ASSESSING T-CELL CROSS-REACTIVITY TO SARS-CoV-2 VARIANTS

by

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The coronavirus pandemic is a major, ongoing public health crisis. COVID-19 has resulted in over 6 million deaths as of April 2023 according to WHO. The COVID-19 mRNA vaccine widely administered in the United States was based on the spike protein of the Washington strain of SARS-CoV-2. Several new studies have indicated that a phenomenon like original antigenic sin (where prior exposure to an antigen leads to an ineffective immune response against related antigens) might be occurring in COVID. In this study, we wanted to determine if a COVID-19 mRNA vaccine-induced memory CTLs cross-react with Delta/Omicron variant epitopes without target cell killing. To test this, we identified HLA-A2 restricted, CD8+ T-cell epitopes in the spike protein that differ between the Washington strain Vs the Delta and/or Omicron variants. We collected T-cells from HLA-A2 positive, MWCCS donors who were vaccinated but not naturally exposed to any variant of SARS-CoV-2 and expanded the antigen-specific T-cells *ex-vivo.* We then assessed the cross-reactive potential of these Washington variant spike-specific CD8+ T-cells (readout was optimized to detect CD8+ T-cells) against the variant peptides generated using IFN-gamma ELISpot assay. We tested 14 peptide pairs and found that vaccine-induced CD8+ T-cells can cross-react with epitope variants. We also observed a decreased T-cell reactivity to the variant epitopes compared to the Washington epitopes in some cases and an increased T-cell reactivity to the variant epitopes compared to the Washington epitopes in some other cases. Another interesting observation was that MHC binding affinity and T-cell binding affinity did not always correlate with the T-cell responses (IFN-g production) observed. Our future efforts will be aimed at testing

the killing capacity of the CTLs when exposed to variant-antigen expressing target cells. The findings from this study has laid a groundwork in determining the efficacy of the COVID vaccine and to determine if there is need for the development of a more efficacious COVID vaccine.

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1.0 Introduction

1.1 COVID-19 Pandemic

The COVID-19 pandemic is a major, ongoing pandemic caused by SARS-CoV-2 virus (severe acute respiratory syndrome coronavirus 2). The coronavirus outbreak started in early December 2019 in the capital city of Wuhan, Hubei province, People's Republic of China, and caused a global pandemic (1). As of March 2023, there has been 761,071,826 confirmed cases of COVID-19 including 6,879,677 deaths reported to WHO. The symptomology of COVID-19 includes fever, malaise, dry cough, fatigue, shortness of breath or difficulty breathing, muscle or body ache, nausea or vomiting, diarrhea (1). Clinical symptoms of COVID-19 include both long-term and short-term respiratory and cardiovascular complications (2). In COVID-19, the disease severity seems to be strongly associated with underlying host conditions including age, sex and overall health. The latter seems to play a critical role in susceptibility and contribute to the risk of infection (3). Conditions such as hypertension, diabetes, cardiovascular and kidney diseases increases the risk of infection two to three-fold (3). In people with co-morbidities, COVID-19 can also lead to life-threatening complications such as acute respiratory distress syndrome (ARDS) which may trigger multi-organ collapse (4). These people are also at a higher risk of developing severe pneumonia with an advanced proinflammatory and prothrombic state (5).

1.2 COVID-19 Vaccine

Until today, no specific drug has been identified for COVID-19 prevention or treatment, and vaccination remains the most economical and effective intervention to limit the spread of SARS-CoV-2. The COVID-19 mRNA vaccine emerged as a revolutionary innovation that played a unique role in controlling the pandemic (6). SARS-CoV-2 is an enveloped positive-sense single-stranded RNA (ssRNA) virus of the Betacoronavirus genus included in the *Coronaviridae* family with four structural proteins namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The mRNA vaccine is comprised of nucleoside-modified mRNA formulated in lipid nanoparticles and encodes the membrane-anchored, full-length SARS-CoV-2 spike protein of the Washington (or ancestral strain). Once inside the host, the mRNA is translated into the SARS-CoV-2 spike protein, which is expressed on the surface of the host cells. The transient expression of this spike antigen induces neutralizing antibody and cellular immune response against it, which confers protection against COVID-19 (7). Individuals who have been vaccinated possess antibodies against the SARS-CoV-2 spike protein. Likewise, individuals who have been naturally exposed to any of the SARS-CoV-2 variants have antibodies against the nucleoprotein of SARS-CoV-2. This is used as an important factor to distinguish donors who have not been naturally exposed to any variant of SARS-CoV-2 (and have only received the vaccine) from those that have been naturally exposed, in this thesis.

1.3 Antibody Escape in COVID-19

The mRNA vaccine codes for the spike protein of the Washington strain of SARS-CoV-2 as previously mentioned. The emergence of these new variants of concern (VOCs) such as Delta and Omicron have raised concerns regarding the effectiveness of the vaccine in offering protection against these variants. Several reports have suggested that frequent mutations in these variants have increasingly challenged effectiveness of the vaccine.

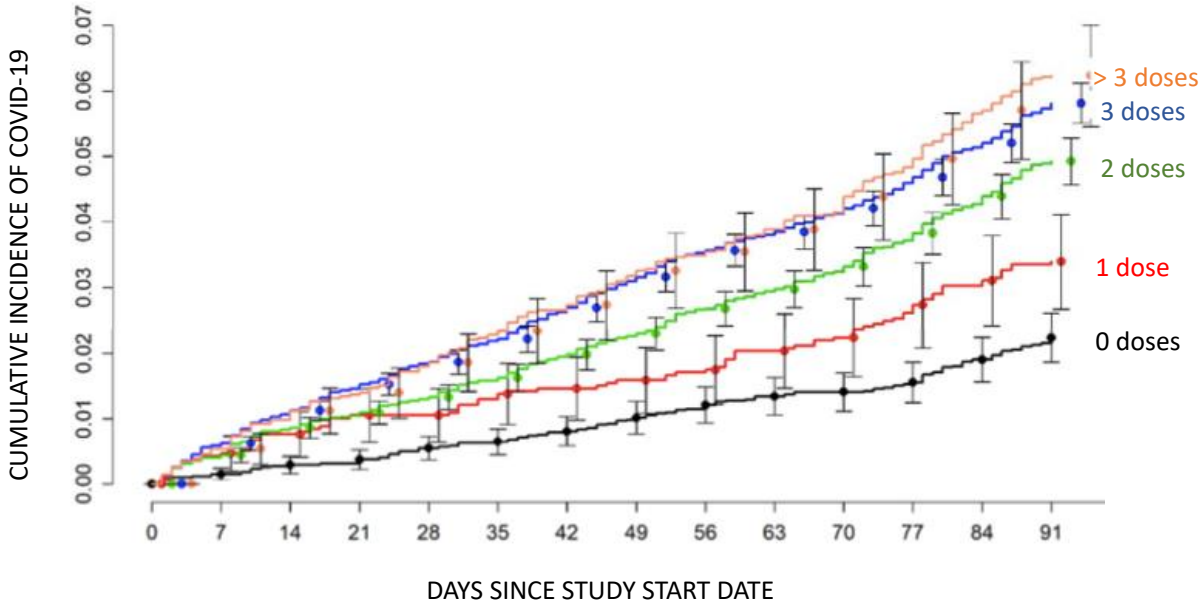
There is plenty of evidence of antibody escape by SARS-CoV-2 variants. The mutations of greatest concern are present in the viral spike (S) protein and include notable mutations in the receptor-binding domain (RBD), the N-terminal domain (NTD), and the furin cleavage site region. Several of these mutations have been known to contribute to immune escape (8). Numerous reports address the effect of variant spike (S) mutations on antibody neutralization capacity. It was found that recurrent substitutions at 452 and 490 such as L452R (present in the Delta variant) impair antibody-antigen association (9). The Delta variant was found to be resistant to neutralization by anti-NTD and anti-RBD monoclonal antibodies, including bamlanivimab, and these antibodies showed impaired binding to the spike protein (10). Omicron subvariants that harbor L452R mutation have been identified (9). Another study found that omicron escapes neutralization from antisera of vaccinated or convalescent individuals by ∼15-fold (11).

1.4 Evidence of Original Antigen Sin In COVID-19

Recent evidence suggests that people receiving more number of doses of vaccine (coding for the Washington strain's spike protein), had a higher risk of contracted SARS-CoV-2 infection

(12) as shown in the graph below (**Figure 1**). These results seemed to suggest that a phenomenon like original antigenic sin might be occurring in COVID-19.

Figure 1. Data from a 2022 preprint (12) showing that people receiving more number of doses of the Washington strain COVID-19 vaccine had a higher risk of getting infected with SARS-CoV-2.



A retrospective cohort study was performed on employees of the Cleveland Clinic to test the effectiveness of the bivalent COVID-19 vaccine. The study subjects were grouped into cohorts based on the number of vaccine doses previously received.

T-cell responses (CD4+ and CD8+ T-cells) have been found to play very important roles in the resolution of SARS-CoV-2 infection and COVID-19 (13). This includes modulating disease severity in humans and reducing viral loads in non-human primates (14). Individuals with agammaglobulinemia or pharmaceutical depletion of B cells generally experience an uncomplicated COVID-19 disease course highlighting the importance of T-cell responses in SARS-CoV-2 infection (15). A 2021 study showed that T-cell responses generated upon

vaccination provide protection in patients with hematologic cancer even in the setting of limited humoral responses (16). Robust T-cell memory is induced after vaccination or following natural exposure to SARS-CoV-2 (8).

Since antibody escape is observed by the SARS-CoV-2 variants, the question of how the SARS-CoV-2 variants-associated mutations affect T-cell responses arose. As a result, several research groups have assessed the vaccine-induced T-cell responses to COVID-19 variants (8), (17),(18).

Furthermore, previous research in our lab on HIV has shown that it is possible for cytotoxic T-cells (CTLs) to cross-react with variant epitopes without target cell killing. In HIV, founder epitopes arising at earlier time points of HIV infection can cross-react to epitope variants arising at later time points of infection. The responses to the variant epitopes were comparable to the founder epitope (19). When these founder epitope specific CTLs (cytotoxic T-cells) were stimulated with the priming peptide, they underwent CD8 downregulation and produced pro-inflammatory cytokines such as IFN-g, TNF-a, and IL-2. However, when these CTLs were stimulated with the variant peptide, pro-inflammatory cytokines were produced but no CD8 downregulation was observed (19). Furthermore, when these CTLs specific to the priming peptide were exposed to target cells expressing the variant peptide, no target cell killing by these CTLs was observed (19). This phenomenon was termed as ‘ALL BARK AND NO BITE’.

No other study to date has looked at whether a phenomenon similar to the one described above (where CTLs cross-react without target cell killing) occurs in COVID-19. With building evidence of immune escape by the SARS-CoV-2 variants, we wanted to address if mutations associated with SARS-CoV-2 variants causes T-cell escape and if T-cells kill target cells infected with variant peptides (or if they lead to a phenomenon like ‘ALL BARK AND NO BITE’)

Furthermore, most studies, such as the study by GeurtsvanKessel et al. (18) have described that CD4+ T-cell responses are more predominant than CD8+ T-cell responses in SARS-CoV-2 infection. Here, we wanted to optimize our readout assay to accurately the capture the role played by CD8+ T-cells in SARS-CoV-2 infection.

2.0 Statement of the Project and Specific Aims

The emergence of new variants of concern such as Delta and Omicron have raised concerns regarding the efficacy of the COVID-19 mRNA vaccine (20), (21). There is plenty of evidence suggesting immune escape by these variants in vaccinated individuals (9), (10). There is also evidence suggesting that a phenomenon like 'original antigenic sin' might be occurring in COVID-19 where individuals who are vaccinated and have immunological memory against the Washington strain of SARS-CoV-2 have an increased susceptibility to getting infected with newer variants (12). Most studies to date that have focused on T-cell responses to SARS-CoV-2 variants have not looked at whether a phenomenon like 'ALL BARK AND NO BITE' (CTL cross-reactivity without target cell killing) occurs in COVID-19 infection. Here, we wanted to address the potential impact of mutations associated with SARS-CoV-2 variants on T-cell cross-reactivity. We first wanted to determine if there is T-cell cross-reactivity to the SARS-CoV-2 (Delta and Omicron) variant epitopes or if there is complete CTL escape. Our future directions involve assessing target cell killing by these CTLs when exposed to variant epitopes. *In this thesis, we wanted to evaluate 1) whether the SARS-CoV-2 variants can cause T-cell immune escape in vaccinated individuals 2) or alternatively, if the COVID-19 mRNA vaccine-induced memory CD8+ T-cells can cross-react with SARS-CoV-2 variant-specific T-cell epitopes.* Therefore, our overarching goal was to characterize the cross-reactive potential of COVID-19 mRNA vaccine-induced memory T-cell responses to SARS-CoV-2 variant epitopes using an *ex-vivo* approach.

To do this, we proposed two specific aims:

1. **To identify HLA-A2 restricted CD8+ T-cell epitopes in the spike protein that differ between the Washington strain and the Delta and Omicron.** In order to identify our

CTL epitope candidates, we pulled the spike protein amino acid sequences of the Washington, Delta and Omicron variants from GENBANK. The sequences were run through NetMHCpan 4.1 server to identify HLA-A2 restricted, 9-mer peptides belonging to the spike protein of Washington, Delta and Omicron variants and 9-mers that differed between the variants were identified. Then, the sequences were run through NetTEpi server to identify 9-mers with the highest predicted increase/decrease in T-cell binding affinity. The final 9-mer pairs were HLA-A2 restricted, had a predicted decrease/increase in T-cell binding affinity and had an amino acid change in Delta/Omicron epitopes compared to Washington epitope.

2. **To assess the vaccine-induced CTL responses to the Washington variant T-cell epitopes and test the nature of their reactivity to the epitope variants (do they cross-react or escape?).** To evaluate this aim we first selected an HLA-A2 positive, vaccinated HIV negative MWCCS study participant with good CTL responses to the Washington spike. Then, we optimized the method used to expand CTLs to achieve adequate numbers for performing an ELISpot assay using dendritic cells and CD40L. Then, the readout was optimized using 9-mer epitopes to measure vaccine-induced CD8+ T-cell responses. Finally, the CTL reactivity to the epitope variants measured (whether the CTLs cross-react or escape) were measured using an overnight IFN-g ELISpot assay using the WA-D/O (Washington- Delta/Omicron) peptide pairs generated.

3.0 Methods

3.1 Donor Identification

MWCCS (MACS/WIHMS Combined Cohort Study) donors who had visits pre- and post-release of the first COVID-19 vaccine were selected. The following criteria were considered -

1. Vaccinated individuals who were not naturally exposed to any SARS-CoV-2 variants (Washington, Delta and Omicron variants) were selected. This was ensured by selecting donors who did not have antibodies against the nucleoprotein of SARS-CoV-2 (presence of antibodies against nucleoprotein in the blood in ELISA assays indicates natural exposure to SARS-CoV-2). This was done as a part of the COVID study.
2. HLA typing was done on these donors to identify HLA-A2 positive donors.
3. Only HIV negative, healthy donors were selected
4. Only donors that had good T-cell responses to the Washington spike epitopes were selected.

3.2 Purification of Monocytes from Blood

PBMCs were isolated following buffy coat processing. PBMCS were separated from the other components of the blood such as blood plasma, erythrocytes, and granulocytes via density gradient centrifugation using Ficoll-Paque or optional Hypaque-Ficoll for further separation of mononuclear cells. Fresh blood diluted with PBS was layered on top of Ficoll. Then, the mixture

was centrifuged for 10 minutes. The sample was removed from the centrifuge carefully so as to not disturb the distinct blood fractions. Then, the PBMCs (buff-colored layer) were pipetted into another centrifuge tube. Then, to isolate monocytes from lymphocytes, anti-human CD14 antibodies were used to isolate monocytes from Peripheral Blood Lymphocytes (PBLs). Anti-CD14-coated microbeads were added to the PBMCs to positively select for the monocytes.

3.3 Generation of Monocyte Derived Dendritic Cells

Human monocytes isolated from the donors were cultured in the presence of rhGM-CSF and rhIL-4 for 5 days to make immature dendritic cells. These immature dendritic cells were subsequently exposed to maturation factors for 48 hours to yield mature aDC1 and DC2. Poly:I:C, IL-1B, TNF-alpha, IFN-gamma, IFN-alpha cytokine cocktail were added to the immature dendritic cells to make aDC1. IL-6, PGE2, TNF-alpha, IL-1B cytokine cocktail were added to immature dendritic cells to make DC2.

3.4 T-Cell Isolation

EasySep Human T-cell Enrichment Kit (STEMCELL Technologies, Cambridge, MA) was used to isolate CD3+ T-cells from frozen PBLs or PBMCs by negative selection. The Enrichment cocktail were added to the cells and incubated at room temperature for 5 minutes. This was followed by the addition of magnetic particles. Unwanted cells were targeted for removal with Tetrameric Antibody Complexes recognizing non-T cells and dextran-coated magnetic particles.

Then the tube was placed in the EasySep magnet and the labelled cells are separated using an EasySep magnet. After incubation at room temperature for 3 minutes, the CD3+ T-cells were poured off into a new tube.

3.5 Expansion of Antigen-Specific T-Cells

Dendritic cell: T-cell co-cultures were done in 48 well-plates. Over 75,000 mature dendritic cells were added to a 48 well plate and they were pulsed with the HLA-A2 restricted peptides of the spike protein of Washington variant of SARS-CoV-2 for an hour. Following the 1-hour incubation at 37C, over 500,000 CD3+ T-cells (DC to T-cell ratio = 1:7.5) were added to the wells containing the dendritic cells. After the addition of T-cells, J558/CD40L were added to some conditions. The Washington variant spike-specific T-cells were expanded under 4 conditions. aDC1s and DC2s were co-cultured with bulk T-cells in the absence and presence of J558/CD40L. The culture was then expanded for 10 days and cytokines IL-2 and IL-7 were added once every 3-4 days. An overnight IFN-g ELISpot assay was done on Day 10. The Washington 9-mers and the variant 9-mers pairs generated previously were used as efferent readout peptides.

3.6 ELISpot for Detecting IFN-g Secreting Cells

In vitro expanded T-cells were harvested, counted, and immediately tested for IFN-g secretion by ELISpot. The IFN-g ELISpot assay was performed following the Mabtech Human IFN-g ELISpot protocol (Mabtech, Cincinnati, OH) using anti-human IFN-g and biotin

monoclonal antibodies and 96-well PVDF ELISpot plates from Millipore. Briefly, 30,000 T-cells (100 uL) were transferred to anti-IFN-g antibody-coated 96-well ELISpot plates. Individual 9-mer dilutions were prepared at 2 ug/mL and added (100 uL) to T-cell containing wells to give a final peptide concentration of 1ug/mL. All ELISpot assays included negative-control wells with expanded T-cells without peptide stimulation (Media only). IFN-g responses to each peptide were tested in duplicate wells. The enumeration of spots was done using the Autoimmun Diagnostika GmbH (AID) ELISpot reader and counting software (AID, Strassberg, Germany). ELISpot data, calculated as the mean of spots in duplicate wells minus the mean and 2 standard deviations (SD) of the negative control values, were shown as IFN-g spot forming units (SFU)/10^6 cells.

4.0 Results

4.1 Aim 1: Identify HLA-A2 Restricted, CD8+ T-Cell Epitopes in the Spoke Protein that Differ Between the Washington and the Delta and Omicron Variants

To identify HLA-A2 restricted, CD8+ T-cell epitopes in the spike protein that differ between the Washington strain and the Delta and Omicron variants, CTL epitope candidates were identified. The spike protein amino acid sequences of WASHINGTON (wild type), DELTA and OMICRON variants were pulled from GENBANK. The sequences were run through NetMHCpan 4.1 server to identify HLA-A2 restricted 9-mer peptides belonging to the spike protein of Washington, Delta and Omicron variants. These 9-mers generated from the 3 sequences (WASHINGTON, DELTA and OMICRON) were compared to identify the ones that had a mutation in DELTA and/or OMICRON compared to the WASHINGTON strain. Then a set of 14 peptide pairs (WA - D/O) pairs that had a predicted decrease/increase in MHC Class I binding capacity were selected as shown below in **Table 1.** NetTEpi program was used to predict the CD8+ T-cell epitopes. These peptides were ordered.

Table 1. HLA-A2 restricted WA-O/D peptide pairs generated from NetMHCpan 4.1 server and NetTEpi server.

	Variant position	sequence	origin	bind level	change?	Epi
1	Spike WA62	VTWFHAIHV	WA	1.043		
2	Spike OM62	VTWFHVISG	O	28.63	decrease	
3	Spike WA135	FCNDPFLGV	WA	1.079		
4	Spike DE 135	FCNDPFLDV	D	3.17	decrease	
5	Spike OM133	FCNDPFLDY	O	17.703	decrease	
6	Spike WA 367	VLYNSASFS	WA	6.305		
7	Spike OM 365	VLYNLAPFF	O	1.858	increase	
8	Spike WA 417	KIADYNYKL	WA	0.067		0.8
9	Spike OM 415	NIADYNYKL	O	0.352	decrease	3
10	Spike WA 444	KVGGNYNYL	WA	2.933		
11	Spike OM 442	KVSGNYNYL	O	1.745	increase	
12	Spike WA 495	YGFQPTNGV	WA	2.882		
13	Spike OM 493	YSFRPTYGV	O	1.313	increase	
14	Spike WA 502	GVGYQPYRV	WA	2.098		
15	Spike OM 500	GVGHQPYRV	O	1.336	increase	
16	Spike WA 505	YQPYRVVVL	WA	1.193		
17	Spike OM 503	HQPYRVVVL	O	31.38	decrease	
18	Spike WA 610	VLYQDVNCT	WA	1.738		
19	Spike D/O 608	VLYQGVNCT	D/O	1.358	increase	
20	Spike WA 612	YQDVNCTEV	WA	0.566		1.5
21	Spike D/O 610	YQGVNCTEV	D/O	2.112	decrease	3
22	Spike WA 762	QLNRALTGI	WA	1.973		
23	Spike OM 769	QLKRALTGI	O	4.43	decrease	
24	Spike WA 943	SALGKLQDV	WA	2.002		
25	Spike DE 941	SALGKLQNV	D	0.467	increase	
26	Spike WA 944	ALGKLQDVV	WA	1.273		

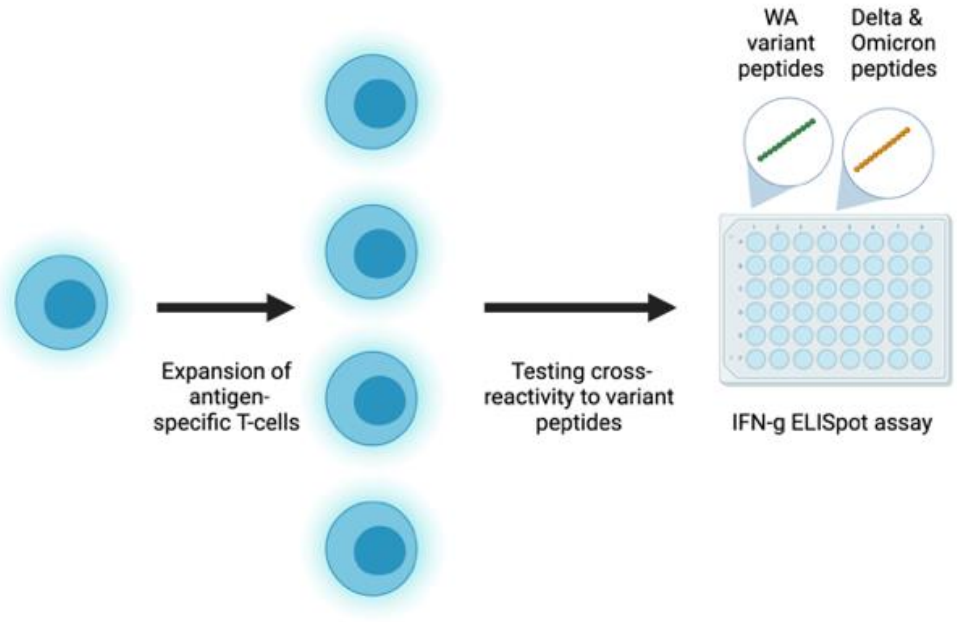
27	Spike DE 942	ALGKLQNVV	D	0.781	increase	
28	Spike WA 976	VLNDILSRL	WA	0.028		0.5
29	Spike OM 974	VLNDIFSRL	O	0.042	decrease	0.8

4.2 Aim 2: To Assess the Vaccine-Induced CTL Responses to the Washington Variant T-Cell Epitopes and to Test the Nature of Their Reactivity to the Epitope Variants (Do They Cross-React or Do They Escape?)

To assess the vaccine-induced CTL responses to the Washington variant T-cell epitopes and to test the nature of their reactivity to the epitope variants, firstly HLA-A2 positive, vaccinated MWCCS study participants with good CTL responses to the Washington spike epitopes were identified. The donors from the MWCCS study who had had visits pre- and post- release of the first COVID vaccine were selected. The donors were HLA-A2 positive, HIV negative, healthy donors who had not been naturally exposed to any SARS-CoV-2 variant (this was ensured by finding donors who did not have antibodies against SARS-CoV-2 nucleoprotein as part of the COVID study) and who had good T-cell responses to Washington spike epitopes. PBMCs from these selected donors were isolated and cultured with the HLA-A2 restricted 9-mers of the Washington variant's spike protein. This culture was expanded for 10 days (to re-stimulate and expand Washington variant spike antigen-specific memory T-cells) followed by an overnight IFN-g ELISpot assay with the Washington and Delta/Omicron 9-mers as readouts. The experimental design was to expand antigen-specific T-cells against the HLA-A2 restricted 9-mers of the Washington variant spike protein and to test the cross-reactivity of these cells to Delta and

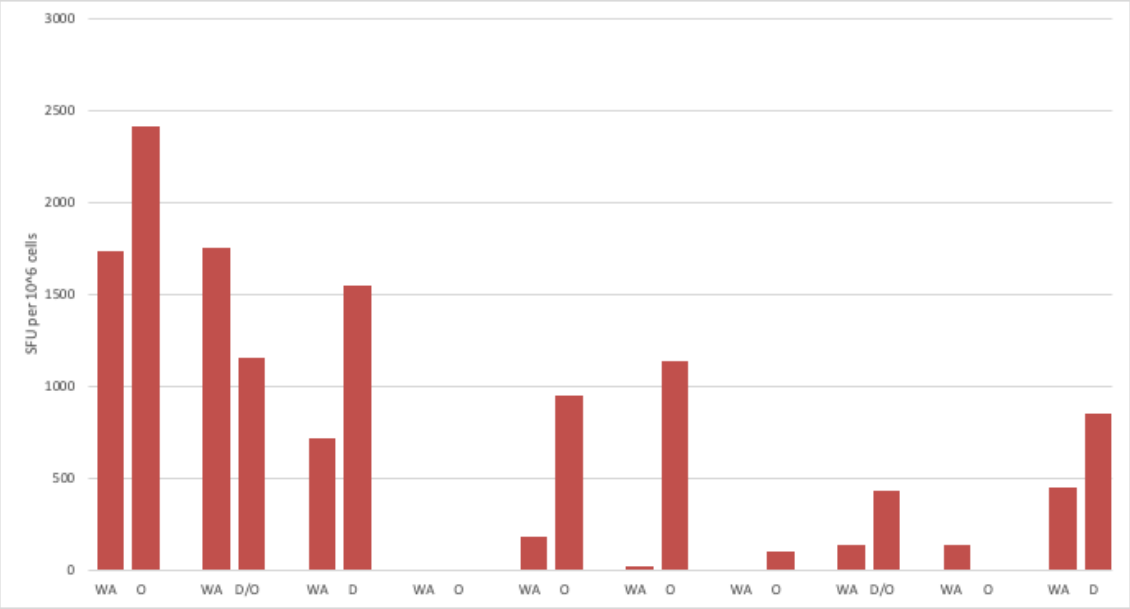
Omicron variant peptides using an ELISpot assay as shown in **Figure 2.** The results of the assay are shown in **Figure 3.**

Figure 2. Experimental design to test cross-reactivity.



Vaccine-induced (SARS-CoV-2 Washington variant strain spike antigen-specific) CD8+ T-cells were expanded against the Washington variant's spike protein and ELISpot assay was done using the WA-variant 9-mer pairs to test the cross-reactive potential of these antigen-specific T-cells to the variant peptides.

Figure 3. ELISpot assay to test cross-reactivity using PBMCs.



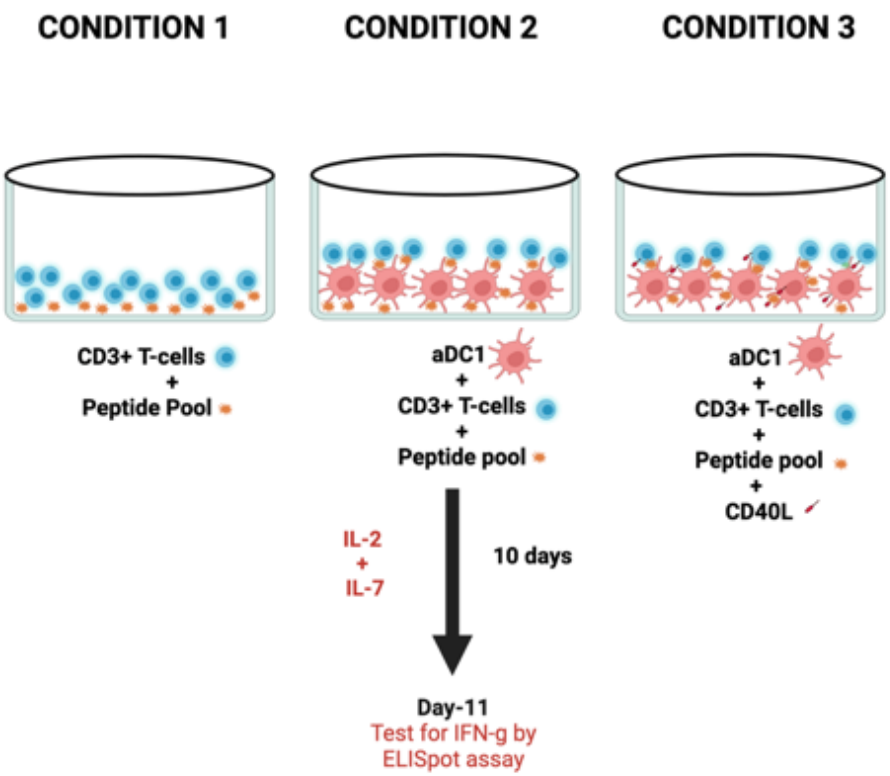
Washington variant antigen-specific T-cells specific cross-react with variant epitopes. In some instances, decreased T-cell reactivity to variant epitopes was observed and in some instances the T-cell reactivity was even higher to the variant epitopes compared to Washington epitopes. The predicted MHC binding affinity and T-cell binding affinity did not correlate with T-cell responses observed in *in-vitro* assays.

The results of the ELISpot assay to look at cross-reactivity suggested that in some cases, there was a decrease in T-cell reactivity to the variant epitopes compared to the Washington epitopes and in some other cases there was an increase in T-cell reactivity to the variant epitopes compared to the Washington epitope. This was similar to what was observed in the HIV study. We also observed that in some instances, the predicted MHC binding affinity and T-cell binding affinity did not correlate with the T-cell responses observed. There was an increased T-cell response when the binding affinity was decreased and a decreased T-cell response when the predicted binding affinity was increased.

Since we observed cross-reactivity in our previous experiment, we wanted to expand the cross-reactive T-cell clones further to perform future assays to look at CD8 downregulation and

target cell killing. So instead of using whole PBMCs, we decided to isolate bulk T-cells from donor PBMCs and co-culture the T-cells in the presence of dendritic cells and CD40L ("surrogate for CD4+ T-cell help") to expand the antigen-specific memory CTLs. The next step was to optimize methods to expand CTLs to achieve adequate number for performing future assays (using dendritic cells/CD40L 'help'). Firstly, an HLA-A2-positive donor was selected. Monocytes were isolated from donor PBMCs using CD14 bead monocyte separation by positive selection. The negatively isolated PBLs were cryopreserved. Then, the monocytes were matured to generate human monocyte-derived dendritic cells. aDC1s were used for this experiment. This was followed by bulk T-cell isolation from the cryopreserved PBLs derived from the same donor using EasySep Human T-cell enrichment kit by negative selection. Then, dendritic cell: T-cell co-cultures were done under 3 different conditions. **Condition 1:** Bulk T-cells with peptides belonging to the Washington variant. **Condition 2:** Bulk T-cells with aDC1s and peptides. **Condition 3:** Bulk T-cells with peptides and CD40L (as a surrogate for CD4+ T-cell 'help'). IL-2 and IL-7 were added once every 3-4 days to promote T-cell expansion. On Day 11, an overnight IFN-gamma ELISpot assay was done where the WA peptides were used as readouts. The experimental design is described in **Figure 4.**

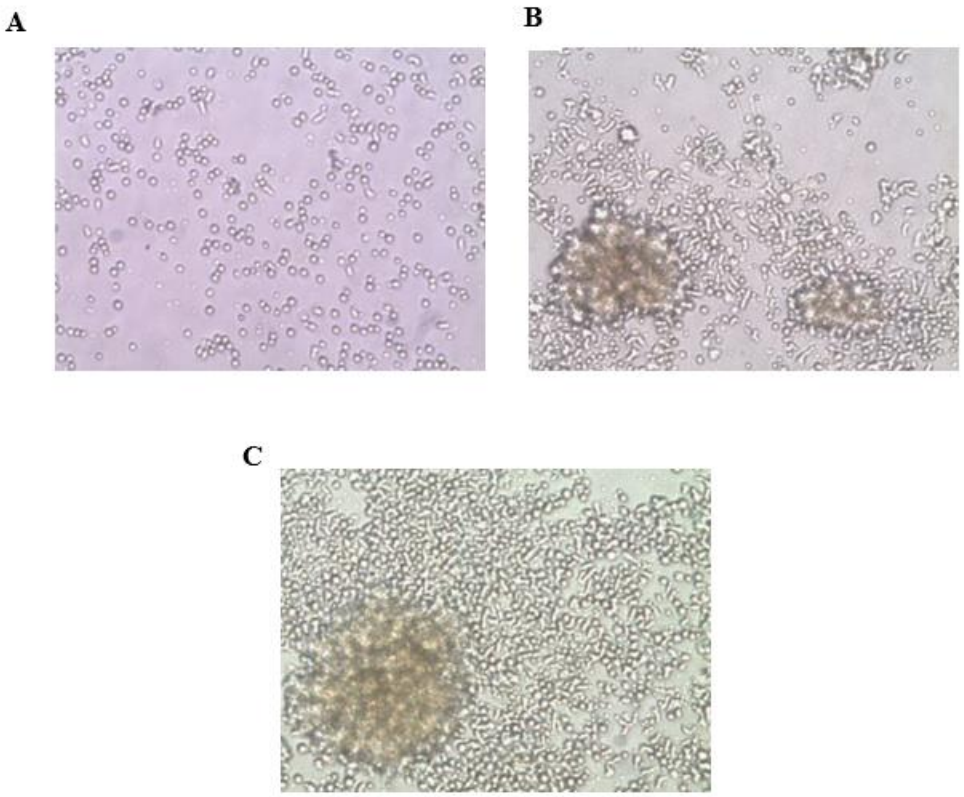
Figure 4. T-cell co-culture and expansion.



In order to expand antigen-specific T-cells *ex-vivo*, DC: T-cell co-culture was done at (DC:T-cell = 1:7.5 ratio) and the culture was expanded for 10 days with the addition of IL-2 and IL-7 every 3-4 days. On Day 11, an overnight IFN-g ELISpot assay was done where the WA peptides were used as readouts.

At the end of 10 days, the cells under each condition has expanded as shown in **Figure 5,** with the highest expansion observed in **Condition 3** where bulk T-cells were co-cultured with aDC1s and CD40L. The highest number of expansion was observed in **Condition 3** where aDC1s were co-cultured with T-cells in the presence of CD40L.

Figure 5. T-cells belonging to each condition on Day 10.

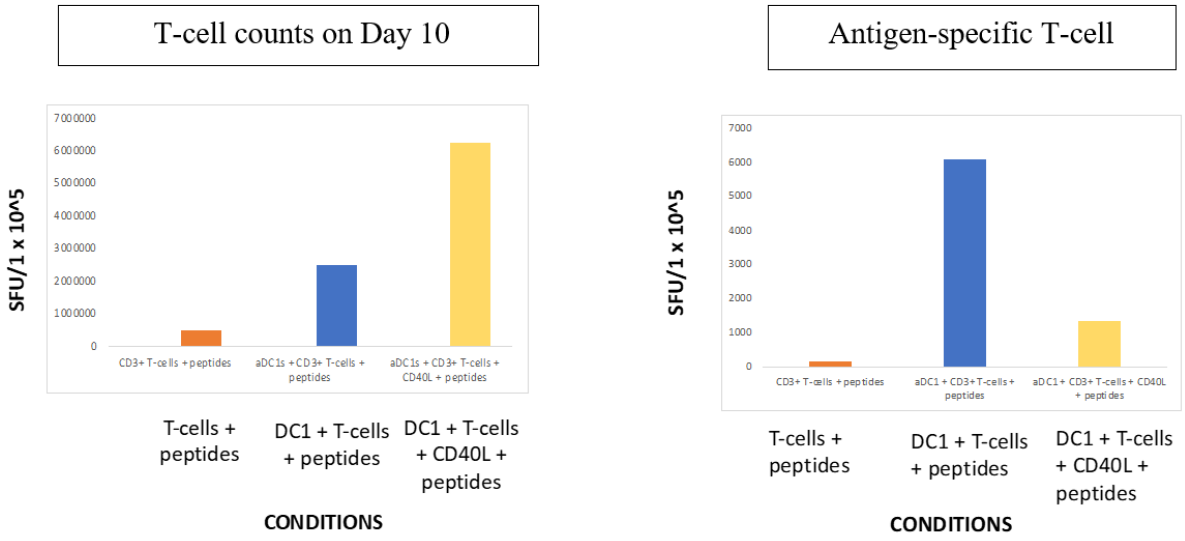


A. CD3+ T-cells with peptides (over 0.5 million cells were counted), B. CD3+ T-cells co-cultured with aDC1s (over 2.5 million cells were counted), C. CD3+ T-cells co-cultured with aDC1s and CD40L (over 6.25 million cells were counted).

However, the ELISpot assay to quantify the antigen-specific T-cells showed that the condition where T-cells were cultured with aDC1s in the absence of CD40L produced the highest number of antigen-specific T-cells as shown in **Figure 6.** Although the highest number of T-cell expansion on day 10 was observed in the condition where T-cells were co-cultured with dendritic cells and CD40L, the highest number of antigen-specific T-cells were present in the condition were T-cells were cultured with dendritic cells in the absence of CD40L. We concluded that dendritic

cells played a positive role in the overall expansion of T-cells *ex-vivo*. We also observed that CD40L promoted non-specific T-cell expansion. This was because although the greatest T-cell expansion was observed in the condition with CD40L, a lower number of antigen-specific T-cells were counted in this condition. In our study, we observed that CD40L (which is a surrogate for CD4+ T-cell 'help') did not help with the generation of antigen-specific T-cells (in fact even played a negative role). We speculated that an increase in PD-L1 expression on CD40L-activated dendritic cells may negatively influence the survival of PD-1 expressing effector memory T-cells, thus limiting expansion during long-term cultures. This highlights the need to consider the context when including CD4+ T-cell 'help' when generating long-term T-cell responders.

Figure 6. Optimize assay to produce antigen-specific T-cells.



Bulk T-cells co-cultured with aDC1s in the absence of CD40L produced the highest number of antigen-specific T-cells. This was followed by the condition where T-cells were co-cultured with aDC1s and CD40L. The least number of antigen-specific T-cells were observed in the condition with only T-cells and peptides.

Since we observed that CD40L in conjugation with DC1s played a negative role in the expansion of antigen-specific T-cells, in our study, we decided to use both DC1s and DC2s in conjugation with J558 (which are cells transfected with CD40L) instead of using soluble CD40L to assess if they had a better effect on antigen-specific T-cell expansion. The 4 different conditions are shown in **Figure 8.** The T-cells and monocytes were obtained from vaccinated donors and the monocytes were used to generate human monocyte-derived dendritic cells. aDC1s and DC2 were generated for this experiment as shown in **Figure 7.**

Figure 7. Mature aDC1s and DC2s appear phenotypically distinct.

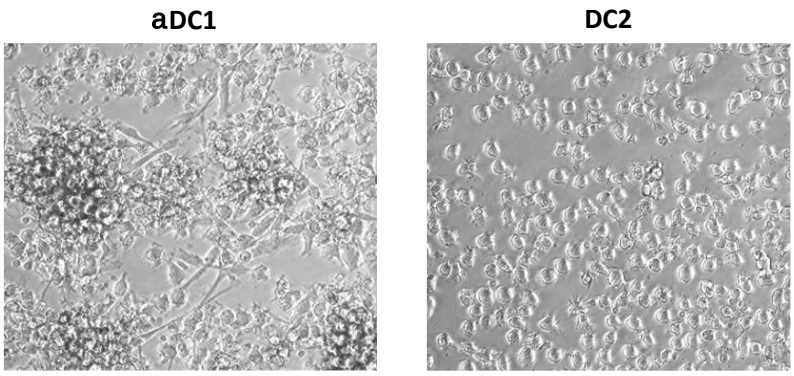
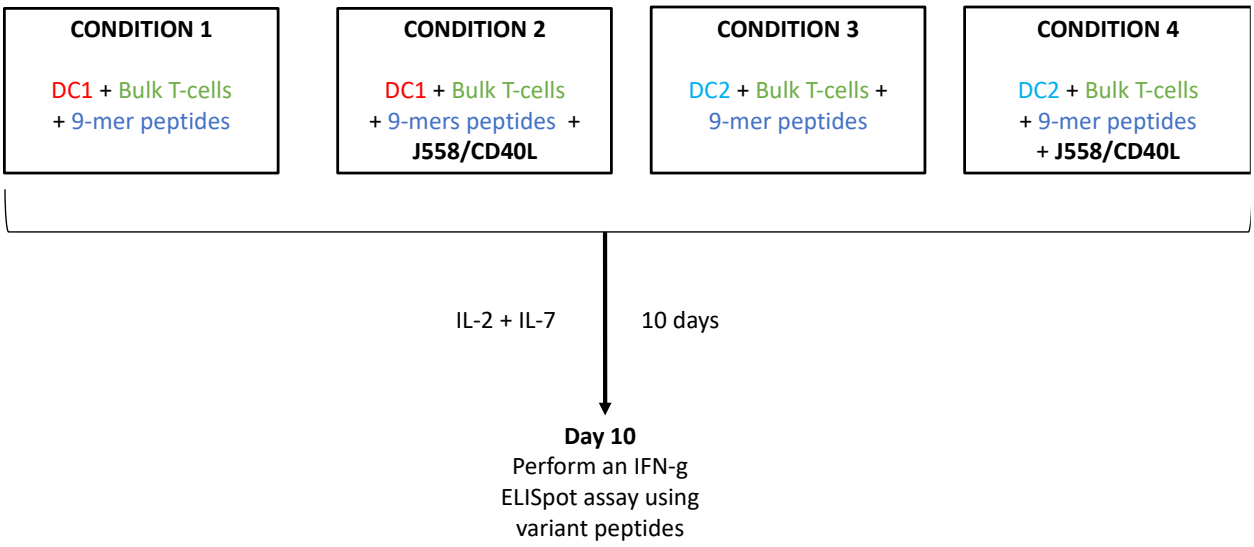


Figure 8. Generation of Washington variant spike-specific T-cells.

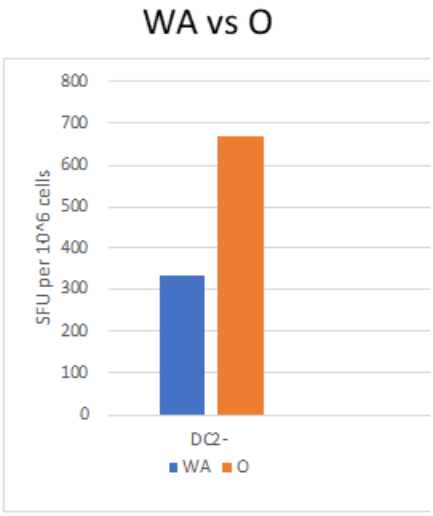


DC:T-cell co-cultures were done to generate Washington variant spike-specific T-cells under 4 different conditions. DC1s and DC2s were co-cultured with bulk T-cells in the absence and presence of J558/CD40L. The cultures were expanded for 10 days followed by an overnight IFN-g ELISpot assay.

The results of the ELISpot assay are shown in **Figure 9.**

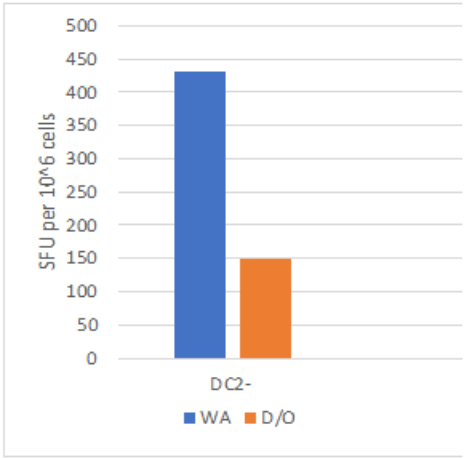
Figure 9. ELISpot assay to test cross-reactivity using T-cells.

A



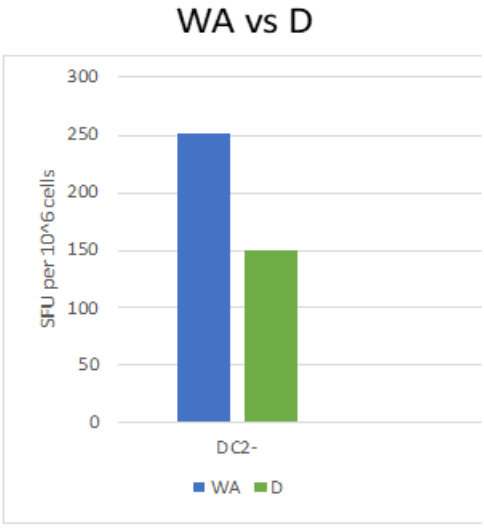
	Variant position	sequence	origin	bind level	change?	Epi
8	Spike WA 417	KIADYNYKL	WA	0.067		0.8
9	Spike OM 415	NIADYNYKL	O	0.352	decrease	3

B



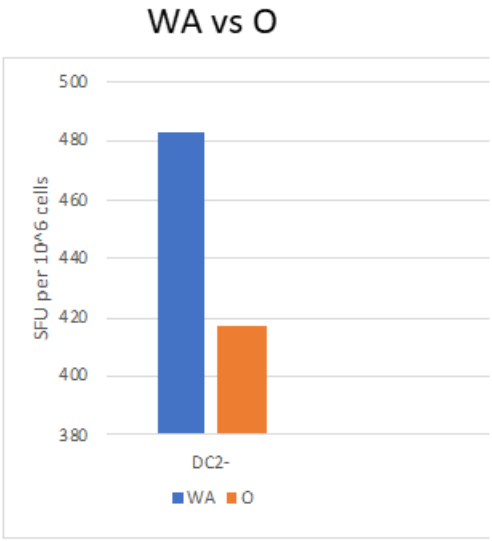
	Variant position	sequence	origin	bind level	change?	Epi
20	Spike WA 612	YQDVNCTEV	WA	0.566		1.5
21	Spike D/O 610	YQGVNCTEV	D/O	2.112	decrease	3

C



	Variant position	sequence	origin	bind level	change?	Epi
24	Spike WA 943	SALGKLQDV	WA	2.002		
25	Spike DE 941	SALGKLQNV	D	0.467	increase	

D



	Variant position	sequence	origin	bind level	change?	Epi
28	Spike WA 976	VLNDILSRL	WA	0.028		0.5
29	Spike OM 974	VLNDIFSRL	O	0.042	decrease	0.8

The DC1 conditions were not taken into consideration due to a very high background (non-specific spots). **A.** In one of the peptide pairs (WA Vs O) where there was a predicted decrease in MHC binding affinity and a predicted decrease in T-cell binding affinity in the variant epitope compared to the Washington epitope, there was an increase in the T-cell response to the variant epitope was higher compared to the Washington epitope. **B.** In another peptide pair (WA Vs D/O), where there was a predicted decrease in MHC binding affinity and predicted decrease in T-cell binding affinity, an expected decrease in T-cell response to the variant epitope compared to the WA epitope was observed. **C.** In another peptide pair (WA Vs D), where there was a predicted increase in MHC binding affinity, a surprising decrease in T-cell response to the variant epitope compared to the WA epitope was observed. **D.** A decrease in T-cell response to variant epitope compared to WA epitope was observed in this peptide pair (WA Vs O) where there was predicted decrease in MHC binding affinity and T-cell binding affinity.

In the peptide pairs tested, there was a decrease in T-cell response to the variant epitope compared to the Washington epitope in 3 of the peptide pairs and an increase in T-cell responses to the variant epitope compared to the Washington epitope in 1 of the peptide pairs.

The results of the ELISpot assay showed a similar result as was observed with the PBMCs. The predicted MHC binding affinity and T-cell binding affinity did not always correlate with the T-cell responses observed. In some peptide pairs where there was a predicted decrease in both MHC binding affinity and T-cell binding affinity, a surprising increase T-cell response was observed *in-vitro.*

5.0 Discussion

This study aimed at addressing a key knowledge gap regarding the potential of emergent SARS-CoV-2 variants to evade immune responses. Due to accumulating evidence of antibody escape in COVID-19, we wanted to determine if a phenomenon such as 'ALL BARK AND NO BITE' occurs in COVID-19 where CTLs cross-react with variant antigen-expressing target cells without target cell killing. In this thesis, we explored the first part of the research question – do the memory CTLs specific to the spike protein of the Washington variant of SARS-CoV-2 cross-react with variant epitopes or do the CTLs fail to bind and respond to the variant epitopes and cause CTL escape? In order to do that, we identified HLA-A2 restricted, CD8+ T-cell epitopes in the spike protein that differed between the Delta/Omicron strains compared to the Washington strain. Then we assessed the vaccine-induced CTL responses to the Washington variant T-cell epitopes and variant epitopes and assessed the nature of CTL reactivity to the epitope variants (to answer the question of whether they cross-react or escape?) using an IFN-g ELISpot assay. We isolated T-cells from HIV negative, healthy MWCCS donors who were vaccinated but unexposed to SARS-CoV-2.

We observed CTL cross-reactivity to SARS-CoV-2 variant epitopes. Certain variant epitopes had an increased reactivity to variant epitopes compared to WA epitopes. One interesting observation was that the predicted MHC binding affinity and T-cell binding affinity to the peptides did not always correlate with the T-cell responses observed. In some of the peptide pairs, although there was a predicted decrease in MHC binding affinity and T-cell binding affinity to the variant epitope compared to the Washington epitope, an increase in T-cell responses were observed and the vice-versa was also observed.

Our future directions involve the identification of more donors that fit the criteria and have good CTL responses. We then aim to expand the T-cell clones (Washington variant spike protein-specific CD8+ T-cells) from these identified and well-characterized donors, to use the T-cell clones generated to test for CD8 downregulation and pro-inflammatory cytokine production when exposed to variant antigen-expressing target cells. Our ultimate goal is to test if CTLs cross-react without target cell killing. For this we aim to establish a killing assay to test the killing potential of these T-cell clones when exposed to variant antigen-expressing target cells. The findings of this study lay a groundwork for achieving our ultimate goal of assessing variant antigen expressing target cell killing by COVID-19 mRNA vaccine-induced memory CTLs.

6.0 Public Health Significance

With recent evidence suggesting that more vaccine doses can increase the risk of individuals getting infected with newer variants of SARS-CoV-2, we wanted to investigate whether a phenomenon like original antigenic sin occurs in COVID-19. We wanted to understand the mechanism behind why more doses of vaccine can increase likelihood of getting COVID. Original antigenic sin or immune imprinting occurs from prior exposure to a related antigen. Since we have seen evidence of a phenomenon like that happening with antibodies in SARS-CoV-2, where antibody binds without neutralizing the virus, we wanted to see if a similar phenomenon occurs with CTLs in COVID. If vaccine-induced memory CTLs do cross-react with newer variants and produce inflammatory cytokines without target cell killing, then bivalent COVID vaccines may not be the best idea. Bivalent vaccines encode antigens represented in the original vaccine as well as the variant antigens. Using variant antigens to design a vaccine might end up doing more harm than good. Since we do not want to be doing more harm than good by administering a vaccine, this is an area of research worth pursuing.

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