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Research paper

Positive selection of B10 cells is determined by BCR specificity and signaling strength

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ABSTRACT

B10 cells, a regulatory B cell subset, negatively regulate immune responses in an IL-10-dependent manner. However, the mechanism of B10 cell development is unclear. We found that B10 cells mainly identified self-antigens. TgV_H3B4 transgenic mice, whose V_H was derived from an actin-reactive natural antibody, exhibit elevated numbers of actin-binding B10 cells. Immunization of TgV_H3B4 mice with actin induced elevated B10 cell numbers in an antigen-specific manner, indicating positive selection of B10 cells by self-antigens. Furthermore, higher BCR signaling strength facilitated B10 cell development. We also observed that actin-reactive IgG levels were unchanged in TgV_H3B4 mice after immunization with actin in contrast to the elevated OVA-reactive IgG level after immunization with OVA, indicating that B10 cells acted in an antigen-specific manner to inhibit the immune response. Our data demonstrate for the first time that B10 cells are positively selected by self-reactivity and that higher BCR signaling strength promotes B10 cell development.

1. Introduction

Certain B cells, termed regulatory B cells, negatively regulate immune responses [1–3]. A subset of regulatory B cells with a $CD19^+CD1d^{hi}CD5^+$ phenotype has been identified in mouse spleens [4]. Cytoplasmic IL-10 expression can be induced in these B cells upon *in vitro* LPS, PMA, ionomycin and monensin (L+PIM) stimulation for 5 h [5,6]. Although they represent only 1–5% of adult mouse splenic B cells, B10 cells are the major IL-10 producing cells [5] and can be discriminated from other regulatory B cell subsets [3,7].

The mechanism of B10 cell development remains unclear. Several signaling pathways have been suggested to be involved in B10 cell development, including TLR [4,8,9], CD40 [10–14] and BCR signaling [5,15–17]. TLR and CD40 stimulation are pivotal for B10 cell development; however, the role of BCR signaling in B10 cell development remains to be formally proven [3]. Many studies have suggested that both BCR specificity and signaling strength might be involved in B10 cell development. Arthritis is prevented after transfer of collagen-simulated [13] or collagen plus anti-CD40 antibody-stimulated [18] arthritogenic splenocytes that produce high IL-10 levels. During experimental autoimmune encephalomyelitis (EAE), self-antigen is required to stimulate autoreactive B cells to produce IL-10 [13]. Type 1 diabetes in NOD mice is protected in an IL-10-dependent manner after transfusion of B cell receptor (BCR)-activated B cells [19]. Yanaba et al. reported that B10 cell development is significantly affected by BCR specificity; a 90% reduction in B10 cell numbers is observed in MD4 transgenic mice with fixed antigen receptors whose V_H is derived from the anti-hen egg lysozyme (HEL) antibody [5]. These studies suggest that recognition of self-antigen by BCR may participate in B10 cell development. BCR signaling strength may also play a role in B10 cell development and activation. Significantly elevated B10 cell numbers are observed in hCD19Tg mice and $CD22^{-/-}$ mice in which BCR signaling is enhanced, and the number of B10 cells decreases dramatically in $CD19^{-/-}$ mice in which BCR signaling is reduced [4,20].

However, there are also contradictions for the role of the BCR in B10 cell development. During *in vitro* cultures, the production of IL-10 decreased after BCR activation was combined with LPS or anti-CD40 antibody [5]. No changes in the LPS-induced production of IL-10 by B cells isolated from the peritoneal cavity (PerC) or spleen were observed after BCR activation using anti-Ig kappa [21]. Mice deficient in nuclear factor of activated T cells c1 (NFATc1) (the expression of NFATc1 is upregulated upon BCR activation) in the B cell compartment exhibit an increased number of IL-10-producing B cells compared with wild-type mice [22]. Additionally,

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mice with a B cell-restricted NFATc1 deficiency develop milder EAE disease symptoms and reduced IFN-γ production compared with control mice. Therefore, the evidence for the requirement of BCR signaling in B10 cell development is currently contradictory, and further studies are needed to elucidate the role of BCR in triggering B10 cell development.

In the present study, TgV_H3B4 transgenic mice, whose V_H was derived from an actin-reactive natural antibody, 3B4 [24,25], were used to explore the role of BCR specificity and signaling strength in B10 cell development. The effects of natural and induced interactions between a self-antigen and B cells on B10 cell development were explored in TgV_H3B4 mice. Our data demonstrate for the first time that B10 cells are positively selected by self-reactivity and that higher BCR signaling strength promotes B10 cell development.

2. Materials and methods

2.1. Ethics statements

The animal husbandry, experiments and welfare were conducted in accordance with the Detailed Rules for the Administration of Animal Experiments for Medical Research Purposes, which is issued by the Chinese Ministry of Health, and were approved by the Fourth Military Medical University Animal Experiment Administration Committee. The mice were raised in specific pathogen-free conditions on the C57BL/6 background and were manipulated with every specific care to reduce the suffering of the mice during the experiments. The mice were euthanized by carbon dioxide asphyxiation.

2.2. Mice

Transgenic TgV_H3B4 mice with VH derived from a monoclonal natural antibody, 3B4, were generated by our group as described previously [24,27,28]. Eight- to twelve-week-old C57BL/6 and TgV_H3B4 mice were used for analysis. Six- to eight-week-old transgenic mice and their littermates were immunized by subcutaneous injection with 100 μg of actin or 100 μg of OVA weekly for 3 weeks. All of the mice were bred under specific pathogen-free conditions and given autoclaved food and water. Animal experiments were performed following guidelines from the Fourth Military Medical University Animal Experiment Administration Committee.

2.3. FACS analysis and antibodies

FACS analysis was performed as described previously [24,27,28]. Single-cell suspensions were prepared from the spleens and PerCs and were treated with buffered 0.14 M NH_4Cl. The cells (5×10^5) were stained with antibodies in PBS containing 3% FCS and 0.1% NaN3 for 30 min on ice, followed by washing and filtrating through nylon mesh, and were then fixed in 1% paraformaldehyde or analyzed immediately on a Coulter Epics XL flow cytometer. The data were analyzed with EXPO32 ADC analysis software (TreeStar, San Carlos, CA). The following antibodies were used in the analyses: anti-CD19 (1D3), anti-CD19 (MB19-1), anti-CD1d (1B1), anti-CD16/CD32 mAb (2.4G2), anti-IL-10 (JES5-16E3), and IgG2b isotype control (eB149/10H5) were from eBioscience (San Diego, CA); anti-CD5 (53–7.3), anti-IgM (R6-60.2), anti-IgM^a (DS-1), anti-CD1d (1B1), anti-CD40 (3/23), anti-CD40 (HM40-3), anti-IgG1 (A85-1), and streptavidin-FITC were from BD Pharmingen (San Diego, CA); and anti-mouse IgM $F(ab')_2$ was from Jackson ImmunoResearch Laboratories. FITC-conjugated actin was prepared according to standardized methods.

2.4. Cell culture and intracellular IL-10 analysis

Intracellular IL-10 detection was performed by flow cytometry [5]. Single cell suspensions (2×10^6 cells/ml) of splenocytes or PerC cells were seeded in 24-well plates with RPMI 1640 medium containing 10% FCS, 4 mM L-glutamine, 200 U/ml streptomycin, 200 μg/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol (Life Technologies). The cells were cultured for 5 h with PMA (50 ng/ml; Sigma–Aldrich), LPS (10 μg/ml, *Escherichia coli* serotype 0111: B4; Sigma-Aldrich), monensin (2 μM; eBioscience), or ionomycin (500 ng/ml; Sigma-Aldrich). For the analysis of the anti-IgM and anti-CD40 effects, the cells were cultured with LPS, soluble anti-mouse IgM $F(ab')_2$ (1 μg/ml), coating anti-mouse IgM $F(ab')_2$ (1 μg/ml or 0.3 μg/ml), and/or anti-mouse CD40 mAb (1 μg/ml, clone 3/23) for 48 h following the last 5-h PIM stimulation. Anti-mouse FcR mAb was used to block the FcRs before cell surface staining with anti-CD19, anti-IgM^a or FITC-conjugated actin. After staining, the cells were fixed with a fixation buffer (Soln4208, Biolegend) and then permeabilized with a permeabilization wash buffer (Soln4210, Biolegend). A PE-conjugated anti-IL-10 mAb was used to detect the IL-10 expression. Background staining was determined with non-reactive isotype-matched control mAbs with gates set with ⩽2% of the positive cells.

2.5. Enzyme-linked immunoSPOT assay

Enzyme-linked immunoSPOT assays were performed as previously described [28]. Ninety-six-well ELISPOT plates (Millipore Corporation) were coated with 20 μg/ml of keratin, myosin, actin, lysophosphatidylcholine (LPC), phosphatidylcholine (PC), LPS or OVA (all from Sigma-Aldrich). Keratin, myosin, or actin were diluted in PBS and left overnight at 4 °C. The LPC and PC were dissolved in ethanol and were allowed to dry overnight at 4 °C. Splenic B cells were purified with anti-CD19 microbeads using BD IMag™ streptavidin-particles (BD Pharmingen), and $CD1d^{hi}CD5^+$ B cells and $CD5^-$ B cells were sorted by a flow cytometry (BD Bioscience Aria II) to ⩾95% purity. After isolation, the $CD1d^{hi}CD5^+$ or $CD5^-$ B cells were seeded at a concentration of 1.5×10^5 cells per well in pre-coated 96-well plates. The cells were stimulated with LPS (20 μg/ml, Sigma-Aldrich) for 48 h. The wells were washed thoroughly with PBS containing 0.05% Tween-20, and the biotin-labeled anti-IgG was added. After 3-h incubation at room temperature and thorough washing, the wells were incubated with streptavidin-alkaline phosphatase (MABTECHAB) for 2 h at room temperature. The spots were developed by adding NCIP/NBT substrate (CWbiotech, China), and the reaction was stopped by extensively washing with water. The spots were counted using the Biosys Bioreader4000 PRO ELISPOT reader (Bio-Sys, German).

2.6. ELISA

IgG against actin or OVA in the mouse sera before and after immunization with actin or OVA were analyzed by ELISA. For antigen coating, actin (10 μg/ml) or OVA (10 μg/ml) were diluted in 100 μl carbonate buffer (pH 9.4) and left overnight at 4 °C. The wells were washed 3 times with PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA at 37 °C for 1 h. Mouse sera (1:100 diluted by PBS) was then added and incubated at 37 °C for 2 h. After 3 washes, the wells were incubated with 1:1000 diluted biotin labeled goat anti-mouse IgG (A85-1, BD Pharmingen,). Following the incubation with streptavidin-horseradish peroxidase (NeoBioscience, China), substrate TMB (NeoBioscience, China) was added. ODs were measured at 450 nm with a Titertek Multiskan MCC/340 ELISA plate reader (Flow, Oy, Finland).

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