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Characterizing the T cell immune response through the receptor-ligand interaction

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Publication Date
2022

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UNIVERSITY OF CALIFORNIA

Los Angeles

Characterizing the T cell immune response through the receptor-ligand interaction

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular Biology

by

Pavlo A. Nesterenko

2022

ABSTRACT OF THE DISSERTATION

Characterizing the T cell immune response through the receptor-ligand interaction

by

Pavlo A. Nesterenko
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2022
Professor Owen N. Witte, Chair

The adaptive immune system provides protection against disease and maintains memory of past exposures. Randomly rearranged antigen receptors can recognize virtually any pathogen. T cells recognize processed peptides that originate from protein fragments and are presented on major histocompatibility complex molecules. Human T cell receptor (TCR) function is mostly studied in the context of model antigens. TCRs against the majority of presented epitopes are not defined. Single cell sequencing allowed for paired TCRα/β sequencing at a scale not previously imaginable, which increased interest in identification of antigen specific TCRs.

Antigen specific TCR discovery efforts are challenging because of the repertoire diversity. Up to 10^{19} possible TCRs can be randomly generated in the thymus. Antigen specific T cells can be selected by a range of techniques based on either physical staining of the TCR or selection of cells activated with specific antigen. Antigen specific T cell rarity and abundance of weakly reactive cells leads to selection of large numbers of false positive clones. The TCRα/β needs to be sequenced and genetically reconstructed in allogeneic T cells to confirm antigen recognition. The qualities of an antigen specific TCR include the ability to direct the killing of target cell lines, stimulating the production of multiple cytokines, and driving cell division. Such data can

then be used to rank TCRs and identify highly functional clones. More sensitive and specific selection prior to sequencing can reduce the amount of labor involved when profiling TCRs.

This thesis work describes a new technology for sequencing antigen specific TCRs, which is used to study T cell responses against SARS-CoV-2. We developed a protocol that allows for TCR sequencing in cells that have been identified as functional based on cytokine production in response to stimulation with specific antigens. TCR engagement drives cytokine production which is one of the fundamental properties of a T cell. For this reason, intracellular cytokine staining is one of the most commonly used techniques to quantify antigen specific T cells. We then identified SARS-CoV-2 polymerase specific TCRs in unexposed human donors. Polymerase specific TCRs are able to recognize multiple human coronaviruses, indicating the potential to provide immunity regardless of the SARS-CoV-2 variant. Broadly reactive T cells provide heterosubtypic immunity against Influenza, another respiratory virus. Based on this work our laboratory, in collaboration with other laboratories at UCLA, is developing a novel COVID-19 vaccine strategy.

Our SARS-CoV-2 work illustrates the utility of studying single TCRs to discover immune responses, but another approach is to use TCRs directly as therapeutics. TCR engineered T cells are effective in the clinic and cure late-stage tumors. Projects described in this thesis are helping drive other efforts in our laboratory that are directed at identifying TCRs against novel prostate cancer antigens.

This dissertation of Pavlo A. Nesterenko is approved.

Lili Yang

Paul C. Boutros

Maureen Su

Hanna Mikkola

Owen N. Witte, committee chair

University of California, Los Angeles

2022

Dedication

to my family

me to think about the best home run experiment, while making sure all necessary controls were in place. The work described in this thesis wouldn't be possible without her.

Dr. John Phillips was an Assistant Adjunct Professor in the lab. John's positive energy was felt by everyone in the lab. He checked on grad students after lab meetings and offered advice and support. John's training in chemistry was a refreshing insight for me, as I mostly viewed things from a cell biology perspective. When I was working on chapter 2 of this thesis, John drew the chemical structure of crosslinking on the whiteboard in the lunchroom, this instantly changed how I thought about the problem. John passed away from glioblastoma in the Fall of 2021. This devastated all of us in the lab and beyond. Cancer is a terrible disease, and as biomedical researchers, we have the opportunity to put a dent in it, while making every moment count.

The most valuable resource of the Witte lab are its people and the culture they create. This work could not have been accomplished without Donghui Cheng, who is so much more than a flow sorter operator. Donghui painstakingly generated single cell clones of cell lines, developed protocols, and offered advice on experimental design. Josh Bangayan taught me how to clone DNA constructs: from plasmid maps to sequencing. Previously I never ran a restriction enzyme digest. Zhiyuan Mao thought about every aspect of antigen presentation and recognition and is continuing to push forward with the work on PAP TCRs. Lisa Ta and Olga Chen are amazing friends with whom we had many great discussions. Brandon Tsai was a technician in our lab and now is an MD/PhD student in the Boutros lab. Brandon has been an incredible resource for anything bioinformatics related and a great friend. I am very thankful to Giselle Burton Sojo and Miyako Noguchi, the two technicians who work on the TCR projects. None of this work would

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BS   Washington State University, Biology, minor in Chemistry, GPA: 3.77, 2014

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In-vivo models: Human, Rhesus Macaque, Mouse (C57BL/6 and NSG)
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Molecular cloning: T cell receptor (TCR), Chimeric Antigen Receptor (CAR) and target gene construct generation *via* Gibson Assembly and PCR
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Tumor immunology training grant (NIH T32 CA-009120-41)

PUBLICATIONS (Peer reviewed)

Adler S.P., Manganello A., Lee R., McVoy M.A., Nixon D.E., Plotkin S., Mocarski E., Cox J.H., Fast P.E., **Nesterenko P.A**., Murray S.E., Hill A.B., Kemble G. A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus–Seronegative Men. *Journal of Infectious Diseases* 214.9 (2016): 1341-1348.

Murray S.E.*, **Nesterenko P.A. *,** Vanarsdall A., Munks M., Smart S., Veziroglu E., Sagario L., Lee R., Cox J., McVoy M., Adler S.P., Hill A.B. Fibroblast-adapted human cytomegalovirus (HCMV) vaccines elicit conventional CD8+ T cell responses in human subjects. *Journal of Experimental Medicine*. 2017

Nesterenko PA, McLaughlin J, Cheng D, Bangayan NJ, Sojo GB, Seet CS, Qin Y, Mao Z, Obusan MB, Phillips JW, Witte ON. Droplet-based mRNA sequencing of fixed and permeabilized cells by CLInt-seq allows for antigen-specific TCR cloning. Proceedings of the National Academy of Sciences. 2021 Jan 19;118(3).

Nesterenko PA, McLaughlin J, Tsai BL, Sojo GB, Cheng D, Zhao D, Mao Z, Bangayan NJ, Obusan MB, Su Y, Ng RH, Chour W., Xie J., Li Y., Lee D., Noguchi M., Carmona C., Phillips J.W., Kim J.T., Yang L., Heath J.R., Boutros P.C., Witte O.N. HLA-A∗ 02: 01 restricted T cell receptors against the highly conserved SARS-CoV-2 polymerase cross-react with human coronaviruses. Cell reports. 2021 Dec 28;37(13):110167.

*Equal contribution

PATENTS

Witte O.N., Nesterenko P.A., Witte J. (2021) METHOD TO SEQUENCE MRNA IN SINGLE CELLS IN PARALLEL WITH QUANTIFICATION OF INTRACELLULAR PHENOTYPE. WO/2021/046121. https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2021046121&tab=PCTBIBLIO

TALKS/ SEMINARS

OHSU Research Week, Portland, OR. May 2016. "Fibroblast-tropic Human Cytomegalovirus (HCMV) vaccines elicit conventional CD8 T-cell responses"

Parker Institute for Cancer Immunotherapy, Los Angeles, CA. November 2019. "Cloning of antigen-specific T cell receptors based on multiplexed cytokine production"

Immuno-Oncology Translational Network, Virtual. July 2021. "TCR Cloning based on intracellular cytokine profiles via CLInt-Seq"

Chapter 1

Function of α/β T cell receptors in infection and vaccination

1. Intro to immunity

The immune system protects organisms from pathogens(*1*). The innate immune system can respond within minutes of infection to highly conserved bacterial and viral motifs using a system of pattern recognition receptors (PRRs). Dendritic cell (DC) stimulation is the bridge between innate and adaptive immune systems. DC PRR stimulation leads to DC maturation and the ability to prime naïve T cells. Adaptive immunity takes days to weeks to build and protects against specific pathogens. Memory cells survey the body and prevent or suppress reinfection by the same pathogen. B cells produce antibodies that recognize surface exposed antigens. Antibodies neutralize viruses and direct cytotoxicity by engaging other immune cells. T cells recognize processed peptide epitopes presented on Major Histocompatibility Complexes (MHC), which can be of self-origin or derived from intracellular and extracellular pathogens. T cells organize the immune response and kill target cells. There are two main classes of T cells, CD4 and CD8, which differ in function and types of antigens recognized. CD4 T cells help to organize both B cell and T cell responses, while CD8 T cells are the cytotoxic effectors. My work focused on CD8+ T cell recognition of specific antigenic determinants.

2. Lymphocyte diversity

Large clonotype diversity allows B cells and T cells to recognize virtually any antigen. A clonotype is a group of cells with the same antigen receptor that is sustained by homeostatic proliferation(*2*). Individual receptors recognize specific biochemical motifs. Theoretical limit for T cell receptor diversity is greater than 10^{19}(*3–5*). T cell clonotype diversity within an average human is only around 10^8, because the absolute number of T cells in the body is limited(*6–8*). For this reason, the T cell repertoire within two individuals with similar HLA alleles rarely

overlaps. The number of possible antigens is much larger than the number of available T cell clonotypes within an individual(*9*). T cell cross-reactivity compensates for this discrepancy by allowing one receptor to recognize many related antigenic determinants with a range of affinities(*9*, *10*). Recognition of some antigens will elicit strong T cell responses and others will only weakly stimulate T cells. This strategy allows for virtually unlimited numbers of antigens to be recognized.

Identifying antigen specific TCRs is a needle in the haystack problem. The frequency of naïve T cells capable of targeting known antigens ranges from 1 in 1,000 to 1 in 1,000,000(*7*, *11*). T cells that target the same epitope are a collection of clonotypes. Low affinity TCRs can be detected by analytical techniques used to quantify reactive cells, potentially leading to overestimation of the number of reactive T cells. Accurate estimates would require a cutoff for the strength of antigen recognition. This cutoff should be the strength that would generate killing in response to recognition of processed antigens. Cytotoxicity assays are not trivial to perform in a high throughput manner, especially when the starting T cell population is rare. Biological and technical limitations make it difficult to approximate the true frequency of reactive T cells.

Understanding which TCRs can target novel antigens is essential to understand diseases and develop therapeutics. All mammalian cells, except red blood cells, present a large variety of antigens on MHC molecules, representative of the total cellular proteome(*1*). If a virus infects a cell or a cell becomes transformed, then additional antigens will be presented, derived from the viral or tumor proteins. Most of these antigens do not have a defined, reactive TCR. Less than 400 epitopes have defined TCRs and most of these epitopes are of viral origin(*12*). Model

antigens are commonly studied epitopes with well-defined TCRs that may have been used to generate mouse transgenic models(*13*). Epitopes that have been used as model antigens come from three sources: 1. Viruses 2. Cancer 3. Normal tissue. TCR transgenic mouse models are used to study immune responses to viruses and cancer, with the caveat that all the data is generated in the context of one TCR. The need remains to uncover the breadth of TCRs involved in human disease and maintenance of tissue homeostasis.

3. TCR structure

The TCR recognizes peptide antigen presented on the surface of MHC molecules through the variable interface of alpha and beta chains(*3*). The TCRα/β heterodimer binds both the antigen as well as the MHC molecule with its complementarity determining regions (CDRs). TCR diversity is derived from somatic DNA recombination in the thymus(*9*). Germline V and J regions recombine to create the alpha chain and V, D and J recombine to generate the beta. Additional diversity is introduced by non-templated deletion and addition of nucleotides at the joining regions during recombination(*14*, *15*). The recombination site creates the CDR3 hypervariable regions that are involved in antigen recognition. The beta chain is the first one to be recombined(*16*). T cell precursor continues to proliferate while the alpha chain is being generated, allowing a single beta chain to be paired with multiple alpha chains(*17*). The combinatorial pairing of alpha and beta chains generates additional diversity(*5*). T cells go through several well characterized stages of development, which is driven by the interactions with multiple cell types in the thymus(*16*, *18*). The end product of thymic maturation is a T cell repertoire that recognizes a large diversity of foreign epitopes and avoids reactivity with self-derived peptides(*4*, *18*). In addition, each clonotype has learned to recognize one MHC allele,

which is called MHC restriction. The mechanisms of how such a repertoire is produced are still not clear. TCRs are selected to bind self MHC by positive selection, where ligands can be weak agonists and structurally mimic potential foreign antigens(*19*). Negative selection deletes self-reactive clones and is primarily driven by medullary thymic epithelial cells that present peptides derived from a large variety of self-proteins, that are otherwise tissue restricted(*18*). Transcription factor Aire has been identified as the driver of this ectopic gene expression(*20*). Mutations in Aire cause autoimmunity in people(*20*).

TCRs bind antigen with relatively weak affinity, such that one pMHC can repeatedly engage many TCRs, which allows for low density ligands to elicit strong T cell responses(*21*). TCR-antigen reactivity is referred to as weak affinity compared to the affinity of antibodies, however within this range some TCRs will recognize target antigens with much higher affinity than others and are called high affinity TCRs. TCR affinity is measured as dissociation rate between TCR and pMHC and ranges from 100-0.1 μM(*22*). Salt bridges can help to stabilize the TCR-pMHC interaction and have been particularly important for increasing the amount of force on the TCR(*23*). The α/β heterodimer lacks independent signaling components. Mechanical piston like movement of the TCR through the CD3 results in CD3 activation(*24*). The CD3 complex is made of four protein chains, ε, γ, δ, and ζ, where ε forms heterodimer pairs with δ and γ and ζ chain forms a homodimer(*25*). Proximal tyrosine kinases, such as LCK, phosphorylate ITAM domains within the CD3(*26*). Most ITAM domains are found in the CD3 ζ heterodimer. Phosphorylated CD3 ζ is then bound by ZAP70 protein which phosphorylates LAT, resulting in phosphorylation of phospholipase C, leading to calcium efflux from the ER which then triggers extracellular calcium flow into the cell(*26, 27*). Calcium signaling is the end result of TCR

engagement and results in T cell activation, this is supported by the observation that calcium influx alone is sufficient for activation(*28*).

4. TCR repertoire sequencing

Clonotype diversity can be profiled by TCR sequencing, which defines clonotypes and maps abundance. Abundance also referred to as frequency can be used to study immune responses. Frequent clones are thought to be undergoing antigen dependent expansion and therefore functional in the immune response(*29*, *30*). Clonal dynamics can be studied by sampling multiple timepoints within the immune response. Typically, beta chain sequencing from genomic DNA is done in bulk population of cells (adaptive biotechnologies). TCRα/β chain sequencing represents the true diversity, because the beta is paired with multiple alpha chains(*29*, *31*). Full length paired TCRs are sequenced from the mRNA, to recover processed sequences that will generate the TCR. Current technologies allow for TCR sequencing in millions of T cells, but paired analysis is limited to thousands.

TCR sequencing alone does not provide information about which antigens are recognized. Computational algorithms can cluster TCRs that are likely to recognize the same antigen, because TCRs that recognize the same antigen are similar in sequence(*32*, *33*). This approach is limited to previously defined antigens and does not allow for discovery of novel TCR reactivities. Analysis of similar TCRs allowed for diagnosis of prior SARS-CoV-2 infection based on TCR sequence data alone(*34*). To further characterize an immune response, TCRs that recognize unknown antigens can be grouped into families(*31*, *32*). It's difficult to extrapolate TCR family performance based on one family member because a subtle difference in the

sequence has been known to dramatically change the function(*35*). Grouping TCRs of unknown

reactivity was used to show that checkpoint blockade in cancer patients induces targeting of new

antigens(*31*). Target antigens of TCRs can be determined by high throughput antigen screening

analysis(*36*, *37*). These techniques require crystallization or over expression of the TCR

construct, which are non-trivial efforts and require the knowledge of the full-length TCR α/β

sequences. Recently, tools have been developed that allow accurate structure prediction using an

inexpensive computer; such technology can potentially predict TCR structure and can inform

efforts on antigen discovery in-silico(*38*).

TCR sequencing in antigen specific T cells defines antigen specific TCRs. Selection of antigen

specific T cells can be done by either physical staining of a specific TCR or selection of

activated T cells. To capture activated cells, T cells are first stimulated with antigen and then

stained for surface markers of activation. CD137 is a well-established activation marker;

however, some concerns exist about specificity(*39*). To increase specificity other markers are

now being combined with CD137. Physical TCR staining is done by peptide-MHC multimer

reagents linked to reporter molecules, which are usually detected by flow cytometry(*40*, *41*).

Peptide-MHC tetramers engage the T cell with four different complexes, to compensate for the

low affinity of the TCR. As would be expected this strategy is highly variable, because

successful construction of the peptide-MHC depends on the epitope. To increase the sensitivity

of the tetramer approach, the dextramer links 10 peptide-MHCs to each other(*42*, *43*). Dextramer

staining allows for better sensitivity; however, the background is higher which affects specificity.

Building on this work, nanoparticle approaches increase the strength of binding by incorporating

larger numbers of peptide-MHCs(*44*, *45*). Peptide-MHC based detection can be scaled up by

using genetically encoded single chain trimer constructs, which covalently link peptide to the MHC molecule(*46*). The addition of oligo barcodes that link epitope identity to the peptide-MHC multimer allows for parallel sequencing of TCRs and epitope identity, which can increase throughput. We developed a TCR sequencing technique based on intracellular cytokine staining (ICS). ICS is a highly specific and sensitive method to quantify functional antigen specific T cells. Droplet-based single-cell RNA analysis was never done, as fixation and permeabilization was thought to decrease the RNA quality. TCR sequencing based on ICS is discussed in Chapter 2. This is a very rapidly evolving field, where high throughput has been one of the primary objectives, to allow for on demand TCR isolation for therapeutic purpose.

Synthetic reconstruction of TCRs from α/β sequences assists discovery of TCRs against novel antigens, by enabling analysis that would be difficult in primary PBMCs(*47*, *48*). TCR α/β constructs can be overexpressed in primary T cells or reporter cell lines for extensive functional analysis. Processed antigen recognition is weaker, necessitating over expression to study low frequency responses. Overexpression also allows for extensive cross-reactivity analysis. Studying one specific TCR removes confounding variables that may otherwise affect recognition. Performance characteristics of high-quality TCRs include recognition of processed epitopes and the ability to stimulate killing of target cells, production of multiple cytokines, and cell division. TCR performance can be combined with other data types, such as gene expression and phenotype.

Linking the TCR sequence to the T cell state informs the role of specific clonotypes within the immune response(*5*, *31*). T cell states are defined by expression of surface markers and genes.

Flow cytometry sorting of specific phenotypes and subsequent TCR beta sequencing allows

exploration of differential TCR usage by distinct T cell subsets. Parallel single-cell gene

expression and TCR sequencing allows for unbiased exploration of clonotype functional

significance. Oligo barcoded antibodies can be used to profile the surfaceome in parallel with

single-cell sequencing(*49*). TCRs with the phenotype of interest can be explored as drivers of the

response. Single-cell immune profiling has been used to show that TCR clonotypes that respond

to checkpoint blockade therapy originate from the blood rather than pre-existing tumor

infiltrating lymphocytes(*31*). Analysis of both TCRs and T cell state can help define both TCR

candidates as well as identify new therapeutic modalities (Figure 1).

5. Discovery of T cell MHC restriction

Tumor graft models were used to study drivers of rejection in transplantation immunology(*50*,

51). The so-called "cellular antibodies" caused graft rejection responses in animals. Anti CD8

sera blocked cytotoxicity, thus CD8 T cells were identified as cytotoxic cells(*52*, *53*). It wasn't

clear why tissue was rejected from some animals but not others(*54*). MHC molecules were

identified as genetic drivers of this segregation(*55*). Viral immunology allowed us to ask the

question of how self is recognized in the context of controlled foreign antigen expression. T cells

recognized viral antigens only if a specific MHC was present(*56*). Two competing hypotheses

stated that either T cells recognize two independent molecules or a molecule that has been

altered to be different, that is an altered self. The prevalent theory state that T cells recognize

virus infected cells by recognizing the MHC molecule and separately binding a viral protein.

Through the study of heterozygous mice, it was determined that T cells recognize a modified

version of the MHC molecule(*57*). The altered self, turned out to be MHC molecules that present foreign peptide epitopes.

6. Antigen processing and presentation

CD4 and CD8 T cells differ dramatically in the types of antigens they recognize. CD4 T cells recognize peptides derived from extracellular proteins presented on MHC class II, which is usually expressed by professional antigen presenting cells. CD8 T cells recognize peptide epitopes presented by MHC Class I. Class I MHCs bind the outer anchor residues of the epitope, which allows for a large diversity of peptides to be presented(*58*). Proteins are degraded into peptides by the proteasome, in addition to directed degradation, many proteins are degraded immediately due to mistakes in translation(*59*, *60*). Only a small minority of peptides generated get presented, so the immune system favors abundant proteins(*61*, *62*). Peptides are loaded into the endoplasmic reticulum, through the TAP protein. In the ER peptides are further trimmed to fit the binding groove of MHC class I, which is often restricted to 9 amino acids(*63–65*). Peptides are then assembled with the polymorphic heavy chain and the constant light chain(*58*). This trimer complex constitutes a stable peptide-MHC.

Peptide presentation is affected by alternative proteasome usage and specific cell conditions, which further complicates antigen presentation(*58*, *66*). The immunoproteasome, which is preferentially used by professional antigen presenting cells, and the thymic proteasome, present different sets of peptides(*67*). In addition, cytokines produced in response to viral infections can affect the abundance of peptides as well as specific components of the proteasome expressed(*62*,

68). Antigen presentation is a highly dynamic process, making it difficult to anticipate the presentation of specific epitopes in defined cell types.

MHC trimers dissociate at the cell surface and reassemble with peptides present in the serum or media(*58*). This quality allows screening of endogenously added peptides for in-vitro T cell recognition. Custom synthetic peptides of varying purity are commercially available. Peptides of up to 20 amino acids have been used to pulse cells in vitro. These studies often used overlapping peptide pools that covered the full length of a protein(*69, 70*). Extracellular proteases can cleave terminal amino acids, allowing the shortened peptide to be loaded onto the MHC.

The immune response is diversified at the population level to increase capacity to respond to diseases(*71*). MHC Class I is highly polymorphic; more than 30,000 alleles exist, (IMGT/HLA). Some MHC alleles are highly overrepresented, indicating their potential importance in protecting against diseases. The HLA-A2 is frequent in all populations and is expressed in 30-50% of individuals of northern European decent(*72*). Each T cell clone only recognizes antigens in the context of one specific MHC molecule. TCR discovery efforts are complicated by the need to perform antigen discovery and characterization in the context of many HLA alleles.

7. Recall memory

Primary infection generates T cell response and establishes memory against the pathogen. Dendritic cells survey peripheral tissues to pick up antigens and subsequently drive the activation and proliferation of antigen specific naïve T cells in the lymph node (Figure 2). Naïve T cells are mostly restricted to secondary lymphoid organs, due to their expression of lymph homing molecules CD62L and CCR7(*73*). Upon encountering antigens, dendritic cells stimulate the TCR

as well as deliver co-stimulation signals (*74*, *75*). Priming dramatically changes naïve T cells to

generate a pool of effector T cells capable of anti-viral function(*74*, *76*). Lymph homing markers

are downregulated and trafficking molecules needed for peripheral tissue entry are

expressed(*77*). Effector T cells traffic into tissues and kill infected cells. Effector cells then go

through a contraction phase and some remain as memory cells. T cell memory stays for decades

and surveys the body for reinfection(*78*, *79*).

CD8+ memory T cells are subdivided into three phenotypes with distinct functional roles: central

memory (Tcm), effector memory (Tem), and resident memory (Trm) (Figure 2)(*80–82*).

Resident memory cells are permanently localized at sites of initial infection to generate a rapid

response(*80*). Trm function by the direct killing of infected cells but also promote an

inflammatory response that recruits other immune cells(*83*, *84*). Respiratory virus control is

associated with presence of Trm cells in the lungs(*85*). Among the cells that Trm cells recruit are

antigen specific Tem cells. Tem cells are localized in the blood and peripheral tissues. Tem and

Trm cells are both designed for immediate antiviral activity(*86*). These cells lack costimulatory

molecules and can be activated by TCR stimulation alone, thus can recognize non-immune cells.

Tcm contains a pool of precursor cells that can expand upon antigen stimulation and are more

similar to naïve T cells(*74*). Tcm circulation is limited to the blood and lymph organs, where

they can be expanded by dendritic cells(*76*). Higher precursor numbers allow for faster

generation of effector pool. Trm and Tem provide protection during the first hours and days of

reinfection, but at day 7 Tcm cells have proliferated and differentiated into the effector pool that

can eliminate the pathogen(*87*, *88*). Memory is compartmentalized where cells that recognize the

same antigen and even have the same TCR acquire multiple phenotypes to provide protection at different layers of infection(*76*).

8. Vaccination

Vaccination allows for the generation of specific memory without the severity of a full infection. In 1796 Edward Jenner experimentally showed that cowpox infection was able to provide protection against the human infection smallpox, establishing the concept of vaccination(*1*). Live attenuated vaccines have been very successful and protect against smallpox, chickenpox and yellow fever (CDC). Inactivated vaccines are killed pathogens and are used against Influenza, Polio, Hepatitis A and Rabies (CDC). Subunit vaccines induce a specific response against a component of the pathogen and are used against hepatitis B, HPV, Whooping cough and Shingles (CDC). Unlike inactivated and attenuated viruses, subunit vaccines require adjuvant to stimulate the innate immune system, because they lack natural viral components. Adjuvant response is not completely understood, so subunit vaccines are generally weaker and require multiple doses and or boosters (*89*). Viral vectored vaccines use viruses that encode the immunogen via cDNA(*89*, *90*). Adenoviruses are the most advanced platforms for viral vectored vaccines, but cytomegalovirus is also entering clinical trials. The advantage of viral vectors is induction of strong immune responses, which includes strong effector memory CD4 and CD8 T cells. Such vectors have been in development for a long time, however viral vectored vaccines are only approved for SARS-CoV-2(*91*). The mRNA vaccines also proved very successful for preventing COVID-19 and are now being considered for other diseases(*92*). The best vaccines are thought to induce sterilizing immunity - blocking any kind of viral proliferation, as can be done by neutralizing antibodies. Sterilizing immunity is an ideal assumption even in cases where

vaccines work well. T cells are likely involved in control of all pathogens to various extents to clear the cells which became infected by viruses that escaped neutralization.

T cells may be critical for preventing diseases where vaccines do not yet exist(*76*). Latent infections proved difficult for vaccine development and may need specialized T cell responses to clear cells infected early in the exposure. Acute infections where natural immunity is not lifelong also proved to be difficult vaccine targets, either because of durability of immune response or changes in the genetic sequence of the pathogen. We do not have effective vaccines against Hepatitis C virus, Human Immunodeficiency virus (HIV) and Respiratory syncytial virus (RSV)(*93*, *94*). Vaccines against Tuberculosis and Influenza have low efficacy(*95*). Influenza is complicated by the need-to-know which strain will become dominant and the difficulty in predicting which viruses may spill over from animal populations, such as the H1N1 in 2009. Human T cells recognize epitopes from different influenza virus family members, a phenomenon called heterosubtypic immunity(*96*, *97*). Heterosubtypic immunity is dependent on CD4 and CD8 T cells, suggesting that T cell cross reactivity is responsible for broadly reactive immune responses(*97*). A live attenuated influenza vaccine can be delivered intra nasally and induces Trm cells in the lung(*98*). The most common influenza vaccine used today is still the injectable form. RSV is difficult to neutralize by antibody because the fusion protein has two different conformations, but most RSV vaccine efforts have been aimed at inducing neutralizing antibodies(*93*). CD8+ T cell response are associated with RSV control in infected humans(*85*). A vaccine that induces Trm in the Lung may protect against RSV(*99*). T cell cross-reactivity and highly functional CD8 T cell responses can help suppress HIV infection for a long time(*100*). Cytomegalovirus vectors protect against HIV and Tuberculosis in rhesus macaque model via

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CD8 dependent immunity(*95*, *101*). A prophylactic vaccine needs to induce Trm cells that are located at the site of pathogen entry or immediately recruited to the site of infection. Alternatively, a vaccine that systemically a induces high frequency of Tem cells may be sufficient, as these cells traffic through tissues and can quickly be recruited upon a pathogen encounter. Development of T cell vaccines will rely on identification of effective T cell responses and the development of a means to induce such responses at the right anatomical site.

The SARS-CoV-2 ongoing pandemic illustrates failures and successes of modern immunology. As of December 2021, there are rising concerns about the efficacy of existing SARS-CoV-2 vaccines against the Omicron variant. The third shot of the vaccine, also called a booster, provides protection against Omicron(*102*). Current boosters do not alter the specificity of the response. Variant specific boosters are in development, but it remains to be seen whether this strategy will work long term. Additional concern about mRNA vaccines is the lack of generation of long-lived plasma cells, which provide constant levels of antibody(*103*, *104*). The inability to generate long-lived plasma cells may mean that the mRNA vaccine technology should be further explored as a T cell vaccine. A more conserved T cell antigen may need to be targeted, which may allow for the targeting of many variants and potentially different human coronaviruses(*105–107*).

Exploring SARS-CoV-2 T cell responses is discussed in more detail in chapter 3.

Figure 1-1

Data Generation

Gene expression UMAP plot

parallel TCR profiling

TCRα	TCRβ

TCR construct synthesis and testing

Allogeneic T cell

1. Peptide recognition
2. Processed antigen recognition
3. Affinity profiling
4. Cross-reactivity profiling
5. Antigen discovery

Reactive TCR candidates

TCRα	TCRβ

Phenotype of interest

Therapeutic discovery

1. Identify signaling pathways involved in antigen specific T cells
(targetable by small molecules, antibodies)
2. Identify TCRs reactvie with novel antigens
(adoptive cell therapeutics, monitoring of effective T cell responses, vaccine antigens)

Figure 1-2

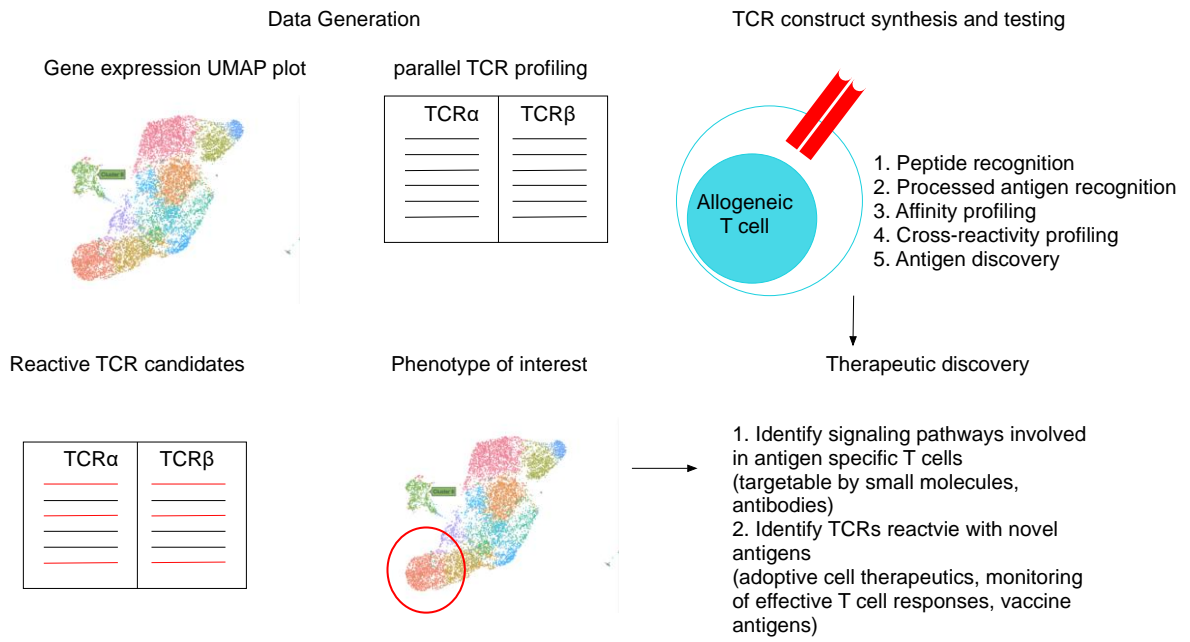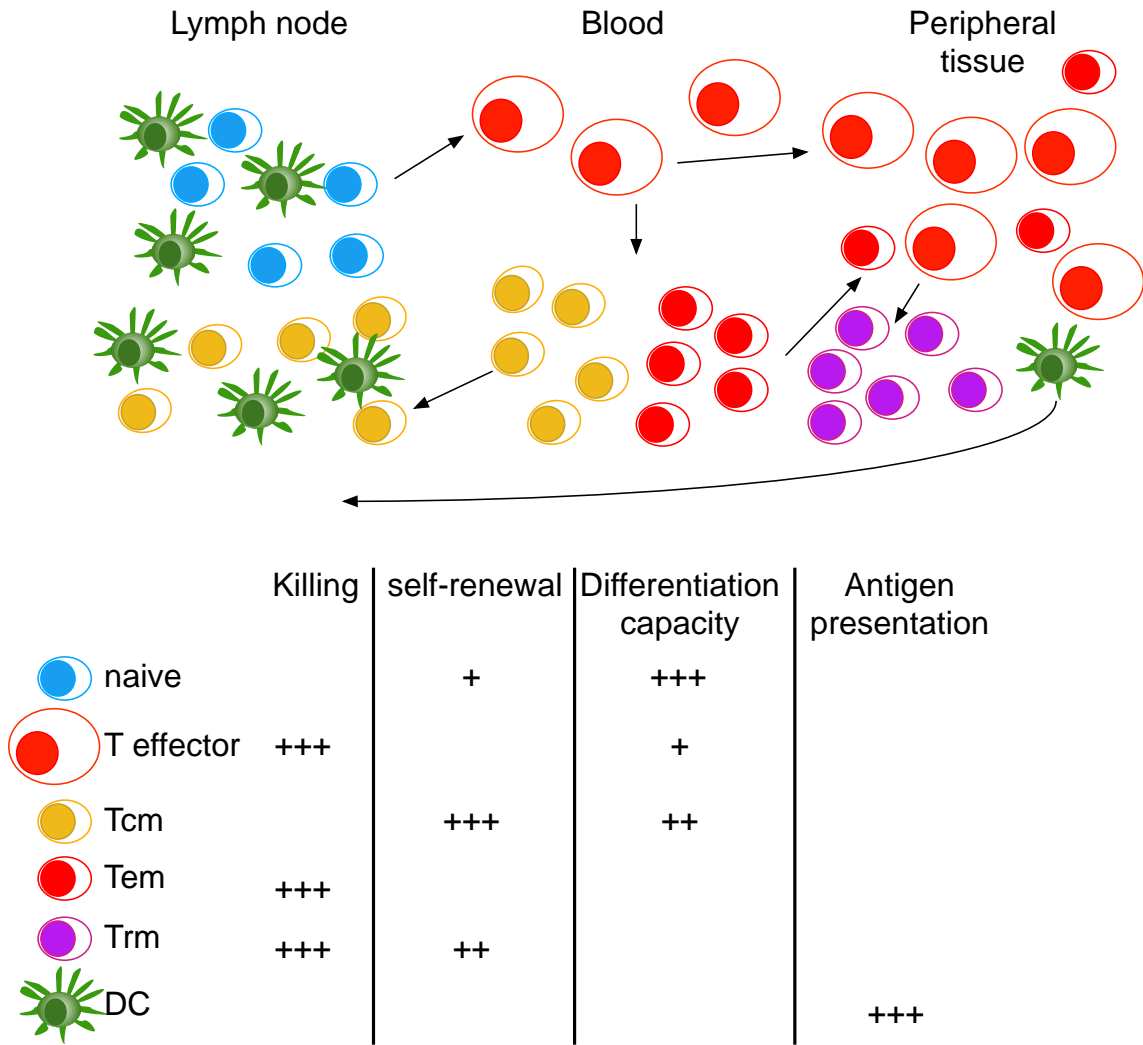|	Killing	self-renewal	Differentiation capacity	Antigen presentation
naive		+	+++	
T effector	+++		+	
Tcm		+++	++	
Tem	+++			
Trm	+++	++		
DC				+++

References:
1. K. (Kenneth M. . Murphy, C. Weaver, *Janeway's Immunobiology* (Garland science, ed. 9th, 2017).
2. I. den Braber, T. Mugwagwa, N. Vrisekoop, L. Westera, R. Mögling, A. Bregje de Boer, N. Willems, E. H. R. Schrijver, G. Spierenburg, K. Gaiser, E. Mul, S. A. Otto, A. F. C. Ruiter, M. T. Ackermans, F. Miedema, J. A. M. Borghans, R. J. de Boer, K. Tesselaar, Maintenance of Peripheral Naive T Cells Is Sustained by Thymus Output in Mice but Not Humans. *Immunity*. **36**, 288–297 (2012).
3. M. M. Davis, P. J. Bjorkman, T-cell antigen receptor genes and T-cell recognition. *Nat. 1988 3346181*. **334**, 395–402 (1988).
4. J. Nikolich-Žugich, M. K. Slifka, I. Messaoudi, The many important facets of T-cell repertoire diversity. *Nat. Rev. Immunol. 2004 42*. **4**, 123–132 (2004).
5. J. A. Pai, A. T. Satpathy, High-throughput and single-cell T cell receptor sequencing technologies. *Nat. Methods 2021 188*. **18**, 881–892 (2021).
6. Q. Qi, Y. Liu, Y. Cheng, J. Glanville, D. Zhang, J.-Y. Lee, R. A. Olshen, C. M. Weyand, S. D. Boyd, J. J. Goronzy, Diversity and clonal selection in the human T-cell repertoire, doi:10.1073/pnas.1409155111.
7. C. Alanio, F. Lemaitre, H. K. W. Law, M. Hasan, M. L. Albert, Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies. *Blood*. **115**, 3718–3725 (2010).
8. I. V. Zvyagin, M. V. Pogorelyy, M. E. Ivanova, E. A. Komech, M. Shugay, D. A. Bolotin, A. A. Shelenkov, A. A. Kurnosov, D. B. Staroverov, D. M. Chudakov, Y. B. Lebedev, I. Z. Mamedov, Distinctive properties of identical twins' TCR repertoires revealed by high-throughput sequencing. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5980–5985 (2014).
9. A. K. Sewell, Why must T cells be cross-reactive? *Nat. Rev. Immunol.* **12** (2012), pp. 669–677.
10. L. Wooldridge, J. Ekeruche-Makinde, H. A. Van Den Berg, A. Skowera, J. J. Miles, M. P. Tan, G. Dolton, M. Clement, S. Llewellyn-Lacey, D. A. Price, M. Peakman, A. K. Sewell, A single autoimmune T cell receptor recognizes more than a million different peptides. *J. Biol. Chem.* **287**, 1168–1177 (2012).
11. W. Yu, N. Jiang, P. J. R. Ebert, B. A. Kidd, S. Müller, P. J. Lund, J. Juang, K. Adachi, T. Tse, M. E. Birnbaum, E. W. Newell, D. M. Wilson, G. M. Grotenbreg, S. Valitutti, S. R. Quake, M. M. Davis, Clonal Deletion Prunes but Does Not Eliminate Self-Specific αβ CD8+ T Lymphocytes. *Immunity*. **42**, 929–941 (2015).
12. D. V. Bagaev, R. M. A. Vroomans, J. Samir, U. Stervbo, C. Rius, G. Dolton, A. Greenshields-Watson, M. Attaf, E. S. Egorov, I. V. Zvyagin, N. Babel, D. K. Cole, A. J. Godkin, A. K. Sewell, C. Kesmir, D. M. Chudakov, F. Luciani, M. Shugay, VDJdb in 2019: Database extension, new analysis infrastructure and a T-cell receptor motif compendium. *Nucleic Acids Res.* **48**, D1057–D1062 (2020).
13. E. W. Newell, M. M. Davis, Beyond model antigens: high-dimensional methods for the analysis of antigen-specific T cells. *Nat. Biotechnol. 2013 322*. **32**, 149–157 (2014).
14. L. NR, S. DG, R. M, B. D, Increased frequency of N-region insertion in a murine pre-B-cell

line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. *Mol. Cell. Biol.* **7**, 3237–3243 (1987).

15.   D. G. Schatz, M. A. Oettinger, D. Baltimore, The V(D)J recombination activating gene, RAG-1. *Cell*. **59**, 1035–1048 (1989).

16.   H. Von Boehmer, I. Aifantis, F. Gounari, O. Azogui, L. Haughn, I. Apostolou, E. Jaeckel, F. Grassi, L. Klein, Thymic selection revisited: how essential is it? *Immunol. Rev.* **191**, 62–78 (2003).

17.   T. P. Arstila, A. Casrouge, V. Baron, J. Even, J. Kanellopoulos, P. Kourilsky, A direct estimate of the human αβ T cell receptor diversity. *Science (80-.).* **286**, 958–961 (1999).

18.   L. Klein, M. Hinterberger, G. Wirnsberger, B. Kyewski, Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol. 2009 912*. **9**, 833–844 (2009).

19.   K. A. Hogquist, S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, F. R. Carbone, T cell receptor antagonist peptides induce positive selection. *Cell*. **76**, 17–27 (1994).

20.   M. S. Anderson, M. A. Su, AIRE expands: new roles in immune tolerance and beyond. **16**, 247–258 (2016).

21.   V. S, M. S, C. M, P. E, L. A, Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*. **375**, 148–151 (1995).

22.   J. D. Stone, D. M. Kranz, Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies. *Front. Immunol.* **4**, 244 (2013).

23.   L. V Sibener, R. A. Fernandes, E. M. Kolawole, C. B. Carbone, F. Liu, D. McAffee, M. E. Birnbaum, X. Yang, L. F. Su, W. Yu, Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell*. **174**, 672-687. e27 (2018).

24.   K. Choudhuri, A. Kearney, T. R. Bakker, P. A. Van Der Merwe, Immunology: How Do T Cells Recognize Antigen? *Curr. Biol.* **15**, R382–R385 (2005).

25.   M. S. Kuhns, M. M. Davis, K. C. Garcia, Deconstructing the Form and Function of the TCR/CD3 Complex. *Immunity*. **24**, 133–139 (2006).

26.   R. T. Abraham, A. Weiss, Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat. Rev. Immunol. 2004 44*. **4**, 301–308 (2004).

27.   N. Joseph, B. Reicher, M. Barda-Saad, The calcium feedback loop and T cell activation: How cytoskeleton networks control intracellular calcium flux. *Biochim. Biophys. Acta - Biomembr.* **1838**, 557–568 (2014).

28.   J. B. Imboden, J. D. Stobo, Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. Exp. Med.* **161**, 446–456 (1985).

29.   J. A. Pai, A. T. Satpathy, High-throughput and single-cell T cell receptor sequencing technologies. *Nat. Methods 2021 188*. **18**, 881–892 (2021).

30.   T. D. Wu, S. Madireddi, P. E. de Almeida, R. Banchereau, Y. J. J. Chen, A. S. Chitre, E. Y. Chiang, H. Iftikhar, W. E. O'Gorman, A. Au-Yeung, C. Takahashi, L. D. Goldstein, C. Poon, S. Keerthivasan, D. E. de Almeida Nagata, X. Du, H. M. Lee, K. L. Banta, S. Mariathasan, M. Das Thakur, M. A. Huseni, M. Ballinger, I. Estay, P. Caplazi, Z. Modrusan, L. Delamarre, I. Mellman, R. Bourgon, J. L. Grogan, Peripheral T cell expansion predicts tumour infiltration and clinical response. *Nature*. **579**, 274–278 (2020).

31.   K. E. Yost, A. T. Satpathy, D. K. Wells, Y. Qi, C. Wang, R. Kageyama, K. L. McNamara, J. M.

Granja, K. Y. Sarin, R. A. Brown, R. K. Gupta, C. Curtis, S. L. Bucktrout, M. M. Davis, A. L. S. Chang, H. Y. Chang, Clonal replacement of tumor-specific T cells following PD-1 blockade. *Nat. Med.* **25**, 1251–1259 (2019).

32. J. Glanville, H. Huang, A. Nau, O. Hatton, L. E. Wagar, F. Rubelt, X. Ji, A. Han, S. M. Krams, C. Pettus, N. Haas, C. S. L. Arlehamn, A. Sette, S. D. Boyd, T. J. Scriba, O. M. Martinez, M. M. Davis, Identifying specificity groups in the T cell receptor repertoire. *Nature*. **547**, 94–98 (2017).

33. H. Huang, C. Wang, F. Rubelt, T. J. Scriba, M. M. Davis, Analyzing the Mycobacterium tuberculosis immune response by T-cell receptor clustering with GLIPH2 and genome-wide antigen screening. *Nat. Biotechnol.* **38**, 1194–1202 (2020).

34. R. Elyanow, T. M. Snyder, S. C. Dalai, R. M. Gittelman, J. Boonyaratanakornkit, A. Wald, S. Selke, M. H. Wener, C. Morishima, A. L. Greninger, M. R. Holbrook, I. M. Kaplan, H. J. Zahid, J. M. Carlson, L. Baldo, T. Manley, H. S. Robins, D. M. Koelle, T-cell receptor sequencing identifies prior SARS-CoV-2 infection and correlates with neutralizing antibody titers and disease severity. *medRxiv* (2021), doi:10.1101/2021.03.19.21251426.

35. P. F. Robbins, Y. F. Li, M. El-Gamil, Y. Zhao, J. A. Wargo, Z. Zheng, H. Xu, R. A. Morgan, S. A. Feldman, L. A. Johnson, A. D. Bennett, S. M. Dunn, T. M. Mahon, B. K. Jakobsen, S. A. Rosenberg, Single and Dual Amino Acid Substitutions in TCR CDRs Can Enhance Antigen-Specific T Cell Functions. *J. Immunol.* **180**, 6116–6131 (2008).

36. A. V. Joglekar, M. T. Leonard, J. D. Jeppson, M. Swift, G. Li, S. Wong, S. Peng, J. M. Zaretsky, J. R. Heath, A. Ribas, M. T. Bethune, D. Baltimore, T cell antigen discovery via Signaling and Antigen presenting Bifunctional Receptors. *Nat. Methods*. **16**, 191 (2019).

37. M. H. Gee, A. Han, S. M. Lofgren, S. R. Quake, M. M. Davis, K. Christopher, G. Correspondence, Antigen Identification for Orphan T Cell Receptors Expressed on Tumor-Infiltrating Lymphocytes In Brief. *Cell*. **172**, 549–563 (2018).

38. M. Baek, F. DiMaio, I. Anishchenko, J. Dauparas, S. Ovchinnikov, G. R. Lee, J. Wang, Q. Cong, L. N. Kinch, R. Dustin Schaeffer, C. Millán, H. Park, C. Adams, C. R. Glassman, A. DeGiovanni, J. H. Pereira, A. V. Rodrigues, A. A. Van Dijk, A. C. Ebrecht, D. J. Opperman, T. Sagmeister, C. Buhlheller, T. Pavkov-Keller, M. K. Rathinaswamy, U. Dalwadi, C. K. Yip, J. E. Burke, K. Christopher Garcia, N. V. Grishin, P. D. Adams, R. J. Read, D. Baker, Accurate prediction of protein structures and interactions using a three-track neural network. *Science (80-.)*. **373**, 871–876 (2021).

39. M. Wolfl, J. Kuball, W. Y. Ho, H. Nguyen, T. J. Manley, M. Bleakley, P. D. Greenberg, Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. *Blood*. **110**, 201–210 (2007).

40. A. H. Bakker, R. Hoppes, C. Linnemann, M. Toebes, B. Rodenko, C. R. Berkers, S. R. Hadrup, W. J. E. van Esch, M. H. M. Heemskerk, H. Ovaa, T. N. M. Schumacher, Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3825 (2008).

41. B. Rodenko, M. Toebes, S. R. Hadrup, W. J. E. van Esch, A. M. Molenaar, T. N. M. Schumacher, H. Ovaa, Generation of peptide–MHC class I complexes through UV-mediated ligand exchange. *Nat. Protoc.* **1**, 1120–1132 (2006).

42. G. Dolton, E. Zervoudi, C. Rius, A. Wall, H. L. Thomas, A. Fuller, L. Yeo, M. Legut, S.

Wheeler, M. Attaf, D. M. Chudakov, E. Choy, M. Peakman, A. K. Sewell, Optimized Peptide–MHC Multimer Protocols for Detection and Isolation of Autoimmune T-Cells. *Front. Immunol.* **9** (2018), doi:10.3389/fimmu.2018.01378.

43. W. Zhang, P. G. Hawkins, J. He, N. T. Gupta, J. Liu, G. Choonoo, S. W. Jeong, C. R. Chen, A. Dhanik, M. Dillon, R. Deering, L. E. Macdonald, G. Thurston, G. S. Atwal, A framework for highly multiplexed dextramer mapping and prediction of T cell receptor sequences to antigen specificity. *Sci. Adv.* **7**, 5835–5849 (2021).

44. V. Mallajosyula, C. Ganjavi, S. Chakraborty, A. M. McSween, A. J. Pavlovitch-Bedzyk, J. Wilhelmy, A. Nau, M. Manohar, K. C. Nadeau, M. M. Davis, CD8+ T cells specific for conserved coronavirus epitopes correlate with milder disease in COVID-19 patients. *Sci. Immunol.* **6**, eabg5669 (2021).

45. S. Peng, J. M. Zaretsky, A. H. C. Ng, W. Chour, M. T. Bethune, J. Choi, A. Hsu, E. Holman, X. Ding, K. Guo, J. Kim, A. M. Xu, J. E. Heath, W. J. Noh, J. Zhou, Y. Su, Y. Lu, J. McLaughlin, D. Cheng, O. N. Witte, D. Baltimore, A. Ribas, J. R. Heath, Sensitive Detection and Analysis of Neoantigen-Specific T CellPopulations from Tumors and Blood. *Cell Rep.* **28**, 2728 (2019).

46. W. Chour, A. M. Xu, A. H. C. Ng, J. Choi, J. Xie, D. Yuan, D. C. DeLucia, R. A. Edmark, L. C. Jones, T. M. Schmitt, M. E. Chaffee, V. R. Duvvuri, K. M. Murray, S. Peng, J. Wallick, H. A. Algren, W. R. Berrington, D. S. O'Mahony, J. K. Lee, P. D. Greenberg, J. D. Goldman, J. R. Heath, Shared antigen-specific CD8+ T cell responses against the SARS-COV-2 spike protein in HLA-A*02:01 COVID-19 participants. *medRxiv* (2020), doi:10.1101/2020.05.04.20085779.

47. A. Jaigirdar, S. A. Rosenberg, M. Parkhurst, A high-avidity WT1-reactive T-cell receptor mediates recognition of peptide and processed antigen but not naturally occurring WT1-positive tumor cells. *J. Immunother.* **39**, 105–116 (2016).

48. P. A. Nesterenko, J. McLaughlin, D. Cheng, N. J. Bangayan, G. B. Sojo, C. S. Seet, Y. Qin, Z. Mao, M. B. Obusan, J. W. Phillips, O. N. Witte, Droplet-based mRNA sequencing of fixed and permeabilized cells by CLInt-seq allows for antigen-specific TCR cloning. *Proc. Natl. Acad. Sci. U. S. A.* **118** (2021), doi:10.1073/pnas.2021190118.

49. M. Stoeckius, C. Hafemeister, W. Stephenson, B. Houck-Loomis, P. K. Chattopadhyay, H. Swerdlow, R. Satija, P. Smibert, Simultaneous epitope and transcriptome measurement in single cells. *Nat. Methods*. **14**, 865–868 (2017).

50. D. Masopust, V. Vezys, E. J. Wherry, R. Ahmed, A brief history of CD8 T cells. *Eur. J. Immunol.* **37**, S103–S110 (2007).

51. R. M. Zinkernagel, P. C. Doherty, MHC-Restricted Cytotoxic T Cells: Studies on the Biological Role of Polymorphic Major Transplantation Antigens Determining T-Cell Restriction-Specificity, Function, and Responsiveness. *Adv. Immunol.* **27**, 51–177 (1979).

52. C. H, B. EA, Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141**, 1376–1389 (1975).

53. S. H, K. P, B. MA, T. T, B. EA, O. HF, O. LJ, Expression of T-cell differentiation antigens on effector cells in cell-mediated cytotoxicity in vitro. Evidence for functional heterogeneity related to the surface phenotype of T cells. *J. Exp. Med.* **141**, 227–241 (1975).

54. G. D. Snell, G. F. Higgins, Alleles at the Histocompatibility-2 Locus in the Mouse as Determined by Tumor Transplantation. *Genetics*. **36**, 306 (1951).

55. P. A. Gorer, S. Lyman, G. D. Snell, J. B. S. Haldane, Studies on the genetic and antigenic basis of tumour transplantation Linkage between a histocompatibility gene and "fused" in mice. *Proc. R. Soc. London. Ser. B - Biol. Sci.* **135**, 499–505 (1948).

56. Z. RM, D. PC, Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature.* **248**, 701–702 (1974).

57. R. M. Zinkernagel, P. C. Doherty, The discovery of MHC restriction. *Immunol. Today.* **18**, 14–17 (1997).

58. J. Neefjes, M. L. M. Jongsma, P. Paul, O. Bakke, Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol. 2011 1112*. **11**, 823–836 (2011).

59. E. A. J. Reits, J. C. Vos, M. Grammé, J. Neefjes, The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nat. 2000 4046779*. **404**, 774–778 (2000).

60. U. Schubert, L. C. Antón, J. Gibbs, C. C. Norbury, J. W. Yewdell, J. R. Bennink, Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nat. 2000 4046779*. **404**, 770–774 (2000).

61. C. A. Herberts, J. J. Neijssen, J. de Haan, L. Janssen, J. W. Drijfhout, E. A. Reits, J. J. Neefjes, Cutting Edge: HLA-B27 Acquires Many N-Terminal Dibasic Peptides: Coupling Cytosolic Peptide Stability to Antigen Presentation. *J. Immunol.* **176**, 2697–2701 (2006).

62. B. Strehl, U. Seifert, E. Krüger, S. Heink, U. Kuckelkorn, P. M. Kloetzel, Interferon-γ, the functional plasticity of the ubiquitin–proteasome system, and MHC class I antigen processing. *Immunol. Rev.* **207**, 19–30 (2005).

63. N. Blanchard, T. Kanaseki, H. Escobar, F. Delebecque, N. A. Nagarajan, E. Reyes-Vargas, D. K. Crockett, D. H. Raulet, J. C. Delgado, N. Shastri, Endoplasmic Reticulum Aminopeptidase Associated with Antigen Processing Defines the Composition and Structure of MHC Class I Peptide Repertoire in Normal and Virus-Infected Cells. *J. Immunol.* **184**, 3033–3042 (2010).

64. S. C. Chang, F. Momburg, N. Bhutani, A. L. Goldberg, The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism. *Proc. Natl. Acad. Sci.* **102**, 17107–17112 (2005).

65. D. Parcej, R. Tampé, ABC proteins in antigen translocation and viral inhibition. *Nat. Chem. Biol. 2010 68*. **6**, 572–580 (2010).

66. Y. Yang, J. B. Waters, K. Fruh, P. A. Peterson, Proteasomes are regulated by interferon gamma: implications for antigen processing. *Proc. Natl. Acad. Sci.* **89**, 4928–4932 (1992).

67. I. Mellman, R. M. Steinman, Dendritic Cells: Specialized and Regulated Antigen Processing Machines. *Cell.* **106**, 255–258 (2001).

68. P. M. Kloetzel, The proteasome and MHC class I antigen processing. *Biochim. Biophys. Acta - Mol. Cell Res.* **1695**, 225–233 (2004).

69. S. G. Hansen, J. B. Sacha, C. M. Hughes, J. C. Ford, B. J. Burwitz, I. Scholz, R. M. Gilbride, M. S. Lewis, A. N. Gilliam, A. B. Ventura, D. Malouli, G. Xu, R. Richards, N. Whizin, J. S. Reed, K. B. Hammond, M. Fischer, J. M. Turner, A. W. Legasse, M. K. Axthelm, P. T. Edlefsen, J. A. Nelson, J. D. Lifson, K. Früh, L. J. Picker, Cytomegalovirus vectors violate CD8+ T cell epitope recognition paradigms. *Science (80-.).* **340** (2013), , doi:10.1126/science.1237874.

70. A. W. Sylwester, B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, J. A. Nelson, L. J. Picker, Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J. Exp. Med.* **202**, 673–685 (2005).

71. A. V. S. Hill, C. E. M. Allsopp, D. Kwiatkowski, N. M. Anstey, P. Twumasi, P. A. Rowe, S. Bennett, D. Brewster, A. J. McMichael, B. M. Greenwood, Common West African HLA antigens are associated with protection from severe malaria. *Nat. 1991 3526336*. **352**, 595–600 (1991).

72. J. M. Ellis, V. Henson, R. Slack, J. Ng, R. J. Hartzman, C. Katovich Hurley, Frequencies of HLA-A2 alleles in five U.S. population groups: Predominance of A∗02011 and identification of HLA-A∗0231. *Hum. Immunol.* **61**, 334–340 (2000).

73. U. Lrich, H. Von A Ndrian, C. Harles, R. M. Ackay, T-Cell Function and Migration — Two Sides of the Same Coin. *http://dx.doi.org/10.1056/NEJM200010053431407*. **343**, 1020–1034 (2009).

74. K. SM, H. S, K. E, A. R, Molecular and functional profiling of memory CD8 T cell differentiation. *Cell*. **111**, 837–851 (2002).

75. R. G. Jones, C. B. Thompson, Revving the Engine: Signal Transduction Fuels T Cell Activation. *Immunity*. **27**, 173–178 (2007).

76. D. Masopust, L. J. Picker, and Early Pathogen Interception Hidden Memories: Frontline Memory T Cells. *J Immunol Ref*. **188**, 5811–5817 (2012).

77. E. C. Butcher, L. J. Picker, Lymphocyte Homing and Homeostasis. *Science (80-.)*. **272**, 60–66 (1996).

78. S. M. Kaech, J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, R. Ahmed, Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol. 2003 412*. **4**, 1191–1198 (2003).

79. S. M. Kaech, E. J. Wherry, R. Ahmed, Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol. 2002 24*. **2**, 251–262 (2002).

80. J. M. Schenkel, D. Masopust, Tissue-Resident Memory T Cells. *Immunity*. **41**, 886–897 (2014).

81. D. Masopust, D. Choo, V. Vezys, E. J. Wherry, J. Duraiswamy, R. Akondy, J. Wang, K. A. Casey, D. L. Barber, K. S. Kawamura, K. A. Fraser, R. J. Webby, V. Brinkmann, E. C. Butcher, K. A. Newell, R. Ahmed, Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* **207**, 553–564 (2010).

82. V. Muruganandah, H. D. Sathkumara, S. Navarro, A. Kupz, A systematic review: The role of resident memory T cells in infectious diseases and their relevance for vaccine development. *Front. Immunol.* **9**, 1574 (2018).

83. T. Gebhardt, L. M. Wakim, L. Eidsmo, P. C. Reading, W. R. Heath, F. R. Carbone, Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol. 2009 105*. **10**, 524–530 (2009).

84. X. Jiang, R. A. Clark, L. Liu, A. J. Wagers, R. C. Fuhlbrigge, T. S. Kupper, Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. *Nat. 2012 4837388*. **483**, 227–231 (2012).

85. A. Jozwik, M. S. Habibi, A. Paras, J. Zhu, A. Guvenel, J. Dhariwal, M. Almond, E. H. C. Wong, A. Sykes, M. Maybeno, J. Del Rosario, M. B. Trujillo-Torralbo, P. Mallia, J. Sidney,

B. Peters, O. M. Kon, A. Sette, S. L. Johnston, P. J. Openshaw, C. Chiu, RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. *Nat. Commun.* **6** (2015), doi:10.1038/ncomms10224.

86. F. Sallusto, J. Geginat, A. Lanzavecchia, Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance. *https://doi.org/10.1146/annurev.immunol.22.012703.104702*. **22**, 745–763 (2004).

87. J. C. Nolz, J. T. Harty, Protective Capacity of Memory CD8+ T Cells Is Dictated by Antigen Exposure History and Nature of the Infection. *Immunity*. **34**, 781–793 (2011).

88. K. M. Huster, M. Koffler, C. Stemberger, M. Schiemann, H. Wagner, D. H. Busch, Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. *Eur. J. Immunol.* **36**, 1453–1464 (2006).

89. B. Pulendran, R. Ahmed, Immunological mechanisms of vaccination. *Nat. Immunol. 2011 126*. **12**, 509–517 (2011).

90. F. Sallusto, A. Lanzavecchia, K. Araki, R. Ahmed, From Vaccines to Memory and Back. *Immunity*. **33**, 451–463 (2010).

91. J. Sadoff, G. Gray, A. Vandebosch, V. Cárdenas, G. Shukarev, B. Grinsztejn, P. A. Goepfert, C. Truyers, H. Fennema, B. Spiessens, K. Offergeld, G. Scheper, K. L. Taylor, M. L. Robb, J. Treanor, D. H. Barouch, J. Stoddard, M. F. Ryser, M. A. Marovich, K. M. Neuzil, L. Corey, N. Cauwenberghs, T. Tanner, K. Hardt, J. Ruiz-Guiñazú, M. Le Gars, H. Schuitemaker, J. Van Hoof, F. Struyf, M. Douoguih, Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19. *N. Engl. J. Med.*, NEJMoa2101544 (2021).

92. F. P. Polack, S. J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, J. L. Perez, G. Pérez Marc, E. D. Moreira, C. Zerbini, R. Bailey, K. A. Swanson, S. Roychoudhury, K. Koury, P. Li, W. V. Kalina, D. Cooper, R. W. Frenck, L. L. Hammitt, Ö. Türeci, H. Nell, A. Schaefer, S. Ünal, D. B. Tresnan, S. Mather, P. R. Dormitzer, U. Şahin, K. U. Jansen, W. C. Gruber, Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* **383**, 2603–2615 (2020).

93. T. J. Ruckwardt, K. M. Morabito, B. S. Graham, Immunological Lessons from Respiratory Syncytial Virus Vaccine Development. *Immunity*. **51**, 429–442 (2019).

94. B. Pulendran, R. Ahmed, Immunological mechanisms of vaccination. *Nat. Immunol. 2011 126*. **12**, 509–517 (2011).

95. S. G. Hansen, D. E. Zak, G. Xu, J. C. Ford, E. E. Marshall, D. Malouli, R. M. Gilbride, C. M. Hughes, A. B. Ventura, E. Ainslie, K. T. Randall, A. N. Selseth, P. Rundstrom, L. Herlache, M. S. Lewis, H. Park, S. L. Planer, J. M. Turner, M. Fischer, C. Armstrong, R. C. Zweig, J. Valvo, J. M. Braun, S. Shankar, L. Lu, A. W. Sylwester, A. W. Legasse, M. Messerle, M. A. Jarvis, L. M. Amon, A. Aderem, G. Alter, D. J. Laddy, M. Stone, A. Bonavia, T. G. Evans, M. K. Axthelm, K. Früh, P. T. Edlefsen, L. J. Picker, Prevention of tuberculosis in rhesus macaques by a cytomegalovirus-based vaccine. *Nat. Med.* **24**, 130–143 (2018).

96. K. M. Grebe, J. W. Yewdell, J. R. Bennink, Heterosubtypic immunity to influenza A virus: where do we stand? *Microbes Infect.* **10**, 1024–1029 (2008).

97. K. A. Benton, J. A. Misplon, C.-Y. Lo, R. R. Brutkiewicz, S. A. Prasad, S. L. Epstein, Heterosubtypic Immunity to Influenza A Virus in Mice Lacking IgA, All Ig, NKT Cells, or γδ T Cells. *J. Immunol.* **166**, 7437–7445 (2001).

98. K. Sun, J. Ye, D. R. Perez, D. W. Metzger, Seasonal FluMist Vaccination Induces Cross-

Reactive T Cell Immunity against H1N1 (2009) Influenza and Secondary Bacterial Infections. *J. Immunol.* **186**, 987–993 (2011).

99. K. M. Morabito, T. R. Ruckwardt, A. J. Redwood, S. M. Moin, D. A. Price, B. S. Graham, Intranasal administration of RSV antigen-expressing MCMV elicits robust tissue-resident effector and effector memory CD8+ T cells in the lung. *Mucosal Immunol. 2017 102*. **10**, 545–554 (2016).

100. S. G. Deeks, B. D. Walker, Human Immunodeficiency Virus Controllers: Mechanisms of Durable Virus Control in the Absence of Antiretroviral Therapy. *Immunity*. **27** (2007), pp. 406–416.

101. S. G. Hansen, M. P. Jr, A. B. Ventura, C. M. Hughes, R. M. Gilbride, J. C. Ford, K. Oswald, R. Shoemaker, Y. Li, M. S. Lewis, A. N. Gilliam, G. Xu, N. Whizin, B. J. Burwitz, S. L. Planer, J. M. Turner, A. W. Legasse, M. K. Axthelm, J. A. Nelson, K. Früh, J. B. Sacha, J. D. Estes, B. F. Keele, P. T. Edlefsen, J. D. Lifson, L. J. Picker, Immune clearance of highly pathogenic SIV infection. *Nature*. **502**, 100 (2013).

102. A. F. Wilfredo Garcia-Beltran, K. J. St Denis, A. Hoelzemer, A. John Iafrate, V. Naranbhai, A. B. Balazs, mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant (2021), doi:10.1016/j.cell.2021.12.033.

103. J. S. Turner, W. Kim, E. Kalaidina, C. W. Goss, A. M. Rauseo, A. J. Schmitz, L. Hansen, A. Haile, M. K. Klebert, I. Pusic, J. A. O'Halloran, R. M. Presti, A. H. Ellebedy, SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans. *Nat. 2021 5957867*. **595**, 421–425 (2021).

104. G. Giannotta, N. Giannotta, mRNA COVID-19 Vaccines and Long-Lived Plasma Cells: A Complicated Relationship. *Vaccines 2021, Vol. 9, Page 1503*. **9**, 1503 (2021).

105. L. Swadling, M. O. Diniz, N. M. Schmidt, O. E. Amin, A. Chandran, E. Shaw, C. Pade, J. M. Gibbons, N. Le Bert, A. T. Tan, A. Jeffery-Smith, C. C. S. Tan, C. Y. L. Tham, S. Kucykowicz, G. Aidoo-Micah, J. Rosenheim, J. Davies, M. Johnson, M. P. Jensen, G. Joy, L. E. McCoy, A. M. Valdes, B. M. Chain, D. Goldblatt, D. M. Altmann, R. J. Boyton, C. Manisty, T. A. Treibel, J. C. Moon, H. Abbass, A. Abiodun, M. Alfarih, Z. Alldis, M. Andiapen, J. Artico, J. B. Augusto, G. L. Baca, S. N. L. Bailey, A. N. Bhuva, A. Boulter, R. Bowles, R. J. Boyton, O. V. Bracken, B. O'Brien, T. Brooks, N. Bullock, D. K. Butler, G. Captur, N. Champion, C. Chan, D. Collier, J. C. de Sousa, X. Couto-Parada, T. Cutino-Mogue, R. H. Davies, B. Douglas, C. Di Genova, K. Dieobi-Anene, A. Ellis, K. Feehan, M. Finlay, M. Fontana, N. Forooghi, C. Gaier, D. Gilroy, M. Hamblin, G. Harker, J. Hewson, L. M. Hickling, A. D. Hingorani, L. Howes, A. Hughes, G. Hughes, R. Hughes, I. Itua, V. Jardim, W.-Y. J. Lee, M. P. Jensen, J. Jones, M. Jones, G. Joy, V. Kapil, H. Kurdi, J. Lambourne, K.-M. Lin, S. Louth, V. Mandadapu, Á. McKnight, K. Menacho, C. Mfuko, O. Mitchelmore, C. Moon, D. Munoz-Sandoval, S. M. Murray, M. Noursadeghi, A. Otter, S. Palma, R. Parker, K. Patel, B. Pawarova, S. E. Petersen, B. Piniera, F. P. Pieper, D. Pope, M. Prossora, L. Rannigan, A. Rapala, C. J. Reynolds, A. Richards, M. Robathan, G. Sambile, A. Semper, A. Seraphim, M. Simion, A. Smit, M. Sugimoto, S. Taylor, N. Temperton, S. Thomas, G. D. Thornton, A. Tucker, J. Veerapen, M. Vijayakumar, S. Welch, T. Wodehouse, L. Wynne, D. Zahedi, L. van Dorp, F. Balloux, Á. McKnight, M. Noursadeghi, A. Bertoletti, M. K. Maini, Pre-existing polymerase-specific T cells expand in abortive seronegative SARS-CoV-2. *Nat. 2021 6017891*. **601**, 110–117 (2021).

106. P. A. Nesterenko, J. McLaughlin, B. L. Tsai, G. Burton Sojo, D. Cheng, D. Zhao, Z. Mao, N. J. Bangayan, M. B. Obusan, Y. Su, R. H. Ng, W. Chour, J. Xie, Y.-R. Li, D. Lee, M. Noguchi, C. Carmona, J. W. Phillips, J. T. Kim, L. Yang, J. R. Heath, P. C. Boutros, O. N. Witte, HLA-A∗02:01 restricted T cell receptors against the highly conserved SARS-CoV-2 polymerase cross-react with human coronaviruses. *Cell Rep.* **37**, 110167 (2021).

107. Z. Chen, E. John Wherry, T cell responses in patients with COVID-19. *Nat. Rev. Immunol.* **20**, 529–536 (2020).

Chapter 2

Droplet-based mRNA sequencing of fixed and permeabilized cells by CLInt-seq allows for antigen-specific TCR cloning

Intro:

The adaptive immune response depends on T Cell Receptor (TCR) diversity to orchestrate a defense against any foreign antigen (Ishizuka et al., 2009; Qi et al., 2014). The αβ TCR is a heterodimer of alpha and beta polypeptides that as a pair recognize peptides, derived from proteins, presented on the Major Histocompatibility (MHC) complex(Sewell, 2012). Alpha and beta chains are generated in the thymus by random somatic recombination of the V, D and J regions to yield a theoretical diversity of over 10^{15} clonotypes(Sewell, 2012). Those TCRs that react with self-derived epitopes or do not recognize MHC are predominantly deleted during development(Takaba and Takayanagi, 2017). One recent estimate of adult TCR diversity in the peripheral blood is 10^8 unique clones(Qi et al., 2014). Isolating rare antigen-specific TCR sequences from such diverse populations remains a significant technical challenge.

Robust techniques have been developed for sequencing TCRs of immunodominant viral and tumor T cell responses(Betts et al., 2003; Dolton et al., 2018; Parkhurst et al., 2017; Rodenko et al., 2006; Wolfl et al., 2007; Wölfl et al., 2008). Immunodominance is the observation that the immune response is focused on a small number of antigenic epitopes and was initially discovered when only one MHC allele allowed the immune recognition of virus infected cells(Zinkernagel and Doherty, 1974). Peptide-MHC multimers representing these epitopes are used for physical staining of the TCR and allow for robust and antigen-specific TCR sequencing, often via FACS sorting of the multimer-positive population(Bethune et al., 2018; Rodenko et al., 2006). Multimer-based isolation of T cells coupled with TCR sequencing revolutionized our

understanding of how viral antigens produce T cell responses(Glanville et al., 2017; Yu et al., 2015b). However, these reagents are epitope-specific and laborious to construct and so are best suited for studying a small number of well defined, dominant epitopes(Alanio et al., 2010; Yu et al., 2015b).

More recently, surface activation markers such as CD137 and CD107a/b have been used to isolate live T cells that have been activated *in vitro*, allowing for isolation and sequencing of antigen-specific TCRs without multimer staining(Betts et al., 2003; Wolfl et al., 2007). These activation marker techniques permit isolation of TCRs reactive with previously undefined epitopes(Parkhurst et al., 2017; Wolfl et al., 2007). The activation marker CD137 has been used for isolation of novel tumor associated, as well as neoantigen-reactive TCRs(Parkhurst et al., 2017; Wolfl et al., 2007). Once cells are isolated, single-cell sequencing can produce TCR alpha/beta pair identity of thousands of cells(Oh et al., 2020; Wu et al., 2020; Yost et al., 2019). Cell surface markers like CD137 are used as proxies for the intracellular effector molecules that respond to T cell activation and can return a false positive signal. These surface activation markers are expressed on up to 0.5% of CD8+ T cells in absence of TCR stimulation. This background expression could make it difficult to specifically identify low frequency responses. Yet many therapeutic T cell responses to viruses and tumors are against undefined, low frequency epitopes.

We sought to establish an mRNA sequencing protocol that could characterize low frequency T cell populations of defined phenotype and function. We surmised that a method based on intracellular protein profiling could provide the desired additional specificity. Such a method

would allow targeting of any protein, including cell type-defining signaling molecules and transcription factors. Transcription factors (TFs), alone or in combination, are lineage defining molecules and have been shown necessary and sufficient for cell differentiation and identity in many contexts(Honaker et al., 2020; Iwafuchi-Doi and Zaret, 2016; Iwafuchi-Doi et al., 2016; Lambert et al., 2018; Takahashi and Yamanaka, 2006). For example, in the neuronal lineage the TFs SOX2, PAX6 and EOMES identify human radial glia progenitors(Thomsen et al., 2016). In T cells, our area of interest, FOXP3 uniquely marks T-regulatory cells (Tregs), which can oppose cytotoxic T cell responses and either prevent destruction of healthy tissue or promote tumor progression(Ali et al., 2014; DeLeeuw et al., 2012; Li and Rudensky, 2016; Nair et al., 2007; Sakaguchi, 2005). Another CD4+ cell type involved in tissue homeostasis is Th17 cells, which produce IL-17 and protect organs from bacteria, but can also mediate autoimmune pathology in IFNγ independent manner(Korn et al., 2009). RORγt transcription factor directs differentiation and identifies Th17 cells(Ivanov et al., 2006; Korn et al., 2009).

Antigen specific CD4+ and CD8+ T cells are often quantified by their intracellular cytokine production capacity(Le Bert et al., 2020; Pitcher et al., 1999; Sylwester et al., 2005). Cytokine secretion is first inhibited and subsequently cells are permeabilized for cytoplasmic cytokine staining (Pitcher et al., 1999; Sylwester et al., 2005; Waldrop et al., 1997) and flow cytometric analysis. Epitope mapping studies have used ICS as a measurement of reactivity and established the antigen scalability of this approach(Le Bert et al., 2020; Hansen et al., 2013; Sylwester et al., 2005). This technique has very low background, in the absence of stimulation through the TCR(Slifka et al., 1999; Waldrop et al., 1997). Different antigen stimulation approaches can be combined with ICS and exact epitope knowledge is not required(Hansen et al., 2013; Linnemann

et al., 2014). For discovery purposes, pools of over 100 peptides can be routinely used(Adler et al., 2016; Hansen et al., 2013; Murray et al., 2017; Sylwester et al., 2005).

Single-cell sequencing of neuronal cells selected based on an intracellular antigen has been described, however that technology has not been performed with the droplet-based sequencing required to rapidly process reactive TCRs (Thomsen et al., 2016). More recently, methanol based permeabilization has been used to select a population of cells based on an intracellular marker for droplet-based sequencing(Katzenelenbogen et al., 2020). In this work, we define a technology for droplet-based mRNA sequencing in cells fixed and permeabilized for ICS. This technology, CLInt-Seq (sequencing via **C**ross**l**inker regulated **Int**racellular phenotype), performs similarly to multimer-based approaches but can be used to discover new peptide-TCR pairs. As proof-of-principle, we used CLInt-Seq to sequence TCRs in EBV-reactive CD8+ T cells selected for TNFα and IFNγ expression. For comparison, selection with multimers was performed in parallel and had comparable efficiency. We also demonstrate that the technology can be applied to sequence Treg cells by profiling the lineage specific transcription factor, FOXP3. Finally, we adapt the method to be compatible with a single cell sequencing platform that allows comprehensive profiling of the αβ TCR response.

Results:

Intracellular staining identifies antigen specific T cells with a lower rate of false positives than cell surface profiling by the activation marker CD137

To compare the specificity of surface activation markers and ICS for the identification of reactive T cells, we tested the ability of both techniques to identify true positive antigen-specific

T cells in an activation assay. Tissue restricted antigen NY-ESO-1, which is overexpressed in

multiple tumors, and a cognate TCR (clone 1G4) co-linked to NGFR were used to create a model

system for estimating the specificity of the ICS approach compared to the CD137

method(Bethune et al., 2018; D'angelo et al., 2018; Robbins et al., 2011b). False positives were

defined by flow cytometry as events that expressed an activation marker but not the TCR

construct. To produce a simulated population that is about 1% positive, a TCR-transduced

population was added into an untransduced population of cells (Fig 1A). Cells were then

stimulated and flow cytometry was performed for CD137 and compared to ICS for TNFα or

IFNγ (Fig 1B). Both assays had comparable sensitivity. However, 3.09% of CD8+ T cells were

in the NGFR⁻/CD137⁺ quadrant (Fig 1B). Illustrating a high background in this assay due to

either constitutive low-level expression or bystander T cell activation. However, only 0.08% and

0.13% of CD8+ T cells were in the NGFR⁻/TNFα⁺ or NGFR⁻/IFNγ⁺ compartments respectively

(Fig 1B). In this model system ICS provided greater specificity than CD137 staining.

TCR alpha/beta pairs can be recovered from primary T cells after intracellular staining
with efficiency equivalent to multimer-based selection

Anticipating that TCR mRNA sequencing would be less robust from fixed and permeabilized

cells, we overexpressed melanoma antigen MART-1 TCR (clone F5) in PBMC to test if TCRs

could be sequenced from IC stained T cells with high level of target mRNA(Bethune et al., 2018;

Johnson et al., 2006a). Cells were then activated with MART-1 peptide and stained for

intracellular TNFα and IFNγ by adapting a published method of single-cell mRNA sequencing in

permeabilized cells that used paraformaldehyde (PFA) as a crosslinker(Thomsen et al., 2016). In

parallel, a control arm was set up where cells were selected with MART-1 multimer. Reactive

cells were then singly deposited by FACS for alpha/beta paired TCR sequencing (See SI

Appendix, fig. S1). TCR clones were isolated from single-cell RT-PCR reactions. Both techniques had equivalent efficiency, measured as a fraction of TCR alpha/beta pairs recovered (75%). This proof-of-concept experiment demonstrated single-cell TCR mRNA could be sequenced from cells that were stained for intracellular antigens.

Having shown ability to sequence TCRs in the setting of overexpression, we sought to measure antigen-specific TCR recovery in a population of normal human PBMCs. As a proof-of-concept, virus-specific T cell responses from two different donors were profiled. Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are common Herpes viruses that infect over 50% of people and generate large memory T cell responses(Klenerman and Oxenius, 2016). Virus-specific CD8+ T cells were singly deposited based on TNFα and IFNγ expression into RT-PCR wells and TCRs were subsequently sequenced (Fig 2B). As a control, we also sorted and sequenced cells based on CD137 production and multimer staining (Fig 2B).

All three techniques (ICS, multimer staining, and CD137 staining) recovered clonal TCRs (Fig 2C). Although the CMV response was oligoclonal, some clones were clearly dominant. In fact, the same dominant clone appeared in all three techniques. In contrast, the EBV-responding TCRs were polyclonal, which is typical of EBV responses. The dominant EBV clone identified by tetramer and CD137 staining appeared only once in ICS, however the subdominant clones appeared 3/55 and 7/55 times as measured by ICS. Cumulatively, this data showed that ICS could enable the isolation and sequencing of TCRs in single cells. This experiment also showed that ICS can be used for TCR cloning with reasonable efficiency, 33-54% compared to 55-94% with live cells (Fig 2C).

The true test for TCR antigen specificity is clonal sequence isolation and transplantation into allogeneic human T cells. This tests if an assay will identify high affinity TCRs, rather than simply cross-reactive clones. Four CMV TCR clones were cloned into retroviral vectors and overexpressed in human PBMCs (Fig 2D) (Dataset S01). Functional capability of these clones was evaluated by cytokine production in a cytotoxicity assay. T cells transduced with the CMV TCRs were cocultured with a PC3 epithelial prostate cancer cell line that expressed HLA-A*02:01 with or without the CMV pp65 protein. All three CMV TCRs that appeared in both tetramer and ICS based selection led to the production of IFNγ when T cells were co-cultured with target cells that express CMV pp65 protein (Fig 2E). Additionally, these same T cells were able to specifically kill target cells that expressed the protein (Fig 2F).

T regulatory cell TCR identification by intra-nuclear profiling of FOXP3.

To explore the ability of ICS based TCR sequencing to characterize immune responses beyond those mediated by cytotoxic CD8+ T cells, we performed single-cell sequencing of T cells that expressed the classic Treg markers: CD3, CD4, CD25 and FOXP3 (Fig 3A)(Raffin et al., 2020). Knowledge of Treg epitopes has been limited to indirect analyses due to the need to phenotype Tregs exclusively by nuclear transcription factor FOXP3(Ahmadzadeh et al., 2019). Our ICS-based cloning efficiently isolated TCRs in this population, as 33 out of 40 single cells deposited returned productive alpha/beta pairs (83%) (Fig. 3A). A specific peptide was not queried, thus the TCRs identified did not show any clonality, unlike the viral antigen specific CD8+ T cells analyzed previously (Fig 3B). Thus, ICS based selection can identify TCRs across T cell phenotypes and functionalities.

Single-cell sequencing of fixed and permeabilized cells in droplet-based format

Very large TCR diversity as well as clonal expansion necessitates sequencing of hundreds of cells to understand the scope of reactivity. Current techniques for parallel single-cell sequencing in thousands of cells are based on microfluidics technology, which encapsulates cells into fluid droplets(Macosko et al., 2015). In our hands, PFA fixed cells performed poorly with a droplet-based sequencing approach (Fig 4B). As an alternative, we optimized the protocol with a chemically reversible crosslinker, DSP (Lomant's Reagent, 3,3'-Dithiodipropionic acid di(Nhydroxysuccinimide ester)) (See SI Appendix, fig. S2)(Subramonia Iyer et al., 1973). DSP reacts with primary amines, has a disulfide bond in the center, and can be cleaved via a reducing agent. This reagent has also been used to preserve cells prior to sequencing, as well as for sequencing of permeabilized cells selected by phospho-protein, but neither example was adapted for droplet-based sequencing (Attar et al., 2018; Gerlach et al., 2019).

CLInt-Seq was used to sequence αβ TCRs in a bulk T cell population via the 10X Genomics V(D)J library construction protocol (Fig 4A). Live cells and PFA-fixed cells were included for comparison. The cDNA profile of CLInt-Seq processed cells was comparable to live cells, but PFA treated cells had a poor cDNA profile (Fig 4B). Subsequently, these samples were sequenced and single-cell data analyzed (Fig 4C). CLInt-Seq cells performed similarly to a live cell control in terms of library cDNA concentration and number of cells successfully sequenced. The proportion of clones with both alpha and beta chains is indicative of the sample quality. CLInt-Seq cells contained only 4% of unpaired clones, compared to 8% in live cells. The PFA crosslinking yielded 81% of unpaired clones. The diversity of live and CLInt-Seq T cells was

similar (Fig 4D). Thus, gene expression analysis via droplet-based sequencing can be performed in permeabilized cells, fixed via DSP crosslinking.

CLInt-Seq coupled to droplet-based sequencing recovers EBV specific TCRs

It is critical that mRNA remains fixed to cellular protein mass prior to single-cell droplet encapsulation. If the mRNA is released prior to a cell being encapsulated into a fluid droplet, then mRNA cellular origin will not be identified correctly and αβ pairing will be lost. This can be estimated by recovery of correctly paired TCR αβ clones. A population of CLInt-Seq processed, EBV specific CD8+ T cells was selected by IFNγ and TNFα expression and sequenced with the 10X V(D)J single cell protocol (Fig 5A). The resulting clonotypes were compared to those derived from live, tetramer-selected cells. Both CLInt-Seq processed cells and live cells returned a similar number of cells captured (Fig 5B). Live cells showed greater recovery of αβ pairs (992 pairs vs 430 pairs), as well as a lower frequency of unpaired clones (21% compared to 37%, Fig 5B). To determine if mRNA identity had been maintained, we compared clonotypes recovered by CLInt-Seq to tetramer-selected live cells. The two clonotype populations overlapped significantly, indicating that TCR mRNA can be faithfully recovered from single cells prepared in this manner (Fig 5C). The shared TCRs also represented a diversity of clonotype frequencies (Fig 5D). This indicated that mRNA cellular origin is maintained when cells are processed per the CLInt-Seq protocol.

Discussion:

We describe a technology for high throughput mRNA sequencing at single-cell level in cells that have been fixed and permeabilized to allow for intracellular staining. Primary amines of protein are crosslinked by DSP, a homo-bifunctional and cleavable crosslinker, which fixes the RNA

inside the cell. Encapsulation of single cells into fluid droplets by the droplet-based cell barcoding, allows for DSP cleaving and release of mRNA for the reverse transcription reaction. This technology can be generalized for any cell type for profiling gene expression in minor populations of cells that cannot be selected by a set of surface markers. As an example, cells could be selected based on expression of one or more transcription factors, which function cooperatively to define cell fate and function. In the lymphocyte lineage, this technology could be used to clone B cell receptors for antibody development. B cells can be identified via activation assays and intracellular activation marker staining. Once the B cell receptor is engaged, phosphorylated form of Bruton's Tyrosine Kinase is generated and could possibly be used as a marker of antigen specific B cells(Nisitani et al., 1999). Multiomics techniques for parallel measurement of gene expression and protein abundance have been described, however so far been limited to the cell surface proteome(Peterson et al., 2017; Stoeckius et al., 2017). These techniques use antibody-oligo complexes that allow oligo barcode sequencing in parallel with cellular mRNA, to quantify protein abundance. Staining for intracellular antigens would allow for such analysis without the cell surface proteome limitation.

In future work in T cells, CLInt-Seq could help improve our understanding of the T cell response to diverse antigens. Currently, only three viruses dominate the landscape of known TCR-epitope reactivities. Of the 193 unique, well-described TCR-epitope reactivities in the published literature, 100 of them are derived from CMV, EBV or HIV(Bagaev et al., 2020). Insights gained are extrapolated to other self or viral antigens and systems, but no two TCR-epitope reactivities are identical(Culina et al., 2018; Leitman et al., 2016; Maness et al., 2007). The uniqueness of each TCR reactivity is illustrated in systems where a large effort has been spent on defining and

characterizing the T cell response, such as HIV. A rare subset of HIV-positive individuals, termed elite controllers, keep viral load low for decades presumably by the CD8+ T cell activity directed against a specific HLA allele, B*57(Collins et al., 2020; Leitman et al., 2016; Walker and Yu, 2013). Subdominant, low frequency HLA-B*57 restricted TCRs are particularly shown to have strong effector activity, by suppressing viral replication(Leitman et al., 2016). Less is known about T cell responses to non-viral antigens; however, existing data suggests that specific TCR reactivities can determine physiological outcomes. Type 1 diabetes is characterized by progressive loss of insulin producing beta cells of the pancreas(Culina et al., 2018). This loss is mediated by CD4+ and CD8+ T cells that recognize self-antigen with low affinity(Bulek et al., 2012; Culina et al., 2018; Delong et al., 2016; Pinkse et al., 2005). CD4+ T cell reactivity against beta cells has been shown to be mediated by recognition of peptide fusions(Delong et al., 2016). Both HIV and diabetes, as an example of self-tissue destruction by T cells that somehow escaped deletion in the thymus, exemplify the need to study specific TCR-epitope pairs, to be able to answer specific questions in health and disease. CLInt-Seq allows stimulation of T cells with over 100 epitopes in parallel and recovers low-frequency, antigen-specific TCRs that could rapidly expand the systematic study of TCR-epitope interactions beyond these viral responses(Le Bert et al., 2020; Hansen et al., 2013).

Rather than focusing on specific antigen reactivities, CLInt-Seq can be used to characterize TCRs in T cell sub-populations with critical roles in immune response. For example, antigen-specific tumor infiltrating lymphocytes (TILs) are found inside the CD39/CD103 compartment(Duhen et al., 2018). However, identifying strongly reactive, anti-tumor TILs remains a challenge. T cells are a dynamic lineage, where differentiation and activation events

are directed by groups of transcription factors(Oestreich and Weinmann, 2012). Nur77 has been shown to be upregulated upon TCR engagement and signaling, and in fact functions as a rheostat to indicate TCR signaling strength(Ashouri and Weiss, 2016; Moran et al., 2011). More recently, the Nur77 family of transcription factors has been implicated in T cell exhaustion, which further confirms its role in T cell receptor signaling(Chen et al., 2019). CLInt-Seq staining for nuclear transcription factor expression could help identify cancer specific CD4+ and CD8+ T cells in the tumor microenvironment(Ashouri and Weiss, 2016; Moran et al., 2011).

We anticipate that CLInt-Seq will speed the identification of candidate therapeutic TCRs immediately suitable for pre-clinical development. As single-cell TCR sequencing maintains alpha/beta pairing of receptors, the reconstructed TCR sequences can be readily tested as potential adoptive T-cell transfer-based therapeutics(Johnson et al., 2006b; Restifo et al., 2012; Robbins et al., 2011a). Adoptive cell therapy involves the infusion of laboratory-modified T cells that can recognize tumor, viral or self-antigens(D'angelo et al., 2018; Robbins et al., 2011a). CLInt-Seq readily identified TCRs reactive to EBV, and could be used to discover TCRs reactive to any antigen of interest, including cancer antigens like NY-ESO-1(D'angelo et al., 2018). In our group, we are pursuing multiple projects to develop TCR reagents for treatment of prostate cancer. We have applied CLInt-Seq to identify TCRs reactive with peptide epitopes of Prostatic Acid Phosphatase, which has been pursued by multiple groups as an immunotherapeutic target in prostate cancer(Becker et al., 2010; Johnson and McNeel, 2012; Kantoff et al., 2010; Olson et al., 2010). Prior work on this target resulted in an FDA approved cellular therapy for prostate cancer, Sipuleucel-T(Kantoff et al., 2010). Analogously, Treg TCR profiling using CLInt-Seq

can query antigen specific TCRs for possible use with Treg adoptive cell therapy to direct an immunosuppressive response(Raffin et al., 2020).

Methods:

Methods on cell culture, cell sorting, TCR cloning from 96well plates, TCR vector construction, virus generation and cytotoxicity testing were adapted from our prior publications and are available in the SI appendix.

PFA based intracellular staining:

PBMCs are washed 2 times with PBS (Fisher Scientific cat. no. MT-46013CM) and once with TCRPMI. Then cells are resuspended in TCRPMI at 500,000 cells/100 µL of media and aliquoted into 96 well plate (Corning cat. no. 353077) for 12-hour rest prior to intracellular staining stimulation. Then, 100 µL of TCRPMI with 20 µg/ml of peptide and 2 µg/ml of CD28/49d antibodies (BD cat. no. 347690) are added to each well. AIM V complete media is used for TCR overexpression experiment. Cells are incubated for one hour at 37C 5% CO_2 and 20 µL of 10X brefeldin A (Biolegened cat no. 420601) is added to each well. Cells are further incubated for 8 hours. For TCR sequencing cells are stained immediately under RNAse free conditions with the FRISCR protocol adapted from (Thomsen et al). Briefly each well is washed twice with 200 µL of wash buffer, which contains nuclease free water (Thermo Fisher, cat. no. 4387936), 10X molecular biology grade PBS, 1% nuclease free BSA (Gemini cat. no. 700-106P)), and 1:400 RNAsin Plus (Promega cat. no. N2615). For TCR overexpression and subsequent sequencing experiment we used RNAsin plus at 1:40000 dilution. The cells are stained with following surface antibodies: CD3-APCCy7 (Thermo Fisher, cat. no. 47-0036-42), CD8a-PE (Thermo Fisher, cat. no. 12-0088-42), CD4-PECy7 (Biolegend, cat. no. 300512). After

staining for 15 minutes at 4C cells are washed with wash buffer and fixed with 100 µL of 4% PFA (EMS cat. no. 15710) for 10 minutes at 4C. Then cells are washed twice and resuspended in 1% BSA buffer with .1% Triton X-100 (Sigma-Aldrich cat. no. T8787) for 10 minutes. Cells are then washed and subsequently stained with intracellular antibodies in wash buffer for IFNγ-APC (Biolegend cat. no. 506510), TNFα-FITC (Biolegend cat. no. 502906), FoxP3-A488 (Biolegend cat. no. 320012), msIgG1-A488 (Thermo Fisher, cat. no. MG120). Cells are then washed and resuspended in wash buffer for FACS analysis. Intracellular staining where we did not plan to do TCR sequencing was done in the absence of RNAsin plus inhibitor.

CLInt-Seq staining:

All buffers except crosslinker step contain 1:400 RNAsin and molecular biology grade PBS to inhibit RNA degradation. Staining is performed in a 96 well U bottom plate. Cells were first washed twice in 200µL of 1% BSA buffer with 1:400 RNAsin (wash buffer) and incubated for 15 minutes on ice with antibodies against surface antigens. DSP (ThermoFisher cat. no. 22585) is stored at -20C in a desiccant filled container. Immediately prior to experiment DSP is left at room temperature for at least 30 minutes and then prepared to a concentration of 50 mg/ml in molecular biology grade DMSO (Sigma cat. no. D8418-50ML). Then 1mg/ml solution is prepared in molecular biology grade PBS, by vortexing 20ul of DSP in a 15ml conical tube and adding 1mL of PBS with P1000. DSP is filtered using a 40µm Flowmi strainer (Sigma cat. no. BAH136800040-50EA). Then .25mg/ml solution is prepared. Cells are washed once with wash buffer and twice with PBS and resuspended in 200 µL of .25mg/ml DSP (Thermo Fisher). Cells are incubated at room temperature for 30 minutes and quenched with 20mM Tris (Thermo Fisher cat. no. AM9850G). Cells are then washed twice and incubated for 10 minutes with 100µL of .05% Triton

X-100 (Thermo Fisher) in wash buffer. Subsequently, cells are washed and resuspended in wash buffer for 20 minutes with antibodies against intracellular antigens. Then, cells are washed again and resuspended in wash buffer for FACS sorting.

10X Genomics single-cell library construction and sequencing:

FACS isolated cells were collected in 2mL Eppendorf tube that contained 400ul of .04% BSA with 1:400 RNAsin. Cells were pelleted at 750g for 3 min. Supernatant was discarded leaving 30-60µL, to reach a final concentration of more than 100 cells/µL. When we knew we could not get enough antigen-specific T cells, we added Jurkat E6.1 carrier cells. 10X genomics human V(D)J libraries were prepared by the UCLA Technology Center for Genomics & Bioinformatics following the standard protocol for 10X library construction. Single cell TCR libraries were sequenced by Illumina MiSeq or NextSeq. Data was analyzed by using 10X genomics pipeline to generate Vloupe files.

CD137 and tetramer staining:

PBMCs were either cultured with TCRPMI as described above and reported previously(Bethune et al., 2018). For TCR overexpression experiments we used AIM V media as described above. PBMCs were washed with PBS two times and once with media, subsequently resuspended at 5×10^5 cells/ 100 µL and aliquoted in 96 well plate for 12 hour rest. Then, cells were stimulated with 20 µg/ml of antigenic peptide and 2 µg/ml of CD28/49d in 100 µL of media for 24 hours. PBMCs were then washed with wash buffer as described above, but RNAsin plus inhibitor was excluded. PBMCs were then stained with CD3-APCCy7 (Thermo Fisher, cat. no. 47-0036-42), CD8a-PE (Thermo Fisher, cat. no. 12-0088-42), CD4-PECy7 and CD137-APC (Biolegend cat. no. 309810) antibody for 20 minutes. Subsequently, cells were washed, resuspended in wash

buffer and 7-AAD (BD cat. no. 559925) or DAPI was added immediately prior to FACS analysis or sorting. Multimer staining was performed with tetramers as previously described and MART-1 (ELAGIGILTV) HLA-A2 tetramer was made in-house(Bethune et al., 2018). Tetramers for NY-ESO-1(MBL cat.no. TB-M011-1), CMV pp65 (MBL cat.no. TB-0010-2), EBV BMLF1(MBL cat.no. TB-M011-2) were purchased.

Author contributions:

P.A.N., J.M.W. and O.N.W. designed experiments. P.A.N., D.C., J.M.W., Y.Q., G.B.S. and M.B.O. performed experiments. P.A.N., D.C., J.M.W., J.W.P., N.J.B, C.S.S, and Z.M. analyzed data. P.A.N., J.W.P. and O.N.W. wrote the paper.

Competing interests statement:

O.N.W, P.A.N. and J.M.W. are inventors on a provisional patent application titled "Method to sequence mRNA in single cells in parallel with quantification of intracellular phenotype"

O.N.W. 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 reported in this article.

Data deposition:

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus(Edgar et al., 2002) and are accessible through GEO Series accession number GSE159927 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159927). Data will be available after Jan 01, 2021.

References:

Adler, S.P., Manganello, A.-M., Lee, R., Mcvoy, M.A., Nixon, D.E., Plotkin, S., Mocarski, E., Cox, J.H., Fast, P.E., Nesterenko, P.A., et al. (2016). The Journal of Infectious Diseases A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus-Seronegative Men.

Ahmadzadeh, M., Pasetto, A., Jia, L., Deniger, D.C., Stevanović, S., Robbins, P.F., and Rosenberg, S.A. (2019). Tumor-infiltrating human CD4 [+] regulatory T cells display a distinct TCR repertoire and exhibit tumor and neoantigen reactivity. Sci. Immunol. *4*, eaao4310.

Alanio, C., Lemaitre, F., Law, H.K.W., Hasan, M., and Albert, M.L. (2010). Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies. Blood *115*, 3718–3725.

Ali, K., Soond, D.R., Piñeiro, R., Hagemann, T., Pearce, W., Lim, E.L., Bouabe, H., Scudamore, C.L., Hancox, T., Maecker, H., et al. (2014). Inactivation of PI(3)K p110δ breaks regulatory T-cell-mediated immune tolerance to cancer. Nature *510*, 407–411.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Anand, S.P., Prévost, J., Nayrac, M., Beaudoin-Bussières, G., Benlarbi, M., Gasser, R., Brassard, N., Laumaea, A., Gong, S.Y., Bourassa, C., et al. (2021). Longitudinal analysis of humoral immunity against SARS-CoV-2 Spike in convalescent individuals up to 8 months post-symptom onset. Cell Reports Med. *2*, 100290.

Ashouri, J.F., and Weiss, A. (2016). B Cells Antigen Receptor Signaling in Human T and Endogenous Nur77 Is a Specific Indicator of.

Attar, M., Sharma, E., Li, S., Bryer, C., Cubitt, L., Broxholme, J., Lockstone, H., Kinchen, J.,

Simmons, A., Piazza, P., et al. (2018). A practical solution for preserving single cells for RNA sequencing. Sci. Rep. *8*, 1–10.

B, R., B, A., S, P., B, P., and M, N. (2020). NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. Nucleic Acids Res. *48*, W449–W454.

Baden, L.R., El Sahly, H.M., Essink, B., Kotloff, K., Frey, S., Novak, R., Diemert, D., Spector, S.A., Rouphael, N., Creech, C.B., et al. (2021). Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. N. Engl. J. Med. *384*, 403–416.

Bagaev, D. V., Vroomans, R.M.A., Samir, J., Stervbo, U., Rius, C., Dolton, G., Greenshields-Watson, A., Attaf, M., Egorov, E.S., Zvyagin, I. V., et al. (2020). VDJdb in 2019: Database extension, new analysis infrastructure and a T-cell receptor motif compendium. Nucleic Acids Res. *48*, D1057–D1062.

Bange, E.M., Han, N.A., Wileyto, P., Kim, J.Y., Gouma, S., Robinson, J., Greenplate, A.R., Hwee, M.A., Porterfield, F., Owoyemi, O., et al. (2021). CD8+ T cells contribute to survival in patients with COVID-19 and hematologic cancer. Nat. Med. 1–10.

Basar, R., Uprety, N., Ensley, E., Daher, M., Klein, K., Martinez, F., Aung, F., Shanley, M., Hu, B., Gokdemir, E., et al. (2021). Generation of glucocorticoid-resistant SARS-CoV-2 T cells for adoptive cell therapy. Cell Rep. *36*, 109432.

Becker, J.T., Olson, B.M., Johnson, L.E., Davies, J.G., Dunphy, E.J., and McNeel, D.G. (2010). DNA vaccine encoding prostatic acid phosphatase (PAP) elicits long-term T-cell responses in patients with recurrent prostate cancer. J. Immunother. *33*, 639–647.

Bernal, J.L., Andrews, N., Gower, C., Gallagher, E., Simmons, R., Thelwall, S., Stowe, J., Tessier, E., Groves, N., Dabrera, G., et al. (2021). Effectiveness of Covid-19 Vaccines against

the B.1.617.2 (Delta) Variant. Https://Doi.Org/10.1056/NEJMoa2108891 NEJMoa2108891.

Le Bert, N., Tan, A.T., Kunasegaran, K., Tham, C.Y.L., Hafezi, M., Chia, A., Chng, M.H.Y., Lin, M., Tan, N., Linster, M., et al. (2020). SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls. Nature *584*, 457.

Bethune, M.T., and Joglekar, A. V. (2017). Personalized T cell-mediated cancer immunotherapy: progress and challenges. Curr. Opin. Biotechnol. *48*, 142–152.

Bethune, M.T., Li, X.-H., Yu, J., McLaughlin, J., Cheng, D., Mathis, C., Moreno, B.H., Woods, K., Knights, A.J., and Garcia-Diaz, A. (2018). Isolation and characterization of NY-ESO-1– specific T cell receptors restricted on various MHC molecules. Proc. Natl. Acad. Sci. *115*, E10702–E10711.

Betts, M.R., Brenchley, J.M., Price, D.A., De Rosa, S.C., Douek, D.C., Roederer, M., and Koup, R.A. (2003). Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J. Immunol. Methods *281*, 65–78.

Bluestone, J.A., Buckner, J.H., Fitch, M., Gitelman, S.E., Gupta, S., Hellerstein, M.K., Herold, K.C., Lares, A., Lee, M.R., Li, K., et al. (2015). Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci. Transl. Med. *7*.

Boehmer, H. Von, Aifantis, I., Gounari, F., Azogui, O., Haughn, L., Apostolou, I., Jaeckel, E., Grassi, F., and Klein, L. (2003). Thymic selection revisited: how essential is it? Immunol. Rev. *191*, 62–78.

Braun, J., Loyal, L., Frentsch, M., Wendisch, D., Georg, P., Kurth, F., Hippenstiel, S., Dingeldey, M., Kruse, B., Fauchere, F., et al. (2020). SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19. Nature *587*, 270–274.

Bulek, A.M., Cole, D.K., Skowera, A., Dolton, G., Gras, S., Madura, F., Fuller, A., Miles, J.J.,

Gostick, E., Price, D.A., et al. (2012). Structural basis for the killing of human beta cells by CD8
+ T cells in type 1 diabetes. Nat. Immunol. *13*, 283–289.

Chen, Z., and John Wherry, E. (2020). T cell responses in patients with COVID-19. Nat. Rev.
Immunol. *20*, 529–536.

Chen, J., López-Moyado, I.F., Seo, H., Lio, C.W.J., Hempleman, L.J., Sekiya, T., Yoshimura,
A., Scott-Browne, J.P., and Rao, A. (2019). NR4A transcription factors limit CAR T cell
function in solid tumours. Nature *567*, 530–534.

Choudhuri, K., Kearney, A., Bakker, T.R., and Van Der Merwe, P.A. (2005). Immunology: How
Do T Cells Recognize Antigen? Curr. Biol. *15*, R382–R385.

Collins, D.R., Gaiha, G.D., and Walker, B.D. (2020). CD8+ T cells in HIV control, cure and
prevention. Nat. Rev. Immunol. *20*, 471–482.

Culina, S., Lalanne, A.I., Afonso, G., Cerosaletti, K., Pinto, S., Sebastiani, G., Kuranda, K., Nigi,
L., Eugster, A., Østerbye, T., et al. (2018). Islet-reactive CD8+ T cell frequencies in the
pancreas, but not in blood, distinguish type 1 diabetic patients from healthy donors. Sci.
Immunol. *3*, 4013.

D'angelo, S.P., Melchiori, L., Merchant, M.S., Bernstein, D., Glod, J., Kaplan, R., Grupp, S.,
Tap, W.D., Chagin, K., Binder, G.K., et al. (2018). Antitumor activity associated with prolonged
persistence of adoptively transferred NY-ESO-1c259T cells in synovial sarcoma. Cancer Discov.
8, 944–957.

D'Angelo, S.P., Melchiori, L., Merchant, M.S., Bernstein, D., Glod, J., Kaplan, R., Grupp, S.,
Tap, W.D., Chagin, K., Binder, G.K., et al. (2018). Antitumor Activity Associated with
Prolonged Persistence of Adoptively Transferred NY-ESO-1 c259T Cells in Synovial Sarcoma.
Cancer Discov. *8*, 944–957.

Davis, M.M., and Bjorkman, P.J. (1988). T-cell antigen receptor genes and T-cell recognition. Nat. 1988 3346181 *334*, 395–402.

DeLeeuw, R.J., Kost, S.E., Kakal, J.A., and Nelson, B.H. (2012). The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: A critical review of the literature. Clin. Cancer Res. *18*, 3022–3029.

Delong, T., Wiles, T.A., Baker, R.L., Bradley, B., Barbour, G., Reisdorph, R., Armstrong, M., Powell, R.L., Reisdorph, N., Kumar, N., et al. (2016). Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. Science (80-.). *351*, 711–714.

Depil, S., Duchateau, P., Grupp, S.A., Mufti, G., and Poirot, L. (2020). 'Off-the-shelf' allogeneic CAR T cells: development and challenges. Nat. Rev. Drug Discov. *19*, 185–199.

Dolton, G., Zervoudi, E., Rius, C., Wall, A., Thomas, H.L., Fuller, A., Yeo, L., Legut, M., Wheeler, S., Attaf, M., et al. (2018). Optimized Peptide–MHC Multimer Protocols for Detection and Isolation of Autoimmune T-Cells. Front. Immunol. *9*.

Duhen, T., Duhen, R., Montler, R., Moses, J., Moudgil, T., De Miranda, N.F., Goodall, C.P., Blair, T.C., Fox, B.A., McDermott, J.E., et al. (2018). Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat. Commun. *9*, 1–13.

Dutta, N.K., Mazumdar, K., and Gordy, J.T. (2020). The Nucleocapsid Protein of SARS–CoV-2: a Target for Vaccine Development. J. Virol. *94*.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. *30*, 207–210.

Einsele, H., Roosnek, E., Rufer, N., Sinzger, C., Riegler, S., Löffler, J., Grigoleit, U., Moris, A., Rammensee, H.-G., Kanz, L., et al. (2002). Infusion of cytomegalovirus (CMV)–specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood *99*, 3916–

3922.

Ferreras, C., Pascual-Miguel, B., Mestre-Durán, C., Navarro-Zapata, A., Clares-Villa, L., Martín-Cortázar, C., de Paz, R., Marcos, A., Vicario, J.L., Balas, A., et al. (2020). SARS-CoV-2 specific memory T lymphocytes from COVID-19 convalescent donors: Identification, biobanking and large-scale production for adoptive cell therapy. BioRxiv 2020.10.23.352294.

Feuchtinger, T., Opherk, K., Bethge, W.A., Topp, M.S., Schuster, F.R., Weissinger, E.M., Mohty, M., Or, R., Maschan, M., Schumm, M., et al. (2010a). Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood *116*, 4360–4367.

Feuchtinger, T., Opherk, K., Bethge, W.A., Topp, M.S., Schuster, F.R., Weissinger, E.M., Mohty, M., Or, R., Maschan, M., Schumm, M., et al. (2010b). Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation.

Forni, D., Cagliani, R., Clerici, M., and Sironi, M. (2017). Molecular Evolution of Human Coronavirus Genomes. Trends Microbiol. *25*, 35–48.

Frank, A.M., Braun, A.H., Scheib, L., Agarwal, S., Schneider, I.C., Fusil, F., Perian, S., Sahin, U., Thalheimer, F.B., Verhoeyen, E., et al. (2020). Combining T-cell-specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. Blood Adv. *4*, 5702–5715.

Garcia, K.C., and Adams, E.J. (2005). How the T Cell Receptor Sees Antigen—A Structural View. Cell *122*, 333–336.

Gauttier, V., Morello, A., Girault, I., Mary, C., Belarif, L., Desselle, A., Wilhelm, E., Bourquard, T., Pengam, S., Teppaz, G., et al. (2020). Tissue-resident memory CD8 T-cell responses elicited by a single injection of a multi-target COVID-19 vaccine. BioRxiv 2020.08.14.240093.

Gerlach, J.P., van Buggenum, J.A.G., Tanis, S.E.J., Hogeweg, M., Heuts, B.M.H., Muraro, M.J., Elze, L., Rivello, F., Rakszewska, A., van Oudenaarden, A., et al. (2019). Combined quantification of intracellular (phospho-)proteins and transcriptomics from fixed single cells. Sci. Rep. *9*, 1469.

Glanville, J., Huang, H., Nau, A., Hatton, O., Wagar, L.E., Rubelt, F., Ji, X., Han, A., Krams, S.M., Pettus, C., et al. (2017). Identifying specificity groups in the T cell receptor repertoire. Nature *547*, 94–98.

Gordon, D.E., Jang, G.M., Bouhaddou, M., Xu, J., Obernier, K., White, K.M., O'Meara, M.J., Rezelj, V. V., Guo, J.Z., Swaney, D.L., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. Nature *583*, 459–468.

Grifoni, A., Sidney, J., Zhang, Y., Scheuermann, R.H., Peters, B., and Sette, A. (2020a). A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2. Cell Host Microbe *27*, 671-680.e2.

Grifoni, A., Weiskopf, D., Ramirez, S.I., Mateus, J., Dan, J.M., Moderbacher, C.R., Rawlings, S.A., Sutherland, A., Premkumar, L., Jadi, R.S., et al. (2020b). Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. Cell *181*, 1489-1501.e15.

Hall, C.B., Powell, K.R., MacDonald, N.E., Gala, C.L., Menegus, M.E., Suffin, S.C., and Cohen, H.J. (1986). Respiratory Syncytial Viral Infection in Children with Compromised Immune Function. N. Engl. J. Med. *315*, 77–81.

Hansen, S.G., Sacha, J.B., Hughes, C.M., Ford, J.C., Burwitz, B.J., Scholz, I., Gilbride, R.M., Lewis, M.S., Gilliam, A.N., Ventura, A.B., et al. (2013). Cytomegalovirus vectors violate CD8+ T cell epitope recognition paradigms. Science (80-.). *340*.

Harty, J.T., Tvinnereim, A.R., and White, D.W. (2000). Cd8+ T cell effector mechanisms in resistance to infection. Annu. Rev. Immunol. *18*, 275–308.

Harvey, W.T., Carabelli, A.M., Jackson, B., Gupta, R.K., Thomson, E.C., Harrison, E.M., Ludden, C., Reeve, R., Rambaut, A., Peacock, S.J., et al. (2021). SARS-CoV-2 variants, spike mutations and immune escape. Nat. Rev. Microbiol. 2021 197 *19*, 409–424.

Hatcher, E.L., Zhdanov, S.A., Bao, Y., Blinkova, O., Nawrocki, E.P., Ostapchuck, Y., Schaffer, A.A., and Rodney Brister, J. (2017). Virus Variation Resource-improved response to emergent viral outbreaks. Nucleic Acids Res. *45*, D482–D490.

Hoffmann, M., Arora, P., Groß, R., Seidel, A., Hörnich, B.F., Hahn, A.S., Krüger, N., Graichen, L., Hofmann-Winkler, H., Kempf, A., et al. (2021). SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. Cell.

Honaker, Y., Hubbard, N., Xiang, Y., Fisher, L., Hagin, D., Sommer, K., Song, Y., Yang, S.J., Lopez, C., Tappen, T., et al. (2020). Gene editing to induce FOXP3 expression in human CD4+ T cells leads to a stable regulatory phenotype and function. Sci. Transl. Med. *12*, 6422.

Hsiue, E.H.-C., Wright, K.M., Douglass, J., Hwang, M.S., Mog, B.J., Pearlman, A.H., Paul, S., DiNapoli, S.R., Konig, M.F., Wang, Q., et al. (2021). Targeting a neoantigen derived from a common TP53 mutation. Science (80-.). *371*.

Huang, H., Wang, C., Rubelt, F., Scriba, T.J., and Davis, M.M. (2020). Analyzing the Mycobacterium tuberculosis immune response by T-cell receptor clustering with GLIPH2 and genome-wide antigen screening. Nat. Biotechnol. *38*, 1194–1202.

Ishizuka, J., Grebe, K., Shenderov, E., Peters, B., Chen, Q., Peng, Y., Wang, L., Dong, T., Pasquetto, V., Oseroff, C., et al. (2009). Quantitating T Cell Cross-Reactivity for Unrelated Peptide Antigens. J. Immunol. *183*, 4337–4345.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. Cell *126*, 1121–1133.

Iwafuchi-Doi, M., and Zaret, K.S. (2016). Cell fate control by pioneer transcription factors. Dev. *143*, 1833–1837.

Iwafuchi-Doi, M., Donahue, G., Kakumanu, A., Watts, J.A., Mahony, S., Pugh, B.F., Lee, D., Kaestner, K.H., and Zaret, K.S. (2016). The Pioneer Transcription Factor FoxA Maintains an Accessible Nucleosome Configuration at Enhancers for Tissue-Specific Gene Activation. Mol. Cell *62*, 79–91.

Johnson, L.E., and McNeel, D.G. (2012). Identification of prostatic acid phosphatase (PAP) specific HLA-DR1-restricted t-cell epitopes. Prostate *72*, 730–740.

Johnson, L.A., Heemskerk, B., Powell, D.J., Cohen, C.J., Morgan, R.A., Dudley, M.E., Robbins, P.F., and Rosenberg, S.A. (2006a). Gene Transfer of Tumor-Reactive TCR Confers Both High Avidity and Tumor Reactivity to Nonreactive Peripheral Blood Mononuclear Cells and Tumor-Infiltrating Lymphocytes. J. Immunol. *177*, 6548–6559.

Johnson, L.A., Heemskerk, B., Powell, D.J., Cohen, C.J., Morgan, R.A., Dudley, M.E., Robbins, P.F., and Rosenberg, S.A. (2006b). Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. J. Immunol. *177*, 6548–6559.

Johnson, L.A., Morgan, R.A., Dudley, M.E., Cassard, L., Yang, J.C., Hughes, M.S., Kammula, U.S., Royal, R.E., Sherry, R.M., Wunderlich, J.R., et al. (2009). Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood *114*, 535–546.

Jozwik, A., Habibi, M.S., Paras, A., Zhu, J., Guvenel, A., Dhariwal, J., Almond, M., Wong, E.H.C., Sykes, A., Maybeno, M., et al. (2015). RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. Nat. Commun. *6*.

Kantoff, P.W., Higano, C.S., Shore, N.D., Berger, E.R., Small, E.J., Penson, D.F., Redfern, C.H., Ferrari, A.C., Dreicer, R., Sims, R.B., et al. (2010). Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. N. Engl. J. Med. *363*, 411–422.

Katzenelenbogen, Y., Sheban, F., Yalin, A., Yofe, I., Svetlichnyy, D., Jaitin, D.A., Bornstein, C., Moshe, A., Keren-Shaul, H., Cohen, M., et al. (2020). Coupled scRNA-Seq and Intracellular Protein Activity Reveal an Immunosuppressive Role of TREM2 in Cancer. Cell *182*, 872-885.e19.

Keller, M.D., Harris, K.M., Jensen-Wachspress, M.A., Kankate, V. V., Lang, H., Lazarski, C.A., Durkee-Shock, J., Lee, P.H., Chaudhry, K., Webber, K., et al. (2020). SARS-CoV-2–specific T cells are rapidly expanded for therapeutic use and target conserved regions of the membrane protein. Blood *136*, 2905–2917.

Khoury, D.S., Cromer, D., Reynaldi, A., Schlub, T.E., Wheatley, A.K., Juno, J.A., Subbarao, K., Kent, S.J., Triccas, J.A., and Davenport, M.P. (2021). Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nat. Med. 2021 277 *27*, 1205–1211.

Killerby, M.E., Biggs, H.M., Haynes, A., Dahl, R.M., Mustaquim, D., Gerber, S.I., and Watson, J.T. (2018). Human coronavirus circulation in the United States 2014–2017. J. Clin. Virol. *101*, 52–56.

Klenerman, P., and Oxenius, A. (2016). T cell responses to cytomegalovirus. Nat. Rev. Immunol. *16*, 367.

Klinger, M., Pepin, F., Wilkins, J., Asbury, T., Wittkop, T., Zheng, J., Moorhead, M., and Faham, M. (2015). Multiplex Identification of Antigen-Specific T Cell Receptors Using a Combination of Immune Assays and Immune Receptor Sequencing. PLoS One *10*, e0141561.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. (2009). IL-17 and Th17 Cells. Annu. Rev. Immunol. *27*, 485–517.

Kuzmina, A., Khalaila, Y., Voloshin, O., Keren-Naus, A., Bohehm, L., Raviv, Y., Shemer-Avni, Y., Rosenberg, E., and Taube, R. (2021). SARS CoV-2 spike variants exhibit differential infectivity and neutralization resistance to convalescent or post-vaccination sera. Cell Host Microbe.

Lambert, S.A., Jolma, A., Campitelli, L.F., Das, P.K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T.R., and Weirauch, M.T. (2018). The Human Transcription Factors. Cell *172*, 650–665.

Leitman, E.M., Willberg, C.B., De Burgh-Thomas, A., Streeck, H., Goulder, P.J.R., and Matthews, P.C. (2016). Subdominant Gag-specific anti-HIV efficacy in an HLA-B∗ 57-positive elite controller. AIDS *30*, 972–974.

Li, M.O., and Rudensky, A.Y. (2016). T cell receptor signalling in the control of regulatory T cell differentiation and function. Nat. Rev. Immunol. *16*, 220–233.

Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., Cheng, L., Li, J., Wang, X., Wang, F., et al. (2020). Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. Nat. Med. *26*, 842–844.

Lineburg, K.E., Grant, E.J., Swaminathan, S., Chatzileontiadou, D.S., Szeto, C., Sloane, H., Panikkar, A., Raju, J., Crooks, P., Rehan, S., et al. (2021). CD8+ T cells specific for an immunodominant SARS-CoV-2 nucleocapsid epitope cross-react with selective seasonal

coronaviruses. Immunity *0.*

Linette, G.P., Stadtmauer, E.A., Maus, M. V., Rapoport, A.P., Levine, B.L., Emery, L., Litzky, L., Bagg, A., Carreno, B.M., Cimino, P.J., et al. (2013). Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood *122*, 863.

Linnemann, C., van Buuren, M.M., Bies, L., Verdegaal, E.M.E., Schotte, R., Calis, J.J.A., Behjati, S., Velds, A., Hilkmann, H., Atmioui, D. el, et al. (2014). High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat. Med. *21*, 81.

Lipsitch, M., Grad, Y.H., Sette, A., and Crotty, S. (2020). Cross-reactive memory T cells and herd immunity to SARS-CoV-2. Nat. Rev. Immunol. *20*, 709–713.

Lumley, S.F., O'Donnell, D., Stoesser, N.E., Matthews, P.C., Howarth, A., Hatch, S.B., Marsden, B.D., Cox, S., James, T., Warren, F., et al. (2020). Antibody Status and Incidence of SARS-CoV-2 Infection in Health Care Workers. Https://Doi.Org/10.1056/NEJMoa2034545 *384*, 533–540.

Lynn, R.C., Weber, E.W., Sotillo, E., Gennert, D., Xu, P., Good, Z., Anbunathan, H., Lattin, J., Jones, R., Tieu, V., et al. (2019). c-Jun overexpression in CAR T cells induces exhaustion resistance. Nat. 2019 5767786 *576*, 293–300.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell *161*, 1202–1214.

Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R.N., Potter, S.C., Finn, R.D., et al. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. *47*, W636–W641.

Mallajosyula, V., Ganjavi, C., Chakraborty, S., McSween, A.M., Pavlovitch-Bedzyk, A.J., Wilhelmy, J., Nau, A., Manohar, M., Nadeau, K.C., and Davis, M.M. (2021). CD8+ T cells specific for conserved coronavirus epitopes correlate with milder disease in COVID-19 patients. Sci. Immunol. *6*, eabg5669.

Maness, N.J., Valentine, L.E., May, G.E., Reed, J., Piaskowski, S.M., Soma, T., Furlott, J., Rakasz, E.G., Friedrich, T.C., Price, D.A., et al. (2007). AIDS virus-specific CD8+ T lymphocytes against an immunodominant cryptic epitope select for viral escape. J. Exp. Med. *204*, 2505–2512.

Mateus, J., Grifoni, A., Tarke, A., Sidney, J., Ramirez, S.I., Dan, J.M., Burger, Z.C., Rawlings, S.A., Smith, D.M., Phillips, E., et al. (2020). Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. Science (80-.). *370*, 89–94.

Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., et al. (2018). Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. Http://Dx.Doi.Org/10.1056/NEJMoa1709866 *378*, 439–448.

McMahan, K., Yu, J., Mercado, N.B., Loos, C., Tostanoski, L.H., Chandrashekar, A., Liu, J., Peter, L., Atyeo, C., Zhu, A., et al. (2021). Correlates of protection against SARS-CoV-2 in rhesus macaques. Nature *590*, 630–634.

McMichael, A.J., Gotch, F.M., Noble, G.R., and Beare, P.A.S. (1983). Cytotoxic T-Cell Immunity to Influenza. N. Engl. J. Med. *309*, 13–17.

Milner, J.J., Toma, C., Yu, B., Zhang, K., Omilusik, K., Phan, A.T., Wang, D., Getzler, A.J., Nguyen, T., Crotty, S., et al. (2017). Runx3 programs CD8+ T cell residency in non-lymphoid tissues and tumours. Nature.

Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. (2011). T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J. Exp. Med. *208*, 1279–1289.

Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalían, S.L., Kammula, U.S., Restifo, N.P., et al. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. Science (80-.). *314*, 126–129.

Muik, A., Wallisch, A.K., Sänger, B., Swanson, K.A., Mühl, J., Chen, W., Cai, H., Maurus, D., Sarkar, R., Türeci, Ö., et al. (2021). Neutralization of SARS-CoV-2 lineage B.1.1.7 pseudovirus by BNT162b2 vaccine–elicited human sera. Science (80-.). *371*, 1152–1153.

Murray, S.E., Nesterenko, P.A., Vanarsdall, A.L., Munks, M.W., Smart, S.M., Veziroglu, E.M., Sagario, L.C., Lee, R., Claas, F.H.J., Doxiadis, I.I.N., et al. (2017). Fibroblast-adapted human CMV vaccines elicit predominantly conventional CD8 T cell responses in humans. J. Exp. Med. *214*, 1889–1899.

Nair, S., Boczkowski, D., Fassnacht, M., Pisetsky, D., and Gilboa, E. (2007). Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity. Cancer Res. *67*, 371–380.

Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., et al. (2017). Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. Https://Doi.Org/10.1056/NEJMoa1707447 *377*, 2531–2544.

Nesterenko, P.A., McLaughlin, J., Cheng, D., Bangayan, N.J., Sojo, G.B., Seet, C.S., Qin, Y., Mao, Z., Obusan, M.B., Phillips, J.W., et al. (2021). Droplet-based mRNA sequencing of fixed and permeabilized cells by CLInt-seq allows for antigen-specific TCR cloning. Proc. Natl. Acad.

Sci. U. S. A. *118*.

Newell, E.W., and Davis, M.M. (2014). Beyond model antigens: high-dimensional methods for the analysis of antigen-specific T cells. Nat. Biotechnol. 2013 322 *32*, 149–157.

Nisitani, S., Kato, R.M., Rawlings, D.J., Witte, O.N., and Wahl, M.I. (1999). In situ detection of activated Bruton's tyrosine kinase in the Ig signaling complex by phosphopeptide-specific monoclonal antibodies. Proc. Natl. Acad. Sci. U. S. A. *96*, 2221–2226.

Nolan, S., Vignali, M., Klinger, M., Dines, J.N., Kaplan, I.M., Svejnoha, E., Craft, T., Boland, K., Pesesky, M., Gittelman, R.M., et al. (2020). A large-scale database of T-cell receptor beta (TCRβ) sequences and binding associations from natural and synthetic exposure to SARS-CoV-2. Res. Sq.

NR, L., DG, S., M, R., and D, B. (1987). Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol. Cell. Biol. *7*, 3237–3243.

Oestreich, K.J., and Weinmann, A.S. (2012). Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. Nat. Rev. Immunol. *12*, 799–804.

Oh, D.Y., Kwek, S.S., Raju, S.S., Li, T., McCarthy, E., Chow, E., Aran, D., Ilano, A., Pai, C.C.S., Rancan, C., et al. (2020). Intratumoral CD4+ T Cells Mediate Anti-tumor Cytotoxicity in Human Bladder Cancer. Cell *181*, 1612-1625.e13.

Olson, B.M., Frye, T.P., Johnson, L.E., Fong, L., Knutson, K.L., Disis, M.L., and McNeel, D.G. (2010). HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase. Cancer Immunol. Immunother. *59*, 943–953.

P'ng, C., Green, J., Chong, L.C., Waggott, D., Prokopec, S.D., Shamsi, M., Nguyen, F., Mak, D.Y.F., Lam, F., Albuquerque, M.A., et al. (2019). BPG: Seamless, automated and interactive

visualization of scientific data. BMC Bioinformatics *20*, 1–5.

Pan, Y., Lee, A.H., Yang, H.T., Wang, Y., Xu, Y., Kadash-Edmondson, K.E., Phillips, J., Champhekar, A., Puig, C., Ribas, A., et al. (2019). IRIS: Big data-informed discovery of cancer immunotherapy targets arising from premRNA alternative splicing. BioRxiv 843268.

Pan, Y., Kadash-Edmondson, K.E., Wang, R., Phillips, J., Liu, S., Ribas, A., Aplenc, R., Witte, O.N., and Xing, Y. (2021). RNA Dysregulation: An Expanding Source of Cancer Immunotherapy Targets. Trends Pharmacol. Sci. *42*, 268–282.

Papadopoulou, A., Gerdemann, U., Katari, U.L., Tzannou, I., Liu, H., Martinez, C., Leung, K., Carrum, G., Gee, A.P., Vera, J.F., et al. (2014). Activity of Broad-Spectrum T Cells as Treatment for AdV, EBV, CMV, BKV, and HHV6 Infections after HSCT. Sci. Transl. Med. *6*, 242ra83-242ra83.

Parkhurst, M., Gros, A., Pasetto, A., Prickett, T., Crystal, J.S., Robbins, P., and Rosenberg, S.A. (2017). Isolation of T-cell receptors specifically reactive with mutated tumor-associated antigens from tumor-infiltrating lymphocytes based on CD137 expression. Clin. Cancer Res. *23*, 2491–2505.

Peng, Y., Mentzer, A.J., Liu, G., Yao, X., Yin, Z., Dong, D., Dejnirattisai, W., Rostron, T., Supasa, P., Liu, C., et al. (2020). Broad and strong memory CD4+ and CD8+ T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19. Nat. Immunol. *21*, 1336–1345.

Peterson, V.M., Zhang, K.X., Kumar, N., Wong, J., Li, L., Wilson, D.C., Moore, R., Mcclanahan, T.K., Sadekova, S., and Klappenbach, J.A. (2017). Multiplexed quantification of proteins and transcripts in single cells. Nat. Biotechnol. *35*, 936–939.

Phillips, J.W., Pan, Y., Tsai, B.L., Xie, Z., Demirdjian, L., Xiao, W., Yang, H.T., Zhang, Y., Lin,

C.H., Cheng, D., et al. (2020). Pathway-guided analysis identifies Myc-dependent alternative pre-mRNA splicing in aggressive prostate cancers. Proc. Natl. Acad. Sci. *117*, 5269–5279.

Pinkse, G.G.M., Tysma, O.H.M., Bergen, C.A.M., Kester, M.G.D., Ossendorp, F., Van Veelen, P.A., Keymeulen, B., Pipeleers, D., Drijfhout, J.W., and Roep, B.O. (2005). Autoreactive CD8 T cells associated with β cell destruction in type 1 diabetes. Proc. Natl. Acad. Sci. U. S. A. *102*, 18425–18430.

Pitcher, C.J., Quittner, C., Peterson, D.M., Connors, M., Koup, R.A., Maino, V.C., and Picker, L.J. (1999). HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat. Med. *5*, 518.

Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M.M., Planchais, C., Porrot, F., Robillard, N., Puech, J., et al. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. Nat. 2021 1–7.

Polack, F.P., Thomas, S.J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J.L., Pérez Marc, G., Moreira, E.D., Zerbini, C., et al. (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. N. Engl. J. Med. *383*, 2603–2615.

Poran, A., Harjanto, D., Malloy, M., Arieta, C.M., Rothenberg, D.A., Lenkala, D., van Buuren, M.M., Addona, T.A., Rooney, M.S., Srinivasan, L., et al. (2020). Sequence-based prediction of SARS-CoV-2 vaccine targets using a mass spectrometry-based bioinformatics predictor identifies immunogenic T cell epitopes. Genome Med. 2020 121 *12*, 1–15.

Qi, Q., Liu, Y., Cheng, Y., Glanville, J., Zhang, D., Lee, J.Y., Olshen, R.A., Weyand, C.M., Boyd, S.D., and Goronzy, J.J. (2014). Diversity and clonal selection in the human T-cell repertoire. Proc. Natl. Acad. Sci. U. S. A. *111*, 13139–13144.

Raffin, C., Vo, L.T., and Bluestone, J.A. (2020). Treg cell-based therapies: challenges and

perspectives. Nat. Rev. Immunol. *20*, 158–172.

Restifo, N.P., Dudley, M.E., and Rosenberg, S.A. (2012). Adoptive immunotherapy for cancer: Harnessing the T cell response. Nat. Rev. Immunol. *12*, 269–281.

Robbins, P.F., Morgan, R.A., Feldman, S.A., Yang, J.C., Sherry, R.M., Dudley, M.E., Wunderlich, J.R., Nahvi, A. V., Helman, L.J., Mackall, C.L., et al. (2011a). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J. Clin. Oncol. *29*, 917–924.

Robbins, P.F., Morgan, R.A., Feldman, S.A., Yang, J.C., Sherry, R.M., Dudley, M.E., Wunderlich, J.R., Nahvi, A. V., Helman, L.J., Mackall, C.L., et al. (2011b). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J. Clin. Oncol. *29*, 917–924.

Rodenko, B., Toebes, M., Hadrup, S.R., van Esch, W.J.E., Molenaar, A.M., Schumacher, T.N.M., and Ovaa, H. (2006). Generation of peptide–MHC class I complexes through UV-mediated ligand exchange. Nat. Protoc. *1*, 1120–1132.

Roth, T.L., Puig-Saus, C., Yu, R., Shifrut, E., Carnevale, J., Li, P.J., Hiatt, J., Saco, J., Krystofinski, P., Li, H., et al. (2018). Reprogramming human T cell function and specificity with non-viral genome targeting. Nat. 2018 5597714 *559*, 405–409.

S, V., S, M., M, C., E, P., and A, L. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature *375*, 148–151.

Sadoff, J., Gray, G., Vandebosch, A., Cárdenas, V., Shukarev, G., Grinsztejn, B., Goepfert, P.A., Truyers, C., Fennema, H., Spiessens, B., et al. (2021). Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19. N. Engl. J. Med. NEJMoa2101544.

Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in

immunological tolerance to self and non-self. Nat. Immunol. *6*, 345–352.

Sant, A.J., and McMichael, A. (2012). Revealing the role of CD4+ T cells in viral immunity. J. Exp. Med. *209*, 1391–1395.

Schatz, D.G., Oettinger, M.A., and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. Cell *59*, 1035–1048.

Seet, C.S., He, C., Bethune, M.T., Li, S., Chick, B., Gschweng, E.H., Zhu, Y., Kim, K., Kohn, D.B., Baltimore, D., et al. (2017). Generation of mature T cells from human hematopoietic stem and progenitor cells in artificial thymic organoids. Nat. Methods *14*, 521–530.

Sewell, A.K. (2012). Why must T cells be cross-reactive? Nat. Rev. Immunol. *12*, 669–677.

Shu, Y., and McCauley, J. (2017). GISAID: Global initiative on sharing all influenza data – from vision to reality. Eurosurveillance *22*, 30494.

Sibener, L. V, Fernandes, R.A., Kolawole, E.M., Carbone, C.B., Liu, F., McAffee, D., Birnbaum, M.E., Yang, X., Su, L.F., and Yu, W. (2018). Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. Cell *174*, 672-687. e27.

Sieling, P., King, T., Wong, R., Nguyen, A., Wnuk, K., Rice, A., Adisetiyo, H., Hermreck, M., Verma, M., Zakin, L., et al. (2021). Single Prime hAd5 Spike (S) + Nucleocapsid (N) Dual Antigen Vaccination of 1 Healthy Volunteers Induces a Ten-Fold Increase in Mean S-and N-T-Cell Responses 2 Equivalent to T-Cell Responses from Patients Previously Infected with SARS-CoV-2 3 4. MedRxiv 2021.04.05.21254940.

Singer, J.B., Gifford, R.J., Cotten, M., and Robertson, D.L. (2020). CoV-GLUE: A Web Application for Tracking SARS-CoV-2 Genomic Variation.

Slifka, M.K., Rodriguez, F., and Whitton, J.L. (1999). Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. Nature *401*, 76.

Smart, A.C., Margolis, C.A., Pimentel, H., He, M.X., Miao, D., Adeegbe, D., Fugmann, T., Wong, K.-K., and Van Allen, E.M. (2018). Intron retention is a source of neoepitopes in cancer. Nat. Biotechnol. 2018 3611 *36*, 1056–1058.

Stoeckius, M., Hafemeister, C., Stephenson, W., Houck-Loomis, B., Chattopadhyay, P.K., Swerdlow, H., Satija, R., and Smibert, P. (2017). Simultaneous epitope and transcriptome measurement in single cells. Nat. Methods *14*, 865–868.

Subramonia Iyer, K., Klee, W.A., and Wooi, M. (1973). Direct Spectrophotometric Measurement of the Rate of Reduction of Disulfide Bonds 'THE REACTIVITY OF THE DISULFIDE BONDS OF BOVINE cr-LACTALBUMIN.

Sun, J.C., and Bevan, M.J. (2003). Defective CD8 T Cell Memory Following Acute Infection Without CD4 T Cell Help. Science (80-.). *300*, 339–342.

Sylwester, A.W., Mitchell, B.L., Edgar, J.B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P.R., Grabstein, K.H., Hosken, N.A., Kern, F., et al. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J. Exp. Med. *202*, 673–685.

Takaba, H., and Takayanagi, H. (2017). The Mechanisms of T Cell Selection in the Thymus. Trends Immunol. *38*, 805–816.

Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell *126*, 663–676.

Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., and Bluestone, J.A. (2004). In Vitro–expanded Antigen-specific Regulatory T Cells Suppress Autoimmune Diabetes. J. Exp. Med. *199*, 1455–1465.

Tarbell, K. V., Yamazaki, S., Olson, K., Toy, P., and Steinman, R.M. (2004). CD25+ CD4+ T

Cells, Expanded with Dendritic Cells Presenting a Single Autoantigenic Peptide, Suppress Autoimmune Diabetes. J. Exp. Med. *199*, 1467–1477.

Tarke, A., Sidney, J., Kidd, C.K., Dan, J.M., Ramirez, S.I., Yu, E.D., Mateus, J., da Silva Antunes, R., Moore, E., Rubiro, P., et al. (2021). Comprehensive analysis of T cell immunodominance and immunoprevalence of SARS-CoV-2 epitopes in COVID-19 cases. Cell Reports Med. *2*.

Thomsen, E.R., Mich, J.K., Yao, Z., Hodge, R.D., Doyle, A.M., Jang, S., Shehata, S.I., Nelson, A.M., Shapovalova, N. V, and Levi, B.P. (2016). Fixed single-cell transcriptomic characterization of human radial glial diversity. Nat. Methods *13*, 87.

Topham, D.J., Tripp, R.A., and Doherty, P.C. (1997). CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J. Immunol. *159*.

Tran, E., Robbins, P.F., Lu, Y.-C., Prickett, T.D., Gartner, J.J., Jia, L., Pasetto, A., Zheng, Z., Ray, S., Groh, E.M., et al. (2016). T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. Https://Doi.Org/10.1056/NEJMoa1609279 *375*, 2255–2262.

Tran, E., Robbins, P.F., and Rosenberg, S.A. (2017). Final common pathway' of human cancer immunotherapy: Targeting random somatic mutations. Nat. Immunol. *18*, 255–262.

Waldrop, S.L., Pitcher, C.J., Peterson, D.M., Maino, V.C., and Picker, L.J. (1997). Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J. Clin. Invest. *99*, 1739–1750.

Walker, B.D., and Yu, X.G. (2013). Unravelling the mechanisms of durable control of HIV-1. Nat. Rev. Immunol. *13*, 487–498.

Wang, P., Nair, M.S., Liu, L., Iketani, S., Luo, Y., Guo, Y., Wang, M., Yu, J., Zhang, B.,

Kwong, P.D., et al. (2021). Antibody Resistance of SARS-CoV-2 Variants B.1.351 and B.1.1.7. Nature 1–6.

Wei, S.C., Duffy, C.R., and Allison, J.P. (2018). Fundamental mechanisms of immune checkpoint blockade therapy. Cancer Discov. *8*, 1069–1086.

Weiskopf, D., Schmitz, K.S., Raadsen, M.P., Grifoni, A., Okba, N.M.A., Endeman, H., van den Akker, J.P.C., Molenkamp, R., Koopmans, M.P.G., van Gorp, E.C.M., et al. (2020). Phenotype and kinetics of SARS-CoV-2-specific T cells in COVID-19 patients with acute respiratory distress syndrome. Sci. Immunol. *5*, 2071.

Wolfl, M., Kuball, J., Ho, W.Y., Nguyen, H., Manley, T.J., Bleakley, M., and Greenberg, P.D. (2007). Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood *110*, 201–210.

Wölfl, M., Kuball, J., Eyrich, M., Schlegel, P.G., and Greenberg, P.D. (2008). Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. Cytom. Part A *73*, 1043–1049.

Wu, T.D., Madireddi, S., de Almeida, P.E., Banchereau, R., Chen, Y.J.J., Chitre, A.S., Chiang, E.Y., Iftikhar, H., O'Gorman, W.E., Au-Yeung, A., et al. (2020). Peripheral T cell expansion predicts tumour infiltration and clinical response. Nature *579*, 274–278.

Xue, S.-A., Gao, L., Ahmadi, M., Ghorashian, S., Barros, R.D., Pospori, C., Holler, A., Wright, G., Thomas, S., Topp, M., et al. (2013). Human MHC Class I-restricted high avidity CD4+ T cells generated by co-transfer of TCR and CD8 mediate efficient tumor rejection in vivo. Https://Doi.Org/10.4161/Onci.22590 *2*.

Yang, L., Bailey, L., Baltimore, D., and Wang, P. (2006). Targeting lentiviral vectors to specific

cell types in vivo. Proc. Natl. Acad. Sci. *103*, 11479–11484.

Yost, K.E., Satpathy, A.T., Wells, D.K., Qi, Y., Wang, C., Kageyama, R., McNamara, K.L., Granja, J.M., Sarin, K.Y., Brown, R.A., et al. (2019). Clonal replacement of tumor-specific T cells following PD-1 blockade. Nat. Med. *25*, 1251–1259.

Yu, W., Jiang, N., Ebert, P.J.R., Kidd, B.A., Müller, S., Lund, P.J., Juang, J., Adachi, K., Tse, T., Birnbaum, M.E., et al. (2015a). Clonal Deletion Prunes but Does Not Eliminate Self-Specific αβ CD8+ T Lymphocytes. Immunity *42*, 929–941.

Yu, W., Jiang, N., Peter, Brian, Müller, S., Peder, Juang, J., Adachi, K., Tse, T., Michael, et al. (2015b). Clonal Deletion Prunes but Does Not Eliminate Self-Specific αβ CD8+ T Lymphocytes. *42*, 929–941.

Zhu, Y., Smith, D.J., Zhou, Y., Li, Y.R., Yu, J., Lee, D., Wang, Y.C., Di Biase, S., Wang, X., Hardoy, C., et al. (2019). Development of Hematopoietic Stem Cell-Engineered Invariant Natural Killer T Cell Therapy for Cancer. Cell Stem Cell *25*, 542-557.e9.

Zinkernagel, R.M., and Doherty, P.C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature *248*, 701–702.

Figure 2-1. Intracellular staining identifies antigen specific T cells with a lower rate of false positives than cell surface profiling by the activation marker CD137

(A) Schematic for experimental design to compare antigen specific activation to bystander T cell activation. Donor PBMC are transduced with a NY-ESO TCR (clone 1G4) construct. Resultant populations are diluted into untransduced population of cells that was treated similarly. Dilution is confirmed by FACS analysis for tetramer staining and secondary transduction marker NGFR. (B) FACS analysis of CD137 upregulation and intracellular staining for IFNγ and TNFα after peptide stimulation.
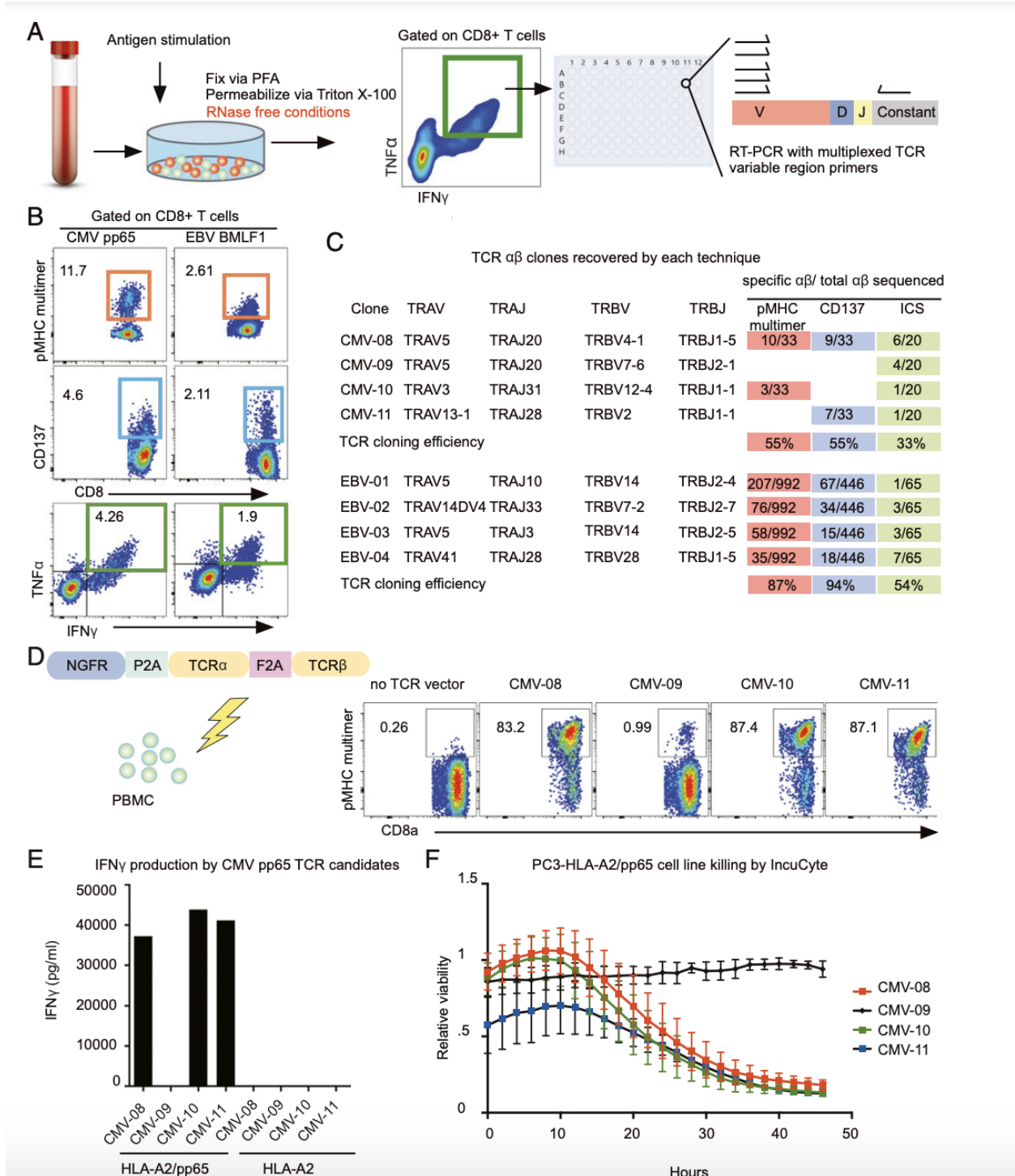
Figure 2-2. TCR alpha/beta pairs can be recovered from primary T cells after intracellular staining with efficiency equivalent to multimer-based selection

(A) Schematic for TCR mRNA sequencing post-intracellular staining of primary human T cells. Human PBMCs are cultured for 9 days, allowed to rest for 12 hours and then intracellular staining is performed. Cytokine producing cells are selected by FACS and single cell deposited into 96 well plates for TCR mRNA alpha and beta chain sequencing. (B) FACS analysis of antigen specific T cells identified by pMHC multimer staining, CD137 staining or intracellular staining. PBMCs are either stimulated with peptide or stained with tetramer carrying the cognate peptide: CMV pp65 HLA-A2 (NLVPMVATV), EBV BMLF1 (GLCTLVAML). Cells that are responsive (colored box) are isolated by FACS. (C) Summary table of TCR clones recovered by each technique and frequency of recovery within each technique. CD137 and tetramer selected cells of the EBV+ subject are analyzed by 10X genomics. Cloning efficiency is compared between the three techniques, defined by frequency of successful recovery of paired TCR alpha and beta chains. (D) Schematic for TCR functional testing in healthy donor PBMCs. CMV-reactive TCRs are overexpressed in healthy PBMC and expression is measured by FACS analysis for multimer binding: CMV pp65 (NLVPMVATV). (E) TCR-transduced PBMCs were stimulated with PC3 cells engineered to express HLA-A2 with or without CMV pp65. Cell supernatants were collected 48 hours after co-culture and secreted IFNγ quantified by ELISA. (F) Cytotoxicity of CMV specific TCRs was evaluated by coculturing TCR-transduced T cells with GFP+ PC3 cells expressing HLA-A2 and CMV pp65. Relative viability was measured by GFP fluorescence using the Incucyte system.
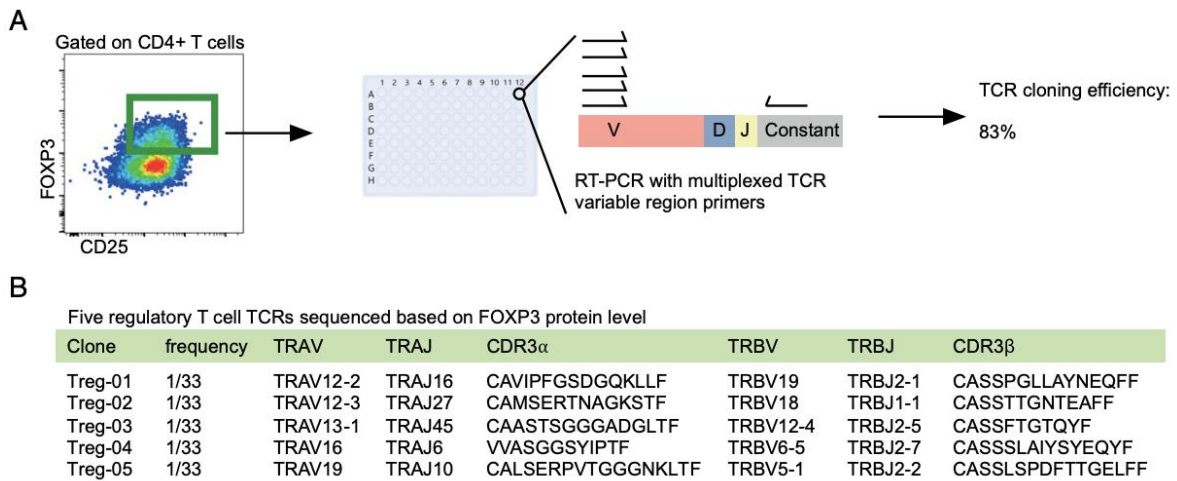
A Gated on CD4+ T cells

FOXP3 / CD25

1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H

RT-PCR with multiplexed TCR variable region primers

V | D | J | Constant

TCR cloning efficiency: 83%

B Five regulatory T cell TCRs sequenced based on FOXP3 protein level

Clone	frequency	TRAV	TRAJ	CDR3α	TRBV	TRBJ	CDR3β
Treg-01	1/33	TRAV12-2	TRAJ16	CAVIPFGSDGQKLLF	TRBV19	TRBJ2-1	CASSPGLLAYNEQFF
Treg-02	1/33	TRAV12-3	TRAJ27	CAMSERTNAGKSTF	TRBV18	TRBJ1-1	CASSTTGNTEAFF
Treg-03	1/33	TRAV13-1	TRAJ45	CAASTSGGGADGLTF	TRBV12-4	TRBJ2-5	CASSFTGTQYF
Treg-04	1/33	TRAV16	TRAJ6	VVASGGSYIPTF	TRBV6-5	TRBJ2-7	CASSSLAIYSYEQYF
Treg-05	1/33	TRAV19	TRAJ10	CALSERPVTGGGNKLTF	TRBV5-1	TRBJ2-2	CASSLSPDFTTGELFF

Figure 2-3. T regulatory cell TCR identification by intra-nuclear profiling of FOXP3.

(A) FOXP3 intracellular staining and subsequent single-cell TCR sequencing in Treg cells. CD4+ PBMCs are expanded in-vitro for 9 days and then stained for surface antigens (CD3, CD4, CD8, CD25), fixed and permeabilized, and stained for FOXP3. Single Treg cells (CD3$^+$, CD4$^+$, CD8$^-$, CD25$^+$, FOXP3$^+$) were FACS deposited into 96 well plates and RT-PCR is performed for TCR sequencing. Cloning efficiency is reported as frequency of successful recovery of full length TCR alpha and beta pairs. (B) Analysis is performed on 40 cells and 33 alpha/beta TCR pairs are generated. Five of the TCRs sequenced are shown.
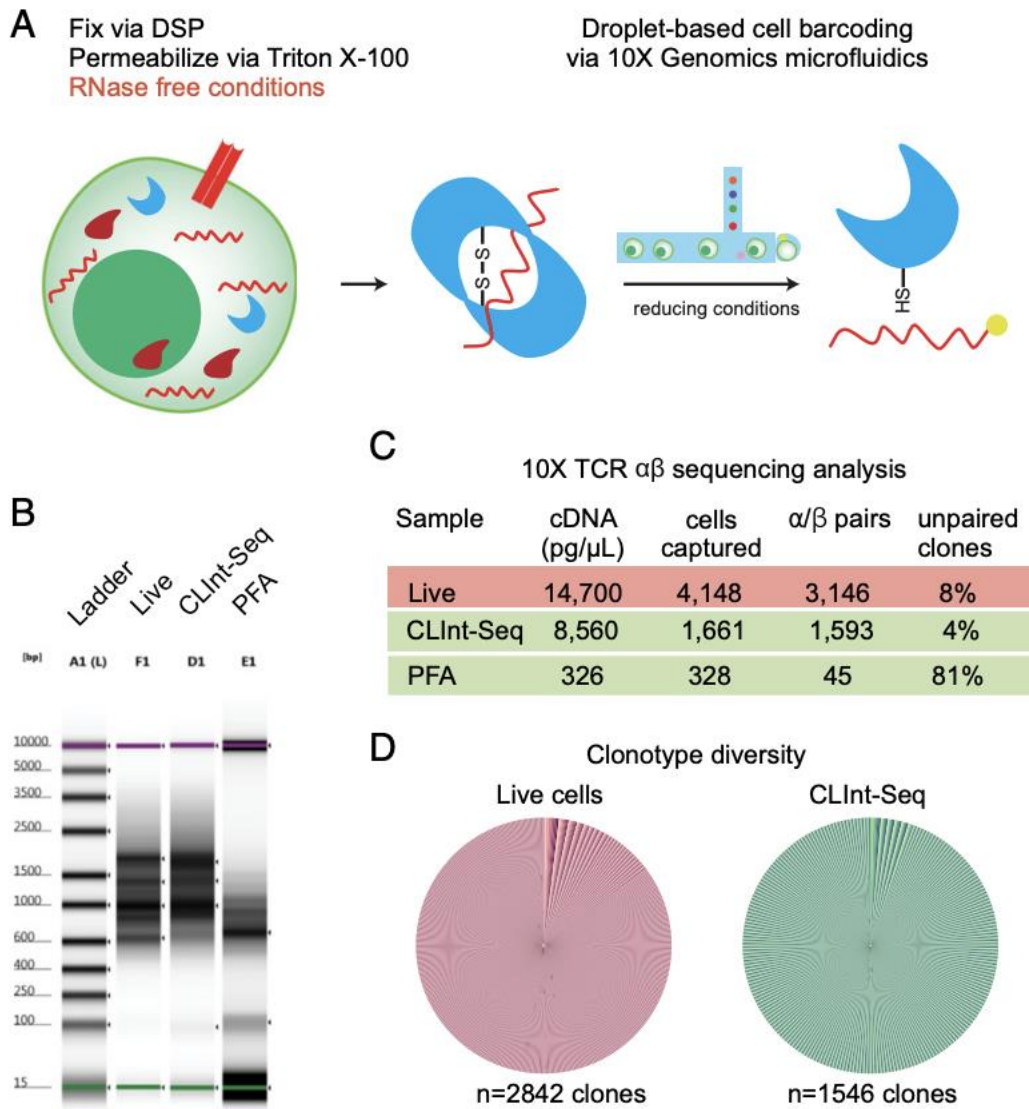
Figure 2-4. Single-cell sequencing of fixed and permeabilized cells in droplet-based format

(A) Schematic for capture of cellular mRNA in cellular protein mass via DSP. The reducing

reagents present in the drop-seq fluidics allow the liberation of mRNA and subsequent RT-PCR.

(B) Electrophoresis analysis of cDNA libraries. Activated human PBMCs and Jurkat cells are

mixed at 5:1 ratio, subsequently fixed with DSP and permeabilized with Triton X-100. Treated

cells are then submitted for 10X genomics TCR V(D)J sequencing. Separate samples of live and

PFA permeabilized cells are processed in the same run as a positive and negative controls. (C)

TCR clone metadata analysis after next generation sequencing (D) Pie chart analysis of TCR

diversity of all paired clones reported in the Loupe VDJ browser (10X Genomics).
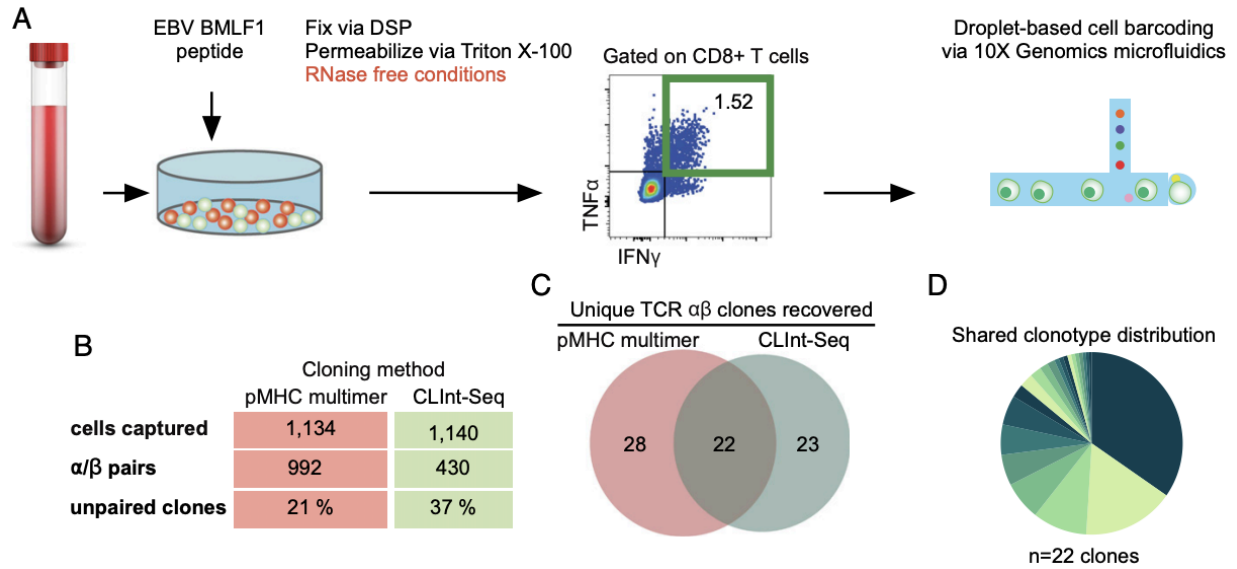
Figure 2-5. CLint-Seq coupled to droplet-based sequencing recovers EBV specific TCRs

(A) Human PBMCs are co-cultured with EBV 9mer epitope (GLCTLVAML), then re-stimulated in the presence of EBV peptide and Brefeldin A and subsequently stained for TNFα and IFNγ cytokines. DSP is used as a crosslinker. Responding cells are FACS sorted into a 2ml Eppendorf tube and submitted for 10X Genomics V(D)J analysis. (B) Metadata for the 10X Genomics TCR sequencing done using CLInt-Seq as well as a historical control generated with tetramer selection. (C) Venn diagram of EBV clonotypes generated by CLInt-Seq and multimer selection, filtered for clones with alpha/beta pair and frequency of 2 or more. (D) Pie chart showing frequency distribution of clonotypes that were found by both techniques.
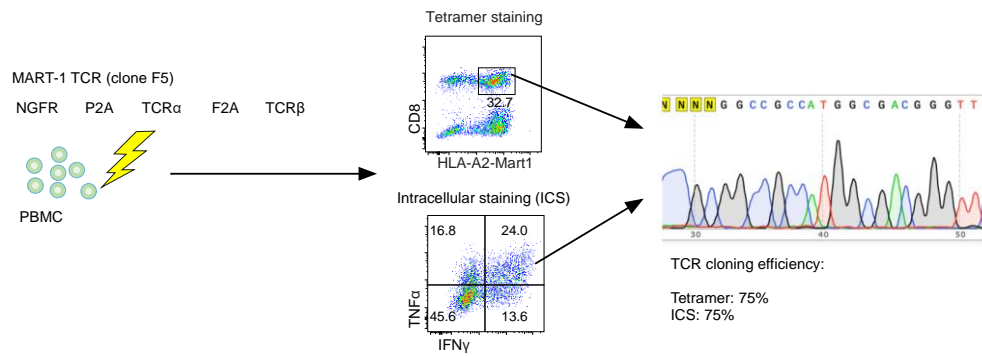
Figure 2-6 (Fig. S1). TCR construct overexpression allows for TCR sequencing post intracellular staining. Donor PBMCs were transduced with TCR (F5), specific for HLA-A*02:01-restricted epitope of MART-1. F5-transduced cells were either stained with cognate tetramer or stimulated with cognate peptide (ELAGIGILTV) and subsequently fixed and stained for intracellular TNFα and IFNγ. Responding cells were deposited by single-cell FACS for TCR sequencing by nested RT-PCR and Sanger sequencing. TCR cloning efficiency is defined as percent of recovered F5 TCR alpha and beta chains from total sorted single cells.
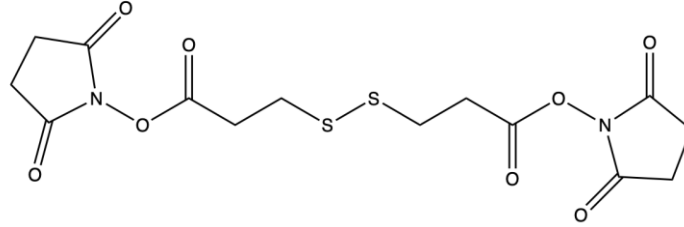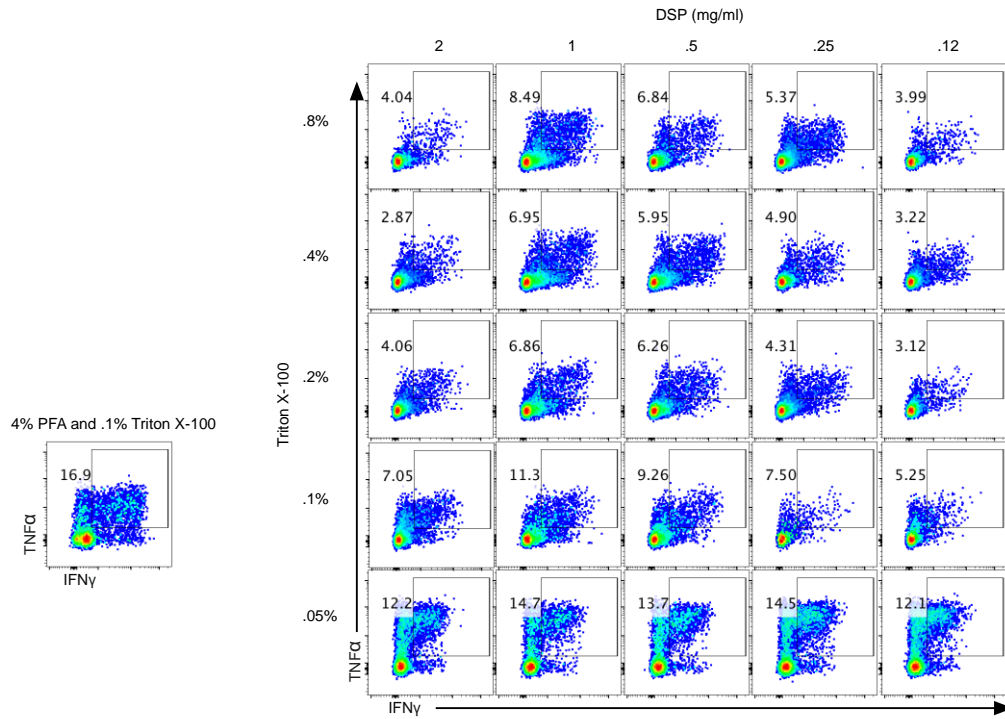
Figure 2-7 (Fig. S2). DSP and Triton X-100 titration. a.DSP chemical structure. b. Human PBMCs are stimulated with PMA/Ionomycin and stained for intracellular IFNγ and TNFα under different conditions of permeabilization with DSP and triton X-100.
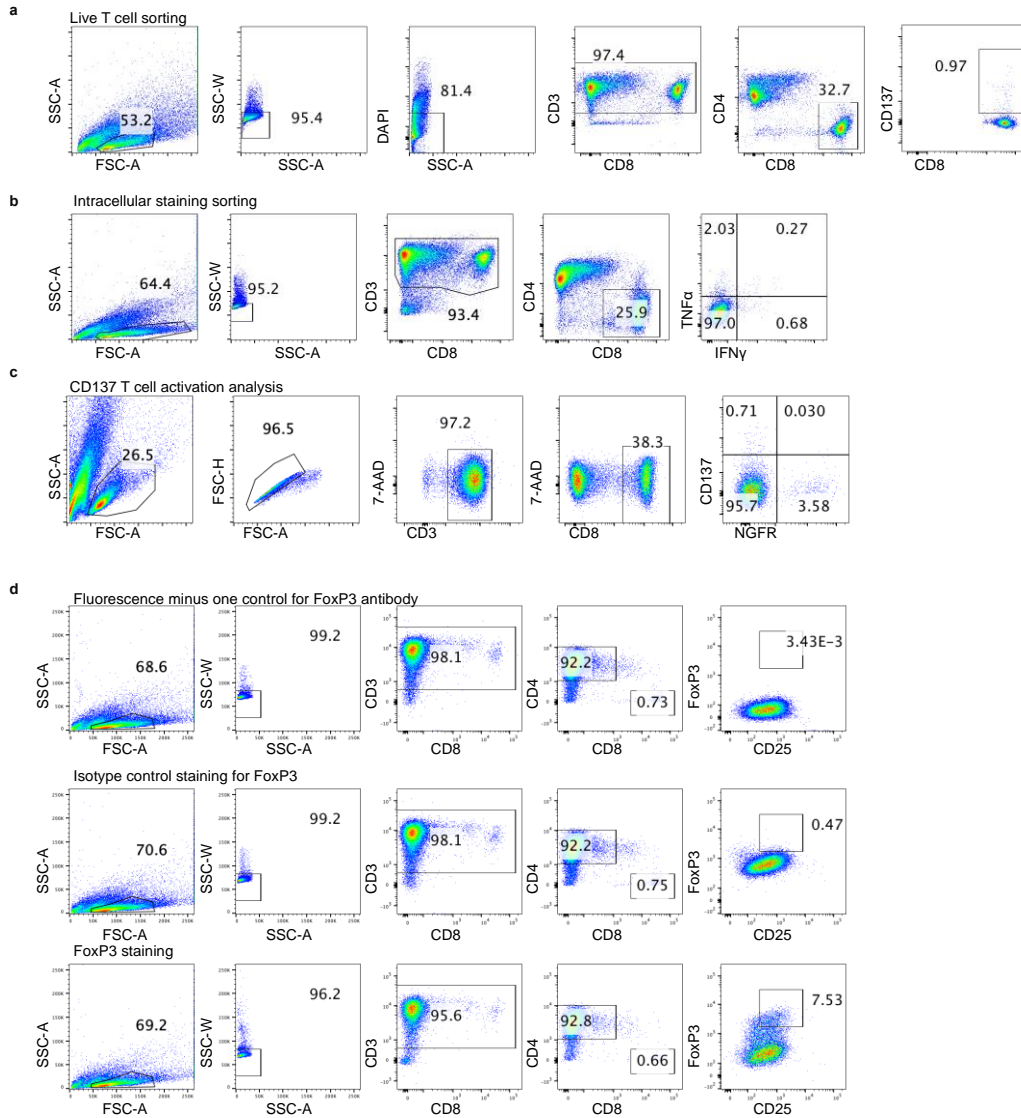
Figure 2-8 (Fig. S3). Gating hierarchy. a. Gating hierarchy used for FACS sorting PBMCs based on CD137 and tetramer staining. b. Gating hierarchy used for FACS sorting of PBMCs based on ICS. c. Gating hierarchy used to analyze CD137 staining. d. Gating hierarchies for FoxP3 level analysis and FACS sorting of T regulatory cells.

Chapter 3

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

Introduction:

Over 4 million people have died from COVID-19 as of August 2021 (World Health

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

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

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

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-

CoV-2 proteins are more conserved than the spike and may present a therapeutic opportunity at

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 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-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., 2010b). 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 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 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 protected against SARS-CoV-2 encounter(Lipsitch et al., 2020; Mallajosyula et al., 2021; Mateus et al., 2020). T

cells interact with target antigens through the T cell receptor (TCR), which is a heterodimer of 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 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.

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 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 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

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 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 (Figure 1B).

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 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.

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 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 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 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).

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 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 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 0.058-7.75%, depending on the TCR.

T cells control viral spread by killing virus infected cells. Cytotoxicity assays showed five out of 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 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 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 showed that this TCR only recognized antigen at high concentration of 10 µg/ml, confirming that this TCR has low specificity 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.

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 affinity against each of the epitopes was profiled via peptide titration assay. RdRp specific TCRαβ constructs exhibited a diverse pattern 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-CoV-1, MERS and HCoVs 229E and NL63 coronaviruses (Figure 4C).

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

COVID-19 caused by circulating variants of SARS-CoV-2. Continuous evolution of SARS-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 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 COVID-19 disease.

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 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 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 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.

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., 2010a; 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., 2006a). There are several advantages to adoptive cell 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). CD4+ T cells that we analyzed here recognized peptide antigen, but not processed

epitopes, likely due to lack of CD8 that could stabilize antigen recognition. Transducing the CD8 molecule as part of the TCR vector can generate Class I restricted CD4+ T cells (Xue et al., 2013). Current approaches for adoptive T cell therapy are expensive and cumbersome (Depil et 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).

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

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Project administration: PAN, JM, ONW

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

Declaration of interests: ONW, JM and PAN are inventors of a patent application in progress 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 reported in this article.
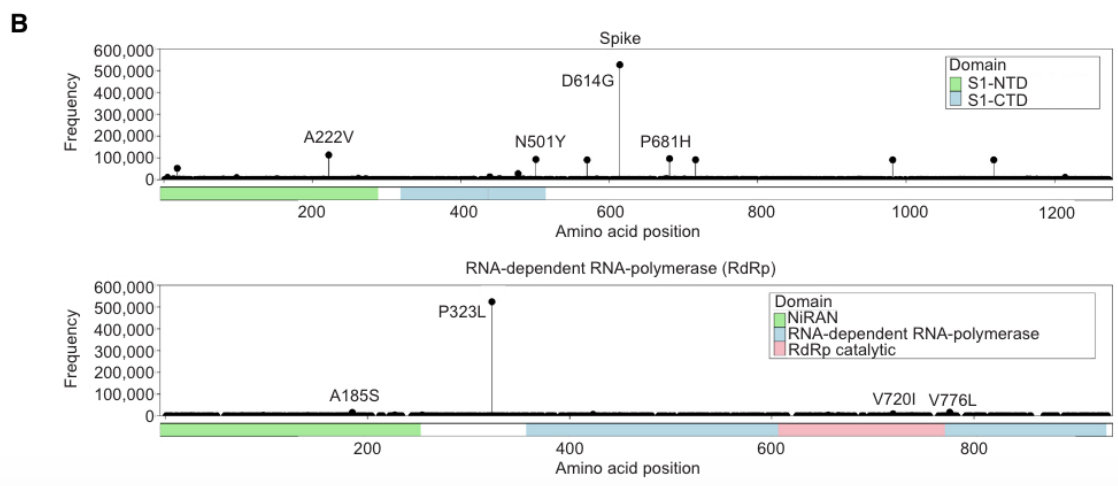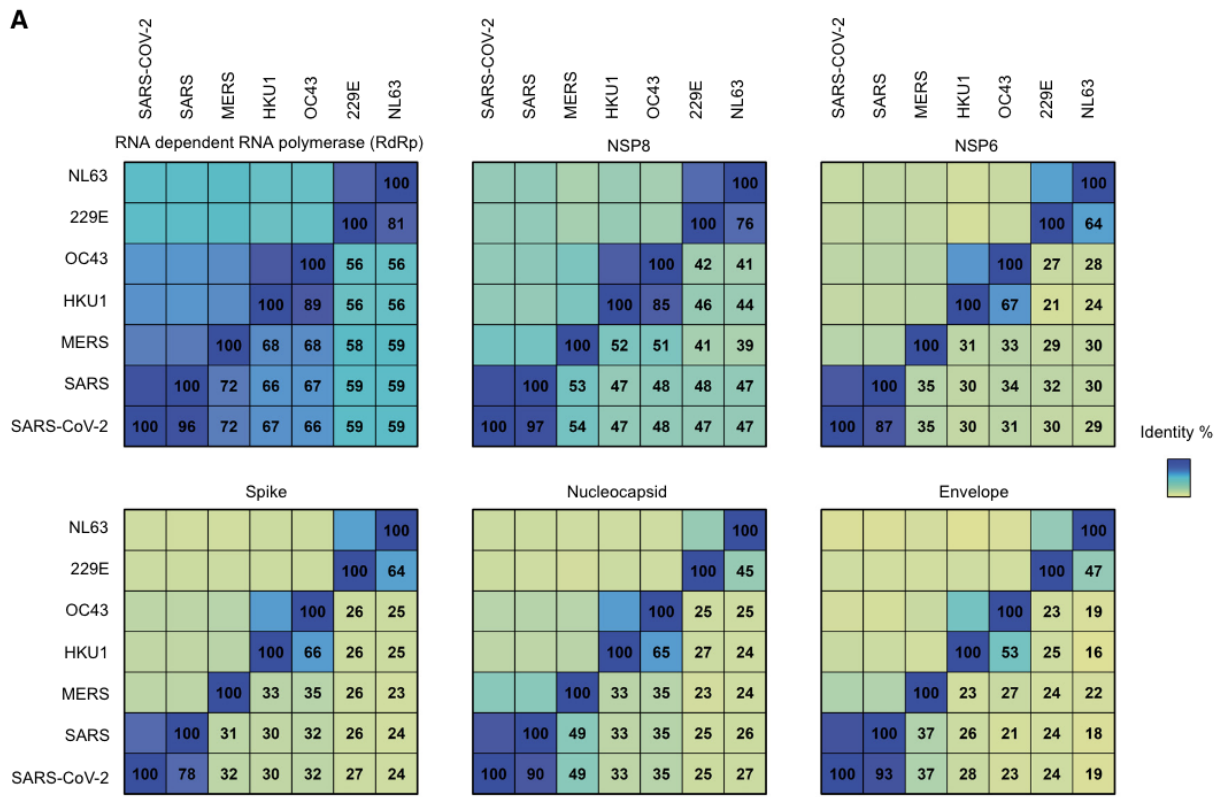
Figure 3-1. RdRp is highly conserved among human coronaviruses and within SARS-CoV-2

(A) Correlation matrix comparing coronavirus protein conservation by amino acid identity across 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.
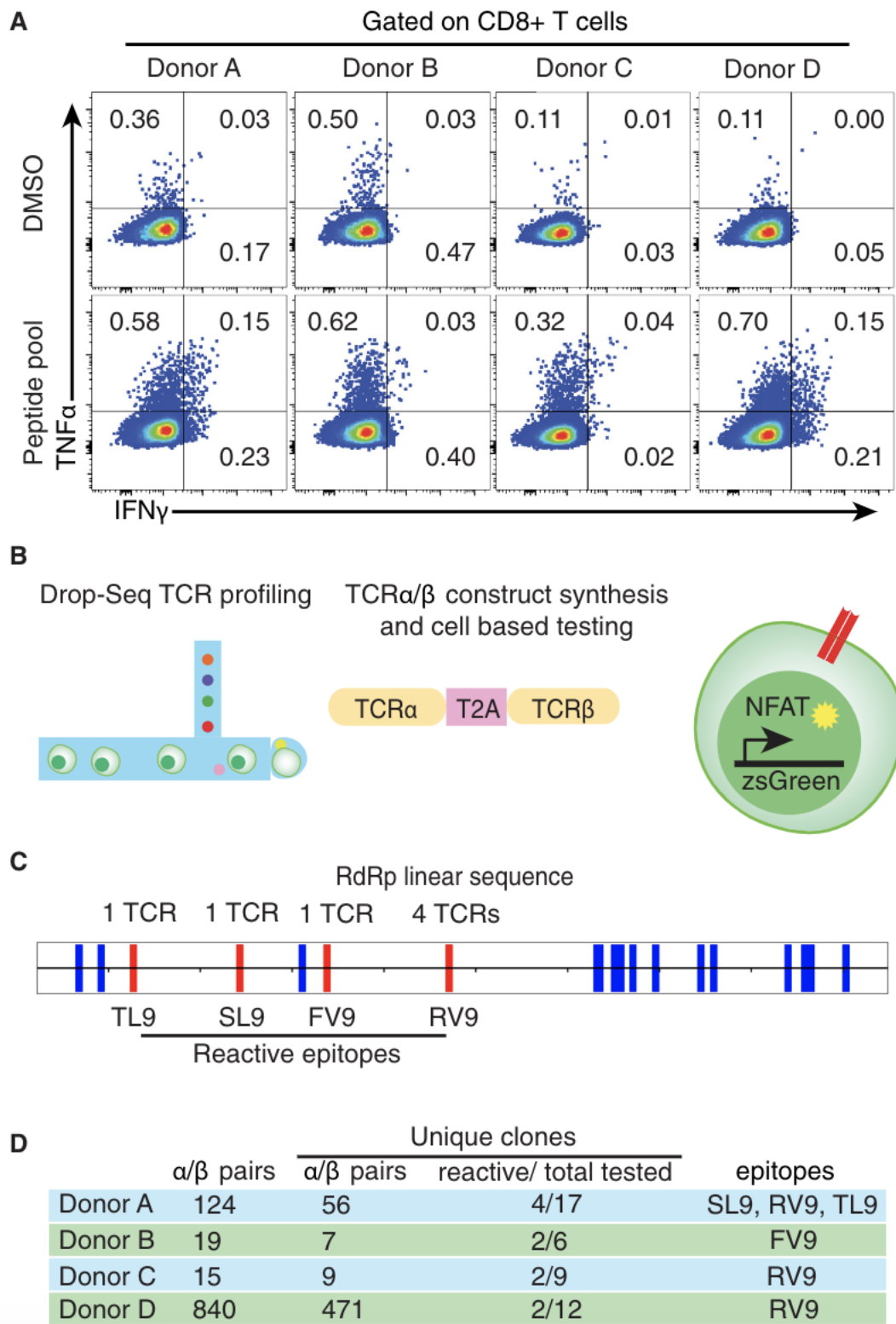
Figure 3-2. T cell receptors from unexposed individuals recognize RdRp epitopes.

(A) FACS sorting analysis of T cells that underwent stimulation by peptide pools and subsequent intracellular staining for TNFα and IFNγ under 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 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.
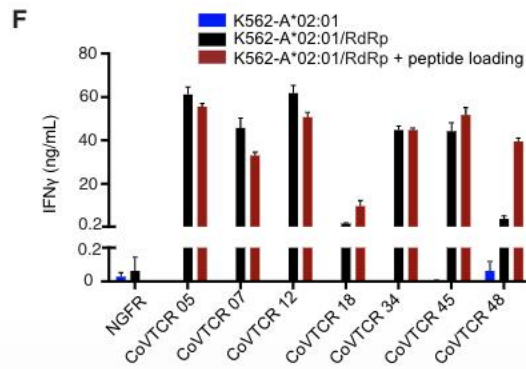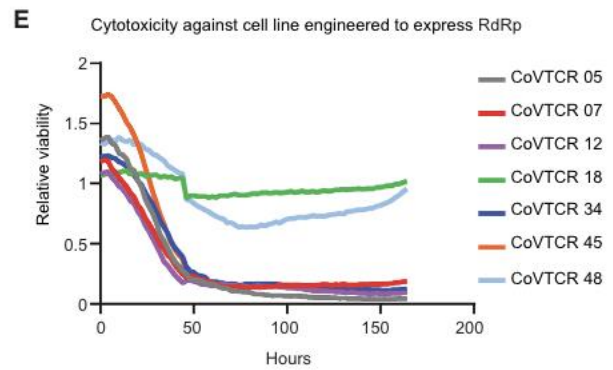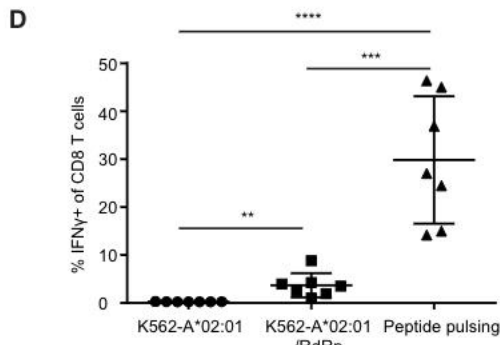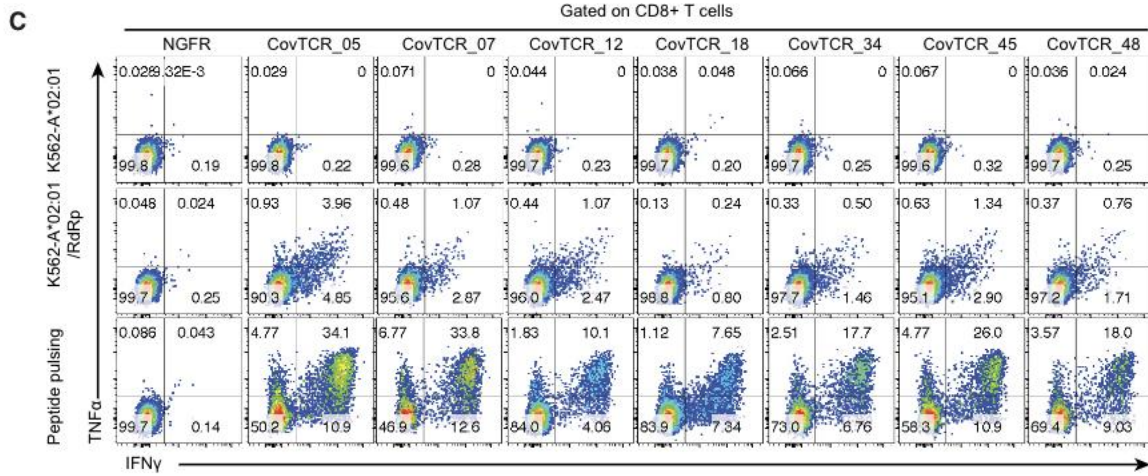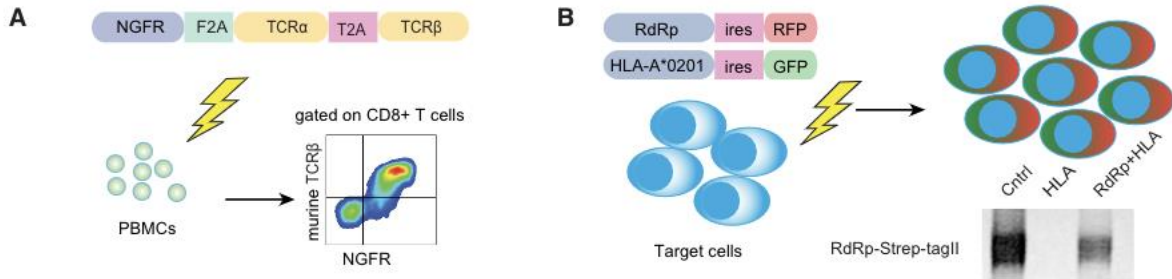
Figure 3-3. Isolated T cell receptors recognize and kill RdRp expressing cells

(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 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. RdRp transfected cells are used as positive control for expression. (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. 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 that expresses the RdRp. HLA-A*02:01 only parental cell line is used as negative control.
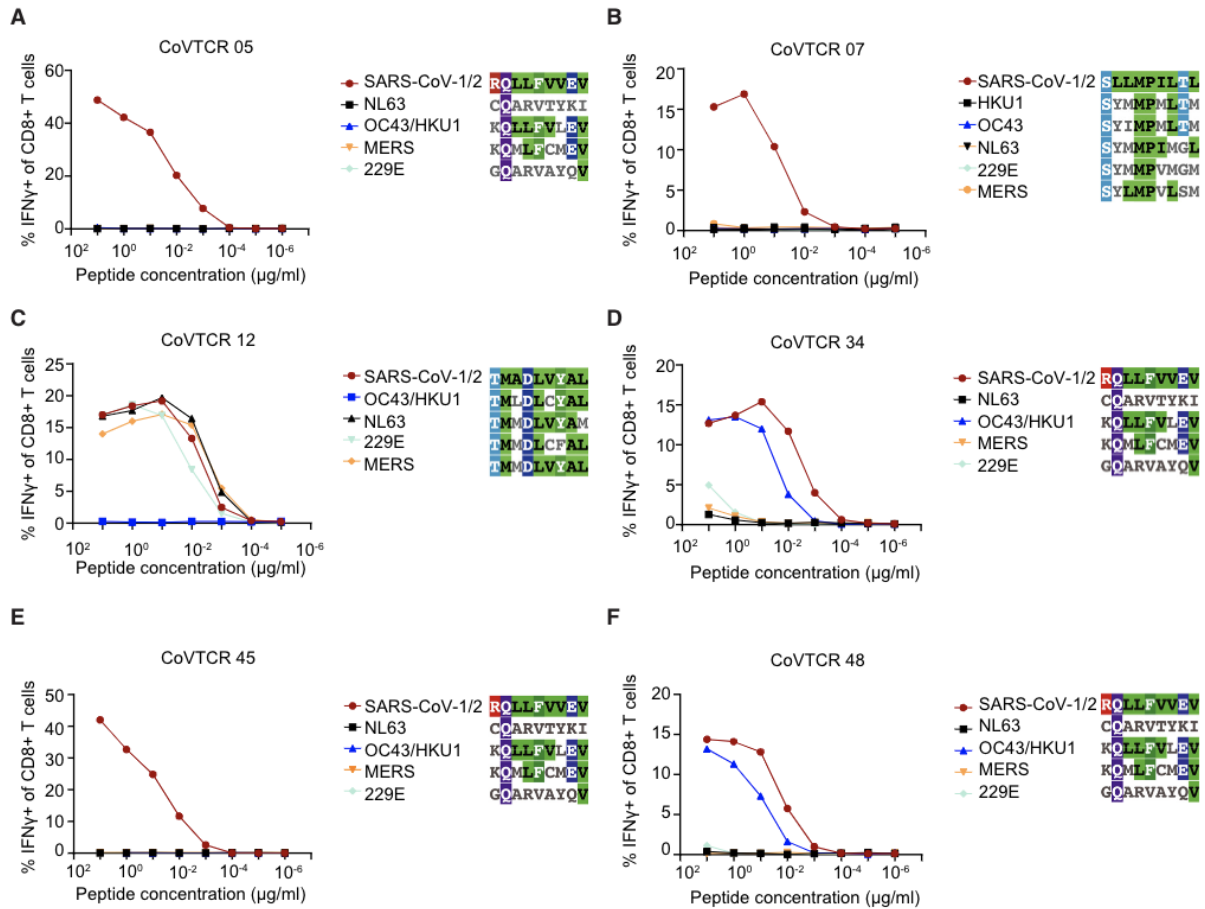
Figure 3-4. SARS-CoV-2 RdRp-targeted T cell receptors broadly recognize human coronaviruses.

Peptide dilution assay to measure affinity 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-A*02:01 to support antigen presentation. Intracellular staining for IFNγ is used to measure T cell activation.
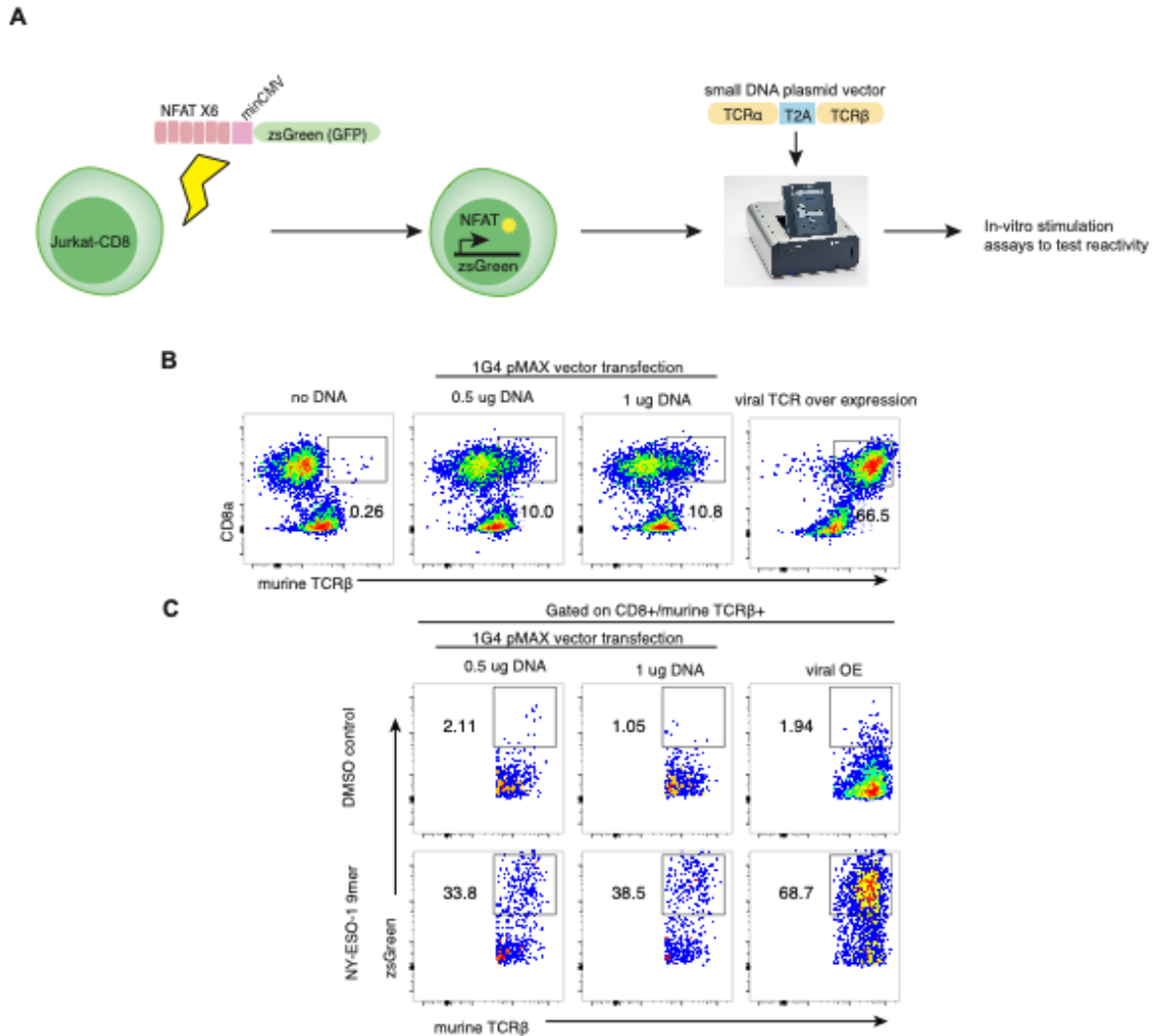
Figure 3-5 (Figure S1). Development of a transfection-based system for TCR construct screening.

A. Diagram for generating a reporter cell line and overexpression of the TCR vector by electroporation. **B.** FACS analysis of 1G4 TCRαβ construct transfection efficiency by staining for the murine beta TCR chain. **C.** FACS analysis of activation in the Jurkat CD8/NFAT-zsGreen cells. Retrovirus transduced Jurkat cells are included as a positive control for the technology. To measure activation transfected or transduced cells are stimulated with NY-ESO-1 HLA-A*02:01 restricted epitope. Related to figure 2
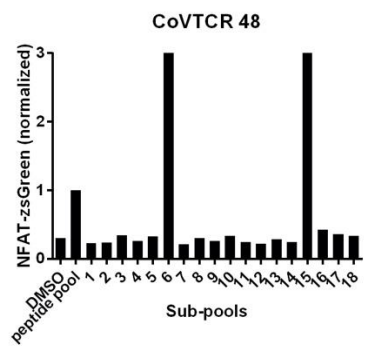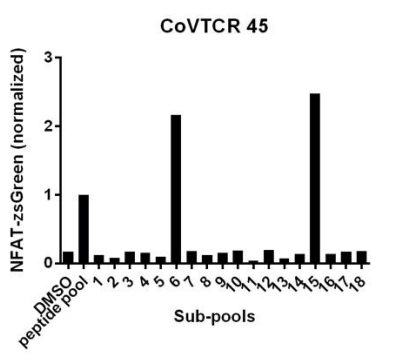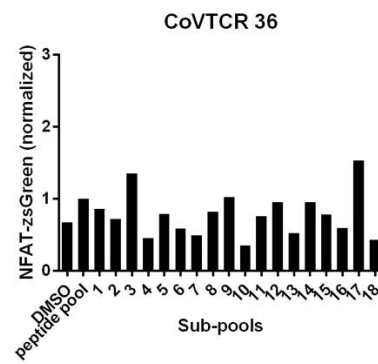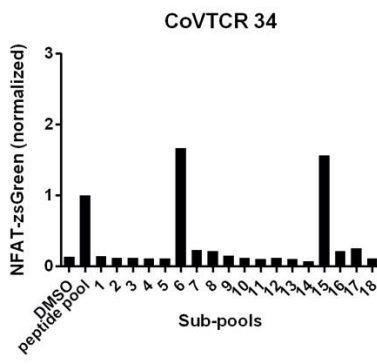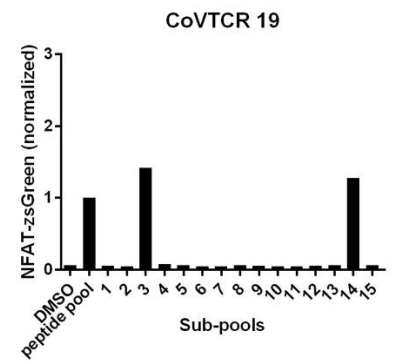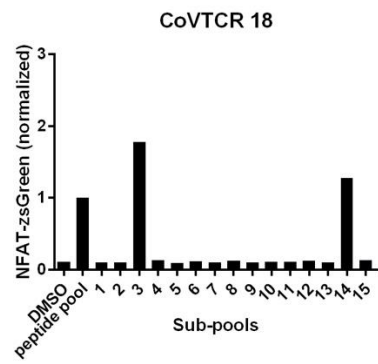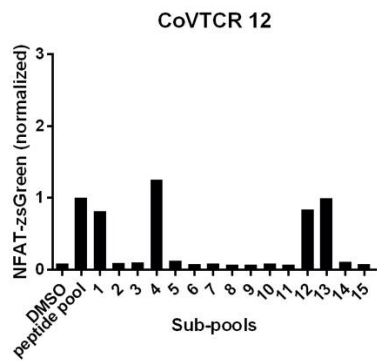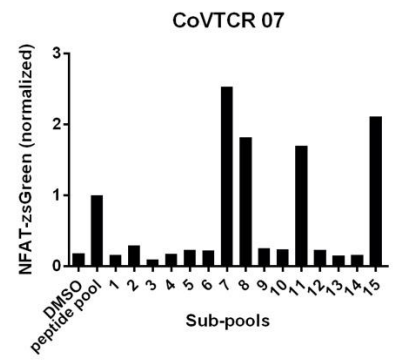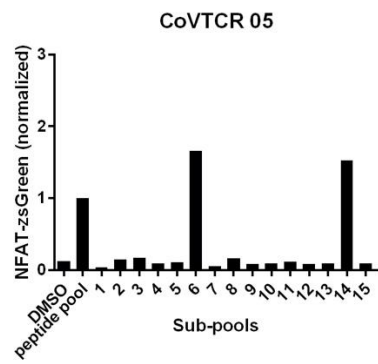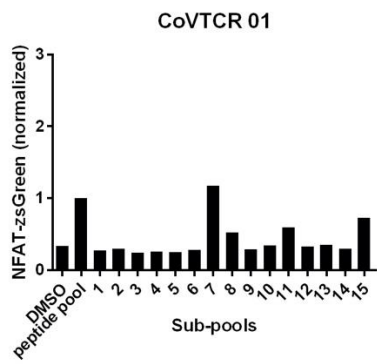
Figure 3-6 (Figure S2). Single reactive epitopes are identified by epitope deconvolution.

Quantification of TCR reactivity to cognate peptide pool and peptide sub-pools by FACS. NFAT-zsGreen (GFP) upregulation is measured by FACS. Signal is normalized to the complete pool. The sub-pool strategy allows for exact epitope determination by comparing multiple sub-pools that elicit response. Related to figure 2
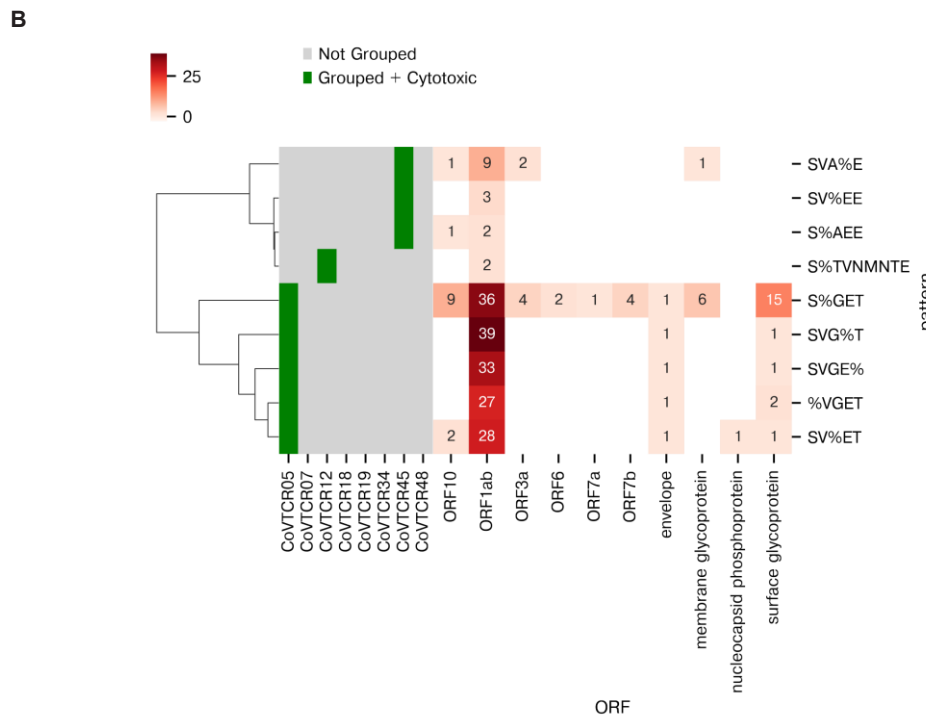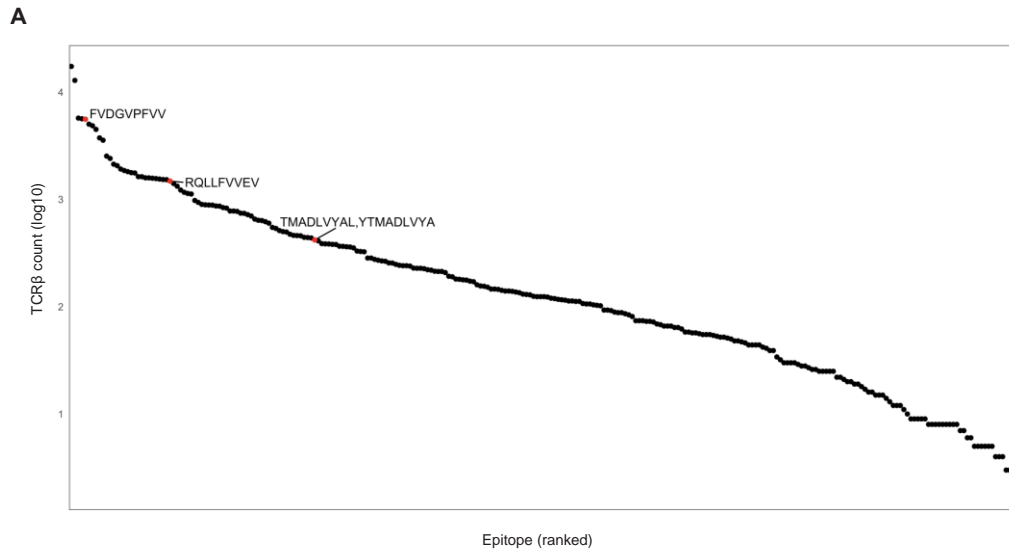
Figure 3-7 (Figure S3). SARS-CoV-2 RdRp epitopes are frequently targeted by unique TCRs from other independent cohorts.

(A)CD8+ T cell epitopes are ranked by the number of unique TCRs in the MIRA data set.

Epitopes identified were ranked and ordered by the number of occurrences (unique TCR count)

in the dataset and plotted. Epitopes that we identified in our own analyses are highlighted in the

plot. Plot was created using ggplot2 in R (v3.6.3). (B) Antigen specificity of global patterns

identified by GLIPH analysis using the combined set of RdRp specific TCRαβ and SARS-CoV-2

antigen specific TCRs from MIRA data set. Heatmap of number of TCRs from MIRA assay

targeting a specific open reading frame (ORF, column) for each GLIPH global pattern (row)

grouped with a CoVTCR ($p < 0.01$ cutoff). TCR groups are included in Table S3. Related to

Figure 3.
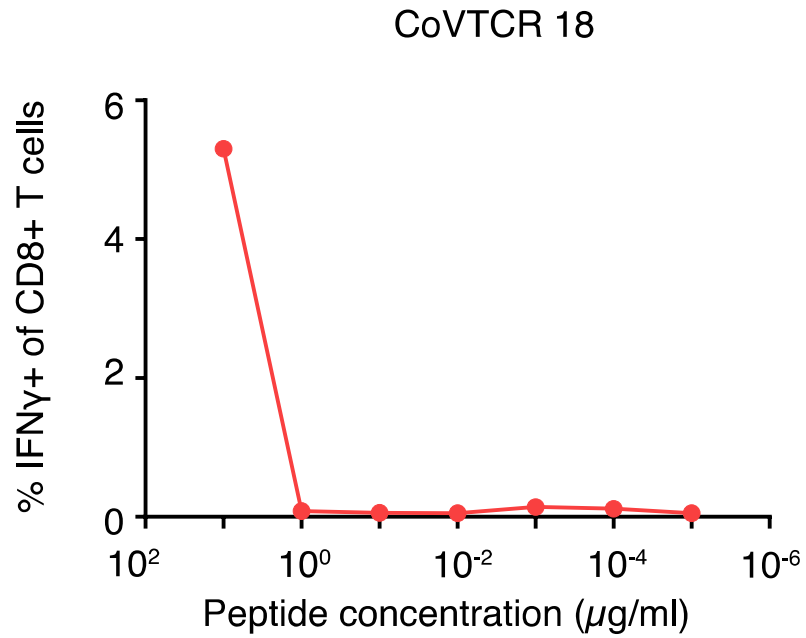
Figure 3-8 (Figure S4). CoVTCR 18 has low affinity for its cognate epitope.

FACS based measurement of intracellular IFNγ production by PBMC engineered to express

CoVTCR 18 in response to 10-fold dilution series of the cognate epitope FV9. Related to Figure

3.

CoVTCR#	Alpha V Region	Alpha J Region	Alpha CDR3 Region	Beta V Region	Beta J Region	Beta CDR3 Region	Epitope	Epitope abbrevation	virus protein	Donor
CoVTCR 01	TRAV12-2	TRAJ54	CAVAQGAQKLVF	TRBV19	TRBJ2-7	CASSTYPAFEQYF	SLLMPILTL, LLMPILTL	SL9	NSP12	A
CoVTCR 05	TRAV13-1	TRAJ12	CAALMDSSYKLIF	TRBV9	TRBJ2-5	CASSVGETQYF	RQLLFVVEV	RV9	NSP12	A
CoVTCR 07	TRAV9-2	TRAJ23	CALGSYNQGGKLIF	TRBV2	TRBJ2-3	CASPTSGKDPDTQYF	SLLMPILTL, LLMPILTL	SL9	NSP12	A
CoVTCR 12	TRAV17	TRAJ57	CATVLPTQGGSEKLVF	TRBV18	TRBJ1-1	CASSLTVNMNTEAFF	TMADLVYAL	TL9	NSP12	A
CoVTCR 18	TRAV12-1	TRAJ45	CVLPSGGGADGLTF	TRBV5-1	TRBJ2-1	CASTGLAGGPYNEQFF	FVDGVPFVV	FV9	NSP12	B
CoVTCR 19	TRAV35	TRAJ43	CAGQLARDMRF	TRBV13	TRBJ2-7	CASRWAGGTAYEQYF	FVDGVPFVV	FV9	NSP12	B
CoVTCR 34	TRAV38-2DV8	TRAJ29	CAYNLGNTPLVF	TRBV7-9	TRBJ2-3	CASSFPLGGSPQLVTQYF	RQLLFVVEV	RV9	NSP12	C
CoVTCR 45	TRAV13-1	TRAJ43	CAATPDDMRF	TRBV9	TRBJ2-1	CASSVAEEQFF	RQLLFVVEV	RV9	NSP12	D
CoVTCR 48	TRAV38-2DV8	TRAJ20	CAYSNDYKLSF	TRBV2	TRBJ2-7	CASSTGPTGLYEQYF	RQLLFVVEV	RV9	NSP12	D

Table S3-1. CoVTCR_epitope_summary

Methods:

Cell culture: Cryo preserved peripheral blood mononuclear cells (PBMCs) were commercially purchased (Allcells and Hemacare). PBMCs were thawed in a water bath set to 37C, transferred to 50 mL conical tube, 1 mL of warm R10 media was added drop wise and then 14 mL R10 was added to the tube. Cells were then centrifuged at 1300 RPM for 7min. To isolate reactive T cells, peripheral blood mononuclear cells (PBMCs) (Allcells and Hemacare) were cultured in TCRPMI media for 8 days in the presence of peptide pools at 1 µg/ml and 25U/ml of IL-2 (Peprotech, CAT# 200-02) as previously described(Nesterenko et al., 2021). PBMCs were then washed two times in PBS (Fisher Scientific, CAT# MT-46013CM), 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, CAT# 31800089) supplemented with 10% FBS (Omega Scientific, CAT# FB-11) and 1X Glutamax (Thermo Fisher, CAT# 35050061). K562 (ATCC CAT# CCL-243) cells were cultured in R10. PBMC for TCR engineering experiments were cultured in AIM V media (Thermo Fisher, CAT# 12055083) supplemented with 5% Human AB serum (Omega Scientific, CAT# HS-20), 50 U/ml of IL-2, 1ng/ml IL-15 (Peprotech, CAT# 200-15), 1X Glutamax and 50µM β-mercaptoethanol. 293T (ATCC) cells were cultured in DMEM (Thermo Fisher CAT# 12100-061) supplemented with 1X Glutamax or Glutamine (Fisher Scientific CAT# BP379-100) and 10% FBS.

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). The PBMCs were stimulated in 96 well U bottom plates with 10 µg/ml of peptide

and 1 µg/ml of CD28/CD49d antibodies (BD Biosciences, CAT# 347690). For peptide titration

assays, serial dilution was set up where the original 10 µg/ml concertation was diluted 10 fold for

every subsequent tube. 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, CAT# 420601) was added. Cells were then incubated for 8 more hours after which

they were either processed or moved to 4C. For analytical assays, cells were stained for surface

markers CD3, CD4, CD8 and intracellular markers TNFα and IFNγ using the Cytofix/Cytoperm

kit (BD Biosciences CAT# 554714). For TCRαβ sequencing via the CLInt-Seq staining we

followed our previously published protocol(Nesterenko et al., 2021). Briefly, this is a technique

for intracellular cytokine staining that is compatible with downstream TCR sequencing by

commercially available single-cell platforms that relies on a reversible crosslinking reagent,

DSP. 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: 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 (RNAse free), 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 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 expression in Jurkat cell line: TCRαβ were constructed from synthetic DNA fragments (IDT and Swift Biosciences). Some TCRs were made as retroviral vectors as described previously(Nesterenko et al., 2021). Some constructs were built into the small pMAX vector (Lonza, CAT# VDC-1040) designed for transfection-based expression. An NFAT-zsGreen reporter construct was overexpressed in the Jurkat CD8 cell line. Both NFAT-szGreen plasmid and the Jurkat CD8 cell line were gifts from the David Baltimore lab. TCRs were infected into the Jurkat cell line using centrifugation at 1350G for 90 minutes at 30C with 5 µg/ml polybrene (Sigma-Aldrich, CAT# H9268). Transfection was done using the Lonza 4D Nucleofactor (Lonza, CAT# AAF-1002B) with the SE cell Line kit S (Lonza CAT# V4XC-1032). Each transfection sample was plated in 20 µL of transfection solution and 2 µL of DNA dissolved in water, was added. DNA minipreps were prepared using the QIAprep miniprep kit (Qiagen CAT# 27106) and concentration was routinely higher than 200 ng/µL. The 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 afterwards they were used for functional assays over the course of three days.

Stimulation of Jurkat cells engineered to express TCRαβ: 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 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+.

PBMC engineering via retrovirus: Retrovirus was produced as described previously(Nesterenko et al., 2021). PBMCs were activated with CD3/CD28 beads (Thermo Fisher, CAT# 11132D) in 24 well plates. After 3 days, TCR construct or empty construct NGFR control retrovirus was added to the cells with 5 µg/ml polybrene (Sigma-Aldrich, CAT# H9268). 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, the infection was repeated, and the following day cells were washed. Cells were 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 TCRs in PBMCs and allow surface TCR staining.

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 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 selected for high levels of RFP and GFP. Stable RdRp protein level was confirmed by Western blot using anti-Strep-tag II antibody (Abcam, ab180957, 1:500) and goat anti-rabbit-IgG HRP(Bio-Rad, 170-6515, 1:5000). These single cell clones were used for the cytotoxicity assay.

Cytotoxicity analysis: K562 target cell lines were co-cultured with TCR engineered PBMCs at a 2:1 effector to target ratio, in R10 media with 1 µg/ml of CD28/CD49d antibodies (BD CAT# 347690). 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 ELISA analysis as described previously(Nesterenko et al., 2021).

Antibodies: NGFR-PECy7 (Biolegend CAT# 345110), murine TCRβ-APC (Biolegend CAT# 109212), CD3-APCCy7 (Thermo Fisher, CAT# 47-0036-42), CD8a-PE (Thermo Fisher, CAT# 12-0088-42), CD4-PECy7 (Biolegend, CAT# 300512). IFNγ-APC (Biolegend, CAT# 506510), TNFα-FITC (Biolegend, CAT# 502906), HLA-A2-APC (Thermo Fisher, CAT# 17-9876-42), anti-Strep-tag II (Abcam CAT# ab180957), secondary goat anti-rabbit IgG HRP (Bio-Rad, CAT#170-6515).

Protein conservation analysis: 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 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). Data 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/).

Lollipop plot generation: SARS-CoV-2 amino acid sequence variations representing 893,589 GISAID 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 numbering, nucleotide location, and amino acid variations. CoV-GLUE excludes certain GISAID sequences; information about the total number of sequences retrieved from GISAID and 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).

Comparison of TCRαβ constructs to a large, publicly available data set: 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). 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 by each MIRA TCR and counted the number of MIRA TCRs per ORF for each GLIPH group.

Data and materials availability: All data are available in the main text or the supplementary information.

Supplemental information:

Figure. S1. Development of a transfection-based system for TCR construct screening.
(A)Diagram for generating a reporter cell line and overexpression of the TCR vector by electroporation. (B) FACS analysis of 1G4 TCRαβ construct transfection efficiency by staining for the murine beta TCR chain. (C) FACS analysis of activation in the Jurkat CD8/NFAT-zsGreen cells. Retrovirus transduced Jurkat cells are included as a positive control for the technology. To measure activation transfected or transduced cells are stimulated with NY-ESO-1 HLA-A*02:01 restricted epitope.

Figure S2. Single reactive epitopes are identified by epitope deconvolution.

Quantification of TCR reactivity to cognate peptide pool and peptide sub-pools by FACS.
NFAT-zsGreen (GFP) upregulation is measured by FACS. Signal is normalized to the complete
pool. The sub-pool strategy allows for exact epitope determination by comparing multiple sub-
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**Figure S3. SARS-CoV-2 RdRp epitopes are frequently targeted by unique TCRs from
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(A)CD8+ T cell epitopes are ranked by the number of unique TCRs in the MIRA data set.
Epitopes identified were ranked and ordered by the number of occurrences (unique TCR count)
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Figure S4. CoVTCR 18 has low affinity for its cognate epitope.

FACS based measurement of intracellular IFNγ production by PBMC engineered to express
CoVTCR 18 in response to 10-fold dilution series of the cognate epitope FV9.

Table S1. Summary of reactive TCR clones and cognate epitopes

Table S2. Nucleotide sequence of TCR clones that have been validated in PBMC

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

References:

Adler, S.P., Manganello, A.-M., Lee, R., Mcvoy, M.A., Nixon, D.E., Plotkin, S., Mocarski, E., Cox, J.H., Fast, P.E., Nesterenko, P.A., et al. (2016). The Journal of Infectious Diseases A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus-Seronegative Men.

Ahmadzadeh, M., Pasetto, A., Jia, L., Deniger, D.C., Stevanović, S., Robbins, P.F., and Rosenberg, S.A. (2019). Tumor-infiltrating human CD4 [+] regulatory T cells display a distinct TCR repertoire and exhibit tumor and neoantigen reactivity. Sci. Immunol. *4*, eaao4310.

Alanio, C., Lemaitre, F., Law, H.K.W., Hasan, M., and Albert, M.L. (2010). Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies. Blood *115*, 3718–3725.

Ali, K., Soond, D.R., Piñeiro, R., Hagemann, T., Pearce, W., Lim, E.L., Bouabe, H., Scudamore, C.L., Hancox, T., Maecker, H., et al. (2014). Inactivation of PI(3)K p110δ breaks regulatory T-cell-mediated immune tolerance to cancer. Nature *510*, 407–411.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Anand, S.P., Prévost, J., Nayrac, M., Beaudoin-Bussières, G., Benlarbi, M., Gasser, R., Brassard, N., Laumaea, A., Gong, S.Y., Bourassa, C., et al. (2021). Longitudinal analysis of humoral immunity against SARS-CoV-2 Spike in convalescent individuals up to 8 months post-symptom onset. Cell Reports Med. *2*, 100290.

Ashouri, J.F., and Weiss, A. (2016). B Cells Antigen Receptor Signaling in Human T and Endogenous Nur77 Is a Specific Indicator of.

Attar, M., Sharma, E., Li, S., Bryer, C., Cubitt, L., Broxholme, J., Lockstone, H., Kinchen, J.,

Simmons, A., Piazza, P., et al. (2018). A practical solution for preserving single cells for RNA sequencing. Sci. Rep. *8*, 1–10.

B, R., B, A., S, P., B, P., and M, N. (2020). NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. Nucleic Acids Res. *48*, W449–W454.

Baden, L.R., El Sahly, H.M., Essink, B., Kotloff, K., Frey, S., Novak, R., Diemert, D., Spector, S.A., Rouphael, N., Creech, C.B., et al. (2021). Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. N. Engl. J. Med. *384*, 403–416.

Bagaev, D. V., Vroomans, R.M.A., Samir, J., Stervbo, U., Rius, C., Dolton, G., Greenshields-Watson, A., Attaf, M., Egorov, E.S., Zvyagin, I. V., et al. (2020). VDJdb in 2019: Database extension, new analysis infrastructure and a T-cell receptor motif compendium. Nucleic Acids Res. *48*, D1057–D1062.

Bange, E.M., Han, N.A., Wileyto, P., Kim, J.Y., Gouma, S., Robinson, J., Greenplate, A.R., Hwee, M.A., Porterfield, F., Owoyemi, O., et al. (2021). CD8+ T cells contribute to survival in patients with COVID-19 and hematologic cancer. Nat. Med. 1–10.

Basar, R., Uprety, N., Ensley, E., Daher, M., Klein, K., Martinez, F., Aung, F., Shanley, M., Hu, B., Gokdemir, E., et al. (2021). Generation of glucocorticoid-resistant SARS-CoV-2 T cells for adoptive cell therapy. Cell Rep. *36*, 109432.

Becker, J.T., Olson, B.M., Johnson, L.E., Davies, J.G., Dunphy, E.J., and McNeel, D.G. (2010). DNA vaccine encoding prostatic acid phosphatase (PAP) elicits long-term T-cell responses in patients with recurrent prostate cancer. J. Immunother. *33*, 639–647.

Bernal, J.L., Andrews, N., Gower, C., Gallagher, E., Simmons, R., Thelwall, S., Stowe, J., Tessier, E., Groves, N., Dabrera, G., et al. (2021). Effectiveness of Covid-19 Vaccines against

the B.1.617.2 (Delta) Variant. Https://Doi.Org/10.1056/NEJMoa2108891 NEJMoa2108891.

Le Bert, N., Tan, A.T., Kunasegaran, K., Tham, C.Y.L., Hafezi, M., Chia, A., Chng, M.H.Y., Lin, M., Tan, N., Linster, M., et al. (2020). SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls. Nature *584*, 457.

Bethune, M.T., and Joglekar, A. V. (2017). Personalized T cell-mediated cancer immunotherapy: progress and challenges. Curr. Opin. Biotechnol. *48*, 142–152.

Bethune, M.T., Li, X.-H., Yu, J., McLaughlin, J., Cheng, D., Mathis, C., Moreno, B.H., Woods, K., Knights, A.J., and Garcia-Diaz, A. (2018). Isolation and characterization of NY-ESO-1–specific T cell receptors restricted on various MHC molecules. Proc. Natl. Acad. Sci. *115*, E10702–E10711.

Betts, M.R., Brenchley, J.M., Price, D.A., De Rosa, S.C., Douek, D.C., Roederer, M., and Koup, R.A. (2003). Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J. Immunol. Methods *281*, 65–78.

Bluestone, J.A., Buckner, J.H., Fitch, M., Gitelman, S.E., Gupta, S., Hellerstein, M.K., Herold, K.C., Lares, A., Lee, M.R., Li, K., et al. (2015). Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci. Transl. Med. *7*.

Boehmer, H. Von, Aifantis, I., Gounari, F., Azogui, O., Haughn, L., Apostolou, I., Jaeckel, E., Grassi, F., and Klein, L. (2003). Thymic selection revisited: how essential is it? Immunol. Rev. *191*, 62–78.

Braun, J., Loyal, L., Frentsch, M., Wendisch, D., Georg, P., Kurth, F., Hippenstiel, S., Dingeldey, M., Kruse, B., Fauchere, F., et al. (2020). SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19. Nature *587*, 270–274.

Bulek, A.M., Cole, D.K., Skowera, A., Dolton, G., Gras, S., Madura, F., Fuller, A., Miles, J.J.,

Gostick, E., Price, D.A., et al. (2012). Structural basis for the killing of human beta cells by CD8 + T cells in type 1 diabetes. Nat. Immunol. *13*, 283–289.

Chen, Z., and John Wherry, E. (2020). T cell responses in patients with COVID-19. Nat. Rev. Immunol. *20*, 529–536.

Chen, J., López-Moyado, I.F., Seo, H., Lio, C.W.J., Hempleman, L.J., Sekiya, T., Yoshimura, A., Scott-Browne, J.P., and Rao, A. (2019). NR4A transcription factors limit CAR T cell function in solid tumours. Nature *567*, 530–534.

Choudhuri, K., Kearney, A., Bakker, T.R., and Van Der Merwe, P.A. (2005). Immunology: How Do T Cells Recognize Antigen? Curr. Biol. *15*, R382–R385.

Collins, D.R., Gaiha, G.D., and Walker, B.D. (2020). CD8+ T cells in HIV control, cure and prevention. Nat. Rev. Immunol. *20*, 471–482.

Culina, S., Lalanne, A.I., Afonso, G., Cerosaletti, K., Pinto, S., Sebastiani, G., Kuranda, K., Nigi, L., Eugster, A., Østerbye, T., et al. (2018). Islet-reactive CD8+ T cell frequencies in the pancreas, but not in blood, distinguish type 1 diabetic patients from healthy donors. Sci. Immunol. *3*, 4013.

D'angelo, S.P., Melchiori, L., Merchant, M.S., Bernstein, D., Glod, J., Kaplan, R., Grupp, S., Tap, W.D., Chagin, K., Binder, G.K., et al. (2018). Antitumor activity associated with prolonged persistence of adoptively transferred NY-ESO-1c259T cells in synovial sarcoma. Cancer Discov. *8*, 944–957.

D'Angelo, S.P., Melchiori, L., Merchant, M.S., Bernstein, D., Glod, J., Kaplan, R., Grupp, S., Tap, W.D., Chagin, K., Binder, G.K., et al. (2018). Antitumor Activity Associated with Prolonged Persistence of Adoptively Transferred NY-ESO-1 c259T Cells in Synovial Sarcoma. Cancer Discov. *8*, 944–957.

Davis, M.M., and Bjorkman, P.J. (1988). T-cell antigen receptor genes and T-cell recognition. Nat. 1988 3346181 *334*, 395–402.

DeLeeuw, R.J., Kost, S.E., Kakal, J.A., and Nelson, B.H. (2012). The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: A critical review of the literature. Clin. Cancer Res. *18*, 3022–3029.

Delong, T., Wiles, T.A., Baker, R.L., Bradley, B., Barbour, G., Reisdorph, R., Armstrong, M., Powell, R.L., Reisdorph, N., Kumar, N., et al. (2016). Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. Science (80-.). *351*, 711–714.

Depil, S., Duchateau, P., Grupp, S.A., Mufti, G., and Poirot, L. (2020). 'Off-the-shelf' allogeneic CAR T cells: development and challenges. Nat. Rev. Drug Discov. *19*, 185–199.

Dolton, G., Zervoudi, E., Rius, C., Wall, A., Thomas, H.L., Fuller, A., Yeo, L., Legut, M., Wheeler, S., Attaf, M., et al. (2018). Optimized Peptide–MHC Multimer Protocols for Detection and Isolation of Autoimmune T-Cells. Front. Immunol. *9*.

Duhen, T., Duhen, R., Montler, R., Moses, J., Moudgil, T., De Miranda, N.F., Goodall, C.P., Blair, T.C., Fox, B.A., McDermott, J.E., et al. (2018). Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat. Commun. *9*, 1–13.

Dutta, N.K., Mazumdar, K., and Gordy, J.T. (2020). The Nucleocapsid Protein of SARS–CoV-2: a Target for Vaccine Development. J. Virol. *94*.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. *30*, 207–210.

Einsele, H., Roosnek, E., Rufer, N., Sinzger, C., Riegler, S., Löffler, J., Grigoleit, U., Moris, A., Rammensee, H.-G., Kanz, L., et al. (2002). Infusion of cytomegalovirus (CMV)–specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood *99*, 3916–

3922.

Ferreras, C., Pascual-Miguel, B., Mestre-Durán, C., Navarro-Zapata, A., Clares-Villa, L., Martín-Cortázar, C., de Paz, R., Marcos, A., Vicario, J.L., Balas, A., et al. (2020). SARS-CoV-2 specific memory T lymphocytes from COVID-19 convalescent donors: Identification, biobanking and large-scale production for adoptive cell therapy. BioRxiv 2020.10.23.352294.

Feuchtinger, T., Opherk, K., Bethge, W.A., Topp, M.S., Schuster, F.R., Weissinger, E.M., Mohty, M., Or, R., Maschan, M., Schumm, M., et al. (2010a). Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood *116*, 4360–4367.

Feuchtinger, T., Opherk, K., Bethge, W.A., Topp, M.S., Schuster, F.R., Weissinger, E.M., Mohty, M., Or, R., Maschan, M., Schumm, M., et al. (2010b). Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation.

Forni, D., Cagliani, R., Clerici, M., and Sironi, M. (2017). Molecular Evolution of Human Coronavirus Genomes. Trends Microbiol. *25*, 35–48.

Frank, A.M., Braun, A.H., Scheib, L., Agarwal, S., Schneider, I.C., Fusil, F., Perian, S., Sahin, U., Thalheimer, F.B., Verhoeyen, E., et al. (2020). Combining T-cell-specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. Blood Adv. *4*, 5702–5715.

Garcia, K.C., and Adams, E.J. (2005). How the T Cell Receptor Sees Antigen—A Structural View. Cell *122*, 333–336.

Gauttier, V., Morello, A., Girault, I., Mary, C., Belarif, L., Desselle, A., Wilhelm, E., Bourquard, T., Pengam, S., Teppaz, G., et al. (2020). Tissue-resident memory CD8 T-cell responses elicited by a single injection of a multi-target COVID-19 vaccine. BioRxiv 2020.08.14.240093.

Gerlach, J.P., van Buggenum, J.A.G., Tanis, S.E.J., Hogeweg, M., Heuts, B.M.H., Muraro, M.J., Elze, L., Rivello, F., Rakszewska, A., van Oudenaarden, A., et al. (2019). Combined quantification of intracellular (phospho-)proteins and transcriptomics from fixed single cells. Sci. Rep. *9*, 1469.

Glanville, J., Huang, H., Nau, A., Hatton, O., Wagar, L.E., Rubelt, F., Ji, X., Han, A., Krams, S.M., Pettus, C., et al. (2017). Identifying specificity groups in the T cell receptor repertoire. Nature *547*, 94–98.

Gordon, D.E., Jang, G.M., Bouhaddou, M., Xu, J., Obernier, K., White, K.M., O'Meara, M.J., Rezelj, V. V., Guo, J.Z., Swaney, D.L., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. Nature *583*, 459–468.

Grifoni, A., Sidney, J., Zhang, Y., Scheuermann, R.H., Peters, B., and Sette, A. (2020a). A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2. Cell Host Microbe *27*, 671-680.e2.

Grifoni, A., Weiskopf, D., Ramirez, S.I., Mateus, J., Dan, J.M., Moderbacher, C.R., Rawlings, S.A., Sutherland, A., Premkumar, L., Jadi, R.S., et al. (2020b). Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. Cell *181*, 1489-1501.e15.

Hall, C.B., Powell, K.R., MacDonald, N.E., Gala, C.L., Menegus, M.E., Suffin, S.C., and Cohen, H.J. (1986). Respiratory Syncytial Viral Infection in Children with Compromised Immune Function. N. Engl. J. Med. *315*, 77–81.

Hansen, S.G., Sacha, J.B., Hughes, C.M., Ford, J.C., Burwitz, B.J., Scholz, I., Gilbride, R.M., Lewis, M.S., Gilliam, A.N., Ventura, A.B., et al. (2013). Cytomegalovirus vectors violate CD8+ T cell epitope recognition paradigms. Science (80-.). *340*.

Harty, J.T., Tvinnereim, A.R., and White, D.W. (2000). Cd8+ T cell effector mechanisms in resistance to infection. Annu. Rev. Immunol. *18*, 275–308.

Harvey, W.T., Carabelli, A.M., Jackson, B., Gupta, R.K., Thomson, E.C., Harrison, E.M., Ludden, C., Reeve, R., Rambaut, A., Peacock, S.J., et al. (2021). SARS-CoV-2 variants, spike mutations and immune escape. Nat. Rev. Microbiol. 2021 197 *19*, 409–424.

Hatcher, E.L., Zhdanov, S.A., Bao, Y., Blinkova, O., Nawrocki, E.P., Ostapchuck, Y., Schaffer, A.A., and Rodney Brister, J. (2017). Virus Variation Resource-improved response to emergent viral outbreaks. Nucleic Acids Res. *45*, D482–D490.

Hoffmann, M., Arora, P., Groß, R., Seidel, A., Hörnich, B.F., Hahn, A.S., Krüger, N., Graichen, L., Hofmann-Winkler, H., Kempf, A., et al. (2021). SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. Cell.

Honaker, Y., Hubbard, N., Xiang, Y., Fisher, L., Hagin, D., Sommer, K., Song, Y., Yang, S.J., Lopez, C., Tappen, T., et al. (2020). Gene editing to induce FOXP3 expression in human CD4+ T cells leads to a stable regulatory phenotype and function. Sci. Transl. Med. *12*, 6422.

Hsiue, E.H.-C., Wright, K.M., Douglass, J., Hwang, M.S., Mog, B.J., Pearlman, A.H., Paul, S., DiNapoli, S.R., Konig, M.F., Wang, Q., et al. (2021). Targeting a neoantigen derived from a common TP53 mutation. Science (80-.). *371*.

Huang, H., Wang, C., Rubelt, F., Scriba, T.J., and Davis, M.M. (2020). Analyzing the Mycobacterium tuberculosis immune response by T-cell receptor clustering with GLIPH2 and genome-wide antigen screening. Nat. Biotechnol. *38*, 1194–1202.

Ishizuka, J., Grebe, K., Shenderov, E., Peters, B., Chen, Q., Peng, Y., Wang, L., Dong, T., Pasquetto, V., Oseroff, C., et al. (2009). Quantitating T Cell Cross-Reactivity for Unrelated Peptide Antigens. J. Immunol. *183*, 4337–4345.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. Cell *126*, 1121–1133.

Iwafuchi-Doi, M., and Zaret, K.S. (2016). Cell fate control by pioneer transcription factors. Dev. *143*, 1833–1837.

Iwafuchi-Doi, M., Donahue, G., Kakumanu, A., Watts, J.A., Mahony, S., Pugh, B.F., Lee, D., Kaestner, K.H., and Zaret, K.S. (2016). The Pioneer Transcription Factor FoxA Maintains an Accessible Nucleosome Configuration at Enhancers for Tissue-Specific Gene Activation. Mol. Cell *62*, 79–91.

Johnson, L.E., and McNeel, D.G. (2012). Identification of prostatic acid phosphatase (PAP) specific HLA-DR1-restricted t-cell epitopes. Prostate *72*, 730–740.

Johnson, L.A., Heemskerk, B., Powell, D.J., Cohen, C.J., Morgan, R.A., Dudley, M.E., Robbins, P.F., and Rosenberg, S.A. (2006a). Gene Transfer of Tumor-Reactive TCR Confers Both High Avidity and Tumor Reactivity to Nonreactive Peripheral Blood Mononuclear Cells and Tumor-Infiltrating Lymphocytes. J. Immunol. *177*, 6548–6559.

Johnson, L.A., Heemskerk, B., Powell, D.J., Cohen, C.J., Morgan, R.A., Dudley, M.E., Robbins, P.F., and Rosenberg, S.A. (2006b). Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. J. Immunol. *177*, 6548–6559.

Johnson, L.A., Morgan, R.A., Dudley, M.E., Cassard, L., Yang, J.C., Hughes, M.S., Kammula, U.S., Royal, R.E., Sherry, R.M., Wunderlich, J.R., et al. (2009). Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood *114*, 535–546.

Jozwik, A., Habibi, M.S., Paras, A., Zhu, J., Guvenel, A., Dhariwal, J., Almond, M., Wong, E.H.C., Sykes, A., Maybeno, M., et al. (2015). RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. Nat. Commun. *6*.

Kantoff, P.W., Higano, C.S., Shore, N.D., Berger, E.R., Small, E.J., Penson, D.F., Redfern, C.H., Ferrari, A.C., Dreicer, R., Sims, R.B., et al. (2010). Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. N. Engl. J. Med. *363*, 411–422.

Katzenelenbogen, Y., Sheban, F., Yalin, A., Yofe, I., Svetlichnyy, D., Jaitin, D.A., Bornstein, C., Moshe, A., Keren-Shaul, H., Cohen, M., et al. (2020). Coupled scRNA-Seq and Intracellular Protein Activity Reveal an Immunosuppressive Role of TREM2 in Cancer. Cell *182*, 872-885.e19.

Keller, M.D., Harris, K.M., Jensen-Wachspress, M.A., Kankate, V. V., Lang, H., Lazarski, C.A., Durkee-Shock, J., Lee, P.H., Chaudhry, K., Webber, K., et al. (2020). SARS-CoV-2–specific T cells are rapidly expanded for therapeutic use and target conserved regions of the membrane protein. Blood *136*, 2905–2917.

Khoury, D.S., Cromer, D., Reynaldi, A., Schlub, T.E., Wheatley, A.K., Juno, J.A., Subbarao, K., Kent, S.J., Triccas, J.A., and Davenport, M.P. (2021). Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nat. Med. 2021 277 *27*, 1205–1211.

Killerby, M.E., Biggs, H.M., Haynes, A., Dahl, R.M., Mustaquim, D., Gerber, S.I., and Watson, J.T. (2018). Human coronavirus circulation in the United States 2014–2017. J. Clin. Virol. *101*, 52–56.

Klenerman, P., and Oxenius, A. (2016). T cell responses to cytomegalovirus. Nat. Rev. Immunol. *16*, 367.

Klinger, M., Pepin, F., Wilkins, J., Asbury, T., Wittkop, T., Zheng, J., Moorhead, M., and Faham, M. (2015). Multiplex Identification of Antigen-Specific T Cell Receptors Using a Combination of Immune Assays and Immune Receptor Sequencing. PLoS One *10*, e0141561.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. (2009). IL-17 and Th17 Cells. Annu. Rev. Immunol. *27*, 485–517.

Kuzmina, A., Khalaila, Y., Voloshin, O., Keren-Naus, A., Bohehm, L., Raviv, Y., Shemer-Avni, Y., Rosenberg, E., and Taube, R. (2021). SARS CoV-2 spike variants exhibit differential infectivity and neutralization resistance to convalescent or post-vaccination sera. Cell Host Microbe.

Lambert, S.A., Jolma, A., Campitelli, L.F., Das, P.K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T.R., and Weirauch, M.T. (2018). The Human Transcription Factors. Cell *172*, 650–665.

Leitman, E.M., Willberg, C.B., De Burgh-Thomas, A., Streeck, H., Goulder, P.J.R., and Matthews, P.C. (2016). Subdominant Gag-specific anti-HIV efficacy in an HLA-B∗ 57-positive elite controller. AIDS *30*, 972–974.

Li, M.O., and Rudensky, A.Y. (2016). T cell receptor signalling in the control of regulatory T cell differentiation and function. Nat. Rev. Immunol. *16*, 220–233.

Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., Cheng, L., Li, J., Wang, X., Wang, F., et al. (2020). Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. Nat. Med. *26*, 842–844.

Lineburg, K.E., Grant, E.J., Swaminathan, S., Chatzileontiadou, D.S., Szeto, C., Sloane, H., Panikkar, A., Raju, J., Crooks, P., Rehan, S., et al. (2021). CD8+ T cells specific for an immunodominant SARS-CoV-2 nucleocapsid epitope cross-react with selective seasonal

coronaviruses. Immunity *0*.

Linette, G.P., Stadtmauer, E.A., Maus, M. V., Rapoport, A.P., Levine, B.L., Emery, L., Litzky, L., Bagg, A., Carreno, B.M., Cimino, P.J., et al. (2013). Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood *122*, 863.

Linnemann, C., van Buuren, M.M., Bies, L., Verdegaal, E.M.E., Schotte, R., Calis, J.J.A., Behjati, S., Velds, A., Hilkmann, H., Atmioui, D. el, et al. (2014). High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat. Med. *21*, 81.

Lipsitch, M., Grad, Y.H., Sette, A., and Crotty, S. (2020). Cross-reactive memory T cells and herd immunity to SARS-CoV-2. Nat. Rev. Immunol. *20*, 709–713.

Lumley, S.F., O'Donnell, D., Stoesser, N.E., Matthews, P.C., Howarth, A., Hatch, S.B., Marsden, B.D., Cox, S., James, T., Warren, F., et al. (2020). Antibody Status and Incidence of SARS-CoV-2 Infection in Health Care Workers. Https://Doi.Org/10.1056/NEJMoa2034545 *384*, 533–540.

Lynn, R.C., Weber, E.W., Sotillo, E., Gennert, D., Xu, P., Good, Z., Anbunathan, H., Lattin, J., Jones, R., Tieu, V., et al. (2019). c-Jun overexpression in CAR T cells induces exhaustion resistance. Nat. 2019 5767786 *576*, 293–300.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell *161*, 1202–1214.

Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R.N., Potter, S.C., Finn, R.D., et al. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. *47*, W636–W641.

Mallajosyula, V., Ganjavi, C., Chakraborty, S., McSween, A.M., Pavlovitch-Bedzyk, A.J., Wilhelmy, J., Nau, A., Manohar, M., Nadeau, K.C., and Davis, M.M. (2021). CD8+ T cells specific for conserved coronavirus epitopes correlate with milder disease in COVID-19 patients. Sci. Immunol. *6*, eabg5669.

Maness, N.J., Valentine, L.E., May, G.E., Reed, J., Piaskowski, S.M., Soma, T., Furlott, J., Rakasz, E.G., Friedrich, T.C., Price, D.A., et al. (2007). AIDS virus-specific CD8+ T lymphocytes against an immunodominant cryptic epitope select for viral escape. J. Exp. Med. *204*, 2505–2512.

Mateus, J., Grifoni, A., Tarke, A., Sidney, J., Ramirez, S.I., Dan, J.M., Burger, Z.C., Rawlings, S.A., Smith, D.M., Phillips, E., et al. (2020). Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. Science (80-.). *370*, 89–94.

Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., et al. (2018). Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. Http://Dx.Doi.Org/10.1056/NEJMoa1709866 *378*, 439–448.

McMahan, K., Yu, J., Mercado, N.B., Loos, C., Tostanoski, L.H., Chandrashekar, A., Liu, J., Peter, L., Atyeo, C., Zhu, A., et al. (2021). Correlates of protection against SARS-CoV-2 in rhesus macaques. Nature *590*, 630–634.

McMichael, A.J., Gotch, F.M., Noble, G.R., and Beare, P.A.S. (1983). Cytotoxic T-Cell Immunity to Influenza. N. Engl. J. Med. *309*, 13–17.

Milner, J.J., Toma, C., Yu, B., Zhang, K., Omilusik, K., Phan, A.T., Wang, D., Getzler, A.J., Nguyen, T., Crotty, S., et al. (2017). Runx3 programs CD8+ T cell residency in non-lymphoid tissues and tumours. Nature.

Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. (2011). T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J. Exp. Med. *208*, 1279–1289.

Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalían, S.L., Kammula, U.S., Restifo, N.P., et al. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. Science (80-.). *314*, 126–129.

Muik, A., Wallisch, A.K., Sänger, B., Swanson, K.A., Mühl, J., Chen, W., Cai, H., Maurus, D., Sarkar, R., Türeci, Ö., et al. (2021). Neutralization of SARS-CoV-2 lineage B.1.1.7 pseudovirus by BNT162b2 vaccine–elicited human sera. Science (80-.). *371*, 1152–1153.

Murray, S.E., Nesterenko, P.A., Vanarsdall, A.L., Munks, M.W., Smart, S.M., Veziroglu, E.M., Sagario, L.C., Lee, R., Claas, F.H.J., Doxiadis, I.I.N., et al. (2017). Fibroblast-adapted human CMV vaccines elicit predominantly conventional CD8 T cell responses in humans. J. Exp. Med. *214*, 1889–1899.

Nair, S., Boczkowski, D., Fassnacht, M., Pisetsky, D., and Gilboa, E. (2007). Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity. Cancer Res. *67*, 371–380.

Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., et al. (2017). Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. Https://Doi.Org/10.1056/NEJMoa1707447 *377*, 2531–2544.

Nesterenko, P.A., McLaughlin, J., Cheng, D., Bangayan, N.J., Sojo, G.B., Seet, C.S., Qin, Y., Mao, Z., Obusan, M.B., Phillips, J.W., et al. (2021). Droplet-based mRNA sequencing of fixed and permeabilized cells by CLInt-seq allows for antigen-specific TCR cloning. Proc. Natl. Acad.

Sci. U. S. A. *118*.

Newell, E.W., and Davis, M.M. (2014). Beyond model antigens: high-dimensional methods for the analysis of antigen-specific T cells. Nat. Biotechnol. 2013 322 *32*, 149–157.

Nisitani, S., Kato, R.M., Rawlings, D.J., Witte, O.N., and Wahl, M.I. (1999). In situ detection of activated Bruton's tyrosine kinase in the Ig signaling complex by phosphopeptide-specific monoclonal antibodies. Proc. Natl. Acad. Sci. U. S. A. *96*, 2221–2226.

Nolan, S., Vignali, M., Klinger, M., Dines, J.N., Kaplan, I.M., Svejnoha, E., Craft, T., Boland, K., Pesesky, M., Gittelman, R.M., et al. (2020). A large-scale database of T-cell receptor beta (TCRβ) sequences and binding associations from natural and synthetic exposure to SARS-CoV-2. Res. Sq.

NR, L., DG, S., M, R., and D, B. (1987). Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol. Cell. Biol. *7*, 3237–3243.

Oestreich, K.J., and Weinmann, A.S. (2012). Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. Nat. Rev. Immunol. *12*, 799–804.

Oh, D.Y., Kwek, S.S., Raju, S.S., Li, T., McCarthy, E., Chow, E., Aran, D., Ilano, A., Pai, C.C.S., Rancan, C., et al. (2020). Intratumoral CD4+ T Cells Mediate Anti-tumor Cytotoxicity in Human Bladder Cancer. Cell *181*, 1612-1625.e13.

Olson, B.M., Frye, T.P., Johnson, L.E., Fong, L., Knutson, K.L., Disis, M.L., and McNeel, D.G. (2010). HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase. Cancer Immunol. Immunother. *59*, 943–953.

P'ng, C., Green, J., Chong, L.C., Waggott, D., Prokopec, S.D., Shamsi, M., Nguyen, F., Mak, D.Y.F., Lam, F., Albuquerque, M.A., et al. (2019). BPG: Seamless, automated and interactive

visualization of scientific data. BMC Bioinformatics *20*, 1–5.

Pan, Y., Lee, A.H., Yang, H.T., Wang, Y., Xu, Y., Kadash-Edmondson, K.E., Phillips, J., Champhekar, A., Puig, C., Ribas, A., et al. (2019). IRIS: Big data-informed discovery of cancer immunotherapy targets arising from premRNA alternative splicing. BioRxiv 843268.

Pan, Y., Kadash-Edmondson, K.E., Wang, R., Phillips, J., Liu, S., Ribas, A., Aplenc, R., Witte, O.N., and Xing, Y. (2021). RNA Dysregulation: An Expanding Source of Cancer Immunotherapy Targets. Trends Pharmacol. Sci. *42*, 268–282.

Papadopoulou, A., Gerdemann, U., Katari, U.L., Tzannou, I., Liu, H., Martinez, C., Leung, K., Carrum, G., Gee, A.P., Vera, J.F., et al. (2014). Activity of Broad-Spectrum T Cells as Treatment for AdV, EBV, CMV, BKV, and HHV6 Infections after HSCT. Sci. Transl. Med. *6*, 242ra83-242ra83.

Parkhurst, M., Gros, A., Pasetto, A., Prickett, T., Crystal, J.S., Robbins, P., and Rosenberg, S.A. (2017). Isolation of T-cell receptors specifically reactive with mutated tumor-associated antigens from tumor-infiltrating lymphocytes based on CD137 expression. Clin. Cancer Res. *23*, 2491–2505.

Peng, Y., Mentzer, A.J., Liu, G., Yao, X., Yin, Z., Dong, D., Dejnirattisai, W., Rostron, T., Supasa, P., Liu, C., et al. (2020). Broad and strong memory CD4+ and CD8+ T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19. Nat. Immunol. *21*, 1336–1345.

Peterson, V.M., Zhang, K.X., Kumar, N., Wong, J., Li, L., Wilson, D.C., Moore, R., Mcclanahan, T.K., Sadekova, S., and Klappenbach, J.A. (2017). Multiplexed quantification of proteins and transcripts in single cells. Nat. Biotechnol. *35*, 936–939.

Phillips, J.W., Pan, Y., Tsai, B.L., Xie, Z., Demirdjian, L., Xiao, W., Yang, H.T., Zhang, Y., Lin,

C.H., Cheng, D., et al. (2020). Pathway-guided analysis identifies Myc-dependent alternative pre-mRNA splicing in aggressive prostate cancers. Proc. Natl. Acad. Sci. *117*, 5269–5279.

Pinkse, G.G.M., Tysma, O.H.M., Bergen, C.A.M., Kester, M.G.D., Ossendorp, F., Van Veelen, P.A., Keymeulen, B., Pipeleers, D., Drijfhout, J.W., and Roep, B.O. (2005). Autoreactive CD8 T cells associated with β cell destruction in type 1 diabetes. Proc. Natl. Acad. Sci. U. S. A. *102*, 18425–18430.

Pitcher, C.J., Quittner, C., Peterson, D.M., Connors, M., Koup, R.A., Maino, V.C., and Picker, L.J. (1999). HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat. Med. *5*, 518.

Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M.M., Planchais, C., Porrot, F., Robillard, N., Puech, J., et al. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. Nat. 2021 1–7.

Polack, F.P., Thomas, S.J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J.L., Pérez Marc, G., Moreira, E.D., Zerbini, C., et al. (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. N. Engl. J. Med. *383*, 2603–2615.

Poran, A., Harjanto, D., Malloy, M., Arieta, C.M., Rothenberg, D.A., Lenkala, D., van Buuren, M.M., Addona, T.A., Rooney, M.S., Srinivasan, L., et al. (2020). Sequence-based prediction of SARS-CoV-2 vaccine targets using a mass spectrometry-based bioinformatics predictor identifies immunogenic T cell epitopes. Genome Med. 2020 121 *12*, 1–15.

Qi, Q., Liu, Y., Cheng, Y., Glanville, J., Zhang, D., Lee, J.Y., Olshen, R.A., Weyand, C.M., Boyd, S.D., and Goronzy, J.J. (2014). Diversity and clonal selection in the human T-cell repertoire. Proc. Natl. Acad. Sci. U. S. A. *111*, 13139–13144.

Raffin, C., Vo, L.T., and Bluestone, J.A. (2020). Treg cell-based therapies: challenges and

perspectives. Nat. Rev. Immunol. *20*, 158–172.

Restifo, N.P., Dudley, M.E., and Rosenberg, S.A. (2012). Adoptive immunotherapy for cancer: Harnessing the T cell response. Nat. Rev. Immunol. *12*, 269–281.

Robbins, P.F., Morgan, R.A., Feldman, S.A., Yang, J.C., Sherry, R.M., Dudley, M.E., Wunderlich, J.R., Nahvi, A. V., Helman, L.J., Mackall, C.L., et al. (2011a). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J. Clin. Oncol. *29*, 917–924.

Robbins, P.F., Morgan, R.A., Feldman, S.A., Yang, J.C., Sherry, R.M., Dudley, M.E., Wunderlich, J.R., Nahvi, A. V., Helman, L.J., Mackall, C.L., et al. (2011b). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J. Clin. Oncol. *29*, 917–924.

Rodenko, B., Toebes, M., Hadrup, S.R., van Esch, W.J.E., Molenaar, A.M., Schumacher, T.N.M., and Ovaa, H. (2006). Generation of peptide–MHC class I complexes through UV-mediated ligand exchange. Nat. Protoc. *1*, 1120–1132.

Roth, T.L., Puig-Saus, C., Yu, R., Shifrut, E., Carnevale, J., Li, P.J., Hiatt, J., Saco, J., Krystofinski, P., Li, H., et al. (2018). Reprogramming human T cell function and specificity with non-viral genome targeting. Nat. 2018 5597714 *559*, 405–409.

S, V., S, M., M, C., E, P., and A, L. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature *375*, 148–151.

Sadoff, J., Gray, G., Vandebosch, A., Cárdenas, V., Shukarev, G., Grinsztejn, B., Goepfert, P.A., Truyers, C., Fennema, H., Spiessens, B., et al. (2021). Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19. N. Engl. J. Med. NEJMoa2101544.

Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in

immunological tolerance to self and non-self. Nat. Immunol. *6*, 345–352.

Sant, A.J., and McMichael, A. (2012). Revealing the role of CD4+ T cells in viral immunity. J. Exp. Med. *209*, 1391–1395.

Schatz, D.G., Oettinger, M.A., and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. Cell *59*, 1035–1048.

Seet, C.S., He, C., Bethune, M.T., Li, S., Chick, B., Gschweng, E.H., Zhu, Y., Kim, K., Kohn, D.B., Baltimore, D., et al. (2017). Generation of mature T cells from human hematopoietic stem and progenitor cells in artificial thymic organoids. Nat. Methods *14*, 521–530.

Sewell, A.K. (2012). Why must T cells be cross-reactive? Nat. Rev. Immunol. *12*, 669–677.

Shu, Y., and McCauley, J. (2017). GISAID: Global initiative on sharing all influenza data – from vision to reality. Eurosurveillance *22*, 30494.

Sibener, L. V, Fernandes, R.A., Kolawole, E.M., Carbone, C.B., Liu, F., McAffee, D., Birnbaum, M.E., Yang, X., Su, L.F., and Yu, W. (2018). Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. Cell *174*, 672-687. e27.

Sieling, P., King, T., Wong, R., Nguyen, A., Wnuk, K., Rice, A., Adisetiyo, H., Hermreck, M., Verma, M., Zakin, L., et al. (2021). Single Prime hAd5 Spike (S) + Nucleocapsid (N) Dual Antigen Vaccination of 1 Healthy Volunteers Induces a Ten-Fold Increase in Mean S-and N-T-Cell Responses 2 Equivalent to T-Cell Responses from Patients Previously Infected with SARS-CoV-2 3 4. MedRxiv 2021.04.05.21254940.

Singer, J.B., Gifford, R.J., Cotten, M., and Robertson, D.L. (2020). CoV-GLUE: A Web Application for Tracking SARS-CoV-2 Genomic Variation.

Slifka, M.K., Rodriguez, F., and Whitton, J.L. (1999). Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. Nature *401*, 76.

Smart, A.C., Margolis, C.A., Pimentel, H., He, M.X., Miao, D., Adeegbe, D., Fugmann, T., Wong, K.-K., and Van Allen, E.M. (2018). Intron retention is a source of neoepitopes in cancer. Nat. Biotechnol. 2018 3611 *36*, 1056–1058.

Stoeckius, M., Hafemeister, C., Stephenson, W., Houck-Loomis, B., Chattopadhyay, P.K., Swerdlow, H., Satija, R., and Smibert, P. (2017). Simultaneous epitope and transcriptome measurement in single cells. Nat. Methods *14*, 865–868.

Subramonia Iyer, K., Klee, W.A., and Wooi, M. (1973). Direct Spectrophotometric Measurement of the Rate of Reduction of Disulfide Bonds 'THE REACTIVITY OF THE DISULFIDE BONDS OF BOVINE cr-LACTALBUMIN.

Sun, J.C., and Bevan, M.J. (2003). Defective CD8 T Cell Memory Following Acute Infection Without CD4 T Cell Help. Science (80-.). *300*, 339–342.

Sylwester, A.W., Mitchell, B.L., Edgar, J.B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P.R., Grabstein, K.H., Hosken, N.A., Kern, F., et al. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J. Exp. Med. *202*, 673–685.

Takaba, H., and Takayanagi, H. (2017). The Mechanisms of T Cell Selection in the Thymus. Trends Immunol. *38*, 805–816.

Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell *126*, 663–676.

Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., and Bluestone, J.A. (2004). In Vitro–expanded Antigen-specific Regulatory T Cells Suppress Autoimmune Diabetes. J. Exp. Med. *199*, 1455–1465.

Tarbell, K. V., Yamazaki, S., Olson, K., Toy, P., and Steinman, R.M. (2004). CD25+ CD4+ T

Cells, Expanded with Dendritic Cells Presenting a Single Autoantigenic Peptide, Suppress Autoimmune Diabetes. J. Exp. Med. *199*, 1467–1477.

Tarke, A., Sidney, J., Kidd, C.K., Dan, J.M., Ramirez, S.I., Yu, E.D., Mateus, J., da Silva Antunes, R., Moore, E., Rubiro, P., et al. (2021). Comprehensive analysis of T cell immunodominance and immunoprevalence of SARS-CoV-2 epitopes in COVID-19 cases. Cell Reports Med. *2*.

Thomsen, E.R., Mich, J.K., Yao, Z., Hodge, R.D., Doyle, A.M., Jang, S., Shehata, S.I., Nelson, A.M., Shapovalova, N. V, and Levi, B.P. (2016). Fixed single-cell transcriptomic characterization of human radial glial diversity. Nat. Methods *13*, 87.

Topham, D.J., Tripp, R.A., and Doherty, P.C. (1997). CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J. Immunol. *159*.

Tran, E., Robbins, P.F., Lu, Y.-C., Prickett, T.D., Gartner, J.J., Jia, L., Pasetto, A., Zheng, Z., Ray, S., Groh, E.M., et al. (2016). T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. Https://Doi.Org/10.1056/NEJMoa1609279 *375*, 2255–2262.

Tran, E., Robbins, P.F., and Rosenberg, S.A. (2017). Final common pathway' of human cancer immunotherapy: Targeting random somatic mutations. Nat. Immunol. *18*, 255–262.

Waldrop, S.L., Pitcher, C.J., Peterson, D.M., Maino, V.C., and Picker, L.J. (1997). Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J. Clin. Invest. *99*, 1739–1750.

Walker, B.D., and Yu, X.G. (2013). Unravelling the mechanisms of durable control of HIV-1. Nat. Rev. Immunol. *13*, 487–498.

Wang, P., Nair, M.S., Liu, L., Iketani, S., Luo, Y., Guo, Y., Wang, M., Yu, J., Zhang, B.,

Kwong, P.D., et al. (2021). Antibody Resistance of SARS-CoV-2 Variants B.1.351 and B.1.1.7. Nature 1–6.

Wei, S.C., Duffy, C.R., and Allison, J.P. (2018). Fundamental mechanisms of immune checkpoint blockade therapy. Cancer Discov. *8*, 1069–1086.

Weiskopf, D., Schmitz, K.S., Raadsen, M.P., Grifoni, A., Okba, N.M.A., Endeman, H., van den Akker, J.P.C., Molenkamp, R., Koopmans, M.P.G., van Gorp, E.C.M., et al. (2020). Phenotype and kinetics of SARS-CoV-2-specific T cells in COVID-19 patients with acute respiratory distress syndrome. Sci. Immunol. *5*, 2071.

Wolfl, M., Kuball, J., Ho, W.Y., Nguyen, H., Manley, T.J., Bleakley, M., and Greenberg, P.D. (2007). Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood *110*, 201–210.

Wölfl, M., Kuball, J., Eyrich, M., Schlegel, P.G., and Greenberg, P.D. (2008). Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. Cytom. Part A *73*, 1043–1049.

Wu, T.D., Madireddi, S., de Almeida, P.E., Banchereau, R., Chen, Y.J.J., Chitre, A.S., Chiang, E.Y., Iftikhar, H., O'Gorman, W.E., Au-Yeung, A., et al. (2020). Peripheral T cell expansion predicts tumour infiltration and clinical response. Nature *579*, 274–278.

Xue, S.-A., Gao, L., Ahmadi, M., Ghorashian, S., Barros, R.D., Pospori, C., Holler, A., Wright, G., Thomas, S., Topp, M., et al. (2013). Human MHC Class I-restricted high avidity CD4+ T cells generated by co-transfer of TCR and CD8 mediate efficient tumor rejection in vivo. Https://Doi.Org/10.4161/Onci.22590 *2*.

Yang, L., Bailey, L., Baltimore, D., and Wang, P. (2006). Targeting lentiviral vectors to specific

cell types in vivo. Proc. Natl. Acad. Sci. *103*, 11479–11484.

Yost, K.E., Satpathy, A.T., Wells, D.K., Qi, Y., Wang, C., Kageyama, R., McNamara, K.L., Granja, J.M., Sarin, K.Y., Brown, R.A., et al. (2019). Clonal replacement of tumor-specific T cells following PD-1 blockade. Nat. Med. *25*, 1251–1259.

Yu, W., Jiang, N., Ebert, P.J.R., Kidd, B.A., Müller, S., Lund, P.J., Juang, J., Adachi, K., Tse, T., Birnbaum, M.E., et al. (2015a). Clonal Deletion Prunes but Does Not Eliminate Self-Specific αβ CD8+ T Lymphocytes. Immunity *42*, 929–941.

Yu, W., Jiang, N., Peter, Brian, Müller, S., Peder, Juang, J., Adachi, K., Tse, T., Michael, et al. (2015b). Clonal Deletion Prunes but Does Not Eliminate Self-Specific αβ CD8+ T Lymphocytes. *42*, 929–941.

Zhu, Y., Smith, D.J., Zhou, Y., Li, Y.R., Yu, J., Lee, D., Wang, Y.C., Di Biase, S., Wang, X., Hardoy, C., et al. (2019). Development of Hematopoietic Stem Cell-Engineered Invariant Natural Killer T Cell Therapy for Cancer. Cell Stem Cell *25*, 542-557.e9.

Zinkernagel, R.M., and Doherty, P.C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature *248*, 701–702.

Chapter 4

Conclusion

The study of how CD8 T cells recognize antigenic determinants can inform therapeutics development. Effective vaccines against many infectious pathogens are lacking(*1*). Some individuals are better at controlling vaccination resistant pathogens and the responses in those individuals can help understand the drivers of control. CD8 T cell responses are often associated with viral control possibly because they can clear infected cells (*2–5*). T cell cross-reactivity also allows for enhanced recognition of viral mutant variants. CD8 T cell role in cancer is very well established. Antibody therapeutics against checkpoint inhibitor molecules, such as PD-1 and CTLA4, are effective against multiple cancers and function by engaging CD8 T cells(*6, 7*). T cell receptors can be directly used as therapeutics in either engineered T cell products or as solubilized bispecific molecules that engage endogenous T cell populations.

In chapter 3, we showed that SARS-CoV-2 internal proteins, particularly the polymerase are more conserved than the Spike, against which the neutralizing antibody response is directed. Currently no respiratory virus vaccine relies on T cell immunity alone. Combining vaccines against spike and nucleocapsid is currently being explored and has been shown to increase protection in the brain(*8*). The work described in this thesis helped to explore the strategy of combining vaccines against the polymerase and the spike. In collaboration with Caius Radu and Ting-Ting Wu, both at UCLA, we showed that an mRNA construct encoding part of the polymerase is immunogenic and mouse challenge experiments are currently being planned.

It would be terrific to see a vaccine against the SARS-CoV-2 polymerase enter clinical trials. The next step, if mouse challenge studies are successful, should be a challenge study in a non-

human primate model. Rhesus macaques are an established model for SARS-CoV-2, but the symptoms are not nearly as severe as for humans, so the pathogenicity and protection studies offer only limited insight(*9*). Many already have anti spike responses either because of vaccination or natural infection, thus vaccine against the polymerase can potentially be given as a monotherapy to people. A T cell vaccine may perform best if delivered in an inhalable form, to induce memory T cells in the airway tract, where most people are exposed to SARS-COV-2. Definition of efficacy will also have to be reviewed as a T cell vaccine is not expected to completely prevent viral replication and may allow for minor symptoms. Reduction of severe illness and quick resolution of symptoms would then be the criteria of protection.

During the writing of this chapter the first TCR was approved as a drug(*10*). Tebentafusp is a soluble TCR linked to an anti-CD3 molecule that can activate T cells. This bispecific recognizes gp100 and improves overall survival in metastatic uveal melanoma by about 6 months. The Tebentafusp TCR has been engineered to have enhanced affinity against the gp100 epitope. Most TCRs are generated for use in cell therapy, however one of the unsolved issues is that the overexpressed TCR construct can pair with endogenously expressed TCR chains. Mispairing has been shown to cause toxicity by creating a novel reactivity leading to recognition of self-antigens(*11*). The fact that the first TCR approved as a drug is a bispecific molecule potentially speaks to the significance of the problem of mispairing. TCRs currently in development as cell therapeutics may need to be considered for use as bispecifics. Development of more TCR therapeutics will require identification of novel antigens that can successfully be targeted.

Effective cancer targeting requires identification of antigens specific to the tumor. Tumor antigens are generally derived from tumor associated (TA) proteins, which are normal proteins enriched in cancer, or neo-antigens, which are derived from somatic mutations(*12*). Cell therapy field predominantly focused on targeting TA antigens, because they are also considered public antigens, that is expressed in many patients. Targeting such proteins can result in toxicity, because of their limited expression in normal tissues(*13*, *14*). Two TA antigens commonly targeted by TCR based therapies are NY-ESO-1 and MART-1. Neo-antigens are predominantly patient specific, which limits their utility for therapeutics development(*15*). One exception are mutations that both activate an oncogene and can be presented by the MHC molecules, such as mutations in KRAS and P53(*16*, *17*). Neo-antigen targeted T cells have induced complete responses in patients with late-stage cancers. This thesis work helped to initiate TCR development against prostate cancer antigen prostatic acid phosphatase (PAP). The next steps will be to test these TCRs for their ability to control cancer cell line growth. These TCRs will most like need to be affinity enhanced. Experiments are underway and being led by Zhiyuan Mao in our lab in collaboration with the lab of Christopher Garcia at Stanford. Many TCRs caused serious adverse events when first tested in the clinic either due to reactivity with the target protein in normal tissue or cross reactivity with an unrelated antigen(*14*). Reactivity against normal tissue will need to be carefully considered, as these TCRs are engineered.

Our lab is collaborating with the group of Yi Xing, an expert on alternative splicing analysis, to generate TCRs that can recognize cancer specific mRNA isoforms. Alternative exon usage in cancer can provide an additional source of antigens(*18*). Up to 95% of human genes undergo alternative splicing to increase protein diversity(*19*). Large scale genomics analysis indicate that

alternative splicing is particularly abundant in cancer and has been suggested to be another hallmark of cancer. Specific mRNA isoforms are responsible for resistance to treatment, such as the ARV7 in prostate cancer(*19*). Oncogenes such as Myc are implicated in driving distinct splicing in cancer. Point mutations within the spliceosome binding sites can inhibit splicing and result in novel exons. Exons and exon junctions that are truly cancer specific and translated into proteins should be also available for T cells to survey and potentially recognize.

The work in this thesis helped develop new technologies and pipelines for TCR discovery and established TCR functional profiling as a method for antigen discovery. I am excited to see how the SARS-CoV-2 polymerase vaccine moves forward. It's very clear that, as SARS-CoV-2 is becoming endemic, the world will need better vaccines to control this virus. The methods established by this thesis were used to discover TCRs against prostatic acid phosphatase (PAP) and cancer specific splice isoforms. These projects are nearing completion and have the potential to transform how prostate cancer is treated.

References:

1. B. Pulendran, R. Ahmed, Immunological mechanisms of vaccination. *Nat. Immunol. 2011 126*. **12**, 509–517 (2011).

2. E. Harrer, T. Harrer, P. Barbosa, M. Feinberg, R. P. Johnson, S. Buchbinder, B. D. Walker, Recognition of the Highly Conserved YMDD Region in the Human Immunodeficiency Virus Type 1 Reverse Transcriptase by HLA-A2-Restricted Cytotoxic T Lymphocytes from an Asymptomatic Long-Term Nonprogressor. *J. Infect. Dis.* **173**, 476–479 (1996).

3. D. R. Collins, G. D. Gaiha, B. D. Walker, CD8+ T cells in HIV control, cure and prevention. *Nat. Rev. Immunol.* **20** (2020), pp. 471–482.

4. K. D. Zens, J. K. Chen, D. L. Farber, Vaccine-generated lung tissue-resident memory T cells provide heterosubtypic protection to influenza infection. *Ref. Inf. JCI Insight*. **1**, 85832 (2016).

5. A. Jozwik, M. S. Habibi, A. Paras, J. Zhu, A. Guvenel, J. Dhariwal, M. Almond, E. H. C. Wong, A. Sykes, M. Maybeno, J. Del Rosario, M. B. Trujillo-Torralbo, P. Mallia, J. Sidney, B. Peters, O. M. Kon, A. Sette, S. L. Johnston, P. J. Openshaw, C. Chiu, RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. *Nat. Commun.* **6** (2015), doi:10.1038/ncomms10224.

6. T. D. Wu, S. Madireddi, P. E. de Almeida, R. Banchereau, Y. J. J. Chen, A. S. Chitre, E. Y. Chiang, H. Iftikhar, W. E. O'Gorman, A. Au-Yeung, C. Takahashi, L. D. Goldstein, C. Poon, S. Keerthivasan, D. E. de Almeida Nagata, X. Du, H. M. Lee, K. L. Banta, S. Mariathasan, M. Das Thakur, M. A. Huseni, M. Ballinger, I. Estay, P. Caplazi, Z. Modrusan, L. Delamarre, I. Mellman, R. Bourgon, J. L. Grogan, Peripheral T cell expansion predicts tumour infiltration and clinical response. *Nature*. **579**, 274–278 (2020).

7. S. C. Wei, C. R. Duffy, J. P. Allison, Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov.* **8** (2018), pp. 1069–1086.

8. T. Dangi, J. Class, N. Palacio, J. M. Richner, P. Penaloza MacMaster, Combining spike- and nucleocapsid-based vaccines improves distal control of SARS-CoV-2. *Cell Rep.* **36**, 109664 (2021).

9. K. McMahan, J. Yu, N. B. Mercado, C. Loos, L. H. Tostanoski, A. Chandrashekar, J. Liu, L. Peter, C. Atyeo, A. Zhu, E. A. Bondzie, G. Dagotto, M. S. Gebre, C. Jacob-Dolan, Z. Li, F. Nampanya, S. Patel, L. Pessaint, A. Van Ry, K. Blade, J. Yalley-Ogunro, M. Cabus, R. Brown, A. Cook, E. Teow, H. Andersen, M. G. Lewis, D. A. Lauffenburger, G. Alter, D. H. Barouch, Correlates of protection against SARS-CoV-2 in rhesus macaques. *Nature*. **590**, 630–634 (2021).

10. P. Nathan, J. C. Hassel, P. Rutkowski, J.-F. Baurain, M. O. Butler, M. Schlaak, R. J. Sullivan, S. Ochsenreither, R. Dummer, J. M. Kirkwood, A. M. Joshua, J. J. Sacco, A. N. Shoushtari, M. Orloff, J. M. Piulats, M. Milhem, A. K. S. Salama, B. Curti, L. Demidov, L. Gastaud, C. Mauch, M. Yushak, R. D. Carvajal, O. Hamid, S. E. Abdullah, C. Holland, H. Goodall, S. Piperno-Neumann, Overall Survival Benefit with Tebentafusp in Metastatic Uveal Melanoma. *N. Engl. J. Med.* **385**, 1196–1206 (2021).

11. G. M. Bendle, C. Linnemann, A. I. Hooijkaas, L. Bies, M. A. De Witte, A. Jorritsma, A. D. M. Kaiser, N. Pouw, R. Debets, E. Kieback, W. Uckert, J. Y. Song, J. B. A. G. Haanen, T. N. M. Schumacher, Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat. Med. 2010 165*. **16**, 565–570 (2010).

12.   M. T. Bethune, A. V. Joglekar, Personalized T cell-mediated cancer immunotherapy: progress and challenges. *Curr. Opin. Biotechnol.* **48** (2017), pp. 142–152.

13.   L. A. Johnson, R. A. Morgan, M. E. Dudley, L. Cassard, J. C. Yang, M. S. Hughes, U. S. Kammula, R. E. Royal, R. M. Sherry, J. R. Wunderlich, C.-C. R. Lee, N. P. Restifo, S. L. Schwarz, A. P. Cogdill, R. J. Bishop, H. Kim, C. C. Brewer, S. F. Rudy, C. VanWaes, J. L. Davis, A. Mathur, R. T. Ripley, D. A. Nathan, C. M. Laurencot, S. A. Rosenberg, Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood.* **114**, 535–546 (2009).

14.   G. P. Linette, E. A. Stadtmauer, M. V. Maus, A. P. Rapoport, B. L. Levine, L. Emery, L. Litzky, A. Bagg, B. M. Carreno, P. J. Cimino, G. K. Binder-Scholl, D. P. Smethurst, A. B. Gerry, N. J. Pumphrey, A. D. Bennett, J. E. Brewer, J. Dukes, J. Harper, H. K. Tayton-Martin, B. K. Jakobsen, N. J. Hassan, M. Kalos, C. H. June, Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood.* **122**, 863 (2013).

15.   E. Tran, P. F. Robbins, S. A. Rosenberg, Final common pathway' of human cancer immunotherapy: Targeting random somatic mutations. *Nat. Immunol.* **18** (2017), pp. 255–262.

16.   E. H.-C. Hsiue, K. M. Wright, J. Douglass, M. S. Hwang, B. J. Mog, A. H. Pearlman, S. Paul, S. R. DiNapoli, M. F. Konig, Q. Wang, A. Schaefer, M. S. Miller, A. D. Skora, P. A. Azurmendi, M. B. Murphy, Q. Liu, E. Watson, Y. Li, D. M. Pardoll, C. Bettegowda, N. Papadopoulos, K. W. Kinzler, B. Vogelstein, S. B. Gabelli, S. Zhou, Targeting a neoantigen derived from a common TP53 mutation. *Science (80-.).* **371** (2021), doi:10.1126/SCIENCE.ABC8697.

17.   E. Tran, P. F. Robbins, Y.-C. Lu, T. D. Prickett, J. J. Gartner, L. Jia, A. Pasetto, Z. Zheng, S. Ray, E. M. Groh, I. R. Kriley, S. A. Rosenberg, T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. *https://doi.org/10.1056/NEJMoa1609279.* **375**, 2255–2262 (2016).

18.   Y. Pan, K. E. Kadash-Edmondson, R. Wang, J. Phillips, S. Liu, A. Ribas, R. Aplenc, O. N. Witte, Y. Xing, RNA Dysregulation: An Expanding Source of Cancer Immunotherapy Targets. *Trends Pharmacol. Sci.* **42**, 268–282 (2021).

19.   J. W. Phillips, Y. Pan, B. L. Tsai, Z. Xie, L. Demirdjian, W. Xiao, H. T. Yang, Y. Zhang, C. H. Lin, D. Cheng, Q. Hu, S. Liu, D. L. Black, O. N. Witte, Y. Xing, Pathway-guided analysis identifies Myc-dependent alternative pre-mRNA splicing in aggressive prostate cancers. *Proc. Natl. Acad. Sci.* **117**, 5269–5279 (2020).

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