

# Bruton's Tyrosine Kinase (Btk) Inhibitor Tirabrutinib Suppresses Osteoclastogenesis and Inflammation in a Mouse Collagen-Induced Arthritis Model

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## Abstract

Rheumatoid arthritis (RA) is characterized by leukocyte infiltration, synovioocyte hyperplasia and osteoclastogenesis, and tyrosine kinases have key roles in the signaling pathways that regulate these processes. Bruton's tyrosine kinase (Btk) is a key regulator of B-cell receptor (BCR) function. BCR signaling is necessary not only for effective antigen-specific humoral immunity, but for B-cell maturation and survival.

Tirabrutinib is a highly potent and selective Btk inhibitor with an  $IC_{50}$  in the nmol/L range. This study was undertaken to investigate the effect of tirabrutinib on a mouse collagen induced arthritis (CIA) model.

Treatment with tirabrutinib resulted in a dose-dependent inhibition of arthritis severity and bone damage in the CIA model. In a cell-based assay, tirabrutinib prevented the production of inflammatory mediators in monocytes, mast cells and osteoclast.

These data support that tirabrutinib inhibits immune-receptor signaling in multiple cells through Btk inhibition and warrant further clinical testing of tirabrutinib for its therapeutic potential in the treatment of inflammatory diseases.

**Keywords:** B-cells; Bruton's Tyrosine kinase inhibitor; Inflammation; CIA; Osteoclasts

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## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint destruction and autoantibody production. Leukocytes (neutrophils), Lymphocytes (B- and T-cells, monocytes, macrophages, dendritic cells) and mast cells are key immune system cells that play a central role in the autoimmune response that occurs during RA. These immune cells release inflammatory mediators, including cytokines, chemokines, proteases and immunoglobulin [1,2]. Biologic therapies that target individual cytokines, such as TNF- $\alpha$ , IL-6 have improved clinical outcomes followed by suppression of osteoclastogenesis and cytokines-induced production of MMPs [3]. Alternative approaches that modulate proinflammatory cytokine production are being explored, especially targeting signal transduction pathways. The

clinical success of compounds that inhibits JAK-STAT signaling is effective in RA therapy [4].

A possible alternative approach to inhibit another signal transduction pathway is to target Bruton's Tyrosine Kinase (Btk). Btk is a member of TEC family kinase that is broadly expressed in cells of several hemopoietic lineages, but not in T-cells, NK cells and plasma cells [5,6]. Genetic evidence supports a critical role for Btk in multiple hematopoietic signaling pathways including the B-cell receptor (BCR), several cytokine receptors and a potential novel role in heterotrimeric G-protein-associated receptors related to B-cell migration and adhesion, such as the CXCR4 and CXCR5 chemokine receptors and adhesion molecules, integrins [7,8]. Btk mutations in humans leads to X-linked agammaglobulinemia (XLA) which is one of the most frequently inherited immunodeficiency diseases and is characterized by

an almost complete arrest of B-cell differentiation at the pre-B cell stage [9]. Mutations in the murine *Btk* gene lead to X-linked immunodeficiency (*xid*) that resembles XLA in humans. The *xid* mice are not able to mount a thymus-independent type II (TI-II) response to antigens [10].

*Btk* has an interesting cysteine residue at the position as Cys481 in ATP-binding pocket and only nine other kinases in human genome have similarly placed cysteine residue. These nine kinases include three EGFR family kinases (EGFR, ErbB2 and ErbB4), four Tec family kinases (Bmx, Itk, Tec and Txk), one Src family member (BLK) and JAK3 [11]. The introduction of the irreversible first-generation *Btk* inhibitor, ibrutinib, to clinical practice has changed outcomes for patients with chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and Waldenström macroglobulinemia (WM) [12-14]. It binds strongly to cysteine 481 in the allosteric inhibitory segment of *Btk* kinase domain, resulting in the complete abrogation of *Btk* activity by inhibition of its autophosphorylation at tyrosine residue 223. However, the kinase of ibrutinib is broad [15] and may cause toxicities. Thus, several selective irreversible and reversible *Btk* inhibitors are now being explored in autoimmune disorders [16,17]. Tirabrutinib is a covalent type inhibitor with comparable efficacy to ibrutinib in the treatment of B-cell malignancies and has greater selectivity for *Btk* (in vitro IC<sub>50</sub>, 2 nmol/L) than other kinases (KINOMEScan platform: 442 kinases) [18]. Tirabrutinib inhibits cell proliferation in some malignant B-cell lines but does not inhibit the activation of T-lymphocytes from human PBMCs [19,20].

Herein, we extended our studies and evaluated the effect of tirabrutinib on a murine collagen-induced arthritis (CIA) model. The data indicate that tirabrutinib could be effective in diseases involving adaptive immunity.

## Materials and Methods

### Animals used

Seven-week-old male of BALB/c and DBA/1JNCrlj mice (Charles River Laboratories Japan, Inc.) were used for TNP-Ficoll and CIA model, respectively. All mice were allowed free access to pelleted CRF-1 diet (Oriental Yeast Co., Ltd.) and tap water. The present study was conducted in compliance with the "Guidance for Animal Experiments," the "Ethical Standards for Experiments using Human Tissues," and the "Standards for Safety Management of Pathogens" established by Ono Pharmaceutical Co., Ltd.

### Reagents

Tirabrutinib was obtained from Ono Pharmaceutical Co., Ltd. (Osaka, Japan) and prednisolone was from LKT Laboratories Inc.

### Thymus-independent type II (TI-II) immunization

40 µg/body of TNP-Ficoll (Biosearch Technologies) in PBS was intraperitoneally administered to male BALB/c mice (Day 0). After 7 days later, TNP-specific Ab titers were determined by ELISA. Tirabrutinib was orally administered for 4 days (Day 0-3) after TNP-Ficoll immunization. ELISA plates were coated overnight with 25 µg/mL TNP-BSA. Several dilutions of both preimmunization and postimmunization serum were added. Isotype-specific Abs

was detected with a HRP-conjugated rat anti-mouse IgM (BD Biosciences). HRP activity was determined with OPD tablets as a substrate (Sigma-Aldrich).

### Collagen-induced arthritis (CIA) mouse model

Male DBA/1J mice were injected intracutaneously at the base of the tail with emulsified type-II collagen (CII/ACF emulsion: 150 µg/0.1mL/body). The mice were boosted 21 days later via an intracutaneous administration of emulsified type-II collagen (CII/AIF emulsion: 100 µg/0.1mL/body). Mice assigned to the "tirabrutinib groups" were given tirabrutinib orally at doses of 1, 3, or 10 mg/kg once daily and twice daily with an interval of 8 hours or longer (daily dose of 2, 6, or 20 mg/kg, respectively) for 14 days (Days 35 to 48) following the initial sensitization. Prednisolone as the positive control was administered orally at a dose of 10 mg/kg once daily for 14 days (Days 35 to 48) following the initial sensitization. Mice assigned to the "pseudo treatment group (sham)" and mice assigned to "vehicle group" were given 0.5% (w/v) methylcellulose (MC) solution orally for 14 days (Days 35 to 48) following the initial sensitization. Clinical arthritis score was assessed for each mouse on Days 35, 38, 42, 45, and 49. The right and left hind limbs were collected from each mouse on Day 49.

### Pro-inflammatory cytokine and MMP gene expression assay by qPCR

Ankles were harvested, frozen, and pulverized, and RNA was isolated using an Rneasy Mini spin column according to the manufacturer's protocol (Qiagen K.K.). cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen K.K.). Cytokine (MIP1-α, IL-1β, KC, TNF-α, IL-6, MCP-1, RANKL, IL-18), Matrix metalloproteinase (MMP3, MMP9) and Actin Beta mRNA expression was measured by quantitative real-time PCR using the LightCycler 480II (Roche Diagnostics K.K.). The data were analyzed by "Advanced Relative Quantification". The quantified mRNA levels of various cytokines in each tissue were converted into relative amounts using the  $\Delta\Delta Ct$  (threshold cycle) method. By subtracting the Ct value of the internal standard (Actin Beta) from the Ct value of each target gene, the  $\Delta Ct$  for each was calculated first (Equation-1). By subtracting the mean  $\Delta Ct$  value of the sham group ( $\Delta Ct_{sham}$  [ave.]) from the  $\Delta Ct$  value of each sample, the  $\Delta\Delta Ct$  for each was then calculated (Equation-2). Relative expression levels of various cytokines were calculated along Equation-3, using the mean  $\Delta\Delta Ct$  value for each experimental group ( $\Delta\Delta Ct$  [ave.]).

Equation-1:  $\Delta Ct = [Ct \text{ value of a target gene}] - [Ct \text{ value of the internal standard}]$

Equation-2:  $\Delta\Delta Ct = [\Delta Ct \text{ value of each sample}] - [\text{Mean } \Delta Ct \text{ value of the sham group } (\Delta Ct_{sham} \text{ [ave.]})]$

Equation-3: Relative expression level =  $2^{-\Delta\Delta Ct \text{ [ave.]}}$

### Plasma cytokine and antibody measurement

Mice were anesthetized and whole blood was collected on day 49. Plasma was extracted from a whole blood by centrifugation for 10 minutes at 1,800 × g using a refrigerated centrifuge. Plasma was isolated as the supernatant and stored frozen at -80°C in an ultra-low temperature freezer until analysis. IL-6 and

KC were measured using a Procarta cytokine assay kit (Veritas). Anti-CII IgG was measured using a Mouse Anti-Type I and Type II collagen IgG Assay Kit (Chondrex).

### Histopathologic evaluation

Left and right hind legs were decalcified with an aqueous solution of EDTA at room temperature. After completing decalcification, a central transversal section of the tarsal joint tissue was excised. Thereafter, the section was embedded in paraffin so that the cut surface would constitute a central transversal section surface by a conventional method. Two thin sections, 4 to 6  $\mu\text{m}$  in thickness, were cut from each paraffin block, then stained with H.E. and TRAP. TRAP staining was performed using a TRAP staining kit (COSMO BIO Co., Ltd.). Eighty sections (in left-and-right pairs) for each of the H.E.-stained sections and TRAP-stained sections were examined histopathologically. Pannus severity, infiltrate, cartilage lesion and bone lesions were scored in a blinded manner on a severity scale ranging from 0 (normal), 1 (minimal), 2 (mild), 3 (moderate) to 4 (severe) [21]. Clinical severity was graded as follows: 0 = normal; 0.5 = erythema and edema in only one digit; 1 = erythema and mild edema of the footpad, or ankle or two to five digits; 2 = erythema and moderate edema of two joints (footpad, ankle, two to five digits); 3 = erythema and severe edema of the entire paw; 4 = reduced swelling and deformation leading to incapacitated limb. The clinical score for each mouse was the sum of the scores in each of the 4 paws, for a maximum score of 16 [22].

TRAP-positive osteoclasts were counted in TRAP-stained sections. The cells having two or more nuclei and TRAP-positive polymorphic cytoplasm were identified as osteoclasts, and osteoclast counts in bone surface layers, medullary cavities, and surrounding connective tissue were counted 3 times per section [22]. The mean was calculated and rounded to the nearest integer.

### Primary cell assay

Primary human cells were purified from three healthy donors. Human peripheral blood mononuclear cells (PBMCs) were treated with tirabrutinib for 10 min and then stimulated with anti-IgM for 18 h. The purified B lymphocyte activation was assessed on cell surface marker expression using the anti-CD69 antibody. Human monocytes were treated with tirabrutinib for 1 h and then cells were inoculated onto an IgG-coated plate for 18 h. Supernatants were assayed for TNF- $\alpha$  and IL-6 and analyzed by Luminex assay (200 xPONENT3.1 System). Mast cells from human bone marrow CD34-positive cells were induced to differentiate. After overnight sensitization with human myeloma IgE, mast cells were treated with tirabrutinib for 1 hr and then added 1  $\mu\text{g}/\text{mL}$  goat anti-human IgE antibody for 30 min. Histamine was measured by ELISA. TNF- $\alpha$ , IL-8 and GM-CSF were measured by Luminex assay (200 xPONENT 3.1 System) after 6h incubation. Human osteoclast precursor cells were treated with tirabrutinib for 7 days and then tartrate-resistant acid phosphatase (TRAP) staining was performed. Cytokines in the media were measured using a Procarta Cytokine Assay (Affymetrix). Bone resorption activity of mature osteoclasts was measured using a Osteolyse Assay Kit

(Lonza). The present study was conducted in accordance with the ethical guideline "Guidance for Human Tissue Experiments" and "Safety procedures of Pathogens" established by ONO Pharmaceutical Co., Ltd.

### Statistics

SAS 9.1.3 (EXSUS Ver. 7.7.1, SAS Institute Japan Ltd. [CAC EXICARE Corporation]) was used for analysis of animal data. Data are expressed as the mean  $\pm$  standard error. All statistical tests were two-sided with a significance level of 5%. For human cells, the  $\text{IC}_{50}$  was estimated by nonlinear regression analysis using the Sigmoid Emax model from three donors.

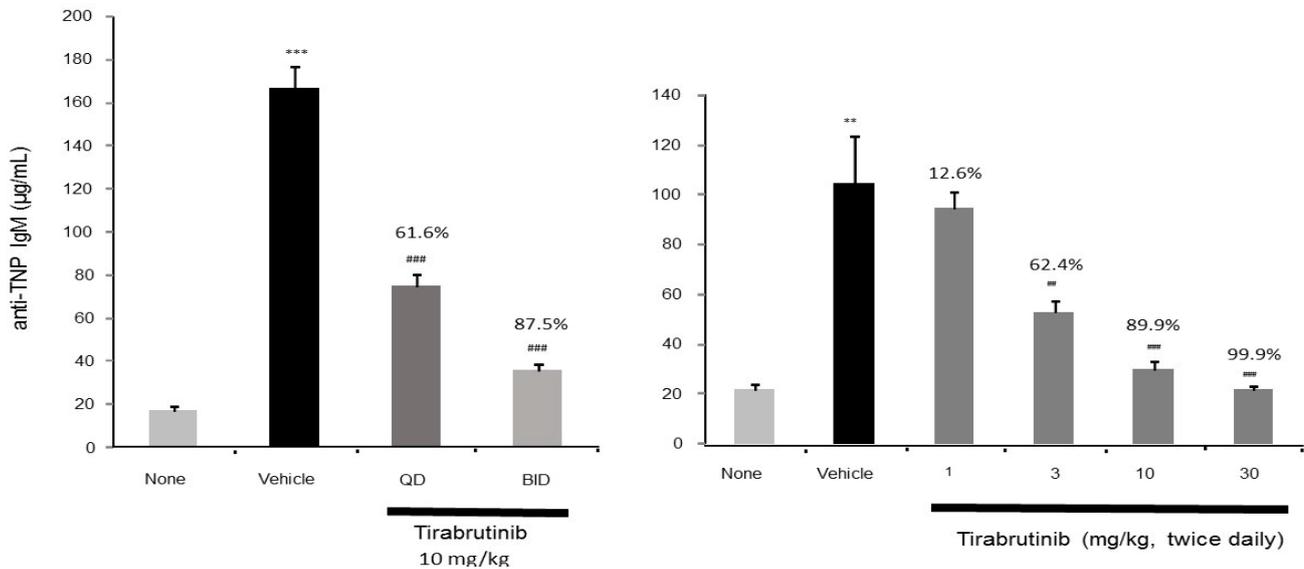
## Results

### Tirabrutinib suppresses TNP-Ficoll induced IgM secretion

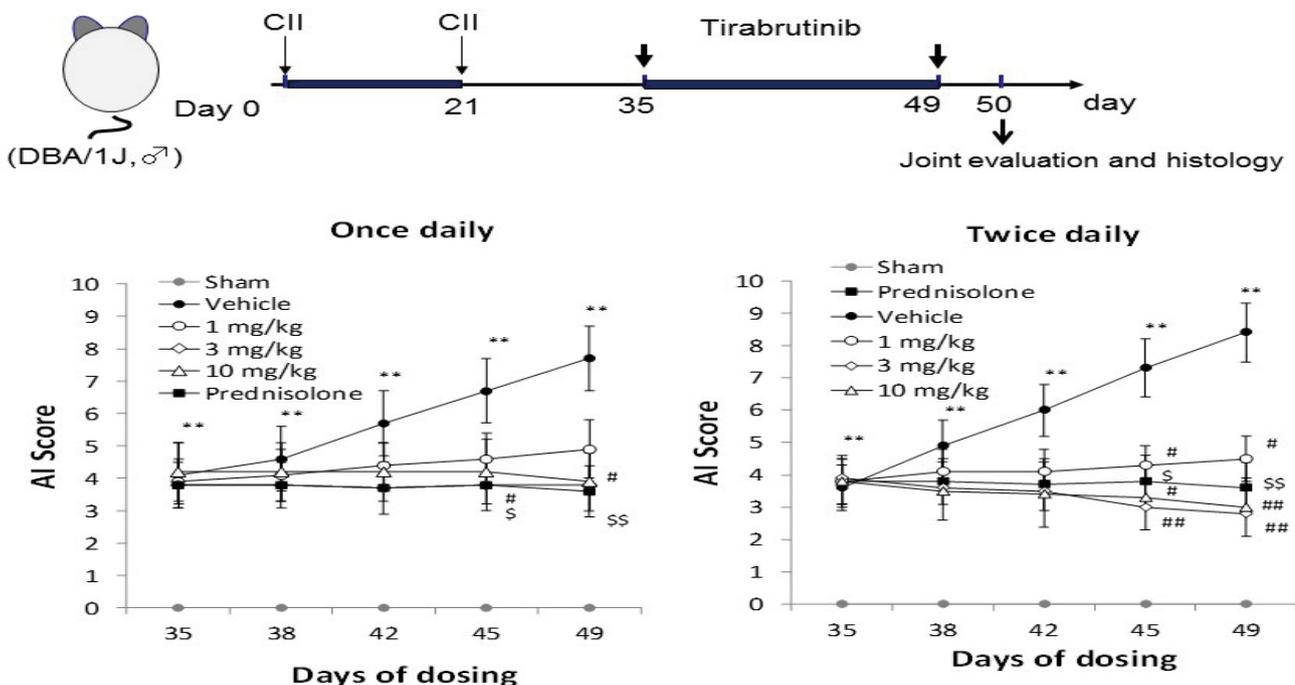
Using TNP-Ficoll as a model TI-II Ag, BTK-deficient mice demonstrated prominent reduction of TNP-specific IgM and IgG3. To test the ability of tirabrutinib to mount an antigen-specific immune reaction, we evaluated the response to TNP-Ficoll antigen. Mice are immunized on day 0 and serum is collected and analyzed for TNP-specific IgM on day 7. Initial experiment to assess a treatment regimen of tirabrutinib in the mice model. Tirabrutinib was administered orally, once or twice daily for 4 days. The twice daily treatment resulted in a better efficacy than compared with once daily (**Figure 1, left**). A second experiment confirmed that twice daily treatment with tirabrutinib suppressed antigen-specific IgM in a dose dependent manner (**Figure 1, right**) and the result was consistent with *Btk* knockout mice [10]. These data indicate that continuous BTK inhibition of B-cells was not achieved by once daily treatment with tirabrutinib in mice.

### Reduction in the arthritis index and proinflammatory genes in mice with CIA after treatment with tirabrutinib

We then evaluated the targeting B-cells by tirabrutinib in a murine collagen-induced arthritis (CIA) model. Treatments were initiated when the disease had partially progressed as measured by an average arthritis index around 4. Tirabrutinib significantly suppressed arthritis severity to the degree of initial level for both regimens, once daily and twice daily (**Figure 2**). Surprisingly, the difference between the two regimens was not statistically significant despite the fact that B-cells function was not blocked continuously by once daily regimen, as shown previously. These data suggest that tirabrutinib may affect not only B-cells but other inflammatory cells which are related to pathogenesis of arthritis. We explored whether tirabrutinib could regulate articular gene expression of proinflammatory cytokines and MMPs. In CIA, ankles were harvested from the left hind limb on Day 49 and gene expression was measured by real-time quantitative PCR. Increases in mRNA levels of MIP-1 $\alpha$ , IL-1 $\beta$ , KC, MMP-3, MMP-9, TNF- $\alpha$ , and IL-6 were significantly higher in vehicle treated mice compared with the pseudo treatment mice. Mice once daily treated with tirabrutinib had lower inflammatory cytokines and MMPs than vehicle treated CIA mice (**Figure 3**).



**Figure 1** Effect of tirabrutinib on TNP-Ficoll-induced IgM secretion in mice. TNP-Ficoll was intraperitoneally administered to male BALB/c mice (Day 0), and serum TNP-specific IgM concentration was determined by ELISA 7 days later (Day 7). Tirabrutinib was orally administered once daily (QD) or twice daily (BID) at indicated doses for 4 days (Day 0, 1, 2 and 3). TNP-Ficoll specific IgM concentration is displayed as the mean  $\pm$  standard error for 6 animals in each group on Day 7. The *t* test was used to compare the groups without and with TNP-Ficoll induction, and Dunnett's test was used to compare the vehicle and tirabrutinib groups. A *p* value of less than 5% was considered statistically significant. \*\*\*:  $p < 0.001$ , \*\*:  $p < 0.01$  vs group without TNP-Ficoll induction (None). ##, ###:  $p < 0.01$ ,  $p < 0.001$  vs group with vehicle. Results of linear regression analysis in the tirabrutinib group:  $p < 0.0001$  for slope.



**Figure 2** Comparison of once daily and twice daily dosing of tirabrutinib in the murine CIA. DBA/1J mice were immunized with 150  $\mu$ g of emulsified type-II collagen (CII/AIF). After 21 days, the mice were given a booster dose (100  $\mu$ g, CII/AIF). CIA was fully developed on day 35. All treatments were initiated when the disease had partially progressed as measured by an average arthritis index around 4.0, and continued for the next 14 days. Study results were presented as the mean  $\pm$  standard error (n=10 animals per group). Wilcoxon rank sum test was performed for comparison between Sham and Vehicle, \*\*:  $P < 0.01$ , Prednisolone and Vehicle, \$:  $P < 0.05$ , \$\$:  $P < 0.01$ . Steel test was performed for comparison between Vehicle and tirabrutinib, #:  $P < 0.05$ , ###:  $P < 0.01$ .

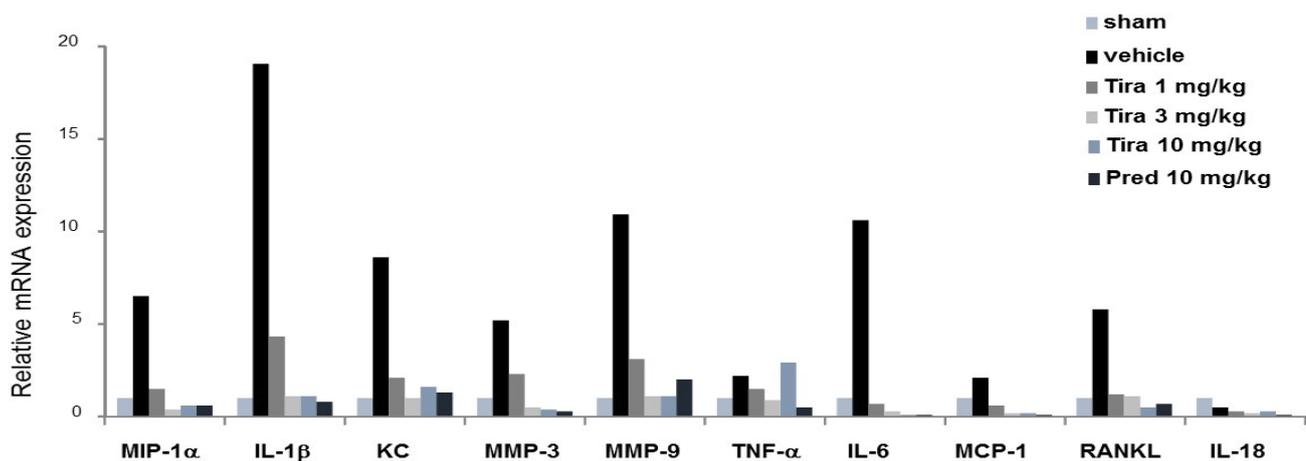
### Effect of tirabrutinib on inflammatory cytokines and anticollagen antibody in plasma

CIA pathogenesis is characterized by the generation of anti-type II collagen antibodies [1,2]. Next, we measured IL-6, KC and anti-type II collagen antibody in serum by ELISA. IL-6 and KC concentrations were significantly reduced in mice once daily treated with tirabrutinib as compared with that in mice treated with vehicle (**Figure 4**). In contrast, anti-type II collagen levels were not dramatically reduced in tirabrutinib treated mice, even though disease severity was not progressed in these mice.

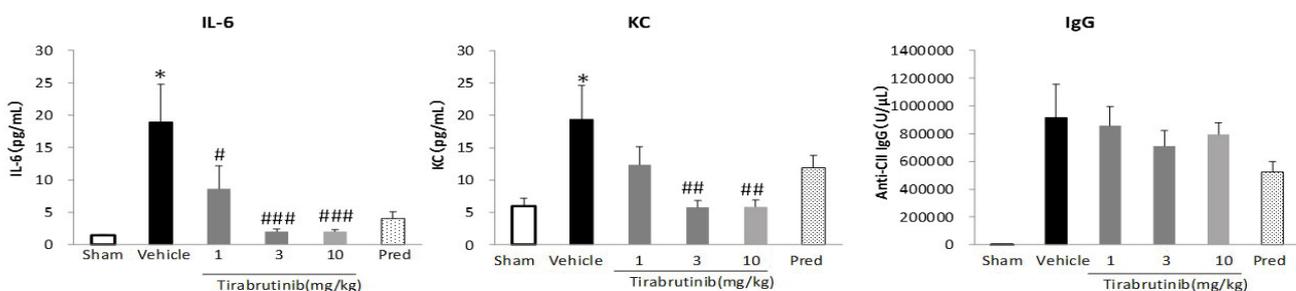
### Biological characteristics of tirabrutinib on various immune effector cells

We then considered the hypothesis that tirabrutinib might suppress other immune cells in addition to B-cells activity. Since Btk is not only expressed in B-cells, but in innate immune

effector cells, including monocytes, macrophages, neutrophils, dendritic cells and mast cells, inhibition of Btk is expected to have pleiotropic anti-inflammatory effects [23]. We evaluated the cellular potency of tirabrutinib (**Table 1**). In primary human B-cells, tirabrutinib did inhibit anti-IgM induced CD69 expression with an IC<sub>50</sub> of 13.8 nM. FcγR-dependent or FcεRI-dependent cytokines production in human monocytes or mast cells were also inhibited by tirabrutinib with a similar potency. In assay using osteoclast precursor cells, tirabrutinib strongly inhibited M-CSF/RANKL-stimulated osteoclast differentiation, chemokines production and resorption, 10-20 fold lower compared to the IC<sub>50</sub> values for other groups. These data support the hypothesis that tirabrutinib might inhibit many immune cells and affect multiple steps in the pathogenesis of CIA.



**Figure 3** Cytokine and MMP profile of arthritic joints. Joints extracts were prepared from mice on day 49. Quantitative real-time PCR was performed to measure intra-articular cytokine and MMP mRNA expression. The quantified mRNA levels of various cytokines in each tissue were normalized to Actin Beta expression. Data are presented as mean  $\pm$  standard error (n =10 animals per group).



**Figure 4** Effect of tirabrutinib on plasma cytokine and anti-type II collagen. Mice were anesthetized and whole blood was collected on day 49. Plasma was extracted from a whole blood and then measured IL-6, KC and anti-CII IgG. Data are presented as mean  $\pm$  standard error (n =10 animals per group). The t test was used to compare the sham and vehicle group, the vehicle and Prednisolone group. Dunnett's test was used to compare the vehicle and tirabrutinib groups. A p value of less than 5% was considered statistically significant. \*: p<0.05 vs Sham group. #, ##, ###: p<0.05, p<0.01, p<0.001 vs group with vehicle.

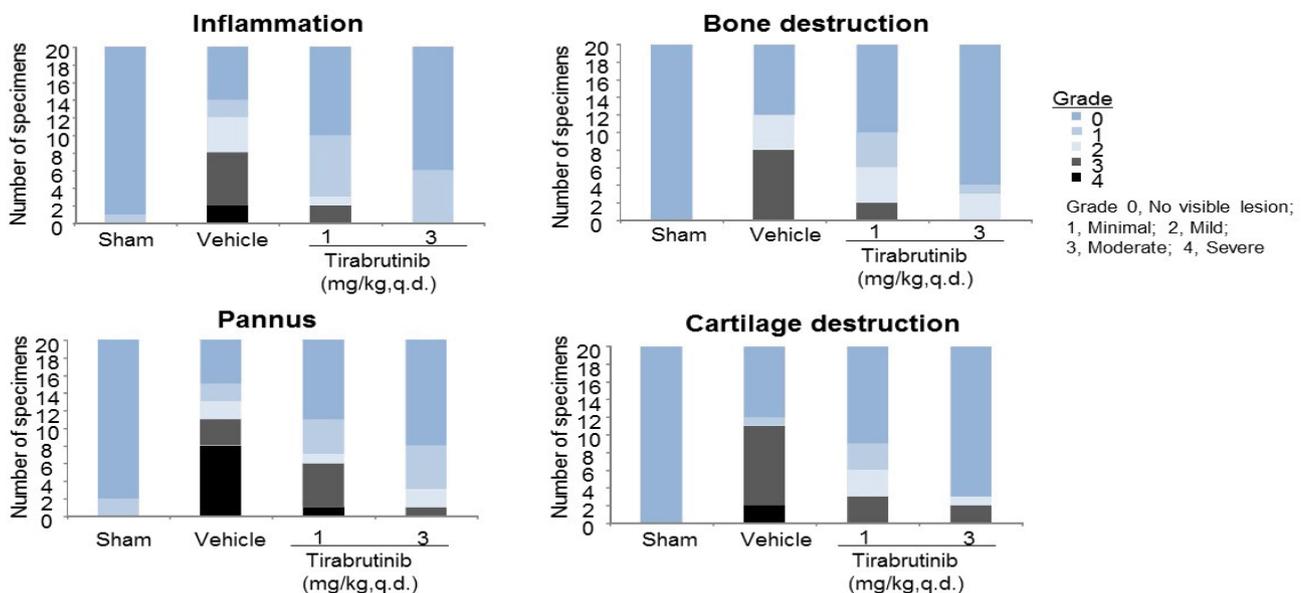
## Reduction in severity of inflammation, pannus, bone and cartilage damage in mice with CIA after treatment with tirabrutinib

Because tirabrutinib regulates various immune cell function, we considered whether it might improve histopathological changes. After once daily treatment of tirabrutinib at dose of 1 and 3 mg/kg, histopathological changes of the joints were evaluated on day 49. Histopathological evaluation of tarsal joints of left and

right hind legs (region including tarsocrural joint, intertarsal joint, and tarsometatarsal joint) was performed for four parameters: inflammation, pannus, bone destruction, and cartilage destruction (**Figure 5**) [21]. In summary of the histopathological evaluation, cartilage destruction was suppressed significantly in the tirabrutinib 1 and 3 mg/kg compared with the vehicle. All findings of inflammation, pannus, and bone destruction were suppressed significantly in the tirabrutinib 3 mg/kg.

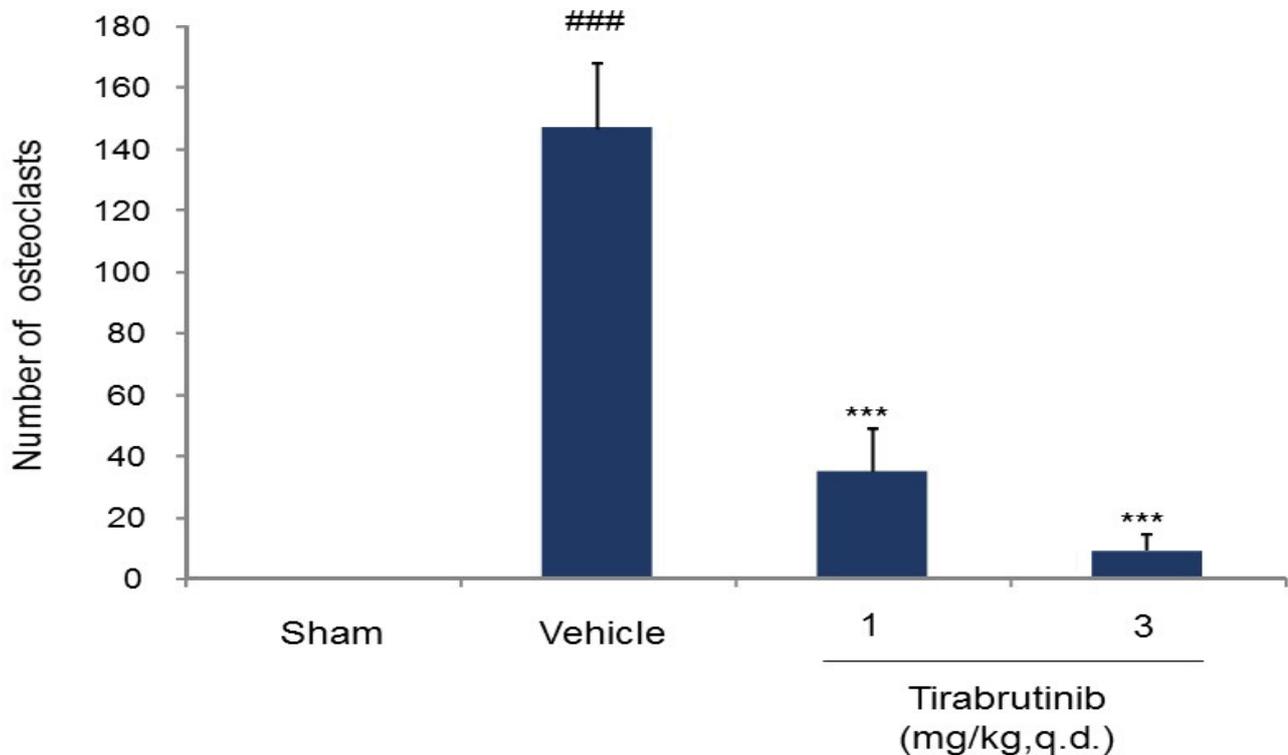
**Table 1:** Potency of tirabrutinib in human isolated primary cell. The IC<sub>50</sub> was estimated by nonlinear regression analysis using the Sigmoid Emax model from three donors or experiments.

Human isolated primary cell	Stimulus	Readout	IC <sub>50</sub> (nM)
B lymphocytes from PBMC	anti-IgM	CD69	13.8
Monocytes	FcγR	TNFα	12.4
		IL-6	49.5
Mast cells	FcεRI	Histamine	32.2
		TNFα	5.6
		IL-8	10.7
		GM-CSF	7.3
Osteoclast precursor cells	M-CSF, RANKL	TRAP (differentiation)	1.4
		MIP-1α, MIP-1β, RANTES, IL-8	1.0-2.1
		Osteolysis (resorption)	2.2



**Figure 5** Tirabrutinib improves anti-inflammation and histologic signs of disease in the murine CIA.

Once daily treatment with tirabrutinib was initiated when the disease had partially progressed as measured by an average arthritis index around 4.0, and continued for the next 14 days. The total number of Inflammation, pannus, bone and cartilage destruction histology scores of the hind paws are presented. Grade 0, No visible lesion; 1, Minimal; 2, Mild; 3, Moderate; 4, Severe. In vehicle treated mice, arthritis-associated findings were observed in 15 out of the 20 sections of left and right joints from 10 animals. The number of sections for each grade for each parameter was as follows: normal (Grade 0); 6, minimal (Grade 1); 2, mild (Grade 2); 4, moderate (Grade 3); 6, severe (Grade 4); 2 for inflammation, normal; 5, minimal; 2, mild; 2, moderate; 3, severe; 8 for pannus, normal; 8, mild; 4, moderate; 8 for bone destruction, and normal; 8, minimal; 1, moderate; 9, severe; 2 for cartilage destruction. In 1 mg/kg of tirabrutinib treated mice, arthritis-associated findings were observed in 11 out of the 20 sections. Normal; 10, minimal; 7, mild; 1, moderate; 2 for inflammation, normal; 9, minimal; 4, mild; 1, moderate; 5, severe; 1 for pannus, normal; 10, minimal; 4, mild; 4, moderate; 2 for bone destruction, and normal; 11, minimal; 3, mild; 3, moderate; 3 for cartilage destruction. In 3 mg/kg of tirabrutinib treated mice, arthritis-associated findings were observed in 8 out of the 20 sections. Normal; 10, minimal; 6 for inflammation, normal; 12, minimal; 5, mild; 2, moderate; 1 for pannus, normal; 16, minimal; 1, mild; 3 for bone destruction, and normal; 17, mild; 1, moderate; 2 for cartilage destruction.



**Figure 6** Effect of tirabrutinib on total number of osteoclasts. In TRAP-stained sections of tarsal joints, cells having two or more nuclei and TRAP-positive polymorphic cytoplasm were identified as osteoclasts, and osteoclast counts in bone surface layers, medullary cavities, and surrounding connective tissue were counted 3 times per section. Study results were presented as the mean  $\pm$  standard error ( $n=10$  animals per group). Wilcoxon rank sum test was performed for comparison between Sham and Vehicle, ### :  $P<0.001$ , Tirabrutinib and Vehicle, \*\*\* :  $P<0.001$ .

### Reduction in number of osteoclast in mice with CIA after treatment with tirabrutinib

In TRAP-stained sections, the osteoclast counts per animal were  $0.1 \pm 0.1$ ,  $146.8 \pm 21.3$ ,  $34.9 \pm 13.9$ , and  $9.2 \pm 5.3$  for the sham, vehicle treated mice, 1 mg/kg and 3 mg/kg of tirabrutinib treated mice, respectively. Osteoclast counts decreased significantly in the 1 and 3 mg/kg of tirabrutinib treated mice compared with the vehicle treated mice (Figure 6).

### Discussion and Conclusion

The pathogenesis of RA is complex and involves the production of inflammatory mediators, cytokines, chemokines, proteases and immunoglobulin. Since B-cells are able to produce a number of proinflammatory cytokines such as IL-6, TNF- $\alpha$  and lymphotoxin- $\beta$  as well as chemokines that can modulate migration and functions of the dendritic cells and CD4 positive Th cells, B-cells play multiple roles in RA pathobiology.

Our data show that twice-daily, not once-, treatment with tirabrutinib markedly suppresses TI-II responses in a dose dependent manner. The result was consistent with *Btk*-deficient mice, suggesting that Btk is the target for B-cells [10]. The success of B-cell targeted therapies in the treatment of RA was rituximab [24,25]. However, the consensus statement on the use

of rituximab in RA has been recommended for patients who have an inadequate response or intolerance to one or more anti-TNF biologics, in conjunction with Methotrexate given the sustained efficacy needs repeated course of treatment [26]. Therefore, the precise contribution of B-cells to the pathogenesis of RA is not well defined.

In CIA model, both twice daily and once daily treatment of tirabrutinib significantly suppressed arthritis severity although the effective suppression of B-cells was not expected by once daily treatment. Production of anti-type II collagen in plasma was not reduced dramatically by once daily treatment. These results indicate that the efficacy of tirabrutinib did not correlate with B-cell suppression. It has been shown that B-cell depletion using a chimeric CD20 mAbs after collagen immunizations did not have a significant effect on arthritis severity. By contrast, B-cell depletion before collagen immunization resulted in significantly reduced severity with lower autoantibody [27], suggesting that the contribution of B-cells to the pathogenesis of CIA model might be small if the treatment of targeting B-cells initiated in disease onset.

The observed efficacy by tirabrutinib may be due to the fact that the activity of various immune effector cells in mice are inhibited by tirabrutinib. In human primary cells, we found that tirabrutinib inhibited the activity of monocytes, mast cells and osteoclast

precursor cells as well as with a similar potency to that of B-cells. Therefore, the antiinflammatory activity of tirabrutinib results at least in part from inhibition of multiple immune effector cells in CIA mice [17]. We then measured the functional effects of tirabrutinib on the expression of key inflammatory mediators. These studies showed that tirabrutinib effectively regulates proinflammatory and protease genes in arthritis mice.

Since these mediators play important roles in the pathogenesis of RA, recent studies have focused on tyrosine kinases as potential targets for the treatment of RA. Among them, targeting JAKs are the most successful and efficacious for RA therapy [28]. Tofacitinib was the first JAK inhibitor approved for the treatment of RA, and a number of related compounds are currently approved or in clinical development. It has been reported that Btk inhibition suppressed the phosphorylation of mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NFkB), and protein kinase C $\alpha$  (PKC $\alpha$ ) in osteoclastic differentiation [29]. MAP kinases, including the p38, ERK and JNK, regulate both cytokine production and cytokine responses in a variety of diseases [30-32]. Partially overlapping activation signals converge on each kinase pathway, which in turn regulate a number of downstream events such as transcription factor activation, cell migration, and proliferation [33]. Therefore, various cytokine profiles in RA patients raise the

possibility that one might potentially select a new target, like Btk, as a therapeutic option.

In conclusion, this study demonstrates that tirabrutinib exhibits potential therapeutic value for the treatment of RA based on the pathogenesis of specific diseases and the genes regulated by a particular kinase.

## Conflict of Interest

Yuko Ariza has no conflict of interest. Masayuki Murata has no conflict of interest. Yoshiko Ueda has no conflict of interest. Shingo Hotta has no conflict of interest. Toshio Yoshizawa has no conflict of interest. The authors are employees of Ono Pharmaceutical Co., Ltd.

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## Author Contributions

Y.A. conceived, designed, performed the experiments, analyzed the data and wrote the manuscript. M.M., Y.U. and S.H. performed experiments and contributed reagents, materials, and analysis tools. T.Y. supervised the study and edited the manuscript.

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