

## REGULATORY ROLE OF PERITONEAL B CELLS IN EAE

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## REGULATORNA ULOGA PERITONEALNIH B LIMFOCITA U EAE

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## ABSTRACT

*B cells play a dual role in the pathogenesis of autoimmune diseases. In experimental autoimmune encephalomyelitis (EAE), an experimental model for multiple sclerosis, B cells contribute to disease progression, while their regulatory role predominates in the initial phases of disease development. Several studies have identified different subsets of regulatory B cells, mostly in the spleen, which are all sources of IL-10. However, peritoneal regulatory B cells are also important producers of IL-10, can migrate towards inflammatory stimuli, and could have an immunoregulatory function. As we have observed expansion of regulatory B cells in the peritoneum of resistant mice after EAE induction, herein we discuss the regulatory roles of B cells in EAE pathogenesis and the possible role of peritoneal regulatory B cells in resistance to EAE induction.*

**Keywords:** multiple sclerosis, EAE, B cells, peritoneal regulatory B cells

## SAŽETAK

*B limfociti imaju dvojnju ulogu u patogenezi autoimunskih bolesti. B limfociti doprinose progresiji eksperimentalnog autoimunskog encefalomijelitisa (EAE), eksperimentalnog modela za multiplu sklerozu, a u inicijalnim fazama razvoja bolesti dominira njihova regulatorna uloga. U nekoliko studija je identifikovano nekoliko subpopulacija regulatornih B limfocita, uglavnom u slezini, a sve proizvode IL-10. Međutim i peritonealni B limfociti proizvode IL-10, migriraju ka inflamatornim stimulusima i mogu da imaju imunoregulatornu funkciju. Pošto smo uočili ekspanziju regulatornih B limfocita u peritoneumu miševa rezistentnih na indukciju EAE, ovde razmatramo regulatorne uloge B limfocita u patogenezi EAE i moguću ulogu peritonealnih regulatornih B limfocita u rezistenciji na indukciju EAE.*

**Ključne reči:** multipla skleroza, EAE, B limfociti, peritonealni regulatorni B limfociti



B cells are effector cells of the adaptive humoral immune system that act by producing specific antibodies. However, B cells express numerous innate immune receptors, including Toll-like receptor (TLR)-3, TLR4, TLR7, TLR8 and TLR9 (1,2); stimulation of these receptors also induces antibody production. It is well established that two populations of B cells exist—B1 and B2 cells—that can be distinguished according to their phenotype, ontogeny, anatomical location and function (3-5). B2 cells, which include follicular and marginal zone B cells, originate from bone marrow precursors and circulate throughout the blood and secondary lymphoid tissues. These cells respond to a broad range of T-dependent and T-independent antigens. In contrast to B2 cells, B1 cells develop during foetal and neonatal develop-

ment, have the capacity to self-renew, and predominantly localize to peritoneal and pleural cavities (3-5). B1 cells are innate immune cells that produce the majority of “natural” immunoglobulins, which are encoded by germline immunoglobulin genes. These natural immunoglobulins act as a first line of defence against pathogens, such as encapsulated polysaccharide-expressing bacteria (4, 5). There are two functionally distinct subsets of B1 cells that can be delineated by differential expression of CD5 (4). B1 cells that express CD5 are known as B1a cells, and those that lack CD5 expression, but have other hallmarks of B1 cells, are known as B1b cells. CD5<sup>+</sup> peritoneal B1a cells produce an abundance of interleukin-10 (IL-10) following stimulation with TLR agonists, such as lipopolysaccharide (LPS) (6).



## B cells in multiple sclerosis

Multiple sclerosis (MS) is classically viewed as a predominantly T cell-mediated autoimmune disease, based on the finding that the disease can be induced in healthy experimental animals by the adoptive transfer of T cells from diseased animals (7,8). T cells can also have a protective role in MS. A specialized population of T cells, CD4<sup>+</sup> regulatory T cells, are critically important for disease attenuation by limiting the activation of T cells during MS and other autoimmune diseases (9,10), in part through the production of IL-10 (11). However, B cells also regulate immune responses and can contribute to MS pathogenesis (12,13) by functioning as antigen-presenting cells for CD4<sup>+</sup> T cell activation (14) and by producing pro-inflammatory cytokines that affect T cell function (15). Recent clinical trials in MS patients that used depleting CD20 monoclonal antibody (Rituximab) suggest that pan-mature B cell depletion has clinical efficacy for the treatment of MS (16,17) in addition to demonstrated efficacy for other autoimmune disorders (13). Additionally, B cells, as with T cells, can have a regulatory role in autoimmune diseases. B cells from patients with multiple sclerosis produce decreased amounts of IL-10 (18). MS patients have significantly higher frequency of CD20<sup>+</sup> B cells, but among B cell subsets they have a reduced frequency of B1 cells, which are known to have a regulatory role in immune responses (19).

Opposite roles of B cells have also been identified during the initiation and progression of an experimental model for MS, experimental autoimmune encephalomyelitis (20). Depletion of mature B cell in mice before EAE induction significantly exacerbates disease symptoms, whereas B cell depletion during EAE progression dramatically inhibits these symptoms. Thereby, the balance between opposing positive and negative regulatory B cell functions shape the normal course of EAE immunopathogenesis. Mice lacking B cells develop an extremely severe and chronic form of EAE, which confirms the regulatory role of B cells in autoimmune diseases (21). The regulatory role of B cells in EAE results from the production of IL-10 (22). A suppressive function for IL-10 produced by B cells has also been demonstrated in a models of inflammatory bowel disease and collagen-induced arthritis (23,24), suggesting a general role for IL-10-producing B cells in immune homeostasis.

## Regulatory B cells

B cell subsets that can down-regulate immune responses by secreting interleukin IL-10 are known as regulatory B cells. These regulatory B cells, which are functionally defined by their immune-suppressive action either *in vitro* or *in vivo* (25), include splenic CD21<sup>hi</sup>CD23<sup>hi</sup>CD1d<sup>hi</sup> transitional 2 marginal zone precursor

or B cells described by the group of Mauri (26,27) and IL-10-producing B cells, termed B10 cells, characterized by the group of Tedder. The latter are mainly found within the CD1d<sup>hi</sup>CD5<sup>+</sup> splenic B cell subset (28,29). Regulatory B cells must be activated to exert suppressive functions, and the activation of regulatory B cells presumably occurs *in vivo* in the context of inflammation. Activated regulatory B cells are more potent suppressors of autoimmunity than their non-activated counterparts (27,30). CD1d<sup>high</sup>CD5<sup>+</sup> B cells can be induced to express cytoplasmic IL-10 following 5 hours of *in vitro* stimulation with LPS, phorbol 12-myristate 13-acetate, and ionomycin, in the presence of monensin to block IL-10 secretion. Splenic B10 pro-B cells have also been functionally identified in mice and are found within the CD1d<sup>high</sup>CD5<sup>+</sup> B cell subpopulation (31). These B10 pro-B cells require 48 hours of *in vitro* stimulation with LPS or via CD40 before they acquire the ability to express cytoplasmic IL-10 (31). Although B10 cells normally represent only 1–2% of splenic B cells, they significantly inhibit the induction of Ag-specific inflammatory reactions and autoimmunity (20,29). Depletion of B10 cells in mice before disease initiation accounts for exacerbated disease, which can be ameliorated by the adoptive transfer of splenic CD1d<sup>high</sup>CD5<sup>+</sup> B cells (32). There is evidence that B regulatory cell-mediated protection in chronic inflammatory diseases is antigen-specific, as B regulatory cells that are *in vivo* activated by one antigen do not protect in inflammatory models induced by a second antigen (27,29). *In vitro*, B regulatory cells can be activated in an antigen-nonspecific manner to secrete IL-10 and suppress immune activation triggered by stimuli, including activation by TLR ligands (31,33–35), CD40 ligation (36,37), a combination of these two pathways (38), or the cytokine IL-21 (39).

## Regulatory B cells in MS

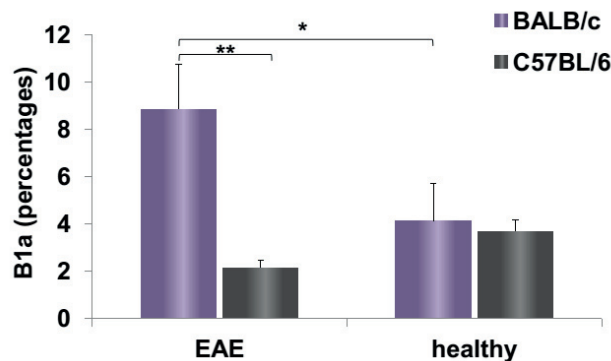
B regulatory cell-mediated amelioration in EAE is dependent upon IL-10. Many reports indicate that B regulatory cells can influence T cell activation. B cell-deficient mice (40) and mice with IL-10-deficient B cells (22) exhibit heightened lymph node T helper 1 (Th1)-cell responses to immunization. B-cell-mediated regulation of EAE is also associated with the suppression of Th17 cells, suggesting that IL-10 from B cells influences disease progression by instructing T cell differentiation (33). The adoptive transfer of B regulatory cells often correlates with a reduction in the frequency of interferon (IFN)- $\gamma$ , IL-17 and/or tumour necrosis factor- $\alpha$  positive T cells (27,39,41,42), and sometimes increased amounts of Foxp3<sup>+</sup> regulatory T cells (43) or IL-10-producing T cells (44). IL-10-producing B cells may contribute to modulation of T-cell responses also indirectly by limiting dendritic cells function and by inhibition of the innate immune responses (45,46). IL-10 produced



by B cells can repress IL-6 and IL-12 production by DCs, thereby inhibiting the differentiation of Th17 and Th1 cells, respectively (33). Additionally, after immunization, DCs from B cell-deficient mice produce higher amounts of IL-12 compared with DCs from wild-type mice (40). Changes in the balance of IL-10 and IL-12 levels have important effects on the pathogenesis of EAE (47). Accordingly, B cell-mediated regulation of EAE begins in the draining lymph nodes within days of immunization. B cells can therefore orchestrate the regulation of autoimmune diseases from within secondary lymphoid organs both directly by inhibiting pathogenic cells (autoreactive T cells and innate immune cells) and indirectly by inducing regulatory activity in different T cell populations.

### B1 cells in EAE

Although splenic regulatory B cells share some functional and phenotypical characteristics with B1a cells, as both cell populations produce IL-10 and express CD5, limited data are available about the regulatory role of B1 cells in EAE. One study showed a reduced severity of demyelination and overall pathology in the brain after the depletion of peritoneal B1 cells during the effector phase of EAE (48). Depletion during the induction phase of disease resulted in an increased incidence of progressive EAE. The experiments in this study were carried out with A.SW mice, which produce anti-MOG antibodies that contribute to EAE pathogenesis, rather than the C57BL/6 mice that are typically used. Attenuation of EAE induced by the depletion of B1 cells could be accounted for by changes in the production of natural antibodies. However, no data are yet available about the IL-10-mediated regulatory role of B1 cells in MS or EAE. It is known that components from *Mycobacterium tuberculosis* that are present in complete Freund's adjuvant, which is used to induce EAE, provide TLR agonists that can stimulate TLRs (22). Furthermore, the *in vitro* stimulation of B cells with TLR agonists produces a cytokine milieu that can inhibit T cell activation in an IL-10-dependent manner, whereas activation of DCs in the same manner induces very low amounts of IL-10 production that is not sufficient to inhibit T cell proliferation (33). Thus, stimulation with TLR agonists that are present in the adjuvants that are used for EAE induction can also trigger regulatory function in B1 cells. Our preliminary findings revealed a significantly higher percentage of CD5<sup>+</sup> B1a lymphocytes in the peritoneum of BALB/c mice, which are resistant to EAE induction with MOG<sub>35-55</sub> peptide (49), compared to susceptible C57BL/6 mice. By contrast, there was no significant difference in the frequencies of B1a or B1b cells in the peritoneum of healthy C57BL/6 versus BALB/c mice (Fig. 1). This finding is in accord with previous findings that *in vivo* stimulation with different microbes (TLR agonists) could



**Figure 1. Immunization with MOG<sub>35-55</sub> induces a significant increase in peritoneal regulatory B1a cells.** BALB/c and C57BL/6 mice were immunized with MOG<sub>35-55</sub>/CFA. Then, 7 days later, mononuclear cells from the peritoneum were isolated and analysed for the cell surface expression of CD19, CD5, and CD11b by flow cytometry. The frequencies of B1a cells (CD19<sup>+</sup>CD11b<sup>+</sup>CD5<sup>+</sup> cells) are presented as the means from three independent experiments; 15 mice per group  $\pm$  SEM; \* $P$ <0.05; \*\* $P$ <0.005. Statistical significance was assessed using Student's *t*-test.

induce the expansion of peritoneal B1 cells. It has been shown that among peritoneal cells, B1a cells are the main source of IL-10 after stimulation with TLR agonists (50). Immunization of susceptible strains, such as C57BL/6, with myelin antigens in adjuvants induces the expansion of inflammatory CD4<sup>+</sup> T cells that gain the capacity to induce inflammation in the CNS. Modulation of immune responses has been suggested to exacerbate or attenuate EAE in susceptible strains of mice (51). Alternatively, our previous study recently showed that alteration of an immunoregulatory pathway by deleting components of the IL-33/ST2 axis may enhance susceptibility to EAE in the resistant BALB/c strain by inducing an inflammatory phenotype in antigen presenting cells (52, 53). In accord with the significantly increased expansion of IL-10-producing peritoneal B1a cells in BALB/c mice compared with susceptible C57BL/6 mice and the fact that peritoneal B cells migrate to lymph nodes (54), it could be assumed that the increased number of peritoneal B1 cells could contribute to the regulatory phenotype of dendritic cells in draining lymph nodes and the resistance of BALB/c mice to the development of EAE.

Further studies will be needed to explore in more detail the regulatory role of B1a cells in EAE and to assess the relative contribution of splenic B regulatory cells and peritoneal regulatory B cells in the pathogenesis of EAE.

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