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The study of gp130/the inflammatory factors regulating osteoclast differentiation in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic immune disease characterized by synovitis and bone destruction. The osteoclasts play a critical role in pathologic bone loss during inflammatory arthritis. In this paper, we report that Interleukin (IL)-6, IL-6Rα/gp130, IL-11, IL-27, and Matrix Metallo Proteinases (MMP)-9 expression results in serum of the RA group were significantly higher than that of the control group. The gp130 positive cells in peripheral blood mononuclear cell (PBMC) and osteoclast-like cells (OLC) which had been induced with receptor activator of nuclear factor κB ligand (RANKL) in RA group were also higher than that in the control group. In addition, after OLC in RA group is cultured with *anti*-gp130 Monoclonal antibody (McAb), the IL-6 and MMP-9 expression in osteoclast supernatant insignificantly decreased. Meanwhile, the expression results of Tartrate Resistant Acid Phosphatase (TRAP)-positive cells and osteoclasts were also decreased significantly. Our study suggests that regulating gp130 receptor can be used to control the differentiation and formation of osteoclasts, which provides a new clinical strategy for RA patients in the future.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune joint disease which is characterized by synovial hyperplasia, joint cartilage destruction, and extra-articular manifestations [1,2]. Many types of cells are involved in the destruction of bone and cartilage, among which osteoclasts are the most important ones [3]. Previous studies have shown that inflammatory factors, especially IL-6、IL-11 and IL-27, are potent fibroblast-like synoviocytes (FLS) activators that can induce FLS to produce other cytokines [4–6]. This in turn promotes autoimmunity, perpetuates inflammatory synovitis, and contributes to joint cartilage destruction [7].

The researches have also revealed that MMP-9 is an important key enzyme reflecting bone resorption and bone remodeling [8]. IL-6 induced monocyte fusion to form multinucleated osteoclast-like cells, and secreted MMP-9 to participate in the induction of bone erosion. When the complex of IL-6/IL6R and gp-130 is formed from binding of IL-6 and IL-6R and homodimerization of gp-130, Janus kinase (JAK) signaling pathway is activated, which subsequently induces tyrosine phosphorylation of STAT3 [9].

Recent reports have indicated that activation of STAT3 can further enhance the high expression of RANKL, thus induce the differentiation and maturation of osteoclast [10]. Moreover, the expression of BCL2, an apoptosis-related gene, can be regulated by IL-6/IL-6R and gp130 through the JAK-STAT signaling pathway that results in an excessive proliferation of synovial tissue and increasing RA occurrence and development [10,11]. In this study, we investigated the expression levels of gp130/inflammatory factors in serum and the supernatant of osteoclast cultured from in vitro in RA and healthy control subjects. We analyzed the correlation between gp130 and the development of RA patients.

In order to study the regulation of the gp130 receptor, which can be used to control osteoclast differentiation and formation, we used anti-human gp130 monoclonal antibodies (mAb) to treat the OLC from RA. The expression of gp130 on the surface of the cells, the number of TRAP positive cells and the phosphorylation of IL-6, MMP-9 were tested [12]. Regulating the gp130 receptor by anti gp130 McAb is used to inhibit the differentiation and formation of osteoclasts, which will provide a new clinical strategy for RA patients.

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2. Materials and methods

2.1. Participants

From January 2015 to June 2018, 47 R A patients were randomly selected as the experimental group from Guanghua Hospital in Shanghai. The experimental group consisted of 15 males and 32 females aged 57 ± 12 with a course of disease for 5 ± 3 years, and met the ACR/EULAR 2010 R A classification standard [13]. The control group, on the other hand, consisted of 40 healthy individuals, 20 males and 20 females, aged from 30 to 60 years old, and with neither history of chronic diseases nor long-term medication or infection within one week of sampling, and no abnormal function of each joint. All the above samples meet the requirement of hemoglobin ≥100 g/L, white blood cells ≥3.5 × 109/L, serum creatinine, serum bilirubin, alanine aminotransferase, aspartate aminotransferase no more than 1.5 times the normal value [14,15]. Consent was obtained for experimentation with all human subjects, and privacy rights of human subjects is always observed.

2.2. Methodology

2.2.1. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunoassay (ELISA) is used to detect the sera of the control group and RA. To extract serum from a blood sample, the following steps are followed: clot at room temperature for 30 min, centrifuge at 3000 RPM for 15 min, pour the serum into separate tubes and cryopreserved at −80 °C, and thaw 30 min before use in the experiment. ELISA performs according to the instructions given by the manufacturer. Take IL-6 as an example, add 100 μl of standard and test sample, wash the plate 4 times after incubating at 37 °C for 120 min, add 50 μl primary antibody. Then, incubate at 37 °C for 60 min, wash the plate 4 times, add 100 μl enzyme-labeled antibody. Subsequently, wash the plate 4 times after incubating at 37 °C for 60 min, add 100 μl substrate color reagent, keep away from light for 10 min. Next, add 50 μl stop solution, measure the absorbance at 450 nm, and the serum IL- 6 content. In the end, the levels of IL-6, IL-6Rα, gp130, IL-11, IL-27, and MMP-9 in RA patients in the control group and RA patients were detected.

2.2.2. Osteoclast-like cells (OLC) induction and culture process

Take 10 ml of whole blood of subjects and dilute 2 to 3 times with RPMI-1640 medium; slowly add the upper layer of Ficoll lymphocyte separation solution; centrifuge at a speed of 2000 RPM for 20 min; collect mononuclear cells layer. Wash the layer twice with RPMI-1640 and centrifuged at a speed of 1500 RPM for 10 min to resuspend the cells [16]. Cultivate the PBMC cells of the control group and RA patients in RPMI-1640 medium supplemented with 20 ng/ml RANKL and M-CSF for 3 days, and then cultivate together with 40 ng/ml RANKL for 7 days. Replenish M-CSF and RANKL for 3 days until a large number of fibrous osteoclast-like cells are visible. Fix the cells with 4% paraformaldehyde, and stain TRAP [17] and label FITC-*anti*-gp130 antibody respectively.

2.2.3. Flow cytometry (FACS)

Flow cytometry (FACS) is used to detect gp130 positive cells. Dilute subjects' PBMC cells and OLC cells induced by M-CSF/RANKL into millions of units. Dispense 100 μl of these solutions into flow tubes, and add 2 μl of FITC-*anti*-gp130 antibody respectively. Then, incubate them at 4 °C for 1 h and wash them twice with PBS. A flow cytometry was used to select lymphocyte population from the scattered light parameter map and gp130 positive cells were sorted and detected.

2.2.4. Preparation of anti-gp130 McAb and procedure of regulating gp130

The *anti*-gp130 McAb is produced according to following steps. Inject intraperitoneally Bagg albino (BALB/c) mice with gp130 recombinant protein mixed with Freund's complete adjuvant, and repeat twice in the second and fourth week to boost immunization. After 3 days in week 5, take the spleen, grind it well, mix the spleen cells and myeloma cells with Hypoxanthin-Aminopterin-Thymidine (HAT) selective medium. Effective cell strains are screened for amplification and purification, which are known as gp130 monoclonal antibodies [18].

The regulation of gp130 steps are followed: While adding 50 μg/ml gp130 monoclonal antibody into the monoclonal antibody intervention group every 3 days during culturing OLC with conditions remaining unchanged, stain TRAP and measure the osteoclast TRAPs. In the meantime IL-6 and MMP-9 expression levels in PBMC and OLC cells is detected using ELISA.

2.3. Statistical analysis

The data is expressed in $\bar{x} \pm S$ and treated with Microsoft Excel. Statistics is further analyzed with Prism, and the differences of the two groups are checked by Student's T test and One-Way ANOVA to analyze the homogeneity in variance. $P < 0.05$ is considered statistically significant.

3. Results

3.1. IL-6, IL-6Rα/gp130, IL-11, IL-27 and MMP-9 expression results in serum

Detected by ELISA, the concentration of IL-6, IL-6Rα and gp130 in serum samples of 40 controls are (18.03 ± 4.77) pg/ml, (48.12 ± 13.67) ng/ml and (249.66 ± 64.99) ng/ml respectively, while that of the 47 R A patients are (43.51 ± 20.53) pg/ml, (53.26 ± 18.36) ng/ml and (226.65 ± 78.54) ng/ml. The results of the two groups are presented in Fig. 1. It was found that IL-6 serum levels are significantly different ($p < 0.01$). The IL-6Rα and gp130 serum levels are not statistically different ($p > 0.05$). Further analyzing the results of the IL-6Rα/gp130 ratio in serum from RA group and control group, it was found that the result of IL- 6Rα/gp130 in RA patients is abnormally higher (0.240 ± 0.050) than the control group (0.195 ± 0.037). The difference is statistically significant ($p < 0.05$). This suggests that in addition to abnormally elevated IL-6, RA patients may also have an imbalance of IL-6Rα/gp130.

The concentration level of IL-11, IL-27, and MMP-9 in serum samples of 47 R A group and control group were tested using ELISA. The IL-11, IL-27, and MMP-9 content in serum for the control group are (18.73 ± 8.35)pg/ml, (82.60 ± 40.16)pg/ml and (102.49 ± 66.81)ng/ml respectively, while that of the 47 R A patients are (24.69 ± 12.22) pg/ml, (123.86 ± 44.23) pg/ml and (194.02 ± 94.10) ng/ml. Compared with control group, the IL-11 content of RA group is statistically different ($P < 0.05$), and the IL-27 and MMP-9 levels are significantly statistically different ($P < 0.01$).

3.2. gp130 positive cells result in PBMC and OLC

Using FACS detection labeled with FITC, about (11.74 ± 1.97) % of cells in PBMC (PBMC -NP) is gp130 positive. On the other hand, the percentage of gp130 positive cells in the control group is about (1.10 ± 0.35) % (Fig. 2A). This difference is statistically significant ($p < 0.05$) as shown in Fig. 2B. After inducing with RANKL and MCSF for 10 days, the result of gp130-positive cells in the OLC of the control group was (15.12 ± 3.88) % while that of RA group was (44.07 ± 5.43) % (Fig. 2A). This difference is also statistically significant ($p < 0.05$) as shown in Fig. 2B. This result suggests that the gp130 content of lymphocytes in RA patients may increase, and the surface of osteoclasts may be abundant in gp130 glycoprotein.

3.3. The expression results of TRAP-positive cells in OLC

When RANKL and M-CSF were added to OLC and co-cultured for 3 days, the cell gap was blurred and merged with each other, multinucleated giant cells appeared, and pseudopods appeared on the irregular

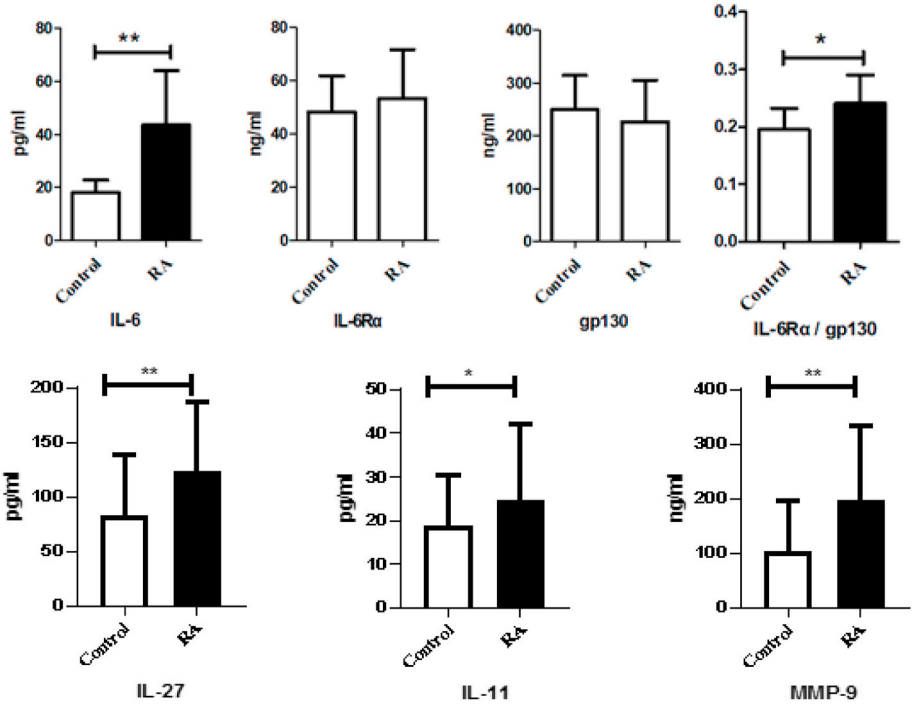


Fig. 1. IL-6 , IL-6Rα , gp130 content, the ratio of IL-6Rα/gp130, IL-11 , IL-27 and MMP-9 in the of serum RA patients.
Note: *. Indicates the group compared with the control group, the difference was significant ($P < 0.05$), **. Indicates the group compared with the control group the difference was significant extremely ($P < 0.01$).

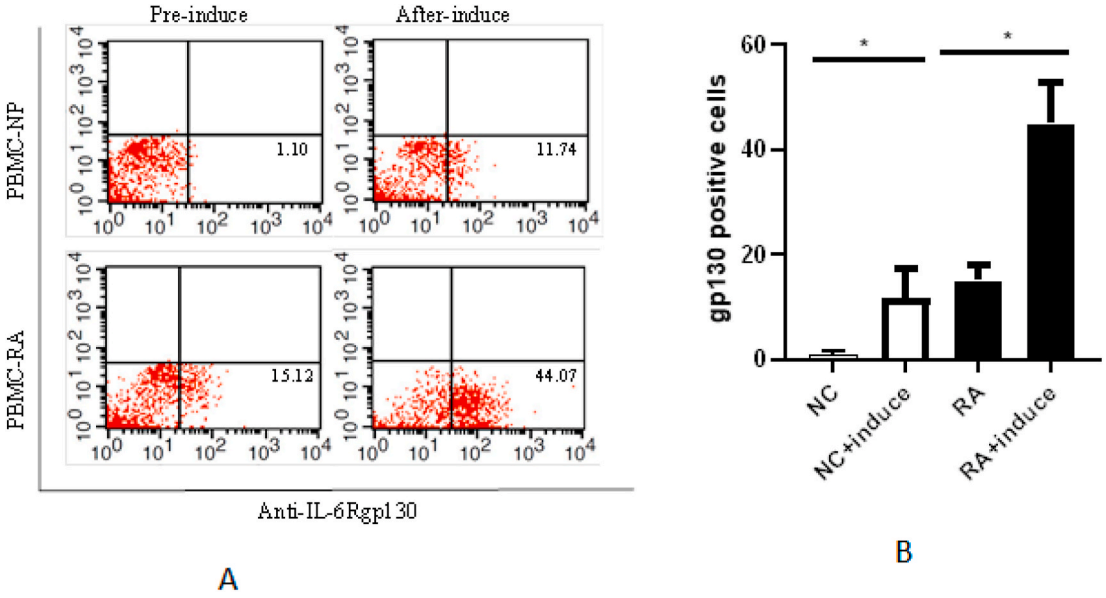


Fig. 2. gp130 expression in the cells from the control group and RA patients after induction with RANKL and M-CSF; Note: the percentage of gp130-positive cells was detected.

edges. After cultivated for 7 days with TRAP staining, the cytoplasm of osteoclast is wine-red observed under a microscope, and the number of nuclei is greater than 3, which can be defined as TRAP + cells [19]. The results of quantitative analysis of cells count show that the number of osteoclasts in RA patients increased significantly ($P < 0.05$) compared with the control group as shown in Fig. 3E.

In order to regulate gp130, cultured anti-human gp130 McAb was added to both the control and the RA group. After 7 days of differentiation and induction, the number of osteoclasts in the RA patient group decreased significantly ($P < 0.05$, Fig. 3E).

3.4. Results of IL-6 and MMP-9 expression in supernatant of OLC

Detected by ELISA, IL-6 and MMP-9 were determined in PBMC, OLC, and treated OLC with anti-gp130 McAb of control group and RA group, and the results are shown in Fig. 4C. The content level of IL-6 and MMP-9 in the osteoclast supernatent of the control group are significantly lower than those of the RA group, and the differences are statistically significant ($P < 0.05$Fig. 4B). The IL-6 and MMP-9 values of osteoclast supernatant of the RA patient group intervened by *anti*-gp130 McAb are significantly lowered, and the differences are statistically significant (P

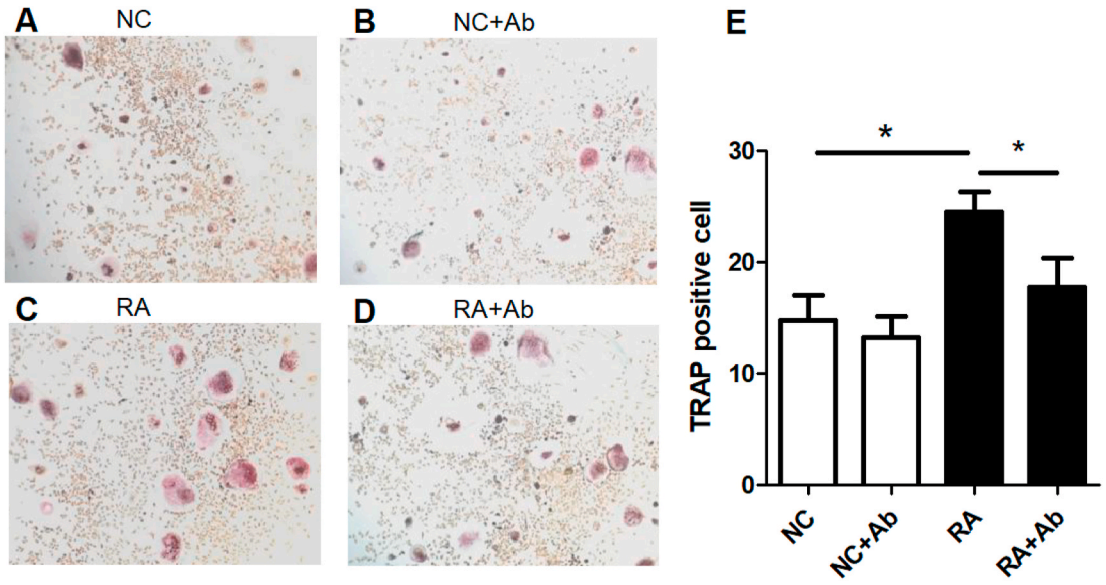
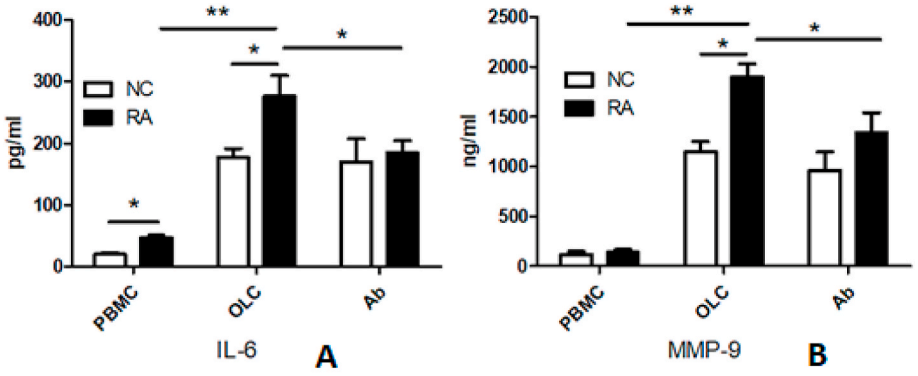


Fig. 3. A: gp130 McAb interferes with the induction of osteoclasts; B: The expression of TRAP-positive cells in normal and RA patients 7 days after induction by RANKL and gp130 McAb.



	IL-6 (pg/ ml)	MMP-9 (ng/ ml)
RA PBMC	47.23±3.71*	142.29±24.50
NC PBMC	20.24±2.28	117.15±30.96
RA OLC	276.07±33.52**	1894.32±133.56**
NC OLC	177.10±14.15	1148.03±102.25
RA gp130 McAb	184.60±19.42*	1340.26±198.59*
NC gp130 McAb	170.02±37.01	962.17±183.71

C

Fig. 4. Effect of anti-human gp130 MaAb on IL-6 and MMP-9 secretion by RA cells. A: Effect of anti-human gp130 MaAb on IL-6. B: Effect of anti-human gp130 MaAb on MMP-9 secretion by RA cells.

< 0.05 Fig. 4B).

4. Discussion

Both IL-6 and IL-11 are the IL-6 family of interleukins, and their main classification is based on the fact that the receptors for these cytokines share gp130 glycoproteins containing signaling receptor subunits. The binding of IL-6 or its family members such as IL-11 to the cell membrane or soluble IL-6R/IL-11R initiates the binding of IL-6R to gp130, which then undergoes homodimerization and signal transduction [20,21]. IL-27 belongs to the IL-12 family of interleukins; it is composed of EB13 and p28 protein, and its transmission pathway also needs to form a heterodimer through IL-27R and gp130 [22]. Studies have shown that the inflammatory response is often driven by cytokines, and the imbalance between proinflammatory and anti-inflammatory cytokines in the body is often the culprit of inducing autoimmune diseases, chronic inflammatory diseases, and related body tissue destruction [23]. According to the experimental results, the high expression of IL-6 and IL-11 in the serum of RA patients suggests that they may regulate immunity response in the systemic circulation to actively participate in the

pathogenesis of RA by transmitting signals through their receptors. IL-6 can induce the proliferation and differentiation of B lymphocytes and T lymphocytes. At synovial lesions, it also induces VEG secretion to promote synovial angiogenesis and RANKL to stimulate osteoclast differentiation and damage joints [24]. It is even related to the BMPs familial level regulation and acts as an ectopic osteogenesis [25]. IL-11 induces the synthesis of CRP with regulating immunity response and bone metabolism. The high expression of IL-27 in the systemic circulation of RA patients reveals the immune imbalance of Th1/Th2 in RA patients. IL-27 circulating regulatory immunity promotes Th1 differentiation and displays its pathogenic effect. It can up-regulate the expression of ICAM-1 on the surface of FLS, strengthen various chemokines such as MMPs etc., excessive activation of JAK/STAT pathway may also increase RA Synovial inflammation and bone destruction in patients [22].

MMP-9 is a representative of matrix metalloproteinase and it participates in extracellular matrix metabolism. MMP-9, an important key enzyme that reflects bone resorption and bone reconstruction, can be highly expressed by osteoclast and plays an important role in the process of osteoclast migration, erosion and anchoring [26]. Experiments show that the high expression of MMP-9 in the systemic circulation is indicative of the severity of bone erosion caused by osteoclast differentiation in RA patients. One of the reasons why the serum levels of IL-6, IL-11, and IL-27 in RA patients were abnormally elevated could be that their induction of osteoclast differentiation a large amount of metal matrix proteases were secreting into the systemic circulation, and hence cause bone erosion. The high expression of IL-6 secretion in the supernatant of mononuclear cells of RA patients also reveals that it may regulate the lymphocyte autocrine IL-6 to induce its monocyte fusion to form multinuclear osteoclast-like cells and secrete MMP-9 to participate in bone erosion.

In PBMC and inducing with M-CSF and RANKL in vitro by in the same conditions, RA patients can generate more osteoclasts than normal people, and there are high expressions of IL-6 and MMP-9. In RA patients, the high expression of gp130 on the surface of osteoclasts suggests that it may have recruited a large amount of IL-6/sIL-6Rα, thereby activating STAT3, and further inducing the expression of RANKL, which is the key to the differentiation and maturation of osteoclast precursors. McAb's intervention also suggested that the presence of gp130 on the surface of osteoclasts is an important target for its bone erosion. Blocking gp130 can significantly reduce the level of IL-6 and MMP-9 secreted by the cells. Its regulation of gp130 can indeed affect osteoclast differentiation in RA patients.

PBMC and induced PBMC by M-CSF and RANKL in vitro in RA patients can generate more osteoclasts than in healthy people, and there are high expressions of IL-6 and MMP-9. In RA patients the high expression of gp130 on the surface of osteoclasts suggests that a large amount of IL-6/sIL-6Rα are recruited and further inducing the expression of RANKL, which is the key to the differentiation and maturation of osteoclast precursors. McAb's intervention also suggested that the presence of gp130 on the surface of osteoclasts is an important target for its bone erosion. Inhibiting gp130 can significantly inhibit the phosphorylation of downstream STAT3 and reduce the level of IL-6 and MMP-9 secreted by the cells. The regulation of gp130 can indeed affect osteoclast differentiation in RA patients.

5. Conclusion

In summary, the results of this experiment demonstrate IL-6, IL-11, or IL-27 in RA patients all participate in the pathogenesis of RA through multiple mechanisms of action, and their specific receptors are required. The formation of homologous or heterodimers with gp130 can activate signal transduction. Granulocytes lacking gp130 do not respond to IL-6 [27]. So gp130 is an important target of key signaling pathways in the pathogenesis of RA. Increasing of gp130 on the cell surface of RA patients may excite its corresponding homo/heterodimeric receptors, recruiting more ligands including IL-6 to induce inflammation and aggravate the condition. The imbalance of gp130 and its ligands in the circulatory system and local lesions of RA patients is the key to the pathogenesis of RA. Therefore, monitoring the dynamic expression of gp130-related receptors and ligands may provide a potentially better laboratory indicator for the diagnosis of RA.

Furthermore, inhibitor antibodies against IL-6, IL-6R and related kinases (JAK) have been used clinically [28], and gp130 has been shown to be highly expressed on the surface of osteoclasts. When it is inhibited, it can reduce osteoclast differentiation, indicating that gp130 will also be a therapeutic target for RA with developmental potential. We applied *anti*-gp130 McAb to inhibit the proliferation and differentiation of OLC in injured joints, and to regulate the signaling pathways of excessive activation of upstream ligands. Regulation of gp130 will be a new method for the RA disease.

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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