EXTENDED REPORT

Chemotaxis of Vγδ2 T cells to the joints contributes to the pathogenesis of rheumatoid arthritis

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ABSTRACT

Objectives To explore the role of Vγδ2 T cells in the pathogenesis of rheumatoid arthritis (RA).

Methods Sixty-eight patients with RA, 21 patients with osteoarthritis and 21 healthy controls were enrolled in the study. All patients with RA fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism criteria for RA. Peripheral Vγδ2 T population, chemokine receptor expression and proinflammatory cytokine secretion were quantified by flow cytometry. The infiltration of Vγδ2 T cells within the synovium was examined by immunohistochemistry and flow cytometry. The effect of tumour necrosis factor (TNF)-α and interleukin (IL)-6 on Vγδ2 T migration was determined by flow cytometry and transwell migration assay.

Results Peripheral Vγδ2 T cells, but not Vδ1 T cells, were significantly lower in patients with RA, which was negatively correlated with disease activity gauged by Disease Activity Score in 28 joints. Vγδ2 T cells from RA accumulated in the synovium and produced high levels of proinflammatory cytokines including interferon-γ and IL-17. Phenotypically, Vγδ2 T cells from RA showed elevated chemotaxis potential and expressed high levels of chemokine receptors CCR5 and CXCR3, which was driven by increased serum TNF-α through nuclear factor kappa B signalling. In vivo, TNF-α neutralising therapy damagedly downregulated CCR5 and CXCR3 on Vγδ2 T cells and repopulated the peripheral Vγδ2 T cells in patients with RA.

Conclusions High levels of TNF-α promoted CCR5 and CXCR3 expression in Vγδ2 T cells from RA, which potentially infiltrated into the synovium and played crucial roles in the pathogenesis of RA. Targeting Vγδ2 T cells might be a potential approach for RA.

INTRODUCTION

γδ T cells are a subset of T cell with distinctive T cell receptor (TCR), which is composed of one γ chain and one δ chain. γδ T cells mainly accumulate in mucosal tissues such as gut, and consist the minor population of peripheral lymphocytes (2%-5%).1,2 Two major subsets of γδ T cells have been defined, namely Vγ9/Vδ2 and Vδ1. Vγ9/Vδ2 T cells, the major population of peripheral blood γδ T cells, express TCR variable regions Vγ9 and Vδ2 and produce high levels of interferon (IFN)-γ and tumour necrosis factor (TNF)-α. They also participate in host defence against intracellular pathogens and haematological malignancies.3,4 Vδ1 T cells, which differ from Vγ9/Vδ2 T cells, mainly reside in the mucosal-associated lymphoid tissues and consist approximately 10%-30% of peripheral γδ T cells.5 Vδ1 cells have lower cytotoxicity compared with Vγδ2 T cells, have regulatory potential6 and produce a broader set of cytokines, including interleukin (IL)-4 and IL-17.7,8 γδ T cells are implicated in many infectious diseases and tumours. In recent years, growing evidence has implicated γδ T cells in human autoimmune disorders such as diabetes, arthritis and multiple sclerosis. Vδ2-expressing circulatory γδ T cells significantly accumulated in the brains of patients with multiple sclerosis.9 In addition, γδ T cells were found to induce Ig secretion in B cell lines and induce autoantibody production in peripheral B cells from patients with systemic lupus erythematosus.10

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes severe joint destruction and deformity. RA is characterised with serum autoantibodies as well as extensive lymphocytes infiltration in the synovia, including T and B cells. CD4+ T cells play crucial roles in the pathogenesis of RA. However, accumulating evidence suggests that γδ T cells are also involved in RA.11,12 In collagen-induced arthritis, an experimental model of RA, preventive depletion of γδ T cells ameliorated the disease severity in DBA1/J mice.12 In human RA, synovial effusions (SF) and synovial membranes have been found to contain a high number of T cells bearing the γδ TCR.11,13 Their percentages in SFs were between twofold and fourfold higher compared with peripheral blood.

In light of this evidence, we investigated Vδ2 T cells in peripheral blood, SF and synovium from patients with RA and their contribution to the pathogenesis of RA.

METHODS

Patients and controls
This study was approved by the Institutional Review Board of Peking Union Medical College Hospital. Written informed consent was obtained from each participating patient and healthy control (HC). Peripheral blood samples were collected from 15 patients with RA before and after treatment with TNF-α antagonist or IL-6 receptor antagonist.

Detection of phosphorylation
Peripheral blood mononuclear cells (PBMCs) were maintained for 24 hours in RPMI 1640 medium reconstituted with 10% fetal bovine serum.
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containing 0.1% serum. To activate cytokine-induced signaling, PBMCs were treated in RPMI 1640 containing TNF-α (100 ng/mL) for 5–30 min at 37°C. Then the cells were fixed and permeabilised according to the instructions of BD Phosflow Protocol. The treated and untreated cells were stained with antibody for 1 hour at room temperature. Stained cells were acquired

![Figure 1](attachment:image)
Statistics
All data were analysed using SPSS V17.0 software. One-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test was used to compare data displaying a normal distribution and homogeneity of variance. Two-way ANOVA was used to examine the influence of two different categorical independent variables. Student’s t-test was used to compare differences between two groups, and paired t-test was used to compare differences before and after treatment. Correlations were calculated using Spearman correlation analysis.

RESULTS
Peripheral blood γδ T cells were lower in patients with RA and negatively correlated with disease activity
To systematically investigate the roles of γδ T cells in the pathogenesis of RA, we first compared the subpopulations of peripheral γδ T cells in RA, patients with osteoarthritis (OA) and HCs. The results showed a significant decrease of peripheral total γδ T cells in RA (3.45%±0.48% vs HC 8.35±1.00% vs OA 7.21%±0.77%; p<0.01) (figure 1A), which resulted from significant reduction of peripheral Vδ2 T cells (1.80%±0.32% vs HC 5.68±0.70% vs OA 4.75%±0.59%; p<0.01) but not Vδ1 T cells (figure 1B,C). In addition, the percentages of peripheral Vδ2 T cells of RA were negatively correlated with the levels of inflammatory markers, including C reactive protein, erythrocyte sedimentation rate as well as the Disease Activity Score in 28 joints (r=−0.6341, n=42, p<0.01; figure 1D). However, no correlation was observed between peripheral Vδ2 T cells and the titres of rheumatoid factor or anticyclic citrullinated peptide antibodies (figure 1D). Taken together, these results suggested peripheral Vδ2 T cells were closely related to RA, which suggested a role in the pathogenesis of RA.

Vδ2 T cells accumulated in RA synovium and were proinflammatory
We then set out to investigate the mechanisms that led to the lower population of peripheral Vδ2 T cells in RA. We found that the proliferation rate of Vδ2 T cells in RA was comparable with that in OA or HC (RA 90.03±7.81% vs HC 82.53±14.97% vs OA 84.77%±6.51%; p>0.05) (online supplementary figure S1A). Also, the apoptosis rates of Vδ2 T cells in RA, OA and HC did not show any significant difference (RA 0.68±0.22% vs HC 0.88±0.56% vs OA 0.96%±0.37%; p>0.05) (online supplementary figure S1B). Therefore, the peripheral reduction of Vδ2 T cells in RA did not result from abnormal proliferation or apoptosis capacity.

Given the previous observation of accumulated γδ T cells in RA SF, we then examined the infiltration of Vδ2 T cells in the joints of RA. Consistently, we found a significantly higher percentage of Vδ2 T cells in RA SF compared with OA SF (5.29%±0.76% vs 1.25%±0.44%; p<0.05 (figure 2A). In addition, we found a significantly higher infiltration of Vδ2 T cells in RA than in OA synovium when examining the cells from enzyme-digested fresh synovium (1.48%±0.19% vs 0.41±0.08%; p<0.05 (figure 2B), as well as immunohistochemical staining of the synovium (36.00%±3.60% vs 2.33%±0.33%; p<0.05 (figure 2C). These findings suggested that peripheral Vδ2 T cells in RA potentially migrated and accumulated in the synovium.

Similar to natural killer cells, Vδ2 T cells possess highly cytotoxic activity and produce proinflammatory cytokines, including IFN-γ and TNF-α (online supplementary figure S2D,E). We
**Figure 3**  CCR5 and CXCR3 upregulation promoted Vδ2 T cell chemotaxis in RA. (A) Transwell migration assay: freshly isolated peripheral blood mononuclear cells from HC, OA and RA were loaded in the upper chamber, and SFs of OA, RA or medium were loaded in the lower chamber in the transwell invasion model. (B) The percentage and MFI of indicated chemokine receptors on Vδ1/Vδ2 T cells of RA. (C) Comparison of the proportion and MFI of indicated chemokine receptors in Vδ2 T cells from RA, HC and OA. (D) Vδ2 T cell migration assay with RA serum in the presence or absence of neutralising antibodies against CCR5, CXCR3 and CXCR6. (E) The concentration of known ligands of CCR5 and CXCR3 in SF of RA (n=22) and OA (n=10), and serum of RA (n=12) and OA (n=7). Data were pooled from three independent experiments (A,D) or five independent experiments (B,C). Results are expressed as mean±SEM. *p<0.05, **p<0.01 by one-way analysis of variance with Tukey-Kramer post-hoc test (A,C,D,E) and Student’s t-test (B). HC, healthy control; MFI, mean fluorescence intensity; OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial effusion.
found that Vδ2 T cells from RA synovium produced higher levels of IFN-γ (57.43±7.63% vs 22.60±2.26%; p<0.05) (figure 2D) and IL-17 (3.23%±0.30% vs 0.33±0.06%; p<0.05) (figure 2F) compared with Vδ2 T cells from OA synovium, although no significant difference in TNF-α production was observed in Vδ2 T cells between RA and OA (56.77%±10.51% vs 46.57±4.90%; p>0.05) (figure 2E). Similarly, peripheral Vδ2 T cells from RA synthesised more IFN-γ and IL-17 compared with those from OA and HC (online supplementary figure S2A-C). In addition, Vδ2 cells produced more TNF-α and IFN-γ than CD3+ T cells that were depleted of Vδ2 (CD3+Vδ2−) as well as total CD3+ T cells. Moreover, CD3+Vδ2− cells produced approximately 20%–35% less TNF-α and 25%–50% less IFN-γ than total CD3+ cells from both PBMC and synovial fluid of patients with RA (online supplementary figure S2D–E). These data suggest Vδ2 T cells from RA aberrantly secrete high levels of IFN-γ and IL-17, and potentially contribute to the pathogenesis of RA.

Chemotaxis of Vδ2 T cells to synovium was driven by CCR5 and CXCR3
To elucidate the mechanism of Vδ2 T cells accumulation in RA synovium, we performed in vitro transwell assay to examine the capacity of Vδ2 T cells chemotaxis in RA. As expected, we found that RA SF significantly promoted the recruitment of RA but not OA Vδ2 T cells (p<0.01, figure 3A). In contrast, neither RA nor OA SFs promoted the recruitment of RA Vδ1 T cells. These results suggest specific recruitment of Vδ2 T cells to the synovium in RA.

The recruitment of leucocytes to target tissues is regulated by chemokines and corresponding receptors. We performed chemokine receptor expression profile screening, and found the expressions of CCR5, CXCR3 and CXCR6 on Vδ2 T cells were significantly higher compared with those on Vδ1 T cells (p<0.05, figure 3B). Moreover, the expressions of CCR5 and CXCR3 on RA Vδ2 T cells were significantly higher than HC or OA Vδ2 T cells (p<0.05, figure 3C). Furthermore, we applied neutralising antibody assay to verify the effect of these upregulated chemokine receptors on Vδ2 T cells recruitment, and we demonstrated that neutralising CCR5 and CXCR3 completely abrogated the migration capacity of RA Vδ2 T cells (figure 3D). Next, we examined the SF and serum levels of the known ligands of CCR5 (MIP-1α, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES)) and CXCR3 (MIG, IP-10, I-TAC). We found all ligands except RANTES were significantly elevated in RA SF compared with OA SF and serum (p<0.01, figure 3E). Taken together, these data indicated that CCR5 and CXCR3 upregulation on Vδ2 T cells, in combination with high levels of their ligands in SF, work cooperatively to promote the recruitment of Vδ2 T cells to the affected joints in patients with RA.

TNF-α and IL-6 upregulated the expression of CCR5 and CXCR3 on Vδ2 T cells
To explore the triggering factors responsible for CCR5 and CXCR3 upregulation in RA, we cultured HC Vδ2 T cells with RA, OA and HC serum. Not surprisingly, we found the expressions of CCR5 and CXCR3 were significantly increased in the presence of RA serum (p<0.05, figure 4A), which were abrogated by administration of neutralising antibody against TNF-α, but not IL-17 (p<0.05, figure 4B). To support this finding, we demonstrated that TNF-α alone could significantly upregulate the expressions of CCR5 and CXCR3 on HC Vδ2 T cells (p<0.05, figure 4C). In addition, similar phenotypes were observed in IL-6-neutralised RA serum or IL-6-treated HC Vδ2 T cells (online supplementary figure S3). These results taken together suggest that TNF-α and IL-6 potentially play a role in the upregulation of CCR5 and CXCR3 expression on RA Vδ2 T cells.

NF-κB signalling pathway was involved in TNF-α-mediated upregulation of chemokine receptors in RA
TNF-α is a multifunctional cytokine involved in apoptosis, cell survival, inflammation and a variety of immune responses via two distinct receptors:17 TNFR-1 (p60) on all cells, and TNFR-2 (p80) that are mainly expressed on immune cells.18 The major signalling pathway of TNFR-associated factor 2 (TRAF-2) is activation of nuclear factor kappa B (NF-κB) via NF-κB-inducing kinase and inhibitor of nuclear factor kappa B kinase (IKK) complex. In addition, TRAF-2 also phosphorylates mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) to activate c-Fos/c-Jun transcription factors.19 To find out which signalling pathway participates in the upregulation of chemokine receptors on Vδ2 T cells, we detected phosphorylation of NF-κB p65 (p55,29), JNK1/2 (pT183/pY185) and p38 MAPK (pT180/pY182) in TNF-α-treated Vδ2 T cells by Phosflow at different time points. The results showed that NF-κB, but not JNK or MAPK, was phosphorylated on TNF-α stimulation (figure 5A). Furthermore, blocking NF-κB signalling with its specific inhibitor (QNZ) completely abrogated the upregulation of CCR5 and CXCR3 on Vδ2 T cells by TNF-α (figure 5B). Together, these findings indicate that NF-κB signalling was involved in the TNF-α regulated expression of CCR5 and CXCR3 on Vδ2 T cells.

TNF-α antagonist therapy restored Vδ2 T cells in patients with RA in vivo
To investigate whether TNF-α regulated the chemotaxis of Vδ2 T cells in vivo, we examined the peripheral Vδ2 T cells from patients with RA treated with etanercept, a kind of TNF-α receptor fusion protein. We found that the percentage of Vδ2 T cells could be restored in patients with RA after treatment with etanercept (figure 6A). Furthermore, TNF-α antagonist treatment downregulated the expression of CCR5 and CXCR3 on Vδ2 T cells in patients with RA (figure 6B,C). We also detected the effect of IL-6 receptor antagonist on modulating peripheral Vδ2 T cell population and the expression of CCR5 and CXCR3. However, no significant effect was observed (online supplementary figure S4). Taken together, these findings further support the conclusion that TNF-α specifically regulates peripheral Vδ2 T cell trafficking to RA synovium by modulating the expressions of CCR5 and CXCR3 in vivo.

DISCUSSION
All the data in this study collectively suggested the hypothesis that in patients with RA, peripheral Vδ2 T cells potentially infiltrated into the synovium and secreted high levels of proinflammatory cytokines, which contributed to the pathogenesis of RA. Mechanistically, we further showed that elevated level of serum TNF-α in patients with RA induced high expressions of CCR5 and CXCR3 on Vδ2 T cells, which promoted Vδ2 T chemotaxis, and NF-κB signalling pathway was involved in this process. More strikingly, anti-TNF-α therapy restored the peripheral Vδ2 T cells population as well as the expression of CCR5 and CXCR3 in patients with RA.

Previous reports show a higher proportion of γδ T cells in RA SFs and synovium compared with peripheral blood. In our study, a significant lower percentage of peripheral Vδ2 T cells in patients with RA was noted, which might be caused by intensive accumulation into synovial tissues but not abnormal cell apoptosis or proliferation potential. Additionally, both Vδ2 cells from peripheral blood and from SF of patients with RA showed proinflammatory phenotype, which produced higher levels of IFN-γ and IL-17 compared with controls. Consistently, peripheral blood and SF from patients with RA contained heterogeneous γδ T cells dominated with effector memory Vγ9/Vδ2 T cells producing inflammatory cytokines including IFN-γ and IL-17. Animal studies also show that γδ T cells are the major source of IL-17 in joints and their increasing numbers are correlated with disease activity. In collagen-induced arthritis model, preventive depletion of γδ T cells significantly delayed the onset and severity of arthritis. Moreover, depletion of Vγ4+ cells, the counterpart of Vδ2 T cells in human and the major population of γδ T cells in mice, significantly attenuates arthritis severity, incidence of arthritis and anticollagen antibodies production. Taken together, our data suggest Vδ2 T cells are involved in the pathogenesis of RA, and targeting Vδ2 T cells might be a promising approach to treat RA.

We further explored the underlying mechanism of abnormal accumulation of Vδ2 T cells in synovial tissues. Chemokine receptors are expressed in various types of cells and interact with chemokines to promote ‘homing’ of cells to target tissues. By profiling chemokine receptors, we found CXCR3 and CCR5,
translation promotes their production. Collectively, these findings are produced locally by synovial fibroblasts and were essential for their migration to synovial tissues. Additionally, we confirmed that the corresponding agonistic ligands and were essential for their migration to synovial tissues. Additionally, TNF-α expression on Vδ2 T cells via NF-κB signalling, which is an important pathway of many inflammatory process. Mechanically, TNF-α regulates CCR5 and CXCR3 αδ2 T population in patients with dramatically restored the Vδ2 T population in vivo. Elevated level of IL-6 after tocilizumab therapy might account for the attenuated repopulation of Vδ2 T cells.

Figure 5 NF-κB signalling pathway was involved in the expression of CCR5 and CXCR3 on Vδ2 T cells. (A) Vδ2 T cells were treated with TNF-α (100 ng/mL) for indicated time. The cells were permeabilised and stained with antibodies against NF-κB p65 (pS529), JNK1/2 (pT183/pY185) or p38 MAPK (pT180/pY182). The data represent one of three independent experiments. The right panels show bar graphs of MFI of Vδ2 T cells pretreated with QNZ (5 μM) or dimethyl sulfoxide (DMSO) for 1 hour. The solid plots represent isotype controls, and the open plots represent indicated staining. Results are expressed as mean±SEM. *p<0.05, **p<0.01 by paired t-test (A) and one-way analysis of variance with Tukey-Kramer post-hoc test (B). HC, healthy control; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; NF-κB, nuclear factor kappa B; TNF-α, tumour necrosis factor α.


The impact of Vδ2 T cells on bone metabolism remains elusive. We showed Vδ2 T cells of RA produced high levels of IFN-γ and IL-17, especially in synovial tissues. IL-17 and receptor activator for nuclear factor-kappa-B ligand (RANKL) are the major cytokines promoting osteoclast differentiation and activation, which ultimately lead to bone erosion. IL-17 stimulates osteoblasts to produce RANKL, which in turn induces differentiation of osteoclast progenitors into mature osteoclasts. Additionally, IL-17-secreting γδ T cells prime δβ T cells to produce IL-17 and enhance the function of Th17 cells. In contrast, the role of IFN-γ in osteoclast genesis is controversial. Although animal studies report that IFN-γ inhibits osteoclast formation, clinical studies have failed to demonstrate the efficacy of IFN-γ administration in bone loss prevention. The implication of elevated IFN-γ of Vδ2 T cells is yet to be further elucidated.
Moreover, Vδ2 T cells potentially contribute to RA pathogenesis in other ways. The majority of adult Vγ9/Vδ2 T cells express the CD45RO memory phenotype with memory CD45RA-CD27+ subset and effector CD45RA-CD27− subset. The effector memory Vγ9/Vδ2 T cells exhibit phenotypic characteristics of specific antigen-presenting cells, including high human leukocyte antigen DR (HLA-DR) and CD80/86 expression, which promotes B cell activation and polarises adaptive immunity towards a Th1 immune response in patients with RA. Both circulating Vδ2 cells and resident Vδ2T cells produce a variety of cytokines and chemokines including MIP, RANTES and IL-8, inducing macrophage aggregation and activated T lymphocytes migration, which may also be involved in the pathogenesis of RA.

In summary, we demonstrate that Vδ2T cells were lower in peripheral blood and accumulated in the RA joint and secreted increasing amounts of proinflammatory cytokines that are involved in the pathogenesis of RA, which resulted from its upregulation of CCR5 and CXCR3 induced by TNF-α via NF-kB signalling pathway. Elucidation of the roles of Vδ2 T cells in RA advances our knowledge in understanding the complex pathogenic mechanism of RA, and provides an alternative mechanism of biological agents to develop new promising biomarker of therapy, and exploring potential Vδ2 cell-targeted therapy.

**Figure 6** TNF-α antagonist therapy repopulated peripheral Vδ2 T cells and downregulated CCR5 and CXCR3 expressions in patients with RA. Treatment-naïve patients with RA (n=12) were treated with etanercept in combination with methotrexate for 3 months. Flow cytometry was performed for the analysis of (A) the percentage of peripheral Vδ2 T cells and the expressions of (B) CCR5 and (C) CXCR3 on Vδ2 T cells before and after treatment. The solid plots represent isotype control, and the open plots represent Vδ2 T cells staining. *p<0.05, **p<0.01 by paired t-test. RA, rheumatoid arthritis; TNF-α, tumour necrosis factor-α.
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