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Research paper

# Loss of WDFY3 ameliorates severity of serum transfer-induced arthritis independently of autophagy

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ABSTRACT

WDFY3 is a master regulator of selective autophagy that we recently showed to interact with TRAF6 and augment RANKL-induced osteoclastogenesis *in vitro* and *in vivo* via the NF-κB pathway. Since the NF-κB pathway plays a major role in inflammation herein, we investigate the role of WDFY3 in an arthritis animal model. Our data show that WDFY3 conditional knockout mice (*Wdfy3*$^{loxP/loxP}$*-LysM-Cre+*) were protected in the K/BxN serum transfer-induced arthritis animal model. These effects were independent of alterations in starvation-induced autophagy as evidenced by Western blot analysis of the autophagy marker LC3, autophagosome formation in osteoclast precursors and lysosome formation in osteoclasts derived from *WDFY3-cKO* mice compared to controls. Moreover, we demonstrate by immunofluorescence and co-immunoprecipitation that WDFY3 interacts with SQSTM1 in macrophages and osteoclasts. Collectively, our data suggest that loss of WDFY3 in myeloid cells leads to reduced severity of inflammatory arthritis independently of WDFY3 function in starvation-induced autophagy.

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that exhibits various clinical manifestations including synovial inflammation and bone loss [1]. Although the development of biologics such as anti-TNF is an effective treatment for the majority of RA patients, approximately 40% of patients do not respond to TNF inhibition, suggesting that RA disease mechanisms are only partly understood. Autophagy, a cellular process that degrades organelles and misfolded proteins and ensures cells survival at homeostatic and stress conditions was recently associated with autoimmunity in multiple studies [2]. Specifically, autophagy plays multiple roles immune functions in macrophages such as clearing intracellular pathogens [3], regulating inflammatory cytokines expression [4], and modulating (M1, M2) macrophage polarization [5]. Recent studies on osteoclasts, the cells responsible for bone and joint destruction in autoimmune diseases, showed that autophagy-related proteins 5 and 7 (ATG5 and ATG7) deficiency leads to defective ruffled border formation and osteoclast function in both *in vitro* and *in vivo* assays [6]. Moreover, mice deficient in autophagy-related protein ATG7 were protected from TNF-mediated joint destruction in experimental arthritis [7]. Mutations in autophagy-related proteins have been associated with autoimmune diseases such as systemic lupus erythematosus [8] and rheumatoid arthritis [9]. WDFY3 is a master regulator of selective autophagy, which can work in concert with adaptor protein SQSTM1 (p62) to recruit and degrade ubiquitinated protein aggregates [10].

During the process of ubiquitinated protein aggregates sequestration, SQSTM1 (p62) works in concert with WDFY3, which is tethered to autophagosomal membranes [11]. Upon the autophagosome formation, cytosolic LC3-I converts into membrane-bound LC3-II. Therefore, the LC3-II to LC3-I ratio has been used as a marker correlated to autophagosome production [12,13]. WDFY3 can also form a complex with SQSTM1 and TNF receptor associated factor 6 (TRAF6) during midbody ring degradation by selective autophagy [14]. SQSTM1 has indispensable roles in osteoclast differentiation since SQSTM1 deficiency leads to defect osteoclast function *in vitro* and osteopetrosis phenotype *in vivo* [15]. Specifically, SQSTM1 acts as a bridge between receptor activator of NF-κB ligand (RANKL)/RANK/TRAF6 mediated NF-κB signaling [16]. Mutations of SQSTM1 at ubiquitin-associated domain lead to increased osteoclast differentiation and function that is

*Abbreviations:* ATG5 and ATG7, Autophagy-related proteins 5 and 7; BEACH, Beige and Chediak-Higashi; co-IP, co-immunoprecipitation; F-actin, filamentous-actin; M-CSF, Macrophage colony-stimulating factor; MNCs, multinucleated cells; PI3P, phosphatidylinositol 3-phosphate; PH, Pleckstrin homology; RANKL, receptor activator of NF-κB ligand

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linked to Paget's disease of bone, a skeletal disorder characterized by focal increased bone remodeling and abnormal bone structure formation [17].

Although the WDFY3/SQSTM1 interaction is important in autophagy, WDFY3 and SQSTM1 have also been associated with synovial fibroblasts and osteoclasts, implicating new roles in rheumatoid arthritis pathology [18,19]. The functional interaction between SQSTM1 and WDFY3 has been clearly documented in human osteoclasts [19], and we recently showed a novel role of WDFY3 in RANKL-induced osteoclastogenesis in the absence of inflammation [20]. To investigate the role of WDFY3 in arthritis we employed the K/BxN serum transfer-induced arthritis model, where anti-glucose-6-phosphate isomerase autoantibodies induce joint specific inflammation that closely resembles the rheumatoid arthritis pathologies in humans [21]. Our data show a new role of WDFY3 protein in the protection of autoimmune arthritis, which is independent of starvation-induced autophagy.

## 2. Methods

### 2.1. Antibodies and reagents

All cell incubations were performed in culture medium consisting of αMEM with 2 mM l-glutamine, 10% heat-inactivated FBS, 100 IU/ml Penicillin and 100 IU/ml Streptomycin (Life Technologies, 10007D). Mouse soluble RANKL (R & D Systems, 462TR) and M-CSF ELISA (R & D Systems, DY416), were used for *in vitro* experiments. CMG14-12 (CMG) media was generated as described before [22]. Anti-Wdfy3 (Abnova, clone 2F12), anti-WDFY3 (Novus, NBP1-03332), anti-SQSTM1 (Progen, GP62-C), LC3 antibody (Novus, NB100-2220), anti-β-actin antibody (Cell Signaling, 4970), 800 or 680 secondary antibodies (Li-Cor) were used for *in vitro* experiments. Cyto-ID kit (Enzo biochem, ENZ-51031) was used for autophagosome staining. LysoTracker DND-99 was used for lysosome staining (Life Technologies, L-7528).

### 2.2. Mice and bone cell culture

Animal experiments were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Eight to twelve weeks old C57BL/6 (The Jackson Laboratory), *Wdfy3* $^{loxP/loxP}$*-LysM-Cre+*, *Wdfy3*$^{loxP/loxP}$ animals [23] were sacrificed to extract bone marrow from both rear femurs and tibias bones. Bone marrow cells were cultured with 1:20 (v/v) CMG media [22] in absence or presence of 30 ng/mL RANKL. Media and cytokines were replenished every other day.

### 2.3. K/BxN serum-transfer arthritis model

Arthritis was induced in 8-week-old male and female mice by i.v. injection of 200 μl of pooled serum from K/BxN mice. Disease severity score was measured as previously described [21]. Briefly, mice were scored for paw swelling, 1 indicates mild swelling of the ankle insufficient to reverse the normal V shape of the foot; 2 indicates swelling sufficient to make the ankle and midfoot approximately equal in thickness to the forefoot; 3 indicates the reversal of the normal V shape of the foot. Every two days, disease severity score was recorded, and ankle thickness was measured using digital calipers [21].

### 2.4. Immunofluorescence staining

Osteoclasts grown on coverslip were imaged by Nikon C1 confocal microscopy. Cells were fixed in 4% paraformaldehyde for 10 min and were permeabilized in 0.2% Triton X-100 and blocked with 10% normal donkey serum buffer in PBS for 2 hours at room temperature. All antibodies were diluted in 5% normal donkey serum buffer in PBS. Primary antibodies were applied on samples overnight at 4 °C. Secondary antibody AF-488 donkey and anti-guinea pig Ig or AF-594 donkey anti-rabbit Ig antibodies were added to samples for one hour incubation at room temperature. TRITC conjugated phalloidin was used for F-actin staining for 30 minutes incubation at room temperature. Coverslips were then mounted in mounting media with DAPI (Vector Laboratories, H1200) to stain nuclei.

### 2.5. Co-Immunoprecipitation

Osteoclast-like cells cultured from 8 to 12 weeks old wild type mice were lysed in lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1%v/v Triton X-100 containing protease inhibitor cocktail tablet (Roche, 4693124001). The clear lysate was incubated with anti-Wdfy3 antibody overnight and was then co-immunoprecipitated by dynabeads protein A immunoprecipitation kit (Life technologies, 10006D).

### 2.6. Western blotting

Bone marrow-derived macrophages and osteoclast-like cells starved in serum-free medium were stimulated with RANKL (100 ng/ml) or M-CSF (100 ng/ml) and lysed at indicated time points. Protein lysates obtained from cell cultures were run on a Nu-Page 3–8% Tris-Acetate gel or 12% Bis-Tris gels (Invitrogen). Proteins were transferred to PVDF membranes and blocked in Odyssey blocking buffer. Membranes were incubated with anti-Wdfy3 primary antibody diluted in Odyssey blocking buffer containing 0.1% Tween-20, overnight at 4 °C. After washing, we incubated with secondary antibody (Li-Cor) in blocking buffer containing 0.1% Tween-20 and 0.02% SDS, washed, and imaged on the Li-Cor Odyssey scanner. The process was repeated with β-actin as loading control. Signal intensity relative to background was determined using Li-Cor Image Studio software.

### 2.7. Statistical analyses

Statistical significance was determined using Student's *t*-test and *p*-values lower than 0.05 were considered significant.

## 3. Results

### 3.1. WDFY3-cKO mice show reduced disease severity in K/BxN serum-transfer induced arthritis

Since reduced WDFY3 expression has been associated with rheumatoid arthritis [18], we used the K/BxN serum-transfer model to determine whether loss of WDFY3 in myeloid cells affected the pathogenesis of inflammatory arthritis. We injected pooled K/BxN serum into *Wdfy3-cKO* and wild type littermates at day 0 and record disease severity scores and ankle thickness every two days throughout the disease course. We observed reduced ankle swelling in *Wdfy3-cKO* at day eight post-serum transfer compared to wild type littermates (Fig. 1A). *Wdfy3-cKO* mice displayed significantly reduced arthritis severity as evidence by disease severity score (Fig. 1B), and measurements of ankle swelling (Fig. 1C).

### 3.2. Macrophages derived from WDFY3-cKO mice exhibit normal autophagosome formation

Since WDFY3 is involved in the autophagy pathway, we next investigated whether loss of WDFY3 affects starvation-induced autophagy. Western blot analysis of total cell lysates derived from wild type and WDFY3 deficient macrophages under starvation for 2 or 4 hours showed no significant differences of LC3-II to LC3-I ratio, an indicator of maturation of autophagosomes (Fig. 2A and B). Blockade of lysosomes and autophagosomes fusion with chloroquine again showed no significant difference in autophagy flux between wild type and WDFY3