

# Nitric oxide produces HLA-G nitration and induces metalloprotease-dependent shedding creating a tolerogenic milieu

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## Summary

Human leucocyte antigen G (HLA-G) is a tolerogenic molecule that protects the fetus from maternal immune attack, may favour tumoral immunoescape and is up-regulated in viral and inflammatory diseases. The aim of this work was to discover if nitric oxide (NO) could affect HLA-G expression or function because NO is an important modulator of innate and adaptive immunity. For this purpose HLA-G expression and function were analysed following treatment with a NO donor or a peroxy-nitrite donor in various cell lines expressing HLA-G either spontaneously or upon transfection. Results showed NO-dependent nitration of both cellular and soluble HLA-G protein, but not all HLA-G moieties underwent nitration. Endogenous biosynthesis of NO by both U-937-HLA-G1 and M8-HLA-G5 stable transfecants also caused HLA-G nitration. The NO decreased total HLA-G cellular protein content and expression on the cell surface, while increasing HLA-G shedding into the culture medium. This effect was post-transcriptional and the result of metalloprotease activity. By contrast, NO pretreatment did not affect HLA-G capability to suppress NK cytotoxicity and lymphocyte proliferation. Our studies show that NO regulates the availability of HLA-G molecules without modifying their biological activities.

**Keywords:** human leucocyte antigen-G; immunosuppression; monocytes; nitration; nitric oxide

## Introduction

Nitric oxide (NO) is a very reactive free radical produced from L-arginine by the enzyme nitric oxide synthase (NOS) – reviewed in [1]. There are three isoforms of this enzyme: two of these, neuronal (nNOS) and endothelial (eNOS), are expressed constitutively and are collectively termed constitutive (cNOS), producing NO in the nanomolar range; and a third form, inducible (iNOS), produces NO in the micromolar range. NO is a versatile signalling mediator<sup>2,3</sup> that interacts with different molecules regulating almost every critical cellular function.<sup>1</sup> In addition, NO reacts with superoxide anions producing the powerful oxidant peroxynitrite. Peroxynitrite and the NO-derived nitrite can react with tyrosine to produce nitrotyrosine,<sup>4</sup> which can change protein function.

Inducible NOS can be induced in the immune cells, mainly in macrophages, modulating the type and extension of the immune response.<sup>1,5</sup> For example, NO interferes with the signalling of the interleukin-2 (IL-2) receptor,<sup>6</sup> decreases the tumour necrosis factor- $\alpha$  receptor expression,<sup>7</sup> and selectively induces apoptosis in T helper type 1 lymphocytes.<sup>8</sup>

Human leucocyte antigen G (HLA-G) is an immunosuppressive molecule with a low degree of polymorphism<sup>9</sup> and a limited tissue distribution, mainly restricted to trophoblasts.<sup>10</sup> However, it can be expressed ectopically by antigen-presenting cells<sup>11,12</sup> and tumour cells.<sup>13</sup> There are four membrane-bound isoforms (HLA-G1, -G2, -G3 and -G4) and three soluble isoforms (HLA-G5, -G6 and -G7) generated by alternative splicing of a primary transcript.<sup>14,15</sup> HLA-G binds at least three surface receptors,

Abbreviations: DETA-NO, N-(4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosophyrazino]butyl) propane-1,3-diamine; SIN-1, 3-[morpholinosydnonimine hydrochloride].

ILT2 (CD85j), ILT4 (CD85d) and KIR2DL4 (CD158d),<sup>16</sup> inhibiting the cytotoxicity of natural killer (NK) and antigen-specific CD8<sup>+</sup> cells,<sup>17</sup> the allogeneic proliferation of CD4<sup>+</sup> cells,<sup>18</sup> and promoting a shift toward a type T helper type 2 cytokine profile.<sup>19</sup> HLA-G1 can be shed from cellular membrane by metalloproteases,<sup>20,21</sup> and this soluble form (sHLA-G1) retains the tolerogenic properties.<sup>22</sup>

Both NOS<sup>23</sup> and HLA-G are expressed in the placenta. In several diseases, such as coeliac disease, multiple sclerosis and HIV infection, HLA-G<sup>24–27</sup> and iNOS<sup>28–30</sup> expression gives rise both to an increase in circulating HLA-G and to protein nitration. It has been shown that NO can influence the function of some tolerogenic molecules such as the enzyme indoleamine 2,3-dioxygenase.<sup>31</sup> There are no data related to the effect of NO on HLA-G. The aim of this work was to know if high NO output could affect HLA-G expression or function. In this work we show that NO to some extent nitrates the HLA-G protein and increases proteolytic shedding of HLA-G, which maintains its suppressive properties in soluble form.

## Materials and methods

### Cell line and cultures

The monocytic cell line U-937, the melanoma cell line M8, the lymphoblastoid cell line LCL and the natural killer cell line NKL were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Gibco, Carlsbad, CA). The transfected cells U-937-HLA-G1 and M8-HLA-G5 were obtained as previously described.<sup>32</sup> The choriocarcinoma cell line JEG-3 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco). Cells were cultured at 37° in a 5% CO<sub>2</sub> humidified atmosphere.

The NO donors were *N*-(4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl)propane-1,3-diamine (DETA-NO; Alexis Corporation, Läufelfingen, Switzerland); and 3-[morpholinosydnonimine hydrochloride] (SIN-1, Alexis Chemicals, San Diego, CA). The broad-spectrum matrix metalloprotease inhibitor GM6001, its negative control, NC-GM6001, and the specific inhibitors of matrix metalloproteases 2/9 (INH 2/9), 3 (INH 3), and 8 (INH 8) were from Calbiochem (Darmstadt, Germany).

Cellular viability measured by trypan blue exclusion was higher than 95% throughout the study.

### Nitrotyrosine immunoprecipitation

Cells were lysed in nonidet P40 0.5% in Tris-HCl buffer with protease inhibitors (Roche Applied Sciences, Upstate, NY) and incubated with anti-nitrotyrosine antibody at a dilution of 1 : 230 for 30 min.<sup>31</sup> Preincubation

of anti-nitrotyrosine antibody with nitrotyrosine 1 mM for 1 hr was used as a control of immune specificity. Immunoprecipitation was performed with an assay kit purchased from Pierce Biotechnology Inc. (Rockford, IL) according to the manufacturer's instructions.

### Western blotting

Protein concentration was quantified by the Bradford assay (BioRad Laboratories, Hercules, CA) using bovine serum albumin as standard. After centrifugation, 20 µg total protein were denatured at 100° for 5 min in a protein sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 30% glycerol, 5% β-mercaptoethanol and 0.4% bromophenol. Proteins were subjected to 10% polyacrylamide gel electrophoresis under denaturing conditions, with subsequent electroblotting transfer onto a nitrocellulose membrane. The membrane was blocked with 0.5% bovine serum albumin in phosphate-buffered saline (PBS)-Tween 0.1% for 1 hr at room temperature, and then incubated for 2 hr with MEM-G1 anti-HLA-G antibody (Abcam, Cambridge, UK) diluted 1 : 2000 in PBS-Tween, or with anti-β-actin (Abcam), diluted 1 : 5000 in PBS-Tween. Immunoblot analysis was performed using a horseradish peroxidase-conjugated anti-mouse antibody (1 : 5000; Amersham Biosciences, Uppsala, Sweden), and developed using the enhanced chemiluminescence kit (Amersham Biosciences). For incubation with additional antibodies, the membranes were previously stripped for 30 min at 56° in 62.5 mM Tris (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol.

### Flow cytometry

Cells were incubated for 30 min at 4° in PBS containing 20% human serum (Sigma-Aldrich, St Louis, MO), and stained with fluorescein isothiocyanate-conjugated anti-HLA-G antibody MEM-G9 (Exbio, Prague, Czech Republic) for 20 min at 4°. After washing, cells were fixed in paraformaldehyde 1%. Control aliquots were stained with the isotype-matched mouse antibody (Beckman Coulter, Fullerton, CA). Fluorescence was detected by an EPICS XL flow cytometer (Beckman Coulter).

### Real time reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from three to five million cells using an RNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Residual DNA was eliminated by DNase I treatment (10–20 units/100 µg, Roche Applied Science, Basel, Switzerland) for 1 hr at 25°. Reverse transcription was carried out using a High-Capacity cDNA Archive Kit according to the

manufacturer's instructions (Applied Biosystems, Foster City, CA). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was used to quantify variations in the amounts of HLA-G transcripts after cell treatment using the *TaqMan* Gene Expression Assay (Applied Biosystem). GAPDH was used as the internal standard.

#### *Enzyme-linked immunosorbent assay*

Specific enzyme-linked immunosorbent assay (ELISA) for HLA-G5 plus sHLA-G1 used anti-HLA-G MEM-G/9 (Exbio) as the capture antibody and anti- $\beta_2$ -microglobulin (DAKO, Glostrup, Denmark) as the secondary antibody as described previously.<sup>12,33</sup> Results were normalized to the absorbance obtained from the control medium without treatment.

#### *Cytotoxic assay*

Cells from the M8 cell line transfected and non-transfected for HLA-G1 were incubated in the presence or absence of 1 mM DETA-NO. After 24 hr at 37°, cells were washed twice and incubated for 1 hr at 37° with  $^{51}\text{Cr}$ . After two washes with complete RPMI-1640 medium, M8 cells were cocultured with NKL cells for 4 hr at 37°. The NKL cells had been previously stimulated with IL-2 (100 U/ml) for 24 hr. Coculture was performed in duplicates and in several M8 : NKL ratios: from 1 : 1 to 1 : 50. Then, 50  $\mu\text{l}$  of each supernatant was mixed with scintillation buffer (Perkin Elmer, Waltham, MA) in a 96-well plate and then read in a  $\beta$ -radiation counter (Wallac 1450; Amersham Biosciences). Specific lysis level was calculated as the  $^{51}\text{Cr}$ -release percentage from the maximum release:

$$\begin{aligned} \% \text{ specific lysis} &= 100 \\ &\times [(\text{sample c.p.m.} - \text{spontaneous release}) \\ &/(\text{maximum release} - \text{spontaneous release})] \end{aligned}$$

The spontaneous release was the counts per min (c.p.m.) measured in  $^{51}\text{Cr}$ -labelled M8 cells cultured in medium without NKL cells. The maximum release was achieved when  $^{51}\text{Cr}$ -labelled M8 cells were incubated with Triton-X-100.

#### *Proliferation assay*

Mixed lymphocyte reaction (MLR) was performed in triplicate, between peripheral blood mononuclear cells (PBMC) as responder cells and cells from the LCL cell line as stimulatory cells. As a third part of this MLR, beads (Ademtech, Pessac, France) were added with or without HLA-G5 attached. The linkage between HLA-G5 and the beads was through two types of anti HLA-G monoclonal antibodies: anti-HLA-G5 5A6G7 (Exbio) and anti-HLA-G 6A10.<sup>34</sup> The beads containing HLA-G5 were incubated

overnight at 4° in the presence or absence of DETA-NO 1 mM. LCL cells were irradiated with 75 Gy and the ratio of culture was one LCL cell to two PBMC. Beads were added in a ratio of 5000 beads per PBMC. After 5 days of culture, 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (Amersham Biosciences) was added to each well. After another 18 hr of culture, cells were collected in a filter, scintillation buffer (Perkin Elmer) was added and the filter was read in a  $\beta$ -radiation counter (Wallac 1450, Amersham Biosciences).

#### *Stimulatory assay*

U-937 and U-937-HLA-G1 transfected cells (500 000 cells/ml) were maintained for 2 days in complete medium. Supernatants were then incubated for 24 hr in the presence or absence of DETA-NO 1 mM at 37°. Then, T lymphocytes from healthy volunteers were incubated for 5 hr in those supernatants. After washing, T cells were stimulated with IL-2 2000 U/ml (Sigma-Aldrich) and anti-CD3 0.27  $\mu\text{g}/\text{ml}$  (Beckman Coulter) and cultured for 4 days. Then the proliferation level of lymphocytes was checked with a bromodeoxyuridine-based colorimetric assay (Roche Molecular Biochemicals, Mannheim Germany).

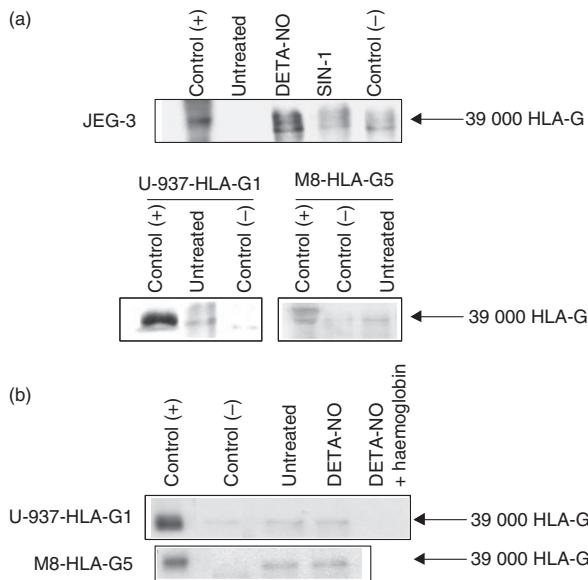
#### *Statistical analysis*

Data were expressed as mean  $\pm$  standard deviation. For statistical analysis the SPSS statistical program for Windows (SPSS Inc., Chicago, IL) was used. Results were compared with the Kruskal-Wallis and Mann-Whitney *U* non-parametric tests, and  $P < 0.05$  was considered significant.

## Results

#### NO can modify HLA-G protein by tyrosine nitration

Analysing the HLA-G sequence and structure, there are several tyrosine residues susceptible to nitration.<sup>35</sup> To address this issue, we initially studied the HLA-G<sup>+</sup> choriocarcinoma cell line JEG-3,<sup>10</sup> which was treated with the peroxynitrite donor SIN-1 or the pure NO donor DETA-NO for 24 hr, followed by immunoprecipitation of the cell extract with anti-nitrotyrosine antibody. Western blot using the anti-HLA-G antibody MEM-G/1 showed that either SIN-1 or DETA-NO produced HLA-G nitration (Fig. 1a). U-937 cells and M8-HLA-G5 cells produced NO that nitrated endogenous proteins (ref. 31 and supplementary data), and interestingly, there was a band of nitrated HLA-G1 and HLA-G5 in the lanes corresponding to cell lysates from U-937-HLA-G1 and M8-HLA-G5 untreated cells, respectively (Fig. 1a). Similarly, culture media from U-937-HLA-G1 and M8-HLA-G5 were also immunoprecipitated with anti-nitrotyrosine antibody, and in the Western blot there was a band corresponding to nitrated, shed



**Figure 1.** (a) Top panel: immunoblot analysis of human leucocyte antigen G (HLA-G) nitration in JEG-3 treated with DETA-NO 100  $\mu$ M or with SIN-1 100  $\mu$ M for 24 hr. Bottom panel: immunoblot analysis of HLA-G nitration in U-937-HLA-G1 and M8-HLAG5 cells. Positive control corresponds to a JEG-3 cellular lysate (top), or U-937-HLA-G1 cellular lysate (bottom). (b) Immunoblot analysis of HLA-G nitration in U-937-HLA-G1 and M8-HLA-G5 culture medium. Cells were treated with DETA-NO 100  $\mu$ M or DETA-NO plus haemoglobin. Control (+) corresponds to a cell lysate of U-937-HLA-G1 cells. Cell lysates and culture media were immunoprecipitated using anti-3-nitrotyrosine antibody. Negative control was preincubation of antibody with 3-nitrotyrosine 1 mM. Immunoprecipitated proteins were separated on sodium dodecyl sulphate-polyacrylamide gel, blotted onto a nitrocellulose membrane, and then probed with anti-HLA-G MEM-G1 antibody. A representative experiment out of three is shown.

HLA-G1 and HLA-G5 (Fig. 1b). These data were confirmed using the NO scavenger haemoglobin, which impairs shed HLA-G1 nitration (Fig. 1b). These results demonstrated that both exogenous and endogenous NO produce HLA-G nitration, which can be detected both in the cell and in the culture medium.

#### NO decreases HLA-G1 levels at the cell surface

Interestingly, the HLA-G cell surface expression decreased after NO treatment. This observation was confirmed by flow cytometric analysis in U937-HLA-G1 transfected cells (Fig. 2a), where treatment with DETA-NO 10  $\mu$ M for 24 hr produced a significant decrease of HLA-G cell surface expression (median fluorescence intensity of control, 10  $\pm$  1.6; median fluorescence intensity after DETA-NO 10  $\mu$ M, 4.5  $\pm$  1.1;  $P < 0.05$ ), and this reduction was more as the concentration of DETA-NO increased. However, haemoglobin as a NO scavenger blocked the DETA-NO-mediated decrease in HLA-G1 expression. In addition,

treatment with SIN-1 for 24 hr decreased HLA-G1 cell surface expression in U-937-HLA-G1-transfected cells (mean fluorescence intensity control: 5.4  $\pm$  0.8; mean fluorescence intensity after SIN-1 10  $\mu$ M: 3.8  $\pm$  0.7;  $P < 0.05$ ) (Fig. 2b). These results demonstrated that NO and peroxynitrite down-regulated HLA-G cell surface expression.

#### HLA-G remains in its native state in the presence of NO

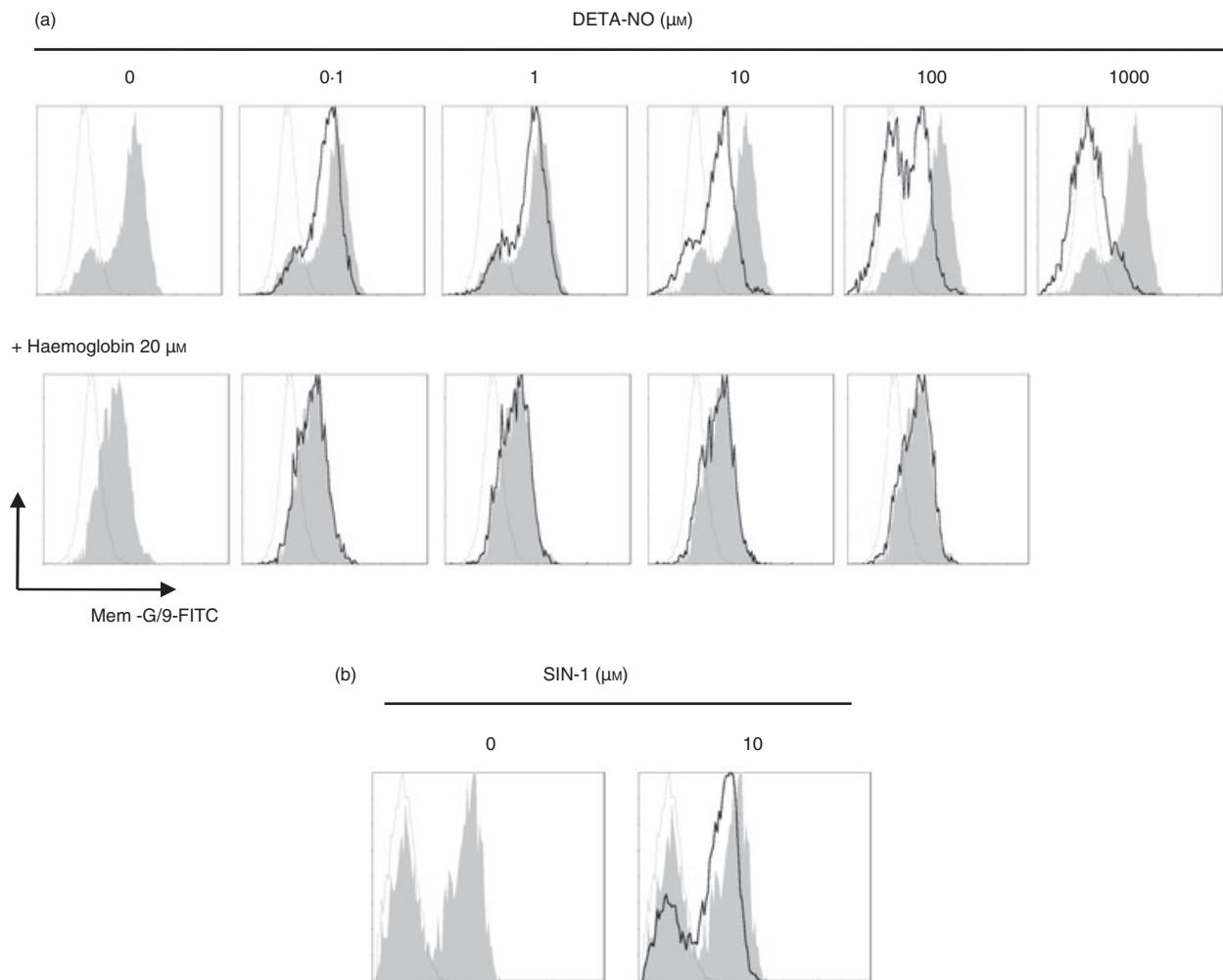
To find out whether this previous observation was the result of an alteration in the HLA-G protein, we analysed the HLA-G binding capacity to MEM-G9, an antibody which only attaches to HLA-G in its native form.<sup>36</sup> We treated culture medium containing sHLA-G1 with increased concentrations of SIN-1 for 12 hr at 37°, and then we measured HLA-G concentrations in the medium by ELISA using MEM-G9 as the capture antibody. We observed a significant ( $P < 0.05$ ), apparently lower concentration of HLA-G1 in the medium treated with SIN-1 at concentrations in the millimolar range (Fig. 3). At NO concentration lower than 1 mM, therefore, HLA-G predominates in its native form. In addition, the 10  $\mu$ M and 1000  $\mu$ M SIN-1-treated HLA-G1 supernatants were immunoprecipitated with anti-nitrotyrosine antibody, and ELISA of the non-nitrated HLA-G1 showed that 85  $\pm$  19% and 65  $\pm$  24% of the initial HLA-G1 did not undergo nitration, respectively. We did not use higher concentrations of SIN-1 because of its pro-apoptotic effect in the millimolar range.<sup>31</sup> We can conclude that part of HLA-G protein remains unnitrated and in the native form at NO concentrations below millimolar.

#### The decrease in HLA-G cell surface expression produced by NO is a post-transcriptional effect

To address the mechanism involved in the NO-mediated change of HLA-G cell surface expression, we initially investigated whether it took place at the level of HLA-G gene expression or was a post-transcriptional effect. For this purpose, JEG-3 cells were treated with DETA-NO for 24 hr. Immunoblot analysis using the anti-HLA-G antibody MEM-G1 showed a dose-dependent reduction in the HLA-G total cellular content (Fig. 4a). However, when HLA-G messenger RNA was evaluated by real-time RT-PCR, we observed that DETA-NO did not change the transcriptional levels significantly (Fig. 4b). We can conclude that the NO-dependent change in HLA-G cell surface expression is a post-transcriptional effect.

#### NO decreases HLA-G cell surface expression employing metalloprotease-mediated shedding

To determine if this decrease of HLA-G cell surface levels produced by NO was the result of HLA-G shedding to

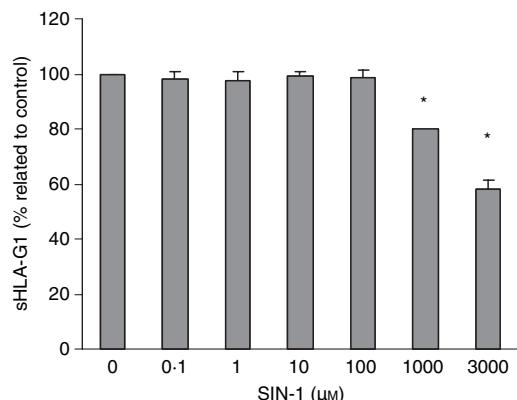


**Figure 2.** (a) Effect of DETA-NO on human leucocyte antigen G (HLA-G) cell surface expression in U-937-HLA-G1 cells for 24 hr in the absence (top) or presence (bottom) of haemoglobin 20  $\mu$ M. (b) Effect of SIN-1 on HLA-G surface expression in U-937-HLA-G1 cells for 24 hr. Cells were stained with the anti-HLA-G antibody MEM-G/9-FITC and analysed by flow cytometry. Grey histograms represent control cells and open histograms represent cells treated with DETA-NO or SIN-1, respectively. Dotted peaks represent irrelevant isotypic antibody. Data are representative of three different experiments.

the medium, we measured the concentration of HLA-G in the conditioned medium by ELISA after 24 hr of NO donor treatment. Figure 5(a) shows that DETA-NO produced a significant increase of HLA-G1 shedding from the U937-HLA-G1 cells. The addition of haemoglobin at a concentration of 20  $\mu$ M impaired this HLA-G shedding to the medium. Similarly to the effect caused by DETA-NO, SIN-1 10  $\mu$ M significantly increased HLA-G1 shedding to the medium ( $P < 0.05$ ) (Fig. 5a).

Metalloproteases are involved in HLA-G1 shedding,<sup>20,21</sup> and to study if the effect of NO in HLA-G1 cell surface expression and shedding was mediated by matrix metalloproteases, we treated U-937-HLA-G1 cells with DETA-NO for 12 hr in the presence of matrix metalloproteases inhibitors (Fig. 5b). Flow cytometry analysis showed that the presence of GM6001, a broad-spectrum inhibitor of metalloproteases, significantly increased HLA-G1 cell

surface expression (mean fluorescence intensity in untreated cells:  $4.4 \pm 0.3$ ; mean fluorescence in GM6001-treated cells:  $5.7 \pm 0.3$ ;  $P < 0.05$ ), and diminished the negative effect of DETA-NO in HLA-G1 cell surface expression (mean fluorescence intensity in DETA-NO-treated cells:  $3.4 \pm 0.1$ ; mean fluorescence intensity in DETA-NO plus GM6001-treated cells:  $4.0 \pm 0.1$ ). Furthermore, GM6001 decreased HLA-G1 shedding to the medium ( $P < 0.05$  related to untreated cells), and blocked the release of HLA-G induced by DETA-NO ( $P < 0.05$  related to cells treated with DETA-NO alone). The GM6001 negative control, NC-GM6001, did not have any effect on HLA-G1 cell surface expression (mean fluorescence intensity in NC-GM6001-treated cells:  $4.6 \pm 0.2$ ) and HLA-G shedding to the medium (Fig. 5b). However, not all metalloproteases participate in HLA-G shedding: while the inhibitors of metalloproteases 3 and 8 decreased



**Figure 3.** Effect of SIN-1 in the measurement of shed human leucocyte antigen G (HLA-G1) by enzyme-linked immunosorbent assay (ELISA). Conditioned medium from U-937-HLA-G1 cells was treated with different concentrations of SIN-1 for 12 hr, and the concentration of sHLA-G1 was measured by ELISA. Results show relative quantities of HLA-G concentration compared to control untreated cells (assigned a value of 100), and are expressed as mean  $\pm$  standard deviation of three different experiments. \* $P < 0.05$  related to control untreated cells.

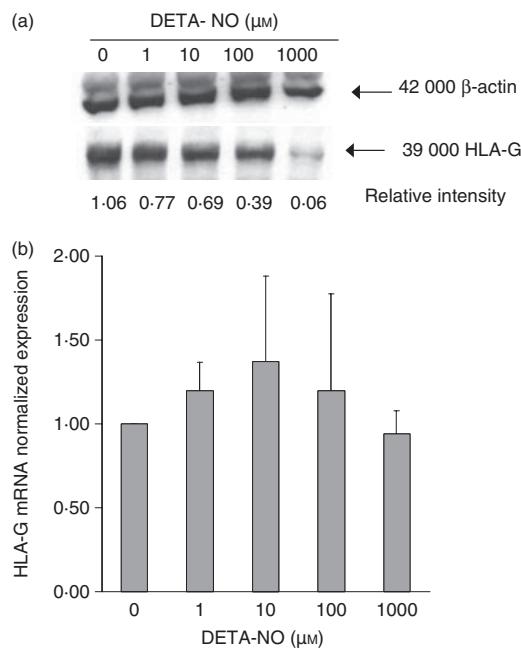
HLA-G1 shedding either in the absence or in the presence of DETA-NO, the inhibitor of metalloproteases 2 and 9 (INH 2/9) blocked neither HLA-G1 release to the medium (Fig. 5b) nor the decrease in cell surface expression (data not shown).

These results show that the effect of NO in HLA-G cell surface expression is through metalloprotease-mediated shedding to the medium.

#### HLA-G maintains its function under the presence of nitric oxide

We have previously demonstrated that M8-HLA-G-transfected cells, which express iNOS, can generate suppression,<sup>33</sup> and here we have shown that the presence of NO does not affect the HLA-G binding to MEM-G/9. For this reason we hypothesized that HLA-G immunosuppressive function was not affected by NO. To address this point we decided to study the HLA-G inhibitory capability in different situations: NK cell cytotoxicity, T-cell alloproliferation in mixed lymphocyte reaction, and T-cell response to IL-2 plus anti-CD3. To achieve a maximum effect of NO in HLA-G modification, we used the highest concentration previously employed.

Natural killer cells induced the lysis of the M8-pcDNA melanoma cell line, and preincubation of M8-pcDNA cells with DETA-NO 1 mM for 24 hr before the cytotoxic assay did not modify the susceptibility of these cells to NK cell cytotoxicity (Fig. 6a). As expected,<sup>37</sup> M8-HLA-G1-transfected cells were significantly more resistant to NK cytotoxicity ( $P < 0.05$  compared to M8-pcDNA cells). Preincubation of M8-HLA-G1-transfected cells with

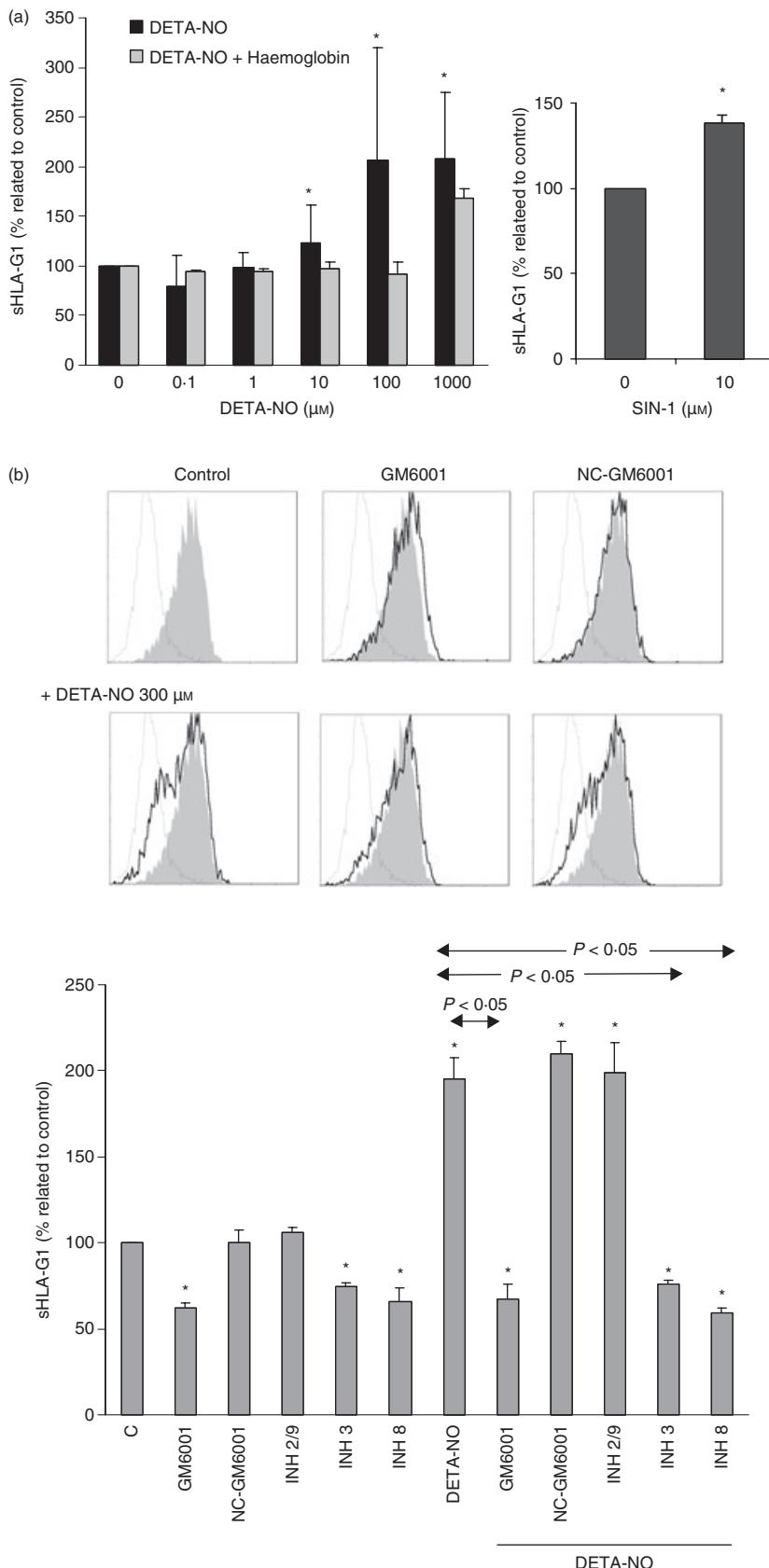


**Figure 4.** Human leucocyte antigen G (HLA-G) expression in JEG-3 cells treated with different concentrations of DETA-NO for 24 hr. (a) Western blot analysis of HLA-G expression in JEG-3 cells. Bands of HLA-G immunodetection with anti-HLA-G antibody MEM-G/1 appeared at 39 000. Loading control was performed using an antibody against  $\beta$ -actin, which produced a band of 42 000. Data indicate the intensity of the HLA-G band related to the  $\beta$ -actin band and are representative of three different experiments. (b) HLA-G messenger RNA expression analysed by real-time reverse transcription–polymerase chain reaction. Data are shown as relative quantities of HLA-G transcripts compared to control untreated JEG-3 cells (assigned a value of 1). Results are expressed as mean  $\pm$  standard deviation of three different experiments.

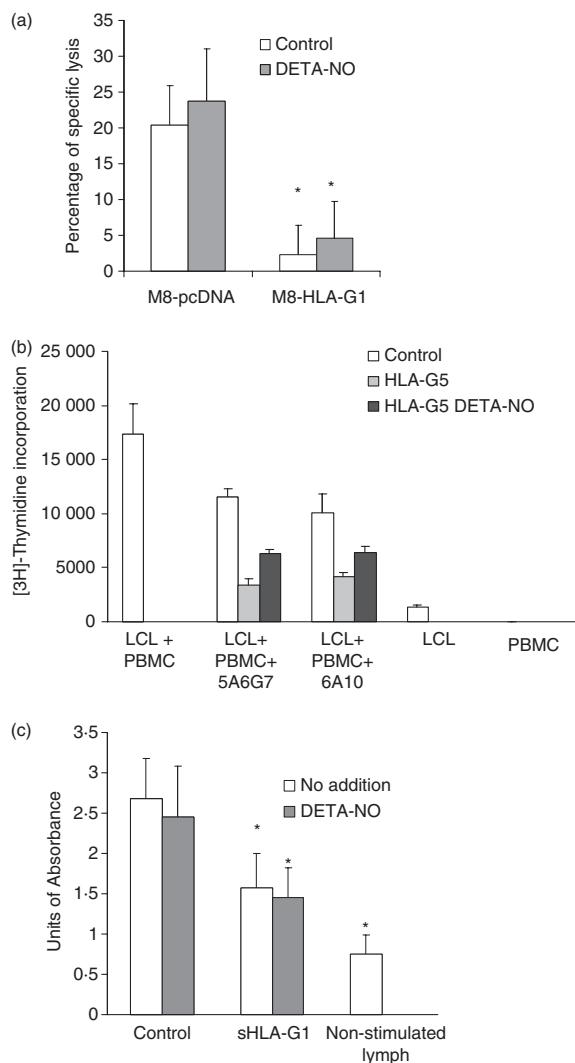
DETA-NO did not abolish the tolerogenic effect of HLA-G1.

The effect of NO in HLA-G suppressive properties of T-cell alloproliferation was checked by a MLR using LCL cells as stimulatory cells and PBMC as responder cells (Fig. 6b). When HLA-G5 attached to beads through the anti-HLA-G5 antibodies 5A6G7 or 6A10 was present in the MLR, there was a clear decrease of lymphocyte alloproliferation. To exclude that the effect was the result of the beads alone, the MLR was also performed in the presence of beads with the antibody attached. The proliferation was much lower when the beads had HLA-G5 attached than when the beads had only the antibody (Fig. 6b). If the HLA-G5-coated beads were previously treated with DETA-NO for 24 hr and then added to the MLR, the proliferative levels were not different compared with those obtained with untreated HLA-G5-beads.

Finally, we studied the proliferation of T cells stimulated with IL-2 plus anti-CD3 in the presence of supernatants containing sHLA-G1 treated or not with DETA-NO. This sHLA-G1-containing supernatant produced a



**Figure 5.** (a) Effect of DETA-NO (left) or SIN-1 (right) on human leucocyte antigen G (HLA-G) shedding from U-937-HLA-G1 cells. Cells were treated with different concentrations of nitric oxide (NO) donor for 24 hr without (dark bars) or with the NO scavenger haemoglobin 20 μM (grey bars). Total HLA-G was quantified by enzyme-linked immunosorbent assay (ELISA). Results show relative quantities of HLA-G concentration compared to control untreated cells (assigned a value of 100), and are expressed as mean ± standard deviation of three different experiments. \* $P < 0.05$  related to control untreated cells. (b) Modulation of the effect of DETA-NO in HLA-G liberation by matrix metalloproteases. U-937-HLAG1 cells were untreated or treated with DETA-NO 300 μM for 12 hr without or with the matrix metalloprotease inhibitor 50 μM: GM6001, its negative control, NC-GM6001, INH 2/9, INH 3, INH 8. Top panel: flow cytometry analysis using MEM-G9/FITC. Shaded histograms represent control cells and open histograms represent cells treated with DETA-NO. Dotted peaks represent irrelevant isotypic antibody. A representative experiment out of three is shown. Bottom panel: shed HLA-G1 in supernatant quantified by ELISA. Results show relative quantities of HLA-G concentration compared to control untreated cells (assigned a value of 100), and are expressed as mean ± standard deviation of three different experiments. \* $P < 0.05$  related to control untreated cells.



significantly lower proliferation compared to the supernatants obtained from the control cells ( $P < 0.05$ ; Fig. 6c). Preincubation of supernatants containing sHLA-G1 with DETA-NO 1 mM for 24 hr did not affect the proliferation induced by IL-2 plus anti-CD3.

These results clearly show that NO does not modify the capability of HLA-G in relation to the inhibition of NK and T-cell response.

## Discussion

We have found that HLA-G protein can be nitrated and, to our knowledge, this is the first report of a post-translational modification of HLA-G protein. However, not all the HLA-G is nitrated, which means that there is some type of hindrance to this reaction,<sup>4</sup> or that only some of the HLA-G molecules have a configuration prone to nitration. Nitration depends on different factors, such as the protein concentration, the proximity to the site of generation of nitrating agents, and the tyrosine environment.<sup>4</sup> The higher degree of nitration associated with a

**Figure 6.** (a) Effect of DETA-NO on the human leucocyte antigen G (HLA-G)-mediated inhibition of cytotoxicity. Data show the percentage of specific lysis achieved by NKL cells in 4 hr of coculture, with M8-pcDNA or M8-HLA-G1 cells as target cells. Target cells were previously incubated without (white bars) or with DETA-NO 1 mM (grey bars) for 24 hr. Results are expressed as mean  $\pm$  standard deviation of three different experiments. \* $P < 0.05$  related to control untreated cells. (b) Effect of DETA-NO on the HLA-G-mediated inhibition of mixed lymphocyte reaction. The proliferation of peripheral blood mononuclear cells was measured by [<sup>3</sup>H]thymidine incorporation in a mixed lymphocyte reaction with LCL cells as stimulatory cells. Beads with HLA-G5 attached were added untreated (shaded bars) or treated with DETA-NO 1 mM for 24 hr (dark bars). Beads with only the corresponding linking antibody (5A6G7 or 6A10) were added as controls (open bars). Results are expressed as mean  $\pm$  standard deviation of a triplicate experiment. (c) Effect of DETA-NO on the HLA-G-mediated inhibition of T-cell proliferation. The proliferation of T lymphocytes after stimulation with interleukin-2 (2000 U/ml) and anti-CD3 (0.25  $\mu$ g/ml) was measured through bromodeoxyuridine incorporation. T lymphocytes were preincubated for 5 hr with culture supernatant from U-937 cells or from U-937-HLA-G1 cells (open bars), or with these supernatants pre-treated with DETA-NO 1 mM for 24 hr (shaded bars). Results are expressed as mean  $\pm$  standard deviation of three different experiments. \* $P < 0.05$  related to control untreated cells.

lower capacity of HLA-G detection by our ELISA method suggest that nitration affects the properly folded HLA-G plus  $\beta_2$ -microglobulin complex.<sup>36</sup> However, noticeable interference by NO in HLA-G measurement using MEM-G9 plus anti- $\beta_2$ -microglobulin antibodies should not be initially expected under physiological NO production.

Nitration can also be produced by endogenous NO, as occurs in the case of U-937-HLA-G1 and M8-HLAG5 cells, where NO production by iNOS nitrates HLA-G in baseline conditions. The intracellular production of NO and the cellular oxidative metabolism lead to intracellular protein modifications.<sup>38</sup> The modified HLA-G can also be found in the medium, suggesting that detection of nitrated HLA-G in biological fluids could be a hallmark of HLA-G production at the inflammatory site.<sup>4</sup>

We showed in this work that NO decreases HLA-G cell surface levels by HLA-G shedding through a matrix metalloprotease-dependent mechanism, as the inhibitor GM6001 blocked NO-induced HLA-G1 shedding. Previous studies have shown that metalloproteases are involved in HLA-G1 shedding,<sup>20,21</sup> and NO can induce the activity and expression of matrix metalloproteases in different cell models, such as macrophages<sup>39</sup> and trophoblasts.<sup>40</sup> There are different metalloproteases that differ in their substrate specificity,<sup>41</sup> and the proteolytic shedding of HLA-G is not common to all matrix metalloproteases, as the inhibitor of matrix metalloproteases 2 and 9 had no effect on HLA-G shedding.

Generation of free radicals and oxidants, like reactive oxygen or nitrogen species, is a common result of metabolism and pathological processes.<sup>1</sup> Concentration of NO

*in vivo* at the site of inflammation is usually in the micromolar range<sup>42</sup> so, increased levels of circulating HLA-G in some inflammatory diseases<sup>24,25</sup> could reflect both an increased HLA-G expression and NO-induced shedding from the cell membrane. NO-dependent HLA-G modulation can be responsible in some circumstances for the negative HLA-G expression at the cell surface, while protein expression is observed inside the cell and in the medium.<sup>43</sup> In preeclampsia there is peroxynitrite production,<sup>44</sup> and lower HLA-G expression in trophoblasts.<sup>45,46</sup> We have also observed in JEG-3 cells that NO decreases HLA-G cell surface expression and increases shedding (data not shown). These findings could be linked in the light of these results; i.e. NO could participate in HLA-G down-regulation and nitration in this disease.

Importantly, the presence of NO did not affect the HLA-G immune suppressive properties, as HLA-G maintained the capability to inhibit NK cytotoxicity and lymphocyte proliferation. It was conceivable that the remaining non-nitrated HLA-G accounted for these inhibitory properties, as we have shown previously that, even in these pro-oxidant conditions, it is not completely nitrated and exists predominantly in its native form. We ruled out the effect of re-expression of un-nitrated HLA-G because in the proliferation assays we used soluble HLA-G pretreated with NO donor, which was still suppressive. NO can inactivate other suppressive molecules such as IDO,<sup>31</sup> and the fact that HLA-G is protective under this type of oxidative stress reinforces the notion of a relevant, and not redundant, tolerogenic role of HLA-G in pregnancy or in tumour immune evasion.<sup>13,47</sup> NO-promoted HLA-G shedding can be a mechanism to extend immunoregulation to the surrounding tissue under stressful conditions, as it could occur in placenta, where both HLA-G<sup>10</sup> and NOS are coexpressed.<sup>23</sup> HLA-G trogocytosis<sup>48</sup> was recently postulated as another mechanism used by tissues to spread immune protection based on intermembrane HLA-G transfer.

In summary, in this work we have demonstrated that NO nitrates HLA-G protein, and decreases HLA-G cell surface expression by increasing metalloprotease-dependent shedding. However, HLA-G keeps its suppressive properties, which could maintain immune protection under oxidative stress, such as that created by oxygen free radicals and NO, while other tolerogenic molecules are inactivated by these agents.

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