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MORPHOLOGICAL CHANGES IN LYMPH NODES AND SPLEEN UPON EAE INDUCTION IN C57BL/6 MIC

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Abstract - Myelin oligodendrocyte glycoprotein (MOG) is a protein widely used in the induction of experimental autoimmune encephalomyelitis (EAE) for studying human multiple sclerosis (MS). In C57BL/6 female mice aged eight weeks, we administered subcutaneously MOG_{35-55} peptide in CFA (complete Freund's adjuvant) along with pertussis vaccine injected intraperitoneally. We observed the sign of flaccid tail as early as thirteen days post-immunization in five of twelve animals. Hematoxylin and eosin staining of paraffin-embedded sections of lymph nodes and spleen revealed the presence of germinal centers in the immunized animals. In the control group of animals, lymphoid follicles without germinal centers were observed. Immunohistochemical staining of spleen sections revealed an expression of MHC II molecules in the EAE-induced group. We would like to point out that even though the clinical signs are mild, the morphological changes are apparent in the lymph nodes and spleen of MOG_{35-55}-immunized mice.

Key words: EAE, MOG, lymph nodes, spleen

INTRODUCTION

The immune system is functionally compartmentalized into primary lymphoid organs responsible for the generation and differentiation of naive T and B cells, and secondary lymphoid organs where immune responses are initiated. Only after activation do T and B cells emigrate from secondary lymphoid organs to seek the antigen in the periphery. Secondary lymphoid organs include the spleen, lymph nodes and organized lymphoid tissues associated with mucosal membranes such as the tonsils, appendix, and Peyer's patches. These highly organized secondary lymphoid organs provide the structures where the antigen is efficiently retained and presented and where ordered cellular interactions between antigen presenting cells (APCs), T cells and B cells take place to initiate and promote efficient immune responses (Karrer et al., 1997, Abbas et al., 1997).

Experimental autoimmune encephalomyelitis (EAE) is an animal model that recapitulates many clinical and pathological features of human multiple sclerosis (Batoulis et al., 2011, Stojkov et al., 2006). EAE induced by immunizing C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 is particularly useful because, like MS, it results in inflammation, demyelination and axon loss, primarily in the spinal cord (Jones et al., 2008; Costa et al., 2003). Neuronal death and gray matter atrophy was documented in mice with EAE (MacKenzie-Graham et al., 2006). Severe exacerbation

was found in mice with abated overall expression of MHC II molecules by plasmacytoid dendritic cells previously immunized with myelin oligodendrocyte glycoprotein (Irla et al., 2010).

We examined the morphological changes in lymph nodes and spleen tissue and the expression of MHCII molecules in sections of the spleen of C57BL/6 mice with EAE induced by MOG_{35-55}.

MATERIALS AND METHODS

Animals

Female C57BL/6 8-week-old mice were purchased from the Military Medical Academy (Serbia). The mice were kept under standard laboratory conditions (room temperature 21±1°C, humidity 30%, 12/12 h light/dark cycle) with food and tap water *ad libitum*. The protocols for the animal experiment were conducted in accordance with the guidelines (86/609/EEC) of the European Community Council Directives and the Serbian Laboratory Animal Science Association – SLASA. The study was performed on 19 mice divided in two groups: EAE-induced (mice immunized with MOG in CFA; n=12) and an IC group (non-treated animals; n=7).

EAE induction

C57BL/6 mice were injected subcutaneously in the right side of the flank with 100 μg of myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}, Sigma Aldrich) in complete Freund's adjuvant (CFA) containing 1 mg/ml of heat-killed and dried *Mycobacterium tuberculosis* H37Ra (Sigma Aldrich) according to Suter et al. (2000). $1x10^9$ of heat-killed *Bordetella pertussis* bacilli in 150 μl of PBS (pertussis vaccine, Institute of Virology, Vaccine and Sera "Torlak") was injected intraperitoneally on the day of immunization and 48 h later.

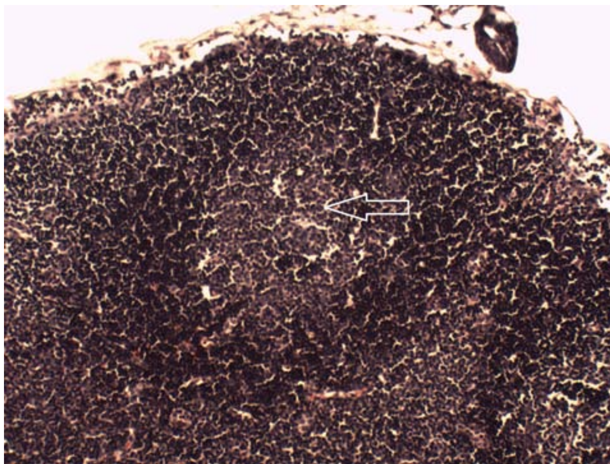
Development of the clinical signs of EAE in the mice was monitored daily, starting at day 7 post immunization according to the following criteria: (0), no clinical signs; (1) flaccid tail; (2) hind leg paresis; (3) full hind leg paralysis; (4) quadriplegia; (5) death. The mice were sacrificed on day 24 after immunization. Over this period of time the mice showed the flaccid tail sign that was less severe than typically observed, and were scored as 0.5 less than the indicated grade.

Tissue collection

All mice were placed under deep anesthesia, and then cardially perfused with PBS pH 7.4 followed by 4% PFA (paraformaldehyde) in 0.1 M phosphate buffer. The organs were preserved in 4% formaldehyde for later histological and immunohistochemical examination. Formalin-fixed paraffin-embedded tissue was cut to 5-μm thick sections of the spleen and lymph nodes by a microtome (Microtome HM 450; Leica). The sections of each experimental group were then stained with hematoxylin and eosin to visualize cell morphology in the tissue sections.

Immunohistochemistry

Paraffin-embedded sections of the spleen (5 μm thick) were harvested onto superfrost slides (ThermoScientific, Menzel, Braunschweig, Germany), deparaffinized in xylene and rehydrated in graded series of alcohols (2x100% 5min, 1x96% 5min, 1x70% 5min). Antigen retrieval was performed by the heat-induced microwave retrieval method, using citrate buffer pH 6. The slides were washed three times in TBS (Tris Buffer saline) and then incubated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. Followed by a series of washes (3× PBS pH 7.4), the sections were then blocked with 10% normal rabbit serum. The sections were incubated for 1 h at room temperature or overnight at +4°C in a humidified chamber with a primary anti-MHC class II antibody (1:50 anti-mouse MHC II; AbD Serotec, Oxford, UK). After three washes with TBS, the sections were incubated with peroxidase-labeled rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) (1:1000) for 1 h at room temperature. Subsequently, all slides were rinsed in distilled water, counterstained with hematoxylin, dehydrated in graded series of alcohols, mounted and cover-slipped.



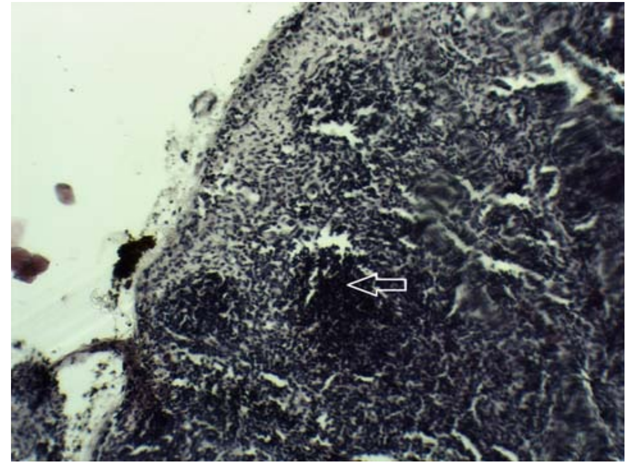
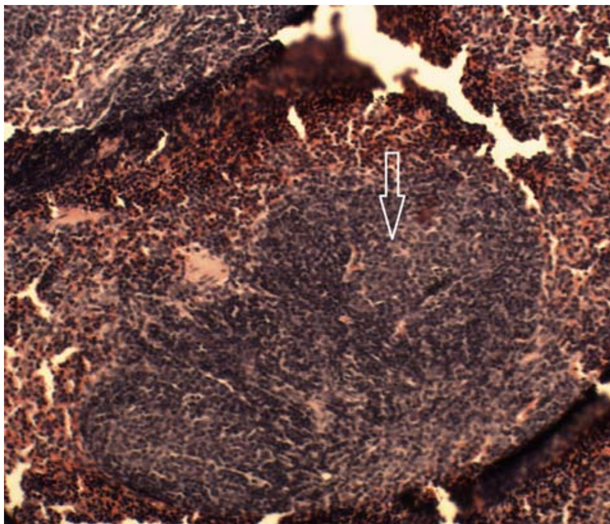


Fig. 1. Hematoxylin and eosin staining of the lymph node. Lymph node cortex of EAE-induced group and control group (photomicroscopic images) (Fig. 1A, Fig. 1B). The germinal center of EAE-induced group and lymphoid follicles of control group is marked by arrows. Magnification x10.



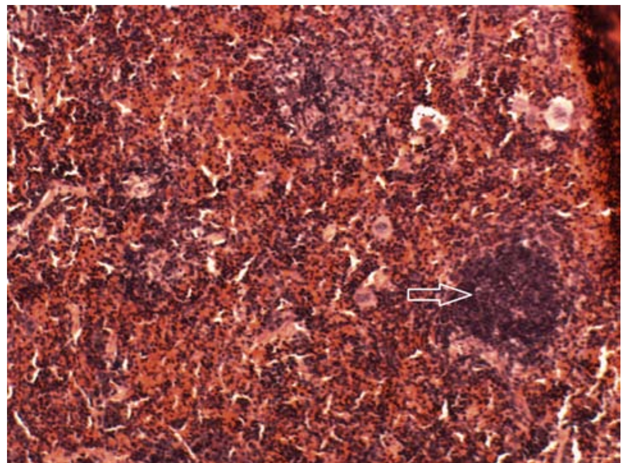


Fig. 2 Hematoxylin and eosin staining of spleen sections. Spleen white pulp of EAE-induced group and control group (photomicroscopic images) (Fig. 2A, Fig. 2B). The enlarged follicle of EAE-induced group with germinal center is marked by vertical white arrow. The follicle without germinal center of the control group is marked by a horizontal white arrow. Magnification x10.

Tissue analysis

The analysis of tissue sections was performed by capturing images of sections using a BH2 research microscope (Olympus Optical Co. LTD. Tokyo, Japan) equipped with a Color View III digital camera (Olympus). Analysis Docu software (Olympus) was used to acquire images. All images were taken under 10x and 40x magnification.

RESULTS AND DISCUSSION

Clinical signs of EAE in C57BL/6 mice

All immunized animals were monitored for clinical signs of EAE development, starting at day 7 post-

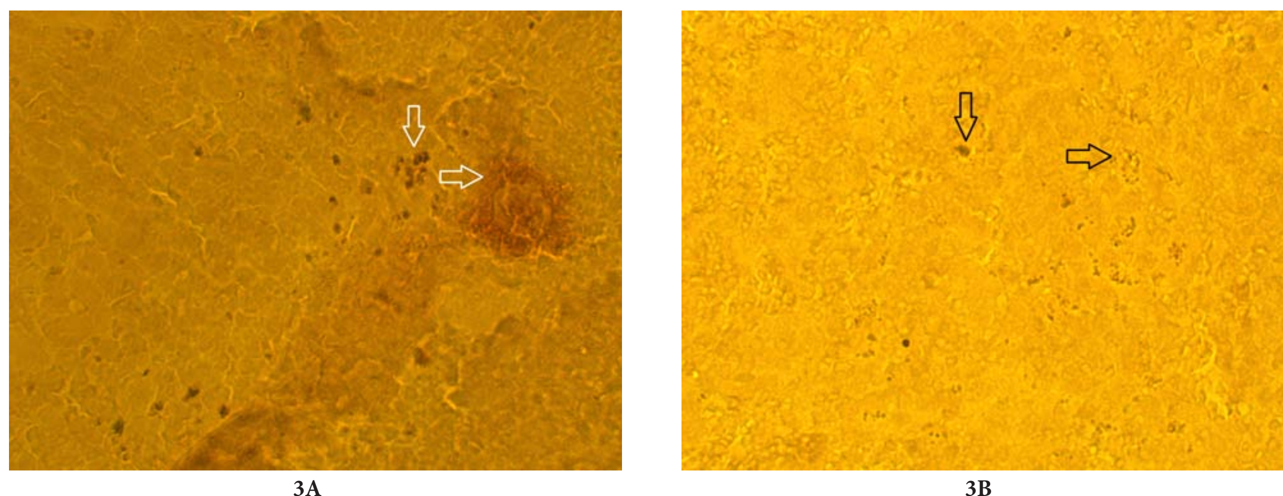


Fig. 3 Immunohistochemical staining of MHC II molecules in spleen sections. EAE-induced group and control group (photomicroscopic images) (Fig. 3A, Fig. 3B). MHC II expression in both groups is marked by arrows. Magnification x40.

MOG immunization. A clinical sign such as a flaccid tail was observed in 5 of 12 animals on day 13 after immunization. Aggravation of clinical signs was not observed on day 24 post immunization when the animals were killed. We further performed histological analysis of the lymph nodes and spleen, and immunohistochemical staining of the MHC II antigen in the sections of the spleen.

Presence of germinal centers in the lymph nodes and spleen in C57BL/6 mice

In the lymph nodes and spleen of both immunized and control groups, lymphoid follicles – B cell areas along with T cells located predominantly between the follicles, were observed. Histological examination of the lymph nodes and spleen from the EAE-induced mice showed an increased number of pyroninophilic cells in the germinal centers of the lymphoid follicles (Fig. 1A, Fig. 2A). T and B cell response to subcutaneously introduced MOG_{35-55} peptide may trigger a cascade of events leading to germinal center formation. Germinal centers that develop in response to antigen stimulation are the sites of B cell clonal expansion. They are functionally polarized into a dark zone in which the B cells divide and a light zone in which the B cells are activated, and selected based on their affinity for the antigen (Victora and Nussenzweig, 2012). In the non-treated group, the presence of lymphoid follicles without germinal centers in the lymph nodes and spleen was observed (Fig. 1B, Fig. 2B). Histological analysis of the spleen showed visible changes in the lymphoid follicle of the spleen white pulp in the MOG_{35-55}-immunized mice (Fig. 2A). The white pulp of the spleen consists of T cell areas and B cell follicles resembling the organization of the lymph nodes. This structure is considered one of the most important inductive sites of the immune system, where trafficking and interactions among dendritic cells, T cells and B cells take place upon antigen challenge (Mebius and Kraal, 2005). We observed a positive correlation between the presence of germinal centers in the lymph nodes and spleen and circulating anti-MOG antibodies in the sera of the EAE-induced mice (unpublished results).

MHCII molecules with significantly higher expression in spleen sections of EAE-induced animals

A significantly higher expression of MHC II molecules was detected in the sections of the spleen from the animals immunized with MOG_{35-55} peptide (Fig. 3A). The sections from the control group (IC) showed a lower expression of MHC class II molecules (Fig. 3B). MHC II molecules play a crucial role in the regulation and induction of immune re-

sponse. Constitutive MHC II expression is the hallmark of three distinct types of cells, dendritic cells, B cells and the cells of monocyte/macrophage lineage (Waldburger et al., 2001). Efficient priming of naive T lymphocytes requires both the presentation of an antigen in the context of MHC molecules and the delivery of accessory signals provided by antigen presenting cells (APCs), such as dendritic cells (DC) (Muraille et al., 2002).

Dendritic cells (DCs) play well-established roles in the induction of immunity and tolerance. Both functions require antigen (Ag)-specific interactions between T cells and DCs in the secondary lymphoid tissues. The outcome of these interactions depends on the modulation and integration of three signals: T cell receptor engagement by peptide-MHC complexes, the recruitment of costimulatory and adhesion molecules, and the delivery of soluble mediators (Lebedeva et al., 2005). Signals associated with inflammation, infections or tissue damage induce DC maturation, a process involving complex phenotypical changes, including the upregulation of MHCII, costimulatory and adhesion molecules, the secretion of inflammatory mediators and altered migratory properties. Activation of naive T cells by mature DCs results in clonal expansion and differentiation into effector and memory T cells (Irla et al., 2010).

Plasmacytoid DCs (pDCs) constitute a unique dendritic cell subtype. They circulate in the blood and access secondary lymphoid organs from the blood, driven mainly by inflammatory stimuli. It is suggested that they take part in both innate and adaptive immunity (Colonna et al., 2004). However, pDCs also express MHCII molecules and undergo a maturation process similar to that of conventional DCs (Villadangos and Young, 2008). Furthermore, pDCs can internalize, process and present antigens to CD4+ T cells and cross-present antigens to CD8+T cells (Hoeffel et al., 2007; Sapoznikov et al., 2007; Di Pucchio et al., 2008; Young et al., 2008). These findings have suggested that pDCs can function as APCs.

EAE induced by immunization with myelin oligodendrocyte glycoprotein (MOG) was found to be severely exacerbated in mice exhibiting a selective abrogation of MHCII expression by pDCs. EAE induction triggered the recruitment of pDCs to the lymph nodes, where they engaged in MHCII-dependent and MOG-specific interactions with CD4+ T cells. It was suggested that MHCII expression by pDCs confers protection against EAE by inhibiting the priming of encephalitogenic T cells in secondary lymphoid tissues but has no evident impact on the subsequent effector phase in the CNS (Irla et al., 2010).

MHC class II antigen positive cells, apparent in the sections of the spleen, might render a link between the mild clinical signs expressed in EAE-induced mice and the expression of MHCII molecules.

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