HLA-G and CD8⁺ regulatory T cells in the inflammatory environment of pre-eclampsia

Priscila Vianna¹, Andressa G Mondadori¹, Moisés E Bauer², Dinara Dornfeld³ and José A B Chies¹

¹Laboratory of Immunogenetics, Department of Genetics, UFRGS, Porto Alegre, RS, Brazil, ²Laboratory of Immunosenescence, Institute of Biomedical Research, PUCRS, Porto Alegre, RS, Brazil and ³Neo-Natal Unit, Nossa Senhora Conceição Hospital, Porto Alegre, RS, Brazil

Correspondence should be addressed to J A B Chies; Email: jabchies@terra.com.br

Abstract

During pregnancy, the maternal immune system is tolerant to foetal antigens via the engagement of immune regulatory mechanisms. Failure in regulating the maternal immunity to foetal antigens may lead to pre-eclampsia (PE). We addressed the role of *HLA-G* gene polymorphisms and protein expression as well as regulatory T cells and Th1/Th2/Th17 cytokines in healthy and pathological pregnancies. Blood samples from 26 pregnant women with PE, 25 non-PE and 7 strictly healthy pregnant women were assessed. PBMCs were phenotyped for early activation markers (CD25 and CD69), regulatory T-cell markers (CD8⁺CD28⁻ and CD4⁺CD25^{high}Foxp3⁺), ILT-2 (HLA-G receptor) and HLA-G. Lymphocyte proliferation was estimated and levels of IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF- α and IL-17 were measured. HLA-G polymorphisms (rs66554220 and rs1063320) were genotyped by PCR. PE women exhibited low levels of HLA-G in PBMCs and low frequency of regulatory CD8⁺CD28⁻ T cells. High amounts of the pro-inflammatory cytokines IL-17, IL-2 and TNF- α as well as IL-4 and IL-10 and an increased proliferative cell activation profile were observed in PE. The allelic and genotypic frequencies of the *HLA-G* gene polymorphisms and the frequency of CD4⁺CD25^{high}Foxp3⁺ T cells did not vary among the groups. Our data suggest that the cytokine imbalance presented in PE is associated with a deficient immune regulatory profile, contributing to an impaired immune tolerance between mother and foetus.

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Introduction

The maternal immunity is tolerant to the presence of the semi-allogeneic foetus during a healthy pregnancy. Adaptive changes, first suggested by Medawar in 1953 (Billingham *et al.* 1953), are related to the maintenance of immune tolerogenic mechanisms that ultimately lead to foetus acceptance. These regulatory mechanisms include *HLA-G* expression by placental and immune tissues, the generation of regulatory T cells and the production of cytokines. Any disturbance in this immune balance may trigger pregnancy complications like pre-eclampsia (PE).

PE is a leading cause of both maternal/foetal complications and mortality, occurring in 5–10% of all pregnancies. It is a major cause of perinatal deaths, premature births and intrauterine growth restriction. PE is characterized by high blood pressure, proteinuria and oedema associated with organ damage and prematurity (Sibai *et al.* 2005). Moreover, PE has been associated with poor placentation and angiogenesis, excessive maternal inflammatory responses, endothelial dysfunction and placental hypoxia, although its aetiology is largely

unknown. The ‘immune system maladaptation theory’ proposes that PE occurs when the maternal immune system does not adapt properly to the presence of the semi-allogeneic foetus (Dekker & Sibai 1999). Dysfunctional molecular and cellular mechanisms have been postulated to be involved with this maladaptation.

The *HLA-G* molecule has an important role during pregnancy. The *HLA-G* is a non-classical class I MHC tolerogenic molecule and its functions are related to an immunosuppressive response via IL-10 production, inhibiting effector T cells, inducing regulatory T-cell (Treg) proliferation and inhibiting the cytotoxic activity of natural killer (NK) cells, although favouring the secretion of pro-angiogenic factors. *HLA-G* also inhibits cytotoxic CD8⁺ T cells function and dendritic cell maturation (Steinborn *et al.* 2003, LeMaoult *et al.* 2004, Carosella *et al.* 2011). This molecule is highly expressed on the maternal–foetus interface (cytotrophoblast, endothelium of chorionic vessels, amniotic membrane) and is also present in some subpopulations of monocytes, CD4⁺ and CD8⁺ T lymphocytes, being sometimes acquired by transfer of membrane-bound forms (Rond 2004, HoWangYin *et al.* 2010). The pregnancy development

has been strictly related to soluble HLA-G molecules (sHLA-G) in maternal blood (Alegre *et al.* 2007, Rizzo *et al.* 2009). Higher serum sHLA-G levels have been described in women with successful pregnancies when compared with PE patients (Fuzzi *et al.* 2002, Yie *et al.* 2004, Hackmon *et al.* 2007). *HLA-G* gene polymorphisms have also been related to this pathology (Larsen *et al.* 2010). *HLA-G* gene expression is regulated by elements at both 5' gene promoter region and at the 3'UTR region. For instance, a microRNA binding site, which has a C/G polymorphism at position +3142 and a deletion/insertion of 14 base pairs (bp) in exon 8 (Veit & Chies 2009), was suggested to strongly interfere on HLA-G expression by affecting mRNA stability.

Failure in peripheral cellular regulatory mechanisms may also lead to pregnancy complications. It has been shown that regulatory CD4⁺CD25^{high}Foxp3 T cells play important roles in the induction of peripheral immune tolerance during pregnancy both in mother and foetus (Aluvihare *et al.* 2004, LeMaout *et al.* 2004, Mold *et al.* 2008). However, the role of CD8⁺CD28⁻ regulatory T cells during healthy and pathological pregnancies is largely unknown. These are highly experienced antigen-specific T cells, generated in response to persistent antigenic stimulation. They are terminally differentiated, low proliferative and senescent. The CD8⁺CD28⁻ T cells were shown to suppress autologous and heterologous CD4⁺ T-cell proliferation by rendering APC tolerogenic through the induction of receptors that transmit negative signals (Strioga *et al.* 2011). Furthermore, CD8⁺CD28⁻ T lymphocytes are able to secrete regulatory cytokines like IL-10 and TGF- β as well as exert cytotoxic activity against CD4⁺ T and APC cells (Smith & Kumar 2008). These cells are often referred as 'Ts' (suppressive CD8⁺ T lymphocytes).

Cytokines have a crucial role in the regulation of foetal and maternal interactions from embryo implantation until birth. Although under physiological conditions, low inflammatory cytokine levels are found in serum, it is important to point out that, during pregnancy, there are several primarily pro-inflammatory states, including placental and parturition. The cytokines also modulate the expression of adhesion molecules on the surface of both maternal and foetal cells (McEwan *et al.*

2009). Healthy pregnancies are often associated with a Th2-related immune response, whereas an exacerbated Th1 response is deleterious and is involved with certain complications such as PE and miscarriages (Marzi *et al.* 1996, Saito *et al.* 1999, Wilczynski 2005). However, it has been shown that IFN- γ , IL-1 and IL-6 are required during embryo implantation, contributing to vascular modification (Ashkar & Croy 2001, McEwan *et al.* 2009). Following embryo implantation, the maternal Th1 immune response is continuously suppressed. Also, regulatory cytokines (e.g. IL-10) control maternal tolerance to foetus by induction of HLA-G expression in the trophoblast (Moreau *et al.* 1999). The Th17 and Treg cells are important players in immune regulation during pregnancy (Saito *et al.* 2010, 2011). Nevertheless, it should be pointed out that while Treg and CD8⁺CD28⁻ T cells are involved with immune tolerance, the Th17 subset is related to autoimmune disorders and induction of chronic inflammation. In this context, there is scarce information regarding the role of IL-17 in PE.

The goal of this study is to assess key molecular and cellular mechanisms implicated in the PE development. Specifically, we addressed (i) the expression of HLA-G on peripheral monocytes/lymphocytes as well as *HLA-G* gene polymorphisms, (ii) regulatory T-cell subsets, (iii) T-cell activation and proliferation, and (iv) the production of Th1/Th2/Th17 cytokines *in vitro*.

Materials and methods

Subjects

Patients were recruited at the Obstetrics Healthy Service at Nossa Senhora Conceição Hospital, Porto Alegre, Brazil. From a total of 58 patients, we identified 26 pregnant women presenting PE (PE group), 25 pregnant women without PE ('non-PE' group) and 7 strictly healthy pregnant women (healthy group). Their clinical characteristics are listed in Table 1. The PE was defined as the presence of hypertension and proteinuria. Hypertension was characterized by blood pressure of at least 140 mmHg (systolic) or at least 90 mmHg (diastolic), on at least two occasions and 4–6 h apart following the 20th week of gestation in women known to be normotensive before. Proteinuria was defined

Table 1 Demographic and clinical characteristics of the study group.

Characteristics	PE (n=26)	Non-PE (n=25)	Healthy group (n=7)	P value
Maternal age at delivery (years) (ME $\pm$ SE)	28.58 $\pm$ 1.22	26.16 $\pm$ 1.01	26.17 $\pm$ 3.01	0.312*
Maternal smoking (n per day) (ME $\pm$ SE)	1.96 $\pm$ 0.9	1.00 $\pm$ 0.5	0	0.451*
Race/ethnicity (n; % Caucasian)	17 (65.4)	17 (68.0)	5 (71.4)	0.950**
Gestational age (weeks) (ME $\pm$ SE)	31.9 $\pm$ 1.48	30.9 $\pm$ 1.09	31.3 $\pm$ 2.29	0.861*
Primiparous women (n; %)	8 (30.7)	12 (48)	5 (71.4)	0.131***
Number of previous miscarriages (n; %)				
0	20 (77.0)	20 (80.0)	7 (100.0)	
1	5 (19.2)	5 (20.0)	0	
≥ 2	1 (3.8)	0	0	

Data are represented as mean (ME) and Standard error (SE) or sample size (n) and frequency (%).

*ANOVA one-way; **Pearson Chi-square; ***Fisher Chi-square.

by the excretion of ≥ 300 mg of protein every 24 h. If 24-h urine samples were not available, proteinuria was defined as a protein concentration of 300 mg/L or more (≥ 1 + on dipstick) in at least two random urine samples taken at least 4–6 h apart following the 20th week of gestation. The inclusion criteria for selecting the 'non-PE' group were: no rise in blood pressure and no hypertension or proteinuria. In the healthy group, besides a rise in blood pressure and/or hypertension and proteinuria, the following exclusion criteria also applied: smoking, gestational diabetes, preterm labour, bleeding, placental dysfunction, hypertension, swelling, pneumonia, toxoplasmosis, urinary tract infection, autoimmune diseases and influenza infections. To ensure the correct assignment, the pregnant women included in the 'healthy group' were clinically evaluated around three months after delivery. Hypertension or proteinuria during this period were exclusion criteria for this study. Women who had chronic hypertension, renal disease, collagen vascular diseases, cancer or thrombosis were not included in the study. All patients participating in this study gave their written informed consent, and the protocol was approved by the ethics committee of the Hospital Conceição Group (Porto Alegre, Brazil) and by the National Research Committee of Ethics.

Blood collection and cell isolation

Peripheral blood was collected by venipuncture and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient. The cell pellet was re-suspended in RPMI-1640 (Sigma-Aldrich) supplemented with 10% of FBS (Fetal Bovine Serum, Sigma-Aldrich), 2% glutamine and 100 U/mL penicillin–0.1 mg/mL streptomycin (Sigma-Aldrich). Cells were counted by means of microscopy (100 $\times$) and viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma-Aldrich). Cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. The concentration of cells in culture was 10⁷ cells/mL.

Table 2 HLA-G allele/genotype overall distribution and frequencies of the 14 bp polymorphism and haplotypes groups between PE, non-PE and pre-eclamptic women.

	PE (n=26) (freq)	Non-PE (n=25) (freq)	Healthy group (n=6) (freq)
Genotypes			
–14 bp/+14 bp	14 (0.66) ^{a,c}	14 (0.66) ^a	4 (0.7) ^c
+14 bp/+14 bp	6 (0.29) ^{a,c}	6 (0.29) ^a	2 (0.3) ^c
–14 bp/–14 bp	1 (0.05) ^{a,c}	1 (0.05) ^a	0 (0) ^c
Alleles			
14 bp deletion	16 (0.38) ^{b,d}	16 (0.38) ^b	4 (0.33) ^d
14 bp presence	26 (0.62) ^{b,d}	26 (0.62) ^b	8 (0.67) ^d
Haplotypes			
del/C	13 (0.3) ^e	9 (0.21) ^e	4 (0.33) ^e
del/G	9 (0.22) ^e	7 (0.17) ^e	1 (0.08) ^e
ins/C	3 (0.08) ^e	7 (0.17) ^e	0 (0) ^e
ins/G	17 (0.40) ^e	19 (0.45) ^e	7 (0.58) ^e

Haplotypes: del/C, deletion of 14 bp/C; del/G, deletion of 14 bp/G; df, degree of freedom; freq, frequency; ins/C, insertion of 14 bp/C; ins/G, insertion of 14 bp/G.

^a $\chi^2=0$, df=2, $P=1.00$; ^b $\chi^2=0$, df=1, $P=1.00$; ^c $\chi^2=0.351$, df=1, $P=0.554$; ^d $\chi^2=0.321$, df=2, $P=0.852$; ^e $\chi^2=2.688$, df=3, $P>0.05$.

Quantification of cytokines

Cells were stimulated with 1% of phytohaemagglutinin (PHA) (Roche Applied Science) during 18 h and harvested for immunological analyses. Following stimulation, supernatants of PHA-stimulated cells were collected and immediately frozen at -70°C before analyses. Multiple cytokines (IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF- α and IL-17) were determined by flow cytometry using the BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit II (BD Biosciences, San Diego, CA, USA). Sample processing and data analysis were performed according to the manufacturer's instructions. Sample data were acquired using a FACSCalibur flow cytometer (BD Biosciences). Sample results were generated in graphical and tabular format using the BD CBA Analysis Software (BD Biosciences).

Immunophenotyping

The PBMCs were isolated and labelled with specific antibodies for cell surface molecules: isotype controls, anti-CD3-FITC, anti-CD4-CY.5, anti-CD8-Cy.5 or PE, anti-CD14-FITC, anti-CD25-FITC, anti-FoxP3-PE, anti-CD28-PE, anti-CD45RA-PE, anti-CD45RO-FITC or PE, anti-CD56-FITC or PE, anti-CD69-FITC and CD85j-FITC (ILT-2) all were purchased from BD Biosciences. We also used the antibody anti-HLA-G-FITC (MEMG/9, from Exbio – Praha,

Figure 1 Low HLA-G expression in PE women. The HLA-G surface expression was evaluated by flow cytometry on PBMCs from pregnant women with or without PE and healthy subjects. (A) PBMCs were phenotyped using specific antibodies against CD14 and HLA-G. Low expression of HLA-G in monocytes (HLA-G⁺CD14⁺) of PE women was observed when compared with the non-PE group and the healthy one. $n=15$ PE, 9 non-PE, 6 healthy women. (B) Besides, low densities of HLA-G on monocytes were observed in PE women (276.32 ± 40.35 in healthy women, 169.82 ± 71.99 in non-PE women and 86.93 ± 34.80 in PE) $n=15$ PE, 9 non-PE, 6 healthy women. (C) Lymphocytes from pregnant women were phenotyped using specific antibodies against CD3 and HLA-G. The results show a decreased expression of HLA-G in lymphocytes (HLA-G⁺CD3⁺) of PE women when compared with the healthy group. (* $P<0.05$ and ** $P<0.01$). $n=19$ PE, 21 non-PE and 7 healthy women. PBMCs, peripheral blood mononuclear cells; PE, women presenting pre-eclampsia.

Table 3 Immunophenotyping of PBMCs between PE, non-PE and healthy women.

	PE ^a	Non-PE ^b	Healthy ^c	P-value
	Mean $\pm$ S.E. (n)	Mean $\pm$ S.E. (n)	Mean $\pm$ S.E. (n)	
% ILT-2+CD14+	50.0 $\pm$ 26.7 (23)	55.1 $\pm$ 28.1 (17)	65.1 $\pm$ 26.6 (7)	NS
% CD4+CD25+	21.22 $\pm$ 1.9 (22)	19.05 $\pm$ 2.8 (20)	22.31 $\pm$ 2.5 (7)	NS
% CD8+CD28+	20.71 $\pm$ 2.08 (21)	14.39 $\pm$ 1.79 (16)	18.93 $\pm$ 5.99 (6)	NS
CD25 (MFI in CD4+)	56.11 $\pm$ 10.51 (24)	43.87 $\pm$ 5.67 (24)	26.90 $\pm$ 1.3 (7)	NS
CD69 (MFI in CD4+)	220.19 $\pm$ 30.89 (22)	205.22 $\pm$ 26.79 (18)	173.10 $\pm$ 9.95 (6)	NS
% CD45RA+CD4+	24.41 $\pm$ 2.66 (24)	19.94 $\pm$ 3.96 (18)	24.18 $\pm$ 2.79 (5)	NS
% CD45RO+CD4+	7.22 $\pm$ 0.86 (24)	7.38 $\pm$ 2.53 (18)	6.02 $\pm$ 0.25 (6)	NS
% CD45RA+CD8+	26.93 $\pm$ 2.59 (20)	20.22 $\pm$ 1.79 (15)	34.78 $\pm$ 2.24 (5)	0.007** _{b,c}
% CD45RO+CD8+	4.83 $\pm$ 1.02 (20)	2.77 $\pm$ 0.53 (15)	5.16 $\pm$ 0.86 (6)	NS
% CD3-CD56 ^{bright}	9.0 $\pm$ 0.3 (22)	8.3 $\pm$ 0.4 (23)	10.4 $\pm$ 0.08 (7)	NS
% CD4+CD25 ^{bright} FoxP3+	6.9 $\pm$ 3.7 (19)	6.1 $\pm$ 2.54 (14)	4.3 $\pm$ 1.0 (6)	NS

ANOVA one-way. NS, not significant.

Czech Republic). The intracellular staining for FoxP3 was performed after cell permeabilization (Perm 2, BD Biosciences). We evaluated the frequency of peripheral NK cells (CD3⁺CD56^{bright}), suppressive CD8⁺CD28⁻ T cells and PBMC expressing HLA-G as well as its cell surface receptor (ILT-2) in pregnancies complicated or not by PE. Furthermore, the mean fluorescence intensity (MFI) of regulatory T cells (CD4⁺CD25^{high}Foxp3⁺ and CD8⁺CD28⁻) was estimated. A minimum of 20,000 events were gated by size (FSC) and granularity (SSC) in the FACSCalibur (BD Biosciences). The frequency of positive cells was calculated from the monocytes/lymphocyte gates. All analyses were performed by CellQuest (BD Biosciences) and FlowJo 7.5.5 (Tree Star Corporation, Ashland, OR, USA).

Proliferation/viability assays

The proliferative/viability responses were determined by colorimetric assays. After 92 h of culture (1% PHA-stimulated PBMCs), 100 μ L supernatant was gently discarded and 40 μ L freshly prepared 3-(4,5-dimethyl 2-thiazolyl) 2,5 diphenyl-2H-tetrazolium (Sigma-Aldrich) solution (5 mg/mL in RPMI-1640) was added to each well. The cell cultures were incubated for 4 h at 37°C in 5% CO₂ atmosphere. After complete removal of the supernatant, 100 μ L dimethyl sulfoxide (Sigma-Aldrich) was added to each well. Optical density (OD) was determined using a BioRad ELISA plate reader at wavelengths of 570 and 630 nm. Proliferation/viability was expressed as OD of stimulated – OD of non-stimulated cultures.

DNA extraction and PCR-RFLP

DNA used for genotyping of molecular variants of the *HLA-G* gene was obtained from samples of PBMC isolated from heparinized blood, previously frozen in RPMI medium supplemented with 10% FBS. DNA extraction was performed using a salting out technique, followed by phenol–chloroform extraction, aimed at obtaining a higher DNA purity and concentration. The *HLA-G* 14 bp insertion/deletion and +3142 polymorphisms were genotyped through polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), and different reactions were performed for each polymorphism, as described previously (Cordero *et al.* 2009).

Genotyping of the 14 bp deletion/insertion polymorphism in exon 8 (3'-UTR) of *HLA-G* gene (rs66554220)

PCR products of exon 8 concerning the 14 bp polymorphism were analysed in 6% polyacrylamide gels containing ethidium bromide and visualized under ultraviolet light. Amplification

Figure 2 High cell activation and T-cell proliferation during PE development. The early cell activation profile and the T-cell proliferation of PBMCs from women with PE, non-PE and healthy one were evaluated. (A) PBMCs were phenotyped using specific antibodies against early cell activation markers, CD25 and CD69. We found high frequencies of CD4⁺CD69⁺ T cells in PE than healthy one. $n=22$ PE, 18 non-PE, 6 healthy women. (B) Cell proliferation/viability was estimated by MTT assays and presented as percentage of basal proliferation among the three groups. The optical densities (ODs) were determined at wavelengths of 570 nm and 620 nm. The results showed a higher mean of basal proliferation in women with PE (OD 0.374 ± 0.096) when compared with the healthy subjects (OD 0.269 ± 0.054) (* $P < 0.05$). $n=11$ PE, 13 non-PE, 7 healthy. (C) High CD28 membrane densities on CD8 cells were observed in PE patients when compared with healthy one. $n=21$ PE, 16 non-PE, 6 healthy. IC, isotype control; PBMCs, peripheral blood mononuclear cells; PE, women presenting pre-eclampsia.

products for the 14bp polymorphism were about 224bp for the allele with the presence of 14bp and 210bp for the allele without 14bp.

Genotyping of nucleotide substitution polymorphism C/G at position +3142 (rs1063320)

The cleavage products of the 3'UTR region amplicon covering the C/G (+3142) *HLA-G* polymorphism were analysed in 1.5% agar gels containing ethidium bromide and were visualized under ultraviolet light. The C allele resulted in an intact 406bp fragment, while the cleavage of the G allele yielded fragments of 316 and 90bp. The 14bp and the microRNA binding site in *HLA-G* gene polymorphisms are in linkage disequilibrium.

Statistical analysis

All variables were tested for normality of distribution by means of the Kolmogorov–Smirnov test. Statistical analysis of data was performed using ANOVA and Kruskal–Wallis tests of SPSS 17.0. Multiple comparisons among levels were checked with Bonferroni *post hoc* test. *HLA-G* genotypic distribution was determined by direct counting. The genotypic frequencies were compared with Hardy–Weinberg expectations. *HLA-G* genotypic and allelic frequencies were compared among the groups using the chi-square test or Fischer exact test. The +3142 and the

14bp polymorphism genotypes were analysed with the software MLocus/WinPEPI, and haplotypes (ins/G, ins/C, del/G e del/C) were inferred and their frequencies determined. The significance level was set at $\alpha = 0.05$ (two-tailed).

Results

Allelic/genotypic frequencies and haplotype groups of the *HLA-G* gene polymorphisms

We sought to investigate genetic polymorphisms in *HLA-G* gene potentially related to PE development. The 14bp insertion/deletion polymorphism of the *HLA-G* gene leads to changes on mRNA structure and stability, altering the profile of *HLA-G* protein expression (Hviid *et al.* 2003). Maternal genotypic and allelic frequencies concerning the presence/absence of the 14-bp on exon 8 were thus compared across the studied groups. The maternal *HLA-G* genotype distribution in PE, non-PE and healthy women were in Hardy–Weinberg equilibrium for the 14-bp polymorphism (*data not shown*). The *HLA-G* allele/genotype, haplotype overall distribution and frequencies are shown in Table 2. No statistically significant differences were observed in allelic and genotypic frequencies among PE women, non-PE and healthy pregnant women. The polymorphisms 3'UTR 14bp (+2960) and 3'UTR C/G (+3142) in *HLA-G* gene are in linkage disequilibrium, and both potentially alter *HLA-G* expression. So, we also compared their haplotype frequencies (del/C, del/G, ins/C e ins/G) among all studied groups. No differences concerning the haplotype frequencies were observed among groups. Interestingly, high frequencies of the ins/G haplotype were observed in all three groups (Table 2).

Figure 3 Proportions of natural Tregs and FoxP3 expression in pregnancy. Phenotypic analysis of PBMC from women presenting or not PE and healthy subjects were performed by flow cytometry. We used specific antibodies against surface CD25, CD4 and intracellular Foxp3. Analysis of Foxp3 densities on Treg cells among PE, non-PE and healthy women were also performed. (A) The lymphocyte population was first defined, (B) the Treg cell population CD4⁺CD25^{high} was identified, and (C) a histogram of the area in which cells are CD4⁺CD25^{high} was created in order to determine the intensity of FoxP3 expression. The results revealed high FoxP3 densities on Treg cells from PE (mfi: 174 ± 17.0) when compared with non-PE women (mfi: 100 ± 22.6; $P = 0.02$). $n = 19$ PE, 14 non-PE, 6 healthy women. IC, isotype control; mfi, mean of fluorescence intensity; PE, pre-eclampsia; Treg, regulatory T cells.

Figure 4 Low levels of CD8⁺CD28⁻ T-suppressor cells in PE. The frequencies of CD8⁺ T-regulatory cells among women suffering PE, non-PE subjects and healthy women were assessed by flow cytometry. The CD8⁺ T suppressor (Ts) population was defined using specific antibodies (CD8 and CD28). (A) Dot plots defining the CD8⁺CD28⁻ cell population were traced. (B) We observed that PE women presented low percentage of CD8⁺CD28⁻ T cells when compared with the healthy women and non-PE (PE: 16.6 ± 8.6 vs healthy: 27.35 ± 8.2 vs non-PE: 15.18 ± 2.7, $*P < 0.05$). $n = 21$ PE, 16 non-PE, 6 healthy women.

Reduced HLA-G expression in PBMCs but unaltered ILT-2 expression in PE

We have also addressed the HLA-G protein expression in the PE development. Although the HLA-G molecule is constitutively expressed by monocytes, it is also expressed at low levels in peripheral lymphocytes (Mitsdoerffer *et al.* 2005, Feger *et al.* 2007). Both monocytes and lymphocytes can also express an HLA-G membrane receptor, ILT-2. Reduced proportions of monocytes (CD14+HLA-G+) and T cells (CD3+HLA-G+) expressing membrane HLA-G were observed in PE patients when compared with non-PE and healthy groups (Fig. 1A and B). Similarly, low membrane HLA-G density, as estimated by MFIs, was observed on peripheral PE monocytes when compared with healthy patients (Fig. 1C). The HLA-G density on lymphocytes and the frequency of ILT-2+-expressing cells did not differ among the studied groups (Table 3).

Effects of PE development on cell activation and T-cell proliferation

PE is a disease associated with inflammatory status that could theoretically modulate the T-cell activation profile. We investigated the CD69 and CD25 expression on Th cells to address early cell activation profiles. We observed higher frequencies of CD4⁺CD69⁺ cells in PE as compared with healthy women (Fig. 2A). In accordance, PBMCs of PE patients were spontaneously more proliferative than other groups, showing a higher basal T-cell proliferation/viability when compared with cells derived from healthy women (Fig. 2B). However, mitogen-induced proliferation/viability did not vary among groups (PE: 0.25 ± 0.03 vs non-PE: 0.31 ± 0.03

vs healthy: 0.28 ± 0.03 ; $P=0.55$). No differences were observed in the frequencies of CD4⁺CD25⁺ and CD8⁺CD28⁺ T-cell populations among groups, and membrane CD25 and CD69 densities on CD4 cells did not vary among the studied groups (Table 3). Nevertheless, we found high CD28 membrane densities on CD8 cells in PE when compared with healthy patients (Fig. 2C). We also screened the profile of naïve (CD45RA) and memory (CD45RO) CD4 and CD8 T cells in women that developed or not PE. We found higher frequencies of CD45RA⁺CD8⁺ cells in healthy subjects than in non-PE (Table 3).

Peripheral NK cells

We have also evaluated frequencies of peripheral NK cells (CD3⁺CD56^{bright}), as they secrete IFN- γ and TNF- α , participating in tissue building, remodelling and formation of new vessels during pregnancy (Vacca *et al.* 2011). In peripheral blood, this subset represents only 10% of all circulating NK cells (Rai *et al.* 2005). However, the frequencies of peripheral NK cells did not differ among PE, non-PE and healthy women (Table 3).

Regulatory T cells

Two major regulatory T-cell populations were also investigated in this study: natural Tregs (CD4⁺CD25^{bright}FoxP3⁺) and CD8⁺CD28[−] T cells. Although frequencies of Tregs did not vary between PE and healthy groups (Table 3), the FoxP3 expression (as estimated by MFI) was found upregulated in PE patients when compared with non-PE individuals (Fig. 3). Interestingly, PE patients had lower frequencies of regulatory T CD8⁺CD28[−] cells when compared with healthy pregnant women (Fig. 4).

Figure 5 High levels of inflammatory cytokines in PE patients. Quantification of Th1/Th2/Th17 cytokine levels was performed following 18 h of 1% PHA *in vitro* stimulation. The profile of cytokine secretion was quantified by flow cytometry among the studied groups (PE, non-PE and healthy women). PE women presented high levels of the pro-inflammatory cytokines IL-17 (D), TNF- α (F) and IL-2 (A), in comparison with the healthy women and TNF- α (F) and IL-2 (A) in comparison with non-PE subjects. The suppressor cytokine IL-10 (C) was elevated in PE and non-PE in comparison with healthy women. In addition, high IL-4 levels, a cytokine that directs towards a Th2 immune response (B), were observed in PE women when compared with the healthy and non-PE subjects. High IFN- γ levels (E) and IL-17 (D) were observed in non-PE when compared with healthy women. Kruskal–Wallis H test $*P<0.05$ and $**P<0.01$. All experiments were performed with 17 PE, 9 non-PE and 6 healthy subjects. PHA, phytohaemagglutinin.

Production of Th1/Th2/Th17 cytokines by mitogen-stimulated PBMCs

Multiple Th1/Th2/Th17 cytokines (IL-2, IL-4, IL-10, IL-17, IFN- γ and TNF- α) were assessed in culture supernatants following *in vitro* PHA stimulation and compared among the studied groups (Fig. 5). PBMCs from PE patients produced higher levels of IL-17, TNF- α , IL-2, IL-4 and IL-10 when compared with healthy or non-PE subjects. The IFN- γ levels did not vary among the studied groups.

Discussion

A comprehensive analysis of cellular and molecular mechanisms involved with PE development was undertaken in this study. We explored the role of the HLA-G molecule, the cell activation profile, regulatory T cells and Th1/Th2/Th17 cytokines in PE development. To note, this was the first work addressing the role of regulatory CD8⁺CD28⁻ T cells ('Ts') in PE development. As multiple immune regulatory networks are involved in the development of tolerance to foetus, a dysregulation on maternal immune responses will potentially lead to pregnancy complications.

Initially, two major polymorphisms in the *HLA-G* gene (14bp del/ins (rs66554220) and C/G +3142 (rs1063320)) previously associated with PE development (Vianna *et al.* 2007) were evaluated. However, no statistical differences were observed in allelic and/or genotypic frequencies among the studied groups. Although these results are in accordance with previous studies showing no association between maternal *HLA-G* genotypes and PE development (Humphrey *et al.* 1995, Iversen *et al.* 2008), this is still a matter of debate, as other studies have already suggested that the 14bp del/ins polymorphism could affect PE development (O'Brien *et al.* 2001, Hylenius *et al.* 2004, Vianna *et al.* 2007, Djuricic *et al.* 2015). Differences in population characteristics (e.g. sample size and ethnic origin) as well as methodologies may explain these discrepancies. As the evaluated polymorphic variants were in linkage disequilibrium, haplotypic analyses were performed. No effect of the haplotypes was observed on PE development. Nevertheless, it should be pointed out that a relationship of the del/G haplotype with low *HLA-G* mRNA levels was already proposed (Castelli *et al.* 2010) and therefore more studies are needed to evaluate the potential involvement of these haplotypes with PE. Importantly, a meta-analysis of the human leucocyte antigen-G (*HLA-G*) 14bp insertion/deletion polymorphism and its relationship to PE revealed a significant association in offspring from primipara and European Caucasian pregnancies (Pabalan *et al.* 2015).

As *HLA-G* has several pivotal immunosuppressive functions during healthy pregnancy including generation of Tregs, IL-10 production, as well as inhibition of effector functions of NK and CD8⁺ T cells and DC

maturation (Steinborn *et al.* 2003, LeMaoult *et al.* 2004, Carosella *et al.* 2011), we have also investigated the expression of the *HLA-G* molecule in PBMCs. In this study, we observed a downregulation of *HLA-G* in PE, with a decreased proportion of *HLA-G*⁺ cells and low *HLA-G* densities in peripheral monocytes. In contrast, surface *HLA-G* was abundantly expressed in healthy pregnant women. This suggests that healthy pregnancies are associated with the generation of more anergic *HLA-G*⁺ monocytes, contributing to adequate immune regulation. The phenomenon of acquisition of regulatory capacity was already demonstrated by cell-to-cell transfer of *HLA-G* (HoWangYin *et al.* 2010). Our findings are in accordance with previous studies that observed low levels of *HLA-G* in the plasma and at the trophoblast of PE women as well as in cases of premature birth (Yie *et al.* 2004, Hackmon *et al.* 2007, Rizzo *et al.* 2009). In addition, Hu *et al.* (2014) demonstrated that *HLA-G* acquisition from decidual dendritic cells by CD4⁺ T cells, a mechanism proposed as important to immune tolerance induction in pregnancy, is impaired in pre-eclampsia. In contrast, Alegre *et al.*, in a longitudinal study evaluating healthy pregnant women, reported an increased *HLA-G* expression (75%) in IFN- γ -stimulated monocytes (Alegre *et al.* 2007). Considering that *HLA-G* favours a suppressor environment during pregnancy, these data together support the immunological maladaptation hypothesis for PE development.

Complicated pregnancies have been associated with an activated immune profile. Here, we observed higher frequencies of CD4⁺CD69⁺ T cells in line with higher basal T-cell proliferation/viability in PE as compared with healthy women. These results are in agreement with a study reporting high numbers of CD4⁺CD69⁺ expressing cells in patients with recurrent spontaneous abortions when compared with healthy pregnant women (Prado-Dryer *et al.* 2008). In addition, Lashley *et al.*, comparing PBMCs from pregnant and non-pregnant women, described a significant increased percentage of activated T cells (CD25^{dim}) in pregnant women (Lashley *et al.* 2011). Thus, the elevated basal proliferative responses observed in our study are compatible with the high expression of early activation markers in PE patients.

NK cells constitute 60–70% of the maternal lymphocytes isolated from the maternal–foetal interface (Ashkar & Croy 2001). NK cytotoxicity is regulated by both cytokines and other regulatory molecules such as *HLA-G*, as stated by the fact that the immunoglobulin-like transcript ILT-2, an *HLA-G* receptor, is expressed by NK cells and was implicated in the regulation of the NK cytotoxicity (Navarro *et al.* 1999). Nevertheless, in our work no significant differences were observed in the frequencies of peripheral circulating NK cells or ILT-2 expression between PE and healthy patients. Probably, the assessment of specific local NK cell populations,

such as those from the decidua, could be a more powerful predictor of the real immune regulatory mechanisms involved in PE development. However, other studies have addressed the role of peripheral NK cells during pregnancy. For instance, an interesting study evaluating peripheral cytokine production and PE development showed that the prevalence of IL-17-expressing NK cells is elevated in PE compared with healthy pregnancy, which might play a role in the aberrant activation of peripheral NK cells in this disorder (Toldi *et al.* 2011). In addition, Fukui showed that women with a history of PE showed immunological abnormalities of natural cytotoxic receptors on peripheral blood NK cells during pregnancy (Fukui 2011).

Pregnancy complications could also occur due to an imbalance in peripheral regulatory cells. Indeed, naturally occurring Tregs (CD4⁺CD25^{bright}Foxp3) have important tolerogenic role during pregnancy (Aluvihare *et al.* 2004, LeMaoult *et al.* 2004, Mold *et al.* 2008), acting in cooperation with the HLA-G molecule in order to down modulate the maternal immune system. Actually, the HLA-G molecule has long-term tolerogenic functions by inducing Tregs (Carosella *et al.* 2011). Although the frequency of Tregs did not vary between PE and healthy groups, we observed an upregulated FoxP3 expression in Tregs of PE when compared with healthy individuals. This elevated Foxp3 expression could be an attempt to control the deleterious or exacerbated inflammatory response present in PE. However, there are conflicting data in the literature with studies suggesting no change (Paeschke *et al.* 2005), increased (Steinborn *et al.* 2012) or even lower Treg numbers (Toldi *et al.* 2008, Prins *et al.* 2009) in PE when compared with healthy subjects or non-pregnant women. Future studies are thus necessary to disentangle these discrepancies by including the analysis of Treg subsets and functional assays. We also observed that PE patients had low frequencies of regulatory CD8⁺CD28⁻ T cells when compared with healthy pregnant women. These results are in line with previous data reporting high frequencies of CD8⁺CD28⁻ T cells in the decidua of healthy pregnant women (Tilburgs *et al.* 2006) and augmented mRNA CD28 levels and Th1 cytokine levels associated with complicated pregnancies (Jin *et al.* 2011). Similarly to natural Tregs, CD8⁺CD28⁻ T cells have a strong suppressor activity on cellular immune responses, inhibiting cytotoxic function and T-cell proliferation (Filaci *et al.* 2007, Strioga *et al.* 2011) via cell-to-cell contact suppression and secretion of anti-inflammatory cytokines. Low counts of peripheral 'Ts' cells (CD8⁺CD28⁻ T cells) were also observed in chronic inflammatory diseases including multiple sclerosis (Mikulkova *et al.* 2010) and rheumatoid arthritis (Scarsi *et al.* 2011). In this sense, we can speculate that a reduced frequency

of CD8⁺CD28⁻ T cells may result in an insufficient control of inflammatory responses in PE. Although the cross-sectional design of this study precludes causal inferences, it indicates that this line of evidence deserves further investigation.

Complicated pregnancies are associated with a pro-inflammatory cytokine profile. In accordance, we observed that PBMCs from PE patients produced higher levels of IL-17, TNF- α and IL-2 when compared with PBMCs from healthy pregnant women. Nonetheless, the levels of so-called anti-inflammatory IL-4 and IL-10 cytokines were also elevated in PE patients. Several studies have already addressed the importance of different cytokine profiles during healthy or complicated pregnancies (Kalkunte *et al.* 2011, Kronborg *et al.* 2011). Some studies have addressed the role of IL-10 in inhibiting the inflammatory effects during PE, inhibiting TNF- α secretion and reducing blood pressure in experimental models (Tinsley *et al.* 2010, Zemse *et al.* 2010). IL-10 is secreted in the decidua during early pregnancy, playing an important role in the Th2 bias at maternal–foetal interface. We speculate that high IL-10, IL-4 levels and increased FoxP3 expression represent regulatory mechanisms involved in an attempt to control the deregulated or excessive inflammation found in PE. Harmon *et al.*, in a rat model of PE, have demonstrated that IL-10 increases Treg cells (Harmon *et al.* 2015). In addition, the high IL-2 levels found in PE patients could be favouring the increased Foxp3 expression, in an attempt to avoid the inflammation presented in this pathology. TNF- α was already involved in PE induction, giving rise to elevated serum uric acid (Zhao *et al.* 2016). In line with our data, Toldi *et al.* reported that NK cells from PE patients produce high levels of IL-17, favouring systemic inflammation (Toldi *et al.* 2011). Another interesting study also described an upregulation of the pro-inflammatory cytokine IL-17, secreted by innate lymphoid cells, in PE (Barnie *et al.* 2015). However, data on the role and source of elevated IL-17 in PE development is still scarce.

In order to select the non-PE group we took into consideration mainly no rise in blood pressure and no hypertension or proteinuria. Nevertheless, it is true that other factors, even environmental ones, can interfere with the immunological features that we would like to evaluate in our study. Thus, in the healthy group, besides a rise in blood pressure and/or hypertension and proteinuria, the following exclusion criteria also applied: smoking, gestational diabetes, preterm labour, bleeding, placental dysfunction, hypertension, swelling, pneumonia, toxoplasmosis, urinary tract infection, autoimmune diseases and influenza infections. These more strict criteria for inclusion in the healthy group could be responsible by the differences observed between this and the PE group, although the non-PE presented

several measures similar to PE women. These results reinforce the need for a stringent sampling procedure in the establishment of the experimental groups. In conclusion, low levels of HLA-G molecule and low frequencies of regulatory CD8⁺CD28⁻ T cells were associated with a pro-inflammatory cytokine profile in PE women. High amounts of the pro-inflammatory cytokines IL-17, IL-2 and TNF- α as well as of IL-4 and IL-10 and an increased proliferative cell activation profile were observed in PE. Taking together, our data suggest the existence of a cytokine imbalance on PE that may be associated with a deficient immune regulatory capacity. Further studies are needed to elucidate the molecular mechanisms and peripheral neuroendocrine events that interact with the immune network involved in the pathophysiology of PE.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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