

Defective production of soluble HLA-G molecules by peripheral blood monocytes in patients with asthma

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Background: HLA-G, a human nonclassic MHC class I molecule, is responsible for complex immunoinhibitory functions. HLA-G is expressed as membrane-bound and is secreted as soluble molecules by the peripheral blood CD14⁺ monocytes activated by IL-10.

Objective: It has been reported that LPS stimulation induces IL-10 production by PBMCs and that IL-10 levels are reduced in patients with severe asthma compared with patients with mild asthma and healthy subjects. The study was designed to investigate whether this impaired IL-10 production can affect the expression and the secretion of soluble HLA-G (sHLA-G)–1/HLA-G5 molecules.

Methods: We investigated the production of sHLA-G1/HLA-G5 and IL-10 by specific ELISAs in the culture supernatants of LPS-activated PBMCs from 24 healthy subjects and 20 patients with moderate to severe persistent asthma.

Results: LPS stimulation induced the secretion of IL-10 and sHLA-G1/HLA-G5 molecules in all healthy subjects, whereas in patients with asthma, the levels of IL-10 were significantly lower ($P < .001$) and the number of cultures exhibiting detectable sHLA-G1/HLA-G5 was reduced (7/20; $P < .001$). The addition of exogenous IL-10 to LPS-stimulated PBMCs from patients with asthma restored normal sHLA-G1/HLA-G5 production.

Conclusion: Our data suggest that a specific deficit of IL-10 secretion in patients with asthma could prevent the normal production of sHLA-G1/HLA-G5 molecules. The reduction of immunosuppressive activity mediated by HLA-G could in turn contribute to the persistence of chronic airway inflammation in asthma. (*J Allergy Clin Immunol* 2005;115:508-13.)

Key words: Asthma, sHLA-G, IL-10, PBMC

Interest in HLA-G antigens has been stimulated recently by the hypothesis that they may play a protective

Abbreviations used

MANOVA: Multiple ANOVA

PBS-Tween: PBS containing 0.05% Tween 20

rIL-10: Recombinant IL-10

sHLA-G: Soluble HLA-G

role in inflammatory diseases.¹ HLA-G molecules are defined as nonclassic HLA class I antigens characterized by different isoforms, low polymorphism, and a restricted tissue distribution. Fifteen HLA-G alleles, providing 7 different transcript isoforms, have been reported; these alleles result from alternative splicing of mRNA and are expressed as membrane-bound proteins (HLA-G1, HLA-G2, HLA-G3, HLA-G4) and soluble molecules (soluble HLA [sHLA-G]–1/HLA-G5, HLA-G6, HLA-G7).²⁻⁶ The constitutive expression of HLA-G antigens in the extra-embryonic cytotrophoblast cells at the feto-maternal interface is implicated in the biological mechanisms that protect the semiallogeneic fetus in pregnancy. Membrane-bound antigens, soluble HLA-G1 molecules from proteolytic shedding, and the corresponding HLA-G5 isoform from alternative splicing have been shown to inhibit lytic activity of natural killer cells, induce apoptosis of CD8⁺ cytotoxic T lymphocytes, and affect CD4⁺ alloproliferation.⁷⁻¹⁰ After identification in cytotrophoblasts, HLA-G products were observed in physiological conditions in thymus and in PBMCs activated by IL-10 or IFNs.¹¹⁻¹³ The expression of HLA-G antigens has been recently reported in some solid tumors,¹⁴⁻¹⁶ virally infected cells,^{17,18} transplanted organs,^{19,20} and cutaneous inflammatory diseases,^{21,22} and soluble molecules have been detected in the cerebrospinal fluid of patients with multiple sclerosis.²³ These findings have increased the knowledge about the tissue distribution of HLA-G antigens and have suggested a generalized immunosuppressive function for this molecule.

It is known that monocytes play a pivotal role in inflammatory response as producers of both proinflammatory (TNF- α , IL-1) and immunosuppressive (IL-10) cytokines. Recently, PBMCs with a CD14⁺ phenotype have been identified as the predominant cell population capable of modulating the expression of sHLA-G1/HLA-G5 molecules.²⁴

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IL-10 is a pleiotropic cytokine with an important role in the regulation of the immune response, mainly by the inhibition of proinflammatory cytokines expression and the alteration of antigen presentation and T-cell activation pathways. In the delicate balance of the proinflammatory and anti-inflammatory processes, the production of sHLA-G1/HLA-G5 molecules by monocytes in response to IL-10 could be considered one of the mechanisms that counteract inflammation. It has been reported that in response to LPS stimulation, the production of IL-10 by PBMCs of patients with severe asthma is decreased compared with that in patients with mild asthma and healthy control subjects.²⁵ On this basis, by using specific ELISAs, we have investigated the production of sHLA-G1/HLA-G5 molecules and the secretion of IL-10 in the culture supernatants of LPS-activated PBMCs from 24 healthy control subjects and 20 patients with moderate to severe persistent asthma. The results obtained have confirmed a deficit in IL-10 production in patients with asthma that in turn prevented a normal production of sHLA-G antigens. However, sHLA-G impairment could be overcome by addition of exogenous IL-10 to the PBMC cultures.

METHODS

Subjects

Twenty subjects with asthma, age $54.5 \text{ years} \pm 12.5 \text{ years}$ (mean $\pm$ SD), were recruited from the outpatient clinics of the Department of Environmental Medicine and Public Health, University of Padova. The subjects included in the study had the following characteristics: (1) a typical history of bronchial asthma for at least 1 year, (2) moderate asthma or severe persistent asthma, and (3) reversibility of bronchial obstruction (increase in FEV₁ of at least 15% after inhaled salbutamol). The mean duration of asthma was $18.8 \text{ years} \pm 12.5 \text{ years}$ (mean $\pm$ SD). Ten patients were atopic; 13 were nonsmokers, and 7 were exsmokers. Classification of asthma severity was standardized according to Global Initiative for Asthma guidelines.²⁶ All patients were regularly treated with inhaled glucocorticoids (fluticasone or budesonide, 200–1600 $\mu\text{g}/\text{d}$) and long-acting inhaled bronchodilators. All patients were given inhaled β_2 -agonists as needed. Five patients with asthma needed oral prednisone (5–50 mg/d).

Twenty-four healthy subjects matched for sex and age were used as controls. Four were atopic, 4 were exsmokers, and 20 were nonsmokers. The subjects had been free of respiratory infections for at least 4 weeks. Informed consent to participate in the study was given by all subjects. The protocol was approved by the ethical committees of the University of Padova and the University of Ferrara.

Pulmonary function tests

Lung function was measured at the recruitment to the study with a dry spirometer (PFT 922; Sensor Medics, Anaheim, Calif). Pulmonary function tests included measurements of baseline FEV₁ and forced vital capacity in all subjects. Subjects were tested at least 12 hours after the last inhaled short-acting bronchodilator. Values were expressed as a percentage of predicted values.²⁷ The mean percentage of FEV₁ was 58.8 ± 3.6 (mean $\pm$ SD) and of forced vital capacity was 76.6 ± 11.2 (mean $\pm$ SD). FEV₁ increased after salbutamol by $25\% \pm 10\%$ (mean $\pm$ SD).

Skin prick tests

Atopy was assessed by skin prick tests performed with common allergen extracts, including grass and tree pollens, animal danders, molds, house dust mites, and storage mites (Lofarma, Milan, Italy). The positive control was histamine (10 mg/mL).

PBMC cultures

A time course–dose-dependence experiment with LPS-stimulated PBMCs was performed on 4 healthy subjects and 4 patients with asthma to evaluate the LPS concentration and culture time. Four different LPS concentrations (2.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL) were used to investigate IL-10 and sHLA-G1/HLA-G5 levels in culture supernatants after 6, 12, 24, and 48 hours of incubation.

PBMCs obtained by Ficoll centrifugation (Cederlane, Hornby, Ontario, Canada) were resuspended in Iscove medium (Biochrom, Berlin, Germany) plus 10% fetal calf serum at the concentration of $1 \times 10^6/\text{mL}$ and cultured for 48 hours. Activated cultures were obtained by 10 ng/mL of LPS (Calbiochem, La Jolla, Calif) while different concentrations (0 ng/mL, 2.5 ng/mL, 5 ng/mL, 7.5 ng/mL) of purified mAb anti-human IL-10²⁸ (Ebioscience, San Diego, Calif) were added for the inhibition of sHLA-G1/HLA-G5 production. To restore sHLA-G1/HLA-G5 secretion in patients with asthma, the LPS-activated cultures were supplemented with 20 ng/mL of recombinant IL-10 (rIL-10)¹² (PeproTech Inc, Rocky Hill, NJ).

CD14⁺ cell viability and percentages were evaluated by cytofluorimetry (FACS Vantage; Becton Dickinson) by using propidium-iodide staining and an anti-human CD14–fluorescein isothiocyanate–conjugated antibody (Cymbus Biotechnology Ltd, Hants, United Kingdom).

sHLA-G1/HLA-G5 levels

sHLA-G1/HLA-G5 levels in culture supernatants were assayed in triplicate as previously reported.²⁹ Briefly, 96-microwell plates (Nunc-Immuno Plate PolySorp, Nunc, Wiesbaden, Germany) were coated with the mAb MEM-G9 (Exbio, Praha, Czech Republic), which recognizes HLA-G molecule, in β_2 -microglobulin–associated form (soluble HLA-G1 and HLA-G5 isoform) at the concentration of 20 $\mu\text{g}/\text{mL}$ in 0.1 mol/L carbonate buffer, pH 9.5, for 1 hour at 37°C and then overnight at 4°C. After 3 washes with PBS containing 0.05% Tween 20 (PBS-Tween), plates were saturated with 100 μL PBS containing 4% BSA overnight at 4°C. Undiluted supernatant samples were added to each well (50 μL) in triplicate. After incubation for 2 hours at 37°C, plates were washed 3 times with PBS-Tween and incubated with 50 μL biotinylated mAb W6/32, which recognizes a framework determinant expressed on β_2 -microglobulin–associated HLA class I heavy chain (ATCC, Rockville, Md), for 1 hour at 37°C. Biotinylation of W6/32 mAb was obtained by using the EZ-Link Sulfo-NHS-LC Biotinylation Kit (Pierce, Rockford, Ill). After 5 washes with PBS-Tween, 100 μL extravidine-peroxidase (Sigma-Aldrich, Milano, Italy) was added, and the plates were incubated for 15 minutes at 37°C. Then, after 5 washes with PBS-Tween, 100 μL o-phenyldiamine peroxidase substrate (Sigma-Aldrich) was added to each well and incubated for 15 minutes at room temperature. The concentration of sHLA-G was estimated by absorbance at 405 nm on a ELISA Microplate Reader 400 (Packard, Meridian, Conn). The standard calibration curves were generated with supernatants from the HLA-G transfected LCL 721.221, purified by affinity chromatography with the anti-HLA class I mAb W6/32. The limit of sensitivity was 1 ng/mL.

IL-10 concentrations

IL-10 concentrations were determined in triplicate of undiluted samples by using the commercially available Human IL-10 Bio-

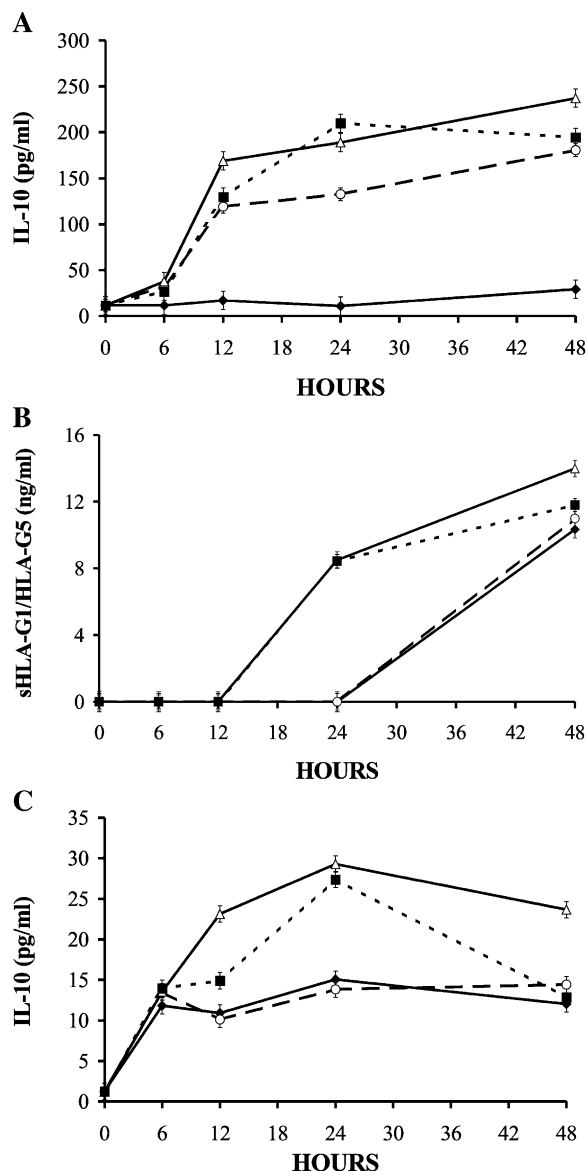


FIG 1. Time course-dose-dependence experiments with LPS-stimulated PBMCs. **(A)** IL-10 and **(B)** sHLA-G1/HLA-G5 levels (mean $\pm$ SD) in 4 healthy subjects; **(C)** IL-10 levels (mean $\pm$ SD) in 4 patients with asthma plotted against hours of incubation. LPS concentrations: (♦) 2.5 ng/mL; (○) 5 ng/mL; (Δ) 10 ng/mL; (■) 20 ng/mL.

Source Immunoassay Kit (Human IL-10 US; BioSource, Camarillo, Calif) with a detection limit of 0.2 pg/mL.

Statistics

Statistical analysis was conducted by using SPSS-X (SPSS Inc, Chicago, Ill) and Stata 8.0 (Stata Corp, College Station, Tex) packages. Normality of continuous numeric data was checked by using the Kolmogorov-Smirnoff 1-sample test, and comparisons between variables were performed by Student *t* test (for paired and independent samples) or by Mann-Whitney *U* test when appropriate. Categorical data were examined by χ^2 tests. A multiple ANOVA (MANOVA) model to evaluate the simultaneous effect of continuous

TABLE I. CD14⁺ percentages and IL-10 and sHLA-G1/HLA-G5 concentrations in the supernatants of unstimulated and LPS-stimulated PBMC cultures from 24 healthy subjects

CD14 ⁺ (%)	Unstimulated		LPS-stimulated	
	IL-10 (pg/mL)	sHLA-G (ng/mL)	IL-10 (pg/mL)	sHLA-G (ng/mL)
7.5	66.3	—	580.0	1.9
15.1	50.0	—	500.0	1.4
7.5	46.7	—	236.9	5.2
11.0	3.9	—	73.2	7.6
11.4	25.2	—	234.6	2.4
13.3	42.8	—	205.8	1.2
6.6	29.9	—	7.7	6.5
11.9	8.3	—	122.9	1.1
9.7	104.2	—	124.6	1.1
12.3	1.6	—	91.8	1.6
10.7	7.4	—	174.9	2.4
12.5	1.6	—	159.4	2.7
6.9	3.6	—	86.2	1.5
4.0	5.3	—	75.0	6.5
11.3	2.8	—	78.2	1.4
6.9	2.4	—	62.4	1.4
6.7	26.9	—	54.2	5.4
5.3	1.7	—	104.7	1.3
5.3	10.3	—	213.3	2.9
10.4	27.7	—	66.1	5.4
9.6*	3.5	—	90.0	1.8
10.9*	4.0	—	63.0	0.9
10.6*	20.0	—	91.0	0.9
9.8*	10.0	—	100.0	3.9

*Atopic.

variables (smoking, atopy, CD14⁺ cell percentage, IL-10 levels in unstimulated cultures, IL-10 and sHLA-G1/HLA-G5 levels in LPS-stimulated supernatants) in the comparison of subjects with asthma versus healthy subjects was also performed. Statistical significance was assumed for *P* < .05 (2-tailed).

RESULTS

On the basis of the data obtained by the time course-dose-dependence experiments reported in Fig 1 and the published study by Tomita et al,²⁵ the LPS concentration of 10 ng/mL with 48 hours of incubation was the optimal stimulus to express sHLA-G1/HLA-G5 in PBMC cultures. LPS induces dose-dependent IL-10 secretion, which precedes the release of sHLA-G1/HLA-G5 in healthy control subjects by at least 12 hours (Fig 1, A and B). The same kinetics of IL-10 production are evident in the patients with asthma tested, but the amount of IL-10 is reduced with an average percentage of 76.3% (Fig 1, C) between 6 and 48 hours at the 10 ng/mL LPS dose. No production of sHLA-G1/HLA-G5 molecules was detectable in supernatants of LPS-stimulated PBMCs from subjects with asthma.

Tables I and II show that LPS stimulation induced the secretion of IL-10 and sHLA-G1/HLA-G5 molecules

TABLE II. CD14⁺ percentages and IL-10 and sHLA-G1/ HLA-G5 concentrations in the supernatants of unstimulated and LPS-stimulated PBMC cultures from 20 patients with asthma

Disease severity	CD14 ⁺ (%)	Unstimulated		LPS-stimulated		LPS + IL-10
		IL-10 (pg/mL)	sHLA-G (ng/mL)	IL-10 (pg/mL)	sHLA-G (ng/mL)	sHLA-G (ng/mL)
Moderate*	20.5	5.9	—	28.0	—	9.2
Moderate	11.0	15.15	—	22.8	—	3.7
Moderate	2.8	4.9	—	16.0	3.5	4.7
Moderate*	5.4	15.3	—	31.1	—	3.3
Moderate*	5.8	8.7	—	23.9	—	8.8
Moderate	13.8	5.1	—	70.0	—	5.4
Severe	7.5	9.0	—	29.1	—	18.5
Severe*	5.7	6.1	—	35.1	1.7	16.0
Severe	16.7	26.3	—	27.4	—	18.5
Severe*	11.3	10.7	—	79.2	8.6	18.5
Severe*	17.7	9.7	—	63.4	—	30.0
Severe*	15.7	8.5	—	29.2	—	9.2
Severe*	10.5	24.0	—	64.9	4.4	4.8
Severe	18.9	69.0	—	28.4	1.2	3.5
Severe	5.5	14.0	—	51.0	—	9.0
Severe*	6.1	5.4	—	65.0	8.8	9.2
Severe*	3.5	4.7	—	22.5	—	7.6
Severe	12.5	10.4	—	45.6	5.4	5.7
Severe	7.2	2.9	—	20.1	—	3.6
Severe	10.3	5.3	—	58.6	—	11.5

*Atopic.

TABLE III. Comparison between CD14⁺ percentages and IL-10 and sHLA-G1/ HLA-G5 concentrations in the supernatants of unstimulated and LPS-stimulated PBMC cultures from 24 healthy subjects and 20 patients with asthma

	Patients with asthma, value	Healthy subjects, value	P
CD14 ⁺ , %	10.4 ± 5.4 (mean ± SD)	9.5 ± 2.8 (mean ± SD)	.455*
IL-10, pg/mL (unstimulated)	8.9/2.9-69.0 (median/range)	9.2/1.6-104.2 (median/range)	.944‡
sHLA-G, ng/mL (unstimulated)	0	0	
IL-10, pg/mL (LPS-stimulated)	30.1/16.0-79.2 (median/range)	95.9/7.7-580.0 (median/range)	<.001‡
sHLA-G-producing (LPS-stimulated)	7/20 (sHLA-G-positive)	24/24 (sHLA-G-positive)	<.001†
sHLA-G, ng/mL (LPS-stimulated)	1.7 ± 2.9 (mean ± SD)	2.8 ± 2.1 (mean ± SD)	.134*
sHLA-G, ng/mL (LPS-stimulated)	10.0 ± 7.0 (mean ± SD) (+ rIL-10)	2.8 ± 2.1 (mean ± SD)	<.001*

*Student *t* test.

† χ^2 Test.

‡Mann-Whitney *U* test.

in all 24 healthy control subjects. Addition of antihuman IL-10 antibody (7.5 ng/mL) to healthy subjects' PBMC cultures completely inhibited the sHLA-G1/HLA-G5 secretion (data not shown) and confirmed the pivotal role of IL-10 in inducing sHLA-G expression. LPS-stimulated PBMC cultures from patients with asthma exhibited lower levels of IL-10 ($P < .001$; Mann-Whitney *U* test) and a reduced number of sHLA-G1/HLA-G5-positive culture supernatants (7/20; $P < .001$; χ^2 test) compared with cultures from healthy subjects (Table III). To control for possible effects of confounding variables, the comparison between healthy subjects and patients with asthma was performed by a MANOVA. The evaluation of simultaneous effects of smoking, atopy, the percentage of CD14⁺ cells, IL-10 levels in unstimulated cultures, and IL-10 and sHLA-G1/HLA-G5 levels in LPS-stimulated supernatants indicated that IL-10 level in LPS-stimulated

cultures was the only variable significantly different between the 2 groups of subjects ($P = 0.0068$; MANOVA test) and confirmed the deficit of IL-10 secretion in patients with asthma. The concentration of sHLA-G1/HLA-G5 molecules in LPS-activated culture supernatants of the 7 patients with asthma in which sHLA-G was detectable was not statistically different ($4.8 \text{ ng/mL} \pm 3.0 \text{ ng/mL}$; mean $\pm$ SD) from that of healthy subjects ($2.8 \text{ ng/mL} \pm 2.1 \text{ ng/mL}$; $P = .0592$; Student *t* test). Furthermore, the concentrations of IL-10 in the 13 sHLA-G-negative supernatants from patients with asthma (45.6 pg/mL ; median) tended to be higher compared with the 7 sHLA-G-positive (27.4 pg/mL) supernatants ($P = .1217$; Mann-Whitney *U* test). No correlations were observed in patients with asthma between the disease severity and sHLA-G1/HLA-G5 levels (severe, $2.2 \text{ ng/mL} \pm 3.3 \text{ ng/mL}$; moderate, $0.6 \text{ ng/mL} \pm 1.4 \text{ ng/mL}$; $P = .2803$; Student

t test) and IL-10 production (severe, 40.3 pg/mL; moderate, 21.0 pg/mL; $P = .1869$; Mann-Whitney *U* test) in LPS-activated cultures. The addition of exogenous rIL-10 to the asthmatic cell cultures restored sHLA-G1/HLA-G5 production in all of the samples (Table II) with an increase in sHLA-G1/HLA-G5 levels compared with healthy subjects ($P < .001$; Student *t* test).

DISCUSSION

Impaired production of IL-10 in LPS-activated PBMC cultures for patients with asthma has been reported previously. We have investigated the possible effect of this impairment on sHLA-G molecule production. Our results have confirmed a generalized deficit in IL-10 production by patients with asthma that in turn prevented a large percentage of subjects (13/20) from secreting detectable levels of sHLA-G1/HLA-G5. Interestingly, the observed mean concentration of sHLA-G1/HLA-G5 molecules in the 7 positive asthmatic supernatants did not differ from that of healthy subjects. Furthermore, an increase in IL-10 concentration, although not significant, was seen in the 13 sHLA-G-negative supernatants from patients with asthma compared with the 7 sHLA-G-positive ones. These data could suggest a possible heterogeneity in the expression of sHLA-G in patients with asthma, but further investigations are required to confirm this hypothesis. To elucidate the precise mechanism responsible for the lack of sHLA-G expression, we cultured PBMCs from patients with asthma in the presence of exogenous rIL-10. Preliminary time course-dose-dependence experiments in healthy subjects showed that IL-10 secretion preceded sHLA-G1/HLA-G5 production and that the addition of anti-IL-10 antibody completely inhibited sHLA-G1/HLA-G5 production. The addition of exogenous rIL-10 to the LPS-activated PBMCs of patients with asthma restored normal sHLA-G1/HLA-G5 production in all subjects, confirming the impairment of IL-10 as the responsible mechanism of the defect. These data indicate that IL-10 secretion was mandatory for the secretion of soluble HLA-G antigens by LPS-stimulated CD14⁺ cells. Because no significant differences were found between the percentage of CD14⁺ cells in cultures from healthy subjects and patients with asthma, we suggest that the differences observed are not related to the percentage of monocytes but rather to their functionality. It is known that the quantitative secretion of IL-10 is under genetic control on the basis of 3 single base change polymorphisms in the promoter sequence.³⁰⁻³² However, similar frequencies of the IL-10 haplotypes have been found in patients with asthma and healthy subjects, indicating that the polymorphism is not primarily related to asthma susceptibility.³³ Furthermore, the results obtained by adding rIL-10 excluded that the lack of sHLA-G production in patients with asthma was related to specific HLA-G polymorphisms in the 3' untranslated region and 5' upstream regulatory region of the gene that seem to regulate the expression of sHLA-G molecules.^{34,35} A

therapeutic role for IL-10 has been proposed because of its anti-inflammatory properties, but controversial results have been reported on its efficacy in the treatment of asthma. However, it has been suggested that IL-10 could play a fundamental role in the polarization of the T_H2 response in pregnancy and dendritic cell maturation³⁶⁻⁴¹ and, consequently, in the induction of antigen-specific tolerance. This could explain the beneficial effects obtained by directly administering IL-10 or inducing the upregulation of the cytokine level in animal models.⁴²⁻⁴⁴ Our data suggest that in healthy subjects, this tolerance could be partly mediated by the monocytes' production of sHLA-G1/HLA-G5 molecules in response to IL-10 induced by inflammatory stimulus. The specific deficit of cytokine secretion observed in patients with asthma⁴⁵⁻⁴⁷ could prevent the normal production of sHLA-G1/HLA-G5 antigens, reducing their immunosuppressive functions. Moreover, it has been reported that in pregnancy, the levels of sHLA-G influence CD14⁺ polarization: high levels of sHLA-G induce an anti-inflammatory T_H2 cytokine response, whereas low levels of sHLA-G enhance a proinflammatory T_H1 response.^{48,49}

Although further investigations are required to define better the mechanism of the impaired production of sHLA-G1/HLA-G5 molecules observed in patients with asthma, this defect could mediate an imbalance in the pro- and anti-inflammatory processes that contribute to the severity and persistence of the asthmatic condition.

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