

## Evaluation of Soluble Human Leukocyte Antigen-G (sHLA-G) Isoforms and Regulatory T Cells in Relapsing-Remitting Multiple Sclerosis

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Received: 10 May 2014; Received in revised form: 1 July 2014; Accepted: 14 July 2014

### ABSTRACT

Soluble forms of nonclassical human leukocyte antigen (HLA)-G have recently been suggested as immunomodulatory factors in multiple sclerosis (MS). HLA-G inhibits the effector function of T cells and natural killer (NK) cells. Also regulatory T cells (Treg) are considered as pivotal players in MS pathogenesis. Thus, we aimed to evaluate the presence of HLA-G molecules and Treg cells in Relapsing-Remitting Multiple Sclerosis (RRMS) patients and compare it to healthy controls.

Patients with RRMS (n=205, mean age=31.32±8.53) and healthy subjects (n=205, mean age=32.2±7.48) were studied. The patients subgrouped to untreated and treated with Interferon beta. Then sHLA-G levels (sHLA-G1 and sHLA-G5) were measured using ELISA method. Treg (CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup>) cells in patients who had sHLA-G>10 U/ml were characterized by using flow cytometry.

Our data showed that there was no significant differences between RRMS patients and healthy controls in sHLA-G concentration ( $p>0.05$ ). Treg cell frequencies were higher in the patients who had sHLA-G >10 U/ml compared to healthy subjects ( $p<0.05$ ).

Collectively, there was significant correlation between sHLA-G and frequency of Treg cells in treated RRMS patients and healthy individuals. It seems that high level sHLA-G has been instrumental in raising frequency of Treg cells in treated patients and could be associated with remission of MS disease.

**Keywords:** Multiple sclerosis; Regulatory T cells; sHLA-G

### INTRODUCTION

Multiple sclerosis (MS) is one of the most

important forms of the central nervous system (CNS) autoimmune inflammatory disorder. T cells play a pivotal role in development of complex events of MS. Currently, genetic, environmental factors and immune

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## Soluble HLA-G Isoforms and Regulatory T Cells in Multiple Sclerosis

deregulation have been identified as major factors in the pathogenesis of MS. The key adventure is breakage of immune tolerance due to CNS self-antigens in genetically predisposed individuals.<sup>1-3</sup> Through this breakage myelin damage is triggered by the activation of infiltrating autoreactive T helper cells (Th1). The predominance of Th1 cells and their related cytokines in relapse events of MS is in parallel with insufficiently counterbalanced Th2 cells. Indeed, dominance of Th2 cells and their cytokines are seen in the stable phase of disease.<sup>4</sup> Classically, MS is categorized in three distinct subtypes of relapsing remitting (RR), secondary progressive (SP), and primary progressive (PP).<sup>5</sup> Relapse is associated with a sudden worsening of symptoms that lasts 24 hours or develops new neurological signs in a month.<sup>6</sup> Among the influencing factors of the disease, human leukocyte antigen G (HLA-G) is considered as one of the basic immune molecules. HLA-G has a limited polymorphism.<sup>7</sup> Messenger RNA of HLA-G molecule endures alternative splicing which leads to the formation of four membrane-anchored (HLA-G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub> and G<sub>4</sub>) and three secreted (HLA-G<sub>5</sub>, G<sub>6</sub>, and G<sub>7</sub>) molecule structures.<sup>8,9</sup> HLA-G1 molecules can interact with T cells, B cells, natural killer (NK) cells, and Antigen Presenting cells (APC) directly by their receptors and employ its immunotolerant tasks at various stages of the immune response.<sup>10</sup> Major isoforms of sHLA-G antigen are HLA-G<sub>1</sub> and HLA-G<sub>5</sub> and Soluble HLA-G (sHLA-G) molecules are derived from shedding of membrane-bound HLA-G<sub>1</sub> and secretion of HLA-G<sub>5</sub> isoforms.<sup>11</sup> The immunosuppressive activities of these HLA-G antigens are mediated through binding to their receptors.<sup>12</sup> It has been demonstrated that peripheral blood monocytes are major cells secreting HLA-G<sub>5</sub> antigens.<sup>13</sup> HLA-G antigen, in both soluble and membrane-anchored forms exhibit effector functions on T cells and NK cells through interaction with immunoglobulin-like transcript (ILT) receptors as ILT2 (LILRB1/CD85j), ILT4 (LILRB2/CD85d), KIR2DL4 (CD158d) and CD8a-chain.<sup>14-18</sup> Since anti-inflammatory cytokines such as interleukin-10 (IL-10) enhance HLA-G expression and consequently up-regulate IL-10 secretion, HLA-G antigens are suggested to act as immunomodulatory molecules by induction of an immune deviation from Th1 to Th2 polarized responses.<sup>19</sup> Regarding MS, the expression of HLA-G antigens is shown on the microglia, macrophages and endothelial cells within and in the

lesions. In microglial cells of MS lesions, HLA-G expression was seen to be enhanced by Th1 proinflammatory cytokines. In addition, low levels of sHLA-G is associated with enhanced risk of autoimmunity.<sup>10</sup>

Besides, regulatory T cells (Treg) play a pivotal role in the pathogenesis of MS and its different disease courses, disease activity and response to various treatment modalities.<sup>20</sup> Treg cells (CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup>) are an essential component of the immune response to induce peripheral tolerance and also play an important role in balance between pro and anti-inflammatory responses.<sup>21</sup> Both sHLA-G and Treg cells have modulator properties in the immune system. Therefore, the aim of this study was to assess whether serum sHLA-G isoforms were related to the frequency of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> (Treg) cells in RRMS patients and healthy controls.

## MATERIALS AND METHODS

### Patients

A total of 205 patients (mean age=31.32±8.53) who were referred to MS clinic with the diagnosis of definite RRMS in stable phase according to the classification of McDonald (2001) were enrolled in this study. All patients were undergoing Expanded Disability Status Scale (EDSS) assessment and brain Magnetic resonance imaging (MRI) on a regular basis and they also completed informed consent form. All RRMS patients were in clinical remission at the time of blood collection. Seventy three patients were untreated (no immunomodulatory drugs), whereas the other patients were under treatment with interferon-β1a (IFN-β). Healthy individuals (n=205, mean age=32.2±9.48) of blood donors (control groups) were also included in this research. This study was approved by the ethical committee of Tarbiat Modares University that it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

### sHLA-G Assay

Firstly peripheral blood samples were allowed to clot and then were centrifuged (for 10 min, 2400 x g). Serums were frozen immediately at -20°C. Then, levels of sHLA-G (sHLA-G1 and sHLA-G5) were determined using a commercial ELISA kit (Glory Science, USA). All sample tests were performed in duplicates. The Optical density (OD) of samples was measured at 450

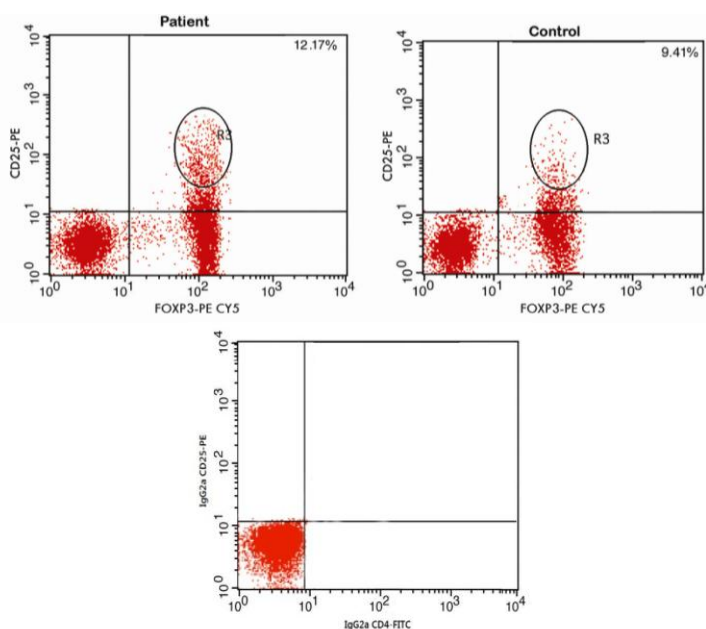
nm by Dynatech plate reader. The quantity of sHLA-G was determined by constructing a calibration curve of HLA-G1 and sHLA-G5.

#### CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg Cell Isolation

Peripheral blood samples from 21 patients and 7 healthy individuals who had sHLA G $\geq$ 10 U/ml (out of 205 participants) were screened for the presence of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg cells by flow cytometry. Since, CD4<sup>+</sup> T cells that co-express high amounts of CD25 exert regulatory functions, Treg counts were characterized as the frequency of FITC-CD4 positive cells with the highest PE-CD25 and PE-CY5-FOXP3 fluorescence intensity. Three-color flow cytometry was used to identify and quantify CD4<sup>+</sup>CD25<sup>high</sup> Foxp3<sup>+</sup> Treg cells in patients treated with IFN- $\beta$  vs. healthy controls (Dot plots one of the RRMS patients, a control

group and isotype control are shown in Figure 1. Cell viability was assessed using Trepan blue exclusion more than 95%.

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated using Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich). Treg cells were stained using human Treg cell kit (anti-human FITC-CD4, PE-CD25, PE-CY5-FOXP3, FITC-IgG2a and PE-IgG2a. BD eBioscience, Belgium) PBMCs were stained (30 min ,4°C) washed and then analyzed using flow cytometry (FACS Calibur ,BD Biosciences) for phenotypic analysis of cells. In order to stain intracellular compartment of FOXP3, cells were fixed and permeabilized before adding anti-human FOXP3 (PE-CY5-clone PCH101, eBioscience) according to the protocol. Data analyses were performed using Cell Quest software (10.5.3) (BD Biosciences).



**Figure 1. A representative plot of CD25<sup>hi</sup> Foxp3<sup>+</sup> Treg cells in a patient and control.**

The x-axis of each dot plots represents specific fluorescence of PE-CY5-Foxp3<sup>+</sup>; the y-axis represents specific fluorescence of PE-CD25<sup>+</sup>. Quadrants were set using appropriate isotype controls for each intra- and extracellular antibody. Peripheral Tregs were identified as CD25<sup>hi</sup> FOXP3<sup>+</sup> cells in CD4<sup>+</sup> lymphocytes gate.

#### Statistical Analysis

We used ANOVA test for comparison of differences of sHLA-G levels among three groups and effect of age on sHLA-G level. Kruskal-Wallis test was used to evaluate the effect of age on the Treg frequency and comparison of differences of Treg cells among the three groups. We also applied Mann-Whitney test for

comparison of Treg frequency and Tukey test for comparison of sHLA-G levels between the two groups. Moreover, data were assessed for the associations of age, sHLA-G and Treg values using Pearson's correlation coefficient. The SPSS software (version 17) was used to calculate statistical significance. Differences were considered significant when  $p < 0.05$ .

## Soluble HLA-G Isoforms and Regulatory T Cells in Multiple Sclerosis

### RESULTS

Demographic characteristics is shown in Table 1. Laboratory Findings include the mean value of sHLA-G and CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg cells based on gender per group, Relationship between the duration of remission after treatment with sHLA-G levels and frequency of Treg cells, correlation between sHLA-G and Treg cells with age of patients and control group which are shown in Table 2. After measuring sHLA-G in participants, Treg cells were measured in subjects with cut off of sHLA-G $\geq$ 10 U/ml (Mean of 100 results of healthy subjects +3SEM. Because there wasn't normal range or cut off for soluble HLA-G.) (Table 2).

In addition, treated patients (n=132) were divided into two groups according to their duration of remission after treatment: remission less than one year and more than one year. The results showed that sHLA-G levels were lower in patients who had remission in less than one year compared to patients with remission in more than one year ( $p=0.040$ ). This comparison was not significant for CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg cells. Furthermore, sHLA-G and CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg cell frequency decreased with long time treatment ( $\rho_{\text{sHLA-G}}=-0.245$ ,  $\rho_{\text{Treg}}=-0.183$ ) (Table 2).

Our results showed a significant difference between RRMS patients (n=21) in Treg cells frequency in comparison to healthy controls (n=7) ( $p=0.048$ ) (Figure. 2a). We assessed the concentration of sHLA-G in treated and untreated RRMS patients compared to healthy controls. Data showed a significant difference for sHLA-G levels in the three groups of control, treated and untreated patients ( $p<0.05$ ). This relation was more significant between control vs. treated patients ( $p<0.000$ ) and control vs. untreated patients ( $P<0.000$ ) (Figure. 2b). On the other hand results were not significant in treated patients by IFN- $\beta$  vs. untreated subjects ( $P=0.127$ ).

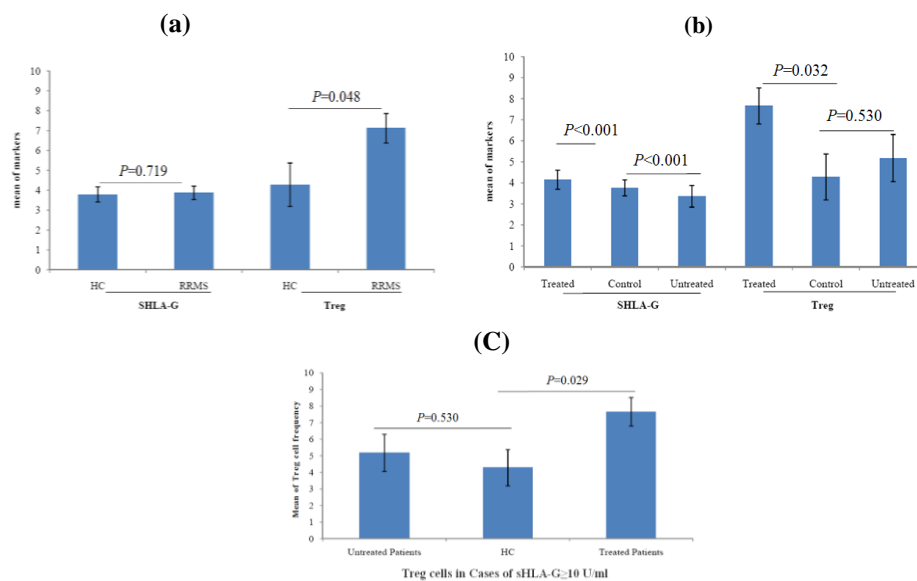
**Table 1. Demographic features of RRMS patients (n=205) and healthy controls (n=205)**

Demographic	RRMSs(Cases)	HCs(Controls)
Age (Mean $\pm$ SD)	31.32 $\pm$ 8.53	32.2 $\pm$ 7.48
Gender		
Male	37(18%)	39(19%)
Female	168(82%)	166(81%)

**Table 2. Laboratory Findings of RRMS patients and healthy controls and Mean of sHLA-G and Treg according to gender and in correlation with age in both groups (n= 205).**

sHLA-G	Gender	RRMS (Cases)		HC		
		(n=205)	P Value	(Controls) (n=205)	P Value	
CD4 <sup>+</sup> CD25 <sup>+</sup> FOXP3 <sup>+</sup> Treg	Male	3.54 $\pm$ 0.75	0.703	6.10 $\pm$ 1.20	0.009	
	Female	3.94 $\pm$ 0.38		3.20 $\pm$ 0.37		
	Female/Male Ratio	4.54		4.26		
	Gender	(n=21)		(n=7)		0.047
	Male	6.93 $\pm$ 0.87		2.64 $\pm$ 0.52		
sHLA-G	Female	7.28 $\pm$ 0.93	0.040	6.48 $\pm$ 1.91	-	
	Female/Male Ratio	4.25		0.75		
	remission					
Treg	$\leq$ 1 year	3.48 $\pm$ 0.41	0.590	-	-	
	$>$ 1 year	6.89 $\pm$ 1.51		-		
	remission					
sHLA-G/ Treg(Correlation)	$\leq$ 1 year	8.12 $\pm$ 1.36	0.034	-	-	
	$>$ 1 year	7.06 $\pm$ 0.58		-		
	remission					
sHLA-G/ Treg(Correlation )	Age	-0.245	0.483	-	-	
		-0.183		-		
		-0.211		-0.150		0.041
		0.011	0.853	0.322	0.482	

Data analysis showed that there were significant differences on sHLA-G level and Treg frequency according to gender in healthy subjects while this was not the same in RRMS patients. Age was effective on sHLA-G level in RRMS ( $p=0.002$ ) and healthy subjects ( $p=0.041$ ) but Kruskal-Wallis test did not show significant differences on age and Treg frequency in the two groups ( $p=0.482$ ). Pearson correlation test indicated that age had an inverse relation with sHLA-G level and Treg frequency in both groups. There were also significant differences in the levels of sHLA-G in patients who had less than 1 year of remission compared to patients who had more than one year remission.



**Figure 2. Comparison sHLA-G level and Treg frequency in RRMS patients and healthy controls (HC). (a) sHLA-G levels compared to RRMS (n=205) and HC (n=205), Treg frequency compared to RRMS (n=21) and HC(n=7), (b): sHLA-G level, in RRMS patients stratified according to treated (n=132), untreated patients(n=73) and HC (n=205) and Treg frequency in RRMS patients who had sHLA-G  $\geq$  10 U which was stratified according to treated (n=16), untreated patients(n=5) and HCs (n=7). (c) Treg frequency in subjects who had sHLA-G  $\geq$  10 U/ml in untreated (n=5), treated patients (n=16) and HCs (n=7)**

We also examined the frequency of Treg cells in the three groups (control, treated and untreated patients). Treg values showed generally non-significant relationship in the three groups ( $p > 0.05$ ). However, the data showed a significant relevance for Treg cells in control vs. treated patients ( $p = 0.029$ ), which was in contrast to the control vs untreated patients ( $p = 0.530$ ) whereas it did not indicate significant difference between treated vs. untreated patients (Figure 2b).

In addition, we found that there was significant relationship between sHLA-G and Treg frequency in treated patients (n=16) and healthy subjects (n=7) who had sHLA-G  $\geq$  10 U/ml ( $p = 0.029$ ) (Figure 2c).

Besides, the gender parameter was effective on sHLA-G levels and Treg frequency in healthy controls, while the sHLA-G levels of males in comparison to females was higher only in healthy individuals. Also, age was effective on sHLA-G level in RRMS patients ( $p = 0.002$ ) and healthy subjects ( $p = 0.041$ ) (Table 2). These data showed an inverse relationship between age and sHLA-G, age and Treg in both case and control groups ( $p < 0.05$ ).

## DISCUSSION

In our current research, we analyzed the involvement of sHLA-G molecules in the stable phase of Iranian RRMS patients in comparison with healthy subjects. In this investigation, sHLA-G level indicated a significant difference in treated patients compared to healthy subjects. Our data were in line with the other findings which have found a link between levels of serum sHLA-G antigen and disease course in MS patients.<sup>22</sup> Mitsdoerffer et al demonstrated that the sHLA-G antigen, arising from monocytes, inhibited both secretion of Th1 and Th2 related cytokines and could potentially have immunoregulatory role in pathogenesis of MS.<sup>22</sup>

sHLA-G generates a tolerogenic phenotype in myeloid dendritic cells through binding to immunoglobulin-like transcript 4 receptor (ILT4) which results in induction of Treg cells.<sup>19</sup> Similarly, we observed the correlation between Treg frequency and sHLA-G level in both healthy subjects and treated patients with sHLA-G  $\geq$  10 U/ml. This study showed the high levels of sHLA-G in treated patients which agreed

## Soluble HLA-G Isoforms and Regulatory T Cells in Multiple Sclerosis

with the results of Mitsdoerffer's study.<sup>23</sup> Since, sHLA-G molecules have diverse functions, their measurement as a plasma biomarker could establish an individualized treatment.

In addition, this study did not find any differences in the frequency of CD4<sup>+</sup> CD25<sup>high</sup> Foxp3<sup>+</sup> cells between untreated RRMS patients and healthy individuals. This finding was in agreement with some studies<sup>24-28</sup> but in contrast to the study of Kumar et al.<sup>29</sup> which reported significantly increased Treg cells (CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>) in whole blood of untreated MS patients compared to healthy individuals. The difference could be related to the type of selective markers that were considered as Treg and because of technical issues. However, a recent study showed reduced relative counts of CD4<sup>+</sup>CD25<sup>+</sup> T cells in untreated RRMS patients compared to healthy controls.<sup>30</sup> The possible explanation could be different subgroup and populations analysed.

Different researches have shown controversial results related to Treg frequency in MS patients compared to healthy subjects. First, some studies have shown that the number of CD4<sup>+</sup> CD25<sup>high</sup> Treg cells is not significantly different in MS patients and HCs.<sup>28</sup> Second, inappropriate peripheral homeostasis of Tregs could explain the lower Treg numbers and leading to evading autoaggressive T cells. Some reports explain an age-suboptimal decrease in changed T-cell homeostasis in a subset of MS patients.<sup>31</sup> A detailed analysis of parameters related to Treg cells in MS patients is necessary to investigate this possibility.

Since both sHLA-G and Treg have modulator properties in the immune system, another aspect of our study was to clarify whether sHLA-G is related with the frequency of Treg cells. The present study found that Treg cell frequency was significantly higher in treated MS patients who had sHLA-G level  $\geq 10$  U/ml vs. healthy subjects. Similarly, other studies have shown that in healthy subjects the number of HLA-G<sup>+</sup> Treg cells was few in the peripheral blood, but they increased in the inflammation sites like CNS.<sup>32</sup>

In this study, the gender parameter was effective on sHLA-G level and frequency of Treg cells in the control group, but in patients' group, the gender parameter was not effective. In fact, sHLA-G level in male participants of the control group was higher than females. This finding did not agree with other studies which could be due to different studied populations.<sup>33</sup> Treg frequency of the control group was higher in females compared to males. On the other hand, age

parameter showed a significant relationship with sHLA-G level in the two groups, while age showed an inverse effect on Treg frequency in the two groups. While, Gregg et al. have shown that increasing of CD4<sup>+</sup>CD25<sup>hi</sup> Treg cells associated with ageing in healthy volunteers.<sup>32</sup>

In this research, sHLA-G level in the control group less than 10 years old was higher than other age groups. In this regard, Fainardi E et al demonstrated no relationship between age parameter and sHLA-G level but they noted higher sHLA-G levels in females than males.<sup>22</sup> Differences in the analysis method used could in part explain the reported discrepancy. The frequency of Treg in patients' age groups (age: 30-40 years) and in control group (age: 10-20 years) were higher than other age groups. Overall, these differences could be due to the putative autoantigen diversity in MS disease and perhaps to be patient-specific owing to epitope spreading with disease progression and differences in genetic background.<sup>34</sup>

In conclusion, our data suggests the increasing of sHLA-G  $>10$  U/ml may influence Treg cell frequency which subsequently leads to induce stable phase in RRMS patients. We suggest, in the early phase of the disease, a reduction in Treg frequency and their function caused by either genetic alterations or reduced thymic Treg output could give rise to suboptimal peripheral tolerance, leading to escape of autoaggressive T cells. Further investigations should be done to resolve these issues possibly performed in the CNS.

### ACKNOWLEDGEMENTS

This work is supported in part by the research center of Tarbiat Modares University. We thank the Isfahan MS and Neuroimmunology Research Centers. We are grateful to all patients and volunteers who participated in this study and appreciate Dr Nahid Eskandari and Dr Mohadase Behjati for critical comments on this article.

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## Soluble HLA-G Isoforms and Regulatory T Cells in Multiple Sclerosis

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