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Title

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Permalink

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Journal

Cellular Immunology, 316

ISSN

0008-8749

Authors

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Publication Date

2017-06-01

DOI

10.1016/j.cellimm.2017.04.001

Peer reviewed



Published in final edited form as:

Cell Immunol. 2017 June ; 316: 61–69. doi:10.1016/j.cellimm.2017.04.001.

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- κ B pathway. Since the NF- κ B 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.

Keywords

Autophagy; Autophagy-linked FYVE containing protein; ALFY; WDFY3; osteoclast; musculoskeletal diseases

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

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Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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- κ B ligand (RANKL)/RANK/TRAF6 mediated NF- κ B signaling [16]. Mutations of SQSTM1 at ubiquitin-associated domain lead to increased osteoclast differentiation and function that is 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 α MEM with 2mM 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 μ l 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 minutes 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 8–12 weeks old wild type mice were lysed in lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM 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 deficient macrophages (Fig. 2C and 2D). We also used commercial available

cationic amphiphilic tracer (CAT) dye to visualize autophagosomes in wild type and WDFY3 deficient macrophages with fluorescent microscopy and detected no differences in autophagosome formation (Fig. 2E). Furthermore, we observed no difference in lysosome formation using LysoTracker dye and fluorescent microscopy in both wild type and WDFY3 deficient macrophages, which suggested WDFY3 deficiency did not influence starvation-induced in macrophages, and lysosomes formation in RANKL-stimulated TRAP⁺ multinucleated giant cells (osteoclasts) (Fig. 2F).

3.3 WDFY3 interacts with SQSTM1 in macrophages and multinucleated giant cells

To examine the interaction between WDFY3 and SQSTM1, we performed immunostaining of WDFY3 and SQSTM1 in mouse bone marrow-derived macrophages and observed SQSTM1 puncta co-localized with WDFY3 (Fig. 3A). We then further differentiated the bone marrow-derived macrophages, in the presence of RANKL into TRAP⁺ multinucleated giant cells/osteoclast (80–100µm) and also observed the presence of SQSTM1 puncta co-localized with WDFY3 in osteoclasts (Fig. 3B). We further confirmed the interaction between WDFY3 and SQSTM1 by co-immunoprecipitation in total cell lysates derived from wild type osteoclasts using anti-WDFY3 antibody and immunoblotted with SQSTM1 (Fig. 3C).

3.4 Macrophages and osteoclasts derived from *WDFY3-cko* mice show decreased level of SQSTM1

We then examined the adaptor protein SQSTM1, which plays multiple roles in both autophagy and RANKL signaling pathway in macrophages. Western blot analysis of total cell lysates from wild type and WDFY3 deficient macrophages treated in the presence or absence of RANKL showed no significant difference of autophagosome formation marker, LC3 but showed a reduced expression of SQSTM1 in WDFY3 deficient macrophages in the presence or absence of RANKL (Fig. 4A). We observed no difference when we quantified the LC3-II to LC3-I ratio and reduced SQSTM1 to β -actin ratio in WDFY3 deficient cells compared to wild type cells (Fig. 4B).

4. Discussion

WDFY3 together with WDFY1, WDFY2, WDFY4 belongs to the WDFY protein family. While all WDFY1, 2, 3 and 4 contain multiple WD40 repeats, their function is further diversified by the presence of an FYVE domain, in WDFY1, 2, 3, which is associated with vesicle trafficking. WDFY1 and WDFY2 are medium size proteins (around 400 amino acids) and have been associated with TLRs signaling and endocytosis respectively [24] and [25]. WDFY3 and WDFY4 are large proteins (>3000 amino acids) and contain PH and BEACH domains. WDFY4 has been associated with rheumatoid arthritis [26] and systemic lupus erythematosus [8] pathology in human genome-wide association studies (GWAS) studies, and WDFY3 has been detected in RA synovial fibroblasts [18] and osteoclasts [19]. Recently we showed that WDFY3 interacts with TRAF6 in RANKL-induced osteoclastogenesis in the absence of inflammation [20].

Herein, we investigated the role of WDFY3 in arthritis using the K/BxN serum transfer arthritis model. The deletion efficiency of WDFY3 in macrophages and osteoclasts derived from *Wdfy3-cKO* mice has been previously described [20]. We observed reduced joint swelling in *Wdfy3-cKO* mice compared to wild type littermates with serum transfer-induced arthritis suggesting that loss of WDFY3 in myeloid cells leads to a protective phenotype in arthritis. The protective effect from arthritis in *Wdfy3-cKO* mice was interesting since we previously observed increased NF- κ B activation in RANKL induced osteoclastogenesis. We anticipated increased NF- κ B activation under inflammatory conditions in the K/BxN serum transfer arthritis model. One plausible explanation for the protective phenotype in *Wdfy3-cKO* mice is that WDFY3 may play different roles in different types of myeloid cells such as neutrophils and mast cells where loss of WDFY3 in these myeloid cells may fail to initiate arthritis pathology due to inhibition of the secretory pathways. The protective effect from arthritis in *Wdfy3-cKO* mice was unlikely due to apoptosis of macrophages or osteoclasts as WDFY3 deletion in macrophages and osteoclasts has minimal effect on their viability [20]. Reduced expression of WDFY3 and the formation of SQSTM1-positive protein aggregates promote cell death in rheumatoid arthritis synovial fibroblasts (RASf) under severe ER stress [18]. Therefore enhanced apoptosis of inflammatory cells other than macrophages in K/BxN serum-transfer *Wdfy3-cKO* mice remains an additional possibility for the reduced pathology observed.

We then examined whether WDFY3 deficient macrophages had a defect in autophagy pathway and therefore lead to protective function in inflammatory arthritis in mice. In ATG5 and ATG7 deficient myeloid cells, autophagosome formation is impaired evidenced by the accumulation of LC3-I and absence of LC3-II [6]. However, WDFY3 deficiency in bone marrow-derived macrophages does not affect starvation-induced autophagy as evidenced by Western blot and immunofluorescence microscopy. These findings are in agreement with other studies that show WDFY3 is dispensable for starvation-induced autophagy [11]. WDFY3 targets specifically ubiquitinated protein aggregates in selective autophagy and has not been shown to play a role in other type of autophagy pathways such as LC3-associated phagocytosis [27].

WDFY3 interaction with SQSTM1 has been clearly documented in human multinucleated giant cells [19]. In agreement with the previous study, we also observed WDFY3 and SQSTM1 interaction in macrophages (pre-osteoclasts) and multinucleated giant cells by immunofluorescence microscopy and co-immunoprecipitation experiments. SQSTM1 has been shown to promote RANKL signaling, and complete ablation of SQSTM1 *in vivo* leads to dysfunctional osteoclastogenesis with increased bone mass [15]. Additionally, mutations of SQSTM1 that disrupt its C-terminal ubiquitin association (UBA) domain caused enhanced RANKL signaling, osteoclast differentiation, and bone resorption, which correlated to Paget's disease of bone in human [28–30]. In this paper, we show that SQSTM1 interacts with WDFY3, a protein that we previously showed to modulate RANKL-induced osteoclastogenesis *in vivo* and *in vitro* [20]. Our new data show that although the interaction of SQSTM1 with WDFY3 is critical for bone remodeling, this interaction may play different roles under non-inflammatory and inflammatory condition as we observed increased osteoclastogenesis but reduced disease severity in inflammatory arthritis in our animal models. Accordingly, we observed a down-regulation of SQSTM1 in WDFY3

deficient macrophages, which may partly contribute to the protective phenotype in K/BxN serum-transfer *Wdfy3-cKO* mice. The reduced expression of SQSTM1 correlates with reduced inflammation as published observations have shown silencing SQSTM1 reduces inflammatory responses in HaCaT cells (keratinocyte cell line) [31]. Interestingly, we recently showed that *Wdfy3-cKO* mice are also protective in a mouse model of psoriasis and have reduced epidermal hyperplasia associated with Munro's microabscess; specifically, WDFY3 deficient neutrophils show reduced NETosis, neutrophil elastase, and ROS signaling but show no difference in neutrophil expansion, circulation and survival [32]. In conclusion, WDFY3 deficiency ameliorates disease severity in the serum transfer-induced arthritis animal model. Further experiments are needed to further address WDFY3 roles in the specific myeloid cell types to uncover the mechanisms of WDFY3 in arthritis pathologies.

Acknowledgments

This work was partly supported by NIH/NIAMS-R01AR062173 and SHC 85700 grants to IEA, and by the UC Davis, graduate group in immunology fellowship to DW. We thank Drs. Bottini and Stanford (LJI) for the gift of K/BxN serum.

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- κ B ligand

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Loss of WDFY3 ameliorates disease severity in an arthritis animal model.

WDFY3 is not essential in starvation-induced autophagy in myeloid cells.

Loss of WDFY3 leads to decreased level of SQSTM1 in myeloid cells.

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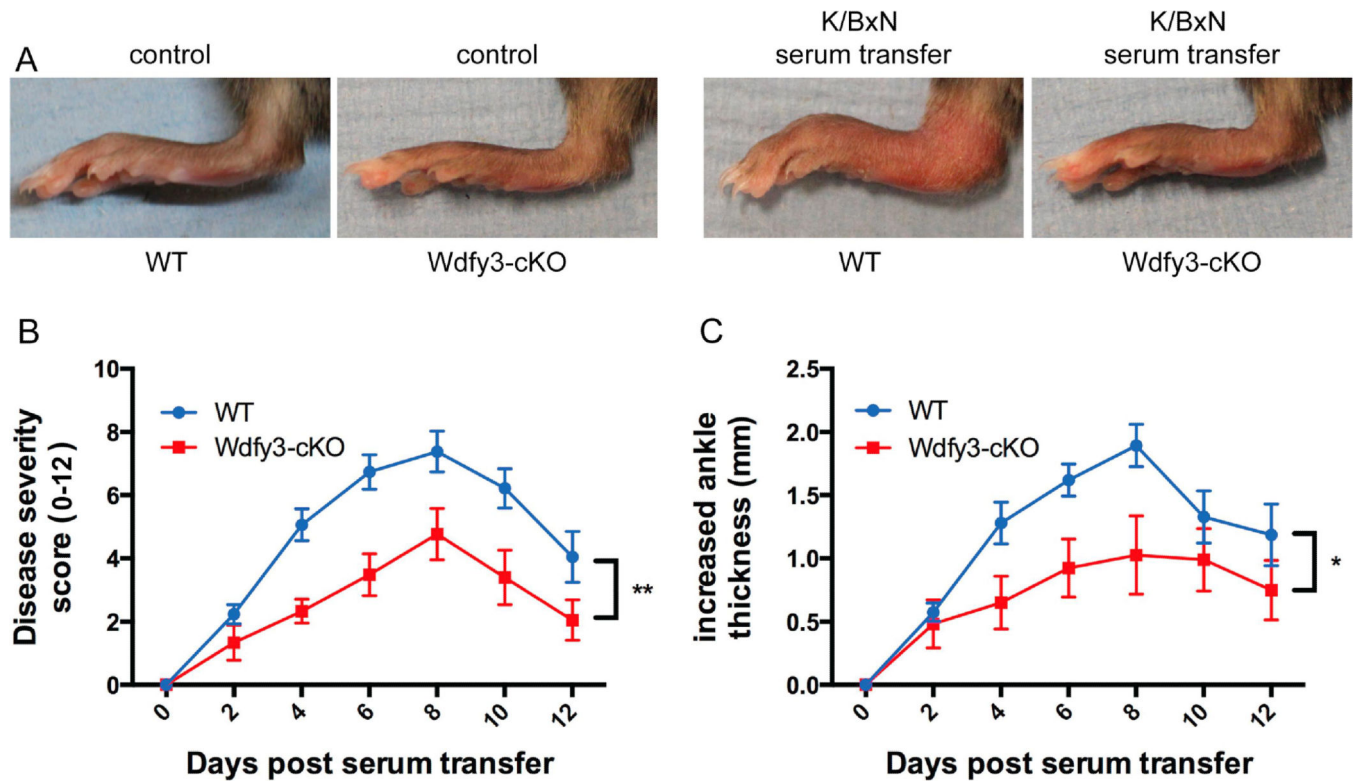
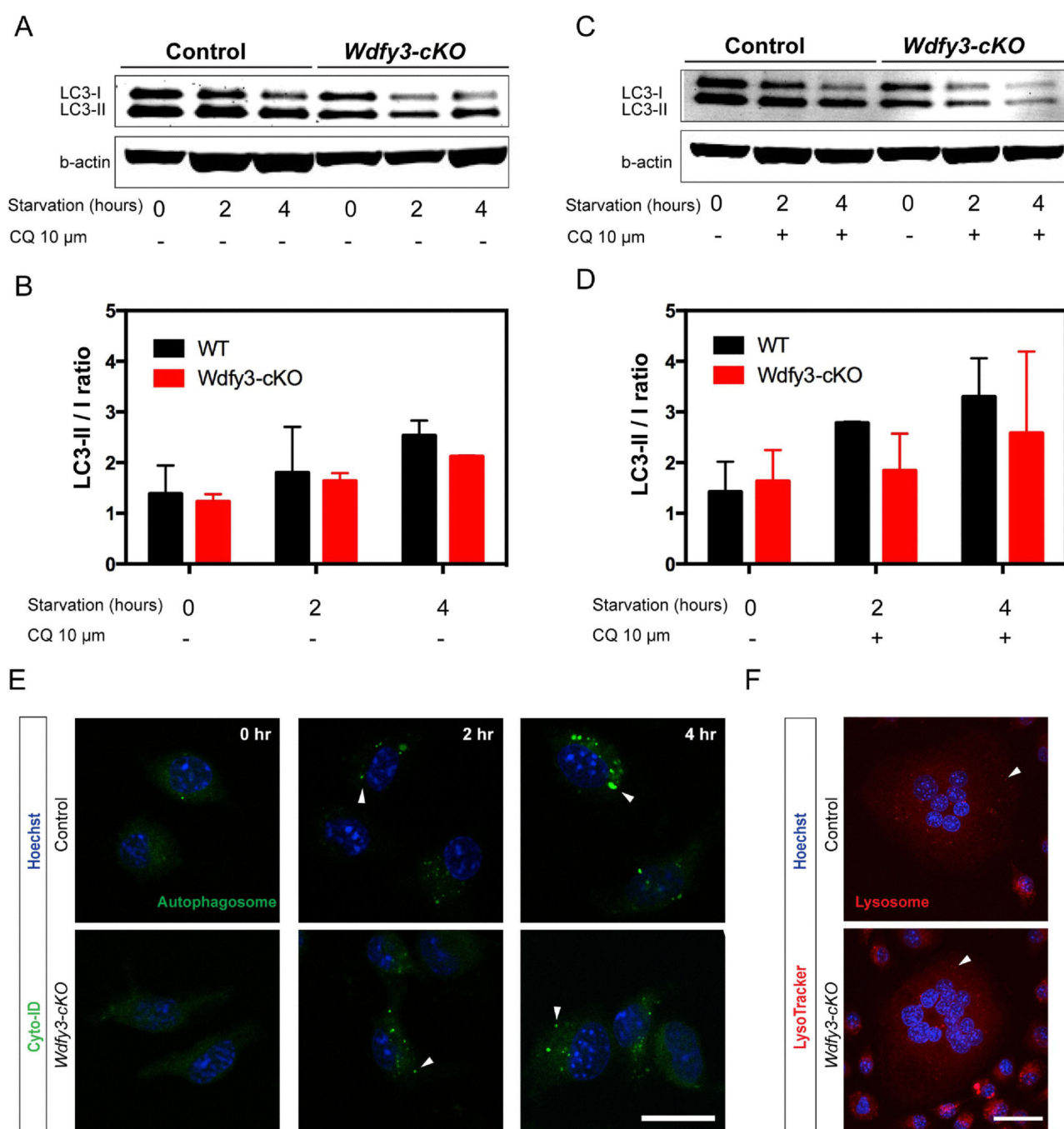


Figure 1.

Reduced disease severity in K/BxN serum-transfer arthritis in *Wdfy3-cKO* mice. Eight weeks old *Wdfy3-cKO* mice and wild type littermates were injected with 200 μ l pooled K/BxN serum. (A) Representative pictures of swollen paws derived from wild type or *Wdfy3-cKO* mice at day 8 post serum transfer. (B) Diseases severity score and (C) increased ankle thickness of the K/BxN serum transferred animals (n=6 for each group). The results were pooled from two independent experiments. Error bars represent mean $\pm$ SEM. 2-way ANOVA is used for statistical analysis in B, C. * $p \leq 0.05$, ** $p \leq 0.01$.

**Figure 2.**

WDFY3 deficient macrophages demonstrate no difference in starvation-induced autophagy, lysosome and autophagosome formation. (A, B) Western blot analysis of total cell lysates from wild type and WDFY3 deficient bone marrow derived macrophages (pre-osteoclasts) starved for 0, 2 and 4 hours in the absence or (C, D) in the presence of 10 μ M chloroquine. (E) Immunofluorescent photomicrographs of wild type or WDFY3 deficient macrophages in serum free HBSS for 2 to 4 hours with 10 μ M chloroquine treatment and stained with Cyto-ID kit to visualized autophagosomes (green) and nuclei (blue). (White arrowheads indicate

autophagosomes). (F) Multinucleated giant cells cultured from wild type or *Wdfy3-cKO* mice were stained with LysoTracker DND-99 (red) and Hoechst (blue) (White arrowheads indicate lysosomes. Scale bars represent 20 μm in E and 30 μm in F.

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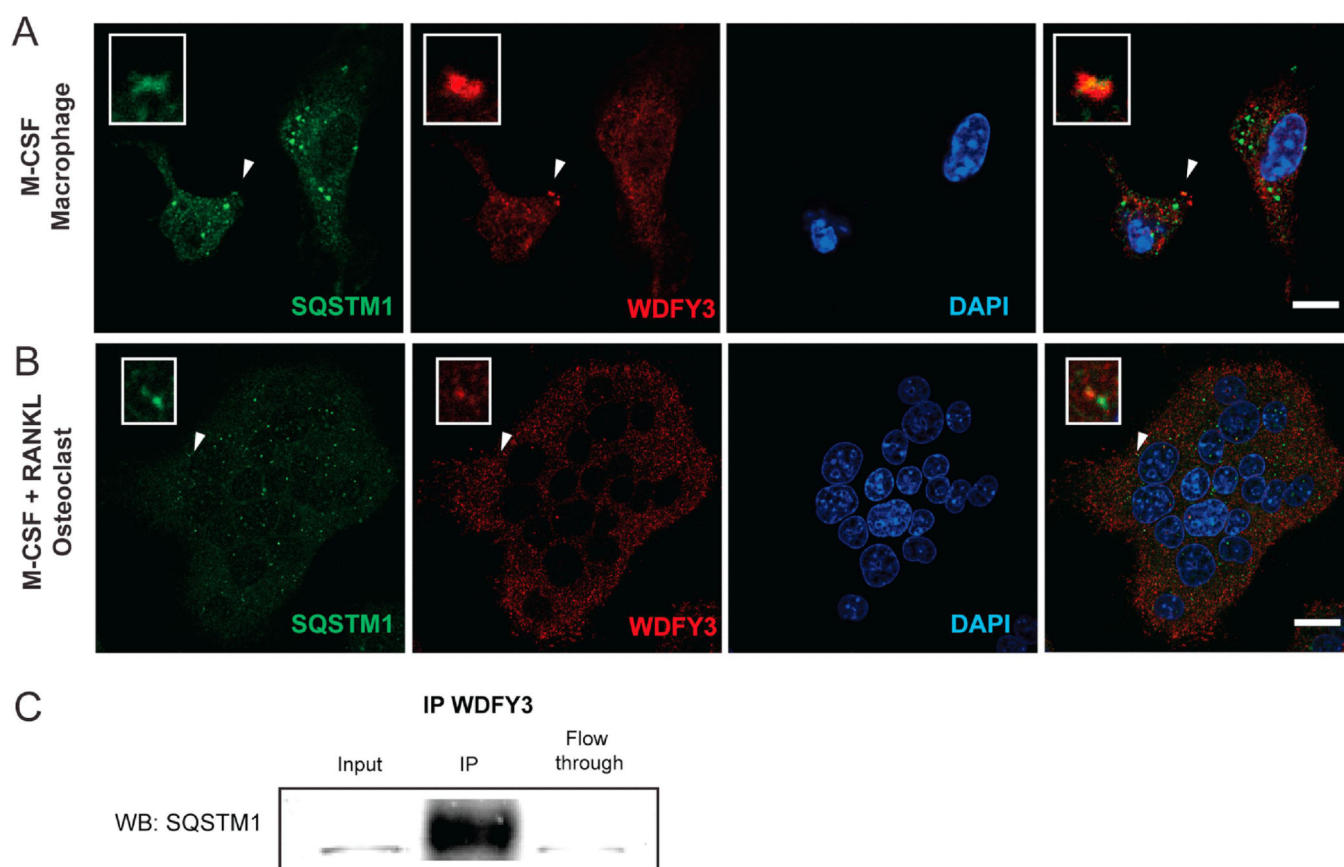


Figure 3. WDFY3 interacts with SQSTM1 in macrophages and multinucleated giant cells. (A) Immunofluorescent photomicrographs of macrophages (pre-osteoclasts) or (B) multinucleated giant cells (osteoclasts) were stained with anti-SQSTM1 (green), anti-WDFY3 (red), and DAPI (blue). (White arrowheads indicate WDFY3 and SQSTM1 co-localization). (C) Total cell lysates from osteoclast cultures co-immunoprecipitated with anti-WDFY3 antibodies and probed with SQSTM1 antibodies. Scale bars represent 10 μ m in A, 20 μ m in B.

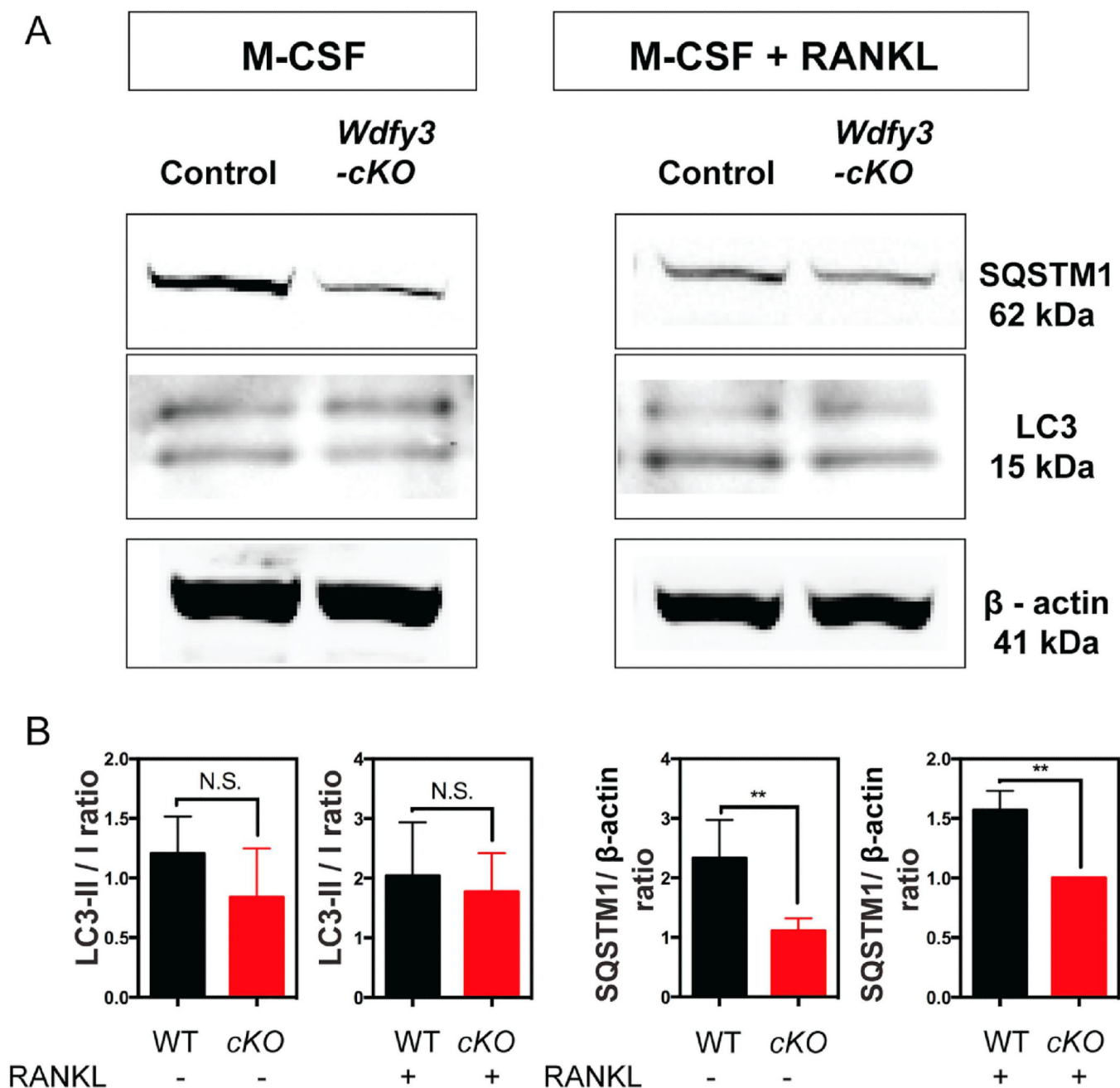


Figure 4.

Loss of WDFY3 leads to decrease level of SQSTM1 in macrophages. (A) Western blot analysis of total cell lysates from macrophages and multinucleated giant cells culture from wild type or *Wdfy3*-cKO mice. (B) Quantification of LC3-II/I ratio and SQSTM1 to β -actin ratio. Student *t*-test is used for statistical analysis in B. ** $p \leq 0.01$.