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.

33 ## Introduction

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

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

51

52 ## Methods

53 ### *Datasets*

54 All datasets used are provided in table 1. Data matrices were obtained from processed data series
55 downloaded from the NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) or Array
56 Express repository (https://www.ebi.ac.uk/arrayexpress/). Probe identifiers were converted to gene symbols
57 using platform annotations provided with each dataset. In circumstances where downloaded datasets were
58 not $\log_2$ transformed, this was performed on the entire processed data matrix. Duplicate genes were removed
59 after the first one identified using Microsoft Excel duplicate remover function.

60 ### *IL-1 and IL-6 module derivation*

61 We previously derived an IL-1 transcriptional module from the transcriptome of fibroblasts stimulated with IL-
62 1β or TNFα (16). We derived a novel IL-6 transcriptional module from a publicly available dataset (Table 1)
63 reporting experiments of human monocyte-derived macrophages (MDM) stimulated with IL-1β (15 ng/ml) or
64 IL-6 (25 ng/ml) for 4 hours (17). In this study the transcriptional programme of cytokine-stimulated MDM was

65 assessed by microarrays and hierarchical clustering was performed using Euclidean distance and average
66 linkage method. This approach identified several unique clusters of genes differentially expressed following
67 stimulation with each cytokine. Genes in clusters D & E showed elevated expression following stimulation by
68 IL-6, but not by IL-1β. We combined the list of genes within these two clusters, removed duplicate or non-
69 annotated genes, and termed this the IL-6 response module.

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

76 ### *Module expression assessment*

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

80 ### *Statistical analysis*

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

84

85 ## Results

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

87 We first sought to derive transcriptional modules that identified and discriminated between the response to
88 IL-1 and IL-6 stimulation. We have previously derived an IL-1 response module from cytokine stimulated
89 fibroblasts (table 2) (16). As in our prior studies (12,13,16), we used the geometric mean of the constituent
90 genes in a module as a summary statistic to describe the relative expression of the module. We demonstrate
91 that in both MDM and PBMC (17,19), IL-1 stimulation induced greater expression of the IL-1 response module
92 than either IL-6 or TNFα stimulation where there was no increased expression above unstimulated cells (Fig
93 1A + B). To identify an IL-6 response module which was able to discriminate from the effects of IL-1, we
94 identified one study that had stimulated human MDM with either IL-1 (15 ng/ml) or IL-6 (25 ng/ml) for 4 hours
95 (17). Hierarchical clustering identified genes induced by IL-6 but not IL-1 (17), and we termed this the IL-6
96 response module (table 2). Internal validation of this module confirmed increased expression in IL-6 stimulated

97 MDM (fig 1A). Testing the IL-6 module in other datasets demonstrated elevated expression following IL-6, but
98 not TNFα, stimulation of human kidney epithelial and macrophage cell lines (20,21) (figs 1C+D), whereas no
99 elevated expression of the IL-6 module was observed following IL-1 or TNFα stimulation of MDM or PBMC
100 (figs 1A+B). These findings demonstrated that the IL-1 and IL-6 response modules could detect the effects of
101 their cognate cytokines, and discriminate these from each other and from an alternative inflammatory
102 cytokine stimulus, TNFα.

103

104 ### *IL-1 and IL-6 module expression in chronic inflammation*

105 To determine whether IL-1 and IL-6 response modules were able to detect elevated cytokine bioactivity in
106 vivo, we assessed the blood transcriptome of juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA)
107 patients. These are conditions in which elevated IL-1 and IL-6 activity are considered to play a key role in
108 disease pathogenesis, evidenced by clinical improvement following therapeutic antagonism of these cytokines
109 (22–25). The blood transcriptome of untreated JIA patients displayed elevated IL-1 and IL-6 bioactivity (fig 2A)
110 (26), but this was not consistently evident in several RA blood transcriptome datasets (fig S1) (27–29).
111 Discrepancies between molecular changes in blood and tissues have been previously described in RA (29), and
112 therefore we tested the hypothesis that in contrast to blood, elevated IL-1 and IL-6 bioactivity was a feature
113 of the synovium in RA. Consistent with this hypothesis, a separate transcriptomic dataset of synovial
114 membrane biopsies from patients with RA (30) showed elevated levels of both IL-1 and IL-6 response module
115 expression compared to non-RA synovium (fig 2C).

116 We used the elevated cytokine activity in the blood of JIA patients to test the hypothesis that therapeutic
117 cytokine modulation would result in changes in cytokine bioactivity as determined by module expression. We
118 made use of the blood transcriptome of JIA patients 3 days following administration of canakinumab, a human
119 monoclonal antibody to IL-1β (26). Patients who had a therapeutic response to canakinumab showed elevated
120 IL-1 module expression which reduced 3 days after canakinumab administration (fig 3A). In contrast, in those
121 who had no treatment response, IL-1 module expression was lower at baseline and was unaffected by
122 canakinumab (fig 3A). Unlike the differences seen in the IL-1 module between responders and non-responders,
123 there were no differences between these groups in IL-6 module expression at baseline (fig 3B). This indicated
124 that these two cytokine response modules quantified two distinct biological processes. Interestingly,
125 expression of the IL-6 module was also diminished after canakinumab treatment in patients who responded
126 to treatment, suggesting that IL-6 activity may be downstream of IL-1 in this context. Of note in these
127 populations, the expression of the *IL1B* gene correlated with that of the IL-1 response module, but the same
128 was not evident between IL-6 module and *IL6* gene expression (fig 3C), illustrating an example in which
129 cytokine gene expression itself may not necessarily reflect the functional activity of that cytokine.

130

131 ### *IL-1 and IL-6 bioactivity in COVID-19*

132 We tested the hypothesis that elevated IL-1 and IL-6 bioactivity is a feature of COVID-19 disease, using the
133 peripheral blood transcriptome of 3 patients with mild-moderate COVID-19 disease who were admitted to
134 hospital and recovered (18). This dataset was generated using the Nanostring system and consisted of 594
135 mRNA targets, which included only 7/57 (12.2%) and 7/41 (17.1%) constituent genes of the IL-1 and IL-6
136 response modules respectively. We demonstrated that IL-1 and IL-6 submodules, generated from these
137 shorter lists of constituent genes, were still able to recapitulate all the findings from fig 3 (fig S2). Assessing
138 the expression of these submodules in the blood transcriptome of COVID-19 patients revealed that IL-1 and
139 IL-6 bioactivity more closely reflected the time-dependent recovery of the patients, compared to the
140 expression of *IL1A*, *IL1B* and *IL6* genes over time (fig 4A).

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

148

149 ## Discussion

150 The protracted clinical course, inverse relationship between viral load and symptom progression, and the
151 association between inflammation and worse clinical outcomes support a hypothesis whereby severe COVID-
152 19 disease is predominantly driven by an exaggerated inflammatory response (1,2). Both IL-1 and IL-6 may
153 play a role in this process (1,4–8), and in this study we utilised transcriptional modules derived from cytokine
154 stimulated cells to demonstrate that their expression, but not that of their cognate cytokine genes, provided
155 a quantitative readout for cytokine bioactivity in vivo, both in the context of COVID-19 and other chronic
156 inflammatory conditions.

157 Studies modulating cytokine activity in COVID-19 have already been initiated, despite inconsistent
158 demonstration of increased activity of these pathways in vivo (10,11). We show that in COVID-19, IL-1 and IL-
159 6 transcriptional module expression is detectable in the blood and at the site of disease in the lung, and that
160 module expression in blood more closely tracks recovery from illness than cognate cytokine gene expression.
161 An interpretation of our findings is that the downstream response to cytokine stimulation is more persistent

162 than the expression of the cytokine gene mRNA, the stability of which is subject to trans-regulatory factors
163 and feedback loops (32,33). Moreover, transcriptional modules are intrinsically composed of genes with co-
164 correlated expression, minimising technical confounding of single gene measurements, demonstrated by the
165 strongly concordant expression between the full and Nanostring subset IL-1 and IL-6 response modules.
166 Therefore, we propose that transcriptional modules integrate the culmination of cytokine signalling events
167 and provide a functional measure of cytokine bioactivity in vivo.

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

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

189 In conclusion, our data support elevated activity of the inflammatory cytokines IL-1 and IL-6 in COVID-19, and
190 demonstrate the power of cytokine transcriptional response modules in providing a dynamic readout of the
191 activity of these pathways in vivo. We propose that use of these modules may enhance efforts to investigate
192 the pathology of COVID-19, support development of methods to stratify patients' risk of clinical progression,
193 and aid quantification of the biological effects of host-directed immunomodulatory therapeutics in COVID-19.

194

195 ## Data and software availability

196 All transcriptional datasets used in this manuscript were derived from public repositories. Their source is
197 detailed in table 1 and software used to analyse the data described in the methods.

198 ## Author contributions

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

202 ## Conflicts of interests

203 No conflicts of interests were disclosed.

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208

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295

296

297 ## Figure legends

298 **Figure 1.** Validation of cytokine response modules. Geometric mean module expression in A) MDM stimulated
299 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)
300 or IL-1β (10 ng/ml) for 6 hours, C) human renal proximal tubular epithelial (HK-2) cells stimulated with IL-6
301 (200 ng/ml) or TNFα (100 ng/ml) for 1.5 hours and D) human macrophage cell lines (THP-1) stimulated with
302 IL-6 (50 ng/ml) or TNFα (10 ng/ml) for 2 hours. Transcriptomic datasets are designated adjacent to figure
303 panels. * = $p < 0.05$ by Mann-Whitney test.

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

308 **Figure 3.** Effect of canakinumab on expression of cytokine response modules and genes. A) Geometric mean
309 expression of IL-1 and IL-6 cytokine response modules in JIA patients before and 3 days after administration
310 of canakinumab. Patients were subdivided into good responders (90-100% improvement) and non-responders
311 (0-30% improvement). Dotted lines indicate median module or gene expression in healthy controls (HC)
312 population in same dataset. * = $p < 0.05$ by Mann-Whitney test. B) Relationship between expression of
313 cytokine response modules and cytokine genes. Statistical assessment of correlation made by Spearman Rank
314 correlation. r^2 = correlation coefficient. Transcriptomic dataset designated adjacent to figure panels.

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

323

324

325 ## Supplementary figure legends

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

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

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

342

343 ## Tables

344 **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

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346

347 **Table 2.** Cytokine response transcriptional modules and constituent genes

Module name	Number of genes	Gene names
IL-1 response	57	*ADORA2A, C15ORF48, C20ORF127, C2CD4B, C7ORF63, CCL20, CCL8, CHMP1B, CSF2, CSF3, CXCL1, CXCL2, CXCL5, CXCL6, DNAJB9, EGLN1, FGF2, FOXO3, GLIS3, GNA15, HAS3, HIATL1, IER3, IL11, IL6, ITPRIP, JARID2, KCNG1, LOC100134000, LRIG1, MAP3K8, MFSD2, MGC87042, MSL3, MT1G, MT1X, MTE, MTHFD2L, NAB1, NAMPT, NFKBIZ, NR4A2, OSGIN2, PFKFB3, PIM2, RCAN1, RNF145, SERPINB4, SGK1, SLC43A3, STEAP1, STEAP2, TFAP2C, TGIF1, TWIST2, ZC3H12A, ZC3H12C*
IL-1 response submodule	7	*CCL20, CCL8, CSF2, CXCL1, CXCL2, IL6, NFKBIZ*
IL-6 response	41	*AAMP, AKIP1, ANKRD10, ARPC2, CCR1, CD14, CSDE1, CTDSP2, CTNNA1, CXADR, DOK1, GADD45B, HAMP, IDH3B, IFI16, IL16, KAT5, LDLRAP1, MAP4, MR1, MSRB2, NCF4, NDUFB8, NPC1, PGD, PI4K2A, PPARD, PSMD4, RAP1GAP, RHOC, RIN2, RNASE1, RREB1, SASH1, SDS, SP110, STIP1, TSC22D3, UBE2M, UFL1, YBX3*
IL-6 response submodule	7	*CCR1, CD14, HAMP, IFI16, IL16, MR1, NCF4*

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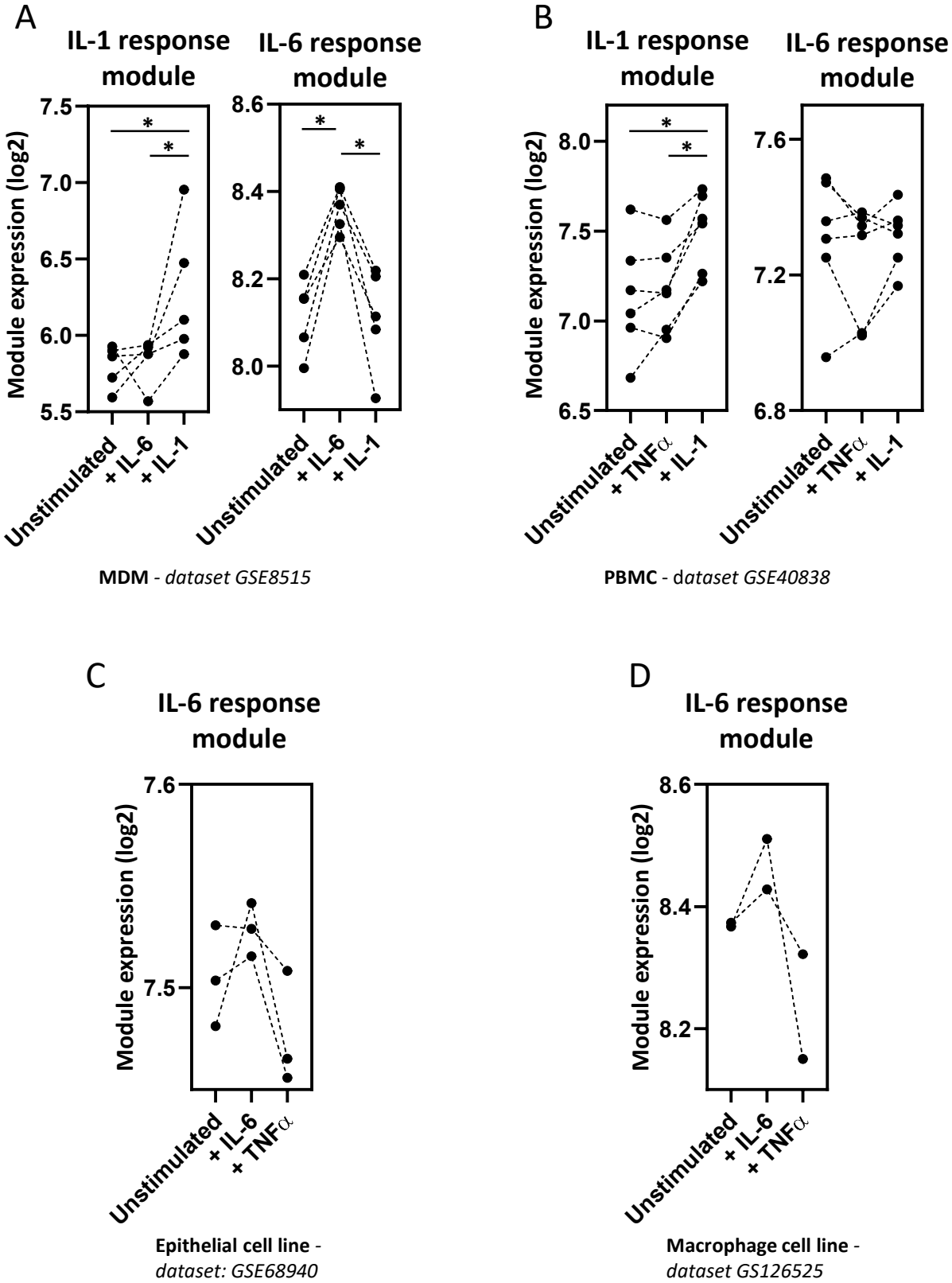
Figure 1



MDM - *dataset GSE8515*

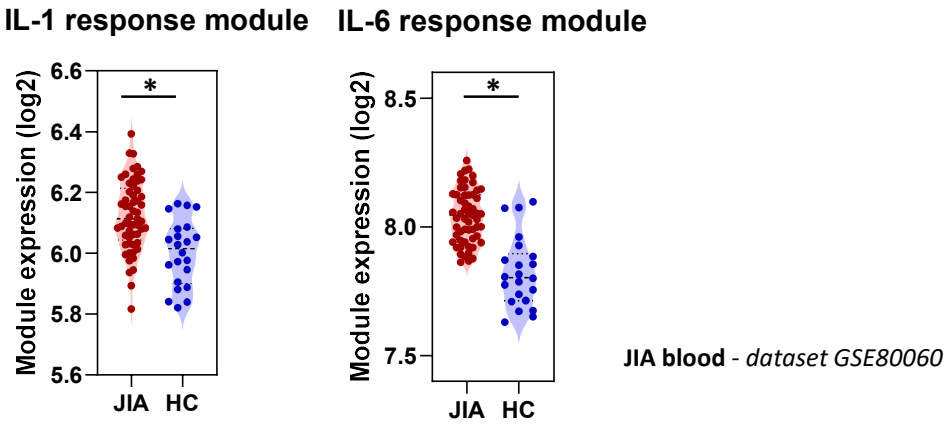
PBMC - d*ataset GSE40838*

Epithelial cell line - *dataset: GSE68940*

Macrophage cell line - *dataset GS126525*

Figure 2

A


IL-1 response module
IL-6 response module
Module expression (log2)
6.6
6.4
6.2
6.0
5.8
5.6
*
JIA
HC
Module expression (log2)
8.5
8.0
7.5
*
JIA
HC
JIA blood - dataset GSE80060

B

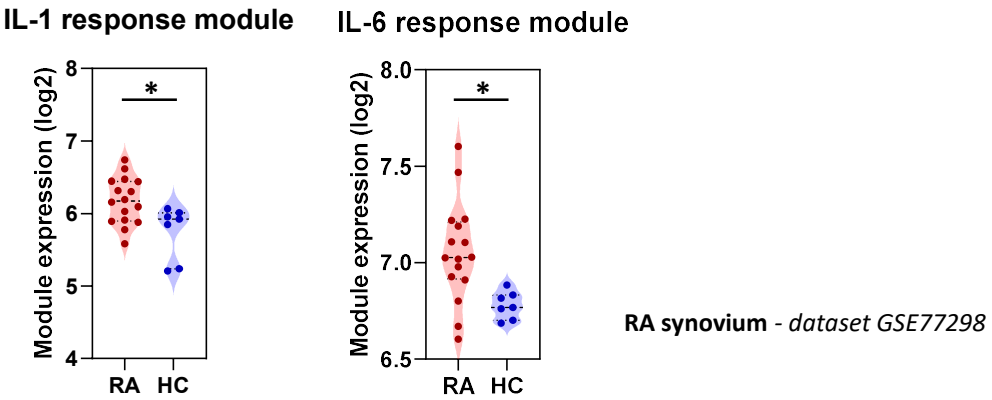
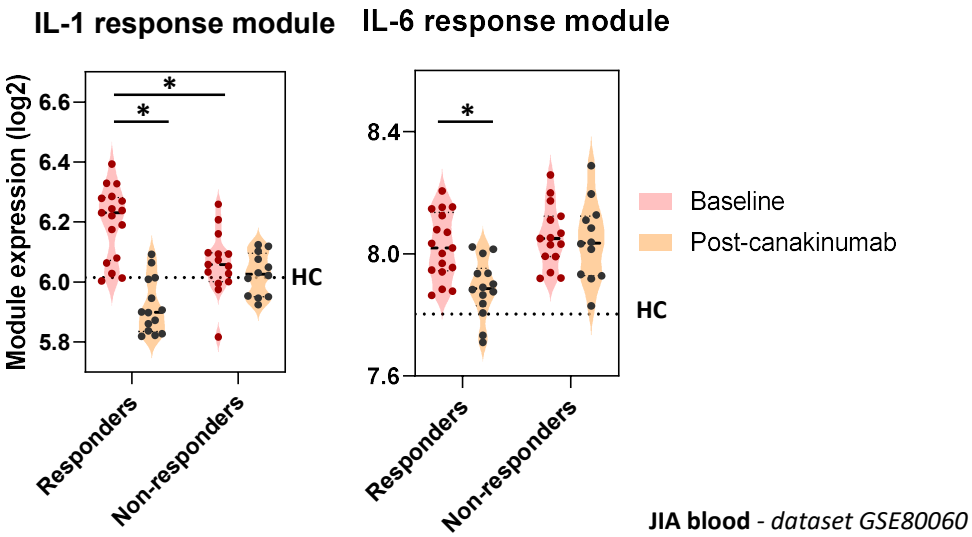

IL-1 response module
IL-6 response module
Module expression (log2)
8
7
6
5
4
*
RA
HC
Module expression (log2)
8.0
7.5
7.0
6.5
*
RA
HC
RA synovium - dataset GSE77298

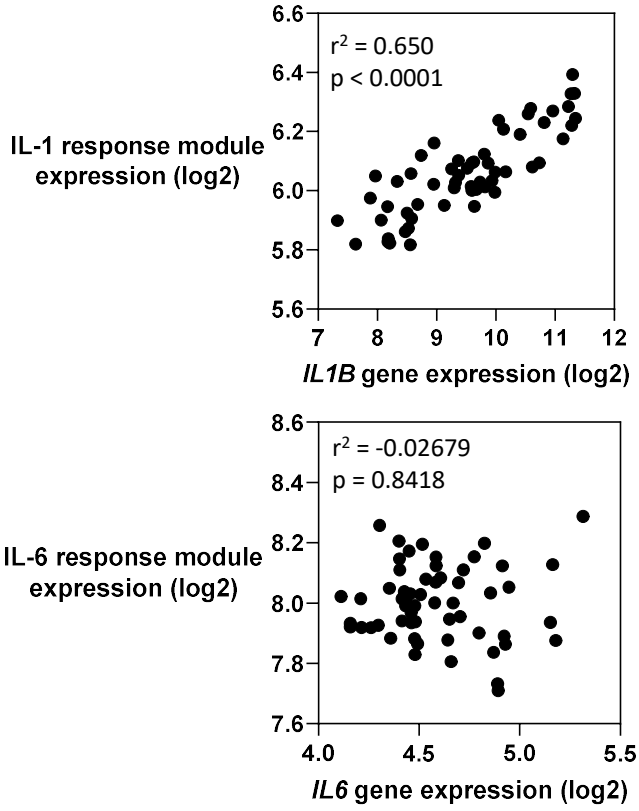
Figure 3

A



JIA blood - *dataset GSE80060*

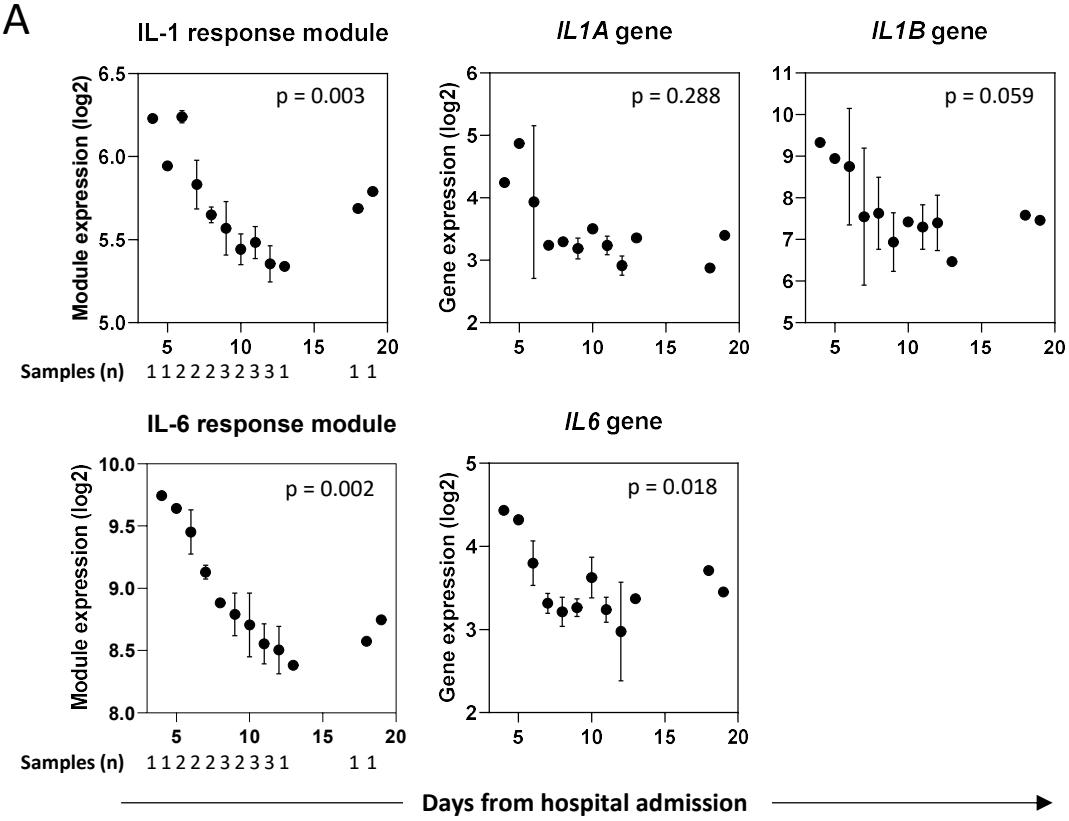
B



JIA blood - *dataset GSE80060*

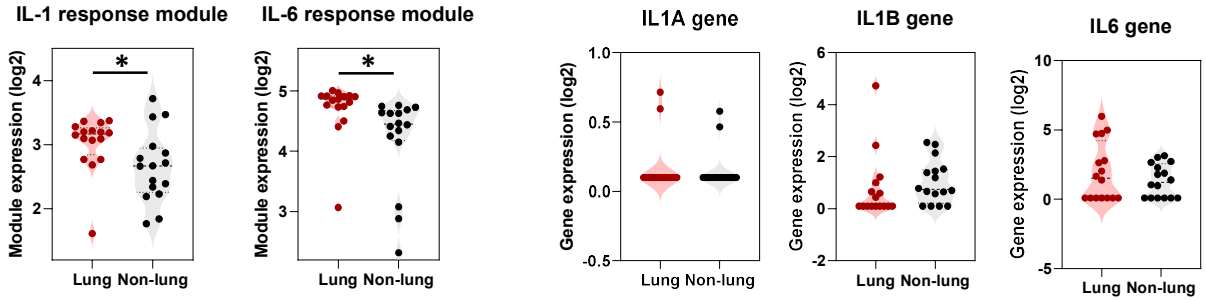
Figure 4

A



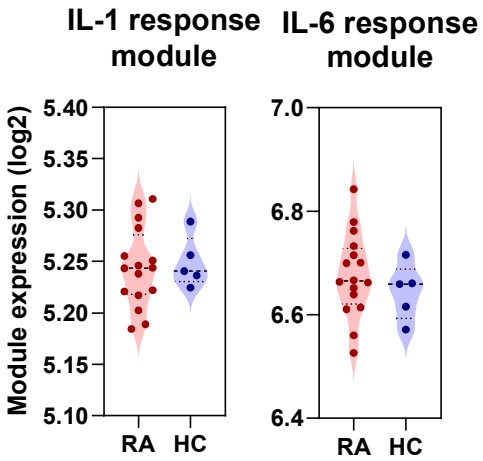
COVID-19 blood - *dataset E-MTAB-8871*

B

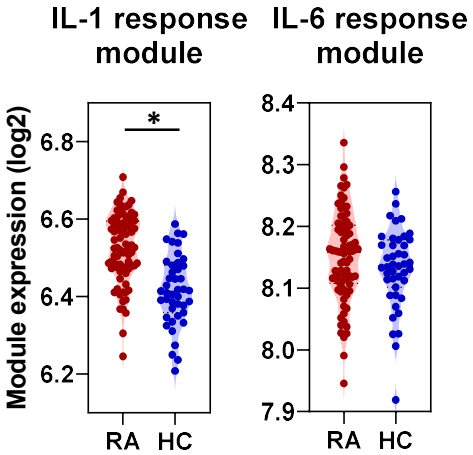


COVID-19 tissues - *dataset GSE150316*

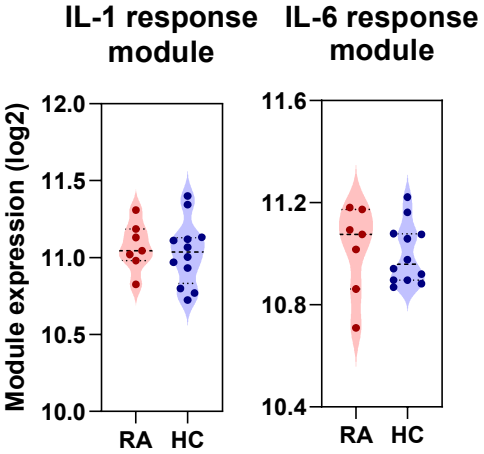
Figure S1



RA blood – *dataset GSE68689*



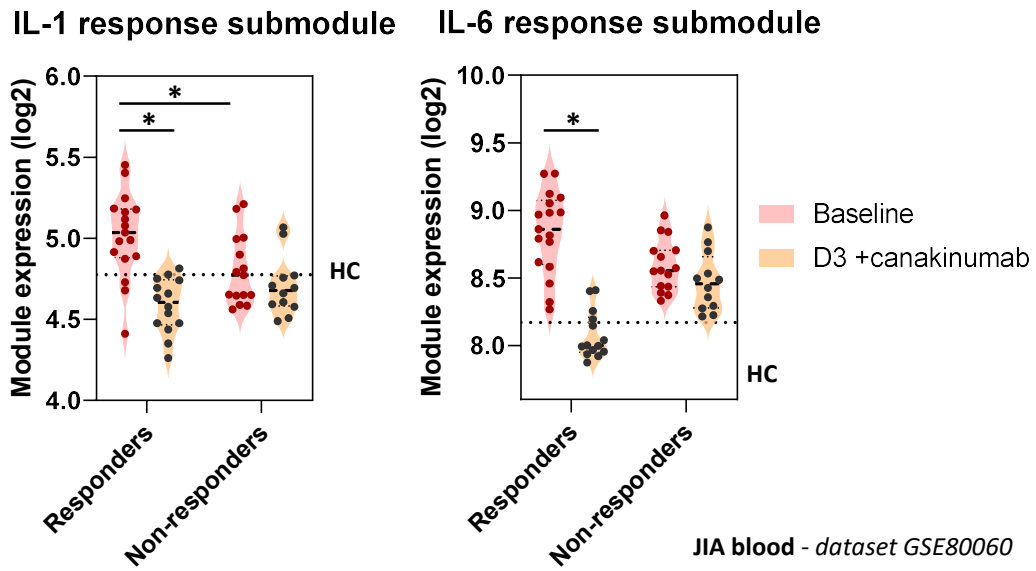
RA blood – *dataset GSE93777*



RA blood – *dataset GSE100191*

Figure S2

A


IL-1 response submodule
IL-6 response submodule
Module expression (log2)
6.0
5.5
5.0
4.5
4.0
10.0
9.5
9.0
8.5
8.0
*
HC
Responders
Non-responders
Baseline
D3 +canakinumab
JIA blood - dataset GSE80060

B

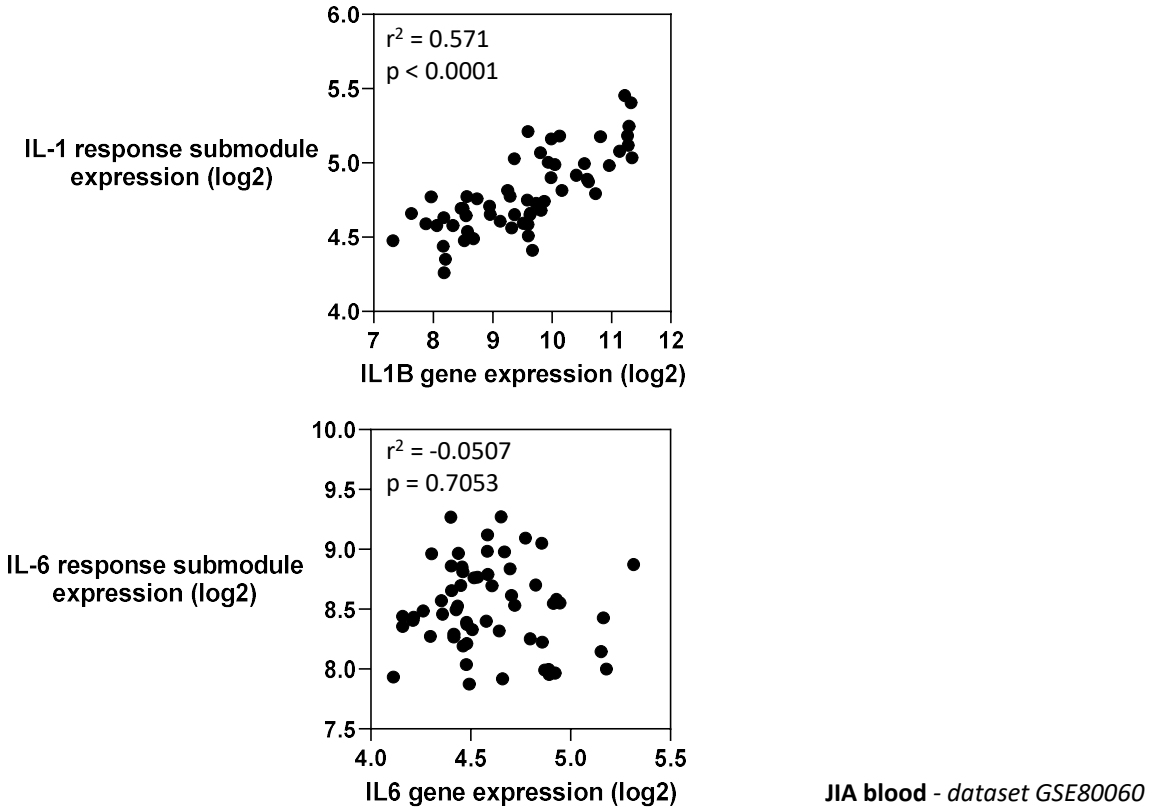
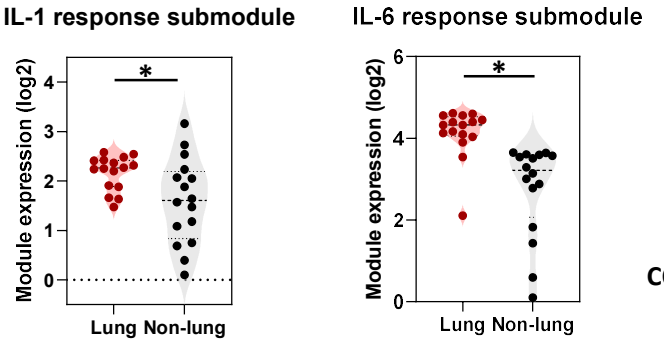

IL-1 response submodule expression (log2)
r2 = 0.571
p < 0.0001
IL1B gene expression (log2)
IL-6 response submodule expression (log2)
r2 = -0.0507
p = 0.7053
IL6 gene expression (log2)
JIA blood - dataset GSE80060

Figure S3



COVID-19 tissues - *dataset GSE150316*