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Original Article

CD38 positive natural killer cells may be involved in progression of inflammation in rheumatoid arthritis patients

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Abstract: This study is to investigate the role of CD38 positive natural killer (NK) cells in peripheral blood (PB) and synovial fluid (SF) in the pathogenesis of rheumatoid arthritis (RA). Paired samples of PB and synovial fluid (SF) were obtained from patients with RA, osteoarthritis (OA), and uratic arthritis (UA), as well as healthy subjects. Frequency and phenotype of peripheral and synovial NK cells were analyzed by flow cytometry. Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were treated with anti-CD38 mAb, alone or together with IL-2. Intracellular staining of granzyme B and perforin, as well as the levels of pro-inflammatory cytokines (i.e., IL-6 and TNF-α), were analyzed and compared. NK cells in the PB were significantly decreased in RA patients, compared with OA patients and healthy controls. However, significantly increased CD38 expression was noted in the NK cells in PB from RA patients. The percentage of $CD38^+$ NK cells was significantly correlated with the simple disease activity index (SDAI), which was greatly expanded in the SF from patients with inflammatory arthritis. Furthermore, treatment of anti-CD38 mAb significantly enhanced the NK cell cytotoxicity and induced secretion of pro-inflammatory cytokines (IL-6 and TNF-α) in PBMCs and SFMCs of RA. However, compared with PB NK cells, SF NK cells expressed higher levels of granzyme B and perforin, in both the control and stimulation groups. Altered NK cell number and phenotype could influence the maintenance and progression of RA, which involves CD38 as a key element.

Keywords: Rheumatoid arthritis (RA), CD38, natural killer (NK) cells, peripheral blood mononuclear cells (PBMCs), synovial fluid mononuclear cells (SFMCs)

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory process of synovial membrane, which induces the destruction of cartilage and bone. Up to now, very little is known about the triggers for inflammatory cascades in the joints. Evidence suggests that the natural killer (NK) cells, as the frontline defense system, may be crucial in the early pathogenesis of RA [1]. Nielsen *et al.* [2] have found that fibroblast-like synoviocytes express numerous ligands for both the activating and inhibitory receptors on NK cells, and the imbalance between these receptors initiates the inflammation in RA joints. Several studies have shown reduced NK cells and impaired cell activity in the peripheral blood (PB) in RA patients [3-9]. Activated NK cells are expanded in the inflammatory sites, triggering osteoclastogenesis and bone destruction in arthritis [10, 11]. NK cell depletion in the PB has been shown to reduce the arthritis severity and prevent the bone erosion in the very model of collagen induced arthritis (CIA) [10]. NK cells participate in the regulation of autoreactive T and B cells, promoting the production of pro-inflammatory cytokines (such as interleukin-6, IL-6, and tumor necrosis factor α, TNF-α) and contributing to the disease development [12]. Despite of these findings, the role of NK cells in the pathogenesis and development of RA has not yet been fully elucidated.

CD38 is a multifunctional transmembrane glycoprotein expressed on most activated immune cells, including lymphocytes and NK cells [13]. It exerts multiple biological functions in modulating the cell proliferation, differentiation, mig-

Table 1. Clinical and demographic data of patients with RA, OA, and UA

	RA (n=36)	OA (n=29)	UA (n=16)
Age, years	47.3±12.4	55.8±9.2	44.6±15.6
Female, n (%)	29 (80.6%)	18 (62.07%)	0
Duration, m	28.2±25.6	18.2±3.6	17.38±23.18
RF positivity, n (%)	22 (61.1%)	NA	NA
ACPA-positive, n (%)	30 (83.3%)	NA	NA
CRP (mg/l)	19.36±11.78	13.01±7.21	26.14±32.40
ESR (mm/h)	62.07±31.19	37.27±10.19	61.15±17.81
SDAI	47.75±15.45	NA	NA
NSAIDs	34 (94.4%)	8 (27.6%)	9 (56.3%)
DMARDs			
Methotrexate n (%), mg/week	33 (91.7%) 10.21±3.35	NA	NA
Leflunomide n (%), mg/day	20 (55.6%) 11±3.08	NA	NA
Hydroxychloroquine n (%), mg/day	7 (19.4%) 228.57±75.59	NA	NA
Tripterysium Glycosides n (%), mg/day	6 (16.7) 56.25±8.06	NA	NA
Prednisone equivalent n (%), mg/day	9 (25%) 6.17±1.21	NA	NA
Glusosamine sulfate N (%)	NA	8 (27.6%)	NA

Abbreviations: RA, rheumatoid arthritis; OA, osteoarthritis; UA, uratic arthritis; NSAIDs, nonsteroidal anti-inflammatory drugs; DMARDs, disease-modifying antirheumatic drugs; NA, information not available.

ration, and apoptosis [13, 14]. Moreover, CD38 could enhance the chemokine responses, adhesion to integrin substrates, and matrix metalloproteinase activities [15-17]. In recent years, the role of CD38 in the pathogenesis of arthritis has been attracting more and more attention. Several studies have demonstrated that CD38-deficient mice display abnormalities in the leukocyte trafficking, in which attenuates the lymphocyte infiltration and activation in joints, in collagen-induced arthritis [18, 19]. However, its relevance with the modulating effects of innate immune cells (such as NK cells) is still unknown.

In our previous study, a high percentage of CD38 expression in the $CD3^-CD56^+$ cell subset in the PB of RA patients has been documented, which is significantly correlated with the rheumatoid factor (RF) level [20]. Co-expression of CD38 and CD56 suggests the potential role of CD38 in the innate immunity in RA patients. In this study, the functional significance of CD38 in regulating the cellular immune responses was investigated, and the induction of cytolytic mediator on NK cells after CD38 activation was analyzed. The activation status and functional capacity of NK cells in the PB and SF from RA patients were analyzed and compared. Moreover, the relationship between CD38 expression and other inflammatory biomarkers in RA was also investigated.

Materials and methods

Study subjects and sample collection

Totally 81 patients with knee effusions who were admitted to the Outpatient Clinic of Arthritis and Rheumatology were included in this study. In these patients, there were 36 cases of RA, 29 cases of OA, and 16 cases of uraticarthritis (UA). RA was diagnosed according to the 2010 ACR/European League Against Rheumatism (EULAR) criteria [21]. Patient characteristics, including age, sex, diagnosis, disease duration, and disease-modifying antirheumatic drugs (DMARDs), were shown in **Table 1**. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Shandong Provincial Qianfoshan Hospital Affiliated to Shandong University.

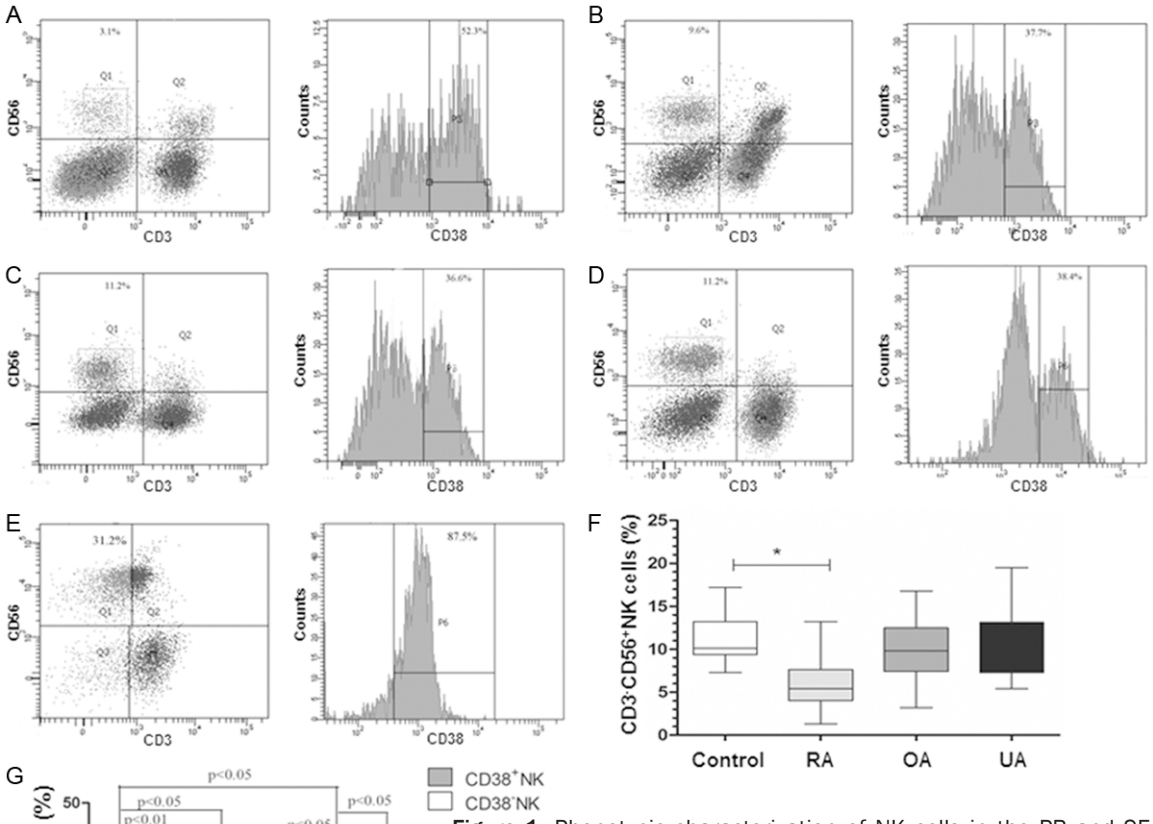

Figure 1. Phenotypic characterization of NK cells in the PB and SF. (A-D) PBMCs were obtained from patients with RA (A), OA (B), and UA (C), as well as healthy controls (D). The expression of CD56, CD3, and CD38 was detected with flow cytometry, and representative pictures were shown. (E) The expression of CD56, CD3, and CD38 in the SFMCs from RA patients was detected with flow cytometry. (F) Percentage of $CD3^-$ $CD56^+$ NK cells out of total lymphocytes in the PB from patients with RA, OA, and UA, as well as healthy controls. (G) Statistical analysis of percentage of NK cells in the PB and SF from patients with RA, OA, and UA. Compared with the control group, $^*P<0.05$.

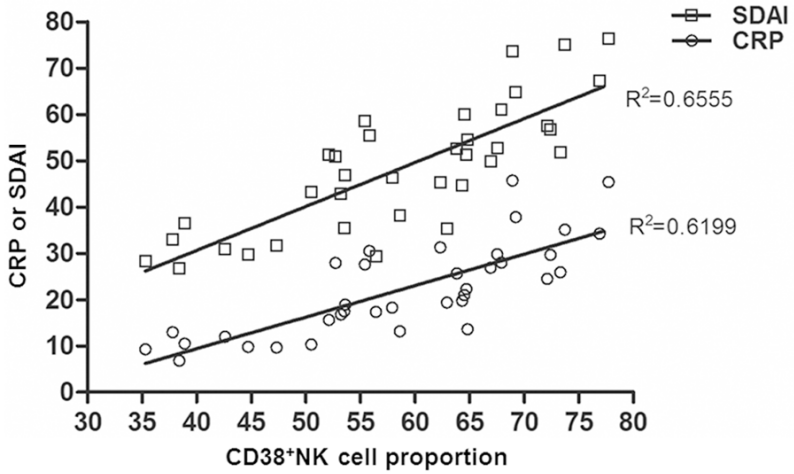

Figure 2. Relationship between the $CD38^+$ NK cell proportion and other biomarkers. Relationship between the proportion of $CD38^+$ NK cells and CRP, as well as SDAI, was analyzed with linear regression analysis.

Paired samples of PB and SF were obtained from each patient. PB samples were also detected from 29 age-matched healthy control subjects from the medical examination center.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were separated using the Ficoll-Hypaque gradient method. After washing with cold fluorescence activated cell

sorter (FACS) flow buffer (Becton Dickinson, San Jose, CA, USA), the cells were re-suspended at the density of 5×10^6 cells/ml, and an aliquot of 200 μl was put into a 10-ml tube. The cells were incubated with appropriate monoclonal antibodies in dark at 4°C for 30 min. The following antibodies were used: anti-CD3 labeled with fluoresceinisothiocyanate (FITC) (Beckman Coulter, Marseille, France), anti-CD56 labeled with PE (Beckman Coulter), anti-CD38 labeled with peridininchlorophyllcyanin5·5 (PerCp-Cy5·5) (Beckman Coulter), anti-CXCR3 with APC (BD Biosciences, Franklin Lakes, NJ, USA), and anti-CCR5 with BV510 (BD Biosciences). The cells were then fixed with polyoxymethylene in PBS. Immunostained samples were analyzed with a FACS Aria TMII flow cytometer (BD Biosciences).

For the detection of granzyme B and perforin, cultured cells were washed twice with PBS supplemented with 2% FCS. Cells were stained for surface markers (i.e., CD3, CD56, and CD38), followed by fixation and permeabilization. Then the cells were stained for mouse anti-granzyme B labeled with Alexa Fluor 647 (BD PharMingen, San Diego, CA, USA) and APC anti-human perforin (Biolegend). The fluorescence was detected with mutiple-color flow cytometry.

Cell isolation, culture, and treatment

Paired samples of PB and SF (at least 20 ml) were obtained from 6 patients with RA. PBMCs and SFMCs were isolated by Lymphoprep gradient centrifugation (Histopaque; Sigma-Aldrich, Poole, UK). After washing, PBMCs or SFMCs were suspended with the RPMI-1640 medium (Gibco Life Technologies, Paisley, UK) containing 10% fetal calf serum (FCS), supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin (HyClone, Logan, Utah, USA). For the stimulation treatment, cells were cultured with the complete medium in the 24-well plate, at the density of 3×10^6 cells/well. Then the cells stimulated with 1 μg/ml anti-CD38 mAb (Biolegend, San Diego, CA, USA), 10 ng/ml IL-2, or anti-CD38 mAb + IL-2 were cultured at 37°C for 24 h.

Enzyme-linked immunosorbent assay (ELISA)

Cells were cultured at 37°C for 24 h. Then the supernatant was collected, and the contents of IL-6 and TNF-α were measured using the ELISA kits (Wuhan Huamei Biotech Co., Ltd., Wuhan, Hubei, China), according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± SD. SPSS17.0 software was used for statistical analysis. After normal distribution test, the independent-sample t-test was performed for comparison between patients and controls. ANOVA was used for the pair-wise comparison. Association analysis was performed with the linear regression model, and the Pearson correlation coefficient was calculated. $P<0.05$ was considered statistically significant.

Results

Proportions and phenotypes of NK cells in PB and SF

NK cells in the PB from the patients with non-infectious arthritis and healthy subjects were first analyzed and compared. Our results showed that, compared with the healthy subjects, no significant distinguishing features were found for OA, and UA (**Figure 1**). For RA, the percentage of $CD3^-CD56^+$ NK cells out of total lymphocytes in the PB was 6.36±2.96% (ranging from 1.3% to 13.2%), which was significantly deceased as compared with the healthy subjects (the percentage of NK cells was 11.27± 2.99%, ranging from 7.3% to 17.2%) ($P<0.01$). On the other hand, CD38, the active marker of immune cells, was expressed in 58.85±11.67% (ranging from 37.8% to 72.8%) of the $CD3^-CD56^+$ NK cells in the PB from RA patients, while CD38 was expressed in 37.59±10.13% (ranging from 25.6% to 61.9%) of the NK cells in the PB from healthy subjects. This phenomenon was not observed for the patients with OA or UA (**Figure 1**).

The proportion and phenotype of NK cells in the PBMCs and SFMCs from the patients were analyzed. Our results showed that, the NK cells were greatly accumulated in the SF as compared to the PB samples (**Figure 1G**). Similar features of synovial NK cells were observed for different arthritis patients. However, the NK cell phenotypes in the SF were strikingly different from those cells in the PB.

The relationship between the $CD38^+$ NK cell proportion and other biomarkers (i.e., C-reactive

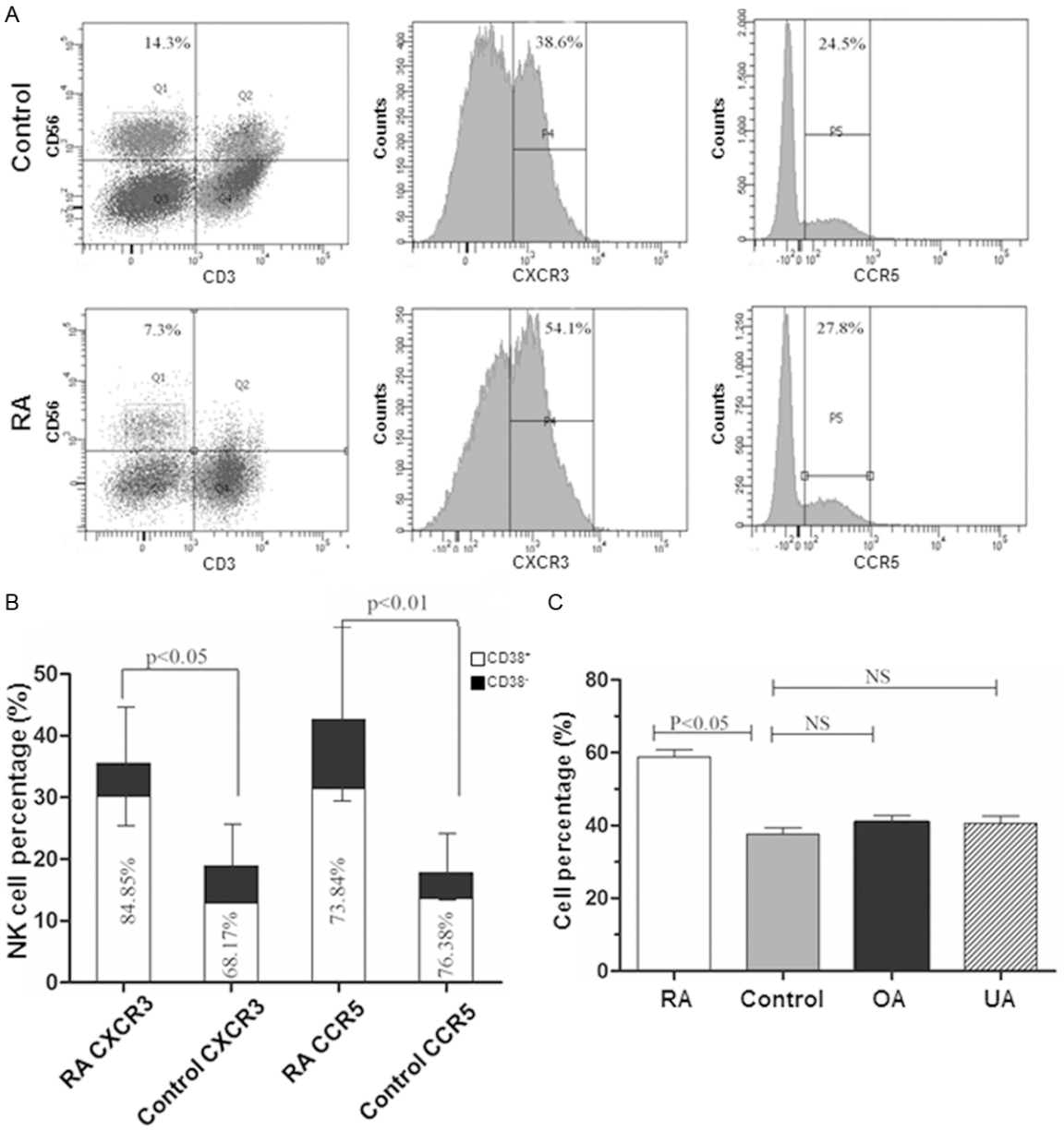

Figure 3. Expression of chemokine receptors in CD38+ and CD38- NK cells. A. PBMCs and SFMCs were obtained from RA patients and healthy controls, and the expression of chemokine receptors (i.e., CXCR3 and CCR5) was detected with flow cytometry. B. Statistical analysis of expression of CXCR3 and CCR5 on CD38+ and CD38- NK cells in the PB from RA patients and healthy controls. C. Statistical analysis of CD38 expression in the SF NK cells from patients with RA, OA, and UA.

protein, CRP, and simple disease activity index, SDAI) was also investigated. Linear regression analysis showed a significant correlation between the expression levels of CD38 and CRP, as well as SDAI (R^2=0.6199 and R^2=0.6555, respectively; P<0.01) (**Figure 2**). Taken together, these results suggest that, the PB or SF NK cells from RA patients display an activated phenotype expressing high level of CD38, while CD38+ NK cells are only assembled in the inflammatory sites in transient arthritis (i.e., OA and UA).

Chemokine receptors in NK cell subsets

Enriched CD38+ NK cells in the SF might reflect a selective recruitment of this kind of NK cells from periphery. Next, the expression of chemokine receptors in different NK cell subsets in the PB from RA patients was investigated. Our

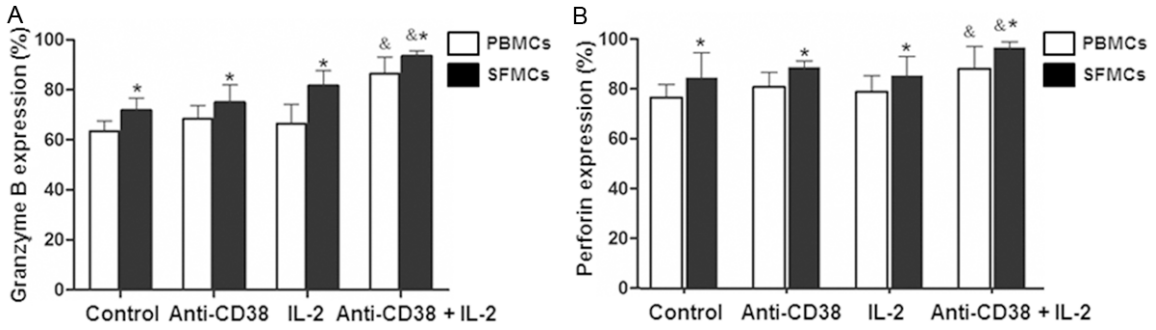

Figure 4. Cytotoxicity assessment of PBMCs and SFMCs. PBMCs and SFMCs were stimulated with anti-CD38 mAb, IL-2, or anti-CD38 mAb +IL-2. The levels of granzyme B (A) and perforin (B) were estimated. Compared with the corresponding PB group, $^{*}P<0.05$; compared with the control group, $^{\&}P<0.05$.

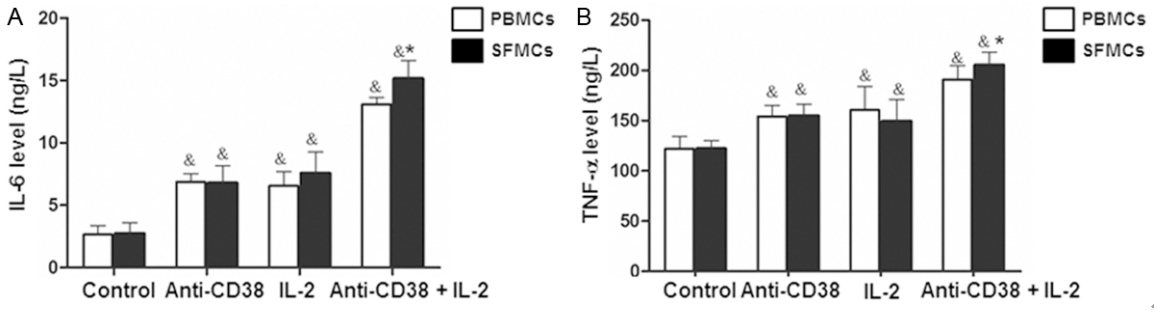

Figure 5. Effect of anti-CD38 mAb on cytokine secretion of PBMCs and SFMCs. PBMCs and SFMCs were stimulated with anti-CD38 mAb, IL-2, or anti-CD38 mAb +IL-2. The levels of IL-6 (A) and TNF-α (B) were estimated with ELISA. Compared with the corresponding PB group, $^{*}P<0.05$; compared with the control group, $^{\&}P<0.05$.

results revealed that significant differences were observed in the expression of CC chemokine receptors (i.e., CXCR3 and CCR5) in the PB NK cells between the RA patients and healthy controls ($P < 0.05$). In addition, $CD38^{+}$ NK cells in the RA patients predominantly expressed CXCR3 and CCR5 (**Figure 3A** and **3B**). For different arthritis types, the expression levels of CD38 in the PB NK cells were extremely high for RA, while comparable expression levels of CD38 in the SF NK cells were observed between different arthritis types (**Figure 3C**). These results represent that $CD38^{+}$ NK cells were preferentially recruited to the inflammatory sites.

Cytotoxicity of NK cells in PBMCs and SFMCs

As *NK cells* represent a relatively *small* fraction *of* mononuclear *cells in the PB,* purified NK cells could not be obtained to examine the activity. Moreover, a previous report has shown that NK cells isolated from either SF or PB alone cannot produce cytokines at detectable levels in the medium [11]. To test whether CD38 mediated the regulation of NK cell cytotoxicity, the PBMCs and SFMCs were stimulated with anti-CD38 mAb and IL-2 alone or together with IL-2, and the levels of intracellular molecules (i.e., granzyme B and perforin) produced by NK cells were estimated. Our results showed that, in the absence of stimulation, moderate levels of granzyme B and perforin were expressed in the $CD3^{-}CD56^{+}$ NK cells (**Figure 4**). When the cells were stimulated with anti-CD38mAb, compared with the control group (without stimulation), slightly increased frequencies of cytolysis-positive NK cells were observed, from either SFMC or PBMC culture ($P>0.05$). Moreover, the treatment of anti-CD38 mAb together with IL-2 resulted in significantly increased frequencies of cytolysis-positive NK cells ($P<0.05$). On the other hand, compared with the PB NK cells, higher expression levels of granzyme B and perforin were detected in the SF NK cells, both in the control and stimulation groups (**Figure 4**). Based on these results, we reason that the SF NK cells have enhanced capacity of cytolysis.

Effect of anti-CD38 mAb on cytokine secretion profile of PBMCs and SFMCs

Dysregulation of cytokine networks in the circulation blood and joints play an essential role in the pathogenesis of RA, which is complex and involves the interaction between various types of cells, including T cells, B cells, and fibroblasts. Paired samples of PBMCs and SFMCs were obtained and cultured from RA patients. Our results showed that, compared with the control group, the secretion levels of pro-inflammatory cytokines (IL-6 and TNF-α) were augmented in the stimulation groups, with the highest secretion levels observed for the combination stimulation (anti-CD38 mAb together with IL-2) group. When stimulated with anti-CD38 mAb alone, no significant difference was observed in the cytokine production between the PBMCs and SFMCs. On the other hand, when stimulated with anti-CD38 mAb together with IL-2, significant differences were observed in the expression levels of IL-6 and TNF-α between the PBMCs and SFMCs (**Figure 5**). Taken together, these results suggest that, the treatment of anti-CD38 mAb, alone or together with IL-2, could lead to de novo production of pro-inflammatory cytokines (i.e., IL-6 and TNF-α) in the mononuclear cellsfrom either PB or SF.

Discussion

In this study, our results showed that, compared with healthy control subjects, markedly reduced frequencies of NK cells were observed in the PB from patients with RA. These findings were in line with many previous reports [17-21]. On the other hand, although the frequency of NK cells was significantly decreased, the proportion of $CD38^+$ NK cells in the PB was interestingly elevated in RA patients, compared with controls and patients with other arthritis. Moreover, positive correlation was demonstrated between the CD38 expression and CRP (a specific marker for inflammation in RA), as well as SDAI. In contrast to the reduced NK cells in the PB, our results showed that, both the total NK cell number and the frequency of $CD38^+$ cells were significantly elevated in the SF from patients with RA and other arthritis, as compared with the PB NK cells. NK cells in the SF expressed high levels of CD38 in the patients with chronic and transient inflammatory arthritis. The similarity between different arthritis types indicated the similar mechanisms underlying the pathogenesis of synovitis. In summary, the vast majority of NK cells in both the PB and SF from RA patients display activated phenotype, as indicated by the high expression of CD38, while in the transient arthritis (including OA and UA), $CD38^+$ NK cells were only assembled in the inflammatory sites.

Recent studies have demonstrated that, the NK cells can experience phenotypical and functional alterations in various microenvironments, such as in the contact with fibroblasts and at the inflammatory sites [22]. Differential expression patterns of CD38 in the NK cells contribute to the detection of abnormal immune cells in inflammation, autoimmunity, and cellular transformation. A recent study has reported that, $CD16^-CD56^+$ NK cells both in the PB and SF may be involved in regulating immune responses, mainly through the CD38-mediated pathway [12]. Our results showed that, the NK cells were activated during inflammatory responses, and the $CD38^+$ NK cells in the SF were accumulated in RA inflammatory joints. CD38 contributes to the leukocyte migration and lead to inflammatory infiltration [19]. Abnormalities in leukocyte trafficking have been reported in CD38-deficient mice, which blocks the leukocyte immigration to the joints in collagen-induced arthritis [18]. Our results demonstrated that, RA patients shared the critical feature of extremely high levels of $CD38^+$ NK cells in the PB and SF, suggesting that CD38 might be involved in NK cell migration from the peripheral tissues to the inflammatory sites. The CXCR3 and CCR5 ligands, including macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES, have been found in the SF [23, 24]. The $CD38^+$ NK cells expressed CXCR3 and CCR5, supporting that this NK cell subsets may be preferentially recruited to the inflammatory joints. CD38 might participate in the migration of blood mononuclear cells through the vascular basement membrane into the rheumatoid synovium.

NK cell cytotoxicity was assessed by measuring the intracellular expression levels of granzyme B and perforin. Our results showed that the NK cell lytic activity was significantly impaired in the PB from RA patients. Although the possibility cannot be excluded that deficient NK cell activity in the PB may be due to the reduced cell number, low expression levels of granzyme B and perforin were found in the PB NK cells,

compared with the SF NK cells. In addition, NK cells in the PB and SF were not associated with the enhanced cytotoxicity in response to CD38 ligation or IL-2 alone, and CD38-mediated lytic function could be observed when IL-2 was involved. IL-2 is probably required for the synthesis of lytic machinery in the NK cells [25]. Another explanation might be that, IL-2 might be essential for the sensitivity of NK cells to the stimuli. It is reported that activation and proliferation of T cells and NK cell induced by CD38 ligation is dependent on IL-2 [26]. Moreover, the survival of NK cells depended on the presence of IL-2. However, the levels of granzyme B and perforin were significantly higher in SFMCs than PBMCs from the same RA patients, either in the presence or absence of stimuli, indicating that NK cells in the SF might be more sensitive to the stimulation with anti-CD38 mAb. Synovial NK cells in the inflammatory synovium presented activated phenotype and enhanced cytotoxicity in RA. Although anti-CD38 mAb only had weak effect on the NK cell cytotoxicity, it may share the same regulator with synovial NK cell cytokine synthesis. In this study, we have demonstrated the functional deficiency of PB NK cells in the patients with RA.

CD38 is not only an adhesion molecule but also a receptor that mediates the production of pro-inflammatory and regulatory cytokines by monocytes [26]. To investigate the relevance of CD38 in the regulation of cellular immune responses, the secretion levels of IL-6 and TNF-α in mononuclear cells stimulated with CD38 were analyzed. De Matos *et al.* [9] have reported that NK cells isolated from either PB or SF cannot produce cytokines at detectable levels. Therefore, in this study, mononuclear cells were used instead of pure NK cells. Our results showed that, stimulation with either anti-CD38 mAb alone, IL-2 alone or anti-CD38 together with IL-2 could significantly increase the secretion levels of IL-6 and TNF-α in both PBMCs and SFMCs. It has been reported that the SF NK cells express higher levels of high-affinity IL-2 receptors, and the significant proportion of SF NK cells could produce TNF-α when stimulated with IL-2 [9]. In the present study, our results indicated significantly elevated secretion level of pro-inflammatory cytokines in SFMCs, compared with PBMCs, when stimulated with anti-CD38 mAb together with IL-2.

In conclusion, our results showed that NK cells modulated the immune responses in RA by expressing various surface molecules. The CD38 signaling pathway was involved in the regulation of IL-6 and TNF-α production in PBMCs and SFMCs, which might contribute to the pathogenesis of RA. Our findings provide evidence for further consideration of CD38 in designing the therapeutic strategies for RA patients in clinic.

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Disclosure of conflict of interest

None.

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