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Neutrophil-Related Gene Expression and Low-Density Granulocytes Associated With Disease Activity and Response to Treatment in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis

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Objective. **To discover biomarkers involved in the pathophysiology of antineutrophil cytoplasmic antibody–associated vasculitis (AAV) and to determine whether low-density granulocytes (LDGs) contribute to gene expression signatures in AAV.**

Methods. **The source of clinical data and linked biologic specimens was a randomized controlled treatment trial in AAV. RNA sequencing of whole blood from patients with AAV was performed during active disease at the baseline visit and during remission 6 months later. Gene expression was compared between patients who met versus those who did not meet the primary trial outcome of clinical remission at 6 months (responders versus nonresponders). Measurement of neutrophil-related gene expression was confirmed in peripheral blood mononuclear cells (PBMCs) to validate the findings in whole blood. A negative-selection strategy isolated LDGs from PBMC fractions.**

Results. **Differential expression between responders (n = 77) and nonresponders (n = 35) was detected in 2,346 transcripts at the baseline visit ($P < 0.05$). Unsupervised hierarchical clustering demonstrated a cluster of granulocyte-related genes, including myeloperoxidase (MPO) and proteinase 3 (PR3). A granulocyte multigene composite score was significantly higher in nonresponders than in responders ($P < 0.01$) and during active disease than during remission ($P < 0.01$). This signature strongly overlapped an LDG signature identified previously in**

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lupus (false discovery rate by gene set enrichment analysis <0.01). Transcription of PR3 measured in PBMCs was associated with active disease and treatment response ($P < 0.01$). LDGs isolated from patients with AAV spontaneously formed neutrophil extracellular traps containing PR3 and MPO.

Conclusion. **In AAV, increased expression of a granulocyte gene signature is associated with disease activity and decreased response to treatment. The source of this signature is likely LDGs, a potentially pathogenic cell type in AAV.**

Granulomatosis with polyangiitis (Wegener's) (GPA) and microscopic polyangiitis (MPA) are two forms of antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) (1). Advances in therapy have transformed AAV from a frequently fatal disease into a chronic illness; however, a proportion of patients with AAV do not achieve remission after an initial course of therapy, and even fewer achieve sustained remission over long-term followup (2). The Rituximab in ANCA-Associated Vasculitis (RAVE) trial expanded the standard treatment options for patients with AAV by demonstrating that rituximab was not inferior to cyclophosphamide as effective remission-induction therapy (3). Despite the success of the trial, only 115 of 197 (58%) study patients in both treatment groups achieved the primary trial outcome, which was defined as complete remission without glucocorticoids at the 6-month study visit, and only 71 patients (36%) remained in stable remission throughout the 18 months of followup (4).

There are currently few clinical and serologic markers that predict clinical outcomes in AAV, and none of these markers have been shown to reliably guide treatment decisions (5–8). While ANCA plays an undisputed diagnostic role in AAV (9), the value of serial measurement of ANCA in predicting clinical outcomes is a subject of controversy (10). Identification of novel therapeutic targets and biomarkers that predict clinical outcomes for use in guiding patient-specific therapeutic decisions is a high priority in AAV.

Neutrophils play an important role in the pathogenesis of AAV (11). Patients with AAV typically have antibodies directed against proteinase 3 (PR3) or myeloperoxidase (MPO), which are neutrophil granular proteins. In vitro studies have shown an activating effect of ANCA on cytokine-primed neutrophils (12), and the pathogenic potential of ANCA has been established in animal models (13,14). Our understanding of the potential roles of neutrophils in AAV has been expanded with the discovery of neutrophil extracellular traps (NETs) (15). NETs are a meshwork of chromatin fibers that contain granule-derived peptides and enzymes and are extruded by neutrophils following various sources of stimulation (16). NETs play a role in host defense against pathogens; however, evidence also implicates NETs in the pathogenesis of many autoimmune diseases, including AAV (17,18). Upon binding to the surface membrane of neutrophils, ANCAs can directly stimulate the extrusion of NETs (NETosis) in vitro (19–21). NETs can be seen at sites of active glomerulonephritis and within disease-associated thrombi in AAV (20,22).

Low-density granulocytes (LDGs) are a distinct subset of neutrophils that colocalize with peripheral blood mononuclear cells (PBMCs) in density gradient preparations (23). Circulating LDGs are not detected in healthy subjects; however, LDGs are abundant in the blood of patients with systemic lupus erythematosus (SLE), where they have been characterized as being proinflammatory, capable of synthesizing type I interferons, and cytotoxic to endothelial cells (24–26). In contrast to normal-density neutrophils, which typically undergo NETosis in ex vivo analyses only upon exposure to proinflammatory stimulants, LDGs undergo spontaneous NETosis without stimulation (24). To date, LDGs have not been described in the blood of patients with AAV; however, 2 whole-genome gene expression profiling studies in AAV have identified granulocyte signatures in PBMC fractions isolated in density gradient preparations, implying that LDGs may be the source cell of these expression signatures (27,28).

The objectives of this study were to identify potential clinical biomarkers in AAV through whole-genome gene expression profiling and to determine whether LDGs are present in AAV and contribute to gene expression signatures derived from blood.

PATIENTS AND METHODS

Patient selection and characterization. Patients were selected from participants in the RAVE trial. Study design details of this trial have been reported elsewhere (3). For the present study, patients were selected based upon clinical outcome data at 6 months. Patients in either treatment assignment group who met the primary outcome in the RAVE trial, which was disease remission without glucocorticoids at month 6, were classified as responders. Patients who did not meet the primary outcome in the RAVE trial were classified as nonresponders.

Ethics and informed consent. All patients enrolled in the RAVE trial or evaluated at the National Institutes of Health (NIH) provided written informed consent for collection and future use of biologic samples and data. Participating ethics boards approved the research.

Sample collection, storage, and processing. Peripheral venipuncture was used to collect 3-ml blood samples directly into Tempus blood RNA tubes at the baseline visit (prior to treatment with rituximab or cyclophosphamide) and

at month 6. RNA was isolated in 2 separate batches using Applied Biosystems RNA chemistry. A complete blood cell count with differential cell count was determined concurrently with collection of each RNA sample. Serum samples were tested for ANCA by means of direct and capture enzyme-linked immunosorbent assays (ELISAs). PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation at each study center and stored in liquid nitrogen until further use.

Deep sequencing, alignment, and quantification of large RNA. Deep sequencing of large RNA (RNA-Seq) was performed by Expression Analysis using an Illumina Genome Analyzer IIx as outlined in the Illumina TruSeq RNA Sample Preparation Guide. Globin reduction was performed on whole blood to reduce the proportion of large RNA from the globin genes, which can interfere with the detection of less abundant gene transcripts (29). Complementary DNA (cDNA) libraries were built for single-read and paired-end sequencing using standard procedures. Starting with 500 ng of total RNA, mRNA was purified by poly(A) selection, chemically fragmented, and reverse transcribed using oligo(dT) primers. Following second-strand synthesis, adapters were ligated to the 3′ end. Polymerase chain reaction (PCR) was performed to amplify and enrich the ligated material to create the final cDNA library.

Sequencing reads were aligned to the human genome using Bowtie2 software (30). Local alignment was applied without overlap, treating overlapping mates as discordant. Discordant or unpaired alignment was not allowed. Bam files were imported into Partek Genomic Suites for RNA quantification analysis. Ensembl transcripts (release 64) were used for the annotation source. Transcript-level raw data without normalization were used for differential expression analyses.

Quantitative reverse transcription–PCR (qRT-PCR) analysis. RNA was isolated from PBMCs using a DNA-Free RNA kit (Zymo Research). Total RNA (500 ng) was reverse transcribed using iScript RT single-strand cDNA (Bio-Rad). We performed qPCR using TaqMan Gene Expression Master Mix (Applied Biosystems), human GAPDH primers (Hs99999905_m1) as internal control, and sequence-specific primers for MPO (Hs00924296_m1), PR3 (Hs01597752_m1), cathelicidin (Hs00189038_m1), and calprotectin S100A8 (Hs00374264_g1). Samples were run in duplicate using a C1000 Touch thermal cycler equipped with a CFX96 Touch real-time PCR detection system (Bio-Rad). Data were analyzed using Bio-Rad CFX Manager software.

Isolation of LDGs. LDGs were isolated from fresh peripheral blood samples obtained from a separate group of 5 patients with AAV sequentially evaluated at the NIH. None of these patients were participants in the RAVE trial. All of the patients fulfilled the American College of Rheumatology (ACR) 1990 classification criteria for Wegener's granulomatosis (31) as modified by the WGET Research Group (32) and were evaluated at random points in the course of disease. Healthy control subjects were recruited through an NIH healthy volunteer program.

AVV LDGs were isolated from the PBMC layer as previously described (24). Briefly, the buffy coat was incubated in sodium chloride solutions to eliminate red blood cells. PBMCs were incubated with a cocktail of biotinylated antibodies for 30 minutes on ice. Cells were then incubated with magnetic beads for 15 minutes on ice and passed through a magnetic-activated cell sorter column (Miltenyi Biotec) according to the manufacturer's instructions. LDG purity was assessed by flow cytometry as described elsewhere (24). Normal-density neutrophils from AAV patients and controls were isolated from the red blood cell layer by Dextran sedimentation.

Immunofluorescence analysis. Cells were seeded on coverslips and stimulated with 40 n*M* phorbol myristate acetate. Neutrophils and LDGs were incubated for 60 minutes at 37°C in an atmosphere of 5% CO_2. Cells were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS). After washing, cells were blocked for 30 minutes with 0.2% porcine skin gelatin (Sigma) in PBS and incubated for 1 hour at 37°C with anti-human elastase (Abcam), anti-MPO (Dako), or anti-PR3 (Santa Cruz Biotechnology) antibodies diluted in blocking buffer. After 3 washes with PBS for 5 minutes each at room temperature, cells were incubated for 30 minutes at 37°C with either Alexa Fluor 555–conjugated anti-rabbit IgG or Alexa Fluor 488–conjugated anti-mouse IgG secondary antibody. Nuclei were costained with a 1:1,000 dilution of the fluorescent dye Hoechst 33342. Coverslips were washed with PBS 3 times for 5 minutes each at room temperature and were mounted on a glass slide using ProLong Gold. Images were acquired on a Zeiss LSM 780 confocal laser scanning microscope, and quantification was performed as previously described (25).

Statistical analysis. The Bioconductor package edgeR (release 2.14) was used for differential expression analyses of read counts (33). Generalized linear models were applied to the non-normally distributed read counts. Since RNA was isolated in 2 separate batches for these experiments, adjustments for batch effect differences were made using an additive model within edgeR. Different cutoffs to define statistical significance for differential gene expression analyses were explored. A multigene composite score was created by calculating Z scores on a per-gene per-sample basis. The mean granulocyte composite score was compared between responders and nonresponders and between active disease and remission using the Wilcoxon rank sum and Wilcoxon signed rank tests.

Linear regression models were used to determine the association between the granulocyte gene composite score (dependent variable) and the following independent variables derived from the baseline study visit: age, ANCA titer by direct or capture ELISA, Birmingham Vasculitis Activity Score modified for Wegener's granulomatosis (BVAS/WG) (34), mean dose of glucocorticoids over the 14 days prior to the baseline visit blood sample collection, absolute neutrophil count, absolute lymphocyte count, hemoglobin level, B cell count, platelet count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) level. The threshold for incorporation of a variable in the multivariable linear regression models was defined as $P < 0.1$, and the threshold for statistical significance in the regression models was defined as $P < 0.05$. Logistic regression was used to determine the association between clinical outcome status (nonresponders versus responders) and the granulocyte gene composite score.

Enrichment of relevant gene set signatures was tested using gene set enrichment analysis (GSEA; v2.1.0) (35). GSEA is a computational method that can be used to determine whether a specific set of genes shows concordant differences between 2 phenotypes (e.g., nonresponders versus responders). Gene sets with an estimated false discovery rate (FDR) of <0.05 were considered significant according to the GSEA

Table 1. Baseline characteristics of the AAV study patients, by response group*

	Nonresponders (n = 35)	Responders (n = 77)
Age, mean ± SD years	51 ± 14.8	52 ± 17.9
Sex, %		
Female	46	56
Male	54	44
Race, % white	91	96
Treatment arm, %		
Cyclophosphamide/azathioprine	66	47
Rituximab	34	53
ANCA subtype, %		
Myeloperoxidase	20	35
Proteinase 3	80	65
Disease type, %		
Microscopic polyangiitis	14	26
Granulomatosis with polyangiitis (Wegener's)	86	74
New diagnosis of AAV, %	31	57†
Organ system involved, %		
Constitutional	60	53
Cutaneous	17	18
Mucous membranes, eyes	23	19
Ear, nose, throat	57	56
Cardiovascular	3	1
Gastrointestinal	3	3
Pulmonary	63	57
Renal	54	65
Nervous	17	22
Received glucocorticoids prior to baseline visit, %	89	87
Cumulative dose of glucocorticoids from 14 days before baseline sample collection, mean ± SD		
Prednisone, mg	290 ± 228.9	332 ± 322.5
Methylprednisolone, gm	0.7 ± 1.11	1.0 ± 1.32
Laboratory values, mean ± SD		
Total white blood cell count, $\times 10^3$/liter	12 ± 5.4	12 ± 4.5
Absolute neutrophil count, $\times 10^3$/liter	9 ± 5.4	10 ± 4.7
Hemoglobin, gm/dl	12 ± 1.5	11 ± 1.8‡
Platelet count, $\times 10^3$/liter	356 ± 129.9	379 ± 133.3
Lymphocyte count, $\times 10^3$/liter	1.3 ± 1.0	1.3 ± 1.0
BVAS/WG score	7.3 ± 2.8	8.3 ± 3.5
ANCA titer	221 ± 145.8	189 ± 122.0
Erythrocyte sedimentation rate, mm/hour	38	44
C-reactive protein, mg/dl	2.2	7.4

* AAV = antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis; BVAS/WG = Birmingham Vasculitis Activity Score for Wegener's Granulomatosis.
† $P = 0.02$ versus nonresponders.
‡ $P = 0.01$ versus nonresponders.

guidelines. Data were then examined via Ingenuity Pathway Analysis (IPA; Qiagen).

In the RAVE trial, PBMCs were collected concurrently with whole blood RNA at some study visits. A subset of 5 nonresponders and 5 responders was selected for gene expression analyses using PBMCs instead of whole blood as the sample source. Nonresponders and responders were matched for age, sex, ANCA specificity (PR3 versus MPO), disease subtype (GPA versus MPA), and treatment assignment (cyclophosphamide versus rituximab). Expression of the following neutrophil-related genes was studied in the PBMC fraction: PR3, MPO, CAMP, and calprotectin (S100A8). Differential expression of mRNA, as measured by qRT-PCR in the PBMC samples, was compared using one-way analysis of variance.

RESULTS

Characteristics of the study patients. The baseline clinical characteristics of the study population are provided in Table 1. There were 112 AAV patients included in the study (77 responders and 35 nonresponders). Specific reasons for inclusion in the nonresponder group included major disease flare before study month 6 (n = 9), treatment crossover for AAV flare (n = 7), BVAS/WG score >0 at month 6 (n = 18), and requirement of prednisone 15 mg/day at month 6 (n = 1). There were few statistically significant differences between responders and nonresponders. The majority of patients

Table 2. Differences in the expression of the 11 genes that make up the granulocyte multigene composite score in nonresponders versus responders

Gene name	Gene symbol	Fold change	Likelihood ratio	*P*
Myeloperoxidase	*MPO*	2.42	14.10	<0.001
Proteinase 3	*PR3*	2.65	10.33	0.001
Elastase, neutrophil expressed	*ELANE*	2.43	11.62	<0.001
Bactericidal/permeability-increasing protein	*BPI*	2.20	12.61	<0.001
Azurocidin 1	*AZU1*	2.64	12.78	<0.001
Cathepsin G	*CTSG*	2.46	12.31	<0.001
Lactoferrin	*LTF*	1.81	6.93	0.008
Defensin A3	*DEFA3*	2.15	9.21	0.002
Defensin A4	*DEFA4*	2.56	13.48	<0.001
Lipocalin 2	*LCN2*	1.93	8.73	0.003
Cathelicidin antimicrobial peptide	*CAMP*	1.66	6.76	0.009

(88%) had received high doses of glucocorticoids in the 2-week period prior to baseline sample collection, but there were no differences between responders and nonresponders in either the proportion of patients who had received glucocorticoids at study entry or the total amount of glucocorticoids received for the episode of disease activity resulting in enrollment in the trial. The percentage of patients with new (versus relapsing) disease at the time of study enrollment and the hemoglobin levels at baseline were significantly higher in the responder group.

Identification of granulocyte gene signature. After filtering transcripts expressed in <50% of both responders and nonresponders, there were 44,532 total aligned reads. Differential expression between responders and nonresponders was seen in 2,346 transcripts at the baseline visit at a threshold of $P < 0.05$. Pathway analysis of differentially expressed genes revealed up-regulation of pathways related to bacterial defense, myeloid differentiation, and neutrophil activation in nonresponders (Supplemental Figure 1 and Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39153/abstract).

Unsupervised hierarchical clustering of differentially expressed genes demonstrated a distinct cluster of 179 genes that predominantly included granulocyte-related genes as well as MPO and PR3, the major autoantigens in AAV (see Supplementary Table 2 for the complete list of genes, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39153/abstract). A multigene composite score was calculated using expression data from 11 granulocyte genes purposefully selected from the cluster of differentially expressed granulocyte genes to represent a spectrum of neutrophil granular proteins. A list of the 11 genes and the differential expression values between nonresponders and responders is provided in Table 2.

The mean granulocyte multigene composite score was higher in nonresponders (0.11; range −0.43 to 7.59) than in responders (−0.27; range −0.46 to 3.70) ($P = 0.02$). Nine of 26 nonresponders (26%) and 4 of 73 responders (5%) had granulocyte composite scores of >1.2 ($P < 0.01$ by Fisher's exact test). Clinical and demographic characteristics of patients with a baseline granulocyte composite score of >1.2 did not differ significantly from patients with a lower granulocyte composite score (data not shown).

Table 3. Linear regression models showing associations between the granulocyte multigene composite score and clinical and laboratory data*

	Univariable model			Multivariable model		
Predictor variable	Parameter estimate	Standard error	*P*	Parameter estimate	Standard error	*P*
Absolute neutrophil count	0.04	0.01	<0.01	0.03	<0.01	0.01
Platelet count	<0.01	<0.01	<0.01	<0.01	<0.01	0.14
BVAS/WG score	0.04	0.02	0.01	0.03	0.02	0.05
Age	0.01	<0.01	0.07	0.01	<0.01	0.09
Hemoglobin	−0.05	0.03	0.12	–	–	–
Absolute lymphocyte count	0.11	0.08	0.15	–	–	–
Erythrocyte sedimentation rate	<0.01	0.10	0.36	–	–	–
Cumulative glucocorticoid use	<0.01	<0.01	0.46	–	–	–
B cell count	<0.01	<0.01	0.49	–	–	–
ANCA titer	<0.01	<0.01	0.76	–	–	–
C-reactive protein	<0.01	0.06	0.80	–	–	–

* The last 7 variables listed (hemoglobin, absolute lymphocyte count, erythrocyte sedimentation rate, cumulative glucocorticoid use, B cell count, antineutrophil cytoplasmic antibody [ANCA], and C-reactive protein) were not included in the multivariable models because of a lack of significant association ($P > 0.1$) in the univariable models. BVAS/WG = Birmingham Vasculitis Activity Score for Wegener's Granulomatosis.

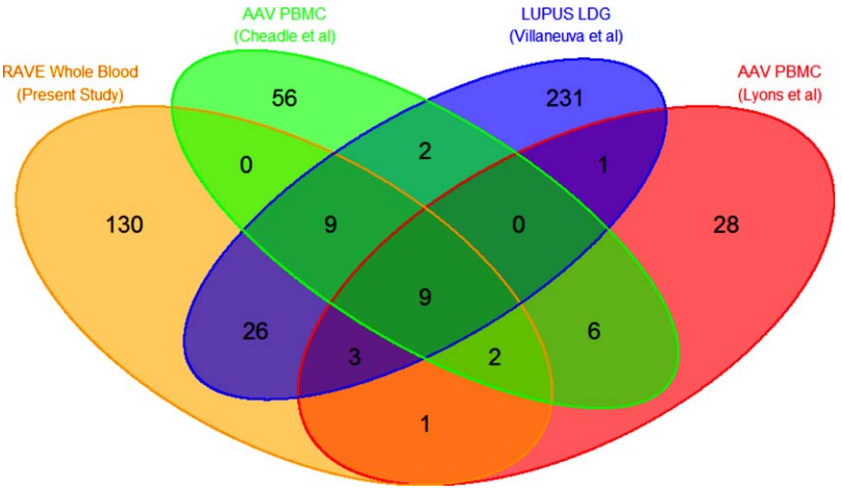

Figure 1. Gene set enrichment analyses. This Venn diagram illustrates overlap in the number of differentially expressed genes identified in 4 independent data sets. The Rituximab in ANCA-Associated Vasculitis (RAVE) trial compared whole blood gene expression between treatment responders and nonresponders in patients with antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV). The Cheadle et al (27) and Lyons et al (28) studies compared expression in peripheral blood mononuclear cells (PBMCs) between patients with AAV and healthy controls. The Villanueva et al (25) study compared expression between low-density granulocytes (LDGs) and autologous normal-density neutrophils in patients with systemic lupus erythematosus (SLE). Overlapping areas indicate genes that are common to the indicated data sets, with the number of genes indicated for each group and each area of overlap.

Whole blood RNA from the month 6 study visit was available only in the responder group. At this time point, all of these patients were in clinical remission without glucocorticoids. The median month 6 granulocyte multigene composite score for responders was higher at the baseline visit during active disease (−0.17; range −0.46 to 3.70) than at the month 6 visit during clinical remission (−0.39; range −0.47 to 0.53) ($P < 0.01$).

Association of granulocyte multigene composite score with clinical outcomes. Linear regression models were used to determine the association between the granulocyte gene composite score (dependent variable) and several clinical variables. Age, BVAS/WG score, absolute neutrophil count, and platelet count were significantly associated with the granulocyte gene composite score on univariable analyses. Only the absolute neutrophil count remained significantly associated in a multivariable regression model (Table 3).

Logistic regression was used to determine the association between clinical outcome status (nonresponders versus responders) and the granulocyte gene composite score. In univariable models, clinical outcome status was significantly associated only with the granulocyte gene composite score and hemoglobin level. A 1-unit increase in the granulocyte gene composite score at baseline was significantly associated with a 1.77 times increased odds of not meeting the primary outcome in the RAVE trial (OR = 1.77 [95% confidence interval (95% CI) 1.15–2.73], $P = 0.01$); this association remained significant after adjustment for age, BVAS/WG score, absolute neutrophil count, hemoglobin level, and glucocorticoid use (OR = 2.13 [95% CI 1.16–3.90], $P = 0.01$). Logistic regression analysis using the granulocyte composite score as a dichotomous outcome variable (>1.2 versus ≤1.2) showed no significant associations with any of the clinical, demographic, or laboratory variables, including ANCA subtype and absolute neutrophil count (data not shown). These analyses demonstrated that the multigene granulocyte composite score was significantly associated with treatment response status in the RAVE trial participants even after adjustment for potential confounding variables.

Enrichment of LDG signature. Given the presence of a granulocyte gene signature detected in whole blood samples from patients with AAV in the RAVE trial, GSEA was used to identify potential cell populations as sources of the signature. Differential expression of granulocyte genes measured in the PMBC fraction from patients with AAV as compared to healthy controls has been reported in 2 independent whole-genome microarray studies (27,28). The list of differentially expressed granulocyte gene sets from both of these prior studies was significantly enriched in the nonresponder group in RAVE (enrichment scores for both studies 0.86, $FDR_{GSEA} < 0.001$). A gene signature derived by comparing differential whole-genome gene expression from isolated LDGs versus autologous normal-density neutrophils in patients with SLE has previously been reported (25). Forty-one of the 281 genes that defined

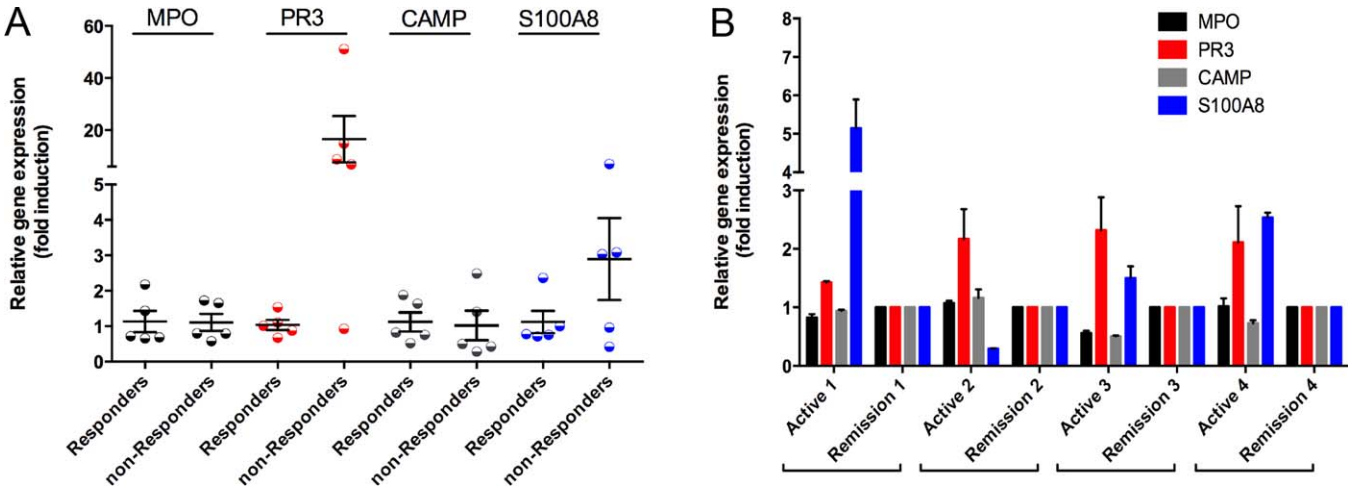

Figure 2. Differential expression of neutrophil-related mRNA in the peripheral blood mononuclear cell (PBMC) fraction. **A,** Expression of proteinase 3 (PR3) and calprotectin (S100A8), but not myeloperoxidase (MPO) or cathelicidin (CAMP), is differentially up-regulated in PBMCs from treatment nonresponders as compared to responders in the Rituximab in ANCA-Associated Vasculitis (RAVE) trial. **B,** Expression of PR3 and S100A8 is differentially up-regulated in PBMCs from the same patients with antineutrophil cytoplasmic antibody–associated vasculitis during active disease as compared to remission.

an LDG signature in the lupus cohort overlapped with differential gene expression in the RAVE trial nonresponders, including 9 of the 11 granulocyte composite score genes (enrichment score 0.79, $FDR_{GSEA} < 0.001$).

There was substantial overlap of differentially expressed genes between the present study, the 2 published studies of PBMCs in AAV, and the study of LDGs in SLE (Figure 1). Nine genes (BPI, CAMP, CEACAM6, DEFA4, HP, MS4A3, PGLYRP1, RETN, and TCN1) were present in all 4 data sets. See Supplementary Figure 2 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39153/abstract) for further information regarding the enrichment analyses.

Validation of granulocyte gene signature in PBMCs. To determine whether the granulocyte gene signature identified in whole blood originated from LDGs, neutrophil-related gene expression was measured in the PBMC fraction in a subset of responders and nonresponders. Significant differential transcription of PR3 between responders and nonresponders was observed. Expression of PR3 was up-regulated 17-fold in 4 of 5 nonresponders as compared to the responders ($P < 0.01$). There was increased expression of S100A8 in nonresponders, although it was not statistically significant ($P = 0.18$). No significant differences were observed for MPO or CAMP (Figure 2A).

PBMCs were available for paired sample analyses in 4 responders from both the baseline (active disease) and month 6 (remission) study visits. Transcription of PR3 was significantly increased in all 4 patients during active disease versus remission ($P < 0.01$). Differences in S100A8 expression were observed in 3 of 4 patients. There were no differences in MPO or CAMP (Figure 2B). These experiments validate the findings in whole blood and provide additional evidence that LDGs are the source of the whole blood granulocyte signature.

Direct isolation of LDGs in the independent cohort. To confirm whether LDGs are present in patients with AAV and to account for the neutrophil signature observed in PBMCs, normal-density neutrophils and LDGs were isolated in 60 ml of blood obtained from a separate group of 5 patients with AAV. Three of these patients were evaluated at 2 separate study visits 6 months apart. The clinical characteristics of these patients are provided (see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39153/abstract). LDGs were identified and quantified in all 5 patients with AAV at every study visit (Supplementary Table 3). Spontaneous NETosis was observed in LDGs from all 5 patients at every visit (Figure 3A). Unstimulated normal-density neutrophils and LDGs from patients with AAV underwent significantly more NET formation compared to unstimulated normal-density neutrophils from healthy controls (Figure 3B). NETs derived from LDGs in patients with AAV, irrespective of ANCA subtype, externalized both PR3 and MPO (Figure 3C).

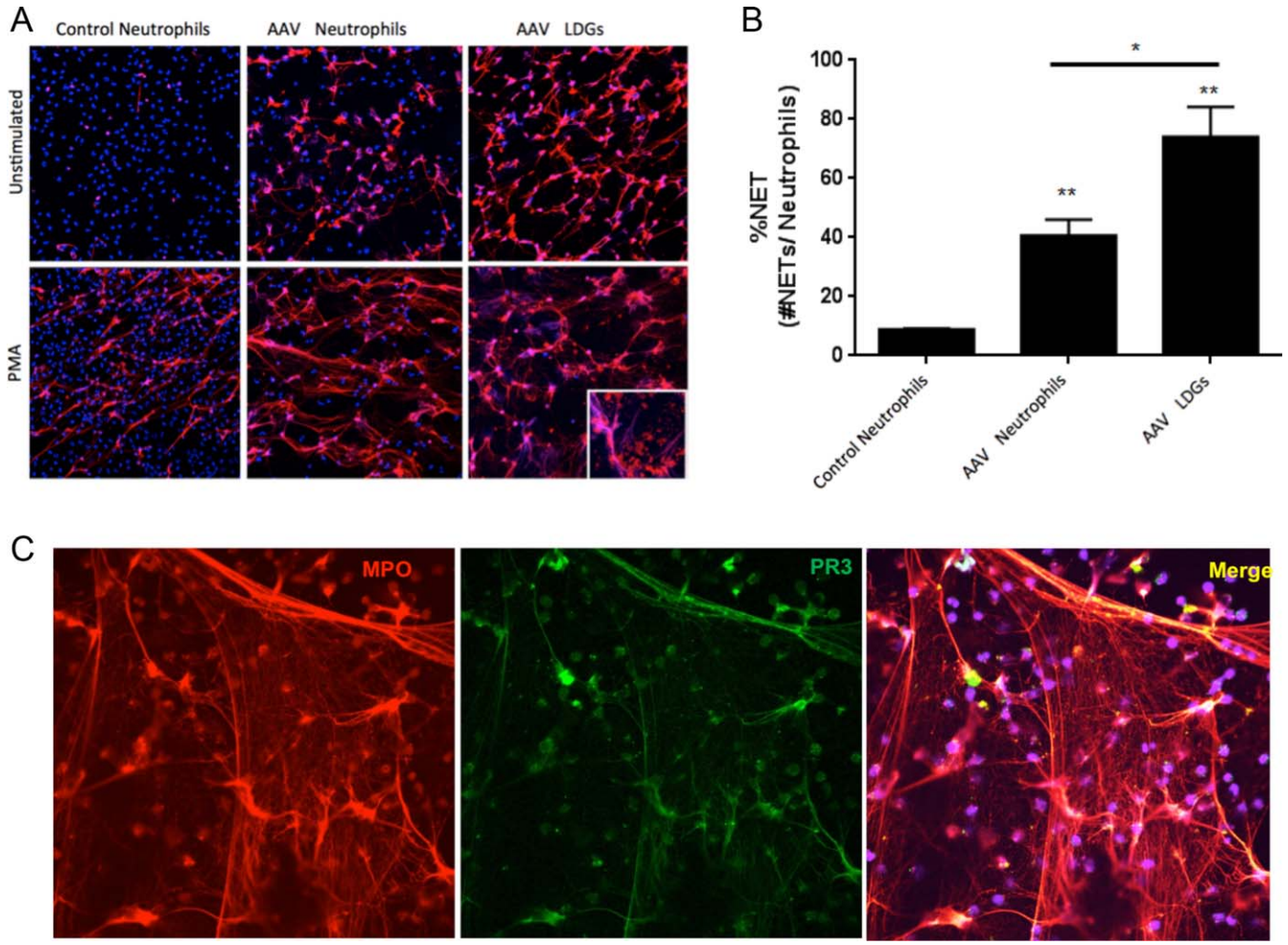

Figure 3. Demonstration of low-density granulocytes (LDGs) in patients with antineutrophil cytoplasmic antibody–associated vasculitis (AAV). **A,** Photomicrographs show immunofluorescence staining of normal-density neutrophils from a healthy control subject and normal-density neutrophils and LDGs from a patient with AAV. Cells were left unstimulated or were stimulated with phorbol myristate acetate (PMA). Formation of neutrophil extracellular traps (NETs; long strands) in the absence of PMA stimulation is observed in both neutrophils and LDGs from the patient with AAV, but not in neutrophils from the control subject. NET formation is enhanced by PMA stimulation. **Inset** shows higher-magnification view. Stained with DAPI (blue) to indicate DNA and with antimyeloperoxidase (anti-MPO) (red). Original magnification × 40. **B,** Unstimulated normal-density neutrophils and LDGs from patients with AAV undergo significantly more ex vivo NET formation than do neutrophils from healthy controls ($** = P < 0.01$). LDGs from patients with AAV undergo significantly more NET formation than do autologous normal-density neutrophils ($* = P < 0.05$). Values are the mean ± SD. **C,** Photomicrographs of LDGs isolated from a patient with AAV demonstrate spontaneous NET formation, and both proteinase 3 (PR3) and MPO colocalize within NETs. Original magnification × 40.

DISCUSSION

Transcriptomic analysis of whole blood from patients with AAV revealed a granulocyte gene expression signature associated with disease activity and treatment response. In the RAVE trial, patients with high levels of granulocyte gene expression during the baseline study visit were less likely to meet the primary outcome of the trial: complete remission at the month 6 study visit. In contrast, clinical features of disease, standard laboratory assessments including ANCA, and disease-specific activity indices did not predict treatment response in these patients.

Differential gene expression of granulocyte-related genes in the PBMC fraction validated the granulocyte signature observed in whole blood and localized the signature to a recently described subset of neutrophils known as LDGs. In particular, expression of PR3 in the PBMC fraction was 17-fold higher in patients who did not meet the primary outcome in RAVE as compared to those who did. LDGs were directly isolated from peripheral

blood in all patients with AAV in an independent cohort of patients, marking the first time these cells have been reported in AAV. LDGs from patients with AAV readily underwent NETosis in the absence of added stimulation and produced NETs containing both PR3 and MPO, the major antigenic targets of ANCA.

The source of a granulocyte signature identified in whole blood could be normal-density neutrophils, LDGs, or both. Comparisons with other transcriptomic studies strongly suggest that LDGs and not normal-density neutrophils are the source of the granulocyte signature identified in this study. Two prior studies examined whole-genome gene expression in sorted normal-density neutrophils versus PBMCs. In the study by Lyons et al (28), granulocyte-related gene expression was detected in the PBMC fraction of blood from patients with lupus and AAV as compared to that from healthy controls and correlated with the quantity of granulocytes identified in PBMCs by flow cytometry. In the study by Cheadle et al (27), transcription of granulocyte genes in the PBMC fraction, including PR3 and MPO, differentiated AAV patients from healthy controls. In both studies, granulocyte expression signatures identified in PBMCs, but not in normal-density neutrophils, strongly overlapped the granulocyte gene signature identified in whole blood in our study.

These data suggest that LDGs, which, by definition, are neutrophils that colocalize in the PBMC fraction, are the source of granulocyte gene expression signatures in AAV. A gene expression signature derived directly from isolated LDGs in patients with SLE (25) also strongly overlaps the granulocyte signatures identified in AAV in each of these studies, providing additional evidence that LDGs are the specific cellular source of these signatures. Although enrichment of degranulated neutrophils could contribute to gene expression signatures identified in this study, electron microscopy studies have demonstrated that LDGs are not simply degranulated neutrophils (23). Spontaneous NET formation observed in LDGs isolated from patients with AAV requires the presence of neutrophil granular proteins, which are abundantly transcribed in LDGs.

Increased transcription of PR3 and, to a lesser extent calprotectin, in PBMCs was associated with disease activity and treatment response. Similar associations were not observed for MPO or CAMP, despite the fact that these genes were components of the granulocyte expression signature identified in whole blood. Other studies have found differences between PR3 and MPO as potential biomarkers in AAV. Independent of ANCA specificity, transcription of PR3, but not MPO, and the presence of neutrophil surface membrane PR3, but not surface membrane MPO, differentiate AAV patients from healthy blood donors and disease controls (36,37). Additionally, increased transcription of PR3 in the PBMC fraction has previously been shown to correlate with disease activity in AAV (27). Whether PR3 expression could be a useful surrogate marker for LDGs needs to be assessed in prospective studies.

A major strength of this study is the use of samples collected within a large clinical trial in AAV linked to systematically recorded clinical and laboratory data. Regression models were used to assess the associations between the granulocyte signature and clinical/laboratory data. The granulocyte signature was weakly associated with the baseline BVAS/WG score and platelet count, suggesting that the signature captures elements of baseline disease activity. Historically, baseline disease activity scores in AAV do not predict treatment response (5). Consistent with this observation, granulocyte gene expression, but not the baseline BVAS/WG score, was significantly associated with treatment response.

Treatment with glucocorticoids is a potential confounder of granulocyte gene expression. Glucocorticoids promote demargination of circulating neutrophils and enhance the release of immature neutrophils from the bone marrow (38). The majority of patients in this study were receiving high doses of glucocorticoids at the time the baseline sample was collected. However, cumulative glucocorticoid use in the 2 weeks prior to the baseline visit was not directly associated with the granulocyte multigene composite score, and no differences in glucocorticoid use were observed between treatment responders and nonresponders, thus lessening concerns for potential confounding by glucocorticoids of the association between the granulocyte expression signature and treatment response.

Use of whole blood as a tissue source for gene expression analysis is potentially a study limitation. Blood comprises many cell populations, and it can be challenging to differentiate changes in transcript abundance in blood caused by regulation of gene transcription activity from changes secondary to the relative abundance of cell populations expressing transcripts at constant levels. However, in this study, the absolute neutrophil and lymphocyte counts at baseline did not differ between treatment responders and nonresponders. Although hemoglobin levels were significantly higher in nonresponders, the association between treatment response and the granulocyte signature score remained significant in multivariable models that adjusted for differences in hemoglobin levels.

Heterogeneity of cell populations in whole blood can also reduce the power of transcriptomic studies to identify significant differential gene expression across phenotypes. In this study, a lenient P value of less than 0.05 was used to define significant differential expression,

thus increasing the potential for false discovery. However, validation of differential granulocyte gene expression in a different tissue source (PBMCs), consistency of findings across different transcriptomic data sets in AAV patients, and functional studies of LDGs lessen the concern for false discovery. The associations between whole blood–derived granulocyte gene expression data and clinical outcomes, while statistically significant, were observed only in a minority of treatment nonresponders (26%). The high doses of glucocorticoids at baseline may have reduced some of the significant differences in gene expression between the responder and nonresponder groups. More-specific measures of LDGs, rather than indirect measures in whole blood, may be required to accurately predict clinical outcomes for individual patients with AAV. LDGs were not isolated directly in fresh samples collected within the RAVE trial, and these cells could not be isolated from frozen PBMCs because they do not survive freeze–thaw cycles.

In conclusion, granulocyte-related gene expression was associated with disease activity and relatively poor response to treatment in AAV. LDGs are the likely source of granulocyte gene expression in this study and previous transcriptomic studies that have identified similar gene expression signatures in AAV. LDGs may play an important role in disease pathogenesis in AAV by promoting direct toxicity to endothelial cells and, potentially, by direct involvement in the causal pathway for the generation of ANCAs through autoantigen externalization due to enhanced NET formation. Future efforts to further characterize the function of LDGs and to explore their potential as biomarkers in AAV are warranted.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Grayson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. Arthritis Rheum 2013;65:1–11.
2. Hoffman GS, Leavitt RY, Fleisher TA, Minor JR, Fauci AS. Treatment of Wegener's granulomatosis with intermittent high-dose intravenous cyclophosphamide. Am J Med 1990;89:403–10.
3. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. N Engl J Med 2010;363:221–32.
4. Specks U, Merkel PA, Seo P, Spiera R, Langford CA, Hoffman GS, et al. Efficacy of remission-induction regimens for ANCA-associated vasculitis. N Engl J Med 2013;369:417–27.
5. Hogan SL, Falk RJ, Chin H, Cai J, Jennette CE, Jennette JC, et al. Predictors of relapse and treatment resistance in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis. Ann Intern Med 2005;143:621–31.
6. McKinney EF, Lyons PA, Carr EJ, Hollis JL, Jayne DR, Willcocks LC, et al. A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. Nat Med 2010;16:586–91.
7. Li ZY, Chang DY, Zhao MH, Chen M. Predictors of treatment resistance and relapse in antineutrophil cytoplasmic antibody–associated vasculitis: a study of 439 cases in a single Chinese center. Arthritis Rheumatol 2014;66:1920–6.
8. Pagnoux C, Hogan SL, Chin H, Jennette JC, Falk RJ, Guillevin L, et al. Predictors of treatment resistance and relapse in antineutrophil cytoplasmic antibody–associated small-vessel vasculitis: comparison of two independent cohorts. Arthritis Rheum 2008;58:2908–18.
9. Hagen EC, Daha MR, Hermans J, Andrassy K, Csernok E, Gaskin G, et al, for the EC/BCR Project for ANCA Assay Standardization. Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. Kidney Int 1998;53:743–53.
10. Tomasson G, Grayson PC, Mahr AD, LaValley M, Merkel PA. Value of ANCA measurements during remission to predict a relapse of ANCA-associated vasculitis—a meta-analysis. Rheumatology (Oxford) 2012;51:100–9.
11. Falk RJ, Jennette JC. ANCA disease: where is this field heading? J Am Soc Nephrol 2010;21:745–52.
12. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. Proc Natl Acad Sci U S A 1990;87:4115–9.
13. Xiao H, Heeringa P, Liu Z, Huugen D, Hu P, Maeda N, et al. The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. Am J Pathol 2005;167:39–45.
14. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J Clinical Invest 2002;110:955–63.
15. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532–5.
16. Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. Cell Death Differ 2011;18:581–8.
17. Kaplan MJ, Radic M. Neutrophil extracellular traps: double-edged swords of innate immunity. J Immunol 2012;189:2689–95.
18. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biology 2012;198:773–83.
19. Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol 2014;25:990–7.
20. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009;15:623–5.
21. Kelley JM, Monach PA, Ji C, Zhou Y, Wu J, Tanaka S, et al. IgA and IgG antineutrophil cytoplasmic antibody engagement of

Fc receptor genetic variants influences granulomatosis with polyangiitis. Proc Natl Acad Sciences U S A 2011;108:20736–41.
22. Nakazawa D, Tomaru U, Yamamoto C, Jodo S, Ishizu A. Abundant neutrophil extracellular traps in thrombus of patient with microscopic polyangiitis. Front Immunol 2012;3:333.
23. Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. Semin Immunopathol 2013;35:455–63.
24. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. J Immunol 2010;184:3284–97.
25. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol 2011;187:538–52.
26. Carmona-Rivera C, Zhao W, Yalavarthi S, Kaplan MJ. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. Ann Rheum Dis 2014. E-pub ahead of print.
27. Cheadle C, Berger AE, Andrade F, James R, Johnson K, Watkins T, et al. Transcription of proteinase 3 and related myelopoiesis genes in peripheral blood mononuclear cells of patients with active Wegener's granulomatosis. Arthritis Rheum 2010;62:1744–54.
28. Lyons PA, McKinney EF, Rayner TF, Hatton A, Woffendin HB, Koukoulaki M, et al. Novel expression signatures identified by transcriptional analysis of separated leucocyte subsets in systemic lupus erythematosus and vasculitis. Ann Rheum Dis 2010;69:1208–13.
29. Vartanian K, Slottke R, Johnstone T, Casale A, Planck SR, Choi D, et al. Gene expression profiling of whole blood: comparison of target preparation methods for accurate and reproducible microarray analysis. BMC Genomics 2009;10:2.
30. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.
31. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990;33:1101–7.
32. WGET Research Group. Design of the Wegener's Granulomatosis Etanercept Trial (WGET). Control Clin Trials 2002;23:450–68.
33. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.
34. Stone JH, Hoffman GS, Merkel PA, Min YI, Uhlfelder ML, Hellmann DB, et al, for the International Network for the Study of the Systemic Vasculitides (INSSYS). A disease-specific activity index for Wegener's granulomatosis: modification of the Birmingham Vasculitis Activity Score. Arthritis Rheum 2001;44:912–20.
35. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50.
36. Ohlsson S, Hellmark T, Pieters K, Sturfelt G, Wieslander J, Segelmark M. Increased monocyte transcription of the proteinase 3 gene in small vessel vasculitis. Clin Exp Immunol 2005;141:174–82.
37. Muller Kobold AC, Kallenberg CG, Cohen Tervaert JW. Leucocyte membrane expression of proteinase 3 correlates with disease activity in patients with Wegener's granulomatosis. Br J Rheumatol 1998;37:901–7.
38. Nakagawa M, Terashima T, D'Yachkova Y, Bondy GP, Hogg JC, van Eeden SF. Glucocorticoid-induced granulocytosis: contribution of marrow release and demargination of intravascular granulocytes. Circulation 1998;98:2307–13.