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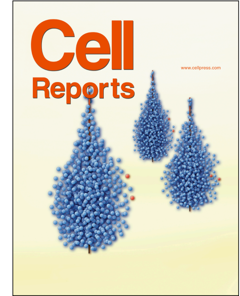

HLA-A*02:01 restricted T cell receptors against the highly conserved SARS-CoV-2 polymerase cross-react with human coronaviruses

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Unexposed Donor

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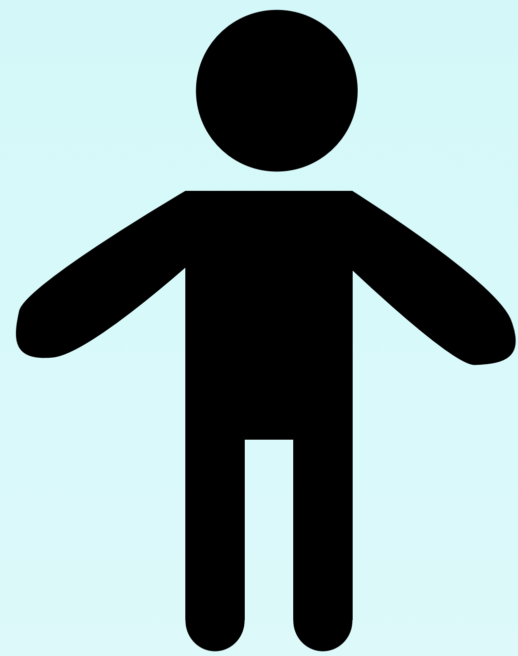
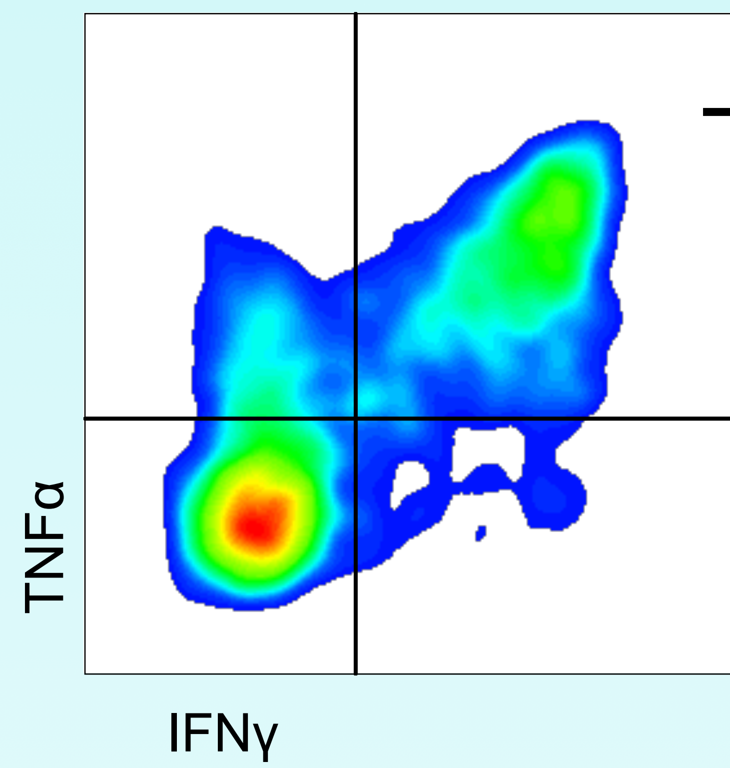
CD8+ T cells



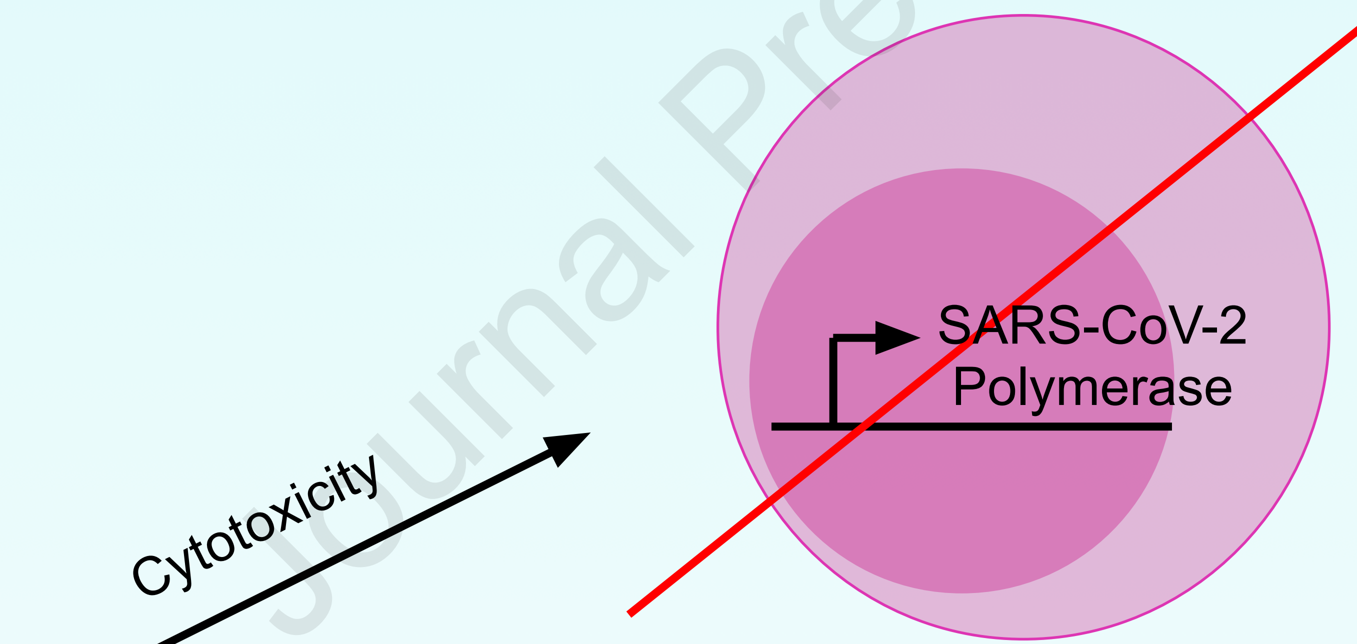


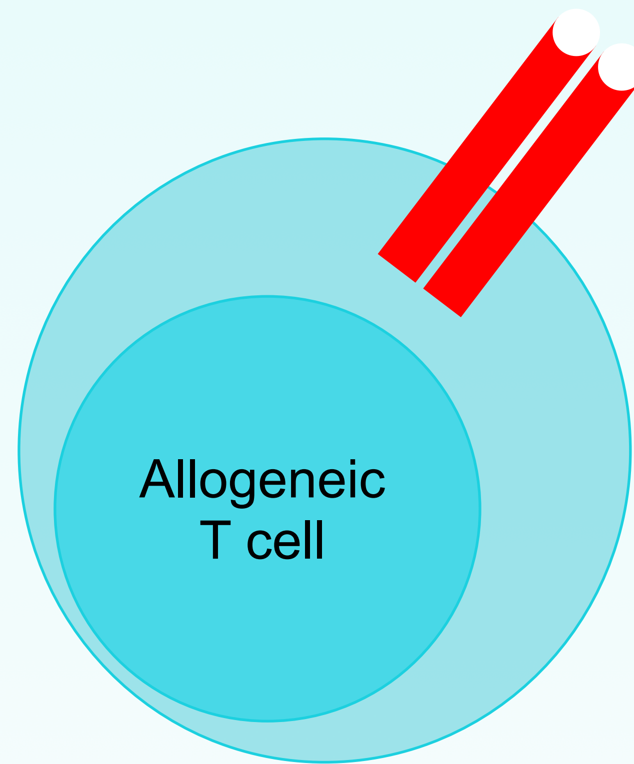
TCRα

TCRβ

Functional analysis

Engineered antigen presenting cell

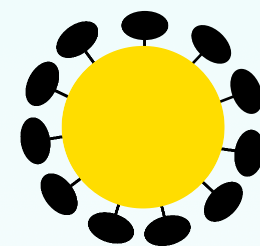




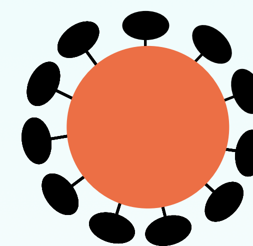
Cytotoxicity

Cross-reactivity

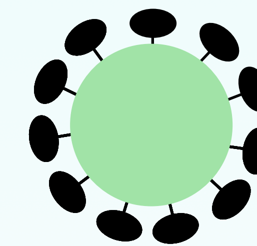
SARS-CoV-2



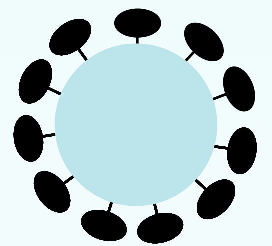
MERS



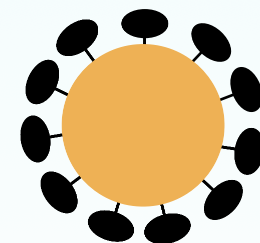
HKU1



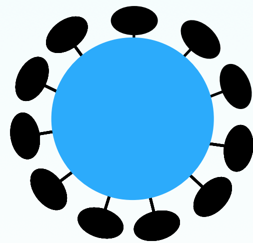
NL63



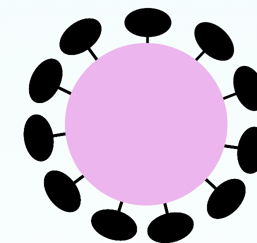
SARS-CoV-1



OC43



229E



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Title: HLA-A*02:01 restricted T cell receptors against the highly conserved SARS-CoV-2 polymerase cross-react with human coronaviruses

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Summary:

35 Cross-reactivity and direct killing of target cells remain underexplored for SARS-CoV-2 specific CD8+ T cells. Isolation of T cell receptors (TCRs) and overexpression in allogeneic cells allows for extensive T cell reactivity profiling. We identify SARS-CoV-2 RNA-dependent RNA-polymerase (RdRp/NSP12) as highly conserved likely due to its critical role in the virus life cycle. We perform single-cell TCRαβ sequencing in HLA-A*02:01 restricted, RdRp specific T
40 cells from SARS-CoV-2 unexposed individuals. Human T cells expressing these TCRαβ constructs kill target cell lines engineered to express full length RdRp. Three TCR constructs recognize homologous epitopes from common cold coronaviruses, indicating CD8+ T cells can recognize evolutionarily diverse coronaviruses. Analysis of individual TCR clones may help define vaccine epitopes that can induce long term immunity against SARS-CoV-2 and other
45 coronaviruses.

Keywords: T cells, SARS-CoV-2, antigen, specific, CD8, TCR, single-cell, COVID-19, immune response, cell therapy

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Introduction:

Over 4 million people have died from COVID-19 as of August 2021 (World Health
50 Organization). Many individuals are now immune as a result of successful vaccination campaigns and protection afforded by the natural infection with SARS-CoV-2 (Anand et al., 2021; Baden et al., 2021; Lumley et al., 2020; Polack et al., 2020; Sadoff et al., 2021). The virus continues to evolve and may escape immune responses generated against the original sequence (Harvey et al., 2021; Planas et al., 2021). The BNT162b2 mRNA vaccine is 88% effective
55 against the new Delta variant compared with 93.7% for the Alpha variant that was circulating previously (Bernal et al., 2021). Increased spread in vaccinated populations necessitates further understanding of the SARS-CoV-2 immune response.

This pandemic can only be controlled by herd immunity against contemporary strains of the
60 virus. Vaccination against the wild type spike protein can prevent COVID-19 (Baden et al., 2021; Polack et al., 2020; Sadoff et al., 2021). SARS-CoV-2 vaccines target the spike protein by generating neutralizing antibodies that prevent host cell infection (Khoury et al., 2021; Lumley et al., 2020). SARS-CoV-2 variants often contain multiple mutations in the spike protein and can resist antibody neutralization creating the possibility that, upon further diversification, viral
65 variants may escape current vaccine defenses (Hoffmann et al., 2021; Kuzmina et al., 2021; Muik et al., 2021; Planas et al., 2021; Wang et al., 2021). Cytotoxic T cells kill infected cells thereby directly limiting viral dissemination once the infection occurred (Hall et al., 1986; Harty et al., 2000; Jozwik et al., 2015; McMichael et al., 1983). T cell recognition is not limited to surface proteins like the spike protein; more conserved proteins can be targeted. Internal SARS-
70 CoV-2 proteins are more conserved than the spike and may present a therapeutic opportunity at

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generating T cell responses that can recognize many coronavirus strains (Grifoni et al., 2020a). T cell vaccine strategies, targeting the nucleocapsid protein, are being explored to generate long term immunity against SARS-CoV-2 (Dutta et al., 2020; Gauttier et al., 2020; Sieling et al., 2021). It remains unknown which epitopes elicit the most effective antiviral responses (Chen and
75 John Wherry, 2020).

Initial evidence for T cell control of respiratory infections was provided by children with genetic defects in T cell development(Hall et al., 1986). Resident memory T cells, which are permanently localized in non-lymphoid tissues, including the lung, are thought to mediate anti-
80 viral responses(Jozwik et al., 2015). In a human RSV infection disease severity was inversely correlated with the preexisting T cells in the lung(Jozwik et al., 2015). Adoptive transfer of highly functional T cell clones can reduce severity of viral diseases as well(Einsele et al., 2002; Feuchtinger et al., 2010a). The mechanism of respiratory viral infection T cell control is thought to happen through FAS and perforin mediated lysis of infected cells(Topham et al., 1997). The
85 efficiency of lysis correlates with the ability to clear an infection(McMichael et al., 1983).

Both convalescent donors and unexposed individuals have SARS-CoV-2 specific T cell responses (Le Bert et al., 2020; Braun et al., 2020; Grifoni et al., 2020b; Mateus et al., 2020; Peng et al., 2020; Tarke et al., 2021; Weiskopf et al., 2020). CD8+ T cell responses have been
90 identified as correlates of protection in SARS-CoV-2 infection (Chen and John Wherry, 2020; Liao et al., 2020; McMahan et al., 2021). Unexposed individuals may have T cell responses that were generated by common cold coronaviruses (HCoVs) and may be partially protective against SARS-CoV-2 encounter(Lipsitch et al., 2020; Mallajosyula et al., 2021; Mateus et al., 2020). T

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cells interact with target antigens through the T cell receptor (TCR), which is a heterodimer of
95 alpha and beta chains. TCRs are inherently cross-reactive to maximize the breadth of ligand recognition, however a single TCR is not guaranteed to recognize related antigens (Sewell, 2012). Several cross-reactive CD8+ T cell responses are known, but specific TCRαβ clones that can drive such reactivity are not defined (Lineburg et al., 2021; Lipsitch et al., 2020; Mallajosyula et al., 2021; Mateus et al., 2020). T cell memory is most often defined as ability to
100 recognize synthetic peptide epitopes in functional assays or peptide-MHC multimer staining. Recognition of processed epitopes derived from full length intracellular antigens is underexplored in SARS-CoV-2. Isolation of specific TCR clones permits unambiguous determination of reactivity and detailed characterization of immune responses such as cytotoxic potential and measurement of cross-reactivity against related viruses.

105

We employ recent technological advances in single-cell sequencing, DNA synthesis and gene transfer to recover antigen specific TCRαβ and subsequently characterize them in allogeneic T cells. The viral polymerase (NSP12/RdRp) was identified as highly conserved within SARS-CoV-2 and other human coronaviruses. RdRp reactive CD8+ T cells were then selected for
110 TCRαβ droplet-based sequencing based on the intracellular level of TNFα and IFNγ via the CLInt-seq, which allows for antigen specific TCR sequencing *via* commercially available Drop-Seq in cells that are stained for intracellular cytokines (Nesterenko et al., 2021). TCRs were initially screened for single epitope recognition in a cell line system *via* the NFAT-GFP reporter system. Reactive TCRs were overexpressed in human PBMCs and killed antigen presenting cells
115 that expressed the full length RdRp. Three TCR constructs were broadly reactive and cross-reacted with epitope homologues from HCoVs.

Results:

RdRp is highly conserved among human coronaviruses and within SARS-CoV-2

120 Antigens derived from highly conserved SARS-CoV-2 proteins should generate immune responses effective against multiple variants. Human coronaviruses are separated by hundreds of years of evolution and serve as a model of the evolutionary constraints that may restrict variant emergence in SARS-CoV-2 (Forni et al., 2017; Killerby et al., 2018). A group of coronavirus proteins involved in RNA synthesis, immune modulation, and structural machinery was selected

125 for further analysis. Sequence identity was compared across all known human coronaviruses. All proteins showed conservation within sub-classes: alpha coronaviruses (229E and NL63), beta coronaviruses (HKU1 and OC43) and both SARS viruses (Figure 1A). The RdRp was most conserved across all coronaviruses (Figure 1A). Across 893,589 SARS-CoV-2 samples sequenced, RdRp was well conserved and had few mutations compared to the spike protein

130 (Figure 1B, Table S4).

T cell receptors from unexposed individuals recognize RdRp epitopes

To generate TCR clones, we screened pooled peptide epitopes predicted to bind HLA-A*02:01 against HLA matched peripheral blood mononuclear cells (PBMCs) collected prior to December

135 of 2019 (Figure 2A). We refer to these samples as unexposed to SARS-CoV-2. CD8+ T cells that responded by production of TNFα and IFNγ were sorted from four different PBMC donors *via* fluorescence activated cell sorting (FACS) (Figure 2A). Responses were low in all donors, around the level of background set based on DMSO control stimulation, as would be expected for donors who were not exposed to a specific pathogen.

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Reactive cells were sorted for single-cell TCRαβ sequencing *via* a highly sensitive technique called CLInt-Seq. Clonally expanded TCR clones were synthesized and tested in an allogeneic cell-based system for evaluation of immune receptor activation (Figure 2B). A High throughput system for TCR reactivity profiling was established (Figure S1A). We utilized a Jurkat cell line

145 that expressed the NFAT-zsGreen T cell activation reporter construct and the CD8 molecule to stabilize MHC Class I interactions. This cell-based reporter system was then optimized with a well characterized TCR, clone 1G4, which is specific for the cancer antigen NY-ESO-1(D'Angelo et al., 2018) (Figure S1B). Comparison of TCR delivery by electroporation or viral integration resulted in similar extent of T cell activation (Figure S1C). SARS-CoV-2 specific

150 TCRs were then electroporated or transduced into the Jurkat cell line and activation was measured by FACS measurement of zsGreen. SARS-CoV-2 reactive epitopes were identified *via* epitope deconvolution using an array of sub-pools (Figures 2C and S2). Of 44 TCR constructs tested in this system, ten recognized the cognate peptide pool (Figure 2D). TCR clones that did not score as reactive in this assay, either did not reach the threshold of the reporter system or

155 were originally expressed in T cells that did not recognize the queried peptide pool. Because the responses sorted were around the level of background, non-reactive TCRs likely represent the background signal. Nine TCRs clearly recognized four unique epitopes of the RdRp (Figure S2 and Table S1).

160 **Isolated T cell receptors recognize and kill RdRp expressing cells**

Processed antigen recognition is critical for vaccine induced priming of naïve T cell responses as well as for lysis of infected cells. To establish potential antiviral efficacy, seven RdRp specific

TCRs were overexpressed in HLA-A*02:01 positive human PBMCs *via* retroviral delivery (Figure 3A and Table S2). Engineered PBMCs were co-cultured with a target cell line

165 engineered to overexpress the full-length SARS-CoV-2 RdRp protein and HLA-A*02:01 (Figure 3B). The engineered T cells were able to produce TNFα and IFNγ in response to recognition of processed antigens. (Figure 3C, 3D). Full length antigen recognition was significantly lower than peptide pulsing, as measured by T cell cytokine production, most likely due to the concentration of peptide during pulsing assays being supraphysiological (Figure 3D). CD4+ T cells the

170 overexpressed the CoVTCRs also responded to peptide pulsing but did not recognize processed antigen. Production of TNFα and IFNγ in CD4+ T cells ranged from 0.058-10.3%, depending on the TCR.

T cells control viral spread by killing virus infected cells. Cytotoxicity assays showed five out of

175 seven TCRs can direct T cells to kill target cell lines (Figure 3E). Recognition of processed epitopes was confirmed by supernatant IFNγ ELISA assay (Figure 3F). At 48 hours, processed antigen recognition was equivalent or better than the peptide pulsing control (Figure 3F).

We sought to determine how common RdRp specific T cells are. Recently a set of more than

180 160,000 TCRβ genes specific for SARS-CoV-2 was made publicly available (Nolan et al., 2020). This data was generated by peptide pool stimulation of PBMCs from 118 donors and subsequent TCRβ gene sequencing in reactive T cells (Klinger et al., 2015). Unique epitopes were ranked by the count of cognate TCRβ sequences (Figure S3A). Three of the four epitopes we identified were frequently targeted by the SARS-CoV-2 specific TCRβ (Figure S3A). GLIPH2, an

185 algorithm for grouping TCRs that recognize the same antigen (Huang et al., 2020), showed three

TCRαβ constructs we defined grouped with other TCRs against ORF1ab, which contains the RdRp (Figure S3B and Table S3). The epitope FV9 was frequently targeted, but its cognate TCR, CoVTCR 18, did not share sequence similarity with any ORF1ab specific TCRβ (Figure S3A and B). This TCR also lacked killing ability in the prior assay (Figure 3E). Peptide titration
190 showed that this TCR only recognized antigen at high concentration of 10 μg/ml, confirming that this TCR has low affinity for this specific target (Figure S4). Two of the four TCRs against the RV9 epitope grouped with ORF1ab specific TCRβ (Figure S3B). CoVTCR 34, specific against RV9, was strongly cytotoxic but did not group with any TCRβ by GLIPH2 analysis.

195 **SARS-CoV-2 RdRp-targeted T cell receptors broadly recognize human coronaviruses**

We then queried RdRp TCR cross-reactivity against the HCoV epitopes. Epitope homologs were identified by alignment of RdRp sequences from all human coronaviruses. Each of the homologous epitopes was synthesized and TCR reactivity against each of the epitopes was profiled via peptide titration assay. RdRp specific TCRαβ constructs exhibited a diverse pattern
200 of coronavirus reactivity (Figure 4 A to F). Three TCRs were highly specific for SARS-CoV-2 (Figure 4 A, B, E). This TCR reactivity may represent the naïve T cell repertoire or could be an immune response to unknown antigen. Only two of the four RV9 reactive TCRs recognized one HCoV (Figure 4 D, F). The TL9 reactive TCR had strong cross-reactivity with SARS, MERS, 229E and NL63 coronaviruses (Figure 4C).

205

Discussion:

This study provides a strong basis for considering the development of vaccines against either specific epitopes or the full length RdRp. Current vaccines provide strong protection against

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COVID-19 caused by circulating variants of SARS-CoV-2. Continuous evolution of SARS-
210 CoV-2 may necessitate updates to the vaccine's spike sequence, selection of a more conserved antigen, or combination of both. One of the challenges of developing booster shots is the need to predict which variant will be the most common when the vaccine is administered. Failure to predict this accurately may decrease the efficacy of the booster. SARS-CoV-2 infection can be recognized by RdRp specific T cells as indicated by strong RdRp CD8+ T cell responses in
215 convalescent donors(Tarke et al., 2021). The RdRp sequence is particularly well conserved within SARS-CoV-2 and among other human coronaviruses. Sequence conservation suggests that the critical functional role of this protein places restriction on its capacity to evolve. We show that RdRp specific T cells are cytotoxic against cells that express full length antigen, which suggests T cell responses against RdRp should help control SARS-CoV-2 infection and prevent
220 COVID-19 disease.

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Inducing broadly reactive T cell responses may be particularly important for generating lifelong immunity against SARS-CoV-2. T cells can recognize the target antigen even after accumulation of point mutations (Sewell, 2012). While we identified RdRp as the most conserved protein, it
225 too is likely to change, as evident from the accumulation of point mutations. Here, we defined two RdRp epitopes that can elicit broadly coronavirus reactive T cell responses. T cells that recognize different human coronaviruses are likely to recognize novel mutation variants as they emerge, due to strong affinity for the antigen. Epitope TL9 reactive T cells have been previously identified as cross-reactive and associated with reduced disease severity, however specific TCR
230 clone driving this response was not identified (Mallajosyula et al., 2021). For the RV9 epitope some TCRs were cross-reactive but others only recognized SARS-CoV-2, showing that TCRs

against the same antigen can have distinct reactivity. The cross-reactive TCRs against RV9 used the same V alpha chain TRAV38-2DV8, implicating that the usage of common alpha chain may allow cross reactivity. Specific TCR sequences that are known to allow for broad reactivity can
235 be used as benchmarks for induction of such immunity. TCR based disease severity correlation will require more TCR characterization to expand the scope of TCRs and HLA allele restrictions. Induction of broadly reactive T cell responses, that are not affected by point mutations in the epitope sequence, as well as benchmarks for measurement of such responses can help guide development of T cell vaccines.

240

Several reports proposed the use of adoptive transfer of antigen specific T cells from convalescent donors, to treat severe COVID-19 disease (Basar et al., 2021; Ferreras et al., 2020; Keller et al., 2020). Viral infections such as CMV and EBV have been previously treated by transfer of highly functional cytotoxic T cells (Einsele et al., 2002; Feuchtinger et al., 2010b;
245 Papadopoulou et al., 2014). It remains to be shown whether adoptive transfer of T cells can control SARS-CoV-2 infection in pre-clinical models, which are complicated by the requirement to be done in the BSL-3 setting. Therapeutic T cell engineering is now routinely done for cancer treatment both in the context of clinical trials as well as FDA approved therapeutics(D'Angelo et al., 2018; Depil et al., 2020; Johnson et al., 2006). There are several advantages to adoptive cell
250 therapy with engineered T cells:1. Large number of antigen specific T cells can be readily produced 2. Well validated TCR specificity 3. T cells have a younger phenotype. TCR engineered T cell also enlist additional CD4+ T cells, which are critical for establishing long term CD8+ T cell memory and antibody production(Sant and McMichael, 2012; Sun and Bevan, 2003). Current approaches for adoptive T cell therapy are expensive and cumbersome (Depil et

255 al., 2020). Technological advances in gene delivery may make T cell engineering a practical approach for viral disease treatment in specific groups of patients (Frank et al., 2020).

Limitations of the Study:

We show that RdRp specific TCRs recognize processed epitopes in a reconstructed system of 260 viral infection, however we do not show direct control of live SARS-CoV-2 virus. Such an experiment is complicated by the requirement to be done in BSL3 setting. In addition, viruses developed complicated mechanisms to escape T cell effector function, which can make it difficult to detect activity and recognition. T cell function in SARS-CoV-2 is still being investigated and direct T cell suppression of viral replication has yet to be established. We did 265 not show correlation between T cell responses we identified and disease severity. Such analysis requires a large patient cohort and could indicate the importance of specific T cell responses.

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290 Methodology: MTB, PAN, PCB, JRH, WC, YS, LY, ZM, NJB, YL, DL, JTK

Investigation: GBS, JM, PAN, JX, RN, BLT, DC, CC, MN

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Software: MBO, BLT, RN, DZ

Resources: JX, WC, YL

295 Visualization: MBO, PAN, BLT, DZ, RN

Project administration: PAN, JM, ONW

Writing – original draft, review & editing: PAN, ONW, JWP

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Declaration of interests: ONW, JM and PAN are inventors of a patent application in progress
300 that will be filed prior to manuscript publication. JRH is a board member of PACT Pharma and Isoplexis. ONW currently has consulting, equity, and/or board relationships with Trethera Corporation, Kronos Biosciences, Sofie Biosciences, Breakthrough Properties, Vida Ventures, Nammi Therapeutics, Two River, Iconovir, Appia BioSciences, Neogene Therapeutics, and Allogene Therapeutics. None of these companies contributed to or directed any of the research
305 reported in this article.

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Figure Legends:

Figure 1. RdRp is highly conserved among human coronaviruses and within SARS-CoV-2

(A) Correlation matrix comparing coronavirus protein conservation by amino acid identity across
310 coronaviruses. (B) Lollipop plots illustrating the distribution and locations of missense variants present in SARS-CoV-2 spike protein and RNA-dependent RNA-polymerase (RdRp). Missense variants are highlighted by lollipops and the most frequent variants are labelled in HGSV nomenclature.

Figure 2. T cell receptors from unexposed individuals recognize RdRp epitopes.

315 (A) FACS sorting analysis of T cells that underwent stimulation by peptide pools and subsequent intracellular staining for TNFα and IFNγ using the CLInt-seq protocol, which allows for downstream single-cell TCRαβ sequencing. In Donors A-C, TNFα and IFNγ double positive cells were selected. For Donor D total fraction of IFNγ positive cells was collected. (B)Schematic for the TCRαβ construct screening in a cell-based reporter system using the
320 NFAT-zsGreen (GFP) reporter. (C) Epitope map of the RdRp epitopes. Epitopes that reacted with TCRαβ constructs are in red, and non-reactive epitopes, that were still part of the pool, are

in blue. Number of cognate TCRαβ constructs for each of the reactive epitopes is indicated. (D) Summary of reactive TCRs from each of the donors.

Figure 3. Isolated T cell receptors recognize and kill RdRp expressing cells

325 (A)Schematic for generation of human PBMCs that express SARS-CoV-2 reactive TCRαβ constructs *via* retrovirus-based construct overexpression. Overexpression is confirmed by NGFR (secondary marker) and murine TCRβ constant region staining by FACS. (B) Schematic for target cell line generation using the K562 cell line and lentiviral overexpression of the major histocompatibility complex (MHC) molecule HLA-A*02:01 and the full length RdRp sequence 330 that is tagged by Strep-tagII. RdRp expression is confirmed by western blot, where 293T cells transfected with the RdRp vector are the positive control. (C) FACS analysis of intracellular staining for TNFα and IFNγ in T cells. Cells are gated on light scatter, CD3+, CD8+. (D) FACS results from panel C. Data from PBMC samples expressing the different TCR constructs are pooled into groups based on the activation stimulus and differences are compared across groups. 335 Student's T test is used to compare the groups **p>0.01, ***p>0.001, ****p>0.0001. (E) PBMC cytotoxicity assay against RdRp overexpressing target cell line. (F). IFNγ production after 48 hours of co-culture of PBMCs and target cell lines expressing RdRp. HLA-A*02:01 only parental cell line is used as negative control. Error bars represent standard deviation.

Figure 4. SARS-CoV-2 RdRp-targeted T cell receptors broadly recognize human 340 coronaviruses.

Peptide dilution assay to measure reactivity against peptide homologs from HCoVs. PBMCs were engineered to overexpress the TCRαβ constructs, expanded, and then pulsed with the indicated concentrations of each of the indicated peptide epitopes in the presence of the K562-

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A*02:01 to support antigen presentation. Intracellular staining for IFNγ is used to measure T cell

345 activation.

STAR Methods:

Lead contact

Further information and requests for resources and reagents should be directed to and will be

350 fulfilled by the lead contact, Owen N. Witte (owenwitte@mednet.ucla.edu).

Materials availability

We generated unique TCR sequences. The full length TCR clone sequences are provided in this paper. Any cell line that we created and used is available to other investigators.

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Data and code availability

- Reactive TCR alpha/beta nucleotide sequences are provided in this paper.
- We have not created any original code.
- Any additional information required to reanalyze the data reported in this paper is

360 available from the lead contact upon request.

Experimental model and subject details

Cell culture

Cryo preserved peripheral blood mononuclear cells (PBMCs) were commercially purchased

365 (Allcells and Hemacare) or obtained from the CFAR Virology Core Laboratory at the UCLA AIDS Institute. PBMCs were thawed in a water bath set to 37C, transferred to 50 mL conical

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tube, 1 mL of warm R10 media was added drop wise and then 14 mL R10 were added. Cells were then centrifuged at 1300 RPM for 7 minutes. To isolate reactive T cells, peripheral blood mononuclear cells (PBMCs) (Allcells and Hemacare) were cultured in TCRPMI media: RPMI
370 1640 (Thermo Fisher), 10% FBS (Omega Scientific), 1X Glutamax (Thermo Fisher), 1X Sodium Pyruvate (Thermo Fisher), 10 mM HEPES (Thermo Fisher), 1X non-essential amino acids (Thermo Fisher), 50μM β-mercaptoethanol (Sigma) and Penicillin-Streptomycin (Omega Scientific). PBMCs were cultured for 8 days in the presence of peptide pools at 1 μg/ml and 25U/ml of IL-2 (Peprotech) as previously described (Nesterenko et al., 2021). PBMCs were then
375 washed two times in PBS (Fisher Scientific), once in TCRPMI and then plated for 12-hour rest in 96-well U bottom plates. Jurkat cells were cultured in R10: 1640 RPMI (Thermo Fisher) supplemented with 10% FBS (Omega Scientific) and 1X Glutamax (Thermo Fisher). An NFAT-zsGreen reporter construct was overexpressed in the Jurkat CD8 cell line. Both NFAT-zsGreen plasmid and the Jurkat CD8 cell line were gifts from the David Baltimore lab. K562 (ATCC)
380 cells were cultured in R10. PBMC for TCR engineering experiments were cultured in AIM V media (Thermo Fisher) supplemented with 5% Human AB serum (Omega Scientific), 50 U/ml of IL-2, 1ng/ml IL-15 (Peprotech), 1X Glutamax and 50μM β-mercaptoethanol. For functional experiments with engineered PBMCs supplemented AIM V media without IL-2 and IL-15 was used. 293T (ATCC) cells were cultured in DMEM (Thermo Fisher) supplemented with 1X
385 Glutamax or Glutamine (Fisher Scientific) and 10% FBS.

RdRp expressing target cell line generation

pLVX-EF1alpha-SARS-CoV-2-nsp12-2xStrep-IRES-Puro was a gift from Nevan Krogan (Addgene plasmid # 141378 ; http://n2t.net/addgene:141378 ; RRID:Addgene_141378)(Gordon

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390 et al., 2020). RNA-dependent RNA-polymerase (RdRp/NSP12) sequence was subcloned into a third-generation lentivirus packaging vector MNDU-3-ires-Strawberry. Lentivirus was produced as described previously(Seet et al., 2017). Lentivirus was used to infect K562-HLA-A*02:01 cells that were described by us previously(Bethune et al., 2018). To ensure stable expression of RdRp and HLA-A*02:01 in K562 cells, single cells were cloned by FACS deposition and 395 selected for high levels of RFP and GFP. Stable RdRp protein level was confirmed by Western blot using anti-Strep-tag II antibody (Abcam, 1:500) and goat anti-rabbit-IgG HRP(Bio-Rad, 1:5000). These single cell clones were used for the cytotoxicity assay.

400 **Method details**

Intracellular staining

RdRp HLA-A*02:01 restricted epitopes were identified by prior publications and or prediction by the netMHCpan4.0 (B et al., 2020; Grifoni et al., 2020a; Poran et al., 2020). PBMCs were stimulated in 96 well U bottom plates with 10 µg/ml of peptide and 1 µg/ml of CD28/CD49d 405 antibodies (BD Biosciences). For peptide titration assays, serial dilution was set up where the original 10 µg/ml concertation was diluted 10 fold for every subsequent dilution. For recognition of processed antigen, PBMCs were stimulated with target cell lines without peptide at a 4:1 effector to target ratio. After one-hour, 1X Brefeldin A (Biolegend) was added. Cells were then incubated for 8 more hours after which they were either processed or moved to 4C. For 410 analytical assays, cells were stained for surface markers CD3, CD4, CD8 and intracellular markers TNFα and IFNγ using the Cytofix/Cytoperm kit (BD Biosciences).

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CLInt-Seq

For TCRαβ sequencing via the CLInt-Seq staining we followed our previously published
415 protocol(Nesterenko et al., 2021). Cells were stimulated as described above for intracellular staining. PBS was made from 10X PBS and nuclease free water (Thermo Fisher). All buffers, except for DSP, contained RNAsin (Promega) at 1:400 dilution. Cells were washed twice with staining buffer: nuclease free PBS (Fisher scientific), 1% nuclease free BSA (Gemini) and 1:400 RNAsin plus (Promega). Cells were then stained for 15 min in buffer for surface antigens and
420 subsequently washed once with staining buffer and twice with PBS. DSP was then added to each well at 0.25 mg/ml in 200 µL of PBS for 30 minutes at room temperature. The reaction was quenched with 20 µL of 200mM TRIS (Thermo Fisher). Cells were then washed twice with buffer and incubated with 100 µL of 0.05% Triton X-100 (Sigma) for 10 minutes on ice. Cells were then washed and incubated with antibodies against TNFα and IFNγ for 20 minutes on ice in
425 100 µL of buffer, followed by a final wash step. Cells were gated on light scatter, CD3+, CD8+/CD4- to analyze TNFα and IFNγ signal. For TCR sequencing cells were either sorted on TNFα/IFNγ double positive cells or IFNγ positive cells.

Single-cell TCRαβ sequencing

430 TNFα and IFNγ-producing CD8+ T cells were sorted by FACS into a 2 ml Eppendorf tube as described previously(Nesterenko et al., 2021). Sorted cells were then resuspended in 30-60 µL in .04% BSA solution with RNAsin, to reach a concentration of more than 100 cells/µL. To achieve such concentration, similarly processed Jurkat cells were added as a carrier cell population. Human TCR VDJ libraries were then constructed by the UCLA Technology Center for

435 Genomics & Bioinformatics using the single-cell VDJ V1.0 and V1.1 (10X Genomics platform). TCR libraries were then sequenced on MiSeq or Nextseq (Illumina).

TCRαβ construct generation and screening

TCRαβ were constructed from synthetic DNA fragments (IDT and Swift Biosciences). Some 440 TCRs were made as retroviral vectors as described previously(Nesterenko et al., 2021). Some constructs were built into the small pMAX vector (Lonza) designed for transfection-based expression. TCRs were infected into the Jurkat cell line using centrifugation at 1350G for 90 minutes at 30C with 5 µg/ml of polybrene (Sigma-Aldrich). DNA minipreps were prepared using the QIAprep miniprep kit (Qiagen) and concentration was routinely higher than 200 ng/µL. Cells 445 were transfected with 2 µL of DNA, dissolved in water, using the Lonza 4D Nucleofactor (Lonza) in 20 µL transfection media from the SE cell Line kit S (Lonza). Lonza pre-installed electroporation protocol for Jurkat clone E6.1 was then used. Cells were rested for 10 minutes and 80 µL of warm R10 media was added. Then total of 100 µL was transferred to a 24 well plate with 400 µL of warm R10 in each well. Cells were then incubated for 12 hours and 450 afterwards they were used for functional assays over the course of three days.

Stimulation of Jurkat

Jurkat cells were plated in 100 µL of R10 media in 96 well U bottom plate. K562 cells expressing HLA-A*02:01 were then added in 100 µL of R10 with 20 µg/ml of peptide. Cells 455 were then incubated at 37C, 5% CO_2. zsGreen (GFP) fluorescence was then measured by FACS analysis. For transfection experiments cells were gated on light scatter, CD8+/murine TCRβ+.

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PBMC engineering via retrovirus

Retrovirus was produced as described previously(Nesterenko et al., 2021). PBMCs were
460 activated with CD3/CD28 beads (Thermo Fisher) in 24 well plates at 1:1 ratio. After 3 days, TCR construct or empty construct NGFR control retrovirus was added to the cells with 5 µg/ml of polybrene (Sigma-Aldrich). Cells were centrifuged at 1350G for 90 minutes at 30C. After transduction, 1 ml from each well was removed and fresh media with 2X cytokines was added. On the next day, infection was repeated, and the following day cells were washed. Cells were
465 then cultured for two more days, at which point the CD3/CD28 beads were magnetically removed. At this stage, cells were expanded further and used for downstream assays. Transduction was always confirmed by FACS quantification of secondary transduction marker NGFR, and TCR surface expression was ensured by staining for the murine TCRβ constant region. Our TCR constructs use mouse constant regions to decrease mis-pairing with endogenous
470 TCRs in PBMCs and allow surface TCR staining.

Cytotoxicity analysis

K562 target cell lines were co-cultured with TCR engineered PBMCs at a 2:1 effector to target ratio, in supplemented AIM V media without cytokines, with 1 µg/ml of CD28/CD49d
475 antibodies (BD). For these experiments, we used K562 target cell line that stably expressed RdRp as well as HLA-A*02:01. The RdRp negative cell line was used as a control for the assay. The IncuCyte system (Sartorius) was used to quantify GFP surface. Because only the K562 target cells expressed GFP, loss of GFP was interpreted as decrease in the number of live cells. 48 hours after addition of effectors to targets, 50 µL of supernatant was collected for IFNγ

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480 ELISA analysis as described previously(Nesterenko et al., 2021) using the OptEIA kit for IFNγ detection (BD Biosciences).

Antibodies

NGFR-PECy7 (Biolegend), murine TCRβ-APC (Biolegend), CD3-APCCy7 (Thermo Fisher,),
485 CD8a-PE (Thermo Fisher), CD4-PECy7 (Biolegend), IFNγ-APC (Biolegend), TNFα-FITC (Biolegend), HLA-A2-APC (Thermo Fisher), anti-Strep-tag II (Abcam), secondary goat anti-rabbit IgG HRP (Bio-Rad).

Protein conservation analysis

490 Coronavirus protein sequences were collected from NCBI Virus(Hatcher et al., 2017). RefSeq assembly accession numbers as follows: SARS-COV-2 (GCF_009858895.2), SARS-COV-1 (GCF_000864885.1), OC43 (GCF_003972325.1), NL63 (GCF_000853865.1), MERS (GCF_000901155.1), HKU1 (GCF_000858765.1), 229E (GCF_000853505.1). For missing protein accessions, we used BLAST(Altschul et al., 1990) to find the sequence most similar to
495 the respective SARS-COV-2 protein sequence. We performed multiple sequence alignment and calculated the percent identity matrix using the MUSCLE algorithm(Madeira et al., 2019).

Lollipop plot generation

SARS-CoV-2 amino acid sequence variations representing 893,589 GISAID (Table S4)
500 sequences were downloaded from CoV-Glue, an online web application for analysis of SARS-CoV-2 virus genome sequences, on 05-01-2021(Shu and McCauley, 2017; Singer et al., 2020). The sequence of Wuhan-Hu-1 (NCBI, NC_045512.2) was used as a reference sequence for

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numbering, nucleotide location, and amino acid variations. CoV-GLUE excludes certain GISAID sequences; information about the total number of sequences retrieved from GISAID and
505 the subset of sequences that passed CoV-GLUE exclusion criteria can be found here (cov-glue.cvr.gla.ac.uk/#/excludedSeqs). Up-to-date information on SARS-CoV-2 proteins and protein domains was queried from UniProt (https://www.uniprot.org/). Data was visualized using the Boutros Plotting Package (v6.0.3) for R(P'ng et al., 2019).

510 **Comparison of TCRs to publicly available data**

The SARS-CoV-2 specific TCRβ dataset (also known as ImmuneCODE MIRA) was downloaded from: https://clients.adaptivebiotech.com/pub/covid-2020. GLIPH2 analysis identifies antigen specificity groups based on enrichment of local motifs or global patterns differing by one amino acid in the TCRβ CDR3 amino acid sequences(Huang et al., 2020).
515 GLIPH2 analysis was performed on the combined set of RdRp specific CoVTCRs and SARS-CoV-2 antigen specific TCRs from ImmuneCODE MIRA dataset(Nolan et al., 2020). TCRs from CD8 T cell experiments (minigene and class I peptide) were included and filtered for productive TCRβs. We filtered for GLIPH groups with V gene bias ($p < 0.01$) that contain both CoVTCRs and MIRA TCRs. We mapped the identity of the open reading frame (ORF) targeted
520 by each MIRA TCR and counted the number of MIRA TCRs per ORF for each GLIPH group.

Quantification and statistical analysis

FlowJo wash used to analyze flow cytometry data and GraphPad Prism was used to generate plots and statistical analyses. Error bars represent standard deviation. Student's T test was used
525 to compare the groups **p>0.01, ***p>0.001, p>0.0001. Protein conservation was visualized

using the Boutros Plotting Package (v6.0.0) for R(P'ng et al., 2019). Epitope homologs were visualized with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Supplemental information:

530 **Table S1. Summary of reactive TCR clones and cognate epitopes. Related to Figure 2.**

Table S2. Nucleotide sequence of TCR clones that have been validated in PBMC. Related to Figure 2.

Table S3. Specificity patterns determined from grouping RdRp specific TCRαβ clones with the MIRA data set by GLIPH2 analysis. Related to Figure 3.

535 **Table S4. Acknowledgement of SARS-CoV-2 sequencing sources. Related to Figure 1.**

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A

RNA dependent RNA polymerase (RdRp)

	SARS-CO	SARS	MERS	HKU1	OC43	229E	NL63
NL63							100
229E						100	81
OC43					100	56	56
HKU1				100	89	56	56
MERS			100	68	68	58	59
SARS		100	72	66	67	59	59
SARS-CoV-2	100	96	72	67	66	59	59

NSP8

	SARS-CO	SARS	MERS	HKU1	OC43	229E	NL63
NL63							100
229E						100	76
OC43					100	42	41
HKU1				100	85	46	44
MERS			100	52	51	41	39
SARS		100	53	47	48	48	47
SARS-CoV-2	100	97	54	47	48	47	47

NSP6

	SARS-CO	SARS	MERS	HKU1	OC43	229E	NL63
NL63							100
229E						100	64
OC43					100	27	28
HKU1				100	67	21	24
MERS			100	31	33	29	30
SARS		100	35	30	34	32	30
SARS-CoV-2	100	87	35	30	31	30	29

Identity %

Spike

	SARS-CO	SARS	MERS	HKU1	OC43	229E	NL63
NL63							100
229E						100	64
OC43					100	26	25
HKU1				100	66	26	25
MERS			100	33	35	26	23
SARS		100	31	30	32	26	24
SARS-CoV-2	100	78	32	30	32	27	24

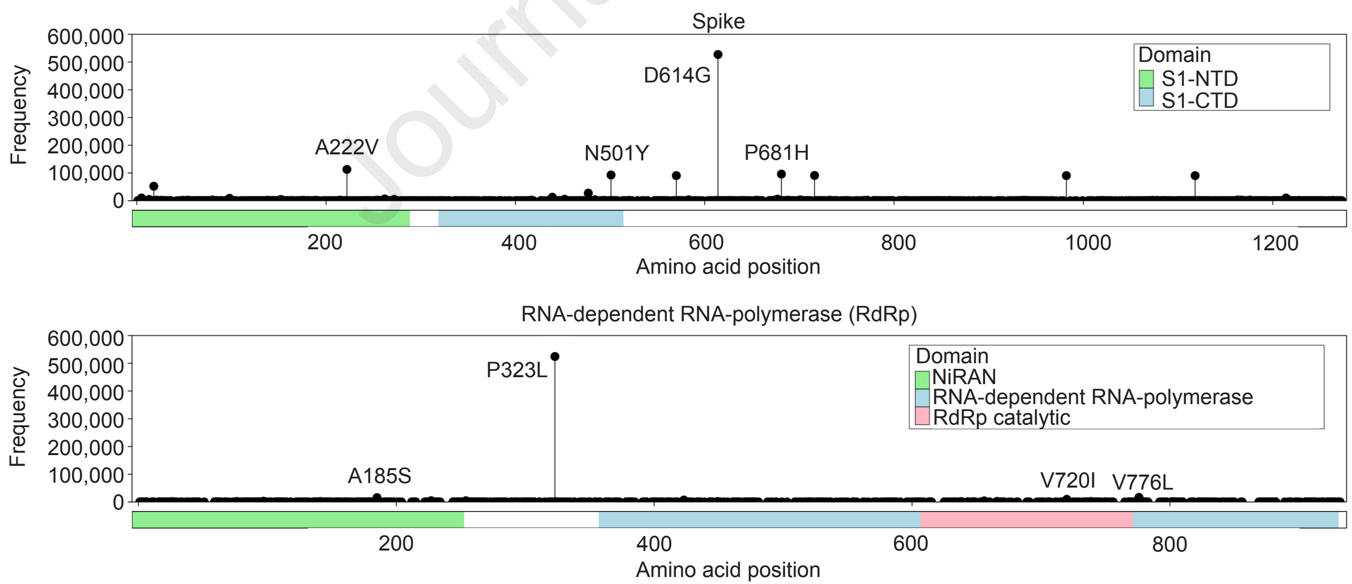
Nucleocapsid

	SARS-CO	SARS	MERS	HKU1	OC43	229E	NL63
NL63							100
229E						100	45
OC43					100	25	25
HKU1				100	65	27	24
MERS			100	33	35	23	24
SARS		100	49	33	35	25	26
SARS-CoV-2	100	90	49	33	35	25	27

Envelope

	SARS-CO	SARS	MERS	HKU1	OC43	229E	NL63
NL63							100
229E						100	47
OC43					100	23	19
HKU1				100	53	25	16
MERS			100	23	27	24	22
SARS		100	37	26	21	24	18
SARS-CoV-2	100	93	37	28	23	24	19

B



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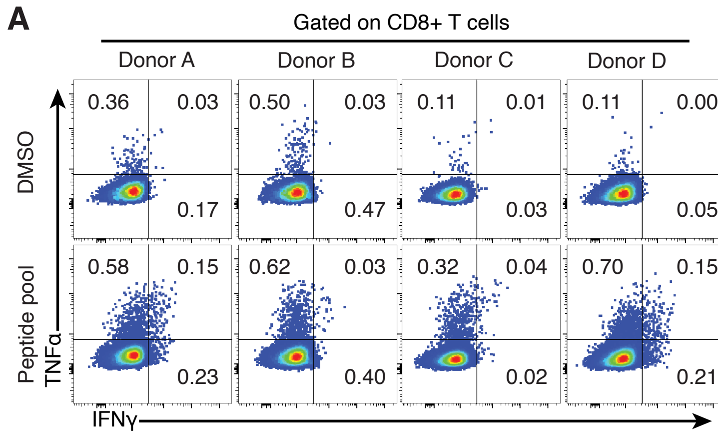

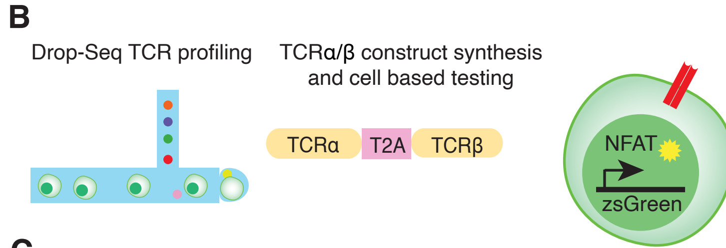

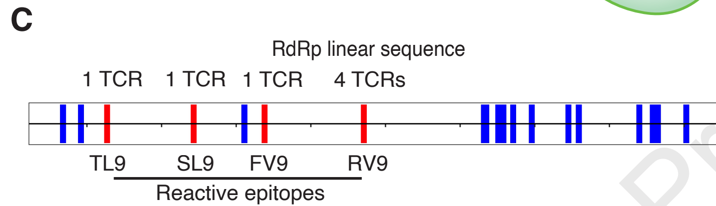

D

		Unique clones		
	α/β pairs	α/β pairs	reactive/ total tested	epitopes
Donor A	124	56	4/17	SL9, RV9, TL9
Donor B	19	7	2/6	FV9
Donor C	15	9	2/9	RV9
Donor D	840	471	2/12	RV9

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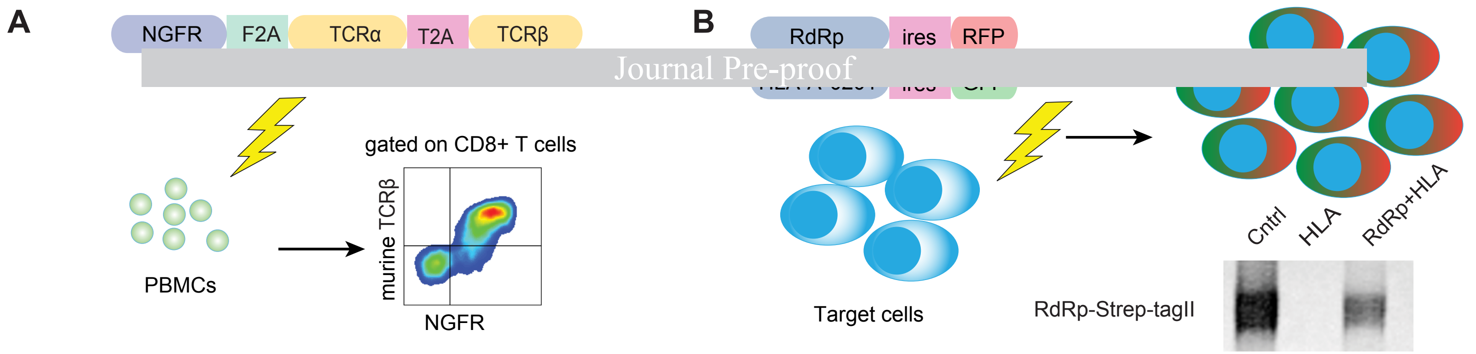

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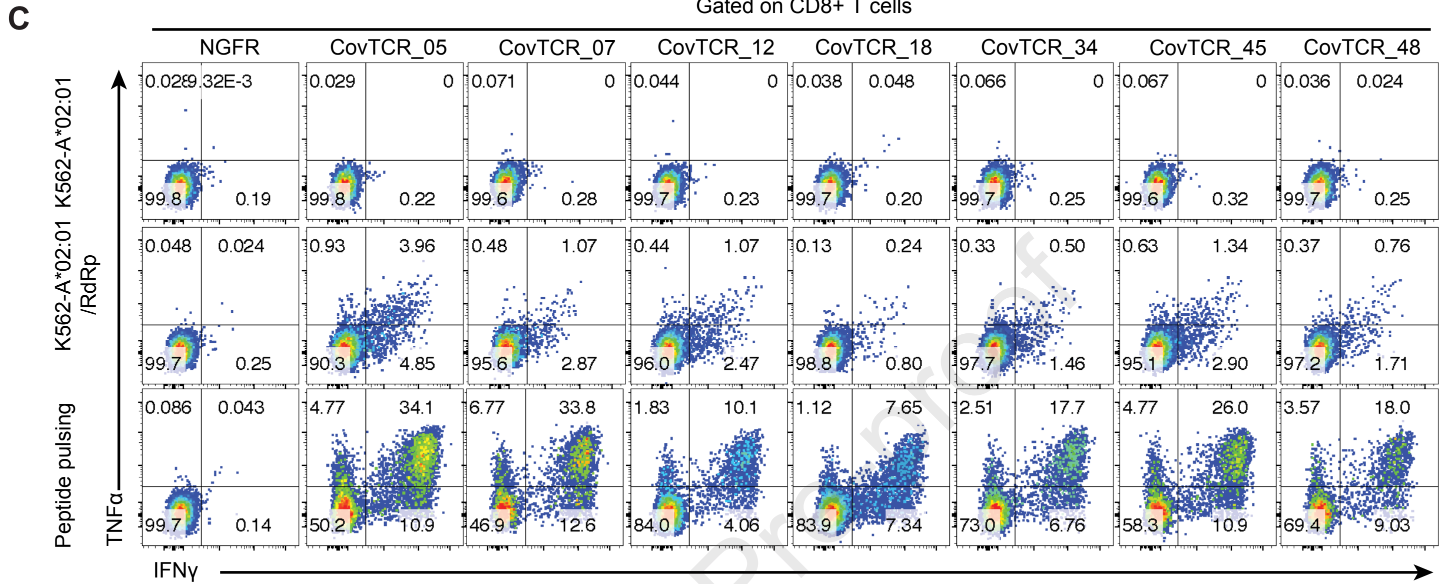

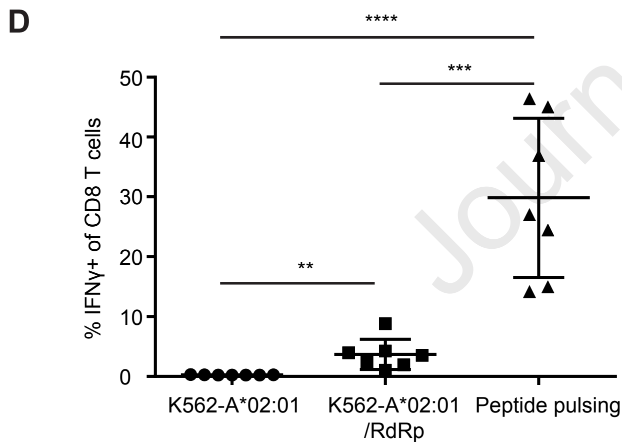

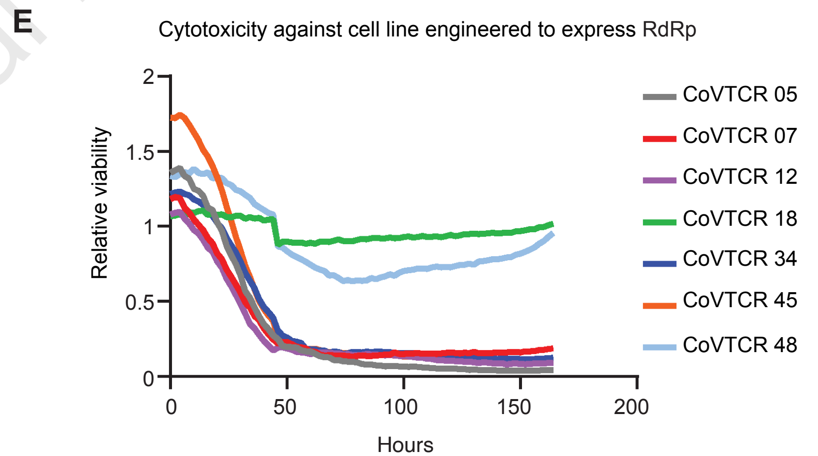

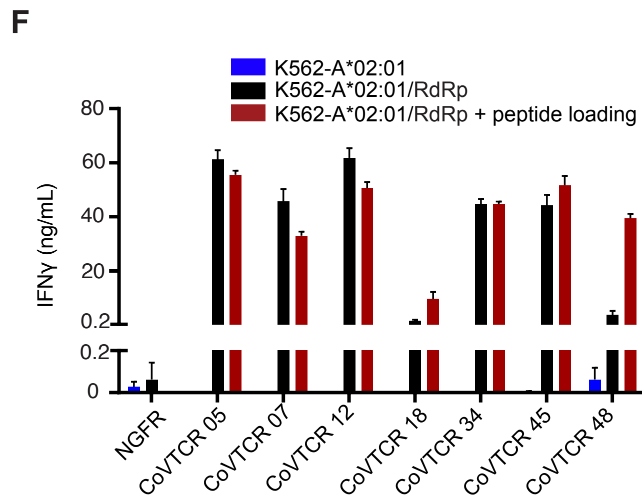

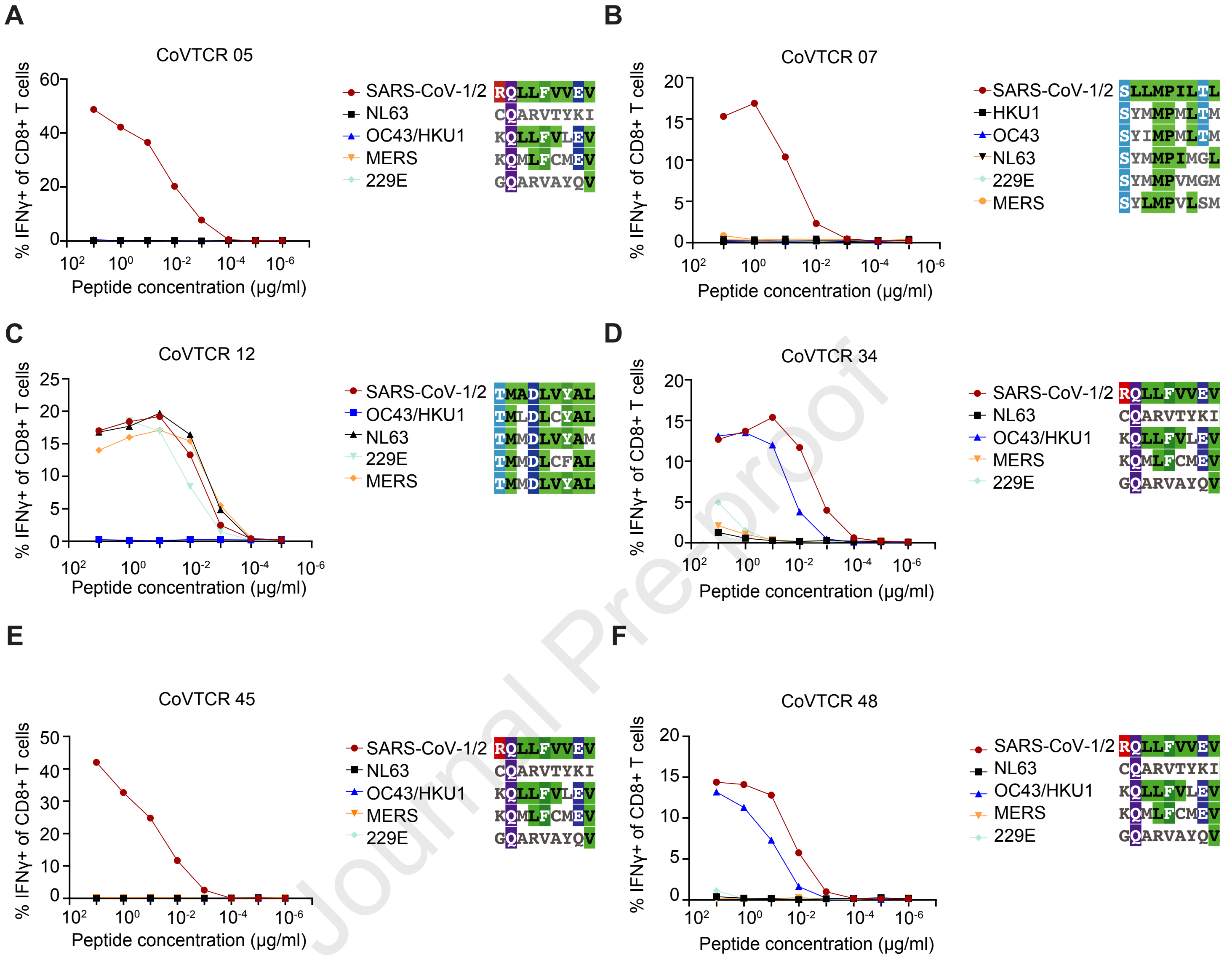

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Highlights

- The RNA-dependent RNA-polymerase is highly conserved among human coronaviruses
- CD8+ T cells from unexposed donors recognize SARS-CoV-2 polymerase epitopes
- TCR engineered T cells kill target cell lines that express the polymerase
- Polymerase reactive TCRs cross-react with seasonal coronaviruses

eTOC blurb

Nesterenko et al. identify T cell responses with potential to confer long term immunity against SARS-CoV-2. The machinery responsible for replicating the viral genome is highly conserved and as shown by Nesterenko et al. can be effectively targeted by CD8+ T cells.

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