CD8 T cells and antibodies drive SARS-CoV-2 evolution in chronic infection

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Abstract

Since its recent zoonotic spill-over severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is constantly adapting to the human host as illustrated by the emergence of variants of concern with increased transmissibility and immune evasion. Prolonged replication in immunosuppressed individuals and evasion from spike-specific antibodies is known to drive intra-host SARS-CoV-2 evolution. Here we show for the first time the major role of CD8 T cells in SARS-CoV-2 evolution. In a patient with chronic, ultimately fatal infection, we observed three spike mutations that prevented neutralisation by convalescent plasma therapy. Moreover, at least four mutations in non-spike proteins emerged that hampered CD8 T-cell recognition of mutant epitopes, two of these occurred before spike mutations. A comparison with worldwide sequencing data showed that several of these T-cell escape mutations had emerged independently as homoplasies in multiple circulating lineages. We propose that human leukocyte antigen class I contributes to shaping the evolutionary landscape of SARS-CoV-2.

Main Text

Prolonged replication of SARS-CoV-2 in immunocompromised hosts under inefficient immune pressure promotes intra-host diversification and selection of immune escape variants. Recently, a number of single case reports of such individuals have shown within host evolution with a focus on the adaptation of the spike protein to neutralising antibodies¹⁻⁴. Selection pressure imposed by CD8 T-cell responses is a major driving force for viral adaptation in other viral infections such as HIV⁵. T-cell responses in SARS-CoV-2 infection are associated with immune control, but so far the potential of this novel coronavirus to escape these responses and its clinical implications remain unclear⁶. Here we investigate the case of a severely immunocompromised host with considerable viral diversification and show that most of the acquired mutations evade recognition by humoral and cellular immunity.

A woman in her seventies was hospitalised in spring 2020 with COVID-19 and severe ARDS. She was managed in our intensive care unit developing multiple complications and multi-organ failure until her death five months later. Her past medical history included follicular lymphoma treated with standard chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine and prednisolone in combination with the B-cell-depleting anti-CD20 antibody obinutuzumab up to a month prior to presentation. As expected, standard ELISAs (Euroimmune) for SARS-CoV-2-specific IgG and IgA were negative throughout the course of disease. COVID-19-directed treatment included steroids and multiple doses of convalescent plasma (Fig. 1a). Therapy with remdesivir was deemed unsafe due to severe renal failure and known hypersensitivity to the vehicle sulfobutylether- β -cyclodextrin. Virological studies revealed persistently high SARS-CoV-2 RNA levels (Extended Data Fig. 1) with replication-competent virus in respiratory tract specimens until death 156 days following COVID-19 diagnosis.

In order to study intra-host viral evolution, sequential upper and lower respiratory tract specimens were used to generate 21 near full length genomes of SARS-CoV-2 spanning 150 days (Supplementary Table 1). The infecting strain was classified as B.1.1.29 (20B) bearing the spike mutation D614G and clustered

with other sequences circulating at that time (Extended Data Fig. 2). Over the following 5 months the virus diversified extensively with 34 different mutations in coding regions emerging at >40% frequency abundance (Extended Data Fig. 3). To study viral evolution in adaptation to the host adaptive immune response, we focused our analyses on the 12 non-synonymous mutations that became fixed in the dominant variants until death (Fig. 1b).

We recognised three mutations located in the spike gene previously associated with antibody escape: A deletion at position 144 in the N-terminal domain (NTD) (mutation I), and S477N (mutation III) and E484K (mutation II) substitutions in the receptor binding domain (RBD) (Fig. 1c). We performed *in vitro* neutralisation assays testing the activity of serum samples taken from the donors of convalescent plasma against patient isolates. All but one donor showed robust neutralising responses against an early pandemic isolate carrying only the D614G spike mutation (MUC-IMB01) and an autologous isolate sampled at day 20, before the emergence of the additional mutations in the spike gene (Fig. 1d). However, the autologous variant isolated at day 83 carrying the del144 and E484K spike mutations was only weakly neutralised by one donor serum and none of the others tested, showing that the combination of these RBD and NTD alterations was sufficient to almost completely abrogate neutralising activity. Structural analysis illustrates that the two mutations in the RBD and the deletion in the NTD are located in epitopes targeted by exemplary neutralising antibodies REGN10993, Ly-COV555, and 4A8 (Fig. 1e). Taken together, these data indicate that the neutralising polyclonal antibodies administered with the convalescent plasma transfusions mediated immune selection pressure that drove adaptation of the viral spike protein to evade these responses.

We investigated whether the lack of an effective T-cell response might have contributed to the failure to clear SARS-CoV-2 infection. An IFN- γ ELISPOT assay that was performed with a fresh peripheral blood sample on day 127 showed that the patient had strong T-cell responses to antigens spike (N-terminal and C-terminal half), nucleocapsid, and ORF3a. These responses tended to be stronger than those of five immunocompetent convalescent donors at a median of 55 days post-infection (Fig. 2a). Flow cytometry on day 145 demonstrated reduced numbers of peripheral T cells (142/ μ l), most of them CD8⁺ (60%). Many CD8 T cells (31%) co-expressed activation markers CD38 and HLA-DR, whereas CD4 T cells were less activated (Fig. 2b).

Since the patient had strong T-cell responses to SARS-CoV-2 antigens and activated CD8 T cells, we hypothesised that mutations in SARS-CoV-2 might have emerged under selective pressure by CD8 T cells and had led to inactivation of T-cell epitopes. Therefore, we examined whether these mutations were located in potential CD8 T-cell epitopes presented by the patient's HLA class I molecules (Supplementary Table 2). For epitope prediction, we used an approach based on simple anchor motifs (Supplementary Table 3). Out of twelve non-synonymous mutations that became fixed in dominant SARS-CoV-2 variants, nine altered the amino acid sequences of putative CD8 T-cell epitopes (Supplementary Table 4), including the eight earliest mutations (day 41-97). Both nucleocapsid mutations affected the same nonameric B*35:01-restricted candidate epitope with pre-mutation sequence TPSGTWLT γ (Figure 3C). Because patient samples were limited, we next studied the effect of mutations on T-cell recognition by

immunocompetent HLA-matched convalescent donors. We established peptide-stimulated T-cell cultures and tested their reactivity against original and altered epitope peptides. In HLA-B*35:01-positive donor 1, stimulation with nucleocapsid TPSGTWLT_Y peptide, but no other HLA-B*35:01 candidates, expanded a viable T-cell culture that strongly recognized TPSGTWLT_Y. The T332I exchange reduced T-cell recognition, whereas subsequent T325K substitution fully abolished recognition (Figure 3D). Thus, two sequential mutations altering this epitope were required to eliminate T-cell recognition.

T cells could also be expanded from two HLA-A*02:01-positive donors with peptide ALWEIQQV_V from nsp8/ORF1ab, but none of the other six HLA-A*02:01 candidates. When these T cells were stimulated with 15-mer peptides encompassing ALWEIQQV_V or its variant ALWEIQQF_V (ORF1ab V4101F), only the original but not the variant was recognized (Figure 3E). From two HLA-A*01:01-positive donors, T-cell cultures were established by stimulation with mixed peptides CTDDNALAY and CTDDNALAY_Y from nsp9/ORF1ab. T cells from donor 2 recognized each peptide, and recognition of their variants was reduced (Fig. 3F). T cells from donor 3 responded to the decameric but not the nonameric peptide, and again mutation reduced recognition (Extended data Fig. 4). Therefore, CTDDNALAY(Y) may represent two T-cell epitopes recognized by the same or different T cells. T-cell responses to each of them are impaired by the ORF1ab T4164I mutation, which replaces an optimal anchor residue threonine in position 2 by suboptimal isoleucine.

To verify HLA restriction and test the impact of mutations on binding of HLA/peptide complexes to T cells, we stained T-cell cultures with HLA/peptide tetramers. The results confirmed that TPSGTWLT_Y was HLA-B*35:01-restricted and CTDDNALAY(Y) were HLA-A*01:01-restricted (Extended data Fig. 5). Cultures from donor 1 were dominated by CD8 T cells that bound to the HLA-B*35:01/TPSGTWLT_Y tetramer (77%), and consecutive mutation strongly reduced binding (down to 1.6% for the double mutant). Similarly, cultures from donor 2 contained substantial proportions of HLA-A*01:01/CTDDNALAY(Y) tetramer-binding CD8 T cells (18–23%), and mutant peptides produced lower tetramer binding. A late-stage cryopreserved PBMC sample of the patient (day 145) was available for tetramer staining. While a large fraction (29.7%) of CD8 T cells bound to the wild type HLA-B*35:01 TPSGTWLT_Y multimer, binding was reduced (12.4%) with the single mutant peptide and nearly abolished (0.5%) with the double mutant (Figure 3G). Thus, the patient had maintained high numbers of CD8 T cells specific for the original epitope 84 days after the second mutation, corroborating strong cellular immune pressure, and two mutations were required to fully prevent these T cells from recognizing their target epitope.

We demonstrate emergence of immune escape mutations against antibodies as well as CD8 T-cell responses in an immunocompromised individual with persistent, ultimately fatal COVID-19. Several previous case reports have described rapid viral diversification in the setting of insufficient immune control^{1–3}. These studies have focused on the adaptation of the SARS-CoV-2 spike protein and identified several mutations located in neutralizing epitopes of the RBD and NTD. Strikingly, also in our case two mutations in the RBD and one in the NTD of spike emerged that have previously been shown *in vitro* to reduce neutralising activity of antibodies targeting these regions^{7–9}. Our data shows sensitivity of the initial autologous virus to neutralising antibodies of transfused convalescent plasma that was abrogated

by the subsequent mutant isolate. This further corroborates that these mutations were selected by and resulted in escape against the transfused polyclonal antibodies. We propose judicious use of convalescent plasma in the management of immunocompromised COVID-19 patients with prolonged infection given its limited therapeutic efficacy and the risk of selection of immune escape variants¹⁰.

Notably, the antibody escape mutations that evolved in this patient are also present in defined variants of concern/interest: E484K in Beta and Gamma, the deletion at position 144 in Alpha, and the mutation S477N in Iota (B.1.526.2) and Kappa (B.1.620)¹¹. The independent emergence of such homoplastic mutations in separate individuals and their selection advantage to become predominant variants in different populations demonstrates convergent evolution in adaptation to the human immune system⁴.

Our results show that selective pressure by CD8 T cells shapes SARS-CoV-2 evolution in the setting of immunosuppression. In our case, CD8 T-cell escape was initiated prior to antibody escape, and prior to administration of convalescent plasma. Subsequent antibody escape may have been facilitated by impaired cellular immunity. It was proposed that mutations in SARS-CoV-2 variants have little impact on overall CD4 or CD8 T-cell reactivity in convalescents or vaccinees¹². However, individual T-cell epitopes and their variants may strongly affect control of infection in immunocompromised patients with reduced T-cell diversity, as observed for other complex viruses¹³. Evidence is now accumulating that CD8 T-cell responses to ancestral SARS-CoV-2 are blunted by mutations occurring in certain viral variants^{14,15}. Of note, most of the CD8 immune escape mutations observed in the present patient have independently emerged in multiple globally circulating lineages (Supplementary Table 6), which strongly suggests that CD8 T cells contribute to SARS-CoV-2 evolution in the wider population.

Recent data has demonstrated the role of CD8 T cells in recovery from COVID-19 in patients with haematological malignancies¹⁶. However, a direct effect of CD8 T cells on virus evolution and immune evasion in a patient has not been shown prior to the present study. It is well established that strong CD8 T-cell responses are directed to nucleoprotein epitopes including HLA-B*35:01/TPSGTWLTY^{6,17-19}. CD8 responses to nsp8/9/10 were less prominent in peptide-screening studies, in contrast to our results suggesting their substantial effect in this patient^{17,18}. However, unbiased screening with intracellular expression fragments covering SARS-CoV-2 identified each of the epitopes HLA-A*02:01/ALWEIQQVV and HLA-A*01:01/CTDDNALAYY among the three most frequently detected CD8 T-cell responses for their HLA restriction¹⁹, consistent with a significant role of these epitopes and the non-structural proteins nsp8 and nsp9 in T-cell control of infection. CD8 T cells specific for epitopes from structural and non-structural proteins of a virus may functionally complement each other, since they recognize cells at different stages and contexts of infection²⁰. In particular, CD8 T cells that recognize epitopes from regulatory proteins expressed early in virus-infected cells may be able to eliminate such cells before virions can be produced.

In conclusion, we find that detailed characterization of the interaction between SARS-CoV-2 and the host in immunocompromised, particularly B-cell-depleted patients, provides a unique opportunity to follow

viral adaptive evolution during the course of chronic infection. Selection pressure shaping SARS-CoV-2 evolution is mediated not only by antibody responses, but importantly also by CD8 T-cell responses.

Declarations

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Author contributions

All authors contributed to the data collection, design of the study, writing of the manuscript and revision of the manuscript. E.K., M.M., A.M., M.A. conceived the study and wrote the manuscript. M.M., J.C.H., C.S. participated in clinical study design and data collection. A.G., H.B., S.K., M.H.A., Alexandra R. performed sequencing and bioinformatic analyses. K.-P.H. performed structural analysis. A.D. performed HLA analysis. S.G. contributed to convalescent plasma collection. P.G., K.M. performed neutralization assays. A.H., A.-W.M., A. L., Anna R., Enrico G., S.Z., A.M. performed laboratory analysis. E.K., J.C.H., Eric G., T.W., S.-S.S., O.W., H.-J.S. acquired patient data and samples and performed medical evaluation. M.S., O.T.K., M.v.B.-B., R.W. contributed resources. All authors read, edited and approved the manuscript.

Competing interests statement

The authors declare no competing interests.

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Figure 1

Intra-host viral evolution and adaptation to neutralising antibodies. a, Chronological order of events in relation to the time point of COVID-19 diagnosis by the first positive SARS-CoV-2 RNA PCR result (day 0). Administration of convalescent plasma and positive viral cultures from patient respiratory samples on

Vero E6 cells are shown. The time points of emergence of the CD8 T-cell (1a, 1b and 2) and antibody escape mutations (I, II and III) which are further investigated in this study are indicated. b, Heatmap with frequencies of emerging de novo mutations in this patient. Only non-synonymous mutations that became fixed in predominant variants are presented (see Table 1 and Fig. 3 in the Supplementary Material for full list of mutations and sequence accession numbers). Nucleotide substitutions, gene and protein names, and amino acid substitutions are displayed. The escape mutations discussed later are numbered as in a. c, Position of the three mutations in the spike gene coloured by domains. The deletion at position 144 (I) is located in the N-terminal domain (NTD) and S477N (III) and E484K (II) are located in the receptor binding domain (RBD). S1: Spike subunit 1, S2: Spike subunit 2, NTD: N-terminal domain, RBD: Receptor-binding domain, FP: fusion peptide, CD: connector domain; TM: transmembrane domain. d, Heatmap with virus neutralization titre (VNT100) corresponding to the reciprocal serum dilution that fully neutralized viral infection in vitro using Vero E6 cells. Neutralising activity of serum samples taken from the donors of convalescent plasma on the day of donation was tested. The time point of each donor's plasma transfusion to the patient is indicated. Neutralising responses were tested against an isolate from the early pandemic phase only with the spike mutation D614G (Muc-IMB01) and autologous isolates of the patient. The time points of isolation of the autologous viruses and additional mutations in the spike protein to D614G are indicated. e, Illustration of the three mutant amino acids using structures of SARS-CoV-2 spike domains in complex with antibodies REGN10993, Ly-COV555 and 4A8. Left and right subpanels: illustration of the positions of the mutation and binding of selected antibodies on the spike trimer. One spike protomer bound by an antibody is shown in blue, the other two in grey. Antigen-binding fragments (Fabs) or single-chain variable fragments (Fvs) are depicted in yellow (heavy chain) and orange (light chain). Middle subpanels: details of the antibody-spike protein interfaces, highlighting the roles of the amino acid changes in these interfaces in antibody escape.

Figure 2

(Note: in-text references to "Figure 3" refer to Figure 2) Recognition and evasion of CD8 T-cell epitopes. a, T-cell responses to selected SARS-CoV-2 antigens in the patient on day 127 after diagnosis and in five convalescent donors (HD1–5) after mild infection were tested in ex vivo IFN- γ ELISPOT. b, Activation markers CD38 and HLA-DR expressed on the patient's peripheral CD8 or CD4 T cells were analyzed on day 145 by flow cytometry. c, Sequences of original (WT) and mutant epitopes (1a, 1b, 2, 3) further

studied below are shown in one-letter amino acid code with mutant residues in red. d-f, Recognition of these three epitopes and their mutant versions by peptide-specific CD8 T-cell cultures from convalescent donors was studied by overnight co-culture of T cells, HLA-matched activated B cells as antigen-presenting cells, and peptide at the indicated concentrations; IFN- γ secreted into the supernatant was measured by ELISA. T-cell cultures were from donor 1 (d) and donor 2 (e and f). g, Peripheral blood cells from the patient at day 145 were stained with HLA-B*35:01/peptide tetramers loaded with the TPSGTWLT γ peptide or its two mutant versions.

Supplementary Files

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