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Development of a New Off-the-Shelf Plasmacytoid Dendritic Cell–Based Approach for the Expansion and Characterization of SARS-CoV-2–Specific T Cells

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Global vaccination against COVID-19 has been widely successful; however, there is a need for complementary immunotherapies in severe forms of the disease and in immunocompromised patients. Cytotoxic CD8⁺ T cells have a crucial role in disease control, but their function can be dysregulated in severe forms of the disease. We report here a cell-based approach using a plasmacytoid dendritic cell line (PDC*line) to expand in vitro specific CD8⁺ responses against COVID-19 Ags. We tested the immunogenicity of eight HLA-A*02:01 restricted peptides derived from diverse SARS-Cov-2 proteins, selected by bioinformatics analyses in unexposed and convalescent donors. Higher ex vivo frequencies of specific T cells against these peptides were found in convalescent donors compared with unexposed donors, suggesting in situ T cell expansion upon viral infection. The peptide-loaded PDC*line induced robust CD8⁺ responses with total amplification rates that led up to a 198-fold increase in peptide-specific CD8⁺ T cell frequencies for a single donor. Of note, six of eight selected peptides provided significant amplifications, all of which were conserved between SARS-CoV variants and derived from the membrane, the spike protein, the nucleoprotein, and the ORF1ab. Amplified and cloned antiviral CD8⁺ T cells secreted IFN- γ upon peptide-specific activation. Furthermore, specific TCR sequences were identified for two highly immunogenic Ags. Hence, PDC*line represents an efficient platform to identify immunogenic viral targets for future immunotherapies. *The Journal of Immunology*, 2024, 212: 1–9.

Coronaviruses have been known to cause several respiratory infections in human for decades. In 2019, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the massive COVID-19 pandemic, infecting more than 770 million people worldwide and causing nearly 7 million deaths (<https://covid19.who.int/>). Although most cases were benign and limited to upper-respiratory tract symptoms, around 20% of the patients presented a severe form characterized by serious oxygen deprivation and inflammatory complications (1, 2). Aside from supportive care, current treatment recommendations include antiviral agents like nirmatrelvir/ritonavir (Paxlovid) or remdesivir. Monoclonal Abs to block virus entry like tixagévimab/cilgavimab (Evusheld) are no longer recommended due to variant emergence. Another treatment option is the class of anti-inflammatory drugs, and it was demonstrated early on that dexamethasone can be beneficial in severe cases (3). Finally, passive therapies, such as convalescent plasma transfusion, showed some encouraging results for patients with acquired immunodeficiencies, but more studies need to be conducted (4). Hence, vaccination remains to date the main option to prevent or mitigate severe forms of the disease.

Aside from viral load, data suggested that dysregulations in the immune response were responsible for clinical deterioration and that severe COVID-19 was as much a viral disease as a disease driven by pathological immune activation. In severe forms, many dysregulations in the immune response have been reported. Among them,

impairments in plasmacytoid dendritic cell functionality (5, 6) and decreases in lymphocyte count have been correlated with disease severity and poor survival (7, 8). In addition, the hyperinflammatory state may lead to a loss of functionality in CD8⁺ T cells, with increased expression of NKG2A, PD-1, or TIM-3 and decreased expression of CD107a, IFN- γ , IL-2, and granzyme B (2, 9–11).

In this context, immunotherapies enhancing antiviral CD8⁺ T cell response could help control disease progression in COVID-19 disease. Indeed, many studies demonstrated the involvement of CD8⁺ T cells in the resolution of COVID-19. Thus, SARS-CoV-2–specific cytotoxic CD8⁺ T cells were found in almost all convalescent donors who have recovered from the disease (12–14). These T cells were found to display a high cytotoxic potential responding to in vitro antigenic stimulation with the expression of CD107a or CD137 (4-1BB) associated with granzyme B, IFN- γ , and TNF- α production (8, 15). In SARS-CoV-2–infected mice and macaques, it has been demonstrated that cytotoxic CD8⁺ T cells enhanced survival and improved clinical outcomes (16, 17). For patients lacking B cell responses, T cells are considered as the main driver of antiviral immunity (18, 19), and it is critical to devise strategies that can efficiently amplify antiviral CD8⁺ T cells or that can lead to effective CD8⁺ T cell adoptive therapy in this context. Altogether, these findings suggest that CD8⁺ T lymphocytes effectively participate in the control of infection by eradicating virus-infected cells. The characterization of these T cells, in terms of expansion potential,

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Abbreviations used in this article: FI, fluorescence index; MFI, median fluorescence intensity; PDC, plasmacytoid dendritic cell; PDC*line, plasmacytoid dendritic cell line.

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specificity, functionality, and elucidation of their TCR sequence, may help improve prophylactic and therapeutic treatments of coronavirus infections.

In this context, we report here the use of an off-the-shelf approach to generate peptide-specific CD8⁺ T cells. This approach is based on an irradiated plasmacytoid dendritic cell line (PDC*line) that has a strong capacity for Ag presentation and that can promote the development of specific CD8⁺ T responses. This provides proof of concept that this strategy enables rapid *in vitro* expansion of autologous CD8⁺ T cells and identification of antiviral TCR sequences.

The efficiency of this plasmacytoid dendritic cell (PDC)-based approach has been demonstrated in *in vitro* and *in vivo* models, in the context of antitumoral immunity with shared Ags or neoantigens (melanoma and lung cancer) and antiviral immune responses (CMV, EBV, hepatitis B virus, and human papillomavirus) (20–24). In addition, this line has been used in a phase I clinical trial (GeniusVac-Mel4, NCT01863108) to treat nine patients with metastatic melanoma, with recently published results demonstrating the safety and biological efficacy of this therapeutic vaccine (25). A new trial in lung cancer is ongoing to treat 64 patients, in combination with immune checkpoint blockers (NCT03970746), with recent results confirming its safety and biological activity (26, 27). This allogenic approach can be rapidly implemented, and this strategy could cover carriers of the HLA-A*02:01 allele, which is used for presentation of Ags of interest by PDC*line (around 45% of the Caucasian population).

The aim of our study was to demonstrate that the PDC*line can be harnessed to amplify *in vitro* and characterize CD8⁺ T cells against immunogenic SARS-CoV-2 targets. SARS-CoV-2-derived peptides were evaluated for their ability to trigger specific CD8⁺ T cell amplification when presented by the PDC*line. With this strategy, we identified candidate epitopes for future vaccines, as well as the isolation and characterization of monoclonal SARS-CoV-2-specific T CD8⁺ that may be harnessed for adoptive therapy based on TCR-transgenic T cells.

Material and Methods

Peptide selection

From the complete reference sequence of SARS-CoV-2 (National Center for Biotechnology Information reference sequence NC_045512.2, isolate Wuhan-Hu-1 from December 24, 2019), all overlapping 9-mer peptides that were restricted by MHC class I HLA-A*02:01 allele with strong affinity ($IC_{50} < 200$ mM, netMHCpan4.1 [28]) were selected (2020/05/28) using the IEDB epitope prediction tool (29). Among them, peptides with a hydrophobicity >40% were excluded (Thermo Fisher Biosciences peptide analyzing tool [30]). Final selection included viral protein location, conservation among coronaviruses, and description in the literature. Absence of similarity to peptides derived from human proteins was verified to avoid cross-reactivity and autoimmune reactions using the BLASTp software from the National Center for Biotechnology Information with default parameters (31). Predicted HLA-A2 binding ability was experimentally validated using the TAP-deficient T2 cell line (American Type Culture Collection, LGC Standards, Molsheim, France). Briefly, lyophilized peptides (synthesized by SmartBiosciences, France) were solubilized in DMSO at 4 mM. Peptides were incubated at 10 μ M in serum-free RPMI culture medium for 3 h at 37°C with T2 cells in presence of β_2 -microglobulin (100 ng/ml, Sigma catalog no. M4890). Peptide binding and HLA-A2 stabilization was assessed by anti-HLA-A2 staining in flow cytometry (PE mouse anti-human HLA-A2, clone BB7.2, BD Pharmingen catalog no. 201019). Peptide binding to HLA-A2 was evaluated by the fluorescence index (FI) calculated using the median fluorescence intensity (MFI) with the following formula: $FI = [MFI(T2 \text{ with peptide}) - MFI(T2 \text{ without peptide})]/MFI(T2 \text{ without peptide})$. The HLA-A*02:01 MelanA peptide (ELAGIGLTV) was used as positive control, and the HLA-B*07:02 CMV peptide (TPRVTGGGAM) was used as negative control.

Immunogenicity testing and generation of peptide-specific CD8⁺ T cells

Blood samples of convalescent donors who have recovered from nonsevere COVID-19 for at least 1 mo were collected from May 2020 to October 2021

at the French Blood Bank of Grenoble ($n = 18$) from the PLASMACOV cohort (French National Ethics Committee approval no. 2020-A00728-31), nine of which were used for *ex vivo* detection of SARS-CoV-2-specific CD8⁺ T cells, and nine of which were used for *in vitro* amplification. Information about which variant infected each donor was not available, but dominant variants during the sampling period were the original Wuhan strain, followed by the Alpha and Delta variants. Biobanked blood samples from unexposed donors included in the biological sample collection (catalog no. DC-2019-3803) prior to 2019 were used as controls ($n = 8$). The HLA-A2 phenotype was assessed by flow cytometry (PE mouse anti-human HLA-A2, clone BB7.2, BD Pharmingen catalog no. 201019). PBMCs were obtained using a density gradient centrifugation with lymphocyte separation medium (Eurobio catalog no. CMSMSL01-01). Cell count and viability were determined using acridine orange/propidium iodide staining (ViaStain, Nexelom catalog no. CS2-0106) in an automated cell counter (Cellometer Auto 2000, Nexelom). For unexposed donors, CD8⁺ T lymphocytes were further purified from PBMCs using negative magnetic bead selection (EasySep Human CD8⁺ T cell enrichment kit, Stemcell Technologies catalog no. 19053). GMP-grade PDCs were derived from an immortalized line of PDCs (PDC*line) (25). PDCs were cultured in X-VIVO 15 medium (Lonza catalog no. BEBP02-054Q) supplemented with gentamicin (20 μ g/ml, Life Technologies catalog no. 15710-049). PDCs were loaded individually with each peptide (10 μ M, 3-h incubation at 37°C), pooled, washed, and irradiated at 60 Gy (BloodXRad, Cegelec) before freezing as subunit product doses in 10% DMSO (Sigma catalog no. D2650). The mix of peptide-loaded PDCs was cocultured in 24-well plates with the donor cells (PBMCs or purified CD8⁺ T cells) at a ratio of 50,000 PDC loaded with each peptide for 2 million donor cells. The cells were cocultured in RPMI 1640 medium containing GlutaMAX (Life Technologies catalog no. 72400-021) and supplemented with gentamicin (20 μ g/ml, Life Technologies catalog no. 15710-049), nonessential amino acids (1 \times , Life Technologies catalog no. 11140-035), and sodium pyruvate (1 mM, Sigma catalog no. 113-24-9). The enriched RPMI was then supplemented with 10% decomplexed FBS (Life Technologies catalog no. A3160801) and 200 UI/ml IL-2 (Proleukin, Promega). The cultures were stimulated at day 0 (D0) and D7, and the cells were harvested at D14. Flow cytometry detection of specific CD8⁺ T cells was performed at D0 and D14 (BD FACS Canto II). The phenotype was assessed using peptide-specific labeled dextramers (FITC, PE, APC, Immudex) combined with anti-CD3 (BV421, clone UCHT1 BD Horizon catalog no. 562426) and anti-CD8 (PerCP-Cy5.5, clone RPA-T8 BD Pharmingen catalog no. 560662) Abs and a viability marker (Live/Dead, Invitrogen catalog no. L34966). The data were analyzed using FACS Diva (version 9.0.1) and FlowJo (version 10.7.2) softwares. Gating strategy included successive gating on lymphocytes, single cells, live cells, and CD3⁺CD8⁺ cells. For qualitative analysis (Fig. 2), a detection threshold was defined as a minimum of 10 dextramer-positive events for an average 500,000 CD8⁺ T cells, corresponding to a frequency of >0.002%.

Cloning of peptide-specific CD8⁺ T cells after *in vitro* culture and TCR sequencing

After 2 wk of expansion by peptide-loaded PDC, peptide-specific CD8⁺ T cells were sorted by flow cytometry upon dextramer staining (FACS Aria II) and one single cell was distributed per well in 10% RPMI medium containing 10% FBS, 300 UI/ml IL-2, 2 μ g/ml PHA (Thermo Fisher Scientific catalog no. R30852801), and feeder cells (100,000 30-Gy-irradiated PBMCs from two different donors per well and 10,000 60-Gy-irradiated ROSI-B-EBV cells per well). The cultures were diluted every 2–3 d with a 50% medium change with 150 UI/ml IL-2. The cultures were restimulated with medium containing IL-2, PHA, and feeders every 2 wk. Peptide-sorted specific clones were amplified, and 150,000 to 200,000 cells of interest were resuspended in lysis binding buffer (Thermo Fisher Scientific, catalog no. A33562). TCR sequencing was performed using the SEQTR as described previously (32). Briefly, a multiplex reverse transcription converted cRNA into single-stranded DNA using a collection of TRAV/TRBV-specific primers, and two rounds of PCR were performed. The PCR products were purified, quantified, and sequenced by next-generation sequencing (MiSeq, Illumina). TCR sequences were further processed using ad hoc NGSPerl scripts (33).

Functionality testing

The functionality of cloned peptide-specific CD8⁺ T cells was assessed with IFN- γ secretion testing by ELISpot (human IFN γ ELISpot kit, Diaclone, France, catalog no. 856.051). A total of 3,000 peptide-specific CD8⁺ T cells were challenged with 6,000 peptide-loaded T2 cells (10 μ M) in culture plates during 18 h at 37°C. Unloaded T2 cells and culture medium were used as a control. Spot revelation used a biotinylated detection Ab and streptavidin-AP substrate with 5-bromo-4-chloro-3-indolyl phosphate/NBT. Spots were counted with an automated counter (Bioreader E β , EazyReader v.20.9). The results are presented as the number of spots for 100,000 cells.

Table I. Description of SARS-CoV-2 selected peptides

Short Name	Sequence	Protein	Position	IC ₅₀ , nM	Hydrophobicity, %	SARS-CoV-1 Homology	Description in SARS-CoV-1
LLL	LLLDRLNQL	Nucleoprotein	222	14.81	33.67	9/9	Refs. 34–38
GMS	GMSRIGMEV	Nucleoprotein	316	50.61	22.16	9/9	Refs. 34–38
VLN	VLNDILSRL	Spike	976	33.57	32.95	9/9	Refs. 35, 37, 39
NLN	NLNESLIDL	Spike	1,192	177.32	30.32	9/9	Refs. 35–39
YLQ	YLQPRTFLL	Spike	269	5.36	37.47	7/9	
RLQ	RLQSLQTYV	Spike	1,000	16.66	24.92	9/9	Refs. 35–39
VLW	VLWAHGFEL	ORF1ab	6,101	5.78	37.32	9/9	
SMW	SMWSFNPET	Membrane	107	19.09	29.06	9/9	

Peptide sequences, proteins, and positions were defined from the reference sequence of SARS-CoV-2 (NC_045512.2). The short name corresponds to the amino acids in positions 1, 2, and 3. IC₅₀ and hydrophobicity were predicted by a bioinformatics algorithm using netMHCpan4.1, IEDB, and the Peptide Analyzing Tool, respectively (28–30). SARS-CoV-1 homology was defined by comparison with the reference sequence of SARS-CoV-1 (GCF_000864885.1).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 9.3.1). Specific CD8⁺ T cell frequencies of unexposed and convalescent donors were compared using a nonparametric unpaired Mann–Whitney *U* test. Amplifications of specific CD8⁺ T cell with the PDC*line in unexposed donors were analyzed with nonparametric paired Wilcoxon test. **p* < 0.05, ***p* < 0.01.

Results

Selection of potential immunogenic peptides derived from SARS-CoV-2 proteins

From the whole SARS-CoV-2 reference sequence, 9,037 overlapping HLA-A*02:01-restricted 9-mer peptides were extracted *in silico* from different proteins (envelope [*n* = 67], membrane [*n* = 214], nucleoprotein [*n* = 411], spike [*n* = 1,265], and ORF1ab [*n* = 7,080]). From this set of peptides, 253 were predicted to be strong HLA-A*02:01 binders with a predicted IC₅₀ < 200 nM. Among them, eight peptides were selected based on different criteria or description in the literature (see *Materials and Methods* and Table I). The two peptides, LLL and GMS, were derived from the nucleoprotein; the four peptides VLN, NLN, YLQ, and RLQ were derived from the spike protein; one peptide, VLW, was derived from the ORF1ab; and one peptide, SMW, was derived from the membrane protein. Most of them showed homology with SARS-CoV-1 (34–39), except YLQ, which was specific to SARS-CoV-2 (Supplemental Fig. 1). Interestingly, two conserved peptides (VLW and SMW) from ORF1ab and from the membrane had not been studied in SARS-CoV-1. In addition, these peptides were conserved between SARS-CoV-2 variants, except VLN, which was mutated in both the Alpha and Omicron variants (Supplemental Fig. 1). All peptides but one (NLN) displayed an IC₅₀ below or equal to 50 nM. Three peptides had a proteasome cleavage

probability below 0.1, but two were described in the literature (GMS and NLN), suggesting *in vivo* generation despite low predicted probability of cleavage. All peptides had a hydrophobicity below 40%, suggesting good solubility and ease of synthesis.

We next verified the ability of the selected peptides to efficiently bind to HLA-A*02:01 molecules. With this aim, we assessed peptide binding with a stabilization assay of HLA-A2 molecules at the surface of the T2 cell line. As shown in Fig. 1, all the selected peptides were able to induce an increase in HLA-A2 surface levels on the T2 cell line, as expressed by higher MFI of the anti-HLA-A2 staining, as measured by flow cytometry (Fig. 1A). This demonstrated their ability to bind to HLA-A2 molecules. The MFI shift from baseline was used to calculate the FI. VLW and YLQ were as efficient HLA-A2-binders as the MelanA peptide, which was chosen as positive control, with respective mean FI of 0.59, 0.64, and 0.64 (average of three experiments). Although lower levels of HLA-A2 molecules were observed with the peptides RLQ, SMW, and GMS, the positive shifts observed clearly demonstrate their capacity to bind to HLA-A2 with respective FIs of 0.24, 0.27, and 0.28. In comparison, the CMV HLA-B7-restricted peptide used as negative control had a negative mean FI of –0.05 (Fig. 1B).

SARS-CoV-2-specific CD8⁺ T cells are detected *ex vivo* in unexposed and convalescent donors at different levels

To evaluate the biological relevance of using these peptides, we next assessed the *ex vivo* frequencies of specific T cells directed against the selected peptides by multimer staining, in a cohort of convalescent (*n* = 9) and unexposed (*n* = 8) donors (Fig. 2A, 2B). We detected *ex vivo* specific CD8⁺ T cells against at least one peptide (with a frequency above the set threshold of 0.002%), for all of

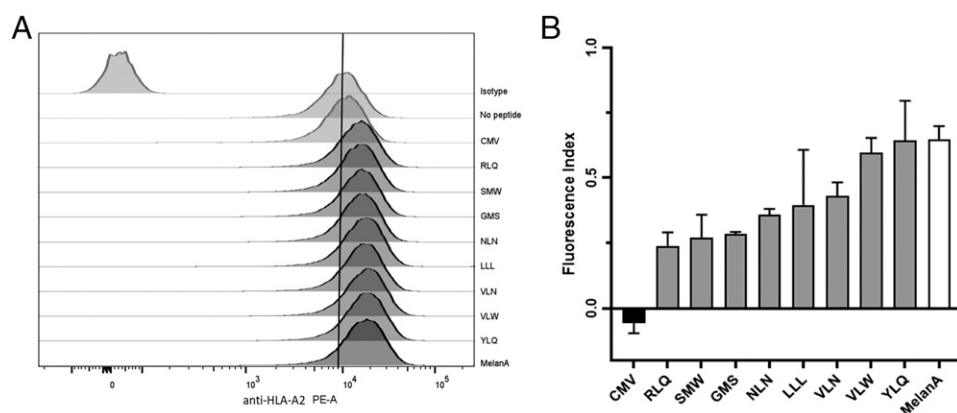
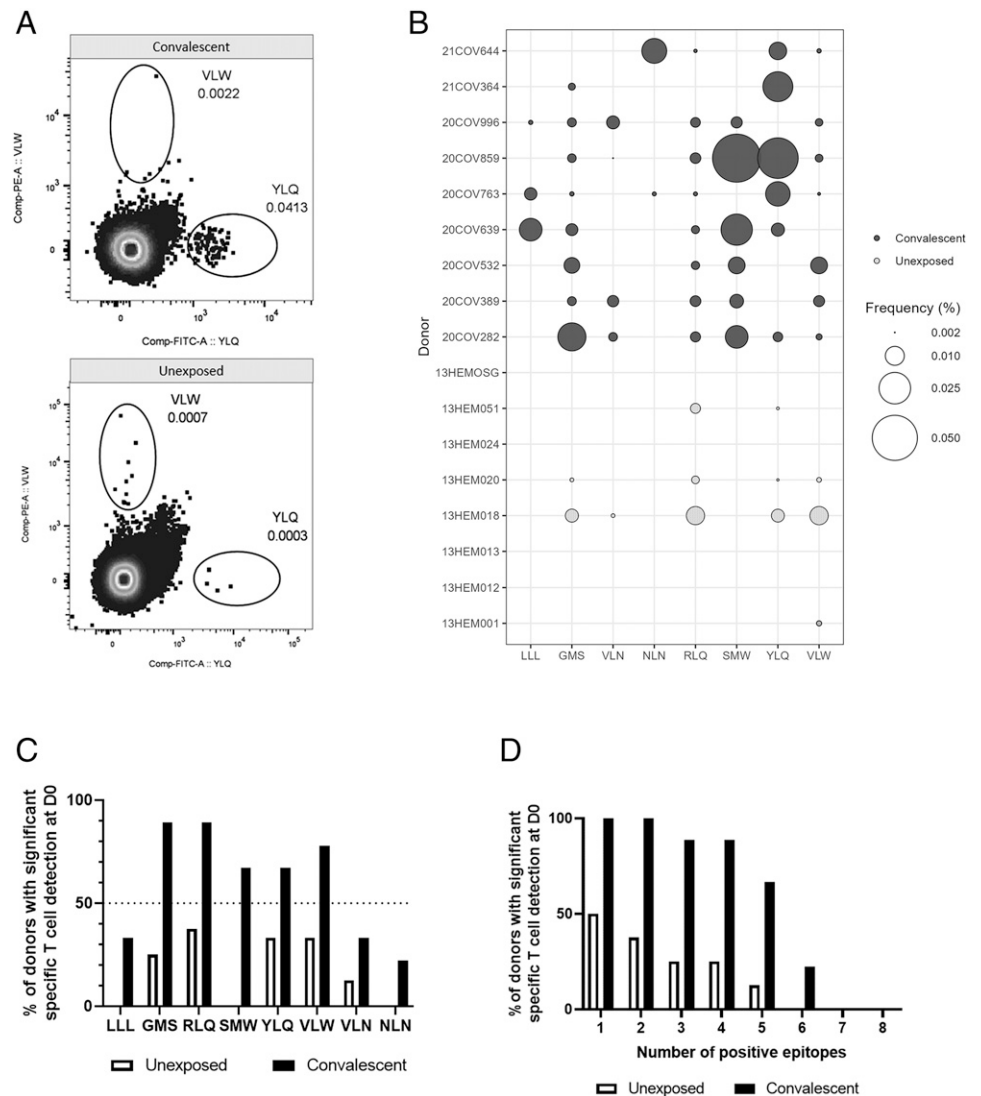


FIGURE 1. Assessment of HLA-A2 peptide binding. **(A)** Representative experiment of the median fluorescence intensities (MFIs) of anti-HLA-A2 staining on peptide-loaded (10 μ M) and peptide-unloaded T2 cells. Positive MFI shifts indicate that peptides bound to HLA-A2 molecules. The MelanA peptide was used as positive control, unloaded T2 (“No peptide”), and the CMV HLA-B7 restricted peptide were used as negative controls. **(B)** Fluorescence index of peptide-loaded T2. Means with SD are represented (*n* = 3).



the nine convalescent donors. In each donor, Ag-specific T cells were detected for two to six peptides (median = 5 of 8). As an indicator of response diversity, basal CD8⁺ T cell frequencies higher than 0.002% for GMS, RLQ, SMW, YLQ, and VLW peptides were detected in more than 50% of convalescent donors (Fig. 2C). T cells directed against the SMW and YLQ peptides reached the highest frequencies. Frequencies of SMW-specific T cells reached 0.056% and 0.025% of CD8⁺ T cells for 20COV859 and 20COV639, respectively. The frequencies of YLQ-specific T cells reached 0.041 and 0.023% of CD8⁺ T cells for 20COV859 and 21COV364, respectively. Interestingly, for unexposed donors, significant frequencies of antiviral specific T cells (>0.002%) were detected in only four of eight individuals toward up to five peptides (median = 0.5 of 8) (Fig. 2D).

As shown in Fig. 3, CD8⁺ T cells directed against LLL, GMS, YLQ, and SMW peptides were found to be significantly more frequent in convalescent than in unexposed donor samples ($p < 0.05$, unpaired nonparametric Mann–Whitney U test). CD8⁺ T cells specific for VLN, RLQ, VLW, and NLN peptides were slightly more frequent in convalescent donors, but the difference was not significant. Overall, the median value of the total frequencies of specific CD8⁺ T cells directed against all the selected peptides was of 0.03% for convalescent donors (range, 0.0237–0.111%). It was significantly higher than for unexposed donors (median = 0.0044; range = 0.0013 to 0.036%; $p = 0.0025$). Altogether, our data demonstrate that specific CD8⁺ T cells directed

against the set of SARS-CoV-2 derived peptides that were chosen for this study can be found both in unexposed and convalescent donors. The higher frequencies of these CD8⁺ T cells observed in convalescent donors suggests their recent activation upon infection. Overall, specific T cells were more frequent in convalescent than unexposed donors (Fig. 3).

Peptide-specific CD8⁺ T cells from unexposed donors expand upon activation with the allogeneic PDC*line

The irradiated and peptide-loaded PDC*line was cocultured with CD8⁺ T lymphocytes purified from unexposed donor PBMCs to evaluate the ability of the cell line to trigger the expansion of virus-specific CD8⁺ T cells. Two stimulations were performed at D0 and D7. Frequencies of peptide-specific CD8⁺ T cells were assessed by flow cytometry using multimer labeling at baseline (D0) and D14 (Fig. 4A). Strikingly, at D14, significant peptide-specific CD8⁺ T cell expansions were found for all the donors (Fig. 4B). The highest amplification rate was observed for the donor 13HEM020 with a 198-fold increase in total peptide-specific CD8⁺ T cells frequencies from baseline to D14. The lowest amplification was observed for the donor 13HEM018 with a 7.5-fold increase from baseline to D14. Significant responses were observed against at least four peptides for every donor: GMS, RLQ, YLQ and VLW. Two donors responded to the eight SARS-CoV-2-derived peptides (Fig. 4C). None of the donors was considered as nonresponsive. This demonstrated that

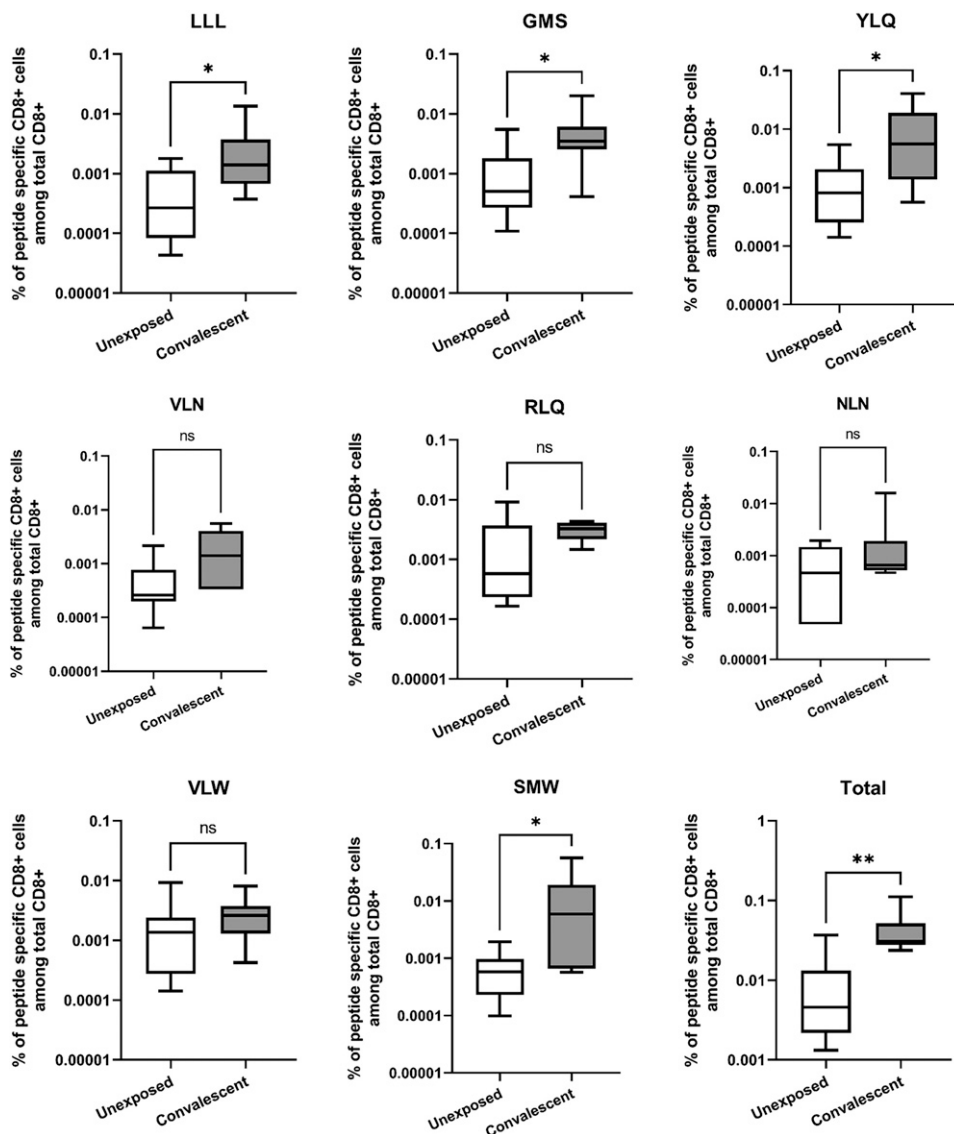


FIGURE 3. Comparison of ex vivo SARS-CoV-2–derived peptide-specific CD8⁺ T cell frequencies in unexposed and convalescent donors. Peptide-specific CD8⁺ T cell frequencies were detected by dextramer labeling in flow cytometry in unexposed (white, $n = 8$) and convalescent (gray, $n = 9$) donors at baseline. Box and whisker plots represent median, minimum, and maximum. Statistics were determined using the Mann–Whitney U test. * $p < 0.05$, ** $p < 0.01$.

robust CD8⁺ T cell responses in unexposed donors against SARS-CoV-2–derived peptides can be generated with the irradiated and peptide-loaded allogeneic PDC*line.

At the peptide level, significantly higher frequencies of peptide-specific CD8⁺ T cells were detected for all the peptides, except for the NLN peptide (Fig. 4D). The peptide that provided the best T amplification was the YLQ peptide with a mean fold increase of 109 times between D0 and D14, followed by RLQ with a mean fold increase of 36 times; both of these peptides were derived from the spike protein. The peptides with the weakest responses were the VLN and NLN peptides with a mean fold increase of 4 and 2.5 times, respectively. These two peptides also derived from the spike protein and were discarded from the study. Thus, the peptides LLL, GMS, VLW, and SMW, derived from the membrane, nucleoprotein, and ORF1ab proteins, in addition to the aforementioned spike peptides RLQ and YLQ, were considered good candidates to stimulate the immune response of SARS-CoV-2–naïve subjects.

SARS-CoV-2 convalescent and unexposed donors reach similar CD8⁺ T cell responses after in vitro plasmacytoid dendritic cell line stimulation

We then wondered whether antiviral specific T cells from convalescent donors could be expanded by the PDC*line. Cocultures were

performed with PBMCs and the irradiated, peptide-loaded PDC*line for 14 d. At the end of the culture, multimer stainings were performed to assess the expansion of six of the eight peptides. Expansions for all convalescent donors were observed and compared with the ones seen with unexposed donors (Fig. 5). Similar responses were observed for all peptides with nonstatistical differences between the two cohorts. Thus, no difference was found postexpansion, which might reflect a characteristic maximal T cell expansion for each peptide due to TCR affinity, telomere shortening upon proliferation, or T cell exhaustion in situ or in vitro.

In vitro expanded CD8⁺ T cells are functional and secrete IFN- γ upon peptide-specific stimulation

RLQ-specific CD8⁺ T cells expand with the PDC*line in all donors, and thus RLQ might be an interesting epitope for immunotherapies. We assessed the functionality of two RLQ-specific CD8⁺ T cell clones in an IFN- γ ELISpot assay with peptide-loaded T2 cells. Both clones secreted IFN- γ upon recognition of RLQ-loaded T2 cells but not with unloaded T2 cells, suggesting their specificity for HLA-A2-RLQ (Fig. 6).

TCR sequencing of peptide-specific CD8⁺ T cell clones

Because the use of the peptide-loaded PDC*line allows the in vitro expansion of peptide-specific CD8⁺ T cells, the line could be used

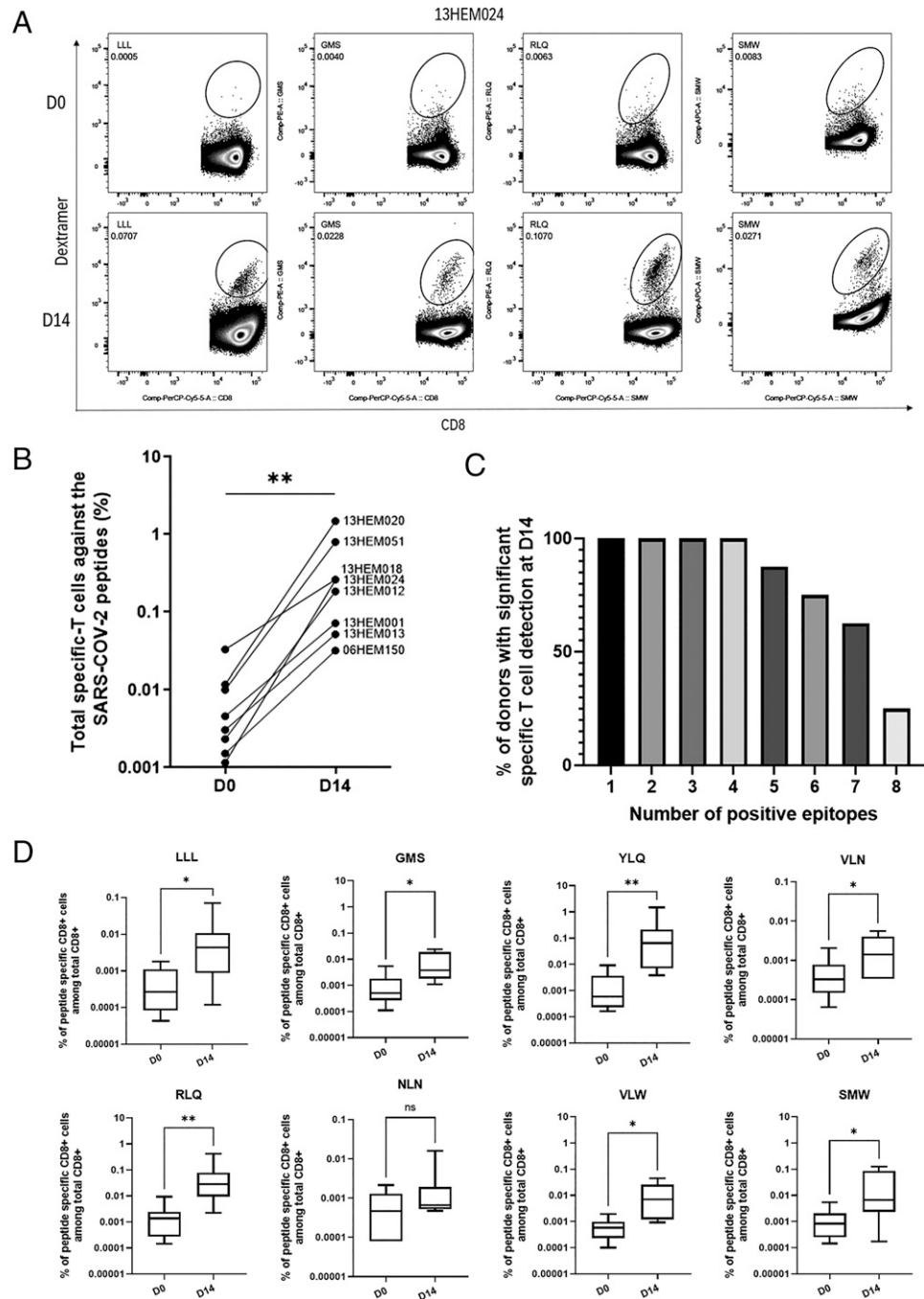


FIGURE 4. Evaluation of the peptide-loaded PDC*line ability to amplify peptide-specific CD8⁺ T cells in unexposed individuals. **(A)** Flow cytometry detection of peptide-specific CD8⁺ T cells using dextramer labeling at days 0 and 14. Dot plot gated on lymphocytes/single cells/alive/CD3⁺CD8⁺. **(B)** Total peptide-specific CD8⁺ T cell amplification from days 0 to 14 after stimulations with PDC in unexposed donors ($n = 8$) (Wilcoxon paired nonparametric test). $**p < 0.01$. **(C)** Percentage of responsive donors according to the number of epitopes. **(D)** Evolution of peptide-specific CD8⁺ T cells frequencies from D0 to D14 in unexposed donors ($n = 8$). Box and whisker plots represent median, maximum, and minimum (Wilcoxon paired nonparametric test). $*p < 0.05$, $**p < 0.01$.

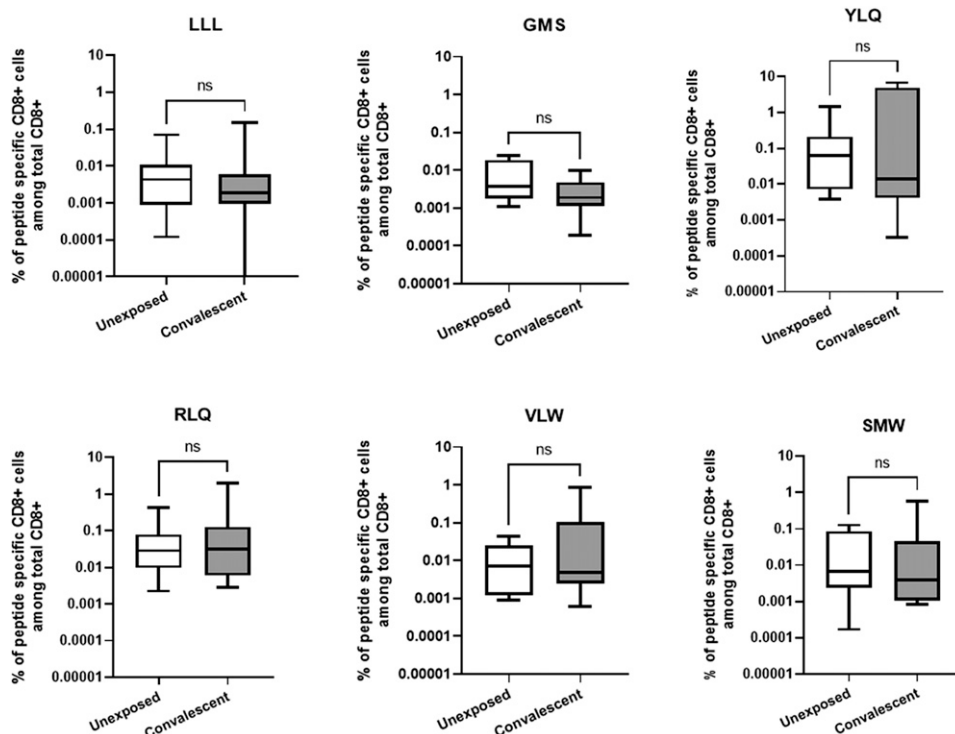
as a powerful tool to identify TCR sequences which target highly immunogenic peptides for further TCR-transgenic applications, in addition to boosting patients' immune responses. We report here the feasibility of TCR sequencing after ex vivo expansion by the PDC*line. Peptide-specific T cells against YLQ and RLQ were cloned by FACS sorting from one unexposed donor having high responses after in vitro expansion. These two specificities were chosen due to the high expansion rate of specific T cells. Cloning of specific cells (>99% specificity by dextramer staining) was followed by TCR sequencing. The identified TCR sequences are presented in Table II. The TCR directed against YLQ made use of TRAV12-1 and TRAJ43, whereas TRBV20 and TRBJ2-2 were used for the TRB chain. The frequencies of each sequence were over 80%, suggesting that the expansion was in majority monoclonal. The identification of

these TCR sequences was efficient and promising for the design of future TCR-based immunotherapies.

Discussion

Cytotoxic T cell-mediated immunity is believed to be important for protection against severe COVID-19 forms, and active or adoptive CD8⁺ T cell therapies might be of particular interest to counteract the impairment in T cell responses observed in severe cases and patients with defective B cell immunity. We herein proposed a systematic method to generate a large number of CD8⁺ T cells directed against on-demand peptides of interest, enabling their cloning, functional characterization, and TCR sequencing for downstream applications. In this study, we investigated the immunogenicity and the ability to generate antiviral CD8⁺ T cells

FIGURE 5. Comparison of responses to PDC stimulation between unexposed and convalescent donors. Shown are the unexposed (white) and convalescent (gray) peptide-specific CD8⁺ T cell frequencies at day 14 after two stimulations with irradiated allogeneic plasmacytoid dendritic cell line loaded with SARS-CoV-2–derived peptides. Box and whisker plots represent median, minimum, and maximum (Mann–Whitney nonparametric unpaired test).



against eight SARS-CoV-2–derived peptides using an innovative plasmacytoid dendritic cell–based approach. Most of these peptides are conserved between SARS-CoV-2 variants and originate from several virus structural and enzymatic components of the viral particle.

As a test for in vitro immunogenicity, a robust proliferation of peptide-specific CD8⁺ T cells was observed for six of the eight SARS-CoV-2–derived peptides selected by bioinformatics analysis after 14 d, namely the nucleoprotein peptides LLL and GMS, the spike peptides RLQ and YLQ, the ORF1ab peptide VLW, and the membrane peptide SMW. We observed that the efficiency of the PDC*line for inducing T cell proliferation was lower for

the spike peptides NLN and VLN. This might be explained by lower TCR affinity or functional impairment of specific T cells in vivo (e.g., exhaustion). Another explanation might rely on the lower frequencies of CD8⁺ precursors against these peptides at baseline. As reported by Shomuradova et al. (40), low levels of CD8 directed against these peptides are detected ex vivo in convalescent donors. On the contrary, they found high frequencies of CD8⁺ T cells directed against the YLQ and RLQ peptides that were detectable in 13 of 14 and 10 of 14 convalescent donors, respectively. Similar observations were made by Schulien et al. (14) and Nielsen et al. (41), and these two peptides induced the largest and most reproducible expansions in our experiments. When measuring the presence of CD8⁺ T cells directed against the SARS-CoV-2 peptides that we selected in convalescent donors, we detected peptide-specific CD8⁺ T cells for all convalescent donors, with a median of five of eight peptide specificities. Altogether, these data suggest that these peptides are processed in vivo by the proteasome and take part in efficient antiviral CD8⁺ T cell responses. Interestingly, responses against these peptides were also generated in unexposed and thus SARS-CoV-2–naïve donors, suggesting an intrinsic immunogenicity for these peptides.

One strength of approaches focusing on CD8⁺ T cell responses also relies on the seemingly lower variability of the peptides they

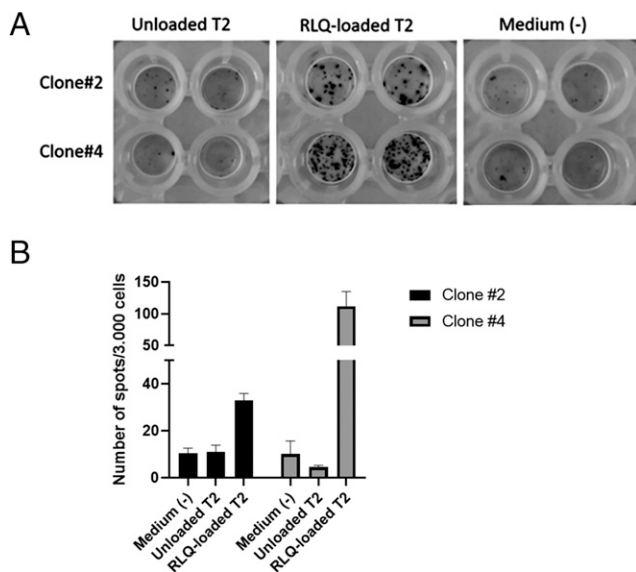


FIGURE 6. ELISpot assays for IFN- γ secretion by RLQ-specific CD8⁺ T cells clones. (A) Photograph of ELISpot plates (2 wells/condition). (B) Number of spots in each condition for the RLQ clones 2 (black bars) and 4 (gray bars).

Table II. TCR sequences analysis

Peptide	Sequence		Frequency
RLQ	TRAV	CDR3 α	TRAJ
	hTRAV21	CAVFGDSNYQLIWGAG	hTRAJ33
	TRBV	CDR3 β	TRBJ
YLQ	hTRBV11-2	CASSGGEAPFNEKLF	hTRBJ01-4
	TRAV	CDR3 α	TRAJ
	hTRAV12-1	CVVNRMDDMRFG	hTRAJ43
TRBV	CDR3 β	TRBJ	
	hTRBV20	CSARSARAQNTGELFFG	hTRBJ02-2
			89.55
			91.6
			84.73
			89.37

TRAV, TRAJ, TRBV, TRBJ, and CDR3 sequences were defined by next-generation sequencing from two CD8⁺ T cell clones specific for RLQ and YLQ peptides.

target, and many studies demonstrated that variant emergence rarely affects CD4⁺ and CD8⁺ T responses (42, 43). Interestingly, most of the selected peptides are conserved in variants of concern, except for VLN, which was mutated in both the Alpha and Omicron variants (Supplemental Fig. 1). Hence, it could be interesting to target conserved coronaviruses epitopes because cross-reactivity might enhance T cell responses and provide further protection, as observed in young children (44). However, it has been reported that the P272L mutation in the frequently recognized YLQPRTFLL–HLA-A*02:01 epitope occurred concomitantly with escape from the immunodominant YLQPRTFLL-directed CD8⁺ T cell responses (45). Nevertheless, the significance of this observation remains unclear, as the P272L mutation is limited to certain lineages and reportedly remains a minor occurrence and was not fixed in successive variants of concern. Focusing on different proteins as T cell targets in future vaccines may contribute to avoid viral immune escape.

Overall, in view of these data, the peptides LLL, GMS, RLQ, YLQ, VLW, and SMW could be interesting targets for immunotherapies, because they are likely to be processed and presented by virus-infected cells and they induce strong T cell expansions in vitro. Positive T cell responses were found for all the viral proteins included (i.e., Spike, M, N, ORB1ab) allowing a broad range of responses covering different parts of the virus and different steps in the infection cycle. Thus, designing a multipeptide vaccine using these six peptides may induce robust CD8⁺ T cell responses in vivo, and we assume that with this combination, anyone should respond against at least one peptide because the combination of four peptides (RLQ, YLQ, SMW, and VLW) led to significant responses in 100% of the tested donors. A clinical study has explored the potential of a multipeptide peptide-based SARS-CoV-2 vaccine from different proteins in a phase I/II clinical trial with promising results founding significant cytokine production in CD8⁺ T cells (46).

The PDC*line platform represents an ideal candidate to generate and study antiviral CD8⁺ T cell responses, both in vitro and in vivo. As stated above, a vaccine focused on specific T cell expansion should be interesting for patients with B cell lymphopenia that can solely develop T cell responses. For example, patients treated with rituximab, an anti-CD20 mAb widely used in hematologic cancers or autoimmune disease, cannot mount humoral responses during the treatment. During the pandemic, these patients faced increased risks of being infected with SARS-CoV-2 and developing severe COVID-19 outcomes (18, 47, 48). Moreover, these patients did not respond to conventional vaccination and hence could benefit from a vaccine stimulating the CD8⁺ T cell arm (49, 50).

In addition, using a PDC-based approach in the context of COVID-19 is highly attractive. Indeed, the use of exogenous PDCs may counterbalance the impaired number of PDCs reported in severe infections (5) and the subsequent reduced Ag presentation. This might help bypass impaired adaptive immunity that contributes to viral dissemination and disease severity in some patients (51). A similar strategy was previously proposed for SARS-CoV-1 treatment. Several peptides, including the LLL and GMS peptides, were loaded on autologous dendritic cells and cocultured with donor-derived CD8⁺ T cells, and significant IFN- γ secretion was observed by ELISPOT (36).

Other important information that can be garnered following PDC-induced CD8⁺ T cell expansion is the functional characterization of specific T cells, as well as their TCR sequences. The TCR sequences of highly expanded CD8⁺ T cells against YLQ and RLQ were identified, and gene usage conformed to previously characterized TCR sequences against HLA-A*02:01- YLQ, which may prove important to understand the immunodominance of this Ag in vivo.

Indeed, consistent with previous study (33), the TCR directed against YLQ made use of TRAV12-1, whereas TRBV20 and TRBJ2-2 were used for the TRB chain, similarly to independently isolated HLA-A*02:01-YLQ-specific TCR (33). RLQ-specific T cell clones were functional and secreted IFN- γ upon specific restimulation. These data highlight the potential for short-term T cell expansion by the PDC*line for specific T cell characterization, enabling molecular specification of antiviral T cells and their potential use in downstream TCR-transgenic applications.

In the current context, in which a large fraction of the population has gained immunity to SARS-CoV-2 through infection or prophylactic vaccination, this platform may not be used for therapeutic vaccination. However, our results provide proof of concept that the PDC*line is a valuable tool to screen candidate epitopes and efficiently expand in vitro antiviral CD8⁺ T cell responses. Candidate peptides can be ranked in terms of expansion potential for inclusion in a T cell-targeted vaccine. Furthermore, thanks to high amplification rates, TCR sequences can be obtained for treatments based on adoptive transfer of TCR-transgenic T cells. The flexibility of the PDC*line platform enables rapid identification of T cell targets in current and future emergent viral diseases.

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Disclosures

Dr. Joel Plumas is cofunder, shareholder and employee of PDC*line Pharma. Dr Marina Deschamps is cofunder, shareholder and part time employee of CanCell Therapeutics. The other authors have no financial conflicts of interest.

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