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

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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
Immune profiling of COVID-19 patients has identified numerous alterations in both innate and adaptive immunity. However, whether those changes are specific to SARS-CoV-2 or driven by a general inflammatory response shared across severely ill pneumonia patients remains unknown. Here, we compared the immune profile of severe COVID-19 with non-SARS-CoV-2 pneumonia ICU patients using longitudinal, high-dimensional single-cell spectral cytometry and algorithm-guided analysis. COVID-19 and non-SARS-CoV-2 pneumonia both showed increased emergency myelopoiesis and displayed features of adaptive immune paralysis. However, pathological immune signatures suggestive of T cell exhaustion were exclusive to COVID-19. The integration of single-cell profiling with a predicted binding capacity of SARS-CoV-2-petides to the patients’ HLA profile further linked the COVID-19 immunopathology to impaired virus recognition. Towards clinical translation, circulating NKT cell frequency was identified as a predictive biomarker for patient outcome. Our comparative immune map serves to delineate treatment strategies to interfere with the immunopathologic cascade exclusive to 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
signatures specific to SARS-CoV-2 and those shared with other pathogen-associated severe RS. Whereas emergency myelopoiesis and adaptive immune paralysis are common features of RS, signs of T cell exhaustion and reduced cytotoxicity were exclusive to COVID-19. Lastly, the identification of circulating NKT frequencies as a predictive biomarker for patient outcome 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 (Tübingen) 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.

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

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

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

In order to interrogate the cytokine polarization, the cells were briefly stimulated in vitro prior to spectral flow acquisition. The resulting cytokine profile of stimulated lymphoid subpopulations from COVID-19 patients showed significantly higher amounts of IL-21, as well as a shift towards a cytotoxic phenotype indicated by high levels of granzyme B and perforin in the T and NK cell compartments, relative to HCs (Fig. S2E, S2F, data not shown). CD107a on T and NK cells was similar in COVID-19s, COVID-19m and HCs (data not shown), suggesting not only equal cytotoxicity but also degranulation capacity across COVID-19 disease severity. The same applies for TNF, IL-4, IL-6, and IL-17A, which failed to reach the cut-off of 0.3 ES when comparing mild and severe COVID-19 (Fig. 2F, S2G-J). However, we found increased production of interferon-γ (IFN-γ), IL-2 and GM-CSF in COVID-19s. Specifically, higher frequencies of IFN-γ-expressing CD8+ EM, TEMRA (CCR7-CD45RA+) and TCRγδ T cells (Fig. 2G) and IL-2-expressing TCRγδ T cells were a common feature of severe RSs shared by COVID-19 and HAP (Fig. 2H). Elevated frequencies of GM-CSF-producing CD4+ and CD8+ TEMRA cells positively correlated with COVID-19 severity in the acute phase of disease (TP 1 and 2) (Fig. 2I, S2K, S2L). Single-cell RNA-seq analysis of blood cells from
COVID-19 patients - in absence of ex vivo stimulation - revealed strong expression of CSF2 (encoding for GM-CSF) particularly in CD4⁺ T cells (Fig. S2M). Detailed differentially expressed gene (DEG) analysis of CSF2 high vs. low expressing CD4⁺ T cells indicated these cells as a hyperinflammatory subset, strongly expressing TNF, IL21, TNFRSF4, GNLY, CD40LG, CCL20, ICAM1 and demonstrating low ANXA1 mRNA levels, among others (Fig. S2N).

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

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

We further characterized the identified DC and NK cell features shared by COVID-19s and HAP (Fig. 3A, Table S3). To reveal the dynamic changes over time, we displayed the COVID-19 cohorts together with HCs as baseline and HAP patients as comparison. Lower expression of HLA-DR in CD56low CD16- NK cells suggest a diminished cytotoxic response in COVID-19s (Fig. 3B-D, S3A) (Erokhina et al., 2020). Similarly, COVID-19s displayed reduced frequency of plasmacytoid DCs (pDC) (Fig. 3E-G, S3B). Although the pDC frequency was also different from HAP, the cut-offs of the ES were not reached. Upregulation of the Fas receptor CD95 was detected in all DC subsets - particularly on pDCs - at early TPs 1 and 2 (Fig. S3C, S3D).

This might play a role in the loss of those cells through Fas-mediated apoptosis.

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

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

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

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

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

The protein expression of CD38, another activation marker, across several T cell subsets positively correlated with COVID-19 severity, with the highest significance (p < 0.0001, R² = 0.24) in CD4- CD8- (TCRγδ enriched) T cells (Fig. 5D, S5D). Furthermore, we observed a loss of the regulatory protein CD161 in CD4+ CD8+ (TCRγδ enriched) T cells in COVID-19s (Fig. 5E, S5E). This phenomenon is especially intriguing, as CD4+ CD8+ (TCRγδ enriched) T cells share the transcriptional signatures of CD161-expressing Mucosa-Associated Invariant T (MAIT) cells, a CD8+ T cell subset resembling innate-like sensors and mediators of antiviral responses (Fergusson et al., 2014, 2016).

Although failing to reach the stringent cut-off for being a unique COVID-19s specific feature (ES vs HAP > 0.4), CD161 was also expressed at a significantly lower level on immature and CD56low CD16+ NK cells in the early phase of severe SARS-CoV-2 related illness compared to mild disease (Fig. S5F). Here, the kinetics of CD161 expression was low at the beginning of disease with a delayed hyperreactivity in COVID-19s (Fig. S5F). Further dissecting the NK cell compartment, CD95 expression in the CD56high NK subset positively correlated with severity of COVID-19 and represents a unique characteristic specific to SARS-CoV-2 infection (Fig. 5F, S5G). As in the DC compartment described before, the significant and specific reduction of this NK subset supports the Fas-mediated, activation-induced apoptosis as the mechanism underlying the shift from effector to immature NK cells (Fig. S5H).

Regarding the cytokine polarization profile, a reduced production of IFN-γ in CD4+ central memory (CM) T cells was found to be COVID-19s specific and reflects the loss of CD4+ CXCR3+ CCR6+ (Th1 Th17-enriched) T cells (Fig. 5G, 5H). All features falling in the COVID-19s specific category and diverging from HAP recovered only partly, both in mild and severe
SARS-CoV-2 infected patients, pointing to a persisting dysfunctional T and NK cell compartment (Fig. 5I).

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

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

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

Because of earlier evidence of SARS-CoV-2 peptide binding to Human Leukocyte Antigen (HLA) molecules differs across genotypes (Nguyen et al., 2020), we introduced next generation sequencing (NGS)-based HLA class I typing of 48 patients of our COVID-19 cohort. We calculated the predicted number of tightly binding (<50nm) SARS-CoV-2-derived peptides per HLA class I gene (based on every single underlying allele genotype) for each of our typed individuals (Fig. S6A, S6B, Table S5). This predicted binding capacity for HLA-A, HLA-B and HLA-C was further called HLA score 50. Next, we integrated this dataset to our single-cell immune profiling analysis and correlated this HLA-A, HLA-B and HLA-C score 50 to all our extracted severe COVID-19-associated immune features (Fig. 6B). This multi-omics approach allowed us to show that the majority of the severe COVID-19-associated immune features of the innate immune system (e.g. NKT frequency, HLA-DR in monocytes and DCs, etc.) was correlating with the SARS-CoV-2 binding strength (Fig. 6B). Meaning, that efficient HLA
binding capacity to SARS-CoV-2 peptides may mitigate the alterations of the innate immune system detected in COVID-19s. Also, the COVID-19s associated GM-CSF production in CD8+ CM T cells positively correlated with high HLA scores. To conclude, the data suggests that weak HLA binding to SARS-CoV-2 peptides may at least in part drive the immunopathology in COVID-19.

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

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

ACE2 expression in a CD4+ T cell subset increases after ex vivo stimulation

SARS-CoV-2 employs the angiotensin-converting enzyme 2 (ACE2) as its receptor for cellular entry (Prompetchara et al., 2020; Zhou et al., 2020). To determine potential entry sites within T cells we measured ACE2 expression across our immune map. We did not identify ACE2 expression in steady state healthy T cell subpopulations, whereas samples from severe RS showed marginal expression, especially in the CD4+ CXCR3+ CCR6+ (Th1 Th17-enriched) subset (Fig. 7A, 7B, 7C, S7A, S7B) which was significantly reduced in COVID-19s (Fig. 7D). When we profiled the stimulated PBMCs mimicking the COVID-19 inflammatory environment, we discovered a CD4+ T cell subpopulation, of which approximately 75-80% expressed ACE2 (Fig. 7E, 7F). This population emerged from samples of both healthy and COVID-19 patients and expressed CD25, PD-1 and CTLA4 (Fig. 7G, 7H, S7C). Further analysis of this subset demonstrated no relevant overlap with a specific cytokine polarization profile or FOXP3
expression (Fig. S7D, S7E). The presence of ACE2 expression on an activated CD4+ T cell subset may provide a mechanism for virus entry and contribution to the immunopathological network of COVID-19.
DISCUSSION

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

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

Alongside signatures shared in severe RS, we also extracted those specific to and unique in COVID-19s. These SARS-CoV-2-induced adaptations were restricted to the T and NK cell compartment. Several studies described an upregulation of PD-1 and CD38 alongside other activation and exhaustion markers, suggesting a hyperactivated and exhausted T cell compartment (De Biasi et al., 2020; Chen and Wherry, 2020). However, again, it was unclear 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
immune alterations were less pronounced in patients with predicted high HLA class I binding capacity to SARS-CoV-2 peptides, GM-CSF production in CD8+ CM T cells – a feature associated with severe COVID-19 disease - was increased. The occurrence of both mild and severe COVID-19-associated immune features in patients with strong SARS-CoV-2 recognition (high HLA score 50) could further explain the inconsistent reports which attempt to link HLA class I binding capacity to SARS-CoV-2 peptides to COVID-19 severity (Ellinghaus et al., 2020; Iturrieta-Zuazo et al., 2020). By combining the single-cell immune mapping with HLA genetics, we uncovered a link between the HLA profile and impaired virus recognition in COVID-19.

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

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

LIMITATIONS OF THE STUDY

While we initially anticipated center-specific batch effects in our multi-center study, this was not the case. However, our HAP cohort consists of patients suffering from severe pneumonia
driven by multiple pathogens, both bacterial and viral. The comparison of COVID-19 patients
to a pure viral pneumonia cohort could help to further specify the unique immune signatures
to SARS-CoV-2 and distinctive to other viruses. Using PBMCs as source of analyzed immune
cells allows for easy implementation of our findings (such as NKT frequency as predictive
biomarker) to the clinics. Even though the simple measurement of circulating NKT cell
frequencies would have predicted all of our COVID-19 patients who developed severe
disease, larger follow-up studies are needed to solidify this measurement as a predictive
biomarker for COVID-19 patient outcomes.
We thank the patients and blood donors who contributed to this study, and the hospitals at Nantes, Toulouse and Tübingen for sample and data collection. Regarding specifically the COVID-BioToul biobank (ClinicalTrials.gov Identifier: NCT04385108) we thank the CRB TBR, the Clinical Research Center 1436 and the Delegation for clinical research and innovation of the Toulouse University Hospital for their highly valuable implication. We thank the biological resource centre for biobanking (CHU Nantes, Nantes Université, Centre de ressources biologiques (BB-0033-00040), F-44000 Nantes, France).

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FIGURE LEGENDS

Figure 1: Immunomonitoring reveals differing immune landscapes in COVID-19m, COVID-19s and HAP patients

A: Schematic of experimental approach.

B: UMAP with FlowSOM overlay showing total CD45^pos^ cells of combined samples. 1000 cells were subsetted from every sample from each cohort.

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

D: Comparison of immune features derived from each leukocyte subpopulation between experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis; threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold 0.3). Each dot represents one immunological feature, colors represent the leukocyte compartment they refer to.

E: Proportion of each immune compartment (normalized to input) in the identified sets of immune features highlighted in Fig. 1D.

See also Figure S1.

Figure 2: Shared T cell features between severe pathogen-induced RSs highlight the emergence of hyperinflammatory and exhausted subsets in COVID-19s

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

B: UMAP with FlowSOM overlay of total T cells of combined samples. 1000 cells were subsetted from every sample from each cohort. T cell subsets with transparent names do not contain immune features highlighted in Fig. 2A.

C: Median frequencies and 25th and 75th percentile of FlowSOM-generated CD4^-^CD8^-^ (TCR_{γδ} enriched) immune cell cluster.

D: Median expression and 25th and 75th percentile of PD-1 in FlowSOM-generated immune cell clusters shown in B.
E: Median expression of CTLA-4 within CD4+ EM T cell subset of HCs shown in grey, of HAP in blue and of mild and severe COVID-19 patients across TPs 1-5 shown in red.

F: Schematic overview of cytokine polarization profile comparing COVID-19s and COVID-19m. UMAP with FlowSOM overlay shows cytokine-producing T cell subpopulations (features reaching an ES > 0.3). 1000 T cells were subsetted from every sample from each cohort.

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

H: Median frequency and 25th and 75th percentile of IL-2 positive cells in FlowSOM-generated immune cell cluster shown in F.

I: Correlation between frequency of GM-CSF expressing CD4+ (left panel) and CD8+ (right panel) TEMRA cells and the severity grade of COVID-19 patients in combined TPs 1 and 2.

J: Heatmap depicting the z-score of each T cell related immune feature (highlighted in Fig. 2A) when compared to HCs for every TP. Both negative and positive changes are visualized by intensity of red color scale. MFI = Mean fluorescence intensity.

Significant p values are depicted using an asterisk (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** = p < 0.0001, Mann-Whitney test, BH correction). See also Figure S2.

Figure 3: Phenotypic alterations in innate immune signatures are shared in severe COVID-19 and HAP

A: Comparison of immune features derived from each leukocyte subpopulation between experimental groups. A dot plot displaying the ES calculated in HAP vs. COVID-19s (x axis; threshold 0.4) compared to the ES calculated in COVID-19m vs. COVID-19s (y axis; threshold 0.3). Each dot represents one immunological feature. The red box highlights immune features, which are associated with severe RS, with a focus on changes within the monocyte, DC and NK cell fraction.

B: UMAP with FlowSOM overlay of total NK cells of combined samples. 1000 cells were subsetted from every sample from each cohort. NK cell subsets with transparent names do not contain immune features highlighted in Fig. 3A.

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

D: Median expression and 25th and 75th percentile of HLA-DR in FlowSOM-generated CD56low CD16+ NK cell cluster shown in B, combined for TP 1 and 2 (left panel) or displayed for every individual TP (right panel).
**E:** UMAP with FlowSOM overlay of total monocytes and DCs of combined samples. 1000 cells were subsetted from every sample from each cohort. Monocyte and DC subsets with transparent names do not contain immune features highlighted in Fig. 3A.

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

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

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

**I:** Heatmap depicting the z-score of each monocyte and DC related immune feature (highlighted in Fig. 3A) when compared to HCs for every TP. Both negative and positive changes are visualized by intensity of red color scale. MFI = Mean fluorescence intensity. Significant p values are depicted using an asterisk (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** = p < 0.0001, Mann-Whitney test, BH correction). See also Figure S3.

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

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

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

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

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

Median expression and the 25th and 75th percentile of HLA-DR (G) or CD86 (H) in FlowSOM-generated monocyte and DC immune cell clusters.
Significant p values are depicted using an asterisk (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** = p < 0.0001, Mann-Whitney test, BH correction). See also Figure S4.

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

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

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

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

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

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

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

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

**H:** Median frequency and 25th and 75th percentile of IFN-\(\gamma\) positive cells in FlowSOM-generated immune cell clusters shown in G.

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

**J:** Median frequencies or expression of indicated populations and markers. Boxplots show the 25th and 75th percentile.
Significant p values are depicted using an asterisk (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 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**

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

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

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

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

See also Figure S6.

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

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

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

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

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

**E:** Representative plot showing ACE2 and isotype staining within the T cell compartment of PMA and ionomycin restimulated (5h) COVID-19 samples.
F: Median frequency and 25th and 75th percentile of ACE2 positive cells in FlowSOM-generated immune cell clusters after PMA and ionomycin restimulation (5h). All TPs have been pooled.

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

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

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

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**Chemicals, peptides, and recombinant proteins**

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**Software and algorithms**

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**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

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

EXPERIMENTAL MODEL AND SUBJECT DETAILS

COVID-19 Patient Samples

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

Human Subjects with HAP and Healthy Samples

Bioresources: IBIS-sepsis (severe septic patients) and IBIS (brain-injured patients), Nantes, France. Patients were enrolled from January 2016 to May 2019 in two French Surgical Intensive Care Units of one University Hospital (Nantes, France) and samples collected in accordance to the guideline of standardization (CoBRA) (Bravo et al., 2015). Patients with immunosuppression were not enrolled to the study. The criteria to diagnose hospital-acquired pneumonia were (1) radiological signs combined with (2) body temperature > 38.3°C without any other cause or leukocytes < 4000/mm³ or > 12000/mm³ and (3) at least two of the following symptoms: purulent sputum, cough or dyspnea, declining oxygenation or increased oxygen-requirement or need for respiratory assistance (Leone et al., 2018). Hospital-acquired pneumonia were microbiologically confirmed with quantitative culture (for patients with antibiotics < 48h) (thresholds of 10⁴ colony-forming units (CFU) per mL for a bronchoalveolar lavage). PCR for Herpes Simplex Virus and Cytomegalovirus were performed in tracheal aspirates at day 1, day 7 and day 15 after ICU admission. The collection of human samples 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**
Frequencies of immune populations, cytokines, median expression values and absolute counts were compared with the non-parametric Mann–Whitney–Wilcoxon test and Benjamini-Hochberg correction for multiple testing, using the R package *rstatix*. For correlation measurements, we used a linear regression model by applying the `lm()` and `summary()` functions. P values of less than 0.05 were considered significant and are indicated by an asterisk (*) or the numerical value on the respective graphs.
SUPPLEMENTAL TABLES

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

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

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

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

HLA types and HLA scores 50 (related to Figure 6B, S6A and S6B): Kreutmair_et_al_Suppl_Table5.xlsx
DECLARATION OF INTERESTS

The authors declare no competing interests.
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promotes accumulation of conventional dendritic cells in the lung during inflammation


Figure 1

a) COVID-19 (three independent cohorts)
- n=86: mild
  - 1: not hospitalized (WHO 1+2)
  - 2: hospitalized (WHO 3)
  - 3: regular oxygen (WHO 4)
- n=35: severe
  - 4: non-invasive ventilation (WHO 5)
  - 5: mechanical ventilation (WHO 6)
  - 6: ECMO/ death (WHO 7+8)

Hospital admission:
- Week 14

Healthy: n=21

Hospital-acquired pneumonia: n=25

b) UMAP plot showing immune cell types:
- Monocytes
- DCs
- NK cells
- CD4+/CD8+ T cells
- B cells
- CD4+ T cells
- CD8+ T cells

c) PC1 vs PC2 plot showing immune phenotype/cohort:
- Healthy
- COVID-19m
- COVID-19s
- HAP

d) Effect size plot:
- COVID-19s in common with HAP
- COVID-19s different from HAP
- COVID-19s different from COVID-19m

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

e) Pie charts showing frequency of immune features:
- COVID-19s specific immune features in common with HAP
- COVID-19s specific immune features different from HAP

Legend:
- 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
**Figure 2**

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

D:

- CD4+ PD-1
- CD4+ CXCR3+ CCR6+
- CD4+ CCR4+
- CD4+ T reg

E:

- CD4+ CM, MFI PD-1
- CD4+ CM, MFI CTLA-4
- CD4+ TEMRA, freq. GM-CSF+
- CD4+ TEMRA, freq. IFNγ+
- TCRγδ, freq. IFNγ+
- TCRγδ, freq. IL2+

**B** T cells

- CD4+ Treg
- CD4+ CM CCR6+
- CD4+ CM CXCR3+CCR6+
- CD4+ CD8-
- CD8+ TEMRA
- CD8+ CM
- CD8+ EM
- CD8+ IFNγ+
- CD8+ TCRγδ

**C** CD4-CD8-

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

Significance:
- Healthy vs. COVID-19m or s
- COVID-19m vs. COVID-19s
- COVID-19s vs. HAP

**D**

- Effect size COVID-19s vs COVID-19m > 0.3
- Effect size COVID-19s vs HAP < 0.4

**E** Stimulated CD4+ EM

- CD4+ CM
- CD4+ EM
- CD4+ TEMRA
- CD8+ EM
- CD8+ CM
- CD8+ TEMRA
- CD8+ TCRγδ

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

- IL-4, IL-6, IL-17A, TNF

**G** Stimulated IFNg

- freq. of positive cells

**H** Stimulated TCRγδ

- IL-2

**I**

- Stimulated CD4+ TEMRA
- Stimulated CD8+ TEMRA

- freq. of GM-CSF pos. cells
- severity grade

**J**

- COVID-19m
- COVID-19s

- z-score

- Healthy
Figure 3

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

B. NK cells

C. CD56 low CD16-

D. HLA-DR

E. Monocytes/DCs

F. pDCs

G. stimulated cDC2s

H. CCR2

I. COVID-19m vs COVID-19s
**Figure 4**

A. Signatures specific for COVID-19s and different from HAP

- Effect size COVID-19s vs COVID-19m > 0.3
- Effect size COVID-19s vs HAP > 0.4

B. Class. Monocytes
   - HLA-DR
   - CD86

C. Class. Monocytes
   - CD86

D. Monocytes
   - DCs
   - p = 0.002
   - R^2 = 0.15

E. Monocytes
   - cDC2s
   - CD86

F. COVID-19m
   - cDC2s, MFI CD86
   - Class. monocytes, MFI CD86
   - DCs, MFI HLA-DR
   - Monocytes, MFI CD86

G. Monocytes
   - HLA-DR

H. Monocytes
   - CD86

- Significance Healthy vs. COVID-19m or s
- Significance COVID-19m vs. COVID-19s
- Significance COVID-19s vs. COVID-19s vs. HAP
**Figure 5**

**A** Signatures specific for COVID-19s and different from HAP

- CD4-CD8- MFICD161
- CD8+ TEMRA, MFICD38
- NKT, freq. of T cells
- CD56 high NK, MFICD95
- CD56 dim CD16+ NK, MFICD95
- CD4+ CM, IFNγ+
- CD4+ T cells
- TCRγδ cells
- CD8+ T cells

**B** Frequency of T cells

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

**C** CD4+ EM

- P = 0.0005
- R² = 0.29

**D** CD4+ EM

- P = 0.0005
- R² = 0.24

**E** CD4+ CD8- CD161

- Median expression t1 + t2

**F** CD6 high NK

- P = 0.0002
- R² = 0.21

**G** Cytokine profile COVID-19s vs. COVID-19m

- CD4+ CM IFNγ

**H** Stimulated CD4+ CM

- Median expression

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

- Time t1-t5

**J** Frequency of T cells

- NKT
- PD-1
- CD38
- CD95

- Time t1-t5

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

Effect size COVID-19s vs HAP > 0.4

Healthy vs. COVID-19m or s

COVID-19m vs. COVID-19s

COVID-19s vs. HAP

+/− z-score

Healthy
Figure 7

A. ACE2 expression in a CD4+ T cell subset

B. unstimulated

C. unstimulated

D. unstimulated

E. ACE2 stimulated gated on CD3+

F. ACE2 stimulated, t1-t5

G. stimulated

H. stimulated CD4+ ACE2-enriched

I. stimulated CD4+ ACE2-enriched