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HLA-E-restricted SARS-CoV-2 epitopes drive CD8$^+$ T cell memory in convalescent and vaccinated individuals: implications for the design of next-generation vaccines and immunotherapeutics

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Abstract

Background Human leukocyte antigen-E (HLA-E) is a non-polymorphic, non-classical HLA class Ib molecule that presents signal peptides from classical HLA class Ia molecules to natural killer (NK) cell receptors. Emerging evidence suggests that HLA-E also presents viral peptides to CD8$^+$ T cells, potentially influencing the control of viral infections. However, CD8$^+$ T cell responses to viral epitopes presented by HLA-E remain largely unexplored.

Objective This study investigates the potential of SARS-CoV-2-derived peptides presented by HLA-E to elicit CD8$^+$ T cell responses and to identify T cell-mediated immunity in SARS-CoV-2 infection.

Methods We describe seven peptides from SARS-CoV-2 that display substantial conservation among the predominant strains from December 2021 to February 2025. These peptides fit within the HLA-E pocket and elicit HLA-E-restricted CD8$^+$ T cell responses by producing cytokines.

Results HLA-E/SARS-CoV-2-restricted CD8$^+$ T cells were identified in the blood of convalescent patients, predominantly expressing TNF-α, with lower levels of IFN-γ and IL-2, in response to predicted immunogenic epitopes. These cytokines were detected at higher frequencies in convalescent patients but were nearly absent in hospitalized patients with severe COVID-19. HLA-E/SARS-CoV-2-restricted CD8$^+$ T cells were induced after BNT162b2 mRNA vaccination, with their frequencies increasing with more vaccine doses. Furthermore, they were significantly elicited in vaccinated individuals after SARS-CoV-2 infection.

Conclusion These findings underscore the translational potential of targeting HLA-E-restricted CD8$^+$ T cell responses in next-generation vaccine design. The high conservation of HLA-E-presented epitopes across SARS-CoV-2 variants suggests that these peptides may offer a potentially valuable basis for expanding studies on vaccineinduced cellular

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immunity, independent of viral sequence drift. This strategy could be extended beyond SARS-CoV-2 to other rapidly evolving pathogens, providing a framework for broad-spectrum, T cell-focused vaccine platforms in precision vaccinology.

Keywords HLA-E, SARS CoV-2, Cytokines, $CD8^+$ T cells, COVID-19 disease

Background

The COVID-19 pandemic, caused by SARS-CoV-2, has had a profound impact on global health and economics [1]. Current vaccines primarily aim to stimulate neutralizing antibodies [2, 3]; however, the emergence of SARS-CoV-2 variants may compromise their long-term efficacy [4, 5]. In contrast, most $CD4^+$ and $CD8^+$ T cell responses remain largely intact [6, 7]. T cells play a critical role in the immune response to SARS-CoV-2 infection and vaccination [8–13]. Studies have shown that most individuals recovering from COVID-19 develop strong and broad SARS-CoV-2-specific $CD8^+$ T cell responses, suggesting these cells may help reduce disease severity [14–16]. Patients with mild symptoms tend to exhibit a higher frequency of polyfunctional, multi-epitope classical $CD8^+$ T cell responses compared to those with severe symptoms, which may contribute to controlling infection [17, 18]. On the contrary, severe COVID-19 disease is associated with the reduction and exhaustion of $CD8^+$ T cells [19, 20]. However, during infection, the expression of classical HLA class I molecules in Calu-3 cells is significantly reduced, while HLA-E expression remains unchanged, enabling recognition by T cells [21].

HLA-E is a nonclassical HLA class Ib molecule expressed on most cell surfaces, primarily through two functional alleles, HLA-E*01:01 and HLA-E*01:03. These alleles differ by a single amino acid at position 107 - arginine in HLA-E*01:01 and glycine in HLA-E*01:03, located outside the peptide-binding groove [22]. This indicates that both alleles have a similar range of peptide-binding abilities, although cell surface stability and peptide affinity vary slightly. The primary role of HLA-E is to present a highly conserved nonameric signal peptide, VMAPRTLVL (VL9-peptides), derived from the leader sequence of classical HLA-Ia molecules to NK cell receptors (CD94–NKG2A/C), thereby regulating NK cell activity [22, 23]. Additionally, HLA-E can also present pathogen-derived peptides to cytotoxic T cells, activating unconventional T cells within the adaptive immune system, and induce solid HLA-E-restricted $CD8^+$ T cell responses [22].

In contrast, the immunogenic potential of HLA-E-restricted $CD8^+$ T cell epitopes remains largely unexplored, despite HLA-E's low polymorphism, which makes it an attractive target for broadly applicable vaccines. Moreover, unlike classical HLA molecules, HLA-E resists downregulation by certain viral immune evasion proteins, including HIV Nef and SARS-CoV-2 ORF8 [21, 24, 25]. Additionally, preclinical studies have shown that rhesus cytomegalovirus (RhCMV) vectors can elicit robust MHC-E–restricted $CD8^+$ T cell responses, capable of controlling simian immunodeficiency virus (SIV) infection in rhesus macaques [26]. This suggests that HLA-E-restricted $CD8^+$ T cells may maintain immune surveillance even when virus-infected cells evade recognition by HLA-A- and HLA-B-restricted counterparts [21, 25, 26].

In this study, we explored the role of HLA-E-restricted $CD8^+$ T cells in the immune response to SARS-CoV-2. Since multi-omics approach is useful in evaluating the host response and in identifying markers of clinical severity [27]; through in silico analysis, we identified seven peptides derived from six distinct SARS-CoV-2 proteins and assessed their binding affinity to the HLA-E molecule. Furthermore, we assessed their ability to elicit antigen-specific HLA-E-restricted $CD8^+$ T cell responses using both in vitro and ex vivo assays. Collectively, our findings underscore the potential of HLA-E-restricted peptides in mediating T cell responses against SARS-CoV-2, highlighting their relevance in the development of peptide-based vaccines and therapeutic strategies.

Materials and methods

Study participants

A total of 89 participants were enrolled and categorized into three groups: convalescent ($n = 40$), hospitalized ($n = 22$), and vaccinated ($n = 27$), according to their COVID-19 status and vaccination history (Table 1). The selection was based on the availability of peripheral blood mononuclear cells (PBMCs) and clinical data. The study adhered to the Declaration of Helsinki and was approved by the Ethical Committee of the A.O.U.P. "Paolo Giaccone" Hospital (protocol code 09/2021). Written informed consent was obtained from all participants. Hospitalized COVID-19 patients were recruited from three medical centers in Palermo, Italy: A.O.U.P. "Paolo Giaccone" University Hospital, A.R.N.A.S. "Civico Di Cristina e Benfratelli" Hospital, and Villa Sofia/Cervello Hospital. Vaccinated individuals were enrolled after receiving two or three doses of the Pfizer/BioNTech BNT162b2 mRNA vaccine.

In silico peptide prediction

The SARS-CoV-2 genome (strain 2019-nCoV/USA-WA1–A12/2020; MT020880) was obtained from the NCBI Protein Database. Peptide binding affinities

Table 1 Subjects enrolled in the study

Characteristic	Hospitalized	Convalescent	Vaccinated (Pfizer/BioNTech BNT162b2 mRNA vaccine: two or three doses)
No. of donors	22	40	27
Median age, years (range)	65 (41–92)	38 (26–66)	28 (20–60)
Sex no. (%)			
Female	10 (45,45)	17 (42,5)	16 (59,3) 12 with 2 doses* 6 with three doses
Male	12 (54,55)	23 (57,5)	11 (40,7) 7 with two doses 4 with three doses

*Two samples were used for blocking antibody assays

were predicted using NetMHC 4.1 [28], which identified cytotoxic T lymphocyte epitopes across ten viral proteins. Strong binders (%Rank < 0.5) and weak binders (%Rank < 2) were selected and filtered for HLA-E specificity via the Immune Epitope Database Analysis Resource (IEDB). NetMHC 4.1 was also used to predict binding affinities for longer peptides and to incorporate structural information from MHC molecules. Sequence homology was assessed using BLAST at an 80% similarity threshold, ensuring specificity and relevance to SARS-CoV-2.

SARS-CoV-2 epitope conservation

The conservation of HLA-E-restricted peptides derived from seven SARS-CoV-2 proteins-Spike, Nucleocapsid, ORF8, 3C-like Protease, RNA-dependent RNA Polymerase, and Exonuclease was assessed using the Los Alamos AnalyzeAlign tool [29]. The analysis was conducted on over 400,000 publicly available SARS-CoV-2 sequences from the GISAID database, collected between June 15, 2021, and February 6, 2025. The tool calculates the frequency of each amino acid and mutation at each position, visualized through sequence logos generated by WebLogo. In these logos, the Y-axis represents residue probability, and the X-axis corresponds to amino acid positions based on the reference genome NC_045512. Stack height indicates overall conservation at each position, while the height of individual symbols reflects the frequency of specific amino acids. A focused analysis was also performed on a subset of over 90,000 sequences from the JN0.1 lineage, collected between December 1, 2021, and February 6, 2025. Mutation frequencies and sequence alignments were examined using a reference genome, with data updates from GISAID on February 12, 2025.

HLA-E/peptide stabilization assay

Peptide binding to HLA-E was evaluated using a stabilization assay in RMA-S cells (RRID: CVCL_2180) transfected with HLA-E*01:03 and human β2-microglobulin. The expression index was calculated based on geometric mean fluorescence intensity (MFI) values. Peptides were obtained from Bio-Fab Research (Rome, Italy) at ≥99% purity. They were reconstituted at 5 mg/mL in DMSO, diluted to 1 mg/mL in Dulbecco's Phosphate Buffered Saline (DPBS, Euroclone), and used at a final concentration of 5 µg/mL. RMA-S/HLA-E01:03 cells (TAP2-deficient murine lymphoma cells stably transfected with HLA-E01:03 and human β2-microglobulin; kindly provided by J.E. Coligan, NIAID, USA) were cultured overnight at 37 °C 5% CO_2 with test peptides at 37 °C for 24 hours in Iscove's medium supplemented with 1% glutamine, 20 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. Peptide binding to HLA-E was assessed by measuring surface HLA-E expression via flow cytometry using a PE-conjugated anti-HLA-E monoclonal antibody (REA1031, Miltenyi Biotec). Data were acquired on a BD FACSAria™ and analyzed with FlowJo software (BD Biosciences). Results were expressed as MFI. The VL9 peptide (from the HLA-C leader sequence) and a non-binding control peptide (WNSNNLDSKVGG) were used as positive and negative controls, respectively.

Blood sampling, PBMC isolation, and stimulation

Peripheral blood was collected in heparin tubes (Greiner Bio-One) from convalescent and hospitalized patients (September 2020), and from vaccinated individuals at 6–9 months post-second dose (January 2021) and 12 months after the booster (November 2023). PBMCs were isolated via density gradient centrifugation using Lympholyte-H (CEDARLANE). Unused cells were cryopreserved in TexMACS Medium (Miltenyi Biotec) with FBS and 20% DMSO, pre-chilled at −80 °C and then stored in liquid nitrogen. Thawed PBMCs were seeded (4×10^5 cells/well) into 96-well U-bottom plates. In preliminary experiments to assess HLA-E peptides immunogenicity, PBMC were incubated with HLA-Class I-negative K562 cells that had been transfected with HLA-E*0103 (K562/HLA-E) [30], kindly provided by E. H. Weiss, Department of Biology, Anthropology and Human Genetics, Ludwig-Maximilians-Universitat, Munich, Germany) pulsed for 1 hour with single peptides

at concentration of 5 µg/ml. Cells were then washed with RPMI to eliminate unbound peptides. Peptide pulsed K562 cells were used as negative control: In subsequent experiments on cytokine production PBMC were stimulated for 18 hrs with PepTivator® SARS-CoV-2 Prot_S (1 µg/mL/peptide), a pool of HLA-E-restricted peptides, or peptide SP09 (5 µg/mL), and incubated at 37 °C with 5% CO_2 in the presence of 5 µg/mL Brefeldin A (Sigma, St. Louis, MO, USA).

Intracellular cytokine staining and CD107 degranulation assay.

PBMCs were stimulated with peptides for 18 hrs, then harvested and washed with PBS (EuroClone) and prepared for intracellular staining procedure. Viability staining was performed using Viobility 405/452 Fixable Dye (Miltenyi Biotec) for 15 minutes at room temperature in the dark. After washing, surface staining was conducted using anti-human CD3 PerCP-Vio 700, CD4 PE-Vio 770, and CD8 APC antibodies (all from Miltenyi Biotec). Cells were fixed with IC Fixation Buffer (eBioscience) and permeabilized twice with Intracellular Staining Permeabilization Wash Buffer (BioLegend). Intracellular cytokine staining was performed using fluorochrome-conjugated antibodies against IFN-γ FITC (REA600), TNF-α PE (REA656), and IL-2 PE-Vio 770 (REA689) (Miltenyi Biotec) in permeabilization buffer. Samples were acquired on a BD FACSLyric and analyzed with FlowJo™ Software v10.9 (BD Biosciences). $CD8^+$ T cells were gated as $CD3^+$ $CD8^+$ $CD4^-$. A positive response was defined as > 0.01% cytokine-producing cells; values below this threshold were considered negative and set to zero [30]. Figure S1 shows the gating strategy.

Cytotoxic activity was assessed by CD107 degranulation assay. Briefly, PBMC were stimulated for 6 hours as described in Sect."Blood sampling, PBMC isolation, and stimulation", and then cells were harvested and washed with PBS (EuroClone) and prepared for the surface staining procedure with viability staining was performed using Viobility 405/452 Fixable Dye (Miltenyi Biotec) for 15 minutes at room temperature in the dark. After washing, surface staining was conducted using anti-human CD3 PerCP-Vio 700, and CD8 APC antibodies (all from Miltenyi Biotec) and anti-human CD107a and b (BD Pharmingen™).

Blocking antibody experiments

To confirm that cytokine production by $CD8^+$ T cells was specifically mediated through HLA-E-restricted TCR-peptide recognition, PBMCs were incubated with blocking monoclonal antibodies (mAbs) in the presence or absence of synthetic SARS-CoV-2-derived peptides (final concentration: 5 µg/mL) in 96-well U-bottom plates. The blocking mAbs included anti-HLA-E (clone unconjugated 3D12HLA-E, eBioscience™) and anti-TCR αβ (clone IP26, eBioscience™), each used at a final concentration of 10 µg/mL. Isotype-matched control antibodies were included as negative controls. Blocking antibodies were added to the PBMC cultures 1 hour prior to peptide stimulation. Following 18 hours of incubation at 37 °C with 5% CO_2, cells were harvested and stained for surface markers CD3 and CD8, along with viability dyes. Intracellular staining for TNF-α was subsequently performed to assess cytokine production.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (RRID: SCR_002798). The Two-way ANOVA or mixed model and the non-parametric tests, Mann-Whitney and Kruskal-Wallis, were used for unpaired samples; p values less than 0.05 were considered statistically significant.

See Supplementary Table S1 for key resources and Supplementary Table S2 for RRIDs of antibodies.

Results

Computational discovery of seven unique SARS-CoV-2 epitopes with exclusive HLA-E binding

To identify potential SARS-CoV-2 epitopes capable of binding to HLA-E, we analyzed the complete genome of the 2019-nCoV/USA-WA1–A12/2020 strain (GenBank accession MT020880) using the Artificial Neural Networks (ANNs) prediction algorithm across all annotated open reading frames (ORFs), including both structural and non-structural proteins, to identify potential HLA-E-restricted epitopes. Using NetMHC 4.1 (19, 20), we predicted a total of 72,572 peptides with the potential to bind to MHC class I molecules (see Supplementary Data Sheet S1). Peptides ranging from 9 to 15 amino acids were evaluated, and 1261 sequences with predicted binding affinities between 0.5 and 0.08 log (IC50) for HLA-E*01:01 and HLA-E*01:03 were selected for further analysis (Fig. 1A).

To assess specificity, we screened all predicted epitopes against 81 HLA-A, HLA-B, HLA-C, and HLA-E alleles. A substantial proportion of peptides showed cross-reactivity with classical MHC class I molecules, particularly HLA-A (49.9%), followed by HLA-B (39%) and HLA-C (9.8%) (Fig. 1B). In contrast, only 1.04% of epitopes were found to have affinity for HLA-E*01:01 and HLA-E*01:03, highlighting the relatively low frequency of HLA-E-restricted SARS-CoV-2 epitopes in the SARS-CoV-2 Predicted Epitope Database (Fig. 1B). From this subset, we identified 216 peptides as unique binders to HLA-E*01:01 and HLA-E*01:03. We then performed homology screening using BLAST with an 80% similarity threshold to exclude peptides with close homologs in other organisms, and we utilized the IEDB for final homology control and epitope selection.

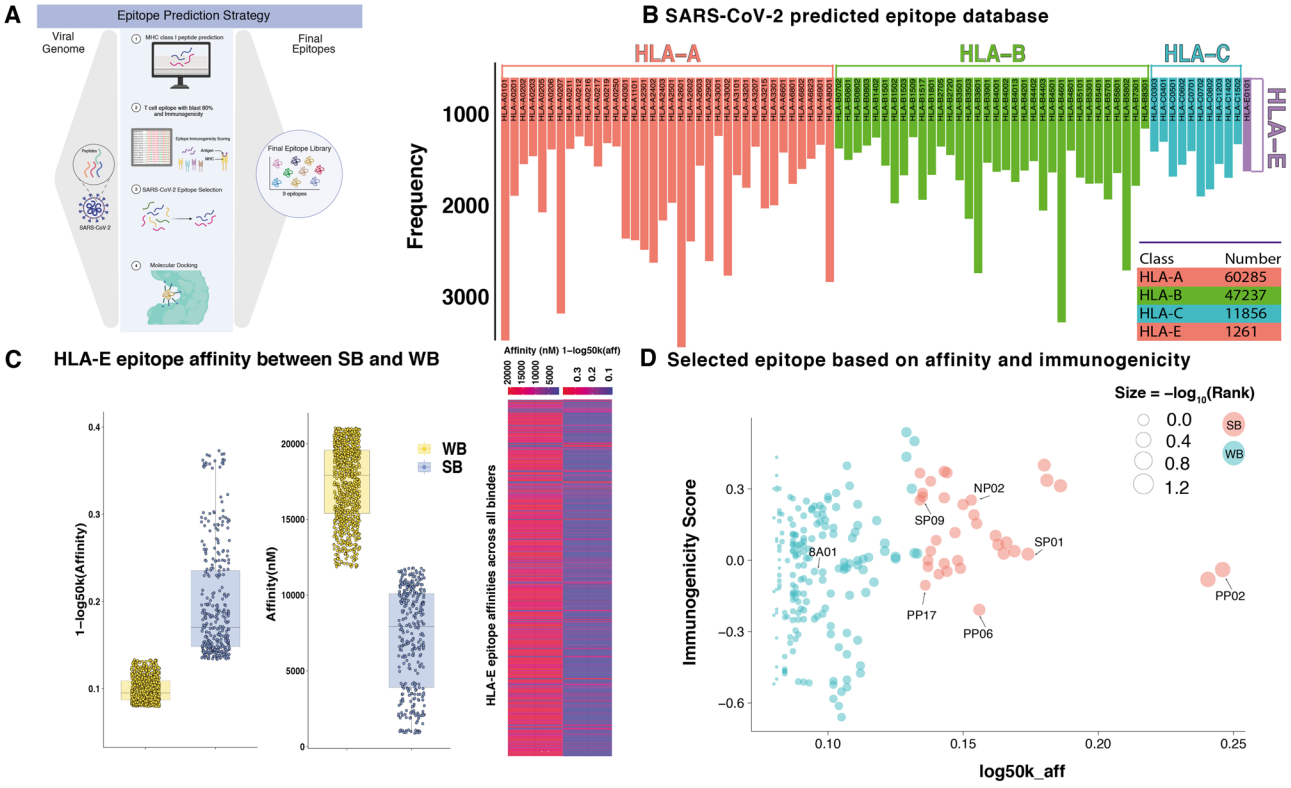


Fig. 1 In silico prediction of unique SARS-CoV-2 epitopes with high affinity for HLA-E*01:01 and HLA-E*0103. **A**) schematic representation of the computational pipeline used to predict HLA-E binding epitopes from SARS-CoV-2 proteins, which included data acquisition, epitope prediction, and affinity analysis. **B**) frequency distribution of SARS-CoV-2 epitopes binding to different MHC class I molecules (HLA-A, HLA-B, HLA-C, and HLA-E). The bar chart represents the number of unique epitopes predicted to bind each HLA allele. The total number of epitopes binding to each HLA allele is indicated at the bottom right. **C**) a total of 120.639 peptides were classified as strong binders (SB) or weak binders (WB) based on their binding affinities, determined using the 1-log50k affinity scale (nM). A heatmap illustrates the predicted binding affinities of the peptides to HLA-E*01:03. **D**) scatter plot showing the distinctiveness of selected epitopes based on their uniqueness to SARS-CoV-2 and strong immunogenicity scores, each colored according to their classification as SB or WB. These seven epitopes were prioritized for further analysis due to their potential effective immunogenicity

Table 2 Characteristics of predicted HLA-E binding SARS-CoV-2 peptides, stabilization and activation assay

Code	protein	position	Peptide sequence	Lenght	Log (IC_{50}) Affinity (core)	Stabilization MFI 24 h	Activation[1]
SP09	SPIKE	320–330	VQPTESIVRFP	11	0.244	5.4 ± 6.5	0.18 ± 0.05
SP01	SPIKE	503–514	VGYQPYRVVVLS	12	0.174	7.8 ± 3.6	0.04 ± 0.04
NP02	N	149–159	RNPANNAAIVL	11	0.156	−1.27 ± 5.6	0.07 ± 0.06
8A01	ORF8a	48–58	RVGARKSAPLI	11	0.143	1.6 ± 2.1	0.21 ± 0.08
PP02	ORF1ab	106–116	IQPGQTFSVLA	11	0.136	4.4 ± 3.1	0.13 ± 0.05
PP06	ORF1ab	565–576	TMTNRQFHQKLL	12	0.134	12.3 ± 4.5	0.16 ± 0.11
PP17	ORF1ab	17–30	GLHPTQAPTHLSVD	14	0.089	1.5 ± 1.5	0.08 ± 0.04

[1]As a readout of activation, we assessed the overall frequency of $CD8^+$ T lymphocytes producing the cytokines IFN-γ, TNF-α, and IL-2 following 24 hrs stimulation of PBMCs with peptide-pulsed or unpulsed RMA-S/HLA-E cells, from seven convalescent individuals

Next, we calculated immunogenicity scores using T-cell epitope prediction tools on the IEDB. Peptides were classified as strong binders (SB) or weak binders (WB) based on predicted log (IC50) affinity values (Fig. 1C). Finally, we selected seven HLA-E-restricted epitopes that were unique to SARS-CoV-2, distinct in their core sequences, and demonstrated positive immunogenicity scores. These peptides were chosen as candidates for downstream validation and analysis (Table 2; Fig. 1D).

Stabilization, and immunogenicity of SARS-CoV-2 epitopes binding to HLA-E

To experimentally validate peptide binding, we performed stabilization assays using TAP-deficient RMA-S cells expressing HLA-E and β2-microglobulin (RMA-S/

HLA-E). Cells were incubated with peptides at 37 °C, and HLA-E surface expression was quantified by flow cytometry after 24 hrs incubation. The stabilization data are shown in Table 2, with cumulative results from five independent experiments (each in duplicate). Normalized MFI was calculated as described in the Methods section. Stabilization was defined by an MFI index ≥ 0.1 [30]. There was no evidence of HLA-E expression in the absence of peptide, while the canonical VL9 peptide, used as a positive control, stabilized HLA-E expression (data not shown). All peptides except NP02 promoted HLA-E stabilization on transfected RMA-S cells, with SP01, SP09, and PP06 showing the highest MFI values.

We next evaluated the immunogenicity of the seven peptides by assessing cytokine responses in $CD8^+$ T cells. PBMCs were stimulated with peptide-pulsed K562 or K562/HLA-E cells, and intracellular cytokine staining was performed. A response was considered positive if cytokine expression exceeded 0.01% above background in at least 30% of donors [30]. According to these criteria, all seven tested peptides elicited at least one cytokine expression amongst IFN-γ, TNF-α, and IL-2 by $CD8^+$ T cells, although at very different extent. Table 2 shows cumulative data from COVID-19 patients, while Fig. 2 shows the global (A) and single (B-D) $CD8^+$ T cell cytokine responses to stimulation with each peptide.

Therefore, and as found for other HLA molecules as well, since actual affinities as predicted by computational approaches do not fully correlate HLA-E cell surface stabilization assay and the $CD8^+$ T cell responses, in

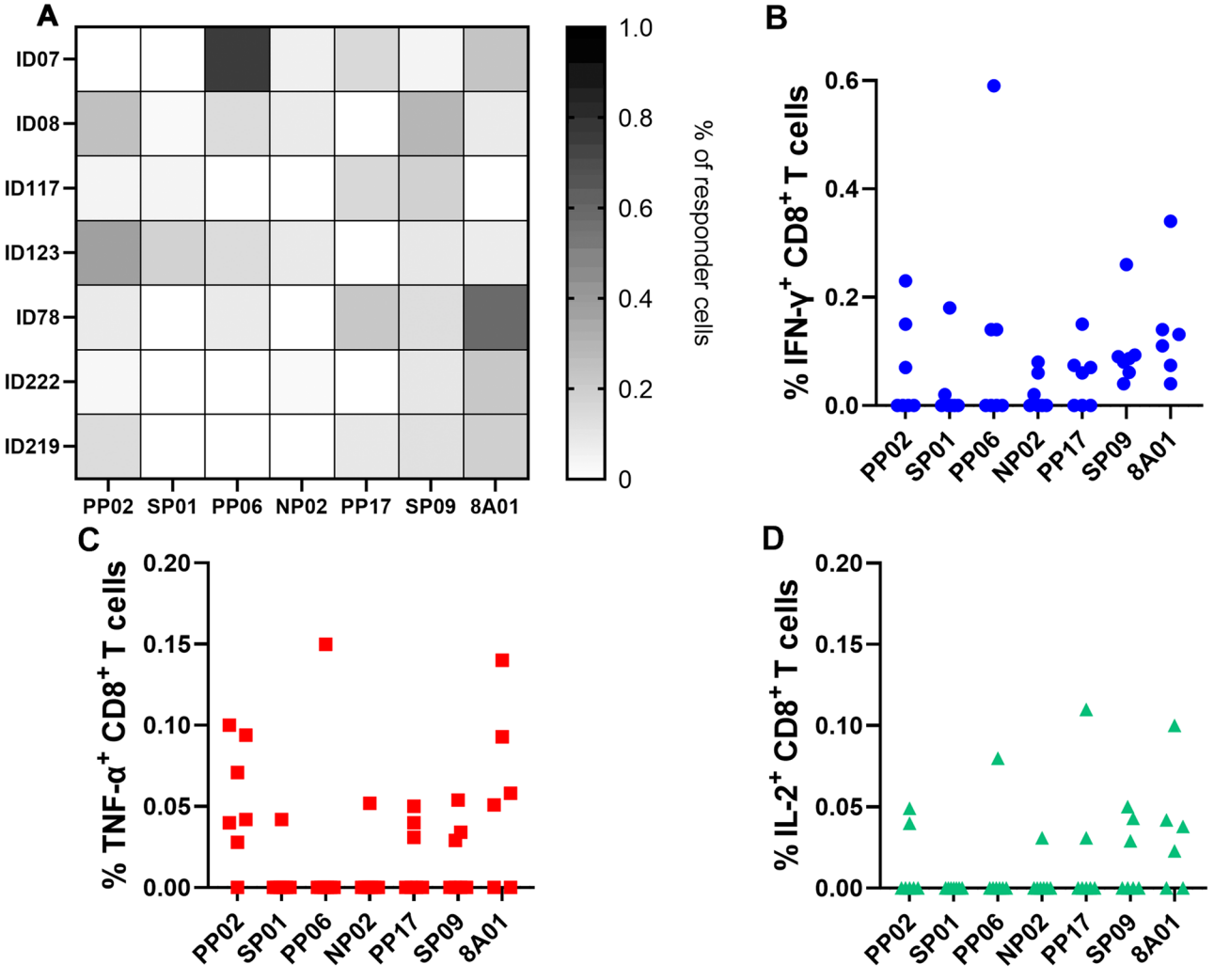


Fig. 2 Representation of the combined cytokine expression results for a discovery cohort of 7 convalescent COVID-19 patients. The panel **A** shows the frequency of the $CD8^+$ T cell cytokine (IFN-γ, TNF-α, and IL-2) response to K562/HLA-E cells pulsed with HLA-E-binding SARS-CoV-2 peptides. White boxes, no cytokine response (the total percentage of cytokine$^+$ $CD8^+$ T cells is < 0.01); light gray boxes, the total percentage of cytokine$^+$ $CD8^+$ T cells ranges from 0.01- 0.2%; gray boxes, the total percentage of cytokine$^+$ $CD8^+$ T cells ranges from 0.4- 0.6%; dark gray boxes, the total percentage of cytokine$^+$ $CD8^+$ T cells ranges from 0.6- 0.8% and black boxes, the total percentage of cytokine$^+$ $CD8^+$ T cells ranges from 0.8–1% or more. Panels **B**, **C**, and **D** show the frequency of $CD8^+$ T cells positive for IFN-γ, TNF-α, and IL-2 respectively, upon stimulation with K562/HLA-E cells pulsed with single peptide

subsequent experiments we chose to use the seven peptides that gave a positive result in either assay.

Blocking TCRαβ or HLA-E significantly reduced $CD8^+$ T cell cytokine responses to SARS-CoV-2 peptides in PBMCs from convalescent and vaccinated donors (Fig. 3), confirming TCRαβ-dependent recognition of HLA-E-bound peptides.

SARS-CoV-2 peptides are conserved in the main SARS-CoV-2 strains

The Alamos AnalyseAlign tool was used to assess the conservation of HLA-E-restricted peptides derived from Spike, Nucleocapsid, ORF8, and ORF1ab proteins in over 400,000 SARS-CoV-2 sequences deposited between June 15, 2021, and February 6, 2025. Our analysis showed a high level of sequence conservation across all major SARS-CoV-2 variants, including Alpha (B0.1.1.7), Beta (B0.1.351), Gamma (P0.1), Delta (B0.1.617.2), and Omicron (B0.1.1.529). Importantly, we also observed substantial sequence conservation within more than 90,000 sequences collected between December 1, 2021, and February 6, 2025, specifically from the JN0.1 SARS-CoV-2 lineage, which is a descendant of BA0.2.86 and is currently the most prevalent SARS-CoV-2 variant globally.

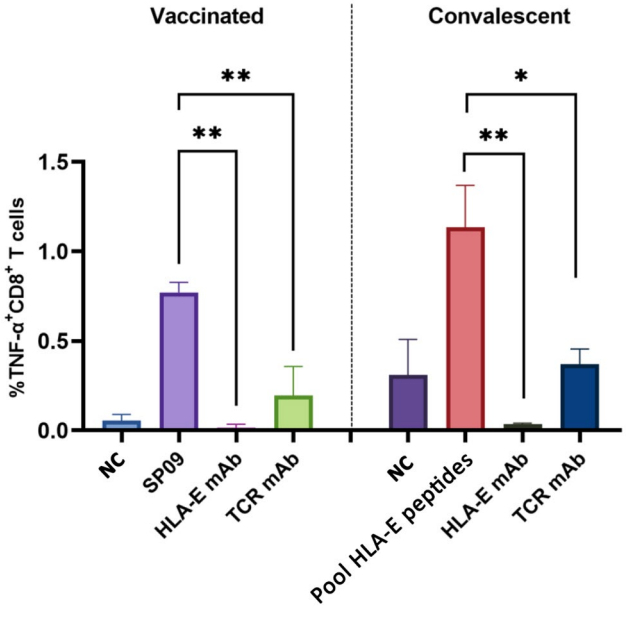


Fig. 3 Recognition requirements of SARS-CoV-2-specific HLA-E-restricted $CD8^+$ T cells. PBMCs from vaccinated individuals (left panel) or COVID-19 convalescent (right panel) patients were cultured for 18 hrs with SP09 peptide or with a pool of the 7 HLA-E/SARS-CoV-2 peptides in the presence of blocking mAbs to HLA-E or the TCR $\alpha\beta$. As negative control (NC) we used unstimulated cells. Intracellular TNF-α expression by $CD8^+$ T cells was estimated by flow cytometry. Data are shown as mean ± SD and are pooled from two independent experiments, each performed in triplicate. $^{**}p < 0.005$ and $^{*}p < 0.05$ when compared to peptide stimulation in the absence of mAbs, using one-way analysis of variance (ANOVA)

As detailed in the Supplementary Data Sheet S2, we observed significant conservation of amino acid sequences across a range of proteins from SARS-CoV-2, with logo frequency by position in the Nucleocapsid, 3C-like protease, RNA-dependent RNA polymerase, and Exonuclease proteins ranging from 98.23% to 99.84%. Furthermore, we noted approximately 96% conservation within the Spike protein, which displayed slightly lower conservation levels in the JN0.1 variant. Across all main SARS-CoV-2 variants, amino acid sequence conservation ranged from 87.54% to 97.58% for Spike, and between 96.99% and 99.64% for other peptides. For example, the Logo frequency for amino acid position 116 in the 3C-like protease (IQPGQTFSVLA) was present in A: 99.63% (379341/380754), X: 0.35% (1342/380754), and V: 0.01% (53/380754) of sequences across all variants (Fig. 4A). In the JN0.1 variant, it was A: 99.84% (90602/90750), X: 0.16% (142/90750), and V: 0.01% (5/90750) (Fig. 4B).

HLA-E-restricted SARS-CoV-2-specific $CD8^+$ T cell cytokine responses in convalescent and hospitalised COVID-19 patients

To evaluate the effector functions of HLA-E-restricted SARS-CoV-2-specific $CD8^+$ T cells, we directly stimulated PBMCs from 44 samples with a pool of HLA-E-binding peptides and measured the response by assessing the frequency of IFN-γ, TNF-α, and IL-2 production by $CD8^+$ T cells. A commercially available peptide pool derived from the entire Spike sequence (PepTivator®) was used as a response control. In convalescent patients, HLA-E restricted $CD8^+$ T cells mainly expressed TNF-α and, although to a lesser extent, IFN-γ (Fig. 5A, left and centre) upon stimulation with the pool of HLA-E-binding SARS-CoV-2 peptides. The results were statistically significant compared to non-stimulated cells within each sample group. $CD8^+$ T cells also produced IL-2 after stimulation with the HLA-E-binding peptide pool compared to unstimulated PBMCs, but the result did not reach statistical significance (Fig. 5A, right). Notably, stimulating PBMCs with PepTivator® caused limited expression of IFN-γ, TNF-α, and IL-2, with cytokine levels lower than those promoted by the HLA-E-binding peptide pool, and these differences were not statistically significant from the cytokine response of unstimulated PBMCs.

We then compared the HLA-E-restricted $CD8^+$ T cell response in 22 convalescent patients with a cohort of 22 hospitalized patients with severe COVID-19. Compared to $CD8^+$ T cells from convalescent individuals, $CD8^+$ T cells from hospitalized COVID-19 patients produced significantly less TNF-α and IFN-γ (Fig. 5B, left and center) after stimulation with the pool of HLA-E-binding peptides, while IL-2 production showed no difference between the $CD8^+$ T cells from both groups (Fig. 5B,

A. Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529)

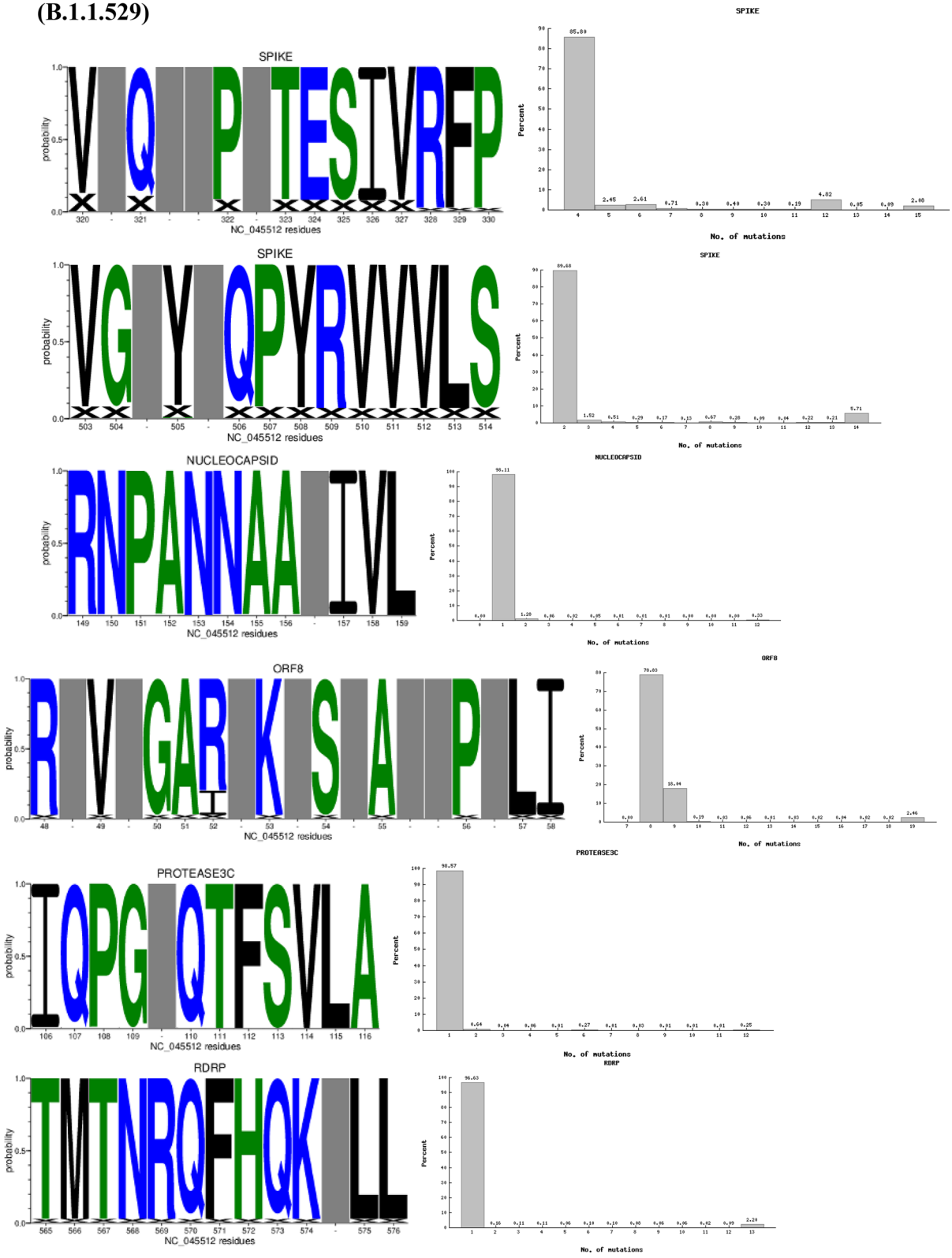


Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 This figure highlights the logo diagrams of each epitope sequence of seven HLA-E binding SARS-CoV-2 peptides. These were checked and analyzed using the Los Alamos AnalyseAlign tool against more than 400,000 sequences collected between (**A**) June 15, 2021, and February 6, 2025 for all main SARS-CoV-2 variants, including Alpha (B0.1.1.7), beta (B0.1.351), gamma (P0.1), Delta (B0.1.617.2), Omicron (B0.1.1.529), and (B) over 90,000 sequences collected from December 1, 2021, to February 6, 2025, for the JN0.1 lineage. All epitopes demonstrated significant conservation of amino acid sequences across the main SARS-CoV-2 variants. The gray boxes in the logos indicate deletions. The colors of each amino acid are categorized based on their hydrophobicity: hydrophilic (RKDENQ, blue), neutral (SGHTAP, green), and hydrophobic (YVMCLFIW, black)

right). Notably, production of IFN-γ by $CD8^+$ T cells from hospitalized patients with severe COVID-19 in response to HLA-E-binding peptides was nearly absent (Fig. 5B, left). Furthermore, there was no statistically significant difference in the production of IFN-γ, TNF-α, and IL-2 in response to PepTivator® between $CD8^+$ T cells from convalescent and severe COVID-19 hospitalized patients.

Collectively, these results clearly demonstrate that HLA-E-restricted $CD8^+$ T cells that preferentially produce TNF-α and IFN-γ form a distinct yet significant population of the SARS-CoV-2-specific $CD8^+$ repertoire in convalescent COVID-19 patients; however, they are poorly represented in hospitalised patients with severe disease.

Vaccination increases cytokine production by HLA-E-restricted Spike peptide-specific $CD8^+$ T cells

We investigated the impact of vaccination on the HLA-E-restricted and SARS-CoV-2-specific $CD8^+$ T cells. First, we assessed the $CD8^+$ T cell response in a cohort of 25 healthy individuals who received two or three doses of the Pfizer/BioNTech BNT162b2 mRNA vaccine. PBMCs were collected from vaccinated donors, on average, 21 days after the second or third dose, and were stimulated with the HLA-E-restricted Spike-derived peptide SP09 or with PepTivator® as a positive control. As shown in Fig. 5C, $CD8^+$ T cell responses to the HLA-E-restricted peptide SP09 increased significantly after vaccination, with significantly higher levels of IFN-γ, TNF-α, and IL-2 compared to unstimulated controls. A similar cytokine profile was observed in response to PepTivator®. Of particular interest, cytokine expression in response to SP09 was higher than that elicited by PepTivator®, although the differences reached statistical significance only for TNF-α.

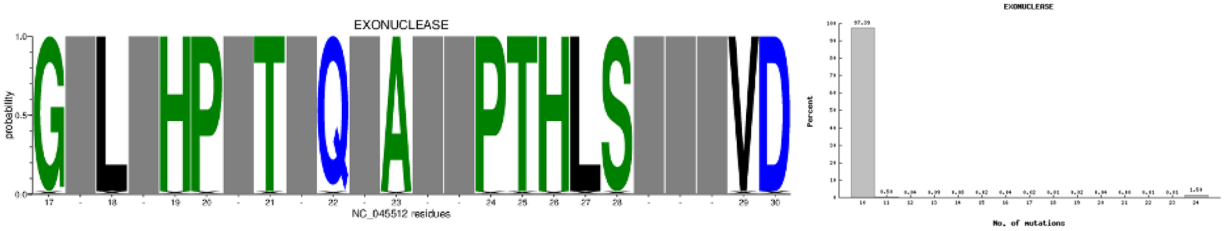
To study the impact of vaccine doses on the development of the HLA-E-restricted $CD8^+$ T cell response, we analysed cytokine production in response to SP09 in 15 individuals who had received two doses and 10 individuals who had received three doses. As shown in Fig. 6A, the IFN-γ, TNF-α, and IL-2 $CD8^+$ T cell responses to SP09 peptide stimulation were statistically significantly lower in donors who had received a second dose compared to those who had received three doses, indicating a dose-dependent increase in the HLA-E-restricted immune response, in terms of cytokine production.

We further evaluated the combined impact of vaccination and natural SARS-CoV-2 infection on the HLA-E-restricted $CD8^+$ T cell response. To this aim, we measured cytokine production in response to the SP09 peptide in healthy donors who received three doses of the BNT162b2 vaccine (Fig. 6A). The TNF-α and IFN-γ $CD8^+$ T cell responses to SP09 peptide stimulation were significantly higher in donors who received three vaccine doses and had been infected with SARS-CoV-2, compared to non-infected, vaccinated individuals (Fig. 6B). These results clearly demonstrate that natural SARS-CoV-2 infection boosts HLA-restricted $CD8^+$ T cell responses in vaccinated individuals, suggesting that this HLA-E-restricted response may contribute to protection.

Cytotoxic potential of HLA-E-restricted SARS-CoV-2-specific $CD8^+$ T cells

We then performed a sensitive CD107a/b flow-cytometry-based degranulation assay to evaluate the cytotoxic potentials of HLA-E-restricted SARS-CoV-2-specific $CD8^+$ T cells. Specifically, we assessed CD107a/b upregulation, in $CD8^+$ T cells from four vaccinated individuals (two doses), four convalescent subjects, and four hospitalized patients. Cumulative data first revealed a significant increase in the frequency of $CD107^+$ $CD8^+$ T cells upon stimulation with the HLA-E peptide pool, as compared to unstimulated cells. This response was comparable to that observed upon stimulation with PepTivator®, which served as a benchmark for activation (Fig. 7A). Notably, no significant differences were detected between cells stimulated with HLA-E peptides and those stimulated with PepTivator®, suggesting that HLA-E peptides are similarly effective in promoting $CD8^+$ T cell degranulation. Moreover, for vaccinated individuals only, we used the Spike-derived peptide SP09 as a stimulus. Also, in this case we observed an increased expression of CD107 due to the action of this peptide, but it does not reach statistical significance compared to the non-stimulated cells (Fig. 7 A).

When CD107a/b expression was analyzed within individual groups, no statistically significant differences emerged in hospitalized and convalescent patients, between unstimulated cells and cells stimulated with either HLA-E peptides or PepTivator®, despite a consistent trend toward increased CD107a/b expression was observed under both stimulation conditions, compared to unstimulated cells across all groups (Fig. 7B,



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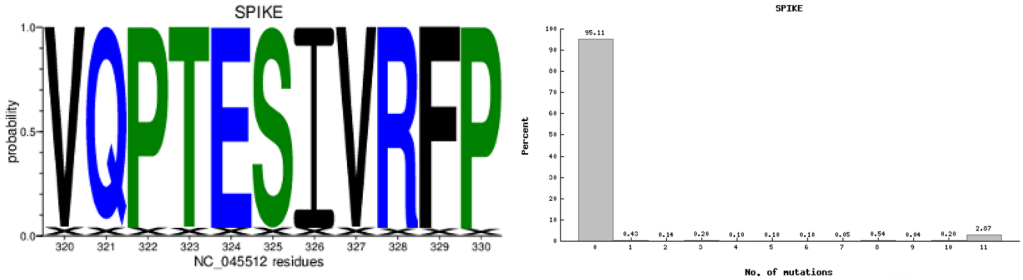

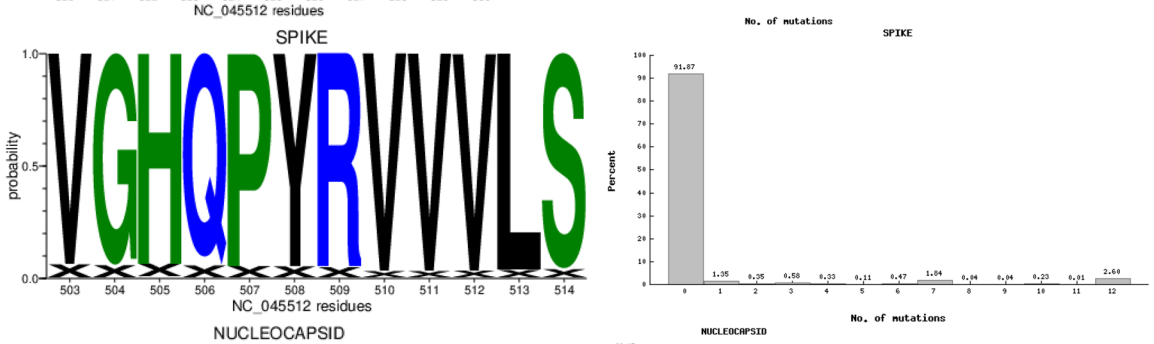

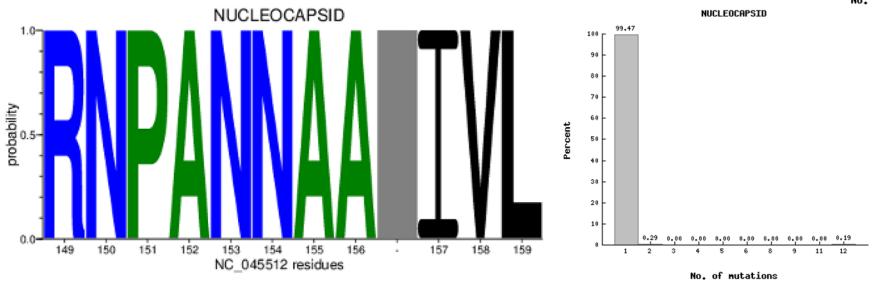

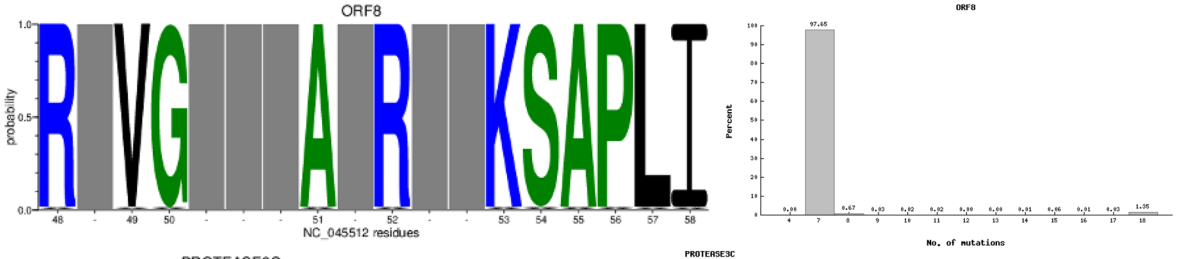

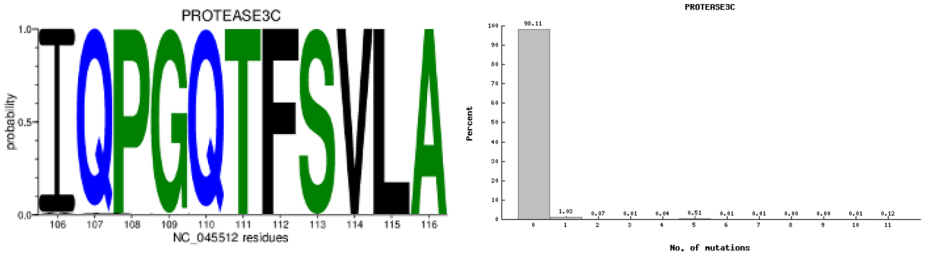

Fig. 4 (continued)

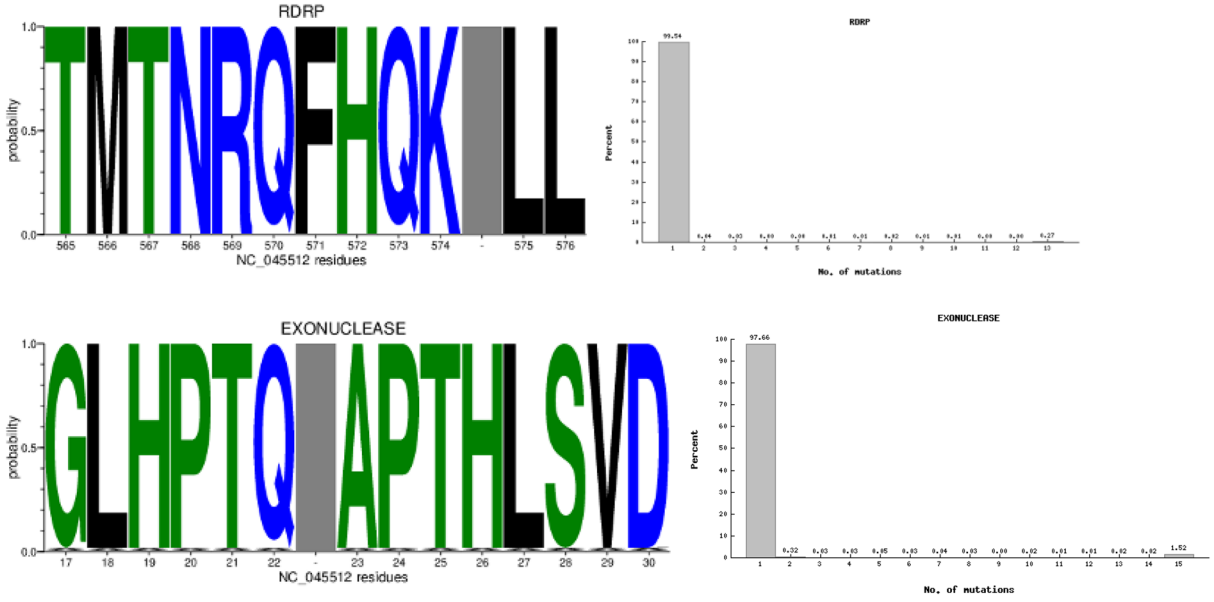

Fig. 4 (continued)

C). Similar results were obtained with CD8⁺ T cells from vaccinated individuals, in which the Spike-derived peptide SP09 only was used as a stimulus (Fig. 7D).

Collectively, these findings indicate that HLA-E–restricted CD8⁺ T cells are capable of exerting cytotoxic activity upon stimulation with HLA-E peptides, demonstrating functional activation that extends beyond cytokine production to include potential cytotoxic mechanisms.

Discussion

The advent of SARS-CoV-2 vaccines has significantly reduced the incidence of severe COVID-19 cases and reduced the mortality rate worldwide [31]. Despite this progress, a comprehensive understanding of the adaptive immune response, particularly the role of CD8⁺ T cells, remains essential for optimising vaccine strategies and improving long-term protection. In this study, we identified a set of HLA-E–restricted epitopes derived from multiple SARS-CoV-2 proteins, including ORF1ab, Spike, ORF8a, and Nucleoprotein. These epitopes were selected based on their predicted binding affinity to HLA-E and their potential to elicit broad CD8⁺ T cell responses, and their unique and exclusive presence in SARS-CoV-2, thereby expanding the scope of non-classical T cell-mediated immunity in SARS-CoV-2 infection. Other peptides that fit HLA-E were identified by in silico analysis, but either they were shared with other pathogens, or their immunogenicity scores were too low to be selected for subsequent in vitro analysis. Nevertheless, they could be considered for future studies to provide a comprehensive evaluation of the role of HLA-E in SARS-CoV-2 infection.

We demonstrated the presence of HLA-E–restricted, peptide-specific CD8⁺ T cells in PBMCs from convalescent individuals, hospitalised patients with severe COVID-19, and vaccinated donors. These findings align with previous studies describing classical HLA-Ia-restricted CD8⁺ T cell responses in SARS-CoV-2 infection and vaccination [9, 17, 18, 32, 33]. Furthermore, using blocking monoclonal antibodies (mAbs) targeting TCR αβ and HLA-E molecules, we show that HLA-E/TCR recognition mediates the activation of HLA-E–restricted CD8⁺ T cells upon stimulation with SARS-CoV-2 peptides. Previous research has highlighted the crucial role of classical CD8⁺ T cell response in SARS-CoV-2 infection and post-vaccination immunity [9, 18, 34]. Although the number of patients tested was limited in our analysis, accumulating evidence from a few studies [21, 34, 35], in addition to our data, demonstrates that peptides derived from different components of SARS-CoV-2 protein antigens can induce HLA-E-restricted and peptide-specific CD8⁺ T cell responses. Additionally, these peptides exhibit a high conservation rate, enabling them to retain their immunogenic potential across the most prevalent SARS-CoV-2 variants.

Interestingly, the characterization of SARS-CoV-2-specific CD8⁺ T cells utilising a stimulation assay and flow cytometry revealed either an absence or a significant reduction of cytokine production (IFN-γ, TNF-α, and IL-2) upon stimulation with the pool of HLA-E-binding peptides during acute, severe, and critical COVID-19 disease among hospitalised patients. The impaired response detected in hospitalized patients may be related to several conditions. These include lymphopenia, which could directly affect the whole CD8 T cell repertoire, including

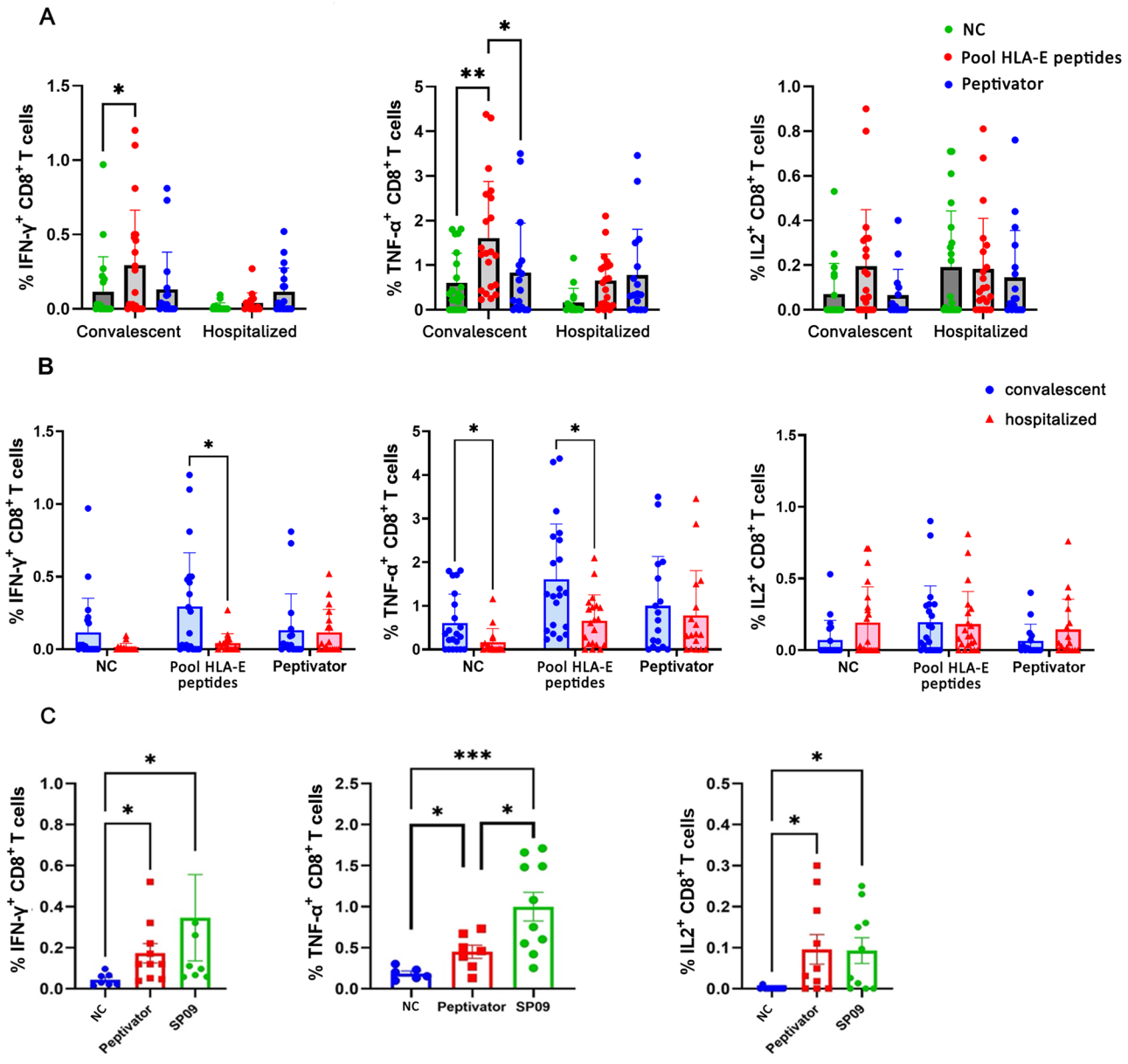

Fig. 5 Frequencies of HLA-E/SARS-CoV-2 peptide-specific CD8$^+$ T cells in convalescent and hospitalized severe COVID-19 patients. A) Frequency of IFN-γ, TNF-α, and IL-2 positive CD8$^+$ T cells in convalescent (n.=21) and hospitalized severe COVID-19 patients (n.=17) upon stimulation with the pool of SARS-CoV-2 peptides. Unstimulated cells were used as a negative control (NC), while the cells stimulated with PepTivator® were considered a positive control of SARS-CoV-2-specific response. The graph represents the median and the error bars. Two-way ANOVA or mixed models were used to compare the three experimental points. *p<0.05; **p<0.01. B) Frequency of IFN-γ, TNF-α and IL-2 positive CD8$^+$ T cells in convalescent and hospitalized severe COVID-19 patients upon stimulation with the pool of HLA-E-SARS-CoV-2 peptides. Unstimulated cells were used as a negative control (NC), while the cells stimulated with PepTivator® were considered as a positive control of SARS-CoV-2-specific response. The graph represents the median and the error bars. Two-way ANOVA or mixed models were used to compare the three experimental points. *p<0.05. C) Frequency of IFN-γ, TNF-α and IL-2 positive CD8$^+$ T cells in vaccinated subjects (n.=10) upon stimulation with SP09 peptide. Unstimulated cells were used as a negative control (NC), while the cells stimulated with PepTivator® were considered as a positive control of SARS-CoV-2-specific response. The graph represents the median and the error bars. The Kruskal-Wallis test was used to compare the values of the groups. *p ≤ 0.05, ***p≤0.001

HLA-E-restricted CD8$^+$ T cells, and CD8$^+$ T-cell exhaustion due to chronic viral antigen exposure. Finally, the expansion of myeloid-derived suppressor cells (MDSC) observed in severe COVID-19 patients could inhibit SARS-CoV-2-specific CD8 T-cell responses, which may contribute to poor disease outcomes [36]. Due to the low polymorphism of HLA-E molecules in the human population, exploring epitopes that bind to HLA-E could be incredibly valuable for developing universal vaccines and/or immunotherapeutic. HLA-E–restricted T cells

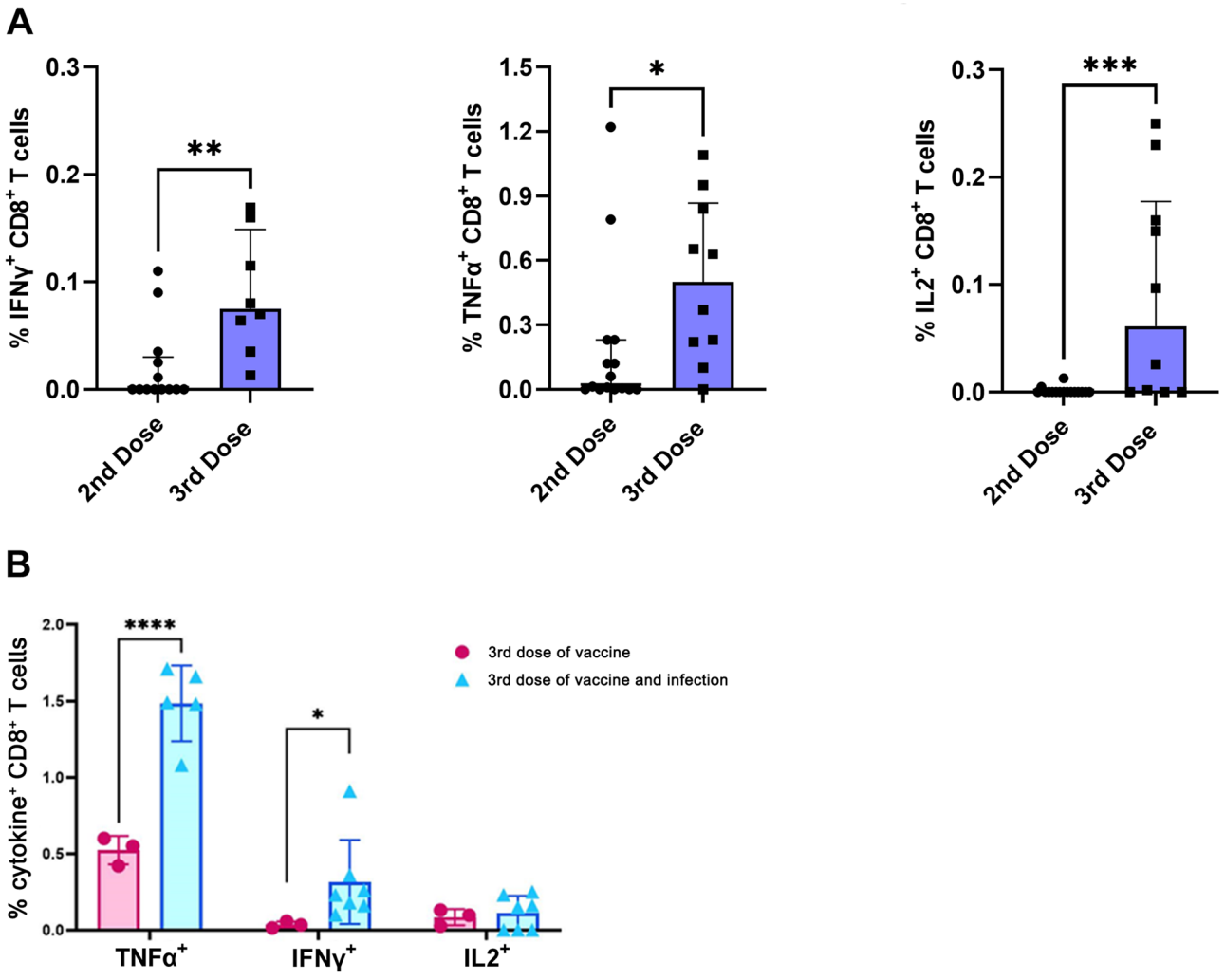

Fig. 6 Cytokine responses of $CD8^+$ T cells stimulated with peptide SP09, in vaccinated individuals, with or without subsequent infection. **A**) frequency of IFN-γ, TNF-α, and IL-2 positive $CD8^+$ T cells after subtraction of the respective NC in 15 vaccinated subjects with two doses, and 10 with three doses. The graph represents the median and the error bars. The Mann-Whitney test was used to compare the values of the groups. *$p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$. **B**) comparison between healthy individuals vaccinated with three doses (n. = 3) of the mRNA BNT162b2 vaccine without infection and individuals vaccinated with three vaccine doses who got infected with SARS-CoV-2 (n. = 7). The Mann-Whitney test was used to compare the values of the groups *$p \le 0.05$, ****$p \le 0.0001$

are detected in the blood in bacterial and viral infections [30, 37–39]. $CD8^+$ T cells expressing HLA-E have been observed in viral infections, but their role in the immune response to SARS-CoV-2 remains underestimated. Yang et al. have recently reported HLA-E–restricted $CD8^+$ T cells in individuals recovering from SARS-CoV-2, which were at levels comparable to conventional HLA-Ia-restricted T cells. More importantly, they showed that, unlike classical HLA-class I molecules, HLA-E was not down-regulated by SARS-CoV-2 infection, and these HLA-E-restricted $CD8^+$ T cells were able to suppress SARS-CoV-2 infection in Calu-3 cells in vitro [21]. This suggests that SARS-CoV-2-specific HLA-E-restricted $CD8^+$ T cells could be beneficial when conventional T cell responses are compromised during SARS-CoV-2 infection. As presented in this study, HLA-E-restricted peptides can induce an even more potent response when compared to cells stimulated with other, non-HLA-E-binding peptides. Moreover, considering that the HLA-E-restricted $CD8^+$ T cell response is greater in convalescent patients and vaccinated subjects than in hospitalised patients with severe disease, we can hypothesise that the HLA-E-restricted response may contribute to the immune response against the disease. Our study also highlights robust immunity to the HLA-E-restricted Spike-derived peptide, as evidenced by the detection of $CD8^+$ T cell responses in the blood of donors following receipt of an mRNA vaccine booster. During the COVID-19 pandemic, vaccines have significantly reduced the link between SARS-CoV-2 infections and hospitalizations

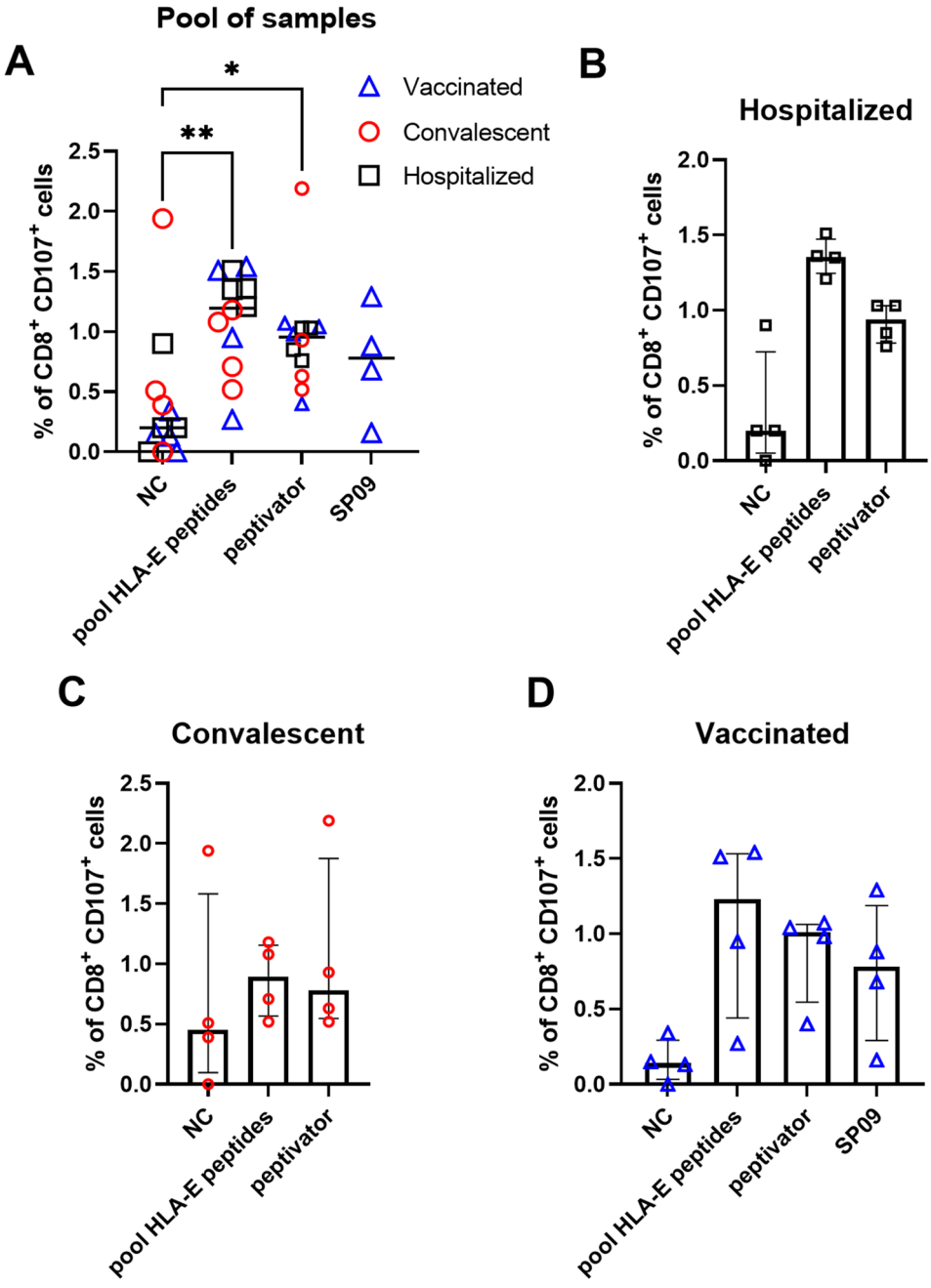

Fig. 7 CD107 expression by HLA-E/SARS-CoV-2 peptide-specific $CD8^+$ T cells in vaccinated, convalescent, and hospitalized COVID-19 patients. (**A**) Cumulative frequency of $CD8^+$ $CD107^+$ T cells across 12 samples (vaccinated $n=4$, convalescent $n=4$, hospitalized $n=4$) after stimulation with a pool of HLA-E-SARS-CoV-2 peptides. Unstimulated cells served as negative controls (NC); PepTivator® stimulation was used as a positive control for SARS-CoV-2-specific response. Bars indicate medians. The Kruskal-Wallis test was used to compare the values of the groups. $*p<0.05$; $**p<0.01$. (**B**–**D**) frequencies of $CD8^+$ $CD107^+$ T cells in hospitalized (**B**), convalescent (**C**), and vaccinated (**D**) subjects stimulated as described in (**A**) (nil, PepTivator®, and HLA-E peptides pool). Bars indicate medians; Kruskal–Wallis test applied for group comparisons

and deaths [40]. Despite the continuous evolution of viral variants and their ability to evade the antibody response to varying degrees [41], emerging evidence shows that T cells may play a protective role against COVID-19 [21, 34, 42]. Memory T cells induced by SARS-CoV-2 vaccines maintain the ability to recognize viral particles [43]. These classical $CD8^+$ T cells are detectable in 41–65% of individuals six months after the second dose, indicating sustained immunity [44]. Notably, Spike-specific classical $CD8^+$ T cell responses are observed in 70–90% of individuals weeks after a second dose mRNA COVID-19 vaccine regimen [43, 45–47]. A study conducted in Finnish

healthcare workers (HCWs) who received three doses of COVID-19 mRNA vaccines showed that the levels of Spike-specific $CD8^+$ T cells remain relatively stable and are detected in 71% of HCWs [48]. Furthermore, there was a significant increase in SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T cell responses after the third vaccine dose [16, 49]. These findings underscore the potential value of vaccines, explicitly highlighting the repeated boosting effect of vaccines on T cell immunity. Although non-classical HLA-specific $CD8^+$ T cell responses in vaccinated individuals have been less explored, the peptides studied here are conserved across the most widespread SARS-CoV-2 variants; considering that HLA-E is also highly conserved, these peptides can also be promising candidates for vaccine formulations targeting T cell immunity.

In the current study, HLA-E-restricted $CD8^+$ T cells exhibit multiple cytokine production at 3–4 weeks post-third vaccine dose, hence retaining a broad memory function spectrum. Notably, there was a significant increase in SARS-CoV-2 Spike-specific $CD8^+$ T cell responses after the third dose. We used TNF-α as a readout of $CD8^+$ T cell response, based on our previous studies demonstrating that this cytokine is broadly produced by all HLA-E-restricted $CD8^+$ T cells, including those $CD8^+$ T cells lacking cytotoxic activities and IFN-γ or IL-2 production capacity, or even showing a type-2 cytokine production profile [30, 38]. Moreover, TNF-α occupies a central, non-redundant position at the interface of antiviral defense and inflammation during SARS-CoV-2 infection. The production of TNF-α among Spike-specific $CD8^+$ T cells linked to HLA-E-restricted peptides suggests a potential mechanism for enhanced antiviral responses. Moreover, patients with a good immune response have been observed to have durable protection from severe disease [50], indicating that long-term immunological memory could play a key role. We also assessed the frequencies and functional qualities of HLA-E-restricted SARS-CoV-2-specific $CD8^+$ T cells in donors with a history of SARS-CoV-2 infection after mRNA vaccination compared to those who had not been naturally infected. We found that natural infection additionally boosts the HLA-E-restricted Spike-specific $CD8^+$ T cell response in vaccinated subjects. Therefore, and similar to the classical T cell responses, HLA-E-restricted SARS-CoV-2 peptide-specific $CD8^+$ T cell responses are detectable after vaccination, especially in those with an induced immunity due to the combination of vaccination and infection.

Finally, the observed upregulation of CD107a/b following stimulation with HLA-E peptides underscores the functional relevance of HLA-E–restricted $CD8^+$ T cells in SARS-CoV-2 immunity. While the lack of statistically significant differences across patient groups may reflect sample size limitations, the consistent trend toward increased degranulation and the statistical significance observed when groups were merged, suggest that these cells possess cytotoxic potential, thus confirming previously reported data showing that a limited number of HLA-E-restricted and SARS-CoV-2 peptide-specific $CD8^+$ T cell clones have cytotoxic activity and suppress viral replication in human lung epithelial cells, although the anti-viral mechanism was not investigated [21]. Whether or not the HLA-E-restricted responses are relevant in controlling viral replication or shaping long-term immunity, is still poorly understood. A very recent study identified multiple cytomegalovirus peptides that bind to Qa-1, the mouse ortholog of human HLA-E, and stimulate Qa-1–restricted $CD8^+$ T cells ex vivo. Adoptive transfer of the Qa-1-restricted $CD8^+$ T cells into RAG-1–deficient mice protects them from cytomegalovirus-induced mortality, underscoring the critical role of these cells in host defense [51].

These findings provide a rationale for further investigation of HLA-E–restricted epitopes in next-generation vaccine platforms, especially those designed for broad population coverage and resilience against variants [21]. In fact, because of the low polymorphism of HLA-E molecules in the human population, exploring HLA-E-binding epitopes could be highly valuable for developing universal vaccines and immunotherapies. Additionally, the application of HLA-E-restricted T cell responses could overcome ineffective adaptive immunity in individuals with impaired classical T cell responses.

In conclusion, we propose that HLA-E, given its ubiquity as an HLA allele locus, represents a promising target for developing peptide-based immunotherapeutics or vaccines. Vaccines focused on universally presented epitopes that induce HLA-E-restricted $CD8^+$ T cell responses could represent a promising approach for developing more specific and effective prophylactic strategies.

Study limitations

We acknowledge that the study involved a relatively small number of participants across different groups (convalescent, hospitalized, and vaccinated), which may undermine the generalizability of the findings. Moreover, the research focused on a limited set of HLA-E-restricted peptides, which may not encompass the full range of potential T-cell responses to SARS-CoV-2, and the assessment of cytokine production relied on specific markers (IFN-γ, TNF-α, IL-2 and CD107), which may not provide a comprehensive view of the immune response. Finally, the study lacks long-term follow-up data to assess the durability of HLA-E-restricted $CD8^+$ T cell responses over time, and the in vitro assays may not fully replicate the in vivo environment, potentially affecting the

relevance of the findings to actual immune responses in patients.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-026-07825-8.

Supplementary material 1

Supplementary material 2

Supplementary material 3

Supplementary material 4

Supplementary material 5

Author contributions

Conceptualization: FD, NC, MPLM, MSA., Methodology: MR, GDB, MB, GP, AMA, MF, NS, SC, VR, EMF., Investigation: MR, GDB, MB, GP, AMA, MF, MSA., Funding acquisition: MPLM, FD, NC., Supervision: MPLM, FD, NC, MSA., Writing original draft: MR, GDB, MPLM, FD, NC, MSA., Writing review and editing: MB, GP, AMA, MF, NS, SC, VR, EMF.

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Data availability

The datasets generated and analyzed for this study can be found in the Zenodo: [Doi: https://doi.org/10.5281/zenodo0.14975295]. These datasets contain raw flow cytometry (FCS) files of CD8+ T cell in vitro studies.

Declarations

Ethics approval and consent to participate

The study adhered to the Declaration of Helsinki and was approved by the Ethical Committee of the A.O.U.P. "Paolo Giaccone" Hospital (protocol code 09/2021). Written informed consent was obtained from all participants.

Consent for publication:

All the authors have approved this manuscript for publication.

Conflict of interest

Mojtaba Shekarkar Azgomi, Marco Pio La Manna, Nadia Caccamo, and Francesco Dieli are inventors on patent application WO/2023/180966 submitted by the University of Palermo entitled "Peptides for the preparation of vaccines against SARS-CoV-2".

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