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Original article

Calcitonin Gene-Related Peptide Effects on Phenotype and IL-12 Production of Monocyte-Derived Dendritic Cells in Rheumatoid Arthritis Patients

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Abstract

Objective(s)
Recent studies on human indicate that the introduction of therapeutic use of tolerogenic dendritic cell (DC) for chronic inflammatory conditions is imminent. For the purpose of defining CGRP potency in tolerogenic DC production, we investigated the phenotype and IL-12 production of DCs generated from the monocytes of rheumatoid arthritis (RA) patients in the presence of the calcitonin gene-related peptide (CGRP), as a multifunctional neuropeptide.

Materials and Methods
DCs were generated from isolated monocytes from four resistant and two early female RA patients using IL-4, GM-CSF, and CGRP at concentrations of 0, 1, and 100 nM. Then, the phenotype of neuropeptide-treated or untreated DCs was determined using flow cytometry and the IL-12 production was measured by ELISA.

Results
Our study showed that, on the last day of the culture, at a concentration of 1 nM CGRP, the mean fluorescence intensity (MFI) for CD80 increased (14.13%) and the MFIs for CD83, CD86, and HLA-DR decreased (14.57%, 5.28%, and 6.88% respectively). Moreover, at 100 nM CGRP concentration, the MFI for CD80 increased (11.10%) and the MFIs for CD83, CD86, and HLA-DR decreased (4.27%, 18.60%, and 19.75% respectively). In addition, our results indicated that the mean concentrations of IL-12 produced at 0, 1, and 100 nm CGRP concentrations measured 13.72±2.41, 11.01±1.61, and 7±1.34 pg/ml respectively.

Conclusion
Decreased CD83, CD86, and HLA-DR expression and reduced IL-12 production by CGRP were found in the RA patients' monocyte-derived DCs. CD83 is a well-defined DC activation marker. HLA-DR and CD86 are appropriate molecules for inducing an immune response. IL-12 promotes cell-mediated immunity. Therefore we suggest that CGRP may be used as an inducer in the production of tolerogenic DCs.

Keywords: Calcitonin gene-related peptide, Dendritic cell, Immune tolerance, Rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a common chronic and progressive autoimmune disease. Joint destruction and functional disability in RA incurs substantial direct and indirect financial costs for patients, their families and society (1). Dendritic cell (DC) contributes to the initiation, induction, maintenance, and complications of RA (2). DC dependent autoreactive $CD4^+$ T cell activation has been accepted as one of the central events in RA pathogenesis (3). Although many advances have been achieved in RA treatment, developing more efficient therapeutic methods with less side effects remains an area of ongoing research (4, 5).

Dendritic cells (DCs) are the professional antigen presenting cells for immune response initiation and effector/memory lymphocyte production (6). On the other hand, they are also known as the potent regulators of immune/inflammatory reactions and can modulate the effector functions of lymphocytes (7). The latter property is dependent on various factors, the most important of which appears to be the stage of their differentiation, activation, and maturation (8). Recent data have shown that immature, semi- or partially mature, and maturation-resistant (alternatively activated) DCs can regulate autoreactive or alloreactive T cell responses and promote or restore tolerance (8, 9). In other words, DC tolerogenicity is not restricted to a specific state or a specific subset of these cells (10). Tolerogenic DCs are deficient in costimulatory molecule (CD80, CD86) expression. Moreover, these DCs produce low levels of IL-12p70, high levels of IL-10 and indoleamine 2,3-dioxygenase (IDO), and are resistant to maturation-inducing factors (8, 10). These regulatory subsets of DCs are able to acquire and present antigen to antigen-specific T cells, but fail to deliver adequate co-stimulatory signals for the activation or proliferation of T cells. Tolerogenic DCs can induce anergy/apoptosis in T cells or promote regulatory T cell generation/proliferation (8, 10). The effective therapeutic application of tolerogenic DCs in animal models of autoimmune diseases, such as collagen-induced arthritis, has been previously presented (8). Additionally, clinical trials for treating rheumatoid arthritis and new-onset diabetes are underway (8).

Neurotransmitters, along with hormones and cytokines, mediate a complex network between neuro-endocrine-immune systems (11). The calcitonin gene- related peptide (CGRP), a 37 amino acid neuropeptide, is mainly produced by unmyelinated postganglionic C-fibers (12, 13). These sensory nerves exist in many tissues, such as blood vessels, the heart, the kidney, the gastrointestinal tract, and lymphoid organs (13). T cells, B cells, mast cells and nerve growth factor stimulated monocytes also secrete CGRP (12-15). CGRP participates in the neuro-immune regulatory network. CGRP can modulate neutrophil (14), monocyte/macrophage (16-18), DC (13, 17, 19, 20), T cell (21, 22), B cell (23), mast cell (24) and eosinophil (25) functions. CGRP inhibits neutrophil recruitment and lowers TNF production by macrophages, and therefore, suppressing acute local and systemic inflammation (14). Antigen presentation, costimulatory molecule expression, and IL-1 and IL-12 production are reduced while IL-10 is increased in Langerhans cells, macrophages and DCs exposed to CGRP (13, 16-20). Moreover, successful experimental therapeutic applications of this neuropeptide for some inflammatory disorders have also been observed (26, 27). In contrast, some other investigations have shown promoting inflammatory effects of CGRP, for instance rise in IL-6 production (28), the enhancement of nitric oxide release and inducible nitric oxide synthase activity in LPS-stimulated mouse peritoneal macrophages (29), or an augmentation of proinflammatory cytokine production (TNF, IL-1) in virus infected macrophages (30). This study was designed to evaluate CGRP's ability to induce tolerogenic phenotype and to reduce IL-12 production in DCs derived from RA patients. This is the first step of a new therapeutic approach that may be applicable to many other similar autoimmune and inflammatory diseases.

Materials and Methods

Patients

Studied participants were composed of six females who had been diagnosed with RA according to the American College of Rheumatology's (ACR) revised criteria. Of these patients, the disease had remained resistant to treatment (four patients) or was in its early state (two patients). The duration of the disease in resistant patients was at least two years and they were defined by: 1) insufficient response to an optimal dose of three or four disease modifying anti-rheumatoid drugs (DMARDs) including methotrexate (maximum dose: 25 mg/week) for at least three months, 2) positive result for the rheumatoid factor (RF), 3) Lack of low disease activity according to Disease Activity Score 28 (DAS 28 > 3.2). Stable low dose prednisone therapy (<10 mg/day) and nonsteroidal anti-inflammatory drug (NSAID) treatment were allowed. None of them had used biologic agents during their course of treatment. Early RA patients were diagnosed, at most, one month before inclusion and had received no steroid therapy. Early patients negative for anti-cyclic citrullinated protein antibodies were excluded. The study protocol was approved by the Medical Ethics Committee of Mashhad University of Medical Sciences. Written informed consents were obtained from all participants.

Generation and culture of monocyte derived dendritic cells

Monocyte-derived DCs were generated as previously described (19, 31). Briefly, mononuclear cells from venous peripheral blood (approximately 50 ml) were isolated by density gradient centrifugation on ficoll (Lympholyte, Cedarlane, Hornby, Ontario, Canada). Then, monocytes were negatively separated using a magnetic activated cell sorting kit (Stemcell Technologies, Vancouver, British Columbia, Canada). This method routinely resulted in more than 80% purity, as assessed by flow cytometry. Isolated monocytes from each patient were cultured for six days at 37 °C in 5% CO_2 in an incubator at $2x10^5$ cells/ml in 24 well plates in a volume of 1 ml RPMI 1640 (Biosera, Ringmer, East Sussex, UK) supplemented with 10% FBS (Gibco/Invitrogen, Burlington, Canada), 2 mM L-gl utamin (Gibco/Invitrogen, Burlington, Canada), 50 µM 2-ME (Gibco/Invitrogen, Burlington, Canada), 100 U/ml penicillin and 100 µg/ml streptomycin. Recombinant human GM-CSF and IL-4 (R&D Systems, Minneapolis, MN) were added at 800 U/ml and 500 U/ml, respectively. After three days, half of the medium was removed and replaced with the same volume of fresh medium containing cytokines. For maturity induction, 1 µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, Saint Louis, M) was added on day 6, and the culture continued for two more days.

Neuropeptide treatment

After six days culture in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml), immature DCs were exposed to human CGRP (Sigma-Aldrich, Saint Louis, MI) for two hours at 37 °C in a CO_2 incubator. Based on previous studies, we applied two CGRP concentrations: 1 and 100 nM. Then, the cells were cultured for two additional days in the above mentioned medium supplemented with LPS (1 µg/ml) and the same concentrations of CGRP.

Immunofluorescence labeling and flow cytometry

Monocytes and mature DCs (treated and untreated with CGRP) were studied for surface markers: CD14, CD11c, CD80, CD83, CD86, and HLA-DR on days 1 and 8. Briefly, the cells were washed in PBS/BSA. The washed cells were then incubated with conjugated monoclonal antibodies (Serotec, Oxford, UK) for 20 minutes at 4 °C, washed in PBS/BSA, and fixed in 1% paraformaldehyde for analysis using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). Isotype-matched conjugated antibodies were used as controls for nonspecific bindings.

Cytokine determination by ELISA

The IL-12 concentration in the supernatants of CGRP treated and untreated-cells harvested on day 8 was determined, according to the

manufacturer's instructions, by an ELISA kit purchased from Bender MedSystems (Vienna, Austria). The minimal sensitivity of the ELISA for human IL-12 was 2.1 pg/ml.

Statistical analysis

Univariate general linear model, independent sample t test, and the Mann-Whitney U test were used, as appropriate, for the comparison of the samples' mean values. Results were expressed as mean±SEM. A *P* value less than 0.05 was judged to be statistically significant.

Results

Decreasing CD14 and increasing CD11c positive cells were clearly observed

On day 1, in comparison to day 8 of the culture, the percentages of CD11c positive cells were significantly enhanced and CD14 positive cells percentages significantly reduced (*P*= 0.000) (Figure 1). On day 1, the mean percentages of CD14 and CD11c positive cells were 87.84±4.45% and 0.44±0.16% respectively. The mean percentages of CD14 positive cells on day 8 were 2.49±0.81%, 2.28±0.71%, and 1.46±0.53% at 0, 1 nM, and 100 nM CGRP concentrations, respectively. Also, CD11c mean percentages at 0, 1 nM, and 100 nM CGRP concentrations changed to 84.88±2.28%, 80.15±6.12%, and 82.05±2.96%, respectively.

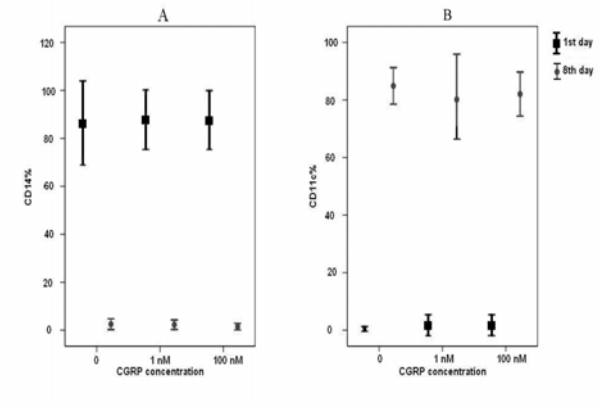

Figure 1. Confidence interval (%95) error bars for the percentage of CD14 (A) and CD11c (B) positive cells in the beginning (1st day) and at the end (8th day) of culture in untreated and CGRP treated groups. Monocytes were isolated from peripheral blood of patients (n= 6) and cultured in the presence of GM-CSF and IL-4 for 8 days. LPS and CGRP were added on day 6. CGRP was applied at concentrations of 0, 1 and 100 nM.

The expression of CD80, CD83, CD86, and HLA-DR increased during the culture period

The mean fluorescence intensity (MFI) for CD80, CD83, CD86, and HLA-DR was determined on days 1 and 8 (Table 1). These values represented the increase in the expression of all makers in both control and treated groups on day 8, in comparison to that of primary monocytes. Although the elevation of CD80, CD86, and HLA-DR expression was significant, the increased expression of CD83 did not show any statistical significance (Figure 2 and Table 1).

CGRP did not reduce CD80 expression in generated DCs from RA patients

On day 8, the expression of CD80 did not decrease in CGRP-treated DCs in comparison to untreated cells (Figures 3 and 4). In early specimens, the MFI means for CD80 increased by 6.95% and 0.78% at 1 nM and 100 nM CGRP concentrations respectively. The resistant sample values were determined as 28.74% and 33.76% at 1 nM and 100 nM concentrations of CGRP respectively. In comparison to untreated cells, these observed elevations were not significant for any CGRP concentration.

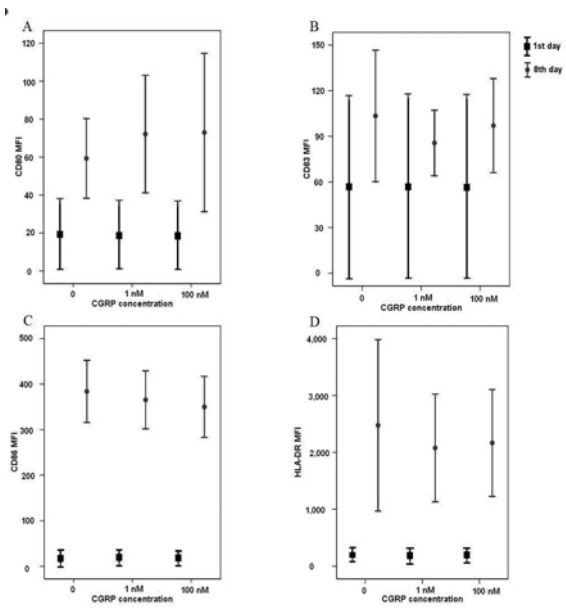

Figure 2. Confidence interval (%95) error bars for MFI of CD80 (A), CD83 (B), CD86 (C) and HLA-DR (D) on days 1 and 8 at CGRP concentrations of 0, 1 and 100 nM. Monocytes were isolated from peripheral blood of patients (n= 6) and cultured in the presence of cytokines (GM-CSF and IL-4) for 8 days. LPS and CGRP were added on day 6.

Table 1. Expression of cell surface markers at different concentrations of CGRP on days 1 and 8.

Marker	CGRP Concentration (nM)	MF1±SEM[a]		*P* value
		1st day	8th day	
CD80	0	19.15±6.53	59.29±8.18	0.005
	1		72.14±12.05	0.005
	100		72.94±16.22	0.019
CD83	0	57.17±21.70	103.42±16.82	0.121
	1		85.60±8.38	0.275
	100		97.04±12.03	0.127
CD86	0	19.17±5.59	384.09±26.51	0.000
	1		365.50±24.79	0.000
	100		349.92±26.07	0.000
HLA-DR	0	188.95±47.92	2475.83±585.90	0.011
	1		2078.01±368.44	0.001
	100		2166.68±365.38	0.003

Abbreviations: CGRP, calcitonin gene related peptide; MFI, mean fluorescence intensity; SEM, standard error of the mean.
[a] For each concentration of CGRP the mean of detected MFIs for all specimens (early and resistant collectively) are shown.

CD83 and CD86 expression were reduced in resistant and early RA patients

In CGRP-treated monocyte-derived DCs, the MFI of CD83 decreased at the CGRP concentration of 1 nM in both early (14.83%) and resistant (18.57%) patients, when compared to their controls (without CGRP) on day 8. At a concentration of 100 nM CGRP, this effect was lower (8.51% and 4.82% for early and resistant specimens respectively) (Figures 3 and 4).

In comparison to untreated DCs on day 8, all treated DCs showed lowered CD86 expression (8.27% and 2.92% at 1 nM and 3.91% and 11.68% at 100 nM concentrations of CGRP in early and resistant samples respectively) (Figures 3 and 5). The reducing effects on CD83 or CD86 were not statistically significant.

HLA-DR expression state was different between early and resistant RA patients

In comparison to the controls on day 8, HLA-DR expression reduced in the treated DCs obtained from early RA cases, at both 1 nM (32.17%) and 100 nM (31.96%) CGRP concentrations (Figure 5). On the other hand, the expression of HLA-DR did not decrease in resistant specimens and showed little increase, in comparison to the controls on day 8 (2.03% and 9.40% at 1 nM and 100 nM CGRP concentrations respectively) (Figure 5).

IL-12 production was reduced in CGRP treated DCs

IL-12 measurement after LPS stimulation demonstrated that CGRP treated cells, in both early and resistant patients, produce lower levels of this cytokine (Figure 6). The means of IL-12 production at 0, 1, and 100 nM concentrations of CGRP were 14.15±3.94, 12.81±0.08, and 8.28±2.28 pg/ml in early and 13.29±4.36, 9.22±3.02 (*P*= 0.051), and 5.73±1.53 pg/ml in resistant patients respectively.

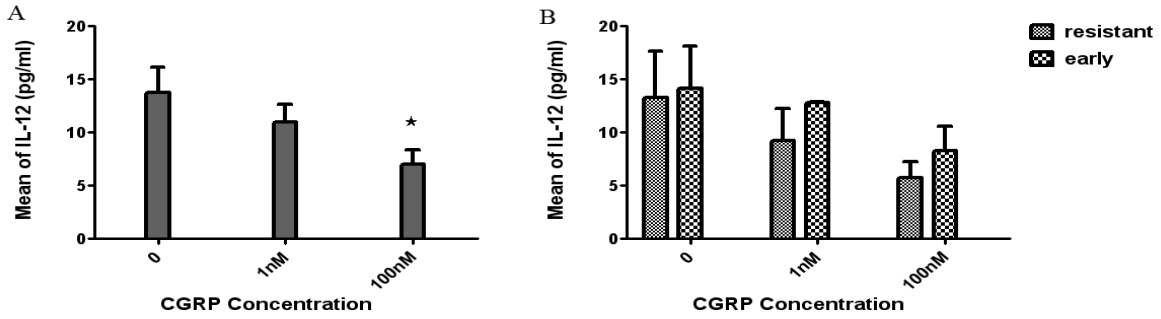

Figure 6. IL-12 production by dendritic cells treated with CGRP, (A) in all patients (early and resistant, collectively) and (B) in two groups of patients (early and resistrant, separately), was altered. Supernatants were harvested on day 8 and IL-12 levels were measured by sandwich ELISA. For DC maturation LPS was applied on day 6. (★*P*= 0.051)

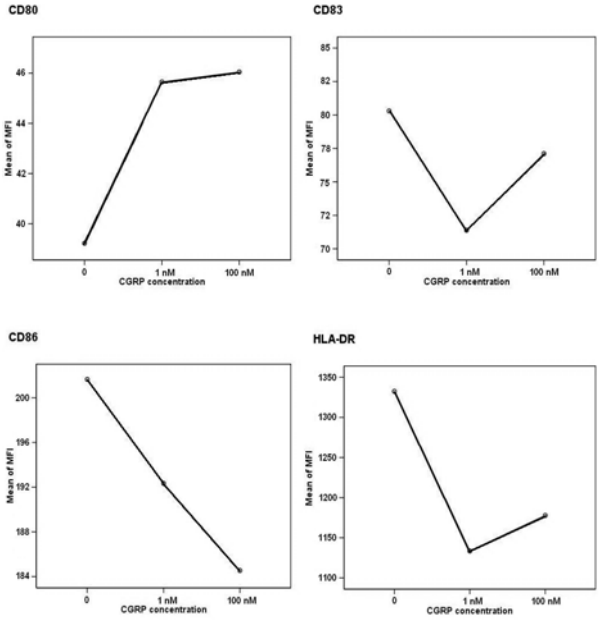

Figure 3. Alterations of cell surface markers expression in generated dendritic cells from monocytes of rheumatoid arthritis patients on day 8 at applied CGRP concentrations. Monocytes were isolated from peripheral blood mononeuclear cells of rheumatoid arthritis patients (early and resistant) and cultured in the presence of GM-CSF and IL-4 for 8 days. LPS and CGRP were added on day 6. CGRP was applied at concentrations of 0, 1 and 100 nM.

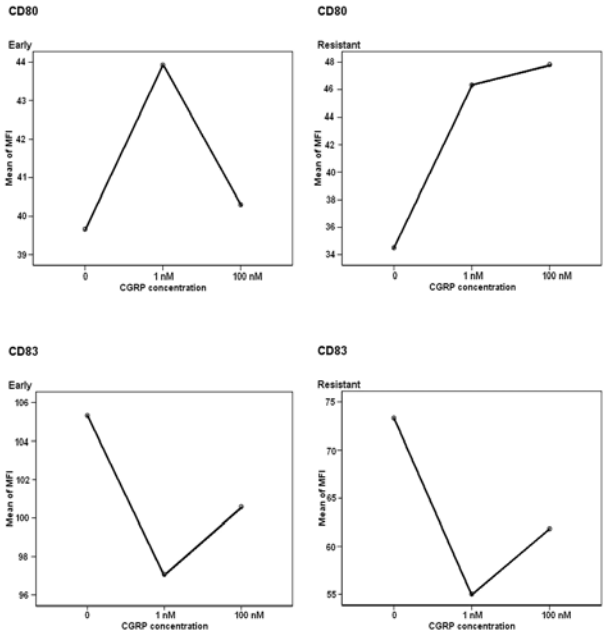

Figure 4. Alterations of CD80 and CD83 expression in generated dendritic cells from monocytes of early and resistant rheumatoid arthritis patients on day 8 at applied CGRP concentrations. Monocytes were isolated from peripheral blood mononeuclear cells of patients and cultured in the presence of GM-CSF and IL-4 for 8 days. LPS and CGRP were added on day 6. CGRP was applied at concentrations of 0, 1 and 100 nM.

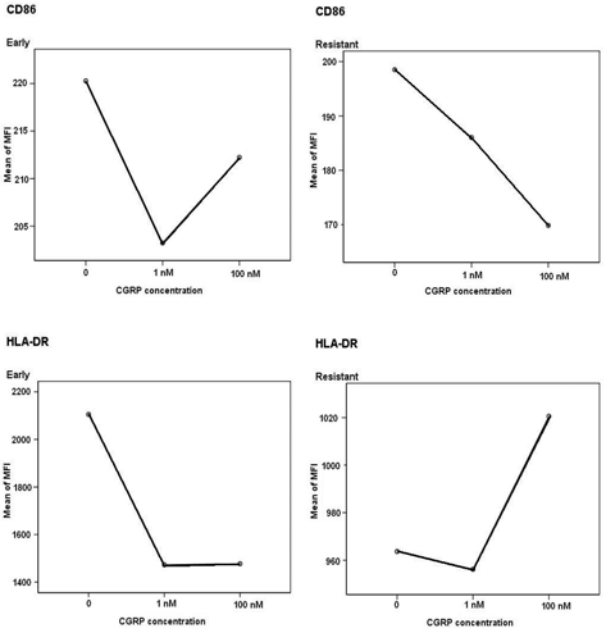

Figure 5. Alterations of CD86 and HLA-DR expression in generated dendritic cells from monocytes of early and resistant rheumatoid arthritis patients on day 8 at applied CGRP concentrations. Monocytes were isolated from peripheral blood mononeuclear cells of patients and cultured in the presence of GM-CSF and IL-4 for 8 days. LPS and CGRP were added on day 6. CGRP was applied at concentrations of 0, 1 and 100 nM.

Discussion

Bidirectional communication between the neuroendocrine and immune systems provides a finely tuned regulatory network that is necessary for health (11). In many inflammatory, infectious or autoimmune diseases, we can see disturbances at any level of these intimate interactions (32, 33). CGRP, as a neuropeptide, is a shared ligand in neuroendocrine and immune systems (34). Many investigators have shown the inflammatory and modulatory effects of this sensory neuropeptide on immune cells (14, 16, 29, 30, 35). In this study, the CGRP tolergenic effects on DCs derived from RA patients was evaluated.

Our results showed both the CGRP-treated cells and the controls differentiate to DCs. CD11c positive cells significantly increased whereas CD14 positive cells significantly decreased. Also, except for CD83, the expressions of other markers (CD80, CD86, and HLA-DR) significantly enhanced at the end of the culture. Non-significant enhancement of CD83 may be correlated to

the increased expression of this molecule in patients' monocytes. It is known that CD83 is pre-formed inside monocytes and rapidly expressed on the cell surface upon activation (36). Additionally, TNF is recognized as an inducer for the human CD83 promoter (37). Therefore, it can be postulated that the inflammatory environment and high production of TNF in RA patients results in monocyte activation and the elevation of CD83 expression.

From a narrower perspective, at a concentration of 1 nM CGRP, the expression of CD83 decreased in comparison to the controls. Although CD83 has long been known as one of the best markers for the evaluation of dendritic cell maturity (37), only a few studies have explored the effect of CGRP on the expression of CD83 in human DCs. Carucci *et al* (19) showed that the DC expression of CD83 was left unchanged by CGRP treatment. It should be noted that their study was performed at a 100 nM concentration of CGRP and on normal human monocytes. On the other hand, we applied two different CGRP concentrations on DCs derived from RA patients.

In the present study, as in other similar works (16, 17, 19), CD80 (B7-1) expression increased and the expression of CD86 (B7-2) reduced in comparison to that of the controls. There are growing evidences that CD80 and CD86 molecules have distinct functional properties, in addition to dissimilar structural features (38-41). These two molecules have different affinities for their ligands, CD28 and CD152 (CTLA-4). CD80 interactions favor CD152 and it can be assumed that CD80 is the major ligand for this molecule. In contrast, CD86 interactions are biased toward CD28. B7-1 and B7-2 have distinct effects on B (42), T, and regulatory T cells. $CD86^{-/-}$ as apposed to $CD80^{-/-}$ DCs could promote TGF-β, IL-10 producing regulatory T cells *in vivo* (39). Moreover, another study has shown that CTLA-4, the major CD80 ligand, plays a critical role in Foxp3 regulatory T cell functions (40). Similar evidences have also been observed in T cell activation (41). Thus, it may be assumed that CD80/CD86 are not interchangeable costimulators. Our finding that CGRP increases CD80 and decreases CD86 expression in DCs in fact supports this theory. This whole process leads to the down regulation of immune responses which is the desired outcome of tolerance induction.

In this study, HLA-DR expression exhibited different behavior in early and resistant patients. As in other similar studies (19, 20), HLA-DR decreased in the early cases of RA compared to the controls. However, in resistant patients, HLA-DR did not decrease in treated specimens when compared to the controls. It is obvious that HLA-DR reduction, as an antigen presenting molecule, can modulate T cell activation and, therefore, control immune reactions. The inability of CGRP to decrease HLA-DR in dendritic cells derived from resistant RA patients could be pertinent to disease progression and associated long term alterations in factors related to cell environmen, particularly cytokines. The various effects of medical treatment should also be considered. It should not be overlooked that even this situation might be helpful in tolerance induction. An increased antigen presentation in the absence of appropriate costimulation (CD86) may lead to the unresponsiveness of T cells.

The CGRP-mediated suppression of IL-12 has been demonstrated by previous similar studies (15-17). The reduced production of IL-12 along with the decreased expression of costimulatory molecules are considered to be important characteristics of tolerogenic DCs (43). Upon maturation, DCs produce high levels of IL-12, an essential $CD4^{+}$ T helper 1 cell-deriving cytokine which promotes inflammatory responses. DC resistance to maturation is necessary for the exhibition of its tolerogenic properties (8, 37).

Less attention has been paid to the neuroimmunoendocrine aspects of RA pathogenesis. Patients with RA show multiple alterations in the endocrine and nervous systems (including CGRP). Both potentially affect different features of the immune system and, therefore, the final disease manifestations (44). The better understanding of complex neuroimmune interactions could lead to

finding novel therapeutic targets for the treatment of many progressive and debilitative inflammatory conditions.

Conclusion

This study demonstrates, for the first time, that CGRP can decrease CD83, CD86, HLA-DR expression, increase CD80 expression and reduce IL-12 production in DCs generated from RA patients' monocytes. These findings indicate that the ability of CGRP to skew *in vitro* generated DCs toward a tolerogenic profile in RA patients. Therefore, we suggest that CGRP may be applicable in the production of tolerogenic DCs in these patients. Further studies are needed to clarify the effects of this neuropeptide on patients' monocyte-derived DC functions.

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