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1 **A monocyte/dendritic cell molecular signature of SARS-** 2 **CoV2-related multisystem inflammatory syndrome in** 3 **children (MIS-C) with severe myocarditis**

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78

79 **Abstract**

80 SARS-CoV-2 infection in children is generally milder than in adults, yet a proportion of cases
81 result in hyperinflammatory conditions often including myocarditis. To better understand these
82 cases, we applied a multi-parametric approach to the study of blood cells of 56 children
83 hospitalized with suspicion of SARS-CoV-2 infection. The most severe forms of MIS-C
84 (multisystem inflammatory syndrome in children related to SARS-CoV-2), that resulted in
85 myocarditis, were characterized by elevated levels of pro-angiogenesis cytokines and several
86 chemokines. Single-cell transcriptomic analyses identified a unique monocyte/dendritic cell
87 gene signature that correlated with the occurrence of severe myocarditis, characterized by
88 sustained NF- κ B activity, TNF- α signaling, associated with decreased gene expression of NF-
89 κ B inhibitors. We also found a weak response to type-I and type-II interferons,

90 hyperinflammation and response to oxidative stress related to increased HIF-1 α and VEGF
91 signaling. These results provide potential for a better understanding of disease
92 pathophysiology.

93

94 **Introduction**

95 In adults, critical forms of COVID-19 are typically characterized by severe pneumonia and
96 acute respiratory distress syndrome (Wiersinga et al., 2020). In children, symptomatic COVID-
97 19 occurs much less frequently and is milder than in adults, with multifactorial reasons for
98 these differences (Brodin, 2020; Castagnoli et al., 2020; Gudbjartsson et al., 2020; Levy et al.,
99 2020; Tagarro et al., 2020). However, in regions with high incidence of SARS-CoV-2
100 infection, some children have presented a postacute hyperinflammatory illness (Datta et al.,
101 2020). In these cases, diagnostic evidence of recent SARS-CoV-2 infection has been
102 consistently reported (Abrams et al., 2020; Jones et al., 2020; Toubiana et al., 2020, 2021).
103 This condition was named multisystem inflammatory syndrome in children (MIS-C) or
104 alternatively PIMS-TS (Pediatric Inflammatory Multisystem Syndrome Temporally
105 Associated with SARS-CoV-2)(Whittaker et al., 2020). MIS-C cases most often present with
106 symptoms similar to Kawasaki Disease (KD), an hyperinflammatory illness characterized by
107 clinical features such as strawberry-like tongue and red and dry lips, bulbar conjunctival
108 injection, diffuse rash, swollen extremities and cervical lymphadenopathy (McCrindle et al.,
109 2017). KD complications can develop as myocarditis or shock syndrome in a minority of cases
110 (Kanegaye et al., 2009). KD is thought to be triggered by viral or bacterial pathogens but the
111 precise pathophysiological mechanisms remain elusive, with one hypothesis proposing an
112 uncontrolled superantigen-driven inflammatory immune response (Chang et al., 2014).
113 Compared to classic KD, MIS-C occurs in patients who are older, have more often

114 gastrointestinal symptoms, myocarditis and shock syndrome, and exhibit higher levels of
115 inflammatory markers (Abrams et al., 2020; Datta et al., 2020; Toubiana et al., 2020, 2021).

116 Inflammatory features of MIS-C are in part overlapping with those of both KD and acute
117 SARS-CoV-2 infection in children, as well as severe COVID-19 in adults (Carter et al., 2020;
118 Consiglio et al., 2020; Datta et al., 2020; Gruber et al., 2020). Very high levels of C-reactive
119 protein (CRP), Procalcitonin (PCT) and IL-6, might reflect a strong immunological response
120 to a pathogenic SARS-CoV-2 superantigen (Cheng et al., 2020). Autoimmune features can also
121 be found in MIS-C patients (Gruber et al., 2020).

122 In order to further decipher SARS-CoV-2-related conditions in children, we have performed a
123 detailed multi-parametric study combining sensitive cytokine measurements, deep immune cell
124 phenotyping and transcriptomic analyses at the single-cell level on peripheral blood
125 mononuclear cells (PBMCs).

126

127 **Results**

128 *Clinical description of the cohort*

129 The study cohort consisted of 56 children hospitalized during the first peak of the SARS-CoV-
130 2 pandemic (from the 6th of April to the 30th of May), and 34 healthy controls (N=26 pediatric
131 and N=8 adults recruited before the COVID-19 pandemic) (**Figure 1, Table S1**). Among the
132 13 children with acute respiratory infection (**Table S1, Figure S1**), 9 had a confirmed SARS-
133 CoV-2 infection (RT-PCR on nasopharyngeal aspiration or swab) (*Acute-inf (CoV2⁺)* group).
134 Six out of these 9 cases had pneumonia, and one had an uncomplicated febrile seizure. One
135 patient with a history of recent bone marrow transplantation for sickle cell disease, required
136 intensive care support. The 4 other patients (*Acute-inf (CoV2⁻)* group) had pneumonia

137 associated with a positive RT-PCR test for either *Mycoplasma pneumoniae* or
138 rhinovirus/enterovirus, and negative RT-PCR for SARS-CoV-2.

139 Forty-three children displayed features of postacute hyperinflammatory illness (**Figure S1**,
140 **Table S1**). SARS-CoV-2 infection status of all samples was confirmed by specific antibody
141 determination (both IgG and IgA) in the plasma, using ELISA and flow cytometry-based
142 technics as described in the methods (**Figure S2A**). Most (n=30) had a confirmed SARS-CoV-
143 2 infection (with 14 also positive for concomitant nasopharyngeal RT-PCR testing) and were
144 therefore considered as cases of MIS-C (*MIS-C (CoV2⁺)* group); all 30 cases of MIS-C
145 presented clinical features of KD, 14 of them fulfilled clinical criteria for a complete form of
146 KD according to the American Heart Association (McCrindle et al., 2017). Of note, 21/30 cases
147 had severe myocarditis (i.e. with elevated high-sensitivity cardiac troponin I and/or regional
148 wall motion abnormalities on echocardiography, and clinical signs of circulatory failure
149 requiring intensive care support; *MIS-C_MYO (CoV2⁺)*). Thirteen tested negative for SARS-
150 CoV-2 and fulfilled clinical criteria for complete (n=6) or incomplete (n=7) Kawasaki disease
151 (KD), and were therefore considered to have KD-like illness (*KD (CoV2⁻)* group) (**Figure S1**,
152 **Table S1**). Clinical and biological characteristics, at time of disease activity and before
153 treatment, or within 24 hours of treatment onset, are presented in **Table S1**. MIS-C cases had
154 low lymphocyte counts and those with severe myocarditis had in addition abnormally increased
155 neutrophil counts as compared to other groups, along with high levels of CRP, PCT, serum
156 alanine transaminases (ALT) and ferritin (**Table S1**). All cases responded favorably to
157 intravenous immunoglobulin injections (IVIG), some (N=12, Table S1) in combination with
158 glucocorticosteroids, received before sampling. Multi-parametric analyses were performed at
159 a median fever persistence of 9-10 days (**Figures 1A, B**).

160

161 ***Elevated inflammatory cytokine levels in pediatric acute infection and***
162 ***postacute hyperinflammatory conditions***

163 We investigated plasma cytokine and chemokine levels in all patients, by Luminex and Simoa
164 assays. Hierarchical clustering analysis and stratification by patient groups revealed overall
165 elevated levels of immune and inflammatory markers, with 40/46 measured proteins
166 significantly elevated ($q < 0.05$) as compared to healthy controls (**Figure 2A**; global heat map).
167 Twelve cytokines were found to be elevated in all groups of patients as compared to healthy
168 controls (**Figure S2B**). High IL-8 and CXCL1 (**Figure S2C**) were more specific to children
169 with acute infection. Cytokine levels did not significantly differ between children with acute
170 infection with or without evidence of SARS-CoV-2 infection (**Figures S2D-F**). IFN γ , IFN α 2,
171 IL-17A, TNF- α , IL-10, Granzyme B, were higher in children with postacute
172 hyperinflammation (*MIS-C (CoV2⁺)*, *MIS-C_MYO (CoV2⁺)*, and *KD (CoV2⁻)* groups), as
173 compared to pediatric healthy donors (CTL) and patients with acute infections (*Acute-inf*
174 (*CoV2⁺*) and *Acute-inf (CoV2⁻*) (**Figure 2A**, top cluster **and 2B**). A slightly higher expression
175 of IFN α 2 and IL-17A was found in MIS-C without myocarditis (*MIS-C (CoV-2⁺)* patients).
176 (**Figure 2C**). In contrast, 11 cytokines and chemokines (CSF2, CCL2, IL-6, CXCL10, FLT3L,
177 VEGF, TGF-A, IL-1RA, PD-L1, CX3CL1, TGF-B1) were higher in MIS-C with severe
178 myocarditis (*MIS-C_MYO (CoV-2⁺)*) (**Figures 2 C, D**). Of note, 8 of them are known to be
179 associated with TNF- α signaling. They are involved in propagation of inflammation (IL-6, IL-
180 15), angiogenesis and vascular homeostasis (VEGF and TGF cytokines) and activation and
181 chemotaxis of myeloid cells (CCL2, CX3CL1, CXCL10) (**Figure 2D**) (Holbrook et al., 2019;
182 Varfolomeev and Ashkenazi, 2004). An increased level of CCL19, CCL20, CCL3 (cell
183 migration and chemotaxis) and IL-1 agonist/antagonist (IL-1 β , IL-1RA) were also observed,
184 as well as increased soluble PD-L1 (**Figure S2G**). Another noticeably elevated cytokine was
185 CSF2, known to be involved in myeloid cell differentiation and migration (**Figure 2D**).

186 Altogether, high inflammatory cytokine levels were detected in both acute infection and
187 postacute inflammatory cases. The strongest inflammatory profile was observed in MIS-C with
188 severe myocarditis (*MIS-C_MYO (CoV2⁺)*) and remarkably, a similar profile was observed
189 when comparing MIS-C without myocarditis (*MIS-C (CoV2⁺)*) and KD-like illness unrelated
190 to SARS-CoV-2 (*KD (CoV2⁻)*). Of note, the inflammatory profile was much reduced in
191 intensity in MIS-C cases with myocarditis under combined glucocorticosteroid and IVIG
192 treatment, as compared to IVIG alone (**Figure S2H**).

193

194 *Low monocyte and dendritic cell frequencies in patients with postacute* 195 *hyperinflammatory illness*

196 To better decipher the blood immune cell composition of each group, PBMCs were analyzed
197 by CyTOF mass spectrometry and by single-cell analyses at the transcriptomic level (SC-RNA-
198 SEQ) (**Figure 1**). Clustering analyses of the data obtained from CyTOF and SC-RNA-SEQ
199 revealed consistent results, with most of the alterations observed in clusters composed of
200 monocytes or dendritic cells (**Figures 3A, B; S3 A-D**). The most drastic changes were a
201 decrease in conventional Dendritic Cells (cDCs) and plasmacytoid Dendritic Cells (pDCs) in
202 patients with a postacute hyperinflammatory illness (*MIS-C_MYO (CoV2⁺)*, *MIS-C (CoV2⁺)*
203 and *KD (CoV2⁻)*). As previously reported (Gruber et al., 2020), we also observed a trend
204 towards a decrease in monocyte clusters in children with postacute hyperinflammatory illness,
205 that was found independent of SARS-CoV-2 status (**Figures 3A, B; S3 A-D**). Of note, some
206 heterogeneity was observed in the proportions of non-classical monocytes in *Acute-Inf (CoV2⁺)*
207 cases. We observed additional heterogeneity in the proportions of classical and intermediate
208 monocytes in patients with severe myocarditis (*MIS-C_MYO (CoV2⁺)*) (**Figures 3A, B**), but
209 there was no correlation with clinical data (**Table S1**), nor cytokine/chemokine measurements
210 (**Figures 2 and S2**). Additional modifications were detected in patients with acute SARS-CoV-

211 2 infection (*Acute-inf (CoV2⁺)* cases), consisting of a decrease of MAIT cells and an excess of
212 naïve and central memory CD4⁺ T cells (**Figures 3 A, B, S3 C, D**).

213

214 ***Overexpression of inflammatory pathways, NF- κ B signaling, and metabolic***
215 ***changes related to hypoxia in acute infection and postacute***
216 ***hyperinflammatory conditions***

217 To gain further insight into the mechanisms behind the different forms of SARS-CoV-2
218 infection in children, we assessed pathways modulated in each group by looking at the
219 differentially expressed genes, obtained from the SC-RNA-SEQ dataset. In patients with acute
220 infection (*Acute-inf (CoV2⁺)* and *Acute-inf (CoV2⁻)*), the numbers of differentially expressed
221 genes were homogeneously distributed among monocytes/DCs, T and B cells (**Figures 4A and**
222 **S4A**). Pathway enrichment analyses (Ingenuity Pathway Analyses; IPA, QIAGEN Inc.)
223 revealed a decrease in oxidative phosphorylation, coupled with an increase of HMGB1
224 signaling, HIF-1 α signaling, and hypoxia signaling. Production of nitric oxide was observed
225 in both groups of acute infections, independently of SARS-CoV-2 infection, as compared to
226 healthy controls (**Figure S4B**). These observations suggest a metabolic switch potentially
227 driven by hypoxic conditions. NF- κ B signaling, VEGF signaling and inflammatory pathways
228 (type-I and type-II IFNs, IL-1, IL-6, and IL-17 signaling pathways) were also found to be
229 overrepresented in both groups of patients (**Figure S4B**).

230 Interestingly, alterations in the same pathways were also identified in all cases of children with
231 SARS-CoV-2-related postacute illnesses (*All MIS-C (CoV2⁺)*: *MIS-C_MYO (CoV2⁺)* and *MIS-*
232 *C (CoV2⁺)*). However, in these cases, alterations were mostly restricted to monocytes and
233 dendritic cells (**Figures 4 A, B**). Comparisons of genes differentially expressed between
234 children with postacute hyperinflammatory illness with or without evidence of SARS-CoV-2
235 infection (*All MIS-C (CoV2⁺)* versus *KD (CoV2⁻)*), did not reveal significant differences with
236 the exception of type-I and type-II interferon signaling (**Figures S4 C, D**).

237 The NF- κ B signaling pathway was identified to be activated in monocytes and DCs of all
238 patients with acute infection and postacute hyperinflammatory illness, independently of SARS-
239 CoV-2 infection (**Figure 5A**). While monocytes and DCs of patients with acute infection
240 (*Acute-inf (CoV2⁺)* and *Acute-inf (CoV2⁻)*) highly expressed genes of the NF- κ B complex
241 (*REL*, *RELA*, *RELB*, *NFKB1*, *NFKB2*; **Figures 5B, D**), monocytes and DCs from all MIS-C
242 patients (*MIS-C_MYO (CoV2⁺)* and *MIS-C (CoV2⁺)*) exhibited a strong decrease in the
243 expression of NF- κ B inhibitors, such as *TNFAIP3* (A20), *TNFAIP2*, *NFKBIA*, *NFKBID*,
244 *NFKBIE* and *NFKBIZ* (**Figures 5C, D**). Strikingly, this decrease in the expression of NF- κ B
245 inhibitors appeared to be specific to the monocytes and DCs of MIS-C patients with severe
246 myocarditis (*MIS-C_MYO (CoV2⁺)*) (**Figures 5 E, S5 A-D**).

247 In conclusion, pathways dysregulated in acute infection or postacute hyperinflammatory
248 illness, reflected an inflammatory status based on NF- κ B signaling combined with changes in
249 metabolism driven by a hypoxic environment. In acute respiratory disease, changes in gene
250 expression reflected involvement of all PBMCs, whereas in postacute hyperinflammatory
251 illnesses, monocytes/DCs were the most affected populations. These results further emphasize
252 the role of monocytes/DCs populations in MIS-C.

253

254 ***Molecular pathways specific to MIS-C with severe myocarditis***

255 To gain further insight into the inflammatory phenotype of monocytes and DCs from MIS-C
256 patients, we compared gene expression between MIS-C patients with or without severe
257 myocarditis and healthy controls. Most gene alterations happened in monocytes/DCs (**Figure**
258 **S6A and S6C**), which lead us to focus on these populations for the following analyses.

259 Pathway enrichment performed both on IPA and EnrichR (Chen et al., 2013; Kuleshov et al.,
260 2016) highlighted the modulation of type-I and type-II interferon signaling pathways, with the
261 upregulation of several interferon stimulated genes (ISGs) (*JAK2*, *STAT1*, *STAT2*, *IFITM1*,

262 *IFITM2, IFI35, IFIT1, IFIT3, MX1, IRF1*) only in the monocytes and DCs of MIS-C patients
263 without myocarditis (**Figures 6 A, B, S6 B, D**). However, both groups of MIS-C patients
264 showed elevated plasma IFN- α 2 and IFN γ proteins (**Figures 2A, B**). Gene expression
265 downregulation in monocytes and DCs of MIS-C patients with severe myocarditis, included
266 most of the MHC class II genes suggesting a decrease in antigen processing and presentation
267 pathways (**Figures 6 C and S6D**).

268 Transcription factor prediction in EnrichR revealed overexpression of targets of the NF- κ B
269 complex in MIS-C patients with severe myocarditis as compared to MIS-C without myocarditis
270 (**Figure S6E**). This activation of NF- κ B signaling in MIS-C patients with severe myocarditis
271 was found to be associated with the strong downregulation of NF- κ B inhibitors as shown in
272 **Figure 5E**.

273 A strong overexpression of genes belonging to TNF- α signaling, as well as inflammatory
274 responses, hypoxia and response to oxidative stress (*HIF1A, HMOX1, HMBG1, etc.*) was found
275 in cases with severe myocarditis (**Figures 6D, E and S6E**). This was associated with a
276 downregulation of genes linked with oxidative phosphorylation, nitric oxide production and
277 iNOS signaling (**Figure S6B**). TGF- β signaling and VEGF signaling were also found enriched
278 in monocytes and DCs of patients with myocarditis and to a lesser magnitude in B cells
279 (**Figures S6 B, E**). Interestingly, an increased expression of several genes encoding S100
280 proteins and calcium-binding cytosolic proteins, all known to serve as danger signals to
281 regulate cell migration, homeostasis and inflammation, were noticed in the cases of severe
282 myocarditis (**Figure S6F**) (Xia et al., 2018).

283 To summarize, NF- κ B activation, a decreased expression of NF- κ B inhibitors, TNF- α
284 signaling, together with a hypoxic response to oxidative stress, VEGF signaling,
285 downregulation of MHC-II genes and low type-I and type-II IFN responses characterize the
286 monocytes and DCs of children with MIS-C and severe myocarditis.

287 ***Identification of a molecular signature specific to MIS-C with severe***
288 ***myocarditis***

289 To identify a potential clinical relevance of our study, we searched for a molecular signature
290 that correlated with the appearance of severe myocarditis among the monocytes/DCs of
291 children with SARS-CoV-2-related MIS-C. By using several SC-RNA-SEQ comparison
292 strategies (**Figure 7A**), we identified 329 genes upregulated in monocytes and DCs of the MIS-
293 C group with myocarditis (N=6) as compared to all other groups (**Figure 7A**). To validate this
294 molecular signature, RNA from PBMCs were sequenced from an independent group of
295 patients. A scoring system was generated, based on normalized expression represented by a Z-
296 score, coupled with hierarchical clustering, in order to identify genes that were overexpressed
297 in children with myocarditis (*MIS-C_MYO (CoV2⁺)* group) as compared to the other groups
298 (**Figure S7A**). Within the 329 genes identified by SC-RNA-SEQ in monocytes and DCs of
299 patients with severe myocarditis, expression of 116 genes were found upregulated in PBMCs
300 from an independent group of 9 patients belonging to the *MIS-C_MYO (CoV2⁺)* group with
301 myocarditis (**Figures 7B**). From these genes, a signature score (SignatureSCORE) was
302 determined for each sample processed by Bulk-RNA-SEQ (**Figure 7C**). We then further
303 developed a RankingSCORE (**Figures S7 A, B**) to identify the top genes that contributed the
304 most to the monocytes and DCs myocarditis signature. This led to the identification of a set of
305 25 genes that clearly segregate patients with severe myocarditis from other MIS-C and *KD*
306 (*CoV2⁻*) (**Figure 7D**). Consistently, most of these 25 genes belong to functional pathways that
307 were previously identified (**Figures 6 and S6**), such as inflammation, oxidative stress, TNF- α
308 and/or NF- κ B signaling, and in some cases already known markers of myocarditis or MIS-C
309 and/or COVID-19, such as genes coding for S100 proteins (**Figures S7 C, D**).

310 **Discussion**

311 Multi-parametric analysis of peripheral blood mononuclear cells from children with acute
312 respiratory infection and postacute hyperinflammation, collected during the COVID-19
313 pandemic, allowed to detect an inflammatory profile associated with a loss of circulating
314 monocytes and dendritic cells (DCs), as well as an upregulation of genes and pathways
315 involving NF- κ B signaling, oxidative stress with establishment of hypoxic conditions and
316 VEGF signaling. These pathways were upregulated in both acute and postacute groups of
317 patients, independently of SARS-CoV-2 infection. However, significant features of MIS-C
318 with severe myocarditis were detected specifically in monocytes and DCs including low type-
319 I and type-II IFN responses, decreased expression of NF- κ B inhibitors, increased TNF- α
320 signaling and overexpression of HIF-1 α .

321 Acute cases were characterized by the detection of inflammatory markers in the plasma with a
322 particularly strong elevation of IL-8 and CXCL1, two chemokines known to mediate neutrophil
323 migration to the lung (Kunkel et al., 1991; Pease and Sabroe, 2002; Sawant et al., 2015) and a
324 modest elevation of IFN α 2 levels. These findings suggest that in some children, a suboptimal
325 anti-viral type-I interferon response, alongside a hyperinflammatory response (IL-6 levels and
326 exacerbation of the NF- κ B pathway), could account for SARS-CoV-2 disease with pneumonia,
327 as compared to the very usual benign or even asymptomatic clinical course of SARS-CoV-2
328 infection in children. This has been previously observed in severe Respiratory Syncytial Virus
329 (RSV) infections (Hijano et al., 2019).

330 In the postacute patients, elevated levels of plasma IFN- γ , IFN α 2, IL-10, IL-15, and, to a lesser
331 extent, TNF- α , were found, as previously described in other cohorts (Brodsky et al., 2020;
332 Carter et al., 2020; Consiglio et al., 2020; Esteve-Sole et al., 2021; Gruber et al., 2020). These
333 findings are typical of an ongoing anti-viral immune response, not directly related to SARS-
334 CoV-2 infection. In addition, elevated chemokines such as CCL2, CCL3 and CCL4 may recruit

335 monocytes and DCs to tissues, possibly accounting for their reduced numbers observed in the
336 blood of those patients. Additional mechanisms such as apoptosis or other cell death pathways
337 may also be involved.

338 Cellular phenotypes that distinguish MIS-C from classical KD have been previously reported
339 (Brodsky et al., 2020; Consiglio et al., 2020; Esteve-Sole et al., 2021). Brodin and colleagues
340 described several key differences such as elevated IL-17, IL-6 and CXCL10 that were only
341 observed in KD, associated with decreased naïve CD4⁺ T cells and increased central memory
342 and effector memory CD4⁺ T cells in MIS-C. In the present study, high levels of IL-17, IL-6
343 and CXCL10, were both found in MIS-C and KD (CoV2⁻) groups. In addition, no major
344 differences in CD4⁺ T cell compartments were detected. Accordingly, only a few differentially
345 expressed genes were found between the MIS-C and KD (CoV2⁻) groups. These data support
346 the hypothesis that MIS-C patients with KD features exhibit a molecular phenotype close to
347 the one seen in KD patients, suggesting overlapping pathogenesis mechanisms (Gruber et al.,
348 2020). Differences observed with previous reports by Brodin and colleagues, may be due to
349 inclusion of only patients with criteria for complete or incomplete KD among the MIS-C cases,
350 or technical differences in the respective studies, such as time of blood sampling relative to
351 admission to hospital and medical treatments.

352 However, we did find noticeable differences in MIS-C cases with severe myocarditis with
353 circulatory failure that required intensive care. The expression of a number of cytokines was
354 further increased in these cases, most of them related to the NF- κ B-TNF- α signaling axis.
355 Elevated VEGF and TGF- α and TGF- β are potential drivers of angiogenesis and vascular
356 homeostasis, whereas elevated chemokines (CCL2, CCL3, CCL20, CX3CL1, CXCL10) could
357 mediate increased cell migration towards inflamed tissues. Molecular analysis confirmed an
358 upregulation of genes belonging to the TNF- α and NF- κ B signaling pathways that were
359 specifically found in monocytes and DCs of MIS-C patients with severe myocarditis. A lower

360 expression of NF- κ B complex inhibitors, including *TNFAIP3* (A20), *TNFAIP2*, *NFKBIA*,
361 *NFKBIZ*, was detected, suggesting a possible mechanism for NF- κ B sustained activation which
362 could then potentially lead to exacerbated TNF- α signaling. Overall, these results point to a
363 potential role of monocytes and DCs in the pathogenesis of MIS-C with severe myocarditis,
364 which might not be directly driven by SARS-CoV-2 infection, but rather due to a tolerance
365 defect in limiting a pathological immune response, as already observed for other pathogens
366 (Goodnow, 2021). It would be interesting to investigate the presence of genetic variants among
367 MIS-C with severe myocarditis, in genes such as *TNFAIP3*, as already discussed (Goodnow,
368 2021). The apparent hypoxic conditions detected in children with myocarditis, could also
369 account for the exacerbation of NF- κ B signaling. HIF-1 α , a sensor of oxidative stress, is well-
370 known for being able to induce a switch from oxidative phosphorylation to glycolysis to limit
371 generation of reactive oxygen species (ROS). It can also activate NF- κ B signaling (D'Ignazio
372 and Rocha, 2016; D'Ignazio et al., 2016). Additional environmental factors and/or genetic
373 predispositions could also be involved. Another striking feature was the low expression of
374 genes involved in type-I and type-II interferon responses, specifically in monocytes and DCs
375 of children with myocarditis, although IFN- γ and IFN α 2 proteins were elevated in the plasma
376 of all MIS-C patients. This reduced response to type-I IFN in the most severe forms of MIS-C
377 (with myocarditis and circulatory failure) is in part reminiscent of the impaired type-I IFN
378 activity observed in the most severe forms of COVID-19 in adults (Bastard et al., 2020; Hadjadj
379 et al., 2020; Zhang et al., 2020). The search for auto-antibodies against IFN α 2 were negative
380 (data not shown) but presence of autoantibodies to ISGs (interferon stimulated genes) cannot
381 be excluded (Combes et al., 2021).

382 Overall, our findings depict a model, supported by previous publications (Amoah et al., 2015;
383 Calabrese et al., 2004; Mann Douglas L., 2001), in which myocarditis is associated with an
384 attenuated negative feedback loop of TNF- α -driven NF- κ B activation, together with an excess

385 of proangiogenic cytokines and chemokines that could attract activated myeloid and T cells to
386 the myocardium tissue (**Figure S8**). Locally, it could lead to the production of inflammatory
387 cytokines known to promote differentiation of cardiac fibroblasts into cardiac myofibroblasts
388 (TNF- α , TGF- β , IL1- β , IL-13, IL-4, VEGF). Cardiac myofibroblasts, as previously reported,
389 may secrete chemokines leading to further activation and recruitment of myeloid cells, creating
390 a feed-forward loop of locally sustained inflammation and myocarditis (**Figure S8**) (Amoah et
391 al., 2015; Angelo and Kurzrock, 2007; Delprat et al., 2020; Hua Xiumeng et al., 2020; Maloney
392 and Gao, 2015).

393 Using SC-RNA-SEQ data, we defined a gene signature specific of SARS-CoV-2-related
394 postacute hyperinflammatory illness with severe myocarditis that was further validated by a
395 global transcriptomic analysis on PBMCs from an independent patient group. The genes
396 defining this signature were consistently enriched in genes associated with inflammation, TNF-
397 α and NF- κ B signaling, oxidative stress and myocarditis (**Figure S7C**). Interestingly, among
398 these genes, the S100 proteins and the calprotectin complex (S100A8/S100A9) in particular,
399 were previously reported and proposed as biomarkers for the most severe adult form of
400 COVID-19 with acute respiratory syndrome (**Figure S7D**) (Silvin et al., 2020).

401 Our study has several limitations, including the relatively low number of cases in each group,
402 the lack of a comparison with asymptomatic or mildly symptomatic non-hospitalized children
403 positive for SARS-CoV-2 and a longitudinal study of children with “classic” KD enrolled
404 before the COVID-19 pandemic. All our cellular data were generated from frozen peripheral
405 mononuclear cells, which does not allow a direct assessment of neutrophils. A parallel analysis
406 of PMN (polymorphonuclear leukocytes) will be required. Endothelial and myocardial cells
407 are at least targets of the disease but may also contribute to the pathophysiology as described
408 above. Also, additional data supporting gene expression findings will be necessary in future
409 studies. Nevertheless, our study provides further in-depth molecular analysis of MIS-C with

410 severe myocarditis. These severe forms were found to be associated with an excessive
411 activation of the TNF- α , NF- κ B signaling axis and poor response to type-I and type-II
412 interferons in monocytes and DCs, secretion of cytokines promoting angiogenesis, chemotaxis
413 and potential migration of activated myeloid cells and neutrophils in the myocardiac tissue.
414 This may help to identify potential new clinical biomarkers and open new therapeutic
415 strategies, including drugs targeting TNF- α or NF- κ B pathways.

416 **Resource Availability**

417

418 **Lead Contact**

419 Further information and requests for resources and reagents should be directed to and will be
420 fulfilled by the Lead Contacts, Mickaël Ménager (mickael.menager@institutimagine.org) and
421 Frédéric Rieux-Laucat (frederic.rieux-laucat@inserm.fr)

422 **Material availability**

423 This study did not generate new unique reagents.

424 **Data and Code availability**

425 The SC-RNA-SEQ data have been deposited in the Gene Expression Omnibus (GEO) database
426 under ID code GEO: GSE167029. The Bulk-RNA-SEQ data have been deposited under ID
427 code GEO: GSE167028. Both can be accessed from the series code GEO: GSE167030

428

429 **Experimental Model and Subject details**

430

431 **Patients and definitions**

432 This prospective multicenter cohort study included children (age \leq 18 years at the time of
433 admission) suspected of infection with SARS-CoV-2 between April 6, 2020 and May 30, 2020.
434 Clinical aspects of 22 of the included patients were previously reported (Toubiana et al., 2020,
435 2021). Children admitted with fever in general pediatric wards or pediatric intensive care units
436 of Tertiary French hospitals involved in the research program, suspected of SARS-CoV-2
437 related illness and who underwent routine nasopharyngeal swabs for SARS-CoV-2 RT-PCR
438 (R-GENE, Argene, Biomerieux, Marcy l'Etoile) or SARS-CoV-2 IgG serology testing
439 (Architect SARS-CoV-2 chemiluminescent microparticle immunoassay; Abbott Core
440 Laboratory, IL, USA), were eligible. The study was approved by the Ethics Committee (Comité
441 de Protection des Personnes Ouest IV, n° DC-2017-2987). All parents provided written
442 informed consent.

443 Case definition for pediatric COVID-19 acute infection was presence of fever, fatigue,
444 neurological abnormalities, gastro-intestinal or respiratory signs, associated with a concomitant
445 nasopharyngeal swab positive for SARS-CoV-2 RT-PCR, and absence of MIS-C criteria
446 (Zimmermann and Curtis, 2020). Case definition for postacute hyperinflammatory illness
447 (**Figure 1**) was presence of fever, laboratory evidence of inflammation and clinically severe
448 illness with multisystem involvement, during the SARS-CoV-2 epidemic period (Datta et al.,
449 2020). This may include children with features of KD; criteria of the American Heart
450 Association was used to define for complete (Fever > 4 days and \geq 4 principal criteria) or
451 incomplete KD (Fever > 4 days and 2 or 3 principal criteria, and without characteristics
452 suggestive of another diagnosis) (McCrindle et al., 2017). Among cases with postacute
453 hyperinflammatory illness, children with a positive SARS-CoV-2 testing (RT-PCR or
454 serology) were considered to have MIS-C according to CDC and WHO criteria to define MIS-
455 C (CDC, 2020). Patients with postacute hyperinflammatory illness, negative SARS-CoV-2
456 testing (RT-PCR or serology), and criteria for KD, were considered as patients with KD-like
457 illness. Patients with MIS-C with clinical signs of circulatory failure requiring intensive care,
458 with elevated high-sensitivity cardiac troponin I levels (>26 ng/mL) and /or decreased cardiac
459 function (diastolic or systolic ventricular dysfunction at echocardiography), were considered
460 to have MIS-C with severe myocarditis (Brissaud et al., 2016; Canter Charles E. and Simpson
461 Kathleen E., 2014).

462 For each included patient, we collected demographic data, symptoms, results of SARS-CoV-2
463 testing and other laboratory tests, echocardiograms, and treatments. All patients with negative
464 initial serology testing were retested after an interval of at least 3 weeks (Architect SARS-CoV-
465 2 chemiluminescent microparticle immunoassay; Abbott Core Laboratory).

466 Healthy controls were recruited before the COVID-19 pandemic (before November 2019).

467

468 **Samples**

469 For each patient and healthy donor, peripheral blood samples were collected on EDTA and
470 lithium heparin tubes. After a centrifugation of the EDTA tube at 2300rpm for 10 minutes,
471 plasma was taken and stored at -80°C before cytokine quantification. PBMCs were isolated
472 from the lithium heparin samples, frozen as described below and stored at -80°C and were used
473 for both bulk and single-cell RNAseq, as well as cell phenotyping by CyTOF. The workflow
474 is summarized in Figure 1B.

475

476 **Isolation of PBMCs**

477 Peripheral blood samples were collected on lithium heparin. PBMCs were isolated by density
478 gradient centrifugation (2200 rpm without break for 30 minutes) using Ficoll (Eurobio
479 Scientific, Les Ulis, France). After centrifugation, cells were washed with Phosphate-buffered
480 saline (PBS) (Thermo Fisher scientific, Illkirch, France). The pellet was resuspended in PBS
481 and cells were centrifuged at 1900 rpm for 5 minutes. Finally, the PBMCs pellet was frozen in
482 a medium containing 90% of Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher scientific,
483 Illkirch, France) and 10% of dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Quentin
484 Fallavier, France).

485

486 **Cytokine measurements**

487 Prior to protein analysis plasma samples were treated in a BSL3 laboratory for viral
488 decontamination using a protocol previously described for SARS-CoV (Darnell and Taylor,
489 2006), which we validated for SARS-CoV-2. Briefly, samples were treated with TRITON
490 X100 (TX100) 1% (v/v) for 2hrs at Room Temperature. IFN α 2, IFN γ , IL-17A, (triplex) and
491 IFN β (single plex) protein plasma concentrations were quantified by Simoa assays developed
492 with Quanterix Homebrew kits as previously described (Rodero et al., 2017). The limit of

493 detection of these assays were 0.6 pg/mL for IFN β , 2 fg/mL for IFN α 2, 0.05 pg/ml for IFN γ
494 and 3 pg/mL for IL17A including the dilution factor. IL-6, TNF α , and IL-10 were measured
495 with a commercial triplex assay (Quanterix). Additional plasma cytokines and chemokines (44
496 analytes) were measured with a commercial Luminex multi-analyte assay (Biotechne, R&D
497 systems).

498

499 **Serology assays**

500 SARS-CoV-2 specific antibodies were quantified using assays previously described (Grzelak
501 et al., 2020). Briefly, a standard ELISA assay using as target antigens the extracellular domain
502 of the S protein in the form of a trimer (ELISA tri-S) and the S-Flow assay, which is based on
503 the recognition of SARS-CoV-2 S protein expressed on the surface of 293T cells (293T-S),
504 were used to quantify SARS-CoV-2 specific IgG and IgA subtypes in plasma. Assay
505 characteristics including sensitivity and specificity were previously described (Grzelak et al.,
506 2020).

507

508 **Cell Phenotyping**

509 To perform high-dimensional immune profiling of PBMCs, we used the Maxpar[®] Direct[™]
510 Immune Profiling System (Fluidigm, Inc France) with a 30-marker antibody panel, for CyTOF
511 (Cytometry by Time Of Flight). Briefly, 3x10⁶ PBMCs resuspended in 300 μ l of MaxPar Cell
512 Staining Buffer were incubated for 20 minutes at room temperature after addition of 3 μ L of
513 10 KU/mL heparin solution and 5 μ l of Human TruStain FcX (Biolegend Europ, Netherland).
514 Then 270 μ L of the samples were directly added to the dry antibody cocktail for 30 minutes. 3
515 mL of MaxPar Water was added to each tube for an additional 10-min incubation. Three
516 washes were performed on all the samples using MaxPar Cell Staining Buffer and they were
517 fixed using 1.6% paraformaldehyde (Sigma-Aldrich, France). After one wash with MaxPar

518 Cell Staining Buffer, cells were incubated one hour in Fix and Perm Buffer with 1:1000 of
519 Iridium intercalator (pentamethylcyclopentadienyl-Ir (III)-dipyridophenazine, Fluidigm, Inc
520 France). Cells were washed and resuspended at a concentration of 1 million cells per mL in
521 Maxpar Cell Acquisition Solution, a high-ionic-strength solution, and mixed with 10% of EQ
522 Beads immediately before acquisition.

523 Acquisition of the events was made on the Helios mass cytometer and CyTOF software version
524 6.7.1014 (Fluidigm, Inc Canada) at the “Plateforme de Cytométrie de la Pitié-Salpetriere
525 (CyPS).” An average of 500,000 events were acquired per sample. Dual count calibration,
526 noise reduction, cell length threshold between 10 and 150 pushes, and a lower convolution
527 threshold equal to 10 were applied during acquisition. Mass cytometry standard files produced
528 by the HELIOS were normalized using the CyTOF Software v. 6.7.1014. For data cleaning, 4
529 parameters (centre, offset, residual and width) are used to resolve ion fusion events (doublets)
530 from single events from the Gaussian distribution generated by each event (Bagwell et al.,
531 2020). Subsequent to data cleaning, the program produces new FCS files consisting of only
532 intact live singlet cells. These data were analyzed in FlowJo v10.7.1 using 3 plugins
533 (DownSampleV3, UMAP and FlowSOM) with R v4.0.2. To increase efficiency of the analysis,
534 samples were downsampled to 50 000 cells, using the DownSample V3 plugin. All samples
535 were concatenated and analyzed in an unsupervised manner. Anti-CD127 antibody had to be
536 excluded due to poor staining. Clustering was performed using FlowSOM (Van Gassen et al.,
537 2015). The number of clusters was set to forty-five in order to overestimate the populations
538 and detect smaller subpopulations. Grid size of the self-organizing map was set to 20x20.
539 Resulting clusters were annotated as cell populations following the kit manufacturer’s
540 instruction. When several clusters were identified as the same cell types, they were
541 concatenated into a single cell population. For visualization purposes, UMAP was computed

542 with the UMAP pluggin (McInnes et al.) with the following parameters: metric (Euclidean),
543 nearest neighbors (15), minimum distance (0.5) and number of components (2).

544

545 **Single-cell transcriptomic (SC-RNA-SEQ)**

546 SC-RNA-SEQ analyses were performed on frozen PBMCs isolated from heparin blood
547 samples. PBMCs were thawed according to 10X Genomics protocol. The SC-RNA-SEQ
548 libraries were generated using Chromium Single Cell 3' Library & Gel Bead Kit v.3 (10x
549 Genomics) according to the manufacturer's protocol. Briefly, cells were counted, diluted at
550 1000 cells/ μ L in PBS+0,04% and 20,000 cells were loaded in the 10x Chromium Controller to
551 generate single-cell gel-beads in emulsion. After reverse transcription, gel-beads in emulsion
552 were disrupted. Barcoded complementary DNA was isolated and amplified by PCR. Following
553 fragmentation, end repair and A-tailing, sample indexes were added during index PCR. The
554 purified libraries were sequenced on a Novaseq 6000 (Illumina) with 28 cycles of read 1, 8
555 cycles of i7 index and 91 cycles of read 2.

556 Sequencing reads were demultiplexed and aligned to the human reference genome (GRCh38,
557 release 98, built from Ensembl sources), using the CellRanger Pipeline v3.1. Unfiltered RNA
558 UMI counts were loaded into Seurat v3.1 (Stuart et al., 2019) for quality control, data
559 integration and downstream analyses. Apoptotic cells and empty sequencing capsules were
560 excluded by filtering out cells with fewer than 500 features or a mitochondrial content higher
561 than 20%. Data from each sample were log-normalized and scaled, before batch correction
562 using Seurat's FindIntegratedAnchors. For computational efficiency, anchors for integration
563 were determined using all control samples as reference and patient samples were projected onto
564 the integrated controls space. On this integrated dataset, we computed the principal component
565 analysis on the 2000 most variable genes. UMAP was carried out using the 20 most significant
566 principal components (PCs), and community detection was performed using the graph-based

567 modularity-optimization Louvain algorithm from Seurat's FindClusters function with a 0.8
568 resolution. Cell types labels were assigned to resulting clusters based on a manually curated
569 list of marker genes as well as previously defined signatures of the well-known PBMCs
570 subtypes (Monaco et al., 2019). Despite filtering for high quality cells, five clusters out of the
571 twenty-six stood out as poor quality clusters and were removed from further analysis, namely:
572 one erythroid-cell contamination; one low UMI cluster from a single control; two clusters of
573 proliferating cells originating from a patient with EBV co-infection and one megakaryocytes
574 cluster. In total 152,201 cells were kept for further analysis.

575 After extraction and reclustering of high-quality cells, differential expression was performed
576 separately on all PBMCs, Monocytes/DCs, T cells or B cells. Differential expression testing
577 was conducted using the FindMarkers function of Seurat on the RNA assay with default
578 parameters. Genes with $\log(\text{FC}) > 0.25$ and adjusted p-values ≤ 0.05 were selected as
579 significant.

580

581 **Bulk RNA-sequencing (Bulk-RNA-SEQ)**

582 Bulk-RNA-SEQ analyses were performed on frozen PBMCs extracted from heparin samples.
583 RNA was extracted from PBMCs following the instructions of RNeasyR Mini kit (Qiagen,
584 Courtaboeuf, France). To note, the optional step with the DNase was performed. RNA integrity
585 and concentration were assessed by capillary electrophoresis using Fragment Analyzer
586 (Agilent Technologies). RNAseq libraries were prepared starting from 100 ng of total RNA
587 using the Universal Plus mRNA-Seq kit (Nugen) as recommended by the manufacturer. The
588 oriented cDNA produced from the poly-A+ fraction was sequenced on a NovaSeq6000 from
589 Illumina (Paired-End reads 100 bases + 100 bases). A total of ~50 millions of passing-filters
590 paired-end reads was produced per library.

591 Paired-end RNA-seq reads were aligned to the human Ensembl genome GRCh38.91 using
592 Hisat2 (v2.0.4)(Kim et al., 2019) and counted using featureCounts from the Subread R
593 package. The raw count matrix was analyzed using DESeq2 (version 1.28.1) (Love et al.,
594 2014). No pre-filtering was applied to the data. Differential expression analysis was performed
595 using the "DESeq" function with default parameters. For visualization and clustering, the data
596 was normalized using the `variant stabilizing transformation` method implemented in the "vst"
597 function. Plots were generated using ggplot2 (version 3.3.2), and pheatmap (version 1.0.12).
598 During exploratory analyses, it was noted that the clustering was mainly driven by the sex of
599 the patients. To remove this effect, it was included in the regression formula for DESeq (~sex
600 + groups), and then removed following vst transformation, using "removeBatchEffect" from
601 the "limma" package (version 3.44.3).

602

603 **Gene signature analysis**

604 To identify genes that could be used as markers of severe myocarditis in the SC-RNA-SEQ
605 dataset, three initial strategies were used, all based on differential expression and selection of
606 the upregulated genes. First, we performed the differential expression between *MIS-C_MYO*
607 (*CoV2*⁺) samples and all other samples. Second, differential analysis was computed between
608 *MIS-C_MYO* (*CoV2*⁺) and other samples with postacute hyperinflammatory illness. In the last
609 strategy, we selected genes that were upregulated between the *MIS-C_MYO* (*CoV2*⁺) and the
610 CTL, but not upregulated in any other group compared to the CTL (Figure 7A). These three
611 strategies allowed us to identify 329 unique genes.

612 To further explore whether these genes could be considered as markers of severe myocarditis,
613 we analyzed their expression profile in our bulk RNA-SEQ dataset. This dataset excluded
614 samples from patients of the *MIS-C_MYO* (*CoV2*⁺) that were included in the SC-RNA-SEQ
615 cohort. Vst-transformed counts were log₂-normalized and converted to z-score using the scale

616 function in R (v 4.0.2). A GeneSCORE was computed for each group as the mean z-score of
617 the samples of a group. Heatmaps representing this $\text{GeneSCORE}_{\text{group}}$ were performed using
618 pheatmap. Hierarchical clustering of the 329 previously identified genes was performed using
619 the complete method on the distance measured using Pearson's correlation, as implemented by
620 pheatmap. The hierarchical clustering was divided into 15 main clusters, 4 of which had the
621 expected pattern of expression: Clusters that had a higher expression in *MIS-C_MYO* (*CoV2+*)
622 than any other group were selected, resulting in 116 genes. A signature score for each sample
623 was performed on these genes, corresponding to the mean expression (z-score) of these N genes
624 in each sample (SignatureSCORE).

625 These genes were subsequently ranked based on the following equation:

$$\begin{aligned} 626 \quad & \textit{RankingSCORE} \\ 627 \quad & = \textit{GeneSCORE}_{\textit{MIS-C_MYO} (\textit{CoV2+})} - (\textit{GeneSCORE}_{\textit{MIS-C} (\textit{CoV2+})} \\ 628 \quad & + \textit{GeneSCORE}_{\textit{KD} (\textit{CoV2-})}) \end{aligned}$$

629 where the SCOREs represent the mean expression (z-score) in each disease groups, and the
630 SignatureScore was computed on the top 25 genes.

631

632 **Quantification and statistical analysis**

633 Cytokine heatmaps were made with Qlucore OMICS explore (version 3.5(26)) and dot plots
634 with GraphPad Prism (version 8). Differentially secreted cytokines were included in the heat
635 maps based on a 1.5 Fold Change (FC) comparison between groups as indicated. Dot plot
636 differences between each group were identified by Kruskal-Wallis tests followed by post-hoc
637 multiple comparison Dunn's test.

638 Statistical tests for cellular composition analysis in both the CyTOF and SC-RNA-SEQ
639 datasets were performed in R v3.6.1. Kruskal-Wallis test followed by post-hoc multiple

640 comparison Dunn's test was applied to assess differences in cell population proportions (*: p
641 ≤ 0.05 ; **: $p \leq 0.01$; ***: $p \leq 0.001$).

642 Differential expression testing in the SC-RNA-SEQ dataset was conducted using the
643 FindMarkers function in Seurat, with default Wilcoxon testing. P-values were controlled using
644 Bonferroni correction. Genes with an absolute $\log(\text{fold-change}) \geq 0.25$ and an adjusted p-value
645 ≤ 0.05 were selected as differentially expressed. Pathways analysis was performed using both
646 the Ingenuity pathway analysis v57662101 software (IPA (QIAGEN Inc.) and EnrichR (Chen
647 et al., 2013; Kuleshov et al., 2016). Heatmaps were extracted from the comparison module in
648 IPA. Pathways with an absolute z-score lower than 2 or a Bonferroni-Hochberg corrected p-
649 values higher than 0.05 were filtered out. Reactome 2016 and Molecular Signature DataBase
650 Hallmark 2020 (MSigDB Hallmark 2020) pathway enrichment analysis were performed using
651 EnrichR. The TRRUST transcription factors 2019 (Han et al., 2018) used for the transcription
652 factors enrichment analysis was performed using Enrich R.

653 **Author contributions:**

654 **CdC, ML, SM, AM, NS, FC, VGP, LB generated and analyzed data. MB, AB, BPP, GA,**
655 **TF analyzed data. MB, LG, PG, JDS, HM, OS, CB, PB, and JLC generated data. CdC,**
656 **ML, AF design figures and wrote manuscript. DD, FRL, JT and MMM conceived the**
657 **study, analyzed data, wrote the manuscript and supervised the study.**

658

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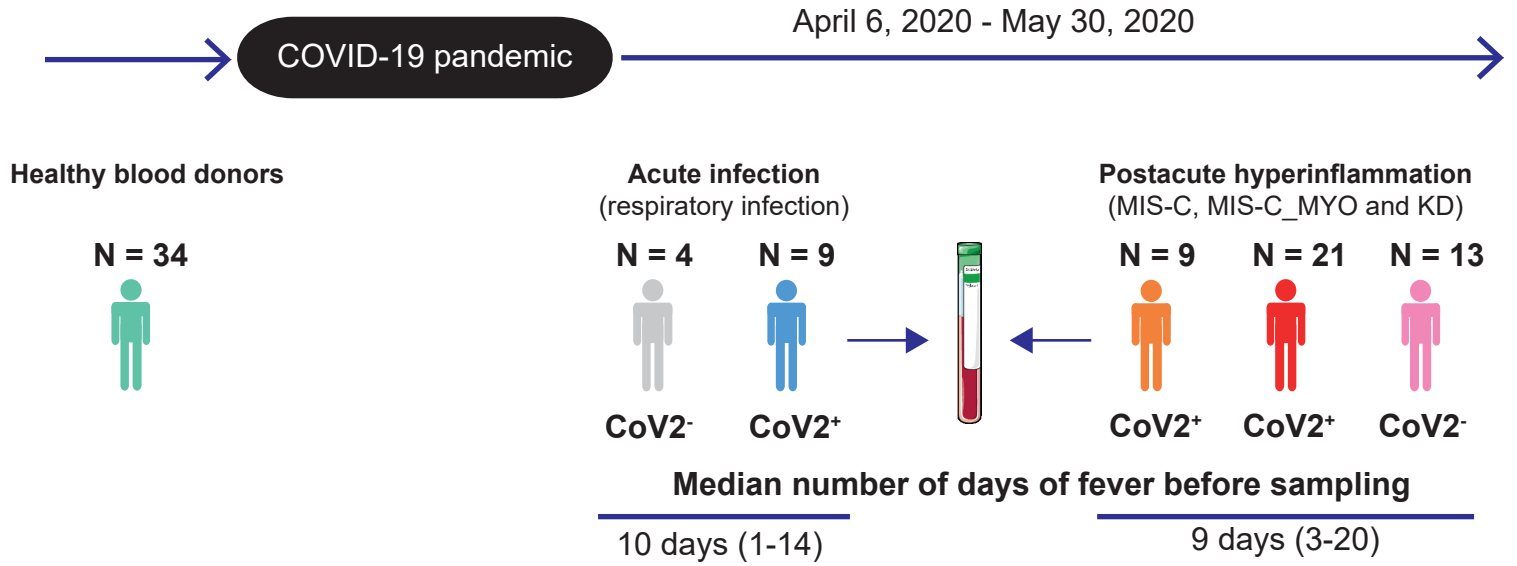
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A



B

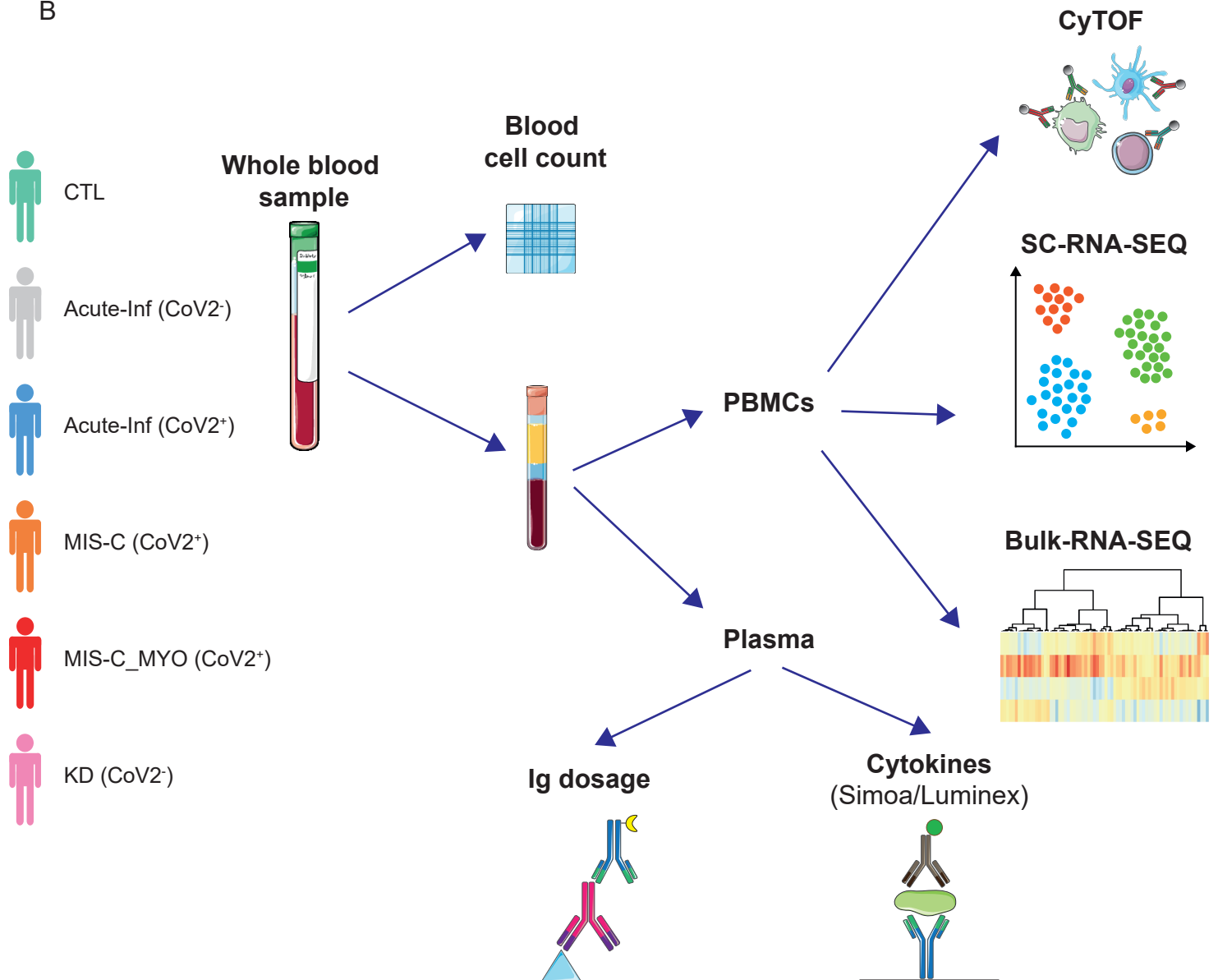


Figure 1

Figure 1: Timeline and experimental designs. **A.** Timeline depicting when the different groups of pediatric patients were enrolled. **B.** Description of the different types of analyses performed on whole blood samples, peripheral blood mononuclear cells (PBMCs) and plasma. CyTOF: Mass cytometry (Cytometry by Time Of Flight). SC-RNA-SEQ: single-cell transcriptomic followed by sequencing. Bulk-RNA-SEQ: transcriptomic bulk level sequencing. Simoa: Single molecule array, digital ELISA. Luminex: cytokine bead array assays. Ig dosage; quantification of SARS-CoV-2 specific immunoglobulins. CTL, healthy donors, in green; *Acute-inf (CoV2⁻)*, patients with acute respiratory infection but no evidence of SARS-CoV-2 infection, in gray; *Acute-inf (CoV2⁺)*, patients with acute respiratory infection and evidence of SARS-CoV-2 infection, in blue; *MIS-C (CoV2⁺)*, patients with postacute multi-inflammatory syndrome and evidence of SARS-CoV-2 infection, in orange; *MIS-C_MYO (CoV2⁺)*, patients with postacute hyperinflammatory syndrome, severe myocarditis and evidence of SARS-CoV-2 infection, in red; *KD (CoV2⁻)*, patients with postacute hyperinflammatory syndrome, no evidence of SARS-CoV-2 infection, but criteria for Kawasaki Disease (KD), in pink. Illustrations were obtained from Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Generic License. <http://smart.servier.com/>.

Diagnosis	Acute infection		Postacute hyperinflammatory illness		
Groups of patients ¹	Acute-Inf (CoV2) n=4	Acute-Inf (CoV2+) n= 9	MIS-C (CoV2+) n=9	MIS-C_MYO (CoV2+) n=21	KD (CoV2) n=13
<i>General</i>					
Male : Female	1:3	6:3	4:5	10:11	6:7
Age; median (range)	5.8 (0.8-16.1)	0.3 (0.05-12)	5.1 (0.5-15)	8.4 (1.7-16.8)	2.3 (0.7-7.2)
Comorbidities	None	1 (BMT)	None	None	None
<i>Clinical features</i>					
Fever	4 (100)	9 (100)	9 (100)	21 (100)	13 (100)
Respiratory manifestations	4 (100)	9 (100)	1 (11)	7 (33)	2 (15)
Pneumonia	4 (100)	6 (67)	1 (11)	7 (33)	2 (15)
Gastrointestinal symptoms	0	1 (11)	6 (67)	19 (95)	6 (46)
Neurological symptoms	1 (25)	6 (67)	1 (11)	6 (29)	0
KD criteria complete	N/A	N/A	4 (44)	10 (48)	6 (46)
Admission to the intensive care unit	0	1 (11)	2 (22)	21 (100)	1 (8)
Ventricular dysfunction ²	N/A	N/A	0	21 (100)	0
Coronary artery dilation or aneurysm ³	N/A	N/A	1 (11)	3 (14)	1 (8)
<i>Biological features ⁴</i>					
Positive NP SARS-CoV-2 RT-PCR	0	9 (100)	4 (44)	10 (48)	0
Positive serum SARS-CoV-2 IgG (Abbott)	0/1 (0)	N/D	9 (100)	21 (100)	0
Troponin I; Median (range) (ref <26 ng/L)	N/D	N/D	10 (10-122) ⁵	324 (30-6900)	51 (10-93) ⁵
Leukocytes x 10 ⁹ /L; Median (range) (ref 5.5-15.5)	N/D	N/D	12.3 (5.6-44)	18.9 (8-42.8)	13.7 (3.3-17.6)
Neutrophils x 10 ⁹ /L; Median (range) (ref 1.8-8.0)	1.6 (1.2-14.5)	1.5 (0.6-7.5)	7.7 (0.2-39)	14.9 (6.0-36.4)	6.5 (0.8-6.4)
Lymphocytes x 10 ⁹ /L; Median (range) (ref 1.5-6.5)	2.7 (2.1-3.4)	2.6 (0.3-7.6)	1.4 (0.6-5.6)	1.0 (0.4-3.7)	2.6 (0.8-6.4)
CRP, mg/L; Median (range) (ref <6.0)	8 (4-16)	7 (4-32)	190 (90-287)	295 (159-448)	161 (31-309)
PCT, ng/mL; Median (range) (ref <0.5)	0.2 (0.02-0.2)	0.16 (0.09-.22)	4.1 (0.2-152)	26.0 (1.7-299)	1.0 (0.1-5.5)
Sodium, mmol/L; Median (range) (ref 136-146)	N/D	131 (130-136)	132 (124-136)	130 (116-135)	135 (133-136)
Albumin, g/L; Median (range) (ref 35-50)	N/D	N/D	23 (15-41)	20 (16-26)	31 (25-40)
ALT, U/L; Median (range) (ref 7-40)	N/D	N/D	28 (6-146)	70 (12-257)	18 (11-277)
Ferritin µg/L; Median (range) (ref)	N/D	N/D	336 (118-1400) ⁵	1123 (172-4490) ⁵	183 (139-390) ⁵
<i>Treatments</i>					
Vasoactive or inotropic agents	0	0	0	17 (81)	0
Antibiotic therapy	3 (64)	3 (33)	5 (56)	21 (100)	4 (31)
IVIG /IVIG before sampling	0	0	8 (89) / 7 (78)	21 (100) / 20 (95)	12 (92) / 11 (85)
Corticosteroids / corticosteroids before sampling	0	0	2 (22) / 1 (11)	10 (48) / 7 (33)	4 (31) / 4 (31)
Other immunomodulatory agents	0	1 (tocilizumab)	0	0	0

¹ Values are numbers (percentages) unless stated otherwise; ² ventricular dysfunction observed at echocardiography; ³ Coronary artery abnormalities observed during hospitalization, Coronary artery dilation was defined as a coronary artery diameter z-score on echocardiography between 2.0 and 2.5, and aneurysm as a z-score ≥ 2.5 (McCindle et al., 2017); ⁴ the most abnormal values before treatment or within 24 hours of treatment onset; ⁵ missing values; BMT: bone marrow transplantation; ALT: alanine transferase; IVIG: intravenous immunoglobulins; N/A : non applicable; N/D: not determined; Ref: reference interval.

Table S1

Table S1. Clinical and biological features of pediatric patients enrolled. All children and adolescents included in the study were suspected of SARS-CoV-2 illness between April 6, 2020 and May 30, 2020 and displayed either an acute respiratory infection or a postacute inflammatory illness.

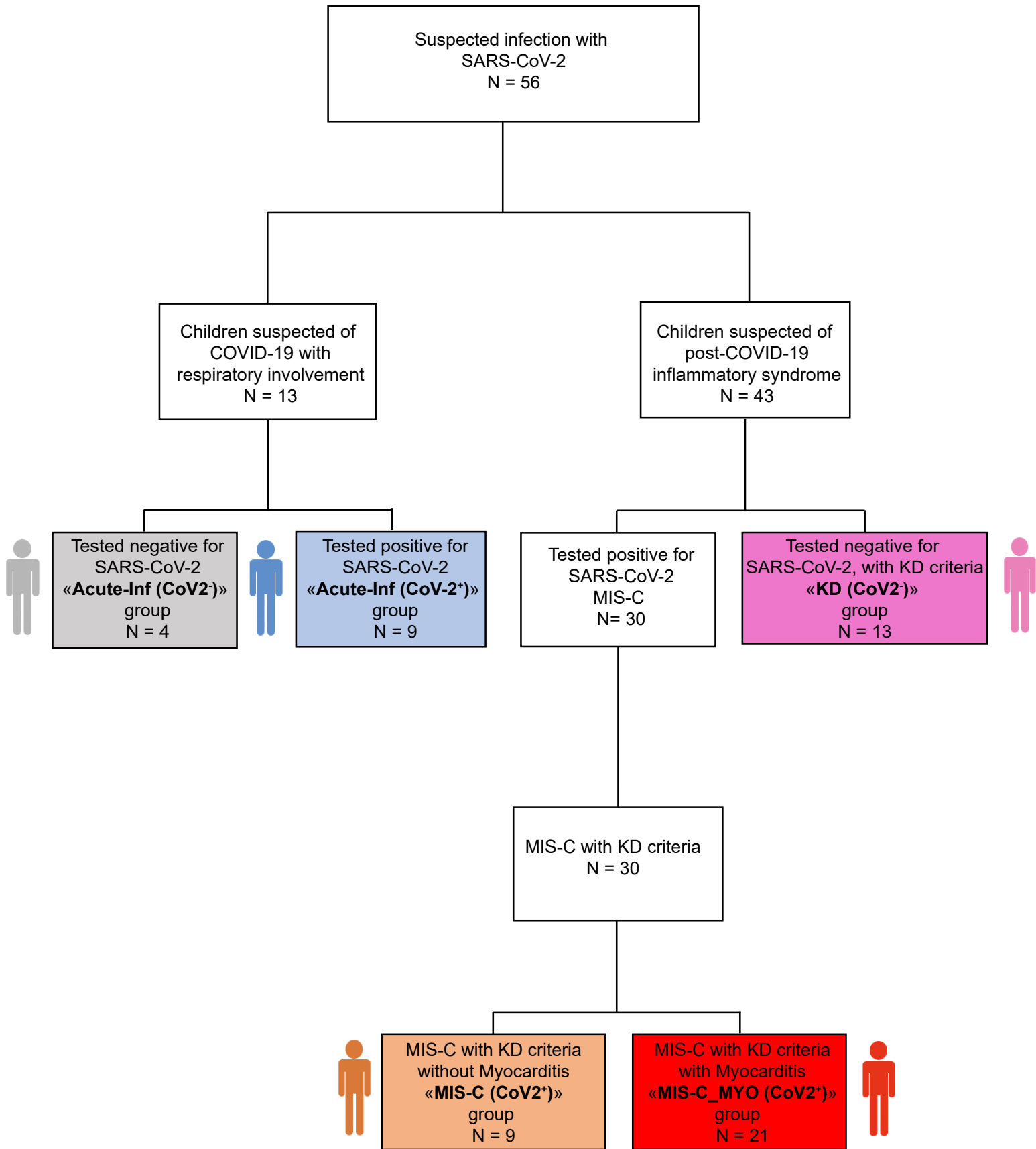


Figure S1

Figure S1: Flowchart describing the patients enrolled. Related to Figure 1. Each group with a name in bold and a color associated corresponds to a group of patients analyzed in this study. Names and colors are used throughout the manuscript. “N” represents the number of patients enrolled in each group.

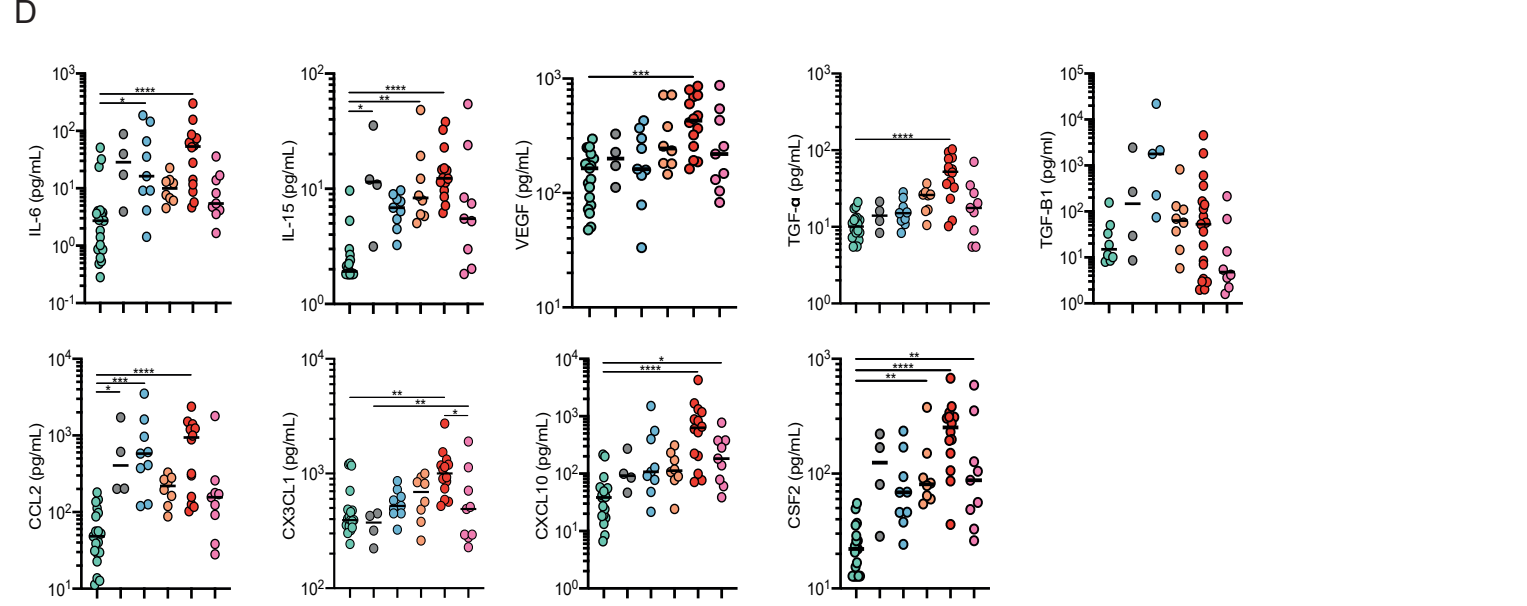
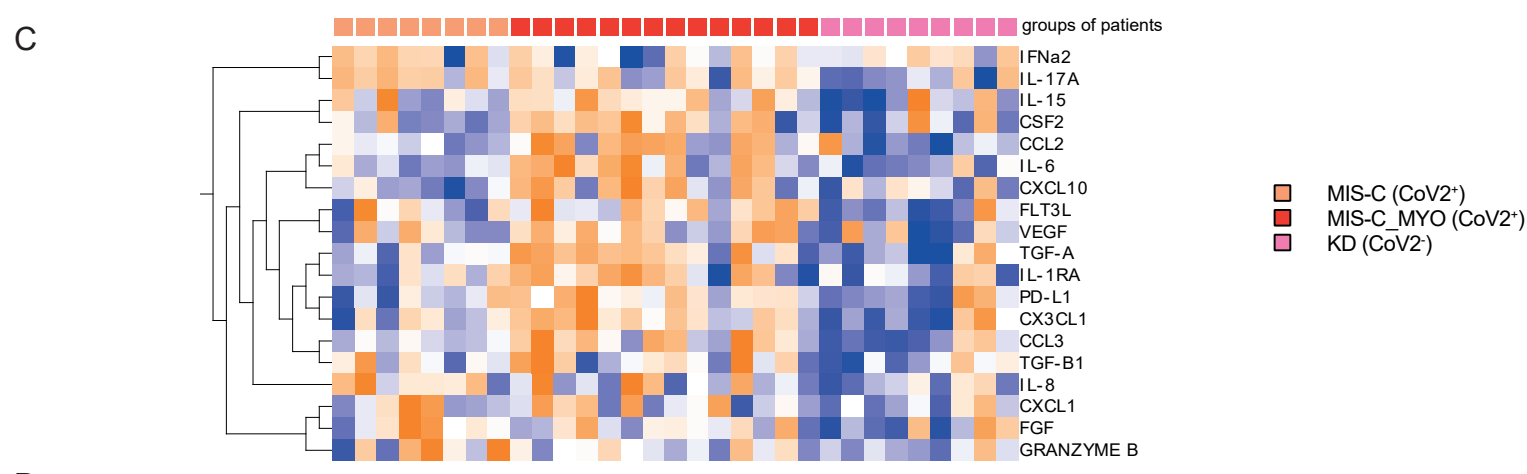
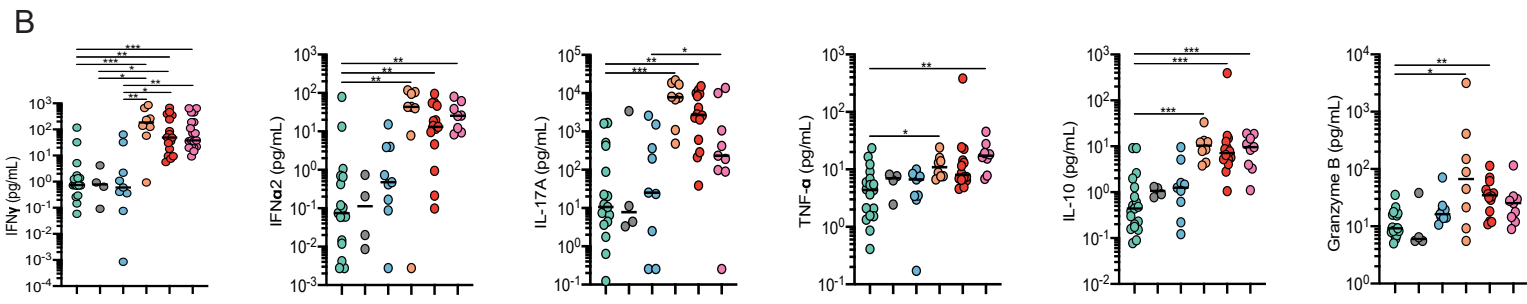
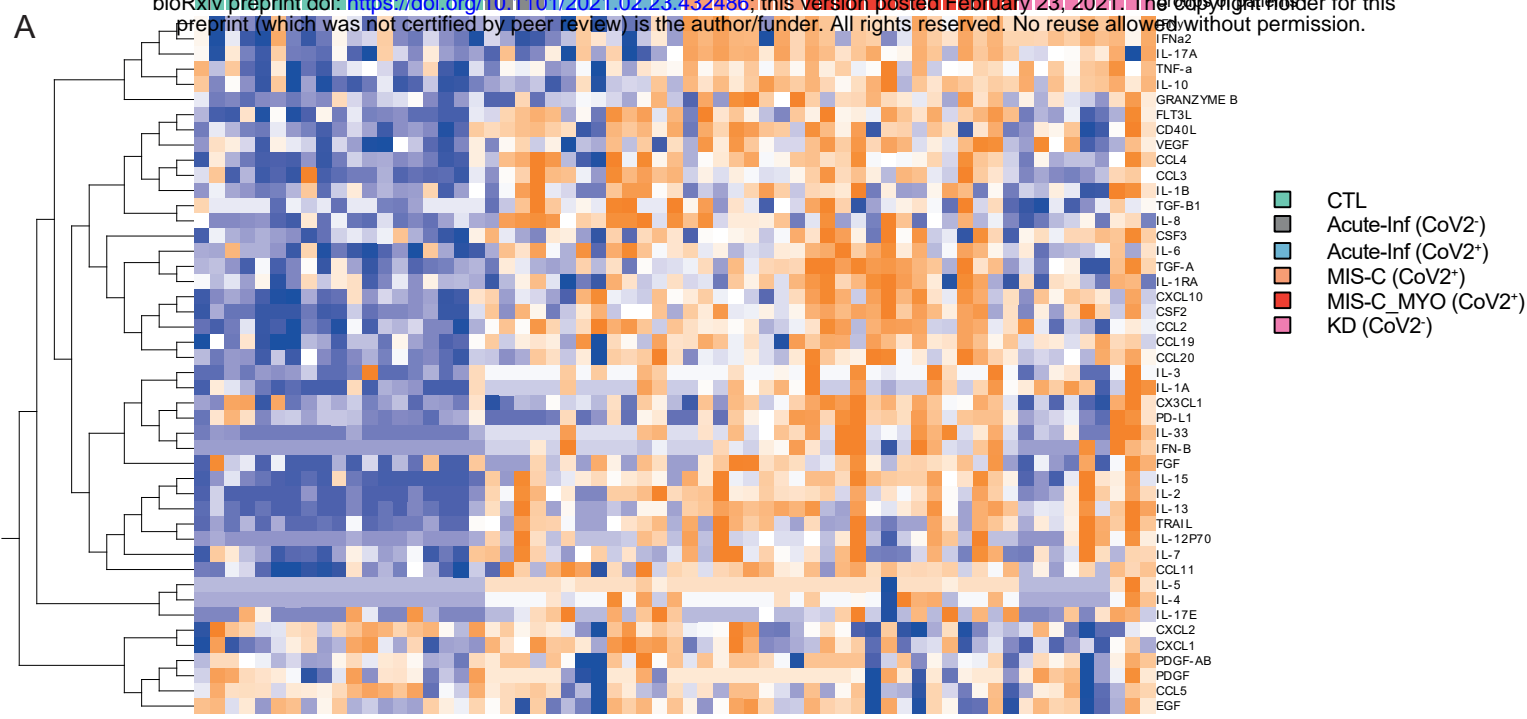


Figure 2

Figure 2: Analyses of cytokine plasma levels. **A.** Heatmap of the cytokines differentially secreted between the different clinical groups: CTL, green; *Acute-inf* (*CoV2*⁻), gray; *Acute-inf* (*CoV2*⁺), blue; *MIS-C* (*CoV2*⁺), orange; *MIS-C_MYO* (*CoV2*⁺), red; *KD* (*CoV2*⁻), pink). On the x axis, blood donors are organized by groups and on the y axis, cytokines are displayed following hierarchical clustering. Cytokines were expressed as pg/ml and log transformed with blue to orange colors representing lower to higher expression respectively. **B.** Dot plots of cytokines elevated in postacute hyper inflammatory groups (*MIS-C* (*CoV2*⁺), *MIS-C_MYO* (*CoV2*⁺) and *KD* (*CoV2*⁻)), as compared to early infection groups (*Acute-inf* (*CoV2*⁺), *Acute-inf* (*CoV2*⁻)) and healthy blood donors (CTL). **C.** Heatmap of the cytokines differentially expressed among the patients with postacute hyper inflammation: *MIS-C* (*CoV2*⁺), *MIS-C_MYO* (*CoV2*⁺) versus *KD* (*CoV2*⁻). **D.** Dot plots of cytokines with a higher expression in the *MIS-C_MYO* (*CoV2*⁺). **B & D.** ρ -values are calculated by Kruskal-Wallis test for multiple comparisons, followed by a post-hoc Dunn's test. * ($\rho < 0.05$), ** ($\rho < 0.01$), *** ($\rho < 0.001$)

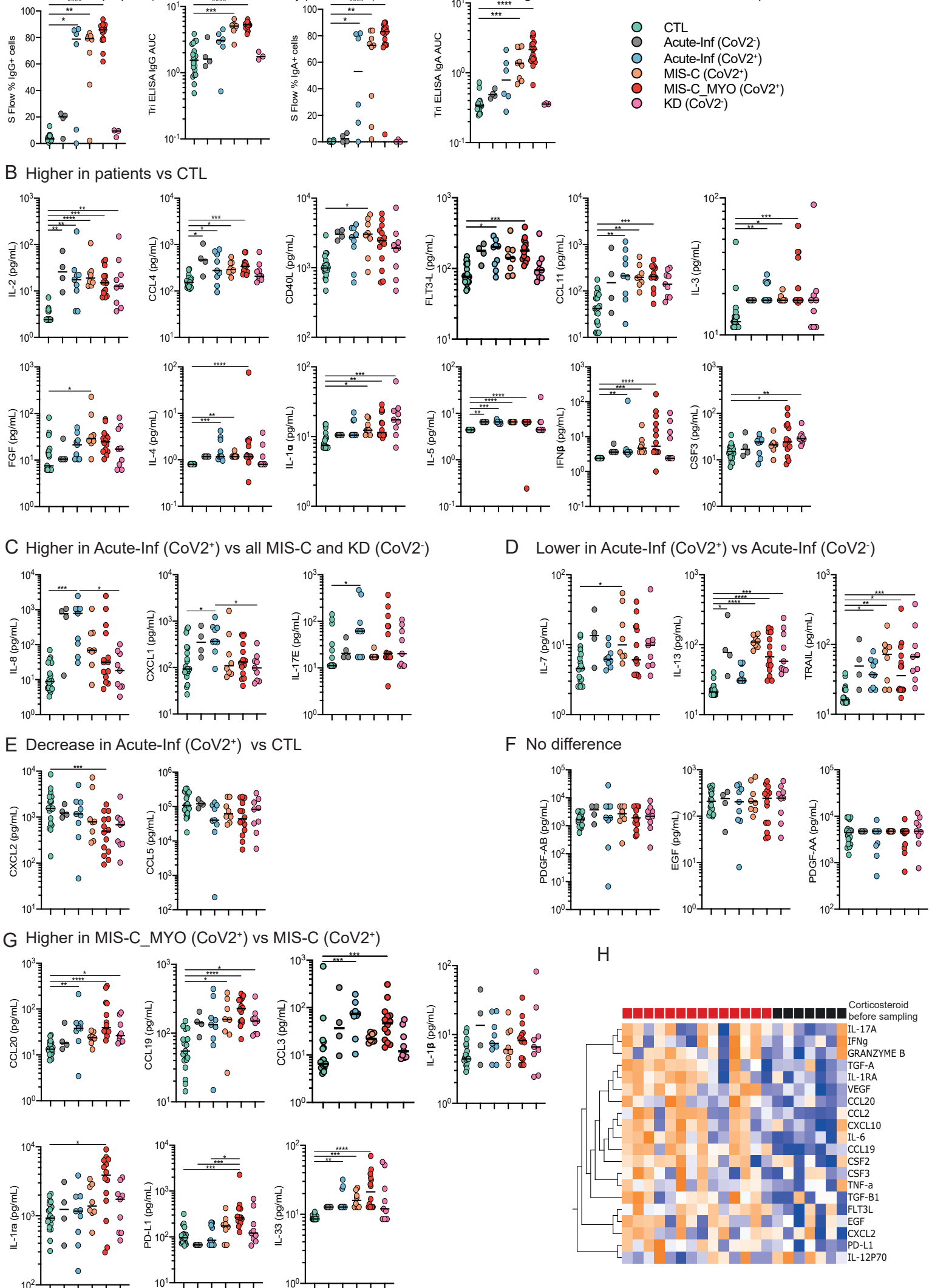


Figure S2

Figure S2: Immunoglobulin and cytokine analyses. Related to Figure 2. **A.** SARS-CoV-2 specific immunoglobulins (IgG, left panels; IgA, right panels) were dosed in the plasma of the different groups, by two methods: S Flow and Tri ELISA. **B - G.** Dot plots of cytokines differentially expressed and grouped based on the description of each sub panel. ρ values are calculated by Kruskal-Wallis test for multiple comparisons, followed by a post hoc Dunn's test. $*(\rho < 0.05)$, $**(\rho < 0.01)$, $***(\rho < 0.001)$. **H.** Hierarchical clustering of cytokines differentially expressed among the patients of the *MIS-C_MYO* (*CoV2*⁺) group without (red square) or with glucocorticosteroid treatment (black square) before blood sampling.

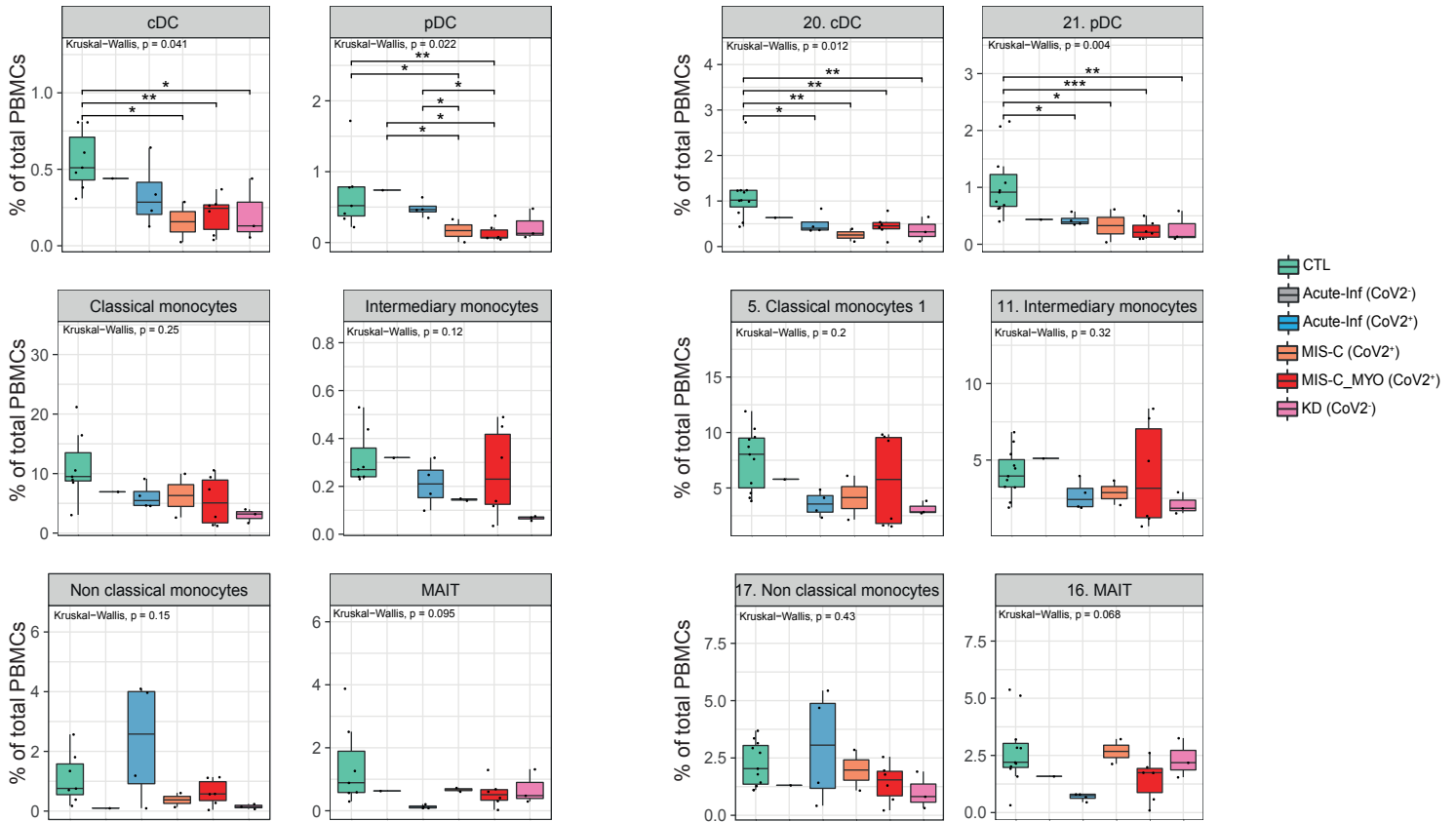
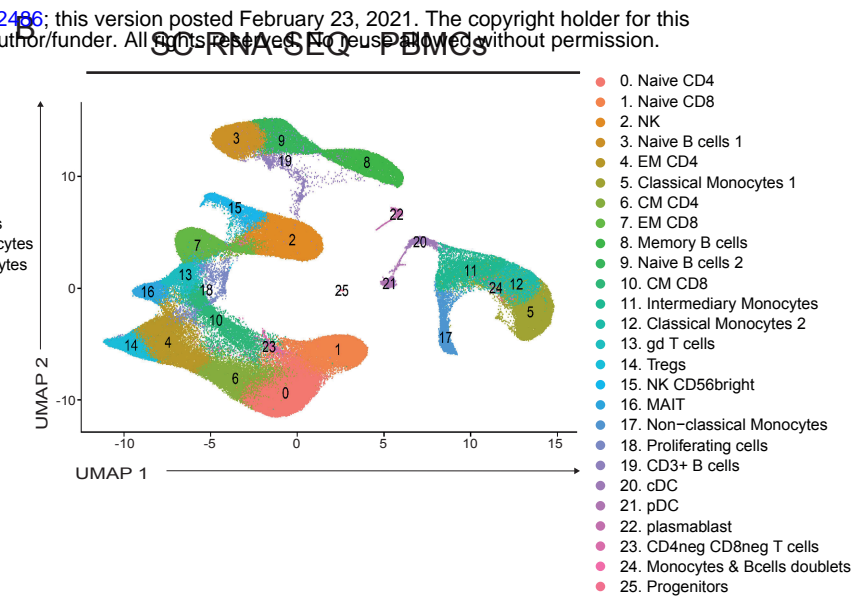
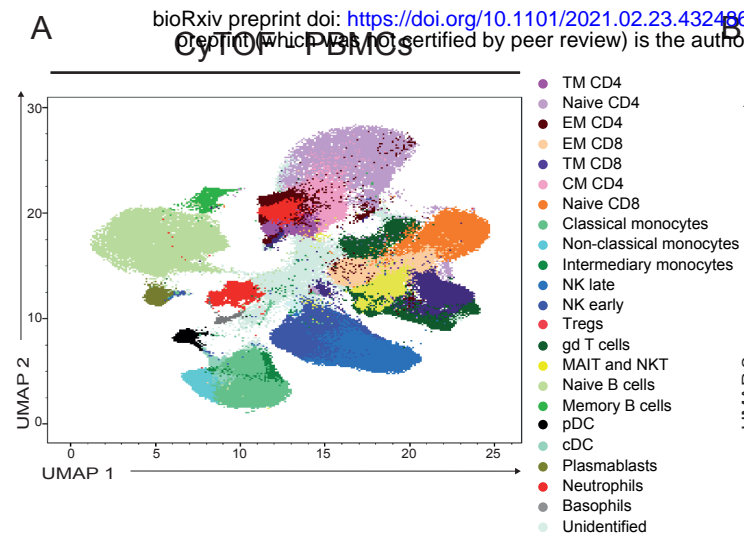
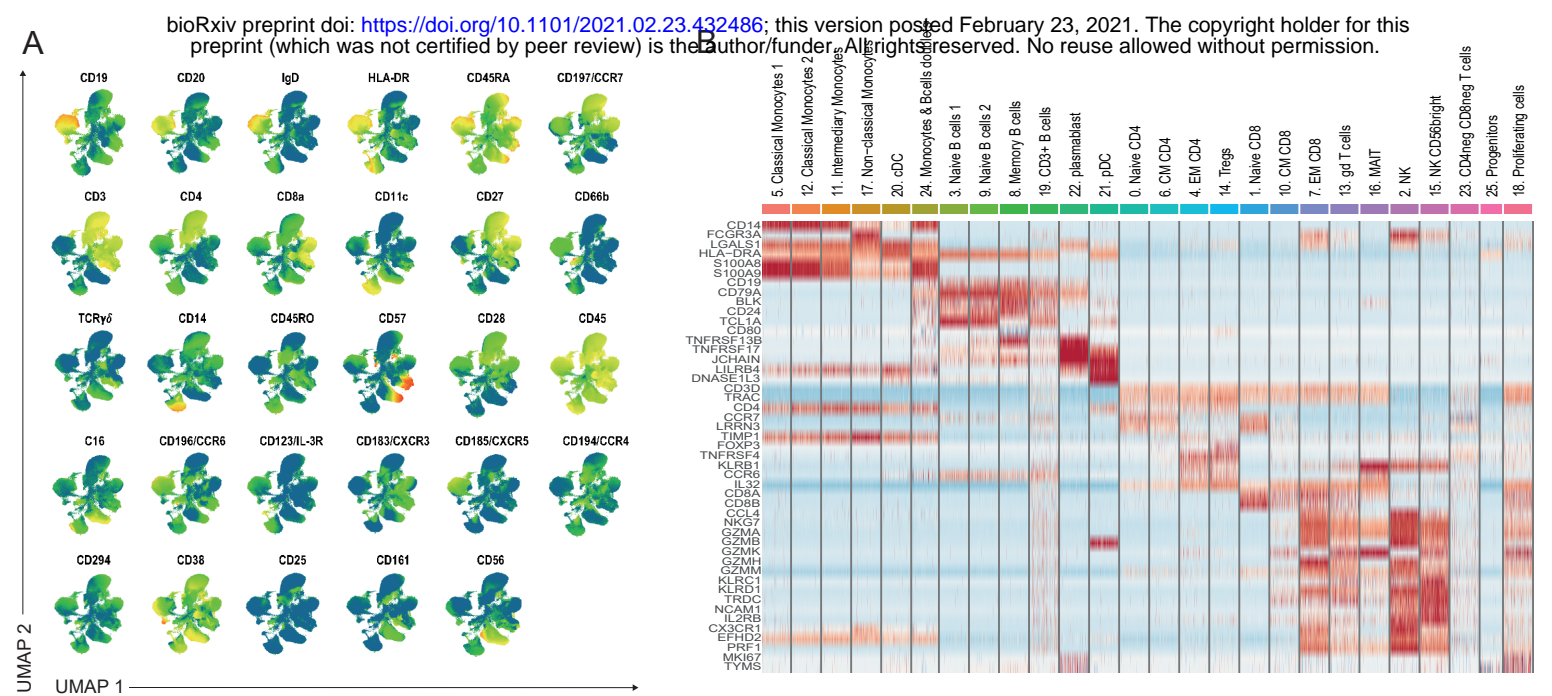


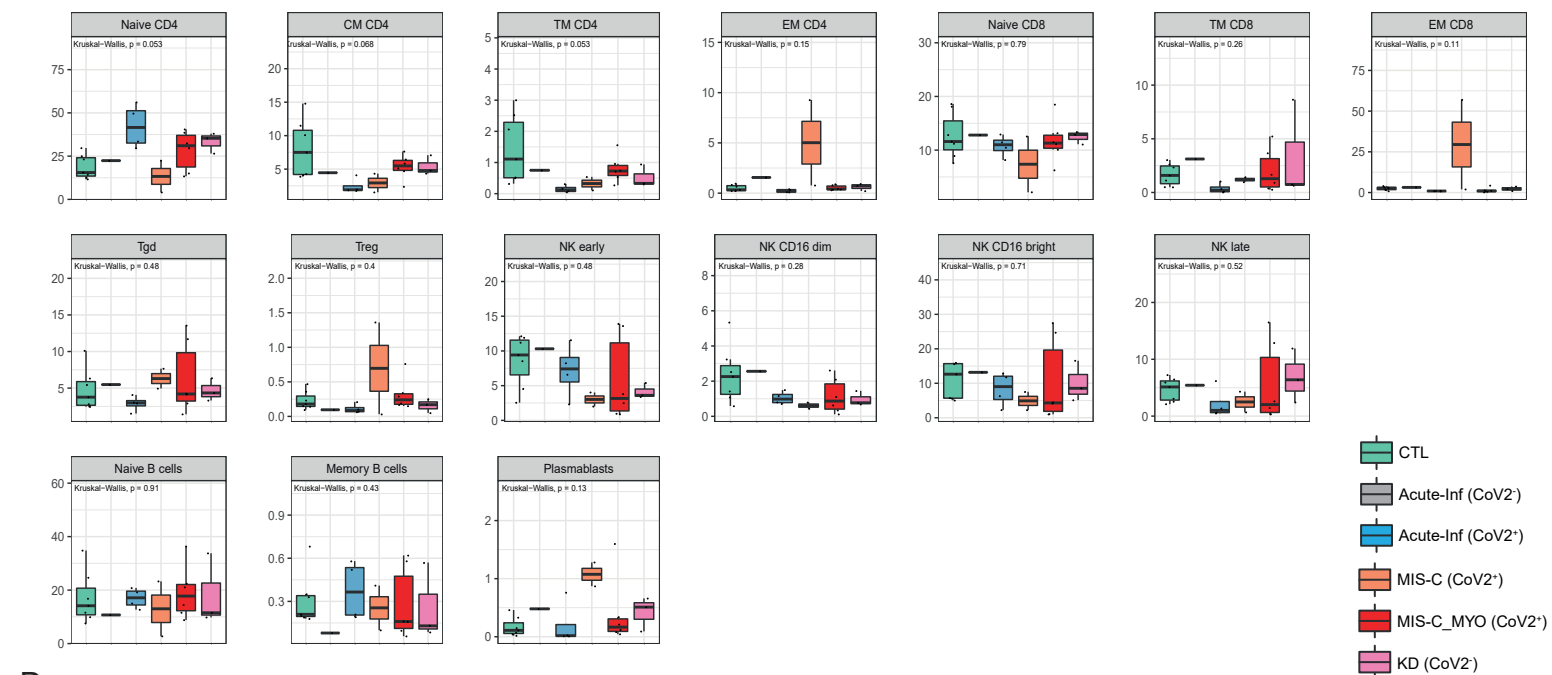
Figure 3

Figure 3: CyTOF and SC-RNA-SEQ characterization of peripheral blood immune cell (PBMCs) distribution. **A.** Upper panel: UMAP of 1,150,000 single cells from PBMCs of 7 CTL, 1 *Acute-inf* (*CoV2*⁻), 4 *Acute-inf* (*CoV2*⁺), 2 *MIS-C* (*CoV2*⁺), 6 *MIS-C_MYO* (*CoV2*⁺) and 3 *KD* (*CoV2*⁻) donors, following analyses by CyTOF and displayed as 23 clusters identified using the individual expression of 29 proteins, as described in Figure S3A. Bottom panel: boxplots of clusters with significant differences observed between groups. **B.** Upper panel: UMAP of 152,201 single cells following extraction from PBMCs (9 CTL, 1 *Acute-inf* (*CoV2*⁻), 4 *Acute-inf* (*CoV2*⁺), 2 *MIS-C* (*CoV2*⁺), 6 *MIS-C_MYO* (*CoV2*⁺), and 3 *KD* (*CoV2*⁻)) and processed by SC-RNA-SEQ. A resolution of 0.8 allows to segregate cells into 26 clusters identified based on the expression of several markers and gene signatures, as shown in Figure S4B. Bottom panel: Boxplots of clusters with significant differences.

A & B. (CTL, green; *Acute-inf* (*CoV2*⁻), gray; *Acute-inf* (*CoV2*⁺), blue; *MIS-C* (*CoV2*⁺), orange; *MIS-C_MYO* (*CoV2*⁺), red; *KD* (*CoV2*⁻), pink). In the boxplots, each dot represents a sample. Boxes range from the 25th to the 75th percentiles. The upper and lower whiskers extend from the box to the largest and smallest values respectively. Any samples with a value at most x1.5 the inter-quartile range of the hinge is considered an outlier and plotted individually. ρ values are calculated by Kruskal-Wallis test for multiple comparisons, followed by a post hoc Dunn's test. * ($\rho < 0.05$), ** ($\rho < 0.01$), *** ($\rho < 0.001$).



C CyTOF - PBMCs



D SC-RNA-SEQ - PBMCs

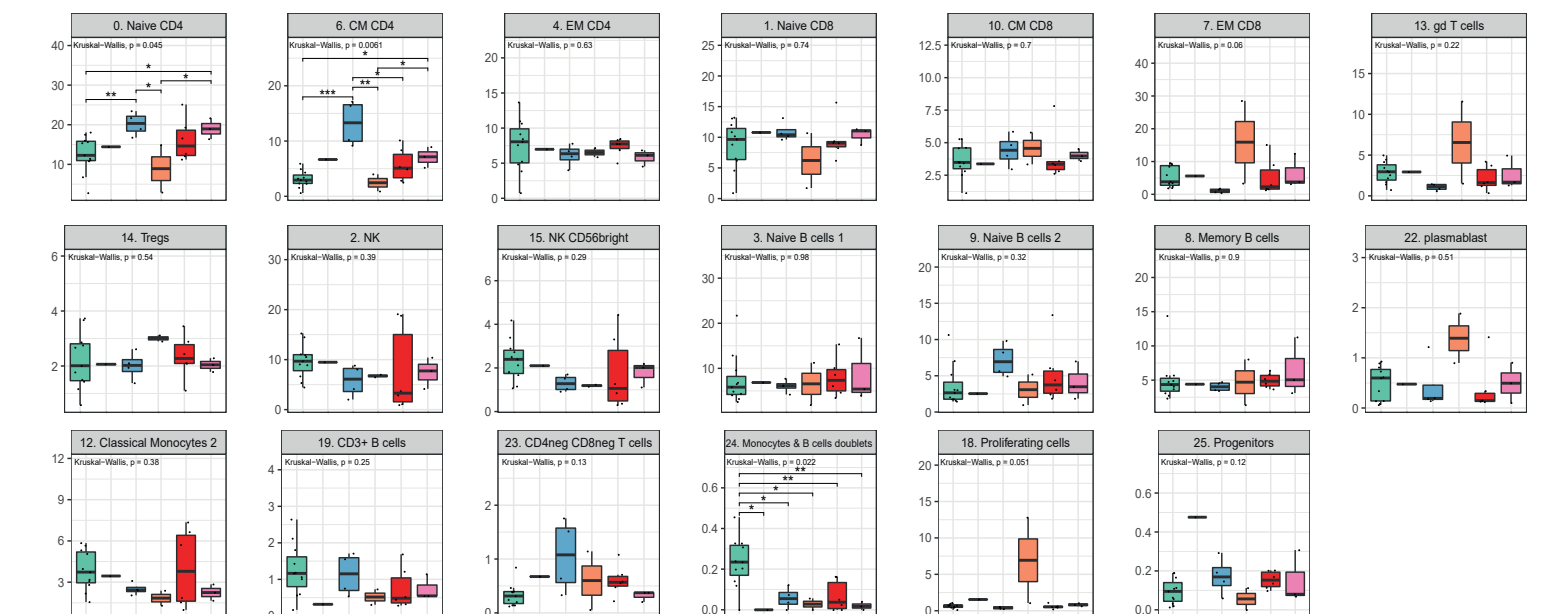


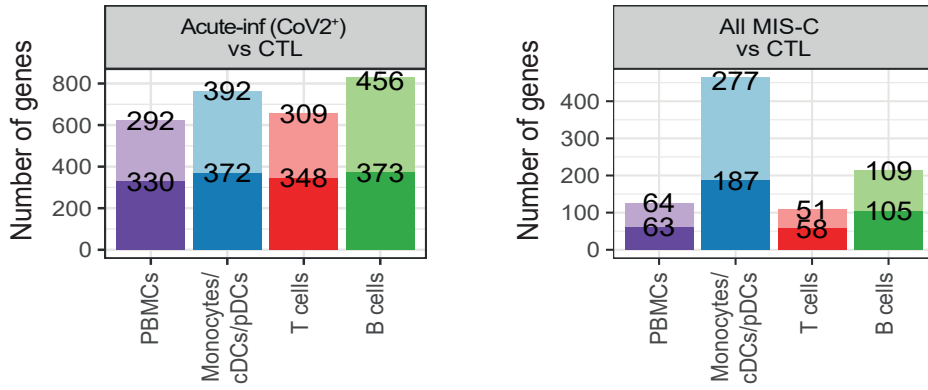
Figure S3

Figure S3: Identification of cell clusters and PBMCs distribution. Related to Figure 3. A.

UMAP showing all cell surface proteins measured by CyTOF to identify clusters displayed in Figure 3A. **B.** Heatmap of the genes (y axis) used in SC-RNA-SEQ to identify clusters of the UMAP in Figure 3B. Color scale represents scaled expression of the genes. Red and blue indicate high and low expression respectively. **C & D.** Boxplots showing the percentage of cell populations from CyTOF (C) and SC-RNA-SEQ (D). Among the *MIS-C* (*CoV2*⁺) group, one patient was diagnosed with a primary Epstein Barr viral infection, characterized at the cellular level by an increase of CD4, CD8 effector memory T cells, Tregs and also proliferating cells.

(CTL, green; *Acute-inf* (*CoV2*⁻), gray; *Acute-inf* (*CoV2*⁺), blue; *MIS-C* (*CoV2*⁺), orange; *MIS-C_MYO* (*CoV2*⁺), red; *KD* (*CoV2*⁻), pink). In the boxplots, each dot represents a sample. Boxes range from the 25th to the 75th percentiles. The upper and lower whiskers extend from the box to the largest and smallest values respectively. Any samples with a value at most x1.5 the interquartile range of the hinge is considered an outlier and plotted individually. ρ values are calculated by Kruskal-Wallis test for multiple comparison, followed by a post hoc Dunn's test. *($\rho < 0.05$), **($\rho < 0.01$), ***($\rho < 0.001$).

A



B

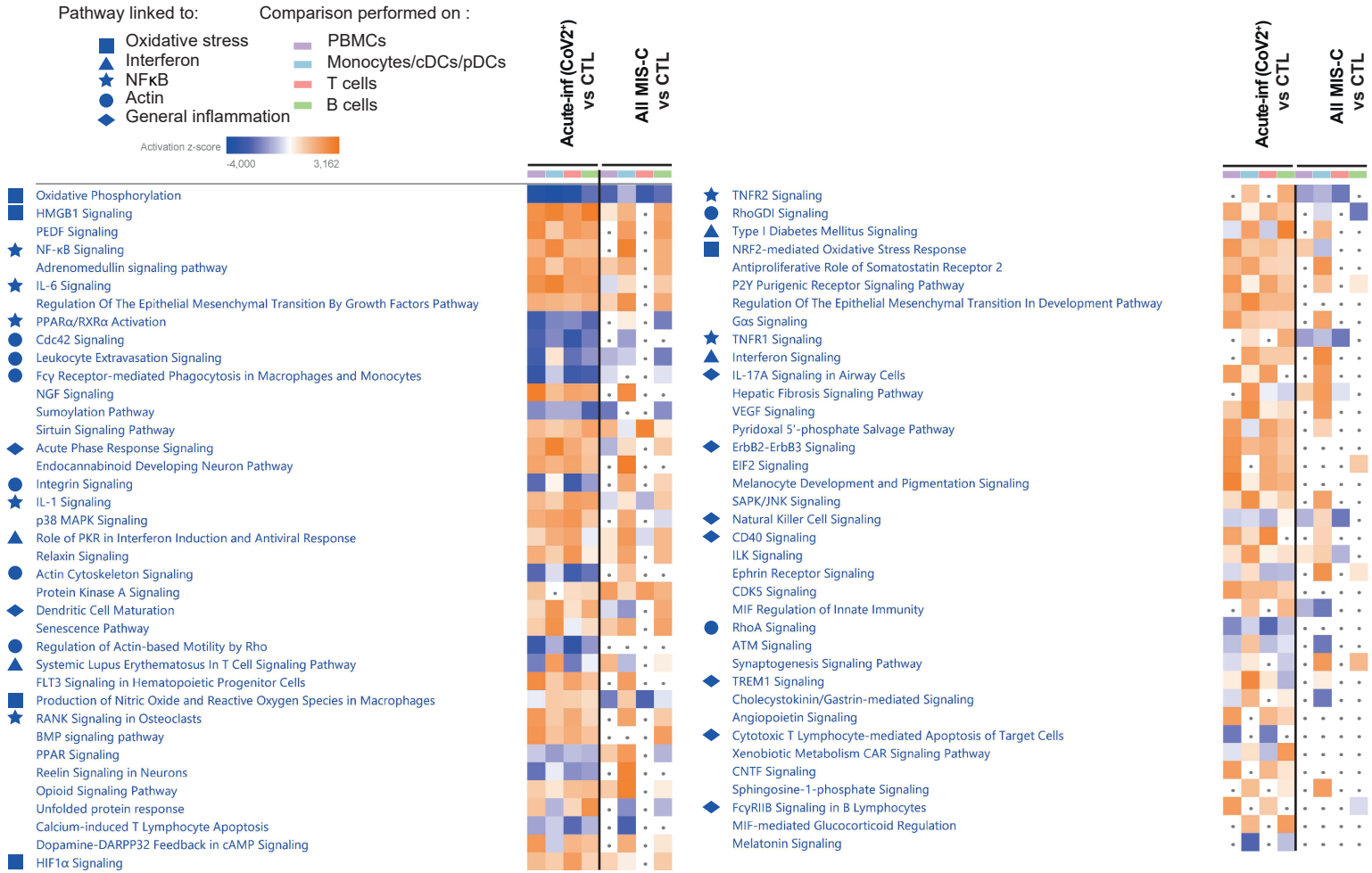
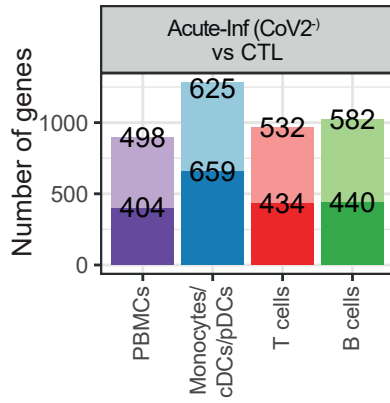


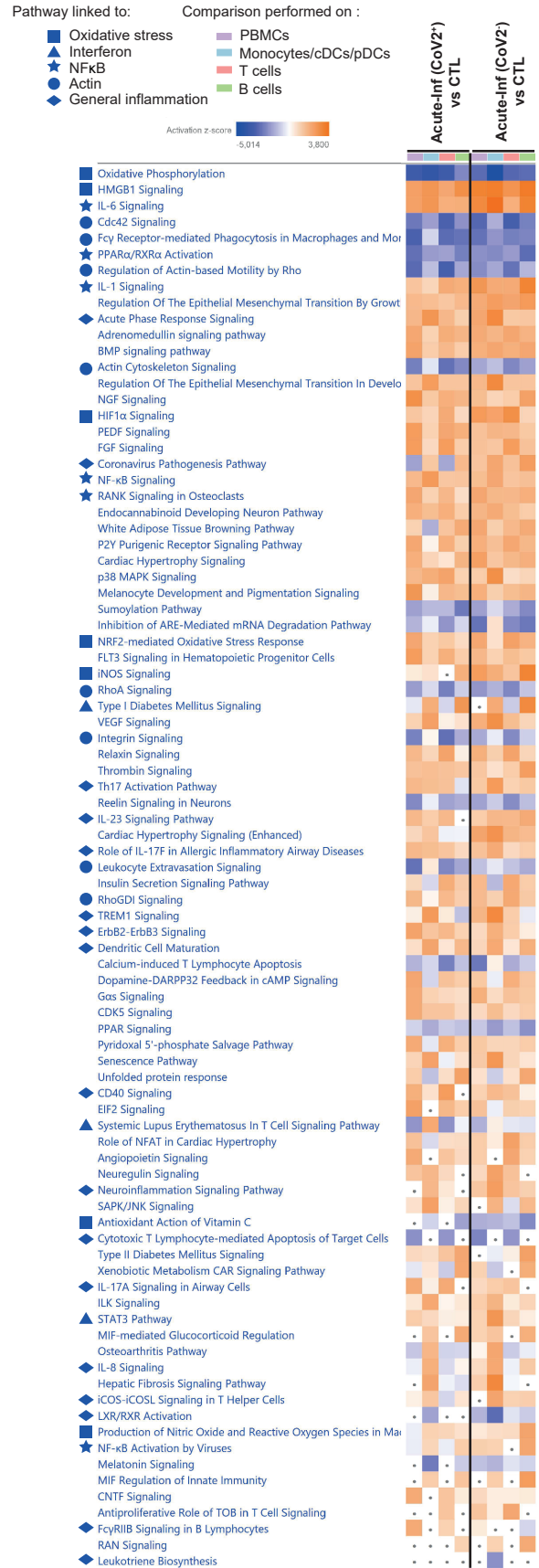
Figure 4

Figure 4: Genes and pathways differentially regulated in acute infection and postacute hyper inflammation following SARS-CoV-2 infection. A & B. Bar charts of the number of up- and downregulated genes in *Acute-inf (CoV2⁺)* (A) and All MIS-C (*MIS-C (CoV2⁺)*) and *MIS-C_MYO (CoV2⁺)* (B), compared to CTL, in PBMCs, monocytes/DCs, T and B cells clusters obtained following SC-RNA-SEQ experiments as displayed in Figure 3B. PBMCs represent all clusters; monocytes/DCs cells, clusters 5, 11, 12, 17, 20, 21, 24; T cells, clusters 0, 1, 2, 4, 6, 7, 10, 13, 14, 15, 16, 18 and 23; and B cells, clusters 3, 8, 9, 19, and 22. The top value on the light-colored bars represent the upregulated genes and the bottom dark represent the downregulated genes. **B.** Heatmap of the canonical pathways, enriched in the differentially expressed genes (DEG) from the comparisons performed in A and B in PBMCs, monocytes/DCs, T and B cells, obtained by using Ingenuity Pathways Analysis (IPA) analyses. Left panel, part 1 and right panel part 2. Symbols are used in front of the pathways to represent pathways belonging to the same functional groups. Pathways with an absolute z-score ≤ 2 or adjusted p-value > 0.05 in all conditions were filtered out. Z-score > 2 means that a function is significantly increased (orange) whereas a Z-score < -2 indicates a significantly decreased function (blue). Grey dots indicate non-significant pathways ($p > 0.05$).

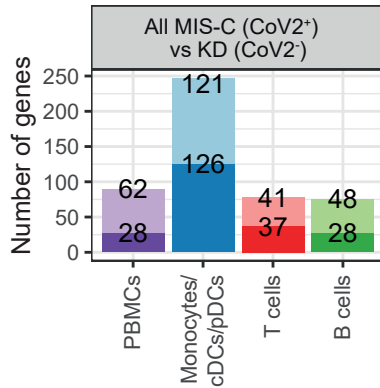
A



B



C



D

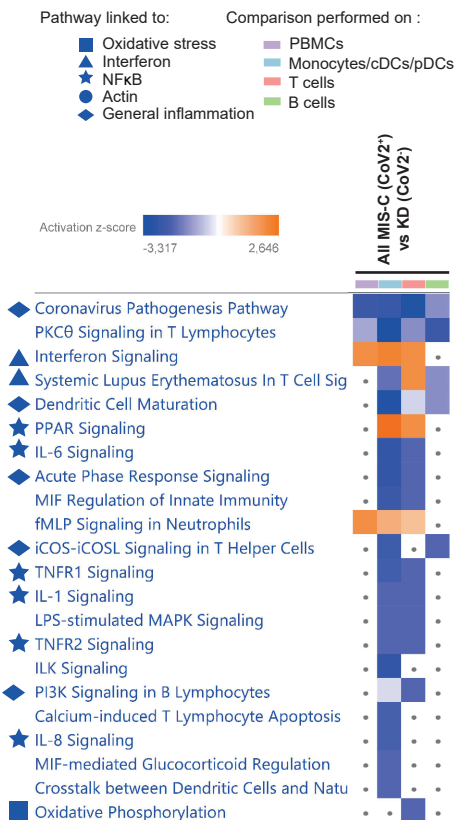
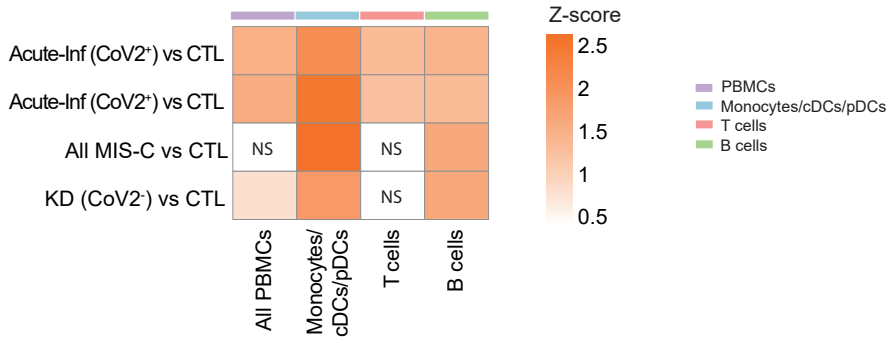


Figure S4: Genes and pathways differentially regulated from SC-RNA-SEQ in acute infection and postacute hyperinflammation when comparing patients with or without evidence of SARS-CoV-2 infection. Related to Figure 4. **A.** Bar charts of the number of up- and downregulated genes in *Acute-inf (CoV2⁻)* compared to CTL, in PBMCs, monocytes/DCs, T and B cell clusters obtained following SC-RNA-SEQ experiments as displayed in Figure 3B. **B.** Heatmap of the canonical pathways obtained with IPA from DEGs of *Acute-inf (CoV2⁺)*, as shown in 4A or of *Acute-inf (CoV2⁻)*, S4A, compared to CTL in PBMCs, monocytes/DCs, T and B cells. **C.** Bar charts of the number of up- and downregulated genes in *All MIS-C (CoV2⁺)* compared directly to *KD (CoV2⁻)*, in PBMCs, monocytes/DCs, T and B cells. **D.** Heatmap of the canonical pathways obtained with IPA from DEGs of *All MIS-C (CoV2⁺)* compared directly to *KD (CoV2⁻)* in PBMCs, monocytes/DCs, T and B cells.

A & C. The top value on the light-colored bars represent the upregulated genes and the bottom dark represent the downregulated genes.

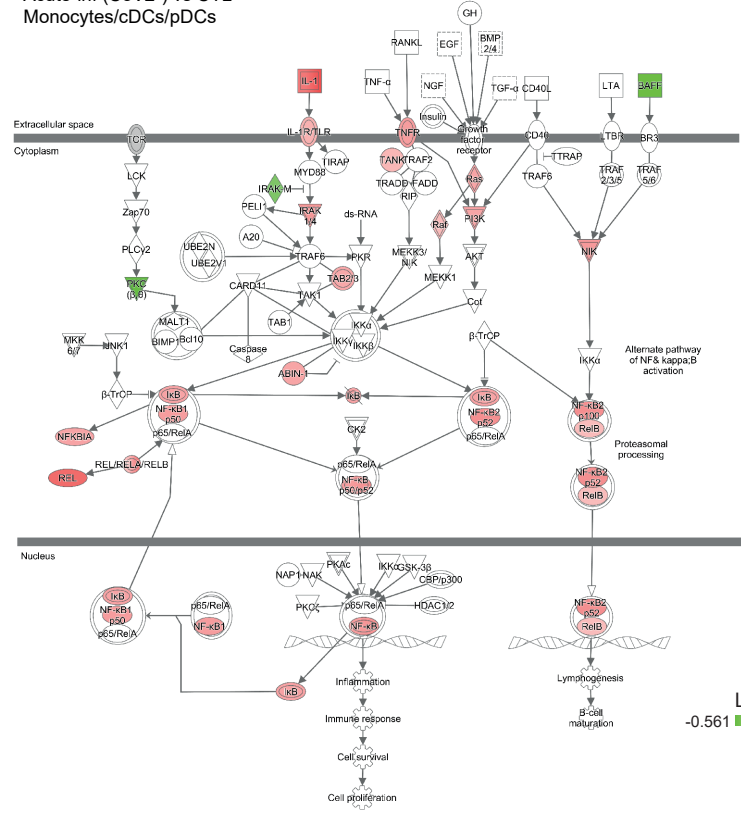
B & D. Symbols are used in front of the pathways to represent pathways belonging to the same functional groups. Pathways with an absolute z-score ≤ 2 or adjusted p-value > 0.05 in all conditions were filtered out. Z-score > 2 means that a function is significantly increased (orange) whereas a Z-score < -2 indicates a significantly decreased function (blue). Grey dots indicate non-significant pathways ($\rho > 0.05$).

A NF- κ B signaling pathway predicted activation



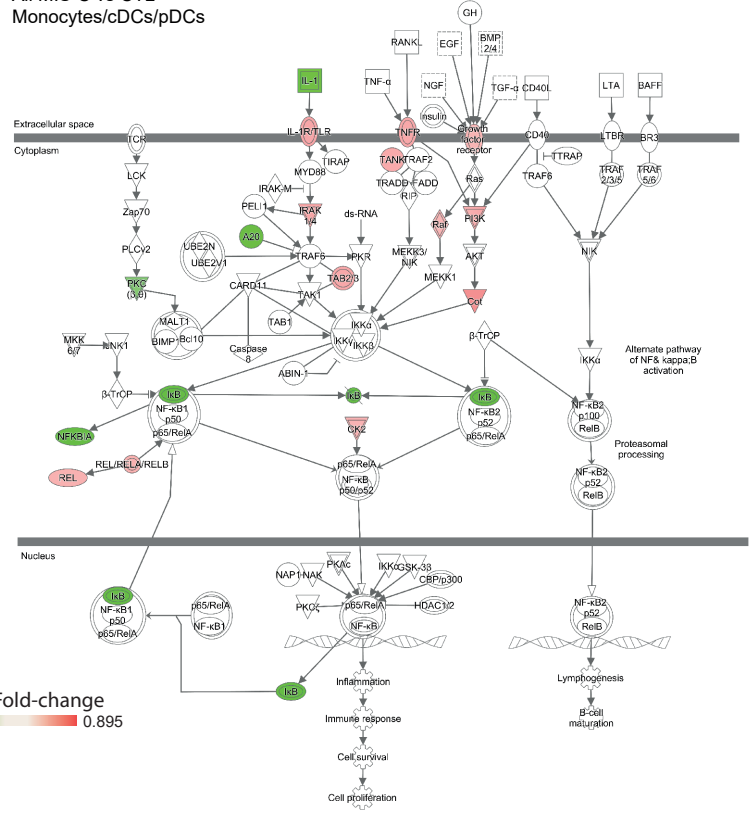
B NF- κ B signaling pathway

Acute-inf (CoV2+) vs CTL
Monocytes/cDCs/pDCs

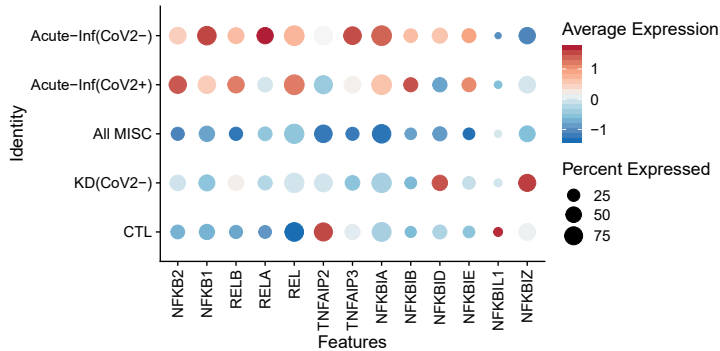


C NF- κ B signaling pathway

All MIS-C vs CTL
Monocytes/cDCs/pDCs



D Expression of NF κ B complex and its inhibitors in monocytes/cDCs/pDCs



E Expression of NF κ B inhibitors in monocytes/cDCs/pDCs

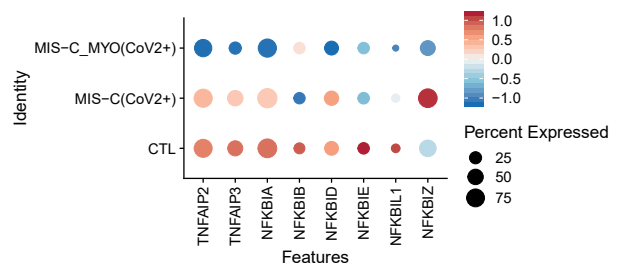


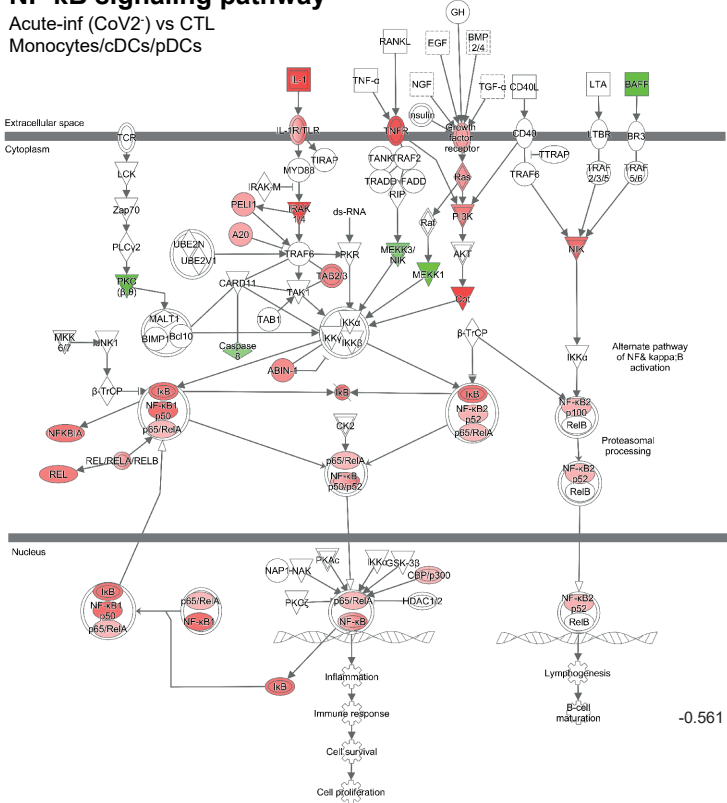
Figure 5

Figure 5: NF- κ B signaling pathway activation in acute infection and postacute hyperinflammatory illness. **A.** Heatmap of the activation of NF- κ B signaling pathway, as predicted by IPA, in each disease group compared to controls. Color scale represents the z-score of the prediction. The higher the score, the more activated the NF- κ B signaling pathway. “NS” indicates non-significant comparisons. **B & C.** NF- κ B signaling pathway from the IPA software, displayed in the monocytes/DCs cells in *Acute-inf* (*CoV2*⁺) versus CTL (B) or *All MIS-C* (*CoV2*⁺) versus CTL (C). Changes in gene expression are represented by the log fold change (increase in red and decreased in green). **D.** Dot plot of the expression in monocytes/DCs cells of the positive (from *NFKB2* to *REL*) and negative regulators (from *TNFAIP2* to *NFKBIZ*) of NF- κ B complex in all disease groups. **E.** Dot plot of the expression in monocytes/DCs of the negative regulators of NF- κ B complex in the MIS-C groups.

A

NF-κB signaling pathway

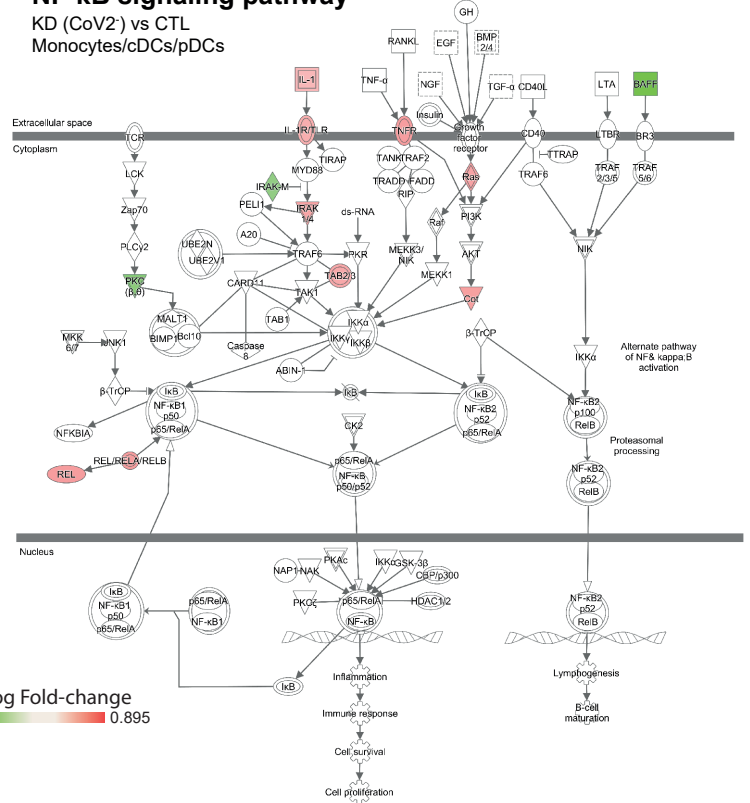
Acute-inf (CoV2-) vs CTL
Monocytes/cDCs/pDCs



B

NF-κB signaling pathway

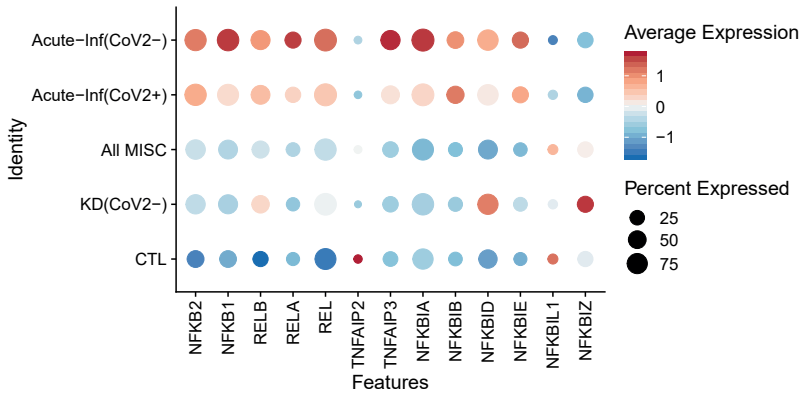
KD (CoV2-) vs CTL
Monocytes/cDCs/pDCs



Log Fold-change
-0.561 0.895

C

Expression of NFκB complex and its inhibitors in B cells



D

Expression of NFκB complex and its inhibitors in T cells

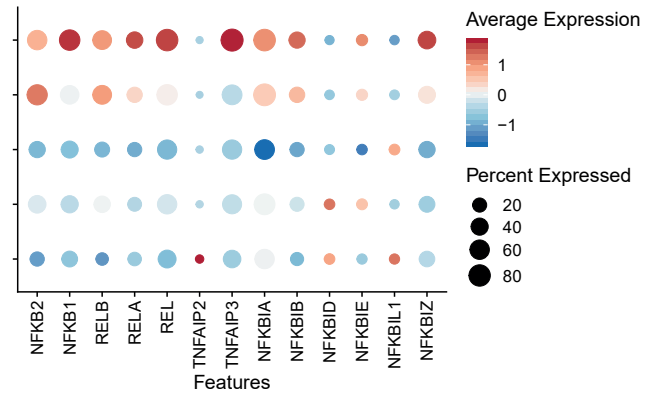
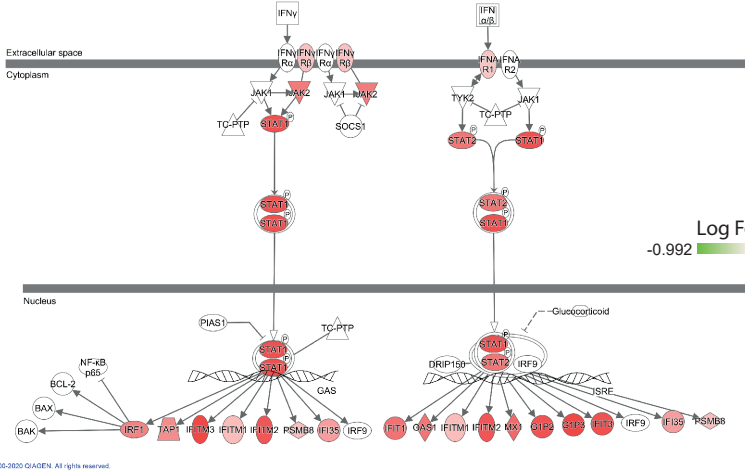


Figure S5: NF- κ B signaling pathway activation in acute infection and postacute hyperinflammatory illness unrelated to SARS-CoV2 infection. Related to Figure 5. A & B. NF- κ B signaling pathway from the IPA software, displayed in the monocytes/DCs cells in *Acute-inf* (CoV2⁻) versus CTL (A) or *KD* (CoV2⁻) versus CTL (B). Changes in gene expression are represented by the log fold change (increase in red and decrease in green). **C & D.** Dot plot of the expression in B cells (C) or T cells (T) of the positive (from *NFKB2* to *REL*) and negative regulators (from *TNFAIP2* to *NFKBIZ*) of NF- κ B complex in all disease groups.

A

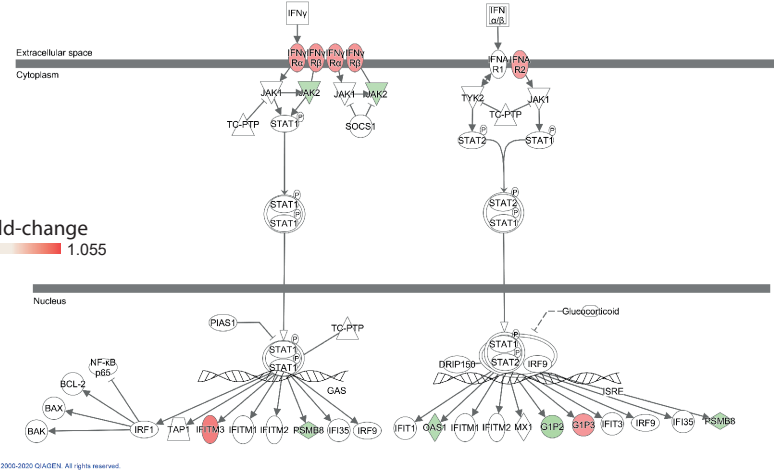
Interferon signaling pathway

MIS-C (CoV2⁺) vs CTL
Monocytes/cDCs/pDCs



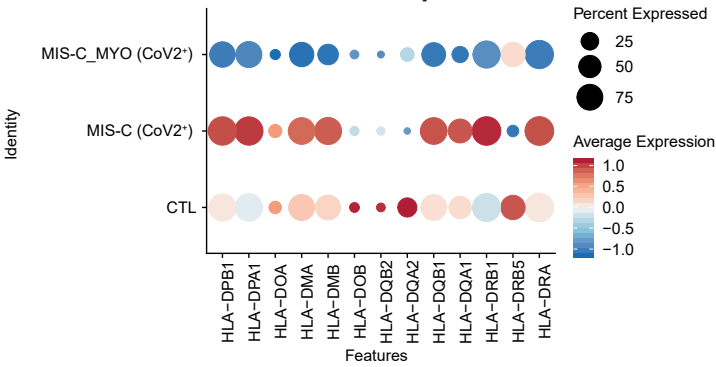
Interferon signaling pathway

MIS-C_MYO (CoV2⁺) vs CTL
Monocytes/cDCs/pDCs



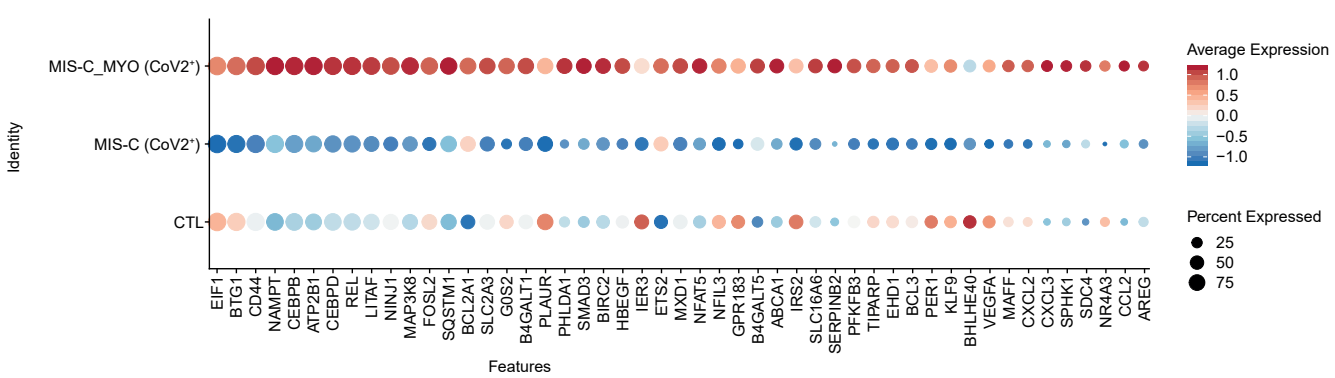
C

MHC class II expression



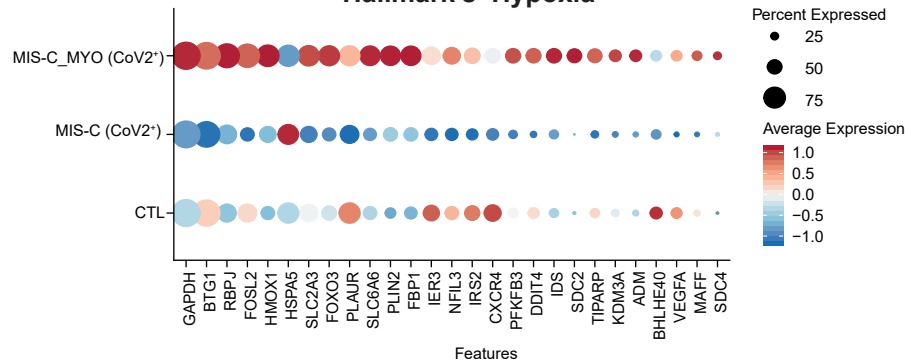
D

Expression of the upregulated genes from Hallmark's 'TNFα signaling via NFκB'



E

Expression of the upregulated genes from Hallmark's 'Hypoxia'



F

Expression of S100 genes

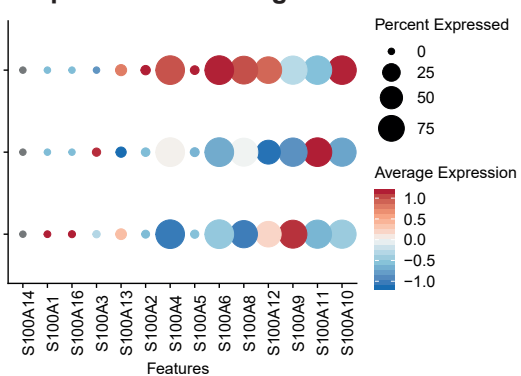


Figure 6: Key pathways and genes deregulated in monocytes/DCs cells of MIS-C_MYO (CoV2⁺) compared to MIS-C (CoV2⁺). **A & B.** Type-I and type-II interferon signaling pathways from the IPA software. Changes in gene expression are represented by the log fold change in monocytes/DCs cells of *MIS-C (CoV2⁺)* (A) or *MIS-C_MYO (CoV2⁺)* (B) compared to CTL (red color represents an upregulation, green a downregulation and white no changes). **C.** Dot plot of the MHC class II-associated genes. **D.** Dot plot of the 49 genes from the TNF-alpha Signaling via NF-κB pathway (pathway enrichment analysis by MSigDB Hallmark 2020 (Figure S6E)). **E.** Dot plot of the 27 genes from the Hypoxia pathway (pathway enrichment analysis by MSigDB Hallmark 2020 (Figure S6E)). **F.** Dot plot of the calcium binding genes of the S100 family in CTL, *MIS-C (CoV2⁺)* and *MIS-C_MYO (CoV2⁺)*

C - F. The expression is represented by the centered scale expression of each gene. The “percent expressed” represents the percentage of cells that express the gene.

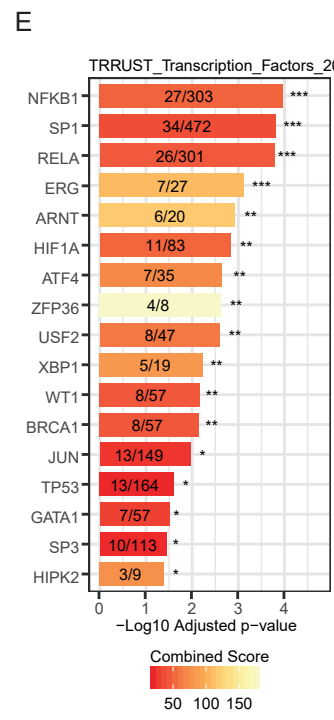
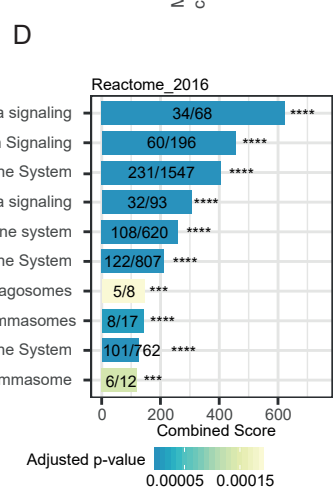
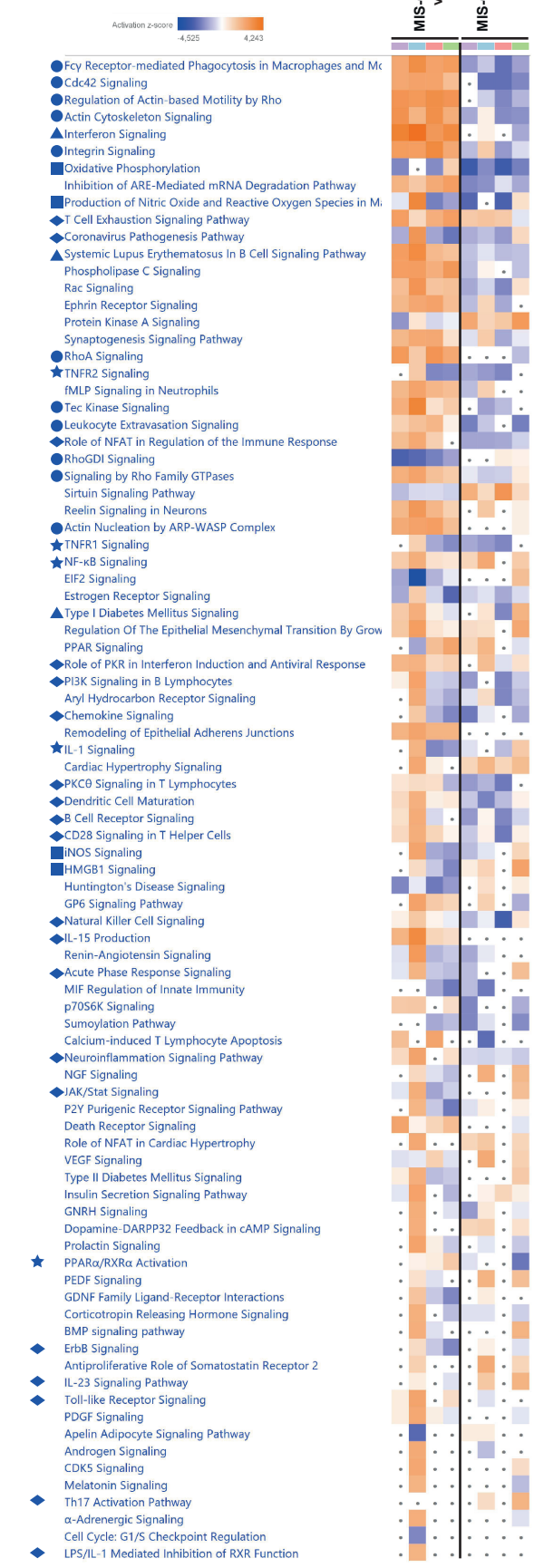
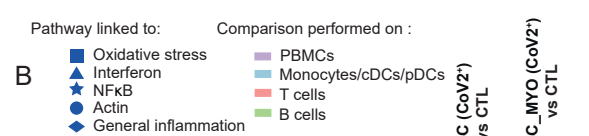
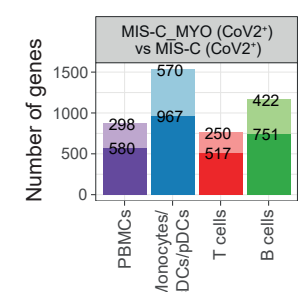
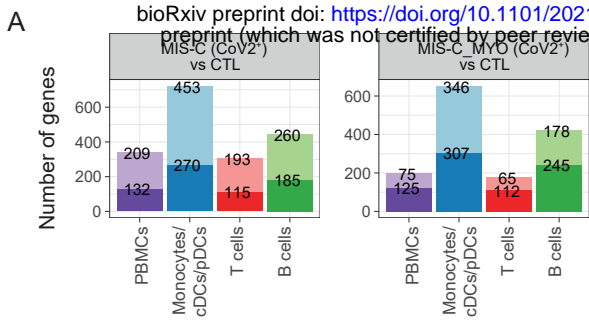


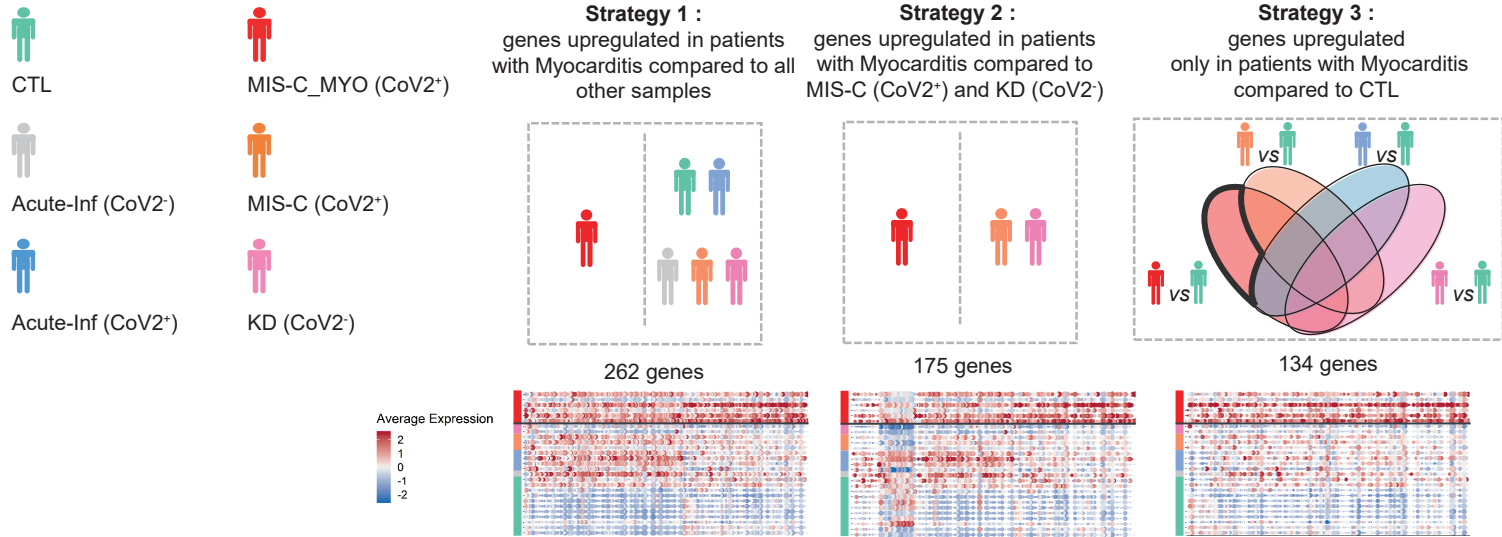
Figure S6

Figure S6: Pathway enrichment analysis in MIS-C (CoV2⁺) with or without severe myocarditis. Related to Figure 6. **A.** Bar charts of the number of up- and downregulated genes in *MIS-C (CoV2⁺)* (left) and *MIS-C_MYO (CoV2⁺)* (right) compared to CTL in PBMCs, monocytes/DCs, T and B cells. **B.** Heatmap of the canonical pathways obtained with IPA from DEGs of *MIS-C (CoV2⁺)* or *MIS-C_MYO (CoV2⁺)* compared to CTL in PBMCs, monocytes/DCs, T and B cells. Symbols are used in front of the pathways to represent pathways belonging to the same functional groups. Pathways with an absolute z-score ≤ 2 or adjusted p-value > 0.05 in all conditions were filtered out. Z-score > 2 means that a function is significantly increased (orange) whereas a Z-score < -2 indicates a significantly decreased function (blue). Grey dots indicate non-significant pathways ($p > 0.05$). **C.** Bar charts of the number of up- and downregulated genes in *MIS-C_MYO (CoV2⁺)* compared to *MIS-C (CoV2⁺)* in PBMCs, monocytes/DCs, T and B cells. **D.** Bar charts of the top 10 pathways from Reactome_2016, predicted in EnrichR to be modulated by the downregulated genes in the monocytes/DCs cells of *MIS-C_MYO (CoV2⁺)* compared to *MIS-C (CoV2⁺)*. Pathways are ranked based on combined score. **E.** Bar charts of the Transcription factors from TRRUST database (top) and the top 10 pathways from MSigDB_Hallmark_2020 (bottom) predicted in EnrichR to be modulated by the genes upregulated in monocytes/DC of *MIS-C_MYO (CoV2⁺)* compared to *MIS-C (CoV2⁺)*. Pathways are ranked based on combined score; Transcription factors are ranked based on adjusted p-values.

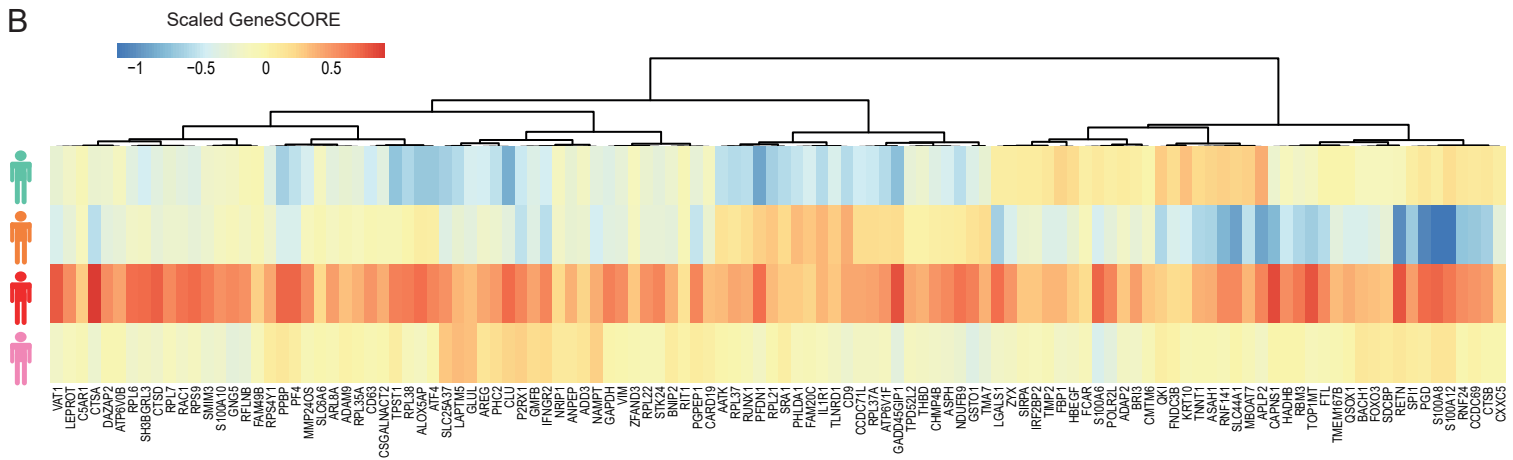
A & C. The top values on the light-colored bars represent the upregulated genes and the bottom dark represent the downregulated genes.

D & E. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$)

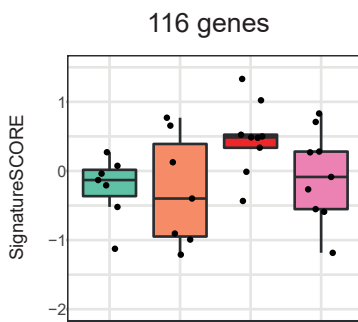
A



B



C



D

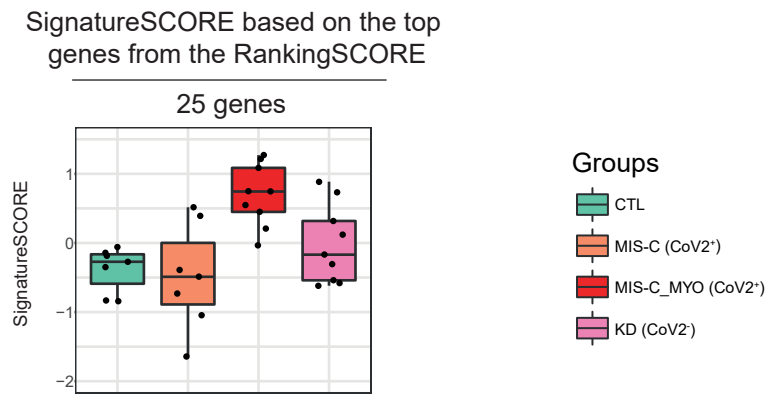


Figure 7

Figure 7: Identification of genes specifically upregulated in monocytes/dendritic cells of children of the MIS-C_MYO (CoV2⁺) group. **A.** Schematic representation of the different strategies used to extract 329 unique markers of the *MIS-C_MYO (CoV2⁺)* group from the monocytes/DCs clusters obtained from the single-cell dataset. Strategy 1: direct comparison of the monocytes/DCs cells of the *MIS-C_MYO (CoV2⁺)* group to all other samples. Strategy 2: direct comparison of the monocytes/DCs cells of *MIS-C_MYO (CoV2⁺)* to other samples with postacute hyperinflammation (*MIS-C (CoV2⁺)* and *KD (CoV2⁻)*). Strategy 3: selection of the genes upregulated only in the monocytes/DCs cells of *MIS-C_MYO (CoV2⁺)* when compared to CTL. Below each strategy, the corresponding dot plot obtained from SC-RNA-SEQ, with the number of upregulated genes. The average expression is represented by the centered scale expression of each gene. On the left, name of each group with its corresponding color is shown. **B.** Heatmap of expression of the 116/329 genes with a higher expression in *MIS-C_MYO (CoV2⁺)* than in other groups in the bulk dataset. Color scale indicates the scaled GeneSCORE (mean z-score of the gene in all samples of a group), with red and blue representing the highest and lowest expressions respectively. Hierarchical clustering of the genes was computed with a Pearson's correlation as a distance. **C.** Box plot of the expression of the 116 genes validated in C, calculated as a SignatureScore. SignatureScore represents for each sample the mean z-score of the 116 genes selected in B in the bulk-RNA-SEQ dataset. (Explained in Figure S7A). **D.** Boxplot showing the SignatureScore computed on the expression of the top 25 genes, as ranked in Figure S7B, in the Bulk-RNA-SEQ dataset.

C & D. Each dot represents a sample. Boxes range from the 25th to the 75th percentiles. The upper and lower whiskers extend from the box to the largest and smallest values respectively. Any samples with a value at most x1.5 the inter-quartile range of the hinge is considered an outlier and plotted individually.

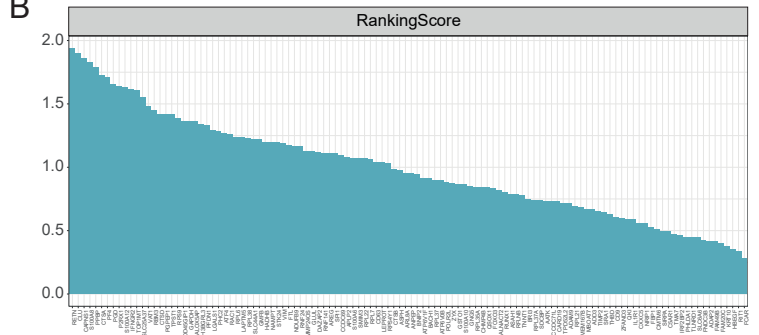
A

$$GeneSCORE = \frac{\sum_{n=1}^N Zscore_n}{N}$$

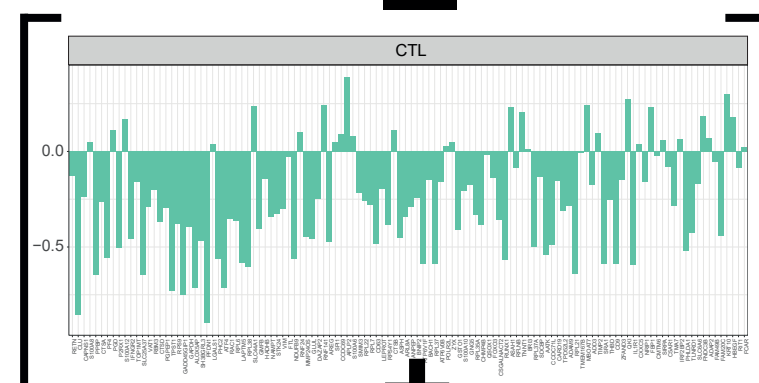
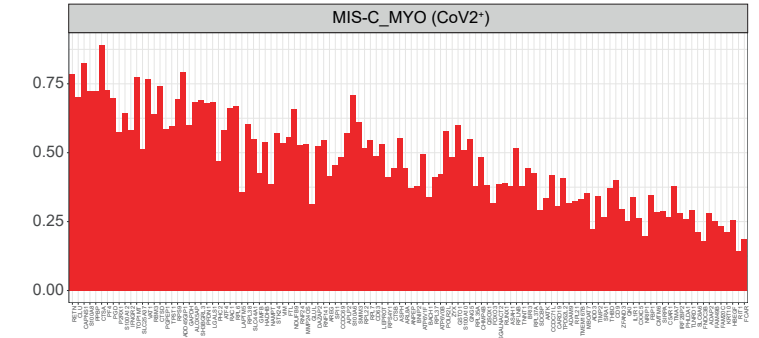
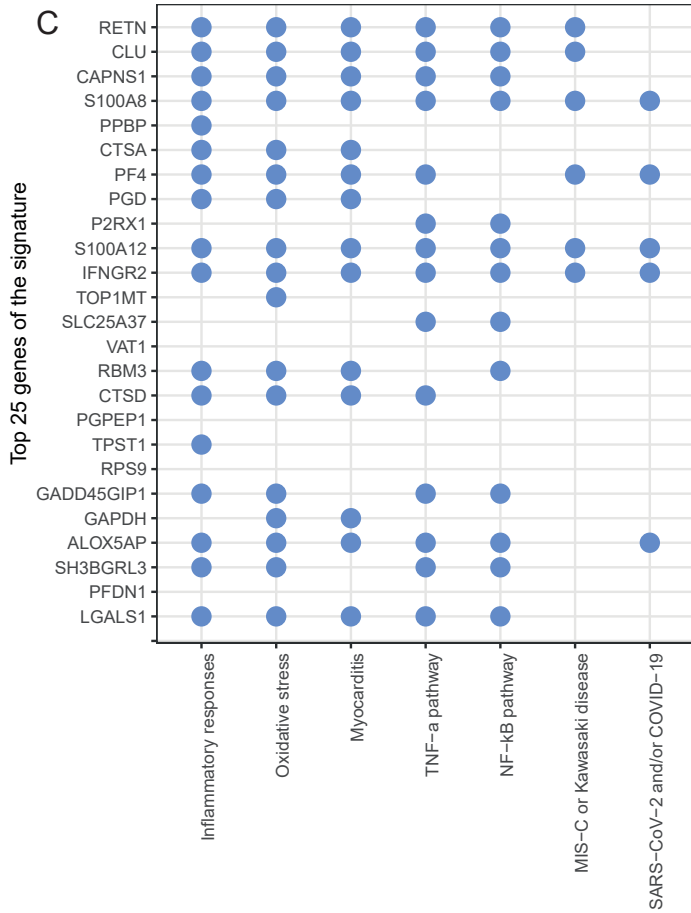
$$SignatureSCORE = \frac{\sum_{m=1}^M Zscore_m}{M}$$

$$RankingSCORE = GeneSCORE_{MIS-C_MYO(CoV2^+)} - (GeneSCORE_{MIS-C(CoV2^+)} + GeneSCORE_{CTL})$$

B



C



D

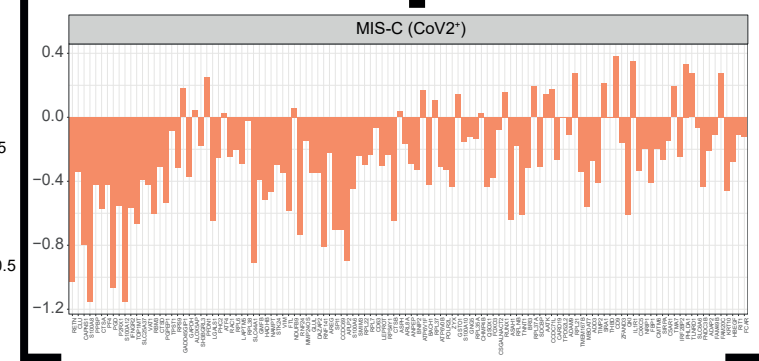
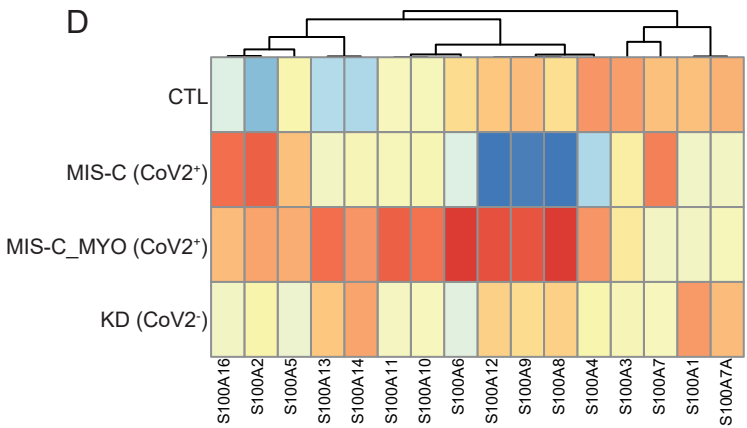
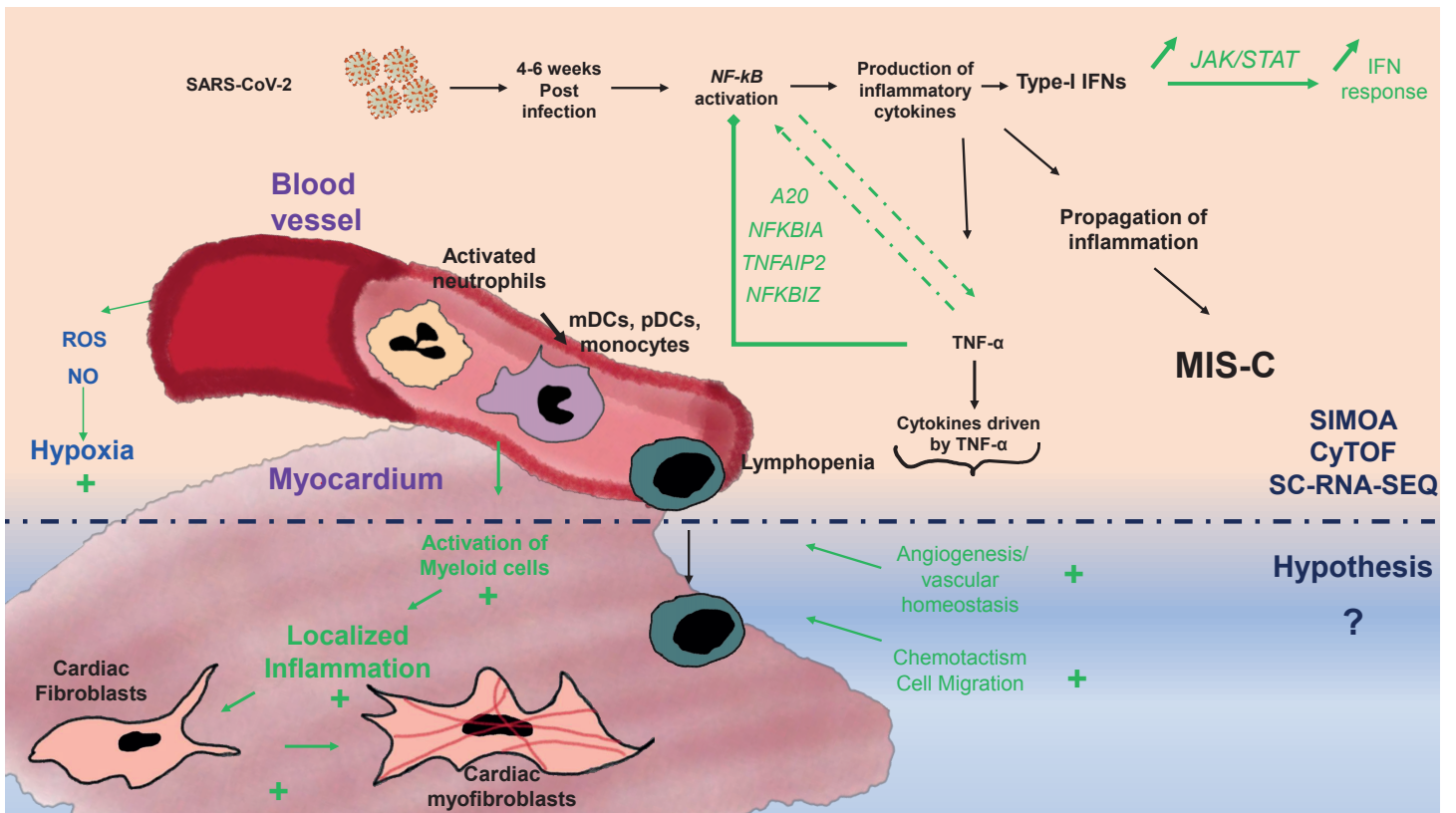


Figure S7

Figure S7: SignatureScore and top genes correlated with the occurrence of myocarditis in MIS-C (CoV2⁺). Related to Figure 7. A. *MIS-C_MYO* (CoV2⁺)-specific genes were scored (GeneSCORE) by adding their expression values (as z-scores) in each sample, divided by the total number of samples. Similarly, a SignatureSCORE was derived for each sample as the mean z-score of these samples. From these two scores, a RankingScore was calculated by subtracting the GeneScore of the *MIS-C* (CoV2⁺) and the CTL groups to the GeneSCORE of the *MIS-C_MYO* (CoV2⁺) group. **B.** Top: Barplot of the RankingScore of each gene. Red, green and orange barplots: GeneScore of the *MIS-C_MYO* (CoV2⁺)-specific genes in the *MIS-C_MYO* (CoV2⁺), CTL and *MIS-C* (CoV2⁺) groups respectively. Genes are ranked based on their RankingScore as explained in S7A **C.** Curated analyses based on literature regarding the known links between the top 25 genes, and inflammatory response, myocarditis, oxidative stress, TNF- α , NF- κ B, MIS-C or Kawasaki disease, SARS-CoV-2 and/or COVID-19. On the Y axis, genes are positioned from top to bottom following their rank (Top = ranked 1). A blue dot means strong links between the gene and the pathway (on the X axis) were found in peer-reviewed scientific publications. **D.** Heatmap of the expression of the genes of the S100A family, extracted from Bulk-RNA-SEQ, from PBMCs. Color scale indicate the scaled GeneSCORE (mean z-score of the gene in all samples of a group), with red and blue representing the highest and lowest expressions respectively. Hierarchical clustering of the genes was computed with a Pearson's correlation as a distance.

A



B

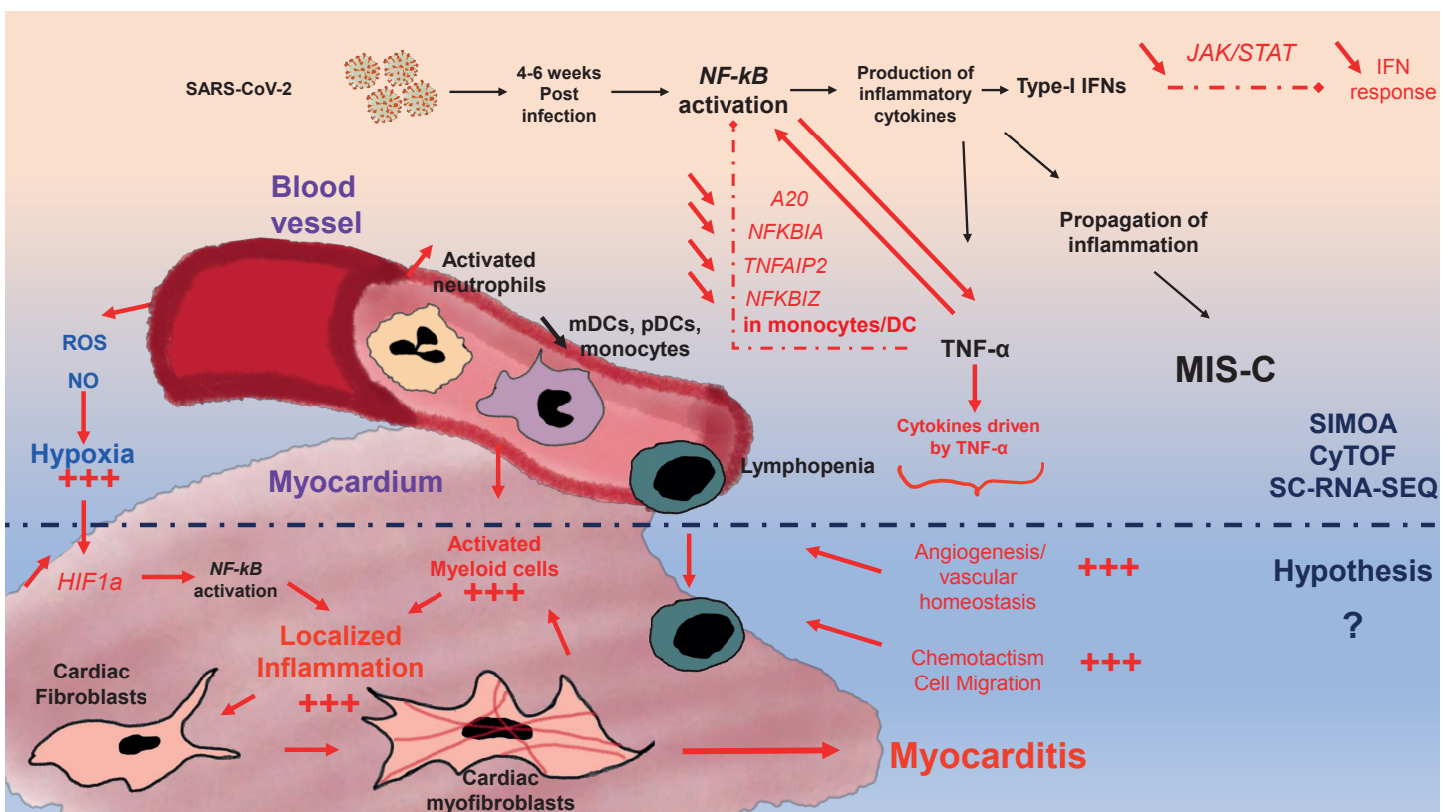


Figure S8

Figure S8. A & B. Graphical representation based on cytokines, cellular and transcriptomic analyses (part above black dotted line), combined with known literature (part under the black dotted line), illustrating a putative model explaining the occurrence of myocarditis among children in the *MIS-C (CoV2⁺)* group. **A.** model based on the *MIS-C (CoV2⁺)* group and **B.** model based on the *MIS-C_MYO (CoV2⁺)* group. Black writing represents genes and functions both modulated in the *MIS-C (CoV2⁺)* and *MIS-C_MYO (CoV2⁺)* groups compared to CTL, whereas green and red are highlighting genes and pathways differentially modulated in the *MIS-C (CoV2⁺)* and *MIS-C_MYO (CoV2⁺)* groups, respectively.