TITLE

Distinct immunological signatures discriminate severe COVID-19 from non-SARS-CoV-2-driven critical pneumonia

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HIGHLIGHTS

GM-CSF+ T cells are a hallmark of severe respiratory syndrome independent of pathogen

T cell exhaustion and impaired early antiviral response is unique in severe COVID-19

Circulating NKT cell frequencies serve as a predictive biomarker for severe COVID-19

HLA profile links COVID-19 immunopathology to impaired virus recognition

eTOC BLURB

The pathogen-specific immune alterations in severe COVID-19 remain unknown. Using longitudinal, high-dimensional single-cell spectral cytometry and algorithm-guided comparison of COVID-19 vs. non-SARS-CoV-2-pneumonia patient samples, Kreutmair et al. identify T and NK cell immune signatures specific to SARS-CoV-2. They furthermore reveal NKT cell frequency as a predictive biomarker for COVID-19 outcome prediction and link impaired virus recognition to HLA genetics.

KEYWORDS

COVID-19, SARS-CoV-2, high-dimensional single cell analysis, immune profiling, immunophenotyping, spectral flow cytometry, biomarker, Hospital-acquired pneumonia, Cytokines, GM-CSF, HLA typing, peptide binding strength

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33 **SUMMARY**

34 Immune profiling of COVID-19 patients has identified numerous alterations in both innate and

35 adaptive immunity. However, whether those changes are specific to SARS-CoV-2 or driven

36 by a general inflammatory response shared across severely ill pneumonia patients remains

37 unknown. Here, we compared the immune profile of severe COVID-19 with non-SARS-CoV-

38 2 pneumonia ICU patients using longitudinal, high-dimensional single-cell spectral cytometry

39 and algorithm-guided analysis. COVID-19 and non-SARS-CoV-2 pneumonia both showed

40 increased emergency myelopoiesis and displayed features of adaptive immune paralysis.

41 However, pathological immune signatures suggestive of T cell exhaustion were exclusive to

42 COVID-19. The integration of single-cell profiling with a predicted binding capacity of SARS-

43 CoV-2-petides to the patients' HLA profile further linked the COVID-19 immunopathology to

44 impaired virus recognition. Towards clinical translation, circulating NKT cell frequency was

45 identified as a predictive biomarker for patient outcome. Our comparative immune map serves

46 to delineate treatment strategies to interfere with the immunopathologic cascade exclusive to

47 severe COVID-19.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has affected over 50 million people worldwide and resulted in more than 3 million deaths as of April 2021 (World Health Organization, 2020a). The causative agent is severe acute RS (RS) coronavirus 2 (SARS-CoV-2) (Lu et al., 2020). The majority of people infected with SARS-CoV-2 are either asymptomatic or develop mild and self-limiting symptoms of fever, cough and shortness of breath. However, approximately 8% of COVID-19 patients go on to experience the severe complications of pneumonia, respiratory failure and acute respiratory distress syndrome (ARDS), frequently requiring admission to the intensive care unit (ICU) and mechanical ventilation (Iype and Gulati, 2020; O'Driscoll et al., 2020). Despite some clinical similarities to other severe respiratory infections causing multi-organ failure, COVID-19 presents unique clinical challenges that we do not yet know how to overcome: at present, the in-ICU mortality rate remains at approximately 50% (Armstrong et al., 2020). Thus there is an urgent need to understand how mild and severe SARS-CoV-2 infection differ from each other, and how they are distinct from other causes of severe RS.

While the factors underpinning severe COVID-19 are not yet completely understood, evidence suggests that extreme respiratory distress in these patients is primarily mediated by immunopathology (Hadjadj et al., 2020; Merad and Martin, 2020). Multiple reports observe differences in the proportions of immune cell populations in the peripheral blood of COVID-19 patients compared to healthy individuals; in particular a marked lymphopenia that is accompanied by changes to the lymphocyte activation and exhaustion phenotypes, some of which are partly associated with severity of the disease (Cao, 2020; Mathew et al., 2020; Su et al., 2020; Zheng et al., 2020). Alongside these cellular characteristics, a cytokine storm, defined by a massive increase in circulating levels of inflammatory cytokines including IL-6, GM-CSF and TNF, drives disease progression and the development of lung immunopathology (Bastard et al., 2020; Bonaventura et al., 2020; Hadjadj et al., 2020; Lucas et al., 2020; Poland et al., 2020; Del Valle et al., 2020; Zhang et al., 2020b). However, due to the lack of large well-controlled studies on the immune responses of hospitalized patients with non-COVID-19 critical pneumonias, the extent to which these immune changes are COVID-19-specific or common to other life-threatening pathogen-induced pneumonias remains unclear. Identifying those immune phenotypes and processes underlying severe COVID-19 would represent an important step forward in the rational development of new and more effective ways of treating this uniquely-challenging disease.

Here, we compared immune profiles in longitudinally collected blood samples from mild and severe COVID-19 patients, alongside a cohort of critically-ill patients suffering from pneumonia triggered by non-SARS-CoV-2 pathogens, and HCs. This enabled us to identify immune

84   signatures specific to SARS-CoV-2 and those shared with other pathogen-associated severe

85   RS. Whereas emergency myelopoiesis and adaptive immune paralysis are common features

86   of RS, signs of T cell exhaustion and reduced cytotoxicity were exclusive to COVID-19. Lastly,

87   the identification of circulating NKT frequencies as a predictive biomarker for patient outcome

88   could immediately serve for early patient stratification and decision-making.

RESULTS

Study participants, sampling protocols and experimental approach

We recruited three cohorts of participants: 57 COVID-19 patients (150 samples) from three independent centers across Germany (Tuebingen) and France (Toulouse and Nantes). COVID-19 patients were categorized into six severity grades based on the World Health Organization's (WHO) ordinal scale (World Health Organization, 2020b), that subdivides mild (severity grade 1-3, COVID-19m) and severe (severity grade 4-6, COVID-19s) disease. The second cohort included 25 patients admitted to the ICU with non-SARS-CoV-2 pneumonia (Hospital-acquired pneumonia (HAP) next to the third cohort: 21 healthy controls (HCs) (Fig. 1A). For the HAP cohort, all episodes of pneumonia were classified as severe and required invasive mechanical ventilation. Comprehensive demographic data was collected and is provided in the Figures S1A and Table S1A and S1B.

COVID-19 patients gave blood samples between days 0 and 96 after their hospital admission (Table S1C, except one patient assigned to severity grade 1), while HAP patients gave a single blood sample at 1-4 days post-diagnosis of pneumonia, and HCs also donated once. In case of COVID-19 patients, the time from infection to hospital admission is on average 6.4 days (Lauer et al., 2020; Li et al., 2020). In total, we collected 196 blood samples across all cohorts. Blood samples were processed for full blood counts and standard biochemistry at the clinical centers, with peripheral blood mononuclear cells (PBMCs) isolated and cryopreserved for later analysis (Fig. 1A, S1A).

The samples were subjected to high-parametric single cell spectral flow cytometry (Fig. 1A, S1A, Table S1A). We employed three overlapping antibody panels targeting a range of cell surface molecules including the SARS-CoV-2 receptor ACE-2 (Table S2A), and cytokines (after short-term stimulation, Tables S2B and S2C). This immunprofiling approach enabled us to assess: (1) the overall lymphocyte and myeloid composition of PBMCs; (2) the relative abundance of T cell subsets and their effector or memory status; (3) levels of B cell differentiation; (4) levels of Natural killer (NK) cell differentiation; (5) the relative abundance of monocyte and DC subsets; (6) signs of lymphocyte activation and exhaustion; (7) production of lymphocyte cytokines; and (8) production of myeloid cytokines. All samples were quality screened (for details see Material and Methods) leading to the inclusion of 167 PBMC samples across all cohorts. Using computational data integration based on 50 markers from the spectral flow cytometry together with 25 clinical measures (e.g. age, sex, Body mass index (BMI), etc.) as well as HLA typing to ultimately define the severe COVID-19-specific immune landscape (Table S1A).

Immunomonitoring reveals differing immune landscapes in COVID-19m, COVID-19s and HAP patients

To generate an overview of the circulating immune compartment in COVID-19m and COVID-19s patients, we analyzed spectral flow cytometry data using FlowSOM-based clustering (Van Gassen et al., 2015) combined with UMAP dimensionality reduction (McInnes L Saul N, Großberger L, 2018) (Fig. 1B, S1B, S1C). Comparison of PBMCs from HCs and COVID-19 patients revealed numerous frequency alterations of canonical immune subsets among CD45$^+$ cells, except CD4$^+$ lymphocytes, NK cells and monocytes which were comparable across all time points (TPs) (Fig. S1D). Compared to COVID-19m, the severe disease was characterized by significantly lower frequencies of CD8$^+$ T cells, coupled with higher frequencies of B cells (Fig. S1D).

We next combined all cytometry parameters of the surface panel (Table S2A) to deeply phenotype T cell, B cell, NK cell, DC and monocyte subsets from each cohort, assessing their differentiation and activation state as well as their exhaustion profile. Following data integration and HAP inclusion, a principal component analysis (PCA) of the resulting immune landscapes showed a clear segregation of cells from HCs compared to both COVID-19 and HAP groups, while COVID-19s patients shared signatures both with COVID-19m and HAP patients (Fig. 1C, S1E). Stratification of the COVID-19 cohort data by sex or age did not reveal marked differences in immune phenotypes (Fig. S1F, S1G).

To uncover the immunological dysregulation of COVID-19s that is distinct from the inflammatory, infectious immune signatures of HAP, we further enriched our dataset with an overall lymphoid and myeloid cytokine profile of the different subpopulations (Tables S2B and S2C). We introduced the statistical measure of the effect size (ES) to combine both significance and fold change in one single statistical value, as proposed for clinical trials before (McGough and Faraone, 2009; Sullivan and Feinn, 2012). We computed the ES of the Mann-Whitney U test between the analyzed groups (Fig. 1D). Applying the interpretation of ES by Cohen (0.1 - 0.3 small effect, >0.3 intermediate and large effect) (Cohen, 1977), we set the threshold for the comparison of mild versus severe COVID to 0.3. Due to the high number of features reaching the threshold of 0.3 in the comparison COVID-19s versus HAP, we applied a more stringent cut-off of 0.4 in order to exclusively filter COVID-19s-specific features. This revealed that mild and severe COVID-19 exhibit distinct immune signatures (represented by an ES > 0.3 and seen in the upper part of the dot plot in Fig. 1D), but in addition, COVID-19s and HAP could be distinguished by a set of immune features (displayed in the upper right square in Fig. 1D; threshold ES > 0.4 vs. HAP). Immune alterations in severe RS (COVID-19s and HAP) occurred within the T cell, NK cell, monocyte and DC compartments (Fig. 1E).

158 Taken together, COVID-19s presents immune features that are both shared and distinct from
159 other pneumonia and affect all immune compartments except for B cells.

160 Shared T cell features between severe pathogen-induced RSs highlight the emergence
161 of hyperinflammatory and exhausted subsets in COVID-19s

162 Following selection of the common immunological trajectories shared across severe RS
163 patients (COVID-19s and HAP) (Fig. 1D, upper left square), we further extracted their dynamic
164 manifestation in the COVID-19 cohort by correlation to disease severity and analysis over
165 time. The identified patterns revealed predominantly the T cell compartment (Fig. 2A, 2B, S2A,
166 S2B, Table S3). The reduction in CD4$^-$ CD8$^-$ (TCR$\gamma\delta$ enriched) T cell frequency appeared to
167 be progressive, reaching its lowest during the second week of hospitalization (TP 3 (day 6-9))
168 (Fig. 2C). Moreover, we observed significantly higher expression levels of PD-1 in COVID-19s
169 patients already during the first 5 days of hospital admission, predominantly affecting the CD4$^+$
170 T cell compartment, pointing to a potential functional deficit in T helper (Th) cell immune
171 responses (Fig. 2D). While in samples from COVID-19m patients PD-1 expression normalized
172 at TP 5 (week 4-14), it remained elevated on memory CD4$^+$ T cell subsets in COVID-19s (Fig.
173 S2C). In contrast to PD-1, the detected upregulation of the inhibitory receptor CTLA-4 on PMA
174 and ionomycin-restimulated CD4$^+$ effector memory (EM) cells occurred only at later stages of
175 disease (TP 3 and 4; day 6-15) (Fig. 2E, S2D). These findings point to a shared altered innate
176 immune response and signs of hyperinflammation and exhaustion within the T cell
177 compartment across all patients with severe RS.

178 In order to interrogate the cytokine polarization, the cells were briefly stimulated *in vitro* prior
179 to spectral flow acquisition. The resulting cytokine profile of stimulated lymphoid
180 subpopulations from COVID-19 patients showed significantly higher amounts of IL-21, as well
181 as a shift towards a cytotoxic phenotype indicated by high levels of granzyme B and perforin
182 in the T and NK cell compartments, relative to HCs (Fig. S2E, S2F, data not shown). CD107a
183 on T and NK cells was similar in COVID-19s, COVID-19m and HCs (data not shown),
184 suggesting not only equal cytotoxicity but also degranulation capacity across COVID-19
185 disease severity. The same applies for TNF, IL-4, IL-6, and IL-17A, which failed to reach the
186 cut-off of 0.3 ES when comparing mild and severe COVID-19 (Fig. 2F, S2G-J). However, we
187 found increased production of interferon-γ (IFN-γ), IL-2 and GM-CSF in COVID-19s.
188 Specifically, higher frequencies of IFN-γ-expressing CD8$^+$ EM, TEMRA (CCR7$^-$ CD45RA$^+$) and
189 TCR$\gamma\delta$ T cells (Fig. 2G) and IL-2-expressing TCR$\gamma\delta$ T cells were a common feature of severe
190 RSs shared by COVID-19 and HAP (Fig. 2H). Elevated frequencies of GM-CSF-producing
191 CD4$^+$ and CD8$^+$ TEMRA cells positively correlated with COVID-19 severity in the acute phase
192 of disease (TP 1 and 2) (Fig. 2I, S2K, S2L). Single-cell RNA-seq analysis of blood cells from

193 COVID-19 patients - in absence of *ex vivo* stimulation - revealed strong expression of *CSF2*
194 (encoding for GM-CSF) particularly in CD4$^+$ T cells (Fig. S2M). Detailed differentially
195 expressed gene (DEG) analysis of CSF2 high vs. low expressing CD4$^+$ T cells indicated these
196 cells as a hyperinflammatory subset, strongly expressing *TNF, IL21, TNFRSF4, GNLY,*
197 *CD40LG, CCL20, ICAM1* and demonstrating low *ANXA1* mRNA levels, among others (Fig.
198 S2N).

199 Overall, these data demonstrate a T cell compartment marked by both hyperinflammatory and
200 exhaustive features shared by patients with severe COVID-19 and non-SARS-CoV-2-induced
201 RSs (HAP). Over time, this phenotype persists, particularly in disease courses of COVID-19s
202 (Fig. 2J).

203 **Phenotypic alterations in innate immune signatures are shared in severe COVID-19 and**
204 **HAP**

205 We further characterized the identified DC and NK cell features shared by COVID-19s and
206 HAP (Fig. 3A, Table S3). To reveal the dynamic changes over time, we displayed the COVID-
207 19 cohorts together with HCs as baseline and HAP patients as comparison. Lower expression
208 of HLA-DR in CD56^{low} CD16$^-$ NK cells suggest a diminished cytotoxic response in COVID-19s
209 (Fig. 3B-D, S3A) (Erokhina et al., 2020). Similarly, COVID-19s displayed reduced frequency
210 of plasmacytoid DCs (pDC) (Fig. 3E-G, S3B). Although the pDC frequency was also different
211 from HAP, the cut-offs of the ES were not reached. Upregulation of the Fas receptor CD95
212 was detected in all DC subsets - particularly on pDCs - at early TPs 1 and 2 (Fig. S3C, S3D).
213 This might play a role in the loss of those cells through Fas-mediated apoptosis.

214 To mimic SARS-CoV-2 infection *in vitro*, the PBMC samples were stimulated for 8h with the
215 TLR7 and TLR8 agonist R848. In response, intermediate and non-classical monocytes as well
216 as conventional DC2s (cDC2s) upregulated expression of the chemokine receptor CCR2 (Fig.
217 S3E, S3F), but only the cDC2-related feature reached the cut-off for being COVID-19s-specific
218 (>0.3 ES vs. COVID-19m) and positively correlated with the severity grade of SARS-CoV-2-
219 mediated disease (Fig. 3H). As CCR2 expression on DCs is a hallmark of inflammation and
220 required for their migration to the inflamed lung (Kvedaraite et al., 2020; Nakano et al., 2017),
221 this could explain the invasion of DCs into the lungs of patients hospitalized with severe
222 COVID-19. The NK and DC-specific dysregulation described here were already apparent
223 during the early phase of the disease, and the vast majority of these changes persisted until
224 TP 5 in severe COVID-19 patients, yet resolved in patients with COVID-19m (Fig. 3I). In line
225 with reduced pDC frequencies, IFN-   levels in the serum of COVID-19s patients showed a
226 robust trend towards reduction, when compared to COVID-19m (Fig. S3G).

227 To summarize, patients with severe RS show signs of diminished cytotoxicity combined with
228 increased cell migration within the NK cell and DC compartment independent of the underlying
229 pathogen.

Impaired antigen-presentation distinguishes the immune response to SARS-CoV-2
versus other respiratory pathogens

232 After defining several common immunological features characterizing the immune landscape
233 of COVID-19s in common with HAP, we next extracted the features specific to SARS-CoV-2
234 infection. We selected all immune traits characterizing COVID-19s (cut-off ES vs. COVID-19m
235 > 0.3) and to further condense the signature uniquely existing in COVID-19s and being
236 different from HAP, we set a strict cut-off ES 0.4 vs. HAP (Fig. 4A, Table S3). Building on the
237 above-described common myeloid features, there were also phenotypic changes within this
238 compartment that were specific to COVID-19s. Specifically, there was significantly lower
239 expression of HLA-DR as well as the co-stimulatory ligand CD86 across antigen presenting
240 cell (APC) subsets, which persisted throughout the duration of our study and were not shared
241 to this extent by patients with mild COVID-19 disease (Fig. 4B, 4C, S4A, S4B). The protein
242 expression of both HLA-DR and CD86 negatively correlated with the severity of COVID-19,
243 with highest significance of this relationship within monocytes (Fig. 4D, 4E, S4C, S4D).
244 Although the apparent paralysis in the APC compartment fulfilled the criteria for COVID-19s-
245 specificity (Fig. 4F), this was largely driven by a more pronounced APC dysfunction in HAP,
246 when compared to HCs (Fig. 4G, H). Taken together, the emerging overall picture of a myeloid
247 compartment characterized by an impaired APC function – most likely due to emergency
248 myelopoiesis - in COVID-19s. However, the data suggest that this is a feature shared across
249 all patients with severe RS and not specific to the immune response against SARS-CoV-2.

Distinct signatures of COVID-19s are exclusive to the lymphocyte compartment

251 While most of the alterations in the monocyte and DC compartment were convergent in the
252 two severe RSs, we identified COVID-19s-specific T and NK cell signatures (Fig. 5A, Table
253 S3; ES COVID-19s vs. COVID-19m > 0.3 and vs. HAP > 0.4). A focused analysis of all T cell
254 subsets (Fig. S5A) revealed a dramatic loss of NKT cells in COVID-19s as one of those
255 signatures (Fig. 5B). This NKT cell reduction was already apparent within the first week after
256 COVID-19-related hospital admission (TP 1 and 2). As shown in the Receiver Operating
257 Characteristic (ROC) curve of Table S4, a cut-off for NKT frequency among T cells of 2.3%
258 can distinguish severe COVID-19 patients from mild disease with a sensitivity of 100% already
259 at day 0-2 after hospital admission. This finding defines NKT frequency as a powerful

260 predictive biomarker for COVID-19s evolution and furthermore suggesting a role of these cells
261 in the first phase of disease.

262 In addition to the above-described upregulation of PD-1 predominantly in CD4$^+$ T cells, higher
263 expression of PD-1 by CD4$^+$ EM cells turned out to be a feature unique to COVID-19s (ES vs
264 HAP > 0.4), which positively correlated with severity grade (Fig. 5C, S5B). Chronically
265 stimulated T cells overexpress inhibitory receptors including PD-1 and display poor effector
266 capacity (Ahmadzadeh et al., 2009; Crawford et al., 2014; Huang et al., 2019a; Pauken and
267 Wherry, 2015; Wu et al., 2014). By comparing *PDCD1* high and low expressing CD4$^+$ T cells
268 using a single-cell RNA-seq dataset (Zhao et al., 2021), we found *PDCD1* high CD4$^+$ T cells
269 to express genes associated with exhaustion (*HAVCR2, LAG3, CTLA4, TIGIT, BATF*) as well
270 as reduced amounts of *TCF7, TNF, IL2RA, TNFRSF4, FAS, MIKI67*, associated with T cell
271 activation (Fig. S5C). This dataset supports the notion that the T cell compartment in COVID-
272 19 patients is impaired or exhausted.

273 The protein expression of CD38, another activation marker, across several T cell subsets
274 positively correlated with COVID-19 severity, with the highest significance ($p < 0.0001$, R^2 =
275 0.24) in CD4$^-$ CD8$^-$ (TCR$\gamma\delta$ enriched) T cells (Fig. 5D, S5D). Furthermore, we observed a loss
276 of the regulatory protein CD161 in CD4$^-$ CD8$^-$ (TCR$\gamma\delta$ enriched) T cells in COVID-19s (Fig.
277 5E, S5E). This phenomenon is especially intriguing, as CD4$^-$ CD8$^-$ (TCR$\gamma\delta$ enriched) T cells
278 share the transcriptional signatures of CD161-expressing Mucosa-Associated Invariant T
279 (MAIT) cells, a CD8$^+$ T cell subset resembling innate-like sensors and mediators of antiviral
280 responses (Fergusson et al., 2014, 2016).

281 Although failing to reach the stringent cut-off for being a unique COVID-19s specific feature
282 (ES vs HAP > 0.4), CD161 was also expressed at a significantly lower level on immature and
283 CD56^{low} CD16$^+$ NK cells in the early phase of severe SARS-CoV-2 related illness compared
284 to mild disease (Fig. S5F). Here, the kinetics of CD161 expression was low at the beginning
285 of disease with a delayed hyperreactivity in COVID-19s (Fig. S5F). Further dissecting the NK
286 cell compartment, CD95 expression in the CD56^{high} NK subset positively correlated with
287 severity of COVID-19 and represents a unique characteristic specific to SARS-CoV-2 infection
288 (Fig. 5F, S5G). As in the DC compartment described before, the significant and specific
289 reduction of this NK subset supports the Fas-mediated, activation-induced apoptosis as the
290 mechanism underlying the shift from effector to immature NK cells (Fig. S5H).

291 Regarding the cytokine polarization profile, a reduced production of IFN-γ in CD4$^+$ central
292 memory (CM) T cells was found to be COVID-19s specific and reflects the loss of CD4$^+$
293 CXCR3$^+$ CCR6$^+$ (Th1 Th17-enriched) T cells (Fig. 5G, 5H). All features falling in the COVID-
294 19s specific category and diverging from HAP recovered only partly, both in mild and severe

295   SARS-CoV-2 infected patients, pointing to a persisting dysfunctional T and NK cell
296   compartment (Fig. 5I).

297   The recorded myeloid features in COVID-19s were even more pronounced in the HAP patients
298   (Fig. 4G, 4H). In contrast, several identified COVID-19s specific T and NK cell features were
299   clearly different from what has been observed in HC and HAP (Fig. 5B, 5E, 5H, 5J). Taken
300   together, whereas changes in the myeloid compartment are shared across severe RS
301   patients, our differential display approach extracted signatures of T cell exhaustion and altered
302   early antiviral innate lymphoid response specific to the immune response to SARS-CoV-2.

303   **HLA profile links COVID-19 immunopathology to impaired virus recognition**

304   After defining the pathological immune landscape specific for SARS-CoV-2 and distinct from
305   other pathogen-induced pneumonias, we next explored the degree of correlation existing
306   across these and other COVID-19s-associated immune features in order to depict the overall
307   immune network underlying COVID-19s. We therefore selected all signatures associated with
308   COVID-19s (ES vs. COVID-19m > 0.3) from TP 1 and 2 and, for each feature, computed
309   Pearson's r correlation values visualized in a heatmap plot for each COVID-19s and HAP (Fig.
310   6A). Given the power of this multi-dimensional, global analysis tool, it was possible to identify
311   correlation patterns within the immune network of the two investigated conditions, namely
312   COVID-19s and HAP. Focusing on the interactive network underlying early and severe SARS-
313   CoV-2 mediated disease, we discovered distinctive associations between different branches
314   of adaptive and innate immunity, translating into correlation clusters between myeloid and T
315   cells (#1), myeloid and NK cells (#2) as well as T and NK cells (#3) (Fig. 6A). These
316   associations were weak in HAP, further supporting our claim of a SARS-CoV-2 specific
317   immune landscape that characterizes severe disease courses.

318   Because of earlier evidence of SARS-CoV-2 peptide binding to Human Leukocyte Antigen
319   (HLA) molecules differs across genotypes (Nguyen et al., 2020), we introduced next
320   generation sequencing (NGS)-based HLA class I typing of 48 patients of our COVID-19 cohort.
321   We calculated the predicted number of tightly binding (<50nm) SARS-CoV-2-derived peptides
322   per HLA class I gene (based on every single underlying allele genotype) for each of our typed
323   individuals (Fig. S6A, S6B, Table S5). This predicted binding capacity for HLA-A, HLA-B and
324   HLA-C was further called HLA score 50. Next, we integrated this dataset to our single-cell
325   immune profiling analysis and correlated this HLA-A, HLA-B and HLA-C score 50 to all our
326   extracted severe COVID-19-associated immune features (Fig. 6B). This multi-omics approach
327   allowed us to show that the majority of the severe COVID-19-associated immune features of
328   the innate immune system (e.g. NKT frequency, HLA-DR in monocytes and DCs, etc.) was
329   correlating with the SARS-CoV-2 binding strength (Fig. 6B). Meaning, that efficient HLA

330   binding capacity to SARS-CoV-2 peptides may mitigate the alterations of the innate immune
331   system detected in COVID-19s. Also, the COVID-19s associated GM-CSF production in CD8$^+$
332   CM T cells positively correlated with high HLA scores. To conclude, the data suggests that
333   weak HLA binding to SARS-CoV-2 peptides may at least in part drive the immunopathology
334   in COVID-19.

335   To translate the complex immune signatures into clinical use, we correlated the COVID-19s-
336   defining immune signatures with routine clinical parameters. In order to identify stratifying
337   biomarkers in the very early phase of disease, we included features significantly associated
338   with COVID-19s at TP 1 only. As every COVID-19 patient was graded according to the
339   maximum severity of disease during the longitudinal follow-up of the study and this grading
340   was allocated to every sample of the same patient, the included features of TP 1 fulfil the
341   criteria to be predictive. Several blood values and BMI (indicated by an arrow) were highly
342   correlated with our COVID-19s-defining immune signatures, thereby translating these
343   immunological findings into clinical routine parameters (Fig. 6C). To further validate these
344   promising candidates for outcome prediction, we linearly correlated them with COVID-19
345   severity grade (Fig. 6D, S6C). Although the number of provided values was limited and several
346   associations turned out to be significant but with a low R squared value, LDH and granulocyte
347   counts showed a strong correlation with worsening of COVID-19, thus presenting easily
348   applicable biomarkers (Fig. 6D, S6D).

349   In conclusion, we provide a translational path forward based on our differential immune map
350   specific for severe SARS-CoV-2 infection combined with predicted HLA class I binding
351   capacity to SARS-CoV-2 peptides, which can be used to guide therapeutic approaches aimed
352   at interrupting the immunopathologic cascade of severe COVID-19.

353   **ACE2 expression in a CD4$^+$ T cell subset increases after *ex vivo* stimulation**

354   SARS-CoV-2 employs the angiotensin-converting enzyme 2 (ACE2) as its receptor for cellular
355   entry (Prompetchara et al., 2020; Zhou et al., 2020). To determine potential entry sites within
356   T cells we measured ACE2 expression across our immune map. We did not identify ACE2
357   expression in steady state healthy T cell subpopulations, whereas samples from severe RS
358   showed marginal expression, especially in the CD4$^+$ CXCR3$^+$ CCR6$^+$ (Th1 Th17-enriched)
359   subset (Fig. 7A, 7B, 7C, S7A, S7B) which was significantly reduced in COVID-19s (Fig. 7D).
360   When we profiled the stimulated PBMCs mimicking the COVID-19 inflammatory environment,
361   we discovered a CD4$^+$ T cell subpopulation, of which approximately 75-80% expressed ACE2
362   (Fig. 7E, 7F). This population emerged from samples of both healthy and COVID-19 patients
363   and expressed CD25, PD-1 and CTLA4 (Fig. 7G, 7H, S7C). Further analysis of this subset
364   demonstrated no relevant overlap with a specific cytokine polarization profile or FOXP3

365 expression (Fig. S7D, S7E). The presence of ACE2 expression on an activated CD4$^+$ T cell

366 subset may provide a mechanism for virus entry and contribution to the immunopathological

367 network of COVID-19.

368 **DISCUSSION**

369 The comparison of two cohorts of severe infectious RSs (COVID-19s and HAP) driven by
370 different pathogens allowed us to uncover unique immune signatures in SARS-CoV-2
371 mediated disease. Recent data describes the immunopathogenesis of HAP as critical illness-
372 related immuno-suppression (Roquilly et al., 2019) mainly characterized by alterations in the
373 IL-12 - IFN-γ axis (Roquilly et al., 2017). Conversely, the COVID-19 immune response includes
374 traits also occurring in other severe RS triggered by other pathogens such as influenza (Lee
375 et al., 2020; Tian et al., 2020). However, mainly due to the small cohort sizes and lack of a
376 comparable control group of patients suffering from non-SARS-CoV-2 driven severe RS, the
377 COVID-19-specific immune signature remains elusive. Within our dataset already, a global
378 PCA analysis of all immunophenotypes allowed for a clear separation between COVID-19s,
379 COVID-19m, HAP and HCs. There was however a partial overlap between COVID-19s and
380 HAP, revealing some core immune features associated with severe RS independent from the
381 disease etiology.

382 Whilst previous studies described an impairment in the monocyte and DC compartment to be
383 decisive for a severe COVID-19 course (Arunachalam et al., 2020; Kuri-Cervantes et al., 2020;
384 Merad and Martin, 2020; Silvin et al., 2020), features which we confirmed here, those were
385 found to not be exclusive to SARS-CoV-2-immunopathology. We confirmed loss of HLA-DR
386 and CD86 expression in APCs, a finding associated with emergency myelopoiesis, where
387 newly emerging myeloid cells show reduced APC capacity (Schulte-Schrepping et al., 2020).
388 Recent data shows the secretion of CCL2 by airway macrophages and a concomitant
389 upregulation of the CCL2-receptor CCR2 in peripheral blood monocytes of SARS-CoV-2
390 infected patients; thus, extensive accumulation of monocytes and macrophages within
391 alveolar spaces in COVID-19 lung autopsies suggests recruitment from circulation (Szabo et
392 al., 2020). Our data support this, but in addition we observed that cDC2s also upregulated
393 CCR2 expression with an even greater ES than monocytes. There is evidence for CCR2 being
394 required for DC migration to the inflamed lung, respectively, while this is not the case in the
395 steady state condition (Nakano et al., 2015, 2017). Thus, the declining number of cDC2s in
396 the systemic circulation of COVID-19 patients may be a reflection of cDC2 extravasation into
397 the affected lungs.

398 Alongside signatures shared in severe RS, we also extracted those specific to and unique in
399 COVID-19s. These SARS-CoV-2-induced adaptations were restricted to the T and NK cell
400 compartment. Several studies described an upregulation of PD-1 and CD38 alongside other
401 activation and exhaustion markers, suggesting a hyperactivated and exhausted T cell
402 compartment (De Biasi et al., 2020; Chen and Wherry, 2020). However, again, it was unclear
403 as to whether this emerging pattern in lymphocytes is the result of severe RS in general, or is

specific to the immunopathology induced by SARS-CoV-2. We here describe an overall picture of T cell exhaustion and altered early antiviral innate lymphoid response unique to COVID-19s.

SARS-CoV-2 entry into the host cells is initiated by binding of the virus to the cell surface transmembrane receptor ACE2, which is predominantly expressed in epithelial cells of the lung, intestine and endothelial cells (Varga et al., 2020). Our analysis revealed the ability of highly activated CD4$^+$ T cells to express ACE2. Others also detected ACE2 positive lymphocytes in lungs COVID-19 patients (Ackermann et al., 2020; chen et al., 2020). Moreover, CD4$^+$ Th cell infection by SARS-CoV-2 occurs in an ACE2 dependent manner (Pierce et al., 2020; Pontelli et al., 2020). Compared to SARS-CoV, SARS-CoV-2 has a 10 - 20-fold higher affinity for host membrane ACE2 (Wrapp et al., 2020). Thus, even low ACE2 expression may be sufficient for viral entry. A direct infection of responding lymphocytes, leading to cell death and impaired SARS-CoV-2 clearance, goes in line with higher peripheral blood viral load positively correlating with COVID-19 severity (Han et al., 2020). In conclusion, the ability for SARS-CoV-2 to directly infect T cells provides yet another potential mechanism to describe the immunopathology of COVID-19.

The cytokine storm in COVID-19 is pronounced as one of the driving immunopathological features in SARS-CoV-2 mediated disease worsening (Merad and Martin, 2020; Moore and June, 2020; Del Valle et al., 2020). Our single-cell profiling of 11 cytokines did not result in higher frequencies of IL-6 and TNF (Del Valle et al., 2020), for which high plasma levels were described in COVID-19 patients, indicating neutrophils, monocytes and endothelial cells at the site of infection likely account for the dysregulated cytokine production. Nevertheless, we identified the cellular sources for GM-CSF as predominantly the CD4$^+$ and CD8$^+$ TEMRA subset - a feature of severe COVID-19 sharing with HAP - and documented correlation of the expression with COVID-19 severity. Our data complement two recent reports, which show that in particular lung invading T cells express GM-CSF (by using scRNA-seq) (Zhao et al., 2021) and that GM-CSF serum levels are elevated in COVID-19 patients (Thwaites et al., 2021). Supportive, elevated circulating GM-CSF$^+$ CD4$^+$ T cell levels are predictive of poor outcomes in sepsis patients (Huang et al., 2019b). Collectively, this suggests GM-CSF to be an early driver of the underlying immunopathological cascade in COVID-19s, thereby being a promising therapeutic target (NRI, GEM TRIAL, Clinical trial identifiers NCT04400929 and NCT04411680, (Bonaventura et al., 2020; Bosteels et al., 2020; Lang et al., 2020; De Luca et al., 2020)).

Using NGS-based HLA-typing and further integration of this dataset into our single-cell immune profiling analysis, this multi-omics approach provides deep insights into the COVID-19 immunopathology and a potential genetic influence: while COVID-19s-associated innate

440   immune alterations were less pronounced in patients with predicted high HLA class I binding
441   capacity to SARS-CoV-2 peptides, GM-CSF production in CD8$^+$ CM T cells – a feature
442   associated with severe COVID-19 disease - was increased. The occurrence of both mild and
443   severe COVID-19-associated immune features in patients with strong SARS-CoV-2
444   recognition (high HLA score 50) could further explain the inconsistent reports which attempt
445   to link HLA class I binding capacity to SARS-CoV-2 peptides to COVID-19 severity (Ellinghaus
446   et al., 2020; Iturrieta-Zuazo et al., 2020). By combining the single-cell immune mapping with
447   HLA genetics, we uncovered a link between the HLA profile and impaired virus recognition in
448   COVID-19.

449   Due to emerging follow-up studies, an increased number of COVID-19 patients are described
450   to experience prolonged symptomatology. This phenomenon, referred to as "long COVID"
451   affects around 10% of the cases. An attributed reason for long-lasting complaints is persistent
452   tissue damage in severe cases. Nevertheless, patients following mild SARS-CoV-2 infections
453   also suffer from prolonged symptoms (Iadecola et al., 2020; Mahase, 2020). We identified
454   several immune features, predominantly of the T and NK compartment, which did not rebound
455   at the end of our study, several weeks after infection. Thus, prolonged immune dysregulation,
456   long after primary pathogen encounter, could play a role in "long COVID".

457   An additional aspect of our study was to identify predictive biomarkers of severe COVID-19
458   patient outcomes. An earlier study identified the frequency of circulating MAIT cells to have
459   predictive value (Flament et al., 2021). Here we identified a dramatic, early loss of NKT cells
460   in the circulating immune compartment of COVID-19s. While others confirmed this observation
461   (Zhang et al., 2020a; Zingaropoli et al., 2020), here we found this phenomenon indeed to not
462   be shared across severe RS patients but being specific to the SARS-CoV-2 immune response.
463   NKT cells are important for the production of an early wave of IL-4 promoting germinal center
464   (GC) formation during viral infection. Delay in GC formation in COVID-19 patients may be a
465   direct consequence of NKT cell migration to the airways (Dempsey, 2018; Fontana and
466   Pepper, 2018; Jouan et al., 2020; Kaneko et al., 2020). Translation of this finding into clinical
467   routine diagnostics can easily be implemented using CD3 and CD56 to calculate NKT cell
468   frequencies upon hospital admission. Across our three independent COVID-19 cohorts, a cut-
469   off set to 2.3% for NKT cell frequencies (among T cells) would have identified all patients who
470   later developed severe disease. Early identification of patients at risk could help to tailor their
471   treatment and improve the outcome.

472   **LIMITATIONS OF THE STUDY**

473   While we initially anticipated center-specific batch effects in our multi-center study, this was
474   not the case. However, our HAP cohort consists of patients suffering from severe pneumonia

475 driven by multiple pathogens, both bacterial and viral. The comparison of COVID-19 patients

476 to a pure viral pneumonia cohort could help to further specify the unique immune signatures

477 to SARS-CoV-2 and distinctive to other viruses. Using PBMCs as source of analyzed immune

478 cells allows for easy implementation of our findings (such as NKT frequency as predictive

479 biomarker) to the clinics. Even though the simple measurement of circulating NKT cell

480 frequencies would have predicted all of our COVID-19 patients who developed severe

481 disease, larger follow-up studies are needed to solidify this measurement as a predictive

482 biomarker for COVID-19 patient outcomes.

504 **AUTHOR CONTRIBUTIONS**

505 Conceptualization, B.B., S.Kreutmair, S.U., N.G.N., C.A., D.D.F. and A.R.; Methodology,
506 S.Kreutmair, S.U., N.G.N. and F.I.; Formal Analysis, S.Kreutmair, S.U., N.G.N., F.I., M.K.,
507 S.B.; Investigation, S.Kreutmair, S.U., N.G.N., F.I., C.A., D.D.F.; Resources, B.B., A.R., M.B.,
508 G.M.-B., R.L., M.C.; Sample and patient data collection and processing, S.Kreutmair, B.G.,
509 I.A., S.A.-H., J.N., M.B., G.M.-B., M.L., N.P.J., M.K., N.P.M., S.G., P.R., H.A.H.; Data Curation,
510 S.Kreutmair, S.U., N.G.N., F.I., C.A., D.D.F., E.F.; Writing – Original Draft, S.Kreutmair,
511 S.Krishnarajah and B.B.; Writing – Review & Editing, S.Kreutmair, S.U., N.G.N., F.I., C.A.,
512 D.D.F., S.Krishnarajah, M.K., E.F., S.B., B.G., M.L., N.P.J., N.P.M., S.G., P.R., H.A.H., I.A.,
513 S.A.-H., J.N., M.C., R.L., G.M.-B., M.B., A.R. and B.B.; Visualization, S.Kreutmair, N.G.N., F.I.
514 and M.K.; Funding Acquisition and Supervision, M.C., R.L., G.M.-B., M.B., A.R., and B.B.

FIGURE LEGENDS

516 **Figure 1: Immunomonitoring reveals differing immune landscapes in COVID-19m,**

517 **COVID-19s and HAP patients**

518 **A:** Schematic of experimental approach.

519 **B:** UMAP with FlowSOM overlay showing total CD45^{pos} cells of combined samples. 1000 cells

520 were subsetted from every sample from each cohort.

521 **C:** PCA of the total immune compartment based on marker expression in the surface panel.

522 **D:** Comparison of immune features derived from each leukocyte subpopulation between

523 experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis;

524 threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold

525 0.3). Each dot represents one immunological feature, colors represent the leukocyte

526 compartment they refer to.

527 **E:** Proportion of each immune compartment (normalized to input) in the identified sets of

528 immune features highlighted in Fig. 1D.

529 See also Figure S1.

530 **Figure 2: Shared T cell features between severe pathogen-induced RSs highlight the**

531 **emergence of hyperinflammatory and exhausted subsets in COVID-19s**

532 **A:** Comparison of immune features derived from each leukocyte subpopulation between

533 experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis;

534 threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold

535 0.3). Each dot represents one immunological feature. The red box highlights immune features,

536 which are associated with severe RS (COVID-19s and HAP), with a focus on changes within

537 the T cell fraction.

538 **B:** UMAP with FlowSOM overlay of total T cells of combined samples. 1000 cells were

539 subsetted from every sample from each cohort. T cell subsets with transparent names do not

540 contain immune features highlighted in Fig. 2A.

541 **C:** Median frequencies and 25th and 75th percentile of FlowSOM-generated CD4$^-$ CD8$^-$

542 (TCR$\gamma\delta$ enriched) immune cell cluster.

543 **D:** Median expression and 25th and 75th percentile of PD-1 in FlowSOM-generated immune

544 cell clusters shown in B.

545 **E:** Median expression of CTLA-4 within CD4$^+$ EM T cell subset of HCs shown in grey, of HAP

546 in blue and of mild and severe COVID-19 patients across TPs 1-5 shown in red.

547 **F:** Schematic overview of cytokine polarization profile comparing COVID-19s and COVID-

548 19m. UMAP with FlowSOM overlay shows cytokine-producing T cell subpopulations (features

549 reaching an ES > 0.3). 1000 T cells were subsetted from every sample from each cohort.

550 **G:** Median frequency and 25th and 75th percentile of IFN-γ positive cells in FlowSOM-

551 generated immune cell clusters shown in F.

552 **H:** Median frequency and 25th and 75th percentile of IL-2 positive cells in FlowSOM-generated

553 immune cell cluster shown in F.

554 **I:** Correlation between frequency of GM-CSF expressing CD4$^+$ (left panel) and CD8$^+$ (right

555 panel) TEMRA cells and the severity grade of COVID-19 patients in combined TPs 1 and 2.

556 **J:** Heatmap depicting the z-score of each T cell related immune feature (highlighted in Fig.

557 2A) when compared to HCs for every TP. Both negative and positive changes are visualized

558 by intensity of red color scale. MFI = Mean fluorescence intensity.

559 Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

560 and **** = $p < 0.0001$, Mann-Whitney test, BH correction). See also Figure S2.

561 **Figure 3: Phenotypic alterations in innate immune signatures are shared in severe**

562 **COVID-19 and HAP**

563 **A:** Comparison of immune features derived from each leukocyte subpopulation between

564 experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis;

565 threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold

566 0.3). Each dot represents one immunological feature. The red box highlights immune features,

567 which are associated with severe RS, with a focus on changes within the monocyte, DC and

568 NK cell fraction.

569 **B:** UMAP with FlowSOM overlay of total NK cells of combined samples. 1000 cells were

570 subsetted from every sample from each cohort. NK cell subsets with transparent names do

571 not contain immune features highlighted in Fig. 3A.

572 **C:** Median expression of various markers in FlowSOM-derived clusters shown in **B**.

573 **D:** Median expression and 25th and 75th percentile of HLA-DR in FlowSOM-generated

574 CD56^{low} CD16$^-$ NK cell cluster shown in **B**, combined for TP 1 and 2 (left panel) or displayed

575 for every individual TP (right panel).

576 **E:** UMAP with FlowSOM overlay of total monocytes and DCs of combined samples. 1000 cells
577 were subsetted from every sample from each cohort. Monocyte and DC subsets with
578 transparent names do not contain immune features highlighted in Fig. 3A.

579 **F:** Median expression of various markers in FlowSOM-derived clusters shown in **E**.

580 **G:** Median frequencies and 25th and 75th percentile of FlowSOM-generated pDC immune cell
581 cluster.

582 **H:** Correlation between median expression of CCR2 in cDC2s following TLR7 and TLR8
583 stimulation against the severity grade of COVID-19 patients. All TPs have been pooled in the
584 left panel, and individual TPs depicted in the right panel.

585 **I:** Heatmap depicting the z-score of each monocyte and DC related immune feature
586 (highlighted in Fig. 3A) when compared to HCs for every TP. Both negative and positive
587 changes are visualized by intensity of red color scale. MFI = Mean fluorescence intensity.

588 Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$
589 and **** = $p < 0.0001$, Mann-Whitney test, BH correction). See also Figure S3.

590 **Figure 4: Impaired antigen-presentation distinguishes the immune response to SARS-**
591 **CoV-2 versus other respiratory pathogens**

592 **A:** Comparison of immune features derived from each leukocyte subpopulation between
593 experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis;
594 threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold
595 0.3). Each dot represents one immunological feature. The red box highlights immune features,
596 which are different in COVID-19s and HAP, with a focus on changes within monocytes and
597 DCs.

598 Median expression of HLA-DR (**B**) or CD86 (**C**) within classical monocytes of HCs shown in
599 grey, HAP patients in blue, and COVID-19m and COVID-19s patients across TPs 1-5 shown
600 in red.

601 Correlation between median expression of HLA-DR (**D**) or CD86 (**E**) in monocytes or DCs (TP
602 1 and 2 pooled) against the severity grade of COVID-19 patients.

603 **F:** Heatmap depicting the z-score of each monocyte and DC related immune feature
604 (highlighted in Fig. 4A) when compared to HCs for every TP. Both negative and positive
605 changes are visualized by intensity of red color scale.

606 Median expression and the 25th and 75th percentile of HLA-DR (**G**) or CD86 (**H**) in FlowSOM-
607 generated monocyte and DC immune cell clusters.

608   Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$
609   and **** = $p < 0.0001$, Mann-Whitney test, BH correction). See also Figure S4.

610   **Figure 5: Distinct signatures of COVID-19s are exclusive to the lymphocyte**
611   **compartment**

612   **A**: Comparison of immune features derived from each leukocyte subpopulation between
613   experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis;
614   threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold
615   0.3). Each dot represents one immunological feature. The red box highlights immune features,
616   which are different in COVID-19s and HAP, with a focus on changes within T and NK cells.

617   **B**: Median frequencies and 25th and 75th percentile of FlowSOM-generated NKT immune cell
618   cluster.

619   **C**: Correlation between median expression of PD-1 in $CD4^+$ EM cells (TP 1 and 2 pooled)
620   against the severity grade of COVID-19 patients.

621   **D**: Correlation between median expression of CD38 in $CD4^-$ $CD8^-$ (TCR$\gamma\delta$ enriched) and $CD4^+$
622   EM T cells (TP 1 and 2 pooled) against the severity grade of COVID-19 patients.

623   **E**: Median expression and 25th and 75th percentile of CD161 in FlowSOM-generated $CD4^-$
624    $CD8^-$ (TCR$\gamma\delta$ enriched) immune cell cluster.

625   **F**: Correlation between median expression of CD95 in $CD56^{high}$ NK cells (TP 1 and 2 pooled)
626   against the severity grade of COVID-19 patients.

627   **G**: Schematic overview of cytokine polarization profile comparing COVID-19s and COVID-
628   19m. UMAP with FlowSOM overlay shows cytokine-producing T cells (features reaching an
629   ES > 0.3 vs COVID-19m and > 0.4 vs HAP). 1000 T cells were subsetted from every sample
630   from each cohort.

631   **H**: Median frequency and 25th and 75th percentile of IFN-γ positive cells in FlowSOM-
632   generated immune cell clusters shown in G.

633   **I**: Heatmap depicting the z-score of each T and NK cell related immune feature (highlighted in
634   Fig. 5A) when compared to HCs for every TP. Both negative and positive changes are
635   visualized by intensity of red color scale. MFI = Mean Fluorescence Intensity.

636   **J**: Median frequencies or expression of indicated populations and markers. Boxplots show the
637   25th and 75th percentile.

638 Significant p values are depicted using an asterisk (* = p < 0.05, ** = p < 0.01, *** = p < 0.001
639 and **** = p < 0.0001, Mann-Whitney test, BH correction). See also Figure S5.

Figure 6: HLA profile links COVID-19 immunopathology to impaired virus recognition

641 **A**: Correlogram of all immune features (TP 1 and 2) with ES COVID-19s vs. COVID-19m >
642 0.3, shown for COVID-19s and HAP. Red arrows highlight immune features unique in COVID-
643 19s (ES vs. HAP > 0.4). The black boxes #1-3 highlight highly correlating immune clusters.

644 **B**: Correlogram of immune features from TP 1 only with ES COVID-19s vs. COVID-19m > 0.3
645 with HLA score 50. HLA score 50 represents the number of predicted tightly binding SARS-
646 CoV-2 peptides of both HLA alleles of a patient. Red arrows highlight SARS-CoV-2-specific
647 immune features (ES COVID-19s vs. HAP > 0.4).

648 **C**: Correlogram of immune features from TP 1 only with ES COVID-19s vs. COVID-19m > 0.3
649 with routinely assessed clinical parameters. Red arrows highlight highly correlating
650 parameters.

651 **D**: Correlation between LDH and granulocyte counts (TP 1 only) against the severity grade of
652 COVID-19 patients.

653 See also Figure S6.

Figure 7: ACE2 expression in a CD4+ T cell subset increases after *ex vivo* stimulation

655 **A**: Comparison of immune features derived from each leukocyte subpopulation between
656 experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis)
657 compared to the ES calculated in COVID-19m vs. COVID-19s (y axis). Each dot represents
658 one immunological feature. The red box highlights the immune feature focused in this figure.

659 **B**: Median expression of indicated markers in FlowSOM-derived clusters of unstimulated
660 samples.

661 **C**: Median frequency and 25th and 75th percentile of ACE2 positive cells in a subset of
662 unstimulated CXCR3$^+$ CCR6$^+$ (Th1 Th17-enriched) CD4$^+$ T cells. All TPs have been pooled.

663 **D**: Median frequency and 25th and 75th percentile of CXCR3$^+$ CCR6$^+$ (Th1 Th17-enriched)
664 CD4$^+$ T cells at each TP.

665 **E**: Representative plot showing ACE2 and isotype staining within the T cell compartment of
666 PMA and ionomycin restimulated (5h) COVID-19 samples.

667 **F**: Median frequency and 25th and 75th percentile of ACE2 positive cells in FlowSOM-
668 generated immune cell clusters after PMA and ionomycin restimulation (5h). All TPs have
669 been pooled.

670 **G**: Median expression of various markers in FlowSOM-derived clusters of PMA and ionomycin
671 restimulated (5h) samples.

672 **H**: Median expression and 25th and 75th percentile of PD-1 (left panel) and CTLA-4 (right
673 panel) in FlowSOM-generated immune cell clusters after PMA and ionomycin restimulation
674 (5h). All TPs have been pooled.

675 Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$
676 and **** = $p < 0.0001$, Mann-Whitney test, BH correction). See also Figure S7.

677 **STAR METHODS**

678 **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-human ACE2 (Biotin) (AC18F)	Adipogen Life sciences	Cat# AG-20A-0032B-C050; RRID: N/A
anti-human CCR2 (K036C2), BV605	BioLegend	Cat# 357213; RRID:AB_2562702
anti-human CCR6 (G034E3), BV711	BioLegend	Cat# 353435; RRID:AB_2629607
anti-human CCR7 (CD197) (G043H7), BV785	BioLegend	Cat# 353229; RRID:AB_2561371
anti-human CD11c (B-ly6), BUV661	BD	Cat# 612968; RRID:AB_2870241
anti-human CD123 (IL-3R) (6H6), APC/Fire 750	BioLegend	Cat# 306041; RRID:AB_2750163
anti-human CD123 (IL-3R) (6H6), BV711	BioLegend	Cat# 306029; RRID:AB_2566353
anti-human CD14 (M5E2), BUV737	BD	Cat# 612763; RRID:AB_2870094
anti-human CD14 (TüK4), Qdot800	Thermo	Cat# Q10064; RRID:AB_2556449
anti-human CD141 (1A4), BB700	BD	Cat# 742245; RRID:AB_2740668
anti-human CD152 (CTLA-4) (BNI3), BB790-P	BD	customized
anti-human CD16 (3G8), BUV496	BD	Cat# 612944; RRID:AB_2870224
anti-human CD161 (HP-3G10), eFluor 450	Thermo	Cat# 48-1619-41; RRID:AB_10854575
anti-human CD19 (HIB19), APC-Cy7	BioLegend	Cat# 302218; RRID:AB_314248
anti-human CD19 (SJ25C1), PE-Cy5.5	Thermo	Cat# 35-0198-42; RRID: AB_11218903
anti-human CD194 (CCR4) (1G1), BUV615	BD	Cat# 613000; RRID:AB_2870269
anti-human CD1c (F10/21A3), BB660-P2	BD	customized
anti-human CD25 (IL-2Ra) (M-A251), PE-Cy7	BioLegend	Cat# 356107; RRID:AB_2561974
anti-human CD27 (M-T271), BUV563	BD	Cat# 741366; RRID:AB_2870866
anti-human CD279 (PD-1) (EH12.2H7), BV421	BioLegend	Cat# 329919; RRID:AB_10900818
anti-human CD279 (PD-1) (EH12.2H7), BV605	BioLegend	Cat# 329924; RRID:AB_2563212
anti-human CD28 (CD28.2), BV605	BioLegend	Cat# 302967; RRID:AB_2800754
anti-human CD3 (HIT3a), APC-Cy7	BioLegend	Cat# 300318; RRID:AB_314054
anti-human CD3 (Oct.03), BV510	BioLegend	Cat# 317332; RRID:AB_2561943
anti-human CD3 (UCHT1), BUV805	BD	Cat# 565515; RRID:AB_2739277
anti-human CD33 (WM53), BUV395	BD	Cat# 740293; RRID:AB_2740032
anti-human CD38 (HIT2), APC-Cy5.5	Thermo	Cat# MHCD3819; RRID:AB_1472718

anti-human CD4 (SK3), Spark Blue 550	BioLegend	Cat# 344656; RRID:AB_2819979
anti-human CD45 (2D1), PerCP	BioLegend	Cat# 368506; RRID:AB_2566358
anti-human CD45 (HI-30), BUV805	BD	Cat# 564915; RRID:AB_2744401
anti-human CD45RA (HI100), BUV395	BD	Cat# 740298; RRID:AB_2740037
anti-human CD56 (HCD56), APC-Cy7	BioLegend	Cat# 318332; RRID:AB_10896424
anti-human CD56 (NCAM16.2), BUV737	BD	Cat# 612766; RRID:AB_2813880
anti-human CD57 (HNK-1), FITC	BioLegend	Cat# 359603; RRID:AB_2562386
anti-human CD8 (3B5), Ax Fluor 700	Thermo	Cat# MHCD0829; RRID:AB_10372957
anti-human CD86 (2331 (FUN-1)), BUV805	BD	Cat# 742032; RRID:AB_2871328
anti-human CD95 (FasR) (DX2), PE/Cy5	Thermo	Cat# 15-0959-42; RRID:AB_11042290
anti-human CXCR3 (G025H7), BV650	BioLegend	Cat# 353729; RRID:AB_2562628
anti-human CXCR5 (CD185) (RF8B2), BV750	BD	Cat# 747111; RRID:AB_2871862
anti-human GM-CSF (BVD2-21C11), PE	BD	Cat# 554507; RRID:AB_395440
anti-human Granzyme B (GB11), FITC	BioLegend	Cat# 515403; RRID:AB_2114575
anti-human HLA-DR (L243), BV570	BioLegend	Cat# 307637; RRID:AB_10895753
anti-human IFN-γ (B27),V450	BD	Cat# 560371; RRID:AB_1645594
anti-human IgD (IA6-2), BV480	BD	Cat# 566138; RRID:AB_2739536
anti-human IgG (polyclonal), Ax Fluor 647	Jackson immuno research	Cat# 109-606-098; RRID:AB_2337899
anti-human IgM (MHM-88), PE/Dazzle594	BioLegend	Cat# 314529; RRID:AB_2566482
anti-human IL-17A (BL168), APC-Cy7	BioLegend	Cat# 512320; RRID:AB_10613103
anti-human IL-1β (H1b-98), Pacific Blue	BioLegend	Cat# 511710; RRID:AB_2124350
anti-human IL-2 (MQ1-17H12), BV711	BioLegend	Cat# 500345; RRID:AB_2616638
anti-human IL-21 (3A3-N2.1), Ax Fluor 647	BD	Cat# 562043; RRID:AB_10896655
anti-human IL-4 (8D4-8), APC	BioLegend	Cat# 500714; RRID:AB_1877159
anti-human IL-6 (MQ2-13A5), PE/Dazzle594	BioLegend	Cat# 501122; RRID:AB_2810622
anti-human IL-8 (E8N1), PE-Cy7	BioLegend	Cat# 511415; RRID:AB_2565290
anti-human TCRγδ (IMMU510), Pe-Cy5	Beckman Coulter	Cat# IM2662U; RRID: N/A
anti-human TNF (MAb11), BV750	BD	Cat# 566359; RRID:AB_2739709
Streptavidin, BB630-P2	BD	customized
Biological samples		

COVID-19 PBMC samples	University Hospital Tuebingen, Germany	N/A
COVID-19 PBMC samples	Toulouse University Hospital, France	N/A
COVID-19 PBMC samples	Nantes University Hospital, France	N/A
HAP PBMC samples	Nantes University Hospital, France	N/A
Healthy PBMC samples	Nantes University Hospital, France	N/A
Chemicals, peptides, and recombinant proteins		
RPMI 1640	Seraglob	Cat# M3413; RRID: N/A
Phosphate-buffered saline	Homemade	N/A
R848	Invivogen	Cat# tlrl-r848; RRID: N/A
Human TruStain FcX	BioLegend	Cat# 422302; RRID:AB_2818986
Formaldehyde 4.0%	PanReac	Cat# 252931.1211; RRID: N/A
Benzonase nuclease	Sigma-Aldrich	Cat# E1014-25KU; RRID: N/A
Fetal bovine serum	Gibco	Cat# A3160802; RRID: N/A
Penicillin Streptomycin	Gibco	Cat# 15140-148; RRID: N/A
GlutaMAX	Gibco	Cat# 35050-038; RRID: N/A
Phorbol 12-myristate 13-acetate	Sigma-Aldrich	Cat# P1585-1MG; RRID: N/A
Ionomycin	Sigma-Aldrich	Cat# I0634-1MG; RRID: N/A
1x Brefeldin A	BD	Cat# 555029; RRID:AB_2869014
1x Monensin	BD	Cat# 554724; RRID:AB_2869012
Live/Dead Fixable Blue	Thermo Scientific	Cat# L23105; RRID: N/A
DNA easy blood and tissue kit	Quiagen	Cat# 69504; RRID: N/A
Deposited data		
spectral flow cytometry data	this study	http://dx.doi.org/10.17632/ffkvft27ds.2
supplemental spreadsheets	this study	http://dx.doi.org/10.17632/ffkvft27ds.2
scRNA-seq data	(Zhao et al., 2021)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167118
Software and algorithms		
Affinity designer	Affinity	https://affinity.serif.com/de/designer/
corrplot	Taiyun Wei and Viliam Simko (2017)	https://github.com/taiyun/corrplot
dplyr	Wickham et al., 2019	https://cran.r-project.org/web/packages/dplyr/index.html

FlowJo V10.6.2.	Tree Star	https://www.flowjo.com/
FlowSOM	(Van Gassen et al., 2015)	https://github.com/SofieVG/FlowSOM
flowStats	Hahne et al., 2020	https://www.bioconductor.org/packages/release/bioc/html/flowStats.html
ggplot2	Wickham et al., 2019	https://cran.r-project.org/web/packages/ggplot2/index.html
Harmony	(Korsunsky et al., 2019)	https://github.com/immunogenomics/harmony
Hmisc	Harrell, 2020	https://cran.r-project.org/web/packages/Hmisc/index.html
pheatmap	Kolde, 2019	https://cran.r-project.org/web/packages/pheatmap/index.html
R studio	(R Studio, 2015)	https://www.rstudio.com/
R version 3.6.1	(R Core, 2013)	https://www.r-project.org/
Seurat (v3.1.4)	(Stuart et al., 2019)	https://satijalab.org/seurat/
SingleR	(Aran et al., 2019)	https://github.com/dviraran/SingleR
Stats	Bolar et al., 2019	https://CRAN.R-project.org/package=STAT
UMAP	(McInnes et al., 2018)	https://github.com/lmcinnes/umap
Other		
Automated cell counter	Bio-Rad	N/A
Cryo thaw devices	Medax	N/A
Cytek Aurora	Cytek Biosciences	N/A
Illumina MiniSeq	Illumina	N/A
LABScan 3D instrument	Luminex	N/A

679

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the

Lead Contact, Burkhard Becher (becher@immunology.uzh.ch).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

687 Spectral flow cytometry data generated during this study and additional supplemental items
688 are available from Mendeley Data at http://dx.doi.org/10.17632/ffkvft27ds.2 (DOI:
689 10.17632/ffkvft27ds.2)

690 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

691 **COVID-19 Patient Samples**

692 Clinical routine data and blood samples for peripheral blood mononuclear cell (PBMC)
693 isolation and cryopreservation were collected at the University Hospital Tuebingen (Germany),
694 the Toulouse University Hospital (France, in the frame of the COVID-BioToul biobank,
695 ClinicalTrials.gov Identifier: NCT04385108) and the Nantes University Hospital (France)
696 (Table S1A). All donors had given written informed consent and the study was approved by
697 the regional ethical review board of Tuebingen (COVID-19), Toulouse (COVID-19) and Nantes
698 (COVID-19, HAP, Healthy) respectively. COVID-19 diagnosis was established by a positive
699 PCR test. PBMC samples were collected longitudinally at the indicated time points post-
700 admission to the hospital (Table S1C). COVID-19 patients were graded according to the
701 maximum severity of disease during the study based on the WHO ordinal scale (World Health
702 Organization, 2020b). The WHO grade 1 and 2 were combined to grade 1 in our scale, the
703 WHO grade 7 and 8 were combined to grade 6 in our categorization. The appropriate severity
704 grade was then allocated to all samples of the same patient. Mean age of COVID-19 patients
705 was 62.2 years, the percentage of females was 40.4.

706 **Human Subjects with HAP and Healthy Samples**

707 Bioresources: IBIS-sepsis (severe septic patients) and IBIS (brain-injured patients), Nantes,
708 France. Patients were enrolled from January 2016 to May 2019 in two French Surgical
709 Intensive Care Units of one University Hospital (Nantes, France) and samples collected in
710 accordance to the guideline of standardization (CoBRA) (Bravo et al., 2015). Patients with
711 immunosuppression were not enrolled to the study. The criteria to diagnose hospital-acquired
712 pneumonia were (1) radiological signs combined with (2) body temperature > 38,3°C without
713 any other cause or leukocytes < 4000/mm^3 or > 12000/mm^3 and (3) at least two of the following
714 symptoms: purulent sputum, cough or dyspnea, declining oxygenation or increased oxygen-
715 requirement or need for respiratory assistance (Leone et al., 2018). Hospital-acquired
716 pneumonia were microbiologically confirmed with quantitative culture (for patients with
717 antibiotics < 48h) (thresholds of 10^4 colony-forming units (CFU) per mL for a bronchoalveolar
718 lavage). PCR for Herpes Simplex Virus and Cytomegalovirus were performed in tracheal
719 aspirates at day 1, day 7 and day 15 after ICU admission. The collection of human samples
720 has been declared to the French Ministry of Health (DC-2011-1399), and it has been approved

by an institutional review board. Written informed consent from a next-of-kin was required for enrolment. Retrospective consent was obtained from patients, when possible. All patients were clinically followed up for 28 days. Control samples were collected from healthy blood donors, recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France). Mean age of HAP patients was 43.8 years, the percentage of females was 8.7. Mean age of healthy controls was 52.0 years, the percentage of both females was 44.4.

METHOD DETAILS

Ex vivo Reactivation of PBMCs

PBMCs collected in clinics were kept in cell culture medium (RPMI-1640, 10% fetal bovine serum (FBS; Gibco), and 1× l-glutamine (Gibco) and 1× penicillin streptomycin (Gibco)) supplemented with 5U ml^{-1} benzonase (Sigma–Aldrich) and frozen in liquid nitrogen until experimental analysis. Then, for spectral flow analysis, cells were thawed using Cryo thaw devices (Medax). Briefly, cells were resuspended in cell culture medium supplemented with 2U ml^{-1} benzonase by centrifugation (300 r.c.f.; 7 min; 24 °C). Cell count was calculated using an automated cell counter (Bio-Rad). Due to the resulting cell count, cells were used for all panels or surface panel only. Subsequent procedure including short-term reactivation of cryopreserved PBMCs and cytometry analysis were performed as described previously (Galli et al., 2019; Hartmann et al., 2016). Briefly, 2 million (mio) cells were directly stained for cytometry analysis (surface panel), while 1 mio cells were restimulated with 50 ng ml^{-1} phorbol 12-myristate 13-acetate (Sigma–Aldrich) and 500 ng ml^{-1} ionomycin (Sigma–Aldrich) in the presence of 1× Brefeldin A and 1x Monensin (both BD Biosciences) for 5 h at 37°C or in case of R848 stimulation, 2.5 mio cells using 2µg ml^{-1} R848 (Invivogen) in the presence of 1× Brefeldin A and 1x Monensin (both BD Biosciences) for 8 h at 37°C.

Surface Labeling for Spectral Flow Cytometry

For spectral cytometry, samples were washed in PBS and then resuspended in 100µl of Live Dead Fixable Blue mixture (Thermo Scientific, 1:500) followed by a washing step. To avoid nonspecific binding, the samples were resuspended in 30 µl of True Stain FcX (BioLegend) and incubated for 10 min at 4°C. Anti-human flow cytometric antibodies were purchased pre-conjugated (Table S2A-C). 70 µl of the first surface-antibody mixture was added and cells were incubated for 15 min at 37°C (Table S2A). After another washing step, the second surface-antibody staining step (100 µl) was performed for 15 min at 4°C (Table S2A). Then, fixation was performed using 150 µl of 2% PFA for 15 min at 4°C.

Intracellular Cytokine Labeling for Spectral Flow Cytometry

For intracellular spectral cytometry, after surface-antibody labeling, cells were fixed and permeabilized using Cytofix Cytoperm reagent (BD Biosciences) for 30 min at 4°C. Intracellular labeling was then performed in 100 µl of 1x permeabilization buffer (Thermo Scientific) for 11 h (Lymphoid cytokine panel, Table S2B) or 10 h (Myeloid cytokine panel, Table S2C) at 4°C.

HLA Typing

For DNA extraction the DNA easy blood and tissue kit from Quiagen was used. HLA typing was performed using next generation sequencing (NGS) with the NGSgo-AmpX v2 HLA kits (GenDx, Utrecht, Netherlands), and sequenced on an Illumina MiniSeq (Illumina, San Diego, CA). Sequence data were analyzed with NGSengine (GenDx, Utrecht, Netherlands). For samples with low DNA amount, HLA typing was also performed using sequence specific oligomers (SSO) with the LABType kits (One Lambda, Canoga Park, CA). The bead-based analysis was run on a LABScan 3D instrument (Luminex, Austin, TX) and analyzed using the Fusion Software (One Lambda, Canoga Park, CA). All assays were performed according to the manufacturer's recommendations.

QUANTIFICATION AND STATISTICAL ANALYSIS

Acquisition and Preprocessing of Spectral Flow Cytometry Data

Spectral cytometry samples were acquired on a Cytek Aurora (Cytek Biosciences). Quality control of the Cytek Aurora was performed daily as instructed by the manufacturer. For downstream analysis, dead cells and doublets were excluded using FlowJo (TreeStar). Samples with viability lower than 10% and fewer than 500 live, CD45 positive cells were excluded. Cytometry data were transformed with an inverse hyperbolic sine (arcsinh) function using the R environment (range 30 - 18000). To balance the influence of markers with different dynamic ranges, we performed background subtraction and channel-based percentile normalization using the 99.9th percentile of each marker across the whole dataset (Bendall et al., 2011). Individual cytokine positivity thresholds were determined based on the 99th percentile of the residual staining in an unstimulated or isotype-stained control sample.

Algorithm-based High-dimensional Analysis of Spectral Flow Cytometry Data

Pre-processed data were downsampled to a maximum of 150'000 cells per donor for the analysis of the main populations, all cells were used for analysis of the specific immune compartments. The high dimensional analysis was carried out using the R environment, based loosely on the workflow described previously (Mair et al., 2016). Two-dimensional UMAP (Uniform Manifold Approximation and Projection) projections were calculated using the *umap* package (McInnes L Saul N, Großberger L, 2018). All FlowSOM-based clustering was

performed on the whole dataset to enable identification of small populations, and the results were overlaid on the dimensionality reduction maps (Van Gassen et al., 2015). Principal component analysis was carried out in the *stats* package using the median activation marker expression of all detected leukocyte subsets. The circles represent the core areas added by the default confidence interval of 68%, which facilitates the separation based on the PC1/2 explanatory rate of the overall difference in measured immune features. For the correlogram, Pearson's r correlation coefficients were computed using the *Hmisc* package and the resulting correlation matrix was visualized using the *corrplot* package. All other plots were drawn using *ggplot2.* For longitudinal visualization, smoothed conditional mean of the feature from the combined COVID-19 cohort was added in light grey.

Calculation of HLA Score 50

Based on the study data of Nguyen et al. (Nguyen et al., 2020), the predicted HLA class I binding capacity to SARS-CoV-2-derived peptides per patient was calculated by counting the number of all SARS-CoV-2-derived peptides which were predicted to be bound by each specific HLA allele. The score 50 includes all SARS-CoV-2 peptides which were predicted for tight binding (<50nm) to the indicated HLA class I allele. The final HLA score 50 per patient represents the total number of tight binding SARS-CoV-2 peptides of both alleles of the patient for HLA-A, HLA-B or HLA-C.

Single-cell RNA-seq Analysis

For single-cell RNA-seq analysis we used a publicly available dataset of sorted CD45^{+} blood cells of COVID-19 patients (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167118), of which 5'-RNA single cell transcriptome (10x genomics) was performed. For preprocessing, the feature-barcode matrices for all the sample were further processed by the R package Seurat (v3.1.4). As a quality-control (QC) step, we first filtered out the cells in which less than 200 genes were detected in the blood samples. To remove potential doublets, we excluded cells with total number of detected genes more than 5000. Low-quality cells with more than 5% mitochondrial genes of all detected genes were removed. The LogNormalize method in Seurat was used to normalize the scRNA-seq and batch effect correction was performed using Harmony. The R package SingleR, an automatic annotation method for single-cell RNA sequencing (Aran et al., 2019) were then used to determine the cell types. The differential expression between selected groups were calculated by the FindAllMarkers function (min.pct = 0.25, logfc.threshold = 0.25, Wilcoxon rank sum tests).

Statistical Analysis

822   Frequencies of immune populations, cytokines, median expression values and absolute

823   counts were compared with the non-parametric Mann–Whitney–Wilcoxon test and Benjamini-

824   Hochberg correction for multiple testing, using the R package *rstatix*. For correlation

825   measurements, we used a linear regression model by applicating the *lm*() and *summary*()

826   functions. P values of less than 0.05 were considered significant and are indicated by an

827   asterisk (*) or the numerical value on the respective graphs.

SUPPLEMENTAL TABLES

828

829 Detailed information about patient cohorts, clinical routine parameter and TPs (related to
830 Figure 1A and S1A): Kreutmair_et_al_Suppl_Table1.xlsx

831 Spectral flow cytometry panels (related to Figure 1-7 and S1-S7):
832 Kreutmair_et_al_Suppl_Table2.xlsx

833 Selected immune features (related to Figure 2-5): Kreutmair_et_al_Suppl_Table3.xlsx

834 ROC curve of NKT cell frequency among T cells (related to Figure 5B):
835 Kreutmair_et_al_Suppl_Table4.xlsx

836 HLA types and HLA scores 50 (related to Figure 6B, S6A and S6B):
837 Kreutmair_et_al_Suppl_Table5.xlsx

838 **DECLARATION OF INTERESTS**

839 The authors declare no competing interests.

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1099

Figure 1

a

COVID-19
(three independent cohorts)

mild
1: not hospitalized (WHO 1+2)
2: hospitalized (WHO 3)
3: regular oxygen (WHO 4)
n=86

severe
4: non-invasive ventilation (WHO 5)
5: mechanical ventilation (WHO 6)
6: ECMO/ death (WHO 7+8)
n=35

25 clinical routine parameters

HLA typing — 48 patients

hospital admission
day 0-2 day 3-5 day 6-9 day 11-15 day 19-96   week 14
t1   t2   t3   t4   t5

PBMC samples

High-dimensional single-cell spectral cytometry — 167 samples

3 panels { unstimulated, PMA/Ionomycin, R848 }

50 individual parameters

Healthy
n=21

Hospital-acquired pneumonia
n=25

12 clinical routine parameters

Integrated data analysis of immune signatures + HLA binding capacity + clinical information

b

Monocytes
DCs
NK cells
B cells
CD4- CD8- T cells
CD8+ T cells
CD4+ T cells
UMAP2
UMAP1

c immune phenotype/ cohort

PC2 (10.8% explained var.)
PC1 (16.7% explained var.)
t1-t5

- Healthy
- COVID-19m
- COVID-19s
- HAP

d

COVID-19s in common with HAP
COVID-19s different from HAP

COVID-19s different from COVID-19m
COVID-19s in common with COVID-19m

Effect size COVID-19m vs COVID-19s
Effect size HAP vs COVID-19s
t1-t5

COVID-19s different from COVID-19m but in common with HAP
COVID-19s different from COVID-19m and different from HAP

- B cell feature
- Monocyte/ DC feature
- NK cell feature
- T cell feature

e

COVID-19s specific immune features in common with HAP

COVID-19s specific immune features different from HAP

frequency (normalized to input)

- Monocyte/ DC feature
- NK cell feature
- T cell feature

Figure 2

A Signatures specific for COVID-19s and in common with HAP

● T cell features

effect size COVID-19s vs COVID-19m > 0.3

effect size COVID-19s vs HAP < 0.4

B T cells

CD4+ Treg
CD4+ CM CCR6+
CD4+ CM CCR4+
CD4+ EM
CD4+ naive
CD4+ CM
CD4+ TEMRA
CD4+ CM CXCR3+CCR6+
CD4- CD8-
NKT
CD8+ naive
CD8+ TEMRA
CD8+ CM
CD8+ EM

C CD4- CD8-

Healthy
COVID-19m
COVID-19s
HAP

* significance Healthy vs. COVID-19m or s

* significance COVID-19m vs. COVID-19s

* significance COVID-19s vs. HAP

D CD4+ PD-1

E stimulated CD4+ EM / CTLA-4

F Cytokine profile COVID-19s vs. COVID-19m

CD8+ T cells
CD8+ CM GM-CSF+
CD4+ TEMRA GM-CSF+
CD8+ EM IFNγ+
CD8+ TEMRA IFNγ+
TCRγδ IFNγ+
CD8+ TEMRA GM-CSF+
TCRγδ cells
TCRγδ IL-2+
CD4+ T cells
IL-4, IL-6, IL-17A,TNF

G stimulated IFNg

H stimulated TCRγδ / IL-2

I stimulated CD4+ TEMRA / stimulated CD8+ TEMRA

p=0.001 R=0.30

p=0.019 R=0.30

J COVID-19m / COVID-19s

+/- z-score

0 = healthy

Figure 3

A Signatures specific for COVID-19s and in common with HAP

- Monocyte/DC features
- NK cell features

0.3

0.4

effect size COVID-19s vs COVID-19m > 0.3

effect size COVID-19s vs HAP < 0.4

B NK cells

Late diff. (CD57+)
CD56 dim CD16+
CD56 high
CD56 low CD16-
CD56 low CD16+
Immature (CD27+)

UMAP2 / UMAP1

C

* significance Healthy vs. COVID-19m or s
* significance COVID-19m vs. COVID-19s
* significance COVID-19s vs. HAP

D CD56 low CD16-

HLA-DR / HLA-DR

median expression

t1+t2

t1 t2 t3 t4 t5

E Monocytes/DCs

pDCs
cDC2s
cDC1s
Non-class. monocytes
Class. monocytes
Int. monocytes

UMAP2 / UMAP1

F

pDCs
Int. Monocytes
Non-class. Monocytes
Class. Monocytes
cDC1s
cDC2s

- Healthy
- COVID-19m
- COVID-19s
- HAP

G pDCs

Frequency of monocytes/DCs

t1 t2 t3 t4 t5

H stimulated cDC2s

p=0.008
R2=0.1

CCR2 median expression

severity grade

t1-t5

stimulated cDC2s CCR2

median expression

t1 t2 t3 t4 t5

I

COVID-19m

CD56 low CD16-, MFI HLA-DR
cDC2s, MFI CCR2
pDCs, freq. of monocytes/DCs

t1 t2 t3 t4 t5

time

COVID-19s

t2 t3 t4 t5

time

+/- z-score

3.5
2.5
2
1.5
1
0.5
0 = healthy

Figure 4

A

Signatures specific for COVID-19s and different from HAP

Monocyte/DC features

effect size COVID-19s vs COVID-19m > 0.3

effect size COVID-19s vs HAP > 0.4

B Class. Monocytes — HLA-DR

C Class. Monocytes — CD86

D Monocytes
p=0.0004
R2=0.19
t1+t2

DCs
p=0.002
R2=0.15
t1+t2

E Monocytes
p<0.0001
R2=0.39
t1+t2

cDC2s
p<0.0001
R=0.26
t1+t2

F

COVID-19m   COVID-19s   +/- z-score

cDC2s, MFI CD86
Class. monocytes, MFI CD86
Class. monocytes, MFI HLA-DR
DCs, MFI HLA-DR
Monocytes, MFI CD86
Monocytes, MFI HLA-DR

time   time   0 = healthy

G Monocytes HLA-DR / DCs HLA-DR

H Monocytes CD86 / DCs CD86

* significance Healthy vs. COVID-19m or s

* significance COVID-19m vs. COVID-19s

* significance COVID-19s vs. HAP

Healthy
COVID-19m
COVID-19s
HAP

Figure 5

Figure 6

Figure 7

A ACE2 expression in a CD4+ T cell subset

0.3
0.4

B unstimulated

CD4+ TEMRA
CD4+ T reg
CD4+ CXCR3+ CCR6+
CD4+ CCR6+
CD4+ CM
CD4+ CCR4+
CD4+ EM
CD8+ TEMRA
NKT
CD4- CD8-
CD8+ CM
CD8+ EM
CD4+ Naive
CD8+ Naive

CD38 CD8 CD1c CD45RA CD16 CD27 CCR4 CD11c CD56 CD86 PD1 HLADR CD28 CXCR3 CCR6 CXCR5 CCR7 CD57 CD95 CD25 CD4 CD161

C unstimulated CXCR3+ CCR6+
ACE2
frequency of positive cells
t1-t5

D unstimulated CD4+ CXCR3+ CCR6+
frequency of T cells
t1 t2 t3 t4 t5

- Healthy
- COVID-19m
- COVID-19s
- HAP

* significance Healthy vs. COVID-19m or s

* significance COVID-19m vs. COVID-19s

* significance COVID-19s vs. HAP

E stimulated
ACE2 Isotype
gated on CD3+
CD4
ACE2

F ACE2 stimulated, t1-t5
frequency of positive cells
ACE2-enriched CM EM Naive TEMRA CM EM Naive TEMRA NK TCRγδ
CD4+ CD8+

G stimulated
CD4+ ACE2-enriched
B cells
NK
TCRγδ
CD4+ CM
CD4+ EM

PD1
CTLA4
CD56
CD16
CD27
CD19
CCR7
CD45RA
TCRgd
CD8
CD25
CD4
CD3
ACE2

H stimulated
CD4+ ACE2-enriched
PD-1
median expression
t1-t5

stimulated
CD4+ ACE2-enriched
CTLA-4
median expression
t1-t5