High Levels of CD38+ NK Cells Contribute to Immune Imbalance of Th1/Th2 and Th17/Treg in Rheumatoid Arthritis

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Research article

Keywords: CD38, collagen-induced arthritis (CIA), cyclic ADP ribose hydrolase (cADPRH), daratumumab, mTOR, NK cells, NKT cells, rheumatoid arthritis (RA), Th1 cells, Th2 cell, Th17 cells, Treg cells

Posted Date: January 10th, 2020

DOI: https://doi.org/10.21203/rs.2.20626/v1

Abstract

Background

Increased CD38 expression and CD38+ cell proportion as well as their importance had been reported in rheumatoid arthritis (RA).

Methods

The proportion of lymphocyte subtypes in RA patients and rats with collagen-induced arthritis (CIA) was examined using flow cytometry. CD38+ NK cells, CD38+ NKT cells and CD4+ T cells as well as mononuclear cells (MNCs) depleted of CD38+ cells were isolated from RA synovial fluid using flow cytometry and cocultured in transwell apparatus.

Results

This study detected a significantly increased CD38+ NK cell proportion and a decreased CD38+ NKT cell proportion in RA peripheral blood and synovial fluid. The CD38+ NK/CD38+ NKT ratio was positively correlated with the disease activity. A similar result was observed in CIA rats. When CD38+ NK cells were cocultured with MNCs, the Treg cell proportion in MNCs and IL-10 level significantly decreased, and Th17 cell proportion and IFN-γ level increased. When the CD38+ NK cells were pretreated with monoclonal anti-CD38 antibody, Treg cell proportion and IL-10 level significantly increased, and the Th17 cell proportion and IFN-γ and IL-6 level decreased. When CD38+ NK cells were cocultured with CD4+ T cells, the Th1/Th2 and Th17/Treg ratios significantly increased, and mTOR signaling was activated in the cells. When the CD38+ NK cells were pretreated with the anti-CD38 antibody, the opposite result was obtained. Coculturing CD38+ NKT cells with MNCs or CD4+T cells showed opposite results. The anti-CD38 antibody also significantly increased TGF-β expression in the CD38+ NK cells.

Conclusions

Our results suggest that a high CD38+ NK and low CD38+ NKT proportion in RA elevates Th1/Th2 and Th17/Treg ratio to contribute to the pathogenesis.

Background

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation. The disrupted balance of Th1 (T helper cell type 1)/Th2 and Th17/Treg (regulatory T cells) are important for RA immune disorders [1–4].

CD38 (cluster of differentiation 38) is a multifunctional ectoenzyme that catalyzes conversion of β-nicotinamide adenine dinucleotide (β-NAD) to cyclic adenosine diphosphoribose (cADPR) and adenosine diphosphoribose (ADPR), and nicotinamide adenine dinucleotide phosphate (NADP) to nicotinic acid adenine dinucleotide phosphate (NAADP) and adenosine diphosphoribose-2'-phosphate (ADPR-P). The

metabolites of NAD and NADP have roles in calcium signaling in different cell types [5, 6]. CD38 is a glycoprotein on the cell membrane as cyclic ADP ribose hydrolase (cADPRH). It is mainly expressed in NK cells, B cells, T cells, monocyte-macrophages and dendritic cells (DCs) and is highly expressed in multiple myeloma cells [7–10]. CD38 is an emerging therapeutic target through which metabolism is altered in relation to infection, aging and tumorigenesis [5]. Through transcriptomic analyses, we discovered that CD38 is specifically overexpressed in synovial tissues in RA compared to samples from ankylosing spondylitis and osteoarthritis; likewise, the proportions of CD38 + cells and CD38 + CD56 + cells are significantly elevated in the peripheral blood of RA patients, and the level of CD38 + cells is positively correlated with the level of rheumatoid factor (RF) in the patients [11]. Moreover, a recent study found that CD38-knockout mice have significantly reduced occurrence and development of collagen-induced arthritis (CIA) [12]. We lately found that CD38 + NK cell inhibits Treg cell differentiation in mononuclear cells (MNCs), which may be a potential cause of the immune imbalance in RA and CIA. We also found that Cyanidin-3-O-glucoside (C3G), is an inhibitor of CD38, decreased the proportion of CD38 + cells. C3G can elevate Sirt6 expression to suppress NKG2D expression, increase TNF-α secretion, and decrease IFN-γ secretion in CD38 + NK cells, which stimulates MNCs to differentiate into Treg cells. Thus, C3G has therapeutic effect on RA [13]. Additionally, other study has shown elevated CD38 expression in B and T cells from patients with autoimmunity [14, 15]. These studies suggest that CD38 may play an important role in RA pathogenesis.

This study investigated the role and mechanism of CD38 + CD56 + cells in RA and CIA. We simultaneously studied CD38 + CD3- CD16 + CD56 + and CD38 + CD3 + CD16 + CD56 + cells, which are also known as CD38 + NK cells and CD38 + NKT cells, respectively. NK cells are granular lymphocytes of the innate immune system that do not express rearranged antigen receptors and are identified in humans by the expression of CD16 and CD56 [16]. NK cells are enriched in RA synovial fluid, resulting in the excessive production of pro-inflammatory cytokines [17]. Moreover, CD38 promotes the NK cell-killing function by interacting with CD16 [18]. NKT cells are a specific lymphoid subpopulation that expresses T cell receptors and NK cell receptors and functions with infection resistance and antitumor activity by producing inflammatory cytokines such as IFN-γ, perforin and granzymes [19–21]. It has been reported that NKT cells show reduced expression or loss of function in various autoimmune diseases such as RA, systemic lupus erythematosus (SLE), systemic sclerosis, multiple sclerosis and insulin-dependent diabetes mellitus [22–25]. This study measured proportions of CD38 + NK and CD38 + NKT cells in peripheral blood and synovial fluid of RA and CIA rats, and investigated the roles and regulatory mechanism of CD38 + NK and CD38 + NKT cell in RA.

Materials And Methods

Sample collection

Peripheral blood from RA patients (n = 30), synovial fluid from RA patients (n = 30) and synovial fluid from OA patients (n = 30) were collected in the Rheumatology Department of the Affiliated Hospital of Qingdao University in Qingdao, China. All enrolled RA patients met the 1987 RA classification criteria of

the American College of Rheumatology (ACR) and the 2010 classification criteria of the American Association of Rheumatology (AAR)/the European League Against Rheumatism (EULAR). Peripheral blood samples from healthy volunteers (n = 30) were collected at the Physical Examination Center of the hospital. The sample collection and experimental design were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University [Approval No. 20190105], and all patients gave written informed consent. Clinical information of the patientis who donated blood samples and synovial fluid samples was shown in Supplementary Table 1 and Supplementary Table 2, respectively.

Generating rat model of bovine type II collagen-induced arthritis

Six-week-old Sprague Dawley (SD) rats (200 ± 16 g) (n = 54, male) were purchased from the Shandong Laboratory Animal Center (China). The breeding and operational procedures of the experimental animals were carried out in accordance with the Helsinki Convention on Animal Protection and the Regulations of the People's Republic of China on the Administration of Experimental Animals. The rats were randomly divided into two groups: the normal control (NC, n = 24) and the collagen-induced arthritis (CIA) model (n = 30) groups. Rats in the CIA model group were intradermally injected at the tail root with an emulsified and equal mixture of bovine type II collagen (Chondrex, USA) (0.2 mg/per rats) and complete Freund's adjuvant (Sigma, USA). One week later, the same method was used to inject the same amount of the emulsified and equally mixed bovine type II collagen and incomplete Freund's adjuvant (Sigma). An inflammation curve was drawn based on changes in thickness of the toe to measure the disease activity. Those rats in the control group were injected with the same volume of physiological saline solution.

Detection of lymphocyte subtypes in rat blood

Rats were euthanized with lethal doses of ketamine and xylazine, and peripheral blood was collected from the inferior vena cava. The cells were collected by centrifugation and lysed using red blood cell lysis buffer (BioLegend, USA). Antibodies and isotype controls for flow cytometry were added (1.0 μg per 10^6 cells in 100 μl volume), and the mixture was incubated at 4 °C for 30 min in the dark. CD3 + T cells were detected with an APC anti-rat CD3 antibody (BioLegend), CD3 + CD4 + T cells with APC anti-rat CD3 and FITC anti-rat CD4 antibodies (BioLegend), CD3 + CD8 + T cells with APC anti-rat CD3 and PerCP anti-rat CD8a antibodies (BioLegend), CD3-CD45RA + B cells with APC anti-rat CD3 and FITC anti-rat CD45RA antibodies (BioLegend), and CD3-CD161 + NK and CD3 + CD161 + NKT cells with APC anti-rat CD3 and FITC anti-rat CD161 antibodies (BioLegend). FITC IgG1, PE IgG1, APC IgG1 and PerCP/Cy5.5 IgG1 antibodies were used for isotype controls. Data were acquired using NovoCyte flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.).

The proportions of CD38 + CD3-CD161 + NK cells, CD38 + CD3 + CD161 + NKT cells, CD38 + CD3 + T cells and CD38 + CD3-CD45RA + B cells were detected using flow cytometry as described above. PE anti-rat CD38 (BioLegend), FITC anti-rat CD161, APC anti-rat CD3 and FITC anti-rat CD45RA were added to the samples.

Detection of rat Treg cells

APC anti-rat CD4 and FITC anti-rat CD25 antibodies (BioLegend) or APC IgG2a and FITC IgG2a isotype control (BioLegend) were added to the blood samples with anticoagulation treatment (1 μg per 10^6 cells in 100 μl volume). The mixture was incubated at room temperature for 15 min in the dark. Then, 1x red blood cell (RBC) lysis/fixation solution (BioLegend) was added, and the mixture was incubated at room temperature for 15 min. After centrifugation, the cell staining buffer (BioLegend) was added to resuspend the pellet, and the mixture was centrifuged at 300 × g for 5 min. The cell pellet was then fixed with fixation buffer (BioLegend) and incubated for 20 min at room temperature. Following centrifugation, the fixed cells were resuspended in permeabilization wash buffer (BioLegend), and PE anti-rat FOXP3 antibody or PE IgG2a isotype control (BioLegend) was added (1 μg per 10^6 cells in 100 μl volume). The mixture was incubated at room temperature for at least 30 min. The mixture was centrifuged and was resuspended in 1x flow cytometry staining buffer. CD4 + CD25 + FOXP3 + Treg cells were detected by flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.)

Detection of lymphocyte subtypes in RA synovial fluid or blood

Peripheral blood of RA patients or healthy volunteers (n = 30, from patients No. 1 to No. 30 in Supplementary Table 1), and RA or OA synovial fluid samples (n = 30, from patients No. 1 to No. 30 in Supplementary Table 2) were collected and washed three times with PBS. The cell pellet was resuspended in PBS. The sample to be tested was incubated with the following antibodies and isotype controls at 4 °C for 30 min (1 μg per 10^6 cells in 100 μl volume). CD45 + lymphocytes, CD3 + T cells, CD3 + CD4 + T cells and CD3 + CD8 + T cells were detected with the FITC anti-CD3/PE anti-CD8/PerCP anti-CD45/APC anti-CD4 detection kit (ACEA Biosciences, China). CD3- CD56 + CD16 + NK cells, CD3 + CD56 + CD16 + NKT cells and CD3- CD19 + B cells were detected using a FITC anti-CD3/PE anti-CD16 + CD56/PerCP anti-CD45/APC anti-CD19 detection kit (ACEA biosciences). FITC IgG1, PE IgG1, APC IgG1 and PerCP/Cy5.5 IgG1 antibodies were used for isotype controls (BioLegend). Lymphocyte subtypes within MNCs were measured using flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.).

The proportions of CD38 + T cells (CD3 + CD38+), CD38 + B cells (CD3-CD19 + CD38+), CD38 + NK cells (CD38 + CD3-CD16 + CD56+) and CD38 + NKT cells (CD38 + CD3 + CD16 + CD56+) were detected using flow cytometry as described above. PerCP anti-human CD38 (BioLegend), FITC anti-human CD3/PE (CD16 + CD56) cocktail (BioLegend), FITC anti-human-CD3 (BioLegend) and APC anti-human-CD19 (BioLegend) were added to the samples. The mixture was incubated at 4 °C for 30 min and centrifuged. The pellet was resuspended in PBS.

Detection of RA Treg cells

This study used a human Treg cell assay kit (Multiscience Biotech). FITC anti-human CD4 and APC anti-human CD25 or FITC IgG2a and APC IgG2a isotype controls were added to blood samples with anticoagulation treatment, synovial fluids, cultured MNCs or CD4 + T cells (1 μg per 10^6 cells in 100 μl

volume). The mixture was incubated at room temperature for 15 min in the dark. Next, 1x flow cytometry lysing solution was added, and the mixtures were incubated at room temperature for 15 min. Following centrifugation, 1x flow cytometry staining buffer was added, and the mixtures were centrifuged. Then, 1x fixation/permeabilization working solution was added, and the mixtures were incubated at room temperature for 30 min. At that time, 1x permeabilization buffer was added, and the mixtures were centrifuged. The pellets were resuspended in 1x permeabilization buffer, and PE anti-human FOXP3 or rat PE IgG2a isotype control was added (1 μg per 10^6 cells in 100 μl volume). The mixture was incubated at room temperature for 30 min and centrifuged. The pellet was resuspended in 1x flow cytometry staining buffer. CD4 + CD25 + FOXP3 + Treg cells were detected using flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.).

Separation of mononuclear cells from synovial fluid or peripheral blood

Heparin sodium-anticoagulated peripheral blood (n = 9, patients No. 22-No. 30 in Supplementary Table 1) was added to an equal volume of PBS. Synovial fluids (n = 9, patients No. 1-No. 9 in Supplementary Table 2) were collected and pretreated with hyaluronidase (10 U/mL) at 37 °C for 30 min, and three volumes of PBS were added. The blood or synovial samples were centrifuged and resuspended with lymphocyte separation solution (Tianjin Haoyang, China). The mixture was centrifuged at 800 × g for 20 min, and the white circular layer was collected to obtain peripheral blood mononuclear cells (PMNCs) or synovial fluid mononuclear cells (SMNCs). The cells were resuspended with PBS.

Flow cytometric preparation of CD38 + NK cells, CD38 + NKT cells and CD38 + CD16 + CD56 + depleted MNCs

The peripheral blood or synovial fluid of RA patients was collected to isolate MNCs as described above. The sample was centrifuged, and the pellet was resuspended in PBS. PerCP anti-human CD38 and PE anti-human CD3 FITC/(CD16 + CD56) (10 μg per 10^7 cells in 500 μl volume) (1:1) were added, and the mixture was incubated for 30 min at 4 °C. The cells were washed, collected by centrifuging, and resuspended in PBS. The labeled cells were sorted using a BD FACS ARIA II with BD FACS Diva V6 software (BD Biosciences, USA). The CD38 and CD16 + CD56-positive and CD16 + CD56-negative gates were established, and the CD38 + NK and CD38 + NKT cells were differentiated by CD3+/- in the CD38 + and CD16 + CD56 + cell populations. The cell population in the negative gate was composed of MNCs depleted of CD38 + CD16 + CD56 + cells. The CD38 + NK cells contained a CD3- CD38 + CD16 + CD56 + population, and the CD38 + NKT cells contained a CD3 + CD38 + CD16 + CD56 + population. The sorted populations were collected into RPMI-1640 media (Gibco) containing 10% human heat-inactivated pooled AB serum (Gibco). The viability of the sorted cells was determined by trypan blue exclusion (Thermo Fisher Scientific) by routine protocol. After sorting, the samples were tested for purity by a BD FACS ARIA II with BD FACS Diva V6 software (BD Biosciences, USA).

Sorting of naïve CD4 + T cells

This study used a human naïve CD4 + T Cell Isolation Kit II (Miltenyi Biotec, Germany) to isolate naïve CD4 + T cells. This method is common for the sorting of naïve CD4 + T Cells [26, 27]. The RA synovial MNCs were obtained as described above and were resuspended in MACS (magnetic-activated cell sorting) buffer (PBS with 5% fetal calf serum) in the kit. CD4 and CD45RA magnetic beads were added to the cells, and the mixture was incubated at 4 °C for 15 min. The sample was loaded into the sorting column, and the negative cells were collected. MACS buffer was added to the sorting column, and the cells were quickly forced into the positive collection tube with the piston. The purity of the freshly sorted naïve CD4 + T cells was determined by flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.).

Coculture of CD38 + NK cells or CD38 + NKT cells with CD38 + CD16 + CD56 + depleted MNCs or CD4 + T cells

CD38 + NK or CD38 + NKT cells were treated with CD38 monoclonal antibody (CD38 mAb, Janssen Pharmaceutical Companies of Johnson & Johnson) at a final concentration of 1 μg/mL for 24 h. The CD38 monoclonal antibody has a trade name of daratumumab and consists of humanized monoclonal antibody. The CD38 antibody was removed by centrifugation at 200 × g for 5 min, and the cells were plated in the upper chamber of a 0.4 μm transwell apparatus (Corning Costar®, USA). Those cells that were treated with an equivalent amount of bovine serum albumin (BSA) (Sigma) were used as controls. Homologous CD38 + CD16 + CD56+-depleted MNCs or CD4 + T cells were seeded into the lower chamber. After 48 h of coculture, the cells were harvested, and the lymphocyte subtypes in the MNCs or CD4 + T cells were detected using flow cytometry. The supernatant was also collected, and cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ) were detected using flow cytometry as following describtion.

Detection of RA Th17 cells

This study used a human Th17 cell assay kit (Multiscience Biotech). Mononuclear cells (MNCs) was placed into a flow cytometry tube, and serum-free medium, PMA/ionomycin mixture and BFA/monensin mixture from the kit were added. The mixture was incubated at 37 °C for 4 h and centrifuged. The pellet was resuspended in medium containing 10% fetal bovine serum. FITC anti-human CD3 and PerCP-Cy5.5 anti-human CD4 antibody were added, and FITC IgG1 and PerCP/Cy5.5 IgG1 isotype control antibodies were added as controls (1 μg per 10^6 cells in 100 μl volume). The mixture was incubated at room temperature for 15 min. The FIX & PERM Medium A from the kit was added, the mixtures were incubated for 15 min at room temperature, and precooled 1x flow cytometry staining buffer was then added. Following centrifugation, FIX & PERM Medium B and PE anti-human IL-17A antibodies from the kit were added, and PE IgG1 isotype control antibody was added as a control (1 μg per 10^6 cells in 100 μl volume). The mixtures were incubated for 15 min at room temperature. Flow cytometry staining buffer was added, and the mixture was centrifuged. The contents were resuspended in 1x flow cytometry staining buffer, and CD3 + CD4 + IL-17A + Th17 cells were detected using flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.).

Detection of cytokines in culture medium

This study used a human Th1/Th2 cytokine detection kit (Hangzhou Cell Gene, China). The corresponding standard in the kit was added to each standard tube, and the samples were added to each sample tube. Fluorescence detection reagents that were conjugated to microcapsules in the kit were added to all tubes, and the mixture was incubated at room temperature for 2.5 h. Following centrifugation, the samples were resuspended in PBS, and the concentrations of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ were measured by flow cytometry (American ACEA BIO, NovoCyte D2040R). The data were analyzed using FCAP_Array_v3 software (BD Biosciences).

Treg cell isolation and sorting

MNCs were obtained from the peripheral blood of RA patients. The MNCs were incubated with FITC anti-human-CD25 (BioLegend), PerCP/Cy5.5 anti-human-CD4 (BioLegend), and FE anti-human-CD127 (BioLegend) antibodies for 30 min at room temperature. The cells were collected by centrifugation and collected in PBS. The labeled cells were sorted using a BD FACS ARIA II with BD FACS Diva V6 software (BD Biosciences, USA). The Treg cells were analyzed for purity.

Cell count kit-8 (CCK8) assay for Treg cells

CD38 + NK or CD38 + NKT cells were treated with CD38 monoclonal antibody (CD38 mAb, TheraMabs Bio-Technology, China) at a final concentration of 1 μg/mL or an equal volume of PBS at 37 °C for 24 h. CD38 + NK or CD38 + NKT cells were then washed and cocultured with Treg cells in a transwell apparatus at 37 °C and 5% CO_2 for 24 h. Treg cells were suspended and washed, and CCK-8 solution (Dojindo, Japan) was added to the 90 μl culture. The OD values were measured at 450 nm using a spectrophotometer (BioTek, USA) after incubation for 3 h.

Detection of TGF−β expression in culture medium

TGF−β expression in the culture medium was detected by flow cytometry using Human TGF−β1 capture bead A4 (BioLegend). The TGF−β1 magnetic beads, TGF−β1 antibody, assay buffer, and standard or tested sample were incubated together for 3 h at room temperature with shaking. Fluorescence detection reagents in the kit were added and incubated together for 0.5 h at room temperature with shaking. Following centrifugation, the samples were resuspended in PBS, and the concentrations of TGF−β were measured using flow cytometry (American ACEA BIO, NovoCyte D2040R). The data were analyzed using Legend_plex_v8.0 software (BioLegend).

Detection of RA Th1 and Th2 cells

This study used a human Th1/Th2 cell assay kit (Multiscience Biotech, China). Serum-free medium and a phorbol myristate acetate (PMA)/ionomycin/brefeldin A (BFA)/monensin mixture in the kit was added to blood samples, peripheral blood MNCs or CD4 + T cells. The mixture was incubated at 37 °C for 4 h and collected by centrifugation. The pellet was resuspended in medium containing 10% fetal bovine serum. FITC anti-human CD3 and PerCP-Cy5.5 anti-human CD4 antibodies were added, and FITC IgG1 isotype

control and PerCP-Cy5.5 antibody were added as controls (1.0 μg per 10^6 cells in 100 μl volume). The mixture was incubated at room temperature for 15 min. The FIX & PERM Medium A in the kit was added, and the mixture was incubated for 15 min at room temperature. Next, 1x flow cytometry staining buffer was added, and the mixture was centrifuged. The FIX & PERM Medium B in the kit, PE anti-human IFN-γ and APC anti-human IL-4 were added, and PE IgG1 isotype control and APC antibody were added as controls (1 μg per 10^6 cells in 100 μl volume). The mixtures were incubated for 15 min at room temperature. The mixtures were centrifuged and were resuspended with flow cytometry staining buffer. CD3 + CD4 + IFN-γ + Th1 cells and CD3 + CD4 + IL-4 + Th2 cells were detected using flow cytometry (American ACEA BIO, NovoCyte D2040R), and the data were analyzed using FlowJo software (Tree Star Inc.). Th1/Th2 ratio was calculated according the proportions of Th1 and Th2 in the CD4 + T cells.

Real-time PCR detection

CD38 + NK or CD38 + NKT cells were collected from the upper chamber of Transwell apparatus. RNA was extracted and reverse transcribed into cDNA (Vazyme, China). Real-time quantitative PCR (Thermo Fisher Scientific, USA) was used to detect the expression levels of CD3γ, CD28 and TGF-β mRNAs. The PCR primer sequences were as follows: CD3 forward 5'-GCATTTTCGTCCTTGCTGTTGGG-3' and reverse 5'-GGTCATCTTCTCGATCCTTGAGG-3'; CD28 forward 5'-GAGAAGAGCAATGGAACCATTATC-3' and reverse 5'-TAGCAAGCCAGGACTCCACCAA-3'; and TGF-β forward 5'-TACCTGAACCCGTGTTGCTCTC-3' and reverse 5'-GTTGCTGAGGTATCGCCAGGAA-3'.

Western blot (WB) analysis

Cocultured CD4 + T cells were collected, and RIPA lysis buffer (Beyotime, China) and PMSF (phenylmethanesulfonyl fluoride) were added. The cells were lysed on ice for 30 min. Following centrifugation at 3000 × g for 15 min at 4 °C, the supernatant was collected, and the protein concentration was determined by a BCA kit (Beyotime). The protein samples were subjected to SDS-PAGE (Beyotime) and transferred to a PVDF membrane (Boster, China). The protein expression changes in the mTOR (mammalian target of rapamycin) pathway were detected using an mTOR substrate antibody sampler kit (Cell Signaling Technology, USA). The membranes were incubated with the phospho-mTOR (Ser2448), total mTOR, phospho-p70S6 kinase (Thr389, Ser371) and phospho-4E-BP1 (Thr37/46) antibodies in the kit at 4 °C overnight. Followed washing three times in Tris buffered saline (TBS), the membrane was incubated with anti-rabbit IgG and HRP-linked antibodies from the kit for 2 h at room temperature. A gel imaging system (GelDoc XR+, Bio-Rad, USA) was used to expose and develop the blots. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference to normalize the expression levels of the target proteins. The expression normalization was performed with ImageJ software (NIH, Bethesda, MD, USA).

The expression of CD38 was examined using a similar protocol, and the antibody was commercially obtained from Santa Cruz Biotechnology. The CD38 antibody is a mouse monoclonal IgG1 (kappa light chain) provided at 200 μg/ml, which is raised against amino acids 1-170 of CD38 of human origin.

Statistical analysis

Normal and variance homogeneity tests were performed using SPSS 17.0 software (IBM, USA). One-way ANOVA was used to perform multigroup significance tests. The least significant difference (LSD) method was used for pairwise comparisons. The Pearson correlation analysis was performed, and $|r| \geq 0.8$ indicated that two variables were highly correlated; $0.8 > |r| \geq 0.5$ indicated that two variables were moderately correlated; $0.5 > |r| \geq 0.3$ indicated low correlation of two variables; and $0.3 > |r|$ indicated that there was no correlation between two variables. The test standard was $\alpha = 0.05$, and $p < 0.05$ indicated statistical significance.

Results

Phenotypic changes in CD38 + NK and CD38 + NKT Cells in CIA peripheral blood

We collected peripheral blood from 30 CIA rats and 24 normal rats. The proportions of CD4 + T cells, CD3-CD19 + B cells and CD3-CD161 + NK cells in total lymphocytes (CD45 + lymphocytes) in CIA group were significantly higher than those of the normal control group ($p = 0.00467$, 0.0415 and 0.0164, respectively), whereas the proportions of CD8 + T cells, CD3 + CD161 + NKT cells and CD4 + CD25 + FOXP3 + Treg cells were significantly lower than those of the normal control group ($p = 0.0037$, 0.0436 and 0.005, respectively). There was no significant difference in the proportion of CD3 + T cells between the two groups ($p = 0.618$). The results are shown in Fig. 1A.

Compared with the normal control group, the proportions of CD38 + CD3- CD19 + B (CD38 + B) cells and CD38 + CD3- CD161 + NK (CD38 + NK) cells in the peripheral blood of CIA rats were significantly increased ($p = 0.0062$ and 0.0016, respectively), the proportion of CD38 + CD3 + CD161 + NKT (CD38 + NKT) cells was significantly decreased ($p = 0.0028$), and there was no significant change in the proportion of CD38 + CD3+ (CD38 + T) cells ($p = 0.3639$) (Fig. 1B). The CD38 + NK/CD38 + NKT ratio values of the CIA rats ranged from 0.83 to 16.28, with an average of 5.16, which was significantly higher than that of the normal control group (range: 0.08–5.62, mean: 1.66, $p = 0.0012$). Meanwhile, the NK/NKT ratio values of the CIA rats ranged from 1.13 to 26.69, with an average of 8.34, which was significantly higher than that of the normal control group (range: 0.18–14.39, mean: 3.86, $p = 0.007$) (Fig. 1C). In addition, the ratio of CD38 + NK/CD38 + NKT in the CIA rats was positively correlated with the CIA inflammation index ($r = 0.76$, $p < 0.0001$), and the NK/NKT ratio was also positively correlated with the disease activity score ($r = 0.69$, $p = 0.0002$) (Fig. 1D).

Phenotypic changes in CD38 + NK and CD38 + NKT cells in RA peripheral blood and synovial fluid

We collected peripheral blood from 30 RA patients and 30 healthy volunteers. The proportions of CD45 + lymphocytes, CD4 + T, CD3- CD19 + B and CD3- CD16 + CD56 + NK cells in total lymphocytes (CD45 + lymphocytes) in the RA group were significantly higher than those in the control group ($p = 0.0007$, 0.0167

and < 0.0001, respectively), whereas the proportions of CD4 + CD25 + FOXP3 + Treg cells in RA group were significantly lower than those in the healthy controls (p = 0.0037 and 0.0029). There were no significant differences in the proportions of CD3 + T, CD8 + T and CD3 + CD16 + CD56 + NKT cells between the two groups (p > 0.05). The results are shown in Fig. 2A.

Compared with healthy volunteers, CD38 + CD3- CD19 + B (CD38 + B) and CD38 + CD3- CD16 + CD56 + NK (CD38 + NK) cells were significantly elevated in the peripheral blood of RA patients (p = 0.0001 and 0.0008, respectively), the proportion of CD38 + CD3 + CD16 + CD56 + NKT (CD38 + NKT) cells was significantly reduced (p = 0.0121), and there was no significant change in the proportion of CD38 + CD3 + cells (p = 0.3689) (Fig. 2B). The ratio of CD38 + NK/CD38 + NKT in RA patients ranged from 0.62 to 23, with an average of 4.545, which was significantly higher than that of the healthy control group (range: 0.31–11.26, mean: 1.153, p = 0.0004). There was no significant difference in NK/NKT ratio between the RA patients and healthy subjects (p = 0.9227) (Fig. 2C). The CD38 + NK/CD38 + NKT ratio was positively correlated with the RA DAS28 (r = 0.716, p < 0.0001), whereas the NK/NKT ratio was slightly correlated with DAS28 (r = 0.465, p = 0.0096) (Fig. 2D).

We collected synovial fluid samples from 30 RA patients and 30 OA patients. The proportions of CD4 + T, CD3- CD19 + B and CD3- CD16 + CD56 + NK cells in total lymphocytes (CD45 + lymphocytes) in RA group were significantly higher than those in OA group (p = 0.0007, 0.0124 and 0.0021, respectively), whereas the proportions of CD3 + CD16 + CD56 + NKT, Treg and CD8 + T cells were significantly lower than those in the control group (p = 0.0019, 0.0002 and 0.0094, respectively). There was no significant difference in the proportions of CD3 + T cells and CD45 + lymphocytes between the two groups (p > 0.05). The results are shown in Fig. 3A.

Compared with the OA control group, the proportions of CD38 + CD3- CD19+ (CD38 + B) and CD38 + CD3- CD16 + CD56 + NK (CD38 + NK) cells in the synovial fluid of RA patients were significantly increased (p = 0.0088 and 0.0002, respectively), the proportion of CD38 + CD3 + CD16 + CD56 + NKT (CD38 + NKT) cells was significantly reduced (p = 0.0189), and there was no significant change in the proportion of CD38 + CD3 + cells (p = 0.7905) (Fig. 3B). The ratio of CD38 + NK/CD38 + NKT cells in RA patients ranged from 0.942 to 14.45, with an average of 3.07, which was significantly higher than that of the OA group (range: 0.234–4.407, mean: 1.58, p < 0.0001). The NK/NKT ratios in RA patients ranged from 1.463 to 9.713, with an average of 2.82, which was significantly higher than that in OA group (range: 0.503–5.326, mean: 1.54, p = 0.0004) (Fig. 3C). The CD38 + NK/CD38 + NKT ratio in RA patients was positively correlated with DAS28 (r = 0.747, p < 0.0001), whereas the NKT/NK ratio was not associated with DAS28 (r=-0.20, p = 0.2875) (Fig. 3D).

Effects of CD38 + NK and CD38 + NKT cells on MNCs

We collected synovial fluid from 9 patients with RA, isolated MNCs from the samples, and then prepared CD38 + NK cells, CD38 + NKT cells and MNCs depleted of CD38 + cells. The purity of CD38 + NK was 99.28%, whereas that of CD38 + NKT was 98.9%. The result is shown in Supplementary Fig. 1A, B. The

CD38 + NK and CD38 + NKT cells were pretreated with CD38 monoclonal antibody (mAb) or BSA as a control and then cocultured with RA synovial fluid MNCs that were depleted of CD16 + CD56 + CD38 + cells. The experimental design is shown in Fig. 4A. Compared with non-cocultured synovial MNCs, the proportion of CD4 + CD25 + FOXP3 + Treg cells in total lymphocytes (CD45 + lymphocytes) in the MNCs was significantly decreased ($p = 0.033$), and the proportion of CD4 + IL-17 + Th17 (Th17) cells in lymphocytes was significantly increased ($p = 0.0055$) in MNCs after coculturing with the CD38 + NK cells. Meanwhile, the proportion of CD4 + CD25 + FOXP3 + Treg cells in MNCs was significantly increased ($p = 0.0021$), and the proportion of CD4 + IL-17 + Th17 cells in lymphocytes was significantly decreased ($p = 0.0152$) in the MNC after coculturing with CD38 + NKT cells. Compared with MNCs cocultured with the CD38 + NK cells, the proportions of CD3 + T cells and CD4 + CD25 + FOXP3 + Treg cells in lymphocytes were significantly higher ($p = 0.0252$ and 0.0021, respectively), and the proportion of CD4 + IL-17 + Th17 cells in lymphocytes was significantly reduced ($p = 0.0025$) in MNCs coculturing with the CD38 + NKT cells. Compared with MNCs cocultured with BSA-pretreated CD38 + NK cells, the proportions of CD4 + T cells and CD4 + IL-17 + Th17 cells were significantly reduced ($p = 0.0402$ and 0.0137, respectively), and the proportion of CD4 + CD25 + FOXP3 + Treg cells was significantly elevated ($p = 0.0479$) in MNCs after coculturing with the antibody-pretreated CD38 + NK cells (CD38 + NK + CD38 mAb). Conversely, compared with MNCs cocultured with BSA-pretreated CD38 + NKT cells, the proportion of CD4 + CD25 + FOXP3 + Treg cells was significantly reduced ($p = 0.0139$), and the proportion of CD4 + IL-17 + Th17 cells was significantly increased ($p = 0.0224$) in MNCs after coculturing with the antibody-pretreated CD38 + NKT cells (CD38 + NKT + CD38 mAb). The above results are shown in Fig. 4B.

Compared with non-cocultured synovial MNCs, the IFN-γ level in the medium was significantly increased ($p = 0.0114$), and IL-10 was significantly decreased ($p = 0.0377$), when MNCs were cocultured with CD38 + NK cells. In contrast, the IL-6 level was significantly decreased ($p = 0.0434$) when MNCs were cocultured with CD38 + NKT. Compared with MNCs cocultured with CD38 + NK cells, the IFN-γ level in the culture medium was significantly decreased ($p = 0.0193$) when MNCs were cocultured with CD38 + NKT cells. Compared with MNCs cocultured with BSA-pretreated with CD38 + NK cells, the levels of IL-2 and IL-10 in the culture medium were significantly increased ($p = 0.0492$ and 0.0275, respectively) when MNCs were cocultured with CD38 antibody-pretreated CD38 + NK cells, and the levels of IL-6 and IFN-γ were significantly reduced ($p = 0.0200$ and 0.0119, respectively). Compared with MNCs cocultured with BSA-pretreated CD38 + NKT cells, the cytokine levels in the culture medium were not changed when MNCs were cocultured with CD38 antibody-pretreated CD38 + NKT cells. The above results are shown in Fig. 4C.

We collected peripheral blood from nine patients with RA, isolated MNCs from the samples, and then prepared CD38 + NK cells, CD38 + NKT cells and MNCs depleted of CD38 + cells using flow cytometry. The CD38 + NK and CD38 + NKT cells were treated with BSA or CD38 mAb. They were then cocultured with MNCs depleted of CD16 + CD56 + CD38 + cells including CD38 + NK and CD38 + NKT cells. The experimental design is also shown in Fig. 4A. Compared with non-cocultured peripheral MNCs, the proportion of CD4 + CD25 + FOXP3 + Treg cells was significantly decreased ($p = 0.0007$), and the proportion of CD4 + IL-17 + Th17 cells in the lymphocytes was significantly increased ($p = 0.0162$) in the blood MNCs after coculturing with CD38 + NK cells. The proportion of CD4 + CD25 + FOXP3 + Treg cells

was significantly increased (p = 0.0269), and the proportion of CD4 + IL-17 + Th17 cells in lymphocytes was significantly decreased (p = 0.0006) in MNCs after coculturing with CD38 + NKT cells. Compared with MNCs cocultured with CD38 + NK cells, the proportion of Treg cells was significantly increased (p = 0.0327) in MNCs after coculturing with CD38 + NKT cells, and the proportion of CD4 + IL-17 + Th17 cells in lymphocytes was significantly decreased (p = 0.0004). Compared with MNCs cocultured with BSA-pretreated CD38 + NK cells, the proportion of Treg cells was significantly increased (p = 0.0454) after coculturing with CD38 antibody-pretreated CD38 + NK cells, and the proportion of CD4 + IL-17 + Th17 cells in lymphocytes was significantly decreased (p = 0.0381). Compared with MNCs cocultured with BSA-pretreated CD38 + NKT cells, the proportion of Treg cells was significantly decreased (p = 0.008), and the proportion of CD4 + IL-17 + Th17 cells in lymphocytes was significantly increased (p = 0.0342) in MNCs after coculturing with CD38 antibody-treated with CD38 + NKT cells. The results are shown in Fig. 5A.

Compared with non-cocultured blood MNCs, the level of IFN-γ in the culture medium was significantly increased (p < 0.0001), and IL-10 was significantly decreased (p = 0.0164), when MNCs were cocultured with CD38 + NK cells. The IL-6 level in the culture medium was significantly decreased (p = 0.0434) when MNCs were cocultured with CD38 + NKT cells. Compared with MNCs cocultured with CD38 + NK cells, IL-6 and IFN-γ levels in the culture medium were significantly decreased (p = 0.0155 and p = 0.0006, respectively) when MNCs were cocultured with CD38 + NKT cells, whereas the IL-10 level was significantly increased (p = 0.0416). Compared with MNCs cocultured with BSA-pretreated with CD38 + NK cells, the levels of IL-2 and IL-10 were significantly increased (p = 0.0499 and p = 0.0013, respectively), and the IL-6 and IFN-γ levels in the culture medium were significantly decreased (p = 0.0051 and p = 0.0454, respectively) when MNCs were cocultured with CD38 antibody-pretreated CD38 + NK cells. Compared to MNCs cocultured with BSA-pretreated CD38 + NKT cells, IL-6 level in the culture medium was significantly increased (p = 0.0341) when MNCs were cocultured with CD38 antibody-pretreated CD38 + NKT cells, but the levels of other cytokines were not significantly changed. The above results are shown in Fig. 5B.

Effect of CD38 + NK and CD38 + NKT cells on CD4 + T cells

To investigate the effects of CD38 + NK and CD38 + NKT cells on CD4 + T cell differentiation, we collected synovial fluid from 9 RA patients and sorted homogenous CD38 + NK cells, CD38 + NKT cells and CD4 + T cells. The CD38 + NK and CD38 + NKT cells were treated with BSA or anti-CD38 antibody and were then cocultured with CD4 + T cells. The experimental design is shown in Fig. 6A. Compared with the non-cocultured CD4 + T cells, the ratios of Th1/Th2 and Th17/Treg cells were increased significantly (p = 0.0086 and 0.0186, respectively) in the CD4 + T cells after coculturing with CD38 + NK cells, whereas the ratios of Th1/Th2 and Th17/Treg cells were significantly decreased (p = 0.0061 and 0.0074, respectively) in the CD4 + T cells after coculturing with CD38 + NKT cells. Compared with CD4 + T cells cocultured with BSA-pretreated CD38 + NK cells, the ratios of Th1/Th2 and Th17/Treg cells were significantly decreased (p = 0.0325 and 0.0259, respectively) in CD4 + T cells after coculturing with CD38 antibody-treated CD38 + NK + CD38 cells. Compared with CD4 + cells with BSA-pretreated CD38 + NKT cells, the ratios of Th1/Th2 and Th17/Treg cells were significantly increased (p = 0.0006 and 0.0002, respectively) in CD4 +

T cells after coculturing with CD38 antibody-pretreated CD38 + NKT cells. The results are shown in Fig. 6B and C.

To determine if CD38 + NK or CD38 + NKT cells had direct effect on Treg cell proliferation, we cocultured CD38 + NK cells or CD38 + NKT cells and Treg cells in place of CD4 + T cells in a transwell apparatus. The experimental design is shown in Supplementary Fig. 2A. We examined the cell proliferation of Treg cells using a CCK-8 kit. Treg cells following coculture with CD38 + NK cells showed no significant change in cell proliferation, compared with the Treg cells without coculture or Treg cells following coculture with anti CD38 antibody-pretreated CD38 + NK cells. The effect of CD38 + NKT on Treg cell proliferation was similar to that of CD38 + NK. The results are shown in Supplementary Fig. 2B. These results indicated that CD38 + NK cells and CD38 + NKT cells as well as CD38 molecular on the cells had no significant effect on Treg cell proliferation.

To investigate the molecular mechanisms by which CD38 + NK and CD38 + NKT cells act on the differentiation of CD4 + T cells, we collected the cocultured CD4 + T cells and then examined changes in the mTOR signaling pathway using Western blot analysis. Compared with CD4 + T cells cocultured with BSA-pretreated CD38 + NK cells, CD4 + T cells showed a significant decrease in the expression levels of phospho-P70S6, phospho-mTOR and total mTOR protein ($p = 0.0473$, 0.0127 and 0.0094, respectively) after coculturing with CD38 antibody-pretreated CD38 + NK cells. Compared with CD4 + T cells cocultured with CD38 + NK cells, CD4 + T cells also showed a significant decrease in phospho-P70S6, phospho-mTOR and total mTOR protein expression levels ($p = 0.0004$, 0.0037 and 0.0012, respectively) after coculturing with CD38 + NKT cells. Compared with CD4 + T cells cocultured with BSA-pretreated with the CD38 + NKT cells, CD4 + T cells showed significant increases in phospho-P70S6, phospho-mTOR and total mTOR protein expression levels ($p = 0.003$, 0.0019 and 0.0059, respectively) after coculturing with CD38 antibody-pretreated CD38 + NKT cells. Compared with non-cocultured CD4 + T cells, CD4 + T cells showed significant increases in the phospho-P70S6, phospho-mTOR, and total mTOR protein expression levels ($p = 0.0075$, 0.0425 and 0.0216, respectively) after coculturing with CD38 + NK cells, while CD4 + T cells also showed significant increases in phospho-P70S6, phospho-mTOR and total mTOR protein levels ($p = 0.0029$, 0.0358 and 0.0116, respectively) after coculturing with CD38 antibody-pretreated CD38 + NKT cells. The above results are shown in Fig. 6D and E.

We also collected CD38 + NK and CD38 + NKT cells from the cocultures described above and used real-time PCR to detect the mRNA levels of CD3, CD28 and TGF-β. Compared with the CD38 + NK cells, the CD38 + NKT cells showed a significant increase in CD3 mRNA expression ($p = 0.0002$), while the CD38 + NKT cells with pretreatment of CD38 antibody showed decreased CD3 mRNA expression ($p = 0.0079$). Regardless of presence or absence of the CD38 antibody, the CD28 level in CD38 + NK and CD38 + NKT cells remained unchanged. Compared with BSA-pretreated CD38 + NK cells, CD38 + NK cells with pretreatment of CD38 antibody showed significantly higher levels of TGF-β expression ($p = 0.0056$), but the antibody pretreatment did not affect TGF-β expression in CD38 + NKT cells. The above results are shown in Fig. 6F. Additionally, flow cytometry analysis of cytokines detected increased TGF-β expression

in the cultured medium of CD38 + NK cells with pretreatment of CD38 antibody. The above results are shown in Fig. 6G and H.

Discussion

This study collected peripheral blood from RA patients and healthy volunteers and synovial fluid from RA and OA patients and then examined changes in lymphocyte subtypes in the samples, especially in the CD38 + cells. We found that the proportions of CD38 + B cells and CD38 + NK lymphocytes in the peripheral blood and synovial fluid of RA patients were significantly increased, and the proportion of CD38 + NKT cells was significantly decreased. Furthermore, the ratio of CD38 + NK/CD38 + NKT cells was positively correlated with DAS28 in the RA patients. Similar results were obtained in CIA rats, and the ratio of CD38 + NK/CD38 + NKT cells was positively correlated with CIA disease activity. The above results suggest that the proportion of CD38 + NK and CD38 + NKT cells and the ratio of CD38 + NK/CD38 + NKT cells are significantly changed in CIA and RA.

To explore the function of CD38 + NK and CD38 + NKT cells on RA, we isolated CD38 + NK and CD38 + NKT cells from RA patient synovial fluid and peripheral blood. We then cocultured CD38 + NK or CD38 + NKT cells with CD38 + CD16 + CD56 + cell-depleted MNCs from the same source. After coculture with CD38 + NK cells, the proportions of Treg cells in peripheral blood MNCs and RA synovial fluid MNCs were significantly decreased, and the proportion of Th17 cells was significantly increased. After coculture with CD38 + NKT cells, the proportion of Treg cells in MNCs was significantly increased, and the proportion of Th17 cells was significantly decreased. The opposite observation was obtained in these MNCs when CD38 + NK or CD38 + NKT cells were pretreated with anti-CD38 antibody. Additionally, IL-10 levels in the culture mdedium were significantly increased, and IFN-γ and IL-6 levels were significantly decreased after coculturing MNCs with CD38 antibody-pretreated CD38 + NK cells. IL-10 levels were also significantly increased after coculture with CD38 + NKT cells, and IFN-γ levels were significantly reduced. There was no change in IL-2, IL-4 and TNF-α production. It has been reported that NK cells aggravate the inflammatory response in RA by secreting IFN-γ [28]. Some studies have found that CD38 promotes the secretion of IFN-γ in NK cells but has no effect on TNF-α production [18, 29]. Their results are consistent with ours. These results suggest that CD38 + NK and CD38 + NKT cells as well as their CD38 molecules can regulate the proportion of Treg and Th17 cells in MNCs.

Treg cells are essential for maintaining peripheral immune tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases by secreting IL-10 [30, 31]. Some studies have reported that the function of Treg cells in RA is partially impaired or that Treg cell number is decreased [32]. Animal studies have shown that Treg cells effectively alleviate the inflammatory response in CIA rats [33] IL-10 is an immunoregulatory cytokine that plays a central role in the pathogenesis of autoimmune diseases. Studies have shown that IL-10 is downregulated in RA synovial fluid and peripheral blood [34]. In vitro studies have shown that IL-10 reduces the inflammatory response in RA [35]. The current study also detected a decreased number of Treg cells in RA patients and CIA rats. Furthermore, decreased proportions of Treg cells in MNCs and CD4 + T cells were detected after coculturing with CD38 + NK cells.

We thus propose that CD38 + NK cells can exert pro-inflammatory effects by inhibiting MNC or their CD4 + T cells to differentiate into Treg cells and secretion of IL-10, whereas CD38 + NKT cells promote differentiation of MNCs or their CD4 + T cells into Treg cells and the secretion of IL-10. A high CD38 + NK/CD38 + NKT cell ratio in RA might contribute to a decreased proportion of Treg cells and decreased IL-10 secretion, an increased proportion of Th17 cells, and elevated levels of IL-6 and IFN-γ, which aggravates RA. This hypothesis can explain why the CD38 + NK/CD38 + NKT ratio is positively correlated with the RA clinical performance DAS28 indicator. Additionally, our experiment indicated that CD38 + NK cells had no direct effect on Treg cell proliferation. Furthermore, we had found that apoptosis of Treg cells was not changed when MNCs were cocultured with CD38 + NK cells [13]. Thus, the decreased Treg cell proportion in MNCs or CD4 + T cells that were cocultured with CD38 + NK cells is due to Treg differentiation rather than the Treg cell-death sensitivity and cell proliferation.

It is believed that the disrupted balance of Th1/Th2 cells and Th17/Treg cells plays an important role in RA. Th17 cells secrete many inflammatory factors to aggravate the inflammatory response of autoimmune diseases [36]. Both Treg cells and Th17 cells are derived from CD4 + cells. Naïve CD4 + Th cells differentiate into at least Th1, Th2, Th17, Treg, Th9 and T follicular helper (Tfh) cells [37, 38]. The present study found that CD38 + NK cells increased the ratios of Th1/Th2 and Th17/Treg cells in CD4 + T cells, and the ratios significantly decreased after the CD38 + NK cells was pretreated with CD38 antibody. CD38 + NKT cells decreased the ratio of Th1/Th2 and Th17/Treg cells in CD4 + T cells following coculture, and the pretreatment of CD38 antibody elevated the ratios. These results suggest that the increased CD38 + NK cell proportion as well as CD38 + NK /CD38 + NKT cell ratio in RA is important reasons for the high Th1/Th2 and Th17/Treg cell ratio in RA, which would aggravate the immune imbalance and disease course. It has been reported that NK cells participate in pneumonia caused by chlamydia by regulating the balance of Th1/Treg and Th17/Treg cells [39].

It had been demonstrated that activation of the mTOR signaling pathway promotes the differentiation of primary T cells into effector Th1 cells, and blocking mTOR can reverse this inhibitory effect on Treg cells [40]. Mice with mTOR knockout have disorders in the differentiation of T cells into Th1, Th2 and Th17 cells [37]. mTOR signaling inhibits the expansion of CD4 + CD25 + FOXP3 + T cells [41]. Blocking agents of the mTOR pathway are being developed for the treatment and prevention of RA [41]. The above studies demonstrated the importance of mTOR in Treg cell differentiation. The present study found that CD38 + NK cells activated mTOR signaling in CD4 + T cells. Treating CD38 + NK cells with CD38 antibody inactivated the mTOR signaling pathway. CD38 + NKT cells and the treatment of CD38 + NKT cells with CD38 antibody had the opposite effects on the mTOR pathway in CD4 + T cells. These observations and studies of others suggest that CD38 + NK cells inhibit Treg cell differentiation by activating the mTOR pathway in CD4 + T cells. The high CD38 + NK/CD38 + NKT ratio in RA may inhibit the differentiation of CD4 + T cells into Treg cells by activating mTOR signaling, resulting in decreased anti-inflammatory capacity and aggravating the course of RA.

This study further explored the molecular mechanisms by which CD38 + NK and CD38 + NKT cells affect CD4 + T cell differentiation into Treg cells. Naïve CD4 + T cells can be induced to differentiate into Treg

cells with TGF-β and into Th17 cells with the combination of TGF-β and IL-6 or IL-21 [42–45]. Anti-CD3 antibodies, CD28, IL-2 and TGF-β are usually used to induce CD4 + T cell differentiation into Treg cells in vitro [44, 45]. Thus, this study focused on investigating CD3, CD28 and TGF-β expression in CD38 + NK cells. We found that the CD38 antibody significantly increased the expression of TGF-β in CD38 + NK cells but not CD3 and CD28 expression. Furthermore, the study found that the CD38 antibody significantly increased IL-2 secretion and decreased IL-6 and IFN-γ secretion in medium of MNCs cocultured with CD38 + NK cells. IFN-γ had been reported to activate the mTOR signaling pathway [43]. Therefore, this study suggests that CD38 + NK cells down-regulates TGF-β and IL-2 expression and up-regulates IFN-γ secretion to decrease CD4 + T cell differentiation into Treg cells. TGF-β plays a pivotal role in maintaining peripheral tolerance against self- and innocuous antigens [46, 47]. A decreased level of serum TGF-β was detected in active RA and RA with low activity [48]. Although the role of TGF-β in RA is not well defined, and the function of TGF-β in murine models has been extensively studied with controversial results. TGF-β has also shown controversial results as an inhibitor or promoter in the inflammatory response [49].

When CD38 + NK cells and MNCs were cocultured, we detected decreased Treg cell proportion and IL-10 levels and increased Th17 cell proportion and IFN-γ levels in MNC and the cultured medium. Another study of ours also detected an increased INF-γ and decreased TNF-α production in the cultured CD38 + NK cells [13]. In that study, we cocultured MNCs with CD38 + NK cells in the presence of TNF-α and an anti-IFN-γ antibody, whereupon the IL-10 + Treg cell proportion significantly increased. When MNCs were cocultured with CD38 + NK cells in the presence of IFN-γ and an anti-TNF-α antibody, the IL-10 + Treg cell proportion sharply decreased [13]. CD38 mediates TNF-α and IFN-γ secretion by regulating Sirtuin 6 (Sirt6) expression in CD38 + NK cells. The two studies of ours suggest that CD38 + NK cells produced low levels of TGF-β and TNF-α and high level of IFN-γ by regulating Sirt6 expression, which supresses Treg cell differentiation via mTOR signaling in CD4 + T cells.

The CD38-blocking antibody daratumumab has been used to treat multiple myeloma [50, 51]. Recent reports indicate that the anti-CD38 antibody consumes plasmablasts in PMNCs of RA patients in a dose-dependent manner in vitro; accordingly, its possible use for RA treatment was proposed [15]. Our study also detected increased proportion CD38 + B cells in RA and CIA. We further found that CD38 antibody treatment stimulates expansion of Treg cells and IL-10 secretion. Probably, CD38 contributes RA pathogenesis via regulating both CD4 + cell and B cell development. These finding supports that anti-CD38 antibody has potential in treating RA.

In conclusion, our study found that CD38 + NK cells were significantly elevated and that CD38 + NKT cells were significantly decreased in RA and CIA, and their ratio was positively correlated with clinical manifestations of the diseases. CD38 + NK cells decreased the proportion of Treg cells and IL-10 levels in MNCs and increased the proportion of Th17 cells and IFN-γ levels. CD38 + NK cells also increased the ratios of Th1/Th2 and Th17/Treg in CD4 + T cells and activated mTOR signaling. The treatment of CD38 antibody as well as CD38 + NKT cells showed the opposite effect on MNCs and CD4 + T cells. The CD38 antibody also stimulated TGF-β expression in CD38 + NK cells. CD38 + NK cells had no effect on Treg cell proliferation. The above results suggest that high level of CD38 + NK as well as the CD38 + NK/CD38 +

NKT ratio in RA reduce TGF-β expression in CD38 + NK cells and activate the mTOR pathway in CD4 + T cells in MNCs, which inhibits the differentiation of CD4 + T cells into Treg cells and enhances Th1/Th2 and Th17/Treg ratios, and finally exacerbates the RA patogenesis. Inhibiting the number of CD38 + NK cells or their function may be a potential diagnostic and therapeutic target for RA.

Abbreviations

cADPR:Cyclic ADP-ribose; cADPRH:Cyclic ADP-ribose hydrolase; CIA:Collagen-induced arthritis; DAS28:Disease Activity Score; IFN-γ:Interferon-γ; IL:Interleukin; MNC:Mononuclear cell; mTOR:mammalian target of rapamycin; NAD+:Nicotinamide adenine dinucleotide; NC:Normal control; NK:Natural killer cells; NKT:natural killer T cells; PBS:Phosphate-buffered saline; PCR:Polymerase chain reaction; PMNC:Peripheral blood mononuclear cell; RA:Rheumatoid arthritis; SMNC:Synovial fluid mononuclear cell;TGF-β:Transforming growth factor-β; TNF-α:Tumor necrosis factor-α; Treg:T regulatory

Declarations

Acknowledgements

This study was supported by the Shandong Provincial Key R & D programs

(2017CXGC1202 and GG201703080038).

Authors' contributions

XTC was the principal investigator, designed the study, supervised

experiments, and wrote the manuscript. HXW and KHF performed

the experiments. KHF provided samples and clinical data. All authors read

and approved the final manuscript.

Funding

This study was supported by the Shandong Provincial Key R & D programs

(2017CXGC1202 and GG201703080038).

Availability of data and materials

All relevant data and materials are included in this published article.

Ethics approval and consent to participate

The study protocol was approved by the Medical Ethics Committee of The

Affiliated Hospital of Qingdao University (Approval number: 20190105,

China). The breeding and care of the experimental animals were carried out

in accordance with the Helsinki Convention on Animal Protection and the

Regulations of the People's Republic of China on the Administration of

Experimental Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Files

Supplementary Table 1. Detailed clinical information on RA patients and healthy volunteers.

Supplementary Table 2. Clinical information on the patients with RA and OA.

Supplementary Figure 1. **Preparation of CD38+ NK cells and CD38+ NKT cells from RA synovial fluids.** (**A**) The purity of CD38+ CD16+ CD56+ cells and CD3- CD16+ CD56+ cells. (**B**) The purity of CD38+ CD16+ CD56+ cells and CD3+ CD16+ CD56+ cells. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

Supplementary Figure 2. Effects of CD38+ NK and CD38+ NKT cells on Treg cell proliferation. (**A**) Illustration of coculture of CD38+ NK cells or CD38+ NKT cells with Treg cells. (**B**) Effects of CD38+ NK and CD38+ NKT cells o- Treg cell proliferation. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

Figures

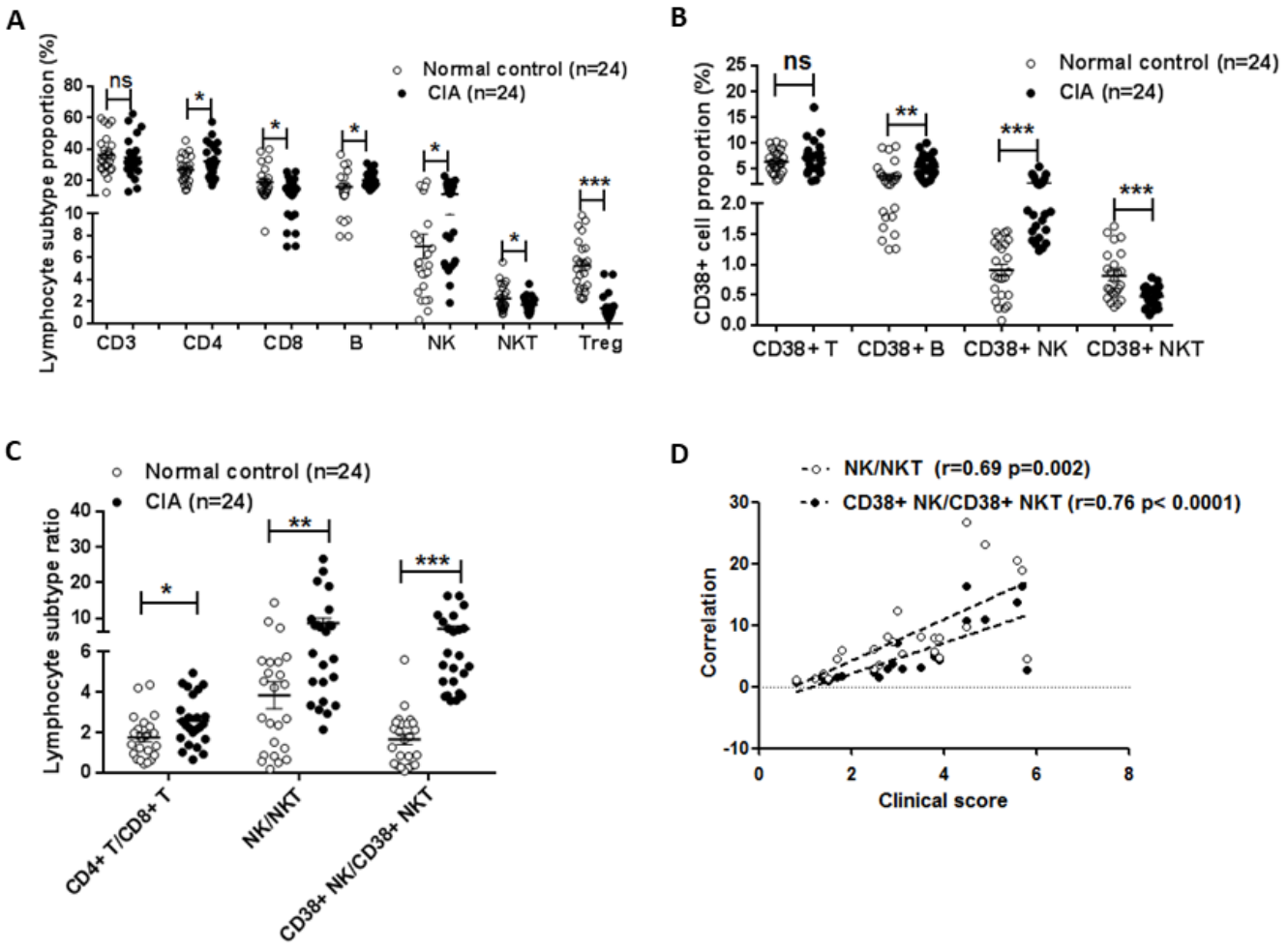

Figure 1

Changes in CD38+ NK and CD38+ NKT cells in peripheral blood lymphocytes of CIA rats. (A) The proportions of lymphocyte subtypes in the peripheral blood of the normal control (n=24) and CIA group (n=24). (B) The proportions of CD38+ cell subtypes in the peripheral blood of the normal control and CIA group. (C) Changes in the ratios of lymphocyte subtypes in the peripheral blood of the normal control and CIA group. (D) Correlation analysis of the CD38+ NK/CD38+ NKT cell ratio in the peripheral blood of the CIA group and disease activity. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

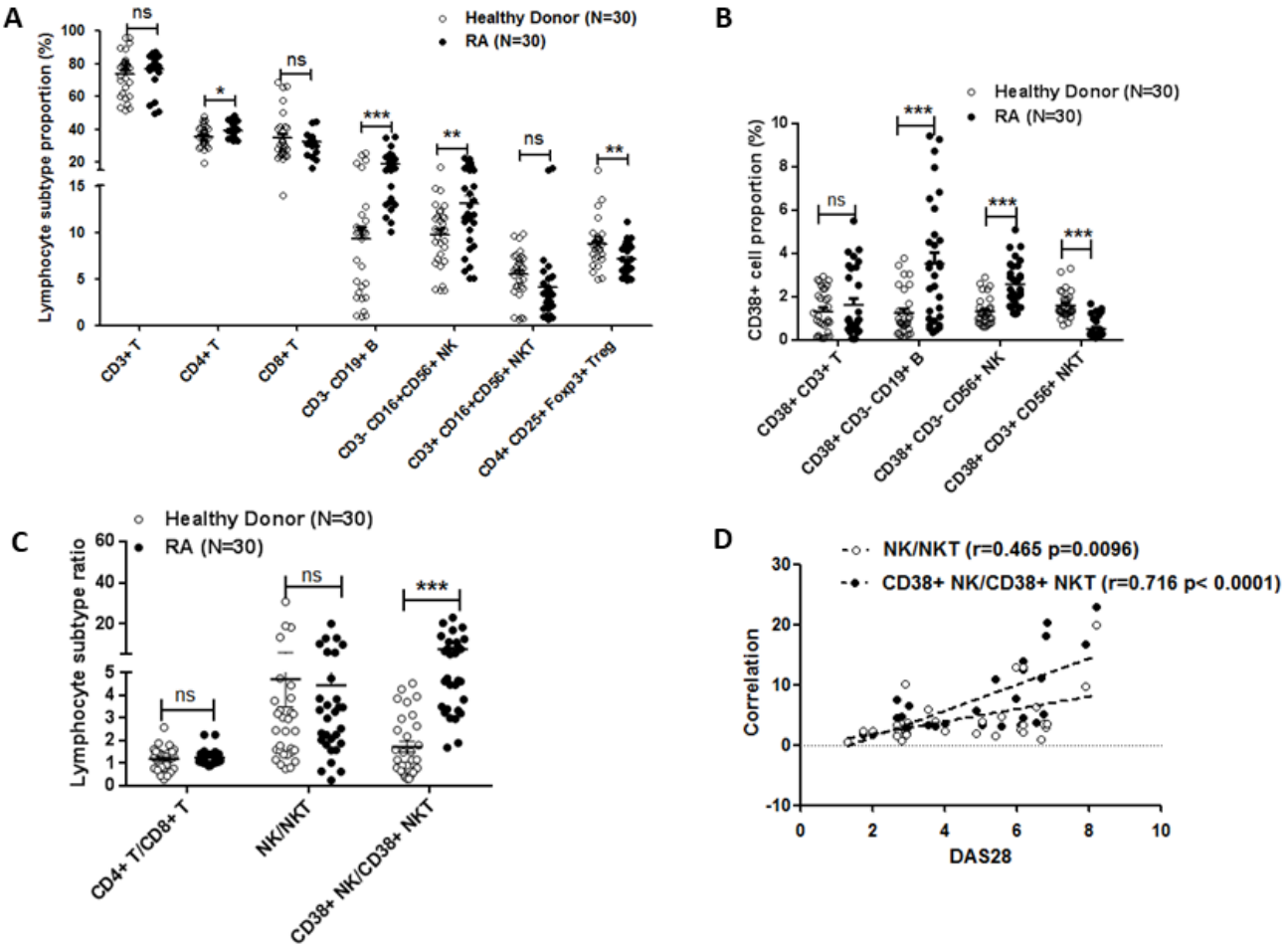

Figure 2

Changes in CD38+ NK and CD38+ NKT cells in peripheral blood lymphocytes of RA patients. (A) The proportions of lymphocyte subtypes in the peripheral blood of healthy individuals (n=30) and RA patients (n=30). (B) The proportions of CD38+ cell subtypes in the peripheral blood of healthy individuals and RA patients. (C) Changes in the ratio of CD38+ NK/CD38+ NKT cells in the peripheral blood of healthy individuals and RA patients. (D) Correlation analysis of the CD38+ NK/CD38+ NKT cell ratio in the peripheral blood of RA patients with DAS28. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

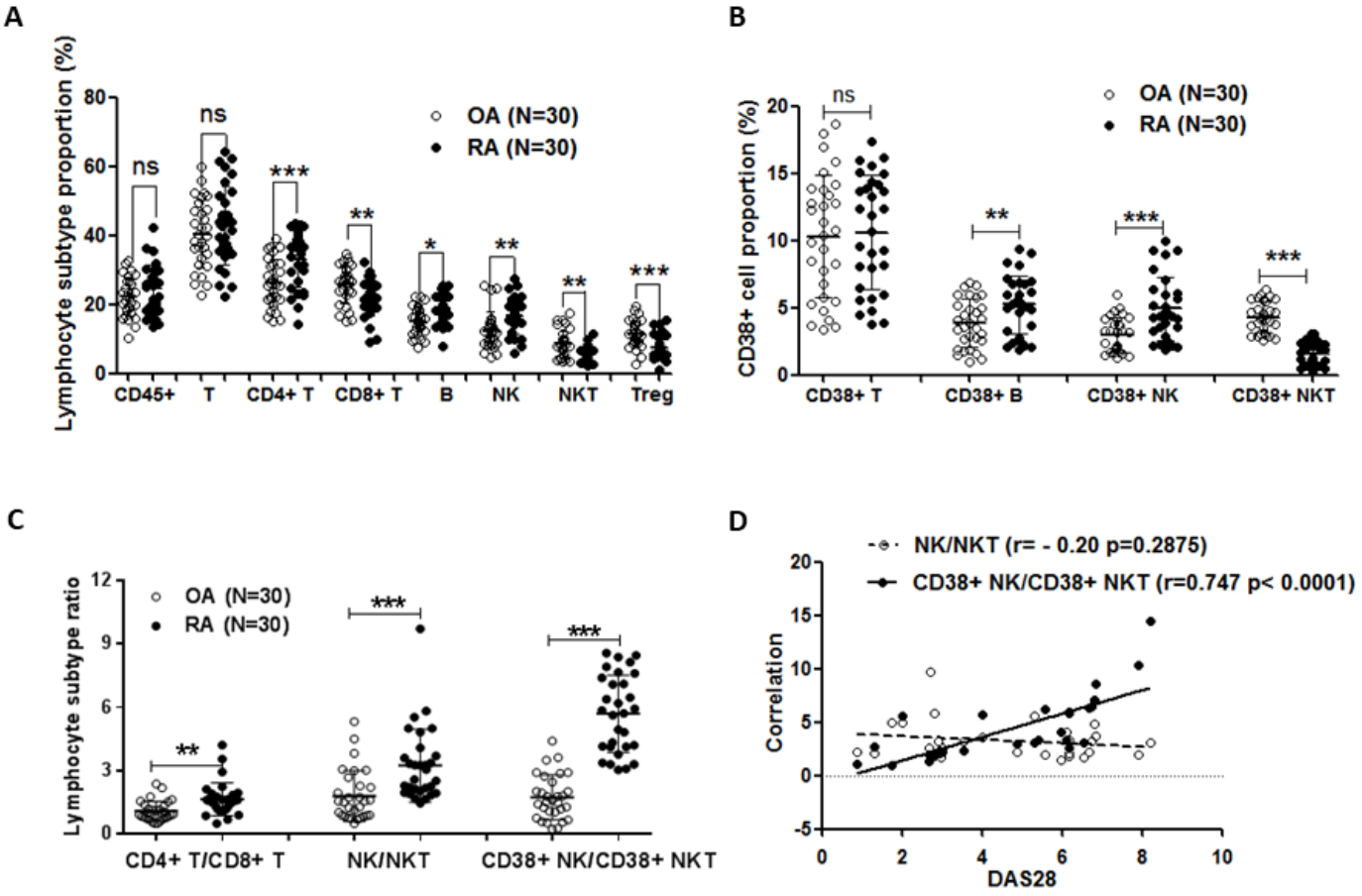

Figure 3

Changes in CD38+ NK and CD38+ NKT cells in synovial fluid lymphocytes of RA patients. (A) The proportions of lymphocyte subtypes in the synovial fluid of OA (n=30) and RA patients (n=30). (B) The proportions of CD38+ cell subtypes in the synovial fluid of OA and RA patients. (C) Changes in the ratios of CD38+ NK/CD38+ NKT cells in the synovial fluid of patients with OA or RA. (D) Correlation analysis of the CD38+ NK/CD38+ NKT cell ratios in the synovial fluid of RA patients with DAS28. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

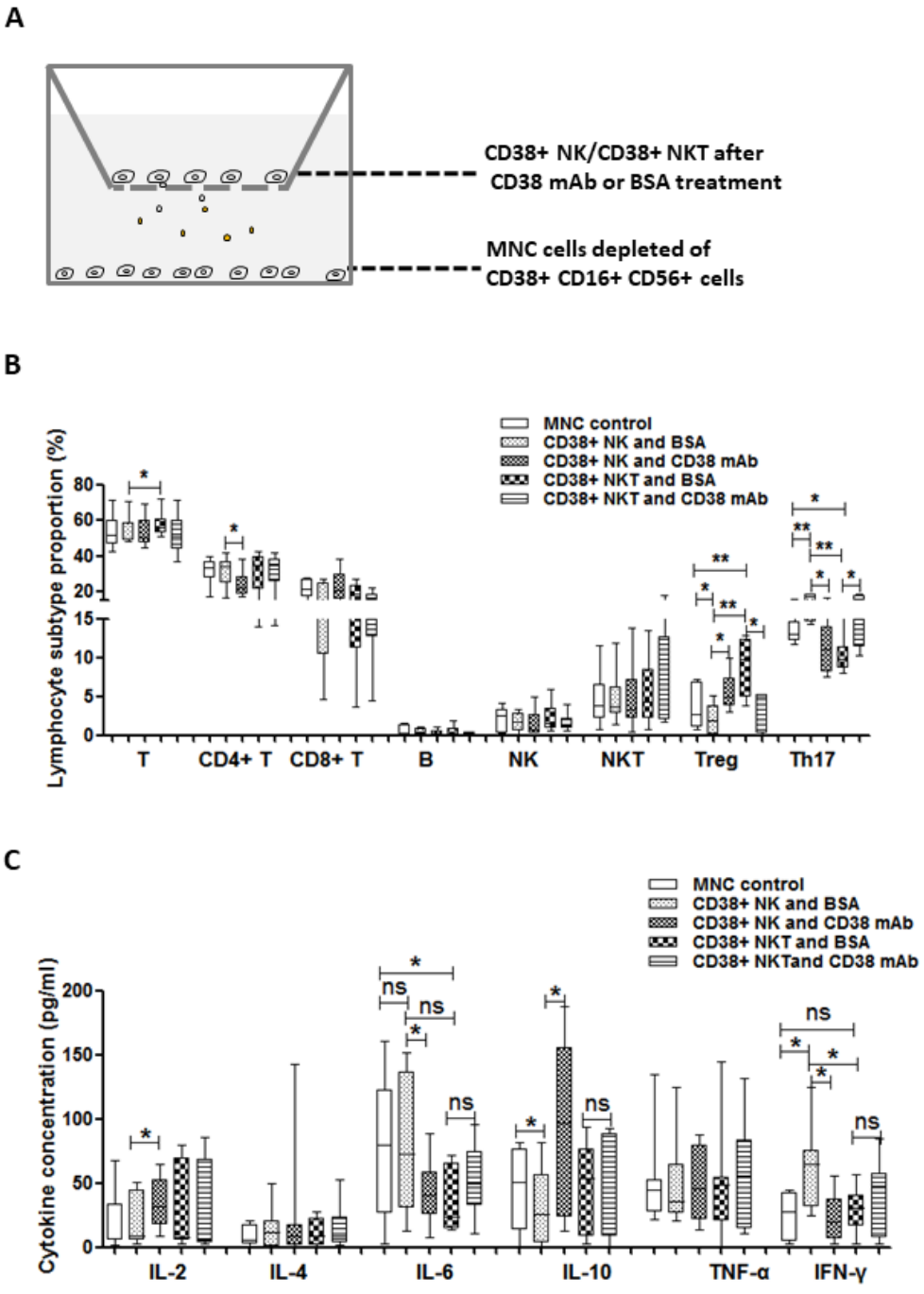

Figure 4

Effect of CD38+ NK and CD38+ NKT cells on lymphocyte subtypes and cytokine secretion in the culture medium. (A) Illustration of coculture of CD38+ NK cells or CD38+ NKT cells with RA synovial fluid MNCs depleted of CD16+ CD56+ CD38+ cells. (B) Effects of CD38+ NK cells and CD38+ NKT cells on lymphocyte subtypes in synovial MNCs. (C) Effects of CD38+ NK cells and CD38+ NKT cells on cytokine

secretion in the coculture medium. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

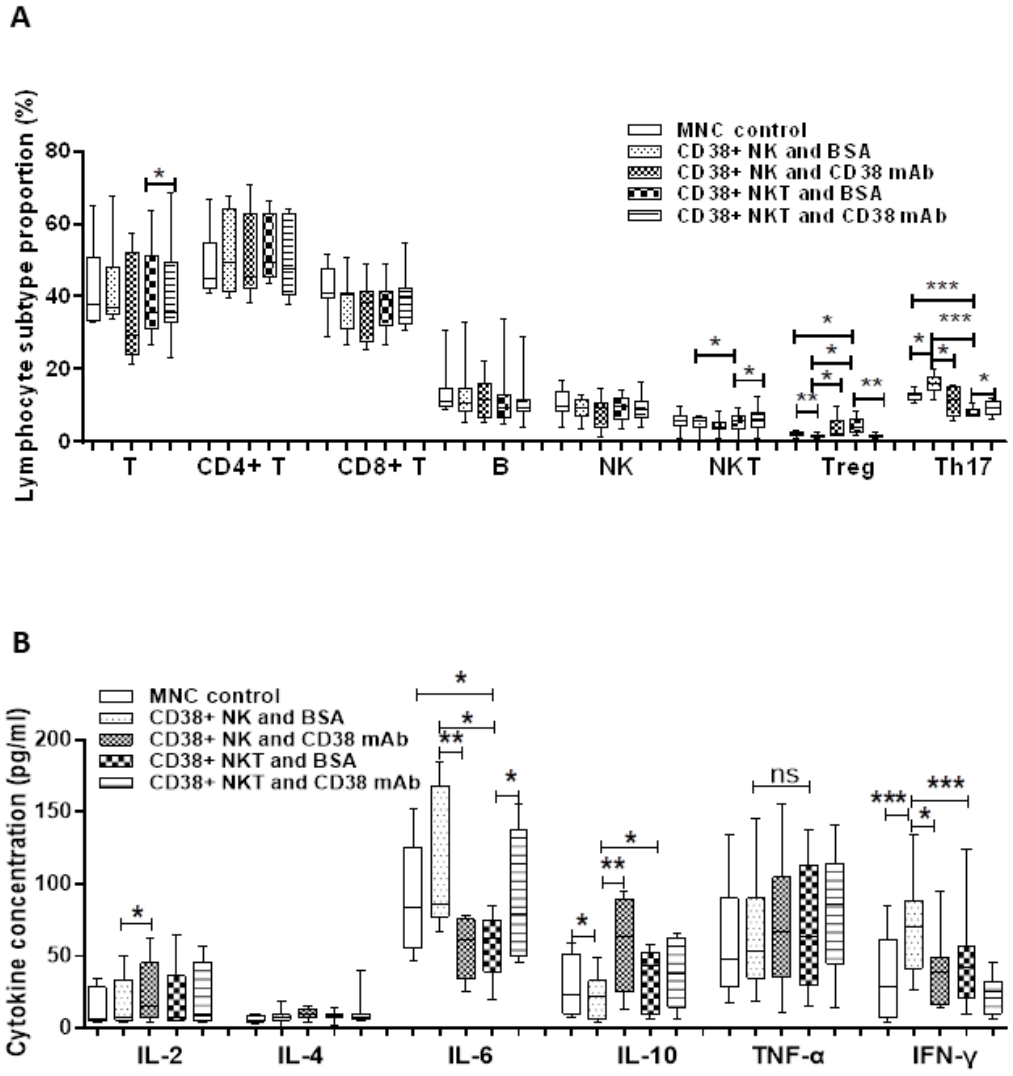

Figure 5

Effect of CD38+ NK and CD38+ NKT cells on lymphocyte subtypes and cytokine secretion in the culture medium. (A) Effects of CD38+ NK cells and CD38+ NKT cells on lymphocyte subtypes in peripheral blood

MNCs. (B) Effects of CD38+ NK cells and CD38+ NKT cells on cytokine secretion in the coculture medium. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

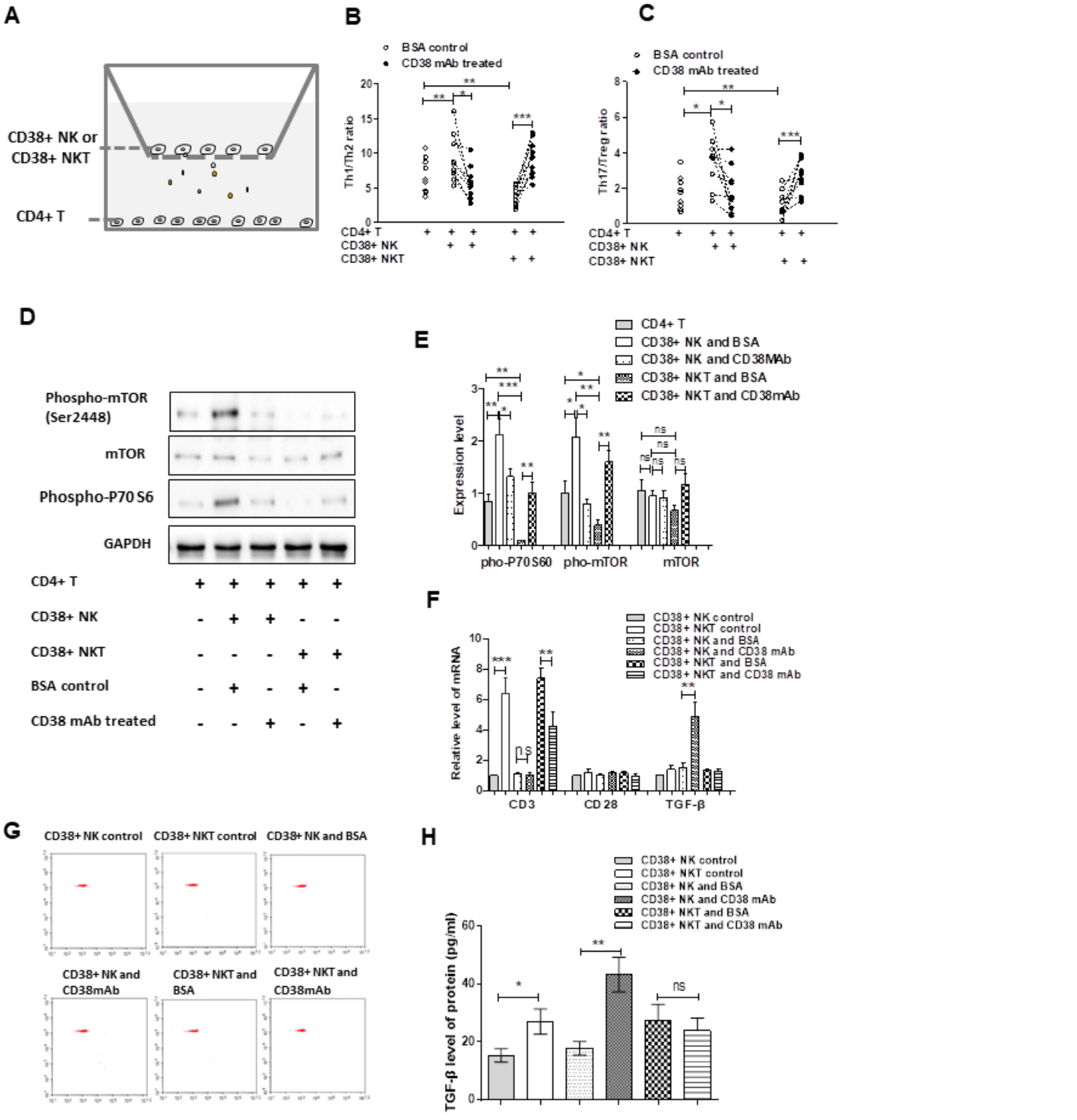

Figure 6

Effect of CD38+ NK and CD38+ NKT cells on CD4+ T cells. (A) Illustration of the coculture of CD38+ NK cells or CD38+ NKT cells with RA synovial fluid-derived CD4+ T cells. (B) Effects of CD38+ NK and CD38+ NKT cells on Th1/ Th2 cell ratio in CD4+ T cells. (C) Effect of CD38+ NK and CD38+ NKT cells on

Th17/Treg cell ratio in CD4+ T cells. (D) Effect of CD38+ NK and CD38+ NKT cells on mTOR signaling in CD4+ T cells using Western blot analysis. (E) Expression levels of phospho-mTOR, mTOR, and phospho-P70 S6 by normalizing densitometry in comparison to their respective GAPDH expression. (F) Expression levels of CD3, CD28 and TGF-β mRNAs in CD38+ NK and CD38+ NKT cells using real-time PCR. (G) Expression of TGF-β protein in culture medium of CD38+ NK and CD38+ NKT cells using flow cytometry analysis. (H) The statistical analysis levels of TGF-β expression. mAb means anti-CD38 antibody. * represents a P value less than 0.05, ** less than 0.01, and *** less than 0.001.

Supplementary Files

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