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# Immunomics analysis of rheumatoid arthritis identified precursor dendritic cells as a key cell subset of treatment resistance

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## ABSTRACT

**Objectives** Little is known about the immunology underlying variable treatment response in rheumatoid arthritis (RA). We performed large-scale transcriptome analyses of peripheral blood immune cell subsets to identify immune cells that predict treatment resistance.

**Methods** We isolated 18 peripheral blood immune cell subsets of 55 patients with RA requiring addition of new treatment and 39 healthy controls, and performed RNA sequencing. Transcriptome changes in RA and treatment effects were systematically characterised. Association between immune cell gene modules and treatment resistance was evaluated. We validated predictive value of identified parameters for treatment resistance using quantitative PCR (qPCR) and mass cytometric analysis cohorts. We also characterised the identified population by synovial single cell RNA-sequencing analysis.

**Results** Immune cells of patients with RA were characterised by enhanced interferon and IL6-JAK-STAT3 signalling that demonstrate partial normalisation after treatment. A gene expression module of plasmacytoid dendritic cells (pDC) reflecting the expansion of dendritic cell precursors (pre-DC) exhibited strongest association with treatment resistance. Type I interferon signalling was negatively correlated to pre-DC gene expression. qPCR and mass cytometric analysis in independent cohorts validated that the pre-DC associated gene expression and the proportion of pre-DC were significantly higher before treatment in treatment-resistant patients. A cluster of synovial DCs showed both features of pre-DC and pro-inflammatory conventional DC2s.

**Conclusions** An increase in pre-DC in peripheral blood predicted RA treatment resistance. Pre-DC could have pathophysiological relevance to RA treatment response.

### WHAT IS ALREADY KNOWN ON THIS SUBJECT

⇒ Limited information is available about the immune cells that are associated with rheumatoid arthritis (RA) treatment resistance.

### WHAT THIS STUDY ADDS

⇒ RA treatment resistance can be predicted by an increase in dendritic cell precursors (pre-DC) in peripheral blood prior to treatment.

⇒ The expression of genes reflecting an increase in pre-DC is negatively correlated to the type I interferon signature, which is associated with good therapeutic response.

⇒ The gene expression of synovial pre-DC-like cells is similar to pro-inflammatory cDC2s.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Stratified treatment of RA might be possible using pre-DC as a biomarker, and it might be possible to develop new therapies for treatment-resistant RA by targeting pre-DC.

## INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune inflammatory disease characterised by persistent synovitis and/or joint destruction. Difficult-to-treat (D2T) RA is recognised as an unsolved problem in the clinical setting.[1–4] In the treatment of RA, both patients and healthcare providers need precision medicine that stratifies treatment based on the predictions of treatment response.

To elucidate the causes of RA and predict therapeutic efficacy, transcriptome analyses have been performed on peripheral blood mononuclear cells[5 6] and synovia.[7 8] To date, type I interferon (IFN) gene signature or activity[5 9 10] has been proposed as predictive factor of treatment response. As for myeloid cells, the evidence suggests a relationship between treatment response and myeloid cells including dendritic cells (DC). An increase in conventional (c) DC in peripheral blood is correlated with treatment response to a tumour necrosis factor (TNF) inhibitor, infliximab.[11] For adaptive immune cells, T cells[12–14] and B cells[15–18] also exhibited association with therapeutic response. In RA synovium, treatment response showed association with expression of DC adhesion molecules.[19] Synovial macrophage and myeloid DC gene signatures were associated

with higher response rates to interleukin (IL)-6 receptor inhibitor, tocilizumab (TCZ).[8]

Dendritic cell precursors (pre-DC) are recently identified subpopulation of DCs. In 2017, See *et al* demonstrated that there are cells that are included in the subset of cells that have conventionally been considered plasmacytoid (p) DC, and that while pre-DC shares many of the same markers as pDC, they differentiate into cDC1 and cDC2.[20] They propose that IL-12 production and naïve $CD4^+$ T cell stimulation, which have traditionally been considered functions of pDC, are in fact functions of pre-DC, not pDC. Compared with pDC, pre-DC characteristically express *CD33 (SIGLEC3)*, *CX3CR1*, *SIGLEC6 (CD327)*, *CD2* and *CD5*. In 2017, Villani *et al* also identified DC subfractions DC1 through DC6 by means of single cell (sc) RNA-sequencing (RNA-seq) of human peripheral blood, and identified AS DC as a DC subfraction that was characterised by the expression of *AXL*, *SIGLEC1* and *SIGLEC6* and that powerfully activated T cells.[21] It is believed that pre-DC and AS DC are largely overlapping populations.[22]

To date, there have been no comprehensive studies of the immune cells that play a pivotal role in treatment-resistant RA. We recently constructed an atlas for the detailed gene expression profiles of peripheral blood immune cells in patients with immune diseases (the Immune Cell Gene Expression Atlas from the University of Tokyo (ImmuNexUT)).[23] In this study, we performed a comprehensive assessment of the transcriptome profiles of immune cells in peripheral blood prior to treatment in a total of 55 patients with RA, 24 of whom had been reported in ImmuNexUT and 31 of whom were newly added, and assessed the gene expressions and subsets that predict treatment resistance. Then, we evaluated the association of pre-DC with treatment-resistance of RA in two independent cohorts.

## MATERIALS AND METHODS

See online supplemental materials and methods.

### Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

## RESULTS

### Clinical features of RA and HC populations

To identify parameters related to the response to molecular targeted therapy, we recruited 55 patients with active RA who required addition or switching of disease-modifying antirheumatic drugs (online supplemental materials and methods). We also included 39 healthy control (HC) volunteers in the analysis (figure 1A). No significant differences in the age or sex were found between the RA and HC populations (online supplemental table 1).

### Overall picture of immune cell subset RNA-seq data

We performed RNA-seq for 55 patients with RA and 39 HC volunteers for each of the 18 peripheral blood immune cell subsets, sorted or isolated based on cell surface antigens (online supplemental table 2). We performed a second RNA-seq analysis for 20 (36.4%) of the 55 patients (of these 20, 15 received abatacept (ABT) and 5 TCZ) at 6 months after treatment initiation to assess effects of treatment on immune system using gene expression. After stringent quality control, 1701 samples with consistent gene expression pattern were included in the analysis (online supplemental figure 1A). In a principal component analysis (PCA), gene expression profiles of the different samples in each subset were similar, with only minor differences between the RA and HC populations (figure 1B, online supplemental figure 1B). Most of the variance in gene expression was explained by the subset (median 74%) and the individual (median 6.1%), although some (median 0.45%) depended on the differences between the RA and HC populations (figure 1C). The explained variance associated with the batches between the datasets was only 0.00055% (median), demonstrating the appropriateness of combining the datasets (online supplemental method).

### Genes with varying expression in RA immune cells and therapeutic medication efficacy

For investigating the differences in each type of immune cell between the RA and HC populations, we performed a Gene Set Enrichment Analysis (GSEA) for each subset of genes with varying expression in the RA population prior to treatment relative to the HC population (figure 2A, online supplemental figure 2). Increased expression of IFN response genes and IL6-Janus kinase (JAK)-signal transducer and activator of transcription (STAT) response genes was found in various subsets with RA population.

Next, we performed GSEA on the immune cells before and after treatment with ABT (n=15, figure 2B and C). Notably, although ABT treatment significantly improved disease activity, ABT did not suppress the expression of IFN-response genes before and after treatment in most immune cells (figure 2C). ABT tended to decrease the expression of genes related to various inflammatory responses including IL6-JAK-STAT3 signalling and cell proliferation in B and $CD4^+$ T cell subsets (figure 2C). A decrease in expression of MYC-associated genes and oxidative phosphorylation-associated genes were observed in plasmablasts after treatment (figure 2C, online supplemental figure 3).

In TCZ-treated patients with RA, inflammatory response genes were downregulated in monocytes, although the result was statistically limited by the small number of patients in the TCZ treatment group (n=5, online supplemental figure 4).

### Pre-DC genes in peripheral blood are associated with poor treatment prognoses

To clarify baseline characteristics of the immune cells in RA with a poor treatment response, we defined patients who had achieved CDAI50 at 6 months as responders.[24 25] We did not use the EULAR criteria of D2T RA for defining treatment response. Power analysis suggested that the total number of patients necessary for the identification of small, medium and large treatment response predictors are 394, 54 and 24, respectively. Therefore, we can expect to identify medium to strong predictors with our cohort of 55 patients with RA. No significant background clinical and treatment differences existed between the responders and the non-responders (online supplemental table 3). We prepared co-expression gene modules based on a weighted gene co-expression network analysis (WGCNA) for each of the immune cell subsets in the patients with RA, and investigated the associations with future treatment response.

Five hundred sixty-four modules were constructed with WGCNA, and the strongest association with treatment resistance was identified for a module 'pDC_M18' (figure 3A). Permutation test showed that this association is not likely to be a coincidence (empirical p=0.070, online supplemental figure 5). The pDC_M18 genes were expressed at higher levels in treatment-resistant RA compared with HC (figure 3B). Notably, treatment did not significantly affect pDC_M18 score (figure 3C). Additionally, baseline pDC_M18 was a better

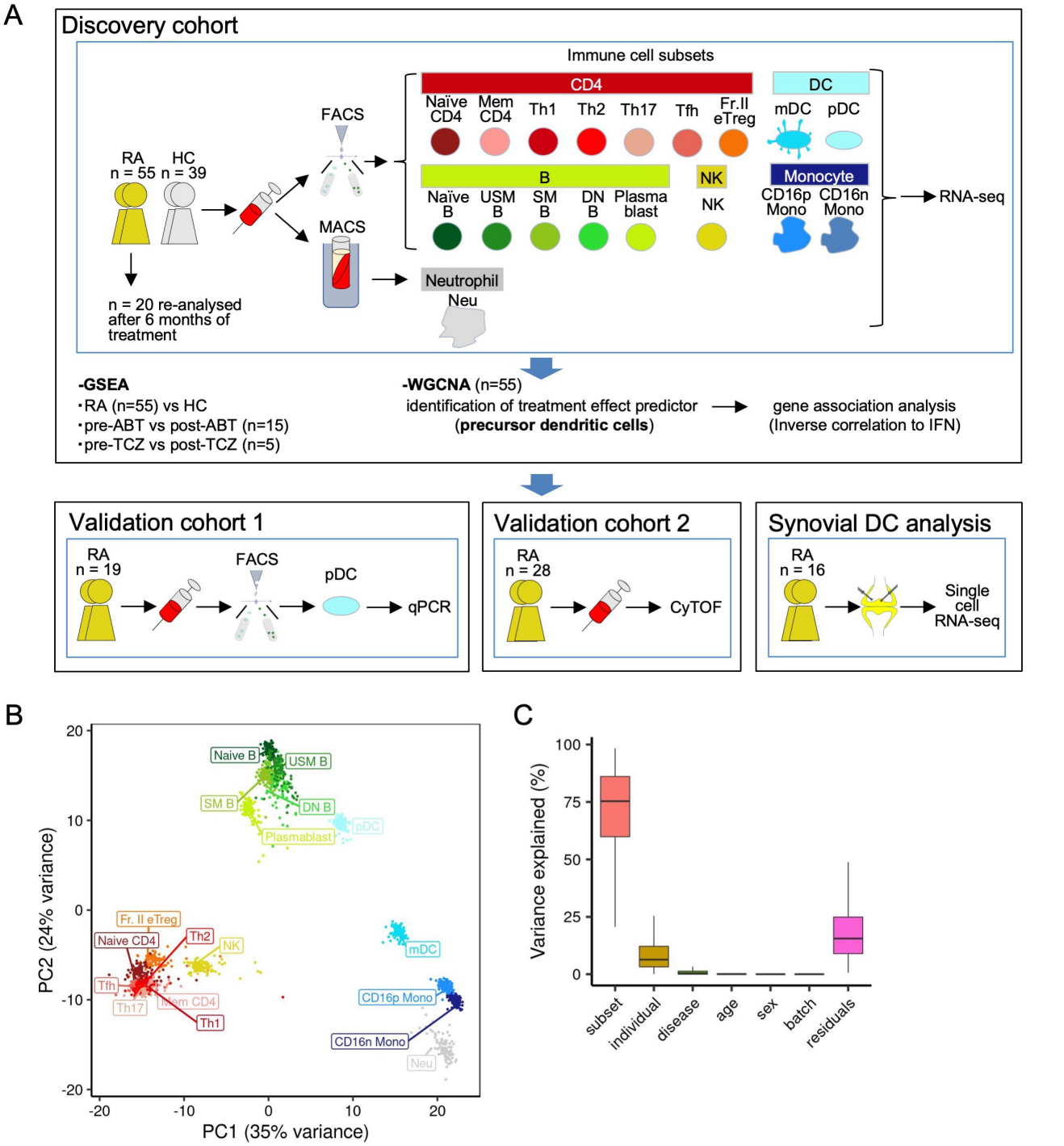


**Figure 1** Overview of the study. (A) Study concept. (B) Using all RNA-seq samples from the RA (n=55) (before treatment/after treatment) and HC (n=39) populations, PCA was performed using the 500 most highly variable genes. (C) A linear mixed model was used to perform gene expression variance decomposition on the RNA-seq samples. The fixed effect or age on gene expression, and the random effects of the immune cell subset, individual, difference between RA and HC (disease), sex and each of the four dataset batches was calculated. ABT, abatacept; CyTOF, cytometry by time of flight; DC, dendritic cell; FACS, fluorescence-activated cell sorting; GSEA, Gene Set Enrichment Analysis; HC, healthy control; IFN, interferon; MACS, magnetic-activated cell sorting; PCA, principal component analysis: qPCR, quantitative PCR;, RA, rheumatoid arthritis; RNA-seq, RNA-sequencing; TCZ, tocilizumab; WGCNA, weighted gene co-expression network analysis. Definitions of the subsets are presented in online supplemental table 2.

predictor of response than anticitrullinated protein antibody (ACPA) or disease duration, which are established clinical parameters for resistance to therapy (figure 3D).[26–28] In addition, we found no significant association between pDC_M18 expression and any clinical measures (online supplemental figure 6A–G), although the pDC_M18 expression tended to be higher in patients with longer disease duration than in patients with shorter disease duration (with the cut-off being defined as 1 year from onset)[29] (p=0.068) (online supplemental figure 6G). Moreover, pDC_M18 tended to predict treatment resistance regardless of the type of molecular targeted drug (online supplemental figure 6H). The pDC_M18 module consisted of

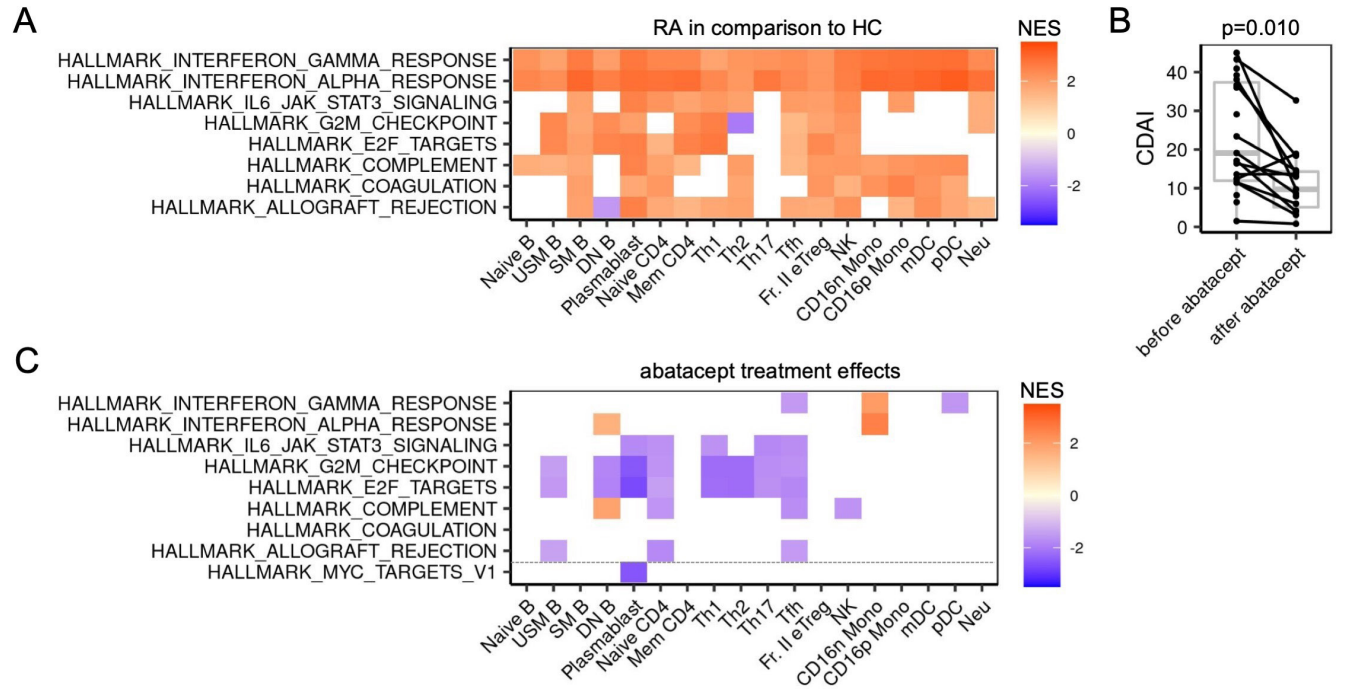


**Figure 2** RA immune cell gene expression and abatacept treatment-induced partial normalisation. (A) The GSEA results in the RA population prior to treatment compared with the HC population for each subset. Gene sets with |NES| >2.5 in at least one subset were targeted; white indicates that enrichment was not significant. (B) Clinical treatment effects of ABT. (C) The GSEA results for RA before and after treatment with ABT. The eight gene sets from (A) with increased expression in the RA population and the gene sets with a change in the |NES| >2.5 in at least one subset are shown. Pathways with false discovery rate <0.05 are coloured. ABT, abatacept; CDAI, clinical disease activity index; GSEA, Gene Set Enrichment Analysis; HC, healthy control; NES, normalised enrichment score; RA, rheumatoid arthritis. Definitions of the subsets are provided in online supplemental table 2.

207 genes (online supplemental table 4) and the network of the top 50 hub genes is shown in figure 3E.

Pre-DC is a subset that shares many of the same markers as pDC and differentiates into cDC1 and cDC2.[20] Unexpectedly, the hub genes of pDC_M18 included a number of pre-DC signature genes, such as *CD33*, *CX3CR1*, *KLF4* and *CD22*. In fact, 13 of the genes were included in the pre-DC signature genes reported by See *et al*[20] (online supplemental table 5) (*CD22, CD244, CD33, CD63, CD93, CLEC10A, CLEC12A, CX3CR1, ITGAX, KLF4, KLF8, RAB32* and *SIGLEC6*; OR=48.8; p<2.2e-16). Of the pDC WGCNA modules, only pDC_M18 exhibited significant overlap with the pre-DC signature genes (OR=27.4, p=1.0e-13; figure 3F). In addition, a clear correlation was found with the expression level of module eigengene (ME) of this module for the proportion of pre-DC in the pDC sample, estimated by deconvolution applying the data from the paper of See *et al* using CIBERSORTx[30] (r=0.70, p=9.0e-8) (figure 3G). Pre-DC is contained in the subset that have conventionally been considered pDC.[20] It was therefore thought that pDC_M18 reflects the proportion of pre-DC in pDC.

AS DC is a subfraction of DC that overlaps with pre-DC[22] and is characterised by the expression of *AXL*, *SIGLEC1* and *SIGLEC6* and powerfully activates T cells.[21] The signature genes of AS DC, defined by Villani *et al*, are enriched in the pDC_M18 module with 13 overlapping genes (OR=12.5; p=4.4e-10; *ACPP, ADAM33, AXL, CD22, CX3CR1, CXCR2, FAM129A, GPR146, HIP1, KLF4, S100A10, SIGLEC1* and *SIGLEC6*) (online supplemental table 5,online supplemental figure 7A). The pDC_M18 module can therefore reflect the proportion of AS DC, similar to that of pre-DC (r=0.69, p=1.3e-7) (online supplemental figure 7B).

In other words, the cells fractionated as pDC contain cell fractions referred to as pre-DC and AS DC. The fact that these cells are increased in treatment-resistant patients with RA is reflected in the gene expression profile.

Next, in a validation cohort (n=19) of patients with RA prior to the initiation of new treatment, we performed quantitative PCR for the pDC in peripheral blood collected before treatment and evaluated the mean Z-scores of the expression of the hub genes in pDC_M18 (figure 1A). No significant differences in pretreatment activity were found between the responders (n=9) and the non-responders (n=10) (online supplemental table 6). The pDC_M18 hub genes were expressed in significantly higher levels in the treatment-resistant group (p=0.043, figure 3H), and the relationship between high expression of pre-DC genes in the pDC fraction and treatment resistance was reproduced.

### Inverse correlation between pre-DC gene expression and IFN gene expression

To investigate the effects of pDC_M18 expression on immune cells, we searched for genes associated with pDC_M18 expression in each immune cell subset in the discovery cohort. A high level of pDC_M18 expression inversely correlated to a low level of expression of IFN-related genes, particularly in pDC and CD16n Mono (figure 4A–4C and online supplemental figure 8). Additionally, in all immune cell subsets studied, an inverse correlation was found between genes included in the pDC_M18 module and the IFN-α response signature (figure 4D,E). The IFN-α response signature expression was increased in responder patients with RA, particularly in CD16n Mono (figure 4F, online supplemental figure 9). Although type I IFN gene signature has been proposed as predictive factor for treatment response,[5 9 10] IFN-α response signature expression in CD16n Mono only showed modest predictive power for CDAI50 at 6 months (figure 4G). Using mediation analysis, we tested whether

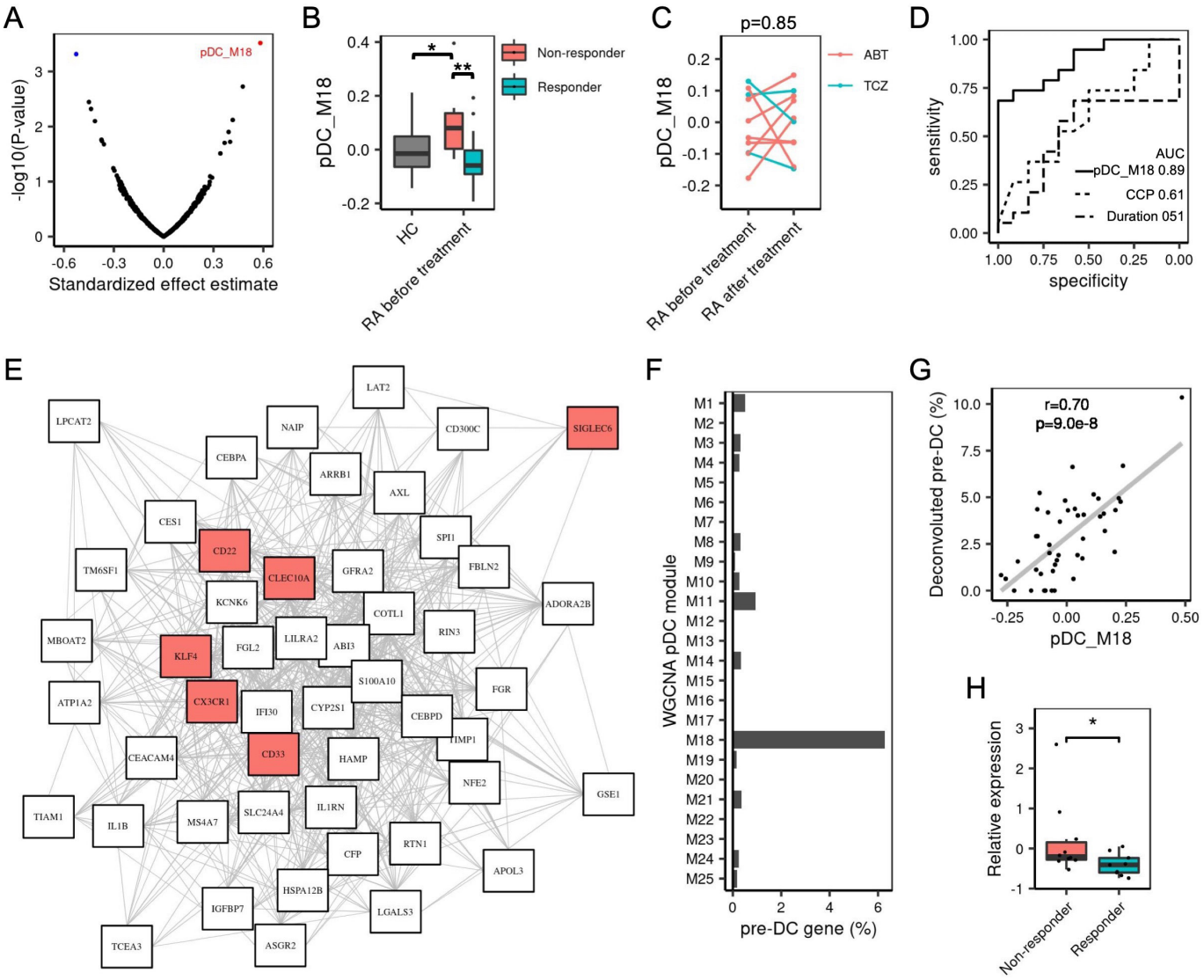


**Figure 3** Pre-DC genes correlate to RA treatment resistance. (A) The assessment was performed using a generalised linear model with failure to achieve CDAI50 at 6 months as the target variable and the eigengene of the module of each immune cell subset as the explanatory variable. Colour-coding was performed with Benjamini-Hochberg false discovery rate <0.10 as the significance level. (B) Comparison of ME expression in the pDC_M18 module in the pretreatment RA populations and the HC population. (C) The change over time in pDC_M18 expression associated with treatment was evaluated in three patients who received TCZ and seven patients who received ABT from whom blood samples were obtained before and after treatment. Paired t-test. (D) ROC curve of pretreatment pDC_M18 expression and treatment prognosis. The dotted line is that of the anti-CCP antibody and treatment prognosis, and the dotted and dashed line is that of disease durations and treatment prognosis. (E) Network figure of gene expression correlations for the top 50 hub genes in the pDC_M18 module. The pre-DC signature genes are colour-coded. Gene pairs with a Pearson's correlation coefficient of expression >0.6 were connected with each other. (F) Match rate with pDC_M18 genes in each pDC WGCNA module. (G) Correlation of the pDC_M18 ME and the proportion of deconvoluted pre-DC in the paper of See *et al*.[20] (H) qPCR was performed on the pDC in peripheral blood before treatment in a separate validation cohort (n=19) of patients with RA before starting a new therapy, and the pDC_M18 expression signatures of the non-responder and responder groups were compared. *P<0.05, **p<0.01. ABT, abatacept; AUC, area under the curve; CCP, citrullinated protein antibody; HC, healthy control; ME, module eigengene; pDC, plasmacytoid dendritic cell; pre-DC, predendritic cells; qPCR, quantitative PCR; RA, rheumatoid arthritis; TCZ, tocilizumab; WGCNA, weighted correlation network analysis.

IFN-α response signature of CD16n Mono has a direct effect on CDAI50 response at 6 months or was indirectly mediated by pDC_M18. pDC_M18 significantly mediated the effects of IFN-α response signature expression on CDAI50 (p=0.010) (figure 4H).

### An increase in the pre-DC cell population in peripheral blood predicts treatment resistance

Next, we used mass cytometric analysis to further validate the relationship between the populations of immune cells in peripheral blood prior to treatment and treatment prognosis in a second validation cohort (n=28) of patients with RA who were going to start ABT therapy (figure 1A). When we compared the clinical characteristics from the responders (n=21) with those from the non-responders (n=7) (online supplemental table 7), no significant differences were found between the two groups in clinical findings except for age, which was lower in the treatment-resistant group (p=0.017).

The cells were clustered based on data on the expression of 36 cell surface proteins obtained through mass cytometry, and

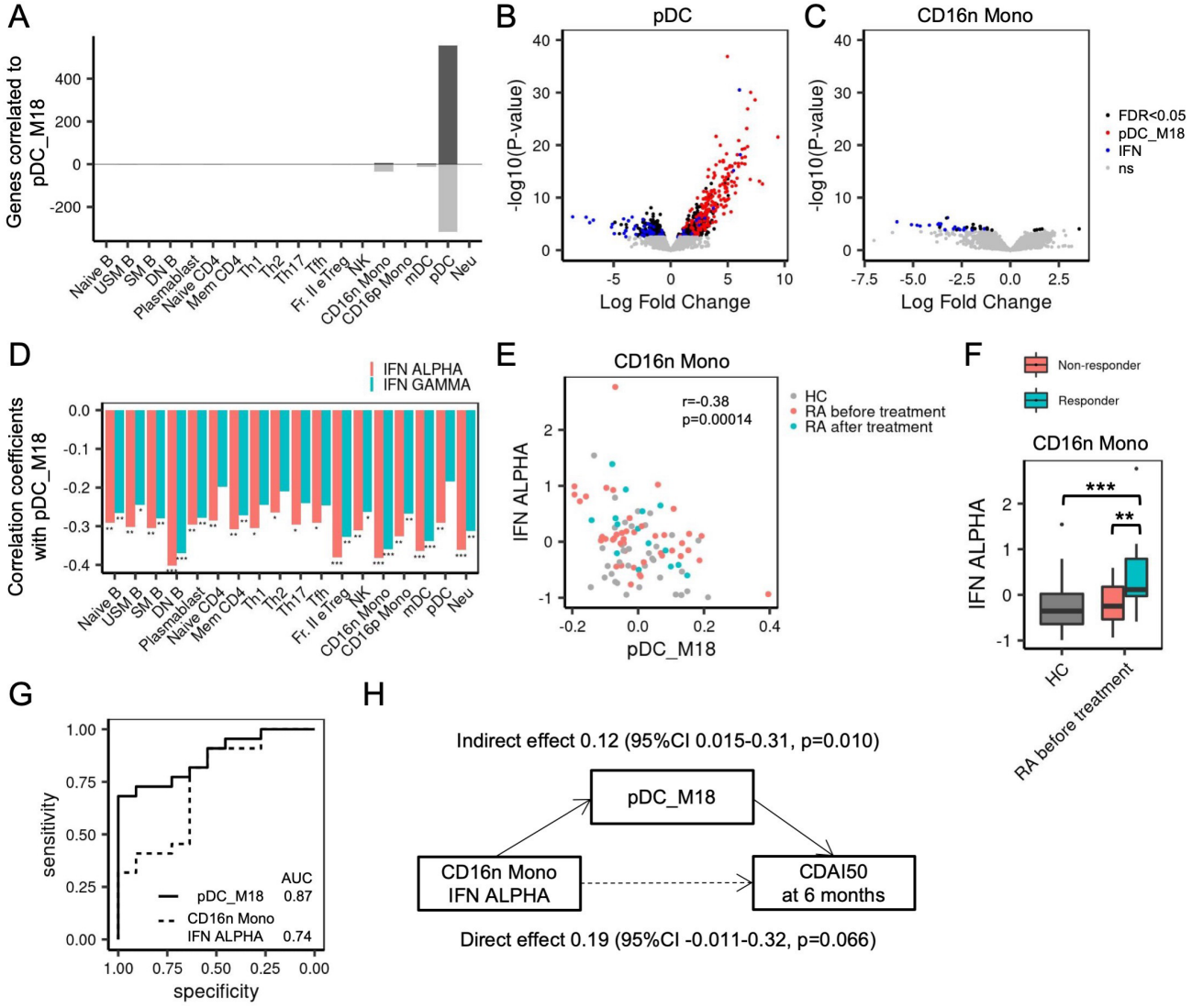


**Figure 4** Inverse correlation of pDC_M18 and IFN response genes. (A) Number of genes for which there was a relationship between the pDC_M18 and gene expression. The number of genes for which there was a positive correlation to pDC_M18 (positive values, shown in dark grey) and the number of genes for which there was a negative correlation to pDC_M18 (negative values, shown in light grey) are shown separately. (B–C) pDC, CD16n Mono volcano plot, showing gene expression correlated to pDC_M18. The genes in pDC_M18 (red) and the genes associated with pDC_M18 (FDR with Benjamini-Hochberg <0.05) that were IFN response genes (blue) were colour-coded separately. (D) Correlation coefficients for the IFN response signature and pDC_M18 for each immune subset. (E) Negative correlation between the IFN-α response signature and pDC_M18 in CD16n Mono. (F) Comparison of the IFN-α response signature in CD16n Mono in the pretreatment RA populations and the HC population. (G) ROC curve of pre-treatment pDC_M18 and CD16n Mono IFN-α response signature expression and treatment prognosis. (H) Mediation model representing the relationships between CD16n Mono IFN-α response signature expression, pDC_M18 and CDAI50 at 6 months. *P<0.05, **p<0.01, ***p<0.001. FDR, false discovery rate; HC, healthy control; IFN, interferon; ns, not significant; pDC, plasmacytoid dendritic cells; RA, rheumatoid arthritis. Subset definitions are provided in online supplemental table 2.

the immune cells in the peripheral blood were classified into 27 populations (figure 5A and B and online supplemental figure 10). However, no significant relationships to treatment prognosis were found for any of these populations (figure 5C).

Because we were not able to identify pre-DC in the automated clustering analysis, we performed manual gating, defining Lin (CD34, CD3, CD14, CD19, CD16)$^-$ HLA-DR$^+$ CD45RA$^+$ CD123$^+$ CD11c$^{nega/mid}$ CX3CR1$^+$ as pre-DC (online supplemental figure 11), which was consistent with the pre-DC gating used in the paper of See *et al*[20] (online supplemental figure 12).

The proportion of pre-DC relative to DC in the peripheral blood obtained prior to treatment was significantly higher in the treatment-resistant group (p=4.0e-06) (figure 5D and online supplemental table 8). The proportion of pre-DC prior to treatment also had superior prognosis predictive performance to ACPA or disease duration, with an area under the curve of 0.95 (figure 5E). With a cut-off value of pre-DC ratio below 2.3%, the sensitivity and specificity of treatment response were 81% and 100%, respectively. Additionally, the proportion of pre-DC was higher in patients with a longer disease duration (p=0.038) (figure 5F).

We performed a meta-analysis using three cohorts of pre-DC signatures by combining the results of gene expression and cell frequency analyses. Forest plots revealed that the direction and strength of the association was consistent between pre-DC signature and treatment non-response (Cochran's Q test p=0.35, $I^2$=3.8%, fixed-effect meta-analysis p<0.0001, online supplemental figure 13).

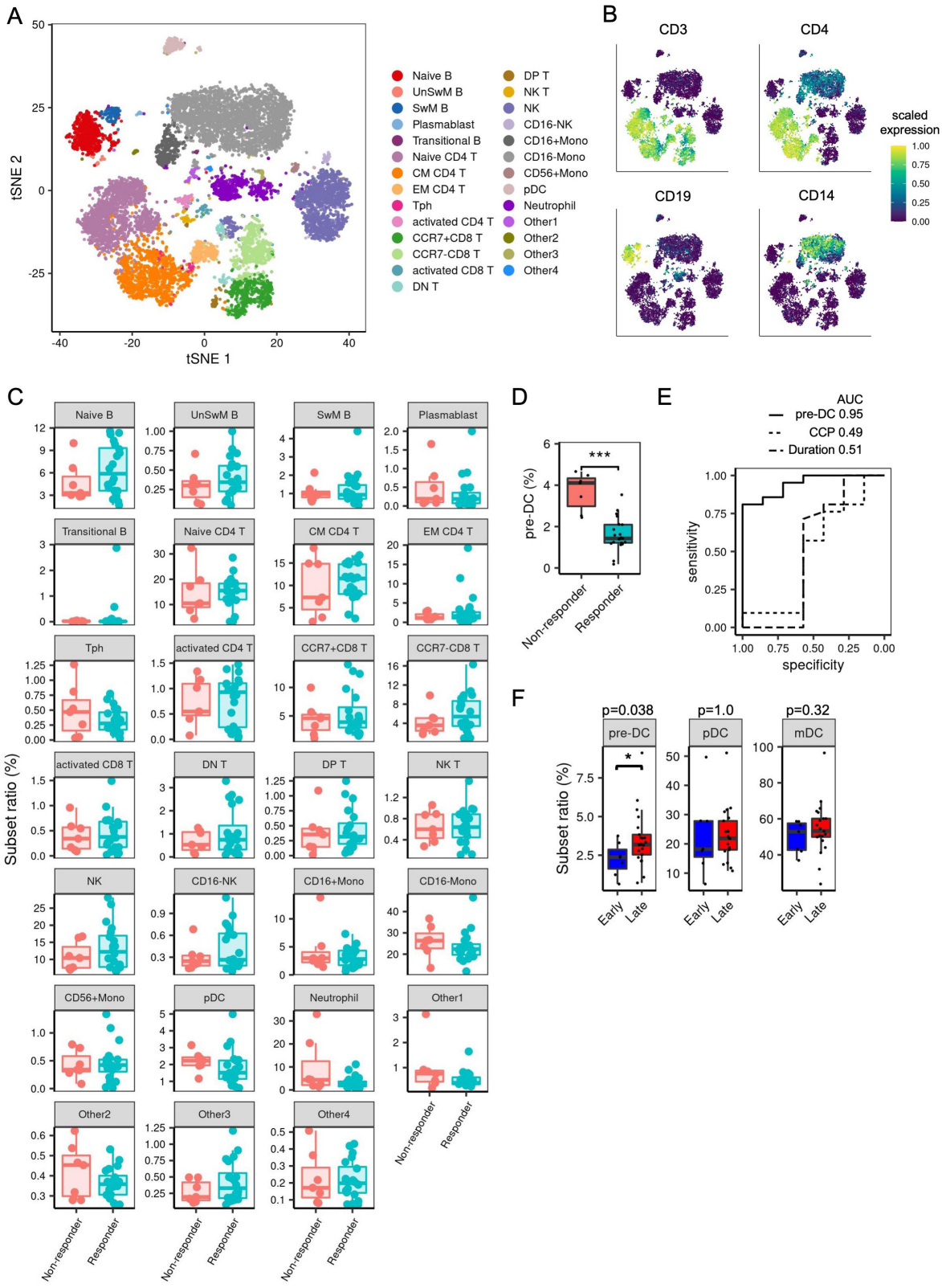


**Figure 5** Search of immune cells associated with prognosis by mass cytometry of patients with RA before ABT treatment initiation. (A) tSNE plot of the peripheral blood immune cell population in 28 patients with RA before ABT therapy. The mass cytometry data for 36 cell surface markers were clustered, and 27 cell populations identified. (B) Representative plots of several cell surface markers. The same tSNE plots as those described in (A) were used. (C) Comparison of the proportion of the 27 cell populations with treatment prognosis (achievement of CDAI50 after 6 months). (D) Comparison of proportions of pre-DC relative to DC with treatment prognosis. (E) ROC curve for the proportion of pre-DC before treatment and treatment prognosis. The dotted line is that of the anti-CCP antibody and treatment prognosis, and the dotted and dashed line is that of disease durations and treatment prognosis. (F) The proportions of pre-DC, pDC and mDC in the longer (>1 year) or shorter (≤1 year) disease duration groups were compared. *P<0.05, ***p<0.001. AUC, area under the curve; CCP, citrullinated protein antibody; mDC, myeloid dendritic cells; pre-DC, predendritic cells; pDC, plasmacytoid dendritic cells; RA, rheumatoid arthritis; tSNE, t-distribution stochastic neighbour embedding.

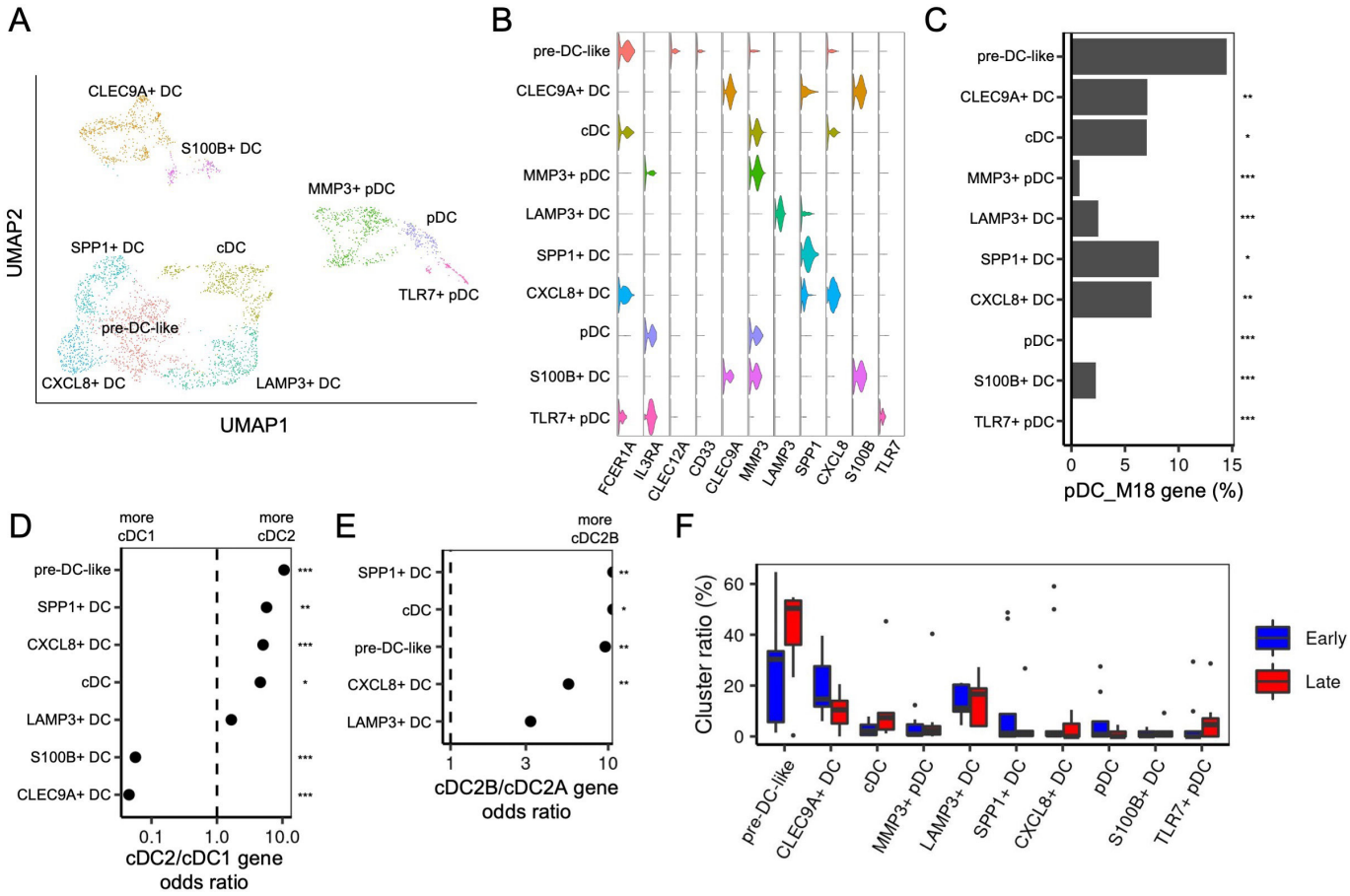


**Figure 6** Synovial dendritic cells single cell RNA-sequencing analysis of untreated RA. (A) UMAP plot of synovial DCs from patients with untreated RA (n=16, 3804 cells). (B) Violin plots of DC cluster marker gene expressions. (C) Match rate with pDC_M18 genes in each DC cluster signature genes. The match rate of pre-DC-like cluster was compared with other DC clusters with Fisher's exact tests. (D–E) ORs of cDC2 and cDC1 signature genes (D) or pro-inflammatory cDC2B and anti-inflammatory cDC2A signature genes (E) in each DC cluster signature genes. (F) DC cluster proportions, stratified by disease duration (duration <3 years=early, n=9). *P<0.05, **p<0.01, ***p<0.001. Pre-DC, predendritic cells; pDC, plasmacytoid dendritic cell; RA, rheumatoid arthritis.

### Synovial pre-DC-like cells are similar to inflammatory cDC2s

Finally, to investigate the pathophysiological relevance of pre-DC in arthritis, we analysed previously reported synovial single cell RNA-seq data of treatment-naïve patients with RA (n=16).[31] We separated DCs from CD45$^+$ synovial immune cells, and clustering analysis identified 10 DC clusters (online supplemental materials and methods figures 1A, 6A,B). The *IL3RA* expression clearly distinguished pDC from cDCs. A cluster of cDCs characteristically expressed pDC_M18 genes, such as *CLEC12A* and *CD33*, and we named them as 'pre-DC-like' cells (figure 6B,C). The signature genes of this pre-DC-like cluster were clearly enriched with pDC_M18 genes with an OR of 9.2 (p<2.2e-16). Cluster marker genes, such as *CLEC9A* for cDC1,[32] clearly distinguished cDC1 clusters (CLEC9A$^+$ DC and S1008B$^+$ DC) from cDC2 clusters (pre-DC-like, SPP1$^+$ DC, CXCL8$^+$ DC and cDC) (figure 6B,D). LAMP3$^+$ DCs uniquely expressed *LAMP3*, a reported marker of regulatory DC that limit antitumour immunity.[32] Recently, cDC2 are subdivided to two clusters; anti-inflammatory cDC2A and pro-inflammatory cDC2B.[33] Synovial pre-DC-like cells and other cDC2 populations expressed signature genes of pro-inflammatory cDC2B (figure 6E). On average, 32% of the synovial DCs were pre-DC-like, and the frequency was higher in patients with RA in longer duration (with the cut-off being defined as 3 years from onset here) (figure 6F, p=0.16), consistent with our peripheral blood data.

## DISCUSSION

In this study, we characterised the gene profiles of the immune cells of patients with RA and demonstrated that treatment resistance can be predicted by an increase in pre-DC in the peripheral blood of patients with RA prior to starting therapy. This result shows the potential for realising a stratified therapy of RA based on analysis of pre-DC in peripheral blood, which is minimally invasive, and also shows that pre-DC may be involved in the immunopathology of treatment-resistant RA.

No significant differences were found between the poor treatment prognosis group and the good treatment prognosis group in rheumatoid factor (RF), ACPA or disease duration (online supplemental table 3) and pre-DC was superior for predicting the prognosis than these known clinical prognosis predictive factors (figures 3D and 5E). Although ACPA has been reported to predict more severe radiographic damage after several years of follow-up,[26 34–36] studies of patients with RA presenting within 3 months or 2 years of symptom onset reported no differences in disease activity according to ACPA status.[37 38] Also, a meta-analysis of randomised controlled trials showed that the effects of disease duration on treatment resistance was modest.[28] In our study, in fact, none of the three cohorts showed significant differences in ACPA status or disease duration between responders and non-responders (online supplemental tables 3,6,7). Pre-DC in peripheral blood may be associated with treatment resistance

via a distinct mechanism and can be a more sensitive prognostic predictor.

Moreover, the results of this study suggest that there is an increase in pre-DC in the peripheral blood prior to treatment in patients with a long (≥1 year) disease duration. This is consistent with reports that the duration of disease tends to be longer in treatment-resistant patients.[28] It is known that in early RA there is a so-called window of opportunity in which treatment response is good.[39] An increase in pre-DC may have an immunological foundation, where treatment response worsens as the disease duration increases. Delay to initial treatment is a risk factor of refractory RA,[3] which can also be associated with pre-DC.

Recently, the human DC have been categorised in greater detail. Villani *et al* used scRNA-seq to establish six populations.[21] AS DC, a population that is comparable to pre-DC, has been shown to be increased in paediatric patients with systemic lupus erythematosus (SLE).[40] Pro-inflammatory cDC2s, which can present antigen to CD4 T cells and are known both as cDC2B or cDC3,[41] were expanded in SLE and were correlated with disease activity.[42]

As for arthritis, there was no difference in the distribution of pDC or mDC in the peripheral blood between juvenile idiopathic arthritis (JIA) and septic arthritis[43] or between JIA and HC.[44] In contrast, the number of $CD141^+$ cDC (cDC1) in the synovial fluid was significantly higher in patients with JIA than in patients with septic arthritis.[43] Synovial cDC1 is also reported to be increased in patients with RA and induce higher levels of $CD4^+$ and $CD8^+$ T cell activation compared with their peripheral blood counterparts.[45] As for cDC2, the same group reported that, in patients with inflammatory arthritis, cDC2 in synovium were increased and more mature, compared with cDC2 in peripheral blood.[46] In our synovial single-cell transcriptome profiling, we identified pre-DC-like DCs occupied around a third of synovial DC population and that were related to pro-inflammatory cDC2Bs (figure 6). cDC2Bs are suggested to mediate T helper 17 responses.[33] In treatment-resistant patients, an increase in pre-DC may affect the synovial acquired immune response in RA by stimulating the $CD4^+$ T cells, either as pre-DC-like cells themselves, or after further differentiating into pro-inflammatory cDC2s. We need to verify this hypothesis with in vitro and in vivo experiments in the future. It has been reported that, in a mouse model of influenza A virus infection, pre-DC enter infected tissues,[47] and it is possible that, in human arthritis, as well, there is a link between an increase in pre-DC, in the blood and at the joint, and inflammation.

In this study, we observed a negative correlation between type I IFN signalling genes and pre-DC gene expression, and pre-DC gene expression had a closer relationship with treatment response than IFN-α signalling (figure 4). It is possible that IFN signalling is inhibitory on pre-DC, or that pre-DC are antagonistic to the pDC that produces IFN-α. The type I IFN stimulation is reported to limit cDC differentiation and promotes pDC differentiation.[4849] Although type I IFN signalling has been reported to be a factor that predicts development of RA,[50] type I IFN signalling predicts a good therapeutic response to TCZ and TNF inhibitors.[5 9 10] The extent of type I IFN signalling moreover differs considerably depending on the individual patient with RA,[23] and it has been reported that around 33% of patients with RA exhibit increased type I IFN signalling.[51] It is possible that there are separate RA subtypes with different immunolopathologies: RA with a relatively good prognosis characterised by increased type I IFN signalling; and RA with a relatively poor prognosis characterised by an increase in pre-DC.

One limitation of this study was that the patients' existing treatments and the new treatments they were going to start receiving were not the same, and the analyses were therefore performed using combined responses to various treatments. The limited number of the discovery cohort might have also prohibited the identification of other potential predictors of treatment-response. In the future, a larger-scale investigation that also takes into account differences in patients' clinical pictures and/or existing treatments should be conducted.

In conclusion, we discovered, through an analysis of the gene expression profiles of immune cells in the peripheral blood of patients prior to treatment, that an increase in pre-DC can predict the prognosis of RA, and the reproducibility of this result was confirmed using two cohorts. We also identified synovial pre-DC-like cells that were similar to pro-inflammatory cDC2 in transcriptome. These results can contribute to our understanding of RA treatment stratification and the pathology of non-responder RA.

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**Data availability statement** Data are available in a public, open access repository. The datasets generated during this study are available at the National Bioscience Database Center (NBDC) with the study accession code of hum0214 and hum0383. We used publicly available software for the analyses.



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