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# Etanercept attenuates collagen-induced arthritis by modulating the association between BAFFR expression and the production of splenic memory B cells

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ABSTRACT

Anti-tumour necrosis factor-α (TNF-α) drugs are approved for the treatment of rheumatoid arthritis (RA). Many studies have investigated the effect of these drugs on the T cell response; however, some clues have indicated that it may also target B cells. This study was carried out to explore the potential effects and mechanisms of etanercept, a soluble TNF-α receptor, on the function of B cells and their development into memory B cells in type II collagen (CII)-induced arthritis (CIA). Beginning on day 24 after CII immunisation, the mice were evaluated every 2–3 days to determine two clinical parameters: their arthritis global assessment and swollen joint count (SJC). The serum concentrations of IgG1, IgG2a and anti-CII antibodies and the splenic pathology and proliferation of B cells were measured. The percentage of total memory B cells in the spleen was analysed with flow cytometry. BAFFR was detected by immunohistochemistry. In CIA mice, etanercept markedly suppressed the arthritis global assessment and the SJC, reduced the production of anti-CII, IgG1 and IgG2a antibodies, and prevented spleen histopathology to varying degrees; however, it had no obvious effect on splenic B cell proliferation. Etanercept also decreased the percentage of total $CD27^+$ memory B cells in the spleen. Treatment with etanercept was associated with a further increase in BAFFR expression, a significant reduction in CD27 expression, and a negative correlation between the levels of BAFFR and the percentage of memory B cells. Our findings showed that increased BAFFR expression has a regulatory effect on the activation of B cells and the generation of memory B cells, which may be one of the mechanisms of the therapeutic effects of etanercept.



## 1. Introduction

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disorder that affects many tissues and organs. However, it principally attacks joints to cause synovitis, which if left untreated will destroy the articular cartilage and immobilise the joint [1,2]. The traditional paradigm of RA's pathogenesis assumes that the disease is driven by antigen-specific T cells that recognise the arthritogenic antigen and perpetuate the chronic inflammatory response [3,4]. However, the success of therapeutic approaches using anti-CD20 monoclonal antibodies (mAbs) has instead linked B cells to the process of RA. Furthermore, the dependence of T cell activation on B cells has been confirmed in B cell depletion studies [5].

Recent progress in B cell studies has revealed two cytokines important for B cell development and function: the B cell activation factor from the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) [6]. They share two receptors—the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and the B cell maturation antigen (BCMA). A third receptor, the BAFF receptor (BAFFR), is specific only for BAFF, and its role in peripheral B cell development is well-recognised [7].

Disease-modifying antirheumatic drugs (DMARDs) such as methotrexate (MTX), sulfasalazine and leflunomide are commonly prescribed to treat RA. For patients with a more severe case of the disease, biological therapies targeting the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), have led to further significant improvements in outcome [8]. There are currently two types of TNF inhibitors that are available: one option is a monoclonal antibody such as infliximab, adalimumab, certolizumab and golimumab, while the other choice is a circulating receptor fusion protein such as the recombinant human tumour necrosis factor

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receptor-Fc fusion protein (rhTNFR:Fc, also called etanercept) [9]. It has long been known that the use of anti-TNF therapy can be associated with the development of anti-nuclear and anti-double-stranded DNA antibodies and, more rarely, a lupus-like syndrome. Recently, some research has focused on the possible effects of anti-TNF agents on B cells, exploring whether these agents could treat RA. Souto-Carneiro et al. have described an increase in the peripheral blood pre-switch memory B cells in patients who underwent specific TNF-blockade with infliximab medication [10,11]. It is still unclear why anti-TNF treatment can affect B cells in RA.

DBA/1J mice are widely used as a model for RA. Immunisation with type II collagen leads to the development of severe polyarthritis mediated by an autoimmune response. Similar to the human condition, mice with collagen-induced arthritis (CIA) display synovitis as well as erosions of cartilage and bone [12,13]. In the present study, the effects of etanercept medication on CIA mice were explored, with MTX used as a suitable positive control. In addition, the potential mechanism of etanercept in regulating splenic memory B cells was elucidated.

## 2. Materials and methods

### 2.1. Reagents

Etanercept (batch number 20110206) was purchased from Shanghai CP Guojian Pharmaceutical Co., Ltd. (Shanghai, China). Methotrexate (MTX) (batch number 110106) was obtained from Shanghai Xinyi Pharmaceutical Co., Ltd. (China). Chicken type II collagen (CII) and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (USA). The Shanghai Institute of Applied Physics at the Chinese Academy of Sciences provided the [$^3$H]-TdR. The ELISA kits for mouse IgG1, IgG2a and anti-CII antibodies were the products of Research & Development Co., Ltd. (Minneapolis, MN, USA). Anti-mouse cluster of differentiation (CD) 27 and BAFFR polyclonal IgG antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-mouse PE-CD19, FITC-CD27 and FITC-anti-rabbit IgG antibodies were the products of eBioscience, Inc. (San Diego, CA).

### 2.2. Mice

Male DBA/1 mice ($18 \pm 2$ g) were purchased from the Shanghai Institute of Materia Medica at the Chinese Academy of Sciences (Certificate No.: SCXK [HU] 2008-0016). The mice were maintained under standard laboratory conditions. All studies adhered to the principles of laboratory animal care guidelines and were approved by the Ethics Review Committee for Animal Experimentation of the Institute of Clinical Pharmacology at Anhui Medical University.

### 2.3. Induction of CIA and treatment

Chicken CII (2 mg/ml) was dissolved in 0.1 mol/l acetic acid overnight at 4 °C under sterile conditions; it was then emulsified with an equal volume of Freund's complete adjuvant (1 mg/ml, Sigma). This emulsion of CII (0.1 ml) was injected intradermally into the back and the base of the tail of the DBA/1 mice on day 0 to induce CIA, followed by a booster injection on day 21 [14]. After the onset of arthritis, CIA mice were randomly divided into 5 groups and treated either subcutaneously with etanercept (1.5, 4.5, or 13.5 mg/kg) or intragastrically with MTX (2 mg/kg) once every 3 days from days 26 to 41. The normal mice were given an equal volume of normal sodium, and the CIA model mice an equal volume of the vehicle (CMC-Na) as that used in the CII emulsion; the volumes were administered at the same time.

### 2.4. Clinical assessment of CIA

An evaluation of the CIA severity was performed by two independent observers with no knowledge of the treatment protocol. Beginning on day 24 after immunisation, the mice were evaluated every 2–3 days using two clinical parameters: an arthritis global assessment and the swollen joint count (SJC).

The arthritis global assessment was based on symptoms in different parts of the CIA mice: the ear (0 = no nodule or redness, 1 = nodule and redness on one ear, and 2 = nodules and redness on both ears); the nose (0 = no connective tissue swelling or redness and 1 = evident connective tissue swelling and redness); the tail (0 = no nodule or redness and 1 = evident nodule and tail redness); and the paw (0 = no swelling or redness, 1 = one front or hind paw swelling and redness, 2 = two paws swelling and redness, 3 = three paws swelling and redness, and 4 = four paws swelling and redness). The above-mentioned scores were tallied to form the arthritis global assessment, and a maximum value of each mouse was 8. Each paw has five phalanx joints and one ankle or wrist joint, so the maximum SJC for each mouse was 24 [15].

### 2.5. Antibodies determination

On day 41, the mice were humanely sacrificed, and their serum was collected and stored at −80 °C until use. The concentrations of IgG1, IgG2a and anti-CII antibodies in the serum were measured using ELISA kits. Each serum sample was assayed in duplicate.

### 2.6. Histopathological examination of the spleen

Paraffin sections of the spleen were stained with haematoxylin and eosin. The micrographs were evaluated histologically by two independent observers. Five compartments were evaluated in the spleen: the cellularity of periarteriolar lymphoid sheaths (PALS), the lymphoid follicles, the marginal zone, the red pulp and the total number of germinal centres (GCs) in each section. The grading scheme consisted of ordinal categories ranging from 0 (no effect) to 4 (severe effect) [16].

### 2.7. Separation and proliferation detection of B cells

A splenic cell suspension was incubated with microbeads that were conjugated to monoclonal rat anti-mouse CD19 antibodies. The B cells were then separated using an LS separation column and a MidiMACS™ separator (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The B cell suspension ($1 \times 10^7$ cells/l, 100 μl) was seeded with lipopolysaccharide (LPS) (100 μl, with a final concentration of 5 mg/l) into a 96-well culture plate and cultured for 48 h. Triplicates were designed. Six hours before terminating the culture, 20 μl of [$^3$H]-TdR was added to each well. The radioactivity was measured by a liquid scintillation counter (LS6500, Beckman, US).

### 2.8. Flow cytometry

Suspensions of single splenic cells or B cells ($1 \times 10^6$/ml) were stained with fluorescence conjugated monoclonal antibodies to either CD19 and CD27 or CD27 alone (eBioscience) for 30 min at 4 °C. The expression of BAFFR in splenic B cells was detected by indirect fluorescent staining. Cell-associated fluorescence was analysed using a FAC scan instrument (FC500, Beckman Coulter) and the affiliated Cell Quest software [6].