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Pressured Cytotoxic T-cell epitope strength among SARS-CoV-2 variants correlates with COVID-19 severity

CTL immune pressure SARS-CoV-2 variants

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Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article

Author Contributions

Author	Role
Vishal Rao	Study design, data analysis, manuscript writing
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Ethical Statement

Ethical approval for this study was obtained from the Institutional Ethics Committee at Bangalore Medical College and Research Institute, Bangalore, India (BMCRI/PS/02/2021-21), and Indian Institute of Science (1-26062020), Bangalore, India. Written informed consent was obtained from all study participants before sample collection.

Abstract

Heterogeneity in susceptibility among individuals to COVID-19 has been evident through the pandemic worldwide. Cytotoxic T lymphocyte (CTL) responses generated against pathogens in certain individuals are known to impose selection pressure on the pathogen, thus driving emergence of new variants. In this study, we probe the role played by host genetic heterogeneity in terms of HLA-genotypes in determining differential COVID-19 severity in patients. We use bioinformatic tools for CTL epitope prediction to identify epitopes under immune pressure. Using HLA-genotype data of COVID-19 patients from a local cohort, we observe that the recognition of pressured epitopes from the parent strain Wuhan-Hu-1 correlates with COVID-19 severity. We also identify and rank list HLA-alleles and epitopes that offer protectivity against severe disease in infected individuals. Finally, we shortlist a set of 6 pressured and protective epitopes that represent regions in the viral proteome that are under high immune pressure across SARS-CoV-2 variants. Identification of such epitopes, defined by the distribution of HLA-genotypes among members of a population, could potentially aid in prediction of indigenous variants of SARS-CoV-2 and other pathogens.

Keywords: SARS-CoV-2 variants; COVID-19; epitopes; CTL epitope strength; immune pressure

Introduction

Severe Acute Respiratory Syndrome Corona-Virus 2 (SARS-CoV-2) which is responsible for the Coronavirus Disease 2019 (COVID-19) pandemic, has infected over 755 million people around the world, as of February 13th, 2023[1]. Despite large-scale vaccination, the pandemic did spread extensively owing to the ability of variants of concern to escape from vaccine-induced immune responses. Infection with SARS-CoV-2 presents a wide range of clinical outcomes ranging from asymptomatic, mild and moderate disease states to severe and critical states associated with severe pneumonia, Acute Respiratory Distress Syndrome (ARDS)[2] and lymphopenia[3]. The immune mechanisms responsible for containing the viral infection have been shown to be dysfunctional in case of severe COVID-19, with sustained cytokine production leading to a hyper-inflamed state[4]. One of the major arms of the immune system implicated in combating viral infections is the Cytotoxic T Lymphocyte (CTL) or CD8+ T cell response, which exhibits a functionally exhausted phenotype in severe COVID-19 cases[5]. Higher frequencies of SARS-CoV-2 specific T cells have been detected previously among convalescent individuals with mild COVID-19 suggesting long term protectivity offered by CTL responses[6,7].

Genetic heterogeneity across humans is known to significantly influence severity of a disease[8]. The Human Leukocyte Antigen (HLA) genotype is one of the host factors which exhibits high sequence variation across individuals. The HLA Class I genotype, composed of 3 sets of HLA alleles

– A, B and C, can directly influence the CTL response which makes individuals with certain HLA genotypes more susceptible to certain diseases. Previous studies have identified many HLA Class-I alleles which are significantly associated with susceptibility to severe COVID-19[9]. Several advances in identifying the viral fragments recognized as non-self by T-cells, known as immunodominant T cell epitopes, have facilitated the quantification of T cell responses through prediction of binding affinities of HLA alleles to pathogenic epitopes using various bioinformatic techniques and machine-learning algorithms[10]. These epitope prediction tools have been used to determine susceptibilities of HLA alleles to severe COVID-19 based on epitope recognition[11] and to identify peptide-based vaccine targets based on high population coverage of epitopes[12,13,14]. In addition, structural prediction of epitopes has also provided insights regarding constrained regions of the viral proteome that are robust to mutations[15,16].

Host immune responses typically impose selection pressure on the pathogen, driving emergence of mutated variants that are capable of escaping the antiviral response. Among the variants of SARS-CoV-2, the Delta variant (B.1.617.2) dominated over other variants during the intense COVID-19 wave in India in April-May 2021, due to its higher transmissibility and partial evasion of the host immune responses[17]. Previous studies have suggested the possibility of T cell immune escape in SARS-CoV-2 variants[18,19,21] as well as in isolates of the parent Wuhan-Hu-1 strain[20] either restricted to certain HLA alleles[18,19,21] or covering larger populations[20,22]. On the contrary, mutations arising in the emerging variants have been reported to facilitate escape, mainly from the neutralizing antibody response[23,24] rather than the T cell responses which are relatively more conserved across variants[25,26]. However, experimental studies showing loss in CTL activity among SARS-CoV-2 variants due to mutations in HLA-restricted epitopes of SARS-CoV-2 proteins ORF3a and Nucleocapsid[27] have necessitated further investigation of CTL immune escape at the global level. BA.1, one of the first sub-variants of Omicron to have emerged, has been known to house upto 37 mutations in the Spike protein that enable escape from antibody mediated neutralization[28]. Further, the BA.2, BA.4 and BA.5 sub-variants of Omicron that have emerged recently, have been shown to escape recognition from BA.1-infected sera[29]. However, the extent of CTL escape among these newly emerged variants remains to be studied. Cross-reactive T-cell responses to BA.1 spike protein due to prior exposure via natural infection or vaccination[30] have been observed and robust T-cell responses against BA.1 in majority of individuals with HLA-dependent escape in a few have been documented previously[31]. An effective T-cell response is highly dependent on presentation of epitopes that show high affinity binding with host HLA molecules. Hence, in order to analyse and explain mutation-driven escape of viral variants from host T cell responses, it becomes necessary to identify the epitopes which offer protectivity to the host and could be under selection pressure.

In this study, we aim to probe the role played by host genetic heterogeneity in determining differential COVID-19 susceptibility and identify epitopes that are more influential in determining disease outcome. From the protein sequence data of SARS-CoV-2 variants available on the NCBI Virus database[32], we bioinformatically obtain CTL epitope sets for each variant using the IEDB epitope prediction tools[33]. We then compare these epitope sets across variants and shortlist epitopes which are under immune pressure across variants, which we refer to as 'pressured epitopes'. We model the epitope-HLA recognition in a COVID-19 cohort from Bangalore, by considering HLA genotypes as a whole and estimate T-cell response due to these pressured epitopes. Our model indicates that the estimated extent of CTL response explains different severity levels of COVID-19. Individuals who have experienced severe COVID-19 have a significantly lower estimated CTL response as compared to individuals who experienced milder symptoms. Finally, we shortlist 6 pressured-protective CTL epitopes that are likely to be pivotal in eliciting an effective CTL response among individuals in our cohort and compute protectivity scores to the epitopes and to the HLA alleles which provide clues about susceptibility.

Methods and materials

SARS-CoV-2 protein sequences and HLA Class-I allele data

A total of 14 SARS-CoV-2 variants including 4 Variants of Concern (B.1.1.7 - Alpha, B.1.351 - Beta, P.1 - Gamma, B.1.617.2 - Delta), 4 Variants of Interest (B.1.525 - Eta, B.1.526 - Iota, C.37 - Lambda, B.1.617.1 - Kappa) as listed in the official WHO webpage as of August, 2021 along with the 4 sub-variants of Omicron - BA.1, BA.2, BA.4, BA.5 and 2 other variants namely, B.1.526.1 and P.2 and the parent Wuhan-Hu-1 strain (A) were considered for our study. Amino acid sequences of 10 proteins, namely - ORF1ab polyprotein, Spike glycoprotein, ORF3a, Envelope protein, Membrane protein, ORF6, ORF7a, ORF8, Nucleocapsid protein and ORF10 were obtained from NCBI Virus[32] (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/).

A total of 1827 HLA Class-I alleles were obtained from 240 ethnic groups with HLA-allele frequency data from the Allele Frequency Net Database (AFND)[34] (http://www.allelefrequencies.net/). The ethnic groups were shortlisted based on i) documentation of frequencies of all 3 HLA Class I alleles - A, B and C, ii) High resolution HLA allele data with polymorphisms denoted for all the alleles documented.

Prediction of CTL epitopes

HLA Class I Epitope predictions for the 10 proteins were performed using the NetMHCPan BA 4.1 tool[35] made available by IEDB (Immune Epitope Database)[33] (http://tools.iedb.org/mhci/). A comprehensive list of 1827 HLA Class I alleles covered by the 240 ethnic groups were considered for pMHC binding predictions. Length of the predicted epitopes was restricted to 9 amino acids. Predicted 9-mers which bind to a given HLA allele with IC50 < 50 nM were considered as strong binding epitopes. Epitope predictions were performed for all 14 variants of SARS-CoV-2.

Immune pressure parameters

For the immune pressure plot, 2 parameters were considered for each protein: the protein sequence pairwise distance and the epitope set pairwise distance between each pair of variants. These parameters are defined as follows:

Let V_1 and V_2 be the two variants being compared and let $P_1 = \{a_{1i}\}_{1\leq i\leq n}$ and $P_2 = \{a_{2j}\}_{1\leq j\leq n}$ be the aligned amino acid sequences of a particular protein belonging to the 2 variants with an alignment length n. Let $E_1 = \{e_{1a}\}_{1\leq a\leq p}$ and $E_2 = \{e_{2b}\}_{1\leq b\leq q}$ be the sets of distinct epitopes of the protein corresponding to the variants V_1 and V_2 respectively, where p and q are the respective number of epitopes of the protein.

Protein sequence pairwise distance $= 1 - \frac{|P_1 \cap P_2|}{|P_1 \cup P_2|}$

Epitope set pairwise distance = 1 - $\frac{|E_1 \cap E_2|}{|E_1 \cup E_2|}$

These 2 parameters are calculated for each pair of variants and are represented as points in a 2D scatter plot (Figure 2B,C)

Shannon Entropy and Epitope Density:

For each residue of a protein, 2 further parameters namely, Shannon Entropy and Epitope Density are calculated as follows:

Let $P_V = \{p_i\}_{1 \le i \le 11}$ be the set of amino acid sequences for a particular protein across the 11 SARS-CoV-2 variants along with Wuhan-Hu-1 considered, where $p_i = \{a_{ij}\}_{1 \le j \le n}$ for all $1 \le i \le 11$ is the amino acid sequence of protein p of variant i with an alignment length of n. Let $R_j = \{r_{ij}\}_{1 \le i \le 11}$ be the set of unique amino acids across variants for protein p at residue j and $M_j = \{m_{kj}\}_{1 \le k \le |R_j|}$ where m_{kj} is the number of variants possessing amino acid a_{kj} at position j.

Propensity of an amino acid i at position j =

$$p_{ij} = \frac{\text{Number of variants possessing amino acid i at position j}}{\text{Total number of variants}} = \frac{m_{ij}}{11}$$

Shannon Entropy at position j = $S_j = -\sum_{k=1}^{|M_j|} p_{kj} . \log p_{kj}$

Epitope density at position j =

E_j = Number of epitopes in the Wuhan strain protein at position j of the particular protein

HLA typing of COVID-19 Bangalore cohort

The patient recruitment is described in detail in separate studies at Banerjee et al, 2022[36] and an unpublished manuscript. In brief, COVID-19 patients with no prior COVID-19 infection or vaccination were recruited during September-December, 2020. A total of 36 Covid-19 patients (median age = 52 $\pm$ 16, both male and female participants) were included in this Bangalore cohort. The disease severity classification was performed based on the clinical symptoms presented by the patient at the hospital, which are as follows: Asymptomatic (n=7), Mild (n=9), Moderate (n=10) and Severe (n=10).

RNA isolation was performed from whole blood using Paxgene Blood RNA kit (Cat# 762164) following manufacturer protocol. Paired-end RNA sequencing was performed in an Illumina Hiseq 4000 instrument. The library preparation and sequencing was performed by Biokart India Private Limited, Bangalore. The raw sequencing data was subjected to adapter trimming with the tool TrimGalore (version 0.6.6) and Class-I HLAs were predicted from the RNA sequencing reads using the software arcasHLA v0.2.0[37] with standard settings.

Epitope and HLA allele protectivity

Protectivity of epitopes and HLA alleles were calculated based on the HLA genotypes corresponding to the clinical samples with varying disease severities.

Let $\{HG_{Aj}\}_{1\le j\le 7}$, $\{HG_{Mij}\}_{1\le j\le 9}$, $\{HG_{Moj}\}_{1\le j\le 10}$ and $\{HG_{Sj}\}_{1\le j\le 10}$ be the sets of individuals with asymptomatic, mild, moderate and severe COVID-19 respectively, represented by their HLA-genotypes. Each HLA genotype is comprised of a set of 6 HLA alleles. Since some HLA-alleles are associated with more than one disease severity, the frequency of association of a HLA allele H with a disease severity DS, $f_{H,DS}$ where $DS \in \{A, Mi, Mo, S\}$ is calculated as follows:

$$\mathrm{f_{H,DS}} = \frac{\text{Number of individuals with HLA allele H and disease severity DS}}{\text{Total number of individuals with HLA allele H}}$$

Each HLA genotype is also associated with a corresponding set of distinct epitopes $\{E_{sj,k}\}_{s\in\{A,Mi,Mo,S\},1\le k\le n,1\le j\le n_s}$ where n is the number of epitopes recognized by the HLA-alleles comprising the genotype and n_s is the number of individuals with disease severity s recognizing the epitope. Since epitope sets recognized by individuals with different disease severities overlap in many cases, the frequency of association of an epitope E with a disease severity DS, $\mathrm{f_{E,DS}}$ where $\mathrm{DS} \in \{\mathrm{A, Mi, Mo, S}\}$ is calculated for each epitope as follows:

$$\mathrm{f_{E,DS}} = \frac{\text{Number of individuals recognizing epitope E with disease severity DS}}{\text{Total number of individuals recognizing epitope E}}$$

A protectivity value is then assigned for each disease state as follows:

$$\text{Protectivity value} = \begin{cases} 3\,;\ \text{if disease severity is Asymptomatic} \\ 2\,;\ \text{if disease severity is Moderate} \\ 1\,;\ \text{if disease severity is Mild} \\ 0\,;\ \text{if diesease severity is Severe} \end{cases}$$

The protectivity score for a HLA-allele H is then calculated as follows:

$$\text{Protectivity score of HLA} - \text{allele H} = \sum_{\mathrm{DS}\in\{\mathrm{A,Mi,Mo,S}\}} \mathrm{f_{H,DS}} * (\text{Protectivity value of disease severity DS})$$

The protectivity score for an epitope E is calculated as follows:

$$\text{Protectivity score of Epitope E} = \sum_{\mathrm{DS}\in\{\mathrm{A,Mi,Mo,S}\}} \mathrm{f_{E,DS}} * (\text{Protectivity value of disease severity DS})$$

Results

Overview

In this study, we identify CTL epitopes that are under immune selection pressure and offer protection against severe COVID-19. We use a bioinformatic tool, NetMHCPanBA to arrive at a preliminary set of strong-binding CTL epitopes of SARS-CoV-2 Wuhan-Hu-1, Variants of Interest (VOIs) ,Variants of Concern (VOCs) and Omicron sub-variants. We then identify those epitopes that are under immune selection pressure through sequence analysis. We studied a cohort of 36 individuals with varying severity of COVID-19 in Bangalore, infected during the first wave of COVID-19 caused by Wuhan-Hu-1 and identified their HLA genotypes. We then identified a shortlist of locally pressured CTL epitopes with respect to the local cohort. Put together, we further shortlist 6 locally pressured Wuhan-Hu-1 epitopes that offer high protection against severe disease (Figure 1).

CTL epitope prediction

Putative CTL epitopes in the SARS-CoV-2 proteome were predicted using the well-established NetMHCPanBA 4.1[35] method available at IEDB[33]. Epitope predictions were carried out for the top 10 abundant proteins in the viral proteome (see Methods), whose complete sequences were documented on the NCBI Virus database for (i) the 5 Variants of Concerns (VOCs) namely B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta) and B.1.1.529 (Omicron) (ii) 4 Variants of Interest (VOIs) namely B.1.525 (Eta), B.1.526 (Iota), B.1.617.1 (Kappa) and C.37 (Lambda) (as documented in the official WHO website as of August 2021) along with the Wuhan strain (A) and (iii) 2 non-virulent variants – B.1.526.1 and P.2. Predicted epitopes binding with their cognate HLA-alleles with IC50 < 50nM were classified as strong binding epitopes. Strong binding epitopes were predicted individually for all HLA Class I alleles documented across various ethnicities around the world in The Allele Frequency Net Database[34]. A total of 2022 strong binding epitopes exhibiting high affinity binding with 1299 HLA-alleles were identified (Appendix File-1).

Immune pressure on CTL epitopes across SARS-CoV-2 variants

Next, we probed if some of the CTL epitopes of SARS-CoV-2 are under higher host selection pressure, resulting in mutations that aid in their escape from host recognition. So, for each pair of SARS-CoV-2 variants, we computed the pairwise distance between epitope sets for each protein and compared it with the whole protein sequence pairwise distance. For most proteins, we observed that the epitope distance was larger than the whole protein sequence distance (Figure 2B, C), showing that CTL epitopes exhibit higher variability, and to be under negative selection pressure leading to immune evasion by the virus. The regions of high variation overlapped significantly (high Shannon entropy), (Figure 2B, C) with regions of high epitope density, further supporting the finding that there was indeed a set of epitopes under immune pressure.

Effective antigen presentation is known to result in a strong CTL response and the corresponding epitopes would be under immune pressure, resulting in their loss of antigenicity in variants that emerge subsequently [27]. To probe this, from the pool of all strong binding epitopes from the parent Wuhan-Hu-1 strain, we identified those that have lost recognition in at least one of the variants, irrespective of the HLA allele. The Spike glycoprotein (Figure 2D) contained a large number of these epitopes. On the other hand, ORF6, a highly conserved protein across SARS-CoV-2 variants, lacked pressured epitopes altogether. Interestingly, HLA alleles, A*02 and B*15 alleles that are globally associated with effective peptide presentation[38], exhibited the highest loss in recognition. A ranked list of HLA-alleles with highest loss in epitope recognition is provided in Appendix Table 1.

Correlating pressured CTL epitope strength with COVID-19 severity

Whole-blood transcriptomes from COVID-19 patients (n=36) with varying severity of the disease (asymptomatic, mild, moderate and severe) were collected from patients in Victoria Hospital, Bangalore, India, for separate studies in the laboratory[36] (unpublished manuscript) which has been utilized here. In this study, we identified HLA Class-I genotypes in these samples from the whole-blood RNA-seq data (GSE196822) using the arcasHLA software[37],and grouped them based on disease severity (Figure 3A). The predicted HLA genotypes for each of the samples in this Bangalore cohort are provided in Appendix Table 2. We set out to identify epitopes that offer the highest protection in the Bangalore cohort. Epitopes recognized by each HLA genotype within the cohort were assigned a protectivity score based on the severity of disease exhibited by the individual (Figure 3B) as follows. We assigned a protectivity value for each disease severity, ranging from 0 for severe to 3 for asymptomatic disease states. We computed a protectivity score for each epitope as the sum of protectivity values of the 4 disease states, weighted by the frequency of occurrence of the epitope in individuals with the particular disease state. Epitopes with a protectivity score ≥2 were shortlisted as 'protective epitopes' (Figure 3B).

Next, we checked for loss in recognition of each protective epitope among SARS-CoV-2 VOCs, VOIs and the Omicron sub-variants BA.1, BA.2, BA.4 and BA.5, with respect to HLA alleles within the Bangalore cohort. Most of the protective epitopes were conserved across variants (Appendix Figure 2) and the trend of epitope loss was largely similar among the Omicron sub-variants (Figure 3C). This indicates that theoretical recognition of BA.2, BA.4 and BA.5 CTL epitopes is similar to the BA.1 variant.

We then checked if epitopes under immune pressure differentiate severity states. For this, we considered 'pressured' CTL epitopes that have lost recognition in at least one of the VOIs and VOCs including Omicron sub-variants. The CTL epitope strength of an individual was calculated as the number of distinct pressured epitopes recognized by the HLA alleles comprising the HLA genotype. We did not observe any significant difference in pressured CTL epitope strength across COVID-19 severity states irrespective of whether we considered the loss in recognition only among VOIs and VOCs excluding Omicron (Appendix Figure 3D) or including Omicron (Appendix Figure 3E). Given the cross-reactivity of T-cell responses to BA.1[30] and the lower severity of Omicron infection, we shortlisted epitopes that have lost recognition in at-least one VOC or VOI but retained recognition in all the sub-variants of Omicron, which we refer to as the locally pressured Omicron conserved (LpOc) epitope set. The number of LpOc epitopes exhibited a decreasing trend with increasing disease severity, which was most pronounced between asymptomatic and severe groups (Figure 3D). This significant difference in recognition was not observed when we considered globally pressured epitopes that have lost recognition with respect to the global HLA allele pool (Appendix Figure 3B,C,F).

We further shortlisted the LpOc epitope set by considering epitopes that are also protective in nature. This resulted in a set of 6 pressured-protective CTL epitopes, which correlate very clearly with disease severity considered as 4 categorical groups (Asymptomatic vs. Moderate p-value = 0.0021, Asymptomatic vs. Severe p-value = 0.0069) and even when considered as a continuous severity spectrum (correlation coefficient (r) = -0.5273, p-value = 0.001) (Figure 3E). A list of these pressured-protective epitopes along with the variant(s) that harbor(s) the escape mutation(s), is provided in Table 1. Interestingly, 4 out of the 6 epitopes had lost recognition in either Kappa (B.1.617.1) or Delta (B.1.617.2) variants.

Protectivity of HLA alleles

HLA allele information of each individual was extracted from the bulk RNA-seq data obtained from whole blood samples of COVID-19 patients in the Bangalore cohort. Using this information, we identified HLA alleles that recognize the set of 6 pressured-protective epitopes (Table 1). As expected, these alleles predominantly occurred in asymptomatic and mild individuals (Figure 4A). However, a few asymptomatic individuals did not express any of these alleles, possibly suggesting the role of non-HLA protective factors in influencing disease severity. This also allowed us to study the association of individual HLA alleles with disease severity. We observed that many HLA alleles mapped to multiple disease states in the cohort (Figure 4B). Here again, we computed a protectivity score to each HLA allele based on disease severity in the individual expressing the allele. HLA alleles A*02:03, A*02:06, A*03:02, A*29:01, B*07:05, B*38:02 and C*15:05 were seen to confer the highest protection while alleles B*07:06, B*49:01 and C*07:01, offered the least protection in our cohort (as they were present in only asymptomatic individuals and severe individuals respectively). A list of HLA genotypes which offer highest protection against COVID-19 is provided in Appendix Table 3. A gradient of disease states from completely asymptomatic in case of alleles with higher protectivity, to purely severe in case of alleles with least protectivity was observed (Figure 4B) suggesting a major role of HLA Class-I mediated presentation of a selected epitope pool to CD8+ T cells in determining disease outcome.

Discussion

Heterogeneity in HLA genotypes is known to influence differential disease susceptibilities among individuals in many diseases[8]. Such heterogeneity in HLA distribution leading to differences in T cell responses, is reflected in the wide range of disease outcomes observed in COVID-19 as well[9]. Susceptibility is mainly attributed to occurrence of certain high-risk HLA alleles which bind poorly to SARS-CoV-2 epitopes thus resulting in a suboptimal CTL response[11,39]. Analysis of CTL epitope sets in an individual at the HLA genotype level is needed to gain insights into the effect of heterogeneity in presentation. Epitope prediction tools such as NetMHCPanBA, coupled with HLA allele data from databases containing HLA allele information (eg., Allele Frequency Net Database) enable the identification of epitopes recognized by indigenous HLA alleles that might be currently infeasible to capture via experiments.

We predicted CTL epitope strength using HLA allele data from the Bangalore cohort and considered variants of interest (B.1.525, B.1.526, C.37, B.1.617.1 - VOIs), variants of concern (B.1.1.7, B.1.351, P.1, B.1.617.2 - VOCs) and Omicron subvariants (BA.1, BA.2, BA.4, BA.5) for our analysis. We observe that several CTL epitope sequences vary more than the non-epitopic regions in the same proteins, possibly indicating that SARS-CoV-2 variants are under CTL immune pressure. We shortlisted protective CTL epitopes which display a protectivity score larger than a threshold value corresponding to mild and asymptomatic disease states. By comparing loss of protective epitope recognition by HLA alleles within the locally generated Bangalore cohort, we showed that level of CTL immune escape is largely similar among the sub-variants of Omicron that have emerged recently. We constructed a pressured epitope set comprising epitopes which are under immune pressure across the SARS-CoV-2 variants considered. We observed that recognition of pressured epitopes could contribute to mild symptoms among Wuhan-Hu-1-infected individuals. However, by pruning the epitope set after considering those preserved in Omicron (LpOc), we identified a smaller set of epitopes which differentiate significantly among the severity groups. Overall, we aimed to estimate the significance of epitopes that offer protection against severe COVID-19 and we found that LpOc epitopes comprise one such set. LpOc epitopes represent a subset of epitopes that are lost among VOCs and VOIs (except Omicron), possibly indicating a role of CTL escape in higher fitness of these variants.

Among the HLA alleles, A*02 and B*15 which have been previously reported to be strong epitope binders[38], showed the highest loss in epitope recognition among variants. It is important to note that the COVID-19 blood samples were collected in September 2020 when Wuhan-Hu-1 was the most prevalent in India. Thus, higher recognition of these locally pressured epitopes by asymptomatic or mild individuals compared to those with moderate or severe COVID-19 possibly implies that the epitopes which initially offered protectivity were under higher immune pressure. This could have led to the emergence of SARS-CoV-2 variants with mutations in these pressured regions as a means of CTL immune escape. This also concurs with previous reports where cross-reactive T cell responses to spike protein were observed in BA.1-infected individuals[30]. This observation indicated two points - (a) potential cross-reactive memory T cell responses to Omicron sub-variants developed over time post natural infection or vaccination could majorly contribute to lower severity of infection and (b) T cell immune pressure might be operating at a cohort level rather than a global level and hence, epitopes which offer protection may differ across populations due to diversity in the cognate HLA alleles which recognize them. We then considered the intersection of protective and locally pressured Omicron conserved epitopes to arrive at a set of 6 pressured-protective epitopes that could differentiate between asymptomatic and moderate/severe states. Among these 6 epitopes, 4 of them lost recognition in either the Kappa or Delta variants that led to the second COVID-19 wave in India during April-May 2021, which was regarded to be more severe than the first wave with the parent Wuhan-Hu-1 strain[17]. In addition, one of these epitopes VMFTPLVPF showed the highest loss in recognition (5 HLA alleles) in the Delta variant, as compared to other Wuhan-Hu-1 epitopes. Given that the Delta and Kappa variants were first documented in India, this possibly suggests cohort-level HLA-driven CTL immune escape of these epitopes, thus contributing to the higher virulence of these variants during the second COVID-19 wave in India.

We used a scoring mechanism similar to that for protective epitopes to define protective HLA alleles in our cohort. Among these, HLA-B*15:03 which has been shown to be protective against viral infections in previous studies[38] appeared among the top ranked HLA alleles in our study in terms of protectivity score as well as magnitude of epitope loss across variants, suggesting that the protection conferred by the allele against Wuhan-Hu-1 epitopes could have placed the specific epitopes under higher immune pressure. In addition, HLA-B*46:01 did not show high affinity binding with any of the

SARS-CoV-2 epitopes in our study, consistent with previous reports[38]. Previous studies have also reported an immunodominant and conserved nucleocapsid epitope $NP_{105-113}$ restricted by the HLA allele B*07:02[40], yet it is not associated with higher protectivity by itself . Although this allele-epitope pair did occur in our strong binding epitope set, the protectivity of the epitope was low since B*07:02 was not a protective allele in our cohort, as also reported by the previous study. We note that similar correlations of CTL epitope strength with COVID-19 severity might not be observed in other cohorts as well as certain individuals within our cohort, where non-HLA dependent immune responses and presence of different comorbidities could dominate and influence severity. Thus, it becomes essential to study HLA-dependent T cell responses in the background of comprehensive host immune responses. In summary, we notice that the protectivity offered by certain epitopes of SARS-CoV-2 against severe COVID-19 by virtue of an effective CTL response generated in the host, potentially subjects these epitopes to selection pressure.

We acknowledge some of the limitations that accompany the approaches undertaken. Some of these include – i) the TAP proteasomal pathway, vesicular trafficking of HLA bound peptides from the ER to the cell surface and absence of TCRs specific to viral epitopes which resemble self-peptides due to thymic negative selection, are further aspects which need to be considered while quantifying CTL epitope strength based on epitope presentation. ii) High affinity binding of certain epitopes could not be validated due to lack of previously available experimental binding affinity studies of indigenous HLA alleles considered in our study. Nevertheless, there is extensive support for the predicted epitopes from literature. Out of 577 experimentally validated epitopes from over 25 publications[41], 482 high binding epitopes appeared in our study. (iii) COVID-19 is known to present a wide range of immune pathologies in patients[42] due to which CTL response need not be a major contributing factor in determining disease severity in some cases. Thus, our hypothesis of lower CTL epitope recognition in severe disease states might not be applicable to all the COVID-19 patient samples considered in our study. Despite these and the small sample size in each category, a clear trend can be observed. Our study shows that although multiple factors could contribute to severity, CTL epitope recognition as a factor by itself is sufficient to show a clear trend. Our framework also allows incorporation of some of these aspects into future studies, once the aforementioned data becomes available.

Large-scale *in silico* analysis of viral and host genomes in recent years has provided a holistic approach towards analysing host-pathogen interactions and ascertaining evolutionary paths adopted by pandemic- or epidemic-causing pathogens. Our current analysis lays emphasis on the role played by host genetic heterogeneity in determining COVID-19 disease outcome and driving evolution of new SARS-CoV-2 variants. The results obtained in our study provide deeper evolutionary insights regarding the prevailing host-pathogen arms race in nature along with potential clinical implications. Identifying pressured epitopes within populations could aid in prediction of possible mutations in future variants of SARS-CoV-2 based on the local selection pressure imposed by highly represented HLA-alleles within the population and assist in better preparedness against the ongoing pandemic.

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Tables

Epitope (Position)	Protein	Variants	HLA allele(s)
VMFTPLVPF (3130-3138)	ORF1ab polyprotein	B.1.525 (VOI) B.1.526 (VOI)	C*03:02 B*15:01 B*15:05 B*15:02 A*32:01
QIYKTPPIK (787-795)	Spike glycoprotein	B.1.617.1 (VOI)	A*03:01
SANLAATKM (1021-1029)	Spike glycoprotein	B.1.617.1 (VOI) B.1.1.7 (VOC)	C*03:02
ASLPFGWLI (39-47)	ORF3a protein	B.1.617.1 (VOI)	B*58:01 A*02:06
TANPKTPKY (3356-3364)	ORF1ab polyprotein	B.1.617.2 (VOC)	C*03:02
YLALYNKYK (3207-3215)	ORF1ab polyprotein	P.1 (VOC)	A*03:02

Table 1: List of 6 pressured-protective LpOc epitopes. Variants harboring the mutated epitope that has lost recognition and the HLA-alleles from the cohort that have lost recognition to the mutated epitope are listed.

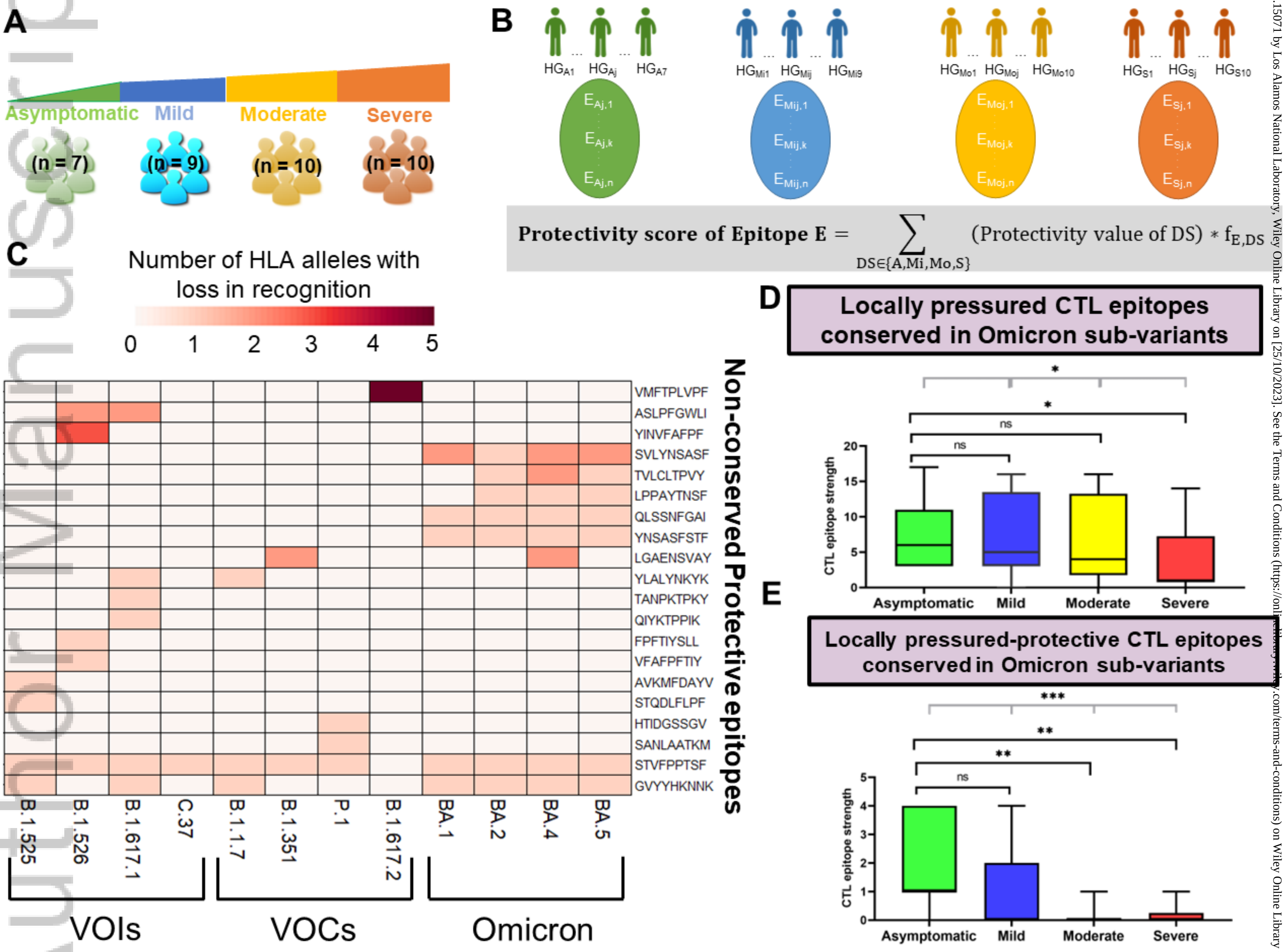
Figure legends

Figure 1: Schematic overview of the study. CTL epitope prediction using NetMHCPanBA was performed for SARS-CoV-2 Wuhan-Hu-1, variants of concern (VOCs), variants of interest (VOIs) and 4 Omicron sub-variants. Strong binding epitopes with predicted IC50 < 50nM were shortlisted. HLA genotypes of 36 individuals from Bangalore infected with Wuhan-Hu-1 with varying severities were obtained. Strong binding epitopes lost in atleast one VOC or VOI with respect to HLA-alleles from our cohort were shortlisted as locally pressured epitopes. These epitopes were further filtered based on conservation among Omicron sub-variants to obtain a set of locally pressured Omicron conserved (LpOc) epitopes. Parallelly, epitopes were assigned a protectivity score based on their recognition across severity states and those above a score of 2 were shortlisted as protective epitopes. Finally, the intersection of LpOc epitopes and protective epitopes was reported as a set of 6 pressured-protective epitopes.

Figure 2: Analysis of CTL epitopes under immune pressure. (A) The set of strong binding epitopes are filtered based on presence of CTL immune selection pressure at these regions, to obtain a set of pressured epitopes. The antigenicity of the protein as well as its epitope set variability in terms of the number of pressured epitopes are then computed. (B) and (C) Multiple sequence alignment of a representative epitope under immune pressure across the 11 variants of SARS-CoV-2, excluding Omicron is depicted (top) for (B) Nucleocapsid protein and (C) ORF3a protein. Sequence variation in the protein is computed using the Shannon entropy index which measures the variability at each residue of the protein (see Methods) (middle - red). Distribution of epitopes within the protein is represented as the epitope density at each residue (middle - green). Symmetric difference between epitope sets for each pair of variants is plotted against the whole-protein sequence distance (bottom). The points in the scatter plot lie above the diagonal (y=x) for both proteins indicating higher selection pressure on epitopic regions. (D) Number of pressured epitopes in each protein of SARS-CoV-2, recognized by the HLA alleles (top 10 based on the number of epitopes lost) are represented as a heat map (top). Contribution of each protein to the pressured epitope set is depicted by an inverted bar plot. Spike glycoprotein and ORF1ab polyprotein are seen to be the major contributors to the pressured epitope set.

Figure 3: Correlation of predicted CTL epitope strength with COVID-19 severity. (A) HLA-genotypes of COVID-19 patients from the Bangalore cohort, with 4 levels of disease severity - asymptomatic, mild, moderate and severe were identified using HLA-typing. CTL epitope strength measured in terms of (B) Schematic representation of computation of epitope protectivity score: Let $[HG_{Aj}]_{1\leq j\leq 7}$, $[HG_{Mij}]_{1\leq j\leq 9}$, $[HG_{Moj}]_{1\leq j\leq 10}$ and $[HG_{Sj}]_{1\leq j\leq 10}$ be the sets of individuals with asymptomatic, mild, moderate and severe COVID-19 respectively, represented by their HLA-genotypes. Each HLA genotype is associated with a corresponding set of distinct epitopes $[E_{sj,k}]_{s\in\{A,Mi,Mo,S\},1\leq k\leq n,1\leq j\leq n_s}$ where n is the number of epitopes recognized by the HLA-alleles comprising the genotype and n_s is the number of individuals with disease severity s. Since epitope sets recognized by individuals with different disease severities overlap in many cases, the frequency of association of an epitope E with a disease severity DS, $f_{E,DS}$ where $DS \in \{A, Mi, Mo, S\}$ is calculated for each epitope. A protectivity value is then assigned for each disease state ranging from 0 for severe cases to 3 for asymptomatic cases. The protectivity score for an epitope is computed as the sum of the 'protectivity value' for each disease severity weighted by the frequency of association of the epitope with the particular disease severity (see Methods). (C) Heatmap representing the extent of loss of recognition of non-conserved protective CTL epitopes among SARS-CoV-2 VOCs, VOIs and the Omicron sub-variants BA.1, BA.2, BA.4 and BA.5. Intensity of the red color is proportional to the number of HLA alleles that have lost recognition of the particular epitope from the parental Wuhan-Hu-1 strain in the particular variant(D,E) Box-plot depicting the number of (D) locally pressured Omicron conserved CTL epitopes and (E) shortlisted pressured-protective CTL epitopes recognized across COVID-19 severity states within the Bangalore cohort. Pairwise comparisons were performed using the Wilcoxon rank sum test (significance marked in black) and Spearman correlation across all 4 severity states was performed by treating disease severity states on an integer scale according to their protectivity values (significance marked in grey). Correlation coefficient (r) = -0.3664 in (D), -0.5273 in (E). (NS - Not Significant, * - p-value $\leq$ 0.05, ** - p-value $\leq$ 0.01, *** - p-value $\leq$ 0.001) .

Figure 4: Association of HLA-alleles with COVID-19 severity. (A) Schematic representation of HLA genotypes of individuals in the Bangalore cohort, sorted and colored based on COVID-19 severity. Each circle represents an HLA allele and a string of 6 HLA-alleles in a line represents an HLA-genotype. The circles (HLA alleles) are shaded with various shades of grey based on the number of pressured-protective epitopes the allele recognizes. (B) Protectivity scores of HLA alleles (top) based on the disease severity they associate with, along with the distribution of disease severity of patients expressing the allele (bottom) is shown. HLA alleles with higher protectivity are largely seen in asymptomatic individuals while those with lower protectivity are observed in individuals with severe disease.



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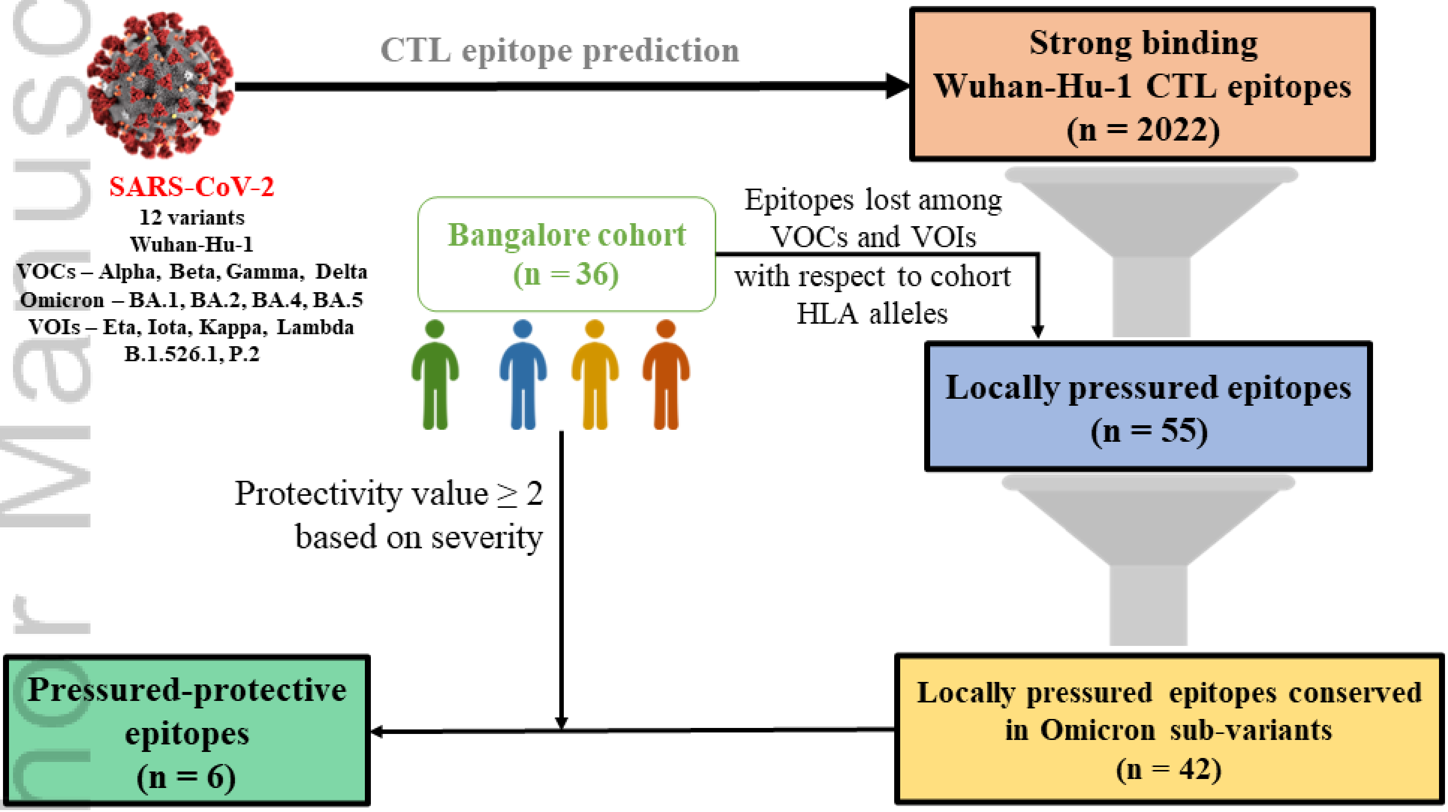

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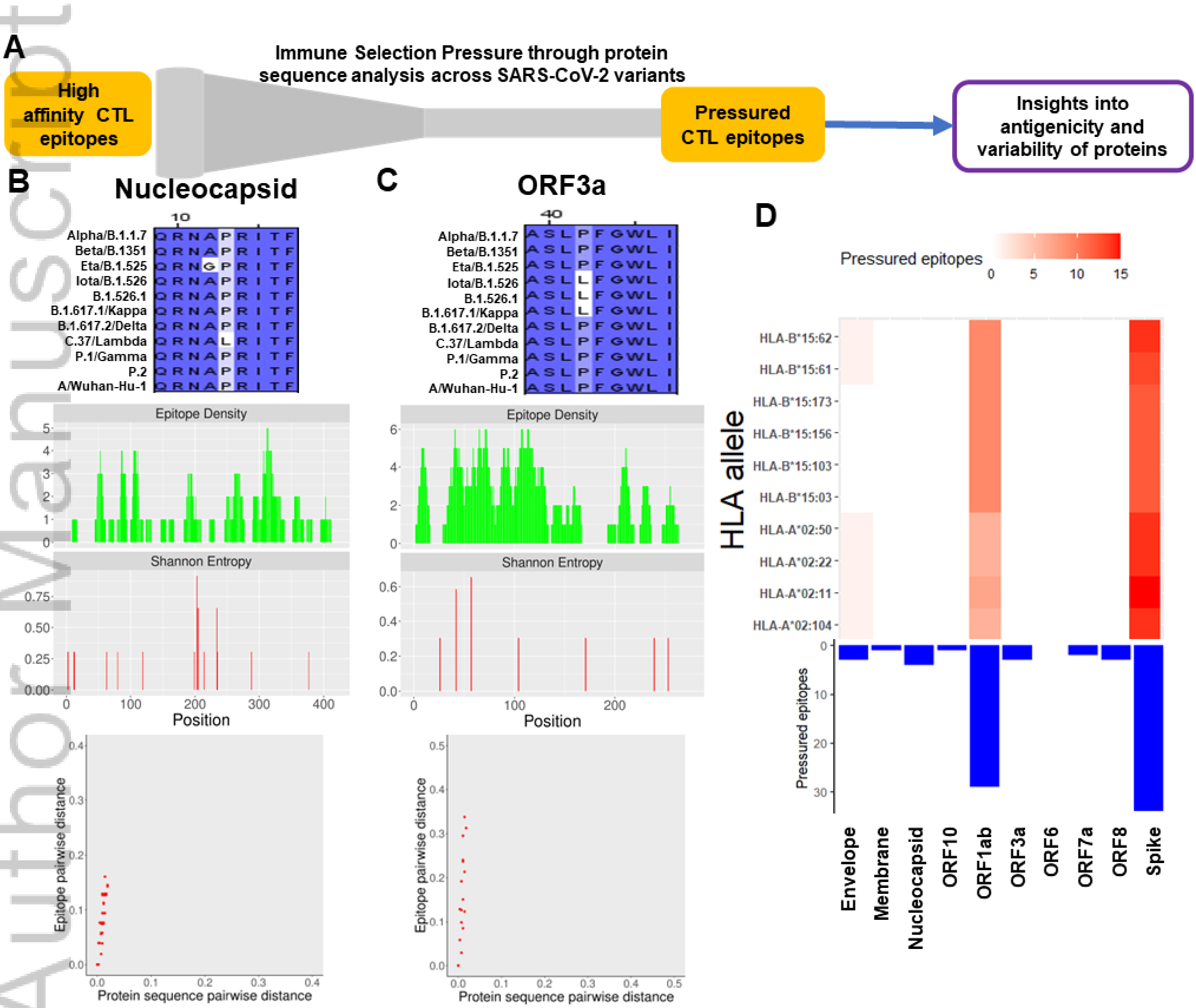

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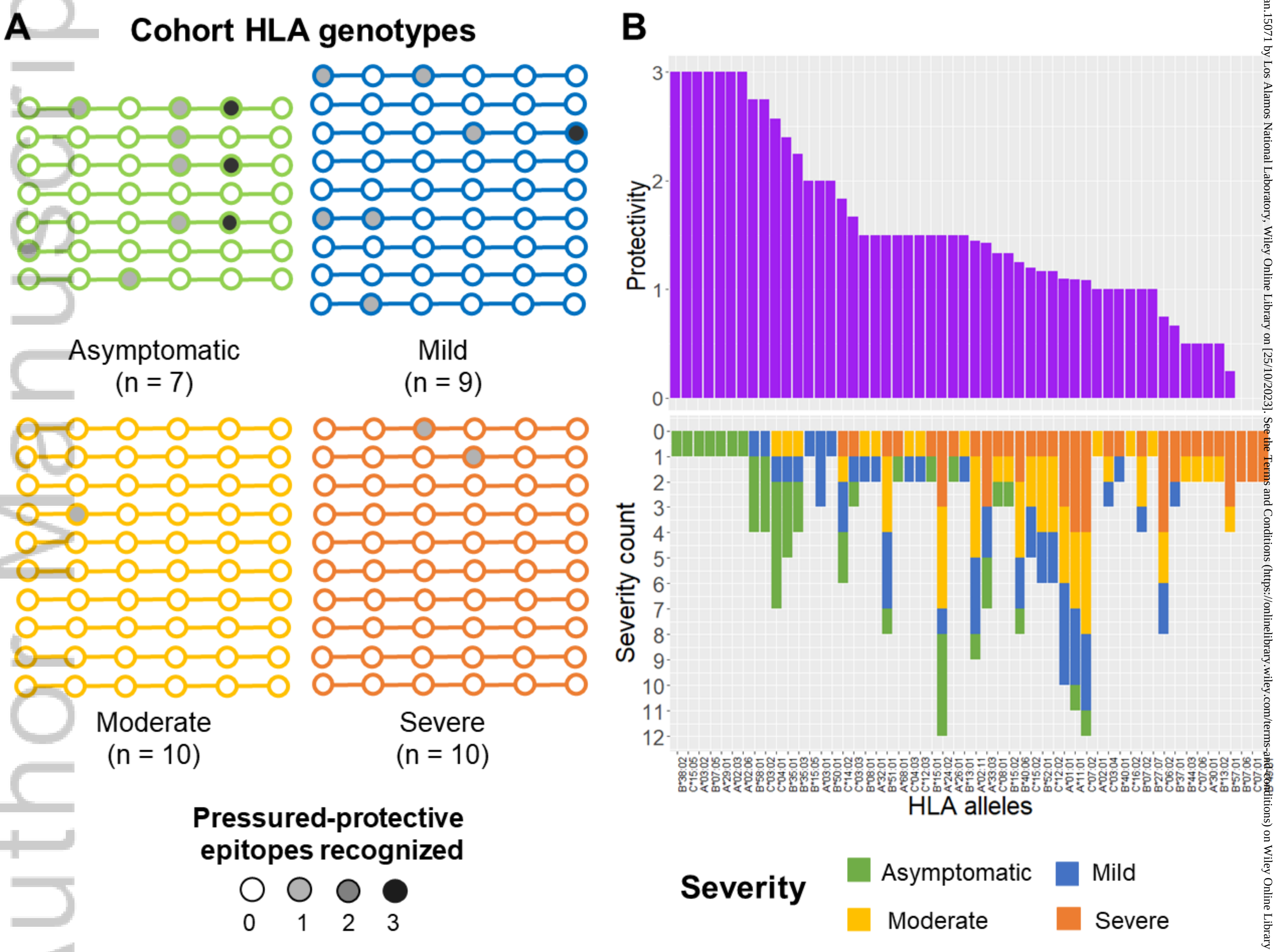

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