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Spleen and Liver Dendritic Cells Differ in their Tolerogenic and Cytokine Induction Potential

Ghasem Mosayebi [1,2] and Seyed Mohammad Moazzeni[2]

[1] Molecular and Medicine Research Center, Department of Immunology, School of Medicine, Arak University of Medical Sciences, Arak, Iran
[2] Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

Dendritic cells (DCs) play an important role in induction of cellular immune responses. It seems that DCs that reside in different organs may be distinct in their ability to induce immune responses. This study was done to address the differences between spleen and liver DCs in induction of immune response and/or tolerance.

$CD11c^+$ DCs were separated from the liver and spleen of C57BL/6 mice and pulsed with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55. 6×10^5 MOG35-55 pulsed spleen or liver DCs were injected in foot pad of different groups of mice. Control groups received unpulsed DCs. After 5 days, the mononuclear cells (MNCs) of the regional lymph nodes were isolated from immunized mice for cytokine assays and lymphocyte transformation test. To study the immunologic or tolerogenic effects of DCs, three weeks after immunization of mice with MOG pulsed liver or spleen DCs, experimental autoimmune encephalomyelitis (EAE) was induced in DC-immunized mice by injection of MOG along with complete Freund's adjuvant. Our results showed that spleen DCs were more potent in stimulating lymph node T cells as illustrated in lymphocyte transformation test. Moreover IL-10 production was higher in mice immunized with liver DCs compared with those immunized with splenic DCs (p=0.017). However, no significant difference in IFN-γ production was observed between two groups. We also found that liver DCs+MOG immunized mice displayed a significantly delayed disease onset compared with spleen DCs+MOG immunized mice and the control groups. The disease score was also milder in liver DCs immunized mice compared with other groups.

It seems that the higher IL-10 production induced by the liver DCs may be one of the main factors in down regulation of immune responses in this organ. It can be concluded also that the liver DCs may inhibit the progress of EAE by shifting the cytokines profile.

Key Words: Cytokines Profile; Experimental Autoimmune Encephalomyelitis; Liver Dendritic Cells; Splenic Dendritic Cells

Corresponding Author: Seyed Mohammad Moazzeni, PhD; Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Tel/ Fax: (+98 21) 8288 3846, E-mail: mozzeni@modares.ac.ir

INTRODUCTION

Dendritic cells (DCs) constitute a specialized cell population that is present at low frequency, in several lymphoid and non-lymphoid organs and is responsible for capturing and presenting antigens to T cells.[1,2] These cells are unique in their ability to stimulate T cells and initiate adaptive immunity.[3] DCs not only induce immunity, but also maintain tolerance to self-antigens.[4-6] The capacity of DCs to initiate or modulate immune responses appears to depend on their lineage development, microanatomical location and stage of phenotypic and functional maturation.[7]

In mice DCs have been classified into at least two populations, $CD8\alpha^+$ and $CD8\alpha^-$ DCs.[8,9] The $CD8\alpha^-$ DCs effectively prime naïve $CD4^+$ T cells and preferentially induce Th2 differentiation in vivo, whereas the $CD8\alpha^+$ DCs induce Th1 differentiation.[10,11]

Although it is well established that DCs in secondary lymphoid tissues (e.g. the spleen) are potent activators of naïve T cells, DCs that reside within non-lymphoid organs (e.g. the liver) are deficient in allostimulatory reactions and can exhibit tolerogenic properties.[12,13] There is evidence that donor-derived DCs may play a role in the immune privilege of liver allografts.[14,15] The regulatory role of DCS in some tissues may be attributed to their subpopulations. However, previous studies have indicated that the ratio of $CD8\alpha^+$ DCs to $CD8\alpha^-$ DCs in the spleen and Peyer's patches are similar but Peyer's patches DCs were capable of differentiating naïve $CD4^+$ T cells *in vitro* into Th cells secreting lower amounts of IFN-γ and a high level of IL-10 and IL-4.[12,16] These findings suggest the role of microenvironment on DCs function as well. Regarding the hepatic DCs, the subpopulation of DCs may not be the only reason for liver tolerogenecity and other factors such as the immaturity of liver DCs or the effect of the liver microenvironment on these cells may explain the lower immune response in this organ and acceptance of hepatic allografts.[17,18]

There are reports regarding the properties of liver derived-DCs from GM-CSF or Flt3-L treated mice however because of inherent difficulties in isolating adequate numbers of DC from the mice liver, limited information is available on the effect of DC of normal mouse liver on tolerance induction.[19,20] In this study, we used experimental autoimmune encephalomyelitis (EAE) as a model for comparison of spleen and liver DCs in the induction of immunity or tolerance.

MATERIALS AND METHODS

Animals

Adult 6-8 weeks old female C57BL/6 mice were prepared from the Razi Vaccine and Serum Research Institute of Iran. Mice were kept under optimal conditions of hygiene, temperature, and humidity with 12 hours light: 12 hours darkness cycle and were allowed food and water *ad libitum*. All experimental procedures on animals were approved by the ethical committee of Faculty of Medical Sciences, Tarbiat Modares University.

Preparation of DCs from the Spleen

The procedure was followed according to our previous report with some modifications.[22] Briefly, spleen fragments were digested for 30 min at 37°C with collagenase D (1 mg/ml) and DNase-I (20 μg/ml, both from Roche, Mannheim, Germany) in RPMI-1640 medium(Sigma-Aldrich, USA) then treated for 5 min with ethylene diamine tetraacetic acid (EDTA) to disrupt T cell-DC complexes.

Low density cells were separated by centrifugation over a 13% nycodenz (Pharma AS, Oslo, Norway) medium and cultured in RPMI 1640 for 90 min at 37°C and 5% CO_2. Afterwards the non-adherent cells were harvested and adherent cells were cultured overnight in the same medium. The floated cells during overnight culture were harvested and used as DC enriched cells. Flow cytometric analysis showed that more than 90% of these cells were positive for CD11c antigen.

Isolation of DCs from the liver

Liver DCs were separated from non-parenchymal cells (NPCs) fraction of the liver. NPCs were isolated from the liver of mice, as previously described by Woo et al, [21] with the following modifications. Mice were anesthetized with ketamine, swabbed with 70% ethanol and an abdominal mid-line incision was performed. The liver was perfused for 3 min in situ via the inferior vena cava, using 20-30 ml ice-cold phosphate buffer saline (PBS) and a 22G intravenous catheter.

Two ml of collagenase D solution (1mg/ml) in RPMI-1640 was then injected. The liver was excised immediately, diced into small pieces and digested in collagenase solution (5 ml/liver) for 30 min at 37°C, with constant stirring. The digested tissue was then filtered through a 0.1 mm sterile wire mesh. Cells from three to four livers were pooled.

The cell suspension was then washed twice with PBS or RPMI-1640 medium by centrifugation at 400g for 5 min at 4°C. The final pellet was re-suspended in 10-15 ml RPMI medium and overlaid on a nycodenz (Pharma AS, Oslo, Norway) gradient 14.5% (w/v) and centrifuged at 600 g for 15 min at 4°C.

The recovered low density NPCs from the interface were collected using a Pasteur pipette and was washed as described above. To enrich the DCs, NPCs were suspended in complete tissue culture medium (RPMI-1640 containing 10% FCS) and incubated overnight (18 hr) at 37°C, 5% CO_2 in tissue culture petri dishes. At the end of the incubation time non-adherent cells were recovered and layered on a 3 ml column of 13% (w/v) nycodenz and centrifuged at 600g for 15 min at 4°C.

Low-density cells referred as "DC-enriched cells", were carefully separated from the interface, washed and used for Ag pulsing and immunization of mice. Flow cytometric analysis of these cells by anti-CD11c antibody revealed their purity to be more than 70%.

***In vitro* Pulsing of DCs**

Dendritic-enriched cells were incubated with MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK; Diapharm Ltd, Russia) (200 µg/ml) for 4–6 h at 37°C and then washed twice to remove the excess antigen.

Immunization of Mice with Peptide Pulsed DCs

6×10^5 spleen or liver MOG pulsed DCs in PBS were injected into the hind footpads of naïve C57BL/6 mice (50 µl/foot pad). Control groups received unpulsed spleen or liver DCs.

Five days post-immunization, mice in each group were sacrificed and cell preparations were made from their popliteal lymph nodes and used as a source of primed lymph node cells in the cytokine assays and lymphocyte transformation test.

To compare the immunity or tolerance induction effects of the liver and spleen DCs other groups of mice (n=6) were immunized s.c. with the same number of spleen or liver MOG pulsed or unpulsed (controls) DCs in the flank region. These mice were monitored daily for EAE symptoms for 3 weeks and finally were used for EAE induction by classical EAE induction method.

Cytokine Assay and Lymphocyte Transformation Test (LTT)

Lymph node cells (2×10^5 cells/well) were cultured in click medium (Sigma-Aldrich, USA) containing 1% normal mouse serum and 200 µg/ml MOG35-55 peptide as antigen. All cultures were done in triplicates with negative control wells containing no antigen. The cells supernatants were harvested after 72 hours of culture for analysis of IFN-γ and IL-10 concentrations by a sandwich ELISA kit with sensitivity of 0.762 pg/ml and 4 pg/ml respectively (BD Biosciences, USA).

For lymphocyte transformation test, the incubation period was extended for 80 hours in CO2 incubator. One µCi of [3H] thymidine (GE healthcare, Sweden) was then added to each culture well and the incubation period continued for 18 hours. The cultures were harvested onto glass fiber filter paper and counted in a liquid scintillation counter (Wallac 1410, GE healthcare). Results were expressed as the mean counts per minute (CPM) of the triplicate cultures.

Induction of EAE in DCs Immunized Mice

After three weeks follow up of pre-immunized mice with MOG pulsed or unpulsed spleen and liver DCs, when no clinical symptoms of EAE was observed, the classical EAE was induced. These mice were inoculated subcutaneous in the flank with 100µl of an emulsion containing 200µg of the encephalitogenic peptide MOG and equal volume of complete Freund's adjuvant (Sigma-Aldrich, USA) supplemented with 4 mg /ml mycobacterium tuberculosis H37RA (Difco, USA). Mice were then injected intra-peritoneally with 400ng of pertussis toxin (Sigma-Aldrich, USA) on the day of immunization; this was repeated 2 days later.

Clinical Evaluation of EAE

Mice were monitored daily and neurological impairments were scored on an arbitrary clinical score as follows: 0, no clinical sign; 1, partial loss of tail tonicity; 2, complete loss of tail tonicity; 3, flaccid tail and abnormal gait; 4, hind leg paralysis; 5, hind leg paralysis with hind body paresis; 6, hind and foreleg paralysis; 7, moribund or death. The day of onset of disease was considered as mean clinical score of 1 for each group and the relapse was defined when a mouse developed an increase of the clinical score (more than 1) accompanied by weight loss.[23]

Statistical Analysis

All results are presented here as Mean±SD of at least six different experiments. U-Mann Whitney test was used for evaluation of statistical differences between the results. The changes in clinical scores in each group were analyzed by Friedman test. *P* values less than 0.05 were considered statistically significant.

RESULTS

EAE Induction

To investigate the role of spleen and liver DCs in induction of immunity or tolerance, these cells were separated from the spleen and liver of C57BL/6 mice by an enzymatic method and pulsed with MOG peptide for 4–6 hours. 6×10^5 pulsed DCs were injected subcutaneous to the flank region of mice. The control groups received unpulsed spleen or liver DCs. The results showed that mice receiving spleen or liver DCs pulsed with the MOG failed to develop clinical EAE even 3 weeks post-immunization. To investigate the probable role of injected DCs in tolerance induction, we induced classical EAE at the end of the third week by injection of MOG peptide along with complete Freund's adjuvant in mice preimmunized with pulsed and unpulsed DCs.

The obtained results indicated that there is no significant difference in the day of onset and mean clinical score of disease between animals immunized with unpulsed spleen or liver DCs (11±1) and classical EAE controls (11±1). While, there is a significant difference in the day of onset of disease between animals immunized with MOG pulsed liver DCs (17±1) compared with MOG pulsed spleen DCs (9±1) and classical EAE control group (11±1) (p=0.01, Figure 1). The disease symptoms were also milder in mice immunized with MOG pulsed liver DCs compared with mice immunized with MOG pulsed spleen DCs and other groups (p=0.01, Figure 1).

***In vivo* Priming of T Cells:**

Spleen and liver DCs were pulsed with the MOG peptide and injected into the footpad of two different groups of mice. Control groups received unpulsed DCs. After 5 days, the mononuclear cells (MNCs) of the regional (popliteal) lymph nodes were isolated and cultured in the presence or absence of MOG35-55 peptide. Results of LTT showed that administration of purified DCs loaded ex vivo with antigen, resulted in T cell priming, as assessed by MOG-dependent proliferation in culture (Table 1).

Totally, the mean CPM of test wells (lymphocytes of mice injected with pulsed DCs) was significantly higher in the presence of antigen than in its absence (p=0.004). As expected, the mean CPM of control wells (lymphocytes of mice injected with unpulsed DCs) was not statistically different in the presence (1240±130) or absence (1000±100) of antigen. Furthermore, these data showed that the basal lymphocyte proliferative response of the test and control groups is approximately the same in the absence of stimulating antigen. The proliferative response of Ag-pulsed splenic DCs injected group, however, was significantly higher than liver DCs injected group when lymphocytes were re-stimulated *in vitro* (p<0.05).

Induced Cytokine Profiles by Liver and Spleen DCs

Mononuclear cells (MNCs) of the regional lymph nodes from mice immunized with MOG pulsed splenic and liver DCs were isolated and cultured in the presence or absence of MOG35-55 peptide for 72 hours. The supernatant of cultured cells was harvested and IFN-γ and IL-10 concentrations were measured by sandwich ELISA.

Table 1. The proliferation response of regional lymph node lymphocytes from mice immunized with MOG35-55-pulsed and unpulsed spleen and liver DCs in the presence and absence of MOG35-55 peptide

Topics	Counts per minute (CPM)	
	+MOG35-55	-MOG35-55
MOG35-55- pulsed splenic DCs	10300±356	1120±150
MOG35-55- pulsed liver DCs	8200±285	800±170
Unpulsed splenic DCs	1240±130	1000±100
Unpulsed liver DCs	800±50	960±60

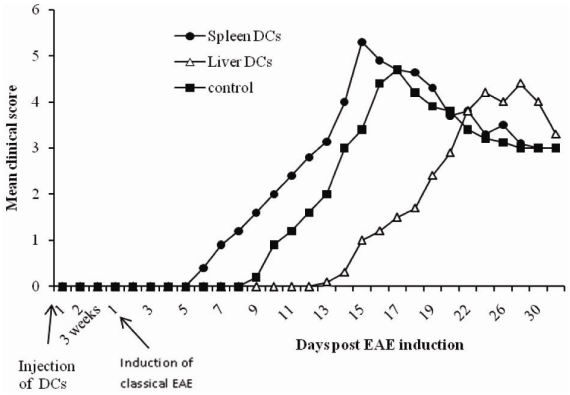

Figure 1. EAE induction in mice pre-immunized by MOG35-55 pulsed liver or spleen DCs.

Investigation of the cytokine profile showed that IFN-γ and IL-10 production in mice immunized with pulsed DCs was significantly higher than control group (p=0.004).

Higher levels of IFN-γ was produced by lymphocytes of mice immunized by spleen DCs compared to liver DCs immunized mice however this difference was not statistically different While the amount of IL-10 production by lymphocytes of liver DCs immunized group was significantly higher than spleen DCs immunized group (p=0.017) (Figure 2).

The ratio of IFN-γ to IL-10 in mice immunized with MOG pulsed splenic DCs (15.6±2) and those immunized with MOG pulsed liver DCs showed also a significant difference (3.7±1, p=0.014).

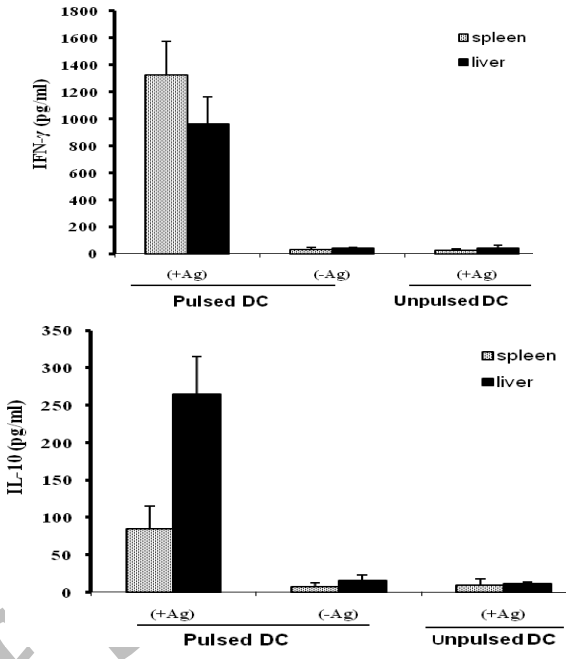

Figure 2. Cytokine secretion by lymph node cells of mice pre-immunized by MOG35-55 pulsed liver or spleen DCs. female normal C57BL/6 mice were immunized with spleen (⊠) and liver (■) MOG35-55-pulsed DCs and unpulsed DCs. Five days post-immunization, IFN-γ and IL-10 secretion by MNCs isolated from the popliteal lymph node of mice in the presence (+Ag) and absence (-Ag) of MOG 35-55 peptide were detected by ELISA.

DISCUSSION

Functional heterogeneity and functional plasticity of DCs provide an effective strategy to manipulate the immune response in a desirable way.[24] There have been some advances in the development of DC-based immunostimulatory and immunosuppressive strategies, which are potentially appropriate to the treatment of cancer, autoimmune disease, allograft rejection, allergy and graft versus host disease.[25-28]

DCs may promote T cell immunity or tolerance, depending not only on their precursors, but also on the microenvironmental factors among which they differentiate.[29,30] There are evidences that donor-derived DCs may play a role in the immune privilege of liver allografts.[15,31]

It is proposed that the function of liver DCs in induction of the immune response is different from that of their splenic counterparts.

Herein, we have analyzed the T cell stimulatory capacity and influence on Th1 and Th2 cytokine production of liver DCs compared with splenic DCs. Our results showed that the ratio of IFN-γ to IL-10 secretions, as representatives of Th1 and Th2 cytokines, by MNCs from mice immunized with splenic DCs was significantly higher than the same ratio in mice immunized with liver DCs. It was reported that DCs isolated from Peyer's patches also secrete higher levels of IL-10 than splenic DC and are able to induce the differentiation of Th2 cells.[16] This findings suggest that high IL-10 production by liver DCs may also contribute to induction of Th2 responses and might be the mechanism underlying the capacity of liver allografts to subvert host immune responses and tolerance induction. It has been reported that resident

DCs are the main population of non-parenchymal liver cells which are responsible for the tolerogenic properties of the liver. These cells mediate immunosuppression by production of anti-inflammatory cytokines such as IL-10 and TGF beta as well as by expression of the negative co-stimulator for T cell activation programmed cell death ligand-1 (PD-L1).[32]

There is a great deal of evidence that DCs in the target organ are central to the immunopathogenesis of EAE and other Th1-mediated autoimmune diseases.[33] Therefore EAE was used as a model in this study to address the role of spleen and liver DCs in induction of immunity or tolerance. In our experimental system, none of the spleen or liver DCs pulsed with the MOG35-55 peptide could induce EAE in naïve C57BL/6 mice by 3 weeks post-immunization. In our previous study we showed that only after 3 subcutaneous injections of MOG pulsed bone marrow derived DCs along with pertussis toxin, mice showed mild clinical symptoms of EAE.[34] However, Weir et al. showed that intravenous injection of bone-marrow derived dendritic cells presenting MOG35-55 peptide into naïve C57BL/6 mice could induce EAE.[35] These finding indicates that DCs presenting a self-peptide possess the ability to activate naïve self-reactive T cells and induce autoimmune disease.

In contrast, Huang et al. showed that pretreatment of Lewis rats with a subcutaneous injection of autoantigen-pulsed plastic adherent DCs induced tolerance to EAE. However the same effect was not observed when using non-adherent floating DCs.[36] It has been shown that targeted expression of MOG to DCs can promote tolerance induction and manifest a significant delay in the development of EAE.[37]

Therefore, to study the probable tolerogenic effect of injected DCs, we induced classical EAE in liver and spleen DC of pre-immunized mice. Our results showed that there is a significant difference in the day of onset of disease between animals immunized with MOG35-55-pulsed liver DCs (17±1) compared with MOG-pulsed spleen DCs (9±1) and classical EAE control group (11±1). The disease symptoms were also milder in mice immunized with pulsed liver DCs compared with mice immunized with pulsed spleen DCs and other groups.

DCs form a heterogeneous cell population.[38] $CD8\alpha^+$ DCs may play a role in maintaining peripheral tolerance, whereas $CD8\alpha^-$ DCs appear to be important for inducing immune responses.[38,39] A difference in the frequency and ratio of DC subtypes appears to be one of the reasons for the difference in immune response in various lymphoid tissues. However, a number of reports have demonstrated that the ratio of $CD8\alpha^-$ DCs to $CD8\alpha^+$ DCs in the liver and spleen is almost identical.[40,41] We also did not observe a significant difference in this ratio between liver and spleen (data not shown).

With reference to the similar percentage of CD11c+/CD8α+ DCs and CD11c+/$CD8\alpha^-$ DCs in the spleen and the liver, it seems that microenvironmental factors and their effect on DCs have a stronger effect on the development of immune responses compared to the phenotype of the DCs. In fact, it was shown that liver sinusoidal endothelial cells impair the DCs ability to induce the proliferation of naïve T cells *in vitro* via an unknown mechanism.[42]

In agreement with the effects of microenvironment, Xia et al. also used the liver fibroblastic stromal cells to mimic the liver microenvironment and found that liver stroma could induce the differentiation of DCs with low CD11c, MHC II but high CD11b expression, high IL-10, but low IL-12 secretion from Lin CD117 + progenitors. Such regulatory DCs could inhibit T-cell proliferation, induce apoptosis of the activated T cells, and dampen the damage of autoimmune hepatitis.[31]

It has also been reported that the route of immunization determines whether immunity or tolerance is induced.[43] Subcutaneous injection of DCs pulsed with MBP was reported to induced tolerance to EAE in rats, whereas intravenous injection of peptide-pulsed DCs in the mouse generated encephalolitogenic T cells.[35,44] We also injected pulsed spleen or liver DCs via the intravenous route but this treatment could not induce any signs of EAE (data not shown). However, the inability of spleen or liver-pulsed DCs to induce EAE may be due to the low number of DCs injected. Although DCs are highly potent activators of T cell immunity and a few DCs appear to be sufficient to induce an immune response.

The frequency of auto-reactive T cells is another important factor in emersion of autoimmune response.[45] Our LTT results showed that spleen derived DCs are more potent stimulator of T cells than liver DCs.

Indeed the intensity and day of onset of EAE induction in mice pre-immunized by different DCs were compatible with T cell stimulatory potential and the induced cytokine profile by these cells. It is

possible that splenic DCs increase EAE severity by higher level of T cell stimulation and shifting the response toward the Th1 type, while liver DCs down regulate EAE intensity by shifting the response toward the Th2 type.

CONCLUSION

It can be concluded that the ability of liver DCs to induce immunity is different from that of splenic DCs and this difference may contributes to immune privilege of liver.

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