Immunology and Inflammation

TCR transgenic clone selection guided by immune receptor analysis and single cell RNA expression of polyclonal responders

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Abstract

Since the precursor frequency of naïve T cells is extremely low, investigating the early steps of antigen-specific T cell activation is challenging. To overcome this detection problem, adoptive transfer of a cohort of T cells purified from T cell receptor (TCR) transgenic donors has been extensively used but is not readily available for emerging pathogens. Constructing TCR transgenic mice from T cell hybridomas is a labor-intensive and sometimes erratic process, since the best clones are selected based on antigen-induced CD69 upregulation or IL-2 production in vitro, and TCR chains are PCR-cloned into expression vectors. Here, we exploited the rapid advances in single cell sequencing and TCR repertoire analysis to select the best clones without hybridoma selection, and generated CORSET8 mice (CORona Spike Epitope specific CD8 T cell), carrying a TCR specific for the Spike protein of SARS-CoV-2. Implementing newly created DALI software for TCR repertoire analysis in single cell analysis enabled the rapid selection of the ideal responder CD8 T cell clone, based on antigen reactivity, proliferation and immunophenotype in vivo. In contrast, a traditional method based on hybridoma technology was unsuccessful. Identified TCR sequences were inserted as synthetic DNA into an expression vector and transgenic CORSET8 donor mice were created. After immunization with Spike/CpG-motifs, mRNA vaccination or SARS-CoV2 infection, CORSET8 T cells strongly proliferated and showed signs of T cell activation. Thus, a combination of TCR repertoire analysis and scRNA immunophenotyping allowed rapid selection of antigen-specific TCR sequences that can be used to generate TCR transgenic mice.

eLife assessment

The paper illustrates a **valuable** approach to generating TCR transgenic mice specific for known epitopes. There is some **solid** evidence for the efficacy of this approach, although only limited evidence is provided that the TCR clone in question successfully recapitulates the functional features of the endogenous response to the same antigen, and the claim that this method is superior to more traditional clone selection methods is **incompletely** substantiated by the data presented.

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Introduction

One of the key features of adaptive immunity is the enormous receptor diversity of lymphocytes, which is estimated to cover over 10^{10} unique sequences for T cells alone ((Cho et al., 2020; Lythe et al., 2016). This empowers the adaptive immune system to tackle an infinite number of invaders. Every single clone cannot expand before antigen encounter because of space constraints and therefore circulates at extremely low precursor frequency. This low precursor frequency has impeded the detailed study of early antigen-specific T cell responses *in vivo*, despite the advent of peptide-MHC tetramers that can detect rare antigen-specific cells after whole body enrichment (Altman and Davis, 2016; Chu et al., 2009; Dileepan et al., 2021; Moon et al., 2009, 2007; Shin et al., 2023). To overcome this detection problem, adoptive transfer of a cohort of naive T cells derived from T cell receptor transgenic mice (TCR Tg), which harbour high numbers of a single clonotypic T-cell population, has been extensively used. This technique allowed the tracking of clonal activation, expansion, differentiation and migration to many model antigens and pathogens, even in early phases of the adaptive immune response, which has greatly contributed to answering key questions in T cell biology in vaccinology, infectious models, cancer, autoimmunity and allergy (Attridge and Walker, 2014; Coquet et al., 2015; Kisielow et al., 1988; Miura et al., 2020; Oxenius et al., 1998).

Although many TCR transgenic mouse models are now commercially available and are extensively used in many fields, it is sometimes still needed to rapidly generate a new TCR transgenic mouse. This is certainly the case for emerging pathogens like SARS-CoV-2 Coronavirus, for which no research tools were readily available in 2019. The generation of TCR Tg mice necessitates the selection of a functionally relevant TCR clone whose TCR Vα- and Vβ-chains are subsequently expressed in a vector that is highly expressed in thymocytes, causing an effective skewing from a polyclonal to a monoclonal repertoire due to allelic exclusion (Irving et al., 1998; Wang et al., 2001). To obtain clonotypic information, the coding sequence for the TCR α- and β-chains are extracted from antigen-reactive T cell clones. The most often used method to find rare antigen-reactive clones relies on immunization of mice with relevant antigen, and the *ex vivo* generation of easily expandable immortal T hybridoma cells from splenocytes. Hybridomas are then selected for reactivity to immunodominant peptides, often independent of cellular phenotype or function, but merely based on upregulation of CD69 or production of IL-2 as a sign for antigen sensitivity. From these hybridomas, the coding regions for the Vα- and Vβ-chain can be amplified by polymerase chain reaction (PCR) and PCR-cloned into a CD4 expression vector (Vanheerswynghels et al., 2018)

The successful selection of a TCR clone that can be used for transgenesis and ultimately as a T cell donor to study antiviral responses depends on two key factors: first, the T cell epitope needs to be antigenic, immunogenic, and stable across pathogenic variants. Second, the TCR clone needs

sufficient affinity to the epitope presented on an MHC molecule, as this determines the strength and kinetics of the immune response. In other words, affinity of the TCR determines the proliferative advantage of an effector cell or the tendency to form long-term memory (Kavazović et al., 2018). Often, the selection process based on hybridoma selection yields multiple epitope specific clones that upregulate CD69 or IL-2, and only minimal functional parameters are checked before prioritizing one clone to proceed with. This can result in the generation of TCR Tg mice whose T cells do not respond optimally in a biological setting, fail to compete with endogenous polyclonal T cells upon adoptive transfer, or fail to form memory responses (Bartleson et al., 2020; Milam et al., 2018; Weber et al., 2012).

Here, we provide an optimized approach for TCR clone selection that no longer requires hybridoma technology but capitalizes on recent technological advances in the single cell sequencing field that combine analysis of cellular heterogeneity of responding T cells with immune receptor profiling (VDJseq). We have recently developed the interactive DALI software tool, which is an R software package which allows for fast identification and analysis of T- and B cell receptor diversity in high-throughput single-cell sequencing data using command line or a graphical user interface (GUI) (Verstaen et al., 2022). Owing to the browser-based interactive GUI, immunologists having limited coding experience can effectively analyze these complex datasets. The DALI tool facilitates linking TCR clonotype information to functional properties, immunophenotype and precursor frequency of individual T cells within a polyclonal response to vaccination or infection *in vivo*. Information like clonotype diversity, clonotype expansion and differentially expressed genes between clonotypes allows for rationalized TCR sequence selection, and for generation of TCR Tg mice.

As a proof of concept that this strategy is helpful, we generated CD8 TCR Tg mice carrying a TCR specific for a Spike Epitope of the SARS-CoV-2 Coronavirus. We first generated a (**COR**ona **S**pike **E**pitope specific CD**8 T** cell) **CORSET8** line based on traditional T cell hybridoma technology and selection, which eventually yielded a poorly reactive TCR transgenic line. As an alternative, rationalized approach, we applied scRNA and TCR sequencing and subsequent analysis of the T cell clones via the DALI tool. This latter approach allowed us to a priori evaluate key characteristics required for the development of TCR Tg T cells, including cytokine production, cellular phenotype, and physiologically relevant clonal expansion. Importantly, the generation of fully functional TCR transgenic mice took only a fraction of the workload and time in comparison to the classical approach. CORSET8 mice and the resulting transgenic T cells were thoroughly evaluated by *ex vivo* and *in vivo* methods following vaccination and SARS-CoV-2 infection. The streamlined method for generating TCR Tg mice, as presented here, offers an attractive approach for future TCR Tg mouse development.

Results

Generation of CORSET8 mice based on hybridoma technology

Designing a TCR transgenic mouse starts with the selection of the antigen epitope to which the TCR is addressed using peptide libraries (Zhuang et al., 2021) and/or computational scanning combined with a cellular activity assay (Erez et al., 2023; Vandersarren et al., 2017; Vanheerswynghels et al., 2018). In a first approach to generate CORSET8 Tg mice, we used the IEDB database (Vita et al., 2018) to predict the most promising MHCI binding Spike epitopes and made use of the classical hybridoma technology to create the transgenic mice. To induce Spike reactive T cells, we immunized C57BL/6 mice by intraperitoneal injection of a mixture of Spike protein and CpG adjuvant every 14 days for a total of three immunisations (**Fig. 1 II** and Suppl. Fig. 1). After fusion of these reactive T cells to the BW5147 lymphoma, the obtained T-cell hybridoma clones were scored for their IL-2 production upon stimulation with a Spike peptide pool (INITRFQTL, IWLGFIAGL, GNYNYLYRL, VVFLHVTYV, FQFCNDPFL). Compared to medium

control, clone 47 had the strongest fold increase in IL-2 (Suppl. Fig. 1A). This clone also upregulated CD69 in the presence of the Spike peptide pool (Suppl. Fig. 1B). Therefore, clone 47 was prioritized and used for the generation of a TCR Tg mouse. However, CD8 T cells from the newly generated CORSET8 mice did not respond to Spike peptide or Spike protein in a BM-DC co-culture experiment (Suppl. Fig. 1C). To check if CORSET8 mice had transgenic T cells with a functioning TCR at all, we crossed these mice with *Rag1*-deficient mice that are unable to generate polyclonal T cells. We readily detected CD8 T cells even on the *Rag1*$^{-/-}$ background (Suppl. Fig. 1D), demonstrating that the TCR was expressed and was of high enough affinity to cause positive selection of CD8 T cells, and therefore functional. Due to the lack of responsiveness, we discontinued this mouse line immediately after it was generated, illustrating that the design of a useful TCR Tg mouse model is an erratic process that can be optimized.

Generation of CORSET8 mice based on single cell and TCR repertoire analysis

Given the unsuccessful generation of CORSET8 mice using the classical hybridoma approach, we decided to rationalize clone selection (**Fig. 1**). To this end, we immunized mice as described in the previous section (**Fig. 1 II**). From these mice, we sorted splenic T cells and used an MHC class I tetramer to discriminate Spike-reactive and non-reactive T cells (Suppl. Fig. 2). The tetramer identifies CD8 T cells directed to a conserved and highly immunogenic epitope (VNFNFNGL) of the SARS-CoV-2 spike protein. The latter has been confirmed by multiple studies (Carmen et al., 2021; Erez et al., 2023; Zhuang et al., 2021). A naïve mouse was taken along as a backdrop for the cellular phenotypes. Ultimately, we subjected 3 samples for single cell sequencing: Tetramer positive T cells originating from the immunised mice, Tetramer negative T cells originating from the immunized mice, and tetramer negative T cells originating from the naïve control mouse. Clustering of these cells revealed different cellular clusters among sorted CD8 T cells (**Fig. 2A**). Tetramer+ cells formed a distinct cluster on the UMAP reduced dimensional space (**Fig. 2B**). The highest differentially expressed genes of the different clusters are plotted in **Figure 2C**. A subgroup of Tetramer+ cells had a distinct phenotype, marked by increased expression of proliferation and activation markers such as *Mki67* and *Gzmb a*nd transcription factors regulating CD8 effector differentiation such as *Tbet* and *Bhlhe40* (Li et al., 2019; Sullivan et al., 2003), highlighting their activated T effector state (**Fig. 2A-C**). Next, TCRseq data was linked to the Seurat object using our DALI tool (Verstaen et al., 2022), which allows for command line or GUI analysis of TCR data (**Fig. 2D**). Upon subsetting and reclustering of the Tetramer+ cells from immunized mice, 3 clusters were defined, of which cluster 1 showed high expression of *Mki67*, while both cluster 0 and 1 showed high expression of *Ifng* and activation markers *Cd69* and *Nr4a1* (**Fig. 2E-F**). Assessing clonal expansion, we found that cluster 0 and 1 contained highly expanded clones (**Fig. 2G**). When assessing clonal expansion in more detail, we found clonotype1 to be highly expanded in cluster 0 and 1 (**Fig. 2H-I**). Given the phenotypic characteristics of the cells harbouring this clonotype, and the observed clonal expansion, we decided to continue using this clonotype for the generation of TCR Tg mice (**Fig 1**; **Fig. 2J**). The selected Vα and Vβ TCR sequences were ordered as gBlocks (IDT) and subsequently cloned into the CD4 expression vector p428 via SalI (Vanheerswynghels et al., 2018). Injection fragments we excised from the p428 backbone by NotI digestion, gel purified and injected into fertilized C57BL/6 oocytes.

CORSET8 mice exhibit normal T cell populations and TCR Tg cells recognize target peptide *ex vivo*

The distribution of splenic CD4 T and CD8 T cells was unaltered in the newly generated CORSET8 mice compared to wildtype mice (**Fig. 3A**). Both CD4 T and CD8 T cells displayed a normal frequency of naïve (CD62L^{+}CD44^{-}), central memory (CD62L^{+} CD44^{+}) and effector (CD62L^{-}CD44^{+}) T cell populations, although there was a small but significant increase in the central memory pool,

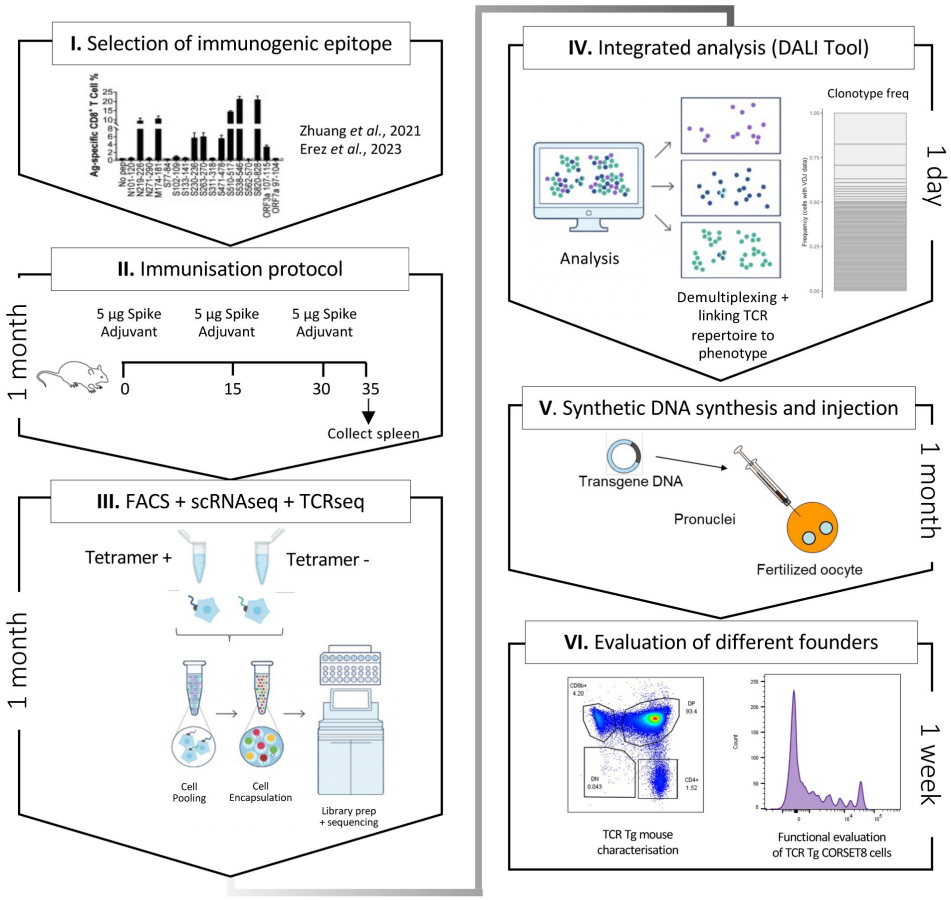

Figure 1

Schematic overview of the generation of rationalized CORSET8 transgenic mice.

I) Selection of the immunogenic epitope: here based on T cell epitope mapping results of Zhuang *et al*. (2021), which were confirmed by Erez *et al*. (2023) (Erez et al., 2023; Zhuang et al., 2021). II) Immunisation protocol to render Spike reactive T cells (for more information see Material and Methods section). III) Fluorescence-activated cell sorting (FACS) followed by single cell RNA and TCR sequencing. IV) Linking the single cell RNA and TCR sequencing data allows an integrated analysis to identify clonotypes and compare functional characteristics. V) Injection of the synthetic DNA into a fertilized oocyte followed by transfer into recipient females for gestation, resulting into the birth of transgenic founder offspring. VI) Dual evaluation of the different founders by phenotypical characterisation of the transgenic mouse and functional testing of the TCR Tg T cells. Parts of the figure adapted from 10xGenomics.

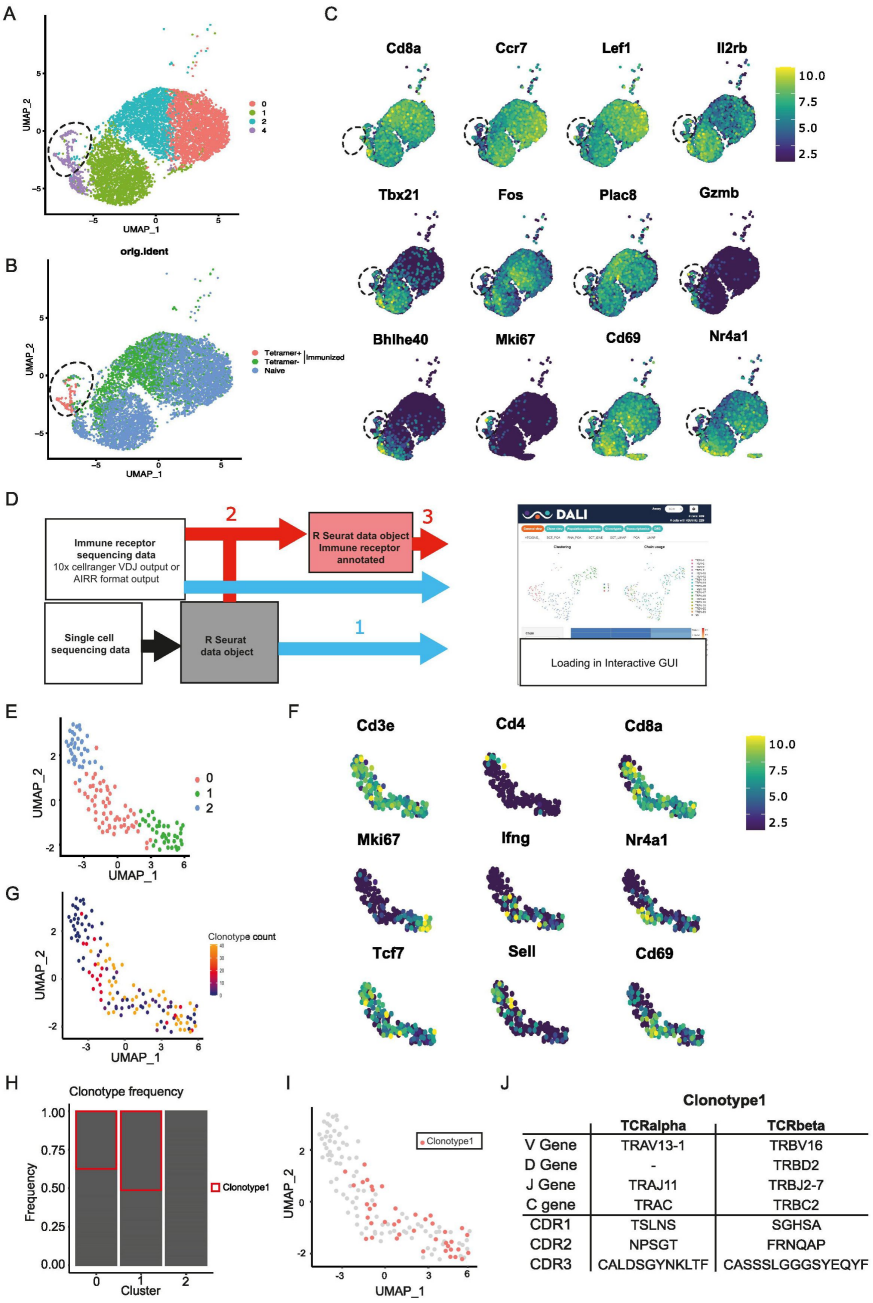

Figure 2

Combined single cell and TCR analysis using DALI identified the most promising T cell clone

A) Uniform manifold approximation and projection (UMAP) of splenic CD8 T cell single-cell RNA sequencing visualizing four different clusters based on single cell analysis. B) Projection of the three sequenced samples on the UMAP: CD8+ Tetramer positive cells of an immunized mouse (sample 1), CD8+ Tetramer negative cells of an immunized mice (sample 2) and total CD8 T cells from a naive mouse (sample 3). C) Gene RNA expression in splenic CD8 T cells. Hallmark genes among the top differentially expressed genes are depicted. D) Overview of DALI pipelines using either -1- loading of the R Seurat object and immune profiling data directly into the interactive Shiny app or -2- generation of an extended R Seurat object containing immune receptor profiling data, which can be loaded into the interactive Shiny app -3-. E) UMAP of subsetted and reclustered Tetramer+ CD8 T cells showing three different clusters. F) RNA expression in Tetramer+ CD8 T cells of curated activation markers. G) UMAP of Tetramer+ CD8 T cells highlighting clonotype expansion. H) Clonotype frequency in the three different clusters of Tetramer+ CD8 T cells. I) Projection of clonotype1 on the UMAP of Tetramer+ CD8 T cells. J) TCRα and TCRβ sequence information of clonotype 1.

as expected based on the phenotype of the cells harboring clonotype 1 (**Fig. 3B**, **Fig. 2F**). Staining of the splenocytes with the Spike Tetramer that was initially used to identify target epitope directed T cells, revealed that about 70% of total CD8 T cells of the CORSET mice on a *Rag1* sufficient background were transgenic cells (**Fig. 3C**). Next, we aimed to functionally evaluate the generated CORSET8 TCR Tg T cells. Therefore, we co- cultured BM-DCs and Cell Tracer Violet (CTV)-labelled CORSET8 T cells in the presence of increasing doses of Spike peptide (VNFNFNGL, 0-10 µg/ml) for 4 days. The dilution of the cell proliferation dye CTV reflects the augmented proliferation of the CORSET8 cells in correspondence with the antigenic dose (**Fig. 3D**). Notably, the CORSET8 cells responded to minute doses of Spike peptide (10 pg/ml), illustrating that the CORSET8 cells express a strong affinity TCR. Furthermore, the CD4 and CD8 T cell distribution in the thymus of the CORSET8 Tg animals showed significant skewing to the CD8+ T cell subset, which also hinted towards successful transgene expression (**Fig. 3E**).

CORSET8 cells divide *in vivo* upon immunization with recombinant spike antigen or with Pfizer BNT162b2 mRNA vaccine

After demonstrating *ex vivo* CORSET8 T cell proliferation, we aimed to assess this phenomenon in an *in vivo* context. CORSET8 T cells were isolated and CTV-labelled to track cellular divisions. Next, 1×10^6 CORSET8 cells were adoptively transferred into C57BL/6 mice that were immunized intratracheally with Spike protein in presence of CpG adjuvant (**Fig. 4A**). Four days after T cell transfer, 95.17% (± 1.61) of CORSET8 cells underwent division in the draining mediastinal lymph node, as demonstrated by the CTV dilution profile (**Fig. 4A, 4C**). In addition, proliferating CORSET8 cells upregulated T cell activation markers such as CD44 and CD5 (**Fig. 4A**). Given that the generated CORSET8 mice might be a very useful tool for vaccine research, we also immunized mice intramuscular with Pfizer BNT162b2 vaccine two days before adoptively transferring 1×10^6 CORSET8 cells (**Fig. 4B**). After four days, 99.63% (± 0.03%) of CORSET8 cells had undergone division and were displaying an activation profile in the draining popliteal lymph node (**Fig. 4B-C**).

CORSET8 cells proliferate in response to SARS-CoV-2 infection

As a final validation of our mouse model, we used the CORSET8 cells in a SARS-CoV-2 infection model, conducted under BSL3 conditions (**Fig. 4D**). As wildtype mice are not susceptible to SARS-CoV-2 infection, we made use of K18-hACE2 Tg mice, the standard mouse model for SARS-CoV-2 research (Moreau et al., 2020; Rathnasinghe et al., 2020; Winkler et al., 2020; Yinda et al., 2021). $hACE2^{Tg/wt}$ mice and their control wildtype littermates were infected with 450 pfu SARS-CoV-2 or mock. Two days after infection, isolated and CTV-labelled CORSET8 T cells were adoptively transferred. Upon analyzing the mediastinal lymph nodes four days post-adoptive transfer, CORSET8 T cells were exclusively detected in the infected $hACE2^{Tg/wt}$ mice, with no presence observed in mock conditions or $hACE2^{wt/wt}$ infected mice (**Fig. 4D**, **Fig. 4F**). After four days, 99.28±0.12% of CORSET8 T wells divided and upregulated activation markers such as CD44 in the draining mediastinal lymph node (**Fig. 4E**). Endogenous CD8 T cells from the recipient SARS-CoV-2 infected mice (CD45.2) did not upregulate CD44, demonstrating that only the transgenic T cells were engaged (**Fig. 4G**). Furthermore, Spike Tetramer staining revealed that CORSET8 T cells had a higher affinity for the Tetramer than Tetramer+ endogenous CD8 T cells (**Fig. 4H**), which again confirms that the generated CORSET8 mice have a high affinity TCR.

Discussion

Adoptive transfer models using TCR transgenic mice as donors of antigen reactive T cells have made it possible to study early T cell responses at clonal level, which is otherwise very strenuous given the extensive variety of polyclonal T cell clones. This broad variety causes low progenitor

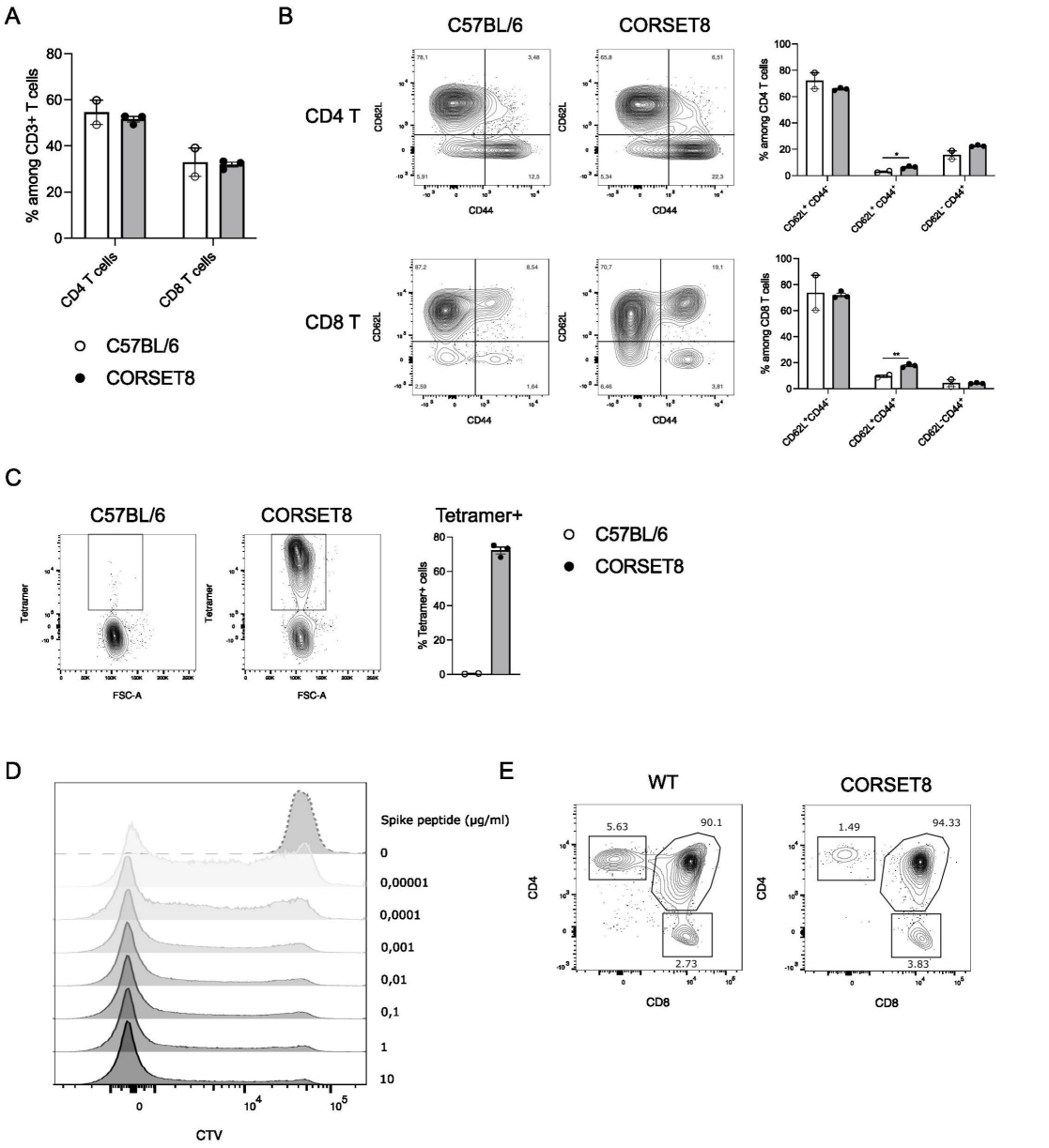

Figure 3

CORSET8 mice exhibit normal T cell populations and TCR Tg cells recognize target peptide *ex vivo*

A) Proportion of CD4 T and CD8 T cells among CD3+ T cells in the spleen of CORSET8 mice and wildtype C57BL/6 littermates. B) Proportion of CD62L^{+} CD44^{-}, CD62L^{+} CD44^{+} and CD62^{-} CD44^{+} T cells among splenic CD4 T and CD8 T cells. C) Spike Tetramer staining on splenic CD8 T cells of CORSET8 mice or wildtype littermates. D) CTV proliferation profile of CORSET8 T cells in co-culture with BM-DCs in presence of increasing doses of Spike peptide. E) Visualization of CD4 and CD8 T cells in the thymus of CORSET8 mice and wildtype littermates. Data information: data are shown as means ± SEM. In **Fig. 3B**, after assessing normality by a Shapiro-Wilk normality test, parametric data were analysed with a t-test *p<0.05, **p<0.01.

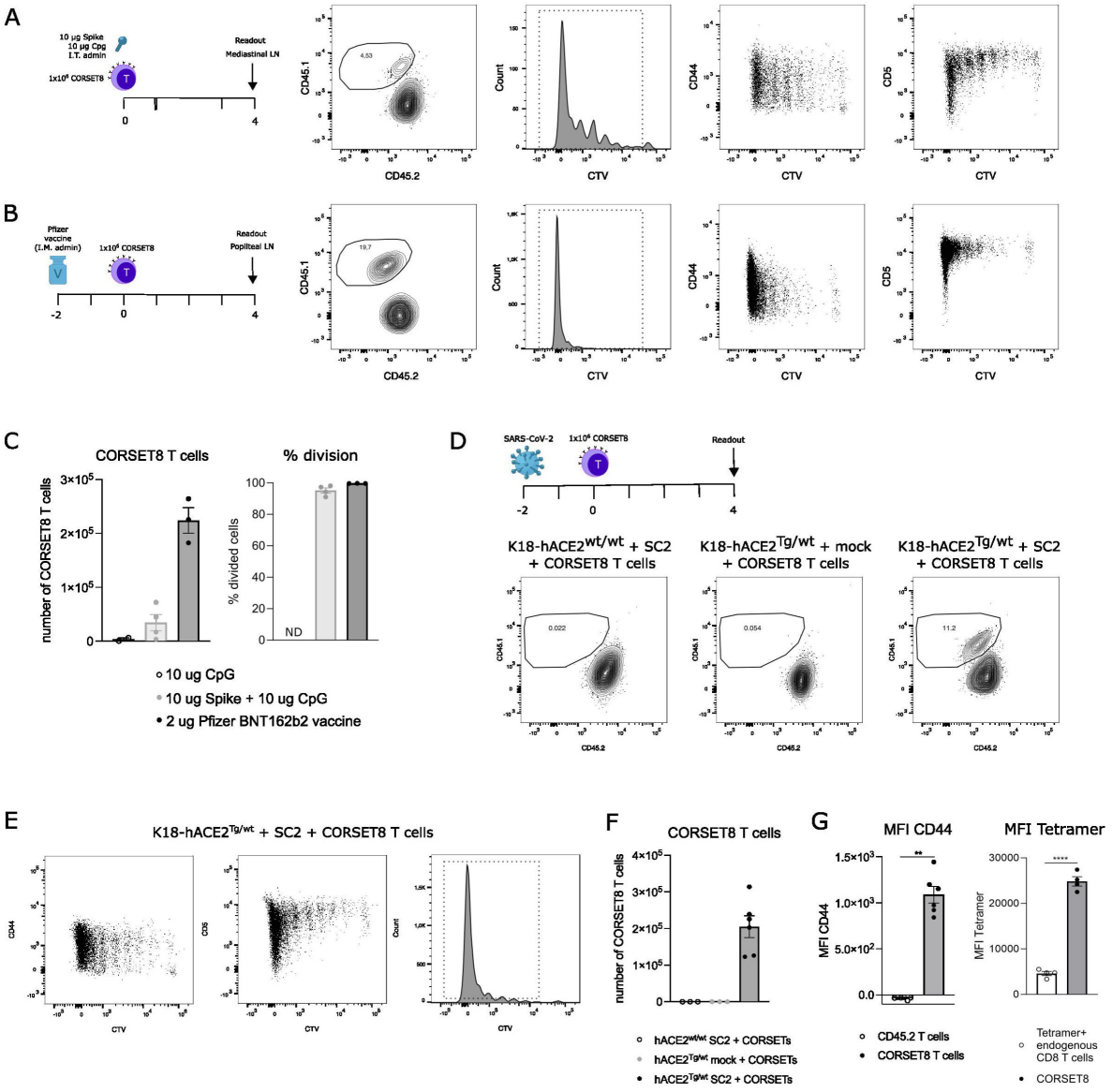

Figure 4

CORSET8 cells proliferate *in vivo* in response to Spike immunization or SARS-CoV-2 infection

A) Schematic set-up of the experiment, gating on CD45.1^{+} CORSET8 cells and their CTV dilution profile 4 days after intratracheal immunization with Spike/CpG. B) Schematic set-up of the experiment, gating on CD45.1^{+} CORSET8 cells and their CTV dilution profile 4 days after intramuscular immunization with Pfizer BNT162b2 vaccine. C) Number of CORSET8 T cells and percentage of divided cells upon immunization with CpG control, Spike/CpG and Pfizer BNT162b2 vaccine. D) Schematic set-up of the experiment and gating on CD45.1^{+} CORSET8 cells in K18-hACE2$^{wt/wt}$ and K18-hACE2$^{Tg/wt}$ mice upon SARS-CoV-2 or mock infection. E) CTV dilution profile in SARS-CoV-2 infected K18-hACE2$^{Tg/wt}$ mice. F) Number of CORSET8 T cells in K18-hACE2$^{wt/wt}$ and K18-hACE2$^{Tg/wt}$ mice upon SARS-CoV-2 or mock infection. G) MFI of CD44 in CD45.1^{+} CORSET8 T cells and CD45.2^{+} T cells of recipient mice. MFI of Tetramer staining on CD45.1+ CORSET8 T cells and CD45.2+ endogenous Tetramer+ T cells. Data information: data are shown as means +/- SEM. In **Fig. 4G**, after assessing normality by a Shapiro-Wilk normality test, non-parametric data were analysed with a Mann-Whitney test. **p<0.01. ****p<0.0001

frequency of any given clone in the pre-immune repertoire, rendering the tracking of early responses exceedingly difficult. In addition, these models confer a significant advantage to T cell research, as they allow to study the functional status of antigen-specific T cells, but also cell-fate decisions to effector cells, Tfh cells or memory cells (Grayson et al., 2000; Mueller et al., 2010; Tubo et al., 2013; West et al., 2011).

The standard method for generating TCR Tg mice comprises immunization of an animal, isolation of T cells out of lymphoid organs and examination of T cell reactivity by i*n vitro* restimulation of T cell hybridoma clones. T cell receptors are then identified crudely using PCR primers and antibodies from the antigen-specific T cell clones, often without knowing the detailed sequence of the Vα and Vβ chains (Barnden et al., 1998; Bosteels et al., 2020; Coquet et al., 2015; Nindl et al., 2012; Oxenius et al., 1998; Plantinga et al., 2013; Vanheerswynghels et al., 2018). The generation of functional TCR Tg mouse models depends on several key steps. A first essential step is the selection of the antigen epitope, which is either done by using peptide libraries (Zhuang et al., 2021) or by computational scanning combined with a cellular activity assay (Erez et al., 2023; Vandersarren et al., 2017; Vanheerswynghels et al., 2018). Second, the selected T cell clones need to be able to mount antigen specific responses. The only way that currently TCRs are screened for this essential characteristic is by stimulating them with antigen peptide or co-culture with antigen-pulsed BM-DCs, whereafter their proliferation, CD69 upregulation and IL-2 cytokine production is assessed (Bosteels et al., 2020; Coquet et al., 2015; Nindl et al., 2012; Oxenius et al., 1998). While these assays report on the potential to activate the TCR bearing clone, it does not inform on fitness/competition of the ensuing clone in a polyclonal environment, nor is it known what the effect of the TCR will be on Tg T cell development into the cell-fate of interest. The importance of the latter has been highlighted by multiple publications. By integrating a single (TCR-transgenic) T cell clone with high- or low-affinity ligands, the impact of affinity on effector and memory potential has been examined (Knudson et al., 2013; Zehn et al., 2009). In fact, whereas effector cells are mainly found amongst high-affinity clones, memory cells recognize antigens with lower affinity and benefit from more clonal diversity (Kavazović et al., 2018). Distinct mechanisms drive effector/memory development in high- and low-affinity T cells, such as regulation of IL-12R signalling, T-bet and Eomes expression (Knudson et al., 2013). It has also been shown that upon weak TCR-ligand interactions, expansion of activated naïve T cells will stop earlier than after strong TCR-ligand interactions (Zehn et al., 2009). On top of that, tonic signalling also determines cell-fate decisions. For instance, using CD4 TCR-transgenic mice, it has been shown that the strength of tonic signalling determines whether the cells will become Tfh cells or not (Bartleson et al., 2020)The same authors showed that the potential to react to tonic MHC signals, reflected by the potential of a clone to upregulate CD5, can also profoundly influence whether a given TCR will induce and immediate effector proliferative response, or rather give a memory response, even when the affinity of two clones for the same immunodominant epitope is nearly identical (Milam et al., 2018; Weber et al., 2012). The affinity bias of the TCR-MHC interaction leading to long term memory and residence, particularly in tissues are still not clear. We predict that the method that we propose here which is based on selection of TCRs based on immunophenotype could lead to selection of clones and generation of TCR Tg T cells whose T cells are biased to become either immediate effectors or long-term memory cells.

Given the costs and time investment to generate TCR Tg mice, other labs have also invested in optimizing the strenuous process. For example, Holst. *et al*. (2006) introduced a new model called a retrogenic ('retro' from retrovirus and 'genic' from transgenic) mouse which significantly speeds up the process by using retrovirus-mediated stem cell gene transfer of the TCR alpha and beta sequence (Holst et al., 2006). Guo et al. (2016) described an efficient system for antigen-specific αβTCR cloning and CDR3 substitution (Guo et al., 2016). Furthermore, they introduced a novel reporter cell line (Nur77-GFP Jurkat 76 TCRα$^-$β$^-$) to characterize functional activity of these αβ and γδ TCRs. More recently, a CRISPR-mediated TCR replacement technique was introduced by Legut *et al*. (2018) where the endogenous TCRβ sequence gets replaced (Legut et al., 2018). In this way, issues like competition with the endogenous TCRs for surface expression or mispairing

between endogenous and transgenic TCRs (mixed dimer formation) are excluded. T cell receptor exchange (TRex) mice, where the TCR sequence is integrated in the T-cell receptor α constant (TRAC) locus, results in the expression steered by the endogenous promoter and regulatory flanking regions and circumvents the problem of random integration of the construct in the mouse genome. Rollins *et al*. (2023) extended the TRex-method by disrupting the expression of the gene to which their Tg TCR was directed to, thereby circumventing T cell tolerance (Rollins et al., 2023).

In an attempt to further rationalize and streamline the selection of functional T cell clones, with high fitness and phenotype of interest, we combined single cell gene expression and TCR analysis. To generate our CORSET8 mice, we selected a T cell clone that expressed proliferation markers (*Mki67*$^{+}$), produced cytokines (*Ifng*$^{+}$), and had the potential to become a memory cell (*Cd69*$^{+}$, *Tcf7*$^{+}$, *Sell*$^{-}$). This approach, in which we rationalize every step of TCR Tg mouse generation, can be used to any antigen of interest. Of note, the newly generated DALI tool is user-friendly and free to use, even for immunologists with limited bioinformatics background. We found this approach to be highly time efficient compared to classical methods (see **Fig. 1** for timelines), albeit with increased reagent costs for single cell sequencing. Nonetheless, the rationalized selection of TCR clones might circumvent the expensive generation of poorly functional TCR Tg mouse lines, which additionally conflicts with the reduction arm of the 3R principle.

Figure legends

Supplemental Figure 1:

Screening of CD8 T cell hybridoma clones and validation of first generation CORSET8 mice

A) MFI of IL-2 for individual CD8 T cell hybridoma clones. Based on the fold increase of IL-2 between medium control and Spike peptide pool stimulation, clone 47 was selected to proceed with. B) CD69 expression in hybridoma clone 47 in medium control and upon Spike peptide pool stimulation. C) BM-DC – CORSET8 T cell co-culture in presence of increasing dose of Spike peptide and Spike protein. D) Representative FlowJo plots gating on CD8 T cells in *Rag1*$^{+/+}$, *Rag1*$^{-/-}$, CORSET8 *Rag1*$^{+/+}$ and CORSET8 *Rag1*$^{-/-}$ mice.

Supplemental Figure 2:

Gating strategy used to sort Spike Tetramer positive and Tetramer negative splenic CD8 T cells. Both a naive and representative immunized mouse are shown.

Materials & methods

Mice

Female wild type (WT) C57BL/6 mice were purchased from Janvier Labs (Saint-Berthevin, France) for the immunization experiments. K18-hACE2 transgenic mice were purchased from The Jackson Laboratory (strain ID 034860). Mice were housed under specific pathogen-free conditions and used between 6 and 12 weeks of age. All experiments were approved by the animal ethics committee at Ghent University (EC2023-097) and were in accordance with Belgian animal protection law.

Generation of CORSET8 mice via classical hybridoma technology

A detailed description of the protocol can be found in Vanheerswynghels *et al*. (2018) (Vanheerswynghels et al., 2018). Briefly, C57BL/6 mice were immunized with Spike protein (5 µg, Bio-Techne) in presence of CpG adjuvant (10 µg, ODN1826, Invivogen) according to the immunization protocol in **Figure 1**. Isolated spleen cells were *in vitro* restimulated with an MHCI peptide pool, based on *in silico*prediction by the IEDB database. After four days of culture, CD8 T cells were positively selected by a CD8 T Mojosort® enrichment kit according to manufacturer instructions and immortalized by fusion with BW5147 T lymphoma cells. From specificity-selected monoclonal T cell clones ($IL2^{+}CD69^{+}$ in DC-T cell co-culture), the Vα and Vβ of the CD8+ TCR were subcloned in a p428 expression vector. The TCR constructs were then micro-injected in fertilized oocytes.

Immunization, infection, and *in vivo* treatments

For the primary immunizations to generate Spike reactive T cells, wildtype C57BL/6 were immunized by intraperitoneal injection of 5 µg Spike protein (5 µg, Bio-Techne) in presence of 10 µg CpG adjuvant (10 µg, ODN1826, Invivogen), followed by two reimmunization steps of 5 µg Spike protein in 10 µg CpG adjuvant every 15 days for a total of three immunizations.

For the adoptive transfer experiments, CORSET8 T cells were purified from the TCR Tg donor mice with the MojoSort® Mouse CD8 T Cell Isolation Kit (Biolegend) and labelled with Cell Proliferation Dye eFluor 450 (0.01 mM; Thermo Fisher Scientific) for 10 min in the dark at 37 °C. Next, 1×10^{6} of these labelled cells (in 100 µl of PBS) were intravenously injected into wildtype or K18-hACE2 Tg C57BL/6 mice. In case of immunizations with Spike protein, C57BL/6 mice were subsequently injected intratracheally with 80µL containing 10 µg Spike protein and 10 µg CpG as adjuvant. In the vaccine immunisation strategy, 2 µg of the COMIRNATY (BNT162b2) vaccine was intramuscularly administered in the thigh muscles of the hind limb (final concentration of 40 µg/ml). To analyse the CORSET8 response to SARS-CoV-2 infection, K18-hACE2 Tg mice were intratracheally infected with a sublethal dose of SARS-CoV-2 virus (450 pfu, SARS-CoV-2 D614G strain SARS-CoV-2/human/FRA/702/2020, obtained from the European Virus Archive (EVAG)). These experiments were performed in Biosafety Level 3 conditions at the VIB-UGent Center for Medical Biotechnology. All intratracheal treatments were given after mice were anesthetized with isoflurane (2 liters/min, 2 to 3%; Abbott Laboratories).

DC – CD8 T cell co-culture

A detailed description of the generation of bone marrow-derived dendritic cells (BM-DCs) can be found in Vanheerswynghels *et al*. (2018) (Vanheerswynghels et al., 2018). Lymph node cells were harvested from TCR Tg donor mice and CD8+ T cells were purified with MojoSort® Mouse CD8 T Cell Isolation Kit (Biolegend) and labelled with Cell Proliferation Dye eFluor 450 (0.01 mM; Thermo Fisher Scientific) for 10 min in the dark at 37 °C. 20.000 BMDCs were co-cultured with purified T cells in a 1:5 DC:T cell ratio in sterile tissue culture medium (TCM; RPMI (GIBCO) containing 10% fetal calf serum (Bodinco), 1.1 mg/ml β-Mercaptoethanol (Sigma-Aldrich), 2 mM L-alanyl-L-glutamine dipeptide (Thermo Fisher Scientific) and 56 µg/ml Gentamicin (Thermo Fisher Scientific)) on ice. A serial dilution of antigen was added to the cells, starting from 10 µg/mL of Spike peptide (GNYNYLYRL). CTV dilution and T cell activation were evaluated by flow cytometry after 4 days of incubation at 37°C and 5% CO_2.

Tissue sampling and processing

Mice were euthanized by cervical dislocation or an overdose of pentobarbital (KELA Laboratoria) and organs were collected. To obtain mediastinal lymph node, splenic or thymic single-cell suspensions for flow cytometry, organs were smashed through a 70 µm filter in PBS. Prior to

staining for flow cytometry, splenic single-cell suspensions were depleted of red blood cells (RBCs) by using RBC lysis buffer [0.15 M NH4Cl (Merck), 1 mM KHCO3 (Merck), and 0.1 mM Na2-EDTA (Merck) in MilliQ H2O] produced in-house.

Flow cytometry and cell sorting

For all flow experiments, single cell suspensions were incubated with a mix of fluorescently labelled monoclonal antibodies (Ab) in the dark for 30 minutes at 4°C. To reduce non-specific binding, 2.4G2 Fc receptor Ab (Bioceros) was added. Dead cells were removed from analysis, using fixable viability dye eFluor506 or eFluor780 (Thermo Fisher Scientific). 123count eBeads Counting Beads (Thermo Fisher Scientific) were added to each sample to determine absolute cell numbers. Before acquisition, photomultiplier tube voltages were adjusted to minimize fluorescence spillover. Single-stain controls were prepared with UltraComp eBeads (Thermo Fisher Scientific) following the manufacturer's instructions and were used to calculate a compensation matrix. Sample acquisition was performed on a LSR Fortessa cytometer equipped with FACSDiva software (BD Biosciences) or on a NovoCyte Quanteon flow cytometer (Agilent) equipped with NovoExpress software. Final analysis and graphical output were performed using FlowJo software (Tree Star, Inc.) and GraphPad Prism 10 (GraphPad Software, Inc.).

The following antibodies were used: anti-CD62L (FITC, eBioscience), anti-Vβ11 (FITC, BD BioScienes, clone RR3-15), anti-CD45.2 (PerCP-Cy5.5, BD Biosciences), anti-TCRβ (BV510, BioLegend), anti-CD45.1 (BV605, BioLegend), anti-CD44 (BV650, BioLegend), anti-CD8 (BV750, BD Biosciences), anti-CD5 (BV786, BD Biosciences), anti-CD44 (RedFluor710, VWR International), anti-CD45 (AF700, BioLegend), anti-CD62L (PE, BD Biosciences), anti-CD8 (PE-Cy7, BioLegend), anti-CD4 (BUV395, BD Biosciences) and anti-CD3 (BUV737, BD Biosciences).

Single cell RNA seq and downstream DALI analysis

Splenocytes of the immunized mice (and naïve control mouse) were stained with an MHCI tetramer recognising our target T cells (H-2K(b) SARS-CoV-2 S 539-546 VNFNFNGL, provided by the NIH Tetramer Core Facility) for 1 hour at 37°C. The cells were washed and stained for surface markers (anti-CD8 (PerCP-eFluor710), Viability dye (eFluor506), anti-CD44 (RedFluor710) and anti-TCRβ (APC-eFluor780)) and a unique TotalSeq-C hashing antibody (BioLegend) for 30 min at 4°C. Afterwards cells were washed and resuspended in PBS + 0,04% BSA. Sorted single-cell suspensions were resuspended at an estimated final concentration of 270, 1150 and 1450 cells/µl and loaded on a Chromium GemCode Single Cell Instrument (10x Genomics) to generate single-cell gel beads-in-emulsion (GEM). The scRNA/Feature Barcoding/BCR/TCR libraries were prepared using the GemCode Single Cell 5' Gel Bead and Library kit, version Next GEM 2 (10x Genomics) according to the manufacturer's instructions. The cDNA content of pre-fragmentation and post-sample index PCR samples was analyzed using the 2100 BioAnalyzer (Agilent). Sequencing libraries were loaded on an Illumina NovaSeq flow cell at VIB Nucleomics core with sequencing settings according to the recommendations of 10x Genomics, pooled in a 75:10:10:5 ratio for the gene expression, TCR, BCR and antibody-derived libraries, respectively.

Demultiplexing of the raw sequencing data was done using bcl2fastq2 (v2.20). Mapping of the gene expression data to the mouse genome (GRCm38; ensembl release 99), barcode processing, unique molecular identifiers (UMI) filtering and gene counting was performed using the Cell Ranger suite (version 6.1.1). TCR data was also processed using the Cell Ranger suite, using the cellranger vdj subcommand. The contig annotation was done with the GRCm38 vdj references provided by 10X Genomics (version 5.0.0).

Preprocessing of the data was done by the scran and scater R package according to workflow proposed by the Marioni lab (Lun et al., 2016). Outlier cells were identified based on 3 metrics (library size, number of expressed genes and mitochondrial proportion) and cells were tagged as outliers when they were 5 median absolute deviation (MADs) away from the median value of each

metric across all cells. Normalization, detecting highly variable genes, finding clusters, and creating UMAP plots and marker gene identification was done using the Seurat pipeline (v3.1.5). Potential doublets were removed from the analysis. TCR data was integrated into the Seurat object using DALI 2.0.0 for downstream analysis.

Generation of CORSET8 mice starting from DALI derived TCR sequences

The selected Vα and Vβ TCR sequences were ordered as gBlocks (IDT) and subsequently cloned into the CD4 expression vector p428 via SalI (Vanheerswynghels et al., 2018). Injection fragments we excised from the p428 backbone by NotI digestion, gel purified and injected in an equimolar solution of 1 ng/μL into fertilized C57BL/6 oocytes.

Statistical analysis

Data information: Data are shown as means ± SEM. All data were analysed with a Shapiro-Wilk normality test to assess whether the data was normally distributed. Parametric data were analysed with a t-test, whereas non-parametric data were analysed with a Mann-Whitney test. *p<0.05, **p<0.01.

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Editors

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Reviewer #1 (Public Review):

Summary:

Debeuf et al. introduce a new, fast method for the selection of suitable T cell clones to generate TCR transgenic mice, a method claimed to outperform traditional hybridoma-based approaches. Clone selection is based on the assessment of the expansion and phenotype of cells specific for a known epitope following immune stimulation. The analysis is facilitated by a new software tool for TCR repertoire and function analysis termed DALI. This work also introduces a potentially invaluable TCR transgenic mouse line specific for SARS-CoV-2.

Strengths:

The newly introduced method proved successful in the quick generation of a TCR transgenic mouse line. Clone selection is based on more comprehensive phenotypical information than traditional methods, providing the opportunity for a more rational T cell clone selection.

The study provides a software tool for TCR repertoire analysis and its linkage with function.

The findings entail general practical implications in the preclinical study of a potentially very broad range of infectious diseases or vaccination.

A novel SARS-CoV-2 spike-specific TCR transgenic mouse line was generated.

Weaknesses:

The authors attempt to compare their novel method with a more conventional approach to developing TCR transgenic mice. In this reviewer's opinion, this comparison appears imperfect in several ways:

• Work presenting the "traditional" method was inadequate to justify the selection of a suitable clone. It is therefore not surprising that it yielded negative results. More evidence would have been necessary to select clone 47 for further development of the TCR transgenic line, especially considering the significant time and investment required to create such a line.

• The comparison is somewhat unfair, because the methods start at different points: while the traditional method was attempted using a pool of peptides whose immunogenicity does not appear to have been established, the new method starts by utilising tetramers to select T cells specific for a well-established epitope.

• Given the costs and time involved, only a single clone could be tested for either method, intrinsically making a proper comparison unfeasible. Even for their new method, the authors' ability to demonstrate that the selected clone is ideal is limited unless they made different clones with varying profiles to show that a particular profile was superior to others.

In my view, there was no absolute need to compare this method with existing ones, as the proposed method holds intrinsic value.

While having more data to decide on clone selection is certainly beneficial, given the additional cost, it remains unclear whether knowing the expression profiles of different proteins in Figure 2 aids in selecting a candidate. Is a cell expressing more CD69 preferable to a cell expressing less of this marker? Would either have been effective? Are there any transcriptional differences between clonotype 1 and 2 (red colour in Figure 2G) that justify selecting clone 1, or was the decision to select the latter merely based on their different frequency? If all major clones (i.e. by clonotype count) present similar expression profiles, would it have been necessary to know much more about their expression profiles? Would TCR sequencing and an enumeration of clones have sufficed, and been a more cost-effective approach?

Lastly, it appears that several of the experiments presented were conducted only once. This information should have been explicitly stated in the figure legends.

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Reviewer #2 (Public Review):

Summary:

The authors seek to use single-cell sequencing approaches to identify TCRs specific for the SARS CoV2 spike protein, select a candidate TCR for cloning, and use it to construct a TCR transgenic mouse. The argument is that this process is less cumbersome than the classical approach, which involves the identification of antigen-reactive T cells in vitro and the construction of T cell hybridomas prior to TCR cloning. TCRs identified by single-cell

sequencing that are already paired to transcriptomic data would more rapidly identify TCRs that are likely to contribute to a functional response. The authors successfully identify TCRs that have expanded in response to SARS CoV2 spike protein immunization, bind to MHC tetramers, and express genes associated with functional response. They then select a TCR for cloning and construction of a transgenic mouse in order to test the response of resulting T cells in vivo following immunization with spike protein of coronavirus infection.

Strengths:

(1) The study provides proof of principle for the identification and characterization of TCRs based on single-cell sequencing data.

(2) The authors employ a recently developed software tool (DALI) that assists in linking transcriptomic data to individual clones.

(3) The authors successfully generate a TCR transgenic animal derived from the most promising T cell clone (CORSET8) using the TCR sequencing approach.

(4) The authors provide initial evidence that CORSET8 T cells undergo activation and proliferation in vivo in response to immunization or infection.

(5) Procedures are well-described and readily reproducible.

Weaknesses:

(1) The purpose of presenting a failed attempt to generate TCR transgenic mice using a traditional TCR hybridoma method is unclear. The reasons for the failure are uncertain, and the inclusion of this data does not really provide information on the likely success rate of the hybridoma vs single cell approach for TCR identification, as only a single example is provided for either.

(2) There is little information provided regarding the functional differentiation of the CORSET8 T cells following challenge in vivo, including expression of molecules associated with effector function, cytokine production, killing activity, and formation of memory. The study would be strengthened by some evidence that CORSET8 T cells are successfully recapitulating the functional features of the endogenous immune response (beyond simply proliferating and expressing CD44). This information is important to evaluate whether the presented sequencing-based identification and selection of TCRs is likely to result in T-cell responses that replicate the criteria for selecting the TCR in the first place.

(3) While I find the argument reasonable that the approach presented here has a lot of likely advantages over traditional approaches for generating TCR transgenic animals, the use of TCR sequencing data to identify TCRs for study in a variety of areas, including cancer immunotherapy and autoimmunity, is in broad use. While much of this work opts for alternative methods of TCR expression in primary T cells (i.e. CRISPR or retroviral approaches), the process of generating a TCR transgenic mouse from a cloned TCR is not in itself novel. It would be helpful if the authors could provide a more extensive discussion explaining the novelty of their approach for TCR identification in comparison to other more modern approaches, rather than only hybridoma generation.

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