Modulation Effects of Human Immature and Mature Dendritic Cells on Glatiramer Acetate Specific T Cell Lines In Vitro

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Abstract: A large body of evidence demonstrates that dendritic cells (DC) play a pivotal role in the control of immunity by priming and tolerizing T cells. In multiple sclerosis (MS), autoreactive T cells are proposed to play a pathogenic role by secreting pro-inflammatory cytokines, but comparison studies on the effects of immature and mature dendritic cells on the cytokines profile of antigen-specific T cell lines are lacking. To evaluate the actions of dendritic cell maturation on T cell polarization, the effects of immature and mature dendritic cells derived from MS patients on in vitro proliferative responses, and cytokine production by glatiramer acetate (GA)-specific T cell lines (TCL) derived from MS patients were analyzed. The results demonstrated that it is easy to derive GA-specific TCLs from MS patients with high specificity; lipopolysaccharide can efficiently induce DC maturation within 24 hours at a concentration of 5 μg/ml; mature DC showed higher co-stimulatory capacity of GA-specific TCLs than immature DC. GA-specific TCLs produced dominantly IL-2, IL-4, IFNγ and IL-10, but low levels of IL-6. In contrast to immature DC, mature DC enhanced capacity to induce IL-6 and IL-10 secretion, but downregulated IL-2, IL-4 and IFNγ production by GA-specific TCLs. It is concluded that DC maturation status modulating proliferation of TCL and production of cytokines may represent another focus for the study on both immunopathogenesis and immunotherapeutic interventions in MS.

Key words: multiple sclerosis; dendritic cells; glatiramer acetate specific T cell line

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autoimmune diseases are the result of inappropriate antigen presentation of either a self-antigen or an antigen with the capacity to mimic a self-antigen in peripheral lymphoid tissues\textsuperscript{[5]}. Before migrating and infiltrating into the CNS, both helper and cytotoxic T cells must be informed and activated by antigen-presenting cells (APC) in the periphery early during an immune response.

It is widely accepted that APC play a central role in the triggering of both innate and acquired immunity. Among APC, dendritic cells (DC) are the most capable inducers of primary and secondary immune responses\textsuperscript{[6]}. DC capture and process foreign antigens through pattern recognition receptors, combine peptide fragments of these antigens with MHC class I and class II molecules, and stimulate naive CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells. In addition, DC can communicate with T cells directly through cell adhesion molecules or indirectly via cytokines. These interactions influence the differentiation of naive CD4\textsuperscript{+} T cells into Th1 or Th2 cells that mediate cellular and humoral immunity respectively.

Since an imbalance of Th1/Th2 immunity causes the onset of various immune diseases\textsuperscript{[7,8]}, and the ability of dendritic cells to initiate immune responses in naive T cells is dependent upon a maturation process that allows the cells to develop their potent antigen-presenting capacity, but the immunostimulatory properties, functions and phenotypes of DC are linked to their maturation state. It is of great importance to investigate how the functional maturation of DC regulates T cell differentiation. In this study, we want to know whether immature and mature DC, and DC derived from patients with multiple sclerosis and healthy subjects differently influence the proliferation and cytokines secretions of antigen-specific T cell lines (TCLs).

**Materials and Methods**

**Patients**

Blood samples were collected from 15 patients (10 females/5 males, mean age 44, range 29 - 63 years) with multiple sclerosis (MS). The diagnosis of MS was based on accepted criteria\textsuperscript{[9]}.

**Antigens**

Gatiramer acetate (GA, Copaxone, Copolymer 1) was obtained from Huddinge University Hospital (Sweden); myelin basic protein (MBP) was purified from human brain by standard methods\textsuperscript{[10]}; tuberculin purified protein derivative (PPD) was purchased from Sigma.

**Generation and maturation of dendritic cells**

Peripheral blood mononuclear cells (PBMC) were prepared from heparinized peripheral blood (20 - 30 ml) samples by centrifugation over Lymphoprep (density 1.077/ml, Nycomed, Norway) density gradient, washed three times with RPMI 1640 medium. DC were generated from PBMC as described previously\textsuperscript{[11]}. Briefly, PBMC were resuspended in RPMI 1640 medium containing 50 U/ml penicillin (Gibco), 50\(\mu\)g/ml streptomycin (Gibco), 1% non-essential amino acids (Gibco), 2 mmol/L L-glutamine (Flow Lab., U K) and 10% fetal calf serum (Gibco) and allowed to adhere to T-25 tissue culture flasks in humidified atmosphere with 5% CO\textsubscript{2} at 37 °C for 2 hours. After 1 hour, the non-adherent cells were removed, and adherent cells were subsequently cultured for 7 days with recombinant human GM-CSF (800 U/ml) (Leucomax, Switzerland) and recombinant human IL-4 (500 U/ml) (Genzyme, USA). Phenotypic changes were monitored by light microscopy. DC on day 7 of culture were analyzed and used immediately. To induce complete maturation of DC, lipopolysaccharide (Sigma, USA) at a concentration of 5\(\mu\)g/ml was added into the cultures after 6 days growth in GM-CSF and IL-4, and the cells were cultured for another 24 hours.

To collect the floating cells that consist mainly of DC, culture flasks were shaken at 220 rpm for 20 min, followed by two washes by centrifugation in medium, resuspension in complete medium, and cell counting under a light microscope in the presence of trypan blue to exclude dead cells. Properties of DC were analyzed by flow cytometry and used for further studies.

**Generation of antigen specific T cell lines**

Antigen specific T cell lines (TCL) were established by using a split-well technique\textsuperscript{[12]}. Briefly, PBMC (5 \(\times\) 10\textsuperscript{6} cells/ml) were seeded into T-25 culture flasks containing RPMI 1640 complete medium with GA (25 \(\mu\)g/ml) or MBP (40\(\mu\)g/ml) or PPD (5\(\mu\)g/ml). After 7 days culture, recombinant human IL-2 (20 \(\mu\)g/ml) (R & D Systems) were added into the cells. The cells were grown continuously and restimulated with antigen and gamma-irradiated (30 Gy) autologous PBMC (2 \(\times\) 10\textsuperscript{5} cells/ml) every 5 - 7 days.

**T cell line antigen specificity assay**

Antigen specificity of TCL induced above was determined by \(^{3}\text{H}\) thymidine (Amersham, U K) incorporation. In brief, after 3 - 4 weeks the cells were transferred in 96 well round bottom microtitre plates (Nunc, Denmark). Wells containing cells of a TCL (1 \(\times\) 10\textsuperscript{4} cells / ml ) and irradiated autologous PBMC (1 \(\times\) 10\textsuperscript{5} cells / ml ) were incubated in the presence or absence of antigen. After 72 hours, 1\(\mu\)Ci / well \(^{3}\text{H}\) thymidine was added and 18 hours later cells were harvested onto glass fibre filters.
(Skatron, Norway) with an automatic cell harvester and read in a beta liquid scintillation counter (Beckman, USA). Incorporation of radioactivity was expressed as mean counts per minute (cpm) for triplicate cultures. Mean values above 2-fold of baseline (without antigens) were considered as antigen-specific TCL.

**Flow cytometric analysis of cell-surface markers**

Cell surface molecules were analyzed by a flow cytometer (FACScan) with CellQuest software (Becton Dickinson, USA). For surface staining, cells were suspended and incubated with conjugated mouse anti-human monoclonal antibodies (McAb) for 20 min at 4 °C in the dark. After staining, the cells were washed and resuspended in 100 µl of PBS. DC were identified by fluorescence-conjugated McAb for CD80-FITC, CD1α-FITC, CD83-FITC, Lineage-FITC, CD86-PE, CD123-PE, CD11c-PE, CD40-PE and HLA-DR-Percp. The cells of TCL were confirmed by CD4-FITC, CD25-FITC, CD80-FITC, CD8-P, CD40-L-PE, CD86-PE and CD3-Percp. All antibodies for FCM analysis were purchased from PharMingen (San Diego, USA). Viable cells were gated out by forward and side light scatter. The results were expressed as percentages of cells stained positively.

**Co-stimulatory assay**

Allogeneic proliferation assay was set up in triplicate wells by coculturing cells of TCL (2 × 10^4 cells / ml) with gammairradiated (30 Gy) either immature or mature DC (2 × 10^5 cells / ml) in 200 µl of RPMI 1640 medium in 96-well round-bottom microtitre plates. As negative controls, cells of TCL (2 × 10^5 cells / ml) in six replicate wells or DC (2 × 10^6 cells / ml) in triplicate wells were cultured in medium alone. The microcultures were incubated in a humidified CO₂ incubator at 37 °C. At day 3, each well was pulsed with 1μCi [³H] thymidine for another 18 hours, and cells were harvested and counted. Results were expressed as the mean counts per minute (cpm).

**Cytokine production assay**

Quantitative assays for IL-2, IL-6, IL-10, IFNγ and IL-4 concentrations in culture supernatants were performed by using enzyme-linked immunoabsorbent assay (ELISA) kits (PharMingen, USA) with antibody pairs and reagents according to the manufacturer’s instructions. Briefly, supernatant was collected after 72 hours of coculture. Polystyrene microtitre plates (Costar, Germany) were coated with the first antibody diluted in coating buffer overnight at 4 °C. Various concentrations of recombinant cytokine were added as external standards. Standards and supernatants were incubated for 2 hours in dilutions of 1:5. Bound cytokine was detected with biotinylated second antibody, streptavidin-peroxidase conjugate and a chromogenic marker served as the detection system. The reaction was stopped with sulfuric acid and quantified by a spectrophotometer. Results were expressed as the mean concentration of duplicate culture supernatants.

**Statistical analysis**

Paired comparisons (immature DC versus mature DC) were done by Wilcoxon’s signed rank test; the other data were compared using the non-parametric Wilcoxon’s test. Reported P-values are two-tailed and considered statistically significant at P < 0.05. Throughout the text, data are expressed as median and range.

**Results**

**Isolation and characterization of GA-specific TCL**

We isolated a panel of 6 GA-reactive TCL from 6 persons (100%). Two GA-reactive TCL were isolated from mononuclear cells (MNC) of two MS patients stimulated by GA (25 µg/ml) at the onset of induction; four TCL from MNC of four MS patients (three IFNβ treated MS patients; one untreated MS patient) induced by MBP (40 µg/ml) at the first time of culture. All GA-specific TCL showed a proliferative response to GA, but none of the tested TCL proliferated significantly in response to MBP (Figure 1). Interestingly, we failed to get MBP-specific TCL from MS patients by using MBP induction.
The phenotype of TCL was analyzed by flow cytometry. The analyzed TCL were predominantly CD4+ cells (71%) (Figure 2). Small part of cells was activated and expressed CD80 (28%), CD86 (27%) and CD25 (16%) surface molecules.

**Generation and characterization of immature DC versus mature DC**

On day 7 of culture, most of cells were becoming non-adherent and expressed low levels of lineage cell markers, such cells showed the characteristics of immature DC with high expression of CD1a (84%) and CD11c (95%), moderate levels of CD86 (11%) and HLA-DR (23%),

![Diagram showing phenotype of T cell after 4 weeks of culture and phenotypes of immature versus mature DC.](image-url)

Figure 2: Phenotype of T cell after 4 weeks of culture. The analyzed TCL were predominantly CD4+ cells (72%). A part of cells was activated and expressed CD80 (28%), CD86 (27%) and CD25 (16%).

Figure 3: Phenotypes of immature versus mature DC. Maturation of DC is reflected by high expression of co-stimulatory molecules (CD40, CD80, CD86), HLA class II molecule, and by turning on expression of CD83; CD11c is not influenced by maturation with LPS. A: immature DC; B: mature DC.
low levels of CD40 (4%) and CD80 (2%), and almost lack of expression of CD83 (0.6%) and CD123 (0.1%) (Figure 3).

DC maturation is associated with higher expression of MHC and costimulatory molecules, as well as de novo expression of CD83. Immature DC incubated for 24 hours with 5μg/ml LPS displayed enhanced levels of CD83 (25%), CD86 (99%), CD80 (97%), CD40 (46%) and HLA-DR (86%) molecules (Figure 3).

Costimulatory capacity of immature DC versus mature DC on T cell lines

Depending on the cytokines used, mature and immature DC can be derived from peripheral blood. Previous reports had shown that DC grown for 7 days in culture containing GM-CSF and IL-4 are not fully mature, but they can be induced to maturation by incubation with LPS. In the presence of LPS, the numbers of free floating DC increased markedly. These LPS-treated DC displayed a more pronounced morphology with highly motile cytoplasmic processes than that of DC on day 7 of culture. To evaluate whether mature DC are more effective to induce the proliferation response in cells of TCL, LPS-treated mature DC were used in the allogeneic proliferation assay of TCL cells. In contrast to immature DC on day 7 of culture, the LPS-treated mature DC can markedly stimulate Ga-specific TCL cell proliferation (P < 0.05) (Figure 4).

Cytokine production of TCL induced by immature DC versus mature DC

We assessed the ability of Ga-specific TCL to produce IL-2, IL-4, IL-6, IFNγ and IL-10 in response to coculture with immature and mature DC derived from MS patients. Statistical differences were calculated based on the mean values in each of MS patients.

IL-2 production. The mature DC from MS patients down-regulated IL-2 secretion of TCL, but immature DC induced from MS patients can slightly increase IL-2 production; mature DC are more effective in decreasing IL-2 production than immature DC (P < 0.001) (Figure 5A).

IL-4 production. Compared to TCL control group, DC down-regulated IL-4 production of TCL, especially mature DC (P < 0.05), but there is no marked difference in the ability of promoting IL-4 secretion of TCL between immature or mature DC (Figure 5B).

IL-6 production. DC can significantly up-regulate IL-6 production of TCL. Compared to immature DC, mature DC has ability to significantly increase the IL-6 level of TCL (P = 0.001) (Figure 5C).

IFNγ production. In contrast to the same derivative mature DC, the immature DC can induce TCL to produce more IFNγ, but there are not any significant difference between them (Figure 5D).

IL-10 production. Compared to the TCL control group, the mature dendritic cells can slightly up-regulate IL-10 secretion of TCL, but there are not any significant difference between immature and mature dendritic cells for the ability to promote TCL to produce IL-10 (data not shown).

Discussion

MS is characterized by elevated levels of pro-inflammatory cytokines such as IFNγ, TNFα, IL-1 and IL-2 in the peripheral blood[13], CSF[14] and in brain lesions[15]. The association of these inflammatory cytokines with disease activity implies that CD4+ T cells of the Th1 helper type I (Th1)[16] play a pivotal role in the immunopathogenesis of the CNS demyelinating disease.

DC originate from bone marrow, travel through the blood and are seeded into non-lymphoid tissues. Immature DC capture and process exogenous antigens and then migrate via the blood and afferent lymph to secondary lymph nodes where mature DC interact with T cells to facilitate activation of helper and killer T cells[17]. DC from blood are heterogeneous, representing both immature and mature populations or perhaps distinct states of activation and non-activation. Immature DC are defined by surface markers like CD1a that represent the function of endocytosis. Following antigen processing, DC become maturing, losing the endocytic activity and expressing high levels of CD83 and MHC class II molecules. Mature DC home to T cell areas of secondary lymph nodes, where they present antigens as peptide-MHC complex to T cells. DC have been further characterized by morphology, function and phenotype.
However, little is known about the cytokine profiles of DC in general, especially in disease states[18]. It has recently been recognized that cytokines produced by DC play a role in T helper cell differentiation toward the Th1 or Th2 cell type[19].

MS is associated with enhanced T cell reactivities to multiple myelin antigens and a massive up-regulation of several cytokines including IFNγ, TNFα and lymphotoxin, and IL-6 and IL-10. We therefore investigated the effects of immature and mature dendritic cells derived from MS patients on in vitro proliferative responses and cytokine production by glatiramer acetate (GA) specific TCL derived from MS patients.

With the presently used method, it is easy to derive GA specific T cell line from healthy donors with high specificity, but difficult to get MBP-specific T cell line from both MS patients in the presence of whole MBP. The prepared TCL express high levels of markers related to T cells (CD3 88 %) and consist mainly of CD4 positive T cells (CD4 71 % and CD8 15 %), and showed only low levels of other markers (CD40L 26 %; CD25 15 %; CD80 29 % and CD86 27 %). DC with a purity that was higher than 80 % were obtained. The prepared mature DC is reflected by high expression of costimulatory molecules (CD40, CD80 and CD86), HLA class II molecule, and by turning on expression of CD83; CD11c is not influenced by maturation with LPS. The DC, especially mature DC, exhibited high capacity to prime T cells in allogeneic MLR (Figure 4). Mature dendritic cells, in addition to providing costimulation, can define the Th1, in contrast to the Th2, nature of a T cell response through the production of cytokines and chemokines[20].

It was also demonstrated that DC have a high capacity to produce and secrete both Th1 and Th2 cytokines. Cytokines generated by DC are important for initiating T cell dependent immune response. For example, production of IL-6[18] and IL-12[21] by DC promotes T cell activation and differentiation into IFNγ producing Th1 cells, which mediate cellular immune responses. The data obtained clearly show that mature DC show higher costimulatory capacity of GA specific T cell lines than immature DC; GA specific T cell lines produce dominantly IL-2, IL-4, IFNγ and IL-10 but low level of IL-6. Mature DC showed enhanced capacity to induce IL-6 and IL-10 secretion, but down-regulate IL-2, IL-4 and IFNγ production by GA specific T cell lines. This observation can also be confirmed by the previous finding[22].

This study adds additional insight into the mechanism of DC immunomodulatory activities in T cell polarization in MS patients and is, to the best our
knowledge, the first report on the effects of DC maturation on GA− specific T cell lines in human. It has been proposed that the predominant mechanism underlying the immunomodulatory activities of glatiramer acetate resides in the immunological cross-reactivity of glatiramer acetate with MBP and its competition with MBP for MHC class II specific T cell activation231. The suppressive activity of glatiramer acetate has, however, been shown to be organ-specific and limited to inflammatory and autoimmune diseases involving CNS-myelin, such as experimental autoimmune encephalitis (EAE), while no effect was demonstrated in SLE or other autoimmune diseases241.

CD4 + T helper (Th) cells can be divided into subsets based on their characteristic cytokines secretion patterns and effector functions. Cytokines produced by Th1 cells (IL-12, IFNγ, IL-2 and TNFα) are inflammatory mediators of various autoimmune process, including autoimmune demyelinating diseases where oligodendrocytes are a target for immune attack16. Cytokines produced by Th2 cells (IL-4 and IL-10) or Th3 cells (TGFB) mediate antibody production, anti-inflammatory cascade and resolution of inflammatory and autoimmune processes25.

In conclusion, DC maturation has an important effect on GA−specific T cell lines cytokines profile. Further understanding of the mechanism implicated in the immunomodulatory effects of DC, may contribute to the design of future immunotherapeutic cocktails for multiple sclerosis.

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