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IMUNORREGULAÇAO DA GESTAÇÃO: RUMO AO SUCESSO

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LISTA DE ABREVIATURAS

APC – Célula apresentadora de antígenos
AR – Artrite Reumatóide
bp – pares de bases
CD – “cluster of differentiation”
Cy – Cychrome
FON – Linhagem celular de melanoma que expressa HLA-G
HLA-A – Antígeno Leucocitário Humano do tipo A
HLA-B – Antígeno Leucocitário Humano do tipo B
HLA-C – Antígeno Leucocitário Humano do tipo C
HLA-E – Antígeno Leucocitário Humano do tipo E
HLA-G - Antígeno Leucocitário Humano do tipo G
IFN-γ - Interferon gama
IL-10 – Interleucina dez
IL-12 – Interleucina doze
IL-4 – Interleucina quatro
IL-5 – Interleucina cinco
IL-6 – Interleucina seis
ILT – “Immunoglobulin like transcript”
JIA – Artrite Idiopática Juvenil
kDa – Kilo dálton
KIR2DL4 – ‘killer cell immunoglobulin-like receptor DL4’
LIF- Fator inibitório de leucemia
LT – Linfócito T
MBL – Lectina ligadora de manose
MEM-G9 – anticorpo usado para detecção de HLA-G de superfície
MHC – Complexo de histocompatibilidade principal
mRNA – RNA mensageiro
NK – Célula “natural killer”
PE – Pre-eclampsia
PHA - Fitoemaglutinina
PBS – Tampão fosfato salina
RNA – Ácido ribonucléico
RSA – “Recurrent Spontaneous Abortion”
TCR – Receptor de células T
TGF-β - Fator de crescimento tumoral beta
TH1 – Células T auxiliares do tipo 1
TH2 – Células T auxiliares do tipo 2
TNF-α - Fator de necrose tumoral alfa
uNK – Célula “Natural Killer” uterina

RESUMO

Durante a gestação, o corpo feminino sofre diversas alterações endócrinas, físicas, psicológicas e imunológicas. O sistema imune materno está em íntimo contato com o feto, que pode ser comparado a um alloenxerto, pois possui 50% de material genético paterno. Em 1953, o cientista e prêmio Nobel Peter Medawar foi o primeiro a formular o conceito de que o embrião representa um transplante para o sistema imune materno estando, portanto, vulnerável à rejeição ou tolerância imunológica. Portanto, como o feto não é rejeitado pela mãe? A interação imunológica que ocorre ao longo da gestação, é mantida até o período de amamentação. Neste trabalho avaliamos alguns componentes do sistema imune que variam durante a gestação, nos focando nos mecanismos que regulam a rejeição do feto pelo sistema imunne materno. Investigamos o papel de polimorfismos nos genes da lectina ligadora de manose (MBL) e da molécula imunomodulatória HLA-G em gestantes saudáveis ou com pre-eclampsia, assim como o balanço no perfil de produção de citocinas TH1 ou TH2 (IFN-γ, IL-4, IL-6, IL-2, IL-10 e TNF-α) na gestação de sucesso. Ainda discutimos o papel da exposição materna aos leucócitos paternos e a ocorrência de atopias na infância. Observamos que variantes alélicas de HLA-G e MBL estão relacionadas ao desenvolvimento e gravidade da pré-eclampsia. Gestantes saudáveis apresentaram perfis de proliferação de celular e freqüências de células NK elevados. Um alto perfil de proliferação celular foi observado também em mulheres saudáveis durante a primeira gestação em comparação a mulheres multíparas. Não observamos diferenças estatísitcas em relação aos níveis de produção de citocinas ao longo de gestações saudáveis. Apesar do óbvio envolvimento e importância de aspectos imunológicos na gravidez, não está claro até o presente momento como o organismo humano consegue tolerar a presença de um feto imuno-incompatível durante o período gestacional completo.

ABSTRACT

Several endocrine, psychological and immunological changes are observed during the gestation. The maternal immune system is in intimate contact with the fetus, which can be understood as an allograft to the mother because it has 50% of paternal genetic material. In 1953, the Nobel prize-winner Peter Medawar was the first to formulate the concept that the embryo represents a transplant to the maternal immune system and is thus vulnerable to rejection or immunological tolerance. Therefore the ultimate remaining question is how the fetus is not rejected by the mother? The immunological interaction that happens during the pregnancy is maintained until the breastfeeding period. Here, we evaluated some immunological aspects that fluctuate during the gestational period, focusing on the mechanisms that regulate the fetus rejection by the maternal immune system. In particular, we investigated the role of polymorphisms of mannose-binding lectin (MBL) and HLA-G genes as well as the balance between TH1/TH2 cytokine profile (IFN-γ, IL-4, IL-6, IL-2, IL-10 and TNF-α) in healthy and pre-eclamptic pregnant women. In addition, we further discussed the role of maternal exposition to paternal leukocytes and the occurrence of atopy in childhood. It was observed that the allelic variants of HLA-G and MBL are associated with the development and severity of pre-eclampsia. The healthy pregnant women had higher T-cell proliferation and NK cell frequency in the first trimester of gestation. A higher proliferation was also observed in the first pregnancy as compared to multiparous women. No differences were observed regarding the cytokine levels during the gestational period of healthy pregnancy. In spite of the obvious involvement and importance of immunological aspects in pregnancy, it is not clear until the present moment as the human organism can tolerate the presence of an imuno-incompatible fetus during all gestational time.

CAPÍTULO 1

INTRODUÇÃO

Capítulo 1 - INTRODUÇÃO

O sistema imune reconhece o próprio e o não-próprio, estabelecendo e mantendo uma irresponsividade ao próprio. O primeiro mecanismo que estabelece tolerância a antígenos próprios é a deleção clonal de células T auto-reativas no timo. No entanto, algumas destas células T auto-reativas escapam deste processo de deleção podendo reconhecer antígenos teciduais periféricos. Células T auto-reativas estão presentes normalmente em todos os indivíduos, mas a incidência de doenças autoimunes é baixa. Isto indica que mecanismos periféricos de auto-tolerância controlam estas células T auto-reativas. Durante a gestação o corpo feminino sofre diversas alterações endócrinas, físicas, psicológicas e também em seu perfil imunológico para se adaptar à presença do feto. O sistema imune materno está em íntimo contato com células e tecidos do feto semi-alogênico, que possui 50% de material genético paterno, o que o caracteriza como estranho ao organismo materno. Desta forma, mecanismos específicos devem agir para modular e moderar o sistema imune materno, impedindo que mulheres grávidas rejeitem seus fetos. Adicionalmente, alterações nestes mecanismos podem levar a complicações durante a gravidez (Hviid, 2006). Em 1953, Medawar propôs a existência da tolerância imunológica materna durante a gravidez, minimizando a ocorrência de uma resposta materna alogênica agressiva ao feto (Medawar, P., 1953). Medawar propôs a existência do mecanismo de tolerância na interface materno-fetal a patir de três observações: (1) a existência da separação física entre tecidos maternos e fetais; (2) a imaturidade antigênica do sistema imune fetal e (3) a “preguiça” imunológica do sistema imune materno. A interação

imunológica entre mãe e feto que ocorre ao longo da gestação é mantida até após o período de amamentação. Dentre os diversos fatores imunológicos que atuam na manutenção da homeostase durante a gestação podemos citar: o perfil de produção de citocinas do tipo TH2 (Lin, Mosmann *et al.*, 1993; Marzi, Vigano *et al.*, 1996), a presença de células T regulatórias (Aluvihare, Kallikourdis *et al.*, 2004; Saito, Sasaki *et al.*, 2005), o papel das células dendríticas (Pollard, 2008), a depleção local de triptofano inibindo a proliferação de células T (Munn, Zhou *et al.*, 1998), a expressão do fator inibitório de leucemia ("Leukemia Inhibitory Factor" - LIF) (Piccinni, Beloni *et al.*, 1998), assim como a importante ação imunossupressora da molécula HLA-G (Hviid, 2006). O feto recebe nutrientes e também células maternas que atravessam a interface materno-fetal através da placenta. Esta transferência de células maternas foi descrita há décadas (Medawar, P. B., 1953). Recentemente, Jeff Mold e colaboradores descreveram o desenvolvimento de um ambiente imunológico regulador fetal gerado a partir da presença das células maternas (Mold, Michaelsson *et al.*, 2008). O sistema imune do feto exerce uma função muito importante ao evitar o "ataque" pelas células maternas que atravessam a placenta através da ação de células T regulatórias fetais que podem permanecer em circulação (após o nascimento) por até 17 anos como memória imunológica, sendo capazes de reconhecer as células maternas. Este evento parece ocorrer através dos mesmos mecanismos utilizados pelo sistema imune materno quando este usa as células T regulatórias maternas evitando a rejeição do feto. Desta forma, mãe e feto estão sob um contato muito mais íntimo do que imaginado anteriormente, e o sistema imune fetal está, apesar de não completamente formado, bastante ativo mesmo antes do nascimento.

Apesar do óbvio envolvimento e importância de aspectos imunológicos na gravidez, não está claro até o presente momento como o organismo humano consegue tolerar a presença de um feto imuno-incompatível durante o período gestacional completo. Problemas de rejeição do feto podem resultar em abortamento. O índice de abortamento na população geral é cerca de 20% (Sotiriadis, Papatheodorou *et al.*, 2004). Apesar de ser considerada como normal, essa taxa de perda gestacional é alta. Uma das grandes causas da ocorrência de aborto é a malformação congênita. As malformações congênitas são definidas como “todo defeito na constituição de algum órgão ou conjunto de órgãos que determina uma anomalia morfológica estrutural presente devido à causa genética ambiental ou mista" e abrangem anomalias estruturais e do sistema nervoso central (Opas, 1984). As anomalias cromossômicas contribuem com 60-70% da ocorrência de abortos espontâneos, sendo as trissomias fortemente associadas a idade materna avançada (Eshre, 2008). Infecções (toxoplasmose, *Helicobacter pylori*), doenças autoimunes, doenças gestacionais, como pre-eclampsia, diabetes gestacional e doenças cardíacas congênitas constituem também importantes causas de aborto (Trogstad, Magnus *et al.*, 2008; Gill, O'brien *et al.*, 2009; Rahangdale, 2009; Sperber, Hom *et al.*, 2009). Além disso, causas não identificadas contribuem para a incidência de abortos que ocorrem principalmente no primeiro trimestre da gestação.

Diferentes trabalhos têm abordado as alterações imunológicas que ocorrem durante o período gestacional, tanto aquelas relacionadas com a implantação embrionária no útero, como aquelas envolvidas na tolerância ao feto durante toda a gestação (Munn, Zhou *et al.*, 1998; Aluvihare, Kallikourdis *et al.*, 2004; Trowsdale e Betz, 2006; Mcewan, Lins *et al.*, 2009). Dentre as

alterações imunológicas que ocorrem durante a gravidez, diferentes moléculas, tipos celulares e eventos imunomodulatórios estão envolvidos, regulando a extensa rede de interações imunológicas.

A molécula HLA-G

Uma molécula de grande importância na regulação da gestação é a molécula HLA-G que desempenha um papel fundamental na manutenção de um ambiente imunossupressor visando a aceitação fetal. Esta molécula está expressa em altos níveis na região materno-fetal, apresentando uma expressão protéica tecido-restrita em tecidos embrionários e adultos: no ovo fertilizado, no citotrofoblasto (Kovats, Main *et al.*, 1990), na membrana amniótica e nas células endoteliais dos vasos do córion durante o primeiro trimestre de gestação e em adultos na córnea (Le Discorde, Moreau *et al.*, 2003), células epiteliais tímicas (Crisa, Mcmaster *et al.*, 1997), em eritroblastos, em monócitos do sangue periférico (Alegre, Diaz-Lagares *et al.*, 2007) e em células T CD4+ e CD8+ (Le Rond, Le Maoult *et al.*, 2004; Feger, Tolosa *et al.*, 2007). O desenvolvimento da gestação somente ocorre quando moléculas solúveis de HLA-G são detectadas (Van Der Ven, Pfeiffer *et al.*, 2000; Fuzzi, Rizzo *et al.*, 2002). Altos níveis séricos de HLA-G solúvel (sHLA-G) já foram descritos em mulheres que apresentam gestações de sucesso, sendo estes níveis superiores aos encontrados em mulheres que apresentaram aborto ou outras complicações como pré-eclampsia (Pfeiffer, Rebmann *et al.*, 2000; Steinborn, Rebmann *et al.*, 2003; Yie, Li *et al.*, 2004; Alegre, Diaz-Lagares *et al.*, 2007).

A molécula HLA-G (Antígeno Leucocitário Humano do tipo G) é distinta de outras moléculas MHC clássicas por apresentar polimorfismos limitados, distribuição tecidual restrita e apenas 7 proteínas variantes. Nosso grupo revisa

o papel imunossupressor da molécula HLA-G na gestação, assim como no desenvolvimento de doenças inflamatórias (Capítulo 9 - **Anexo 1**). Esta molécula foi primeiramente identificada em células de coriocarcinoma (Ellis, Sargent *et al.*, 1986; Geraghty, Koller *et al.*, 1987; Ellis, Palmer *et al.*, 1990). O processamento alternativo do transcrito primário de HLA-G pode gerar quatro isoformas de membrana (G1, G2, G3 e G4) e três isoformas solúveis (G5, G6 e G7), que exercem função imunossupressora. A molécula HLA-G1 de membrana e a isoforma solúvel HLA-G5 são as mais estudas. Ao contrário das outras moléculas de HLA classe I, as moléculas de HLA-G parecem não exercer funções imunes estimulatórias significantes e mesmo respostas contra HLA-G alogenéico não são relatadas. Porém, assim como as outras moléculas de HLA, a molécula de HLA-G pode exercer efeito imunossupressor via interação a receptores de inibição como ILT-2 (CD85j/LILR B1), ILT-4 (CD85d/LILRB2) e KIR2DL4 (CD158d), diferencialmente expressos na superfície de células NK, células T, linfócitos B e células apresentadoras de antígenos (APC) (Carosella, Moreau *et al.*, 2003). A interação da molécula HLA-G com receptores de inibição ou ativação na superfície de céluas NK impede a lise de células do trofoblasto (Ponte, Cantoni *et al.*, 1999) ou ativa a produção de citocinas importantes na promoção do remodelamento da vascularização na região materno-fetal importante para um correto suprimento de oxigênio ao feto (Loke e King, 2000; Moffett-King, 2002; Parham, 2004). Recentemente, uma nova via de imunossupressão por HLA-G foi descrita onde linfócitos T e células NK ativados adquirem HLA-G por um mecanismo de transferência de membrana denominado “trogocitose”, assim, estas células passam a exercer função imunossupressora (Caumartin, Favier *et al.*, 2007;

Lemaoult, Caumartin *et al.*, 2007). A expressão de HLA-G tem sido associada a diferentes tipos de câncer atuando como meio de escape da vigilância do sistema imune e no crescimento de tumores (Rouas-Freiss, Moreau *et al.*, 2005). Além disso, vários estudos relatam uma expressão defeituosa da molécula HLA-G no citotrofoblasto extraviloso de mulheres com pré-eclampsia (Colbern, Chiang *et al.*, 1994; Hara, Fujii *et al.*, 1996; Lim, Zhou *et al.*, 1997).

Gene HLA-G

O gene HLA-G situa-se no complexo de histocompatibilidde principal (MHC do inglês “Major Histocompatibility Complex”), que abrange um conjunto de genes no braço curto do cromossoma 6, (região 6p21.3) recebendo este nome devido sua associação com a rejeição de enxertos. Um grande número de genes neste complexo apresenta funções importantes na regulação do sistema imune. Os genes do MHC são de extrema importância na imunidade adaptativa, tanto para a resposta humoral mediada por anticorpos quanto para a resposta celular (através das células T). Em humanos as moléculas de MHC são denominadas antígenos leucocitários humanos, HLA (do inglês “Human Leukocyte Antigens”). As moléculas HLA são primordialmente divididas em classes I e II. As moléculas de classe I são expressas praticamente em todos tipos celulares e apresentam antígenos endógenos pequenos para células T CD8+ (células T citotóxicas). Em humanos, a classe I de HLA ainda pode ser dividida na classe Ia, denominada clássica e representada pelas moléculas HLA-A, B e C e pela classe Ib, denominada não-clássica e representada pelas moléculas HLA-E, F e G. As moléculas “clássicas” de HLA tipo Ia são caracterizadas por serem altamente polimórficas e agirem direcionando células T para eliminar células infectadas por vírus ou outros patógenos intracelulares.

A molécula HLA-G é uma molécula não clássica do tipo Ib cuja estrutura se assemelha a molécula clássica de HLA, constituída de três domínios alfa ligados não-covalentemente e uma cadeia β2-microglobulina. Diferente de suas "parceiras" clássicas, a molécula HLA-G apresenta poucos polimorfismos em sua região codificadora, um perfil de expressão tecidual restrito em condições saudáveis e uma característica única de formar multímeros. HLA-G é a molécula HLA não-clássica mais estudada. Há apenas 36 alelos descritos, que codificam 14 proteínas diferentes quando comparados aos 673, 1077 e 360 alelos descritos para os HLAs A, B e C respectivamente (Nolan, 2008). Este perfil limitado de polimorfismos da molécula HLA-G é distribuído ao longo dos três domínios alfa, enquanto que nas moléculas clássicas de HLA estes polimorfismos estão concentrados nas fendas peptídicas. Conforme anteriormente citado, as formas protéicas de HLA-G se diferem em 7 isoformas: 4 formas associadas a membrana (G1-G4) e três formas secretadas. Estas isoformas são formadas a partir do processamento alternativo do gene HLA-G (Carosella, Moreau *et al.*, 2003). De acordo com estudos anteriores, dependendo do tipo celular e condições fisiológicas, formas diferentes de HLA-G são produzidas (Lila, Carpentier *et al.*, 2000; Morales, Pace *et al.*, 2003; Rouas-Freiss, Bruel *et al.*, 2005). Todas as isoformas contem pelo menos o domínio alfa-1 e a forma HLA-G1 é mais completa isoforma. Nas isoformas G5-G7, o intron 4 não é eliminado, o que introduz um códon de parada ("stop codon") e desta forma os domínios de membrana e citoplasmáticos não são traduzidos, resultando em formas secretadas, ou seja, proteínas solúveis (Ishitani e Geraghty, 1992; Fujii, Ishitani *et al.*, 1994; Kirszenbaum, Moreau *et al.*, 1994; Moreau, Carosella *et al.*, 1995; Paul,

Cabestre *et al.*, 2000). Já foi demonstrado que um polimorfismo no gene HLA-G, envolvendo a deleção/inserção de 14 pares de bases (bp) no éxon 8, está correlacionado com a manutenção da estabilidade do RNA mensageiro (mRNA) deste gene (Rousseau, Le Discorde *et al.*, 2003). A deleção dos 14bp está associado a baixos níveis de mRNA de HLA-G, extendendo-se a baixos níveis protéicos de HLA-G (Rebmann, Van Der Ven *et al.*, 2001; Hviid, Hylenius *et al.*, 2003). O envolvimento deste polimorfismo na manutenção da gestação, na fertilização *in vitro* e na pré-eclampsia já foi intensamente estudado (Hviid, Hylenius *et al.*, 2002; Hviid, Hylenius *et al.*, 2004; Hylenius, Andersen *et al.*, 2004; Tripathi, Abbas *et al.*, 2004; Vianna P., 2008). Em um estudo avaliando o papel de HLA-G no desenvolvimento de doenças inflamatórias como a Artrite Idiopática Juvenil (JIA) e Artrite Inflamatória (RA), nosso grupo demonstrou que variantes alélicas no gene HLA-G podem influenciar o desenvolvimento destas desordens. Mulheres jovens com JIA, apresentaram uma maior freqüência do alelo com 14 pb deletados em relação aos controles (Veit, Vianna *et al.*, 2008). Estes resultados sugerem que o alelo de deleção de 14pb é um fator de risco para o desenvolvimento de JIA, principalmente em mulheres.

Perfil de citocinas TH1 x TH2

A síntese de citocinas é disparada em resposta à ativação celular decorrente ao desafio antigênico ou fisiologicamente como, por exemplo, na hematopoiese, na maturação de células T ou na proliferação homeostática (Boyman, Letourneau *et al.*, 2009). As citocinas são mediadores protéicos solúveis envolvidos na emissão de sinais entre as células durante uma resposta imune. As células T $CD4^+$ auxiliares do sistema imune respondem a estímulos podendo se diferenciar em subpopulações que produzem e secretam

grupos distintos de citocinas e que, conseqüentemente, desempenham diferentes funções efetoras. Classicamente, podemos dividir as duas principais subpopulações efetoras de células T auxiliares em TH1 e TH2. As células da subpopulação TH1 produzem principalmente IFN-γ e IL-2, mas não IL-4 e IL-5, e desempenham importante papel nas respostas imunes celulares contra patógenos intracelulares. Já as células TH2 secretam IL-4, IL-5 e IL-10, mas não IFN-γ, e estão envolvidas na imunidade humoral e respostas alérgicas (Reiner e Seder, 1995; Abbas, Murphy *et al.*, 1996; Mosmann e Sad, 1996). Em humanos, IL-10 é uma citocina chave na desativação das funções efetoras de macrófagos e sua produção não é restrita aos tipos celulares TH2 ou TH1, exercendo uma atividade imunorregulatória juntamente com TGF-β. (Sornasse, Larenas *et al.*, 1996; Aoki, Borchers *et al.*, 2005). As subpopulações TH1 e TH2 se regulam mutuamente, sendo que a produção de IL-10 inibe o desenvolvimento e atividade das células TH1, enquanto estas são capazes de inibir o desenvolvimento de subpopulações TH2 a partir da produção de IFN-γ (Gajewski e Fitch, 1988; Morel e Oriss, 1998). Este controle mútuo é de importância crucial para o estabelecimento, manutenção e regulação das respostas imunes. Múltiplos fatores podem influenciar no desenvolvimento de precursores TH1 ou TH2, como a natureza das células apresentadoras de antígeno, a forma de administração do antígeno, o background genético do hospedeiro, e o ambiente de citocinas durante a ativação de células T (Scott, Natovitz *et al.*, 1986; Coffman, Varkila *et al.*, 1991; Duncan e Swain, 1994; Gollob e Coffman, 1994; De Becker, Moulin *et al.*, 1998). Citocinas regulatórias, como IL-2, IL-12, IL-10, TNF-α e TGF-β, são importantes na modulação da ativação de células T e no estabelecimento da resposta imune antígeno-

específica (Gajewski, Joyce *et al.*, 1989; Ahmadzadeh e Rosenberg, 2005; Katz, Pillarisetty *et al.*, 2005).

Como a gestação é um processo que requer intervenções imunológicas para permitir o crescimento e desenvolvimento do feto, uma gestação de sucesso apresenta dentre outras características, um perfil de regulação da produção de citocinas. As citocinas estão envolvidas em vários aspectos da gestação incluindo: implantação, placentação, ativação uterina e no amadurecimento cervical (Keelan e Mitchell, 2007). O padrão de resposta adaptativa parece afetar diretamente o sucesso gestacional. TNF-α é inicialmente necessário para a implantação embrionária no útero através da produção de VEGF ("vascular endothelial growth factor"), o qual modula a permeabilidade placentária e a angiogênese necessária para uma implantação e placentação eficientes (Chung *et al.* 2000). No entanto, esta resposta inflamatória inicial deverá ser regulada pela indução da expressão de IL-10. As células T regulatórias $CD4^{+}CD25^{high}$Foxp3+ são importante fonte de citocinas imunorregulatórias como IL-10 e TGF-β (Saito, Sasaki *et al.*, 2005). Tem sido demonstrado em mulheres com aborto recorrente, que apresentam falha após fertilização *in vitro* e transferência de embriões, que, apesar do aumento nos níveis de TNF-α não ocorre aumento da expressão de IL-10 (Ng *et al.* 2002). Diversos estudos indicam que um padrão efetor TH2 está associado ao sucesso gestacional e que a manutenção de um padrão TH1 será prejudicial para a gravidez (Marzi *et al.* 1996; Raghupathy *et al.* 2000; Clark & Croitoru 2001). Citocinas como IL-1 e IL-6 estão relacionadas à regulação das etapas da implantação, favorecendo a invasão do trofoblasto mediada por

metaloproteases, interferindo na receptividade do endométrio ao blastocisto (Mcewan, Lins *et al.*, 2009).

Sugere-se que o ajuste do sistema imune direcionado para o padrão TH2 seria responsável pela melhora clínica observada em doenças autoimunes de padrão TH1 durante a gestação (Khan *et al.* 2001). No entanto, contrariamente a outras doenças, a diabetes tipo 1, mesmo sendo considerada uma doença autoimune caracterizada principalmente por perfil de citocinas TH1, não paresenta melhora em seu perfil durante a gestação. Este aparente paradoxo nos mostra que a tolerância ao feto durante a gestação envolve inúmeros mecanismos, além de uma simples inibição da resposta efetora TH1 e direcionamento para TH2. Uma das possibilidades de indução de tolerância é a ocorrência de uma imunossupressão no organismo. Porém, uma imunossupressão geral (mesmo em baixo nível) poderia potencialmente afetar a homeostase do sistema imune através da interferência em subpopulações de células regulatórias. Além disto, a capacidade de desenvolvimento de uma resposta imune do organismo, como um todo e, conseqüentemente, as redes de interação entre as células do sistema imune podem ser afetadas por uma imunossupressão geral.

Células regulatórias CD4+CD25^{high+}Foxp3+

A noção de uma subpopulação de células T supressoras regulatórias (Treg) tem sido revista com importante função na estruturação de diferentes respostas imunes (Sakaguchi, Sakaguchi *et al.*, 1995; Read, Malmstrom *et al.*, 2000; Sakaguchi, 2000, 2005; Curotto De Lafaille e Lafaille, 2009). A subpopulação T que parece reunir as características necessárias para desempenhar o papel de supressão em uma resposta imune pertence a um

grupo de células T $CD4^{+}CD25^{high}$ que expressa ainda o fator de transcrição Foxp3. Estas células são consideradas naturalmente ativadas e presentes no organismo em freqüências relativamente baixas. Apenas 2-6 % das células T $CD4^{+}$ em humanos são células que expressam altos níveis de CD25, tornando-se regulatórias (Treg). As células $CD4^{+}CD25^{high}Foxp3^{+}$ inibem a imunoestimulação de células T convencionais através do contato célula-célula ou através da produção de citocinas imunorregulatórias tais como IL-10 e TGF-β. As células T $CD4^{+}$ humanas que expressam baixos níveis de CD25 não possuem atividade imunorreguladora. Anomalias genéticas de Foxp3 afetam o desenvolvimento e a funcionalidade das células T $CD4^{+}CD25^{high}$ (Sakaguchi, Wing *et al.*, 2007). A importância das células T $CD4^{+}CD25^{high}Foxp3^{+}$ na prevenção de doenças autoimunes *in vivo* foi revelada por diferentes experimentos envolvendo técnicas de timectomia e transferência de células (Berthelot & Maugars 2004, Sakaguchi 2004, Bluestone & Abbas 2003). Estes estudos demonstraram a importância das células Treg $CD4^{+}CD25^{high}Foxp3^{+}$ na tolerância natural periférica e no controle negativo de respostas imunes patológicas/fisiológicas. A remoção desta subpopulação de células Treg não leva apenas ao aumento da incidência de doenças autoimunes, como também ao aumento da resposta a antígenos não-próprios incluindo proteínas xenogenéicas e aloenxertos (Wood e Sakaguchi, 2003). A utilização de citometria de fluxo nos permite avaliar precisamente a subpopulação de células T regulatórias através da análise de expressão de marcadores de superfície das mesmas ($CD4^{+}CD25^{high}Foxp3^{+}$). Os principais marcadores fenotípicos para estas células são CTLA-4, CD45RO e HLA-DR que são expressos

consitutivamente nesta subpopulação celular ao contrário de células T $CD4^+$ não regulatórias onde estes são expressos temporariamente.

Inibição das células NK na região de contato materno-fetal

O endométrio forma a interface mãe/placenta e apresenta contato direto com as células e tecidos fetais durante a gravidez. Nesta região, existe uma grande quantidade de linfócitos, e seria de se esperar que o feto, que representa o equivalente a um aloenxerto (pois expressa também antígenos herdados de origem paterna) sofresse rejeição. Para que a rejeição não ocorra, diversos mecanismos imunossupressores são colocados em funcionamento, sendo um destes, a ausência da expressão de moléculas do MHC ("major histocomatibility complex") de classe I e II nas células trofoblásticas (as quais formam o tecido fetal mais intimamente em contato com as células maternas), evitando assim a citotoxicidade mediada por células T CD8+. No entanto, a não expressão de MHC de classe I pelas células trofoblásticas as torna alvos potenciais para a lise mediada por células NK. A expressão nas células trofoblásticas da molécula não clássica do MHC, HLA-G a qual é reconhecida por diferentes receptores de inibição das células NK, tais como CD94/NKG2A e LIR-1, parece ser responsável pela inibição da lise celular por células NK (Rouas-Freiss, Goncalves *et al.*, 1997; Szereday, Barakonyi *et al.*, 2003; Varla-Leftherioti, Spyropoulou-Vlachou *et al.*, 2003). A maioria dos trabalhos avalia exaustivamente os genes de classe Ib, principalmente HLA-G, que apresentam sua distribuição tecidual restrita, baixos níveis de expressão e polimorfismos limitados. Embora extensa especulação, as funções exatas dos genes de classe Ib ainda não foram completamente elucidadas. As células do trofoblasto, originadas do feto, não expressam as clássicas moléculas de antígeno de HLA

classe I e II, exceto por uma fraca expressão de HLA-C(Redman, Mcmichael *et al.*, 1984; Hunt, Andrews *et al.*, 1987).

Sendo assim, destacamos a grande importância do papel imunorregulador da molécula HLA-G durante a gestação. O desenvolvimento saudável da gestação ocorre somente quando moléculas solúves de HLA-G são detectadas (Pfeiffer *et al.* 2000; Fuzzi *et al.* 2002). Mulheres que desenvolvem gestações de sucesso, apresentam um alto nível sérico de HLA-G solúvel (sHLA-G), sendo estes níveis superiores aos encontrados em mulheres que sofreram aborto. Concomitante a este fato, diversos experimentos sugerem que citocinas efetoras do tipo TH2 estão associadas a altos níveis de sHLA-G (HLA-G solúvel) - e conseqüentemente ao sucesso gestacional, ao passo que baixas concentrações de sHLA-G parecem induzir a expressão de citocinas efetoras do tipo TH1 (Maejima *et al.* 1997; Kapasi *et al.* 2000). Estudos de polimorfismos no gene de HLA-G em diferentes populações de países industrializados têm reportado pouca variação de seqüências dentro destes genes (Steffensen, Christiansen *et al.*, 1998; Van Der Ven, Skrablin *et al.*, 1998). Porém, sugere-se que, em estudos em países em desenvolvimento, há uma maior variabilidade de seqüência destes genes, provavelmente devido à maior exposição populacional a infecções por patógenos (Van Der Ven e Ober, 1994).

Pré-Eclampsia

Dentre diversas complicações gestacionais, a pré-eclampsia atua como agravante em uma gestação. Esta patologia responsável por mais de 7% de mortalidade materno-fetal, representando a segunda causa de morte materna, é de grande importância para uma correta compreensão dos fenômenos

imunológicos que regulam o processo da gestação. A etiologia e patogênese da doença envolvem uma combinação de fatores de predisposição materno-fetal e fatores ambientais (Roberts, Taylor *et al.*, 1989). A pré-eclampsia é caracterizada por elevada pressão sanguínea, proteinúria e edema associados a danos em órgãos e prematuridade. Uma das hipóteses da patogênese da pré-eclampsia é a teoria da maladaptação imunológica, que propõe que o sistema imune materno não se adapte adequadamente ao feto semi-alogênico (Dekker e Sibai, 1998).

Muitas citocinas e fatores de crescimento têm sido caracterizados no útero e placenta de gestantes normais e com pré-eclampsia (Wilczynski, Tchorzewski *et al.*, 2002; Taylor, Grimwood *et al.*, 2003; El-Baradie, Mahmoud *et al.*, 2009) e as funções do trofoblasto são modificadas pela ação de várias destas citocinas (Hayashi, M *et al*. 2003). É relatada uma inibição da atividade imunossupressora de células T regulatórias em condições patológicas como a pré-eclampsia (Sasaki, Darmochwal-Kolarz *et al.*, 2007). Uma variedade de peptídeos encontrados na placenta como hormônio liberador de corticotropina, gonadotropina coriônica e inibina-A são marcadores de interesse para a predisposição à pré-eclampsia (Zeeman, Alexander *et al.*, 2003). Considerando a hipótese de maladaptação imunológica, supõe-se que os genes candidatos que contribuem para o desenvolvimento da pré-eclampsia são os genes de função imunológica. Muita atenção tem sido relevante na avaliação dos genes do complexo de histocompatibilidade principal (MHC) no braço curto do cromossoma 6, o HLA. Estes estudos têm investigado os loci do HLA clássico Ia e II e seus produtos. Vários resultados contraditórios têm sido obtidos. No entanto, a relevância destes loci gênicos como candidatos atuais é importante

porque, ao contrário de outros tecidos, o trofoblasto não expressa antígenos clássicos de MHC (HLA-A, HLA-B e HLA-DR), mas sim antígenos HLA-C, E e mais proeminente, a molécula não-clássica HLA-G (HLA de classe Ib). Após a caracterização do gene de HLA-G e seu perfil de expressão, tornou-se óbvio que este gene é candidato na região do MHC, caracterizando perfis imunológicos de sucesso ou não-sucesso gestacional. Em um estudo populacional, nosso grupo demonstrou que variantes no gene da molécula HLA-G podem influenciar o desenvolvimento de desordens gestacionais como a pré-eclampsia (Vianna, Dalmaz *et al.*, 2007). Sendo assim, aspectos imunológicos como o papel imunossupressor da molécula HLA-G durante a gestação, relacionam-se com o desenvolvimento desta patologia.

Exposição repetida ao esperma

Dentre as várias causas de ocorrência de aborto ou complicações gestacionais, fatores paternos também são de extrema importância (Hamamah, Royere *et al.*, 1997). A ocorrência de pré-eclampsia, por exemplo, é um fator de complicação da gestação e especula-se que uma de suas causas de surgimento seja o curto período de tempo de exposição ao esperma paterno, sugerindo que casais com maior tempo de relacionamento e cohabitação apresentam chances menores de desenvolver complicações como a pré-eclampsia (Einarsson, Sangi-Haghpeykar *et al.*, 2003). A exposição materna repetida ao esperma deve condicionar o sistema imune materno, induzindo tolerância a uma futura presença do feto que se constitui de antígenos paternos. Em uma hipótese desenvolvida, nosso grupo sugere que a exposição materna repetida aos antígenos paternos através do esperma pode modular o sistema imune fetal, induzindo um ambiente regulatório, reduzindo as chances

de ocorrência de atopias na prole (Vianna P., 2008). Esta hipótese corrobora com a idéia de que a exposição prévia materna a infecções gera proteção a seus filhos contra doenças respiratórias, como alergias e vai também a favor da "hipótese da higiene", que defende que condições de vida mais higiênicas com menos exposição à patógenos na infância induz a um aumento da incidência de alergia. Mães em contato com outras crianças, expostas a infecções ou sujeiras, passarão para sua prole uma condição imune com características regulatórias, protegendo seus filhos contra infecções. Ainda, em uma família com vários filhos, estas crianças estarão sempre desafiando seu sistema imune devido ao contato direto com diversas crianças, que também estabelecem contatos fora de casa, em creches, por exemplo. Estas crianças trarão para casa vírus adquiridos fora que poderão ser passados para os irmãos ou para a mãe grávida, estimulando o sistema imune materno e gerando um ambiente regulatório que pode ser passado ao feto.

Papel da molécula MBL na gestação

Vários estudos sugerem que um perfil de inflamação excessiva durante a gestação está associado ao desenvolvimento de complicações como a pré-eclampsia, ocorrência de partos prematuros e abortos (Lin, Moss *et al.*, 2007; De Man, Dolhain *et al.*, 2008). Desta forma, o controle do perfil de uma resposta inflamatória desempenha um papel fundamental na manutenção de uma gestação de sucesso. Porém, a indução de um ambiente pro-inflamatório no útero é essencial para permitir uma correta angiogênese na interface da região materno-fetal nas primeiras semanas de gestação (Hu, Yang *et al.*, 1992; Lambropoulou, Tamiolakis *et al.*, 2006). Mediadores imunológicos como os componentes do sistema do complemento estão presentes no ambiente

uterino e garantem a manutenção de um ambiente inflamatório no útero, primordial para uma boa placentação (Wells, Bennett *et al.*, 1987).

Dentre estes reguladores da inflamação presentes no útero, a lectina de ligação a manose (MBL), um componente da imunidade inata, é relacionada como importante mediador da inflamação durante a gestação (Van De Geijn, Dolhain *et al.*, 2007). A MBL é uma das proteínas mais estudadas da família das colectinas humanas, que são oligômeros formados por cadeias polipeptídicas caracterizados por um domínio de reconhecimento de carboidratos (CRD) ligado a uma região colagenosa (Sorensen, Thiel *et al.*, 2005). A MBL desempenha um papel importante na primeira linha de defesa do organismo e teve seu papel inicialmente avaliado em 1968, em um caso de uma criança com infecção bacteriana recorrente relacionada com baixos níveis desta proteína (Worthley, Bardy *et al.*, 2005; Bouwman, Roep *et al.*, 2006). Esta proteína está presente no soro sob as formas oligo ou poli associadas e iniciam e ativam a via do complemento dependente da lectina, se ligando a motivos na superfície microbiana, promovendo a fagocitose e efeitos antibacterianos.

Além da ativação do complemento, a MBL pode ser considerada uma proteína pro-inflamatória, modulando a inflamação e ainda induzindo apoptose (Turner, 2003). Alguns estudos demonstraram que altos níveis de MBL estão associados com gestações de sucesso e que estes níveis são aumentados principalmente no primeiro trimestre das gestações de sucesso (Van De Geijn, Roos *et al.*, 2007). Além disso, mulheres grávidas portadoras de uma variante polimórfica de MBL apresentam um risco aumentado de desenvolver diabetes mellitus gestacional e seus filhos nascem com peso acima da média (Megia, Gallart *et al.*, 2004). Porém, há resultados contraditórios em relação aos níveis

protéicos de MBL e sua associação com gestações de sucesso ou não. Desta forma, as funções exatas das alterações dos níveis de MBL na gestação não estão totalmente elucidadas (Van De Geijn, Dolhain *et al.*, 2008).

A estrutura da MBL é formada por multímeros de cadeias polipeptídicas idênticas de 32kDa, cada uma compreendendo quatro regiões distintas codificadas pelos diferentes éxons do gene *MBL2*, que será analisado posteriormente em um artigo do nosso grupo (**capítulo 5**). Cada cadeia tem uma região C-terminal com um domínio de reconhecimento de carboidratos cálcio-dependente; uma pequena região hidrofóbica, denominada pescoço, em formato de hélice; uma região colagenosa contendo glicina e uma região N-terminal rica em cisteína. Três cadeias polipeptídicas formam uma hélice tripla com a região colagenosa, estabilizada pelas interações hidrofóbicas e pontes dissulfídicas entre as cadeias com a região N-terminal. Essa é a forma molecular básica da MBL circulante. No soro, a MBL consiste de oligômeros, de dímeros até hexâmeros, em formato de buquê (Ezekowitz, 2003; Dommett, Klein *et al.*, 2006).

Essa proteína de fase aguda e de origem hepática é capaz de se ligar multivalentemente à manose terminal, N-acetilglicosamina, glicose e frutose, em leveduras e bactérias gram-negativas (Worthley, Bardy *et al.*, 2005). Após se ligar a um patógeno, a MBL sofre uma mudança conformacional ativando moléculas associadas, como as serino proteases associadas à MBL (MASP-1, MASP-2 e MASP-3), resultando na iniciação da ativação do complemento pela via das lectinas (Ip, To *et al.*, 2004; Selander, Martensson *et al.*, 2006). A MBL é a única colectina conhecida capaz de ativar o sistema do complemento (iniciando a via das lectinas) e, desse modo, promover a fagocitose dos

microorganismos sem o envolvimento de anticorpos (Zimmermann-Nielsen, Baatrup *et al.*, 2002; Fiane, Ueland *et al.*, 2005; Zimmermann-Nielsen, Gronbaek *et al.*, 2005).

A MBL é considerada um paradigma da imunidade inata, sendo referenciada por muitos autores como uma “faca-de-dois-gumes” ou apelidada de “*Jekyl-and-Hyde*”, ou seja, a MBL tem um papel duplo na patofisiologia humana: de um lado, baixos níveis de MBL estão associados com maior susceptibilidade a infecções por microorganismos extracelulares, pois a MBL reconhece esses patógenos e desencadeia resposta imune, eliminando-os. Por outro lado, baixos níveis de MBL têm caráter protetor contra infecções por microorganismos intracelulares, pois a proteína realiza a opsonização e a fagocitose desses microorganismos, transportando-os para dentro da célula, o que facilita a ação e aumenta a infectividade de alguns patógenos que atuam dentro das células (Ezekowitz, 2003; Fiane, Ueland *et al.*, 2005; Worthley, Bardy *et al.*, 2005; Bouwman, Roep *et al.*, 2006).

Gene MBL2

O gene *MBL2*, localizado no cromossomo 10, (região 10q11.2-q21) codifica a lectina de ligação à manose (Ip, To *et al.*, 2004). O éxon 1 codifica a região rica em cisteína e parte da região colagenosa rica em glicina, o éxon 2 codifica o restante da região colagenosa, o éxon 3 codifica a estrutura helicoidal, conhecida como a região do pescoço e o éxon 4 codifica o CRD. A região promotora do gene contém elementos regulatórios que afetam a transcrição da proteína (Dommett, Klein *et al.*, 2006). Os níveis séricos de MBL são geneticamente determinados, mas alterados de acordo com as variantes

polimórficas expressas a partir de alterações estruturais (“single nulceotide polimorphisms” - SNP*s*) (Madsen, Garred *et al.*, 1995).

Polimorfismos de um único nucleotídeo (SNPs) localizados no éxon 1 desse gene estão relacionados com alteração na conformação da proteína, resultando na deformação da estrutura em hélice da região colagenosa e, conseqüentemente, interferem na formação dos oligômeros. Esse prejuízo na polimerização leva a baixos níveis séricos da proteína, os quais estão associados com deficiência na fagocitose e susceptibilidade aumentada a doenças infecciosas e auto-imunes (Thio, Mosbruger *et al.*, 2005; Bouwman, Roep *et al.*, 2006; Maury, Aittoniemi *et al.*, 2007).

As regiões polimórficas mais estudadas encontradas no éxon 1 compreendem os seguintes polimorfismos: R52C, que representa a troca de uma arginina por uma cisteína no códon 52, causada pela substituição CGT → TGT, denominado alelo “*D*”; G54D, que significa a troca de uma glicina por um aspartato no códon 54, representando a substituição GGC → GAC, e é denominado alelo “*B*”; e G57E, caracterizando a troca de uma glicina por um glutamato no códon 57, devido à substituição GGA → GAA, denominado de alelo “*C*”. Uma região codificadora contendo qualquer uma das três variantes não selvagens é coletivamente designada “*O*”, enquanto o alelo selvagem é referido como “*A*” (Wiertsema, Herpers *et al.*, 2006; Muller, Keil *et al.*, 2007). Ainda na região do éxon 1 encontra-se outro polimorfismo, Cd52L, que é a troca de uma arginina por uma leucina também no códon 52. Por fim, a IVS-I-5 é uma variante pouco estudada, localizada na região do quinto nucleotídeo do primeiro íntron (G→A) (Neonato, Lu *et al.*, 1999).

Três outros polimorfismos foram descritos na região promotora do gene da MBL, também relacionados com a diminuição dos níveis de MBL sérica (Madsen, Garred *et al.*, 1995). Estes sítios polimórficos estão localizados nos nucleotídeos -550 (alelo H/L, substituindo G→C), -221 (alelo X/Y, com a troca G→C) e +4 (alelo P/Q, onde, C→T). Estes três *loci* estão intimamente ligados e, devido a forte desequilíbrio de ligação, apenas sete haplótipos (HYPA, LYQA, LYPA, LXPA, LYPB, LYQC e HYPD) são comumente encontrados. (Madsen, Satz *et al.*, 1998; Parrella, Seripa *et al.*, 2007; Wang, Saito *et al.*, 2007).

Os níveis de MBL já foram estudados relacionando essa proteína a diferentes patologias, como infecções, complicações pós-transplantes, auto-imunidade, imunossupressão (crianças, portadores de fibrose cística e de HIV, pacientes sob quimioterapia) e gestação. O papel da MBL como modulador de inflamação é complexo e seu mecanismo de ação ainda não é totalmente conhecido. Uma possível explicação para a associação das variantes de MBL com diferentes doenças leva em conta que a MBL seria capaz de ativar citocinas pró-inflamatórias, induzindo sua liberação a partir de monócitos. A indução de liberação de TNF-α, IL-1β e IL-6 a partir de monócitos, mediada por MBL, foi confirmada em concentrações de MBL abaixo de 4mg/ml, no entanto, altas concentrações desta mesma proteína suprimem esta mesma expressão (Dommett, Klein *et al.*, 2006).

As alterações nos níveis de MBL podem ser um dos indutores do desenvolvimento da pre-eclampsia (PE). Como a PE é uma doença multifatorial, caracterizada por inúmeras alterações, inclusive imunológicas, o estudo de polimorfismos em genes de caráter imunológico são de extrema valia

para a compreensão da patologia da doença. Neste caso, as análises dos polimorfismos nas regiões estutural e promotora do gene MBL podem ser úteis para elucidar a patogênese da pré-eclampsia.

Baseados nestas informações avaliamos neste trabalho a rede de interações imunológicas durante o processo de gestação, visando um melhor entendimento dos mecanismos de aceitação e tolerância materna ao feto.

CAPÍTULO 2

OBJETIVOS

Capítulo 2 - OBJETIVOS

O presente trabalho tem o objetivo de avaliar parâmetros imunológicos de gestantes saudáveis e gestantes com pré-eclampsia (PE), através de análise e caracterização de possíveis alterações imunológicas relacionadas com o desfecho favorável ou não da gestação. Para que o objetivo proposto possa ser alcançado, delineamos os objetivos específicos descritos a seguir:

I. Estabelecer o padrão qualitativo e quantitativo de citocinas TH1 e TH2 no soro de mulheres durante (1^0, 2^0 e 3^o trimestres gestacionais) e após a gestação (amamentação);

II. Estabelecer o padrão de expressão de moléculas de superfície (CD3, CD4, CD8, CD56, CD161, CD45RO, CD45RA, CD25 e CD69) em células mononuclerares do sangue periférico de mulheres antes, durante (1^0, 2^0 e 3^o trimestres) e após a gestação (amamentação);

III. Caracterizar as subpopulações de células T regulatórias CD4+CD25^{high}+ ao longo da gestação;

IV. Caracterizar o perfil fenotípico das células NK em gestantes saudáveis durante todo período da gestação;

V. Caracterizar a o perfil de expressão de superfície de HLA-G em células mononucleares do sangue periférico de gestantes saudáveis nos diferentes tempos gestacionais;

VI. Analisar a capacidade proliferativa de células T do sangue periférico ao longo da gestação;

VII. Genotipar uma variante do gene HLA-G que está associada com o crescimento fetoplacentário, comparando gestantes com eventos de pré-eclampsia *versus* sem eventos de pré-eclampsia;

VIII. Analisar os polimorfismos presentes no gene de MBL (*MBL2*) que codifica a proteína ligadora de manose, na região promotora e estrutural, comparando gestantes com eventos de pré-eclampsia *versus* sem eventos de pré-eclampsia;

IX. Sugerir aspectos adicionais que podem interferir no sucesso gestacional, modulando o sistema imune materno e fetal, como por exemplo, a intensidade e freqüência de exposição materna ao esperma.

Capítulo 3 - Artigo 1

Immunogenetics of pregnancy: Role of a 14-bp deletion in the maternal HLA-G gene in primiparous pre-eclamptic Brazilian women

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ABSTRACT

The etiology and pathogenesis of pre-eclampsia (PE) involve a combination of maternal-fetal genetic and immunological factors. The immunological maladaptation theory of PE predicts that the maternal immune system does not tolerate the semi-allogeneic foetus. Human leukocyte antigen-G (HLA-G) is expressed in some types of immune cells as well as in the foetus-maternal interface by trophoblasts, playing an immunoregulatory role. Here, we have evaluated a 14-bp deletion polymorphism in the 3'-untranslated region of exon 8 of HLA-G gene in pregnant PE women and controls. HLA-G genotypes in both control and PE women were in Hardy-Weinberg equilibrium. The healthy pregnant and PE women had similar genotype frequencies (p=0.789). This was similarly observed when PE women were sub grouped accordingly to the severity disease (p=0.646). However, the primiparous PE women presented a tendency to higher frequency of the 14-bp deletion allele (0.422) compared to the primiparous healthy women (0.286), p= 0,09. Our data suggest that the maternal 14-bp deletion of HLA-G is not associated with the risk for PE but could interfere on the PE development of primiparous women.

Abbreviations: **PE** Pre-eclampsia; **HLA-G** Human leukocyte antigen-G; **TH1** T helper lymphocyte type 1; **TH2** T helper lymphocyte type 2; **UTR** Untranslated region; **IL-10** Interleukin 10.

INTRODUCTION

Pre-eclampsia (PE) is a systemic disorder of unknown origin and is characterized by abnormal vascular response to placentation, increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation cascade, and endothelial cell dysfunction [Working Group Report on High Blood Pressure in Pregnancy,1]. The etiology and pathogenesis of PE involve a combination of maternal-fetal genetic and immunological factors. The disorder is heterogeneous and pathogenesis can differ in women according to the presence of different risk factors. Pathogenesis of PE in primiparous women may differ to that in women with pre-existing vascular disease, multifetal gestation, diabetes mellitus, or previous pre-eclampsia events [2, 3]. PE women are usually diagnosed with hypertension and associated proteinuria. Pre-eclampsia can be serious if severe hypertension is associated with proteinuria or if hypertension is associated with severe proteinuria (≥5 g per day) [4, 5]. In general, maternal and perinatal outcomes are usually favorable in women with mild pre-eclampsia developing beyond of 36 weeks of gestation. In contrast, maternal and perinatal morbidities and mortalities are increased in women who develop the disorder before the 33^{th} gestational week [5-7]. The PE is usually regarded as a disease of first pregnancy.

The immunological maladaptation hypothesis of PE predicts that the maternal immune system does not tolerate the semiallogeneic foetus. During pregnancy, the maternal immune system is in close contact with cells and tissues of the semiallogeneic foetus. Therefore, there must be specific mechanisms engaged in modulating the maternal immune system in order to prevent the foetus rejection. Women with healthy pregnancies tend to present a

Th2 type of immune response while a Th1 type response is incompatible with a successful pregnancy, and plays a role in certain complications like PE development [8-12]. The Human leukocyte antigen-G (HLA-G) is a non-classical HLA class Ib molecule predominantly expressed in the foetus-maternal interface and plays an important role during implantation and maternal acceptance of the foetus. HLA-G mRNA has been detected in many different tissues but HLA-G protein expression is limited to a few specific cells, such as trophoblasts in placenta, monocytes, lymphocytes, and in the thymus [13-15]. Some immunological interactions can contribute for the foetus maintenance. Expression of HLA-G by trophoblasts inhibits activation of maternal T cells, natural killer (NK) and antigen-specific CTL cytolysis via specific receptors [16-19], and IL-10 secreting cells may stimulate the HLA-G expression [20]. Interestingly, some effectors CD4+ and CD8+ T lymphocytes acquire immunosuppressive HLA-G1 from antigen-presenting cells (APCs) and reverse their function from effectors to regulatory cells [21]. Recently, Fanchin et al., 2007 reviewed the possible relationship between HLA-G and human embryo implantation, exploring the HLA-G expression on human preimplantation embryos and in the endometrium, as well as its levels in embryo culture supernatants and circulating maternal blood [22]. HLA-G has few polymorphic alleles and shows a limited pattern of expression, contrasting to the highly polymorphic HLA class Ia and II antigens [15, 23-32]. A very interesting variation of HLA-G involves a 14 bp deletion/insertion polymorphism in the 3′UTR of the HLA-G gene located at position 3741 at exon 8. The 14-bp sequence at the beginning of exon-8 is suggested to be responsible for the alternative splicing of the HLA-G transcript. This 14-bp polymorphism has been

associated with HLA-G isoforms that lack 92 base sequences in the first part of exon 8 (3'UTR) [33]. During mRNA processing, this sequence functions as a cryptic branch point for mRNA splicing and is thus more stable in nature [34, 35]. However, the 14-bp deletion causes the retention of 92 bases in the mature transcript, resulting into an unstable transcript. The 14-bp deletion/insertion polymorphism may influence both the HLA-G isoform splicing patterns and HLA-G mRNA stability. This may change the HLA-G function and could be of pivotal importance in certain pregnancy complications like PE [35, 36]. The allele +14 bp has been associated with lower levels of soluble HLA-G and was implicated in the development of PE and recurrent abortions [35, 37, 38]. Several studies have been suggested the importance of the maternal HLA-G expression during cleavage embryo development and during the course of pregnancy. Yao *et al.,* 2005 described a disparity between HLA-G mRNA isoforms and protein expression in embryos. They suggested that in some stage embryos, this difference might be due to HLA-G protein remaining from maternal oocyte stores produced before embryonic genome activation. Thus, HLA-G expressed at this stage may be more a marker of oocyte rather than embryo's quality [39]. In addition, Menezo et al., 2006 reported that the levels of secreted soluble HLA-G protein are higher than the capacity proposed for soluble HLA-G release by the embryo, so the signals secreted by the embryos are not in the order of the magnitude of estimated HLA-G protein concentrations [40]. These findings shed some light into the contribution of the maternal HLA-G protein for a successful pregnancy. Here, we analyzed the maternal 14-bp HLA-G polymorphism, investigating both allelic and genotypic frequencies in Brazilian women that developed PE in a case-control study. The hypothesis of

immune maladaptation in PE was studied here, evaluating the importance of maternal HLA-G genotype during successful pregnancy.

MATERIAL AND METHODS

Individuals: The patients were recruited at the Maternity Unit of at public hospital in Southern Brazil (Hospital Nossa Senhora Conceição, Porto Alegre). We identified 162 healthy pregnant women with uncomplicated pregnancies (controls) and 157 pregnant with PE. The inclusion criteria for selecting controls included: no rise in blood pressure, no hypertension or proteinuria, similar age (healthy women 28.08 yrs ± 7.37 and PE women 30.32 yrs ± 7.46), no biological relationship and a delivery date as close as possible to the delivery date(s) of the matched patient group. Controls were followed up for at least three months after delivery. If hypertension and/or proteinuria were observed during this follow up period, this specific control individual was excluded. Pre-eclampsia was defined as the presence of hypertension and proteinuria. Hypertension is characterized by blood pressure of at least 140 mm Hg (systolic) or at least 90 mm Hg (diastolic), on at least two occasions and 4–6 h apart following the 20^{th} week of gestation in women known to be normotensive beforehand [5, 41]. Proteinuria is defined as an excretion of 300 mg or more 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 20^{th} week of gestation [5]. The PE was classified as severe when there was a blood pressure ≥ 160/110 mmHg; or urinary protein excretion ≥5g per 24h; a platelet count of <100 000mm^{-3} in at least two samples; the combination of haemolysis, abnormal liver enzymes associated with persistent epigastric or upper right quadrant pain; persistent and severe symptoms as altered mental status, headaches, blurred vision or blindness; presence of multiorgan involvement

such as pulmonary edema, oliguria (<500mL per day) [42]. 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 Hospitalar Conceição Group (Porto Alegre, Brazil) and by the National Research Committee of Ethics.

Polymerase chain reaction (PCR) amplification of exon 8 of the HLA-G gene: DNA was isolated from whole blood using a salting-out procedure [43]. The 14-bp polymorphism at exon 8 of HLA-G gene was detected through PCR analysis: 200 ng of genomic DNA were added to a final volume of 25 μL containing 10mM Tris-HCl (pH 8.8), 50mM KCl, 0.08% (v/v) NonidetP40, 1.5mM $MgCl_2$; 0.2mM of each dNTPs; 10pmol of each primer and 0.75 units of *Taq*-polymerase. Thermocycling conditions: The 35 cycles of 94^0C for 30 s, 64^0C for 60 s and 72^0C for 120s were preceded by an initial denaturation cycle of 94 ^{0}C for 2 min and followed by a final extension step at 72^0C for 10min. The samples were genotyped by PCR using specific primers (5'GTGATGGGCTGTTTAAAGTGTCACC3' and 5'GGAAGGAATGCAGTTCAGCATGA-3') as described previously [26].

Genotyping of the 14bp deletion polymorphism in exon 8 (the 3'-untranslated region) of the HLA-G gene: The PCR products of exon 8 were analyzed by 6% polyacrylamide gel electrophoresis containing ethidium bromide, and visualized under ultraviolet light. The amplicon sizes for the 14-bp polymorphism were 224 bp for the + 14 bp allele and 210 bp for the allele.

Statistical analysis: HLA-G genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. HLA-G allelic frequencies and HLA-G genotypes based upon the 14bp deletion polymorphism in exon 8 of control and PE women were compared using the χ^2 test. The p values were corrected for multiple comparisons when necessary. The significance level was set at α = 0.05 (two-tailed) and all statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Maternal HLA-G allele frequencies and genotype distribution

The 14-bp polymorphism in HLA-G exon 8 is associated with different HLA-G mRNA isoforms that may change patterns and quantity of HLA-G mRNA expression and induce alternative splicing. In this way, maternal genotypic and allelic frequencies concerning the presence/absence of the 14-bp on exon 8 were compared among PE women and healthy control. One hundred and fifty seven women who had experienced PE and one hundred and sixty two healthy control pregnant women were HLA-G genotyped. The maternal HLA-G genotype distribution in both PE and control women were in Hardy-Weinberg equilibrium for the 14-bp deletion polymorphism **(Table 1)**. The HLA-G allele/genotype overall distribution and frequencies are shown in **Table 2**. No statistically significant differences were observed in allelic and genotypic frequencies between PE women and control pregnant women, however there was a tendency of a lower frequency of homozygous individuals for the +14 bp allele (+14bp/+14bp) (0.146-0.172) when compared with the heterozygous (0.491-0.519) and homozygous (0.335-0.337) for the deletion genotype (**Table 2)**. There were 75 women who developed gestational diabetes following the PE. However, all analyses performed excluding these subjects showed the same level of statistical significance (data not shown), indicating that the gestational diabetes did not interfere on the PE development.

Association between specific maternal HLA-G polymorphism and different forms of pre-eclampsia

Pre-eclampsia is a multifaceted disorder that can present mild and severe forms. We thus analyzed the patients sub-grouped by the development of mild or severe PE. Patients who had previous hypertension and subsequent PE were excluded from this sub-grouping (n=47). No statistically significant differences were observed on genotypic frequencies between these two subgroups, as shown in **Table 3** (Chi-square test, healthy *versus* mild PE p=0.646 and healthy *versus* severe PE p=0.953). Patients were also characterized by clinical parameters including ethnic origin, number of aborts, number of gestations and weight. No significant differences were observed in genotypic frequencies between the control group and PE women sub-grouped accordingly the aforementioned characteristics (**Table 4**). However, it is interesting to note that the primiparous PE women presented higher frequency of the -14/-14bp genotype (n=19; freq=0.442) as compared to the primiparous healthy women (n=15; freq=0.283), although this only approached statistical significance (p=0.09) (**Table 4**).

Association between pre-eclampsia and dominating genotypes

In order to evaluate the role of maternal 14-bp deletion genotype (-14bp/-14bp) in PE development, we aggregated women that were heterozygous for the 14-bp deletion/insertion polymorphism (-14bp/+14bp) with those that were homozygous for the presence of the 14-bp segment (+14bp/+14bp) as shown in **Table 5**. No statistically significant differences were observed between the control and PE women with mild and severe clinical forms for this analysis (**Table 5**).

DISCUSSION

During pregnancy, the maternal immune system is in close contact with cells and tissues from the semiallogeneic foetus. This suggests that specific mechanisms must be operating to modulate and moderate the maternal immune system in order to prevent the foetus rejection - i.e, promoting the acceptance of the semiallogeneic foetus. A successful pregnancy has been called by several authors as a ´Th2 phenomenon`, characterized by a shifted Th2 cytokine profile. Indeed, certain complications during pregnancy, such as PE, have been associated with a Th1 response [12, 44, 45]. The etiology and pathogenesis of the PE involve a combination of maternal-fetal genetic and immunological factors. One hypothesis for the pathogenesis of PE is the immunological maladaptation theory, taking into consideration that the maternal immune system does not adapt to the semiallogeneic foetus. Considering the immunological maladaptation hypothesis, candidate genes with immunological functions should be sought to predispose for PE development. HLA-G genes code for important transmembrane proteins involved in the modulation of the maternal immune system during pregnancy and therefore in the maternal acceptance of the semiallogeneic foetus. It has been shown that IL-10 (an anti-inflammatory cytokine) is able to activate HLA-G expression [20, 44]. In PE placentas, the HLA-G extravillous cytotrophoblast invasion is reduced and this defect was associated with a lack of HLA-G expression [46-48]. Such defective HLA-G expression may contribute to the immune and vascular abnormalities associated with this pathology [31, 46]. The strong HLA-G expression by invasive trophoblasts may, in part, explain maintenance of the semi-allogeneic foetus during pregnancy. It is thought that HLA-G inhibits the activation of

maternal T cells and natural killer cells resident in deciduas favoring a Th2 type cytokine response [17, 19, 31]. Here, we aimed to investigate the underlying role of maternal 14-bp HLA-G gene polymorphism during successful pregnancy and PE.

It was observed that both the allelic and genotypic distributions of the 14-bp polymorphism were similarly represented between healthy and PE patients. Hviid *et al*., [26], in a study correlating the 14-bp HLA-G polymorphism and recurrent spontaneous abortions, did not observe significant differences in the frequencies of HLA-G alleles and/or genotypic distribution between control women and women with RSA. Hylenius *et al* 2004 in a family triads study correlating the risk of PE in women did not find significant differences in the maternal 14-bp genotype distribution between PE and the control group. However, differences regarding the maternal allele distribution suggested that the mother HLA-G genotype may confer risk for PE development in the primiparous women. Further analyses described an overrepresentation of the +14/+14 bp genotype in offspring from primiparous women with severe PE and suggested that the differences found in the distributions of the 14 bp polymorphism between PE cases and control could be related with a differential transmission of the paternal 14 bp polymorphism in exon 8 [49]. O'Brien et al., 2001 described low levels of HLA-G expression in placentas from primiparous pre-eclamptic women in comparison to normal placentas and observed differential distribution of HLA-G polymorphisms between normal and PE samples. They also observed an excess of the +14bp allele in PE samples, suggesting a possible role of HLA-G in susceptibility to PE development [50]. Therefore, these previous studies have suggested the importance of differences

in HLA-G mRNA alternative splicing and levels of HLA-G in complicated pregnancies, demonstrating the pivotal role of HLA-G in maintenance of a successful pregnancy.

When the samples were sub grouped accordingly to clinical parameters, no significant differences were observed in the maternal 14-bp polymorphism genotypic frequencies between control and PE women. However, it is interesting to note that the primiparous PE women presented a tendency to higher frequencies of the 14-bp deletion allele as compared to the primiparous healthy women. This tendency can generate less and more unstable HLA-G molecules. The instability of the HLA-G molecule could lead to an increased pro-inflammatory profile and possible foetus rejection. In addition, PE is generally associated to the first pregnancy. Thus, in primiparous women, both the 14-bp deletion allele and a limited sperm exposure with the same partner before conception would induce an increased risk of PE [51]. Prolonged exposure to paternal sperm through sexual intercourse has been suggested to be protective again PE [52]. Women younger than 20 years presented a high risk of PE, thus the protective effects of a long-term sperm exposure with the same partner might explain this fact. A previous abortion (spontaneous or induced) or a healthy pregnancy with the same partner is associated with a reduced risk of PE, although this protective effect is lost by changing the partner [2, 51, 53, 54]. Previous studies have produced conflicting results regarding the 14-bp HLA-G polymorphism and pregnancy complications. Hviid *et al*., [26, 55] showed high levels of homozygous for the presence of the 14-bp sequence in women with reduced fertility and recurrent spontaneous abortion (RSA). In contrast, Tripathi *et al*., [35] demonstrated increasing number of hetererozygous

for the 14-bp polymorphism in RSA women. HLA-G has previously been shown to be codominantly expressed [56]; however, HLA-G alleles containing the 14-bp sequence have been found to be associated with a lower HLA-G mRNA level for most isoforms in heterozygous (first trimester) trophoblast samples [24, 26, 33]. In this study, we did not find a dominancy of HLA-G genotyped concerning the deletion of 14-bp in exon 8 of HLA-G gene as previously suggested.

It has been suggested that the presence of the 14-bp sequence in exon 8 of HLA-G gene is important for the maintenance of stable forms of mRNA and HLA-G molecules. In particular, our results showed a tendency of higher frequency of the maternal 14-bp deletion in primiparous PE women. The 14-bp deletion has been postulated to generate more unstable HLA-G transcripts, influencing both HLA-G isoform splicing patterns and HLA-G mRNA stability as well as changing HLA-G function leading to certain complications of pregnancy like PE [35, 36]. Our data suggest that the maternal 14-bp polymorphism may be important in the outcome of pregnancy and address the possibility that the maternal HLA-G polymorphisms could influence functional parameters. However, further studies are necessary to clarify the molecular mechanisms and the specific cellular networks involved in HLA-G function.

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HLA-G genotype (3'UTR polymorphism)		Healthy pregnant women (n=162) [(freq)]	Chi-square (χ^2)	Pre-eclamptic pregnant women (n=157) [(freq)]	Chi-square (χ^2)
-14bp/+14bp	Observed Count	79.0 (0.491)	0.01	81.0 (0.519)	0.47
	Expected Count	82.3		79.7	
+14bp/+14bp	Observed Count	28.0 (0.172)	0.001	23.0 (0.146)	0.32
	Expected Count	25.9		25.1	
-14bp/-14bp	Observed Count	55.0 (0.337)	0.002	53.0 (0.335)	0.13
	Expected Count	54.8		53.2	
Total			**0.013**		**0.92**
χ^2 tabulated (α=0.05; df =1) = 3.84 (p>0.05)					

Table 1 Hardy- Weinberg equilibrium for HLA-G genotype between control and pre-eclamptic women.

HLA-G genotype (3'UTR polymorphism) [a]	Healthy pregnant women (n=162) [(freq)]	Pre-eclamptic pregnant women (n=157) [(freq)]
-14bp/+14bp	79 (0.491) [a]	81 (0.519) [a]
+14bp/+14bp	28 (0.172) [a]	23 (0.146) [a]
-14bp/-14bp	55 (0.337) [a]	53 (0.335) [a]
HLA-G allele (3'UTR polymorphism) [b]		
Minus 14bp	189 (0.583) [b]	188 (0.599) [b]
Plus 14bp	135 (0.417) [b]	126 (0.401) [b]

[a] $\chi^2 = 0.474$; df=2; p=0.789

[b] $\chi^2 = 0.156$; df=1; p=0.693

Table 2 HLA-G allele/genotype overall distribution and frequencies in control and pre-eclamptic women.

HLA-G genotype (3'UTR polymorphism)	Healthy pregnant women (n=162) [(freq)]	Mild Pre-eclamptic pregnant women (n=57) [(freq)]	Severe Pre-eclamptic pregnant women (n=53) [(freq)]
-14bp/+14bp	79 (0.491) [a]	31 (0.544) [b]	25 (0.472) [c]
+14bp/+14bp	28 (0.172) [a]	7 (0.123) [b]	10 (0.188) [c]
-14bp/-14bp	55 (0.337) [a]	19 (0.333) [b]	18 (0.340) [c]

[a , b] χ^2 = 8.74; df=2; p=0.646/CP = > 1.00

[a, c] χ^2 = 0.96; df=2; p=0.953/CP=>1.00

CP= corrected p value for multiple comparisons

Table 3 HLA-G genotype frequencies according characteristics of development of the mild or severe pre-eclampsia.

Clinical Parameters		Control women n (freq)			Pre-eclamptic pregnant women n (freq)		
		HLA-G genotype (+14bp/-14bp)	HLA-G genotype (+14bp/+14bp)	HLA-G genotype (-14bp/-14bp)	HLA-G genotype (+14bp/-14bp)	HLA-G genotype (+14bp/+14bp)	HLA-G genotype (-14bp/-14bp)
Ethnic origin	Euro-derived	63 (0.512)[a]	22 (0.179) [a]	38 (0.309) [a]	55 (0.524) [a]	15 (0.143) [a]	35 (0.333) [a]
	Afro-derived	16 (0.444)[b]	5 (0.139) [b]	15 (0.417) [b]	25 (0.500) [b]	8 (0.160) [b]	17 (0.340) [b]
Abortion	<2	75 (0.490)[c]	26 (0.170) [c]	52 (0.340) [c]	75 (0.517) [c]	23 (0.155) [c]	50 (0.338) [c]
	>2	5 (0.625)[d]	1 (0.125) [d]	2 (0.250) [d]	4 (0.500) [d]	none	4 (0.500) [d]
Weight	Normal	24 (0.585)[e]	3 (0.073) [e]	14 (0.341) [e]	14 (0.500) [e]	7 (0.250) [e]	7(0.250) [e]
	Obesity	45 (0.450)[f]	21 (0.210) [f]	34 (0.340) [f]	51 (0.515) [f]	12 (0.121) [f]	36 (0.364) [f]
Gestation	First	26 (0.490)[g]	12 (0.226)[g]	15 (0.283) [g]	15 (0.349) [g]	9 (0.209) [g]	19 (0.442) [g]
	> 1	61 (0.492)[H]	20 (0.161) [H]	43 (0.347) [H]	65 (0.586) [H]	14 (0.126) [H]	32 (0.288) [H]

The number in parenthesis show the genotype frequencies distribution according the clinical parameter described.

[a] $\chi^2 = 0.573$; df=2; p=0.751., [b] $\chi^2 = 0.528$; df=2; p=0.768., [c] $\chi^2 = 0.140$; df=2; p=0.932., [d] $\chi^2 = 1.778$; df=2; p=0.411., [e] $\chi^2 = 4.267$; df=2; p=0.118., [f] $\chi^2 = 2.882$; df=2; p=0.237., [g] $\chi^2 = 5.662$; df=2; p=0.093., [H] $\chi^2 = 2.086$; df=2; p=0.352

Table 4 – Genotype frequencies in both control and PE women classified accordingly clinical parameters.

	HLA-G genotypes (+14bp/-14bp) and (+14bp/+14bp)	HLA-G genotypes (-14bp/ -14bp)
Healthy pregnant women (n=162) [(%)]	108 (0.663)[a]	55 (0.337)[a]
Mild pre-eclamptic pregnant women (n=57) [(%)]	38 (0.667)[b]	19 (0.333)[b]
Severe pre-eclamptic pregnant women (n=53) [(%)]	35 (0.660)[c]	18 (0.340)[c]
Total pre-eclamptic pregnant women (n=157) [(%)]	105 (0.665)	52 (0.335)

[a, b] Fisher's exact test; p=0.546/CP= >1.00

[a, c] Fisher's exact test; p=0.551/CP= >1.00

CP= corrected p value for multiple comparisons

Table 5 HLA-G aggregated genotype overall distribution and frequencies in control and pre-eclamptic women.

Capítulo 4 - Artigo 2

Maternal exposure to paternal antigens can modulate the foetus immune system and is associated with reduced atopy in the childhood

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ABSTRACT

The influence of the maternal immune system on pregnancy and on the foetus immune system have give rise to a variety of observations and interesting hypotheses. For example, the higher prevalence of atopy in first-born children as compared to their brotherhood is known as the “birth order effect”. The “hygienic hypothesis” states that more hygienic live conditions, and consequently reduced exposure to pathogens in young age (included the period of foetal development), increases the risk of atopy. In addition, the manipulation of the maternal immune system through maternal vaccination with paternal leukocytes (paternal leukocyte immunization) was suggested as a treatment of Recurrent Spontaneous Abortion. Here we review the ideas concerning maternal exposure to paternal antigens. In particularly, we discuss the idea that this phenomenon may induce a regulatory environment in women that interfere with the developing foetal immune system. This regulatory environment could be responsible for protecting children to the development of atopy during adulthood. We propose that maternal exposure to paternal antigens through different situations, such as pregnancy, repeated exposure to sperm or Paternal Leukocyte Immunization (PLI) would combine the “birth order effect” and the “hygienic hypothesis” and thus lower the risk to atopy in children through the transference of a regulatory environment to the foetus.

INTRODUCTION

The “hygienic hypothesis” sustains that more hygienic live conditions and consequently lower pathogen exposure, in young age, increases the risk of atopy (Strachan 1989). It has been proposed that the increasing prevalence of childhood allergic diseases in the Western World could be related to two aspects: more hygienic live conditions and smaller families (Bernsen et al. 2006). In addition, it was hypothesized that the sibling order could be essential in reducing the prevalence of allergy and asthma. Following these ideas, the challenge of the immune system and protection against atopy during the childhood occurs frequently in families with several children, since children often acquire infections through the contact with older siblings (Williams et al. 1994; Strachan 1995). It is also true that, in a large family, virus brought home by the children, frequently boosts the mother immune system. Thus, repeated contact with infections would change the maternal immune system, giving rise to a regulatory environment that could affect next pregnancies. An extension of such ideas is that maternal exposure to infections could provide protection of her children against allergic respiratory diseases, and that first children of mothers working (e.g day care employees) would have lowered risks of allergy when compared to first children of other mothers (Hersoug 2006). Moreover, if a child is infected with a respiratory virus that the mother already has been exposed to, the infection shows a less severe profile.

Several cellular subsets, cell surface molecules and intracellular signaling networks regulate an immune response against any given antigen. Therefore, the maternal immune system is under a constant process of reshaping. It was suggested that women with healthy pregnancies tend to

present a Th2 type of immune response whereas women with a history of recurrent spontaneous abortions are generally biased towards a Th1 type response (Lim et al. 1996; Marzi et al. 1996). Recently, regulatory T cells (Treg) have been pointed as important mediators in immune tolerance to self and allogeneic antigens (Sakaguchi 2005). In pregnancy, Treg cells have a critical role in the maintenance and tolerance of the semiallogeneic foetus, modulating the maternal immune system in order to prevent the foetus rejection (Aluvihare et al. 2004). The CD4+CD25+ regulatory T cells are a naturally occurring population of T cells that suppress the development of a variety of immune responses (Maloy et al. 2005). Bersen et al. 2006 suggested that nulliparous women have a lower activity of Treg cells specific to paternal antigens compared to parous women and this lower activity could affect the foetus immune system and consequently the child risk of allergic diseases. We believe that a similar effect of modulation of the foetus immune system can result from other situations involving maternal exposure to paternal antigens.

A tolerogenic environment can be induced by exposure to paternal antigen

Paternal antigens can modulate the maternal immune system by several ways including pregnancy, repeated exposure to sperm or through paternal leukocyte immunization (PLI). Primiparous women usually show lower tolerance directed to paternal antigens when compared to multiparous women. This effect could be partly explained by the limited exposure to partner sperm before conception. A limited exposure to partner sperm has already been linked to pre-eclampsia development, while a long history of sexual cohabitation before

conception has been related to a reduced risk of pre-eclampsia (Einarsson et al. 2003; Saftlas et al. 2003).

One of the therapeutic interventions used in attempt to induce a successful pregnancy, avoiding the fetal rejection and the occurrence of Recurrent Spontaneous Abortion (RSA) is PLI (Orgad et al. 1999). This intervention, in some of the treated women, leads to changes in the maternal immune system in a way that immune tolerance is induced and a successful pregnancy is achieved. This induced immune tolerance is thought to generate a regulatory environment that induces the acceptance of the semi-allogeneic foetus. It is quite logical that this immune regulation would also interfere with the foetus immune system, possibly turning it tolerant to a variety of antigens and, in a similar phenomenon as the “birth order effect”, protecting the child against the development of atopy. Although many factors can interfere with this method such as women’s age and medical history (Chaichian et al. 2007), it is important to point out that the efficacy of the procedure, *per se*, is not under discussion here, but the changes occurring at the immune system of a foetus developing in a woman that was submitted to paternal leukocyte immunotherapy.

Also, the finding that repeated exposure to seminal fluids before conception may result in the regulation of the mother immune response runs in parallel with the fact that previous maternal exposure to infections provides protection of her children against allergic respiratory diseases. Therefore, a protective memory response induced by paternal vaccination could generate a regulatory immune system already at the first successful pregnancy, and we would be able to observe a protective effect on the development of childhood atopy in subjects born after such treatment. Hence, we can hypothesize that

maternal exposure to paternal antigens through deliberate PLI can be used as a therapeutic intervention to reduce atopy.

Epidemiological studies can approach our hypothesis that the maternal tolerogenic environment induced by exposure to paternal antigens could affect the childhood. Firstly, it would be interesting to study the immune system of children born from RSA women treated with PLI that had a successful pregnancy. It would also be interesting to characterize the immune response of children born from mothers who had not lived with the first child (because of divorce and separation or death). This approach would allow us to focus on changes of the maternal immune system due to exposure to paternal antigens during the first pregnancy at the same time that it minimizes the interference of other environmental factors, such as all the virus brought home by other children. In addition, one would compare children from mothers who received blood transfusions or organ transplants before the conception, with children from women treated with PLI.

The understanding and control of atopy is important to prevent the high incidence of allergies in the childhood. A correlation between maternal exposure to paternal antigens and childhood atopy will shed light on the mechanisms that underline these phenomena.

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Capítulo 5 - Artigo 3

Association between Mannose-Binding Lectin gene polymorphisms and pre-eclampsia development in Brazilian women

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Association between Mannose-Binding Lectin gene polymorphisms and pre-eclampsia development in Brazilian women

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Key words: MBL polimorphisms, pre-eclampsia, inflammation.

ABSTRACT

A balance between inflammatory and anti-inflammatory mediators is required in the maternal-fetus interface. Mannose-binding lectin (MBL) found in the uterine environment is involved with the maintenance of an inflammatory environment in uterus and higher MBL levels have been associated with successful pregnancies. However, the roles of elevated levels are not yet completely understood. MBL serum concentrations are genetically determined and disrupted by single nucleotide polymorphisms (SNPs) in the structural and promoter MBL gene (*MBL2*). In contrast, lower MBL levels could be involved with the pre-eclampsia (PE) development. Here we evaluated the MBL gene polymorphisms in the structural and promoter regions and addressed their associations with PE. Higher frequencies of OO genotype, associated with a lower MBL serum producer status, were found in PE women when compared to healthy controls (0.11 *versus* 0.04, p=0.07). In addition, the MBL-grouped genotypes did not influence the time of gestation. Hence, women that developed PE were more likely to present preterm birth when compared to healthy women (28-36 weeks) (p<0.01). Besides, PE women carriers of OO genotype had a reduced probability of maintaining pregnancy. Our data suggest that some MBL polymorphisms could be potential risk factors for PE development.

Abbreviations: PE Pre-eclampsia; **MBL** Mannose-binding lectin; **SNPs** Single Nucleotide Polymorphisms;

INTRODUCTION

The correct immunological balance is required to allow the fetus growth during pregnancy. A successful pregnancy is characterized by a bias toward TH2 response (Marzi, Vigano *et al.*, 1996; Raghupathy, 1997; Reinhard, Noll *et al.*, 1998). Several studies have demonstrated that an excessive inflammatory profile during the pregnancy is associated with disorders like pre-eclampsia (PE), short gestation and miscarriage (Lin, Moss *et al.*, 2007; De Man, Dolhain *et al.*, 2008). Nevertheless some inflammation is required to allow a correct angiogenesis in the maternal-fetal interface. Inflammatory cells and immunological mediators like components of the complement system are present in the uterine environment. They may ensure the maintenance of a pro-inflammatory environment in uterus, pivotal to a good placentation. The development of PE, a disorder that leads to higher maternal and fetal mortalities, involves inflammatory events and a spectrum of clinical presentations, including an incorrect placentation (Redman, Sacks *et al.*, 1999; Duckitt e Harrington, 2005; Redman e Sargent, 2005; Fekete, Ver *et al.*, 2006; Venkatesha, Toporsian *et al.*, 2006). PE is characterized by abnormal vascular response to placentation, increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation cascade, and endothelial cell dysfunction, besides a systemic activation of maternal inflammatory cell responses (Redman, Sacks *et al.*, 1999; Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy, 2000; Sibai, Dekker *et al.*, 2005; Rusterholz, Hahn *et al.*, 2007; Gu, Lewis *et al.*, 2008). The etiology and pathogenesis of PE involve a combination of maternal–fetal genetic and immunologic factors. NK cell activation during

early pregnancy is of benefit for the placentation and is supported by genetic studies on PE development (Hiby, Walker *et al.*, 2004). We had previously demonstrated that PE development is associated to polymorphisms of the HLA-G gene, largely studied in the regulation of pregnancy (Vianna, Dalmaz *et al.*, 2007). Among the inflammatory mediators found in the uterus, the mannose-binding lectin (MBL), a component of innate immune system, could be importantly involved with the maintenance of pregnancy. MBL is present in the serum under oligo- or polymeric association and initiates and activates the lectin pathway of complement, binding to specific bacterial motifs and promoting phagocytosis. MBL could be also considered as a pro-inflammatory protein, modulating the inflammation and further inducing apoptosis (Turner, 2003). Previous studies demonstrated that higher MBL levels were associated with successful pregnancies and MBL levels were increased in the first trimester of a healthy pregnancy (Van De Geijn, Roos *et al.*, 2007). However, there are contradictory results regarding the MBL levels during pregnancy (Van De Geijn, Dolhain *et al.*, 2008), and their functions are not yet completely understood. MBL serum concentrations are genetically determined but ascribed to accordingly single nucleotide polymorphisms (SNPs) in the structural (exon 1 of *MBL2*: AA, AO or OO genotype) or promoter region (H/L- rs11003125 or X/Y-rs7096206) of the *MBL2* (Madsen, Garred *et al.*, 1995). These polymorphisms are related to MBL deficiency and the development of several diseases and healthy complications (Madsen, Videm *et al.*, 1998; Garred, Madsen *et al.*, 1999; Kilpatrick, 2002; Tsutsumi, Ikegami *et al.*, 2003).

SNPs in exon 1, codon 54 (allele *B*- rs1800450*)* and codon 57 (allele *C*-rs1800451*)* are resulted by the substitution of one glycine residue with aspartic

acid and glutamic acid, respectively. A nucleotide substitution in codon 52 (allele *D*- rs5030737*)* leads exchange of an arginine residue with a cysteine. All three variants inhibit the correct oligomerization of MBL chains into the basic trimer structure, in this manner reducing the amount of functional MBL subunits in heterozygous individuals. In concerning the mutations in exon 1, the wild type allele is referred as A, and the “O-allele” represents the variant alleles B, C, or D. The AA wild type genotype produced the highest MBL serum concentrations and the OO genotype the lowest, disrupting multimer formation and resulting in impaired function (Madsen, Garred *et al.*, 1995; Roos, Bouwman *et al.*, 2003). The variants in the first exon were never found together in the same chromosome and they are in strong linkage disequilibrium with the variants in the promoter region. In this way, to better evaluate the role of MBL polymorphisms on the correct formation of MBL multimer, is also interesting to study the polymorphisms of the promoter region of the MBL gene. Mutations in the promoter region prevent the binding of transcriptions factors, generating defectives protein forms and lowered secreted levels (Terai, Kobayashi *et al.*, 2003; Larsen, Madsen *et al.*, 2004). Lower MBL levels were also related to pregnancy complications (Kilpatrick, 1996; Christiansen, Kilpatrick *et al.*, 1999; Kilpatrick, Starrs *et al.*, 1999; Kruse, Rosgaard *et al.*, 2002; Annells, Hart *et al.*, 2004; Annells, Hart *et al.*, 2005) and altered MBL levels could be importantly involved in the PE development. The study of gene polymorphisms of immunological interest could be of great value in the understanding of disease pathology. Of particular note, the MBL polymorphisms could be useful to elucidate the pathogenesis of PE.

MATERIAL AND METHODS

Individuals: The patients were recruited at the Maternity Unit of at public hospital in Southern Brazil (Hospital Nossa Senhora Conceição, Porto Alegre). We identified 162 healthy pregnant women with uncomplicated pregnancies (controls) and 157 pregnant women with PE. The inclusion criteria for selecting controls included: no rise in blood pressure, no hypertension or proteinuria, similar age (healthy women 28.08 yrs $\pm$ 7.37 and PE women 30.32 yrs $\pm$ 7.46), no biological relationship and a delivery date as close as possible to the delivery date(s) of the matched patient group. Controls were followed up for at least three months after delivery. If hypertension and/or proteinuria were observed during this follow up period, this specific control individual was excluded. Pre-eclampsia was defined as the presence of hypertension and proteinuria. Hypertension is characterized by blood pressure of at least 140mmHg (systolic) or at least 90mmHg (diastolic), on at least two occasions and 4–6h apart following the 20^{th} week of gestation in women known to be normotensive beforehand (Brown, Hague *et al.*, 2000; Sibai, 2003). Proteinuria is defined as an excretion of 300 mg or more of protein every 24h. If 24h 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 20^{th} week of gestation (Sibai, 2003). The PE was classified as severe when there was a blood pressure $\geq$ 160/110mmHg; or urinary protein excretion ≥5g per 24h; a platelet count of $<$100.000mm^{-3} in at least two samples; the combination of haemolysis, abnormal liver enzymes associated with persistent epigastric or upper right quadrant pain; persistent and severe symptoms as altered mental status, headaches, blurred vision or blindness;

presence of multiorgan involvement such as pulmonary edema, oliguria (<500mL per day) (Sibai, Dekker *et al.*, 2005). 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 Conceição Hospital (Porto Alegre, Brazil) and by the National Research Ethics Committee.

Genomic DNA: DNA was isolated from whole blood using a salting-out procedure according to Lahiri e Nurnberger (Lahiri e Nurnberger, 1991).

Polymerase chain reaction PCR RFLP: For the genotyping of two SNPs in exon 1 (*codon 54* and *57*) of MBL*2* gene, we performed the *Polymerase Chain Reaction (PCR) - Restriction Fragment Length Polymorphism (*RFLP). In each reaction was also included a sequence-verified control. The forward primer 5'-ACCCAGATTGTAGGACAGAG -3' and reverse primer 5'-CAGGCAGTTTCCTCTGGAAGG -3' were used for amplification. Generating an amplified product of 349-bp. The **B** *(codon 54)* and **C** (codon 57) alleles were detected respectively by ***Ban*I** (New England - BioLabs) and ***Mbo*II** (Fermentas, Life Sciencies) restriction enzymes digestions of the 349-bp product amplified by the MBL exon 1 PCR primers, followed by a 3% agarose gel electrophoresis. ***Ban*I** cleaves the **A** allele into two fragments (260 bp and 89 bp) and leaves the **B** allele undigested, while ***Mbo*II** specifically cleaves the **C** allele into two fragments (279 bp and 70 bp). MBL genotyping was performed through PCR analysis: 200ng of genomic DNA were added to a final volume of 50μL containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 0.08%, 2 mM $MgCl_2$; 0.2mM of each dNTPs; 10pmol of each primer and 0.75 units of *Taq*-polymerase.

Thermocycling conditions: The 35 cycles of 94°C for 30s, 54°C for 30s and 72°C for 30s were preceded by an initial denaturation cycle of 94°C for 5 min and followed by a final extension step at 72°C for 5min.

Polymerase chain reaction - site-directed mutagenesis (SDM): The SDM-PCR was employed to evaluate the SNP in the exon 1 (codon 52) of MBL gene. For amplification, we employed the forward primer 5'-CATCAACGGCTTCCCAGGCAAAGACGCG -3', while the reverse primer was the same for the codons 54 and 57. The restriction enzyme ***Hha*I** (New England - BioLabs) cleaves the **A, B**, and **C** alleles while ***Mlu*I** (New England - BioLabs) cleaves the 125-bp PCR product specific for the **D** (codon 57) allele into two fragments (100 bp and 25 bp). The O-allele represents the variant alleles B, C, or D because all have similar effects on MBL functions.

MBL haplotyping: The detection of the promoter variants (-550) **H/L** and (-221) **X/Y** was determined using sequence-specific primers (PCR-SSP). We performed four reactions for the same sample using four primers in each reaction, being two specific primers and other two internal control primers.

Primer **L** forward: 5'-GCTTACCCAGGCAAGCCTGTC-3';
Primer **X** reverse: 5'-GGAAGACTATAAACATGCTTTCG-3';
Primer **H** forward: 5'-GCTTACCCAGGCAAGCCTGTG-3';
Primer **Y** reverse: 5'-GGAAGACTATAAACATGCTTTCC-3'.

The *cis/trans* location of the promoter variant L relative to the X and Y variants was determined by PCR-SSP, using the MBL **cis-LX** and MBL **cis-LY** primer pairs, which combine a downstream specificity for the L allele with upstream specificities for the X and Y alleles, respectively. In the same way, the genotyping of the H allele, relative to the X and Y variants was also determined using the MBL **cis-HX** and MBL **cis-HY** primer pairs, which combine a

downstream specificity for the H allele with upstream specificities for the X and Y alleles, respectively.

Division in MBL levels groups: The SNPs of the exon 1 of the MBL gene are in linkage disequilibrium with the promoter polymorphisms, resulting in six more frequent haplotypes: HYA, HYD, LYA, LYB, LYC and LXA (Madsen, Garred *et al.*, 1995). Moreover, the individuals were categorized into three groups based upon the genotypes: the high MBL genotype **group 1** associated with high MBL serum levels ((H/L)YA/(H/L)YA and (H/L)YA/LXA); the intermediate MBL genotype **group 2** associated with intermediate MBL serum levels (LXA/LXA and (H/L)YA/O); and the low MBL genotype **group 3** associated with MBL deficiency resulting in the lowest MBL serum levels (LXA/O and O/O) (Frakking, Brouwer *et al.*, 2006).

Statistical analysis: MBL genotypic distribution was determined by direct counting. The genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. MBL allelic frequencies as well the presence/absence of the allele were compared using the Chi-square-test or Fisher exact test if appropriate. The significance level was set at α =0.05 (two-tailed). We evaluated the gestational age in weeks and its correlation with the three MBL genotype groups in the overall cohort, using the Kaplan–Meier method. All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Maternal MBL allele frequencies and genotype distribution

MBL genotype distribution in both PE and control women were in Hardy-Weinberg equilibrium for the SNPs in exon and promoter MBL*2* regions (*data*

not shown). We compared control and PE women to evaluate the MBL allelic/genotypic overall distribution and frequencies of the SNPs in exon 1 and promoter region (**Table 1**). Concerning the SNPs in the Exon 1, pre-eclamptic women presented a higher frequency of the **C** and **D** alleles (Allele C: freq=0.08, p=0.01 and Allele D: freq=0.10 p=0.005, respectively) when compared to healthy women (freq=0.04 for both the C and D alleles). We also found that PE women had higher frequencies of the genotypes **AC** and **AD** when compared with healthy women (genotype AC: PE women freq=0.16 *versus* healthy women freq=0.07; p=0.01 and genotype AD: PE women freq=0.20 *versus* healthy women freq=0.08; p<0.01). No differences in allelic and genotypic frequencies in the MBL*2* promoter region were found between PE and control pregnant women.

Definition of haplotype and genotype groups accordingly to MBL polymorphisms

We sought to investigate whether PE was related to any specific MBL genotype/haplotype group by analyzing both the structural and promoter MBL polymorphisms in these individuals (**Table 2**). The SNPs in exon 1: codon 54 (allele *B),* codon 57 (allele *C)* and in codon 52 (allele *D)* lead to a disrupted oligomerization of MBL chains into the basic structure, reducing the amount of functional MBL. By this way, in concerning the mutations in exon 1, the wild type allele is referred as A, and the O-allele represents the variant alleles B, C, or D. The AA wild type genotype produced the highest MBL serum concentrations, whereas the OO genotype produced the lowest, resulting in impaired functions. Since the SNPs in the structural gene of MBL are in linkage disequilibrium with the two polymorphisms in promoter region, we grouped the

individuals accordingly to the haplotypes previously described in literature. Based on the genotypes, the individuals were categorized as “high-producer MBL”, “intermediate producer” and “low MBL producer”.

No statistically significant differences were observed in haplotype groups frequencies between PE women and control pregnant women (Chi-square, p=0.65). However, concerning the genotypic frequencies, there was a trend for a higher frequency of the **OO** genotype (lower MBL producers) in PE women (freq=0.11) when compared with the healthy women (freq=0.04) (p=0.07). We found no differences of the SNPs in the MBL*2* structural gene, taken together the O-allele, in PE women and control group (O-allele 0.30 and 0.25 respectively) (**Table 2**).

Association of genotype/haplotype groups and the disease severity

Considering that PE can be presented in either mild or severe forms, we analyzed patients accordingly to disease severity and subgrouped by the haplotype and genotype frequencies (**Table 3**)**.** No differences were observed between the haplotype/genotype distribution and different PE forms. However, it is interesting to note that as the severity of PE increased, there was a trend for a higher frequency of the OO genotype. The severe PE women presented a higher frequency of the OO genotype (freq=0.15) when compared with the healthy women (freq=0.04, p=0.07) or with the mild PE women (freq=0.11, p=0.07).

MBL genotypes/haplotypes and their association with clinical parameters

Patients were also characterized by clinical and socioeconomic parameters including ethnic origin, number of aborts, number of gestations and

weight (**Table 4**). No significant differences were observed in genotypic / haplotypic frequencies between the control group and PE women sub-grouped accordingly the mentioned characteristics (**Table 4**). However, it is interesting to note that the Afro-derived women with PE had higher frequency of the **OO** genotype (freq=0.14) as compared to the Afro-derived healthy women (freq=0.06), although this only approached statistical significance (p=0.09) (**Table 4**). Also, pre-eclamptic multiparous women presented a higher frequency of the **OO** genotype (freq=0.10) as compared to the healthy multiparous women (freq=0.04), although this only approached statistical significance (p=0.08).

Time of delivery and MBL genotype

Many factors are suggested to play a role in gestational age at delivery including immunological and genetics factors (Crider et al., 2005). Events involving increasing inflammatory profile are related with pre-term birth. Therefore it is interesting to associate polymorphisms of inflammatory genes like MBL*2* and their contribution to pre-term birth. Our data suggest that PE women are more likely to have a preterm birth (28 to 31 weeks) than healthy women (PE women freq=0.13 *versus* healthy women freq=0.03, Fisher's Exact Test p=0.01, *data not shown*). To analyze the influence of MBL genotypes in the time of delivery, we compared gestational age at delivery between the different MBL genotype groups (**Table 5**)**.** Healthy pregnant women that delivered at term (37 to 41 weeks) presented higher frequencies of the AA genotype (freq=0.83, n=65), associated with high levels of MBL secretion, when compared with PE women at the same gestational time (freq=0.52, n=31, p=0.07). Conversely, there was a higher frequency of the MBL genotype group AA in PE pregnant women (freq=0.35, n=21) than healthy women (freq=0.12,

n=9) at a shorter gestational age (32-36 weeks) (Fisher's Exact Test, $p<0.01$). Pre-eclamptic women are also more likely to present higher frequencies of the intermediate MBL genotype AO (freq=0.16) and increased chance of having pre-term birth (28-31 weeks) when compared to healthy women (freq=0.04) (Fisher's Exact Test, $p<0.01$) (**Table 5**).

We also evaluated the cumulative probability of maintaining a pregnancy and the effect of MBL genotypes groups on gestation age as shown in **Figure 1**. MBL genotype groups were associated with the length of gestational age in a survival test. Further analysis revealed that both the PE and healthy women carriers of the MBL genotype group OO showed a tendency to present shorter gestational age than women with the MBL genotype groups AA and AO. However, the healthy women presented an extended time for higher probability of maintaining pregnancy (until 37 weeks) when compared with PE women (until 28 weeks).

DISCUSSION

Inflammation plays a fundamental role in the maintenance of the pregnancy. Inflammatory responses are stimulated by any form of tissue injury as well as by the innate immune system (Redman, Sacks *et al.*, 1999). A TH2-biased cytokine profile response is correlated with a successful pregnancy (Lin, Mosmann *et al.*, 1993) but some inflammatory environment in the uterus is pivotal to a good placentation. Since PE is a disease characterized by several immunological and genetic factors, the study of polymorphisms in genes of immunological interest is of great value in the understanding of the disease pathology. Here we described the polymorphisms in the structural and promoter MBL gene regions as well as their relation with the pathogenesis of PE.

MBL plays a fundamental role on the regulation of inflammation and it is important for the maintenance of pregnancy. MBL is a component of the innate immune system that initiates and activates the lectin pathway of complement. Besides it acts as a pro-inflammatory protein, modulating the inflammation and further inducing apoptosis. Expression of paternal antigens on fetal cells can induce activation of the maternal complement cascade (Faulk, Jarret *et al.*, 1980; Tedesco, Radillo *et al.*, 1990). The activation of the complement by MBL contributes to the destruction of trophoblast cells at the maternal-fetal interface, increasing the possibility of a insufficient vascularization at time of implantation (Sziller, Babula *et al.*, 2007). Besides the harmful effects of the MBL super activation, inflammation induced by MBL has an important role during a successful pregnancy. Maternal MBL serum levels are increased from the first trimester of pregnancy suggesting a role of MBL in nidation, placentation and maintenance of pregnancy (Van De Geijn, Roos *et al.*, 2007). The rise in MBL

levels during pregnancy is related to the maternal genotype. Nevertheless, a higher deposition of complement components have been identified in placentas from PE women (Sinha, Wells *et al.*, 1984; Tedesco, Radillo *et al.*, 1990).

Several studies have showed that low maternal MBL levels (or low MBL producer genotypes) in association with adverse pregnancy outcome, including recurrent miscarriages, PE, risk for chorioamnionitis and preterm delivery (Kilpatrick, 1996; Christiansen, Kilpatrick *et al.*, 1999; Kruse, Rosgaard *et al.*, 2002; Annells, Hart *et al.*, 2004; Annells, Hart *et al.*, 2005). Conversely, others suggested that reduced MBL levels in the serum could decrease the capacity of MBL-mediated complement activation and also limiting the complement-mediated destruction of semi-allogeneic fetal cells during pregnancy (Sziller, Babula *et al.*, 2007). In this study we found a trend toward high frequencies of MBL maternal genotype OO in PE. Importantly, disease severity was related to a particular MBL genotype: women with the most severe form of PE had the highest frequencies of the maternal MBL genotype OO.

The OO genotype is associated with the lowest MBL serum concentrations, generating disturbed multimer and impaired MBL function (Madsen et al., 1995; Roos et al., 2003). A successful pregnancy is biased toward a TH2-related cytokine profile, but some inflammation is also required to allow a correct angiogenesis in the maternal-fetus interface. MBL is a key factor in enhancing inflammation, acting as a pro-inflammatory molecule. However, other risk factors not evaluated here may also contribute and interact with the MBL genotype for the pregnancy outcome. However, there is not a consensus for the altered MBL levels in pregnancy until now. Some authors associated higher MBL levels with a successful pregnancy (Kilpatrick, Starrs *et al.*, 1999;

Annells, Hart *et al.*, 2004), while others authors found higher MBL levels in association with pregnancy complications (Sziller, Babula *et al.*, 2007; Van De Geijn, Dolhain *et al.*, 2008). Regarding the MBL haplotypes groups and PE development, we did not find any association between PE and healthy women. In a case control study, Van De Geij, 2007 also did not observe any association between MBL haplotypes and presence/absence of PE (Van De Geijn, Dolhain *et al.*, 2007).

We also investigated the role of different clinical parameters that would interfere in the development of PE. We associated lower MBL levels (according to the OO genotype group) with the severe form of PE. In this way, it is hypothesized that low MBL-related producer genotypes could be considered as disease form modifiers, worsen the disease profile. In another study, Kilpatrick found an association between lower MBL levels and the presence of PE (Kilpatrick, 1996). Our data further suggest that OO genotype could be over represented accordingly to disease severity. In relation to the PE development and gestational age, in all three genotype groups, we found that PE women were more likely to developed preterm birth (28-31 and 32-36 weeks) when compared to healthy women. In addition, even if the pregnant women were high MBL producers but they presented PE, the chances in having a preterm birth are increased, suggesting the association between PE development and gestational age. Many risk factors are suggested to play a role in gestational age at delivery including infections, increased inflammation, genetic and environmental factors (such as stress, smoking, obesity and heavy work) (Crider, Whitehead *et al.*, 2005; Lin, Moss *et al.*, 2007; De Man, Dolhain *et al.*, 2008). Some data were published on preterm delivery and MBL levels. Annels

et al. (2004) found association between low or intermediate MBL production and preterm birth (Annells, Hart *et al.*, 2004). Conversely, Van de Geijn (2008) found that women with the high MBL production genotype are more likely to have preterm birth when compared with the intermediate genotype group (Van De Geijn, Dolhain *et al.*, 2008), suggesting that higher levels of inflammation or infection could increase the risk of pre-term birth (Bowden, Barrett *et al.*, 2001; Wolfberg, Lee-Parritz *et al.*, 2004; Boggess, 2005; French, Mcgregor *et al.*, 2006; De Man, Dolhain *et al.*, 2008). We also evaluated the cumulative probability of maintaining a pregnancy and the effect of MBL genotype groups on gestation age in PE and healthy women. We found that the genotype OO in PE women lead to a reduced time of probability of maintaining a pregnancy (28 weeks) when compared with healthy women (36 weeks). This decreasing time of probability of maintaining a pregnancy can be explained by clinical effects caused by the PE development like hypertension, reduced uteroplacental blood flow and reduced fetal nutrition (Vatten e Skjaerven, 2004). In addition, low levels of MBL could affect the inflammatory profile, worsen the angiogenesis process in maternal-fetal interface. Our data corroborate with the fact that low MBL levels have been associated with adverse pregnancy outcomes like PE (Kilpatrick, 1996; Christiansen, Kilpatrick *et al.*, 1999; Kilpatrick, Starrs *et al.*, 1999; Kruse, Rosgaard *et al.*, 2002; Annells, Hart *et al.*, 2004; Annells, Hart *et al.*, 2005). Considering that PE is a pregnancy disease and higher MBL levels are essential in the first trimester of a successful pregnancy, our data suggest that women presenting low levels of MBL could be potential PE developers. However, further studies should be performed to better understand the complex relationships between pre-eclampsia and genetic variations of MBL.

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Table 1 - MBL allele/genotype overall distribution and frequencies of the SNPs in exon 1 and promoter region between control and pre-eclamptic women

			Genotypes			Alleles
Exon 1		**A/B**	**AA**	**AB**	**BB**	**B**
	PE [freq(n)]		0.78 (125)[a]	0.22 (34)[a]	0 (1)[a]	0.11 (36)[f]
	Control [freq(n)]		0.67 (111)[a]	0.32 (54)[a]	0.01 (2)[a]	0.17 (58)[f]
A/C			**AA**	**AC**	**CC**	**C**
	PE [freq(n)]		0.84 (134)[b]	0.16 (25)[b]	0 (1)[b]	0.08 (27)[g]
	Control [freq(n)]		0.93 (154)[b]	0.07 (11)[b]	0 (0)[b]	0.04 (11)[g]
		A/D	**AA**	**AD**	**DD**	**D**
	PE [freq(n)]		0.80 (128)[c]	0.20 (32)[c]	0 (0)[c]	0.10 (32)[h]
	Control [freq(n)]		0.92 (150)[c]	0.08 (14)[c]	0 (1)[c]	0.04 (16)[h]
Promoter Region		**L/H**	**LL**	**LH**	**HH**	**H**
	PE [freq(n)]		0.45 (66)[d]	0.38 (56)[d]	0.17 (26)[d]	0.33 (108)[i]
	Control [freq(n)]		0.48 (76) [d]	0.41 (65)[d]	0.11 (18)[d]	0.32 (101)[i]
		Y/X	**YY**	**YX**	**XX**	**X**
	PE [freq(n)]		0.72 (106)[e]	0.26 (39)[e]	0.02 (3) [e]	0.15 (45)[j]
	Control [freq(n)]		0.72 (114) [e]	0.24 (38)[e]	0.04 (7) [e]	0.16 (52)[j]

[a]χ^2 p=0.06; [b]χ^2 p=0.01; [c]χ^2 p=0.003; [d]χ^2 p=0.23; [e]χ^2 p=0.45; [f]χ^2 p>0.05; [g]χ^2 p=0.01; [h]χ^2 p=0.005
[i]χ^2 p=0.12; [j]χ^2 p=0.39

Table 2 - MBL haplotype and genotypic/allelic distribution of the promoter and exon 1 polymorphisms between control and pre-eclamptic women

		Pre-eclamptic women [freq(n)]	Control Women [freq(n)]
Haplotype			
	1*	0.49 (77)[a]	0.51 (81)[a]
	2**	0.46 (54)[a]	0.54 (64)[a]
	3***	0.45 (14)[a]	0.55 (17)[a]
A/O			
	AA	0.52 (83)[b]	0.54 (89)[b]
	AO	0.37 (60)[b]	0.42 (70)[b]
	OO	0.11 (17)[b]	0.04 (7)[b]
Allele	A	0.70 (226)[c]	0.75 (248)[c]
	O	0.30 (94)[c]	0.25 (84)[c]

Haplotype 1* high serum MBL levels
Haplotype 2** intermediate serum MBL levels
Haplotype 3*** lower serum MBL levels

Allele O = alleles B+C+D; Allele A = wild type

[a] χ^2 p=0.65; [b] χ^2 p=0.07; [c] χ^2 p=0.22

Table 3 - Haplotypes of promoter/exon regions and genotypes frequencies in control and pre-eclamptic women sub-grouped by the development of mild or severe PE

	Haplotypes [freq(n)]			Genotypes [freq(n)]		
	1*	**2****	**3*****	**AA**	**AO**	**OO**
Healthy pregnant women	0.51 (81)[a]	0.40 (64)[a]	0.09 (17)[a]	0.54 (89)[e]	0.42 (70)[e]	0.04 (7)[e]
Total pre-eclamptic pregnant women	0.52 (77)[b]	0.37 (54)[b]	0.11(14)[b]	0.52 (83)[f]	0.37 (60)[f]	0.11 (17)[f]
Mild pre-eclamptic pregnant women	0.60 (32)[c]	0.33 (18)[c]	0.07 (4)[c]	0.61 (35)[g]	0.32 (18)[g]	0.07 (4)[g]
Severe pre-eclamptic pregnant women	0.48 (23)[d]	0.37 (18)[d]	0.15 (7)[d]	0.44 (24)[h]	0.40 (22)[h]	0.15 (8)[h]

Haplotype **1*** high serum MBL levels Allele O = alleles B+C+D; Allele A = wild type
Haplotype **2**** intermediate serum MBL levels
Haplotype **3***** lower serum MBL levels

[a, b] χ^2 p=0.65 df=2; [a, c] χ^2 p=0.57 df=2; [a, d] χ^2 p=0.27 df=2
[e, f] χ^2 p=0.07 df=2; [e,g] χ^2 p=0.30 df=2; [e,h] χ^2 p=0.07 df=2

Table 4 - Haplotypes of promoter-exon regions and genotypes frequencies in both control and pre-eclamptic women classified accordingly clinical parameters.

Clinical Parameters		Haplotypes [freq(n)]			Genotypes [freq(n)]		
		1*	**2****	**3*****	**AA**	**AO**	**OO**
Healthy Women							
Ethnic Origin	Euro-derived	0.48 (58)	0.42 (51)	0.10 (11)	0.51 (64)	0.45 (57)	0.04 (5)
	Afro-derived	0.58 (21)	0.33 (12)	0.08 (3)	0.62 (23)[a]	0.32 (12)[a]	0.06 (2)[a]
Abortion	0	0.52 (58)	0.39 (43)	0.09 (10)	0.55 (65)	0.40 (47)	0.05 (6)
	=1	0.53 (18)	0.38 (13)	0.09 (3)	0.56 (19)	0.41 (14)	0.03 (1)
	>2	0.25 (2)	0.75 (6)	0 (0)	0.25 (2)	0.75 (6)	0 (0)
Weight	Normal	0.57 (26)	0.39 (18)	0.04 (2)	0.55 (27)	0.40 (20)	0.04 (2)
	Obesity	0.47 (45)	0.42 (41)	0.11 (10)	0.52 (52)	0.44 (44)	0.04 (4)
Gestation	First	0.56 (19)	0.32 (11)	0.12 (4)	0.63 (22)	0.31 (11)	0.06 (2)
	> 1	0.50 (60)	0.42 (52)	0.08 (10)	0.51 (65)[b]	0.45 (58)[b]	0.04 (5)[b]
Pre-eclamptic Women							
Ethnic Origin	Euro-derived	0.57 (57)	0.33 (33)	0.10 (10)	0.57 (60)[j]	0.36 (38)[j]	0.07 (8)[j]
	Afro-derived	0.44 (20)	0.44 (20)	0.12 (5)	0.45 (23)[a]	0.41 (21)[a]	0.14 (7)[a]
Abortion	0	0.53 (60)	0.36 (41)	0.10 (12)	0.54 (65)	0.36 (44)	0.10 (12)
	=1	0.55 (11)	0.30 (6)	0.15 (3)	0.50 (12)	0.37 (9)	0.13 (3)
	>2	0.25 (2)	0.75 (6)	0 (0)	0.25 (2)	0.75 (6)	0 (0)
Weight	Normal	0.48 (14)	0.38 (11)	0.14 (4)	0.45 (15)	0.45 (15)	0.10 (3)
	Obesity	0.52 (46)	0.38 (33)	0.10 (9)	0.54 (51)	0.36 (34)	0.11 (10)
Gestation	First	0.53 (21)	0.40 (16)	0.07 (3)	0.56 (24)	0.37 (16)	0.07 (3)
	> 1	0.53 (55)	0.36 (37)	0.11 (12)	0.51 (58)[b]	0.38 (43)[b]	0.10 (12)[b]

Haplotype 1* high serum MBL levels
Haplotype 2** intermediate serum MBL levels
Haplotype 3*** lower serum MBL levels
[a]χ^2 p=0.09 df=2; [b]χ^2 p=0.08 df=2

Allele O = alleles B+C+D; Allele A = wild type

Table 5 – Analysis of gestational age at delivery (in weeks) between the genotype groups.

		Gestational age at delivery [freq(n)]				Total (n)
Genotype		22 to 27 weeks	28 to 31 weeks	32 to 36 weeks	37 to 41 weeks	
AA	Healthy pregnant women	(0.04) 3[a]	(0.01)1[b]	(0.12) 9[c]	(0.83) 65[d]	78
	Pre-eclamptic pregnant women	(0.03) 2[a]	(0.10) 6[b]	(0.35) 21[c]	(0.52) 31[d]	60
AO	Healthy pregnant women	(0) 0[e]	(0.04) 2[f]	(0.13) 7[g]	(0.83) 44[h]	53
	Pre-eclamptic pregnant women	(0.02) 1[e]	(0.16) 7[f]	(0.21) 9[g]	(0.61) 26[h]	43
OO	Healthy pregnant women	(0) 0[i]	(0) 0[j]	(0.17) 1[k]	(0.83) 5[l]	6
	Pre-eclamptic pregnant women	(0) 0[i]	(0.17) 2[j]	(0.25) 3[k]	(0.58) 7[l]	12

[c] Fisher's Exact Test p=0.002; [d] Fisher's Exact Test p=0.07; [f] Fisher's Exact Test p=0.02

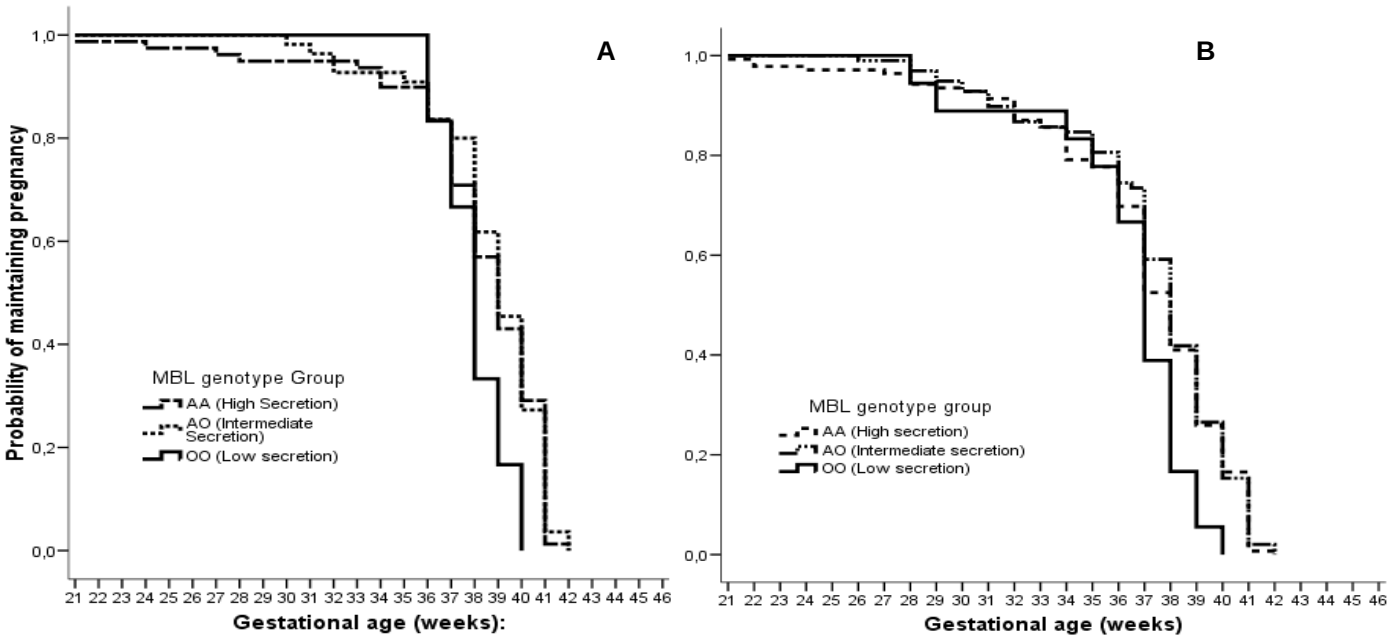

Figure 1 - The gestational age in healthy (**A**) and pre-eclamptic (**B**) women between the different MBL genotype groups was evaluated by the Kaplan–Meier survival curve. MBL genotype AA is associated with high MBL serum levels; MBL genotype AO with intermediate MBL serum levels and MBL genotype OO with the lowest MBL serum levels.

Capítulo 6 - Artigo 4

GESTAÇÃO DE SUCESSO: Entenda o papel do sistema imune materno na aceitação ou rejeição do feto

Priscila Vianna e José Artur Bogo Chies

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GESTAÇÃO DE SUCESSO:

Entenda o papel do sistema imune materno na aceitação ou rejeição do feto

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RESUMO

Como evitar a rejeição do feto pelo sistema imune da mãe? Durante a gestação, o sistema imunológico materno está em contato direto com o feto, que é constituído de material genético materno e paterno. Para evitar uma possível rejeição do feto pelo corpo da mãe, o sistema imunológico materno deve se adaptar, induzindo alterações que permitam a manutenção de uma gestação e um desfecho de sucesso: o nascimento! Vamos conhecer um pouco mais sobre o sistema de defesa do nosso corpo e como ele atua durante a gestação.

A evolução da gestação, o nascimento do bebê e a produção de leite para alimentá-lo constituem uma seqüência natural e bem planejada de acolher um novo ser. A interação imunológica que ocorre ao longo da gestação, é mantida até o período de amamentação. O aleitamento permite a transferência de anticorpos da mãe para o filho e estes anticorpos protegerão a criança durante seu desenvolvimento, permanecendo até um ano após o nascimento. Porém, neste artigo, vamos rever os aspectos imunológicos que oscilam ao longo da gestação, enfocando a rejeição do feto pelo sistema imune materno e demonstrando que toda relação, inclusive as mais harmônicas, são susceptíveis a ‘crises’.

Durante a gestação, o corpo feminino sofre diversas alterações endócrinas, físicas e também em seu perfil imunológico. O sistema imune materno está em íntimo contato com o feto, que pode ser comparado a um transplante, pois possui 50% de material genético paterno, tornando-se estranho ao organismo. Em 1953, o cientista e prêmio Nobel Peter Medawar

(1915-1987) foi o primeiro a formular o conceito de que o embrião se comporta no organismo materno como um transplante estando, portanto, vulnerável à rejeição ou tolerância imunológica. Portanto, como o feto não é rejeitado pela mãe?

O SISTEMA IMUNE

O sistema imune é constituído por um conjunto de células (leucócitos, que são diferentes tipos de glóbulos brancos) e moléculas (anticorpos, citocinas, quimiocinas e proteínas do complemento, entre outras) que circulam por todo o corpo através da linfa e do sangue. O sistema imunológico defende o organismo contra elementos estranhos, reconhecendo o que pode ser lesivo ao organismo no intuito de manter sua integridade. Estes elementos estranhos são denominados “imunógenos”, pois são capazes de iniciar uma resposta imune, como a resposta a infecção por bactérias, fungos, vírus e parasitas ou a presença de tumores. Agentes extrínsecos como transplantes também podem ser reconhecidos levando a rejeição do mesmo. Portanto, é muito importante que nosso sistema imune esteja em equilíbrio, visando manter sua integridade. As células do sistema imune (leucócitos) estão organizadas de forma que, diferentes tipos celulares desempenham funções distintas, dentre elas: reconhecer uma área infectada, recrutar outras células para esta área, capturar e apresentar o imunógeno para as células responsáveis, neutralizar o imunógeno, além de eliminar células infectadas. Estas células são produzidas diariamente na medula óssea e incluem vários tipos celulares com funções distintas. Dentre os diversos tipos de leucócitos, podemos citar os linfócitos T e os linfócitos B, sendo que os primeiros passam por um processo de

'amadurecimento' no timo, órgão linfóide situado na caixa torácica, logo acima do coração. Desta forma, as respostas imunológicas podem ser classificadas como celulares (as respostas diretas realizadas pelos leucócitos) ou humorais (através da produção de anticorpos e proteínas do complemento). No sistema imune, células especializadas (células apresentadoras de antígeno) são encarregadas de capturar um imunógeno, englobando-o, digerindo-o e em seguida apresentando-o para as células T que vão secretar proteínas sinalizadoras e reguladoras de uma resposta imunológica.

Os linfócitos T são peças centrais do sistema imune, responsáveis pela imunidade celular (cuja resposta é diretamente realizada por células). De acordo com o padrão de citocinas (proteínas sinalizadoras solúveis) que estas células secretam, elas desempenham funções diferentes. Os tipos principais de células T são: as células T citotóxicas, as células T auxiliares (do inglês, *T helper*) e as células T regulatórias. As células T citotóxicas agem no combate a infecções por patógenos intracelulares e no combate às células cancerígenas. As células T auxiliares são responsáveis pela formação de dois tipos principais de respostas celulares efetoras no nosso organismo frente a ocasiões distintas: resposta TH1 (resposta *T helper 1*) ou TH2 (resposta *T helper 2*). A diferença entre estas duas respostas consiste no perfil das citocinas produzidas (tanto em termos de quantidade como tipo de citocina) frente ao antígeno encontrado (**veja Figura 1**). Ainda de grande importância, temos em circulação uma importante população de linfócitos conhecidos como T reguladores, capazes de produzir citocinas que suprimem várias respostas imunes excessivas, inibindo a proliferação de outras células T.

COMO O SISTEMA IMUNE DA MÃE ‘ENXERGA’ A GESTAÇÃO

Para que uma gestação de sucesso ocorra, é importante que o sistema imune materno reconheça o feto, sem rejeitá-lo e induza uma resposta imunológica de aceitação, gerando um ambiente essencial para uma boa evolução da gravidez. A relação harmoniosa entre mãe e filho envolve a interação de aspectos da imunologia celular e humoral (através das citocinas e anticorpos) assim como de outros componentes. Existem vários mecanismos protetores que modulam a resposta imune materna ao feto e garantem a sua aceitação, como por exemplo: (1) a presença da placenta (tecido de origem fetal) que isola fisicamente e imunologicamente (veja a seção ‘A molécula HLA-G’) o feto da mãe, e (2) a presença de um estado efetor do tipo TH2 na mãe que evita um forte ataque ao feto. A placenta isola o embrião em um ambiente semi-permeável, induzindo a aceitação materna do feto, protegendo-o de um possível ‘ataque’ do sistema imune materno e permitindo uma troca de nutrientes entre mãe e feto (**Figura 2**). Algumas alterações imunológicas sofridas pelo corpo da mulher ao longo da gestação incluem: modificações no padrão de secreção de citocinas, inibição da proliferação de certos tipos celulares ou indução da expressão de moléculas na superfície das células.

As citocinas são proteínas que auxiliam na comunicação entre as células em um organismo. Elas são produzidas e secretadas, sinalizando para as células do corpo em resposta a vários estímulos (como por exemplo, a presença de um patógeno). As citocinas liberadas pelos linfócitos T exercem um importante papel na aceitação do feto dentro do organismo materno. O desenvolvimento de um perfil de resposta imune efetora, com a secreção de citocinas do tipo TH2, permite ao feto se desenvolver corretamente, sem ser

visto como um corpo estranho, garantindo ao feto um maior ambiente de aceitação (tolerância) devido às características moduladoras das citocinas produzidas durante esta resposta. Vários estudos demonstram que uma resposta materna com citocinas do tipo TH1, está relacionada a complicações na gestação, podendo levar a rejeição do feto. Os linfócitos T reguladores também exercem papel fundamental neste processo, inibindo a atividade inflamatória do sistema imune em busca de um ambiente supressor, excelente para o desenvolvimento fetal. **(Figura 3).** Porém, todo este equilíbrio na produção de citocinas e inibição de respostas celulares, precisa ser finamente controlado ao longo de toda a gestação. Momentos distintos do tempo gestacional requerem perfis diferentes de equilíbrio entre estes vários fatores. O atraso na ativação ou inibição de qualquer uma destas vias pode resultar em complicações gestacionais ou, mesmo, em aborto.

CONVERSA SÉRIA ENTRE MÃE E FETO

Um estudo inédito publicado na revista *Science* em dezembro de 2008, e coordenado pelo imunologista norte-americano Joseph McCune, demonstra que o sistema imune do feto exerce também uma função muito importante ao evitar o ‘ataque’ pelas células maternas que atravessam a placenta, através da ação de células T regulatórias fetais. Estas células T regulatórias fetais são induzidas pela presença das células maternas. Além disso, estas células regulatórias fetais podem permanecer em circulação (após o nascimento) por até 17 anos como memória imunológica, sendo capazes de reconhecer as células maternas. Este evento parece ocorrer através dos mesmos mecanismos utilizados pelo sistema imune materno quando este usa as células

T regulatórias maternas evitando a rejeição do feto. Esse estudo inovador mostra como mãe e feto estão sob um contato muito mais íntimo do que imaginado anteriormente. Além disso, é uma prova de que o sistema imune fetal está, apesar de não completamente formado, já bastante ativo mesmo antes do nascimento.

A MOLÉCULA HLA-G

Um fator de grande importância para a manutenção de uma gestação de sucesso é a molécula denominada HLA-G (do inglês, *Human Leukocyte Antigen-G*). Esta molécula é expressa por genes não clássicos do complexo de histocompatibilidade principal *(MHC – major histocompatibility complex)* do tipo I. Os genes presentes do MHC desempenham funções muito importantes na regulação do sistema imune. A molécula HLA-G apresenta um perfil de expressão restrito a apenas alguns tecidos e tipos celulares. Interessantemente, esta molécula está presente em grande quantidade na interface materno-fetal, nas células do trofoblasto (tecido embrionário que dá origem a placenta). Esta molécula possui uma capacidade única de não apresentar antígenos fetais para o sistema imune materno, isolando imunologicamente o feto de um possível ataque. O HLA-G age efetivamente na inibição de células que poderiam 'atacar' o feto, como por exemplo, as células *natural killer (NK)*, ou assassinas naturais. As células assassinas naturais são leucócitos que tem atividade citotóxica sem a necessidade do reconhecimento prévio de um antígeno. Elas reconhecem padrões moleculares e são reguladas por citocinas, além de receptores de ativação e inibição em sua superfície. A expressão da molécula HLA-G na interface materno-fetal pode impedir a

reatividade das células assassinas naturais ao trofoblasto, enviando um aviso de inibição para um possível ataque ao feto **(Figura 4)**.

Um estudo de doutorado desenvolvido pelos autores deste artigo no laboratório de Imunogenética da UFRGS desde 2005 demonstra que gestantes apresentam flutuações no perfil imunológico dependendo do tempo gestacional. Neste estudo, foram coletadas amostras sanguíneas de mulheres que tinham intenção de engravidar e de gestantes em diferentes períodos da gestação (1º, 2º e 3º trimestres gestacionais), assim como no primeiro trimestre de amamentação. Os resultados mostram que, dentre os linfócitos, as células T regulatórias têm seu número aumentado no primeiro trimestre de gestação, fase mais crítica para o desenvolvimento fetal, favorecendo uma gestação de sucesso, pois agem inibindo uma ativação excessiva do sistema imune e buscando a manutenção de um equilíbrio.

Porém, neste mesmo período de gestação, as células assassinas naturais encontram-se também em maior número, indicando perigo à gestação. Estas células assassinas naturais não somente podem atacar as células derivadas do feto (como anteriormente discutido), como também secretam citocinas que induzem a inflamação. Apesar de inicialmente pensarmos em uma resposta prejudicial ao feto mediada pelas células assassinas naturais, estudos recentes coordenados pelo cientista Van der Meer, da Universidade de Radboud, confirmaram que as células assassinas naturais desempenham um importante papel no inicio da gestação, secretando citocinas inflamatórias que favorecem uma correta vascularização da região materno-fetal, permitindo o suporte de oxigênio necessário ao desenvolvimento do feto. No segundo trimestre de

gestação o número de linfócitos T reguladores e de células assassinas naturais diminui **(Figura 5)**.

COMO PREVENIR DOENÇAS COMO A PRÉ-ECLAMPSIA

Uma das complicações gestacionais freqüentes, que se desenvolve principalmente no primeiro trimestre em primigestas (mulheres durante a primeira gestação) é a pré-eclâmpsia. A pré-eclâmpsia é uma doença que envolve aumento de pressão sangüínea e excreção de proteínas na urina, colocando em risco a sobrevivência tanto do feto quanto da mãe. A pré-eclâmpsia é uma complicação comum da gravidez, representando a segunda causa de morte materna, sendo responsável por até 7% das causas de óbito fetais e maternos durante a gravidez. Dentre as origens do desenvolvimento da pré-eclâmpsia, inclui-se uma origem imunológica. A teoria da maladaptação imunológica que induz o desenvolvimento desta doença propõe que, como o feto representa um transplante para o sistema imunológico materno, torna-se óbvio que ele deva ser rejeitado. Desta forma, se o organismo materno não consegue readaptar suas respostas para a manutenção do feto, uma das alterações geradas, na tentativa de 'eliminar' o feto, envolveria o aumento da pressão sangüínea. Porém, se diagnosticada corretamente, o tratamento da pré-eclâmpsia é simples, consistindo em muito repouso, diminuição da ingestão de sal e aumento da ingestão de líquidos. Como a pré-eclâmpsia abrange aspectos imunológicos, torna-se óbvio que moléculas de perfil imunológico como a HLA-G possam interferir nesta doença. Um estudo realizado pelos autores no Laboratório de Imunogenética da Universidade Federal do Rio Grande do Sul demonstrou que, uma deleção no gene da molécula HLA-G está

associada a uma menor expressão desta molécula, relacionando-se ao desenvolvimento de pré-eclâmpsia em mulheres primigestas **(Figura 6)**. Esta relação entre a deleção no gene de HLA-G e o desenvolvimento de pré-eclampsia pode ser útil como um mecanismo de prognóstico em uma gestação. Uma análise laboratorial a partir de uma simples coleta de sangue pode sugerir uma maior ou menor tendência ao desenvolvimento de patologias como a pré-eclâmpsia durante a gestação.

UMA GESTAÇÃO TRANQUILA E FELIZ

Considerando que a gravidez pode ser influenciada pelas inúmeras alterações imunológicas que ocorrem no corpo materno durante este período, torna-se de grande importância conhecer os parâmetros imunológicos e a cronologia destas alterações ao longo de uma gravidez. Conhecer o nosso corpo e suas alterações pode auxiliar na prevenção de perdas gestacionais associadas a respostas imunológicas excessivas ou inadequadas, levando ao desenvolvimento de uma gestação de sucesso, tanto para a mãe quanto para o bebê.

SUGESTOES PARA LEITURA

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INDICE DE FIGURAS

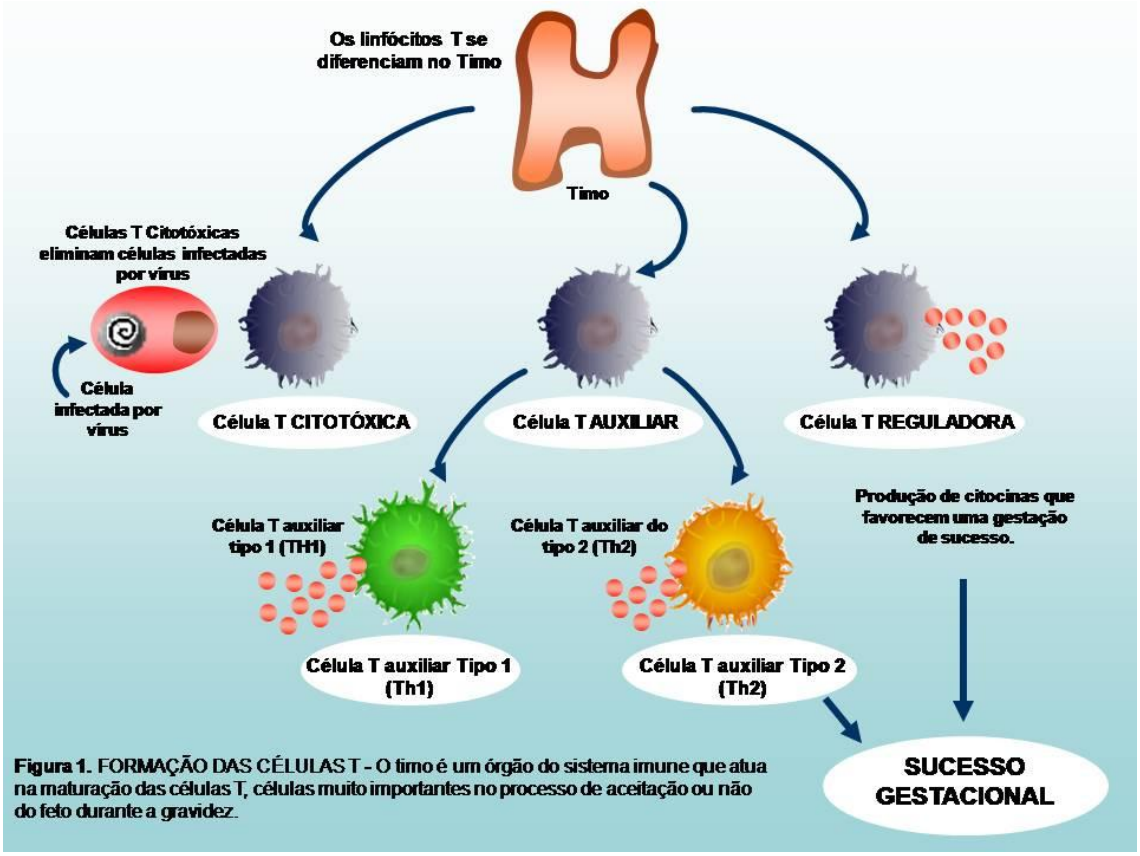

Figura 1. FORMAÇÃO DAS CÉLULAS T - O timo é um órgão do sistema imune que atua na maturação das células T, células muito importantes no processo de aceitação ou não do feto durante a gravidez.

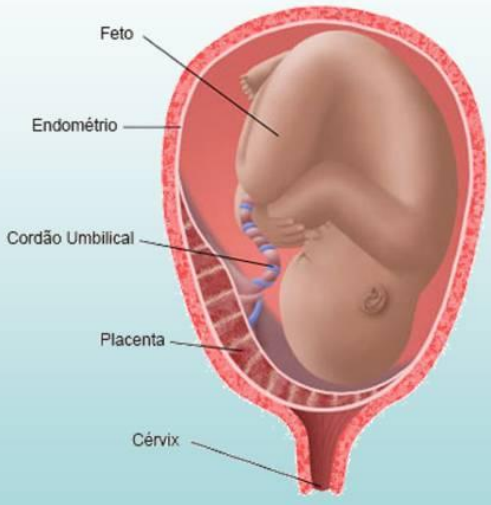

Figura 2. A PLACENTA - Na gestação, a placenta isola parcialmente o feto da mãe, para protege-lo, porém permite a troca de oxigênio e nutrientes assim como uma comunicação imunológica ao longo da gestação.

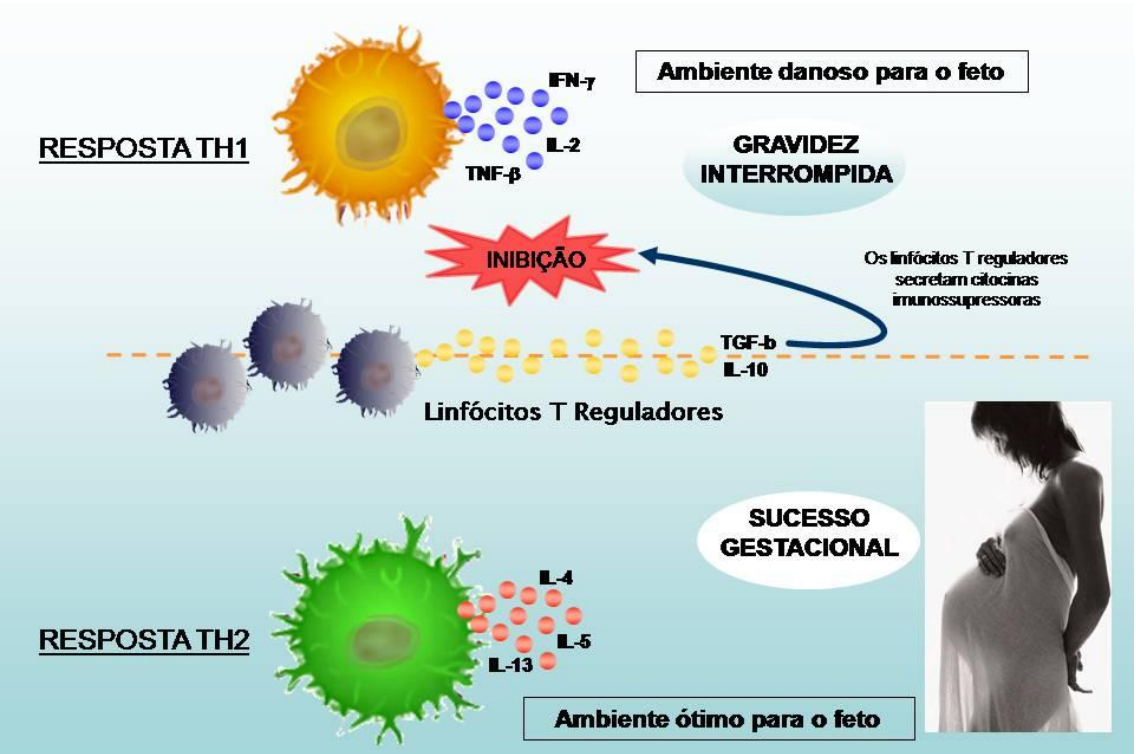

Figura 3. AS DIFERENTES RESPOSTAS IMUNES - O sistema imune de uma gestante pode desenvolver diferentes respostas que favorecem ou não uma gestação. Durante uma resposta imune, as células T reguladoras tentam inibir um ambiente desfavorável ao desenvolvimento do feto, este ambiente é composto pelas células T auxiliares do Tipo 1 e pelas citocinas que elas produzem.

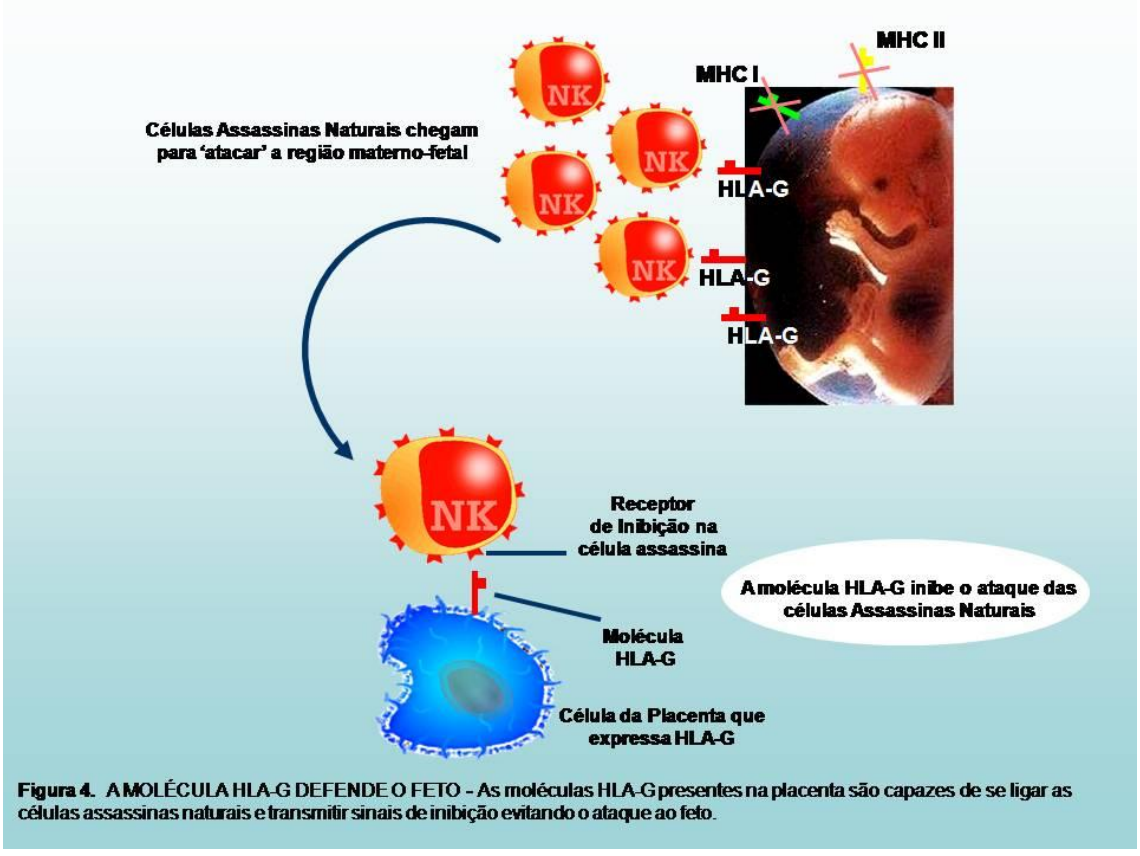

Figura 4. A MOLÉCULA HLA-G DEFENDE O FETO - As moléculas HLA-G presentes na placenta são capazes de se ligar as células assassinas naturais e transmitir sinais de inibição evitando o ataque ao feto.

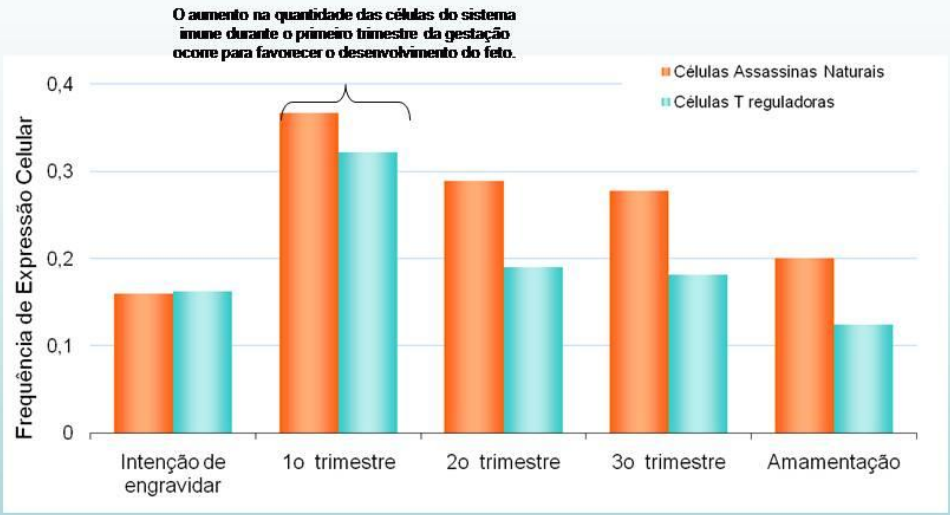

Figura 5. AS CÉLULAS IMUNES DURANTE A GESTAÇÃO - No primeiro trimestre da gestação, é observado um aumento no número de células do sistema imune que agem para criar um ambiente agradável para o desenvolvimento do feto. Logo depois que o feto está bem estabelecido estas células diminuem de quantidade no decorrer da gestação.

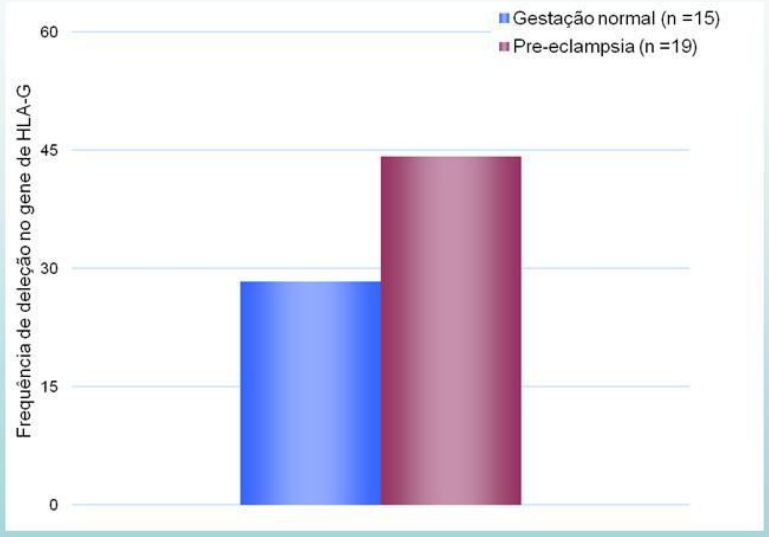

Figura 6. DELEÇÃO NA MOLÉCULA HLA-G - As gestantes que desenvolvem pré-eclampsia tem tendência a apresentar uma maior taxa de deleção na molécula HLA-G, podendo ser um fator de complicação durante a gestação. Isso demonstra como as doenças gestacionais podem afetar o sistema imune materno.

CAPÍTULO 7

DISCUSSÃO

Capítulo 7 - DISCUSSÃO

Durante a gestação, o corpo feminino sofre diversas alterações endócrinas, físicas, psicológicas e também em seu perfil imunológico. O sistema imune materno está em íntimo contato com o feto, que pode ser comparado a um alloenxerto, pois possui 50% de material genético paterno, tornando-se estranho ao organismo. Em 1953, Medawar propôs a existência da tolerância imunológica materna durante a gravidez, impedindo desta forma, uma resposta materna alogênica agressiva ao feto. Dentre os diversos fatores imunológicos que atuam na manutenção da homeostase durante a gestação, podemos citar: o perfil de produção de citocinas do tipo TH2 (Lin, Mosmann *et al.*, 1993; Marzi, Vigano *et al.*, 1996), a presença de células T regulatórias (Aluvihare, Kallikourdis *et al.*, 2004; Saito, Sasaki *et al.*, 2005), o papel das células dendríticas (Pollard, 2008), a depleção local de triptofano inibindo a proliferação de células T (Munn, Zhou *et al.*, 1998), a expressão do fator inibitório de leucemia (“Leukemia Innhibitory Factor” - LIF) (Piccinni, Beloni *et al.*, 1998), assim como a importante ação imunorregulatória da molécula HLA-G (Hviid, 2006). Neste trabalho avaliamos alguns componentes do sistema imune que variam durante a gestação, nos focando nos mecanismos que regulam a rejeição do feto pelo sistema imunne materno. Investigamos o papel de polimorfismos nos genes da lectina ligadora de manose (MBL) e da molécula imunomodulatória HLA-G em gestantes saudáveis ou com pre-eclampsia. Também avaliamos o balanço no perfil de produção de citocinas TH1 ou TH2 (IFN-γ, IL-4, IL-6, IL-2, IL-10 e TNF-α) durante a gestação saudável. Ainda discutimos o papel da exposição materna aos leucócitos paternos e a

ocorrência de atopias na infância. Observamos que variantes alélicas de HLA-G e MBL estão relacionadas ao desenvolvimento e gravidade da pré-eclampsia. Gestantes saudáveis apresentaram perfis de proliferação de celular e freqüências de células NK elevados. Um alto perfil de proliferação celular foi observado também em mulheres saudáveis durante a primeira gestação em comparação a mulheres multíparas. Porém não observamos diferenças estatísitcas em relação aos níveis de produção de citocinas ao longo de gestações saudáveis.

Imunorregulação da Pre-eclampsia

A relação dos níveis de HLA-G e o desenvolvimento de pre-eclampsia são bastante estudados. A etiologia e patogênese desta doença gestacional envolvem uma combinação de predisposição materno-fetal e fatores ambientais (Roberts, Taylor *et al.*, 1989). Uma das teorias do desenvolvimento da pré-eclampsia é a teoria da maladaptação imunológica que propõe que o sistema imune materno não tolera o feto semi-alogênico. As funções do trofoblasto são também modificadas pela ação de várias citocinas durante a pre-eclampsia (Hayashi, Ohkura *et al.*, 2003). Desta forma, propusemo-nos a estudar alguns aspectos imunogenéticos que pudessem contribuir para a elucidação da patogênese da pré-eclampsia. Avaliamos a freqüência de variantes do gene HLA-G e sua relação com o desenvolvimento de PE. Ao longo deste trabalho (**capítulo 3**), observamos que gestantes primíparas que desenvolveram pré-eclampsia apresentaram uma maior freqüência de expressão da variante alélica de HLA-G com a deleção de 14 pares de bases. Sugere-se que a presença dos 14 pares de base no gene de HLA-G é importante para a manutenção de formas estáveis das moléculas e RNA

mensageiro de HLA-G. Desta forma, a deleção destes 14 pares de bases, geraria transcritos instáveis, influenciando as funções de HLA-G e favorecendo o desenvolvimento de certas complicações como a PE (Abbas, Tripathi *et al.*, 2004; Hviid, 2006). Nossos dados estão de acordo com estes achados, demonstrando a importância da molécula HLA-G na regulação da gestação.

Exposição limitada ao esperma e ocorrência de pré-eclampsia

A pré-eclampsia é uma doença relatada como sendo mais freqüente na primeira gestação e seu desenvolvimento está também correlacionada à exposição limitada ao esperma com o mesmo parceiro antes da concepção (Einarsson, Sangi-Haghpeykar *et al.*, 2003). A exposição prolongada ao esperma do mesmo parceiro tem sido sugerida com fator de proteção contra o desenvolvimento da pré-eclampsia (Robillard, Hulsey *et al.*, 1994). Apoiados na "Hipótese da Higiene" que propõe que condições de vida mais higiênicas e consequente baixa exposição a patógenos levam a um aumento na incidência de alergias, estabelecemos uma teoria. Seguindo estas idéias, sugerimos que a exposição repetida e prolongada ao esperma do mesmo parceiro pode gerar um ambiente imunorregulador materno durante a gestação que será transferido ao feto, induzindo após nascimento, menores taxas de alergia durante a infância (**capítulo 4**). Como o contato repetido com patógenos gera um ambiente imune tolerizado, sugerimos que a exposição repetida ao esperma possa gerar também um ambiente imune regulador que será transferido para a prole. As células T regulatórias desempenham funções importantes na regulação das respostas imunes, como por exemplo, inibindo as respostas alérgicas do tipo TH2 (Provoost, Maes *et al.*, 2009). Bersen e colaboradores acreditam que mulheres na primeira gestação apresentam baixos níveis de

atividade de células T regulatórias específica a antígenos paternos quando comparadas as mulheres que já tiveram mais de um filho com o mesmo pai, e ainda, que estes baixos níveis de atividade podem afetar o sistema imune da prole, e consequentemente, o risco do desenvolvimento de alergias (Bernsen, Nagelkerke *et al.*, 2006). Nós acreditamos que a mesma situação de modulação do sistema imune da prole pode ser vista envolvendo a exposição materna a antígenos paternos.

Lectina Ligadora de Manose na regulação da Gestação

Como a patogênese da pré-eclampsia envolve múltiplos fatores imunológicos, continuamos a avaliar os aspectos imunogenéticos que influenciam a manutenção da gestação (**capítulo 5**). Investigamos então, a influência do mediador imunológico componente do sistema do complemento, a lectina ligadora de manose (MBL), presente no ambiente uterino e sua relação com a patogênese da pre-eclampsia. A MBL, um componente da imunidade inata, é relacionada como importante mediador da inflamação durante a gestação (Van De Geijn, Dolhain *et al.*, 2007), garantindo a manutenção de um ambiente inflamatório primordial para uma boa placentação (Redman, Mcmichael *et al.*, 1984; Roberts, Taylor *et al.*, 1989). A presença de MBL na interface materno-fetal pode contribuir para a ativação de fagócitos engajados na eliminação de restos celulares e células apoptóticas (Ogden, Decathelineau *et al.*, 2001; Nauta, Castellano *et al.*, 2004). Nossos dados demonstram que, mulheres que desenvolvem pré-eclampsia apresentam maiores freqüências do genótipo relacionado a baixos níveis séricos de MBL, mostrando a relação protetora dos níveis elevados de MBL na gestação (**Tabela 2** – capítulo 5). Estes dados estão de acordo com vários estudos publicados que demonstram

altos níveis de MBL associados com gestações de sucesso e que estes níveis são aumentados principalmente no primeiro trimestre destas gestações (Roos, Bouwman *et al.*, 2003; Van De Geijn, Roos *et al.*, 2007). Além disso, sugerimos que o genótipo OO de MBL (correspondente a baixos níveis de seceção de MBL no plasma) contribui para a gravidade da pré-eclampsia e não necessariamente para seu desenvolvimento (**Tabela 3** – capítulo 5). Van de Geijn e colaboradores em um estudo caso controle também demonstraram associação entre o genótipo "baixo secretor de MBL" e a forma grave da pré-eclampsia (Van De Geijn, Dolhain *et al.*, 2007). Porém, um estudo avaliando os níveis séricos de MBL demonstrou que, mulheres com a forma grave da pré-eclampsia apresentaram maiores níveis séricos de MBL (Celik e Ozan, 2008). A diferença entre estes resultados pode ser explicada pelo fato de um estudo se focar no nível gênico e o outro avaliar a secreção da proteína, sugerindo diferenças pós-transcricionais. Uma outra possibilidade é a forma de classificação da pré-eclampsia como forma grave, podendo diferir de acordo com os parâmetros clínicos utilizados na classificação. Alguns estudos relatam diferentes níveis de MBL de acordo com a gravidade da pré-eclampsia (Van De Geijn, Dolhain *et al.*, 2007; Celik e Ozan, 2008), porém os dados são contraditórios.

Papel das células NK e molécula HLA-G durante a gestação

A subpopulação leucocitária mais abundante na interface materno-fetal durante os primeiros trimestres de gestação é a subpopulação de células NK (Starkey, Sargent *et al.*, 1988; Trundley e Moffett, 2004). O papel preciso das células NK uterinas (uNK) envolve a secreção de citocinas como IFN-γ, essencial no remodelamento dos vasos sanguíneos na interface materno-fetal.

Avaliando o papel das células NK periféricas durante a gestação saudável, observamos uma alta freqüência de células NK (CD3-CD16+CD56+) durante o primeiro trimestre gestacional (**Figura 2A** – capítulo 6). Esta freqüência é diminuída ao longo da gestação alcançando os menores valores de expressão no fim da gestação (terceiro trimestre). Estes resultados demonstram a importância do papel das células NK no início da gestação, fornecendo um ambiente inflamatório ótimo para uma correta vascularização e suprimento de oxigênio para o desenvolvimento fetal. A molécula HLA-G desempenha um papel fundamental na manutenção de um ambiente imunossupressor visando a aceitação fetal. Esta molécula participa na indução de uma resposta imune regulatória, induzindo a produção de IL-10, inibindo o ataque das células NK maternas contra o feto, inibindo a ativação de células CD4+ e CD8+, induzindo células T regulatórias e induzindo apoptose (Maejima, Fujii *et al.*, 1997; Rouas-Freiss, Goncalves *et al.*, 1997; Feger, Tolosa *et al.*, 2007; Lemaoult, Caumartin *et al.*, 2007). Além disto, diversos experimentos sugerem que citocinas TH2 estão associadas a altos níveis de HLA-G solúvel (sHLA-G)- e conseqüentemente ao sucesso gestacional - ao passo que baixas concentrações de sHLA-G parecem induzir a expressão de citocinas do tipo TH1 (Maejima, Fujii *et al.*, 1997; Kapasi, Albert *et al.*, 2000).

HLA-G age principalmente se ligando à receptores de ativação/inibição das células NK. As células NK uterinas são também capazes de atacar as células do trofoblasto em razão da falta de expressão de moléculas clássicas de MHC (Witt, Goodridge *et al.*, 2004). Desta forma, HLA-G age se ligando aos receptores de inibição das células NK. Entretanto, Rajagopalan e colaboradores, demonstraram que as moléculas de HLA-G também se ligam a

receptores de ativação em células NK para favorecer o mecanismo de angiogênese durante a gestação (Rajagopalan, Bryceson *et al.*, 2006). Nossos dados mostram uma alta freqüência de células CD3+ expressando HLA-G no primeiro trimestre da gestação (**Figura 2B** – capítulo 6), evidenciando o papel imunorregulador desta molécula no período crítico de implantação. Estes altos níveis de expressão de HLA-G associadas com a alta freqüência na expressão de células NK sugerem como a molécula HLA-G pode, ao mesmo tempo, atuar ativando as células NK através da interação com seus receptores, além de atuar no controle da inibição de um possível ataque ao feto pelas células NK.

Regulação das funções efetoras celulares na gestação saudável: papel das citocinas

Sob condições fisiológicas normais as citocinas encontram-se geralmente em níveis séricos bastante baixos, ou mesmo indetectáveis. As células T CD4+ auxiliares do sistema imune respondem a estímulos podendo se diferenciar em subpopulações que produzem e secretam grupos distintos de citocinas e que, conseqüentemente, desempenham diferentes funções efetoras. As duas principais subpopulações efetoras de células T auxiliares se dividem em TH1 e TH2. Estas subpopulações são distintas de acordo com as citocinas que expressam: a subpopulação TH1 produz IFN-γ, TNF-α e IL-2 e a subpopulação TH2 é caracterizada pela produção de IL-4, IL-5, IL-13 e IL-10 (Abbas *et al.* 1996). Múltiplos fatores podem influenciar no desenvolvimento de precursores TH1 ou TH2, como a natureza das células apresentadoras de antígeno, a forma de administração do antígeno, o background genético do hospedeiro, e o ambiente de citocinas durante a ativação de células T (De Becker *et al.,* 1998;

Beebee *et al.,* 1997; Duncan *et al.,* 1994; Gollob & Coffman, 1994; Hsieh *et al.,* 1993; Coffman *et al.,* 1991; Scott *et al.,* 1986).

Os mecanismos efetores imunológicos maternos ativados durante a gestação são de extrema importância para tolerância ao feto e para manutenção de uma gestação de sucesso. O padrão de resposta adaptativa parece afetar diretamente o sucesso gestacional. O TNF-α é inicialmente necessário para a implantação embrionária no útero através da produção de VEGF ("vascular endothelial growth factor"), o qual modula a permeabilidade placentária e a angiogênese necessária para uma implantação e placentação eficiente (Chung, Yelian *et al.*, 2000). No entanto, esta resposta inflamatória inicial deverá ser regulada pela indução da expressão de IL-10 posteriormente. As células T regulatórias CD4+CD25+FoxP3+ são importantes produtoras de citocinas imunorregulatórias como IL-10 e TGF-β (Saito, Sasaki *et al.*, 2005). As respostas efetoras do tipo TH1 desempenham um papel central na rejeição de transplantes. A citocina IL-2 pode estimular a proliferação de células T CD4+, de maneira autócrina, levando a ativação das células T CD8+ citotóxicas, importantes efetoras no mecanismo de rejeição de tumores. Ainda, IFN-γ pode ativar monócitos e macrófagos na região de enxerto, amplificando a resposta de rejeição. Por isso é de extrema importância a regulação do balanço de produção de citocinas durante a gestação, já que o feto age como um enxerto para o sistema imune materno. Tem sido demonstrado em mulheres com aborto recorrente, que apresentam falha após fertilização *in vitro* e transferência de embriões, que, apesar do aumento nos níveis de TNF-α, não ocorre aumento da expressão de IL-10 (Ng, Gilman-Sachs *et al.*, 2002). Diversos trabalhos indicam que um padrão efetor de citocinas do tipo TH2 está

associado ao sucesso gestacional e que a manutenção de um padrão TH1 será prejudicial para a gravidez (Marzi, Vigano *et al.*, 1996; Raghupathy, 1997; Clark e Croitoru, 2001). Na interface materno-fetal, as células T CD3+ representam 10-20% de todas as células (Slukvin, Merkulova *et al.*, 1996) e estão em íntima associação com células com morfologia de células dendríticas (Pollard, 2008). As citocinas secretadas por células T na interface materno-fetal desempenham um papel importante no desenvolvimento do embrião durante a pré-implantação, na implantação do embrião e no mecanismo de tolerância fetal pelo sistema imune materno (Piccinni, 2002). Ao investigarmos amostras de plasma e sobrenadante de culturas estimuladas de gestantes saudáveis e sem complicações (**Figuras 5 e 6**, capítulo 6) em relação à produção de citocinas efetoras do tipo TH1 (IFN-γ, TNF-α, IL-2) e do tipo TH2 (IL-4 e IL-10), não observamos diferenças significativas nos níveis de produção destes mediadores solúveis pelas células do sangue periférico destas pacientes nos diferentes tempos gestacionais (1º, 2º e 3º trimestres). Piccini e colaboradores também não observaram diferenças significativas nos níveis de produção de citocinas através de medidas em sangue periférico (Piccinni, 2002). Apesar disso, verificamos que os valores médios dos níveis de IL-2 são mais baixos durante a gestação aumentando apenas no último trimestre, sugerindo a manutenção de um ambiente regulador ideal para uma gestação de sucesso, sem ativação de células T CD8+ citotóxicas danosas ao feto (**Figura 5B** – capítulo 6). Também observamos uma tendência para a redução dos níveis de IL-10 no primeiro trimestre de gestação (**Figura 5E** – capítulo 6), permitindo uma resposta inflamatória no intuito de promover a correta vascularização da interface mateno-fetal no início da gestação. Estes níveis de IL-10 estão

potencialmente aumentados após o primeiro trimestre, favorecendo a tolerância ao feto durante o resto da gestação. Outros autores relataram um aumento significativo de citocinas do tipo TH2 através de medidas no sangue periférico, em mulheres que tiveram gestações de sucesso e um aumento de citocinas efetoras do tipo TH1 no sangue periférico de mulheres que sofreram abortos recorrentes (Hill, Polgar *et al.*, 1995). Nossos dados, apesar de estatisticamente não significativos, sugerem que altos níveis de IL-6 estão presentes em todas as etapas como demonstrado nas análises em gestantes saudáveis (**Figura 5D**- capítulo 6). Citocinas como IL-1 e IL-6 estão relacionadas à regulação das etapas da implantação, favorecendo a invasão das células do trofoblasto mediada por metaloproteases (Mcewan, Lins *et al.*, 2009).

Divulgação Nacional da Imunorregulação da Gestação

No âmbito da divulgação nacional de nossos resultados, submetemos dados preliminares (**capítulo 7**) à revista Ciência Hoje que abrange leitores de todas as classes sociais e vários níveis de formação no Brasil. É de extrema importância a divulgação dos dados produzidos na pesquisa nacional para a população. Neste artigo, esclarecemos como é regulada a interação mãe-feto durante a gestação e relacionamos o papel das células do sistema imune com o desenvolvimento de complicações como a pré-eclampsia. Ainda citamos outros trabalhos nacionais e trabalhos internacionais de alto impacto.

Limitações do Estudo

Apesar de avaliar inúmeros marcadores imunológicos de interesse que influenciam o perfil de uma gestação de sucesso ou não, este trabalho apresenta uma série de limitações que devem ser consideradas. Na avaliação

do perfil de produção citocinas no sobrenadante de culturas de PBMC estimuladas durante a gestação saudável, podemos especular que o tamanho amostral reduzido é um fator contribuinte para a ausência de significância estatística. Da mesma forma, podemos citar a ausência da avaliação da relação entre os níveis protéicos de HLA-G e seu perfil gênico. Amostras de DNA poderiam ser extraídas no intuito de comparar dentre os diferentes tempos gestacionais o perfil de ativação do gene HLA-G. Ainda, amostras de placenta ou cordão umbilical poderiam ser obtidas visando comparar os componentes imunológicos avaliados neste trabalho em sangue periférico,.com os expressos diretamente na interface materno-fetal. Outros fatores que interferem no perfil imunológico da regulação da gestação e não foram abordados neste trabalho abrangem o balanço hormonal e a ocorrência de estresse, ansiedade ou depressão. Todos estes fatores devem ser levados em consideração em futuras abordagens, visando correlacionar os diferentes mecanismos que interagem na imunorregulação da gestação.

Considerações finais

Neste trabalho avaliamos as alterações imunológicas durante a gestação focando o envolvimento das alterações imunogenéticas e efetoras em todo processo, assim como o desenvolvimento de complicações como a pre-eclampsia. Torna-se relevante o potencial imunorregulador das moléculas HLA-G e MBL, contribuindo para um melhor entendimento do processo da patogênese da pré-eclampsia. Além disso, fatores extrínsecos como o tempo e intensidade de exposição materna ao esperma paterno afetam o perfil de sucesso ou não de uma gestação. Os dados aqui apresentados reforçam o fato de que componentes imunológicos desempenham papel fundamental no

controle da aceitação materna ao feto e agem em conjunto, numa delicada rede de interações.

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Capítulo 8 - Anexos

ANEXO 1: HLA-G – From fetal tolerance to a regulatory molecule in inflammatory diseases

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ABSTRACT

The Human Leukocyte Antigen G (HLA-G) is a non-classical class I MHC which is characterized by low polymorphism at DNA level, limited tissue distribution in non-pathological conditions and the expression of both membrane-bound and soluble isoforms by alternative splicing. This molecule has become object of interest for its possible role in pregnancy maintenance. HLA-G seems to be involved in the induction and maintenance of tolerance between the mother immune system and the semi-allogeneic fetus at the fetal-placentary interface and also seems to play an important role in embryo implantation. Besides, several studies point out to a broader immunoregulatory role for this molecule. Here we revise the potential roles of the HLA-G molecule on the immune system, the unique regulatory region of its gene, the influence of gene polymorphisms on HLA-G expression as well as the several situations in which this molecule has been involved, such as pregnancy, transplantation, cancer, viral infections and, more recently, inflammatory diseases.

Keywords: HLA-G, immune regulation, genetics, pregnancy, cancer, inflammatory diseases.

INTRODUCTION

"*Although experimental evidence is needed before a final decision can be reached regarding functionality of nonclassical class I gene products, the data presented here favor the hypothesis that nonclassical genes are largely nonfunctional and are essentially pseudogenes. It is possible, however, that certain nonclassical genes have acquired new functions different from that of classical genes."*

This passage, extracted from an article written by Hughes and Nei in 1989 [1], reflects the relative incredulity of scientists about the role of nonclassical HLA on human biology twenty years ago. At that time, there had been 31 years since the discovery of the first HLA allele, 21 years since the adoption of the HLA nomenclature and six years since the mapping of the HLA gene region at 6p21.3. The HLA classic molecules were fairly well studied and their role on tissue rejection was already well established. Since nonclassical HLA molecules did not seem to present foreign antigens and were shown to be almost monomorphic, it would be reasonable to think of them as essentially nonfunctional genes. Two years later, HLA-G, which had been firstly named 6.0 because of the fragment length in which it was found [2], had its expression detected in trophoblast [3,4] revealing the tip of the iceberg that represents the importance of this molecule on the immune system. Eight years later, the HLA-G expression was described in tumor cells [5], making evident that this molecule was not exclusively expressed during on pregnancy. Further descriptions of the expression of HLA-G in transplants and its induction on viral infections have widened even more the spectrum of situations in which this

molecule has a potential role. More recently, HLA-G research is beginning to focus on another potential area of interest, which is inflammatory diseases. Here we discuss the intriguing features of the HLA-G molecule, including the unique regulatory regions of this gene and the consequent diverse roles this molecule has in the immune system.

THE HLA-G GENE AND MOLECULE

The HLA-G gene is located at the Major Histocompatibility Complex (MHC), which comprises a collection of genes at the short arm of chromosome 6, region 6p21.3 that received this name because of its association to graft rejection. A large number of the genes within that region have been shown to have important functions in the biology of the immune system. The MHC genes represent the front end of the immune system adaptive response, since both antibody (B cell mediated) and T cell mediated immune responses are initiated through genes contained within the MHC. In humans, MHC molecules are named HLA (Human Leukocyte Antigens). The HLA molecules are typically divided in class I and II molecules. The latter are expressed mainly on antigen-presenting cells (i.e. macrophages, dendritic cells, B cells), which have the function of presenting peptides mainly derived from extracellular proteins, to the T cell receptor on CD4+ helper T cells. The class I molecules are expressed in nearly all types of cells and have the function to present short endogen (intracellular derived) peptides to CD8+ cytotoxic T cells. In humans, class I molecules can be further divided in Ia, represented by HLA-A, B and C, which are called “classical”, and Ib (nonclassical), such as HLA-E, F and G. Classical

class I molecules are characterized as highly polymorphic and act directing T cells to eliminate cells infected by viruses or other intracellular pathogens.

HLA-G is a class Ib molecule, whose structure resembles that of classic HLA class I molecules, constituted by three alpha domains non-covalently associated to a β2-microglobulin chain. Nevertheless, unlike its classic counterparts, HLA-G exhibits low polymorphism at its coding region, it presents a restricted expression pattern in healthy conditions and it possesses a unique characteristic among HLA molecules, which is to form HLA-G multimers. Also, by alternative splicing the HLA-G gene can give rise to seven different protein isoforms. All these characteristics undoubtfully contribute to the increasing interest of scientists to this molecule and some of these features have proved to influence HLA-G biological functions.

As previously mentioned, HLA-G, the most studied non-classic HLA molecule, has limited polymorphism as compared to classic HLA molecules: it possesses 36 alleles described to date, which codify 14 different proteins, as compared to 673, 1077 and 360 alleles from HLAs A, B and C (Anthony Nolan Research Institute, September 2008, http://www.anthonynolan.org.uk/research/hlainformaticsgroup). This limited polymorphism is distributed along the three alpha domains, while in classic HLA molecules, it is concentrated around the peptide binding groove. The HLA-G limited polymorphism clearly restricts the peptide repertoire capable of binding it and, indeed, it was shown that in placenta, a single peptide accounts for 15% of eluted peptides from HLA-G molecules [6]. The bound peptide sits deeper in the cleft than in classic HLA molecules, and its buried area seems to be even higher than other non-classic MHC molecules, such as HLA-E and Qa-2 (a mouse non-classic molecule) [7].

These special characteristics of the HLA-G make unlikely to this molecule to perform a role on antigen presentation. However, more studies are needed to address the real influence of the bound peptide on receptor binding.

HLA-G proteins can occur in different isoforms: four membrane-bound (G1-G4) and three secreted (G5-G7) forms have been described to date [8], and are generated by alternative splicing, which is a regulable phenomenon. According to previous studies, depending on the cell type and physiological condition, different HLA-G isoforms are produced [9-11]. All isoforms contain at least the alpha-1 domain and HLA-G1 is the complete isoform. In G5-G7 isoforms, intron 4 is not spliced out, which introduces a stop codon and thus the transmembrane and cytoplasmic domains are not translated, resulting in secretable, soluble proteins [12-16]. HLA-G also possesses a cytoplasmic tail that is shorter than those from HLA-A, B and C, due to a frameshift mutation at exon 6 [2]. This feature has important implications for HLA-G expression as it unfolds an endoplasmic reticulum (ER) retrieval motif that results in a slower turnover and prolonged expression of HLA-G at the cell surface [17] as compared to classic HLA molecules.

IMMUNOTOLEROGENIC FEATURES OF HLA-G

The description of a suppressive role

HLA-G expression was first described at the cytotrophoblast and therefore the first studies on this molecule concentrated on its role on pregnancy. During pregnancy, the maternal immune system is in close contact with cells and tissues from the semiallogeneic fetus. This suggests that specific mechanisms must be operating to modulate and moderate the maternal immune system in order to prevent the fetus rejection - i.e, promoting the acceptance of the semiallogeneic fetus. A successful pregnancy has been called by several authors as a “TH2 phenomenon”, characterized by a shifted TH2 cytokine profile. Indeed, certain complications during pregnancy, such as pre-eclampsia, have been associated with a TH1 response [18-20]. The etiology and pathogenesis of pre-eclampsia involve a combination of maternal-fetal genetic and immunological factors. Some immunological interactions can contribute for the fetus maintenance. To protect the fetus from the mother’s immune system, preventing T-cell mediated cytolysis, the cytotrophoblasts are devoid of HLA-A or B and express very low HLA-C. In addition, expression of HLA-G by these cells inhibits activation of maternal T cells, natural killer (NK) and antigen-specific CTL (cytotoxic T cell) cytolysis via specific receptors [21,22], and IL-10 secreting cells may stimulate the HLA-G expression [23]. HLA-G expression is highly tissue-restricted: besides being expressed in fetal tissues, such as trophoblast cells, HLA-G constitutive expression was found only in adult thymic medulla, cornea, pancreatic islets, and erythroid and endothelial-cell precursors. However, HLA-G expression can be induced in

situations such as transplantation, inflammatory diseases, in tumor cells, multiple sclerosis and in viral infections.

Immunoregulatory functions of HLA-G

The role of HLA-G in the maintenance of pregnancy as an immunosuppressive molecule, the various reports of its expression in different types of cancer, as well as the emphasis in recent years in analyzes of the mechanisms used by tumors to avoid the immune system recognition and destruction provided the impetus to investigate the mechanisms through which HLA-G exerts its regulatory functions. Several mechanisms have been identified (Figure 1). As it was previously mentioned that HLA-G was shown to inhibit the cytotoxic activity of CTL and NK cells [24,25]. Similarly, HLA-G is able to protect class I-negative cells or allogeneic tumors from NK-mediated anti-tumor immunity [26] and even tumor cells that express ligands for NK activator receptors such as MICA [27,28]. Also, it was shown to inhibit CD4+ T cell alloproliferative responses [29], the proliferation of T and NK cells [30-32], and also to act on APCs by inhibiting their maturation and function [33]. From now on, we will discuss some of the reasons why HLA-G seems to be capable of performing a major regulator role in on the immune system: (i) HLA-G was reported to bind many types of receptors, some of which are widely distributed among immune cells; (ii) HLA-G may exert long-term immunotolerogenic effects through the generation of suppressor cells; and (iii) even cells which do not transcribe HLA-G may temporarily become HLA-G+ acquiring a suppressive profile through intercellular uptake of HLA-G containing membrane patches, a mechanism also known as trogocytosis.

Receptors for HLA-G

HLA-G exerts its effects through binding to specific receptors present at various immune cell types (Figure 1). The leukocyte receptor complex of chromosome 19 includes two polymorphic gene families: leukocyte immunoglobulin-like receptors (LILR) and killer cell immunoglobulin-like receptors (KIR). Certain LILR and KIR molecules that are expressed at both APC and NK cells are thought to bind HLA-G. Among LILR molecules, LILR1 (ILT-2, CD85j) and LILR2 (ILT4, CD85d) are inhibitory receptors that recognize all HLA class I molecules [34]. ILT-2 is expressed by B cells, some T and NK cells, and all monocytes, whereas ILT-4 is specific of myeloid lineages. LILRB1 and LILRB2 have been convincingly shown to bind HLA-G [35-38]. Co-crystal structures show that LILRB1 binds HLA-G predominantly in the α3 domain [39]. This binding pattern may be different for LILRB2 as its binding to HLA-A (which has a similar crystal structure as HLA-G) was shown to include contacts with both the α3 domain and β2m chain [40,41].

HLA-G has been found in different conformations at the cell surface. An interesting fact that HLA-G can exist as dimers of two β2m-associated HLA-G complexes. The HLA-G homodimer is linked by disulfide bonds between cysteines located at position 42 of the HLA-G chain, at the α1 domain [42,43]. This cysteine is unique among HLA-I molecules, implying that the formation of these structures in this group of molecules is restricted to HLA-G. It has been shown that this homodimeric complex dramatically increases LILRB1 binding, with a higher affinity and slower dissociation rates than monomers. Recent data show that the ILT-2 and ILT-4 binding sites of HLA-G dimers are more

accessible than those of HLA-G monomers [44]. This increased avidity resulted in augmented signaling through LILRB1 [45] and ~100 -fold lower concentration of dimers needed to induce signaling [44].

KIR2DL4 (CD158d) is a member of the KIR gene family which is thought to bind HLA-G. It is located at the center of the KIR gene complex and is present in all KIR haplotypes. Its expression by a variety of NK subsets has been reported [46-49], although its expression in vivo has proven difficult to detect. Structural analyses indicate that this molecule can function as an activator or inhibitory molecule, and signaling assays in vitro suggested that both activities are possible [50,51]. Whether KIR2DL4 can actually bind HLA-G is still controversial. Although a number of different works have reported KIR2DL4 binding HLA-G [47,48, 52-54], either they lack replicability by other works or they have not presented the appropriate control molecules [55]. A possible explanation for these contradictory findings may be that KIR2DL4 binds HLA-G through a low affinity interaction that only takes place when the ligand is concentrated in endosomal compartments [47,55]. Although the binding site of KIR2DL4 on HLA-G remains unknown, the α1-domain residues Met76 and Gln79, both unique to HLA-G, seem to be involved. There is some evidence that KIR2DL4 binds HLA-G in a similar orientation to other KIRs and HLA-I [53]. However, this would not be possible for HLA-G dimers since the juxtaposition of the two protomers would lead to steric clashes with KIR2DL4 [56]. Nevertheless, it is possible that the interaction with this molecule is primarily the role of soluble HLA-G [47], which is predominantly monomeric [44]. There are evidences that, like classical sHLA class I antigens [57,58], sHLA-G induces apoptosis of activated CD8+ T cells and NK cells through binding to

CD8, leading to ligand (L) upregulation, soluble FasL secretion and activated CD8+ cell apoptosis by Fas/sFasL interaction [59]. However, there is conflicting data in the literature about the potency of sHLA-G in relation to classical sHLA class I molecules in triggering apoptosis through CD8 [59,60]. In fact, it is likely that sHLA-G molecules do not play a major role in physiological conditions, since their level in serum is about one order of magnitude below that required to induce CD8+ T and NK cell apoptosis in vitro [59]. Thus, the potential role of HLA-G in inducing apoptosis through CD8 would be restricted to pathological conditions associated with a marked increase in the level of sHLA-G in serum or in a given anatomic site. Also, whether sHLA-G cooperates with classical sHLA class I antigens in inducing apoptosis and whether this effect is additive or synergistic remains to be determined [61].

Other receptors have been implicated in HLA-G binding, although further studies are needed to confirm these findings. NK receptors KIR2DL1, KIR2DL2/3 and KIR3DL1 were reported as capable of binding HLA-G but these results have never been replicated [62,63]. Also, sHLA-G1 has been shown to inhibit in vitro and in vivo angiogenesis by inducing endothelial cell apoptosis upon binding to the CD160/BY55 receptor [63,64]. CD160 is expressed by endothelial cells and also T and NK cells. However, blocking with specific monoclonal antibodies to CD160 or HLA-G was not shown, and other HLA-I molecules might bind CD160 [63, 65, 55]. HLA-G also seems to have an influence on CD94/NKG2 binding to HLA-E: the affinity of both CD94/NKG2A and CD94/NKG2C for HLA-E is highest when a leader peptide derived from HLA-G is bound, as compared with a leader peptide from a classical HLA-I molecule [66]. This could be a potential mechanism of discrimination between

fetal and maternal cells by NK cells; however, the binding of HLA-E presenting the HLA-G leader peptide to decidual leukocytes expressing CD94/NKG2 receptors has not been functionally investigated [55].

HLA-G suppressor cells

There is increasing evidence that, besides its direct inhibitory effects, HLA-G may exert long-term immunotolerogenic effects through the generation of suppressor cells. Several HLA-G-related suppressor cells have been identified [reviewed in 67]. Naturally occurring regulatory T cells are present in the peripheral blood under physiological conditions. These cells can be CD4+ or CD8+ and constitutively express HLA-G1 at its surface. HLA-G1+ T cells are hyporesponsive and mediate their suppressive functions through soluble factors that include HLA-G, but not IL-10 or TGF-B. Their occurrence has been identified also in sites of inflammation [68]. HLA-G+ T cells can also be induced through allostimulation and produce soluble HLA-G5 and, at rare occasions, HLA-G1 [69,70]. Although their origin is unclear, these cells are suppressive and limit the alloproliferation of autologous CD4 T cells. HLA-G-induced regulatory T cells were first described in vitro after allogeneic stimulation by HLA-G1+ APCs. These cells were hyporesponsive and inhibit the proliferation of autologous T cells. They are not characterized by a particular phenotype and their mechanisms of action remain largely unknown but, although HLA-G is directly responsible for their induction, they do not exert their regulatory functions through HLA-G [71-73]. HLA-G-induced tolerogenic dendritic cells (DC) are DC matured in the presence of HLA-G tetramers, in which the stimulation ability is markedly reduced. Moreover, these cells are capable of

inducing the generation of CD4+C25+CTLA4+ and IL-10-producing regulatory T cells [74,75]. APCs can also express HLA-G under pathological conditions. These cells were found in transplanted tissues, in tumors, and during inflammatory diseases and viral infections [76-79]. They have been shown to block the reactivity of T cells and to induce suppressor T cells [71], and seem to have an important physiologic role in acute B-chronic lymphocytic leukemia [80]. Adult bone marrow mesenchimal stem cells (MSC) are multipotent cells capable of differentiating into several lineages and which possess strong immunomodulatory properties. Recently, it was shown that HLA-G was a key contributor to MSC immunosuppressive functions [81].

In conclusion, HLA-G-dependent regulatory cells are diverse, and given such different origins, modes of induction, phenotypes and mechanisms of action. It is very unlikely that all these cells might play the same role at the same situations.

Trogocytosis and HLA-G

Trogocytosis is a cell-to-cell contact-dependent uptake of membranes and associated molecules. Nevertheless, during trogocytosis, all molecules contained within a certain membrane area are transferred, including some that do not participate in the cell-to-cell crosstalk, and are therefore transferred nonspecifically. Most of the studies on trogocytosis was carried out on murine T cells and shows that CD4+ and CD8+ T cells can respectively acquire MHC Class II and MHC Class I molecules from antigen presenting cells (APC) in an antigen-specific manner [82-84]. Recently, trogocytosis of HLA-DR, CD80, and HLA-G1 from APC by T cells was evidenced in humans, and was shown to

follow the same rules as in the murine system [85,31,86]. Functionally, CD8+ T cells that acquired their MHC Class I ligands became susceptible to antigen-specific cytolysis [83,84]. Then, after HLA-DR and CD80 acquisition, T cells stimulated resting T cells in an antigen-specific manner, behaving as APCs themselves [85-87] whereas acquisition of HLA-G1 rendered T cells immunosuppressive [31]. This might constitute a cheap and efficient way of modulating presentation/stimulation capabilities of the immune system. It was shown that NK cells can acquire MHC Class I proteins [88-92] and viral receptors [89] from their targets.

As HLA-G is commonly expressed by tumor cells in vivo, it is interesting to investigate whether it could be acquired by NK cells, and if this acquisition is able to interfere in their function and constitutes a mechanism of immune escape. Indeed, it was demonstrated that HLA-G1 can be acquired from tumor cells by activated but not resting NK cells by trogocytosis, and this can be a mechanism of immune escape for HLA-G-negative tumor cells [31]. Almost all activated NK cells can acquire detectable levels of HLA-G1 in a few minutes by a cell-to-cell contact-dependent mechanism. Therefore, differently from HLAG1-expressing cells, NK cell-surface expression of acquired HLA-G1 is temporary, as these cells do not transcribe HLA-G. Functionally, NK cells that acquire HLA-G1 stop proliferating, are no longer cytotoxic, and behave as suppressor cells capable of inhibit cytotoxic functions of other NK cells. All these functional changes are due to acquired HLA-G1, and could be abrogated by blocking HLA-G1 or its receptor ILT2 at the NK cell surface.

HLA-G REGULATION AND POLYMORPHISMS AT REGULATORY REGIONS

Despite the efforts to clarify the mechanisms of regulation of HLA-G expression, the mechanisms underlying HLA-G expression remain largely unknown. Since the description of the differential expression of HLA-G and classic HLA molecules in trophoblast cells, a different pattern of expression control was suggested to these genes. A clue to the differences in protein expression, as compared to another HLA genes, is given by the HLA-G unique promoter region, which exhibits several differences in relation to the promoters of classic HLA genes (and also to other non-classic HLAs). The HLA-G promoter region presents many typical elements deleted or modified, rendering HLA-G expression unresponsive to classical HLA stimulator factors such as nf-kB, IRF1 and CIITA [93]. In addition, while in classic HLA genes the promoter elements are located within 220 bp upstream the ATG start codon, the regulatory elements of HLA-G are located on a region that spans ~1.5 kb upstream from the start codon [Reviewed in 94] (Figure 2). Several factors have been shown to influence HLA-G gene expression: stress [95] and treatment with leukemia inhibitory factor are able to activate the HLA-G gene [96], and IL-10 [23], IFNs [97,98], GM-CSF [99], glucocorticoids [100] and progesterone [101,102] are able to stimulate HLA-G expression.

Another striking characteristic of the HLA-G promoter is it high polymorphism. To date, more than 30 SNPs have been identified in this region (National Center for Biotechnology Information, October 2008 http://www.ncbi.nlm.nih.gov/), many of which within or very close to known transcription factor binding sites or regulatory elements [103]. Thirteen

haplotypes have been identified based on 27 polymorphisms in three different ethnic groups, and evidences for selection for maintaining two different promoter haplotype lineages, consistent with a history of balancing selection at this region, were reported. These different haplotype lineages probably present different promoter activity patterns [104]. The research group of Carole Ober assessed the influence of five promoter haplotypes on HLA-G expression through luciferase reporter assays on a cell model (JEG-3 choriocarcinoma cells), which constitutively express the molecule: they shown that a subset of promoter haplotypes associated to the allele G*010101 (G*010101b and G*010101c) presented higher promoter activity than another subset (G*010101a, G*010301 and G*010102). The enhanced expression of these alleles was mainly due to a polymorphism at position -725 (rs1233334). This site, when artificially mutated from G to a C or T, at the G*010101c haplotype, reduced its activity by 44% and 58%, respectively. Conversely, a C to G mutation at this same point, on the G*010102 allele resulted on a 37% increase on promoter expression [105]. Interestingly, the -725G allele was previously associated to recurrent miscarriage [103], contradicting results which had associated a lower HLA-G expression with miscarriage rates [106,107].

In addition to promoter polymorphisms, epigenetic factors might contribute to HLA-G transcriptional regulation. Methylation status of the HLA-G promoter has been inversely correlated to HLA-G expression [108]. Still, ovarian carcinomas presented high levels of methylation at the surrounding region of a hypoxia response element (HRE), the binding site of HIF-1, which has recently been reported as a negative regulator of HLA-G expression in human tumor cell

lines [109]. This data suggests that methylation may be a mechanism used by tumors to avoid HLA-G inactivation.

The 3' untranslated region (3'UTR) also seems to play an important role on HLA-G expression, mainly through post-transcriptional regulatory mechanisms. A 14bp insertion/deletion polymorphism located at position +2960 at exon 8 (rs16375) has attracted the attention of several scientific groups due to its potential role both on HLA-G alternative splicing and on RNA stability. It was previously shown that transcripts with the 14pb sequence (ins) could undergo an additional splicing step which removes 92 bases from the region in which this sequence is located. This deletion is thought to influence mRNA stability as the HLA-G transcripts with the 92 bases spliced out were shown to be more stable than the "complete" mRNAs in placental cells after actinomycin treatment [110]. However, in heterozygote trophoblasts the measure of mRNA originated from each allele revealed that the ins allele was less expressed than the 14bp deletion allele (del) [111], and several studies have repeatedly reported the association of the ins allele and lower soluble HLA-G levels and even the lack of detectable HLA-G expression in the plasma of homozygotes for the ins allele [112-115]. This fact is consistent if we consider that the ins allele is in linkage disequilibrium (LD) with the G*010102, whose promoter activity was reported as low, while the del allele is in LD with the G*010101 allele, in which are included the high activity promoters described above [105]. The 14 bp polymorphism is so far the most studied HLA-G polymorphism, and its implications on pregnancy and disease will be discussed later. Another interesting element at the 3'UTR is a microRNA binding site, which is a potential target for miR-148a, miR-148b and miR-152 [116]. Inside this 20 nucleotide

region lies a C/G polymorphism, at position +3142 (rs1063320). Tan and cols. performed in silico and in vitro tests which showed that the G allele favors microRNA targeting therefore favoring the repression of HLA-G expression. We hypothesized that the +3142 polymorphism is directly responsible for the regulation of the HLA-G expression at the translation level, and a co-responsible for HLA-G protein levels, together with the HLA-G promoter region [117].

HLA-G AND PREGNANCY

The human Embryo implantation

Human embryo implantation is a complex process that requires both the ability of the embryo to implant into the uterus and adequate endometrial receptivity. All evidence indicates that during this process, several embryo- and endometrium-led adaptations in the maternal immune system are needed to allow establishment of a viable pregnancy. The study of HLA-G and the observation of a predominant profile of expression in the trophoblast have opened a novel perspective in the understanding of the immunomodulation of human reproduction during healthy, pathologic pregnancies and also in the embryo implantation. The relationship between HLA-G and human embryo implantation acts as a driver in the immune tolerance of the semiallogeneic fetus by the mother. The 14 bp deletion/insertion polymorphism may influence both HLA-G isoform splicing patterns and HLA-G mRNA stability. This may alter the HLA-G function and could be of pivotal importance in certain pregnancy complications such as pre-eclampsia [118,119]. The insertion allele has been associated with lower levels of soluble HLA-G and was implicated in the

development of pre-eclampsia and recurrent abortions [118,120,121]. Differences regarding maternal allele distribution suggested that the maternal HLA-G genotype might confer risk for pre-eclampsia development in primiparous women [122]. Several studies have suggested the importance of the maternal HLA-G expression during cleavage-stage embryo development and during the course of pregnancy. Yao and cols. (2005) described a disparity between HLA-G mRNA isoforms and protein expression in embryos. They suggested that in some cleavage-stage embryos, this difference might be due to HLA-G protein remaining from maternal oocyte stores produced before embryonic genome activation. Thus, HLA-G expressed at this stage may be more a marker of oocyte rather than embryo's quality [123]. Some researchers reported that the levels of secreted soluble HLA-G protein are higher than the capacity proposed for soluble HLA-G release by the embryo, so the signals secreted by the embryos are not in the order of the magnitude of estimated HLA-G protein concentrations [124]. These findings shed some light into the contribution of the maternal HLA-G protein for a successful pregnancy. Therefore, it is important to study the hypothesis of the immune maladaptation in pre-eclampsia development, evaluating the importance of the maternal HLA-G genotype during a successful pregnancy.

Immunological Maladaptation, HLA-G and Pre-eclampsia

Pre-eclampsia (PE) is a systemic disorder of unknown origin that is characterized by abnormal vascular response to placentation, increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation cascade, and endothelial cell dysfunction [125]. The etiology and

pathogenesis of PE involves a combination of maternal–fetal genetic and immunologic factors. The disorder is heterogeneous and pathogenesis can differ in women according to the presence of different risk factors. One hypothesis for the pathogenesis of pregnancies complications such pre-eclampsia is the immunological maladaptation theory, that states a failure of the maternal immune system in tolerate the semiallogeneic fetus. Considering the immunological maladaptation hypothesis, candidate genes with immunological functions should be sought to predispose for pathologic conditions in pregnancy. HLA-G genes code for important transmembrane proteins involved in the modulation of the maternal immune system during pregnancy and therefore in the maternal acceptance of the semiallogeneic fetus. It has been shown that IL-10 [an anti-inflammatory cytokine] is able to activate HLA-G expression [23,19]. In pre-eclamptic placentas, the HLA-G extravillous cytotrophoblast invasion is reduced and this defect was associated with a lack of HLA-G expression [126-128]. Such defective HLA-G expression may contribute to the immune and vascular abnormalities associated with this pathology [126, 129]. The strong HLA-G expression by invasive trophoblasts may, in part, explain maintenance of the semi-allogeneic fetus during pregnancy. It is thought that HLA-G inhibits the activation of maternal T cells and natural killer cells resident in deciduas favoring a TH2 type cytokine response [22,129, 25]. Pathogenesis of PE in primiparous women may differ to that in women with preexisting vascular disease, multifetal gestation, diabetes mellitus, or previous pre-eclampsia events [130,131]. It was demonstrated a tendency of higher frequency of the maternal 14-bp deletion in primiparous pre-eclamptic women as compared to primiparous healthy women [122]. This can

be due to less and more unstable HLA-G molecules. The instability of the HLA-G molecule could lead to an increased pro-inflammatory profile and possible fetus rejection. In addition, pre-eclampsia is generally associated to the first pregnancy. Thus, in primiparous women, both the 14-bp deletion allele and a limited sperm exposure with the same partner before conception would induce an increased risk of pre-eclampsia [132]. However, several studies have produced conflicting results regarding the 14-bp HLA-G polymorphism and pregnancy complications.

HLA-G, TRANSPLANTATION AND ONCOLOGY

Transplantation and oncology are two particularly clear situations in which HLA-G is concerned. Since the first description of HLA-G expression by tumor cells in 1998 [5], numerous studies have been performed on more than one thousand malignant lesions, showing that although turned off in surrounding normal areas, HLA-G gene transcription and protein expression is switched on in various tumor lesions [133]. In the context of transplantation, HLA-G expression might be beneficial and promote tolerance to grafts. The expression of HLA-G was studied in more than 1000 patients after heart, [134,9,135] kidney, [136] liver, [73] and liver-kidney [73,72,9] transplantation, with those expressing HLA-G in the graft and/or at the plasma exhibiting significantly better graft acceptance. Thus, in transplanted patients, titration of HLA-G might be used as a monitoring tool to determine and follow tolerance status, which could then be used to adjust immunosuppressive therapies. In this context, patients with high HLA-G titers could be candidates for a reduction in immunosuppressive treatment, whereas HLA-G–negative (or with low levels)

patients would have a comparatively higher risk of rejection. Furthermore, HLA-G itself might be used as a therapeutic tolerogenic agent, exogenously provided to HLA-G–negative patients as complementary and/or alternative therapy [137].

In the context of oncology, studies on several malignant lesions showed that HLA-G transcription and protein expression may be switched on in tumor lesions and protect them from NK cytolysis. It was later shown that HLA-G expression by tumor lesions protected against cytolysis [138,139] correlated with malignancy in ovarian and breast carcinomas [140], as well as in melanocytic lesions [141], and with unfavorable outcome in chronic lymphocytic leukemia [80], and gastric and colorectal cancers [142]. High HLA-G plasma levels were also recently observed in patients with neuroblastoma and correlated with relapse [143]. Expression of HLA-G has been evidenced in different malignant hematopoietic diseases, but most particularly in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and B chronic lymphocytic leukemia (B-CLL).

Thus, HLA-G expression would favor tumor development by impairing antitumor immunity. In this way, HLA-G titration in peripheral blood might be used for diagnosis and/or monitoring, but in this context, high titers of HLA-G would represent a negative factor [140,80]. In HLA-G positive patients, HLA-G itself might finally constitute a therapeutic target: if expressed as a membrane-bound protein, as observed in some hematological malignancies [80], HLA-G could be used as a tumor marker to deliver therapy. Alternatively, HLA-G could be blocked or deleted as a contributor to tumor immunosuppression and/or tumoral escape [144].

Recently, new aspects of HLA-G biology have been reported that are critical to HLA-G pathologic relevance and should help design HLA-G based diagnosis and therapeutic strategies. First is the highly inhibitory function by HLA-G multimers. Second is the demonstration that HLA-G is not only a shield against immune aggression but can also have a long-term inhibitory function through regulatory cells. Third is the demonstration that HLA-G can transfer from cell to cell, cause effector cells to behave as regulatory cells, and so spread HLA-G inhibitory function beyond the reach of HLA-G– expressing cells. These data reinforce the potential clinical significance of even a few HLA-G– expressing cells [137].

Based on all of this information, HLA-G expression might constitute a target for antitumor therapy. One might interfere on HLA-G transcription, act on HLA-G alternative splicing, or block the function of HLA-G or of HLA-G-driven suppressor cells [139]. In this regard, the development of an animal model would allow the in vivo validation of the concept that the immunogenicity of HLA-G+ tumor cells might be enhanced by blocking the expression and function of HLA-G. This approach might define whether strategies aimed at blocking HLA-G by using a specific antibody affect the clinical course of HLA-G+ malignant diseases.

HLA-G AND VIRAL INFECTIONS

One of the several mechanisms adopted by viruses to evade the human immune system is the interference with the expression of HLA antigens. Normally, alterations in the self HLA cell profile are easily detected by immune surveillance and these cells are depleted. In viral infections, such as HIV, the

virus down regulate cell surface class I classical antigens (HLA-A and HLA-B) to avoid HIV-specific CTL responses, but the expression of HLA-G remains unaffected or at least not decreased. The HIV infected cells are resistant to lysis by NK cells, interaction of HLA-G1 with KIR of NK cells can inhibit the antigen-specific HLA-restricted CTL response [60]. Various NK cell receptors that recognize MHC-independent ligands can regulate important cytolytic NK functions. This immunoregulation could be achieved by increased expression of HLA-G during viral infections. When HIV Nef down regulates the surface class I antigens by interacting with their cytoplasmic domain [145], it may not be able to interact with non-classical HLA-I antigens such as HLA-G, which has a truncated domain [2]. In spite of the inability of viral Nef to downregulate HLA-G, some changes could indirectly influence the expression of HLA-G, particularly increased interleukin 10 [146]. It has been shown that this cytokine upregulates HLA-G expression [23]. In the immunoregulation of viral infections, the stage of infection has a profound effect on the microenvironment, which, in turn, could alter HLA-G expression.

Other acute viral infections such as human cytomegalovirus and herpes simplex both decrease cell surface expression of HLA-G1, but can increase HLA-G1 expression upon reactivation [147,148]. Further, HLA-G polymorphisms are also associated with the risk of HIV infection. Many studies have shown the importance of some HLA-G alleles in the development of HIV and have found significant association with protection from HIV-1 infection and impaired production of a functional HLA-G molecule [149,150,41]. Besides their immunoregulatory properties, the impact of this non-classical class I antigen HLA-G in the susceptibility to viral infections needs further investigations.

A NEW FIELD OF STUDY – INFLAMMATORY DISEASES

HLA-G expression in inflammatory diseases is a relatively recent issue concerning HLA-G research. The first studies on this area described the HLA-G expression in muscle fibers in various inflammatory myopathies [151], in atopic dermatitis [78] and psoriatic skin [152]. It was promptly proposed, based on the findings that HLA-G seems to shift T-helper responses towards a TH2-type response, [153-156] that HLA-G would act as a tissue-protective molecule in inflammatory responses and numerous studies have been conducted since then.

An interesting finding concerning inflammatory bowel disease is that HLA-G expression was observed in ulcerative colitis (UC) patients, but not in Crohn's disease (CD), which constitutes a potential differentiation factor for the two diseases [157]. Moreover, a genetic association between the 14bp del allele and UC had been described, indicating that this allele might constitute a risk factor for UC. The same study also reported an increased frequency of the allele in CD patients who underwent ileocecal resection, suggesting that HLA-G may also have a role on CD course [158]. In celiac disease, an immune-mediated disorder characterized by an immune response to ingested gluten, HLA-G expression was higher in the co-occurrence of autoimmune or genetic diseases, and also depended on the transgressions in the diet with gluten ingested, possibly indicating that the enhanced expression of sHLA-G in this disease could be related to a mechanism to try restore the tolerance process towards oral antigens [159].

HLA-G has been studied in Multiple Sclerosis [MS] in some detail. MS is considered an autoimmune inflammatory demyelinating disease of the central nervous system. HLA-G was shown to be strongly expressed at MS lesions and in areas with lymphocytic and monocytic inflammation [160]. Cerebrospinal fluid and intrathecal synthesis of sHLA-G was higher in MS than in controls. In this context, HLA-G levels directly correlated with IL-10 levels and to better prognosis markers [161,162]. Moreover, HLA-G expression was shown to be increased in patients after treatment with Interferon-β (IFN-β) the major immunomodulatory agent used in the treatment of MS. HLA-G derived from monocytes, the primary source of HLA-G in MS, was shown to inhibit both TH1 (IFN-g, IL-2) and TH2 (IL-10) cytokine production by antigen-stimulated autologous CD4 T cells [160]. Recently, it was shown a trend towards an inverse correlation between CSF concentrations of sHLA-G and sHLA-I and between CSF levels of sHLA-G and sFas in relapsing-remitting MS, suggesting that sHLA-G could play an immunomodulatory role in MS through Fas/FasL-mediated mechanisms [163]. Another study addressed the possible genetic influence of HLA-G polymorphisms on multiple sclerosis, but no significant differences were observed for the analyzed polymorphisms [164].

In Rheumatoid Arthritis (RA), lower plasma levels of HLA-G were reported. However, HLA-G levels in these patients correlated with the presence of disease-associated epitopes, which could represent a link to genetic factors or may merely be an indirect consequence of disease activity or a combination of both [165]. To date, attempts to find a genetic association between HLA-G and susceptibility to RA have failed [114,166]. However, an interesting finding concerning RA therapy is that metothrexate (MTX) was shown to induce HLA-G

expression in vitro and that the response to MTX seems to be influenced by the HLA-G 14-bp genotype, with the del/del genotype being the most favorable. Interestingly, in Juvenile Idiopathic Arthritis, a significant association between the deletion allele and disease susceptibility in girls was reported by our group [166].

An interesting work revealed evidences of the potential importance of HLA-G in controlling situations of acute inflammation: it was reported that HLA-G5 had a marked and persisting elevation in septic shock and a significantly higher concentration of this molecule was present in survivors in comparison with nonsurvivors, therefore being identified as a potential predictor of survival in this situation [167]. Other disease studies concerning HLA-G presented controversial results. In Asthma, the HLA-G gene had been marked as a susceptibility gene in four independent samples [168], but these results were not replicated by a second study [169]. Still, polymorphisms at the promoter region and also at the 3' UTR were implicated in asthma susceptibility [116]. However, further studies trying to characterize HLA-G expression produced confusing results: although HLA-G plasma levels were elevated in atopic asthma patients [170], another study reported a decreased production of HLA-G (and IL-10) by peripheral blood mononuclear cells stimulated with lipopolysaccharide, which was reversed by exogenous IL-10, indicating that a specific deficit of IL-10 secretion in patients with asthma could prevent the normal production of sHLA-G1/HLA-G5 molecules [171]. In Systemic Lupus Erythematosus (SLE), one study reported higher levels of HLA-G in the patients' plasma [172] while another study reported lower levels [115]. Also, this last study reported a genetic association with the 14-bp polymorphism, with the

ins/ins genotype as a risk factor for SLE development. However, this result was not replicated by our research group, which instead, observed an increased frequency of the heterozygous genotype, with genotypic frequencies departing from HW equilibrium [173]. It is possible that environmental and/or ethnicity-specific factors are contributing for such conflicting results.

The 14-bp polymorphism has been studied in other situations and has been associated to several diseases: the insertion allele had been observed more frequently in patients with sarcoidosis [174] and in Behcet's disease [175], while the deletion allele was reported as a risk factor for idiopathic dilated cardiomyopathy [176] and for Pemphigus vulgaris (PV) [177]. HLA-G expression at the skin of PV patients has been recently reported [178] However, in order to establish these associations, these results must be replicated in future works.

Although the number of studies in autoimmune an/or pro-inflammatory diseases is still limited, these studies put in evidence the broad spectrum of pathologic situations in which HLA-G may be involved. There are promising evidences that therapeutic strategies which include control (stimulation or inhibition) of HLA-G expression in such situations may influence disease activity and prognosis.

CONCLUSIONS AND FUTURE PERSPECTIVES

After two decades of studies, research on HLA-G has achieved a mature stage. From the status of a monomorphic molecule, whose utility in the biology of humans was neglected and even put into doubt, HLA-G has turned into an intriguing molecule with unique structural features, and there is a consistent

amount of information indicating that it is capable of exerting direct and long-lasting effects on the immune system. As we have shown throughout this review, HLA-G expression is a two-edged sword. While beneficial in pregnancy and in transplantation, its expression in tumors and viral infections would be detrimental by impairing antiviral and antitumor immunity respectively. Given the broad immunoregulatory functions of this molecule and the wide spectrum of situations in which it might be involved, it is tempting thinking of it as a potential tool for clinical purposes. This would include not only its use as a diagnostic and prognostic tool, but also as a molecular target to deliver therapy, as a therapeutic agent administered exogenously or by controlling its endogenous expression. However, some steps still must be undertaken in order that the accumulated knowledge on HLA-G reverts into clinical benefits for patients.

One important issue yet to be clarified is the role of the multiple isoforms and structures that HLA-G can form. When approaching HLA-G isoforms, the majority of the papers analyzed HLA-G1 and G5 whereas the other isoforms have been poorly assessed. Still, the most important fact that is yet to be taken into account by future studies is that multimers may be responsible for the majority of HLA-G inhibitory function. It is now clear that the relationship between HLA-G structure and its functional relevance needs to be established in vivo beyond doubt. The development of new tools for analyzing HLA-G structures will allow a reanalysis of data, which might greatly strengthen the relevance of HLA-G in disease. Also, future studies should concentrate on the investigation of different types of HLA-G-dependent suppressor cells, probably the main elements responsible for the long lasting effects of HLA-G on the immune system. Is seems unlikely that all HLA-G-dependent suppressor cells

play the same roles and are present at the same contexts; therefore, the identification of those cells and its roles at a given (pathological) context will greatly help in elucidating mechanisms of disease onset and progression, as well as the development of strategies for counteracting those mechanisms.

Another important step to accomplish is a better understanding of the HLA-G gene regulation. Although many regulatory elements have been identified at the HLA-G promoter and 3' UTR regions the relevance of these elements and possible interaction that may exist between them remains elusive. Assessing this issue will be crucial to understanding how HLA-G expression is up-regulated in certain tumors and which could be the better approaches to influence HLA-G expression according to what would be required for each situation.

Finally, there are many situations where the possible influence of HLA-G has been reported by association studies, or even by measuring its levels in blood or in situ, but there is still poor evidence of the direct influence of HLA-G. The majority of inflammatory diseases previously cited can be included as examples. For them, further studies will be needed to evaluate the real influence of HLA-G. Although the various accumulated evidences of HLA-G broad regulatory functions may have caused frenzy among scientists, it is worth to keep in mind that "HLA-G is no panacea and does not have to be correlated with everything to be useful and important in some given situations" [137].

"Increasingly complex, HLA-G? Yes, but not increasingly chaotic because the field of HLA-G research benefits from a true asset: focus. Indeed, there might be disagreements over such technical subtlety, or such particular result, but

there are few arguments over what HLA-G is, can do, and should be used for: HLA-G is at minima a tolerogenic molecule that should be used to monitor and cure. This is not bad for a molecule that barely existed 10 years ago."

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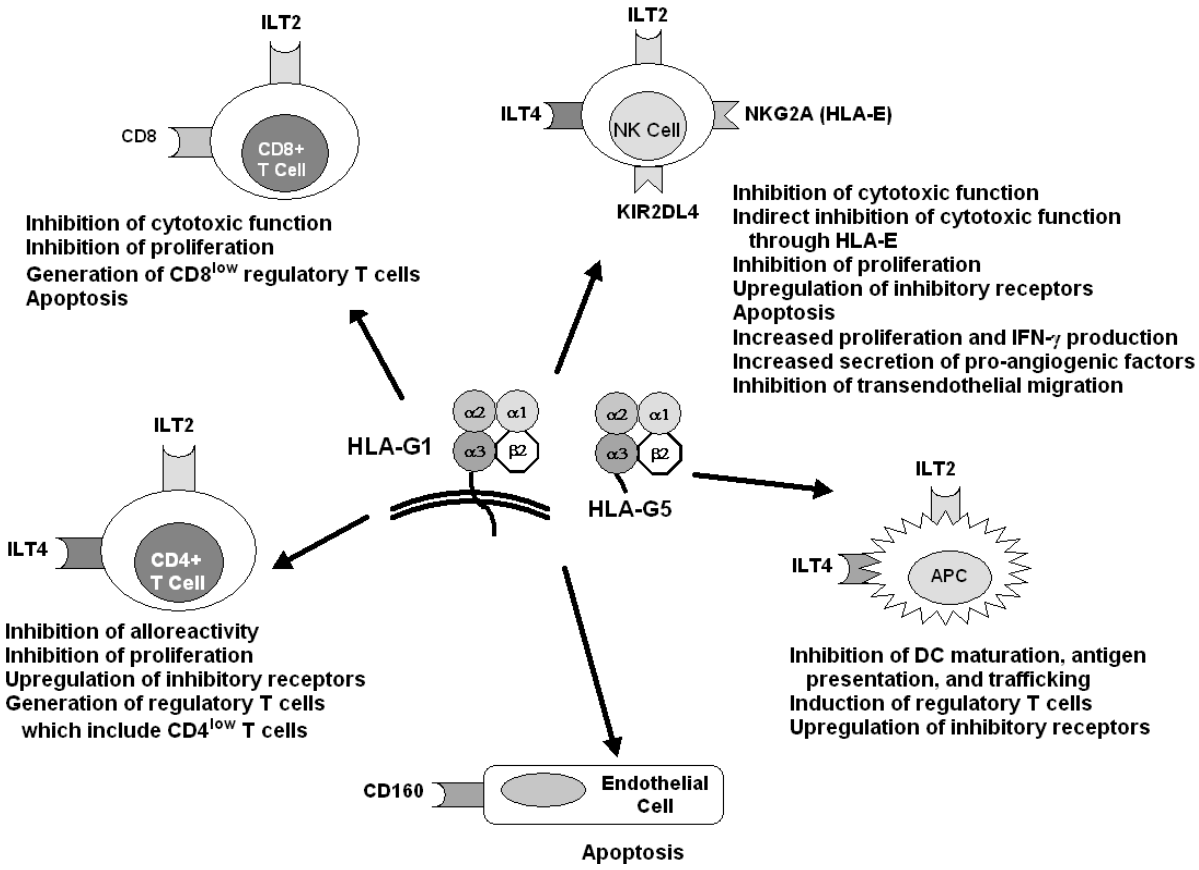

Figure 1. Immunoregulatory activities mediated by HLA-G, Target cells and receptors. Modified from (61).

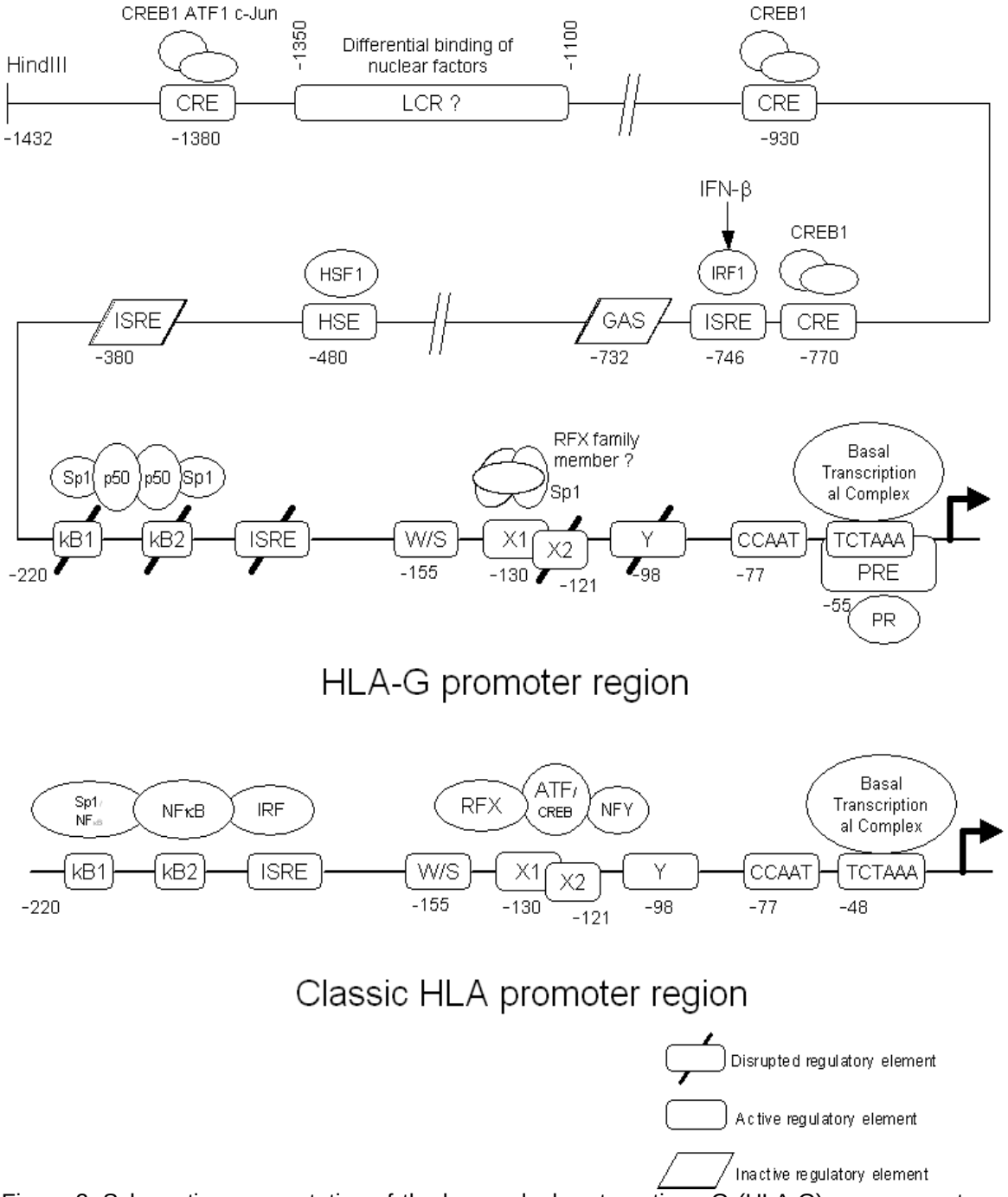

Figure 2. Schematic representation of the human leukocyte antigen-G (HLA-G) gene promoter [updated from (179)]. Numbers indicate location of regulatory boxes relative to ATG (bp). LCR ?, putative locus control region; CRE, three functional CRE/TRE sites bound by CREB1, ATF1, and c-Jun; ISRE, interferon (IFN) sequence responsive element. HSE, heat shock element that binds heat shock factor-1; GAS, non-functional IFN-gactivated site. kB2 and kB1, referred to as enhancer A within classical HLA class I promoter, are disrupted within the HLA-G promoter and display affinity for P50, a subunit of nuclear factor-kB (NF-kB). The conserved X1 half of X box associates with RFX and Sp1 in vitro;_?_indicates that RFX member factor is not yet identified. X2 and Y boxes are mutated, thus avoiding CIITA-induced trans-activation of HLA-G gene. PRE, a novel progesterone response element which is juxtaposed to the TCTAAA box and, through bindong of PR, is thought to enhance HLA-G transcription.

ANEXO 3 – Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Cara senhora,

A chance de uma mulher ganhar um bebê com saúde e levá-lo para casa depende, entre outras coisas, do meio ambiente (comida, poluição, etc.) e das características genéticas dos pais. Algumas mulheres não conseguem ter esses bebês, pois abortam. Os motivos que levam ao aborto ainda não estão muito claros. Acredita-se que mãe não consiga "segurar" a gravidez por problemas imunes e genéticos (rejeição feita pelo sistema de defesa do corpo humano).

Estamos convidando a senhora a nos ajudar a compreender esses problemas imunogenéticos, através de um estudo que estamos realizando. Para tanto, estamos solicitando a sua permissão para realizarmos colheita do seu sangue e saliva para estudar e avaliar substâncias (partículas ligadas às células do sangue) que estão relacionadas com a chance de ter um bebê. Nós iremos realizar **1 (uma) coleta de sangue.** O sangue será processado imediatamente após coleta para ser analisado e descartado logo após análise, não sendo armazenado para manipulações posteriores. Com ele, iremos fazer estudos das células do sistema de defesa da gestante. Iremos comparar os mecanismos de defesa em gestantes durante diferentes períodos da gestação. O risco associado com essa coleta de sangue é mínimo (hematoma no local onde foi inserida a agulha).

Se houver necessidade de ficar sabendo qual o é resultado do seu exame, a senhora poderá saber, quando o mesmo estiver disponível, sem penalidades ou prejuízo ao seu cuidado. O material obtido não será utilizado para fins comerciais. Fica garantido o sigilo e a privacidade das pacientes quanto aos dados confidenciais envolvidos na pesquisa. Os dados gerados serão armazenados por 5 anos e estarão à inteira disposição da senhora para acompanhá-los. A doadora tem a total liberdade de não querer entrar no estudo, ou de sair do mesmo, quando achar necessário, sem que isso traga prejuízos ao seu cuidado. Não há formas de ressarcimento ou de indenização decorrentes da participação na pesquisa.

O professor José Artur Bogo Chies e a aluna de doutorado Priscila Vianna são os biólogos responsáveis pela análise do material cedido pela paciente. **Caso haja a necessidade de maiores explicações, a senhora poderá nos conectar através do número (51) 3308-6740** (com Professor José Artur Bogo Chies), através do endereço: UFRGS, Av. Bento Gonçalves, 9500 – Campus do Vale, prédio 43.323 – sala 221 ou ainda pelos emails: priscila.vianna@ufrgs.br e jabchies@terra.com.br. "Qualquer questão ética poderei entrar em contato com a Coordenação do Comitê de Ética em Pesquisa do GHC pelo telefone (51) 3357-2407".

Este documento se encontra em duas vias de igual conteúdo e valor.

Eu, ----------, abaixo assinada, ciente dos termos acima descritos, permito a colheita de meu sangue para a pesquisa das células CD4+CD25+ e do balanço Th1/Th2 para o estudo do sucesso da minha gravidez.

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(Assinatura da doadora) (Assinatura do pesquisador)

(Assinatura Testemunha)

Porto Alegre, ---------- de ---------- de 200