SARS-CoV-2 VACCINATION RESPONSES IN VULNERABLE POPULATIONS

SARS-CoV-2 VACCINATION RESPONSES IN VULNERABLE POPULATIONS

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Lay Abstract

Rheumatoid arthritis (RA) and scleroderma (SSc) are autoimmune disorders that are commonly treated with drugs that suppress the immune response. While this helps reduce disease symptoms, it can make vaccinations, which stimulate the immune system, less effective. The original COVID-19 vaccine trials did not include people on immunosuppressive drugs, leaving patients and their doctors to wonder if they will have weaker immune responses to COVID-19 vaccination. The goal of this study was to determine if participants with RA or SSc, on immunosuppressive drugs, produce similar immune responses following vaccination to healthy adults without these conditions. We determined that while participants with SSc did not have impaired responses, participants with RA did have impaired responses to COVID-19 vaccination. We discovered which immunosuppressive drugs contributed to these weaker immune responses. Receiving multiple COVID-19 vaccinations, even in vulnerable people like those with RA or older adults, did not overwhelm or exhaust immune cells.

Abstract

Immunosuppressive medications are commonly prescribed to minimize the aberrant inflammatory responses in the autoimmune disorders rheumatoid arthritis (RA) and systemic sclerosis (SSc). It has been previously shown that immunosuppressive drugs can negatively impact responses to vaccination, though the impact differs by vaccine type. With the advent of the SARS-CoV-2 pandemic, and the development of new mRNA vaccines, it was unclear how well participants with RA and SSc, on immunosuppressive drugs, would response to these vaccinations. We hypothesized that people with RA or SSc, on immunomodulatory drugs, would have weaker humoral and cellular responses to SARS-CoV-2 vaccination, even following subsequent vaccinations, compared with healthy controls. To explore this, we collected blood and serum from participants with RA, SSc, and controls at various timepoints after multiple SARS-CoV-2 vaccinations. In Chapter 2, we determined that participants with RA on immunosuppressive drugs had weaker humoral and spike-specific $CD4^+$ T cell responses, but not weaker spike-specific $CD8^+$ T cell responses, than controls around the second, third, and fourth SARS-CoV-2 vaccinations. While costimulation inhibitors negatively impacted the humoral responses, the inclusion of JAK inhibitors in the immunosuppressive drug regimen was associated with weaker spike-specific $CD4^+$ T cell responses, demonstrating that different immunosuppressive drugs impact unique arms of the memory response. In Chapter 3, we discovered that participants with SSc did not have lower humoral or spike-specific T cell responses to SARS-CoV-2 vaccination than controls. In

Chapter 5, we determined that repeated SARS-CoV-2 vaccination did not cause T cell exhaustion in multiple vulnerable populations, including immunosuppressed participants with RA and older adults in long-term care, or healthy younger adults. Overall, our research has provided insight into SARS-CoV-2 vaccination responses in participants with RA and SSc, allowing patients and their doctors to make informed decisions regarding vaccination timelines and medication-specific concerns.

Acknowledgements

I would like to thank my supervisor Dr. Dawn Bowdish for her guidance and support during my PhD. Dawn is an excellent mentor and helped me grow as both a scientist in the lab, and as a scientific communicator to different audiences. Her love of science, and genuine desire to improve the lives of people around her, inspires me and has brought joy and meaning to my studies. Whenever she gives a talk, it reminds me of the curiosity and passion that led me to pursue this path. Dawn has been incredibly supportive of my endeavors to learn new techniques, and she has provided me with opportunities to share my research and learn about the latest advances in the field at conferences. I cannot thank you enough for the encouragement and learning opportunities that you provided.

I would also like to thank Dr. Jessica Breznik. I have probably asked her about 1,000 questions since the start of my PhD, and she has patiently answered my questions and taught me many new techniques in the lab. Jessica's wealth of knowledge, and commitment to teaching new lab members, has helped many graduate students become better researchers, and better scientific writers. Jessica has a nearly unparalleled attention to detail, and I greatly appreciate the time she has taken to proofread my papers, scholarship applications, and abstracts over the years. Her support has helped me become the scientist I am today.

I am also thankful to my committee members: Dr. Maggie Larché, Dr. Matthew Miller, Dr. Jonathan Bramson, and Dr. Chris Verschoor. They have supported my research endeavors, challenged me to think critically about my work, and helped me to improve my knowledge of statistics and immunology. It was a privilege to work with and learn from them. I am also grateful for the support of the many wonderful coordinators, technicians, students, and team members at the McMaster Immunology Research Center and our collaborating departments and institutes. Science is a team sport, and without this dedicated and passionate team the studies in this thesis would not have been possible.

I have to give a huge shoutout to my labmates and friends. We've laughed, cried, and learned together. A PhD isn't just about the destination, it's about the journey, and y'all have made the journey that much better. Whether it was troubleshooting in the lab, or a trivia night to decompress, you all helped me push through, expand my knowledge, and kept a smile on my face. I'm lucky to have met you all, and I know you're going to accomplish great things.

My final thanks go out to my family. You've never doubted my ability to accomplish whatever I set my mind to, even if I myself have had doubts. From the grade school science fairs and speech competitions all the way to my PhD, you've supported me and cheered me on. You fostered my curiosity about the world, and encouraged me to follow the path that makes me happy. I wouldn't be where I am today without your support.

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List of Abbreviations and Symbols

ACE2	Human angiotensin converting enzyme 2
ACR	American College of Rheumatology
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
BAL	Bronchoalveolar lavage fluid
CAR	Coxsackie–adenovirus receptor
CD	Cluster of differentiation
cGAS/STING	Cyclic GMP–AMP synthase–stimulator of interferon genes
COVID-19	Coronavirus disease 2019
DC	Dendritic cell
DMARD	Disease modifying anti-rheumatic drugs
DMSO	Dimethyl sulfoxide
EMRA	Effector memory re-expressing CD45RA
EULAR	European League Against Rheumatism
GR	Glucocorticoid receptor
ICOS	Inducible T-cell Co-stimulator

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILD	Interstitial lung disease
JAK	Janus kinase
LAG-3	Lymphocyte activation gene 3
LTC	Long-term care
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK	Natural killer (cells)
PD-1	Programmed cell death protein 1
RA	Rheumatoid arthritis
RBD	Receptor binding domain (of SARS-CoV-2 spike protein)
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SSc	Systemic sclerosis
T_{FH}	T follicular helper (cell)
Th	T helper (cell)
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domain
TIM-3	T-cell immunoglobulin and mucin-domain containing molecule-3

TLR	Toll-like receptors
TNF	Tumor necrosis factor
Treg	T regulatory cell
VOC	Variant of concern

List of Appendices

Declaration of Academic Achievement

This thesis provides an accurate summary of my research and novel findings during my time as a doctoral candidate. I wrote the thesis, and the manuscripts, with edits and input from my supervisor, Dr. Dawn Bowdish, and co-authors of the published works. The studies involved collaborations with other researchers, as such, I have detailed my contributions along with those of my collaborators in the space below.

Chapter 2: I am the primary author of this manuscript. I processed the blood samples with the help of Braeden Cowbrough, conducted the majority of the activation-induced marker (AIM) assays, performed the flow cytometry gating, analyzed the data, generated the figures, and wrote the manuscript. Dr. Jessica A. Breznik conducted a portion of the activation-induced marker assays. I analyzed the data from these experiments. Dr. Jessica A. Breznik also contributed to data interpretation and manuscript editing. Jann C. Ang conducted the neutralization assays, and Hina Bhakta and Angela Huynh designed and performed the ELISAs, with help from Rumi Clare. Barbara Baker was the clinical coordinator for the study and collected participant samples and metadata. Lauren Heessels, Sumiya Lodhi, and Elizabeth Yan collected participant metadata for the study. Joycelyne Ewusie, Jake Szamosi, and Myanca Rodrigues, helped to generate the code for the linear mixed models. Dr. Ishac Nazy, Dr. Jonathan Bramson, and Dr. Matthew Miller contributed to study designs and interpretation.

Dr. Sasha Bernatsky, Dr. Maggie J. Larché, and Dr. Dawn Bowdish obtained funding for the study, contributed to the study design, and manuscript editing.

Chapter 3: I am the primary author of the manuscript in 3.1 and the additional data in 3.2. I processed the collected blood samples, conducted the majority of the AIM assays, performed all of the ELLA automated ELISAs, autoantibody profiling, and the flow cytometry gating, analyzed the data, generated the figures, and wrote the manuscript. Kayla Zhang provided expertise on autoantibody profiling. Braeden Cowbrough also collected and processed blood samples. Dr. Jessica A. Breznik conducted a portion of the activation-induced marker assays. I analyzed the data from these experiments. Dr. Jessica A. Breznik also contributed to data interpretation and manuscript editing. Angela Huynh, Hina Bhakta, and Rumi Clare designed and conducted the ELISAs for this study. Barbara Baker was the clinical coordinator for the study and collected participant samples and metadata. Lauren Heessels, Sumiya Lodhi, and Elizabeth Yan collected participant metadata for the study. Dr. Ishac Nazy and Dr. Jonathan Bramson contributed to assay designs and interpretation. Dr. Maggie J. Larché, and Dr. Dawn Bowdish obtained funding for the study, contributed to the study design, and manuscript editing.

Chapter 4: I am the primary author of this manuscript. I designed the exhaustion marker panels for the AIM assays and surface immunophenotyping flow cytometry stains, conducted the immunophenotyping stains and some of the AIM and intracellular cytokine stain (ICS) assays, performed the flow cytometry gating

for all assays (AIM, ICS, immunophenotyping), analyzed the data and generated the code for the multivariable linear mixed models, generated the figures, and wrote the manuscript. Dr. Jessica A Breznik, Ying Wu, Dr. Allison Kennedy, and Li-Min Liu conducted the majority of the AIM and ICS experiments and contributed to data interpretation. Dr. Jessica A. Breznik also contributed to manuscript editing. Braeden Cowbrough collected many of the participant samples, and we both processed the blood samples. Barbara Baker was the clinical coordinator for the RA study and collected participant samples and metadata. Catherine M. Andary also collected and organized participant metadata for the RA study. Maha Mushtaha and Nora Adballa were the clinical coordinators for the HA cohort study, and collected participant samples and metadata. Gail Gauvreau and Paul Y. Kim were also involved in project administration for the HA study. Jamie D. McNicol and Hesam Hafezalseheh contributed to data interpretation and recommended statistical approaches. Judah A. Denburg, Andrew P. Costa, Darryl P. Leong, Ishac Nazy, and MyLinh Duong were all involved in designing the studies and obtaining funding. Jonathan Bramson, Maggie J. Larché, Chris P. Verschoor, and Dawn Bowdish also designed the studies and obtained funding, as well as contributing to data interpretation and experimental design. Chris P. Verschoor, in consultation with Jamie D. McNicol, put together the COMPASS code pipeline for Figure 2, and advised on statistical approaches. Chris P. Verschoor and Dawn Bowdish contributed to manuscript editing and aided in revisions.

Chapter 5: I conceptualized and created this protocol to assess spike-specific B cells in peripheral blood using flow cytometry. Allyssa Phelps and Dr. Joshua Koenig provided advice and reagents. Dr. Jessica Breznik and Dr. Dawn Bowdish contributed to data interpretation involved in optimizing the protocol.

Appendix 2: I conducted all AIM assays, designed and conducted the T follicular helper cell stain, and designed and conducted the majority of the B cell spike stains. I performed all the flow cytometry gating, data analysis, and generated the figures. Lan Chen also conducted a portion of the B cell spike stains, to both aid the project and learn the protocol. Braeden Cowbrough collected the participant samples, and we both processed the blood. Hina Bhakta, Rumi Clare, and Angela Huynh designed and conducted the ELISAs. Jann C. Ang conducted the neutralization assays. Dr. Jessica A. Breznik and Dr. Dawn Bowdish contributed to data interpretation.

Chapter 1. Introduction

1.1 Rheumatic Diseases

1.1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases[1]. According to the Public Health Agency of Canada, approximately 1.2% of Canadians are living with the condition, and RA is more common in females than in males[2]. In RA patients, the immune system aberrantly targets citrullinated (or to a lesser extent carbamylated) self-proteins, culminating in inflammatory immune invasion of sites such as the joints, causing severe pain and pathological tissue remodeling in the affected sites[3]. The exact causes of this loss of self-tolerance are not entirely elucidated, however there is a strong association between certain major histocompatibility complex class II (MHC II) alleles and risk of developing RA[4,5]. Genetic risk factors are not entirely responsible for the condition however, and environmental factors, such as smoking, further increase the risk of developing RA[6,7]. The initial breakdown in tolerance typically leads to the production of autoantibodies, such as rheumatoid factor, and antibodies targeting citrullinated and carbamylated proteins[5]. These autoantibodies can develop many years before the onset of clinical RA symptoms[8,9].

Progression from this asymptomatic state to active disease occurs in some individuals, and is tied to the influx of various immune cell populations into the synovial tissue, though the triggers for progression are not well understood[5,7]. The invading immune cells include T cells, which produce inflammatory cytokines

such as TNF, and eventually die by pyroptosis, contributing to an inflammatory feedback loop and local tissue damage[5]. Aberrant inflammatory cytokine and matrix metalloproteinase production by macrophages in the synovial tissue further exacerbate tissue pathology [5,7,10]. Synoviocytes from people with RA also display aggressive, tissue-invasive properties that favor the destruction of cartilage and bone in the joints[11,12].

1.1.2 Immunomodulatory drugs used to treat RA

The involvement of antibodies, B cells, and T cells in the pathology of RA has led to the use of immunomodulatory drugs such as disease modifying anti-rheumatic drugs (DMARDs) and biologics that dampen these inflammatory cells and pathways[13,14]. Conventional DMARDs include drugs such as methotrexate, which leads to the accumulation of the immunosuppressive molecule adenosine[14]. Glucocorticoids such as prednisone, which inhibit the activity of NF-kB, a pro inflammatory transcription factor used by many immune cell types, can also be used to treat RA[15]. Compared to conventional DMARDs, biologics tend to more specifically target certain cell types, cytokines, or pathways, such as abatacept which blocks T cell activation, or adalimumab which neutralizes TNF-α[16,17]. Recently, janus kinase (JAK) inhibitors have also shown promise in the treatment of RA[18]. A breakdown of commonly used immunomodulatory drugs in RA, and their mechanisms of action, can be found in Table 1.

Table 1. Immunomodulatory drugs commonly used to treat RA

Immunomodulatory Drug	Mechanism

Glucocorticoids:	
Prednisone Methylprednisolone	• Bind to glucocorticoid receptor (GR), causing it to translocate to the nucleus of the cell[19,20] • Inhibits activity of the transcription factors NF-κB and AP-1 through directly binding the transcription factors[20–23] • GR also binds AP-1 and NF-κB DNA response elements to repress their activity[21,23] • GR treatment can reduce inflammatory cytokine production[24,25]
DMARDs:	
Methotrexate	• Leads to increased adenosine production, which broadly suppresses inflammatory responses in multiple immune cell types[26] • Adenosine engagement with the A_{2a} receptor on T cells leads to inhibition of effector functions downstream of T cell receptor engagement[26,27] • Interferes with NF-κB signaling[26] • Sensitizes T cells to apoptosis[26] • Significantly reduces serum IFN-γ, IL-8, and IL-6 levels in people with RA[28,29]
Sulfasalazine	• Inhibits activity of NF-κB[30] • Interferes with TNF-α production by macrophages, and antibody production by B cells[31,32] • Also leads to adenosine accumulation[33]
Leflunomide	• Interferes with de novo pyrimidine biosynthesis, which is required by activated

	lymphocytes during proliferation[29]
Hydroxychloroquine	• Mechanism is not entirely elucidated[34] • Alters lysosomal pH, which potentially interferes with antigen-presentation and thus endosomal TLR signaling, leading to reduced cytokine production[34]
Mycophenolate mofetil	• Inhibits purine biosynthesis by inosine-5'-monophosphate dehydrogenase, preferential for the isoform found in B and T cells[35] • Similar to leflunomide, this interferes with T and B cell proliferation[35]
TNF Inhibitors:	
Adalimumab	• Anti-TNF-α monoclonal antibody[36] • Binds to soluble and transmembrane TNF-α to interfere with ability of this cytokine to engage its receptor[36]
Etanercept	• TNF-receptor fusion protein. Binds to TNF-α, therefore interfering with its ability to bind cell surface TNF-receptors[37]
Infliximab	• Anti-TNF-α monoclonal antibody, binds to soluble and transmembrane TNF-α[38]
Golimumab	• Anti-TNF-α monoclonal antibody, binds to soluble and transmembrane TNF-α[39]
JAK inhibitors:	
Upadacitinib	• Specifically inhibits JAK1[18]

Tofacitinib	• Mainly targets JAK1 and JAK3, some activity against JAK2[18]
Baricitinib	• Mainly targets JAK1 and JAK2[18]
Other biologics:	
Rituximab	• Anti-CD20 monoclonal antibody[40] • CD20 is expressed on B cells, but not typically on plasma cells or other cell types, resulting in preferential depletion of B cells[40]
Tocilizumab	• Anti-IL-6 receptor monoclonal antibody[41] • Can bind to soluble and membrane bound IL-6R, thus interfering with IL-6 signalling[41]
Abatacept	• A cytotoxic T-lymphocyte associated protein 4 (CTLA-4) fusion protein that binds to CD80 and CD86[42] • Acts as a costimulation inhibitor by interfering with the ability of CD28 to bind CD80/86, thus impairing T cell activation[42]

1.1.3 Infection and vaccination responses in people with RA

Individuals with RA may be at an increased risk of developing various infections, and this risk is particularly pronounced in those with higher disease activity (i.e., Higher Disease Activity Score 28 (DAS28) and Clinical Disease Activity Index (CDAI), composite scores including measures such as tender and swollen joint counts, and erythrocyte sedimentation rate), and those on certain immunosuppressive medications, such as methotrexate and glucocorticoids[43–47].

Infection risk varies by immunomodulatory drug, with DMARDs such as methotrexate conferring minor increases in infection risks[43,47]. Conversely, TNF, JAK, and IL-6 inhibitors have been reported to potentially confer higher risks of infection in participants with RA[43]. The risk of infection associated with glucocorticoids increases with the dose used[43,44].

In addition to impacting infection risks, immunomodulatory drugs may also impact vaccination responses. Conflicting evidence exists in the literature regarding whether or not methotrexate and glucocorticoids negatively impact humoral responses to pneumococcal and influenza vaccination[48–51]. The B cell depleting agent rituximab is consistently associated with poor responses to influenza vaccination[52,53]. While TNF-inhibitors are generally not reported to negatively impact pneumococcal or influenza vaccination responses, some studies have reported deficits in the responses to certain vaccine antigens, and differences in responses between classes of TNF-inhibitors[49–51,54,55]. Interestingly, spondyloarthritis patients treated with TNF-inhibitors displayed lower seroconversion rates than controls following influenza vaccination, while RA patients treated with TNF-inhibitors did not have lower seroconversion rates[54]. The lower seroconversion rate in participants with spondyloarthritis was associated with monoclonal antibody TNF-inhibitors, but not etanercept, while participants with RA on either TNF-inhibitor class did not display deficits in their responses[54]. Given that the participants with RA and spondyloarthritis were receiving similar, standard doses of the TNF-inhibitors, this data suggests that autoimmune conditions may interact with immunomodulatory drugs to

differentially impact vaccination responses. The impact of a given immunomodulatory drug class on vaccination responses in one autoimmune condition may therefore not be applicable to other autoimmune conditions. This necessitates the evaluation of vaccination responses in a variety of autoimmune disorders, without the assumption of cross-applicability of findings between conditions. Vaccine type is also an important consideration when evaluating the impact of immunomodulatory drugs on vaccination responses, as evidenced by reports that the JAK inhibitor tofacitinib may impair responses to pneumococcal, but not influenza vaccination[56].

1.1.4 Systemic Sclerosis

Systemic sclerosis (SSc) is a rare autoimmune condition characterized by excessive fibrosis of multiple organ systems, and effects approximately 19/100,000 Canadians[57–59]. Similar to the pattern observed in RA, SSc is more common in females than in males[58,60,61]. Patients with SSc can be described as having limited or diffuse disease[57]. In limited cutaneous SSc, skin fibrosis is typically localized to the distal parts of the limbs, while diffuse cutaneous SSc can include skin fibrosis on the chest and abdomen[57,62,63]. Both limited and diffuse SSc can include fibrosis of internal organs, which increases morbidity and mortality[57,62,63]. As a result of the fibrosis, patients with SSc can develop pulmonary arterial hypertension, gastrointestinal dysfunction, digital ulcers, and interstitial lung disease (ILD)[59,64–66]. While survival has improved in recent years for patients with SSc, most of the deaths in this population are still due to

disease-related complications[67]. Of note, 30-90% of patients are estimated to develop ILD, which accounts for 35% of SSc-related deaths[67,68].

Although the exact causes of SSc are unclear, perturbations are seen in multiple leukocyte populations and in the serum cytokine profile, suggesting broad immune dysregulation[59,69,70]. Multiple studies have reported altered levels of T regulatory cells (Tregs) in patients with SSc, coupled with deficits in their immunosuppressive capabilities[71]. Serum IL-6 is elevated in patients with SSc, and B cell overproduction of this cytokine is thought to contribute to SSc disease severity[64,72]. IL-6 can increase collagen production by fibroblasts, exacerbating fibrosis[73–75]. Patients with SSc who have higher levels of serum IL-6 were therefore more likely to have pulmonary hypertension, ILD, and higher skin scores from the Medsger Damage Index[76]. IL-6 also promotes differentiation of $CD4^+$ T cells towards a Th17 phenotype and away from a Treg phenotype[77]. Th2 responses are similarly dysregulated in SSc, with elevated levels of IL-13 inducing increased deposition of extracellular matrix proteins, and further linked to pulmonary hypertension[64,76].

1.1.5 Immunomodulatory drugs used to treat SSc

Given the unclear etiology and complicated mechanisms behind SSc, current treatment options are limited. Patients with SSc can be treated with broadly immunosuppressive drugs, such as mycophenolate mofetil, which sensitizes T cells to apoptosis and interferes with antibody production[59,78]. In recent years, biologics targeting IL-6, and JAK inhibitors have been explored with some

success[79,80]. In severe cases, biologics such as the B cell depleting rituximab have also shown promise[59]. Individuals with SSC can also have arthritis, in which case methotrexate is often prescribed[59]. Altogether, given the heterogeneity in SSc disease manifestations and underlying immune perturbations, many different immunomodulatory drugs can be employed in this population, which may in turn impact their responses to infections and vaccinations.

1.1.6 Infection and vaccination responses in people with SSc

Disease-related factors remain a predominant cause of death in people with SSc, with pulmonary fibrosis, pulmonary arterial hypertension, and cardiac and renal issues among the main culprits[67,81–84]. Nonetheless, infections in people with SSc are also a common reason for hospitalization, and contributor to mortality[67,81,85–87]. Furthermore, many of the immunosuppressive drugs used to treat RA are also used to treat SSc, and may present similar risks for infection. Participants with SSc who are on DMARDs have been reported to mount lower humoral responses to pneumococcal vaccination than both controls and participants with SSc that were not on DMARDs[88]. Conversely, participants with SSc and controls mount comparable humoral responses to influenza vaccinations, and this response was not largely impacted by the use of immunomodulatory drugs[89,90]. This further supports the notion that immunomodulatory drugs may divergently impact responses to different vaccine platforms. It is therefore critical that the impacts of immunosuppressive drugs, and the responses to different vaccination platforms, are evaluated in people with

different autoimmune conditions without the assumption that findings for one condition, vaccine type, or drug, hold true for others.

1.2 SARS-CoV-2, the virus and the vaccine

1.2.1 SARS-CoV-2 origin and virology

In December of 2019, a novel positive sense single stranded RNA virus from the *Coronaviridae* family was detected in Wuhan, China[91,92]. This novel coronavirus shared the ability of its relative, SARS-CoV, to cause severe acute respiratory syndrome eventually earning it the moniker SARS-CoV-2[93]. The genetic similarity between SARS-CoV-2 and coronaviruses found in the horseshoe bats of southeast Asia, coupled with the link between early cases and a live animal market, led researchers to conclude the virus was of zoonotic origin[93–97]. Interestingly, the bat coronaviruses that are related to SARS-CoV-2 do not have extremely high sequence identity, suggesting they did not directly give rise to SARS-CoV-2[97]. Furthermore, bats were not sold at the live animal market in question[98]. Thus, it is likely that SARS-CoV-2 used an intermediate animal reservoir before making the jump to humans, a modus operandi shared by SARS-CoV[91,97,99,100].

The similarity between SARS-CoV-2 and the bat coronaviruses, as well as SARS-CoV, was useful in the identification of coding regions and structural proteins in the new virus. The SARS-CoV-2 genome encodes the membrane (M), nucleocapsid (N), envelope (E), and spike (S) proteins (Figure 1) in addition to various other proteins such as an RNA-dependent RNA polymerase[101]. The

spike protein of SARS-CoV-2 can be divided into functional regions, with the S1 domain containing the receptor binding domain (RBD), while the S2 domain mediates membrane fusion (Figure 1)[97]. The S2 domain displays remarkable conservation in sequence identity with the S2 domains of SARS-CoV and other bat coronaviruses[101]. As a whole, the genome of SARS-CoV-2 is more similar to that of bat coronaviruses than that of SARS-CoV[97]. The RBD however maintains expression of key amino acids used by SARS-CoV to bind human angiotensin converting enzyme 2 (ACE2)[102]. This led to the eventual discovery of ACE2 as the cellular receptor targeted by SARS-CoV-2[102]. ACE2 is expressed by many cell types throughout the body, including those in the respiratory tract, the intestines, and the heart[103]. Within the respiratory tract, the expression level of ACE2 is reported to be higher within cells of the upper respiratory tract[103,104]. Accordingly, the primary transmission route of SARS-CoV-2 is through respiratory droplets and aerosols[104–106].

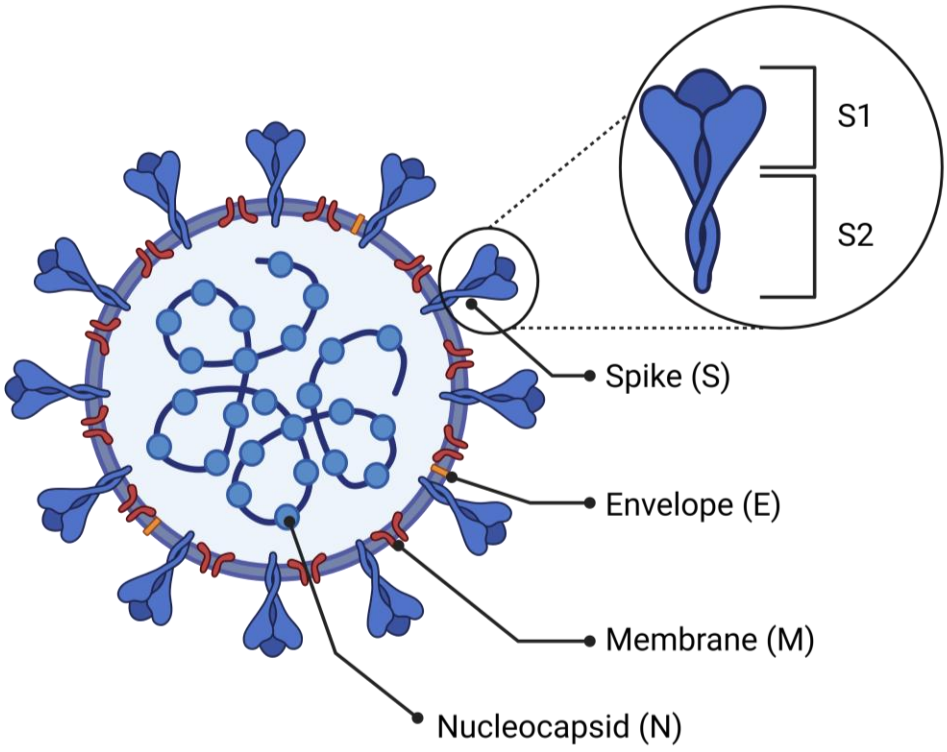

Figure 1. SARS-CoV-2 immunogenic proteins of interest. Created with BioRender.com

1.2.2 SARS-CoV-2 morbidity and mortality

Though SARS-CoV and SARS-CoV-2 utilize the same receptor to infect cells, particularly in the respiratory tract, SARS-CoV-2 has spread globally in a way its predecessor never did. Since the declaration of the SARS-CoV-2 pandemic in March 2020, there have been over 700 million confirmed cases, and over 7 million deaths by July 2025[91,107]. In 2021, SARS-CoV-2 infections were the second leading cause of death globally[108]. In the United States and Canada, SARS-CoV-2 infections remained among the top ten causes of death in both 2022 and 2023[109–112]. While some individuals infected with SARS-CoV-2 are asymptomatic, the most common symptoms include cough, fever, and shortness

of breath[113–115]. In cases requiring hospitalization, high rates of pneumonia have been reported, with a portion of these cases progressing to acute respiratory distress syndrome, organ failure, and death[113–115]. The risk of poor outcomes associated with SARS-CoV-2 infection differs greatly between populations, with older adults and individuals with comorbidities at increased risk of hospitalization and death[115,116]. Although media coverage of SARS-CoV-2 infections, morbidity, and mortality has declined over time, SARS-CoV-2 remains one of the top respiratory viruses contributing to hospital bed occupancy and outbreaks in congregate living settings in Ontario[117].

1.2.3 Vulnerable populations

Individuals with rheumatic diseases, such as rheumatoid arthritis, are likely at increased risk of SARS-CoV-2 infections and are more likely to have poor outcomes with these infections, than people without rheumatic conditions[118–120]. This risk is particularly pronounced in patients with higher disease activity, and those with ILD[119,121,122]. The risk of poor SARS-CoV-2 infection outcomes in individuals with rheumatic diseases is further compounded by the very medications used to treat the conditions. Inclusion of glucocorticoids, rituximab, or JAK inhibitors, in the drug regimens of individuals with rheumatic diseases is associated with an increased risk of severe SARS-CoV-2 infection, and death[118,121,123]. Importantly, the incidence and prevalence of RA increases with age, and thus the interplay of susceptibility to poor SARS-CoV-2 outcomes due to both age and rheumatic condition must be considered[2].

1.2.4 Potential correlates of protection in SARS-CoV-2 infections

Early animal studies provided evidence for the importance of humoral and T cell responses in the clearance of SARS-CoV-2[124,125]. Mice lacking T and B cells were unable to clear their SARS-CoV-2 infections, indicating that the innate immune system alone is not sufficient for protection[124]. While removal of either B cells, $CD4^+$ T cells, or $CD8^+$ T cells individually from the mice significantly impaired viral clearance, humoral immunity was sufficient to clear the virus in the absence of cellular immunity, and vice versa, during primary infections[124]. In the absence of B cells, the depletion of $CD8^+$ T cells impaired viral clearance more notably than the depletion of $CD4^+$ T cells[124]. This coincides with the understanding that $CD8^+$ T cells directly kill virus infected cells, while $CD4^+$ T cells are more commonly involved in assisting and fine-tuning humoral and other cellular responses[124]. Antibodies, in turn, employ a variety of mechanisms to interfere with SARS-CoV-2 infection and spread, including both neutralizing and non-neutralizing functions[124,126]. In people with SARS-CoV-2 infections, early $CD8^+$ T cell responses, and SARS-CoV-2 specific $CD4^+$ and $CD8^+$ T cell levels were also correlated with less severe disease[127,128]. Conversely, deficits in SARS-CoV-2-specific antibody, $CD4^+$ T cell, or $CD8^+$ T cell responses were associated with severe disease and poor outcomes[128,129]. Similarly, the potency, not just the levels, of the neutralizing antibodies in individuals with COVID-19 was a predictor of survival[130].

1.2.5 Immune responses to SARS-CoV-2 infections

Long-lasting humoral and cellular responses are generally detectable in the blood of participants who had mild, not just severe, SARS-CoV-2 infections[131–133]. Anti-spike and anti-RBD antibodies are detectable in the blood of people with SARS-CoV-2 infections within 1-2 weeks of symptom onset, peaking in the first month[134]. The levels of anti-spike and anti-RBD IgG are reported to be relatively stable or decline slightly with time post infection, though the rate of this decline is faster during the first six months, before plateauing[131,132,134–136]. Conversely, blood anti-spike and anti-RBD IgA and IgM may decline following SARS-CoV-2 infections[131,132,134]. Neutralizing titers also peaked within the first month following SARS-CoV-2 infection symptom onset and remain relatively stable for 3-6 months, though they may decline, yet remain detectable, by 6-8 months post infection[131,134,135]. It has been suggested that this initial decline may be due to the loss of short-lived plasmablasts, and the shift to relying on long-lived plasma cells, that occurs in the months following SARS-CoV-2 infections[131].

In contrast to antibody levels, SARS-CoV-2-specific memory B cell responses took 3-6 months to reach maximum levels in the blood, and did not decline by 6-8 months post symptom onset[131,132,134,136]. SARS-CoV-2-specific T cell responses are detectable within the first month following infection, and are durable with multiple studies reporting detectable responses 8-12 months after SARS-CoV-2 infection[132,134,136,137]. SARS-CoV-2-specific $CD4^+$ T cells tend to be more abundant than SARS-CoV-2-specific $CD8^+$ T cells, though the reason for

this remains unclear[132,133,136]. The SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells also differ in phenotype, with the reports of $CD4^+$ T cells primarily displaying a central memory phenotype, while the $CD8^+$ T cells were largely effector memory or effector memory re-expressing CD45RA (EMRA)[132,136].

In addition to the systemic responses generated following SARS-CoV-2 infection, anti-spike and anti-RBD IgG and IgA antibodies are also detectable in the respiratory mucosa[138]. Similarly, neutralizing antibodies are detectable in the respiratory mucosa following SARS-CoV-2 infection, albeit at lower levels than the titers observed in the blood[138]. RBD-specific B cells are also detectable in the bronchoalveolar lavage fluid (BAL) and lungs of people with a previous SARS-CoV-2 infection, as are SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells[138,139]. Despite the generation of local and systemic memory responses, and their relative durability, following SARS-CoV-2 infections, however, the rate of reinfection with SARS-CoV-2 increases with time from the previous infection[140].

1.2.6 SARS-CoV-2 mRNA vaccine formulations and efficacy

The months following the pandemic outbreak were characterized by unprecedented levels of scientific collaboration, allowing the rapid development of novel SARS-CoV-2 spike-encoding mRNA vaccines[141,142]. The initial trials and preliminary reports for Pfizer's BNT162b2 and Moderna's mRNA-1273 demonstrated that both vaccines were safe and effective in the prevention of COVID-19 following administration of the primary series two dose regimen, although individuals with autoimmune disorders and those on

immunosuppressive drugs were excluded from the trials[141–143]. The vaccine efficacy of the primary mRNA vaccination series against the ancestral strain of SARS-CoV-2 was over 90%, with protection lasting at least 6 months[141,142,144].

Both Pfizer's BNT162b2 and Moderna's mRNA-1273 use mRNA encoding the full-length, prefusion stabilized, ancestral SARS-CoV-2 spike protein, encapsulated in lipid nanoparticles[141,142]. The most notable difference between the BNT162b2 and the mRNA-1273 vaccines was the dose, at 30 μg and 100 μg respectively, and thus the mechanism of the immune responses to the vaccines would likely be similar[141,142]. The modifications to the mRNA in the vaccines serve to stabilize the molecule, increase translation, and decrease recognition by Toll-like receptors (TLRs)[145–147]. The lipid nanoparticles provide further stability by protecting the mRNA, and have been suggested to possess an inherent immunostimulatory capacity[147,148].

Once injected, the lipid nanoparticle-encapsulated mRNA can be phagocytosed by local tissue dendritic cells (DCs), or travel to the lymph nodes to be engulfed by DCs[147]. Murine studies have indicated that the spike mRNA levels are higher in the lymph node than other tissues such as the spleen, lungs, muscles, and liver, following vaccination, suggesting much of the vaccine mRNA ends up here[149]. The mRNA is then rapidly translated into the spike protein, and processed by the DCs for presentation to naïve T cells[147]. In murine models, the serum spike protein levels are already increased by one day post vaccination, and decline by one week post vaccination, while the spike mRNA levels in the

lymph node decline by 3 days post vaccination[149]. The presence of the spike-encoding mRNA and spike protein itself are thus transient.

1.2.7 Immune responses to the mRNA vaccination primary series

Initially, it was recommended that individuals receive two doses of the mRNA vaccines, delivered weeks to months apart, in what was termed 'the primary series'[150–152]. In both individuals without a previous SARS-CoV-2 infection, and those with previous infections, their first SARS-CoV-2 mRNA vaccination led to increased anti-spike and anti-RBD IgG[153,154]. Similarly, the first dose of a SARS-CoV-2 mRNA vaccine also induced detectable spike-specific $CD4^+$ and $CD8^+$ T cell responses in the majority of participants[153,155]. The second dose of an mRNA vaccine further increased the anti-spike IgG and spike-specific T cell levels[155–158]. The anti-spike and anti-RBD IgG levels peaked rapidly, within 1-2 weeks of the second SARS-CoV-2 vaccination, before declining over the next few months[156,157,159,160]. Neutralizing titers also peaked early after the second vaccination, and declined with time post-vaccination[156,159–161]. Despite this decline, anti-spike IgG and neutralizing activity was still detectable in the majority of vaccinated individuals by 6 months post vaccination[156,159,160].

SARS-CoV-2-specific B cell responses displayed different kinetics than that of antibody levels. Akin to what is observed in the cases of SARS-CoV-2 infections, SARS-CoV-2-specific B cell levels do not decline the first few months after the second mRNA vaccination[159]. Rather, these SARS-CoV-2-specific B cells actually increase at the 3-6 month post vaccination interval[159]. Spike-

specific T cell populations do contract in the first few months after vaccination, though the spike-specific $CD4^+$ T cell levels then plateau and remain detectable for at least 6 months[159]. Conversely, it has been reported that spike-specific $CD8^+$ T cells can further decline, and become undetectable in some individuals by 6 months post vaccination[159].

The dosing interval between the first and second mRNA vaccinations may also impact the kinetics and magnitude of the resulting memory responses. The manufacturer recommended dosing interval for the mRNA vaccines was between 21-28 days[152]. In order to strategically provide some protection against SARS-CoV-2 infection to a larger proportion of the population, Canadian public health units and hospitals prioritized providing more healthy adults with a single dose of an mRNA vaccine, as opposed to fewer individuals with two doses. To accomplish this more widespread vaccination coverage, they moved from providing vaccinations at the 21-28 day dosing interval, and favored longer dosing intervals, for healthy adults[162–164]. Interestingly, a longer interval between the first and second SARS-CoV-2 mRNA vaccines led to higher anti-spike IgG, neutralizing titers, and RBD-specific memory B cells[165–167]. The literature is somewhat conflicted regarding the impact of primary series dosing interval on spike-specific T cell responses, with some studies reporting no impact of longer versus shorter dosing intervals, and others reporting that extended dosing intervals did not increase spike-specific T cell responses[166,167].

Despite the similarity in the formulations of the BNT162b2 and mRNA-1273 vaccines, some studies have reported higher antibody levels and protection against infection in recipients of the mRNA-1273 vaccine, compared with those that received a BNT162b2 vaccine[161,168]. While antibody levels and neutralization capacity have been tied to protection against symptomatic SARS-CoV-2 infections and poor outcomes, non-neutralizing antibodies may also contribute to protection against severe SARS-CoV-2 infections, through mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP)[126,129,130,169,170]. The mRNA-1273 and BNT162b2 vaccines induce similar levels of ADCP by monocytes, but the mRNA-1273 formulation induces higher antibody-dependent neutrophil and natural killer cell activity[171]. The mRNA-1273 vaccine also induced higher levels of serum anti-spike and anti-RBD IgA[171].

Although mRNA vaccination can induce serum anti-spike and anti-RBD IgA, the respiratory mucosa anti-RBD IgA levels and the neutralizing titers are lower than those found following SARS-CoV-2 infections[138,172]. Additional SARS-CoV-2 vaccinations can boost nasal and saliva neutralizing antibody titers, but in uninfected individuals few RBD-specific B cells are found in the respiratory mucosa, and further studies suggest that these antibodies are not being produced locally[138,173–175]. Tissue-resident SARS-CoV-2 specific T cells are also infrequent in samples from the respiratory tracts of uninfected vaccinated individuals, compared with those that have had previous SARS-CoV-2 infections[138,175,176]. Thus, while the mRNA SARS-CoV-2 vaccines induce strong

systemic memory immune responses, they do not produce strong respiratory mucosa-resident memory populations.

1.2.8 SARS-CoV-2 adenovirus vector vaccines

Replication-deficient adenovirus vector vaccines, including AstraZeneca's chimpanzee adenovirus vector vaccine (ChAdOx1 nCoV-19), and Janssen's human adenovirus vector vaccine (Ad26.COV2.S), encoding the SARS-CoV-2 spike protein were also developed[177–179]. Once injected, ChAdOx1 can bind to the coxsackie-adenovirus receptor (CAR) on host cells, while Ad26.COV2.S employs CD46 as its cellular receptor[180–182]. These receptors are expressed on many cell types, including non-immune cells and antigen-presenting cells (APCs)[183]. After binding to their cellular receptors, the adenoviruses enter the cell by clathrin-mediated endocytosis[183]. Once inside the cell, the DNA encoding the spike protein of SARS-CoV-2 is translated into mRNA, leading to the production of the spike protein. APCs, including DCs, can then process the spike protein either from their own endogenous production or use spike protein acquired by phagocytosis, and present the peptides to naïve T cells in the lymph nodes[183]. The adenoviral vectors themselves also act as adjuvants during this immune response, activating TLRs and the cGAS/STING pathways, eventually leading to inflammatory cytokine production[183].

The Ad26.COV2.S vaccine was originally recommended as a single dose, and provided lower protection against infection and poor outcomes than its mRNA counterparts, with about 66% efficacy against severe COVID-19[177,184].

The ChAdOx1 nCoV-19 vaccine was recommended in a two-dose primary series, with a vaccine efficacy of 74%[179,185]. In Canada, the most commonly used vaccines were BNT162b2, followed by mRNA-1273, and fewer ChAdOx1 nCoV-19, with only a small number of individuals receiving Ad26.COV2.S[186]. Both adenoviral vector vaccines were associated with rare clotting events, and by the end of 2023, authorization for both vaccines was cancelled in Canada[183–185,187].

1.2.9 SARS-CoV-2 adenovirus vector vaccine immune responses

It has been previously reported that a single dose of the Ad26.COV2.S or ChAdOx1 nCoV-19 vaccine induces detectable neutralizing antibodies within the first 2-4 weeks after vaccination, in the majority of participants[178,188,189]. For the Ad26.COV2.S single dose vaccine, these neutralizing titers were significantly lower than those induced by the mRNA vaccines, but the levels did not decline over the next 6-8 months[161,189,190]. Unlike the Ad26.COV2.S vaccine, the ChAdOx1 nCoV-19 vaccine was recommended for a two dose primary series, and the second dose further boosts the anti-spike IgG levels, but not the spike-specific T cell responses[191–193]. Nonetheless, the peak antibody levels following the second ChAdOx1 nCoV-19 vaccination are lower than those observed after the second mRNA vaccination, but show a similar trend of waning by 6 months post vaccination[190,192,194].

Spike-specific B cells are also detectable following Ad26.COV2.S vaccination, and increase in frequency from 3-6 months after vaccination, a kinetic pattern shared with the mRNA vaccines[189]. Despite the common kinetics between the

vaccine formulations for spike-specific B cell levels, the Ad26.COV2.S vaccination did lead to lower frequencies of spike-specific B cells, and altered B cell phenotypes, compared to the mRNA vaccines[189]. Spike-specific T cell responses are also detectable in the majority of participants within 2 weeks of the first Ad26.COV2.S or ChAdOx1 nCoV-19 vaccination[178,188]. The spike-specific $CD4^+$ T cell levels and their cytokine-producing capacity following Ad26.COV2.S may be lower than that observed following mRNA vaccination[189]. Conversely, the spike-specific $CD8^+$ T cell responses were more similar between vaccine formulations[189]. The spike-specific T cell responses were still detectable by 6-8 months post dose 1 for the Ad26.COV2.S vaccine, and post dose 2 for the ChAdOx1 nCoV-19 vaccine[161,189,190].

1.2.10 The third mRNA vaccination

Both the induction and the longevity of humoral and cellular responses to SARS-CoV-2 vaccination are associated with vaccine related protection. Although the primary series demonstrated long-lasting protection against SARS-CoV-2 infection, this vaccine efficacy declined with time after vaccination[140,144]. The observed decline in vaccine efficacy coincided with reports of declines in humoral responses from peak levels over time following vaccination[156,157,159–161]. In vulnerable populations such as older adults, lower antibody levels were associated with mortality in individuals hospitalized due to COVID-19, highlighting the importance of strong humoral responses in maintaining protection against poor outcomes associated with SARS-CoV-2 infections[129]. These data led to recommendations for a third mRNA vaccination[195,196].

Within one month of the third mRNA vaccination, neutralizing antibody titers were reportedly increased[197–201]. The impact of the third mRNA vaccination on humoral responses was particularly pronounced in some vulnerable groups, such as immunosuppressed organ transplant recipients. In this population, the third dose was able to induce a humoral response in approximately half of the participants who had not demonstrated detectable humoral responses following the primary series[202]. The third mRNA vaccination also increased anti-spike antibody levels and neutralizing titers in older adults in long-term care homes[203,204]. Although antibody levels declined from their peak with time following the third SARS-CoV-2 vaccination, the rate of this waning is slower than after the primary series[199,205,206]. Conversely, the third mRNA vaccination may only induce a transient increase in spike-specific T cell levels[200]. Spike-specific T cell levels can thus decline from their transient peak within 30-90 days after the third vaccination[199,200,206].

Following recommendations for the third dose, studies from Israel, England, Qatar, and the USA, demonstrated that the third dose displayed a higher vaccine effectiveness against symptomatic and severe disease than the primary series alone[207–212]. The vaccine effectiveness did, however, decline with time following the third mRNA vaccination[208,211,213]. This decline in effectiveness could be seen in as little as one month after the third vaccination, and continued on this downward trajectory with each passing month[208,211,213]. This waning vaccine effectiveness was not simply due to changes in antibody or T cell levels

with time but was also influenced by a new emerging threat: SARS-CoV-2 variants.

1.2.11 SARS-CoV-2 variants and updated vaccinations

As SARS-CoV-2 spread across the globe, mutated variants with altered transmissibility emerged[214,215]. Many of these variants contain mutations in the spike protein, which enhanced binding to the ACE2 receptor and promoted evasion of existing humoral immune responses[214–216]. The WHO monitored the emergence of new variants, and designated them as a variant of concern (VOC) if they were anticipated to cause more severe disease, or generally negatively impact the population to a greater degree than the currently circulating SARS-CoV-2 lineages[217,218]. One of the earliest mutations to emerge in the spike protein was the D614G substitution (Figure 2)[219]. In the months that followed the discovery of the D614G mutation, VOCs containing numerous mutations in the spike protein began to emerge.

Some of the earliest VOCs emerged in Fall of 2020, namely the Alpha variant (B.1.1.7) and the Beta variant (B.1.351) (Figure 2)[215,220,221]. Although the Alpha variant contained multiple mutations in the spike protein, neutralizing titers in vaccinated individuals were only moderately lower against the alpha variant compared with the ancestral strain[216,222,223]. Thus, although some studies suggested that the alpha variant could cause greater mortality than the previous dominant strains, real world data demonstrated that the primary SARS-CoV-2 vaccination series still provided a high level of protection against symptomatic

disease and severe infection[224–226]. Conversely, neutralization of the Beta variant in vaccinated individuals was significantly reduced compared with neutralization of the ancestral or D614G strains[160,190,216,223,227]. Accordingly, the vaccine effectiveness of a primary mRNA vaccination series against infection with the Beta variant was lower than that reported for the alpha variant, though protection against severe disease was maintained[226]. A third mRNA vaccine dose did, however, increase neutralizing titers against the Beta variant in both the general population and in older adults in long-term care[197,203].

By December of 2020, the Delta variant (B.1.617.2) had emerged, and quickly outcompeted both the Alpha and Beta variants with its enhanced transmission ability (Figure 2)[220,228,229]. The Delta variant was also associated with an increased risk of hospital admission compared with the Alpha variant, and led to breakthrough infections in vaccinated people[230–232]. Although the protection against infection with the Delta variant provided by a primary mRNA vaccination series was lower than the protection against Alpha, the primary series still provided a high level of protection against severe outcomes[225,232–234]. The mutations in the spike protein of the Delta variant led to reduced neutralization by the serum antibodies of vaccinated individuals, likely contributing to the breakthrough infections[156,235,236]. A third mRNA SARS-CoV-2 vaccination was able to increase the neutralizing titers against the Delta variant, and the vaccine effectiveness of 3 doses against hospitalization with the Delta variant was high[204,210]. The vaccine effectiveness did decline with time following vaccination,

raising questions about the duration of protection against a variant known to cause breakthrough infections[210,234].

In November of 2021, the eventual usurper of Delta's global dominance, and the variant with sublineages that continue to be the dominant VOCs to this day, emerged: Omicron (B.1.1.529) (Figure 2)[218,237]. The Omicron variant, with over 30 mutations in it's spike protein, displayed a higher transmissibility than the Delta variant, and eventually gave rise to the BA.1, BA.2, and BA.3 sublineages[237–240]. While the BA.1 sublineage was initially the dominantly circulating sublineage, it came be to replaced by the even more transmissible BA.2 sublineage[239–241]. In vaccinated individuals, neutralizing titers against the original Omicron variant and BA.1 were markedly lower than those against D614G or the ancestral strains[240,242,243]. Neutralizing titers against the BA.2 variant were reported to be either similar to or slightly lower than those against the BA.1 variant[240,244,245]. While a third mRNA vaccination was reported to increase the neutralizing titers against Omicron and it's sublineages, the titers remained significantly lower than those against the ancestral or D614G strains[204,242,244,245].

Unsurprisingly, vaccine effectiveness against infection with the Omicron variants was notably lower than the protection afforded against the Delta variant[244]. The vaccine effectiveness of a primary mRNA vaccination series against symptomatic infection or hospitalization with an Omicron variant was estimated to be around 65% and 70%, respectively, and declined from this upper

bound rapidly with time post vaccination[246,247]. It should be noted, however, that estimates of vaccine effectiveness varied greatly between studies depending on their definition of effectiveness (against infection, symptomatic infection, or severe outcomes). Nonetheless, a third dose of an mRNA vaccine was reported to provide greater protection against infection and hospitalization than the primary series alone[209,210,244,246,248,249].

Soon after the emergence of Omicron and the BA.1 and BA.2 sublineages, two new Omicron sublineages emerged: BA.4 and BA.5[250]. These lineages possessed the same spike protein, which was similar to the spike protein sequence of BA.2[250]. Despite this similarity, the additional mutations in the spike protein of BA.4/BA.5 led to a significant drop in neutralization by serum of individuals previously infected with BA.1 or BA.2[251–253]. Compared to BA.2, BA.4 and BA.5 were also less well neutralized by the serum of vaccinated individuals[252–254]. This heightened evasion of neutralization likely contributed to BA.4 and BA.5 to overtaking BA.2 in many regions, and subvariants of BA.5 were also detected as the sublineage spread[251,254,255]. Despite BA.4/BA.5 overtaking BA.2 in many regions, two BA.2 subvariants eventually recombined, producing the XBB.1 variant, with notable evasion of neutralization by serum from people previously infected with either BA.2 or BA.5[255].

While receiving two or three SARS-CoV-2 vaccinations reduced the likelihood of developing severe disease upon infection with BA.5 or XBB.1, it was becoming woefully apparent that vaccines eliciting immune responses against the ancestral

SARS-CoV-2 spike protein would not be able to keep up with the ever-evolving spike proteins of the variants[256]. Although the Omicron variants were reported to cause less severe disease than the Delta variant, the transmissibility of the Omicron variant still posed a significant problem for both case numbers and the development of the sublineages[257–261]. Interestingly, despite the evasion of humoral responses by many of the aforementioned variants, T cell responses remained robust. Spike-specific T cell responses to the Alpha, Beta, Delta, and Omicron variants, compared with the responses to the ancestral strain, were largely preserved in vaccinated individuals[190,204,216]. Cross-reactive T cells may therefore aid in providing cross-variant protection, even if a variant manages to slip by the humoral responses, and comprehensive analyses of SARS-CoV-2 vaccination responses should evaluate both arms of immunity.

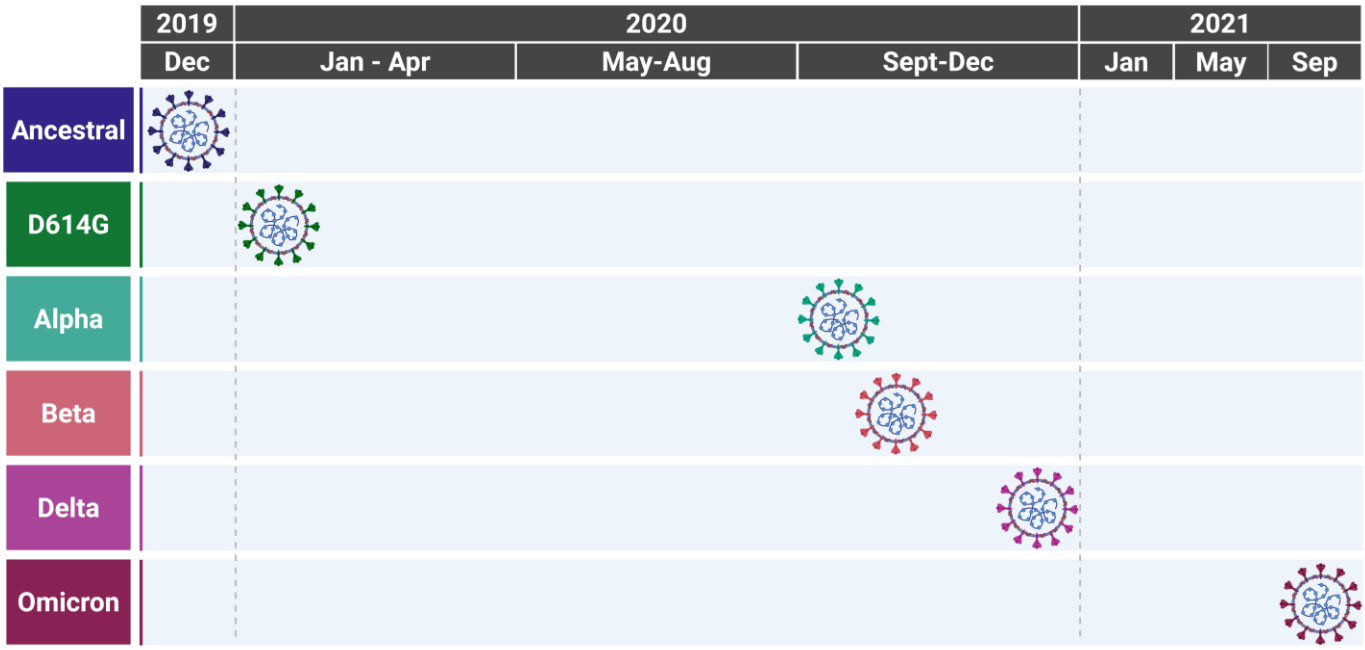

Figure 2. Timeline of the emergence of different SARS-CoV-2 variants of concern. Created with BioRender.com

1.2.12 Recommendations for additional SARS-CoV-2 vaccinations and the development of variant-specific vaccines

The continued emergence of variants of concern, coupled with the immune evasiveness of these variants, led to the recommendation for additional SARS-CoV-2 vaccinations in many jurisdictions[262–265]. In some areas, the fourth dose was with Pfizer's BNT162b2 or Moderna's mRNA-1273, encoding the spike protein from the ancestral SARS-CoV-2 strain. Compared with individuals who received 3 doses, those that received a fourth dose displayed elevated serum neutralizing antibodies and antibody titers[157,266]. In the age of Omicron, the fourth dose displayed greater vaccine effectiveness against infection and severe

disease than the three dose series, though this protection waned beyond 1 month post vaccination[266–268]. As recommendations for a fourth dose were being provided, new bivalent mRNA vaccines were being developed. The bivalent mRNA vaccines, originally encoding the spike proteins for the ancestral and BA.1 variant, and later encoding the spike for the BA.4/BA.5 variants, then became the recommended vaccine type, with the goal of providing better protection against the emerging variants than their monovalent predecessors[262–264,269].

Compared with fourth dose monovalent vaccines, multiple studies reported that receiving a bivalent BA.1 vaccine for the fourth dose was associated with higher neutralizing titers against the BA.1 variant, while others found no significant difference between the monovalent and bivalent formulation induction of BA.1 neutralizing antibodies[270–272]. The literature was also conflicted on whether or not the BA.1 bivalent vaccine induced higher levels of neutralizing titers against BA.4/BA.5 than the monovalent vaccine[270–272]. Similarly, while general consensus was that receipt of a BA.4/BA.5 bivalent vaccine increased neutralizing titers against BA.4/BA.5 compared with pre-bivalent vaccination levels, there were conflicting reports regarding whether these neutralizing titers were higher than in participants who received a monovalent fourth dose[270,273–276]. Despite the conflicting reports on neutralizing antibody titers, fourth dose bivalent vaccines displayed a greater effectiveness against symptomatic and severe infections than either a 3-dose vaccination series, or a 4th dose with a monovalent vaccine[277–279].

Following BA.4/BA.5 bivalent vaccination, however, neutralizing antibody responses remained low against the XBB.1 variant, leading to the development of monovalent vaccines encoding the XBB variant spike protein[270,276,280,281]. Compared with other older vaccine formulations, the XBB vaccine provided better protection against SARS-CoV-2 infection and poor outcomes such as hospitalization[282–284]. The molecular arms race between increasingly immune evasive SARS-CoV-2 variants and vaccines which target them has not ended. New mRNA vaccines continue to be developed to target the circulating dominant SARS-CoV-2 variants, and increase variant-specific neutralization[285,286]. As of February 2025, the Public Health Agency of Canada recommends that various vulnerable groups continue to receive yearly updated SARS-CoV-2 vaccinations to maintain protection against the latest variants, while less vulnerable groups may also receive additional vaccinations[287].

1.2.13 Hybrid immunity

With the increasing vaccination coverage, coupled with the increases in infections, many individuals had antigenic exposure to SARS-CoV-2 through both infection and vaccination, leading to the generation of hybrid immunity. Initial studies showed that individuals with a prior SARS-CoV-2 infection had higher spike-specific antibody levels following primary series vaccination than their uninfected counterparts[157]. In one study, the anti-spike IgG levels of participants with a previous SARS-CoV-2 infection and one vaccination were similar to those seen in uninfected individuals who received two SARS-CoV-2 vaccinations[288]. The primary vaccination series, however, did not largely increase the spike-

specific $CD4^+$ or $CD8^+$ T cell responses in previously infected individuals, and spike-specific T cell levels were similar between previously infected and uninfected participants following the primary vaccination series[155,159]. Despite the similar levels of spike-specific T cells in the individuals with hybrid immunity and the uninfected vaccinated participants, individuals with hybrid immunity had more T cells capable of producing Granzyme B and IFN-γ, as well as IL-2[289]. The rate of waning in anti-spike IgG following the primary series was similar between individuals with a previous infection and uninfected participants, though cellular responses were largely maintained in both groups[159,204].

In the era before Omicron, risk of SARS-CoV-2 infection was generally lower in vaccinated individuals with a previous infection, compared to vaccinated individuals without a previous infection, though this protection waned with time since last exposure[140,290]. This information led the Public Health Agency of Canada to advise that people with a SARS-CoV-2 infection, and who were previously vaccinated, wait 3 months before receiving their next vaccination[291]. While infection with a previous variant could provide moderate protection against symptomatic infection with Omicron BA.2, additional vaccinations of previously infected individuals increased their omicron neutralizing titers and generally provided better protection against reinfection[256,292,293]. The protective effect of previous infection on the risk of reinfection may not be consistent in all groups of people, or for all emerging variants. In older adults in long-term care, it has been reported that infection with BA.1/BA.2 actually increased the risk of reinfection with BA.5[294]. This surprising finding indicates that there may be differences in

immune responses to some variants that may not confer protection to subsequent variants, at least in older frail individuals.

1.2.14 The impacts of immunomodulatory drugs on SARS-CoV-2 vaccination responses

Given that immunosuppressive drugs can impact the immune responses to other vaccine types, coupled with the exclusion of people with autoimmune conditions from initial SARS-CoV-2 vaccine trials, the efficacy and immunogenicity of SARS-CoV-2 mRNA vaccinations in people with autoimmune disorders who were on immunosuppressive drugs presented a critical knowledge gap. Additionally, the susceptibility of people with autoimmune disorders and on immunosuppressive drugs to poor outcomes associated with SARS-CoV-2 infections further highlighted the importance of understanding their immune responses to the novel mRNA vaccine platform, to provide protection against severe disease. In order to address the paucity of research into the impact of immunomodulatory drug use on SARS-CoV-2 vaccination responses many research teams pivoted to explore these responses.

Early studies of responses to the first SARS-CoV-2 vaccination, with either mRNA or viral-vector vaccines, found that participants with psoriasis or RA, who were receiving methotrexate alone or in combination with other medications, had lower seroconversion rates and neutralizing antibody titers than those receiving other drugs, such as TNF inhibitors, as well as controls[295,296]. Methotrexate and TNF inhibitors did not, however, negatively impact spike-specific T cell responses in participants with psoriasis or RA[295–297]. In a mixed cohort comprised of people

with different autoimmune conditions, including RA, ankylosing spondylitis, psoriasis, and inflammatory bowel disease, treatment with TNF inhibitors alone or concurrently with methotrexate was associated with lower humoral responses to the primary series of vaccinations than controls and participants with autoimmune conditions who were not on immunosuppressive drugs[298]. Unlike in the previous studies with cohorts of only participants with psoriasis or RA, treatment with methotrexate or TNF inhibitors in the mixed autoimmune cohort was associated with deficits in spike-specific T cell responses compared to controls[298]. These studies thus reaffirm that the impact of a given immunosuppressive drug on vaccination responses may differ depending on the autoimmune conditions examined.

The use of anti-CD20 also led to lower seroconversion rates in participants with RA compared to controls following the first SARS-CoV-2 vaccination[296]. In participants with multiple sclerosis, treatment with anti-CD20 was also associated with lower anti-spike IgG, but not impaired spike-specific $CD4^+$ or $CD8^+$ T cell responses, after the first and second SARS-CoV-2 vaccinations[299]. Use of costimulation inhibitors in participants with RA was also associated with deficits in the humoral responses to the first, second, and third SARS-CoV-2 vaccinations[296,297,300]. Costimulation inhibitors were also reported to impair T cell responses to the second and third SARS-CoV-2 vaccinations in participants with RA[297,300]. Altogether, these studies demonstrated the importance of evaluating the impact of immunosuppressive drugs on both humoral and cellular responses to SARS-CoV-2 vaccination, as the responses can be disparate.

Literature on SARS-CoV-2 vaccination responses in participants with SSc was more scarce, due to the rarity of this condition. In participants with SSc who were not on immunosuppressive drugs, seroconversion rates and anti-spike IgG levels were similar to those of controls by 3 months post dose 2[301,302]. The use of methotrexate or mycophenolate mofetil in participants with SSc were associated with weaker humoral responses to SARS-CoV-2 vaccination[301,303,304]. T cell responses to SARS-CoV-2 vaccination in participants with SSc were not largely investigated.

Despite the weaker SARS-CoV-2 vaccination responses associated with the use of different immunosuppressive in people with autoimmune diseases, vaccination still provided protection. In the eras of the Alpha and Delta variants, two doses of an mRNA vaccine displayed a vaccine effectiveness of 83% against infection in participants with RA[305]. For individuals with psoriasis, ankylosing spondylitis, or inflammatory bowel disease, the vaccine effectiveness against infection was similarly high[305]. The vaccine effectiveness against severe outcomes was even higher, >90% for all the aforementioned conditions[305]. Generally, the third dose afforded similar or better protection against infection than the primary series[305]. A study published in early 2024, on data collected during the Omicron variant era, found that in a cohort of participants with various autoimmune conditions, a fourth mRNA vaccine dose was associated with a reduced risk of infection or poor outcomes associated with infection, compared with those who only received three doses[306].

1.2.15 Concerns about vaccination by people with autoimmune conditions

It has been previously reported that immunosuppressive drugs such as methotrexate can negatively impact influenza and pneumococcal vaccination responses, and that temporarily pausing treatment with methotrexate around the vaccinations can improve immune responses, at the cost of an increased risk of disease flares[307,308]. While acknowledging the potential risk of flares associated with vaccination and pausing immunosuppressive medications in people with rheumatic diseases, the American College of Rheumatology (ACR) still strongly recommended SARS-CoV-2 vaccination for patients with rheumatic diseases[308]. This recommendation applied regardless of current disease activity, provided that the patient was not currently hospitalized with severe illness, as the benefits of SARS-CoV-2 vaccination were deemed to outweigh the risks[308]. Considering the previous vaccinology data on the negative impact of certain immunosuppressive drugs on vaccination responses, the ACR also recommended that methotrexate and JAK inhibitors should be withheld for one week after each primary series vaccine dose[308]. The costimulation inhibitor abatacept was also recommended to be withheld around the first SARS-CoV-2 vaccination, for 1 week prior to, and 1 week after vaccination[308]. Other medications such as hydroxychloroquine, leflunomide, and sulfasalazine were not recommended to be withheld[308]. The goal of these recommendations was to improve SARS-CoV-2 vaccination responses in these vulnerable populations, while minimizing the risks of disease flares that could be further elevated by longer periods without immunosuppressive medications[308].

Early in the pandemic, some people living with autoimmune conditions expressed concerns that vaccination could lead to disease flares[309,310]. Multiple studies explored the risks of disease flares in participants with autoimmune diseases following SARS-CoV-2 vaccination in order to address these concerns. In cohorts of participants with different autoimmune diseases that predominantly received 2 doses of an mRNA vaccine, the frequency of disease flares was relatively low, and classified as mild[311–313]. In these studies however, the majority of participants did not hold their medications around vaccination[312,313]. In a cohort of patients with SSc, including both those who continued taking their immunosuppressive medication and those on methotrexate who paused their medication, none of the patients had disease flares after the second SARS-CoV-2 vaccination[301]. Participants with RA that received either two doses of an mRNA or inactivated SARS-CoV-2 vaccine also did not have an elevated risk of disease flare following vaccination, when compared to unvaccinated participants on similar medications[314].

In studies on mixed autoimmune disease cohorts, and on participants with RA, a third SARS-CoV-2 mRNA vaccination was also not associated with an increased risk of disease flares[300,315]. Per the ACR recommendations, the participants with RA did not pause their medications around the third SARS-CoV-2 vaccination in this study[300]. Another study in participants with RA, however, found that pausing methotrexate treatment around the third SARS-CoV-2 vaccination was associated with increased disease flares compared to participants that did not pause their medications[316]. It is therefore likely that

SARS-CoV-2 vaccination in participants taking immunosuppressive drugs is not associated with a high frequency of flares, while withholding medication may increase this risk for the tradeoff of improved immunogenicity[316]. Exploring the impact of different immunomodulatory drugs on SARS-CoV-2 vaccination responses therefore remains a pertinent concern, in order to inform recommendations on which medications should potentially be held around vaccination to improve responses, and if these medication pauses may provide benefit around doses beyond the primary series.

1.3 Rationale

Previous studies have demonstrated that immunosuppressive drugs can negatively impact humoral and cellular responses to different vaccinations, and that the impact of the same drugs may differ depending on vaccine type and autoimmune condition[48–55,88]. With the advent of the SARS-CoV-2 pandemic, new vaccine formulations, including mRNA vaccines, were introduced to the population. Given the susceptibility of people with autoimmune disorders on immunosuppressive drugs to poor outcomes associated with SARS-CoV-2 infection, there is a vested interest in determining how well they respond to SARS-CoV-2 vaccination, and if these immune responses are comparable to those seen in healthy controls[118–123]. We hypothesized that people with RA or SSc, on immunomodulatory drugs, would have weaker cellular and humoral responses to SARS-CoV-2 vaccination, compared with healthy controls, and that additional vaccinations would increase antibody levels. We also predicted that

certain immunomodulatory drug classes, such as costimulation inhibitors, would be associated with poorer vaccination responses.

Our study therefore sought to expand on the existing literature regarding the impact of immunosuppressive drugs on SARS-CoV-2 vaccination responses in participants with RA. We extended our exploration to include the fourth SARS-CoV-2 vaccination and delineated the impact of different immunosuppressive drugs on not only humoral and cellular responses, but on $CD4^+$ and $CD8^+$ T cell responses. The longitudinal nature of this study, with repeated measures from the same participants over time, also allowed us to track if additional SARS-CoV-2 vaccinations still increased humoral or cellular responses in participants with RA, even if it was not to the level observed in controls. For participants with SSc, the literature on SARS-CoV-2 vaccination responses was rather sparse, and typically focused on humoral responses, without investigating T cell responses[301–304]. We therefore sought to address this knowledge gap, by investigating both humoral and T cell responses after the second, third, and fourth SARS-CoV-2 vaccinations in participants with SSc.

The recommendations for additional SARS-CoV-2 vaccinations, particularly in vulnerable populations including immunosuppressed individuals, has raised concerns from the public that this may negatively impact or overwhelm their immune systems. In order to address this concern, we also examined the impact of repeated SARS-CoV-2 vaccinations on T cell exhaustion in multiple vulnerable

groups (individuals with RA on immunosuppressive drugs and older adults in long-term care), and in healthy younger adults.

The goals of this study can thus be broken into 3 overarching aims:

1. Examine the magnitude of the humoral and cellular responses to multiple SARS-CoV-2 vaccinations in participants with RA, compared to controls.
    a. Within the RA cohort, determine which immunosuppressive drugs, if any, are associated with weaker humoral or cellular responses to SARS-CoV-2 vaccination
2. Determine if participants with SSc mount comparable, or weaker, humoral and cellular responses to SARS-CoV-2 vaccination, compared to controls.
3. Elucidate if repeated SARS-CoV-2 vaccination is associated with T cell exhaustion or large changes to the T cell compartment in vulnerable populations such as immunosuppressed individuals with RA and older adults in long-term care, as well as controls.

During our exploration of these aims, we also sought to expand our ability to examine and characterize SARS-CoV-2 infection and vaccination responses. This led to the development of a protocol to identify spike-specific B cells using flow cytometry, which would allow phenotyping of these B cells and thus greater granularity than the existing ELISPOT method.

Chapter 2. Immunomodulatory drugs have divergent effects on humoral and cellular immune responses to SARS-CoV-2 vaccination in people living with rheumatoid arthritis

2.1 Preface

Chapter 2.1 is composed of a manuscript published in *Scientific Reports**. This manuscript aimed to determine if SARS-CoV-2 vaccination in people with rheumatoid arthritis, who were on immunosuppressive drugs, led to comparable humoral and cellular responses to those observed in controls. It also sought to extend this exploration to determine which immunosuppressive drugs impact the response to vaccination, and whether or not different drugs affect the humoral and cellular (T cell) responses after the second, third, and fourth SARS-CoV-2 vaccinations. Prior to our study, studies published in the literature primarily evaluated humoral responses, and did not often explore how different drugs may disparately affect not only the cellular and humoral arms of immunity, but also the spike-specific $CD4^+$ vs $CD8^+$ T cell responses. Our study therefore extended the evaluation of humoral and cellular responses to SARS-CoV-2 vaccinations in participants with RA to include fourth dose responses, and delineated $CD4^+$ and $CD8^+$ T cell responses.

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Immunomodulatory drugs have divergent effects on humoral and cellular immune responses to SARS-CoV-2 vaccination in people living with rheumatoid arthritis

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Understanding the efficacy of SARS-CoV-2 vaccination in people on immunosuppressive drugs, including those with rheumatoid arthritis (RA), is critical for their protection. Vaccine induced protection requires antibodies, CD4⁺ T cells, and CD8⁺ T cells, but it is unclear if these are equally affected by immunomodulatory drugs. Here, we determined how humoral and cellular SARS-CoV-2 vaccination responses differed between people with RA and controls, and which drug classes impacted these responses. Blood was collected from participants with RA on immunomodulatory drugs and controls after their second, third, and fourth SARS-CoV-2 vaccinations. Receptor binding domain (RBD)-specific antibodies were quantified by ELISA. Spike-specific memory T cells were quantitated using flow cytometry. Linear mixed models assessed the impact of age, sex, and immunomodulatory drug classes on SARS-CoV-2 vaccination responses. Compared to non-RA controls (n = 35), participants with RA on immunomodulatory drugs (n = 62) had lower anti-RBD IgG and spike-specific CD4⁺ T cell levels, but no deficits in spike-specific CD8⁺ T cells, following SARS-CoV-2 vaccination. Use of costimulation inhibitors was associated with lower humoral responses. JAK inhibitors were associated with fewer spike-specific CD4⁺ T cells. Participants with RA on immunomodulatory drugs mounted weaker responses to SARS-CoV-2 vaccination, with different drug classes impacting the cellular and humoral compartments.

Immunosuppressive medications such as steroids, disease modifying anti-rheumatic drugs (DMARDs), and biologics are commonly prescribed to treat autoimmune disorders including rheumatoid arthritis (RA). DMARDs, which include methotrexate, broadly suppress inflammatory responses, whereas biologics target and block specific inflammatory mediators or pathways (e.g. interleukin-6 and tumor necrosis factor inhibitors)[1]. Multiple large-scale studies have reported an increased risk of SARS-CoV-2 infection, hospitalization, and death in people with rheumatic diseases such as RA[2,3]. Furthermore, certain immunomodulatory drug classes, such as TNF and

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costimulation (i.e. Cytotoxic T-lymphocyte Antigen 4, CTLA-4 Ig) inhibitors, are associated with weaker humoral responses to SARS-CoV-2 vaccination[4–6].

Antibodies targeting the spike protein of SARS-CoV-2, and the neutralization capacity of these antibodies, have been widely explored as potential correlates of protection following SARS-CoV-2 vaccination[7,8]. Vaccine-elicited neutralizing antibodies were protective against symptomatic infection with the ancestral strain of SARS-CoV-2, and maintained efficacy against earlier variants of concern[7,8]. SARS-CoV-2 variants of concern, such as omicron variants, have the ability to evade humoral immunity due to the accumulation of mutations, particularly in the spike protein[9]. While antibodies are often used as a marker of protection following SARS-CoV-2 vaccination, spike-specific T cells are also critical for vaccine induced protection[10,11]. Omicron variants in particular have accumulated mutations in the spike protein that contribute to their ability to evade humoral immune responses generated by vaccination against the ancestral strain, while T cell responses are largely conserved[9,12,13]. Thus, when assessing SARS-CoV-2 vaccination responses it is necessary to evaluate both cellular and humoral immunity.

Given the likely role of T cells in long-term and cross variant protection, it is important to explore if different immunomodulatory drug classes impact T cell responses compared with humoral responses, or even differentially impact $CD4^+$ vs $CD8^+$ responses. Furthermore, the question of whether different immunomodulatory drug classes affect the subpopulations (Th1, Th2, Th17, T regulatory) of spike-specific $CD4^+$ T cells could offer insight into how protective their responses will be in the context of SARS-CoV-2 infection. In order to better understand the impact of different drug classes on humoral and cellular responses to SARS-CoV-2 vaccination, we explored both arms of immunity after 2, 3, and 4 doses of SARS-CoV-2 vaccines in people with RA, who are on immunomodulatory drugs. We found that while costimulation inhibitors are associated with weaker humoral responses in terms of antibody levels, JAK inhibitors are associated with fewer spike-specific $CD4^+$ but not $CD8^+$ T cells. Participants on JAK inhibitors also displayed an altered spike-specific $CD4^+$ T cell skew, with a greater proportion of T regulatory (Treg) cells. This study therefore highlights that different drug classes may affect both the development and the functional skew of humoral and cellular responses to SARS-CoV-2 vaccination.

Results

Participant and control demographics

Altogether 62 participants on immunomodulatory drugs for RA (median age 63.0 years [interquartile range (IQR) 55.0–68.0]; 84% female sex), and 35 control participants (median age 64.0 [IQR 54.0–70.3]; 66% female sex) who did not have autoimmune conditions and were not on immunomodulatory drugs, provided samples at multiple timepoints surrounding their second, third, and fourth SARS-CoV-2 vaccinations (Table 1). Age did not differ significantly between groups, but the RA group was much more predominantly female than controls. Most participants with RA were on DMARDs (n = 49, 79%), 40% (n = 25) were on TNF inhibitors, 17% (n = 11)

	Rheumatoid arthritis	Controls	P value
Total participants	62	35	N/A
Age, median years (IQR)	63.0 (55.0–68.0)	64.0 (54.0–70.3)	ns[a]
Sex, % female subjects (n)	84 (52)	66 (23)	0.045[b]
First dose vaccine type	48 BNT162b2 5 mRNA-1273 9 ChAdOx1	23 BNT162b2 4 mRNA-1273 7 ChAdOx1	ns[b]
Second dose vaccine type	44 BNT162b2 14 mRNA-1273 4 ChAdOx1	27 BNT162b2 6 mRNA-1273 1 ChAdOx1	ns[b]
Third dose vaccine type	39 BNT162b2 12 mRNA-1273	18 BNT162b2 12 mRNA-1273	ns[b]
Fourth dose vaccine type	18 BNT162b2 23 mRNA-1273 1 bivalent	6 BNT162b2 6 mRNA-1273 2 bivalent	ns[b]
Days between dose 1 and dose 2 (median ± SD)	71.0 ± 20.2	72.0 ± 20.6	ns[a]
Days between dose 2 and dose 3 (median ± SD)	152.5 ± 33.3	183.0 ± 27.5	<0.0001[a]
Days between dose 3 and dose 4 (median ± SD)	119.0 ± 48.5	203.0 ± 67.5	0.0004[a]
Steroids[c], % (n)	19 (12)	N/A	N/A
DMARDs[d], % (n)	79 (49)	N/A	N/A
TNF (tumor necrosis factor) and TNF receptor inhibitors[e], % (n)	40 (25)	N/A	N/A
Janus Kinase Inhibitors, % (n)	17 (11)	N/A	N/A
Costimulation inhibitor (Abatacept), % (n)	19 (12)	N/A	N/A
SARS-CoV-2 Infections, % (n)	24 (15)	14 (5)	ns[b]

Table 1. Participant demographics and immunomodulatory drugs. Vaccine type information is unknown for one control participant, and the fourth dose for one participant with RA. Vaccination history reported for all participants who provided samples after that dose. *SD* standard deviation, *DMARDs* disease modifying anti-rheumatic drugs. [a]Student's *t* test. [b]Fisher's exact test. [c]Prednisone and methylprednisolone. [d]Sulfasalazine, leflunomide, methotrexate, mycophenolate mofetil, and hydroxychloroquine. [e]Includes adalimumab, etanercept, infliximab, and golimumab.

were on JAK inhibitors, and 19% (n = 12) were on costimulation inhibitors. A small number (n = 12, 19%) were on oral steroids. Many participants were on more than one class of immunomodulatory drug. Participants with RA had a shorter interval between their second and third SARS-CoV-2 vaccinations, and third and fourth SARS-CoV-2 vaccinations, than controls (Table 1).

Participants with RA exhibit lower antibody responses to SARS-CoV-2 vaccination

Anti-receptor binding domain (RBD) antibodies are associated with neutralization and early viral control of SARS-CoV-2[14]. Using a multivariable linear mixed model with timepoint as a time-varying covariate, we found that participants living with RA had lower levels of anti-RBD IgG overall than controls following SARS-CoV-2 vaccination (Fig. 1a,b, Table 2). Unlike other statistical tests, the multivariable linear mixed does not identify specific timepoints where responses differ between RA and control cohorts, but rather determines whether the controls and RA groups are different when all other factors including time are accounted for. While age did not clearly associate with antibody levels, males had lower levels of anti-RBD IgG than females when looking in both the RA and control cohorts (Fig. 1b, Table 2). As expected, participants with a previous SARS-CoV-2 infection (COVID-19 +) had higher levels of serum anti-RBD IgG (Fig. 1b). Compared with anti-RBD IgG levels at 2–6 weeks post dose 2, antibody levels were significantly increased following dose 3 and dose 4 in controls and participants with RA (Table 2, Supplemental Fig. 1a,b). Since the participants with RA had significantly weaker humoral responses to SARS-CoV-2 vaccination, with notable variation within the cohort, we next examined which drug classes contributed to this deficit. Using a multivariable linear mixed model, which looked only within the RA cohort, it was found that individuals with RA on costimulation inhibitors had lower anti-RBD IgG responses to vaccination than those taking other classes of drugs (Fig. 1c,d, Table 2). While the model suggests that costimulation inhibitors may have a detrimental impact on anti-RBD IgG levels following SARS-CoV-2 vaccination, the limited sample size was not sufficiently powered to definitively determine drug impacts, highlighting the need for larger scale studies to validate these findings. To provide further insight into the impact costimulation inhibitors have on anti-RBD IgG levels, we broke down the participants with RA into different drug groups at 2–6 weeks post dose 3 (the timepoint with the most samples) and graphed their antibody levels (Fig. 1d). Note only four participants were on costimulation inhibitors at this timepoint, as drug regimens changed throughout the study and different participants provided samples at different timepoints. The heterogeneity in drug regimens among participants with RA, and the changes in these regimens throughout the study, do however reflect the reality of treating autoimmune disorders. Interestingly, at 2–6 weeks post dose 3 and 3 months after dose 4, no drug class impacted the neutralization capacity of the antibodies in participants with RA, against either the ancestral SARS-CoV-2 or the omicron BA.1 variant (Fig. 2a–c). This is in accordance with our finding that anti-RBD IgG levels only moderately correlated with ancestral SARS-CoV-2 neutralization at 3 months post dose 4 (Fig. 2d).

Participants with RA have lower spike-specific CD4$^+$ T cells than controls following SARS-CoV-2 vaccination

Spike-specific T cells are also thought to be critical for vaccine induced protection[10,11,15]. Participants with RA had lower levels of spike-specific CD4$^+$ T cells than controls (Fig. 3a,b, Table 2). Age, sex, and previous SARS-CoV-2 infection did not significantly influence the levels of spike-specific CD4$^+$ T cells in either participants with RA or controls (Fig. 3b, Table 2). Levels of spike-specific CD4$^+$ T cells were also not increased after the third and fourth vaccinations, compared with post dose 2, in either controls or participants with RA (Table 2, Supplemental Fig. 1c,d). Given the lower numbers of spike-specific CD4$^+$ T cells in participants with RA, a multivariable linear mixed model was used to investigate which drugs were associated with this deficit. The use of JAK inhibitors in the RA drug regimens was associated with lower spike-specific CD4$^+$ T cells (Fig. 3c,d, Table 2). We visualized this in Fig. 3d using the 2–6 weeks post dose 3 timepoint. At 2–6 weeks post dose 3, participants on costimulation inhibitors trended towards lower levels of spike-specific CD4$^+$ T cells, however by 3 months post dose 4 this gap had closed (Supplemental Fig. 2a). A trend towards lower antigen-specific CD4$^+$ T cell levels in participants treated with JAK inhibitors was also seen when we measured the influenza-specific (Agriflu) CD4$^+$ T cell levels at the 2–6 week post dose 3 timepoint (Fig. 3e). JAK inhibitors did not however clearly alter the CD4$^+$ T cell response to polyclonal TCR specificity-independent stimulation using Cytostim (Supplemental Fig. 2b).

Spike-specific CD4$^+$ T cell skew may differ by drug class

Considering that spike-specific CD4$^+$ T cell levels were lower in participants with RA on JAK inhibitors, we also wondered if the phenotypic skew of these cells differed depending on drug classes. When looking 2–6 weeks after dose 3, we found that the frequencies of spike-specific Th1 (CXCR3$^+$CCR6$^-$CCR4$^-$), Th2 (CXCR3$^-$CCR6$^-$CCR4$^+$), and Th17 (CXCR3$^+$CCR6$^+$CCR4$^+$) CD4$^+$ T cells did not differ between those on different RA drug treatments, or controls (Fig. 4a–c). Surprisingly, the frequency of spike-specific T regulatory cells (Tregs, CD25$^+$CD39$^+$) was significantly higher in participants with RA on JAK inhibitors than in those on TNF inhibitors, other drugs, or controls (Fig. 4d). In contrast, participants with RA on JAK inhibitors had lower spike-specific CD4$^+$ Th2 and Th17 cell numbers (cells per mL of whole blood) than controls but did not display higher numbers of spike-specific Tregs (Supplemental Fig. 3a–d). This suggests that the increased frequency of spike-specific Tregs in this population is not due to an increase in the numbers of these cells, but rather due to the lower levels of the other CD4$^+$ subpopulations. The influence of this change in spike-specific CD4$^+$ T cell skew on vaccine-induced protection against SARS-CoV-2 remains to be determined.

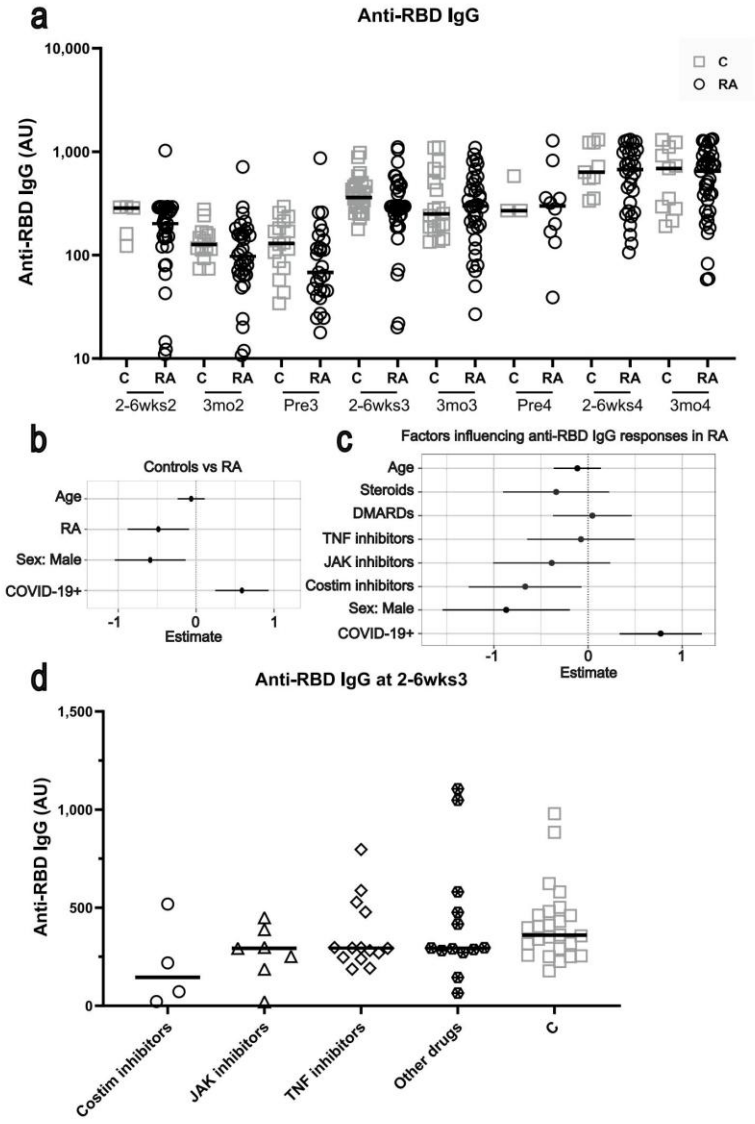

Figure 1. Serum anti-RBD IgG in participants with RA and controls following SARS-CoV-2 vaccination. (**a**) Anti-RBD IgG in the serum was measured by ELISA for participants with RA (open circles), and controls (C, grey squares). The solid line represents the median. PreX is pre dose X, 2–6wksX is 2–6 weeks post dose X, 3moX is 3 months post dose X. (**b**) Multivariable linear mixed model estimates (Log-twofold changes ± SD) comparing RA and C cohorts, and the effects of different parameters on anti-RBD IgG levels. (**c**) Multivariable linear mixed model estimates (Log-2-fold changes ± SD) within the RA cohort of the effects of different parameters, including immunomodulatory drugs, on anti-RBD IgG levels following SARS-CoV-2 vaccination. Costim denotes costimulation. (**d**) Anti-RBD IgG levels in participants with RA on costimulation inhibitors, JAK inhibitors, TNF inhibitors, other drug classes (steroids ± DMARDs), and controls (C, grey squares) at 2–6 weeks post dose 3.

Participants with RA and controls have similar numbers of spike-specific CD8⁺ T cells following SARS-CoV-2 vaccination

Variable	Effect estimate (±SD)	Parameter	P value[a]
Rheumatoid arthritis	−0.48 ± 0.20	Anti-RBD IgG	< 0.05
	−1.07 ± 0.34	Spike-specific CD4+[b]	< 0.05
	0.14 ± 0.41	Spike-specific CD8+	0.74
Age	−0.06 ± 0.09	Anti-RBD IgG	0.47
	−0.09 ± 0.15	Spike-specific CD4+	0.54
	−0.22 ± 0.18	Spike-specific CD8+	0.23
Male sex	−0.59 ± 0.23	Anti-RBD IgG	0.01
	−0.46 ± 0.39	Spike-specific CD4+	0.25
	0.92 ± 0.48	Spike-specific CD8+	0.06
COVID-19 infection (positive)	0.58 ± 0.17	Anti-RBD IgG	< 0.001
	0.49 ± 0.30	Spike-specific CD4+	0.10
	−0.001 ± 0.35	Spike-specific CD8+	0.99
Timepoint[c] (compared with 2–6 weeks post dose 2)			
3-months post dose 2	−0.75 ± 0.16	Anti-RBD IgG	< 0.001
	−0.52 ± 0.27	Spike-specific CD4+	0.06
	−0.22 ± 0.30	Spike-specific CD8+	0.47
Pre-dose 3	−0.88 ± 0.18	Anti-RBD IgG	< 0.001
	−0.62 ± 0.30	Spike-specific CD4+	< 0.05
	−0.07 ± 0.33	Spike-specific CD8+	0.83
2–6 weeks post dose 3	0.93 ± 0.16	Anti-RBD IgG	< 0.001
	0.47 ± 0.27	Spike-specific CD4+	0.09
	0.61 ± 0.31	Spike-specific CD8+	< 0.05
3-months post dose 3	0.69 ± 0.17	Anti-RBD IgG	< 0.001
	0.32 ± 0.28	Spike-specific CD4+	0.25
	0.96 ± 0.32	Spike-specific CD8+	< 0.05
Pre dose 4	0.79 ± 0.26	Anti-RBD IgG	< 0.05
	0.59 ± 0.43	Spike-specific CD4+	0.17
	1.04 ± 0.49	Spike-specific CD8+	< 0.05
2–6 weeks post dose 4	1.88 ± 0.19	Anti-RBD IgG	< 0.001
	0.57 ± 0.32	Spike-specific CD4+	0.07
	1.67 ± 0.36	Spike-specific CD8+	< 0.001
3-months post dose 4	1.51 ± 0.18	Anti-RBD IgG	< 0.001
	0.59 ± 0.31	Spike-specific CD4+	0.05
	1.30 ± 0.34	Spike-specific CD8+	< 0.001
Immunomodulatory drugs			
Steroids	−0.34 ± 0.29	Anti-RBD IgG	0.24
	0.04 ± 0.43	Spike-specific CD4+	0.93
	−0.83 ± 0.50	Spike-specific CD8+	0.09
DMARDs	0.04 ± 0.21	Anti-RBD IgG	0.83
	−0.58 ± 0.33	Spike-specific CD4+	0.08
	−0.31 ± 0.37	Spike-specific CD8+	0.40
TNF inhibitors	−0.08 ± 0.29	Anti-RBD IgG	0.80
	0.41 ± 0.43	Spike-specific CD4+	0.34
	0.48 ± 0.50	Spike-specific CD8+	0.34
JAK inhibitors	−0.39 ± 0.32	Anti-RBD IgG	0.23
	−2.57 ± 0.48	Spike-specific CD4+	< 0.001
	0.05 ± 0.56	Spike-specific CD8+	0.93
Costimulation inhibitor	−0.67 ± 0.30	Anti-RBD IgG	< 0.05
	−0.60 ± 0.47	Spike-specific CD4+	0.21
	−0.14 ± 0.53	Spike-specific CD8+	0.80

Table 2. Multivariable linear mixed modelling of the impact of variables on humoral and cellular responses following COVID-19 vaccination. [a]Significance cut-off $\alpha = 0.05$. [b]Spike-specific $CD4^+$ and $CD8^+$ refer to T cells. [c]Estimates from mixed model including both controls and participants with RA.

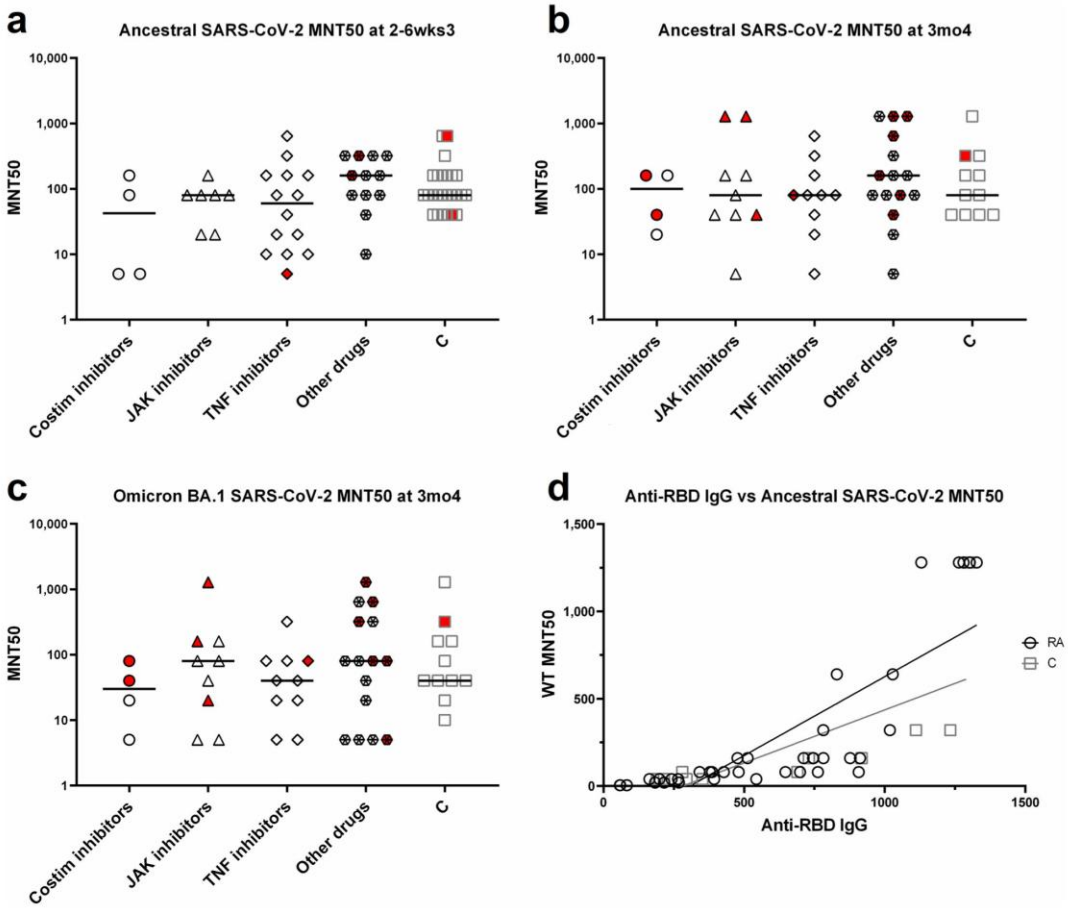

Figure 2. Neutralization capacity against ancestral and omicron BA.1 SARS-CoV-2 in participants with RA and controls. MNT50 against ancestral SARS-CoV-2 in participants with RA on costimulation inhibitors, JAK inhibitors, TNF inhibitors, other drug classes (steroids ± DMARDs), and controls (C, grey squares) at 2–6 weeks post dose 3 (**a**), and 3 months post dose 4 (**b**). (**c**) MNT50 against the omicron BA.1 variant of SARS-CoV-2 in participants with RA and controls at 3 months post dose 4. Comparisons were made by Brown-Forsythe tests with Dunnett's T3 post-hoc tests. (**d**) Relationship between anti-RBD IgG levels and ancestral (WT) MNT50 in participants with RA and controls assessed using simple linear regression and the Pearson correlation coefficient (R = 0.81 for RA, R = 0.71 for C). Symbols filled in red indicate participants who have previously had a SARS-CoV-2 infection.

In contrast to what was observed with the spike-specific $CD4^+$ T cell numbers, participants with RA overall did not exhibit any deficits in numbers of spike-specific $CD8^+$ T cells (Fig. 5a,b, Table 2). When looking at both the control and RA samples, spike-specific $CD8^+$ T cell numbers increased after the third and fourth SARS-CoV-2 vaccinations, compared with post dose 2 (Table 2, Supplemental Fig. 1e,f). Age and previous SARS-CoV-2 infection did not clearly associate with levels of spike-specific $CD8^+$ T cells in either the control or RA samples (Fig. 5b,c). When looking within the RA cohort only, no drug class was significantly associated with spike-specific $CD8^+$ T cell numbers (Fig. 5c). Among participants with RA, males did however exhibit more robust spike-specific $CD8^+$ T cell responses than females (Fig. 5b,c, Table 2). Finally, to illustrate any differences, or lack thereof, in antigen-specific $CD8^+$ T cell responses between drug groups, we plotted the samples from the RA cohort at 2–6 weeks post dose 3 (Fig. 5d,e). The spike- and influenza-specific $CD8^+$ T cell responses were not significantly different between drug groups (Fig. 5d,e). There were also no drug class associated deficits in the $CD8^+$ T cell response to polyclonal TCR specificity-independent stimulation using Cytostim (Supplemental Fig. 2c).

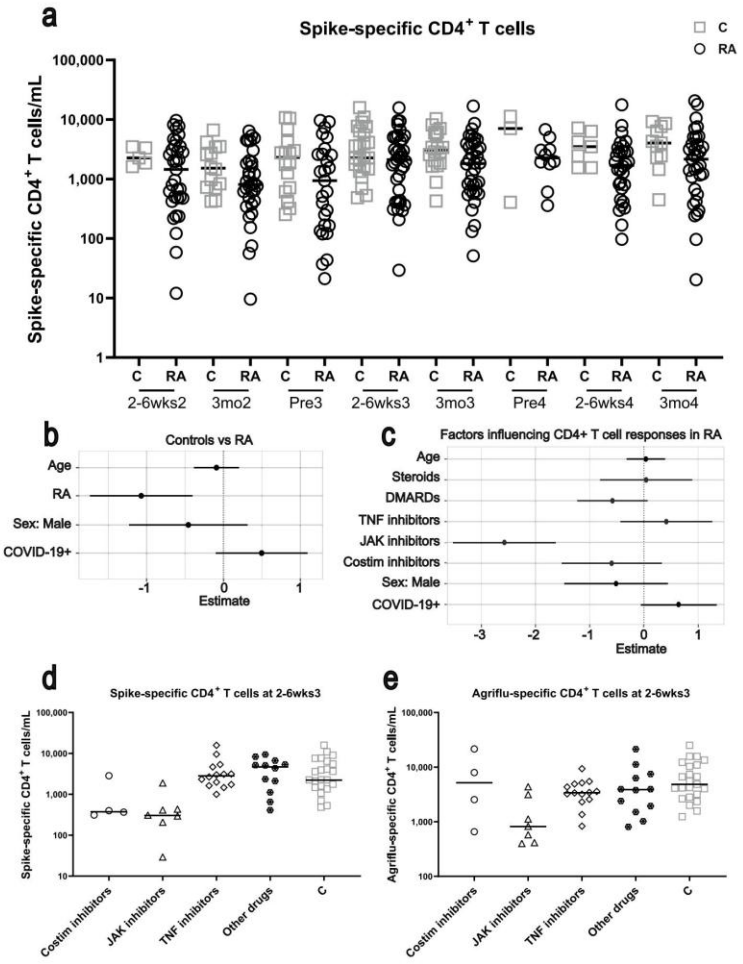

Figure 3. Levels of spike-specific CD4+ T cells in participants with RA on immunomodulatory drugs, and controls. (**a**) The numbers of spike-specific CD4+ T cells were measured using AIM assays in participants with RA (open circles), and controls (C, grey squares). The solid line represents the median. PreX is pre dose X, 2–6wksX is 2–6 weeks post dose X, 3moX is 3 months post dose X. (**b**) Multivariable linear mixed model estimates (Log-twofold changes ± SD) comparing RA and C cohorts, and the effects of different parameters on spike-specific CD4+ T cell numbers. (**c**) Multivariable linear mixed model estimates (Log-twofold changes ± SD) within the RA cohort of the effects of different parameters, including immunomodulatory drugs, on spike-specific CD4+ T cells following SARS-CoV-2 vaccination. (**d**) Spike-specific CD4+ T cell levels in participants with RA on costimulation inhibitors, JAK inhibitors, TNF inhibitors, other drug classes (steroids ± DMARDs), and controls (C, grey squares) at 2–6 weeks post dose 3. (**e**) Influenza-specific (Agriflu-specific) CD4+ T cell levels in participants with RA on different immunomodulatory drug classes at 2–6 weeks post third SARS-CoV-2 vaccination. Comparisons were made by Brown-Forsythe tests with Dunnett's T3 post-hoc tests.

Discussion

Understanding the impact of different immunomodulatory drug classes on humoral and cellular responses to SARS-CoV-2 vaccination is critical for the protection of vulnerable populations, including those with RA. In particular, whether certain drug classes affect only cellular or only humoral responses to SARS-CoV-2 vaccination, or affect the skew of the cellular responses, still needs to be fully elucidated. In this study we found that participants on RA treatments had lower anti-RBD IgG levels than non-RA controls following their second, third, and fourth SARS-CoV-2 vaccinations. Additional vaccine doses did boost the antibodies levels, as did previous SARS-CoV-2 infection. Inclusion of costimulation inhibitors in their immunomodulatory drug regimen was associated with weaker humoral responses. This finding is consistent with other studies that showed the costimulation inhibitor abatacept weakened humoral responses to pneumococcal and SARS-CoV-2 vaccination[6,16,17].

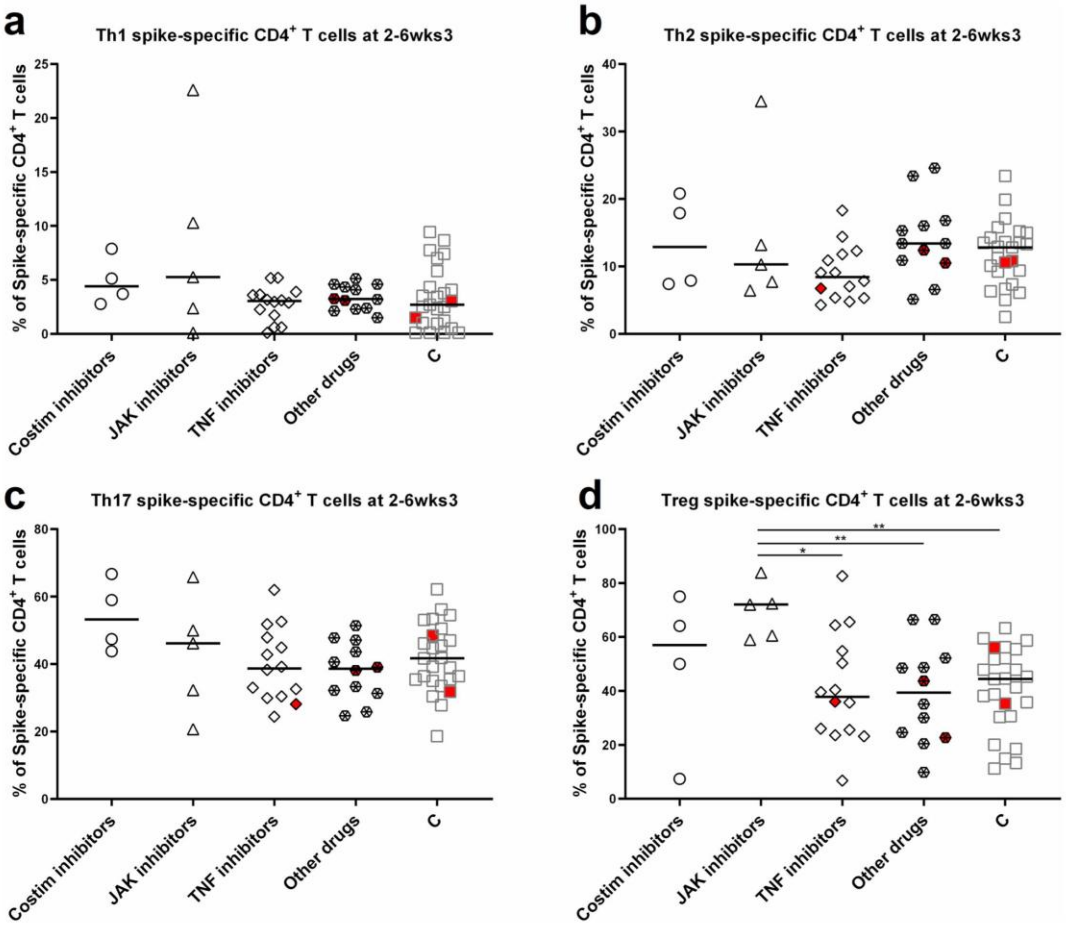

Figure 4. Skew of spike-specific CD4⁺ T cells in participants with RA, on immunomodulatory drugs, and controls at 2–6 weeks post dose 3. (**a**) The frequency of spike-specific CD4⁺ T cells, determined by AIM assays, displaying a Th1 skew ($CXCR3^+CCR6^-CCR4^-$) in participants with RA and controls (C, grey squares). Participants with RA were broken down by drug class into those taking costimulation inhibitors, JAK inhibitors, TNF inhibitors, or other drug classes (steroids ± DMARDs). Participants were only plotted if there were >20 $CD4^+AIM^+$ events, allowing accurate determination of phenotype. (**b**) The frequency of spike-specific CD4⁺ T cells displaying a Th2 skew ($CXCR3^-CCR6^-CCR4^+$) in participants with RA and controls. (**c**) The frequency of spike-specific CD4⁺ T cells displaying a Th17 skew ($CXCR3^-CCR6^+CCR4^+$) in participants with RA and controls. (**d**) The frequency of spike-specific CD4⁺ T cells displaying a T regulatory phenotype (Tregs, $CD25^+CD39^+$) in participants with RA and controls. Symbols filled in red indicate participants who have previously had a SARS-CoV-2 infection. Comparisons were made by Brown-Forsythe tests with Dunnett's T3 post-hoc tests. $p<0.05$ *, $p<0.01$ **.

Interestingly, we were unable to establish if any RA drug significantly affected neutralization capacity of the antibodies against either the ancestral or omicron BA.1 SARS-CoV-2. The primary limitation of our study is the small overall sample size, with low numbers of participants on certain drug classes, which we addressed by using the minimum number of clinically relevant covariates. Age and sex are known to impact immune responses, and we were able to account for this impact on humoral and cellular responses to SARS-CoV-2 vaccination[18–20]. Our study highlights the need for studies with larger sample sizes in the future to detect differential drug treatment effects on vaccination responses with a higher degree of certainty.

Though antibody levels are commonly examined following vaccination, spike-specific T cells are also thought to be critical for vaccine induced protection, and protection against variants of concern[10,11,15,21]. We found that participants on RA treatments had lower numbers of spike-specific CD4⁺ T cells following SARS-CoV-2 vaccination. Use of JAK inhibitors was associated with this significantly weaker response[22]. Other studies have reported decreased IFN-γ responses upon stimulation with the spike protein in participants with immune-mediated

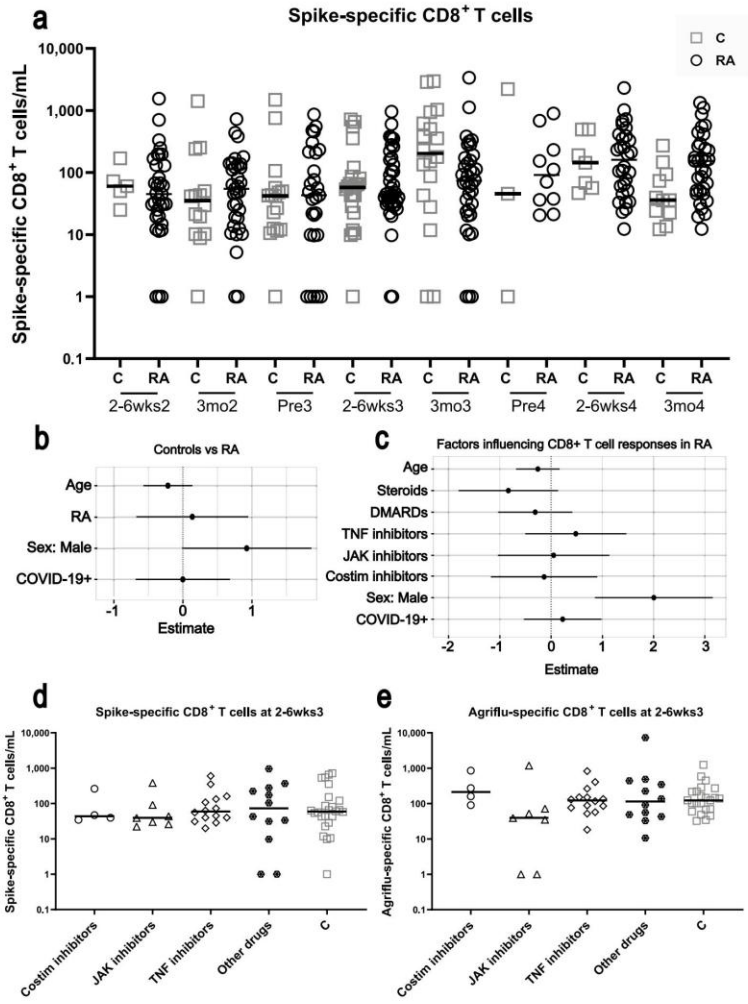

Figure 5. Levels of spike-specific CD8⁺ T cells in participants with RA on immunomodulatory drugs, and controls. (**a**) The number of spike-specific CD8⁺ T cells were measured using AIM assays in participants with RA (open circles), and controls (C, grey squares). The solid line represents the median of a group. PreX is pre dose X, 2-6wksX is 2–6 weeks post dose X, 3moX is 3 months post dose X. (**b**) Multivariable linear mixed model estimates (Log-twofold changes ± SD) comparing RA and C cohorts, and the effects of different parameters on spike-specific CD8⁺ T cell numbers. (**c**) Multivariable linear mixed model estimates (Log-twofold changes ± SD) within the RA cohort of the effects of different parameters, including immunomodulatory drugs, on spike-specific CD8⁺ T cells following SARS-CoV-2 vaccination. (**d**) Spike-specific CD8⁺ T cell levels in participants with RA on costimulation inhibitors, JAK inhibitors, TNF inhibitors, other drug classes (steroids ± DMARDs), and controls (C, grey squares) at 2–6 weeks post dose 3. (**e**) Influenza-specific (Agriflu-specific) CD8⁺ T cell levels in participants with RA on different immunomodulatory drug classes at 2–6 weeks post third SARS-CoV-2 vaccination. Comparisons were made by Brown-Forsythe tests with Dunnett's T3 post-hoc tests.

inflammatory disorders on DMARDs, costimulation inhibitors, and TNF inhibitors[22,23]. These studies looked at earlier timepoints, following the first and second SARS-CoV-2 vaccinations, highlighting that booster vaccinations may be critical for participants on these drugs to mount responses comparable to those seen in controls. We saw this in our own study, as participants on costimulation inhibitors appeared to have lower spike-specific CD4⁺ T cell levels at 2–6 weeks post dose 3, but these levels had become comparable to controls following dose 4.

JAK inhibitors not only impacted the number of spike-specific CD4⁺ T cells, but also the phenotypic subpopulations. While the frequencies of Th1, Th2, and Th17 cells were similar to other drug classes, the frequency

of Tregs was increased in participants with RA on JAK inhibitors. This was not due to an increase in the number of Tregs, but likely due to the decrease in the number of Th17 and Th2 cells in this group, allowing Tregs to account for more of the remaining spike-specific CD4+ T cell population. To our knowledge, the impact of JAK inhibitors on the skew of CD4+ T cell responses following SARS-CoV-2 vaccination has not been previously reported. However, JAK inhibitors have been reported to alter T cell skews in graft-versus-host disease[24]. The shift of the spike-specific CD4+ T cell population towards a regulatory phenotype may decrease the viral clearance of SARS-CoV-2 in these participants upon infection, further dampening their vaccine-induced protection[25,26]. The impact of JAK inhibitors on SARS-CoV-2 infection remains to be definitively determined, with conflicting reports in the literature[27,28]. In contrast, previous studies have shown that the presence of high numbers of CD8+ T cells correlates with improved SARS-CoV-2 infection survival in patients with hematologic cancer, and in non-human primate models[10,29]. We observed that participants with RA on various classes of immunomodulatory drugs mounted spike-specific CD8+ T cell responses that were comparable to those found in non-RA controls, similar to what has been previously reported in participants with RA and controls after the third SARS-CoV-2 vaccination[22].

In summary, our study determined that participants with RA on immunomodulatory drugs generally mount weaker humoral and CD4+ T cell responses to SARS-CoV-2 vaccination than controls. However, the drug classes which impact these arms of immunity differ. Costimulation inhibitors were associated with weaker humoral responses, while JAK inhibitors were associated with lower levels of spike-specific CD4+ T cells. Moving forward, it will be critical to consider which drug classes patients are taking, as the drugs could differentially impact infection risk and long-term cross variant protection.

Methods

Participant recruitment and study design

From May 2021 to February 2023, adult (18+) participants classified as having RA (n = 62) according to the criteria from the European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) were recruited from a tertiary care rheumatology clinic in Hamilton, Ontario. Adult (18+) controls C, (n = 35) without autoimmune disease and not on immunomodulatory drugs, were recruited in the local community. Exclusion criteria (for both RA and controls) were significant immunodeficiency syndromes such as HIV, any prior/current treatment with rituximab, acute febrile illness on the scheduled blood collection date, pregnancy, anyone involved in another new vaccine clinical trial, and anyone undergoing chemotherapy. The age distribution of the RA and control groups were similar.

Sample collection

Participants had their blood drawn at up to 8 timepoints: 2–6 weeks (RA n = 33, C n = 5) and 3-months after their second vaccination (RA n = 34, C n = 14), before dose 3 (RA n = 27, C n = 16), 2–6 weeks post dose 3 (RA n = 40, C n = 25), 3-months post dose 3 (RA n = 38, C n = 19), before dose 4 (RA n = 10, C n = 3), 2–6 weeks post dose 4 (RA n = 31, C n = 9), and 3 months post dose 4 (RA n = 39, C n = 11). Study design was cross-sectional based on sample availability, in that not all participants provided samples for all timepoints. Table 1 reports demographic information for all enrolled participants.

Venous blood was collected in sodium heparin vacutainer tubes. One non-heparinized vacutainer tube was also collected and centrifuged to separate the serum as per manufacturer directions (Becton Dickinson). Serum was stored at − 80 °C until use.

ELISAs

Serum was thawed and anti-SARS-CoV-2 receptor binding domain (RBD) immunoglobulins were evaluated by ELISA as previously described, with serum diluted within the linear range[30,31]. SARS-CoV-2 infections were determined by a positive PCR or rapid test, or by seroconversion using an anti-nucleocapsid IgG ELISA[32].

Neutralization assays

Serum was thawed and antibody neutralization capacity assessed using live ancestral and omicron BA.1 SARS-CoV-2 cell culture assays as previously described[33]. Results reported as MNT50 (geometric microneutralization titers at 50%).

Activation induced marker assays

To quantitate SARS-CoV-2 memory CD4+ and CD8+ T cells, activation induced marker assays were used as previously described[30]. Briefly, peptides derived from the SARS-CoV-2 spike protein (SARS-CoV-2 S Immunodominant Peptivator, Miltenyi Biotec, 1 µg/ml), influenza peptides (AgriFlu, Afluria Tetra inactivated influenza vaccine 2020–2021 season, Seqirus, 4 µl of 0.12 µg/µl), or Cytostim (Miltenyi Biotec, 0.5 µl/well) were used to stimulate 100 µl of whole blood for 48 h at 37 °C. IMDM was added to each well to bring the final volume to 200 µl.

Characterization of T cell populations were then performed using fluorochrome-conjugated antibodies against CD3 (1:50, clone UCHT1, BD Biosciences), CD4 (1:50, clone SK3, BD Biosciences), CD25 (1:50, clone M-A251, BD Biosciences), CD134 (1:100, clone ACT-35, BioLegend), CD39 (1:25, clone A1, BioLegend), CD8 (1:50, clone RPA-T8, BD Biosciences), CD137 (1:50, clone 4B4-1, BioLegend), CD69 (clone FN50, BioLegend), CXCR3 (1:25, clone G025H7, BioLegend), CCR6 (1:25, clone G034E3, BioLegend), and CCR4 (1:50, clone L291H4, BioLegend) suspended in Brilliant Stain Buffer Plus (BD Biosciences) and phosphate buffered saline (PBS). Samples were fixed using 1xFix/Lyse solution (eBioscience), washed with PBS, and resuspended in FACS Wash (0.5% (w/v) bovine serum albumin (BSA), 5 mM EDTA (pH 7.4–7.6) in PBS). AIM+CD8+ and AIM+CD4+ T cells that responded to the presented peptides (antigen-specific) and thus expressed activation markers (i.e.,

$CD69^+CD137^+$ for CD8; $CD25^+CD134^+$ for CD4) were quantitated by flow cytometry. We refer to the cells as spike-specific due to their activated signature in response to exposure to the spike protein. $CD4^+$ T cell subsets were identified by chemokine receptor expression: Th1 ($CXCR3^+CCR6^-CCR4^-$), Th2 ($CXCR3^-CCR4^+CCR6^-$), and Th17 ($CXCR3^-CCR4^+CCR6^+$)[32]. Tregs were identified as CD4+ CD39+[34]. Cell numbers were quantitated using CountBright Absolute Counting Beads (Invitrogen).

The flow cytometry gating strategy can be seen in Supplemental Fig. 4. Samples were acquired on a Cytoflex LX (4 laser, Beckman Coulter) using CytExpert software. Data analysis was conducted with FlowJo version 10 (TreeStar).

Statistics

Data were analyzed in R version 4.1.2 (R Core Team 2022) using the tidyverse, lmerTest, and emmeans packages. Antibody and T cell data were log transformed and then analyzed using multivariable linear mixed models. The first objective was to determine if there were differences in the antibody or T cell responses to SARS-CoV-2 vaccination between participants with RA and controls. To address objective 1, the first mixed models compared the humoral and cellular responses between participants with RA and controls, not considering drug classes. The second objective was to determine which drugs may impact the humoral or cellular responses to SARS-CoV-2 vaccination in participants with RA, with the associated mixed model looking only within the RA cohort. Given our primary limitation being sample size, we selected the minimum number of appropriate, clinically relevant covariates for the mixed models (age, sex, vaccination types, any prior COVID-19 infection). Changes in drug regimens of participants throughout the study were accounted for in the model, and assigned based on whether or not a patient was on that drug at the timepoint. Fisher's exact tests and Student's *t* tests used to compare group demographics. In GraphPad version 8, Brown-Forsythe tests (one-way ANOVAs accounting for unequal standard deviations) with Dunnett's T3 post-hoc tests were used to assess differences in T cell responses to influenza peptides and polyclonal stimulation (cytostim), as these measures were collected at one timepoint. $P < 0.05$ was considered statistically significant.

Study approval

Study recruitment and procedures were reviewed and approved by the Hamilton Integrated Research Ethics Board (HiREB) protocol #13307. Participants provided informed consent for sample and data collection and publication prior to participation. All procedures were performed in accordance with the approved study protocol.

Data availability

Data and statistical code for analyses are available upon request to Dawn Bowdish.

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Author contributions
J.M.B.—processed samples, performed AIM assays, analyzed data and generated figures, wrote manuscript. J.A.B.—processed samples, performed AIM assays, contributed to data interpretation and manuscript editing. J.C.A.—conducted neutralization assays. A.H.—developed and optimized ELISAs for antibody detection, performed ELISAs. B.C.—collected and processed samples, collected participant metadata, aided in recruitment of controls. B.B.—clinical coordinator, recruited and contacted patients, collected samples. L.H.—collected participant metadata and helped schedule participant blood draws. S.L.—collected participant metadata. E.Y.—collected participant metadata. H.B.—conducted ELISAs for anti-RBD IgG and anti-nucleocapsid antibodies. J.E.—provided input on appropriate statistical modelling, coding, and covariate inclusion. I.N.—provided aid for ELISAs, provided critical input to study design and interpretation. J.B.—provided critical input to study design and data interpretation. Developed cellular assays. S.B.—obtained funding, contributed to data interpretation and manuscript editing. M.J.L.—created study design and obtained funding, contributed to data interpretation and analysis. Aided manuscript editing. D.M.E.B.—created study and obtained funding, contributed to data interpretation, experimental design, and manuscript writing. All authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

Additional information
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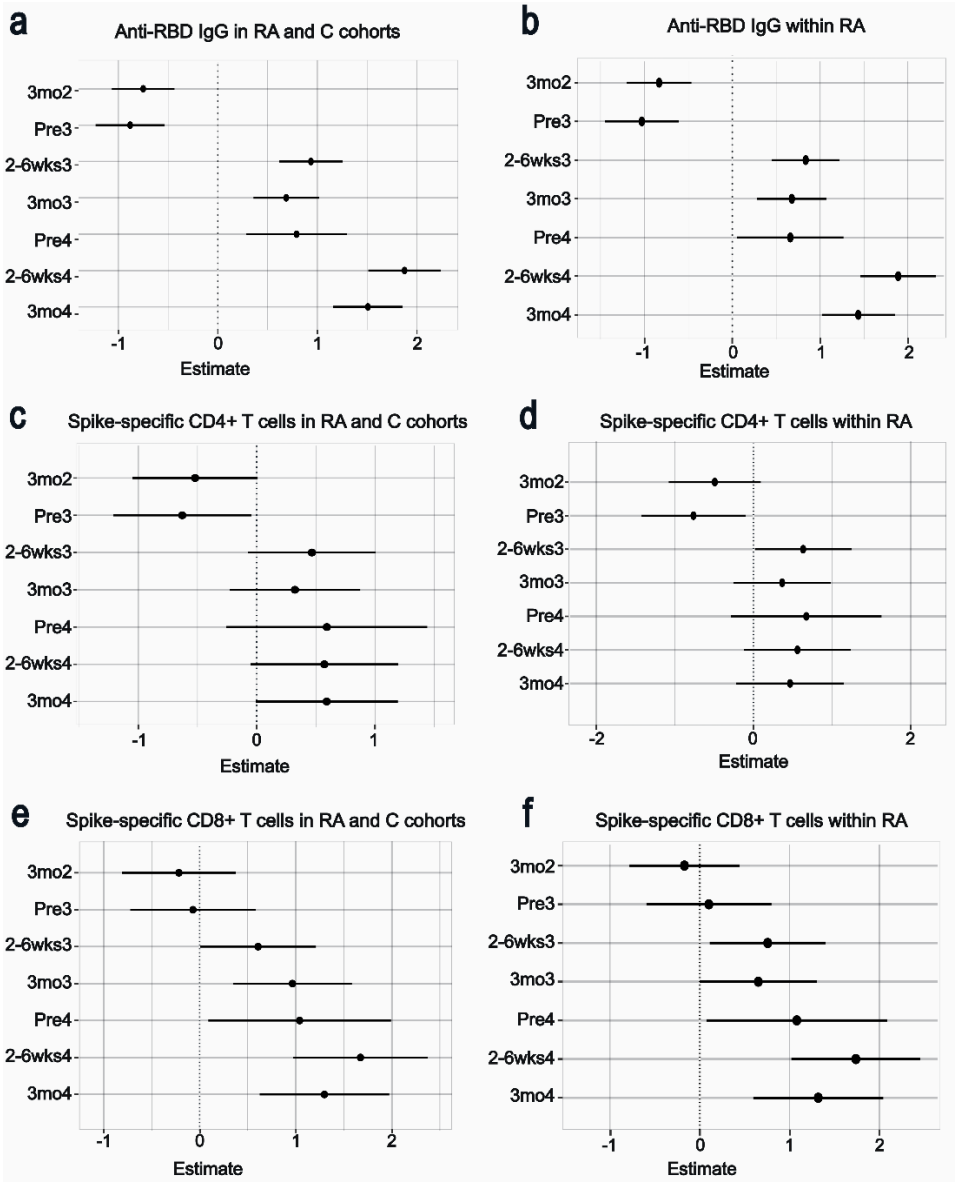
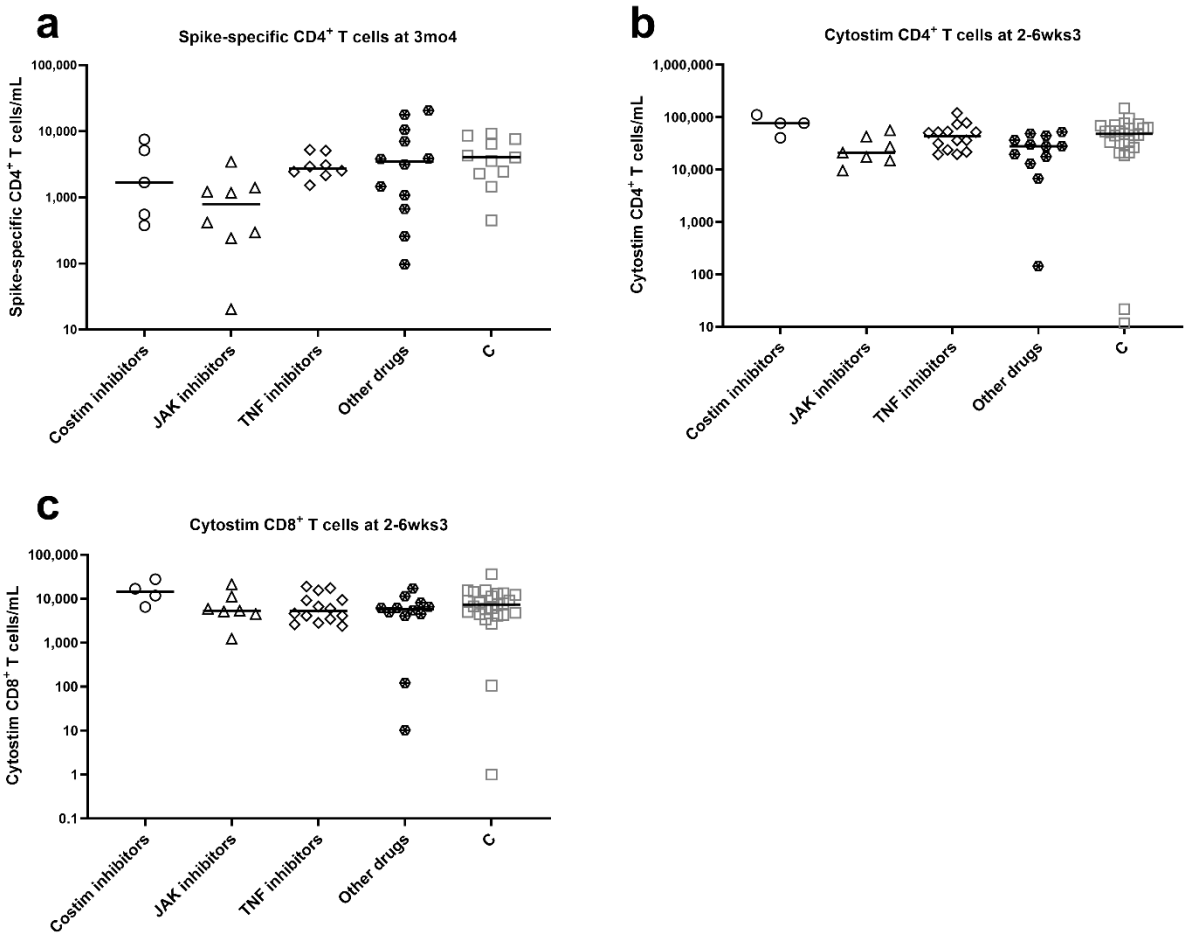
Supplemental Materials

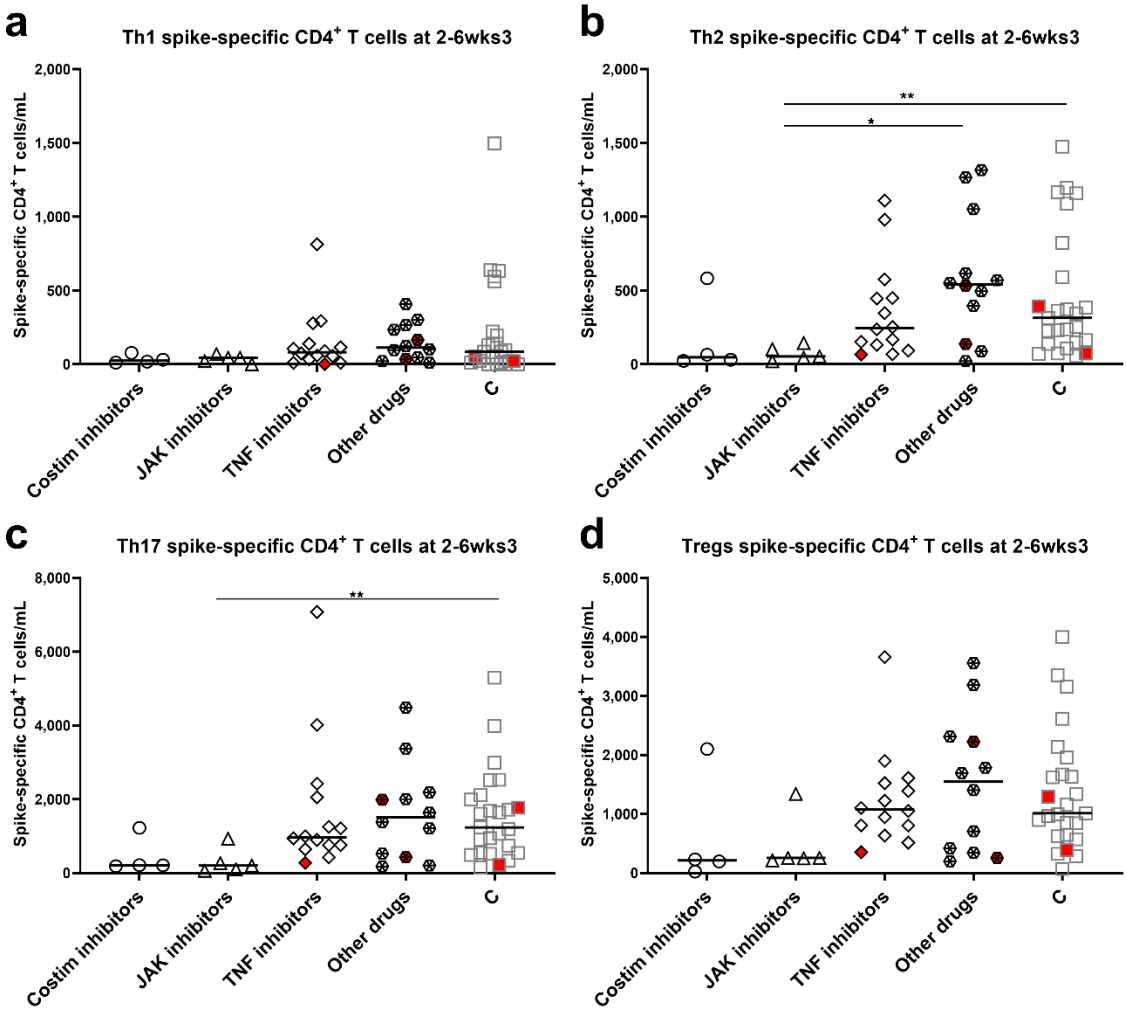


Supplemental Figure 1. Impact of timepoint and dose on SARS-CoV-2 vaccination responses. (a) Multivariable linear mixed model estimates (Log-2 fold changes) examining RA and C cohorts, and the impact of timepoint on anti-RBD IgG levels. (b) Multivariable linear mixed model estimates (Log-2 fold changes) examining the impact of timepoint and vaccine doses on anti-RBD IgG levels within the RA cohort. (c, d) Multivariable linear mixed model estimates (Log-2 fold changes) examining the impact of timepoint and vaccine doses on spike-specific $CD4^+$ T cell levels in RA and C cohorts (c), and within the RA cohort only (d). (e, f) Multivariable linear mixed model estimates (Log-2 fold

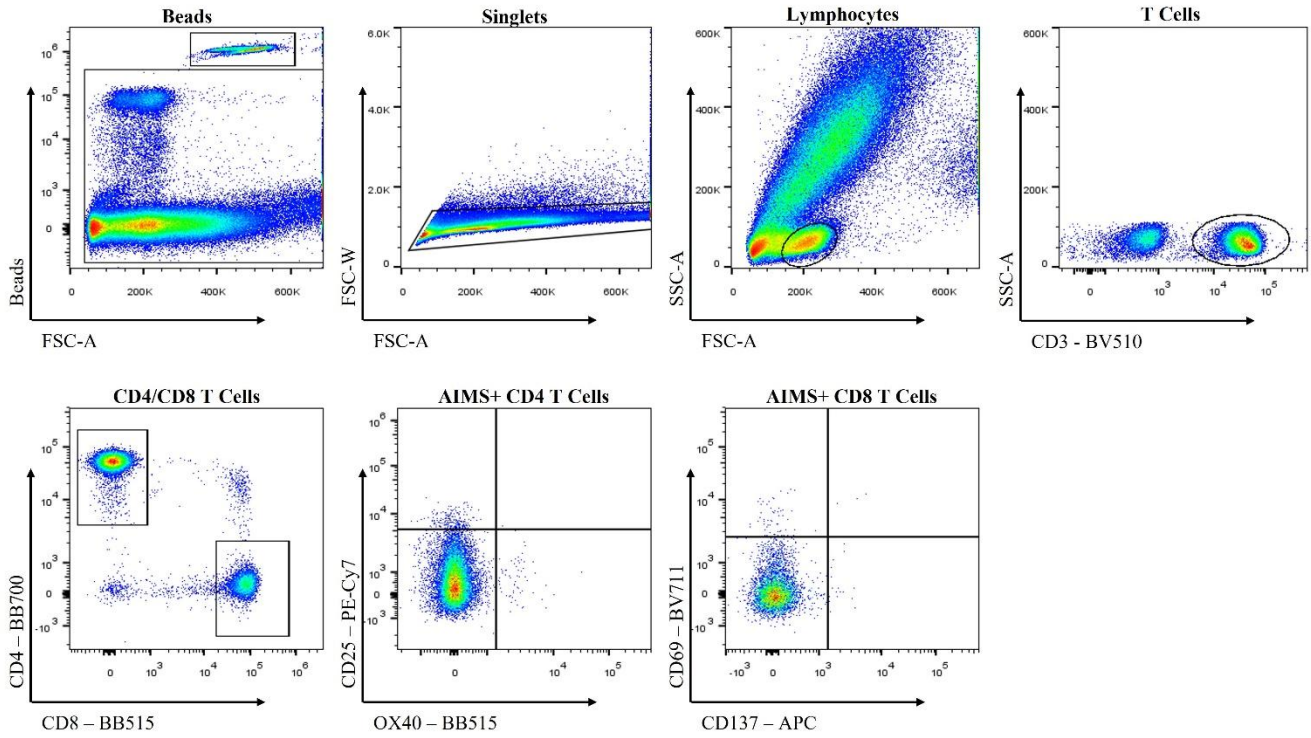
changes) examining the impact of timepoint and vaccine doses on spike-specific $CD8^+$ T cell levels in RA and C cohorts (e), and within the RA cohort only (f).



Supplemental Figure 2. $CD4^+$ and $CD8^+$ T cell responses to polyclonal stimulation broken down by drug group. (a) Spike-specific $CD4^+$ T cell levels in participants with RA on costimulation inhibitors, JAK inhibitors, TNF inhibitors, other drug classes (steroids +/- DMARDs), and controls (C, grey squares) at 3 months post dose 4. (b) The number of $CD4^+$ T cells and $CD8^+$ T cells (c) activated in response to Cytostim were measured using AIM assays in participants with RA on costimulation inhibitors, JAK inhibitors, TNF inhibitors, other drug classes (steroids +/- DMARDs), and controls (C, grey squares) at 2-6 weeks post dose 3. The solid line represents the median of a group. Comparisons were made by Brown-Forsythe tests with Dunnett's T3 post-hoc tests.



Supplemental Figure 3. Skew of spike-specific CD4+ T cells in participants with RA, on immunomodulatory drugs, and controls at 2-6 weeks post dose 3. (a) The number of spike-specific $CD4^+$ T cells per mL of whole blood, determined by AIM assays, displaying a Th1 skew ($CXCR3^+CCR6^-CCR4^-$) in participants with RA and controls (C, grey squares). Participants with RA were broken down by drug class into those taking costimulation inhibitors, JAK inhibitors, TNF inhibitors, or other drug classes (steroids +/- DMARDs). Participants were only plotted if there were >20 $CD4^+AIM^+$ events, allowing accurate determination of phenotype. (b) The number of spike-specific $CD4^+$ T cells displaying a Th2 skew ($CXCR3^-CCR6^-CCR4^+$) in participants with RA and controls. (c) The number of spike-specific $CD4^+$ T cells displaying a Th17 skew ($CXCR3^-CCR6^+CCR4^+$) in participants with RA and controls. (d) The number of spike-specific $CD4^+$ T cells displaying a T regulatory skew (Tregs, $CD25^+CD39^+$) in participants with RA and controls. Symbols filled in red indicate participants who have previously had a SARS-CoV-2 infection. Comparisons were made by Brown-Forsythe tests with Dunnett's T3 post-hoc tests. $p < 0.05$ *, $p < 0.01$ **.



Supplemental Figure 4. AIM assay gating strategy. Count beads were separated from the other events, followed by doublet exclusion and gating on the lymphocyte population. T cells were then identified by expression of CD3 and divided into $CD4^+$ and $CD8^+$ subsets. AIM^+ $CD4^+$ T cells co-expressed CD25 and OX40, while AIM^+ $CD8^+$ T cells co-expressed CD69 and CD137.

2.2 Hybrid Immunity, Fourth, and Fifth SARS-CoV-2 vaccination responses in participants with RA

Following the previously published manuscript in *Scientific Reports*[317], we continued to collect samples from participants with RA and controls after the fourth and fifth SARS-CoV-2 vaccinations (Appendix 2). The goal was to expand on our exploration of hybrid immunity around the fourth and fifth vaccinations, as well as generally report the fifth dose vaccination responses. These samples were collected during a time when the Omicron variant was circulating, and thus more infections were anticipated. The number of infected participants ended up being quite small, as did the number of samples collected after the fifth vaccination. As such, this was an observational study that was not powered to make definitive conclusions. We have, however, included the data and methods for the new readouts here. Samples collected at 2-6 weeks and 3 months post vaccination have been pooled for post dose 4 (pd4) and post dose 5 (pd5), and each participant only contributed one sample to pd4 and/or pd5. Participants were reported as having a previous SARS-CoV-2 infection if they tested positive by either PCR, rapid antigen test, or seroconverted to become positive for anti-N IgG[317].

Chapter 3. Reassuring humoral and cellular immune responses to SARS-CoV-2 vaccination in participants with systemic sclerosis

3.1 Preface

This chapter contains a manuscript published in *Immunology Letters**. Continuing our investigations of SARS-CoV-2 vaccination responses in people with autoimmune conditions, who are typically on immunosuppressive drugs, we also explored the humoral and cellular responses in participants with SSc, compared to controls, following the second, third, and fourth SARS-CoV-2 vaccinations. Previous studies on SARS-CoV-2 vaccination responses in participants with SSc focused on humoral responses to the first and second doses[301–304]. Our study therefore evaluated the responses to the, at the time, more recent vaccine doses, and to our knowledge was the first to include a comprehensive analysis of $CD4^+$ and $CD8^+$ T cell responses to SARS-CoV-2 vaccination in participants with SSc.

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Reassuring humoral and cellular immune responses to SARS-CoV-2 vaccination in participants with systemic sclerosis

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SUMMARY

Individuals with systemic sclerosis (SSc) are particularly susceptible to SARS-CoV-2 infections, yet it remains to be determined if they generate humoral and cellular responses comparable to controls following SARS-CoV-2 vaccinations. Herein, we collected blood and serum after second, third, and fourth SARS-CoV-2 vaccinations in patients with SSc and controls. Following each dose, participants with SSc mounted comparable serum anti-RBD IgG, anti-RBD IgA, and spike-specific $CD4^+$ and $CD8^+$ T cell responses to those found in controls. At 3 months post dose 2, the frequencies of Th1, Th2, Th17, and Treg spike-specific $CD4^+$ T cells in participants with SSc did not differ from controls. At 2-6 weeks post dose 3, participants with SSc displayed reduced frequencies, but not numbers, of Th17-polarized spike-specific $CD4^+$ T cells. Thus, participants with SSc did not display significantly weaker humoral or cellular responses to SARS-CoV-2 vaccination than controls, enabling reassurance of vaccine immunogenicity in participants with SSc.

KEYWORDS: Systemic sclerosis; SARS-CoV-2; COVID-19; Vaccines; Autoimmune diseases

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disorder characterized by vasculopathy and excessive fibrosis of multiple organ systems, including the skin and lungs[1]. Prior to vaccination, patients with SSc may have a greater risk of SARS-CoV-2 infection than the general population, with more severe SSc disease activity correlating with higher SARS-CoV-2 prevalence and mortality[2].

For example, interstitial lung disease (ILD), a severe manifestation of SSc, is linked to a greater prevalence of SARS-CoV-2 infections[2]. Given the likely susceptibility of patients with SSc to SARS-CoV-2 infections and poorer outcomes, it is important to understand whether vaccination is effective in people living with SSc.

SSc pathology is characterized by perturbations in various leukocyte populations and in circulating serum cytokines, suggesting broad immune dysregulation[3–5]. Immunomodulatory drugs are commonly used to help control this immune dysregulation[3]. Unfortunately, the use of immunosuppressive drugs can suppress not only the autoimmune response, but also responses to novel pathogens and the generation of protective immunity after vaccination, leaving patients with SSc vulnerable to infection[6–9]. In particular, mycophenolate mofetil is often used to treat SSc and has been associated with both severe SARS-CoV-2 infection and weaker responses to SARS-CoV-2 vaccination[2,10]. Their intrinsic immune dysregulation coupled with immunomodulatory drug treatment raises concerns regarding the efficacy of SARS-CoV-2 vaccination in this population.

While many studies focus on humoral responses after SARS-CoV-2 vaccination, the importance of cellular immunity, in particular T cells, in long term- and cross-variant- protection cannot be underestimated[11–13]. In this study we aimed to determine if participants with SSc, including those on immunomodulatory drugs, mounted weaker humoral or cellular immune responses to SARS-CoV-2 vaccination compared to controls. We evaluated SARS-CoV-2 antibody levels, in

addition to measuring spike-specific $CD4^+$ and $CD8^+$ T cell responses, to provide a thorough overview of vaccine immunogenicity.

RESULTS

This study included 22 patients with SSc with a median age of 57.0 years (IQR 46.5 - 64.5), who were predominantly female (95%). Demographic data are included in Table 1. The control cohort included 33 participants who did not have autoimmune disorders and were not on immunomodulatory drugs. The control cohort did not significantly differ in age from the patients with SSc (median age 65.0, IQR 54.5 - 70.5). The control group was also predominantly female (64%), though the sex composition significantly differed from that of the SSc cohort. The median time intervals between the first and second, second and third, and third and fourth SARS-CoV-2 vaccinations did not significantly differ between participants with SSc and controls. Of the participants with SSc, 12 (55%) were on some form of immunomodulatory drug. The most common immunomodulatory drugs used to treat participants with SSc were disease modifying antirheumatic drugs (DMARDs), followed by steroids and denosumab.

Table 1. Participant Demographics and Vaccine Information

	Controls	Systemic Sclerosis	*P*
Total participants	33	22	N/A
Age, yrs, median (IQR)	65.0 (54.5-70.5)	57.0 (46.5-64.5)	ns[a]
Sex, % female subjects (n)	64 (21)	95 (21)	0.009
First dose vaccine type			ns[b]
BNT162b2	22	16	
mRNA-1273	4	2	
ChAdOx1	7	4	
Second dose vaccine type			ns[b]
BNT162b2	26	15	
mRNA-1273	6	7	
ChAdOx1	1	0	
Third dose vaccine type			ns[b]
BNT162b2	19	11	
mRNA-1273	11	4	
Fourth dose vaccine type			ns[b]
BNT162b2	3	2	
mRNA-1273	4	1	
Bivalent	1	3	
Days between dose 1 and dose 2, median (IQR)	72.0 (64.0-80.0)	64.5 (48.8-74.5)	ns[a]
Days between dose 2 and dose 3, median (IQR)	182.0 (177.0-191.0)	180.0 (146.8-192.3)	ns[a]

Days between dose 3 and dose 4, median (IQR)	225.0 (151.5-279.5)	289.0 (187.0-301.5)	ns[a]
Steroids[c], % (n)	N/A	18 (4)	N/A
DMARDs[d], % (n)	N/A	36 (8)	N/A
Rituximab, % (n)	N/A	9 (2)	N/A
IL-6 inhibitors, % (n)	N/A	5 (1)	N/A
Privigen, % (n)	N/A	5 (1)	N/A
Denosumab, % (n)	N/A	14 (3)	N/A
Pentoxifylline, % (n)	N/A	5 (1)	N/A
Tacrolimus, % (n)	N/A	5 (1)	N/A
JAK inhibitors, % (n)	N/A	5 (1)	N/A
Total SARS-CoV-2 Infections, % (n)	15 (5)	9 (2)	ns[b]

SD- standard deviation, DMARDs- disease modifying anti-rheumatic drugs.

[a] Student's *t* test. $P < 0.05$ is significant.

[b] Fisher's exact test. $P < 0.05$ is significant.

[c] prednisone (average 10 mg daily)

[d] methotrexate, mycophenolate mofetil, leflunomide, and hydroxychloroquine.

Not all participants received their third or fourth SARS-CoV-2 vaccination or provided samples at all timepoints. Vaccination types are reported as n, since not all participants received all vaccinations.

Participants with SSc mount comparable humoral responses to controls following SARS-CoV-2 vaccination

Following the second SARS-CoV-2 vaccination, participants with SSc mounted comparable anti-receptor binding domain (RBD) IgG responses to controls (Figure 1A). There were also no significant differences in the levels of anti-RBD IgG between participants with SSc and controls following their third or fourth SARS-CoV-2 vaccinations (Figure 1A). Similarly, participants with SSc developed comparable anti-RBD IgA levels to controls following the second, third, and fourth SARS-CoV-2 vaccinations (Figure 1B).

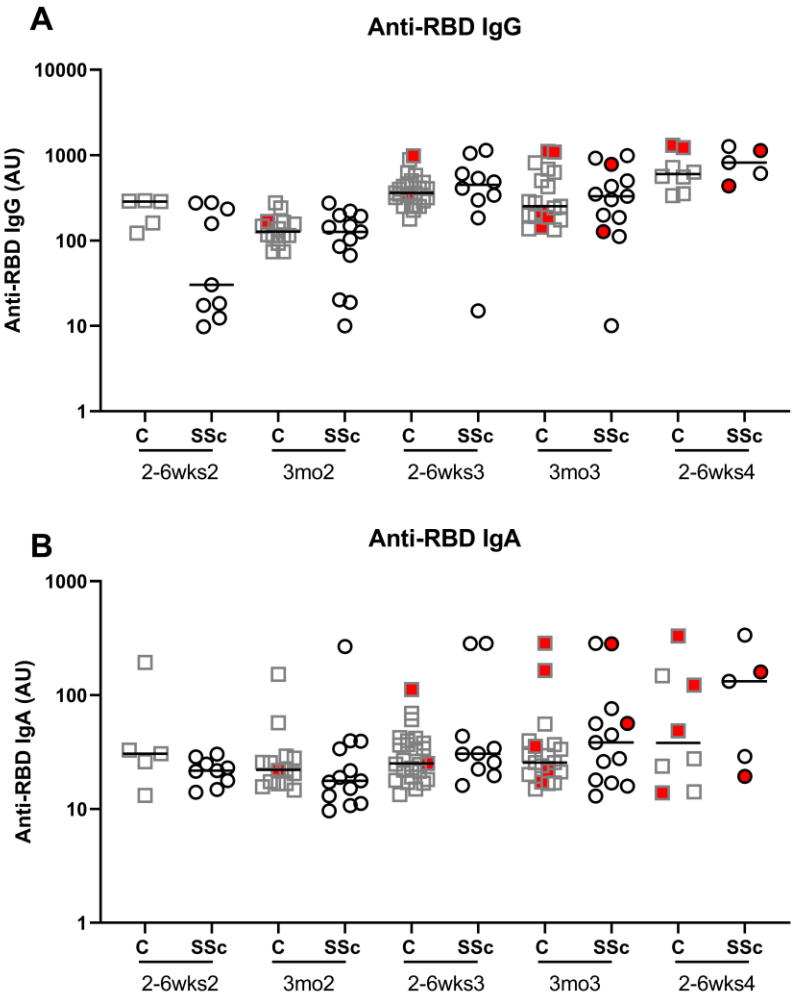

Figure 1. Participants with SSc mount comparable humoral responses to controls following SARS-CoV-2 vaccination. Serum anti-RBD IgG (A) and IgA (B) were measured using ELISAs for people living with SSc (open circles) and controls (C, open grey squares). Filled symbols denote participants who previously had a SARS-CoV-2 infection. The solid lines indicate the median of each group. 2-6wksX denotes 2-6 weeks post dose X, 3moX denotes 3 months post dose X. Data were plotted on a log scale. Potential differences between

participants with SSc and controls were evaluated with mixed-effects models followed by Šidák post-hoc tests; no significant differences were found.

Participants with SSc mount comparable spike-specific CD4$^+$ T cell responses to controls following SARS-CoV-2 vaccination

SARS-CoV-2 spike-specific CD4$^+$ T cell responses also play a pivotal role in long term vaccine-induced protection[12,14]. Participants with SSc mounted comparable spike-specific CD4$^+$ T cell responses to controls following their second, third, and fourth SARS-CoV-2 vaccinations (Figure 2A, B). The levels of spike-specific CD4$^+$ T cells were similar between participants with SSc and controls both when looking at the total numbers of spike-specific CD4$^+$ T cells, and their frequencies out of all CD4$^+$ T cells (Figure 2A, B). To further evaluate antigen-specific responses in participants with SSc, we stimulated the T cells using influenza HA antigens (Agriflu), which people can respond to due to previous seasonal influenza exposures and vaccinations. The participants with SSc had comparable influenza HA-specific CD4$^+$ T cell responses to those observed in controls, suggesting that antigen-specific T cell responses to non-SARS-CoV-2 seasonal respiratory viruses are likely equivalent in magnitude (Figure 2C). Finally, to assess the overall response capacity of the CD4$^+$ T cell compartment, we conducted a TCR specificity-independent polyclonal stimulation using Cytostim (Figure 2D). Participants with SSc and controls displayed similar levels of activated CD4$^+$ T cells following this polyclonal stimulation, indicating that they have equivalent responses to antigen-independent stimulation (Figure 2D).

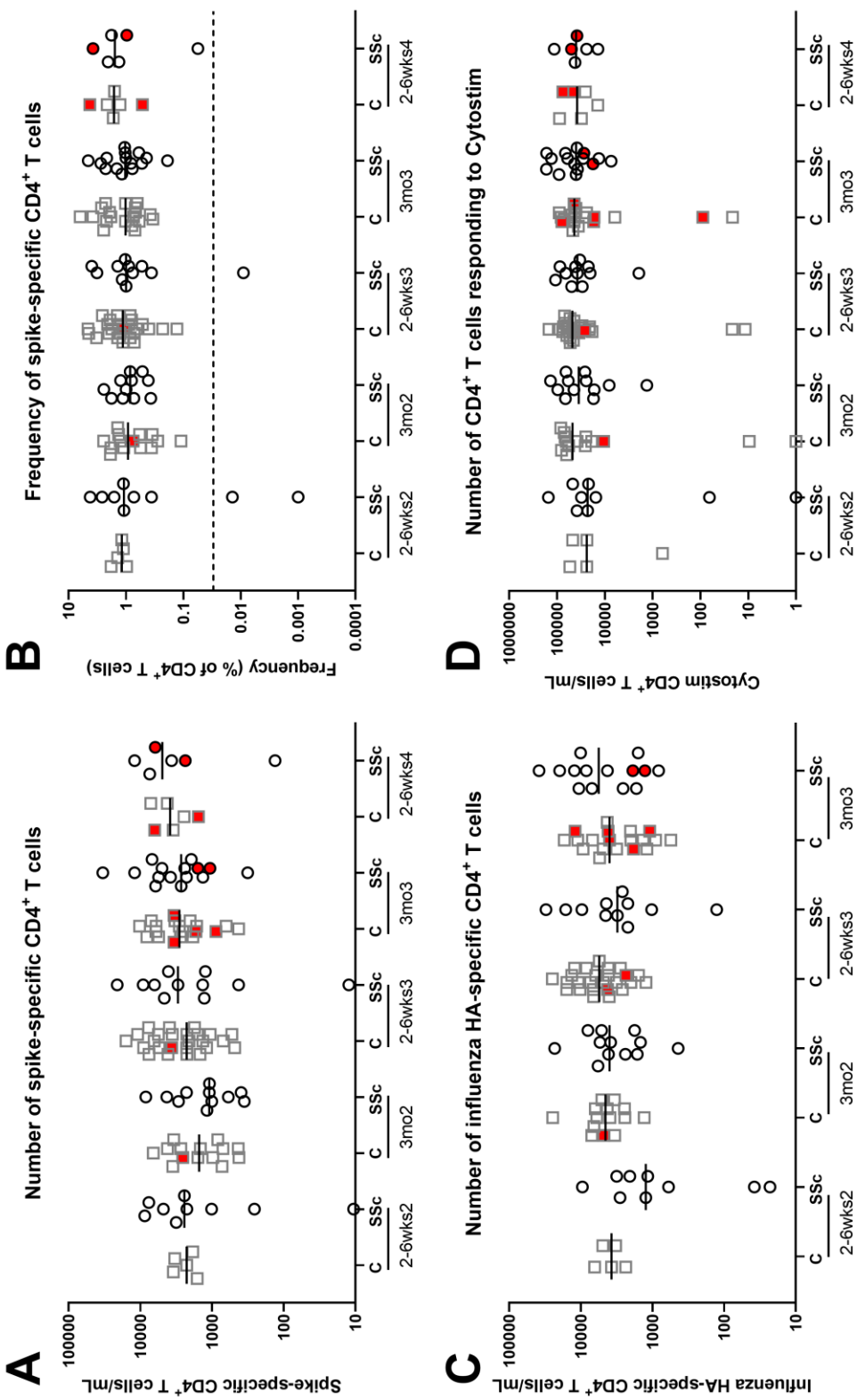
A
Number of spike-specific CD4+ T cells
Spike-specific CD4+ T cells/mL
100000
10000
1000
100
10
C SSc C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3 2-6wks4
B
Frequency of spike-specific CD4+ T cells
Frequency (% of CD4+ T cells)
10
1
0.1
0.01
0.001
0.0001
C SSc C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3 2-6wks4
C
Number of influenza HA-specific CD4+ T cells
Influenza HA-specific CD4+ T cells/mL
100000
10000
1000
100
10
C SSc C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3
D
Number of CD4+ T cells responding to Cytostim
Cytostim CD4+ T cells/mL
1000000
100000
10000
1000
100
10
1
C SSc C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3 2-6wks4

Figure 2. Participants with SSc mount comparable spike-specific CD4$^+$ T cell responses to controls following SARS-CoV-2 vaccination. The number (A) and frequency (B) of spike-specific CD4$^+$ T cells were evaluated using AIM assays in participants with SSc (open circles) and controls (C, open grey squares). The solid lines indicate the median of the group, while the dashed line represents a threshold of positivity (0.03%). Filled symbols denote participants who previously had a SARS-CoV-2 infection. (C) AIM assays were used to evaluate influenza HA-specific (Agriflu) CD4$^+$ T cell numbers as well as non-antigen-specific (Cytostim) polyclonal responses (D). Data were plotted on a log scale. Comparisons between participants with SSc and controls were evaluated with mixed-effects models followed by Šidák post-hoc tests; no significant differences were observed.

Spike-specific CD4$^+$ T cell polarization is largely similar in participants with SSc and controls following SARS-CoV-2 vaccination

Participants with SSc can display elevated levels of serum cytokines, which contribute to polarizing CD4$^+$ T cells preferentially towards Th1, Th2, or Th17 fates, and ultimately impact responses to infections[4,15–17]. Participants with SSc produce equivalent numbers of spike-specific CD4$^+$ T cells to controls; however, we hypothesized that the cytokine milieu could result in post-vaccination differences in T cell polarization (Figure 3). Three months after the second SARS-CoV-2 vaccination, participants with SSc and controls had similar frequencies of spike-specific CD4$^+$ Th1, Th2, Th17, and T regulatory cells (Figure

3A-D). The numbers of these cells did not significantly differ between groups (Supplementary Figure S2A-D). Interestingly, at 2-6 weeks after the third SARS-CoV-2 vaccination, participants with SSc displayed lower frequencies of Th17-polarized spike-specific $CD4^+$ T cells (Figure 3G). Although the frequencies of Th17 spike-specific $CD4^+$ T cells were altered at 2-6 weeks post dose 3, the numbers of these cells did not significantly differ from those in controls (Supplementary Figure S2G). The frequency changes observed thus did not exert profound changes on the overall spike-specific $CD4^+$ T cell responses. There were also no significant differences in the frequencies or numbers of Th1, Th2, or T regulatory spike-specific $CD4^+$ T cells between participants with SSc and controls at 2-6 weeks after dose 3 (Figure 3E, F, H, Supplementary Figure S2E, H).

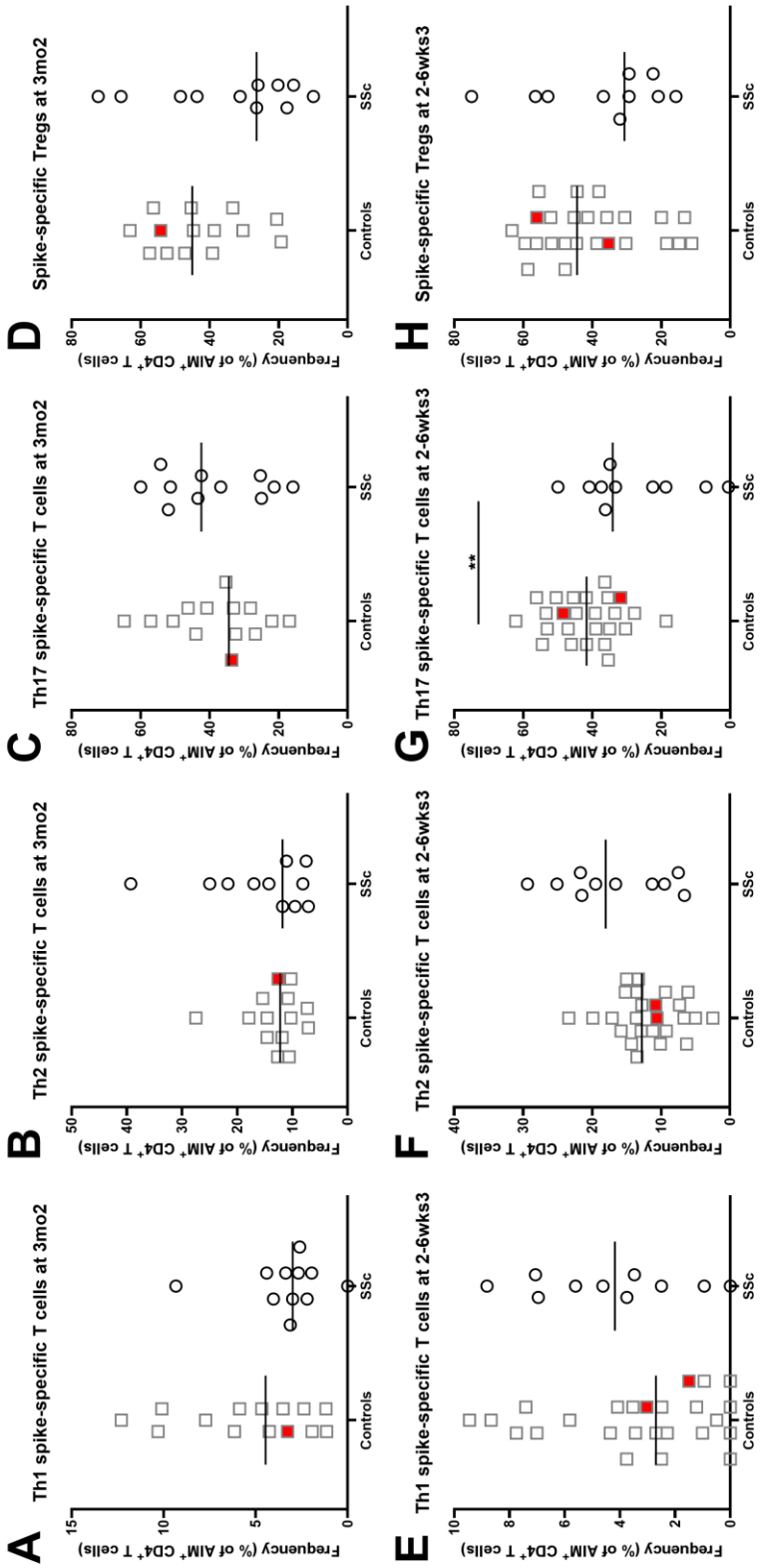
A
Th1 spike-specific T cells at 3mo2
B
Th2 spike-specific T cells at 3mo2
C
Th17 spike-specific T cells at 3mo2
D
Spike-specific Tregs at 3mo2
E
Th1 spike-specific T cells at 2-6wks3
F
Th2 spike-specific T cells at 2-6wks3
G
Th17 spike-specific T cells at 2-6wks3
H
Spike-specific Tregs at 2-6wks3
Frequency (% of AIM+ CD4+ T cells)
Controls
SSc
**

Figure 3. Spike-specific CD4$^+$ T cell polarization is largely similar in participants with SSc and controls following SARS-CoV-2 vaccination. The frequency of spike-specific CD4$^+$ T cells was evaluated using AIM assays. The spike-specific T cell population was then broken down into Th1 (CXCR3$^+$CCR6$^-$ CCR4$^-$), Th2 (CXCR3$^-$CCR6$^-$CCR4$^+$), Th17 (CXCR3$^-$CCR6$^+$CCR4$^+$), or T regulatory cells (Tregs, CD25$^+$CD39$^+$). T cell phenotypes are shown for samples collected 3 months after dose 2 (A-D) and 2-6 weeks after dose 3 (E-H). Filled symbols denote participants who previously had a SARS-CoV-2 infection. The solid lines indicate the median of each group. Comparisons were made by Student's *t* tests, or Welch's *t* tests for those with unequal variation. $P < 0.05$ *, $P < 0.01$ **.

Spike-specific CD8$^+$ T cell responses to SARS-CoV-2 vaccination are similar between participants with SSc and controls

CD8$^+$ T cells play an essential role in antiviral responses due to their ability to directly kill virus-infected cells[11]. Previous studies have also demonstrated the importance of CD8$^+$ T cells in SARS-CoV-2 vaccination-induced protection[11]. Following the second, third, and fourth SARS-CoV-2 vaccinations, participants with SSc had equivalent numbers and frequencies of spike-specific CD8$^+$ T cells to their control counterparts (Figure 4A-B). While some participants had frequencies of spike-specific CD8$^+$ T cells below the limit of detection (0.03%), the SSc cohort did not have a greater proportion of non-responders than the control cohort (Figure 4B). Similarly, the antigen-specific CD8$^+$ T cell response to

influenza HA (Agriflu) was comparable between those with SSc and controls, suggesting there are no differences in T cell responses to other non-SARS-CoV-2 viral antigens (Figure 4C). The $CD8^+$ T cells from participants with SSc also displayed comparable responses to those of controls after polyclonal stimulation (Figure 4D).

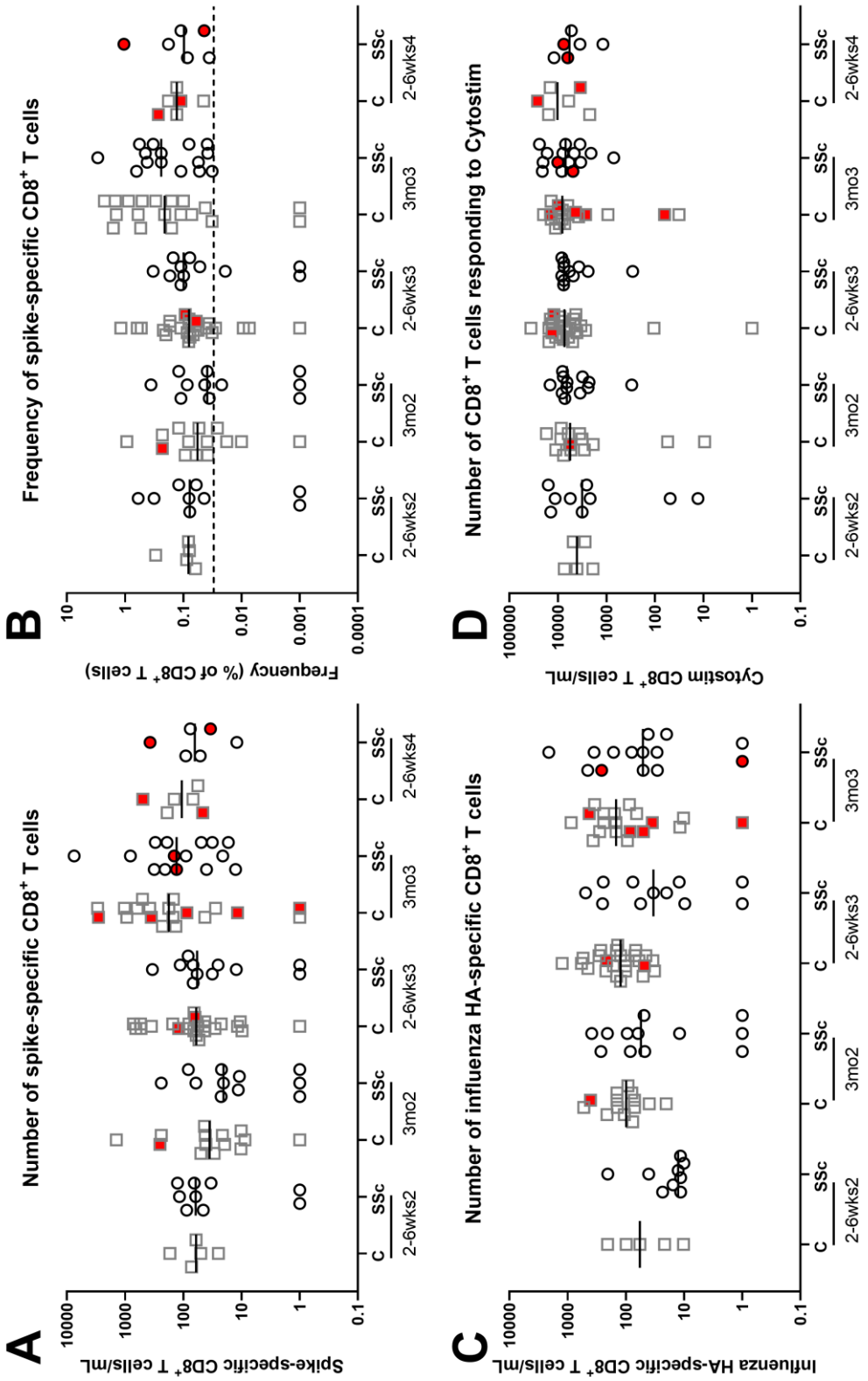
A
Number of spike-specific CD8+ T cells
Spike-specific CD8+ T cells/mL
10000
1000
100
10
1
0.1
C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3 2-6wks4
B
Frequency of spike-specific CD8+ T cells
Frequency (% of CD8+ T cells)
10
1
0.1
0.01
0.001
0.0001
C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3 2-6wks4
C
Number of influenza HA-specific CD8+ T cells
Influenza HA-specific CD8+ T cells/mL
10000
1000
100
10
1
0.1
C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3
D
Number of CD8+ T cells responding to Cytostim
Cytostim CD8+ T cells/mL
100000
10000
1000
100
10
1
0.1
C SSc C SSc C SSc C SSc C SSc
2-6wks2 3mo2 2-6wks3 3mo3 2-6wks4

Figure 4. Spike-specific CD8$^+$ T cell responses to SARS-CoV-2 vaccination are similar between participants with SSc and controls. The number (A) and frequency (B) of spike-specific CD8$^+$ T cells were evaluated using AIM assays in participants living with SSc (open circles) and controls (C, open grey squares). The solid line indicates the median of the group. The dashed line represents a threshold of positivity (0.03%). (C) AIM assays were used to evaluate influenza HA-specific (Agriflu) CD8$^+$ T cell numbers as well as non-antigen-specific (Cytostim) polyclonal responses (D). Filled symbols denote participants who previously had a SARS-CoV-2 infection. Data were plotted on a log scale. Comparisons between participants with SSc and controls were evaluated with mixed-effects models followed by Šidák post-hoc tests; no significant differences were observed.

Predominance of female participants in the SSc cohort does not skew comparisons to controls

SSc is more common in females; thus our SSc cohort was predominantly female[18]. Given that females can mount stronger humoral responses than males to other vaccines, we wanted to ensure that the different sex breakdowns of the SSc and controls cohorts was not artificially inflating the SSc vaccination responses [19,20]. We therefore performed subanalyses on only female participants with SSc and female controls, to determine if there were differences in their humoral or cellular responses to SARS-CoV-2 vaccination (Supplementary Figure 3). We did not find significant differences between female SSc participant

and female control vaccination responses at any time points (Supplementary Figure 3). This therefore suggests that the significant difference in sex distribution between cohorts is not skewing the findings of the study.

Given the small SSc cohort size, the study was not powered to compare humoral or cellular responses by immunomodulatory drug class. However, as we recognize that immunomodulatory drugs may influence immune responses after vaccination, a figure has been included which compares SSc participants who were on any immunomodulatory drugs, SSc participants who were not on immunomodulatory drugs, and controls at each timepoint in the study (Supplementary Figure 4). Participants with SSc who were on immunomodulatory drugs displayed heterogenous humoral and cellular responses after SARS-CoV-2 vaccination, comparable to the responses observed in participants with SSc who were not on any immunomodulatory drugs (Supplementary Figure 4).

DISCUSSION

In this study, we evaluated humoral and cellular responses after SARS-CoV-2 vaccinations in participants with SSc. Participants with SSc and controls produced similar levels of anti-RBD IgG and anti-RBD IgA following the second, third, and fourth SARS-CoV-2 vaccinations. Spike-specific $CD4^+$ and $CD8^+$ T cell levels were also comparable between participants with SSc and controls, suggesting no deficit in this arm of cellular immunity. Furthermore, the polarization of the spike-specific $CD4^+$ T cells (Th1, Th2, Th17, Treg) did not

differ following the second SARS-CoV-2 vaccination, and only displayed minor frequency alterations early after the third dose.

There are few studies focusing on the SARS-CoV-2 vaccination responses of participants with SSc, with one reporting that SSc patients and controls produced similar levels of total anti-SARS-CoV-2 IgG by 3 months after the second vaccination[21]. Interestingly, this study noted that participants with SSc displayed lower levels of anti-SARS-CoV-2 IgG at 1 month after their second SARS-CoV-2 vaccination[21]. While we did not observe a significant difference in anti-RBD IgG or IgA levels between participants with SSc and controls at this timepoint, there was a bimodal distribution of high and low levels of anti-RBD IgG within the SSc cohort, which was not observed following the third or fourth SARS-CoV-2 vaccinations.

Our current study also assesses the cellular responses to SARS-CoV-2 vaccination, which are postulated to be critical for long term protection[12,14]. In particular, we discovered that following SARS-CoV-2 vaccinations, participants with SSc do not have reduced spike-specific $CD4^+$ or $CD8^+$ T cell levels compared with controls. This finding coincided with the observation that influenza HA-specific and polyclonal-stimulation responsive T cells did not differ between groups, suggesting that there is no overall deficit in the functional capacity of the T cell compartment in people with SSc. Interestingly, the bimodal distribution of anti-RBD IgG levels at 2-6 weeks post dose 2 was not as apparent in the T cell

compartment. The factors contributing to the delayed rise in antibody levels therefore do not appear to impact T cell responses.

Participants with SSc often have perturbed serum cytokine profiles, which could polarize $CD4^+$ T cells towards different fates than those in controls[4,15,16]. Furthermore, overall Treg levels are lower in participants with SSc, raising the question of if they would be prone to altered Treg responses upon vaccination or infection[22]. In an ideal infection response, Tregs exert a fine balance between preventing inflammation-related tissue damage, and avoiding over-suppression of antiviral functions[23]. Given that different polarizations of $CD4^+$ T cells are optimal for responding to intracellular or extracellular pathogens, and avoiding excessive tissue damage, we sought to examine if the polarization of the spike-specific $CD4^+$ T cells in participants with SSc was similar to that observed in controls. The frequencies and numbers of Th1, Th2, Th17, and Treg spike-specific $CD4^+$ T cells were largely equivalent between participants with SSc and controls following their second and third SARS-CoV-2 vaccinations. The only difference observed was that participants with SSc had lower frequencies of Th17 spike-specific $CD4^+$ T cells than controls at 2-6 weeks post dose 3. This difference was not significant when looking at the numbers of Th17 spike-specific $CD4^+$ T cells at this timepoint, suggesting that the difference in frequency only had a minor impact on the overall compartment. The existing literature on SARS-CoV-2 vaccination responses, and in particular cellular responses, in participants with SSc is sparse. One previous study in a cohort of participants with other autoimmune conditions found that those with untreated conditions, as well as

participants on various classes of immunomodulatory drugs, did not display altered production of IL-17A following the first or second SARS-CoV-2 vaccinations[8]. While these participants did not have SSc, this may suggest that the Th17-polarized responses following SARS-CoV-2 vaccination are not largely perturbed in various autoimmune conditions compared with controls. This is in line with our observation that the Th17 polarization of spike-specific $CD4^+$ T cells was largely similar between controls and participants with SSc.

One of the limitations of our study was our smaller cohort size. This small cohort negated the possibility of examining the impact of different immunosuppressive drugs on SARS-CoV-2 vaccination responses in participants with SSc. SSc is a rare autoimmune disorder, which coupled with skin fibrosis can make study enrollment and blood collection difficult. Despite this limitation, we provided what is to our knowledge the first investigation of both humoral and cellular immune responses, including T cell polarization, after SARS-CoV-2 vaccination in participants with SSc.

Overall, this study determined that participants with SSc and controls mount comparable humoral and cellular responses to SARS-CoV-2 vaccination. Given the susceptibility of participants with SSc to SARS-CoV-2 infections and poor outcomes, vaccination poses a viable option for the continued protection of this vulnerable population, though more evidence will be required to determine how different immunomodulatory drug classes used to treat SSc may alter vaccine efficacy.

METHODS

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dawn Bowdish (bowdish@mcmaster.ca).

Materials availability

This paper does not report newly generated reagents.

Data and code availability

- Deidentified data is available upon request to the lead contact.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

Experimental model and study participant details

Participant recruitment and ethics

Study recruitment and procedures were reviewed and approved by the Hamilton Integrated Research Ethics Board (HiREB) protocol #13307. Participants gave informed consent for sample collection, data analysis, and publication prior to their participation. Participants with SSc (age 18+, n=22) were recruited from a

rheumatology clinic in Hamilton, Ontario, Canada, from July 2021 to December 2022. Adult (18+) controls (C, n=33) without autoimmune disease were recruited from the surrounding community. Exclusion criteria (applying to both SSc and controls) were significant immunodeficiency syndromes (e.g., HIV), pregnancy, acute febrile illness on the scheduled blood collection date, enrollment in a new vaccine clinical trial, and chemotherapy treatment. SARS-CoV-2 infections were determined by a positive rapid antigen test, PCR, or by seroconversion to anti-nucleocapsid+ (anti-N+) as determined by an anti-nucleocapsid IgG ELISA (detailed below) [24]. SSc is more common in females, thus the cohort was predominantly female. Sub-analyses were included to account for this. The control cohort used in this study was the same as in another paper from our lab, where we compared responses between those who had rheumatoid arthritis and those who did not (control)[25].

Peripheral blood was collected in sodium heparin vacutainer tubes from participants at 2-6 weeks (SSc n=9, C n=5) and 3 months (SSc n=13, C n=14) after their second SARS-CoV-2 vaccination, 2-6 weeks (SSc n=11, C n=25) and 3 months (SSc n=15, C n=19) after their third vaccination, and 2-6 weeks after their fourth vaccination (SSc n=6, C n=8). Blood was also collected in coagulant free collection tubes, allowing serum isolation and cryopreservation at -80°C. The study design was cross-sectional based on sample availability, as not all participants provided samples at all timepoints.

Method details

Enzyme immunoassays for the measurement of SARS-CoV-2 antibodies

Plasmids encoding mammalian cell codon optimized sequences for the SARS-CoV-2 spike protein RBD as well as the Nucleocapsid protein (R&D Systems, Cat#11033-CV) were used to produce protein antigens. Enzyme immunoassays (EIA) to measure human anti-IgG and IgA SARS-CoV-2 antibodies were then conducted as previously described[26–28]. Briefly, microtiter well plates (384 wells, Nunc Maxisorp, Cat#464718) were coated with 25 µL/well of RBD (2 µg/mL) or Nucleocapsid (2 µg/mL) in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 4ºC. Plates were washed twice using phosphate buffered saline (PBS) supplemented with 0.05% Tween 20 and washed three times with PBS alone. Plates were then blocked with 100 µL/well PBS supplemented with 3% skim milk for 2 hours at room temperature. After washing, serum samples were diluted in PBS supplemented with 1% skim milk and 0.05% Tween 20 (1/400 and 1/800 for RBD IgG, 1/100 for RBD IgA and 1/100 for Nucleocapsid IgG), then added 25 µL/well in duplicate wells and incubated for 1 hr at room temperature. Next, the plates were washed and bound antibodies were detected using 25 µL/well alkaline phosphatase conjugated goat anti-human IgG (Fcγ-fragment-specific; 1/2000, Jackson ImmunoResearch Laboratories Inc, Cat#109-056-098) or alkaline phosphatase conjugated goat anti-human IgA (α-chain-specific; 1/1000, Jackson ImmunoResearch Laboratories Inc, Cat#109-056-011) prepared in PBS with 1% skim milk and 0.05% Tween 20. Plates were washed again followed by the addition of 50 µL/well substrate, 4-nitrophenylphosphate disodium salt hexahydrate (pNPP) in diethanolamine (Millipore Sigma,

Cat#N9389). Finally, the optical density (OD) was read at 405 nm with 490 nm as reference and measured using a BioTek 800TS microplate reader. The assay cut-off was determined as the mean plus 3 standard deviations (SD) of the pre-COVID-19 control population. Results were reported as arbitrary units (AU), defined as the optical density multiplied by the dilution factor.

T cell Activation-Induced Marker (AIM) assays and flow cytometry

The AIM assay was carried out as previously described [28]. To assess $CD4^+$ and $CD8^+$ T cells specific to SARS-CoV-2, peptides from the spike protein of SARS-CoV-2 were used to stimulate whole blood (SARS-CoV-2 S Immunodominant Peptivator, Miltenyi Biotec, 1 µg/ml) for 48 hrs at 37ºC. Influenza hemagglutinin (HA)-specific $CD4^+$ and $CD8^+$ T cell responses were evaluated using influenza HA peptides (AgriFlu inactivated influenza vaccine 2020-2021 season, Seqirus, 4µl of 0.12µg/µl), while Cytostim (Miltenyi Biotec, 0.5µl/well) was used to evaluate non-antigen-specific polyclonal response capacity. AIM^+CD8^+ and AIM^+CD4^+ T cells that responded to the presented spike peptides (or influenza HA peptides in the case of AgriFlu) expressed activation markers (i.e., $CD69^+CD137^+$ for CD8; $CD25^+CD134^+$ for CD4) which were quantitated by flow cytometry. The spike-specific $CD4^+$ T cell population was also broken down into Th1 ($CXCR3^+CCR6^-CCR4^-$), Th2 ($CXCR3^-CCR6^-CCR4^+$), Th17 ($CXCR3^-CCR6^+CCR4^+$), or T regulatory cells (Tregs, $CD25^+CD39^+$)[24,29].

The stain included antibodies against CD3 (1:50, clone UCHT1, BD Biosciences), CD4 (1:50, clone SK3, BD Biosciences), CD8 (1:50, clone RPA-T8,

BD Biosciences), CD25 (1:50, clone M-A251, BD Biosciences), CD134 (1:100, clone ACT-35, BioLegend), CD39 (1:25, clone A1, BioLegend), CD137 (1:50, clone 4B4-1, BioLegend), CD69 (clone FN50, BioLegend), CXCR3 (1:25, clone G025H7, BioLegend), CCR6 (1:25, clone G034E3, BioLegend), and CCR4 (1:50, clone L291H4, BioLegend) suspended in Brilliant Stain Buffer Plus (BD Biosciences) and phosphate buffered saline (PBS). After staining, samples were fixed using 1xFix/Lyse solution (eBioscience), washed with PBS, and resuspended in FACS Wash (0.5% (w/v) bovine serum albumin, 5mM EDTA (pH 7.4-7.6) in PBS. CountBright Absolute Counting Beads (Invitrogen) were used to determine cell numbers.

Samples were acquired on a Cytoflex LX (4 laser, Beckman Coulter).

Quantification and statistical analysis

Statistical analyses were completed using GraphPad Prism version 8, and statistical test details are included in Figure and Table legends. Fisher's exact test and Student's *t* tests were used where appropriate to compare demographic factors between participants with SSc and controls. Fisher's exact tests with more than 2 groups (i.e. vaccination types) were conducted in R version 4.1.2 (R Core Team 2022). Mixed-effects models with Šidák post-hoc tests were used to evaluate differences in antibody and T cell levels between participants with SSc and controls across timepoints (assessed main effects of cohort and timepoint). Comparisons of spike-specific $CD4^+$ T cell phenotypes between participants with SSc and controls at either 3 months post dose 2 or 2-6 weeks post dose 3 were

conducted using Student's *t* tests, or Welch's *t* tests for those that displayed significantly different variation between cohorts. *P* values less than 0.05 were considered statistically significant, shown graphically as $p < 0.05$ *, $p < 0.01$ **. *P* values are two-tailed. The number of participants in the study at a given timepoint is denoted by 'n'. The exact values of 'n' for the SSc cohort and control were as follows: 2-6 weeks post dose 2 (SSc n=9, C n=5), 3 months (SSc n=13, C n=14) post dose 2, 2-6 weeks (SSc n=11, C n=25) and 3 months (SSc n=15, C n=19) after post dose 3, and 2-6 weeks post dose 4 (SSc n=6, C n=8).

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AUTHOR CONTRIBUTIONS

JMB- conducted experiments, analyzed data and generated figures, wrote manuscript.

JAB- conducted experiments, contributed to data interpretation and manuscript editing.

AH- developed and optimized ELISAs for antibody detection, performed ELISAs.

BC- collected and processed samples, collected participant metadata, aided in recruitment of controls.

BB- clinical coordinator, recruited and contacted patients, collected samples.

LH- collected participant metadata and helped schedule participant blood draws.

SL- collected participant metadata.

EY- collected participant metadata.

HB- conducted ELISAs for anti-RBD IgG and anti-nucleocapsid antibodies.

RC- conducted ELISAs for anti-RBD IgG and anti-nucleocapsid antibodies.

IN- provided aid for ELISAs, provided critical input to study design and interpretation.

JLB- Provided critical input to study design and data interpretation. Developed cellular assays.

MJL – created study design and obtained funding, contributed to data interpretation and DMEB- created study and obtained funding, contributed to data interpretation, experimental design, and manuscript writing.

DECLARATION OF INTERESTS

DMEB has received honorarium for consulting on the topic of vaccines from Pfizer-Canada and AstraZeneca. MJL is on the Advisory Boards/Speakers Bureaus for AbbVie, Actelion, Amgen, Astra-Zeneca, Boehringer-Ingelheim, BMS, Fresenius-Kabi, Gilead, GSK, Lilly, Mallinckrodt, Novartis, Pfizer, Sanofi, SOBI, UCB, and Scleroderma Society of Ontario/Canada. None of the other authors have competing interests.

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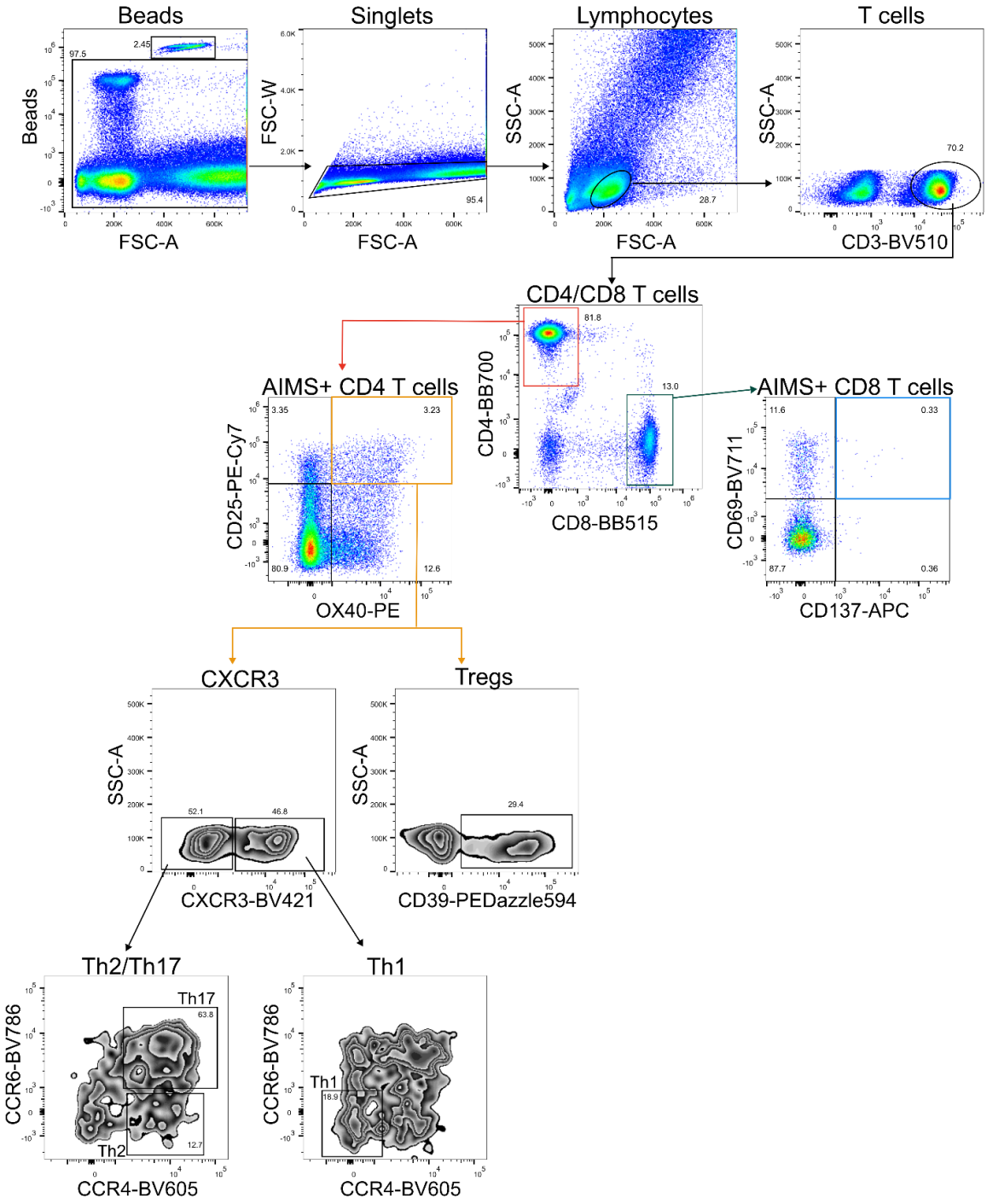
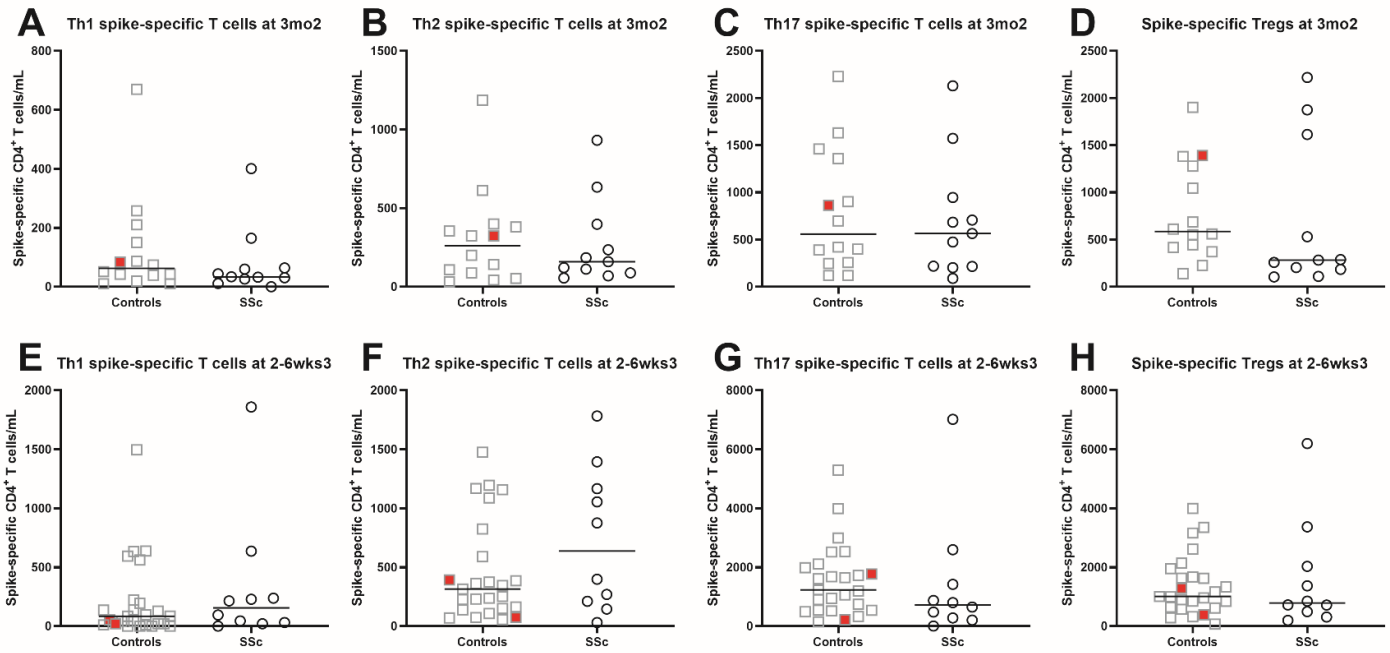
Data Supplement

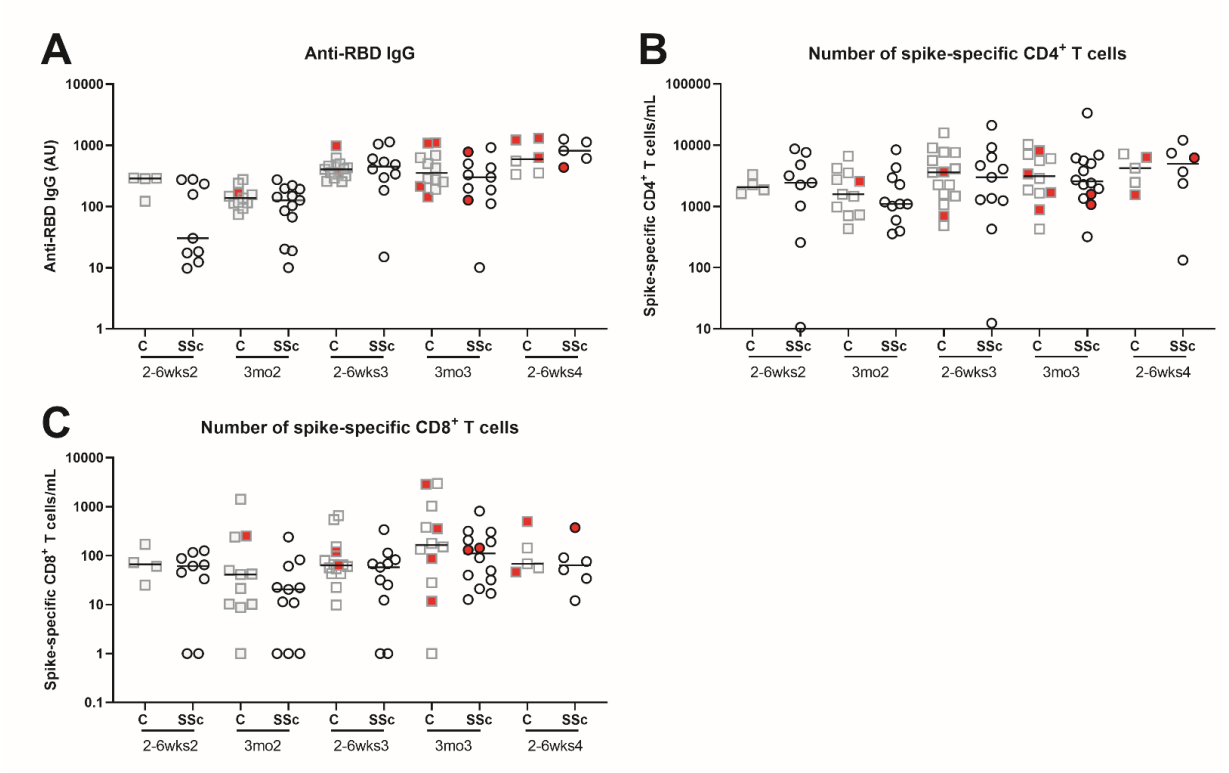


Supplementary Figure 1. Activation-induced marker (AIM) assay gating strategy. Count beads were gated out first. Non-bead events then underwent

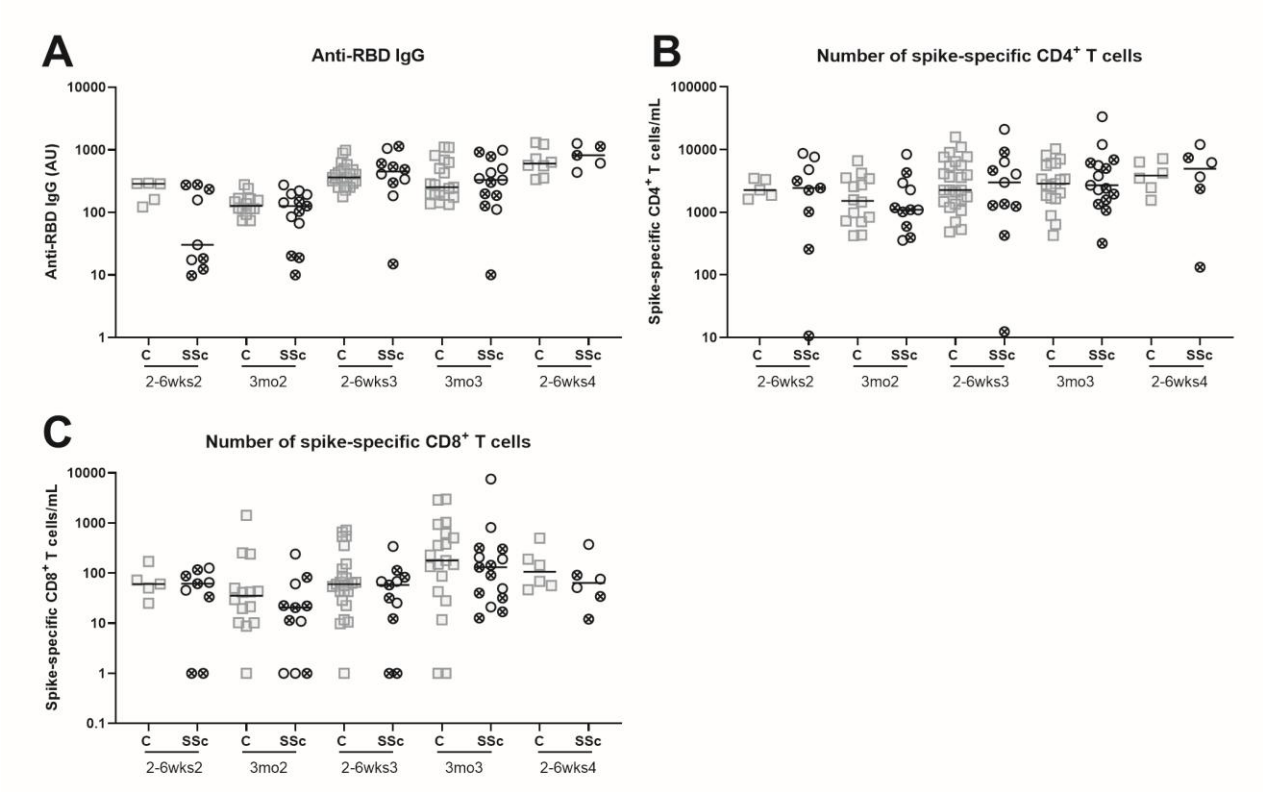
doublet exclusion, followed by gating on lymphocytes. T cells were identified by expression of CD3 and divided into $CD4^+$ and $CD8^+$ subsets. AIM^+ $CD4^+$ T cells co-expressed CD25 and OX40, while AIM^+ $CD8^+$ T cells co-expressed CD69 and CD137. Within the AIM^+ $CD4^+$ T cell compartment, cells were separated into Th1 ($CXCR3^+CCR6^-CCR4^-$), Th2 ($CXCR3^-CCR6^-CCR4^+$), Th17 ($CXCR3^-CCR6^+CCR4^+$), or Tregs ($CD39^+$).



Supplementary Figure 2. Spike-specific CD4$^+$ T cell skew numbers in participants with SSc and controls. The number of spike-specific CD4$^+$ T cells was evaluated using AIM assays. The spike-specific T cell population was then broken down into Th1 (CXCR3$^+$CCR6$^-$CCR4$^-$), Th2 (CXCR3$^-$CCR6$^-$CCR4$^+$), Th17 (CXCR3$^-$CCR6$^+$CCR4$^+$), or T regulatory cells (Tregs, CD25$^+$CD39$^+$). T cell phenotypes are reported for samples collected 3 months after dose 2 (A-D) and 2-6 weeks after dose 3 (E-H). Filled symbols denote participants who previously had a SARS-CoV-2 infection. The solid line indicates the median of the group. Comparisons were made by Student *t* tests, or Welch *t* tests for those with unequal variation. No significant differences were detected.



Supplementary Figure 3. SARS-CoV-2 vaccination responses in female SSc participants and controls. (A) Serum anti-RBD IgG was measured using ELISAs for female participants living with SSc (open circles) and female controls (C, open grey squares). The number of spike-specific $CD4^+$ T cells (B) and spike-specific $CD8^+$ T cells (C) were evaluated using AIM assays in female participants. Filled symbols denote participants who previously had a SARS-CoV-2 infection. The solid lines indicate the median of each group. 2-6wksX denotes 2-6 weeks post dose X, 3moX denotes 3 months post dose X. Data were plotted on a log scale. Potential differences between participants with SSc and controls were evaluated with mixed-effects models followed by Šidák post-hoc tests. $P < 0.05$ *.



Supplementary Figure 4. SARS-CoV-2 vaccination responses in participants with SSc highlighting those on immunomodulatory drugs. (A) Serum anti-RBD IgG was measured using ELISAs for participants with SSc (open circles) and controls (grey squares). Open circles with a cross through them indicate participants with SSc who were on an immunomodulatory drug at that time in the study period. The number of spike-specific $CD4^+$ T cells (B) and spike-specific $CD8^+$ T cells (C) were evaluated using AIM assays. The solid lines indicate the median of each group. 2-6wksX denotes 2-6 weeks post dose X, 3moX denotes 3 months post dose X. Data were plotted on a log scale.

3.2 Unusual Kinetics of Humoral Immune Responses to SARS-CoV-2 Vaccination in Patients with Systemic Sclerosis

3.2.1 Introduction

Humoral responses to SARS-CoV-2 vaccination were previously anticipated to be an important correlate of protection against SARS-CoV-2, due in part to the ability of some antibodies to neutralize the virus[318]. As SARS-CoV-2 continues to mutate to evade recognition by antibodies, however, SARS-CoV-2-specific T cells largely maintain their ability to recognize the virus, highlighting their potential role in long-term protection[319,320]. In our investigations of humoral and cellular immunity after SARS-CoV-2 vaccination in participants with SSc, we made the surprising observation that the kinetics of vaccine responses differed in some participants with SSc after the second SARS-CoV-2 vaccination, compared to both controls and other participants with SSc. Specifically, we observed that approximately half of the participants with SSc in our study exhibited a delayed rise in antibodies following the second SARS-CoV-2 vaccination. In this section, we investigated whether this delay was tied to other immunological or demographic factors.

3.2.2 Additional Materials and Methods

Study population

This study represents a secondary analysis on a subset of individuals who provided samples after the second SARS-CoV-2 vaccination (complete primary series vaccination)[317,321]. Protocols were therefore conducted as previously described[321].

Autoantibody Profiles

Positivity for anti-centromere (CENP-B), anti-topoisomerase (Scl-70), and anti-ribonucleoprotein (U1-sRNP) antibodies were evaluated using an IMTEC line immunoassay according to manufacturer instructions (IMTEC-ANA-LIA XL).

ELLA Automated ELISA

A custom Simple 5-Plex Cartridge Kit (SPCKE-PS-008047) from Bio-Techne was used for the detection of IL-13, IL-4, TNF-α, IL-6, and IL-11 within the same sample. Serum was thawed, diluted, and added to the cartridge per the manufacturer's instructions. Cartridges were then run using the Ella™ automated ELISA instrument. IL-4 data were not included as all samples were below the limit of detection.

Statistical Analysis

Student's t-tests, one-way ANOVAs, or Brown-Forsythe ANOVAs for measures with unequal standard deviations (SDs) between groups, and mixed-effects models were performed using GraphPad version 8. Fisher's exact tests were performed using R version 4.4.1 (R Core Team) with the stats package. Main effects of cohort and timepoint in spike-specific T cell levels were analyzed using mixed-effects models. Spike-specific $CD4^+$ T cell phenotypes were compared using one-way ANOVAs as they were shown for a single timepoint. Student's t-tests or Fisher's exact tests were used to compare demographic factors between groups. Fisher's exact tests were used to compare the proportions of participants

with SSc with serum concentrations of IL-11 and IL-13 above the limit of detection. Student's t-tests were used to compare the levels of IL-6, and TNF-α at 2-6wks and 3mo.

3.2.3 Results

We had previously reported that levels of anti-receptor binding domain (RBD) and anti-spike antibodies did not differ between people living with SSc and controls without autoimmune disease following the second, third, and fourth SARS-CoV-2 vaccinations[321]. Despite the fact that people living with SSc are able to mount comparable responses by 3 months after the second SARS-CoV-2 vaccination, herein we report that there were two distinct kinetic patterns of response. Interestingly, within the month following their second vaccination, approximately half of the participants with SSc developed anti-RBD and anti-spike IgG responses comparable to those seen in controls (SSc Responders: SSc-R, n=4), while the other half did not have detectable antibodies within this first month (SSc Non-Responders: SSc-NR, n=5) (Figure 3a, b). At 3 months after their second SARS-CoV-2 vaccination, however, the majority of these SSc-NR participants produced detectable anti-spike IgG, and some also produced detectable anti-RBD IgG (Figure 3a, b). None of the SSc participants tested positive for SARS-CoV-2 by RT-PCR, rapid antigen test, or seropositivity for anti-nucleocapsid IgG (which can indicate recent SARS-CoV-2 infection) at any point in the study period. Therefore, the difference between the SSc-R and SSc-NR groups was not the result of prior SARS-CoV-2 infection (Table 2).

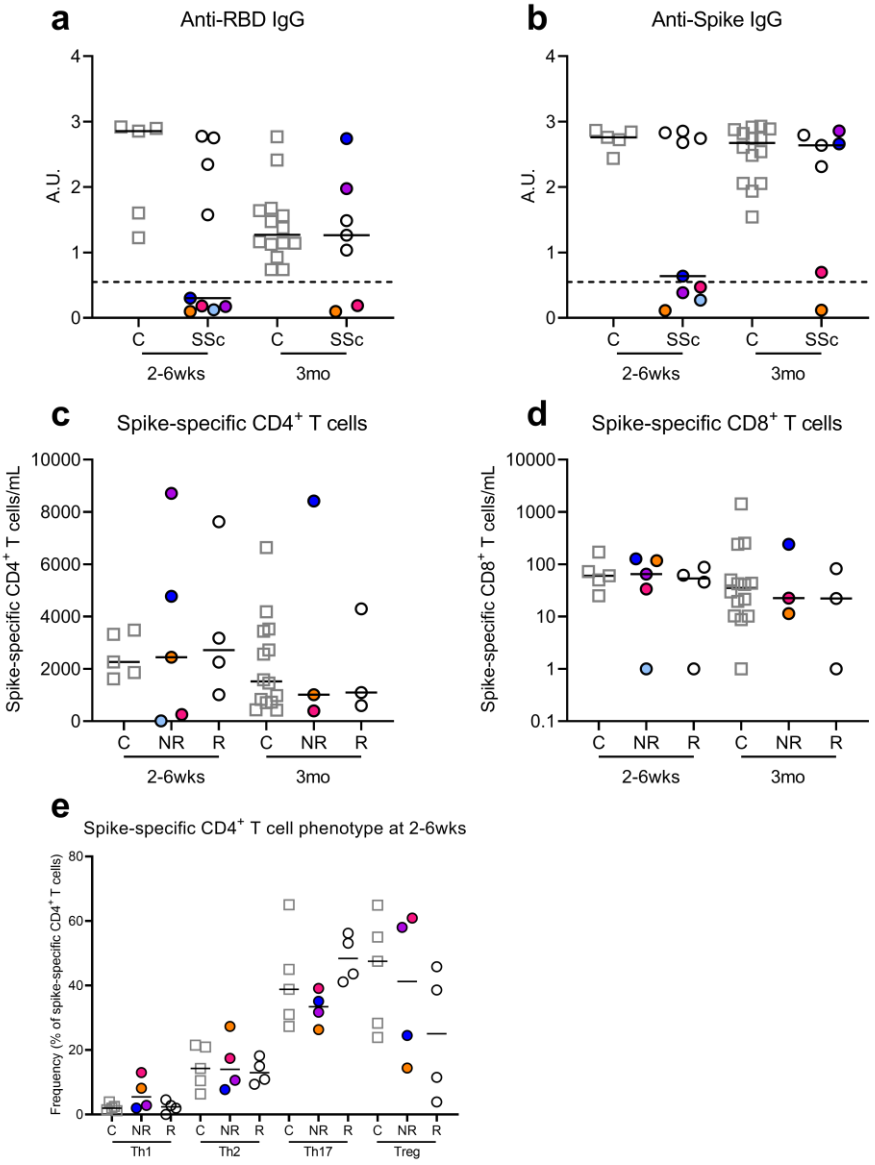


Figure 3. Humoral and cellular responses in participants with SSc and controls following the second SARS-CoV-2 vaccination.

ELISAs were used to measure levels of **(a)** anti-RBD and **(b)** anti-Spike IgG in the serum of participants with SSc (open circles) and controls (C, grey squares). A.U. = arbitrary units. The dashed line represents the seropositivity threshold. 2-6wks indicates 2-6 weeks post dose 2. One SSc Non-Responder was lost to

follow-up at 3 months post dose 2 (3mo). Peripheral blood was collected from participants with SSc and controls, and AIM assays were used to determine the numbers of spike-specific **(c)** $CD4^+$ and **(d)** $CD8^+$ T cells. **(e)** Helper phenotypes of spike-specific $CD4^+$ T cells. NR refers to SSc Non-Responders, R is SSc Responders. Two NR and 1 R did not provide sufficient samples to measure T cell responses at 3 months post dose 2. SSc-R, SSc-NR, and control spike-specific T cell levels were compared at 2-6 weeks post dose 2, and 3 months post dose 2 using mixed-effects models **(c, d)**. SSc-R, SSc-NR, and control spike-specific $CD4^+$ T cell phenotypes were compared using one-way ANOVAs as they were shown for a single timepoint **(e)**. The colored points represent the same non-responder participant over time, solid lines depict the median.

Table 2. SSc Responder, SSc Non-Responder, and control group demographics and laboratory findings

	Controls	SSc-NR	SSc-R[a]	P value
Total participants	15	5	4	N/A
Age, median years (IQR)	66.0 (41.0-71.0)	48.0 (45.5-69.0)	57.0 (50.0-61.8)	ns[b]
Sex, % female subjects (n)	80 (12)	100 (5)	100 (4)	ns[c]
CMV status, % positive (n)	47 (7)	20 (1)	50 (2)	ns[c]
First dose vaccine type, % (n)				ns[c]
BNT162b2	67 (10)	80 (4)	75 (3)	
mRNA-1273	20 (3)	20 (1)	0 (0)	
ChAdOx1	13 (2)	0 (0)	25 (1)	
Second dose vaccine type, % (n)				ns[c]
BNT162b2	73 (11)	60 (3)	100 (4)	
mRNA-1273	20 (3)	40 (2)	0 (0)	
ChAdOx1	7 (1)	0 (0)	0 (0)	
Days between dose 1 and dose 2 (median ± SD)	69.0 ± 21.3	48.0 ± 27.0	60.5 ± 19.7	ns[b]
Days between dose 2 and the 2-6-week post dose 2 draw (median ± SD)	32.0 ± 10.6	35.0 ± 18.4	29.0 ± 2.2	ns[b]
SARS-CoV-2 Infections, % positive (n)[d]	7 (1)	0 (0)	0 (0)	ns[c]

Immunomodulatory Drug Classes, % (n)				
Steroids[e]	NA	40 (2)	0 (0)	ns[c]
Mycophenolate	NA	20 (1)	0 (0)	ns[c]
Rituximab	NA	20 (1)	0 (0)	ns[c]
Hydroxychloroquine	NA	0 (0)	25 (1)	ns[c]
IL-6 inhibitors	NA	20 (1)	(0)	ns[c]
Privigen	NA	0 (0)	25 (1)	ns[c]
Methotrexate	NA	20 (1)	(0)	ns[c]
Pentoxifylline	NA	0 (0)	25 (1)	ns[c]
Autoantibody classes, % positive (n)	NA			
Anti-centromere	NA	60 (3)	0 (0)	ns[c]
Anti-topoisomerase	NA	20 (1)	75 (3)	ns[c]
Anti-ribonucleoprotein	NA	0 (0)	25 (1)	ns[c]

[a]Responder indicates detectable anti-spike and anti-RBD IgG at 2-6 weeks post dose 2.

[b]One-way ANOVA.

[c]Fisher's exact test.

[d]Recent SARS-CoV-2 infection determined by either positive RT-PCR, rapid antigen test, or seropositivity for anti-nucleocapsid IgG.

[e]Prednisone, both participants prescribed 7.5 mg daily.

[f]Student's t-test.

Antibody responses to vaccination are intimately tied with cellular responses, particularly those of $CD4^+$ T cells. Thus, we next examined the SARS-CoV-2 spike-specific T cell responses in the participants with SSc and controls. At both 2-6 weeks and 3-months post dose 2 there were no differences in spike-specific $CD4^+$ and $CD8^+$ T cell numbers between SSc-R, SSc-NR, and control participants (Figure 3c, d**).** The bimodal distribution observed for antibody levels in participants with SSc (Figure 3a, b) was not observed in either T cell compartment (Figure 3c, d). Within the spike-specific $CD4^+$ T cell compartment, there were no differences in the frequencies of Th1, Th2, Th17, or Treg cells between SSc-R, SSc-NR, and controls groups (Figure 3e). Thus, a delayed rise in spike-specific T cells, or differences in their phenotypes, did not likely contribute to the delayed antibody responses in the SSc-NR group.

We next explored if there were demographic differences between the SSc-R, SSc-NR, and the control groups (Table 2). All participants in both SSc groups were female, while 80% (12/15) of controls were female. These groups did not differ in age, first or second SARS-CoV-2 vaccines, or the interval between vaccine doses. Furthermore, the interval between the second SARS-CoV-2 vaccinations and the 2–6 week post dose 2 collections did not differ between groups (Table 2). There were also no significant differences in immunomodulatory drug classes used to treat the SSc-R and SSc-NR groups, or in their serum autoantibody profiles (Table 2).

Cytomegalovirus (CMV) seropositivity impacts multiple immune cell compartments, and has been previously associated with increases in EM re-expressing CD45RA (EMRA) and terminally differentiated T cell subsets[322–325]. Although CMV seropositivity was not associated with impaired SARS-CoV-2 vaccination responses in older adults, it has been associated with impaired responses to influenza vaccination and poor outcomes upon SARS-CoV-2 infection[324,326–328]. Given the impact of CMV seropositivity on various immune cell compartments and other vaccine responses, we next examined if the CMV serostatus differed between the SSc-R and SSc-NR groups. The proportion of CMV seropositive participants did not significantly differ between the SSc-R and SSc-NR groups (Table 2).

The autoantibody profile and pattern of organ involvement in SSc patients has previously been found to be associated with elevation of specific cytokines in the serum [69,70,76]. We measured levels of the fibrosis-associated cytokines IL-11, IL-4, and IL-13, and the pro-inflammatory cytokines IL-6 and TNF-α. Most participants in the SSc-R and the SSc-NR groups fell below the limit of detection for their serum levels of IL-11 and IL-13, and there were no significant differences in the proportions of participants with detectable levels of these cytokines at either 2-6 weeks or 3 months post dose 2 (Figure 4a, b). Serum IL-6 and TNF-α levels did not significantly differ between SSc-R and SSc-NR individuals at either 2-6 weeks or 3 months post dose 2 (Figure 4c, d). Levels of IL-4 were also evaluated, but all participants fell below the limit of detection (data not shown).

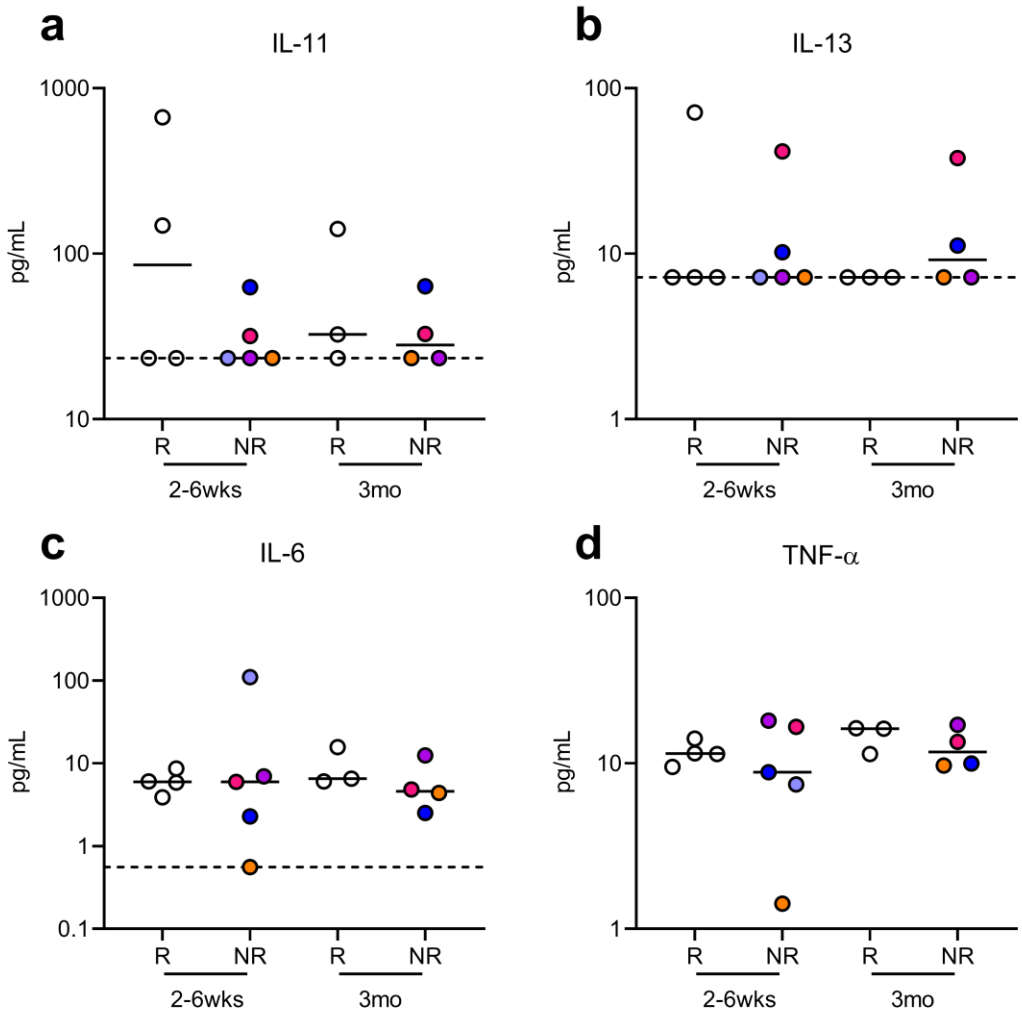


Figure 4. Serum cytokine levels in participants with SSc after the second SARS-CoV-2 vaccination. ELLA Automated ELISAs were used to determine the levels of serum cytokines **(a)** IL-11, **(b)** IL-13, **(c)** IL-6, **(d)** TNF-α. The dashed lines represent the limits of detection (LOD) for each cytokine. Participants with points on the dashed line fell below the LOD. One participant with a concentration of IL-6 below the limit of detection was assigned the LOD value. NR refers to SSc Non-Responders, R is SSc Responders. 2-6wks indicates 2-6 weeks post dose 2, 3mo indicates 3 months post dose 2. One SSc-NR was lost

to follow-up at 3mo. The colored points represent the same participant over time, solid lines depict the median. Fisher's exact tests were used to compare the proportions of SSc-R and SSc-NR with concentrations of IL-11 **(a)** and IL-13 **(b)** above the limit of detection at 2-6wks, and 3mo. Student's t-tests were used to compare the levels of IL-6 **(c)**, and TNF-α **(d)**, between SSc-R and SSc-NR at 2-6wks, and 3mo.

3.2.4 Discussion

Herein, we discovered that some individuals with SSc may exhibit delayed humoral responses to the second SARS-CoV-2 vaccination, taking 1-3 months to catch up to the levels seen in controls. This delayed humoral response was not tied to differences in spike-specific $CD4^+$ or $CD8^+$ T cell levels, spike-specific $CD4^+$ T cell skew, age, sex, CMV serostatus, vaccine type, dosing interval, immunomodulatory drug classes, autoantibody profiles, or serum IL-11, IL-13, IL-6, or TNF-α levels. The mechanisms behind this delay thus remain to be elucidated. Validation of this phenomenon in a larger cohort of participants with SSc will be necessary to identify specific disease, drug, biological, or demographic features associated with this phenomenon.

Previous studies have not reported a delayed antibody response in participants with other autoimmune conditions, like rheumatoid arthritis, after the second SARS-CoV-2 vaccination [298,301,317]. Given the paucity of data in the literature focusing on vaccination responses in participants with SSc, and the potential disparity between the humoral response kinetics of participants with

SSc and individuals with other autoimmune conditions, it also remains unclear if this delayed response in some participants is unique to SARS-CoV-2 vaccination, or whether it occurs following other primary vaccination series and primary infections. As new vaccines continue to be introduced, understanding the kinetics of humoral responses in vulnerable populations, and thus the period in which they remain vulnerable prior to antibody generation, will be essential for identifying new strategies to ensure protection is in place during these vulnerable periods. The potential for delayed humoral responses in some participants with SSc is a nuance that must be considered when communicating with patients so that they can continue to take precautionary measures to avoid infections during this period.

Chapter 4. No evidence of immune exhaustion after repeated SARS-CoV-2 vaccination in vulnerable and healthy populations

4.1 Preface

Chapter 4 contains a manuscript published in *Nature Communications**. As the pandemic continued and new variants emerged, vulnerable populations in particular were recommended to receive additional SARS-CoV-2 vaccinations. The accelerated and frequent nature of these additional doses, compared with other vaccinations, led to concerns from the public that these vaccinations could overwhelm or exhaust their immune system. In order to alleviate public concern, and in particular the concern of vulnerable populations who are targeted for these vaccinations, we explored if repeated SARS-CoV-2 vaccination contributed to T cell exhaustion in multiple vulnerable populations and healthy younger adults. Prior to this study, immune exhaustion in the context of repeated SARS-CoV-2 vaccination had not largely been comprehensively examined, especially in vulnerable populations. Our study incorporated not only phenotypic markers associated with exhaustion, but also evaluated the functional capacity of the T cells after the second, third, and fourth SARS-CoV-2 vaccinations in older adults in long-term care (LTC), participants with RA on immunosuppressive drugs, and healthy younger adults (HA).

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No evidence of immune exhaustion after repeated SARS-CoV-2 vaccination in vulnerable and healthy populations

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Abstract

Frequent SARS-CoV-2 vaccination in vulnerable populations has raised concerns that this may contribute to T cell exhaustion, which could negatively affect the quality of immune protection. Herein, we examined the impact of repeated SARS-CoV-2 vaccination on T cell phenotypic and functional exhaustion in frail older adults in long-term care (n=23), individuals on immunosuppressive drugs (n=10), and healthy adults (n=43), in Canada. Spike-specific $CD4^+$ and $CD8^+$ T cell levels did not decline in any cohort following repeated SARS-CoV-2 vaccination, nor did the expression of exhaustion markers on spike-specific or total T cells increase. T cell production of multiple cytokines (i.e. polyfunctionality) in response to the spike protein of SARS-CoV-2 did not decline in any cohort following repeated vaccination. None of the cohorts displayed elevated levels of terminally differentiated T cells following multiple

SARS-CoV-2 vaccinations. Thus, repeated SARS-CoV-2 vaccination was not associated with increased T cell exhaustion in older frail adults, immunosuppressed individuals, or healthy adults.

Introduction

One of the unique features of the COVID-19 pandemic was the recommendation for multiple vaccines in a relatively short period of time[1–3]. In Canada, the primary vaccination series typically included 2 mRNA vaccines administered weeks to months apart, and additional doses were recommended for protection against emerging variants[1,4,5]. As increasingly immune evasive variants continue to emerge, it is expected that vulnerable populations such as older adults and the immunocompromised will continue to be offered multiple updated SARS-CoV-2 vaccinations in relatively short intervals to maintain protective immunity[1,2]. However, accelerated and frequent boosting in vulnerable patients has raised concerns that this may contribute to detrimental long term effects, such as on the immune system itself[6,7]. Furthermore, SARS-CoV-2 infection is known to cause changes in the T cell compartment, including differences in expression of receptors associated with exhaustion[8,9]. While immune responses to infection and vaccination are not equivalent, in the eyes of the public, this concern of immune exhaustion after infection can carry over to vaccination.

Immune exhaustion is a nuanced term, typically used to describe the consequences of chronic stimulation of T cells in the context of infection or malignancy[10]. Long-term or high levels of exposure to their cognate antigen, and

thus repeated stimulation through the T cell receptor, can result in the upregulation of exhaustion markers such as PD-1, LAG-3, TIM-3, and TIGIT[10–13]. Sustained co-expression of multiple exhaustion markers produces stronger inhibitory signals which dampen T cell activation and cytokine production, and thus can be used to identify more severely exhausted T cells[10,13]. The delineation between activation and exhaustion, however, can be unclear. As an example, in convalescent COVID-19 patients, a higher proportion of IFN-γ producing T cells were found in the PD-1^{+} population than the PD-1^{-} population, which is more consistent with a state of activation rather than exhaustion[14]. It is therefore critical to consider the co-expression of multiple exhaustion markers, as well as the cytokine-producing functional capacity of T cells when considering cells to be activated or exhausted. While vaccination is not a chronic stimulation condition, the novelty of the mRNA SARS-CoV-2 vaccines, and the frequency of vaccination in vulnerable populations, have prompted consideration of the intricacies of T cell immune exhaustion in the context of mRNA vaccination.

In this study, we investigated the impacts of repeated SARS-CoV-2 vaccination on circulating and spike-specific T cells, including expression of exhaustion markers, and explored their functional capacities after the second, third and fourth doses of mRNA vaccines in vulnerable cohorts of older adults in long-term care facilities and individuals with rheumatoid arthritis on immunosuppressive drugs, and in healthy community-dwelling adults. We show that within each cohort between the second, third, and fourth vaccinations, there were no significant declines in spike-specific T cell levels, their cytokine production

capacity, or major changes in exhaustion associated marker expression, indicating that immune exhaustion of T cells does not occur following repeated SARS-CoV-2 vaccination.

Results

Study population

We assessed the impact of repeated SARS-CoV-2 vaccination on T cell phenotypes and function in frail older adults living in long-term care facilities (LTC), people living with rheumatoid arthritis and taking immunosuppressive drugs (RA), and healthy younger adults (HA). To ensure that changes in immune phenotype were not due to SARS-CoV-2 infection, participants were excluded before or during the study period if they had a positive PCR test, rapid antigen test, or seroconverted to become positive for anti-nucleocapsid IgG.. The LTC cohort included frail older adults living in long-term care homes (n=23, median age 84.0 yrs, 60.9% female) (Table 1). The RA cohort included participants with rheumatoid arthritis, who were on immunosuppressive drugs (n=10, median age 68.0 yrs, 70.0% female) (Table 1). A detailed description of the immunosuppressive drug classes used by participants with RA is available in Supplementary Data 1. The HA cohort was comprised of younger healthy individuals (n=43, median age 47.0 yrs, 60.5% female) (Table 1). The cohorts did not significantly differ in sex distribution but did significantly differ in age (Table 1). The most common first, second, and third vaccine type was BNT162b2 in the HA and RA cohorts, whereas mRNA-1273 was more commonly used in the LTC

cohort (Table 1). mRNA-1273 was the most common fourth dose vaccine type in the LTC and RA cohorts, while the HA cohort had a more even distribution of mRNA-1273 and BNT162b2 (Table 1). The intervals between successive vaccinations significantly differed between the cohorts (Table 1). Blood samples were collected from participants 3 months after their second, third, and fourth SARS-CoV-2 vaccinations.

Table 1. Participant Demographics

	Frail older adults (LTC)	Immunosuppressed (RA)	Younger Adults (HA)	*p* value
Sample size	23	10	43	N/A
Age, median ± SD	84.0 ± 12.9	68.0 ± 6.9	47.0 ± 10.3	<0.0001[a]
Sex, % female (n)	60.9 (14)	70.0 (7)	60.5 (26)	0.8922[b]
First dose vaccine type	11 BNT162b2 12 mRNA-1273	8 BNT162b2 2 mRNA-1273	43 BNT162b2	<0.0001[b]
Days between dose 1 and dose 2 (median ± SD)	27.0 ± 3.4	76.5 ± 18.5	28.0 ± 28.7	<0.0001[a]

Second dose vaccine type	11 BNT162b2 12 mRNA-1273	8 BNT162b2 2 mRNA-1273	41 BNT162b2 2 mRNA-1273	<0.0001[b]
Days between dose 2 and dose 3 (median ± SD)	218.0 ± 9.2	138.0 ± 23.0	254.5 ± 45.1	<0.0001[a]
Third dose vaccine type[c]	11 BNT162b2 12 mRNA-1273	8 BNT162b2 2 mRNA-1273	15 BNT162b2 13 mRNA-1273 2 unknown	0.2609[b]
Days between dose 3 and dose 4 (median ± SD)	133.0 ± 9.0	104.0 ± 38.8	245.0 ± 15.2	<0.0001[a]
Fourth dose vaccine type[c]	1 BNT162b2 17 mRNA-1273	3 BNT162b2 5 mRNA-1273	4 BNT162b2 3 mRNA-1273	0.0091[b]

[a]One-way ANOVA

[b]two-tailed Fisher's exact test

[c]Not all participants provided samples post dose 3 and post dose 4.

Expression of markers associated with exhaustion on spike-specific CD4$^+$ and CD8$^+$ T cells

We first examined if repeated SARS-CoV-2 vaccination impacted spike-specific CD4$^+$ and CD8$^+$ T cell levels using activation-induced marker (AIM) assays, and multivariable linear mixed models accounting for age and sex. Compared with post dose 2, older adults (LTC) displayed elevated frequencies of spike-specific CD4$^+$ T cells following the fourth SARS-CoV-2 vaccination (Fig. 1a, Supplementary Data 2). The frequencies of spike-specific CD8$^+$ T cells were also elevated in older adults (LTC) after the third and fourth SARS-CoV-2 vaccinations, compared with the second dose (Fig. 1b, Supplementary Data 2). The immunosuppressed RA and the HA cohorts did not display significant changes in the frequencies of spike-specific CD4$^+$ or spike-specific CD8$^+$ T cells following repeated SARS-CoV-2 vaccination (Fig. 1a, 1b, Supplementary Data 2).

To further elucidate the impact of repeated SARS-CoV-2 vaccinations on the spike-specific CD4$^+$ and CD8$^+$ T cell populations, we investigated their surface co-expression of the exhaustion markers LAG-3, PD-1, and TIM-3. Exhaustion markers can also be markers of activation, and therefore it is expected that there will be some increase in expression on spike-specific T cells after stimulation with the spike peptide[14]. If the frequencies of cells expressing these markers remained the same after repeated vaccination, this would indicate normal activation; whereas if the frequencies of cells expressing exhaustion markers

increased after each dose, this would suggest, but not definitively demonstrate, a progression toward cellular exhaustion. No significant changes in combined exhaustion marker expression on spike-specific $CD4^+$ T cells were observed in older adults (LTC), immunosuppressed individuals with RA, or the HA cohort, following the third or fourth SARS-CoV-2 vaccination, compared with the second vaccination (Fig. 1c, Supplementary Data 3). Compared with post dose 2, older adults living in LTC displayed elevated frequencies of PD-1^+LAG-3^+TIM-3^- spike-specific $CD8^+$ T cells following the third and fourth SARS-CoV-2 vaccinations (Fig. 1d, Supplementary Data 3). No significant changes in expression of exhaustion markers on spike-specific $CD8^+$ T cells were observed in immunosuppressed individuals with RA or the HA cohort after repeated SARS-CoV-2 vaccination (Fig. 1d, Supplementary Data 3).

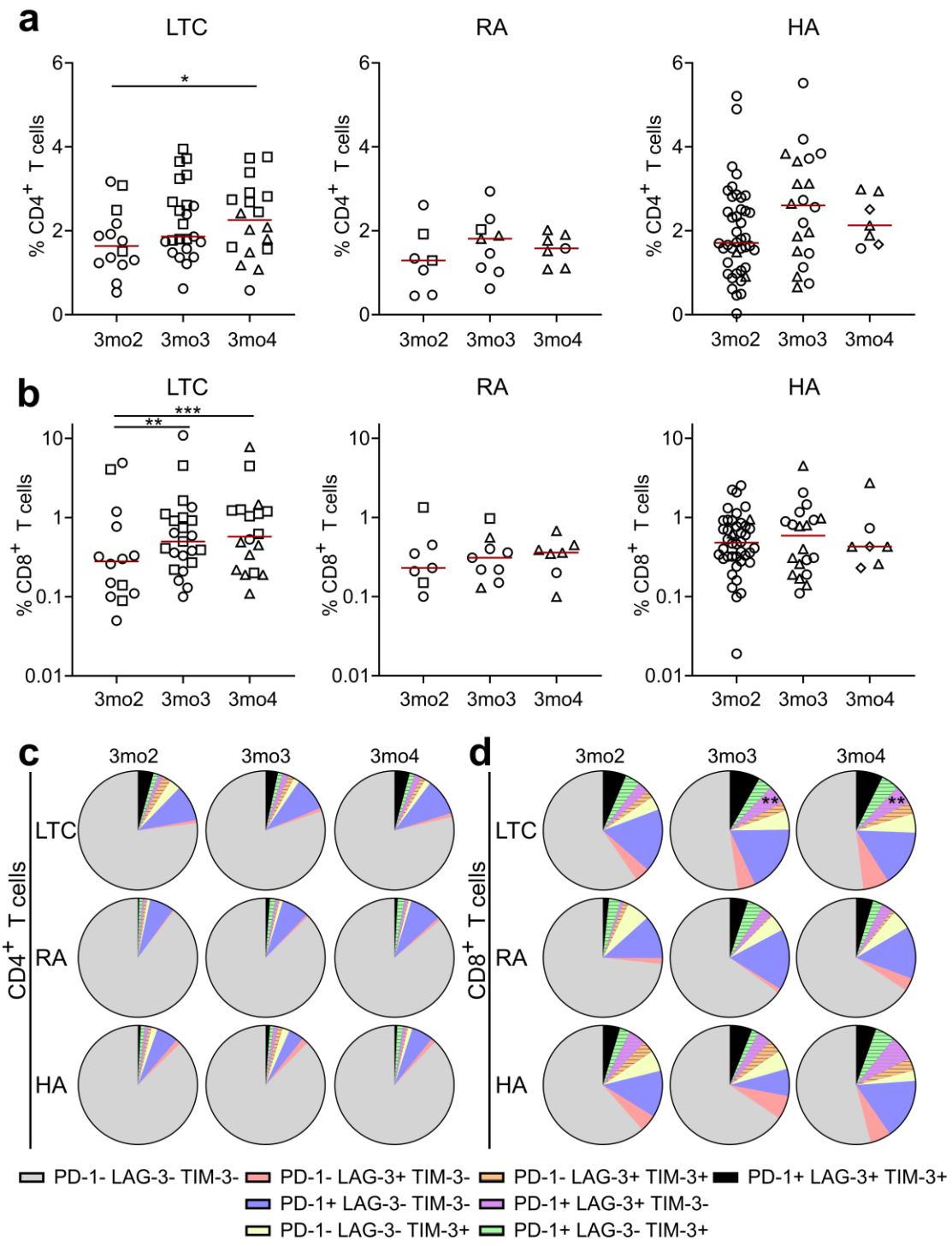


Figure 1. Spike-specific CD4⁺ and CD8⁺ T cell levels and expression of PD-1, TIM-3, and LAG-3 following subsequent SARS-CoV-2 vaccinations. Spike-

specific T cell levels and surface phenotypes were assessed by AIM assay and flow cytometry in the LTC, RA, and HA cohorts. (a) Frequencies of spike-specific $CD4^+$ T cells. $p = 0.0420$*. (b) Frequencies of spike-specific $CD8^+$ T cells. $p = 0.0048$**, $p = 0.0002$***. The solid red lines indicate the median of each group. (c) Frequencies of spike-specific $CD4^+$ T cells expressing each combination of PD-1, TIM-3, and LAG-3, displayed as the mean of each combination as a proportion of all combinations. (d) Frequencies of spike-specific $CD8^+$ T cells expressing each combination of PD-1, TIM-3, LAG-3, displayed as the mean of each combination as a proportion of all combinations. LTC: 3mo2 n=14, 3mo3 n=23, 3mo4 n=18. RA: 3mo2 n=7, 3mo3 n=9, 3mo4 n=7. HA: 3mo2 n=42, 3mo3 n=20, 3mo4 n=7. For (a) and (b), participant vaccination history is indicated by circles (BNT162b2 only), squares (mRNA-1273 only), triangles (mixed BNT162b2 and mRNA-1273), or diamonds (mRNA only but one vaccine unknown). 3moX denotes 3 months post dose X. Multivariable linear mixed models accounting for age and sex were used to assess changes in spike-specific T cells levels within each cohort, and changes in the frequencies of PD-1, TIM-3, and LAG-3, within each cohort (a-d). FDR adjusted p values were obtained within each cohort for (c), and (d), to account for multiple testing on cells from the same parent population. If *p* values are not indicated, the result was not significant.

T cell functionality and polyfunctionality following repeated SARS-CoV-2 vaccination

After observing minimal changes in the surface expression of the exhaustion markers PD-1, TIM-3, and LAG-3 on spike-specific T cells following repeated SARS-CoV-2 vaccination, we investigated whether there were deficits in T cell cytokine production capabilities. In particular, the loss of polyfunctionality — the ability of a T cell to produce multiple different cytokines — is a characteristic of functional exhaustion[10]. COMPASS employs Bayesian statistics to determine the posterior probabilities of antigen-specific production of various combinations of cytokines, based on examination of flow cytometry data[15]. The posterior probability of spike-specific $CD4^+$ (Fig. 2a) and $CD8^+$ T cell (Fig. 2b) responses expressing any combination of TNF-α, IL-2, GM-CSF, and IFN-γ was evaluated using COMPASS[15]. The COMPASS functionality scores (FS), representing the proportion of expressed cytokine combinations out of all the possible combinations in each participant, did not change following repeated SARS-CoV-2 vaccinations, in any cohort, for either $CD4^+$ or $CD8^+$ T cells (Fig. 2c, 2d, Supplementary Data 4). Therefore, repeated vaccination did not result in a loss of diversity of expressed cytokine combinations[10,13]. To ensure the lack of change in the FS was not masking movement from higher states of polyfunctionality (i.e., producing 3-4 cytokines) to previously unoccupied states of lower polyfunctionality (i.e., producing 1-2 cytokines), we also employed the COMPASS polyfunctionality score (PFS). The PFS is similar to the FS, but weighs polyfunctional T cell subsets producing 3-4 cytokines more highly than those that produce fewer cytokines[15]. We found that the PFS for $CD4^+$ or $CD8^+$ T cell cytokine expression did not significantly decline in any cohort between

second, third, or fourth vaccinations, indicating that there was no reduction in the most highly polyfunctional T cell subsets following repeated SARS-CoV-2 vaccinations (Fig. 2e, 2f, Supplementary Data 4).

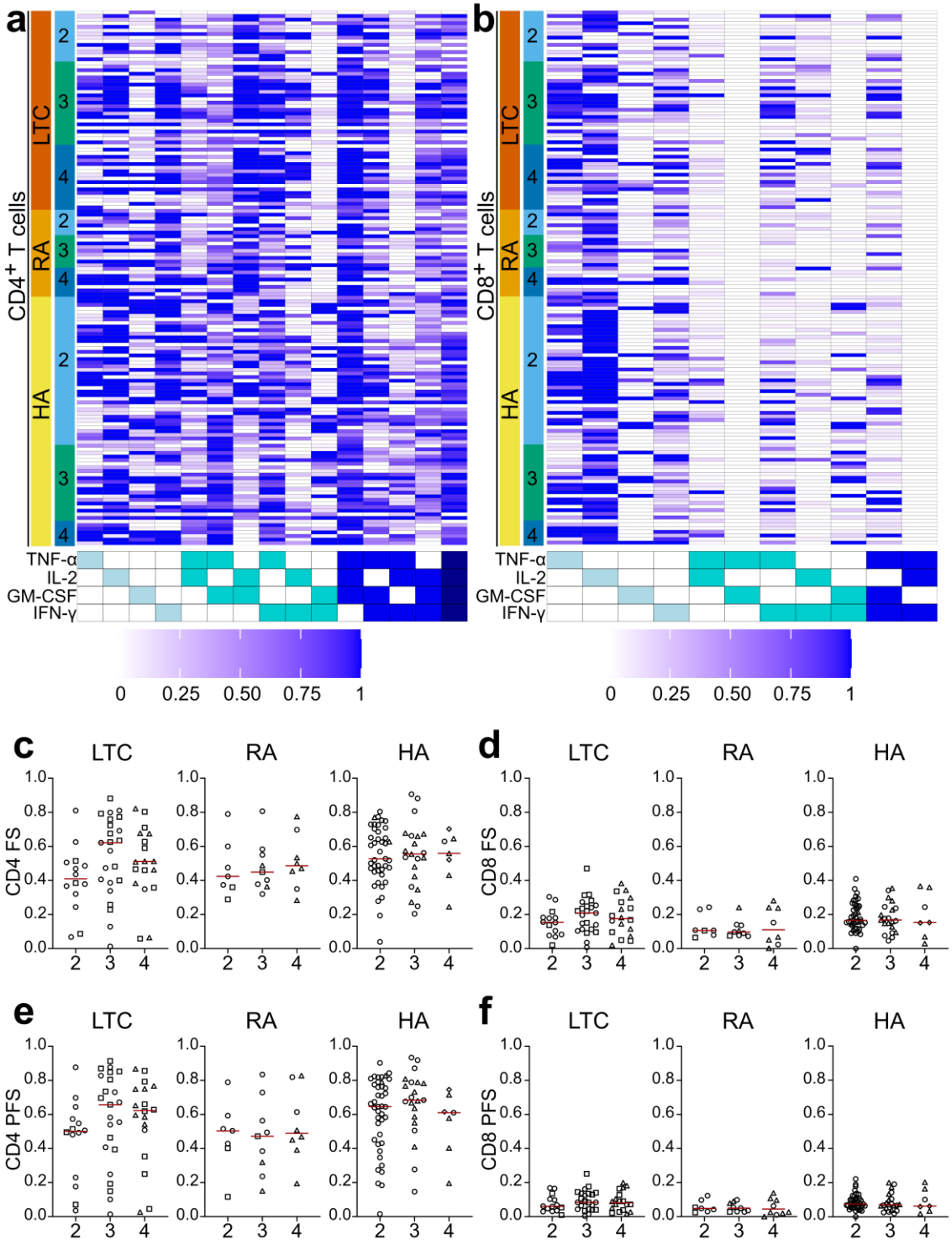


Figure 2. T cell functionality and polyfunctionality following repeated SARS-CoV-2 vaccination. PBMCs were stimulated for 24 hours using a peptide

pool derived from the spike protein of SARS-CoV-2, and intracellular cytokine stains were conducted to evaluate cytokine production using flow cytometry. COMPASS heatmap of posterior probabilities of a spike-specific response in (a) $CD4^+$ T cells (b) and $CD8^+$ T cells for each cytokine combination. COMPASS functionality score (FS) for (c) $CD4^+$ T cells and (d) $CD8^+$ T cells in each cohort following repeated SARS-CoV-2 vaccinations. COMPASS polyfunctionality score (PFS) for (e) $CD4^+$ T cells and (f) $CD8^+$ T cells in each cohort following repeated SARS-CoV-2 vaccinations. For (a) and (b), each column represents a different cytokine combination as indicated by the shaded legend beneath the heatmap, while each row is a unique participant. The blue scaling within the heatmaps indicates the posterior probabilities. Colored bars on the left side denote the cohorts associated with the rows, and timepoint within each cohort. For (c-f), participant vaccination history is indicated by circles (BNT162b2 only), squares (mRNA-1273 only), triangles (mixed BNT162b2 and mRNA-1273), or diamonds (mRNA only but one vaccine unknown). The solid red lines indicate the median of each group. 2 indicates 3mo2, 3 is 3mo3, and 4 is 3mo4. LTC: 3mo2 n=14, 3mo3 n=23, 3mo4 n=18. RA: 3mo2 n=7, 3mo3 n=9, 3mo4 n=8. HA: 3mo2 n=41, 3mo3 n=21, 3mo4 n=7. Multivariable linear mixed models accounting for age and sex were used to assess changes in the $CD4^+$ and $CD8^+$ T cell FS and PFS within a given cohort following additional SARS-CoV-2 vaccinations. For heatmaps, columns of cytokine combination subsets with posterior probabilities less than 0.005 for all participants are not displayed. If *p* values are not indicated, the result was not significant.

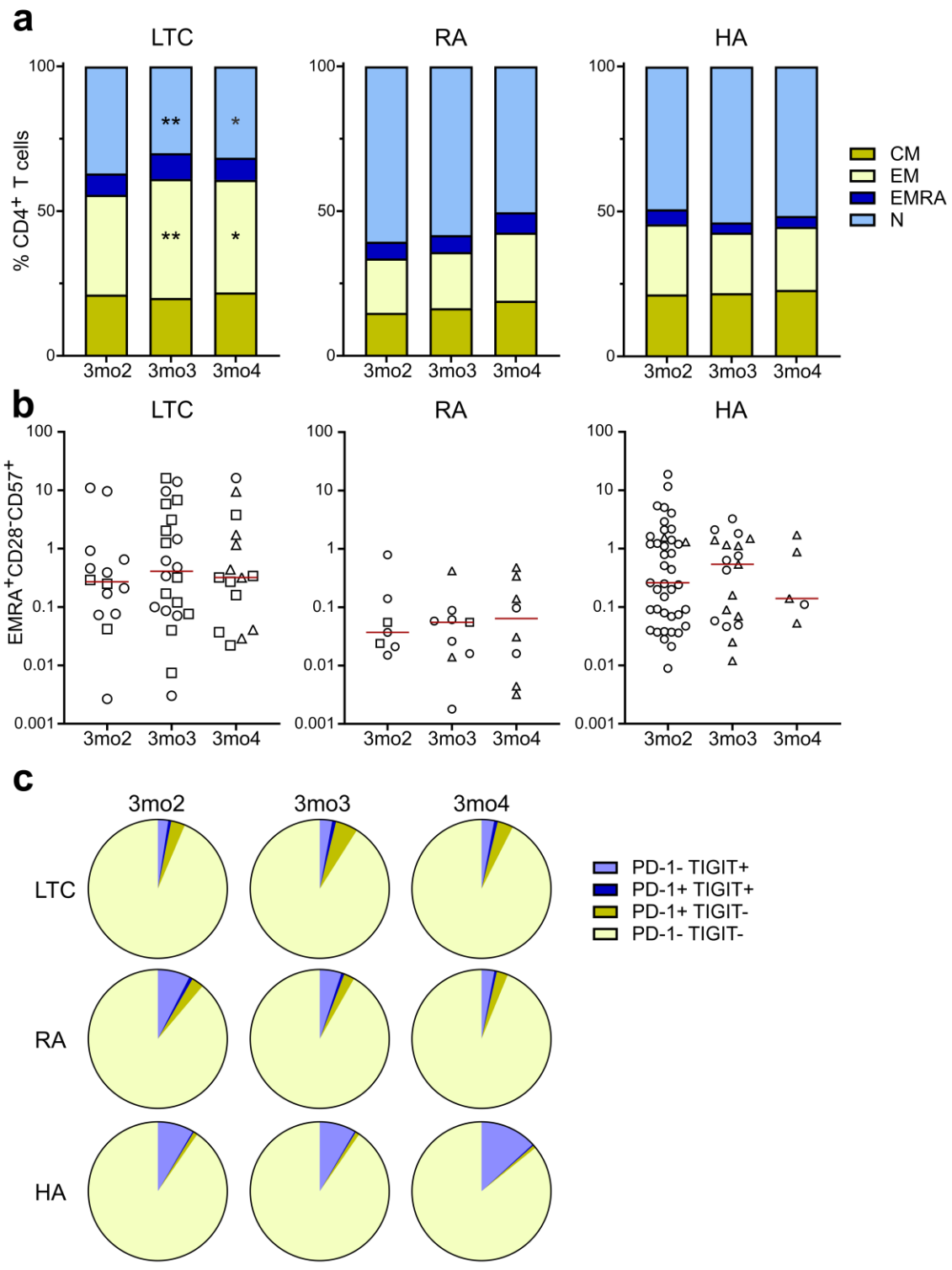
Immunophenotyping of the circulating T cell compartment following repeated SARS-CoV-2 vaccination

Next, we evaluated whether the circulating T cell compartment, in the absence of *ex vivo* stimulation, changed in any of the cohorts following repeated SARS-CoV-2 vaccination. Using flow cytometry, we assessed frequencies of $CD4^+$ and $CD8^+$ T cell naïve, effector memory (EM), central memory (CM), and EM re-expressing CD45RA (EMRA) subsets. The older adults in LTC displayed significantly higher frequencies of EM $CD4^+$ T cells following the third and fourth SARS-CoV-2 vaccinations, as compared with post dose 2 (Fig. 3a, Supplementary Data 5). The increase in EM $CD4^+$ T cells in older adults (LTC) was coupled with a decrease in naïve $CD4^+$ T cells following the third and fourth SARS-CoV-2 vaccinations (Fig. 3a, Supplementary Data 5). Interestingly, this change in $CD4^+$ T cell distribution was not observed in the immunosuppressed RA or the HA cohorts (Fig. 3a, Supplementary Data 5).

It is well recognized that chronic infections can result in persistent stimulation of T cells, which can push them towards a terminally differentiated phenotype, characterized by expression of CD57 coupled with a loss of CD28 expression, especially within the EMRA compartment[16–19]. In contrast, vaccination is an acute stimulus, multiple times, and would not be expected to act as a chronic stimulation[20]. As such, we evaluated the levels of $EMRA^+CD28^-CD57^+$ T cells within the $CD4^+$ and $CD8^+$ T cell compartments. There were no significant changes in the frequencies of $EMRA^+CD28^-CD57^+$ $CD4^+$ T cells in older adults in

LTC, immunosuppressed individuals with RA, or the HA cohort following repeated SARS-CoV-2 vaccinations (Fig. 3b, Supplementary Data 5).

We next examined whether repeated SARS-CoV-2 vaccination was associated with changes in overall T cell phenotypic exhaustion, characterized by expression of multiple exhaustion markers (i.e., PD-1, TIM-3, and TIGIT) on unstimulated T cells following the second, third, and fourth SARS-CoV-2 vaccinations. All cohorts displayed minimal levels of TIM-3$^+$ CD4$^+$ T cells, which varied little following additional SARS-CoV-2 vaccinations (Supplementary Fig. 1, Supplementary Data 6). Older adults in LTC, immunosuppressed individuals with RA, and the HA cohort also did not display any significant changes in the frequencies of PD-1$^+$ or TIGIT$^+$ CD4$^+$ T cells following the third or fourth SARS-CoV-2 vaccinations, compared with post dose 2 (Fig. 3c, Supplementary Data 5).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PD-1+ TIGIT-
PD-1- TIGIT-

Figure 3. Immunophenotyping of circulating $CD4^+$ T cell compartment following repeated SARS-CoV-2 vaccination. Flow cytometry was used to assess the frequencies of naïve (N, $CCR7^+CD45RA^+$), central memory (CM, $CCR7^+CD45RA^-$), effector memory (EM, $CCR7^-CD45RA^-$) and EM re-expressing CD45RA (EMRA, $CD45RA^+CCR7^-$) $CD4^+$ T cells. (a) The mean frequency of each T cell subset was determined and plotted in a stacked bar format. (b) The frequencies of terminally differentiated $CD4^+$ T cells ($EMRA^+CD57^+CD28^-$), out of all $CD4^+$ T cells, within each cohort following the second, third, and fourth SARS-CoV-2 vaccinations. The solid red lines indicate the median of each group. (c) The frequency of $CD4^+$ T cells expressing each combination of the exhaustion markers PD-1 and TIGIT, displayed as the mean value within each cohort at each timepoint. For (b), participant vaccination history is indicated by circles (BNT162b2 only), squares (mRNA-1273 only), triangles (mixed BNT162b2 and mRNA-1273), or diamonds (mRNA only but one vaccine unknown). 3moX denotes 3 months post dose X. LTC: 3mo2 n=14, 3mo3 n=22, 3mo4 n=15. RA: 3mo2 n=7, 3mo3 n=9, 3mo4 n=8. HA: 3mo2 n=41, 3mo3 n=19, 3mo4 n=5. Multivariable linear mixed models accounting for age and sex were used to assess changes in the frequencies of each T cell subset within a given cohort following repeated SARS-CoV-2 vaccinations. FDR adjusted p values were obtained within each cohort for (a) and (c) to account for multiple testing on cells from the same parent population. If *p* values are not indicated, the result was not significant. $p<0.05$ *, $p<0.01$ **.

Older adults in LTC and immunosuppressed individuals with RA did not display significant changes in the $CD8^+$ T cell compartment following repeated SARS-CoV-2 vaccination (Fig. 4a, Supplementary Data 7). The HA cohort had significantly lower frequencies of EMRA $CD8^+$ T cells following the third SARS-CoV-2 vaccination compared with post dose 2, but this observation disappeared following dose 4 (Fig. 4a, Supplementary Data 7). There were no significant changes in the frequencies of $EMRA^+CD28^-CD57^+$, or PD-1^+ and $TIGIT^+$ $CD8^+$ T cells in older adults in LTC, immunosuppressed individuals with RA, or the HA cohort following repeated SARS-CoV-2 vaccinations (Fig. 4b, 4c, Supplementary Data 7). Similar to what was observed for $CD4^+$ T cells, the $CD8^+$ T cells also expressed only minimal levels of TIM-3 (Supplementary Fig. 1, Supplementary Data 6).

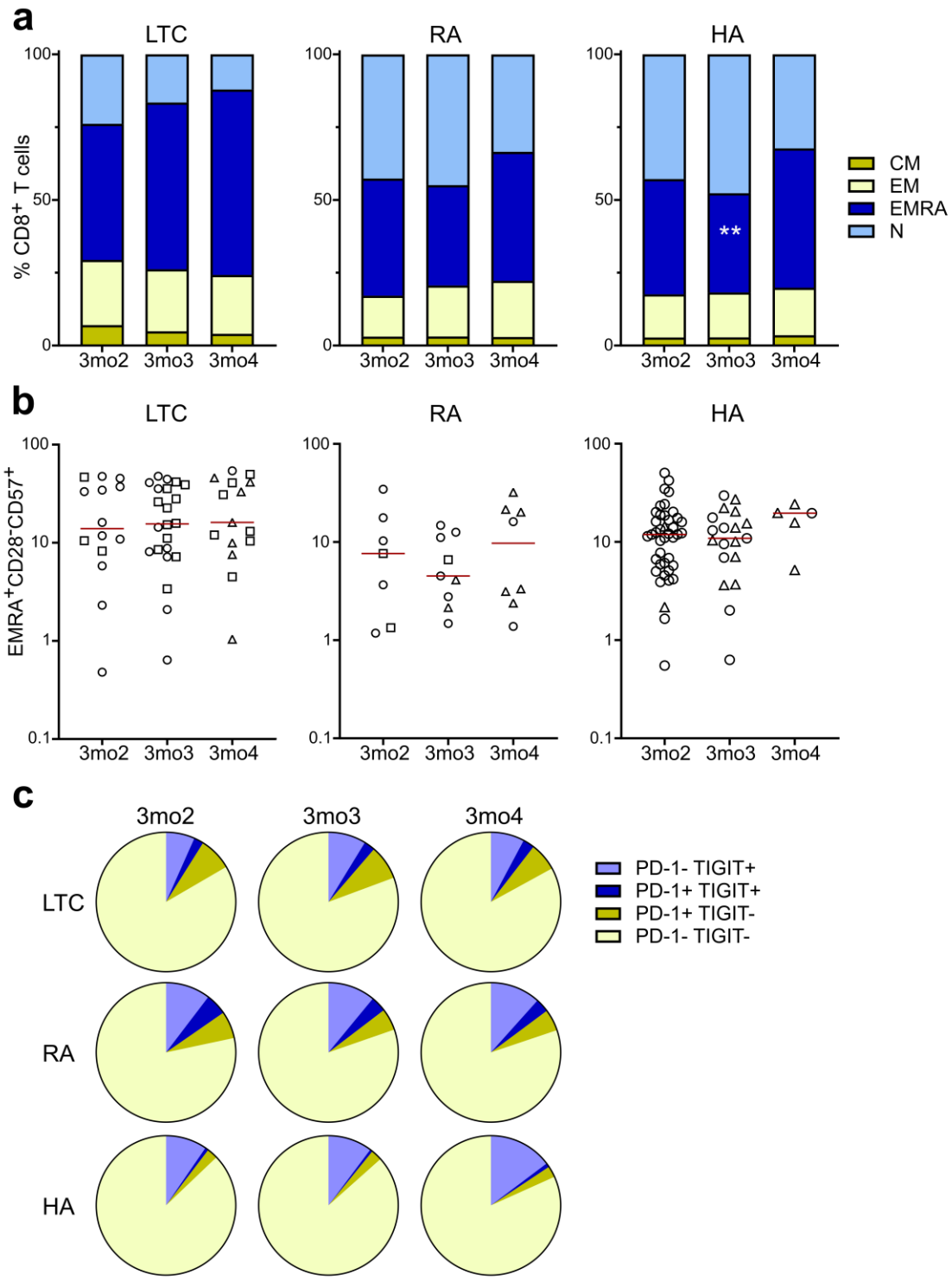

Figure 4. Immunophenotyping of circulating CD8⁺ T cell compartment following repeated SARS-CoV-2 vaccination. Flow cytometry was used to

assess $CD8^+$ T cell phenotypes. (a) The mean frequencies of naïve (N), CM, EM, and EMRA $CD8^+$ T cells were determined and plotted in a stacked bar format. (b) The frequencies of terminally differentiated $CD8^+$ T cells ($EMRA^+CD57^+CD28^-$), out of all $CD8^+$ T cells, within each cohort following the second, third, and fourth SARS-CoV-2 vaccinations. The solid red lines indicate the median of each group. (c) The frequency of $CD8^+$ T cells expressing each combination of the exhaustion markers PD-1 and TIGIT, displayed as the mean value within each cohort at each timepoint. For (b), participant vaccination history is indicated by circles (BNT162b2 only), squares (mRNA-1273 only), triangles (mixed BNT162b2 and mRNA-1273), or diamonds (mRNA only but one vaccine unknown). 3moX denotes 3 months post dose X. LTC: 3mo2 n=14, 3mo3 n=22, 3mo4 n=15. RA: 3mo2 n=7, 3mo3 n=9, 3mo4 n=8. HA: 3mo2 n=41, 3mo3 n=19, 3mo4 n=5. Multivariable linear mixed models accounting for age and sex were used to assess changes in the frequencies of each T cell subset within a given cohort following repeated SARS-CoV-2 vaccinations. FDR adjusted p values were obtained within each cohort for (a) and (c) to account for multiple testing on cells from the same parent population. If *p* values are not indicated, the result was not significant. $p<0.05$ *.

Comparisons of spike-specific T cells, exhausted phenotypes, and functional capacity between the LTC, RA, and HA cohorts

To assess if functional differences in cellular immunity are present between the LTC, RA, and HA cohorts, we examined if the three cohorts differed in their

spike-specific T cell responses, T cell functional capacity, and exhausted phenotypes, following the second, third, and fourth SARS-CoV-2 vaccinations. The spike-specific $CD4^+$ and $CD8^+$ T cell levels were similar between the three cohorts after each vaccine dose (Supplementary Data 8). Expression of exhaustion markers on spike-specific $CD4^+$ and $CD8^+$ T cells significantly differed between the cohorts after the second and third vaccinations, but not after the fourth SARS-CoV-2 vaccination (Supplementary Data 8). Changes in exhaustion marker expression between the cohorts varied by vaccine dose (Supplementary Data 8). At all timepoints, the CD4 PFS, CD8 FS, and CD8 PFS were similar between the cohorts (Supplementary Data 8). The CD4 FS of the HA cohort was significantly higher than that of the LTC cohort following the second SARS-CoV-2 vaccination, though this difference was not observed following subsequent vaccinations. Collectively, these data demonstrate that there are subtle differences in T cell responses to vaccination across cohorts.

Consistent with well-documented changes in the T cell compartment that occur with aging, participants in the LTC cohort had higher frequencies of EM, EMRA, and terminally differentiated $CD4^+$ T cells, and lower levels of naïve $CD4^+$ T cells, compared to the HA and/or RA cohorts (Supplementary Data 8). The LTC cohort also had lower levels of naïve $CD8^+$ T cells but higher frequencies of EMRA $CD8^+$ T cells compared to either the HA or RA cohort (Supplementary Data 8). Terminally differentiated $CD8^+$ T cell frequencies were similar between all cohorts after the second vaccination but higher in the LTC cohort than the RA cohort following the third SARS-CoV-2 vaccination (Supplementary Figure 8). Similar to

what was observed for the spike-specific T cell exhaustion marker expression, the frequencies of overall $CD4^+$ and $CD8^+$ T cells co-expressing PD-1 and TIGIT differed between cohorts following the second and third SARS-CoV-2 vaccinations (Supplementary Data 8). These differences between the cohorts in overall T cell exhaustion marker expression continued following the fourth SARS-CoV-2 vaccination for $CD4^+$ but not $CD8^+$ T cells (Supplementary Data 8). Combined LTC, RA, and HA cohort correlation analyses were also conducted to assess the relationships of the exhaustion parameters (Supplementary Figure 2).

Impact of dosing interval on T cell exhausted phenotypes and functional capacity

To maximize the number of healthy adults vaccinated with a single dose when SARS-CoV-2 vaccines initially became available, Canadian public health units shifted from using the relatively short manufacturer-recommended dosing interval of 21 days to either a ‘delayed’ interval (35-42 days between dose 1 and 2) or an ‘extended’ interval (>45 days between dose 1 and 2) for primary series vaccinations[2,3,21]. We[22] and others[23] found that a longer dosing interval between first and second vaccinations resulted in higher levels of neutralizing antibodies. To expand those observations to cellular immunity, herein, we investigated whether differences in primary series dosing interval in the HA cohort impacted spike-specific T cell levels, exhaustion, and functional capacity, as well as the overall phenotypes of the T cell compartment at 3 months post dose 2.

The ‘recommended’ (Rec) cohort received the primary series as per the recommended dosing interval (i.e. <35 days between their first and second vaccination), the ‘delayed’ (Del) cohort had 35-42 days between dose 1 and 2, and the ‘extended’ (Ext) cohort had >45 days between the first and second dose[22]. The Rec, Del, and Ext cohorts did not significantly differ in age, sex, or dose 1 and dose 2 vaccine types (Supplementary Data 9). We found no significant differences in spike-specific $CD4^+$ T cell levels or exhaustion marker expression between any of the HA dosing interval groups (Fig. 5a, b, Supplementary Data 10). There were also no significant differences in spike-specific $CD8^+$ T cell levels or their surface exhaustion marker expression (Fig. 5c, d, Supplementary Data 10). Although $CD4^+$ T cell functionality and polyfunctionality did not differ between dosing intervals (Fig. 5e, Supplementary Data 10), the HA cohort with the shortest dose interval (Rec) had significantly higher $CD8^+$ T cell functionality and polyfunctionality scores than the longest dosing interval (Ext) (Fig. 5f, Supplementary Data 10). $CD4^+$ and $CD8^+$ central memory, effector memory, EMRA, and naïve T cell frequencies did not differ between the dosing interval groups (Fig. 5g, Supplementary Data 10). Similarly, there were no significant differences in the frequencies of terminally differentiated $CD4^+$ or $CD8^+$ T cells, or the surface expression of the exhaustion markers PD-1 and TIGIT on unstimulated $CD4^+$ T cells as whole, between the dosing intervals (Fig. 5h-l, Supplementary Data 10). Participants in the Rec and Ext dosing intervals had significantly higher frequencies of PD-1^+ $TIGIT^+$ $CD8^+$ T cells than participants in the Del cohort (Fig. 5j, Supplementary Data 10).

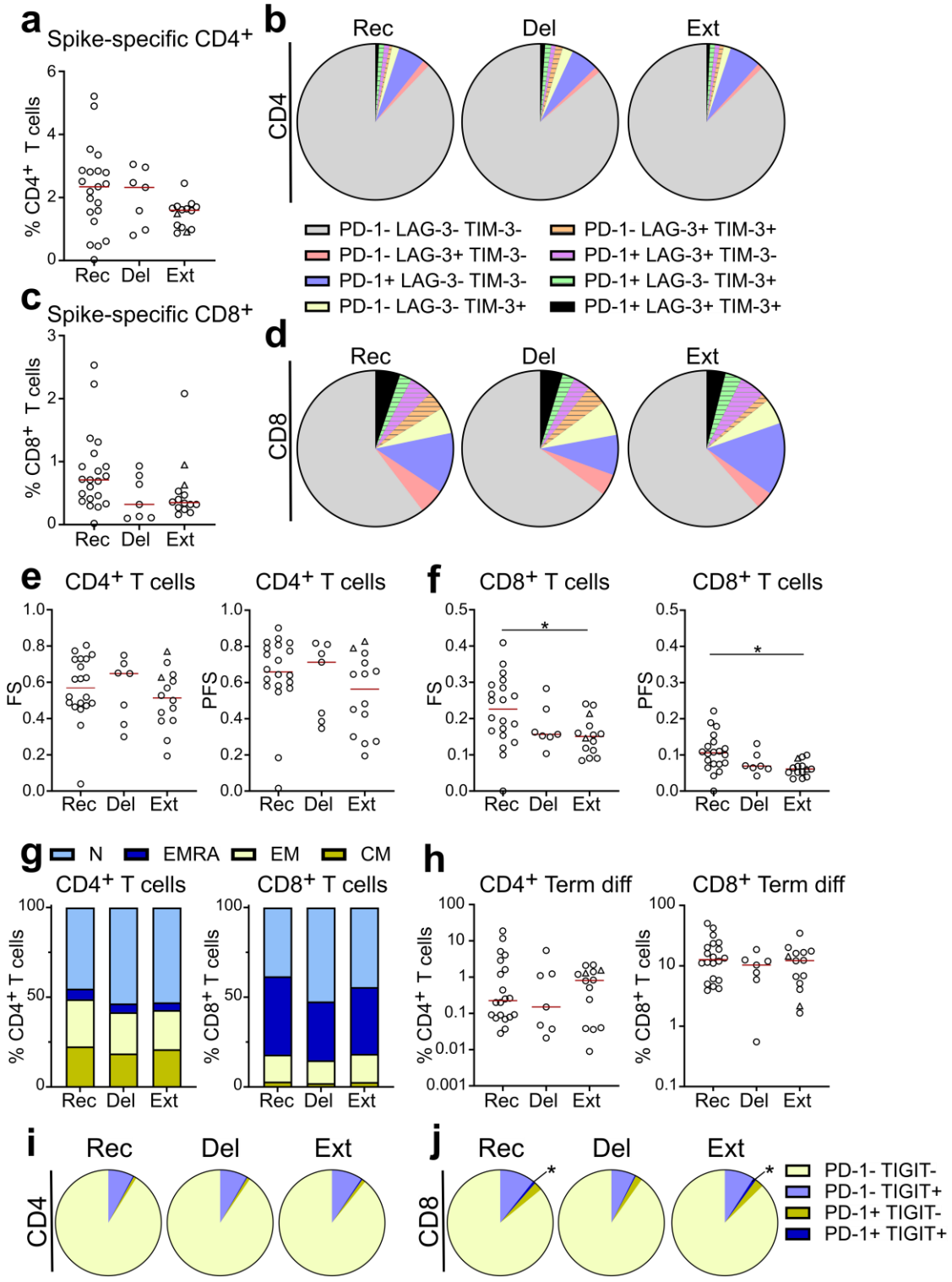

Figure 5. Immune phenotype and exhaustion in HA with different dose intervals between the first and second SARS-CoV-2 vaccination. Flow

cytometry was used to assess T cell phenotypes and functional capacity in HA at 3 months post dose 2. The HA 'recommended' (Rec) cohort had <35 days between dose 1 and 2, the 'delayed' (Del) cohort had 35-42 days between dose 1 and dose 2, and the 'extended' (Ext) cohort had >42 days between dose 1 and dose 2. (a) Frequencies of spike-specific $CD4^+$ T cells in each HA cohort. (b) Frequencies of spike-specific $CD4^+$ T cells expressing each combination of PD-1, TIM-3, and LAG-3, displayed as mean for each combination. (c) Frequencies of spike-specific $CD8^+$ T cells in each HA cohort. (d) Frequencies of spike-specific $CD8^+$ T cells expressing each combination of PD-1, TIM-3, LAG-3, displayed as mean for each combination. COMPASS functionality score (FS) and polyfunctionality score (PFS) for (e) $CD4^+$ T cells and (f) $CD8^+$ T cells in response to stimulation with peptides from the spike protein of SARS-CoV-2. (g) Frequencies of naïve, central memory (CM), effector memory (EM) and EM re-expressing CD45RA (EMRA) $CD4^+$ and $CD8^+$ T cells, displayed as mean of each combination as a proportion of all combinations, in each cohort. (h) The frequencies of terminally differentiated $CD4^+$ and $CD8^+$ T cells. The frequency of (i) $CD4^+$ T cells and (j) $CD8^+$ T cells expressing each combination of PD-1 and TIGIT, displayed as mean of each combination as a proportion of all combinations, in each cohort. For (a), (c), (e), (f), and (h), participant vaccination history is indicated by circles (BNT162b2 only), squares (mRNA-1273 only), triangles (mixed BNT162b2 and mRNA-1273), or diamonds (mRNA only but one vaccine unknown). The solid red lines indicate the median of each group. HA Rec n=20 (21 for AIMs), HA Del n=7, HA Ext n=14. ANOVAs were used to

assess differences between dosing interval cohorts. FDR adjusted p values were obtained for (b), (d), (g), (i), and (j) to account for multiple testing on cells from the same parent population. If the ANOVA was significant, after FDR correction if applicable, post-hoc tests were used to determine which dosing intervals differed from one another. If *p* values are not indicated, the result was not significant. $p<0.05$ *.

Discussion

Given the concern in the general public that repeated SARS-CoV-2 vaccination may compromise immune protection, we first examined the impact of repeated SARS-CoV-2 vaccination on the spike-specific T cell compartment. Spike-specific $CD4^+$ and $CD8^+$ T cell levels did not decline following repeated vaccination in any cohort. In fact, older adults in LTC displayed elevated spike-specific $CD4^+$ and $CD8^+$ T cell frequencies after additional vaccinations. There are conflicting reports as to whether spike-specific T cell levels increase after additional SARS-CoV-2 vaccinations beyond the primary series, at least in uninfected individuals[24–26]. Others have reported that increases in spike-specific T cell levels following the third SARS-CoV-2 vaccination in healthy donors may be transient, with spike-specific T cell frequencies declining to pre-third dose levels within 60 days post vaccination[27]. Considering that our samples were collected after this contraction period, when frequencies have returned to pre-boost levels, it was not surprising that we did not observe significant differences in the spike-specific T cell levels of the HA cohort following repeated SARS-CoV-

2 vaccination[27]. The stable spike-specific $CD4^+$ T cell levels we observed in participants with RA following repeated SARS-CoV-2 vaccination aligns with the previous findings of our lab and others[28,29]. Levels of spike-specific $CD8^+$ T cells were lower than spike-specific $CD4^+$ T cells in all cohorts, which is consistent with prior observations[30,31]. Overall, evidence suggests that repeated SARS-CoV-2 vaccination does not cause declines in spike-specific T cell levels.

There are previous reports of SARS-CoV-2 infection-associated changes in the expression of the exhaustion markers PD-1, TIM-3, LAG-3, and TIGIT, although it is unclear whether these changes exert functional consequences, or merely indicate a resolving immune response[8,9,13,32]**.** In people with a previous SARS-CoV-2 infection, levels of $TIM\text{-}3^+$ and $PD\text{-}1^+$ $CD8^+$ T cells are elevated after additional SARS-CoV-2 vaccinations[24]. Although another report demonstrated that repeated exposure to SARS-CoV-2 antigens does not induce a notable $CD8^+$ T cell exhausted phenotype in healthy donors[33], concerns have persisted that repeated SARS-CoV-2 vaccination could elicit similar effects to infection on the T cell compartment. While immune responses to SARS-CoV-2 vaccination have some similarity to those after infection, they are not identical, and the distinct conditions of SARS-CoV-2 antigen exposure can result in differences within the SARS-CoV-2-specific T cell compartment[34]. With this distinction in mind, it was perhaps unsurprising that we did not see elevation of these exhaustion markers in any of the cohorts on total $CD4^+$ T cells, $CD8^+$ T cells, or spike-specific $CD4^+$ T cells following repeated SARS-CoV-2 vaccination. The only change in exhaustion marker expression observed was an increase in

PD-1^{+}LAG-3^{+}TIM-3^{-} spike-specific CD8^{+} T cells in older frail adults, but the functional significance of this change is unclear.

To delineate T cell activation from exhaustion, both exhaustion marker expression and cytokine producing function must be evaluated, as a loss of the ability to produce multiple different cytokines (i.e. polyfunctionality) is associated with T cell exhaustion rather than activation[13,14]. In healthy adults, the primary SARS-CoV-2 vaccination series has been reported to induce functional and polyfunctional T cell responses, and these T cell cytokine responses have not been reported to decline following the third SARS-CoV-2 vaccination[24,27,35,36]. Importantly, when we extended the examination of polyfunctional T cell responses to participants from vulnerable populations, and following the fourth SARS-CoV-2 vaccination, we found that repeated SARS-CoV-2 vaccinations were not associated with decreased spike-specific T cell functionality scores or polyfunctionality scores in any of the cohorts. Thus, despite our observation of an increase in PD-1^{+}LAG-3^{+}TIM-3^{-} spike-specific CD8^{+} T cells in older frail adults, none of the cohorts displayed decreased cytokine production that would be characteristic of true exhaustion, as opposed to activation.

Severe SARS-CoV-2 infection can also lead to elevated frequencies of terminally differentiated T cells, and decreased frequencies of naïve T cells[8,9]. To further contrast the immunological impacts of repeated SARS-CoV-2 vaccination from the negative effects observed following SARS-CoV-2 infection, we examined if there were any changes in the composition of the CD4^{+} or CD8^{+} T

cell compartments after repeated SARS-CoV-2 vaccinations. The RA and HA cohorts did not display changes in their naïve $CD4^+$ or $CD8^+$ T cell levels after repeated vaccination, though the LTC cohort had higher frequencies of EM $CD4^+$ T cells and decreased levels of naïve $CD4^+$ T cells across timepoints. The absence of this change in the other cohorts, and in the $CD8^+$ T cells of the LTC cohort, suggests that this change is not a result of repeated SARS-CoV-2 vaccination, but rather cohort specific to frail older adults living in LTC. Chronic stress, older age, and frailty are all associated with reduced levels of naïve T cells[37–39]. Long-term care residents tend to be frail, and their frailty increases with the length of their stay[40,41]. Thus, it is possible that the reduction in naïve $CD4^+$ T cells is merely a product of increasing frailty and age over the course of LTC residence rather than repeated vaccinations, but future studies using pre-pandemic samples would be required to disentangle these factors. None of the cohorts displayed elevated levels of terminally differentiated $CD4^+$ or $CD8^+$ T cells following repeated SARS-CoV-2 vaccination, again contrasting repeated SARS-CoV-2 vaccination with the changes observed in SARS-CoV-2 infection and chronic infections[8,16–19].

Considering that, compared to healthy adults, older adults and individuals on immunomodulatory drugs are at risk of poor outcomes associated with SARS-CoV-2 infection[42–46], we compared T cell vaccination responses between these three cohorts. We did observe the expected age- and frailty-related changes in the LTC cohort, including lower levels of naïve T cells than their younger counterparts in the HA and RA cohorts[38,47]. While the spike-specific T cell

responses were similar between the cohorts at each timepoint, there were differences in their exhausted phenotype profiles. Only the spike-specific $CD4^+$ T cell FS was significantly higher in the HA cohort than the LTC cohort, while the other FS and PFS did not differ between cohorts, which suggests that the exhaustion marker profile differences do not largely influence the functional capacity of the spike-specific T cells. There are numerous differences in T cell exhaustion marker profiles between the vulnerable cohorts and healthy adults following repeated SARS-CoV-2 vaccination. Whether these influence vaccine effectiveness, or contribute to the severity of SARS-CoV-2 infection, remains a subject for future exploration.

Given that one of the primary concerns regarding immune exhaustion was the frequency of SARS-CoV-2 vaccinations, we explored the impact of primary series dosing interval on overall T cell phenotypes and spike-specific T cell responses in the HA cohort. Dosing interval did not impact spike-specific T cell levels or their expression of exhaustion markers. There is debate regarding the impact of dosing interval on spike-specific T cell functional responses. Some reports indicate that a longer interval between the first and second dose fails to impact polyfunctional spike-specific $CD4^+$ T cells responses[48]. However, others report that longer dosing intervals did not increase spike stimulation associated IFN-γ production by ELISPOT, but did increase spike-specific $CD4^+$ T cell cytokine production within ELISPOT positive individuals[23]. This suggests that a longer dosing interval may not elicit higher $CD4^+$ T cell responses in all individuals, but rather may exert the greatest impact among a subset of

individuals with strong overall responses. For spike-specific $CD8^+$ T cell responses, previous studies found that the frequencies of polyfunctional spike-specific $CD8^+$ T cells did not differ between dosing intervals[23,48]. Our study differs from previous publications by including GM-CSF in addition to TNF-α, IFN-γ, and IL-2, which may contribute to our finding that the shortest primary series dosing interval was associated with higher spike-specific $CD8^+$ T cell FS and PFS. While polyfunctionality is implicated in protection against severe disease and disease progression in the context of other pathogens, it remains to be determined if functionality and polyfunctionality are correlates of immune protection against SARS-CoV-2, and if they can explain differences in infection rates or disease severity attributed to dosing interval[15,49–51]. We also found that the total $CD8^+$ T cell compartment of participants in the shortest and longest dosing intervals displayed higher frequencies of PD-1^+ $TIGIT^+$ cells than those within the intermediate dosing interval. However, the frequencies of PD-$1^-$$TIGIT^-$ $CD8^+$ T cells were not significantly different between the vaccine interval groups, and it is therefore unlikely that this difference in exhaustion marker expressing $CD8^+$ T cells impacts overall immune function.

There are a few limitations to our study including the small size of our RA cohort, and the high number of participants in the HA cohort lost to follow-up, as it became increasingly difficult to obtain samples from individuals without previous SARS-CoV-2 infections after the second dose. Our study included only individuals without previous SARS-CoV-2 infections, to avoid confounding of infection associated changes in T cells with those of vaccination. Consistent with

other reports in the literature, we found that repeated SARS-CoV-2 vaccination did not result in enhanced cytokine production in individuals without a previous SARS-CoV-2 infection[24]. Studies that incorporated individuals with hybrid immunity, or that did not assess history of SARS-CoV-2 infections, could see different trends than those focusing on uninfected individuals. Additionally, previous studies have demonstrated that the majority of double positive cells in the AIM assays are antigen-specific, with little bystander activation[52–54]. However, we cannot eliminate the possibility that bystander activation may contribute to changes in the expression of surface markers. We also did not evaluate the Th1, Th2, Th17, or Treg phenotypes within the population of $CD4^+$ T cells expressing exhaustion markers. The results and conclusions herein can only be definitively applied to the vaccine types and doses included in this study. Future research may seek to extend the vaccine types and doses examined, as well as explore the interplay of exhaustion, T cell receptor sequences, and affinity, as sequencing and specific measures of affinity were not within the scope of the current study.

In conclusion, we have demonstrated that repeated SARS-CoV-2 vaccinations do not increase T cell exhaustion or lead to substantive alterations in the T cell compartment in frail older adults in LTC, immunosuppressed individuals with RA, or healthy adults. Spike-specific T cell levels did not decline upon additional vaccinations, nor did their expression of exhaustion markers increase. T cells also did not display functional deficits in response to stimulation with the spike protein from SARS-CoV-2 following additional SARS-CoV-2

vaccinations. Finally, the overall T cell compartment did not display increases in exhaustion marker expression or terminally differentiated T cells. Repeated SARS-CoV-2 vaccination, as recommended particularly for vulnerable cohorts, is therefore not cause for concern with regards to the T cell compartment, as it does not induce phenotypic or functional characteristics of T cell exhaustion.

Methods

Ethics Statement and Blood Collection

Study recruitment and procedures were reviewed and approved by the Hamilton Integrated Research Ethics Board (HiREB) for protocols #13059 (Long-Term Care, LTC), #13229 (TIMING, HA), and #13307 (SUCCEED, RA). Informed consent for sample and data collection and publication was obtained from all participants or their substitute decision makers. Participants in the LTC cohort were part of the COVID in Long-Term Care Study, a longitudinal observational cohort study of residents of long-term care facilities in Ontario, Canada[55]. Frailty scores were calculated using the Clinical Frailty Scale, which combines measures of basic activities of daily living, along with health status and comorbidities, as previously described[56–58]. The scoring system ranges from 1-9, with 9 indicating terminally ill, very frail, individuals. Participants in the SUCCEED (immunosuppressed RA) cohort were classified as having rheumatoid arthritis based on criteria from the European League Against Rheumatism (EULAR)/ American College of Rheumatology (ACR) and were prescribed immunomodulatory drugs (Supplementary Data 1). The participants in the RA

cohort were part of the SUCCEED study and recruited from clinics in Ontario, Canada[28,59]. The HA cohort included healthcare workers recruited in Ontario[22]. Previous COVID-19 infection was determined based on a positive PCR or rapid antigen test, or seroconversion for anti-nucleocapsid IgG antibodies[60]. Only participants without prior SARS-CoV-2 infection, and who received 2, 3, or 4 SARS-CoV-2 mRNA vaccinations were included in the study cohort. Cohort demographic information can be found in Table 1.

Peripheral blood was collected in sodium heparin vacutainers 3 months after the second, third, and fourth SARS-CoV-2 vaccinations. Within the HA cohort, peripheral blood was also collected for a subset of individuals at 3 weeks post dose 2. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient. The PBMCs were cryopreserved in liquid nitrogen before thawing for use in T cell assays. For some participants there were not enough cells to perform all assays at all assessment timepoints.

Increased expression of exhaustion-associated markers can accompany early T cell expansion, but this is likely indicative of activation rather than a state of exhaustion[33,61,62]. Using matched samples collected from the HA cohort at 3 weeks and 3 months post dose 2, we compared the prevalence of $CD4^+$ and $CD8^+$ T cell subsets (naïve, central memory, effector memory, EMRA) as well as T cell expression of exhaustion markers (Supplementary Figure 3, Supplementary Data 11). Although others have reported that peak SARS-CoV-2-specific T cell responses occur within a few weeks of SARS-CoV-2 vaccination

and decline in the following 1-3 months, we did not observe a significant decline in the spike-specific T cell frequencies from 3 weeks to 3 months post dose 2[27,36]. As expected, we saw that the prevalence of $CD4^+$ central memory T cells increased between 3 weeks and 3 months post dose 2. Frequencies of PD-1^+TIGIT$^-$ and PD-1^+TIGIT$^+$ $CD4^+$ T cells were reduced at 3 months post dose 2, compared with 3 weeks post dose 2 (Supplementary Figure 3, Supplementary Data 11). The changes in overall T cell exhaustion marker expression following the second SARS-CoV-2 vaccination suggests that T cells are still activated at 3 weeks post vaccination, so 3 months post vaccination was the appropriate time to measure exhaustion

Activation Induced Marker (AIM) Assay

Spike-specific memory T cell responses were quantified using an activation-induced marker (AIM) assay[63]. Briefly, cryopreserved PBMCs were thawed, washed, counted, and resuspended in RPMI supplemented with 10% fetal bovine serum (#12483020, Gibco), 1% Pen/Strep (#15140122, Gibco), 1% HEPES (1M, #7365-45-9, Sigma), 1% GlutaMAX (#35050061, Gibco), and 0.5% β-mercaptoethanol (#21985023, Gibco) (cRPMI), then rested overnight in a tissue culture incubator at 37°C/5% CO_2. The following day the cells were counted, and the concentration was adjusted so that 100µL of cRPMI containing $0.5x10^6$ cells was added to each well of a 96-well U-bottom plate. Peptides from the spike protein of SARS-CoV-2 (S complete Peptivator, #130-127-951, Miltenyi Biotec) were diluted in cRPMI and used to stimulate PBMCs at a final concentration of

1µg/mL for 44 h at 37°C/5% CO_2. Cytostim (#130-092-172, Miltenyi Biotec) was used as a positive stimulation control for each sample at 0.25µL per well, and a media only unstimulated control was also included for each sample.

After 44 h, cells were washed with PBS, then incubated for 30 minutes at room temperature in the dark with Zombie Near-IR (#423105, BioLegend) to identify live cells. Cells were washed with PBS, then washed with FACS Wash (0.5% (w/v) bovine serum albumin (BSA, #A3912, Sigma), 5 mM EDTA (pH 7.4–7.6, #E5134-500G, Sigma) in PBS). The staining cocktail was prepared in PBS and Brilliant Stain Buffer Plus (#566385, BD Biosciences) as detailed in Supplementary Data 12. Antibodies were titrated by lot to determine optimal concentration. Samples were incubated with the antibody cocktail for 30 minutes at room temperature in the dark, washed with FACS Wash, and resuspended in FACS Wash to run on the cytometer.

AIM+ (spike-specific) T cells were identified by co-expression of CD134 (PE) and CD25 (PE-Cy7) on $CD4^+$ T cells (CD3-BV510, CD4-BB700) and CD137 (APC) and CD69 (BV711) on $CD8^+$ T cells (BB515)[52,64,65]. Expression of LAG-3 (PE/Dazzle594), TIM-3 (BV605), and PD-1 (BV421) on the AIM+ T cells was assessed. The media control well was used to set the gates for co-expression of activation markers (AIM+ T cells), and isotype controls were used to set the gates for each exhaustion marker within the AIM+ $CD4^+$ or AIM+ $CD8^+$ T cells. The gating strategy for the AIM assay is depicted in Supplementary Figure 4.

Intracellular cytokine stain (ICS)

To evaluate cytokine production, $0.5x10^6$ cells were seeded into a 96-well U-bottom plate. Peptides from the spike protein of SARS-CoV-2 (S complete Peptivator, #130-127-951, Miltenyi Biotec) were used to stimulate PBMCs for 19 h. Golgi plug (#555029, BD) was diluted 125x in cRPMI before adding 50µL to each well. The plate was then incubated at 37°C/5% CO_2 for 5 hours. Cells were then washed with PBS, and stained with Zombie Aqua (#423102, BioLegend) for 30 minutes at room temperature in the dark to detect live cells. Cells were washed with PBS, followed by FACS Wash. The surface stain was prepared in FACS Wash, and included CD4 (Pacific Blue), and CD8 (AF700). Cells were incubated with the surface stain for 30 minutes in the dark at room temperature, fixed and permeabilized by incubation with Cytofix/Cytoperm (#51-2091KZ, BD Biosciences), and washed twice with 1x Perm/Wash buffer (#51-2091KZ, BD Biosciences).

The intracellular stain antibody cocktail was prepared in 1x Perm/Wash buffer, and included IFN-γ (APC), TNF-α (PE-Cy7), GM-CSF (APCVio770), IL-2 (PE), and CD3 (BV605), as detailed in Supplementary Data 12. Samples were stained with intracellular stain antibody cocktail for 30 minutes at room temperature in the dark, washed with 1x Perm/Wash buffer, then resuspended in FACS Wash to be run on the cytometer.

Immunophenotyping

$CD4^+$ and $CD8^+$ T cell populations were identified by surface staining and flow cytometry analysis of unstimulated PBMCs as previously described[60]. Briefly,

naïve T cells were classified as $CCR7^{+}CD45RA^{+}$, central memory were $CCR7^{+}CD45RA^{-}$, effector memory were $CCR7^{-}CD45RA^{-}$, and EM re-expressing CD45RA (EMRA) were $CD45RA^{+}CCR7^{-}$. Terminally differentiated cells were classified as $EMRA^{+}CD57^{+}CD28^{-}$. T cells within the $CD4^{+}$ and $CD8^{+}$ compartments were also assessed for expression of PD-1 (FITC), TIGIT (BV510), and TIM-3 (BV785). Isotype controls were also used to determine positivity for the exhaustion markers. The gating strategy for the surface immunophenotyping is depicted in Supplementary Figure 5, details for the antibody staining cocktail can be found in Supplementary Data 12.

All samples assessed by flow cytometry were acquired on a CytoFLEX (Beckman Coulter) using CytExpert software (version 2.4). FCS files were gated using FlowJo™ version 10 software (BD Life Sciences).

Combinatorial polyfunctionality analysis of antigen-specific T cell subsets (COMPASS)

ICS samples were manually gated down to the $CD4^{+}$ and $CD8^{+}$ T cell nodes using FlowJo™ version 10 (BD Life Sciences) and then exported from these nodes for analysis using the COMPASS package in R (version 1.42.0)[15], as well as the flowCore (v2.8.0), and openCyto (v2.8.4) packages. COMPASS employs Bayesian statistics to determine the posterior probability of antigen-specific responses for each possible cytokine combination (of the explored cytokines GM-CSF, IFN-γ, TNF-α, and IL-2) within the $CD4^{+}$ or $CD8^{+}$ T cell population of each participant, based on differences in cytokine production between unstimulated,

and antigen-stimulated wells. The parameters were set to 40,000 iterations with 8 replications. The COMPASS package also produces two different summary scores to summarize the polyfunctionality of the T cells. The functionality score (FS) uses the posterior probabilities to determine the proportions of different cytokine combinations that are present in each individual for their $CD4^+$ and $CD8^+$ T cells (separately), out of the total possible cytokine combinations. The polyfunctionality score weighs more heavily the cytokine combinations which are positive for more unique cytokines (i.e. weighs more heavily triple positive, and quadruple cytokine-positive combinations, over those that only produce one or two unique cytokines).

Statistics

Fisher's exact tests and multivariable linear mixed models were run in R version 4.4.1 (R Core Team) using stats (v4.4.1), usethis (v2.2.3), tidyverse (v2.0.0), tidytext (v0.4.2), devtools (v2.4.5), sjPlot (v2.8.16), lme4 (v 1.1.35.4), and lmerTest (v3.1.3) packages. Data were $\log_2$ transformed for analysis using mixed models. Fisher's exact test was used to compare demographics between the LTC, RA, and HA cohorts for categorical variables. Two-tailed multivariable linear mixed models, accounting for age and sex, were used to assess changes in T cell populations within each cohort after additional SARS-CoV-2 vaccinations. Within each cohort, FDR adjusted p values were calculated for surface stain T cell compartment breakdowns (CM, EM, EMRA, N), surface exhaustion marker expression, and spike-specific $CD4^+$ and $CD8^+$ T cell exhaustion markers, to

account for multiple mixed models/repeated testing run on T cells stemming from the same parent population, using the p.adjust function in R. For the three dosing intervals (Rec, Del, Ext) within the HA cohort, and the comparisons between the LTC, RA, and HA cohorts D'Agostino & Pearson tests were used with p <0.05 indicating a significant departure from normality. Ordinary one-way ANOVAs, or Brown-Forsythe ANOVAs for parameters with unequal standard deviations between cohorts, or Kruskal-Wallis tests for nonparametric measures, were used to assess differences between dosing interval cohorts and the differences between the LTC, RA, and HA cohorts at each post vaccination timepoint. If the ANOVA was significant, after FDR correction if applicable, Tukey's, Dunnett's T3, or Dunn's post-hoc tests were used to determine which dosing intervals differed from one another (for ordinary one-way ANOVAs, Brown-Forsythe ANOVAs, and Kruskal-Wallis tests respectively). COMPASS heatmaps displaying samples clustered by group and timepoint were produced using the cowplot (v1.1.3) package. GraphPad version 8 was used to plot flow cytometry data, and for ANOVAs assessing demographic differences between the cohorts for age and dosing intervals. Correlation coefficients were determined using linear mixed models, accounting for the random effect of cohort, and FDR adjusted p values were obtained to account for multiple testing. The correlation plot was created using the corrplot (v0.92) package. Female sex was entered as 2, male sex was entered as 1. 3mo2 was 2, 3mo3 was 3, 3mo4 was 4. Only complete cases were considered.

Data Availability

The data generated in this study for the LTC and RA cohorts are provided in the Source Data File. Data for the HA cohort are available under restricted access, as consent was not obtained for disclosure of individualized participant information. Data from the HA cohort can only be disclosed following the submission and approval of the request to a Population Health Research Institute (PHRI) Hamilton Review Committee[22]. Requests for access or further information can be sent to the corresponding author, with an anticipated response time of 2 weeks. If access is approved, data would be available to the requesting party for the duration of their study. Measures of central tendency for the HA cohort are provided in the source data file.

Code Availability

The COMPASS code used in this study is available on Zenodo[66].

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Author contributions

JMB- conducted experiments, analyzed data and generated figures, wrote manuscript.

JAB- conducted experiments, contributed to experiment design, data interpretation and manuscript editing.

YW- conducted experiments, contributed to data interpretation.

AK- conducted experiments, contributed to data interpretation.

LL- conducted experiments.

BC- collected and processed blood samples, collected participant metadata.

BB- clinical coordinator, recruited and contacted patients, collected samples.

MH- contributed to experiment design and project management.

CMA- collected and organized participant metadata.

MM- clinical coordinator, recruited and contacted patients, collected participant metadata.

NA- Clinical coordinator, recruited and contacted patients, collected participant metadata.

JDM- contributed to data interpretation and statistics.

GG- Project administration for HA study, sample collection.

PYK- Project administration for HA study, sample collection.

JAD- Designed HA study and obtained funding.

APC- Designed LTC study and obtained funding.

DPL- Designed HA study and obtained funding.

IN- Designed HA and LTC studies and obtained funding.

MD- Designed HA study and obtained funding.

JLB- Designed LTC study and obtained funding. Contributed to data interpretation and study design.

MJL- Designed RA study and obtained funding. Contributed to data interpretation.

CPV-Designed LTC study and obtained funding. Created pipeline for COMPASS analysis, aided in statistical analyses and data interpretation.

DMEB- Designed LTC, HA, and RA studies, and obtained funding. Contributed to data interpretation, experimental design, and manuscript writing and revision.

Competing interests statement

MJL is the Director of the Canadian Scleroderma Research Group and has received honoraria for consulting or speakers fees from AbbVie, Actelion, Amgen, AstraZeneca, Boehringer-Ingelheim, BMS, Fresenius-Kabi, GSK, Lilly, Mallinckrodt, Novartis, Pfizer, Sanofi, SOBI, UCB, and Scleroderma Society of Ontario/Canada. DMEB has received honorarium from Pfizer-Global, Pfizer Canada, and AstraZeneca for consulting on the topics of vaccines. DMEB is on the Board of Directors of the Lung Health Foundation (unpaid, volunteer), and speaks on the topic of adult vaccination and other lung health issues to policymakers, knowledge users, and the general public. DMEB was an expert witness for the below court cases on the topic of vaccination;

i) Nabil Ben Naoum vs. L'honorable Maxime Bernier, Canada. Provided written affidavit. and was cross examined. June-September, 2022.

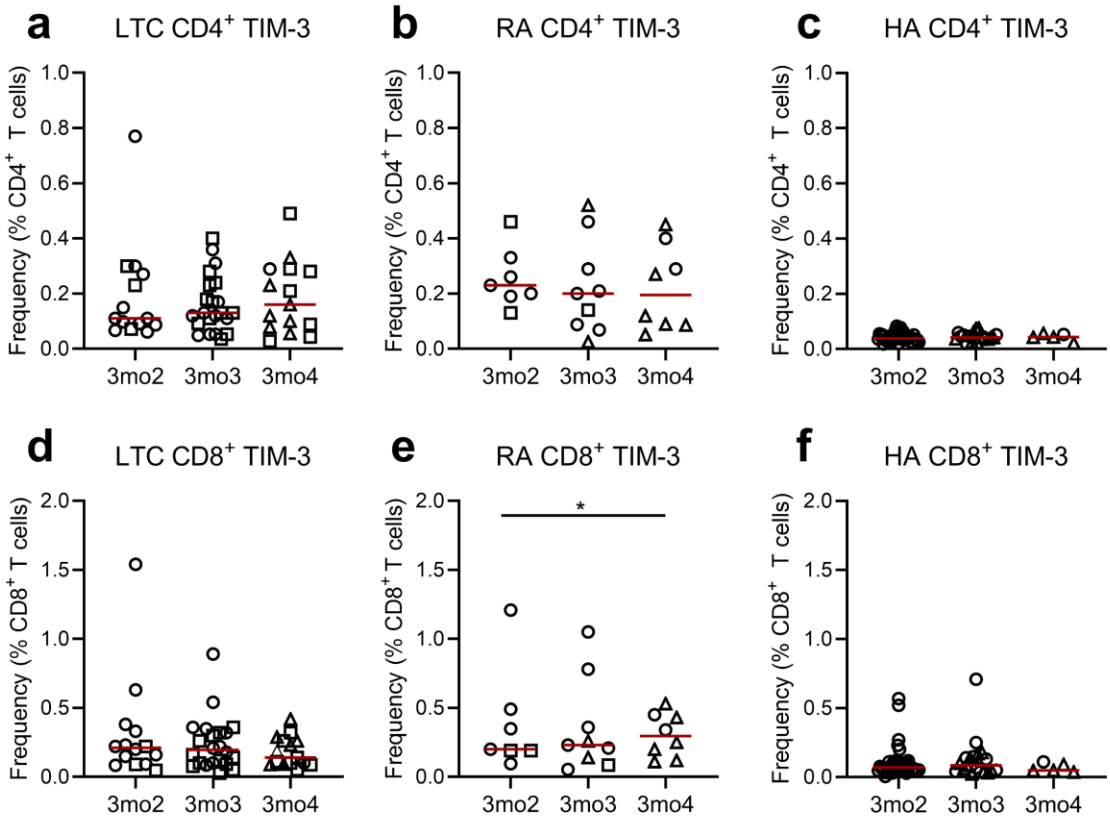
ii) David Lavergne-Poitras vs. PMG Technologies Inc., Canada, Montreal, QC. Provided written affidavit. and was cross examined. May-July 2022.

iii) Syndicat des Metallos S.L., 2008, 9599, 2004, 9344, 9554, 1976, 9449, 9519, 5778, 9996 et als. vs. Procureur General du Canada, Canada, Montreal, QC. Provided written affidavit. March-May 2022.

iv) Fisman et al ats Bridle.DM-LSDOCS.FID1093293. Provided written affidavit.

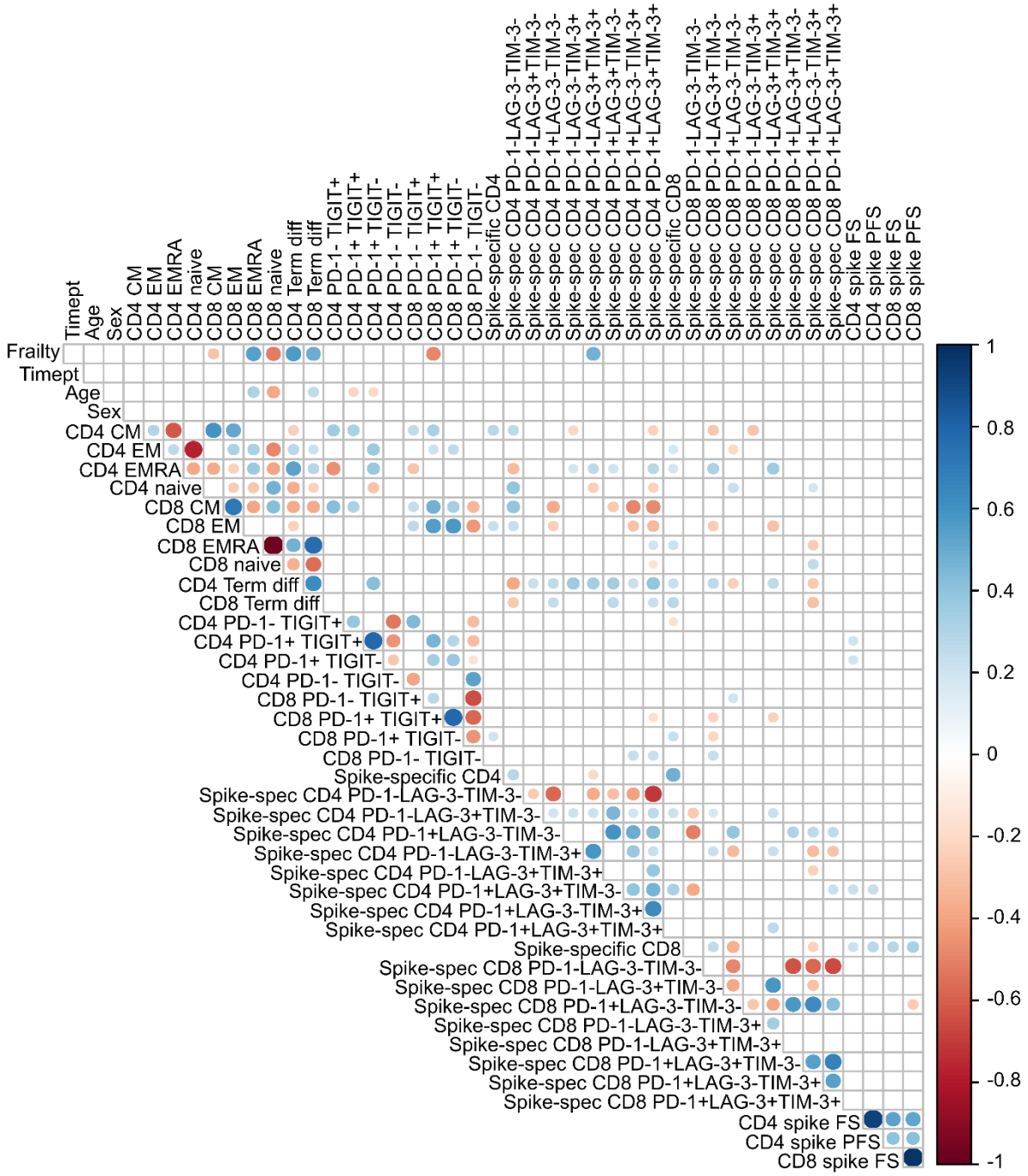
The remaining authors declare no competing interests.

Supplementary Information



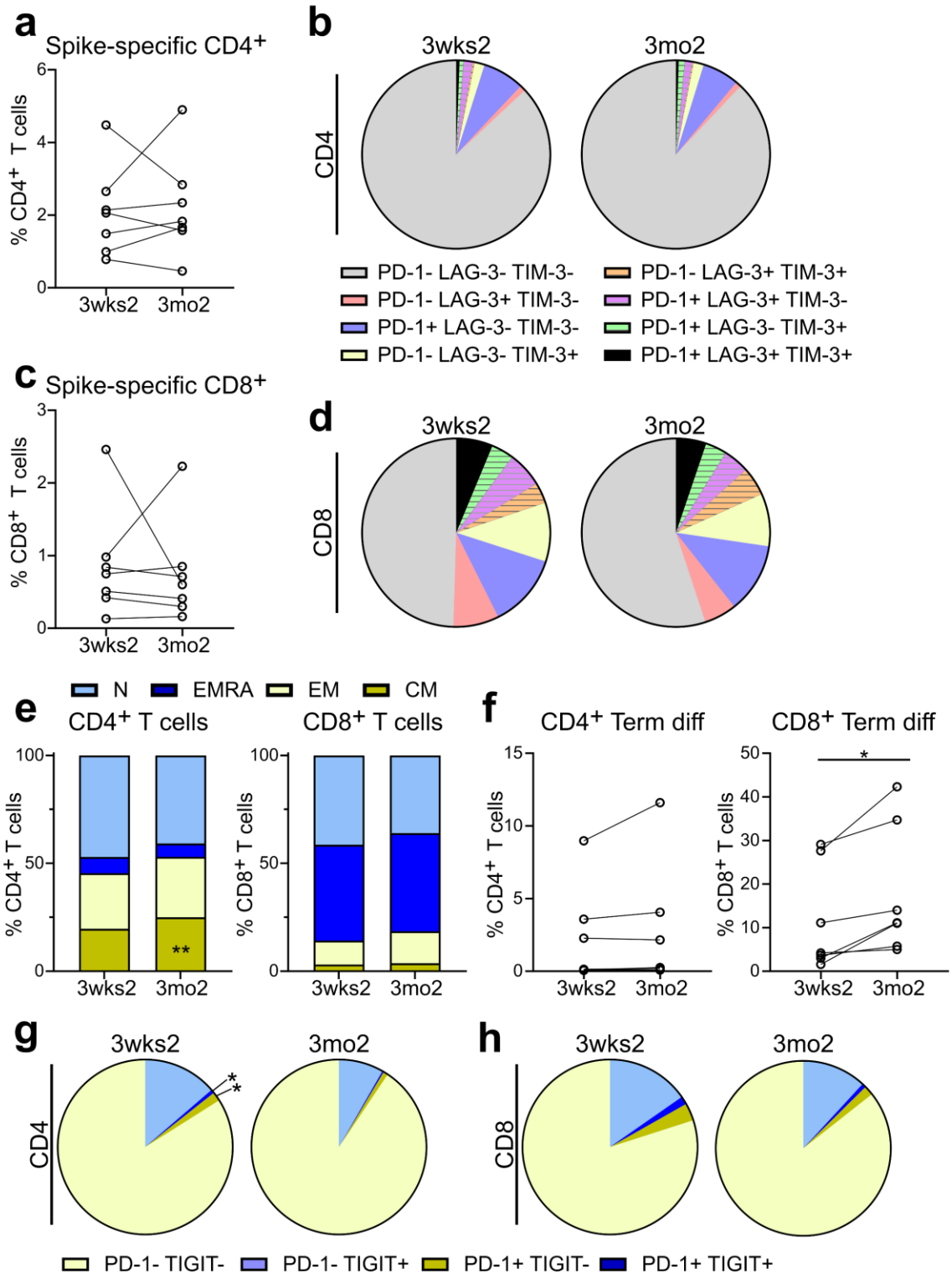
Supplementary Figure 1. Frequencies of TIM-3$^+$ T cells following repeated SARS-CoV-2 vaccination. Flow cytometry was used to assess the frequencies of unstimulated CD4$^+$ T cells expressing TIM-3 in each cohort (a-c), and the frequencies of CD8$^+$ T cells expressing TIM-3 in each cohort (d-f). LTC: 3mo2 n=14, 3mo3 n=22, 3mo4 n=15. RA: 3mo2 n=7, 3mo3 n=9, 3mo4 n=8. HA: 3mo2 n=41, 3mo3 n=19, 3mo4 n=5. Circle points indicate participants whose previous vaccinations were BNT162b2 only, square points indicate participants with

mRNA-1273 only, triangle points indicate participants that received both BNT162b2 and mRNA-1273, and diamond points indicate participants for whom one mRNA vaccination type was unknown. 3moX denotes 3 months post dose X. Multivariable linear mixed models accounting for age and sex were used to assess changes in the frequencies of each T cell subset within a given cohort following additional SARS-CoV-2 vaccinations. If not displayed, result was not significant. $p<0.05$ *.

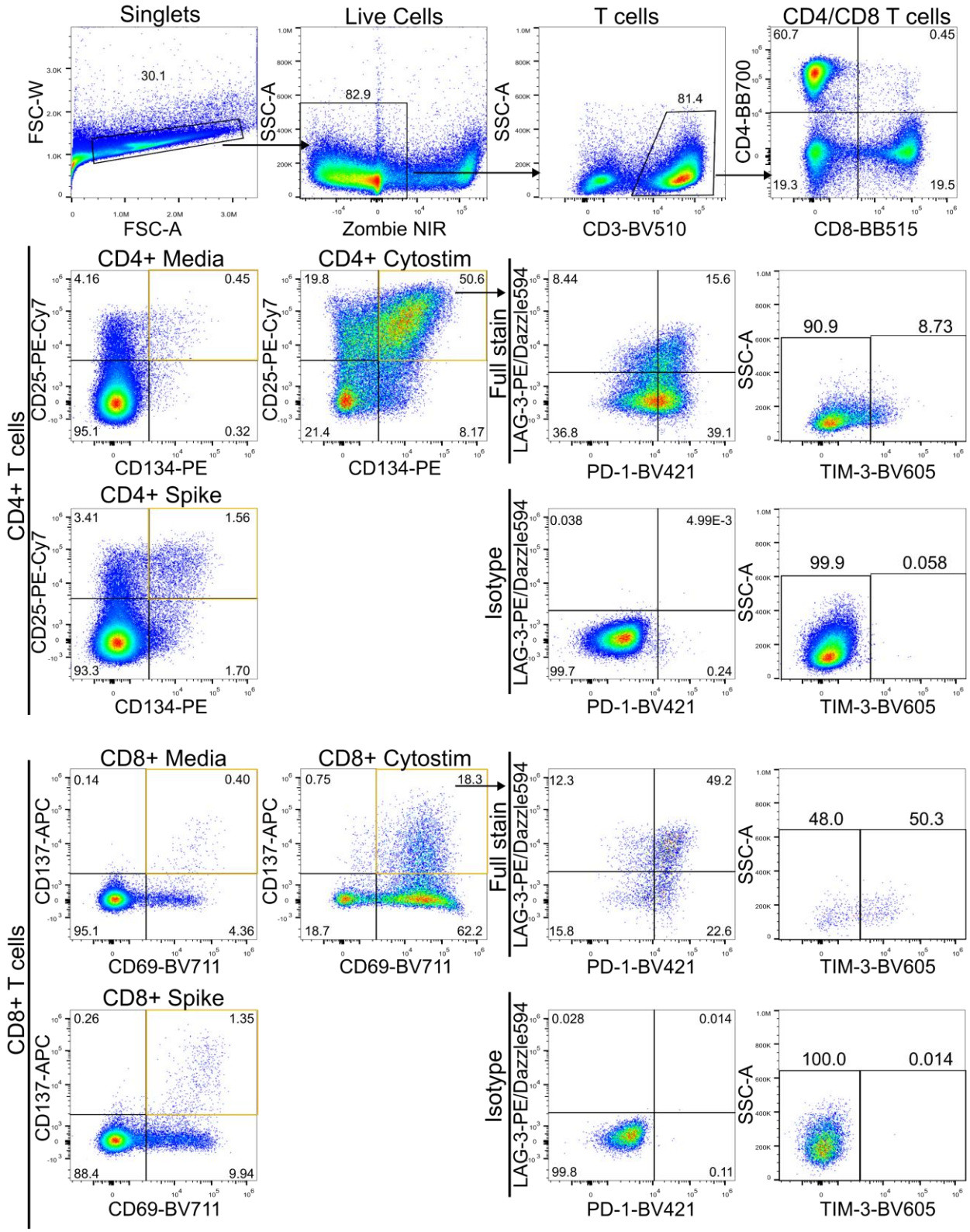


Supplementary Figure 2. Correlation of immune exhaustion parameters. Flow cytometry was used to assess T cell phenotypes and functional capacity at 3 months post second, third, and fourth SARS-CoV-2 vaccinations. The LTC (n=23), RA (n=10), and HA (n=43) cohorts were included in the analysis. Frailty scores were only available for 18/23 participants in the LTC cohort. Spike-spec means spike-specific, FS is the COMPASS functionality score, PFS is the COMPASS polyfunctionality score. Correlation coefficients were determined

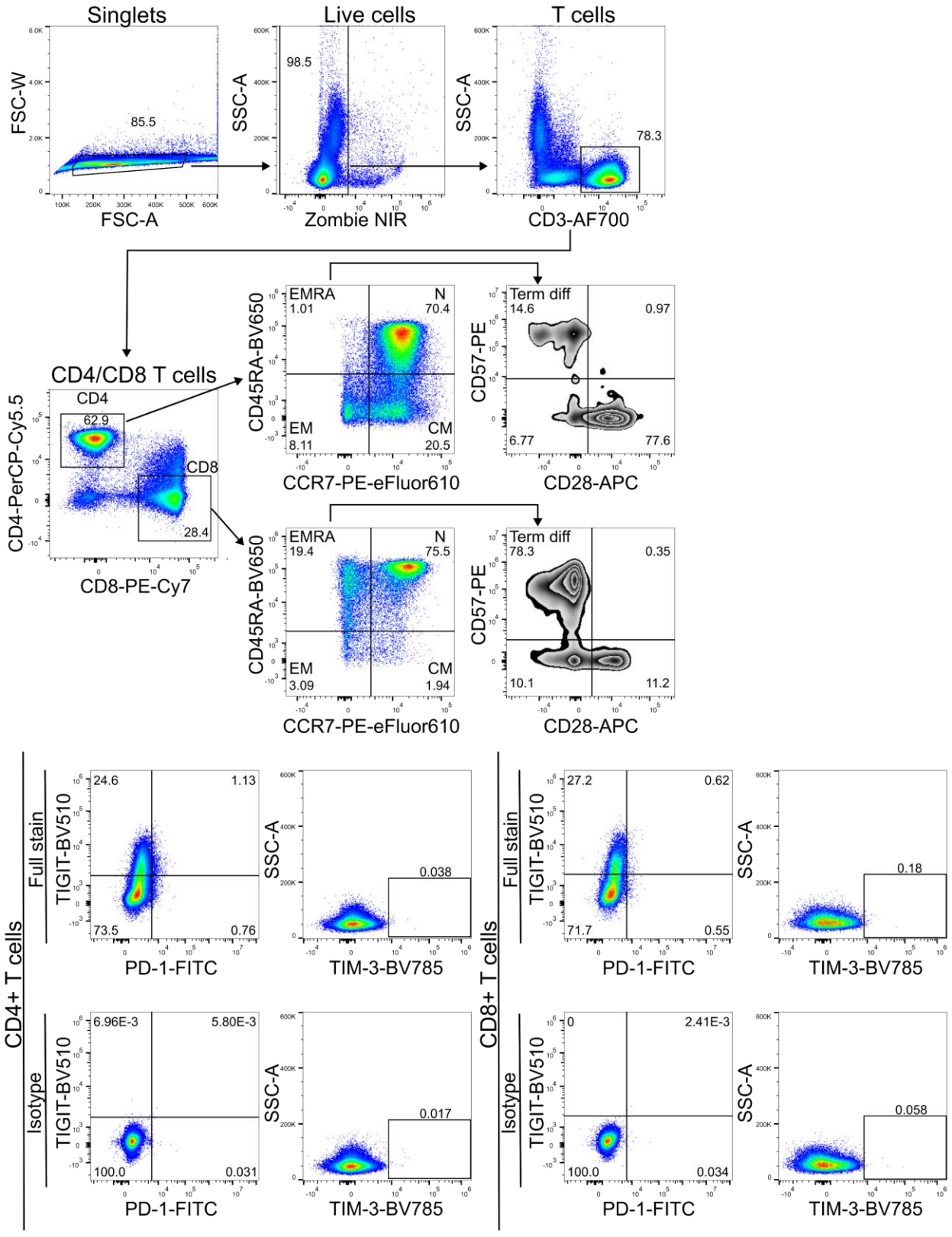
using linear mixed models, accounting for the random effect of cohort, and FDR adjusted p values were obtained to account for multiple testing. Colored points are only shown for significant pairs. The size and color of the points indicates the strength and directionality of the correlation. $p<0.05$ is significant.


a
Spike-specific CD4+
% CD4+ T cells
3wks2
3mo2
b
3wks2
3mo2
CD4
PD-1- LAG-3- TIM-3-
PD-1- LAG-3+ TIM-3-
PD-1+ LAG-3- TIM-3-
PD-1- LAG-3- TIM-3+
PD-1- LAG-3+ TIM-3+
PD-1+ LAG-3+ TIM-3-
PD-1+ LAG-3- TIM-3+
PD-1+ LAG-3+ TIM-3+
c
Spike-specific CD8+
% CD8+ T cells
d
CD8
e
N
EMRA
EM
CM
CD4+ T cells
CD8+ T cells
f
CD4+ Term diff
CD8+ Term diff
g
h
PD-1- TIGIT-
PD-1- TIGIT+
PD-1+ TIGIT-
PD-1+ TIGIT+

Supplementary Figure 3. Kinetics of immune phenotype and exhaustion in HA following the second SARS-CoV-2 vaccination. T cell phenotypes were assessed in matched samples within the HA cohort at 3 weeks post dose 2 (3wks2) and 3 months post dose 2 (3mo2) by flow cytometry. **(a)** Frequencies of spike-specific $CD4^+$ T cells. **(b)** Mean frequencies of spike-specific $CD4^+$ T cells expressing each combination of PD-1, TIM-3, and LAG-3. **(c)** Frequencies of spike-specific $CD8^+$ T cells. **(d)** Mean frequencies of spike-specific $CD8^+$ T cells expressing each combination of PD-1, TIM-3, LAG-3. **(e)** Mean frequencies of naïve, central memory (CM), effector memory (EM) and EM re-expressing CD45RA (EMRA) $CD4^+$ and $CD8^+$ T cells. **(f)** Frequencies of terminally differentiated $CD4^+$ and $CD8^+$ T cells. **(g)** Mean frequencies of $CD4^+$ T cells and **(h)** $CD8^+$ T cells expressing each combination of PD-1 and TIGIT. Lines between points indicate matched samples, n=7. Two-tailed paired t-tests were used to assess differences between 3wks2 and 3mo2. FDR adjusted p values were obtained for **(b)**, **(d)**, **(e)**, **(g)**, and **(h)** to account for multiple testing on cells from the same parent population. If p values are not indicated, the result was not significant. $p<0.05$ *, $p<0.01$ **.


Singlets
Live Cells
T cells
CD4/CD8 T cells
FSC-W
FSC-A
30.1
SSC-A
Zombie NIR
82.9
SSC-A
CD3-BV510
81.4
CD4-BB700
CD8-BB515
60.7
0.45
19.3
19.5
CD4+ T cells
CD4+ Media
CD4+ Cytostim
CD4+ Spike
CD25-PE-Cy7
CD134-PE
4.16
0.45
95.1
0.32
19.8
50.6
21.4
8.17
3.41
1.56
93.3
1.70
Full stain
Isotype
LAG-3-PE/Dazzle594
PD-1-BV421
TIM-3-BV605
8.44
15.6
36.8
39.1
90.9
8.73
0.038
4.99E-3
99.7
0.24
99.9
0.058
CD8+ T cells
CD8+ Media
CD8+ Cytostim
CD8+ Spike
CD137-APC
CD69-BV711
0.14
0.40
95.1
4.36
0.75
18.3
18.7
62.2
0.26
1.35
88.4
9.94
12.3
49.2
15.8
22.6
48.0
50.3
0.028
0.014
99.8
0.11
100.0
0.014

Supplementary Figure 4. AIM assay gating strategy. Doublets and debris were first excluded, followed by gating on live cells. T cells were then identified as $CD3^+$ events. Within the T cell compartment, cells were divided into $CD4^+$ or $CD8^+$ single positive events. In the $CD4^+$ T cell compartment, AIM+ cells (those responding to the stimulus, orange outlined quadrant) were detected as $CD25^+CD134^+$. In the $CD8^+$ T cell compartment, AIM+ cells were detected as $CD137^+CD69^+$. The unstimulated (media) well was used to help determine the position of this gate. The media well, spike-stimulated well, and Cytostim positive control wells are shown to demonstrate how the AIM+ population looks between conditions. Within the AIM+ $CD4^+$ or $CD8^+$ T cell compartment, quadrant gating for PD-1^+LAG-3^+, PD-1^+LAG-3^-, PD-1^-LAG-3^-, and PD-1^-LAG-3^+ events was conducted based on an isotype control (shown above, from the Cytostim well). Each of these quadrants was then further gated into TIM-3^+ and TIM-3^- events based on an isotype control, to obtain each possible exhaustion marker combination.


Singlets
Live cells
T cells
FSC-W
FSC-A
85.5
SSC-A
Zombie NIR
98.5
SSC-A
CD3-AF700
78.3
CD4/CD8 T cells
CD4
CD8
CD4-PerCP-Cy5.5
CD8-PE-Cy7
62.9
28.4
CD45RA-BV650
CCR7-PE-eFluor610
EMRA
1.01
N
70.4
EM
8.11
CM
20.5
CD57-PE
CD28-APC
Term diff
14.6
0.97
6.77
77.6
EMRA
19.4
N
75.5
EM
3.09
CM
1.94
Term diff
78.3
0.35
10.1
11.2
CD4+ T cells
CD8+ T cells
Full stain
Isotype
TIGIT-BV510
PD-1-FITC
TIM-3-BV785
SSC-A
24.6
1.13
73.5
0.76
0.038
6.96E-3
5.80E-3
100.0
0.031
0.017
27.2
0.62
71.7
0.55
0.18
0
2.41E-3
100.0
0.034
0.058

Supplementary Figure 5. Surface immunophenotyping gating strategy. Doublets and debris were first excluded, followed by gating on live cells. T cells were then detected as $CD3^+$ events. T cells were divided into $CD4^+$ or $CD8^+$ single positive events. Within the $CD4^+$ and within the $CD8^+$ T cell compartments, naïve T cells were $CCR7^+CD45RA^+$, central memory were $CCR7^+CD45RA^-$, effector memory were $CCR7^-CD45RA^-$, and EM re-expressing CD45RA (EMRA) were $CD45RA^+CCR7^-$. Terminally differentiated cells were classified as $EMRA^+CD57^+CD28^-$. $CD4^+$ T cells, and $CD8^+$ T cells, were also assessed for co-expression of PD-1 and TIGIT, and expression of TIM-3. Gates for each exhaustion marker were set using isotype controls (shown above).

Chapter 5. Protocol for the identification and characterization of human SARS-CoV-2 spike-specific B cells using flow cytometry

5.1 Preface

SARS-CoV-2 spike-specific memory B cells are an important aspect of long-term memory responses to both SARS-CoV-2 infection and vaccination. Upon reactivation, these cells can differentiate to produce antibodies, and undergo further somatic hypermutation to fine-tune their specificity[329–331]. Compared with B cell ELISPOT, flow cytometry-based evaluation of antigen-specific B cell responses would allow for immunophenotyping of the spike-specific B cells, and direct detection of B cells that are not secreting antibodies[332,333]. An advantage of using the full-length spike protein for these assays, as opposed to subunits of the spike, is the ability to quantitate spike-specific B cells encompassing those that recognize all of the major domains of the spike. We have optimized a protocol using the full-length ancestral spike-protein to detect SARS-CoV-2 spike-specific memory B cells in the peripheral blood using flow cytometry.

It is, however, possible for B cells to recognize and bind non-spike epitopes in the spike-fluorophore complex, such as the streptavidin or fluorophore themselves[333]. This non-spike-specific recognition of the spike-fluorophore complex could lead to an inflated estimate of the numbers of true spike-specific B cells. To avoid non-specific binding of B cells to the spike protein-streptavidin-PE, we used a decoy molecule consisting of an irrelevant protein-streptavidin conjugated to the tandem dye PEDylight594. This PE tandem dye was made with

the same PE lot that was used to tag the spike protein, thus the decoy molecule binds B cells specific for streptavidin or PE moieties. After the decoy serves to bind the non-specific B cells, spike-PE is added to each sample, increasing the likelihood that the spike-PE+ events are truly spike-specific. This protocol therefore thoroughly accounts for and minimizes instances of non-specific binding, increasing reliability of results and reducing artificial inflation of spike-specific B cell numbers that may be present in other approaches.

Multiple different B cell subpopulations can be found in circulation, including memory B cells, naïve B cells, and antibody-secreting cells such as plasmablasts and plasma cells[159,334,335]. Within $CD19^+$ B cells, memory B cell can be identified by expression of CD27, and antibody-secreting cells based on their coexpression of CD27 and CD38[159,334,335]. Naïve B cells lack CD27 expression, but express IgD and IgM[159,334]. Memory B cells, which have previously encountered their antigen, can remain un-class switched (IgM^+), or switch to express other isotypes such as IgG or IgA[159,334]. This protocol therefore includes other fluorescently-tagged antibodies in the staining panel to characterize the isotype of the spike-specific B cells, and differentiate between antibody-secreting cells, memory B cells, and naïve B cells, to track class-switching and antigen-experienced subpopulations after SARS-CoV-2 vaccination and infection[334].

The protocol is formatted with headings, list levels, timing, and details based on a template from STAR protocols.

5.2 Before you begin

This protocol describes a flow cytometry staining workflow to identify ancestral SARS-CoV-2 spike-specific memory B cells in human peripheral blood mononuclear cells. The biotinylated beta lactoglobulin (BLG) for the decoy molecule was prepared according to a previously published protocol, and the generation of the decoy molecule herein is based upon this protocol[333]. The timing of this protocol requires that the biotinylated BLG be prepared in advance according to the referenced protocol, as the current protocol does not include a step for preparing the biotinylated BLG.

5.2.1 Institutional permissions

Blood was collected from healthy individuals and peripheral blood mononuclear cells (PBMCs) were isolated. Isolated PBMCs were suspended in human AB serum with 10% Dimethyl sulfoxide (DMSO), then stored in liquid nitrogen. Study recruitment and procedures were reviewed and approved by the Hamilton Integrated Research Ethics Board (HiREB) protocol #13307. Participants gave informed consent for sample collection and publication prior to their participation. All procedures followed the approved protocols. In order to collect and utilize human blood, researchers must obtain permission from the relevant institutions and regulatory boards of their area.

5.2.2 Preparation of decoy molecule

Timing: 2 h

In this step, the decoy molecule will be created using previously prepared biotinylated BLG as the irrelevant biotinylated protein.

1. Pipette 250 µL of Streptavidin-PE (SA-PE) into the column insert of a 100 kDA Amicon Ultra-15 centrifugal filter.
    a. Add 15 mL of 1x phosphate buffered saline (PBS) to the column insert.
    b. Centrifuge at 2000 x g for 10 minutes at 4°C. Discard flowthrough.
        i. Repeat the wash steps two more times.
2. Mix the remaining liquid by gentle pipetting above the column membrane. Transfer this liquid to a new Eppendorf tube.
3. Add 1x PBS to the Eppendorf tube, bringing the final volume to 500 µL.
4. Pipette 40 µl of 0.67M borate buffer into the Eppendorf tube.
5. Remove DL594 NHS ester labelling tube from the freezer and bring to room temperature.
6. Transfer all liquid from the Eppendorf tube into the DL594 NHS ester tube.
7. Invert tube to mix until solution appears homogenous.
8. Cover tube to protect from the light and leave a room temperature for 1h.
9. Pipette entire solution into a new 100 kDA Amicon Ultra-15 filter
    a. Add 15 mL of 1x PBS to the column insert
    b. Centrifuge at 2000 x g for 10 minutes at 4°C.
    c. Repeat this wash step once more.
10. Collect the remaining liquid from above the membrane. This is the PE-DL594.
11. Measure the volume of the PE-DL594.
12. Using a nanodrop, calculate the concentration of the PE and the DL594 by dividing the absorbance at the appropriate wavelength (OD 566 and OD 595 respectively) by the micromolar extinction coefficient (1.96 and 0.08 respectively)
13. Load the PEDL594 with biotinylated BLG.
    a. Calculate the pmoles of PE present in the PEDL594 vial

b. Multiply this value by 6 to ensure oversaturation of the SA. This will give the pmoles of biotinylated BLG that need to be added to the tube.

c. Add the volume of biotinylated BLG containing the required pmoles detailed in step 13b.

14. Dilute the PEDL594-BLG solution using 1x PBS to a final concentration of 2.0 µM of PE.

15. Add an equal volume of glycerol to the tube to create a 50% glycerol PE-protein solution.

16. This procedure creates the decoy molecule at a final concentration of 1µM. It can be stored at -20°C for up to one year.

Note: If liquid does not flow through Amicon filter after 10 minutes of centrifugation, return the tube to the centrifuge for an additional 5 minutes.

5.2.3 Preparation of buffers and reagents

Timing: 1.5 h

1. Reconstitute biotinylated spike protein.

   a. Pipette 1000 µL of sterile deionized water into the vial with the lyophilized protein. This creates a solution at 200 µg/mL.

   b. Allow protein to solubilize for 60 mins, mixing every 20 mins gently with a pipette.

   c. Aliquot 30 µL (6µg) of the spike protein into Eppendorf tubes.

   d. The tubes can be stored at -80°C for up to 3 months per the company's recommendations.

2. Autoclave water for use diluting Fix/Lyse buffer.

3. Prepare FACS + EDTA buffer.

4. On the day that samples will be stained, prepare fresh 1x Fix/Lyse buffer.

   a. Dilute the 10x stock Fix/Lyse buffer to 1x in autoclaved water.

5. Reconstitute Zombie NearIR according to manufacturer's instructions.

CRITICAL: Do not vortex or shake the biotinylated spike protein to solubilize it. Mixing must be gentle.

5.2.4 Preparation of spike-PE

Timing: 1.5 h

1. Thaw spike trimer at room temperature.
2. Mix spike trimer aliquot with streptavidin-PE. You will need 12 ng of streptavidin-PE for every 200 ng of spike.
    a. Dilute the streptavidin-PE in sterile 1x PBS to a concentration of 12 ng/µL.
    b. Each aliquot of spike protein contains 6 µg in 30 µL, and is thus at a concentration of 200 ng/µL.
    c. Add 30 µL of diluted streptavidin-PE to the Eppendorf tube containing the thawed spike protein (30 µL).
    d. Mix gently with a pipette.
    e. Incubate at room temperature in the dark for 45 minutes.
    f. Add 15 µL of 1xPBS.
    g. Add 75 µL of pure glycerol to the Eppendorf. This makes a 50% glycerol solution with a total volume of 150 µL. The spike concentration will now be 40 ng/µL.
    h. 5 µL will thus be used for each sample.
    i. Store the spike-PE at -20°C.

Note: The spike-PE can be stored for one month at -20°C.

5.3 Key resources table

Table 3. Spike-specific B cell stain key resources table

Reagent or Resource	Source	Identifier
Antibodies		
IgD-BV421	BioLegend	348226
CD45RB-APC	Molecular probes	A15702

CD19-AF700	BioLegend	363034
CD38-PE-Cy7	BioLegend	356608
CD14-BV711	BioLegend	301838
CD24-BV785	BioLegend	311141
IgG-FITC	BioLegend	410720
CD3 BV711	BioLegend	317328
CD16 BV711	BioLegend	302044
CD27-BV605	BioLegend	302830
IgM-BV510	BioLegend	314522
Biological samples		
Peripheral blood mononuclear cells from healthy donors vaccinated against SARS-CoV-2	McMaster University, Hamilton, Ontario, Canada	N/A
Chemicals, peptides, and recombinant proteins		
Dylight 594 NHS ester labelling kit	Thermo Fisher	46413
Glycerol	Sigma-Aldrich	G5516-500ML
Biotinylated SARS-CoV-2 S protein, His, Avitag, Super stable trimer	Acrobiosystems	SPN-C82E9
Streptavidin-PE (SA-PE)	Agilent	PJRS25-1
Fix/Lyse Buffer	eBioscience	00-53333-57
Ethylenediaminetetraacetic acid (EDTA) 0.5M	Sigma	E5134-500G
Bovine Serum Albumin	Sigma	A3912
Zombie Near IR	BioLegend	423105
Brilliant Stain Buffer Plus	BD	566385

CytoFLEX Daily QC Fluorospheres	Beckman Coulter	B53230* (has been replaced with C65719 by company)
VersaComp Antibody Capture Kit	Beckman Coulter	B22804
Software and algorithms		
FlowJo	BD Life Sciences	https://www.flowjo.com/
Other		
100 kDA Amicon Ultra-15 centrifugal filters	MilliporeSigma	UFC910008

5.4 Materials and equipment

- 0.5M EDTA: add 93.06 g of EDTA to 500 mL ddH2O.

Store 0.5M EDTA at room temperature. Expiry date is 2 months after creation, or the expiry date of the EDTA if said date occurs within 2 months.

Table 4. FACS + EDTA buffer preparation

Reagent	**Final concentration**	**Amount**
Bovine Serum Albumin (BSA)	0.5% w/v	2.5 g
Ethylenediaminetetraacetic acid (EDTA) 0.5M	5mM	5 mL
1x PBS	n/a	500 mL
Total	**n/a**	**505 mL**

Store at 4°C. Expiry date is 2 months after creation, or the expiry date of any reagent in this list if said date occurs within 2 months.

Table 5. Spike-specific B cell staining mix

Reagent	Volume (µL/sample)
ancestral-spike-specific B cell stain	
IgD-BV421	0.5
CD45RB-APC	0.5
CD19-AF700	1
CD38-PE-Cy7	1
CD14-BV711	1
CD24-BV785	1
IgG-FITC	1
CD3 BV711	2.5
CD16 BV711	2.5
CD27-BV605	2.5
IgM-BV510	2.5
Spike-PE	5
Brilliant Stain Buffer Plus	10
1x PBS	69
FMO-spike-specific B cell stain	
IgD-BV421	0.5
CD45RB-APC	0.5
CD19-AF700	1
CD38-PE-Cy7	1
CD14-BV711	1
CD24-BV785	1

IgG-FITC	1
CD3 BV711	2.5
CD16 BV711	2.5
CD27-BV605	2.5
IgM-BV510	2.5
Brilliant Stain Buffer Plus	10
1x PBS	74

5.5 Step-by-step method details

5.5.1 Thawing cryopreserved PBMCs

Timing: [1 h]

During this step, PBMCs will be thawed after previous cryopreservation in liquid nitrogen.

1. Remove a bottle of sterile 1x PBS from the fridge at least 30 minutes before thawing cells to warm to room temperature.
2. In a 15 mL conical tube, add 5 mL of 1xPBS.
    a. A separate 15 mL tube will be required for each vial of PBMCs that will be thawed.
3. Thaw PBMC vials
    a. Remove cryovials of PBMCs from liquid nitrogen and place on dry ice.
    b. Remove a cryovial from dry ice and transfer to the 37°C water bath.
        i. Do not submerge the cryovial. You can use a foam flotation device, or hold the cryovial by the cap, so that water covers the section of the vial containing frozen liquid.
        ii. Once the PBMCs in the cryovial are largely thawed, with only a sliver of ice remaining, remove from the water bath.

4. Using a 1 mL serological pipette, gently mix the PBMCs in the cryovial to ensure cells are resuspended.
5. Using the 1 mL serological pipette, transfer the PBMCs from the cryovial to the 15 mL conical tube containing 5 mL of 1xPBS.
6. Repeat the above steps for each vial of PBMCs that you thaw.
7. Centrifuge the 15 mL conical tubes at 485 x g for 6 minutes at 4°C.
8. Decant the supernatant and resuspend the pellet in 1 mL of 1xPBS.
9. Count the live cells using your preferred method.
10. Adjust the live cell concentration to 10 million cells/mL in 1xPBS.

5.5.2 Plate setup and staining PBMCs

Timing: [3-4 h]

In this step, PBMCs will be added to a 96-well U-bottom plate and stained for analysis using flow cytometry, including measuring cell viability. The decoy molecule is added to bind B cells specific for biotin, streptavidin, or PE. By adding the decoy molecule before the spike-PE, B cells specific for the streptavidin or PE will bind the decoy, and thus non-specific binding to the spike-PE will be reduced. The tandem dye (PEDL594) used to label the decoy molecule is made with the same base fluorophore used to label the spike (PE), to maximize binding to B cells specific for PE epitopes. After staining with the decoy, the spike-PE is added, which will bind spike-specific B cells. Fluorescently tagged antibodies are then added to identify B cells, measure the types of B cells (e.g. antibody-secreting cells, memory B cells, naïve B cells) and the isotype they are expressing (e.g., IgG, IgM, IgD). For each participant, a fluorescence minus one (FMO) well will be included, which is stained with all components except the spike-PE, to allow later gating to identify the spike-PE+ population.

11. Each participant PBMC sample will require the following 3 conditions: unstained, FMO, and ancestral spike.

    a. Add 100 µL of PBMCs in 1xPBS to each of 3 wells. Each well now contains 1 x 10^6 cells.

    b. Repeat for each participant sample as needed.

Optional: If the PBMC cell count is low, the unstained well can receive 0.5 x 10^6 cells. In this case, ensure that the initial volume in the well is topped up to 100 µL with 1xPBS.

12. Add 100 µL of 1xPBS to each well. Pipette gently to mix.

13. Centrifuge the plate at 485 x g for 5 minutes at 21°C.

14. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.

15. Add 200 µL of 1xPBS to each well. Pipette gently to mix.

16. Centrifuge the plate at 485 x g for 5 minutes at 21°C.

17. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.

18. Prepare your viability dye.

    a. Dilute Zombie NearIR 1:10,000 in 1xPBS.

19. Add 100 µL of diluted Zombie NearIR to the designated FMO and ancestral spike wells.

20. Do not add Zombie NearIR to the designated unstained wells.

    a. To the unstained wells, add 100 µL of 1xPBS.

21. Cover the plate to protect from the light. Incubate at room temperature for 30 minutes while covered.

22. After 30 minutes, add 100 µL of 1xPBS to each well. Pipette gently to mix.

23. Centrifuge the plate at 485 x g for 5 minutes at 21°C.

24. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.

25. Add 200 µL of 1xPBS to each well. Pipette gently to mix.

26. Centrifuge the plate at 485 x g for 5 minutes at 21°C.

27. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.

28. Add 9.5 µL of 1xPBS to each well. Pipette gently to mix.

29. Add 0.5 µL of decoy to the FMO and ancestral spike wells. Pipette gently to mix.

30. Do not add decoy to the unstained wells.

    a. To the designated unstained wells, add 0.5 µL of 1xPBS. Pipette gently to mix.

31. Cover the plate to protect from the light and put on a plate rocker on the lowest setting for 15 minutes.

Note: The purpose of the rocker is to ensure the decoy is distributed evenly over the samples.

32. After 15 minutes, add 150 µL of 1xPBS to each well. Pipette gently to mix.

33. Centrifuge the plate at 485 x g for 5 minutes at 21°C.

34. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.

35. Add 200 µL of 1xPBS to each well. Pipette gently to mix.

36. Centrifuge the plate at 485 x g for 5 minutes at 21°C.

37. Prepare the surface stains as described in Table 5.

38. Add 100 µL of the ancestral spike stain to each designated ancestral spike well. Pipette gently to mix.

39. Add 100 µL of the FMO stain to each designated FMO well. Pipette gently to mix.

40. Add 100 µL of 1xPBS to each designated unstained well. Pipette gently to mix.

41. Cover the plate to protect from light.

42. Incubate the plate at room temperature for 30 minutes, while covered.

43. After 30 minutes, add 100 µL of 1x Fix/Lyse to each well. Pipette gently to mix.

44. Cover the plate to protect from the light and incubate at room temperature for 10 minutes.
45. Centrifuge the plate at 485 x g for 5 minutes at 21°C.
46. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.
47. Add 200 µL of 1xPBS to each well. Pipette gently to mix.
48. Centrifuge the plate at 485 x g for 5 minutes at 21°C.
49. Remove supernatants from the wells using a multichannel pipette or vacuum manifold.
50. Resuspend cells in 240 µL of FACS+EDTA buffer.
51. Filter cells before running on flow cytometer.
52. Samples should be run on the cytometer on the same day that they were stained.

5.6 Expected outcomes

FCS files from the flow cytometer can be analyzed using FlowJo. The gating strategy with a representative sample is included in Figure 5. Events were gated to remove doublets and dead cells, then on lymphocytes based on forward scatter (FSC-A) and side scatter (SSC-A) properties. Events that were $CD3^+$, $CD14^+$, and/or $CD16^+$, representing T cells, monocytes, and NK cells, were removed. B cells were identified as $CD19^+$, and memory B cells as $CD27^+$ within the $CD19^+$ compartment[334]. Quadrant gates for the spike-PE vs decoy were set based on the FMO stained condition, which lacks only the spike-PE. Memory B cells that are positive for the decoy are therefore not spike-specific, but those that are single positive for spike-PE are spike-specific. Antibody-secreting cells can then be examined within the spike-specific B cell compartment based on CD38 expression[334]. The panel also includes IgM, IgG, and IgD, for further phenotyping

within the B cell compartment, or spike-specific B cells depending on population size, if desired. The sample used to create this gating strategy was from a healthy, 65 year old female, collected 2-6 weeks after her fifth SARS-CoV-2 vaccination. The sample collection was also >6 months after a SARS-CoV-2 infection.

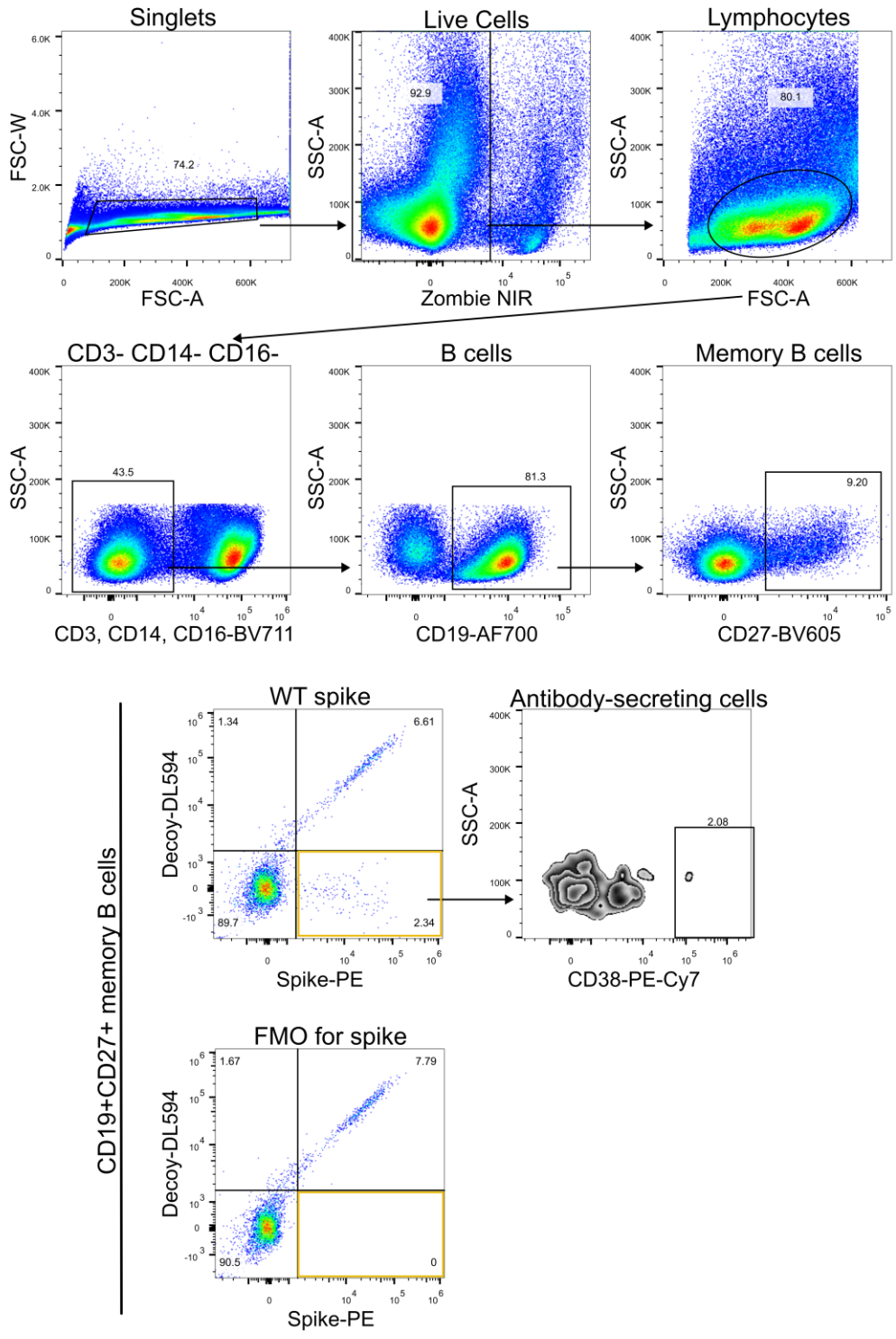


Figure 5. Flow cytometry gating strategy for spike-specific B cells. Doublets were excluded, then events were gated on live cells. Lymphocytes were identified by FSC-A and SSC-A properties. Cells were then gated for BV711

negative ($CD3^-CD16^-CD14^-$) to minimize levels of T cells, monocytes, and NK cells. B cells were identified by CD19 expression in the BV711 negative compartment. Within the B cell compartment, memory B cells were defined as $CD27^+$. In the memory B cell compartment, positivity for the spike-PE and/or decoy-DyLight594 were determined. Gates were set based on the FMO stained condition, which is FMO for the spike-PE. Events which are positive for the decoy molecule are not spike-specific, but rather are binding to either the fluorophore, streptavidin, or biotinylated BLG. Events that are positive for spike-PE, but negative for decoy, are the spike-specific B memory B cells. Antibody-secreting cells can be evaluated within the spike-specific B cell compartment based on high expression of CD38.

5.7 Limitations

Spike-specific B cell numbers are relatively low, even in SARS-CoV-2 vaccinated donors. While this stain can be conducted with less than 1 x 10^6 cells per well following the same protocol, this is not recommended, as the spike-specific B cell population would be difficult to detect. Furthermore, given the low numbers of spike-specific B cells, phenotyping of the spike-specific B cells would become increasingly difficult with lower cell counts.

This protocol is optimized for use with the ancestral SARS-CoV-2 spike protein, the authors cannot comment on if this protocol can be applied to the spike proteins of SARS-CoV-2 variants of concern, or used for proteins other than the spike.

Even with daily QC on the CytoFlex, day-to-day variation in lasers can occur. We therefore recommend that a positive control sample from an individual with a relatively large spike-specific B cell population be run each time an experiment is

conducted, to track variation over time and identify anomalies. Additionally, if possible and if the study is longitudinal in nature, samples from the same participant at different timepoints should be run together on the same day, to avoid confounding variability from time between runs. If multiple cohorts are involved in the study, participants from different cohorts should be run on the same day, as opposed to distinct days being designated for one cohort at a time, to optimize cross-comparability.

5.8 Troubleshooting

This section includes potential problems that may be encountered during the staining procedure, and how to address them.

5.8.1 Problem 1: Low cell viability

This problem is related to steps 1-10. If cell viability is low upon initial thawing and counting, this may be due to improper media preparation for freezing cells, or issues in upstream PBMC isolation and processing. It is also possible that some of this cell death occurred during the thawing process. DMSO is toxic to cells, we therefore recommend during thawing that the cryovials be removed from the water bath when only a sliver of ice is left in the vial. This ensures that by the time the liquid in the cryovial is transferred to the 15mL conical tube, the cells have not been sitting in thawed DMSO-containing media for an extended period of time.

5.8.2 Problem 2: Decoy molecule not staining consistently

This problem is related to steps 28-31. If the decoy molecule is not staining consistently, ensure that the liquid in the wells has descended to the bottom of the well and is not on one of the sides. The liquid should cover the bottom of the well. Additionally, ensure that the plate rocker position and speed causes the liquid to move around the bottom of the well, and that it is not tipped to one particular side. When adding the decoy molecule and 1x PBS, mix gently by pipetting up and down 3-4 times.

5.8.3 Problem 3: Low overall event counts on cytometer

Some cell loss is expected during the washing and staining process. If the event count on the cytometer is much lower than anticipated, ensure that the cells have pelleted to the bottom of the well each time they are centrifuged by visual inspection. Carefully remove the supernatant by moving the multichannel pipette or vacuum manifold down the side of the plate wells, do not place the end directly into the middle of the well. After removing the supernatant, visually inspect the bottom of the wells to determine if the cell pellet is still visible, this will help confirm if the technique used during supernatant removal is contributing to cell loss.

Chapter 6. Discussion

The immunosuppressive drugs used to treat autoimmune conditions can contribute to weaker humoral and cellular responses to vaccinations[48–55,88]. Further complicating matters, the same drug classes, at similar doses, may negatively impact vaccination responses in people with certain autoimmune conditions, but not impair the responses in people with other autoimmune conditions[54]. Additionally, the same drug class may negatively affect responses to only certain vaccine types (e.g., tofacitinib impairs humoral responses to pneumococcal vaccination, but not influenza vaccination)[56]. The complex interplay of autoimmune condition, immunosuppressive drug class and dose, and vaccine type, necessitates studies of the impact of immunosuppressive drugs on vaccination responses in people with various autoimmune diseases, and for emerging vaccine platforms with novel antigens. The rapid roll out of the SARS-CoV-2 mRNA vaccines, and the susceptibility of people with autoimmune conditions and on immunosuppressive drugs to poor outcomes upon SARS-CoV-2 infection, led to an urgent need to understand how these individuals would respond to the SARS-CoV-2 vaccines[118–123].

The overarching aims of our study were therefore to examine the strength of the humoral and cellular responses to SARS-CoV-2 vaccination in participants with RA or SSc and determine if these levels were comparable to controls. If there were deficits in the response, we sought to determine which drug classes were associated with these impairments. In addition to evaluating the strength of the humoral and cellular responses to SARS-CoV-2 vaccination in participants

with RA or SSc on immunosuppressive drugs, we also sought to determine if repeated SARS-CoV-2 vaccination itself would negatively impact the T cell compartment, by examining T cell exhaustion after repeated SARS-CoV-2 vaccination. We hypothesized that participants with RA or SSc, on immunosuppressive drugs, would have weaker humoral and cellular responses to SARS-CoV-2 vaccinations than controls. We also predicted that repeated SARS-CoV-2 vaccination would act as multiple acute exposures, rather than a chronic stimulus, and thus not lead to T cell exhaustion.

In Chapter 2, we found that participants with RA, on immunosuppressive drugs, mounted weaker humoral and spike-specific $CD4^+$ T cell responses, but not weaker $CD8^+$ T cell responses, than those observed in controls, after the second, third, and fourth SARS-CoV-2 vaccinations[317]. The inclusion of costimulation inhibitors in the drug regimen was associated with impaired humoral responses to SARS-CoV-2 vaccination, a finding that matched previous literature reports of responses to the second[297,336] and third doses[300]. Conversely, while other studies had found that costimulation inhibitors were associated with impaired T cell responses after the second[297,336] and third[300] SARS-CoV-2 mRNA vaccinations in people with RA, we did not find costimulation inhibitors as a contributor to poor spike-specific T cell responses[317]. It is therefore possible that people with RA on costimulation inhibitors require additional SARS-CoV-2 vaccinations beyond the first three doses in order to "catch up" to the spike-specific $CD4^+$ T cell levels observed in controls. This is not an unprecedented concept. It has been previously reported that T cell

cytokine production deficits in people with autoimmune disorders on certain immunomodulatory drug classes, such as anti-IL-17, following the second dose are ameliorated after the third SARS-CoV-2 vaccination[337]. In our study, however, even including post dose 4 samples, participants with RA on costimulation inhibitors still had lower antibody levels. The number of SARS-CoV-2 vaccinations required to elicit responses in participants with RA on costimulation inhibitors that are comparable to controls, or participants with RA on other immunosuppressive medications, thus differs between the humoral and cellular compartments. It is also possible that future studies after the 5th SARS-CoV-2 vaccination and beyond will find that costimulation inhibitors are no longer associated with weaker humoral responses. As the spike-specific CD4^{+} T cell levels increase, and potentially catch up to the levels seen in controls following the fourth SARS-CoV-2 vaccination, this T cell compartment could support B cell class switching and antibody production. In support of this concept, it has been shown that costimulation inhibitor treatment reduces the levels of RBD-specific B cells class switched to IgG in circulation following the primary vaccination series[336], and that costimulation inhibitors may reduce overall T_{FH} levels[338,339].

In the age of Omicron, even when adjusting for number of vaccinations, individuals with RA are at an increased risk of poor outcomes associated with SARS-CoV-2 infections, with JAK inhibitors in particular associating with an increased risk of hospitalization[340]. A previous study in participants with RA had reported that those on JAK inhibitors had lower antibody levels after the third SARS-CoV-2 vaccination[300]. JAK inhibitors were not associated with lower

antibody levels in our cohort, suggesting that, like the T cell responses of participants on costimulation inhibitors, the number of SARS-CoV-2 vaccinations may overcome deficits in the memory response associated with different immunosuppressive drugs. This previous study found that participants with RA on JAK inhibitors had a similar positivity rate for spike-specific T cells (based on IFN-γ release) as controls, following the third SARS-CoV-2 vaccination[300]. While costimulation inhibitors were not associated with lower spike-specific $CD4^+$ T cell levels in participants with RA in our study, JAK inhibitors were associated with lower spike-specific $CD4^+$ T cell levels[317]. A difference in methodology could account for the discrepancy between our study and the aforementioned study. While rates of positivity for spike-specific T cells may not differ, the magnitude of the response in terms of actual T cell numbers may differ. Nonetheless, when considering spike-specific $CD4^+$ T cell numbers, four doses may be sufficient to ameliorate previously weaker responses in participants with RA on costimulation inhibitors, but not participants with RA on JAK inhibitors. It is therefore probable that the number of SARS-CoV-2 vaccine doses required to “catch up” to controls may also differ by immunosuppressive drug class.

Early in the pandemic, studies largely focused on if the magnitude of the humoral and cellular responses were similar between participants on immunosuppressive drugs and controls[295,296,299,336]. Later, attention shifted towards additional SARS-CoV-2 vaccinations to correct waning humoral responses with time post vaccination or to address new circulating variants[297,298,337,341,342]. Other researchers have found that the third and fourth

SARS-CoV-2 vaccinations reduce antibody waning in participants with autoimmune disorders on immunosuppressive drugs, compared with the waning observed following the second dose[337]. While T cell levels may be relatively stable after additional vaccinations, doses beyond the primary series ameliorate declines in T cell cytokine production with time after vaccination[337]. We also found that SARS-CoV-2 vaccinations beyond the primary series led to elevated anti-RBD IgG levels, but not spike-specific $CD4^+$ T cell levels, in participants with RA[317]. Thus, even if additional SARS-CoV-2 vaccinations do not increase a facet of humoral or cellular memory responses, or cause these levels to become similar to controls, they can increase the longevity of these responses. A study published shortly after our manuscript found that a fourth SARS-CoV-2 vaccination was associated with increased protection against infection in participants with autoimmune conditions, supporting the importance of additional vaccine doses[306]. Reduced humoral or cellular responses in participants with autoimmune disorders on certain immunomodulatory drug classes, compared with controls, therefore does not mean SARS-CoV-2 vaccination is without benefit in these populations. This nuance suggests that the benefits of SARS-CoV-2 vaccination should focus on reduction of poor outcomes associated with infection and ameliorating waning, and that “catching up” to the magnitude of humoral or cellular responses observed in controls is not the only important metric.

In Chapter 3, we pivoted to evaluate the humoral and cellular responses to the second, third, and fourth SARS-CoV-2 vaccinations in participants with SSc,

compared to controls[321]. In contrast to what was found for participants with RA, the participants with SSc did not mount weaker humoral, spike-specific $CD4^+$ T cell, or spike-specific $CD8^+$ T cell responses than controls[321]. We had initially hypothesized that participants with RA or SSc, on immunosuppressive drugs, would have impaired humoral and cellular responses to SARS-CoV-2 vaccination. While this was what we found in participants with RA, it was initially somewhat surprising that participants with SSc did not have impaired humoral or cellular responses to SARS-CoV-2 vaccinations. Upon further examination, however, the differences in SARS-CoV-2 vaccination responses between the two cohorts are likely related to the immunosuppressive drug classes they were on. In the RA study, costimulation inhibitors were associated with weaker humoral responses to SARS-CoV-2 vaccination, while other drugs such as DMARDs and steroids did not have an impact[317]. The SSc cohort contained participants on DMARDs and steroids, but did not include anyone on costimulation inhibitors[321]. Furthermore, JAK inhibitors, which negatively impacted spike-specific $CD4^+$ T cell responses in participants with RA, were only used by one participant in the SSc cohort[317,321]. Although the impact of immunosuppressive drugs on vaccination responses may differ depending on autoimmune condition and drug dose used in those conditions, the fact that the participants with RA and the participants with SSc had unique breakdowns of participants on certain drug classes likely contributed to the disparate findings between the two cohorts. Further, larger scale, studies would be required to definitively determine if

costimulation or JAK inhibitors exerted a similarly negative impact on vaccination responses in participants with SSc as they do in participants with RA.

Although the humoral responses to SARS-CoV-2 vaccination in participants with SSc were not lower than those observed in controls, we noticed that some participants with SSc had a delayed rise in antibody levels after the second SARS-CoV-2 vaccination. We explored this delayed response in Chapter 3.2, but as a consequence of small samples size were unable to determine which factors may be contributing to this delayed response. It is possible that this delayed response was an artifact of small sample size and does not occur in many people with SSc. Alternately, the delayed response may be related to a parameter that was not evaluated in the current study. For instance, it has been previously shown that individuals with SSc have alterations in the lymphatic vessels in their skin, with reports of lower numbers of lymphatic vessels and impaired drainage[343,344]. If there was impaired lymphatic drainage in the area around the vaccine intramuscular injection site, perhaps this led to delayed responses to vaccination as APCs struggled to reach the lymph nodes. Previous studies on SARS-CoV-2 vaccination responses in participants with SSc had focused primarily on humoral responses[301,303,304], but this delayed humoral response was not something other studies reported, nor was such a bimodal distribution in antibody responses observed in our RA cohort after the second SARS-CoV-2 vaccination. The implications of this delayed humoral response, and if participants with delayed responses are particularly vulnerable to SARS-CoV-2

infections during this period before their antibody levels rise, remains to be determined.

Taken together, Chapters 2 and 3 provide a comprehensive examination of humoral and cellular responses to SARS-CoV-2 vaccination in participants with RA and SSc. They extend the findings of the existing literature to incorporate additional SARS-CoV-2 vaccinations, and evaluations of T cell compartment parameters that were not previously reported. For instance, our study was the first, to our knowledge, to investigate spike-specific $CD4^+$ and $CD8^+$ T cell responses to SARS-CoV-2 vaccination in participants with SSc, and further evaluate the phenotypes of the spike-specific $CD4^+$ T cells as Th1, Th2, Th17, or Tregs[321]. The findings of our studies with participants with RA and SSc emphasize the importance of examining SARS-CoV-2 vaccination responses in populations with different autoimmune conditions, and considering the different drug classes these participants may be on, as certain drug classes may impair the vaccination responses and thus could confer a greater risk of subsequent SARS-CoV-2 infection. They also highlight that additional SARS-CoV-2 vaccinations are immunogenic in both participants with RA and SSc, and different numbers of SARS-CoV-2 vaccinations may be required to elicit responses comparable to controls.

The persistent threat of SARS-CoV-2, and the emergence of immune evasive variants, has also led to the recommendation that individuals receive additional SARS-CoV-2 vaccinations over time to maintain protection against severe

disease. This recommendation is especially strong for vulnerable populations, such as older adults in congregate living facilities, and immunosuppressed people[150,162,345]. Compared to many other vaccines, however, SARS-CoV-2 vaccinations are offered at relatively short intervals[150,162,163]. This vaccination schedule, and the novel aspect of the mRNA vaccine platform, led to concerns from the public and vulnerable populations regarding potential negative impacts of repeated SARS-CoV-2 vaccination[346,347]. We therefore explored if repeated SARS-CoV-2 vaccination actually led to T cell exhaustion in participants with RA on immunosuppressive drugs, older adults in long-term care, and healthy younger adults.

In Chapter 4, we found that repeated SARS-CoV-2 vaccination did not lead to reduced levels of spike-specific T cells, an increase in exhaustion markers on spike-specific and bulk T cells, changes in cytokine production capacity, or overall changes to the T cell compartment (in terms of CM, EM, EMRA, and naïve subsets), in individuals with RA on immunosuppressive drugs, older adults in long-term care, or healthy younger adults. In further support of the concept that multiple vaccinations in a relatively short period of time were not overwhelming the immune system, we also demonstrated in the healthy younger adult cohort that a shorter primary series dosing interval was not associated with higher levels of exhausted T cells, compared with a longer dosing interval. Before this study, immune exhaustion following SARS-CoV-2 vaccination had not been largely examined, with the available studies focusing on exhaustion marker expression or polyfunctionality in healthy cohorts, and not largely examining responses in

older adults or individuals with autoimmune conditions who were on immunosuppressive drugs[158,199,200,348]. Our study was the first to explicitly examine immune exhaustion following SARS-CoV-2 vaccination in multiple vulnerable populations, and to consider exhaustion marker expression, functional capacity, and overall T cell compartment breakdowns in our evaluation.

The findings in Chapter 4 support the notion that repeated SARS-CoV-2 vaccination is not acting as a chronic stimulation, but rather, multiple acute exposures. Although this study extended to four SARS-CoV-2 vaccinations, we would anticipate that additional doses would not cause T cell exhaustion in any of the populations studied, given the acute nature of the exposures. Further supporting the notion that multiple vaccinations in a relatively short period of time do not overwhelm the immune system are the childhood routine immunization schedules[349]. Children who receive multiple vaccinations in this relatively short time period are not at an increased risk of hospitalization due to infectious diseases not covered by the vaccines[350,351]. The vaccine schedule is therefore not overwhelming the immune system and leaving children vulnerable to other pathogens. Although not recommended, an individual in Germany received 217 SARS-CoV-2 vaccinations in less than 2.5 yrs[352]. This individual did not experience adverse events due to the repeated vaccinations, nor did he have impaired T cell cytokine production[352]. Receiving over 200 does did not impair the memory responses in this person, thus the comparatively lower number of additional SARS-CoV-2 vaccinations recommended by public health authorities would not be expected to cause impairment. We hope that having the concrete

data from Chapter 4 to support general vaccinology knowledge will help alleviate the concerns of the public and vulnerable populations regarding repeated vaccination, provide clinicians and policy makers with information that will encourage vaccination, and thus ensure continued protection for vulnerable populations. Chapter 4 also complements our earlier studies on the strength of the humoral and cellular responses to SARS-CoV-2 vaccination in participants with autoimmune conditions who are on immunosuppressive drugs. We have now determined that vaccination is immunogenic in these populations, and that this immunogenicity is not overwhelming or detrimental to the T cell compartment.

Limitations and future directions

The primary limitations of the studies included in this thesis are the relatively small cohort sizes. In Chapter 2, we disentangled which drug classes may be associated with weaker humoral and cellular responses to SARS-CoV-2 vaccination in participants with RA, though we acknowledge that validation of these findings in other larger cohorts would be beneficial. We were not powered to disaggregate the DMARD class into the individual drugs within it, and it is possible that some of the drugs in the DMARD class may have a negative effect on vaccination responses, while others do not. We were also not powered to pull out a dose effect for steroids, and previous studies have found that higher steroid doses may be more immunosuppressive[43,44]. The SSc cohort in Chapter 3 was smaller than the RA cohort, with participants on a wide variety of medications, and thus there were small sample sizes for each individual medication. If the SSc

cohort had turned out to have weaker humoral or cellular responses than the controls following SARS-CoV-2 vaccination, we would have been unable to determine which drug classes were associated with this deficit. Similarly, it is possible that participants with SSc on certain drug classes, that were not largely represented in this study, may have weaker SARS-CoV-2 vaccination responses[303,304]. The small cohort size also interfered with our ability to determine which factors may be related to the delayed humoral response post dose 2 in some participants with SSc (Chapter 3.2). Until this phenomenon is validated in a larger cohort, we cannot completely exclude the possibility that it may be an artifact of sample size.

Another limitation of the studies in Chapters 2 and 3 is that the T helper cell compartments were defined solely based on chemokine receptor expression, and not cytokine production. This method, and the markers used to identify the $CD4^+$ T cells as Th1, Th2, or Th17, has however been previously used in various studies of SARS-CoV-2 infection and vaccination responses[133,299,324]. Future studies would be strengthened by the inclusion of intracellular cytokine stains to evaluate T cell cytokine production and skew. Additionally, Tregs were defined as $CD39^+CD25^+CD134^+CD4^+$ T cells as this strategy has been reported to accurately identify Tregs even in the context of stimulation[353]. We recognize that expression of the transcription factor FoxP3 is one of the most widely used methods to identify Tregs, however, this transcription factor can be upregulated in T cells that are not Tregs upon stimulation[353]. Given that our identification of spike-specific T cells employed an activation induced marker assay, and thus

stimulation, FoxP3 would not have been a completely reliable marker for Treg identification. Additionally, staining for transcription factors requires the fixation and permeabilization of the T cells, and thus if this method were employed, we would be unable to sort and further characterize the Tregs if desired. Conversely, the fixation step in our current AIMs protocol is not critical for staining, and thus this step could be removed if we wanted to sort any of the antigen-specific T cell subpopulations for further study.

Although we initially intended to explore SARS-CoV-2 vaccination responses around the fifth, primarily bivalent, vaccination in participants with RA, SSc, and controls, as well as the hybrid immunity in previously infected individuals around this timepoint, we unfortunately were unable to collect a sufficient number of samples for these analyses. The bivalent vaccinations, at the time, encoded spike proteins from the Omicron BA.1 or the Omicron BA.4/5 variants[262–264,269]. We had therefore wanted to determine how these bivalent vaccinations augment responses to ancestral and Omicron spike proteins by both antibodies and T cells, particularly in our participants with autoimmune conditions who were on immunosuppressive drugs, and how these responses would compare to controls. We had previously found that participants with RA had lower levels of anti-RBD IgG, and spike-specific $CD4^+$ T cells against the ancestral SARS-CoV-2 spike protein, raising the question of whether their variant-specific responses would be even further impaired. Recently, a study using a cohort of individuals with various autoimmune diseases, all treated with TNF-inhibitors, found that a fifth dose bivalent vaccine was able to increase neutralizing antibody titers against the

ancestral and various Omicron BA strains, but only in individuals who had not had a previous Omicron BA strain infection[342]. In individuals with hybrid immunity, the fifth dose did not increase neutralizing antibody titers, nor did the fifth dose alter spike-specific $CD4^+$ or $CD8^+$ T cell levels compared with the frequencies post dose 4[342]. This study therefore provided additional insight into the importance of considering previous infection status and vaccine doses when evaluating humoral and cellular responses, but the question of how the responses of people on these immunosuppressive drugs compares to controls, and which drugs, if any, may augment fifth dose bivalent vaccination responses, remains to be determined.

Even when adjusting for previous infection and vaccination status, in the age of Omicron variants, participants with RA who are on immunosuppressive drugs are at an increased risk of hospitalization and death upon SARS-CoV-2 infection[340]. In people with autoimmune conditions, inclusion of methotrexate or rituximab in their drug regimens has also been associated with a greater risk of SARS-CoV-2 reinfection[354]. When considering hybrid immunity, it has been previously reported that recent SARS-CoV-2 infection in healthy adults may boost antibody levels and provide protection against reinfection with a similar variant[140,256,290,292,293]. We and others have found that hybrid immunity increases antibody levels, but not spike-specific T cell levels, in individuals with autoimmune conditions, who are on immunosuppressive drugs, compared with vaccination alone[317,342,355,356]. Interestingly, a recent study reported that in a cohort of individuals with various autoimmune conditions, who were on

immunosuppressive drugs, recent previous SARS-CoV-2 infections may not be protective against reinfection[357]. The interplay of how different immunosuppressive drugs may impact hybrid immune responses in participants with RA compared to controls, especially for other non-spike SARS-CoV-2 protein responses, and the implications this has on protection against reinfection, remains to be determined. While we had initially aimed to include analyses of spike-specific and other SARS-CoV-2 antigen-specific T cell responses in previously infected and uninfected participants with RA and controls around the fourth and fifth SARS-CoV-2 vaccinations to explore this factor, we were underpowered in this endeavor. Future studies may seek to collaborate with other investigators in the SUCCEED study, as grouping samples with different autoimmune conditions, a commonly employed approach, may provide the opportunity to disentangle these factors.

The studies herein examined antibody levels and neutralizing capacity, but did not examine other Fc-mediated antibody effector functions, such as ADCC. Previous studies have, however, demonstrated that although neutralizing antibodies may be a correlate of protection, non-neutralizing antibody functions may also provide protection[126,129,130,169,170,358]. SARS-CoV-2 mRNA vaccination has been reported to increase ADCC[153]. As mentioned in the introduction, the BNT162b2 and mRNA-1273 vaccines induce similar levels of ADCP by monocytes, but mRNA-1273 induces higher antibody-dependent NK cell activity and neutrophil phagocytosis[171]. As SARS-CoV-2 continues to mutate and evade neutralizing antibody responses, it is possible that the importance of non-

neutralizing antibodies will increase, particularly for those that target more conserved regions of the spike protein of SARS-CoV-2. As such, examining Fc-mediated antibody effector functions in participants with RA or SSc on immunosuppressive drugs, and comparing those to the responses observed in controls following various SARS-CoV-2 vaccinations, could offer additional insight into mechanisms and correlates of protection. It would be of particular interest if certain immunosuppressive drugs negatively impacted Fc-mediated antibody effector functions, and if the pattern of related drugs differed from that observed for overall antibody levels.

Certain immunosuppressive drug classes have also been implicated in altering the class switching of the humoral responses to SARS-CoV-2 vaccination. In particular, TNF inhibitors and monoclonal antibodies that interfere with IL-4/IL-13 have been associated with reduced IgG4 class switching[359]. In an additional layer of complexity, the production of spike-specific IgG4 antibodies appears to occur primarily after mRNA vaccination, but is relatively infrequent following adenoviral vector SARS-CoV-2 vaccination[359,360]. The implications of altered IgG subclass profiles on long-term protection against severe SARS-CoV-2 infection and VOCs remain to be determined. In individuals with high overall anti-spike IgG levels, high levels of IgG4 may interfere with the Fc-mediated antibody effector functions of other subclasses[360,361]. It would therefore be important to evaluate IgG subclass distributions in the future studies of Fc-mediated effector functions in participants with autoimmune conditions, who are

on immunosuppressive drugs, as it is likely that the IgG subclasses and Fc-mediated effector functions are highly related.

On a related note, future studies may also seek to employ the spike-specific B cell flow cytometry staining protocol to explore memory B cell class switching after repeated SARS-CoV-2 vaccinations in people with RA and SSc, as well as controls. Our *Scientific Reports* paper had found that inclusion of costimulation inhibitors in the drug regimen was associated with lower levels of anti-RBD IgG in participants with RA[317]. It would be interesting to examine if the memory B cells in these participants had altered distributions of IgG and IgM expression. If the spike-specific memory B cells in participants on costimulation inhibitors were primarily IgM+ as opposed to IgG+, and if this distribution significantly differed from participants with RA on other drug classes, this could suggest that the reduced anti-RBD IgG levels are related to impaired class switching. This finding would further strengthen our conclusion regarding the negative impact of costimulation inhibitors on humoral responses to SARS-CoV-2 vaccination, and would align with a previous study that showed altered memory B cell class switching in participants with RA on this medication[336].

As new mRNA vaccines for other pathogens begin to enter the market, it would be prudent to consider if the aforementioned immunomodulatory drugs will similarly negatively impact vaccination responses to these new vaccines, and if these deficits can be overcome by multiple vaccine doses. For instance, while we did not find that costimulation inhibitors were associated with impaired T cell

responses in our study, which included samples collected after the second, third, and fourth SARS-CoV-2 vaccinations, other studies did find deficits in the T cell responses after fewer doses[297,300,336]. It is therefore possible that additional doses of new mRNA vaccines entering the market will be required in populations on costimulation inhibitors, if a similar dose-dependent effect is observed. Furthermore, if existing vaccine types, such as non-mRNA influenza vaccinations, are converted to mRNA vaccines, it is possible that individuals on immunosuppressive drugs who previously mounted strong immune responses to the other vaccine formulation may have impaired responses to the mRNA vaccine formulation, as the impacts of immunosuppressive drugs on responses can vary by vaccine type.

Overall concluding remarks

The studies included in this thesis have determined how individuals with different autoimmune diseases respond to SARS-CoV-2 vaccination, and which immunosuppressive drug classes negatively impact different aspects of the memory immune response. Even though participants with RA may have weaker humoral and $CD4^+$ T cell responses to SARS-CoV-2 vaccination than controls, repeated SARS-CoV-2 vaccination still increased their humoral responses and spike-specific $CD8^+$ T cell levels. We have also determined that repeated SARS-CoV-2 vaccination does not lead to T cell exhaustion, even in vulnerable populations like older adults in long-term care, and individuals with RA on immunosuppressive drugs. SARS-CoV-2 vaccinations are therefore immunogenic in participants with RA and SSc, and this immunogenicity is not

negatively impacting the T cells in participants with RA. Altogether, this work provides a better understanding of SARS-CoV-2 vaccination responses in people with autoimmune conditions, and the impacts of immunosuppressive drugs, including for the novel mRNA vaccine platform. The data generated herein thus provides policy makers, clinicians, and patients with the evidence to make informed vaccine decisions and recommendations.

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Appendices

Appendix 1. SUCCEED Investigator Group non-author collaborator list for Scientific Reports paper

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Appendix 2. Hybrid Immunity, Fourth, and Fifth SARS-CoV-2 vaccination responses in participants with RA

Additional Methods

Sample timepoints

Following the same protocol as previously described[317], as a continuation of that study, samples were collected from participants with RA and controls at 2-6 weeks and 3 months after the fifth SARS-CoV-2 vaccination. Due to the small sample size, these timepoints were combined for display into the umbrella of post dose 5 (pd5). Participant samples from either 2-6 weeks or 3 months post dose 4, or pre dose 5, were included where PBMCs were available.

T cell Activation-Induced Marker (AIM) assays

SARS-CoV-2-antigen-specific memory T cells were identified using an activation-induced marker (AIM) assay. Briefly, cryopreserved PBMCs were thawed, washed, counted, and resuspended in RPMI supplemented with 10% fetal bovine serum (#12483020, Gibco), 1% Pen/Strep (#15140122, Gibco), 1% HEPES (1M, #7365-45-9, Sigma), 1% GlutaMAX (#35050061, Gibco), and 0.5% β-mercaptoethanol (#21985023, Gibco) (cRPMI). The cells were then rested at 37°C/5% CO_2 in the incubator overnight. The next day, cells were counted again and resuspended in cRPMI to a concentration of $5x10^6$ cells/mL. 100µL of media containing $0.5x10^6$ cells were added to each well of a 96-well U-bottom plate. Peptides from the ancestral spike protein (SARS-CoV-2 S complete Peptivator, #130-127-951, Miltenyi Biotec), Omicron BA.1 spike protein (PepMix™ SARS-CoV-2 Spike B.1.1.529, #PM-SARS2-SMUT08-1, JPT), nucleocapsid (SARS-

CoV-2 N Peptivator, 130-126-699, Miltenyi Biotec), matrix protein (SARS-CoV-2 M Peptivator, 130-126-703), and RNA polymerase (PepMix^{TM} SARS-CoV-2 NSP-12, #PM-WCPV-NSP12-2, JPT), were each diluted in cRPMI and used to stimulate the PBMCs at a final concentration of 1µg/mL for 48 h at 37°C/5% CO_2. Cytostim (#130-092-172, Miltenyi Biotec) was used as a positive stimulation control for each sample at 0.50µL per well, and a media only unstimulated control was also included for each sample (received 100µL of media). The final volume in each well was 200µL.

After 48 hours, the cells were washed with PBS, then stained with Zombie Near-IR (1:10000, Cat. #423105, BioLegend) for 30 minutes in the dark at room temperature. Cells were then washed again with PBS, followed by FACS Wash. Cells were then stained for 30 minutes at room temperature in the dark, with a cocktail of fluorochrome conjugated antibodies against CD3 (BV510), CD4 (BB700), CD25 (PE-Cy7), CD134 (PE), CD8 (BB515), CD137 (APC), CD69 (BV711) in Brilliant Stain Buffer Plus (BD Biosciences) and PBS, the details of which can be found in Supplementary Table 1. The flow cytometry gating strategy is the same as that depicted in the manuscript in Chapter 2. While antigen-specific $CD4^+$ and $CD8^+$ T cells were evaluated, the data for antigen-specific $CD8^+$ T cells were extremely low and often below background media control thresholds for positivity, and thus were not displayed herein.

T follicular helper (T_{FH}) cell stain

Cryopreserved PBMCs were thawed, washed, counted, and resuspended to $5x10^6$ cells/mL in PBS. 100µL of PBS containing $0.5x10^6$ cells were added to each well of a 96-well U-bottom plate. Each sample had a well for the unstained control, full stain, and isotype well. Cells were washed with PBS, then the full stain and isotype wells were stained with Zombie Near-IR (1:10,000, #423105, BioLegend) for 30 minutes in the dark at room temperature. The unstained well received 100µL of PBS. Samples were then washed with PBS, followed by a wash with FACS Wash. Cells were then stained for 30 minutes at room temperature in the dark with a cocktail containing fluorochrome conjugated antibodies against CD3, CD4, CD25, CD8, CD39, CXCR3, CCR6, CCR4, CXCR5, PD-1, and ICOS, in Brilliant Buffer and PBS, the details of which are in Supplementary Table 1. Cells were then washed and resuspended in FACS Wash to be run on the cytometer. The flow cytometry gating strategy is included in Supplementary Figure 1. T_{FH} were defined as $CD3^+CD4^+CXCR5^{+}$[133,319,362,363].

Spike-specific B cell stain

Details on the spike-specific B cell stain methodology can be found in Chapter 5.

Flow cytometry

Samples were acquired on a Cytoflex LX (4 laser, Beckman Coulter) using CytExpert software. Data analysis was conducted with FlowJo version 10 (BD Life Sciences).

Results

Participant Demographics

In order to explore of hybrid immunity around the fourth and fifth vaccinations, as well as generally report the fifth dose vaccination responses, samples were collected from participants with RA and controls with and without previous SARS-CoV-2 infections at various timepoints following the fourth and fifth SARS-CoV-2 vaccinations (Table 6). Due to the small samples size, samples collected 2-6 weeks post dose 4, 3 months post dose 4, and pre dose 5, were all grouped into the heading ‘post dose 4’. Samples collected 2-6 weeks or 3 months post dose 5 were grouped into the heading ‘post dose 5’. If participants developed a SARS-CoV-2 infection between their fourth and fifth dose sample collections, they were moved to the appropriate ‘infected’ group at the sample collection after infection. The age, sex, first and second vaccine types, and dose 1-2 interval of the previously infected and uninfected RA and control cohorts did not significantly differ from one another (Table 6). While the third and fourth dose vaccine types did not differ between cohorts, the dose 2-3 interval was significantly longer in uninfected controls than infected or uninfected participants with RA (Table 6). The dose 3-4 interval was longer in the previously infected controls than the previously infected or uninfected participants with RA (Table 6). While the available sample collection timepoints differed between cohorts following the fourth dose, the distribution did not differ after the fifth dose (Table 6). While the majority of participants received mRNA vaccines for their first, second, third, and

fourth doses, the fifth doses were primarily bivalent mRNA vaccines (Table 6). Infections in the control cohort were primarily detected by seroconversion to become anti-N+ (Table 6). In contrast, infections in participants with RA were more commonly detected by positive PCR or rapid antigen tests, though not all participants with a positive test seroconverted to become anti-N+ (Table 6).

Table 6. Participant Demographics

	Previously infected controls	Uninfected controls	Previously infected RA	Uninfected RA	*p* value
Overall cohort size	6	12	9	12	N/A
Sample size post dose 4[a]	2	10	7	12	N/A
Sample size post dose 5[a]	5	9	7	10	N/A
Age, median ± SD	64.0 ± 5.6	69.5 ± 11.7	65.0 ± 9.7	67.5 ± 6.7	0.8345[b]
Sex, % female (n)	66.7 (4)	58.3 (7)	77.8 (7)	75.0 (9)	0.8644[c]
First dose vaccine type	4 BNT162b2 1 mRNA-1273 1 ChAdOx1	11 BNT162b2 1 mRNA-1273	8 BNT162b2 1 ChAdOx1	10 BNT162b2 2 ChAdOx1	0.4435[c]
Days between dose 1 and dose 2 (median ± SD)	78.0 ± 27.5	74.0 ± 18.7	68.0 ± 21.7	80.0 ± 19.9	0.2010[b]

Second dose vaccine type	4 BNT162b2 2 mRNA-1273	12 BNT162b2	8 BNT162b2 1 ChAdOx1	10 BNT162b2 1 mRNA-1273 1 ChAdOx1	0.1316[c]
Days between dose 2 and dose 3 (median ± SD)	175.0 ± 10.6	191.0 ± 39.6	153.0 ± 22.7	152.5 ± 25.9	0.0025[b]
Third dose vaccine type	5 BNT162b2 1 mRNA-1273	7 BNT162b2 5 mRNA-1273	8 BNT162b2 1 mRNA-1273	9 BNT162b2 3 mRNA-1273	0.5117[c]
Days between dose 3 and dose 4 (median ± SD)[d]	233.0 ± 82.9	142.0 ± 56.7	129.0 ± 29.2	115.0 ± 35.9	0.0026[b]
Fourth dose vaccine type[d]	3 BNT162b2 1 mRNA-1273 1 bivalent	3 BNT162b2 7 mRNA-1273	3 BNT162b2 6 mRNA-1273	8 BNT162b2 4 mRNA-1273	0.1267[c]
Post dose 4 sample collection timepoints	1 at 2-6wks4 1 at 3mo4	5 at 2-6wks4 4 at 3mo4 1 pre dose 5	2 at 2-6wks4 6 at 3mo4	10 at 2-6wks4 2 at 3mo4	0.06115[c]

Days between dose 4 and dose 5 (median ± SD)[e]	144.0 ± 27.6	158.5 ± 34.9	203.0 ± 21.7	204.5 ± 54.2	0.1367[b]
Fifth dose vaccine type	5 bivalent	8 bivalent 1 novavax	7 bivalent	9 bivalent 1 BNT162b2	>0.9999[c]
Post dose 5 sample collection timepoints	4 at 2-6wks5 1 at 3mo5	5 at 2-6wks5 4 at 3mo5	6 at 2-6wks5 1 at 3mo5	9 at 2-6wks5 1 at 3mo5	0.3671[c]
Method of SARS-CoV-2 infection identification[f]	4 anti-N+ 2 positive test and anti-N+	N/A	3 anti-N+ 2 positive test only 4 positive test and anti-N+	N/A	0.4825[c]
Prednisone, % (n)	N/A	N/A	0 (0)	8.3 (1)	>0.9999[c]
DMARDs[g], % n	N/A	N/A	66.7 (6)	66.7 (8)	>0.9999[c]
TNF and TNF receptor inhibitors[h], % (n)	N/A	N/A	11.1 (1)	8.3 (1)	>0.9999[c]
Janus Kinase Inhibitors, % (n)	N/A	N/A	22.2 (2)	25.0 (3)	>0.9999[c]
Costimulation inhibitor	N/A	N/A	33.3 (3)	16.7 (2)	0.6108[c]

(Abatacept), % (n)					

[a]Not all participants provided samples both post dose 4 and post dose 5.

[b]Ordinary one-way ANOVA

[c]Two-tailed Fisher's Exact Test

[d]Dose 3-4 interval and fourth dose vaccine type unavailable for 1 infected control, and 2 uninfected controls

[e]Dose 4-5 interval unavailable for 1 infected control, 3 uninfected controls, and 2 infected RA

[f]Anti-N+ indicates participant seroconverted from anti-nucleocapsid negative to positive for anti-N IgG. 'Test' denotes a positive PCR or rapid antigen test.

[g]Includes methotrexate, leflunomide, hydroxychloroquine, and sulfasalazine.

[h]Adalimumab

Humoral Responses

We, and others, have reported that previous SARS-CoV-2 infections increase anti-spike/anti-RBD IgG levels, though it remained to be determined if the fifth vaccination, which was primarily bivalent in our cohort, would alter this trend and variant-specific (Omicron BA.1) neutralization[157,288,317]. We therefore evaluated anti-RBD IgG levels, and the neutralization capacity against the ancestral SARS-CoV-2 and Omicron BA.1 variant, in our participants after the fifth vaccination (Figure 6). We also included anti-RBD IgA (Figure 6B), as previous studies have reported that prior infection can elevate anti-RBD IgA levels, though it can decline rapidly with time following infection[131,172]. Unlike the other figures in this section, antibody responses post dose 4 are not displayed, as this data has previously been published in our *Scientific Reports* manuscript[317].

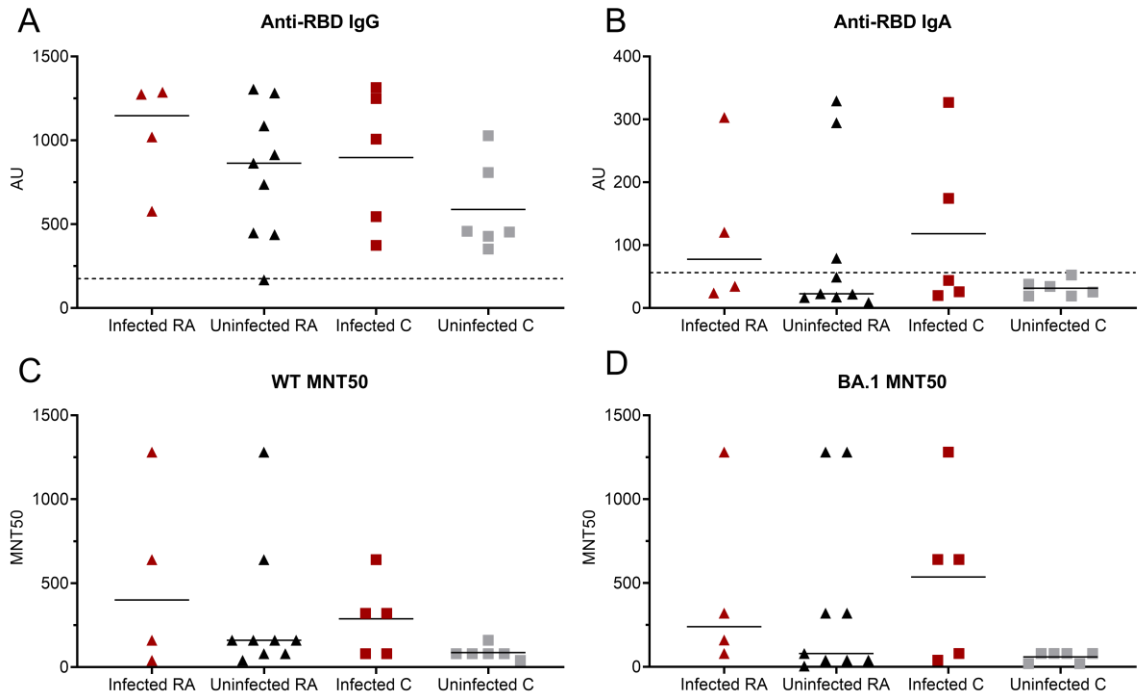


Figure 6. Serum anti-RBD antibody levels and neutralization capacity in participants with RA and controls following the fifth SARS-CoV-2 vaccination. Anti-RBD IgG **(A)** and IgA **(B)** levels in the serum were evaluated using ELISAs for previously infected and uninfected participants with RA and controls. The dashed line marks the limit of detection. AU stands for arbitrary units, based on the optical density value multiplied by the serum dilution factor. MNT50 against ancestral SARS-CoV-2 and **(C)** the Omicron BA.1 variant **(D)** were also measured in participants with RA and controls. The solid lines indicate the median of each group. Infected refers to participants with a previous SARS-CoV-2 infection. Infected RA n=4, Uninfected RA n=9, Infected C n=5, Uninfected C n=6.

Spike-specific B cells

It has been previously reported that both SARS-CoV-2 vaccination and SARS-CoV-2 infection lead to increased frequencies of spike-specific B cells, which remain detectable for months afterward[131,134,159,189]. At earlier timepoints after the second SARS-CoV-2 vaccination, individuals with previous SARS-CoV-2 infections have higher frequencies of spike-specific B cells than those without a previous infection[159]. While hybrid immunity may therefore influence spike-specific B cells responses, immunosuppressive drugs may further influence these cells. Following the primary vaccination series, participants with RA on the costimulation inhibitor abatacept were found to have lower frequencies class-switched IgG+ RBD-specific and overall IgG+ memory B cells than controls[297,336]. In contrast, participants with RA who were on methotrexate did not display a deficit in their IgG+ RBD-specific B cell levels[336]. There is however a paucity of data exploring the interplay of hybrid immunity and immunosuppressive drug treatments and their effect on spike-specific B cells in participants with RA, particularly after the bivalent vaccinations, leading us to assess this population in our study (Figure 7A, B). Although SARS-CoV-2-specific memory B cells can produce antibodies targeting SARS-CoV-2 upon restimulation, plasma cells are generally recognized as the predominant antibody-secreting cells, and thus we also evaluated the levels of spike-specific antibody-secreting cells (plasma cells and plasmablasts) in circulation (Figure 7C, D)[131,159].

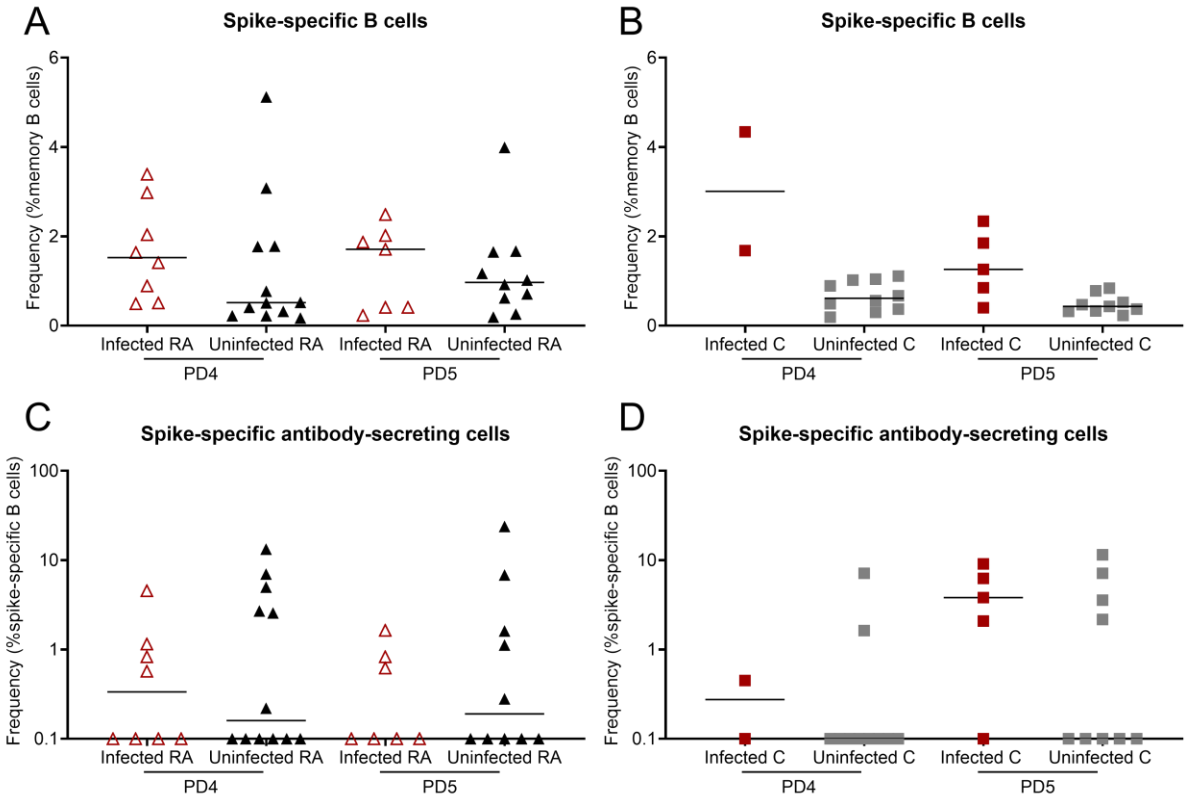


Figure 7. Spike-specific B cell frequencies following fourth and fifth SARS-CoV-2 vaccinations in participants with RA and Controls. Flow cytometry was used to assess the frequencies of spike-specific memory B cells (Spike trimer^{+}Decoy^{-} events) out of all memory B cells (CD27^{+}CD19^{+}) in participants with **(A)** RA and **(B)** controls. Frequencies of spike-specific antibody-secreting cell types (CD38^{+} events within spike-specific memory B cells compartment, includes plasmablasts and plasma cells) were also assessed in participants with RA **(C)** and controls **(D)**. Frequencies of zero are shown as 0.1 for display on the log axis. The solid lines indicate the median of each group. PD4 indicates samples collected post dose 4, PD5 indicates samples collected post dose 5. Infected refers to participants with a previous SARS-CoV-2 infection. PD4:

Infected RA n=8, Uninfected RA n=12, Infected C n=2, Uninfected C n=10. PD5: Infected RA n=7, Uninfected RA n=10, Infected C n=5, Uninfected C n=9.

Spike-specific T cells

In a continuation of the parameters evaluated in the *Scientific Reports* manuscript, we assessed the levels of ancestral-SARS-CoV-2-spike-specific T cells in our participants with RA and controls after the fifth SARS-CoV-2 vaccination (Figure 8A, B). Given that these assays were conducted with PBMCs instead of peripheral blood, samples from post dose 4 were also included for comparison to the fifth dose samples. Furthermore, we expanded our assessment of spike-specific T cells to include frequencies of Omicron BA.1 spike-specific T cells, since multiple infections took place during the time that Omicron BA variants were circulating, and the bivalent vaccinations at that time encoded the spike protein of Omicron variants (Figure 8C, D).

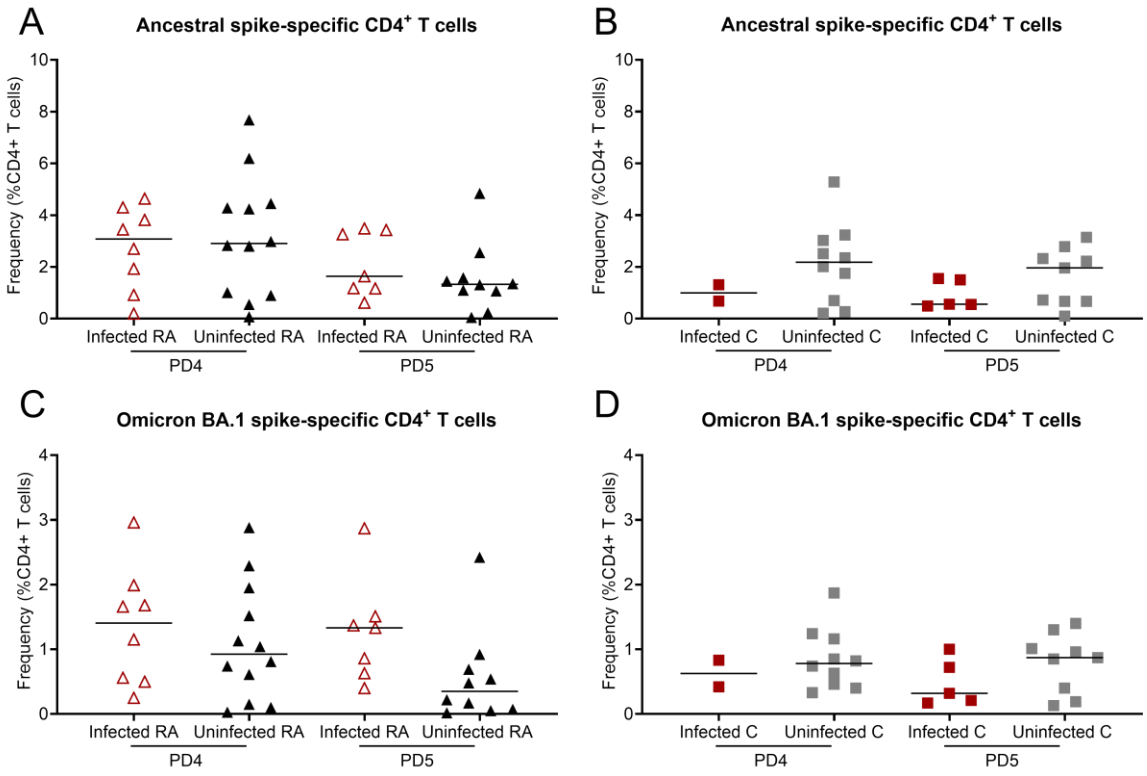


Figure 8. Ancestral and Omicron BA.1 spike-specific CD4⁺ T cell frequencies following fourth and fifth SARS-CoV-2 vaccinations in participants with RA and Controls. AIM assays were used to determine the frequencies of ancestral SARS-CoV-2 spike-specific CD4⁺ T cells in participants with **(A)** RA and **(B)** controls, as well as the frequencies of Omicron BA.1 spike-specific CD4+ T cells in participants with **(C)** RA and **(D)** controls. The solid lines indicate the median of each group. PD4 indicates samples collected post dose 4, PD5 indicates samples collected post dose 5. Infected refers to participants with a previous SARS-CoV-2 infection. PD4: Infected RA n=8, Uninfected RA n=12, Infected C n=2, Uninfected C n=10. PD5: Infected RA n=7, Uninfected RA n=10, Infected C n=5, Uninfected C n=9.

Other SARS-CoV-2 antigen-specific T cells

We also examined SARS-CoV-2 M-specific and N-specific T cell responses (Figure 9A-D). Studies have demonstrated that many patients with previous SARS-CoV-2 infections have M-specific and N-specific $CD4^+$ T cells, and that infection may elevate the frequencies of these cells above the levels seen in uninfected donors[128,132,136,364,365]. While the frequencies of these cells may wane with time post infection, they remain detectable for months afterward[365]. Given the deficits in spike-specific $CD4^+$ T cell responses to SARS-CoV-2 vaccination in participants with RA, we aimed to determine if their infection responses also differed from controls.

Interestingly, previous studies have determined that people without previous SARS-CoV-2 infections can have T cells that respond to proteins involved in viral replication and transcription[366,367]. Levels of T cells recognizing the NSP12 protein of SARS-CoV-2 (RNA polymerase) have also been reported to be higher in healthy individuals who do not develop detectable SARS-CoV-2 infection compared with those that do develop detectable infections[367]. We therefore also included peptides from the RNA polymerase protein of SARS-CoV-2 in our AIM assay, to evaluate if the levels of these cells differed between infected and uninfected participants with RA, and infected and uninfected controls (Figure 9E, F).

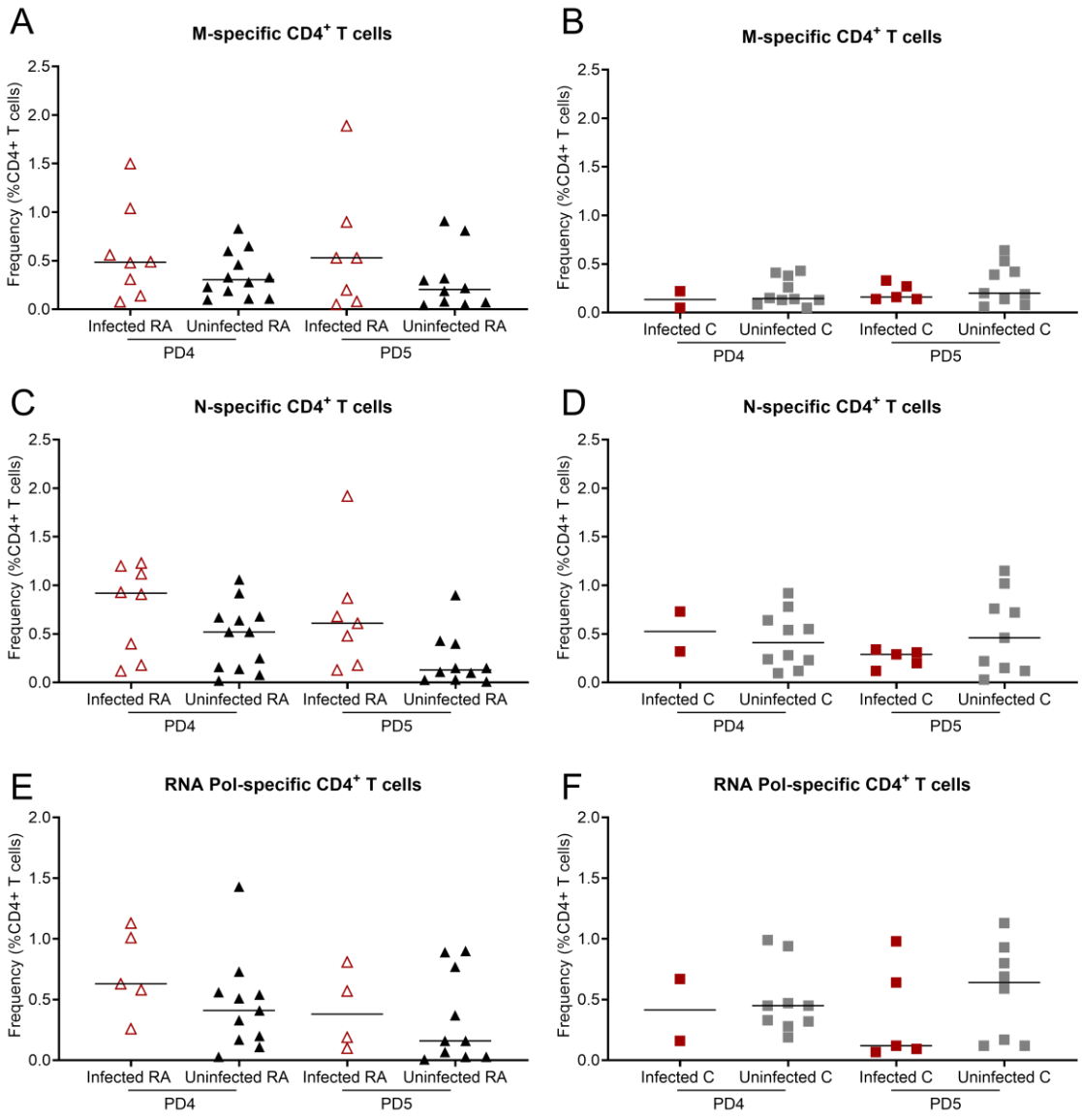


Figure 9. SARS-CoV-2 antigen-specific CD4⁺ T cell frequencies following fourth and fifth SARS-CoV-2 vaccinations in participants with RA and Controls. AIM assays were used to determine the frequencies of M-protein-specific, N-protein-specific, and RNA polymerase-specific CD4⁺ T cells in **(A, C, E)** participants with RA and **(B, D, F)** controls. The solid lines indicate the median of each group. PD4 indicates samples collected post dose 4, PD5 indicates

samples collected post dose 5. Infected refers to participants with a previous SARS-CoV-2 infection. M- and N-specific $CD4^+$ T cells: PD4: Infected RA n=8, Uninfected RA n=12, Infected C n=2, Uninfected C n=10. PD5: Infected RA n=7, Uninfected RA n=10, Infected C n=5, Uninfected C n=9. RNA polymerase-specific $CD4^+$ T cells: PD4: Infected RA n=5, Uninfected RA n=11, Infected C n=2, Uninfected C n=9. PD5: Infected RA n=4, Uninfected RA n=10, Infected C n=5, Uninfected C n=8.

T follicular helper cells

T follicular helper (T_{FH}) cells are a subset of $CD4^+$ helper T cells that provide aid to germinal center B cells in tasks such as class-switching[368,369]. While T_{FH} cells are primarily found in secondary lymphoid organs, where they express CXCR5, PD-1, and Inducible T-cell Co-stimulator (ICOS), circulating blood T_{FH} cells have also been reported[363,368]. These circulating T_{FH} cells are clonally similar to the T_{FH} cells in secondary lymphoid organs, though their phenotype differs as they are largely $CXCR5^+$ but negative for PD-1 and ICOS[363,368]. As such, studies on SARS-CoV-2 vaccination and infection responses that looked at T_{FH} cells in the peripheral blood typically defined them as $CXCR5^+CD4^+$ T cells[128,133,155,319,364]. Circulating SARS-CoV-2-specific T_{FH} cells, including spike-specific cells, have been reported following both SARS-CoV-2 infection and vaccination, where they have been associated with long-lived antibody production and elevated neutralizing antibody titers, respectively[128,133,136,155,364]. Considering the importance of circulating T_{FH} cells in the humoral responses to SARS-CoV-2 infections and vaccination, we saw value

in determining their frequencies in our participants with RA and controls after the fourth and fifth SARS-CoV-2 vaccinations (Figure 10).

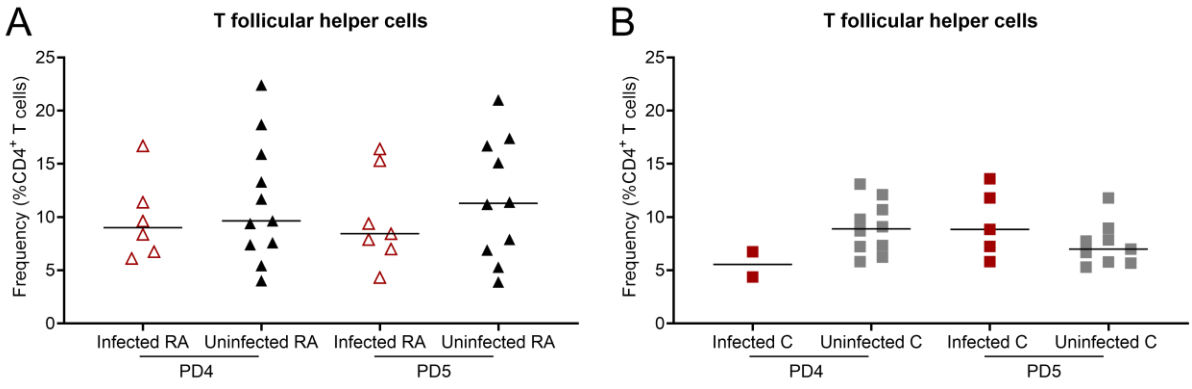
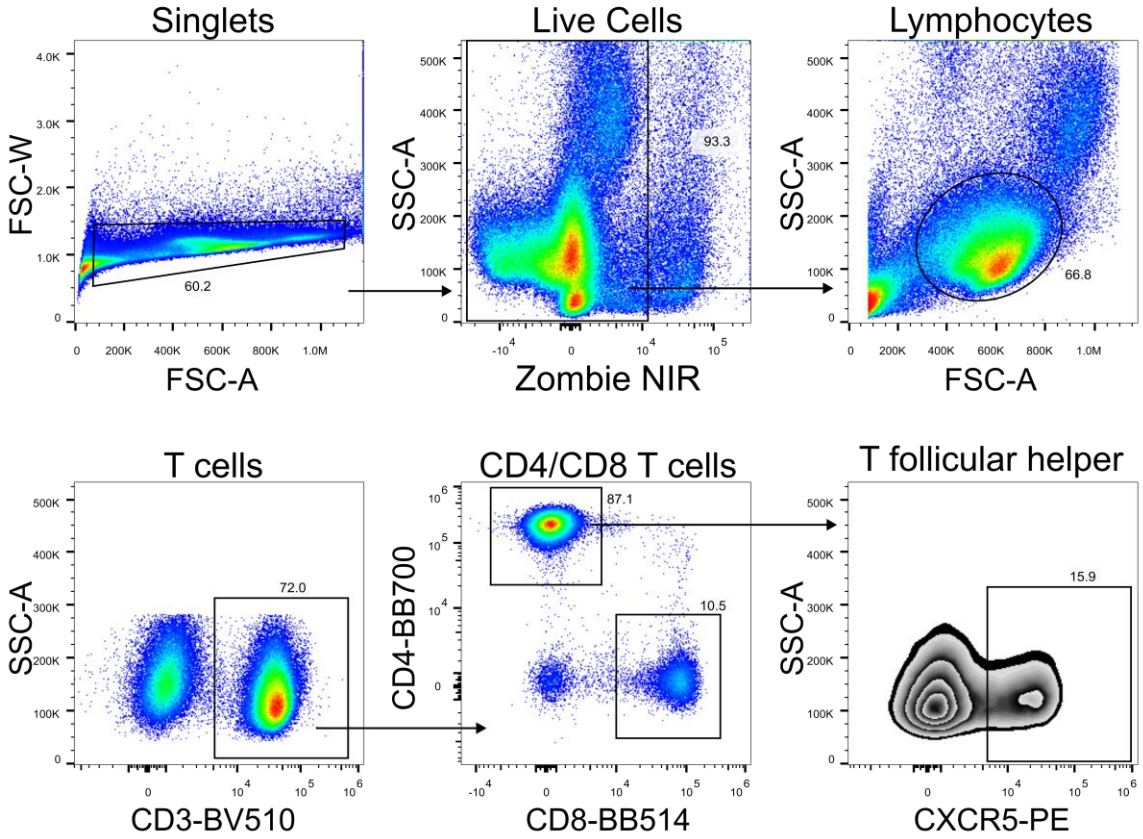


Figure 10. T follicular helper cell frequencies following fourth and fifth SARS-CoV-2 vaccinations in participants with RA and Controls. Flow cytometry was used to determine the frequencies of T_{FH} cells, defined as $CXCR5^{+}CD4^{+}$ T cells, in participants with **(A)** RA and **(B)** controls. The solid lines indicate the median of each group. PD4 indicates samples collected post dose 4, PD5 indicates samples collected post dose 5. Infected refers to participants with a previous SARS-CoV-2 infection. PD4: Infected RA n=6, Uninfected RA n=11, Infected C n=2, Uninfected C n=10. PD5: Infected RA n=7, Uninfected RA n=10, Infected C n=5, Uninfected C n=9.

Supplemental information



Supplementary Figure 1. T follicular helper cell gating strategy. Debris and doublets were excluded, live cells gated on, and then lymphocytes were identified by scatter properties within the live cell gate. Within lymphocytes, $CD3^+$ events were identified as T cells, and these T cells were gated into $CD4^+$ and $CD8^+$ single positive cells. Within the $CD4^+$ T cell compartment, T follicular helper cells were identified as $CXCR5^+$ events.

Supplementary Table 1. Flow Cytometry Antibodies for Hybrid Immunity study

Panel	Specificity	Clone	Fluorophore	Manufacturer	Cat. Number	Dilution (1:X)
Tfh, AIM	Live/Dead	NA	Zombie Near IR	BioLegend	423105	10,000
Tfh, AIM	CD3	UCHT1	BV510	BD Biosciences	563109	50
Tfh, AIM	CD4	SK3	BB700	BD Biosciences	566392	50
Tfh, AIM	CD25	M-A251	PECy7	BD Biosciences	557741	50
Tfh, AIM	CD8	RPA-T8	BB515	BD Biosciences	564526	50
AIM	CD134	ACT-35	PE	BioLegend	350004	100
AIM	CD137	4B4-1	APC	BioLegend	309810	50
AIM	CD69	FN50	BV711	BioLegend	310944	50
Tfh	CD39	A1	PEDazzle594	BioLegend	328224	100
Tfh	CXCR3	G025H7	BV421	BioLegend	353716	25
Tfh	CCR6	G034E3	BV785	BioLegend	353422	25
Tfh	CCR4	L291H4	BV605	BioLegend	359418	25
Tfh	CXCR5	J252D4	PE	BioLegend	356904	50
Tfh	PD-1	EH12.2H7	APC	BioLegend	329908	25
Tfh	ICOS	C398.4A	BV711	BioLegend	313548	25
Tfh	mIgG1k (PD-1 isotype)	MOPC-21	APC	BioLegend	400120	*
Tfh	Armenian Hamster IgG (ICOS isotype)	HTK888	BV711	BioLegend	400963	*

*Dilution for isotypes is lot dependent and should match final µg of corresponding antibody.

Appendix 3. Licensing Agreement for *Immunology Letters* paper

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Reassuring humoral and cellular immune responses to SARS-CoV-2 vaccination in participants with systemic sclerosis

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Journal	Immunology Letters
Article number	106929
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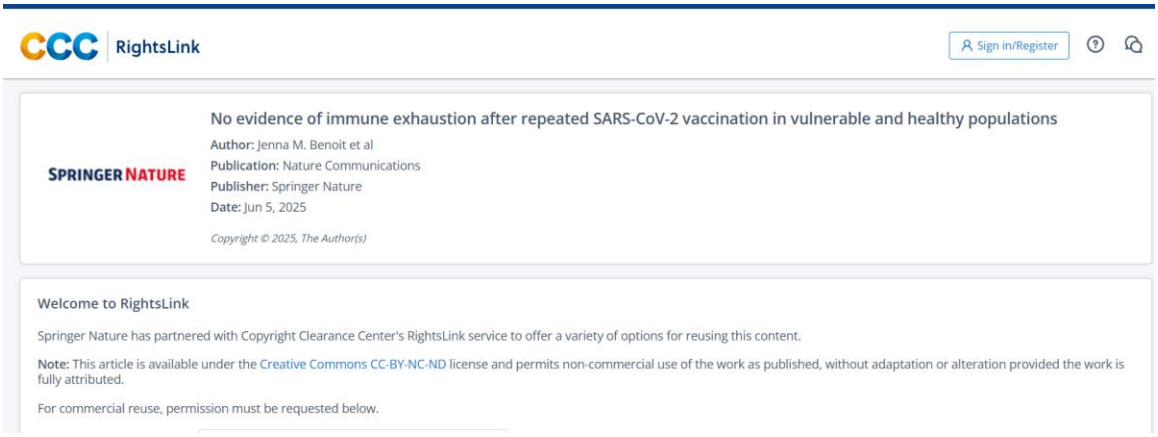

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SPRINGER NATURE

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