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Increased soluble HLA in COVID-19 present a disease-related, diverse immunopeptidome associated with T cell immunity

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Highlights

Soluble HLA serum levels are elevated in COVID-19 patients

First evidence for the association of sHLA with T cell immunity and disease outcome

COVID-19-associated increased diversity in sHLA-presented peptides

Identification of a nucleocapsid-derived sHLA-presented peptide from COVID-19 plasma

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Increased soluble HLA in COVID-19 present a disease-related, diverse immunopeptidome associated with T cell immunity

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SUMMARY
HLA-presented antigenic peptides are central components of T cell-based immunity in infectious disease. Beside HLA molecules on cell surfaces, soluble HLA molecules (sHLA) are released in the blood suggested to impact cellular immune responses. We demonstrated that sHLA levels were significantly increased in COVID-19 patients and convalescent individuals compared to a control cohort and positively correlated with SARS-CoV-2-directed cellular immunity. Of note, patients with severe courses of COVID-19 showed reduced sHLA levels. Mass spectrometry-based characterization of sHLA-bound antigenic peptides, the so-called soluble immunopeptidome, revealed a COVID-19-associated increased diversity of HLA-presented peptides and identified a naturally presented SARS-CoV-2-derived peptide from the viral nucleoprotein in the plasma of COVID-19 patients. Of interest, sHLA serum levels directly correlated with the diversity of the soluble immunopeptidome. Together, these findings suggest an inflammation-driven release of sHLA in COVID-19, directly influencing the diversity of the soluble immunopeptidome with implications for SARS-CoV-2-directed T cell-based immunity and disease outcome.

INTRODUCTION
Tremendous amount of knowledge on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) humoral and cellular immunity has been obtained since the onset of the coronavirus disease 2019 (COVID-19) pandemic.1 Human leukocyte antigen (HLA) molecules presenting virus-derived peptides are essential for an effective anti-viral cellular immunity to enable the eradication of infected cells by T lymphocytes.2 Aside from membrane-bound HLA molecules on cell surfaces, the presence of soluble HLA-peptide complexes (sHLA) has been detected in a variety of body fluids.3 Mechanisms of active release or secretion include proteolytic shedding of membrane proteins and alternative splicing events.4,5 sHLA serum levels are altered in various pathologies, including infectious, malignant, inflammatory, and autoimmune diseases, and were previously reported to be associated with disease stage or prognosis.5–10 However, the immunological function of sHLA molecules remains poorly understood and debated.11 Several studies have demonstrated an inhibitory role of sHLA, presumably by T cell receptor-based signaling in the absence of costimulatory signals, the induction of apoptosis, or down-regulation of natural killer cell cytotoxicity,12–14 whereas others reported sHLA-mediated T cell activation.15,16 Antigen-specific activation of CD8+ T cells might occur through passive peptide transfer from sHLA complexes to membrane-bound HLA molecules.15,16

Alike their membrane-bound counterparts, sHLA molecules can carry their antigenic peptides. This entirety of sHLA-bound peptides is referred to as the soluble immunopeptidome, which can be analyzed and characterized by mass spectrometry-based approaches.17–19 Especially in the context of cancer, the soluble immunopeptidome as a potential source of tumor-associated antigens came into focus to enable liquid biopsies instead of often poorly accessible tumor samples.17–19 Similarly, this could be applied to viral diseases to investigate the entire infection-modulated immunopeptidome in comparative profiling approaches. So far, the role of classical sHLA in COVID-19 has not been investigated. Recent publications that analyzed levels of non-classical sHLA-G in COVID-19 patients reported contradictory results.

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COVID-19-specific treatment: ○ no △ Remd ▽ Dexa □ Remd, Dexa
Thus, the role of sHLA in COVID-19 remains unclear. In this study, we investigated sHLA serum levels in COVID-19 patients and SARS-CoV-2 convalescent individuals and evaluated the soluble COVID-19 immunopeptidome by screening for SARS-CoV-2-derived HLA-presented peptides and profiling SARS-CoV-2-mediated changes in the sHLA peptide repertoire.

RESULTS
Soluble HLA levels are increased in COVID-19 patients and SARS-CoV-2 convalescent individuals

Quantification of sHLA class I serum levels of COVID-19 patients with acute infection (n = 31) and SARS-CoV-2 convalescent individuals (n = 290) was conducted 1 to 27 days (median 12 days) and 28 to 170 days (median 44 days) after positive SARS-CoV-2 polymerase chain reaction (PCR) testing, respectively and compared to a cohort of healthy volunteers (control, n = 80, Table S1). No correlation of sHLA class I levels with the time from positive SARS-CoV-2 PCR testing to sample collection was observed (Figure S1A). 35%, 51%, and 65% of COVID-19 patients, convalescent individuals, and healthy control donors were female, respectively. The median age was 62 (range 24–88), 44 (range 18–84), and 35 (range 22–71) years for COVID-19 patients, convalescents, and healthy controls, respectively. Neither for COVID-19 patients, convalescent individuals nor control donors sHLA class I serum levels correlated with demographics, including age and body mass index, and did not differ between genders (Figures S1B–S1D). The sHLA class I serum levels were significantly increased in COVID-19 patients (median 3.05 μg/mL, p = 0.0006) and SARS-CoV-2 convalescents (median 2.02 μg/mL, p = 0.0135) compared to the control cohort (control, n = 80, median 1.56 μg/mL, Figure 1A). COVID-19-specific drug treatment (remdesivir, dexamethasone) within three days before sample collection did not influence sHLA class I levels in the COVID-19 cohort (Figures 1A and S2A).

Longitudinal follow-up analysis of 53 SARS-CoV-2 convalescent donors 4 months (T2, median sHLA class I 1.95 μg/mL, Table S2) after the first sample collection (T1, median sHLA class I 2.29 μg/mL) revealed a persisting increase of sHLA class I serum levels (Figure 1B, p = 0.3742) compared to the healthy control cohort with a decreasing trend over time (Figure 1C). Of note, SARS-CoV-2 convalescents, reporting long-term persistence of disease symptoms at the time of sample collection, showed significantly increased sHLA class I levels (n = 51, median 2.04 μg/mL) compared to the control cohort (median 1.56 μg/mL, p = 0.0329, Figure 1D).

Therefore, we investigated sHLA class I levels and the relation to different symptoms in more detail. The majority of COVID-19 patients (90%) and convalescent individuals (95%) showed SARS-CoV-2-specific symptoms during acute infection including fever (55 and 57%), headache (26 and 75%), sore throat (33 and 51%), or loss of smell and taste (32 and 65%). 61 and 7% of COVID-19 patients and convalescent individuals were hospitalized during acute infection, respectively. Elevated sHLA class I levels were significantly associated with COVID-19 symptoms, such as cough (p = 0.0388), headache (p = 0.0285), and fatigue (p = 0.0018), reported by convalescent individuals (Table S3). Within the COVID-19 cohort no difference in sHLA class I levels with single disease symptoms (e.g. cough, sore throat, loss of smell or taste, and fever) nor a correlation with laboratory values, comprising white blood cell counts, lymphocyte counts, hemoglobin or C-reactive protein, was observed (Tables S3 and S4). Of note, COVID-19 patients with a severe course of disease requiring invasive ventilation (n = 4) showed significantly reduced sHLA class I levels (p = 0.0142, Figure 1E).
Investigation of the correlation between sHLA class I serum levels and SARS-CoV-2-directed humoral and cellular immune responses did not reveal any relation of sHLA class I levels with anti-SARS-CoV-2 antibody titers (Figures S2B and S2C). However, the intensity of SARS-CoV-2-specific T cell responses showed a weak positive correlation with sHLA class I levels in convalescent individuals (p = 0.0413, Figure 1F).

In addition, we analyzed serum levels of sHLA-DR in COVID-19 patients (n = 31), SARS-CoV-2 convalescent individuals (n = 222) and healthy control donors (n = 80). Overall, sHLA-DR levels (range 0.0–966 pg/mL) were considerably lower compared to sHLA class I levels (0.26–9.13 μg/mL) with a subset of samples even being in the non-measurable range. In line with sHLA class I, sHLA-DR levels were significantly increased in COVID-19 patients (median 36.1 pg/mL, p< 0.0001) and SARS-CoV-2 convalescents (median 0.0 pg/mL, p = 0.0343) compared to the control cohort (control, n = 80, median 0.0 pg/mL, Figure S3A). 74% of COVID-19 patients and 46% of convalescent individuals exhibited measurable sHLA-DR serum levels, whereas only 30% of healthy control donors showed measurable levels (Figure S3B). Similar to sHLA class I, COVID-19-specific drug treatment did not influence sHLA-DR levels in the COVID-19 cohort (Figure S3C).

The soluble immunopeptidome in COVID-19 patients and healthy volunteers represents a huge repertoire of different HLA class I- and HLA class II-presented peptides

For the identification and characterization of sHLA-presented COVID-19-associated peptides, we analyzed plasma samples from 21 COVID-19 patients (1 – 22 days after positive SARS-CoV-2 PCR test, Tables S5 and S6) and 20 healthy control donors (Tables S5 and S7) on state-of-the-art orbitrap mass spectrometers. The COVID-19 cohort covered a total of 35 different HLA class I allotypes with HLA-A*02 (n = 12, 57%), HLA-C*07 (n = 9, 43%), and HLA-A*03 (n = 8, 38%) as the most frequent allotypes. Among the world population,99.5% of the individuals carry at least one of the allotypes covered by the COVID-19 cohort (Figure 2A). The healthy volunteer control cohort matched 73% of HLA-A, 79% of HLA-B, and 90% of HLA-C allotypes of the COVID-19 cohort. The majority of HLA allotypes (97.7%) showed no significant difference in frequency between the two cohorts (Figure S4A). 99.7% of the world population carries at least one allotype of the control cohort (Figure S4B).

Mass spectrometry-based analysis of COVID-19 plasma samples revealed a total of 24,266 unique HLA class I ligands (median 2,727) from 9,746 different source proteins and 8,639 unique HLA class II peptides (median 852) from 1,621 source proteins (Figure 2B). A total of 13,934 unique HLA class I ligands (median 1,300) from 6,530 source proteins and 2,953 unique HLA class II peptides (median 302) from 666 source proteins were identified in plasma samples of the control cohort (Figure S4C). The peptide length distribution of HLA class I ligands for both the COVID-19 and control cohort showed a clear peak at nine amino acids (median 74% 9mers for COVID-19, 81% 9mers for control) with up to 99% of identified ligands being eight to ten amino acids long (Figures 2C and S5A). For the HLA class II-derived immunopeptidomes, the peptide length distribution centered around 15 amino acids (median 62% 13 – 17mers for COVID-19, 56% 13 – 17mers for control, Figures 2D and S5B). Gibbs cluster analysis showed clearly distinguishable motifs, revealing the HLA-specific patterns expected for the donor-specific HLA alleles (Figure 2E). Unique HLA class I and HLA class II peptide identifications did not correlate with the amount of utilized plasma in both cohorts (Figures S5C–S5F).

Comparative immunopeptidome profiling reveals a SARS-CoV-2-associated enhancement of immunopeptidome diversity and a COVID-19-associated source protein signature

The donor-individual diversity of the soluble immunopeptidome in terms of different HLA class I- and HLA class II-presented peptides per sample was significantly increased in COVID-19 patients compared to the control cohort (HLA class I: median COVID-19 2,727, median control 1,300, p = 0.0001, HLA class II: median COVID-19 852, median control 302, p< 0.0001, Figure 3A), but does not cohere with remdesivir or dexamethasone treatment (Figures S6A and S6B). Furthermore, the diversity of the soluble COVID-19 HLA class I immunopeptidome significantly correlated with the quantified sHLA class I serum levels (p = 0.0016, Figure 3B). Comparative immunopeptidome profiling of COVID-19 and control immunopeptidomes revealed exclusive presentation for 71% (24,227/34,266) of HLA class I-presented peptides within the COVID-19 immunopeptidomes, whereas only 28% (3,895/13,934) of HLA class I-restricted peptides of control immunopeptidomes showed exclusive representation (Figure S6C). 96% of HLA class I ligands in each cohort are presented by HLA allotypes that are represented in both cohorts. Considering only HLA-restricted peptides presented on HLA allotypes matching both cohorts, 70% (22,910/32,763) of the
peptides still showed exclusive presentation in the COVID-19 soluble immunopeptidome (Figures S6D and S6E). In addition, this broad cohort-wide diversity of the COVID-19 immunopeptidome could be observed for HLA class II-presented peptides where 79% (6,824/8,639) and 39% (1,138/2,953) of the peptides were exclusively identified in COVID-19 and control immunopeptidomes, respectively (Figures S6F and S6G).

Next, we aimed to examine which factors contribute to this increased immunopeptidome complexity in COVID-19. Therefore, we mapped the HLA class I- and HLA class II-presented peptides to their source proteins and performed comparative profiling on source protein level considering only proteins that were

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**Figure 2.** Mass spectrometry-based characterization of the soluble immunopeptidome in COVID-19 patients

(A) HLA class I allotype population coverage within the COVID-19 immunopeptidome cohort compared to the world population (calculated with the IEDB population coverage tool, [www.iedb.org](http://www.iedb.org)). The frequencies of individuals within the world population carrying up to six HLA allotypes (x axis) of the COVID-19 cohort are indicated as gray bars on the left y axis. The cumulative percentage of population coverage is depicted as black dots on the right y axis. See Figure S4B for the Control cohort.

(B) HLA class I and HLA class II peptide yields of COVID-19 plasma samples (n = 21) as identified by mass spectrometry are indicated in light and dark gray bars, respectively. See Figure S4C for the Control cohort.

(C and D) Peptide length distribution of (C) HLA class I and (D) HLA class II peptides in the COVID-19 immunopeptidome cohort (n = 21). Each line represents one single donor with the relative abundance of peptides depicted on the y axis. See Figures S5A and S5B for Control cohort.

(E) HLA-specific motifs derived from 9mers identified from plasma of UPN03. Motifs were created by submitting all 9mers to the GibbsCluster - 2.0 server.
In-depth immunopeptidome screening revealed the sHLA-restricted presentation of a SARS-CoV-2-derived nucleocapsid peptide in the plasma of COVID-19 patients

For the in-depth screening of naturally presented HLA class I- and HLA class II-restricted peptides derived from the SARS-CoV-2 genome within the soluble plasma immunopeptidome of COVID-19 patients, we analyzed the COVID-19 plasma samples on two different state-of-the-art mass spectrometers (Orbitrap Fusion Lumos and timsTOF Pro). The data processing against the SARS-CoV-2 proteome revealed three peptide sequences originating from the SARS-CoV-2 proteome within the applied FDR of 1% within the timsTOF Pro data with each peptide sequence identified in a different donor. Spectral validation using synthetic peptides was performed for all three sequences. Of these, the sHLA class II-restricted viral peptide KQQTVTLLPAADLD (NCAP_SARS2388-402) derived from the C-terminal region of the nucleoprotein of SARS-CoV-2 could be validated as true identification (Figures 4A and 4B).

DISCUSSION

Several studies have reported elevated levels of SHLA molecules in different pathologies, including infectious, malignant, and autoimmune diseases, and their association with disease severity or outcome. In this study, we quantified total sHLA class I and -DR serum levels in COVID-19 patients, convalescent individuals and a cohort of healthy control donors showing elevated sHLA levels in COVID-19 patients compared to the control cohort. This is in line with recently reported elevated levels of the non-classical sHLA-G in COVID-19 patients. In line with previous publications, the amount of sHLA varied considerably among different individuals. Of note, sHLA class I levels even remained elevated in SARS-CoV-2 convalescent individuals up to six months after infection indicating long-term distorted sHLA release with elevated sHLA class I serum levels associated with persisting COVID-19 symptoms. Patients with post- or long-COVID syndrome suffer from a huge variety of different symptoms including fatigue, shortness of breath or cognitive dysfunction. First evidence suggests that uncontrolled inflammation and autoimmunity plays a role in these patients. In this context, sHLA serum levels might also be elevated in these patients, which would need to be further investigated in large cohort studies.
We further analyzed the soluble immunopeptidome of a COVID-19 and control cohort to characterize disease-associated alterations in the antigenic peptide repertoire carried by sHLA molecules. Thereby, an increased diversity of sHLA-presented peptides, attributable to a COVID-19 signature of related source proteins, was observed compared to the healthy control cohort independently of COVID-19-specific therapies. This indicates that the state of intracellular virus replication, inflammation, and immune activation in COVID-19 is directly mirrored in the soluble immunopeptidome. No direct influence on antigen presentation of the COVID-19-specific therapies remdesivir and dexamethasone was observed. Of note, our study demonstrates a direct correlation of sHLA class I serum levels with the diversity of the soluble immunopeptidome.

Furthermore, we were able to detect a naturally in vivo presented SARS-CoV-2-derived peptide from the nucleoprotein within the soluble plasma of a COVID-19 patient. Thus far, the identification of naturally presented SARS-CoV-2-derived HLA peptides was limited to immunopeptidomics approaches that use artificial systems with in vitro infected, protein-loaded or protein-overexpressed cells. A critical factor for the detection of in vivo presented SARS-CoV-2-derived HLA ligands might be the timepoint of sample collection after infection, which might explain the low frequent detection of SARS-CoV-2-derived peptides within our cohort, in which the samples were collected several days after infection. A recent study detected SARS-CoV-2-derived HLA peptides in cell lines 3 to 24 h post infection with a peak of viral peptides at 6 h indicating rapid HLA presentation of virus-derived peptides that decreases over time. Furthermore, the cells that are primarily targeted by SARS-CoV-2 are not well exposed to the plasma, which might impede the release of sHLA molecules carrying virus-derived peptides by these cells in the plasma. However, especially sHLA class II molecules might not be derived from the SARS-CoV-2 target cells directly, but from antigen-presenting cells and are therefore more reliable to detect within the plasma of the patients. However, we have to emphasize that absence of evidence does not equal evidence of absence as the sensitivity of shotgun mass spectrometric discovery approaches, even in the context of immense technical improvements in the last decades, remains for sure limited as the immunopeptidome is a highly dynamic, rich,
and complex assembly of peptides. Nevertheless, the detection of the nucleoprotein-derived peptide within the soluble immunopeptidome proves the soluble immunopeptidome as an interesting and so far under-investigated source for the detection of in vivo presented virus-derived HLA-presented peptides. For tumor patients the soluble immunopeptidome was previously described as a source for the detection of tumor-associated antigens presented by sHLA molecules, which might serve as potential biomarkers.17–19

It is still a matter of intense debate to what extent sHLA complexes exert pro- or anti-immunomodulatory effects and whether shifts in sHLA levels contribute to disease outcome or are observed as a consequence of disease.11 We here demonstrated elevated sHLA class I and -DR serum levels in COVID-19 representing a highly diverse and disease-associated soluble immunopeptidome reflecting ongoing SARS-CoV-2-induced inflammation. Furthermore, we could demonstrate the association of long-term elevated sHLA class I serum levels with persisting COVID-19 symptoms. On the other hand, we provided evidence for a positive correlation of anti-SARS-CoV-2-specific T cell responses with sHLA class I levels suggesting a positive role of sHLA molecules for anti-viral cellular immunity and T cell activation.15,16 The induction of a potent SARS-CoV-2-directed T cell responses is essential to prevent severe courses of COVID-19.34–41 In line, patients with critical illness, in terms of required invasive ventilation, exhibited conspicuous low sHLA class I levels. Future large cohort studies are needed to further delineate and characterize the functional role of sHLA for T cell immunity and COVID-19 outcome.

Together, the findings of this study indicate an inflammation-driven higher release of sHLA molecules in COVID-19, which directly influence the diversity of the soluble immunopeptidome and positively modulate anti-SARS-CoV-2 cellular immunity.

Limitations of the study
Limitations and caveats of our study include the high inter-individual heterogenicity of sHLA levels requiring large cohorts to determine differences in sHLA levels, the non-matched age distribution between the cohorts as well as the use of different instrument platforms for the immunopeptidome analysis. Furthermore, the individual HLA allotype might influence the sHLA levels,42,43 which should be further analyzed in large cohort studies. Future studies are also required to investigate in controlled and longitudinal settings the influence of COVID-19-specific drug treatments on the immunopeptidome.

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AUTHOR CONTRIBUTIONS
A.N. and J.S.W. designed the study. A.N., J.R., and M.R. performed sample preparation. A.N., N.H.G., J.B., and M.R. performed immunopeptidome experiments. A.N., J.R., and R.K. conducted ELISA experiments. S.H. and A.P. accomplished analysis of SARS-CoV-2 antibody responses. M.R., J.S.H., T.H., H.M., S.G., M.B., and J.S.W. conducted patient data and sample collection as well as medical evaluation. A.N., J.R., J.H., and M.L.D. analyzed data and performed statistical analyses. A.N. visualized data and created the figures. A.N. drafted the manuscript; all authors edited and reviewed the manuscript. J.S.W. supervised the study.

DECLARATION OF INTERESTS
A.N., J.S.H., and J.S.W. hold patents on peptides described in this manuscript secured under the numbers 20_169_047.6 and 20_190_070.1. The other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-human HLA-A,-B,-C | Biolegend | Cat# 311402; RRID: AB_314871; Clone W6/32 |
| HRP anti-human β2 microglobulin | Biolegend | Cat# 280303; RRID: AB_2617014; Clone 2M2 |
| Anti-human HLA class II | In-house production | Clone Tu-39 |
| Anti-human HLA-DR | In-house production | Clone L243 |
| Chemicals, peptides, and recombinant proteins | | |
| HLA monomer | Biolegend | Cat# 280301 |
| TMB substrate | Biolegend | Cat# 421101 |
| Critical commercial assays | | |
| Human HLA Class II Histocompatibility Antigen, DR Alpha Chain (HLA-DRA) ELISA kit | Cusabio | Cat# CSB-EL010497HU |
| Complete™ Protease Inhibitor Cocktail (Roche) | Merck | Cat# 11836145001 |
| Elecsys® anti-SARS-CoV-2 assay | Roche Diagnostics GmbH | Cat# 09203095190 |
| Atellica CH C-Reactive Protein_2 | Siemens Healthineers | Cat# 11097631 |
| Deposited data | | |
| Mass Spectrometry Data | Data generated for this study deposited in PRIDE repository | PXD029567 |
| Supplemental Items | Additional Supplemental Items are available from Mendeley Data | http://doi.org/10.17632/8w3yh5dwx7.1 |
| Software and algorithms | | |
| PEAKS 8.5 | Bioinformatic Solutions | www.bioinfor.com |
| IEDB population coverage tool | IEDB | www.iedb.org |
| GraphPad Prism 9.2.0 | GraphPad Software | www.graphpad.com |
| R version 4.1.1 | R | www.cran.r-project.org |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Juliane S. Walz (juliane.walz@med.uni-tuebingen.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- The mass spectrometry immunopeptidomics data has been deposited to the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD029567.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plasma and serum samples

Peripheral blood (EDTA tubes) and serum (serum tubes) samples from COVID-19 patients (n = 31, 1 - 27 days after first positive SARS-CoV-2 PCR test) were collected at the Department of Internal Medicine I, University Hospital Tübingen, Germany. Serum samples as well as questionnaire-based assessment of donor characteristics and disease symptoms during acute infection from convalescent volunteers after SARS-CoV-2 infection (n = 290, 28 - 170 days after positive SARS-CoV-2 PCR testing, PCR negative and/or symptom-free at the time of sample collection) were collected at the University Hospital Tübingen, Clinical Cooperation Unit Translational Immunology, Germany. Convalescent volunteers’ recruitment was performed by online and paper-based calls. SARS-CoV-2 infection was confirmed by PCR test after nasopharyngeal swab. Peripheral blood or serum samples from healthy volunteers (controls) were collected at the University Hospital Tübingen (n=80). Informed consent was obtained in accordance with the Declaration of Helsinki protocol. The study was approved by and performed according to the guidelines of the local ethics committees (179/2020/BO2, 431/2012BO2). HLA typing was carried out by the Department of Hematology and Oncology, University Hospital Tübingen, Germany. Serum was separated by centrifugation for 10 min at 3,220 \( \times g \) and the supernatant was stored at \(-80^\circ C\). Peripheral blood samples were used for the isolation of plasma after clearing from cells by centrifugation for 10 min at 1,200 \( \times g \) at room temperature without brake. Complete \( ^\text{TM} \) Protease Inhibitor Cocktail (Roche) was added to the plasma followed by centrifugation at 4 \( ^\circ C \) for 10 min at 12,000 \( \times g \). The supernatant was stored at \(-80^\circ C\). Plasma samples were used for subsequent immunopeptidome analysis, serum samples for sHLA quantification. Detailed donor characteristics are provided in Table S1 for the sHLA quantification cohorts and in Tables S4–S6 for the immunopeptidomics cohorts.

METHOD DETAILS

Quantification of sHLA class I molecules by ELISA

For the quantitative determination of sHLA class I concentration in serum samples, flat-bottomed high binding 96-well microplates (Greiner, Microlon\textsuperscript{\textregistered}, 655061) were coated with 100 \( \mu L \) of anti-human HLA-A,-B,-C antibody (clone W6/32, Biolegend) diluted 1:1000 in 100 mM carbonate-bicarbonate coating buffer (\( \text{Na}_2\text{CO}_3, 3.53 \text{ mg/mL} \) and \( \text{NaHCO}_3, 5.6 \text{ mg/mL} \)) per well and incubated over night at 4 \( ^\circ C \). The plates were washed four times with phosphate-buffered saline (PBS) supplemented with 0.5% Tween-20 and subsequently blocked with 200 \( \mu L \) 3% bovine serum albumin (BSA)-PBS solution per well for 2 h at room temperature. Serum samples were diluted 1:200 in 0.5% BSA-PBS. An HLA monomer (Biolegend, 280301) starting at a concentration of 100 ng/mL diluted 1:2 in seven steps was used as standard. After washing four times with 0.5% Tween-20-PBS, 100 \( \mu L \) of the diluted samples, standards and blank samples were loaded per well in triplicates. After incubation for 2 h at room temperature, the plates were washed four times with 0.5% Tween-20-PBS. The horseradish peroxidase (HRP) anti-human \( \beta_2\)-microglobulin antibody (clone 2M2, Biolegend, 280303) was used as detection antibody in a 1:800 dilution (100 \( \mu L \) per well) in 0.5% BSA-PBS with an incubation time of 90 min at room temperature. The plates were washed six times with 0.5% Tween-20-PBS. 100 \( \mu L \) per well TMB substrate (1:1 mixture of TMB substrate A and TMB substrate B) was added and incubated for 15 to 30 min at room temperature in the dark. After stopping the reaction with 100 \( \mu L \) per well 1 M phosphoric acid (\( \text{H}_3\text{PO}_4 \)), the optical density was measured at 450 nm wave length using an ELISA reader (SpectraMax Plus 384, Molecular Devices). The analyses were performed in triplicates and measurement was repeated for coefficient of variation (CV) values >15%. The standard curve was created by plotting the mean absorbance of standards against the known concentration of standards in logarithmic scale, using the four-parameter algorithm. Results were depicted as \( \mu g/mL \).

Quantification of sHLA-DR molecules by ELISA

Serum level of sHLA-DR was determined using the Human HLA Class II Histocompatibility Antigen, DR Alpha Chain (HLA-DRA) ELISA kit (CSB-EL010497HU, Cusabio) following the manufacturer’s protocol. The standard ranges from 18.75 pg/mL to 1,200 pg/mL. Samples were measured in duplicates and measurement was repeated for CV values >15%. The standard curve was created by plotting the mean absorbance of standards against the known concentration of standards in logarithmic scale, using the four-parameter algorithm. Results were depicted as \( \mu g/mL \).
Elecsys® anti-SARS-CoV-2 immunoassay

The Elecsys® anti-SARS-CoV-2 assay is an ECLIA (electrogenerated chemiluminescence immunoassay) assay designed by Roche Diagnostics GmbH and was used according to manufacturer’s instructions. It is intended for the detection of high affinity antibodies (including IgG) directed against the nucleocapsid protein of SARS-CoV-2 in human serum. Readout was performed on the Cobas e 411 analyzer. Negative results were defined by a cut-off index (COI) of <1.0. Quality control was performed following the manufacturer’s instructions on each day of testing.

Determination of laboratory values

C-reactive protein (CRP, wide range) concentration was measured in lithium-heparinized plasma samples using an immunoturbidimetric assay on an ADVIA XPT clinical chemistry analyzer (Siemens Healthineers). Complete blood counts, including white blood cells (WBC), lymphocytes and hemoglobin concentration, were determined with the Sysmex XN 9000 haematology analyzer (Sysmex).

T cell response in convalescent individuals

Data on SARS-CoV-2-specific T cell responses of convalescent individuals assessed by interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assay after 12-day in vitro expansion were retrieved from a previous publication. For this analysis, we considered SARS-CoV-2-specific T cell response intensities against the previously described SARS-CoV-2-specific HLA class I-restricted epitope composition. This SARS-CoV-2-specific epitope composition was designed from immunogenic SARS-CoV-2-derived T cell epitopes derived from different open reading frames (ORF), including spike, nucleocapsid, and membrane proteins, and recognized exclusively in convalescent patients after SARS-CoV-2 infection and not in SARS-CoV-2-unexposed individuals. The epitope composition covers several different HLA class I allotypes to allow for standardized evaluation and determination of intensities of SARS-CoV-2-specific T cell responses. The intensity of T cell responses is depicted as calculated spot counts, which are measured mean spot counts of duplicates in the ELISpot assay normalized to 5 × 10⁶ cells subtracting the normalized mean spot count of the respective negative control.

Isolation of HLA ligands

HLA class I and HLA class II molecules were isolated from snap-frozen plasma samples by standard immunoaffinity purification using the pan-HLA class I-specific W6/32, the pan-HLA class II-specific Tu-39, and the HLA-DR-specific L243 monoclonal antibodies (all produced in-house, University of Tübingen, Department of Immunology) to extract HLA ligands. Isolation was performed as previously described for tissue applying the centrifugation step directly after thawing without a homogenization and sonification step.

Mass spectrometric data acquisition

For comparative immunopeptidome profiling of COVID-19 and control samples, the mass spectrometric analysis was performed on orbitrap mass spectrometers. Peptides were separated by nanoflow high-performance liquid chromatography using a 50 μm × 25 cm PepMap rapid separation column (Thermo Fisher) and a gradient ranging from 2.4 to 32.0% acetonitrile over the course of 90 min. Eluting peptides were analyzed in the online-coupled Orbitrap Fusion Lumos (COVID-19 patient samples) or Q Exactive HF Orbitrap (control samples) mass spectrometer equipped with a nano electro spray ion source using a data dependent acquisition mode employing a top speed (3 s, Fusion Lumos) or top 35 (Q Exactive HF) collisional-induced dissociation (CID, Fusion Lumos HLA class I peptides) or higher-energy collisional dissociation (HCD, Fusion Lumos HLA class II peptides and Q Exactive HF) fragmentation method (normalized collision energy 25 - 35%). Mass range for HLA class I peptide analysis was set to 400 - 650 m/z with charge states 2+ and 3+ selected for fragmentation. For HLA class II peptide analysis mass range was limited to 400 - 1,000 m/z with charge states 2+ to 5+ selected for fragmentation. Regular quality controls ensure the equal performance of both mass spectrometers.

COVID-19 patient samples were additionally analyzed on a timsTOF Pro mass spectrometer (Bruker Daltonics) for in-depth screening of SARS-CoV-2-derived peptides within the immunopeptidome. Peptides were separated on a Bruker nanoElute LC system using a 75 μm × 25 cm Aurora Series emitter column (Ionopticks) and a gradient ranging from 0 to 95% acetonitrile over the course of 60 min with consecutive ramps from 0 to 32% (30 min) and 32 to 40% (15 min), followed by two 5-min ramps to 60 and 95%, respectively. Eluting peptides were analyzed in the online-coupled trapped ion mobility spectrometry (tims)
time-of-flight (TOF) mass spectrometer timsTOF Pro equipped with a CaptiveSpray ion source using a data dependent acquisition mode employing 6 parallel accumulation-serial fragmentation (PASEF) MS/MS scans per cycle using CID fragmentation (collision energy 20 - 59 eV). Ramp time was set to 200 ms, transfer time to 60 ms, and pre-pulse storage to 6 ms. A range of 0.6–1.6 Vs/cm² were used as ion mobility range. The total acquisition time was 60 min. Mass range was set to 600 - 2,000 m/z with charge states 1+ and to 100 - 2,000 m/z with charge states 2+ and 3+ selected for fragmentation, respectively.

Database search
Data processing was performed with PEAKS 8.5 (Bioinformatic Solutions). The data were searched against a FASTA database containing 20,395 reviewed human UniProt entries downloaded on 17/05/2021, supplemented with the 16 reviewed SARS-CoV-2 UniProt entries downloaded on 17/05/2021, 13 novel unannotated virus ORFs whose translation is supported by Ribo-seq, and 230 recurrent or variant-defining mutations from several different SARS-CoV-2 variants, including the variants of concern alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2) and the variants of interest iota (B.1.526) and epsilon (B.1.429). No enzyme specificity was set, peptide mass error tolerances were set to 5 ppm (Orbitrap data) or 20 ppm (TOF data) for precursors and 0.02 Da for fragment ions, oxidized methionine was considered as variable modification with three allowed variable modifications per peptide. Peptide lengths were set to 8 - 20 amino acids for HLA class I and 12 - 30 amino acids for HLA class II. A 1% false discovery rate (FDR) was calculated using a decoy database search approach. The integrated tool PEAKS PTM was used to identified peptides with unspecified post-translational modifications.

Peptide synthesis and spectrum validation
For all experimentally eluted potential SARS-CoV-2-derived peptide sequences synthetic peptides were produced for spectrum validation. Peptides were produced by the peptide synthesizer Liberty Blue (CEM) using the 9 fluorenylmethyl-oxycarbonyl/tert-butyl strategy. Spectrum validation of the experimentally eluted peptides was performed by computing the similarity of the spectra with corresponding synthetic peptides measured in a complex matrix. The spectral correlation was calculated between eluted peptide spectra and synthetic peptide spectra using the intensities of annotated b- and y-ion peaks.

HLA annotation and Gibbs clustering
HLA class I annotation was performed using the donor-specific HLA allotypes depicted in Tables S2 and S3 supplemented with HLA-G*01 in NetMHCpan 4.1 and SYFPEITHI 1.0 annotating peptides with percentile rank below 2% and ≥60% of the maximal score, respectively. Gibbs clustering was performed for all 9mers using the GibbsCluster - 2.0 Server using the default settings.

Comparative profiling and functional enrichment
For comparative profiling of HLA-presented source proteins, we excluded proteins identified exclusively by multi-mapping peptides i.e. peptides that map to different source proteins. These source proteins were annotated based on their UniProt identifiers, which were then loaded into R (version 4.1.1) to generate the belonging KEGG annotations. UniProt identifiers were translated to their respective entrez identifiers using the AnnotationDbi (version 1.54.1) and org.Hs.eg.db (version 3.13.0) packages. Ultimately, the information was used as input for the clusterProfiler package (version 4.0.5) to create a cnetplot image of the annotated KEGG processes.

QUANTIFICATION AND STATISTICAL ANALYSIS
Overlap analysis was performed using BioVenn. The population coverage of HLA allotypes was calculated by the IEDB population coverage tool (www.iedb.org). All figures and statistical analyses were generated using GraphPad Prism 9.2.0 (GraphPad Software). Data are displayed as mean ± SD, boxplots as median with 25% or 75% quantiles and min/max whiskers. Continuous data were tested for distribution and individual groups were tested by use of two-sided Fisher’s exact test, unpaired t-test, unpaired Mann-Whitney-U-test, Kruskal-Wallis test, or paired Wilcoxon signed rank test, all performed as two-sided tests. If applicable adjustment for multiple testing was done. p values of <0.05 were considered statistically significant.