Revealed heterogeneity in rheumatoid arthritis based on multivariate innate signature analysis

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Abstract

Objective

A growing body of evidence highlights the persistent activation of the innate immune system and type I interferon (IFN) signature in the pathogenesis of rheumatoid arthritis (RA) and its association with disease activity. Since the recent study revealed heterogeneity in the IFN signature in RA, we investigated for the first time the heterogeneity in innate signature in RA.

Methods

The innate gene expression signature (10 TLRs, 7 IL1/IL1R family members, and CXCL8/IL8) was assessed in peripheral blood mononuclear cells from RA patients (n=67), both with active (DAS28≥3.2, n=32) and inactive disease (DAS28<3.2, n=35), and in healthy control subjects (n=55).

Results

Of the 13 deregulated innate genes (TLR2, TLR3, TLR4, TLR5, TLR8, TLR10, IL1B, IL1RN, IL18, IL18R1, IL1RAP, and SIGIRR/IL1R8) associated with RA, TLR10 and IL1RAP are being reported for the first time. Multivariate analysis based on utilising patient similarity networks revealed the existence of four patient's subsets (clusters) based on different TLR8 and IL1RN expression profiles, two in active and two in inactive RA. Moreover, neural network analysis identified two main gene sets describing active RA within an activity-related innate signature (TLR1, TLR2, TLR3, TLR7, TLR8, CXCL8/IL8, IL1RN, IL18R1). When comparing active and inactive RA, upregulated TLR2, TLR4, TLR6, and TLR8 and downregulated TLR10 (P<0.04) expression was associated with the disease activity.

Conclusion

Our study on the comprehensive innate gene profiling together with multivariate analysis revealed a certain heterogeneity in innate signature within RA patients. Whether the heterogeneity of RA elucidated from diversity in innate signatures may impact the disease course and treatment response deserves future investigations.

Key words

rheumatoid arthritis, heterogeneity, IL1 family, Toll-like receptors, disease activity

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterised by synovial inflammation and the progressive destruction of joint cartilage and bones (1). The pathogenesis of RA is complex with a growing body of evidence of a major impact of innate immunity and type I interferons (IFNs), respectively (2-4).

The major players in innate immunity are Toll-like receptors (TLRs) and members of the interleukin (IL)-1/IL-1R family, both of which share the same intracellular signalling Toll-IL-1-receptor (TIR) homology domain. Thus, a strong pro-inflammatory signal leading to NF-κB activation is indistinguishable in both the TLR and IL-1 ligands (5). The TLRs may be activated by i) *Proteus* infection of the urinary tract and oral and gut dysbiosis, ii) Epstein-Barr virus and parvovirus B19, and iii) endogenous TLR ligands such as the heat shock protein gp96 and tenascin in RA (reviewed in (6, 7)). Similarly, several members of the IL-1 family were found to be overexpressed in the synovial membrane in RA, making a substantial contribution to the alteration of cartilage and bone homeostasis (8). Importantly, RA was the first disease in which IL-1 inhibition was successfully applied, leading to reduced inflammation and articular damage (9).

Recently, a heterogeneity within genes regulated by IFN type I (IFN signature) has been reported in RA (10). Although there is a lack of knowledge of the exact mechanisms leading to aberrant IFN activation in autoimmunity, the activation of the IFN signature has been linked to TLRs and other innate genes (11, 12). The IFN signature is believed to prompt the tolerance breakdown and the subsequent autoimmune perpetuation (13). From the clinical point of view, an enhanced IFN signature has been associated with clinical outcome, treatment response and disease activity in RA (10, 14-16), although some controversy still exists concerning its clinical relevance (10).

Based on the existing linkage between IFN and innate signatures, we were wondering whether innate signature shows the heterogeneity in RA. We, therefore, analysed the complex expression pattern of innate genes including *TLR1-10*, seven members of the *IL1/IL1R* family and interleukin 8 (*CXCL8/IL8*) in peripheral blood mononuclear cells (PBMCs) of patients with RA. Using the multivariate data mining analysis, we evaluated the diversity of the innate signatures in RA and its relationship to the disease activity.

Materials and methods

Study subjects

The study cohort consisted of 67 Czech patients who met the 2010 ACR/EULAR classification criteria for RA (17) and were recruited at a single tertiary rheumatology centre. All the patients were treated according to the national Czech guidelines and standard protocols (18); for the medication used, duration of the disease, and the demographic and clinical features see Table I. Subgroups were formed on the basis of the disease activity as assessed by means of the Disease Activity Score in 28 joints (DAS28), with a DAS28 of ≥3.2 being taken as active RA (inactive RA, n=35; active RA, n=32). The baseline demographic and clinical data, as well as a type of medications, its duration and cumulative steroid dosage, did not differ between subgroups of active and inactive patients ($p>0.05$). The age- and gender-matched healthy control subjects comprised 55 medical staff members or their relatives (mean age 54 yrs, range 41–90 yrs, female/male 45/10) in whom autoimmune and inflammatory diseases, recent vaccination, infection, and usage of immunosuppressive drugs were excluded by means of questionnaires.

The patients and control subjects provided written informed consent in accordance with the Helsinki Declaration about the use of peripheral blood for the purpose of this study, which was approved by the ethics committee of the University Hospital and Palacký University Olomouc.

Real-time reverse transcription-polymerase chain reaction (qRT-PCR)

The PBMC were isolated from blood collected in EDTA tubes by Ficoll den-

sity gradient centrifugation (Sigma-Aldrich, Germany), then lysed in Tri reagent (Sigma-Aldrich, Germany) and frozen at -80°C. Total RNA was extracted using a Direct-zol RNA kit (Zymo Research, USA) according to the manufacturer's recommendations. Reverse transcription was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) as reported previously (19).

qPCR was performed using a high-throughput SmartChip Real-Time-qPCR system (WaferGen, USA) allowing 5,184 reactions per chip. The reactions were carried out in 100 nl reaction volume containing LightCycler 480 SYBR Green I Master mix (Roche, Switzerland) with 1.6 μM (each) of gene-specific exon-spanning primers and 0.27 ng of cDNA in quadruplicates. The primer sequences are listed in Table S1 (Integrated DNA Technologies, USA). Each run included a no-template control, in which RNA was replaced by water, and human universal reference RNA (Stratagene, USA) which was used in quadruplicates as a calibrator at a 0.27 ng/reaction mix. The thermal cycler parameters were as follows: one cycle of 95°C for 5 min followed by 40 cycles of 34 s at 95°C and 1 min 4 s at 60°C. Melting curve analysis was performed from 97°C to 60°C (0.4°C/step) immediately after amplification. The relative mRNA expression was calculated using Phosphoglycerate kinase 1 as the reference gene (20).

In order to assess the innate immunity gene expression signature in RA, we investigated the expression of *TLR* (*TLR1-10*), the *IL1/IL1R* family (21 members), and *CXCL8/IL8* in PBMC. On the basis of the pilot evaluation of qPCR assays on a cohort of 20 RA patients, 14 assays of *IL1/IL1R* family members (*IL1A, IL36RN, IL36A, IL36B, IL36G, IL37, IL38, IL33, IL1R2, IL18RAP, IL1RL1, IL1RL2, IL1RAPL1, IL1RAPL2*) were below the detection limit of the system and were, therefore, excluded from further analysis. The study continued with gene expression profiling of 18 innate immunity genes: *TLR1-10* and seven members of the *IL1/IL1R* family, together with *CXCL8/IL8*.

Table I. Demographic and clinical characteristics of RA patients.

Demographic and clinical features	RA (n=67)	Inactive RA (n=35)	Active RA (n=32)
Female/Male	56/11	31/4	25/7
Age (years) mean (min-max)	55 (27-80)	52 (27-73)	57 (44-61)
Age at the onset of the disease (years) mean (min-max)	39 (5-65)	40 (5-65)	38 (15-57)
Duration of the disease (years) mean (min-max)	16 (1-58)	13 (1-33)	19 (1-58)
Disease activity:			
DAS28 mean (min-max)	3.44 (0.60-6.70)	2.41 (0.60-3.14)	4.56 (3.20-6.70)
ESR (mm/hr) mean (min-max)	19 (2-116)	12 (2-40)	27 (3-116)
CRP (mg/l) mean (min-max)	8.0 (0.6-65.0)	3.0 (0.6-9.2)	13.4 (0.6-65.0)
ACPA positive, % (n)	76 (51)	77 (27)	75 (24)
RF positive, % (n)	66 (44)	69 (24)	63 (20)
Medications, % (n)			
Steroids	70 (47)	51 (18)	91 (29)
NSAIDs	64 (43)	51 (18)	78 (25)
Methotrexate	85 (57)	86 (30)	84 (27)
Other DMARDs*	19 (13)	8 (2)	34 (11)
Biologics#	46 (31)	49 (17)	44 (14)

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ACPA: anticitrullinated protein antibodies; RF: rheumatoid factor; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs.

*Other DMARDs taken were hydroxychloroquine (n=3), leflunomide (n=7), sulfasalazine (n=2), and combination of leflunomide and sulfasalazine (n=1).

#Biologics taken were TNF-α inhibitors (n=18), tocilizumab (n=6), abatacept (n=4) and rituximab (n=3).

Statistical analysis and data mining methods

Statistical analysis (Mann-Whitney U-test, Kruskal-Wallis test, Benjamini-Hochberg correction, Shapiro-Wilk test) of relative gene expression values were calculated using GraphPad Prism 5.01 (GraphPad Software, USA) and the R statistical software package, a free software environment for statistical computing and graphics (http://www.r-project.org/). Spearman correlation between gene expression and continuous DAS28 values were performed using Genex (MultiD Analyses AB, Sweden). A *p*-value <0.05 was considered significant.

Firstly, the LRNet algorithm (21) was used to construct a patient similarity network (PSN) to show the similarities of the gene expression profiles among individual patients. The nearest neighbours within the network have the highest similarity in terms of gene expression levels and colours distinguish the particular subgroups of patients with similar profiles. To obtain a set of the most characteristic genes, we constructed these networks based on different combinations of a small number of genes. The selection of the best gene combination for active and inactive RA patients was evaluated by measured values of weighted modularity (the network partitioning ability) and silhouette (evaluation of the internal quality of clusters) (22, 23). For more details, see On-line supplementary file.

Secondly, a neural network-based algorithm (ANN), together with 10-fold cross-validation (Neuralnet package (https://cran.r-project.org/package_neuralnet), from the R software) was applied to a learning set of 57 RA patients with known disease activity status. For the pre-selection of the most informative genes for ANN, the Random Forest machine learning classifier was applied. The selection of the best combination of ANN markers and ANN structure was performed on the basis of the root mean square error (RMSE) and classification error. The classification error for the top marker sets and final ANN was calculated on a validation cohort of 10 patients in whom the activity status was hidden from the bioinformaticians (MR, MK). A flowchart of the process is documented in Supplementary Fig. S1. For more details, see On-line supplementary file. Next,

Table II. Relative mRNA expression levels of genes differentially expressed between A) RA *vs.* healthy controls, B) active *vs.* inactive RA.

A: RA *vs.* healthy controls

Gene	Mean (95 % CI)		FC	*p*	p_{corr}
	Healthy controls	RA			
SIGIRR	0.196 (0.167-0.225)	0.367 (0.329-0.405)	1.87	3.9×10^{-10}	0.1×10^{-9}
IL18	0.036 (0.031-0.042)	0.060 (0.054-0.067)	1.56	4.1×10^{-8}	3.7×10^{-7}
IL1RN	0.018 (0.013-0.024)	0.039 (0.034-0.044)	2.75	1.4×10^{-7}	8.6×10^{-7}
TLR5	0.029 (0.020-0.037)	0.060 (0.052-0.067)	3.20	4.4×10^{-7}	2.0×10^{-6}
IL18R1	0.006 (0.004-0.007)	0.011 (0.009-0.012)	1.99	3.4×10^{-6}	1.2×10^{-5}
TLR3	0.003 (0.002-0.004)	0.006 (0.005-0.007)	6.59	1.8×10^{-5}	5.4×10^{-5}
IL1RAP	0.008 (0.006-0.010)	0.014 (0.012-0.017)	2.08	4.2×10^{-5}	1.1×10^{-4}
TLR8	0.040 (0.032-0.049)	0.062 (0.053-0.071)	1.59	4.2×10^{-4}	9.5×10^{-4}
IL1B	0.035 (0.002-0.067)	0.062 (0.033-0.091)	1.79	8.2×10^{-4}	1.6×10^{-3}
TLR2	0.049 (0.035-0.062)	0.067 (0.057-0.077)	1.91	1.3×10^{-3}	2.3×10^{-3}
CXCL8/IL8	0.108 (0.025-0.191)	0.145 (0.096-0.195)	2.48	2.2×10^{-3}	3.7×10^{-3}
TLR10	0.007 (0.006-0.008)	0.010 (0.008-0.011)	1.41	2.1×10^{-2}	3.2×10^{-2}
TLR4	0.050 (0.043-0.057)	0.041 (0.036-0.046)	0.86	3.2×10^{-2}	4.5×10^{-2}

B: Active *vs.* inactive RA

Gene	Mean (95 % CI)		FC	*p*	p_{corr}
	Inactive RA	Active RA			
TLR10	0.011 (0.009-0.013)	0.008 (0.005-0.011)	0.49	6.5×10^{-3}	1.2×10^{-1}
TLR8	0.057 (0.042-0.072)	0.067 (0.056-0.077)	1.37	1.4×10^{-2}	1.2×10^{-1}
TLR6	0.023 (0.017-0.028)	0.030 (0.024-0.036)	1.57	2.1×10^{-2}	1.3×10^{-1}
TLR2	0.057 (0.046-0.068)	0.078 (0.061-0.095)	1.40	3.3×10^{-2}	1.5×10^{-1}
TLR4	0.039 (0.031-0.048)	0.043 (0.037-0.049)	1.34	4.1×10^{-2}	1.5×10^{-1}

p_{corr} value corrected for multiple comparisons (Benjamini-Hochberg correction)
FC (Fold change) between group medians of relative mRNA expression levels.

a gene expression similarity network was constructed by means of the LR-Net algorithm using nearest neighbour- and representativeness analysis (21) in subgroups of active and inactive RA patients. The network vertices represent the individual genes and the size of each vertex corresponds to the local importance of the expression of a particular gene on the basis of the number of its nearest neighbours (= other genes). The links (edges) between vertices and their strength represent the similarities between pairs of vertices. For more details, see On-line supplementary file.

Results

Innate immune gene expression signature of RA

In order to gain a deeper insight into the innate immune system associated with RA, we investigated the innate immunity expression signature in the RA patients and healthy controls. Since our data did not meet the assumption of normality as assessed by the Shapiro-Wilk test, the non-parametric Mann-Whitney U-test was used for the comparison of data distribution between two groups. Of the thirteen deregulated genes in RA were six TLRs: upregulated *TLR2*, *TLR3*, *TLR5*, *TLR8*, and *TLR10*, and downregulated *TLR4* comparing to controls (p_{corr}<0.05; Table IIA; Fig. 1). Of IL-1/IL-1R family, six members were upregulated *IL1B*, *IL1RN*, *IL18*, *IL18R1*, *IL1RAP*, and *SIGIRR*/*IL1R8*, as well as upregulated chemokine *CXCL8*/*IL8* (P_{corr}<0.05; Table IIA; Fig. 1) in RA. The expression of *IL1R1*, *TLR1*, *TLR6*, *TLR7*, and *TLR9* were not different between RA and controls (p_{corr}>0.05; Suppl. Table S2A).

To exclude the differences in gene profiles between patients treated with different drugs, we compared subgroups formed on the basis of the medications used. No difference was observed between the profiles in the subgroups of patients based on various medications (p>0.05).

Innate immune gene expression signature associated with active and inactive RA

Next, we wondered which genes or their combinations characterise patients with active and inactive RA. When active and inactive RA were compared, upregulated expression of *TLR2*, *TLR4*, *TLR6*, and *TLR8* and downregulation of *TLR10* was observed in patients with active disease (p<0.05; Table IIB, Suppl. Table S2B, Fig. 2). Moreover, the DAS28 score correlated negatively with the expression of *TLR10* (r=-0.367, p=0.002, Fig. S2A) and positively with the expression of *TLR8* (r=0.236, p=0.05, Fig. S2B). Although the mRNA expression of *TLR4* was downregulated in the RA patients as a whole when compared to the healthy controls, subanalysis in subgroups according to the disease activity revealed upregulation of *TLR4* in active RA (Fig. 3). Regarding TLR10, the mRNA expression of *TLR10* was upregulated in the RA patients as a whole when compared to the healthy controls, while subanalysis revealed *TLR10* mRNA downregulation in those patients with active RA (Fig. 3). Concerning the IL-1/IL-1R family, no difference was observed in the expression of the genes that were studied between the patients with active and inactive disease when basic statistics were applied (Suppl. Table S2B).

Then, combinations of multiple genes for the discrimination of active and inactive RA were investigated by multivariate data analysis. The gene expression similarity network for active RA was characterised by the expression of *TLR2*, *TLR3*, *TLR8*, and *IL18R1*, and inactive RA was associated with the *TLR2*, *TLR5*, *TLR7*, *IL18R1*, and *IL1RAP* genes (Fig. 4). The selected genes had the highest representativeness in the individual networks.

To investigate and visually assess the complex expression innate signatures in our patients, we performed analysis by utilising the abovementioned patient similarity networks. This analysis revealed the existence of four patient's subsets (clusters) based on different *TLR8* and *IL1RN* expression profiles, two in active and two in inactive RA. The high modularity and the good per-

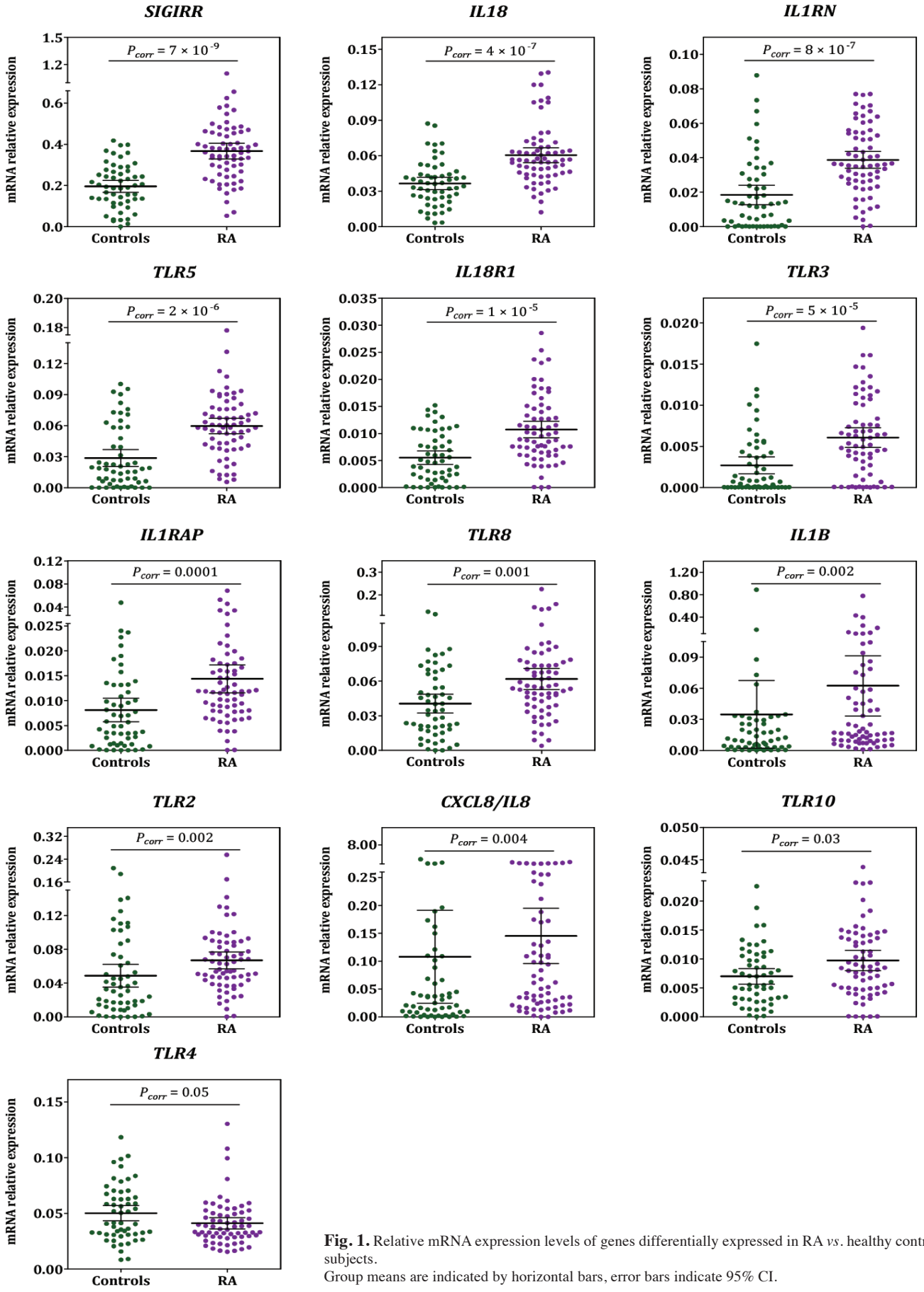

Fig. 1. Relative mRNA expression levels of genes differentially expressed in RA *vs*. healthy control subjects.
Group means are indicated by horizontal bars, error bars indicate 95% CI.

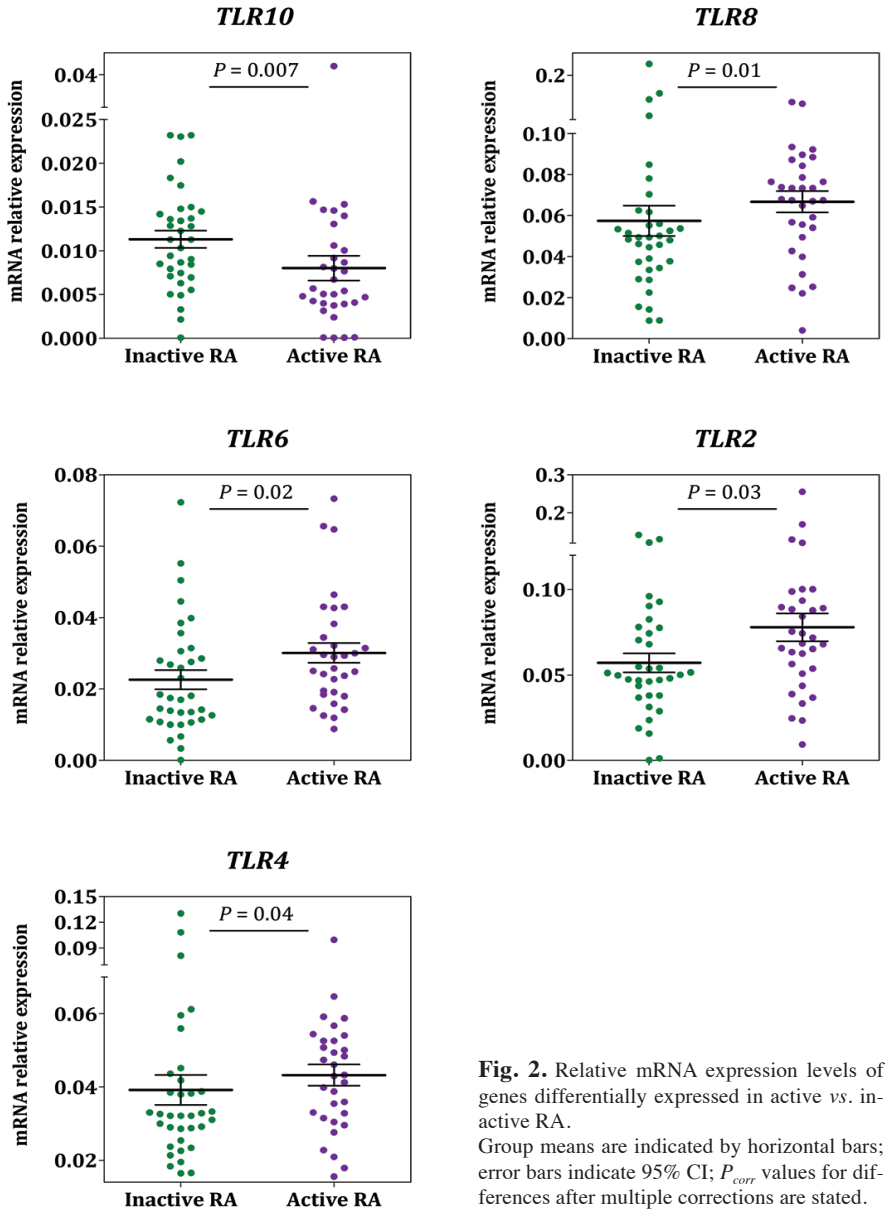

Fig. 2. Relative mRNA expression levels of genes differentially expressed in active *vs*. inactive RA.
Group means are indicated by horizontal bars; error bars indicate 95% CI; P_{corr} values for differences after multiple corrections are stated.

formance of silhouette analysis were observed across the combinations that were tested (Fig. 5). To exclude the differences in gene profiles due to the different treatment regimen, the distributions of used drugs in particular subgroups were compared. As shown in Figure S3, the proportion of patients treated with a particular drug did not differ among revealed subsets (clusters). Further, using ANN we identified two combinations of genes: *TLR1*, *TLR2*, *TLR7*, *TLR8*, *IL1RN*, *IL18R1*, and *CXCL8/IL8*, and *TLR1*, *TLR2*, *TLR3*, *IL1RN*, and *IL18R1*, whose co-expression discriminates between patients with active and inactive RA. With these combinations used as an input to a classifier containing ten neural networks, 80% overall agreement was achieved for blinded patient data on the basis of five-fold cross-validation. Furthermore, two combinations of genes were needed for successful characterisation of the subgroups of patients, showing that within the active RA subgroup, there are at least two different gene expression signatures. When ANN was constructed for the combination of only TLR genes associated with disease activity on the basis of classical statistics (*TLR2*, *TLR4*, *TLR6*, *TLR8*, and *TLR10)*, this combination reached only 40% overall agreement for blinded patient data based on five-fold cross-validation. Moreover, the observed existence of two main subsets with different expression signatures within the active RA patients confirmed the result from patient similarity network analysis.

Discussion

A growing body of evidence highlights a persistent activation of the innate immune system and IFN signature in the pathogenesis of RA as well as its relationship with the disease activity. In addition to recent studies that revealed heterogeneity in the IFN signature in RA (10, 14), our study for the first time also highlighted the heterogeneity in the innate signature within RA patients.

To analyse the innate signature in our patients, we used two multivariate data mining approaches that have excellent properties for analysing gene expression patterns. Firstly, gene expression and patient similarity networks exploration enables visual assessment of the most informative markers within a sample set and shows the relationship between patients with similar gene profiles (24-26). Also applied neural network approach takes into account the intrinsic characteristics of gene expression data (27, 28), confirms the most informative gene subsets, and improves classification accuracy with best parameters based on datasets (14, 24, 29, 30). Using patient similarity network analysis, four patient's subsets based on the innate signature were detected, two in active and two in inactive RA. The applied network exploration identified expression of *TLR8* and *IL1RN* as the most discriminant among detected subgroups. Importantly, the heterogeneity in RA patients was further supported by the neural network analysis which identified two main gene sets describing active RA within an innate signature (*TLR1, TLR2, TLR3, TLR7, TLR8, CXCL8/IL8, IL1RN, IL18R1*). Our data for the first time identified certain heterogeneity in innate signature in RA, which may have a significant impact on the disease course and treatment response, thus deserving future investigations.

From our results, *TLR8* and *IL1RN* appear to be key genes whose expressions characterise diversity in RA and active and inactive RA subgroups. TLR8 is

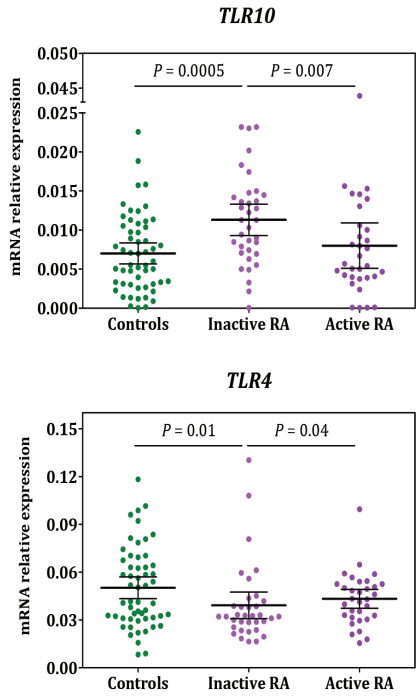

Fig. 3. Relative mRNA expression levels of *TLR4* and *TLR10* in active RA, inactive RA, and healthy control subjects.
Group means are indicated by horizontal bars, error bars indicate 95 % CI. Kruskal-Wallis test revealed differences among all tested subgroups (*TLR4: p*=0.02; *TLR10*: *p*=0.001).
The horizontal connecting lines show significant differences between two particular subgroups (controls *vs*. inactive RA, inactive RA *vs*. active RA, respectively); comparison between controls and active RA did not reach significance for both *TLR4* and *TLR10*.

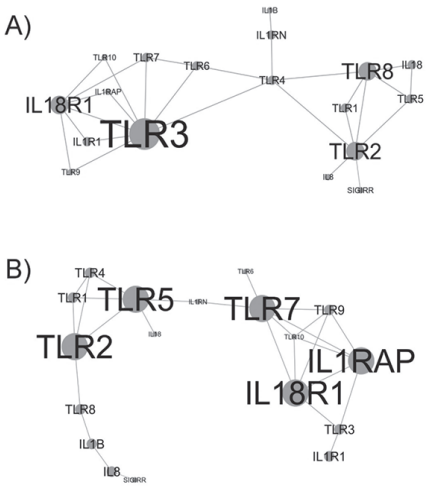

Fig. 4. Gene expression similarity network in **A**) active and **B**) inactive RA.
Vertices represent the individual genes, and the size of each vertex corresponds to the local importance (representativeness) of the expression of a particular gene on the basis of the number of its nearest neighbours. Links (edges) between vertices and their strength represent similarities between pairs of vertices.

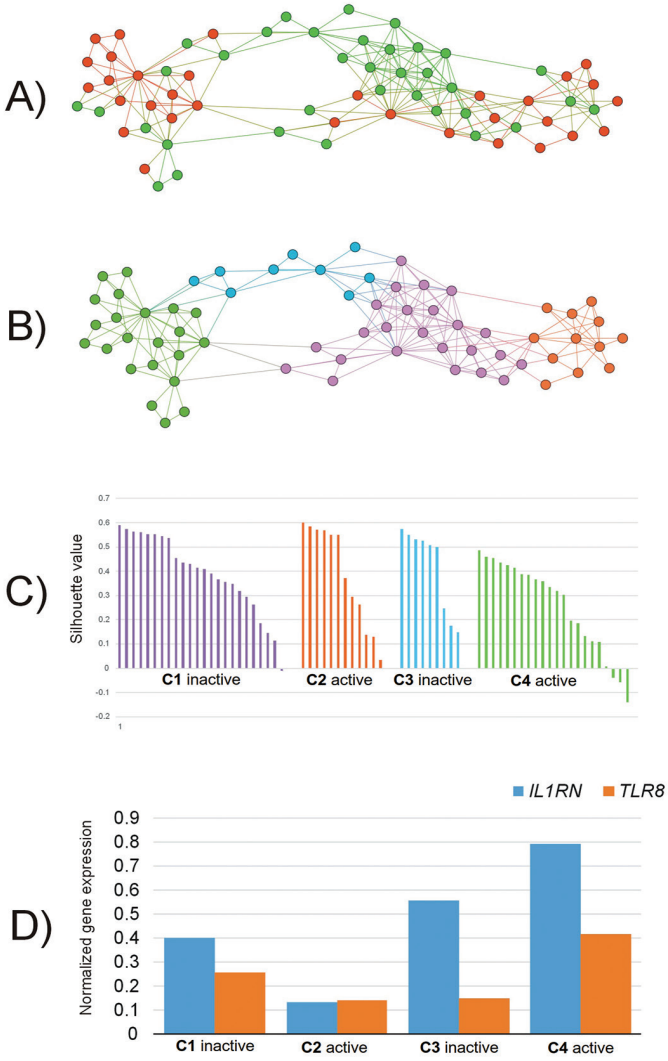

Fig. 5. Patient similarity network analysis based on *TLR8* and *IL1RN* expression in RA patients **A**) with active (red) and inactive disease (green).
B) Of four well-separated clusters, two clusters included predominantly inactive RA patients (C1 - violet, C3 - blue) and two predominantly active RA (C2 - orange, C4 - green).
C) The silhouette analysis of detected clusters. The bars represent individual patients, and high values for them indicate that the patient is well matched to their own cluster and poorly matched to neighbouring clusters.
D) Characteristics of observed clusters showing the normalised gene expression values of *IL1RN* and *TLR8*.

able to recognise viral single-stranded and bacterial RNAs and induce both NF-κB-dependent cytokines and type I IFNs (31). Studies on the role of the TLR8 in arthritic inflammation showed that its increased expression correlates with the elevation of IL-1β levels and disease status (32). Another study proved that the activation of the TLR8 signalling pathway in human blood results in a predominant pro-inflammatory gene signature (33). Importantly, recent studies demonstrated that the activation profile of individual TLRs may be influenced by the complex TLR-TLR interactions (34, 35). Regarding TLR8, a distinct immune activation profile was observed by co-signalling of TLR8 together with TLR2 when compared to TLR8 alone (36). Moreover, TLR2 was shown to suppress IFNβ production induced by TLR8 activation (37). Additionally, a cross-talk of TLR8 with other endosomal TLRs has been identified crucially involved in the generation of autoimmunity (31).

Next, key gene within innate signature was *IL1RN* which codifies IL-1 receptor antagonist (IL-1Ra) that blocks IL-1 signalling. The importance of *IL1RN* in the RA pathogenesis has been demonstrated by Il1rn(-/-) mice, which spontaneously

develop autoimmune arthritis that is dependent on TLR activation (38). In human, associations of sequence variants *IL1RN* VNTR (rs2234663) and +2018 SNP with RA disease activity was reported (39, 40). Other VNTR variant (allele *IL1RN**2) was found to influence not only the plasma levels of IL-1Ra, but also the response to infliximab therapy (41). However, contrary to the animal studies (38), we and others observed elevated expression of *IL1RN* in RA patients compared to healthy subjects (39). Our multivariate analysis revealed very high variability in *IL1RN* expression among our patient subgroups, irrespective of the disease activity. This led us to suggest that *IL1RN* may act in a dose-dependent manner and most likely in interaction with complex TLR interactions, thus deserving future investigations. Our data further supports the crucial role of IL-1Ra together with TLR8 in the RA pathogenesis, thus nominating them as candidates for future studies.

Also, other genes or their protein products of an innate signature identified from neural network analysis have been associated with RA disease activity in previous studies, such as TLR3 (42), IL18R1 (43), TLR7 (44), and CXCL8/IL8 (45). Although individual innate genes have been already linked to RA, a more complex picture may be observed when using multivariate analysis. Overall, our findings revealed heterogeneity in RA in innate signature including *TLR* and *IL1*/*IL1R* genes. Whether the heterogeneity of innate signature contributes to the reported variability in IFN signature in RA (11) deserves future investigations.

In our study, we also comprehensively explored the RA-associated signature when compared to healthy controls. Of 13 innate deregulated genes (*TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR8*, *TLR10*, *IL1B*, *IL1RN*, *IL18*, *IL18R1*, *IL1RAP*, and *SIGIRR*/*IL1R8*) associated with RA, *TLR10* and *IL1RAP* are being reported for the first time. In line with our results, the upregulation of *TLR2*, *TLR5*, and *TLR8* in peripheral blood monocytes (46-48) and *TLR3* in synovial tissue (49) was reported. Regarding *TLR4,* which is highly expressed in the synovium (50), we and others (51) observed the downregulation of *TLR4* expression in RA PBMC. We also detected the upregulated expression of *TLR10* in the PBMC of RA patients as a whole for the first time. The first evidence about the possible involvement of TLR10 in RA already exists; it is based on the association of a *TLR10* I473T allelic variant with RA (52). However, there are controversies regarding its function. Some studies have demonstrated that TLR10 is a pro-inflammatory receptor activating NF-κB signalling (53, 54), while others have reported NF-κB inhibitory activity (52, 55) and inflammation suppression (56, 57). Further studies are needed to clarify its function, ligands, and the influence of the genetic background in RA on its regulation. Our study also confirmed the results from analyses of individual members of the IL-1 family: increased expression of the pro-inflammatory members *IL1B*, *IL1RN*, and *IL18* was demonstrated in the peripheral blood cells (39, 58) and of *IL18R1* and *SIGIRR* in the synovial tissue (59, 60) of RA patients. Here, we report for the first time upregulated gene expression of *IL1RAP* in RA. IL-1RAP is a co-receptor involved in several signalling pathways, including IL-1, IL-33, IL-36G, and SCF (61, 62), and a lack of IL-1RAP was shown to abrogate the cellular response to IL-1 (63). The contribution of this co-receptor to the RA pathogenesis deserves future investigation. Additionally, elevation in the expression of the chemokine *CXCL8*/*IL8* was observed in our study, which is in line with the reported elevation of IL-8 in synovial fluids and serum in RA patients (64). When active and inactive disease were compared, the upregulation of *TLR2*, *TLR4*, *TLR6*, and *TLR8* and downregulation of *TLR10* were revealed in active RA. Concerning TLR10, an association of the I473T allelic variant (rs11466657) with disease severity and a low response to infliximab has been reported (52). Functional studies have shown that the *TLR10* I473T variant lacks inhibitory activity on the NF-κB inflammatory pathway in comparison to the wild-type allele (52). Similarly, downregulation of *TLR10* was also observed in our patients with active disease. These observations nominate TLR10 as a candidate target molecule able to attenuate the inflammation in active RA.

The authors are aware of some limitations. First, the study was performed in a real-world cohort of patients treated with different medications, however, the distribution of various medications, its duration and dosage did not differ between compared subgroups of active and inactive patients. Second, the innate gene signature should be completed on a protein level of functionally active cytokines in future studies. However, we believe that this multivariate approach highlighted for the first time the heterogeneity of innate molecules in RA and nominated combinations of key innate molecules for further functional studies. To conclude, our study on comprehensive innate gene profiling together with multivariate data mining analysis revealed a certain heterogeneity in innate signature within RA patients. Moreover, *TLR8* and *IL1RN* were identified as the key genes whose expressions contribute to the heterogeneity of innate signature in RA. The clinical consequences of the observed heterogeneity of innate signature in RA should be addressed in future studies. We believe that this integrated approach is likely to generate insights into the heterogeneity of innate signature in RA.

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