

Allergic Women have Reduced sHLA-G Plasma Levels at Delivery

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Problem

HLA-G antigen maintains a tolerogenic condition at the foeto-maternal interface, counteracts inflammation in autoimmune diseases and soluble HLA-G (sHLA-G) levels decrease in allergic-asthmatics. Taking into consideration these findings, we analyzed if sHLA-G and interleukin-10 (IL-10) could be influenced by pregnancy and labour in allergic and non-allergic women.

Method of study

sHLA-G isoforms and IL-10 levels were determined in the plasma samples of 43 women (15 non-allergic, 28 allergic) during third trimester, at delivery and 2 years after pregnancy by immunoenzymatic assays.

Results

A significant increase in sHLA-G and IL-10 levels was documented at delivery in both allergic and non-allergic women. Allergic women showed lower sHLA-G concentrations. sHLA-G1 was evidenced as the predominant plasma isoform.

Conclusion

The data showed increased sHLA-G and IL-10 concentrations at delivery, regardless of the allergic status. The sHLA-G1 isoform is mainly responsible for the increased sHLA-G levels at delivery.

Introduction

In the last few years, it has been demonstrated that HLA-G antigens are likely to play a pivotal role during pregnancy.^{1,2} The HLA-G antigens are currently defined as non-classical MHC Ib antigens characterized by low allelic polymorphism and restricted tissue distribution. Furthermore, the presence of alternative mRNA splicing can generate different membrane-bound (HLA-G1, -G2, -G3, -G4) and soluble isoforms (HLA-G5, -G6, -G7).³ The expression

of HLA-G molecules was first described in extravillous cytotrophoblast cells, and it was further associated with the development and maintenance of a tolerogenic condition against the semiallogenic foetus.⁴ Later, HLA-G expression was also identified in adult thymic medulla, cornea, nail matrix, immature erythroid cells and pancreas.⁵ Several reports have confirmed the possibility of membrane and soluble HLA-G molecules (sHLA-G) in the protection of the foetus from deleterious effects of maternal immune cells. HLA-G molecules are able to induce apoptosis

in CD8⁺ T cells via a Fas-FasL-dependent mechanism,^{6,7} to inhibit natural killer cell activity⁸ and allo-specific CD4⁺ T-cell proliferation,⁹ to enhance regulatory CD4⁺ T cells¹⁰ and to inhibit the differentiation of dendritic cells.¹¹ Overall, these effects propose HLA-G molecules as efficient mediators of immunosuppressive functions against innate and adaptive immune responses.

HLA-G expression itself is also regulated by the immune system. For example, interleukin 10 (IL-10) up-regulates HLA-G surface expression on decidual stromal cells, while it is down-regulated by IL-2.¹²

sHLA-G concentrations in serum/plasma of pregnant women have been associated to clinical outcome.¹ sHLA-G levels in plasma from women who subsequently develop preeclampsia (PE) are lower than control patients, as early as in the first trimester.¹³ In comparison with non-pregnant women, sHLA-G levels strongly increase during the first trimester. Interestingly, women with significantly decreased sHLA-G levels in the second trimester have an increased risk of developing PE and/or intrauterine growth retardation (IUGR).¹³

Pregnancy is further associated with alterations in cytokine levels. We have previously demonstrated an increase in spontaneous IL-10 production during pregnancy compared with 2 years after pregnancy.¹⁴ IL-10 is one of the main enhancers of HLA-G production, with a clear feedback loop interaction between these two molecules.¹⁵

We have further demonstrated the relationship between sHLA-G and IL-10 in a study where we described a clear decrease in sHLA-G and IL-10 levels in asthmatic patients.¹⁶ This indicates that sHLA-G levels could be affected in conditions where there is an altered cytokine balance.

The aim of this study was to analyze possible differences in sHLA-G production in allergic and non-allergic pregnant women. We analyzed the levels of soluble HLA-G, the two isoforms sHLA-G1 and sHLA-G5, as well as IL-10 in plasma from 43 women (28 allergic and 15 non-allergic) at two time-points during pregnancy (third trimester, delivery) as well as 2 years after pregnancy.

Materials and methods

Study Subjects and Diagnosis of Allergy

Plasma samples were randomly selected from women participating in a larger prospective study

Table I Demographic data of allergic and non-allergic women

	Allergic women (n = 28)	Non-Allergic women (n = 15)	P-value*
Maternal age at delivery [years; mean (range)]	32 (24–44)	32 (27–40)	0.30
Gestation length [months; mean (range)]	40 (37–42)	40 (38–43)	0.58
Mode of delivery	ECS: 4/28 VD: 24/28	ECS: 2/15 VD: 13/15	0.93
Sex of the child	Girl: 14/28 Boy: 14/28	Girl: 10/15 Boy: 5/15	0.30
Number of children [mean (range)]	1 (1–4)	2 (1–3)	0.64

ECS, elective caesarean section; VD, vaginal delivery.

*Mann-Whitney sum of rank test.

on allergic heredity including 281 infants and their parents, described in detail elsewhere.¹⁴ The women were classified as allergic ($n = 28$) or non-allergic ($n = 15$) (Table I) based on their clinical history (allergic bronchial asthma and/or allergic rhinoconjunctivitis to animal dander and/or to pollen) together with skin prick test (SPT) results. The same nurse performed SPT according to the manufacturer's recommendation (ALK, Copenhagen, Denmark) against the following inhalant allergens: birch, timothy, mugwort, horse, rabbit, cat, dog and *Dermatophagoides farinae* (Soluprick 10 Histamine Equivalent Prick). Histamine chloride (10 mg/mL) served as the positive control and the allergen diluent as the negative control. The SPT was considered positive if the weal diameter after 15 min was ≥ 3 mm.

Demographic data are shown in Table I. All pregnancies were term pregnancies (>37 weeks) and all infants had birth weights within the normal range. There were no statistically significant differences regarding maternal age, mode of delivery, sex of the child, or the number of children between the two groups. The clinician (CN) who met all families both before and after the delivery recorded that the women unwillingly used inhalation steroids, antihistamines or other drugs. None of the women used systemic steroids. Approval was granted by the Human Ethics Committee at Huddinge University Hospital, Stockholm, Sweden. All families gave their informed consent to the study.

Samples

Plasma samples were obtained from peripheral blood samples collected from the same women at three time points: during the 3rd trimester of pregnancy, at delivery and at a non-pregnant state 2 years postpartum. All the samples were stored at -20°C until analysis. The samples were thawed prior to the ELISA assay, and freezing and thawing of the samples was avoided.

Cytometric Bead Array

Levels of IL-10 in plasma were measured using the Cytometric Bead Array (CBA; BD Biosciences Pharmingen, San Diego, CA, USA) technique according to the recommendations from the manufacturer. Briefly, the standards and samples were diluted in assay diluent mixed with the PE-labelled beads and incubated for 3 hr. After incubation, the samples and standards were washed to remove unbound material and analyzed using the BD CBA software (BD Biosciences Pharmingen). Calibration of the flow cytometer was performed using BD FACS-CompTM (BD Biosciences) and BD CaliBRITETM Beads (BD Biosciences Pharmingen). The sensitivity of the assay was 3.3 pg/mL.

Measurement of Total sHLA-G Levels by Enzyme-linked Immunosorbent Assay

Total sHLA-G (sHLA-G1 and HLA-G5) antigen concentrations were investigated by enzyme-linked immunosorbent assay (ELISA), as reported in the Essen Workshop on sHLA-G quantification.¹⁷ Briefly, 20 $\mu\text{g/mL}$ of MEM-G9 MoAb (Exbio, Praha, Czech Republic) was used as capture antibody and anti-beta 2 microglobulin-HRP-conjugated MoAb, (Dako, Glostrup, Denmark) as detection antibody. The concentration of sHLA-G was estimated as a mean of triplicate plasma samples by absorbance at 450 nm on a microplate reader (Wallac Victor 3; PerkinElmer, Waltham, MA, USA). HeLa cell wild-type culture supernatants were used as negative controls and transfected HeLa-G5 cells (kindly provided by Prof. E. Weiss, Institut für Anthropologie und Genetik, LMU, München, Germany) as positive controls. Culture supernatants were collected at 90% cell confluence and concentrated by the lyophilization procedure. Following depletion of albumin by the Albumin depletion kit (Enchant Life Science kit; Pall Corpora-

tion, East Hills, NY, USA), purification of the sHLA-G proteins was carried out as reported previously.¹⁸ The sHLA-G molecules obtained were used as standards at different dilutions (30, 25, 20, 15, 6, 3, 1.5, 1 ng/mL). The standard curve is shown in Fig. 1a. The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV was 4.0%. The limit of sensitivity was 1.0 ng/mL.

Measurement of HLA-G5 Levels by ELISA

Concentrations of HLA-G5 in plasma samples were quantified following the protocol proposed by Rebmann et al.¹⁷ Briefly, 20 $\mu\text{g/mL}$ of 5A6G7 MoAb (Exbio) was used as capture antibody, and biotinylated anti-HLA class I W6/32 MoAb, (Dako) and extravidin-peroxidase (Sigma-Aldrich, Milan, Italy) were used for detection. The concentration of sHLA-G was estimated as a mean of triplicate plasma samples by absorbance at 450 nm on a microplate reader (Wallac Victor 3; PerkinElmer). We used the same standards as for the total sHLA-G ELISA. The standard curve is shown in Fig. 1b. The intra-assay coefficient of variation (CV) was 2.0% and the inter-assay CV was 3.5%. The limit of sensitivity was 1.0 ng/mL.

Determination of Non-classical sHLA-G1

After ELISA measurements of plasma levels of total sHLA-G and HLA-G5, the amount of sHLA-G1 was expressed as the difference between total sHLA-G (sHLA-G1 and HLA-G5) and HLA-G5 concentrations.

Statistical Analysis

Statistical analyses were performed with STATISTICA 7.1 software (Statsoft Inc., Tulsa, OK, USA), by the Division of Mathematics at Stockholm University (Dr. Jan-Olov Persson). To analyze HLA-G variation over time, Friedman's test was used and the influence of background variables was measured by logistic regression. Fishers's exact test was used to test for differences in the proportion of HLA-G-positive individuals between the two groups. For all results, the Mann-Whitney *U*-test was used to test for differences in median values between groups. Correlations between HLA-G levels and IL-6 and IL-10, respectively, were analyzed with Spearman rank test. A difference was considered significant if $P < 0.05$.

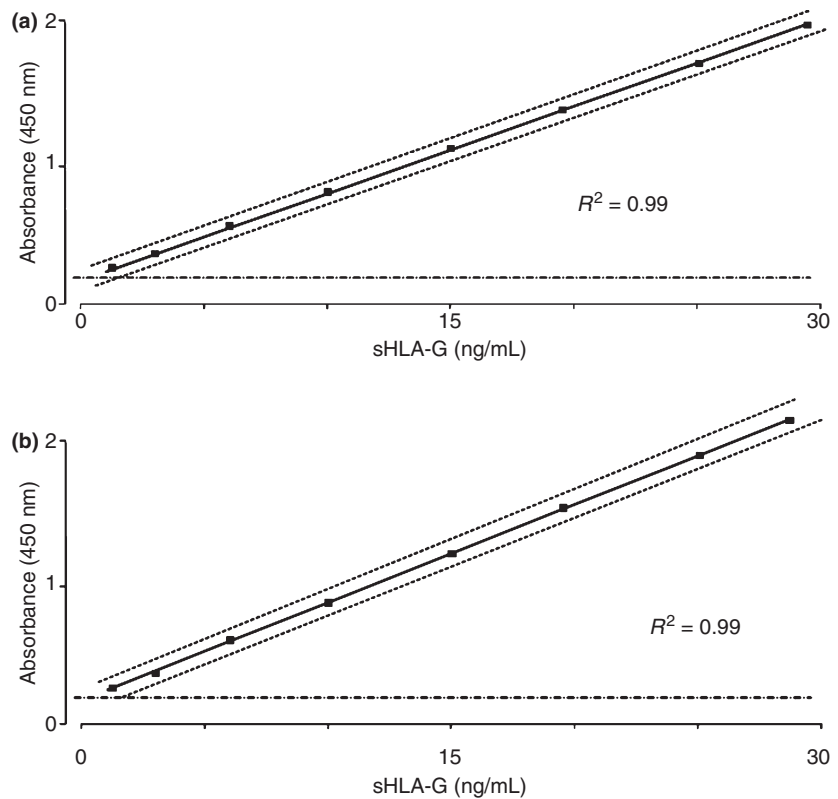


Fig. 1 Standard curve of the ELISA assays for total sHLA-G (a) and HLA-G5 isoform (b). The standard sHLA-G was used at different dilution (30, 25, 20, 15, 6, 3, 1.5, 1 ng/mL) in triplicate. The enzyme-linked immunosorbent assays were performed as described in the Materials and methods section. The detection limit, 3.29 SD added to the mean optical density of repeated negative control measurements, is reported (Dotted line). The bold line indicates the linear regression and the dashed lines the 95% confidence interval.

Results

Labour Influences sHLA-G and IL-10 Plasma Levels

To evaluate the effect of pregnancy and labour on the plasma sHLA-G levels, we measured sHLA-G in 43 women (15 non-allergic and 28 allergic) at three occasions, during the 3rd trimester, at labour and at 2 years postpartum. Soluble HLA-G plasma levels were significantly increased during labour in comparison with the third trimester and 2 years after delivery (Fig. 2a). The IL-10 plasma levels followed the same pattern (Fig. 2b).

The Spearman correlation coefficient between sHLA-G and IL-10 plasma levels was calculated for all the individuals at the different time points, but no significant correlations between these factors were observed.

Allergy is associated with reduced sHLA-G plasma levels during pregnancy and at delivery

When subdividing the women according to allergic status, plasma levels of both sHLA-G (Fig. 3a) and

IL-10 (Fig. 3b) presented the same behaviour towards labour. In both groups of women, sHLA-G (non-allergic, $P < 0.001$; allergic: $P < 0.001$, Friedman test) and IL-10 (non-allergic, $P < 0.05$; allergic, $P < 0.01$, Friedman test) levels were elevated at delivery in comparison with the third trimester and 2 years after delivery. Comparison at each time point showed that there was a significant difference in the proportion of sHLA-G-positive samples between allergic (11/28) and non-allergic women (14/15) during the 3rd trimester ($P = 0.0009$, Fisher exact test) with lower HLA-G levels in allergic women ($P < 0.01$; Mann-Whitney *U*-test). In addition, allergic women had lower sHLA-G levels (38.0 ng/mL) than non-allergic women (62.0 ng/mL) at delivery (Fig. 3a) ($P < 0.05$, Mann-Whitney *U*-test). Interestingly, no significant differences were evidenced 2 years after pregnancy ($P = \text{NS}$; Mann-Whitney *U*-test). These differences between sHLA-G levels in allergic and non-allergic women are not modified by background variables in both third trimester ($P = 0.006$, Logistic regression) and delivery ($P = 0.007$, Logistic regression).

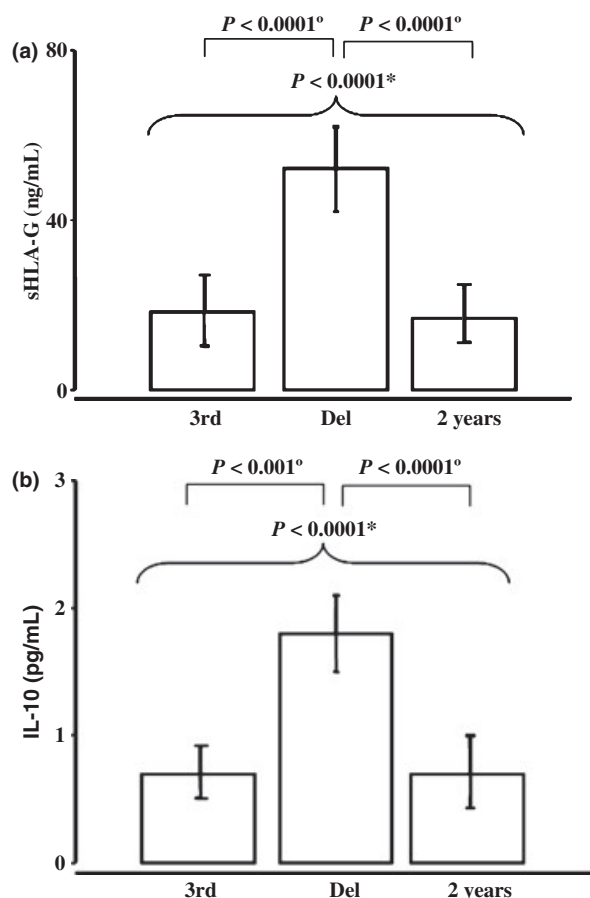


Fig. 2 Molecule levels in plasma samples from 43 pregnant women (allergic and non-allergic) at the third trimester (3rd), delivery (del) and 2 years after delivery (2 ys) measured by enzyme-linked immunosorbent assay. (a) sHLA-G; (b) IL-10. *Difference between time-points (Friedman test); $^{\circ}$ Mann-Whitney *U*-test was used to test for differences in median values between groups.

IL-10 levels increased at delivery in both allergic and non-allergic women, but no significant differences were found between the two groups (Fig. 3b).

sHLA-G Isoform Analysis

The analysis of sHLA-G isoforms (sHLA-G1, HLA-G5) demonstrated that sHLA-G1 was the predominant isoform responsible for the increase in sHLA-G levels in plasma during delivery (Fig. 4a,b).

Subdividing the women according to the allergic status revealed that sHLA-G1 increased at delivery both in allergic (from 0 ng/mL in the 3rd trimester to 36.0 ng/mL at delivery) and non-allergic (from 22.0 ng/mL in the 3rd trimester to 42.2 ng/mL at

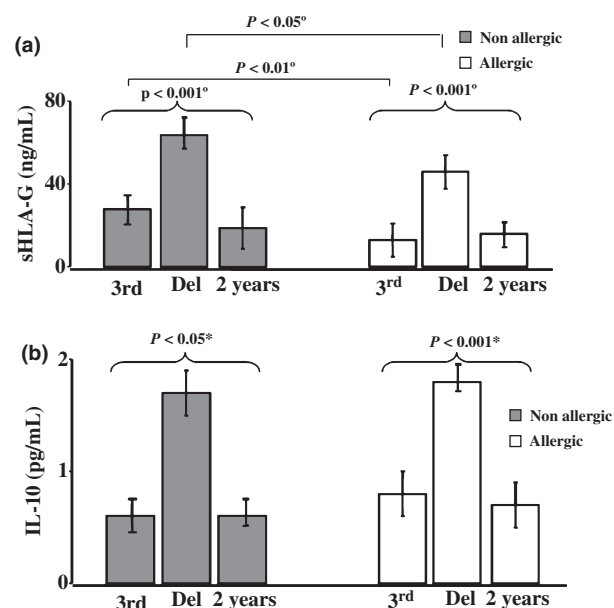


Fig. 3 Soluble HLA-G (a) and IL-10 (b) levels in plasma samples from 15 non-allergic (grey) and 28 allergic (white) women at the third trimester (3rd), delivery (del) and 2 years after delivery (2 ys) measured by enzyme-linked immunosorbent assay. *Difference between time-points (Friedman test); $^{\circ}$ Mann-Whitney *U*-test was used to test for differences in median values between groups.

delivery) women (Fig. 4a), with a clear increase during labour in comparison with the third trimester and 2 years after delivery (non-allergic, $P < 0.0001$; allergic, $P < 0.0001$, Friedman test) and higher levels in non-allergic women both in the third trimester ($P = 0.001$; Mann-Whitney *U*-test) and at delivery ($P < 0.05$; Mann-Whitney *U*-test). Moreover, there was a significant difference in the proportion of sHLA-G1-positive samples between allergic (10/28) and non-allergic (14/15) women during the third trimester ($P = 0.0003$, Fisher exact test).

HLA-G5 levels presented the same concentrations in atopic and non-atopic women at all the three time-points (Fig. 4b), with no differences between time points (non-allergic, $P = \text{NS}$; allergic, $P = \text{NS}$, Friedman test).

Discussion

We focused our study on the comparison of sHLA-G plasma concentrations at third trimester, delivery and 2 years after pregnancy in allergic and non-allergic women. The results demonstrated an impressive increase in sHLA-G molecules at delivery in

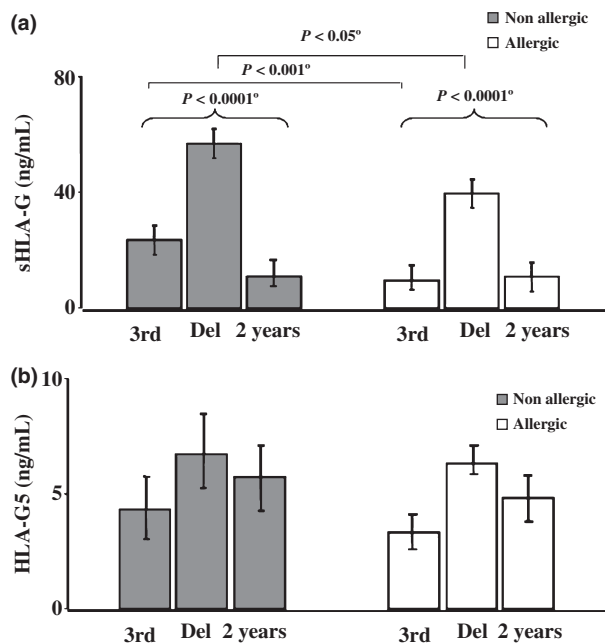


Fig. 4 Soluble HLA-G1 (a) and HLA-G5 (b) isoform levels in plasma samples from 15 non-allergic (grey) and 28 allergic (white) women at the third trimester (3rd), delivery and 2 years after delivery (2 ys) measured by enzyme-linked immunosorbent assay. *Difference between time-points (Friedman test); *Mann-Whitney *U*-test was used to test for differences in median values between groups.

both allergic and non-allergic women. Furthermore, our data revealed significantly reduced levels of sHLA-G molecules at delivery in allergic women in comparison with non-allergic women. A similar pattern was observed for plasma IL-10, with increased levels at delivery, but without any difference between allergic and non-allergic women. Our results demonstrate that the delivery process is able to affect both sHLA-G and systemic cytokine levels in both allergic and non-allergic women.

Interestingly, sHLA-G levels were lower in allergic women than in non-allergic women at delivery. The reduced sHLA-G levels were not caused by deficient IL-10 production, as allergic and non-allergic women presented equal amounts at all three time-points investigated. This indicates that other factors involved in sHLA-G production and/or regulation differ between these two groups of women. It is possible that the Th2 cytokine microenvironment present in an allergic individual¹⁹ differently influences the sHLA-G secretion. To definitely prove that allergic status affects the sHLA-G production, it should have been evaluated in these women prior to preg-

nancy also. As the women enrolled in the study while visiting the maternity ward, i.e. only during pregnancy, this was not feasible.

Reduced sHLA-G levels have been described in pregnancies complicated by PE and intra-uterine growth retardation.^{20,21} HLA-G molecules have been implicated in the regulation of uterine natural killer (uNK) cells. The uNK cells are supposed to participate in the process of placentation and being particularly important for the process of uterine spiral artery transformation. A dysregulation of uterine NK cells has been suggested in pregnancy-associated pathological conditions like PE, and modified HLA-G expression on trophoblasts and/or altered levels of sHLA-G have been implicated as possible mechanisms for this dysregulation. Soluble HLA-G may contribute locally and further away from the invading trophoblast to trigger functional maturation of the uNK cells and thereby contribute to vascular remodelling and decidualization. Soluble HLA-G levels in plasma from women who subsequently developed PE were lower than in control patients, as early as the first trimester.¹³ The reduced release of sHLA-G into the maternal circulation in PE may alter the maternal-foetal immune relationship and thus be involved in the cause of this disorder.

However, the absence of any complication in allergic women and their babies suggests a non-significant clinical influence of the observed differences in sHLA-G plasma levels in comparison with non-allergic women. It is interesting in relation to the old Th1/Th2 pregnancy dogma,²² suggesting that allergic women with their Th2 profile would have higher parity compared with non-allergic women. This is highly questionable, but the supposed Th2 profile of allergic women could perhaps compensate for the lower sHLA-G levels.

Non-allergic women have higher sHLA-G plasma levels at delivery, while 2 years after pregnancy, the two groups presented equal levels. Allergic women seem to experience a prime during pregnancy that is still evident 2 years after pregnancy. These data are in agreement with our recent study that demonstrated the presence of immunological changes imposed by pregnancy still evident 2 years after labour.¹⁶

The difference in the levels of sHLA-G between allergic and non-allergic women could be also genetically determined. The HLA-G gene is characterized by a polymorphism of deletion/insertion of 14 base pairs at the 3' untranslated region of the exon 8.²³

This polymorphism influences the stability of the HLA-G mRNA, and particularly the presence of the 14-bp sequence destabilizes the mRNA and consequently reduces the protein production.^{24,25} This insertion/deletion polymorphism is in linkage disequilibrium with particular alleles in the classical HLA loci including HLA-DR3.²⁶ Some allergic disorders may be associated with polymorphisms in the HLA region that may be in linkage disequilibrium with this polymorphism predisposing to low sHLA-G levels. Moreover, the HLA-G protein production seems to be controlled also by HLA-G gene polymorphisms at the 5' upstream regulatory region,²⁷ mutations²⁸ and microRNAs.²⁹ It would be interesting to evaluate all these mechanisms in an attempt to explain the different HLA-G production by allergic and non-allergic women.

To investigate better the nature of sHLA-G increase, we analyzed the sHLA-G1 and HLA-G5 isoforms, which represent the main components of the total sHLA-G in plasma. Our data demonstrated that sHLA-G1 molecules were the most frequent isoform in plasma (75–80%) in both allergic and non-allergic women during labour. As sHLA-G1 molecules are considered as mainly originated by metalloproteinase (MMP)-dependent shedding at post-translational level of the membrane antigens,³⁰ it could be hypothesized that sHLA-G1 could derive from the placenta disruption during labour that is characterized by an increase in MMP-9 levels.³¹

On the contrary, the production of HLA-G5 soluble isoform is mainly ascribed to splicing mechanisms at gene levels that occur in the IL-10-activated CD14⁺ peripheral blood monocyte population. IL-10 represents the main activator of CD14⁺ cells, and it is well accepted that HLA-G5 production mediated by IL-10 represents a mechanism to counteract inflammation. In our study, we observed a significant increase in plasma IL-10 levels at delivery, without any significant difference between allergic and non-allergic women. The increased levels of IL-10 at delivery could affect the HLA-G5 production, associated to an anti-inflammatory response that tries to counteract the inflammatory status of delivery.³²

In summary, our results demonstrate an increase in sHLA-G plasma concentrations at delivery compared with the 3rd trimester and 2 years after delivery. sHLA-G plasma levels in allergic women followed the same pattern as in non-allergic women with the highest levels at delivery. However, at

delivery, sHLA-G levels were lower in allergic women than in non-allergic women. The reduced sHLA-G levels were not caused by deficient IL-10 production, as allergic and non-allergic women produced equal amounts at all three time-points investigated. Further experiments are needed to understand the mechanisms responsible for the reduced production of sHLA-G in allergic women that, however in this study, did not represent a significant hazard for a normal delivery.

Acknowledgments

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