ORIGINAL ARTICLE

Interleukin-1 pathway in active large vessel vasculitis patients with a poor prognosis: a longitudinal transcriptome analysis

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Abstract

Objectives. Large vessel vasculitis (LVV) is characterised by a high relapse rate. Because accurate assessment of the LVV disease status can be difficult, an accurate prognostic marker for initial risk stratification is required. We conducted a comprehensive longitudinal investigation of next-generation RNA-sequencing data for patients with LVV to explore useful biomarkers associated with clinical characteristics. **Methods.** Key molecular pathways relevant to LVV pathogenesis were identified by examining the whole blood RNA from patients with LVV and healthy controls (HCs). The data were examined by pathway analysis and weighted gene correlation network analysis (WGCNA) to identify functional gene sets that were differentially expressed between LVV patients and HCs, and associated with clinical features. We then compared the expression of the selected genes during week 0, week 6, remission and relapse. **Results.** The whole-transcriptome gene expression data for 108 samples obtained from LVV patients ($n = 27$) and HCs ($n = 12$) were compared. The pathway analysis and WGCNA revealed that molecular pathway related to interleukin (IL)-1 was significantly upregulated in LVV patients compared with HCs, which correlated with the positron emission tomography vascular activity score, a disease-extent score based on the distribution of affected arteries. Further analysis revealed that the expression levels of genes in the IL-1 signalling pathway remained high after conventional treatment and were associated with disease relapse. **Conclusion.** Upregulation of the IL-1 signalling pathway was a characteristic of LVV patients and was associated with the extent of disease and a poor prognosis.

Keywords: gene expression, giant cell arteritis, interleukin-1, large vessel vasculitis, Takayasu's arteritis

INTRODUCTION

Large vessel vasculitis (LVV) is characterised by granulomatous inflammation of medium- and large-sized arteries.[1] At the Chapel Hill Consensus Conference of 2012, giant cell arteritis (GCA) and Takayasu's arteritis (TAK) were defined as different types of primary LVV.[2] Although several potential mechanisms have been implicated in the development of inflammatory vascular system reactions, including microbial infection and autoimmune reactions,[3,4] the precise mechanism underlying the development of vasculitis remains to be determined.

Most patients with LVV enter remission after prednisolone (PSL) therapy. However, relapse during the maintenance phase is common,[5] implying that conventional treatments ameliorate the symptoms of vasculitis, but may not fully inhibit the molecular mechanisms responsible for LVV. Although physicians monitor disease activity based on patient symptoms and inflammatory markers – primarily the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level – accurate assessment of vascular disease activity status can be difficult. Although clinical studies have identified several risk factors for relapse, including a high body mass index (BMI),[6] a high positron emission tomography (PET) vascular activity score (PETVAS)[7] and thoracic aorta involvement,[8–10] the risk assessment for relapse in these patients is still incomplete.

Several studies have been conducted to examine biomarkers of interest, including cytokines and hormones.[11–16] Potential biomarkers identified to date include pentraxin 3,[11] matrix metalloproteinase 3,[12] vascular endothelial growth factor,[13] osteopontin[14] and tissue inhibitor of matrix metalloproteinase 1.[15] Our previous broad immunophenotyping study revealed that helper T (Th) 1, Th17, follicular helper T and CD8-positive T cells were associated with disease activity, but had less utility for predicting disease relapse.[17] As a next step, RNA sequencing should provide further valuable information. Previous RNA-sequencing data from de Smit *et al*. and Régnier *et al*. suggested that the JAK/STAT pathway was activated in CD4 and CD8 T cells,[18,19] and biomarkers with greater sensitivity and specificity have been strongly anticipated.

Here, to identify a specific biomarker of LVV, we conducted a longitudinal examination of gene expression data of patients with LVV, focusing on the immunological characteristics of LVV and their clinical significance.

RESULTS

Baseline clinical characteristics and therapeutic responses in patients with LVV

We collected gene expression data and clinical profiles from 27 patients with LVV (GCA, $n = 17$; TAK, $n = 10$) who were followed longitudinally across 96 visits. Nine patients experienced disease relapse during the 60 weeks of treatment and were defined as non-responder patients. The baseline characteristics, treatments and effects of treatment in LVV patients and healthy controls (HCs) are summarised in Table 1. Age, sex, race, smoking history, BMI and major comorbidities were not different between LVV patients and HCs. To extract the characteristics of non-responders, we compared baseline clinical data between responders and non-responders. In the non-responder group, the time from symptom onset to diagnosis tended to be longer (17 vs. 11 weeks, $P = 0.18$) and the PETVAS tended to be higher (24 vs. 6, $P = 0.11$). The area under the curve (AUC) of the duration from symptom onset to diagnosis and the PETVAS for predicting disease relapse were 0.67 ($P = 0.074$) and 0.76 ($P = 0.10$) respectively. Responders used biologics more frequently before relapse than did non-responders (50% vs. 22%, $P = 0.16$).

Clinical courses of patients with LVV in response to treatment

The clinical course after induction therapy is shown in Figure 1. The first relapse occurred from weeks 10 to 53 after treatment initiation (Figure 1a). Relapse was diagnosed based on symptoms and abnormal imaging findings. The CRP levels (Figure 1b) and the PSL dosage (Supplementary figure 1) in responders and non-responders are shown. Samples obtained at week 0 (responders, $n = 16$; non-responders, $n = 7$), week 6 (responders, $n = 11$; non-responders, $n = 7$), remission (PSL treatment, $n = 22$; PSL and infliximab (IFX) treatment, $n = 5$; and PSL and tocilizumab (TCZ) treatment, $n = 15$) and relapse ($n = 13$) were analysed by performing RNA-sequencing and cytokine assays (Figure 1c).

Table 1. Baseline clinical characteristics of patients with LVV

Variable	LVV $n = 27$	Responder $n = 18$	Non-responder $n = 9$	HC $n = 12$
Age, years	69 (46–74)	70 (47–76)	64 (41–71)	62 (58–73)
Male, n (%)	13 (48)	9 (50)	4 (44)	5 (42)
Race, Japanese, n (%)	25 (93)	16 (89)	9 (100)	12 (100)
Body mass index, kg m^{-2}	21 (18–25)	20 (17–23)	21 (20–24)	22 (20–25)
Smoking history, n (%)	10 (37)	7 (39)	3 (33)	2 (17)
Comorbidities				
Hypertension, n (%)	7 (26)	5 (28)	2 (22)	2 (17)
Diabetes mellitus, n (%)	3 (11)	3 (17)	0 (0)	0 (0)
Dyslipidaemia, n (%)	7 (26)	5 (28)	2 (22)	2 (17)
Fatty liver disease, n (%)	1 (4)	0 (0)	1 (11)	2 (17)
Chronic kidney disease, n (%)	4 (15)	2 (11)	2 (22)	1 (8)
Polymyalgia rheumatica, n (%)	7 (26)	5 (28)	2 (22)	0 (0)
Inflammatory bowel disease, n (%)	2 (7)	1 (6)	1 (11)	0 (0)
Aortic regurgitation, n (%)	3 (11)	2 (11)	1 (11)	0 (0)
Diagnosis (GCA/TAK), n	17/10	12/6	5/4	–
GCA with positive TAB finding, n (%)	10 (59), $n = 17$	8 (67), $n = 12$	2 (40), $n = 5$	–
Large vessel involvement, n (%)	22 (81)	13 (72)	9 (100)	–
Time from symptom onset to diagnosis, weeks	13 (6.5–29)	11 (5.6–22)	17 (8.4–52)	–
Imaging and laboratory tests at first observation				
PETVAS	12 (6–25), $n = 13$	6 (5–25), $n = 7$	24 (11–25), $n = 6$	–
ESR, mm h^{-1}	109 (54–129)	115 (58–131)	101 (72–116)	–
White blood cells, 10^3 cells μL^{-1}	7.3 (6.4–9.3)	7.7 (6.4–9.2)	6.9 (6.3–9.3)	–
Neutrophils, 10^3 cells μL^{-1}	5.5 (4.5–6.9)	5.9 (4.3–7.4)	5.2 (4.7–6.6)	–
Lymphocytes, 10^3 cells μL^{-1}	1.3 (1.1–1.7)	1.3 (1.1–1.8)	1.2 (1.0–1.6)	–
Monocytes, cells μL^{-1}	438 (331–563)	435 (283–565)	442 (336–540)	–
Eosinophils, cells μL^{-1}	100 (77–193)	99 (62–209)	122 (93–247)	–
Haemoglobin, g dL^{-1}	11 (10–13)	11 (9.5–12)	11 (10–13)	–
Platelets, 10^4 μL^{-1}	36 (30–40)	39 (25–51)	34 (32–37)	–
CRP, mg dL^{-1}	3.2 (1.8–6.4)	5.4 (2.1–7.0)	3.2 (2.7–6.3)	–
Treatment				
PSL dose at week 0, mg day^{-1}	50 (40–60)	50 (40–60)	50 (40–60)	–
PSL dose at week 6, mg day^{-1}	20 (15–20)	20 (15–20)	20 (15–30)	–
Biologics use prior to relapse, n (%)	11 (41)	9 (50)	2 (22)	–

Continuous data are expressed as median (IQR) and categorical data as number (percentage). CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GCA, giant cell arteritis; HC, healthy control; LVV, large vessel vasculitis; PETVAS, positron emission tomography vascular activity score; PSL, prednisolone; TAB, temporal artery biopsy; TAK, Takayasu's arteritis.

Samples at week 0 were obtained prior to induction therapy.

Identification of the molecular profile of patients with active LVV

To identify the molecular biological features of LVV, we performed a comprehensive gene expression analysis of samples from LVV patients at week 0 ($n = 23$) and HCs ($n = 12$). At a significance threshold of false discovery rate (FDR) < 0.05 and fold change in expression > 1.5, we identified 468 differentially expressed genes (DEGs). Among these, 267 were upregulated and 201 were downregulated compared with HCs, as shown in the volcano plot (Figure 2a). The identities of all DEGs are provided in Supplementary table 1.

Hierarchical cluster analysis

The selected 468 DEGs were then subjected to hierarchical cluster analysis to examine characteristic differences among GCA, TAK and HCs. Hierarchical cluster analysis showed that LVV patients and HCs were in separate clusters but did not separate patients with GCA and TAK (Figure 2b). Considering the results of cluster analysis, we analysed samples from patients with GCA and TAK as being in the same cluster.
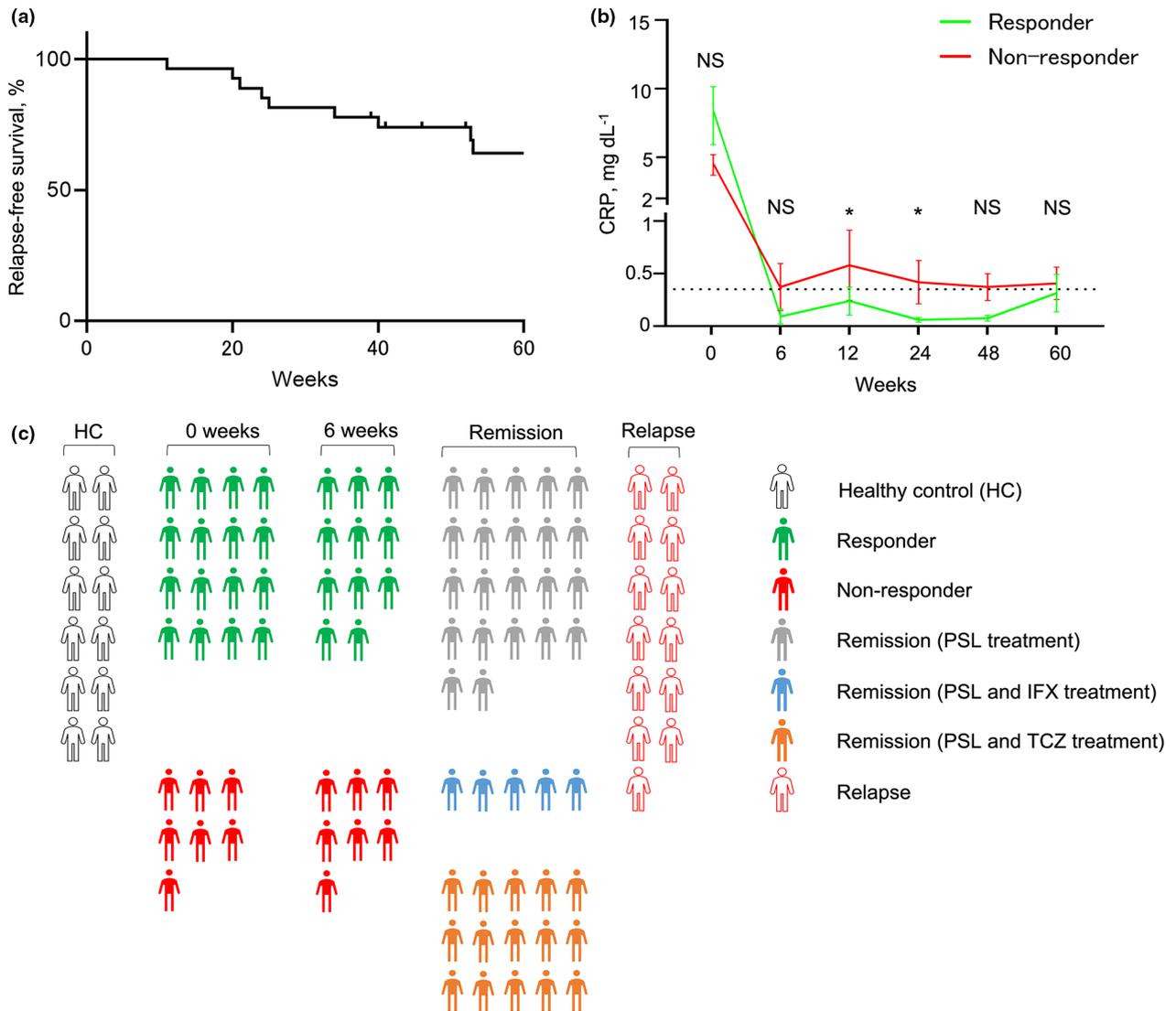
Figure 1. Clinical course of LVV in treatment-responsive and treatment-non-responsive patients. Patients were divided into responder and non-responder groups based on whether they experienced relapse during the 60 weeks of treatment. Relapse was defined as the reappearance of vasculitis-related manifestations or exacerbation of imaging findings requiring an increase in the prednisolone (PSL) dose or additional immunosuppressive agents. **(a)** The Kaplan–Meier curves for LVV patients ($n = 27$). **(b)** C-reactive protein (CRP) levels for responders ($n = 17$) and non-responders ($n = 10$). The dotted line represents the upper normal range of CRP (0.35 mg dL^{-1}). **(c)** Sample collection design. The data shown represent the mean $\pm$ SEM. *$P < 0.05$. Statistical analysis was performed by **(a)** constructing a Kaplan–Meier curve or **(b)** performing the Mann–Whitney U-tests.

Pathway enrichment analysis

We expected that, compared with HCs, LVV patients would show enriched immune- and inflammation-related pathways. Pathway enrichment analysis showed that pathways associated with non-alcoholic fatty liver disease and interleukin (IL)-1 signalling were over-represented among the 267 upregulated genes (Figure 2c). Pathways associated with endochondral ossification, hair follicle development, development and heterogeneity of the innate lymphoid cell family, white fat cell differentiation, allograft rejection and T-cell antigen receptor signalling were included among the 201 downregulated genes (Figure 2d).
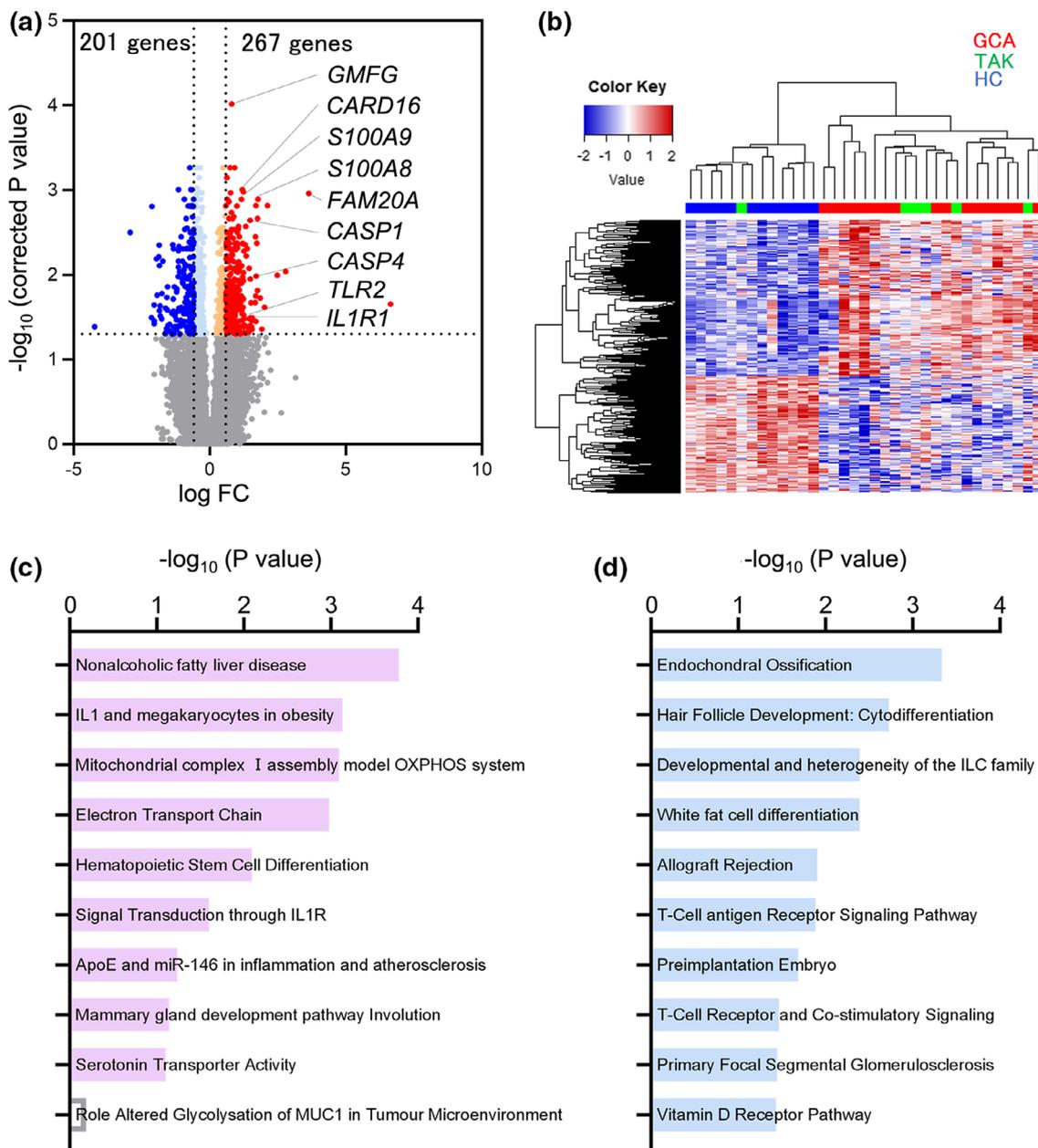
Figure 2. Differentially expressed transcripts and pathways in LVV (week 0) and healthy controls (HCs). **(a)** A volcano plot showing differentially expressed genes (DEGs) between LVV and HCs (FC > 1.5, FDR < 0.05). **(b)** A hierarchical clustering heatmap of 267 upregulated DEGs, represented by normalised gene expression values. Red, giant cell arteritis; green, Takayasu's arteritis (TAK); and blue, healthy control (HC). Top 10 **(c)** upregulated and **(d)** downregulated pathways in LVV compared with HCs, as determined by the WikiPathways analysis of differentially expressed transcripts. **c** and **d**: pink and blue, *P* < 0.05; grey, *P* ≥ 0.05.

Correlations with clinical disease parameters

Next, we performed a weighted gene correlation network analysis (WGCNA) to assess how the LVV signature changes with disease activity. Gene expression data from patients with active LVV before induction therapy and HCs were explored by dividing them into 49 transcript co-expression modules (Figure 3a). WGCNA revealed specific modules that correlated with the Indian Takayasu clinical activity score (ITAS), PETVAS, serum CRP
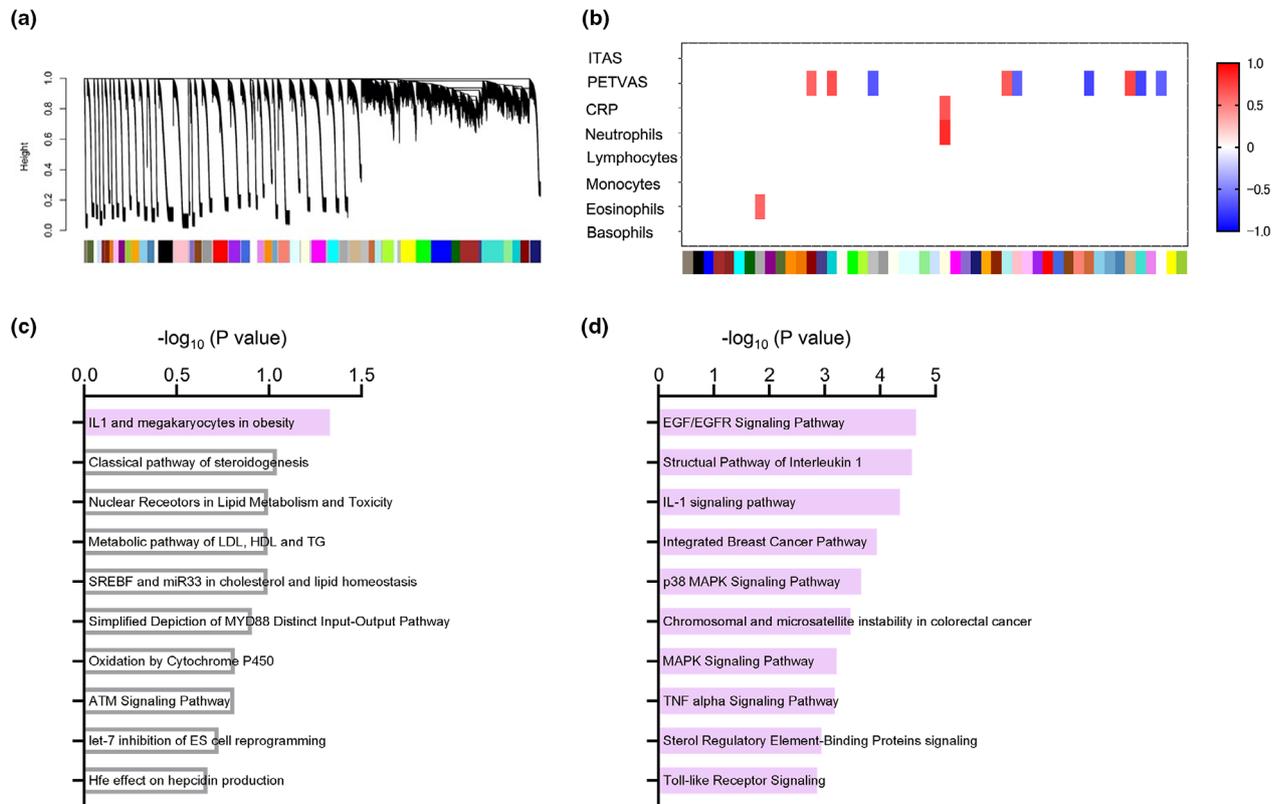
(a)

(b)

(c)

(d)

Figure 3. Molecular characteristics correlated with clinical parameters. **(a)** Forty-nine transcript co-expression modules were identified using the weighted gene correlation network analysis (WGCNA). **(b)** Modules correlated with the Indian Takayasu clinical activity score (ITAS), positron emission tomography vascular activity score (PETVAS), serum C-reactive protein (CRP) level, neutrophil count, lymphocyte count, monocyte count, eosinophil count, and basophil count at LVV diagnosis. Top 10 pathways in **c** CRP/neutrophil count-correlated modules and **(d)** PETVAS-correlated modules. **c** and **d**: pink, $P < 0.05$; grey, $P \geq 0.05$.

level, neutrophil count, lymphocyte count, monocyte count, eosinophil count and basophil count at LVV diagnosis (Figure 3b). CRP levels and neutrophil counts correlated with the same module, which was related to the IL-1 pathway ($P = 0.047$; Figure 3c). The PETVAS correlated significantly with several modules, none of which overlapped with the CRP/neutrophil count-correlated module. The PETVAS correlated positively with modules related to the Toll-like receptor (TLR) ($P = 0.0014$) and IL-1 pathways ($P < 0.001$), as shown in Figure 3d.

Residual gene expression during treatment with PSL, IFX and TCZ

The overarching goal of our study was to understand the extent to which drug treatment returns the molecular phenotypes of patients with LVV to a healthy state. We compared DEGs in LVV patients and HCs at week 0, week 6 and remission

to assess how treatment influenced the blood transcriptional signatures of patients with LVV (Figure 4a and b). Of 267 genes upregulated in active LVV, 150 remained high during remission in patients treated with PSL (Figure 4a). Genes associated with IL-1 signalling pathways were residually expressed during PSL treatment (Figure 4c). Genes that were residually upregulated during treatment are shown in Supplementary table 2. Combined treatment with PSL plus IFX or TCZ normalised the molecular profiles in patients with LVV more efficiently than treatment with PSL monotherapy did, at the transcriptome level (Figure 4a and b).

Identification of the initial gene signature associated with treatment responses

Patients were categorised into responder and non-responder groups, as defined in the Methods
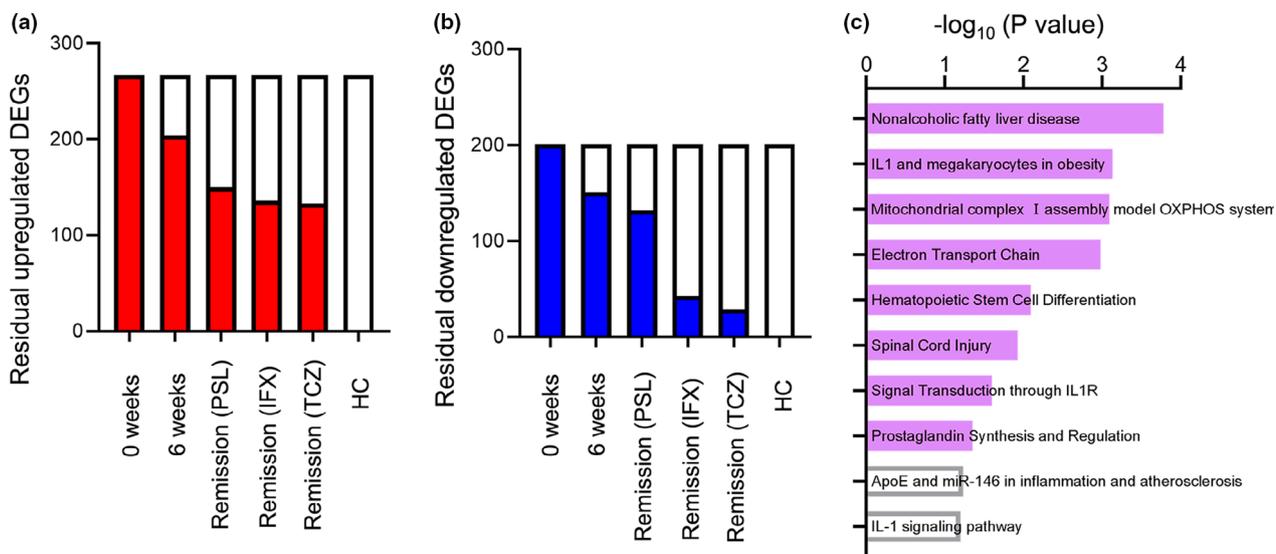
Figure 4. Identification of residual molecular profiles across treatments. **(a)** Red and **(b)** blue bars show the number of upregulated and downregulated differentially expressed genes (DEGs), respectively, at weeks 0 and 6 and during remission under treatment with prednisolone (PSL), infliximab (IFX) and tocilizumab (TCZ), in comparison with healthy controls. **(c)** The WikiPathways analysis of 150 genes with higher residual expression under treatment with PSL. Pink, $P < 0.05$; grey, $P \geq 0.05$.

section. We calculated the AUC to test the validity of gene expression levels at weeks 0 and 6 in discriminating responders from non-responders. The expression levels of 2 and 37 protein-coding genes were significantly higher in non-responders than in responders at week 0 and week 6 respectively (all $P < 0.05$; Supplementary table 3). The top 20 genes with high AUC values are shown in Figure 5a and b. We also compared serum cytokine levels between responders and non-responders at week 0 (Figure 5c) and week 6 (Figure 5d). The AUC values related to gene expression levels were significantly higher at week 6 than those at week 0, as were the serum cytokine levels. For example, the AUC values of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A for predicting disease relapse were 0.71 at week 0 ($P = 0.056$) and 0.91 at week 6 ($P = 0.0019$). Considering the AUC levels shown in Figure 5a–d, 37 gene expression levels at week 6 predicted relapse with high sensitivity and specificity. The expression levels of these 37 genes were higher in non-responders than in responders. Network analysis for the selected 37 genes using GeneMANIA via Cytoscape 3.8.0 (National Institute of General Medical Sciences, Bethesda, MD, USA) highlighted the TLR2–IL-1 pathways (Figure 5e),[20] which showed significantly greater predictive power for relapse.

Longitudinal expression of genes and serum cytokines related to the IL-1 signalling pathway

Expression levels of representative genes and serum cytokines included in the IL-1 signalling pathway are shown in Figure 6. Among them, higher expression of TLR2, IL-1 receptor type 1 (IL-1R1), caspase-1 and caspase-4 was observed at week 6 in non-responders compared with responders (Figure 6a). The expression levels of these genes gradually increased from week 0 to week 6 in non-responders, but not in responders. Furthermore, the gene expression levels of IL-1R1 and caspase-4 were higher during relapse than during remission (all $P < 0.05$). Serum levels of IL-1β, IL-6, IL-8, IL-10, IL-13, IL-18 and tumor necrosis factor (TNF)-α were increased in patients with LVV; however, analysis of their longitudinal expression did not reveal any significant differences between responders and non-responders (Figure 6b and Supplementary figure 2).

DISCUSSION

Conventionally, LVV is diagnosed and monitored by performing clinical assessment of acute-phase reactants. While protein measurements and
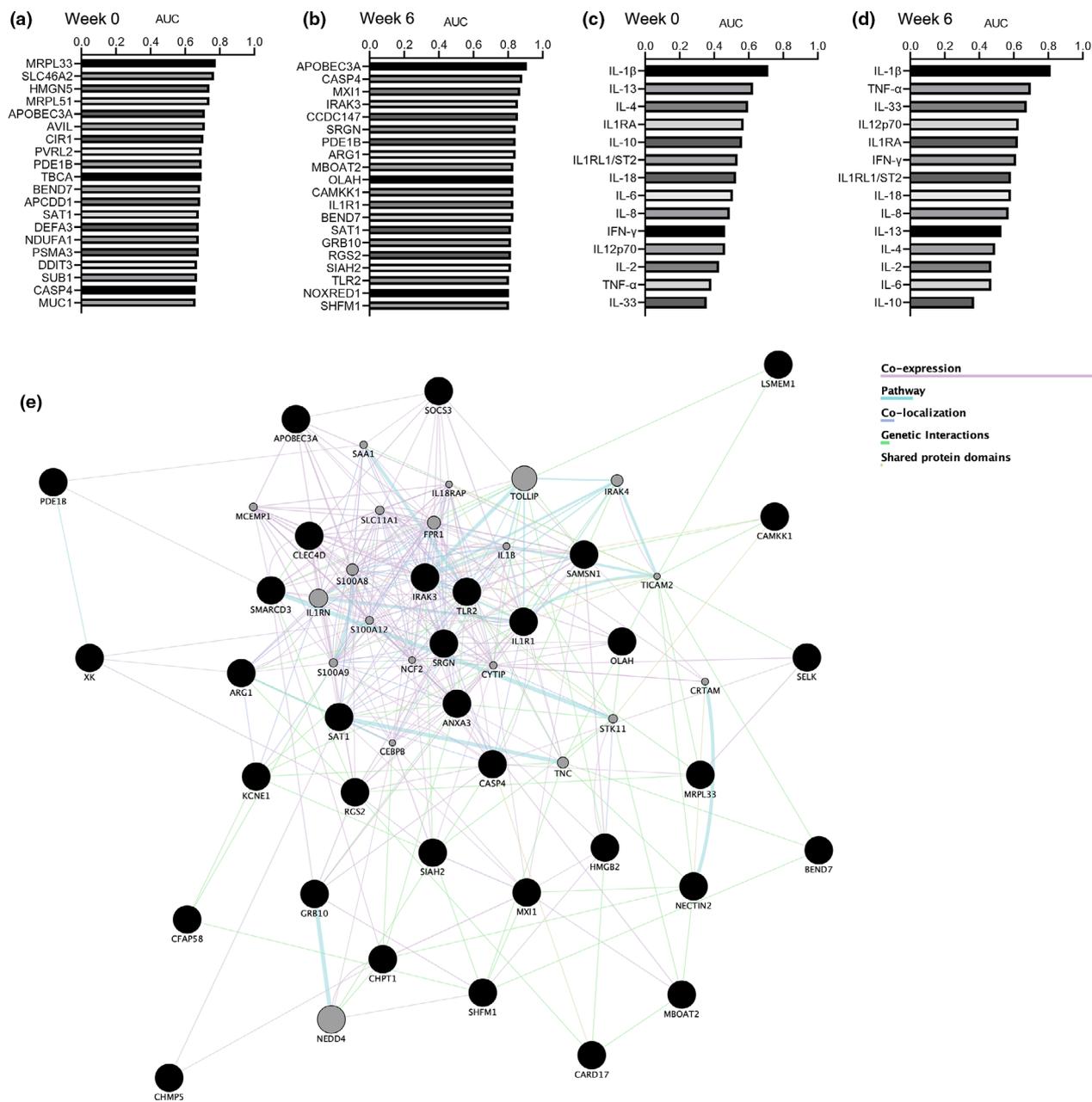
Figure 5. Significance of genes and cytokines associated with disease relapse. Rank of the area under the curve (AUC) to test the validity of gene expression at **(a)** week 0 and **(b)** week 6 in discriminating responders from non-responders. Rank of the AUC to test the validity of cytokine expression at **(c)** week 0 and **(d)** week 6 in discriminating responders from non-responders. **(e)** Interactions among 37 significantly upregulated genes in non-responders at week 6.

profiling peripheral blood cells and vasculitis tissue have identified potential biomarkers associated with LVV,[11–19] most studies to date have focused on preselected cell types or limited molecular pathways. Although CD4 and CD8 T cells have been implicated in LVV pathogenesis,[21] our longitudinal transcriptome analysis suggests that LVV pathogenesis is prominently associated with innate inflammation. In this study, we showed that levels of gene expression associated with IL-1 signalling and non-alcoholic fatty liver disease were upregulated in LVV. The IL-1 signalling was characteristic for LVV and was associated with the PETVAS (a disease-extent
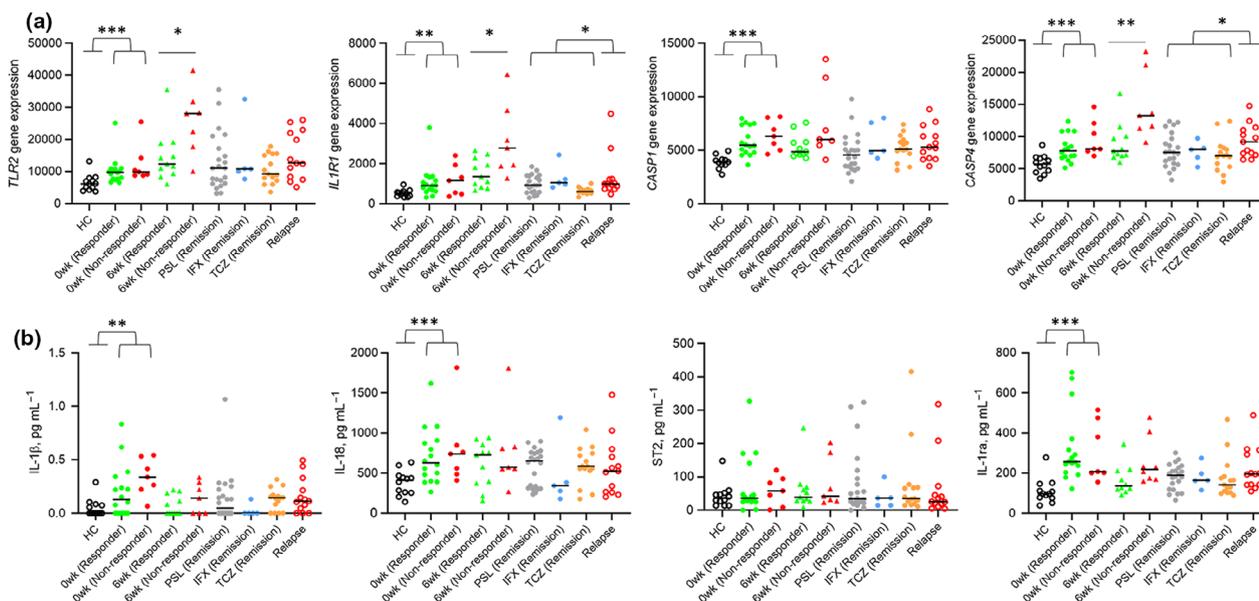
Figure 6. Longitudinal expression of genes and serum cytokines related to the interleukin (IL)-1 signalling pathway. Expression levels of representative **(a)** genes and **(b)** serum cytokines (*n* = 3) in the IL-1 signalling pathway at weeks 0 or 6; remission under treatment with prednisolone (PSL), infliximab (IFX) and tocilizumab (TCZ); and relapse. *P < 0.05, **P < 0.01 and ***P < 0.001, as determined using the Mann–Whitney *U*-test.

score). We also found that they had greater sensitivity and specificity to predict a poor prognosis compared with serum cytokines. Non-alcoholic fatty liver disease pathway includes ubiquinol-cytochrome *c* reductase binding protein, ubiquinol-cytochrome c reductase hinge protein and NADH dehydrogenase family proteins, which are associated with mitochondrial metabolism. Considering that the prevalence of fatty liver disease was not different between LVV patients and HCs, upregulation of the non-alcoholic fatty liver disease pathway may reflect the activation of mitochondrial metabolism.

Whether LVV should be classified as an autoimmune disease or an autoinflammatory disease has been widely discussed. Mutoh *et al.* recently identified new anti-endothelial cell autoantibodies in patients with TAK using an expression cloning system. However, at least 30% of TAK cases and most cases of GCA are autoantibody-negative.[22] Upregulation of IL-1 signalling was also reported in patients with Kawasaki disease and Behçet's disease,[23–25] but not in vasculopathy in patients with systemic sclerosis.[26] LVV patients often show high fever, high CRP level and vessel wall thickening (aneurysms), which are common symptoms of Kawasaki disease and Behçet's disease known as

autoinflammatory diseases caused by abnormalities of innate immune systems, suggesting that acceleration of innate immunity may be involved in the pathogenesis of LVV.

Our longitudinal transcriptome analysis revealed that the levels of molecules associated with TLR and IL-1 signalling remained high in LVV patients who achieved remission with PSL treatment. Further analysis revealed that the inflammasome pathway represented by TLR2 and IL-1 signalling was particularly associated with disease relapse. Pryshchep *et al.*[27–29]; reported that vessel-specific TLRs were involved in vascular inflammation in particular, TLR2 was ubiquitously expressed on inflamed temporal, carotid, subclavian, thoracic, mesenteric and iliac arteries.[29] Acute-phase serum amyloid A, an endogenous TLR2 ligand, was highly elevated in patients with active LVV.[16] Serum amyloid A showed proinflammatory and proangiogenic effects in an *ex vivo* temporal artery culture model.[29] Recent evidence showed that severe acute respiratory syndrome coronavirus 2 infection activates TLR signalling in innate immune cells, which may lead to the development of LVV.[30,31]

Immune cells capable of producing large amounts of IL-1β include monocytes, macrophages, dendritic cells and neutrophils. IL-1β

binds to IL-1R1 on target cells, leading to the activation of IL-1 receptor-associated kinase (IRAK) and NF-κB. In our study, caspase-1, caspase-4, IRAK3 and IL-1R1 were upregulated in samples from patients with LVV. Inflammasome-dependent IL-1β release and its downstream pathway may contribute to LVV pathogenesis. Excessive transduction of IL-1β signalling may alter the phenotype of the endothelial vascular system and subsequently induce lymphocyte activation.[32] This hypothesis is supported by the finding that IL-1 receptor antagonist-deficient (IL-1rn$^{-/-}$) mice spontaneously develop aortitis, similar to TAK.[33,34] These results are inconsistent with many previous findings related to acquired immunity. The IL-1 signalling pathway links innate and adaptive immunity, facilitating the differentiation between lymphocytes and innate immune cells.

Recent clinical trials of TCZ have revealed that therapies targeting IL-6 can effectively reduce LVV relapse and the dose of PSL required for effective treatment.[35,36] Furthermore, TNF-α inhibition-based therapies with, for example, IFX, etanercept and adalimumab are effective against TAK.[37,38] Like IL-6 and TNF-α, IL-1β is highly expressed in inflamed arterial walls of LVV patients and may contribute to its pathogenesis.[39,40] Ly *et al.* showed that anakinra was effective in refractory GCA patients, improving inflammatory biomarker levels and symptoms, and promoting the disappearance of arterial inflammation on PET-CT images.[41] Thus, using targeted therapeutics against the IL-1 signalling may be a promising approach for treating LVV.

This study had several limitations. First, since we used whole blood RNA, transcriptional changes were likely to reflect alterations in cell compositions in whole blood. Second, since the data from healthy subjects were not evaluated at multiple time points, natural variation in mRNA expression could not be completely excluded. Third, because of the small sample size, we were unable to separate patients into training and test sets for validation. Fourth, we analysed patients with GCA and TAK together, even though these represent different forms of LVV. Further investigation may clarify the role of the inflammasome represented by the TLR2–IL-1 pathway in LVV and contribute to the development of innovative medicines for personalised treatment, consistent with the underlying molecular mechanisms and the achievement of deeper molecular remission.

METHODS

Patients with LVV and HCs

Consecutive patients with LVV presenting at Keio University Hospital between August 2013 and May 2019, who fulfilled the American College of Rheumatology criteria for GCA and TAK,[42,43] were enrolled in this study. The whole blood samples were obtained from patients during week 0 (at the time of diagnosis prior to initiating induction therapy); after 6 weeks of treatment; at remission following treatment with PSL, IFX and TCZ; and at the time of disease relapse. All patients received daily PSL therapy at an initial dose equivalent to 0.6–1.0 mg per kg body weight, which was used as PSL monotherapy or in combination with other biologics. We recruited 12 individual HCs for comparison purposes with the LVV patients.

This study was approved by the Institutional Review Board of Keio University School of Medicine (approval number #20140479) and was conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from all participating individuals.

Clinical assessment

Clinical information was obtained from the patients' records and annual medical check-up reports. We collected information pertaining to age; sex; race; BMI; smoking habits; comorbidities including hypertension, diabetes mellitus, dyslipidaemia, fatty liver disease, chronic kidney disease, polymyalgia rheumatica, inflammatory bowel disease and aortic regurgitation; the time from symptom onset to diagnosis; laboratory data on the ESR; white blood cell counts, haemoglobin and platelet counts; CRP levels at diagnosis and each visit; and treatment during follow-up. Arterial involvement was evaluated using, for example, any or all of the following histological and/or radiological examinations: ultrasonography, CT, ^{18}F-fluorodeoxyglucose PET-CT and magnetic resonance imaging. PETVASs at LVV diagnosis were calculated based on the PET-CT scan findings.

Disease assessment was performed at each visit. Remission was defined as the absence of clinical symptoms with a normal CRP level that was maintained for at least 12 weeks.[35] Relapse was defined as the reappearance of vasculitis-related manifestations or exacerbation of imaging findings requiring an increase in the PSL dose or additional immunosuppressive agents.[35] Patients were divided into responder and non-responder groups based on whether they experienced relapse during the 60 weeks of treatment.

Sample preparation

Blood samples were collected in PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland). Total RNA was extracted from each whole blood sample using a PAXgene Blood miRNA Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The integrity and quantity of the total RNA were measured with an Agilent 2100 Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). All RNA samples fulfilled the criterion of an RNA integrity value of > 7.

Library preparation and sequencing

Sequencing libraries were constructed using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) with a NEBNext Poly(A) mRNA Magnetic Isolation Module, according to the manufacturer's protocols. We used a QIAseq FastSelect RNA Removal Kit (human globin RNA; Qiagen) to remove globin mRNAs during library construction. The quality of the libraries was assessed using an Agilent 2200 TapeStation High Sensitivity D1000 System (Agilent Technologies). The pooled libraries of samples were sequenced using a HiSeq 500 instrument (Illumina, Inc., San Diego, CA, USA) with 76-base pair single-end reads.

Alignment with the whole transcriptome

Sequencing adaptors and low-quality reads and bases were trimmed with the Trimmomatic-0.38 tool.[44] The sequence reads were aligned to the human reference genome (hg19) using STAR, version 2.7.1a.[45]

Quantifying gene expression levels and detecting DEGs

The aligned reads were subjected to downstream analyses using the StrandNGS 3.2 software (Agilent Technologies). The read counts allocated for each gene and transcript (Ensembl Genes 2014.01.02) were quantified using the trimmed mean of the M-value (TMM) method.[46] To identify DEGs between the LVV and HCs, transcripts below the FDR < 0.05 with a fold change in expression > 1.5 between cases and controls were considered significantly differentially expressed.

Pathway analysis

The pathway statistical analysis was performed on a pathway collection of the WikiPathways database using the PathVisio tool to determine pathways that were dysregulated the most.[47,48] The analysis factored in the number of genes in each pathway that was measured and that was differentially expressed.

Hierarchical cluster analysis

To elucidate differences in gene expression levels among LVV patients and HCs, hierarchical cluster analysis was performed. To explore and visualise the subgroups identified based on the gene expression data, TMM-normalised data were analysed based on matrices of mean Euclidean distances using Ward's method.[49]

WGCNA

We used the WGCNA method to identify specific gene networks. Correlation modules were identified using the WGCNA method and the affinity propagation algorithm, as published previously.[50] Pearson's correlation coefficients were used to analyse the correlations between each module and different clinical parameters.

Genes associated with clinical response to treatment with PSL, IFX and TCZ

LVV is primarily managed by treatment with PSL, IFX and TCZ in our hospital. To identify novel biomarkers associated with clinical responses to treatment with PSL, IFX and TCZ, we compared gene expression levels in patients in remission with those in HCs. We defined DEGs as genes showing differential expression (versus HCs) even after treatment with PSL, IFX and TCZ.

Association of the initial gene signature with the treatment response

We hypothesised that the gene expression differences before and after treatment may indicate a potential risk for disease relapse in patients with LVV. We compared gene expression levels at weeks 0 and 6 between responders and non-responders and calculated the AUC by the receiver operating characteristic (ROC) curve analysis. We also investigated serum cytokine levels and compared them between responders and non-responders at weeks 0 and 6.

Serum inflammatory cytokine assay

Serum levels of IL-1β, IL-1 receptor antagonist, IL-1 receptor-like 1/ST2, IL-2, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-18, IL-33, interferon-γ and TNF-α were determined using a commercial electrochemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD, USA), according to the manufacturer's protocol. Three technical replicate experiments were carried out. Serum samples were stored at −80°C prior to the assay. We then compared the expression levels of proteins and genes at weeks 0 and 6 between responders and non-responders, with the optimal cut-off value for discrimination determined by the ROC analysis.

Statistical analysis

All analyses were conducted using the R statistics package version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria), SPSS Statistics version 26.0 (IBM Corp., Armonk, New York, USA), JMP software version 14.0 (SAS Institute, Cary, NC, USA) or GraphPad Prism software version 8.0 (GraphPad, San Diego, CA, USA). Continuous data were expressed as the median (interquartile range), and categorical data were expressed as the number and/or percentage. Descriptive statistics were used to summarise the data. Continuous variables were compared using the Mann–Whitney *U*-test, and categorical variables were compared using the chi-squared test. The threshold for statistical significance was set at *P* < 0.05.

CONFLICT OF INTEREST

AUTHOR CONTRIBUTIONS

Kotaro Matsumoto: Data curation; Formal analysis; Investigation; Methodology; Writing-review & editing. **Katsuya Suzuki:** Data curation; Formal analysis; Writing-review & editing. **Keiko Yoshimoto:** Data curation; Methodology. **Sho Ishigaki:** Data curation; Methodology. **Hiroto Yoshida:** Methodology; Project administration. **Mayu Magi:** Methodology; Project administration. **Yoshihiro Matsumoto:** Methodology; Project administration. **Yuko Kaneko:** Data curation; Formal analysis; Writing-review & editing. **Tsutomu Takeuchi:** Conceptualization; Funding acquisition; Project administration; Supervision.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.