CD14+CD16- Monocytes Are the Main Precursors of Osteoclasts in Rheumatoid Arthritis via Expressing Tyro3TK

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Abstract

Background Monocytes as precursors of osteoclasts in rheumatoid arthritis (RA) were well demonstrated, while monocyte subsets in osteoclast formation were still controversial. Tyro3 tyrosine kinase (Tyro3TK) is a member of the receptor tyrosine kinase family involved in immune homeostasis, the role of which in osteoclast differentiation was reported recently. This study aimed to compare the osteoclastic capacity of $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes in RA and determine the potential involvement of Tyro3TK in their osteoclastogenesis.

Methods Osteoclasts were induced in $CD14^+CD16^+$ and $CD14^+CD16^-$ monocyte subsets isolated from healthy control (HC) and RA patients *in vitro* and evaluated by tartrate-resistant acid phosphatase (TRAP) staining. Then, the expression of Tyro3TK on $CD14^+CD16^+$ and $CD14^+CD16^-$ monocyte subsets in peripheral blood of RA, osteoarthritis (OA), and HC were evaluated by flow cytometry, and their correlation with RA patient clinical and immunological features were analyzed. The role of Tyro3TK on $CD14^+CD16^-$ monocytes in RA patient osteoclastogenesis was further investigated by osteoclast differentiation assay with Tyro3TK blockade.

Results The results revealed that $CD14^+CD16^-$ monocytes were the main source of osteoclasts. The expression of Tyro3TK on $CD14^+CD16^-$ monocytes was significantly upregulated in RA patients compared with HC and OA patients, and positively correlated with the disease manifestations, such as IgM level, tender joint count and the disease activity score. Moreover, anti-Tyro3TK antibody could dose-dependently inhibited Gas6-mediated osteoclast differentiation in $CD14^+CD16^-$ monocytes.

Conclusions These findings indicate that elevated Tyro3TK on $CD14^+CD16^-$ monocytes serves as a critical signal for osteoclast differentiation in RA.

Background

Rheumatoid arthritis (RA) is one of the most common chronic systemic inflammatory rheumatic disease hallmarked by synovitis, aggressive lesions of articular cartilage and bone, which leads to irreversible joint deformity and loss of function [1–3]. Bone erosion is a main pathological change in RA, which can even be observed in more than 45% of RA patients at an early stage [4]. It has been proved that excessive activation of local osteoclasts is involved in focal bone erosion in RA [5]. Osteoclasts are multinucleated cells which derived from the monocyte/macrophage lineage, especially from $CD14^+$ monocyte [6].

Monocytes are plastic cells that can differentiate into macrophages, dendritic cells, and osteoclasts, which can accumulate in the blood and continuously migrate to inflammatory joints. Overexpression of monocytes in RA patients can lead to chronic joint inflammation and bone destruction [7]. Recently, based on differential surface expression of CD14 and CD16, human monocytes could be subdivided into two major subsets: $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes, accounting for 5–10% and 90–95% of monocytes in healthy individuals, respectively [8].

However, the role of $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes in osteoclasts formation is still controversial. Bolzoni M et al. demonstrated that bone marrow $CD14^+CD16^+$ monocytes from patients with multiple myeloma could differentiate into more osteoclasts than $CD14^+CD16^-$ monocytes [9]. Chiu YG et al. also suggested that $CD16^+$ monocytes from psoriatic arthritis patients were more prone to differentiate to osteoclasts [10]. In contrast, several studies illustrated that the osteoclasts were mainly derived from the $CD14^+CD16^-$ monocytes in healthy donors [10–12]. Komano Y et al. further demonstrated that $CD14^+CD16^-$ monocytes rather than $CD14^+CD16^+$ monocytes were the circulating osteoclast precursors of osteoclasts in RA recently [11]. All these suggest that peripheral blood monocyte subsets may be directly involved in exacerbated osteoclast formation in RA. However, it is still controversial which monocyte subsets are the major sources of osteoclasts.

Tyro3 tyrosine kinase (Tyro3TK) is one of the family members of TAM (Tyro3TK, AxlTK, MerTK) receptor tyrosine kinases (RTKs) [13], which could be expressed on the plasma membrane of a variety of cells, such as monocytes/macrophages, dendritic cells, NK cells and nerve cells [14]. Tyro3TK could regulate the clearance of apoptotic cells, cytokine production, cell proliferation, thrombus formation, and hematopoiesis by binding to its ligands growth arrest-specific protein 6 (Gas6) and protein S (ProS1) [15, 16]. It was reported that Gas6 is expressed in RA synovium tissue and fluid and plays a role in RA synovium endothelial cell survival [17]. Furthermore, the expression of Gas6 appears to be stimulated by an inflammatory response, since elevated serum Gas6 levels were shown in sepsis and other systemic inflammation [18].

In 1998, Nakamura YS et al. firstly identified that Tyro3TK could be expressed in multinucleated osteoclasts, and the bone resorption activity of mature osteoclasts can be enhanced when binding with the ligand Gas6. However, Tyro3TK did not affect the differentiation of osteoclasts from bone marrow cells [19]. Katagiri M et al. also found that Tyro3TK can be detected in mature osteoclasts while they showed that Gas6 demonstrated no apparent effect on osteoclast formation in mouse osteoclast progenitor cells [20]. Kawaguchi H et al. found that Tyro3TK can only be detected in mouse mature osteoclasts among bone cells, while Gas6 is widely expressed in bone cells, stimulating the function of osteoclasts [21]. Recently, Ruiz-Heiland G et al. illustrated that Tyro3TK deficient mice showed an increased bone mass and impaired osteoclast differentiation in the arthritis model, suggesting the involvement of Tyro3TK in the differentiation and functional maturation of osteoclasts [22]. All these indicated that Tyro3TK might play a critical role in bone destruction in inflammatory arthritis. Despite these findings, the expression and osteogenic function of Tyro3TK on monocyte subsets in RA remain largely unknown.

In this study, we compared the osteoclastic capacity of $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes in RA and determined the levels as well as the potential involvement of Tyro3TK in their osteoclastogenesis, aiming to further understand the mechanism of RA bone destruction.

Methods

Patients and controls

53 patients with RA (Table 1), 28 osteoarthritis (OA) patients, as well as 45 age- and sex-matched healthy control (HC) were enrolled in this study. All the patients met the 2010 American College of Rheumatology (ACR) revised criteria for RA [23] and 1986 ACR criteria for OA [24]. The study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital. Moreover, all participants provided informed consent.

Table 1
Demographic and clinical characteristics of RA patients

Characteristics	RA (n = 53)
Age, mean (range), years	53 (23–83)
Sex, no, female/male	40/13
Duration, mean (range), years	14.4 (0.25–58)
SJC, median (range) of 28 joints	2 (0–28)
TJC, median (range) of 28 joints	5 (0–28)
RF, mean (range), IU/ml	297.5 (20–5660)
Anti-CCP antibody, mean (range), IU/ml	171 (2.72–311)
ESR, mean (range), mm/h	46.9 (6–115)
CRP, mean (range), mg/l	29.8 (0.27–124)
DAS28-ESR, mean (range)	6.47 (1.25–11.94)

RA, rheumatoid arthritis; SJC, swollen joint count; TJC, tender joint count; RF, rheumatoid factor; Anti-CCP antibody, anti-cyclic citrullinated peptide antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score 28.

Clinical and laboratory indices of RA

The following data of patients with RA were recorded: gender, age, duration, swollen joint count (SJC), tender joint count (TJC) and laboratory parameters including white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), platelets (PLT), immunoglobulin (Ig) A, IgG, IgM, anti-cyclic citrullinated peptide antibody (anti-CCP antibody), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP). Disease activity scores were calculated using the 28-joint Disease Activity Score-erythrocyte sedimentation rate (DAS28-ESR) in patients with RA. DAS28-ESR > 5.1 was considered a high disease activity according to the recommendations from the European League Against Rheumatism (EULAR).

Antibodies And Reagents

Recombinant human macrophage colony-stimulating factor (rhM-CSF) was obtained from PeoproTech GmbH (Rocky Hill, CT). Recombinant human RANKL (rhRANKL), recombinant human Gas6 (rhGas6), human anti-Tyro3TK antibody, human Tyro3TK PE-conjugated antibody, Mouse IgG2B PE-conjugated antibody were purchased from R&D Systems (Minneapolis, MN). Human TruStain FcX™ (Fc Receptor Blocking Solution) was purchased from BioLegend (San Diego, CA). Human CD14 FITC-conjugated antibody and human CD16 APC-conjugated antibody were purchased from eBioscience (San Diego, CA). Leukocyte Acid Phosphatase Kit was purchased from Sigma-Aldrich (St Louis, MO). α-minimum essential medium (α-MEM), 1% penicillin/streptomycin, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA).

Flow cytometry analysis and sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh EDTA blood samples using Ficoll density-gradient centrifugation. Before staining with antibodies, single-cell suspensions were incubated with human Fc Receptor Blocking Solution for 15 min at room temperature.

To detect the expression of Tyro3TK on $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes, cells were stained with CD14 FITC-conjugated antibody, CD16 APC-conjugated antibody, and Tyro3TK PE-conjugated antibody. Corresponding negative isotype and fluorochrome-matched control (FMO) staining were also performed. The cells were then analyzed on FACS Aria II.

For $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes sorting, cells were stained with CD14 FITC-conjugated antibody and CD16 APC-conjugated antibody. Then the stained cells were sorted with FACS Aria II. The purified $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes were further analyzed after sorting; the purity of which used for experiments was about 95% - 99%.

In vitro osteoclast differentiation

$CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes from freshly isolated PBMCs were purified by FACS sorting. Then the cells were cultivated 17 days separately in 96-well plates (5×10^4 cells/200 μl per well) in α-MEM with 1% PenStrep, 10% heat-inactivated fetal bovine serum, 30 ng/ml rhM-CSF and 50 ng/ml rhRANKL. Different concentrations of rhGas6 and/or human anti-Tyro3TK antibody were added as indicated. The medium was changed with fresh medium every 6 days. Osteoclast differentiation was evaluated by staining cells for TRAP using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions. TRAP-positive multinucleated cells were counted by an inverted fluorescence microscope (Olympus IX71-141, Tokyo, Japan).

Statistical analysis

All data were analyzed on the statistical software program SPSS 24.0 for windows (SPSS, Chicago, IL). Differences between groups were evaluated by Student's t-test, non-parametric Mann-Whitney U test, one-way ANOVA test, and Spearman's correlation test. *P* value less than 0.05 was considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant).

Results

$CD14^+CD16^-$ monocytes are the main precursors of osteoclasts in RA

To reveal which monocyte subset plays a significant role in osteoclast formation in RA, we performed osteoclast differentiation assay with monocyte subpopulation *in vitro*. $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes were isolated from 5 HC and 5 RA patients by FACS sorting, respectively, the purity of which was confirmed by FACS (Fig. 1). Then osteoclast differentiation and TRAP staining were performed. Interestingly, the results showed that the number of TRAP-positive osteoclasts differentiated from $CD14^+CD16^-$ monocytes were much more than that from $CD14^+CD16^+$ monocytes in HC (Fig. 2A). Moreover, $CD14^+CD16^-$ monocytes demonstrated upregulated capacity of osteoclast differentiation in RA patients (Fig. 2B). However, there was no distinct difference for $CD14^+CD16^+$ monocytes between RA patients and HC (Fig. 2C).

Expression of Tyro3TK is enriched on $CD14^+CD16^-$ monocytes and upregulated in RA patients

Then, we tried to reveal the effects of Tyro3TK on monocyte subsets-mediated osteoclast differentiation. The expression of Tyro3TK on monocyte subsets in RA patients, OA patients, and HC were first analyzed and presented as mean fluorescence intensity (MFI). The gating strategy was demonstrated in Fig. 3A. We identified that there was no apparent difference in the expression of Tyro3TK on $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes in HC and OA (Fig. 3B-C). Interestingly, the expression of Tyro3TK on $CD14^+CD16^-$ monocytes in patients with RA was significantly higher than that of $CD14^+CD16^+$ monocytes (Fig. 3D). Moreover, the expression of Tyro3TK on $CD14^+CD16^-$ monocytes was significantly increased in RA patients as compared with OA patients and HC. However, no significant difference was found for Tyro3TK expression on $CD14^+CD16^+$ monocytes between RA patients, OA patients, and HC (Fig. 3E-F).

Tyro3TK on $CD14^+CD16^-$ monocytes are associated with RA patient clinical and immunological features

Then, we analyzed the correlation of Tyro3TK on $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes with RA patient clinical and immunological features, respectively. The results revealed substantial associations (Table 2). Notably, the levels of Tyro3TK on $CD14^+CD16^-$ monocytes were found to be positively correlated with DAS28-ESR, TJC, and serum IgM (Fig. 4A-C). Detailed analyses showed that RA patients

with high disease activity (DAS28-ESR > 5.1) showed higher levels of Tyro3TK on $CD14^+CD16^-$ monocytes (Fig. 4D). Similar results were also seen in RA patients with tender joints and RF positivity (Fig. 4E-F). However, no apparent association was found between the levels of Tyro3TK on $CD14^+CD16^-$ monocytes and RA patient gender, anti-CCP, or swollen joints (Fig. 4G-I).

Table 2
Correlation of Tyro3TK expression on monocyte subsets with RA patient clinical and immunological features.

Features	Tyro3TK on $CD14^+CD16^+$monocytes		Tyro3TK on $CD14^-CD16^-$monocytes	
	r	*P*	*r*	*P*
Age	-0.005	0.974	0.071	0.664
Duration	-0.077	0.637	-0.071	0.664
WBC	0.15	0.357	0.097	0.554
RBC	0.03	0.853	0.041	0.803
Hb	-0.023	0.889	0.035	0.831
PLT	0.092	0.572	-0.012	0.941
ESR	0.088	0.59	0.104	0.522
CRP	0.118	0.469	0.072	0.659
IgA	0.222	0.169	0.196	0.225
IgG	0.106	0.52	0.099	0.547
IgM	**0.348***	**0.028**	**0.432****	**0.005**
RF	0.136	0.402	0.108	0.509
Anti-CCP antibody	0.192	0.243	0.172	0.295
TJC	**0.459****	**0.003**	**0.514****	**0.001**
SJC	0.054	0.741	0.043	0.793
DAS28-ESR	0.28	0.08	**0.323***	**0.042**

WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; PLT, platelets; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IgA/G/M, immunoglobulin A/G/M; RF, rheumatoid factor; Anti-CCP antibody, anti-cyclic citrullinated peptide antibody; TJC, tender joint count; SJC, swollen joint count; DAS, disease activity score. The date was analyzed by Spearman's correlation coefficient test. *P < 0.05, **P < 0.01.

Upregulated Tyro3TK on $CD14^+CD16^-$ monocytes promotes their osteoclast differentiation in RA

To further illustrate the osteoclast-priming effects of Tyro3TK on $CD14^+CD16^-$ monocytes in RA patients, we performed osteoclast differentiation assay with or without Tyro3TK blockade. As shown in Fig. 5A, the co-culture of $CD14^+CD16^-$ monocytes isolated from RA patients with rhGas6 promoted TRAP-positive osteoclast formation, especially at the dose of 50 ng/ml. Strikingly, anti-Tyro3TK antibody significantly compromised this rhGas6-mediated exacerbation of osteoclast differentiation in a dose-dependent manner. At the dose of 200 ng/ml, anti-Tyro3TK antibody could almost abolish the formation of osteoclasts(Figure 5B). Collectively, these results revealed the critical role of Tyro3TK in mediating $CD14^+CD16^-$ monocyte differentiation into osteoclasts.

Discussion

In this study, we found that $CD14^+CD16^-$ monocytes were more potent in osteoclast differentiation in HC, the capacity of which was more powerful in RA patients. The expression of Tyro3TK on $CD14^+CD16^-$ monocytes was elevated in RA, correlating with the clinical features of the patient. Moreover, upregulated Tyro3TK on $CD14^+CD16^-$ monocytes promotes their osteoclast differentiation in RA.

Peripheral blood monocytes played an essential role in secreting inflammatory factors, regulating innate immunity, and inducing osteoclast formation [25]. Monocyte heterogeneity has been recognized in humans for a long time. Based on phenotypic characteristics, human monocytes can be divided into $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes [7]. Furthermore, these two monocyte subsets might possess different functions. Our previous study showed that $CD14^+CD16^+$ monocytes in patients with systemic lupus erythematosus showed inflammatory phenotype, with increased CD80, CD86, HLA-DR, and CX3CR1, which could promote Th17 response [26]. IL-17 is a pro-inflammatory cytokine mainly produced by $CD4^+$ T cells and plays a critical role in RA synovitis [27]. Kotake S et al. illustrated that the level of cytokine IL-17 was significantly increased in RA synovial fluid, and IL-17 could promote $CD14^+$ monocytes differentiated into osteoclasts [28]. $CD14^+CD16^+$ monocytes can also migrate to RA synovium and produce high levels of TNF-α, IL-6, and IL-1β. These cytokines could promote the production of cytokine IL-17, thus playing a critical role in synovial inflammation and osteoclasts formation [29–31]. Here, we showed that $CD14^+CD16^-$ monocytes were more prone to differentiate into osteoclasts than $CD14^+CD16^+$ monocytes in healthy controls. Moreover, the osteoclastic capacity of $CD14^+CD16^-$ monocytes was significantly enhanced in RA patients. Although with controversial, these results were consistent with most previous studies [10–12]. Therefore, we speculate that $CD14^+CD16^-$ monocytes are the main osteoclast precursors in RA, while $CD14^+CD16^+$ monocytes are more competent in producing proinflammatory cytokines. Detailed mechanistic studies are still needed to reveal the differential functions of these two monocyte subsets.

Tyro3TK was initially discovered as a therapeutic target in tumors [32]. More and more studies have focused on their critical role in autoimmune disease [33, 34]. Barth ND et al. demonstrated that Tyro3TK could express in monocytes [35]. As the ligand of Tyro3TK, Gas6 was evaluated in RA synovium tissue and fluid [17]. It can promote RA synovial hyperplasia, which is hallmarked by the abundant synovial

fibroblasts and associated with bone destruction in RA [36]. While Gas6-Tyro3TK interaction may play a critical osteoclast-priming role [19–22]. In this study, we showed that Tyro3TK on $CD14^+CD16^-$ monocytes of RA patients was significantly upregulated, which was associated with clinical features and disease activity. Furthermore, Gas6 can promote the osteoclasts formation of $CD14^+CD16^-$ monocytes, while disrupts Gas6-Tyro3TK interaction, the number of osteoclasts differentiated from $CD14^+CD16^-$ monocytes decreased significantly with a dose-dependent anti-Tyro3TK antibody. The study also extends our findings, demonstrating that Tyro3TK has a distinct role in regulating $CD14^+CD16^-$ monocytes osteoclastogenesis, suggesting that Tyro3TK might be a possible therapeutic target for RA bone destruction. Therefore, it is intriguing to propose that targeting Tyro3TK and $CD14^+CD16^-$ monocytes at the same time may have a more apparent inhibitory effect on bone destruction of RA. However, the detailed signal mechanisms of Tyro3TK on $CD14^+CD16^-$ in RA need to be further studied.

Conclusion

In summary, this study reveals that $CD14^+CD16^-$ monocytes are the main precursors of osteoclasts in RA. Moreover, upregulated Tyro3TK expression on these cells provides a pivotal role for osteoclastogenesis, which might serve as therapeutic targets for the persistent disease.

Abbreviations

RA, rheumatoid arthritis; OA, osteoarthritis; HC, healthy control; TRAP tartrate-resistant acid phosphatase; Gas6, growth arrest-specific protein 6; ProS1, protein S; RTKs, receptor tyrosine kinases; ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; SJC, swollen joint count; TJC, tender joint count; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; PLT, platelets; IgA/G/M, immunoglobulin A/G/M; anti-CCP antibody, anti-cyclic citrullinated peptide antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffer saline; FMO, fluorochrome-matched controls; MFI, mean fluorescence intensity; α-MEM, α-minimum essential medium; M-CSF, macrophage colony-stimulating factor; RANKL, nuclear factor-κB ligand.

Declarations

Ethics approval and consent to participate

All subjects gave written informed consent under the Declaration of Helsinki. The protocol was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Performed the experiments: JM.X. and LL.X.; Analyzed the data: HQ.Z., X.L.; Contributed reagents/materials/analysis tools: MX.B., Z.Z., H.Z., G.C., X.L.; Wrote the manuscript: JM.X. and LL.X.; Conceived the study, reviewed, and edited the manuscript: FL.H. and Y.S.

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Figures

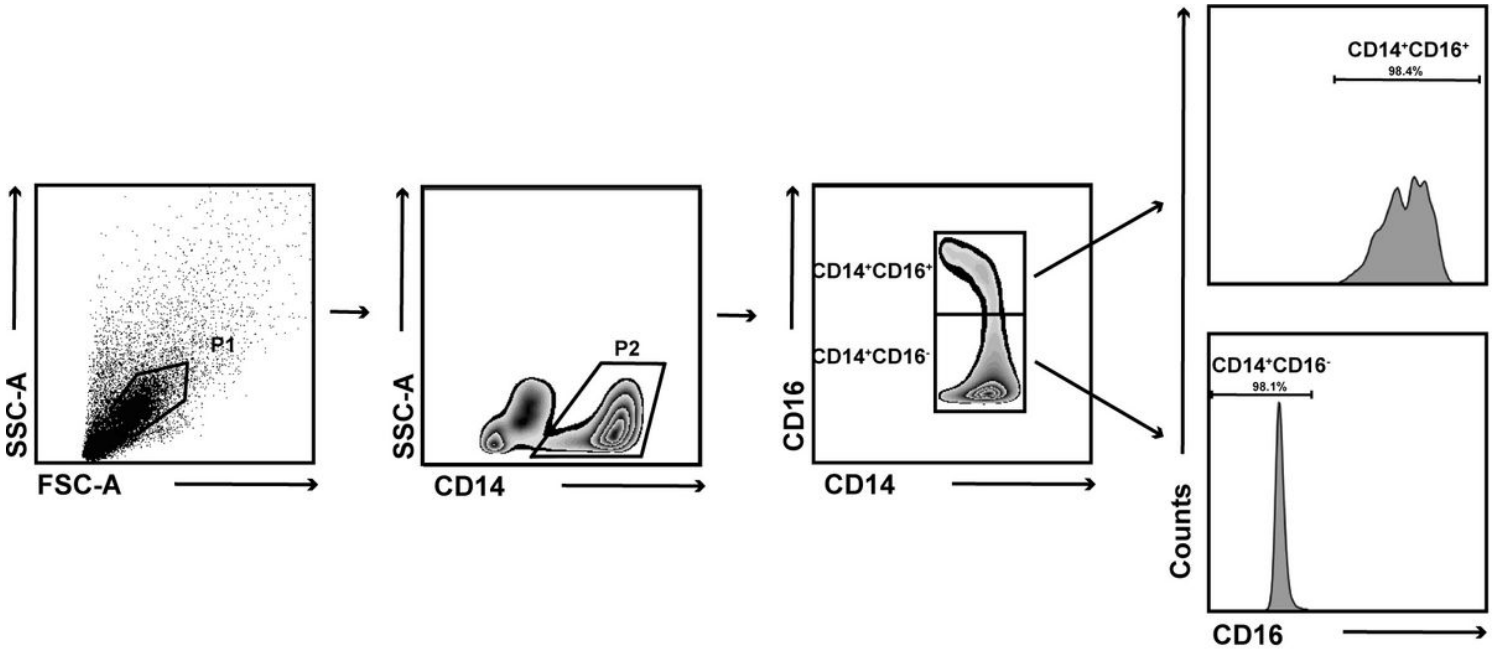

Figure 1

Gating strategy for flow cytometry sorting of human CD14+CD16+ and CD14+CD16- monocytes. Peripheral blood mononuclear cells from RA and HC were stained with FITC-conjugated anti-CD14 antibody and APC-conjugated anti-CD16 antibody. CD14+CD16+ and CD14+CD16- monocytes were analyzed and sorted by flow cytometric, the purity of sorted CD14+CD16+ and CD14+CD16- monocytes used for experiments was about 95% - 99%.

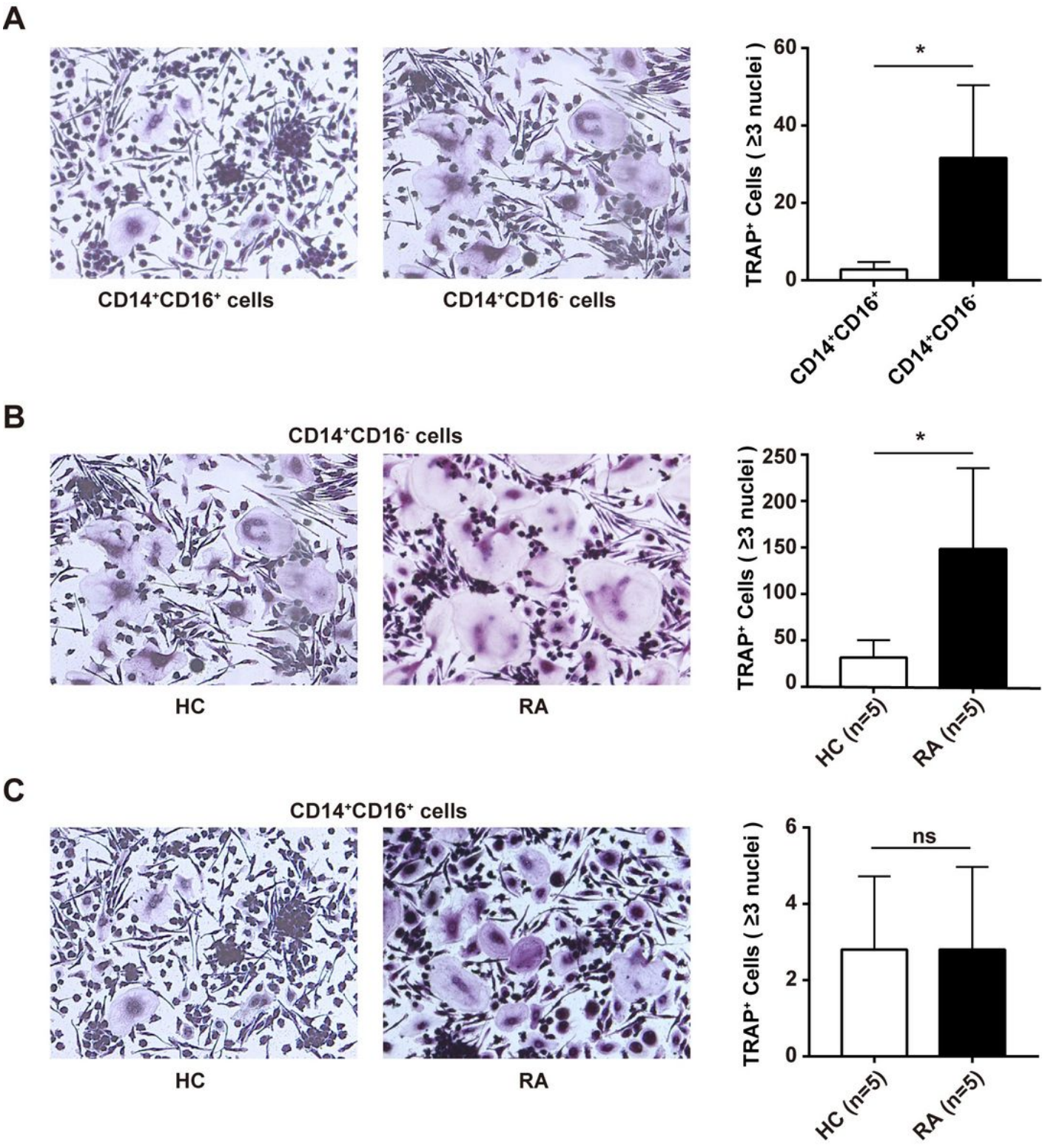

Figure 2

CD14+CD16- monocytes are main osteoclast precursors in RA. Purified CD14+CD16+ and CD14+CD16- monocytes from RA (n = 5) and HC (n = 5) were cultured with rhM-CSF (30 ng/ml) and rhRANKL (50 ng/ml) for osteoclast differentiation. The cells were detected for tartrate-resistant acid phosphatase (TRAP) staining on day 17, and the TRAP-positive multinuclear cells were osteoclasts. The representative charts and the statistical results were shown, respectively. (A) CD14+CD16+ versus CD14+CD16-

monocytes in HC (*P = 0.026); (B) RA versus HC for CD14+CD16- monocytes (*P = 0.019); (C) RA versus HC for CD14+CD16+ monocytes. *P < 0.05, ns, not significant (Student's t test A-C).

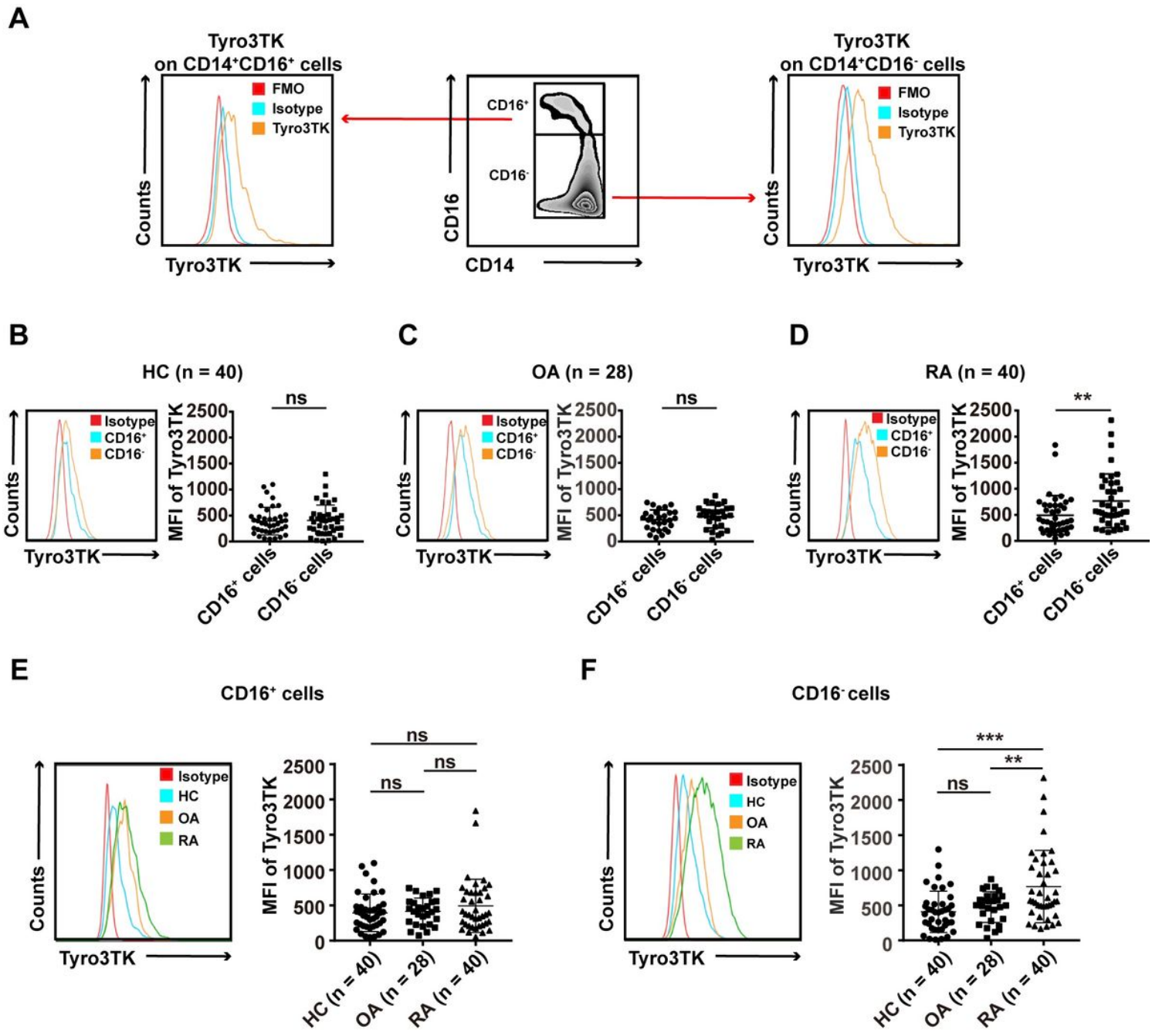

Figure 3

The expression of Tyro3TK on CD14+CD16- monocytes is increased in RA. (A) Gating strategy for identifying the expression of Tyro3TK on CD14+CD16+ and CD14+CD16- monocytes. Accordingly, the expression of Tyro3TK on CD14+CD16+ and CD14+CD16- monocytes in HC (n = 40) (B), OA (n = 28) (C), and RA patients (n = 40, **P = 0.008) were analyzed, respectively, and presented as mean fluorescence intensity (MFI). (E) The expression of Tyro3TK on CD14+CD16+ monocytes were compared between HC, OA, and RA patients (**P = 0.008, ***P < 0.001). (F) The expression of Tyro3TK on CD14+CD16-

monocytes were compared between HC, OA, and RA patients. **P < 0.01, ***P < 0.001, ns, not significant (Mann-Whitney U test B and D, Student's t test C, one-way ANOVA test E and F).

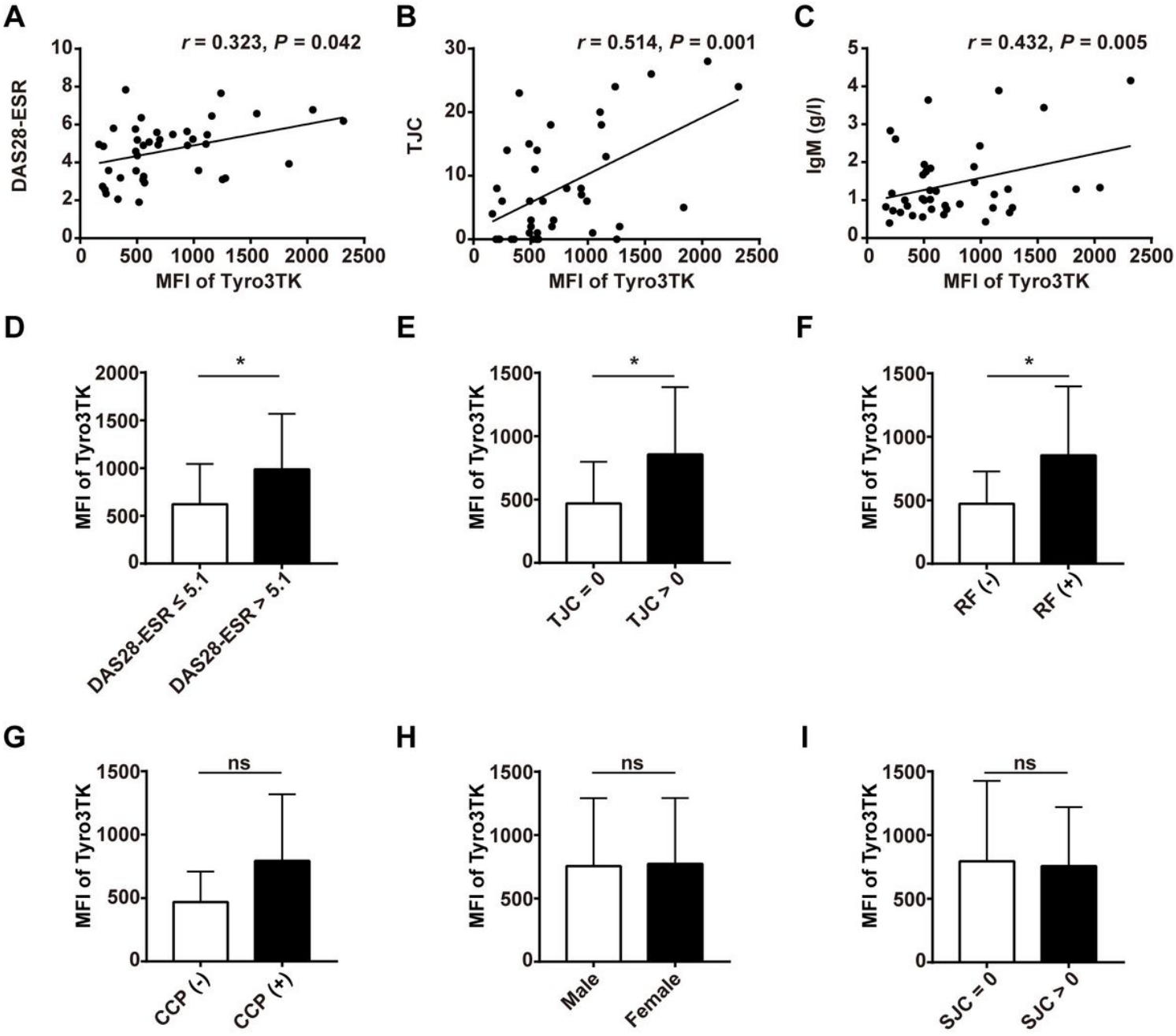

Figure 4

Correlation analysis of Tyro3TK on CD14+CD16- monocytes with RA patient clinical manifestations. The associations of Tyro3TK on CD14+CD16- monocytes with RA patient DAS28-ESR (r = 0.323, *P = 0.042) (A), tender joint counts (TJC) (r = 0.514, **P = 0.001) (B), and IgM (r = 0.432, **P = 0.005) (C) were analyzed, respectively. The expression of Tyro3TK on CD14+CD16- monocytes were also compared between different RA patient groups: (D) RA with high disease activity (DAS28-ESR > 5.1) and non-high disease activity (DAS28-ESR ≤ 5.1) (*P = 0.034), (E) RA with and without tender joints (*P = 0.031), (F) rheumatoid factor (RF) positive and negative RA (*P = 0.024), (G) anti-CCP antibody positive and negative

RA, (H) male and female RA, (I) RA with and without swollen joints. (Spearman's rank correlation test A-C, and Mann-Whitney U test D-I, *P < 0.05, **P < 0.01, ns, not significant).

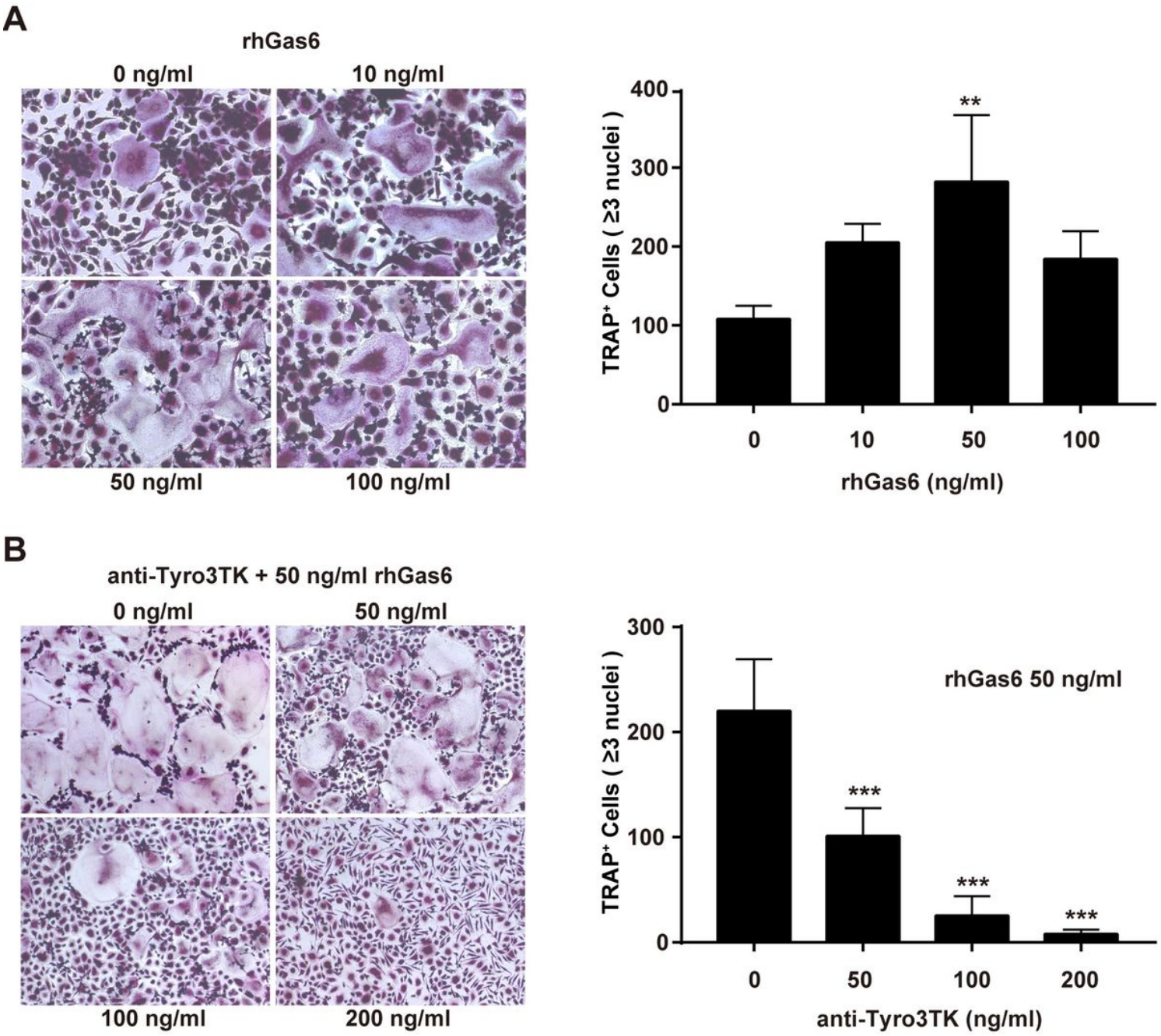

Figure 5

Tyro3TK promotes CD14+CD16- monocytes mediated osteoclastogenesis in RA. Purified CD14+CD16- monocytes from RA patients (n = 8) were cultured with rhM-CSF (30 ng/ml) and rhRANKL (50 ng/ml) under different conditions for osteoclast differentiation. 17 days later, the cells were harvested for TRAP staining. The representative charts and the statistical results were shown, respectively. (A) Different concentrations of rhGas6 (0 ng/ml, 10 ng/ml, 50 ng/ml and 100 ng/ml) were supplemented for osteoclast differentiation (n = 3 per group, **P = 0.006). (B) Different concentrations of anti-Tyro3TK antibody (0 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml) and 50 ng/ml rhGas6 were supplemented for

osteoclast differentiation (n = 5 per group, ***P < 0.001). **P < 0.01, ***P < 0.001 (One-way ANOVA test A and B).