Molecular Remission at T cell level in Patients with Rheumatoid Arthritis

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Abstract

Background. While numerous disease-modifying anti-rheumatic drugs (DMARDs) has brought about a dramatic paradigm shift in the management of rheumatoid arthritis (RA), unmet needs remain such as small proportion of achievement drug-free patients. The aim of this study is to explore key molecules for remission at T cell level, which is known to involve the pathogenesis of RA deeply, and investigate disease course of patients who achieved molecular remission (MR).

Methods. We enrolled a total of 46 patients with RA and 10 healthy controls (HCs). We performed gene expression profiling and selected remission signature genes in $CD4^+$ T cells and $CD8^+$ T cells of patients with RA using machine learning methods. In addition, we investigated the benefit of achieving MR on disease control thereafter.

Results. We identified 9 and 23 genes that were associated with clinical remission in $CD4^+$ and $CD8^+$ T cells. Principal component analysis (PCA) demonstrated expression profiling of them was similar with HCs. As to remission signature genes in $CD4^+$ T cells, PCA result was reproduced using validation cohort, indicating their robustness. There was a trend towards better disease control during 12 months follow-up in patients treated with tocilizumab in deep MR than those in non-deep MR, although it was not significant.

Conclusion. We identified robust genes which represented remission status in $CD4^+$ T cells using machine learning techniques. The current study will promote our understandings about molecular mechanism to achieve deep remission in the management of RA.

Trial registration: Not required.

Background

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial tissue [1]. The advent of disease-modifying anti-rheumatic drugs (DMARDs) has brought about a dramatic paradigm shift in the management of RA. Currently, the goal for RA treatment is to achieve clinical remission (CR), which has been facilitated by the development of various types of biological agents. However, unmet needs in the management of RA remain in not a small proportion of patients with remission. More than half of patients with CR experience a flare following DMARD cessation [2,3]. Moreover, the existence of patients with difficult-to-treat RA [4], who ultimately become resistant to multi-types of DMARDs, raises requirement of understanding about molecular status of remission.

Thus, molecular remission (MR) has been proposed for distinguishing patients with “deep” remission from others [5,6]. Proteome study demonstrated that a low multi-biomarker disease activity score, which they developed, was associated with limited radiographic progression over the following 12 months [5]. We recently investigated molecular signatures that were associated with deep remission at multi-omics level [6]. In the previous report, drug treatments altered the molecular profile closer to that of healthy

controls (HCs) at the transcriptome, serum proteome, and immunophenotype level. In addition, a longitudinal monitoring suggests that the achievement of MR by DMARDs was associated with long-term stable CR. However, as to transcriptome, how each remission signature molecule links with clinical trait was unclear because we used whole blood specimen and expression profile was various according to cell subsets, indicating necessity of further study using each cell subpopulation [6].

It is well known that T cells contribute to the pathogenesis of RA. Susceptible genes for RA outside the MHC locus are highly expressed in $CD4^+$ T cells [7,8]. The evidence that subset of $CD8^+$ T cells is also critical contributor for development of RA is accumulating. $CD8^+$ T cells are required for development of ectopic germinal center in synovium, which is considered to be the home of core immune response in RA [9]. Recently, to investigate clarify the comprehensive characteristics of T cells in RA, we conducted multi-dimensional, immunophenotyping analysis according to developmental stage: $CD4^+$ T cells were classified into four subsets (naïve (TN), stem cell memory (TSCM), central memory (TCM) and effector memory (TEM)), whereas $CD8^+$ T cells were classified into five stages (TN, TSCM, TCM, TEM and CD45RA-positive effector memory (TEMRA)). The study demonstrated that $CD8^+$ TEMRA increased in patients with RA compared with HCs, and TEM-follicular helper (Tfh) cells and TEM-T helper 17(Th17) cells were correlated with disease activity, suggesting T cells in patients with remission may represent MR state of RA [10].

Here, we report key molecules for remission at T cell level, and investigate disease course of patients who achieved MR.

Methods

Patients and control subjects

The current study utilized cohorts from our previous report [10]. The detailed information was described previously. Briefly, there were 2 cohorts in this study (cohort 6 in Supplementary Table S9 and cohort 7 in Supplementary Table S10 of the previous report): cross-sectional gene expression profiling was performed in derivation cohort, and longitudinal gene expression profiling was performed at pre-treatment and post-treatment in validation cohort (Figure 1). Patients in validation cohort were treated by tocilizumab (TCZ) for 6 months and achieved remission after treatment. Peripheral blood samples and synovial fluid (SF) samples were collected before treatment from patients with drug-naïve RA and during treatment from other patients, respectively. We separated each T cell subpopulation from specimens, and RNA was isolated. Then, we conducted RNA-sequencing and performed bioinformatic analysis. Read count normalization was performed using the transcripts per million (TPM) method [13]. Disease activity was assessed by standard composite indices, such as DAS28-ESR (disease activity score 28-ESR) and DAS28-CRP, and remission was defined as a DAS28-CRP < 2.4 [14].

Development of RA remission signature models

To select genes that had a strong relationship with the remission, we applied least absolute shrinkage and selection operator (lasso) to normalized gene expression data of patients with drug-naïve RA and treatment in derivation cohort. Lasso is one of machine learning methods, and suitable for detecting key variables without prior feature selection from multi-variate dataset that contains only a few covariates which are associated with the outcome, improving the prediction accuracy and interpretability of regression models [15]. To avoid overfitting, we first divided gene expression dataset of derivation cohort to training data and test data (with ratio = 7:3), and conducted lasso using training data with seven-fold cross-validation. Then, a partial least-squares regression (PLS-R) was utilized to weight values to selected genes and construct a model that was useful for separating remission and non-remission patients. Variable importance in projection (VIP) score obtained by PLS-R is a significant measurement of each predictor variable. Genes with VIP score with greater than 1 was considered to be related with remission [16]. Thus, we can evaluate the importance of each gene. Receiver operating characteristics (ROC) analysis was used to evaluate the prediction accuracy in test data by statistical model generated from training set. Then, the statistical model was applied to whole set data to enumerate “remission odds” for each sample. The caret and ropls R package were used for lasso and PLS-R modeling, respectively [17].

Principal component analysis and pathway mapping

Principal component analysis was performed using normalized data with the FactoMineR R package [18]. To gain a functional annotation of selected genes, Enrichr’s plugin [19] KEGG pathways [20] was used.

Statistics

Continuous data were presented as the median and interquartile range or as a number with a percentage value, as appropriate. The Wilcoxon rank sum test was used to examine the differences between continuous variables. Fisher’s exact test was used to compare proportions in categorical data between groups. All statistical analyses were performed with R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Remission signature genes in RA

The primary objective is to explore genes that are relevant to remission status in RA (so called, remission signature genes). Therefore, we first selected genes that were useful for separating patients with remission and those with non-remission using training dataset of derivation cohort, with dividing for $CD4^+$ and $CD8^+$ T cells. By lasso, of whole 15304 transcripts, 17 and 46 genes were selected as important molecules in $CD4^+$ and $CD8^+$ T cells for classifying patients with remission or non-remission, respectively.

Then, we weighted genes selected by lasso and construct statistical model that separate remission and non-remission (so called, RA remission signature model) by applying PLS-R. As a result, ROC analysis by applying test set that wasn’t used for selection separated them with good accuracy (area under the curve

[AUC], 0.947 and 0.929 for $CD4^+$ and $CD8^+$ T cells, respectively) (Figure 2A). This result indicated that the combination of lasso and PLS-R captured genes that were informative in our data. In addition, 9 (e.g., *MST1, ASB2, SULT2B1* and *SOCS3*) and 23 (e.g., *CRLF2, NIM1* and *ID1*) genes were passed through criteria (VIP > 1) in $CD4^+$ and $CD8^+$ T cells, respectively (Figure 2B and 2C and Supplementary Table S1). Hereafter, we refer to these genes as remission signature genes. To understand the function of remission signature genes, pathway analysis was performed (Figure 2B and 2C). In $CD4^+$ T cells, molecules involved in various metabolic pathways (*Vitamin B6 metabolism* and *Glycine, serine and threonine metabolism*), endocrine pathways (*steroid hormone biosynthesis, adipocytokine signaling pathway, prolactin signaling pathway* and *insulin resistance*) and *TNF signaling pathway* were enriched. As to $CD8^+$ T cells, as well, molecules involved in metabolic pathways (*taurine and hypotaurine metabolism* and *fatty acid degradation*) and *JAK-STAT signaling pathway* were enriched.

Remission odds of T cell subpopulation

To investigate the effects of different DMARDs and difference of T cell subpopulation (TN, TCM, TEM and TEMRA) on remission signature genes, we compared remission odds according to each subgroup. Using RA remission signature model generated by the combination of lasso and PLS-R, remission odds of each subject were produced: if patients were in remission status, remission odds toward 1 (>0.5), and if patients were far apart from remission, remission odds toward 0 (<0.5). Since T cells in SF are considered to reflect pathological status, we calculated remission odds of T cell subpopulation in SF from some patients.

In $CD4^+$ T cells, remission odds of patients with DMARDs, all of whom were in remission except one patient (Figure 1), were significantly higher compared with drug-naïve patients (Figure 3A). Of note, although there was no significance due to limited samples, SF samples had trend toward low remission odds as well as drug-naïve samples from peripheral blood, suggesting remission signature genes might represent pathogenic status of RA. Conversely, in comparison with HCs, they were similar values without difference of type of drugs, indicating all drugs pushed pathogenic gene expression profile of remission signature genes back to healthy state. Correspondingly, principal component analysis (PCA) using remission signature genes in $CD4^+$ T cells demonstrated only drug-naïve samples made different cluster from others except naïve subset (Figure 3B). To validate classification ability of remission signature genes, PCA analysis was conducted using expression data in validation cohort. Along with earlier results, samples from patients in remission created cluster apart from those in non-remission, supporting robustness of remission signature genes of $CD4^+$ T cells (Figure 3C).

Regarding to $CD8^+$ T cell subpopulations, like $CD4^+$ T cells, all of remission odds of patients with DMARDs were significantly higher compared with those of drug-naïve (Figure 4A). However, remission odds of some samples in groups of DMARDs were also significantly higher than those of HCs, suggesting selected genes in $CD8^+$ T cells might not represent healthy state correctly. In addition, PCA demonstrated all clusters overlapped except TEM subpopulation (Figure 4B). Further, PCA using

validation cohort also couldn't show validity of selected genes, indicating vulnerable ability of remission signature genes in $CD8^+$ T cells (Figure 4C).

Relations between molecular remission and following disease activity

To elucidate the benefits of MR, we next addressed whether there were any differences between patients in "deep" MR and non-deep MR. To achieve this goal, we conducted a follow-up study of consecutive 29 patients (MTX, n=10; IFX, n=10; TCZ, n=9) treated with DMARDs in derivation cohort for up to 12 months after the time point at measurement of gene expression. We defined MR of each cell subset as remission odds greater than average value of remission odds in each cell subset, and deep MR of each patient as the number of cell subsets in MR greater than 4 (maximum 7). Of the 29 patients treated with DMARDs, 12 and 17 patients were classified as deep MR and non-deep MR. Disease activity did not have a statistically significant difference at any time point (Figure 5A). Cumulative DAS28-ESR (described as AUC), however, of patients treated with TCZ in deep MR had a trend lower than those with TCZ in non-deep MR (12.48 [11.25-13.82] vs 18.26 [17.07-18.36], p = 0.19) (Figure 5B). In comparison among drugs, the difference was significant between patients treated with TCZ and those with MTX in deep MR. Although we conducted sensitivity analysis by changing outcome (e.g., DAS28-CRP and each component of DAS28) and definition of deep MR (e.g., the cut-off number of cell subpopulation, limited to $CD4^+$ T cell subpopulations), we couldn't find significant benefit of deep MR in our data (data not shown).

Discussion

In the current study, we identified remission signature genes of RA in $CD4^+$ and $CD8^+$ T cells. Although those in $CD8^+$ T cells were vulnerable when applying to other cohort, those in $CD4^+$ T cells had robust ability to classify remission and non-remission patients in both cross-sectional and longitudinal cohorts. Further, remission odds calculated by RA remission model of $CD4^+$ T cells showed similar value among patients in remission and HCs, suggesting selected 9 genes represent whether $CD4^+$ T cells of RA is pushed back to healthy state or not. In addition, deep MR by TCZ had the potential to be associated with better disease control thereafter.

As shown in previous reports, obviously T cells were involved in the development and chronicity of RA [7-10]. Compared with $CD4^+$ T cells, evidence of a role for $CD8^+$ T cells in RA are emerging [9]. Clonal expansion was observed for CD8+ T cells but not for CD4+ T cells in newly diagnosed patients with RA, indicating requirement of them for initial phase of RA [21]. However, RA risk alleles were preferentially expressed in $CD4^+$ T cells, not $CD8^+$ T cells [7,8], and proportion of only $CD4^+$ T cell subpopulations were positively associated with disease activity [10], suggesting $CD4^+$ T cells more deeply contribute to the activity state of RA than $CD8^+$ T cells.

Remission signature genes identified in $CD4^+$ T cells were downregulated in patients with remission. *MST1* promotes migration of T cells via activating LFA-1 [22,23]. In addition, *MST1* deficient T cells prone to apoptosis [24]. *ASB2* is known to promote NF-κB activation, leading suppression of apoptosis of T

cells [25]. *SULT2B1* is involved in cholesterol homeostasis and expressed in activated T cells, prompting proliferation via inhibition of LXR signaling [26]. As to *SOCS3*, since it inhibits STAT3 that is downstream molecule of JAK-STAT pathway which is inhibited by TNFα- and IL-6- inhibitor [27], downregulation of *SOCS3* may be assumed to lead to activation of inflammatory pathway [28]. However, indeed, loss of *SOCS3* in $CD4^+$ T cells promotes anti-inflammatory cytokines, such as interleukin 10 and transforming growth factor-beta 1, and suppresses inflammatory responses [29], suggesting decrease in expression of *SOCS3* itself has benefit to control RA. Regarding to other 5 genes identified as remission signature genes in $CD4^+$ T cells (*ABHD11, KCNK1, PSAT1, CYB5D2* and *CABP4*), function in T cells is unknown and further functional study is needed to clarify its significance in RA.

However, we couldn't show the significant benefit of achieving deep MR defined by remission signature genes on clinical course, while those treated with TCZ in deep MR had a favorable trend. To date, although several studied tried to predict following change of disease activity using only transcriptome data, they failed to show robust predictability [6,30]. In our data, most patients even in non-deep MR we could follow were under good control during follow-up as shown in Figure 5A, which might make hard to detect statistical significance. In addition, definition of "deep" MR in the current study lacks of evidence. Therefore, to explore impact of MR on clinical trait and association between MR and type of drugs, we need to plan prospective study with larger sample size.

This study suffers from several limitations. First, we didn't examine association between functional subpopulations and MR. We reported previously that Tfh and Th17 cells in TEM were correlated with disease activity, suggesting various extent of contribution of T cells to disease status according to their function [10]. Second, another benefit of MR is not considered in the current study, such as lower risk of flare. Because concept of MR in RA is emerging recently, we need to validity of definition and effect of MR on real-world patients in future research.

Conclusions

We identified robust remission signature genes in $CD4^+$ T cells. The current study will highlight the utility of transcriptome in $CD4^+$ T cells for classifying remission and non-remission in RA, and promote development of novel therapeutic target against RA.

Declarations

Ethics approval and consent to participate

Ethics approval was obtained from the Institutional Review Board of Keio University School of Medicine. Consent to participate was obtained from all subjects in the current study before blood specimen was collected.

Consent for publication

Not applicable.

Availability of data and materials

Transcriptome data are available at the GEO database. The accession codes are GSE113156 and GSE118829. All custom computer codes in the generation or processing of the described data are available upon reasonable request.

Competing interests

YO, KK, YK, MT and RK are employees of Takeda Pharmaceutical Company Limited. KS has received research grants from Eisai, Bristol-Myers Squibb, Kissei Pharmaceutical, and Daiichi Sankyo, and speaking fees from Abbie Japan, Astellas Pharma, Bristol-Myers Squibb, Chugai Pharmaceutical, Eisai, Fuji Film Limited, Janssen Pharmaceutical, Kissei Pharmaceutical, Mitsubishi Tanabe Pharmaceutical, Pfizer Japan, Shionogi, Takeda Pharmaceutical, and UCB Japan, consulting fees from Abbie, and Pfizer Japan. AY has received speaking fees from Chugai Pharmaceutical, Mitsubishi Tanabe Pharmaceutical, Pfizer Japan, Ono Pharmaceutical, Maruho, and Novartis, and consulting fees from GSK Japan. TT has received research grants from Astellas Pharma Inc, Bristol-Myers KK, Chugai Pharmaceutical Co. Ltd., Daiichi Sankyo Co. Ltd, Takeda Pharmaceutical Co. Ltd, Teijin Pharma Ltd, AbbVie GK, Asahikasei Pharma Corp, Mitsubishi Tanabe Pharma Co, Pfizer Japan Inc, and Taisho Toyama Pharmaceutical Co. Ltd, Eisai Co. Ltd, AYUMI Pharmaceutical Corporation, and Nipponkayaku Co. Ltd, and speaking fees from AbbVie GK, Bristol-Myers KK, Chugai Pharmaceutical Co. Ltd, Mitsubishi Tanabe Pharma Co, Pfizer Japan Inc, and Astellas Pharma Inc, and Diaichi Sankyo Co. Ltd, and consultant fees from Astra Zeneca KK, Eli Lilly Japan KK, Novartis Pharma KK, Mitsubishi Tanabe Pharma Co, Abbivie GK, Nipponkayaku Co. Ltd, Janssen Pharmaceutical KK, Astellas Pharma Inc, and Taiho Pharmaceutical Co. Ltd. JI, MT, YK and RM declare no potential conflict of interest.

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Authors' Contributions

Conceptualization: JI, KS and MT. Funding acquisition: AY and TT. Data acquisition: MT, YK, KK, YK, MT and RK. Formal analysis: JI and YO. Supervision: KS. Writing and original draft preparation: JI. Writing review and editing: KS and TT.

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Abbreviations

area under the curve (AUC); CD45RA-positive effector memory T cells (TEMRA); central memory T cells (TCM) ; clinical remission (CR); disease activity score-28 ESR (DAS28-CRP); disease activity score-28 ESR (DAS28-ESR); disease-modifying anti-rheumatic drugs (DMARDs); effector memory T cells (TEM); healthy controls (HCs); infliximab (IFX); least absolute shrinkage and selection operator (lasso); methotrexate (MTX); molecular remission (MR); naïve T cells (TN); partial least-squares regression (PLS-R) ; principal component analysis (PCA); Receiver operating characteristics (ROC); rheumatoid arthritis (RA); stem cell memory T cells (TSCM); synovial fluid (SF) ; T helper 17 cell (Th17); T-follicular helper cells (Tfh) ; tocilizumab (TCZ) ; transcripts per million (TPM) ; Variable importance in projection (VIP).

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Figures

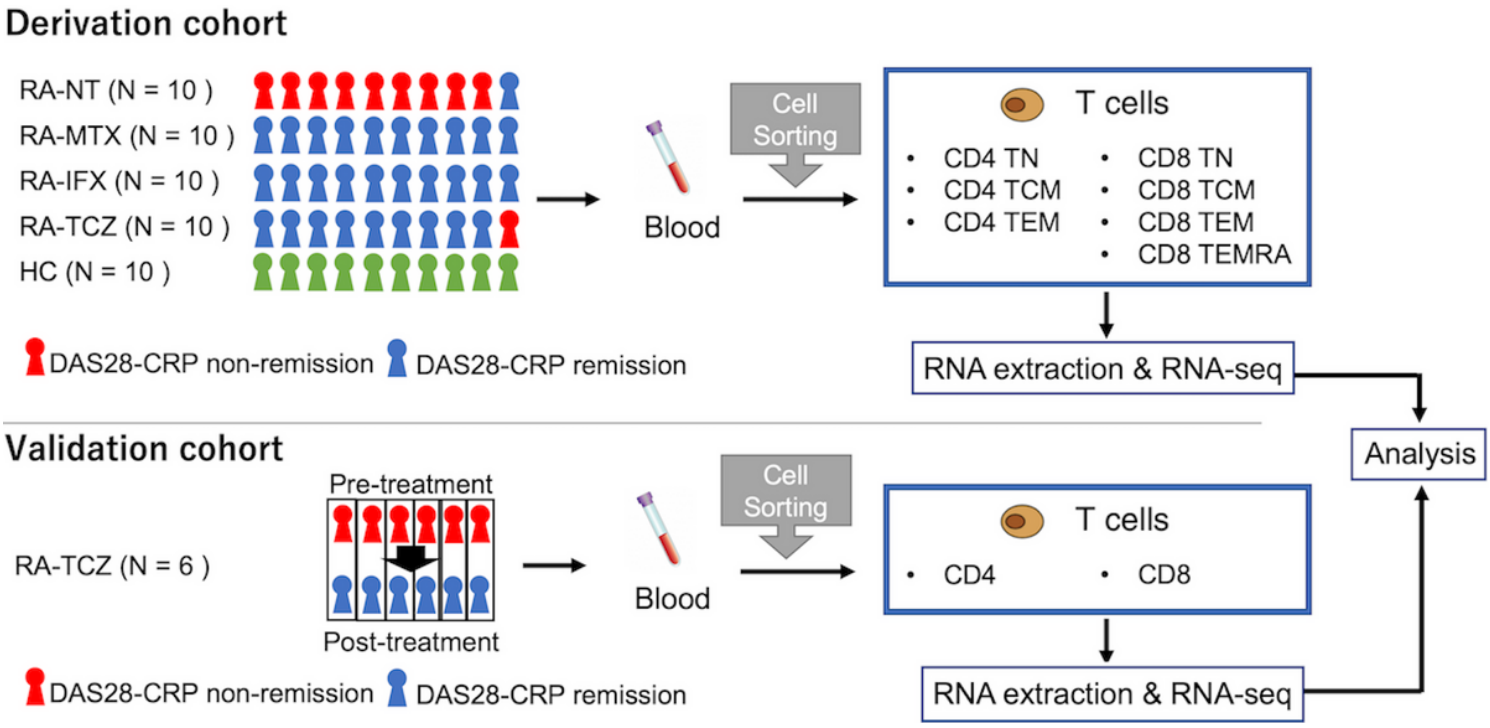

Figure 1

Work flow of the current study. We used 2 cohorts: derivation cohort is cross-sectional subjects, and validation cohorts is longitudinal subjects treated by TCZ. In derivation cohort, we measured gene expression according to cell subpopulation and summarized to whole CD4+ T cells and CD8+ T cells in expression analysis. HC, healthy control; IFX, infliximab; MTX, methotrexate; RA, rheumatoid arthritis; NT, non-treatment; TCM, central memory T cell; TCZ, tocilizumab; TEM, effector memory T cell; TEMRA, CD45RA-positive effector memory T cell; TN, naïve T cell.

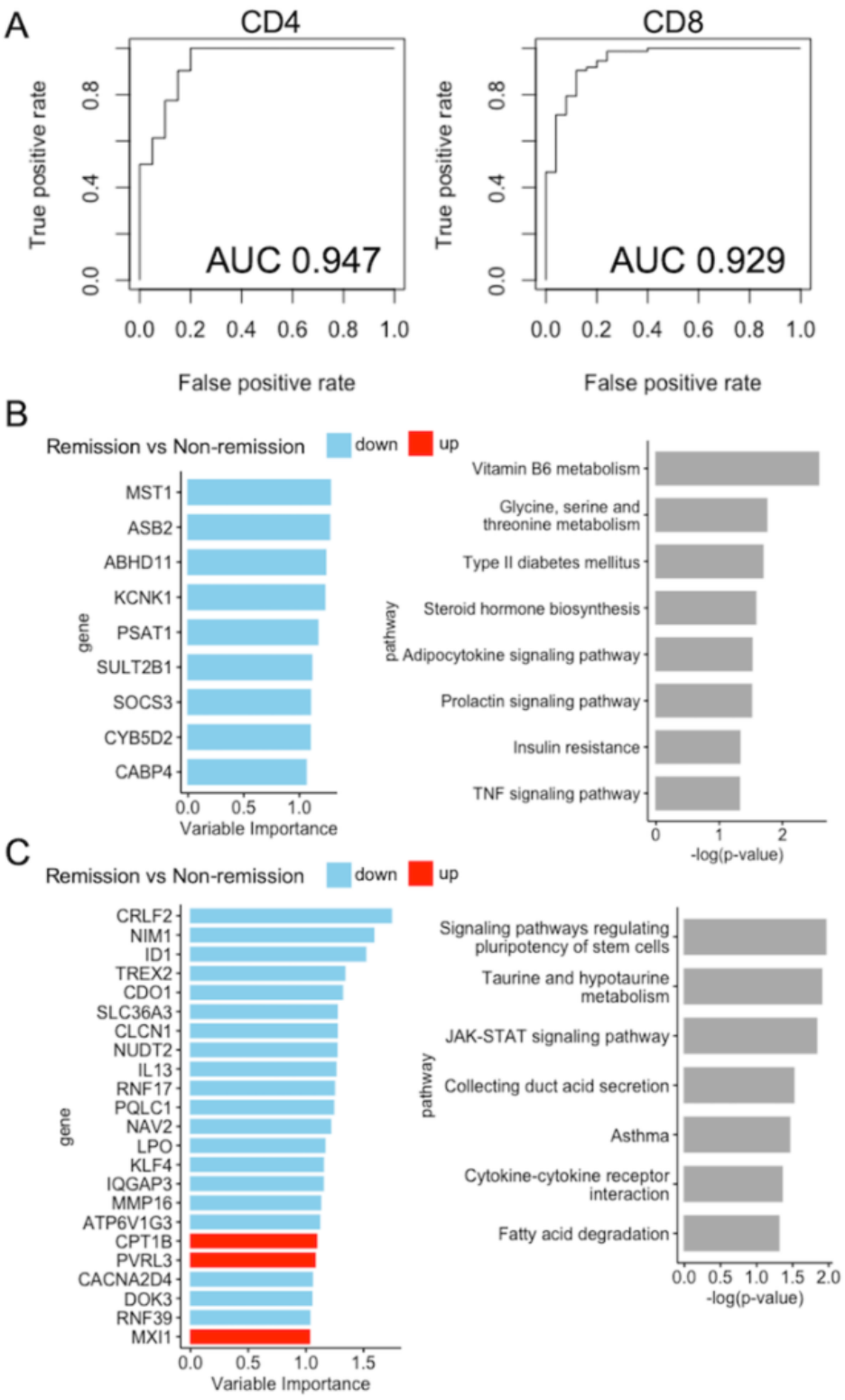

Figure 2

Identification of molecular signatures associated with remission in RA. A) ROC analysis by the statistical model generated from the combination of lasso and PLS-R. B, C) Remission signature genes (left) and enrichment analysis (right) of CD4+ T cells and CD8+ T cells (B). By PLS-R, expression of genes with blue bar towards low and red bar towards high in remission patients, respectively. AUC, area under the curve; PLS-R, a partial least-squares regression; RA, rheumatoid arthritis; ROC, receiver operating characteristic.

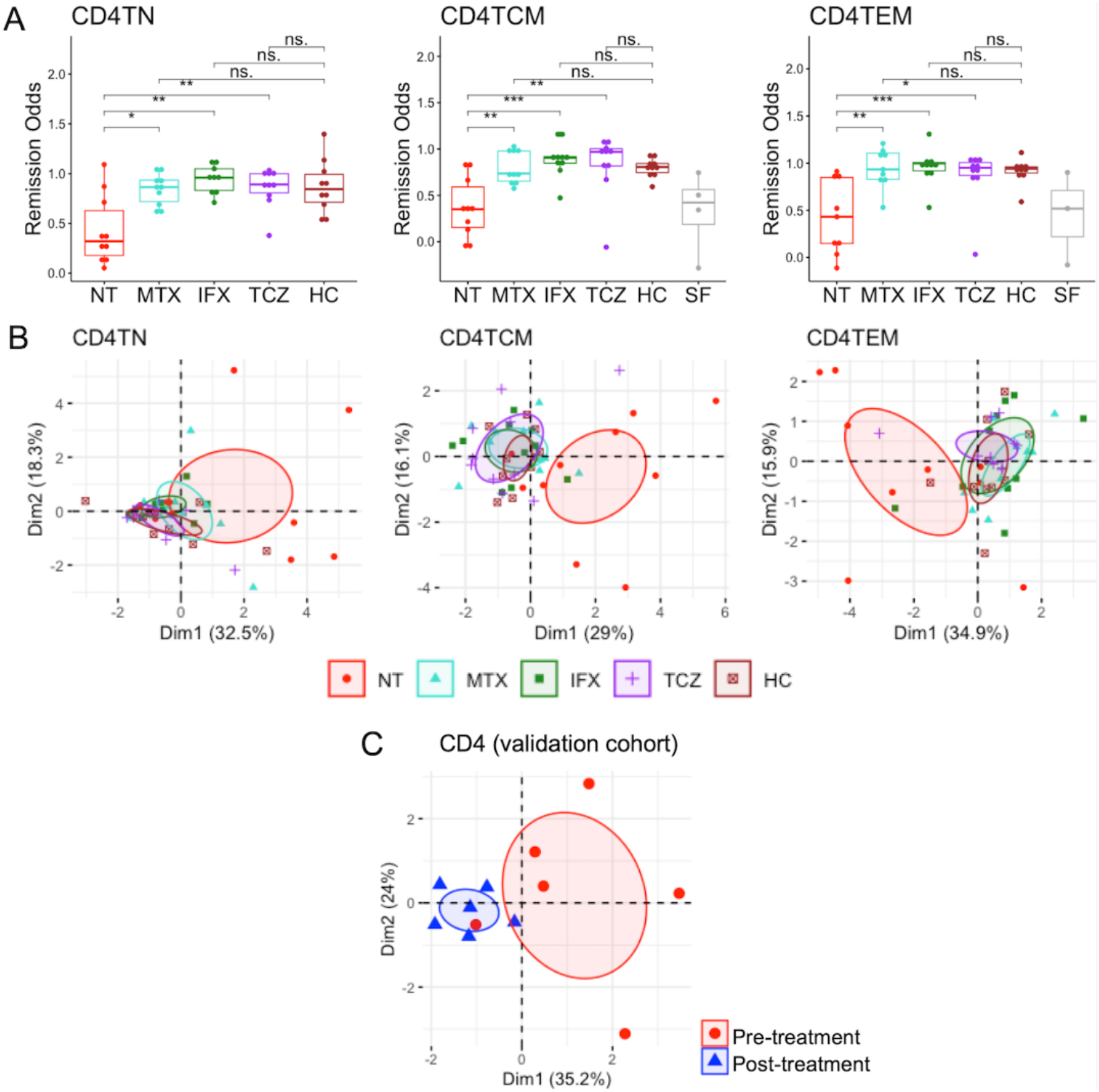

Figure 3

Remission odds and gene expression profiling of remission signature genes in CD4+ T cells. A) Remission odds generated by RA remission model. '*': $p < 0.05$, '**': $p < 0.01$, '***': $p < 0.001$. B, C) Principal component analysis using remission signature genes in derivation cohort (B) and validation cohort (C). The ellipse shows the 95% confidence interval of the value of principal component analysis. Patients in validation cohort were treated by TCZ for 6 months and achieved remission after treatment. HC, healthy control; IFX, infliximab; MTX, methotrexate; RA, rheumatoid arthritis; NT, non-treatment; SF,

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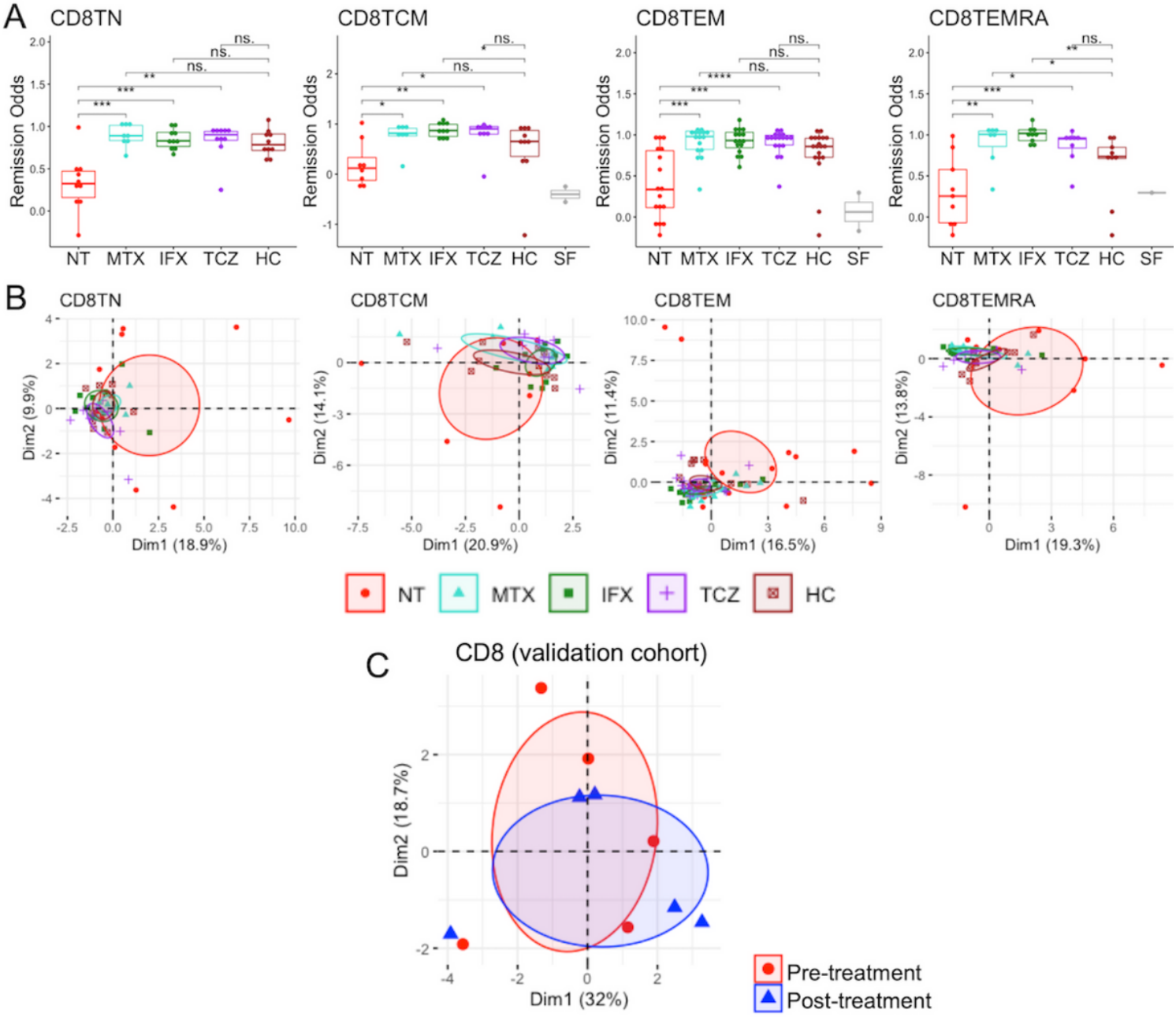

Figure 4

Remission odds and gene expression profiling of remission signature genes in CD8+ T cells. A) Remission odds generated by RA remission model. '*': $p < 0.05$, '**': $p < 0.01$, '***': $p < 0.001$, '****': $p < 0.0001$. B, C) Primary component analysis using remission signature genes in derivation cohort (B) and validation cohort (C). The ellipse shows the 95% confidence interval of the value of principal component analysis. Patients in validation cohort were treated by TCZ for 6 months and achieved remission after treatment. HC, healthy control; IFX, infliximab; MTX, methotrexate; RA, rheumatoid arthritis; NT, non-treatment; SF, synovial fluid; TCM, central memory T cell; TCZ, tocilizumab; TEM, effector memory T cell; TEMRA, CD45RA-positive effector memory T cell; TN, naïve T cell.

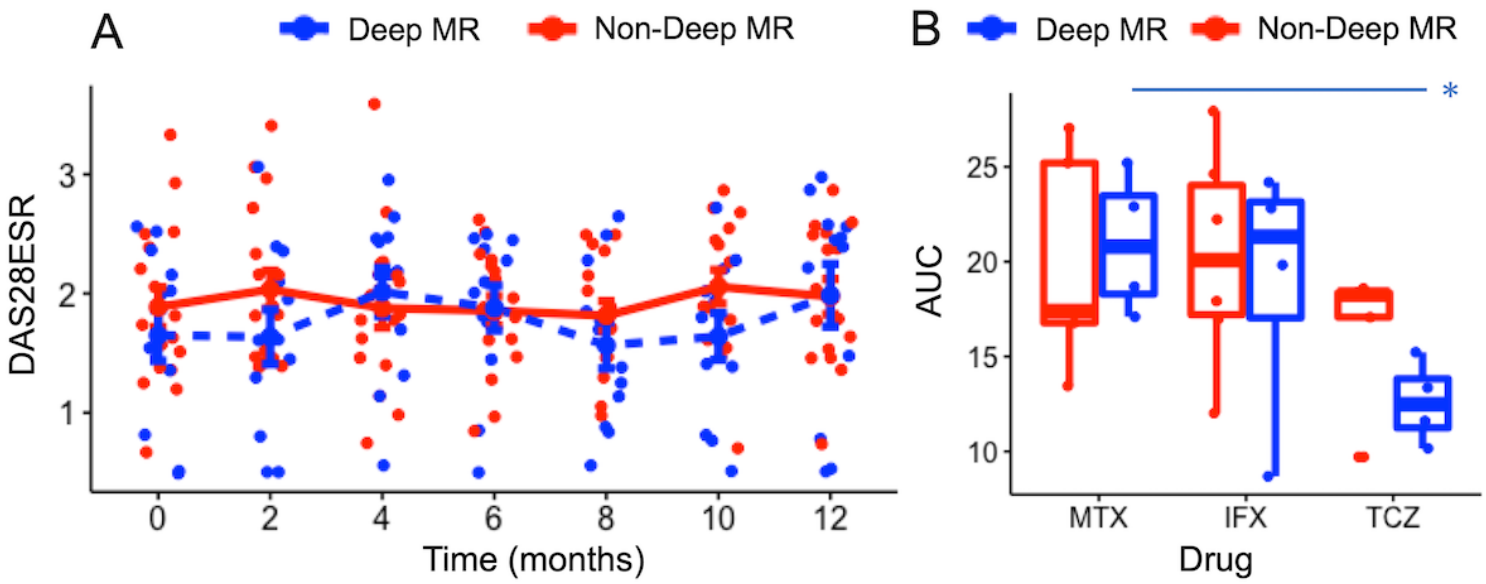

Figure 5

Relationship between MR and disease activity. A) The changes in DAS28-ESR during the follow-up period, which was split into two-week intervals. For each group, the average ± standard error of DAS28-ESR within the same interval was shown by line. B) Comparison of AUC (commutative DAS28-ESR during 12 months follow-up) according to type of drugs. '*': $p < 0.05$. AUC, area under the curve; DAS28-ESR, disease activity score-28 ESR; IFX, infliximab; MTX, methotrexate; TCZ, tocilizumab.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx