85j), from R&D Systems (Minneapolis, MN; anti-CD85d), from Tebu-Peprotech (W6/32) and from Eurobio (Les Ulis, France; secondary mAb anti-mouse Fab).

The B lymphoblastoid cell lines LCL.721.221-G5 and LCL.721.221-B7, kindly provided by D. Geraghty (Fred Hutchinson Cancer Research, Seattle, WA), were obtained by transfection of HLA-G and HLA-B7, respectively [7].

**shLA-G purification**

Soluble HLA class I molecules were obtained using Hi Trap N-hydroxysuccimide-activated high performance columns (Amersham Pharmacia Biosciences) coated with W6/32 mAb (5 mg/mL) as previously described [12]. Then columns were blocked with 100 mM ethanolamine, pH 9.0, overnight before use. After washing with phosphate-buffered saline, 100 mL of LCL-HLA-G or LCL-HLA-B7 supernatants were applied onto the columns overnight at 4°C for HLA-G and HLA-B7 purification, respectively. After washing in phosphate-buffered saline, bound antigen was eluted with 0.1 M glycine buffer, pH 11.0, and neutralized with 1 M Tris, pH 7.5, buffer solution. Presence of HLA-G5 or HLA-B7 in eluted fractions was determined using ELISA as previously described [12]. Four positive fractions were obtained and were then pooled. Buffer was exchanged with culture medium using PD-10 desalting columns (Amersham Pharmacia Biosciences).

**Cell culture**

Monocytes and NK cells were isolated from blood buffy coat after separation of mononuclear cells on Ficoll gradient (Lymphoprep; Axis-Shield, Oslo, Norway) and three low-speed centrifugations (200 x g) to remove platelets. Human CD14 microbeads from Miltenyi Biotec (Paris, France) were used according to the manufacturer’s instructions. Autologous NK cells were isolated using StemSep NK enrichment cocktail (StemCell, Grenoble, France) according to the manufacturer’s instructions. Purities of monocyte and NK cell preparations were evaluated by FCM and were respectively >95% CD14+ cells and >95% CD56+/CD3- cells.

Monocytes (1 x 10^6/mL) were cultured 3 days in six-well plates in RPMI Glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% FCS in presence of 800 U/mL GM-CSF and 500 U/mL IL-4. At day 3, medium was replaced and 800 U/mL GM-CSF and 250 U/mL IL-4 was added. At day 5, immature DC were phenotyped by FCM (>90% CD1a+/CD14- cells) and were matured in renewed medium in presence of 500 ng/mL LPS, 250 U/mL IFN-γ, 800 U/mL GM-CSF and 250 U/mL IL-4.

In the indicated conditions, purified shLA-G or shLA-B7 molecules were added at 1 µg/mL and 2 µg/mL, during the 48 h of maturation.

Mature DC at day 7 and autologous NK cell co-cultures at different ratios were performed 1–3 days in 96-well round-bottom plates, with 1 x 10^5 NK cells per well, in 200 µL RPMI, 10% FCS medium. No purified shLA-G was added during the co-culture.

**Flow cytometry studies**

Phenotypic analysis of monocyte-derived DC was performed using flow cytometric direct immunofluorescence except for CD85d staining for which an indirect staining protocol was used. Cells were first incubated for 30 min in human AB serum at 4°C to avoid nonspecific binding. Then, cells were analyzed by double staining with a FACSCalibur cytometer (Becton Dickinson) for the indicated maturation markers. CD69 expression on NK cells after 1 day of co-culture with autologous DC was analyzed by double staining with CD56 mAb.

**Enzyme-linked immunosorbent assays for IL-10, IL-12p70 and IFN-γ**

IL-10 and IL-12p70 concentrations were assayed after 48 h of DC maturation in culture supernatants at day 7 using ELISA assay kits from Becton Dickinson/Pharmingen, according to the manufacturer’s instructions. IFN-γ levels were assayed after 48 h of DC/NK co-cultures in supernatants using IFN-γ module set from Bender/Tebu (Le Perray-en-Yvelines, France) and Microtiter plates (Corning Costar, Issy-les-Moulineaux, France).

**Cytotoxicity assay**

NK cell cytotoxicity was evaluated in standard 4-h 51Cr-release assays. Briefly, Daudi cells (5 x 10^5) labelled with 51Cr sodium dichromate (1 mCi/10^6 cells) were co-cultured in RPMI 1640 medium in 96-well U-bottomed plate for 4 h with NK cells at a 50/1, 25/1, 2.5/1 effector-to-target ratio. 51Cr release was evaluated using a Top-Count gamma counter (Packard Instruments, Rungis, France). The percent specific lysis was calculated by the formula: (mean experimental cpm – mean spontaneous cpm) / (mean maximum lysis cpm – mean spontaneous cpm) x 100. Spontaneous cpm and maximum cpm represent 51Cr release without NK cells and release in presence of triton and HCl, respectively.

**Statistical analysis**

Variations of surface marker expression are calculated as MFI/isotype MFI. Cytokine secretions and MFI mean variations between untreated and shLA-G-treated DC were analyzed by Wilcoxon’s paired ranked test and considered significant when p<0.05.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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