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Altered peripheral B lymphocyte homeostasis and functions mediated by IL-27 via activating the mammalian target of rapamycin signaling pathway in patients with rheumatoid arthritis

Yawei Tang[1,2] | Ziran Bai[1] | Jingjing Qi[1] | Zhimin Lu[1] | Ahmad[1] | Guan Wang[1] | Minli Jin[1] | Bing Wang[1] | Haifeng Chen[1,3] | Xia Li[1]

[1]Department of Immunology, College of Basic Medical Science, Dalian Medical University, Dalian, China

[2]Department of Clinical Laboratory, Second Hospital of Dalian Medical University, Dalian, China

[3]Department of Rheumatology, Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi, China

Correspondence
Xia Li, Department of Immunology, College of Basic Medical Science, Dalian Medical University, Dalian, 116044, China.
Email: lixia416@163.com

Haifeng Chen, Department of Rheumatology, Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi, 214023, China.
Email: haifengchen357@163.com

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Abstract

B cell dysfunction and inflammatory cytokine over-production participate in the pathogenesis of rheumatoid arthritis (RA). Here we compared peripheral B cell homeostasis and immune functions between RA patients and healthy controls (HC) and explored vital signaling pathways involved in altered RA B cells. We found that RA patients showed significantly decreased frequencies of peripheral $CD19^+CD27^+CD24^{high}$ regulatory B (Breg) cells but increased frequencies of $CD19^+CD27^+CD38^{high}$ plasmablasts and $CD19^+CD138^+$ plasma cells, and higher levels of serum immunoglobulin (Ig)M and IgG. Compared to HC peripheral B cells, RA peripheral B cells had more increased proliferation and higher expression of activation markers. Importantly, our results showed that RA peripheral B cells displayed the mTOR signaling pathway to be more activated, and inhibition of mTOR could restore RA B cell homeostasis and functions. RA serum-treated B cells exhibited more increased expressions of mTOR, which could be restored with the addition of anti-interleukin (IL)-27 neutralizing antibody. Serum IL-27 levels were significantly increased in RA patients and positively correlated with disease activity, the frequencies of plasma cells and the levels of autoantibodies. In vitro, IL-27 notably promoted immune dysfunction of RA B cells, which were inhibited by anti-IL-27 neutralizing antibody. Also, the mTOR pathway was more activated in IL-27-treated RA B cells, and mTOR inhibition apparently reversed abnormalities of RA B cells mediated by IL-27. These results suggest that increased serum IL-27 levels could promote peripheral B cell dysfunction in RA patients via activating the mTOR signaling pathway. Thus, IL-27 may play a pro-pathogenic role in the development of RA, and antagonizing IL-27 could be a novel therapy strategy for RA.

KEYWORDS
B cells, IL-27, mTOR, rheumatoid arthritis

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IL-27 PROMOTES B CELL DYSFUNCTION VIA ACTIVATING THE MTOR PATHWAY IN RA

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease, characterized by over-expression of inflammatory cytokines and autoantibodies, as well as over-activation of several immune cells, including T cells, B cells, macrophages, mast cells, dendritic cells and natural killer cells [1,2]. The imbalance of immune cells and inflammatory cytokines in RA results in synovial inflammation and joint destruction, which play an important role in the pathogenesis of RA.

There are several studies on the immune dysregulation of B cells in RA, which are responsible for the production of autoantibodies and function as efficient antigen-presenting cells, as well as cytokine and chemokine-producing cells [3]. RA patients showed decreased frequencies of circulating CD27^{+} memory B cells, which could be restored by effective therapies [4]. Patients with B cell-rich synovitis exhibited higher levels of disease activity and autoantibody seropositivity in early RA [5]. B cell-expressed inducible co-stimulator ligand (ICOSL) and interferon (IFN)-γ were essential for autoimmune arthritis via inducing germinal center (GC) B cells, but not plasma cells [6,7]. GC formation and antibody production were key pathogenic functions of B cells in arthritic mice [8]. B cell depletion enhanced regulatory T (Treg) cell activity and suppressed arthritis [9,10]. Additionally, regulatory IL-10-producing B (B10) cells were correlated inversely with disease activity, biological inflammation and autoantibody levels in mice and RA patients [11]. These studies demonstrate the crucial role of B cells in the pathogenesis of RA.

The immune function of B cells depends upon their surrounding micro-environment. For instance, hypoxia exposure to GC and inflammatory cytokines [B cell activating factor (BAFF) and IL-4] can up-regulate frequencies of GC B cells and plasma cells, as well as the glucose metabolism of B cells [12–14]. IL-27, a cytokine of the IL-12 family, can bind to a cell surface receptor (gp130 and IL-27Rα) to activate the Janus kinase/signal transducer and activator of transcription-1 (JAK/STAT) signaling pathway [15,16]. Recently, several studies report the effects of IL-27 in RA. IL-27 was highly expressed in both serum and synovial tissue of RA patients [17]. Increased levels of IL-27 in RA synovial tissue were associated with B cell-dominated synovial inflammation. IL-27 was also detected in nodules of RA patients. However, the effect of nodule-derived IL-27 on the systemic or synovial inflammation in RA remains unknown [18]. In recent researches, some studies showed that IL-27 could ameliorate arthritis mainly through suppressing IL-17-mediated inflammation, exhibiting a protective role in RA [19]. Conversely, others found that increased IL-27 expression induced the production of inflammatory cytokines and chemokines, and was linked with the disease activity of RA patients [20]. In an arthritis mouse model, IL-27R deficiency reduced the severity of the disease by inhibiting IFN-γ-producing T cells [21]. These results suggest that IL-27 acts as a detrimental factor in the pathogenesis of RA. However, the exact role of IL-27 is still a matter of discussion in RA, and the relationship between immune dysfunction of B cells and IL-27 in RA currently remains unclear.

In this study, we compared immune function and homeostasis of peripheral B cells in RA patients and healthy control and explored vital signaling pathways involved in altered B cells. Moreover, the potential role of IL-27 signal in immune dysregulation of RA B cells was further studied. Our results will be of significant importance for understanding the role of B cell dysfunctions mediated by the IL-27 signal in the pathogenesis of RA.

MATERIALS AND METHODS

Patients and healthy controls

Serum was collected from RA patients ($n = 149$) who fulfilled the American College of Rheumatology criteria for RA [22] from the Department of Rheumatology and Immunology of the Second Hospital of Dalian Medical University. The patients had no other autoimmune or systemic diseases. Age- and sex-matched healthy controls (HC, $n = 64$) were obtained from the medical examination center in this hospital. All participants signed informed consent statements to approve the use of their blood. The study protocol was approved by the ethics committee of the Second Hospital of Dalian Medical University. Detailed clinical characteristics and laboratory features are shown in Supporting information, Table S1.

Sorting and stimulation of T/B cells

Peripheral blood mononuclear cells (PBMCs) were isolated from RA patients and HC with Ficoll density-gradient centrifugation, and CD19^{+} B cells or CD4^{+} T cells were purified from PBMCs (> 95% purity), according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). To study the effects of human serum on B cells, CD19^{+} B cells (2.5×10^5/well) from HC were cultured in RPMI-1640 medium supplemented with 2% fetal bovine serum (FBS), 2% HC serum, or 2% RA serum in the presence of 0.5 μg/ml anti-CD40 antibody (R&D Systems, Minneapolis, Minnesota, USA) and 0.1 μM cytosine–phosphate–guanosine (CpG) (Miltenyi Biotech)

for 24 h. To explore the roles of IL-27 in RA B cells, peripheral CD19$^+$ B cells (2.5×10^5/well) were added to 24-well plates with or without 50 ng/ml human recombinant IL-27 (Peprotech, Cranbury, New Jersey, USA) in the presence of 0.5 μg/ml anti-CD40 antibody and 0.1 μM CpG for 3 or 5 days. To study the effect of IL-27 on RA T cells, CD4$^+$ T cells were treated with 50 ng/ml human recombinant IL-27 in the presence of 2 μg/ml soluble anti-CD3/CD28 antibody for 3 days. In some experiments, 50 μg/ml anti-IL-27 neutralizing antibody (R&D Systems) or 1 nM rapamycin (Solarbio, Beijing, China) was added to the cell cultures.

Flow cytometric analysis

Briefly, the above cells were washed and suspended in 100 μl of phosphate-buffered saline (PBS) solution and surface antigens were stained for 30 min at 4°C. For intracellular nuclear protein staining, cells were fixed and permeabilized with an intracellular fixation and permeabilization buffer set (eBioscience, San Diego, California, USA) for 1 h, and washed three times with ×1 permeabilization buffer, followed by staining with intracellular nuclear antibodies for another 30 min at 4°C. For intracellular staining, the cells were treated with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (1000 ng/ml) for 5 h in the presence of brefeldin A (10 μg/ml). The cells were then stained with surface antigens antibodies for 30 min, fixed and permeabilized, followed by intracellular staining. Frequencies of staining-positive cells were detected using flow cytometry (Accuri C6; BD Biosciences, San Diego, California, USA). Fluorochrome conjugated anti-human antibodies were used as follows: allophycocyanin (APC)-CD19, peridinin chlorophyll/cyanin (PerCp/Cy) 5.5-CD27, fluorescein isothiocyanate (FITC)-CD38, phycoerythrin (PE)-gp130, PE-CD138, PE-CD86 and CD80-FITC (Biolegend, San Diego, California, USA). PE-IgD, PE-IL-17A, APC-IFN-γ, APC-C-X-C chemokine receptor type 5 (CXCR5), PE-programmed cell death 1 (PD-1), PE-forkhead box protein 3 (Foxp3), PE-phospho-mTOR (eBioscience), AlexaFluor 488-WSX-1 (R&D), phospho-P70S6K and phospho-4E-BP1 (Cell Signaling Technology, Danvers, Massachusetts, USA). The second antibody anti-rabbit IgG (H+L) was conjugated with APC and isotype-matched mouse IgG controls (eBioscience).

Proliferation and apoptosis assays

CD19$^+$ B cells from RA patients and HC were labeled with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience) in PBS for 10 min at room temperature. An excess of ice-cold RPMI-1640 medium with 10% FBS was added to the cells to quench the reaction and it was washed extensively. CFSE-labeled B cells (1×10^5/well) were cultured using the above methods. Following 5 days of culture, cells were collected and stained with anti-APC-CD19 antibody. B cell proliferation was determined with flow cytometry analysis of CFSE fluorescence intensity. To detect apoptotic cells, PBMCs or CD19$^+$ B cells were collected and resuspended in ×1 binding buffer, and stained with FITC-annexin V (BD Biosciences) and anti-APC-CD19 antibody in the dark. After 15 min, cells were washed and data were analyzed with flow cytometry analysis.

Array protein biochip for cytokines in serum

Serum molecules from new-onset RA patients ($n = 3$) and age- and sex-matched HC ($n = 5$) were analyzed with Quantibody Human Cytokine Antibody Array 440 (GAH-CAA-Q440; RayBiotech, Peachtree Corners, Georgia, USA), according to the manufacturer's specifications. All RA patients fulfilled the American College of Rheumatology criteria for RA and had no other autoimmune or systemic diseases. An Axonscanner 4000B with GenePix software was used to collect fluorescence intensities. Detailed clinical characteristics and laboratory features of RA patients are shown in Supporting information, Table S2.

Enzyme-linked immunosorbent assay (ELISA)

Serum IL-27 levels in 149 RA patients and 64 HC were measured with human IL-27 ELISA kits (R&D), according to the manufacturer's instructions. The plate was read at 450 nm and the sensitivity of ELISA kits used in the experiment was 156 pg/ml. The levels of immunoglobulin (IgM and IgG) and inflammatory cytokines (IL-6 and IL-10) in cell culture supernatants or serum were quantitated with ELISA kits, according to the manufacturer's instructions.

Statistical analysis

Data were summarized as means ± standard error of the mean (SEM). Statistical significance was performed with Student's t-test and the correlations between serum IL-27 levels and clinical features in RA patients were analyzed with Spearman's rank test. Serum IL-27 concentrations

between RA patients and HC were analyzed with the Mann–Whitney rank-sum test. All statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, California, USA). A p-value < 0.05 was considered significantly different.

RESULTS

RA patients showed altered peripheral B cell homeostasis and functions

The imbalance of effector/regulatory B cells and B cell dysfunction contributes to the over-production of autoantibodies of RA. In this study we found that, compared to HC, RA patients showed significantly decreased frequencies of peripheral $CD19^+CD27^+CD24^{high}$ Breg cells ($8.04 \pm 0.35\%$ versus $11.64 \pm 0.53\%$, $p < 0.001$, Figure 1b), whereas these RA patients exhibited increased frequencies of $CD19^+CD27^+CD38^{high}$ plasmablasts ($9.16 \pm 0.50\%$ versus $6.81 \pm 0.34\%$, $p < 0.001$, Figure 1c) and $CD19^+CD138^+$ plasma cells ($8.93 \pm 0.39\%$ versus $5.95 \pm 0.40\%$, $p < 0.001$, Figure 1d) and higher levels of serum IgM (11.08 ± 0.55 µg/ml versus 7.11 ± 0.36 µg/ml, $p < 0.001$) and IgG (53.84 ± 1.47 µg/ml versus 41.41 ± 1.47 µg/ml, $p < 0.001$, Figure 1e). In addition, there was more increased proliferation ($23.92 \pm 0.81\%$ versus $12.90 \pm 1.29\%$, $p < 0.01$, Figure 1f) and higher expression of activation markers (CD80: $12.55 \pm 0.84\%$ versus $4.61 \pm 0.39\%$, $p < 0.01$; CD86: $10.65 \pm 0.39\%$ versus $5.67 \pm 0.50\%$, $p < 0.01$, Figure 1h) in RA $CD19^+$ B cells than that in HC B cells, although there was no significant difference in B cell apoptosis ($16.29 \pm 0.59\%$ versus $15.64 \pm 0.41\%$, $p = 0.167$, Figure 1g) between RA patients and HC. These data suggest that altered peripheral B cell homeostasis and functions may responsible for the pathogenesis of RA.

The mTOR signaling pathway was involved in altered RA peripheral B cell homeostasis and functions

It was demonstrated that the mTOR signaling pathway could regulate B cell-mediated immune functions and germinal center B cell growth [23]. To clarify the roles of mTOR pathway in the homeostasis and immune functions of RA B cells, we first compared the mean fluorescence intensity (MFI) of the phosphorylated (p) mTOR and its subunit in RA and HC B cells, and found that RA B cells showed higher expression of p-mTOR (MFI = $27.99 \pm 0.88 \times 10^2$ versus $21.59 \pm 0.66 \times 10^2$, $p < 0.01$), mTORC1 subunits p-P70S6K (MFI = $19.49 \pm 0.41 \times 10^3$ versus $15.95 \pm 0.35 \times 10^3$, $p < 0.01$) and p-4E-BP1 (MFI = $17.60 \pm 0.23 \times 10^3$ versus

$15.46 \pm 0.17 \times 10^3$, $p < 0.01$) in comparison to HC B cells (Figure 2A).

In order to further evaluate the effects of mTOR pathway on RA peripheral B cells, we treated RA B cells with mTOR-specific inhibitor rapamycin. The results showed that rapamycin-treated RA B cells exhibited a significantly up-regulated percentage of Breg cells compared to untreated RA B cells ($12.00 \pm 0.40\%$ versus $7.43 \pm 0.36\%$, $p < 0.01$, Figure 2B). Conversely, the percentages of plasmablasts ($8.70 \pm 0.81\%$ versus $15.38 \pm 1.04\%$, $p < 0.01$, Figure 2C) and plasma cells ($3.69 \pm 0.36\%$ versus $6.88 \pm 0.15\%$, $p < 0.01$, Figure 2D) and the levels of supernatant IgM (18.35 ± 1.36 µg/ml versus 32.05 ± 5.98 µg/ml, $p < 0.01$) and IgG (18.02 ± 0.99 µg/ml versus 21.46 ± 1.24 µg/ml, $p < 0.05$, Figure 2E) were more down-regulated in the culture system of rapamycin-treated RA B cells than that of untreated B cells. In addition, compared to untreated RA B cells, rapamycin-treated B cells showed more decreased levels of inflammatory cytokines, including IL-6 (216.1 ± 51.91 pg/ml versus 406.1 ± 62.59 pg/ml, $p < 0.01$) and IL-10 (204.7 ± 71.07 pg/ml versus 425.8 ± 79.53 pg/ml, $p < 0.01$, Figure 2F). Rapamycin-treated B cells also displayed significantly decreased proliferation ($14.47 \pm 1.05\%$ versus $20.78 \pm 0.77\%$, $p < 0.001$, Figure 2G) and lower expression of CD80 ($7.43 \pm 0.45\%$ versus $14.76 \pm 0.39\%$, $p < 0.01$) and CD86 ($6.28 \pm 0.47\%$ versus $16.37 \pm 1.03\%$, $p < 0.001$, Figure 2H) in comparison to untreated B cells. These results demonstrate that the mTOR signaling pathway may be involved in altered RA peripheral B cell homeostasis and functions.

IL-27 signal might be a key factor in RA serum-activated mTOR pathway of B cells

Given that the activities of immune cells are associated with internal milieu, we wondered whether RA inflammatory microenvironment could result in B cell abnormality. HC B cells were cultured with human serum from RA patients or HC and higher expressions of p-mTOR (MFI = $23.09 \pm 0.76 \times 10^2$ versus $19.74 \pm 0.69 \times 10^2$, $p < 0.01$), p-P70S6K (MFI = $21.79 \pm 0.51 \times 10^3$ versus $19.32 \pm 0.41 \times 10^3$, $p < 0.05$) and p-4E-BP1 (MFI = $20.82 \pm 0.20 \times 10^3$ versus $18.27 \pm 0.40 \times 10^3$, $p < 0.001$, Figure 3A) were found in RA serum-treated B cells than in HC serum-treated B cells, suggesting that RA serum may activate the mTOR pathway in B cells, which may further mediate B cell abnormality.

In order to illustrate whether there are key molecules in RA serum, which play important roles in activating mTOR pathway and mediating B cell abnormality, we detected the levels of 440 inflammatory molecules in the serum of RA patients ($n = 3$) and age- and gender-matched
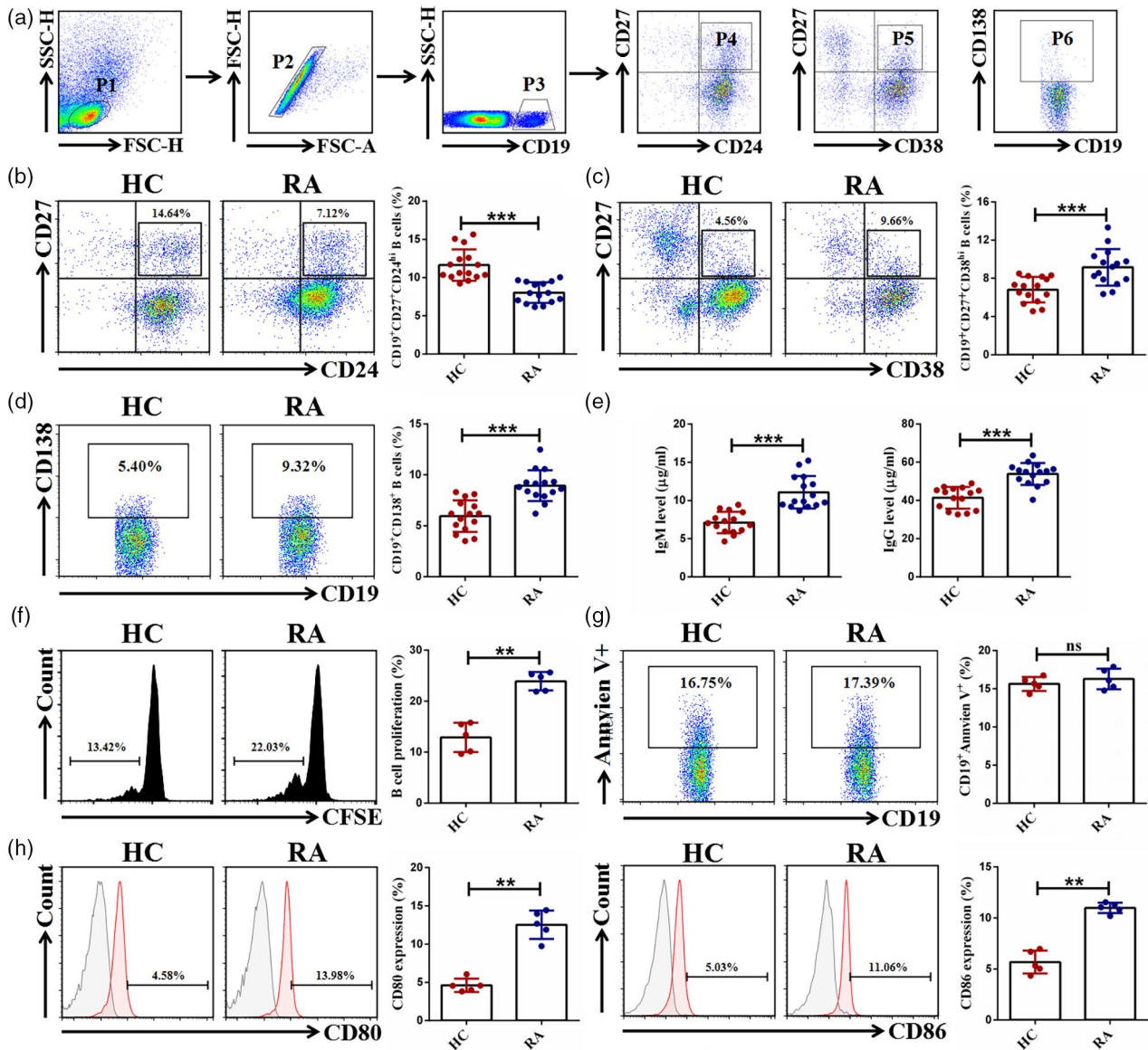
FIGURE 1 Rheumatoid arthritis (RA) patients showed altered peripheral B cell homeostasis and functions. (a) Representative flow cytometry charts depicting the gating strategy for $CD19^+CD27^+CD24^{high}$ regulatory B cells, $CD19^+CD27^+CD38^{high}$ plasmablasts and $CD19^+CD138^+$ plasma cells. (b,c) Peripheral blood mononuclear cells (PBMCs) were isolated from RA patients and healthy controls (HC), and the frequencies of $CD19^+CD27^+CD24^{high}$ regulatory B cells (b), $CD19^+CD27^+CD38^{high}$ plasmablasts (c) and $CD19^+CD138^+$ plasma cells (d) were determined with flow cytometry assay ($n = 15$). (e) The serum levels of immunoglobulin (Ig)M and IgG in RA patients and HC were measured with enzyme-linked immunosorbent assay (ELISA) assay ($n = 15$). (f) $CD19^+$ B cells were sorted with magnetic beads from PBMCs of RA patients and HC and carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled B cells were analyzed with flow cytometry assay at 5 days ($n = 5$). (g,h) PBMCs were isolated from RA patients and HC and the frequency of $CD19^+$ Annvien V^+ B cells (g) and the expressions of CD80 and CD86 (h) on $CD19^+$ B cells were detected with flow cytometry assay ($n = 5$). $**p < 0.01$; $***p < 0.001$; NS = no significance

HC ($n = 5$) with protein array analysis. The differences of serum 440 inflammatory molecules in RA patients relative to HC were depicted with the volcano plot, where the x-axis represents the adjusted p-value and the y-axis represents a fold change of 440 inflammatory molecules in RA patients relative to HC. In addition, the top highly elevated serum molecules in RA were further revealed by the heat-map and planar arrays (Supporting information,

Figure S1). These results indicated that the levels of serum IL-27 were most increased in RA patients compared to those in HC [30162 ± 7768 versus 677.80 ± 1212 optical density (OD) value, $p < 0.05$]. To further verify the differences of serum IL-27 levels between RA patients and HC, we tested serum IL-27 levels in 149 RA patients and 64 HC with ELISA analysis. As expected, serum IL-27 levels were significantly increased in RA than in HC
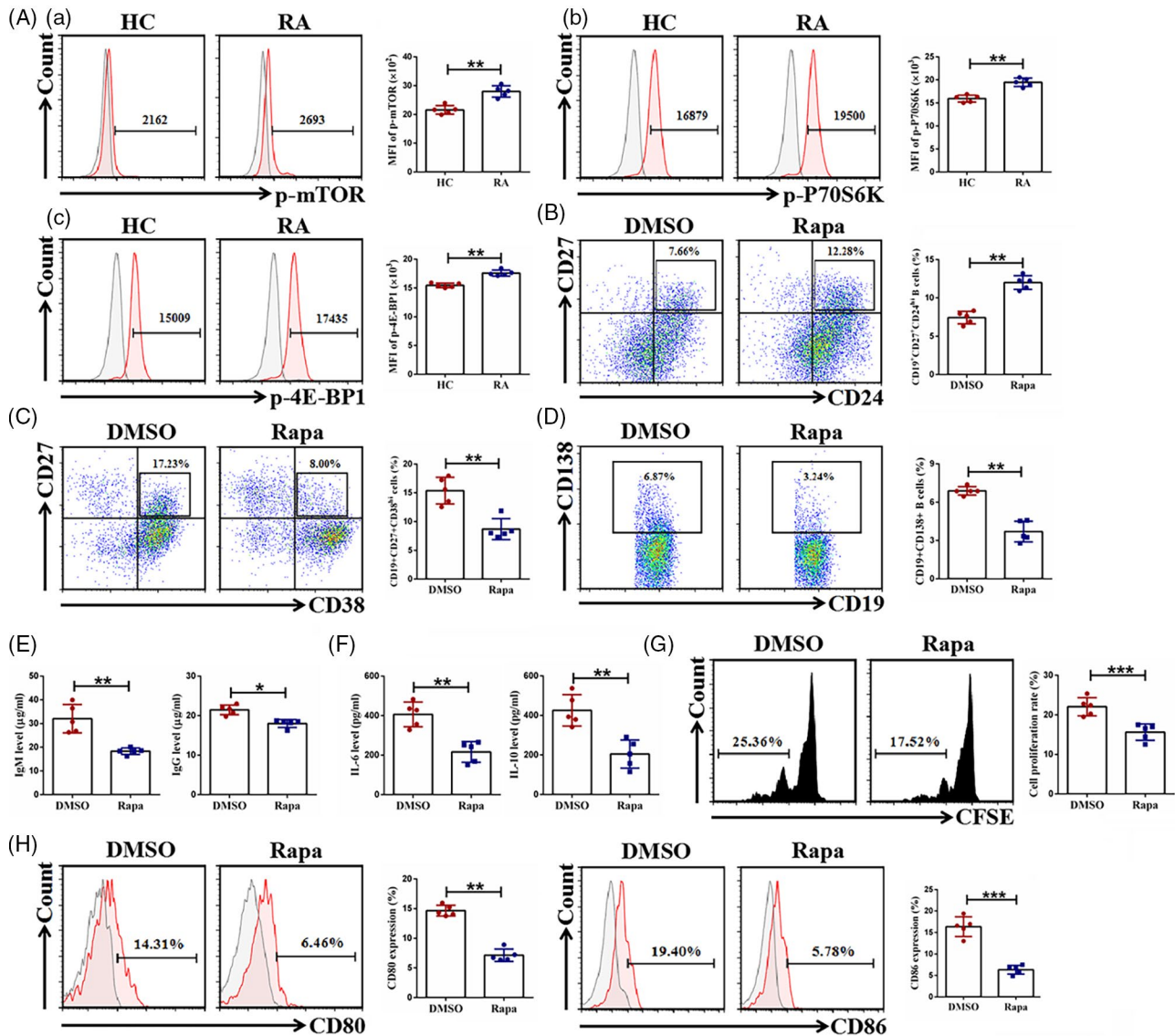
FIGURE 2 The mammalian target of rapamycin (mTOR) signaling pathway was involved in altered rheumatoid arthritis (RA) peripheral B cell homeostasis and functions. (A) Peripheral blood mononuclear cells (PBMCs) were isolated from RA patients and healthy controls (HC), and the levels of phosphorylated (p)-mTOR (a), p-P70S6K (b) and p-4E-BP1 (c) on $CD19^+$ B cells were analyzed with flow cytometry assay ($n = 5$). (B–D) RA $CD19^+$ B cells were sorted with magnetic beads and stimulated with or without rapamycin in the presence of anti-CD40/CpG for 72 h, and the frequencies of $CD19^+CD27^+CD24^{high}$ regulatory B cells (B), $CD19^+CD27^+CD38^{high}$ plasmablasts (C) and $CD19^+CD138^+$ plasma cells (D) were determined with flow cytometry assay ($n = 5$). (E,F) RA $CD19^+$ B cells were stimulated for 72 h; the supernatants were collected and the levels of IgM, IgG, IL-6 and IL-10 were measured with enzyme-linked immunosorbent assay (ELISA) assay ($n = 5$). (G) RA $CD19^+$ B cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured for 5 days, and B cell proliferation was analyzed with flow cytometry assay ($n = 5$). (H) The expression of CD80 and CD86 on B cells was quantified with flow cytometry assay at 72 h ($n = 5$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

(1902.00 ± 111.80 versus 1100.00 ± 44.00 pg/ml, $p < 0.001$, Figure 3C). Importantly, increased IL-27 levels were positively correlated with disease activity, the frequencies of plasma cells and the concentration of IgM and IgG in RA patients (Supporting information, Figures S2 and S3), implying the potential pathogenic role of IL-27 in RA B cells.

To further clarify whether the IL-27 signal contributes to the RA serum-activated mTOR pathway in B cells, HC B cells were cultured with RA serum in the presence or

absence of anti-IL-27 neutralizing antibody. As shown in Figure 3D, in the presence of anti-IL-27 neutralizing antibody the expression of p-mTOR (MFI = 17.96 ± 0.28 × 10² versus 20.44 ± 0.42 × 10², $p < 0.05$), p-P70S6K (MFI = 17.90 ± 0.24 × 10³ versus 20.58 ± 0.36 × 10³, $p < 0.05$) and p-4E-BP1 (MFI = 18.26 ± 0.40 × 10³ versus 20.39 ± 0.32 × 10³, $p < 0.05$) were significantly downregulated in the RA serum-treated B cell culture system. These data indicate that IL-27 might be a key cytokine in
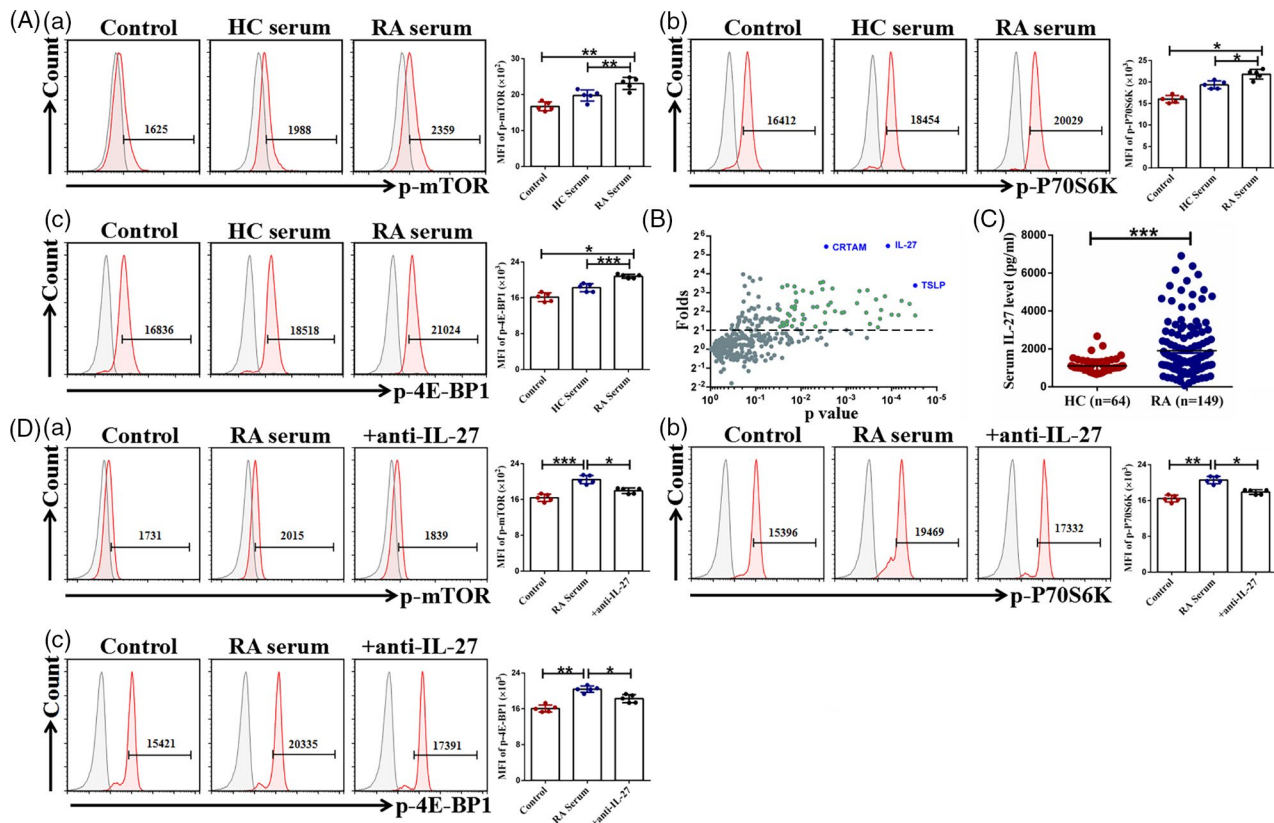
FIGURE 3 Interleukin (IL)-27 signal might be a key factor in rheumatoid arthritis (RA) serum-activated mammalian target of rapamycin (mTOR) pathway of B cells. (A) CD19$^+$ B cells were sorted with magnetic beads from peripheral blood mononuclear cells (PBMCs) of healthy controls (HC) and cultured with RPMI-1640 supplemented 2% fetal bovine serum (FBS), 2% serum from RA patients or HC in the presence of anti-CD40/CpG for 24 h. The levels of phosphorylated (p)-mTOR (a), p-P70S6K (b) and p-4E-BP1 (c) on CD19$^+$ B cells were detected with flow cytometry assay ($n = 5$). (B) The differences of serum 440 inflammatory molecules in RA patients relative to HC were depicted with the volcano plot, where the *x*-axis represents the adjusted *p*-value and the *y*-axis represents the fold change of 440 inflammatory molecules in RA patients relative to HC. (C) Interleukin (IL)-27 levels in RA patients ($n = 149$) and HC ($n = 64$) were detected with enzyme-linked immunosorbent assay (ELISA) assay. (D) CD19$^+$ B cells were sorted with magnetic beads from HC PBMCs and cultured with RPMI-1640 supplemented 2% fetal bovine serum (FBS), 2% RA serum with or without anti-IL-27 neutralizing antibody in the presence of anti-CD40/CpG for 24 h, and the expressions of p-mTOR (a), p-P70S6K (b) and p-4E-BP1 (c) on CD19$^+$ B cells were detected with flow cytometry assay ($n = 5$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

RA serum, which participated in the activation of mTOR pathway in B cells.

IL-27 signal directly induced the alteration of RA peripheral B cell homeostasis and functions

In order to explore the direct effects of IL-27 signal on the alteration of RA peripheral B cell homeostasis and functions, we first examined IL-27 receptor expression on B cells. As shown in Figure 4a, RA B cells showed higher expression of IL-27 receptor subunits gp130 ($4.72 \pm 0.45\%$ versus $2.66 \pm 0.23\%$, $p < 0.01$) and WSX-1 ($6.03 \pm 0.6\%$ versus $4.40 \pm 0.30\%$, $p < 0.01$) compared to HC B cells. In addition, recombinant IL-27 further up-regulated the expression of gp130 and

WSX-1 on RA B cells (gp130 = $13.86 \pm 0.32\%$ versus $9.44 \pm 0.29\%$, $P < 0.001$; WSX-1 = $7.63 \pm 0.69\%$ versus $4.74 \pm 0.40\%$, $p < 0.01$), which could be blocked with the addition of anti-IL-27 neutralizing antibody (gp130 = $11.11 \pm 0.27\%$, $p < 0.01$; WSX-1 = $5.36 \pm 0.36\%$, $p < 0.01$, Figure 4b).

Next, we investigated the effects of IL-27 signal on RA B cell activities. In our preliminary studies, purified RA B cells were cultured in the presence of various concentrations of human recombinant IL-27 (0, 25, 50, 75 and 100 ng/ml) for 3 or 5 days and the proliferation and apoptosis of B cells were analysed with flow cytometry assay. As shown in Supporting information, Figure S4, IL-27 at a concentration of 50 ng/ml induced higher proliferation of RA B cells, but had no effect on B cell apoptosis. Therefore, in subsequent studies, we selected 50 ng/ml of human recombinant IL-27 to stimulate RA B cells *in vitro*.
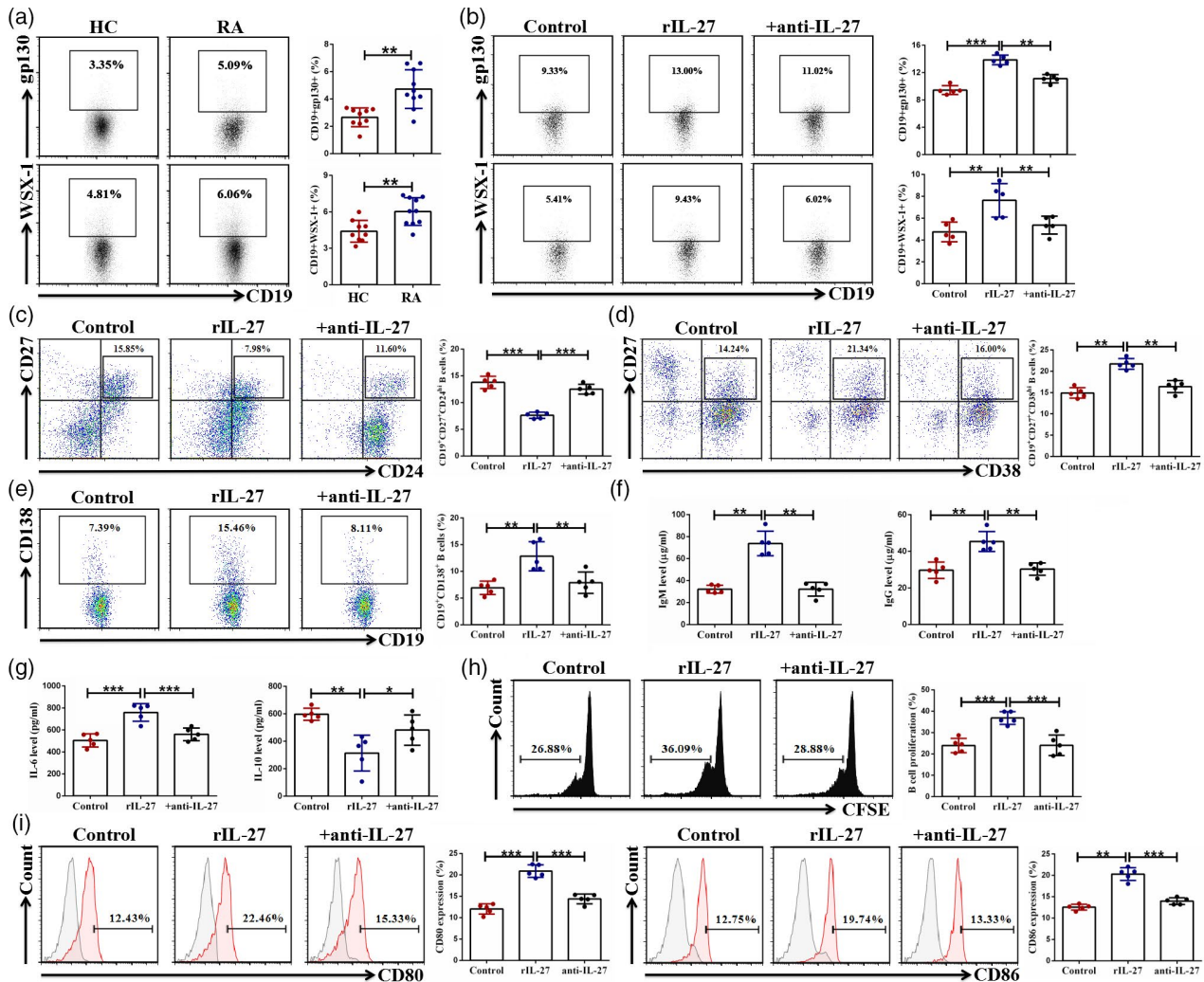
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FIGURE 4 The effects of the interleukin (IL)-27 signal on the alteration of rheumatoid arthritis (RA) peripheral B cell homeostasis and functions. (a) Peripheral blood mononuclear cells (PBMCs) were isolated from RA patients ($n = 10$) and healthy controls (HC, $n = 9$), and the expressions of gp130 and WSX-1 (IL-27 receptors) on CD19$^+$ B cells were analyzed with flow cytometry assay. (b–e) RA CD19$^+$ B cells were sorted with magnetic beads and stimulated with IL-27 and anti-CD40/cytosine–phosphate–guanosine (CpG) in the presence or absence of anti-IL-27 neutralizing antibody for 72 h, and the expressions of gp130 and WSX-1 on CD19$^+$ B cells (b), the frequencies of CD19$^+$CD27$^+$CD24^{high} regulatory B cells (c), CD19$^+$CD27$^+$CD38^{high} plasmablasts (d) and CD19$^+$CD138$^+$ plasma cells (e) were determined with flow cytometry assay ($n = 5$). (f,g) RA CD19$^+$ B cells were stimulated for 72 h and the supernatants were collected, and the levels of immunoglobulin (Ig)M, IgG, IL-6 and IL-10 were measured with enzyme-linked immunosorbent assay (ELISA) assay ($n = 5$). H, RA CD19$^+$ B cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured for 5 days, and B cell proliferation was analyzed with flow cytometry assay ($n = 5$). (i) The expression of CD80 and CD86 on B cells was quantified with flow cytometry assay at 72 h ($n = 5$). **$p < 0.01$; ***$p < 0.001$

We found that recombinant IL-27 significantly decreased the frequency of Breg cells ($7.62 \pm 0.26\%$ versus $13.79 \pm 0.51\%$, $p < 0.001$, Figure 4c), while it increased the frequencies of plasmablasts ($21.71 \pm 0.56\%$ versus $14.90 \pm 0.54\%$, $p < 0.01$, Figure 4d) and plasma cells ($12.84 \pm 1.22\%$ versus $6.91 \pm 0.56\%$, $p < 0.01$, Figure 4e) and the levels of supernatant IgM and IgG (IgM = 73.81 ± 5.00 μg/ml versus 32.16 ± 1.59 μg/ml, $p < 0.01$; IgG = 45.37 ± 2.43 μg/ml versus (9.68 ± 2.00 μg/ml, $p < 0.01$, Figure 4f) in the RA B cells in-vitro culture system. IL-27 treatment also increased the production of

IL-6 (759.2 ± 80.27 pg/ml versus 504.9 ± 59.46 pg/ml, $p < 0.001$), but deceased IL-10 production (313.5 ± 130.8 pg/ml versus 596.5 ± 44.02 pg/ml, $p < 0.01$, Figure 4g). In addition, our results showed that recombinant IL-27 promoted the proliferation ($36.05 \pm 1.0\%$ versus $0.34 \pm 1.60\%$, $p < 0.001$, Figure 4h) and activation (CD80 = $20.88 \pm 0.65\%$ versus $12.00 \pm 0.53\%$, $p < 0.001$; CD86 = $20.65 \pm 0.43\%$ versus $12.55 \pm 0.30\%$, $p < 0.01$, Figure 4i) of RA B cells. However, these effects induced by IL-27 could be blocked with the addition of anti-IL-27 neutralizing antibody.

It has been reported that IL-27 could modulate the number and functions of several T cell subpopulations, including T helper type 1 (Th1), Th17, regulatory T cells (Treg) and CD4$^+$CXCR5$^+$PD-1$^+$ follicular T helper cell (Tfh) cells [24–26]. However, in our study, IL-27 had no effects on frequencies of Th1, Th17, Treg and Tfh cells (Supporting information, Figure S5), which need more studies to be further verified. Taken together, our results suggest that IL-27 could directly mediate the alteration of RA peripheral B cell homeostasis and functions.

The mTOR pathway played a central role in RA peripheral B cell abnormalities induced by IL-27

The aforementioned results showed that mTOR signaling pathway was involved in altered RA peripheral B cell homeostasis and functions. Does the mTOR signaling pathway directly contribute to IL-27-induced RA peripheral B cell dysfunction?

As shown in Figure 5a, compared to untreated RA B cells, IL-27-treated RA B cells showed higher expressions of p-mTOR (MFI = $19.60 \pm 0.52 \times 10^2$ versus $14.11 \pm 0.36 \times 10^2$, $p < 0.01$), mTORC1 subunits p-P70S6K (MFI = $20.65 \pm 0.41 \times 10^3$ versus $16.18 \pm 0.35 \times 10^3$, $p < 0.001$) and p-4E-BP1 (MFI = $18.55 \pm 0.35 \times 10^3$ versus $14.35 \pm 0.34 \times 10^3$, $p < 0.01$), which can be partially suppressed by anti-IL-27 neutralizing antibody. Next, RA B cells were stimulated with recombinant IL-27 in the presence or absence of rapamycin. We found that rapamycin treatment significantly up-regulated the percentage of Breg cells ($13.66 \pm 0.56\%$ versus $9.03 \pm 0.51\%$, $p < 0.05$, Figure 5B) but down-regulated the frequencies of plasmablasts ($14.16 \pm 0.56\%$ versus $19.08 \pm 0.91\%$, $p < 0.01$, Figure 5C) and plasma cells ($8.29 \pm 0.20\%$ versus $11.63 \pm 0.20\%$, $p < 0.001$, Figure 5D) and levels of supernatant IgM (35.33 ± 2.41 μg/ml versus 62.75 ± 6.23 g/ml,
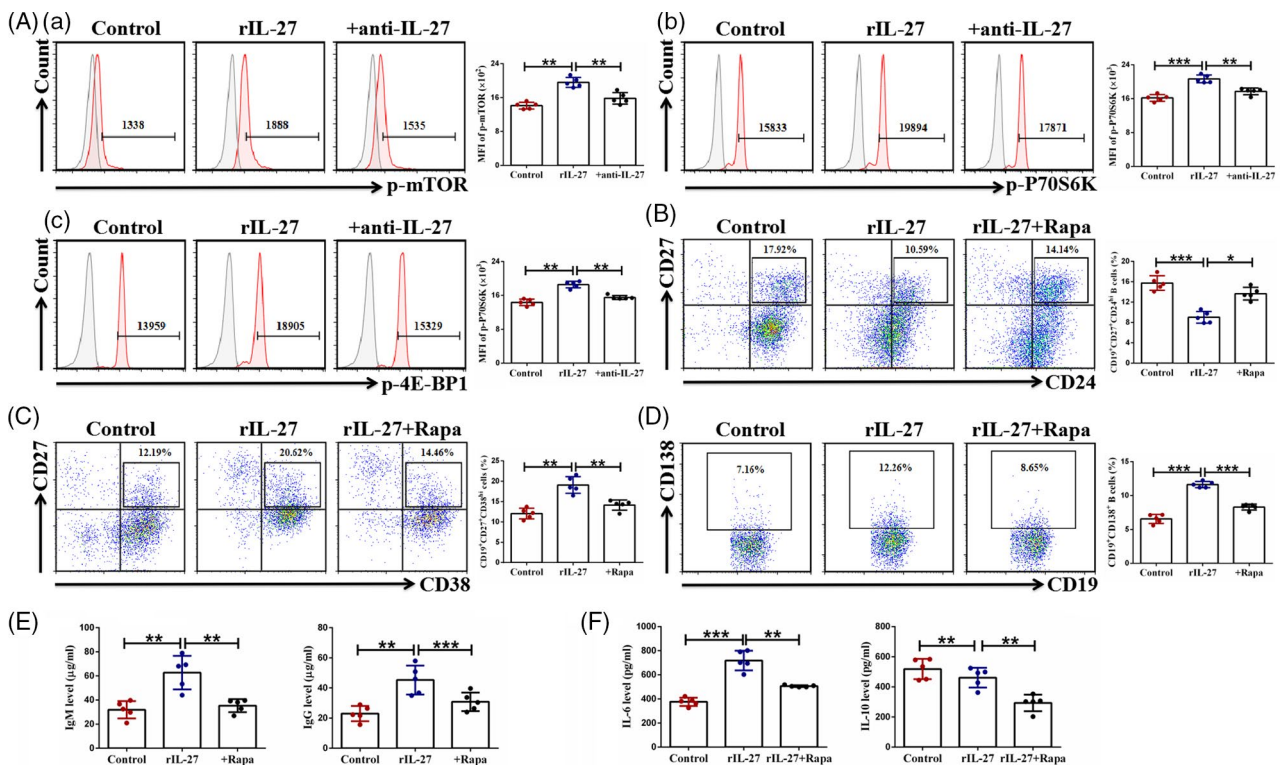

FIGURE 5 Altered rheumatoid arthritis (RA) B cell homeostasis and functions mediated by interleukin (IL)-27 signal via activating mammalian target of rapamycin (mTOR) pathway. (A) RA CD19$^+$ B cells were sorted with magnetic beads and stimulated with IL-27 and anti-CD40/cytosine–phosphate–guanosine (CpG) in the presence or absence of anti-IL-27 neutralizing antibody for 72 h, and the levels of phosphorylated (p)-mTOR (a), p-P70S6K (b) and p-4E-BP1 (c) on CD19$^+$ B cells were detected with flow cytometry assay ($n = 5$). (B–D) RA CD19$^+$ B cells were sorted with magnetic beads and stimulated with IL-27 antibodies with or without rapamycin in the presence of anti-CD40/CpG, and the frequencies of CD19$^+$CD27$^+$CD24^{high} regulatory B cells (b), CD19$^+$CD27$^+$CD38^{high} plasmablasts (C) and CD19$^+$CD138$^+$ plasma cells (D) were determined with flow cytometry assay ($n = 5$). (E,F) RA CD19$^+$ B cells were stimulated for 72 h and the supernatants were collected, and the levels of immunoglobulin (Ig)M, IgG, IL-6 and IL-10 were measured with enzyme-linked immunosorbent assay (ELISA) assay ($n = 5$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

$p < 0.01$), IgG (30.85 ± 2.75 µg/ml versus 45.27 ± 4.3 µg/ml, $p < 0.001$, Figure 5E) and IL-6 (506.1 ± 7.76 pg/ml versus 718.7 ± 81.31 pg/ml, $p < 0.01$, Figure 5F) in IL-27-treated RA B cell culture system. However, rapamycin treatment further enhanced the levels of supernatant IL-10 from IL-27-treated RA B cells (293.5 ± 54.93 pg/ml versus 461.2 ± 65.71 pg/ml, $p < 0.01$, Figure 5F). Taken together, these results suggest that the mTOR pathway may play a central role in RA B cell dysfunction induced by IL-27.

DISCUSSION

In this study, we found that RA patients showed altered peripheral B cell homeostasis and immune functions via activation of the mTOR signaling pathway. IL-27, as an obviously increased cytokine in RA serum was positively correlated with RA disease activity, the frequencies of plasma cell and the levels of autoantibodies. Importantly, we showed that IL-27-IL-27 receptors axis notably enhanced immune dysfunction of B cells from RA patients via activating the mTOR signaling pathway.

It is well known that B cells are linked to innate and adaptive immunity and characterized as positive regulators of humoral immune responses [27]. In addition to their well-defined role in antibody production, B cells may also regulate immune responses to microbes and participate in inflammation through T cell activation and cytokine production [28]. In RA patients, dysregulation of B cells enhanced effector T cells and suppressed Tregs, which could be restored by B cell depletion [29]. Also, the patients with a low frequency of CD27$^+$ memory B cells showed a better response to B cell depletion therapy [4]. These studies suggest that hyperactivated B cells may contribute to the development of RA. Here, we found that RA patients showed increased plasmablasts and plasma cells, as well as enhanced proliferation and activation of RA B cells. Recently, Bregs have been recognized as an important new B cell subset of the immune system. Breg cells expressing IL-10 restrain the excessive inflammatory responses that occur during innate immunity, inflammation and autoimmune diseases [30]. Importantly, impairment of Breg cells was correlated with exacerbated RA [31]. In our present study, the frequency of Breg cells was significantly down-regulated in RA patients, revealing that the imbalance of effector and regulatory B cells may be involved in the pathogenesis of RA.

The mTOR signaling pathway is an evolutionarily conserved serine/threonine protein kinase, which plays an important role in regulating cellular growth and metabolism [32]. It was demonstrated that the mTOR signaling pathway mediated the immune activities of CD4$^+$, CD8$^+$ T cells and APC [33]. Recently, the mTOR pathway has

been reported to regulate germinal center B cell growth, selection and affinity maturation [34]. In addition, inhibition of the mTOR pathway in B cells suppressed mitogen-dependent blast cell growth and inhibited cell proliferation by blocking cell cycle progression at the G1 phase [35]. These studies indicate the importance of the mTOR pathway in B cell-mediated immune function. In our study, we showed that peripheral B cells from RA patients had higher expressions of p-mTOR and p-mTORC1. Rapamycin, as a specific inhibitor of the mTOR pathway, significantly inhibited the differentiation and secretory functions of B cells but increased the frequency of Breg cells. Accordingly, the mTOR signaling pathway might be critical in the process of B cell immune dysfunction in RA patients.

As the functions of immune cells are closely related to the internal environment, we further detected the effect of RA inflammatory microenvironment on B cells abnormality. In the present study, serum from RA patients and healthy controls was used to represent the complicated environmental factors, and our data demonstrated that mTOR pathway was remarkably activated in RA serum-stimulated B cells. A variety of substances in RA serum, including cytokines and chemokines, play important roles in innate and adaptive immune responses. To search for the components that are differentially expressed between RA serum and normal serum, we performed a protein chip analysis and showed that IL-27 was most increased among 440 inflammatory molecules in RA serum. Furthermore, blocking IL-27 could partially restore the effect that enhanced mTOR activation of B cells induced by RA serum. Based on these data, we concluded that IL-27 might be a key cytokine in RA serum, which participated in activation of the mTOR pathway in B cells.

Recently, it has been demonstrated that IL-27 displayed both pro- and anti-inflammatory effects in infection, autoimmune disease and tumors through regulating immune cells. However, there are few studies on the relationship of IL-27 and B cells. Here, we mainly focused upon the exact role of IL-27 signal in the alteration of peripheral B cells in RA. We confirmed that IL-27 receptors (gp130 and WSX-1) were detectable on B cells. Recombinant IL-27 promoted the proliferation, activation differentiation and secretory functions of B cells *in vitro*. Previous studies demonstrated that IL-27 binding with the gp130/WSX-1 complex could activate the JAK/STAT signaling pathway and mTOR pathway [16,36]. In our study, we further showed that recombinant IL-27 could activate the mTOR signaling pathway in RA B cells, which were inhibited by anti-IL-27 neutralizing antibody. In addition, rapamycin treatment reduced the differentiation and antibodies/cytokines secretion in IL-27-stimulated RA B cells. Consequently, the

mTOR pathway may be vital in regulating the immune dysfunction of IL-27-induced B cells in RA patients.

More studies were reported regarding the role of IL-27 on T cells. For instance, IL-27 was known to promote Th1 differentiation, induce Tfh cell differentiation, maintain Treg function and suppress the development and secretion of Th17 and Th2 cells [37–41]. The contrary effects of IL-27 on Th1 (decrease), Treg (increase) and Th17 (increase) cell were also observed [24–26]. However, our present data have not shown significant effects of IL-27 on the frequencies of T cell subpopulations, including Th1, Th2, Treg and Tfh cells, despite certain trends. These results might be partly attributed to fewer samples, thus more samples will be needed to verify the roles of IL-27 in T cell subsets in RA.

In conclusion, altered peripheral B cell homeostasis and functions in RA might be mediated by increased serum IL-27. IL-27 can promote hyperactivities and enhance secretory functions of B cells in RA mainly via activation of the mTOR signaling pathway, implying that IL-27 may play a pro-pathogenic role in the development of RA. In future, we need to explore whether targeting IL-27 treatment can improve the disease severity of RA patients or animal models.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

X.L. and H.C. designed and supervised the study. Y.T. and Z.B. performed the experiments and Y.T. wrote the manuscript. Z.L. and B.W. revised the manuscript. J.Q., G.W. and M.J. participated in the sample collection. All authors edited and approved the manuscript.

ETHICS APPROVAL

The study protocol was approved by the ethics committees of Second Hospital of Dalian Medical University.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included this published article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of the article at the publisher's website.

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