Longitudinal Analysis of CD4 and CD8 T Cell Receptor Repertoires Associated with Newcastle Disease Virus Infection in Layer Birds

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ABSTRACT

Velogenic Newcastle disease, also known as exotic Newcastle disease, is a contagious and fatal viral avian disease for both wild and domestic bird species. The World Health Organization for Animals (OIE) defined this disease as one of the most important avian diseases worldwide. This study used integrative genetics, genomics, and immunological approaches to study the chicken host response to Newcastle Disease Virus (NDV) with aim to sustainably enhance innate resistance in chickens. The T-cell receptor (TCR) is a highly polymorphic surface receptor on the T cells that recognize antigens presented by the major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). To investigate the temporal TCR dynamics under Newcastle disease virus infection, we performed high-throughput sequencing of CD4 and CD8 TCRβ chains on longitudinal peripheral blood mononuclear cell (PBMC) samples collected from pre-infection, 7-, 14-, and 21-days post infection (dpi), and the lung and harderian gland samples collected at 21 dpi from two commercial egg laying hens. From the Vβ and Jβ segment usage heatmap data, we found the most frequently used Vβ and Jβ segments are relatively common across all samples, reflecting a potential intrinsic bias in the VDJ rearrangement process. Repertoire overlap analysis revealed that in one bird the CD8 TCRβ sequences that were found with a high frequency at one time point were also found with a high frequency at all other time points, showing that frequencies of dominant TCRβs were largely consistent over time. Moreover, diversity rarefaction plot data indicated for both birds that CD4 TCRβ diversity at 21 dpi was lower than before infection, providing evidence for the appearance of clonally expanded repertoires during infection with NDV. In addition, at different timepoints the diversity of PBMC CD4 repertoires was higher than their counterparts in the CD8 groups. Finally, we tracked from pre-infection to 21 dpi the CD8 TCR clonotypes in the bird used for repertoire overlap analysis and

found most of the TCR clonotypes are time persistent. For example, CAKRGNRNERLIF, CASSSTGSGTPLNF, and CAKQDNERLIF were observed at all these timepoints and had the highest clonotype abundance at 7 dpi. In general, our study will expand knowledge about antigen receptor repertoire functions in cell-mediated and humoral immunity to enable better understanding of the role of T cell immune repertoires in health and disease.

INTRODUCTION

Newcastle disease virus

Newcastle disease virus (NDV) is a member of the genus *Orthoavulavirus* within the family *Paramyxoviridae* in the order *Mononegavirales*. At present, the genus *Avulavirus* includes 21 serotypes of avian paramyxoviruses (APMV), and NDV is the serotype 1 (APMV-1) (de Leeuw and Peeters 1999). NDV has a single-stranded, negative sense and non-segmented RNA genome with a length of 15,186 nucleotides. The genome contains six genes encoding six major structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin neuraminidase protein (HN), large RNA-dependent RNA polymerase protein (L), and they are arranging in the order of 3'-NP-P-M-F-HN-L-5'. In addition, two nonstructural proteins V and W are encoded by the P gene and expressed after RNA editing (Steward et al. 1993). NDV infection starts with the recognition of receptors, then virion binds to the sialyglycoconjugates on the host cell surface. After that the viral lipid envelope fuses with the membrane of the host cell, where upon the interaction of two viral surface glycoproteins, HN and F, plays an important role (Connolly et al. 2009; Connaris et al. 2002). The sequence composition at the F protein cleavage site has been proven to be a reliable indicator of NDV pathogenicity, where virulent NDV strains usually have several basic residues, while avirulent strains do not (Connolly et al. 2009; Rue et al. 2010).

Newcastle disease

Velogenic Newcastle disease (ND), also known as exotic Newcastle disease, is a contagious and fatal viral avian disease for both wild bird and domestic bird species. The World Health Organization for Animals (OIE) defined this disease as one of the most important poultry diseases worldwide. ND virus affects bird's digestive, nervous and respiratory system, moreover

the highly virulent strains can kill birds without obvious clinical signs. Very virulent NDV (vNDV) can cause significant economic losses with up to 100% mortality in unvaccinated poultry flocks, thus this disease is severely impacting poultry industry, especially Asia, Africa, Central America, parts of South America (Miller, Decanini, and Afonso 2010; Dimitrov et al. 2016). In the past fifty years, there are three large-scale Newcastle disease outbreaks in the US which have significantly influenced the commercial poultry industry. The first outbreak happened in the early 1970's. vNDV was introduced by an importation of infected parrots into U.S., including New York, Florida, Texas, New Mexico and California, but mainly transmitted among southern California (Burridge, Riemann, and Utterback 1975; Hanson, Spalatin, and Jacobson 1973; H. Kinde et al. 2005; Utterback and Schwartz 1973). The virus spread rapidly between chicken flocks from 1971 to 1973 and was eradicated three years later at the cost of 11.9 million bird destruction and 56 million USD of economic loss (Hanson, Spalatin, and Jacobson 1973). The second major outbreak occurred during 2002-2003 in southern California again caused by the illegally imported privately-owned game fowl (Hietala et al. 2004). The pandemic was eradicated at a cost of the 3.21 million bird depopulation and 160 million USD (Nolen 2003). After that, in May of 2018, vNDV was detected again in Southern California. From 2018 to 2020, according to U.S. Department of Agriculture (USDA) record, NDV affected 476 positive premises including 4 commercial premises in six counties of California, one county of Utah and one country of Arizona (Ferreira et al. 2020)

NDV infection can generate different levels of virulence in birds, from inapparent infection to 100% mortality. Thus, depending on the severity of this disease shown in chickens, NDV strains are categorized into four major pathotypes: asymptomatic enteric, lentogenic, mesogenic and velogenic strains, which show avirulence, low virulence, intermediate virulence and highly

virulence respectively (Hanson and Brandly 1955). Lentogenic strain present with mild or subclinical respiratory infection signs with negligible mortality. Mesogenic strain presents with some respiratory signs, temporary nervous signs, lower egg quality and production and result in ≤ 10% mortality. Viscerotropic and neurotropic velogenic stains are highly virulent strains and present with severe nervous and respiratory signs, frequently with hemorrhagic intestinal lesion, and spread rapidly to cause up to 90% mortality (Hines and Miller 2012; Al-Garib et al. 2003).

Control methods of Newcastle disease

The poultry industry relies mainly on strict biosecurity and vaccination protocols to control the spread of NDV. Stringent biosecurity can prevent NDV from being introduced and transmitted into a susceptible poultry flock before they have gotten sufficient protective immunity or can keep them away from any viral exposure (Kapczynski, Afonso, and Miller 2013). The main components of biosecurity is to protect poultry housing from the potential viral transmission from wild birds or their excretions, since those sources have been demonstrated previously to be the cause of many NDV outbreaks in the world (Aldous et al. 2004; D. J. Alexander, Wilson, et al. 1985; D. J. Alexander, Russell, et al. 1985; Biancifiori and Fioroni 1983; Dennis J. Alexander 2011). Effective biosecurity method includes preventing the interaction of wild birds with commercial birds. In addition, small flocks need adhere to the permission required by the federal and state authorities when importing from other countries or states.

Up to now, the most efficient method is to vaccinate birds. When administered to healthy birds in a correct and timely fashion, the ND vaccination is able to decrease or eliminate clinical both symptoms and mortality from virulent NDV infection. Vaccination also decreases the duration, incidence and amount of virus shedding, and increases the titer of NDV needed for breakthrough infection of the vaccinated birds (Kapczynski, Afonso, and Miller 2013; Miller et al.

2007; Kapczynski, Wise, and King 2006). Moreover, if more than 85% of the birds in flock receive appropriate dose of vaccines that yields a high antibody titer, the ideal status of herd immunity can be achieved (van Boven et al. 2008). Live and inactivated ND vaccines are the two main vaccines used in the poultry industry since the 1950's (Gallili and Ben-Nathan 1998). Other types of vaccines, such as recombinant and antigenically matched engineered vaccines have also been developed (Izquierdo-Lara et al. 2019; Dimitrov et al. 2017). Other issues such as incomplete or improper immunization can affect the efficacy of ND vaccination. For example, it is challenging to have vaccine applied evenly and equally to every bird in large commercial flocks. In many developing countries, additional challenges are faced, such as a scavenge system with different bird ages in a flock, limited refrigeration for vaccine storage and transportation etc.

Advanced integrative genetics, genomics, and immunology approaches, which aim to both identify genetic markers and biomarkers associated with resilience to NDV and to sustainably enhance innate resistance in chickens, can provide an alternative way to prevent ND (Kapczynski, Afonso, and Miller 2013). Genetics plays an important role in studying disease resistance to pathogens in birds, and examples include the application of transgenic and genomic selection in poultry (Bumstead 1998; Jie and Liu 2011; Zekarias et al. 2002). Different degrees of resistance to NDV infection among different breeds or genetic lines was observed in 1940s (Alkiston and Gorrie 1942). Later on, differences in mortality rates between chicken breeds or lines suggested the effect of genetic variations on viral resistance to ND infection (Cole and Hutt 1961; Seal, King, and Bennett 1996). Then, Cole and Hutt found that different chicken strains had different antibody response against NDV, and further studies indicated that the antibody titer against NDV has low to moderate heritability (Lwelamira, Kifaro, and Gwakisa 2009; Luo et al. 2013). With the development of high-quality chicken genome assembly in chickens, as well as high-throughput

genotyping and sequencing methods, there have been several studies on the identification of quantitative trait loci (QTL) affecting NDV antibody levels in chickens (Luo et al. 2013; Saelao, Wang, Chanthavixay, et al. 2018; Yonash et al. 2001), and additional research is still needed to further narrow the genomic regions and identify genes that confer causative variants associated with immune responses against NDV.

Chicken immune system

In general, birds have a similar immune system and immune responses than mammals. However, some differences can be noticed especially between immune organs, cells, and molecules. In addition to the primary lymphoid organs (bone marrow and thymus) found in mammals, birds have a special primary lymph organ, the bursa of Fabricius. The bursa of Fabricius is a diverticulum of the cloaca only present only in birds. It is the single site of B cell receptor (BCR) repertoires development that accompanies B cell differentiation and maturation (Fellah et al. 2014). In chickens, the bursa only shows activity only in young birds, it reaches its maximum size at about 8 weeks, and usually atrophies at six months of age (Ratcliffe et al. 1986). Because of these characteristics, many early studies used bursectomy in chickens, either by surgical or chemical means to confirm that lymphocytes from the bursa were responsible for antibody maturation (Kincade, Lawton, and Cooper 1971). Moreover, as a part of the primary lymphoid tissues, the bursa of Fabricius plays an important role in the response to gut microbiota and pathogens (Ratcliffe 2006; Kozuka et al. 2010).

Birds have three major classes of Immunoglobulins produced in bursa, IgM, IgY (also called avian IgG), and IgA, which represent the functional homologues of mammalian IgM, IgG and IgA, respectively. Birds lack the immunoglobulin isotypes of IgD and IgE, due to the unique process of antibody repertoire generation in birds. Unlike humans and mice, each avian BCR chain

just has one variable (V), one joining (J), and fifteen diversity (D) gene segments. However, birds have many V pseudogenes upstream of the V genes, and portions of these V pseudogenes will be exchanged with the homologous sequences in V genes to produce great diversity in BCR repertoires during the gene conversion.

The thymus is another primary lymphoid organ for birds. T cells are born from hematopoietic stem cells then developing in bone marrow, migrate to thymus to mature, then go to all over the body through blood circulation. However, the location of avian thymus is different from mammals. In chicken, thymus is composed of 7-8 separated lobes of ovoid tissues located on both sides of neck, distributed from the third cervical vertebra to the upper thoracic vertebrae, parallel to the vagus nerve and the internal jugular vein (Ciriaco et al. 2003). The lobes reach their maximum size at about 3-4 months of age, then begin to involute. Lobes are covered by a fine fibrous connective tissues capsule that is embedded in fat tissues. T cells recognize different antigens via several T cell receptors (TCR). Like mammals, birds also have TCR1 (gamma and delta TCR) and TCR2 (alpha and beta TCR) isotypes. However, birds have another receptor isotype that is also generated in thymus and neither expressed TCR1 nor TCR2, called TCR3. This special receptor was first detected in the blood at one week of age and was found in both cytotoxic T cells and helper T cells (Chen, Bucy, and Cooper 1990).

Another major anatomical difference in immune-related organs between birds and mammals in immune-related organs is the absence of encapsulated lymph nodes in birds. Birds usually complete their local immune responses in well-developed mucosa-associated lymphoid tissue (MALT), such as in respiratory tract, reproductive tract, gut, and eyes (Oláh, Nagy, and Vervelde 2014). The harderian gland (HG) is one of the essential secondary lymphoid tissues in birds. The HG is an immune-endocrine organ located in the orbit behind the eyes. Although other

species, such as rodents, also have HGs, only avian HGs can be considered as the secondary lymphoid tissue since they have an incomparable large amount of plasma cells (Maynard and Downes 2019). Also, the number of plasma cells in HGs increases dramatically with bird's age These plasma cells can proliferate *in situ*, and include IgM-producing, IgY-producing and IgA-producing plasma cells; consequently, all of these three immunoglobulins can be found in avian tears (Jeurissen et al. 1989; Jalkanen et al. 1984; Oláh, Kupper, and Kittner 1996).

T cells depend on a diversity of T cell receptors to recognize antigens presented in the major histocompatibility complex (MHC) molecules of antigen presenting cells (APCs) surface. Like mammals, avian TCRs are highly diverse heterodimers composed with two chains, α and β chains on αβ T cells, or γ and δ chains shown on γδ T cells. Each TCR consists of two immunoglobulin domains, a variable region on the top that is important for antigen recognition, and a constant region close to T cell membrane which determines the class of TCR. The variable regions of α and δ chains are encoded by variable (V) and joining (J) gene segments and the variable regions of β and γ chains are encoded by variable (V), diversity (D) and joining (J) gene segments. The diversity of TCRs is mainly attributable to VDJ segments rearrangement which occurs during somatic recombination process in developing lymphocytes. Briefly, one allele of each segment is recombined with others to form a complete variable region, which then binds with a constant region to form a functional TCR chain transcript. Of note, different numbers of D gene segments can be inserted between the V and J segments. Also, random nucleotides might be added or deleted at the junction parts between these segments. Thus, VJD recombination as well as use of random nucleotides will theoretically lead to an infinite number of clonotypes that can ensure the TCR to recognize a huge diversity of antigens. Previous studies have confirmed that these processes are not random but rather influenced by negative and positive selection during

lymphocyte development. Negative selection refers to the elimination of autoreactive cells and positive selection refers to the rescue of cells otherwise destined to die by neglect. Several factors were confirmed to be involved in negative and positive selections, such as special V segment usage (Meng et al. 2011), chain pairing structure (Wardemann et al. 2003), self-antigen reaction (Madi et al. 2014), interaction with MHC (Gao et al. 2019; Houston and Fink 2009), length of CDR3 region (C. Wang et al. 2017).

Each TCR chain contains three main hyper-variable regions called complementarity determining regions (CDR 1-3). Among these, CDR3 has highest variability in amino acid residues of each antigen biding site since it is encoded either by the junctions of VDJ segments or VJ segments. Also, CDR3 is the only region of the whole TCR that has a direct contact with the antigen peptides. For the reasons described above, CDR3 is often used in many studies to determine T cell clonotypes (Turner et al. 2006; Miles, Douek, and Price 2011).

T cells have two main subsets, CD4 and CD8 T cells. CD8 T cells are also called cytotoxic T cells because they can directly kill virus-infected cells or cancer cells. CD4 T cells are also called T helper cells since they can determine how the host immune system will respond to the infected cells then indirectly kill them. CD4 T cells produce small signaling molecules to regulate other immune cells' behaviors, support CD8 responses, help B cells to form the antigen-specific B cells, and eventually result in the generation of specific antibodies to bind cognate antigens.

The TCR repertoire (or TCR profile) is the sum of all TCRs produced by T cells in one individual. During the onset and progression of diseases, the TCR repertoire can change dramatically due to the exposure to foreign antigens (Wolf et al. 2018; Venturi et al. 2016). Immunologists are increasingly interested in the study of TCR repertoire changes under different disease conditions, in order to track an individual's immune responses to a specific pathogen. In

chickens, prior studies mainly used spectratyping and Sanger sequencing to profile the TCR repertoire. Upon infection with Marek's disease, both public and private CDR3 sequences were found in the oligoclonal expanded CD8 T cells (Mwangi et al. 2011). Also, frequent CDR3 sequences from CD8 TCR Vβ repertoire were found under multiple repeated infections with the parasite *Eimeria maxima* (Ren et al. 2014). CD4 and CD8 T cells use the same genetic elements to generate functional TCRs, during positive selection T cells with receptor specific an antigen presented by MHC class I will become into CD8 T cells, and T cells with receptor specific an antigen presented by MHC class II will become into CD4 T cells. In humans, previous studies found low overlap between CD4 and CD8 T cells that shared same CDR3 sequences (Li et al. 2016; Yin et al. 2011). However, it is currently still unclear to what extent the TCR repertoire of CD4 and CD8 T cells is different in chicken.

The development of high-throughput sequencing has helped reveal the dynamic changes in immune receptors during viral infections. High-throughput sequencing has been widely used in experiments involving human and mice, however few studies have been conducted in the chickens (Chaudhary and Wesemann 2018; Hou et al. 2016). The specific objective of this study was to profile dynamic repertoire changes under Newcastle disease virus infection from different T cell subsets in blood and different lymphoid tissues in commercial egg laying hens.

MATERIALS AND METHODS

Chickens, viral strain and sample process

Two Hy-Line W-36 birds (#2549 and #2553) were used in this study. Hy-Line W-36 is a commercial egg laying hen line maintained in the Avian Facilities at the University of California, Davis. Animal experiments were performed according to the guidelines approved by the

Institutional Animal Care and Use Committee, University of California, Davis (IACUC #21886). At 21 days post hatch, these birds were challenged through both nasal and ocular routes with 4×10^8 NDV La Sota strain (EID_{50}). Peripheral blood samples were collected before infection, and at 7-, 14- and 21-days post infection (DPI). Lungs and Harderian glands were collected at 21 DPI. Lung and HG tissues were dissociated and filtered through 40-um mesh cell strainers to remove debris then resuspended into single cell suspensions. Peripheral blood mononuclear cells (PBMCs) and lung mononuclear cells were separated using Histopaque®-1077 (Sigma) then resuspended into single cell suspensions.

Separation of CD4 and CD8 by Flow-cytometry

Single cell samples were stained with antibodies to CD3 (Mouse Anti-Chicken CD3-PE, Southern Biotech), CD4 (Mouse Anti-Chicken CD4-FITC, BioRad) and CD8 (Mouse Anti-Chicken CD8 Beta, BioRad). Cells were sorted by fluorescence-activated cell sorting (FACS Beckman Coulter) into CD3+CD4+ and CD3+CD8+ cell samples. Recovered cells were lysed by lysis buffer RA1 from RNA isolation kit (NucleoSpin® RNA XS, Takara Bio USA) and stored at -80°C for further processing.

Library preparation and sequencing

Total RNA was isolated from sorted samples using NucleoSpin® RNA XS isolation kit. Since the 5' end of the TCR beta germline DNA is variable region in which a specific primer cannot bind, we used SMARTer® RACE 5'/3' Kit (Takara Bio USA, USA) to amplify the complementary-determining region (CDR) of the TCR beta chain from the 5' end to its constant region. The first step was to generate first-strand cDNA, which was synthesized from 500ng

starting RNA with 5' CDS Primer A from the kit, incubating at 72□ for 3 minutes, then cooling to 42□ for 2 minutes. The second step is to produce 5'-RACE ready cDNA, we used SMARTer II A Oligonucleotide and SMARTScribe Reverse Transcriptase from the kit, incubating at 42□ for 90 minutes and heating at 70□ for 10 minutes. The last step was to rapid amplify 5' RACE-ready cDNA ends, we added the custom primer (GSP), 2.5ul of the resulting cDNA from second step and other reagents from the kit with a program of 40 cycles of 94 □ for 30 seconds and 72□ for 2 minutes. The nucleotide sequence of our custom primer, also known as gene-specific primer was 5'-GTTCAGGGAAGAAACCAGAGGCCAGG-3'. The PCR products were purified using QIAquick Gel Extraction kit (Qiagen, Germany) viand then were examined by Nanodrop Spectrophotometer with criteria of 260/280 ratio less than 1.9 and 260/230 larger than 1.0. In addition, the GSPs were designed together with unique 8-nucleotide barcodes for individual sample to put in the same high-throughput sequencing pool. We pooled 10ul of 8 purified DNA samples with different barcodes into one group: all CD4 PBMCs in one group, all CD8 PBMCs in one group, and all lung and HG samples in one group. The pooled DNA samples were submitted for sequencing using Illumina MiSeq with PE250 reads at the Genome Center at University of California, Davis.

Pre-processing and basic statistics

The sequence data were pre-processed using VDJtools version 1.2.1 (Shugay et al. 2015). We filtered out the potential cross-sample contamination generated by FACS, PCR, or sequencing processes by command line "Decontaminate". We then removed the non-coding clonotypes, the ones that contain stop codons or frameshifts in their receptor sequences using "FilterNonFunctional".

For basic statistical analysis, we calculated the following basic parameters: number of reads per sample (count), number of clonotypes per sample (diversity), mean and geometric mean of clonotype frequency, mean length of CDR3 nucleotide sequence, mean number of unique CDR3 nucleotide sequences that code for the same CDR3 amino acid sequence (convergence).

CDR3 alignment

There is no publicly available database for chicken TCR V and J gene segments, so we aligned and mapped our Illumina sequencing results to an unpublished database assembled based on the previous works of many researchers.

We de-multiplexed the sequencing data by Molecular Identifier Guided Error Correction (MIGEC) pipeline version 1.2.9 (Shugay et al. 2014). The reptools package from Python v2.7.13 was used to process the demultiplexed FASTQ files, and classify the reads into V, J and C gene segments. Then CDR3 segments was identified depending on the alignment of V and J segments. An "expected error" rate greater than 0.2 according to the QUAL string was used to further filter CDR3 segments. In addition, CDR3s were further filtered out if frameshifts or STOP codon were present, or either the conserved N-terminal cysteine residue or the conserved C-terminal phenylalanine/tryptophan residue were absent.

The USEARCH in reptools was used to identify gene segments based on the custom sequence databases in a call using UBLAST algorithm (Edgar 2010), then in the local alignment mode, identified the ends of the CDR3 in a call using USEARCH. Quality filtering and de-replication were performed using calls to USEARCH (Edgar and Flyvbjerg 2015). In alignment for CDR3 extraction, a sequence identity with no less than 75% was required. For the process of segment identification, a sequence identity with no less than 80% (for the V segment) or no less than 90% (for the J and C segments) was required.

TCR Vβ and Jβ segment usage

Vβ and Jβ segments usage was calculated based on the frequency of associated reads matching with each Vβ and Jβ segments in our custom database and hierarchically clustered by samples and V or J usage vectors.

Repertoire diversity and rarefaction analysis

Diversity and rarefaction analysis of repertoires were calculated using VDJtools version 1.2.1. For this analysis, data was split into two groups: CD4 and CD8. Diversity estimates were calculated by sample-size-based rarefaction and extrapolation using VDJtools using R package ggplot2. Rarefaction plots display the dependencies between sample diversity and sample size. Those curves were interpolated from zero to the current sample size and then extrapolated up to the size of the largest of samples depending on multinomial models, allowing comparison of diversity estimates.

Repertoire overlap analysis

A comprehensive analysis of clonotype sharing for pairs of samples was conducted using VDJtools version 1.2.1 and R version 3.4.1.

Pairwise distance: an all-versus-all pairwise distance for the sample repertoires using "ClacPairwiseDistance" in VDJtools was performed and repertoire similarity was calculated.

Cluster analysis: A multi-dimensional scaling (MDS) plot was performed for repertoire cluster analysis, and Euclidean distance between points reflects the distance between repertoires. Different colors represent different time points.

Overlap pair analysis: For the CD4 or CD8 PBMC group, clonotype scatterplots with linear regression were performed to compare overlaps between T cell sequences over time for CD4 and CD8 groups. Each dot on the scatterplots indicates a single TCR clonotype, and point size is scaled to the geometric mean of this clonotype. X and Y axes denote log10 clonotype frequencies in each sample. Two marginal histograms show the overlapping (red) and total clonotype (grey) abundance distributions in corresponding sample. Histograms were weighted by clonotype abundance.

Joint clonotypes: The number of shared and non-shared TCR sequences from different samples were calculated. We firstly compared the CD4 with CD8 group in the same bird with the same time point, then we compared all the CD4 (or CD8) samples across different time points within the same bird.

Clonotypes tracking analysis: Clonotype tracking stackplot contains detailed profiles for top clonotypes, as well as collapsed (Not-shown) and non-overlapping (Non-overlapping) clonotypes. Clonotype CDR3 amino acid sequence was plotted against the sample where the clonotype reaches maximum abundance. The read count, frequency (both non-symmetric) and the total number of clonotypes shared between samples from different time points were summarized.

RESULTS

Basic statistics

After filtering out the potential contaminations and non-coding clonotypes, the basic statistics for each sample are shown in Table 1. Counts represent the number of CDR3 reads in each sample and it ranged from 9,199 to 1,801,999 reads per sample depending on the sequencing read depth. Diversity is the number of unique clonotypes in each sample, and it ranged from 477 to 19,235 clonotypes per sample. Average clonotype frequencies includes mean and geometric mean of frequencies. The mean frequencies among samples ranged from 5.20E-05 to 2.10E-03. Mean lengths of CDR3 nucleotide sequences ranged from 39bp to 48bp. Convergence is the mean number of unique CDR3 nucleotide sequences that code for the same CDR3 amino acid sequence, and it ranged from 1.16 to 1.67.

TCR Vβ and Jβ segment usage

Figure 1 shows the distributions of Vβ gene usage across samples from different time points within CD4 or CD8 group. For the CD4 group (Figure 1A), TRBV 1.2, 1.4, and 1.11 clonotypes were the top three abundant ones in bird #2549, while TRBV 1.9, 1.11, and 1.4 were the top three in the bird #2553. For the CD8 group (Figure 1B), TRBV 1.11, 1.4, 1.2, 1.3 and 1.7 were the most abundant in #2549 while TRBV 1.11, 1.4, 1.8, 1.3 and 1.7 were the most abundant in #2553. In order to quantify the relative similarity of Vβ gene segment usage across all samples, we calculated the Vβ segment Jensen-Shannon divergence (vJSD) between them and generated a heatmap (Figure 1C). The vJSD ranges from 0 to 1. A vJSD of 0 indicates identical Vβ segment usage, while a vJSD of 1 indicates that the Vβ segment usages are distinct between two samples. When comparing the vJSD between samples within the same chicken, especially from bird #2549,

the results revealed that the usage of Vβ segments for both CD4+ and CD8+ T-cells was consistent over time. But samples from different chicken displayed a higher divergence between them.

Figure 2 shows the distributions of Jβ gene usage across samples from different timepoints within CD4 or CD8 group. For the CD4 group, TRBJ 3 and TRBJ 4 were the most abundant, while TRBJ 3 and TRBJ 2 were the most abundant in the CD8 group.

Repertoire diversity and rarefaction analysis

Figure 3 shows the rarefaction plots for both CD4 and CD8 samples in each bird. Because different samples had different number of sequencing reads, which affected the diversities of each sample. Therefore, we down-sampled each sample to 1,250,000 reads to compare repertoire diversities in each bird. For bird #2549, the diversities of PBMC CD4 repertoires from different timepoints were greater than their counterparts in the CD8 group, for example, diversity of CD4 7dpi PBMC from #2549 (2549_CD4_07P) was greater than #2549 CD8 7dpi PBMC (2549_CD8_07P). For bird #2553, for a similar pattern was observed at 0dpi and 7dpi for PBMC samples. Additionally, for the CD4 group, the diversity of pre-infection PBMC (0P) was greater than 21dpi PBMC (21P) in both birds, which suggested potential clonal expansion with NDV infection.

Repertoire overlap analysis

Besides diversity, we also quantified the relative similarities in TCR repertoire samples.

We first performed multi-dimensional scaling (MDS) plots for all the samples to cluster samples by their pairwise distances. The similarities of TCR repertoires from different birds, tissues, timepoints, and cell subsets are presented in Figure 4.

In general, three clusters were identified: TCR clonotypes from lung and secondary immune organ HG were separated from the ones from peripheral blood, PBMC samples from #2549, and PBMC samples from #2553.

Next, we examined whether the frequencies of TCRβ sequences were consistent over time with NDV infection by separately plotting samples from the CD4 and CD8 PBMC groups from different birds were separately plotted. In general, frequencies of TCRβ sequences across different time points in both CD4 and CD8 groups were highly correlated (r^2 from 0.37 to 0.88) except 2549CD4_14P versus 2549CD4_21P ($r^2 = 0.01$) (Figure 5A,B).

Joint Clonotypes

Next, we examined the number of shared and non-shared TCR clonotypes from PBMCs at different timepoints within each chicken for the CD4 and CD8 T groups, respectively. In general, the number of clonotypes that were overlapped within each bird over time (i.e., persistent clonotypes) were low, however, these shared clonotypes had greater abundances (Figure 5), such as #2549 CD4 7P 14P, #2549 CD8 7P, 14P, and 21P. These results suggest that their TCR repertoires were largely dominated by shared clonotypes across time points in these samples. Furthermore, the shared and non-shared TCR clonotypes between CD4 and CD8 samples from PBMCs were analyzed. Whether before or after NDV infection, there was very small numbers of TCR CDR3 clonotypes shared between chicken CD4 and CD8 T cells.

Track clonotypes

Finally, based on the results from Figure 5b, we tracked the clonotypes from #2549 CD8 PBMCs from pre-infection to 21 days post infection to identify any specific time-persistent clonotypes (clone expansion) and their amino acids. The tracking stackplot and heatmap of the top 100 clonotypes from different timepoints and the clonotype tracking summary heatmap are

presented in Figure 8. Many shared clonotypes were shared across different time points, for example CAKRGNRNERLIF, CASSSTGSGTPLNF, CAKQDNERLIF had the highest clonotype abundance at 7 dpi.

DISCUSSION

The first aim of our study was to improve the methodologies for using high-throughput sequencing to investigate the TCR CDR3 diversities in NDV infected chickens. We have successfully isolated total RNAs from low fluorescence-activated sorted mononuclear cells with very limited sample amounts, amplified CDR3 regions with our custom primers, and performed the analysis of TCR CDR3s repertoires using a custom chicken TCR database.

The next aim of our study was to investigate the temporal TCR clonotype dynamics overtime with NDV infection. The high frequency of some specific Vβ and Jβ segment usage indicates temporal persistence of TCRβ clonotypes usage in chickens, and these segments might be more commonly used in the V-D-J rearrangement process or were strongly favored by positive selection by multiple MHC alleles (Park et al. 2020). Moreover, certain clonotypes were highly abundant across multiple time points after NDV infection suggesting that these specific clonotypes had clonally expanded due to NDV infection. The persistent frequency of dominant TCR clonotypes was potentially due to specific responses to antigens that selectively skew the TCR repertoire (Attaf, Huseby, and Sewell 2015) or convergent recombination (Venturi et al. 2006; Madi et al. 2014). A human epitope-specific T-cell dataset, called Optimized Likelihood estimate of immunoglobulin Amino-acid sequences (OLGA) (Sethna et al. 2019) was used to calculate the probability of generating a given CDR3 amino acid sequence, in order to examine if the TCR clonotypes were identified as expected. If not, the data would support that this persistent expansion

was more likely a result of antigen selection. Our results suggest that the CD4 TCR diversity from 21 dpi peripheral blood samples was lower than the one in pre-infection peripheral blood samples in the same bird. This provides another partial evidence for a clonally expanded repertoire during infection with NDV. During a successful immune response, T cells with antigen-specific clonotypes would be activated and begin expanding whcih results in a decreasing overall diversity of TCR repertoires (Galson et al. 2014). In previous experiments in humans, primates and mouse, the expansion of TCR clonotypes with specificity for antigens after vaccination or infection have also been observed (Strauli and Hernandez 2016; Miles, Douek, and Price 2011). In human autoimmune diseases, compared to healthy controls, lower clonotype diversities of TCR repertoires have also been observed from people with the disease (Thapa et al. 2015; Chang et al. 2019; Cui et al. 2018).

Finally, CD4 and CD8 TCR clonotypes during infection with NDV were compared. CD4 and CD8 have some common V segment usages, such as TRBV 1.11, 1.4, and 1.7, but TRBV 1.3 and 1.8 were frequent only in CD8 samples. For J segment usage, most of the CD4 samples used TRBJ 3. In contrast, CD8 samples not only frequently used TRBJ3, but also used TRBJ 2, which was not shown in the CD4 group. For TCR clonotype diversity, the diversities of PBMC CD4 repertoires from different timepoints were higher than their counterparts in CD8 groups, a finding also shown in human studies (Robins et al. 2009; Qi et al. 2014; Lindau et al. 2019). When comparing pairwise distances between CD4 and CD8 samples, the repertoires from different T cell subsets became separated from each other after NDV infection. Also, the overlap of TCRbeta sequences between CD4 and CD8 T cells are very low, this was not a result of the different lengths of CDR3s, since both displayed a similar range (Table 1) and similar distribution (not shown here). This was not related to biased V and J gene usage since most V and J segment usages were similar.

This illuminates that most of CD4 and CD8 T cells have distinct TCR β s and there is very small number of TCR β s may be capable of reaching with both MHC I and MCH II peptide ligands. A similar phenomenon was also found previously when TCR β s paired with a suitable TCR α chain (Yin et al. 2011).

This study has provided some interesting observations on the dynamic TCR repertoires changes during NDV infection in chickens and further study on the potential functional role of antigen specific time persistent TCRβ clonotypes to NDV identified in this study is warranted. As a pilot experiment, our study only included a limited number of birds. More in-depth work including more birds will be needed in the future studies.

FUTURE DIRECTION

Future work should further examine the functional role of T cells repertoires during NDV infection in different chicken lines. Fayoumi and Leghorn are two highly inbred chicken lines, and Fayoumis have been shown to be more resistant to NDV while the Leghorn line was more susceptible to NDV (Saelao, Wang, Gallardo, et al. 2018; Y. Wang et al. 2014). In preliminary experiments, blood, lung, and HG samples from infected and healthy Fayoumi and Leghorn birds have been collected. These samples were processed into single cell suspension and stored in liquid nitrogen. However, when the frozen samples were thawed to count live cells, the cell viability from some samples was very low. Some possible would be to improve thawing methods, such as using warm staining media to dilute cells. In addition, if live cell numbers are still low, when staining cells for flow-cytometry, the Fluorescence Minus One Controls (FMOs) could be discarded to reduce cell waste. Alternatively, rather than freezing cells at all, collected blood samples could be sorted directly on the same day.

In addition, different antibodies, such as CD25, CD28, CD44 and also CD45 could be used to compare TCR repertoires from naïve versus activated or memory T cells. Such an analysis could generate a deeper understanding of immune cell specific TCR repertoires in chickens (Naghizadeh et al. 2021).

For data analysis, alternative methods could be tried. With higher bird numbers, clusters of similar TCRβ sequences with shared CDR3 sequence or shared motifs could be identified. These clusters would potentially recognize the same epitope and might represent NDV-specific TCR clonotypes. In addition, antigen-specific TCR clonotypes will expand and reach the peak of expansion, then the responding TCR clonotypes will significantly contract afterwards, although naïve or non-specific T cells will not show this phenomenon (Robinson et al. 2016). Our future work can also focus on the contraction process of NDV-specific TCR clonotypes and identify key clonotypes responsible for the expansion and contraction in responding to NDV infection in chickens.

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TABLE AND FIGURES

Table 1 Basic statistics of TCR repertoire sequencing.

Sample_ID	Count	Diversity	Mean_Frequency	Geomean_Frequency	Mean_Cdr3nt_Length	Convergence
2549CD4_0P	114796	4175	2.40E-04	1.94E-05	43.83	1.34
2549CD4_7P	202584	7758	1.29E-04	1.11E-05	43.91	1.37
2549CD4_14P	51951	3415	2.93E-04	3.87E-05	43.89	1.28
2549CD4_21P	780074	3856	2.59E-04	5.32E-06	46.85	1.54
2549CD4_HG	20011	784	1.28E-03	1.80E-04	44.49	1.23
2549CD4_Lung	9199	477	2.10E-03	3.84E-04	42.75	1.16
2549CD8_0P	18343	1148	8.71E-04	1.23E-04	44.04	1.25
2549CD8_7P	20698	981	1.02E-03	9.52E-05	42.66	1.32
2549CD8_14P	29283	1872	5.34E-04	6.76E-05	43.07	1.29
2549CD8_21P	19402	1404	7.12E-04	9.84E-05	43.23	1.27
2549CD8_Lung	76932	2720	3.68E-04	3.89E-05	43.71	1.29
2553CD4_0P	950626	10840	9.23E-05	4.64E-06	42.88	1.42
2553CD4_7P	384084	12012	8.33E-05	7.13E-06	43.91	1.33
2553CD4_14P	512333	4009	2.49E-04	9.58E-06	48.08	1.41
2553CD4_21P	117125	2966	3.37E-04	2.56E-05	45.50	1.30
2553CD4_HG	523674	4856	2.06E-04	9.44E-06	48.37	1.50
2553CD4_Lung	1801999	19235	5.20E-05	2.79E-06	43.42	1.44
2553CD8_0P	839997	6218	1.61E-04	5.57E-06	42.36	1.46
2553CD8_7P	925008	8119	1.23E-04	5.11E-06	45.25	1.44
2553CD8_14P	936543	5689	1.76E-04	5.25E-06	44.05	1.57
2553CD8_21P	1219804	7168	1.40E-04	3.87E-06	41.31	1.52
2553CD8_Lung	596568	3977	2.51E-04	8.58E-06	38.59	1.67
2553CD8_HG	660861	4182	2.39E-04	7.69E-06	41.87	1.47

Figure 1. TCR Vβ segment usage. (A) Heatmap of Vβ segment usage for CD4 samples. (B) Heatmap of Vβ segment usage for CD8 samples. "V" means gene TRBV. Hierarchical clustering with Euclidian distance is shown for samples above and for segments to the left of the plots. (C) Heatmap of Jensen-Shannon divergence of Vβ gene usage between different samples. Lower divergence indicates similar Vβ/Jβ gene usage.

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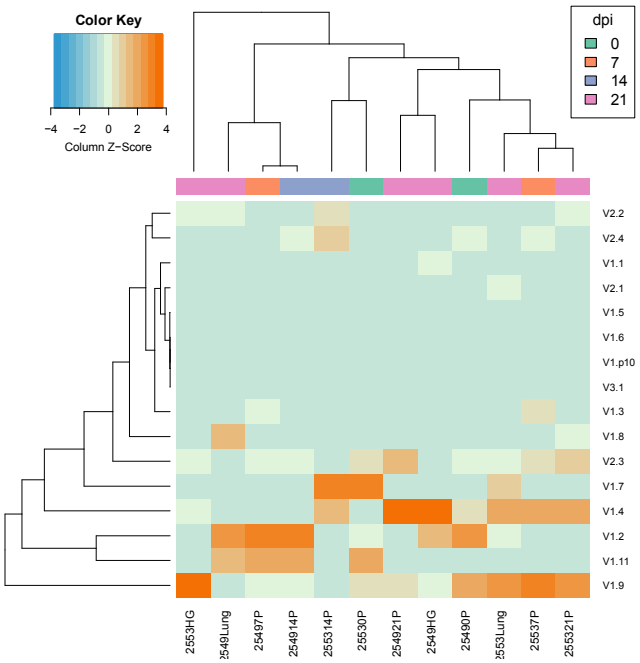

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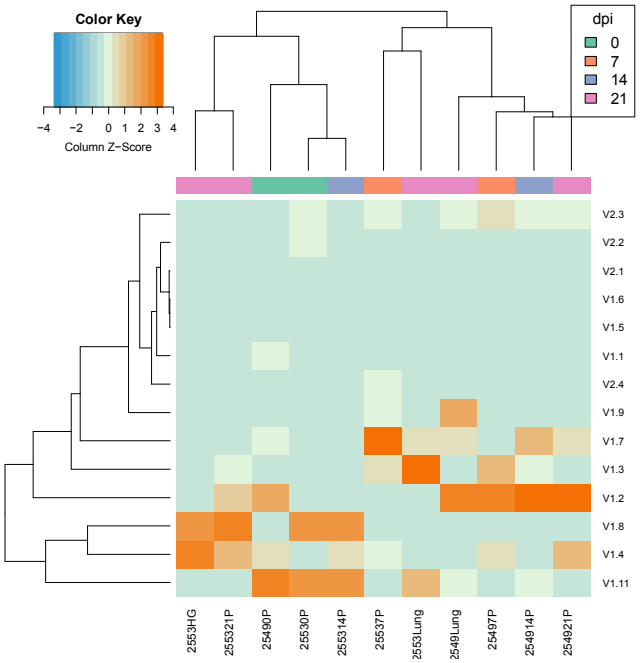

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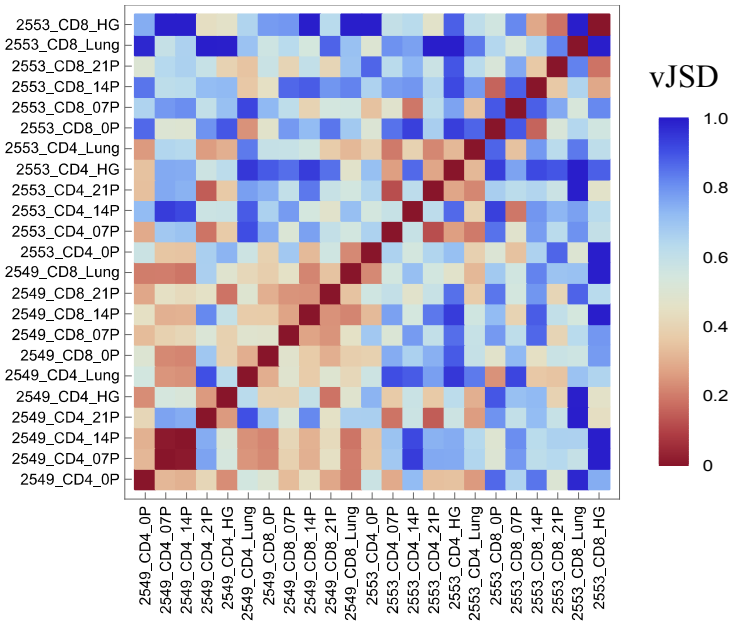
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2553_CD8_21P
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2553_CD8_07P
2553_CD8_0P
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2553_CD4_HG
2553_CD4_21P
2553_CD4_14P
2553_CD4_07P
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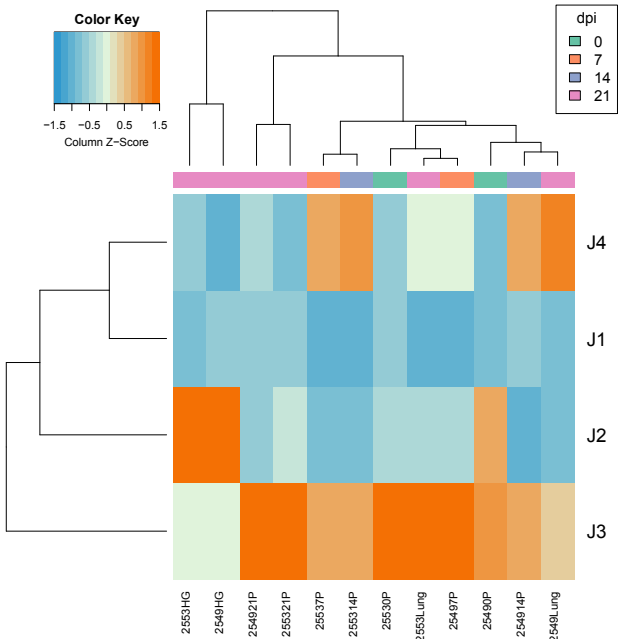
2553_CD4_Lung
2553_CD8_0P
2553_CD8_07P
2553_CD8_14P
2553_CD8_21P
2553_CD8_Lung
2553_CD8_HG
vJSD
1.0
0.8
0.6
0.4
0.2
0

Figure 2. TCR Jβ segment usage. (A) Jβ segment usage for CD4 samples. (B) Jβ segment usage for CD8 samples. "J" means gene TRBJ. Hierarchical clustering with Euclidian distance is shown for samples above and for segments to the left of the plots.

(A)



(B)

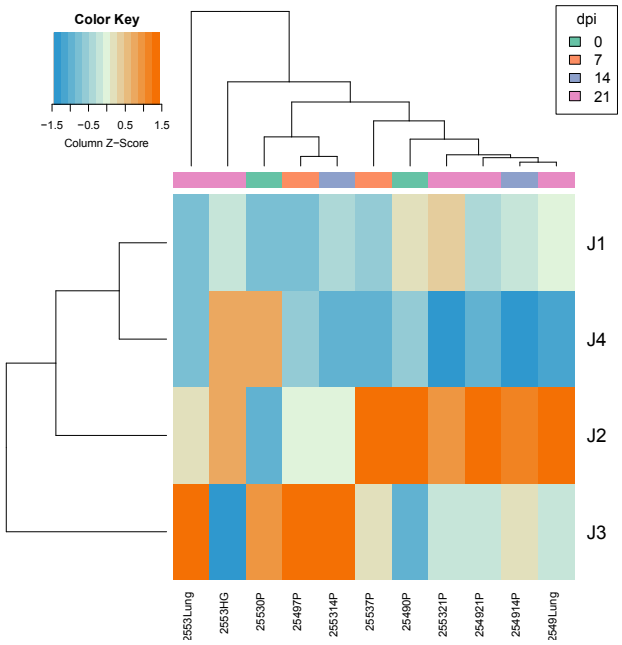
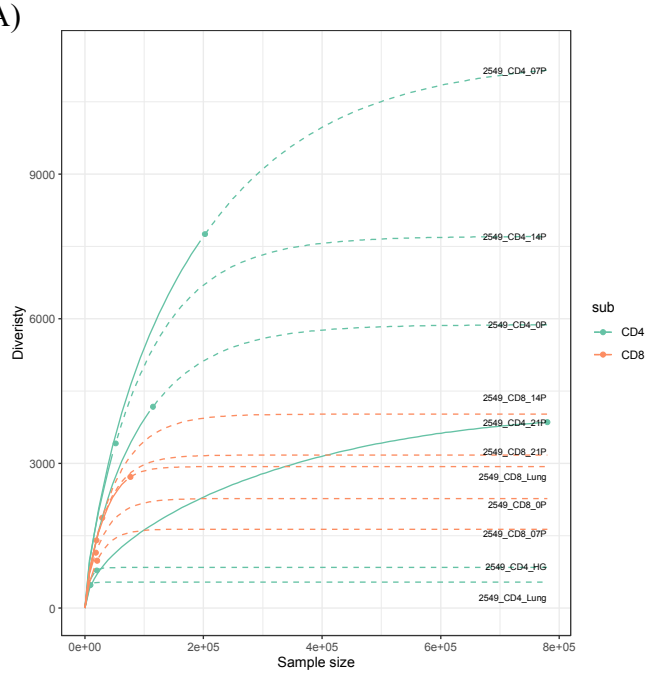


Figure 3. Rarefaction plots for bird #2549 samples (A) and #2553 samples (B). Sample size on the X axis means CDR3 read count, and diversity on the Y axis means the number of different clonotypes in each sample. The point on each curve indicates the observed sample size and diversity. The solid lines indicate interpolated values, and the dotted lines indicate extrapolated values. Grey shaded areas indicate 95% confidence intervals.

(A)



(B)

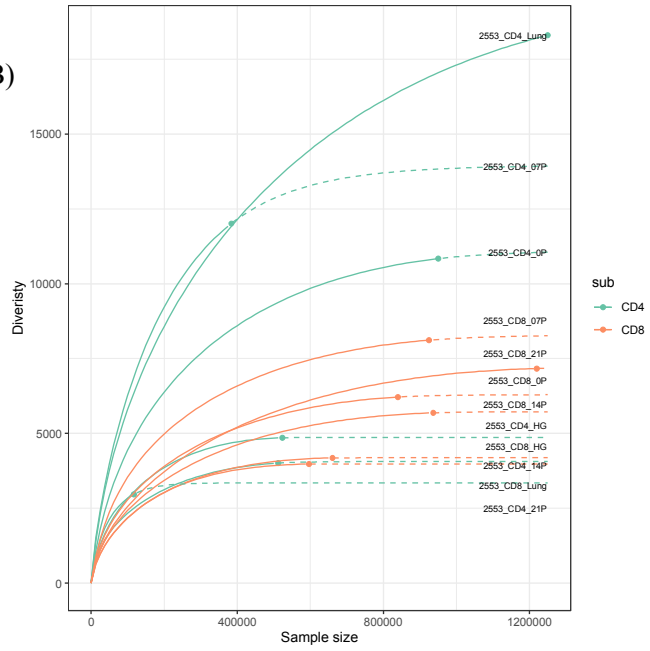


Figure 4. Multi-dimensional scaling (MDS) plots for all samples. Euclidean distance between points reflects the distance between repertoires. Points are colored by days post infection (dpi).

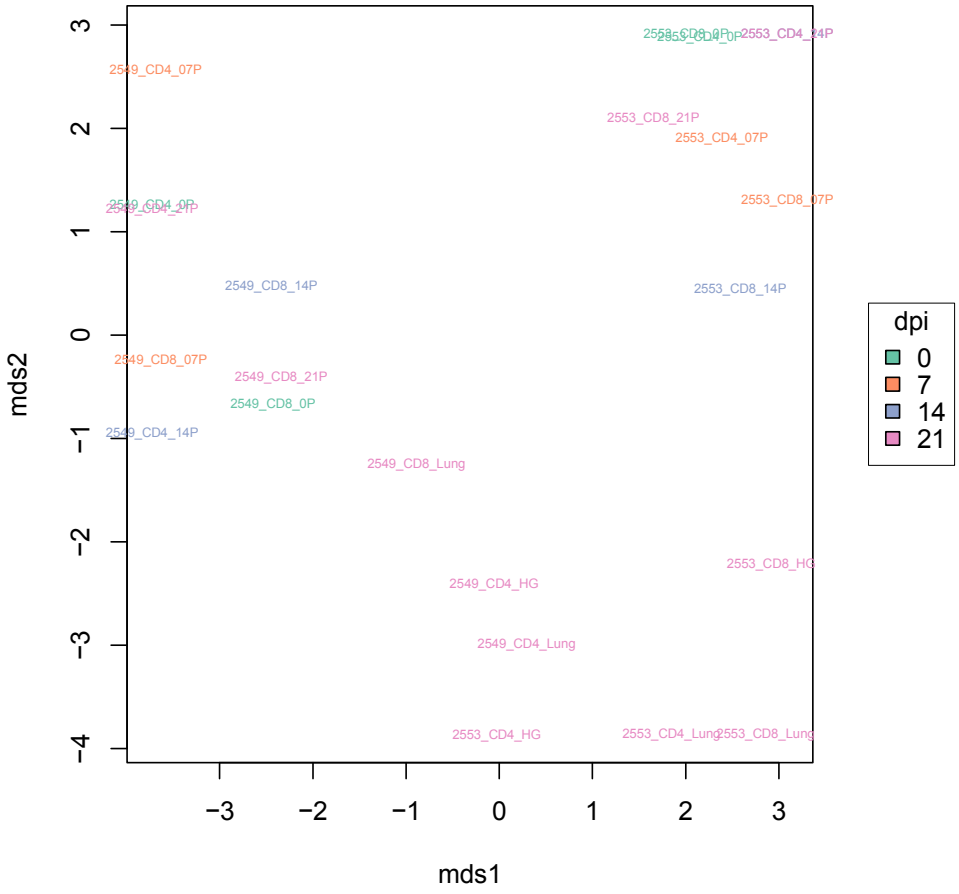
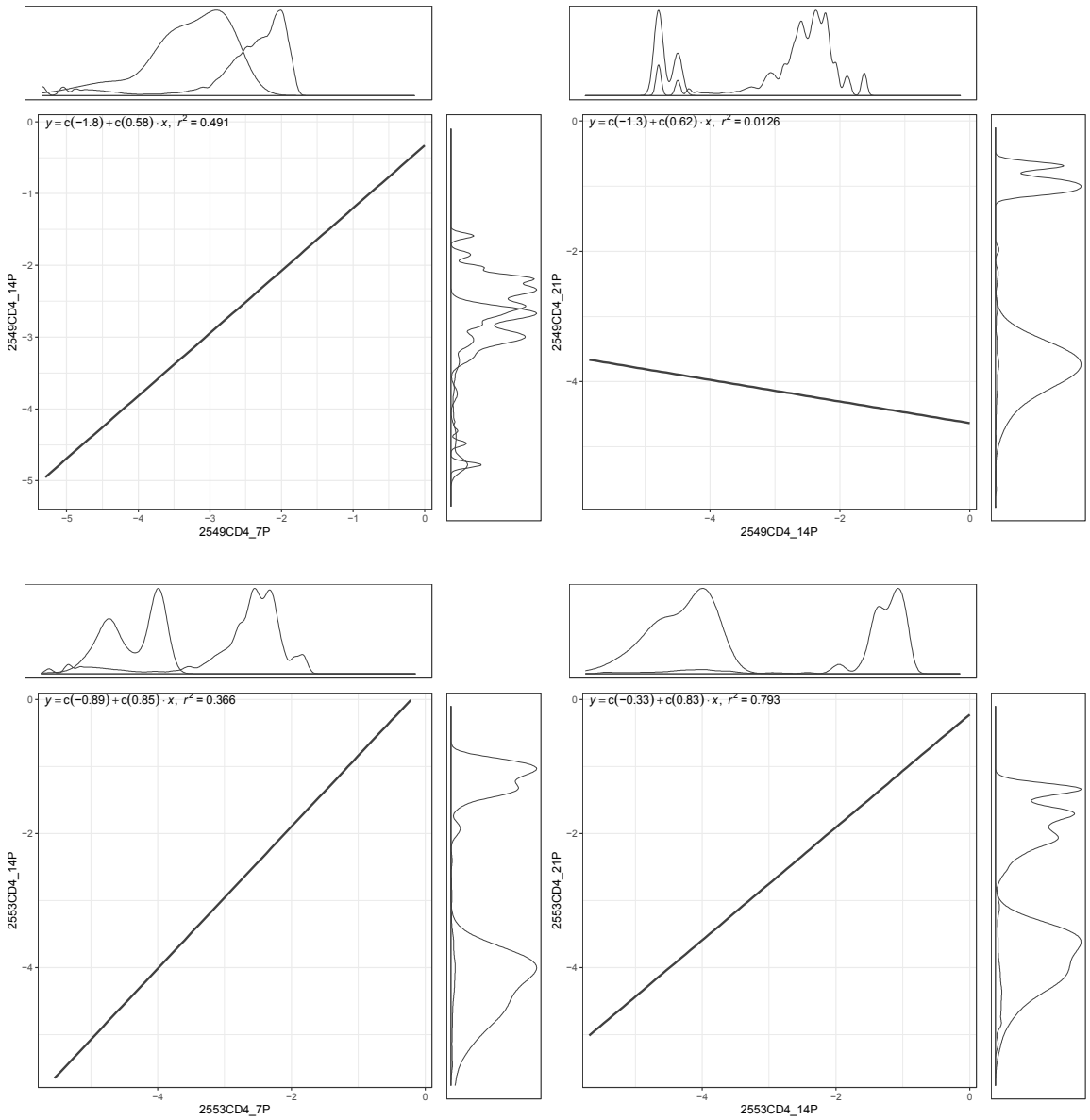


Figure 5. Overlaps between TCR clonotypes over time for CD4 (A) and CD8 (B) groups. Each dot on the scatterplots indicates a single TCR clonotype, and point size is scaled to the geometric mean of this clonotype. X and Y axes denote log10 clonotype frequencies in each sample. Two marginal histograms show the overlapping (red) and total clonotype (grey) abundance distributions in corresponding sample. Histograms are weighted by clonotype abundance, in another word, they display read distribution by clonotype size.

(A)



(B)

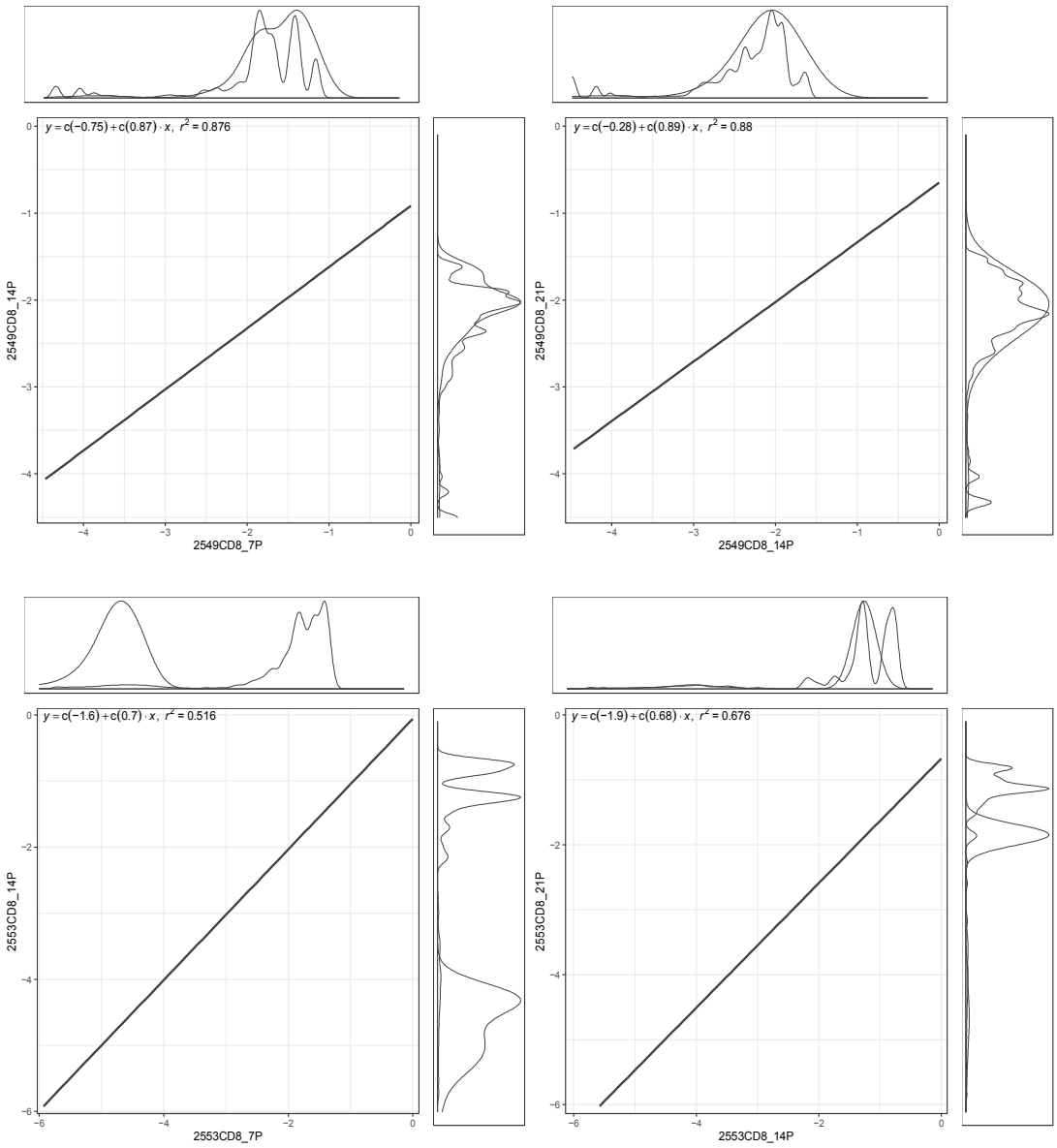
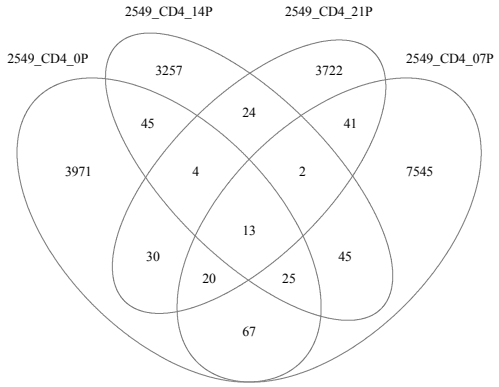
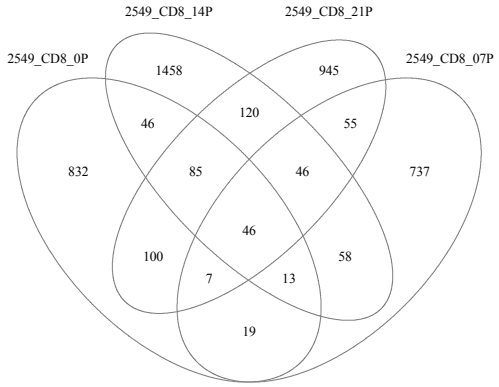

y = c(−0.75) + c(0.87) · x, r² = 0.876
2549CD8_14P
2549CD8_7P
y = c(−0.28) + c(0.89) · x, r² = 0.88
2549CD8_21P
2549CD8_14P
y = c(−1.6) + c(0.7) · x, r² = 0.516
2553CD8_14P
2553CD8_7P
y = c(−1.9) + c(0.68) · x, r² = 0.676
2553CD8_21P
2553CD8_14P

Figure 6. Venn diagrams show the number of shared and non-shared TCR clonotypes from PBMCs at different timepoints within each bird for CD4 and CD8 T cells.

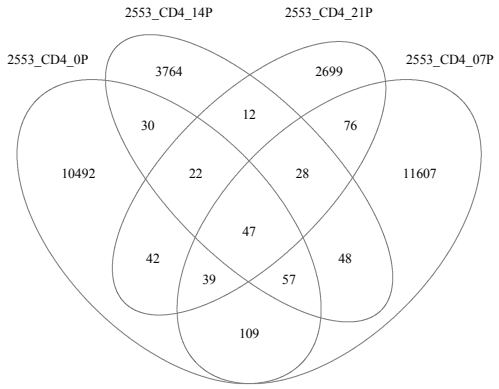
2549 CD4



2549 CD8



2553 CD4



2553 CD8

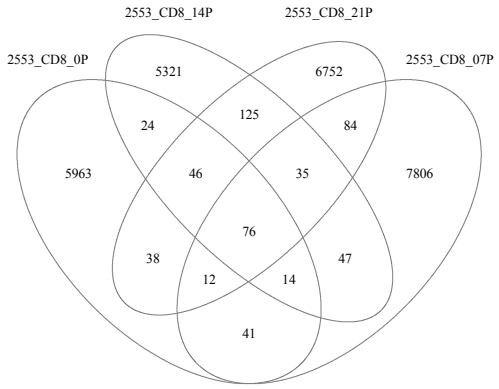


Figure 7. Venn diagrams of the number of shared and non-shared TCR clonotypes between the CD4 and CD8 PBMC samples.

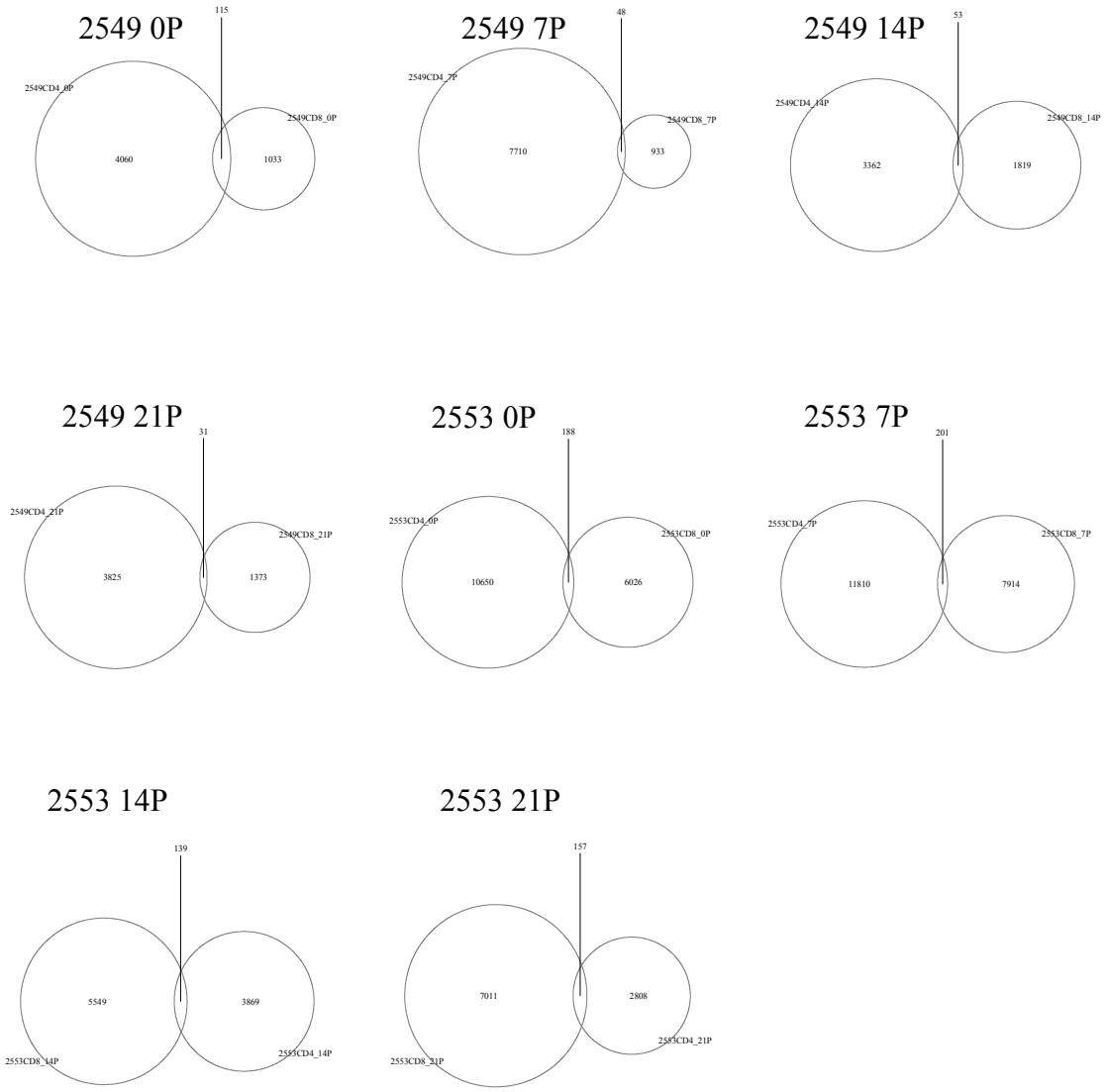
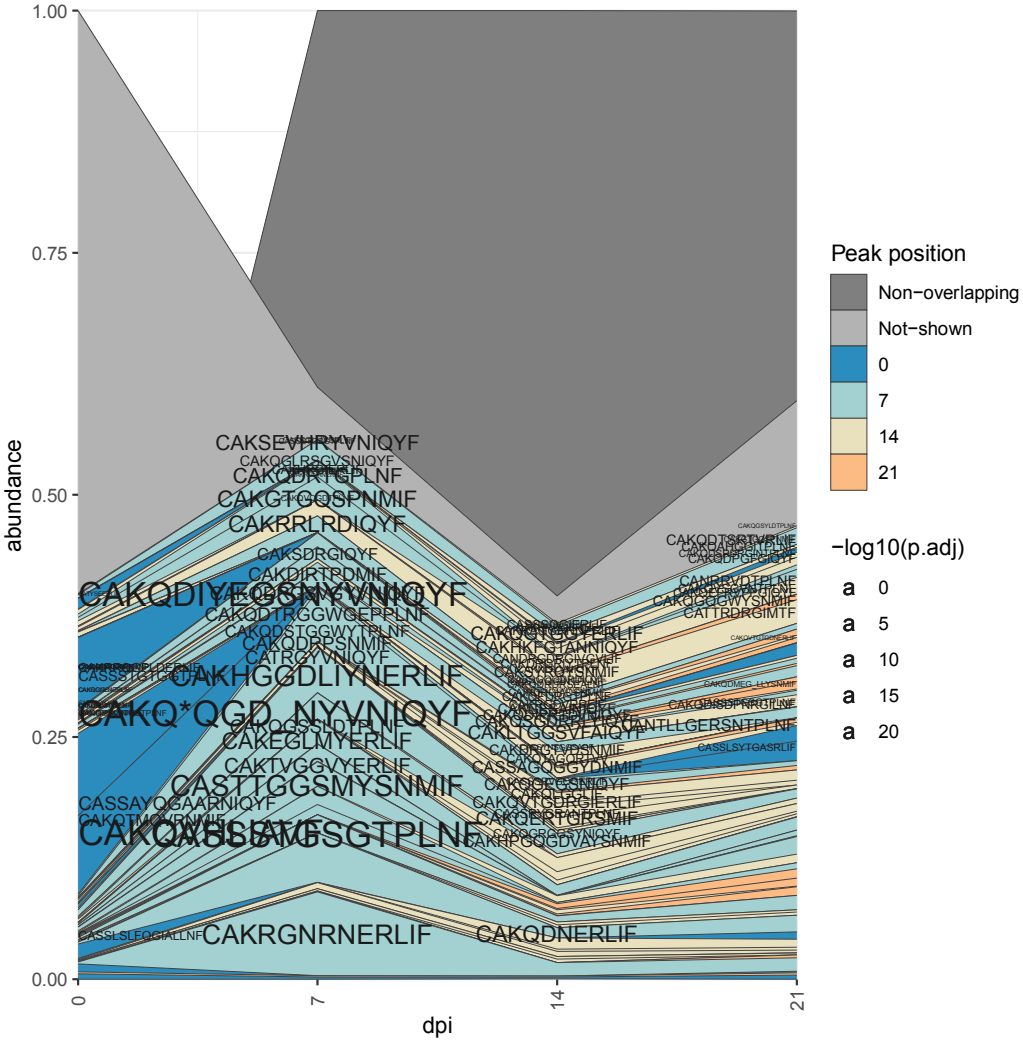
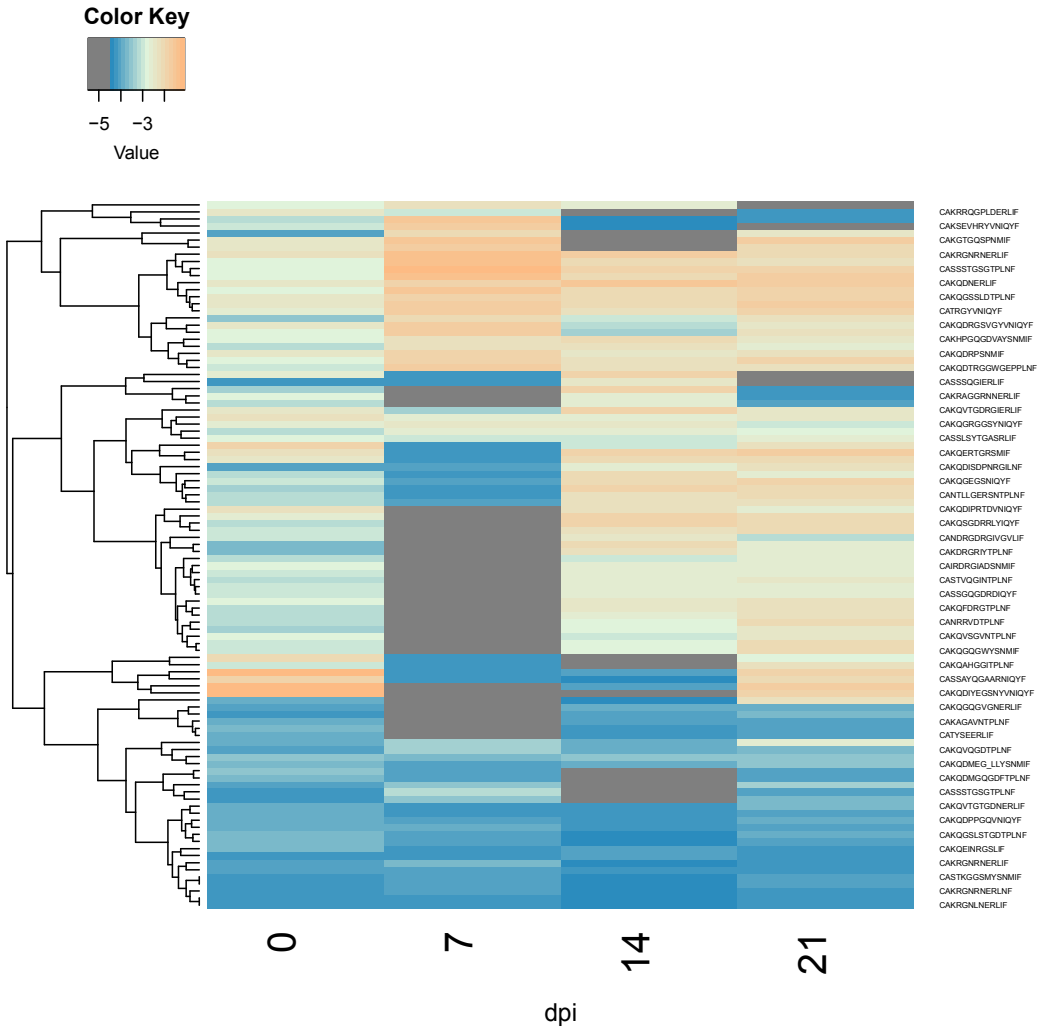


Figure 8. Tracked clonotypes for #2549 CD8 PBMC samples over time. (A) the clonotype tracking stackplot for the top 100 clonotypes, as well as collapsed (Not-shown) and non-overlapping (Non-overlapping) clonotypes. Clonotype CDR3 amino acid sequence was plotted against the sample where the clonotype reaches maximum abundance. Clonotypes were colored by the peak position of their abundance profile. (B) Clonotype tracking heatmap for the top 100 joint clonotype abundances. (C) The clonotype tracking summary. Count, diversity, and frequency panels correspond to the read count, the total number, and the frequency of clonotypes that were shared between samples.

(A)



(B)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−3
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CAKQDNERLIF
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CAKDRGRIYTPLNF
CAIRDRGIADSNMIF
CASTVQGINTPLNF
CASSGQGDRDIQYF
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CANRRVDTPLNF
CAKQVSGVNTPLNF
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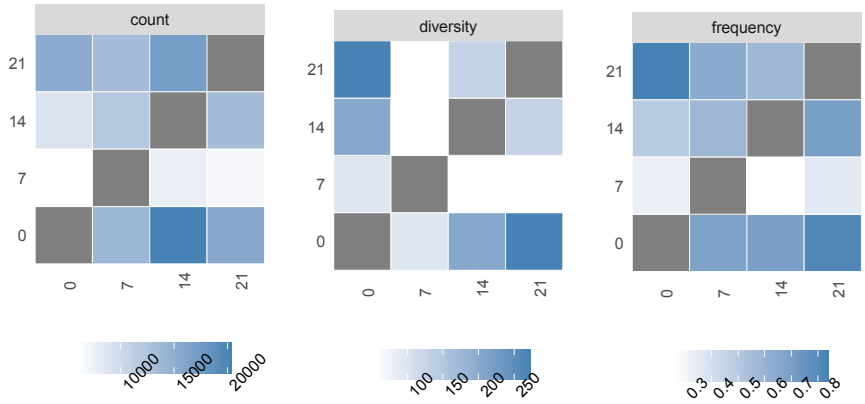
7
14
21
dpi

(C)


count
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frequency
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14
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15000
20000
100
150
200
250
0.3
0.4
0.5
0.6
0.7
0.8