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Hannah Soloff April 8, 2023

Viral Evolution During Monoclonal Antibody Treatment of Immunocompromised SARS-CoV-2 Patients

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Abstract

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SARS-CoV-2 mutations conferring immune escape can arise during prolonged infections of immunocompromised patients. Without an effective endogenous immune response, pressures such as monoclonal antibody treatment may select for such mutations, some of which could serve as a key element in the emergence of new variants of concern. To explore the role of monoclonal antibody treatment in the emergence of immune escape mutations during prolonged immunocompromised patient infections, we combined two longitudinal case studies with an extensive literature review. Our case studies utilized viral genomic sequencing of longitudinal nasopharyngeal samples to analyze intra-host viral evolution of patients treated with either single-agent or combination monoclonal antibodies. We observed the emergence of multiple immunologically important mutations, emphasizing the importance of closely monitoring viral evolution at the individual level. Additionally, the literature review identified 77 immunocompromised patients with immune escape mutation emergence during monoclonal antibody treatment. Interestingly, mutations associated with neutralization evasion and those found in variants of concern were frequently observed among the literature and primary data. Therefore, this study underscores the importance of closely monitoring SARS-CoV-2 intra-host evolution and understanding the implications of antibody treatment in immunocompromised patients.

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Chapter 1

Introduction

SARS-CoV-2 is the rapidly mutating virus responsible for Coronavirus disease 2019 (COVID-19) [1]. This respiratory illness results in clinical outcomes ranging from mild illness to severe acute respiratory syndrome and death, and its evolution remains a major global health concern. The virus's highly adaptive nature provides a cause for concern because the emergence of mutations and new variants can occur readily. For example, major Variants of Concern (VOC) have been identified and are proven to possess increased transmissibility and escape from immune recognition, thus making some vaccination and therapeutic measures less effective[2]. Therefore, genomic surveillance of SARS-CoV-2 is crucial in identifying mutations, VOC, and factors that contribute to their emergence[3].

Immunocompromised patients can develop prolonged infection, providing opportunity for new mutations to arise. These mutations can then be subject to selective pressure from a partial or incomplete endogenous immune response, and exogenous treatment, which are often found in immunocompromised patients. Many of the mutations identified during immunocompromised patient infections closely resemble those found in variants of concern (VOC), warranting the ongoing investigation into the role of immunocompromised individuals in the continued evolution of SARS-CoV-2 [4–18]. An exogenous treatment that is used often in clinical practice is monoclonal antibody treatment. These antibodies target specific epitopes of the SARS-CoV-2 spike protein and are thought to select for immune evasion mutations [17,19–21]. Therefore, continued investigation into the evolutionary outcomes of monoclonal antibody treatment in immunocompromised patients is imperative.

1.1 SARS-CoV-2 in Immunocompromised Patients

Immunocompromised patients include individuals with increased risk of SARS-CoV-2 disease morbidity and mortality due to an underlying condition, receipt of immunosuppressive medications, or a combination of the two conditions [22]. These patients are more likely to experience prolonged infection and an increased risk of disease severity, presenting major public health implications. Prolonged infection not only increases the risk of transmission due to the ongoing presence of a significant viral load, but it may allow SARS-CoV-2 to mutate more readily within the host [4–18].

1.2 Monoclonal Antibody Treatment

Monoclonal antibodies are a frequently used exogenous therapeutic intervention in the treatment of viral infections such as SARS-CoV-2. The antibodies work by binding to the receptor binding domain (RBD) of the spike (S) protein, which blocks the virus from binding to its ACE2 receptor target and entering human cells.

Single agent monoclonal antibodies target one epitope, typically within the RBD of the spike protein, thus providing high specificity but lacking the ability to target more than one epitope. Many reports have indicated an increasing frequency of escape mutations following single agent mAb treatments, many of which are consistent with mutations found in VOC. For example, a previous longitudinal analysis of the within-host evolution of the virus in a cohort of immunocompromised patients revealed emergence of the E484K mutation in the patients treated with mAb [23,24]. Due to the limitations of single agent mAb treatment, the development of combination mAb treatment appeared to be an effective method to target multiple epitopes simultaneously, thus yielding fewer incidents of escape. However, escape from combination antibody cocktails has recently been reported [25].

The significance of studying escape from mAb treatment in immunocompromised patients can be further exemplified through the analysis of the Omicron variant. For example, multiple mutations found within the Omicron variant, including E484K and N501Y, have emerged *in vivo* during the infections of immunocompromised patients[26]. Furthermore, these mutations occur at sites that interact with the binding to multiple antibody treatments, and may have emerged as a response to

these selective pressures[27]. Therefore, at the population level, monoclonal antibody treatments may become less effective over time as SARS-CoV-2 evolves and develops escape mechanisms.

1.3 Study Rationale

Despite growing numbers of studies observing immune escape mutation emergence during mAb treatment in immunocompromised SARS-CoV-2 patients, few have investigated the overall trends in clinical mAb treatments in these patients and the emergence of new VOC. To elucidate more information on the impact of monoclonal antibodies as a potential selective pressure that drives viral evolution, we aim to investigate the current trends in the literature while placing our experimental findings in the context of these trends. The relevant projects that will be integrated include single agent and combination mAb analyses, which both revealed compelling evidence of significant immune escape during mAb treatments. Our single mAb study discovered that patients treated with mAb incurred mutations conferring immune escape, compared to patients treated with convalescent plasma or no exogenous treatment [28]. The combination mAb study, which included a longitudinal evolutionary analysis of SARS-CoV-2 during infection in a person with HIV (PWH), unveiled one of the first discoveries of immune escape mutations during combination monoclonal antibody use *in vivo*.

By integrating our primary experimental findings with the current literature, we aim to generate a comprehensive summary of the evolution of SARS-CoV-2 in response to mAb treatment in immunocompromised patients to elucidate crucial information that may guide clinical use of mAbs. This multimodal analysis will demonstrate the dynamic relationship between monoclonal antibody therapies, weakened endogenous immune response, and within-host viral evolution. By strengthening our understanding of this interplay, we aim to determine the importance of selective pressures on viral mutation and their role in the emergence of new VOC.

Chapter 2

Literature Review

2.1 Introduction

I identified primary literature describing within-host evolution of the SARS-CoV-2 virus in immunocompromised patients following treatment with monoclonal antibody therapy. I then extracted relevant patient data from each study to create a comprehensive list of all identified patients, focusing on immune escape mutation emergence.

Extraction of patient data from the 18 articles yielded 77 patients that fit the criteria of 1) SARS-CoV-2 infection, 2) immunocompromised, 3) treated with monoclonal antibodies, and 4) experienced significant viral evolution during infection.

By focusing on the emergence of immune escape mutations thought to confer escape from monoclonal antibodies, we aim to emphasize the importance of genomic surveillance in immunocompromised patients treated with mAbs.

2.2 Methods

2.2.1 Building a Comprehensive Literature Search

I conducted a search and review of peer-reviewed articles to obtain current information about the evolutionary trends of SARS-CoV-2 in relation to monoclonal antibody treatment.

To build a comprehensive and inclusive literature search in *Pubmed*, I implemented the methodology from a Johns Hopkins University Welsch Medical Library video lecture [29]. I first derived key concepts from the research question: “What role do monoclonal antibody therapies play in driving resistance mutation emergence in immunocompromised patients infected with SARS-CoV-2? The resulting concepts included 1) immunocompromised patients, 2) SARS-CoV-2, 3) monoclonal antibodies, and 4) resistance mutations. Within each category, appropriate key

words and Medical Subject Headings (MeSH) terms were compiled and transformed into searchable queries by applying truncation, quotes, connecting words, and field tags (Table 2.1). To check for typos or syntax errors that may negatively affect search results, I input each query group into *Pubmed* individually and then checked for errors indicated by the history and search details

one-by-one while checking advanced search options for errors. Finally, I combined each query group into a single comprehensive search, ensuring that the results only applied to all relevant categories. Additional articles were identified by referring to primary search article citations. Article primary inclusion criteria include publication before January 1st, 2023, available in full text through public or institutional access, and publication in English.

Step	**Search Queries Entered into *Pubmed***
#1	immunocompromis*[tw] OR immunosuppress*[tw] OR immunodeficien*[tw] OR "Immunocompromised Host"[Mesh]
#2	SARS-CoV-2[tw] OR COVID-19[tw] OR Coronavirus-19[tw] OR "SARS-CoV-2"[Mesh] OR "SARS-CoV-2 variants"[Supplementary Concept] OR "COVID-19 rebound"[Supplementary Concept]
#3	“therapeutic antibod*”[tw] OR “native human monoclonal antibod*”[tw] OR “monoclonal antibod*”[tw] OR mAb[tw] OR mAbs[tw] OR “monoclonal antibody cocktail” [tw] OR “combination monoclonal antibod*”[tw] OR “combination therapy”[tw] OR monotherapy[tw] OR “single-agent monoclonal antibod*”[tw] OR "Antibodies, Monoclonal, Humanized"[Mesh] OR "Antibodies, Neutralizing"[Mesh] OR "Antigen-Antibody Reactions"[Mesh] OR "Antibody Affinity"[Mesh]
#4	“immune escape”[tw] OR variant*[tw] OR “virus evolution”[tw] OR “viral evolution”[tw] OR “within-host evolution”[tw] OR “intra-host evolution”[tw]

	"Drug Resistance, Viral"[Mesh] OR "Mutation"[Mesh] OR "Mutation Rate"[Mesh] OR “Mutation, Missense"[Mesh] OR "Immune Evasion"[Mesh]
#5	#1 AND #2 AND #3 AND #4

Table 0.1. Sequential Steps for Building a Comprehensive Literature Search.

After identifying relevant keywords and MeSH (Medical Subject Headings) terms, truncation, quotation marks, and field tags were applied where appropriate. The lists were then combined using “OR” and “AND” when relevant.

2.2.2 Analyzing Immune Escape Probability

After inputting and sorting relevant patient and virus evolution data into an excel document, I isolated the immune escape mutations that were most prevalent within the dataset. I then utilized the interactive web tool “Sites in SARS-CoV-2 genome where mutations escape antibody binding[20]” to visualize the escape probability of these common mutations. The resulting figure (Figure 2.3) was generated by selecting the LY-CoV555 (bamlanivimab) option and manually highlighting and labeling mutations observed in the patients treated with bamlanivimab.

2.3 Results

This literature review focused on obtaining SARS-CoV-2 intra-host viral evolutionary data for immunocompromised patients treated with either single-agent or combination monoclonal antibody therapy. Patients included in this study were either immunocompromised due to underlying health conditions or immunosuppressive treatments.

2.3.1 Literature Search Findings

The *Pubmed* search resulted in the identification of 20 articles, and citation and reference tracking led to the discovery of 11 additional articles (31 articles total). Following exclusion of duplicates and articles lacking eligibility, relevance, and quality, 18 records remained for detailed analysis

(Figure 2.2). The 18 article titles, publication dates, first author, and journal of publication are listed in (Table 2.2).

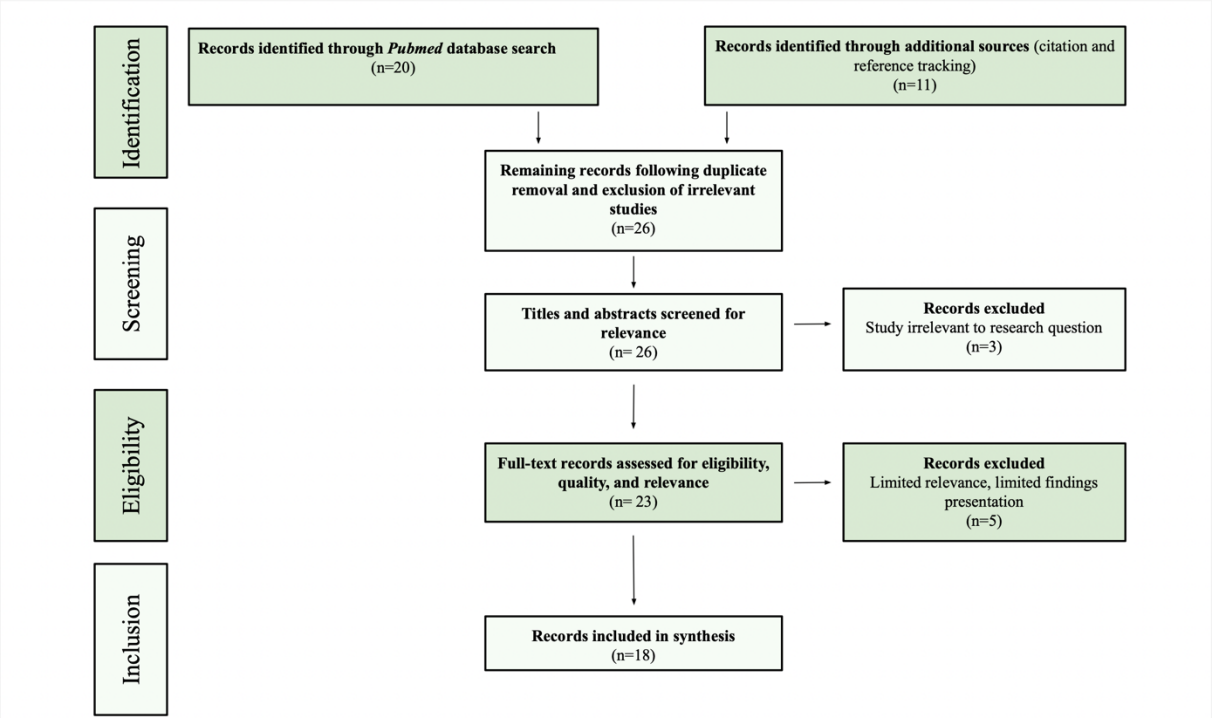


Figure 0.1. Identification and Screening Methodology for Literature Review.

Screening for duplicates and irrelevant sources began once all articles were identified via *Pubmed* search and citation and reference tracking. The remaining 26 articles were screened for relevance based on the abstract, resulting in three exclusions. The remaining 23 articles were assessed for eligibility, quality, and relevance based on full-text screening, resulting in 5 exclusions and 18 inclusions of articles for synthesis.

Article Title	Publication Date	First Author	Journal of Publication
Emergence of Q493R mutation in SARS-CoV-2 spike protein during bamlanivimab/etesevimab treatment and resistance to viral clearance	10/26/22	Manish C. Choudhary	Nature
Emergence of Delta and Omicron variants carrying resistance-associated mutations in immunocompromised patients undergoing sotrovimab treatment with long-term viral excretion	09/03/22	Cristina Andrés	ESCMID
Emergence of SARS-CoV-2 resistance mutations in a patient who received anti-SARS-COV2 spike protein monoclonal antibodies: a case report	12/07/21	Honorine Fenaux	BMC Infectious Diseases
De novo emergence of SARS-CoV-2 spike mutations in immunosuppressed patients	07/28/22	Lacy M. Simons	Transplant Infectious Disease
Emergence of SARS-COV-2 Spike Protein Escape Mutation Q493R after Treatment for COVID-19	07/27/21	Daniele Focosi	Emerging Infectious Diseases
Emergence of E484K Mutation Following Bamlanivimab Monotherapy among High-Risk Patients Infected with the Alpha Variant of SARS-CoV-2	08/19/21	Nathan Peiffer-Smadja	Virus
Emergence of the E484K mutation in SARS-COV-2-infected immunocompromised patients treated with bamlanivimab in Germany	07/14/21	Bjoern Jensen	The Lancet
Bamlanivimab Treatment Leads to Rapid Selection of Immune Escape Variant Carrying the E484K Mutation in a B.1.1.7-Infected and Immunosuppressed Patient	12/01/21	Benedikt Lohr	Clinical Infectious Diseases

Bamlanivimab as monotherapy in two immunocompromised patients with COVID-19	07/28/21	Grégory Destras	The Lancet (Microbe)
Persistence and Evolution of SARS-CoV-2 in an Immunocompromised Host	12/03/20	Bina Choi	New England Journal of Medicine
Rapid Selection of Sotrovimab Escape Variants in Severe Acute Respiratory Syndrome Coronavirus 2 Omicron-Infected Immunocompromised Patients	02/01/23	Smaranda Gliga	Clinical Infectious Diseases
Resistance Mutations in SARS-CoV-2 Delta Variant after Sotrovimab Use	04/14/22	Rebecca Rockett	New England Journal of Medicine
Resistance mutations in SARS-CoV-2 omicron variant in patients treated with sotrovimab	05/17/22	Camille Vellas	ESCMID
SARS-CoV-2 Variants in Immunocompromised Patient Given Antibody Monotherapy	10/01/22	Aurélie Truffo	Emerging Infectious Diseases
Evolution of spike mutations following antibody treatment in two immunocompromised patients with persistent COVID-19 infection	11/09/21	Yotam Bronstein	Journal of Medical Virology
Influence of treatment with neutralizing monoclonal antibodies on the SARS-CoV-2 nasopharyngeal load and quasispecies	09/16/21	Camille Vellas	Clinical Microbiology and Infection
Spike Gene Evolution and Immune Escape Mutations in Patients with Mild or Moderate Forms of COVID-19 and Treated with Monoclonal Antibodies Therapies	01/24/22	Aude Jary	Viruses
Sotrovimab drives SARS-CoV-2 omicron variant evolution in immunocompromised patients	05/27/22	Grégory Destras	The Lancet (Microbe)

Table 0.2. Papers Isolated from Literature Review for Further Analysis.

Eighteen papers qualified for inclusion based on relevance to the research inquiry, quality, and credibility. ESCMID=European Society of Clinical Microbiology and Infectious Diseases.

2.3.2 Clinical Characteristics

The average age of the patients, after removing unclear or absent data, was 59.4 years old. Of the patients whose biological sex was reported, 15 were female and 36 were male. All patients were immunocompromised either due to an underlying condition, immunosuppressive treatment for an underlying condition, or a combination of the two. The average length of infection was 29.2 days (about 4 weeks).

The parent variant linages identified in the patients at infection onset included Pre-Alpha (n=3), Alpha (n=24), Delta (n=5), and Omicron (n=43). The monoclonal antibodies used in these reports include both single-agent therapies such as bamlanivimab (n=17) and sotrovimab (n=48), as well as antibody cocktails casirivimab/imdevimab (n=3) and bamlanivimab/etesevimab (n=9). Two patients received more than one type of mAb treatment, administered at different infection timepoints.

2.3.3 Viral Evolution

Viruses in all patients included for analysis developed mutations that were absent at baseline and are thought to elicit immune escape from monoclonal antibodies. The most common of these mutations was E340A/G/K (n=35), which is thought to confer escape from sotrovimab [30,31]. Twenty-one patients developed the P337L/R/T mutation, which is also thought to confer escape from sotrovimab. E484A/KQ, located in the spike protein of SARS-CoV-2, emerged during the infection of 21 patients, and has been found to significantly reduce the potency of earlier mAbs such as bamlanivimab and casirivimab. To confirm whether the observed mutations are likely to elicit immune escape from monoclonal antibodies, most studies (15 studies) utilized literature citations, while others combined literature citations with pseudovirus neutralization assays (3 studies). Other spike positions with frequent mutations include Q493K/R (n=12), K356T (n=3), S371F (n=1), and K417N (n=1). Each of these mutations is known to confer immune escape based on previous studies observing *in vivo* emergence, as well as emergence in *in vitro* experiments such as deep mutational scanning[32]. By utilizing yeast libraries that express proteins with SARS-CoV-2 RBD mutations and exposing them to antibody treatments, deep mutational scanning identifies escape mutations and maps them to the SARS-CoV-2 genome.

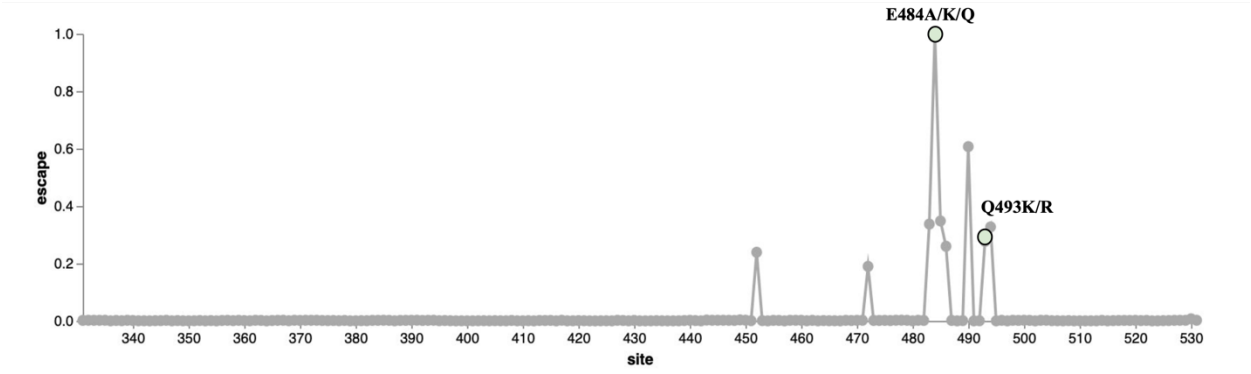


Figure 0.2. Potential of escape mutation emergence at each amino acid position on the spike protein RBD against bamlanivimab (LY-CoV555).

The X-axis represents amino acid position along the RBD of the spike protein, and the Y-axis represents likelihood of immune escape mutation emergence at each amino acid position during exposure to bamlanivimab [19,20,32]. Mutations E484A/K/Q (n=21) and Q493K/R (n=12) are highlighted and labeled on the graph.

Overall, the literature review methods successfully identified 18 articles for analysis, including a total of 77 patients with a wide range of underlying conditions, viral strains of infection, monoclonal antibodies received, dates of infection onset, and significant viral evolution (Table 2.3).

Patient Number and Source	Patient Age and Sex	Immunosuppressive Underlying Condition	Immunosuppressive Medication	Viral Strain	mAbs Administered	Days to Last Positive SARS-CoV-2 qPCR	Date of Infection Onset	Observed Mutations	Method of Immune Escape Mutation Confirmation
1 (Guigon et al. 2021)	63, N/A	cutaneous T-cell lymphoma (mycosis fungoides)	N/A	B.1.1.7	bamlanivimab/etesevimab	40	2021-04-21	E484K, Q493R	Literature Citation
2 (Jensen et al. 2021)	Early 70's, M	ANCA-associated vasculitis with end-stage renal disease	rituximab, Prednisolone	B.1	bamlanivimab	20	Before 04-2021	E484K, E484Q	Literature Citation
3 (Jensen et al. 2021)	Early 40's, F	AIDS, cerebral toxoplasmosis	N/A	B.1.1	bamlanivimab	32	Before 04-2021	E484K	
4 (Jensen et al. 2021)	Early 60's, M	relapsed follicular lymphoma	obinutuzumab, thiotepa, cytarabine, etoposide	B.1.177	bamlanivimab	103	2020-11-01	E484K	
5 (Jensen et al. 2021)	Late 60's, M	Heart transplant recipient (about 30 years ago)	cyclosporine, azathioprine, prednisolone	B.1.177	bamlanivimab	40	Before 2021-04	E484K	
6 (Jensen et al. 2021)	Late 60's, M	Chronic lymphatic leukemia	tacrolimus, mycophenolate mofetil, prednisolone	B.1.258	bamlanivimab, imdevimab/casirivimab	91	2020-12-01	E484K	
7 (Choi et al. 2020)	45, M	severe antiphospholipid syndrome complicated by diffuse alveolar hemorrhage	severe antiphospholipid syndrome complicated by diffuse alveolar hemorrhage	N/A	casiriviamb/indevimab	154	Before 2020-12	Q493K	Literature Citation
8 (Gliga et al. 2022)	37, F	Allogeneic SCT (AML)	Allogeneic SCT (AML)	BA.1	sotrovimab	21	Between 2022-20-01 and 2022-02-25	P337S, E340A, E340V	Pseudovirus Neutralization Assay and Literature Citation
9 (Gliga et al. 2022)	42, M	CVID	CVID	BA.1.1		N/A		P337S, P337L	
10 (Gliga et al. 2022)	45, F	KTx	KTx	BA.2		71		E340K, E340A	
11 (Gliga et al. 2022)	28, F	ALL	ALL	BA.1.1		35		E340D	
12 (Gliga et al. 2022)	72, F	Liver cirrhosis Child A, polyarthritis	Liver cirrhosis Child A, polyarthritis	BA.1.13		14		P337S, E340A	
13 (Gliga et al. 2022)	74, F	Cryoglobulinemic vasculitis 2015	Prednisolone (5 mg/die), Rituximab	BA.1		21		E340K	
14 (Gliga et al. 2022)	45, F	HTx	MMF, Tacrolimus, Prednisolone	BA.1		N/A		P337S, P337H	
15 (Gliga et al. 2022)	67, M	HTx	MMF, Tacrolimus, Prednisolone	BA.1		28		P337S, P337P, E340D, E340K	
16 (Gliga et al. 2022)	74, M	KTx	MMF, Tacrolimus, Prednisolone	BA.1.18		21		E340K, E340A	
17 (Gliga et al. 2022)	76, M	KTx+ PancreasTx	Tacrolimus, Prednisolone	BA.1		14		P337R, E340D, E340K, E340A	
18 (Gliga et al. 2022)	37, M	Liver fibrosis with portal hypertension, SLE	Prednisolone, Hydroxychloroquine	BA.1		28		P337S, E340V, E340K, E340D, E340A	
19 (Gliga et al. 2022)	73, M	KTx	MMF, Tacrolimus	BA.1		21		P337S, P337H, E340K	
20 (Gliga et al. 2022)	16, M	Allogeneic SCT (ALCL)	Ruxolitinib	BA.1.1		N/A		P337S	

Patient Number and Source	Patient Age and Sex	Immunosuppressive Underlying Condition	Immunosuppressive Medication	Viral Strain	mAbs Administered	Days to Last Positive SARS-CoV-2 qPCR	Date of Infection Onset	Observed Mutations	Method of Immune Escape Mutation Confirmation
21 (Peiffer-Smadja et al. 2021)	87, M	Peripheral arterial obstructive disease, diabetes, hypertension, coronary heart disease, grade III chronic renal disease	N/A	B.1.1.7	bamlanivimab	27	N/A	E484K	Literature Citation
22 (Peiffer-Smadja et al. 2021)	35, M	Diabetes, hypertension, end-stage renal disease on dialysis, restrictive ventilatory disorder, juvenile idiopathic arthritis				38		E484A,E484K	
23 (Peiffer-Smadja et al. 2021)	61, M	Stroke, peripheral arterial obstructive disease, diabetes, hypertension, coronary heart disease, and end-stage kidney disease on dialysis				18		E484K	
24 (Peiffer-Smadja et al. 2021)	97, M	Hypertension, diabetes				14		E484K	
25 (Peiffer-Smadja et al. 2021)	64, M	Diabetes, hypertension, coronary heart disease, heart transplantation				48		E484K	
26 (Peiffer-Smadja et al. 2021)	66, M	Stroke, tuberculosis, diabetes, hypertension, rheumatoid arthritis, end-stage renal disease, kidney transplantation				40		E484K, Q493R	
27 (Destras et al. 2021)	62, M	Angioimmunoblastic T-cell lymphoma	N/A	20A.EU2	bamlanivimab	20	Between 2021-12 and 2022-03	E484K, E484A, E484Q	Literature Citation
28 (Destras et al. 2021)	87, M	Mantle cell lymphoma		B.1.1.7	bamlanivimab	17		E484K, E484A, E484Q	
29 (Focosi et al. 2021)	73, M	Cholangiocarcinoma	N/A	B.1.1.8	bamlanivimab/etesevimab	28	2021-04-12	Q493R	Literature Citation
30 (Rockett et al. 2022)	30's , F	Lung Tx	N/A	B.1.617.2	sotrovimab	23	Between 2021-08 and 2021-11	E340K, E340A	Literature Citation
31 (Rockett et al. 2022)	30's, F	CVID, Bronchiectasis		B.1.617.2		24		E340A, P337L	
32 (Rockett et al. 2022)	20's, M	Renal Tx, PCKD, CMV, BK viraemia		B.1.617.2		14		E340K	
33 (Rockett et al. 2022)	70's, F	MDS/myeloproliferative disorder		B.1.617.2		37		E340K, E340A, E340V	
34 (Truffot et al. 2021)	72, M	Chronic lymphocytic leukemia, hypogammaglobinemia	venetoclax, rituximab	B.1.1.7	bamlanivimab	56	N/A	E484Q	Literature Citation
35 (Bronstein et al. 2021)	68, M	chronic lymphocytic leukemia	venetoclax, rituximab	B.1.1.7	bamlanivimab	43	2021-01	E484Q	Literature Citation

Patient Number and Source	Patient Age and Sex	Immunosuppressive Underlying Condition	Immunosuppressive Medication	Viral Strain	mAbs Administered	Days to Last Positive SARS-CoV-2 qPCR	Date of Infection Onset	Observed Mutations	Method of Immune Escape Mutation Confirmation
36 (Vellas et al. 2022)	N/A	Liver SOT	N/A	BA.1	sotrovimab	14	Between 2022-20-01 and 2022-02-25	P337H	Literature Citation
37 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1.1		21		E340D	
38 (Vellas et al. 2022)		Renal SOT	N/A	BA.1.1		14		E340G	
39 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1.1		21		E340K	
40 (Vellas et al. 2022)		IS treatment	Unspecified	BA.2		7		E340A	
41 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1.1		7		P337S	
42 (Vellas et al. 2022)		Renal SOT	N/A	BA.1.1		14		P337L	
43 (Vellas et al. 2022)		Renal SOT	N/A	BA.1		14		E340D	
44 (Vellas et al. 2022)		Pulmonary SOT	N/A	BA.1		14		E340D	
45 (Vellas et al. 2022)		Primary immunodeficiency	N/A	BA.1.1		7		E340D	
46 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1.1		7		E340K	
47 (Vellas et al. 2022)		Liver SOT	N/A	BA.1.1		7		E340K	
48 (Vellas et al. 2022)		Renal SOT	N/A	BA.1.1		7		S371F	
49 (Vellas et al. 2022)		Chronic kidney disease	N/A	BA.1.1		7		P337R	
50 (Vellas et al. 2022)		Chronic kidney disease	N/A	BA.1		7		E340D	
51 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1.1		7		E340D	
52 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1.1		7		E340K	
53 (Vellas et al. 2022)		IS treatment	Unspecified	BA.1		7		K356T	
54 (Destras et al. 2022)	60, M	Diffuse large B- cell lymphoma R-DHAOx, CAR-T-cells (D26)	N/A	BA.1	sotrovimab	16	After 2021-02-27	P337S	Literature Citation
55 (Destras et al. 2022)	70, M	Heart transplant	N/A	BA.1		9		E340A	
56 (Destras et al. 2022)	72, F	Kidney transplant	N/A	BA.1		26		P337S	
57 (Destras et al. 2022)	52, F	Gougerot Sjögren	rituximab	BA.1		5		P337S, E340D	
58 (Destras et al. 2022)	19, M	Germinal embryonal carcinoma	N/A	BA.1		13		P337R, E340D, P337S, E340K	
59 (Destras et al. 2022)	59, M	Systemic scleroderma	N/A	BA.1		43		E340D, P337S, E340K	
60 (Destras et al. 2022)	59, M	Heart transplant	N/A	BA.1		23		E340Q	
61 (Destras et al. 2022)	75, M	Multiple sclerosis	N/A	BA.2		6		E340D	

Patient Number and Source	Patient Age and Sex	Immunosuppressive Underlying Condition	Immunosuppressive Medication	Viral Strain	mAbs Administered	Days to Last Positive SARS-CoV-2 qPCR	Date of Infection Onset	Observed Mutations	Method of Immune Escape Mutation Confirmation
62 (Vellas et al. 2021)	N/A	Solid Organ Transplant, lymphoma, leukemia, OR cerebral lymphoma	None, diethyl fumarate, mycophenolate mofetil, chemotherapy for lymphoma, leukemia, OR cerebral lymphoma	B.1.1.7	bamlanivimab/etesevimab	14	Before 2021-09	Q493R	Literature Citation
63 (Vellas et al. 2021)					bamlanivimab/etesevimab	7	Before 2021-09	Q493R	
64 (Vellas et al. 2021)					bamlanivimab/etesevimab	14		Q493R	
65 (Vellas et al. 2021)					bamlanivimab/etesevimab	7		Q493K	
66 (Vellas et al. 2021)					bamlanivimab/etesevimab	21		E484K	
67 (Andrés et al. 2022)	57, M	Follicular lymphoma	Epcoritamab	BA.1	sotrovimab	90	2022-02-03	E340K, E340Q, E340R, R346T, K356T	Literature Citation
68 (Andrés et al. 2022)	91, M	Mantle lymphoma, with CR	Rituximab	AY.100		N/A	2022-01-17	P337L, E340V	
69 (Andrés et al. 2022)	65, M	Refractory diffuse large B-cell lymphoma	Obinutuzumab + Glofitamab + CA RO7227166	BA.1.1		9	2022-02-14	E340V	
70 (Andrés et al. 2022)	75, F	Chronic lymphatic leukaemia	N/A	BA.1.1		N/A	2022-02-01	K356T	
71 (Andrés et al. 2022)	65, F	Chronic lymphatic leukaemia	Obinutuzumab + venetoclax	BA.1.1		11	2022-01-31	P337L	
72 (Fenaux et al. 2021)	55, M	stage I follicular lymphoma	CHOP chemotherapy	B.1.1.7	bamlanivimab/etesevimab	51	2021-04-13	K417N, E484K, Q493R	Literature Citation
73 (Simons et al. 2022)	64, M	mantle cell lymphoma	chemotherapy, rituximab,	B.1.1	bamlanivimab	130	2021-01-05	E484Q, E484K	Pseudovirus Neutralization Assay and Literature Citation
74 (Simons et al. 2022)	48, M	chronic lymphocytic leukemia with Richter's transformation	venetoclax, ubituximab, and umbralisib	B.1.2	bamlanivimab, casiriviamb/imdevimab	117	2021-03-04	E484K	
75 (Lohr et al. 2021)	55, F	acute myeloid leukemia	chemotherapy	B.1.1.7	bamlanivimab	27	2021-01-09	E484K, Q493R	Literature Citation
76 (Lohr et al. 2021)	54-79, N/A	Necrotizing myopathy	N/A		bamlanivimab	6	2021-01-28	Q493R	
77 (Lohr et al. 2021)		Bi-phenotypic acute leukemia, GVHD	systemic corticosteroids		bamlanivimab/etesevimab	68	2021-05-01	Q493R	

Table 0.3. Table of Immunocompromised Patients Treated with Monoclonal Antibodies for SARS-CoV-2 Infection.

ALL= acute lymphoblastic leukemia, CR= complete response;,CA= Clinical Assay; F, female; GVHD= graft-versus-host disease, IS= immunosuppressive, mAB=monoclonal anti-body, M=male, R -CHOP = rituximab, cyclophosphamide, hydroxydanorubicin, vincristine sulfate, prednisone, SOT= solid organ transplant

2.3.4 Monoclonal Antibody Authorization and VOC Prevalence

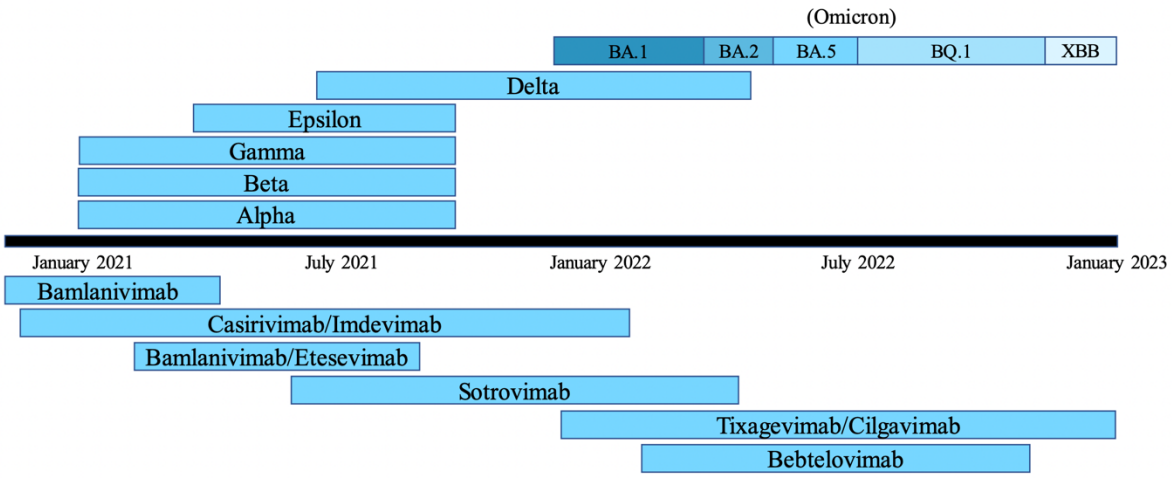


Figure 0.3. Monoclonal antibodies authorized by the FDA relative to Variant of Concern designation by the CDC and time.

Variants including Alpha (B.1.1.7 and Q lineages), Beta (B.1.351 and descendent lineages), Gamma, (P.1and descendent lineages), Epsilon (B.1.427 and B.1.429), and Delta (B.1.617.2 and AY lineages) are represented above the x-axis and are present above the time in which the CDC officially designated each as a VOC [33] . Omicron (BA.1, BA.2, BA.5, BQ.1, and XBB) are represented during the times in which each subvariant's distribution in the U.S. was above 50% according to the CDC National SARS-CoV-2 Strain Surveillance Program [34]. Monoclonal antibodies including bamlanivimab (LY-CoV555), cairivimab/imdevimab (REGN10933+ REGN10987 or REGN-COV2), bamlanivimab/etesevimab (LY-CoV555+ LY-CoV016),

sotrovimab (S309), tixagevimab/cilgavimab (COV2-2130+ COV2-2196 or AZD7442), and bebtelovimab are represented beneath the x-axis and represent the time of FDA authorization for respective use in the general public [35].

At the population level, the prevalence of specific VOC lineages have changed over the course of the COVID-19 pandemic. These VOC usually emerge because their new mutations provide a selective advantage for the virus, including immune escape from treatments and increased transmissibility[26]. Therefore, certain monoclonal antibody treatments that provide strong efficacy against one SARS-CoV-2 lineage may be less successful in neutralizing a new VOC with immune escape mutations. Based on the constantly changing efficacy of antibody treatments in relation to prevalent viral lineages, the FDA has made multiple authorizations, revocations, and modifications to their recommendations for clinical use over time.

In addition to our search for individual cases of immune escape mutation emergence during monoclonal antibody treated SARS-CoV-2 infections in immunocompromised patients, I also sought information regarding population-level monoclonal antibody use and immune escape. By searching authorization records of the FDA and the history of VOC designation by the CDC, I created a comparison of monoclonal antibody use and variant prevalence over time.

The CDC closely monitors SARS-CoV-2 variants as they emerge, classifying them by attributes and prevalence in the United States. Variants are classified as VOC if they are thought to significantly decrease the accuracy of diagnostic tests, decrease the efficacy of treatments and vaccines, possess increased transmissibility, and result in increased disease severity. VOC are reclassified into lower classes if they no longer pose a significant risk to United States public health, or if there is a sustained reduction in variant prevalence. These lower classes include variants being monitored (VBM) and variants of interest (VOI). VBM include variants that are capable of resistance to public health countermeasures or are circulating at very low levels, while VOI include variants that contain genetic indications of increased transmission and immune escape and decreased responsiveness to therapeutics or diagnostics, while showing little spread across the U.S. and in other countries [33].

The CDC designated the Alpha (B.1.1.7 and Q Pango lineages), Beta (B.1.351 and descendent Pango lineages), and Gamma (P.1 and descendent Pango lineages) variants as VOC on December

29, 2020. Alpha, Beta and Gamma were all reclassified as VBM on September 21, 2021. The Epsilon (B.1.427 and B.1.429 Pango lineages) variant was designated as a VOC on March 19, 2021 and reclassified as a VBM on September 21, 2021. The Delta (B.1.617.2 and AY Pango lineages) variant as a VOC on June 15, 2021 and reclassified it as a VBM on April 14, 2022 [33].

The Food and Drug Administration (FDA) monitors the current data regarding viral evolution and subsequent responsiveness to monoclonal antibody treatments. Bamlanivimab was the first monoclonal antibody developed to treat SARS-CoV-2 infections. It was developed by Eli Lilly and targets the RBD of the spike protein on the SARS-CoV-2 genome. Eli Lilly also developed etesevimab for use in conjunction with bamlanivimab, which targets the RBD on the spike protein of the SARS-CoV-2 genome. In April of 2021, the FDA recalled bamlanivimab as a single agent mAb due to decreased efficacy against circulating Gamma and Beta variants, while maintaining approval of the bamlanivimab/etesevimab cocktail. However, over time, the cocktail also proved to be less effective against these circulating variants and was thus restricted by the FDA to use in patients suspected to be infected with a susceptible viral lineage.

Monoclonal antibody cocktail casirivimab (REGN10933)/imdevimab (REGN10987) (REGN-COV2) was developed by Regeneron. Like Eli Lilly's bamlanivimab/etesevimab cocktail, REGN-COV2 combined two monoclonal antibodies to target different SARS-CoV-2 spike protein RBD regions. After multiple clinical trials revealed reduced risk of COVID-19 disease severity, hospitalization, and viral load in both low and high-risk patients [3,36,37], the FDA authorized it for emergency use in November of 2020. The cocktail was later restricted to use only when the patient is thought to be infected with a susceptible viral lineage due to the increase prevalence of the Omicron variant, which was highly unlikely to be neutralized by the cocktail.

Sotrovimab (S309) was developed by GSK and Vir Biotechnology. It was authorized for emergency use by the FDA in May of 2021 after studies showed reduced risk of severe disease outcomes and efficacy against circulating variants at the time.

Tixagevimab/cilgavimab (COV2-2130+ COV2-2196, AZD7442, or EVUSHELD) was a long-acting monoclonal antibody that was authorized by the FDA in December of 2021 as a pre-exposure prophylaxis for COVID-19 infection in immunocompromised patients. It was recalled by the FDA in January of 2023 due to increased Omicron variant prevalence and its lack of susceptibility to the cocktail.

Bebtelovimab was authorized in February of 2022 by the FDA after data showed that the mAb had potent neutralizing efficacy against all SARS-CoV-2 variants except for Mu. Therefore, this mAb was effective against the Omicron variant and its sublineages at the time of authorization. After new studies suggested that bebtelovimab was not expected to neutralize Omicron subvariants BQ.1 and BQ.1.1, the FDA restricted emergency use authorization.

These findings provide meaningful insight into the changes in monoclonal antibody authorization in the U.S. over time as certain mAbs were deemed ineffective against primary circulating variants.

2.4 Discussion

To explore the broader implications of monoclonal antibody use in the treatment of immunocompromised SARS-CoV-2-infected patients, I conducted an extensive literature review in search of both individual patient data and public health recommendations across time. I identified consistent examples of immune escape mutation emergence during mAb use, which is consistent with our hypothesis that the SARS-CoV-2 genome will continue to evolve in response to these antibodies' selective pressure. I identified 77 immunocompromised patients treated with mAbs for SARS-CoV-2 infections in which mutations thought to confer immune escape emerged. Along with supporting our hypothesis that SARS-CoV-2 develops escape mutations from monoclonal antibodies, the average length of infection of our patient list was nearly four weeks long (compared to the average one to two weeks in immunocompetent individuals)[38], supporting the hypothesis that immunocompromised patients are particularly likely to experience longer duration of infection, and that immune escape may contribute to this.

This study was limited in scope, since it relied exclusively on *Pubmed* as a search engine for the literature review for the patient data portion. Future exploration could combine sources located on other databases. Additionally, the SARS-CoV-2 virus continues to evolve along with recommended treatments during the time of writing, requiring that this literature review be limited to dates up until January 2023.

At the time of writing, multiple studies explore the implications of many monoclonal antibody therapies [39,40], but few combine the longitudinal studies of immunocompromised patients into a centralized meta-analysis. Our discovery of 18 studies with varying methods of immune escape

mutation confirmation, monoclonal antibodies administered to patients, date of infection, reason for immunosuppression, and lineage responsible for each SARS-CoV-2 infection supports the fact that, at the population level, immune escape mutations emerge frequently, particularly in prolonged immunocompromised patient infections. Furthermore, by layering these findings with public health contextual data about monoclonal antibodies and viral evolution, this study provides strong support for SARS-CoV-2 evolution in response to selective pressures such as monoclonal antibody therapies.

Interestingly, the mutations at amino acid position E484, found in the Omicron variant, were repeatedly discovered in patients infected with earlier strains of the virus (n=20) that did not contain this mutation at baseline. Moreover, the FDA decided to recall multiple monoclonal antibody therapies, many of which were administered to these patients, due to reduced efficacy against the Omicron variant. Thus, contextual analysis of both individual longitudinal cases and the public health recommendations based on evolutionary trends provides a unique perspective on the relationship between monoclonal antibodies and viral evolution.

Overall, this literature review aimed to elucidate the current data about intra-host evolution of the SARS-CoV-2 virus to identify a likely source of mutation emergence. We provided additional support to the hypothesis that monoclonal antibody administration during immunocompromised SARS-CoV-2 infections could provide the selective pressures necessary to favor these mutations. Since immune escape mutations provide selective advantages for the virus, it is possible for them to appear in new variants that may possess increased infectivity, transmissibility, and treatment evasion risks that may affect the global population. Based on our findings, maintenance of genomic surveillance and observation of intra-host evolution during the monoclonal antibody treatment of immunocompromised patients is crucial to prevent the possible emergence and spread of new variants.

Chapter 3

SARS-CoV-2 Evolution and Immune Escape in Immunocompromised Patients Treated with Bamlanivimab

(The following chapter is based on the published manuscript Scherer EM, Babiker A, Adelman MW, et al. SARS-CoV-2 Evolution and Immune Escape in Immunocompromised Patients. N Engl J Med. 2022 [28].)

3.1 Introduction

Immunocompromised patients are hypothesized to be a possible reservoir for VOC emergence due to the combination of prolonged infection, weakened endogenous immune response, and exogenous antibody treatment that may create selective pressures for SARS-CoV-2[20]. Weakened endogenous immunity is thought to contribute to delayed viral clearing and persistent infection in immunocompromised patients[10]. As infection persists, viruses remain active for longer periods, and therefore have ample opportunity to evolve within the host. This evolution may be exacerbated by the presence of selective pressures such as those from monoclonal antibodies, which target the spike protein of the SARS-CoV-2 virus to prevent its binding to the ACE2 receptor in human cells. Neutralization by monoclonal antibodies may be avoided if the virus acquires advantageous mutations in regions targeted by this treatment [41]. Therefore, examining the within-host evolution of immunocompromised patients treated with exogenous antibodies is crucial in identifying mutations that may lead to new VOC.

This study analyzed the within-host evolution of the SARS-CoV-2 virus during the infection of five immunocompromised patients, including two patients treated with the monoclonal antibody therapy bamlanivimab (P2, P3). P1 received no exogenous immune treatments, and P4 and P5

received convalescent plasma. Out of the five patients, only the patients treated with monoclonal antibodies (P2, P3) developed immune escape mutations, supporting the hypothesis that mAbs could selective for immune evasion. This chapter will focus on the viral evolution observed within the patients treated with monoclonal antibodies (P2 and P3) to explore the relationship between weakened endogenous immune response, prolonged infection, and selective pressures due to monoclonal antibodies.

3.2 Methods

The overall methodology of this study involved SARS-CoV-2 genome sequencing from samples from five immunocompromised patients to identify within-host evolution. To do so, we analyzed the changes to the genomes across infection in the patients with available samples across multiple timepoints. By comparing the later timepoint genomes to the original genome in each respective patient, we identified intra-host single nucleotide variants (iSNVs), which indicate significant viral evolution.

Additionally, we aimed to investigate whether these mutations emerged due to immune escape by conducting neutralization assays. These assays involved creating analogs of the SARS-CoV-2 genomes present in each patient called pseudoviruses, and testing whether the analogs escaped neutralization by patient serum (containing bamlanivimab) *in vitro.* Thus, we combined our findings with knowledge in the current literature to determine immune escape capabilities of the variants identified in these immunocompromised patients.

3.2.1 Sample Collection

Clinical data was sourced from electronic medical review and samples including residual nasopharyngeal (NP) swabs and serum samples were collected in the Emory Medical Labs. After patients enrolled in the study and consented, whole blood samples were collected.

3.2.2 Genomic Sequencing and Analysis of SARS-CoV-2

To sequence the genomes of the virus during patient infection, we isolated the RNA from the nasopharyngeal samples using total nucleic acid extraction and treated the sample with DNase, which degrades any remaining DNA fragments that could interfere with the sequencing reads. We then generated cDNA using random hexamer primer cDNA synthesis via qPCR before cleaning the samples of any impurities and excess cDNA fragments using SPRI ampure beads. To prepare the purified sample for sequencing, cDNA libraries were tagmented with specific primers designed to produce amplified samples compatible with the Ilumina sequencing platform using Nextera XT. Indexed cDNA libraries were PCR amplified, cleaned with ampure beads, and quantified again with a KAPA qPCR to confirm accurate dilution during library pooling before sequencing on the Ilumina platform.

Once the samples were sequenced, we assembled the consensus sequence of the SARS-CoV-2 genome, which is the sequence comprised of the most frequently occurring amino acids at each position. Comparison of consensus sequences against the reference sequence Wuhan-1 was performed to identify SNPs (single nucleotide polymorphisms), which are nucleotides that differ at one position on the genome compared to the reference sequence. Identifying these differences from the reference genome allowed us to assign the lineages present at the onset of infection in each patient. Analysis of intra-host single nucleotide variants (iSNVs) was then conducted to observe the significant viral mutations that developed at different timepoints of infection. iSNV analysis differs from identification of SNPs in that iSNVs compare the viral evolution against the genome present at the onset of infection and identifies minor variants in the virus population. Such analysis provides insight into the viral evolution during individual patient infection. The process began with creating duplicate libraries for patient samples with multiple timepoints available and merging the duplicate libraries. We then assembled the merged library to the reference SARS-CoV-2 genome using NC_045512.1 (viral-ngs version 2.1.19.0-rc119). The reads containing iSNVs were manually inspected in geneious.com. To limit the likelihood of confounding variables, iSNVs were removed if they were found in the same position across all reads, which suggests a potential artefact of the library construction process. iSNVs were only reported if they were found in both duplicate libraries and if they occurred at frequencies above 2% to reduce false positives.

3.2.3 Pseudovirus Neutralization Assay

In order to determine the immune escape capabilities of the spike protein mutations in P1, P2, and P3, our laboratory partners constructed a SARS-CoV-2 pseudovirus assay using incompetent lentiviruses, including those expressing spike from Wuhan-Hu-1 and the identified spike variants. This assay was adapted from previously published methodology [42]. The neutralization experiment involves adding respective pseudoviruses to a series of diluted patient blood plasma samples and observing whether viral neutralization by plasma antibodies successfully occurs. Neutralization occurs when the antibodies in the serum experience a strong affinity for the pseudovirus, therefore binding to it and preventing it from infecting cells. Next, the mixtures of pseudovirus-sera samples were plated onto a human cell line engineered expressing ACE2 to observe binding affinity between the virus and the ACE2 region. If pseudoviruses were properly neutralized by the monoclonal antibodies provided by exogenous treatment, binding of the pseudovirus to the cell line should not occur. The experimental positive control was the anti-SARS-CoV-2 mAb CC12.1 [43], which is known to effectively neutralize SARS-CoV-2 and prevent ACE2 binding. The negative control included pooled sera from patients lacking a history of COVID-19 infection in Atlanta, GA between March and April of 2020.

3.3 Results

Our study focused on five patients, all with underlying health conditions. All patients were immunocompromised due to either immunosuppressive treatments or an underlying health condition. All patients experienced prolonged infection (>30 days), with a range of 42-302 days of infection. P2 and P3were treated with bamlanivimab (mAb) and P6 and P7 were treated with convalescent plasma. Bamlanivimab is a monoclonal antibody therapy that was approved by the FDA for clinical use on November 9th, 2020, via Emergency Use Authorization (EUA) [44]. The mAb works by targeting the SARS-CoV-2 spike protein to prevent its binding to human cell ACE2 receptors, therefore inhibiting viral proliferation [45].

All patients were treated with remdesivir, and all but P1were treated with steroids. Outcomes include recovery for all patients besides P2, who unfortunately succumbed to the infection. Phylogenetic analysis confirmed that all patients contracted a viral strain that was consistent with community spread, and each sample collected throughout infection was monophyletic, suggesting that the detected mutations were most likely due to within-host variation, as opposed to reinfection.

Patient	Age, Sex	Underlying Condition	Immunosuppressive Treatment	Monoclonal Antibody Use	Observed iSNVs	Viral Lineage	Clinical Outcome
P1	65, M	B-cell ALL, PBSCT, cGVHD	rituximab, tacrolimus	N	Q677P	B.1.2	Recovery
P2	46, F	DLBCL	R-CHOP	Bamlanivimab (d4)	Q493R, E484K	B.1.2	Death
P3	38, F	MDS, PBSCT, cGVHD	rituximab, MMF, prednisone	Bamlanivimab (d8)	E484K	B.1.2	Recovery
P4	44, M	thymoma, thymectomy, Good Syndrome	N/A	N	N/A	B.1.568	Recovery
P5	46, M	marginal zone lymphoma	rituximab	N	N/A	B.1.493	Recovery

Table 0.1. Clinical and Viral Features of Five Immunocompromised Patients with Persistent SARS-CoV-2 Infection.

Spike protein mutations in SARS-CoV-2 genome that are associated with immune escape were only found in the patients who received monoclonal antibodies. Abbreviations: ALL= acute lymphoblastic leukemia; cGVHD = chronic graft versus host disease; DLBCL = diffuse large B cell lymphoma; MDS = myelodysplastic syndrome; PBSCT = peripheral blood stem cell

transplantation; R-CHOP = rituximab, cyclophosphamide, hydroxydanorubicin, vincristine sulfate, prednisone; MMF= mycophenolate mofetil; IVIG = intravenous immunoglobulin.

For the purposes of this study, the following portion of this chapter will highlight details in the patients treated with bamlanivimab (P2, P3).

3.3.1 Clinical Courses Overview of P2 and P3

Patient 2 was a 46-year-old female who was treated with 2 cycles of R-CHOP (rituximab, cyclophosphamide, hydroxydaunomycin, oncovin, prednisone) due to her diagnosis of stage IV diffuse large B-cell lymphoma two months prior to COVID-19 infection. She received rituximab, an antibody treatment that targets the CD20 protein on cancerous blood cells. However, this therapy can also target healthy blood cells, which results in overall immunosuppression[46]. A few days after her last RCHOP cycle, she developed a cough and shortness of breath, and tested positive for COVID-19 via SARS-CoV-2 NP PCR (C_T unknown). On day 4 of illness, she received bamlanivimab, resulting in clinical improvement. She received a third RCHOP cycle 17 days later. The patient was readmitted to the hospital seven days after this treatment due to fever and intensifying shortness of breath. A SARS-CoV-2 NP PCR was positive (C_T 18) and a chest CT scan revealed poor lung condition. She received a five-day course of the SARS-CoV- antiviral remdesivir, and due to suspicion of organizing pneumonia, she was also treated with pulse steroids before tapering. She was discharged after seven days but was readmitted seven days following discharge due to dyspnea and hypoxia to 86%. Upon readmission, a SARS -CoV-2 NP PCR was positive (C_T 16) and another chest CT showed worsened lung condition. She was then treated with methylprednisolone, but her clinical condition worsened. On day 22 of hospitalization, another SARS -CoV-2 NP PCR was positive (C_T 16), and she unfortunately passed away on day 33. Overall, this 46-year-old woman undergoing chemotherapy experienced prolonged SARS-CoV-2 infection for 33 days, with persistently low Ct, indicating that she experienced inefficient endogenous and exogenous immunity to clear the virus.

Patient 3 was a 38-year-old female who was previously diagnosed with myelodysplastic syndrome. Three years prior to COVID-19 diagnosis, she underwent matched related peripheral blood stem cell transplant (PBSCT), resulting in complications including graft versus host disease

(GVHD) of the gastrointestinal tract, eyes, and skin. Approximately three months prior to COVID-19 diagnosis, she received her last dose of rituximab. Around this time, she was also treated with mycophenolate mofetil, prednisone, and monthly immunoglobulin transfusions. Following exposure to a known positive case, she tested positive for COVID-19 via SARS -CoV-2 NP PCR (C_T unknown) while asymptomatic. Eight days later, she received bamlanivimab, and two weeks after treatment she experienced shortness of breath and hypoxia, leading to hospitalization. Upon admission, a SARS -CoV-2 NP PCR was positive (C_T unknown) and a chest CT revealed poor lung condition. During her hospitalization, no COVID-19 treatment was administered and no changes to her immunosuppressive treatments were made. One-week post-discharge, she was readmitted due to progressive shortness of breath and fever. She then received remdesivir for five days and dexamethasone for 10 days as treatment for SARS-CoV-2. Another SARS -CoV-2 NP PCR (C_T 19) was positive after five days of remdesivir treatment. Upon clinical improvement, she was discharged and initially continued to improve. However, symptoms of shortness of breath and fever worsened following steroid tapering. Three weeks after discharge, she was readmitted, with chest CT revealing worsened lung condition, and a positive SARS -CoV-2 NP PCR (C_T 23). Clinical judgement concluded that she was experiencing a SARS-CoV-2 related inflammatory response, so she was discharged on a dexamethasone taper for 4 weeks which was converted into a maintenance dose of prednisone. Following steroid treatment, her pulmonary pathology improved, but she was readmitted both three (day 260 since initial COVID-19 diagnosis) and seven months later due to CMV enteritis-related symptoms. A clinical lymphocyte panel revealed T and B cell immune deficiencies and her SARS-CoV-2 NP PCR tests were both positive (C_T 34 and 26), although she did not have any respiratory symptoms at the time. Following final discharge, she continued follow up with Infectious Diseases and tested negative at twelve months since initial diagnosis via home rapid antigen test. Overall, this 38-year-old woman treated with immunosuppressive drugs experienced prolonged SARS-CoV-2 infection for 302 days, with persistently low Ct, indicating that the patient also lacked the immune response to clear the SARS-CoV-2 virus.

3.3.2 Immune Responses

The impact of exogenous antibody treatment and lack of endogenous antibody response was observed using multiple immune measurements including immunoglobulin G (IgG), neutralizing antibody titers, and lymphocyte frequencies.

IgG antibodies are among the most common humoral antibodies and arise following acute infection and persist for long periods. High serum IgG titers were present in the patients who received monoclonal antibody treatment (P2 and P3) against a stabilized spike trimer of reference Wuhan-Hu-1 at the earliest timepoints (d33 and d55, respectively). The titers persisted throughout the infection and were detected at decreased levels at the last timepoints tested (d77 and d83, respectively). Similarly, the neutralizing serum titers against the reference pseudovirus remained potent and persistent in the samples at the first timepoints (d33 and d55, respectively), while decreasing but elevated above baseline in the final timepoints (d77 and d83, respectively).

Lymphocytes are crucial immune response cells found in the blood and lymphatic system. They include B and T cells, which operate in conjunction to both develop antibodies and eliminate pathogens from the body. Lymphopenia is a deficiency in lymphocytes often observed during viral infection [47]. Of the patients with available blood samples (P2, P4, and P5), all had lower lymphocyte frequencies than healthy controls, which is consistent with clinical lymphopenia associated with COVID-19 infection [48,49]. Furthermore, very low to undetectable B cell counts in all three patients indicate that the antibody responses against the reference pseudovirus were due to exogenous treatment rather than endogenous immune response.

In contrast, all patients exhibited detectable levels of T cell concentrations, although P2 had very low SARS-CoV-2-specific T cell concentrations similar to the healthy control population, indicating insignificant concentrations. P4 and P5, however, had robust T cell concentrations, with significantly higher responses against SARS-CoV-2 spike peptides than age-matched COVID-19 controls. All samples elicited T cell responses to a positive control antigen.

Overall, the higher titers of IgG and neutralizing antibodies against the pseudovirus in the patients who received mAb, combined with their low B cell concentrations, indicate that neutralizing responses were provided by the exogenous mAb treatment.

Patient	Exogenous Treatment Administered for COVID-19 Treatment	IgG and Neutralizing Antibody Titers Against Reference Pseudovirus	Detectable B Cell Concentrations	Detectable T Cell Concentrations
P1	None	N	N/A	N/A
P2	**Bamlanivimab**	**Y**	**N**	**Y (low)**
P3	**Bamlanivimab**	**Y**	**N/A**	**N/A**
P4	Convalescent Plasma	N	N	Y (high)
P5	Convalescent Plasma	Y (low)	N	Y (high)

Table 0.2. Immune responses of immunocompromised patients infected with SARS-CoV-2. Only the patients treated with bamlanivimab possessed a detectable antibody response against the reference Wuhan-Hu-1 pseudovirus. Of the patients with available blood samples, P2 had no B cell and low T cell concentrations, while P4 and P5 both had no B cell and high T cell concentrations.

3.3.3 Intra-Host Viral Evolution

We assessed within-host evolution of the SARS-CoV-2 virus in each patient by using high-depth viral sequencing on samples collected across available timepoints. First, sequencing allowed us to conduct phylogenetic analysis and identify the viral lineage each patient was infected with; B1.2 (P1, P2, and P3), B.1.568 (P4), and B.1.493 (P5). Of the patients with longitudinal samples available, phylogenetic analysis revealed that the samples were monophyletic, meaning that the viral genomes at each timepoint were descendants from a single shared ancestor, indicating within-host evolution rather than coinfection or reinfection. Additionally, each phylogenetic analysis utilizing reference sequences from the Emory Healthcare system between January 1, 2021, and April 30, 2021, indicate infection consistent with community spread.

Additionally, between 4 and 26 consensus single nucleotide polymorphisms (SNPs), most of which were nonsynonymous and located in the spike protein, were detected following the first available timepoint. Therefore, viral evolution compared to Wuhan-Hu-1 continued to occur after the initial sample timepoint.

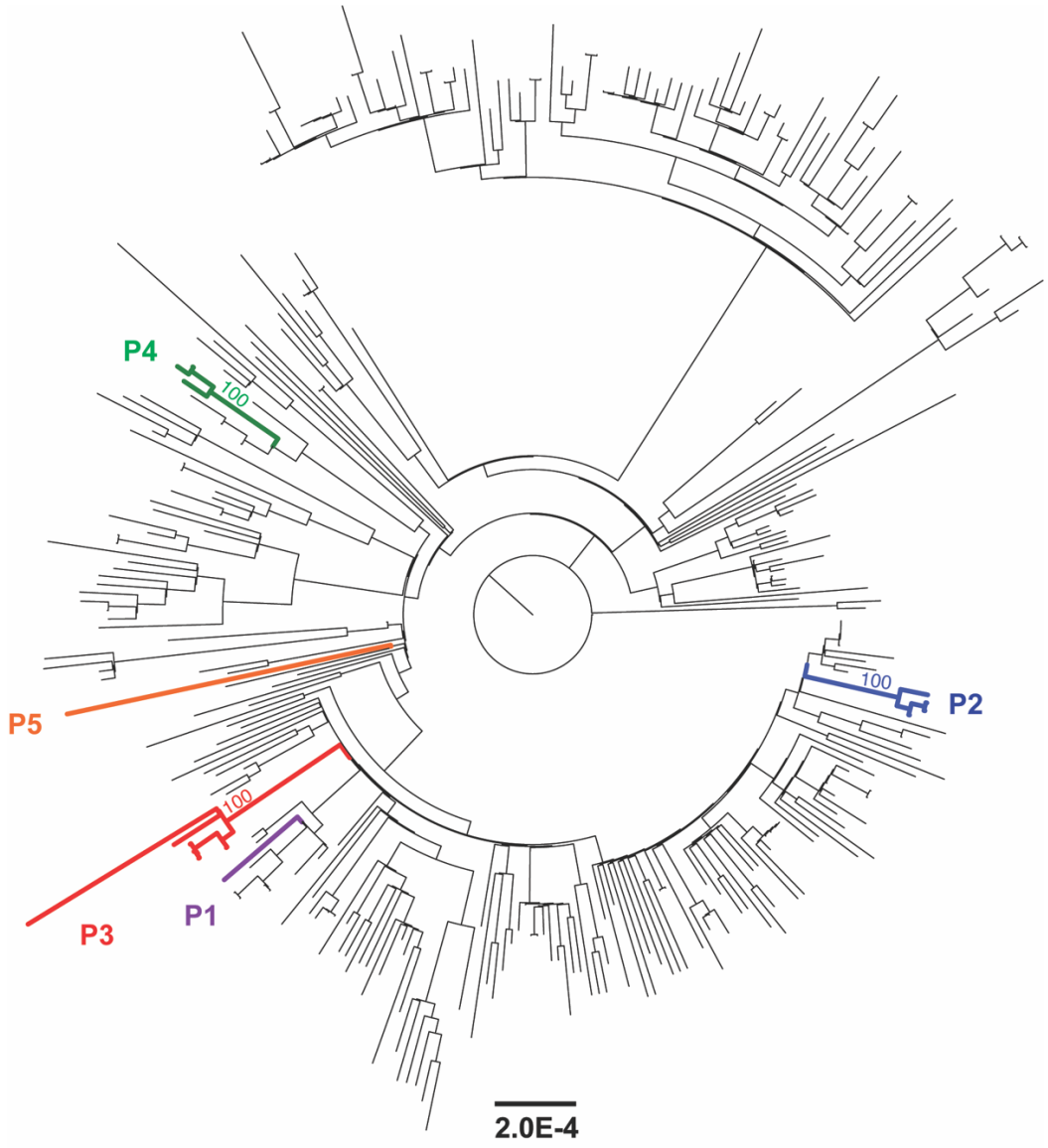


Figure 0.1. SARS-CoV-2 consensus sequences from each immunocompromised patient are consistent with community spread and indicate no evidence for reinfection.

All patient sequences underwent phylogenetic analysis along with 301 reference patient samples in the Emory Healthcare system between January 1, 2021, and April 30, 2021. Patient sequences with longitudinal samples are monophyletic, indicating viral evolution as opposed to reinfection [28].

High-depth viral sequencing identified intra-sample single nucleotide variants (iSNVs) at greater than 2% frequency in P2, P3, and P4. Of these patients with available longitudinal samples, P2

and P3 developed iSNVs and fixed mutations associated with immune escape, as well as rapid mutations within the receptor binding domain (RBD) of the SARS-CoV-2 virus (the region targeted by bamlanivimab). For example, P2 developed the RBD substitution Q493R and S494P, which both emerged, fell, then reemerged in frequency. E484K was also observed consistently after d28 but at intermediate frequencies in P2. In P3, variability at position 484 also occurred, and was observed as E484Q emergence followed by transient E484K, before becoming fixed at E484K at the final timepoint. Therefore, P2 and P3 exhibited mutation E484K, a spike mutation associated with immune escape, at either consistent and intermediate or intermittent frequencies.

Changes in the N-terminal domain (NTD) were also observed in P2 and P3, although this region is not a target of neutralizing antibodies form bamlanivimab. Many of these changes occurred in the NTD recurrent deletion region 2 (RDR2) [50], which corresponds with similar deletions also found in other immunocompromised patients[4–6,8,9].

In contrast to the rapid mutation detected in the RBD and NTD regions of the spike protein in P2 and P3, no such iSNVs were detected in P4 during any sampling timepoints, despite CP treatment. P1, who received no exogenous treatment, also did not develop any consensus-level mutations in the RBD and NTD regions at d30, although the substitution Q677P was present. Despite substantial infection length, P5 also did not develop consensus-level spike mutations in the RBM or NTD at d192. Therefore, just one iSNV in P1 and none in P5 were observed, although this could be attributed to low sequencing depth. Therefore, significant consensus-level mutations associated with immune escape only emerged in the patients treated with bamlanivimab.

3.3.4 Neutralization Assay

Interestingly, sera from P2 and P3 were able to neutralize pseudoviruses with reference spikes but were unable to neutralize pseudoviruses containing autologous spikes, or those containing the spike mutations present in the patient. Moreover, pseudoviruses containing P2 autologous spikes from both d28 and d56 were not neutralized by five sera samples between d33 and d77, and P3 samples from d55 and d83 were unable to neutralize either the pseudovirus containing d51 or d56 spikes. Since the SARS-CoV-2 virus was unable to be neutralized by the patient sera in both cases, the spike mutations conferring antibody neutralization resistance likely emerged during infection,

following bamlanivimab administration. P1 sera was unable to neutralize both the reference and autologous spikes, suggesting that no anti-SARS-CoV-2 antibodies developed during infection.

3.4 Discussion

Overall, our findings suggest that ongoing infection itself may not be enough to promote significant viral evolution. Rather, long-term infection in immunocompromised patients can become problematic when a selection pressure is introduced, such as mAb therapy, with a lack of efficient endogenous immune response. Therefore, this study highlights the importance of the relationship between endogenous immune response, exogenous treatments, and within-host evolution of SARS-CoV-2 in immunocompromised patients. Our comprehensive study analyzed both viral evolution and immune response to infection in the patients, leading to crucial findings.

The two patients treated with mAb (P2 and P3) experienced significant viral evolution. One of the notable sights of mutation was the RBM region of the spike protein, which is the region mAb targets. These results indicate that the mAb treatment can serve as an important ongoing selective pressure, causing neutralization escape. The direct correlation of the target site of mAb (RBD region) and the high variance reported in this area indicates that exogenous immune-aiding therapies such as bamlanivimab can select for mutations associated with immune escape, and therefore results in persistent infection that contributes to within-host evolution.

However, the same two patients also experienced notable evolution in the NTD region, which is unusual because it is the target of some neutralizing antibodies, but not mAb. The specific area of the NTD region that experienced evolution is called the recurrent deletion region 2 (RDR2), which is also known to vary in other immunocompromised patients outside of this study. Evolution of the RDR2 region in immunocompromised patients could indicate that, despite their impaired immune systems, they still had some presence of anti-SARS-CoV-2 antibodies. This evolution also highlights the importance of the RDR2 region in genome analysis, as this tends to be a common area of mutation in both immunocompromised and immunocompetent patients.

This study was limited by the small sample size, and therefore should be expanded upon in the future in larger-scale clinical settings to identify the selective pressures that cause immune escape in SARS-CoV-2 infection. However, our findings were significant in that they consistently

demonstrate that certain exogenous treatments, such as mAb, combined with weakened immune response, can result in significant within-host evolution that could lead to VOC. Our work, along with the work of others, stresses the need for both preventing infection in immunocompromised patients, as well as treating them accordingly to prevent the potential genesis of new VOC.

Chapter 4

PWH with SARS-CoV-2 Infection Treated with Monoclonal Antibody Cocktail

(The following chapter is based on the published manuscript Khosravi D, Soloff H, Langsjoen RM, et al. Severe Acute Respiratory Syndrome Coronavirus 2 Evolution and Escape From Combination Monoclonal Antibody Treatment in a Person With HIV. Open Forum Infect Dis. 2023 [51].)

4.1 Introduction

The preceding investigation focused on immune escape mutation emergence during the single-agent monoclonal antibody treatment of immunocompromised SARS-CoV-2 patients [24,52,53]. In contrast, this chapter describes the evolution of SARS-CoV-2 in an immunocompromised person living with HIV (PWH) treated with combination monoclonal antibody therapy. Combination monoclonal antibody cocktails are thought to result in fewer escape mutations than single-agent antibodies since cocktails target multiple domains within the spike region [52]. However, the frequency of escape from combination antibody cocktails is currently unknown, making detailed longitudinal studies crucial for monitoring viral evolution and responsiveness to treatment. Additionally, although emergence of spike mutations found in VOC has been reported in PWH with persistent SARS-CoV-2 infection, little is known about the impact of selective pressure from exogenous antibodies in the treatment of PWH. Therefore, this study also yields results that could impact the treatment of PWH during SARS-CoV-2 infection.

4.2 Methods

We generated full-length viral genome sequences from residual nasopharyngeal samples from three infection timepoints to analyze the intra-sample nucleotide variants (iSNVs) and characterize the within-host evolution of SARS-CoV-2 during the patient's infection.

4.2.1 Sample Collection

Nasopharyngeal (NP) swab samples were collected at Grady Hospital on day 0, day 31, and day 44 of infection. This study was approved by the Emory University and Grady Health System Institutional Review Boards.

4.2.2 SARS-CoV-2 Library Assembly

To sequence the genomes of the virus during patient infection, we first isolated the RNA from the nasopharyngeal samples. This process involved total nucleic acid extraction, testing the samples for SARS-CoV-2 via qRT-PCR [54], followed by addition of HL-dsDNAse to the samples to degrade any remaining DNA. The remaining RNA was then converted to cDNA using random primer annealing and first strand cDNA synthesis. Next, samples underwent both amplicon-based and metagenomic library construction to ensure reporting of high-confidence iSNVs and reduce false-positives. Errors may occur during PCR and sequencing from both methods, so utilizing both techniques decreases the likelihood of identifying overlapping false-positive iSNVs. Amplicon-based library construction followed the manufacturer instructions for the xGen SARS-CoV-2 Amplicon Panels kit (IDT).

Metagenomic library construction performed an additional cDNA synthesis and Ampure bead purification of the cDNA. Purified cDNA libraries were tagmented and amplified using Nextera XT (Illumina) and repurified using additional Ampure beads. Duplicate metagenomic libraries were constructed from RNA to accurately identify iSNVs. These libraries were quantified using KAPA qPCR, pooled, purified with Ampure beads, and sequenced on the Illumina platform.

4.2.3 iSNV Analysis

We assembled each library to the reference SARS-CoV-2 genome using NC_045512 (viralecon v2.4.4 for amplicon-based libraries and Viral-ngs v2.1.12.0 for metagenomic libraries). We then aligned the consensus sequence to reference Wuhan-Hu-1 using Geneious Prime and manually inspected for insertions, deletions, and mutations.

The reads containing iSNVs were manually inspected and verified in genious.com. To limit the likelihood of artefacts, iSNVs were removed if they were found in the same position across all reads, which suggests a potential artefact of the library construction process. iSNVs were only reported if they were found in both amplicon-based and metagenomic libraries and if they occurred at frequencies above 2% to reduce false positives.

4.3 Results

4.3.1 Clinical Synopsis

The patient was a 44-year-old Hispanic male diagnosed with SARS-CoV-2 10 months before presentation at the hospital, where he was admitted due to chills, night sweats, significant unintentional weight loss, intermittent fevers, and fatigue throughout the preceding 6 months. Additionally, in the weeks leading up to hospital admission, the patient was experiencing worsening productive cough and difficulty breathing. The patient had low blood pressure, accelerated heart rate, and accelerated respiration with relatively normal concentrations of blood oxygen (>90%).

The patient had a recent HIV diagnosis, and a test within the hospital confirmed a very high HIV viral load and low CD4 count, indicating impaired immunity. On day 0, the patient's nasopharyngeal swab was positive for SARS-CoV-2 (C_T 25.7). On day 2, the patient was treated with casirivimab/imdevimab (REGEN-COV™) monoclonal antibodies, which was followed by improved respiratory stability, although fever remained. Around this time, the patient also tested positive for disseminated Mycobacterium avium complex (DMAC), which is a group of common environmental bacteria that primarily infect immunocompromised individuals[55]. He began treatment for HIV infection with combination antiretroviral therapy (ART) as well as combination antibiotics for DMAC infection and an additional antibiotic for pneumonia prophylaxis before being discharged from the hospital.

The patient returned to the hospital one month later, presenting with low blood pressure, accelerated heart rate, and fever. Due to concern for complications related to his DMAC infection, he was admitted. A SARS-CoV-2 nasopharyngeal swab was positive with C_T of 15.2 on day 31 since initial presentation. He was then discharged but returned two weeks later due to persistent fevers and presented again with accelerated heart rate and low blood pressure, along with a positive SARS-CoV-2 nasopharyngeal swab (C_T 14.9), although his respiratory symptoms significantly improved. During this admission, the patient continued treatment for HIV and DMAC. He was then discharged after remaining afebrile for multiple days.

Unfortunately, the patient lost contact with the clinical team. However, a family member reported that following discharge, he stopped his medication regimen due to difficulties swallowing, stopped eating and drinking, and passed away roughly two months later.

4.3.2 Consensus Mutations and iSNVs

We successfully sequenced high depth complete genomes at the three timepoints of this patient's infection (d0, d31, and d44). The viral lineage was Delta (AY.119), with significant evolution in the genome occurring throughout infection, especially within the spike region. Across the three timepoints, we observed 89 iSNVs via metagenomic sequencing and 134 iSNVs by amplicon sequencing. 78 iSNVs were detected in both sequencing methods and were used in the final analysis. Of these iSNVs, 9 were determined to be consensus-level, and 8 of these were nonsynonymous, meaning that they altered the amino acid coded for at that position, therefore potentially altering the function of the protein. Additionally, 12 other mutations were observed to change in frequency across the timepoints by at least 10%.

Nucleotide Change	Amino Acid Change	Gene	Allele Frequency at Day 0	Allele Frequency at Day 31	Allele Frequency at Day 44
T17442C	Synonymous	Helicase	0%	9%	70%
G18181T	D44Y	Exoribonuclease	0%	5%	71%
C21855T	S98F	S	71%	7%	61%
G22289T	A243S	S	0%	4%	61%
T22896C	**V445A**	**S**	**0%**	**54%**	**89%**
A22920T	**Y453F**	**S**	**7%**	**96%**	**96%**
C25460T	A23V	orf3a	5%	88%	98%
C29466T	A398V	N	90%	0%	0%

Table 0.1. Consensus-level SARS-CoV-2 mutations arising during infection in a PLWH.

Mutations are reported relative to reference sequence Wuhan-Hu-1 and exclude those only detected via one of the two library construction processes. Fixed mutations present at all timepoints were excluded. 6 of the 9 mutations occurred in the spike gene, and 8 of the 9 mutations were nonsynonymous. The bolded mutations (V445A, Y453F) are associated with immune escape from either casirivimab or imdevimab, and both increased significantly in frequency following casirivimab/imdevimab administration between d0 and d31[51].

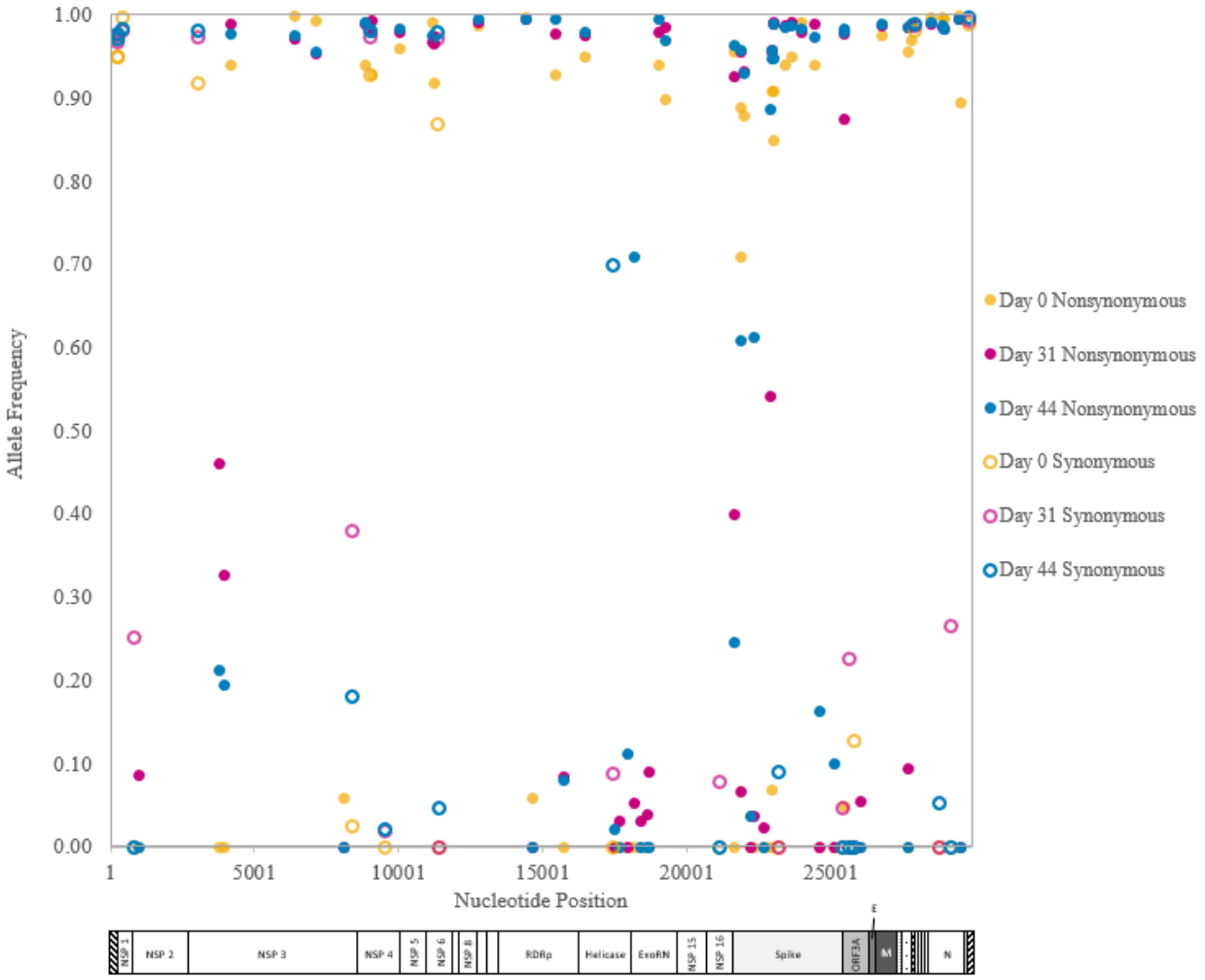


Figure 0.1. iSNV frequency relative to nucleotide position and sampling time point. iSNV allele frequency (y-axis) is plotted against nucleotide position (x-axis). Points are colored by time point, and open or closed circles represent synonymous or nonsynonymous mutations, respectively. The SARS-CoV-2 genome map below the x-axis highlights genes of interest[51].

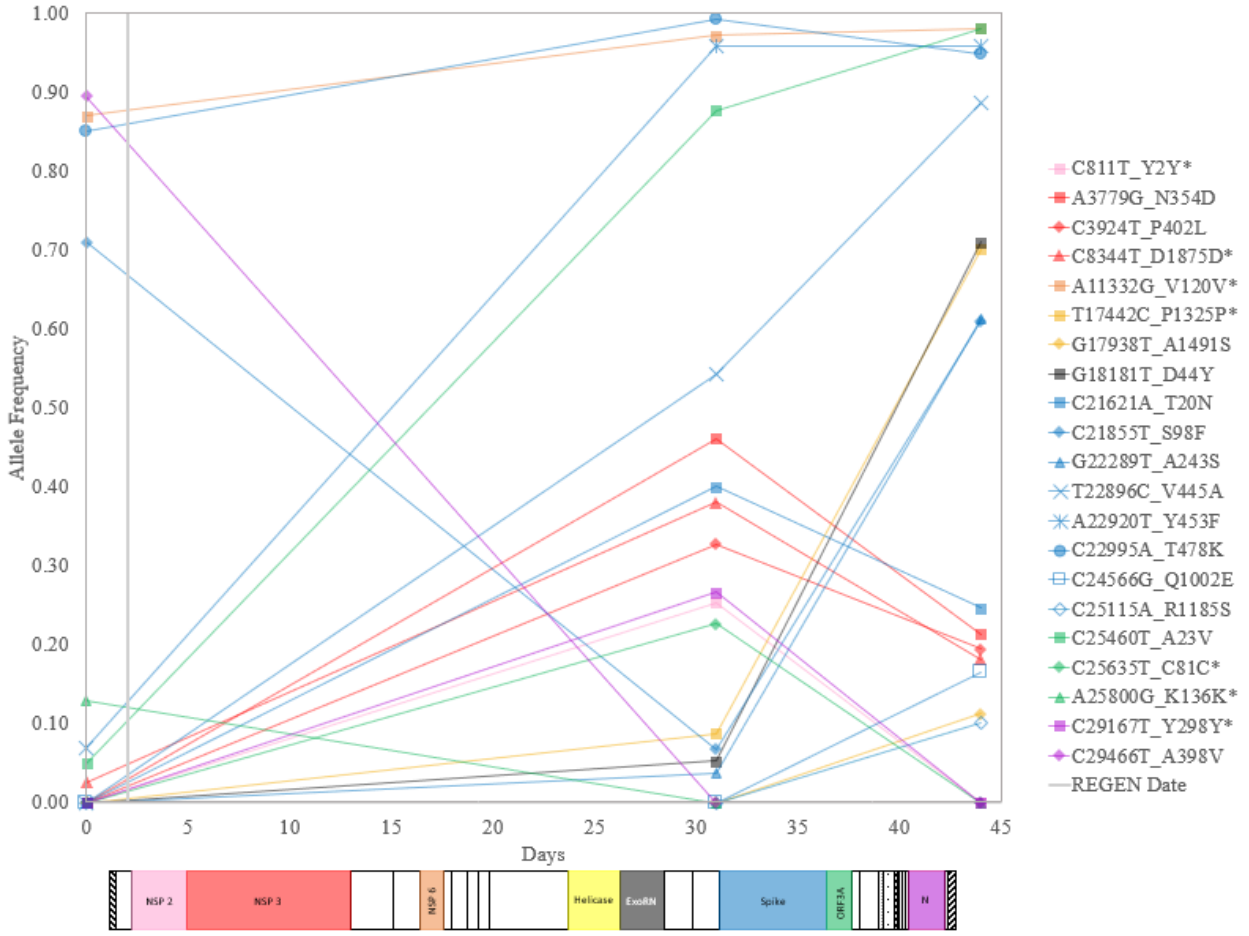


Figure 0.2. iSNV allele frequency of samples experiencing more than 10% frequency change between any two timepoints.

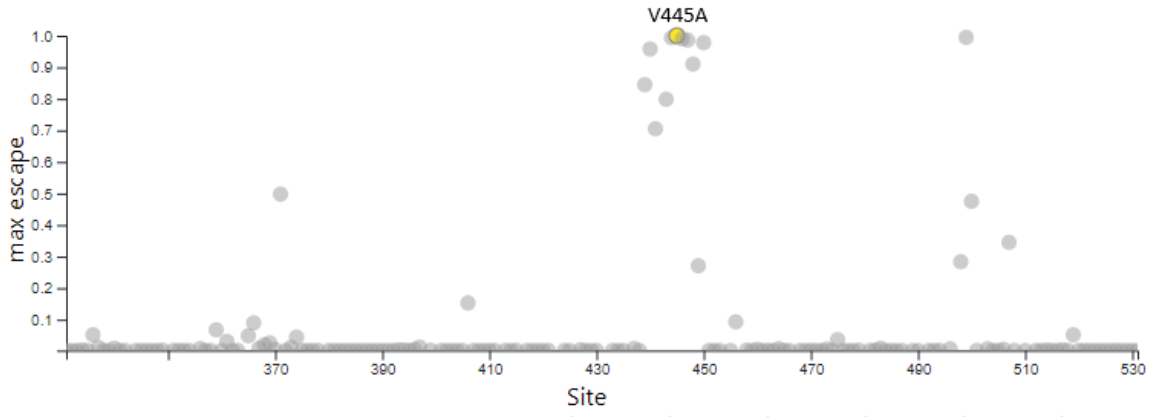
Includes consensus-level iSNVs (n=9) and non-consensus iSNVs (n=12) that differ in allele frequency by more than 10% between any two time points (total=21). iSNV allele frequency (y-axis) is plotted against time (x-axis). Points and lines are color-coded by gene, and asterisks mark synonymous changes. Day 1 (gray vertical line) indicates treatment with casirivimab/imdevimab.

4.3.3 Deep Mutational Scanning Analysis

Interestingly, two amino acid mutations in the spike protein thought to confer escape from either casirivimab or imdevimab emerged during infection. These mutations arose between d0 and d31 of infection, and the patient received casirivimab/imdevimab treatment between these two timepoints. To confirm the immune escape capacity of these two mutations, we consulted an

experimental study that utilized scanning mutagenesis to map escape mutations to casirivimab, imdevimab, and the casirivimab/imdevimab cocktail. Scanning mutagenesis reveals regions along the SARS-CoV-2 genome in which mutations are likely to confer escape from specific antibodies. According to these findings, the V445A spike mutation confers strong escape from imdevimab (REGN10987) and the Y453F spike mutation confers moderate escape from casirivimab (REGN10933). Mutation G476S also emerged and is associated with immune escape from casirivimab, although the relevance to this patient is unclear since the mutation was fixed at all three timepoints.

A



B

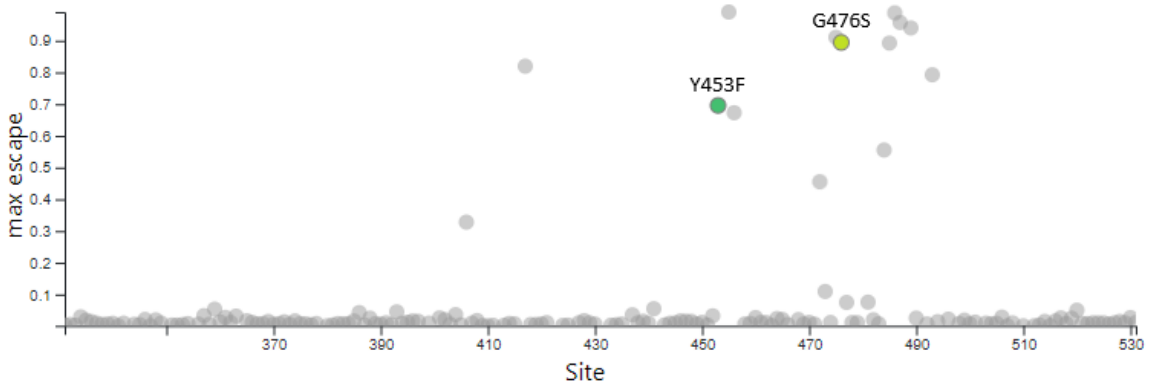


Figure 0.3. Maximum potential escape of mutations occurring at each amino acid position in the Spike RBD, against monoclonal antibodies imderivimab (REGN10987) (A) and casirivimab (REGN10933) (B)[51].

Figures were generated using dms-view[56]. Mutations observed in this study are highlighted and labeled in each plot.

4.4 Discussion

We identified significant viral evolution during the monoclonal antibody cocktail treatment of a PWH infected with SARS-CoV-2. This evolution included two spike mutations that are thought to confer immune escape from one of the two antibodies included in the casirivimab/imdevimab cocktail. At the time of writing, this was the first known *in vivo* report of immune escape mutation emergence from combination monoclonal antibody treatment. The emergence of these two significant mutations suggests possible selection for escape by casirivimab/imdevimab, thus contributing to the importance of monitoring SARS-CoV-2 evolution in immunocompromised individuals treated with monoclonal antibody cocktails.

Age, Sex	**Underlying Condition**	**Immuno-suppressive Treatment**	**Monoclonal Antibody Use**	**Consensus-Level iSNVs Conferring Immune Escape**	**Viral Lineage**	**Clinical Outcome**
44, M	HIV, DMAC	N/A	casirivimab/imdevimab	V445A, Y453F	AY.119	Death

Table 0.2. Clinical and Evolutionary Data of a PWH Treated with Casirivimab/Imdevimab for SARS-CoV-2.

Chapter 5

Conclusions

This thesis aims to explore the potential for SARS-CoV-2 to select for immune escape mutations during the monoclonal antibody treatment of immunocompromised patients. We conducted two case studies and an extensive literature review to observe relevant evolutionary trends. Our studies successfully identified significant viral evolution during the monoclonal antibody treatment of immunocompromised SARS-CoV-2 patients, including 77 patients from the literature and 3 in our primary case studies. Immune escape mutation emergence was observed in the viral samples of the 80 patients described in this study. The repeated emergence of mutations associated with immune escape against the monoclonal antibodies received by each patient strongly suggests that SARS-CoV-2 selects for mutations that escape monoclonal antibody binding.

Additionally, the literature analysis at the population level provided meaningful insight into SARS-CoV-2 variants of concern and monoclonal antibodies. Furthermore, the FDA and CDC timeline analysis identified repeated FDA revocation or restriction of specific monoclonal antibodies due to inefficacy against circulating variants. These trends suggest that SARS-CoV-2 may select for resistance mutations at the population level.

Interestingly, the E484K/A/Q emerged during the infections of 21 patients identified in the literature review. Mutations at this amino acid position are associated with immune escape against bamlanivimab: the monoclonal antibody administered to most of these patients. Based on the timeline analysis and CDC data, E484K rose to consensus level in Gamma and Beta variants during the FDA authorization of bamalnivimab, followed by later restrictions due to the mAb's inefficacy against the variants. Therefore, the combination of individual and population-level trends provides multifaceted evidence about SARS-CoV-2 evolution and monoclonal antibody treatments.

Based on the findings within this study, continued monitoring of SARS-CoV-2 evolution during monoclonal antibody treatment of immunocompromised patients is imperative. Consistent identification of mutations associated with immune escape strongly suggests that the interplay between weak endogenous immunity, selective pressures from monoclonal antibodies, and persistent infection may result in selection for SARS-CoV-2 immune escape mutations during immunocompromised patient infections. Monitoring these treatments in immunocompromised patients may serve as a key factor in preventing the emergence of new global variants of concern.

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