Cytotoxic T Lymphocytes Expressing GPR56 are Up-regulated in the Peripheral Blood of Patients with Active Rheumatoid Arthritis and Reflect Disease Progression

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\textbf{ABSTRACT}

\textbf{Objective:} This study aims to elucidate the changes in the percentage of GPR56 and/or granzyme B (GZMB) positive cells in rheumatoid arthritis (RA) CD4 and CD8 T lymphocytes, and to explore their clinical value in diagnosing and reflecting the progression of RA.

\textbf{Methods:} The percentages of GPR56 and/or GZMB positive cells were analyzed in peripheral blood (PB) and spleen T cells in a collagen-induced arthritis (CIA) model established in DBA/1 mice. The percentages of GPR56+ and/or GZMB+ cells were further analyzed in PBs from RA patients and healthy controls. Correlation analysis was performed between clinical indicators and GPR56+, GZMB+, and GPR56+ GZMB+ T cells. Receiver operating characteristic (ROC) curves were used to evaluate the value of GPR56 and GZMB in differentiating active and stable remitting RA.

\textbf{Results:} GPR56+ levels were increased in CD4 and CD8 T cells in the PB of CIA mice. The percentages of GPR56+ and GZMB+ cells were increased in both CD4 and CD8 T cell subsets in patients with active RA. GPR56+, GZMB+, and GPR56+ GZMB+ cells were positively correlated with rheumatoid factor and DAS28. ROC analysis revealed that AUCs for GPR56+, GZMB+, and GPR56+ GZMB+ cell percentages to distinguish active RA from stable remission RA were 0.7106, 0.6941, 0.7024, with cut-off values of 16.35, 16.40, 14.80 in CD4 + T cells, and 0.8031, 0.8086, 0.8196 with cut-off values 60.25, 62.15, 40.15 in CD8 + T cells, respectively.

\textbf{Conclusions:} GPR56+ and/or GZMB+ T cells are up-regulated in patients with active RA and reflect their condition. The detection of GPR56 and GZMB is helpful for RA disease assessment.

\textbf{KEYWORDS}

Cytotoxic T lymphocytes; GPR56; granzyme B; rheumatoid arthritis

\textbf{Introduction}

Rheumatoid arthritis (RA) is a common chronic autoimmune disease characterized by progressive joint inflammation and the presence of multiple autoantibodies in serum, resulting in joint and bone damage and dysfunction (Lin et al. 2020; Scott et al. 2010). Although the pathogenesis of RA has not been fully elucidated, many researchers have

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found that genetic and environmental factors favor the production of autoantibodies and the development of inflammation (Buch et al. 2021; Derksen et al. 2017). When a genetically susceptible individual develops inflammation due to viral or bacterial infection or tissue damage, the individual is likely to be predisposed to RA (Lin et al. 2020). The immune balance of individuals with RA is disrupted, resulting in an overactive immune system and less immune suppression (Chen et al. 2019). As immune cells infiltrate and leukocytes migrate into synovial joints, pro-inflammatory cytokines can be secreted, and autoantibodies reactive with citrulline and carbamyl proteins can be produced, thereby interfering with immune tolerance (Lin et al. 2020; O’Neil and Kaplan 2019; Weyand et al. 2017). In addition, Treg cells, which characterize the body’s immunosuppressive capacity and immune tolerance, are attenuated by increased DNA methylation at the Foxp3 locus, which is critical for the maintenance and enhancement of Treg cell function (Ospelt et al. 2017).

In addition to damaged Treg cells, other specific T cell subsets that contribute to the development of RA are also of increasing interest. Cytotoxic T lymphocytes (CTLs), formerly commonly referred to as CD8 T cells, secrete perforin, granzyme A, and granzyme B (GZMB) to perform cytotoxic functions, including damage to cell membranes and induction of apoptosis (Lopez et al. 2013). Reports of CD8 CTLs counts in the peripheral blood of RA patients vary widely due to differences in the progression of RA in different studies, and CD8 CTLs may be depleted in some RA patients because CD8 CTLs are continuously stimulated (Coulthard et al. 2012; Lübbers et al. 2016; Wong and Pamer 2003). However, there is no doubt that CD8 CTLs can induce inflammatory responses by secreting pro-inflammatory cytokines or by activating and recruiting CD4 T cells into inflamed tissues (Carvalheiro et al. 2013). The exhaustion of CD8 T cells is thought to be associated with improved outcomes in autoimmune and inflammatory diseases (McKinney et al. 2015), and the unexhausted CD8 T cell phenotype can be involved in the pathogenesis of RA (Wasén et al. 2017). In recent years, CD4 CTLs with cytolytic ability have also been identified (Namekawa et al. 1998; Soghoian and Streeck 2010). The increased frequency of CD4 CTLs in peripheral blood and synovial fluid of RA patients can promote inflammation and tissue damage in RA by secreting perforin and granzymes (Chemin et al. 2019; Lin et al. 2020; Namekawa et al. 1998). Therefore, granzymes and perforin play essential roles in the development of RA.

As a cytotoxic particle, GZMB induces the apoptotic pathway of downstream cells by co-secreting with perforin, and stimulates CTL proliferation and activity by cleaving its self-proteins (Darrah et al. 2017; Kumar et al. 2015; Niland et al. 2010; Trapani and Smyth 2002). Granzymes and perforin were reported to be co-expressed with GPR56 in T and NK cells (Peng et al. 2011; Tian et al. 2017). In CTL cell-related studies, GPR56 has received more and more attention. As a member of the G protein-coupled receptor superfamily, GPR56 is expressed on cytotoxic cells, and GPR56 expression continues to increase as cytotoxicity matures (Peng et al. 2011). However, there is currently no study of the clinical significance of GPR56 in autoimmune diseases. Therefore, the relevant role of GPR56 in GZMB+ CTL cells of patients with rheumatoid arthritis deserves further study and exploration.
In this study, the CIA model was used to analyze the changes in the proportions of GPR56+ and GZMB+ subsets in T cells in the spleen and peripheral blood. On this basis, we collected peripheral blood samples from RA patients to further study GPR56 and/or GZMB positive proportions in T cells. We also focused on the roles of GPR56 and GZMB in assisting the diagnosis and reflecting the progression of RA.

**Materials and methods**

**Mice and CIA model**

Ten-week-old female DBA/1 mice purchased from Charles River (Charles River Laboratory Animal, Beijing, China) were used in this research. All mice received artificial care, and the research protocol complied with the animal research: in vivo experiment report (ARRIVE) guidelines, and was approved by the Ethics Committee of Peking University People’s Hospital (2018PHC060). Bovine type II collagen (Chondrex, Redmond, WA, USA) was emulsified in equal volumes in Freund’s complete adjuvant (FCA; Sigma-Aldrich, St. Louis, MO). The DBA/1 mice were immunized intracutaneously with 100 μl emulsion (150 μg bovine CII) at the base of the tail. After 21 days, the mice received a second intradermal immunization with 150 μg CII emulsified in Freund’s incomplete adjuvant (FIA; Sigma-Aldrich, St. Louis, MO). CIA mice with disease scores greater than 10 were used in the experiments. Mice were sacrificed 4 weeks after the second immunization, and healthy female DBA/1 mice of the same age were used as controls. Peripheral blood and spleen lymphocytes were extracted, and flow cytometry was used to detect the proportion of cell subsets.

**Patients**

In total, 47 RA patients were enrolled from Peking University People’s Hospital between October 2020 and June 2021. All RA patients were diagnosed according to the American College of Rheumatology criteria (Aletaha et al. 2010). These 47 patients included 26 active RA patients (any joint with active disease or any sign of systemic disease) and 21 patients in stable remission according to the 2014 update of the treat-to-target recommendations (absence of signs and symptoms of significant inflammatory disease activity)(Smolen et al. 2016), and remission lasted for no less than 3 months. And 43 healthy controls (HCS) were recruited from the Physical Examination Center. None of these enrolled individuals had any other autoimmune disease or tumor or infection, and none of these patients had received any glucocorticoid and/or immunosuppressive medication within the last 3 weeks. All studies were conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Peking University People’s Hospital (2018PHB210-01), and informed consent was obtained from each participant.

**Flow cytometry**

Lymphocytes from mouse spleen and peripheral blood were obtained from mouse lymphocyte separation solution (Dakeweii, Shenzhen, China). Peripheral blood mononuclear cells (PBMCs) were acquired by Ficoll density gradient centrifugation using separation
solution (Dakewei, Shenzhen, China). Then these cells were washed twice with PBS, stained with fluorescent antibodies against surface markers including CD4 (mouse: ThermoFisher eBioscience; human: Biolegend), CD8a (mouse: Biolegend; human: BD Biosciences), CD45RA, CCR7 and GPR56 (all from Biolegend; anti-GPR56, clone: CG4, with both human and mouse reactivity) for 30 minutes. After two washes, the Transcription Factor Staining Buffer Kit (TONBO, San Diego, CA) was used for intracellular staining. After fixation and permeabilization, cells were incubated with anti-granzyme B antibody (Biologend) for 30 min. Human peripheral blood T cells were divided into CD45RA+CCR7+ naive (TN), CD45RA-CCR7+ central memory (TCM), CD45RA-CCR7-effector memory (TEM) and CD45RA+CCR7-effector memory re-expressing CD45RA (TEMRA) T cells (Peng et al. 2011; Tian et al. 2017), and GPR56 and GZMB positive percentages in these subsets were analyzed. FACSCanto cytometer (BD Biosciences, San Jose, CA, USA) was used for analysis. Fluorescence minus one (FMO) controls were used in flow cytometry analysis.

Clinical parameters measurement

WBCs and lymphocytes were measured using Sysmex XE-2100 (TOA Medical Electronics, Kobe, Japan). CRP was detected using i-CHROMA (Boditech Med Inc., Chuncheon, Korea). IgG, IgA, IgM and rheumatoid factor (RF) were measured using IMMAGE800 (Beckman Coulter Inc., CA, USA). Anti-cyclic citrullinated peptide antibody (anti-CCP) was determined by indirect immunofluorescence testing using Sprinter XL (Euroimmun, Lubeck, Germany). Disease activity was assessed by the Disease Activity Score in 28 joints, using the CRP level, as previously reported (Vander Cruyssen et al. 2005).

Statistics

GraphPad Prime 5.5 software (GraphPad, La Jolla, CA, USA) was used for statistical analysis. Student’s t test was used to compare levels between two groups and ANOVA with multiple comparisons was used to compare more than two groups. Spearman’s correlation coefficient (r) was used for correlation analysis. Receiver operating characteristic (ROC) curves were used to evaluate the efficiency of cell levels for the diagnosis of active RA. Areas under the curve (AUCs) were calculated, and optimal cut-off values were analyzed according to the maximum Youden index (sensitivity% + specificity% –1). All statistical tests are two-tailed. The p value less than 0.05 was considered statistically significant.

Results

The percentages of GPR56+ cells in peripheral blood T cells of CIA mice were significantly increased

We firstly used the CIA model to study the changes of GPR56+ cell percentages in peripheral blood and spleen in the development of rheumatoid arthritis. Analysis of the peripheral blood and spleen of CIA mice at the peak of the disease showed that the proportion of GPR56+ in peripheral blood CD4+ and CD8+ T cells of CIA mice was
significantly higher than that of healthy controls. Although the GPR56 ratio was also significantly higher in spleen CD8 cells than controls, it was not different in spleen CD4 + T cells (Figure 1a–b). The GZMB+ ratio was up-regulated in CD8 cells in spleen and peripheral blood, but the difference was not significant in CD4 cells (Figure 1a–b). GZMB and GPR56 co-expressing cells showed the same trend as GZMB+ cells. In addition, the proportion of GPR56 + T cells in peripheral blood was significantly higher than that in the spleen. According to the mice experiments, the co-expression level of GPR56 and GZMB in peripheral blood was significantly higher than that in the spleen, by comparing GZMB+ ratios in GPR56+ subsets (Figure 1c). The absolute numbers were also compared and found to be consistent with the trend in the percentage of cells (data not shown).
GPR56+ and GZMB+ percentages in CD4 and CD8 T cells in active RA patients were increased

We further analyzed peripheral blood from 43 healthy controls (HC), 26 active RA patients and 21 RA patients in stable remission, with the age and gender distributions matched. We analyzed GPR56+ and GZMB+ percentages in CD4 and CD8 T cells by flow cytometry. As shown in Figure 2, the proportions and absolute numbers (per liter) of GPR56+, GZMB+, and GPR56+ GZMB+ CD4 T cells in active RA patients showed a significant increase compared with healthy controls, and the percentage of GPR56+ CD4 T cells was also higher in patients with active RA than in patients with RA in stable remission.

In terms of CD8 + T cells, HC and stable remission RA patients had lower levels of GPR56+, GZMB+, and GPR56+ GZMB+ cells than active RA patients, except that absolute numbers of GPR56+ and GZMB+ CD8 + T cells did not differ between HC and patients with active RA.

Naïve T cells were decreased in active RA patients while CD8+ TEMRA cells were increased in stable remission RA patients

We explored the changes in functional T cell subsets among the three groups. CD4 and CD8 T cells were divided into CD45RA+CCR7+ naïve (TN), CD45RA−CCR7+ central memory (TCM), CD45RA−CCR7− effector memory (TEM), and CD45RA+CCR7− effector memory re-expressing CD45RA (TEMRA) T cells (Peng et al. 2011; Tian et al. 2017). The results showed that the percentages of TN in CD4 and CD8 T cells in active RA patients were decreased. In addition, compared with HCs, the proportion of TEMRA in CD8 T cells in RA patients in stable remission was significantly up-regulated, and other comparisons among the three groups were not significantly different (Figure 3).

![Figure 2. Analysis of GPR56 and granzyme B positive percentages in CD4 and CD8 T cells in the peripheral blood of RA patients. GPR56 and granzyme B (GZMB) positive percentages in CD4 and CD8 T cells in the peripheral blood of active RA patients (n = 26), stable remission RA patients (n = 21) and healthy controls (HC, n = 43) were measured through staining for CD4, CD8, GPR56 and GZMB. Representative FACS plots of are shown. Numbers in the figure show the percentage of each quadrant. The percentages and absolute numbers of GPR56+, GZMB+, and GPR56+ GZMB+ cells were analyzed and compared among the three groups. Data are presented as the mean with SD. *p < .05; **p < .01; ***p < .001.](image-url)
Percentages of GPR56+ and GZMB+ cells in functional T cell subsets were up-regulated in active RA patients

We further analyzed GPR56 and GZMB positive percentages in functional T cell subsets in the three groups. In CD4 T cell subsets, the percentages of GPR56+, GZMB+, and GPR56+ GZMB+ cells in TCM, TEM, and TEMRA cells were increased in active RA patients compared with HC and RA patients in stable remission, with no significant difference for TN cells among the three groups. In CD8 T cell subsets, percentages of GPR56+, GZMB+, and GPR56+ GZMB+ cells were significantly higher in TN, TEM, and TEMRA cells in active RA patients than in HC and RA patients in stable remission. GPR56+, GZMB+, and GPR56+ GZMB+ percentages in CD8+ TCM cells were also higher in active RA patients than in stable remission RA patients (Figure 4).

GPR56+ and GZMB+ T cell subsets were positively correlated with RF and DAS28

We next conducted correlation analyses between T cells expressing GPR56 and/or GZMB and clinical indicators related to the progression and severity of RA. Both the numbers and proportions of CD4 and CD8 T cells expressing GPR56 and GZMB in the peripheral blood of RA patients were positively correlated with RF (Table 1). In contrast, only GPR56+, GZMB+, and GPR56+ GZMB+ percentages in CD8+ T cells manifested a significantly positive correlation with anti-CCP (Table 1). However, there was no significant difference

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**Figure 3.** Comparison of the CD4 and CD8 subpopulation levels in peripheral blood T cell subsets between RA patients and healthy controls. PBMCs of 47 RA patients (26 active patients and 21 stable remissions) and 43 healthy controls (HC) were analyzed by CD4, CD8, CD45RA, and CCR7 staining. According to CD45RA and CCR7, human CD4 and CD8 T cells were divided into CD45RA+CCR7+ naive (TN), CD45RA-CCR7+ central memory (TCM), CD45RA-CCR7- effector memory (TEM) and CD45RA+CCR7– effector memory-expressing CD45RA (TEMRA) T cells. Representative FACS plots of CD45RA versus CCR7 in CD4 and CD8 T cells are shown. The mean percentages of each cell subset in the three groups were compared. Results are expressed as mean values of SD. *p < .05; **p < .01.
between T cells expressing GPR56 and/or GZMB and CRP as well as ESR. The DAS28 score was found to be significantly positively correlated with the proportions of GPR56+, GZMB+, GPR56+ GZMB+ in CD4 cells, and positively correlated with the proportions and absolute numbers of GPR56+, GZMB+, GPR56+ GZMB+ in CD8 cells (Table 1).

**Figure 4.** Comparison of GPR56 and granzyme B positive percentages in CD4 and CD8 T cell subsets between different groups. PBMCs from 47 RA patients, including 26 active and 21 in stable remission (SR) and 43 healthy controls (HC) were analyzed through staining for CD4, CD8, CD45RA, CCR7, GZMB, and GPR56. The percentages of GPR56+, GZMB+, and GPR56+ GZMB+ cells in each CD4 and CD8 subpopulation, including CD45RA+CCR7+ naive (TN), CD45RA−CCR7+ central memory (TCM), CD45RA−CCR7− effector memory (TEM), and CD45RA+CCR7− effector memory re-expressing CD45RA (TEMRA), were compared among the three groups. The results are presented as the mean with SD. *p < .05; **p < .01; ***p < .001.
Table 1. Correlation analysis between the level of cell subsets and clinical indicators

|                                | ESR | RF  | anti-CCP | CRP | DA528 |
|--------------------------------|-----|-----|----------|-----|-------|
|                                | r   | P   | r        | P   | r     | P     |
| % of GPR56+ in CD4+ T cells    | -0.005 | 0.974 | 0.333 | 0.022 | 0.071 | 0.637 |
| % of GZMB+ in CD4+ T cells     | -0.009 | 0.953 | 0.316 | 0.030 | 0.053 | 0.724 |
| % of GPR56+GZMB+ in CD4+ T cells | -0.012 | 0.935 | 0.327 | 0.025 | 0.062 | 0.678 |
| # of CD4+GPR56+ cells(x10^5/L) | 0.012 | 0.937 | 0.422 | 0.003 | 0.048 | 0.748 |
| # of CD4+GZMB+ cells(x10^5/L)  | 0.001 | 0.996 | 0.374 | 0.010 | 0.017 | 0.910 |
| # of CD4+GPR56+GZMB+ cells(x10^5/L) | 0.017 | 0.911 | 0.393 | 0.006 | 0.028 | 0.850 |
| % of GPR56+ in CD8+ T cells    | 0.215 | 0.146 | 0.303 | 0.038 | 0.302 | 0.099 |
| % of GZMB+ in CD8+ T cells     | 0.234 | 0.113 | 0.315 | 0.031 | 0.294 | 0.045 |
| % of GPR56+GZMB+ in CD8+ T cells | 0.219 | 0.140 | 0.311 | 0.034 | 0.297 | 0.043 |
| # of CD8+GPR56+ cells(x10^5/L) | 0.257 | 0.081 | 0.362 | 0.012 | 0.179 | 0.229 |
| # of CD8+GZMB+ cells(x10^5/L)  | 0.253 | 0.086 | 0.372 | 0.010 | 0.174 | 0.242 |
| # of CD8+GPR56+GZMB+ cells(x10^5/L) | 0.254 | 0.084 | 0.370 | 0.010 | 0.184 | 0.216 |

Bold represents correlations with p-values less than 0.05. r, correlation coefficient.
The positive percentages of GPR56 and GZMB in CD4 and CD8 T cells can be used to differentiate different stages of RA

Subsequently, we investigated whether GPR56 and GZMB could play a role in the diagnosis of different stages of RA. We assessed the area under the ROC curve (AUC) to analyze the significance of the percentages of GPR56 and/or GZMB positive cells in CD4 and CD8 T cells for RA diagnosis. The results showed that the percentages of GPR56+, GZMB+, and GPR56+ GZMB+ cells had AUCs of 0.7106, 0.6941, and 0.7024 (in CD4+ cells) and 0.8031, 0.8086, and 0.8196 (in CD8+ cells) to differentiate active RA from stable remitting RA, respectively (Figure 5). We next calculated the cut-off values of GPR56 and GZMB sunset percentages to distinguish active from stable remission RA. According to the maximum Youden’s index (sensitivity% + specificity% –1), the optimal cut-off values for T cell subsets expressing GPR56 were 16.35% (in CD4 T cells) and 60.25% (in CD8 T cells); cutoff values for T cell subsets expressing GZMB were 16.40% (in CD4 T cells) and 62.15% (in CD8 T cells); cut-off values for T cell subsets expressing both GPR56 and GZMB were 14.80% (in CD4 T cells) and 40.15% (in CD8 T cells), as shown in Table 2. Accordingly, GPR56 and GZMB are helpful in assisting the diagnosis to differentiate between active RA and stable remission RA.

Discussion

In this study, we comprehensively investigated changes in GPR56+ and GZMB+ subsets in CD4 and CD8 T cells in a mouse model of CIA and RA patients. We found that the percentage of GPR56+ cells was significantly up-regulated in the peripheral blood of CIA mice and patients with active RA. We performed a correlation analysis and found that GPR56 and/or GZMB-positive CD4 and CD8 CTLs were positively associated with clinical indicators of RA. Finally, we demonstrated the clinical value of GPR56 and GZMB in differentiating active and stable remitting RA using ROC curves.

We compared changes in GPR56+ cell subsets in peripheral blood and spleen T cells by the CIA model. We performed this part of the experiment to investigate whether the changes in GPR56 in CIA development are localized to peripheral blood or are widely increased in peripheral immune organs. In patient studies, we could only obtain peripheral blood but not spleen. Our study demonstrated that the percentage of GPR56+ cells in peripheral blood was significantly higher than that in spleen, and that GPR56 was highly expressed in GZMB+ cells only in peripheral blood T cells, suggesting that the situation in peripheral blood and spleen is very different, although CIA mouse spleen GPR56 was also significantly up-regulated in CD8 T cells. The up-regulation of GPR56 in peripheral blood of CIA mice and its characteristic expression in CTL cells suggest that the changes and diagnostic significance of GPR56 in peripheral blood can be explored by detecting GPR56 in peripheral blood of RA patients.

GZMB has been reported to dose-dependently induce chondrocyte apoptosis, an important factor involved in RA progression (Saito et al. 2008). Research on the significance of GPR56 in RA is limited. The level of soluble GPR56 was reported to be up-regulated in RA, and GPR56 could represent the cytotoxic capacity of CTLs (Jacquier et al. 2021; Peng et al. 2011; Tseng et al. 2018). Therefore, we further analyzed the difference in the proportions of GPR56 and GZMB positive cells between RA patients in active and stable remission, and
found that the proportion and count of CD4 and CD8 T cells expressing GPR56 and GZMB were higher in patients with active RA than in HCs and patients in stable remission. It has been suggested that GPR56 and GZMB can support the judgment of RA severity.
Table 2. The optimal cut-off values for cell subset levels to distinguish active RA from stable emission RA.

| CD4 GPR56+(%) | Cutoff | Sensitivity% | Specificity% |
|---------------|--------|--------------|--------------|
| CD8 GPR56+(%) | > 16.35 | 42.31        | 100          |
| CD4 GZMB+(%)  | > 60.25 | 61.54        | 85.71        |
| CD8 GZMB+(%)  | > 16.40 | 46.15        | 100          |
| CD4 GPR56+GZMB+(%) | > 62.15 | 69.23        | 80.95        |
| CD8 GPR56+GZMB+(%) | > 14.80 | 46.15        | 100          |
|                | > 40.15 | 100          | 52.38        |

The cut-off value is calculated from the maximum value of the Youden index.

CD4 and CD8 T cells can be accurately classified into TCM, TN, TEMRA, and TEM by staining with CD45RA and CCR7 (Tian et al. 2017). TN and TCM circulate between blood and lymph and have strong proliferation and self-renewal capacity (Truong et al. 2019). Our results showed that there were lower CD4 and CD8 TN levels in active RA patients than in HC and RA patients in stable remission, suggesting that the proliferation and self-renewable ability were decreased in active RA patients. In addition, studies on TEMRA have mainly focused on CD8 TEMRA (Sathaliyawala et al. 2013), and our findings suggest that RA patients in stable remission have a trend of up-regulation of CD8 TEMRA compared with HCs. Considering that TEMRA and TEM can migrate to peripheral tissues to exert cytotoxic effects (Truong et al. 2019), our findings suggest that CD8 CTLs-mediated tissue damage is involved in the pathogenesis of RA, which is consistent with previous studies (Huseby et al. 2001).

We further measured GPR56+ and GZMB+ percentages in TN, TCM, TEM, and TEMRA cells. The proportions of GPR56+, GZMB+, and GPR56+ GZMB+ cells in the four functional CD4 and CD8 T cell subsets in active RA patients were significantly higher than those in HC or stable remission RA patients. Granzyme, perforin, and interferon production is accompanied by phenotypic switching of TN and TCM for an efficient phenotype, and efficient CD8 CTLs can express more GZMB (Carvalheiro et al. 2013; Wherry and Ahmed 2004). This corroborates with our results that increased GPR56+ and GZMB+ T cell subsets in active RA patients are more cytotoxic and may contribute to RA tissue damage and inflammation.

To clarify whether GPR56 and GZMB are involved in the pathogenesis of RA, the relationship between GPR56/GZMB and clinical indicators reflecting the severity and progression of RA was analyzed. RF, anti-CCP, and DAS28 scores were significantly positively correlated with CD4 and CD8 T cells expressing GPR56 and/or GZMB. RF and anti-CCP are antibodies with key indications and diagnostic significance in RA pathogenesis (Derksen et al. 2017). They are used to diagnose RA and their titers can also help judge the severity and progression of RA (Aletaha et al. 2010; Infantino et al. 2014). Increased soluble GPR56 in serum has been reported to be positively associated with up-regulation of RF and tumor necrosis factor, both of which are predictors of poor disease progression in RA patients (Tseng et al. 2018). Serum levels of GZMB have been reported to be consistent with RF titers (Qiao et al. 2020). Therefore, we hypothesized that GPR56 and GZMB might serve as potential biomarkers to help diagnose RA and reflect the condition of RA.
Considering that GPR56+ and GZMB+ percentages are up-regulated in patients with active RA, we focused on whether GPR56 and GZMB could be useful diagnostic biomarkers for RA. The AUCs of GPR56+, GZMB+ and GPR56+ GZMB+ CD4 and CD8 CTLs were found to be high by ROC curve analysis, ranging from approximately 0.7 to 0.8, suggesting that GPR56 and GZMB can help distinguish active and stable remission RA patients. We initially demonstrated that GPR56 and GZMB can be used to differentiate active RA from stable remitting RA with high specificity (>80%) and relatively slightly lower sensitivity (<70%). This is consistent with previous studies showing higher levels of GZMB in patients with active RA (Qiao et al. 2020).

There are still some shortcomings in our research. Our study has a limited sample size and lacks in-depth molecular studies, so in-depth studies are needed to elucidate the reasons why GPR56 and GZMB are associated with RF and anti-CCP and their exact roles in the occurrence and progression of RA. The difference in the percentage of GPR56+ subsets in the spleen and peripheral blood is also an aspect that needs further analysis, which may involve factors such as differences in the microenvironment and differences in terminal differentiation. As for the pathogenic mechanism of GPR56 up-regulation in the pathogenesis of RA, our present manuscript does not involve them. We believe that it cannot be considered that the up-regulation of GZMB and GPR56 can lead to RA, and it may be that the occurrence of RA indirectly leads to the up-regulation of GZMB and GPR56. To elucidate this issue, a series of in-depth mechanistic experiments using animal models, including in vivo adoptive transfer and gene knockout models, will be carried out in the future.

In conclusion, our study demonstrates for the first time that GPR56+ and/or GZMB + CD4 and CD8 CTL cells are up-regulated in the peripheral blood of patients with active RA and can reflect autoimmune status. Detection of GPR56 and/or GZMB levels in CD4 and CD8 CTL cells is helpful in diagnosing disease progression of RA patients.

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**Contributors**

CL designed the research and drafted the manuscript. XZ and MZ carried out most of the experiments. TL, AB and SS performed some of the cellular and molecular experiments. RK and XA did part of the analysis work. SL and YS did part of the clinical measurements. All authors read and approved the final manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).
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**Data availability statement**

The data associated with this paper was available from the corresponding author upon reasonable request.

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