Title

Transcriptional response modules characterise IL-1 and IL-6 activity in COVID-19

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Abstract

Dysregulated IL-1 and IL-6 responses have been implicated in the pathogenesis of severe Coronavirus Disease 2019 (COVID-19). Innovative approaches for evaluating the biological activity of these cytokines in vivo are urgently needed to complement clinical trials of therapeutic targeting of IL-1 and IL-6 in COVID-19. We show that the expression of IL-1 or IL-6 inducible transcriptional signatures (modules) reflects the bioactivity of these cytokines in juvenile idiopathic arthritis (JIA) and rheumatoid arthritis, and discerns the effect of therapeutic cytokine blockade in JIA. In COVID-19, elevated expression of IL-1 and IL-6 response modules, but not these cytokines per se, is a feature of disease both in blood and in affected organs. We propose that IL-1 and IL-6 transcriptional response modules can provide a dynamic readout of the activity of these cytokine pathways in vivo, with potential applications for identifying COVID-19 patients who may benefit from IL-1 or IL-6 blocking therapy, and to aid quantification of the biological effects of these treatments.
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Introduction

Severe Coronavirus Disease 2019 (COVID-19) typically occurs over a week from symptom onset, when viral titres have diminished, suggesting an unregulated host inflammatory response may be driving the pathogenesis of severe disease (1–3). Elevated IL-1 and IL-6 responses have each been associated with disease severity (1,4–8). In addition, the hyperinflammatory state in COVID-19 is reported to resemble some aspects of haemophagocytic lymphohistiocytosis (HLH), a condition that may benefit from therapeutic IL-1 blockade (9). These observations have generated hypotheses that IL-1 and / or IL-6 may be key drivers of pathology in severe COVID-19, and led to clinical trials of IL-1 and IL-6 antagonists in this context (10,11).

The measurement of individual cytokines at the protein or RNA level may not reflect their biological activity accurately within multivariate immune systems that incorporate redundancy and feedback loops. To address this limitation, we have previously derived and validated gene expression signatures, or modules, representing the transcriptional response to cytokine stimulation, using them to measure functional cytokine activity within genome-wide transcriptomic data from clinical samples (12–15). No such transcriptional modules have been published and tested for detection of human IL-1 or IL-6 bioactivity. In the present study, we have sought to address this gap to provide urgently needed analysis tools in COVID-19 research. We describe the derivation and validation of IL-1 and IL-6 inducible transcriptional modules. We test the hypothesis that these modules can be used in the molecular assessment of the pathophysiology and the response to therapeutic cytokine blockade of inflammatory conditions, including COVID-19.

Methods

Datasets

All datasets used are provided in table 1. Data matrices were obtained from processed data series downloaded from the NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) or ArrayExpress repository (https://www.ebi.ac.uk/arrayexpress/). Probe identifiers were converted to gene symbols using platform annotations provided with each dataset. In circumstances where downloaded datasets were not log<sub>2</sub> transformed, this was performed on the entire processed data matrix. Duplicate genes were removed after the first one identified using Microsoft Excel duplicate remover function.

IL-1 and IL-6 module derivation

We previously derived an IL-1 transcriptional module from the transcriptome of fibroblasts stimulated with IL-1β or TNFα (16). We derived a novel IL-6 transcriptional module from a publicly available dataset (Table 1) reporting experiments of human monocyte-derived macrophages (MDM) stimulated with IL-1β (15 ng/ml) or IL-6 (25 ng/ml) for 4 hours (17). In this study the transcriptional programme of cytokine-stimulated MDM was
assessed by microarrays and hierarchical clustering was performed using Euclidean distance and average linkage method. This approach identified several unique clusters of genes differentially expressed following stimulation with each cytokine. Genes in clusters D & E showed elevated expression following stimulation by IL-6, but not by IL-1β. We combined the list of genes within these two clusters, removed duplicate or non-annotated genes, and termed this the IL-6 response module.

We applied the above IL-1 and IL-6 response modules to one study where transcriptional profiling was performed using the Nanostring system, which assesses the expression of a subset of the whole genome (594 genes) (18). Consequently, only a subset of the modules’ constituent genes were present in this dataset (table 2). Therefore, to verify the validity of applying our method to this dataset, we generated new cytokine response submodules using only genes from this subset, and showed them to provide the same discrimination of IL-1 and IL-6 responses as the parent modules (figs S2-3).

**Module expression assessment**

The expression of transcriptional modules was derived by calculating the geometric mean expression of all constituent genes, as previously described (13). The scripts used allowed the absence of a constituent gene in the analysed dataset, a scenario that did not affect geometric mean calculation.

**Statistical analysis**

All module score calculations were calculated in R v3.6.1 and RStudio v1.2.1335, using scripts generated and deposited in our previous publication ([https://github.com/MJMurray1/MDIScoring](https://github.com/MJMurray1/MDIScoring)) (13). Mann-Whitney tests, Spearman rank correlations and Kruskal-Wallis tests were calculated in GraphPad Prism v8.4.

**Results**

**Identification and validation of IL-1 and IL-6 transcriptional modules**

We first sought to derive transcriptional modules that identified and discriminated between the response to IL-1 and IL-6 stimulation. We have previously derived an IL-1 response module from cytokine stimulated fibroblasts (table 2) (16). As in our prior studies (12,13,16), we used the geometric mean of the constituent genes in a module as a summary statistic to describe the relative expression of the module. We demonstrate that in both MDM and PBMC (17,19), IL-1 stimulation induced greater expression of the IL-1 response module than either IL-6 or TNFα stimulation where there was no increased expression above unstimulated cells (Fig 1A + B). To identify an IL-6 response module which was able to discriminate from the effects of IL-1, we identified one study that had stimulated human MDM with either IL-1 (15 ng/ml) or IL-6 (25 ng/ml) for 4 hours (17). Hierarchical clustering identified genes induced by IL-6 but not IL-1 (17), and we termed this the IL-6 response module (table 2). Internal validation of this module confirmed increased expression in IL-6 stimulated
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MDM (fig 1A). Testing the IL-6 module in other datasets demonstrated elevated expression following IL-6, but not TNFα, stimulation of human kidney epithelial and macrophage cell lines (20,21) (figs 1C+D), whereas no elevated expression of the IL-6 module was observed following IL-1 or TNFα stimulation of MDM or PBMC (figs 1A+B). These findings demonstrated that the IL-1 and IL-6 response modules could detect the effects of their cognate cytokines, and discriminate these from each other and from an alternative inflammatory cytokine stimulus, TNFα.

IL-1 and IL-6 module expression in chronic inflammation

To determine whether IL-1 and IL-6 response modules were able to detect elevated cytokine bioactivity in vivo, we assessed the blood transcriptome of juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) patients. These are conditions in which elevated IL-1 and IL-6 activity are considered to play a key role in disease pathogenesis, evidenced by clinical improvement following therapeutic antagonism of these cytokines (22–25). The blood transcriptome of untreated JIA patients displayed elevated IL-1 and IL-6 bioactivity (fig 2A) (26), but this was not consistently evident in several RA blood transcriptome datasets (fig S1) (27–29). Discrepancies between molecular changes in blood and tissues have been previously described in RA (29), and therefore we tested the hypothesis that in contrast to blood, elevated IL-1 and IL-6 bioactivity was a feature of the synovium in RA. Consistent with this hypothesis, a separate transcriptomic dataset of synovial membrane biopsies from patients with RA (30) showed elevated levels of both IL-1 and IL-6 response module expression compared to non-RA synovium (fig 2C).

We used the elevated cytokine activity in the blood of JIA patients to test the hypothesis that therapeutic cytokine modulation would result in changes in cytokine bioactivity as determined by module expression. We made use of the blood transcriptome of JIA patients 3 days following administration of canakinumab, a human monoclonal antibody to IL-1β (26). Patients who had a therapeutic response to canakinumab showed elevated IL-1 module expression which reduced 3 days after canakinumab administration (fig 3A). In contrast, in those who had no treatment response, IL-1 module expression was lower at baseline and was unaffected by canakinumab (fig 3A). Unlike the differences seen in the IL-1 module between responders and non-responders, there were no differences between these groups in IL-6 module expression at baseline (fig 3B). This indicated that these two cytokine response modules quantified two distinct biological processes. Interestingly, expression of the IL-6 module was also diminished after canakinumab treatment in patients who responded to treatment, suggesting that IL-6 activity may be downstream of IL-1 in this context. Of note in these populations, the expression of the IL1B gene correlated with that of the IL-1 response module, but the same was not evident between IL-6 module and IL6 gene expression (fig 3C), illustrating an example in which cytokine gene expression itself may not necessarily reflect the functional activity of that cytokine.
We tested the hypothesis that elevated IL-1 and IL-6 bioactivity is a feature of COVID-19 disease, using the peripheral blood transcriptome of 3 patients with mild-moderate COVID-19 disease who were admitted to hospital and recovered (18). This dataset was generated using the Nanostring system and consisted of 594 mRNA targets, which included only 7/57 (12.2%) and 7/41 (17.1%) constituent genes of the IL-1 and IL-6 response modules respectively. We demonstrated that IL-1 and IL-6 submodules, generated from these shorter lists of constituent genes, were still able to recapitulate all the findings from fig 3 (fig S2). Assessing the expression of these submodules in the blood transcriptome of COVID-19 patients revealed that IL-1 and IL-6 bioactivity more closely reflected the time-dependent recovery of the patients, compared to the expression of IL1A, IL1B and IL6 genes over time (fig 4A).

Finally, we determined the expression of IL-1 and IL-6 response modules at the site of COVID-19 disease, using autopsy samples collected from pulmonary and non-pulmonary tissues (31). We tested the hypothesis that cytokine activity would be highest in the lungs, the site of predominant disease. This approach revealed elevated expression of IL-1 and IL-6 response modules in the lungs compared to other organs, using both full length modules and the abbreviated submodules (fig 4B & fig S3). In contrast, IL1A, IL1B and IL6 expression did not show such differences, further underlying the increased sensitivity of the transcriptional modules in quantifying cytokine bioactivity in COVID-19 infection (fig 4B).

Discussion

The protracted clinical course, inverse relationship between viral load and symptom progression, and the association between inflammation and worse clinical outcomes support a hypothesis whereby severe COVID-19 disease is predominantly driven by an exaggerated inflammatory response (1,2). Both IL-1 and IL-6 may play a role in this process (1,4–8), and in this study we utilised transcriptional modules derived from cytokine stimulated cells to demonstrate that their expression, but not that of their cognate cytokine genes, provided a quantitative readout for cytokine bioactivity in vivo, both in the context of COVID-19 and other chronic inflammatory conditions.

Studies modulating cytokine activity in COVID-19 have already been initiated, despite inconsistent demonstration of increased activity of these pathways in vivo (10,11). We show that in COVID-19, IL-1 and IL-6 transcriptional module expression is detectable in the blood and at the site of disease in the lung, and that module expression in blood more closely tracks recovery from illness than cognate cytokine gene expression. An interpretation of our findings is that the downstream response to cytokine stimulation is more persistent.
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than the expression of the cytokine gene mRNA, the stability of which is subject to trans-regulatory factors and feedback loops (32,33). Moreover, transcriptional modules are intrinsically composed of genes with co-correlated expression, minimising technical confounding of single gene measurements, demonstrated by the strongly concordant expression between the full and Nanostring subset IL-1 and IL-6 response modules. Therefore, we propose that transcriptional modules integrate the culmination of cytokine signalling events and provide a functional measure of cytokine bioactivity in vivo.

Our findings have several implications. First, they support the rationale for investigating the therapeutic benefit of neutralising IL-1 and IL-6 in COVID-19 (10,11), aiming to reduce cytokine induced pathology at the site of disease. Second, IL-1 and IL-6 response modules may be used to measure cytokine bioactivity following therapeutic immunomodulation with drugs that target these cytokines, such as anakinra, canakinumab and tocilizumab (9–11), permitting correlation between clinical responses and levels of cytokine activity. This may be key, as individual protein level measurements after cytokine blockade in vivo do not necessarily reflect bioactivity, exemplified by the rise in IL-6 cytokine in blood following administration of tocilizumab, a humanised monoclonal antibody against the IL-6 receptor (34). Finally, IL-1 and IL-6 response modules may be sensitive biomarkers to stratify for disease severity in COVID-19. This hypothesis will need to be tested in larger datasets with a greater range of clinical severity, alongside other gene signatures derived from unsupervised analyses of the blood transcriptome in COVID-19.

Our study has some clear limitations. Foremost, the sample sizes in the COVID-19 blood dataset utilised were small, and as this cohort did not have severe COVID-19 disease, we were not able to assess how module expression tracks more prolonged clinical syndromes. Thus, our findings will need to be validated in larger datasets with a wider range of COVID-19 severity when these become available in the public domain. Equally, assessing neutralisation of IL-1 and IL-6 with biologic agents was limited by the paucity of available datasets that assessed the impact of these drugs in the days, not months, following administration, analogous to the analyses planned in COVID-19 (10). Finally, determining the specificity of the IL-1 and IL-6 response modules was limited to the available datasets and the range of cytokine stimulation conditions performed in those experiments. Comparing the expression of these modules across a wider range of biologically paired cytokine stimulations will allow refinement of their sensitivity and specificity.

In conclusion, our data support elevated activity of the inflammatory cytokines IL-1 and IL-6 in COVID-19, and demonstrate the power of cytokine transcriptional response modules in providing a dynamic readout of the activity of these pathways in vivo. We propose that use of these modules may enhance efforts to investigate the pathology of COVID-19, support development of methods to stratify patients’ risk of clinical progression, and aid quantification of the biological effects of host-directed immunomodulatory therapeutics in COVID-19.
Data and software availability
All transcriptional datasets used in this manuscript were derived from public repositories. Their source is detailed in table 1 and software used to analyse the data described in the methods.

Author contributions
GP and LCKB conceived the study and performed the analyses. GP, LCKB and MN critically appraised the results, drafted the manuscript, and agreed on the data presented and the conclusions reached in the final version.

Conflicts of interests
No conflicts of interests were disclosed.

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Figure legends

**Figure 1.** Validation of cytokine response modules. Geometric mean module expression in A) MDM stimulated in vitro with either IL-1β (15 ng/ml) or IL-6 (25 ng/ml) for 4 hours, B) PBMC stimulated with TNFα (20 ng/ml) or IL-1β (10 ng/ml) for 6 hours, C) human renal proximal tubular epithelial (HK-2) cells stimulated with IL-6 (200 ng/ml) or TNFα (100 ng/ml) for 1.5 hours and D) human macrophage cell lines (THP-1) stimulated with IL-6 (50 ng/ml) or TNFα (10 ng/ml) for 2 hours. Transcriptomic datasets are designated adjacent to figure panels. * = p < 0.05 by Mann-Whitney test.

**Figure 2.** Cytokine response module expression in chronic inflammatory conditions. Geometric mean expression of IL-1 and IL-6 cytokine response modules in A) blood of patients with JIA compared to healthy controls, and B) in the synovium of RA patients compared to that of healthy controls. Transcriptomic datasets are designated adjacent to figure panels. * = p < 0.05 by Mann-Whitney test.

**Figure 3.** Effect of canakinumab on expression of cytokine response modules and genes. A) Geometric mean expression of IL-1 and IL-6 cytokine response modules in JIA patients before and 3 days after administration of canakinumab. Patients were subdivided into good responders (90-100% improvement) and non-responders (0-30% improvement). Dotted lines indicate median module or gene expression in healthy controls (HC) population in same dataset. * = p < 0.05 by Mann-Whitney test. B) Relationship between expression of cytokine response modules and cytokine genes. Statistical assessment of correlation made by Spearman Rank correlation. \( r^2 \) = correlation coefficient. Transcriptomic dataset designated adjacent to figure panels.

**Figure 4.** Cytokine response module and gene expression in COVID-19. A) Geometric mean expression of IL-1 and IL-6 response module and \( IL1A \), \( IL1B \) and \( IL6 \) gene expression in patients admitted for COVID-19 disease. Number of patient samples at each timepoint designated on first plot of each row, but applicable for all panels. Where more than one sample available at any time point, the mean expression +/- SEM is plotted. p value represents Kruskal-Wallis test with time since hospital admission as the independent variable. B) Geometric mean expression of cytokine response modules and expression of cytokine genes in samples taken from lungs and non-lung organs at autopsy from patients infected with COVID-19. * = p<0.05 by Mann-Whitney test. Transcriptomic datasets assessed are designated adjacent to each figure panel.
Supplementary figure legends

**Figure S1.** Cytokine response module expression in the blood of rheumatoid arthritis (RA) patients. Geometric mean expression of IL-1 and IL-6 cytokine response modules in the transcriptome of blood samples from RA patients compared to healthy controls. * = p < 0.05 by Mann-Whitney test. Transcriptomic datasets assessed are designated adjacent to each figure panel.

**Figure S2.** Effect of canakinumab on expression of cytokine genes and response submodules. A) Geometric mean expression of IL-1 and IL-6 cytokine response submodules in JIA patients before and 3 days after administration of canakinumab. Patients were subdivided into good responders (90-100% improvement) and non-responders (0-30% improvement). Dotted lines indicate median module or gene expression in healthy controls (HC) population in same dataset. * = p < 0.05 by Mann-Whitney test. B) Relationship between expression of cytokine response modules and cytokine genes. Statistical assessment of correlation made by Spearman Rank correlation. $r^2$ = correlation coefficient. Transcriptomic dataset designated adjacent to figure panels.

**Figure S3.** Cytokine response submodules and gene expression in COVID-19 infected tissues. Geometric mean expression of cytokine response submodules in samples taken from lungs and non-lung organs at autopsy from patients infected with COVID-19. * = p<0.05 by Mann-Whitney test. Transcriptomic datasets assessed are designated adjacent to each figure panel.
### Tables

**Table 1.** Transcriptional datasets used in this manuscript

| Title of dataset                                                                 | Accession number | Repository   |
|---------------------------------------------------------------------------------|------------------|-------------|
| Identification of IL-1 and IL-6-responsive genes in human monocyte-derived macrophages | GSE8515          | NCBI GEO    |
| Transcriptome analysis in peripheral blood mononuclear cells (PBMC) from HOIL-1-deficient patients upon TNF-α or IL-1β stimulation | GSE40838         | NCBI GEO    |
| Response of HK-2 cells to stimulation with IL6 and TNF-alpha                     | GSE68940         | NCBI GEO    |
| Comparative gene expression in response to various inflammatory stimuli in vitro: infection-mediated versus systemic inflammation | GSE126525        | NCBI GEO    |
| Gene expression data of whole blood of systemic juvenile idiopathic arthritis (SJIA) patients treated with canakinumab or placebo and age matched healthy controls | GSE80060         | NCBI GEO    |
| Gene expression from the whole blood of rheumatoid arthritis patients and normal controls. | GSE68689         | NCBI GEO    |
| Multi-omics monitoring of drug response in rheumatoid arthritis.                | GSE93777         | NCBI GEO    |
| Transcriptional Signature Associated with Early Rheumatoid Arthritis and Healthy Individuals at high risk to develop the disease. | GSE100191        | NCBI GEO    |
| Synovial biopsies of rheumatoid arthritis and healthy controls                  | GSE77298         | NCBI GEO    |
| Transcriptomic analysis of immune response in healthy controls and COVID-19 cases using the NanoString Human Immunology Panel | E-MTAB-8871      | ArrayExpress|
| Spectrum of Viral Load and Host Response Seen in Autopsies of SARS-CoV-2 Infected Lungs | GSE150316        | NCBI GEO    |
| Module name         | Number of genes | Gene names                                                                 |
|---------------------|-----------------|-----------------------------------------------------------------------------|
| IL-1 response       | 57              | ADOR2A, C15ORF48, C200RF127, C2CD48, C70RF63, CCL20, CCL8, CHMP1B, CSF2,     |
|                     |                 | CSF3, CXCL1, CXCL2, CXCL5, CXCL6, DNAJB9, EGLN1, FGF2, FOXO3, GLI1S3, GNA15, |
|                     |                 | HAS3, HIATL1, IER3, IL11, IL6, ITPR1P, JARID2, KCNG1, LOC100134000, LRIG1,  |
|                     |                 | MAP3K8, MFSD2, MGC87042, MSL3, MT1G, MT1X, MTE, MTHFD2L, NAB1, NAMPT,       |
|                     |                 | NFKBIZ, NR4A2, OSGIN2, PFKFB3, PIM2, RCAN1, RNF145, SERPINB4, SGK1,        |
|                     |                 | SLCA3A3, STEAP1, STEAP2, TFAP2C, TGF1, TWIST2, ZC3H12A, ZC3H12C            |
| IL-1 response       | 7               | CCL20, CCL8, CSF2, CXCL1, CXCL2, IL6, NFKBIZ                                |
| submodule            |                 |                                                                             |
| IL-6 response       | 41              | AAMP, AKIP1, ANKR1D10, ARPC2, CCR1, CD14, CSDE1, CTDSP2, CTNNA1, CXADR,    |
|                     |                 | DOK1, GADD45B, HAMP, IDHI3B, IFI16, IL16, KAT5, LDLRAP1, MAP4, MR1, MRSB2, |
|                     |                 | NCF4, NDUF8B, NPC1, PGD, PI4K2A, PPARD, PSMD4, RAP1GAP, RHOC, RIN2,        |
|                     |                 | RNASE1, RREB1, SASH1, SDS, SP110, STIP1, TSC22D3, UBE2M, UFL1, YBX3         |
| IL-6 response       | 7               | CCR1, CD14, HAMP, IFI16, IL16, MR1, NCF4                                   |
| submodule            |                 |                                                                             |
**Figure 1**

A. **IL-1 response module**

| Condition          | Module Expression (log2) |
|--------------------|-------------------------|
| Unstimulated       | 6.0                     |
| + IL-6             | 6.5                     |
| + IL-1             | 7.5                     |

B. **IL-6 response module**

| Condition          | Module Expression (log2) |
|--------------------|-------------------------|
| Unstimulated       | 7.0                     |
| + IL-6             | 7.5                     |
| + IL-1             | 8.0                     |

MDM - dataset GSE8515  
PBMC - dataset GSE40838

C. **IL-6 response module**

| Condition          | Module Expression (log2) |
|--------------------|-------------------------|
| Unstimulated       | 7.5                     |
| + IL-6             | 8.0                     |
| + TNF_{α}           | 8.5                     |

Epithelial cell line - dataset: GSE68940

D. **IL-6 response module**

| Condition          | Module Expression (log2) |
|--------------------|-------------------------|
| Unstimulated       | 8.2                     |
| + IL-6             | 8.4                     |
| + TNF_{α}           | 8.6                     |

Macrophage cell line - dataset GS126525
Figure 2

A

IL-1 response module  IL-6 response module

JIA blood - dataset GSE80060

B

IL-1 response module  IL-6 response module

RA synovium - dataset GSE77298
Figure 3

A

IL-1 response module

| Module expression (log2) |
|-------------------------|
| 6.6 | 6.4 | 6.2 | 6.0 | 5.8 |
| Responders | * | | | |
| Non-responders | | | | |

IL-6 response module

| Module expression (log2) |
|-------------------------|
| 8.4 | 8.0 | 7.6 | 7.2 | 6.8 |
| Responders | * | | | |
| Non-responders | | | | |

Baseline

Post-canakinumab

HC

**JIA blood - dataset GSE80060**

B

**JIA blood - dataset GSE80060**

r^2 = 0.650
p < 0.0001

r^2 = -0.02679
p = 0.8418

IL1B gene expression (log2)

IL6 gene expression (log2)
Figure 4

A

IL-1 response module

IL1A gene

IL1B gene

IL-6 response module

IL6 gene

Days from hospital admission

COVID-19 blood - dataset E-MTAB-8871

B

COVID-19 tissues - dataset GSE150316
Figure S1

RA blood – dataset GSE68689

RA blood – dataset GSE93777

RA blood – dataset GSE100191
Figure S2

A

IL-1 response submodule  IL-6 response submodule

| Module expression (log2) |
|--------------------------|
| Responders               |
| Nonresponders            |

- Baseline
- D3 + canakinumab

B

JIA blood - dataset GSE80060

IL-1 response submodule expression (log2)

- $r^2 = 0.571$
- $p < 0.0001$

IL-6 response submodule expression (log2)

- $r^2 = -0.0507$
- $p = 0.7053$

JIA blood - dataset GSE80060
**Figure S3**

IL-1 response submodule

COVID-19 tissues - dataset GSE150316