

Dendritic Cell Stimulation by IFN- β Alters T Cell Function via Modulation of Cytokine Secretion in Diabetes Type 1

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Abstract- During antigen capture and processing, mature dendritic cells (DC) express large amounts of peptide-MHC complexes and accessory molecules on their surface. We investigated the role of IFN- β in induction HLA-G expression on the monocyte derived DC and cytokine profile in diabetes type 1. We accomplished secretory pattern and total cytokine production of the Th1 cytokine (IL-2, γ IFN) and Th2 cytokines (IL-4, IL-10) before and after mixed leukocyte reaction (MLR) of 30 diabetic patients and 30 normal subjects. In this study a significant increase of IL-10 and γ IFN reduction after IFN- β Therapy in culture in presence of HLA-G bearing DC as compared to control were seen. It is seen that dendritic cell causes IL-10 production of T cell in vitro that reduce T cell activation from diabetes patients and normal subjects resulted to the production and expression of HLA-G on these cells from both groups. Using mixed leukocyte reaction, it was found that IFN- β -treated dendritic cell mediated the inhibition of autologous T cell activation via IL-10 production and level of HLA-G on dendritic cell may be correlated to disease activity in diabetes patients and it could also serve as a useful marker for disease progress and treatment.

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Key words Cytokine, HLA-G, IFN- β (Interferon β), diabetes type 1, autoimmunity, dendritic cell

Introduction

Diabetes type 1 is associated with an imbalance between T helper (Th) 1 and Th2 cytokines (1,2). The pathophysiological mechanism initiating this autoimmune response remains to be determined. Human studies suggested that cytokines play important roles in initiating the pathogenesis of type 1 diabetes. Cytokines are associated with destruction of pancreatic cells and development of diabetes type 1 (3). In diabetes type 1, the Th1/Th2 ratio varied and characterized by a lower production of the Th2 cytokines IL-4 and IL-10 and a higher secretion of Th1 cytokines IFN- γ and IL-2 (4-6). It demonstrated that IFN- γ stimulate antigen presenting cell (APC) to produce inflammatory monokines to destroy beta cells (7,8). The important mechanisms of how T lymphocytes are activated and the tolerance to the beta cells antigen is diminished remain to be elucidated. However, an exact event in the antigen recognition and activation is presentation of antigen by APC to T cells.

Variety of positive as well as negative regulatory signals is provided by APC in particular by dendritic cells (9-11). Interferon-beta (IFN- β), a type I Interferon, is normally induced by viruses or ds RNA (12) which is considered an immunomodulatory molecule for dendritic cells (13). The immune response is naturally regulated by various mechanisms aimed at controlling hyperactivity and preventing self destruction. An association between HLA and type 1 diabetes is generally accepted (9,10,14). HLA-G a non-classical MHC class I antigen is characterized by a limited polymorphism and is encoded by an altered native transcription of spliced mRNAs resulting in at least seven different isoforms, including membrane bound HLA-G1, G2, G3, G4 and HLAG5 (formerly HLAG15), G6 (formerly HLA-G25), and G7 proteins (15). These were first detected on extravillous cytotrophoblast cells (16). These antigens play an important role in cancer, pregnancy and transplantation. Moreover functional studies have identified HLA-G as a key mediator in immune tolerance (17-20).

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Whereas, dendritic cell might have tolerogenic function even in their mature form, we studied effect of IFN-beta with GM-CSF (Granulocyte- Monocyte colony stimulating factor) and IL-4 (Interleukin 4) on DCs in regulation of T cells.

In this study we showed that IFN-beta-GM-CSF-IL-4-LPS activated dendritic cells mediate inhibition of T cell activation by producing IL-10 and IL-4 in MLR. Thus this cell may be involved in the immune regulatory pathway in the pathogenesis of type I diabetes.

Patients and Methods

The patients were those admitted in the endocrinology department of Shariati Hospital, a teaching hospital affiliated to Tehran University of Medical Sciences. The diagnosis of illness was made, according to clinical and laboratory findings, by consultant endocrinologist. The patients who took part in this study were informed clearly of the nature of this investigation and they gave the consultant endocrinologist a written consent for taking blood sample for measuring certain variables as well as using the blood cells for the present study. The blood samples which served as normal control were obtained from blood transfusion services.

To study the immune phenotype and cell surface expression of monocyte derived DC bearing HLA-G, 50ml blood samples were collected from 30 patients with diabetes type 1, with median age 29.3 and 30 normal controls with median age 30.76. All diabetic patients were under treatment with insulin and showed a mean fasting blood sugar of 162 mg/dl (Table 1).

Blood mononuclear cells (MNC) were isolated from heparinized blood by centrifugation on a Ficoll Histopaque 1.077 (sigma, U.S.A). The cells from the interphase were collected and washed three times with RPMI 1640 medium (sigma). Cell viability was determined by trypan blue exclusion. Monocytes 90% isolated from MNC by plastic flasks adherence. $5-8 \times 10^6$ /ml of MNC were then cultured in 5ml RPMI 1640 medium supplemented with 10% fetal calf serum, 50units/ml penicillin and 50 μ g/ml streptomycin. After 2h of incubation at 37°C in an atmosphere of 5% CO₂, the adherent cells were cultured in complete medium containing 1000 units /ml rhGM-CSF (Serotec, UK), 500unit/ml rhIL-4 (serotec, UK) and IFN- β (1000 units/ml, Avonex; Biogen, Cambridge) to generate DC.

Non adherent cells were Harvested for T cell isolation. The T cells (> 95%) were isolated. CD³⁺ T cells were purified respectively using MACS CD³⁺ magnetic beads, CD³⁺ T cell isolation kit (Miltenyi Biotec). These

cells were counted and then frozen in 10% dimethylsulfoxide containing a mixture of 60% RPMI and 30% FCS.

We stimulated DCs with 50 μ g/ml insulin (Actrapid Novo Nordisk, Bagsvared, Denmark) on day 4 for antigen processing by these cells for 72h at 37°C, in 5% CO₂.

The immature monocyte-derived DCs were then activated by adding TNF- α (10ng/ml) into the culture to promote maturation of these cells and HLA-G expression.

Expressions of molecular markers on the immune cells were determined by flowcytometry on a fluorescent activated cell sorter (B.D).

Harvested cells were washed twice with PBS supplemented with 1% BSA. Fc receptors on cells were pre-blocked with excess human IgG (Sigma-Aldrich) on ice for 15 min. Cells were stained for 30 min at 4°C with the following FITC-conjugated Abs: anti-HLR-DR and anti-CD83 and PE-conjugated Abs: CD1a (eBioscience) or isotype controls (eBioscience)

Finally in order to identify and enumerate the cells, the cell suspensions were analyzed by flowcytometry.

Total RNA was extracted from treated DCs with IFN β and from jeg-3 coriocarcinoma cell line which was used as a positive control for HLA-G expression (National cell bank of country), using RNazol Bee reagent (Biosite, TABY, Sweden) based on the manufacture instructions. RNA was checked for Quality by electrophoresis in 1.5% agarose gel. The transcriptional levels of HLA-G were evaluated by reverse transcriptase PCR in comparison of those of jeg-3 cells.

First strand cDNA was synthesized using 3 μ g of total RNA in 20 μ l reaction mixture consisting of 5X reaction buffer 4 μ l; 10mM dNTPs 2 μ l; 100 μ M dithiotheritol (DTT) 1.5 μ l; 10pMol/ml random hexamer(N6)1 μ l, and M-MLV reverse transcriptase 200units (metabion). Thereafter, the mixture was incubated at 42°C for 45 min.

Table 1. Features of diabetic patients and normal subjects who participated in the study

Features	Diabetes	Normal
Total Numbers	30	30
Gender	M(19),F(11)	M(25),F(5)
Age in years(Range)	28.5(13-49)	28.86(22-44)
Insulin dependent	+	-
Familial History of Diabetes	+	-
Fasting Blood Sugar	160.6 \pm 57.4	88.6 \pm 9.3

Primers used in this study are 5-TCATGCTGAGA TGGAAGCAG-3; 5-TCTCCACAGCACAGCCAGC-3 for HLA-G and 5-TGGCCACGGCTGCTTCCAGC-3; CAGGAGGAGCAATGATCTTGAT-3 for beta actin.

Briefly, 25µl reaction mixture of PCR was prepared using 2.5 µl of 10 × buffer , 3µl of 25mM mgcl₂ , 1.5µl dNTPs (10Mm), 10 pmol of each primer and 1 unit of Amplify Tag Gold DNA polymerase(Perkin Elmer Biosystems, USA) . PCR was performed in 35 cycles, initiated by 1 cycle at 95°C for activating the tag Gold's DNA polymerase followed by 94°C; 30sec,60°C ; 30sec , 72°C; 30sec and final extension of 10minutes at 72°C. Amplification of β actin was performed using 26 cycles.PCR amplification products and 100bp molecular weight markersVIII (Roche) was separated on 1% agarose gels containing etidium bromide. The primers for HLA-G produced PCR amplification products of 118bp, whereas the β actin primers gave a product of 321bp.Control PCR amplifications without cDNA were always carried out in parallel were consistently negative. All PCR amplification was performed in duplicate.

Then, isolated T cells and autologous DC were co cultured in 96 U-shaped-bottomed plates that 10×10⁴ Tcell were cultured in each well with 1×10⁴ DC in a final volume of 200µl per well. All samples were run in triplicates .Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere for 5 days and then pulsed with 200 µl 3-[4,5-dimethylthiazolyl]-2,5-diphenyl-tetrazolium bromide(MTT; Sigma) as a color indicator of metabolic activity , denoting cell proliferation (21). Supernatant harvested at 4h later, and then dimethylsulfoxide (DMSO) was added (200 µl). The color change was read in an Elisa reader at 550nm wave length.

Cytokines concentrations were determined in duplicate in serum and culture supernatants using commercially available human IL-2, IL-10, IL-4 and γIFN Bender MedSystems Immunoassay Kit (Bender MedSystems GmbH Campus Vienna Biocenter 2 A-1030 Viena,Austria Europe). Serum- free supernatant of Dc-Tcell cultures with and without HLA-G were harvest 5 days of incubation and frozen down for subsequent cytokine analysis.

Statistical analysis

For statistical analysis, we used paired *t*-tests. Each paired *t*-test was calculated for diabetic patients as well as the normal donors .The *P* values were determined in all cases and they were considered statistically significant at *P*<0.05.

Results

Phenotype analysis of monocyte-derived DCs was carried out using flowcytometry.

Analysis of phenotype showed the expression of HLA-DR and minimal level of CD-83 on their surface before stimulation with Ag.Expression of the CD83 marker and HLA-DR after antigenic stimulation indicating the maturation of dendritic cells (Figure1).

The functional potential of mature monocyte-derived DC to induce proliferation of T lymphocytes was investigated by MLR in presence or absence of HLA-G. Proliferation of T lymphocytes co-cultured with mature DC was found to decrease by about 40% in presence of DC bearing HLA-G (Figure 2, Table 2).

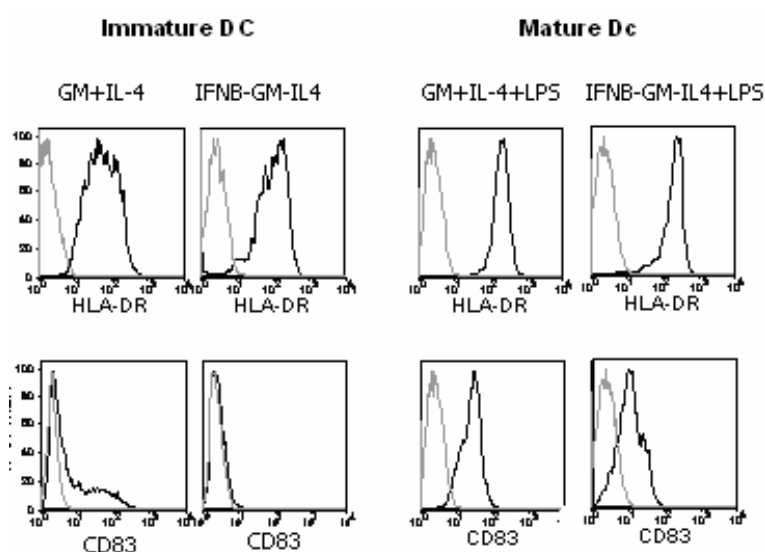


Figure 1. Flowcytometry results of immature and mature dendritic cells treated with IFN-beta in comparison with controls

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DC bearing HLA-G in MLR inhibited T cell proliferation in the normal subjects as well as diabetic patients. Since we compared the T cell proliferation responses in DC expressing HLA-G and in DC lacking HLA-G, upon addition of MTT, T-Cell proliferation was inhibited in co-culture system containing DC bearing HLA-G in both diabetic as well as normal subjects as indicated by metabolic activity assay (MTT) (Table 3, Figure 3).

Interferon-γ, IL-2, IL-10 and IL-4 cytokines release assays were carried out in MLR supernatant and serum using commercially available Elisa kits. The results are illustrated in table 4.

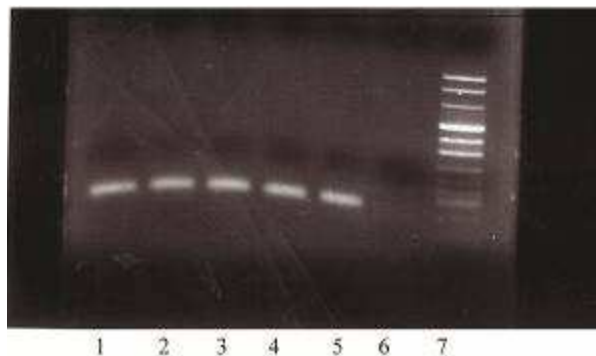


Figure 2. RT-PCR analyses of HLA-G and β actin gene expression in diabetes type 1 and controls after treatment with IFN-β. The cDNA samples were from: jeg-3 cell line as positive control (lane 1); dendritic cell from patients with diabetes type 1 (Lane 2, 3) DCs from a normal (Lane 4, 5); negative control with out cDNA (lane 6); DNA weight marker VIII (lane 7)

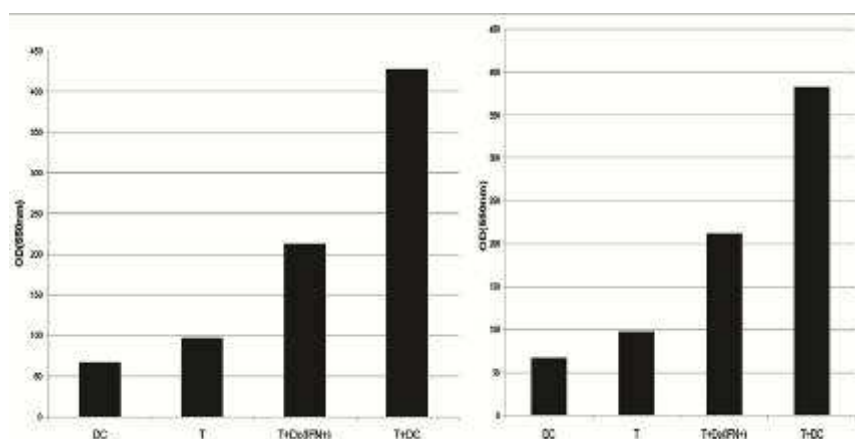


Figure 3. Mean of Mixed leukocyte reaction in diabetes patients (left) and normal subjects (right) with control evaluated by MTT assay. Dendritic cells were cultured with T cells (1 to 10).

Table 2. Effect of IFN-β on expression of HLA-G on DCs in vitro as evaluated by flowcytometry. The results are percent of DC bearing HLA-G

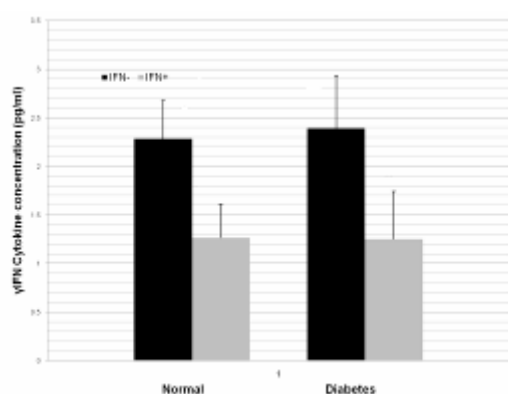
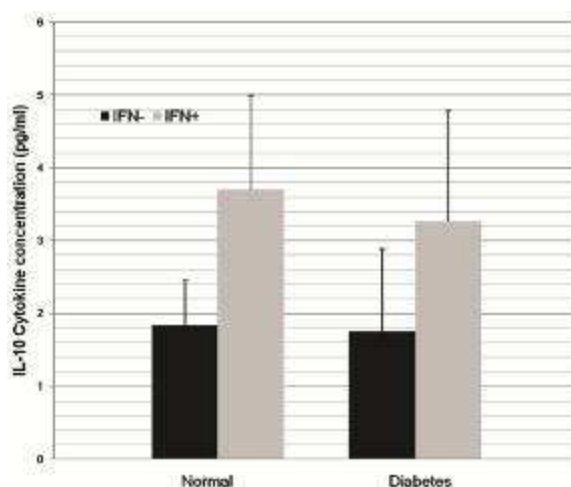
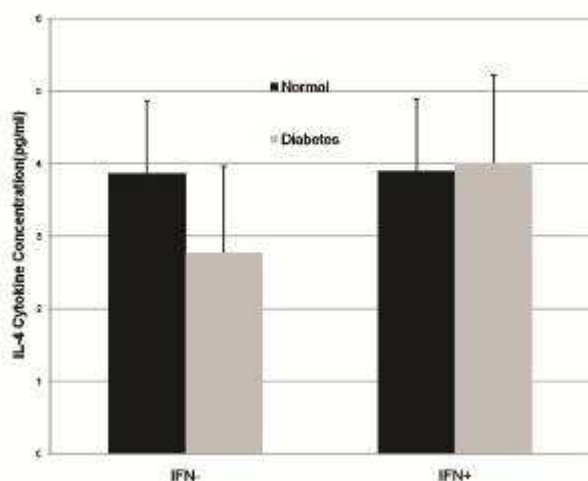
Cells	Total Samples	Dc without IFN-β		Dc + 1000units IFN-β		P value
		Mean	SD	Mean	SD	
Diabetic DC	30	0.2	0.06	33%	0.11	< 0.05
Normal DC	30	0.5	0.15	35%	0.09	

Table 3. Effect of DC-bearing HLA-G on CD3⁺T-Cell proliferation in MLR, evaluated by MTT assay. The data are OD of MTT at 550 nm read in Elisa reader

MLR	Total Samples	DC Without HLA-G	Dc with HLA-G	P value
		Mean ± SD	Mean ± SD	
Diabetic	30	427.26±56.58	213.06±62.17	< 0.05
Normal	30	382.36±81.86	211.3±58.9	

Table 4. Cytokine production profile (γ IFN, IL-2, IL-4, IL-10 [pg/ml]) in supernatant of culture media after 6 days and serum. Values are mean \pm SD.

Cytokines	γ IFN	IL-2	IL-4	IL-10
Supernatant of MLR(Dc without HLA-G +TCell) in Diabetes patients	2.39 \pm 0.53	10.4 \pm 4.26	2.76 \pm 1.25	1.47 \pm 1.13
Supernatant of MLR(Dc without HLA-G +TCell) in normal subjects	2.28 \pm 0.39	9 \pm 3.8	3.87 \pm 1.09	1.83 \pm 0.62
Supernatant of MLR(Dc with HLA-G +TCell) in Diabetes patients	1.25 \pm 0.49	3.51 \pm 2.03	4.01 \pm 1.39	3.26 \pm 1.51
Supernatant of MLR(Dc with HLA-G +TCell) in Normal subjects	1.26 \pm 0.34	5.2 \pm 1.80	3.89 \pm 1.24	3.69 \pm 1.30
Serum cytokines concentration in Diabetes patients	12.56 \pm 0.44	5.01 \pm 0.63	4.52 \pm 0.37	1.57 \pm 0.66
Serum cytokines concentration in Normals subjects	5.88 \pm 1.24	6.71 \pm 0.91	5.61 \pm 1.35	4.32 \pm 1.67

**Figure 4.** γ IFN concentration in the supernatant of culture media in diabetes patients and normal. Dendritic cells were cultured with T cells. Values are mean \pm SD. Statistically significant at $p < 0.05$.**Figure 6.** IL-10 concentration in the supernatant of culture media in diabetes patients and normal. Dendritic cells were cultured with T cells. Statistically significant at $p < 0.05$.**Figure 5.** IL-4 concentration in the supernatant of culture media in diabetes patients and normal. Dendritic cells were cultured with T cells. Values are mean \pm SD. Statistically significant at $p < 0.05$.

Discussion

We studied a comprehensive and follow up analysis of Th1/Th2 secretion in MLR supernatant in presence IFN- β -treated dendritic cell and without IFN- β in diabetes patients and controls. An important step in the present study was to induce HLA-G on dendritic cell derived from monocytes. This was achieved by treatment of dendritic cells in vitro with IFN- β . This cytokine has been shown to promote the differentiation of blood monocytes to DC and to contribute to DC maturation (22). It was also demonstrated in this study that IFN- β up regulated considerably HLA-G expression on dendritic cells (Figure 2 and Table 2). In a variety of research it has been demonstrated that DC bearing HLA-G have the potential to down regulate and to inhibit T cell ac-

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tivities (23-27). The cells which are inhibited by HLA-G bearing DC carry specific receptors for HLA-G. The presence of HLA-G receptors on CD4 T cells has also been shown for immunoglobulin like transcript 2 (ILT-2) indicating this molecule a possible candidate mediating inhibitory effects (28-30). In the present study, the inhibition of T cells proliferation was observed only when dendritic cells were treated with IFN- β (Table 3, Figure 3). Thus IFN- β and HLA-G molecules on the dendritic cells are two components contributing to this phenomenon, limiting cells proliferation; thereby reducing the potential of T-cells. We demonstrated that DC bearing HLA-G in MLR causes higher production of IL-4, IL-10 and lower production of IL-2, IFN γ in diabetes patient and controls (Figure 4, 5 and 6). The serum increased secretion of Th1 cytokines and higher Th1/Th2 ratio in our diabetes group is consistent with others, before treatment with IFN β (Figure 7). It is also interesting that several evidences suggest that autoimmune diabetes is associated with decreased IL-4 secretion rather than enhanced secretion of Th1 cytokines (31). In this view IL-10 can directly inhibit T cell activation, through inhibition of IL-2 production. Functionally; IL-10 inhibits the capacity of peripheral blood DC to stimulate alloreactive T cells (32). Wilson and et al indicated that increased γ IFN and decreased IL-4 secretion resulted a progression of type 1 diabetes (33). Our results suggest that some desirable immune modulator effects of IFN- β may be accompanied via the up regulation of the immune tolerogenic molecule of HLA-G and IL-10.

It is quite evident that the cells bearing HLA-G are an important negative immune-regulatory constituent for this suppressing effect. It is speculated that IFN- β by increasing HLA-G on dendritic cells can also down regulate inflammatory response in vivo mediated by T cells in islet tissues of pancreatic gland. Levels of HLA-G on dendritic cells may also be correlated to disease activity in diabetes patients and it could also serve as a useful marker for disease progress and treatment. We would like to propose that HLA-G may be a factor limiting autoimmune response, thus potentially constituting a new component for controlling and suppressing the detrimental role of T-cells in the pathogenesis of diabetes type 1.

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